UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

INSTITUTO DE BIOTECNOLOGIA

PURIFICACION Y CARACTERIZACION QUIMICA-FUNCIONAL DE TOXINAS DEL VENENO DEL ALACRAN *Pandinus imperator* QUE TIENEN ACCION SOBRE CANALES DE CALCIO

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Que   para   obtener   el   grado   de

DOCTOR EN BIOTECNOLOGIA

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"The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science".

Albert Einstein, 1930

Testimonio de Gratitud

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Con cariño, amor y respeto,
Para Georgina, a quien debo
La terminación de este trabajo.

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Prefacio

Los receptores de rianodina son una familia de canales que liberan calcio de almacenes intracelulares. Este catión regula una gran variedad de funciones celulares, incluyendo contracción muscular, secreción de hormonas, activación de linfocitos, etc. (Meissner, 1994). La rianodina es un alcaloide de origen vegetal, que se ha utilizado hasta el momento para purificar y caracterizar el funcionamiento de este canal, de ahí su nombre (Rogers y col. 1948).

El veneno de los alacranes, es una fuente de diferentes clases de péptidos que afectan la función de canales iónicos (Koppenhofer y Smidth 1968; Catteral, 1977; Possani, 1984; García y col. 1997), alterando así la función de diversos tejidos animales.

Los péptidos mas ampliamente conocidos son aquellos que específicamente reconocen canales de sodio y potasio (Catteral y col. 1977; Carbone y col. 1982; Miller, 1995; Gordon y col. 1998); sin embargo también se han reportado toxinas que reconocen canales de calcio y cloro (Olivera, 1985; Valdivia y Possani, 1998; Debin, 1993). Estos polipéptidos han sido muy útiles para identificar y caracterizar canales iónicos en diversas células.

De acuerdo a lo antes mencionado, en esta tesis se reporta el trabajo realizado con veneno de un alacrán africano, con miras a encontrar compuestos que ayuden a un mejor entendimiento del funcionamiento de un canal de calcio intracelular, también llamado receptor de rianodina. De esta manera:

a) Se buscó purificar a homogeneidad algunas toxinas presentes en el veneno del alacrán *Pandinus imperator*, que tuvieran algún efecto sobre el receptor de rianodina.

b) Se buscó caracterizar químicamente estas toxinas.

c) Por último, se pretendió explicar el mecanismo de acción de una de estas toxinas en el receptor de rianodina.

Por lo que respecta a la presentación de la investigación y sus resultados, dividí la tesis en cinco apartados, como se señala en el índice-contenido. En la introducción se describe el cuadro completo de los elementos estudiados, como una referencia. A continuación se presentan los objetivos a realizar, los cuales están avalados en los resultados que aparecen aquí en formato de artículos, antecedidos por su respectiva ficha bibliográfica.

En la última sección se presenta una discusión, en la que se incluyen los aspectos más relevantes del trabajo de tesis, cuyos detalles se localizan en los artículos incluidos en el apartado III.

Al final del trabajo se incluye, a manera de apéndice, un artículo ya publicado, que si bien no forma parte del tema central de este trabajo, fue realizado durante mi entrenamiento doctoral.

Resumen

En el presente trabajo se reporta la purificación a homogeneidad y la determinación de la secuencia de aminoácidos de la imperatoxina A (IpTxa) y de la imperatoxina I (IpTxi). La IpTxa es un péptido de 4 kDa que con una Kd de 6 nM incrementa específicamente el pegado de $[^3H]$rianodina a membranas de músculo esquelético, no así a membranas de músculo cardiaco. La secuencia de aminoácidos de la IpTxa no muestra similitud con otras toxinas de alacrán hasta ahora descritas, aunque sí se encontró similitud con la secuencia de aminoácidos de las toxinas de araña Tx2-9 y con la angelenina, toxinas que actúan sobre canales de Ca^{2+} tipo P.

La IpTxi, una proteína de 15 kDa, inhibió el pegado de $[^3H]$rianodina a membranas de músculo esquelético y cardiaco, con una Kd aparente de 20 nM

La IpTxi es una proteína heterodimérica con acción lipolítica; la subunidad larga de esta toxina tuvo una similitud de 35% con fosfolipasas de tipo A2 de abeja (*Apis mellifera*) y del escorpión (*Heloderma horridum*). La subunidad pequeña no tiene ninguna similitud con otros péptidos conocidos. El efecto de la IpTxi sobre el canal de rianodina parece ser indirecto. El pegado de rianodina, marcada radioactivamente, al canal es inhibido por los ácidos grasos liberados por acción lipolítica de IpTxi.

Utilizando concentraciones más altas que las que se necesitan para regular el receptor de rianodina, la IpTxa e IpTxi no afectan otros canales de calcio o transportadores iónicos en músculo y cerebro.

Summary

In this thesis, the purification to homogeneity and the complete amino acid sequences of Imperatoxin A (IpTx$_a$) and Imperatoxin I (IpTx$_i$) are reported. Imperatoxin A, a 4 kDa peptide specifically increased [^{3}H]-ryanodine binding to the skeletal but not the cardiac ryanodine receptor (RyR), with a Kd of 6 nM . At concentrations higher than those needed to regulate RyRs, neither IpTx$_i$ nor IpTx$_a$ affected other Ca^{2+} channels or ion transporters of muscle and brain. IpTxi, a 15 kDa protein, inhibited [^{3}H]-ryanodine binding to skeletal and cardiac rianodine receptor, with an apparent Kd of 20 nM.

The amino acid sequence of IpTx$_a$ shows no homology to any scorpion toxin so far described, but shares some similarities to the amino acid sequence of Tx2-9 and angelenin, two spider toxins that target neuronal P-type Ca^{2+} channels. IpTx$_i$ is a heterodimeric protein with lipolytic action, a property that is only shared with β-bungarotoxins. The large subunit of IpTx$_i$ was about 35% identical to PLA2 from the bee (*Apis mellifera*) and the lizard (*Heloderma horridum*) venoms. The small subunit has no significant similarity to any other known peptide. The effect of IpTxi on the ryanodine channel seems to be indirect, mediated by the fatty acids liberated by the lipolytic action of IpTxi.

I.- INTRODUCCION.

La contracción simultánea de las células musculares es esencial para un movimiento eficiente de nuestros músculos. La contracción muscular depende del estímulo del tejido muscular a través de los nervios motores. La unión sincronizada entre una depolarización y la actividad contráctil (acoplamiento excitación-contracción) son críticas para un control preciso de los músculos. A nivel molecular ocurren otros mecanismos para la acción coordinada de éstos.

Una célula muscular, llamada miofibra, es cilíndrica y multinucleada, tiene muchos racimos de miofilamentos, las miofibrillas. Cada miofibrilla esta constituida de arreglos repetidos llamados sarcómeros. Los sarcómeros contienen dos tipos de filamentos, los gruesos formados de miosina y los delgados que contienen actina.

Cada miofibrilla esta rodeada de una red de membranas denominadas retículo sarcoplásmico (RS). El RS forma vesículas continuas llamadas cisternas terminales. Las cisternas terminales están separadas por solo 16 nm de la membrana de los túbulos transversos (túbulos-t) que no son mas que invaginaciones de la membrana plasmática (sarcolema) Las cisternas terminales están asociadas con los túbulos-t formando las diádas si solo una cisterna terminal es la asociada o triádas si son dos las cisternas terminales asociadas (fig 1).

El RS sirve como almacén de los iones Ca^{2+} tomados del citoplasma y de las miofibrillas. La membrana del RS tiene una ATPasa de Ca^{2+} la que toma los iones Ca^{2+} del citoplasma hacia el interior del RS. La depolarización de la membrana plsmática de las células musculares produce la apertura de canales de Ca^{2+} en las membranas del RS y la liberación
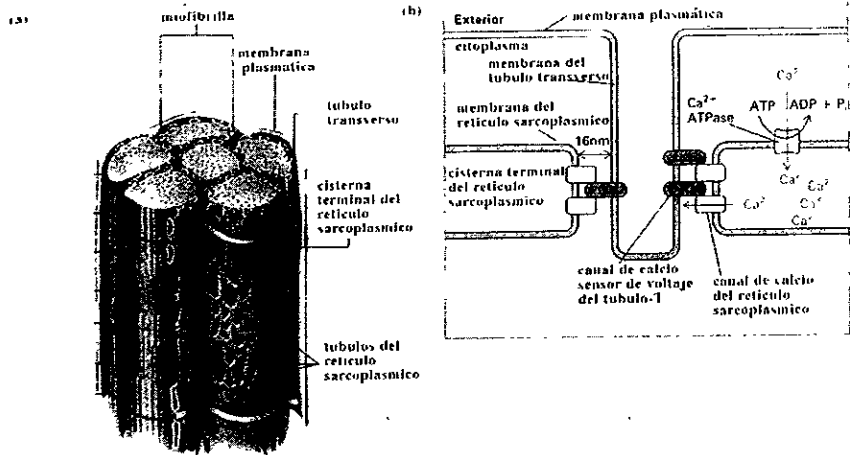
FIGURA 1. a) Estructura tridimensional de una miofibra. Los túbulos transversos (túbulos-t) que están formados por invaginaciones de la membrana plsmática quedan muy cerca de las cisternas terminales del retículo sarcoplásmico, b) Diagrama de una tríada (formada por el túbulo-t asociado a dos cisternas terminales del RS) mostrando la relación del túbulo-t con la cisterna terminal. Los canales de calcio sensibles a voltaje (canales de tipo L, receptor de dihidropiridina) que se encuentra en la membrana del túbulo-t tocan el "pie" del canal liberador de calcio (RyR) que se encuentra en la membrana del RS. Cuando el músculo se estimula los iones calcio almacenados en el RS se liberan al citosol a través del RyR. La ATPasa de Ca^{2+} que se encuentra también en la membrana del RS es la encargada de tomar al calcio del citoplasma y almacenarlo nuevamente en el RS. (Molecular Cell Biology)

de iones calcio del RS hacia el citoplasma, disparando así la contracción muscular. Esta liberación de calcio ocurre a través de arreglos tetraméricos de receptores de rianodina (RyR) que hacen un puente entre la membrana del túbulo-t y el RS.

1.1 El receptor de rianodina.

Se sabe desde hace varias décadas, que la madera proveniente de árboles del género *Ryania* contiene productos tóxicos. En la década de los 40s, antes de la proliferación del uso de insecticidas como fosfatos orgánicos e hidrocarburos clorados, E. F. Rogers y colaboradores realizaron un estudio para encontrar nuevos insecticidas a partir de plantas. Este trabajo reveló que extractos del tallo y raíces de *Ryania speciosa* Vahl contenían actividad insecticida. Rogers y colaboradores (1948) purificaron a partir del tallo de esta planta lo que llamaron rianodina, la cual tenia 700 veces más actividad insecticida comparado con el material inicial.

Podemos decir, a la fecha, que la rianodina tiene un significado histórico dado que, sus propiedades y acción biológica, han permitido la identificación y caracterización molecular de una familia de canales intracelulares liberadores de calcio, ahora comúnmente llamados receptores de rianodina (RyR)

Los receptores de rianodina son una familia de canales de Ca^{2+} que participan en la liberación de Ca^{2+} intracelular. A la fecha se sabe que el almacén de Ca^{2+} intracelular más importante es el retículo sarcoplásmico en músculo y el retículo endoplásmico en células no musculares (Sutko y Airey, 1996). Se han realizado una gran cantidad de estudios utilizando rianodina, gracias a los cuales se logró, entre otras cosas, purificar y clonar el canal (Inui y col 1987; Imagawa y col 1987; Lai y col 1988) Hasta el momento sabemos que este canal es un tetrámero, de peso molecular aproximado de 560 kDa por monómero y

que se encuentra localizado en la membrana del retículo sarcoplásmico, formando una estructura que se le ha llamado "pie". Esta estructura se localiza en el espacio citoplásmico entre la membrana del retículo sarcoplásmico y el túbulo-t (fig. 1). El receptor funcional es un homotetrámero, el cual tiene forma de coliflor y mide aproximadamente 29x29x12 nm del lado citoplasmático y un pequeño dominio transmembranal que se extiende 7 nm a través de la membrana del RS para formar el poro del canal (Wagenknecht y col, 1989). El centro de la coliflor incluye un poro, con un diámetro de 1-2 nm que parece ser el poro del canal. Existen evidencias estructurales y funcionales de que el poro central esta conectado a cuatro canales radiales incluidos en la porción periférica de cada monómero. Esta estructura sugiere un posible camino de los iones calcio: el calcio puede entrar del lumen del RS a través del canal central y pasar al citoplasma por los canales radiales (fig. 2). La región del poro corresponde a la porción carboxilo terminal de cada monómero del RyR que incluye de acuerdo a diferentes modelos, cuatro (Takeshima y col, 1989; Nakai y col, 1990; Hakamata y col, 1992) o 10 a 12 (Zorzato y col, 1990; Otsu y col, 1990) segmentos transmembranales. El resto de la molécula forma la gran región extramembranal que corresponde a la estructura de "pie".

A la fecha se conocen tres isoformas del receptor de rianodina en mamíferos, los cuales son codificados por tres genes diferentes, ryr1, ryr2, ryr3. Los receptores de rianodina 1,2 y 3, correspondientes, también son llamados de músculo esquelético, cardiaco y de cerebro, respectivamente, por ser estos tejidos donde fueron aislados e identificados (Meissner, 1994; Ogawa, 1994). A partir de músculo de organismos vertebrados no mamíferos, se han aislado dos isoformas más a las que se les ha llamado alfa y beta, que a su vez son muy parecidas por su secuencia de aminoácidos a las formas 1 y 3 de mamíferos, respectivamente (Oyamada y col.1994).

