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UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

INSTITUTO DE BIOTECNOLOGÍA

DEPARTAMENTO DE GENÉTICA Y FISIOLOGÍA MOLECULAR

Modulación y farmacología de la corriente de Ca^{2+} tipo T en células espermatogénicas de ratón

T E S I S

que para obtener el grado de

Doctor en Ciencias Bioquímicas

presenta

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Cuernavaca, Morelos. **2002**

Este trabajo se realizó bajo la asesoría del Dr. Alberto Darszon, en el Departamento de Genética y Fisiología Molecular del Instituto de Biotecnología de la Universidad Nacional Autónoma de México. Cuernavaca, Morelos.

Agradecemos el apoyo económico de CONACyT, DGAPA-UNAM, DGEP-UNAM y el Howard Huges Medical Institute otorgado al laboratorio para la realización de este proyecto.

A mis padres, Ignacio y Mary,

por su amor y motivación. Gracias por obsequiarnos la libertad de ser.

A mis hermanos Salomé, Alejandro y Omar,

por su apoyo incondicional y tantas experiencias juntos.

AGRADECIMIENTOS

A mi familia académica:

Al Dr. Alberto Darszon por su dirección y apoyo,
gracias por su confianza y consejos.

Al Dr. Arturo Liévano por iniciarme en esta hermosa profesión y darnos ejemplo de perseverancia y fortaleza. Gracias.

A las Drs. Carmen Beltrán, Claudia Treviño y Ricardo Felix por su apoyo y consejos.

A mis queridos amigos y compañeros de laboratorio: Carlos Muñoz, gracias por la invitación a conocer este instituto. Felipe, Daniel, Blanca, Yuyú, Oti, Lalo, Esme, Carla, Gise, José Luis, Takuya, Gabriel, Lucy, Harumi y Laura por su amistad, su ayuda y hacer más agradable mi estancia en este laboratorio.

A mis amigos Vicky, Magaly, Marco Tulio, Roald, Cesar, Carlos, Pedro y Enrique. Gracias por su amistad

A Francisca Calendario, María de la Paz Colín, Antonia Gama, José Luis de la Vega Beltrán y Juan Monroy por su amistad y apoyo técnico en el laboratorio.

Al Dr. Lourival Possani, a la Dra. Alejandra Bravo, al Dr. Jean Louis Charli, al Dr. David Naranjo y al Dr. Arturo Hernández que formaron parte del honorable jurado. Gracias por sus comentarios y sugerencias.

Al Dr. Froylan Gómez y al Dr. Omar Pantoja por su asesoría como miembros del comité tutoral.

A CONACyT por la beca-credito otorgada para la realización de mis estudios de posgrado.

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ABREVIATURAS

A, amplitud inicial de la corriente

AA, ácido araquidónico

ADAM, familia de las adesin metalo proteinasas (por sus siglas en inglés)

ADN, *ácido deoxiribonucléico*

AMPc, adenosin monofosfato cíclico

[AMPc]i, concentración de AMPc intracelular

AN, ácido niflúmico

ATPasas, adenosin trifosfato sintasas

ATP-Mg, adenosin trifosfato sal de Mg^{2+}

A-23187, ionóforo de Ca^{2+}

BSA, sero albúmina bovina

CaM, calmodulina

[Ca^{2+}]i, concentración de Ca^{2+} intracelular

CCDV, canales de Ca^{2+} dependientes de voltaje

CE, células espermatogénicas

CSO_4, sulfato de colesterol

DHPs, dihidropiridinas

DIDS, ácido 4,4'-diisotiocianatoestilbeno-2-2'-disulfónico

DMSO, dimetilsulfóxido

DNAsa, ácido deoxiribonucleasa

DS, desviación estándar

EGTA, ácido tetra acético etilen glicol-bis(β-aminoetil eter)

Em, potencial de membrana

E.S.M., error estándar de la media

F, constante de Faraday

GABA, ácido γ-aminobutírico

HEPES, ácido (N-[2-hidroxietil] piperazina–N-[2-etanosulfónico])

HP, potencial de mantenimiento

HVA, alto umbral de activación (por sus siglas en inglés)

H89, N-[2-((p-bromocinamil)amino)etil]-5-isoquinolinesulfamida

ICa_T, corriente de Ca^{2+} tipo T

InsP3R, receptor de inositol trifosfato

IP3, inositol trifosfato

[IP3]i, concentración intarcelular de IP3

K_D, constante de disociación

KDa, kilodaltones

KHz, kiloHertz

KLI, kurtoxin like I

KLII, kurtoxin like II

KN62, 1-[N,O-bis-(5-isoquinolina-sulfonil)-N-metil-L-tirosil]-4-fenilpiperazina

LVA, bajo umbral de activación (por sus siglas en inglés)

M, molar

mg, miligramos

ml, mililitros

mM, milimolar

mOsm, miliosmoles

ms, milisegundos.

mV, milivoltios.

MΩ, megaOhms.

nA, nanoamperios

nM, nanomolar.

nm, nanómetros.

NPPB, ácido 5-nitro-2(3-fenilpropilamino) benzóico.

[Na^+]i, concentración de Na^+ intracelular

pA, picoamperios.

PAS, ácido periódico de Schiff

Patch-Clamp, fijación de voltaje en microáreas de membrana.

pF, picofaradios

pHi, pH intracelular

PKA, proteína cinasa dependiente de AMPc

PTK, proteína tirosin cinasa.

PTX, toxina Pertussis.

pS, picosiemens.

Q, carga

R, constante de los gases.

RA, reacción acrosomal

RNA, ácido ribonucléico

rpm, revoluciones por minuto.

s, segundo.

s, intervalo de potencial para un cambio de *e*-veces cercano a $V_{1/2}$.

SOCs, canales operados por el vaciamiento de pozas internas (por sus siglas en inglés).

t, tiempo.

TRP o *trp*, transient receptor potential (proteína o gen, respectivamente)

TFP, trifluoperazina.

V_m, potencial de prueba.

$V_{1/2}$, potencial al cual la corriente alcanza la mitad de su amplitud máxima

W5, N-(6-aminohexil)-1-naftalenosulfamida.

W7, N-(6-aminohexil)-5-cloro-1-naftaleno-sulfamida.

ZP, zona pelúcida.

ZP3h, ZP3 humana.

μm, micra.

μl, microlitros.

μM, micromolar.

τ, constante de tiempo

τ_{inact}, constante de inactivación

ω-CTX, ω-conotoxina

2-OH-β-CD, 2-OH-β-ciclodextrina.

RESUMEN

Durante la fecundación, el flujo de iones a través de la membrana plasmática del espermatozoide está involucrado en diferentes procesos fisiológicos indispensables para la fertilidad de esta célula. Uno de los iones más importantes en la fecundación es el Ca^{2+}. Este ion participa de manera fundamental en dos eventos previos a la fusión de los gametos: la capacitación y la reacción acrosomal (RA). En ambos eventos, ocurre un incremento en la concentración de Ca^{2+} intracelular ($[Ca^{2+}]i$), que en el caso de la RA depende de Ca^{2+} externo. Poco se sabe de las vías de entrada de Ca^{2+} durante la capacitación, a diferencia de la RA. El uso de fluoróforos sensibles a Ca^{2+} han permitido identificar dos fases en el incremento de la $[Ca^{2+}]i$ durante la RA inducida por la zona pelúcida (ZP), lo cual es consistente con la participación de al menos dos tipos de canales de Ca^{2+} diferentes durante la RA de mamífero. La primera entrada corresponde a la activación de la corriente de Ca^{2+} tipo T (ICa_T) dependiente de voltaje sensible a dihidropiridinas (DHPs), registrado tanto en células espermatogénicas (CE) como en el espermatozoide maduro, y cuyas características farmacológicas coinciden con la inhibición del incremento de la $[Ca^{2+}]i$ en el gameto maduro y de la RA. De la activación de la ICa_T depende la apertura de la segunda entrada de Ca^{2+}, la cual podría corresponder a un canal capacitativo. La secuencia de estos eventos sugiere la importancia de los canales de Ca^{2+} dependientes de voltaje en la fecundación. A pesar de la relevancia de la corriente T en este proceso, aún se desconocen las vías de señalización que la modulan y su identidad molecular. En el presente trabajo se describe, por primera vez, la modulación de la ICa_T por la albúmina (BSA), uno de los componentes esenciales para la capacitación. La BSA induce un incremento en la densidad de la corriente de manera dependiente de la concentración, y corre significativamente la voltaje dependencia de la activación y la inactivación en estado estacionario de los canales. Los efectos de la BSA no están relacionados a su capacidad para remover colesterol de la membrana plasmática de las CE y son dependientes de voltaje. Los resultados nos permiten especular sobre la posible participación de la corriente de Ca^{2+} tipo T en el incremento de la $[Ca^{2+}]i$ durante la capacitación del espermatozoide maduro. En contraste, el 17-β-estradiol inhibe significativamente la actividad de los canales de Ca^{2+} T de manera dependiente de la concentración e independiente de voltaje. Los resultados presentados en este trabajo sugieren que la modulación de la ICa_T por la BSA y el 17-β-estradiol no están relacionados. De la misma forma, en este estudio analizamos la posible modulación de la ICa_T por calmodulina (CaM) y su participación en la RA. El W7 y la trifluoperazina (TFP), dos inhibidores específicos de CaM,

reducen la densidad de la ICa_T de manera dependiente de la concentración con una IC_{50} de 10 y 12 μM, respectivamente. El W7 altera la voltaje dependencia de la activación de la corriente T y retarda las cinéticas de activación e inactivación. Este antagonista de CaM promueve la inactivación de la ICa_T en estados cerrados, lo que explica la disminución de la amplitud de la corriente. Consistente con lo anterior, el W7 inhibe al transitorio de Ca^{2+} inducido por la zona pelúcida (ZP) en el espermatozoide capacitado. De la misma forma, el W7 y la TFP inhibieron la RA con una IC_{50} de 10 μM. En contraste, dos inhibidores de proteína cinasa dependientes de CaM y proteína cinasa A, así como un inhibidor de fosfatasas dependientes de CaM, no tuvieron efecto en la ICa_T de las CE ni en la RA del espermatozoide maduro. En conjunto, estos resultados sugieren la modulación de la ICa_T por CaM y son consistentes con la participación de los canales T en la RA. Por otra parte, se muestran evidencias de que, al menos en nuestras condiciones experimentales, ni la progesterona ni el ácido γ-aminobutírico (GABA) modulan a la ICa_T. En la parte farmacológica, se describe el bloqueo reversible de la corriente T por inhibidores de canales aniónicos tales como el ácido niflúmico (AN) y el ácido 5-nitro-2(3-fenilpropilamino) benzóico (NPPB). Para ambos bloqueadores aniónicos, el bloqueo de la ICa_T es dependiente de voltaje y de la concentración. La IC_{50} para el AN fue 43 μM, mientras que los canales T parecen tener dos sitios de afinidad para NPPB. Estos resultados sugieren que el AN no inhibe a la RA inducida por ZP por bloquear a la ICa_T, mientras que la inhibición de la RA por el NPPB podría deberse en parte al bloqueo de la ICa_T. Por último, en este trabajo se reporta la caracterización electrofisiológica preliminar de dos toxinas de alacrán (KLI y KLII) similares a la kurtoxina que bloquean a la ICa_T con alta afinidad (200 nM). El bloqueo de la ICa_T por KLI y KLII es poco reversible y poco dependiente de voltaje. Estas toxinas no afectan la corriente de K^+ presente en las CE, lo que sugiere que el bloqueo de la ICa_T es específico. El uso de estas toxinas nos permitió identificar un nuevo componente dependiente de voltaje de la corriente de Ca^{2+} de las CE. Este nuevo componente de la corriente de Ca^{2+} es resistente a Ni^{2+} (1 mM), a Cd^{2+} (20 μM), a la KLII (200 nM) reportada en este trabajo y no se modula por BSA. Estas características sugieren que este componente no pertenece a canales de Ca^{2+} HVA ni a canales T. De manera consistente, ambas toxinas inhiben parcialmente la RA inducida por la ZP lo que sugiere la posible participación de otros canales en este proceso. Este trabajo constituye el primer reporte de la inhibición de la RA de mamífero con toxinas peptídicas.

SUMMARY

The sperm acrosome reaction (AR) is an exocytic event in which hydrolytic enzymes are released allowing the penetration of the egg coat. During AR, the influx of Ca^{2+} through the sperm plasma membrane is essential. Although the T-type Ca^{2+} current (ICa_T) plays a key role in this process, its mechanisms of regulation are unknown. This work shows the modulation of the ICa_T by serum albumin (BSA), which is essential for sperm capacitation. BSA increases the current density. This effect is not related to the BSA capacity to remove cholesterol nor 17-β-estradiol, the latter inhibits the activity of ICa_T. We also analyzed the regulation of the ICa_T by calmodulin (CaM). The W7 and the trifluoperazine (TFP), two specific inhibitors of CaM, reduce the ICa_T density with IC_{50} of 10 and 12 μM, respectively. W7 promotes the inactivation of the ICa_T from closed states that explains the decrease of the current amplitude. Consistently, W7 inhibits the Ca^{2+} transient induced by the zona pellucida in the capacitated sperm and blocked the AR, supporting the participation of the T-type Ca^{2+} channels in the AR. In the pharmacological studies, the reversible blocking of the ICa_T by anionic channel blockers, niflumic acid (NA) and NPPB, is shown. The IC_{50} for NA is 43 μM. At last, this study shows the characterization of two scorpion toxins (KLI and KLII), which block the ICa_T with high affinity (200 nM). The blocking effect of the ICa_T by both toxins is specific. The application of these toxins revealed a new component of the Ca^{2+} current

I.- INTRODUCCIÓN

1.1.- Aspectos Generales de la Fecundación.

Desde el punto de vista biológico, la reproducción es un evento fundamental del cual depende la preservación de las distintas especies debido a la generación de nuevos organismos. Más aún, la reproducción incrementa la variabilidad genética de una población al incorporar nuevas combinaciones de alelos durante la recombinación genética (en los organismos que la presentan). Este hecho favorece la capacidad de adaptación de una especie a un ambiente altamente cambiante y por lo tanto asegura la supervivencia de la misma.

En general, existen dos formas de reproducción: la reproducción asexual y la reproducción sexual. Clásicamente, se define a la reproducción asexual como la generación de nuevos organismos genéticamente idénticos a partir de un solo progenitor, sin que este proceso involucre la participación de gametos o recombinación genética. Por otra parte, la reproducción sexual implica la participación de gametos, con la finalidad de recombinar el material genético de dos individuos de la misma especie. Así, el nuevo organismo difiere genéticamente no solo de sus progenitores, sino que posee características que lo distinguen del resto de los individuos de su especie. Clásicamente, se ha considerado que la reproducción sexual posee grandes ventajas evolutivas como consecuencia de la creación de nuevas combinaciones de genes. Así, de manera consistente con la consideración antes mencionada, las especies que preferentemente se reproducen asexualmente son organismos con menor diversidad genética en comparación con aquellas de reproducción sexual (Alberts *et al*, 1994).

El estudio de la fisiología de la reproducción sexual abarca desde los mecanismos detallados de la gametogénesis, incluyendo la fisiología propia del espermatozoide y el óvulo, hasta el proceso de la fecundación durante el cual los gametos se fusionan para formar un nuevo organismo diploide.

Hace casi un siglo del inicio del estudio de la fisiología de la reproducción por Lillie (revisado en Darszon *et al*, 1999) y, sin embargo, a pesar de la vital importancia de la fecundación muchos de los eventos moleculares involucrados en este proceso permanecen aún sin aclararse. Actualmente, sabemos que ciertas señales químicas emitidas por el óvulo influyen directamente en la interacción entre los gametos, y que los canales iónicos presentes en el espermatozoide maduro juegan un papel clave en la fecundación. Más aún, existen evidencias que demuestran que los componentes de la capa externa del óvulo afectan profundamente la fisiología del espermatozoide tanto en invertebrados marinos como en mamíferos, de tal manera que inducen una serie de cambios ordenados que lo preparan para llevar a cabo la fecundación. Uno de los eventos indispensables para la fecundación es la reacción acrosomal (RA). J.C. Dan describió este fenómeno por primera vez en 1954 cuando estudiaba la entrada del espermatozoide al óvulo de erizo de mar (revisado en Darszon *et al*, 2001). El acrosoma es una vesícula secretoria derivada del aparato de Golgi, localizada en la parte anterior de la cabeza del espermatozoide, la cual contiene a las enzimas necesarias para la penetración de la capa externa del óvulo (Knobil *et al*, 1994). Así, la RA es un evento de exocitosis que consiste en la fusión de la membrana acrosomal y la celular en múltiples sitios de la cabeza del espermatozoide, con la subsecuente liberación de las enzimas líticas presentes en el acrosoma. Además, durante la RA el espermatozoide expone nuevos elementos membranales que se requieren para la penetración de la capa externa de óvulo y para la fusión de ambos gametos. Como sabemos, Dan reportó que la RA depende de Ca^{2+} externo (revisado en Darszon *et al*, 2001) e involucra un influjo de Ca^{2+} a través de canales de Ca^{2+} dependientes de voltaje (CCDV) (Darszon *et al*, 1999; Shackmann, 1989). Sin embargo, el estudio de las corrientes de Ca^{2+} presentes en el espermatozoide se ha visto limitado por la morfología y el tamaño de esta célula. Por lo anterior, como una estrategia alternativa para el estudio de los CCDV involucrados en la fecundación, se ha caracterizado a las corrientes de

Ca^{2+} presentes en las células precursoras del mismo (Santi *et al*, 1996; Arnoult *et al*, 1996) y/o se han utilizado técnicas fluorométricas para registrar el influjo de Ca^{2+} en la célula madura (Arnoult *et al*, 1999). Esta estrategia experimental ha permitido describir que los canales de Ca^{2+} presentes en las células espermatogénicas (CE) son principalmente de bajo umbral de activación (LVA, por sus siglas en inglés) o tipo T. Más aún, las dihidropiridinas (DHPs), el Ni^{2+} y el Cd^{2+} bloquean a los canales de Ca^{2+} tipo T con la misma afinidad con la que inhiben al influjo transitorio de Ca^{2+} en el espermatozoide maduro (Arnoult *et al*, 1999) y a la RA inducida por el agonista fisiológico (Santi *et al*., 1996; Arnoult *et al*., 1996). Todos estos datos sugieren que la corriente de Ca^{2+} tipo T (ICa_T) permanece de manera funcional en la célula madura, y que este tipo de canales tienen un papel preponderante en el proceso de la fecundación. A pesar de las evidencias experimentales antes mencionadas, poco sabemos acerca de los mecanismos moleculares que regulan a estas proteínas membranales.

El presente trabajo aborda el estudio de la RA en mamífero, y en particular se enfoca en el estudio de la modulación del canal de Ca^{2+} tipo T debido a su relevancia en la fisiología de la fecundación.

1.1.1 - Espermatogénesis.

La espermatogénesis, o formación de los gametos masculinos, consiste en una serie de divisiones celulares que concluyen en la formación de células haploides (meiosis), las cuales llevan a cabo un proceso de especialización celular denominada espermiogénesis que da lugar a la formación de los espermatozoides maduros. En los mamíferos, la espermatogénesis se lleva a cabo de forma continua durante toda la vida del organismo adulto (Fig. 1) y se caracteriza por las siguientes etapas, las cuales involucran a tipos celulares perfectamente definidos:

Etapa mitótica

La fase de mitosis tiene como objetivo la autorenovación de las células germinales denominadas espermatogonias tipo A1 (Krester y Kerr 1988; Garner y Hafez, 1987). Las espermatogonias son las células responsables de generar a los siguientes tipos celulares que se diferenciarán durante la meiosis (esto ocurre a partir de espermatogonias tipo B). Este tipo celular posee una forma esférica de 12 a 14 µm de diámetro, con múltiples nucléolos cercanos a la

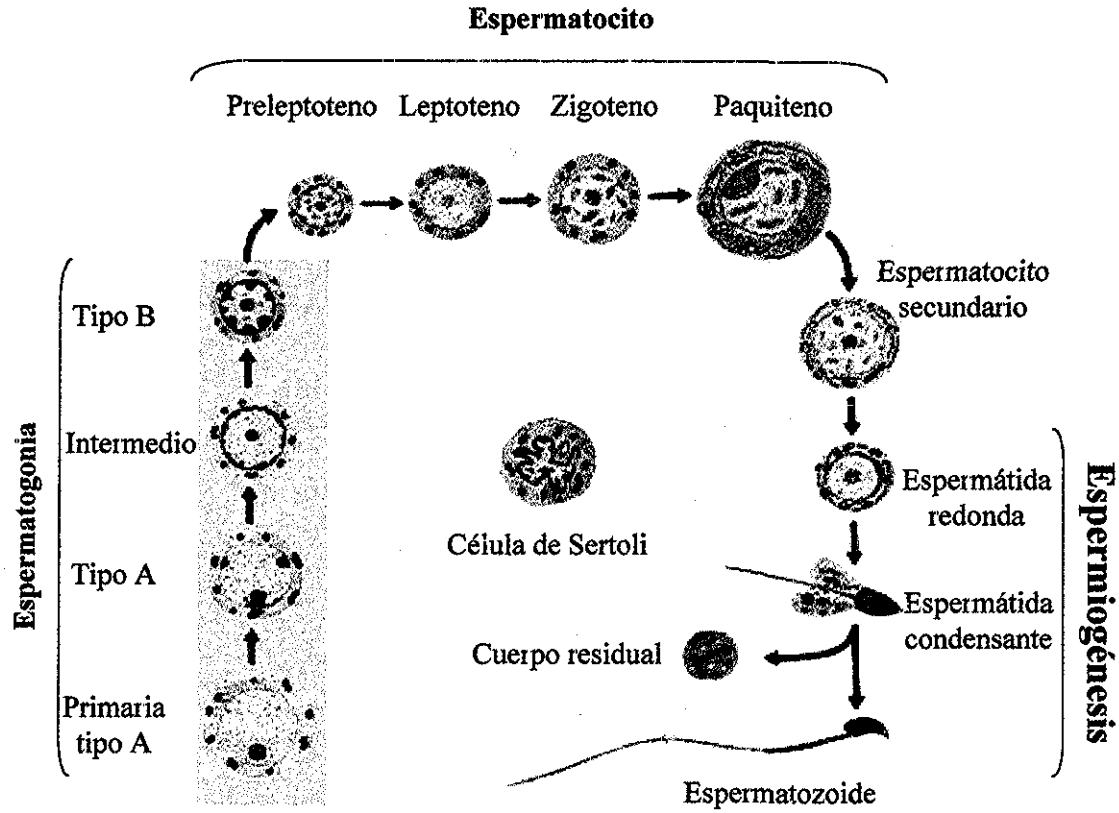

Figura 1.- Diagrama esquemático de la espermatogénesis. Las principales fases de la espermatogénesis incluyen la proliferación y la renovación de las espermatogonias por mitosis (línea azul), la división meiótica de los espermatocitos (línea amarilla) y la transformación morfológica durante la espermiogénesis (modificado de Bellvé, 1993).

membrana nuclear interna (Bellvé, 1993) (Fig 1; eje ascendente). Durante esta etapa, las espermatogonias están interconectadas por puentes citoplasmáticos que resultan de una citocinesis incompleta y que permiten el paso de metabolitos de alto peso molecular como proteínas y RNA mesajeros. Gracias a estas estructuras, a pesar de las diferencias genéticas, las células de una misma etapa meiótica son fenotípicamente equivalentes, lo que permite que las células germinales se desarrollen como un grupo (Braun *et al.*, 1989).

Etapa meiótica

Durante la fase de meiosis ocurre la reducción del número de cromosomas que contienen cada una de las células como consecuencia de las dos divisiones celulares consecutivas características de este tipo de división celular (Alberts *et al.*, 1994). La meiosis inicia después de las espermatogonias tipo B (figura 1, eje horizontal amarillo), las cuales son células redondas de 8 a 10 μm de diámetro, con cúmulos de cromatina adyacentes a la membrana nuclear (Bellvé, 1993). En esta etapa también es posible identificar a los espermatocitos primarios y secundarios que darán origen a las espermátidas haploides (Bellvé, 1993). En general, los espermatocitos son células redondas cuyo diámetro varía de 8 a 18 μm dependiendo del avance de la división celular (preleptoteno, leptoteno, zigoteno y paquiteno). De la misma forma, la condensación de la cromatina varía dependiendo del grado de diferenciación en el que se encuentren, y va desde finas hebras de cromatina (preleptoteno) hasta núcleos grandes con cromosomas gruesos y prominente heterocromatina adyacente a la envoltura nuclear (paquiteno) (Bellvé, 1993). El último tipo celular característico de esta etapa es la espermátida redonda. Esta célula tiene un diámetro de 8 a 10 μm, con cromatina homogénea y un núcleo central prominente. Posee un cuerpo cromatoide cercano al núcleo y se puede observar la formación del acrosoma en el polo apical del núcleo (Bellvé, 1993).

En la primera división meiótica, los espermatocitos primarios duplican su ADN y el número de cromosomas (4n) lo que favorece la formación de cromátidas dobles. Estas cromátidas se aparean con sus homólogas para llevar a cabo el entrecruzamiento o recombinación genética. Posteriormente, al dividirse, cada célula hija (los espermatocitos secundarios) reciben la mitad de los cromosomas por lo que conservan una carga genética diploide (2n). Los espermatocitos secundarios a su vez se dividen, sin que ocurra otra duplicación de ADN, por lo que las células resultantes (espermátidas redondas) serán haploides (n).

Espermiogénesis

La espermiogénesis es un proceso de diferenciación celular que inicia con las espermátidas condensantes y que concluye con la formación de los espermatozoides (Krester y Kerr 1988; Garner y Hafez, 1987). Inicialmente, las espermátidas condensantes son células esféricas con un diámetro de 8 a 10 μm, de citoplasma claro, núcleo pequeño con cromatina homogénea y con un prominente nucléolo central. En esta etapa aparecen los flagelos, las cuales son sensibles a digestión enzimática (Bellvé, 1993). La observación del desarrollo acrosómico utilizando la tinción de PAS (ácido periódico de Schiff) ha permitido describir 4 fases de la espermiogénesis: la fase de Golgi, la de capuchón, la acrosómica y la de espermiación (Krester y Kerr 1988; Duane *et al*, 1989). La serie de cambios morfológicos durante la espermiogénesis, resulta en la transformación de una célula esférica (la espermátida condensante, figura 1 eje descendente) en una célula alargada con la formación de nuevos organelos como el acrosoma y el flagelo, en la reorganización y eliminación de la mayor parte del citoplasma y de los organelos, y en la supercondensación del material genético. El material genético supercondensado junto con los cambios en el citoesqueleto dan al núcleo y al acrosoma la forma característica de cada

especie. Al final de la espermiogénesis, el espermatozoide se libera al lumen del túbulo para comenzar su travesía por el tracto reproductor masculino.

Morfología del espermatozoide

Una vez concluida la espermiogénesis, el espermatozoide maduro está formado por dos regiones principales: la cabeza y el flagelo, las cuales están unidas a través de una pieza conectora o cuello (Fig 2). En el caso del espermatozoide de ratón, la cabeza es aplanada y mide aproximadamente 5x3x1 μm (largo, ancho y espesor). La cabeza incluye al núcleo, y al acrosoma, rodeados por un poco de citoplasma. El núcleo del espermatozoide ocupa la mayor parte del volumen de la cabeza y contiene el DNA perfectamente compactado gracias a la intervención de histonas básicas específicas del espermatozoide denominadas protaminas (Hecht, 1990). El tamaño del acrosoma y la superficie de contacto de este organelo con el núcleo varían entre las diferentes especies. El acrosoma comprende dos regiones, la cefálica y la ecuatorial. Se sabe que la región ecuatorial del acrosoma posee una proteína que participa en la fusión del espermatozoide con el óvulo, la fertilina (Yanagimachi, 1994). Además, la membrana acrosomal interna contiene una fracción membranal que contiene hialorunidasa, la proteína PH-20 y proacrosina (Yanagimachi, 1994). El contenido del acrosoma esta compuesto por fosfolipasas, colagenasas, catepsina D, calpaína II, fosfatasa ácida y otras glicosidasas diferentes de la hialorunidasa. Estas sustancias degradan la capa externa del óvulo homólogo durante la fecundación. El flagelo o cola puede dividirse en cuatro segmentos: el segmento conector o cuello, la pieza media, la principal y la pieza final o terminal. A lo largo de los tres últimos segmentos se localiza el axonema, que es un grupo de microtúbulos dispuestos en un orden de 9+2 pares y que son parte del aparato mótil del espermatozoide. La energía necesaria para el

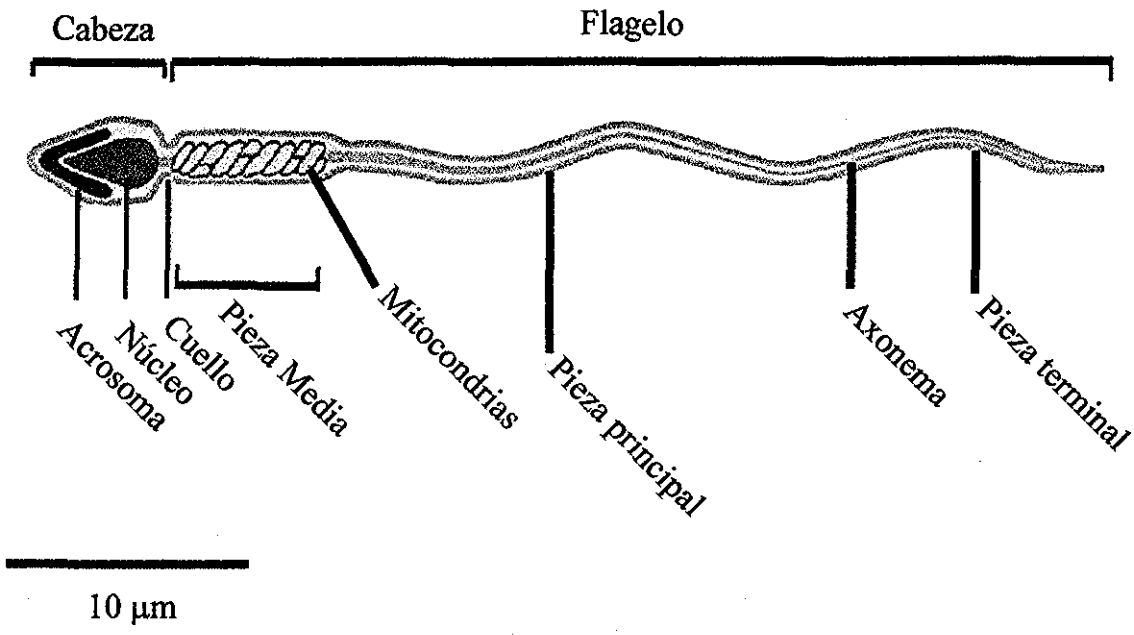

Fig. 2.- Esquema de un espermatozoide humano. Corte longitudinal de un espermatozoide (modificado de Alberts *et al*., 1994).

movimiento del flagelo se obtiene de un grupo de mitocondrias localizadas en la pieza media del flagelo.

1.1.2.- Fisiología del espermatozoide

Capacitación

La capacitación es una serie de procesos dinámicos mediante los cuales el espermatozoide maduro adquiere la capacidad de responder a los componentes de la capa externa del óvulo y como consecuencia llevar a cabo la RA (revisado en Darszon *et al*., 2001). Chang y Austin describieron la capacitación por primera vez en la década de los 50s, tras observar que los espermatozoides de mamífero recién eyaculados o recolectados del epidídimo carecen de la capacidad de fecundar al óvulo (Chang, 1951; Austin, 1951; revisado en Yanagimachi, 1994). Fisiológicamente, la capacitación se lleva acabo en el tracto genital femenino, específicamente en

el istmo del oviducto, donde las secreciones sitio específicas de los componentes del fluido del tracto genital promueven este proceso de maduración (Lakoski *et al.*, 1988; Yanagimachi, 1994). No todos los espermatozoides se capacitan y salen del istmo al mismo tiempo. Esto podría deberse a las diferencias fisiológicas propias de cada espermatozoide (Yanagimachi, 1994, revisado en Knobil *et al.*, 1994). De manera experimental, es posible inducir la capacitación al incubar a los espermatozoides de ratón en un medio definido que contenga al menos tres de los componentes clave: Ca^{2+}, $NaHCO_3$ y albúmina sérica (Yanagimachi, 1994; Visconti *et al*, 1995a,b). Los requerimientos para inducir la capacitación *in vitro* varían entre las especies. Por ejemplo, el uso de heparina como complemento facilita la capacitación de los espermatozoides de bovino, mientras que para el caso de dos especies de mono la adición de AMPc y cafeína favorece la capacitación (revisado en Yanagimachi, 1994).

Hasta la fecha, aún se desconocen muchos de los mecanismos moleculares involucrados en la capacitación (Baldi *et al*, 1996; Visconti y Kopf, 1998; Wassarman *et al*, 2001). Sin embargo, sabemos que este proceso involucra cambios en las concentraciones internas de diferentes iones (entre ellos el aumento de la $[Ca^{2+}]_i$), en la composición de los lípidos de la membrana, así como también una reestructuración de la misma e involucra eventos de fosforilación de diferentes proteínas del espermatozoide (Visconti *et al*, 1999; Baldi *et al.*, 2000; Flesch y Gadella, 2000) (Figura 3).

De manera general, durante el transcurso del espermatozoide por el tracto genital femenino la extracción del colesterol de la membrana plasmática por moléculas aceptoras, como la albúmina, da inicio a una reorganización de la membrana celular que culmina en la redistribución o inserción de distintos componentes membranales y en la capacitación de los gametos masculinos (Baldi *et al*, 2000). Se ha propuesto que la extracción del colesterol propicia un incremento en la fluidez de la membrana celular, lo cual favorece la permeabilidad al Ca^{2+} y al

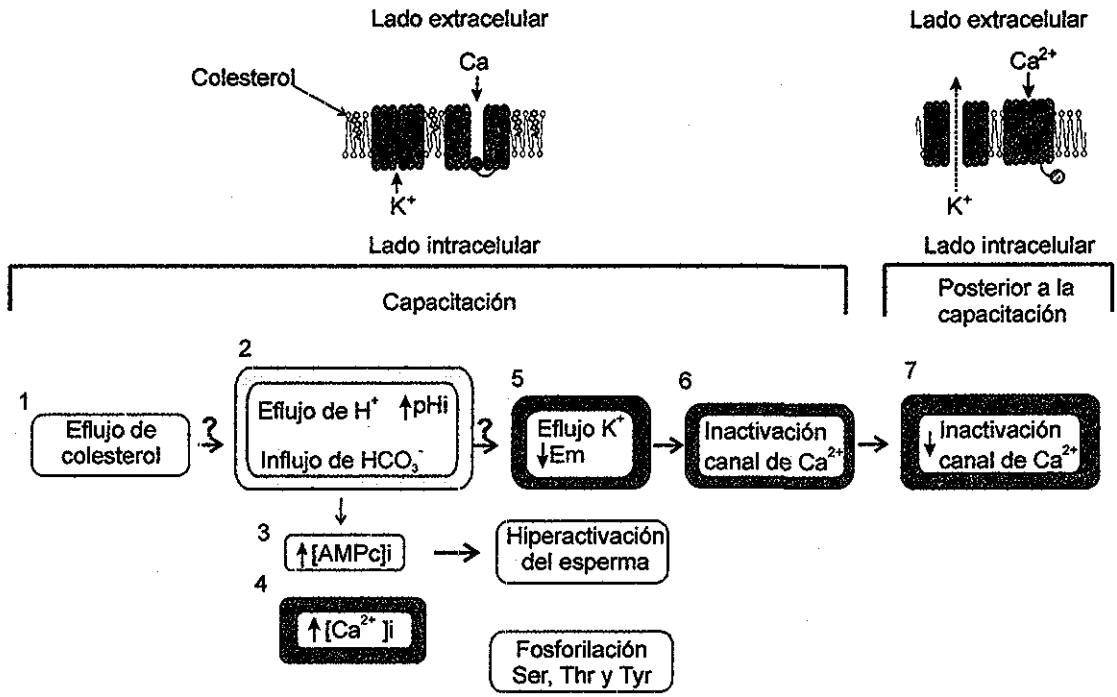

Figura 3.- Capacitación espermática en mamíferos. La capacitación ocurre en el tracto genital femenino, durante la fecundación. Este proceso inicia con el eflujo de colesterol inducido por la albúmina presente en el itsmo del oviducto, lo cual origina una reorganización de la membrana plasmática. La extracción de colesterol, de alguna manera aún no determinada, causa una alcalinización (2) que induce un incremento en la [AMPc]i y, probablemente, activa a un canal de K^+ del tipo rectificador entrante (5, canal rojo en los esquemas superiores). El incremento de AMPc y el influjo de HCO_3^- activarían a una proteína cinasa dependiente de AMPc (PKA) y a una proteína tirosin cinasa (PTK), respectivamente. Estos eventos son necesarios para la fosforilación de diversas proteínas y la hiperactivación del espermatozoide observadas durante la capacitación. Asimismo, durante este proceso ocurre un incremento en la $[Ca^{2+}]i$ por mecanismos aún no determinados (4). La apertura del canal de K^+ favorece una hiperpolarización de la membrana, la cual liberaría a los canales de Ca^{2+} tipo T de su estado inactivado (7, representados en color verde en los esquemas superiores) (modificado de Darszon *et al.*, 2001).

HCO_3^-, dos compuestos esenciales para la capacitación (Shi y Roldán, 1995; Visconti *et al*, 1995; Visconti y Kopf, 1998). Existen evidencias experimentales que sugieren la participación de la albúmina como aceptor de colesterol durante la capacitación (Visconti *et al*, 1999). Así, se ha demostrado que es posible inhibir la fosforilación de proteínas y la capacitación *in vitro* de espermatozoides de ratón si se preincuba la BSA con sulfato de colesterol en una relación 1:1.

Por otra parte, también se ha visto que en condiciones *in vitro*, el uso de heptasacáridos (moléculas que unen colesterol tales como las ciclodextrinas), promueve la liberación de colesterol de la membrana celular del espermatozoide de ratón en ausencia de BSA, e inducen un incremento en la fosforilación en tirosinas y la capacitación. Mas aún, sabemos que durante la capacitación decrece el cociente molar colesterol:fosfolípidos en la membrana del espermatozoide (revisado en Baldi *et al*., 2000). En conjunto, estos datos sugieren que la liberación del colesterol de la membrana del espermatozoide es, al menos, una de las señales iniciales que activan los mecanismos de transducción de señales involucrados en la capacitación. La salida de colesterol podría modificar a los microdominios de membrana denominados *rafts*. Se sabe que al menos existen dos tipos de *rafts* presentes en el espermatozoide, uno asociado a caveolina y el segundo asociado al gangliosido GM1 (Treviño *et al*., 2001). Sin embargo, solo se ha demostrado una redistribución de los microdominios (*rafts*) que contienen GM1 en espermatozoides reaccionados, aún no existen evidencias de este fenómeno durante la capacitación (Treviño *et al*., 2001).

La extracción del colesterol, de manera aún no determinada, induce un incremento en el pH_i del espermatozoide probablemente mediado por un intercambiador Cl^-/HCO_3^- dependiente de Na^+ y un exportador de protones sensible a ácido flufenámico (Zeng *et al*., 1996; ver figura 3). Durante su almacenamiento en el epidídimo, el pH_i del espermatozoide es relativamente ácido (6.54 ± 0.08) lo que probablemente contribuye a mantener el estado no capacitado (Parrish *et al*., 1989; Zeng *et al*., 1996; Muñoz-Garay *et al*., 2001) y a prolongar la viabilidad del gameto (Yanagimachi, 1994). Sin embargo, una vez iniciada la capacitación el pH_i se incrementa (hasta 6.73 ± 0.09) por la participación del intercambiador de Cl^-/HCO_3^- dependiente de Na^+ (Zeng *et al*., 1996). Se ha propuesto que el influjo de HCO_3^- estimularía a una adenilato ciclasa soluble dependiente de bicarbonato e independiente de proteínas G o pHi (revisado en Darszon *et al*.,

2001), la cual es específica del espermatozoide (Sinclair *et al*., 2000). Como consecuencia de lo anterior, se ha reportado un incremento en la $[AMPc]_i$ asociado a la capacitación (Visconti y Tezon, 1989; Visconti *et al*., 1995). Este incremento activaría a una proteína cinasa dependiente de AMPc (PKA), que podría estar participando en los eventos de fosforilación de distintas proteínas presentes en el espermatozoide involucradas en una o más de las cascadas de señalización que aún quedan por definir (revisado en Gadella y Harrison, 2000). Más aún, la PKA probablemente también participe en la activación de una proteína tirosina cinasa (revisado en Darszon *et al*., 2001) y en los eventos implicados en la reorganización de la composición lipídica de la membrana del espermatozoide inducida por el HCO_3^-, uno de los componentes claves para la capacitación (Gadella y Harrison, 2000). Así pues, la fosforilación durante la capacitación del espermatozoide de ratón y humano depende de Ca^{2+} y HCO_3^- externos (Visconti *et al*., 1995a,b; Baldi *et al*., 1996; Luconi *et al*., 1996; Naz, 1996; Emiliozzi y Fenichel, 1997).

Con respecto a los mecanismos involucrados en el incremento de la $[Ca^{2+}]_i$ durante la capacitación hay poca información disponible. Se sabe que la $[Ca^{2+}]_i$ del espermatozoide humano en estado de reposo se eleva durante el proceso de capacitación *in vitro* y alcanza una meseta estable en ~100 min (revisado en Darszon *et al*., 2001). Este incremento de Ca^{2+} depende de Ca^{2+} externo ya que al menos se necesita una concentración de 90 µM de Ca^{2+} en el medio extracelular para favorecer este cambio de concentración en ratón (Fraser, 1987). Aunque este requerimiento parece no ser necesario para el espermatozoide humano (Emiliozzi y Fenichel, 1997). Al igual que en otras células, en los espermatozoides de distintas especies de mamíferos, la $[Ca^{2+}]_i$ podría estar regulada por la participación de los intercambiadores de Na^+/Ca^{2+} y de Ca^{2+}/H^+, ATPasas de Ca^{2+}, CCDV y, probablemente, por pozas de Ca^{2+} intracelulares (Fraser, 1995; Baldi *et al* ,2000; Darszon *et al*., 2001). Son varias las evidencias que sugieren la presencia

de estos mecanismos de regulación del Ca^{2+} interno en el espermatozoide (Blackmore, 1993; Walensky y Snyder, 1995; Dragileva *et al*, 1999; O'Toole *et al*., 2000).

Sin embargo, la participación de los diferentes mecanismos mencionados anteriormente es motivo de debate. En el caso de la participación de las pozas internas en la determinación de la $[Ca^{2+}]_i$ no existen evidencias sólidas que apoyen esta hipótesis ya que el espermatozoide no posee un retículo endoplásmico como tal (Knobil *et al*., 1994), aunque se ha sugerido que el acrosoma podría constituir una reserva intracelular de Ca^{2+} (Walensky y Snyder, 1995; Treviño *et al*., 1998). Así, el trabajo de diferentes grupos sugiere que el acrosoma no es capaz de mantener una concentración elevada significativamente de Ca^{2+} (Kirkman-Brown *et al*, 2000; Kobori *et al*., 2000), miemtras otras evidencias sugieren una posible participación de las ATPasas de Ca^{2+} presentes en el acrosoma. Se ha demostrado que el uso de compuestos que inhiben a las ATPAsas de Ca^{2+}, tales como la quercetina o tapsigargina, aceleran el proceso de capacitación debido a que favorecen el incremento del Ca^{2+} intracelular (Mendoza y Tesarik, 1993; revisado en Baldi *et al*, 2000). Más aún, la calreticulina, una proteína que une Ca^{2+} (Nakamura *et al*., 1993), y el receptor de inositol trifosfato ($InsP_3R$) (Walensky y Snyder, 1995; Treviño *et al*, 1998) están presentes en el acrosoma de varias especies de mamíferos, lo que sugiere que el acrosoma puede almacenar Ca^{2+}.

De la misma forma, no se cuenta con evidencias claras que permitan apoyar la hipótesis de una posible participación de los intercambiadores de Na^+/Ca^{2+} y Ca^{2+}/H^+ en la modulación del Ca^{2+} interno durante la capacitación del espermatozoide. Como se mencionó anteriormente, hasta la fecha se sabe de la presencia de un intercambiador Na^+/Ca^{2+} en el espermatozoide pero no existen pruebas suficientes que apoyen que este intercambiador participe en el incremento de la $[Ca^{2+}]_i$ (Baldi *et al*., 2000).

Con respecto a los CCDV, si bien diversos grupos han aportado evidencias que muestran la presencia de canales de Ca^{2+} en el espermatozoide de mamífero (Liévano *et al*., 1996; Arnoult *et al*., 1996; Serrano *et al*., 1999; Westenbroek y Babcock, 1999; López-González *et al*., 2001), su participación en la regulación de la $[Ca^{2+}]_i$ durante la capacitación esta lejos de estar comprendida Se ha propuesto que durante la capacitación, la fosforilación en tirosinas podría modular a los canales de Ca^{2+} tipo T (Arnoult *et al*., 1999). De hecho, se piensa que la hiperpolarización que ocurre durante la capacitación debida a la participación de canales de K^+ (Arnoult *et al*., 1999; Muñoz-Garay *et al*., 2000), remueve la inactivación de los canales de Ca^{2+} tipo T y favorece su transición al estado cerrado, lo que permitiría que los canales de Ca^{2+} respondieran a la depolarización inducida por el agonista fisiológico durante la RA (Santi *et al*., 1996; Florman *et al*., 1998; Arnoult *et al*., 1999) Resulta interesante mencionar que en algunas especies de mamífero, compuestos como la heparina, indispensable para la capacitación del espermatozoide de bovino, regulan la $[Ca^{2+}]_i$ a través de CCDV probablemente a través de su receptor membranal respectivo (revisado en Darszon *et al*., 2001). Hasta la fecha, se desconoce si los canales de Ca^{2+} tipo T pudieran estar modulados por alguno de los componentes indispensables para la capacitación (como la albúmina o el HCO_3^-). Estudios preliminares hechos en nuestro laboratorio, mostraron que al menos la albúmina es capaz de modular a la ICa_T de las CE Sin embargo, nada se sabía del mecanismo mediante el cual ocurre dicha modulación por lo que nos dimos a la tarea de realizar un estudio más detallado al respecto en colaboración con el Dr. Felipe Espinosa (Centro de Neurociencia Básica, Universidad de Texas).

Además del Ca^{2+}, otros iones parecen ser importantes para la capacitación espermática. Las concentraciones intracelulares de K^+, Na^+ y Cl^- influyen en este proceso. Por ejemplo, el incremento de Na^+ intracelular inducido con monensina, un ionóforo de Na^+, acelera la capacitación en el espermatozoide de ratón. Por otra parte, se sabe que la concentración de Zn^{2+}

disminuye durante la capacitación en el espermatozoide de hámster. Más aún, si se incuba al espermatozoide en un medio que contenga Zn^{2+}, se inhibe la capacitación. El mismo trabajo sugiere que el Zn^{2+} podría estar jugando un papel importante en la desestabilización de la membrana plasmática del espermatozoide durante la capacitación (revisado en Baldi *et al.*, 2000).

Reacción Acrosomal

Como se mencionó al principio de este trabajo, el acrosoma es una vesícula secretora derivada del aparato de Golgi, localizada en la parte anterior de la cabeza del espermatozoide, la cual contiene a las enzimas necesarias para la penetración de la capa externa del óvulo (Knobil *et al*, 1994) Así, la reacción acrosomal (RA) es un evento de exocitosis que consiste en la fusión de la membrana acrosomal y la celular en múltiples sitios, con la subsecuente liberación de las enzimas líticas presentes en el acrosoma Además, durante la RA el espermatozoide expone nuevos elementos membranales que se requieren para la fusión de ambos gametos. Así, el segmento ecuatorial de la cabeza del espermatozoide adquiere sus propiedades fusogénicas solo después de que ocurre la RA.

En mamíferos, la RA inducida con la ZP también requiere de Ca^{2+} externo (Yanagimachi, 1988) e involucra un influjo de Ca^{2+} a través de CCDV (Darszon *et al* , 1999; Shackmann, 1989). Se sabe que la ZP solubilizada causa un incremento en el pHi y en la $[Ca^{2+}]i$, los cuales son indispensables para la exocitosis acrosomal (Florman y First, 1988; Florman *et al.*, 1989). Estudios previos han demostrado que el incremento en la $[Ca^{2+}]i$ inducida por la ZP, ocurre antes de la exocitosis (Florman *et al.*, 1989; Storey *et al.*, 1992; Florman, 1994), como resultado de la unión de la ZP3 a su receptor. Hasta el momento no existen pruebas contundentes acerca de la naturaleza de este receptor, como se discutirá más adelante. Sin embargo, uno de los candidatos

es una proteína denominada PKDREJ, la cual es un homólogo de mamífero del receptor del agonista fisiológico del erizo de mar (Hughes *et al.*, 1999). Esta proteína podría activar a un canal catiónico o constituir ella misma un canal catiónico que depolarizaría el potencial de membrana (Darszon *et al.*, 2001). Se ha demostrado que la unión del agonista fisiológico a su receptor induce cambios en el potencial de membrana del espermatozoide, un incremento en la $[Ca^{2+}]i$ y la alcalinización indispensables para la RA (Darszon *et al.*, 2001; ver figura 4). Es probable que los cambios en el potencial de membrana causados por la unión del agonista provoquen la apertura

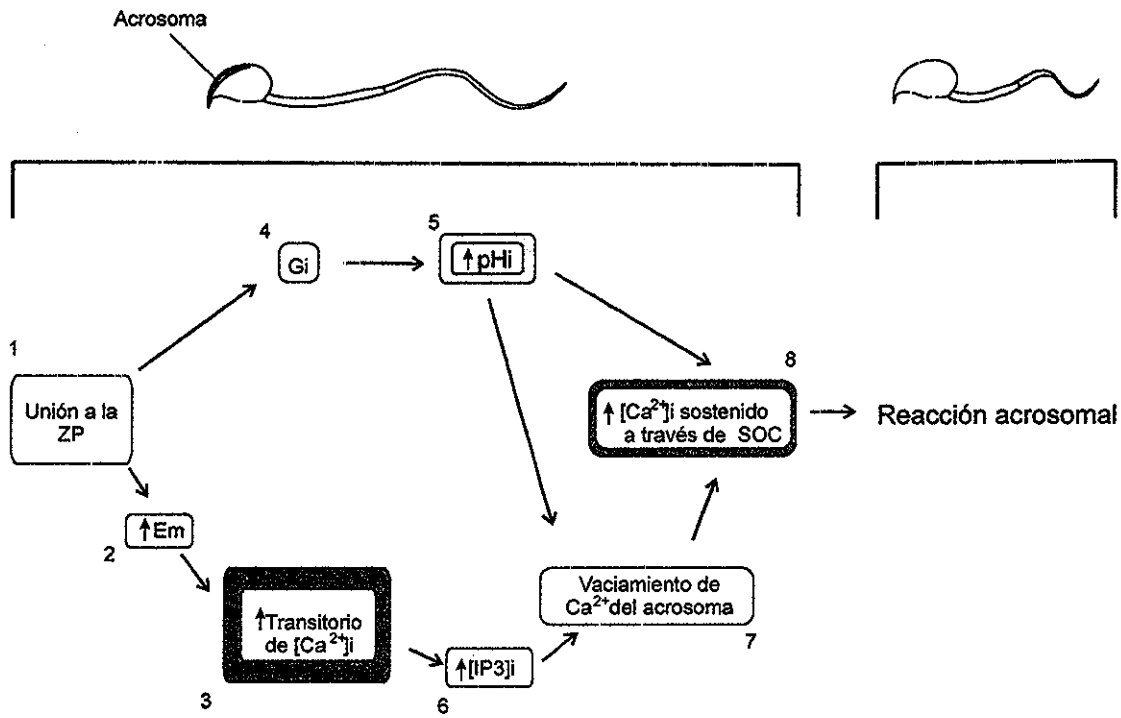

Figura 4.- La reacción acrosomal en mamífero. La unión del agonista fisiológico al receptor (o receptores) (1) induce una depolarización del potencial de membrana (2), el cual favorece un incremento transitorio en la $[Ca^{2+}]i$ (3) y una alcalinización (5) regulada por proteínas G sensibles a la toxina Pertussis (4). Estos cambios son indispensables para el incremento sostenido de la $[Ca^{2+}]i$ (8) causado por el aumento de la [IP3]i (6), el cual induce el vaciamiento de las pozas internas de Ca^{2+} (7). Como consecuencia de estos procesos, el espermatozoide libera el contenido acrosomal (representado en color azul en el dibujo del espermatozoide de ratón a la izquierda) (modificado de Darszon *et al.*, 2001).

de los canales de Ca^{2+} tipo T presentes en el espermatozoide maduro, los cuales se abrirían tras la eliminación de la inactivación durante la capacitación. Existen evidencias que sugieren que, al menos, parte del incremento de la $[Ca^{2+}]_i$ que se observa durante la RA del espermatozoide de ratón, bovino y humano es sensible a la toxina Pertussis (PTX), un inhibidor de proteínas G de la clase G_i (Endo *et al*, 1987; Florman *et al*, 1989). Más aún, se ha reportado la presencia de múltiples proteínas G en el espermatozoide de mamífero tales como las proteínas G_i y la G_z, (Glassner *et al*., 1991). De manera consistente, la ZP activa a las proteínas G_{i1} y G_{i2} en el espermatozoide de ratón (Ward *et al*., 1994), y el incremento transitorio de pHi necesario para la RA inducida por la ZP es sensible a PTX (Arnoult *et al*., 1996a). Sin embargo, aún no está claro si las proteínas Gi pudieran modular a algunos canales iónicos o cual es la relación entre el incremento entre el pHi y la $[Ca^{2+}]i$ Por otra parte, se ha propuesto que la calmodulina (CaM) pudiera participar en la RA en mamífero debido a su localización en la región acrosomal (Jones *et al*, 1980; Kann *et al*., 1991) y a su redistribución durante esta reacción (Hernández *et al*., 1994). Más aún, la adición de antagonistas de CaM inhibe a la RA y al influjo de Ca^{2+} asociado a la exocitosis acrosomal en el espermatozoide de erizo de mar (Sano, 1983; Guerrero y Darszon, 1989). Estos hechos sugieren que la CaM podría estar modulando a los canales de Ca^{2+} del espermatozoide de mamífero, por lo que parte de este proyecto se enfocó al estudio de la posible modulación de la corriente de Ca^{2+} T por CaM.

El uso de fluoróforos sensibles a Ca^{2+} ha permitido identificar dos fases en el incremento de la $[Ca^{2+}]i$ inducida por la ZP, lo cual ha mostrado ser consistente con la participación de al menos dos tipos de canales de Ca^{2+} diferentes durante la RA de mamífero (Florman, 1994). La primera entrada corresponde a la activación por ZP del canal de Ca^{2+} tipo T (Florman *et al*., 1998) sensible a DHPs, registrado tanto en células espermatogénicas (Arnoult *et al*, 1996; Santi *et al*., 1996) como en el espermatozoide maduro (Arnoult *et al*, 1999), y cuyas características

farmacológicas coinciden con la inhibición del incremento de la $[Ca^{2+}]_i$ y de la RA (Darszon *et al.*, 2001). A pesar de la relevancia de la corriente T en la fecundación, aún se desconoce su identidad molecular. Se ha propuesto que el influjo de Ca^{2+} a través de los canales T activaría a la fosfolipasa C presente en el espermatozoide (Vanha-Perttula y Kasurinen, 1989; Breitbart y Noar,1999; Baldi *et al.*, 2000). Consistente con esta idea, se ha reportado que la ZP induce un incremento en el IP3 (Tomes *et al.*, 1996) y se han dado evidencias de la presencia del receptor de IP3 en el acrosoma de estas células (Walensky y Snyder, 1995; Treviño *et al.*, 1998). Más aún, recientemente se demostró que la PLCδ4 participa en los eventos primarios de la RA inducida por ZP3 (Fukami *et al*, 2001) Así, se ha propuesto que el incremento del IP3 intracelular provocaría un vaciamiento de las pozas internas (Walensky y Snyder, 1995), lo cual activaría al segundo componente del infujo de Ca^{2+}. Este incremento lento y sostenido en la $[Ca^{2+}]i$ tiene características propias de un canal de Ca^{2+} de tipo capacitativo (Santi *et al*, 1999; O'Toole *et al.*, 2000). En general, la salida de Ca^{2+} de las pozas internas ocasiona la apertura de canales operados por el vaciamiento de pozas internas (SOCs por sus siglas en inglés) presentes en la membrana plasmática de las células (Parekh y Penner, 1997). A pesar de que la identidad molecular de los canales capacitativos no se conoce, se ha propuesto que la familia de los genes *trp* codifican para este tipo de canales (Darszon *et al.*, 2001). Hasta la fecha, se ha demostrado la presencia de siete genes *trp* en las CE de ratón (Vannier *et al*, 1999; Treviño *et al.*,2001) Recientemente Jungnickel y colaboradores (2001) mostraron que al menos TRP2 está involucrado en la entrada de Ca^{2+} inducida por la ZP, lo cual constituye una evidencia más a favor de la participación de canales capacitativos en la RA.

Por otra parte, se ha propuesto una explicación alterna a la activación de los canales T por la ZP. Se sabe que la concentración intracelular de Cl^- es lo suficientemente alta como para inducir una depolarización de la membrana plasmática del espermatozoide cuando este canal

aniónico se abre, y de esta manera, inducir la RA (revisado en Darszon *et al.*, 2001). Reportes anteriores han demostrado que la RA inducida por ZP es sensible a antagonistas del canal de Cl^- receptor de glicina, y que la glicina induce la RA (Melendrez y Meizel, 1995; Melendrez y Meizel, 1996; Llanos *et al.*, 2001). Más aún, ratones que poseen un receptor de glicina mutado son incapaces de llevar a cabo la RA inducida por ZP (Sato *et al*, 2000).

Por último, la relevancia fisiológica de otros agonistas de la RA distintos de la ZP aún no esta demostrada. Uno de estos agonistas es la progesterona. Esta hormona induce un incremento en la $[Ca^{2+}]i$ y de $[Na^+]i$, un eflujo de Cl^- y la depolarización de la membrana plasmática (revisado en Darszon *et al.*, 2001)

1.1.3.- Fecundación en mamíferos

En esencia, cuando el óvulo y el espermatozide maduros se encuentran en el tracto genital femenino, la fecundación en mamíferos puede dividirse en cinco pasos los cuales se ejecutan en un orden estricto: 1.- La unión especie-específica entre el espermatozoide y el óvulo, 2.- La reacción acrosomal inducida por ZP3, 3.- La penetración de la zona pelúcida por el espermatozoide, 4.- La unión del espermatozoide a la membrana celular del óvulo homólogo, y 5.- La fusión de las membranas celulares del espermatozoide y el óvulo (Wassarman *et al*, 2001). A manera de resumen, una vez en el oviducto el encuentro del espermatozoide y el óvulo es un evento químicamente dirigido más que un fenómeno aleatorio. Existen ciertas evidencias que sugieren que el espermatozoide nada hacia el óvulo atraído por un quimioatractante emitido por las células del folículo que rodean al óvulo, por lo que podríamos hablar de un fenómeno de "quimiotaxis" durante la fecundación (revisado en Wassarman *et al*, 2001). En cada evento de fecundación, el espermatozoide capacitado debe unirse de manera especie-especifica a proteínas glicosiladas presentes en la gruesa capa externa del óvulo denominada zona pelúcida (ZP). La ZP

de los mamíferos está compuesta de tres glicoproteínas: la ZP1, ZP2 y ZP3. La ZP2 y la ZP3 forman filamentos que rodean al óvulo y la ZP1 sirve de conección entre la ZP2 y la ZP3 (Alberts *et al*., 1994). Una vez unido a la ZP, el espermatozoide reacciona a la ZP3 y se induce la RA. Es entonces cuando el espermatozoide penetra la capa externa de óvulo para alcanzar el espacio perivitelino localizado entre la ZP y la membrana celular del óvulo. Una vez en el espacio perivitelino, el espermatozoide debe unirse a la membrana del óvulo y fusionarse con este. La fusión de un solo espermatozoide al óvulo es suficiente para prevenir la fusión subsecuente de cualquier otro espermatozoide que haya penetrado la ZP (Yanagimachi, 1994), debido a la depolarización de la membrana del óvulo y a la reacción cortical (Alberts *et al*., 1994). La reacción cortical consiste en la liberación de las enzimas hidrolíticas de los gránulos corticales unidos a la cara interna de la membrana plasmática del óvulo, lo cual modifica la estructura de la ZP al cortar parcialmente a la ZP2 y a los carbohidratos de la ZP3. Este proceso evita la unión de otros espermatozoides a la ZP (Alberts *et al*., 1994). En este punto, el óvulo fecundado inicia su transformación a cigoto. Todo el proceso hasta aquí descrito se lleva a cabo en 90 minutos en condiciones de fecundación *in vitro* en el caso de óvulos de ratón (revisado en Wassarman *et al*., 2001). A continuación describiremos de manera breve cada uno de los cinco pasos de la fecundación antes mencionados.

La unión especie-específica entre el espermatozoide y el óvulo

Es un hecho bien documentado que la unión del espermatozoide al óvulo es un evento especie-específico (Yanagimachi, 1994; Wassarman *et al*., 2001). En general, cuando el espermatozide y el óvulo provienen de diferentes especies de mamífero la unión del espermatozoide a la ZP no ocurre en ensayos *in vitro*. Tal restricción se puede eliminar al

remover la ZP del óvulo (utilizando proteasas o con el uso de soluciones salinas de pH bajo), de tal forma que la interacción entre el espermatozoide y el óvulo de una especie distinta ocurra directamente en la membrana plasmática del óvulo. Un ejemplo de la interacción directa del espermatozoide al óvulo en ausencia de ZP es el ensayo utilizado en la fecundación clínica asistida (*in vitro*) en la cual, para determinar la capacidad de fecundación de un donante humano, se utilizan óvulos de hámster carentes de ZP ("hamster test") (Yanagimachi, 1984; revisado en Wassarman *et al.*, 2001). En condiciones control, los espermatozoides humanos no se unen a óvulos de hámster que posean una ZP intacta. Por lo tanto, resulta evidente que la cubierta extracelular del óvulo evita la unión de espermatozoides de especies distintas al mismo, y como consecuencia, la ZP sirve como una barrera para evitar la fecundación del óvulo por espermatozoides ajenos a la especie. Este hecho se cumple tanto en mamíferos como en el resto de los vertebrados.

La especie-especificidad de la unión entre el espermatozoide y el óvulo sugiere la posible existencia de elementos transmembranales o receptores que determinan esta característica. Se ha propuesto que el espermatozoide reconoce receptores específicos en el óvulo homólogo debido a que este posee proteínas específicas afines para dichos receptores. Sin embargo, existe la posibilidad de que especies evolutivamente cercanas compartan algunos motivos estructurales en los receptores de la ZP. Lo anterior podría explicar la unión entre los espermatozoides de ratón y los óvulos de hámster, o viceversa.

Para el caso del óvulo, dentro de los candidatos moleculares propuestos como responsables del reconocimiento especie-específico se han considerado a la ZP3, la cual es una de las tres glicoproteínas que forman a la ZP (Wassarman, 1990; Wassarman, 1999). Las bases que apoyan esta hipótesis se basan en el hecho de que la incubación de los espermatozoides con ZP3 purificada inhibe la unión del espermatozoide al óvulo homólogo. Más aún, las evidencias

experimentales indican que los oligosacáridos presentes en los residuos de serina localizados en el carboxilo terminal de la ZP3 son los responsables de la unión del espermatozoide (revisado en Wassarman *et al*, 2001). Consistente con esta posibilidad, ciertos oligosacáridos a concentraciones micromolares inhiben la unión del espermatozoide a la ZP en ensayos *in vitro*. Así, tal como ocurre en otros procesos de adhesión, la unión del espermatozoide y la ZP parece ser un evento mediado por carbohidratos. Otra evidencia más que apoya esta hipótesis es que la expresión de ZP3 humana (ZP3h) en óvulos de ratón ZP3$^{-/-}$ no basta para favorecer la unión de espermatozoides humanos (Rankin *et al*, 1998; Rankin y Dean, 2000). Sin embargo, los espermatozoides de ratón si se adhieren al óvulo de ratón que expresa ZP3h. Esto probablemente se debe a que el proceso de glicosilación de la ZP3h en el óvulo de ratón sigue el patrón propio del hospedero más que el correspondiente al patrón del óvulo humano (Wassarman *et al*., 2001).

Mucho más complejo ha resultado ser el estudio de las proteínas del espermatozoide involucradas en el reconocimiento de los receptores de la ZP. Hasta la fecha, se han propuesto, al menos, a 24 proteínas involucradas en este proceso (Wassarman *et al*, 2001). Dentro de este amplio grupo de candidatos, se encuentran algunas enzimas, tales como la β-galactosiltranferasa (Gong *et al*, 1995), la α-fucosiltransferasa, la proteína tirosina cinasa ZRK (Leyton *et al*, 1992) y la fosfolipasa A_2. También se han propuesto a algunas proteínas del tipo de las lectinas (proteínas de unión a manosa o a galactosa), a la zonadhesina o a la proteína-56, además de dos miembros de la familia de las metaloproteínas (ADAM), la β-fertilina del espermatozoide y la ceritestina (revisado en Wassarman *et al*., 2001). Otros candidatos propuestos son: una hialorunidasa (Gmachl y Kreil, 1993), proteínas similares a tripsina (Boettger-Tong *et al*, 1993) y las espermadhesinas (Gao y Garbers, 1998).

La explicación al porque de esta amplia gama de candidatos en este paso de la fecundación en mamíferos, se debe a que en ninguno de los casos anteriores se han encontrado

pruebas contundentes que demuestren que al menos uno de ellos es completamente indispensable para la unión especie-específica del espermatozoide a la ZP. Un caso distinto es el que se presenta para algunas especies de erizo de mar, en las cuales la manipulación de los alelos responsables de la bindina, una proteína del espermatozoide que participa en la unión al óvulo, afecta enormemente la especie-especificidad de la fecundación (revisado en Wassarman *et al* , 2001).

La reacción acrosomal inducida por ZP3.

Inmediatamente después de la unión del espermatozoide a la ZP, el espermatozoide inicia un proceso de exocitosis dependiente de Ca^{2+}, denominado reacción acrosomal (RA). Como se mencionó anteriormente, este proceso de exocitosis es consecuencia de la fusión, en múltiples puntos, de la membrana acrosomal y la membrana plasmática del espermatozoide, lo que origina la liberación del contenido acrosomal (para mayor detalle ver la sección 1.1.2 correspondiente a este punto).

La penetración de la zona pelúcida por el espermatozoide.

Después de la reacción acrosomal, el espermatozoide permanece unido a la ZP a través de la ZP2 (Yanagimachi, 1994; Wassarman *et al* , 2001). Como paso siguiente, el espermatozoide debe penetrar la ZP para alcanzar la membrana plasmática del óvulo y fusionarse a él. En este paso son de vital importancia la motilidad del espermatozoide (Yanagimachi, 1994; Nishigaki *et al* , 2001) y una activa hidrólisis enzimática, en la cual participa la acrosina y otras serin proteasas (revisado en Wassarman *et al* ., 2001)

La unión del espermatozoide a la membrana celular del óvulo homólogo.

Al igual que en el caso de la unión del espermatozoide a la ZP del óvulo homólogo, se han propuestos varios candidatos involucrados en la unión de las membranas celulares de ambos gametos. A la fecha, es ampliamente aceptado que el espermatozoide reaccionado se une a la membrana plasmática del óvulo en la región postacrosomal (Yanagimachi, 1994). Con respecto a las proteínas involucradas en esta interacción se han propuesto a tres proteínas de la familia ADAM presentes en el espermatozoide, llamadas fertilinaα, fertilinaβ y ciritestina. Como contraparte, en el óvulo se ha propuesto que el receptor α6β1 integrina es el responsable de esta unión (Wassarman *et al.*, 2001) Sin embargo, experimentos realizados con ratones que carecen de fertilinaα, β o ciritestina reducen la fertilidad en un 50% al afectar la unión del espermatozoide a la ZP o la migración del espermatozoide en el útero más que por una reducción de la unión de las membranas celulares de los gametos (Wassarman *et al.*, 2001).

La fusión de las membranas celulares del espermatozoide y el óvulo.

El último paso en la fecundación corresponde a la fusión de los gametos después de la unión de las membranas celulares. El resultado de varias investigaciones sugiere que la proteína CD9, miembro de la superfamilia de integrinas membranales del ovocito, tiene una función crucial en la fusión de ambas células. La falta de esta proteína ocasiona que las hembras presenten una capacidad reproductiva reducida (~21%), pero no afecta a los machos (Miyado *et al*, 2000; Wassarman *et al*, 2001).

1.2.- Canales de Ca^{2+} dependientes de voltaje.

Los canales de Ca^{2+} participan en varios procesos fisiológicos de distintos tipos celulares, entre los que se encuentran la espermatogénesis, la capacitación y la RA de los espermatozoides. Durante la espermatogénesis, ocurre un incremento en la $[Ca^{2+}]_i$ el cual se ha sugerido podría ser importante para la proliferación, diferenciación y maduración del espermatozoide (Santi *et al.*, 1996; Abou-Haila y Tulsiani, 2000). De manera similar, la capacitación involucra cambios en las concentraciones internas de diferentes iones, entre ellos el aumento de la $[Ca^{2+}]_i$ (Visconti *et al.*, 1999; Baldi *et al*, 2000; Flesch y Gadella, 2000). Además, al menos dos canales de Ca^{2+} diferentes participan en el aumento de la $[Ca^{2+}]_i$, el cual es esencial para la RA (Darszon *et al*, 1999; Publicover y Barrat; 1999; Florman *et al*, 1998). De lo anterior, es posible deducir la importancia que tienen los canales de Ca^{2+} en la fecundación. En el presente trabajo, nos enfocamos en el estudio de los canales de Ca^{2+} dependientes de voltaje tipo T debido a su relevancia en la fecundación

1.2.1.- Estructura y función de las subunidades de los canales de Ca^{2+} dependientes de voltaje

Los CCDV, al igual que el resto de los canales iónicos, son proteínas de membrana que, de manera general, están compuestas de una subunidad transmembranal denominada $\alpha 1$, una subunidad intracelular β, un complejo formado por las subunidades δ y $\alpha 2$, y en algunos casos, por una subunidad transmembranal γ (Liu y Campbell, 1998) (Fig. 5). La subunidad $\alpha 1$ es una polipéptido inusualmente grande (con una masa relativa de 190 a 250 KDa), responsable de formar el poro del canal, y esta constituida por dominios extracelulares (que pueden estar o no glicosilados), dominios intracelulares (que poseen sitios de regulación por diferentes moléculas) y dominios hidrofóbicos (intramembranales). Esta subunidad contiene al filtro de selectividad y

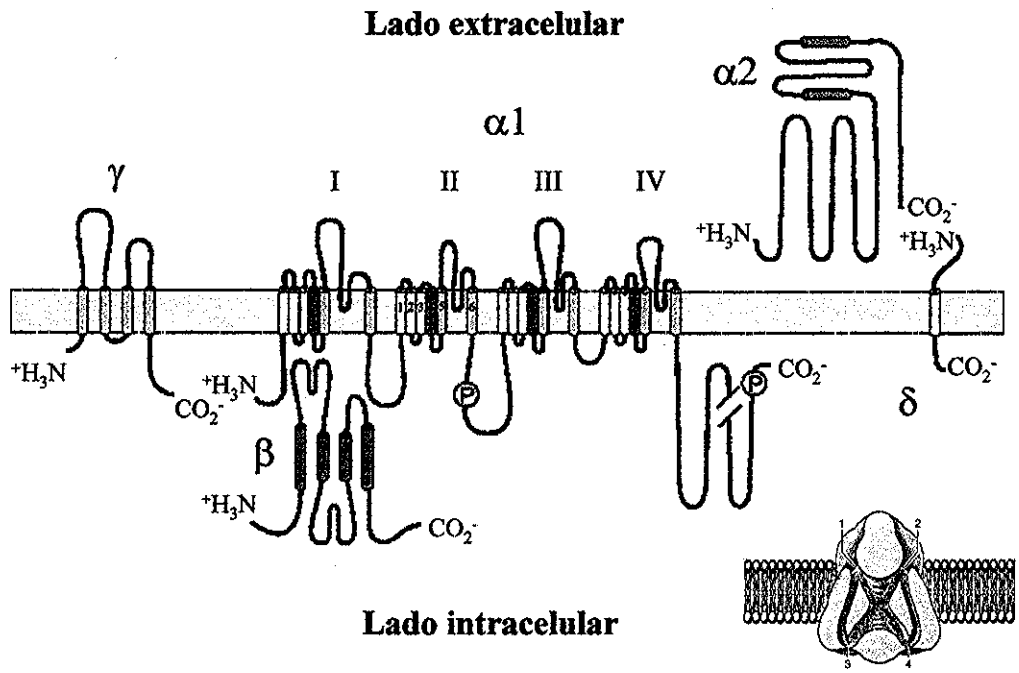

Figura 5.- Esquema de un canal de Ca^{2+} dependiente de voltaje. La subunidad α1 esta compuesta de cuatro dominios homólogos (I al IV), cada uno de los cuales contiene seis segmentos transmembranales (1 al 6). Los cilindros representan los segmentos transmembranales α-hélice. El cilindro rojo representa al segmento S4, el cual forma parte del sensor de voltaje. Los cilindros verdes representan a los segmentos S5 y S6, conectados por el asa P, los cuales forman las paredes del poro del canal. La subunidad β es citoplasmática, mientras que la subunidad γ es membranal. El complejo α2-δ se ancla a la membrana (representada en azúl) a través de la subunidad δ (modificado de Catterall, 2000). El inserto corresponde al modelo propuesto, basado en los datos cristalográficos de Sato *et al.* (2001), para la proteína α inserta en la membrana plasmática para canales de Na^+. Con base en la similitud estructural entre los canales de Na^+ y los de Ca^{2+}, Sato y colaboradores propusieron que la subunidad α1 de los canales de Ca^{2+} podría tener la misma estrucura tridimensional. La región en verde indica el poro conductor del canal (1-2), mientras que la región en rojo indica los poros (3-4) donde se localiza el segmento S4 del sensor de voltaje (modificado de Sato *et al.*, 2001).

los sitios de unión a los agonistas o antagonistas que estimulan o inhiben su actividad (Catterall, 2000). La secuencia de aminoácidos de la subunidad α1 está organizada en cuatro dominios repetidos (I al IV), donde cada dominio consta de seis segmentos transmembranales (S1 al S6) y un asa asociada a la membrana entre los segmentos S5 y S6, la cual forma el poro (Catterall, 2000; Fig. 5). Por otra parte, la subunidad β no posee segmentos transmembranales y está involucrada en el tráfico de la subunidad α1 hacia la membrana plasmática y en su modulación (Bichet *et al.*, 2000; Restituito *et al.*, 2000). En general, la subunidad β incrementa los niveles de

expresión de α1, modifica la voltaje dependencia de la activación y de la inactivación e incrementa la velocidad de la inactivación, aunque estos efectos dependen del tipo de subunidad α1 y β involucradas en la interacción (Catterall, 2000). La subunidad γ es una glicoproteína con cuatro segmentos transmembranales, mientras que el complejo α2-δ posee muchos sitios de glicosilación y varias secuencias hidrofóbicas. Se sabe que las subunidades α2-δ están codificadas por el mismo gen, y que las formas maduras de este complejo se producen por proteólisis post-traduccional y por la creación de puentes disulfuro (De Jongh *et al.*, 1990, Jay *et al*, 1991). Se sabe poco acerca de las implicaciones funcionales de la subunidad γ y del complejo α2-δ. Por ejemplo, la coexpresión de las subunidades α2-δ incrementa los niveles de expresión de la subunidad α1, de forma más discreta que la subunidad β, y le confiere propiedades de apertura normales (revisado en Catterall, 2000). Por otra parte, la subunidad γ modifica ligeramente la cinética de activación e inactivación de canales de Ca^{2+} de alto y bajo umbral de activación (Klugbauer *et al.*, 2000; Kang *et al.*, 2001).

1.2.2 - Clasificación funcional y molecular de corrientes de Ca^{2+} dependientes de voltaje, definidas por sus propiedades fisiológicas y farmacológicas

Clasificación funcional

En general, las corrientes de Ca^{2+} identificadas en las células de mamíferos se han clasificado en dos grandes grupos con base en su umbral de activación: las corrientes de Ca^{2+} de bajo umbral de activación (o LVA, por sus siglas en inglés), cuya activación es alrededor de –60 mV, y las corrientes de Ca^{2+} de alto umbral de activación (o HVA, por sus siglas en inglés) que se activan a partir de –30 mV. Las corrientes de Ca^{2+} HVA se subdividen en cuatro tipos denominados L, N, P/Q, y R (ver tabla 1). La corriente de Ca^{2+} tipo L tiene como características

un umbral de activación alto (alrededor de –30 mV), una inactivación lenta (completa en ~300-500 ms), una deactivación rápida, una conductancia unitaria aproximada de 20 pS y se bloquea con concentraciones de DHPs en el rango submicromolar. Este tipo de corriente se localiza en casi todos los tejidos excitables (neuronas, músculo liso y cardiaco, células endocrinas, etc.) y algunas células no excitables (Hille, 1992; Tabares y López-Barneo, 1996). La corriente de Ca^{2+} tipo N posee un umbral de activación alto, una inactivación completa en menos de 300 ms, una deactivación rápida y es sensible a la ω-conotoxina (fracción GVIA), SNX-482 y SNX-194. Este tipo de corriente de Ca^{2+} se expresa principalmente en el sistema nervioso central y periférico (Tabares y López-Barneo, 1996; Newcomb *et al*, 1998). Los canales de Ca^{2+} tipo P/Q son los canales HVA con inactivación más lenta (completa en más de 500 ms), poseen una deactivación rápida y son sensibles a la ω-agatoxina (fracción IVA) y a la ω-conotoxina (fracción MVIIC) (Newcomb *et al*, 1998). Las corrientes P/Q se expresan principalmente en el sistema nervioso central (Tabares y López-Barneo, 1996). Por otra parte, las corrientes de Ca^{2+} tipo R se caracterizan por ser resistentes a las DHPs, ω-conotoxina GVIA, ω-agatoxina IVA (Hille, 1992) y son sensibles a la SNX-482 y a la agatoxina IIIA (Newcomb *et al*., 1998). La corriente de Ca^{2+} tipo R se localiza principalmente en el sistema nervioso y cardiaco (Tabares y López-Barneo, 1996).

Canales de Ca^{2+} de bajo umbral de activación (LVA)

Por otra parte, la corriente de Ca^{2+} de bajo umbral de activación (LVA) posee, hasta la fecha, un solo subtipo de canales de Ca^{2+} dependientes de voltaje y se conoce como corriente de Ca^{2+} tipo T. Estos canales comúnmente se encuentran inactivados al potencial de reposo de las células, y se activan después de una hiperpolarización de la membrana (Kostyuk, 1999). A pesar de que los canales LVA no forma un grupo homogéneo, estos comparaten varias características biofísicas en

común. Como su nombre lo indica, la corriente de Ca^{2+} tipo T se activa alrededor de –60 mV a concentraciones fisiológicas de Ca^{2+} extracelular (figura 6). Sin embargo, aún a potenciales negativos puede observarse una substancial inactivación en estado estacionario (Kostyuk, 1999; Serrano *et al*., 1999). La activación de los canales de Ca^{2+} LVA es muy rápida y depende de voltaje. Esta característica es la responsable de que en una familia de corrientes macroscópicas, registradas con pulsos depolarizantes, se presente el patrón de entrecruzamiento característico de los canales T (revisado en Perez-Reyes *et al*., 1998). Los canales LVA poseen una inactivación rápida (completa en menos de 50 ms, por lo cual se le denomina transitoria o T) que en algunos tipos celulares puede contener dos componentes (Kostyuk, 1999). Una caraterística propia de los canales T es la diferencia entre las cinéticas de activación y de deactivación. La deactivación de los canales LVA es extremadamente lenta (de 1.9 a 6.5 ms a –80 mV), lo que las diferencia de los canales de alto umbral de activación (Hille, 1992; Tabares y López-Barneo, 1996; Kostyuk, 1999). Una de las características biofísicas más importantes es una conductacia unitaria baja (de 8 pS aproximadamente) (Hille, 1992; Tabares y López-Barneo, 1996). Dentro de sus propiedades farmacológicas, se ha descrito que esta corriente de Ca^{2+} es sensible a mibefradil (Clozel *et al*, 1997; Pitt, 1997), poliaminas de arginina (Scott *et al*., 1992) y a la kurtoxina (Chuang *et al*., 1994). Es importante resaltar que la kurtoxina es, hasta la fecha, la única toxina peptídica específica para canales de Ca^{2+} LVA (Chuang *et al*., 1994). Sin embargo, existe evidencias de canales de Ca^{2+} T sensibles a DHPs en el rango μM (Santi *et al*., 1996; Arnoult *et al*., 1996; Kostyuk, 1999). Mas aún, se sabe que el ácido niflúmico (AN) y otros bloqueadores de canales aniónicos pueden inhibir a CCDV (Walsh y Wang, 1996; Doughty *et al*., 1998) y a distintos tipos de canales catiónicos de manera inespecífica (Gögelein *et al*., 1990; Lerma y Martín del Rio, 1992).

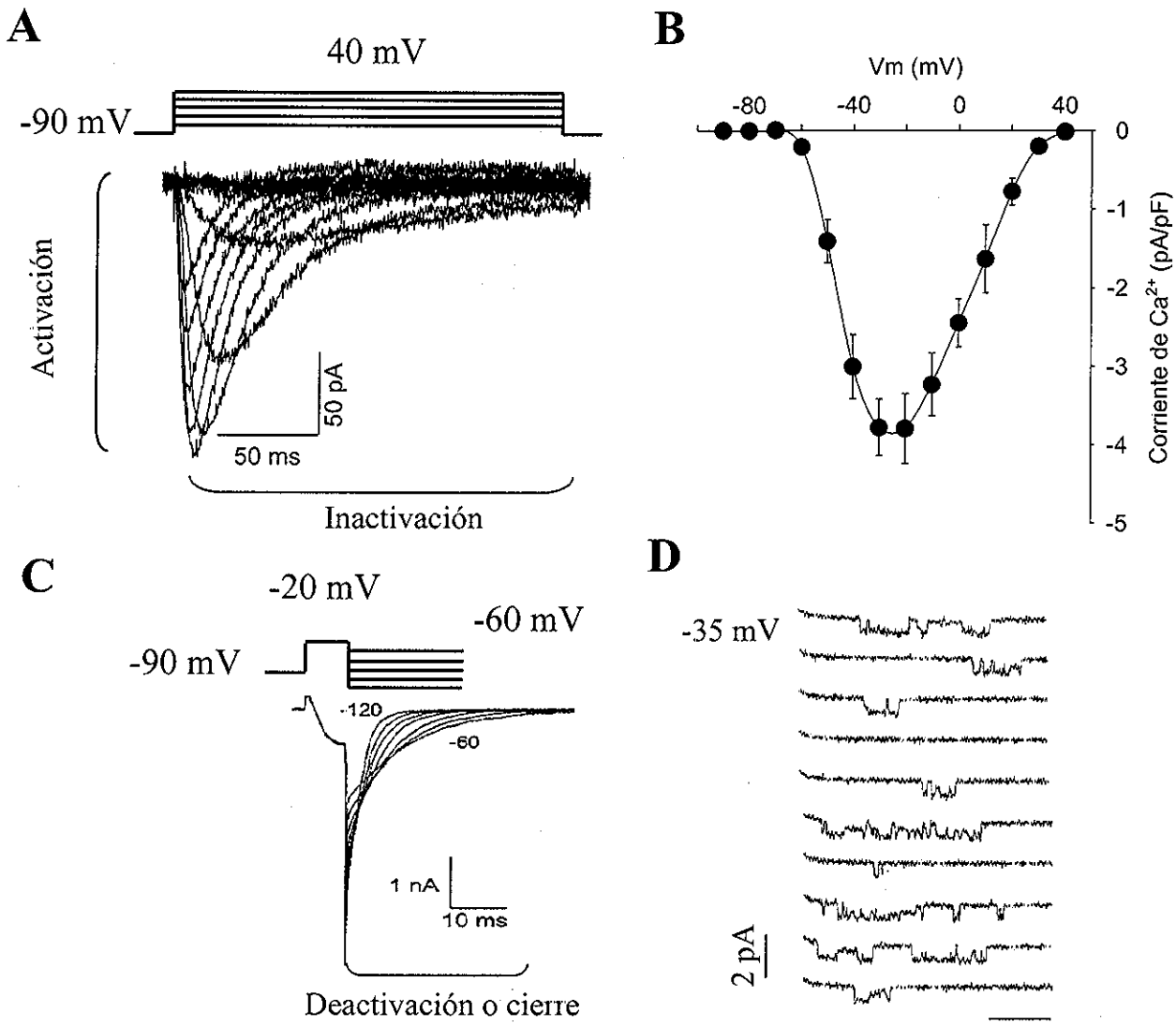

Figura 6.- Características biofísicas de una corriente de Ca^{2+} de bajo umbral de activación (LVA). A: Corrientes de Ca^{2+} tipo T nativas registradas en células espermatogénicas. La aplicación de un pulso depolarizante produce una corriente con activación e inactivación rápida (τ = 3 y 20 ms a-20 mV, respectivamente). El patrón de entrecruzamiento de las corrientes es una característica distintiva de los canales LVA (modificado de López-González *et al.*, 2000). **B:** Relación corriente-voltaje de los canales LVA, en donde se observa que la corriente se activa a voltajes cercanos al potencial de reposo (-60 mV) (modificado de López-González *et al*, 2000). **C:** La deactivación o cierre de los canales posterior a la aplicación de un pulso depolarizante corto (10 ms) es extremadamente lenta (τ= 6 ms a –80 mV) (Cribbs *et al*, 1998). **D:** Trazos correspondientes al registro de canal unitario LVA en células adrenales bovinas a –35 mV. El valor de conductancia unitaria es 9 pS. Escala de la barra de tiempo: 40 ms (Barrett *et al*, 2000).

Clasificación molecular

Como se mencionó anteriormente, los canales de Ca^{2+} de alto umbral de activación (HVA) están formados por cuatro subunidades: α_1, β, $\alpha_2\delta$ y γ. A la fecha, se han descrito siete genes que codifican para la subunidad $\alpha 1$ de los distintos canales de Ca^{2+} HVA ($Ca_v1.1$-1.4 y $Ca_v2.1$-2.3) (Walker y De Waard, 1998) la cual es responsable de la conducción de los iones, la sensibilidad al voltaje y de la unión específica de los distintos fármacos y toxinas (ver tabla 1). Con respecto a los canales de Ca^{2+} de bajo umbral de activación, la reciente clonación de tres nuevas subunidades α_1 ($Ca_v3.1$-3.3, ver tabla 1) por Perez-Reyes y colaboradores ha permitido empezar a dilucidar la estructura molecular de estos canales de Ca^{2+} (Perez-Reyes *et al.*, 1998; Cribbs *et al.*, 1998; Lambert *et al.*, 1998). De acuerdo con diferentes estudios, los canales LVA podrían estar integrados por una subunidad $\alpha 1$ y, al menos, una subunidad γ (Klugbauer *et al.*, 2000) sin la participación de las subunidades β clásicas (Perez-Reyes *et al.*, 1998; Leuranguer *et al.*, 1998).

1.2.3.- Identificación molecular de los canales de Ca^{2+} dependientes de voltaje en las células espermatogénicas y el espermatozoide de ratón

A pesar del importante papel de los canales de Ca^{2+} dependientes de voltaje en la fecundación, aún se desconoce la identidad molecular de las corrientes de Ca^{2+} del espermatozoide (Babcock y Pfeiffer, 1987; Florman *et al.*, 1992). El tamaño y la compleja naturaleza del espermatozoide han evitado la caracterización electrofisiológica directa de los canales de Ca^{2+} dependientes de voltaje expresados en su membrana plasmática. Por otra parte, la incapacidad de síntesis de proteínas, propia del espermatozoide, ha contribuido a dificultar la aplicación de las estrategias moleculares adecuadas para el estudio de los canales iónicos con técnicas de biología molecular. Por estas

Tabla 1.- Clasificación funcional y molecular de los canales de Ca^{2+} dependientes de voltaje.

	Nombre del canal	Tipo de corriente	Nombre de la subunidad	Inactivación (ms).	Deactivación.	Conductancia unitaria (pS)	Farmacología
Canales de Ca^{2+} de alto umbral de activación (HVA).	Cav1.1(α1.1)	L	α1S[1]	~ 300-500[1]	Rápida[1]	ND	DHP[1]
	Cav1.2(α1.2)	L	α1C[1]	~ 300-500[1]	Rápida[i]	22.7[13]	DHP[1]
	Cav1.3(α1.3)	L	α1D[1]	~ 300-500[1]	Rápida[i]	ND	DHP[i]
	Cav1.4(α1.4)	L	α1F[1]	~ 300-500[1]	Rápida[i]	ND	DHP[i]
	Cav2.1(α2.1)	P/Q	α1A[1]	> 500[i]	Rápida[i]	ND	ω-agatoxina (fracción IVA)[1], ω-conotoxina (MVIIC, IC_{50}= 300 nM)[16]
	Cav2.2(α2.2)	N	α1B[i]	< 300[i]	Rápida[i]	19.7[13]	ω-conotoxina (fracción GVIA)[1], SNX-482 (IC_{50}= 500 nM) y SNX-194 (IC_{50}= 1 nM)[16]
	Cav2.3(α2.3)	R	α1E[4-6]	19-28[8]	Lenta[8] (0.19 ms a 100 mV)[17]	12-14[13]	SNX-482 (IC_{50}= 30 nM) y AgatoxinaIIIA (IC_{50}= 3-10 nM)[16]
Canales de Ca^{2+} de bajo umbral de activación (LVA).	Cav3.1(α3.1)	T	α1G[2,15]	5-6 (a –30 mV)[2]	Lenta (2.5 ms a –80 mV)[2]	7.5[2]	Mibefradil (Ro 40-5967)[3,12], poliaminas de arginina (PA)[11] y kurtoxina[14]
	Cav3.2(α3.2)	T	α1H[9,10,15]	20 (a –30 mV)[9,10]	Lenta (~6.5 ms a –80 mV)[9,10]	5.3[9]	Mibefradil (IC_{50}= 1.4 μM)[9,10,12], PA (10 nM)[11] y kurtoxina[14]
	Cav3.3(α3.3)	T	α1I[9,10,15,17]	70 (a –30 mV)[17]	Lenta (1.9 ms a –80 mV)[17]	3.9-11[17]	Mibefradil (IC_{50}= 1.5 μM)[18]

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razones, los esfuerzos más recientes se han enfocado en la utilización de las células de la línea germinal de la cual se origina el espermatozoide Las CE conservan la capacidad de sintetizar proteínas, lo que las hace idóneas para la aplicación de estrategias de biología molecular, además de poseer una morfología más adecuada para realizar registros electrofisiológicos (Hagiwara y Kawa, 1984; Liévano *et al* , 1996; Arnoult *et al* , 1996a; 1996b; Santi *et al*., 1996).

Caracterización electrofisiológica y óptica de los canales de Ca^{2+} dependientes de voltaje en las células espermatogénicas y en el espermatozoide de mamífero.

Experimentos en células individuales cargadas con colorantes fluorescentes sensibles a Ca^{2+} indican que la ZP induce un incremento en la $[Ca^{2+}]i$ que precede a la exocitosis (Florman *et al* , 1989; Florman, 1994; Storey *et al*., 1992). Estos experimentos revelaron dos fases en el incremento de la $[Ca^{2+}]i$ inducido por la ZP3, consistentes con la participación de al menos dos tipos diferentes de canales de Ca^{2+} en la RA del espermatozoide de mamífero (Florman, 1994). La adición de ZP3 eleva transitoriamente la $[Ca^{2+}]i$ a nivel micromolar en un lapso de 40-50 ms, la cual se relaja al valor de reposo en 200 ms (Arnoult *et al*., 1999). La duración temporal y la farmacología de este transitorio de Ca^{2+} son consistentes con las propiedades de los canales de Ca^{2+} tipo T. Por otra parte, distintos estudios electrofisiológicos sugieren que el único tipo de corrientes de Ca^{2+} dependiente de voltaje presente en las células espermatogénicas de las fases tardías de la espermatogénesis de ratón es de tipo T (Liévano *et al* , 1996; Arnoult *et al*., 1996). Consistentemente, tanto la corriente de Ca^{2+} tipo T, como la RA y el incremento transitorio de Ca^{2+} intracelular se inhiben con concentraciones micromolares de dihidropiridinas (DHPs), pimozida y Ni^{2+} (Darszon *et al* , 2001). Sin embargo, a pesar de que el componente transitorio del incremento de la $[Ca^{2+}]i$ durante la RA inducida por ZP3 probablemente se deba a la activación de canales de Ca^{2+} tipo T (Darszon *et al* , 1999; Arnoult *et al*., 1998), aún queda por establecer la

identidad molecular definitiva del canal de Ca^{2+} (Benoff, 1998; Wennemuth *et al.*, 2000; Serrano *et al.*, 1999). Recientemente, la aplicación de estrategias fluorométricas ha sugerido la expresión funcional de otros tipos de canales de Ca^{2+} dependientes de voltaje en el espermatozoide maduro. Wennemuth y colaboradores (2000) reportaron la presencia de canales de Ca^{2+} tipo N y R ($Ca_v2.2$ y $Ca_v2.3$, respectivamente) que participan en la entrada de Ca^{2+} inducida por depolarizaciones. Sin embargo, no se ha demostrado que estos tipos de canales de Ca^{2+} dependientes de voltaje participen en la RA inducida por ZP. Más aún, el bloqueo de la expresión funcional de α1E ($Ca_v2.3$) no modifica las propiedades biofísicas, ampliamente reportadas, de la corriente de Ca^{2+} tipo T de las células espermatogénicas de ratón involucrada en la RA (Sakata *et al.*, 2001) por lo que la participación de los canales de Ca^{2+} tipo R en la RA aún está en duda.

Por último, durante la RA es necesaria la participación de otro canal para explicar el componente lento durante el incremento de la $[Ca^{2+}]_i$ de la RA. La elevación sostenida de la $[Ca^{2+}]_i$ inducida por ZP3 involucra la liberación de Ca^{2+} de pozas intracelulares sensibles a IP3 (Walensky y Snyder, 1995) y la subsecuente entrada de Ca^{2+} a través de canales operados por el vaciamiento de pozas internas (SOCs) presentes en la membrana plasmática (O'Toole et al, 2000; Jungnickel et al., 2001). De lo anterior podemos concluir que, hasta la fecha, la única corriente de Ca^{2+} dependiente de voltaje directamente implicada en la RA es la corriente de Ca^{2+} tipo T (ver tabla 2)

Transcritos para canales de Ca^{2+} dependientes de voltaje presentes en las células espermatogénicas de mamífero.

A pesar de que la principal corriente de Ca^{2+} registrada por métodos electrofisiológicos en las células espermatogénicas de rata y ratón pertenece al tipo T (LVA) (Hagiwara y Kawa; 1984; Liévano *et al*, 1996; Arnoult *et al*, 1996; Santi *et al.*, 1996; Arnoult *et al.*, 1997), se ha detectado

Tabla 2. Expresión de canales de Ca^{2+} en las células espermatogénicas y el espermatozoide de mamífero.

	Nombre del canal	Tipo de corriente	Nombre de la subunidad	RNAm	Proteína	Actividad funcional	RA
Canales de Ca^{2+} de alto umbral de activación (HVA).	Cav1.1(α1.1)	L	α1S	ND	ND	-	ND
	Cav1.2(α1.2)	L	α1C	+	+	-	ND[1-6]
	Cav1.3(α1.3)	L	α1D	-	-	-	ND
	Cav1.4(α1.4)	L	α1F	ND	ND	-	ND
	Cav2.1(α2.1)	P/Q	α1A	+	+	-	ND[7]
	Cav2.2(α2.2)	N	α1B	+	+	+	ND[5]
	Cav2.3(α2.3)	R	α1E	+	+	+	ND[5,7]
Canales de Ca^{2+} de bajo umbral de activación (LVA)	Cav3.1(α3.1)	T	α1G	+	+	+	+[2]
	Cav3.2(α3.2)	T	α1H	+	ND	+	+[2,8]
	Cav3.3(α3.3)	T	α1I	ND	ND	ND	ND

ND= no determinado
RA= evidencias de participación en la reacción acrosomal.
Actividad funcional= registrada por electrofisiología o técnicas fluorométricas.

1.- Goodwin *et al*., 1997. 2.- Espinosa *et al*., 1999. 3.- Goodwin *et al*., 1998. 4 - Westenbroek *et al*., 1999. 5.- Wennemuth *et al*., 2000. 6.- Serrano *et al*., 1999. 7 - Liévano *et al*., 1996. 8.- Son *et al*., 2000.

la presencia de RNA mensajeros para la subunidad α_1 de diferentes canales de Ca^{2+} dependientes de voltaje en estas células. Dentro de los transcritos identificados se encuentran los mensajeros para los canales de Ca^{2+} $Ca_v1.2$ (α1C) (Goodwin *et al*., 1997; Espinosa *et al*., 1999), $Ca_v2.1$ (α1A), $Ca_v2.2$ (α1B) y $Ca_v2.3$ (α1E) (Liévano *et al*., 1996), así como transcritos para $Ca_v3.1$ (α1G) y $Ca_v3.2$ (α1H) (Espinosa *et al*., 1999) (ver tabla 2). Las tres primeras subunidades α_1 codifican para canales de Ca^{2+} de alto umbral de activación (HVA) y las dos restantes codifican para canales de bajo umbral de activación (LVA) o tipo T. Más aún, análisis de RTPCR demostraron la expresión del mensajero de la subunidad $Ca_v3.2$ (α1H) en testículo humano (Son *et al*., 2000).

Inmunolocalización de los canales de Ca^{2+} dependientes de voltaje en las células espermatogénicas y el espermatozoide

Fue hasta fechas recientes que se obtuvieron las evidencias inmunocitoquímicas de la expresión de la proteína α_1. Las pruebas experimentales indican que cuatro subunidades α1 para canales de Ca^{2+} ($Ca_v1.2$, y $Ca_v2.1$-2.3) están presentes en el espermatozoide maduro y se distribuyen regionalmente (Goodwin *et al.*, 1998; Westenbroek y Babcock, 1999; Wennemuth *et al.*, 2000) Así, por ejemplo, $Ca_v2.2$ (α1B) se localiza en la cabeza, en la pieza media y en la parte proximal de la pieza principal del flagelo del espermatozoide (Wennemuth *et al*, 2000). Más aún, el uso de anticuerpos específicos ha permitido demostrar que las subunidades α1 para los canales $Ca_v1.2$ y $Ca_v2.1$ se expresan desde estadios previos al espermatozoide maduro, al igual que cuatro subunidades β de canales de Ca^{2+} (Serrano *et al.*, 1999). El resumen de la información correspondiente a la expresión de las proteínas de los canales de Ca^{2+} HVA aparece en la tabla 2. Desafortunadamente, hasta la fecha no se cuenta con anticuerpos de buena calidad contra las subunidades de $Ca_v3.1$ y $Ca_v3.2$ (α1G y α1H, respectivamente) de los canales de Ca^{2+} LVA presentes en las CE (Espinosa *et al.*, 1999), lo que ha impedido su inmunolocalización en el espermatozoide maduro. Sin embargo, se ha reportado la expresión de la proteína de $Ca_v3.1$ en células espermatogénicas de rata (Weiergräber *et al*, 2000).

II.- OBJETIVOS

2.1.- Objetivo general del proyecto:

El objetivo general de este proyecto fue estudiar la modulación y la farmacología de la corriente de Ca^{2+} tipo T en las células espermatogénicas de ratón, debido a su papel relevante en la fecundación durante la capacitación y la reacción acrosomal.

2.2.- Objetivos particulares:

Para cumplir con los objetivos generales, se plantearon los siguientes objetivos particulares:

2.2.1.- Modulación

A) Caracterizar electrofisiológicamente la modulación de la corriente de Ca^{2+} tipo T por albúmina (BSA), debido a que es un elemento clave en la capacitación del espermatozoide que induce un incremento de Ca^{2+} intracelular.

B) Estudiar la posible modulación de la corriente T por β-estradiol, ya que existen evidencias que sugieren la participación de este compuesto en la regulación de canales T en otros tejidos.

C) Determinar si la regulación de la corriente T por BSA se debía a su capacidad de extraer colesterol de la membrana plasmática como se sabe ocurre durante la capacitación.

D) Evaluar, preliminarmente, si la BSA y el β-estradiol modulan a la corriente de Ca^{2+} tipo T a través de los mismos mecanismos moleculares.

E) Explorar si la calmodulina (CaM) modula a la corriente de Ca^{2+} de bajo umbral de activación en las células espermatogénicas, utilizando inhibidores específicos para la CaM. Este objetivo se basa en las evidencias experimentales que sugieren la participación de la CaM en el influjo de Ca^{2+} observado durante la RA.

F) Determinar si la CaM participa en el incremento transitorio de Ca^{2+} y en la RA inducidos por la ZP en el espermatozoide maduro.

G) Evaluar, preliminarmente, si la posible modulación de la corriente T por CaM involucraba a cinasas o fosfatasas dependientes de CaM.

H) Investigar si la corriente T de las células espermatogénicas se modula por progesterona o por ácido γ-aminobutírico (GABA), ya que reportes previos indican que estos compuestos pueden inducir un influjo de Ca^{2+} en el espermatozoide y la RA.

2.2.2.- Estudios farmacológicos

A) Estudiar si la corriente de Ca^{2+} tipo T es sensible a bloqueadores de canales aniónicos tales como el ácido niflúmico (AN) y el ácido 5-nitro-2(3-fenilpropilamino) benzóico (NPPB), debido a que estos inhibidores pueden afectar a distintos tipos de canales catiónicos.

B) Comparar la dependencia de la concentración de los bloqueadores aniónicos sobre la inhibición de la RA y sobre la corriente T, para determinar si el bloqueo de la RA se debe al efecto de los fármacos antes mencionados sobre los canales de Ca^{2+} tipo T o sobre los canales aniónicos presentes en el espermatozoide.

C) Identificar toxinas de alacrán con alta afinidad para la corriente de Ca^{2+} tipo T de las células espermatogénicas, tomando como base la secuencia de la kurtoxina.

D) Caracterizar electrofisiológicamente el efecto de las posibles toxinas sobre la corriente T.

E) Evaluar el efecto de dichas toxinas en la RA inducida por la ZP.

(16.7±3.5% de la corriente). De la misma forma, este componente de la corriente de Ca^{2+} es insensible a una combinación de Ni^{2+} complementada con albúmina (20.6±2.3% de la corriente, Fig. 30B), un compuesto que incrementa a la actividad del canal de Ca^{2+} tipo T en las células espermatogénicas (Espinosa *et al*, 2000). En conjunto, estos resultados descartan la participación de TRP sensibles a 1 mM de Ni^{2+} (O'Toole *et al*., 2000), de canales de Ca^{2+} HVA sensibles a 20 μM de Cd^{2+} (Hille, 1992) o de canales de Ca^{2+} LVA sensibles a KLI (200 nM) o BSA (0.5%) (este trabajo). Así, nuestros resultados sugieren que este componente de la corriente de Ca^{2+} corresponde a un tipo de canal distinto a los reportados previamente en las células espermatogénicas.

V.- DISCUSIÓN.

5.1.- El posible papel fisiológico de la regulación dual de la corriente de Ca^{2+} tipo T por albúmina y β-estradiol durante la capacitación.

El espermatozoide maduro posee una sola vesícula secretoria (el acrosoma) por lo que la exocitosis debe coordinarse en respuesta al contacto con el óvulo homólogo para favorecer una fecundación eficiente. Previo a la fecundación, el espermatozoide debe adquirir su capacidad de fecundar al óvulo a través de un proceso de maduración (la capacitación). En este proceso, la albúmina juega un papel determinante al inducir un incremento en el influjo de Ca^{2+} y bicarbonato, los cuales son indispensables para la capacitación (Shi y Roldán, 1995; Visconti *et al.*, 1995; Visconti y Kopf, 1998). Sin embargo, hasta el presente trabajo no se había probado directamente la posibilidad de que la albúmina (BSA) pudiera modular a la corriente de Ca^{2+} tipo T presente en el espermatozoide y/o en las células espermatogénicas. Resultados previos en nuestro laboratorio demostraron que la BSA (0.5%) induce un incremento en la amplitud de la corriente de Ca^{2+} tipo T de manera dependiente de la concentración (ver apéndice; Espinosa *et al.*, 2000). Estudiando con más detalle este fenómeno, los resultados del presente trabajo muestran que el efecto de la BSA es dependiente de voltaje (Fig. 7A), y que además acelera la cinética de activación de la corriente T (Fig. 7B) ocasionando una disminución en el valor de la constante de tiempo de activación. De la misma forma, la aplicación de BSA favorece que la corriente T se active a potenciales más negativos y que se inactive a potenciales más positivos (Fig. 7C). El corrimiento en ambas curvas en estado estacionario (activación e inactivación), podría ocasionar un incremento en la corriente ventana en presencia de la BSA. Así, en el pico de la ventana (~ -52 mV) el 28 y el 44% de los canales estan disponibles para abrirse en ausencia (control) y en presencia de BSA, respectivamente

(Fig 7C). Es importante destacar que el incremento en la corriente ventana podría tener relevancia en la fisiología del espermatozoide, en particular durante el inicio de la capacitación. Existe evidencia que apunta a que el canal T está presente en el espermatozoide maduro (Arnoult *et al*., 1999) donde el potencial de reposo es cercano a -50 mV (Espinosa y Darszon, 1995). Se sabe que la membrana del espermatozoide alcanza el punto máximo de hiperpolarización (~-70 mV) a los 45 minutos después de iniciada la capacitación inducida por ciclodextrinas (Dr. Pablo Visconti, comunicación personal). Considerando lo anterior, es posible que los canales de Ca^{2+} T participen en el influjo de Ca^{2+} inducido por la BSA durante los primeros 30 minutos, el cual es un prerrequisito para la capacitación. Los resultados sugieren que los canales de Ca^{2+} tipo T pudieran contribuir al incremento de la $[Ca^{2+}]_i$ durante la capacitación, debido al incremento del número de los canales disponibles para abrirse al potencial de reposo en el espermatozoide no capacitado (~ -50 mV), lo que ocasionaría el incremento del influjo de Ca^{2+}.

Es posible que el efecto de la BSA sobre la corriente T resulte de la acción de remover alguna molécula hidrofóbica presente en la membrana plasmática del espermatozoide, la cual pudiera modular la ICa_T y así evitar una exocitosis prematura. Más aún, el efecto de la BSA en la ICa_T de las CE es irrevesible y depende de Ca^{2+} externo (Espinosa, 1999a), lo cual es consistente con la propuesta arriba mencionada. Así, en el momento en que la capacitación se lleva a cabo y el espermatozoide esta listo para viajar al sitio donde ocurre la fecundación, la albúmina removería esta molécula hidrofóbica lo que permitiría que los canales T quedaran libres de esta inhibición y se incrementara la $[Ca^{2+}]_i$ en el espermatozoide. Inesperadamente, los datos indican que la BSA no está modulando a la ICa_T a través de su capacidad de quitar colesterol de la membrana celular, como lo muestran los experimentos con BSA preincubada con una concentración saturante de un

análogo del colesterol (sulfato de colesterol; CSO_4) y con 2-OH-β-ciclodextrina (2-OH-β-CD) (Fig. 8B).

Otra posible explicación para el incremento de la ICa_T inducido por la BSA se basa en la capacidad antioxidante de la BSA (Guermonprez *et al.*, 2001). Se sabe que las cadenas laterales de la cisteína, tirosina y/o histidina de la albúmina pueden actuar como reductores potenciales (Ivanov *et al.*, 2000). Por otra parte, recientemente se describió la modulación de canales de Ca^{2+} LVA por agentes oxidantes (DTNB, oxido nitroso) o reductores (DTT, L-cisteína) en nociceptores de rata (Todorovic *et al*, 2001a) y en canales recombinantes (Todorovic *et al*, 2001b). De hecho, los agentes reductores incrementan la amplitud de la ICa_T en los nociceptores (Todorovic *et al.*, 2001a). Considerando los trabajos arriba mencionados, nuestros resultados no nos permiten descartar totalmente la posibilidad de un efecto directo de la BSA en la corriente T de las CE. Sin embargo, el hecho de que el efecto de la BSA en la ICa_T sea irreversible sugiere que la modulación de la corriente es indirecta.

Con la idea de explorar que molécula hidrofóbica pudiera estar inhibiendo a la corriente T, estudiamos el efecto del 17-β-estradiol en las CE basados en los reportes previos sobre la modulación de canales T por estradiol (Mermelstein *et al*, 1996; Ogata *et al*, 1996; Zhang *et al.*, 1994; Nakajima *et al.*, 1995; Yamamoto *et al.*, 1995; Kitazawa *et al*, 1997; Ruehlmann *et al*, 1998), y considerando que el 17-β-estradiol está presente en la membrana del espermatozoide (Martínez y Morros, 1985). La BSA presaturada con 17-β-estradiol no indujo el incremento de la corriente al pico sino que produjo una discreta disminución de la amplitud de corriente la cual se anuló al agregar BSA por segunda vez (Fig. 9D). Este hecho es consistente con la hipótesis que sugiere que el incremento de la

ICa_T inducido por la BSA pudiera deberse a la disminución de la concentración de 17-β-estradiol de la membrana celular más que de colesterol. Sin embargo, es importante resaltar que el efecto de la BSA sobre la corriente T es dependiente de voltaje (Fig. 7A) mientras que el efecto del estradiol no lo es (Fig. 9B). Esta diferencia en la dependencia de voltaje indica que el efecto de cada uno de estos compuestos es independiente uno del otro, por lo que aún persiste la interrogante acerca del compuesto hidrofóbico que estaría inhibiendo tónicamente a los canales T antes de la capacitación y que sería el blanco de la albúmina. Un posible candidato como inhibidor de los canales T de las células espermatogénicas es el ácido araquidónico (AA). Estudios recientes demostraron que el AA modula a la subunidad α1H (Ca_V3 2) (Zhang *et al.*, 2000), la cual codifica para un canal de Ca^{2+} tipo T (Cribbs *et al.*, 1998). En este estudio se demostró que el AA directamente induce una lenta atenuación de la corriente de α1H por la disminución de la corriente ventana (Zhang *et al.*, 2000). Tal como se mencionó en la introducción, parte de nuestro trabajo demostró que α1H es una de las dos subunidades que codifican para canales de Ca^{2+} de bajo umbral de activación presentes en las CE (ver apéndice Espinosa *et al*, 1998). Más aún, resulta interesante saber que varios trabajos han aportado evidencias que sugieren la participación del AA en la RA (Domínguez *et al*., 1999; Sistina y Rodger, 1997).

Por otra parte, el mecanismo mediante el cual el 17-β-estradiol reduce a la corriente de Ca^{2+} requiere de un estudio más detallado. Como se mencionó, estudios previos han demostrado que la aplicación aguda de β-estradiol inhibe a los canales de Ca^{2+} tipo T en las células del músculo liso (Mermelstein *et al.*, 1996), en neuronas (Ogata *et al*, 1996), y en miocitos cardiacos o lisos (Zhang *et al*, 1994; Nakajima *et al*, 1995; Yamamoto *et al.*, 1995; Kitazawa *et al.*, 1997; Ruehlmann *et al.*, 1998). Sin embargo, a diferencia de lo

reportado para el caso de la inhibición de canales de Ca^{2+} de alto umbral (HVA) (Ogata *et al*, 1996; Mermelstein *et al*., 1996), esta inhibición no involucraría la participación de proteínas G, ya que la corriente de Ca^{2+} tipo T de las CE es básicamente independiente de la modulación por dichas proteínas (Arnoult *et al*., 1997) Nuestros datos indican que el efecto del 17-β-estradiol es específico ya que el uso de un isómero hormonalmente inactivo, el 17-α-estradiol, causó una menor reducción en la respuesta del canal T (Fig 9C) Resulta interesante que las observaciones de diferentes estudios sugieren que la presencia de diferentes compuestos hidrofóbicos pueden afectar las propiedades físicas de la membrana a través de la alteración de las interacciones entre las proteínas de membrana y la bicapa lipídica (Andersen *et al*., 1999). Los resultados presentados en esta sección muestran un efecto diferencial entre el 17-β-estradiol y el 17-α-estradiol en la corriente T, lo cual es inconsistente con un efecto basado en la hidrofobicidad de ambas moléculas. Considerando que el 17-β-estradiol está presente en la membrana del espermatozoide (Martínez y Morros, 1985), queda abierta la pregunta de una posible interacción directa entre esta hormona y alguna subunidad accesoria de los canales de Ca^{2+} T presentes en la membrana de las CE que pudiera modular a la ICa_T, tal como ocurre con canales de K^+ (Valverde *et al*., 1999) Por último, recientemente se demostró que la aplicación de 17-β-estradiol puede inducir un incremento en la $[Ca^{2+}]_i$ por la activación de un receptor funcional de estrógenos que se expresa en la superficie de la membrana celular del espermatozoide humano (Luconi *et al*, 1999) Los resultados mostrados en esta parte de esta tesis sugieren que en este incremento de la $[Ca^{2+}]_i$ no participan los canales de Ca^{2+} tipo T. Por otra parte, estudios recientes demostraron que el 17-β-estradiol induce un incremento en la concentración intracelular de oxido nítrico a través de un receptor de

estrógenos tipo β (Zhu *et al*., 2002). Resulta interesante que el oxido nitroso aplicado al medio externo bloquea la corriente de Ca^{2+} tipo T de la subunidad $Ca_v3.2$ (α1H) expresada heterólogamente (Todorovic *et al*., 2001). Este podría ser el mecanismo mediante el cual el 17-β-estradiol inhibe la corriente T de las CE.

5.2.- La corriente de Ca^{2+} tipo T de las células espermatogénicas podría estar modulada por calmodulina.

Previo a la fecundación, el espermatozoide de los mamíferos debe llevar a cabo la reacción acrosomal (RA). Aún en la actualidad, se desconocen varios de los pasos involucrados en la transducción de señales que conducen a la RA del espermatozoide maduro. Este evento de exocitosis inducido por la zona pelúcida (ZP) que rodea al óvulo homólogo, depende de la entrada de Ca^{2+} externo (Wassarman, 1999). La evidencia experimental sugiere que los canales de Ca^{2+} dependientes de voltaje son una vía importante en la regulación de la entrada de Ca^{2+} al espermatozoide durante este proceso (Darszon *et al*., 1999; Arnoult *et al*., 1996a; Arnoult *et al*., 1999). El trabajo de diferentes grupos ha demostrado que las CE expresan funcionalmente canales de Ca^{2+} tipo T (Hagiwara y Kawa, 1984; Liévano *et al*., 1996; Arnoult *et al*., 1996a; 1996b; Santi *et al*., 1996) con características farmacológicas consistentes con las reportadas para la RA y el influjo de Ca^{2+} asociado a la misma (Arnoult *et al*., 1998). De la misma forma, se ha demostrado que el canal T está presente en el espermatozoide maduro (Arnoult *et al*., 1999) y por lo tanto, este canal debe ser un elemento importante en la cascada de señales que participan en la RA inducida por la ZP en el espermatozoide de mamífero (Arnoult *et al*., 1996a; Arnoult *et al*., 1999; Darszon *et al*., 1999).

A pesar de que la corriente de Ca^{2+} tipo T de las células espermatogénicas se ha caracterizado detalladamente en trabajos previos, se sabe poco sobre su regulación. Dicha información es necesaria para profundizar en el estudio de los mecanismos moleculares que conducen a la RA del espermatozoide. Trabajos recientes indican que la regulación de canales de Ca^{2+} de alto umbral de activación (HVA) por calmodulina (CaM) podría contribuir en la determinación de los niveles de Ca^{2+} intracelular (Peterson *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). Curiosamente, observaciones previas obtenidas al usar inhibidores de CaM en el espermatozoide de erizo de mar (Sano, 1983; Guerrero y Darszon, 1989) y en mamífero (Courtot *et al.*, 1999) sugieren la participación de CaM en la RA. Con esto en mente y considerando las ventajas del modelo biológico con el que contamos, se decidió explorar la posible regulación por CaM de los canales de Ca^{2+} tipo T presentes en las células espermatogénicas. Los resultados presentados en este trabajo muestran que el W7, un antagonista específico para CaM, reduce marcadamente la magnitud de la corriente T a concentraciones mayores a 2 μM (Figs. 10 a 12). Este efecto depende de la concentración y fue esencialmente independiente de voltaje (Fig. 10C y D). La TFP, otro antagonista de CaM, también redujo significativamente la amplitud de la corriente (Fig. 11A) a concentraciones que inhiben a la CaM (Massom *et al.*, 1990). Más aún, la adición de CaM en la solución interna de registro redujo el efecto inhibitorio del W7, sugiriendo una potencial participación de esta proteína en la regulación del canal de Ca^{2+} tipo T (Fig. 11B). Nuestros resultados son consistentes con trabajos recientes que demuestran que CaM modula a canales de Ca^{2+} de bajo umbral de activación en células nativas (Barrett *et al.*, 2000) o expresados ($Ca_V3.2$ o α1H) en sistemas heterólogos (Wolfe *et al.*, 2002). Sin embargo, en los dos casos arriba citados la regulación de los canales de

Ca^{2+} de bajo umbral de activación por CaM es a través de la protein cinasa II dependiente de CaM.

Los resultados presentados en este estudio sugieren la participación de la CaM en la regulación del canal de Ca^{2+} tipo T de las células espermatogénicas, pero no son una demostración definitiva. El W7, el W5 y la TFP pueden tener otros efectos menos específicos tales como una inhibición directa de canales iónicos membranales o intracelulares, o de la fosfolipasa C (Ehrlich *et al.*, 1988; Schlatterer y Schaloske, 1996; Scholf *et al.*, 1999). Sin embargo, las concentraciones inhibitorias usadas en este trabajo concuerdan con las descritas para la inhibición de la CaM y son menores a las asociadas a efectos menos específicos de estos compuestos (Ichikawa *et al.*, 1991). Además, la dependencia parcial de Ca^{2+} del efecto inhibitorio del W7 apoya la idea de la regulación de la corriente T por el complejo Ca^{2+}/CaM (Fig. 11C). Más aún, los efectos directos de los inhibidores de la CaM sobre distintos tipos de canales iónicos son parcial o completamente reversibles (Laver *et al.*, 1997), mientras que los efectos de estos compuestos en nuestros experimentos fueron irreversibles (datos no mostrados), lo que nos sugiere un efecto indirecto sobre los canales. Finalmente, cuando el W7 y la TFP actúan bloqueando directamente a un canal iónico, la adición de CaM a la solución interna es incapaz de inhibir dicho bloqueo (Kleene, 1994), a diferencia de lo observado en nuestros experimentos. En conjunto, estas consideraciones nos permiten proponer que el W7 y la TFP afectan a la corriente de Ca^{2+} por inhibir la función de la CaM.

Por otra parte, se ha sugerido que la inactivación de los canales de Ca^{2+} HVA implica la participación del Ca^{2+} que entra a través del poro del canal, la unión del ion a la CaM y el cambio de conformación de la misma. El complejo Ca^{2+}/CaM interactúa con el canal iniciando un proceso intramolecular que culmina en una inactivación más rápida del

canal de Ca^{2+} (Zühlke y Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). De acuerdo con lo anterior, la aplicación aguda del antagonista de CaM, el W7, resultó en un enlentecimiento parcial de la inactivation de la ICa_T en las células espermatogénicas (Fig. 13A, B, C y D). Sin embargo, este efecto de los antagonistas de CaM sobre la inactivación contrastan con la disminución observada de la amplitud de la corriente T en las células espermatogénicas. En el presente trabajo, se muestra que, además de la reducción de la amplitud de la corriente, la aplicación de distintas concentraciones de W7 cambia drásticamente otras propiedades biofísicas de la corriente. Estos cambios incluyen un corrimiento de la voltaje dependencia de la activación a valores positivos y un enlentecimiento significativo de la cinética de activación (Figs. 12 y 14). Considerando que la activación y la inactivación están estrechamente acopladas en los canales T (Serrano *et al.*, 1999), los resultados sugieren la reducción en la constante de tiempo de la activación en presencia del W7 podría indirectamente enlentecer el curso temporal aparente de la inactivación de la corriente T. Además de los efectos antes descritos, el W7 parece promover la inactivación a voltajes en los cuales los canales T no están activos (Fig. 14A y B). Sin embargo, no está claro si la CaM participa en este efecto.

En resumen, el presente estudio muestra que los antagonistas de CaM, como el W7 y la TFP, inhiben con una potencia similar a la corriente T de las CE monitoreada por métodos electrofisiológicos, y a la RA inducida por la ZP. Notablemente, una concentración similar de W7 (10 μM) disminuyó el influjo transitorio de Ca^{2+} disparado por la ZP en el espermatozoide maduro registrado con un fluoróforo sensible a Ca^{2+} (Fig. 15A). En contraste, el W5, un análogo del W7, antagonista de la CaM pero mucho menos potente, tuvo un efecto mucho menor sobre la corriente T de las CE (Fig. 11A), sobre la RA

y en el influjo transitorio de Ca^{2+} asociado a esta (Fig. 15A y B). Más aún, la sustitución del Ca^{2+} externo por Ba^{2+} (Fig. 11C) o la adición de CaM a la solución interna de registro redujo la inhibición de la corriente T de las CE causada por el W7 (Fig. 11B). En conjunto, estos resultados son consistentes con un vínculo directo entre la activación del canal de Ca^{2+} T y la RA inducida por la ZP, y sugiere que la CaM podría regular al canal T del espermatozoide maduro. En el espermatozoide maduro esto es particularmente importante porque durante la RA ocurre una delicada redistribución y modificaciones de las moléculas presentes en la membrana plasmática (Wassarman, 1999; Flesch y Gadella, 2000; Darszon *et al.*, 2001). Por ejemplo, recientemente se demostró que después de la RA se redistribuyen las microdominios de membrana (*rafts*) que contienen GM1, un gangliosido presente en un tipo de microdominios membranales (*rafts*) (Treviño *et al*, 2001). Este fenómeno de reorganización membranal también es importante durante la capacitación, donde el uso de inhibidores de la CaM, como el W7 y el calmidazolio, inhiben significativamente el porcentaje de espermatozoides capacitados y su capacidad de llevar a cabo la RA inducida por lisofosfatidilcolina (Si y Olds-Clarke, 2000).

5.3.- La corriente de Ca^{2+} tipo T no participa en el influjo de Ca^{2+} inducido por progesterona o ácido γ-aminobutírico (GABA) en mamífero.

En todas las especies animales estudiadas hasta la fecha, la inducción de la RA por los agonistas fisiológicos implica un influjo de Ca^{2+} extracelular. En el erizo de mar, los polímeros de fucosa sulfatada provocan un influjo bifásico de Ca^{2+}, en el cual participan dos tipos de canales iónicos. La primera fase es sensible a dihidropiridinas (DHPs) y verapamil, antagonistas de canales de Ca^{2+} tipo L (Ca_V1) dependientes de voltaje. La segunda fase, la cual se activa 5 segundos después de la primera, es insensible a bloqueadores de canales de Ca^{2+} dependientes de voltaje pero es sensible a Ni^{2+} (IC_{50} = 10

μM) (Guerrero y Darszon, 1989; Schackman, 1989; González-Martínez *et al.*, 2001). En mamíferos, la inducción de la RA por la ZP también activa dos vías de entrada de Ca^{2+}. La primera entrada corresponde a la activación del canal de Ca^{2+} tipo T sensible a DHPs, y cuyo bloqueo inhibe el incremento de la concentración intracelular de Ca^{2+} ($[Ca^{2+}]_i$) y a la RA (Arnoult *et al*, 1996; Darszon *et al*, 2001). Posterior a la activación del canal T, ocurre un incremento en la $[Ca^{2+}]_i$ propiciado por la participación de un canal de Ca^{2+} de tipo capacitativo, que probablemente este formado por la combinación de TRP2 y otros TRPs (Santi *et al.*, 1999; O'Toole *et al.*, 2000; Jungnickel *et al.*, 2001).

Varios reportes han demostrado que la progesterona induce un influjo transitorio de Ca^{2+} y a la RA en el espermatozoide humano (Aitken *et al.*, 1996; Baldi *et al.*, 1991; Blackmore *et al.*, 1990; Meizel *et al.*, 1997; Plant *et al.*, 1995), probablemente a través de la activación de un receptor membranal de progesterona (Blackmore *et al.*, 1991; Meizel y Turner, 1991; Sabeur *et al.*, 1996; Tesarik *et al*, 1992; Benoff *et al*, 1995; Luconi *et al*, 1998). Debido a que la progesterona induce ambos procesos a concentraciones en el orden micromolar, similar a la concentración de esta hormona en el *cumulus oophorus* (Osman *et al*, 1989), se ha propuesto que la progesterona pudiera tener un papel importante durante la fecundación *in vivo* (Fisher *et al*, 1998; Garcia y Meizel, 1999)

Como se mencionó anteriormente, se ha demostrado que el influjo de Ca^{2+} a través de canales dependientes de voltaje es necesario para la RA inducida por la ZP (Florman *et al*, 1992; Florman, 1994; Arnoult *et al.*, 1996a; Arnoult *et al*, 1996b). Específicamente, las evidencias electrofisiológicas sugieren que, al menos, el canal de Ca^{2+} tipo T está involucrado en la RA (Santi *et al.*, 1996; Arnoult *et al*, 1996a; Arnoult *et al.*, 1999). Con

base en los datos anteriores, resultaba interesante explorar si la corriente de Ca^{2+} tipo T estaba involucrada en la RA inducida con progesterona o GABA.

Los resultados sugieren que la aplicación de 50 μM de progesterona o 125 μM de ácido γ-aminobutírico (GABA) no modifican las características biofísicas fundamentales de la corriente de Ca^{2+} tipo T tales como la amplitud de la corriente, la cinética de activación o de inactivación (Figs. 18 y 19). De estos resultados podemos concluir que la corriente de Ca^{2+} tipo T no participa en el influjo de Ca^{2+} ni en la RA inducida por la progesterona o el GABA. Esta es, a nuestro conocimiento, la primera evidencia directa de que la progesterona o el GABA no modulan a la corriente T de las células espermatogénicas ya que las condiciones experimentales utilizadas en trabajos previos dificultan una evaluación apropiada (García y Meizel, 1999; Kirkman-Brown *et al.*, 2000). Sin embargo, considerando que las CE se encuentran en un proceso de diferenciación (Bellvé, 1993), existe la posibilidad de que la cascada de segundos mensajeros involucrados en la modulación de la corriente de Ca^{2+} T por progesterona o GABA aún no estuviera funcional a diferencia de la célula madura. Por otra parte, estudios recientes sugieren que el canal de Ca^{2+} tipo T tiene una mínima participación en el influjo de Ca^{2+} y en la RA inducida por progesterona en el espermatozoide maduro, ya que se necesitan concentraciones altas de mibefradil para inhibir ambos procesos (García y Meizel, 1999; Bonaccorsi *et al.*, 2001). Más aún, consistente con los resultados de este trabajo se ha descrito que los canales de Ca^{2+} tipo T están involucrados en el incremento de Ca^{2+} inducido por la neoglicoproteína α-D-manosa-sero albúmina bovina (manosa-BSA) pero no participan en el incremento de Ca^{2+} inducido por la progesterona (Blackmore y Eisoldt, 1999). Lo anterior se explica al

considerar que la BSA modula a los canales de Ca^{2+} T, tal como lo mostramos en la primera parte de esta tesis (discutido en la sección 6.1).

Por último, se sabe que la progesterona promueve la RA en distintas especies. Considerando que la progesterona puede activar a la fosfolipasa C presente en el espermatozoide, es posible que dicha activación produzca el vaciamiento de las pozas internas de Ca^{2+} y como consecuencia active al segundo canal de Ca^{2+} involucrado en la RA, el cual depende del vaciamiento de las pozas internas (O'Toole *et al.*, 2000). Esto podría explicar porque la progesterona y la ZP actúan de manera cooperativa en la RA, aún cuando activan a distintos elementos de regulación involucrados en la exocitosis (revisado en Darszon *et al*, 2001).

5.4.- La inhibición de la RA inducida con la ZP por bloqueadores aniónicos es independiente de la corriente de Ca^{2+} tipo T.

Estudios previos han demostraron la localización de un receptor ionotrópico para GABA en el espermatozoide de mamífero (Erdo y Werkele, 1990; Rabow *et al.*, 1995). Como se mencionó anteriormente, el GABA o la glicina pueden inducir la RA en el espermatozoide humano, de ratón y de cerdo, y dicha reacción se puede inhibir con antagonistas de canales de Cl^- tales como el ácido niflúmico (Meizel, 1997). Con la finalidad de evaluar la posible participación de canales aniónicos en la RA inducida por la ZP, nuestro grupo realizó registros electrofisiológicos en el espermatozoide directamente y en las células espermatogénicas de ratón como una primera aproximación a esta interesante pregunta. Nuestros resultados demostraron la presencia de canales aniónicos funcionales sensibles a ácido niflúmico (AN) tanto en el espermatozoide maduro (ver apéndice; Espinosa *et al.*, 1998) como en sus células precursoras (Fig. 20). Sin embargo, debido a que

el AN y otros bloqueadores de canales aniónicos pueden inhibir a distintos canales iónicos de manera inespecífica, resultó necesario descartar que la inhibición de la RA inducida por la ZP fuera consecuencia del bloqueo inespecífico de la ICa_T.

Los resultados de esta serie de experimentos mostraron que la aplicación de concentraciones micromolar de ácido niflúmico (AN) o de ácido 5-nitro-2-(3-fenilpropilamina) benzóico (NPPB) inhiben a la corriente T de las células espermatogénicas (Fig. 22) de manera dependiente de voltaje (Fig. 23). Se sabe que el AN inhibe a la RA inducida por la ZP y a los canales aniónicos expresados en el espermatozoide de ratón (ver apéndice, Espinosa *et al*., 1998). De hecho, se considera que el AN y otros compuestos, tales como el NPPB y el DDF, bloquean canales de Cl^- activados por Ca^{2+} (Baron *et al*., 1991; Cotton *et al*., 1997; Lamb *et al*., 1994), al canal de Cl^- CFTR (Walsh y Wang, 1993; Doughty *et al*., 1998), a canales de Cl^- regulados osmóticamente (Diener *et al*., 1996), y a canales de Cl^- rectificadores entrantes de los espermatozoides de *Caenorhabditis elegans* (Machaca *et al*., 1996). Sin embargo, estos compuestos también afectan a algunos canales catiónicos tales como el receptor de NMDA (Lerma y Martín del Río, 1991), a canales catiónicos pobremente selectivos (Gögelein *et al*., 1990), y a CCDV (Walsh y Wang, 1993; Doughty *et al*., 1998). Como sabemos, se ha documentado ampliamente que la corriente de Ca^{2+} presente en las CE de ratón pertenece al tipo T (Liévano *et al*., 1996; Santi *et al*., 1996; Arnoult *et al*., 1996). Más aún, estos canales permanecen presentes de manera funcional en el espermatozoide maduro (Arnoult *et al*., 1999; López-González *et al*., 2001), donde son de vital importancia durante la RA (Darszon *et al*., 2001). Debido a que el AN inhibe a la RA en el espermatozoide maduro, resultaba relevante el determinar si la corriente T era sensible a estos bloqueadores aniónicos. Los resultados obtenidos muestran que el AN bloqueó a la corriente de Ca^{2+} con una IC_{50} de 43 μM (Fig. 22A). Por otra parte,

el NPPB parece tener dos sitios de unión distintos con afinidades aproximadas de 25 nM y 75 µM (Fig. 22B). En contraste, la RA inducida por la ZP es más sensible a estos bloqueadores con una IC_{50} de 1 y 6 µM para el AN (ver apéndice, Espinosa *et al*, 1998) y el NPPB (ver apéndice, Espinosa *et al*., 1999), respectivamente. Considerando estos datos, para el caso del AN, la inhibición de los canales de Ca^{2+} tipo T no es determinante en el bloqueo de la RA inducida por la ZP y se puede atribuir a una efecto del AN sobre canales aniónicos involucrados en este proceso. El razonamiento en el que se basa la conclusión anterior tiene como sustento trabajos farmacológicos en los cuales se ha descrito la correlación existente entre la inhibición de la corriente T por bloqueadores de canales de Ca^{2+} dependientes de voltaje en las células espermatogénicas, con una IC_{50} similar a la del bloqueo de la RA del espermatozoide maduro y del influjo de Ca^{2+} asociado a este fenómeno (Arnoult *et al*., 1996). Desde este punto de vista, solamente el NPPB podría estar contribuyendo en la inhibición de la RA inducida por la ZP a través del sitio de alta afinidad (Fig. 22B). Más aún, el bloqueo por ambos compuestos es dependiente de voltaje (Fig. 23). Considerando que el potencial de reposo del espermatozoide capacitado es aproximadamente –70 mV (Florman *et al*., 1998; Arnoult *et al*, 1999), el bloqueo de la corriente T por los antagonistas de canales aniónicos, el cual es más potente a potenciales depolarizados, sería mínimo. De igual manera, los datos sugieren que la corriente de Ca^{2+} no es modulada por GABA (discutido en la sección 6.2), por lo que durante la inhibición de la RA inducida por GABA, el AN estaría actuando sobre canales aniónicos principalmente.

Con respecto al mecanismo de bloqueo de la corriente T por los antagonistas de canales aniónicos, se puede proponer lo siguiente: varios reportes sugieren la interacción directa de estos bloqueadores con canales aniónicos y catiónicos (Gögelein *et al*., 1990; Oba *et al*., 1997; Ottolia y Toro, 1994; Tilman *et al*., 1993; White y Aylwin, 1991). Así, el

sitio de unión al canal T podría ser el poro y posiblemente la unión ocurra en el lado externo de la membrana como se ha sugerido para otros canales (Evoniuk y Skolnick, 1988; Tilman *et al.*, 1993), ya que el efecto de bloqueo en la corriente T es reversible (Fig. 21A y B). Para poder sentir el voltaje, el sitio de unión del bloqueador debería localizarse hacia el interior del poro, inmerso en la membrana (Hille, 1992). Sin embargo, con el advenimiento de los estudios estructurales recientes de los canales iónicos (MacKinnon *et al.*, 1998; Doyle *et al.*, 1998; Sato *et al.*, 2001; Zhou *et al.*, 2001), en particular de K^+ y Na^+, esta idea ha cambiado sustancialmente. Así, se puede especular que estos bloqueadores podrían interactuar directamente con partes estructurales externas involucradas en la conducción de los iones, las cuales de manera alostérica, afectarían el flujo de iones a través del poro. Otra posible explicación podría ser que en lugar de unirse directamente al poro del canal, estos bloqueadores pudieran interactuar con el sitio de unión de alguna subunidad accesoria y de esta manera influir en la corriente T. Una explicación alternativa se basa en la permeabilidad de los antagonistas de canales aniónicos. En este caso, los bloqueadores podrían entrar a la célula y afectar alguna ruta de regulación de los canales T. Por ejemplo, los bloqueadores usados en el presente trabajo también inhiben a las cicloxigenasas y a las lipoxigenasas que participan en el metabolismo del ácido araquidónico (AA) (Civelli *et al.*, 1991) y a la 3α–hidroxisteroide dehidrogenasa involucrada en el metabolismo de esteroides sexuales (Penning *et al.*, 1985). Los metabolitos de las rutas metabólicas de la lipoxigenasa y la cicloxigenasa modulan positivamente canales catiónicos y aniónicos (Diener *et al.*, 1996; Kanli y Norderhus, 1998). Más aún, estudios recientes muestran que los canales de Ca^{2+} $Ca_V3.2$ (α1H) se modulan por ácido araquidónico (Zhang *et al.*, 2000), de manera que la inhibición de las cicloxigenasas y/o lipoxigenasas induciría un incremento en la

concentración del AA, el cual reduciría la amplitud de la corriente T. De la misma forma que en el caso del AA, los bloqueadores de canales aniónicos podrían modificar el metabolismo de las hormonas esteroides (Penning *et al.*, 1985). Sabemos que el estradiol puede modular negativamente la corriente de Ca^{2+} T presente en las células espermatogénicas de ratón (resultados discutidos en la sección 6.1 de esta tesis) y a canales de Ca^{2+} dependientes de voltaje en músculo liso (Nakajima *et al.*, 1995). Más aún, hormonas como la testosterona y las progestinas pueden modular positivamente a canales de Ca^{2+} dependientes de voltaje (Takeuchi y Guggino, 1996; Bukusoglu and Sarlak, 1996). Así, además de la acción genómica inducida por las hormonas esteroideas, estos compuestos podrían modular canales iónicos que pudieran ser críticos en la diferenciación de las CE. Sería interesante, por lo tanto, investigar más detalladamente sí la inhibición (directa o indirecta) de enzimas como la 3α–hidroxisteroide dehidrogenasa afecta la modulación de los canales de Ca^{2+} T de las células espermatogénicas.

5.5.- KLI, una nueva toxina de alacrán bloquea a la corriente de Ca^{2+} tipo T y revela otro componente de la corriente macroscópica de Ca^{2+} de las células espermatogénicas.

A pesar de que la corriente de Ca^{2+} tipo T de las CE se ha caracterizado en detalle, se desconoce que subunidad α1 codifica para esta corriente. Más aún, hay controversia en cuanto a la naturaleza molecular de la corriente T debido a la expresión de numerosas subunidades que codifican para diferentes canales de Ca^{2+} dependientes de voltaje. La aplicación de estrategias de biología molecular ha permitido detectar la presencia de RNA mensajeros que codifican para las subunidades α1 de $Ca_V1.2$ (Goodwin *et al*., 1997), $Ca_V2.1$ y $Ca_V2.3$ (Liévano *et al*., 1996), así como también para $Ca_V3.1$ y $Ca_V3.2$ (Espinosa *et al.*, 1999). De las subunidades mencionadas, los primeros tres genes codifican para las

subunidades de los canales de Ca^{2+} tipo L, P/Q y R, respectivamente, mientras que los dos últimos codifican para canales de Ca^{2+} tipo T. Asimismo, el uso de anticuerpos ha demostrado la expresión de $Ca_V1.2$, $Ca_V2.1$ y $Ca_V2.3$ (Goodwin *et al.*, 1998; Serrano *et al.*, 1999; Westenbroek y Babcock, 1999; Wennemuth *et al*, 2000), así como de las subunidades auxiliares $Ca_V\beta_1$-$Ca_V\beta_3$ (Serrano *et al*, 1999) El trabajo electrofisiológico de diferentes grupos ha demostrado que las células espermatogénicas expresan principalmente solo una corriente de Ca^{2+} tipo T (Hagiwara y Kawa, 1984; Liévano *et al.*, 1996; Arnoult *et al*, 1996a; 1996b; Santi *et al.*, 1996; Wennemuth *et al.*, 2000), lo cual resulta inconsistente con la expresión de las diferentes subunidades $\alpha1$ mencionadas anteriormente. Sin embargo, algunos resultados obtenidos con el uso de estrategias alternativas como la fluorometría sugieren la expresión funcional de canales de Ca^{2+} tipo N y R en las células espermatogénicas de ratón, a pesar de las evidencias obtenidas con electrofisiología (Wennemuth *et al.*, 2000).

Aunque es difícil correlacionar los resultados electrofisiológicos obtenidos en el ratón con las evidencias moleculares obtenidas en diferentes especies (incluido el humano), resulta determinante identificar a la entidad molecular que codifica para la corriente T, así como entender su regulación, para aclarar los mecanismos involucrados en la RA. El progreso hecho en la comprensión de la composición estructural de los canales de Ca^{2+} dependientes de voltaje ha demostrado que la expresión heteróloga de las subunidades $Ca_V3.1$ y $Ca_V3.2$ origina canales de Ca^{2+} con propiedades biofísicas y farmacológicas propias de los canales de Ca^{2+} tipo T nativos (Perez-Reyes *et al.*, 1999). Recientemente, Chuang *et al.* (1999) aprovecharon la expresión heteróloga de estas subunidades para buscar toxinas con alta afinidad y especificidad para canales de Ca^{2+} tipo T. En ese trabajo,

los autores reportaron una toxina de alacrán (la kurtoxina) que se une a $Ca_V3.1$ y $Ca_V3.2$ con alta afinidad e inhibe su actividad como canal debido a que modifica los mecanismos de apertura y cierre de los canales ("gating"). En este trabajo describimos lo que es a nuestro conocimiento la primera caracterización biofísica reportada del bloqueo de la corriente de Ca^{2+} nativa de las CE por dos nuevas toxinas de alacrán (KLI y KLII). De manera similar a la kurtoxina, la aplicación extracelular de KLI o KLII reduce significativamente la corriente de Ca^{2+} tipo T a todos los voltajes examinados. Sin embargo, a pesar de la alta homología estructural (arriba del 80%) de KLI y KLII con la kurtoxina, se encontraron diferencias en el modo de acción de estas toxinas en la corriente T nativa. La inhibición de la corriente macroscópica de Ca^{2+} en las CE causada por KLI o la KLII en el intervalo de -40 a +20 mV fue poco dependiente de voltaje (Fig. 27A, panel derecho), además de parcialmente reversible, lo que sugiere que estas toxinas pudieran actuar como bloqueadores del poro más que como modificadoras del "gating". Sin embargo, hay que hacer notar que en la inhibición de la corriente a voltajes más positivos a -20 mV, la fracción de la corriente bloqueada por 200 nM de KLI o de KLII parece ser mayor, sin llegar a ser estadísticamente significativo. Para un análisis detallado de los efectos de la KLI y la KLII arriba mencionados, sería necesario un estudio a nivel de canal unitario. Más aún, la creación de mutantes complementarias de las toxinas y de los canales recombinantes podría ayudar a identificar los sitios de contacto entre la toxina y el canal aumentando la comprensión del modo de acción de estos péptidos. No obstante, la homología en la secuencia entre estos péptidos (KLI y KLII) y la kurtoxina sugiere que la inhibición de la corriente de Ca^{2+} T de las CE se debe al bloqueo de los canales de Ca^{2+} $Ca_V3.1$ y/o $Ca_V3.2$ (Chuang *et al.*, 1999). A pesar de que los mecanismos moleculares del bloqueo podrían ser diferentes, esta hipótesis se apoya en nuestros resultados (ver apéndice,

Espinosa *et al*, 1999), que demuestran la expresión de los RNAs mensajeros para $Ca_V3.1$ y $Ca_V3.2$ y en las diferencias farmacólogicas entre la corriente T nativa de las CE y la subunidad $Ca_V2.3$ expresada heterológamente. Como sabemos, la subunidad α1E ($Ca_V2.3$) es el transcrito con mayor abundancia relativa y se había propuesto como el mejor candidato para codificar a la corriente T en las CE (Liévano *et al*, 1996). Sin embargo, recientemente se demostró que la falta del canal $Ca_V2.3$ (α1E) no modifica ninguna de las características biofísicas fundamentales de la corriente de Ca^{2+} T de las CE (densidad de corriente, activación, inactivación, etc.), lo que descarta la participación de $Ca_V2.3$ en dicha corriente de Ca^{2+} (Sakata *et al*, 2001) y corrobora los resultados anteriores mostrados en este trabajo (ver apéndice, Espinosa *et al*, 1999).

De manera consistente con trabajos previos, el bloqueo de la corriente de Ca^{2+} tipo T por KLI o la KLII inhibió la RA inducida por la ZP (Fig. 28). Con base en las características farmacológicas de los canales de Ca^{2+} dependientes de voltaje, se ha sugerido que la corriente de Ca^{2+} tipo T ($Ca_V3.1$ y/o $Ca_V3.2$) participa, de manera importante, en la RA del espermatozoide de ratón (Wassarman *et al*.,2001; Darszon *et al*, 2001), aún cuando se han propuesto explicaciones alternativas (Benoff, 1998). Este problema se ha debido a la falta de inhibidores específicos para canales dependientes de voltaje tipo T. Sin embargo, el hecho de que KLI y KLII inhiban significativamente a la RA inducida por la ZP en el espermatozoide maduro sugiere fuertemente que los canales T juegan un papel importante en el proceso de señalización celular iniciado por la ZP. Sin embargo, estos resultados indican que aún cuando los canales T son un elemento de vital importancia durante la RA, es posible que otras vías de entrada de Ca^{2+} también estén involucradas en este proceso. De hecho, la diferencia observada entre el porcentaje de

inhibición de la RA (Fig. 28) y la fracción de corriente resistente a 200 nM de las toxinas (Fig. 24B) pudiera explicarse con un incremento en los niveles de expresión del segundo componente de la corriente de Ca^{2+} en el espermatozoide maduro. Lo anterior, hace necesario considerar el posible papel de otros canales de Ca^{2+} durante la RA.

El uso de métodos bioquímicos y moleculares para estudiar a las células espermatogénicas de ratón y humano ha permitido detectar la presencia de al menos dos subunidades de canales de Ca^{2+} distintas al tipo T ($Ca_V1.2$ y $Ca_V2.1$). El reciente reporte de la activación de los canales de Ca^{2+} $Ca_V2.2$ y $Ca_V2.3$ (tipo N y R, respectivamente) en el espermatozoide de ratón en respuesta a depolarizaciones inducidas por alto K^+ externo, ha complicado aún más la identificación molecular de la corriente de Ca^{2+} (Wennemuth *et al*, 2000). Además, es altamente posible que durante la fecundación se requiera de vías alternas de entrada de Ca^{2+} que probablemente sean necesarias para la RA y/o para la adquisición de la motilidad progresiva del espermatozoide (O'Toole *et al*, 2000; Jungnickel *et al*, 2001; Ren *et al*, 2001; Quill *et al*., 2001). Estos datos en su conjunto, sugieren que el espermatozoide posee una diversidad de canales de Ca^{2+} más amplia de lo que se pensaba inicialmente. Más aún, contrario a nuestras expectativas, KLI y/o la KLII no bloquearon totalmente a la corriente de Ca^{2+} observada en las CE (Figs. 24B y 25B). La interpretación más simple a esta observación es que la porción de la corriente insensible a KLI o a la KLII (200 nM) podría estar codificada por una subunidad $\alpha 1$ distinta de la subfamilia de canales de Ca^{2+} T. De manera consistente con esta propuesta, la presencia de un pequeño hombro en la parte descendiente de la curva I-V (alrededor de 0 a +10 mV) detectado en presencia de las toxinas, apoya la idea de trabajos previos que sugieren la expresión funcional de diversos tipos de canales de Ca^{2+} (Goodwin *et al*, 1998; Serrano *et al*., 1999; Westenbroek

y Babcock, 1999; Wennemuth *et al.*, 2000; O'Toole *et al.*, 2000; Jungnickel *et al.*, 2001; Ren *et al.*, 2001; Quill *et al.*, 2001). La expresión funcional del segundo componente es muy baja en este tipo de células, lo que ha evitado en gran medida su identificación previa en la corriente de Ca^{2+}. La presencia de diferentes componentes en la corriente macroscópica de Ca^{2+} de las CE podría ayudar a explicar la polémica sensibilidad de esta corriente de Ca^{2+} a bloqueadores de canales de Ca^{2+} HVA tales como la ω-CTX GVIA (Wennemuth *et al.*, 2000). Sin embargo, es necesario un examen más detallado para determinar si la farmacología de la corriente residual pudiera corresponder a un canal de alto umbral de activación.

Considerando este nuevo componente de la corriente de Ca^{2+} de las CE es necesario reevaluar los resultados presentados en las secciones previas, lo que nos permite hacer varias interpretaciones: resulta interesante que la aplicación de 100 μM de W7, un antagonista de CaM, no inhiba completamente la corriente de Ca^{2+} registrada en las CE (Fig. 10C). Esto podría explicarse con base en el conocimiento de que varias vías de señalización influyen en la actividad de los canales T, lo cual pudiera evitar una inhibición absoluta. Sin embargo, esta corriente residual podría explicarse con la presencia de un canal distinto a la corriente T que no se regule por CaM, hecho que puede ser factible con base en los registros obtenidos en presencia de las toxinas descritas en este trabajo. De la misma forma, se puede señalar que los dos sitios de unión para el NPPB sugeridos por los resultados de la curva dosis-respuesta (Fig. 22B), pudieran ser en realidad evidencia de la presencia de dos componentes de la corriente de Ca^{2+} de las CE. Para responder a estas incognitas sería necesario hacer una serie de experimentos en los que se combine el uso de las toxinas KLI y KLII y los agentes antes mencionados para distinguir entre los efectos

directos de los fármacos sobre los parámetros biofísicos de cada componente de la corriente de Ca^{2+} de manera más apropiada.

Como una primera aproximación para tratar de identificar la naturaleza de este segundo componente de la corriente de Ca^{2+}, se aprovechó la alta sensibilidad de los canales LVA por el Ni^{2+} (Lee *et al.*, 1999) para separar a los dos componentes de la corriente de Ca^{2+}. La aplicación de 1 mM de Ni^{2+} redujo la corriente de Ca^{2+} total a un 25±1.7%, el cual correspondería al componente resistente a las toxinas (Fig. 31A; panel superior) ya que comparte ciertas propiedades biofísicas con la corriente resistente a las toxinas descrito anteriormente (Fig 31A; panel inferior). La incubación de las células en Ni^{2+}/KLII (1 mM/200 nM), permitió descartar que el segundo componente registrado en presencia de Ni^{2+} correspondiera a una corriente de Ca^{2+} de bajo umbral (Fig. 31B). Otra evidencia más que apoya que el componente resistente a Ni^{2+} es distinto al canal T se basa en la insensibilidad de este componente a la BSA (0.5%), la cual incrementa a la corriente T presente en las CE como lo demuestran resultados discutidos previamente en este trabajo (ver sección de modulación y/o apéndice, Espinosa *et al.*, 2000). Debido a que el RNA mensajero de mayor abundancia relativa presente en las CE es α1E ($Ca_V2.3$ o tipo R; Liévano *et al.*, 1996), y considerando que no existe un bloqueador comercial específico para canales $Ca_V2.3$ (tipo R), se decidió utilizar Cd^{2+} (20 μM) el cual bloquea preferencialmente a canales de Ca^{2+} HVA (Hille, 1992). La aplicación de Ni^{2+}/Cd^{2+} (1 mM/20 μM, respectivamente), no bloqueó al componente resistente a Ni^{2+} lo cual sugiere que este componente no pertenece a canales de Ca^{2+} HVA. Este último resultado es consistente con el trabajo reciente de Sakata y colaboradores (2001), en el que reportan que el bloqueo de la expresión de $Ca_V2.3$ (α1E) no altera las propiedades biofísicas ni la

densidad de la corriente T en las células espermatogénicas. En el mismo trabajo, la aplicación de 5 μM de ω-conotoxina GVIA en presencia de 300 μM de Cd^{2+} revela un componente resistente a estos dos bloqueadores, lo que sugiere que en el espermatocito en paquiteno no se expresa funcionalmente el canal de Ca^{2+} $Ca_V2.2$ (tipo N) o algún otro canal de Ca^{2+} HVA sensible a la concentración de Cd^{2+} mencionada (Sakata *et al.*, 2001). Sin embargo, es necesaria una caracterización electrofisiológica y farmacológica más detallada de la corriente resistente a Ni^{2+} y a la KLI para descartar la posible participación de un canal de Ca^{2+} HVA.

Otro posible candidato para producir este segundo componente de la corriente de Ca^{2+} resistente a las toxinas (KLI o KLII) es una nueva proteína llamada CatSper. Hasta la fecha se han clonado dos genes que codifican para CatSper, cuya estructura es similar a la de canales de Ca^{2+} dependientes de voltaje (Ren *et al.*, 2001; Quill *et al.*, 2001). La expresión del RNA mensajero de CatSper es específica de testículo y la proteína se localiza en el flagelo del espermatozoide. Resulta interesante que la interrupción del gene (*Knockout*) que codifica para el CatSper da como resultado un ratón estéril, debido a la incapacidad del gameto para mantener patrones de motilidad normales, lo que reduce su capacidad de penetrar la zona pelúcida del óvulo homólogo (Ren *et al*., 2001).

En resumen, en esta parte de la presente tesis hemos descrito dos nuevas toxinas de alacrán que inhiben específicamente a la corriente T de las CE de ratón y a la RA inducida por la ZP. Además, hemos dado evidencia de que las CE podrían poseer otro componente funcional de la corriente macroscópica de Ca^{2+} cuya presencia ha estado enmascarada por su aparente similitud en cuanto a sus propiedades biofísicas con las características propias de los canales LVA. De la misma forma, hemos aportado lo que es a nuestro conocimiento

la primera evidencia de la inhibición de la RA de mamíferos con toxinas peptídicas. La aplicación de estas toxinas podría tener una gran importancia para evaluar la importancia de los canales T en la señalización eléctrica y bioquímica del espermatozoide. Más aún, considerando que se desconoce la subunidad $\alpha 1$ que codifica para la corriente T nativa de las CE, el uso de la KLI y la KLII como herramientas farmacológicas permitiría definir la identidad molecular no solo de la corriente T de estas células sino de otros tipos celulares de diferentes tejidos, tal como ha ocurrido con toxinas específicas para otro tipo de canales de Ca^{2+}.

VI.- CONCLUSIONES.

6.1.- Modulación:

6.1.1.- Modulación por albúmina y β-estradiol.

A) La albúmina (BSA) modula a la corriente de Ca^{2+} tipo T de las células espermatogénicas, de manera dependiente de la concentración.
B) La BSA modifica la voltaje dependencia de la activación e inactivación de la corriente T, lo cual podría incrementar la corriente ventana de las células espermatogénicas.
C) Nuestros resultados sugieren que los canales de Ca^{2+} tipo T, debido al aumento en la corriente de ventana, pudieran contribuir al incremento de $[Ca^{2+}]i$ en el reposo durante la capacitación.
D) La capacidad de la BSA para inducir la capacitación depende de la extracción de colesterol de la membrana, pero en la modulación de la corriente de Ca^{2+} tipo T pudiera estar quitando otras moléculas hidrofóbicas de la membrana aún no determinadas.
E) Los mecanismos que median los efectos del β-estradiol y la BSA sobre la corriente T son distintos.
F) Es necesario un estudio más profundo para determinar el mecanismo mediante el cual el β-estradiol inhibe a la corriente de Ca^{2+} T.

6.1.2.- Posible modulación por calmodulina.

A) El W7, un inhibidor específico de calmodulina (CaM), reduce a la corriente de Ca^{2+} tipo T de manera dependiente de la concentración e independiente de voltaje.
B) Nuestros resultados sugieren que el efecto del W7 sobre la corriente T se debe a la acción de este fármaco sobre la CaM, ya que otros inhibidores de esta proteína como la TFP producen alteraciones semejantes. Más aún, la adición de CaM a la solución interna de registro o la sustitución de Ca^{2+} por Ba^{2+} inhiben parcialmente el efecto del W7.
C) El W7 afecta tanto a la cinética de activación como a la de inactivación, y promueve la inactivación desde el estado cerrado.
D) El W7 inhibió con una IC_{50} similar a la corriente T de las células espermatogénicas, y al transitorio de Ca^{2+} y a la RA inducidos por el agonista fisiológico en el espermatozoide maduro. Por lo que, nuestros resultados sugieren que el bloqueo de la RA se debe a la inhibición de los canales de Ca^{2+} tipo T.
E) La CaM parecería modular directamente el canal responsable de la corriente T. Sin embargo, todavía no es posible descartar la participación de cinasas o fosfatasas dependientes de CaM en esta regulación.

6.1.3.- Progesterona y ácido γ-aminobutírico (GABA).

A) Nuestros resultados sugieren que ni la progesterona (50 μM) ni el ácido γ-aminobutírico (GABA) (125 μM) modulan a la corriente de Ca^{2+} tipo T de las células espermatogénicas. Por lo tanto, es probable que este tipo de canales de Ca^{2+} no participen en el incremento de Ca^{2+} durante la RA inducida por progesterona o GABA.

6.2.- Estudios farmacológicos:

6.2.1 -Bloqueadores aniónicos.

A) La corriente de Ca^{2+} tipo T es sensible al AN y al NPPB, de manera dependiente de la dosis y del voltaje.
B) El bloqueo de la corriente T por estos compuestos es parcialmente reversible en todos los casos.
C) Las curvas dosis-respuesta sugieren que para el AN (IC_{50}= 43 μM) existe solo un sitio de unión al canal. Para el caso del NPPB, nuestros resultados sugieren dos sitios de unión, alcanzando una primer meseta de inhibición (45%) a una concentración entre 0.5 y 10 μM.
D) Debido a que la concentración para inhibir a la RA por AN (IC_{50}= 11 μM) fue menor que la necesaria para bloquear a la corriente de Ca^{2+} T, nuestros resultados sugieren que el canal T tiene una pobre participación en este proceso. La inhibición de la RA por el AN se debe probablemente a la inhibición de los canales aniónicos presentes en el espermatozoide.
E) Por otra parte, nuestros resultados sugieren que la inhibición de la RA por NPPB (IC_{50}= 6 μM) podría deberse, en parte, al bloqueo de la corriente de Ca^{2+} tipo T.

6.2.2.- Toxinas de alacrán

A) Tomando como base la secuencia de la kurtoxina, identificamos dos toxinas (KLI y KLII) que presentan una alta homología con esta misma.
B) La aplicación de 200 nM de KLI y KLII inhibe a la corriente de Ca^{2+} tipo T en un 65 y 75%, respectivamente, sin modificar la curva I-V.
C) Nuestros resultados sugieren que la inhibición de la corriente T es poco dependiente del voltaje.
D) El análisis de la voltaje dependencia de la inactivación en estado estacionario sugiere que 200 nM de KLII no tiene efecto alguno sobre este parámetro.
E) El efecto de KLII es específico sobre la corriente de Ca^{2+} T, ya que 200 nM de KLI no inhibió a la corriente de K^+ presente en las células espermatogénicas.
F) De manera consistente, KLI y KLII (200 nM) inhibieron a la RA inducida por el agonista fisiológico en un 39.3 y 46.1 %, respectivamente.
G) El uso de la KLI reveló un componente minoritario de la corriente de Ca^{2+} resistente a Ni^{2+} (1 mM), Cd^{2+} (20 μM) y a KLII (200 nM), lo que sugiere que no pertenece a canales LVA o HVA clásicos.
H) El componente resistente a KLI y KLII (200 nM) tiene una cinética de inactivación más lenta que la corriente de Ca^{2+} total.

VII.- PERSPECTIVAS.

La corriente de Ca^{2+} tipo T tiene un papel preponderante en la fecundación en mamífero, tal como lo sugieren los resultados presentados en este trabajo. Sin embargo, aún quedan muchas preguntas interesantes por resolver en cuanto a la modulación y la identidad molecular del canal.

Con respecto a la modulación, aún falta explorar si la corriente de Ca^{2+} T de las células espermatogénicas se regula por ácido araquidónico (AA), tal como se ha reportado para la subunidad α1H expresada en sistemas heterólogos (Zhang *et al*, 2000). De ocurrir esta modulación, podría probarse nuestra hipótesis que sugiere al AA como un posible blanco en la modulación de la corriente T por albúmina. Más aún, existen reportes que sugieren la participación del AA en la RA (Breitbart y Spungin, 1997; Sistina y Rodger, 1999), lo que hace aún más interesante explorar esta posibilidad. De la misma forma, resultaría interesante explorar si la inhibición de la corriente de Ca^{2+} T por 17-β-estradiol involucra la síntesis de oxido nítrico, ya que se sabe que los canales de Ca^{2+} de bajo umbral de activación se bloquean por otros agentes oxidantes como el DTNB o el oxido nitroso (Todorovic *et al*., 2001a; Todorovic *et al*, 2001b).

Por otra parte, los resultados presentados en este estudio son consistentes con la hipótesis de la participación de la calmodulina (CaM) en la regulación de la corriente de Ca^{2+} tipo T de las células espermatogénicas, pero no son una demostración definitiva. Para complementar esta parte proponemos explorar la secuencia de las subunidades α1G (Perez-Reyes *et al*, 1998) y α1H (Cribbs *et al*., 1998) clonadas para buscar posibles sitios de unión a CaM (Rhoads y Friedberg, 1997), y realizar ensayos de unión entre la posible secuencia de unión para CaM de la subunidad α1 y la CaM. Esta estrategia nos permitiría encontrar evidencias directas de la interacción entre estas dos proteínas fortaleciendo nuestra hipótesis.

Otro aspecto interesante por evaluar es el posible efecto de los bloqueadores de canales aniónicos sobre las cicloxigenasas y a las lipoxigenasas que participan en el metabolismo del ácido araquidónico (Civelli *et al.*, 1991) y a la 3α–hidroxisteroide dehidrogenasa involucrada en el metabolismo de esteroides sexuales (Penning *et al*, 1985), ya que los metabolitos de las rutas metabólicas de la lipoxigenasa y la cicloxigenasa modulan positivamente a canales catiónicos y aniónicos (Diener *et al*,1996; Kanli y Norderhus, 1998). De manera similar, los inhibidores de canales aniónicos podrían modificar el metabolismo de las hormonas esteroides (Penning *et al*, 1985), las cuales pueden modular positivamente a canales de Ca^{2+} dependientes de voltaje (Takeuchi y Guggino, 1996; Bukusoglu and Sarlak, 1996).

Por último, considerando que se desconoce la subunidad α1 que codifica para la corriente T *nativa de las células espermatogénicas*, el uso de las toxinas de alacrán (KLI y la KLII) descritas en este trabajo, permitiría definir la identidad molecular no solo de la corriente T de estas células sino de otros tipos celulares de diferentes tejidos, tal como ha ocurrido con toxinas específicas para otro tipo de canales de Ca^{2+}.

VIII. BIBLIOGRAFÍA

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IX.- APÉNDICE

Artículos generados a partir de esta tesis doctoral:

A) **López-González, I.**, Olamendi-Portugal, T., de la Vega-Beltrán, J.L., García, B.I., Van Der Walt, J., Dayson, K., Tytgat, J., Felix, R., Possani, L.D. y Darszon, A. (2002). Two new scorpion toxins inhibit T-type Ca^{2+} channels in mouse male germ cells and inhibit the sperm acrosome reaction. (en preparación).

B) **López-González, I.**, de la Vega-Beltrán, J.L., Santi, C.M., Florman, H.M., Felix, R. y Darszon, A. (2001). Calmodulin antagonists inhibit T-type Ca^{2+} currents in mouse spermatogenic cells and the zona pellucida-induced sperm acrosome reaction. *Dev. Biol.* **236**, 210-219.

C) Espinosa, F., **López-González, I.**, Muñoz-Garay, C., Felix, R., de la Vega-Beltrán, J.L., Kopf, G.S., Visconti, P.E., y Darszon, A. (2000). Dual regulation of the T-type Ca^{2+} current by serum albumin and beta-estradiol in mammalian spermatogenic cells. *FEBS Lett* **475**, 251-256.

D) Espinosa, F., **López-González, I.**, Serrano, C.J., Gasque, G., de la Vega-Beltrán, J.L., Treviño, C.L., y Darszon, A. (1999). Anion channel blockers differentially affect T-type Ca^{2+} currents of mouse spermatogenic cells, alpha1E currents expressed in Xenopus oocytes and the sperm acrosome reaction. *Dev. Genet* **25**, 103-114.

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Developmental Biology 236 210–219 (2001)
doi:10 1006/dbio 2001 0314 available online at http://www idealibrary com on IDEAL®

Calmodulin Antagonists Inhibit T-Type Ca^{2+} Currents in Mouse Spermatogenic Cells and the Zona Pellucida-Induced Sperm Acrosome Reaction

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The sperm acrosome reaction (AR) is a regulated exocytotic process required for gamete fusion. It depends on an increase in $[Ca^{2+}]_i$ mediated by Ca^{2+} channels. Although calmodulin (CaM) has been reported to regulate several events during the AR, it is not known whether it modulates sperm Ca^{2+} channels. In the present study we analyzed the effects of CaM antagonists W7 and trifluoroperazine on voltage-dependent T-type Ca^{2+} currents in mouse spermatogenic cells and on the zona pellucida-induced AR in sperm. We found that these CaM antagonists decreased T-currents in a concentration-dependent manner with IC_{50} values of ~10 and ~12 μM, respectively. W7 altered the channels' voltage dependence of activation and slowed both activation and inactivation kinetics. It also induced inactivation at voltages at which T-channels are not activated, suggesting a promotion of inactivation from the closed state. Consistent with this, W7 inhibited the ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. Likewise, W7 and TFP inhibited the AR with an IC_{50} of ~10 μM. In contrast, inhibitors of CaM-dependent kinase II and protein kinase A, as well as a CaM-activated phosphatase, had no effect either on T-currents in spermatogenic cells or on the sperm AR. Together these results suggest a functional interaction between CaM and the sperm T-type Ca^{2+} channel. They are also consistent with the involvement of T-channels in the AR. © 2001 Academic Press

Key Words: **Ca^{2+} channel regulation; T-type Ca^{2+} channel; spermatogenic cells; sperm; CaM; W7; TFP; acrosome reaction**

INTRODUCTION

Voltage-dependent Ca^{2+} channels (VDCC) are required components for the elaborate functioning of excitable and nonexcitable cells. Because of this, their regulation is of capital importance to the control of cellular activity. Extracellular ligands, membrane potential, phosphorylation, Ca^{2+} itself, and diffusible second messengers are all well-established regulators of Ca^{2+} channel activity (Hofmann *et al.* 1999). Recently, several studies demonstrated that calmodulin (CaM), a 17-kDa highly conserved EF hand protein which constitutes the classical Ca^{2+} receptor inside cells (Means *et al.* 1982), is implicated in the regulation of cardiac L-type (Zühlke and Reuter 1998; Peterson *et al.* 1999; Qin *et al.*, 1999) and neuronal P/Q-type (Lee *et al.*, 1999) VDCC. A deletion in the C-terminal of the ion-conducting α_{1C} subunit of the cardiac L-type VDCC prevents Ca^{2+}-dependent inactivation of the channel (Zühlke and Reuter 1998). The deletion includes an amino acid sequence called the IQ motif, which in many other proteins binds CaM (Rhoads and Friedberg 1997). Furthermore, mutant forms of CaM which cannot bind Ca^{2+}, have a dominant-negative action on recombinant L-type Ca^{2+}-channel α_1-subunit inactivation (Peterson *et al.* 1999;

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0012-1606/01 $35.00

Zühlke *et al.* 1999). Likewise, biochemical studies indicated that CaM binds to the IQ motif of the L-type channels and mutational analysis showed that Ca^{2+}-dependent inactivation was greatly slowed by substitution of amino acids in the IQ sequence (Peterson *et al.* 1999; Qin *et al.* 1999; Zühlke *et al.* 1999).

Ca^{2+} influx through VDCC in sperm is required for the acrosome reaction (AR), an exocytotic event essential for sperm–egg fusion (Darszon *et al.*, 1999; Wassarman, 1999). Although the molecular identity of the VDCC involved in this event is currently unresolved, several studies indicate that this channel belongs to the T-type (or the low-voltage activated) family (Arnoult *et al.*, 1996a, 1999; Liévano *et al.* 1996; Santi *et al.* 1996). CaM is believed to participate in the AR because of its localization in the acrosome region (Jones *et al.*, 1980; Kann *et al.* 1991) and its redistribution during this reaction (Hernández *et al.* 1994). Furthermore, CaM antagonists inhibit the AR in sea urchin sperm (Sano, 1983; Guerrero and Darszon 1989). These findings motivated us to explore whether CaM could regulate sperm VDCC. However, the study of VDCC in sperm is complicated by difficulties in applying molecular biology and electrophysiology techniques directly to these small, terminal cells. Indeed, only rarely have Ca^{2+} currents been recorded from mature sperm (Weyand *et al.* 1994; Espinosa *et al.* 1998). An alternative approach has been to use spermatogenic cells, developmental precursors that synthesize proteins for later use in the sperm (Liévano *et al.* 1996; Arnoult *et al.*, 1996a, 1999; Santi *et al.*, 1996). In the present study we examined the effects of specific CaM inhibitors on VDCC activity in mouse spermatogenic cells and on the zona pellucida (ZP)-induced AR in sperm.

MATERIALS AND METHODS

Materials

The following were all from Calbiochem (San Diego, CA): CaM inhibitors *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5); and protein kinase inhibitors: *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89) and 1-[*N,O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN62). CaM inhibitor trifluoperazine dihydrochloride (TFP), calcineurin inhibitory fragment, adenosine 5′-triphosphate (magnesium salt; ATP-Mg_2) phosphocreatine (Di-Tris salt) trypsin (bovine pancreas, type XI), and deoxyribonuclease (DNase; bovine pancreas, type I) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Cell Preparation

Spermatogenic cells were obtained as previously described from 3-month-old CD1 mice testis (Espinosa *et al.*, 1998). In the cell suspension pachytene spermatocytes and round and condensing spermatids were most frequently observed as individual cells or synplasts. Inasmuch as similar results were obtained from these stages, data were pooled for presentation. Caudal epididymal mature mouse sperm were mechanically collected from CD1 mice and placed in 1.5-ml microcentrifuge tubes containing medium 199 supplemented with BSA (0.1% w/v), Na^+ pyruvate (30 mg/L), and $NaHCO_3$ (2.2 g/L) at 37°C (Lee and Storey, 1985). After ~10 min the fraction of motile sperm was determined by visual inspection and preparations with <75% motile cells were discarded.

Assay for the Acrosome Reaction

Sperm aliquots (4–5 × 10^6/ml) were incubated at 37°C for 30 min for *in vitro* capacitation. Thereafter, 5 ZP equiv/μl, prepared as previously indicated (Cross and Meizel, 1989), was added to the sperm suspension to induce AR, which was assayed 30 min later using Coomassie blue G-250 (Visconti *et al.* 1999). The CaM antagonists or the control solvents were added 5 min prior to ZP. To calculate the percentage of AR, at least 100 sperm were assayed per experimental condition for the presence or the absence of the characteristic dark blue acrosomal crescent.

Electrophysiology

Ca^{2+} currents were recorded according to the whole-cell patch-clamp technique (Hamill *et al.*, 1981). All recordings were performed at room temperature using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2- to 4-MΩ borosilicate glass micropipettes. Except when indicated, cells were clamped at a holding potential (HP) of −90 mV. Currents were evoked by 14- to 200-ms depolarizing voltage steps (0.25–0.1 Hz), to test potentials ranging from −80 to 40 mV. Capacity transients were electronically compensated, and linear leak and residual capacity currents were subtracted online using a P/4 standard protocol. Currents were captured online and digitized at a sampling rate of 10 kHz following filtering of the current record (5 kHz) using a personal computer attached to a TL-1 interface (Axon). Pulse protocols, data capture, and analysis of recordings were performed using pCLAMP software (Axon). To isolate Ca^{2+} currents cells were bathed in a solution containing (in mM) $CaCl_2$ (10); NaCl (130); KCl (3); $MgCl_2$ (2); $NaHCO_3$ (1); NaH_2PO_4 (0.5); Hepes (5); and glucose (10) (pH 7.3/NaOH). The internal (patch pipette) solution consisted of (in mM) $CsMeSO_3$ (110); CsF (10); CsCl (15); $CaCl_2$ (4.6); EGTA (10); Hepes (5); ATP-Mg_2 (4); and phosphocreatine (10) (pH 7.3/CsOH). All drugs were prepared as 10–80 mM stock solutions in dimethylsulfoxide (DMSO) and diluted in the bath solution for each experiment to give the desired final concentration.

$[Ca^{2+}]_i$ Determination

Capacitated sperm (1 × 10^7/ml) were loaded with fluo-3 during a 20-min incubation in a BSA-free medium containing 0.0125% Pluronic F-127 and 5 μM acetoxylmethyl ester dye precursor (Molecular Probes, Eugene, OR). Sperm were immobilized on Cell-Tak-coated coverslips and drugs were added by gentle superfusion. $[Ca^{2+}]_i$ values were determined in single cells with excitation illumination (490 nm, 7 nm slit width) from a 75-W Xe arc selected by a Polychrome II monochrometer (TILL Photonics) and fluorescent emission (>530 nm) collected by photomultiplier tubes at 2-ms intervals, as described previously (Arnoult *et al.* 1999). Solubilized ZP solutions (5 μg/ml) and other solutions were added by a drug-delivery pipette positioned near the sperm head. $[Ca^{2+}]_i$ concentrations were determined using a K_D value for this dye in mouse sperm of 636 nM (Rockwell and Storey, 1999).

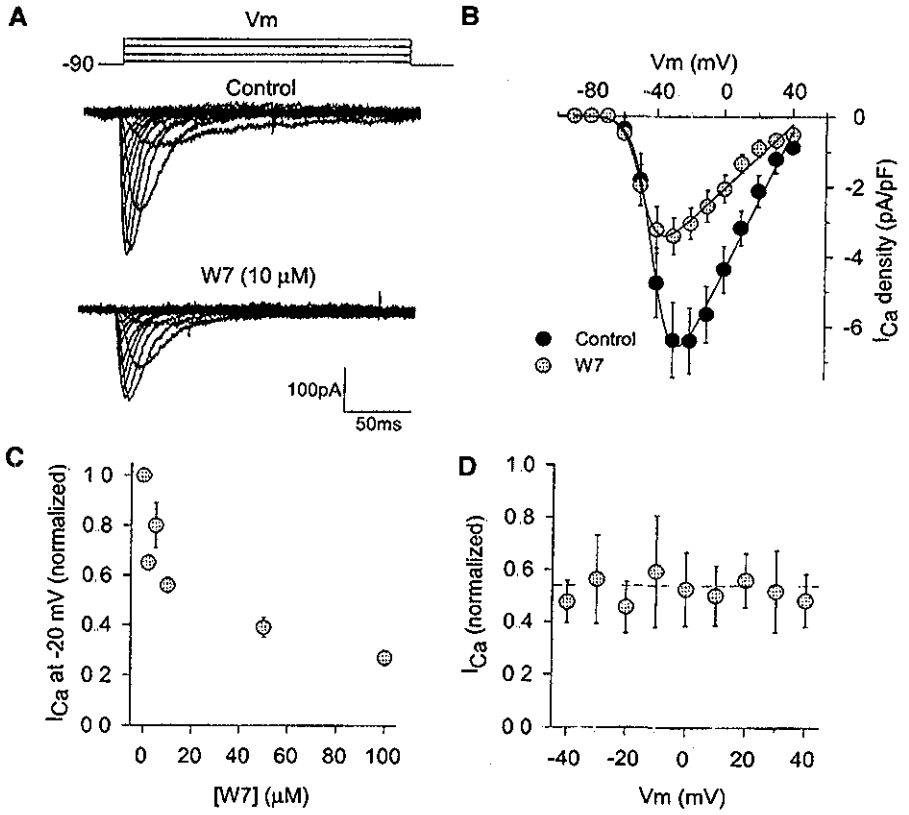

FIG. 1. W7 inhibits Ca^{2+} T-currents in spermatogenic cells. (A) Traces in the upper panel illustrate Ca^{2+} currents evoked by 200-ms depolarizations from a HP of −90 mV to test potentials ranging from −90 to 40 mV with a 10-mV increase in the pulse amplitude per step. During 5-min control experiments the rundown of peak current was frequently negligible and always <10%. Following addition of 10 μM of the specific CaM antagonist W7 to the bath-recording solution a significant reduction in current amplitude was observed (lower panel). (B) Average peak current plotted against test potential (V_m) from cells before and after W7 application (n = 4). (C) Inhibition of T-channel activity by W7 was dose dependent. Peak currents were normalized by their value before the cells were exposed to W7. (D) W7-induced inhibition of T-currents was voltage independent. Fraction of inhibited current for various depolarizing pulses after W7 treatment. Peak currents in the presence of the drug were normalized, averaged, and plotted as a function of test potential.

Statistical Analysis

Unless otherwise noted, data are given as means ± SE. The number of experiments is indicated in the figure legends. Statistical differences between two means were determined by Student's t tests. Means were considered significantly different at $P < 0.05$ and are indicated by an asterisk.

RESULTS

As previously reported (Liévano *et al.*, 1996; Arnoult *et al.*, 1996a, 1998; Santi *et al.*, 1996; Hagiwara and Kawa, 1984) patch-clamp experiments revealed that the only VDCC in mouse spermatogenic cells is of the T-type. The upper traces in Fig. 1A illustrate a family of representative Ca^{2+} currents elicited in a control cell by 10-mV depolarizing pulses from a HP of −90 mV. W7, a CaM antagonist, was used to explore whether CaM regulates sperm VDCC. The lower traces in Fig. 1A show a significant inhibition of the T-current (>45%) after the same cell was superfused with 10 μM W7. Figure 1B summarizes the corresponding current–voltage (I–V) relationships measured at the time of peak current during each record obtained before and during application of W7 in several cells. Figure 1C shows the dose dependence of the W7 inhibition. Currents were elicited by a 20-ms pulse to −20 mV from a HP of −90 mV, measured at the peak and normalized by traces recorded before drug application. Although up to 100 μM W7 could not completely abolish the currents, application of 10 μM W7 to the bath solution inhibited ~50% of the total macroscopic peak current. Thus, this concentration was used thereafter to explore in more detail the actions of W7. As can be seen in Fig. 1D, the inhibition of the T-currents induced by W7 did not exhibit noticeable voltage dependence in the ±40-mV range.

To test whether CaM was indeed involved in the inhibi-

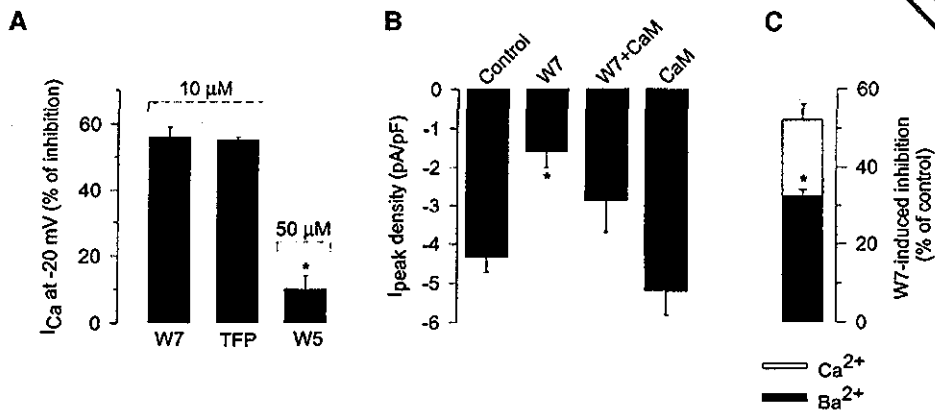

FIG. 2. Inhibition of T-currents by W7 in spermatogenic cells is mimicked by TFP (A) T-channel activity was measured in cells treated with different CaM antagonists Following exposure to the indicated concentration of the inhibitors peak current amplitude was normalized to the control and the percentage of inhibition was compared TFP (10 μM) inhibited the current in a fashion similar to that of W7 whereas 50 μM of W5 (a much less potent CaM antagonist) was unable to cause a significant inhibition of the T-current (B) Comparison of average Ca^{2+} current density after addition of W7 (10 μM) to the bath-recording solution and CaM (10 μM) to the internal solution alone or in combination Peak currents were measured during test pulses to −20 mV from a HP of −90 mV, normalized to cell capacitance and then averaged (n = 5–21) (C) Shown superimposed is the average value of W7-induced inhibition of T-currents in Ca^{2+}- and Ba^{2+}-containing solutions Currents were evoked by 200-ms pulses to −20 mV from a HP of −90 mV Asterisks represent significant differences with respect to the control

tion induced by W7 on the T-currents we next examined the effects of its structural homolog W5 (same as W7 but lacking a Cl^-) a much less potent CaM antagonist Even high concentrations of W5 (50 μM) were unable to cause a significant inhibition of the T-currents (Fig. 2A) In contrast, another potent antagonist of CaM trifluoroperazine (TFP), inhibited the current in a fashion similar to that of W7. To further test whether W7 was directly blocking the T-channel the inhibition caused by W7 in the absence and in the presence of CaM inside the recording pipette was compared (Fig 2B) The percentage of current inhibited by 10 μM W7 was significantly smaller when an equimolar concentration of CaM was included in the internal solution CaM by itself had no effect on the magnitude of the Ca^{2+} currents, which could indicate that endogenous CaM occupies the regulation sites of the channel In addition, with Ba^{2+} as the permeant ion we found that W7-induced inhibition was significantly reduced (Fig 2C), which implies that this action is dependent on Ca^{2+}

Tail currents were measured to further characterize the inhibitory effect of W7 Repolarization of the plasma membrane to −90 mV after a short depolarization to a test potential of −20 mV caused a rapid increase in the amplitude of the inward Ca^{2+} current resulting from the larger driving force for Ca^{2+} influx. Representative superimposed traces of currents obtained before and during the application of W7 clearly illustrate the inhibition caused by the drug (Fig 3A inset) Figure 3A summarizes normalized results from several cells showing that W7 (10 μM) suppressed maximal tail current density by ~46%. Data points were fit with Boltzmann equations to determine activation parameters. Under control conditions, T-current was half-maximally activated at −40 7 ± 1.6 mV In contrast acute application of W7 induced a small but significant ($P < 0$ 05) ~6 3-mV rightward shift in the voltage dependence of activation (Fig 3B) Figure 3C compares the apparent time course of current activation in the absence and the presence of the drug It can be seen that in addition to decreasing amplitude, W7 treatment resulted in a slight delay in the onset of the current and, consequently a significant increase in the time required for Ca^{2+} currents to reach the peak (7 ± 0 3 and 9.2 ± 1 5 ms, respectively).

It was recently established that the binding of Ca^{2+}/CaM to IQ regions inhibits high-voltage-activated (HVA) Ca^{2+} channels by promoting inactivation This mode of CaM regulation was examined in the T-channels of spermatogenic cells. W7 caused a shift of ~3.8 mV toward the depolarizing direction in the average steady-state inactivation curve as well as a slight change in its slope from a control value of 4.6 ± 0.7 to 5.4 ± 0 8 in the treated cells (not shown) Although the differences in both parameters were not statistically significant W7 altered the waveform (Fig 4A) The fraction of current remaining after 80 ms (solid line in Fig. 4A) during 200-ms-long activating pulses (residual current) was significantly increased by W7, suggesting an effect on the inactivation of the T-currents (Fig 4B) Thus W7 may restrain inactivation from the open state of the channels or indirectly slow down inactivation as a consequence of a decreased rate of activation. As illustrated in Fig 4C, the time constant of inactivation significantly increased from 14 1 ± 0.9 to 25.9 ± 1 9 ms after W7 treatment This observation is consistent with the view

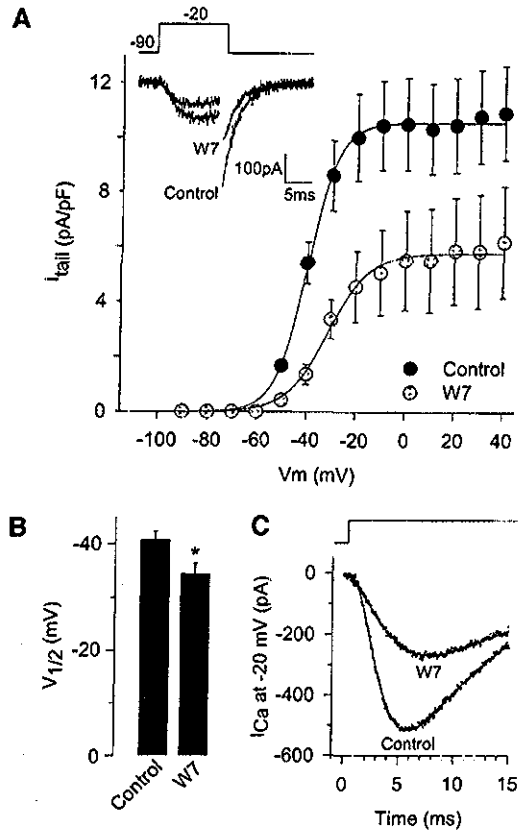

FIG. 3. W7 modulates T-current activation kinetics (A) Control and inhibited tail currents in a number of cells were normalized by capacitance averaged and plotted against membrane potential. Fits of the curves were obtained using a Boltzmann function $I_{Ca} = I_{max}/(1 + \exp[-(V_m - V_{1/2})/s])$ where V_m is the test potential $V_{1/2}$ is the potential at which the current has reached half-maximal amplitude and s is the range of potential for an e-fold change around $V_{1/2}$ Inset: Superimposed traces illustrating Ca^{2+} tail currents obtained before and after application of W7 (10 μM) by subjecting the cell to a depolarizing pulse from a HP of −90 mV to −20 mV for 14 ms and repolarizing the membrane to the HP. (B) Comparison of average $V_{1/2}$ derived from the Boltzmann fits (n = 6–11). (C) Time course of activation of T-currents in both the absence and the presence of W7 was examined in cells with uncompensated series resistance of <6 MΩ Currents were activated by 20 ms (to −20 mV from a HP of −90 mV) with the best fit of the Hodgkin–Huxley equation (Hille 1992) superimposed

that W7 may be acting via CaM, given that this protein induces inactivation of HVA-Ca^{2+} channels. Current inactivation at a step voltage of −20 mV becomes progressively slower as a function of W7 concentration (Fig. 4D, open bars) Although the slowdown of T-channel inactivation caused by W7 should increase current magnitude, this CaM antagonist actually reduces it We calculated the integral of the currents measured before and after W7 and, as illustrated in Fig 4D (filled bars), the influx of Ca^{2+} was significantly smaller upon drug application

Finally, to gain insight into the mechanisms by which CaM antagonists inhibit T-currents in spermatogenic cells we studied the state-dependent inactivation of the channels. Patch-clamp whole-cell experiments suggested that inactivation could occur from closed states of the T-channel given that long prepulses (ranging from −120 to −80 mV, voltages at which no channel opening was detectable) decreased current amplitude in response to a test pulse to −20 mV (compare currents at −120 and −80 in Fig. 5A). This difference became greater after the addition of W7. On average, W7 decreased currents to 80 ± 3 78 ± 2, and 72 ± 5% of the control value after prepulses to −120 −100, and −80 mV, respectively (Fig. 5B).

Recent results indicate that functional T-channels do remain in mature sperm after testicular differentiation and that they play a key role in determining the AR (Arnoult *et al*. 1999) Inasmuch as CaM antagonists proved to be potent regulators of Ca^{2+} channel activity in spermatogenic cells, these agents could also affect the sperm AR To test this, we first determined whether sperm T-channels were also inhibited by CaM antagonists. In sperm ZP triggers a $[Ca^{2+}]_i$ transient that has the kinetic and pharmacological characteristics anticipated of a T-channel (Arnoult *et al*. 1999) An example of a rapid ZP-evoked $[Ca^{2+}]_i$ transient is shown in Fig. 6A. ZP-dependent $[Ca^{2+}]_i$ transients were observed in 65% of cells (22/34) with $[Ca^{2+}]_i$ rising to peak levels of 7.09 ± 1 1 μM and relaxing to basal levels within 250 ms The rising phase of this response is described by a single exponential (τ = 15 2 ± 1.9 ms for 10–90% peak values). In contrast, buffer-treated controls exhibited only monotonic rises to 0.27 ± 0 05 μM during this time period. ZP also evoked $[Ca^{2+}]_i$ responses in 57% (12/21) of sperm treated with 10 μM W7. However, the peak $[Ca^{2+}]_i$ response was inhibited by 73% (2.21 ± 0.89 μM) and the activation time course was slowed (τ = 33 6 ± 4.1 ms). In contrast, 10 μM W5 had only minor effects on ZP-evoked $[Ca^{2+}]_i$ transients (Fig. 6B): responses were observed in 59% of cells (10/17), the maximal response was reduced by only 12% (6.21 ± 0 94 μM), and only a minor slowing of activation time was observed (τ = 17.7 ± 3 1 ms).