FIGURA 2. Representación tridimensional del receptor de rianodina reconstruido y visto de forma paralela al plano de la bicapa. (a) el receptor de rianodina completo. (b) una rebanada por la mitad que revela la estructura interna del RyR . Las líneas horizontales indican la localización putativa de la bicapa lipídica. Abreviaturas: TA, estructura transmembranal; CA, estructura citoplásmica "pie"; p, tapón; c, canal lleno de solvente, los diferentes dominios estructurales se asignan con números. Los dominios 5-6 están localizados del lado de los túbulos-t, los dominios 7-8 se encuentran del lado del RS, 3 es el dominio llamado asa o manija el dominio 1 es el que conecta a la parte transmembranal con los dominios 2 y 3 (Wagenknecht 1995).

Los receptores de rianodina tienen, una muy amplia distribución, tanto desde el punto de vista filogenético como por tejidos. Existen evidencias bioquímicas y farmacológicas de su presencia en langostas, en insectos como *Drosophila* y nemátodos como *C. Elegans*, así también en vertebrados (mamíferos, aves, reptiles, pescados, y ánfibios) (Sutko y Airey 1996). Algunos tipos celulares que expresan receptores de rianodina son neuronas, tanto del sistema nervioso central como periférico, músculo liso, células endoteliales, hepatocitos, osteocitos, y células pancreáticas (Sutko y Airey 1996). En muchas de estas células, la identidad de la isoforma específica expresada, la distribución intracelular de estas proteínas y la forma en que responden para la mediación de la liberación del Ca^{2+} aún no se conoce, por lo que representa una área de estudio importante.

El receptor de rianodina regula el flujo de Ca^{2+} del retículo sarcoplásmico hacia el citoplasma. En músculo estriado, tiene un papel central en el acoplamiento del mecanismo "excitación-contracción", específicamente, en el acoplamiento entre la depolarización del sarcolema y la liberación de Ca^{2+} del retículo sarcoplásmico.

A la fecha existen dos mecanismos propuestos para el acople excitación-contracción; el modelo del acoplamiento directo en el que la liberación de Ca^{2+} del retículo sarcoplásmico se produce por una interacción directa entre el receptor de dihidropiridina (DHPR) que esta localizado en la superficie de la membrana de los túbulos-t y el receptor de rianodina. En particular, se propone que la depolarización de la membrana plasmática produce un cambio conformacional en el receptor de dihidropiridina, que es transmitido al receptor de rianodina e induce la liberación de Ca^{2+} del retículo sarcoplásmico (Rios y col. 1993). En este modelo, el receptor de dihidropiridina actúa como detector de voltaje y no como canal,

ya que la entrada de calcio proveniente del medio extracelular no se requiere para que haya contracción muscular (fig. 3).

De manera alterna, el acople excitación-contracción puede ser mediado por un proceso conocido como liberación de calcio inducida por calcio, (Fabiato, 1993). El canal del retículo sarcoplásmico se activa por un aumento en la concentración de calcio citoplasmático, sin embargo, las corrientes de Ca^{2+} debidas a la activación del canal de DHP (canal de calcio tipo L) son insuficientes para activar el proceso de contracción de manera directa, pero podrían inducir liberación adicional de Ca^{2+} del retículo sarcoplásmico. Este proceso puede favorecerse por la existencia de un gradiente de Ca^{2+} en el citosol, dado que la entrada de iones Ca^{2+} a la célula a través del receptor de dihidropiridina tiene acceso preferencial al receptor de rianodina, estableciéndose una especie de acoplamiento funcional (Sham y col. 1995) (fig. 4)

Modulación del receptor de rianodina

La actividad de los canales liberadores de calcio esta modulada por una gran variedad de agentes (revisado por Coronado y col, 1994). El calcio tiene una gran importancia en la regulación del RyR, se cree que es el activador "fisiológico" del canal, ya que otros ligandos requieren de calcio para poder ejercer su efecto. El calcio en el rango de concentración micromolar y los nucleótidos de adenina a concentraciones milimolares funcionan como activadores, incrementan la probabilidad de apertura del canal e inducen la liberación de calcio del RS, el magnesio en concentraciones milimolares funciona como un inhibidor.

En músculo esquelético la dependencia de calcio para la salida de iones calcio del RS tiene forma de campana, con un máximo a pCa 6 ($-\log[Ca^{2+}]$). La salida de calcio disminuye a
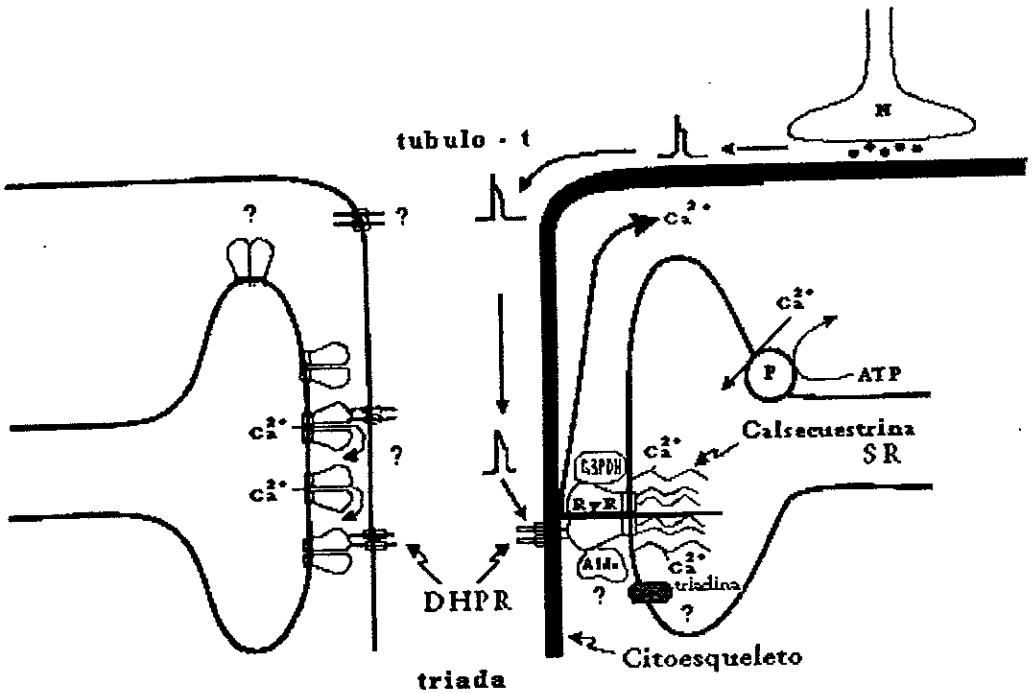
FIGURA 3. Representación esquemática de una triada, donde se muestran algunos aspectos de organización y funcionamiento de esta estructura. Los receptores de dihidropiridina (DHPR) y rianodina (RyR) que participan en el proceso de liberación de calcio están localizados en el espacio entre las membranas del retículo sarcoplásmico y el túbulo-t; se ejemplifica el mecanismo de liberación de calcio inducida por voltaje, donde la membrana sufre una depolarización producida por una neurona motora (N) que es sentida por el DHPR el cual sufre un cambio conformacional que le permite interaccionar con el RyR que a su vez se activa y permite el paso de los iones Ca^{2+} del RS al citosol para iniciar la contracción muscular. Calsecuestrina y triadina son proteínas asociadas al RyR que se cree participan en el acoplamiento excitación–contracción. (Sutko y Airey, 1996).

FIGURA 4. Representación esquemática del mecanismo de liberación de calcio inducido por calcio en músculo cardiaco (calcio induce liberación de calcio, CICR) Los iones calcio entran a través de los canales de calcio tipo L (DHPR) y se unen al sitio de alta afinidad a calcio del RyR activando a éste que a su vez permite la salida de los iones calcio del retículo sarcoplásmico al citoplasma.(Bers, 1991)

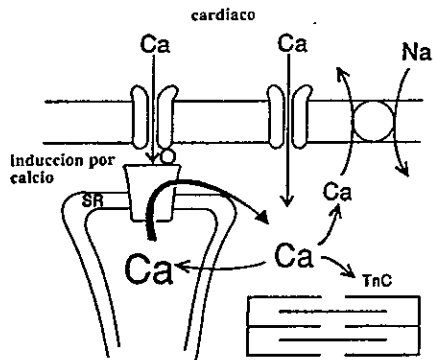pCa 9 o 3. Se cree que esta dependencia de calcio se debe a la presencia de dos sitios de unión a calcio sobre el canal, un sitio de alta afinidad que estimula la liberación de calcio y otro de baja afinidad que inhibe la liberación.

El RyR2 es más sensible a la activación por concentraciones micromolares de calcio y menos sensible a la inactivación por concentraciones milimolares que el RyR1 y esto también se observa en el efecto diferencial del Ca^{2+} sobre la unión de rianodina en los dos tipos de canal.

El magnesio inhibe la liberación de calcio con una EC_{50} de 70 μM. El mecanismo de inhibición parece ser por desplazamiento del Ca^{2+} de su sitio de alta afinidad. En contraste a su efecto sobre el RyR1, el Mg^{2+} en concentraciones milimolares no bloquea completamente al RyR2.

Los nucleótidos de adenina estimulan la liberación de Ca^{2+} de manera más efectiva en el RS esquelético que en el cardiaco. La respuesta a diferentes agentes farmacológicos también difiere entre estos dos tipos de receptor: El RyR1 es más fácilmente bloqueado por rojo de rutenio mientras que el RyR2 se activa más fácilmente por cafeína.

La rianodina, dependiendo de su concentración, estimula o inhibe la liberación de calcio de RS esquelético y cardiaco. A concentraciones en el rango de 0.01-10 μM estimula la liberación de calcio mientras que a concentraciones mayores (10-100 μM) la liberación se inhibe. La activación e inhibición parecen ser producidas por unión de la rianodina a sitios funcionalmente independientes (Humerickhouse y col, 1993). A bajas concentraciones (menos de 10 μM) la rianodina coloca a los canales parcialmente abiertos o sea en un estado de subconcuctancia.

Los ligandos, que abren el canal y estimulan la liberación del calcio del RS (μM Ca^{2+}, mM ATP, cafeína) aumentan la unión de rianodina. El calcio que tiene efecto activador e inhibidor sobre el canal también presenta estos dos efectos sobre la unión de rianodina. Se cree que estos efectos son debidos a que el sitio de alta afinidad de la rianodina esta más accesible cuando el canal esta abierto. Conforme la concentración de rianodina aumenta, la afinidad del receptor por la rianodina disminuye. Este efecto se describe como una interacción alostérica negativa entre cuatro sitios idénticos de unión (uno por monómero) La primera molécula de rianodina se une con alta afinidad al canal abierto bloqueándolo en una configuración parcialmente abierta y reduciendo la unión a los otros sitios. Siguen tres pasos mas de unión cada uno disminuyendo la afinidad, indicando un estado conformacional diferente hasta que una rianodina por monomero esta unida y el canal está totalmente bloqueado. (Buck y col, 1992; Carrol y col, 1991)

Las funciones del receptor de rianodina son afectadas por un gran número de compuestos no relacionados estructuralmente (Coronado y col, 1994), como por ejemplo, proteínas, nucleótidos y toxinas.

A. Proteínas.

A.1. FK 506 *binding proteins* (FKBP12), es una proteína de aproximadamente 12 kDa, la cual copurifica con el receptor de rianodina. La naturaleza de la interacción entre la FKBP12 y el receptor de rianodina no se conoce. La FKBP12 tiene actividad cis/trans-peptidil-prolil isomerasa, por consiguiente, existe la probabilidad que la FKBP12 pudiera inducir cambios en la conformación del receptor de rianodina gracias a esta actividad. Sin embargo, esto no parece ser el caso, ya que cuando se bloquea la actividad isomerasa de la

FKBP12 en presencia del receptor de rianodina, este no muestra ningún cambio en sus propiedades (Timerman y col, 1995). La relación molar de la FKBP:RyR es 1:1; una proteína FKBP se une a cada uno de los cuatro monómeros del RyR. Una proteína FKBP12 con un peso molecular ligeramente mayor (FKBP12.6) esta asociada con el RyR2 en músculo cardiaco. (Lam y col, 1995) En ausencia de FKBP12 los canales muestran un incremento en la probabilidad de apertura y estados más largos de subconductancia, se aumenta la sensibilidad a cafeína pero mantiene sus propiedades farmacológicas básicas, indicando un efecto estabilizador de la FKBP12 sobre el canal (Ahern y col, 1994; Chen y col, 1994). Por otro lado, la coexpresión de FKBP12 y RyR resulta en canales que presentan eventos de apertura completa (no se presentan subestados) y de mayor duración (75 ms) a los presentados en ausencia de FKBP12 (4.4 ms) (Brillantes y col, 1994) La sensibilidad a cafeína es mayor en canales libres de la proteína FKBP12, indicando que esta proteína también regula el estado cerrado del canal. Por crio-microscopia electrónica se ha localizado la proteína FKBP12 en el dominio citoplasmático a una distancia aproximada de 10 nm del dominio transmembranal del RyR. (Wagenknecht y col, 1996).

A.2. Calmodulina. La calmodulina (CaM) es un regulador citoplasmático de enzimas dependientes de Ca^{2+}. A concentraciones milimolares esta proteína inhibe la liberación de calcio de RS cardiaco y esquelético. El efecto ocurre en ausencia de ATP, por lo que no se debe a una fosforilación del canal (Fuentes y col, 1994) El efecto de CaM depende de las concentraciones de calcio: a bajas concentraciones de Ca^{2+} este agente activa el canal, mientras que a altas concentraciones de Ca^{2+} el canal se inhibe (Tripathy y col 1995; Ikemoto, 1995). Por lo tanto, la calmodulina puede servir como un apagador que controla la liberación de Ca^{2+} mediada por el receptor de rianodina en una forma dependiente de las

necesidades de calcio. Además de la acción directa de CaM sobre el RyR, ésta puede afectar la actividad del canal a través de la proteína cinasa dependiente de calmodulina. La fosforilación del canal por la cinasa dependiente de CaM disminuye la unión de rianodina, dado que la rianodina se une preferentemente al canal abierto, estos resultados implican a la fosforilación en la modulación de apertura y cierre del canal (Takasago y col, 1991).

A.3. Triadina. La triadina es una glicoproteína integral del retículo sarcoplásmico, con un peso molecular de 95 kDa que colocaliza con el RyR en músculo esquelético y cardiaco. La liberación de calcio del RS es afectada por anticuerpos anti-triadina (Brandt y col, 1992) Se ha propuesto que la triadina interacciona con el receptor de dihidropiridina, transmitiendo una señal de activación entre este receptor y el de rianodina (Caswell y col. 1991) El análisis hidrofílico de la secuencia de aminoácidos de la triadina sugiere que ésta contiene un segmento transmembranal que separa a la proteína en dos dominios, uno pequeño (47 aminoácidos de la región amino terminal) citoplamático y uno mayor (683 residuos) que se encuentra en el lumen del RS. Se cree que la triadina interacciona con la calsecuestrina y que posiblemente funciona como conexión entre la calsecuestrina y el RyR (fig. 5) (Guo y Campbell, 1995).

A.4. Sorcina. La sorcina es una proteína de 22 kDa que une Ca^{2+}, se ha encontrado asociada con el receptor de rianodina en el retículo sarcoplásmico de músculo cardiaco. La naturaleza de las interacciones entre estas proteínas y las consecuencias que estas tienen en la función del receptor de rianodina aún no se saben (Meyers y col. 1995).

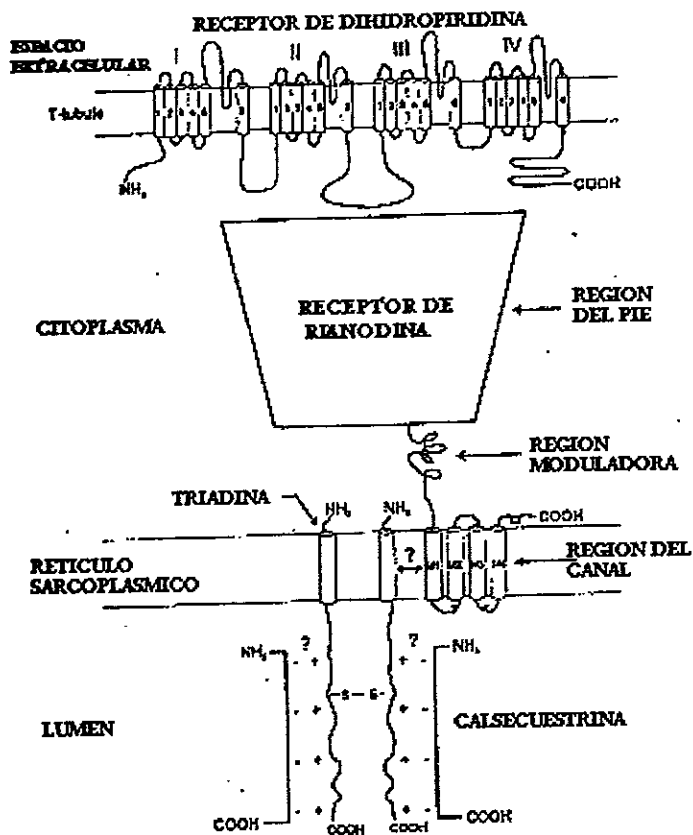**FIGURA 5.** Modelo molecular del receptor de rianodina acoplado al receptor de dihidropiridina y proteínas asociadas; se observa en el receptor de dihidropiridina solo la subunidad α1 y una de las subunidades del receptor de rianodina en donde se muestra la región del pie y el poro del canal, la triadina se representa con su extremo carboxilo terminal asociado a la calsecuestrina (tomado de McPherson y Cambell 1993).

A.5. Calsecuestrina. La calsecuestrina es una proteína ácida que se encuentra en el lumen del RS, que tiene una alta capacidad para unir calcio y permanece asociada la RyR, se propone que los segmentos cargados positivamente de la triadina son los intermediarios en esta asociación (Guo y Campbell, 1995) (fig. 5) Se cree que además de la capacidad de almacenar calcio en el RS, la calsecuestrina juega un papel activo en la regulación de la liberación de calcio (Ikemoto y col, 1991).

B. Ribosa de adenosina difosfato cíclico (cADPR)

cADP-ribosa es un metabolíto de nicotinamida adenin dinucleotido (NAD^+) el cual se cree que actúa como segundo mensajero en varios tejidos (Lee y col, 1989)

Los estudios de varios investigadores, iniciando en la década de los 80s (Clapper y col 1987), hasta la fecha (Lee, 1996), han demostrado que la cADPR es un mensajero intracelular capaz de provocar la liberación de Ca^{2+} de almacenes celulares, en una gran variedad de tejidos Los resultados estos trabajos sugieren que el cADPR produce este efecto, activando el proceso de liberación de Ca^{2+} mediado por el receptor de rianodina. Este es el caso en músculo cardiaco, donde se ha propuesto ser un regulador endógeno de la liberación de Ca^{2+} del retículo sarcoplásmico (Meszaros y col. 1993).

C. Acidos Grasos

Se sabe que los ácidos grasos y sus derivados forman parte de algunas señales de transducción. Varios de estos procesos celulares pueden tener efecto sobre el metabolismo del calcio. Por lo que no es sorprendente que varios metabolítos de lípidos tengan efecto sobre la actividad del canal liberador de calcio. A concentraciones micromolares la palmitoil carnitina y palmitoil CoA estimulan la unión de [^{3}H]rianodina y la liberación de

calcio de músculo esquelético de mamífero (El-Hayek y col 1993) y de aves (Dumonteil y col 1994) Su acción parece ser específica y limitada al RyR esquelético, ya que la palmitoil carnitina no tuvo efecto sobre la unión de [^{3}H]rianodina en membranas de RS cardiaco.

D. Toxinas peptídicas.

D.1 Miotoxina a. La miotoxina a es un polipéptido de 42 aminoácidos aislado del veneno de la serpiente de cascabel *Crotalus viridis viridis*, que provoca un incremento en la liberación de Ca^{2+} de membranas del retículo sarcoplásmico de músculo esquelético (Furukawa y col 1994) La liberación de calcio inducida por miotoxina es inhibida por Mg^{2+} y por rojo de rutenio. Experimentos de unión muestran que la miotoxina no afectó la unión de rianodina y que la miotoxina marcada no se une al RyR, sugiriendo que su blanco es una proteína diferente al RyR (Okhura y col 1995).

D.2 Helotermina. La helotermina es un polipéptido de 25.5 kDa purificada a partir del veneno del heloderma mexicano (*Heloderma horridum horridum*); se encontró que bloquea tanto el receptor de rianodina cardíaco como el esquelético. En experimentos de unión la helotermina muestra desplazar a la rianodina reversiblemente (Morrissette y col., 1995).

D.3 Rianotoxina. La rianotoxina es un polipéptido de 11.4 kDa aislado del veneno del alacrán *Bouthotus judaicus*, encontrándose que tiene efectos sobre la actividad del receptor de rianodina similares a los producidos por la rianodina (Morrissette y col., 1996). En particular, la rianotoxina incrementa la liberación de Ca^{2+} de vesículas de membranas del retículo sarcoplásmico e induce estados largos de subconductancia en el receptor de

rianodina aislado. Así también, estimula el pegado de rianodina marcada de una forma dependiente de la concentración y de manera reversible.

Muchas preguntas importantes, en relación al papel que juegan los receptores de rianodina en la mediación de los eventos de liberación de calcio en los diferentes tipos de células permanecen aún sin ser contestadas. Las respuestas a estas preguntas requerirán al menos en parte, el uso de agentes farmacológicos que modifiquen la actividad de los receptores de rianodina, tanto en las propiedades del canal como en especificidad, de acuerdo a la isoforma de que se trate.

1.2 Uso de toxinas para el estudio de canales de calcio.

Sabemos que los niveles de calcio intracelular sirven para regular muchos procesos, tales como: secreción de neurotransmisores y hormonas, para activar algunos canales iónicos y enzimas, proliferación celular, etc. También sabemos que los canales de calcio sensibles a voltaje, se encuentran entre los más heterogéneos de los canales iónicos. Una sola neurona generalmente tiene varios tipos de canales de calcio, esenciales para la integración y expresión de la actividad en el sistema nervioso. Es por demás señalar la importancia de entender el significado funcional de tal diversidad.

A la fecha, dos tipos de ligandos polipeptídicos, las ω-conotoxinas y ω-agatoxinas han sido utilizados para definir varias clases de canales de calcio (Olivera y col.1991; Sher y col. 1991) Las ω-conotoxinas constituidas de 24-29 aminoácidos, aisladas del veneno del caracol *Conus*; y las ω-agatoxinas, que son péptidos un poco más largos aislados del veneno de la araña *Agelenopsis aperta*. Como se puede ver en ambos casos los ligandos

para canales de calcio son producidos en venenos, y por ello puede considerarse que péptidos similares pueden encontrarse en organismos parecidos, o semejantes, al caracol y araña.

Los venenos de alacrán, por ejemplo, contienen gran cantidad de péptidos básicos, muchos de los cuales modifican el mecanismo de apertura y cierre de canales de sodio o bloquean con alta afinidad canales de potasio. (Ménez y col. 1992). Estas toxinas han sido una herramienta muy importante en la identificación, purificación y caracterización funcional de los canales iónicos correspondientes. La existencia de toxinas que tienen acción sobre canales intracelulares de calcio (receptor de rianodina) fue descrita por primera vez en el veneno del alacrán africano *Buthotus hottentota* (Valdivia y col. 1991); más tarde, se encontraron en el veneno del alacrán *Pandinus imperator*, péptidos con actividad sobre el RyR (Valdivia y col. 1992).

A partir de esta información, este trabajo presenta la purificación, caracterización química y funcional de dos péptidos aislados del veneno del alacrán *Pandinus imperator,* con efecto sobre el receptor de rianodina

II.- OBJETIVOS.

El objetivo propuesto en esta tesis es encontrar nuevas herramientas para el estudio del receptor de rianodina, abarcando los siguientes aspectos:

1.- La purificación a partir del veneno del alacrán *Pandinus imperator*, de compuestos que tengan algún efecto sobre el receptor de rianodina.

2.- La caracterización química de estos compuestos.

3.- La valoración de los posibles cambios funcionales de estas toxinas, por tratamientos con reactivos químicos

4.- La síntesis química de fragmentos de esta(s) toxina(s) y el ensayo sobre el receptor de rianodina.

5.- Por último, tomando junta toda esta información, analizar el mecanismo de acción de esta toxina(s) sobre el receptor de rianodina.

Esta información es importante para ayudar a entender el funcionamiento del canal de calcio intracelular (receptor de rianodina).

III.- RESULTADOS.

A continuación presentamos los resultados obtenidos de nuestra investigación.

La manera de reportar estos resultados será en el formato de artículos publicados, dado que además de ser un requisito del proyecto de doctorado, deja patente la participación de nuestro laboratorio en la caracterización de las nuevas toxinas encontradas.

Los resultados quedan divididos en dos artículos. El primer artículo describe la purificación y caracterización de un péptido encontrado en el veneno del alacrán *Pandinus imperator*, que tiene un efecto inhibitorio sobre el receptor de rianodina.

El segundo trabajo describe también la purificación y caracterización de un segundo péptido encontrado en el mismo veneno, pero que tiene un efecto contrario al péptido anterior, ya que éste activa al canal intracelular de calcio.

Los trabajos se presentan antecedidos por su ficha bibliográfica respectiva y por un resumen. Ambos trabajos describen a péptidos que actúan sobre el receptor de rianodina, que servirán como herramientas para el estudio de este canal, cumpliéndose así el objetivo propuesto en este trabajo. Finalmente, se incluye a manera de apéndice un último trabajo, que si bien no forma parte del tema central de esta tesis, fue realizado durante mi entrenamiento doctoral.

3.1 ARTICULOS PUBLICADOS.

Fernando Z. Zamudio, Renaud Conde, Carolina Arévalo, Baltazar Becerril, Brian M. Martin, Héctor H. Valdivia, and Lourival D. Possani. (1997) "The mechanism of inhibition of Ryanodine Receptor channels by Imperatoxin I, a heterodimeric protein from the scorpion *Pandinus imperator*". en *The Journal of Biological Chemistry*, **272**, No. 18, 11886-11894p.

En este trabajo nos propusimos purificar y caracterizar uno de los componentes del veneno del alacrán *Pandinus imperator* que inhibe la unión de rianodina a su receptor.

Utilizando tres pasos de cromatografía, exclusión molecular, intercambio iónico y fase reversa, logramos purificar a homogeneidad un componente al que llamamos toxina inhibitoria del veneno de *Pandinus imperator*, abreviado IpTxi. Este componente de naturaleza peptídica resultó estar constituido por dos cadenas polipeptídicas, una corta de 27 aminoácidos y una larga de 104 aminoácidos, esta última con gran parecido a fosfolipasas de tipo A2 (abeja y heloderma). En la literatura no se encontraron otros péptidos parecidos a la cadena corta.

Se preparó una biblioteca de cDNA a partir de las glándulas del alacrán y se clono el gene que codifica para la IpTxi confirmándose así la secuencia de aminoácidos de IpTxi obtenida por secuenciación directa, utilizando la técnica de degradación automatizada de Edman.

Este componente del veneno del alacrán *Pandinus imperator* tiene la capacidad de inhibir la unión de [^{3}H]rianodina a su receptor, tanto tipo 1 como tipo 2 (esquelético y cardiaco), mediante un mecanismo que aparentemente no involucra la interacción directa de este péptido con el receptor.

Con el fin de investigar el mecanismo de inhibición de la unión de rianodina al receptor, se procedió a separar las dos cadenas y probar su actividad por separado, encontrando que ninguna de las dos cadenas por sí sola inhibía el pegado de rianodina a su receptor. Para descartar sí estos resultados se debían al procedimiento químico usado para separar las cadenas (reducción y carboximetilación) se decidió sintetizar químicamente la cadena corta

con tres variaciones, la cisteína de la posición 4 se cambió por metionina (C4M) o por alanina (C4A) o se conservo la cisteína (C). Los dos primeros se sintetizaron para evitar la formación de dímeros, encontrando que ninguno de estos péptidos tienen actividad sobre el receptor de rianodina.