CaM antagonists (W7 and TFP) also significantly inhibited the ZP-induced AR in capacitated sperm with an IC_{50} value of ~10 μM (Fig. 7A). Notably, this concentration also inhibits ~50% of the total macroscopic peak current through T-channels in spermatogenic cells (see Fig. 2A). In addition Fig. 7A shows that as anticipated from the pharmacology of the T-currents (see Fig. 2A), W5 caused a less-important inhibition of the sperm AR Furthermore, W7 appears to block AR by altering Ca^{2+} uptake through Ca^{2+} channels because A23187, a Ca^{2+} ionophore that triggers AR in the absence of ZP (Visconti *et al*., 1999), was able to overcome the inhibition caused by W7 (Fig. 7B).

It has been proposed that T-type Ca^{2+} in mouse spermatogenic cells is enhanced by inhibition of protein phosphorylation (Arnoul *et al*., 1997). Because CaM participates in some cell signaling events regulating protein kinases, we therefore thought it pertinent to investigate whether CaM

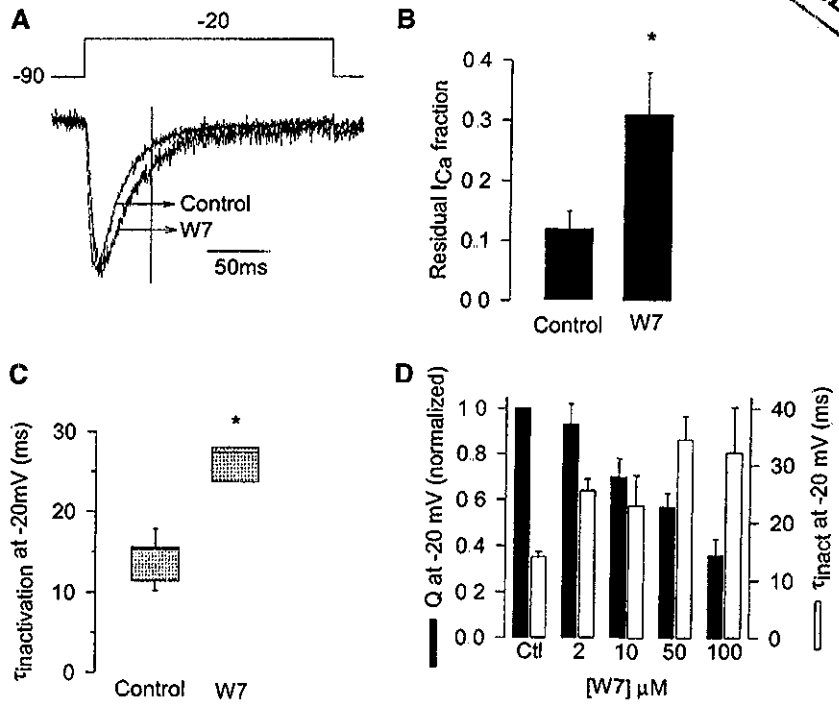

FIG. 4. W7 affects current inactivation (A) Comparison of the time course of typical current traces in both the absence and the presence of W7 (10 μM) The voltage protocol used to measure inactivation is shown above and the traces were normalized to allow kinetic comparison. (B) The percentage of current remaining after 80 ms (solid line in A) into a depolarizing pulse before and after W7 application is compared Currents were recorded in response to 200-ms depolarizing pulses from −90 to −20 mV (C) The inactivating phase of the currents was fitted with a single exponential: $I_{Ca} = A \exp(-t/\tau) + c$ where A is the initial amplitude, t is time τ is the time constant for inactivation and c is a constant Values of τ of inactivating currents obtained at −20 mV before and after W7 treatment are compared (n = 6–11) (D) Integral of the current (Q) measured during 200-ms command steps to −20 mV from a HP of −90 mV Currents measured at this command step after W7 application were normalized to the current observed before drug application in each cell (Ctl), averaged, and plotted as a function of W7 concentration (filled bars) Open bars compare τ of inactivation as a function of the concentration of W7 (n = 3–7)

might be involved in this mechanism of Ca^{2+} channel regulation Whole-cell patch-clamp experiments indicated that micromolar concentrations of the CaM kinase inhibitors KN62 and H89 did not diminish T-currents in spermatogenic cells (not shown) Consistent with this, the sperm AR was not affected by these inhibitors (Fig 7C) In addition the use of an inhibitory peptide derived from the CaM-dependent serine/threonine protein phosphatase calcineurin, did not alter current density through T-type Ca^{2+} nor the time constant of inactivation (data not shown) suggesting that a phosphatase activity is not required for modulation of Ca^{2+} channels in spermatogenic cells

DISCUSSION

Mammalian sperm must undergo the AR prior to fertilization The signal transduction steps that lead to the sperm AR are not well understood This exocytotic event, induced by the ZP surrounding the oocyte, depends on the uptake of external Ca^{2+} (Wassarman 1999) Growing experimental evidence suggests that a voltage-gated Ca^{2+} channel is an important pathway responsible for modulating Ca^{2+} entry into sperm during this process (Darszon *et al.* 1999; Arnoult *et al.*, 1996a 1999) Previous electrophysiological studies of Ca^{2+} currents in spermatogenic cells demonstrated the presence of T-currents (Hagiwara and Kawa, 1984; Liévano *et al.* 1996; Arnoult *et al.* 1996a,b; Santi *et al.* 1996) with pharmacological attributes consistent with those of the AR and the Ca^{2+} influx associated with it (Arnoult *et al.*, 1998) Likewise it was shown that this channel is retained in the mature sperm (Arnoult *et al.* 1999) and, therefore it must be an important element in the transduction events leading to the ZP-induced AR in mammalian sperm (Arnoult *et al.* 1996a 1999; Darszon *et al.* 1999).

Although the T-type Ca^{2+} currents of spermatogenic cells have been characterized, their regulation is poorly understood Such information is needed to unveil the molecular mechanisms leading to the sperm AR Recent findings indicate that CaM regulates HVA-channels, contributing to the determination of the intracellular levels of Ca^{2+} (Peter-

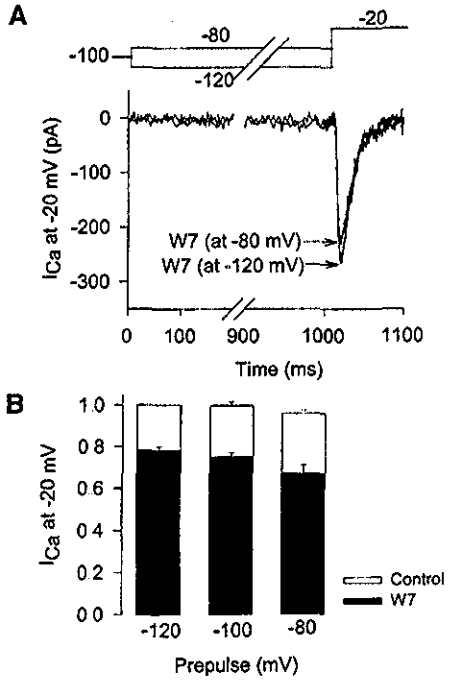

FIG. 5. W7 promotes T-channel inactivation from the closed state. (A) Voltage clamp traces recorded in a cell after application of W7 at two different potentials. Currents were evoked applying 1-s voltage prepulses to −120 and −80 mV followed by a 75-ms test pulse to −20 mV. The amount of inactivation after W7 addition at −80 mV exceeded that observed at −120 mV. (B) Inactivation measured from the test pulses is compared in different cells in both the absence and the presence of W7 during prepulses to −120 −100 and −80 mV (n = 5). Open bars represent the average currents in response to the test potential normalized to that evoked after application of the prepulse to −120 mV in the control cells. Filled bars denote current amplitude after addition of W7 normalized to that obtained before drug application.

son *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). Previous observations using CaM inhibitors in sea urchin (Sano, 1983; Guerrero and Darszon, 1989) and mammalian sperm (Courtot *et al.*, 1999) suggested the participation of CaM in the AR. With this in mind and considering the advantages of studying ion channels in spermatogenic cells, we explored the possible regulation of the T-type Ca^{2+} channels present in these cells by CaM. The results presented herein show that the specific CaM antagonist W7, at concentrations above 2 μM, markedly reduced T-current magnitude (Figs. 1–3). This effect is concentration dependent and essentially voltage independent (Figs. 1C and 1D). TFP, another CaM antagonist, also significantly reduced current amplitude at concentrations that inhibit CaM (Massom *et al.*, 1990). Furthermore, addition of CaM inside the recording pipette decreased the inhibitory effect of W7, suggesting a potential participation of this protein in the regulation of T-type Ca^{2+} channels (Figs. 2A and 2B).

The results presented here are in line with the involvement of CaM in T-type Ca^{2+} channel regulation in spermatogenic cells; though, they are not a definitive demonstration. W7, W5, and TFP can have other less-specific effects such as direct inhibition of plasma membrane and intracellular Ca^{2+} channels and phospholipase C (Ehrlich *et al.*, 1988; Schlatterer and Schaloske, 1996; Schofl *et al.*, 1999). However, the inhibitor concentrations used in this study are fitting for CaM inhibition and are lower than those associated with less-specific effects (Ichikawa *et al.*, 1991). In addition, the Ca^{2+} dependence of the inhibitory effects of W7 (Fig. 2C) supports the role of a Ca^{2+}/CaM complex in T-channel regulation. Moreover, the direct effects of CaM inhibitors on ion channel activity have been shown to be partly or fully reversible (Laver *et al.*, 1997), whereas the actions of these compounds in our experiments resulted in a nonreversible inhibition (not shown), suggesting an indirect effect on the channels. Finally, when W7 and TFP act directly on ion channels, addition of CaM

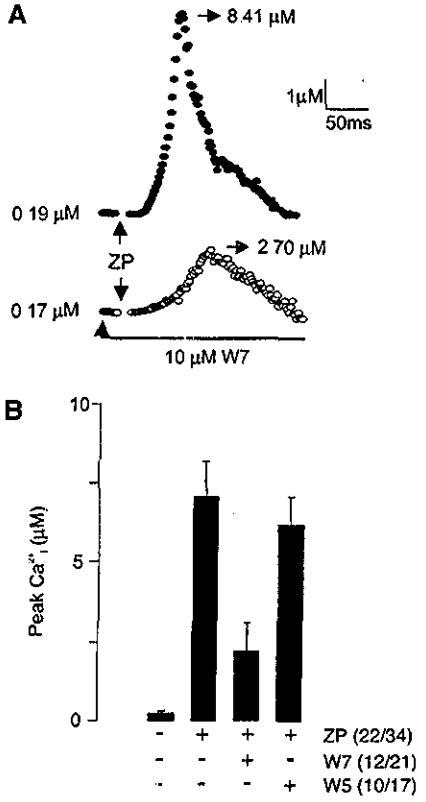

FIG. 6. W7 inhibits ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. (A) Examples of $[Ca^{2+}]_i$ responses evoked by ZP (30 μg/ml) in sperm treated with buffer and with W7. Initial and peak $[Ca^{2+}]_i$ values are indicated. (B) Sperm were incubated for 10 min with buffer (n = 22), 10 μM W7 (n = 12) or 10 μM W5 (n = 10) prior to the addition of ZP. Data represent the peak $[Ca^{2+}]_i$ levels (± SD) during transients and the fraction of total cells exhibiting responses is shown.

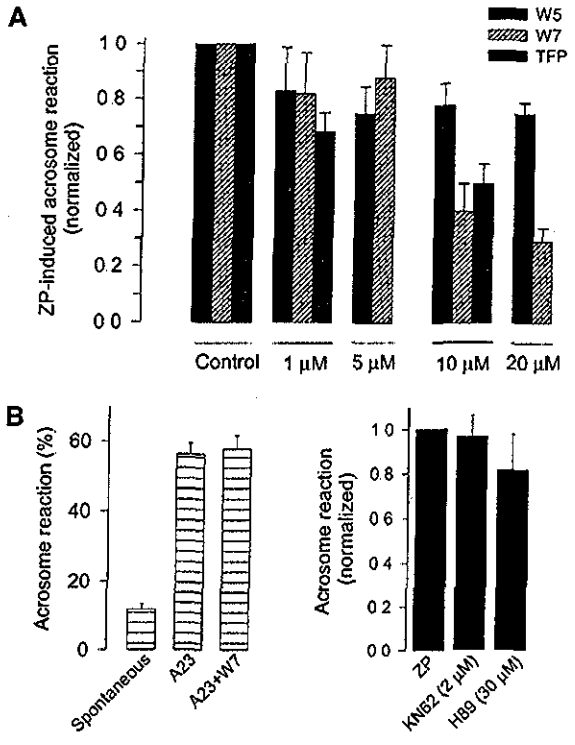

FIG. 7. CaM antagonists affect the sperm AR (A) Effect of W7 on the percentage of sperm undergoing ZP-induced AR Sperm were incubated in the presence of increasing concentrations of W7 After capacitation, the percentage of the AR was monitored Data represent means ± SEM of at least five independent experiments Sperm were also incubated in the presence of different concentrations of TFP (n = 6) and W5 (n = 7) as listed, and ZP-induced AR was measured (B) AR inhibition caused by W7 is overcome by A23187 Samples were incubated for 35 min in the presence (15 μM) of the Ca^{2+} ionophore A23187 (A23) alone or in combination with W7 (n = 4) (C) CaM-dependent protein kinase inhibitors do not affect AR The effects of KN62 and H89 on the mouse sperm AR are compared Sperm were incubated in both the absence and the presence of these two protein kinase inhibitors at the indicated concentrations Data represent means ± SEM of five separate determinations

inside the recording pipette appears to be unable to reverse this interaction (Kleene, 1994). Altogether, these considerations lead us to believe that W7 and TFP influence T-channel function by inhibiting CaM

It has been proposed that inactivation in HVA-channels involves Ca^{2+} entering through the pore, binding to CaM, and changing its conformation. The Ca^{2+}/CaM complex interacts with the channel, initiating an intramolecular process that culminates in a down-regulation of Ca^{2+} channel activity (Zühlke and Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.* 1999; Zühlke *et al.*, 1999; Lee *et al.* 1999) In agreement with this, acute application of CaM antagonist W7 results in a partial slowdown of inactivation of the T-type Ca^{2+} channels in spermatogenic cells (Figs 4C and 4D) However more striking is the decrease in T-type current amplitude caused by CaM antagonists in these cells. Here we show that, in addition to decreasing current amplitude, submaximal concentrations of W7 drastically change other basic biophysical properties of the current (Figs. 3 and 5). These changes include a positive shift in the voltage dependence of activation and a significant slowing of the apparent activation kinetics. Because activation and inactivation are tightly coupled in T-channels (Serrano *et al.* 1999), we suggest that the decrease in the rate of activation in the presence of W7 may indirectly slow down the apparent inactivation time course of the channels. In addition W7 appears to promote inactivation from voltages at which T-channels are not apparently activated (Figs. 5A and 5B) Whether CaM directly mediates this mechanism is still not clear

In summary, the present study shows that CaM antagonists W7 and TFP inhibit with similar potency T-channels in spermatogenic cells monitored by electrophysiological methods and the ZP-induced AR. Notably a similar concentration of W7 (10 μM) represses the Ca^{2+} transient triggered by ZP in mature sperm assessed by using a fluorescent probe. In contrast W5, a close analog of W7 but a much less potent CaM antagonist, is a poor inhibitor of the T-currents in spermatogenic cells and of the sperm AR and the Ca^{2+} transient associated with it. Furthermore, replacement of external Ca^{2+} by Ba^{2+} or inclusion of CaM inside the recording pipette decreases the inhibition of the T-currents in spermatogenic cells caused by W7. The overall results are consistent with a direct link between T-Ca^{2+} channel activation and the ZP-induced sperm AR, and suggest that CaM may regulate sperm T-channels. In mature sperm this is particularly important because during AR a delicate redistribution and modification of plasma membrane molecules takes place and intracellular Ca^{2+} dynamics are drastically changed (Darszon *et al.*, 1999; Wassarman, 1999; Flesch and Gadella, 2000) In addition this regulation may be important for sperm capacitation, where micromolar concentrations of CaM antagonists W7 and calmidazolium have been shown to significantly inhibit the percentage of capacitated sperm assessed by the chlortetracycline staining method (Si and Olds-Clarke, 2000) and by their ability to undergo AR in response to lysophosphatidylcholine

ACKNOWLEDGMENTS

This work was supported by grants from CONACyT to A.D and R.F.; DGAPA (UNAM), HHMI, and ICGEB to A.D; and NIH (HD32177 and GM56479) to H.M.F. I.L.G was the recipient of a CONACyT graduate fellowship. We thank Dr. F. Gómez-Lagunas for valuable discussions and commenting on the manuscript and Dr. Arturo Hernández-Cruz in whose laboratory the initial experiments were performed.

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Received for publication March 15, 2001
Revised April 27, 2001
Accepted May 2, 2001
Published online June 27, 2001

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Two new scorpion toxins inhibit T-type Ca^{2+} channels in mouse male germ cells and inhibit the sperm acrosome reaction.

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Abstract

Polypeptide toxins that specifically interact with ion channels and modulate their activity are useful pharmacological tools to investigate their function. Recently kurtoxin, a 63 amino acid toxin isolated from the venom of the scorpion *Parabuthus transvaalicus*, was isolated and shown to be active on recombinant T-type Ca^{2+} channels (Chuang *et al.*, 1998, Nature Neurosci 1, 668-674). In this report we characterize two homologous scorpion toxins from the scorpion *Parabuthus granulatus* named kurtoxin-like I and II (KLI and KLII, respectively). KLII and Kurtoxin are the same, and KLI is different in 5 amino acids. Both toxins affect native voltage-gated T-type Ca^{2+} channels in mouse spermatogenic cells and inhibit the physiologically-induced sperm acrosome reaction (AR). KLI and KLII significantly inhibit Ca^{2+} currents in isolated spermatogenic cells without affecting their basic kinetic properties. The effect of the toxins on the channels was weakly voltage-dependent and only partially reversible. A maximum inhibition of ~60% could be reached at saturating concentrations suggesting the presence of an additional toxin-resistant component in the whole-cell Ca^{2+} current in spermatogenic cells. Notably, KLI and KLII produced an inhibition of ~45% in the sperm AR induced by the egg's *zona pellucida*. These results are consistent with the participation of the T-type Ca^{2+} channels in the mammalian sperm AR. On the other hand KLI and KLII are potentially excellent tools to localize and isolate the T-type Ca^{2+} channels from mouse sperm

Developmental Biology 236, 210–219 (2001)
doi:10.1006/dbio.2001.0314 available online at http://www.idealibrary.com on IDEAL®

Calmodulin Antagonists Inhibit T-Type Ca^{2+} Currents in Mouse Spermatogenic Cells and the Zona Pellucida-Induced Sperm Acrosome Reaction

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The sperm acrosome reaction (AR) is a regulated exocytotic process required for gamete fusion. It depends on an increase in $[Ca^{2+}]_i$ mediated by Ca^{2+} channels. Although calmodulin (CaM) has been reported to regulate several events during the AR, it is not known whether it modulates sperm Ca^{2+} channels. In the present study we analyzed the effects of CaM antagonists W7 and trifluoroperazine on voltage-dependent T-type Ca^{2+} currents in mouse spermatogenic cells and on the zona pellucida-induced AR in sperm. We found that these CaM antagonists decreased T-currents in a concentration-dependent manner with IC_{50} values of ~10 and ~12 μM, respectively. W7 altered the channels' voltage dependence of activation and slowed both activation and inactivation kinetics. It also induced inactivation at voltages at which T-channels are not activated, suggesting a promotion of inactivation from the closed state. Consistent with this, W7 inhibited the ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. Likewise, W7 and TFP inhibited the AR with an IC_{50} of ~10 μM. In contrast, inhibitors of CaM-dependent kinase II and protein kinase A, as well as a CaM-activated phosphatase, had no effect either on T-currents in spermatogenic cells or on the sperm AR. Together these results suggest a functional interaction between CaM and the sperm T-type Ca^{2+} channel. They are also consistent with the involvement of T-channels in the AR. © 2001 Academic Press

Key Words: Ca^{2+} channel regulation; T-type Ca^{2+} channel; spermatogenic cells; sperm; CaM; W7; TFP; acrosome reaction.

INTRODUCTION

Voltage-dependent Ca^{2+} channels (VDCC) are required components for the elaborate functioning of excitable and nonexcitable cells. Because of this, their regulation is of capital importance to the control of cellular activity. Extracellular ligands, membrane potential, phosphorylation, Ca^{2+} itself, and diffusible second messengers are all well-established regulators of Ca^{2+} channel activity (Hofmann *et al.*, 1999). Recently, several studies demonstrated that calmodulin (CaM), a 17-kDa highly conserved EF hand protein which constitutes the classical Ca^{2+} receptor inside cells (Means *et al.*, 1982), is implicated in the regulation of cardiac L-type (Zühlke and Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.*, 1999) and neuronal P/Q-type (Lee *et al.*, 1999) VDCC. A deletion in the C-terminal of the ion-conducting α_{1C} subunit of the cardiac L-type VDCC prevents Ca^{2+}-dependent inactivation of the channel (Zühlke and Reuter, 1998). The deletion includes an amino acid sequence, called the IQ motif, which in many other proteins binds CaM (Rhoads and Friedberg, 1997). Furthermore, mutant forms of CaM which cannot bind Ca^{2+} have a dominant-negative action on recombinant L-type Ca^{2+}-channel α_1-subunit inactivation (Peterson *et al.*, 1999;

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0012-1606/01 $35.00

Zühlke *et al.*, 1999). Likewise, biochemical studies indicated that CaM binds to the IQ motif of the L-type channels and mutational analysis showed that Ca^{2+}-dependent inactivation was greatly slowed by substitution of amino acids in the IQ sequence (Peterson *et al* 1999; Qin *et al*., 1999; Zühlke *et al*., 1999).

Ca^{2+} influx through VDCC in sperm is required for the acrosome reaction (AR) an exocytotic event essential for sperm-egg fusion (Darszon *et al*., 1999; Wassarman, 1999). Although the molecular identity of the VDCC involved in this event is currently unresolved, several studies indicate that this channel belongs to the T-type (or the low-voltage activated) family (Arnoult *et al*., 1996a, 1999; Liévano *et al* 1996; Santi *et al* 1996). CaM is believed to participate in the AR because of its localization in the acrosome region (Jones *et al*., 1980; Kann *et al*., 1991) and its redistribution during this reaction (Hernández *et al* 1994). Furthermore, CaM antagonists inhibit the AR in sea urchin sperm (Sano, 1983; Guerrero and Darszon, 1989). These findings motivated us to explore whether CaM could regulate sperm VDCC. However, the study of VDCC in sperm is complicated by difficulties in applying molecular biology and electrophysiology techniques directly to these small, terminal cells. Indeed, only rarely have Ca^{2+} currents been recorded from mature sperm (Weyand *et al*., 1994; Espinosa *et al* 1998). An alternative approach has been to use spermatogenic cells developmental precursors that synthesize proteins for later use in the sperm (Liévano *et al* 1996; Arnoult *et al*., 1996a, 1999; Santi *et al*., 1996). In the present study we examined the effects of specific CaM inhibitors on VDCC activity in mouse spermatogenic cells and on the zona pellucida (ZP)-induced AR in sperm.

MATERIALS AND METHODS

Materials

The following were all from Calbiochem (San Diego, CA): CaM inhibitors *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5); and protein kinase inhibitors: *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89) and 1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN62). CaM inhibitor trifluoperazine dihydrochloride (TFP), calcineurin inhibitory fragment, adenosine 5′-triphosphate (magnesium salt; ATP-Mg_2) phosphocreatine (Di-Tris salt), trypsin (bovine pancreas, type XI), and deoxyribonuclease (DNase; bovine pancreas, type I) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Cell Preparation

Spermatogenic cells were obtained as previously described from 3-month-old CD1 mice testis (Espinosa *et al*., 1998). In the cell suspension pachytene spermatocytes and round and condensing spermatids were most frequently observed as individual cells or synplasts. Inasmuch as similar results were obtained from these stages, data were pooled for presentation. Caudal epididymal mature mouse sperm were mechanically collected from CD1 mice and placed in 1.5-ml microcentrifuge tubes containing medium 199 supplemented with BSA (0.1% w/v), Na^+ pyruvate (30 mg/L) and $NaHCO_3$ (2.2 g/L) at 37°C (Lee and Storey, 1985). After ~10 min the fraction of motile sperm was determined by visual inspection and preparations with <75% motile cells were discarded.

Assay for the Acrosome Reaction

Sperm aliquots (4–5 × 10^6/ml) were incubated at 37°C for 30 min for *in vitro* capacitation. Thereafter 5 ZP equiv/μl prepared as previously indicated (Cross and Meizel, 1989), was added to the sperm suspension to induce AR, which was assayed 30 min later using Coomassie blue G-250 (Visconti *et al*., 1999). The CaM antagonists or the control solvents were added 5 min prior to ZP. To calculate the percentage of AR, at least 100 sperm were assayed per experimental condition for the presence or the absence of the characteristic dark blue acrosomal crescent.

Electrophysiology

Ca^{2+} currents were recorded according to the whole-cell patch-clamp technique (Hamill *et al*., 1981). All recordings were performed at room temperature using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2- to 4-MΩ borosilicate glass micropipettes. Except when indicated, cells were clamped at a holding potential (HP) of −90 mV. Currents were evoked by 14- to 200-ms depolarizing voltage steps (0.25–0.1 Hz), to test potentials ranging from −80 to 40 mV. Capacity transients were electronically compensated and linear leak and residual capacity currents were subtracted online using a P/4 standard protocol. Currents were captured online and digitized at a sampling rate of 10 kHz following filtering of the current record (5 kHz) using a personal computer attached to a TL-1 interface (Axon). Pulse protocols, data capture, and analysis of recordings were performed using pCLAMP software (Axon). To isolate Ca^{2+} currents cells were bathed in a solution containing (in mM) $CaCl_2$ (10); NaCl (130); KCl (3); $MgCl_2$ (2); $NaHCO_3$ (1); NaH_2PO_4 (0.5); Hepes (5); and glucose (10) (pH 7.3/NaOH). The internal (patch pipette) solution consisted of (in mM) $CsMeSO_3$ (110); CsF (10); CsCl (15); $CaCl_2$ (4.6); EGTA (10); Hepes (5); ATP-Mg_2 (4); and phosphocreatine (10) (pH 7.3/CsOH). All drugs were prepared as 10–80 mM stock solutions in dimethylsulfoxide (DMSO) and diluted in the bath solution for each experiment to give the desired final concentration.

$[Ca^{2+}]_i$ Determination

Capacitated sperm (1 × 10^7/ml) were loaded with fluo-3 during a 20-min incubation in a BSA-free medium containing 0.0125% Pluronic F-127 and 5 μM acetoxylmethyl ester dye precursor (Molecular Probes, Eugene, OR). Sperm were immobilized on Cell-Tak-coated coverslips and drugs were added by gentle superfusion. $[Ca^{2+}]_i$ values were determined in single cells with excitation illumination (490 nm, 7 nm slit width) from a 75-W Xe arc selected by a Polychrome II monochrometer (TILL Photonics) and fluorescent emission (>530 nm) collected by photomultiplier tubes at 2-ms intervals, as described previously (Arnoult *et al*., 1999). Solubilized ZP solutions (5 μg/ml) and other solutions were added by a drug-delivery pipette positioned near the sperm head. $[Ca^{2+}]_i$ concentrations were determined using a K_D value for this dye in mouse sperm of 636 nM (Rockwell and Storey, 1999).

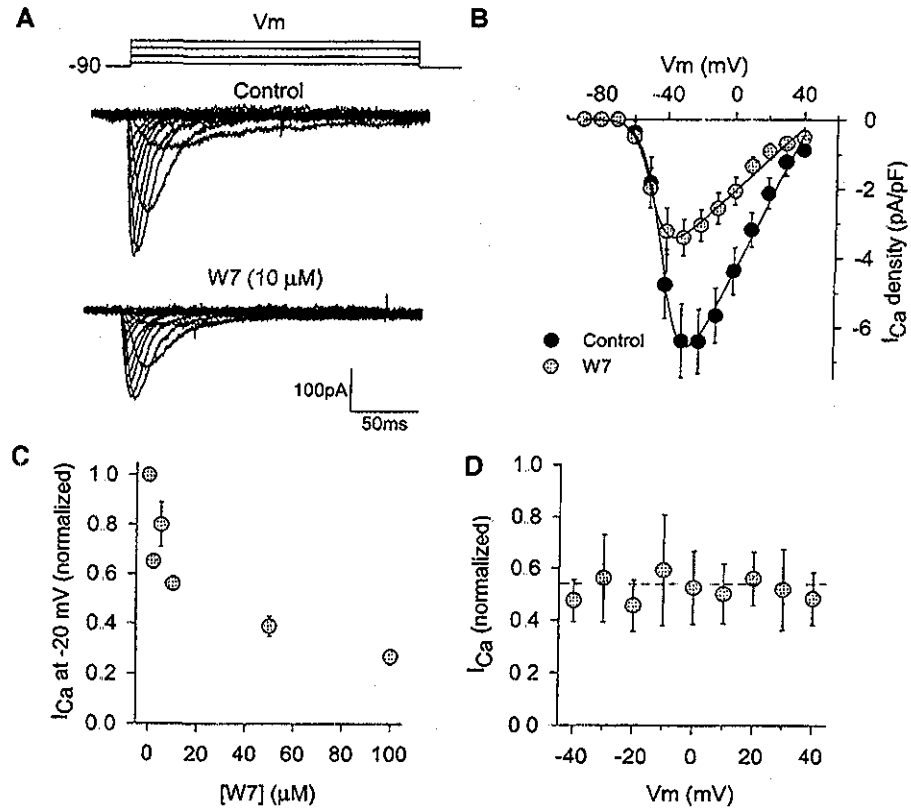

FIG. 1. W7 inhibits Ca^{2+} T-currents in spermatogenic cells (A) Traces in the upper panel illustrate Ca^{2+} currents evoked by 200-ms depolarizations from a HP of −90 mV to test potentials ranging from −90 to 40 mV with a 10-mV increase in the pulse amplitude per step. During 5-min control experiments the rundown of peak current was frequently negligible and always <10%. Following addition of 10 μM of the specific CaM antagonist W7 to the bath-recording solution a significant reduction in current amplitude was observed (lower panel). (B) Average peak current plotted against test potential (V_m) from cells before and after W7 application ($n = 4$) (C) Inhibition of T-channel activity by W7 was dose dependent. Peak currents were normalized by their value before the cells were exposed to W7. (D) W7-induced inhibition of T-currents was voltage independent. Fraction of inhibited current for various depolarizing pulses after W7 treatment. Peak currents in the presence of the drug were normalized, averaged and plotted as a function of test potential

Statistical Analysis

Unless otherwise noted, data are given as means ± SE. The number of experiments is indicated in the figure legends. Statistical differences between two means were determined by Student's t tests. Means were considered significantly different at $P < 0.05$ and are indicated by an asterisk.

RESULTS

As previously reported (Liévano *et al.*, 1996; Arnoult *et al.*, 1996a, 1998; Santi *et al.* 1996; Hagiwara and Kawa 1984) patch-clamp experiments revealed that the only VDCC in mouse spermatogenic cells is of the T-type. The upper traces in Fig. 1A illustrate a family of representative Ca^{2+} currents elicited in a control cell by 10-mV depolarizing pulses from a HP of −90 mV. W7, a CaM antagonist, was used to explore whether CaM regulates sperm VDCC. The lower traces in Fig. 1A show a significant inhibition of the T-current (>45%) after the same cell was superfused with 10 μM W7. Figure 1B summarizes the corresponding current-voltage (I–V) relationships measured at the time of peak current during each record obtained before and during application of W7 in several cells. Figure 1C shows the dose dependence of the W7 inhibition. Currents were elicited by a 20-ms pulse to −20 mV from a HP of −90 mV, measured at the peak and normalized by traces recorded before drug application. Although up to 100 μM W7 could not completely abolish the currents, application of 10 μM W7 to the bath solution inhibited ~50% of the total macroscopic peak current. Thus, this concentration was used thereafter to explore in more detail the actions of W7. As can be seen in Fig. 1D, the inhibition of the T-currents induced by W7 did not exhibit noticeable voltage dependence in the ±40-mV range.

To test whether CaM was indeed involved in the inhibi-

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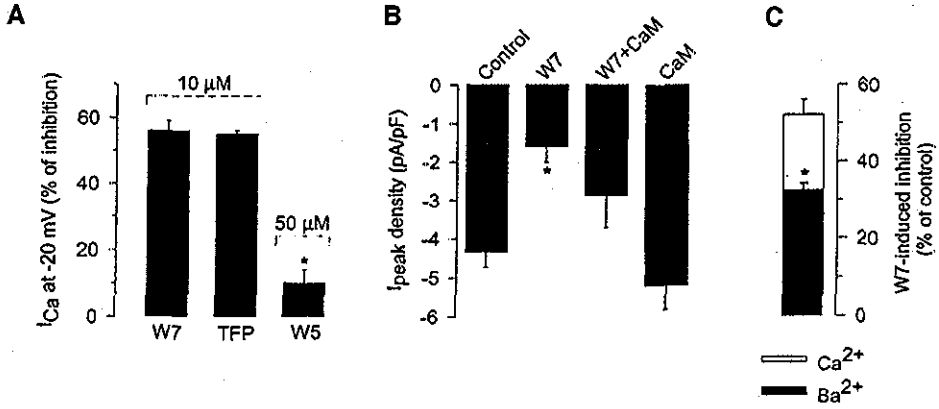

FIG. 2. Inhibition of T-currents by W7 in spermatogenic cells is mimicked by TFP (A) T-channel activity was measured in cells treated with different CaM antagonists. Following exposure to the indicated concentration of the inhibitors, peak current amplitude was normalized to the control and the percentage of inhibition was compared. TFP (10 μM) inhibited the current in a fashion similar to that of W7, whereas 50 μM of W5 (a much less potent CaM antagonist) was unable to cause a significant inhibition of the T-current. (B) Comparison of average Ca^{2+} current density after addition of W7 (10 μM) to the bath-recording solution and CaM (10 μM) to the internal solution, alone or in combination. Peak currents were measured during test pulses to −20 mV from a HP of −90 mV, normalized to cell capacitance and then averaged (n = 5–21). (C) Shown superimposed is the average value of W7-induced inhibition of T-currents in Ca^{2+}- and Ba^{2+}-containing solutions. Currents were evoked by 200-ms pulses to −20 mV from a HP of −90 mV. Asterisks represent significant differences with respect to the control.

tion induced by W7 on the T-currents, we next examined the effects of its structural homolog W5 (same as W7 but lacking a Cl^-), a much less potent CaM antagonist. Even high concentrations of W5 (50 μM) were unable to cause a significant inhibition of the T-currents (Fig. 2A). In contrast, another potent antagonist of CaM, trifluoroperazine (TFP), inhibited the current in a fashion similar to that of W7. To further test whether W7 was directly blocking the T-channel, the inhibition caused by W7 in the absence and in the presence of CaM inside the recording pipette was compared (Fig. 2B). The percentage of current inhibited by 10 μM W7 was significantly smaller when an equimolar concentration of CaM was included in the internal solution. CaM by itself had no effect on the magnitude of the Ca^{2+} currents, which could indicate that endogenous CaM occupies the regulation sites of the channel. In addition, with Ba^{2+} as the permeant ion, we found that W7-induced inhibition was significantly reduced (Fig. 2C), which implies that this action is dependent on Ca^{2+}.

Tail currents were measured to further characterize the inhibitory effect of W7. Repolarization of the plasma membrane to −90 mV after a short depolarization to a test potential of −20 mV caused a rapid increase in the amplitude of the inward Ca^{2+} current resulting from the larger driving force for Ca^{2+} influx. Representative superimposed traces of currents obtained before and during the application of W7 clearly illustrate the inhibition caused by the drug (Fig. 3A, inset). Figure 3A summarizes normalized results from several cells showing that W7 (10 μM) suppressed maximal tail current density by ~46%. Data points were fit with Boltzmann equations to determine activation parameters. Under control conditions, T-current was half-maximally activated at −40.7 ± 1.6 mV. In contrast, acute application of W7 induced a small but significant ($P < 0.05$) ~6.3-mV rightward shift in the voltage dependence of activation (Fig. 3B). Figure 3C compares the apparent time course of current activation in the absence and the presence of the drug. It can be seen that, in addition to decreasing amplitude, W7 treatment resulted in a slight delay in the onset of the current and, consequently, a significant increase in the time required for Ca^{2+} currents to reach the peak (7 ± 0.3 and 9.2 ± 1.5 ms, respectively).

It was recently established that the binding of Ca^{2+}/CaM to IQ regions inhibits high-voltage-activated (HVA) Ca^{2+} channels by promoting inactivation. This mode of CaM regulation was examined in the T-channels of spermatogenic cells. W7 caused a shift of ~3.8 mV toward the depolarizing direction in the average steady-state inactivation curve, as well as a slight change in its slope from a control value of 4.6 ± 0.7 to 5.4 ± 0.8 in the treated cells (not shown). Although the differences in both parameters were not statistically significant, W7 altered the waveform (Fig. 4A). The fraction of current remaining after 80 ms (solid line in Fig. 4A) during 200-ms-long activating pulses (residual current) was significantly increased by W7, suggesting an effect on the inactivation of the T-currents (Fig. 4B). Thus, W7 may restrain inactivation from the open state of the channels or indirectly slow down inactivation as a consequence of a decreased rate of activation. As illustrated in Fig. 4C, the time constant of inactivation significantly increased from 14.1 ± 0.9 to 25.9 ± 1.9 ms after W7 treatment. This observation is consistent with the view

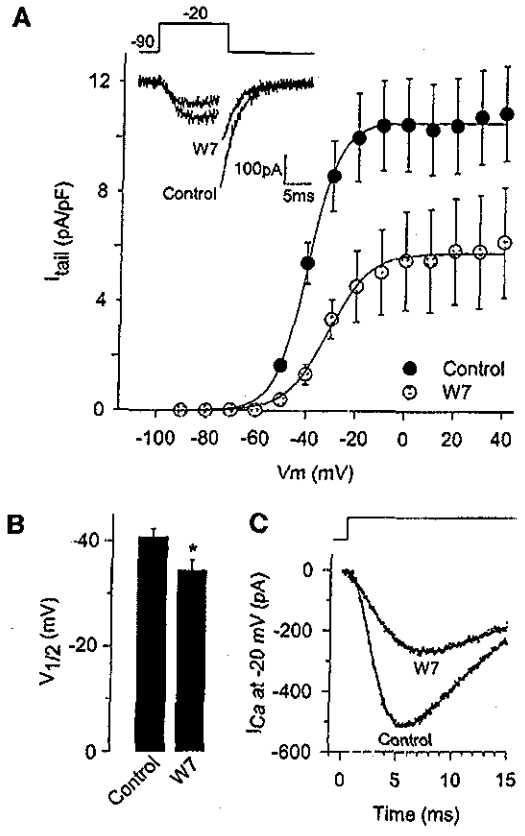

FIG. 3. W7 modulates T-current activation kinetics. (A) Control and inhibited tail currents in a number of cells were normalized by capacitance, averaged, and plotted against membrane potential. Fits of the curves were obtained using a Boltzmann function, $I_{Ca} = I_{max}/(1 + \exp[-(V_m - V_{1/2})/s])$, where V_m is the test potential, $V_{1/2}$ is the potential at which the current has reached half-maximal amplitude, and s is the range of potential for an e-fold change around $V_{1/2}$. Inset: Superimposed traces illustrating Ca^{2+} tail currents obtained before and after application of W7 (10 μM) by subjecting the cell to a depolarizing pulse from a HP of −90 mV to −20 mV for 14 ms and repolarizing the membrane to the HP. (B) Comparison of average $V_{1/2}$ derived from the Boltzmann fits (n = 6–11). (C) Time course of activation of T-currents in both the absence and the presence of W7 was examined in cells with uncompensated series resistance of <6 MΩ. Currents were activated by 20 ms (to −20 mV from a HP of −90 mV) with the best fit of the Hodgkin–Huxley equation (Hille, 1992) superimposed.

that W7 may be acting via CaM, given that this protein induces inactivation of HVA-Ca^{2+} channels. Current inactivation at a step voltage of −20 mV becomes progressively slower as a function of W7 concentration (Fig. 4D, open bars). Although the slowdown of T-channel inactivation caused by W7 should increase current magnitude, this CaM antagonist actually reduces it. We calculated the integral of the currents measured before and after W7 and, as illustrated in Fig. 4D (filled bars), the influx of Ca^{2+} was significantly smaller upon drug application.

Finally, to gain insight into the mechanisms by which CaM antagonists inhibit T-currents in spermatogenic cells, we studied the state-dependent inactivation of the channels. Patch-clamp whole-cell experiments suggested that inactivation could occur from closed states of the T-channel, given that long prepulses (ranging from −120 to −80 mV, voltages at which no channel opening was detectable) decreased current amplitude in response to a test pulse to −20 mV (compare currents at −120 and −80 in Fig. 5A). This difference became greater after the addition of W7. On average, W7 decreased currents to 80 ± 3, 78 ± 2, and 72 ± 5% of the control value after prepulses to −120, −100, and −80 mV, respectively (Fig. 5B).

Recent results indicate that functional T-channels do remain in mature sperm after testicular differentiation and that they play a key role in determining the AR (Arnoult *et al*., 1999). Inasmuch as CaM antagonists proved to be potent regulators of Ca^{2+} channel activity in spermatogenic cells, these agents could also affect the sperm AR. To test this, we first determined whether sperm T-channels were also inhibited by CaM antagonists. In sperm, ZP triggers a $[Ca^{2+}]_i$ transient that has the kinetic and pharmacological characteristics anticipated of a T-channel (Arnoult *et al*., 1999). An example of a rapid ZP-evoked $[Ca^{2+}]_i$ transient is shown in Fig. 6A. ZP-dependent $[Ca^{2+}]_i$ transients were observed in 65% of cells (22/34), with $[Ca^{2+}]_i$ rising to peak levels of 7.09 ± 1.1 μM and relaxing to basal levels within 250 ms. The rising phase of this response is described by a single exponential (τ = 15.2 ± 1.9 ms for 10–90% peak values). In contrast, buffer-treated controls exhibited only monotonic rises to 0.27 ± 0.05 μM during this time period. ZP also evoked $[Ca^{2+}]_i$ responses in 57% (12/21) of sperm treated with 10 μM W7. However, the peak $[Ca^{2+}]_i$ response was inhibited by 73% (2.21 ± 0.89 μM) and the activation time course was slowed (τ = 33.6 ± 4.1 ms). In contrast, 10 μM W5 had only minor effects on ZP-evoked $[Ca^{2+}]_i$ transients (Fig. 6B): responses were observed in 59% of cells (10/17), the maximal response was reduced by only 12% (6.21 ± 0.94 μM), and only a minor slowing of activation time was observed (τ = 17.7 ± 3.1 ms).

CaM antagonists (W7 and TFP) also significantly inhibited the ZP-induced AR in capacitated sperm with an IC_{50} value of ~10 μM (Fig. 7A). Notably, this concentration also inhibits ~50% of the total macroscopic peak current through T-channels in spermatogenic cells (see Fig. 2A). In addition, Fig. 7A shows that, as anticipated from the pharmacology of the T-currents (see Fig. 2A), W5 caused a less-important inhibition of the sperm AR. Furthermore, W7 appears to block AR by altering Ca^{2+} uptake through Ca^{2+} channels because A23187, a Ca^{2+} ionophore that triggers AR in the absence of ZP (Visconti *et al*., 1999), was able to overcome the inhibition caused by W7 (Fig. 7B).

It has been proposed that T-type Ca^{2+} in mouse spermatogenic cells is enhanced by inhibition of protein phosphorylation (Arnoul *et al*., 1997). Because CaM participates in some cell signaling events regulating protein kinases, we therefore thought it pertinent to investigate whether CaM

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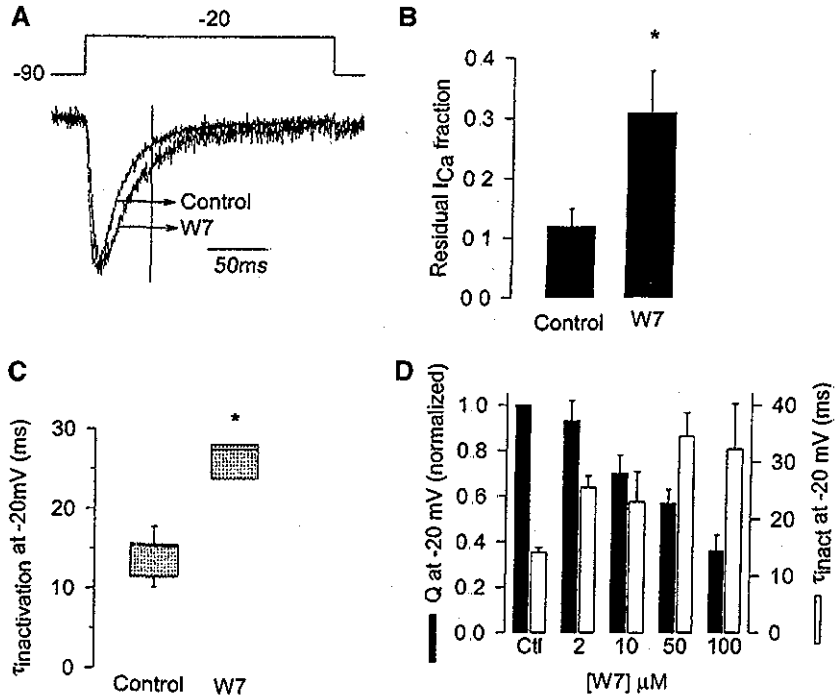

FIG. 4. W7 affects current inactivation. (A) Comparison of the time course of typical current traces in both the absence and the presence of W7 (10 μM). The voltage protocol used to measure inactivation is shown above, and the traces were normalized to allow kinetic comparison. (B) The percentage of current remaining after 80 ms (solid line in A) into a depolarizing pulse before and after W7 application is compared. Currents were recorded in response to 200-ms depolarizing pulses from −90 to −20 mV. (C) The inactivating phase of the currents was fitted with a single exponential: $I_{Ca} = A \exp(-t/\tau) + c$, where A is the initial amplitude, t is time, τ is the time constant for inactivation, and c is a constant. Values of τ of inactivating currents obtained at −20 mV before and after W7 treatment are compared (n = 6–11). (D) Integral of the current (Q) measured during 200-ms command steps to −20 mV from a HP of −90 mV. Currents measured at this command step after W7 application were normalized to the current observed before drug application in each cell (Ctl), averaged, and plotted as a function of W7 concentration (filled bars). Open bars compare τ of inactivation as a function of the concentration of W7 (n = 3–7).

might be involved in this mechanism of Ca^{2+} channel regulation. Whole-cell patch-clamp experiments indicated that micromolar concentrations of the CaM kinase inhibitors KN62 and H89 did not diminish T-currents in spermatogenic cells (not shown). Consistent with this, the sperm AR was not affected by these inhibitors (Fig. 7C). In addition the use of an inhibitory peptide derived from the CaM-dependent serine/threonine protein phosphatase calcineurin did not alter current density through T-type Ca^{2+} nor the time constant of inactivation (data not shown), suggesting that a phosphatase activity is not required for modulation of Ca^{2+} channels in spermatogenic cells.

DISCUSSION

Mammalian sperm must undergo the AR prior to fertilization. The signal transduction steps that lead to the sperm AR are not well understood. This exocytotic event, induced by the ZP surrounding the oocyte, depends on the uptake of external Ca^{2+} (Wassarman, 1999). Growing experimental evidence suggests that a voltage-gated Ca^{2+} channel is an important pathway responsible for modulating Ca^{2+} entry into sperm during this process (Darszon *et al.*, 1999; Arnoult *et al.*, 1996a, 1999). Previous electrophysiological studies of Ca^{2+} currents in spermatogenic cells demonstrated the presence of T-currents (Hagiwara and Kawa, 1984; Liévano *et al.*, 1996; Arnoult *et al.*, 1996a,b; Santi *et al.*, 1996) with pharmacological attributes consistent with those of the AR and the Ca^{2+} influx associated with it (Arnoult *et al.*, 1998). Likewise, it was shown that this channel is retained in the mature sperm (Arnoult *et al.*, 1999) and, therefore, it must be an important element in the transduction events leading to the ZP-induced AR in mammalian sperm (Arnoult *et al.*, 1996a, 1999; Darszon *et al.*, 1999).

Although the T-type Ca^{2+} currents of spermatogenic cells have been characterized, their regulation is poorly understood. Such information is needed to unveil the molecular mechanisms leading to the sperm AR. Recent findings indicate that CaM regulates HVA-channels, contributing to the determination of the intracellular levels of Ca^{2+} (Peter-

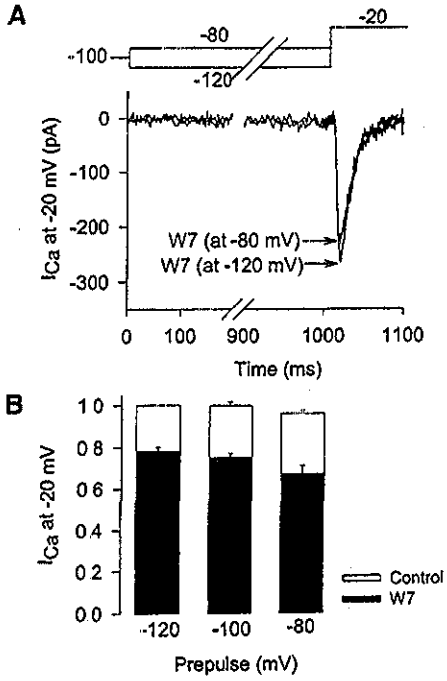

FIG. 5. W7 promotes T-channel inactivation from the closed state. (A) Voltage clamp traces recorded in a cell after application of W7 at two different potentials. Currents were evoked applying 1-s voltage prepulses to −120 and −80 mV followed by a 75-ms test pulse to −20 mV. The amount of inactivation after W7 addition at −80 mV exceeded that observed at −120 mV. (B) Inactivation measured from the test pulses is compared in different cells in both the absence and the presence of W7 during prepulses to −120 −100 and −80 mV (n = 5). Open bars represent the average currents in response to the test potential normalized to that evoked after application of the prepulse to −120 mV in the control cells. Filled bars denote current amplitude after addition of W7 normalized to that obtained before drug application.

son *et al.*, 1999; Qin *et al.* 1999; Zühlke *et al.* 1999; Lee *et al.*, 1999). Previous observations using CaM inhibitors in sea urchin (Sano, 1983; Guerrero and Darszon, 1989) and mammalian sperm (Courtot *et al.*, 1999) suggested the participation of CaM in the AR. With this in mind and considering the advantages of studying ion channels in spermatogenic cells, we explored the possible regulation of the T-type Ca^{2+} channels present in these cells by CaM. The results presented herein show that the specific CaM antagonist W7, at concentrations above 2 μM, markedly reduced T-current magnitude (Figs. 1–3). This effect is concentration dependent and essentially voltage independent (Figs. 1C and 1D). TFP, another CaM antagonist, also significantly reduced current amplitude at concentrations that inhibit CaM (Massom *et al.* 1990). Furthermore, addition of CaM inside the recording pipette decreased the inhibitory effect of W7, suggesting a potential participation of this protein in the regulation of T-type Ca^{2+} channels (Figs. 2A and 2B).

The results presented here are in line with the involvement of CaM in T-type Ca^{2+} channel regulation in spermatogenic cells; though, they are not a definitive demonstration. W7, W5, and TFP can have other less-specific effects such as direct inhibition of plasma membrane and intracellular Ca^{2+} channels and phospholipase C (Ehrlich *et al.*, 1988; Schlatterer and Schaloske, 1996; Schofl *et al.* 1999). However, the inhibitor concentrations used in this study are fitting for CaM inhibition and are lower than those associated with less-specific effects (Ichikawa *et al.*, 1991). In addition the Ca^{2+} dependence of the inhibitory effects of W7 (Fig. 2C) supports the role of a Ca^{2+}/CaM complex in T-channel regulation. Moreover, the direct effects of CaM inhibitors on ion channel activity have been shown to be partly or fully reversible (Laver *et al.*, 1997), whereas the actions of these compounds in our experiments resulted in a nonreversible inhibition (not shown), suggesting an indirect effect on the channels. Finally, when W7 and TFP act directly on ion channels, addition of CaM

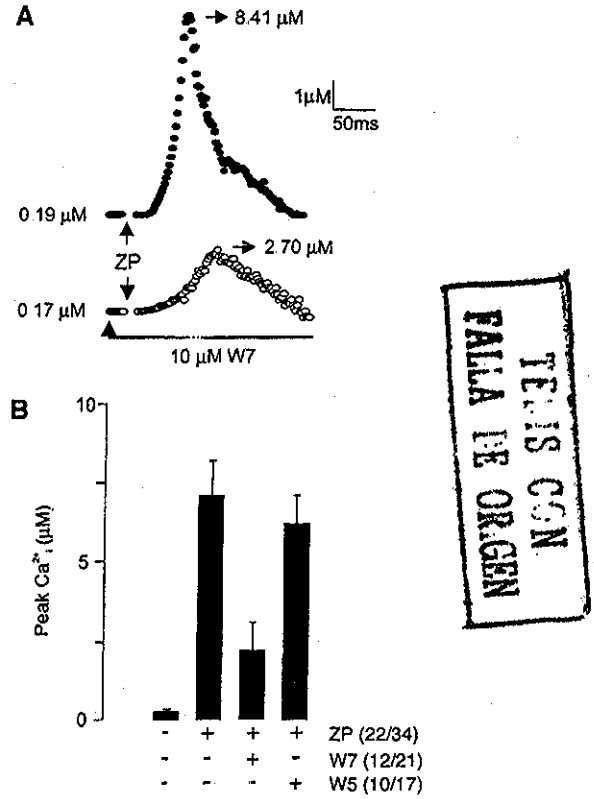

FIG. 6. W7 inhibits ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. (A) Examples of $[Ca^{2+}]_i$ responses evoked by ZP (30 μg/ml) in sperm treated with buffer and with W7. Initial and peak $[Ca^{2+}]_i$ values are indicated. (B) Sperm were incubated for 10 min with buffer (n = 22), 10 μM W7 (n = 12), or 10 μM W5 (n = 10) prior to the addition of ZP. Data represent the peak $[Ca^{2+}]_i$ levels (± SD) during transients and the fraction of total cells exhibiting responses is shown.

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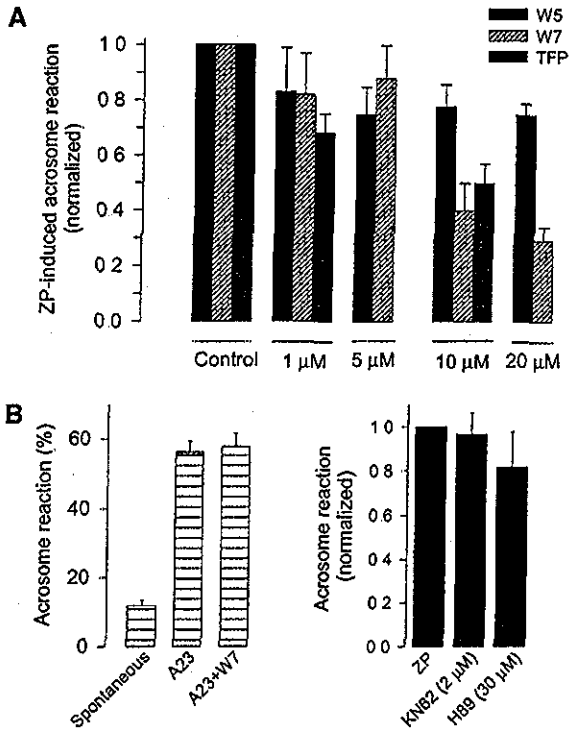

FIG. 7. CaM antagonists affect the sperm AR. (A) Effect of W7 on the percentage of sperm undergoing ZP-induced AR. Sperm were incubated in the presence of increasing concentrations of W7. After capacitation, the percentage of the AR was monitored. Data represent means ± SEM of at least five independent experiments. Sperm were also incubated in the presence of different concentrations of TFP (n = 6) and W5 (n = 7) as listed, and ZP-induced AR was measured. (B) AR inhibition caused by W7 is overcome by A23187. Samples were incubated for 35 min in the presence (15 μM) of the Ca^{2+} ionophore A23187 (A23) alone or in combination with W7 (n = 4). (C) CaM-dependent protein kinase inhibitors do not affect AR. The effects of KN62 and H89 on the mouse sperm AR are compared. Sperm were incubated in both the absence and the presence of these two protein kinase inhibitors at the indicated concentrations. Data represent means ± SEM of five separate determinations.

inside the recording pipette appears to be unable to reverse this interaction (Kleene, 1994). Altogether, these considerations lead us to believe that W7 and TFP influence T-channel function by inhibiting CaM.

It has been proposed that inactivation in HVA-channels involves Ca^{2+} entering through the pore, binding to CaM, and changing its conformation. The Ca^{2+}/CaM complex interacts with the channel, initiating an intramolecular process that culminates in a down-regulation of Ca^{2+} channel activity (Zühlke and Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). In agreement with this, acute application of CaM antagonist W7 results in a partial slowdown of inactivation of the T-type Ca^{2+} channels in spermatogenic cells (Figs. 4C and 4D). However, more striking is the decrease in T-type current amplitude caused by CaM antagonists in these cells. Here we show that, in addition to decreasing current amplitude, submaximal concentrations of W7 drastically change other basic biophysical properties of the current (Figs. 3 and 5). These changes include a positive shift in the voltage dependence of activation and a significant slowing of the apparent activation kinetics. Because activation and inactivation are tightly coupled in T-channels (Serrano *et al.*, 1999), we suggest that the decrease in the rate of activation in the presence of W7 may indirectly slow down the apparent inactivation time course of the channels. In addition W7 appears to promote inactivation from voltages at which T-channels are not apparently activated (Figs. 5A and 5B). Whether CaM directly mediates this mechanism is still not clear.

In summary, the present study shows that CaM antagonists W7 and TFP inhibit with similar potency T-channels in spermatogenic cells monitored by electrophysiological methods and the ZP-induced AR. Notably, a similar concentration of W7 (10 μM) represses the Ca^{2+} transient triggered by ZP in mature sperm assessed by using a fluorescent probe. In contrast W5, a close analog of W7 but a much less potent CaM antagonist, is a poor inhibitor of the T-currents in spermatogenic cells and of the sperm AR and the Ca^{2+} transient associated with it. Furthermore, replacement of external Ca^{2+} by Ba^{2+} or inclusion of CaM inside the recording pipette decreases the inhibition of the T-currents in spermatogenic cells caused by W7. The overall results are consistent with a direct link between T-Ca^{2+} channel activation and the ZP-induced sperm AR, and suggest that CaM may regulate sperm T-channels. In mature sperm this is particularly important because during AR a delicate redistribution and modification of plasma membrane molecules takes place and intracellular Ca^{2+} dynamics are drastically changed (Darszon *et al.*, 1999; Wassarman, 1999; Flesch and Gadella, 2000). In addition this regulation may be important for sperm capacitation, where micromolar concentrations of CaM antagonists W7 and calmidazolium have been shown to significantly inhibit the percentage of capacitated sperm assessed by the chlortetracycline staining method (Si and Olds-Clarke, 2000) and by their ability to undergo AR in response to lysophosphatidylcholine.

ACKNOWLEDGMENTS

This work was supported by grants from CONACyT to A. D. and R.F.; DGAPA (UNAM), HHMI, and ICGEB to A.D.; and NIH (HD32177 and GM56479) to H.M.F. I.L.G. was the recipient of a CONACyT graduate fellowship. We thank Dr. F. Gómez-Lagunas for valuable discussions and commenting on the manuscript, and Dr. Arturo Hernández-Cruz in whose laboratory the initial experiments were performed.

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Received for publication March 15, 2001
Revised April 27, 2001
Accepted May 2, 2001
Published online June 27, 2001

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Dual regulation of the T-type Ca^{2+} current by serum albumin and β-estradiol in mammalian spermatogenic cells

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Received 9 May 2000

Edited by Maurice Montal

Abstract **This study provides evidence for a novel mechanism of voltage-gated Ca^{2+} channel regulation in mammalian spermatogenic cells by two agents that affect sperm capacitation and the acrosome reaction (AR). Patch-clamp experiments demonstrated that serum albumin induced an increase in Ca^{2+} T current density in a concentration-dependent manner, and significant shifts in the voltage dependence of both steady-state activation and inactivation of the channels. These actions were not related to the ability of albumin to remove cholesterol from the membrane. In contrast, β-estradiol significantly inhibited Ca^{2+} channel activity in a concentration-dependent and essentially voltage-independent fashion. In mature sperm this dual regulation may influence capacitation and/or the AR.** © **2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words I-Ca^{2+} channel; Spermatogenic cell; Serum albumin; Bovine serum albumin; β-Estradiol; Sperm capacitation

1. Introduction

Ca^{2+} channels in the mammalian sperm plasma membrane govern Ca^{2+} uptake and thus regulate intracellular signaling. Capacitation, a poorly understood maturational process, renders sperm responsive to physiological agents such as the zona pellucida or progesterone leading to the acrosome reaction (AR). This exocytotic event is required for successful fertilization. Capacitation, while occurring in vivo in the female reproductive tract, can also be accomplished in vitro by incubating sperm in defined medium containing appropriate concentrations of three key components: Ca^{2+}, $NaHCO_3$ and serum albumin [1,2]. In vitro studies have demonstrated that capacitation of mouse sperm is accompanied by metabolic alterations, a time-dependent and cAMP-regulated increase in the protein tyrosine phosphorylation of a subset of proteins [2,3], and changes in the distribution and composition of plasma membrane lipids and phospholipids. The requirement for serum albumin in capacitation has been related to its ability to remove cholesterol from the sperm membrane [4,5]. It has been proposed that cholesterol efflux then leads to changes in the membrane architecture and fluidity that give rise to the capacitated state [6,7]. In addition, the presence of a functional 29 kDa estrogen receptor on the human sperm surface has been recently documented. Its activation results in a rapid and sustained increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), dependent on the presence of extracellular Ca^{2+}, that causes increased tyrosine phosphorylation of sperm proteins and reduced progesterone-induced AR [8].

Extracellular Ca^{2+} appears also to be necessary for the completion of capacitation and for the increase of $[Ca^{2+}]_i$ required during capacitation [2]. In line with this, electrophysiological studies carried out in spermatogenic cells, a developmental precursor stage of mature sperm, have revealed that the voltage-gated T-type Ca^{2+} channel in the plasma membrane [9,10] is a major mediator of Ca^{2+} entry into these cells [11]. The pharmacology of T channels from spermatogenic cells resembles that of the Ca^{2+} entry mechanisms thought to be responsible for the AR in sperm [12]. These findings suggest that the Ca^{2+} T channels play an important role in the AR and could participate in the regulation of $[Ca^{2+}]_i$ during capacitation. Unfortunately, the size, complex geometry and highly differentiated and motile nature of sperm preclude its systematic electrophysiological characterization. In this report we used spermatogenic cells as a model system to study whether compounds that regulate capacitation and the AR are able to modulate T channels. Our results show that albumin and β-estradiol can regulate Ca^{2+} T channels in spermatogenic cells, and suggest that in mature sperm they may influence capacitation and the AR.

2. Materials and methods

2.1. Materials

Bovine albumin (essentially fatty acid-free prepared from bovine albumin fraction V), 17β-estradiol (1,3,5[10]-estratriene-3,17β-diol), and cholesterol 3-sulfate (sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

2.2. Cell preparation

Spermatogenic cells were obtained as described previously [13]. Briefly, testes from adult CD1 mice were excised and suspended in ice-cold dissociation solution. The tunica albuginea was removed and the seminiferous tubules were separated. Tissue fragments were extruded from a single tubule and dispersed into individual cells or

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PII: S0014-5793(00)01688-4

symplasts (aggregates), collected by centrifugation, resuspended in external recording solution (see below) without Ca^{2+} and stored on ice until assayed. Spermatogenic cells or symplasts at two different stages of differentiation (pachytene spermatocytes and round spermatids) were preferentially observed and used in electrophysiological recordings. Inasmuch as similar results were obtained from both stages, data were pooled for presentation.

2.3. Electrophysiology

Ca^{2+} currents were recorded using the whole-cell patch-clamp technique [14] at room temperature using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2–4 MΩ micropipettes. Cells were clamped at a holding potential (HP) of −90 mV and capacity transients were electronically compensated. Currents were evoked by 50–125 ms depolarizing voltage steps to test potentials ranging from −70 to +20 mV. To measure Ca^{2+} channel inactivation at steady state, cells were held for 210 ms at potentials ranging successively from −75 through −36 mV prior to a 90-ms step depolarization to a test potential of −25 mV. Linear leak and residual capacity currents were subtracted on-line using a P/4 standard protocol. Current records were captured on-line and digitized at a sampling rate of 5 kHz following filtering of the current record (2 kHz). Series resistance was compensated by ≥50%. Pulse protocols, data capture and analysis of recordings were performed using pCLAMP software (Axon). To isolate Ca^{2+} currents, cells were bathed in a solution containing (in mM): $CaCl_2$ 10; NaCl 130; KCl 3; $MgCl_2$ 2; $NaHCO_3$ 1; NaH_2PO_4 0.5; HEPES 5; glucose 10 (pH 7.3). The internal (patch pipette) solution consisted of (mM): CsCl 100; CsF 10; EGTA 5; HEPES 5; ATP-Mg_2 4; phosphocreatine 4 (pH 7.3). Bovine serum albumin (BSA) was diluted in the bath solution to give the desired final concentration. Cholesterol 3-sulfate and 17β-estradiol were prepared as 100 mM stocks in dimethylsulfoxide (DMSO) and stored at −20°C. A fresh aliquot was diluted to its final concentration in the bath solution for each experiment. Final concentration of DMSO typically was <0.001%.

3. Results and discussion

The possibility that serum albumin, a required component in media that supports capacitation, may modulate sperm Ca^{2+} channels has not been directly examined. Inasmuch as electrophysiological experiments are very difficult to perform in mature sperm, we used spermatogenic cells as a model system to determine the influence of albumin on their Ca^{2+}

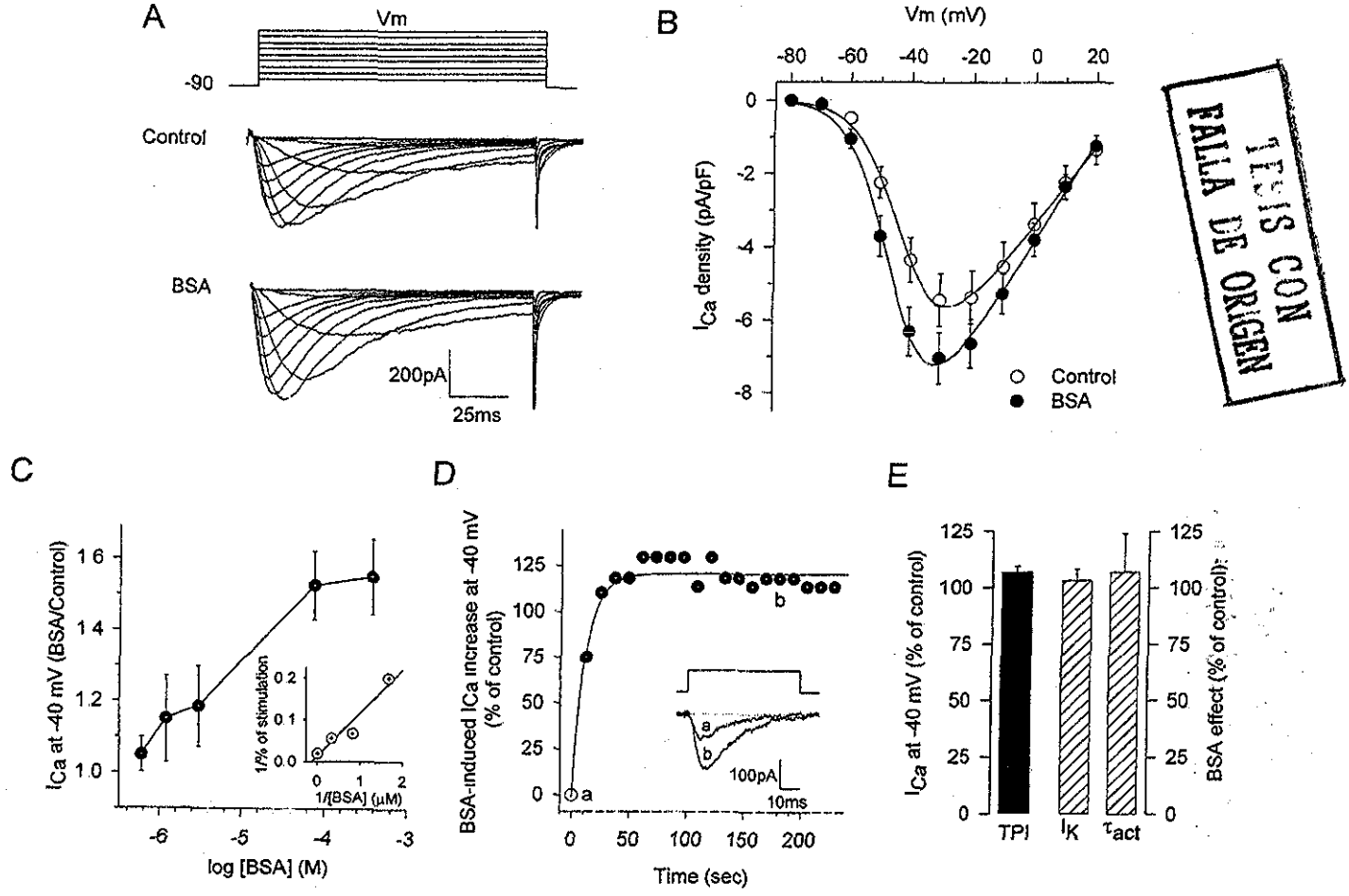

Fig. 1. BSA enhances Ca^{2+} current in mouse spermatogenic cells. A: Voltage-gated T currents (I_{Ca}) recorded, according to the protocol shown above, from a representative cell in the absence (upper traces) and in the presence of 0.5% BSA (lower traces). B: Current–voltage (I–V) relationships for steady-state activation of I_{Ca} before and after BSA application. Values represent mean ± S.E.M. from peak currents normalized by cell capacitance. C: Semilogarithmic plot of current density increase as a function of BSA concentration. Data were obtained in response to 100 ms depolarizations to −40 mV from a HP of −90 mV. Inset: Lineweaver–Burk plot derived from the BSA concentration–response data. D: Representative plot of peak current as a function of time. Data were approximated by an exponential function (solid line). Inset: Individual traces taken from the time course shown; letters designate the regions of the curves represented. E: BSA specifically increases T current in spermatogenic cells. Application of 75 μM triosephosphate isomerase (TPI) was not effective in increasing Ca^{2+} current amplitude. The solid bar corresponds to the summary of normalized peak current in cells subjected to TPI ($n=4$). Hatched bars summarize a comparison of current amplitude (I_K) and time constant of activation (τ_{act}) before and after BSA (75 μM) application ($n=4$). Currents were recorded in response to 200 ms depolarizing pulses from −90 to +50 mV.

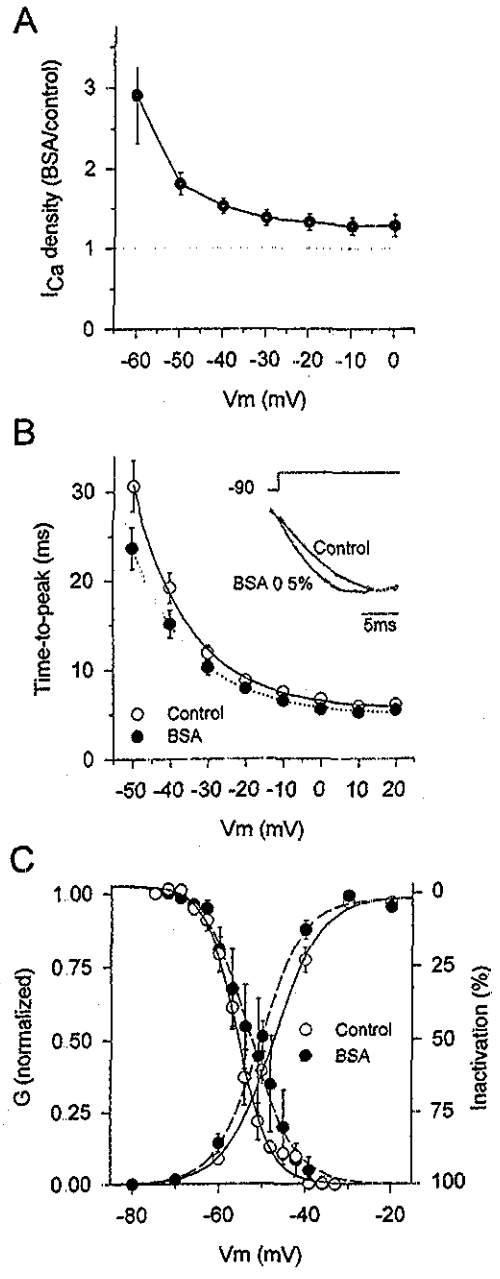

Fig. 2. Bovine serum albumin affects the kinetics of I currents in spermatogenic cells. A: Voltage dependence of the relative increase in current density induced by BSA. The ratios of current density after BSA application over the control at different voltages from the same cells in Fig. 1B are shown. B: Time-to-peak–voltage relationship for activation of I_{Ca}. Smooth lines correspond to exponential fits of data ($n = 11$). Inset: Example traces taken from a representative cell included in the time-to-peak–voltage relationship. Currents are shown normalized and only the first 15 ms are displayed to allow comparison of kinetics. The activating phase of the currents was fitted with single exponential equations (superimposed lines). C: BSA significantly increases the window current in spermatogenic cells. Comparison of steady-state activation and inactivation in control and BSA-treated cells. The fraction of inactivated channels was plotted as a function of prepulse voltage and data were fitted with Boltzmann equations (control, solid line; BSA-treated cells, dotted line). Symbols represent mean ± S.E.M. values of 3–5 cells in each condition. Conductance (G) was calculated from the Goldman–Hodgkin–Katz equation with an equilibrium potential for Ca^{2+} calculated using the MaxChelator (MAXC v5.0) software. Data were averaged and then fitted with Boltzmann equations (smooth curves). *Symbols represent mean ± S.E.M. values of 11 cells in each condition.*

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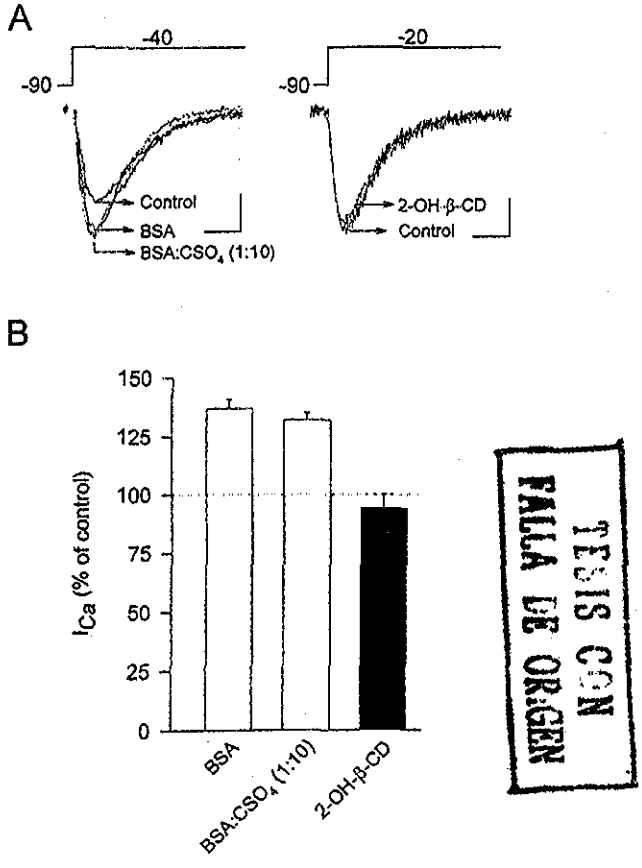

Fig. 3. The effects of BSA on I-channels in spermatogenic cells are not related to its ability to serve as cholesterol acceptor. A: Montage of current traces obtained first in the absence of BSA (control) and then treated with 15 μM BSA presaturated with cholesterol, followed by a third incubation in the presence of BSA alone. The scale bars represent 50 pA and 25 ms (left panel). The right panel shows representative superimposed current traces obtained before and after application of 500 μM of 2-OH-β-cyclodextrin (2-OH-β-CD). B: Histograms comparing the effect of a 2 min incubation with BSA alone, BSA presaturated with cholesterol sulfate (CSO_4), and 2-OH-β-CD on Ca^{2+} current amplitude ($n = 4$–6).

I currents. A pachytene symplast subjected to the patch-clamp technique showed typical Ca^{2+} currents (Fig. 1A) in response to 10 mV voltage steps from a HP of −90 mV before (upper traces) and 2 min after incubation with 75 μM of BSA (lower traces). Test pulses elicited a rapidly activating and inactivating inward current (I_{Ca}) whose amplitude was significantly increased in the BSA-treated cells. The magnitude of these currents was normalized by cell capacitance and the averaged results plotted as a function of membrane potential in Fig. 1B. Having established significant differences in I current density, we next determined the concentration dependence and time course of the BSA action. The effects of BSA were concentration-dependent with a maximal increment of I current occurring at 75 μM. This increase was normalized with respect to the control, averaged and plotted against the logarithm of concentration (Fig. 1C). A concentration–

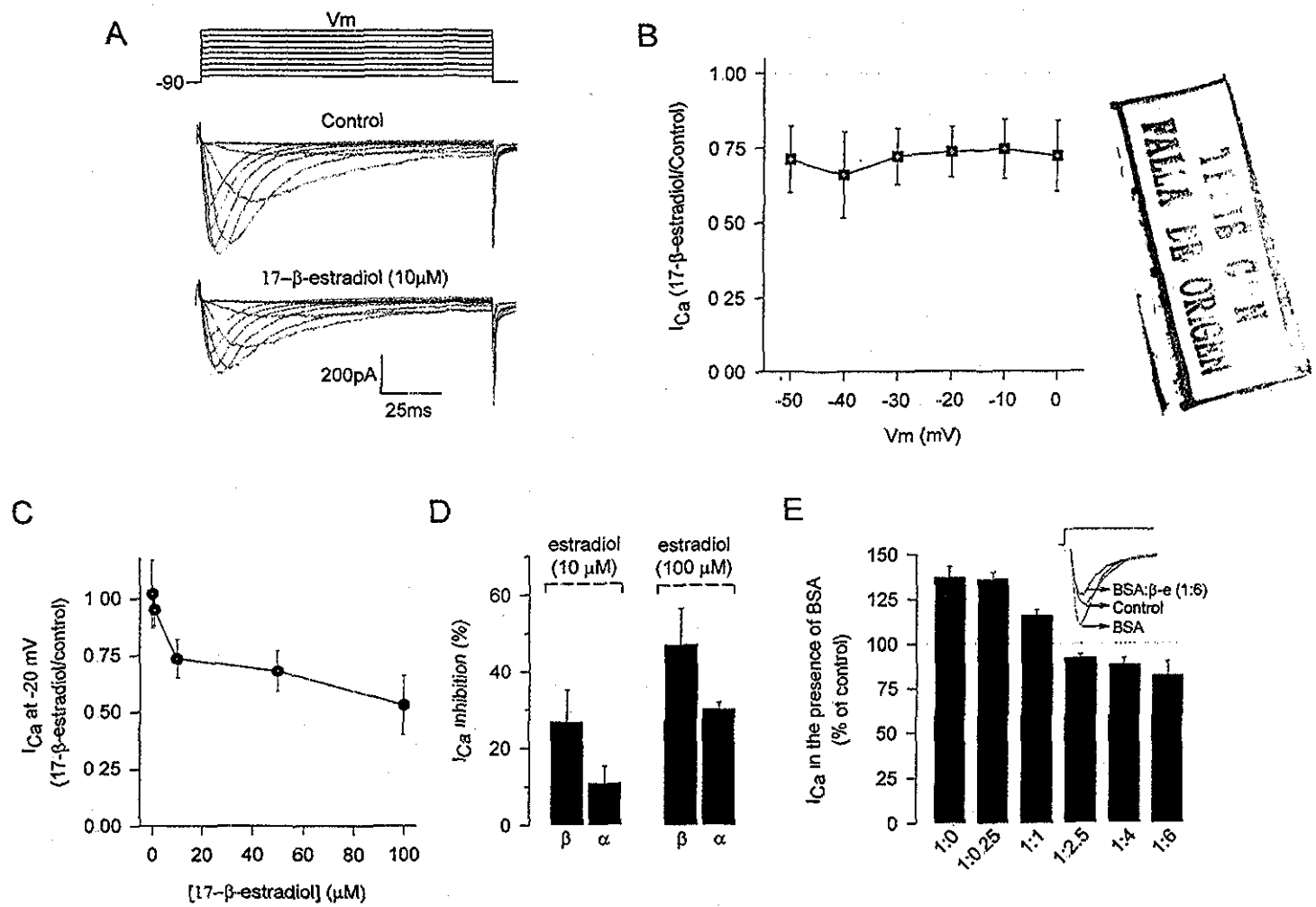

Fig. 4. 17β-Estradiol inhibits Ca^{2+} current in spermatogenic cells. A: I currents recorded in a representative cell before (upper panel) and after a 2 min incubation in 17β-estradiol. B: Peak currents after β-estradiol application at each test potential were averaged and normalized to control. C: Currents measured in response to 200 ms depolarizing pulses from a HP of −90 mV to a test potential of −20 mV before and after application of different concentrations of estradiol normalized to the control. Data represent mean ± S.E.M. of 3–8 experiments. D: Comparison of current inhibition after a 2 min incubation in the presence of β or α isomers ($n = 3$ and 8, respectively). Currents were evoked with depolarizing pulses from a HP of −90 to a test potential of −20 mV and the concentration of estradiol was 10 (left bars) or 100 μM (right bars). E: The ratio of current before and after presaturating BSA with various concentrations of 17β-estradiol is shown. Inset: Superimposed current traces evoked by a voltage step from −90 to −40 mV. 17β-Estradiol-presaturated BSA was added at a concentration of 15 μM. After incubation for 2 min, cells were treated with BSA alone. Bars denote pooled results showing the effect of BSA presaturated with increasing concentrations of 17β-estradiol on the T current.

response curve was generated (Fig. 1C, inset), where the reciprocal of the normalized average current in response to BSA was plotted against the reciprocal of the BSA concentration. Data were then fitted using a Lineweaver–Burk equation and a K_D of ~8 μM was calculated. Fig. 1D illustrates that BSA increased the Ca^{2+} T current within seconds of administration. Peak current was measured every 12 s in response to depolarizations from a HP of −90 mV to a test potential of −40 mV (inset), and the time course was approximated by a single exponential expression with $\tau \approx 11$ s. This modulation was specific since the use of a non-related protein such as triosephosphate isomerase did not significantly affect Ca^{2+} current amplitude (Fig. 1E, solid bar). Furthermore, control experiments demonstrated that a 2 min incubation with BSA had no effect on the magnitude and kinetics of whole-cell K^+ currents recorded in spermatogenic cells (Fig. 1E, hatched bars).

The enhancement of Ca^{2+} current by BSA was voltage-dependent. Fig. 2A compares current–voltage (I–V) curves obtained before and after BSA application and shows that albumin increased Ca^{2+} current density at negative voltages most markedly (from −60 to −40 mV). We next examined current activation kinetics. Records were obtained at different potentials and both the time-to-peak and time constant of activation were measured. In the control cells, time-to-peak decreased monotonically with voltage from 30.6 ± 2.8 ms at −50 mV to 6.1 ± 0.4 ms at +20 mV, reaching a voltage-independent minimum of about 6 ms at 0 mV. Treatment with BSA decreased the time-to-peak to 23.6 ± 2.3 and 5.5 ± 0.3 ms at −50 and +20 mV, respectively, with values statistically different in the voltage range below −30 mV (Fig. 2B). In addition, the time constant of activation after BSA decreased 1.3-fold on average with respect to the control at −40 mV. In these experiments currents were recorded in response to depolarizations from a HP of −90 mV to a test potential of −40 mV and the traces were fitted to single exponential equations

(Fig. 2B, inset) with time constants of 9 ± 1.1 and 6.9 ± 1.1 ms for the cells before and after BSA treatment, respectively.

Calculating whole-cell conductance and fitting the average normalized data with Boltzmann equations (Fig. 2C) we found that T currents after BSA treatment activated at lower test potentials ($V_{1/2} = -47.5$ in the control and -50.5 mV in the treated cells). In addition, inactivation was studied by applying 210 ms pulses followed by a test pulse to −25 mV, as described in Section 2, to measure channel availability. Average data were fitted to Boltzmann equations and comparison of the T current before and after BSA treatment indicated significant differences in the voltage-dependent inactivation at steady state ($V_{1/2} = -58.5$ versus -52.9 mV). Notably, there were voltages at which channels were activated during the prepulse but they were not completely inactivated, as evidenced by currents evoked during the test pulses. This activity is referred to as a window current and is typically illustrated by the overlap in the steady-state activation and inactivation curves (Fig. 2C). It can be clearly seen that T currents after treatment had a larger window region. At the peak of the window (~ −52 mV) 28 and 44% of the channels are available to be open in the control condition and after BSA administration, respectively.

To gain insight into the mechanisms underlying the albumin regulation of T channels, we next investigated whether this effect was a consequence of its well-known ability to serve as a cholesterol binding molecule. Fig. 3A (left panel) shows that the addition of BSA presaturated with a high concentration (1:10 molar ratio) of an analog of cholesterol (cholesterol-SO_4^-) or cholesterol (not shown) produced a significant increase in current amplitude (~ 1.3-fold) that was not modified by a second application of BSA alone. Neither cholesterol nor cholesterol-SO_4^- by itself modified T current properties (not shown). In addition, a 2 min incubation with 2-OH-β-cyclodextrin, a cyclic heptasaccharide able to promote a rapid cholesterol efflux from the sperm membrane and to induce an increase in tyrosine phosphorylation patterns similar to those seen in response to BSA during sperm capacitation [7], had no effect on the magnitude and kinetics of the T current (right panel). Together these results, summarized in Fig. 3B, provide evidence that BSA is not functioning here through its ability to remove cholesterol from the cell membrane.

Previous studies have shown that acute application of β-estradiol inhibits T-type channels in smooth muscle cells [17] as well as L-type channels in neurons [15], and cardiac and smooth muscle myocytes [18–22]. Thus it is possible that serum albumin could be enhancing T currents by removing a hydrophobic molecule such as β-estradiol. To test this hypothesis, spermatogenic cells were first directly superfused with increasing concentrations of 17β-estradiol and patch-clamp Ca^{2+} currents were recorded (Fig. 4A). As anticipated, currents in treated cells were smaller at all voltages tested (Fig. 4B). A small inhibition (~ 10%) was observed at nM concentrations and became significant at μM concentrations (Fig. 4C). This inhibition was voltage-independent (Fig. 4B) and too fast (< 2 min) to be attributed to genomic activation. Unlike what has been reported for high voltage-activated channels [15,16], this inhibition probably does not involve the activation of a G protein signaling pathway since spermatogenic T channels are basically G protein-independent [17]. A hormonally inactive isomer of 17β-estradiol (17α-estradiol) caused less reduction in T channel activity (Fig. 4D) indicating some specificity in the response. We next explored whether BSA stimulation could be antagonized by 17β-estradiol. As shown in Fig. 4E, BSA presaturated with 17β-estradiol did not increase peak current but induced a small reduction of current amplitude which was reversed by a second addition of BSA alone (inset). This finding is consistent with the hypothesis that the enhancement of T current caused by BSA might be due to the removal of 17β-estradiol rather than cholesterol from the plasma membrane. However, as shown in Fig. 4E, a progressive increase in 17β-estradiol inhibited T channel activity only at molar ratios > 1:2.5, raising the question whether this effect was caused by the presence of free 17β-estradiol. In addition, since BSA affects the T currents in a voltage-dependent manner while estradiol does not, it is likely that their effects are independent. However, these results suggest a potential physiological regulation of estrogens on spermatogenic cells. On the other hand, recently it was shown that 17β-estradiol can increase $[Ca^{2+}]_i$ by activating a novel functional estrogen receptor on the cell surface [8]. Our data suggest that this effect of estradiol is not mediated through T channels.

Mature sperm have only a single secretory vesicle (acrosome) and exocytosis must be coordinated with egg contact for efficient fertilization. Our results suggest that T-type Ca^{2+} channels, due to their window current, may contribute to setting $[Ca^{2+}]_i$ at the resting potential and therefore influence sperm capacitation. Since BSA increases the window current by modifying the voltage dependence of activation and inactivation, it could increase Ca^{2+} influx. As there is evidence that T channels are present in mature sperm [12] where resting potential is about −50 mV [23], BSA could facilitate an increase in Ca^{2+} entry, a prerequisite to capacitation. It is also possible that the actions of BSA on the T current may result from the removal of a hydrophobic molecule present in the sperm plasma membrane which suppresses premature secretion until the completion of capacitation, at which time sperm have arrived near the site of fertilization. The signaling mechanism by which 17β-estradiol reduces Ca^{2+} currents must await further investigation. Interestingly, numerous studies have shown that the function of membrane-spanning proteins can be affected by the material properties of the lipid bilayer through hydrophobic interactions between the protein and the bilayer [24]. Since 17β-estradiol is present in the sperm plasma membrane [6], the question arises as to whether changes in its concentration may be translated into an increase in the structural stress in the bilayer, and this force may be transmitted to sperm Ca^{2+} channels residing therein.

Acknowledgements: This work was supported by grants from DGAPA (UNAM), HHMI, ICGEB and CONACyT to A.D. F.E. was the recipient of a DGEP (UNAM) predoctoral fellowship. P.E.V. and G.S.K. were supported by NIH HD06274. We are grateful to *Dr. C.L. Treviño for critically reading the manuscript as well as to* J. Monroy for expert technical assistance.

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DEVELOPMENTAL GENETICS 25:103–114 (1999)

Anion Channel Blockers Differentially Affect T-Type Ca^{2+} Currents of Mouse Spermatogenic Cells, α1E Currents Expressed in *Xenopus* Oocytes and the Sperm Acrosome Reaction

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ABSTRACT The direct electrophysiological characterization of sperm Ca^{2+} channels has been precluded by their small size and flat shape. An alternative to study these channels is to use spermatogenic cells, the progenitors of sperm, which are larger and easier to patch-clamp. In mouse and rat, the only voltage-dependent Ca^{2+} currents displayed by these cells are of the T type. Because compounds that block these currents inhibit the zona pellucida-induced Ca^{2+} uptake and the sperm acrosome reaction (AR) at similar concentrations, it is likely that they are fundamental for this process. Recent single channel recordings in mouse sperm demonstrated the presence of a Cl^- channel. This channel and the zona pellucida (ZP)-induced AR were inhibited by niflumic acid (NA), an anion channel blocker [Espinosa *et al.* (1998): FEBS Lett. 426:47–51]. Because NA and other anion channel blockers modulate cationic channels as well, it became important to determine whether they affect the T-type Ca^{2+} currents of spermatogenic cells. These currents were blocked in a voltage-dependent manner by NA, 1,9-dideoxyforskolin (DDF), and 5-nitro-2-(3-phenylpropylamine)benzoic acid (NPPB). The IC_{50} values at −20 mV were 43 μM for NA, 28 μM for DDF and 15 μM for NPPB. Moreover, DDF partially inhibited the ZP-induced AR (40% at 1 μM) and NPPB displayed an IC_{50} value of 6 μM for this reaction. These results suggest that NA and DDF do not inhibit the ZP-induced AR by blocking T-type Ca^{2+} currents, while NPPB may do so. Interestingly, 200 μM NA was basically unable to inhibit α1E Ca^{2+} channels expressed in *Xenopus* oocytes, questioning that this α subunit codes for the T-type Ca^{2+} channels present in spermatogenic cells. Evidence for the presence of α1C, α1G, and α1H in mouse pachytene spermatocytes and in round and condensing spermatids is presented. Dev. Genet. 25:103–114, 1999. © 1999 Wiley-Liss, Inc.

Key words: anion channel blockers; Ca^{2+} channels; acrosome reaction

INTRODUCTION

The acrosome reaction (AR) is an exocytotic event triggered when sperm contact the extracellular layers of the oocyte. This reaction allows sperm to penetrate and fuse with the egg [Wassarman and Litscher, 1995]. Ionic fluxes are fundamental for the AR to occur, both in mammals and in marine invertebrates [reviewed in Schackmann, 1989; Florman *et al.*, 1998; Darszon *et al.*, 1999]. The AR requires an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), which depends on external Ca^{2+} [Florman *et al.*, 1998]. Ca^{2+} channel antagonists preclude the $[Ca^{2+}]_i$ increase and inhibit AR. These results demonstrate the fundamental participation of voltage-dependent Ca^{2+} channels (VDCCs) in the AR [Darszon *et al.*, 1999].