Por otro lado, debido a que la actividad fosfolipasa es altamente dependiente de su estructura tridimensional se usó un reactivo especifico para inhibir la actividad fosfolipásica sin modificar su estructura. Se encontró que cuando la IpTxi es tratada con este reactivo (bromuro de p-bromo fenacilo), pierde tanto la actividad fosfolipásica como su capacidad de inhibir la unión de rianodina a su receptor. Estos resultados parecían indicar que la acción de esta toxina sobre el canal se debía al efecto fosfolipásico de la toxina y no a una unión directa al receptor.

Con el fin de confirmar esta hipótesis, se procedió a incubar la IPTxi con membranas de músculo esquelético y probar la capacidad del sobrenadante de esta reacción (ácidos grasos libres, producto de la actividad fosfolipásica de la toxina) sobre la unión de [^{3}H]rianodina a su receptor. Se encontró que este sobrenadante es capaz de inhibir la unión de rianodina marcada a membranas de músculo esquelético. A fin de confirmar estos resultados, se probaron diferentes concentraciones de diversos ácidos grasos, tanto en el bloqueo del canal en bicapas como en la unión de rianodina marcada al receptor de rianodina. Observando que los ácidos grasos bloquean el canal en forma dosis dependiente. Los resultados parecen apoyar la hipótesis de que el efecto inhibitorio de IpTxi es indirecto.

The Mechanism of Inhibition of Ryanodine Receptor Channels by Imperatoxin I, a Heterodimeric Protein from the Scorpion *Pandinus imperator**

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From the §Department of Physiology, University of Wisconsin Medical School, Madison, Wisconsin 53706, the ‡Department of Molecular Recognition and Structural Biology, Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca, Morelos 62271, Mexico, and the ¶National Institute of Mental Health, Unit on Molecular Structures, Bethesda, Maryland 20892

We present an in-depth analysis of the structural and functional properties of Imperatoxin I (IpTx$_i$), an ~15-kDa protein from the venom of the scorpion *Pandinus imperator* that inhibits Ca^{2+} release channel/ryanodine receptor (RyR) activity (Valdivia, H. H., Kirby, M. S., Lederer, W. J., and Coronado, R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 12185–12189). A cDNA library was prepared from the venomous glands of this scorpion and used to clone the gene encoding IpTx$_i$. From a single continuous messenger RNA, the information coding for the toxin is translated into two mature polypeptide subunits after elimination of a basic pentapeptide. The IpTx$_i$ dimer consists of a large subunit (104-amino acid residues) with phospholipase A$_2$ (PLA$_2$) activity covalently linked by a disulfide bond to a smaller (27 amino acid residues), structurally unrelated subunit. Thus, IpTx$_i$ is a heterodimeric protein with lipolytic action, a property that is only shared with β-bungarotoxins, a group of neurotoxins from snake venoms. The enzymatic subunit of IpTx$_i$ is highly homologous to PLA$_2$ from bee (*Apis mellifera*) and lizard (*Heloderma horridum*) venoms. The small subunit has no significant similarity to any other known peptide, including members of the Kunitz protease inhibitors superfamily that target the lipolytic effect of β-bungarotoxins. A synthetic peptide with amino acid sequence identical to that of the small subunit failed to inhibit RyR. On the other hand, treatment of IpTx$_i$ with *p*-bromophenacylbromide, a specific inhibitor of PLA$_2$ activity, greatly reduced the capacity of IpTx$_i$ to inhibit RyRs. These results suggested that a lipid product of PLA$_2$ activity, more than a direct IpTx$_i$-RyR interaction, was responsible for RyR inhibition.

Scorpion venoms contain families of small basic proteins that modify the gating mechanism of Na$^+$ channels or block with

high affinity K$^+$ channels of excitable cells (1, 2). These toxins have been invaluable tools in the identification, purification, structural mapping, and functional characterization of the corresponding ionic channels The study of other ionic channels have also been aided by toxins from poisonous animals. For instance, snake venoms contain potent neurotoxins that block acetylcholine receptors (3), and snail and spider venoms contain small molecular weight proteins directed against neuronal Ca^{2+} channels (4, 5). A Cl$^−$ channel-specific blocker peptide was also recently isolated from a scorpion venom (6). Thus, there exist a vast array of natural ligands useful for structural and functional characterization of ionic channels

The Ca^{2+} release channel of SR[1] constitutes the major pathway for Ca^{2+} release during the process of excitation-contraction coupling in cardiac and skeletal muscle (7) The Ca^{2+} release channel binds the plant alkaloid ryanodine with nanomolar affinity, hence the name ryanodine receptor (RyR) Ryanodine has been an invaluable tool in the structural and functional characterization of RyR The alkaloid binds to a conformationally sensitive domain on the RyR protein and may be used in binding assays as an index of the functional state of the channel (8, 9). However, ryanodine displays extremely slow dissociation kinetics that make its effect practically irreversible. Furthermore, certain concentrations of ryanodine may open RyRs while others may block them (10), leading to ambiguous results.

From the venom of the African scorpion *Pandinus imperator*, we isolated Imperatoxin I (IpTx$_i$), a ~15-kDa protein that inhibited [^{3}H]ryanodine binding to cardiac and skeletal SR by blocking RyR channels (11). At concentrations well above the half-maximal effective concentrations (ED$_{50}$) exhibited for RyR, IpTx$_i$ did not modify the binding of ligands targeted against other transporters and ionic channels of striated muscle (11). IpTx$_i$ blocked RyR rapidly and reversibly, and when injected in ventricular cells it decreased twitch amplitude and intracellular Ca^{2+} transients, suggesting a selective blockade of Ca^{2+} release from the SR (11).

In this study, we carried out an in-depth analysis of the mechanism of action of IpTx$_i$ on RyRs of cardiac and skeletal muscle. We determined the complete amino acid and nucleotide sequence of IpTx$_i$ and show that, like β-bungarotoxins, IpTx$_i$ is a heterodimeric protein composed of a high molecular weight subunit with PLA$_2$ activity and a small, structurally unrelated

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; *p*BPB, *p*-bromophenacyl bromide; PLA$_2$, phospholipase A$_2$; RyR, ryanodine receptor; lyso-PC, lysophosphatidylcholine; HPLC, high performance liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.

This paper is available on line at http://www-jbc.stanford.edu/jbc/

subunit. We also show that free fatty acids, lipid products of IpTx$_i$-PLA$_2$ activity, are involved in the inhibition of RyR. IpTx$_i$ thus offers an alternative way to block RyR distinct from ryanodine and other ligands that require a physical interaction with the RyR protein.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents — Protease lysine C (Lys-C) and restriction enzymes were from Boehringer Mannheim. Chemicals and solvents for peptide sequencing were from Millipore Co. Primers for polymerase chain reaction (lambda gt11 forward and reverse; 1218 and 1222) and Vent^R polymerase were from New England BioLabs. DNA sequencing kit (Sequenase version II), was from U. S. Biochemical Corp. Subcloning and sequencing vector (pBluescript phagemid; pKS), M13'−20, and M13 reverse sequencing primers were from Stratagene. Brain phosphatidylethanolamine and brain phosphatidylserine were from Avanti Polar Lipids. [³H]Ryanodine was from DuPont NEN. *p*-Bromophenacyl bromide (pBPB) was from Sigma.

Purification of IpTx$_i$ — *P. imperator* venom was obtained by electric stimulation of scorpions maintained alive in the laboratory. Venom (120 mg per batch) was suspended in double distilled water and centrifuged at 15,000 × *g* for 30 min. The supernatant was applied onto a column (0.9 × 190 cm) of Sephadex G-50 superfine (Pharmacia Biotech Inc.). Fractions were eluted with 20 mM NH$_4$OAc (pH 4.7) at a flow rate of 20 ml/h. Fraction II containing IpTx$_i$ was applied to a column (0.9 × 30 cm) of carboxymethyl (CM)-cellulose 32 (Whatman) equilibrated with 20 mM NH$_4$OAc (pH 4.7). Peptides were eluted at a flow rate of 20 ml/h with a linear gradient of 250 ml of 20 mM NH$_4$OAc (pH 4.7) and 250 ml of the same buffer containing 0.55 M NaCl. Peptides displaying capacity to inhibit [³H]ryanodine binding and phospholipase activity were dialyzed against deionized water (3 × 30 min), concentrated by lyophilization, and injected into a C$_4$ reverse-phase HPLC column (Vydac). IpTx$_i$ was eluted with a linear gradient of 0−60% acetonitrile in 0.1% trifluoroacetic acid running at 1 ml/min for 60 min. The purity and identity of IpTx$_i$ was confirmed by amino acid sequence analysis, as described for Na$^+$ channel-blocking peptides (12). IpTx$_i$ was quantified either by amino acid analysis or by absorbance at 280 nm ($A_{280\,nm}$) using an extinction coefficient (ϵ) = 14,952.

Amino Acid Analysis and Microsequencing of IpTx$_i$ — Amino acid analysis was performed on samples hydrolyzed in 6 N HCl with 0.5% phenol at 110 °C in evacuated, sealed tubes as described (13). Reduction of IpTx$_i$ with dithiothreitol and alkylation with iodoacetic acid was performed as described (13). Reduced and alkylated IpTx$_i$ was cleaved with protease Lys-C in 200 µl of 25 mM Tris-HCl (pH 7.2), and 1.0 mM EDTA, at a enzyme:peptide ratio = 1:100. Microsequence determination of native, reduced, and carboxymethylated toxin, and their peptide fragments was carried out with a 6400/6600 MilliGen/Biosearch Prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes.

Cloning and Sequencing of the cDNA Encoding IpTx$_i$ — Two oligonucleotides encoding for two different regions of IpTx$_i$ were synthesized as described (13). Oligonucleotide 1 (5'-AC(N)ATGTGGGG(N)AC(N)AA(C)G/A)TGGTG-3'; where *N* means any nucleotide) encoded for the first 8 amino acids of the amino terminus of the large subunit of IpTx$_i$. Oligonucleotide 2 (5'-GA(G/A)GC(N)GG(N)TA(T/C)GG(N)GC(N)TGGGC-3') encoded for residues 14−21 of the small subunit. Total RNA was purified from the venomous glands (telsons) of 30 scorpions. The telsons were pulverized in liquid nitrogen and poured into 10 ml of GT buffer (4 M guanidinium isothiocyanate, 0.025 M sodium citrate (pH 7.0), 0.5% *N*-lauryl sarcosine, and 0.03 M β-mercaptoethanol), vortexed, and centrifuged at 5,000 × *g* for 10 min. One ml of 2 M sodium acetate (pH 4) was added to the recovered supernatant. The resulting solution was extracted with 10 ml of water-saturated phenol plus 2 ml of chloroform. isoamyl alcohol (49:1, v:v), allowing to sit on ice for 15 min. After centrifuging at 10,000 × *g* for 20 min at 4 °C, the aqueous layer was precipitated with 1 volume of isopropyl alcohol, incubated at −20 °C for 1 h, and centrifuged at 10,000 × *g* for 20 min at 4 °C. The pellet was dissolved in 0.3 ml of GT buffer and 0.3 ml of isopropyl alcohol, incubated at −20 °C for 1 h, and pelleted again. The pellet was washed with 95% ethanol, briefly dried under vacuum, and resuspended in RNase-free water. Messenger RNA was purified following the instructions of the Hybond-mAP protocol (messenger affinity paper; Amersham Corp., RPN.1511). cDNA synthesis was performed as described (13) from 5 µg of mRNA. The cloning of the cDNA library was also performed as described (13). The screening of the cDNA library was performed with oligonucleotides 1 and 2 separately, but the clones detected with oligonucleotide 1 were analyzed first. Inserts of cDNA from positive clones

were polymerase chain reaction-amplified using λ gt11 forward and reverse primers. Polymerase chain reaction products were purified from agarose gel and ligated into the *Eco*RV site of pBluescript (pKS) phagemid The ligation reactions were used to transform *Escherichia coli* DH5-α cells. Plasmid DNA from white colonies was digested with *Bam*HI and *Hind*III to verify the size of the original inserts. Clones of interest were sequenced using the Sequenase^R kit version 2 (U. S. Biochemical Corp.) on both strands. Oligonucleotides λ gt11 forward, λ gt11 reverse, M13 -20, and M13 reverse were used for sequencing.

Sequence Comparisons — The amino acid sequence of the large and small subunits of IpTx$_i$ were compared with those of other proteins deposited in the protein data base of GenBank (Los Alamos National Laboratory, Los Alamos, NM) by computer analysis using the program Blitz version 1.5 (Biocomputing Research Unit, University of Edinburgh, UK).

Determination of Phospholipase A$_2$ Activity — Phospholipase A$_2$ activity was determined by the titration method of Shiloah *et al.* (14), using dilute egg yolk (1:20 in saline solution) as substrate. Liberation of acid was measured at pH 8.0 and 37 °C by titration with 5 mM NaOH under a constant stream of N$_2$. One unit of enzyme is defined as the amount of enzyme that liberates 1 µmol of free fatty acid/min under the above conditions. Inhibition of phospholipase A$_2$ with pBPB was carried out as described by Díaz *et al.* (15). One hundred µl of 200 µM IpTx$_i$ in 35 mM Tris (pH 8 0) were mixed with 10 µl of 2 mM *p*-BPB in acetone and incubated for 16 h at room temperature under dim light. The reaction was stopped by filtration in a Sephadex G-10 column. pBPB-treated IpTx$_i$ elutes in the void volume of the column, whereas the excess of pBPB is retarded.

Synthesis of the Small Subunit of IpTx$_i$ — The small subunit of IpTx$_i$ was synthesized by the solid phase method of Merrifield (16), using *t*-butyloxycarbonyl-amino acids. This subunit contains a Cys residue at position 4. To avoid formation of inter-peptide disulfide bonds, Cys^4 was substituted by Ala (peptide A), Met (peptide M), or Cys (peptide C) protected with 3-nitro-2-pyridinesulfonyl (thiol-protecting group that will not allow formation of disulfide bonds). At the end of the synthesis all three peptides were separated in a C$_{18}$ reverse-phase HPLC column, using the conditions described above. The purity of the synthetic peptides was confirmed by both amino acid analysis and microsequencing, as described above.

[³H]Ryanodine Binding Assays — [³H]Ryanodine binding to pig cardiac and rabbit skeletal SR vesicles was carried out for 90 min at 36 °C in 0.1 ml of 0.2 M KCl, 1 mM Na$_2$EGTA, 0.995 mM CaCl$_2$, 10 mM Na-Pipes (pH 7 2). The calculated free Ca^{2+} was 10 µM [³H]Ryanodine (68.4 Ci/mmol) was diluted directly in the incubation medium to a final concentration of 7 nM. Protein concentration was 0.2−0.4 and 0.3−0.5 mg/ml for skeletal and cardiac SR, respectively. Samples were filtered on Whatman GF/B glass fiber filters and washed twice with 5 ml of distilled water. A Brandel M24R cell harvester was used for filtration. Nonspecific binding was determined in the presence of 10 µM unlabeled ryanodine and has been subtracted from each sample.

Planar Bilayer Technique — Recording of single RyR in lipid bilayers was performed as described previously (17). Briefly, a phospholipid bilayer of phosphatidylethanolamine:phosphatidylserine (1:1 dissolved in *n*-decane to 20 mg/ml) was formed across an aperture of ∼300 µm diameter in a delrin cup. The *cis* chamber (900 µl) was the voltage control side connected to the head stage of a 200 A Axopatch amplifier, and the *trans* chamber (800 µl) was held at virtual ground. Both chambers were initially filled with 50 mM cesium methane sulfonate and 10 mM Tris/Hepes (pH 7.2). After bilayer formation, cesium methane sulfonate was raised to 300 mM in the *cis* side, and 100−200 µg of SR vesicles were added. After detection of channel openings, Cs$^+$ in the *trans* chamber was raised to 300 mM to collapse the chemical gradient. Single channel data were collected at steady voltages (−30 mV) for 2−5 min. Channel activity was recorded with a 16-bit VCR-based acquisition and storage system at a 10-kHz sampling rate. Signals were analyzed after filtering with an 8-pole Bessel filter at a sampling frequency of 1.5−2 kHz. Data acquisition and analysis were done with Axon Instruments software and hardware (pClamp v6.0, Digidata 200 AD/DA interface).

RESULTS

Purification of IpTx$_i$ — Purification of IpTx$_i$ from *P. imperator* venom was performed in three chromatographic steps as described under "Experimental Procedures" and shown in Fig. 1. After fractionation of whole venom in Sephadex G-50 (Fig. 1A), *five fractions were collected and assayed for effects on [³H]ry-anodine binding. Fraction 2 contained polypeptides in the*
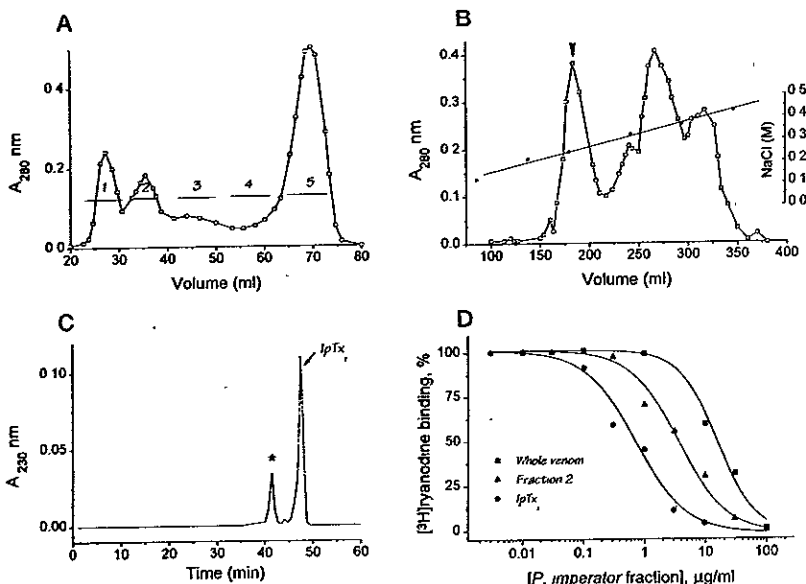
FIG. 1. **Purification of IpTx$_i$.** *A, P. imperator* soluble venom (120 mg of protein) was applied to a Sephadex G-50 column (0.9 × 190 cm) equilibrated and run with 20 mM ammonium acetate buffer (pH 4.7). 5-ml samples were collected, and tested for their capacity to inhibit [³H]ryanodine binding. Only fraction 2 totally inhibited [³H]ryanodine binding. *B*, fraction 2 was further separated through a CM-cellulose column (0 9 × 30 cm), equilibrated, and run with 20 mM ammonium acetate buffer (pH 4.7). A linear gradient of sodium chloride resolved 5 subfractions, the first of which (indicated by the *arrow*) contained IpTx$_i$. *C*, the subfraction from the CM-cellulose column containing IpTx$_i$ was lyophilized and injected into a C₄ reverse-phase HPLC column and eluted with a 0–100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The peak labeled with an *asterisk* was not studied further. *D*, dose-response curve for whole venom and purified components [³H]Ryanodine (7 nM) was incubated with cardiac SR protein in 0.2 M KCl, 10 μM CaCl$_2$, 10 mM Na-Hepes (pH 7.2) in the absence (control, 100%) and the presence of indicated concentrations of venom components. Nonspecific binding was determined in the presence of 20 μM ryanodine and has been subtracted from this and subsequent results.

range of 8–16 kDa and was the only fraction that inhibited [³H]ryanodine binding. The second step in the purification of IpTx$_i$ consisted of ion-exchange chromatography in CM-cellulose (Fig. 1B). The fraction containing IpTx$_i$ (indicated by the *arrow*) eluted early in the run, when the concentration of NaCl was ~160 mM. Fig. 1C shows the chromatographic profile of IpTx$_i$ after elution from a reverse-phase C₄ HPLC column. Only a minor contaminant was present (labeled with *asterisk*), which was discarded for further studies of IpTx$_i$ structure. However, amino acid sequence analysis of purified IpTx$_i$ yielded two different amino acids per reaction cycle, with an apparent equivalent stoichiometry. This indicated either that a contaminant was still present at the end of our purification procedure or that IpTx$_i$ was composed of two different peptide subunits.

We determined the dose-response relationship for each of the active venom components to assess their potency to inhibit RyRs (Fig. 1D). Whole *P. imperator* venom and fraction 2 inhibited [³H]ryanodine binding to cardiac SR with a concentration of 20.2 and 4.3 μg/ml, respectively, yielding the half-maximal effect (IC$_{50}$); IC$_{50}$ for pure IpTx$_i$ was 0.7 μg/ml. Based on this value and the molecular mass of IpTx$_i$ (~15 kDa), the estimated apparent dissociation constant (K_d) was 46 nM. Essentially identical results were obtained when *skeletal* SR was used for displacement studies (not shown).

*Electrophoretic Analysis of IpTx$_i$—*To elucidate whether IpTx$_i$ was composed of two subunits, an aliquot of IpTx$_i$ was reduced and alkylated. The modified toxin was then chromatographed in a Bio-Gel P30 column (Fig. 2A) to eliminate reaction

by-products and excess of reagents. Three peaks were obtained (Fig. 2A) and analyzed by SDS-PAGE (Fig. 2B). Peak 1 (labeled *Red*, abbreviation for reduced) had lower molecular mass than native IpTx$_i$ (labeled *Nat*); peak 2 could not be resolved in the same gel (not shown), but direct sequencing indicated that it corresponds to a ~3-kDa peptide (Fig. 3A); peak 3 was not peptidic in nature, indicating that it corresponded to the reducing and alkylating reagents. Fig. 2C shows that the apparent molecular mass of native IpTx$_i$ was ~15 kDa and that of the reduced IpTx$_i$ was ~12 kDa. Thus, treatment of IpTx$_i$ with thiol-reducing agents separates a large (~12 kDa) from a small (~3 kDa) subunit.

*Peptide Sequence Determination—*The reduced and alkylated derivatives of IpTx$_i$ (peak 1 and 2 of Fig. 2A) were sequenced by direct Edman degradation. As shown in Fig. 3A, we identified the first 27 amino acid residues of the large subunit, and all 28 amino acid residues of the small subunit To identify the remaining residues, the large subunit was cleaved with Lys-C endopeptidase, which yielded three peptide fragments that eluted with different retention times in an HPLC column (data not shown). The amino acid sequence of each of these peptides, obtained also by Edman degradation, is *underlined* in Fig. 3A

*Cloning and Sequencing of the cDNA Encoding IpTx$_i$—*Two oligodeoxynucleotides synthesized according to the amino acid sequence of stretch 1–8 of the large subunit and stretch 14–21 of the small subunit were used to screen a cDNA library, constructed from the venomous glands of *P. imperator* scorpi-

FIG. 2. **Separation of large and small subunits of IpTx₁.** *A,* IpTx₁ (100 μg) was reduced and alkylated as described under "Experimental Procedures," applied to a Bio-Gel P30 column (0.9 × 27 cm), and eluted with 10% acetic acid. Peak 1 corresponds to a ~12-kDa peptide, peak 2 to a ~3-kDa peptide (determined by direct sequencing), and peak 3 to reaction by-products. *B,* SDS-PAGE analysis of 3 μg of native IpTx₁ *(Nat)* and 3 μg of peak 1 *(Red)*. Gel was 10% polyacrylamide. Proteins were stained with Coomassie Blue. The running gel *(R.G.)* and the tracking dye *(T.D.)* positions are indicated by *arrows.* *C,* the retention factor *(R_f)* for various molecular weight markers is plotted as a function of log₁₀ molecular weight *(M.W.).* The apparent molecular weight for native and reduced IpTx₁ was extrapolated from a linear regression to the data points.
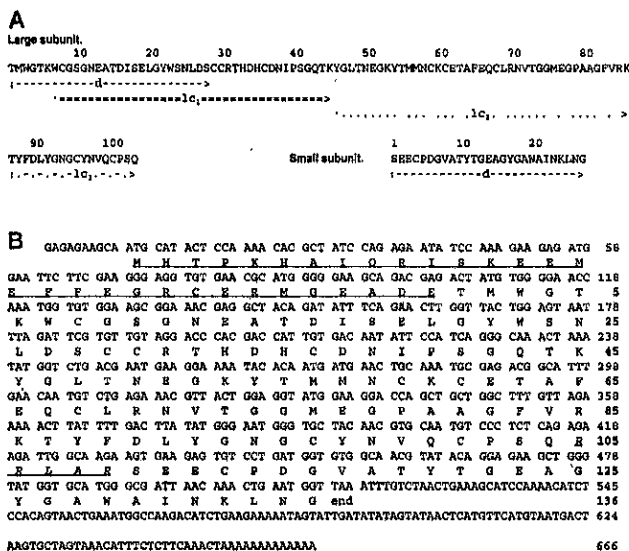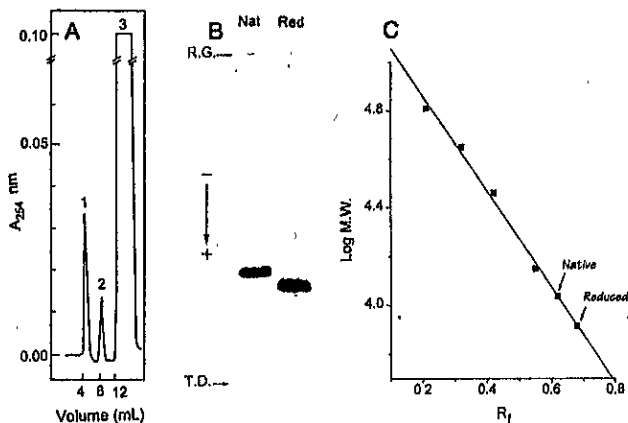

FIG. 3. **Amino acid and nucleotide sequences of IpTx₁.** *A,* the complete amino acid sequence of IpTx₁ was obtained from direct Edman degradation using peak 1 (large subunit) and peak 2 (small subunit) of Fig. 2A (labeled *d*) and from proteolytic fragments *(lc₁, lc₂,* and *lc₃)* of peak 1. To obtain the proteolytic fragments, peak 1 (100 μg) was incubated for 4 min at 36 °C with 1 μg of Lys-C endopeptidase in Tris buffer as specified in the Boehringer catalog. The reaction products were separated by HPLC, essentially as described in the legend to Fig. 1A. *Numbers on top* of the amino acid sequence correspond to the amino acid positions in the primary structure. *B,* nucleotide sequence of the cDNA gene encoding for the entire IpTx₁ molecule with the deduced amino acid sequence below each base triplet. The putative signal peptide (relative position −31 to −1) is *underlined*. The pentapeptide *RRLAR* (relative position 105–109) is not found in the mature protein and is assumed to be cleaved during processing. *Numbers on right* refers to the nucleotide and amino acid positions.