The electrophysiological characterization of mammalian sperm Ca^{2+} channels has been very difficult. Sperm are tiny morphologically complex cells, unable to synthesize proteins [Hetch, 1988]. Their progenitors, the spermatogenic cells, synthesize the ion channels that will end up in mature sperm. Pachytene spermatocytes and round and condensing spermatids are at the later stages of differentiation, translationally active, and much larger than sperm, making them easier to patch-clamp [Hagiwara and Kawa, 1984; Liévano *et al.*, 1996; Santi *et al.*, 1996; Arnoult *et al.*, 1996b]. Tran-

Contract grant sponsor: CONACyT; Contract grant sponsor: DGAPA; Contract grant sponsor: Howard Hughes Medical Institute; Contract grant sponsor: International Centre for Genetic Engineering and Biotechnology

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Received 13 January 1999; Accepted 31 March 1999

scripts for α1E, and to a minor extent α1A, subunits that code for the pore and voltage sensor of different voltage-activated Ca^{2+} channels, were found in pachytene spermatocytes and in round spermatids [Liévano *et al.*, 1996]. More recently, an α1C isoform was detected by reverse transcription (RT) in situ polymerase chain reaction (PCR) in spermatogenic cells [Goodwin *et al.*, 1998; Benoff, 1998]. Pachytene spermatocytes and round spermatids only display T-type Ca^{2+} channels [Santi *et al.*, 1996; Arnoult *et al.*, 1996]. The T-type Ca^{2+} currents of spermatogenic cells are blocked by Ni^{2+}, dihydropyridines, and amiloride, at concentrations that inhibit the zona pellucida (ZP)-induced sperm Ca^{2+} uptake and AR, which suggests that they play a key role in this process [Liévano *et al.*, 1996; Arnoult *et al.*, 1996]. Although T-type Ca^{2+} channels from brain and heart were recently cloned [Perez-Reyes *et al.*, 1998; Cribbs *et al.*, 1998], it is unclear which α1 subunit codes for T-type Ca^{2+} channels in spermatogenic cells and other tissues [Meir and Dolphin, 1998; Piedras-Rentería *et al.*, 1997; Piedras-Rentería and Tsien, 1998].

Cl^- channels have been implicated in the mammalian sperm AR [Wistrom and Meizel, 1993; Meizel, 1995]. The first single channel recordings in mouse sperm showed the presence of a niflumic acid (NA)-sensitive Cl^- channel [Espinosa *et al.*, 1998]. Interestingly, the zona pellucida (ZP) and γ-aminobutyric acid (GABA)-induced AR were inhibited by similar μM concentrations of NA, suggesting the participation of this anion channel in the reaction. However, NA and other anion channel blockers [White and Aylwin, 1990; Tilman *et al.*, 1991] have been shown to modulate diverse cationic channels both positively or negatively. For example, NA increases the open probability of single channel transitions [Ottolia and Toro, 1994], and the macroscopic currents [Gribkoff *et al.*, 1996] of heterologously expressed Ca^{2+}-dependent K^+ channels. By contrast, N-methyl-D-aspartate (NMDA) [Lerma and Martín del Rio, 1991] and nicotinic [Séguéla *et al.*, 1993] receptors are negatively modulated by NA. T-type Ca^{2+} channels could also be targets of this blockers. In this study, the anion channel antagonists, NA, dideoxyforskolin (DDF), and nitrophenylprolylamine benzoic acid (NPPB) were found to block the T-type Ca^{2+} currents of pachytene spermatocytes and the mouse sperm AR with different potencies. α1E channels expressed in *Xenopus* oocytes were basically insensitive to 200 μM NA. Evidence is presented for the presence of α1C, α1G, and α1H in spermatogenic cells.

MATERIALS AND METHODS

Materials

Essential and nonessential amino acids 1×, M-199, collagenase, bovine serum albumin (BSA), NA, and DDF, were from Sigma (St. Louis, MO). Trizol, Superscript preamplification system, *Taq* DNA polymerase, 1-kb DNA ladder from Gibco-BRL (Gaithersburg MD). Other reagents and salts were from the highest quality commercially available.

Spermatogenic Cell Dissociation and Purification

Testis from adult CD-1 mice were used as a source of germ cells. They were decapsulated, and the seminiferous tubules suspended in EKRB (in mM: 120.1 NaCl, 4.8 KCl, 25.2 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 11 glucose, 1 glutamine, 1× essential and nonessential amino acids, adjusted to pH 7.2 with CO_2). Spermatogenic cells for patch-clamp studies were obtained by mechanical dissociation. DNase (100 μg/ml) was added to the EKRB dissociation solution (the external recording solution but depleted of Ca^{2+}) immediately before the dissociation procedure. After discarding the *Tunica albuginea*, seminiferous tubules were separated using forceps under the stage of a stereoscopic microscope. Cells were extruded from the seminiferous tubules by holding one extreme of the tubule with forceps and "squeezing" the rest of the tubule in the opposite direction with another forceps. The cells were then pipetted repeatedly to separate them. The cells were dissociated and stored on ice. Spermatogenic cells remained healthy for ≥12 h. Spermatogenic cells for purification were obtained as described [Liévano *et al.*, 1996]. The resulting cell suspension was resuspended into EKRB 0.5% BSA and filtered through a 80-μm mesh Nytex nylon filter (Tetko, Lancaster NY), and stored at 4–10°C. The single-cell suspension of germ cells was sedimented at unit gravity through a 2–4% BSA linear gradient generated on a staput chamber (Johns Scientific, Ontario). Gradients were collected after 2.5 h in 10-ml fractions. Fractions were assessed for cell morphology and purity by light microscopy using Nomarski optics, and similar fractions were pooled.

Patch-clamp of Pachytene Spermatocytes

An aliquot of the mechanically dissociated spermatogenic cell suspension was placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon) with recording medium (in mM: 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-Hepes, 5 glucose, 10 $CaCl_2$, pH 7.35) at 20–23°C. All cells used in this study were at the pachytene stage of differentiation, either as single cells or as synplasts [see Santi *et al.*, 1996]. Whole-cell currents were recorded with an Axopatch 1-B amplifier in the voltage-clamp mode under the command of the pClamp program (Axon Instruments, Foster City, CA), connected to the pipette and the bath by Ag-AgCl wires. The composition of the pipette internal solution was (in mM: 110 Cs-methanesulfonate, 10 CsF, 15 CsCl, 5 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 10 Cs-Hepes, pH 7.35). Glass borosilicate pipettes were pulled to tip diameters of about 1.8 μm, with resistances of 2–3.5 MΩ, when filled with pipette solution. Records were low-pass

filtered at 2 kHz (4-pole Bessel filter), digitized at 10 kHz, and analyzed off-line. A P/4 pulse protocol was used routinely to minimize leak and capacitive currents from current records; series resistance was compensated by ≥50%. Anion channel blocker stock solutions (200 mM NA, 100 mM DDF and NPPB (ICN, Costa Mesa, CA), in dimethylsulfoxide (DMSO) were added to the recording chamber to reach the concentrations indicated Figures 1–5, without changing pH. For each cell tested, controls were recorded; the blocker was then added to the bath and 2–5 min later, the pulse protocol repeated and recorded. In some experiments, the blocker was washed with >50 times the chamber volume (~200 µl) and records obtained again. To achieve final DDF concentrations of 200 µM, it was necessary to sonicate the bath solution.

AR Inhibition Experiments

Sperm were obtained from CD-1 mice >3½ months killed by cervical dislocation. The cells were collected from excised epididymides and suspended (3.6×10^6/ml) in M-199 media supplemented with 25 mM $NaHCO_3$, 1 mM sodium pyruvate, and 0.4% BSA, were capacitated at 37°C for 30 min in a 5% CO_2 atmosphere. Thereafter, the heat-solubilized ZP was added and cells left to acrosome react for 30 min. Inhibition was studied with the same procedure except the blocker was added 5 min before the ZP. At the end of the experiment, AR was determined by established procedures [Boettger-Tong *et al.*, 1992]. A minimum of 100 sperm were counted under light-field microscopy. Results were normalized by substracting the spontaneous AR and considering the A-23187 induced AR as 1.

Expression of Recombinant α1E Ca^{2+} Channels

Stage V and VI oocytes were surgically removed from tricaine-anesthetized *Xenopus laevis* and treated for 1.5 h with 1.5 mg/ml collagenase in Ca-free ND-96 medium (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 Hepes, 2 pyruvic acid, pH 7.6). After recovering for 18–24 h, oocytes were injected in the nucleus with 10 nl of cDNA coding for α1E Ca^{2+} channel subunit [Soong *et al.*, 1993] at a concentration of 0.22 ng/nl (a gift from Dr. Snutch). Oocytes were incubated at 17°C for 3–5 days in ND-96 containing 1.8 mM $CaCl_2$ and 50 mg/ml gentamicin. Macroscopic currents were recorded at 16–18°C with a two-electrode voltage-clamp amplifier (Dagan CA-1; Dagan Corp.). Acquisition and data analysis were performed using pClamp (v6.0) software (Axon Instruments). Leak currents were subtracted using a P/4 protocol. Voltage and currents electrodes (0.3–2 MΩ tip resistance) were filled with 1 M KCl, 10 mM EGTA, and 10 mM HEPES (pH 7.4). Oocytes were placed in a 500-µl recording chamber and whole cell currents were measured with Ba^{2+} in the external solution (in mM: 10 $BaCl_2$, 1 KOH, 110 NaOH, 10 Hepes, titrated to pH 7.2, with methane sulfonic acid). To minimize kinetic contamination by endogenous Ca^{2+}-activated Cl^- currents, oocytes were injected with 50 nl of a BAPTA solution (in mM: 10 BAPTA, 10 Hepes, titrated to pH 7.2, with

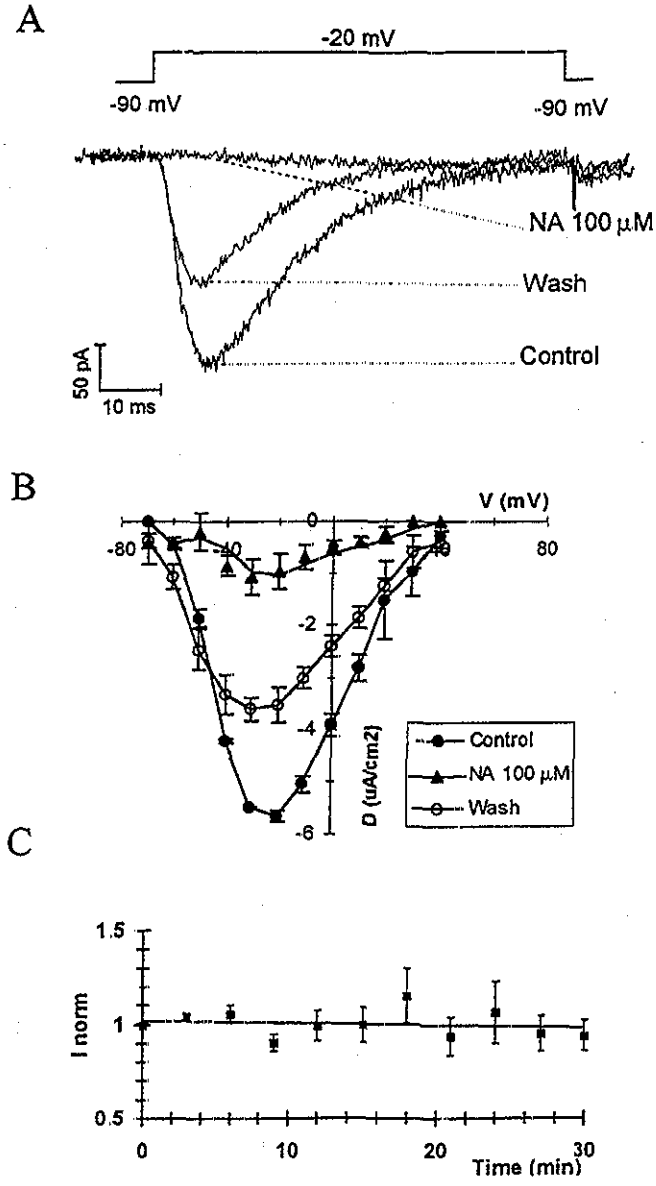


Fig. 1. Niflumic acid (NA) blocks T-type Ca^{2+} currents in spermatogenic cells. Mouse spermatogenic cells were mechanically obtained and whole-cell currents recorded in pachytene spermatocytes as described under Methods. **A:** Representative traces of currents obtained applying pulses to −20 mV from a holding potential of −90 mV in the absence (Control) and presence of 100 µM NA (NA) and after washing the blocker (Wash). **B:** I-V curves of control (*filled circles*), 100 µM NA (*closed triangles*) and recovery after blocker removal (*open circles*). Current density was obtained dividing the peak current at each voltage by the capacitance [Santi *et al.*, 1996]. **C:** Time stability of peak T-type Ca^{2+} currents at −20 mV. Results are averages ±SEM; n = 4

CsOH) 1–1.5 h before the experiment [Parent *et al*, 1997]. Voltage pulses were applied from a holding potential of −90 mV at 10-mV steps until +40 mV. Data were sampled at 10 kHz and filtered at 1 kHz.

For blockage experiments, the chamber solution was changed, superfusing with 14–40 times the chamber volume, with the 10 mM Ba^{2+} solution containing 200 μM NA or 20 μM nifedipine, and incubating 5–7 min before recording.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from seminiferous tubules and purified spermatogenic cells were extracted with Trizol reagent. Before each RT, 5 μg of each total RNA was dissolved in 10 μl water and digested with 1 U RNase-free DNase (RQ1; Promega, Madison, WI) in the transcription buffer (in mM: 50 KCl, 2.5 $MgCl_2$, Tris-Cl 20, pH 8.4) for 10 min at 37°C, and the DNase was heat-inactivated at 65°C for 15 min. At this point, random hexamers were added to a final concentration of 12.5 ng/μl, and quickly chilled on ice. RT was achieved with the Superscript pre-amplification system, according to the manufacturer's instructions. Similar results were obtained by priming the cDNA synthesis with oligo-dT_{12-18}. Control reactions were done in the same conditions, but omitting the RT enzyme. For PCR, we designed oligonucleotides against α1C, forward (5′-GCGAATTCAC-NGGNGARGAYTGGAAY) and reverse (5′-GCGG-ATCCCATYTCNGGYTCYTCYTCRTC), α1G, forward (5′-CGGGATCCAATAAGGACTGACTCCCTGGA) and reverse (5′-CCCAAGCTTGGTGGGCTGAGTTTITT-CTTA) and α1H, forward (5′-CGGGATCCGAYAAYTG-GAAYGGNATHATG) and reverse (5′-CGGAATTCNG-TYTCCATYTCNACYTCYTG) Ca^{2+} channel subunits. Amplifications were performed on a Mastercycler 5330 programmable thermal controller (Eppendorf) with *Taq* DNA polymerase. PCR reactions were carried out at a final volume of 50 μl, having 200 μM dNTP, 0.25 μM each primer, 1.5 mM $MgCl_2$. Mixtures were overlaid with mineral oil, and PCR was initiated by adding 2.5 U of *Taq* DNA polymerase per reaction. Program was for α1C 94°C 5 min, and cycling as follows, denaturation at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 2 min, during 35 cycles, final extension 72°C for 5 min; for α1G 94°C 5 min, and cycling as follows, denaturation at 94°C for 45 s, annealing at 55°C for 90 s, extension at 72°C for 1 min, during 40 cycles, final extension 72°C for 5 min and for α1H 94°C 5 min, and cycling as follows, denaturation at 94°C for 45 s, annealing at 43°C for 90 sec, extension at 72°C for 30 s, during 40 cycles, final extension 72°C for 5 min. Amplimer identity was obtained by double-strand sequencing of the cloned fragment into Bluescript KS^+, using the Termosequenase kit (Amersham), according to the supplier's instructions.

RESULTS

Anion Channel Blockers Inhibit T-Type Ca^{2+} Currents

Whole-cell recordings were obtained on mechanically dissociated mouse spermatogenic cells. As previously reported in rat [Hagiwara and Kawa, 1984] and mouse [Liévano *et al*, 1996; Santi *et al*, 1996; Arnoult *et al*, 1996], T-type currents were the only voltage-dependent Ca^{2+} currents these cells display. T-type Ca^{2+} currents are classified as low-voltage-activated Ca^{2+} currents with fast inactivation kinetics [Perez-Reyes *et al*, 1998a]. Accordingly, T-type Ca^{2+} currents were elicited upon ~125-ms depolarizations from a holding potential of −90 mV to potentials of −70 to +40 mV, using 10-mV increments. Peak currents were measured both before and after adding the anionic channel blocker. Figure 1A depicts T-type Ca^{2+} currents obtained by depolarizing to −20 mV before, after exposure to 100 μM NA, and posterior to washing the blocker. This concentration of NA completely blocked the Ca^{2+} current. Current recovery after washing was partial for the blockers tested. Figure 1B shows the complete I-V curves for the three conditions described above. Control currents at −20 mV are stable for at least 30 min (Fig. 1C), ruling out current washout as a source of current reduction.

Figure 2 shows concentration curves for T-type Ca^{2+} current blockade by NA, DDF, and NPPB. NA and DDF inhibited peak current (Imax) at −20 mV with IC50s of 43 μM and 28 μM, respectively (Fig. 2A,B). NPPB seems to have two different binding sites, I_{max} decreased reaching a plateau at 45% inhibition of 0.5–10 μM, higher concentrations increased blockage reaching 100% at 100 μM (Fig. 2C).

Figure 3 illustrates the voltage-dependent blockade by these anion channel blockers (ACBs). At low voltages, the T-type Ca^{2+} currents from spermatogenic cells are poorly blocked. More depolarized potentials enhance blockage. For example, at −50 mV, 50 μM DDF blocked 34% of the current, whereas at +10 mV, the block was 76%.

DDF and NPPB Inhibit the ZP-Induced Acrosome Reaction

It was previously reported that NA blocks a Cl^- channel (IC_{50} = 11 μM) and the ZP (IC_{50} = 1 μM) induced AR with similar affinities [Espinosa *et al*, 1998]. The sensitivity of T-type Ca^{2+} currents to ACBs suggests that their blockage could cause NA to inhibit the sperm AR. Therefore, it was important to compare the potency of these ACBs to inhibit the AR induced by ZP with their ability to block T-type Ca^{2+} channels.

DDF only partially inhibits the mouse sperm ZP-induced AR (Fig. 4A). At 1 μM, it inhibits ~45% of the AR, with higher concentrations apparently damaging the cells. By contrast, NPPB inhibited AR with an IC_{50} of ~6 μM (Fig. 4B). Thus, smaller concentrations of NA and DDF were required to inhibit the ZP-induced AR

than to block T-type Ca^{2+} currents from spermatogenic cells.

α1E Ba^{2+} Currents Expressed in *Xenopus* Oocytes Are Very Mildly Sensitive to Niflumic Acid

Although currently under active debate, the α1E gene has been considered one of the candidates to code for the T-type Ca^{2+} channel pore subunit [Soong *et al.*, 1993; Liévano *et al.*, 1996; Ertel and Ertel, 1997]. To test this proposal further, cDNA coding for the α1E Ca^{2+} channel subunit from rat brain was expressed in *X. laevis* oocytes. Figure 5 shows Ba^{2+} currents elicited by a depolarization from a holding potential of −90 mV to 10 mV both in the absence and in the presence of 200 μM NA. It turns out that α1E-expressed Ba^{2+} currents were basically insensitive to NA. Only a 7% blockage was seen with 200 μM of this compound, a concentration that totally inhibits T-type Ca^{2+} currents in spermatogenic cells. Furthermore, experiments done with 20 μM nifedipine, which completely blocks spermatogenic T-type Ca^{2+} currents [Liévano *et al.* 1996; Arnoult *et al.*, 1996, 1998], corroborated the insensitivity of α1E-expressed Ca^{2+} currents in *Xenopus* oocytes to this blocker [Soong *et al.*, 1993] (not shown). These results question the possibility that α1E codes for T-type Ca^{2+} channels in spermatogenic cells (see under Discussion)

α1C, α1G, and α1H Are Present in Spermatogenic Cells

The work by Liévano *et al.* [1996] on the α1 subunits present in spermatogenic cells was done with probes to the α1 subunits known at the time. Then, the only α1 subunit that, under certain conditions, displayed some similarities to T-type Ca^{2+} currents was α1E. Liévano and colleagues found that only α1E, and to a minor extent α1A, were present in spermatogenic cells. Recently, using RT in situ PCR, Goodwin *et al.* [1998] showed the presence of α1C in spermatogenic cells. In addition, Meir and Dolphin [1998] reported that α1C expressed in HEK293 cells produces L-type as well as T-type single channel transitions. Furthermore, two α1 subunits named G and H were recently cloned from rat brain and human heart respectively, which when expressed yield Ca^{2+} currents with T-type characteristics [Perez-Reyes *et al.*, 1998a; Cribbs *et al.*, 1998]. This findings and the results described in the previous section motivated our group to determine whether α1G and α1H were present in spermatogenic cells, and to use a more sensitive assay to detect α1C

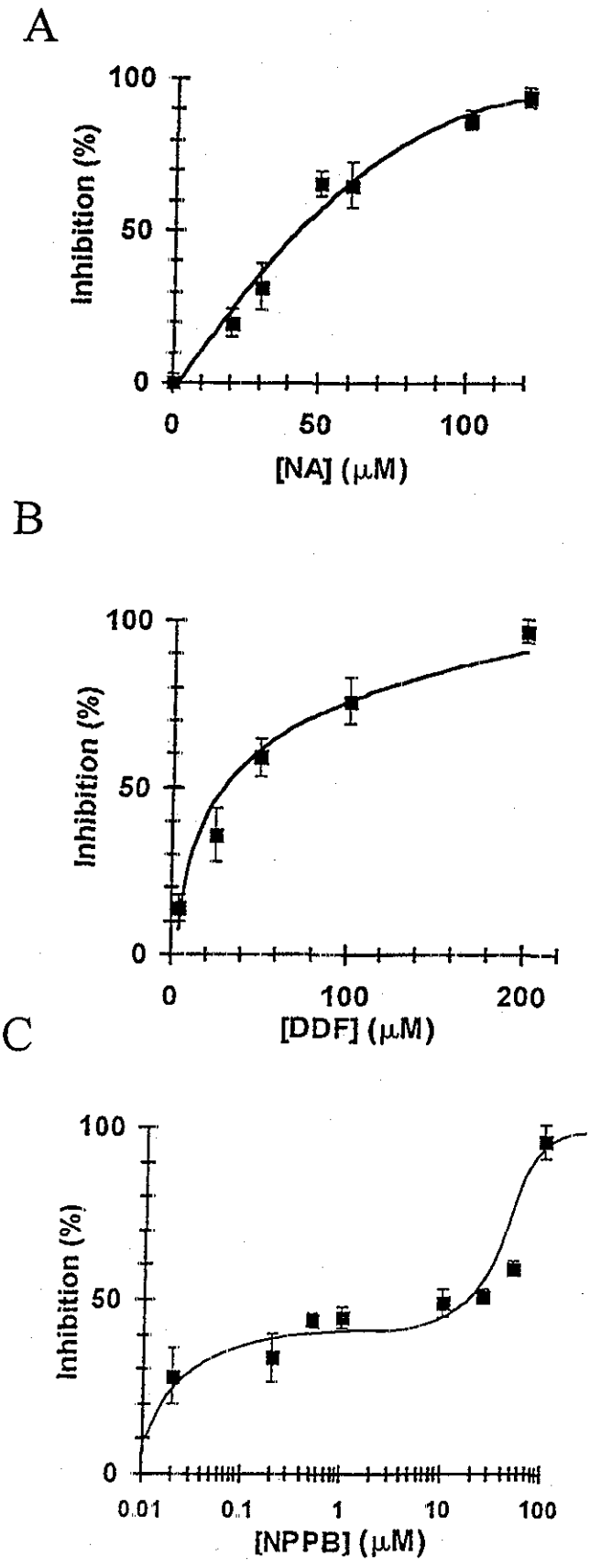
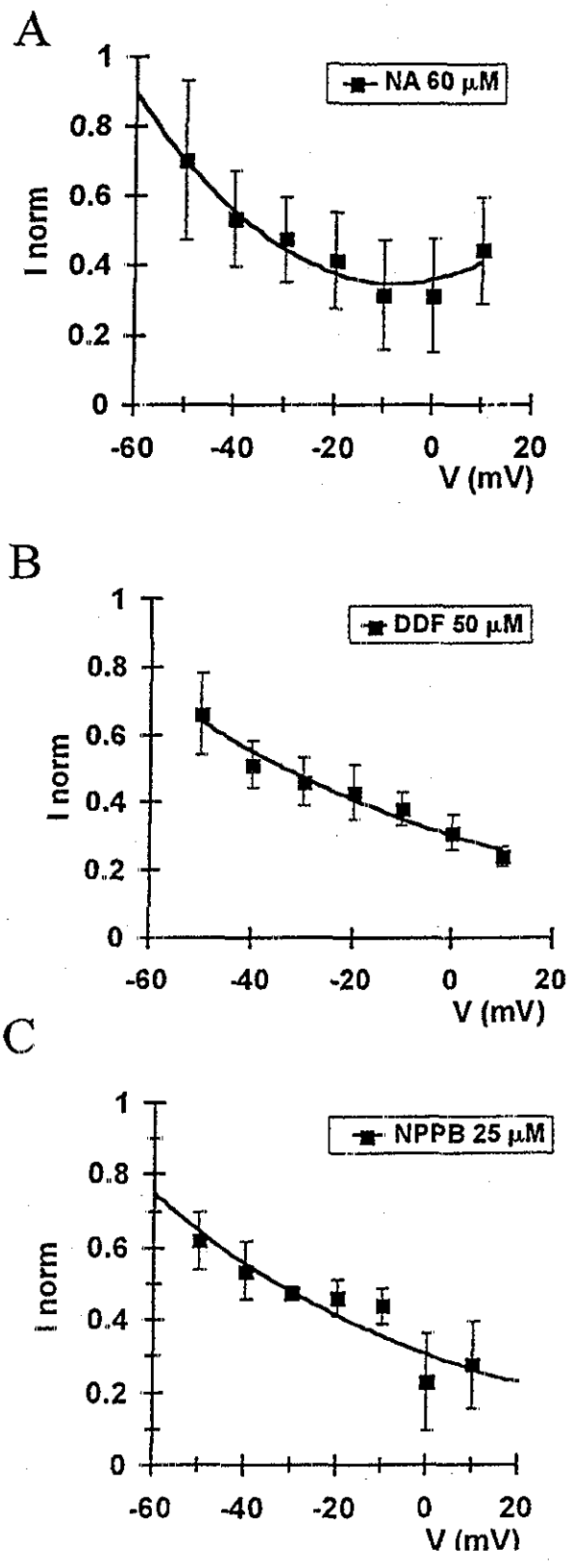


Fig. 2. Dose-response curves of peak T-type Ca^{2+} current blockade in individual pachytene spermatocytes or synplasts by anion channel blockers. Peak currents were normalized with respect to the control. IC_{50} values for niflumic acid (NA) (A) and dideoxyforskolin (DDF) (B) were 43 μM and 28 μM, respectively. Nitrophenylpropylamine benzoic acid (NPPB) (C) appears to have two different binding sites with apparent affinities of 25 nM and 75 μM. Results are averages ±SEM; n = 4.



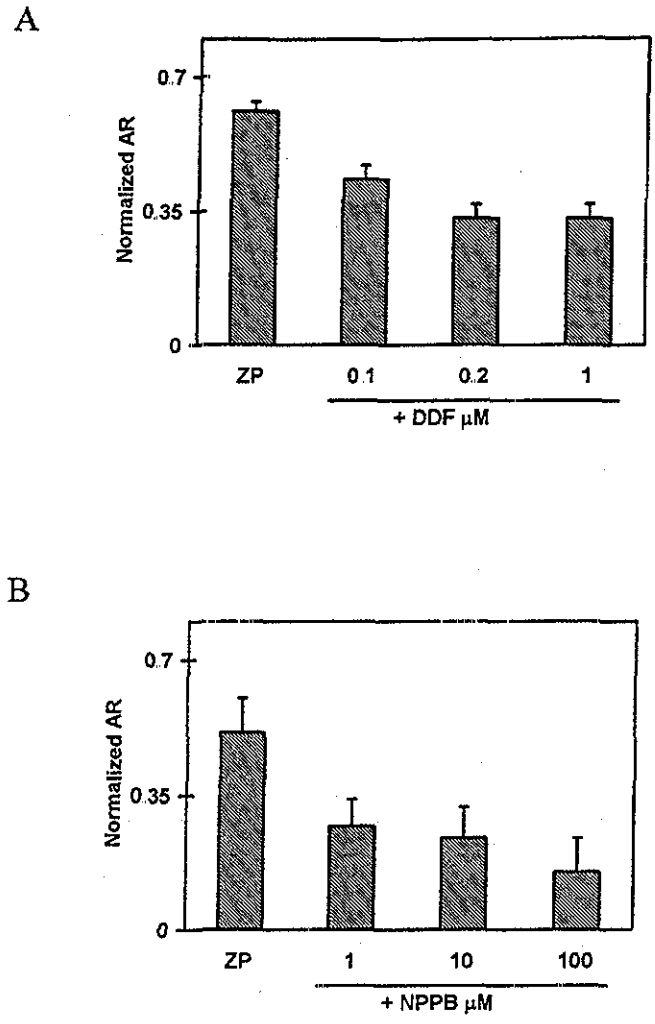
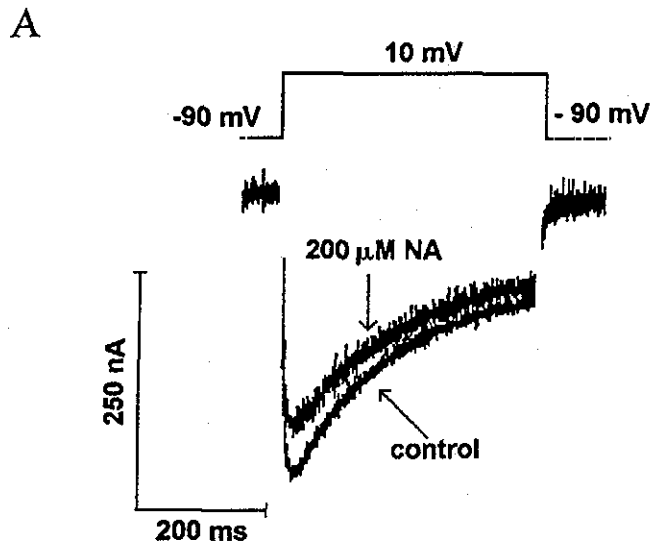


Fig. 4. Dideoxyforskolin (DDF) (A) and nitrophenylpropylamine benzoic acid (NPPB) (B) inhibit the mouse sperm acrosome reaction (AR) induced by solubilized zona pelucida (5 ZP/µl) Acrosome reaction (AR) and its inhibition were determined as indicated under Methods Bars = mean ±SEM; n ≥ 3 Results were normalized considering the A-23187 induced AR as 1 and the spontaneous AR substracted

Figure 6 illustrates the electrophoretic pattern of the amplimers obtained in the RT-PCR experiments using oligonucleotides for α1G. It was possible to amplify a fragment of the expected size in mouse brain, testis, pachytene spermatocytes, and round and condensing spermatids. The amplimer identity was confirmed by double-strand sequencing. Similarly, amplimers of the

Fig. 3. Blockage by (A) niflumic acid NA (60 µM), (B) dideoxyforskolin DDF (50 µM), and (C) nitrophenylpropylamine benzoic acid NPPB (25 µM) is voltage dependent Results are the averages of the normalized peak T-type Ca^{2+} currents of individual pachytene spermatocytes or synplasts as a function of applied voltage Results are averages ±SEM; n = 4



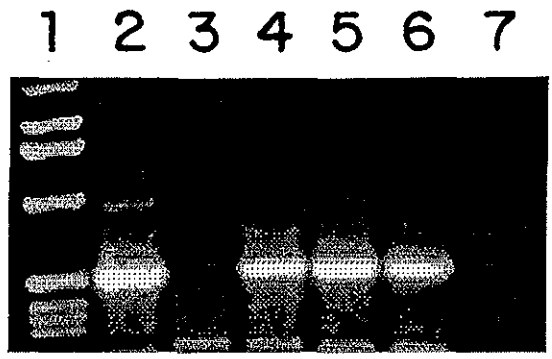


Fig. 6. RT-PCR experiments showing the expression of Ca^{2+} channel α1G subunit in brain (*lane 2*), testis (*lane 3*), pachytene spermatocytes (*lane 4*), round (*lane 5*) and condensing (*lane 6*) spermatids and the reaction without cDNA (*lane 7*); 1-kb DNA ladder (*lane 1*). Amplicon sizes were ~460 bp.

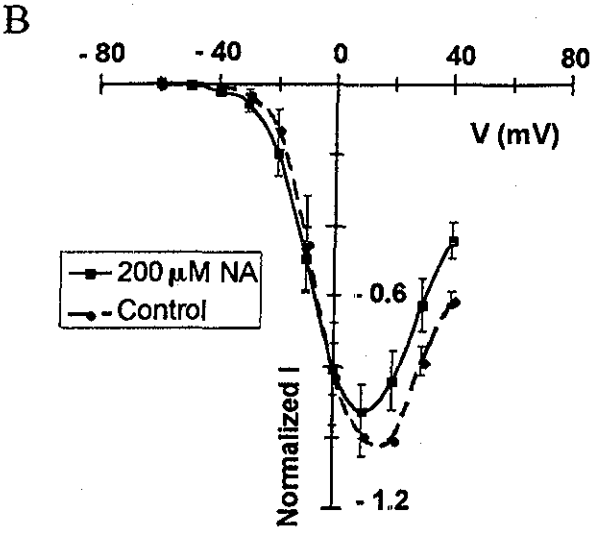


Fig. 5. Expression of α1E in *Xenopus* oocytes yields macroscopic Ba^{2+} currents basically insensitive to niflumic acid (NA). Oocytes were nuclear injected with ~2 ng cDNA coding for the α1E subunit. **A:** Representative current traces evoked at test pulses of +10 mV (pulse protocol shown above), before (control) and after exposing the oocyte to 200 μM NA. **B:** I-V curves for each cell before (-●-) or (---) and after exposure to 200 μM NA (-■-) or (---) were normalized to the current evoked at +20 mV and then averaged. Error bars = SEM; n = 5

expected size were obtained when oligonucleotides for α1H were used (not shown); double-strand sequencing confirmed the identity of the fragment. Control reactions in which the RT enzyme was omitted in the cDNA synthesis yielded no product. These experiments show that α1G and α1H are expressed during the last stages of spermatogenesis. Figure 7 shows an alignment of the DNA sequences obtained during this work for α1G (SP α1G) and α1H (SP α1H) and those reported by Perez-Reyes [1998a] (RB α1G) and Cribbs [1998] (MM α1H), respectively. The deduced amino acid sequence for SP α1G and for SP α1H have a 97% and 99% identity with RB α1G and MM α1H, respectively.

To test again for the presence of α1C with a more sensitive assay, RT-PCR analysis was performed with a different set of oligonucleotides than previously used [Liévano *et al.*, 1996]. A single band of the expected size was obtained for mouse brain cDNA. This amplimer was sequenced and determined to be a fragment from α1C. For testis and spermatogenic cells only a smear around the expected size was observed. These smears were blotted and probed with the brain ^{32}P-labeled amplimer [Sambrook *et al.*, 1989]. A sharp signal was obtained in testis and spermatogenic cells, indicating the expression, although low, of α1C in these cells (not shown).

DISCUSSION

Micromolar NA was found to inhibit the ZP-induced AR and single anion channel transitions in mouse sperm [Espinosa *et al.*, 1998]. NA and other compounds, such as NPPB and DDF, are considered blockers of anion channels. They block Ca^{2+}-activated Cl^- channels [Baron *et al.*, 1991; Cotton *et al.*, 1997; Lamb *et al.*, 1994], the CFTR Cl^- channel [Walsh and Wang, 1993; Doughty *et al.*, 1998], osmotically regulated Cl^- channels [Diener *et al.*, 1996], and an inward rectifying Cl^- channel studied in *Caenorhabditis elegans* spermatocytes [Machaca *et al.*, 1996]. However, these compounds also affect some cation channels, such as the NMDA-R [Lerma and Martín del Rio, 1991], nonselective cation channels [Gögelein *et al.*, 1990], and voltage dependent Ca^{2+} channels (VDCCs) [Walsh and Wang, 1993; Doughty *et al.*, 1998]. In addition, NA and NPPB can increase macroscopic currents of K^+ channels expressed in *Xenopus* oocytes [Gribkoff *et al.*, 1996] and

A

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SP α1G          1 CAATAAGGACTGACTCCCTGGACGTGCAGGGCCTGGGTAGCCGGGAAGAC 50
                  |||||||||||||||||||||||||| ||||||||||||||||||||||||||
RB α1G       6520 caataaggactgactccctggatgtgcagggcctgggtagccgggaagac 6569

SP α1G         51 CTGTTGTCAGAGGTGAGTGGGCCCTCCTGCCCTCTGACCCGCTCCTCATC 100
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
RB α1G       6570 ctgttgtcagaggtgagtgggccctcctgccctctgacccggtcctcatc 6619

SP α1G        101 CTTCTGGGGCGGGTCGAGCATCCAGGTGCAGCAGCGCTCCGGCAGCCAGA 150
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
RB α1G       6620 cttctggggcgggtcgagcatccaggtgcagcagcgttccggcatccaga 6669

SP α1G        151 GCAAAGTCTCCAAGCACATCCGCCTGCCAGCCCCTTGCCCAGGCCTGGAA 200
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
RB α1G       6670 gcaaagtctccaagcacatccgcctgccagccccttgcccaggcctggaa 6719

SP α1G        201 CCCAGCTGGGCCAAGGACCCTCAAGAGACCAGAAGCAGCTTAGAGCTGGA 250
                  |||||||||||||||||||||| ||||||||||||||||||||||||||||
RB α1G       6720 cccagctgggccaaggaccctccagagaccagaagcagcttagagctgga 6769

SP α1G        251 CACGGAGCTGAGCTGGATTTCAGGAGACCTCCTGCCCAGCAGTCAGGAAG 300
                  |||||||||||||||||||||||||||||||||| |||||||| |||||||
RB α1G       6770 cacggagctgagctggatttcaggagacctccttcccagcagccaggaag 6819

SP α1G        301 AACCCCTGTCCCCACGGGACTTGAAAAAATGCTACAGTGTAGAGGCCCAG 350
                  |||||||||| |||||||||| |||| || ||||||||||||||| |||||
RB α1G       6820 aaccccctgttcccacgggacctgaagaagtgctacagtgtagagacccag 6869

SP α1G        351 AGCTGCCGGCGCAGGCCTGGGTCCTGGCTAGACGAACAGAGGAGACACTC 400
                  |||||| |||||||||||||||| |||||||| |||||| ||||||||||
RB α1G       6870 agctgcaggcgcaggcctgggttctggctagatgaacagcggagacactc 6919

SP α1G        401 CATCGCTGTCAGCTGCCTGGACAGCGGCTCCCAGCCCCGCCTATGTCCAA 450
                  ||| |||||||||||| |||||||||||||||| ||||||||||||||||
RB α1G       6920 cattgctgtcagctgtctggacagcggctcccaaccccgcctatgtccaa 6969

SP α1G        451 GCCCCTCAAGCCTCGGGGGCCAACCTCTTGGGGGCCCTGGGAGCCGGCCT 500
                  |||||||||||||||||||||||||||||||||||| ||||||||||||||
RB α1G       6970 gcccctcaagcctcgggggccaacctcttgggggtcctgggagccggcct 7019

SP α1G        501 AAGAAAAAACTCAGCCCACC 520
                  ||||||||||||||||||||
RB α1G       7020 aagaaaaaactcagcccacc 7039
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B

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SP α1H          1 GAAGGATACTCTCCGTGAGTGTACCCGTGAAGACAAGCACTGCCTCAGCT 50
                  |||||||||||||||||| ||||||||||||||||||||||||||||||||
MM α1H         10 gaaggatactctccgtgaatgtacccgtgaagacaagcactgcctcagct 59

SP α1H         51 ACCTGCCCGCGCTCTCCCCCGTCTACTTCGTCACCTTCGTGCTGGTCGCC 100
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H         60 acctgcccgcgctctcccccgtctacttcgtcaccttcgtgctggtcgcc 109

SP α1H        101 CAGTTCGTGCTGGTCAACGTGGTGGTGGCCGTGCTCATGAAGCACCTGGA 150
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H        110 cagttcgtgctggtcaacgtggtggtggccgtgctcatgaagcacctgga 159

SP α1H        151 GGAGAGCAACAAGGAGGCCCGGGAGGATGCGGAGATGGATGCCGAGATCG 200
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H        160 ggagagcaacaaggaggcccgggaggatgcggagatggatgccgagatcg 209

SP α1H        201 AGCTGGAGATCGCACAGGGGTCCACAGCCCAGCCCCCGTCCACAGCACAG 249
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H        210 agctggagatcgcacaggggtccacagcccagccccgtccacagcacag 259

SP α1H        250 GAAAGCCAAGGTACCGATCCAGACACCCCGAACCTCCTGGTCGTGCGCAA 299
                  |||||||||||||||||| ||||||||||||||||||||||||||||||||
MM α1H        260 gaaagccaaggtaccgagccagacaccccgaacctcctggtcgtgcgcaa 309

SP α1H        300 AGTATCTGTGTCCAGGATGCTCTCGCTACCCAATGACAGCTACATGTTCC 349
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H         10 agtatctgtgtccaggatgctctcgctacccaatgacagctacatgttcc 359

SP α1H        350 GG 351
                  ||
MM α1H        360 gg 361
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Fig. 7. Alignment of the nucleotide sequence. **A:** Clone obtained from spermatogenic cells for α1G (SP α1G) compared with the rat brain α1G (RB α1G) previously described by Perez-Reyes *et al.* [1998a], Genbank accession No. AF027984. **B:** Clone obtained from spermatogenic cells for α1H (SP α1H) with *Mus musculus* T-type Ca^{2+} channel α1 subunit mRNA, partial cDNAs (MM α1H), described by Cribbs *et al.* [1998], Genbank accession No. AF051947. Sequence identity is indicated by a *vertical line* between the sequences. The nucleotide sequences showed a 96.34% and 99.7% identity between the α1G and the α1H respectively.

the open probability of $_{Ca}K^+$ channels reconstituted in planar bilayers [Ottolia and Toro, 1994] The neuronal $GABA_A$-R, itself a channel, is potentiated at low NA concentrations and inhibited at high concentrations [Woodward *et al*, 1994].

The only type of voltage-dependent Ca^{2+} currents found in mouse spermatogenic cells are of the T-type [Liévano *et al*, 1996; Santi *et al*, 1996; Arnoult *et al*, 1996] These channels are probably still present in mature sperm, as they are unable to synthesize proteins. The T-type Ca^{2+} channels from spermatogenic cells and the ZP-induced sperm Ca^{2+} uptake and AR are inhibited by similar concentrations of dihydropyridines and other blockers. These observations strongly suggest that T-type Ca^{2+} channels play a fundamental role in the sperm AR [Arnoult *et al*, 1996, 1998; Liévano *et al*, 1996].

Because NA inhibited the mouse sperm AR, it seemed important to determine whether T-type Ca^{2+} currents from spermatogenic cells were sensitive to this blocker. NPPB and DDF were also tested. The three compounds blocked these Ca^{2+} currents with IC_{50} values of: NA 43 μM and DDF 28 μM and, as shown in Fig. 2C, NPPB appears to have two distinct binding sites with approximate affinities of 25 nM and 75 μM. By contrast, the ZP-induced sperm AR was more sensitive to these blockers, with IC_{50} values of: NA 1 μM [Espinosa *et al*, 1998], DDF 1 μM, and NPPB 6 μM (Fig. 4). Considering that, until now, blockers of the T-type Ca^{2+} currents from spermatogenic cells have been shown to have very similar potencies to inhibit the sperm AR and its associated Ca^{2+} uptake [Arnoult *et al*, 1996], these results suggest that it is unlikely that T-type Ca^{2+} channels are involved in the inhibition of the ZP-induced AR caused by NA and DDF and suggest that other channels, possibly anionic, may influence this reaction. From this point of view, only in the case of the high affinity binding site for NPPB could T-type Ca^{2+} channel blockade contribute to AR inhibition. Furthermore, at the resting membrane potential of capacitated sperm (~−55 mV or less) [Florman *et al*, 1998], the voltage dependence of the blockage by Anion channel blockers (ACBs) (more potent at depolarized potentials; Fig 3) would even decrease their efficacy to inhibit T-type Ca^{2+} currents. However, there is still the possibility that in contrast to the T-type Ca^{2+} channel blockers described so far, ACBs could behave differently in spermatogenic cells and in mature sperm, complicating the comparison between the IC_{50} values of channel blockage and the AR.

Several reports suggest a direct interaction of these ACBs with either anion or cation channels [Gögelein *et al*, 1990; Oba *et al*, 1997; Ottolia and Toro, 1994; Tilman *et al*, 1993; White and Aylwin, 1991]. The binding site could be within the pore [Evoniuk and Skolnick, 1988] and possibly reached only from one side of the membrane [Tilman *et al*, 1993]. In order to feel the voltage, the blocker binding site must be inside the membrane [Hille, 1992] Thus, these compounds may directly interact within the pore of T-type channels NPPB could be acting elsewhere since it displays two binding sites

Although there is no precedent, instead of directly binding to the pore of T-type Ca^{2+} channels, these blockers could interact with channel accessory subunits. Alternatively, the ACBs may exert an indirect effect over T-type Ca^{2+} currents. For example, some models suggest the direct interaction between cation and anion channels [Kanli and Norderhus, 1998; Sonders and Amara, 1996]. So far, no direct interaction between Cl^- and VDCCs has been documented, making it unlikely that the inhibition of T-type Ca^{2+} currents of spermatogenic cells by ACBs is due to Cl^- channel inhibition

ACBs are membrane permeable and could also affect enzymatic pathways that may influence ion channels function. ACBs used in this work also inhibit cycloxygenases and lipoxygenases that participate in arachidonic acid metabolism [Civelli *et al*, 1991] and the 3α-hydroxysteroid dehydrogenase involved in sex steroid metabolism [Penning *et al*, 1985]. Metabolites of the lipoxygenase, and cycloxygenase pathways positively modulate cation and anion channels [Diener *et al*, 1996; Kanli and Norderhus, 1998]. Intracellular Ca^{2+} increases and Ca^{2+} currents can be modulated negatively (by estradiol) [Nakajima *et al*, 1995] or positively (by testosterone or progestins) [Takeuchi and Guggino, 1996; Bukusoglu and Sarlak, 1996]. In addition to their known genomic actions, steroid hormones could modulate VDCCs on spermatogenic cells that might be critical for their differentiation. Further investigation is needed to evaluate the possible co-modulation (direct or through shared transduction pathways) of T-type Ca^{2+} channels and Cl^- channels of spermatogenic cells.

α1E Expressed Currents in *Xenopus* Oocytes Are Very Weakly Sensitivity to NA

Voltage-dependent Ca^{2+} channels have been classified according to their voltage-threshold of activation as high (HVA) or low-voltage-activated (LVA). HVA Ca^{2+} currents were subclassified as L, N, P, or Q; and LVA Ca^{2+} currents as R or T [Tabares and López-Barneo, 1996]. VDCCs are constituted by the α1, α2δ, β, and γ subunits. The α1 subunit contains the pore and the voltage sensor [Catterall, 1995]. Several genes coding for α1 subunits have been cloned (A, B, C, D, E, G, H, I, and S). Their heterologous expression has revealed, for instance, that L-type currents are coded by α1S, α1C, and α1D By contrast, the molecular identity of T-type currents in different tissues is yet under debate [Ertel and Ertel, 1997]. A rat α1E clone expressed in *Xenopus* oocytes yielded Ca^{2+} channels exhibiting functional properties compatible to those of low-voltage-activated Ca^{2+} channels [Soong *et al*, 1993; Stephens *et al*, 1997], while other clones display characteristics closer to R-type Ca^{2+} currents, which are HVA [Ertel and Ertel, 1997] In addition, depending on the cell type,

antisense oligonucleotides against α1E inhibited both T-type currents [Piedras-Rentería *et al*, 1997] and R-type currents [Piedras-Rentería and Tsien, 1998]. By contrast, Meir and Dolphin [1998] found that expression of α1B, E, or C in COS7 cells, which do not display endogenous Ca^{2+} channel subunits or Ca^{2+} channels, can yield low conductance, low-voltage-activated Ca^{2+} channels whose voltage dependence and kinetics of activation and inactivation makes them indistinguishable from native T-type Ca^{2+} channels. These apparently conflicting observations reveal the necessity of establishing which α1 subunit codes for the T-type Ca^{2+} currents of spermatogenic cells that appear to be crucial for the mouse sperm AR.

The most prevalent α1 transcript of VDCCs in spermatogenic cells so far is type E [Liévano *et al*., 1996]. Therefore, a rat brain α1E clone was expressed in *X. laevis* oocytes, and its sensitivity to NA determined. The expressed currents were essentially insensitive to 200 μM NA which completely blocks T-type Ca^{2+} currents of spermatogenic cells. This result questions the possibility that α1E codes for the T-type currents, though alternative explanations must be considered. ACBs different from those used here, inhibited Ca^{2+} activated Cl^- currents in smooth muscle cells, where the $[Ca^{2+}]_i$ increase driven through T-type channels was not affected [Baron *et al*., 1991]. There are contrasting findings on the effect of ACBs on other VDCC currents. In some reports, L-type currents were inhibited by μM ACBs [Asai *et al*, 1996; Doughty *et al*, 1998; Walsh and Wang, 1996] but in others, they were insensitive up to 100 μM [Cotton *et al*. 1997; Lamb *et al*., 1994]. The differential sensitivity to ACBs could be due to the fact that L-type Ca^{2+} currents can be coded by three different α1 subunits (S, C, or D). It is therefore still possible that sequence variations between the rat brain α1E and that of mouse spermatogenic cells explain the lack of sensitivity to NA. In addition, subunits other than α1 that constitute VDCCs can modify their affinity for blockers [Varadi *et al*, 1995; Gurnett *et al*, 1997].

Initially, the findings of Soong *et al*. [1993] showing that α1E expressed in *X. laevis* oocytes yielded currents insensitive to 10 μM nifedipine was consistent with the fact the T-type Ca^{2+} currents were believed to be insensitive to dihydropyridines [Hess, 1990]. However, it is now known that T-type Ca^{2+} currents are sensitive to μM dihydropyridines in some cells [Liévano *et al*, 1995], and for sure in spermatogenic cells [Liévano *et al*., 1996; Santi *et al*, 1996; Arnoult *et al*, 1996, 1998]. Experiments done in this work with 20 μM nifedipine corroborated the insensitivity of α1E expressed Ca^{2+} currents in *Xenopus* oocytes to this blocker (not shown). A straightforward explanation, also consistent with the NA results described above, would be that T-type Ca^{2+} currents of spermatogenic cells are not driven through α1E-coded channels.

Detection of α1C, α1G, and α1H in Spermatogenic Cells

Recently Perez-Reyes *et al*. [1998a] cloned a novel α1 subunit, α1G. Expression of this subunit in *Xenopus* oocytes yielded Ca^{2+} currents with the properties expected of T-type currents. Moreover, α1H and α1I are two newly cloned α1 subunits belonging to what may be a family of α1 subunits coding for T-type Ca^{2+} currents [Cribbs *et al*, 1998; Perez-Reyes *et al*., 1998b; Lee *et al*., 1999]. These reports and the lack of sensitivity to nifedipine and NA of the heterologously expressed α1E channel prompted a search for α1G and α1H in spermatogenic cells. RT-PCR experiments indicate the presence of α1G and α1H in mouse pachytene spermatocytes and in round and condensing spermatids. A more sensitive assay than that used previously [Liévano *et al*., 1996] allowed the detection of low levels of α1C mRNA, too. The presence of a rat cardiac muscle related α1C in mouse sperm [Goodwin *et al*., 1997] and in spermatogenic cells [Goodwin *et al*., 1998] had been reported. Which of these subunits codes for the T-type Ca^{2+} currents in spermatogenic cells? Why do spermatogenic cells express different α1 subunits if only T-type currents are functionally expressed? These interesting and fundamental questions remain to be answered.

ACKNOWLEDGMENTS

The authors thank T. Snutch for the α1E clone, J. Alvarez-Leefmans and H. Pasantes for anion channel blockers, E. Mata for help with mice, P. Gaytan and E. López-Bustos for oligonucleotide synthesis, and P. Labarca and R. Felix for comments. This work was supported by grants from Consejo Nacional de Ciencia y Tecnologia, DGAPA-UNAM, the Howard Hughes Medical Institute, and the International Centre for Genetic Engineering and Biotechnology.

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Mouse sperm patch-clamp recordings reveal single Cl^- channels sensitive to niflumic acid, a blocker of the sperm acrosome reaction

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Received 11 February 1998

Abstract **Ion channels lie at the heart of gamete signaling. Understanding their regulation will improve our knowledge of sperm physiology, and may lead to novel contraceptive strategies. Sperm are tiny (~3 μm diameter) and, until now, direct evidence of ion channel activity in these cells was lacking. Using patch-clamp recording we document here, for the first time, the presence of cationic and anionic channels in mouse sperm. Anion selective channels were blocked by niflumic acid (NA) (IC_{50} = 11 μM). The blocker was effective also in inhibiting the acrosome reaction induced by the zona pellucida, GABA or progesterone. These observations suggest that Cl^- channels participate in the sperm acrosome reaction in mammals.**

Key words: Acrosome reaction; Ion channel; Niflumic acid; Sperm patch-clamp recording

1. Introduction

Ion channels are deeply involved in the egg-sperm dialogue. Components from the outer layer of the egg induce changes in sperm permeability to ions which, depending on the species, regulate sperm motility, chemotaxis and the acrosome reaction (AR). This reaction is required for sperm to fertilize the egg in many species, including man. The egg's extracellular matrix, the zona pellucida (ZP), induces the AR in mammals [1]. Nonetheless, other agents like γ-aminobutiric acid (GABA) and progesterone can induce this reaction [2–6]. The sperm AR is inhibited by ion channel blockers, evidencing their predominant role in this process, and their suitability as targets for contraception [7].

Due to the small size of sperm thus far only planar bilayer techniques have yielded direct information about their ion channels [8–12]. To explore how ion channels participate in the AR we have recorded single channel currents directly from mouse sperm. Here, using patch-clamp recording we document, for the first time, the presence of cation and anion channels in mammalian sperm head plasma membrane. Anion selective channels were blocked by niflumic acid (NA) (IC_{50} = 11 μM). The blocker inhibits also the acrosome reaction induced by the zona pellucida, GABA or progesterone, suggesting that Cl^- channels participate in the sperm AR in mammals. Voltage-gated Cl^- currents, blocked by μM NA (IC_{50} = 100 μM), were monitored also in pachytene spermatocytes [13–15], indicating that NA sensitive Cl^- channels are expressed early in spermatocyte differentiation.

2. Materials and methods

2.1. Sperm collection and AR induction

Sperm were obtained from CD-1 mice >3½ months killed by cervical dislocation. The cells were collected from excised epididymides as described [16]. Cells (3.6×10^6/ml) were suspended in M-199 media (from Sigma) supplemented with 25 mM $NaHCO_3$, 1 mM sodium pyruvate and 0.4% glucose, and were capacitated at 37°C for 30 min in 5% CO_2 atmosphere. Thereafter the agents (heat-solubilized ZP, progesterone or GABA) were added and cells were left to acrosome react for 30 min. Inhibition was studied with the same procedure except that the blocker was added 5 min prior to the agonist. At the end of the experiment AR was determined by established procedures [17,18]. A minimum of 100 sperm were counted under light field microscopy and expressed as the fraction of reacted sperm induced by the agonist vs. a maximum of reacted sperm obtained with the Ca^{2+} ionophore A-23187 (AR/ARA-23) at 15 μM.

2.2. Electrophysiology

2.2.1. Sperm. Seals were obtained using Kimax or 7052 glass pipettes having >5 megaohms resistance and <1 μm of tip diameter. Sperm were rinsed for 10 min in (mM): 60 NaCl, 150 sucrose, 20 sodium lactate, 1 sodium pyruvate, 5.6 glucose, 1 EDTA, 10 benzamidine and 20 μg/ml aprotinin; pH 8.4, pelleted at 325×*g* for 10 min at room temperature in cylindrical 2 ml Eppendorf tubes before patch-clamping them. The pelleted cells were resuspended, left for 10 min at 20–23°C and then placed on ice. An aliquot was allowed to settle for 5–10 min on a coverslip and placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon Corp.) endowed with Hoffman Optics. Experiments were done at 20–23°C and currents monitored with and Axoclamp 1-B amplifier (Axon instruments, Foster City, CA), sampling at 10–25 kHz, and filtering at 2–5 kHz. Data analysis was performed with Pclamp6 routines. Patch-clamping the sperm head is laborious due to its small size and flatness. A perpendicular approach between pipette and cell surface is necessary to achieve high resistance seals (>1 gigaohms, Fig. 1A). This occurred only in ~7% of trials, out of which ~80% displayed single channel activity. In 25% of the high resistance seals spontaneous excised patches were obtained. In this cases the polarity of the membrane patch was not known. Excised patches were lost very rapidly, therefore, ion substitution experiments were not possible and data were acquired only with the solution used during sealing. Analysis was done only on records with >5 megaohms of seal resistance. Apparent permeability on excised patches were calculated according to [19]. The solutions in pipette and in bath employed in sperm patch-clamp experiments are indicated in the figure legends.

2.2.2. Spermatogenic cells. Testis from adult CD-1 mice were used as a source of germ cells. Spermatogenic cells were obtained as described [13,14]. The dissociated cells were stored at 4–10°C and remained healthy for at least 12 h. An aliquot of the cell suspension was placed in a recording chamber superfused with recording medium (in mM: 130 NaCl, 3 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucose, 0.16 amiloride, pH 7.35) at 20–

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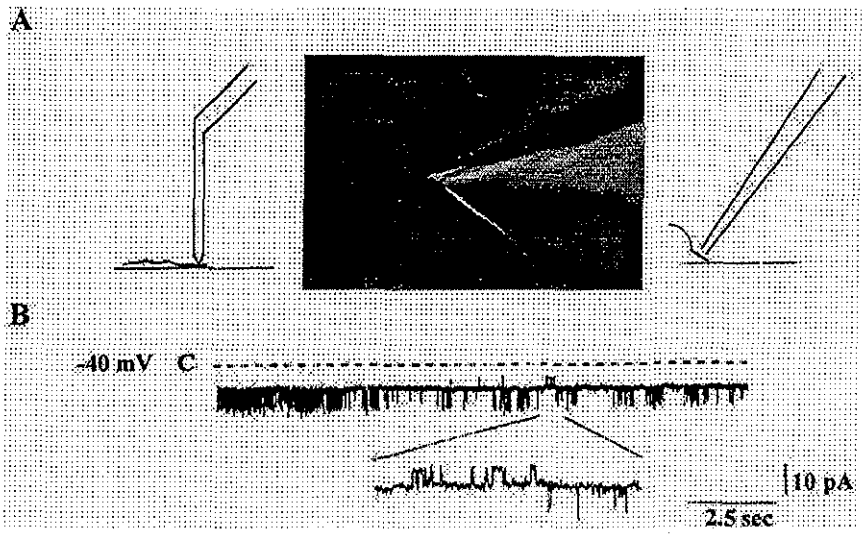

Fig. 1. Direct single channel recording in mouse sperm. A: The center shows a micro-photograph of a patch-clamped mouse sperm (head sperm width = ~3 μM). Schematic representations of perpendicular approaches to sperm are illustrated on both sides of the photo. B: Single channel activity in an excised patch. The pipette was filled with (in mM): 30 NF, 120 NaCl, 0.1 GABA, 10 HEPES, pH 7.2. The bath solution was (in mM): 145 NaCl, 10 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, pH 7.4. The trace obtained at −40 mV shows the presence of mainly two types of transitions (see text). The discontinuous line indicates zero current (C, closed state).

23°C. The solution in the pipette was (in mM): 110 Cs-Methanesulfonate, 10 CsF, 15 CsCl, 2 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 5 Cs-HEPES, pH 7.35. Glass borosilicate pipettes were pulled to tip diameters of about 1.5 μm, having resistances between 2 and 5 megaohms when filled with pipette solution. Records were low-pass filtered at 2 kHz (4-pole Bessel filter). A p/4 pulse protocol was used routinely to minimize leak and capacitive currents from current records [20].

2.3. Membrane potential measurements

In cell attached experiments (Fig. 3) real ionic cytoplasmic concentrations are not known for the patched cell. Membrane potential (V_m)

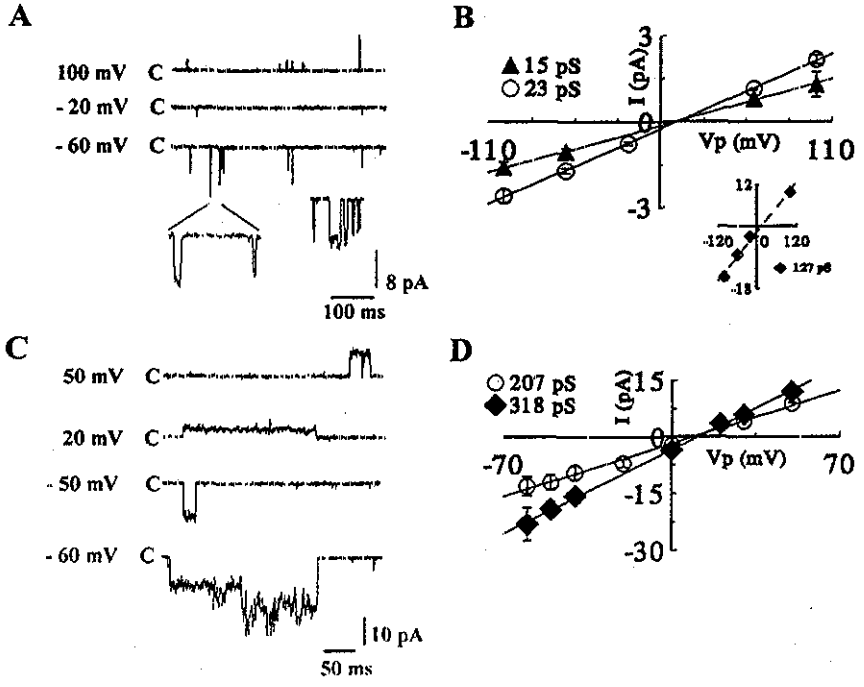

Fig. 2. Cationic channels. The traces shown in this figure were recorded on spontaneous excised patches at the indicated voltages. A fast mouse sperm cation channel displaying several conductance levels. Kinetic analysis yielded basically a monoexponential closing time constant of 0.82 ± 0.27 ms at −60 mV, and opening time constants of 4.3 ± 1 ms and 53 ± 12 ms. Left inset shows a 20-fold time expansion of the indicated area of the burst at −60 mV. Right inset shows a burst from the same recording at −60 mV (identical scales). Solutions were (in mM): 145 NaCl, 2.5 $CaCl_2$, 10 $MgCl_2$, 10 HEPES-Na, pH 7.4 (in bath); and, 120 CsCl, 0.05 $CaCl_2$, 10 EGTA-Cs, 10 HEPES-Cs, pH 7.2 (in pipette). Similar transitions were observed in 12 experiments. B: I–V relations of the channel in A, E_{rev} = 10 mV. The inset shows the I–V relation of the largest conductance. C: Representative traces of a slow high-conducting channel showing two main conductance levels. Similar transitions were observed in 11 experiments. D: I–V relations of the channel in C. Solutions were (in mM): bath as in A; and in the pipette; 30 KF, 90 KCl, 0.05 $CaCl_2$, 10 EGTA-K and 10 HEPES-K, pH 7.2. Smaller fast cationic transitions similar to those in A are also present.

180

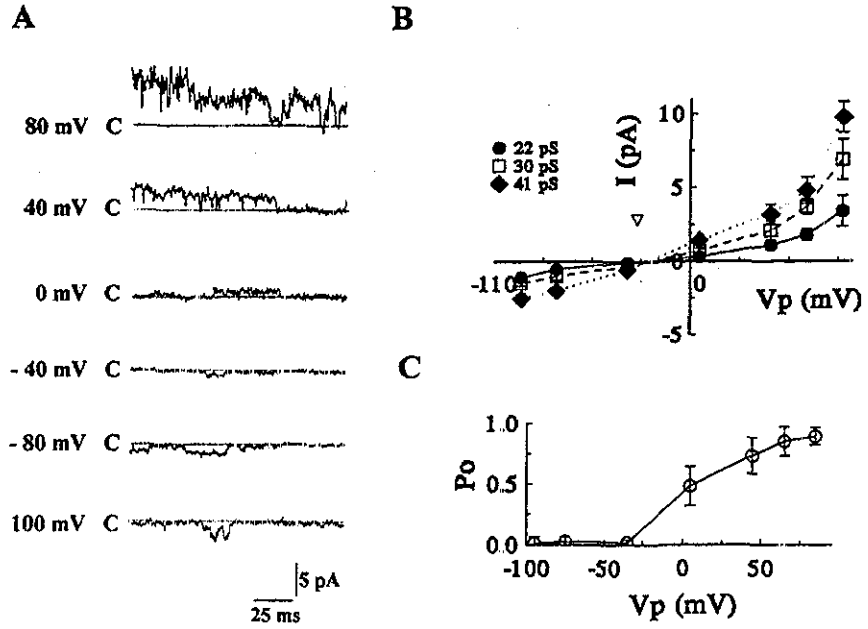

Fig. 3 Mouse sperm anion channels. A: Anion single channel transitions at the indicated pipette potentials recorded in the cell attached configuration The pipette contained (mM): 120 NaCl, 30 NaF, 10 HEPES-Na, pH 7 6 B: I–V relations from A showing outward rectification at high positive applied potentials Slope conductances of 22, 30 and 41 pS were measured in the linear region. E_{rev} averaged -30 ± 5 mV ($n=3$), which considering the resting potential of sperm under equivalent conditions (−85 mV, see Section 2) and 10 mM Cl^- in the sperm cytoplasm [22], indicates anion selectivity C: Open probability (P_o) of the anion channel measured during 6.4 s at each pipette potential (V_p)

determinations in sperm suspensions were done as in [21] using DiS-$C_3(5)$, a V_m sensitive fluorophore. The resting potential of sperm suspended in the external solution (TEA-Cl 60, TEA-$MeSO_4$ 70, 10 $CaCl_2$, pH 7.4) used for cell attached experiments was estimated to be -85 ± 7 mV ($n=8$) The internal K^+ concentration used for this calculations was 120 mM [22]

3. Results and discussion

To explore the in situ characteristics of sperm ion channels, we recorded single channel currents directly from mouse sperm Single channel currents could be recorded in 42

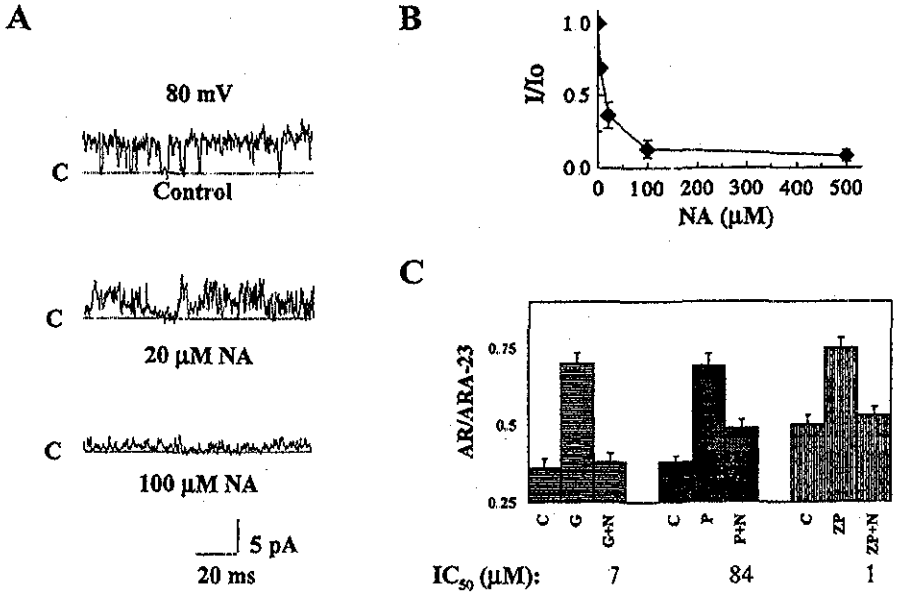

Fig. 4 Niflumic acid (NA) blocks a mouse sperm Cl^- channel and the AR induced by ZP3, GABA and progesterone. A: Conditions were as in Fig. 3. Voltage in pipette was 80 mV in all cell attached records shown. The control trace is at the top, the following traces were taken 10 min apart, after perfusing the external chamber with control solution plus 20 µM (middle trace) or 100 µM (lower trace) NA. B: Dose dependence of channel blockade by NA (average ± S.E.M., $n=3$) C: NA inhibits the mouse sperm acrosome reaction (AR) induced by GABA (0.1 µM), progesterone (45 µM) and solubilized zona pellucida (5 ZP/µl) AR and its inhibition were determined as indicated in Section 2. The bars represent the average ± S.E.M ($n=5$) of the ratio % AR under a certain condition (C, control; G, GABA; N, NA; P, progesterone; ZP, zona pellucida) and the AR achieved with the Ca^{2+} ionophore A-23187 (ARA-23) The numbers under the bars are the IC_{50} determined in five concentration dependence experiments.

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patches, 14 of which were excised patches, the remaining ones corresponding to cell attached patches Fig 1B shows single channel activity in an excised patch at −40 mV. Mainly, two types of current transitions can be observed: (1) fast transitions that occur in bursts; and (2) smaller upward transitions (inset below). In addition, a slow high-conducting channel similar to the one of Fig. 2C, was also observed during this recording (not shown).

Single channel currents shown in Fig 2A were monitored in an excised patch at different applied voltages. These transitions have fast kinetics with spike shaped transitions being the most common, although square shaped transitions are also present (see record at higher resolution and the bursting period in Fig 2A). From the I–V plots, which have an $E_{rev} = 10$ mV, a PNa^+/PCs^+ of 1.5 (150 Na^+_{bath}/145 $Cs^+_{pipette}$) was calculated In a separate experiment PNa^+/PK^+ was 0 71, indicating a permeability sequence $K^+ > Na^+ > Cs^+$, analogous to that seen in bilayers ($K^+ > Rb^+ > Na^+ > Cs^+$) for a cAMP-activated sea urchin sperm cationic channel [12]. Similar single channel currents were recorded in four excised patches and eight cell attached ones In three experiments where transitions were recorded at various voltages, P_o did not vary significantly (0.012 ± 0.022 in the ± 100 mV range) In ~25% of the experiments where fast cationic transitions were seen, burst periods with $P_o > 0.3$ were observed between voltages ≥+40 mV or ≤−40 mV (inset in Fig. 2A). The pattern of the transitions is also reminiscent of a mouse sperm cationic channel detected in planar bilayers [11]. Moreover, the poor selectivity of this channel suggests it could participate in the ZP induced depolarization during mouse and bull sperm AR [23].

Fig. 2C shows a record of an excised patch displaying slower cationic transitions with main conductance substrates of 207 and 318 pS ($E_{rev} = 11$ mV; $PCa^{2+}/PNa^+ = 6$; assuming $PNa^+/PK^+ = 1$). Similar transitions were recorded in 11 experiments (eight cell attached and three excised patches) Planar bilayer studies indicated the presence of a similar high conductance, poorly selective cationic channel that allows divalents through, in sea urchin [24] and mouse sperm plasma membranes [10,11]. Patch-clamp recording shows that this cation selective channel displays slow kinetics with long open times (tenths of milliseconds to seconds), resembling the behavior of the Ca^{2+} selective channel reconstituted in planar lipid bilayers.

In three experiments it was possible to record channel currents that were blocked by NA (a Cl^- channel blocker, Figs 3 and 4). Fig. 3A shows single channel currents monitored at different applied voltages. Inspection of the records revealed the presence of open substrates with conductances of 22, 30 and 41 pS, measured in the ohmic region of the I–V relation. The reversal potential, −30 mV, is consistent with that predicted for an anion selective channel, assuming a resting potential of −85 mV (see Section 2, Fig. 3B). At more positive pipette potentials, the channel rectifies, probably due to the presence of 30 mM F^- in the pipette Some Cl^- channels are known to have a low F^- permeability ($PCl^-/PF^- = 50$; [25]) P_o of the channel increases as the applied potential becomes more positive (Fig 3C). Blockade by NA was dose-dependent ($IC_{50} = 11$ μM, Fig 4A, B). Lipid bilayer studies had also shown the presence of anion channels in sea urchin [26] and mouse sperm plasma membranes [11,27].

Several reports indicate the participation of Cl^- channels in sperm physiology. NA was reported to inhibit a Ca^{2+} induced

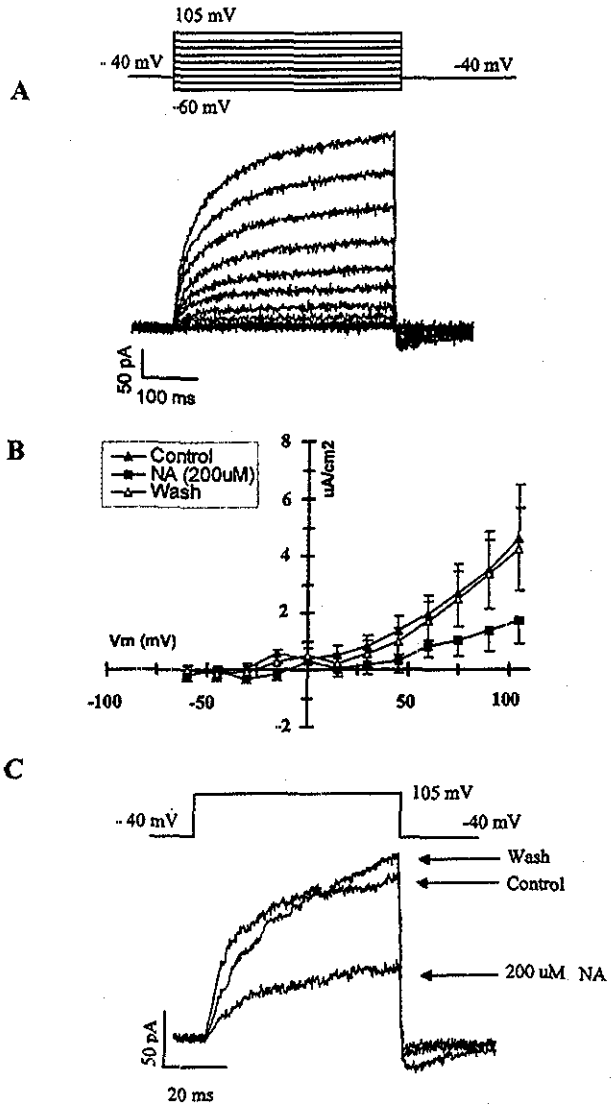

Fig 5 NA blocks outward whole cell currents resulting from Cl^- influx in pachytene spermatocytes Spermatogenic cells and whole-cell recordings were obtained as described in Section 2. Patch pipettes were filled with (in mM): 110 Cs-Methanesulfonate, 10 CsF, 15 CsCl, 2 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 5 Cs-HEPES, pH 7.35; the bath solution contained (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucose, 10 $CaCl_2$, 0.16 amiloride, pH 7 35 Currents were triggered by depolarizations from −40 mV holding potential up to 100 mV in 15 mV steps A: Cl^- currents elicited at 40 mV (top shows voltage protocol) and their block by 200 μM NA B: I–V relation of the Cl^- currents in mouse pachytene spermatocytes (filled triangles), and their blockade (squares) and recovery (empty triangles) after exposure to 200 μM NA. The points represent the average of three experiments, the standard deviation was smaller than the symbols The estimated IC_{50} was 100 μM

hyperpolarization which is partially driven by Cl^- in mouse sperm [21]. External Cl^-, and putative $GABA_A$-R channels have been implicated in the GABA and progesterone induced AR in human and mouse sperm [4,5,28]. In porcine and human sperm, Glycine receptor activation was reported to elicit AR [29] and Cl^- efflux [30]. In other cell systems, NA and other fenamates (non-steroidal anti-inflammatory drugs) have been shown to block Ca^{2+}-activated Cl^- channels [31,32], as well as an heterologously expressed $GABA_A$-R [33]. More-

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over, Sigel et al (1989), reported the expression of a rat β homopentamer $GABA_A$-R which opens in the absence of GABA [34]. Future experiments will have to be done to determine if the sperm Cl^- channel reported here is of the $GABA_A$-R type.

Since NA blocks Cl^- channels in mouse sperm and in other systems, the effect of this compound was tested on the AR. NA blocked the AR induced by GABA (0.125 μM), progesterone (45 μM) or solubilized zona pellucida (5 ZP/μl) (Fig. 4C). The ZP3 and GABA induced AR was more sensitive to NA (IC_{50} of 1 and 7 μM, respectively) than that induced by progesterone (84 μM). This was unexpected since it has been proposed that progesterone triggers the AR by potentiating the $GABA_A$-R channel [2,4,5]. This result may indicate that distinct Cl^- channels participate in AR induced by the different agonists. Alternatively, progesterone could modulate two different surface sperm receptors one coupled to a Ca^{2+} channel [2,35,36] and the other, a $GABA_A$-R type, which upon progestin binding decreases its affinity for NA [29,37].

We investigated further whether spermatogenic cells are endowed with NA sensitive Cl^- channels. Cl^- currents, activated by depolarization, and blocked by NA (IC_{50} 100 μM), could be monitored in pachytene spermatocytes (Fig. 5). These results show that NA sensitive Cl^- channels are expressed early in spermatocyte differentiation. However, at difference with anion channels present in the sperm, Cl^- channels in spermatocytes display a lower affinity for this blocker.

At the least, our results lead to the conclusion that mouse sperm, and spermatocytes, as early as pachytene, are endowed with Cl^- channels blocked by NA. Furthermore, the cumulated experimental evidence suggests that mouse sperm Cl^- channels may participate in the AR induced by ZP3 and GABA. It is possible that one of these Cl^- channels is an isoform of the $GABA_A$-R, and that mammalian sperm posses more than one mechanism capable of triggering the events leading to the fusion of the acrosomal vesicle.

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IV.- RESULTADOS.

4.1.- Modulación de la corriente de Ca^{2+} tipo T.

4.1.1.- Efecto de la albúmina y β-estradiol en la corriente de Ca^{2+} de las células espermatogénicas.

Como se mencionó en los antecedentes, durante la capacitación la extracción del colesterol de la membrana plasmática por diferentes moléculas aceptoras (como la albúmina) da inicio a una reorganización de la membrana celular lo cual favorece la permeabilidad al Ca^{2+} y al HCO_3^-, dos compuestos esenciales para este proceso (Shi y Roldán, 1995; Visconti *et al.*, 1995; Visconti y Kopf, 1998). Sin embargo, hasta el presente trabajo no se había probado directamente la posibilidad de que la albúmina (BSA) pudiera modular a la corriente de Ca^{2+} tipo T presente en el espermatozoide y las células espermatogénicas. Debido a que las características morfológicas del espermatozoide dificultan realizar los experimentos electrofisiológicos directamente en el gameto maduro, utilizamos a las células espermatogénicas como un modelo para determinar la influencia de la BSA en la corriente de Ca^{2+} tipo T. Resultados previos en nuestro laboratorio demostraron que la BSA (0.5%) induce un incremento en la amplitud de la corriente de Ca^{2+} tipo T de manera dependiente de la concentración, sin afectar a las corrientes de K^+ presentes en las CE. El curso temporal del efecto de la BSA presentó una $\tau = 11$ s (ver apéndice; Espinosa *et al.*, 2000).

Tomando como base las observaciones previas, nos dimos a la tarea de hacer un estudio más detallado de esas primeras observaciones. Nuestros resultados demostraron que el efecto de la BSA es dependiente de voltaje como se muestra en el análisis de la densidad de corriente de la ICa_T incubada en 0.5% de BSA a diferentes voltajes (figura 7A). La línea

discontinua representa la densidad de corriente obtenida en condiciones control para el intervalo de voltajes mostrado, mientras que los puntos representan el cociente del promedio de la densidad de corriente en presencia de BSA sobre el promedio control a diferentes voltajes. El efecto de la BSA fue más marcado a voltajes negativos (de –60 a –40 mV) como se observa en el panel A de la figura 7. A continuación, examinamos el efecto de la BSA sobre la cinética de activación. Nosotros medimos el tiempo al pico y la constante de activación obtenidos a partir de registros hechos a diferentes potenciales. En las células control, el tiempo al pico disminuyó monotónicamente de acuerdo al voltaje aplicado de 30.6 ± 2.8 ms a –50 mV a 6.1 ± 0.4 ms a +20 mV, alcanzando un valor mínimo independiente de voltaje de 6 ms a 0 mV. En el caso de las células tratadas con BSA el tiempo al pico disminuyó a 23.6 ± 2.3 y 5.5 ± 0.3 ms a –50 y +20 mV, respectivamente. Los valores obtenidos para ambas condiciones de registro fueron estadísticamente diferentes a pulso de voltaje menores a -30 mV (Fig. 7B). De manera consistente, la constante de tiempo de activación fue menor 1.3 veces, después de la aplicación de la BSA, con respecto al control a –40 mV. Comúnmente, estos experimentos se registraron en respuesta a depolarizaciones a partir de un potencial de mantenimiento de -90 mV hasta un pulso de prueba de -40 mV. Posteriormente, ajustamos los trazos obtenidos a una exponencial simple (Fig. 7B, *inserto*) cuya constante temporal fue de 9 ± 1.1 y 6.9 ± 1.1 ms antes y después del tratamiento con BSA, respectivamente.

Al analizar la conductancia macroscópica de la ICa_T y ajustando los promedios normalizados con la ecuación de Boltzmann (Fig. 7C), encontramos que después de la aplicación de BSA la ICa_T se activaba a potenciales más negativos ($V_{½}$=-47.5 en el control

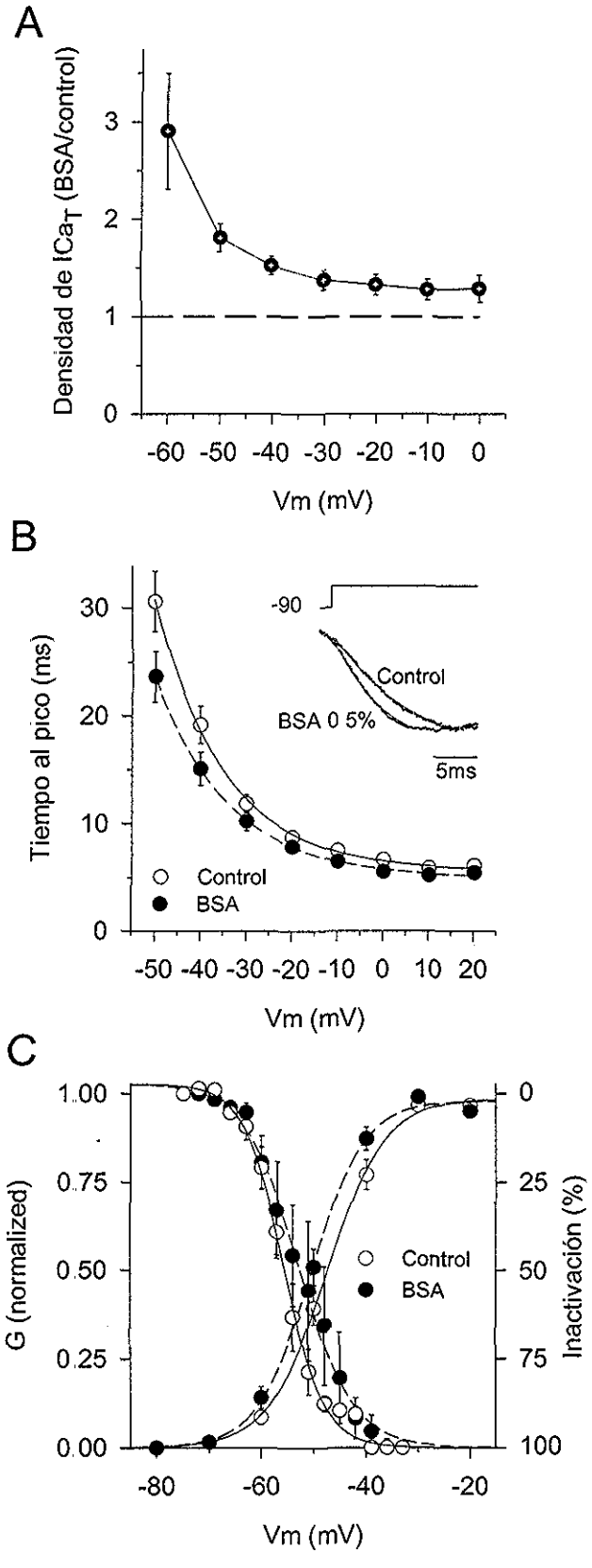

Figura 7.- La seroalbúmina bovina (BSA) afecta la cinética de la corriente de Ca^{2+} tipo T de las células espermatogénicas. A: Dependencia de voltaje del incremento relativo en la densidad de corriente inducido por BSA. La gráfica nos muestra los cocientes de la densidad de corriente después de la aplicación de BSA entre el control a diferentes voltajes aplicados a la misma célula. **B:** Relación tiempo al pico-voltaje de la activación de la ICa_T. Las líneas corresponden al ajuste de los datos a una exponencial simple (n=11). Inserto: trazos representativos de la relación tiempo al pico-voltaje mostrada. Las corrientes se normalizaron para facilitar la comparación cinética de ambas condiciones, solo se muestran los primeros 15 ms. La fase de activación de la corriente en cada condición se ajustó con la ecuación de una exponencial simple (líneas superpuestas) **C:** La BSA incrementa significativamente la corriente ventana de las células espermatogénicas. Comparación de la activación e inactivación en estado estacionario en las células control y células tratadas con BSA. La gráfica nos muestra la fracción de canales inactivados como función del voltaje del prepulso aplicado y el ajuste de los datos correspondientes con la ecuación de Boltzmann (control, línea continua; células tratadas con BSA, línea discontinua). n= 3-5 células para cada condición. La conductancia (G) se calculó con la ecuación de Goldman-Hodgkin-Katz utilizando un potencial de equilibrio para Ca^{2+} calculado con el programa MaxChelator (MAXC v5.0). El promedio de los datos se ajustó de acuerdo a la ecuación de Boltzmann (n=11 para cada condición). En todos los casos los símbolos representan la media ± E.S.M.

y -50.5 mV en las células tratadas). Más aún, el estudio de la inactivación en estado estacionario se llevo acabo con prepulsos largos (de 210 ms) seguidos de un pulso de prueba a -25 mV, tal como se describió en la sección de métodos, para medir la disponibilidad para la apertura del canal. Los datos promedio se ajustaron a la ecuación de Boltzmann. La comparación de la inactivación en estado estacionario antes y después del tratamiento con BSA demostró diferencias estadísticamente significativas ($V_{½}$=-58.5 para el control versus -52.9 mV para las células tratadas). Es importante destacar que en algunos voltajes, a pesar de la activación de los canales durante el prepulso, estos no se inactivaron completamente tal como se puede ver en la corriente registrada durante los pulsos de prueba. Esta actividad corresponde a la llamada corriente ventana, y se ilustra típicamente en la sobreposición de las curvas de activación e inactivación en estado estacionario (Fig. 7C). Los resultados mostrados en esta figura sugieren que el tratamiento de la ICa_T con BSA produjo un incremento en la corriente de ventana estadísticamente significativo. Así, podría ser que en el pico de la ventana (~ -52 mV) el 28 y el 44% de los canales estaban disponibles para abrirse en ausencia (control) y en presencia de BSA, respectivamente.

El mecanismo por el cual la BSA regula a la ICa_T podría estar relacionado con su capacidad para quitar colesterol de la membrana. La figura 8A (panel izquierdo) muestra que la adición de BSA preincubada con una concentración saturante (cociente molar de 1:10) de un análogo del colesterol (sulfato de colesterol; CSO_4) produjo un incremento significativo en la amplitud de la corriente (de ~1.3 veces) que no se aumentó por una segunda aplicación de BSA sin colesterol. Lo que sugiere que la BSA-CSO_4 ya había inducido la respuesta máxima posible a la concentración aplicada. Más aún, la aplicación de CSO_4 por si mismo no modificó las propiedades de la ICa_T (datos no mostrados).

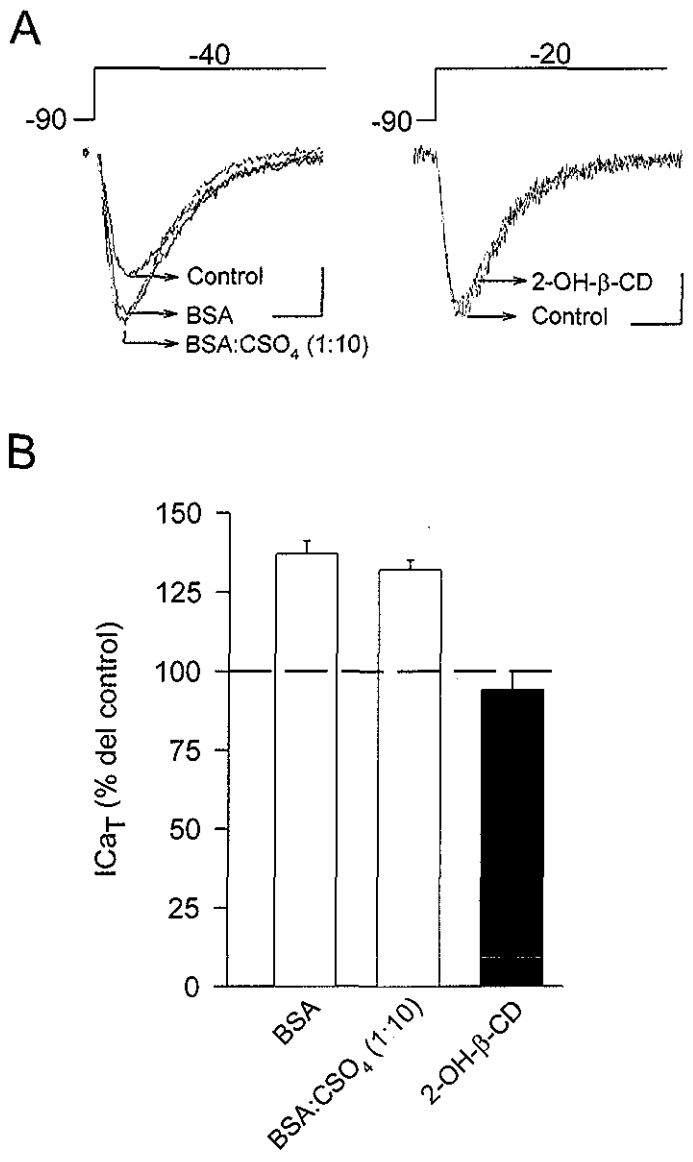

Figura 8.- Los efectos de la albúmina bovina (BSA) en la ICa_T de las células espermatogénicas no están relacionados a su capacidad como aceptor de colesterol. **A:** montaje de los trazos de corriente obtenidos en ausencia (control) y presencia de 15 μM de BSA presaturada con colesterol (CSO_4), seguidos de una segunda adición de BSA sin CSO_4 (panel izquierdo). El panel derecho muestra los trazos de corriente superimpuestos representativos antes y después de la aplicación de 500 μM de 2-OH-β-ciclodextrina (2-OH-β-CD). Las barras de escala representan 50 pA y 25 ms para ambos paneles. **B:** Histogramas de la comparación del efecto de 2 minutos de incubación con BSA, BSA presaturada con CSO_4 y 2-OH-β-CD en la amplitud de la ICa_T (n=4-6 células). La línea discontinua representa el control. Las barras representan la media ± E.S.M.

De manera consistente con los datos anteriores, el uso de 2-OH-β-ciclodextrina (2-OH-β-CD), un heptasacárido cíclico capaz de promover un eflujo rápido de colesterol de la membrana del espermatozoide y que induce un incremento en los patrones de fosforilación

en tirosina similares a los reportados en respuesta a BSA durante la capacitación (Visconti *et al*., 1999), no tuvo efecto alguno en la magnitud y en la cinética de la ICa_T (fig. 8A, panel derecho). En conjunto, estos resultados (resumidos en la figura 8B), son evidencia de que la BSA no está modulando a la ICa_T a través de su capacidad de quitar colesterol de la membrana celular.

Estudios previos han demostrado que la aplicación aguda de β-estradiol inhibe a los canales de Ca^{2+} tipo T de las células de músculo liso (Mermelstein *et al*., 1996), en neuronas (Ogata *et al*., 1996), y en miocitos cardiacos o lisos (Zhang *et al*., 1994; Nakajima *et al*., 1995; Yamamoto *et al*., 1995; Kitazawa *et al*., 1997; Ruehlmann *et al*., 1998). Así que cabría la posibilidad de que la albúmina pudiera estar incrementando a la ICa_T al extraer de la membrana celular a alguna molécula hidrofóbica tal como el β-estradiol. Para probar esta hipótesis, perfundimos a las células espermatogénicas con concentraciones crecientes de 17-β-estradiol y registramos a la corriente de Ca^{2+} (Fig. 9A). Tal como anticipábamos, la corriente registrada de las células tratadas fue menor en todos los voltajes de prueba aplicados (Fig. 9B). A concentraciones nM, la inhibición de la ICa_T fue mínima (~10%) y se hizo más evidente a concentraciones μM (ver apéndice; Espinosa *et al*., 2000). Esta inhibición fue independiente de voltaje (Fig. 9B) y muy rápida (< 2 min) como para ser atribuida a una activación genómica. A diferencia de lo reportado para canales de Ca^{2+} de alto umbral de activación (Ogata *et al*., 1996; Mermelstein *et al*., 1996), esta inhibición probablemente no involucre la activación de una vía de señalización a través de proteínas G ya que la corriente T de las células espermatogénicas es básicamente independiente de proteínas G (Arnoult *et al*., 1997). Un isómero del 17-β-estradiol (17-α-estradiol) hormonalmente inactivo causó una menor reducción en la actividad de la corriente T (Fig.

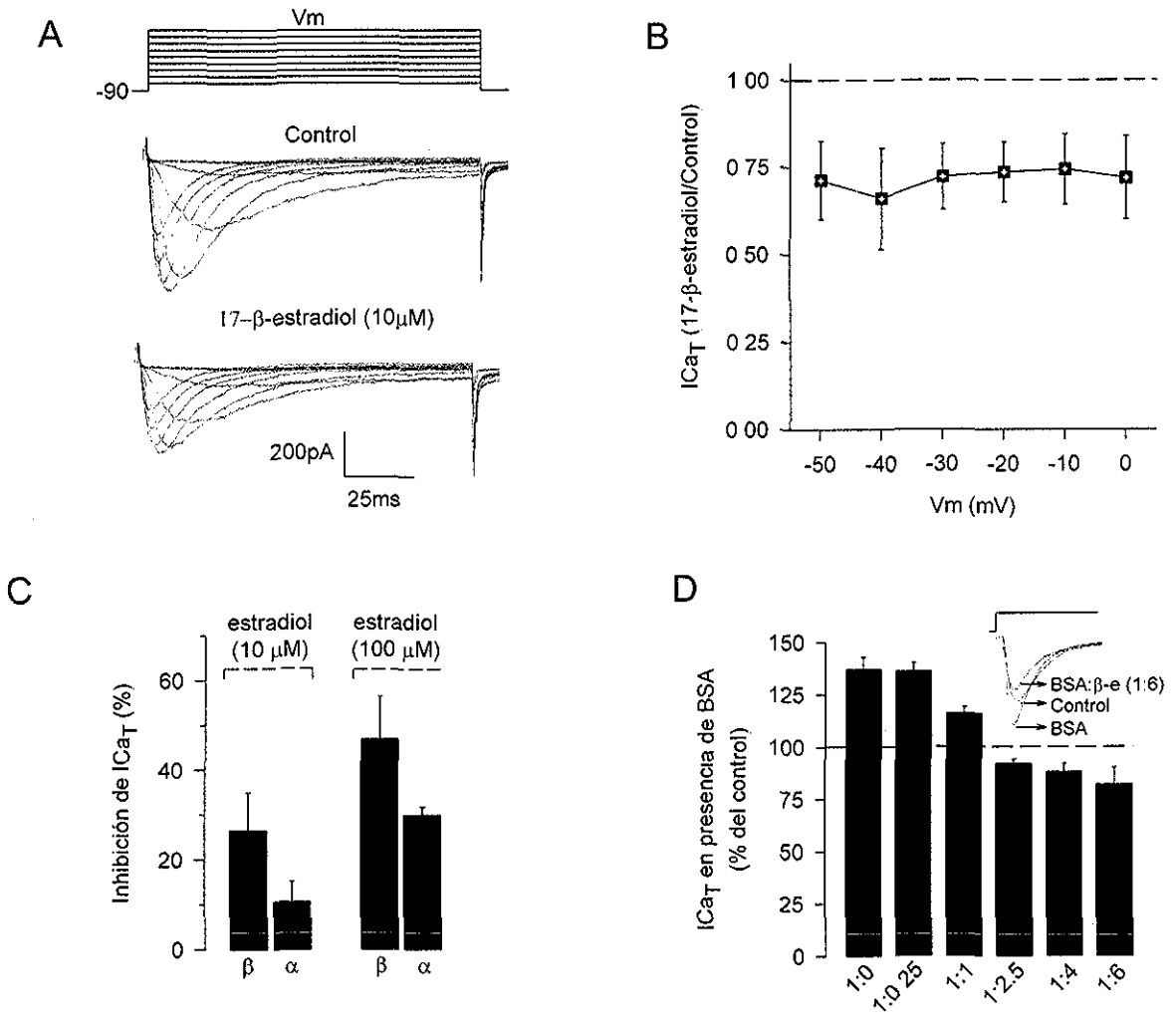

Figura 9.- el 17-β-estradiol inhibe la corriente de Ca^{2+} tipo T de las células espermatogénicas. A: registros representativos de corrientes T antes (panel superior) y después de 2 minutos de incubación con 17-β-estradiol (panel inferior). **B:** corriente al pico después de la aplicación del 17 -β-estradiol a diferentes pulsos de prueba normalizados con respecto al control (línea discontinua) **C:** comparación de la inhibición de la ICaT después de 2 minutos de incubación en presencia de los isómeros β y α del estradiol (n=3 y 8, respectivamente). Las corrientes se registraron con pulso de prueba de –20 mV a partir de un potencial de reposo de –90 mV, y la concentración de estradiol fue de 10 (barras a la izquierda) y 100 μM (barras a la derecha). **D:** Efecto del β-estradiol sobre el incremento de la ICaT inducido por BSA. Cocientes de la corriente tratada con albúmina presaturada con diferentes concentraciones de β-estradiol. La línea discontinua representa el promedio del control. Inserto: trazos de corriente superimpuestos obtenidos a pulsos de prueba a –40 mV. La BSA se presaturó con 15 μM de 17 -β-estradiol. Después de 2 minutos de incubación, se aplicó una solución de BSA libre de estradiol a las células tratadas con BSA-estradiol. Las barras representan el promedio del efecto de la BSA pretratada con concentraciones crecientes de β-estradiol ± E.S.M.

9C) indicando cierta especificidad en la respuesta. Como paso siguiente, exploramos sí la estimulación de la ICaT por BSA pudiera ser antagonizada por 17-β-estradiol. Como se muestra en la figura 9D, la BSA presaturada con 17-β-estradiol no indujo el incremento de

la corriente al pico sino produjo una discreta disminución de la amplitud de corriente la cual fue anulada por una segunda adición de BSA (*inserto*). Este hecho es consistente con la hipótesis que sugiere que el incremento de la ICa_T inducido por la BSA pudiera deberse a la disminución de la concentración de 17-β-estradiol de la membrana celular más que de colesterol. Sin embargo, tal como se muestra en la Fig 9D, el incremento progresivo de 17-β-estradiol inhibió la actividad del canal T solamente a cocientes molares > 1:2.5, dejando abierta la pregunta de si la inhibición de la ICa_T en estas condiciones experimentales era causada por la presencia de 17-β-estradiol libre. Además, es importante hacer resaltar que el efecto de la BSA en la corriente T fue dependiente de voltaje mientras que el efecto del estradiol no. Esta diferencia en la dependencia de voltaje indica que el efecto de cada uno de estos compuestos es independiente uno del otro, y sugiere una posible regulación fisiológica potencial por estrógenos en las células espermatogénicas. Por otra parte, estudios recientes demostraron que el 17-β-estradiol puede inducir un incremento en la $[Ca^{2+}]_i$ a través de la activación de un receptor de estrógenos localizado en la superficie celular (Luconi *et al.*, 1999). Nuestros datos sugieren que este incremento de Ca^{2+} inducido por estrógenos no estaría relacionado a la activación de los canales de Ca^{2+} tipo T.

4.1.2 - Antagonistas de calmodulina inhiben a la corriente de Ca^{2+} tipo T en las células espermatogénicas y a la reacción acrosomal inducida por la zona pelúcida

Reportes previos (Liévano *et al.*, 1996; Arnoult *et al*, 1996a; Santi *et al*, 1996; Hagiwara y Kawa, 1984; Arnoult *et al*, 1998) de registros electrofisiológicos revelaron que las células espermatogénicas expresan funcionalmente solo un tipo de corriente de Ca^{2+} dependiente de voltaje, una corriente de Ca^{2+} tipo T. Los trazos del panel superior en la Fig.

10A ilustran una familia de corrientes de Ca^{2+} representativa de una célula control obtenidas al aplicar pulsos depolarizantes con incrementos de +10 mV a partir de un potencial de mantenimiento (HP) de -90 mV. Con estas condiciones experimentales usamos el W7, una antagonista selectivo de calmodulina (CaM), para explorar si la CaM pudiera estar participando en la regulación de los canales de Ca^{2+} dependientes de voltaje (CCDV) del espermatozoide. Los trazos inferiores de la Fig. 10A muestran la evidente inhibición de la corriente T (>45%) después de perfundir a la célula con 10 μM de W7. La Fig. 10B resume las curvas corriente-voltaje (*I-V*) correspondientes al tiempo al pico de la corriente durante cada registro obtenido antes y después de la aplicación de W7 en varias células. La Fig. 10C presenta la curva dosis-respuesta de la inhibición de la ICa_T por W7. Las corrientes se obtuvieron al aplicar un pulso de 20 ms a -20 mV a partir de un HP de -90 mV, en los cuales se midió la corriente al pico, cada trazo se normalizó con respecto al control (en ausencia del fármaco). Aunque 100 μM de W7 no abolió completamente a la corriente de Ca^{2+}, la aplicación de 10 μM de W7 fue suficiente para inhibir aproximadamente el 50% del total de la corriente macroscópica. Así que, de aquí en adelante utilizamos la concentración de 10 μM para evaluar con más detalle los efectos del W7. Tal como se puede apreciar en la Fig. 10D, la inhibición de la corriente T inducida por el W7 no mostró una notable dependencia de voltaje en el intervalo de ±40 mV.

Para probar si la CaM estaba involucrada en la inhibición de la corriente T inducida por el W7, a continuación examinamos el efecto de un homólogo estructural del W7, el W5 (el cual es similar al W7 pero carece de un Cl^- y es mucho menos potente como antagonista

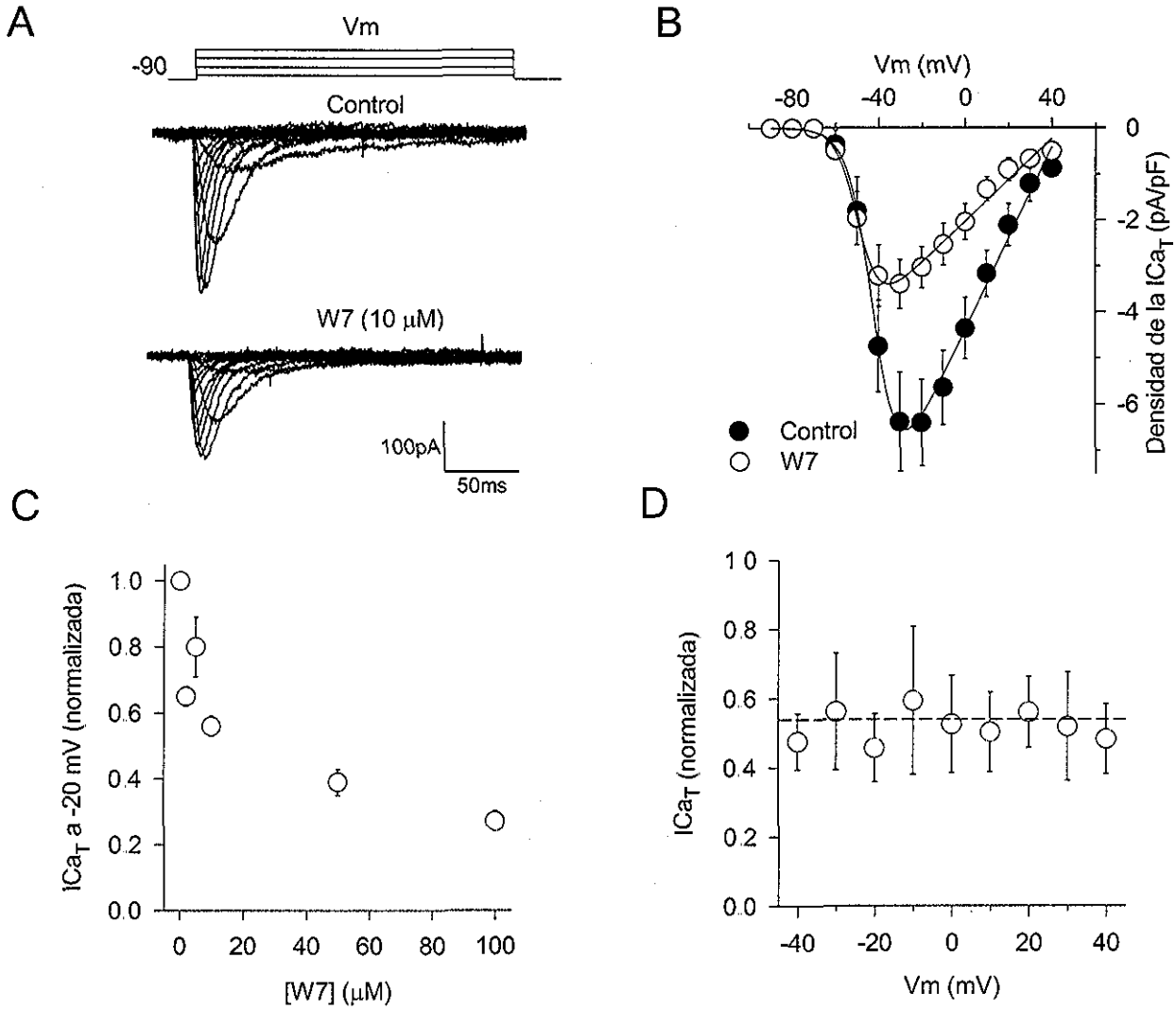

Figura 10.- El W7 inhibe la corriente de Ca^{2+} T de las células espermatogénicas. A: Los trazos del panel superior ilustran la familia de corrientes obtenidas por un protocolo de pulso de 200 ms a partir de un potencial de mantenimiento de –90 mV con pulsos de prueba que van desde –90 mV hasta 40 mV con incrementos de 10 mV por pulso. Durante los experimentos control (5 min), el lavado de la corriente al pico fue mínimo y siempre menor al 10%. Después de la adición de 10 µM del W7, un antagonista específico de CaM; a la solución externa de la cámara de registro, se observó una disminución significativa de la amplitud de la corriente (panel inferior). **B:** Curvas I-V correspondientes a las células antes y después de la aplicación del W7 (n= 4). **C:** Curva dosis-respuesta de la inhibición de la ICa_T por W7. El pico de la corriente se normalizó con respecto a su valor antes de la aplicación del W7. **D:** La inhibición inducida por el W7 fue independiente de voltaje. La gráfica presenta la fracción de la corriente inhibida a varios pulsos depolarizantes después del tratamiento con W7. La corriente al pico se normalizó, promedió y se graficó en función al pulso de prueba aplicado. En todos los casos, los símbolos representan el promedio ± E.S.M.

de CaM). Consistentemente, la aplicación de una alta concentración de W5 (50 μM) fue incapaz de causar una inhibición significativa de la corriente T (Fig. 11A). En contraste, otro potente antagonista de CaM, la trifluoperazina (TFP) inhibió a la corriente de manera similar al W7. Para descartar que el W7 estuviera bloqueando directamente al canal T, comparamos la inhibición de la corriente causada por W7 en ausencia y presencia de CaM incluida en la solución interna de registro (Fig. 11B). El porcentaje de inhibición de la corriente por W7 (10 μM) fue significativamente menor cuando se incluía una concentración equimolar de CaM en la solución interna. La aplicación CaM en la solución interna, por si misma, no tuvo efecto sobre la magnitud de la corriente de Ca^{2+}, lo cual indica que la CaM endógena ocupa los sitios de regulación del canal. Además, al utilizar Ba^{2+} como acarreador de carga, encontramos que la inhibición inducida por el W7 se redujo

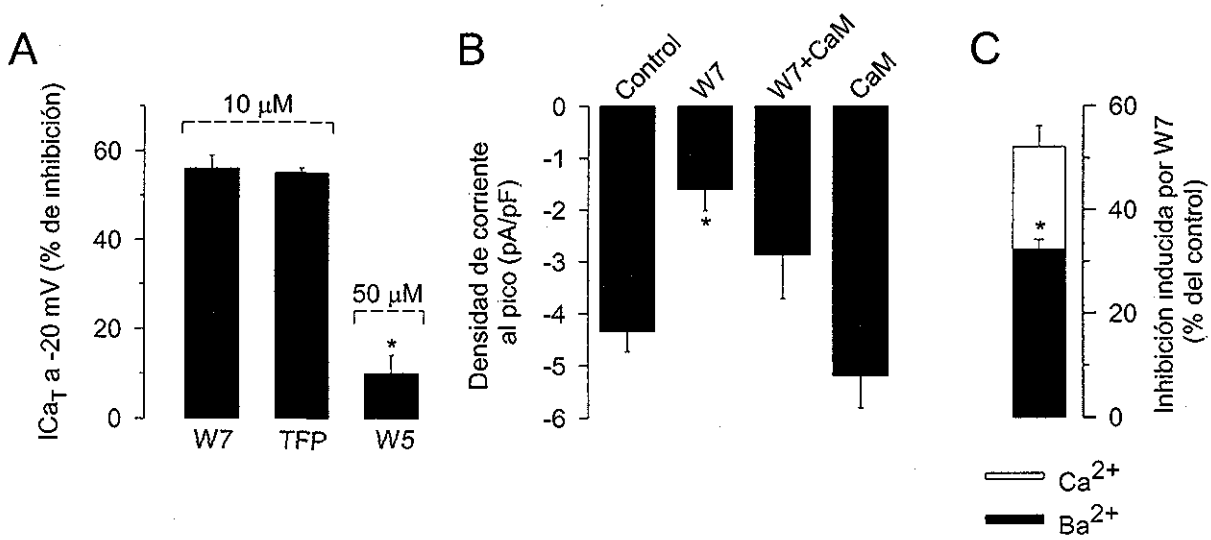

Figura 11.- La TFP induce una inhibición de la ICa_T similar a la producida por el W7. A: TFP (10 μM) inhibió a la corriente de Ca^{2+} de manera similar al W7, mientras que 50 μM de W5 (un antagonista de CaM mucho menos potente) fue incapaz de inducir una inhibición significativa de la corriente. **B:** comparación de la densidad promedio de la corriente de Ca^{2+} antes (control) y después de la adición de W7 (10 μM) a la solución externa, en presencia o ausencia de CaM (10 μM) en la solución interna (n=5-21). **C:** Barras superpuestas correspondientes a los promedios ± E.S.M. de la inhibición inducida por W7 en presencia de Ca^{2+} o Ba^{2+} como acarreadores de carga. Los asteriscos representan una diferencia estadística con respecto al control ($p< 0.05$)

significativamente (Fig. 11C) lo cual implica que la acción del W7 depende de Ca^{2+} parcialmente.

Continuando con la caracterización del efecto del W7, analizamos a las corrientes de cola. La repolarización de la membrana plasmática a -90 mV antecedida por una depolarización corta a -20 mV causó un rápido incremento en la amplitud de la corriente entrante de Ca^{2+} debido al gran gradiente electroquímico que favorece el influjo de Ca^{2+}. La Fig. 12A (*inserto*) muestra los trazos superimpuestos representativos de la corriente obtenida antes y durante la aplicación de W7, e ilustra la inhibición causada por este fármaco. De la misma forma, la figura 12*A* resume los resultados normalizados de varias células y hace evidente que el W7 (10 μM) disminuye la densidad máxima de la corriente

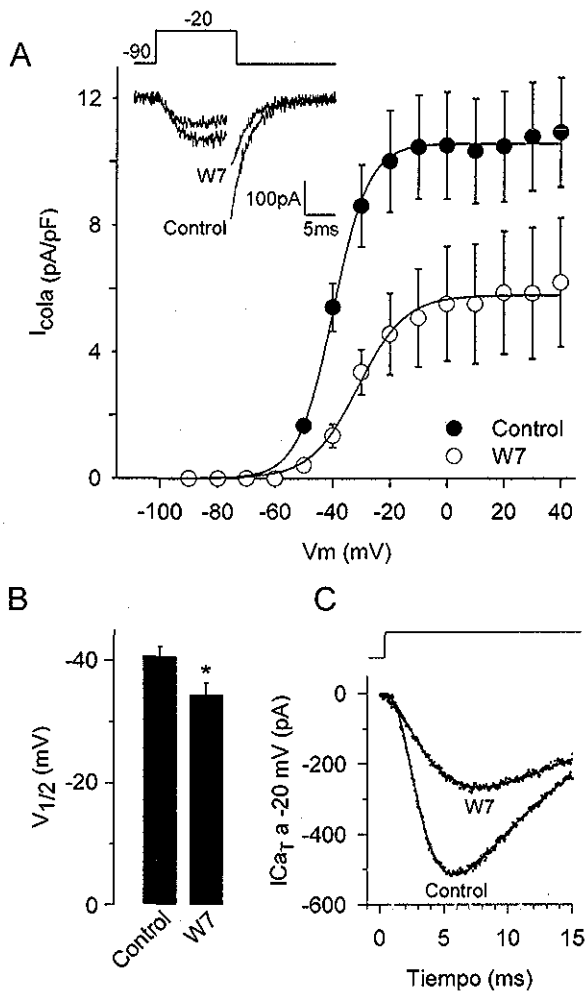

Figura 12.- El W7 modula la cinética de activación de la corriente T. A: Datos normalizados del control y de la inhibición de las corrientes de cola de varias células, promediados y graficados en contra del potencial de membrana aplicado. El ajuste de los datos se hizo de acuerdo a una función de Boltzmann, $I_{Ca} = I_{max}/(1 + \exp[-(V_m-V_{1/2})/s])$, donde V_m es el potencial de prueba, $V_{1/2}$ es el potencial al cual la corriente alcanza la mitad de su amplitud máxima, y s es el intervalo de potencial para un cambio de e-veces cercano a $V_{1/2}$. *Inserto*: Los trazos superimpuestos ilustran las corrientes de colas de Ca^{2+} antes y después de la aplicación de W7 (10 μM), obtenidas al despolarizar a la membrana celular a-20 mV durante 14 ms y repolarizarla a –90 mV (HP). **B:** Comparación de las $V_{1/2}$ promedio derivadas del ajuste con la ecuación de Boltzmann (n=6-11 células). **C:** curso temporal de la activación de la corriente T en ausencia y presencia de W7 en células con una resistencia en serie <6 MΩ sin compensar. Las corrientes se activaron durante 20 ms a –20 mV a partir de un potencial de mantenimiento de –90 mV. Los trazos incluyen el mejor ajuste a las corrientes obtenidos con la ecuación de Hodgkin-Huxley (Hille, 1992).

de cola en un 46% aproximadamente. Los datos se ajustaron a una ecuación de Boltzmann para determinar los parámetros de la activación. En las condiciones control, el voltaje al cual la corriente alcanzó la mitad de su amplitud máxima ($V_{1/2}$) fue -40.7 $\pm$ 1.6 mV. En contraste, la aplicación aguda de W7 indujo un pequeño pero significativo corrimiento en la dependencia de voltaje de la activación de ~6.3 mV ($p<0.05$) hacia potenciales positivos (Fig. 12*B*). La figura 12*C* compara el curso temporal aparente de la activación de la corriente en ausencia y presencia de W7. De nuestros resultados podemos observar que además de disminuir la amplitud de la corriente, el tratamiento con W7 también resulta en un ligero retraso en el inicio de la activación de la corriente, y como consecuencia en un incremento significativo en el tiempo necesario para alcanzar el pico de la corriente de Ca^{2+} (7 $\pm$ 0.3 y 9.2 $\pm$ 1.5 ms, respectivamente).

Recientemente se demostró que la unión de Ca^{2+}/CaM a la región IQ, sitio consenso de unión a CaM, inhibe a canales de Ca^{2+} HVA debido a que promueve la inactivación de los mismos (Zühlke *et al*, 1998; Lee *et al*., 1999; Peterson *et al*., 1999; Zühlke *et al*, 1999). Con base en lo anterior, examinamos si este modo de regulación se presentaba en el canal I de las células espermatogénicas. El W7 causó un corrimiento de ~3.8 mV en el sentido depolarizante en la curva de inactivación en estado estacionario, así como también produjo un cambio en la pendiente de la curva de un valor de 4.6 $\pm$ 0.7 (control) a 5.4 $\pm$ 0.8 en las células tratadas (datos no mostrados). Aunque las diferencias en ambos parámetros no fueron estadísticamente significativas, el W7 si modificó a la cinética de inactivación de la corriente (Fig. 13*A*). Dicha modificación se hace evidente al analizar la fracción de la corriente residual después de 80 ms (línea continua en la Fig. 13*A*) durante un pulso de activación de 200 ms. La corriente residual se incremento significativamente al aplicar W7

sugiriendo un efecto en la inactivación de la corriente T (Fig. 13*B*). Así, el W7 podría limitar a la inactivación del estado abierto de los canales o bien indirectamente enlentecer a la inactivación como consecuencia de su efecto sobre la activación como se mostró anteriormente. Tal como se ilustra en la fig. 13*C*, la constante de tiempo de la inactivación se incremento significativamente de 14.1 ± 0.9 a 25.9±1.9 ms después del tratamiento con W7. Esta observación es consistente con la hipótesis de que el W7 podría estar actuando a través de la CaM, ya que esta proteína induce inactivación en distintos tipos de canales iónicos incluyendo en canales de Ca^{2+} HVA. La inactivación de la corriente de Ca^{2+} registrada con un pulso de prueba de –20 mV se fue haciendo más lenta en función de la concentración del W7 aplicado (Fig. 13*D*, barras blancas). El hecho de que el W7 incremente la constante de tiempo de la inactivación debería favorecer un aumento en la amplitud de corriente de Ca^{2+} y no reducir la amplitud de la misma como lo muestran nuestros resultados. Una posible explicación a esta observación consiste en la hipótesis de que el W7 estuviera afectando principalmente a la carga total de la corriente a pesar de enlentecer a la inactivación. Con la idea de probar está hipótesis, calculamos la integral de la corriente medida antes y después de la aplicación de W7 y, como se ilustra en la Fig. 13*D* (barras negras), el influjo de Ca^{2+} fue significativamente menor después de la aplicación del fármaco. Así pues, el enlentecimiento de la inactivación no fue suficiente para contrarrestar el efecto del W7 sobre el influjo total de Ca^{2+} lo que provoca una amplitud de corriente menor en presencia del fármaco.

Por último, para profundizar en el estudio de los mecanismos involucrados en el efecto inhibitorio de los antagonistas de CaM sobre la corriente T de las células espermatogénicas, analizamos la inactivación dependiente del estado del canal T. Trabajos

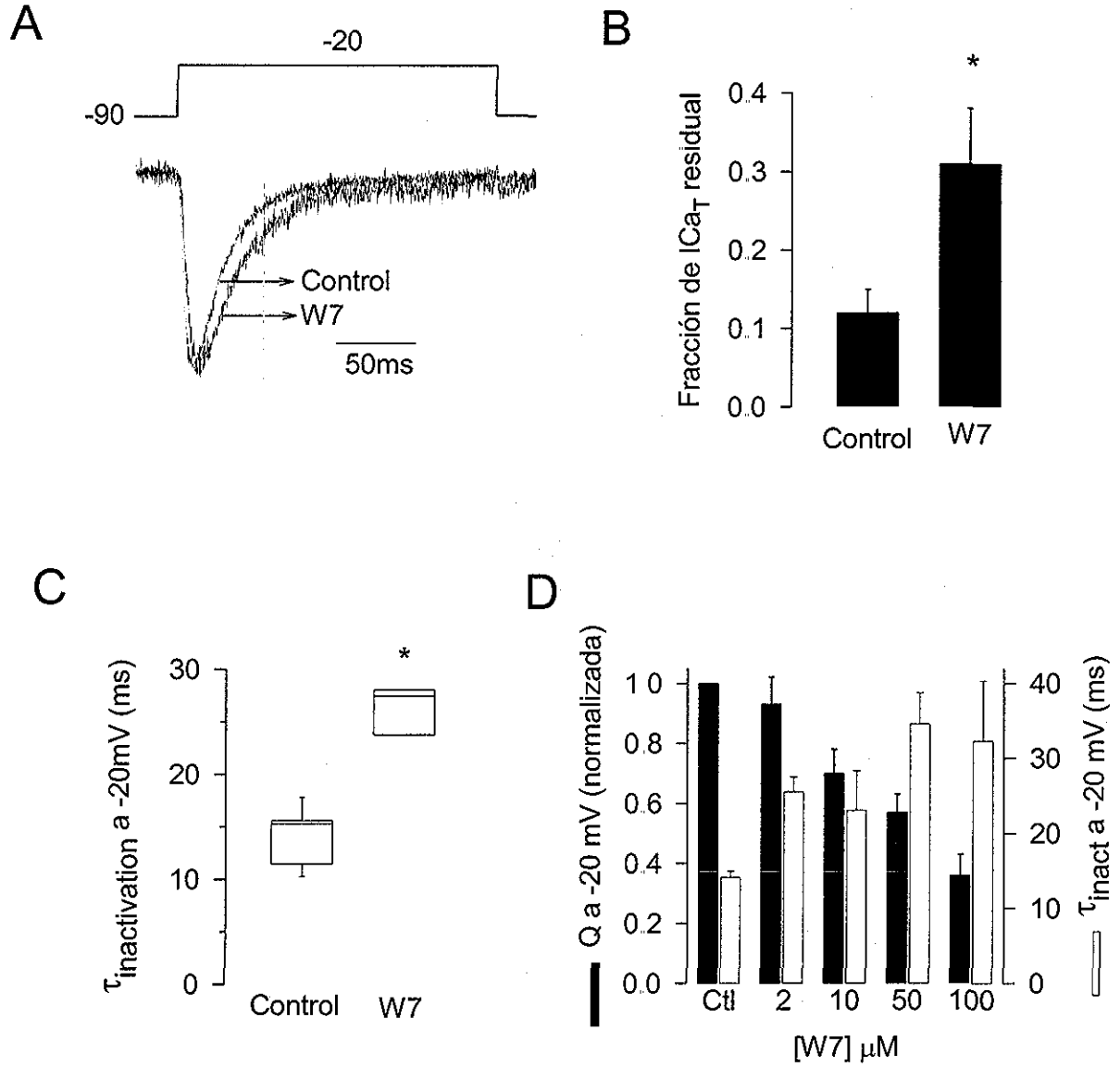

Figura 13.- El W7 afecta la inactivación de la corriente de Ca^{2+}. A: Comparación del curso temporal de un trazo de la corriente de Ca^{2+} típica en ausencia y presencia de W7 (10 μM). En la parte superior del panel A se muestra el protocolo de voltaje utilizado para el estudio de la inactivación. Normalizamos los trazos de corriente para hacer la comparación de los mismos. **B:** Comparación de la fracción de la corriente residual después de 80 ms (línea continua en A) de un pulso depolarizante antes y después de la aplicación de W7. Las corrientes se obtuvieron en respuesta a pulsos depolarizantes de 200 ms a –20 mV a partir de un potencial de mantenimiento de –90 mV. **C:** La fase de inactivación de la corriente se ajustó a una exponencial simple: I_{Ca}= A exp (-t/τ) + *c*, donde A es la amplitud inicial, t es el tiempo, τ es la constante de tiempo para la inactivación y *c* es una constante. La gráfica muestra la comparación de la *t* de inactivación de las corrientes obtenidas antes y después del tratamiento con W7 (n = 6-11). **D:** gráfica de la integral de la corriente (Q) calculada a partir de la corriente obtenida con un pulso de voltaje de 200 ms a –20 mV desde un potencial de mantenimiento de –90 mV. La carga calculada con este protocolo en presencia de W7 se normalizó con respecto a la carga obtenida en condiciones control. Los datos promedio se graficaron en función de la concentración de W7 (barras negras). Las barras blancas corresponden a los valores promedio obtenidos para la τ de inactivación de la corriente en función de la concentración de W7 (n= 3-7). Las barras representan el promedio ± E.S.M.

anteriores sugieren que los canales de Ca^{2+} tipo T presentan inactivación en estados cerrados, ya que al aplicar prepulsos largos (de –120 a –80 mV, voltajes en los cuales no se detectan aperturas del canal) disminuyen la amplitud de corriente en respuesta a un pulso de prueba de –20 mV (comparación de las corrientes registradas a –120 y –80 mV en la Fig. 14A). Esta inactivación es más evidente después de la aplicación de W7. En promedio, el W7 disminuye a la corriente en un 80 ± 3, 78 ± 2 y 72 ± 5 % en comparación con su control (99 ± 1, 97 ± 2, 95 ± 1 %) después de aplicar prepulsos de –120, –100 y –80 mV, respectivamente (Fig. 14B). Resultados recientes indican que los canales T permanecen de manera funcional en el espermatozoide maduro después de la diferenciación en el testículo y que estos juegan un papel clave en la RA (Arnoult *et al.*, 1999). Debido a que los antagonistas de CaM resultaron ser potentes reguladores de la actividad de los canales de

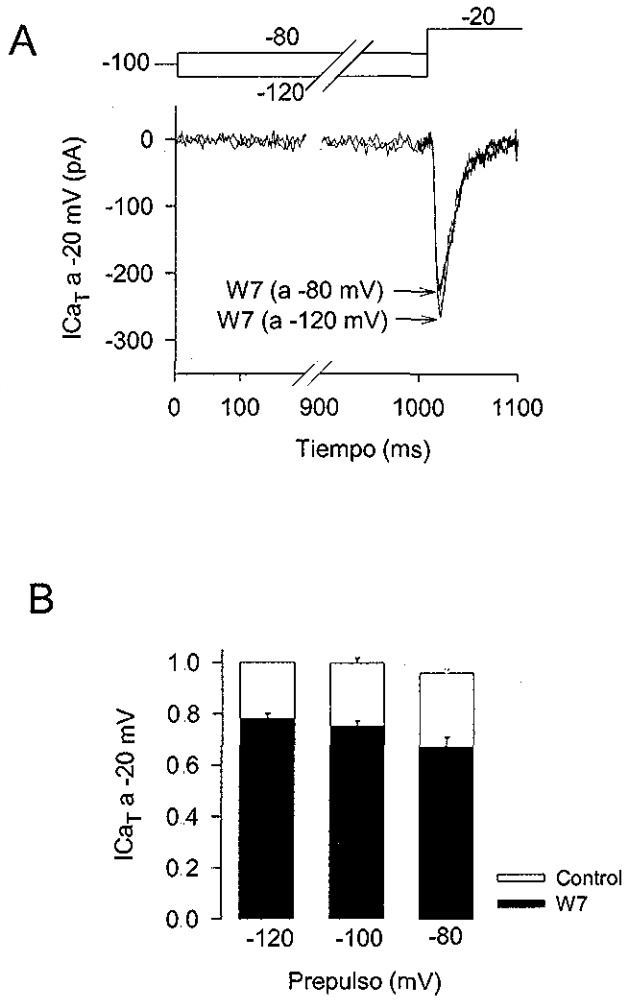

Figura 14.- El W7 promueve la inactivación de los canales T desde el estado cerrado. A: trazos de corriente registrados en fijación de voltaje en una célula después de la aplicación de W7 (10 μM) a dos diferentes potenciales. La corriente se registró con un prepulso de 1 s a –120 y –80 mV seguido de un pulso de prueba de –20 mV durante 75 ms. La inactivación en estado cerrado fue mayor a –80 mV que al aplicar un prepulso de –120 mV. **B:** comparación de la inactivación en estado estacionario obtenida en ausencia o presencia de W7 con prepulsos de –120, -100 y –80 mV (n = 5). Las barras blancas representan la corriente promedio en respuesta al potencial del pulso de prueba normalizadas con respecto a la amplitud obtenida con un prepulso de –120 mV en las células control. Las barras negras representan la amplitud de corriente obtenida después de la adición de W7, normalizadas con respecto al control.

Ca^{2+} T en las células espermatogénicas, estos agentes pudieran tener un efecto en la RA del espermatozoide maduro. Para probar esta hipótesis, primero determinamos si los canales T del espermatozoide también se inhibían con los antagonistas de CaM. La ZP dispara un incremento transitorio de Ca^{2+} intracelular en el espermatozoide, cuya cinética y características farmacológicas son propias del canal T (Arnoult *et al.*, 1999). La figura 15A muestra un ejemplo del transitorio de Ca^{2+} intracelular inducido por la ZP. Estos transitorios de Ca^{2+} intracelular dependientes de ZP se observaron en el 65% de las células observadas (22/34), con un valor de Ca^{2+} intracelular al pico de 7.09 ± 1.1 μM. Este incremento transitorio de Ca^{2+} regresa a su nivel basal en 250 ms. La fase de elevación de

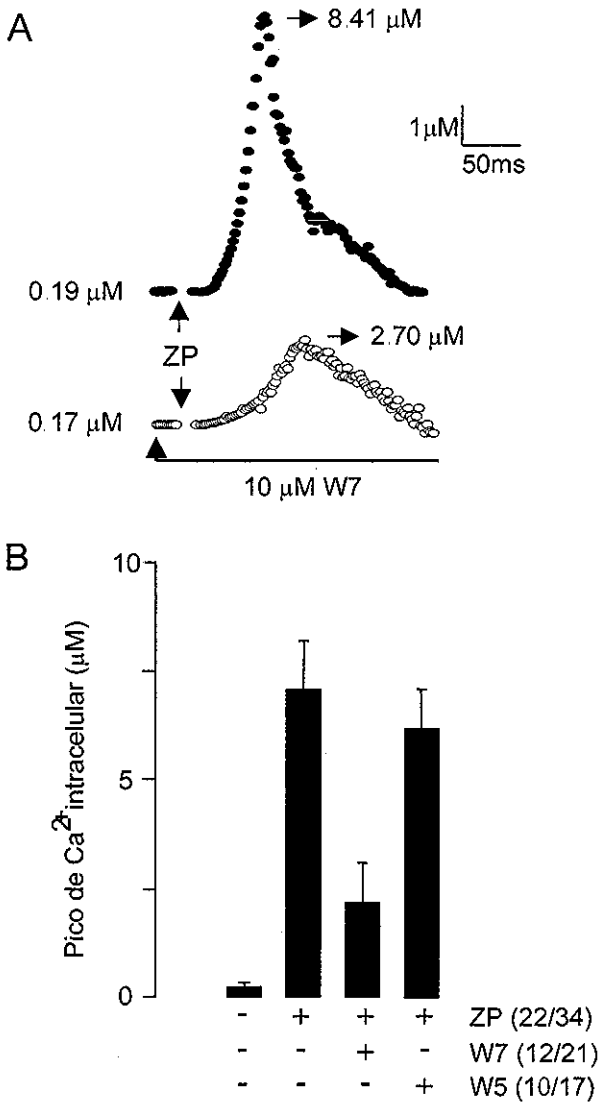

Figura 15.- El W7 inhibe el incremento de Ca^{2+} intracelular inducido por la ZP. **A:** trazos representativos del incremento de Ca^{2+} intracelular inducido por la ZP (30 μg/ml) en condiciones control (círculos negros) y en presencia de W7 (10 μM, círculos blancos). En los trazos se muestran el valor inicial de la $[Ca^{2+}]i$ y el valor al pico. **B:** Los espermatozoides se incubaron durante 10 min en asusencia (n= 22), o presencia de 10 μM de W7 (n= 12) o de 10 μM de W5 (n= 10) antes de la adición de la ZP. Las barras representan el promedio de los valores al pico ± DS durante los incrementos transitorios de Ca^{2+}. También se incluyen los valores de la fracción de células que respondieron al estímulo. Estos experimentos se hicieron en colaboración con el Dr. Harvey M. Florman.

esta respuesta se puede describir con una exponencial simple (τ=15.2±1.9 ms para el incremento de Ca^{2+} entre el 10 y el 90% del valor al pico). En contraste, las células control tratadas con la solución de registro solo presentaron un incremento monotónico a 0.27 ± 0.05 μM durante este periodo de tiempo. La ZP también es capaz de inducir el incremento transitorio de Ca^{2+} en el 57% de los espermatozoides (12/21) tratados con 10 μM de W7. Sin embargo, el pico de la respuesta transitoria de Ca^{2+} intracelular se inhibió en un 73% (2.21 ± 0.89 μM) y el tiempo de activación del curso temporal fue más lento (τ= 33.6 ± 4.1 ms). En contraste, 10 μM de W5 tuvo efectos mínimos sobre el transitorio de Ca^{2+} intracelular inducido por la ZP (Fig. 15B): el 59% de las células respondieron al estímulo con ZP (10/17), el W5 solamente redujo el valor máximo del transitorio en un 12% (6.21 ± 0.94 μM), y tuvo un efecto menor en el tiempo de activación observado (τ= 17.7 ± 3.1 ms).

Consistentemente, los antagonistas de CaM (W7 y TFP) también inhibieron significativamente a la RA inducida por la ZP en espermatozoides capacitados con una IC_{50} de ~10 μM (Fig. 16*A*). Es importante hacer notar que esta concentración también inhibe ~50% del pico de la corriente macroscópica de los canales T en las células espermatogénicas (ver Fig. 11A). Más aún, la Fig. 16*A* muestra que, tal como se anticipaba de la farmacología de las corrientes T (ver Fig. 11A), el W5 causó una inhibición menos importante de la RA en el espermatozoide maduro. Por otra parte, nuestros resultados sugieren que el bloqueo de la RA por el W7 parece deberse a la disminución del influjo de Ca^{2+} a través del canal de Ca^{2+} dependiente de voltaje ya que el uso de A23187, un ionóforo de Ca^{2+} que dispara la RA en ausencia de ZP (Visconti *et al.*, 1999), fue capaz de sobrepasar la inhibición causada por el W7 (Fig. 16*B*).

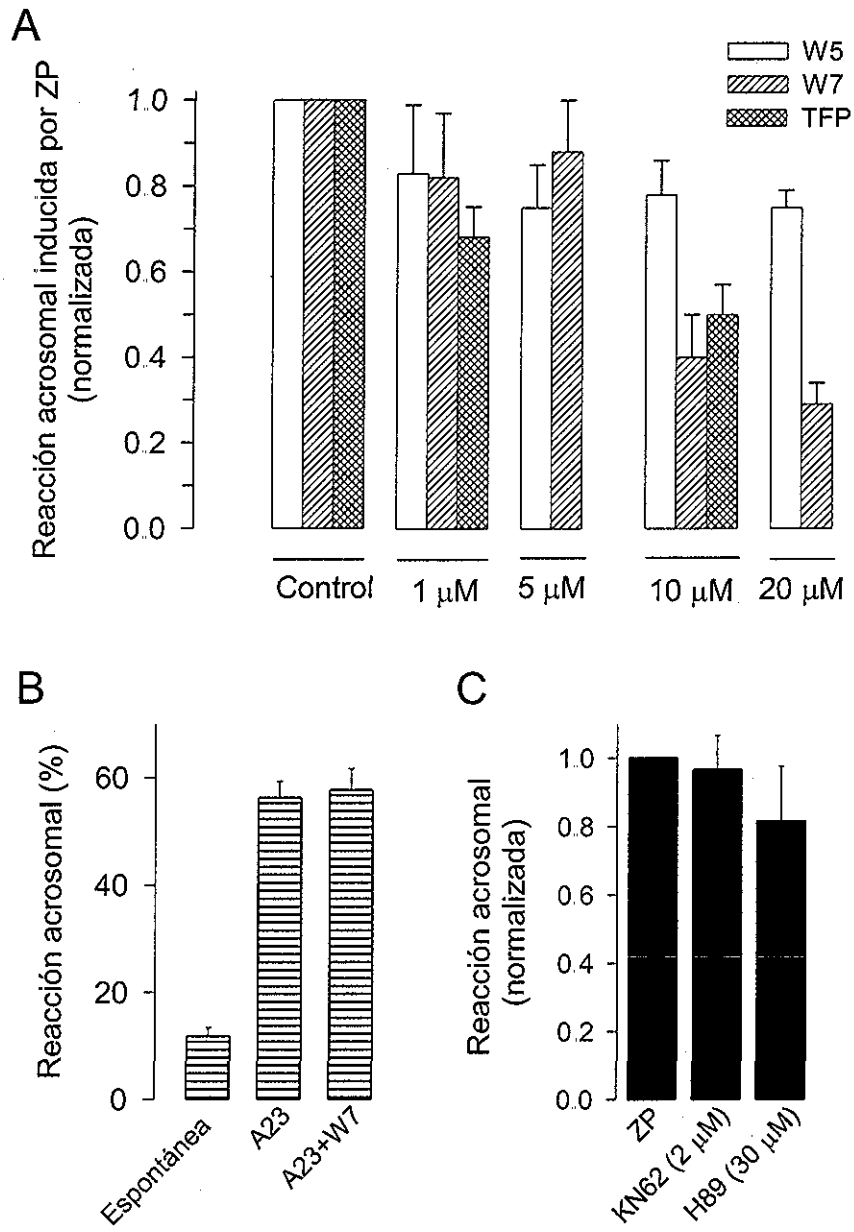

Figura 16.- Los antagonistas de CaM afectan la RA en los espermatozoides capacitados. A: efecto del W7 en el porcentaje de espermatozoides que sufren la RA inducida por la ZP. Los espermatozoides se incubaron en presencia de concentraciones crecientes de W7. Después de la capacitación, se monitoreó el porcentaje de RA inducida. Los datos representan el promedio ± E.S.M. de al menos cinco experimentos independientes. De la misma forma, los espermatozoides se incubaron en presencia de diferentes concentraciones de TFP (n= 6) y W5 (n= 7) para probar su efecto en la RA. **B:** La RA inducida por A23187 es insensible a W7. Las muestras se incubaron durante 35 min en presencia del ionóforo de Ca^{2+} A23187 (A23, 15 µM) solo o en combinación con W7 (n= 4). **C:** Dos inhibidores de protein cinasas dependientes de CaM no afectan a la RA. La gráfica nos muestra la comparación de la RA en ratón en presencia de KN62 y H89. Los espermatozoides se incubaron en ausencia o presencia de estos dos inhibidores de protein cinasas en las concentraciones indicadas. Los datos representan el promedio ± E.S.M. de cinco experimentos por separado.

Por último, se sabe que la CaM participa en distintos eventos de señalización a través de protein cinasas (Soderling, 1999). En el caso de la corriente de Ca^{2+} tipo T de las células espermatogénicas, se ha propuesto que la amplitud de la corriente T aumenta debido a la inhibición de la fosforilación (Arnoult *et al* , 1997). Debido a lo anterior, consideramos pertinente investigar si la modulación por CaM pudiera involucrar este tipo de regulación de canales de Ca^{2+}. Sin embargo, nuestros experimentos de fijación de voltaje indicaron que en este caso, la posible modulación de la corriente T es independiente de la participación de cinasas ya que el uso de concentraciones micromolares de dos inhibidores de CaM cinasas como el KN62 y el H89 no diminuyeron a la corriente T ni afectaron la cinética de la misma en las células espermatogénicas (datos no mostrados). De manera consistente, estos inhibidores no afectaron a la RA de los espermatozoides (Fig. 16*C*). Por otra parte, el uso de un péptido inhibidor, derivado de la fosfatasa dependiente de CaM calcineurina, tampoco afectó a la densidad de corriente ni a la constante de inactivación de la corriente T sugiriendo que en esta vía de modulación de la corriente T no se requiere la actividad de una fosfatasa dependiente de CaM (Fig 17A y B).

4.1.3 - La corriente de Ca^{2+} tipo T de las células espermatogénicas no se modula por progesterona o ácido γ-aminobutírico (GABA)

La iniciación de la RA por progesterona es un ejemplo de activación no genómica que induce una elevación del Ca^{2+} intracelular ($[Ca^{2+}]_i$) (Meizel *et al.*, 1990, revisado en Thomas y Meizel, 1988). Varios reportes han demostrado un influjo transitorio de Ca^{2+} inducido por progesterona en el espermatozoide humano (Aitken *et al.*, 1996; Baldi *et al* ,

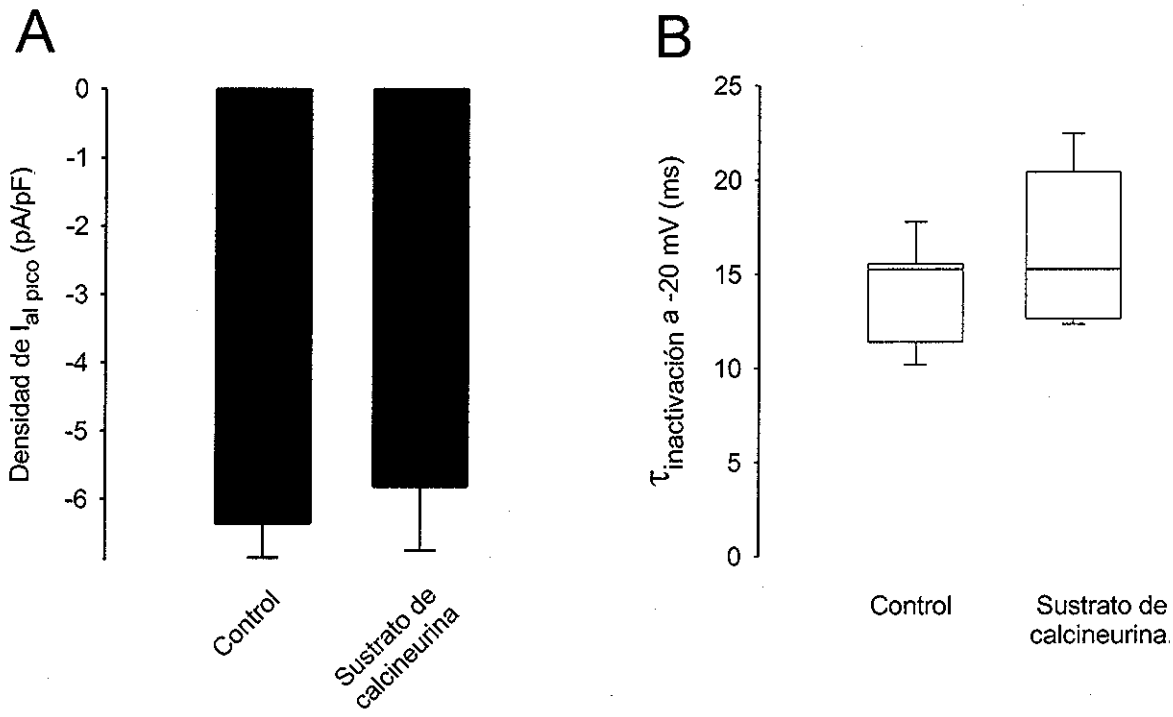

Figura 17.- La modulación de la ICa_T es independiente de la participación de una fosfatasa dependiente de CaM. El uso de un péptido inhibidor de la calcineurina (10 μM), una fosfatasa dependiente de CaM, no modificó ni a la densidad de la corriente al pico (**A**; n= 5) ni a la constante de inactivación de la corriente T (**B**; n= 5). Las barras representan el promedio ± E.S.M.

1991; Blackmore *et al.*, 1990; Meizel *et al.*, 1997; Plant *et al.*, 1995) que involucra a un receptor membranal de progesterona (Blackmore *et al.*, 1991; Meizel y Turner, 1991; Sabeur *et al.*, 1996; Tesarik *et al.*, 1992; Benoff *et al.*, 1995; Luconi *et al.*, 1998). A pesar de que es un hecho bien documentado que la mayoría de los espermatozoides responden a progesterona (Aitken *et al.*, 1996; Meizel *et al.*, 1997; Plant *et al.*, 1995), aún se desconocen las rutas de entrada de Ca^{2+}.

Como se mencionó anteriormente, se ha demostrado que el influjo de Ca^{2+} a través de canales dependientes de voltaje es necesario para la RA inducida por la ZP (Florman *et al.*, 1992; Florman, 1994; Arnoult *et al.*, 1996a; Arnoult *et al.*, 1996b). Específicamente, las evidencias electrofisiológicas sugieren que, al menos, el canal de Ca^{2+} tipo T está

involucrado en la RA (Santi *et al.*, 1996; Arnoult *et al*, 1996a; Arnoult *et al*, 1999). En el caso de la RA inducida por progesterona, la pregunta de una posible participación del canal de Ca^{2+} T está sin resolver. Sin embargo, si dicho canal estuviera involucrado en el influjo de Ca^{2+} inducido por progesterona entonces el potencial de membrana sería un factor clave y determinante. Algunos estudios han sugerido que la progesterona induce una depolarización de la membrana dependiente de cationes, pero la relación de esta depolarización y el incremento de Ca^{2+} no se ha investigado (Calzada *et al*, 1988; Foresta *et al.*, 1993). De igual forma, varios estudios apoyan la idea de la participación del receptor de $GABA_A$, un receptor ionotrópico de Cl^-, en la RA inducida por progesterona (Wistron y Meizel, 1993; Turner y Meizel, 1995; Turner et al, 1994; Shi y Roldan, 1995; Roldan *et al.*, 1994). Estudios recientes han sugerido que la participación del receptor de $GABA_A$ durante la RA inducida por progesterona posiblemente altere el potencial de membrana y, de esta manera, induce el influjo de Ca^{2+} a través de canales de Ca^{2+} dependientes de voltaje (Meizel *et al.*, 1997).

El objetivo de esta parte del trabajo fue explorar si la progesterona o el ácido γ-aminobutírico (GABA) pudieran inducir el influjo de Ca^{2+} que dispara a la RA a través de la modulación del canal de Ca^{2+} tipo T. Como se observa a continuación, nuestros resultados sugieren que ni la progesterona ni el GABA modulan a la corriente de Ca^{2+} tipo T. La figura 18A nos muestra los trazos de la corriente T obtenidos al aplicar un pulso de prueba de –20 mV de 60 ms, a partir de un potencial de mantenimiento de –90 mV, en ausencia y presencia de 50 μM de progesterona. La incubación de las células con progesterona no modificó la cinética de activación de la corriente ni la cinética de inactivación de la misma.

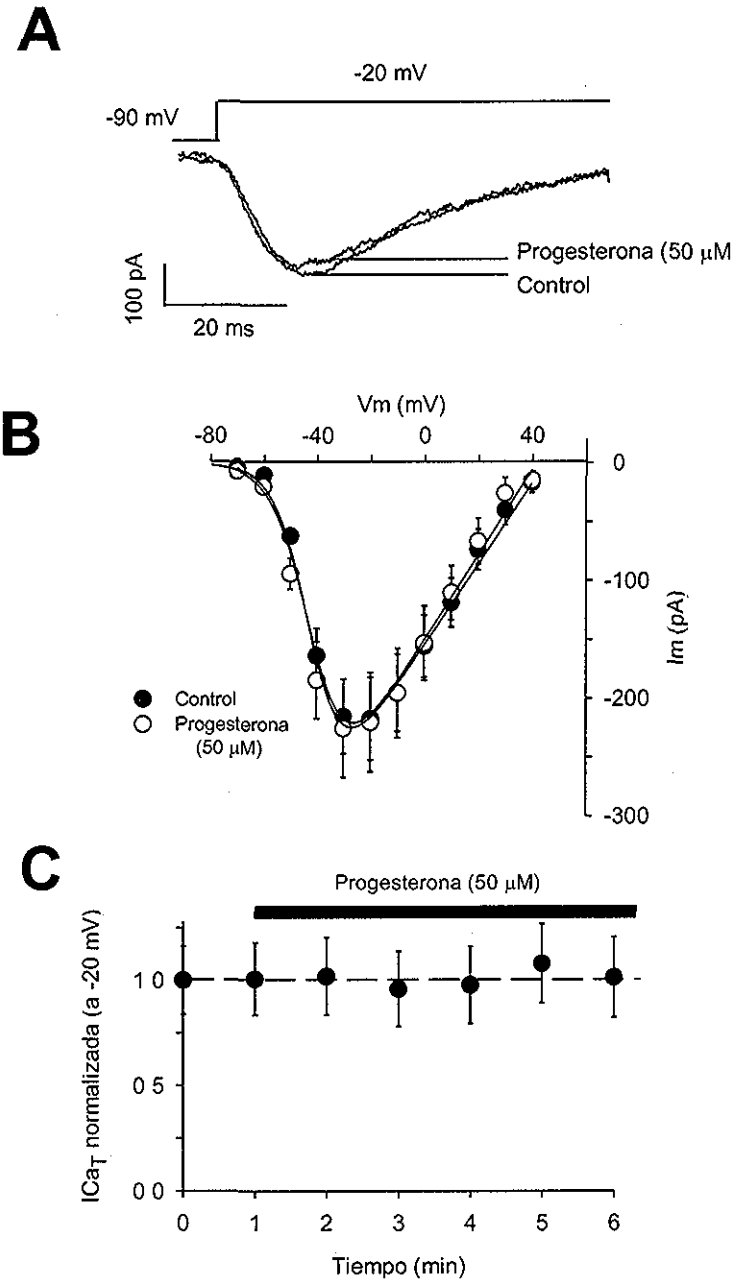

Figura 18.- La progesterona (50 µM) no modula la corriente de Ca^{2+} tipo T de las células espermatogénicas. A: Trazos de corriente de Ca^{2+} obtenidos al aplicar un pulso de –20 mV a partir de un potencial de mantenimiento de –90 mV antes y 5 minutos después de la aplicación de 50 µM de progesterona. **B:** Curvas I-V correspondientes a la corriente de Ca^{2+} tipo T en ausencia (control; círculos negros) o 5 minutos después de la adición de progesterona (círculos blancos) **C:** Curso temporal de la adición de progesterona. Los datos representan el promedio de la corriente al pico a –20 mV normalizada ± E.S.M. (n= 4-9)

Consistente con los datos anteriores, la presencia de progesterona (50 μM) tampoco alteró la forma de la curva I-V de la corriente de Ca^{2+} T de las células espermatogénicas (Fig. 18B). El curso temporal de la incubación con progesterona nos mostró que la amplitud del pico de la corriente de Ca^{2+} se mantuvo estable durante la aplicación de la hormona (Fig. 18C), de manera similar a la corriente en condiciones control (línea punteada en Fig. 18C).

Con la finalidad de explorar si el GABA pudiera estar modulando a la corriente de Ca^{2+} T y de esta manera contribuir a la RA inducida por progesterona (Wistron y Meizel, 1993; Turner y Meizel, 1995; Turner *et al.*, 1994; Shi y Roldan, 1995; Roldan *et al.*, 1994), aplicamos este compuesto a la corriente registrada con un pulso de prueba de –20 mV. La incubación de las células con 125 μM de GABA no modificó a las cinéticas de activación o de inactivación de la corriente como se puede observar en la figura 19A. Consistentemente,

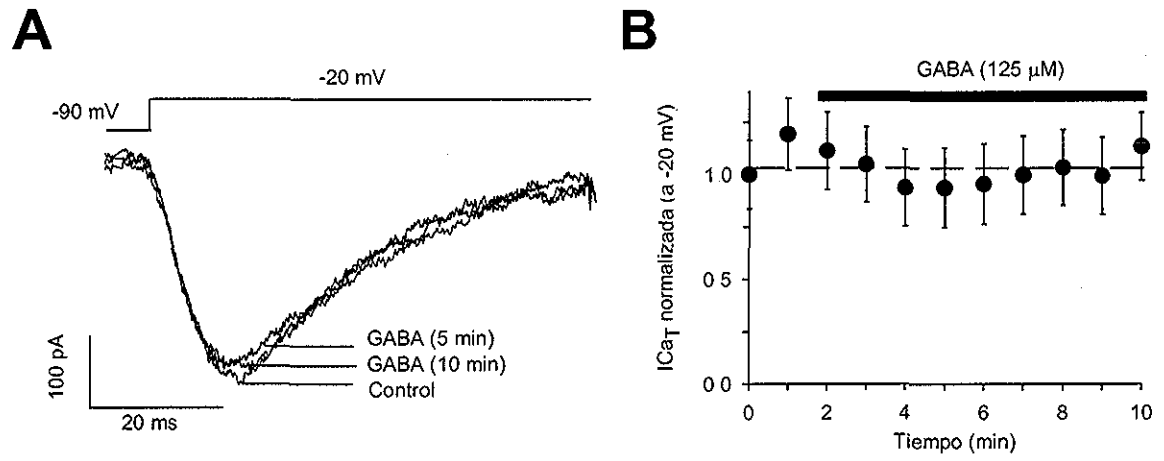

Figura 19.- El ácido γ-aminobutírico (GABA) no modula la corriente de Ca^{2+} tipo T de las células espermatogénicas. A: Trazos de corriente de Ca^{2+} obtenidos al aplicar un pulso de –20 mV a partir de un potencial de mantenimiento de –90 mV antes y 10 minutos después de la aplicación de 125 μM de GABA. **B:** Curso temporal de la adición de GABA (125 μM). Los datos representan el promedio de la corriente al pico a –20 mV normalizada ± E.S.M. (n= 4-9).

el estudio del curso temporal de la aplicación de GABA reveló que la corriente de Ca^{2+} al pico no se modifica por la aplicación de GABA (Fig. 19B). En conjunto, estos resultados sugieren que el canal de Ca^{2+} tipo T no participa en la RA inducida por progesterona

4.2.- Estudios farmacológicos de la corriente de Ca^{2+} tipo T.

4.2.1 - Efecto de bloqueadores de canales aniónicos sobre la ICa_T de las CE de ratón.

Estudios previos demostraron la presencia de un canal aniónico en la membrana plasmática del espermatozoide de erizo de mar el cual se bloquea parcialmente por DIDS, un inhibidor de canales aniónicos. De manera interesante, el DIDS bloquea la RA en *Strongilocentrotus purpuratus*, lo que sugiere la posible participación de canales de Cl^- en la RA (Morales *et al.*, 1993). Con respecto al espermatozoide de mamífero, sabemos que en la membrana celular del espermatozoide de cerdo y carnero se localiza un receptor a GABA ionotrópico (Erdo y Wetkele, 1990; Rabow *et al.*, 1995). Más aún, GABA o glicina puede inducir la RA en el espermatozoide humano, de ratón y cerdo, y dicha RA se puede inhibir con antagonistas de canales de Cl^- tales como el ácido niflúmico (Meizel, 1997). Con la finalidad de evaluar la posible participación de canales aniónicos en la RA inducida por la ZP, nuestro grupo realizó registros electrofisiológicos en el espermatozoide directamente y en las células espermatogénicas de ratón como una primera aproximación a esta interesante pregunta. Nuestros resultados demostraron la presencia funcional de canales aniónicos sensibles a ácido niflúmico (AN) tanto en el espermatozoide maduro (ver apéndice; Espinosa *et al.*, 1998) como en sus células precursoras (Fig. 20).

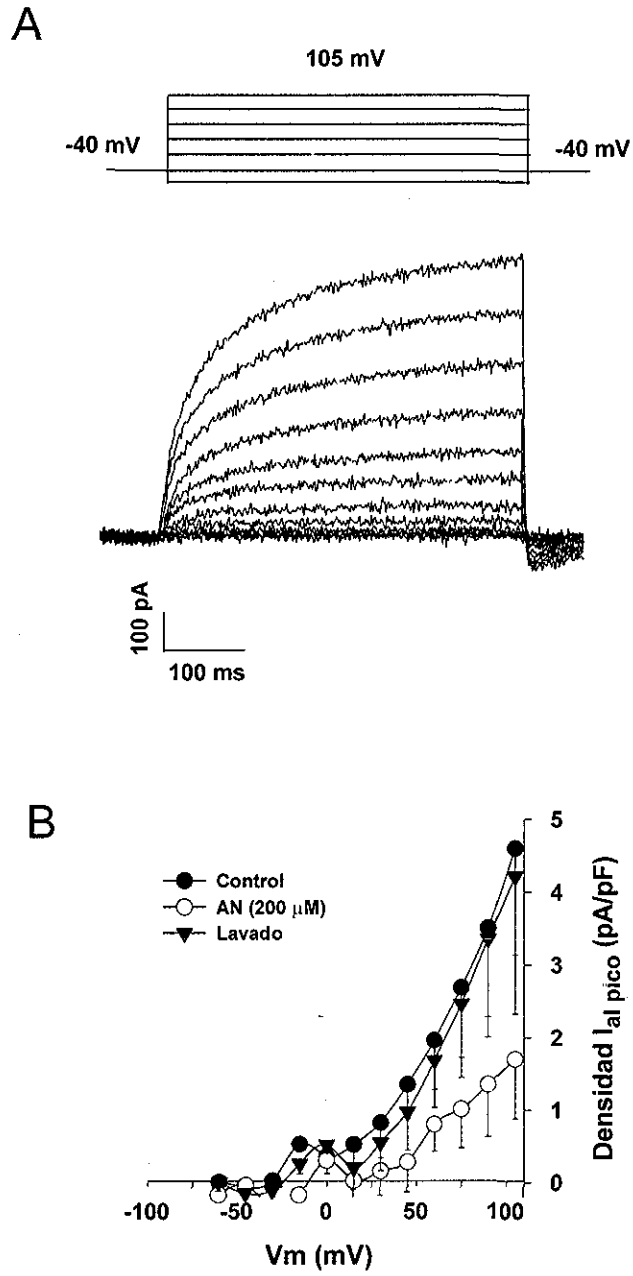

Figura 20.- El ácido niflúmico (AN) bloquea la corriente macroscópica saliente producida por el influjo de Cl^- en las células espermatogénicas. A: Familia de corrientes de Cl- registradas a partir de un potencial de mantenimiento de –40 mV con pulso de prueba depolarizantes desde –60 mV hasta 105 mV. La solución interna estaba compuesta de (en mM): 110 Cs-metanosulfonato, 10 CsF, 15 CsCl, 2 Cs-EGTA, 4 ATP-Mg, 10 fosfocreatina, 5 Cs-HEPES, pH 7.35. La solución externa contenía (en mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucosa, 10 $CaCl_2$, 0.16 amilorida, pH 7.35. **B:** curvas corriente-voltaje de la corriente de Cl- de espermatocitos en paquiteno en ausencia (círculos negros), presencia de 200 µM de AN (círculos blancos) y su recuperación (triángulos negros). Los puntos representan el promedio de 5 experimentos ± E.S.M.

Sin embargo, debido a que el AN y otros bloqueadores de canales aniónicos pueden inhibir a distintos canales iónicos de manera inespecífica, era posible que estos fármacos pudieran estar bloqueando a la RA inducida por la ZP debido a un efecto inespecífico sobre la ICa_T, la cual es un elemento clave en una RA exitosa como se mencionó anteriormente. Con la intención de encontrar evidencias que nos ayudaran a distinguir si el bloqueo de la RA por los inhibidores de canales aniónicos se debía al efecto de dichos compuestos sobre los canales aniónicos o como consecuencia de un efecto inespecífico sobre la corriente T, decidimos probar dos diferentes inhibidores de canales aniónicos en la ICa_T de las células espermatogénicas. Los fármacos utilizados fueron: el ácido niflúmico (AN) y el ácido 5-nitro-2-(3-fenilpropilamina) benzóico (NPPB).

La aplicación de 100 μM de AN en la solución de registro inhibió el 90% de la corriente T, de manera parcialmente reversible tal como se muestra en la figura 21A. La figura 21B nos muestra la relación corriente-voltaje de la ICa_T en ausencia (control), presencia y lavado de 100 μM de AN. Por otra parte, los registros control demostraron que la corriente T a –20 mV es estable al menos durante los primeros 30 min, lo que descarta la posibilidad de un decaimiento propio de la corriente en nuestras condiciones experimentales (Fig. 21C). De igual forma, el NPPB bloqueó a la corriente T de manera parcialmente reversible (datos no mostrados).

Los paneles 22A y B nos muestran la dosis dependencia del bloqueo de la corriente T por AN y NPPB, respectivamente. De los datos anteriores podemos concluir que el AN inhibe a la ICa_T con una IC_{50} de 43 μM. La forma de la curva de la inhibición de la ICa_T por AN nos sugiere un solo sitio de unión para este compuesto (Fig. 22A). Para el caso del NPPB, la curva dosis-respuesta nos sugiere dos posibles sitios de unión, ya que la

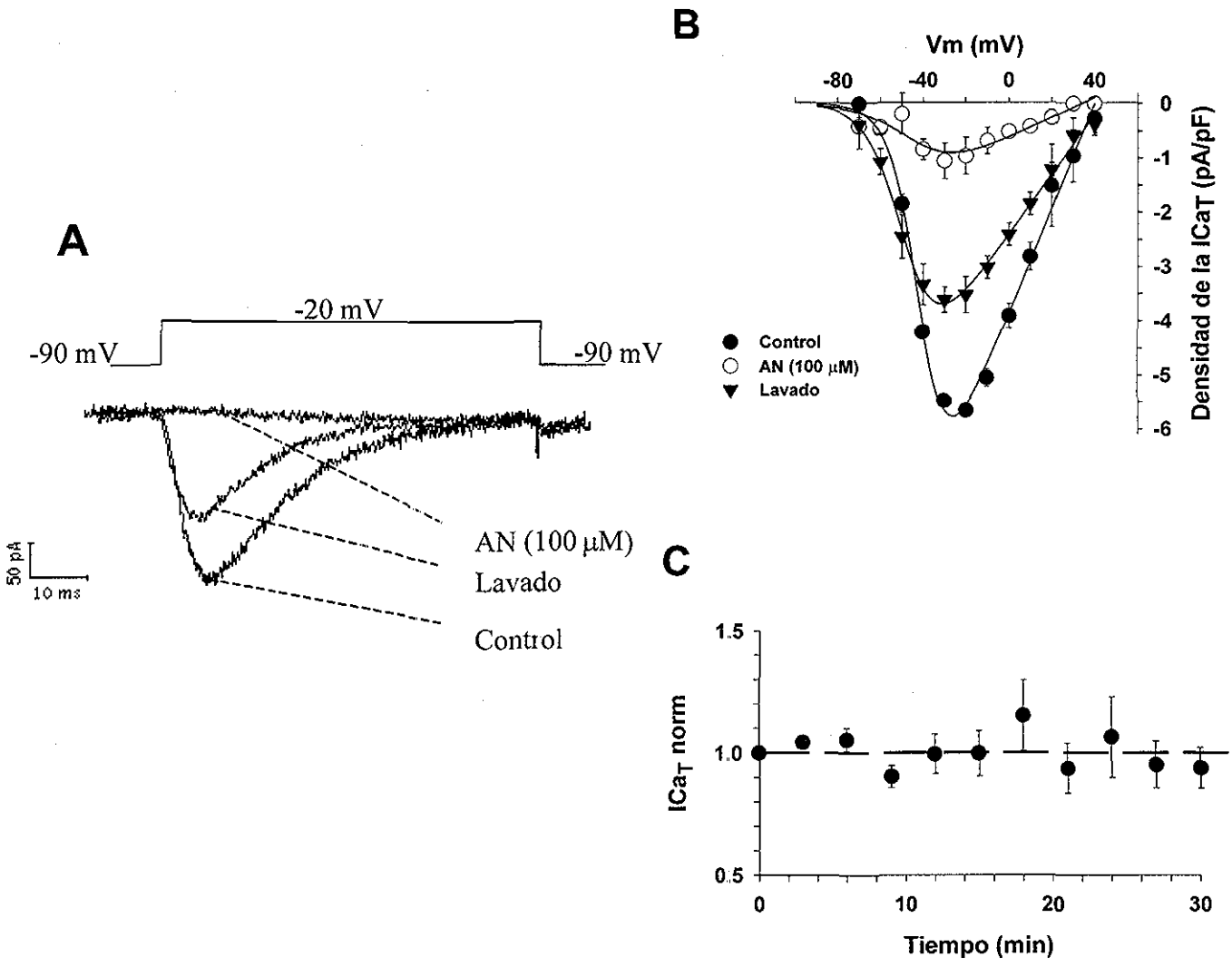

Figura 21.- El ácido niflúmico (AN) bloquea la corriente de Ca^{2+} tipo T en las células espermatogénicas. **A:** trazos representativos de la corriente obtenida al aplicar pulsos de prueba de –20 mV a partir de un potencial de mantenimiento de –90 mV en ausencia (control), presencia y lavado de 100 µM de AN. **B:** Curvas I-V correspondientes al control (círculos negros), a la aplicación de 100 µM de AN (círculos blancos) y a la recuperación de la corriente después del lavado del fármaco (triángulos negros) El promedio corresponde a cuatro experimentos independientes. **C:** estabilidad temporal del pico de la corriente T a –20 mV. Los símbolos representan el promedio ± E.S.M. La línea discontinua representa el ajuste promedio de los datos (n= 4)

inhibición de la corriente máxima alcanza un primer nivel de bloqueo del 45% de la corriente al aplicar concentraciones entre 0.5 y 10 µM. Concentraciones más altas de NPPB incrementaron el bloqueo de la ICa_T, alcanzando un bloqueo del 100% a una concentración de 100 µM (Fig.22B).

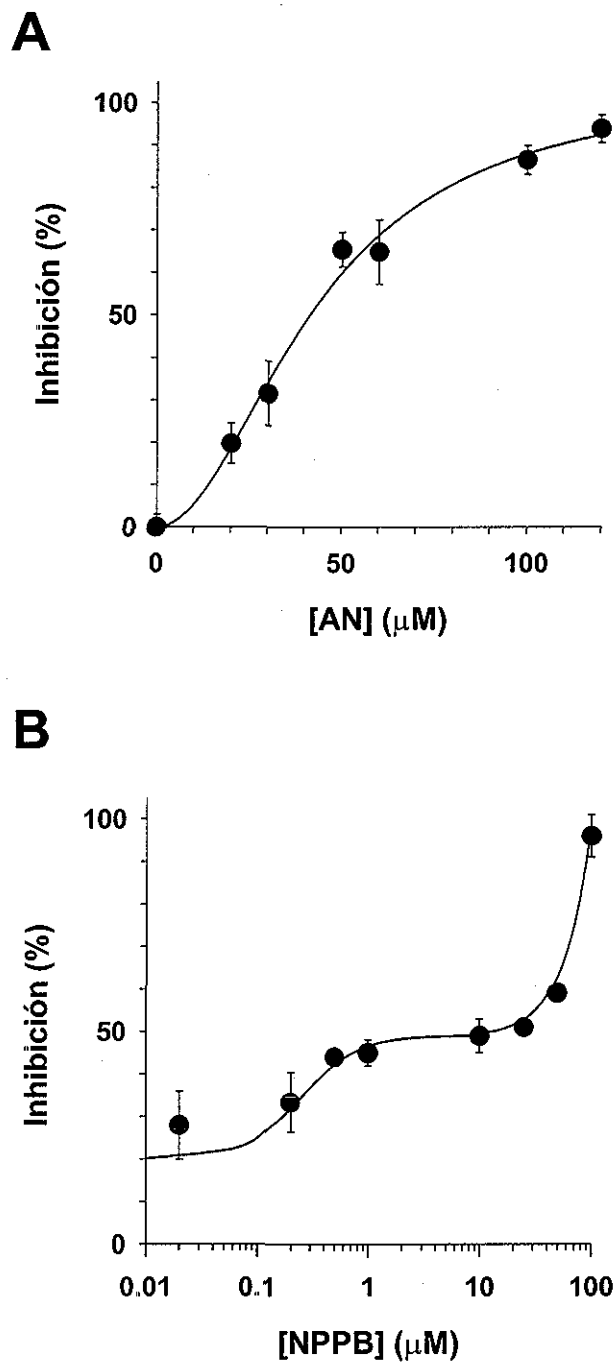

Figura 22.- Curvas dosis-respuesta del bloqueo de la corriente de Ca^{2+} tipo T por los inhibidores de canales aniónicos. Las corrientes al pico (a -20 mV) se normalizaron con respecto al control. El valor de la IC_{50} para el ácido niflúmico (AN) fue de 43 μM (**A**). Por otra parte, el ácido nitrofenilpropilamina benzóico (NPPB) parece tener dos sitios de unión al canal con afinidades aparentes de 25 nM y 75 μM (**B**). Los símbolos representan el promedio ± E.S.M. (n= 4).

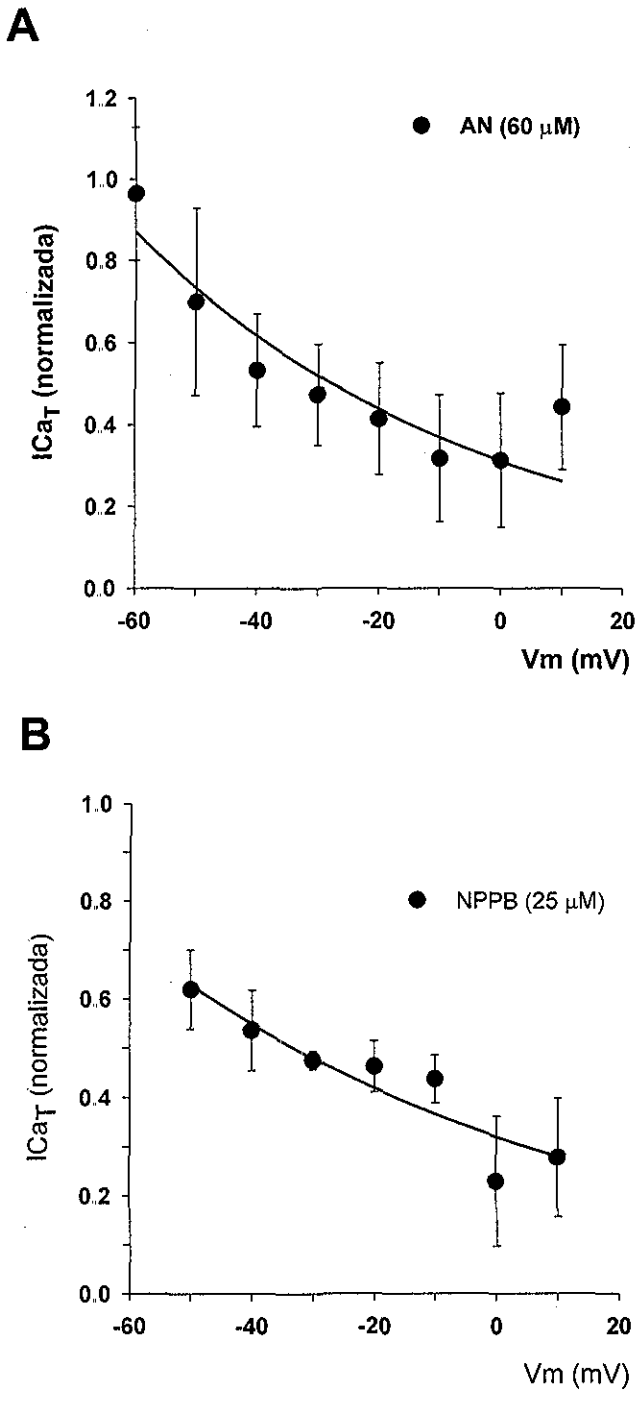

Figura 23.- El bloqueo de la ICa_T por ácido niflúmico (AN) y ácido nitrofenilpropilamino benzoico (NPPB) es dependiente de voltaje. Voltaje dependencia del bloqueo de la corriente por la aplicación de 60 µM de AN (**A**) o 25 µM de NPPB (**B**). Los resultados son el promedio de la corriente al pico normalizada y se graficaron en función del voltaje aplicado. Los símbolos representan el promedio ± E.S.M. (n= 4).

El bloqueo de la ICa_T por ambos inhibidores (AN y NPPB) es dependiente de voltaje. A voltajes negativos, el bloqueo es pequeño. Sin embargo, voltajes más depolarizantes incrementaron la inhibición de la corriente (Fig. 23A y B, respectivamente). Por ejemplo, a –70 mV, el AN bloqueó el 70% de la corriente T, mientras que a 0 mV, el bloqueo fue de 35% (Fig. 23A).

De acuerdo a nuestros datos (ver apéndice, Espinosa *et al.*, 1999), la RA inducida por la ZP es más sensible al AN que la corriente T por lo que podemos concluir que los canales T no son el principal blanco del AN en este proceso, como se discutirá más adelante. En el caso del NPPB, nuestros resultados indican que parte de la inhibición de la RA inducida por ZP podría deberse al efecto de este fármaco sobre la corriente T, como consecuencia del sitio de alta afinidad para NPPB sugerido en la figura 22B.

4.2.2.- Una nueva toxina de alacrán inhibe a la corriente de Ca^{2+} tipo T de las células espermatogénicas y modifica la función del espermatozoide durante la reacción acrosomal.

Clásicamente, el uso de toxinas peptídicas que interactúan con canales iónicos y que modifican la actividad de los mismos ha permitido profundizar en el conocimiento de los mecanismos y los motivos estructurales involucrados en el funcionamiento de los canales. Más aún, las herramientas farmacológicas también han permitido evaluar la relevancia fisiológica de los canales iónicos en distintos tejidos y procesos celulares. Recientemente, se aisló una toxina de 63 amino ácidos (kurtoxina) que bloquea específicamente a canales de Ca^{2+} tipo T expresados en sistemas heterólogos (Chuang *et al.*, 1998). Aunque la kurtoxina también afecta a canales de Na^+ dependientes de voltaje, esta toxina presentó una alta afinidad (Kd=15 nM) por canales de Ca^{2+} de bajo umbral ($Ca_V3.1$ y $Ca_V3.2$) sin

bloquear a canales de Ca^{2+} de alto umbral de activación incluido el canal tipo L (CaV1.3) (Chuang *et al*, 1998). En este trabajo, caracterizamos los efectos de dos toxinas extraídas del veneno de otro alacrán africano (*Parabuthus granulatus*), las cuales inhiben significativamente a la corriente de Ca^{2+} de las células espermatogénicas de ratón, y atenúan a la RA inducida por el agonista fisiológico en el espermatozoide maduro.

En colaboración con el grupo del Dr. Lourival Possani, purificamos recientemente dos nuevas toxinas aisladas por HPLC de las fracciones del veneno del alacrán *Parabuthus granulatus*. La comparación de la estructura primaria de estas nuevas toxinas con otras toxinas ya reportadas reveló que estos péptidos están relacionados a la kurtoxina de *P transvaalicus*, única toxina específica para canales de Ca^{2+} de bajo umbral de activación (Ca_V3). A estas nuevas toxinas se les denominó kurtoxin-like I y Kurtoxin-like II (KLI y KLII, respectivamente)

KLI y KLII inhiben la corriente T de las células espermatogénicas. Una vez más, consistente con investigaciones previas (Arnoult *et al*., 1996; 1999; Liévano *et al*., 1996; Santi *et al*, 1996), nuestros resultados demostraron que la corriente de Ca^{2+} presente en las células espermatogénicas es esencialmente de tipo T. Los trazos presentados en la figura 24A corresponden a la corriente macroscópica registrada en las CE con pulsos depolarizantes de 200 ms a –20 mV, a partir de un potencial de mantenimiento de –90 mV (HP). Al aplicar pulso depolarizantes a partir de este HP, pudimos observar que las corrientes tuvieron un umbral de activación aparente de –50 mV, alcanzaron un pico de corriente cercano a –20 mV y exhibieron una pronunciada dependencia de voltaje (ver Fig. 26). Tal como se puede apreciar en los trazos de la figura 24A, la aplicación de

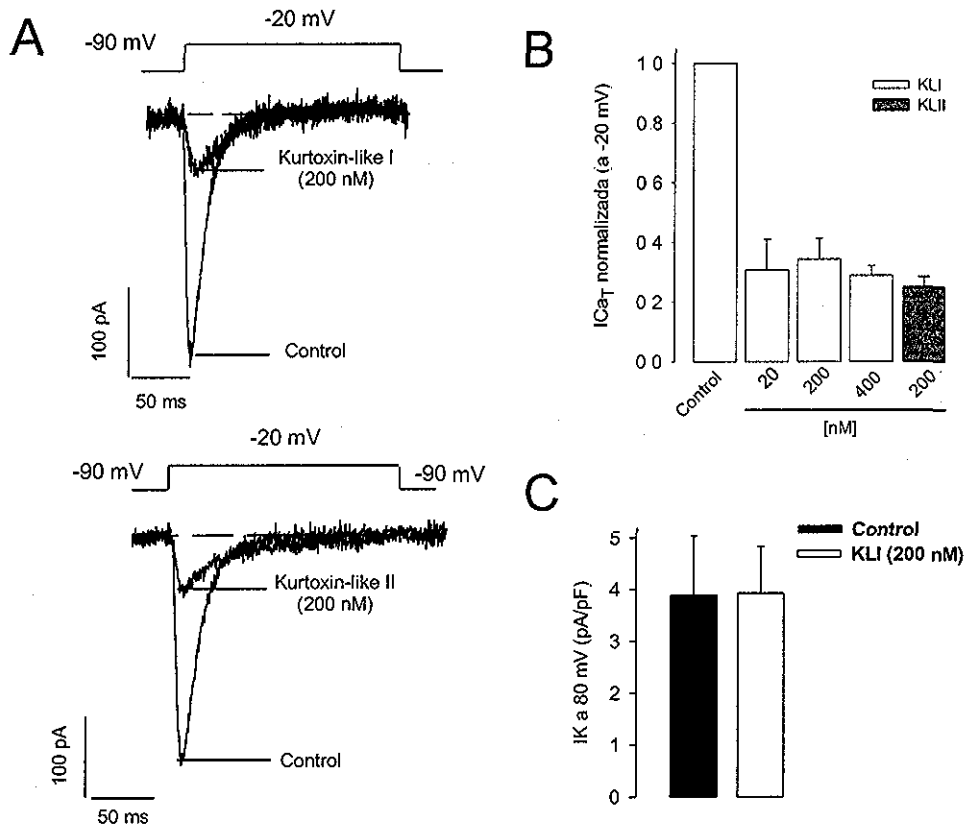

Figura 24.- Dos nuevas toxinas similares a la kurtoxina inhiben específicamente a la corriente T de las células espermatogénicas. A: *Trazos superimpuestos obtenidos al aplicar un pulso de* prueba de –20 mV a partir de un potencial de mantenimiento de –90 mV en ausencia (control) o presencia de 200 nM de KLI (panel superior) o KLII (panel inferior) **B:** Comparación de la inhibición de la corriente de Ca^{2+} T por KLI o KLII La corriente en presencia de las toxinas se normalizó con respecto al control (n= 4). **C:** KLI (200 nM) no inhibió a la corriente de K^+ presente en las células espermatogénicas. En todos los casos, las barras representan el promedio ± E.S.M.

200 nM de KLI o KLII abolió más del 60% de la ICa_T (Fig. 24B). La aplicación de una concentración más alta de KLI (400 nM) no incrementa significativamente la fracción de corriente bloqueada (Fig. 24B), por lo que consideramos que 200 nM de KLI es suficiente para alcanzar el bloqueo máximo de la ICa_T por estas toxinas. Más aún, en canales LVA recombinantes la aplicación de 350 nM de kurtoxina bloquea el 95% de la corriente T (Chuang *et al*., 1998). Considerando el antecedente previo, los resultados presentados en la

figura 24B sugieren la presencia de una fracción de la corriente de Ca^{2+} (~ 0.25) insensible a KLI. La inhibición de la ICa_T por estas toxinas fue específica ya que experimentos control demostraron que la incubación con KLI (Fig 24C) o KLII (dato no mostrado) no afectó ni la magnitud ni la cinética de la corriente macroscópica de K^+ registrada en las células espermatogénicas.

La figura 25B resume la comparación del curso temporal del bloqueo de la corriente T en presencia de KLI y KLII. El curso temporal del bloqueo fue similar para ambas toxinas, aunque ligeramente más rápido para la KLI. Estos datos nos dan un panorama real de la inhibición de la corriente de Ca^{2+}, ya que el efecto de las toxinas fue significativamente mayor que el decaimiento propio de la corriente (Fig. 25A), el cual se corrigió de los registros hechos en presencia de las toxinas de acuerdo al método previamente descrito por Randall y Tsien (1995). Esta corrección consiste en calcular la pendiente del lavado de la corriente en condiciones control y restar este valor de la pendiente de los registros en presencia de las toxinas (Randall y Tsien, 1995; fig. 25A). De manera similar a lo reportado para los canales T recombinantes expresados en ovocitos de *Xenopus* (Chuang *et al*, 1998), el bloqueo de la corriente T es pobremente reversible ya que a pesar de lavados prolongados (8 min) la recuperación de la corriente fue mínima (datos no mostrados).

Debido a que la KLI y la KLII están altamente relacionadas a la kurtoxina, la cual actúa como un modificador de la apertura ("gating") dependiente del voltaje, examinamos los efectos de estas toxinas a diferentes voltajes. La figura 26 muestra las curvas corriente-voltaje correspondientes (*I-V*) medidas al tiempo al pico de la corriente de cada registro obtenido antes y durante la aplicación de 200 nM de KLI (panel A) y KLII (panel B) en las células espermatogénicas. Inesperadamente, la inhibición de la corriente T inducida por las

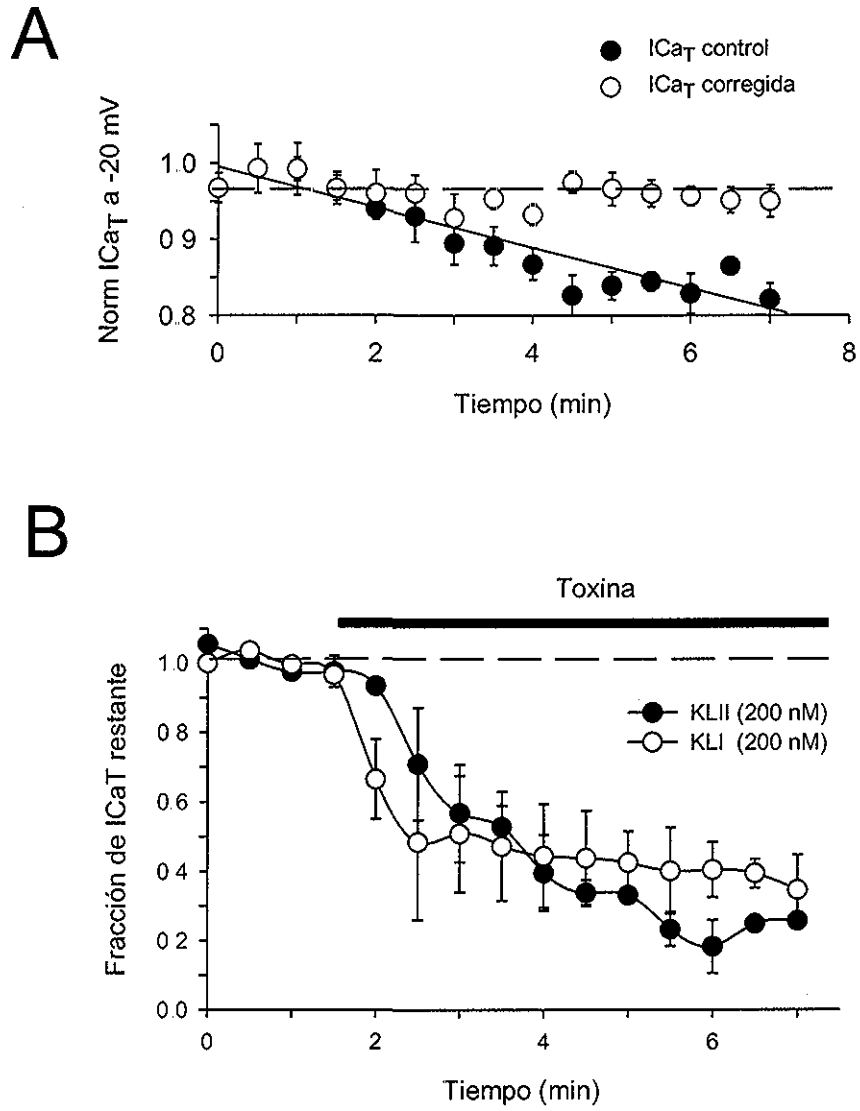

Figura 25.- Curso temporal del bloqueo de la corriente T por KLI y KLII. A: Corrección del decaimiento de la corriente de Ca^{2+} tipo T de acuerdo al método descrito por Randall y Tsien (1995). **B:** Curso temporal del bloqueo de la corriente T por 200 nM de KLI (círculos blancos) o KLII (círculos negros). La línea discontinua representa el promedio de la corriente control. Los datos obtenidos se corrigieron como en **A**. Los símbolos representan el promedio ± E.S.M. (n= 4)

toxinas no parece ser muy dependiente de voltaje en el intervalo de ±40 mV. Sin embargo, la forma de la curva *I-V* se modificó ligeramente en presencia de las toxinas. La aplicación de las toxinas, evidenció la aparición de un pequeño hombro en la fase descendente de la curva I-V (alrededor de 0-10 mV) el cual no estaba presente en el control. Vale la pena mencionar que esta concentración de KLI o KLII (200 nM) es suficiente para alcanzar la

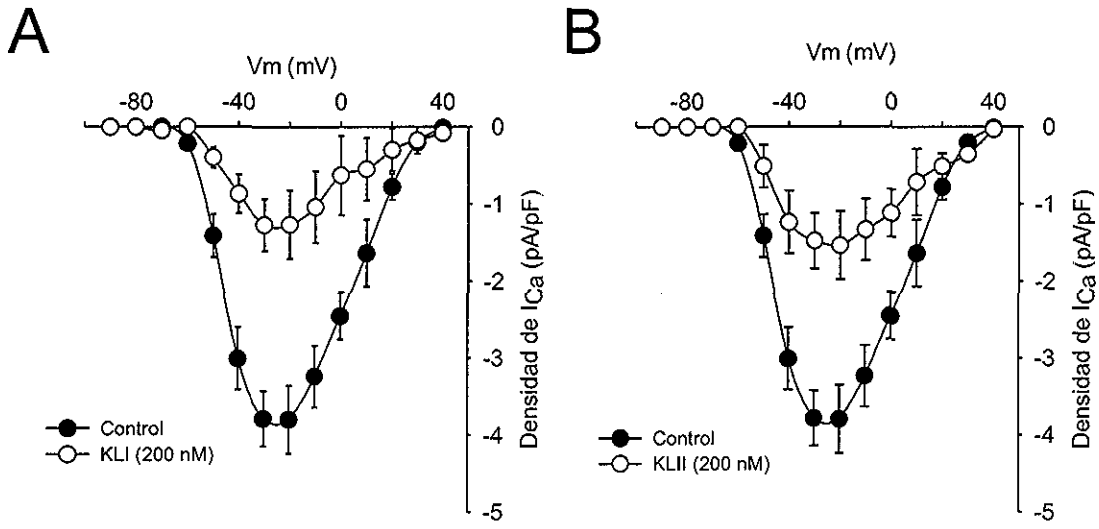

Figura 26.- La KLI y KLII no modifican significativamente a la curva corriente-voltaje de la corriente T pero evidencian un componente minoritario de la corriente de Ca^{2+}. A: curva I-V en ausencia (círculos negros) o presencia de KLI (círculos blancos). **B:** curvas I-V correspondientes a los experimentos en ausencia (círculos negros) o presencia de KLII (círculos blancos). Los símbolos representan el promedio ± E.S.M. (n= 4-5).

máxima inhibición posible de la fracción de corriente sensible a las toxinas, la cual podría corresponder a la corriente T. Este pequeño componente de la corriente macroscópica podría explicarse, al menos, por dos hipótesis: la primera sería que las toxinas, además de inhibir a la corriente T, modificaran la curva I-V de una fracción de canales de bajo umbral resistentes a 200 nM de las toxinas. Esta hipótesis sería consistente con un posible efecto diferencial de las toxinas sobre alguna de las dos subunidades α1 que codifican para canales de bajo umbral de activación ($Ca_V3.1$ o α1G, $Ca_V3.2$ o α1H) presentes en las células espermatógenicas de ratón (ver apéndice, Espinosa *et al.*, 1999). La segunda explicación sería que este otro componente de la corriente de Ca^{2+} podría deberse a la actividad de un canal de Ca^{2+} no LVA, y sería consistente con reportes previos que sugieren la presencia de distintos tipos de canales de Ca^{2+} en el espermatozoide de ratón (Serrano *et*

al., 1999; Westenbroek y Babcock, 1999; Wennemuth *et al.*, 2000; O'Toole *et al.*, 2000; Jungnickel *et al.*, 2001) De cualquier manera, ambas hipótesis apuntan a que la corriente macroscópica de Ca^{2+} de las células germinales pudiera estar compuesta por diferentes tipos de canales de Ca^{2+}.