A

Large subunit.

```
        10         20         30         40         50         60         70         80
TMHGTKWCGSGNEATDISELGYWSNLDSCCRTHDHCDNIPSGQTKYGLTNEGKYTHQNCKCETAFEQCLRNVTGGMEGPAAGFVRK
(------------d------------->
        '===================lc₁===================>
                                        '........ ... ... ,lc₁, ...... . .... . >

        90        100                                    1        10         20
TYFDLYGNGCYNVQCPSQ             Small subunit.   SEECPDGVATYTGEAGYGANAINKLNG
(.-.-.-.-lc₃.-.-.>                              (-------------d------------->
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B

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GAGAGAAGCA ATG CAT ACT CCA AAA CAC GCT ATC CAG AGA ATA TCC AAA GAA GAG ATG   56
            M   H   T   P   K   H   A   I   Q   R   I   S   K   E   E   M
GAA TTC TTC GAA GGG AGG TGT GAA CGC ATG GGG GAA GCA GAC GAG ACT ATG TGG GGA ACC 118
 E   F   F   E   G   R   C   E   R   M   G   E   A   D   E   T   M   W   G   T    5
AAA TGG TGT GGA AGC GGA AAC GAG GCT ACA GAT ATT TCA GAA CTT GGT TAC TGG AGT AAT 178
 K   W   C   G   S   G   N   E   A   T   D   I   S   E   L   G   Y   W   S   N   25
TTA GAT TCG TGT TGT AGG ACC CAC GAC CAT TGT GAC AAT ATT CCA TCA GGG CAA ACT AAA 238
 L   D   S   C   C   R   T   H   D   H   C   D   N   I   P   S   G   Q   T   K   45
TAT GGT CTG ACG AAT GAA GGA AAA TAC ACA ATG ATG AAC TGC AAA TGC GAG ACG GCA TTT 298
 Y   G   L   T   N   E   G   K   Y   T   M   M   N   C   K   C   E   T   A   F   65
GAA CAA TGT CTG AGA AAC GTT ACT GGA GGT ATG GAA GGA CCA GCT GCT GGC TTT GTT AGA 358
 E   Q   C   L   R   N   V   T   G   G   M   E   G   P   A   A   G   F   V   R   85
AAA ACT TAT TTT GAC TTA TAT GGG AAT GGG TGC TAC AAC GTG CAA TGT CCC TCT CAG AGA 418
 K   T   Y   F   D   L   Y   G   N   G   C   Y   N   V   Q   C   P   S   Q   R  105
AGA TTG GCA AGA AGT GAA GGC TGT CCT GAT GGT GTG GCA ACG TAT ACA GGA GAA GCT GGG 478
 R   L   A   R   S   E   E   C   P   D   G   V   A   T   Y   T   G   E   A   G  125
TAT GGT GCA TGG GCG ATT AAC AAA CTG AAT GGT TAA ATTTGTCTAACTGAAAGCATCCAAAACATCT 545
 Y   G   A   W   A   I   N   K   L   N   G  end                                 136
CCACAGTAACTGAAATGGCCAAGACATCTGAAGAAAAATAGTATTGATATATAGTATAACTCATGTTCATGTAATGACT 624
AAGTGCTAGTAAACATTTCTCTTCAAACTAAAAAAAAAAAAAA                                    666
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ons. These oligodeoxynucleotides were used as probes to isolate a full-length IpTx₁ cDNA clone. Both probes led to the isolation of the same cDNA clone. This complete nucleotide sequence is shown in Fig. 3*B*. This sequence contained an open reading frame of 167 amino acids encompassing 1) a putative signal peptide (first underlined 31 amino acids, positions −31 to −1); 2) the mature ~12-kDa, large subunit of IpTx₁ (104 amino acids, positions 1–104); 3) a putative connector pentapeptide *(RRLAR,* positions 105–109); and 4) the mature small subunit (27 amino acids; positions 110–136). Thus, a single continuous cDNA clone encoded the two polypeptide subunits of IpTx₁. This finding confirmed that IpTx₁ is a heterodimeric protein. Since treatment of IpTx₁ with thiol-reducing agents (Fig. 2A) effectively separates both subunits, this suggests that they are covalently linked by a disulfide bridge.

Sequence Homology of IpTx₁ Subunits—A comparison of the amino acid sequence of the two subunits of IpTx₁ with se-

quences available from GenBank revealed intriguing results. First, the small subunit shared no significant similarity with sequences of scorpion peptide blockers of Na⁺, K⁺, or Cl⁻ channels, with members of the Kunitz protease inhibitor superfamily, or with any other sequence deposited in the protein data base. Thus, this small subunit constitutes a new class of scorpion peptides. On the other hand, Fig. 4 shows that the large subunit of IpTx₁ was 38% homologous to PLA₂ from honey bee *(A. mellifera)* venom and 35% homologous to PLA₂ from heloderma *(H. horridum)* venom. These two PLA₂s compose group III of secreted PLA₂ (18), whose main features are low molecular mass (~14 kDa), high disulfide bond content, and strict dependence on Ca²⁺ for lipolytic effect. Since the major criterion for classification is sequence homology more than function, IpTx₁ may be classified within this group with the remarkable difference, however, of possessing an accessory protein.

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 -1    1                                       50
      TWNGTKWCQSGWEATDISELGYNSHLDSCCRTHDHC-DNIPS-GQTKYGL
      IIYPOTLWCQHGNKSSGPNELGRFKHTDACCRTHDHCPDVH-SAGESKHGL
      GAFIKPOTLWCQAGWAASDYSQLGTEKDTDWCCRDHDHC-ERWISALEYKHGH

      51                                      100
      TWZGKYTWWCKCETAFEQCLRNVTGGWEGPAAGFVRKTYFD-LYGNGCY
      TWTASHTRLSCDCDDKFYDCLKNSADTISSY---FVGKHYFN-LIDTKGY
      RWYYPSTISHCDCDWQFRSCLWKLKDGTADY----VGQTYFNVLKIP-CF

      101                                     150
      BVQCPSQ ...........................................
      KLEHPVTGCGERTEGR-CLHYTVDKSKPK-VYQWFDLRKYXXNSRG-GSX
      ELEEGE-GCVDWNFWLECTESKIMPVAKLVSAAPYQAQAETQSGEG ...

      151
      ........ (IpTx₁)
      SYHFEIVY (Bee phospholipase)
      ........ (Heloderma phospholipase)
```

FIG. 4. **Amino acid sequence homology between the large subunit of IpTx₁ and PLA₂s from group III.** The amino acid sequence of the large subunit of IpTx₁ (*first row*) was aligned with that of bee (*A. mellifera, second row*) and lizard (*H. suspectum, third row*) venom PLA₂. A few gaps (*dashes*) were introduced to enhance similarities. *Bold letters* indicate exact homology for the three PLA₂s. *Dots* indicate end of the sequence, whereas *X* indicates unknown residues, as indicated in the reference source. Amino acid sequences taken from IpTx₁, this work; bee PLA₂, GeneBank Accession No. X16709; *Heloderma suspectum* PLA₂, Gomez *et al.* (32).

*Lack of Inhibition by the Short Subunit of IpTx₁—*We tested if the small subunit of IpTx₁ was responsible for blocking RyR. Reduced and carboxymethylated small subunit (peak 2 of Fig. 2A), at concentrations up to 15 µM, had no effect on the binding of [³H]ryanodine to cardiac or skeletal RyR (data not shown). To discard the presence of spurious reagents as being the cause of negative results, we synthesized the small subunit in three distinctive forms as follows: peptide C was similar to the native small subunit, and peptides A and M were also similar to the native small subunit, except that Cys⁴ was substituted by Ala and Met, respectively. This avoided the formation of interpeptide disulfide bridges caused by oxidation of Cys⁴. The three peptides were HPLC-purified before testing. Table I shows that none of the purified synthetic peptides had significant effect on the binding of [³H]ryanodine to cardiac RyR or on the binding of [³H]PN200–110 to voltage-dependent Ca²⁺ channels of sarcolemma (19).

*The Phospholipase A₂ Activity of IpTx₁ Is Important to Inhibit RyR—*We next investigated if the large subunit of IpTx₁, or its enzymatic activity, was involved in RyR inhibition. The reduced and carboxymethylated form of the large subunit of IpTx₁ (peak 1) was incapable of inhibiting [³H]ryanodine binding to cardiac or skeletal RyR. However, this was expected given that internal disulfide bridges are essential for preservation of the toxin's tridimensional structure as well as for expression of its enzymatic activity (20). We thus resorted to *p*BPB, a covalent modifier of His residues that specifically blocks phospholipase A₂ activity (15) without affecting disulfide bonds The rate of fatty acid liberation by 1 mol of IpTx₁ was 384 ± 26 and 41 ± 12 mol min⁻¹ in control and after incubation with *p*BPB, respectively (*n* = 4; data not shown). Thus, treatment of IpTx₁ with *p*BPB decreased substantially its PLA₂ activity. Fig. 5 shows that *p*BPB-treated IpTx₁ decreased dramatically its capacity to inhibit the binding of [³H]ryanodine to cardiac and skeletal RyR (*open symbols*). This was in contrast to control IpTx₁ (a batch of IpTx₁ that underwent the same treatment as *p*BPB-treated IpTx₁, except that *p*BPB was omitted), which retained its capacity to inhibit cardiac and skeletal RyR with high affinity (*filled symbols*).

*Ca²⁺ Dependence of IpTx₁ Effect—*The enzymatic activity of PLA₂ from group III requires Ca²⁺ for catalysis (20). Therefore, we expected that if the inhibition of RyRs by IpTx₁ resided in its enzymatic activity, inhibition should be apparent only in the presence of micromolar [Ca²⁺] Fig. 6 shows that Ca²⁺ was

TABLE I

Effect of synthetic peptide analogs of the small subunit of ipTx₁ on the binding of [³H]ryanodine and [³H]PN200–110 to cardiac sarcolemma and SR vesicles

Peptide concentration tested was 10 µM. Results are the mean ± S.D of three independent determinations.

	[³H]Ryanodine binding[a]	[³H]PN200–110 binding[b]
	%	%
Control	100	100
Peptide A	116 ± 8	100 ± 6
Peptide M	110 ± 4	111 ± 12
Peptide C	106 ± 5	111 ± 7

[a] Binding of [³H]ryanodine to SR vesicles was carried out as described in the text.
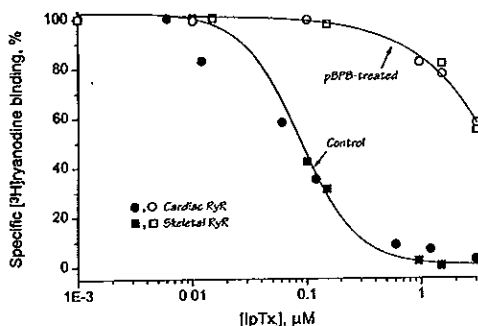[b] Binding of [³H]PN200–110 to cardiac sarcolemma was performed as described (19).

FIG. 5. **Treatment with a PLA₂ inhibitor lowers the potency of IpTx₁ to inhibit RyR.** IpTx₁ (200 µM) was incubated with 2 mM *p*BPB (*filled symbols*) for 16 h as described under "Experimental Procedures" or with acetone (*open symbols*), the drug vehicle, as control. Binding of [³H]ryanodine to cardiac (*circles*) or skeletal (*squares*) SR was performed in the absence (defined as 100%) or in the presence of indicated concentrations of IpTx₁.

essential for binding of [³H]ryanodine to RyRs and for detection of the IpTx₁ effect. The *left panel* shows the Ca²⁺ dependence of [³H]ryanodine binding to *skeletal* SR and the effect of IpTx₁. Specific binding in control (*open circles*) had a threshold for detection at 100 nM [Ca²⁺] (*p*Ca 7) and was optimal at 10–100 µM [Ca²⁺]. Higher [Ca²⁺] inhibited binding, giving rise to a *bell-shaped curve*. In the presence of IpTx₁ (*filled circles*), the binding curve was dramatically decreased in absolute values. The percentage of IpTx₁ inhibition was 5.5, 32, and 71% at *p*Ca 7, 6, and 5, respectively. No further inhibition was observed at higher [Ca²⁺]. Thus, the degree of IpTx₁ inhibition increased with [Ca²⁺].

In *cardiac* SR (Fig. 6, *right panel*), the Ca²⁺ dependence of [³H]ryanodine binding also had a threshold for detection at *p*Ca 7 and a maximum at *p*Ca 5 (control, *open circles*). However, unlike skeletal SR, inactivation of binding by high [Ca²⁺] was not pronounced. Binding decreased only by 30% with respect to maximum in cardiac RyRs, and it decreased by 80% in skeletal RyRs. This distinctive response of RyR to Ca²⁺ is also displayed by individual RyR reconstituted in lipid bilayers (8, 21), indicating that the binding assay effectively tracks the activity of the receptor. In the presence of IpTx₁, the binding curve was markedly decreased The percentage of inhibition was 10, 38, and 83% at *p*Ca 7, 6, and 5, respectively. Thus, although cardiac and skeletal RyRs respond differently to Ca²⁺, the inhibitory effect of IpTx₁ increased equally with [Ca²⁺] in both isoforms.
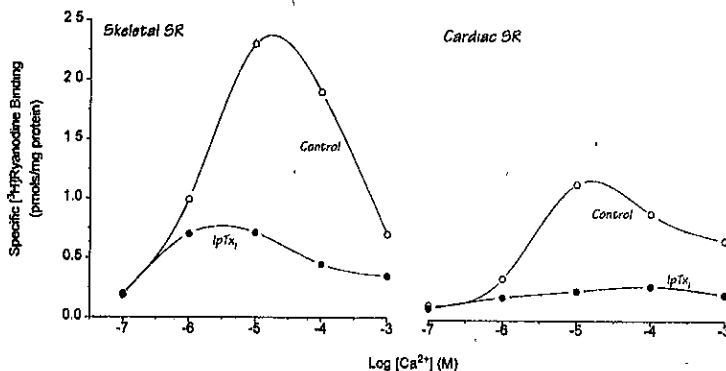
Inhibition of RyR by Supernatant of IpTx₁-treated SR Vesi-

FIG. 6. **Ca^{2+} dependence of IpTx$_i$ inhibition of [^{3}H]ryanodine binding.** Rabbit skeletal (0.3 mg/ml) or pig cardiac (0.4 mg/ml) SR vesicles were incubated for 90 min at 36 °C with 7 nM [^{3}H]ryanodine in the absence *(open circles)* or the presence *(filled circles)* of 200 nM IpTx$_i$. The incubation medium consisted of 0.2 M KCl, 10 mM Na-Hepes (pH 7.2), 1 mM EGTA, and CaCl$_2$ necessary to bring free [Ca^{2+}] to the desired value. The Ca^{2+}:EGTA ratios were calculated by a computer program using affinity constants given in Fabiato (33).

cles — The membranes of SR vesicles contain different classes of phospholipids that may serve as substrates for the PLA$_2$ activity of IpTx$_i$. We reasoned that if the inhibitory properties of IpTx$_i$ were at least partly due to its enzymatic activity, then inhibition should be observed by incubating RyR with supernatant of IpTx$_i$-treated SR vesicles. Control SR vesicles were diluted in binding medium to 1 mg/ml and incubated at 36 °C with 100 nM IpTx$_i$. After 30 min, the IpTx$_i$-treated SR vesicles were pelleted at 32,000 × *g* for 15 min, and a clear supernatant was obtained. The supernatant was then tested for its capacity to inhibit the binding of [^{3}H]ryanodine to cardiac SR vesicles. A control tube that contained only IpTx$_i$ in binding medium without SR vesicles was run in parallel to determine the percentage of inhibition caused by the toxin alone. Fig. 7 shows that increasing concentrations of IpTx$_i$-treated SR supernatant inhibited the binding of [^{3}H]ryanodine dose-dependently *(filled circles)*. This inhibition could not be attributed to IpTx$_i$ because the control supernatant reduced binding only marginally *(open circles)*. For instance, in the most extreme case, 10 μl of IpTx$_i$-treated SR supernatant inhibited 100% of specific binding; the estimated concentration of IpTx$_i$ in this tube was 10 nM, which inhibited binding only by 22%. Hence, the lipolytic activity of IpTx$_i$ releases from SR vesicles a phospholipid product that is capable of inhibiting RyR activity.