El bloqueo de la corriente T por ambas toxinas (KLI y KLII) es poco dependiente del voltaje en un intervalo de –50 a 10 mV, tal como se muestra en la figura 27A. La incubación con KLII no tuvo efecto alguno sobre la inactivación en estado estacionario de la corriente T (Fig. 27B), donde la $V_{1/2}$ de la curva de inactivación en estado estacionario en condiciones control y en presencia de 200 nM de KLII fue la misma (~ -58 mV, Fig. 27C).

Estudios recientes indican que el canal de Ca^{2+} tipo T permanece de manera funcional en el espermatozoide maduro después de la diferenciación testicular, y que juegan un papel central en la RA (Arnoult *et al.*, 1999). Debido a que tanto la KLI como la KLII son potentes inhibidores de la actividad del canal de Ca^{2+} en las células espermatogénicas, estas toxinas podrían afectar a la RA del espermatozoide. Nosotros exploramos esta posibilidad al estudiar el efecto de estos péptidos en el porcentaje de RA inducida con ZP utilizando el método de azul de Coomasie (ver la sección de Materiales y Métodos para mayor información). A una concentración de 200 nM ambas toxinas, KLI y KLII, inhibieron significativamente a la RA (~39.3 y ~46.1%, respectivamente) en el espermatozoide capacitado de ratón (Fig. 28A y B). De manera interesante, la aplicación de esta concentración bloqueó entre el 65 y 75% del total de la corriente macroscópica a través del canal de Ca^{2+} tipo T de las células espermatogénicas (ver Fig. 24B).

Como se mencionó con anterioridad, la kurtoxina afecta selectivamente a los miembros de la subfamilia de canales de Ca^{2+} Ca_V3 sin alterar a las corrientes producidas

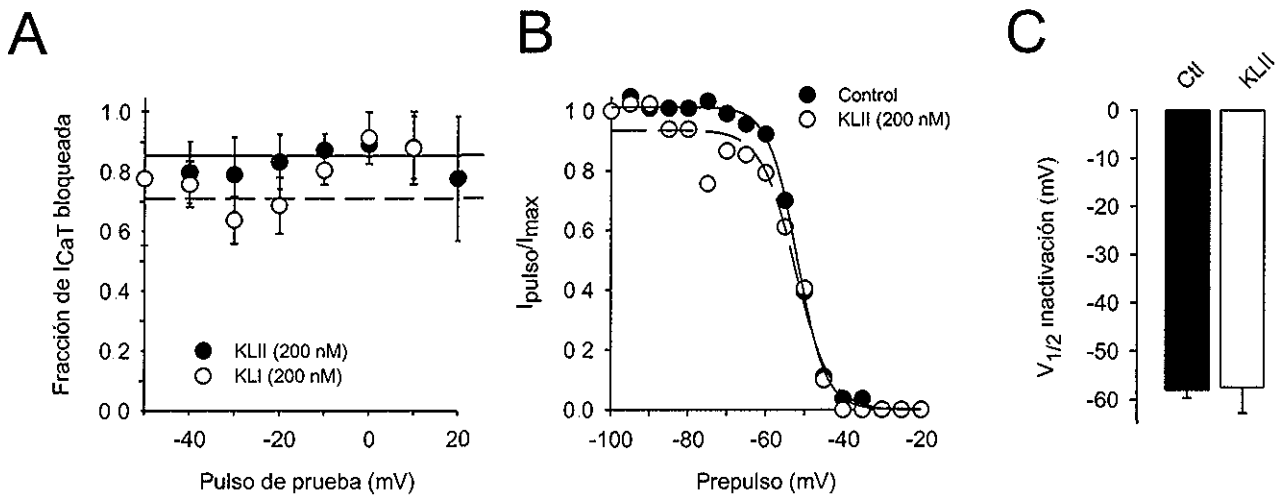

Figura 27.- El bloqueo de la corriente T por KLI y KLII es poco dependiente de voltaje y no afecta la inactivación en estado estacionario. A: el panel muestra la poca dependencia de voltaje del bloqueo de la corriente T por KLI (círculos blancos) o KLII (círculos negros). **B:** Curva de inactivación en estado estacionario representativa de la corriente T en ausencia (círculos negros) o presencia de KLII (círculos blancos). **C:** comparación de la $V_{1/2}$ de la curva de inactivación de la ICa_T en condiciones control (barra negra) y durante la aplicación de KLII (barra blanca). En todos los casos, se graficó el promedio ± E.S.M. (n= 5).

por los canales de Ca^{2+} $Ca_V1.2$ y Ca_V 2.1-2.3 (tipo L, N, Q/P, y R , respectivamente), por lo que se puede utilizar como una herramienta específica para evaluar la presencia de canales de Ca^{2+} tipo T en diferentes tejidos y tipos celulares (Chuang *et al.*, 1998). Debido a que KLI y KLII bloquearon una proporción significativa de la corriente de Ca^{2+}, es factible sugerir que la corriente de Ca^{2+} tipo T constituye el componente mayoritario de la corriente macroscópica de Ca^{2+} registrada en las células espermatogénicas. Por otra parte, al aplicar una concentración de 200 nM de las toxinas (KLI o KLII), observamos que para ambos casos un porcentaje similar de la corriente de Ca^{2+} (34±7 y 25±3%, respectivamente) fue resistente a la adición de las toxinas (Fig. 24B). Los datos anteriores sugieren la presencia de un componente adicional de la corriente de Ca^{2+} en las CE. Esta idea se apoya en las diferencias encontradas al comparar la forma de las curvas I-V (ver Fig. 26A y B) y en la

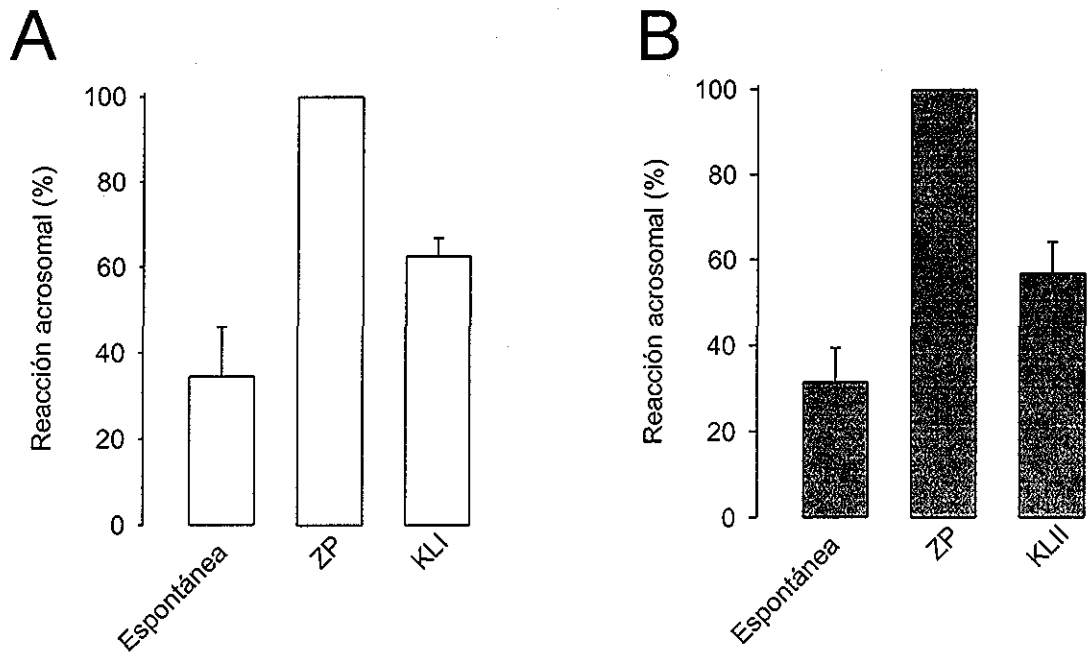

Figura 28.- KLI y KLII inhiben la RA inducida por ZP. A: Efecto de la KLI (200 nM) en el porcentaje de espermatozoides que sufren la RA. Después de la capacitación, el porcentaje de RA se monitoreó en condiciones control (espontánea), y posterior a la inducción con ZP en ausencia y presencia de la toxina. Los datos representan el promedio ± E.S.M., de al menos tres experimentos independientes por duplicado. **B:** Efecto de KLII (200 nM) sobre la RA inducida por ZP medida como en **A** (*n*=5).

cinética de la corriente de Ca^{2+} antes y después de la aplicación de las toxinas (Fig. 29A). Para la comparación cinética de la corriente, utilizamos pulsos de voltaje a –20 mV a partir de un potencial de mantenimiento de –90 mV. Los trazos de la corriente registrados en presencia de la toxina se normalizaron con respecto a los trazos de corriente control obtenidos de las mismas células. Con estas condiciones experimentales, la corriente de Ca^{2+} tuvo una forma similar en ausencia y presencia de las toxinas, caracterizada por un pico inicial seguido de un decaimiento exponencial. Sin embargo, la fase de decaimiento de la corriente del componente insensible a las toxinas fue más lento que la corriente control (Fig. 29A). Este hallazgo podría explicarse, al menos, por dos hipótesis: el retardo de la

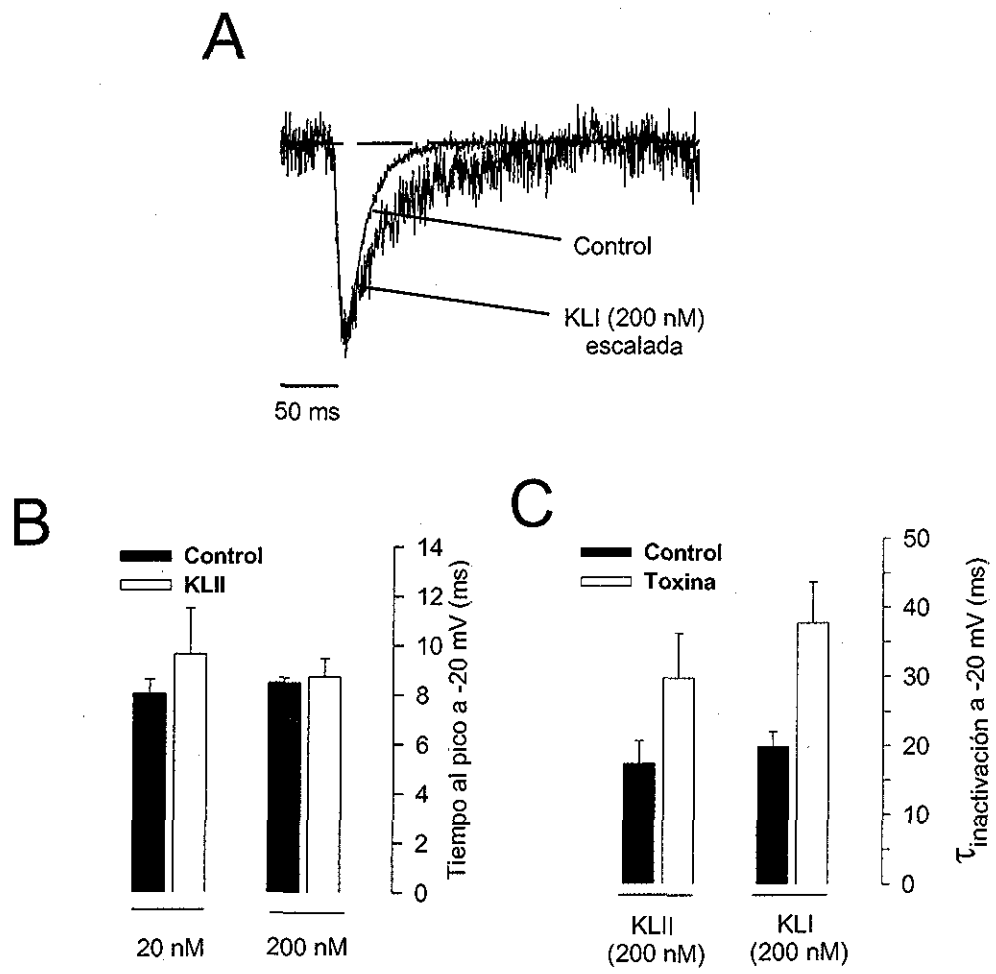

Figura 29.- El componente resistente a KLI y KLII tiene una cinética de inactivación más lenta. A: trazos de corriente representativos de la corriente de Ca^{2+} en presencia y ausencia de KLI (200 nM) a –20 mV a partir de un potencial de mantenimiento de –90 mV. La corriente en presencia de la toxina se normalizó para facilitar la comparación de la cinética. **B:** el componente resistente a la KLII tiene la misma cinética de activación. Comparación del tiempo al pico de la corriente de Ca^{2+} en ausencia y presencia de KLII. **C:** la constante de tiempo de inactivación de la corriente resistente a las toxinas (KLI y KLII) es mayor. Comparación de la τ de inactivación en ausencia y presencia de las toxinas. Las barras representan el promedio ± E.S.M. (n=5).

cinética de inactivación podría deberse al efecto de las toxinas sobre la corriente, o bien podría explicarse al asumir que la depolarización abre al menos dos tipos de canales de Ca^{2+} con una activación similar pero con diferentes propiedades de inactivación (Fig. 29A). El análisis de la duración del tiempo al pico nos confirmó que la activación de la corriente de Ca^{2+} en las células espermatogénicas requiere entre 9-10 ms a -20 mV en ausencia y en

presencia de 200 nM de KLII (Fig. 29B), lo que sugiere una activación similar. Sin embargo, con respecto a la inactivación, nuestros resultados discutidos previamente sugieren que las toxinas están actuando como bloqueadores de la corriente no como modificadores de la apertura ("gating"). Es un hecho bien documentado que cuando un compuesto actúa como bloqueador induce un aceleramiento aparente de la inactivación de la corriente, no un enlentecimiento (Gomora *et al*, 2000). Por lo tanto, nuestros resultados apoyan la hipótesis de la participación de otro tipo de canal de Ca^{2+} en la corriente macroscópica de las células espermatogénicas. Aunque ambas corrientes se inactivan exponencialmente, la figura 29C sugiere que la corriente macroscópica de las células espermatogénicas es generada por canales de Ca^{2+} con diferente inactivación. Así, la corriente total tuvo una constante de tiempo (τ_{inact}) de 19.9±2.2 ms mientras que la τ_{inact} fue de 37.8±5.9 ms para la corriente resistente a 200 nM de KLI. Estos datos, junto con los estudios de la inactivación en estado estacionario (Fig. 27B y C) indican que la nueva corriente insensible a las toxinas podría estar compartiendo algunas propiedades biofísicas con la corriente total lo cual pudo haber evitado su identificación previa.

En un esfuerzo por identificar a que tipo de canal pertenece este componente resistente a las toxinas, utilizamos Ni^{2+} para bloquear selectivamente a la ICa_T presente en las CE. Se sabe que el Ni^{2+} bloquea preferencialmente a las corrientes de Ca^{2+} tipo T de diferentes tipos de neuronas y a canales LVA recombinantes (Lee *et al.*, 1999). La figura 30 muestra la curva dosis-respuesta obtenida de la aplicación de Ni^{2+} a las CE. Los datos obtenidos se ajustaron de acuerdo a la ecuación de Hill de la cual se estimaron una IC_{50} aproximada de 30 μM, un coeficiente de Hill de 1.4±0.11 y una corriente resistente a Ni^{2+} que correponde al 25±5 % de la corriente total. Esta corriente resistente a 1 mM de Ni^{2+} podría corresponder al

componente resistente a las toxinas (Fig. 31A; panel superior). Notablemente, la comparación de las cinéticas demostró que la fracción de la corriente de Ca^{2+} resistente a 1 mM de Ni^{2+} tiene una fase de inactivación lenta análoga al componente de la corriente resistente a las toxinas descrito anteriormente (Fig. 31A; panel inferior), lo que sugiere que la corriente de Ca^{2+} resistente en ambas condiciones experimentales podría originarse por el mismo tipo de canal. Por otra parte, los valores obtenidos para la IC_{50} y el coeficiente de Hill, sugieren que el componente bloqueado por Ni^{2+} podría corresponder a canales de Ca^{2+} LVA (Lee et al., 1999).

La figura 31B muestra que este nuevo componente de la corriente de Ca^{2+} total es resistente a la aplicación combinada de Ni^{2+}/Cd^{2+} (26.5±2.5% de la corriente) y Ni^{2+}/KLII

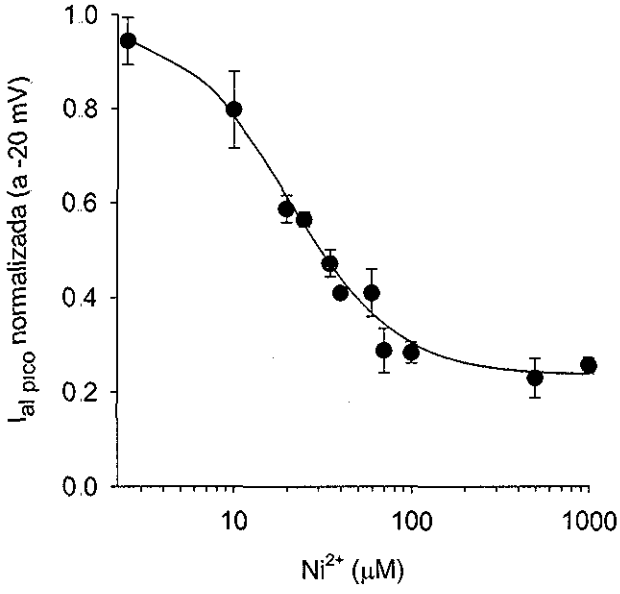

Figura 30.- Curva dosis-respuesta a Ni^{2+} en las células espermatogénicas. Los registros se obtuvieron a partir de un potencial de mantenimiento de –90 mV. Los trazos de corriente en presencia y ausencia de 1 mM de Ni^{2+} se muestran en la fig. 31A. La línea continua representa el ajuste de los datos experimentales a la ecuación de Hill. Los símbolos representa el promedio ± E.S.M. (n=5).

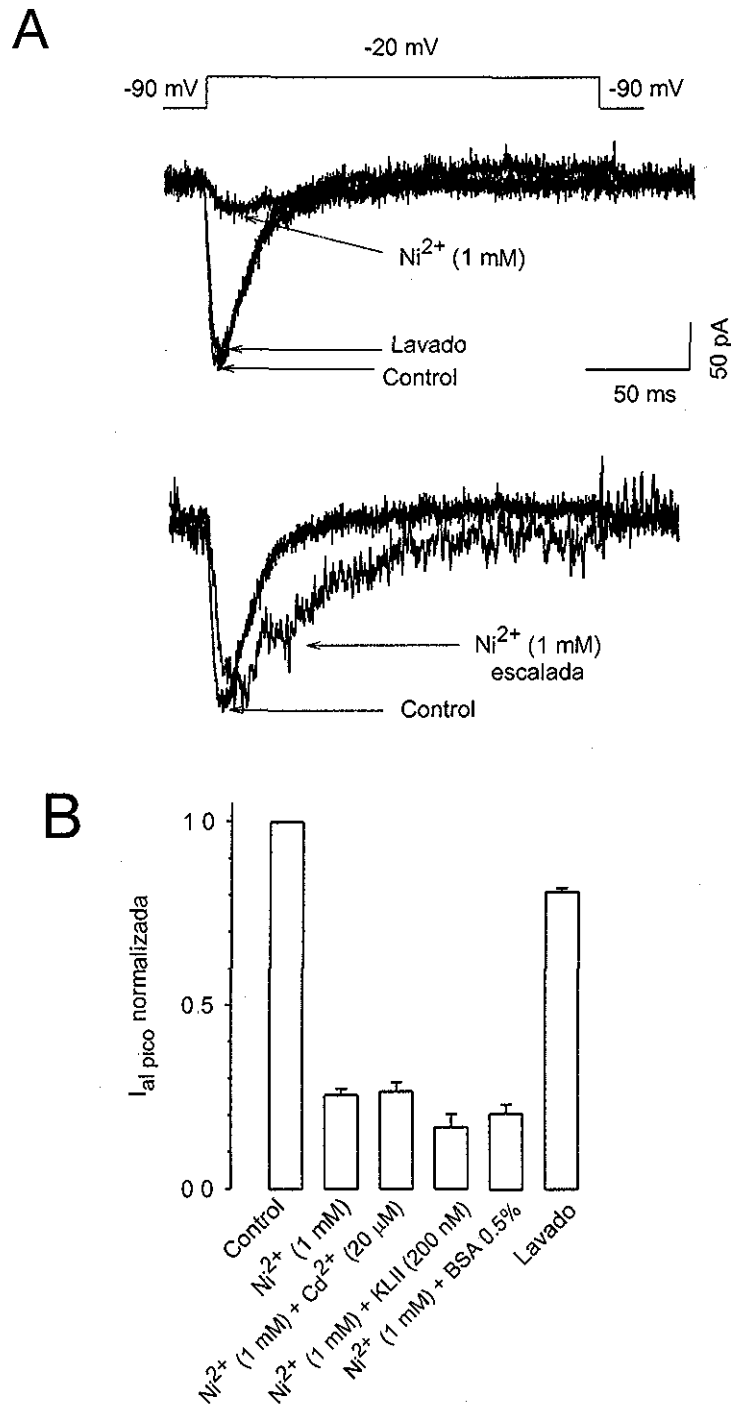

Figura 31.- El nuevo componente de la corriente total de Ca^{2+} no corresponde a canales de Ca^{2+} LVA, HVA o TRP. A: trazos de corriente representativos obtenidos a –20 mV a partir de un potencial de mantenimiento de -90 mV en ausencia, presencia y lavado de 1 mM de Ni^{2+} (panel superior). Los trazos de corriente en ausencia y presencia de Ni^{2+} se normalizaron para hacer la comparación de la cinética de la corriente resistente a 1 mM de Ni^{2+} y el control (panel inferior). **B:** la corriente residual es resistente a Ni^{2+} (1 mM), Ni^{2+}/Cd^{2+} (1 mM/20 µM, respectivamente), Ni^{2+}/KLII (1 mM/200 nM, respectivamente), y no se estimula por la aplicación de BSA (0.5%) en presencia de 1 mM de Ni^{2+}. Las barras representan el promedio ± E.S.M. (n=3-5).

(16.7±3.5% de la corriente). De la misma forma, este componente de la corriente de Ca^{2+} es insensible a una combinación de Ni^{2+} complementada con albúmina (20.6±2.3% de la corriente, Fig. 30B), un compuesto que incrementa a la actividad del canal de Ca^{2+} tipo T en las células espermatogénicas (Espinosa *et al*, 2000). En conjunto, estos resultados descartan la participación de TRP sensibles a 1 mM de Ni^{2+} (O'Toole *et al*., 2000), de canales de Ca^{2+} HVA sensibles a 20 μM de Cd^{2+} (Hille, 1992) o de canales de Ca^{2+} LVA sensibles a KLI (200 nM) o BSA (0.5%) (este trabajo). Así, nuestros resultados sugieren que este componente de la corriente de Ca^{2+} corresponde a un tipo de canal distinto a los reportados previamente en las células espermatogénicas.

V.- DISCUSIÓN.

5.1.- El posible papel fisiológico de la regulación dual de la corriente de Ca^{2+} tipo T por albúmina y β-estradiol durante la capacitación.

El espermatozoide maduro posee una sola vesícula secretoria (el acrosoma) por lo que la exocitosis debe coordinarse en respuesta al contacto con el óvulo homólogo para favorecer una fecundación eficiente. Previo a la fecundación, el espermatozoide debe adquirir su capacidad de fecundar al óvulo a través de un proceso de maduración (la capacitación). En este proceso, la albúmina juega un papel determinante al inducir un incremento en el influjo de Ca^{2+} y bicarbonato, los cuales son indispensables para la capacitación (Shi y Roldán, 1995; Visconti *et al.*, 1995; Visconti y Kopf, 1998). Sin embargo, hasta el presente trabajo no se había probado directamente la posibilidad de que la albúmina (BSA) pudiera modular a la corriente de Ca^{2+} tipo T presente en el espermatozoide y/o en las células espermatogénicas. Resultados previos en nuestro laboratorio demostraron que la BSA (0.5%) induce un incremento en la amplitud de la corriente de Ca^{2+} tipo T de manera dependiente de la concentración (ver apéndice; Espinosa *et al.*, 2000). Estudiando con más detalle este fenómeno, los resultados del presente trabajo muestran que el efecto de la BSA es dependiente de voltaje (Fig. 7A), y que además acelera la cinética de activación de la corriente T (Fig. 7B) ocasionando una disminución en el valor de la constante de tiempo de activación. De la misma forma, la aplicación de BSA favorece que la corriente T se active a potenciales más negativos y que se inactive a potenciales más positivos (Fig. 7C). El corrimiento en ambas curvas en estado estacionario (activación e inactivación), podría ocasionar un incremento en la corriente ventana en presencia de la BSA. Así, en el pico de la ventana (~ -52 mV) el 28 y el 44% de los canales estan disponibles para abrirse en ausencia (control) y en presencia de BSA, respectivamente

(Fig 7C). Es importante destacar que el incremento en la corriente ventana podría tener relevancia en la fisiología del espermatozoide, en particular durante el inicio de la capacitación. Existe evidencia que apunta a que el canal T está presente en el espermatozoide maduro (Arnoult *et al*., 1999) donde el potencial de reposo es cercano a -50 mV (Espinosa y Darszon, 1995). Se sabe que la membrana del espermatozoide alcanza el punto máximo de hiperpolarización (~-70 mV) a los 45 minutos después de iniciada la capacitación inducida por ciclodextrinas (Dr. Pablo Visconti, comunicación personal). Considerando lo anterior, es posible que los canales de Ca^{2+} T participen en el influjo de Ca^{2+} inducido por la BSA durante los primeros 30 minutos, el cual es un prerrequisito para la capacitación. Los resultados sugieren que los canales de Ca^{2+} tipo T pudieran contribuir al incremento de la $[Ca^{2+}]_i$ durante la capacitación, debido al incremento del número de los canales disponibles para abrirse al potencial de reposo en el espermatozoide no capacitado (~ -50 mV), lo que ocasionaría el incremento del influjo de Ca^{2+}.

Es posible que el efecto de la BSA sobre la corriente T resulte de la acción de remover alguna molécula hidrofóbica presente en la membrana plasmática del espermatozoide, la cual pudiera modular la ICa_T y así evitar una exocitosis prematura. Más aún, el efecto de la BSA en la ICa_T de las CE es irrevesible y depende de Ca^{2+} externo (Espinosa, 1999a), lo cual es consistente con la propuesta arriba mencionada. Así, en el momento en que la capacitación se lleva a cabo y el espermatozoide esta listo para viajar al sitio donde ocurre la fecundación, la albúmina removería esta molécula hidrofóbica lo que permitiría que los canales T quedaran libres de esta inhibición y se incrementara la $[Ca^{2+}]_i$ en el espermatozoide. Inesperadamente, los datos indican que la BSA no está modulando a la ICa_T a través de su capacidad de quitar colesterol de la membrana celular, como lo muestran los experimentos con BSA preincubada con una concentración saturante de un

análogo del colesterol (sulfato de colesterol; CSO_4) y con 2-OH-β-ciclodextrina (2-OH-β-CD) (Fig. 8B).

Otra posible explicación para el incremento de la ICa_T inducido por la BSA se basa en la capacidad antioxidante de la BSA (Guermonprez *et al.*, 2001). Se sabe que las cadenas laterales de la cisteína, tirosina y/o histidina de la albúmina pueden actuar como reductores potenciales (Ivanov *et al.*, 2000). Por otra parte, recientemente se describió la modulación de canales de Ca^{2+} LVA por agentes oxidantes (DTNB, oxido nitroso) o reductores (DTT, L-cisteína) en nociceptores de rata (Todorovic *et al*, 2001a) y en canales recombinantes (Todorovic *et al*, 2001b). De hecho, los agentes reductores incrementan la amplitud de la ICa_T en los nociceptores (Todorovic *et al.*, 2001a). Considerando los trabajos arriba mencionados, nuestros resultados no nos permiten descartar totalmente la posibilidad de un efecto directo de la BSA en la corriente T de las CE. Sin embargo, el hecho de que el efecto de la BSA en la ICa_T sea irreversible sugiere que la modulación de la corriente es indirecta.

Con la idea de explorar que molécula hidrofóbica pudiera estar inhibiendo a la corriente T, estudiamos el efecto del 17-β-estradiol en las CE basados en los reportes previos sobre la modulación de canales T por estradiol (Mermelstein *et al*, 1996; Ogata *et al*, 1996; Zhang *et al.*, 1994; Nakajima *et al.*, 1995; Yamamoto *et al.*, 1995; Kitazawa *et al*, 1997; Ruehlmann *et al*, 1998), y considerando que el 17-β-estradiol está presente en la membrana del espermatozoide (Martínez y Morros, 1985). La BSA presaturada con 17-β-estradiol no indujo el incremento de la corriente al pico sino que produjo una discreta disminución de la amplitud de corriente la cual se anuló al agregar BSA por segunda vez (Fig. 9D). Este hecho es consistente con la hipótesis que sugiere que el incremento de la

ICa_T inducido por la BSA pudiera deberse a la disminución de la concentración de 17-β-estradiol de la membrana celular más que de colesterol. Sin embargo, es importante resaltar que el efecto de la BSA sobre la corriente T es dependiente de voltaje (Fig. 7A) mientras que el efecto del estradiol no lo es (Fig. 9B). Esta diferencia en la dependencia de voltaje indica que el efecto de cada uno de estos compuestos es independiente uno del otro, por lo que aún persiste la interrogante acerca del compuesto hidrofóbico que estaría inhibiendo tónicamente a los canales T antes de la capacitación y que sería el blanco de la albúmina. Un posible candidato como inhibidor de los canales T de las células espermatogénicas es el ácido araquidónico (AA). Estudios recientes demostraron que el AA modula a la subunidad α1H (Ca_V3 2) (Zhang *et al.*, 2000), la cual codifica para un canal de Ca^{2+} tipo T (Cribbs *et al.*, 1998). En este estudio se demostró que el AA directamente induce una lenta atenuación de la corriente de α1H por la disminución de la corriente ventana (Zhang *et al.*, 2000). Tal como se mencionó en la introducción, parte de nuestro trabajo demostró que α1H es una de las dos subunidades que codifican para canales de Ca^{2+} de bajo umbral de activación presentes en las CE (ver apéndice Espinosa *et al*, 1998). Más aún, resulta interesante saber que varios trabajos han aportado evidencias que sugieren la participación del AA en la RA (Domínguez *et al*., 1999; Sistina y Rodger, 1997).

Por otra parte, el mecanismo mediante el cual el 17-β-estradiol reduce a la corriente de Ca^{2+} requiere de un estudio más detallado. Como se mencionó, estudios previos han demostrado que la aplicación aguda de β-estradiol inhibe a los canales de Ca^{2+} tipo T en las células del músculo liso (Mermelstein *et al.*, 1996), en neuronas (Ogata *et al*, 1996), y en miocitos cardiacos o lisos (Zhang *et al*, 1994; Nakajima *et al*, 1995; Yamamoto *et al.*, 1995; Kitazawa *et al.*, 1997; Ruehlmann *et al.*, 1998). Sin embargo, a diferencia de lo

reportado para el caso de la inhibición de canales de Ca^{2+} de alto umbral (HVA) (Ogata *et al*, 1996; Mermelstein *et al*., 1996), esta inhibición no involucraría la participación de proteínas G, ya que la corriente de Ca^{2+} tipo T de las CE es básicamente independiente de la modulación por dichas proteínas (Arnoult *et al*., 1997) Nuestros datos indican que el efecto del 17-β-estradiol es específico ya que el uso de un isómero hormonalmente inactivo, el 17-α-estradiol, causó una menor reducción en la respuesta del canal T (Fig 9C) Resulta interesante que las observaciones de diferentes estudios sugieren que la presencia de diferentes compuestos hidrofóbicos pueden afectar las propiedades físicas de la membrana a través de la alteración de las interacciones entre las proteínas de membrana y la bicapa lipídica (Andersen *et al*., 1999). Los resultados presentados en esta sección muestran un efecto diferencial entre el 17-β-estradiol y el 17-α-estradiol en la corriente T, lo cual es inconsistente con un efecto basado en la hidrofobicidad de ambas moléculas. Considerando que el 17-β-estradiol está presente en la membrana del espermatozoide (Martínez y Morros, 1985), queda abierta la pregunta de una posible interacción directa entre esta hormona y alguna subunidad accesoria de los canales de Ca^{2+} T presentes en la membrana de las CE que pudiera modular a la ICa_T, tal como ocurre con canales de K^+ (Valverde *et al*., 1999) Por último, recientemente se demostró que la aplicación de 17-β-estradiol puede inducir un incremento en la $[Ca^{2+}]_i$ por la activación de un receptor funcional de estrógenos que se expresa en la superficie de la membrana celular del espermatozoide humano (Luconi *et al*, 1999) Los resultados mostrados en esta parte de esta tesis sugieren que en este incremento de la $[Ca^{2+}]_i$ no participan los canales de Ca^{2+} tipo T. Por otra parte, estudios recientes demostraron que el 17-β-estradiol induce un incremento en la concentración intracelular de oxido nítrico a través de un receptor de

estrógenos tipo β (Zhu *et al*., 2002). Resulta interesante que el oxido nitroso aplicado al medio externo bloquea la corriente de Ca^{2+} tipo T de la subunidad $Ca_v3.2$ (α1H) expresada heterólogamente (Todorovic *et al*., 2001). Este podría ser el mecanismo mediante el cual el 17-β-estradiol inhibe la corriente T de las CE.

5.2.- La corriente de Ca^{2+} tipo T de las células espermatogénicas podría estar modulada por calmodulina.

Previo a la fecundación, el espermatozoide de los mamíferos debe llevar a cabo la reacción acrosomal (RA). Aún en la actualidad, se desconocen varios de los pasos involucrados en la transducción de señales que conducen a la RA del espermatozoide maduro. Este evento de exocitosis inducido por la zona pelúcida (ZP) que rodea al óvulo homólogo, depende de la entrada de Ca^{2+} externo (Wassarman, 1999). La evidencia experimental sugiere que los canales de Ca^{2+} dependientes de voltaje son una vía importante en la regulación de la entrada de Ca^{2+} al espermatozoide durante este proceso (Darszon *et al*., 1999; Arnoult *et al*., 1996a; Arnoult *et al*., 1999). El trabajo de diferentes grupos ha demostrado que las CE expresan funcionalmente canales de Ca^{2+} tipo T (Hagiwara y Kawa, 1984; Liévano *et al*., 1996; Arnoult *et al*., 1996a; 1996b; Santi *et al*., 1996) con características farmacológicas consistentes con las reportadas para la RA y el influjo de Ca^{2+} asociado a la misma (Arnoult *et al*., 1998). De la misma forma, se ha demostrado que el canal T está presente en el espermatozoide maduro (Arnoult *et al*., 1999) y por lo tanto, este canal debe ser un elemento importante en la cascada de señales que participan en la RA inducida por la ZP en el espermatozoide de mamífero (Arnoult *et al*., 1996a; Arnoult *et al*., 1999; Darszon *et al*., 1999).

A pesar de que la corriente de Ca^{2+} tipo T de las células espermatogénicas se ha caracterizado detalladamente en trabajos previos, se sabe poco sobre su regulación. Dicha información es necesaria para profundizar en el estudio de los mecanismos moleculares que conducen a la RA del espermatozoide. Trabajos recientes indican que la regulación de canales de Ca^{2+} de alto umbral de activación (HVA) por calmodulina (CaM) podría contribuir en la determinación de los niveles de Ca^{2+} intracelular (Peterson *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). Curiosamente, observaciones previas obtenidas al usar inhibidores de CaM en el espermatozoide de erizo de mar (Sano, 1983; Guerrero y Darszon, 1989) y en mamífero (Courtot *et al.*, 1999) sugieren la participación de CaM en la RA. Con esto en mente y considerando las ventajas del modelo biológico con el que contamos, se decidió explorar la posible regulación por CaM de los canales de Ca^{2+} tipo T presentes en las células espermatogénicas. Los resultados presentados en este trabajo muestran que el W7, un antagonista específico para CaM, reduce marcadamente la magnitud de la corriente T a concentraciones mayores a 2 μM (Figs. 10 a 12). Este efecto depende de la concentración y fue esencialmente independiente de voltaje (Fig. 10C y D). La TFP, otro antagonista de CaM, también redujo significativamente la amplitud de la corriente (Fig. 11A) a concentraciones que inhiben a la CaM (Massom *et al.*, 1990). Más aún, la adición de CaM en la solución interna de registro redujo el efecto inhibitorio del W7, sugiriendo una potencial participación de esta proteína en la regulación del canal de Ca^{2+} tipo T (Fig. 11B). Nuestros resultados son consistentes con trabajos recientes que demuestran que CaM modula a canales de Ca^{2+} de bajo umbral de activación en células nativas (Barrett *et al.*, 2000) o expresados ($Ca_V3.2$ o α1H) en sistemas heterólogos (Wolfe *et al.*, 2002). Sin embargo, en los dos casos arriba citados la regulación de los canales de

Ca^{2+} de bajo umbral de activación por CaM es a través de la protein cinasa II dependiente de CaM.

Los resultados presentados en este estudio sugieren la participación de la CaM en la regulación del canal de Ca^{2+} tipo T de las células espermatogénicas, pero no son una demostración definitiva. El W7, el W5 y la TFP pueden tener otros efectos menos específicos tales como una inhibición directa de canales iónicos membranales o intracelulares, o de la fosfolipasa C (Ehrlich *et al.*, 1988; Schlatterer y Schaloske, 1996; Scholf *et al.*, 1999). Sin embargo, las concentraciones inhibitorias usadas en este trabajo concuerdan con las descritas para la inhibición de la CaM y son menores a las asociadas a efectos menos específicos de estos compuestos (Ichikawa *et al.*, 1991). Además, la dependencia parcial de Ca^{2+} del efecto inhibitorio del W7 apoya la idea de la regulación de la corriente T por el complejo Ca^{2+}/CaM (Fig. 11C). Más aún, los efectos directos de los inhibidores de la CaM sobre distintos tipos de canales iónicos son parcial o completamente reversibles (Laver *et al.*, 1997), mientras que los efectos de estos compuestos en nuestros experimentos fueron irreversibles (datos no mostrados), lo que nos sugiere un efecto indirecto sobre los canales. Finalmente, cuando el W7 y la TFP actúan bloqueando directamente a un canal iónico, la adición de CaM a la solución interna es incapaz de inhibir dicho bloqueo (Kleene, 1994), a diferencia de lo observado en nuestros experimentos. En conjunto, estas consideraciones nos permiten proponer que el W7 y la TFP afectan a la corriente de Ca^{2+} por inhibir la función de la CaM.

Por otra parte, se ha sugerido que la inactivación de los canales de Ca^{2+} HVA implica la participación del Ca^{2+} que entra a través del poro del canal, la unión del ion a la CaM y el cambio de conformación de la misma. El complejo Ca^{2+}/CaM interactúa con el canal iniciando un proceso intramolecular que culmina en una inactivación más rápida del

canal de Ca^{2+} (Zühlke y Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). De acuerdo con lo anterior, la aplicación aguda del antagonista de CaM, el W7, resultó en un enlentecimiento parcial de la inactivation de la ICa_T en las células espermatogénicas (Fig. 13A, B, C y D). Sin embargo, este efecto de los antagonistas de CaM sobre la inactivación contrastan con la disminución observada de la amplitud de la corriente T en las células espermatogénicas. En el presente trabajo, se muestra que, además de la reducción de la amplitud de la corriente, la aplicación de distintas concentraciones de W7 cambia drásticamente otras propiedades biofísicas de la corriente. Estos cambios incluyen un corrimiento de la voltaje dependencia de la activación a valores positivos y un enlentecimiento significativo de la cinética de activación (Figs. 12 y 14). Considerando que la activación y la inactivación están estrechamente acopladas en los canales T (Serrano *et al.*, 1999), los resultados sugieren la reducción en la constante de tiempo de la activación en presencia del W7 podría indirectamente enlentecer el curso temporal aparente de la inactivación de la corriente T. Además de los efectos antes descritos, el W7 parece promover la inactivación a voltajes en los cuales los canales T no están activos (Fig. 14A y B). Sin embargo, no está claro si la CaM participa en este efecto.

En resumen, el presente estudio muestra que los antagonistas de CaM, como el W7 y la TFP, inhiben con una potencia similar a la corriente T de las CE monitoreada por métodos electrofisiológicos, y a la RA inducida por la ZP. Notablemente, una concentración similar de W7 (10 μM) disminuyó el influjo transitorio de Ca^{2+} disparado por la ZP en el espermatozoide maduro registrado con un fluoróforo sensible a Ca^{2+} (Fig. 15A). En contraste, el W5, un análogo del W7, antagonista de la CaM pero mucho menos potente, tuvo un efecto mucho menor sobre la corriente T de las CE (Fig. 11A), sobre la RA

y en el influjo transitorio de Ca^{2+} asociado a esta (Fig. 15A y B). Más aún, la sustitución del Ca^{2+} externo por Ba^{2+} (Fig. 11C) o la adición de CaM a la solución interna de registro redujo la inhibición de la corriente T de las CE causada por el W7 (Fig. 11B). En conjunto, estos resultados son consistentes con un vínculo directo entre la activación del canal de Ca^{2+} T y la RA inducida por la ZP, y sugiere que la CaM podría regular al canal T del espermatozoide maduro. En el espermatozoide maduro esto es particularmente importante porque durante la RA ocurre una delicada redistribución y modificaciones de las moléculas presentes en la membrana plasmática (Wassarman, 1999; Flesch y Gadella, 2000; Darszon *et al.*, 2001). Por ejemplo, recientemente se demostró que después de la RA se redistribuyen las microdominios de membrana (*rafts*) que contienen GM1, un gangliosido presente en un tipo de microdominios membranales (*rafts*) (Treviño *et al*, 2001). Este fenómeno de reorganización membranal también es importante durante la capacitación, donde el uso de inhibidores de la CaM, como el W7 y el calmidazolio, inhiben significativamente el porcentaje de espermatozoides capacitados y su capacidad de llevar a cabo la RA inducida por lisofosfatidilcolina (Si y Olds-Clarke, 2000).

5.3.- La corriente de Ca^{2+} tipo T no participa en el influjo de Ca^{2+} inducido por progesterona o ácido γ-aminobutírico (GABA) en mamífero.

En todas las especies animales estudiadas hasta la fecha, la inducción de la RA por los agonistas fisiológicos implica un influjo de Ca^{2+} extracelular. En el erizo de mar, los polímeros de fucosa sulfatada provocan un influjo bifásico de Ca^{2+}, en el cual participan dos tipos de canales iónicos. La primera fase es sensible a dihidropiridinas (DHPs) y verapamil, antagonistas de canales de Ca^{2+} tipo L (Ca_V1) dependientes de voltaje. La segunda fase, la cual se activa 5 segundos después de la primera, es insensible a bloqueadores de canales de Ca^{2+} dependientes de voltaje pero es sensible a Ni^{2+} (IC_{50} = 10

μM) (Guerrero y Darszon, 1989; Schackman, 1989; González-Martínez *et al.*, 2001). En mamíferos, la inducción de la RA por la ZP también activa dos vías de entrada de Ca^{2+}. La primera entrada corresponde a la activación del canal de Ca^{2+} tipo T sensible a DHPs, y cuyo bloqueo inhibe el incremento de la concentración intracelular de Ca^{2+} ($[Ca^{2+}]_i$) y a la RA (Arnoult *et al*, 1996; Darszon *et al*, 2001). Posterior a la activación del canal T, ocurre un incremento en la $[Ca^{2+}]_i$ propiciado por la participación de un canal de Ca^{2+} de tipo capacitativo, que probablemente este formado por la combinación de TRP2 y otros TRPs (Santi *et al.*, 1999; O'Toole *et al.*, 2000; Jungnickel *et al.*, 2001).

Varios reportes han demostrado que la progesterona induce un influjo transitorio de Ca^{2+} y a la RA en el espermatozoide humano (Aitken *et al.*, 1996; Baldi *et al.*, 1991; Blackmore *et al.*, 1990; Meizel *et al.*, 1997; Plant *et al.*, 1995), probablemente a través de la activación de un receptor membranal de progesterona (Blackmore *et al.*, 1991; Meizel y Turner, 1991; Sabeur *et al.*, 1996; Tesarik *et al*, 1992; Benoff *et al*, 1995; Luconi *et al*, 1998). Debido a que la progesterona induce ambos procesos a concentraciones en el orden micromolar, similar a la concentración de esta hormona en el *cumulus oophorus* (Osman *et al*, 1989), se ha propuesto que la progesterona pudiera tener un papel importante durante la fecundación *in vivo* (Fisher *et al*, 1998; Garcia y Meizel, 1999)

Como se mencionó anteriormente, se ha demostrado que el influjo de Ca^{2+} a través de canales dependientes de voltaje es necesario para la RA inducida por la ZP (Florman *et al*, 1992; Florman, 1994; Arnoult *et al.*, 1996a; Arnoult *et al*, 1996b). Específicamente, las evidencias electrofisiológicas sugieren que, al menos, el canal de Ca^{2+} tipo T está involucrado en la RA (Santi *et al.*, 1996; Arnoult *et al*, 1996a; Arnoult *et al.*, 1999). Con

base en los datos anteriores, resultaba interesante explorar si la corriente de Ca^{2+} tipo T estaba involucrada en la RA inducida con progesterona o GABA.

Los resultados sugieren que la aplicación de 50 μM de progesterona o 125 μM de ácido γ-aminobutírico (GABA) no modifican las características biofísicas fundamentales de la corriente de Ca^{2+} tipo T tales como la amplitud de la corriente, la cinética de activación o de inactivación (Figs. 18 y 19). De estos resultados podemos concluir que la corriente de Ca^{2+} tipo T no participa en el influjo de Ca^{2+} ni en la RA inducida por la progesterona o el GABA. Esta es, a nuestro conocimiento, la primera evidencia directa de que la progesterona o el GABA no modulan a la corriente T de las células espermatogénicas ya que las condiciones experimentales utilizadas en trabajos previos dificultan una evaluación apropiada (García y Meizel, 1999; Kirkman-Brown *et al.*, 2000). Sin embargo, considerando que las CE se encuentran en un proceso de diferenciación (Bellvé, 1993), existe la posibilidad de que la cascada de segundos mensajeros involucrados en la modulación de la corriente de Ca^{2+} T por progesterona o GABA aún no estuviera funcional a diferencia de la célula madura. Por otra parte, estudios recientes sugieren que el canal de Ca^{2+} tipo T tiene una mínima participación en el influjo de Ca^{2+} y en la RA inducida por progesterona en el espermatozoide maduro, ya que se necesitan concentraciones altas de mibefradil para inhibir ambos procesos (García y Meizel, 1999; Bonaccorsi *et al.*, 2001). Más aún, consistente con los resultados de este trabajo se ha descrito que los canales de Ca^{2+} tipo T están involucrados en el incremento de Ca^{2+} inducido por la neoglicoproteína α-D-manosa-sero albúmina bovina (manosa-BSA) pero no participan en el incremento de Ca^{2+} inducido por la progesterona (Blackmore y Eisoldt, 1999). Lo anterior se explica al

considerar que la BSA modula a los canales de Ca^{2+} T, tal como lo mostramos en la primera parte de esta tesis (discutido en la sección 6.1).

Por último, se sabe que la progesterona promueve la RA en distintas especies. Considerando que la progesterona puede activar a la fosfolipasa C presente en el espermatozoide, es posible que dicha activación produzca el vaciamiento de las pozas internas de Ca^{2+} y como consecuencia active al segundo canal de Ca^{2+} involucrado en la RA, el cual depende del vaciamiento de las pozas internas (O'Toole *et al.*, 2000). Esto podría explicar porque la progesterona y la ZP actúan de manera cooperativa en la RA, aún cuando activan a distintos elementos de regulación involucrados en la exocitosis (revisado en Darszon *et al*, 2001).

5.4.- La inhibición de la RA inducida con la ZP por bloqueadores aniónicos es independiente de la corriente de Ca^{2+} tipo T.

Estudios previos han demostraron la localización de un receptor ionotrópico para GABA en el espermatozoide de mamífero (Erdo y Werkele, 1990; Rabow *et al.*, 1995). Como se mencionó anteriormente, el GABA o la glicina pueden inducir la RA en el espermatozoide humano, de ratón y de cerdo, y dicha reacción se puede inhibir con antagonistas de canales de Cl^- tales como el ácido niflúmico (Meizel, 1997). Con la finalidad de evaluar la posible participación de canales aniónicos en la RA inducida por la ZP, nuestro grupo realizó registros electrofisiológicos en el espermatozoide directamente y en las células espermatogénicas de ratón como una primera aproximación a esta interesante pregunta. Nuestros resultados demostraron la presencia de canales aniónicos funcionales sensibles a ácido niflúmico (AN) tanto en el espermatozoide maduro (ver apéndice; Espinosa *et al.*, 1998) como en sus células precursoras (Fig. 20). Sin embargo, debido a que

el AN y otros bloqueadores de canales aniónicos pueden inhibir a distintos canales iónicos de manera inespecífica, resultó necesario descartar que la inhibición de la RA inducida por la ZP fuera consecuencia del bloqueo inespecífico de la ICa_T.

Los resultados de esta serie de experimentos mostraron que la aplicación de concentraciones micromolar de ácido niflúmico (AN) o de ácido 5-nitro-2-(3-fenilpropilamina) benzóico (NPPB) inhiben a la corriente T de las células espermatogénicas (Fig. 22) de manera dependiente de voltaje (Fig. 23). Se sabe que el AN inhibe a la RA inducida por la ZP y a los canales aniónicos expresados en el espermatozoide de ratón (ver apéndice, Espinosa *et al*., 1998). De hecho, se considera que el AN y otros compuestos, tales como el NPPB y el DDF, bloquean canales de Cl^- activados por Ca^{2+} (Baron *et al*., 1991; Cotton *et al*., 1997; Lamb *et al*., 1994), al canal de Cl^- CFTR (Walsh y Wang, 1993; Doughty *et al*., 1998), a canales de Cl^- regulados osmóticamente (Diener *et al*., 1996), y a canales de Cl^- rectificadores entrantes de los espermatozoides de *Caenorhabditis elegans* (Machaca *et al*., 1996). Sin embargo, estos compuestos también afectan a algunos canales catiónicos tales como el receptor de NMDA (Lerma y Martín del Río, 1991), a canales catiónicos pobremente selectivos (Gögelein *et al*., 1990), y a CCDV (Walsh y Wang, 1993; Doughty *et al*., 1998). Como sabemos, se ha documentado ampliamente que la corriente de Ca^{2+} presente en las CE de ratón pertenece al tipo T (Liévano *et al*., 1996; Santi *et al*., 1996; Arnoult *et al*., 1996). Más aún, estos canales permanecen presentes de manera funcional en el espermatozoide maduro (Arnoult *et al*., 1999; López-González *et al*., 2001), donde son de vital importancia durante la RA (Darszon *et al*., 2001). Debido a que el AN inhibe a la RA en el espermatozoide maduro, resultaba relevante el determinar si la corriente T era sensible a estos bloqueadores aniónicos. Los resultados obtenidos muestran que el AN bloqueó a la corriente de Ca^{2+} con una IC_{50} de 43 μM (Fig. 22A). Por otra parte,

el NPPB parece tener dos sitios de unión distintos con afinidades aproximadas de 25 nM y 75 µM (Fig. 22B). En contraste, la RA inducida por la ZP es más sensible a estos bloqueadores con una IC_{50} de 1 y 6 µM para el AN (ver apéndice, Espinosa *et al*, 1998) y el NPPB (ver apéndice, Espinosa *et al*., 1999), respectivamente. Considerando estos datos, para el caso del AN, la inhibición de los canales de Ca^{2+} tipo T no es determinante en el bloqueo de la RA inducida por la ZP y se puede atribuir a una efecto del AN sobre canales aniónicos involucrados en este proceso. El razonamiento en el que se basa la conclusión anterior tiene como sustento trabajos farmacológicos en los cuales se ha descrito la correlación existente entre la inhibición de la corriente T por bloqueadores de canales de Ca^{2+} dependientes de voltaje en las células espermatogénicas, con una IC_{50} similar a la del bloqueo de la RA del espermatozoide maduro y del influjo de Ca^{2+} asociado a este fenómeno (Arnoult *et al*., 1996). Desde este punto de vista, solamente el NPPB podría estar contribuyendo en la inhibición de la RA inducida por la ZP a través del sitio de alta afinidad (Fig. 22B). Más aún, el bloqueo por ambos compuestos es dependiente de voltaje (Fig. 23). Considerando que el potencial de reposo del espermatozoide capacitado es aproximadamente –70 mV (Florman *et al*., 1998; Arnoult *et al*, 1999), el bloqueo de la corriente T por los antagonistas de canales aniónicos, el cual es más potente a potenciales depolarizados, sería mínimo. De igual manera, los datos sugieren que la corriente de Ca^{2+} no es modulada por GABA (discutido en la sección 6.2), por lo que durante la inhibición de la RA inducida por GABA, el AN estaría actuando sobre canales aniónicos principalmente.

Con respecto al mecanismo de bloqueo de la corriente T por los antagonistas de canales aniónicos, se puede proponer lo siguiente: varios reportes sugieren la interacción directa de estos bloqueadores con canales aniónicos y catiónicos (Gögelein *et al*., 1990; Oba *et al*., 1997; Ottolia y Toro, 1994; Tilman *et al*., 1993; White y Aylwin, 1991). Así, el

sitio de unión al canal T podría ser el poro y posiblemente la unión ocurra en el lado externo de la membrana como se ha sugerido para otros canales (Evoniuk y Skolnick, 1988; Tilman *et al.*, 1993), ya que el efecto de bloqueo en la corriente T es reversible (Fig. 21A y B). Para poder sentir el voltaje, el sitio de unión del bloqueador debería localizarse hacia el interior del poro, inmerso en la membrana (Hille, 1992). Sin embargo, con el advenimiento de los estudios estructurales recientes de los canales iónicos (MacKinnon *et al.*, 1998; Doyle *et al.*, 1998; Sato *et al.*, 2001; Zhou *et al.*, 2001), en particular de K^+ y Na^+, esta idea ha cambiado sustancialmente. Así, se puede especular que estos bloqueadores podrían interactuar directamente con partes estructurales externas involucradas en la conducción de los iones, las cuales de manera alostérica, afectarían el flujo de iones a través del poro. Otra posible explicación podría ser que en lugar de unirse directamente al poro del canal, estos bloqueadores pudieran interactuar con el sitio de unión de alguna subunidad accesoria y de esta manera influir en la corriente T. Una explicación alternativa se basa en la permeabilidad de los antagonistas de canales aniónicos. En este caso, los bloqueadores podrían entrar a la célula y afectar alguna ruta de regulación de los canales T. Por ejemplo, los bloqueadores usados en el presente trabajo también inhiben a las cicloxigenasas y a las lipoxigenasas que participan en el metabolismo del ácido araquidónico (AA) (Civelli *et al.*, 1991) y a la 3α–hidroxisteroide dehidrogenasa involucrada en el metabolismo de esteroides sexuales (Penning *et al.*, 1985). Los metabolitos de las rutas metabólicas de la lipoxigenasa y la cicloxigenasa modulan positivamente canales catiónicos y aniónicos (Diener *et al.*, 1996; Kanli y Norderhus, 1998). Más aún, estudios recientes muestran que los canales de Ca^{2+} $Ca_V3.2$ (α1H) se modulan por ácido araquidónico (Zhang *et al.*, 2000), de manera que la inhibición de las cicloxigenasas y/o lipoxigenasas induciría un incremento en la

concentración del AA, el cual reduciría la amplitud de la corriente T. De la misma forma que en el caso del AA, los bloqueadores de canales aniónicos podrían modificar el metabolismo de las hormonas esteroides (Penning *et al.*, 1985). Sabemos que el estradiol puede modular negativamente la corriente de Ca^{2+} T presente en las células espermatogénicas de ratón (resultados discutidos en la sección 6.1 de esta tesis) y a canales de Ca^{2+} dependientes de voltaje en músculo liso (Nakajima *et al.*, 1995). Más aún, hormonas como la testosterona y las progestinas pueden modular positivamente a canales de Ca^{2+} dependientes de voltaje (Takeuchi y Guggino, 1996; Bukusoglu and Sarlak, 1996). Así, además de la acción genómica inducida por las hormonas esteroideas, estos compuestos podrían modular canales iónicos que pudieran ser críticos en la diferenciación de las CE. Sería interesante, por lo tanto, investigar más detalladamente sí la inhibición (directa o indirecta) de enzimas como la 3α–hidroxisteroide dehidrogenasa afecta la modulación de los canales de Ca^{2+} T de las células espermatogénicas.

5.5.- KLI, una nueva toxina de alacrán bloquea a la corriente de Ca^{2+} tipo T y revela otro componente de la corriente macroscópica de Ca^{2+} de las células espermatogénicas.

A pesar de que la corriente de Ca^{2+} tipo T de las CE se ha caracterizado en detalle, se desconoce que subunidad α1 codifica para esta corriente. Más aún, hay controversia en cuanto a la naturaleza molecular de la corriente T debido a la expresión de numerosas subunidades que codifican para diferentes canales de Ca^{2+} dependientes de voltaje. La aplicación de estrategias de biología molecular ha permitido detectar la presencia de RNA mensajeros que codifican para las subunidades α1 de $Ca_V1.2$ (Goodwin *et al*., 1997), $Ca_V2.1$ y $Ca_V2.3$ (Liévano *et al*., 1996), así como también para $Ca_V3.1$ y $Ca_V3.2$ (Espinosa *et al.*, 1999). De las subunidades mencionadas, los primeros tres genes codifican para las

subunidades de los canales de Ca^{2+} tipo L, P/Q y R, respectivamente, mientras que los dos últimos codifican para canales de Ca^{2+} tipo T. Asimismo, el uso de anticuerpos ha demostrado la expresión de $Ca_V1.2$, $Ca_V2.1$ y $Ca_V2.3$ (Goodwin *et al.*, 1998; Serrano *et al.*, 1999; Westenbroek y Babcock, 1999; Wennemuth *et al*, 2000), así como de las subunidades auxiliares $Ca_V\beta_1$-$Ca_V\beta_3$ (Serrano *et al*, 1999) El trabajo electrofisiológico de diferentes grupos ha demostrado que las células espermatogénicas expresan principalmente solo una corriente de Ca^{2+} tipo T (Hagiwara y Kawa, 1984; Liévano *et al.*, 1996; Arnoult *et al*, 1996a; 1996b; Santi *et al.*, 1996; Wennemuth *et al.*, 2000), lo cual resulta inconsistente con la expresión de las diferentes subunidades $\alpha1$ mencionadas anteriormente. Sin embargo, algunos resultados obtenidos con el uso de estrategias alternativas como la fluorometría sugieren la expresión funcional de canales de Ca^{2+} tipo N y R en las células espermatogénicas de ratón, a pesar de las evidencias obtenidas con electrofisiología (Wennemuth *et al.*, 2000).

Aunque es difícil correlacionar los resultados electrofisiológicos obtenidos en el ratón con las evidencias moleculares obtenidas en diferentes especies (incluido el humano), resulta determinante identificar a la entidad molecular que codifica para la corriente T, así como entender su regulación, para aclarar los mecanismos involucrados en la RA. El progreso hecho en la comprensión de la composición estructural de los canales de Ca^{2+} dependientes de voltaje ha demostrado que la expresión heteróloga de las subunidades $Ca_V3.1$ y $Ca_V3.2$ origina canales de Ca^{2+} con propiedades biofísicas y farmacológicas propias de los canales de Ca^{2+} tipo T nativos (Perez-Reyes *et al.*, 1999). Recientemente, Chuang *et al.* (1999) aprovecharon la expresión heteróloga de estas subunidades para buscar toxinas con alta afinidad y especificidad para canales de Ca^{2+} tipo T. En ese trabajo,

los autores reportaron una toxina de alacrán (la kurtoxina) que se une a $Ca_V3.1$ y $Ca_V3.2$ con alta afinidad e inhibe su actividad como canal debido a que modifica los mecanismos de apertura y cierre de los canales ("gating"). En este trabajo describimos lo que es a nuestro conocimiento la primera caracterización biofísica reportada del bloqueo de la corriente de Ca^{2+} nativa de las CE por dos nuevas toxinas de alacrán (KLI y KLII). De manera similar a la kurtoxina, la aplicación extracelular de KLI o KLII reduce significativamente la corriente de Ca^{2+} tipo T a todos los voltajes examinados. Sin embargo, a pesar de la alta homología estructural (arriba del 80%) de KLI y KLII con la kurtoxina, se encontraron diferencias en el modo de acción de estas toxinas en la corriente T nativa. La inhibición de la corriente macroscópica de Ca^{2+} en las CE causada por KLI o la KLII en el intervalo de -40 a +20 mV fue poco dependiente de voltaje (Fig. 27A, panel derecho), además de parcialmente reversible, lo que sugiere que estas toxinas pudieran actuar como bloqueadores del poro más que como modificadoras del "gating". Sin embargo, hay que hacer notar que en la inhibición de la corriente a voltajes más positivos a -20 mV, la fracción de la corriente bloqueada por 200 nM de KLI o de KLII parece ser mayor, sin llegar a ser estadísticamente significativo. Para un análisis detallado de los efectos de la KLI y la KLII arriba mencionados, sería necesario un estudio a nivel de canal unitario. Más aún, la creación de mutantes complementarias de las toxinas y de los canales recombinantes podría ayudar a identificar los sitios de contacto entre la toxina y el canal aumentando la comprensión del modo de acción de estos péptidos. No obstante, la homología en la secuencia entre estos péptidos (KLI y KLII) y la kurtoxina sugiere que la inhibición de la corriente de Ca^{2+} T de las CE se debe al bloqueo de los canales de Ca^{2+} $Ca_V3.1$ y/o $Ca_V3.2$ (Chuang *et al.*, 1999). A pesar de que los mecanismos moleculares del bloqueo podrían ser diferentes, esta hipótesis se apoya en nuestros resultados (ver apéndice,

Espinosa *et al*, 1999), que demuestran la expresión de los RNAs mensajeros para $Ca_V3.1$ y $Ca_V3.2$ y en las diferencias farmacólogicas entre la corriente T nativa de las CE y la subunidad $Ca_V2.3$ expresada heterológamente. Como sabemos, la subunidad α1E ($Ca_V2.3$) es el transcrito con mayor abundancia relativa y se había propuesto como el mejor candidato para codificar a la corriente T en las CE (Liévano *et al*, 1996). Sin embargo, recientemente se demostró que la falta del canal $Ca_V2.3$ (α1E) no modifica ninguna de las características biofísicas fundamentales de la corriente de Ca^{2+} T de las CE (densidad de corriente, activación, inactivación, etc.), lo que descarta la participación de $Ca_V2.3$ en dicha corriente de Ca^{2+} (Sakata *et al*, 2001) y corrobora los resultados anteriores mostrados en este trabajo (ver apéndice, Espinosa *et al*, 1999).

De manera consistente con trabajos previos, el bloqueo de la corriente de Ca^{2+} tipo T por KLI o la KLII inhibió la RA inducida por la ZP (Fig. 28). Con base en las características farmacológicas de los canales de Ca^{2+} dependientes de voltaje, se ha sugerido que la corriente de Ca^{2+} tipo T ($Ca_V3.1$ y/o $Ca_V3.2$) participa, de manera importante, en la RA del espermatozoide de ratón (Wassarman *et al*.,2001; Darszon *et al*, 2001), aún cuando se han propuesto explicaciones alternativas (Benoff, 1998). Este problema se ha debido a la falta de inhibidores específicos para canales dependientes de voltaje tipo T. Sin embargo, el hecho de que KLI y KLII inhiban significativamente a la RA inducida por la ZP en el espermatozoide maduro sugiere fuertemente que los canales T juegan un papel importante en el proceso de señalización celular iniciado por la ZP. Sin embargo, estos resultados indican que aún cuando los canales T son un elemento de vital importancia durante la RA, es posible que otras vías de entrada de Ca^{2+} también estén involucradas en este proceso. De hecho, la diferencia observada entre el porcentaje de

inhibición de la RA (Fig. 28) y la fracción de corriente resistente a 200 nM de las toxinas (Fig. 24B) pudiera explicarse con un incremento en los niveles de expresión del segundo componente de la corriente de Ca^{2+} en el espermatozoide maduro. Lo anterior, hace necesario considerar el posible papel de otros canales de Ca^{2+} durante la RA.

El uso de métodos bioquímicos y moleculares para estudiar a las células espermatogénicas de ratón y humano ha permitido detectar la presencia de al menos dos subunidades de canales de Ca^{2+} distintas al tipo T ($Ca_V1.2$ y $Ca_V2.1$). El reciente reporte de la activación de los canales de Ca^{2+} $Ca_V2.2$ y $Ca_V2.3$ (tipo N y R, respectivamente) en el espermatozoide de ratón en respuesta a depolarizaciones inducidas por alto K^+ externo, ha complicado aún más la identificación molecular de la corriente de Ca^{2+} (Wennemuth *et al*, 2000). Además, es altamente posible que durante la fecundación se requiera de vías alternas de entrada de Ca^{2+} que probablemente sean necesarias para la RA y/o para la adquisición de la motilidad progresiva del espermatozoide (O'Toole *et al*, 2000; Jungnickel *et al*, 2001; Ren *et al*, 2001; Quill *et al*., 2001). Estos datos en su conjunto, sugieren que el espermatozoide posee una diversidad de canales de Ca^{2+} más amplia de lo que se pensaba inicialmente. Más aún, contrario a nuestras expectativas, KLI y/o la KLII no bloquearon totalmente a la corriente de Ca^{2+} observada en las CE (Figs. 24B y 25B). La interpretación más simple a esta observación es que la porción de la corriente insensible a KLI o a la KLII (200 nM) podría estar codificada por una subunidad $\alpha 1$ distinta de la subfamilia de canales de Ca^{2+} T. De manera consistente con esta propuesta, la presencia de un pequeño hombro en la parte descendiente de la curva I-V (alrededor de 0 a +10 mV) detectado en presencia de las toxinas, apoya la idea de trabajos previos que sugieren la expresión funcional de diversos tipos de canales de Ca^{2+} (Goodwin *et al*, 1998; Serrano *et al*., 1999; Westenbroek

y Babcock, 1999; Wennemuth *et al.*, 2000; O'Toole *et al.*, 2000; Jungnickel *et al.*, 2001; Ren *et al.*, 2001; Quill *et al.*, 2001). La expresión funcional del segundo componente es muy baja en este tipo de células, lo que ha evitado en gran medida su identificación previa en la corriente de Ca^{2+}. La presencia de diferentes componentes en la corriente macroscópica de Ca^{2+} de las CE podría ayudar a explicar la polémica sensibilidad de esta corriente de Ca^{2+} a bloqueadores de canales de Ca^{2+} HVA tales como la ω-CTX GVIA (Wennemuth *et al.*, 2000). Sin embargo, es necesario un examen más detallado para determinar si la farmacología de la corriente residual pudiera corresponder a un canal de alto umbral de activación.

Considerando este nuevo componente de la corriente de Ca^{2+} de las CE es necesario reevaluar los resultados presentados en las secciones previas, lo que nos permite hacer varias interpretaciones: resulta interesante que la aplicación de 100 μM de W7, un antagonista de CaM, no inhiba completamente la corriente de Ca^{2+} registrada en las CE (Fig. 10C). Esto podría explicarse con base en el conocimiento de que varias vías de señalización influyen en la actividad de los canales T, lo cual pudiera evitar una inhibición absoluta. Sin embargo, esta corriente residual podría explicarse con la presencia de un canal distinto a la corriente T que no se regule por CaM, hecho que puede ser factible con base en los registros obtenidos en presencia de las toxinas descritas en este trabajo. De la misma forma, se puede señalar que los dos sitios de unión para el NPPB sugeridos por los resultados de la curva dosis-respuesta (Fig. 22B), pudieran ser en realidad evidencia de la presencia de dos componentes de la corriente de Ca^{2+} de las CE. Para responder a estas incognitas sería necesario hacer una serie de experimentos en los que se combine el uso de las toxinas KLI y KLII y los agentes antes mencionados para distinguir entre los efectos

directos de los fármacos sobre los parámetros biofísicos de cada componente de la corriente de Ca^{2+} de manera más apropiada.

Como una primera aproximación para tratar de identificar la naturaleza de este segundo componente de la corriente de Ca^{2+}, se aprovechó la alta sensibilidad de los canales LVA por el Ni^{2+} (Lee *et al.*, 1999) para separar a los dos componentes de la corriente de Ca^{2+}. La aplicación de 1 mM de Ni^{2+} redujo la corriente de Ca^{2+} total a un 25±1.7%, el cual correspondería al componente resistente a las toxinas (Fig. 31A; panel superior) ya que comparte ciertas propiedades biofísicas con la corriente resistente a las toxinas descrito anteriormente (Fig 31A; panel inferior). La incubación de las células en Ni^{2+}/KLII (1 mM/200 nM), permitió descartar que el segundo componente registrado en presencia de Ni^{2+} correspondiera a una corriente de Ca^{2+} de bajo umbral (Fig. 31B). Otra evidencia más que apoya que el componente resistente a Ni^{2+} es distinto al canal T se basa en la insensibilidad de este componente a la BSA (0.5%), la cual incrementa a la corriente T presente en las CE como lo demuestran resultados discutidos previamente en este trabajo (ver sección de modulación y/o apéndice, Espinosa *et al.*, 2000). Debido a que el RNA mensajero de mayor abundancia relativa presente en las CE es α1E ($Ca_V2.3$ o tipo R; Liévano *et al.*, 1996), y considerando que no existe un bloqueador comercial específico para canales $Ca_V2.3$ (tipo R), se decidió utilizar Cd^{2+} (20 μM) el cual bloquea preferencialmente a canales de Ca^{2+} HVA (Hille, 1992). La aplicación de Ni^{2+}/Cd^{2+} (1 mM/20 μM, respectivamente), no bloqueó al componente resistente a Ni^{2+} lo cual sugiere que este componente no pertenece a canales de Ca^{2+} HVA. Este último resultado es consistente con el trabajo reciente de Sakata y colaboradores (2001), en el que reportan que el bloqueo de la expresión de $Ca_V2.3$ (α1E) no altera las propiedades biofísicas ni la

densidad de la corriente T en las células espermatogénicas. En el mismo trabajo, la aplicación de 5 μM de ω-conotoxina GVIA en presencia de 300 μM de Cd^{2+} revela un componente resistente a estos dos bloqueadores, lo que sugiere que en el espermatocito en paquiteno no se expresa funcionalmente el canal de Ca^{2+} $Ca_V2.2$ (tipo N) o algún otro canal de Ca^{2+} HVA sensible a la concentración de Cd^{2+} mencionada (Sakata *et al.*, 2001). Sin embargo, es necesaria una caracterización electrofisiológica y farmacológica más detallada de la corriente resistente a Ni^{2+} y a la KLI para descartar la posible participación de un canal de Ca^{2+} HVA.

Otro posible candidato para producir este segundo componente de la corriente de Ca^{2+} resistente a las toxinas (KLI o KLII) es una nueva proteína llamada CatSper. Hasta la fecha se han clonado dos genes que codifican para CatSper, cuya estructura es similar a la de canales de Ca^{2+} dependientes de voltaje (Ren *et al.*, 2001; Quill *et al.*, 2001). La expresión del RNA mensajero de CatSper es específica de testículo y la proteína se localiza en el flagelo del espermatozoide. Resulta interesante que la interrupción del gene (*Knockout*) que codifica para el CatSper da como resultado un ratón estéril, debido a la incapacidad del gameto para mantener patrones de motilidad normales, lo que reduce su capacidad de penetrar la zona pelúcida del óvulo homólogo (Ren *et al*., 2001).

En resumen, en esta parte de la presente tesis hemos descrito dos nuevas toxinas de alacrán que inhiben específicamente a la corriente T de las CE de ratón y a la RA inducida por la ZP. Además, hemos dado evidencia de que las CE podrían poseer otro componente funcional de la corriente macroscópica de Ca^{2+} cuya presencia ha estado enmascarada por su aparente similitud en cuanto a sus propiedades biofísicas con las características propias de los canales LVA. De la misma forma, hemos aportado lo que es a nuestro conocimiento

la primera evidencia de la inhibición de la RA de mamíferos con toxinas peptídicas. La aplicación de estas toxinas podría tener una gran importancia para evaluar la importancia de los canales T en la señalización eléctrica y bioquímica del espermatozoide. Más aún, considerando que se desconoce la subunidad $\alpha 1$ que codifica para la corriente T nativa de las CE, el uso de la KLI y la KLII como herramientas farmacológicas permitiría definir la identidad molecular no solo de la corriente T de estas células sino de otros tipos celulares de diferentes tejidos, tal como ha ocurrido con toxinas específicas para otro tipo de canales de Ca^{2+}.

VI.- CONCLUSIONES.

6.1.- Modulación:

6.1.1.- Modulación por albúmina y β-estradiol.

A) La albúmina (BSA) modula a la corriente de Ca^{2+} tipo T de las células espermatogénicas, de manera dependiente de la concentración.
B) La BSA modifica la voltaje dependencia de la activación e inactivación de la corriente T, lo cual podría incrementar la corriente ventana de las células espermatogénicas.
C) Nuestros resultados sugieren que los canales de Ca^{2+} tipo T, debido al aumento en la corriente de ventana, pudieran contribuir al incremento de $[Ca^{2+}]i$ en el reposo durante la capacitación.
D) La capacidad de la BSA para inducir la capacitación depende de la extracción de colesterol de la membrana, pero en la modulación de la corriente de Ca^{2+} tipo T pudiera estar quitando otras moléculas hidrofóbicas de la membrana aún no determinadas.
E) Los mecanismos que median los efectos del β-estradiol y la BSA sobre la corriente T son distintos.
F) Es necesario un estudio más profundo para determinar el mecanismo mediante el cual el β-estradiol inhibe a la corriente de Ca^{2+} T.

6.1.2.- Posible modulación por calmodulina.

A) El W7, un inhibidor específico de calmodulina (CaM), reduce a la corriente de Ca^{2+} tipo T de manera dependiente de la concentración e independiente de voltaje.
B) Nuestros resultados sugieren que el efecto del W7 sobre la corriente T se debe a la acción de este fármaco sobre la CaM, ya que otros inhibidores de esta proteína como la TFP producen alteraciones semejantes. Más aún, la adición de CaM a la solución interna de registro o la sustitución de Ca^{2+} por Ba^{2+} inhiben parcialmente el efecto del W7.
C) El W7 afecta tanto a la cinética de activación como a la de inactivación, y promueve la inactivación desde el estado cerrado.
D) El W7 inhibió con una IC_{50} similar a la corriente T de las células espermatogénicas, y al transitorio de Ca^{2+} y a la RA inducidos por el agonista fisiológico en el espermatozoide maduro. Por lo que, nuestros resultados sugieren que el bloqueo de la RA se debe a la inhibición de los canales de Ca^{2+} tipo T.
E) La CaM parecería modular directamente el canal responsable de la corriente T. Sin embargo, todavía no es posible descartar la participación de cinasas o fosfatasas dependientes de CaM en esta regulación.

6.1.3.- Progesterona y ácido γ-aminobutírico (GABA).

A) Nuestros resultados sugieren que ni la progesterona (50 μM) ni el ácido γ-aminobutírico (GABA) (125 μM) modulan a la corriente de Ca^{2+} tipo T de las células espermatogénicas. Por lo tanto, es probable que este tipo de canales de Ca^{2+} no participen en el incremento de Ca^{2+} durante la RA inducida por progesterona o GABA.

6.2.- Estudios farmacológicos:

6.2.1 -Bloqueadores aniónicos.

A) La corriente de Ca^{2+} tipo T es sensible al AN y al NPPB, de manera dependiente de la dosis y del voltaje.
B) El bloqueo de la corriente T por estos compuestos es parcialmente reversible en todos los casos.
C) Las curvas dosis-respuesta sugieren que para el AN (IC_{50}= 43 μM) existe solo un sitio de unión al canal. Para el caso del NPPB, nuestros resultados sugieren dos sitios de unión, alcanzando una primer meseta de inhibición (45%) a una concentración entre 0.5 y 10 μM.
D) Debido a que la concentración para inhibir a la RA por AN (IC_{50}= 11 μM) fue menor que la necesaria para bloquear a la corriente de Ca^{2+} T, nuestros resultados sugieren que el canal T tiene una pobre participación en este proceso. La inhibición de la RA por el AN se debe probablemente a la inhibición de los canales aniónicos presentes en el espermatozoide.
E) Por otra parte, nuestros resultados sugieren que la inhibición de la RA por NPPB (IC_{50}= 6 μM) podría deberse, en parte, al bloqueo de la corriente de Ca^{2+} tipo T.

6.2.2.- Toxinas de alacrán

A) Tomando como base la secuencia de la kurtoxina, identificamos dos toxinas (KLI y KLII) que presentan una alta homología con esta misma.
B) La aplicación de 200 nM de KLI y KLII inhibe a la corriente de Ca^{2+} tipo T en un 65 y 75%, respectivamente, sin modificar la curva I-V.
C) Nuestros resultados sugieren que la inhibición de la corriente T es poco dependiente del voltaje.
D) El análisis de la voltaje dependencia de la inactivación en estado estacionario sugiere que 200 nM de KLII no tiene efecto alguno sobre este parámetro.
E) El efecto de KLII es específico sobre la corriente de Ca^{2+} T, ya que 200 nM de KLI no inhibió a la corriente de K^+ presente en las células espermatogénicas.
F) De manera consistente, KLI y KLII (200 nM) inhibieron a la RA inducida por el agonista fisiológico en un 39.3 y 46.1 %, respectivamente.
G) El uso de la KLI reveló un componente minoritario de la corriente de Ca^{2+} resistente a Ni^{2+} (1 mM), Cd^{2+} (20 μM) y a KLII (200 nM), lo que sugiere que no pertenece a canales LVA o HVA clásicos.
H) El componente resistente a KLI y KLII (200 nM) tiene una cinética de inactivación más lenta que la corriente de Ca^{2+} total.

VII.- PERSPECTIVAS.

La corriente de Ca^{2+} tipo T tiene un papel preponderante en la fecundación en mamífero, tal como lo sugieren los resultados presentados en este trabajo. Sin embargo, aún quedan muchas preguntas interesantes por resolver en cuanto a la modulación y la identidad molecular del canal.

Con respecto a la modulación, aún falta explorar si la corriente de Ca^{2+} T de las células espermatogénicas se regula por ácido araquidónico (AA), tal como se ha reportado para la subunidad α1H expresada en sistemas heterólogos (Zhang *et al*, 2000). De ocurrir esta modulación, podría probarse nuestra hipótesis que sugiere al AA como un posible blanco en la modulación de la corriente T por albúmina. Más aún, existen reportes que sugieren la participación del AA en la RA (Breitbart y Spungin, 1997; Sistina y Rodger, 1999), lo que hace aún más interesante explorar esta posibilidad. De la misma forma, resultaría interesante explorar si la inhibición de la corriente de Ca^{2+} T por 17-β-estradiol involucra la síntesis de oxido nítrico, ya que se sabe que los canales de Ca^{2+} de bajo umbral de activación se bloquean por otros agentes oxidantes como el DTNB o el oxido nitroso (Todorovic *et al*., 2001a; Todorovic *et al*, 2001b).

Por otra parte, los resultados presentados en este estudio son consistentes con la hipótesis de la participación de la calmodulina (CaM) en la regulación de la corriente de Ca^{2+} tipo T de las células espermatogénicas, pero no son una demostración definitiva. Para complementar esta parte proponemos explorar la secuencia de las subunidades α1G (Perez-Reyes *et al*, 1998) y α1H (Cribbs *et al*., 1998) clonadas para buscar posibles sitios de unión a CaM (Rhoads y Friedberg, 1997), y realizar ensayos de unión entre la posible secuencia de unión para CaM de la subunidad α1 y la CaM. Esta estrategia nos permitiría encontrar evidencias directas de la interacción entre estas dos proteínas fortaleciendo nuestra hipótesis.

Otro aspecto interesante por evaluar es el posible efecto de los bloqueadores de canales aniónicos sobre las cicloxigenasas y a las lipoxigenasas que participan en el metabolismo del ácido araquidónico (Civelli *et al.*, 1991) y a la 3α–hidroxisteroide dehidrogenasa involucrada en el metabolismo de esteroides sexuales (Penning *et al*, 1985), ya que los metabolitos de las rutas metabólicas de la lipoxigenasa y la cicloxigenasa modulan positivamente a canales catiónicos y aniónicos (Diener *et al*,1996; Kanli y Norderhus, 1998). De manera similar, los inhibidores de canales aniónicos podrían modificar el metabolismo de las hormonas esteroides (Penning *et al*, 1985), las cuales pueden modular positivamente a canales de Ca^{2+} dependientes de voltaje (Takeuchi y Guggino, 1996; Bukusoglu and Sarlak, 1996).

Por último, considerando que se desconoce la subunidad α1 que codifica para la corriente T *nativa de las células espermatogénicas*, el uso de las toxinas de alacrán (KLI y la KLII) descritas en este trabajo, permitiría definir la identidad molecular no solo de la corriente T de estas células sino de otros tipos celulares de diferentes tejidos, tal como ha ocurrido con toxinas específicas para otro tipo de canales de Ca^{2+}.

VIII. BIBLIOGRAFÍA

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IX.- APÉNDICE

Artículos generados a partir de esta tesis doctoral:

A) **López-González, I.**, Olamendi-Portugal, T., de la Vega-Beltrán, J.L., García, B.I., Van Der Walt, J., Dayson, K., Tytgat, J., Felix, R., Possani, L.D. y Darszon, A. (2002). Two new scorpion toxins inhibit T-type Ca^{2+} channels in mouse male germ cells and inhibit the sperm acrosome reaction. (en preparación).

B) **López-González, I.**, de la Vega-Beltrán, J.L., Santi, C.M., Florman, H.M., Felix, R. y Darszon, A. (2001). Calmodulin antagonists inhibit T-type Ca^{2+} currents in mouse spermatogenic cells and the zona pellucida-induced sperm acrosome reaction. *Dev. Biol.* **236**, 210-219.

C) Espinosa, F., **López-González, I.**, Muñoz-Garay, C., Felix, R., de la Vega-Beltrán, J.L., Kopf, G.S., Visconti, P.E., y Darszon, A. (2000). Dual regulation of the T-type Ca^{2+} current by serum albumin and beta-estradiol in mammalian spermatogenic cells. *FEBS Lett* **475**, 251-256.

D) Espinosa, F., **López-González, I.**, Serrano, C.J., Gasque, G., de la Vega-Beltrán, J.L., Treviño, C.L., y Darszon, A. (1999). Anion channel blockers differentially affect T-type Ca^{2+} currents of mouse spermatogenic cells, alpha1E currents expressed in Xenopus oocytes and the sperm acrosome reaction. *Dev. Genet* **25**, 103-114.

E) Espinosa, F., de la Vega-Beltrán, J.L., **López-González, I.**, Delgado, R., Labarca, P., y Darszon, A. (1998). Mouse sperm patch-clamp recordings reveal single Cl^- channels sensitive to niflumic acid, a blocker of the sperm acrosome reaction. *FEBS Lett.* **426**, 47-51.

Developmental Biology 236 210–219 (2001)
doi:10 1006/dbio 2001 0314 available online at http://www idealibrary com on IDEAL®

Calmodulin Antagonists Inhibit T-Type Ca^{2+} Currents in Mouse Spermatogenic Cells and the Zona Pellucida-Induced Sperm Acrosome Reaction

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The sperm acrosome reaction (AR) is a regulated exocytotic process required for gamete fusion. It depends on an increase in $[Ca^{2+}]_i$ mediated by Ca^{2+} channels. Although calmodulin (CaM) has been reported to regulate several events during the AR, it is not known whether it modulates sperm Ca^{2+} channels. In the present study we analyzed the effects of CaM antagonists W7 and trifluoroperazine on voltage-dependent T-type Ca^{2+} currents in mouse spermatogenic cells and on the zona pellucida-induced AR in sperm. We found that these CaM antagonists decreased T-currents in a concentration-dependent manner with IC_{50} values of ~10 and ~12 μM, respectively. W7 altered the channels' voltage dependence of activation and slowed both activation and inactivation kinetics. It also induced inactivation at voltages at which T-channels are not activated, suggesting a promotion of inactivation from the closed state. Consistent with this, W7 inhibited the ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. Likewise, W7 and TFP inhibited the AR with an IC_{50} of ~10 μM. In contrast, inhibitors of CaM-dependent kinase II and protein kinase A, as well as a CaM-activated phosphatase, had no effect either on T-currents in spermatogenic cells or on the sperm AR. Together these results suggest a functional interaction between CaM and the sperm T-type Ca^{2+} channel. They are also consistent with the involvement of T-channels in the AR. © 2001 Academic Press

Key Words: **Ca^{2+} channel regulation; T-type Ca^{2+} channel; spermatogenic cells; sperm; CaM; W7; TFP; acrosome reaction**

INTRODUCTION

Voltage-dependent Ca^{2+} channels (VDCC) are required components for the elaborate functioning of excitable and nonexcitable cells. Because of this, their regulation is of capital importance to the control of cellular activity. Extracellular ligands, membrane potential, phosphorylation, Ca^{2+} itself, and diffusible second messengers are all well-established regulators of Ca^{2+} channel activity (Hofmann *et al.* 1999). Recently, several studies demonstrated that calmodulin (CaM), a 17-kDa highly conserved EF hand protein which constitutes the classical Ca^{2+} receptor inside cells (Means *et al.* 1982), is implicated in the regulation of cardiac L-type (Zühlke and Reuter 1998; Peterson *et al.* 1999; Qin *et al.*, 1999) and neuronal P/Q-type (Lee *et al.*, 1999) VDCC. A deletion in the C-terminal of the ion-conducting α_{1C} subunit of the cardiac L-type VDCC prevents Ca^{2+}-dependent inactivation of the channel (Zühlke and Reuter 1998). The deletion includes an amino acid sequence called the IQ motif, which in many other proteins binds CaM (Rhoads and Friedberg 1997). Furthermore, mutant forms of CaM, which cannot bind Ca^{2+}, have a dominant-negative action on recombinant L-type Ca^{2+}-channel α_1-subunit inactivation (Peterson *et al.* 1999;

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0012-1606/01 $35.00

Zühlke *et al.* 1999). Likewise, biochemical studies indicated that CaM binds to the IQ motif of the L-type channels and mutational analysis showed that Ca^{2+}-dependent inactivation was greatly slowed by substitution of amino acids in the IQ sequence (Peterson *et al.* 1999; Qin *et al.* 1999; Zühlke *et al.* 1999).

Ca^{2+} influx through VDCC in sperm is required for the acrosome reaction (AR), an exocytotic event essential for sperm–egg fusion (Darszon *et al.*, 1999; Wassarman, 1999). Although the molecular identity of the VDCC involved in this event is currently unresolved, several studies indicate that this channel belongs to the T-type (or the low-voltage activated) family (Arnoult *et al.*, 1996a, 1999; Liévano *et al.* 1996; Santi *et al.* 1996). CaM is believed to participate in the AR because of its localization in the acrosome region (Jones *et al.*, 1980; Kann *et al.* 1991) and its redistribution during this reaction (Hernández *et al.* 1994). Furthermore, CaM antagonists inhibit the AR in sea urchin sperm (Sano, 1983; Guerrero and Darszon 1989). These findings motivated us to explore whether CaM could regulate sperm VDCC. However, the study of VDCC in sperm is complicated by difficulties in applying molecular biology and electrophysiology techniques directly to these small, terminal cells. Indeed, only rarely have Ca^{2+} currents been recorded from mature sperm (Weyand *et al.* 1994; Espinosa *et al.* 1998). An alternative approach has been to use spermatogenic cells, developmental precursors that synthesize proteins for later use in the sperm (Liévano *et al.* 1996; Arnoult *et al.*, 1996a, 1999; Santi *et al.*, 1996). In the present study we examined the effects of specific CaM inhibitors on VDCC activity in mouse spermatogenic cells and on the zona pellucida (ZP)-induced AR in sperm.

MATERIALS AND METHODS

Materials

The following were all from Calbiochem (San Diego, CA): CaM inhibitors *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5); and protein kinase inhibitors: *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89) and 1-[*N,O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN62). CaM inhibitor trifluoperazine dihydrochloride (TFP), calcineurin inhibitory fragment, adenosine 5′-triphosphate (magnesium salt; ATP-Mg_2) phosphocreatine (Di-Tris salt) trypsin (bovine pancreas, type XI), and deoxyribonuclease (DNase; bovine pancreas, type I) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Cell Preparation

Spermatogenic cells were obtained as previously described from 3-month-old CD1 mice testis (Espinosa *et al.*, 1998). In the cell suspension pachytene spermatocytes and round and condensing spermatids were most frequently observed as individual cells or synplasts. Inasmuch as similar results were obtained from these stages, data were pooled for presentation. Caudal epididymal mature mouse sperm were mechanically collected from CD1 mice and placed in 1.5-ml microcentrifuge tubes containing medium 199 supplemented with BSA (0.1% w/v), Na^+ pyruvate (30 mg/L), and $NaHCO_3$ (2.2 g/L) at 37°C (Lee and Storey, 1985). After ~10 min the fraction of motile sperm was determined by visual inspection and preparations with <75% motile cells were discarded.

Assay for the Acrosome Reaction

Sperm aliquots (4–5 × 10^6/ml) were incubated at 37°C for 30 min for *in vitro* capacitation. Thereafter, 5 ZP equiv/μl, prepared as previously indicated (Cross and Meizel, 1989), was added to the sperm suspension to induce AR, which was assayed 30 min later using Coomassie blue G-250 (Visconti *et al.* 1999). The CaM antagonists or the control solvents were added 5 min prior to ZP. To calculate the percentage of AR, at least 100 sperm were assayed per experimental condition for the presence or the absence of the characteristic dark blue acrosomal crescent.

Electrophysiology

Ca^{2+} currents were recorded according to the whole-cell patch-clamp technique (Hamill *et al.*, 1981). All recordings were performed at room temperature using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2- to 4-MΩ borosilicate glass micropipettes. Except when indicated, cells were clamped at a holding potential (HP) of −90 mV. Currents were evoked by 14- to 200-ms depolarizing voltage steps (0.25–0.1 Hz), to test potentials ranging from −80 to 40 mV. Capacity transients were electronically compensated, and linear leak and residual capacity currents were subtracted online using a P/4 standard protocol. Currents were captured online and digitized at a sampling rate of 10 kHz following filtering of the current record (5 kHz) using a personal computer attached to a TL-1 interface (Axon). Pulse protocols, data capture, and analysis of recordings were performed using pCLAMP software (Axon). To isolate Ca^{2+} currents cells were bathed in a solution containing (in mM) $CaCl_2$ (10); NaCl (130); KCl (3); $MgCl_2$ (2); $NaHCO_3$ (1); NaH_2PO_4 (0.5); Hepes (5); and glucose (10) (pH 7.3/NaOH). The internal (patch pipette) solution consisted of (in mM) $CsMeSO_3$ (110); CsF (10); CsCl (15); $CaCl_2$ (4.6); EGTA (10); Hepes (5); ATP-Mg_2 (4); and phosphocreatine (10) (pH 7.3/CsOH). All drugs were prepared as 10–80 mM stock solutions in dimethylsulfoxide (DMSO) and diluted in the bath solution for each experiment to give the desired final concentration.

$[Ca^{2+}]_i$ Determination

Capacitated sperm (1 × 10^7/ml) were loaded with fluo-3 during a 20-min incubation in a BSA-free medium containing 0.0125% Pluronic F-127 and 5 μM acetoxylmethyl ester dye precursor (Molecular Probes, Eugene, OR). Sperm were immobilized on Cell-Tak-coated coverslips and drugs were added by gentle superfusion. $[Ca^{2+}]_i$ values were determined in single cells with excitation illumination (490 nm, 7 nm slit width) from a 75-W Xe arc selected by a Polychrome II monochrometer (TILL Photonics) and fluorescent emission (>530 nm) collected by photomultiplier tubes at 2-ms intervals, as described previously (Arnoult *et al.* 1999). Solubilized ZP solutions (5 μg/ml) and other solutions were added by a drug-delivery pipette positioned near the sperm head. $[Ca^{2+}]_i$ concentrations were determined using a K_D value for this dye in mouse sperm of 636 nM (Rockwell and Storey, 1999).

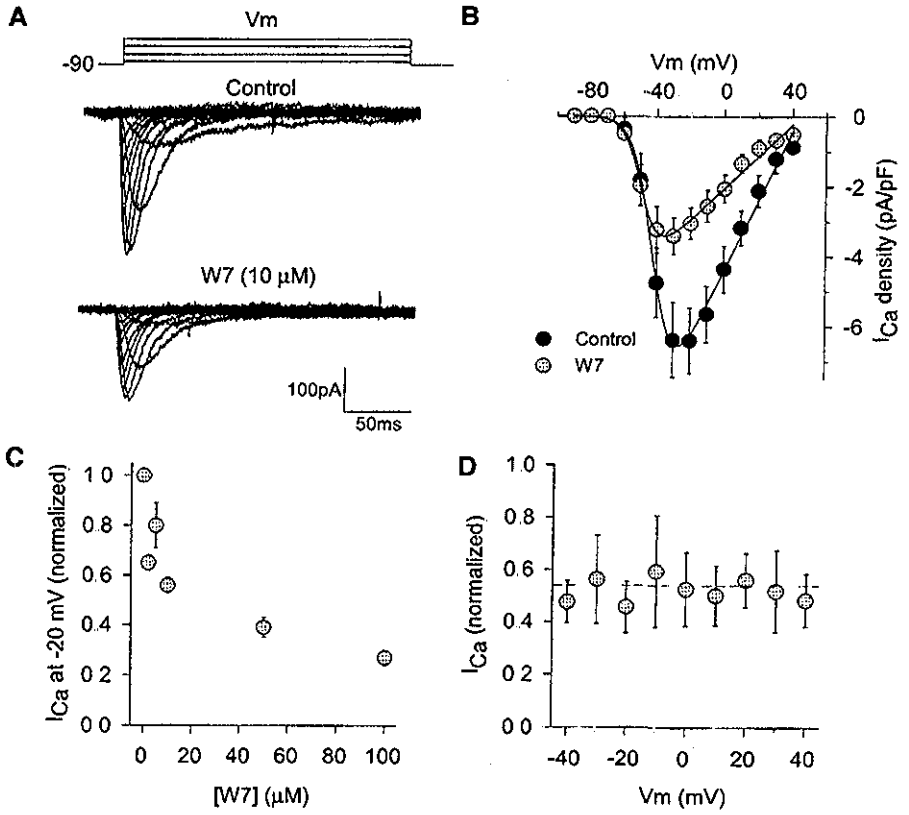

FIG. 1. W7 inhibits Ca^{2+} T-currents in spermatogenic cells. (A) Traces in the upper panel illustrate Ca^{2+} currents evoked by 200-ms depolarizations from a HP of −90 mV to test potentials ranging from −90 to 40 mV with a 10-mV increase in the pulse amplitude per step. During 5-min control experiments the rundown of peak current was frequently negligible and always <10%. Following addition of 10 μM of the specific CaM antagonist W7 to the bath-recording solution a significant reduction in current amplitude was observed (lower panel). (B) Average peak current plotted against test potential (V_m) from cells before and after W7 application (n = 4). (C) Inhibition of T-channel activity by W7 was dose dependent. Peak currents were normalized by their value before the cells were exposed to W7. (D) W7-induced inhibition of T-currents was voltage independent. Fraction of inhibited current for various depolarizing pulses after W7 treatment. Peak currents in the presence of the drug were normalized, averaged, and plotted as a function of test potential.

Statistical Analysis

Unless otherwise noted, data are given as means ± SE. The number of experiments is indicated in the figure legends. Statistical differences between two means were determined by Student's t tests. Means were considered significantly different at $P < 0.05$ and are indicated by an asterisk.

RESULTS

As previously reported (Liévano *et al.*, 1996; Arnoult *et al.*, 1996a, 1998; Santi *et al.*, 1996; Hagiwara and Kawa, 1984) patch-clamp experiments revealed that the only VDCC in mouse spermatogenic cells is of the T-type. The upper traces in Fig. 1A illustrate a family of representative Ca^{2+} currents elicited in a control cell by 10-mV depolarizing pulses from a HP of −90 mV. W7, a CaM antagonist, was used to explore whether CaM regulates sperm VDCC. The lower traces in Fig. 1A show a significant inhibition of the T-current (>45%) after the same cell was superfused with 10 μM W7. Figure 1B summarizes the corresponding current–voltage (I–V) relationships measured at the time of peak current during each record obtained before and during application of W7 in several cells. Figure 1C shows the dose dependence of the W7 inhibition. Currents were elicited by a 20-ms pulse to −20 mV from a HP of −90 mV, measured at the peak and normalized by traces recorded before drug application. Although up to 100 μM W7 could not completely abolish the currents, application of 10 μM W7 to the bath solution inhibited ~50% of the total macroscopic peak current. Thus, this concentration was used thereafter to explore in more detail the actions of W7. As can be seen in Fig. 1D, the inhibition of the T-currents induced by W7 did not exhibit noticeable voltage dependence in the ±40-mV range.

To test whether CaM was indeed involved in the inhibi-

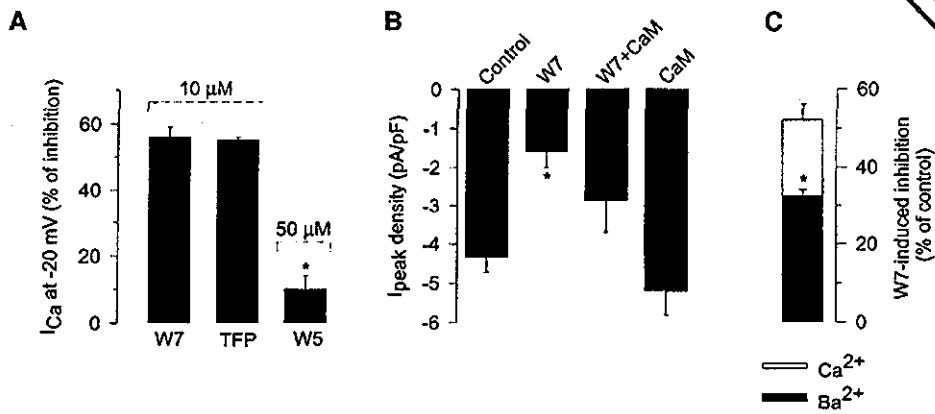

FIG. 2. Inhibition of T-currents by W7 in spermatogenic cells is mimicked by TFP (A) T-channel activity was measured in cells treated with different CaM antagonists Following exposure to the indicated concentration of the inhibitors peak current amplitude was normalized to the control and the percentage of inhibition was compared TFP (10 μM) inhibited the current in a fashion similar to that of W7 whereas 50 μM of W5 (a much less potent CaM antagonist) was unable to cause a significant inhibition of the T-current (B) Comparison of average Ca^{2+} current density after addition of W7 (10 μM) to the bath-recording solution and CaM (10 μM) to the internal solution alone or in combination Peak currents were measured during test pulses to −20 mV from a HP of −90 mV, normalized to cell capacitance and then averaged (n = 5–21) (C) Shown superimposed is the average value of W7-induced inhibition of T-currents in Ca^{2+}- and Ba^{2+}-containing solutions Currents were evoked by 200-ms pulses to −20 mV from a HP of −90 mV Asterisks represent significant differences with respect to the control

tion induced by W7 on the T-currents we next examined the effects of its structural homolog W5 (same as W7 but lacking a Cl^-) a much less potent CaM antagonist Even high concentrations of W5 (50 μM) were unable to cause a significant inhibition of the T-currents (Fig. 2A) In contrast, another potent antagonist of CaM trifluoroperazine (TFP), inhibited the current in a fashion similar to that of W7. To further test whether W7 was directly blocking the T-channel the inhibition caused by W7 in the absence and in the presence of CaM inside the recording pipette was compared (Fig 2B) The percentage of current inhibited by 10 μM W7 was significantly smaller when an equimolar concentration of CaM was included in the internal solution CaM by itself had no effect on the magnitude of the Ca^{2+} currents, which could indicate that endogenous CaM occupies the regulation sites of the channel In addition, with Ba^{2+} as the permeant ion we found that W7-induced inhibition was significantly reduced (Fig 2C), which implies that this action is dependent on Ca^{2+}

Tail currents were measured to further characterize the inhibitory effect of W7 Repolarization of the plasma membrane to −90 mV after a short depolarization to a test potential of −20 mV caused a rapid increase in the amplitude of the inward Ca^{2+} current resulting from the larger driving force for Ca^{2+} influx. Representative superimposed traces of currents obtained before and during the application of W7 clearly illustrate the inhibition caused by the drug (Fig 3A inset) Figure 3A summarizes normalized results from several cells showing that W7 (10 μM) suppressed maximal tail current density by ~46%. Data points were fit with Boltzmann equations to determine activation parameters. Under control conditions, T-current was half-maximally activated at −40 7 ± 1.6 mV In contrast acute application of W7 induced a small but significant ($P < 0$ 05) ~6 3-mV rightward shift in the voltage dependence of activation (Fig 3B) Figure 3C compares the apparent time course of current activation in the absence and the presence of the drug It can be seen that in addition to decreasing amplitude, W7 treatment resulted in a slight delay in the onset of the current and, consequently a significant increase in the time required for Ca^{2+} currents to reach the peak (7 ± 0 3 and 9.2 ± 1 5 ms, respectively).

It was recently established that the binding of Ca^{2+}/CaM to IQ regions inhibits high-voltage-activated (HVA) Ca^{2+} channels by promoting inactivation This mode of CaM regulation was examined in the T-channels of spermatogenic cells. W7 caused a shift of ~3.8 mV toward the depolarizing direction in the average steady-state inactivation curve as well as a slight change in its slope from a control value of 4.6 ± 0.7 to 5.4 ± 0 8 in the treated cells (not shown) Although the differences in both parameters were not statistically significant W7 altered the waveform (Fig 4A) The fraction of current remaining after 80 ms (solid line in Fig. 4A) during 200-ms-long activating pulses (residual current) was significantly increased by W7, suggesting an effect on the inactivation of the T-currents (Fig 4B) Thus W7 may restrain inactivation from the open state of the channels or indirectly slow down inactivation as a consequence of a decreased rate of activation. As illustrated in Fig 4C, the time constant of inactivation significantly increased from 14 1 ± 0.9 to 25.9 ± 1 9 ms after W7 treatment This observation is consistent with the view

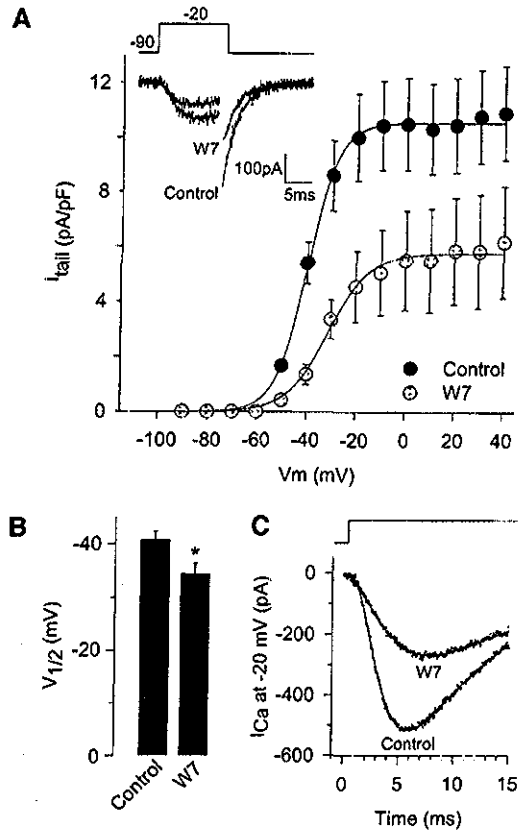

FIG. 3. W7 modulates T-current activation kinetics (A) Control and inhibited tail currents in a number of cells were normalized by capacitance averaged and plotted against membrane potential. Fits of the curves were obtained using a Boltzmann function $I_{Ca} = I_{max}/(1 + \exp[-(V_m - V_{1/2})/s])$ where V_m is the test potential $V_{1/2}$ is the potential at which the current has reached half-maximal amplitude and s is the range of potential for an e-fold change around $V_{1/2}$ Inset: Superimposed traces illustrating Ca^{2+} tail currents obtained before and after application of W7 (10 μM) by subjecting the cell to a depolarizing pulse from a HP of −90 mV to −20 mV for 14 ms and repolarizing the membrane to the HP. (B) Comparison of average $V_{1/2}$ derived from the Boltzmann fits (n = 6–11). (C) Time course of activation of T-currents in both the absence and the presence of W7 was examined in cells with uncompensated series resistance of <6 MΩ Currents were activated by 20 ms (to −20 mV from a HP of −90 mV) with the best fit of the Hodgkin–Huxley equation (Hille 1992) superimposed

that W7 may be acting via CaM, given that this protein induces inactivation of HVA-Ca^{2+} channels. Current inactivation at a step voltage of −20 mV becomes progressively slower as a function of W7 concentration (Fig. 4D, open bars) Although the slowdown of T-channel inactivation caused by W7 should increase current magnitude, this CaM antagonist actually reduces it We calculated the integral of the currents measured before and after W7 and, as illustrated in Fig 4D (filled bars), the influx of Ca^{2+} was significantly smaller upon drug application

Finally, to gain insight into the mechanisms by which CaM antagonists inhibit T-currents in spermatogenic cells we studied the state-dependent inactivation of the channels. Patch-clamp whole-cell experiments suggested that inactivation could occur from closed states of the T-channel given that long prepulses (ranging from −120 to −80 mV, voltages at which no channel opening was detectable) decreased current amplitude in response to a test pulse to −20 mV (compare currents at −120 and −80 in Fig. 5A). This difference became greater after the addition of W7. On average, W7 decreased currents to 80 ± 3 78 ± 2, and 72 ± 5% of the control value after prepulses to −120 −100, and −80 mV, respectively (Fig. 5B).

Recent results indicate that functional T-channels do remain in mature sperm after testicular differentiation and that they play a key role in determining the AR (Arnoult *et al*. 1999) Inasmuch as CaM antagonists proved to be potent regulators of Ca^{2+} channel activity in spermatogenic cells, these agents could also affect the sperm AR To test this, we first determined whether sperm T-channels were also inhibited by CaM antagonists. In sperm ZP triggers a $[Ca^{2+}]_i$ transient that has the kinetic and pharmacological characteristics anticipated of a T-channel (Arnoult *et al*. 1999) An example of a rapid ZP-evoked $[Ca^{2+}]_i$ transient is shown in Fig. 6A. ZP-dependent $[Ca^{2+}]_i$ transients were observed in 65% of cells (22/34) with $[Ca^{2+}]_i$ rising to peak levels of 7.09 ± 1 1 μM and relaxing to basal levels within 250 ms The rising phase of this response is described by a single exponential (τ = 15 2 ± 1.9 ms for 10–90% peak values). In contrast, buffer-treated controls exhibited only monotonic rises to 0.27 ± 0 05 μM during this time period. ZP also evoked $[Ca^{2+}]_i$ responses in 57% (12/21) of sperm treated with 10 μM W7. However, the peak $[Ca^{2+}]_i$ response was inhibited by 73% (2.21 ± 0.89 μM) and the activation time course was slowed (τ = 33 6 ± 4.1 ms). In contrast, 10 μM W5 had only minor effects on ZP-evoked $[Ca^{2+}]_i$ transients (Fig. 6B): responses were observed in 59% of cells (10/17), the maximal response was reduced by only 12% (6.21 ± 0 94 μM), and only a minor slowing of activation time was observed (τ = 17.7 ± 3 1 ms).

CaM antagonists (W7 and TFP) also significantly inhibited the ZP-induced AR in capacitated sperm with an IC_{50} value of ~10 μM (Fig. 7A). Notably, this concentration also inhibits ~50% of the total macroscopic peak current through T-channels in spermatogenic cells (see Fig. 2A). In addition Fig. 7A shows that as anticipated from the pharmacology of the T-currents (see Fig. 2A), W5 caused a less-important inhibition of the sperm AR Furthermore, W7 appears to block AR by altering Ca^{2+} uptake through Ca^{2+} channels because A23187, a Ca^{2+} ionophore that triggers AR in the absence of ZP (Visconti *et al*., 1999), was able to overcome the inhibition caused by W7 (Fig. 7B).

It has been proposed that T-type Ca^{2+} in mouse spermatogenic cells is enhanced by inhibition of protein phosphorylation (Arnoul *et al*., 1997). Because CaM participates in some cell signaling events regulating protein kinases, we therefore thought it pertinent to investigate whether CaM

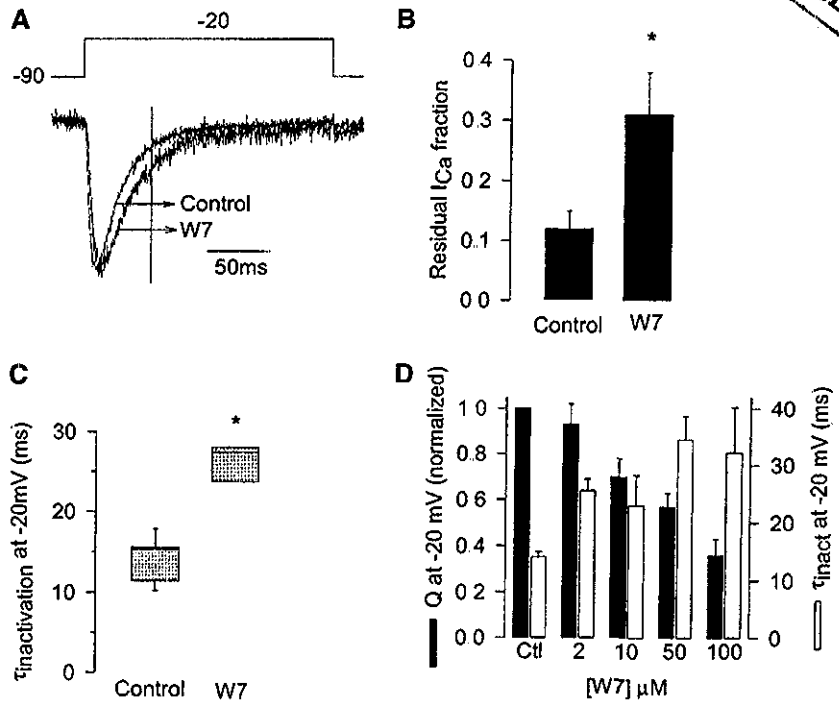

FIG. 4. W7 affects current inactivation (A) Comparison of the time course of typical current traces in both the absence and the presence of W7 (10 μM) The voltage protocol used to measure inactivation is shown above and the traces were normalized to allow kinetic comparison. (B) The percentage of current remaining after 80 ms (solid line in A) into a depolarizing pulse before and after W7 application is compared Currents were recorded in response to 200-ms depolarizing pulses from −90 to −20 mV (C) The inactivating phase of the currents was fitted with a single exponential: $I_{Ca} = A \exp(-t/\tau) + c$ where A is the initial amplitude, t is time τ is the time constant for inactivation and c is a constant Values of τ of inactivating currents obtained at −20 mV before and after W7 treatment are compared (n = 6–11) (D) Integral of the current (Q) measured during 200-ms command steps to −20 mV from a HP of −90 mV Currents measured at this command step after W7 application were normalized to the current observed before drug application in each cell (Ctl), averaged, and plotted as a function of W7 concentration (filled bars) Open bars compare τ of inactivation as a function of the concentration of W7 (n = 3–7)

might be involved in this mechanism of Ca^{2+} channel regulation Whole-cell patch-clamp experiments indicated that micromolar concentrations of the CaM kinase inhibitors KN62 and H89 did not diminish T-currents in spermatogenic cells (not shown) Consistent with this, the sperm AR was not affected by these inhibitors (Fig 7C) In addition the use of an inhibitory peptide derived from the CaM-dependent serine/threonine protein phosphatase calcineurin, did not alter current density through T-type Ca^{2+} nor the time constant of inactivation (data not shown) suggesting that a phosphatase activity is not required for modulation of Ca^{2+} channels in spermatogenic cells

DISCUSSION

Mammalian sperm must undergo the AR prior to fertilization The signal transduction steps that lead to the sperm AR are not well understood This exocytotic event, induced by the ZP surrounding the oocyte, depends on the uptake of external Ca^{2+} (Wassarman 1999) Growing experimental evidence suggests that a voltage-gated Ca^{2+} channel is an important pathway responsible for modulating Ca^{2+} entry into sperm during this process (Darszon *et al.* 1999; Arnoult *et al.*, 1996a 1999) Previous electrophysiological studies of Ca^{2+} currents in spermatogenic cells demonstrated the presence of T-currents (Hagiwara and Kawa, 1984; Liévano *et al.* 1996; Arnoult *et al.* 1996a,b; Santi *et al.* 1996) with pharmacological attributes consistent with those of the AR and the Ca^{2+} influx associated with it (Arnoult *et al.*, 1998) Likewise it was shown that this channel is retained in the mature sperm (Arnoult *et al.* 1999) and, therefore it must be an important element in the transduction events leading to the ZP-induced AR in mammalian sperm (Arnoult *et al.* 1996a 1999; Darszon *et al.* 1999).

Although the T-type Ca^{2+} currents of spermatogenic cells have been characterized, their regulation is poorly understood Such information is needed to unveil the molecular mechanisms leading to the sperm AR Recent findings indicate that CaM regulates HVA-channels, contributing to the determination of the intracellular levels of Ca^{2+} (Peter-

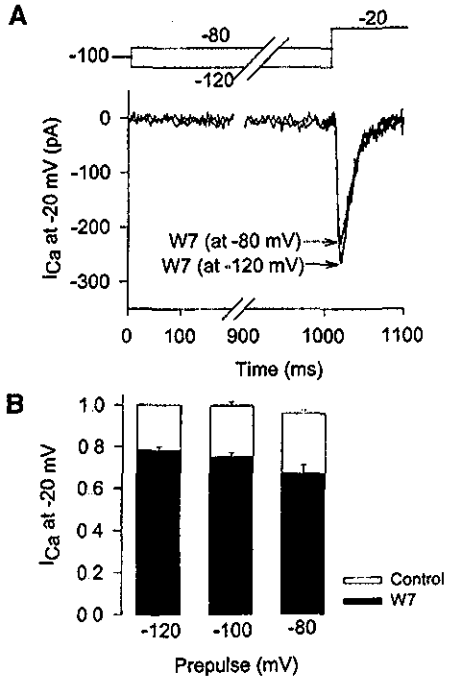

FIG. 5. W7 promotes T-channel inactivation from the closed state. (A) Voltage clamp traces recorded in a cell after application of W7 at two different potentials. Currents were evoked applying 1-s voltage prepulses to −120 and −80 mV followed by a 75-ms test pulse to −20 mV. The amount of inactivation after W7 addition at −80 mV exceeded that observed at −120 mV. (B) Inactivation measured from the test pulses is compared in different cells in both the absence and the presence of W7 during prepulses to −120 −100 and −80 mV (n = 5). Open bars represent the average currents in response to the test potential normalized to that evoked after application of the prepulse to −120 mV in the control cells. Filled bars denote current amplitude after addition of W7 normalized to that obtained before drug application.

son *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). Previous observations using CaM inhibitors in sea urchin (Sano, 1983; Guerrero and Darszon, 1989) and mammalian sperm (Courtot *et al.*, 1999) suggested the participation of CaM in the AR. With this in mind and considering the advantages of studying ion channels in spermatogenic cells, we explored the possible regulation of the T-type Ca^{2+} channels present in these cells by CaM. The results presented herein show that the specific CaM antagonist W7, at concentrations above 2 μM, markedly reduced T-current magnitude (Figs. 1–3). This effect is concentration dependent and essentially voltage independent (Figs. 1C and 1D). TFP, another CaM antagonist, also significantly reduced current amplitude at concentrations that inhibit CaM (Massom *et al.*, 1990). Furthermore, addition of CaM inside the recording pipette decreased the inhibitory effect of W7, suggesting a potential participation of this protein in the regulation of T-type Ca^{2+} channels (Figs. 2A and 2B).

The results presented here are in line with the involvement of CaM in T-type Ca^{2+} channel regulation in spermatogenic cells; though, they are not a definitive demonstration. W7, W5, and TFP can have other less-specific effects such as direct inhibition of plasma membrane and intracellular Ca^{2+} channels and phospholipase C (Ehrlich *et al.*, 1988; Schlatterer and Schaloske, 1996; Schofl *et al.*, 1999). However, the inhibitor concentrations used in this study are fitting for CaM inhibition and are lower than those associated with less-specific effects (Ichikawa *et al.*, 1991). In addition, the Ca^{2+} dependence of the inhibitory effects of W7 (Fig. 2C) supports the role of a Ca^{2+}/CaM complex in T-channel regulation. Moreover, the direct effects of CaM inhibitors on ion channel activity have been shown to be partly or fully reversible (Laver *et al.*, 1997), whereas the actions of these compounds in our experiments resulted in a nonreversible inhibition (not shown), suggesting an indirect effect on the channels. Finally, when W7 and TFP act directly on ion channels, addition of CaM

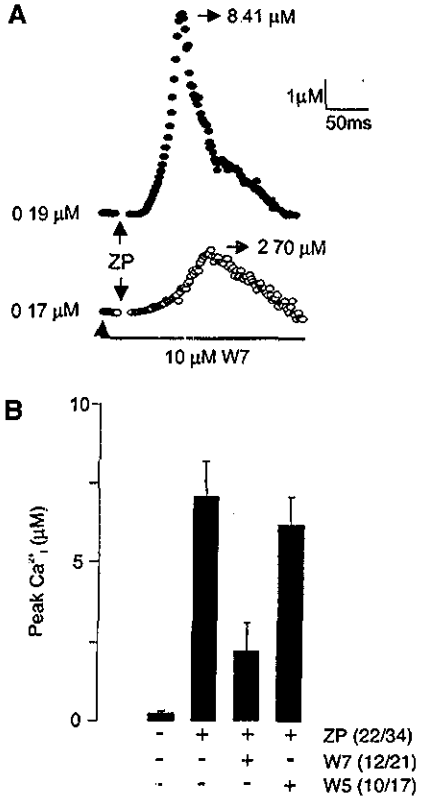

FIG. 6. W7 inhibits ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. (A) Examples of $[Ca^{2+}]_i$ responses evoked by ZP (30 μg/ml) in sperm treated with buffer and with W7. Initial and peak $[Ca^{2+}]_i$ values are indicated. (B) Sperm were incubated for 10 min with buffer (n = 22), 10 μM W7 (n = 12) or 10 μM W5 (n = 10) prior to the addition of ZP. Data represent the peak $[Ca^{2+}]_i$ levels (± SD) during transients and the fraction of total cells exhibiting responses is shown.

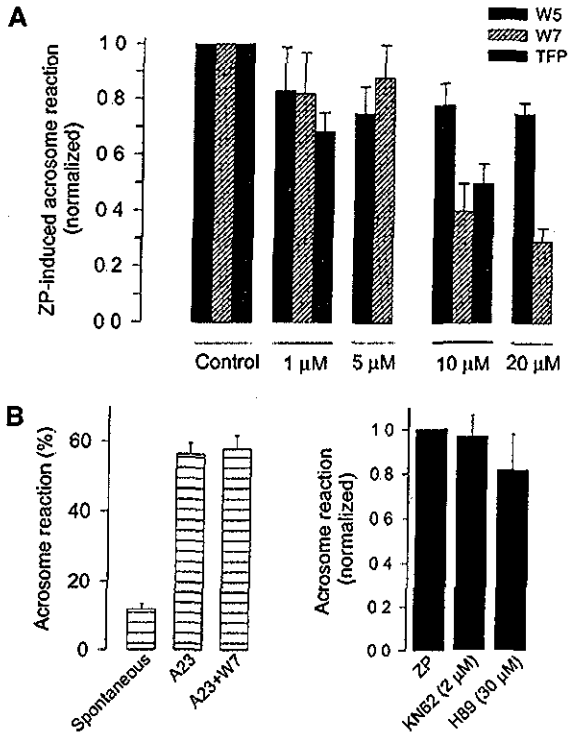

FIG. 7. CaM antagonists affect the sperm AR (A) Effect of W7 on the percentage of sperm undergoing ZP-induced AR Sperm were incubated in the presence of increasing concentrations of W7 After capacitation, the percentage of the AR was monitored Data represent means ± SEM of at least five independent experiments Sperm were also incubated in the presence of different concentrations of TFP (n = 6) and W5 (n = 7) as listed, and ZP-induced AR was measured (B) AR inhibition caused by W7 is overcome by A23187 Samples were incubated for 35 min in the presence (15 μM) of the Ca^{2+} ionophore A23187 (A23) alone or in combination with W7 (n = 4) (C) CaM-dependent protein kinase inhibitors do not affect AR The effects of KN62 and H89 on the mouse sperm AR are compared Sperm were incubated in both the absence and the presence of these two protein kinase inhibitors at the indicated concentrations Data represent means ± SEM of five separate determinations

inside the recording pipette appears to be unable to reverse this interaction (Kleene, 1994). Altogether, these considerations lead us to believe that W7 and TFP influence T-channel function by inhibiting CaM

It has been proposed that inactivation in HVA-channels involves Ca^{2+} entering through the pore, binding to CaM, and changing its conformation. The Ca^{2+}/CaM complex interacts with the channel, initiating an intramolecular process that culminates in a down-regulation of Ca^{2+} channel activity (Zühlke and Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.* 1999; Zühlke *et al.*, 1999; Lee *et al.* 1999) In agreement with this, acute application of CaM antagonist W7 results in a partial slowdown of inactivation of the T-type Ca^{2+} channels in spermatogenic cells (Figs 4C and 4D) However more striking is the decrease in T-type current amplitude caused by CaM antagonists in these cells. Here we show that, in addition to decreasing current amplitude, submaximal concentrations of W7 drastically change other basic biophysical properties of the current (Figs. 3 and 5). These changes include a positive shift in the voltage dependence of activation and a significant slowing of the apparent activation kinetics. Because activation and inactivation are tightly coupled in T-channels (Serrano *et al.* 1999), we suggest that the decrease in the rate of activation in the presence of W7 may indirectly slow down the apparent inactivation time course of the channels. In addition W7 appears to promote inactivation from voltages at which T-channels are not apparently activated (Figs. 5A and 5B) Whether CaM directly mediates this mechanism is still not clear

In summary, the present study shows that CaM antagonists W7 and TFP inhibit with similar potency T-channels in spermatogenic cells monitored by electrophysiological methods and the ZP-induced AR. Notably a similar concentration of W7 (10 μM) represses the Ca^{2+} transient triggered by ZP in mature sperm assessed by using a fluorescent probe. In contrast W5, a close analog of W7 but a much less potent CaM antagonist, is a poor inhibitor of the T-currents in spermatogenic cells and of the sperm AR and the Ca^{2+} transient associated with it. Furthermore, replacement of external Ca^{2+} by Ba^{2+} or inclusion of CaM inside the recording pipette decreases the inhibition of the T-currents in spermatogenic cells caused by W7. The overall results are consistent with a direct link between T-Ca^{2+} channel activation and the ZP-induced sperm AR, and suggest that CaM may regulate sperm T-channels. In mature sperm this is particularly important because during AR a delicate redistribution and modification of plasma membrane molecules takes place and intracellular Ca^{2+} dynamics are drastically changed (Darszon *et al.*, 1999; Wassarman, 1999; Flesch and Gadella, 2000) In addition this regulation may be important for sperm capacitation, where micromolar concentrations of CaM antagonists W7 and calmidazolium have been shown to significantly inhibit the percentage of capacitated sperm assessed by the chlortetracycline staining method (Si and Olds-Clarke, 2000) and by their ability to undergo AR in response to lysophosphatidylcholine

ACKNOWLEDGMENTS

This work was supported by grants from CONACyT to A.D and R.F.; DGAPA (UNAM), HHMI, and ICGEB to A.D; and NIH (HD32177 and GM56479) to H.M.F. I.L.G was the recipient of a CONACyT graduate fellowship. We thank Dr. F. Gómez-Lagunas for valuable discussions and commenting on the manuscript and Dr. Arturo Hernández-Cruz in whose laboratory the initial experiments were performed.

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Received for publication March 15, 2001
Revised April 27, 2001
Accepted May 2, 2001
Published online June 27, 2001

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Two new scorpion toxins inhibit T-type Ca^{2+} channels in mouse male germ cells and inhibit the sperm acrosome reaction.

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Abstract

Polypeptide toxins that specifically interact with ion channels and modulate their activity are useful pharmacological tools to investigate their function. Recently kurtoxin, a 63 amino acid toxin isolated from the venom of the scorpion *Parabuthus transvaalicus*, was isolated and shown to be active on recombinant T-type Ca^{2+} channels (Chuang *et al.*, 1998, Nature Neurosci 1, 668-674). In this report we characterize two homologous scorpion toxins from the scorpion *Parabuthus granulatus* named kurtoxin-like I and II (KLI and KLII, respectively). KLII and Kurtoxin are the same, and KLI is different in 5 amino acids. Both toxins affect native voltage-gated T-type Ca^{2+} channels in mouse spermatogenic cells and inhibit the physiologically-induced sperm acrosome reaction (AR). KLI and KLII significantly inhibit Ca^{2+} currents in isolated spermatogenic cells without affecting their basic kinetic properties. The effect of the toxins on the channels was weakly voltage-dependent and only partially reversible. A maximum inhibition of ~60% could be reached at saturating concentrations suggesting the presence of an additional toxin-resistant component in the whole-cell Ca^{2+} current in spermatogenic cells. Notably, KLI and KLII produced an inhibition of ~45% in the sperm AR induced by the egg's *zona pellucida*. These results are consistent with the participation of the T-type Ca^{2+} channels in the mammalian sperm AR. On the other hand KLI and KLII are potentially excellent tools to localize and isolate the T-type Ca^{2+} channels from mouse sperm

Developmental Biology 236, 210–219 (2001)
doi:10.1006/dbio.2001.0314 available online at http://www.idealibrary.com on IDEAL®

Calmodulin Antagonists Inhibit T-Type Ca^{2+} Currents in Mouse Spermatogenic Cells and the Zona Pellucida-Induced Sperm Acrosome Reaction

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The sperm acrosome reaction (AR) is a regulated exocytotic process required for gamete fusion. It depends on an increase in $[Ca^{2+}]_i$ mediated by Ca^{2+} channels. Although calmodulin (CaM) has been reported to regulate several events during the AR, it is not known whether it modulates sperm Ca^{2+} channels. In the present study we analyzed the effects of CaM antagonists W7 and trifluoroperazine on voltage-dependent T-type Ca^{2+} currents in mouse spermatogenic cells and on the zona pellucida-induced AR in sperm. We found that these CaM antagonists decreased T-currents in a concentration-dependent manner with IC_{50} values of ~10 and ~12 μM, respectively. W7 altered the channels' voltage dependence of activation and slowed both activation and inactivation kinetics. It also induced inactivation at voltages at which T-channels are not activated, suggesting a promotion of inactivation from the closed state. Consistent with this, W7 inhibited the ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. Likewise, W7 and TFP inhibited the AR with an IC_{50} of ~10 μM. In contrast, inhibitors of CaM-dependent kinase II and protein kinase A, as well as a CaM-activated phosphatase, had no effect either on T-currents in spermatogenic cells or on the sperm AR. Together these results suggest a functional interaction between CaM and the sperm T-type Ca^{2+} channel. They are also consistent with the involvement of T-channels in the AR. © 2001 Academic Press

Key Words: Ca^{2+} channel regulation; T-type Ca^{2+} channel; spermatogenic cells; sperm; CaM; W7; TFP; acrosome reaction.

INTRODUCTION

Voltage-dependent Ca^{2+} channels (VDCC) are required components for the elaborate functioning of excitable and nonexcitable cells. Because of this, their regulation is of capital importance to the control of cellular activity. Extracellular ligands, membrane potential, phosphorylation, Ca^{2+} itself, and diffusible second messengers are all well-established regulators of Ca^{2+} channel activity (Hofmann *et al.*, 1999). Recently, several studies demonstrated that calmodulin (CaM), a 17-kDa highly conserved EF hand protein which constitutes the classical Ca^{2+} receptor inside cells (Means *et al.*, 1982), is implicated in the regulation of cardiac L-type (Zühlke and Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.*, 1999) and neuronal P/Q-type (Lee *et al.*, 1999) VDCC. A deletion in the C-terminal of the ion-conducting α_{1C} subunit of the cardiac L-type VDCC prevents Ca^{2+}-dependent inactivation of the channel (Zühlke and Reuter, 1998). The deletion includes an amino acid sequence, called the IQ motif, which in many other proteins binds CaM (Rhoads and Friedberg, 1997). Furthermore, mutant forms of CaM which cannot bind Ca^{2+} have a dominant-negative action on recombinant L-type Ca^{2+}-channel α_1-subunit inactivation (Peterson *et al.*, 1999;

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0012-1606/01 $35.00

Zühlke *et al.*, 1999). Likewise, biochemical studies indicated that CaM binds to the IQ motif of the L-type channels and mutational analysis showed that Ca^{2+}-dependent inactivation was greatly slowed by substitution of amino acids in the IQ sequence (Peterson *et al* 1999; Qin *et al*., 1999; Zühlke *et al*., 1999).

Ca^{2+} influx through VDCC in sperm is required for the acrosome reaction (AR) an exocytotic event essential for sperm-egg fusion (Darszon *et al*., 1999; Wassarman, 1999). Although the molecular identity of the VDCC involved in this event is currently unresolved, several studies indicate that this channel belongs to the T-type (or the low-voltage activated) family (Arnoult *et al*., 1996a, 1999; Liévano *et al* 1996; Santi *et al* 1996). CaM is believed to participate in the AR because of its localization in the acrosome region (Jones *et al*., 1980; Kann *et al*., 1991) and its redistribution during this reaction (Hernández *et al* 1994). Furthermore, CaM antagonists inhibit the AR in sea urchin sperm (Sano, 1983; Guerrero and Darszon, 1989). These findings motivated us to explore whether CaM could regulate sperm VDCC. However, the study of VDCC in sperm is complicated by difficulties in applying molecular biology and electrophysiology techniques directly to these small, terminal cells. Indeed, only rarely have Ca^{2+} currents been recorded from mature sperm (Weyand *et al*., 1994; Espinosa *et al* 1998). An alternative approach has been to use spermatogenic cells developmental precursors that synthesize proteins for later use in the sperm (Liévano *et al* 1996; Arnoult *et al*., 1996a, 1999; Santi *et al*., 1996). In the present study we examined the effects of specific CaM inhibitors on VDCC activity in mouse spermatogenic cells and on the zona pellucida (ZP)-induced AR in sperm.

MATERIALS AND METHODS

Materials

The following were all from Calbiochem (San Diego, CA): CaM inhibitors *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5); and protein kinase inhibitors: *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89) and 1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN62). CaM inhibitor trifluoperazine dihydrochloride (TFP), calcineurin inhibitory fragment, adenosine 5′-triphosphate (magnesium salt; ATP-Mg_2) phosphocreatine (Di-Tris salt), trypsin (bovine pancreas, type XI), and deoxyribonuclease (DNase; bovine pancreas, type I) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Cell Preparation

Spermatogenic cells were obtained as previously described from 3-month-old CD1 mice testis (Espinosa *et al*., 1998). In the cell suspension pachytene spermatocytes and round and condensing spermatids were most frequently observed as individual cells or synplasts. Inasmuch as similar results were obtained from these stages, data were pooled for presentation. Caudal epididymal mature mouse sperm were mechanically collected from CD1 mice and placed in 1.5-ml microcentrifuge tubes containing medium 199 supplemented with BSA (0.1% w/v), Na^+ pyruvate (30 mg/L) and $NaHCO_3$ (2.2 g/L) at 37°C (Lee and Storey, 1985). After ~10 min the fraction of motile sperm was determined by visual inspection and preparations with <75% motile cells were discarded.

Assay for the Acrosome Reaction

Sperm aliquots (4–5 × 10^6/ml) were incubated at 37°C for 30 min for *in vitro* capacitation. Thereafter 5 ZP equiv/μl prepared as previously indicated (Cross and Meizel, 1989), was added to the sperm suspension to induce AR, which was assayed 30 min later using Coomassie blue G-250 (Visconti *et al*., 1999). The CaM antagonists or the control solvents were added 5 min prior to ZP. To calculate the percentage of AR, at least 100 sperm were assayed per experimental condition for the presence or the absence of the characteristic dark blue acrosomal crescent.

Electrophysiology

Ca^{2+} currents were recorded according to the whole-cell patch-clamp technique (Hamill *et al*., 1981). All recordings were performed at room temperature using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2- to 4-MΩ borosilicate glass micropipettes. Except when indicated, cells were clamped at a holding potential (HP) of −90 mV. Currents were evoked by 14- to 200-ms depolarizing voltage steps (0.25–0.1 Hz), to test potentials ranging from −80 to 40 mV. Capacity transients were electronically compensated and linear leak and residual capacity currents were subtracted online using a P/4 standard protocol. Currents were captured online and digitized at a sampling rate of 10 kHz following filtering of the current record (5 kHz) using a personal computer attached to a TL-1 interface (Axon). Pulse protocols, data capture, and analysis of recordings were performed using pCLAMP software (Axon). To isolate Ca^{2+} currents cells were bathed in a solution containing (in mM) $CaCl_2$ (10); NaCl (130); KCl (3); $MgCl_2$ (2); $NaHCO_3$ (1); NaH_2PO_4 (0.5); Hepes (5); and glucose (10) (pH 7.3/NaOH). The internal (patch pipette) solution consisted of (in mM) $CsMeSO_3$ (110); CsF (10); CsCl (15); $CaCl_2$ (4.6); EGTA (10); Hepes (5); ATP-Mg_2 (4); and phosphocreatine (10) (pH 7.3/CsOH). All drugs were prepared as 10–80 mM stock solutions in dimethylsulfoxide (DMSO) and diluted in the bath solution for each experiment to give the desired final concentration.

$[Ca^{2+}]_i$ Determination

Capacitated sperm (1 × 10^7/ml) were loaded with fluo-3 during a 20-min incubation in a BSA-free medium containing 0.0125% Pluronic F-127 and 5 μM acetoxylmethyl ester dye precursor (Molecular Probes, Eugene, OR). Sperm were immobilized on Cell-Tak-coated coverslips and drugs were added by gentle superfusion. $[Ca^{2+}]_i$ values were determined in single cells with excitation illumination (490 nm, 7 nm slit width) from a 75-W Xe arc selected by a Polychrome II monochrometer (TILL Photonics) and fluorescent emission (>530 nm) collected by photomultiplier tubes at 2-ms intervals, as described previously (Arnoult *et al*., 1999). Solubilized ZP solutions (5 μg/ml) and other solutions were added by a drug-delivery pipette positioned near the sperm head. $[Ca^{2+}]_i$ concentrations were determined using a K_D value for this dye in mouse sperm of 636 nM (Rockwell and Storey, 1999).

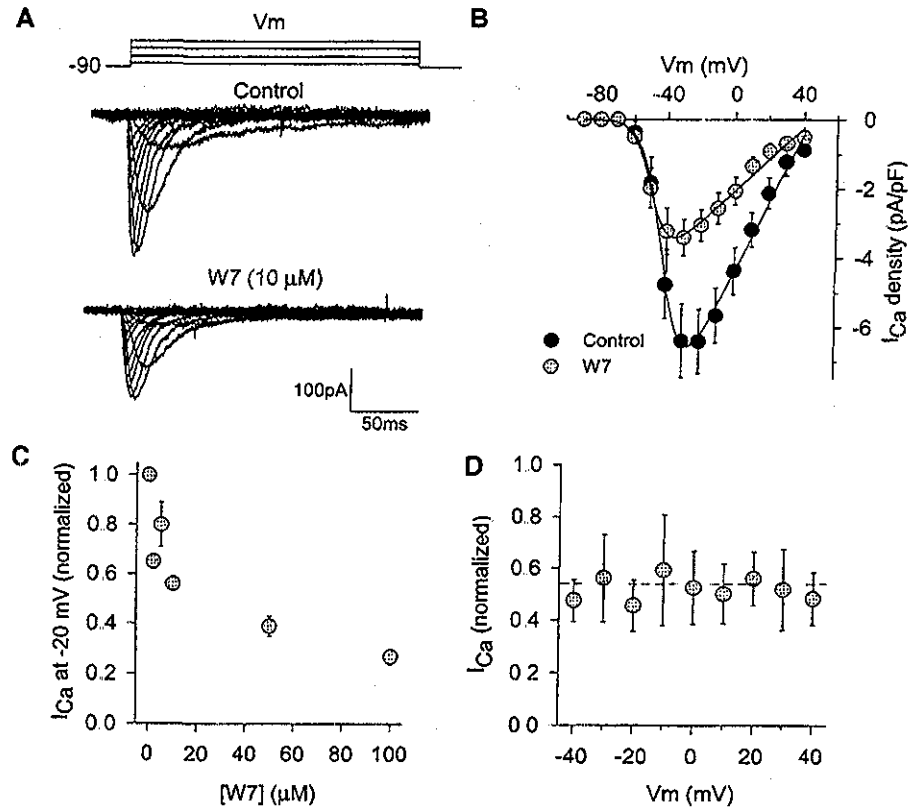

FIG. 1. W7 inhibits Ca^{2+} T-currents in spermatogenic cells (A) Traces in the upper panel illustrate Ca^{2+} currents evoked by 200-ms depolarizations from a HP of −90 mV to test potentials ranging from −90 to 40 mV with a 10-mV increase in the pulse amplitude per step. During 5-min control experiments the rundown of peak current was frequently negligible and always <10%. Following addition of 10 μM of the specific CaM antagonist W7 to the bath-recording solution a significant reduction in current amplitude was observed (lower panel). (B) Average peak current plotted against test potential (V_m) from cells before and after W7 application ($n = 4$) (C) Inhibition of T-channel activity by W7 was dose dependent. Peak currents were normalized by their value before the cells were exposed to W7. (D) W7-induced inhibition of T-currents was voltage independent. Fraction of inhibited current for various depolarizing pulses after W7 treatment. Peak currents in the presence of the drug were normalized, averaged and plotted as a function of test potential

Statistical Analysis

Unless otherwise noted, data are given as means ± SE. The number of experiments is indicated in the figure legends. Statistical differences between two means were determined by Student's t tests. Means were considered significantly different at $P < 0.05$ and are indicated by an asterisk.

RESULTS

As previously reported (Liévano *et al.*, 1996; Arnoult *et al.*, 1996a, 1998; Santi *et al.* 1996; Hagiwara and Kawa 1984) patch-clamp experiments revealed that the only VDCC in mouse spermatogenic cells is of the T-type. The upper traces in Fig. 1A illustrate a family of representative Ca^{2+} currents elicited in a control cell by 10-mV depolarizing pulses from a HP of −90 mV. W7, a CaM antagonist, was used to explore whether CaM regulates sperm VDCC. The lower traces in Fig. 1A show a significant inhibition of the T-current (>45%) after the same cell was superfused with 10 μM W7. Figure 1B summarizes the corresponding current-voltage (I–V) relationships measured at the time of peak current during each record obtained before and during application of W7 in several cells. Figure 1C shows the dose dependence of the W7 inhibition. Currents were elicited by a 20-ms pulse to −20 mV from a HP of −90 mV, measured at the peak and normalized by traces recorded before drug application. Although up to 100 μM W7 could not completely abolish the currents, application of 10 μM W7 to the bath solution inhibited ~50% of the total macroscopic peak current. Thus, this concentration was used thereafter to explore in more detail the actions of W7. As can be seen in Fig. 1D, the inhibition of the T-currents induced by W7 did not exhibit noticeable voltage dependence in the ±40-mV range.

To test whether CaM was indeed involved in the inhibi-

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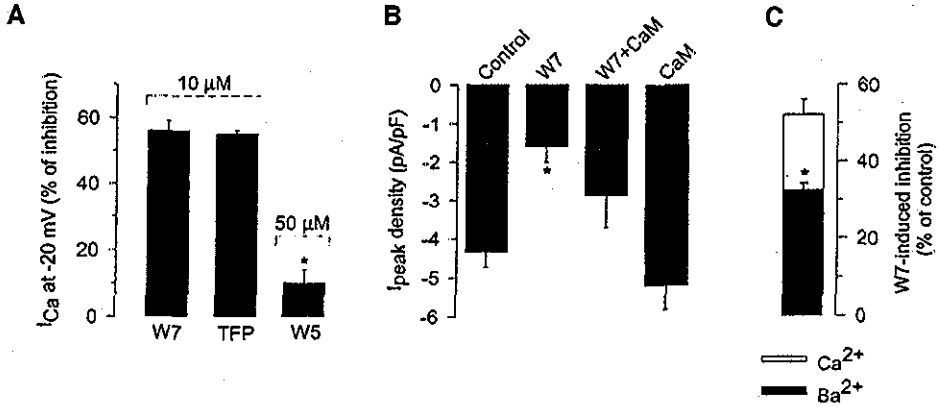

FIG. 2. Inhibition of T-currents by W7 in spermatogenic cells is mimicked by TFP (A) T-channel activity was measured in cells treated with different CaM antagonists. Following exposure to the indicated concentration of the inhibitors, peak current amplitude was normalized to the control and the percentage of inhibition was compared. TFP (10 μM) inhibited the current in a fashion similar to that of W7, whereas 50 μM of W5 (a much less potent CaM antagonist) was unable to cause a significant inhibition of the T-current. (B) Comparison of average Ca^{2+} current density after addition of W7 (10 μM) to the bath-recording solution and CaM (10 μM) to the internal solution, alone or in combination. Peak currents were measured during test pulses to −20 mV from a HP of −90 mV, normalized to cell capacitance and then averaged (n = 5–21). (C) Shown superimposed is the average value of W7-induced inhibition of T-currents in Ca^{2+}- and Ba^{2+}-containing solutions. Currents were evoked by 200-ms pulses to −20 mV from a HP of −90 mV. Asterisks represent significant differences with respect to the control.

tion induced by W7 on the T-currents, we next examined the effects of its structural homolog W5 (same as W7 but lacking a Cl^-), a much less potent CaM antagonist. Even high concentrations of W5 (50 μM) were unable to cause a significant inhibition of the T-currents (Fig. 2A). In contrast, another potent antagonist of CaM, trifluoroperazine (TFP), inhibited the current in a fashion similar to that of W7. To further test whether W7 was directly blocking the T-channel, the inhibition caused by W7 in the absence and in the presence of CaM inside the recording pipette was compared (Fig. 2B). The percentage of current inhibited by 10 μM W7 was significantly smaller when an equimolar concentration of CaM was included in the internal solution. CaM by itself had no effect on the magnitude of the Ca^{2+} currents, which could indicate that endogenous CaM occupies the regulation sites of the channel. In addition, with Ba^{2+} as the permeant ion, we found that W7-induced inhibition was significantly reduced (Fig. 2C), which implies that this action is dependent on Ca^{2+}.

Tail currents were measured to further characterize the inhibitory effect of W7. Repolarization of the plasma membrane to −90 mV after a short depolarization to a test potential of −20 mV caused a rapid increase in the amplitude of the inward Ca^{2+} current resulting from the larger driving force for Ca^{2+} influx. Representative superimposed traces of currents obtained before and during the application of W7 clearly illustrate the inhibition caused by the drug (Fig. 3A, inset). Figure 3A summarizes normalized results from several cells showing that W7 (10 μM) suppressed maximal tail current density by ~46%. Data points were fit with Boltzmann equations to determine activation parameters. Under control conditions, T-current was half-maximally activated at −40.7 ± 1.6 mV. In contrast, acute application of W7 induced a small but significant ($P < 0.05$) ~6.3-mV rightward shift in the voltage dependence of activation (Fig. 3B). Figure 3C compares the apparent time course of current activation in the absence and the presence of the drug. It can be seen that, in addition to decreasing amplitude, W7 treatment resulted in a slight delay in the onset of the current and, consequently, a significant increase in the time required for Ca^{2+} currents to reach the peak (7 ± 0.3 and 9.2 ± 1.5 ms, respectively).

It was recently established that the binding of Ca^{2+}/CaM to IQ regions inhibits high-voltage-activated (HVA) Ca^{2+} channels by promoting inactivation. This mode of CaM regulation was examined in the T-channels of spermatogenic cells. W7 caused a shift of ~3.8 mV toward the depolarizing direction in the average steady-state inactivation curve, as well as a slight change in its slope from a control value of 4.6 ± 0.7 to 5.4 ± 0.8 in the treated cells (not shown). Although the differences in both parameters were not statistically significant, W7 altered the waveform (Fig. 4A). The fraction of current remaining after 80 ms (solid line in Fig. 4A) during 200-ms-long activating pulses (residual current) was significantly increased by W7, suggesting an effect on the inactivation of the T-currents (Fig. 4B). Thus, W7 may restrain inactivation from the open state of the channels or indirectly slow down inactivation as a consequence of a decreased rate of activation. As illustrated in Fig. 4C, the time constant of inactivation significantly increased from 14.1 ± 0.9 to 25.9 ± 1.9 ms after W7 treatment. This observation is consistent with the view

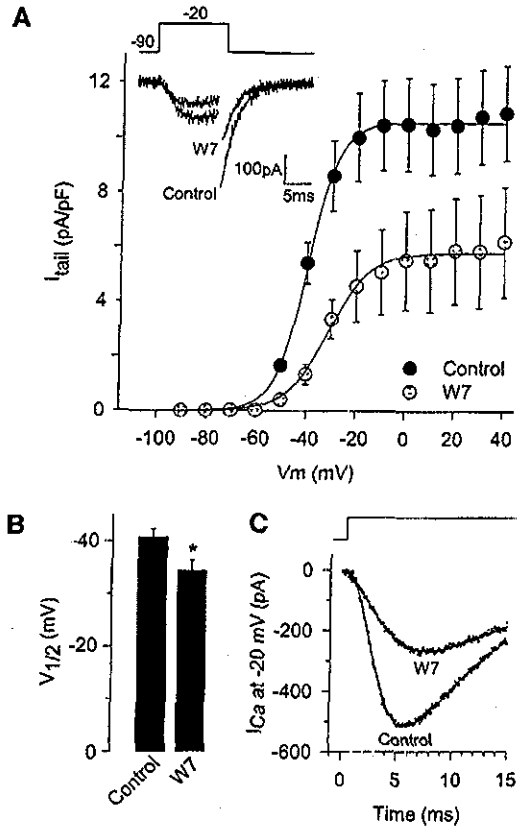

FIG. 3. W7 modulates T-current activation kinetics. (A) Control and inhibited tail currents in a number of cells were normalized by capacitance, averaged, and plotted against membrane potential. Fits of the curves were obtained using a Boltzmann function, $I_{Ca} = I_{max}/(1 + \exp[-(V_m - V_{1/2})/s])$, where V_m is the test potential, $V_{1/2}$ is the potential at which the current has reached half-maximal amplitude, and s is the range of potential for an e-fold change around $V_{1/2}$. Inset: Superimposed traces illustrating Ca^{2+} tail currents obtained before and after application of W7 (10 μM) by subjecting the cell to a depolarizing pulse from a HP of −90 mV to −20 mV for 14 ms and repolarizing the membrane to the HP. (B) Comparison of average $V_{1/2}$ derived from the Boltzmann fits (n = 6–11). (C) Time course of activation of T-currents in both the absence and the presence of W7 was examined in cells with uncompensated series resistance of <6 MΩ. Currents were activated by 20 ms (to −20 mV from a HP of −90 mV) with the best fit of the Hodgkin–Huxley equation (Hille, 1992) superimposed.

that W7 may be acting via CaM, given that this protein induces inactivation of HVA-Ca^{2+} channels. Current inactivation at a step voltage of −20 mV becomes progressively slower as a function of W7 concentration (Fig. 4D, open bars). Although the slowdown of T-channel inactivation caused by W7 should increase current magnitude, this CaM antagonist actually reduces it. We calculated the integral of the currents measured before and after W7 and, as illustrated in Fig. 4D (filled bars), the influx of Ca^{2+} was significantly smaller upon drug application.

Finally, to gain insight into the mechanisms by which CaM antagonists inhibit T-currents in spermatogenic cells, we studied the state-dependent inactivation of the channels. Patch-clamp whole-cell experiments suggested that inactivation could occur from closed states of the T-channel, given that long prepulses (ranging from −120 to −80 mV, voltages at which no channel opening was detectable) decreased current amplitude in response to a test pulse to −20 mV (compare currents at −120 and −80 in Fig. 5A). This difference became greater after the addition of W7. On average, W7 decreased currents to 80 ± 3, 78 ± 2, and 72 ± 5% of the control value after prepulses to −120, −100, and −80 mV, respectively (Fig. 5B).

Recent results indicate that functional T-channels do remain in mature sperm after testicular differentiation and that they play a key role in determining the AR (Arnoult *et al*., 1999). Inasmuch as CaM antagonists proved to be potent regulators of Ca^{2+} channel activity in spermatogenic cells, these agents could also affect the sperm AR. To test this, we first determined whether sperm T-channels were also inhibited by CaM antagonists. In sperm, ZP triggers a $[Ca^{2+}]_i$ transient that has the kinetic and pharmacological characteristics anticipated of a T-channel (Arnoult *et al*., 1999). An example of a rapid ZP-evoked $[Ca^{2+}]_i$ transient is shown in Fig. 6A. ZP-dependent $[Ca^{2+}]_i$ transients were observed in 65% of cells (22/34), with $[Ca^{2+}]_i$ rising to peak levels of 7.09 ± 1.1 μM and relaxing to basal levels within 250 ms. The rising phase of this response is described by a single exponential (τ = 15.2 ± 1.9 ms for 10–90% peak values). In contrast, buffer-treated controls exhibited only monotonic rises to 0.27 ± 0.05 μM during this time period. ZP also evoked $[Ca^{2+}]_i$ responses in 57% (12/21) of sperm treated with 10 μM W7. However, the peak $[Ca^{2+}]_i$ response was inhibited by 73% (2.21 ± 0.89 μM) and the activation time course was slowed (τ = 33.6 ± 4.1 ms). In contrast, 10 μM W5 had only minor effects on ZP-evoked $[Ca^{2+}]_i$ transients (Fig. 6B): responses were observed in 59% of cells (10/17), the maximal response was reduced by only 12% (6.21 ± 0.94 μM), and only a minor slowing of activation time was observed (τ = 17.7 ± 3.1 ms).

CaM antagonists (W7 and TFP) also significantly inhibited the ZP-induced AR in capacitated sperm with an IC_{50} value of ~10 μM (Fig. 7A). Notably, this concentration also inhibits ~50% of the total macroscopic peak current through T-channels in spermatogenic cells (see Fig. 2A). In addition, Fig. 7A shows that, as anticipated from the pharmacology of the T-currents (see Fig. 2A), W5 caused a less-important inhibition of the sperm AR. Furthermore, W7 appears to block AR by altering Ca^{2+} uptake through Ca^{2+} channels because A23187, a Ca^{2+} ionophore that triggers AR in the absence of ZP (Visconti *et al*., 1999), was able to overcome the inhibition caused by W7 (Fig. 7B).

It has been proposed that T-type Ca^{2+} in mouse spermatogenic cells is enhanced by inhibition of protein phosphorylation (Arnoul *et al*., 1997). Because CaM participates in some cell signaling events regulating protein kinases, we therefore thought it pertinent to investigate whether CaM

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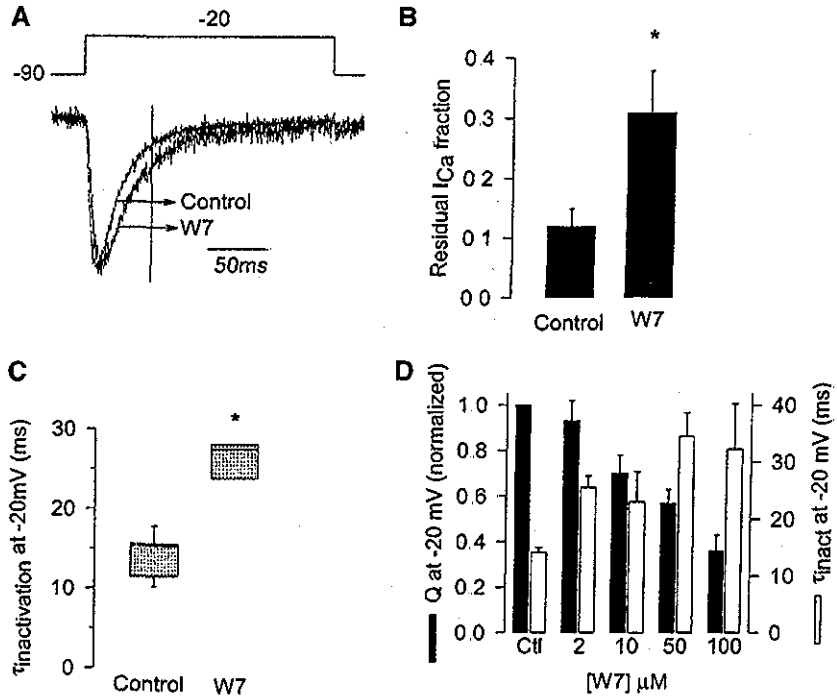

FIG. 4. W7 affects current inactivation. (A) Comparison of the time course of typical current traces in both the absence and the presence of W7 (10 μM). The voltage protocol used to measure inactivation is shown above, and the traces were normalized to allow kinetic comparison. (B) The percentage of current remaining after 80 ms (solid line in A) into a depolarizing pulse before and after W7 application is compared. Currents were recorded in response to 200-ms depolarizing pulses from −90 to −20 mV. (C) The inactivating phase of the currents was fitted with a single exponential: $I_{Ca} = A \exp(-t/\tau) + c$, where A is the initial amplitude, t is time, τ is the time constant for inactivation, and c is a constant. Values of τ of inactivating currents obtained at −20 mV before and after W7 treatment are compared (n = 6–11). (D) Integral of the current (Q) measured during 200-ms command steps to −20 mV from a HP of −90 mV. Currents measured at this command step after W7 application were normalized to the current observed before drug application in each cell (Ctl), averaged, and plotted as a function of W7 concentration (filled bars). Open bars compare τ of inactivation as a function of the concentration of W7 (n = 3–7).

might be involved in this mechanism of Ca^{2+} channel regulation. Whole-cell patch-clamp experiments indicated that micromolar concentrations of the CaM kinase inhibitors KN62 and H89 did not diminish T-currents in spermatogenic cells (not shown). Consistent with this, the sperm AR was not affected by these inhibitors (Fig. 7C). In addition the use of an inhibitory peptide derived from the CaM-dependent serine/threonine protein phosphatase calcineurin did not alter current density through T-type Ca^{2+} nor the time constant of inactivation (data not shown), suggesting that a phosphatase activity is not required for modulation of Ca^{2+} channels in spermatogenic cells.

DISCUSSION

Mammalian sperm must undergo the AR prior to fertilization. The signal transduction steps that lead to the sperm AR are not well understood. This exocytotic event, induced by the ZP surrounding the oocyte, depends on the uptake of external Ca^{2+} (Wassarman, 1999). Growing experimental evidence suggests that a voltage-gated Ca^{2+} channel is an important pathway responsible for modulating Ca^{2+} entry into sperm during this process (Darszon *et al.*, 1999; Arnoult *et al.*, 1996a, 1999). Previous electrophysiological studies of Ca^{2+} currents in spermatogenic cells demonstrated the presence of T-currents (Hagiwara and Kawa, 1984; Liévano *et al.*, 1996; Arnoult *et al.*, 1996a,b; Santi *et al.*, 1996) with pharmacological attributes consistent with those of the AR and the Ca^{2+} influx associated with it (Arnoult *et al.*, 1998). Likewise, it was shown that this channel is retained in the mature sperm (Arnoult *et al.*, 1999) and, therefore, it must be an important element in the transduction events leading to the ZP-induced AR in mammalian sperm (Arnoult *et al.*, 1996a, 1999; Darszon *et al.*, 1999).

Although the T-type Ca^{2+} currents of spermatogenic cells have been characterized, their regulation is poorly understood. Such information is needed to unveil the molecular mechanisms leading to the sperm AR. Recent findings indicate that CaM regulates HVA-channels, contributing to the determination of the intracellular levels of Ca^{2+} (Peter-

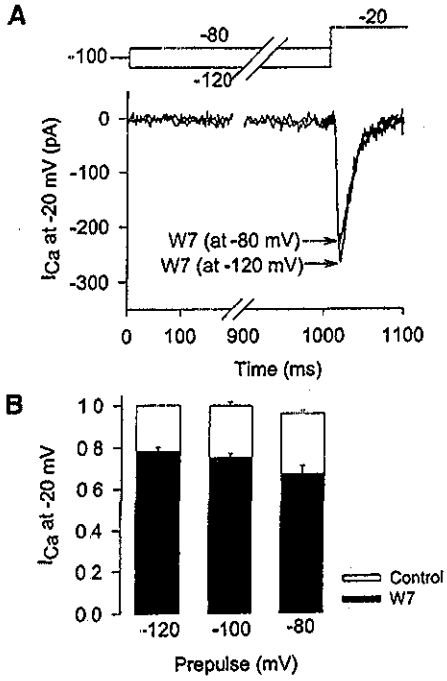

FIG. 5. W7 promotes T-channel inactivation from the closed state. (A) Voltage clamp traces recorded in a cell after application of W7 at two different potentials. Currents were evoked applying 1-s voltage prepulses to −120 and −80 mV followed by a 75-ms test pulse to −20 mV. The amount of inactivation after W7 addition at −80 mV exceeded that observed at −120 mV. (B) Inactivation measured from the test pulses is compared in different cells in both the absence and the presence of W7 during prepulses to −120 −100 and −80 mV (n = 5). Open bars represent the average currents in response to the test potential normalized to that evoked after application of the prepulse to −120 mV in the control cells. Filled bars denote current amplitude after addition of W7 normalized to that obtained before drug application.

son *et al.*, 1999; Qin *et al.* 1999; Zühlke *et al.* 1999; Lee *et al.*, 1999). Previous observations using CaM inhibitors in sea urchin (Sano, 1983; Guerrero and Darszon, 1989) and mammalian sperm (Courtot *et al.*, 1999) suggested the participation of CaM in the AR. With this in mind and considering the advantages of studying ion channels in spermatogenic cells, we explored the possible regulation of the T-type Ca^{2+} channels present in these cells by CaM. The results presented herein show that the specific CaM antagonist W7, at concentrations above 2 μM, markedly reduced T-current magnitude (Figs. 1–3). This effect is concentration dependent and essentially voltage independent (Figs. 1C and 1D). TFP, another CaM antagonist, also significantly reduced current amplitude at concentrations that inhibit CaM (Massom *et al.* 1990). Furthermore, addition of CaM inside the recording pipette decreased the inhibitory effect of W7, suggesting a potential participation of this protein in the regulation of T-type Ca^{2+} channels (Figs. 2A and 2B).

The results presented here are in line with the involvement of CaM in T-type Ca^{2+} channel regulation in spermatogenic cells; though, they are not a definitive demonstration. W7, W5, and TFP can have other less-specific effects such as direct inhibition of plasma membrane and intracellular Ca^{2+} channels and phospholipase C (Ehrlich *et al.*, 1988; Schlatterer and Schaloske, 1996; Schofl *et al.* 1999). However, the inhibitor concentrations used in this study are fitting for CaM inhibition and are lower than those associated with less-specific effects (Ichikawa *et al.*, 1991). In addition the Ca^{2+} dependence of the inhibitory effects of W7 (Fig. 2C) supports the role of a Ca^{2+}/CaM complex in T-channel regulation. Moreover, the direct effects of CaM inhibitors on ion channel activity have been shown to be partly or fully reversible (Laver *et al.*, 1997), whereas the actions of these compounds in our experiments resulted in a nonreversible inhibition (not shown), suggesting an indirect effect on the channels. Finally, when W7 and TFP act directly on ion channels, addition of CaM

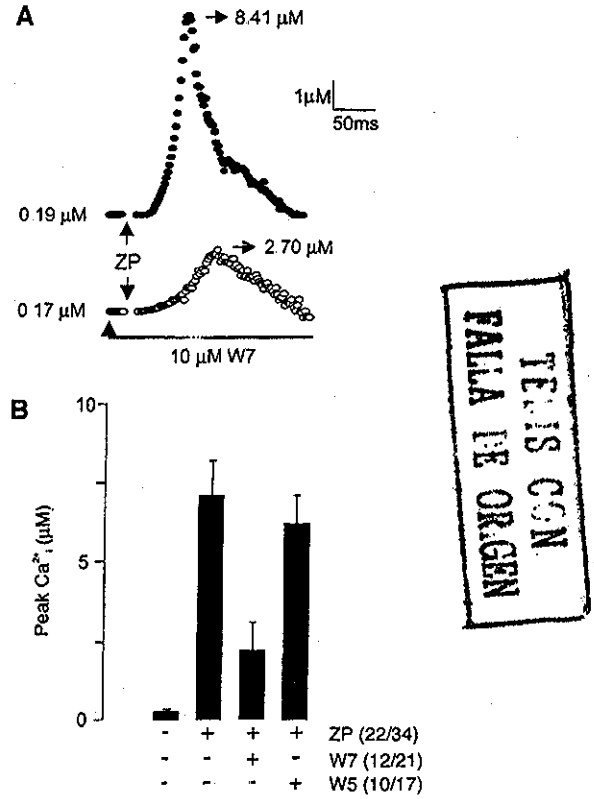

FIG. 6. W7 inhibits ZP-induced $[Ca^{2+}]_i$ transients in capacitated sperm. (A) Examples of $[Ca^{2+}]_i$ responses evoked by ZP (30 μg/ml) in sperm treated with buffer and with W7. Initial and peak $[Ca^{2+}]_i$ values are indicated. (B) Sperm were incubated for 10 min with buffer (n = 22), 10 μM W7 (n = 12), or 10 μM W5 (n = 10) prior to the addition of ZP. Data represent the peak $[Ca^{2+}]_i$ levels (± SD) during transients and the fraction of total cells exhibiting responses is shown.

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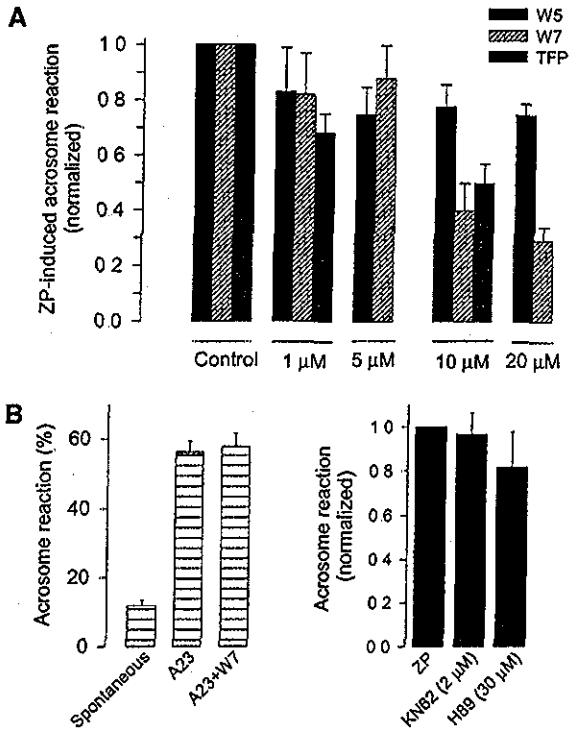

FIG. 7. CaM antagonists affect the sperm AR. (A) Effect of W7 on the percentage of sperm undergoing ZP-induced AR. Sperm were incubated in the presence of increasing concentrations of W7. After capacitation, the percentage of the AR was monitored. Data represent means ± SEM of at least five independent experiments. Sperm were also incubated in the presence of different concentrations of TFP (n = 6) and W5 (n = 7) as listed, and ZP-induced AR was measured. (B) AR inhibition caused by W7 is overcome by A23187. Samples were incubated for 35 min in the presence (15 μM) of the Ca^{2+} ionophore A23187 (A23) alone or in combination with W7 (n = 4). (C) CaM-dependent protein kinase inhibitors do not affect AR. The effects of KN62 and H89 on the mouse sperm AR are compared. Sperm were incubated in both the absence and the presence of these two protein kinase inhibitors at the indicated concentrations. Data represent means ± SEM of five separate determinations.

inside the recording pipette appears to be unable to reverse this interaction (Kleene, 1994). Altogether, these considerations lead us to believe that W7 and TFP influence T-channel function by inhibiting CaM.

It has been proposed that inactivation in HVA-channels involves Ca^{2+} entering through the pore, binding to CaM, and changing its conformation. The Ca^{2+}/CaM complex interacts with the channel, initiating an intramolecular process that culminates in a down-regulation of Ca^{2+} channel activity (Zühlke and Reuter, 1998; Peterson *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999; Lee *et al.*, 1999). In agreement with this, acute application of CaM antagonist W7 results in a partial slowdown of inactivation of the T-type Ca^{2+} channels in spermatogenic cells (Figs. 4C and 4D). However, more striking is the decrease in T-type current amplitude caused by CaM antagonists in these cells. Here we show that, in addition to decreasing current amplitude, submaximal concentrations of W7 drastically change other basic biophysical properties of the current (Figs. 3 and 5). These changes include a positive shift in the voltage dependence of activation and a significant slowing of the apparent activation kinetics. Because activation and inactivation are tightly coupled in T-channels (Serrano *et al.*, 1999), we suggest that the decrease in the rate of activation in the presence of W7 may indirectly slow down the apparent inactivation time course of the channels. In addition W7 appears to promote inactivation from voltages at which T-channels are not apparently activated (Figs. 5A and 5B). Whether CaM directly mediates this mechanism is still not clear.

In summary, the present study shows that CaM antagonists W7 and TFP inhibit with similar potency T-channels in spermatogenic cells monitored by electrophysiological methods and the ZP-induced AR. Notably, a similar concentration of W7 (10 μM) represses the Ca^{2+} transient triggered by ZP in mature sperm assessed by using a fluorescent probe. In contrast W5, a close analog of W7 but a much less potent CaM antagonist, is a poor inhibitor of the T-currents in spermatogenic cells and of the sperm AR and the Ca^{2+} transient associated with it. Furthermore, replacement of external Ca^{2+} by Ba^{2+} or inclusion of CaM inside the recording pipette decreases the inhibition of the T-currents in spermatogenic cells caused by W7. The overall results are consistent with a direct link between T-Ca^{2+} channel activation and the ZP-induced sperm AR, and suggest that CaM may regulate sperm T-channels. In mature sperm this is particularly important because during AR a delicate redistribution and modification of plasma membrane molecules takes place and intracellular Ca^{2+} dynamics are drastically changed (Darszon *et al.*, 1999; Wassarman, 1999; Flesch and Gadella, 2000). In addition this regulation may be important for sperm capacitation, where micromolar concentrations of CaM antagonists W7 and calmidazolium have been shown to significantly inhibit the percentage of capacitated sperm assessed by the chlortetracycline staining method (Si and Olds-Clarke, 2000) and by their ability to undergo AR in response to lysophosphatidylcholine.

ACKNOWLEDGMENTS

This work was supported by grants from CONACyT to A. D. and R.F.; DGAPA (UNAM), HHMI, and ICGEB to A.D.; and NIH (HD32177 and GM56479) to H.M.F. I.L.G. was the recipient of a CONACyT graduate fellowship. We thank Dr. F. Gómez-Lagunas for valuable discussions and commenting on the manuscript, and Dr. Arturo Hernández-Cruz in whose laboratory the initial experiments were performed.

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Received for publication March 15, 2001
Revised April 27, 2001
Accepted May 2, 2001
Published online June 27, 2001

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Dual regulation of the T-type Ca^{2+} current by serum albumin and β-estradiol in mammalian spermatogenic cells

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Received 9 May 2000

Edited by Maurice Montal

Abstract **This study provides evidence for a novel mechanism of voltage-gated Ca^{2+} channel regulation in mammalian spermatogenic cells by two agents that affect sperm capacitation and the acrosome reaction (AR). Patch-clamp experiments demonstrated that serum albumin induced an increase in Ca^{2+} T current density in a concentration-dependent manner, and significant shifts in the voltage dependence of both steady-state activation and inactivation of the channels. These actions were not related to the ability of albumin to remove cholesterol from the membrane. In contrast, β-estradiol significantly inhibited Ca^{2+} channel activity in a concentration-dependent and essentially voltage-independent fashion. In mature sperm this dual regulation may influence capacitation and/or the AR.** © **2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words I-Ca^{2+} channel; Spermatogenic cell; Serum albumin; Bovine serum albumin; β-Estradiol; Sperm capacitation

1. Introduction

Ca^{2+} channels in the mammalian sperm plasma membrane govern Ca^{2+} uptake and thus regulate intracellular signaling. Capacitation, a poorly understood maturational process, renders sperm responsive to physiological agents such as the zona pellucida or progesterone leading to the acrosome reaction (AR). This exocytotic event is required for successful fertilization. Capacitation, while occurring in vivo in the female reproductive tract, can also be accomplished in vitro by incubating sperm in defined medium containing appropriate concentrations of three key components: Ca^{2+}, $NaHCO_3$ and serum albumin [1,2]. In vitro studies have demonstrated that capacitation of mouse sperm is accompanied by metabolic alterations, a time-dependent and cAMP-regulated increase in the protein tyrosine phosphorylation of a subset of proteins [2,3], and changes in the distribution and composition of plasma membrane lipids and phospholipids. The requirement for serum albumin in capacitation has been related to its ability to remove cholesterol from the sperm membrane [4,5]. It has been proposed that cholesterol efflux then leads to changes in the membrane architecture and fluidity that give rise to the capacitated state [6,7]. In addition, the presence of a functional 29 kDa estrogen receptor on the human sperm surface has been recently documented. Its activation results in a rapid and sustained increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), dependent on the presence of extracellular Ca^{2+}, that causes increased tyrosine phosphorylation of sperm proteins and reduced progesterone-induced AR [8].

Extracellular Ca^{2+} appears also to be necessary for the completion of capacitation and for the increase of $[Ca^{2+}]_i$ required during capacitation [2]. In line with this, electrophysiological studies carried out in spermatogenic cells, a developmental precursor stage of mature sperm, have revealed that the voltage-gated T-type Ca^{2+} channel in the plasma membrane [9,10] is a major mediator of Ca^{2+} entry into these cells [11]. The pharmacology of T channels from spermatogenic cells resembles that of the Ca^{2+} entry mechanisms thought to be responsible for the AR in sperm [12]. These findings suggest that the Ca^{2+} T channels play an important role in the AR and could participate in the regulation of $[Ca^{2+}]_i$ during capacitation. Unfortunately, the size, complex geometry and highly differentiated and motile nature of sperm preclude its systematic electrophysiological characterization. In this report we used spermatogenic cells as a model system to study whether compounds that regulate capacitation and the AR are able to modulate T channels. Our results show that albumin and β-estradiol can regulate Ca^{2+} T channels in spermatogenic cells, and suggest that in mature sperm they may influence capacitation and the AR.

2. Materials and methods

2.1. Materials

Bovine albumin (essentially fatty acid-free prepared from bovine albumin fraction V), 17β-estradiol (1,3,5[10]-estratriene-3,17β-diol), and cholesterol 3-sulfate (sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

2.2. Cell preparation

Spermatogenic cells were obtained as described previously [13]. Briefly, testes from adult CD1 mice were excised and suspended in ice-cold dissociation solution. The tunica albuginea was removed and the seminiferous tubules were separated. Tissue fragments were extruded from a single tubule and dispersed into individual cells or

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PII: S0014-5793(00)01688-4

symplasts (aggregates), collected by centrifugation, resuspended in external recording solution (see below) without Ca^{2+} and stored on ice until assayed. Spermatogenic cells or symplasts at two different stages of differentiation (pachytene spermatocytes and round spermatids) were preferentially observed and used in electrophysiological recordings. Inasmuch as similar results were obtained from both stages, data were pooled for presentation.

2.3. Electrophysiology

Ca^{2+} currents were recorded using the whole-cell patch-clamp technique [14] at room temperature using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2–4 MΩ micropipettes. Cells were clamped at a holding potential (HP) of −90 mV and capacity transients were electronically compensated. Currents were evoked by 50–125 ms depolarizing voltage steps to test potentials ranging from −70 to +20 mV. To measure Ca^{2+} channel inactivation at steady state, cells were held for 210 ms at potentials ranging successively from −75 through −36 mV prior to a 90-ms step depolarization to a test potential of −25 mV. Linear leak and residual capacity currents were subtracted on-line using a P/4 standard protocol. Current records were captured on-line and digitized at a sampling rate of 5 kHz following filtering of the current record (2 kHz). Series resistance was compensated by ≥50%. Pulse protocols, data capture and analysis of recordings were performed using pCLAMP software (Axon). To isolate Ca^{2+} currents, cells were bathed in a solution containing (in mM): $CaCl_2$ 10; NaCl 130; KCl 3; $MgCl_2$ 2; $NaHCO_3$ 1; NaH_2PO_4 0.5; HEPES 5; glucose 10 (pH 7.3). The internal (patch pipette) solution consisted of (mM): CsCl 100; CsF 10; EGTA 5; HEPES 5; ATP-Mg_2 4; phosphocreatine 4 (pH 7.3). Bovine serum albumin (BSA) was diluted in the bath solution to give the desired final concentration. Cholesterol 3-sulfate and 17β-estradiol were prepared as 100 mM stocks in dimethylsulfoxide (DMSO) and stored at −20°C. A fresh aliquot was diluted to its final concentration in the bath solution for each experiment. Final concentration of DMSO typically was <0.001%.

3. Results and discussion

The possibility that serum albumin, a required component in media that supports capacitation, may modulate sperm Ca^{2+} channels has not been directly examined. Inasmuch as electrophysiological experiments are very difficult to perform in mature sperm, we used spermatogenic cells as a model system to determine the influence of albumin on their Ca^{2+}

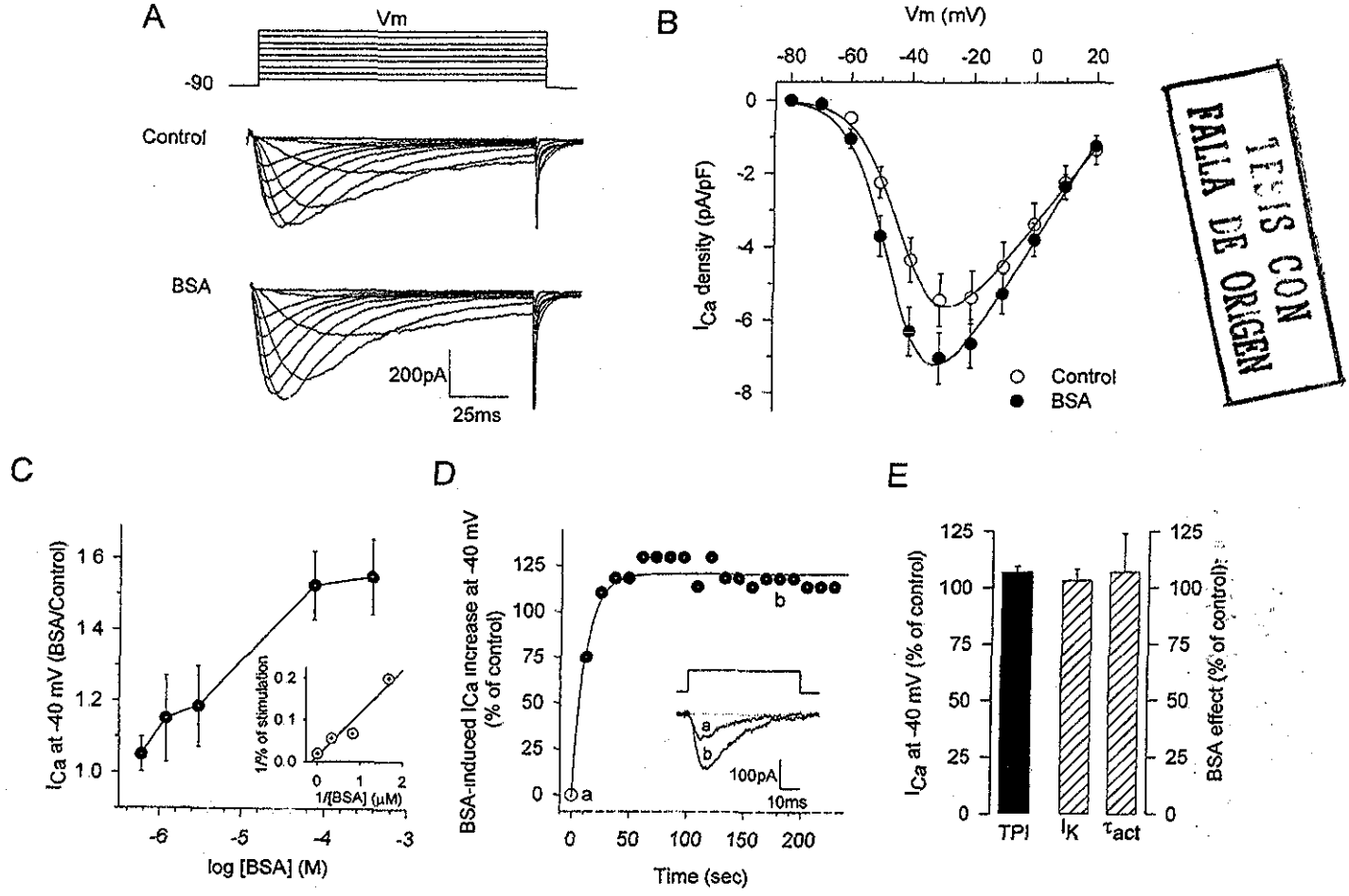

Fig. 1. BSA enhances Ca^{2+} current in mouse spermatogenic cells. A: Voltage-gated T currents (I_{Ca}) recorded, according to the protocol shown above, from a representative cell in the absence (upper traces) and in the presence of 0.5% BSA (lower traces). B: Current–voltage (I–V) relationships for steady-state activation of I_{Ca} before and after BSA application. Values represent mean ± S.E.M. from peak currents normalized by cell capacitance. C: Semilogarithmic plot of current density increase as a function of BSA concentration. Data were obtained in response to 100 ms depolarizations to −40 mV from a HP of −90 mV. Inset: Lineweaver–Burk plot derived from the BSA concentration–response data. D: Representative plot of peak current as a function of time. Data were approximated by an exponential function (solid line). Inset: Individual traces taken from the time course shown; letters designate the regions of the curves represented. E: BSA specifically increases T current in spermatogenic cells. Application of 75 μM triosephosphate isomerase (TPI) was not effective in increasing Ca^{2+} current amplitude. The solid bar corresponds to the summary of normalized peak current in cells subjected to TPI ($n=4$). Hatched bars summarize a comparison of current amplitude (I_K) and time constant of activation (τ_{act}) before and after BSA (75 μM) application ($n=4$). Currents were recorded in response to 200 ms depolarizing pulses from −90 to +50 mV.

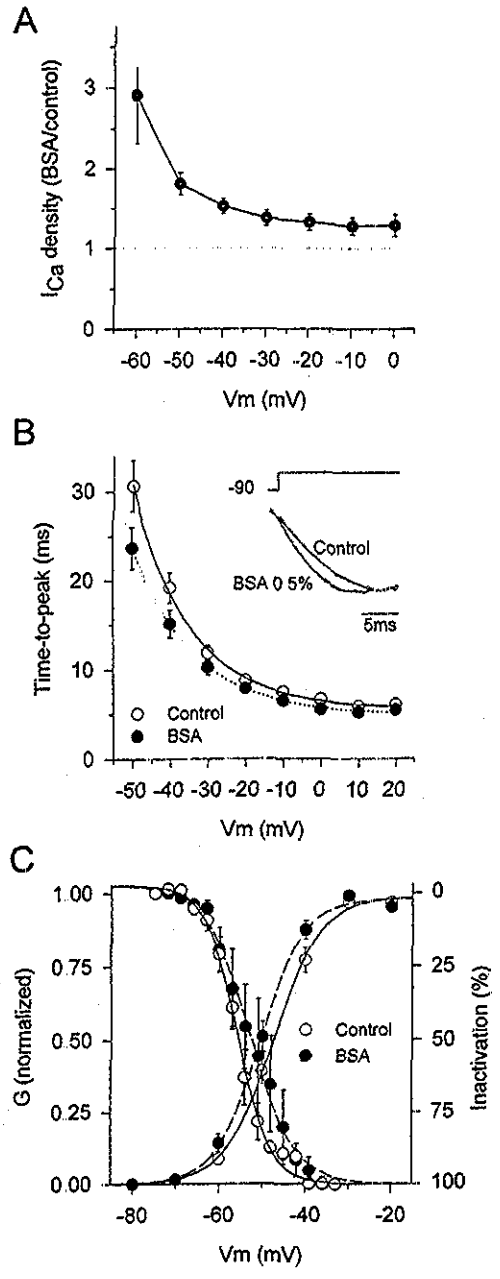

Fig. 2. Bovine serum albumin affects the kinetics of I currents in spermatogenic cells. A: Voltage dependence of the relative increase in current density induced by BSA. The ratios of current density after BSA application over the control at different voltages from the same cells in Fig. 1B are shown. B: Time-to-peak–voltage relationship for activation of I_{Ca}. Smooth lines correspond to exponential fits of data ($n = 11$). Inset: Example traces taken from a representative cell included in the time-to-peak–voltage relationship. Currents are shown normalized and only the first 15 ms are displayed to allow comparison of kinetics. The activating phase of the currents was fitted with single exponential equations (superimposed lines). C: BSA significantly increases the window current in spermatogenic cells. Comparison of steady-state activation and inactivation in control and BSA-treated cells. The fraction of inactivated channels was plotted as a function of prepulse voltage and data were fitted with Boltzmann equations (control, solid line; BSA-treated cells, dotted line). Symbols represent mean ± S.E.M. values of 3–5 cells in each condition. Conductance (G) was calculated from the Goldman–Hodgkin–Katz equation with an equilibrium potential for Ca^{2+} calculated using the MaxChelator (MAXC v5.0) software. Data were averaged and then fitted with Boltzmann equations (smooth curves). *Symbols represent mean ± S.E.M. values of 11 cells in each condition.*

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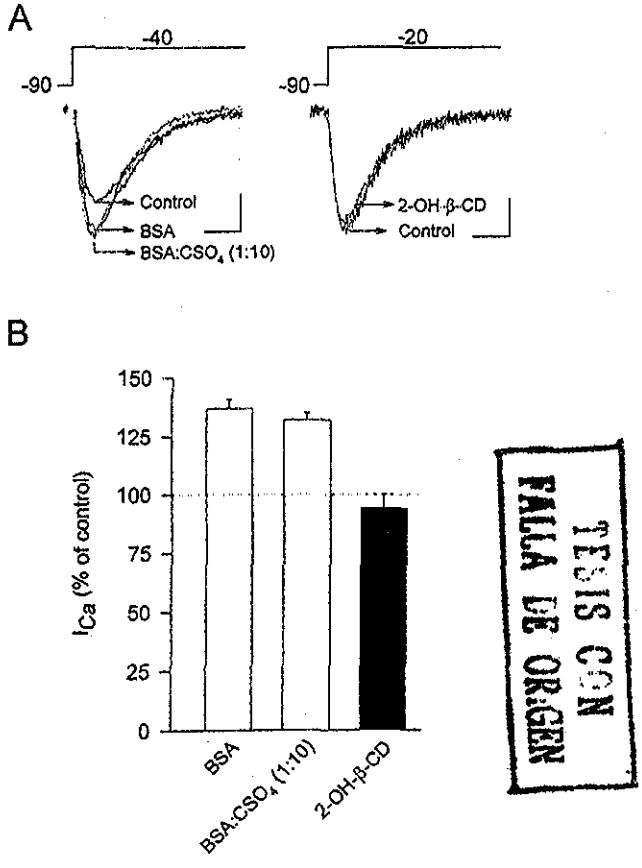

Fig. 3. The effects of BSA on I-channels in spermatogenic cells are not related to its ability to serve as cholesterol acceptor. A: Montage of current traces obtained first in the absence of BSA (control) and then treated with 15 μM BSA presaturated with cholesterol, followed by a third incubation in the presence of BSA alone. The scale bars represent 50 pA and 25 ms (left panel). The right panel shows representative superimposed current traces obtained before and after application of 500 μM of 2-OH-β-cyclodextrin (2-OH-β-CD). B: Histograms comparing the effect of a 2 min incubation with BSA alone, BSA presaturated with cholesterol sulfate (CSO_4), and 2-OH-β-CD on Ca^{2+} current amplitude ($n = 4$–6).