Reversibility of IpTx$_i$ Effect—To rule out the possibility that inhibition of RyR by IpTx$_i$ was caused by an irreversible disruption of the channel protein, we incubated SR vesicles (1 mg/ml) with water (control) or with 1 μM IpTx$_i$ (IpTx$_i$-treated SR). After 30 min at 36 °C, an aliquot was taken from each sample. Then, both SR samples were washed twice, pelleted, and prepared for [^{3}H]ryanodine binding. Fig. 8 shows that binding of [^{3}H]ryanodine to IpTx$_i$-treated vesicles was decreased with respect to control after 30 min of incubation. However, these vesicles displayed essentially the same binding as control after washing off IpTx$_i$. Thus, RyRs recover their capacity to bind [^{3}H]ryanodine after treatment with IpTx$_i$. Assuming that the [^{3}H]ryanodine binding assay followed changes in channel gating (8–10, 17, 21), the changes described above suggest that IpTx$_i$ released a phospholipid product that bound to RyRs (or a closely associated regulatory protein) and decreased open probability (p_o); after removal of the phospholipid product, p_o could recover to control levels.

Single Channel Effects of IpTx$_i$—To test directly the above hypothesis and to gain insight on the manner in which IpTx$_i$
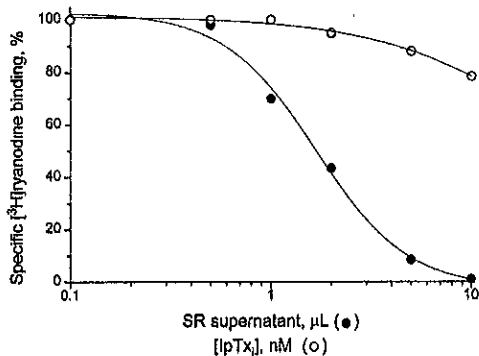

FIG. 7. **Inhibition of RyR by supernatant of IpTx$_i$-treated SR.** SR supernatant was prepared as described in the text. Binding of [^{3}H]ryanodine to cardiac SR vesicles was carried out in the absence (control, 100%) or presence of indicated volumes of SR supernatant *(filled symbols)*. To keep the reaction volume constant, binding medium was added to SR supernatant to a final volume of 10 μl. To determine the inhibition caused by IpTx$_i$ alone, control vials containing IpTx$_i$ without SR vesicles were spun in parallel. The final concentration of IpTx$_i$ in the binding assays and the inhibition associated with it is shown by the *open symbols*.

inhibits RyRs, we reconstituted swine cardiac RyR in planar lipid bilayers, as described (11, 17). Fig. 9 shows traces from continuous recording at −30 mV holding potential in the absence (control) and the presence of increasing concentrations of IpTx$_i$. Channel activity was monitored over 80 s in each condition, and the mean p_o was obtained from current histograms. The traces show that IpTx$_i$ blocks RyR dose-dependently: at low concentrations (≤200 nM) IpTx$_i$ decreases the lifetime of the open events, converting long openings into brief, frequently unresolved open events; at higher concentrations, both long and brief openings progressively decrease in frequency until they disappear. A quantitative description of this effect is presented in the open time histograms of Fig. 9B. In control, 2,034 events could be fitted with three exponentials with mean open time (τ) = 0.7 ms (66%), 1.9 ms (29%), and 9.8 ms (5%). In the presence of 500 nM IpTx$_i$, the histogram for 801 openings collected during the same period as control was monoexponential
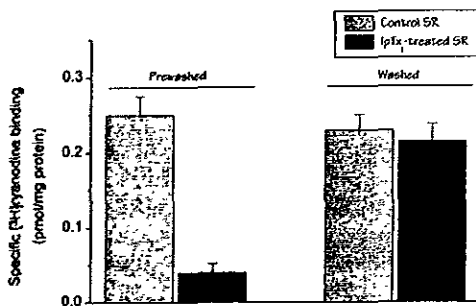
Fic 8 **Reversibility of IpTx$_i$ effect.** Prewashed vesicles are car-
diac SR vesicles incubated with water (control) or with 1 μM IpTx$_i$
(IpTx$_i$-treated SR) for 30 min at 36 °C. Washed vesicles are the same
control and IpTx$_i$-treated vesicles after being pelleted and washed twice
with binding medium. Binding of [³H]ryanodine was conducted in all
vesicles and is expressed in absolute values (pmol/mg).

with $\tau = 0.8$ ms. Both the decrease in the frequency and
duration of the open events contributed to the decrease in p_o.
Fig. 9C shows the log dose-response relation of p_o as a function
of IpTx$_i$ concentration. The IC$_{50}$ for IpTx$_i$ inhibition was 140
nM. Since this value is in fairly good agreement with that
determined in binding experiments (50–80 nM), this indicates
that [³H]ryanodine, at the concentrations used, does not inter-
fere with the inhibitory capacity of IpTx$_i$. More importantly,
the results show that the lipolytic product of IpTx$_i$ is capable of
interacting directly with the RyR or with a closely associated
protein that regulates channel gating.

Inhibition of RyR by Fatty Acids—PLA$_2$ catalyzes the hydrol-
ysis of the S_n2 fatty acyl bond of phospholipids to release free
fatty acids and lysophospholipids (22). We tested the effect of
each of these lipids separately on RyR activity. Fig. 10A shows
that lysophosphatidylcholine (lyso-PC), at concentrations up to
300 μM, does not modify substantially the open lifetime or the
unitary conductance of cardiac RyR. On the other hand, linoleic
acid, an 18-carbon unsaturated fatty acid, blocks RyR at lower
concentrations and in a manner that is reminiscent of the
blockade produced by IpTx$_i$. Addition of 30 μM linoleic acid to
the cytosolic face of the channel decreases the lifetime and the
frequency of open events; similar to IpTx$_i$, the fatty acid in-
duces the appearance of openings that are too fast to be re-
solved by our recording bandwidth.

The potency of several fatty acids to inhibit RyRs was deter-
mined by their capacity to inhibit [³H]ryanodine binding to
cardiac SR vesicles (Fig. 10B). Palmitic acid, a 16-carbon sat-
urated fatty acid, partially inhibited RyR but at concentrations
$\geq$100 μM. Arachidonic acid, linoleic acid, and oleic acid, unsat-
urated fatty acids abundant in SR membranes (23), totally
inhibited RyR with an IC$_{50}$ = 25,
55, and 70 μM, respectively. In agreement with single channel
results, lyso-PC and lysophosphatidylethanolamine were un-
able to modify [³H]ryanodine binding. Thus, not all PLA$_2$ prod-
ucts are capable of inhibiting RyRs. Among the fatty acids, the
potency to inhibit RyRs increases with the carbon chain length
and the number of unsaturations.

DISCUSSION

In the present study, we determined the complete amino acid
and nucleotide sequence of IpTx$_i$, a heterodimeric protein from
the venom of the scorpion *P. imperator*, and unraveled the
molecular mechanism by which it inhibits RyRs of cardiac and
skeletal muscle.

The deduced amino acid sequence from the cDNA encoding

IpTx$_i$ and the amino acid sequence determined by Edman deg-
radation strongly suggests that IpTx$_i$ is synthesized as a pre-
cursor of the pre-pro form. Putative signal and connector pep-
tides must be removed to produce mature IpTx$_i$. The proposed
signal peptide does have a large content of acidic residues (8
out of the last 18 residues are acidic), a property that is rarely
seen in signal peptides. For this reason, we cannot exclude
whether the proposed signal peptide codes for another peptide
before coding for the large subunit of IpTx$_i$. Regarding the
connecting pentapeptide Arg-Arg-Leu-Ala-Arg (positions 105 to
109 in the cDNA sequence of Fig. 3), we gather that several
enzymes must be required to eliminate it from the mature
protein. Initially, a cleavage must occur at Arg[109] (monobasic
site), as it occurs in the maturation of prosomatostatin and
other prohormones (24). Two characteristics distinguish this
cleavage site and both are present in the cDNA of IpTx$_i$: (i) Arg
(or other basic residue) is immediately preceded by Ala or Leu
(or both); (ii) Arg is located three or five residues upstream the
basic amino acid involved in the cleavage. Next, another cleav-
age must occur at Arg[106] (dibasic site) as is the case during the
maturation of diverse peptidic hormones (25). A third enzyme
with carboxypeptidase activity must intervene to remove
Arg[105] and Arg[106]. Because of this complicated pattern of mat-
uration, we assume that the linking of the small and large
subunit of IpTx$_i$ must be important for the function of IpTx$_i$.

Mature IpTx$_i$ is composed of a ~3-kDa peptide covalently
linked to a ~12-kDa peptide with PLA$_2$ activity. The large
subunit of IpTx$_i$ conserves the most important substructures of
secreted PLA$_2$ from group III (Fig. 4) The His/Asp pair, essen-
tial for Ca^{2+} binding, is in position 33/34 of IpTx$_i$ and is pre-
ceded in the three PLA$_2$s by a Cys-Cys-Arg motif; the amino
terminus (roughly residues 4–12 of IpTx$_i$), presumably in-
volved in substrate binding, is also highly conserved in the
three PLA$_2$s; lastly, the Cys residues, essential for maintaining
proper folding through disulfide bonds, may be used in this
group as the elemental frame to align homologous sequences
and to identify stretches of sequence that have been inserted or
deleted through evolution. Only Cys[101] of IpTx$_i$ (relative posi-
tion 104 in Fig. 4) does not match with Cys residues present at
the carboxyl terminus of the other PLA$_2$ shown. It is likely,
therefore, that Cys[101] is involved in the disulfide bridging with
Cys^4 of the small subunit.

The presence in IpTx$_i$ of a PLA$_2$ subunit covalently linked to
a small, structurally unrelated peptide is reminiscent of the
bipartite arrangement of β-bungarotoxins (26), a group of
snake neurotoxins that inhibit neurotransmitter release (27).
Each β-bungarotoxin dimer has a PLA$_2$ subunit covalently
bound to a smaller subunit related to Kunitz-type protease
inhibitors. Members of the Kunitz superfamily bind to and
block voltage-dependent K$^+$ and Ca^{2+} channels (28). Thus,
although the small subunit of IpTx$_i$ showed no homology to
members of the Kunitz superfamily, its distinctive arrange-
ment within the toxin suggested that it might act as a blocker.
For this reason, it was surprising at first to realize that the
small subunit was not directly responsible for inhibiting RyRs.
Direct addition of the small subunit to our [³H]ryanodine bind-
ing assays failed to inhibit RyRs, as did a synthetic peptide
with amino acid sequence similar to that of the small subunit
and two additional derivatives designed to avoid interpeptide
disulfide bridge formation (Table I). Hence, the target site for
the small subunit, or its biological function, remains to be
determined

Given our failure to detect inhibition by the small subunit,
we turned our attention to the large subunit of IpTx$_i$. In a
reduced and carboxymethylated form, the large subunit of
IpTx$_i$ failed to inhibit RyRs (not shown). However, this was not