I currents. A pachytene symplast subjected to the patch-clamp technique showed typical Ca^{2+} currents (Fig. 1A) in response to 10 mV voltage steps from a HP of −90 mV before (upper traces) and 2 min after incubation with 75 μM of BSA (lower traces). Test pulses elicited a rapidly activating and inactivating inward current (I_{Ca}) whose amplitude was significantly increased in the BSA-treated cells. The magnitude of these currents was normalized by cell capacitance and the averaged results plotted as a function of membrane potential in Fig. 1B. Having established significant differences in I current density, we next determined the concentration dependence and time course of the BSA action. The effects of BSA were concentration-dependent with a maximal increment of I current occurring at 75 μM. This increase was normalized with respect to the control, averaged and plotted against the logarithm of concentration (Fig. 1C). A concentration–

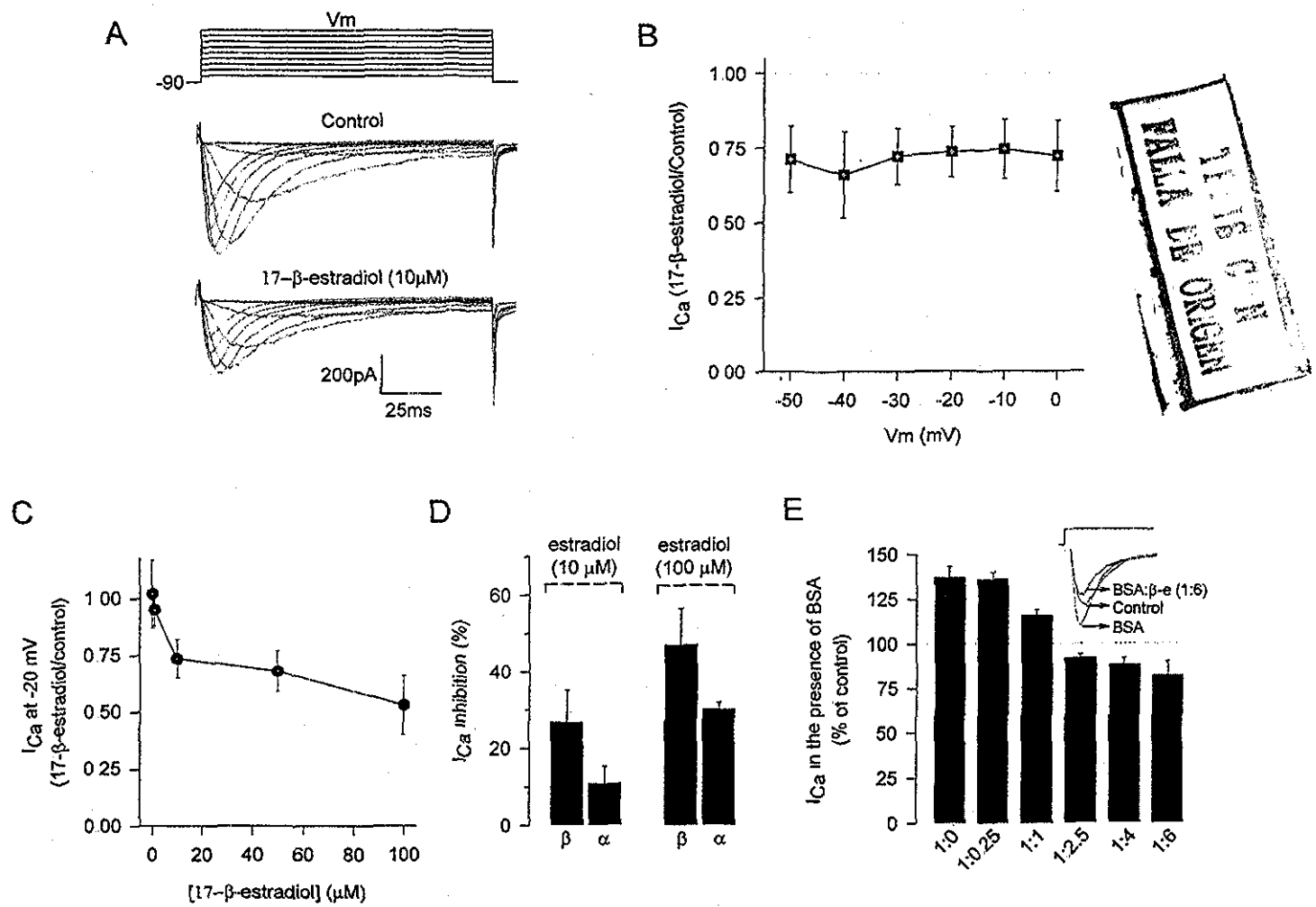

Fig. 4. 17β-Estradiol inhibits Ca^{2+} current in spermatogenic cells. A: I currents recorded in a representative cell before (upper panel) and after a 2 min incubation in 17β-estradiol. B: Peak currents after β-estradiol application at each test potential were averaged and normalized to control. C: Currents measured in response to 200 ms depolarizing pulses from a HP of −90 mV to a test potential of −20 mV before and after application of different concentrations of estradiol normalized to the control. Data represent mean ± S.E.M. of 3–8 experiments. D: Comparison of current inhibition after a 2 min incubation in the presence of β or α isomers ($n = 3$ and 8, respectively). Currents were evoked with depolarizing pulses from a HP of −90 to a test potential of −20 mV and the concentration of estradiol was 10 (left bars) or 100 μM (right bars). E: The ratio of current before and after presaturating BSA with various concentrations of 17β-estradiol is shown. Inset: Superimposed current traces evoked by a voltage step from −90 to −40 mV. 17β-Estradiol-presaturated BSA was added at a concentration of 15 μM. After incubation for 2 min, cells were treated with BSA alone. Bars denote pooled results showing the effect of BSA presaturated with increasing concentrations of 17β-estradiol on the T current.

response curve was generated (Fig. 1C, inset), where the reciprocal of the normalized average current in response to BSA was plotted against the reciprocal of the BSA concentration. Data were then fitted using a Lineweaver–Burk equation and a K_D of ~8 μM was calculated. Fig. 1D illustrates that BSA increased the Ca^{2+} T current within seconds of administration. Peak current was measured every 12 s in response to depolarizations from a HP of −90 mV to a test potential of −40 mV (inset), and the time course was approximated by a single exponential expression with $\tau \approx 11$ s. This modulation was specific since the use of a non-related protein such as triosephosphate isomerase did not significantly affect Ca^{2+} current amplitude (Fig. 1E, solid bar). Furthermore, control experiments demonstrated that a 2 min incubation with BSA had no effect on the magnitude and kinetics of whole-cell K^+ currents recorded in spermatogenic cells (Fig. 1E, hatched bars).

The enhancement of Ca^{2+} current by BSA was voltage-dependent. Fig. 2A compares current–voltage (I–V) curves obtained before and after BSA application and shows that albumin increased Ca^{2+} current density at negative voltages most markedly (from −60 to −40 mV). We next examined current activation kinetics. Records were obtained at different potentials and both the time-to-peak and time constant of activation were measured. In the control cells, time-to-peak decreased monotonically with voltage from 30.6 ± 2.8 ms at −50 mV to 6.1 ± 0.4 ms at +20 mV, reaching a voltage-independent minimum of about 6 ms at 0 mV. Treatment with BSA decreased the time-to-peak to 23.6 ± 2.3 and 5.5 ± 0.3 ms at −50 and +20 mV, respectively, with values statistically different in the voltage range below −30 mV (Fig. 2B). In addition, the time constant of activation after BSA decreased 1.3-fold on average with respect to the control at −40 mV. In these experiments currents were recorded in response to depolarizations from a HP of −90 mV to a test potential of −40 mV and the traces were fitted to single exponential equations

(Fig. 2B, inset) with time constants of 9 ± 1.1 and 6.9 ± 1.1 ms for the cells before and after BSA treatment, respectively.

Calculating whole-cell conductance and fitting the average normalized data with Boltzmann equations (Fig. 2C) we found that T currents after BSA treatment activated at lower test potentials ($V_{1/2} = -47.5$ in the control and -50.5 mV in the treated cells). In addition, inactivation was studied by applying 210 ms pulses followed by a test pulse to −25 mV, as described in Section 2, to measure channel availability. Average data were fitted to Boltzmann equations and comparison of the T current before and after BSA treatment indicated significant differences in the voltage-dependent inactivation at steady state ($V_{1/2} = -58.5$ versus -52.9 mV). Notably, there were voltages at which channels were activated during the prepulse but they were not completely inactivated, as evidenced by currents evoked during the test pulses. This activity is referred to as a window current and is typically illustrated by the overlap in the steady-state activation and inactivation curves (Fig. 2C). It can be clearly seen that T currents after treatment had a larger window region. At the peak of the window (~ −52 mV) 28 and 44% of the channels are available to be open in the control condition and after BSA administration, respectively.

To gain insight into the mechanisms underlying the albumin regulation of T channels, we next investigated whether this effect was a consequence of its well-known ability to serve as a cholesterol binding molecule. Fig. 3A (left panel) shows that the addition of BSA presaturated with a high concentration (1:10 molar ratio) of an analog of cholesterol (cholesterol-SO_4^-) or cholesterol (not shown) produced a significant increase in current amplitude (~ 1.3-fold) that was not modified by a second application of BSA alone. Neither cholesterol nor cholesterol-SO_4^- by itself modified T current properties (not shown). In addition, a 2 min incubation with 2-OH-β-cyclodextrin, a cyclic heptasaccharide able to promote a rapid cholesterol efflux from the sperm membrane and to induce an increase in tyrosine phosphorylation patterns similar to those seen in response to BSA during sperm capacitation [7], had no effect on the magnitude and kinetics of the T current (right panel). Together these results, summarized in Fig. 3B, provide evidence that BSA is not functioning here through its ability to remove cholesterol from the cell membrane.

Previous studies have shown that acute application of β-estradiol inhibits T-type channels in smooth muscle cells [17] as well as L-type channels in neurons [15], and cardiac and smooth muscle myocytes [18–22]. Thus it is possible that serum albumin could be enhancing T currents by removing a hydrophobic molecule such as β-estradiol. To test this hypothesis, spermatogenic cells were first directly superfused with increasing concentrations of 17β-estradiol and patch-clamp Ca^{2+} currents were recorded (Fig. 4A). As anticipated, currents in treated cells were smaller at all voltages tested (Fig. 4B). A small inhibition (~ 10%) was observed at nM concentrations and became significant at μM concentrations (Fig. 4C). This inhibition was voltage-independent (Fig. 4B) and too fast (< 2 min) to be attributed to genomic activation. Unlike what has been reported for high voltage-activated channels [15,16], this inhibition probably does not involve the activation of a G protein signaling pathway since spermatogenic T channels are basically G protein-independent [17]. A hormonally inactive isomer of 17β-estradiol (17α-estradiol) caused less reduction in T channel activity (Fig. 4D) indicating some specificity in the response. We next explored whether BSA stimulation could be antagonized by 17β-estradiol. As shown in Fig. 4E, BSA presaturated with 17β-estradiol did not increase peak current but induced a small reduction of current amplitude which was reversed by a second addition of BSA alone (inset). This finding is consistent with the hypothesis that the enhancement of T current caused by BSA might be due to the removal of 17β-estradiol rather than cholesterol from the plasma membrane. However, as shown in Fig. 4E, a progressive increase in 17β-estradiol inhibited T channel activity only at molar ratios > 1:2.5, raising the question whether this effect was caused by the presence of free 17β-estradiol. In addition, since BSA affects the T currents in a voltage-dependent manner while estradiol does not, it is likely that their effects are independent. However, these results suggest a potential physiological regulation of estrogens on spermatogenic cells. On the other hand, recently it was shown that 17β-estradiol can increase $[Ca^{2+}]_i$ by activating a novel functional estrogen receptor on the cell surface [8]. Our data suggest that this effect of estradiol is not mediated through T channels.

Mature sperm have only a single secretory vesicle (acrosome) and exocytosis must be coordinated with egg contact for efficient fertilization. Our results suggest that T-type Ca^{2+} channels, due to their window current, may contribute to setting $[Ca^{2+}]_i$ at the resting potential and therefore influence sperm capacitation. Since BSA increases the window current by modifying the voltage dependence of activation and inactivation, it could increase Ca^{2+} influx. As there is evidence that T channels are present in mature sperm [12] where resting potential is about −50 mV [23], BSA could facilitate an increase in Ca^{2+} entry, a prerequisite to capacitation. It is also possible that the actions of BSA on the T current may result from the removal of a hydrophobic molecule present in the sperm plasma membrane which suppresses premature secretion until the completion of capacitation, at which time sperm have arrived near the site of fertilization. The signaling mechanism by which 17β-estradiol reduces Ca^{2+} currents must await further investigation. Interestingly, numerous studies have shown that the function of membrane-spanning proteins can be affected by the material properties of the lipid bilayer through hydrophobic interactions between the protein and the bilayer [24]. Since 17β-estradiol is present in the sperm plasma membrane [6], the question arises as to whether changes in its concentration may be translated into an increase in the structural stress in the bilayer, and this force may be transmitted to sperm Ca^{2+} channels residing therein.

Acknowledgements: This work was supported by grants from DGAPA (UNAM), HHMI, ICGEB and CONACyT to A.D. F.E. was the recipient of a DGEP (UNAM) predoctoral fellowship. P.E.V. and G.S.K. were supported by NIH HD06274. We are grateful to *Dr. C.L. Treviño for critically reading the manuscript as well as to* J. Monroy for expert technical assistance.

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DEVELOPMENTAL GENETICS 25:103–114 (1999)

Anion Channel Blockers Differentially Affect T-Type Ca^{2+} Currents of Mouse Spermatogenic Cells, α1E Currents Expressed in *Xenopus* Oocytes and the Sperm Acrosome Reaction

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ABSTRACT The direct electrophysiological characterization of sperm Ca^{2+} channels has been precluded by their small size and flat shape. An alternative to study these channels is to use spermatogenic cells, the progenitors of sperm, which are larger and easier to patch-clamp. In mouse and rat, the only voltage-dependent Ca^{2+} currents displayed by these cells are of the T type. Because compounds that block these currents inhibit the zona pellucida-induced Ca^{2+} uptake and the sperm acrosome reaction (AR) at similar concentrations, it is likely that they are fundamental for this process. Recent single channel recordings in mouse sperm demonstrated the presence of a Cl^- channel. This channel and the zona pellucida (ZP)-induced AR were inhibited by niflumic acid (NA), an anion channel blocker [Espinosa *et al.* (1998): FEBS Lett. 426:47–51]. Because NA and other anion channel blockers modulate cationic channels as well, it became important to determine whether they affect the T-type Ca^{2+} currents of spermatogenic cells. These currents were blocked in a voltage-dependent manner by NA, 1,9-dideoxyforskolin (DDF), and 5-nitro-2-(3-phenylpropylamine)benzoic acid (NPPB). The IC_{50} values at −20 mV were 43 μM for NA, 28 μM for DDF and 15 μM for NPPB. Moreover, DDF partially inhibited the ZP-induced AR (40% at 1 μM) and NPPB displayed an IC_{50} value of 6 μM for this reaction. These results suggest that NA and DDF do not inhibit the ZP-induced AR by blocking T-type Ca^{2+} currents, while NPPB may do so. Interestingly, 200 μM NA was basically unable to inhibit α1E Ca^{2+} channels expressed in *Xenopus* oocytes, questioning that this α subunit codes for the T-type Ca^{2+} channels present in spermatogenic cells. Evidence for the presence of α1C, α1G, and α1H in mouse pachytene spermatocytes and in round and condensing spermatids is presented. Dev. Genet. 25:103–114, 1999. © 1999 Wiley-Liss, Inc.

Key words: anion channel blockers; Ca^{2+} channels; acrosome reaction

INTRODUCTION

The acrosome reaction (AR) is an exocytotic event triggered when sperm contact the extracellular layers of the oocyte. This reaction allows sperm to penetrate and fuse with the egg [Wassarman and Litscher, 1995]. Ionic fluxes are fundamental for the AR to occur, both in mammals and in marine invertebrates [reviewed in Schackmann, 1989; Florman *et al.*, 1998; Darszon *et al.*, 1999]. The AR requires an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), which depends on external Ca^{2+} [Florman *et al.*, 1998]. Ca^{2+} channel antagonists preclude the $[Ca^{2+}]_i$ increase and inhibit AR. These results demonstrate the fundamental participation of voltage-dependent Ca^{2+} channels (VDCCs) in the AR [Darszon *et al.*, 1999].

The electrophysiological characterization of mammalian sperm Ca^{2+} channels has been very difficult. Sperm are tiny morphologically complex cells, unable to synthesize proteins [Hetch, 1988]. Their progenitors, the spermatogenic cells, synthesize the ion channels that will end up in mature sperm. Pachytene spermatocytes and round and condensing spermatids are at the later stages of differentiation, translationally active, and much larger than sperm, making them easier to patch-clamp [Hagiwara and Kawa, 1984; Liévano *et al.*, 1996; Santi *et al.*, 1996; Arnoult *et al.*, 1996b]. Tran-

Contract grant sponsor: CONACyT; Contract grant sponsor: DGAPA; Contract grant sponsor: Howard Hughes Medical Institute; Contract grant sponsor: International Centre for Genetic Engineering and Biotechnology

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Received 13 January 1999; Accepted 31 March 1999

scripts for α1E, and to a minor extent α1A, subunits that code for the pore and voltage sensor of different voltage-activated Ca^{2+} channels, were found in pachytene spermatocytes and in round spermatids [Liévano *et al.*, 1996]. More recently, an α1C isoform was detected by reverse transcription (RT) in situ polymerase chain reaction (PCR) in spermatogenic cells [Goodwin *et al.*, 1998; Benoff, 1998]. Pachytene spermatocytes and round spermatids only display T-type Ca^{2+} channels [Santi *et al.*, 1996; Arnoult *et al.*, 1996]. The T-type Ca^{2+} currents of spermatogenic cells are blocked by Ni^{2+}, dihydropyridines, and amiloride, at concentrations that inhibit the zona pellucida (ZP)-induced sperm Ca^{2+} uptake and AR, which suggests that they play a key role in this process [Liévano *et al.*, 1996; Arnoult *et al.*, 1996]. Although T-type Ca^{2+} channels from brain and heart were recently cloned [Perez-Reyes *et al.*, 1998; Cribbs *et al.*, 1998], it is unclear which α1 subunit codes for T-type Ca^{2+} channels in spermatogenic cells and other tissues [Meir and Dolphin, 1998; Piedras-Rentería *et al.*, 1997; Piedras-Rentería and Tsien, 1998].

Cl^- channels have been implicated in the mammalian sperm AR [Wistrom and Meizel, 1993; Meizel, 1995]. The first single channel recordings in mouse sperm showed the presence of a niflumic acid (NA)-sensitive Cl^- channel [Espinosa *et al.*, 1998]. Interestingly, the zona pellucida (ZP) and γ-aminobutyric acid (GABA)-induced AR were inhibited by similar μM concentrations of NA, suggesting the participation of this anion channel in the reaction. However, NA and other anion channel blockers [White and Aylwin, 1990; Tilman *et al.*, 1991] have been shown to modulate diverse cationic channels both positively or negatively. For example, NA increases the open probability of single channel transitions [Ottolia and Toro, 1994], and the macroscopic currents [Gribkoff *et al.*, 1996] of heterologously expressed Ca^{2+}-dependent K^+ channels. By contrast, N-methyl-D-aspartate (NMDA) [Lerma and Martín del Rio, 1991] and nicotinic [Séguéla *et al.*, 1993] receptors are negatively modulated by NA. T-type Ca^{2+} channels could also be targets of this blockers. In this study, the anion channel antagonists, NA, dideoxyforskolin (DDF), and nitrophenylprolylamine benzoic acid (NPPB) were found to block the T-type Ca^{2+} currents of pachytene spermatocytes and the mouse sperm AR with different potencies. α1E channels expressed in *Xenopus* oocytes were basically insensitive to 200 μM NA. Evidence is presented for the presence of α1C, α1G, and α1H in spermatogenic cells.

MATERIALS AND METHODS

Materials

Essential and nonessential amino acids 1×, M-199, collagenase, bovine serum albumin (BSA), NA, and DDF, were from Sigma (St. Louis, MO). Trizol, Superscript preamplification system, *Taq* DNA polymerase, 1-kb DNA ladder from Gibco-BRL (Gaithersburg MD). Other reagents and salts were from the highest quality commercially available.

Spermatogenic Cell Dissociation and Purification

Testis from adult CD-1 mice were used as a source of germ cells. They were decapsulated, and the seminiferous tubules suspended in EKRB (in mM: 120.1 NaCl, 4.8 KCl, 25.2 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 11 glucose, 1 glutamine, 1× essential and nonessential amino acids, adjusted to pH 7.2 with CO_2). Spermatogenic cells for patch-clamp studies were obtained by mechanical dissociation. DNase (100 μg/ml) was added to the EKRB dissociation solution (the external recording solution but depleted of Ca^{2+}) immediately before the dissociation procedure. After discarding the *Tunica albuginea*, seminiferous tubules were separated using forceps under the stage of a stereoscopic microscope. Cells were extruded from the seminiferous tubules by holding one extreme of the tubule with forceps and "squeezing" the rest of the tubule in the opposite direction with another forceps. The cells were then pipetted repeatedly to separate them. The cells were dissociated and stored on ice. Spermatogenic cells remained healthy for ≥12 h. Spermatogenic cells for purification were obtained as described [Liévano *et al.*, 1996]. The resulting cell suspension was resuspended into EKRB 0.5% BSA and filtered through a 80-μm mesh Nytex nylon filter (Tetko, Lancaster NY), and stored at 4–10°C. The single-cell suspension of germ cells was sedimented at unit gravity through a 2–4% BSA linear gradient generated on a staput chamber (Johns Scientific, Ontario). Gradients were collected after 2.5 h in 10-ml fractions. Fractions were assessed for cell morphology and purity by light microscopy using Nomarski optics, and similar fractions were pooled.

Patch-clamp of Pachytene Spermatocytes

An aliquot of the mechanically dissociated spermatogenic cell suspension was placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon) with recording medium (in mM: 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-Hepes, 5 glucose, 10 $CaCl_2$, pH 7.35) at 20–23°C. All cells used in this study were at the pachytene stage of differentiation, either as single cells or as synplasts [see Santi *et al.*, 1996]. Whole-cell currents were recorded with an Axopatch 1-B amplifier in the voltage-clamp mode under the command of the pClamp program (Axon Instruments, Foster City, CA), connected to the pipette and the bath by Ag-AgCl wires. The composition of the pipette internal solution was (in mM: 110 Cs-methanesulfonate, 10 CsF, 15 CsCl, 5 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 10 Cs-Hepes, pH 7.35). Glass borosilicate pipettes were pulled to tip diameters of about 1.8 μm, with resistances of 2–3.5 MΩ, when filled with pipette solution. Records were low-pass

filtered at 2 kHz (4-pole Bessel filter), digitized at 10 kHz, and analyzed off-line. A P/4 pulse protocol was used routinely to minimize leak and capacitive currents from current records; series resistance was compensated by ≥50%. Anion channel blocker stock solutions (200 mM NA, 100 mM DDF and NPPB (ICN, Costa Mesa, CA), in dimethylsulfoxide (DMSO) were added to the recording chamber to reach the concentrations indicated Figures 1–5, without changing pH. For each cell tested, controls were recorded; the blocker was then added to the bath and 2–5 min later, the pulse protocol repeated and recorded. In some experiments, the blocker was washed with >50 times the chamber volume (~200 µl) and records obtained again. To achieve final DDF concentrations of 200 µM, it was necessary to sonicate the bath solution.

AR Inhibition Experiments

Sperm were obtained from CD-1 mice >3½ months killed by cervical dislocation. The cells were collected from excised epididymides and suspended (3.6×10^6/ml) in M-199 media supplemented with 25 mM $NaHCO_3$, 1 mM sodium pyruvate, and 0.4% BSA, were capacitated at 37°C for 30 min in a 5% CO_2 atmosphere. Thereafter, the heat-solubilized ZP was added and cells left to acrosome react for 30 min. Inhibition was studied with the same procedure except the blocker was added 5 min before the ZP. At the end of the experiment, AR was determined by established procedures [Boettger-Tong *et al.*, 1992]. A minimum of 100 sperm were counted under light-field microscopy. Results were normalized by substracting the spontaneous AR and considering the A-23187 induced AR as 1.

Expression of Recombinant α1E Ca^{2+} Channels

Stage V and VI oocytes were surgically removed from tricaine-anesthetized *Xenopus laevis* and treated for 1.5 h with 1.5 mg/ml collagenase in Ca-free ND-96 medium (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 Hepes, 2 pyruvic acid, pH 7.6). After recovering for 18–24 h, oocytes were injected in the nucleus with 10 nl of cDNA coding for α1E Ca^{2+} channel subunit [Soong *et al.*, 1993] at a concentration of 0.22 ng/nl (a gift from Dr. Snutch). Oocytes were incubated at 17°C for 3–5 days in ND-96 containing 1.8 mM $CaCl_2$ and 50 mg/ml gentamicin. Macroscopic currents were recorded at 16–18°C with a two-electrode voltage-clamp amplifier (Dagan CA-1; Dagan Corp.). Acquisition and data analysis were performed using pClamp (v6.0) software (Axon Instruments). Leak currents were subtracted using a P/4 protocol. Voltage and currents electrodes (0.3–2 MΩ tip resistance) were filled with 1 M KCl, 10 mM EGTA, and 10 mM HEPES (pH 7.4). Oocytes were placed in a 500-µl recording chamber and whole cell currents were measured with Ba^{2+} in the external solution (in mM: 10 $BaCl_2$, 1 KOH, 110 NaOH, 10 Hepes, titrated to pH 7.2, with methane sulfonic acid). To minimize kinetic contamination by endogenous Ca^{2+}-activated Cl^- currents, oocytes were injected with 50 nl of a BAPTA solution (in mM: 10 BAPTA, 10 Hepes, titrated to pH 7.2, with

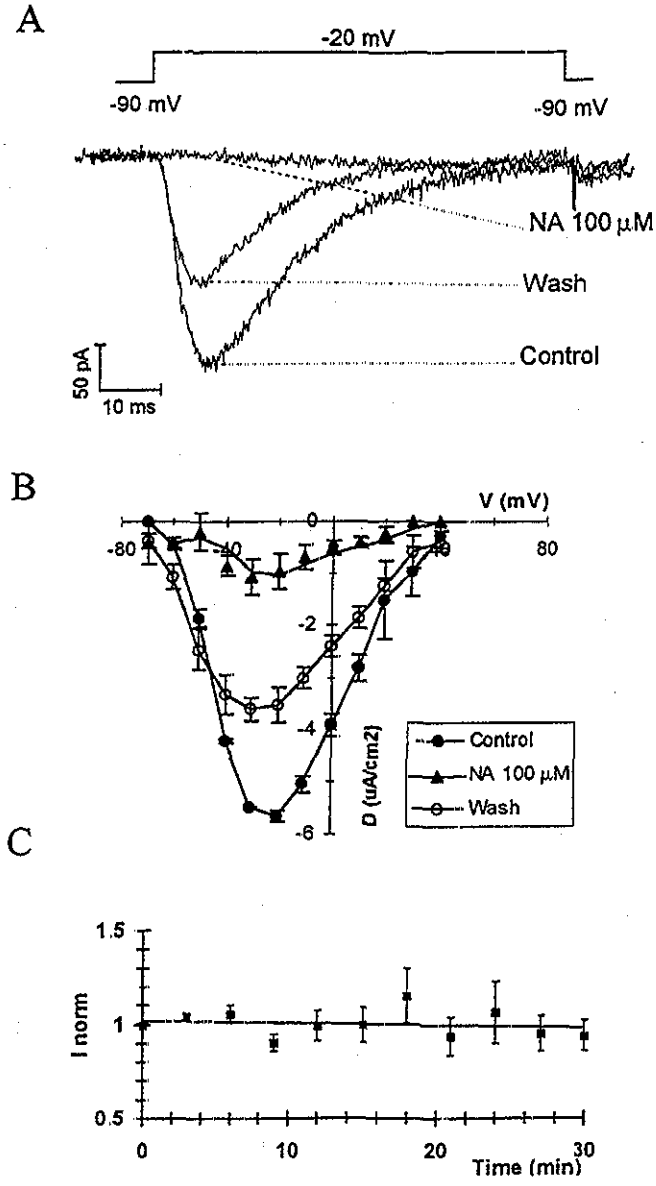


Fig. 1. Niflumic acid (NA) blocks T-type Ca^{2+} currents in spermatogenic cells. Mouse spermatogenic cells were mechanically obtained and whole-cell currents recorded in pachytene spermatocytes as described under Methods. **A:** Representative traces of currents obtained applying pulses to −20 mV from a holding potential of −90 mV in the absence (Control) and presence of 100 µM NA (NA) and after washing the blocker (Wash). **B:** I-V curves of control (*filled circles*), 100 µM NA (*closed triangles*) and recovery after blocker removal (*open circles*). Current density was obtained dividing the peak current at each voltage by the capacitance [Santi *et al.*, 1996]. **C:** Time stability of peak T-type Ca^{2+} currents at −20 mV. Results are averages ±SEM; n = 4

CsOH) 1–1.5 h before the experiment [Parent *et al*, 1997]. Voltage pulses were applied from a holding potential of −90 mV at 10-mV steps until +40 mV. Data were sampled at 10 kHz and filtered at 1 kHz.

For blockage experiments, the chamber solution was changed, superfusing with 14–40 times the chamber volume, with the 10 mM Ba^{2+} solution containing 200 μM NA or 20 μM nifedipine, and incubating 5–7 min before recording.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from seminiferous tubules and purified spermatogenic cells were extracted with Trizol reagent. Before each RT, 5 μg of each total RNA was dissolved in 10 μl water and digested with 1 U RNase-free DNase (RQ1; Promega, Madison, WI) in the transcription buffer (in mM: 50 KCl, 2.5 $MgCl_2$, Tris-Cl 20, pH 8.4) for 10 min at 37°C, and the DNase was heat-inactivated at 65°C for 15 min. At this point, random hexamers were added to a final concentration of 12.5 ng/μl, and quickly chilled on ice. RT was achieved with the Superscript pre-amplification system, according to the manufacturer's instructions. Similar results were obtained by priming the cDNA synthesis with oligo-dT_{12-18}. Control reactions were done in the same conditions, but omitting the RT enzyme. For PCR, we designed oligonucleotides against α1C, forward (5′-GCGAATTCAC-NGGNGARGAYTGGAAY) and reverse (5′-GCGG-ATCCCATYTCNGGYTCYTCYTCRTC), α1G, forward (5′-CGGGATCCAATAAGGACTGACTCCCTGGA) and reverse (5′-CCCAAGCTTGGTGGGCTGAGTTTITT-CTTA) and α1H, forward (5′-CGGGATCCGAYAAYTG-GAAYGGNATHATG) and reverse (5′-CGGAATTCNG-TYTCCATYTCNACYTCYTG) Ca^{2+} channel subunits. Amplifications were performed on a Mastercycler 5330 programmable thermal controller (Eppendorf) with *Taq* DNA polymerase. PCR reactions were carried out at a final volume of 50 μl, having 200 μM dNTP, 0.25 μM each primer, 1.5 mM $MgCl_2$. Mixtures were overlaid with mineral oil, and PCR was initiated by adding 2.5 U of *Taq* DNA polymerase per reaction. Program was for α1C 94°C 5 min, and cycling as follows, denaturation at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 2 min, during 35 cycles, final extension 72°C for 5 min; for α1G 94°C 5 min, and cycling as follows, denaturation at 94°C for 45 s, annealing at 55°C for 90 s, extension at 72°C for 1 min, during 40 cycles, final extension 72°C for 5 min and for α1H 94°C 5 min, and cycling as follows, denaturation at 94°C for 45 s, annealing at 43°C for 90 sec, extension at 72°C for 30 s, during 40 cycles, final extension 72°C for 5 min. Amplimer identity was obtained by double-strand sequencing of the cloned fragment into Bluescript KS^+, using the Termosequenase kit (Amersham), according to the supplier's instructions.

RESULTS

Anion Channel Blockers Inhibit T-Type Ca^{2+} Currents

Whole-cell recordings were obtained on mechanically dissociated mouse spermatogenic cells. As previously reported in rat [Hagiwara and Kawa, 1984] and mouse [Liévano *et al*, 1996; Santi *et al*, 1996; Arnoult *et al*, 1996], T-type currents were the only voltage-dependent Ca^{2+} currents these cells display. T-type Ca^{2+} currents are classified as low-voltage-activated Ca^{2+} currents with fast inactivation kinetics [Perez-Reyes *et al*, 1998a]. Accordingly, T-type Ca^{2+} currents were elicited upon ~125-ms depolarizations from a holding potential of −90 mV to potentials of −70 to +40 mV, using 10-mV increments. Peak currents were measured both before and after adding the anionic channel blocker. Figure 1A depicts T-type Ca^{2+} currents obtained by depolarizing to −20 mV before, after exposure to 100 μM NA, and posterior to washing the blocker. This concentration of NA completely blocked the Ca^{2+} current. Current recovery after washing was partial for the blockers tested. Figure 1B shows the complete I-V curves for the three conditions described above. Control currents at −20 mV are stable for at least 30 min (Fig. 1C), ruling out current washout as a source of current reduction.

Figure 2 shows concentration curves for T-type Ca^{2+} current blockade by NA, DDF, and NPPB. NA and DDF inhibited peak current (Imax) at −20 mV with IC50s of 43 μM and 28 μM, respectively (Fig. 2A,B). NPPB seems to have two different binding sites, I_{max} decreased reaching a plateau at 45% inhibition of 0.5–10 μM, higher concentrations increased blockage reaching 100% at 100 μM (Fig. 2C).

Figure 3 illustrates the voltage-dependent blockade by these anion channel blockers (ACBs). At low voltages, the T-type Ca^{2+} currents from spermatogenic cells are poorly blocked. More depolarized potentials enhance blockage. For example, at −50 mV, 50 μM DDF blocked 34% of the current, whereas at +10 mV, the block was 76%.

DDF and NPPB Inhibit the ZP-Induced Acrosome Reaction

It was previously reported that NA blocks a Cl^- channel (IC_{50} = 11 μM) and the ZP (IC_{50} = 1 μM) induced AR with similar affinities [Espinosa *et al*, 1998]. The sensitivity of T-type Ca^{2+} currents to ACBs suggests that their blockage could cause NA to inhibit the sperm AR. Therefore, it was important to compare the potency of these ACBs to inhibit the AR induced by ZP with their ability to block T-type Ca^{2+} channels.

DDF only partially inhibits the mouse sperm ZP-induced AR (Fig. 4A). At 1 μM, it inhibits ~45% of the AR, with higher concentrations apparently damaging the cells. By contrast, NPPB inhibited AR with an IC_{50} of ~6 μM (Fig. 4B). Thus, smaller concentrations of NA and DDF were required to inhibit the ZP-induced AR

than to block T-type Ca^{2+} currents from spermatogenic cells.

α1E Ba^{2+} Currents Expressed in *Xenopus* Oocytes Are Very Mildly Sensitive to Niflumic Acid

Although currently under active debate, the α1E gene has been considered one of the candidates to code for the T-type Ca^{2+} channel pore subunit [Soong *et al.*, 1993; Liévano *et al.*, 1996; Ertel and Ertel, 1997]. To test this proposal further, cDNA coding for the α1E Ca^{2+} channel subunit from rat brain was expressed in *X. laevis* oocytes. Figure 5 shows Ba^{2+} currents elicited by a depolarization from a holding potential of −90 mV to 10 mV both in the absence and in the presence of 200 μM NA. It turns out that α1E-expressed Ba^{2+} currents were basically insensitive to NA. Only a 7% blockage was seen with 200 μM of this compound, a concentration that totally inhibits T-type Ca^{2+} currents in spermatogenic cells. Furthermore, experiments done with 20 μM nifedipine, which completely blocks spermatogenic T-type Ca^{2+} currents [Liévano *et al.* 1996; Arnoult *et al.*, 1996, 1998], corroborated the insensitivity of α1E-expressed Ca^{2+} currents in *Xenopus* oocytes to this blocker [Soong *et al.*, 1993] (not shown). These results question the possibility that α1E codes for T-type Ca^{2+} channels in spermatogenic cells (see under Discussion)

α1C, α1G, and α1H Are Present in Spermatogenic Cells

The work by Liévano *et al.* [1996] on the α1 subunits present in spermatogenic cells was done with probes to the α1 subunits known at the time. Then, the only α1 subunit that, under certain conditions, displayed some similarities to T-type Ca^{2+} currents was α1E. Liévano and colleagues found that only α1E, and to a minor extent α1A, were present in spermatogenic cells. Recently, using RT in situ PCR, Goodwin *et al.* [1998] showed the presence of α1C in spermatogenic cells. In addition, Meir and Dolphin [1998] reported that α1C expressed in HEK293 cells produces L-type as well as T-type single channel transitions. Furthermore, two α1 subunits named G and H were recently cloned from rat brain and human heart respectively, which when expressed yield Ca^{2+} currents with T-type characteristics [Perez-Reyes *et al.*, 1998a; Cribbs *et al.*, 1998]. This findings and the results described in the previous section motivated our group to determine whether α1G and α1H were present in spermatogenic cells, and to use a more sensitive assay to detect α1C

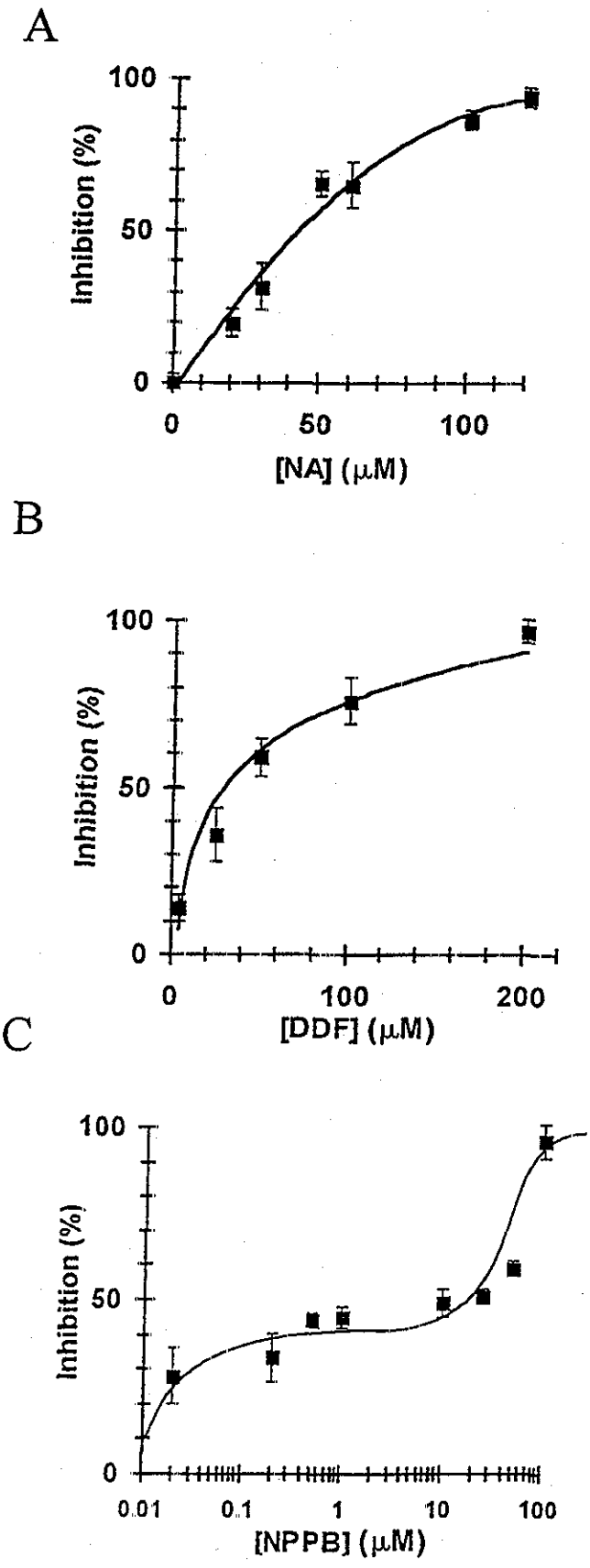
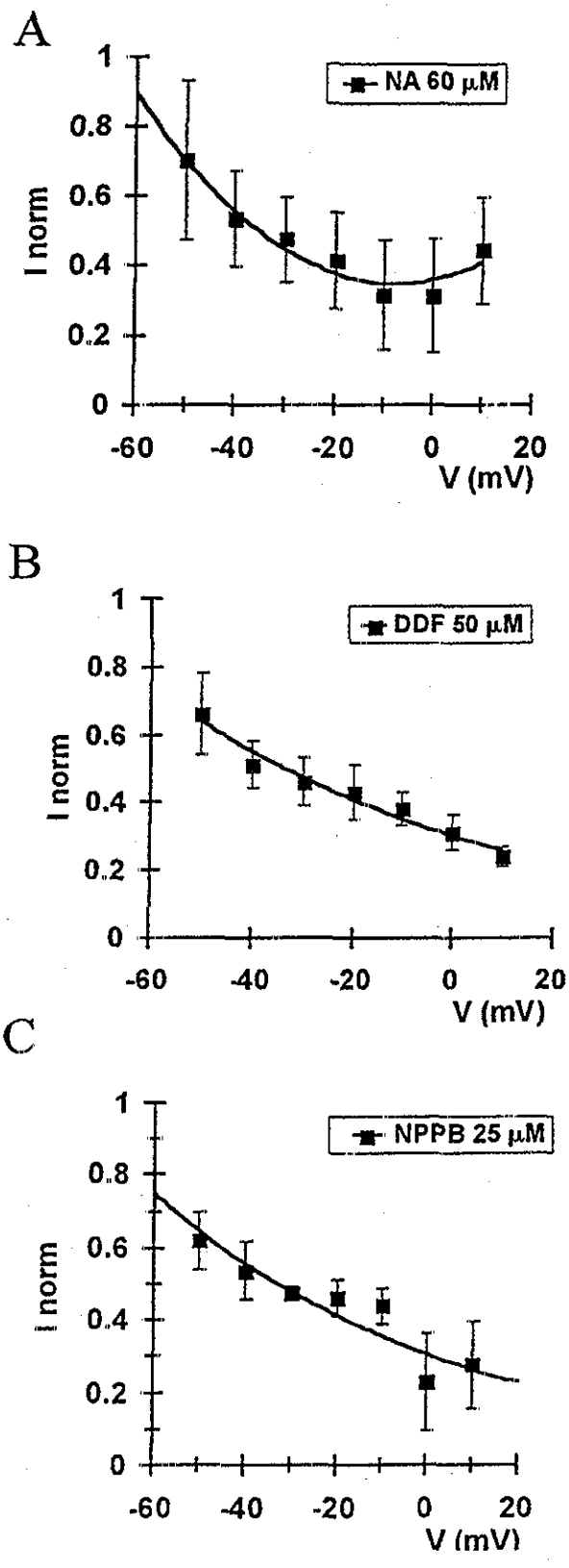


Fig. 2. Dose-response curves of peak T-type Ca^{2+} current blockade in individual pachytene spermatocytes or synplasts by anion channel blockers. Peak currents were normalized with respect to the control. IC_{50} values for niflumic acid (NA) (A) and dideoxyforskolin (DDF) (B) were 43 μM and 28 μM, respectively. Nitrophenylpropylamine benzoic acid (NPPB) (C) appears to have two different binding sites with apparent affinities of 25 nM and 75 μM. Results are averages ±SEM; n = 4.



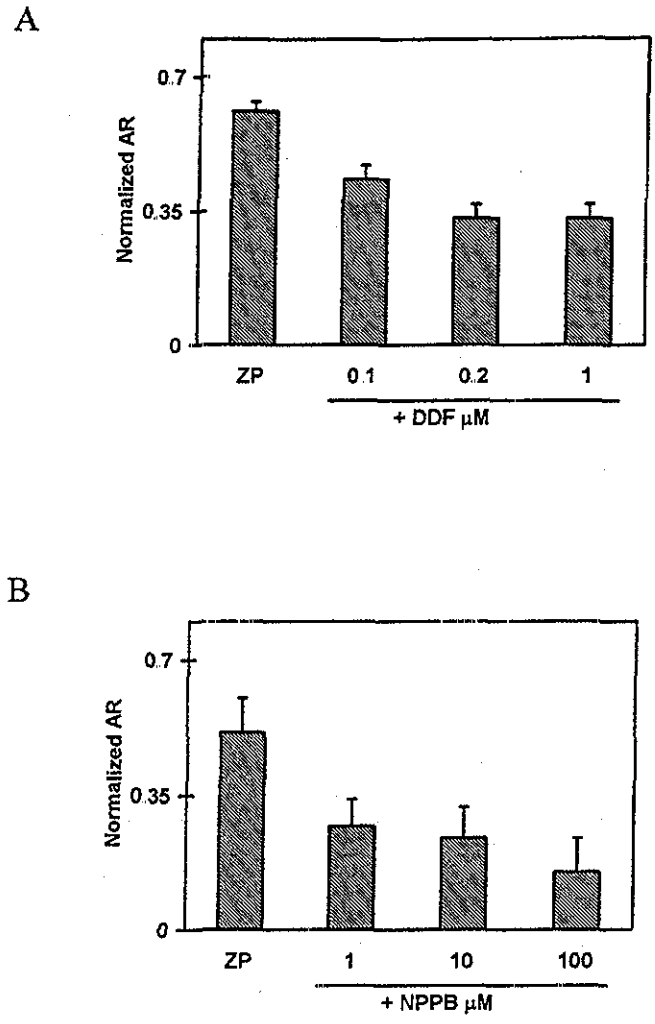
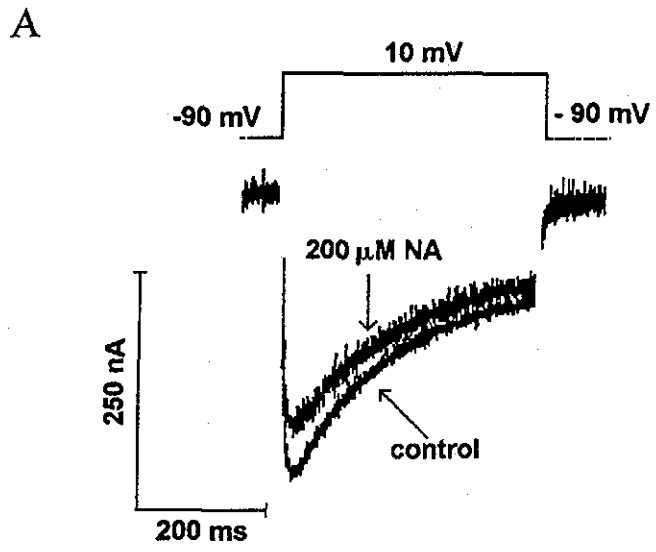


Fig. 4. Dideoxyforskolin (DDF) (A) and nitrophenylpropylamine benzoic acid (NPPB) (B) inhibit the mouse sperm acrosome reaction (AR) induced by solubilized zona pelucida (5 ZP/µl) Acrosome reaction (AR) and its inhibition were determined as indicated under Methods Bars = mean ±SEM; n ≥ 3 Results were normalized considering the A-23187 induced AR as 1 and the spontaneous AR substracted

Figure 6 illustrates the electrophoretic pattern of the amplimers obtained in the RT-PCR experiments using oligonucleotides for α1G. It was possible to amplify a fragment of the expected size in mouse brain, testis, pachytene spermatocytes, and round and condensing spermatids. The amplimer identity was confirmed by double-strand sequencing. Similarly, amplimers of the

Fig. 3. Blockage by (A) niflumic acid NA (60 µM), (B) dideoxyforskolin DDF (50 µM), and (C) nitrophenylpropylamine benzoic acid NPPB (25 µM) is voltage dependent Results are the averages of the normalized peak T-type Ca^{2+} currents of individual pachytene spermatocytes or synplasts as a function of applied voltage Results are averages ±SEM; n = 4



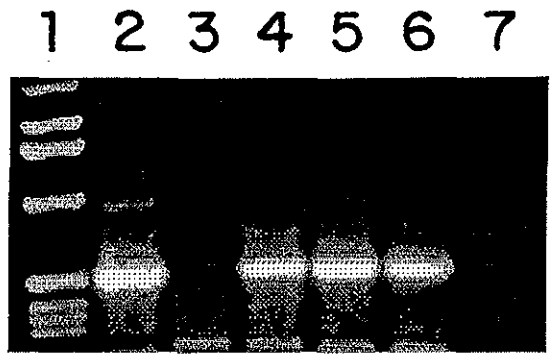


Fig. 6. RT-PCR experiments showing the expression of Ca^{2+} channel α1G subunit in brain (*lane 2*), testis (*lane 3*), pachytene spermatocytes (*lane 4*), round (*lane 5*) and condensing (*lane 6*) spermatids and the reaction without cDNA (*lane 7*); 1-kb DNA ladder (*lane 1*). Amplicon sizes were ~460 bp.

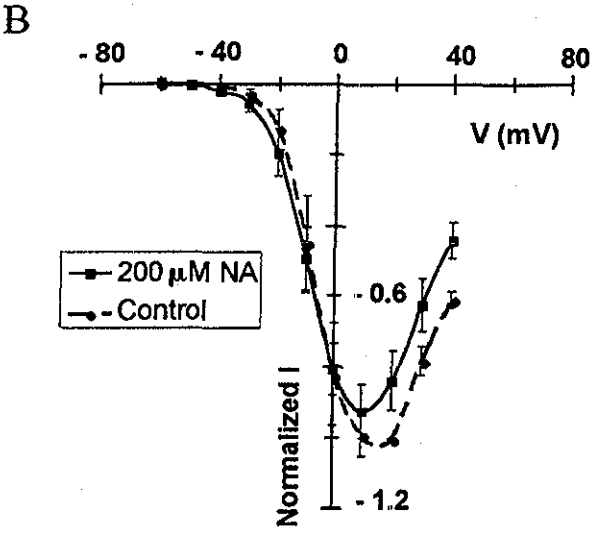


Fig. 5. Expression of α1E in *Xenopus* oocytes yields macroscopic Ba^{2+} currents basically insensitive to niflumic acid (NA). Oocytes were nuclear injected with ~2 ng cDNA coding for the α1E subunit. **A:** Representative current traces evoked at test pulses of +10 mV (pulse protocol shown above), before (control) and after exposing the oocyte to 200 μM NA. **B:** I-V curves for each cell before (-●-) or (---) and after exposure to 200 μM NA (-■-) or (---) were normalized to the current evoked at +20 mV and then averaged. Error bars = SEM; n = 5

expected size were obtained when oligonucleotides for α1H were used (not shown); double-strand sequencing confirmed the identity of the fragment. Control reactions in which the RT enzyme was omitted in the cDNA synthesis yielded no product. These experiments show that α1G and α1H are expressed during the last stages of spermatogenesis. Figure 7 shows an alignment of the DNA sequences obtained during this work for α1G (SP α1G) and α1H (SP α1H) and those reported by Perez-Reyes [1998a] (RB α1G) and Cribbs [1998] (MM α1H), respectively. The deduced amino acid sequence for SP α1G and for SP α1H have a 97% and 99% identity with RB α1G and MM α1H, respectively.

To test again for the presence of α1C with a more sensitive assay, RT-PCR analysis was performed with a different set of oligonucleotides than previously used [Liévano *et al.*, 1996]. A single band of the expected size was obtained for mouse brain cDNA. This amplimer was sequenced and determined to be a fragment from α1C. For testis and spermatogenic cells only a smear around the expected size was observed. These smears were blotted and probed with the brain ^{32}P-labeled amplimer [Sambrook *et al.*, 1989]. A sharp signal was obtained in testis and spermatogenic cells, indicating the expression, although low, of α1C in these cells (not shown).

DISCUSSION

Micromolar NA was found to inhibit the ZP-induced AR and single anion channel transitions in mouse sperm [Espinosa *et al.*, 1998]. NA and other compounds, such as NPPB and DDF, are considered blockers of anion channels. They block Ca^{2+}-activated Cl^- channels [Baron *et al.*, 1991; Cotton *et al.*, 1997; Lamb *et al.*, 1994], the CFTR Cl^- channel [Walsh and Wang, 1993; Doughty *et al.*, 1998], osmotically regulated Cl^- channels [Diener *et al.*, 1996], and an inward rectifying Cl^- channel studied in *Caenorhabditis elegans* spermatocytes [Machaca *et al.*, 1996]. However, these compounds also affect some cation channels, such as the NMDA-R [Lerma and Martín del Rio, 1991], nonselective cation channels [Gögelein *et al.*, 1990], and voltage dependent Ca^{2+} channels (VDCCs) [Walsh and Wang, 1993; Doughty *et al.*, 1998]. In addition, NA and NPPB can increase macroscopic currents of K^+ channels expressed in *Xenopus* oocytes [Gribkoff *et al.*, 1996] and

A

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SP α1G          1 CAATAAGGACTGACTCCCTGGACGTGCAGGGCCTGGGTAGCCGGGAAGAC 50
                  |||||||||||||||||||||||||| ||||||||||||||||||||||||||
RB α1G       6520 caataaggactgactccctggatgtgcagggcctgggtagccgggaagac 6569

SP α1G         51 CTGTTGTCAGAGGTGAGTGGGCCCTCCTGCCCTCTGACCCGCTCCTCATC 100
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
RB α1G       6570 ctgttgtcagaggtgagtgggccctcctgccctctgacccggtcctcatc 6619

SP α1G        101 CTTCTGGGGCGGGTCGAGCATCCAGGTGCAGCAGCGCTCCGGCAGCCAGA 150
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
RB α1G       6620 cttctggggcgggtcgagcatccaggtgcagcagcgttccggcatccaga 6669

SP α1G        151 GCAAAGTCTCCAAGCACATCCGCCTGCCAGCCCCTTGCCCAGGCCTGGAA 200
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
RB α1G       6670 gcaaagtctccaagcacatccgcctgccagccccttgcccaggcctggaa 6719

SP α1G        201 CCCAGCTGGGCCAAGGACCCTCAAGAGACCAGAAGCAGCTTAGAGCTGGA 250
                  |||||||||||||||||||||| ||||||||||||||||||||||||||||
RB α1G       6720 cccagctgggccaaggaccctccagagaccagaagcagcttagagctgga 6769

SP α1G        251 CACGGAGCTGAGCTGGATTTCAGGAGACCTCCTGCCCAGCAGTCAGGAAG 300
                  |||||||||||||||||||||||||||||||||| |||||||| |||||||
RB α1G       6770 cacggagctgagctggatttcaggagacctccttcccagcagccaggaag 6819

SP α1G        301 AACCCCTGTCCCCACGGGACTTGAAAAAATGCTACAGTGTAGAGGCCCAG 350
                  |||||||||| |||||||||| |||| || ||||||||||||||| |||||
RB α1G       6820 aaccccctgttcccacgggacctgaagaagtgctacagtgtagagacccag 6869

SP α1G        351 AGCTGCCGGCGCAGGCCTGGGTCCTGGCTAGACGAACAGAGGAGACACTC 400
                  |||||| |||||||||||||||| |||||||| |||||| ||||||||||
RB α1G       6870 agctgcaggcgcaggcctgggttctggctagatgaacagcggagacactc 6919

SP α1G        401 CATCGCTGTCAGCTGCCTGGACAGCGGCTCCCAGCCCCGCCTATGTCCAA 450
                  ||| |||||||||||| |||||||||||||||| ||||||||||||||||
RB α1G       6920 cattgctgtcagctgtctggacagcggctcccaaccccgcctatgtccaa 6969

SP α1G        451 GCCCCTCAAGCCTCGGGGGCCAACCTCTTGGGGGCCCTGGGAGCCGGCCT 500
                  |||||||||||||||||||||||||||||||||||| ||||||||||||||
RB α1G       6970 gcccctcaagcctcgggggccaacctcttgggggtcctgggagccggcct 7019

SP α1G        501 AAGAAAAAACTCAGCCCACC 520
                  ||||||||||||||||||||
RB α1G       7020 aagaaaaaactcagcccacc 7039
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B

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SP α1H          1 GAAGGATACTCTCCGTGAGTGTACCCGTGAAGACAAGCACTGCCTCAGCT 50
                  |||||||||||||||||| ||||||||||||||||||||||||||||||||
MM α1H         10 gaaggatactctccgtgaatgtacccgtgaagacaagcactgcctcagct 59

SP α1H         51 ACCTGCCCGCGCTCTCCCCCGTCTACTTCGTCACCTTCGTGCTGGTCGCC 100
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H         60 acctgcccgcgctctcccccgtctacttcgtcaccttcgtgctggtcgcc 109

SP α1H        101 CAGTTCGTGCTGGTCAACGTGGTGGTGGCCGTGCTCATGAAGCACCTGGA 150
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H        110 cagttcgtgctggtcaacgtggtggtggccgtgctcatgaagcacctgga 159

SP α1H        151 GGAGAGCAACAAGGAGGCCCGGGAGGATGCGGAGATGGATGCCGAGATCG 200
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H        160 ggagagcaacaaggaggcccgggaggatgcggagatggatgccgagatcg 209

SP α1H        201 AGCTGGAGATCGCACAGGGGTCCACAGCCCAGCCCCCGTCCACAGCACAG 249
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H        210 agctggagatcgcacaggggtccacagcccagccccgtccacagcacag 259

SP α1H        250 GAAAGCCAAGGTACCGATCCAGACACCCCGAACCTCCTGGTCGTGCGCAA 299
                  |||||||||||||||||| ||||||||||||||||||||||||||||||||
MM α1H        260 gaaagccaaggtaccgagccagacaccccgaacctcctggtcgtgcgcaa 309

SP α1H        300 AGTATCTGTGTCCAGGATGCTCTCGCTACCCAATGACAGCTACATGTTCC 349
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||
MM α1H         10 agtatctgtgtccaggatgctctcgctacccaatgacagctacatgttcc 359

SP α1H        350 GG 351
                  ||
MM α1H        360 gg 361
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Fig. 7. Alignment of the nucleotide sequence. **A:** Clone obtained from spermatogenic cells for α1G (SP α1G) compared with the rat brain α1G (RB α1G) previously described by Perez-Reyes *et al.* [1998a], Genbank accession No. AF027984. **B:** Clone obtained from spermatogenic cells for α1H (SP α1H) with *Mus musculus* T-type Ca^{2+} channel α1 subunit mRNA, partial cDNAs (MM α1H), described by Cribbs *et al.* [1998], Genbank accession No. AF051947. Sequence identity is indicated by a *vertical line* between the sequences. The nucleotide sequences showed a 96.34% and 99.7% identity between the α1G and the α1H respectively.

the open probability of $_{Ca}K^+$ channels reconstituted in planar bilayers [Ottolia and Toro, 1994] The neuronal $GABA_A$-R, itself a channel, is potentiated at low NA concentrations and inhibited at high concentrations [Woodward *et al*, 1994].

The only type of voltage-dependent Ca^{2+} currents found in mouse spermatogenic cells are of the T-type [Liévano *et al*, 1996; Santi *et al*, 1996; Arnoult *et al*, 1996] These channels are probably still present in mature sperm, as they are unable to synthesize proteins. The T-type Ca^{2+} channels from spermatogenic cells and the ZP-induced sperm Ca^{2+} uptake and AR are inhibited by similar concentrations of dihydropyridines and other blockers. These observations strongly suggest that T-type Ca^{2+} channels play a fundamental role in the sperm AR [Arnoult *et al*, 1996, 1998; Liévano *et al*, 1996].

Because NA inhibited the mouse sperm AR, it seemed important to determine whether T-type Ca^{2+} currents from spermatogenic cells were sensitive to this blocker. NPPB and DDF were also tested. The three compounds blocked these Ca^{2+} currents with IC_{50} values of: NA 43 μM and DDF 28 μM and, as shown in Fig. 2C, NPPB appears to have two distinct binding sites with approximate affinities of 25 nM and 75 μM. By contrast, the ZP-induced sperm AR was more sensitive to these blockers, with IC_{50} values of: NA 1 μM [Espinosa *et al*, 1998], DDF 1 μM, and NPPB 6 μM (Fig. 4). Considering that, until now, blockers of the T-type Ca^{2+} currents from spermatogenic cells have been shown to have very similar potencies to inhibit the sperm AR and its associated Ca^{2+} uptake [Arnoult *et al*, 1996], these results suggest that it is unlikely that T-type Ca^{2+} channels are involved in the inhibition of the ZP-induced AR caused by NA and DDF and suggest that other channels, possibly anionic, may influence this reaction. From this point of view, only in the case of the high affinity binding site for NPPB could T-type Ca^{2+} channel blockade contribute to AR inhibition. Furthermore, at the resting membrane potential of capacitated sperm (~−55 mV or less) [Florman *et al*, 1998], the voltage dependence of the blockage by Anion channel blockers (ACBs) (more potent at depolarized potentials; Fig 3) would even decrease their efficacy to inhibit T-type Ca^{2+} currents. However, there is still the possibility that in contrast to the T-type Ca^{2+} channel blockers described so far, ACBs could behave differently in spermatogenic cells and in mature sperm, complicating the comparison between the IC_{50} values of channel blockage and the AR.

Several reports suggest a direct interaction of these ACBs with either anion or cation channels [Gögelein *et al*, 1990; Oba *et al*, 1997; Ottolia and Toro, 1994; Tilman *et al*, 1993; White and Aylwin, 1991]. The binding site could be within the pore [Evoniuk and Skolnick, 1988] and possibly reached only from one side of the membrane [Tilman *et al*, 1993]. In order to feel the voltage, the blocker binding site must be inside the membrane [Hille, 1992] Thus, these compounds may directly interact within the pore of T-type channels NPPB could be acting elsewhere since it displays two binding sites

Although there is no precedent, instead of directly binding to the pore of T-type Ca^{2+} channels, these blockers could interact with channel accessory subunits. Alternatively, the ACBs may exert an indirect effect over T-type Ca^{2+} currents. For example, some models suggest the direct interaction between cation and anion channels [Kanli and Norderhus, 1998; Sonders and Amara, 1996]. So far, no direct interaction between Cl^- and VDCCs has been documented, making it unlikely that the inhibition of T-type Ca^{2+} currents of spermatogenic cells by ACBs is due to Cl^- channel inhibition

ACBs are membrane permeable and could also affect enzymatic pathways that may influence ion channels function. ACBs used in this work also inhibit cycloxygenases and lipoxygenases that participate in arachidonic acid metabolism [Civelli *et al*, 1991] and the 3α-hydroxysteroid dehydrogenase involved in sex steroid metabolism [Penning *et al*, 1985]. Metabolites of the lipoxygenase, and cycloxygenase pathways positively modulate cation and anion channels [Diener *et al*, 1996; Kanli and Norderhus, 1998]. Intracellular Ca^{2+} increases and Ca^{2+} currents can be modulated negatively (by estradiol) [Nakajima *et al*, 1995] or positively (by testosterone or progestins) [Takeuchi and Guggino, 1996; Bukusoglu and Sarlak, 1996]. In addition to their known genomic actions, steroid hormones could modulate VDCCs on spermatogenic cells that might be critical for their differentiation. Further investigation is needed to evaluate the possible co-modulation (direct or through shared transduction pathways) of T-type Ca^{2+} channels and Cl^- channels of spermatogenic cells.

α1E Expressed Currents in *Xenopus* Oocytes Are Very Weakly Sensitivity to NA

Voltage-dependent Ca^{2+} channels have been classified according to their voltage-threshold of activation as high (HVA) or low-voltage-activated (LVA). HVA Ca^{2+} currents were subclassified as L, N, P, or Q; and LVA Ca^{2+} currents as R or T [Tabares and López-Barneo, 1996]. VDCCs are constituted by the α1, α2δ, β, and γ subunits. The α1 subunit contains the pore and the voltage sensor [Catterall, 1995]. Several genes coding for α1 subunits have been cloned (A, B, C, D, E, G, H, I, and S). Their heterologous expression has revealed, for instance, that L-type currents are coded by α1S, α1C, and α1D By contrast, the molecular identity of T-type currents in different tissues is yet under debate [Ertel and Ertel, 1997]. A rat α1E clone expressed in *Xenopus* oocytes yielded Ca^{2+} channels exhibiting functional properties compatible to those of low-voltage-activated Ca^{2+} channels [Soong *et al*, 1993; Stephens *et al*, 1997], while other clones display characteristics closer to R-type Ca^{2+} currents, which are HVA [Ertel and Ertel, 1997] In addition, depending on the cell type,

antisense oligonucleotides against α1E inhibited both T-type currents [Piedras-Rentería *et al*, 1997] and R-type currents [Piedras-Rentería and Tsien, 1998]. By contrast, Meir and Dolphin [1998] found that expression of α1B, E, or C in COS7 cells, which do not display endogenous Ca^{2+} channel subunits or Ca^{2+} channels, can yield low conductance, low-voltage-activated Ca^{2+} channels whose voltage dependence and kinetics of activation and inactivation makes them indistinguishable from native T-type Ca^{2+} channels. These apparently conflicting observations reveal the necessity of establishing which α1 subunit codes for the T-type Ca^{2+} currents of spermatogenic cells that appear to be crucial for the mouse sperm AR.

The most prevalent α1 transcript of VDCCs in spermatogenic cells so far is type E [Liévano *et al*., 1996]. Therefore, a rat brain α1E clone was expressed in *X. laevis* oocytes, and its sensitivity to NA determined. The expressed currents were essentially insensitive to 200 μM NA which completely blocks T-type Ca^{2+} currents of spermatogenic cells. This result questions the possibility that α1E codes for the T-type currents, though alternative explanations must be considered. ACBs different from those used here, inhibited Ca^{2+} activated Cl^- currents in smooth muscle cells, where the $[Ca^{2+}]_i$ increase driven through T-type channels was not affected [Baron *et al*., 1991]. There are contrasting findings on the effect of ACBs on other VDCC currents. In some reports, L-type currents were inhibited by μM ACBs [Asai *et al*, 1996; Doughty *et al*, 1998; Walsh and Wang, 1996] but in others, they were insensitive up to 100 μM [Cotton *et al*. 1997; Lamb *et al*., 1994]. The differential sensitivity to ACBs could be due to the fact that L-type Ca^{2+} currents can be coded by three different α1 subunits (S, C, or D). It is therefore still possible that sequence variations between the rat brain α1E and that of mouse spermatogenic cells explain the lack of sensitivity to NA. In addition, subunits other than α1 that constitute VDCCs can modify their affinity for blockers [Varadi *et al*, 1995; Gurnett *et al*, 1997].

Initially, the findings of Soong *et al*. [1993] showing that α1E expressed in *X. laevis* oocytes yielded currents insensitive to 10 μM nifedipine was consistent with the fact the T-type Ca^{2+} currents were believed to be insensitive to dihydropyridines [Hess, 1990]. However, it is now known that T-type Ca^{2+} currents are sensitive to μM dihydropyridines in some cells [Liévano *et al*, 1995], and for sure in spermatogenic cells [Liévano *et al*., 1996; Santi *et al*, 1996; Arnoult *et al*, 1996, 1998]. Experiments done in this work with 20 μM nifedipine corroborated the insensitivity of α1E expressed Ca^{2+} currents in *Xenopus* oocytes to this blocker (not shown). A straightforward explanation, also consistent with the NA results described above, would be that T-type Ca^{2+} currents of spermatogenic cells are not driven through α1E-coded channels.

Detection of α1C, α1G, and α1H in Spermatogenic Cells

Recently Perez-Reyes *et al*. [1998a] cloned a novel α1 subunit, α1G. Expression of this subunit in *Xenopus* oocytes yielded Ca^{2+} currents with the properties expected of T-type currents. Moreover, α1H and α1I are two newly cloned α1 subunits belonging to what may be a family of α1 subunits coding for T-type Ca^{2+} currents [Cribbs *et al*, 1998; Perez-Reyes *et al*., 1998b; Lee *et al*., 1999]. These reports and the lack of sensitivity to nifedipine and NA of the heterologously expressed α1E channel prompted a search for α1G and α1H in spermatogenic cells. RT-PCR experiments indicate the presence of α1G and α1H in mouse pachytene spermatocytes and in round and condensing spermatids. A more sensitive assay than that used previously [Liévano *et al*., 1996] allowed the detection of low levels of α1C mRNA, too. The presence of a rat cardiac muscle related α1C in mouse sperm [Goodwin *et al*., 1997] and in spermatogenic cells [Goodwin *et al*., 1998] had been reported. Which of these subunits codes for the T-type Ca^{2+} currents in spermatogenic cells? Why do spermatogenic cells express different α1 subunits if only T-type currents are functionally expressed? These interesting and fundamental questions remain to be answered.

ACKNOWLEDGMENTS

The authors thank T. Snutch for the α1E clone, J. Alvarez-Leefmans and H. Pasantes for anion channel blockers, E. Mata for help with mice, P. Gaytan and E. López-Bustos for oligonucleotide synthesis, and P. Labarca and R. Felix for comments. This work was supported by grants from Consejo Nacional de Ciencia y Tecnologia, DGAPA-UNAM, the Howard Hughes Medical Institute, and the International Centre for Genetic Engineering and Biotechnology.

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Mouse sperm patch-clamp recordings reveal single Cl^- channels sensitive to niflumic acid, a blocker of the sperm acrosome reaction

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Received 11 February 1998

Abstract **Ion channels lie at the heart of gamete signaling. Understanding their regulation will improve our knowledge of sperm physiology, and may lead to novel contraceptive strategies. Sperm are tiny (~3 μm diameter) and, until now, direct evidence of ion channel activity in these cells was lacking. Using patch-clamp recording we document here, for the first time, the presence of cationic and anionic channels in mouse sperm. Anion selective channels were blocked by niflumic acid (NA) (IC_{50} = 11 μM). The blocker was effective also in inhibiting the acrosome reaction induced by the zona pellucida, GABA or progesterone. These observations suggest that Cl^- channels participate in the sperm acrosome reaction in mammals.**

Key words: Acrosome reaction; Ion channel; Niflumic acid; Sperm patch-clamp recording

1. Introduction

Ion channels are deeply involved in the egg-sperm dialogue. Components from the outer layer of the egg induce changes in sperm permeability to ions which, depending on the species, regulate sperm motility, chemotaxis and the acrosome reaction (AR). This reaction is required for sperm to fertilize the egg in many species, including man. The egg's extracellular matrix, the zona pellucida (ZP), induces the AR in mammals [1]. Nonetheless, other agents like γ-aminobutiric acid (GABA) and progesterone can induce this reaction [2–6]. The sperm AR is inhibited by ion channel blockers, evidencing their predominant role in this process, and their suitability as targets for contraception [7].

Due to the small size of sperm thus far only planar bilayer techniques have yielded direct information about their ion channels [8–12]. To explore how ion channels participate in the AR we have recorded single channel currents directly from mouse sperm. Here, using patch-clamp recording we document, for the first time, the presence of cation and anion channels in mammalian sperm head plasma membrane. Anion selective channels were blocked by niflumic acid (NA) (IC_{50} = 11 μM). The blocker inhibits also the acrosome reaction induced by the zona pellucida, GABA or progesterone, suggesting that Cl^- channels participate in the sperm AR in mammals. Voltage-gated Cl^- currents, blocked by μM NA (IC_{50} = 100 μM), were monitored also in pachytene spermatocytes [13–15], indicating that NA sensitive Cl^- channels are expressed early in spermatocyte differentiation.

2. Materials and methods

2.1. Sperm collection and AR induction

Sperm were obtained from CD-1 mice >3½ months killed by cervical dislocation. The cells were collected from excised epididymides as described [16]. Cells (3.6×10^6/ml) were suspended in M-199 media (from Sigma) supplemented with 25 mM $NaHCO_3$, 1 mM sodium pyruvate and 0.4% glucose, and were capacitated at 37°C for 30 min in 5% CO_2 atmosphere. Thereafter the agents (heat-solubilized ZP, progesterone or GABA) were added and cells were left to acrosome react for 30 min. Inhibition was studied with the same procedure except that the blocker was added 5 min prior to the agonist. At the end of the experiment AR was determined by established procedures [17,18]. A minimum of 100 sperm were counted under light field microscopy and expressed as the fraction of reacted sperm induced by the agonist vs. a maximum of reacted sperm obtained with the Ca^{2+} ionophore A-23187 (AR/ARA-23) at 15 μM.

2.2. Electrophysiology

2.2.1. Sperm. Seals were obtained using Kimax or 7052 glass pipettes having >5 megaohms resistance and <1 μm of tip diameter. Sperm were rinsed for 10 min in (mM): 60 NaCl, 150 sucrose, 20 sodium lactate, 1 sodium pyruvate, 5.6 glucose, 1 EDTA, 10 benzamidine and 20 μg/ml aprotinin; pH 8.4, pelleted at 325×*g* for 10 min at room temperature in cylindrical 2 ml Eppendorf tubes before patch-clamping them. The pelleted cells were resuspended, left for 10 min at 20–23°C and then placed on ice. An aliquot was allowed to settle for 5–10 min on a coverslip and placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon Corp.) endowed with Hoffman Optics. Experiments were done at 20–23°C and currents monitored with and Axoclamp 1-B amplifier (Axon instruments, Foster City, CA), sampling at 10–25 kHz, and filtering at 2–5 kHz. Data analysis was performed with Pclamp6 routines. Patch-clamping the sperm head is laborious due to its small size and flatness. A perpendicular approach between pipette and cell surface is necessary to achieve high resistance seals (>1 gigaohms, Fig. 1A). This occurred only in ~7% of trials, out of which ~80% displayed single channel activity. In 25% of the high resistance seals spontaneous excised patches were obtained. In this cases the polarity of the membrane patch was not known. Excised patches were lost very rapidly, therefore, ion substitution experiments were not possible and data were acquired only with the solution used during sealing. Analysis was done only on records with >5 megaohms of seal resistance. Apparent permeability on excised patches were calculated according to [19]. The solutions in pipette and in bath employed in sperm patch-clamp experiments are indicated in the figure legends.

2.2.2. Spermatogenic cells. Testis from adult CD-1 mice were used as a source of germ cells. Spermatogenic cells were obtained as described [13,14]. The dissociated cells were stored at 4–10°C and remained healthy for at least 12 h. An aliquot of the cell suspension was placed in a recording chamber superfused with recording medium (in mM: 130 NaCl, 3 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucose, 0.16 amiloride, pH 7.35) at 20–

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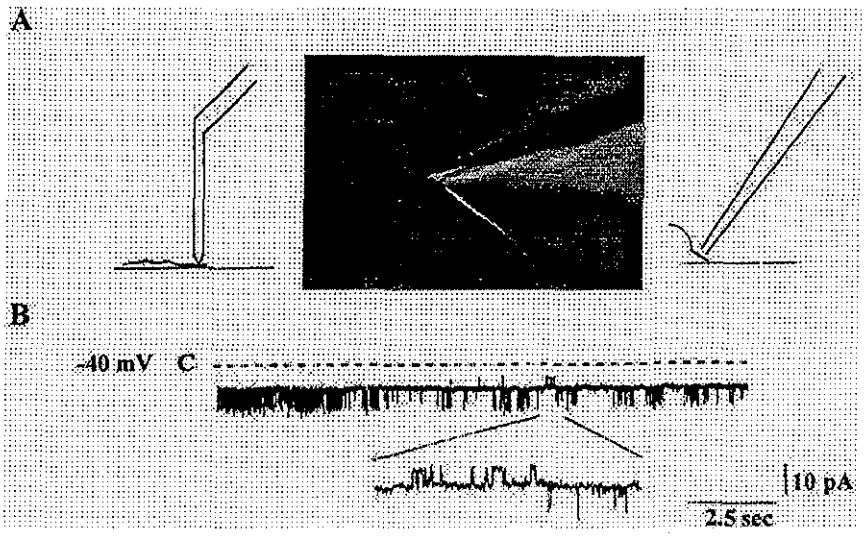

Fig. 1. Direct single channel recording in mouse sperm. A: The center shows a micro-photograph of a patch-clamped mouse sperm (head sperm width = ~3 μM). Schematic representations of perpendicular approaches to sperm are illustrated on both sides of the photo. B: Single channel activity in an excised patch. The pipette was filled with (in mM): 30 NF, 120 NaCl, 0.1 GABA, 10 HEPES, pH 7.2. The bath solution was (in mM): 145 NaCl, 10 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, pH 7.4. The trace obtained at −40 mV shows the presence of mainly two types of transitions (see text). The discontinuous line indicates zero current (C, closed state).

23°C. The solution in the pipette was (in mM): 110 Cs-Methanesulfonate, 10 CsF, 15 CsCl, 2 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 5 Cs-HEPES, pH 7.35. Glass borosilicate pipettes were pulled to tip diameters of about 1.5 μm, having resistances between 2 and 5 megaohms when filled with pipette solution. Records were low-pass filtered at 2 kHz (4-pole Bessel filter). A p/4 pulse protocol was used routinely to minimize leak and capacitive currents from current records [20].

2.3. Membrane potential measurements

In cell attached experiments (Fig. 3) real ionic cytoplasmic concentrations are not known for the patched cell. Membrane potential (V_m)

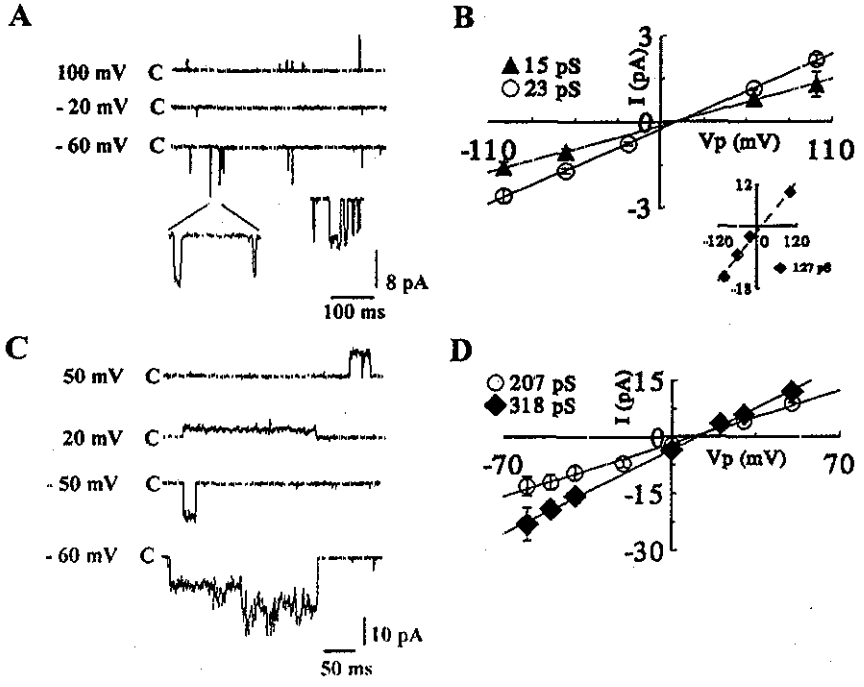

Fig. 2. Cationic channels. The traces shown in this figure were recorded on spontaneous excised patches at the indicated voltages. A fast mouse sperm cation channel displaying several conductance levels. Kinetic analysis yielded basically a monoexponential closing time constant of 0.82 ± 0.27 ms at −60 mV, and opening time constants of 4.3 ± 1 ms and 53 ± 12 ms. Left inset shows a 20-fold time expansion of the indicated area of the burst at −60 mV. Right inset shows a burst from the same recording at −60 mV (identical scales). Solutions were (in mM): 145 NaCl, 2.5 $CaCl_2$, 10 $MgCl_2$, 10 HEPES-Na, pH 7.4 (in bath); and, 120 CsCl, 0.05 $CaCl_2$, 10 EGTA-Cs, 10 HEPES-Cs, pH 7.2 (in pipette). Similar transitions were observed in 12 experiments. B: I–V relations of the channel in A, E_{rev} = 10 mV. The inset shows the I–V relation of the largest conductance. C: Representative traces of a slow high-conducting channel showing two main conductance levels. Similar transitions were observed in 11 experiments. D: I–V relations of the channel in C. Solutions were (in mM): bath as in A; and in the pipette; 30 KF, 90 KCl, 0.05 $CaCl_2$, 10 EGTA-K and 10 HEPES-K, pH 7.2. Smaller fast cationic transitions similar to those in A are also present.

180

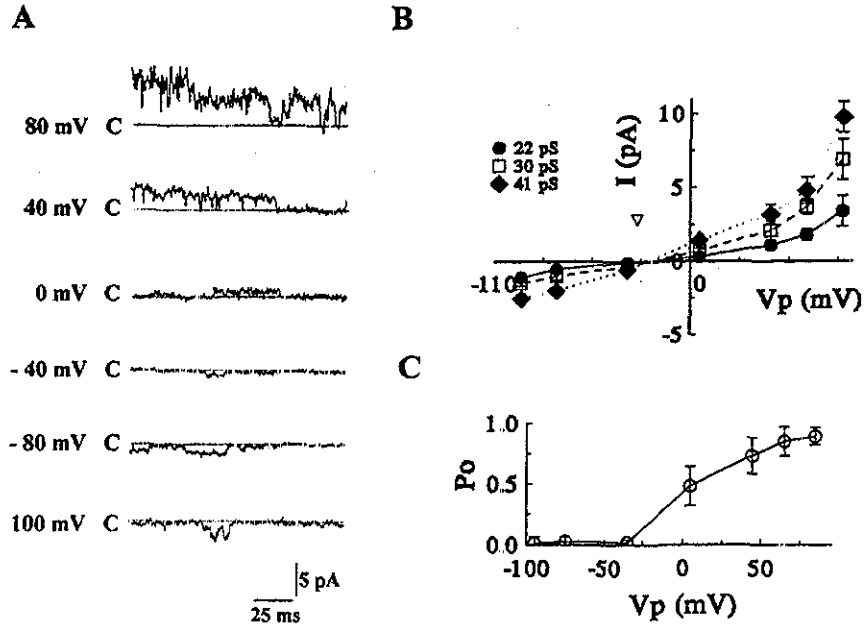

Fig. 3 Mouse sperm anion channels. A: Anion single channel transitions at the indicated pipette potentials recorded in the cell attached configuration The pipette contained (mM): 120 NaCl, 30 NaF, 10 HEPES-Na, pH 7 6 B: I–V relations from A showing outward rectification at high positive applied potentials Slope conductances of 22, 30 and 41 pS were measured in the linear region. E_{rev} averaged -30 ± 5 mV ($n=3$), which considering the resting potential of sperm under equivalent conditions (−85 mV, see Section 2) and 10 mM Cl^- in the sperm cytoplasm [22], indicates anion selectivity C: Open probability (P_o) of the anion channel measured during 6.4 s at each pipette potential (V_p)

determinations in sperm suspensions were done as in [21] using DiS-$C_3(5)$, a V_m sensitive fluorophore. The resting potential of sperm suspended in the external solution (TEA-Cl 60, TEA-$MeSO_4$ 70, 10 $CaCl_2$, pH 7.4) used for cell attached experiments was estimated to be -85 ± 7 mV ($n=8$) The internal K^+ concentration used for this calculations was 120 mM [22]

3. Results and discussion

To explore the in situ characteristics of sperm ion channels, we recorded single channel currents directly from mouse sperm Single channel currents could be recorded in 42

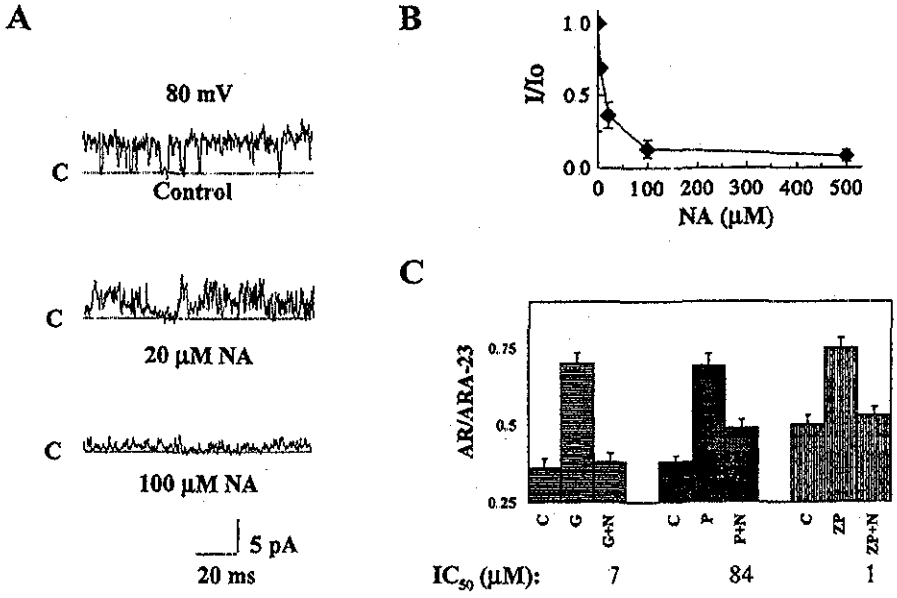

Fig. 4 Niflumic acid (NA) blocks a mouse sperm Cl^- channel and the AR induced by ZP3, GABA and progesterone. A: Conditions were as in Fig. 3. Voltage in pipette was 80 mV in all cell attached records shown. The control trace is at the top, the following traces were taken 10 min apart, after perfusing the external chamber with control solution plus 20 µM (middle trace) or 100 µM (lower trace) NA. B: Dose dependence of channel blockade by NA (average ± S.E.M., $n=3$) C: NA inhibits the mouse sperm acrosome reaction (AR) induced by GABA (0.1 µM), progesterone (45 µM) and solubilized zona pellucida (5 ZP/µl) AR and its inhibition were determined as indicated in Section 2. The bars represent the average ± S.E.M ($n=5$) of the ratio % AR under a certain condition (C, control; G, GABA; N, NA; P, progesterone; ZP, zona pellucida) and the AR achieved with the Ca^{2+} ionophore A-23187 (ARA-23) The numbers under the bars are the IC_{50} determined in five concentration dependence experiments.

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patches, 14 of which were excised patches, the remaining ones corresponding to cell attached patches Fig 1B shows single channel activity in an excised patch at −40 mV. Mainly, two types of current transitions can be observed: (1) fast transitions that occur in bursts; and (2) smaller upward transitions (inset below). In addition, a slow high-conducting channel similar to the one of Fig. 2C, was also observed during this recording (not shown).

Single channel currents shown in Fig 2A were monitored in an excised patch at different applied voltages. These transitions have fast kinetics with spike shaped transitions being the most common, although square shaped transitions are also present (see record at higher resolution and the bursting period in Fig 2A). From the I–V plots, which have an $E_{rev} = 10$ mV, a PNa^+/PCs^+ of 1.5 (150 Na^+_{bath}/145 $Cs^+_{pipette}$) was calculated In a separate experiment PNa^+/PK^+ was 0 71, indicating a permeability sequence $K^+ > Na^+ > Cs^+$, analogous to that seen in bilayers ($K^+ > Rb^+ > Na^+ > Cs^+$) for a cAMP-activated sea urchin sperm cationic channel [12]. Similar single channel currents were recorded in four excised patches and eight cell attached ones In three experiments where transitions were recorded at various voltages, P_o did not vary significantly (0.012 ± 0.022 in the ± 100 mV range) In ~25% of the experiments where fast cationic transitions were seen, burst periods with $P_o > 0.3$ were observed between voltages ≥+40 mV or ≤−40 mV (inset in Fig. 2A). The pattern of the transitions is also reminiscent of a mouse sperm cationic channel detected in planar bilayers [11]. Moreover, the poor selectivity of this channel suggests it could participate in the ZP induced depolarization during mouse and bull sperm AR [23].

Fig. 2C shows a record of an excised patch displaying slower cationic transitions with main conductance substrates of 207 and 318 pS ($E_{rev} = 11$ mV; $PCa^{2+}/PNa^+ = 6$; assuming $PNa^+/PK^+ = 1$). Similar transitions were recorded in 11 experiments (eight cell attached and three excised patches) Planar bilayer studies indicated the presence of a similar high conductance, poorly selective cationic channel that allows divalents through, in sea urchin [24] and mouse sperm plasma membranes [10,11]. Patch-clamp recording shows that this cation selective channel displays slow kinetics with long open times (tenths of milliseconds to seconds), resembling the behavior of the Ca^{2+} selective channel reconstituted in planar lipid bilayers.

In three experiments it was possible to record channel currents that were blocked by NA (a Cl^- channel blocker, Figs 3 and 4). Fig. 3A shows single channel currents monitored at different applied voltages. Inspection of the records revealed the presence of open substrates with conductances of 22, 30 and 41 pS, measured in the ohmic region of the I–V relation. The reversal potential, −30 mV, is consistent with that predicted for an anion selective channel, assuming a resting potential of −85 mV (see Section 2, Fig. 3B). At more positive pipette potentials, the channel rectifies, probably due to the presence of 30 mM F^- in the pipette Some Cl^- channels are known to have a low F^- permeability ($PCl^-/PF^- = 50$; [25]) P_o of the channel increases as the applied potential becomes more positive (Fig 3C). Blockade by NA was dose-dependent ($IC_{50} = 11$ μM, Fig 4A, B). Lipid bilayer studies had also shown the presence of anion channels in sea urchin [26] and mouse sperm plasma membranes [11,27].

Several reports indicate the participation of Cl^- channels in sperm physiology. NA was reported to inhibit a Ca^{2+} induced

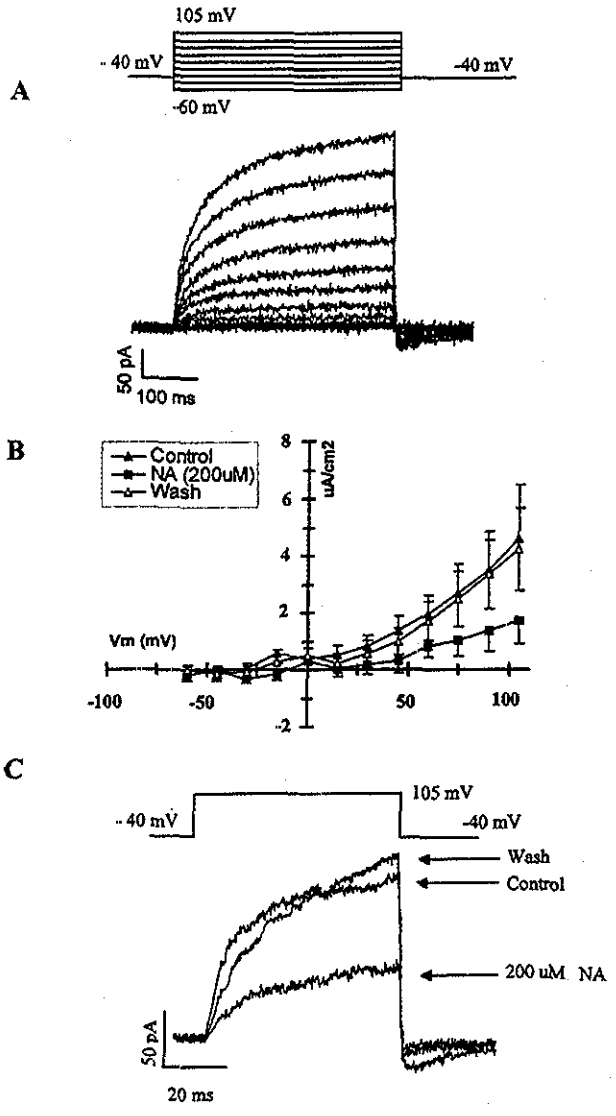

Fig 5 NA blocks outward whole cell currents resulting from Cl^- influx in pachytene spermatocytes Spermatogenic cells and whole-cell recordings were obtained as described in Section 2. Patch pipettes were filled with (in mM): 110 Cs-Methanesulfonate, 10 CsF, 15 CsCl, 2 Cs-EGTA, 4 ATP-Mg, 10 phosphocreatine, 5 Cs-HEPES, pH 7.35; the bath solution contained (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucose, 10 $CaCl_2$, 0.16 amiloride, pH 7 35 Currents were triggered by depolarizations from −40 mV holding potential up to 100 mV in 15 mV steps A: Cl^- currents elicited at 40 mV (top shows voltage protocol) and their block by 200 μM NA B: I–V relation of the Cl^- currents in mouse pachytene spermatocytes (filled triangles), and their blockade (squares) and recovery (empty triangles) after exposure to 200 μM NA. The points represent the average of three experiments, the standard deviation was smaller than the symbols The estimated IC_{50} was 100 μM

hyperpolarization which is partially driven by Cl^- in mouse sperm [21]. External Cl^-, and putative $GABA_A$-R channels have been implicated in the GABA and progesterone induced AR in human and mouse sperm [4,5,28]. In porcine and human sperm, Glycine receptor activation was reported to elicit AR [29] and Cl^- efflux [30]. In other cell systems, NA and other fenamates (non-steroidal anti-inflammatory drugs) have been shown to block Ca^{2+}-activated Cl^- channels [31,32], as well as an heterologously expressed $GABA_A$-R [33]. More-

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over, Sigel et al (1989), reported the expression of a rat β homopentamer $GABA_A$-R which opens in the absence of GABA [34]. Future experiments will have to be done to determine if the sperm Cl^- channel reported here is of the $GABA_A$-R type.

Since NA blocks Cl^- channels in mouse sperm and in other systems, the effect of this compound was tested on the AR. NA blocked the AR induced by GABA (0.125 μM), progesterone (45 μM) or solubilized zona pellucida (5 ZP/μl) (Fig. 4C). The ZP3 and GABA induced AR was more sensitive to NA (IC_{50} of 1 and 7 μM, respectively) than that induced by progesterone (84 μM). This was unexpected since it has been proposed that progesterone triggers the AR by potentiating the $GABA_A$-R channel [2,4,5]. This result may indicate that distinct Cl^- channels participate in AR induced by the different agonists. Alternatively, progesterone could modulate two different surface sperm receptors one coupled to a Ca^{2+} channel [2,35,36] and the other, a $GABA_A$-R type, which upon progestin binding decreases its affinity for NA [29,37].

We investigated further whether spermatogenic cells are endowed with NA sensitive Cl^- channels. Cl^- currents, activated by depolarization, and blocked by NA (IC_{50} 100 μM), could be monitored in pachytene spermatocytes (Fig. 5). These results show that NA sensitive Cl^- channels are expressed early in spermatocyte differentiation. However, at difference with anion channels present in the sperm, Cl^- channels in spermatocytes display a lower affinity for this blocker.

At the least, our results lead to the conclusion that mouse sperm, and spermatocytes, as early as pachytene, are endowed with Cl^- channels blocked by NA. Furthermore, the cumulated experimental evidence suggests that mouse sperm Cl^- channels may participate in the AR induced by ZP3 and GABA. It is possible that one of these Cl^- channels is an isoform of the $GABA_A$-R, and that mammalian sperm posses more than one mechanism capable of triggering the events leading to the fusion of the acrosomal vesicle.

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