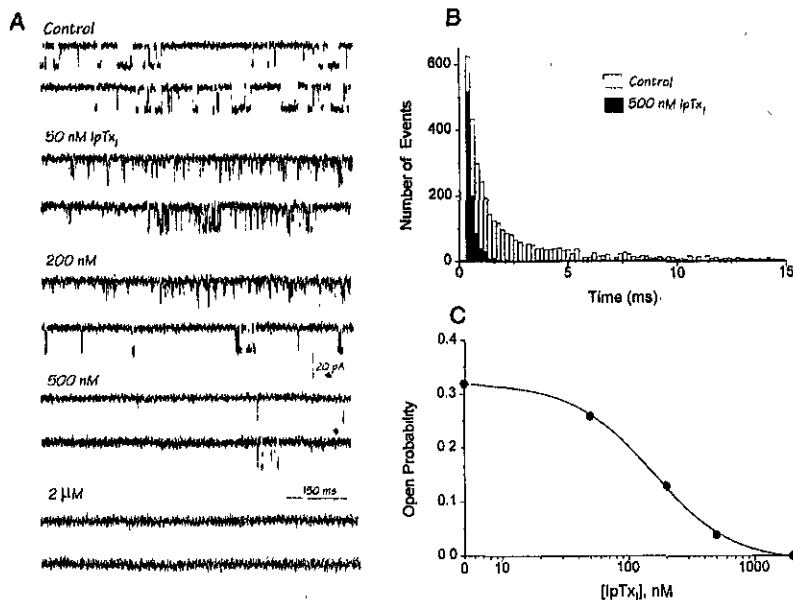FIG. 9. **Inhibition of cardiac RyR activity by IpTx$_i$.** *A*, pig cardiac RyR were reconstituted in planar lipid bilayers and recorded in the absence (control) and the presence of indicated concentrations of IpTx$_i$. Holding potential: −30 mV. Recording solution, symmetrical 300 mM cesium methane sulfonate and 10 mM Na-Hepes (pH 7.2). Under these conditions, Cs$^+$ flows from the luminal (trans) to the cytosolic (cis) side of the channel, and openings are represented by downward deflections of the base-line current. *B*, open time histograms in the absence (*open bars*) and the presence (*filled bars*) of 500 nM IpTx$_i$. *C*, plot of open probability (P_o) as a function of [IpTx$_i$]. *Smooth line* is a fit to data points using the expression $p_o = (p_o$ control$)/1 + (($IpTx$_i$]/IC$_{50})^{n_H}$. Where p_o control is the p_o in the absence of IpTx$_i$ (0.32); IC$_{50}$ is the concentration of IpTx$_i$ that produces half-maximal inhibition (140 nM), and n_H is the Hill coefficient (1.22).
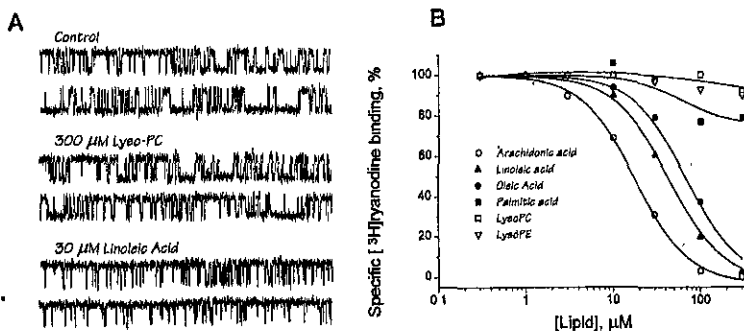

FIG. 10. **Selective inhibition of RyR by fatty acids.** *A*, representative traces of cardiac RyR activity in control (*top trace*) and in the presence of 300 μM lyso-PC (*middle trace*) or 30 μM linoleic acid (*bottom trace*). Openings are represented by downward deflections of the base-line current. For the experiment shown plus two additional experiments, mean p_o was 0.43 ± 0.09 (control), 0.46 ± 0.11 (lyso-PC), and 0.12 ± 0.06 (linoleic acid). *B*, the binding of 7 nM [^{3}H]ryanodine to cardiac SR vesicles was determined in the presence of methanol (maximal concentration = 1%) and this value defined as 100%, or in the presence of indicated concentrations of lipids dissolved in methanol.

surprising given that disulfide bridges are essential to maintain the toxin's three-dimensional structure (20). It was the treatment of IpTx$_i$ with *p*BPB, *a covalent modifier of His residues* that inhibits PLA$_2$ activity without affecting disulfide bridges (15), that decreased substantially the capacity of IpTx$_i$ to inhibit RyRs (Fig. 5). This suggested that a lipid product of PLA$_2$ activity was involved in the inhibition of RyRs. Three

separate lines of evidence supported this notion. 1) Inhibition of RyR by IpTx$_i$ was favored by the presence of Ca^{2+} (≥1 μM; Fig. 6), as expected from the Ca^{2+} dependence of enzymatic activity of PLA$_2$ from group III and several other groups (20). 2) The supernatant of IpTx$_i$-treated SR vesicles could inhibit RyRs even before IpTx$_i$ was present in a concentration large enough to do so (Fig. 7); this indicated that RyR inhibition was brought

about by a lipid product of IpTx$_i$ released from SR vesicles during the incubation period. 3) The kinetics of RyR inhibition by IpTx$_i$ were mimicked by direct addition of linoleic acid (but not of lyso-PC) to the cytoplasmic side of the channel (Figs. 9 and 10); other long chain, unsaturated fatty acids could also inhibit RyRs (Fig. 10). Thus, a potential scenario for inhibition of RyR by IpTx$_i$ is as follows: in the presence of micromolar [Ca^{2+}], the large subunit of IpTx$_i$ catalyzes the hydrolysis of the S_n2 fatty acyl bond of the phospholipids of the SR membrane. The reaction yields free fatty acids and lysophospholipids; the former are released into the incubation medium, and the latter may be liberated or remain embedded into the SR membrane. Free fatty acids bind to RyR or to a closely associated protein that controls gating. At low concentrations, they produce an incomplete block of RyR; higher concentrations gradually block the RyR completely, giving rise to the dose-response relationship of channel activity and [^{3}H]ryanodine binding *versus* IpTx$_i$ concentration of Figs 1, 5, and 9.

PLA$_2$s are abundant components of snakes, scorpions, and bee venoms and often constitute the main toxic component to mammals. Although all PLA$_2$s induce a variety of pathological symptoms including neurotoxicity and myotoxicity (29), they differ in their mechanism of action and in their molecular targets. For example, the snake neurotoxin crotoxin is composed of a PLA$_2$ subunit and an inhibitory subunit, which keeps the phospholipase inactive until binding to a presynaptic receptor triggers the dissociation of the inhibitory subunit (30). In contrast, notexin from *Netechis scutatus scutatus* is a PLA$_2$ without an associated subunit that produces muscle paralysis by binding to a specific receptor in the neuromuscular junction (31). IpTx$_i$ is the first example of a scorpion toxin in which a PLA$_2$ is found chaperoned by a smaller, structurally unrelated subunit. In a previous study (11), we found that several transporters and ion channels of striated muscle including the inositol trisphosphate receptor, the muscarinic receptor, voltage-sensitive Na$^+$ channels, and the Ca^{2+}-ATPase of SR were insensitive to micromolar concentrations of IpTx$_i$. These results argue against an indiscriminate effect on key molecules of excitation-contraction coupling by the lipolytic action of IpTx$_i$. However, an important question that still remains to be answered concerns the presence of the small subunit of IpTx$_i$. What is its molecular target? What function does it perform to enhance the PLA$_2$ activity or to suppress it while in transit to the specific receptor? Regardless of the primary site affected, a major contribution of this study was to establish the fact that the molecular mechanism involved in the toxicity of IpTx$_i$ will most likely involve abnormalities in intracellular Ca^{2+} mobilization due to blockade of RyRs.

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En este trabajo se describe la purificación, caracterización química y síntesis química de otra toxina del veneno del alacrán *Pandinus imperator*.

Este nuevo péptido llamado Imperatoxina a (IpTxa), por su efecto activador, a diferencia de la IpTxi, tiene la capacidad de aumentar la unión de [^{3}H]rianodina al receptor de tipo 1 (esquelético) y no tiene efecto sobre el receptor de rianodina tipo 2, lo que hace a esta toxina un valioso candidato para el estudio de este tipo de receptor, dado que hasta el momento no se ha descrito un compuesto que presente selectividad para alguna de las isoformas del receptor de rianodina. A las concentraciones en las que la IpTxa es efectiva en el RyR1, ésta no tiene efecto sobre otros canales de calcio o transportadores presentes en músculo o en cerebro (Valdivia y col 1992)

La Imperatoxina a es un péptido de 33 aminoácidos estructurado por 3 puentes disulfuro, carece de aminoácidos de carácter aromático y tiene la desventaja de encontrarse en muy pequeña proporción en el veneno total (0.03%), es por esto y por su tamaño que decidimos sintetizarlo químicamente. Se obtuvo un péptido que cumple con todas las características funcionales de la toxina nativa, contando ahora con una cantidad suficiente de toxina para utilizarla en el estudio del receptor, así como con una metodología que permite la síntesis de péptidos análogos para un estudio relación estructura-función mas completo.

Hasta el momento no se conoce otro péptido relacionado a la IpTxa. La máxima similitud se encuentra con la angelenina y la Tx2-9, ambas toxinas de araña, de las que se ha definido su acción sobre canales de calcio tipo P.

Primary structure and synthesis of Imperatoxin A (IpTxₐ), a peptide activator of Ca²⁺ release channels/ryanodine receptors

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Abstract We present the complete amino acid sequence of Imperatoxin A (IpTxₐ), a 33-amino-acid peptide from the venom of the scorpion *P. imperator* which activates Ca²⁺ release channels/ryanodine receptors (RyR) of sarcoplasmic reticulum (SR). The amino acid sequence of IpTxₐ shows no homology to any scorpion toxin so far described, but shares some homology to the amino acid sequence of Tx2-9 and agelenin, two spider toxins that target neuronal P-type Ca²⁺ channels. We also describe the total synthesis of IpTxₐ, and demonstrate that it efficiently activates RyRs with potency and affinity identical to those of native IpTxₐ. The use of synthetic IpTxₐ should help in the identification of the structural motifs of RyR critical for channel gating.
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Key words: Ca²⁺ release channel; Sarcoplasmic reticulum; *Pandinus imperator* scorpion venom; Caffeine; Synthetic peptide

1. Introduction

Ryanodine receptors (RyR) are essential for maintaining the intracellular Ca²⁺ homeostasis in striated muscle and in a variety of non-excitable cells [1,2]. Their role in excitation-contraction coupling of cardiac and skeletal muscle is well established [3], and their participation in stimulus-secretion coupling of secretory cells [4], and elevation of [Ca²⁺]ᵢ in neurons [5], is increasingly evident. In all of these cells, RyR releases massive amounts of Ca²⁺ from intracellular Ca²⁺ pools in response to a variety of triggering signals.

To this point, ryanodine has been the major probe used for structural and functional studies of RyRs. The alkaloid binds to RyRs with high affinity and specificity, and there is a consensus that [³H]ryanodine binding is an indicator of the number of receptors that are in the open conformational state [6]. However, ryanodine also displays some undesirable features that limit its use in experiments with intact cells, such as its slow association and dissociation rates, and its behavior as both an agonist and a blocker depending on the concentration used [6,7].

Scorpion venoms have traditionally represented excellent sources of ionic channel-blocking peptides. In the venom of the scorpion *P. imperator* we found Imperatoxin A (IpTxₐ), a short peptide that specifically and with high affinity increased

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[³H]ryanodine binding and enhanced the activity of RyRs reconstituted in planar lipid bilayers [8,9]. At concentrations well above the half-maximal effective concentration (ED₅₀) exhibited for RyRs, IpTxₐ did not affect other Ca²⁺ channels or ion transporters of muscle and brain [8]. Moreover, all of these effects could be seen only on skeletal-type RyR, suggesting that IpTxₐ preferentially affects this particular RyR isoform [9]. However, the use of this promising agent has been hampered by the extremely small amount of peptide obtained from the whole venom.

In this paper we communicate the entire amino acid sequence of IpTxₐ and describe the synthesis of an IpTxₐ analog that displays functional properties identical to those of the authentic native IpTxₐ. The design of a fully functional synthetic IpTxₐ analog should alleviate the problem associated with the scarcity of IpTxₐ and accelerate its use as a peptide probe of RyR function.

2. Materials and methods

2 1. Purification of IpTxₐ

IpTxₐ was purified from *P. imperator* scorpion venom in three chromatographic steps Crude venom was extracted from CO₂-anesthetized scorpions kept alive in the laboratory, recovered with deionized water, and lyophilized Batches (100 mg) of crude venom were dissolved in 2-3 ml of deionized water and applied onto a column (1.5×125 cm) of Sephadex G-50 fine Fractions were eluted with 20 mM NH₄AcOH (pH 4.7) at a flow rate of 10 ml/h. Fraction 3 containing IpTxₐ was applied to a column (1×25 cm) of carboxymethyl cellulose 32 (Pharmacia) equilibrated with 20 mM NH₄AcOH (pH 4.7). Peptides were eluted at a flow rate of 12 ml/h with a linear gradient of 250 ml of 20 mM NH₄AcOH (pH 4 7) and 250 ml of the same buffer containing 0.5 M NaCl. The peak containing IpTxₐ eluted when the NaCl concentration at the top of the column reached 340 mM. This fraction was concentrated by vacuum centrifugation and injected into an Aquapore C₈ reverse-phase HPLC column (Pierce). IpTxₐ was eluted with a linear gradient of 0-100% acetonitrile in 0.075% trifluoroacetic acid (TFA) run at 1 ml/min for 60 min. IpTxₐ was quantified by absorbance at 280 nm (A₂₈₀ₙₘ) using an extinction coefficient (ε) = 1852 M⁻¹ cm⁻¹.

2 2. Preparation of sarcoplasmic reticulum vesicles and [³H]ryanodine binding assay

Heavy sarcoplasmic reticulum (SR) was prepared from rabbit white back and leg muscle using the procedure of Meissner [10]. [³H]ryanodine binding to rabbit skeletal SR was carried out as previously described [8,9]. Briefly, the standard incubation medium contained 0.2 M KCl, 1 mM Na₂EGTA, 10 mM Na-Pipes, pH 7.2 and CaCl₂ necessary to set [free Ca²⁺] in the range of 1 nM to 100 μM. Ca²⁺/EGTA ratios were calculated using the stability constants of Fabiato [11]. [³H]Ryanodine (68.4 Ci/mmol, Dupont NEN) was diluted directly in the incubation medium to a final concentration of 7 nM. Protein concentration was in the range of 0.2-0 4 mg/ml and was determined by the Bradford method. Incubations lasted 90 min at 36°C. Samples (0.1 ml) were always run in duplicate, filtered on What-

man GF/B glass fiber filters and washed twice with 5 ml of distilled water using a Brandel M24-R cell harvester (Gaithersburg, MD) Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine and has been subtracted from each sample. Equilibrium binding data were fitted by nonlinear regression analysis with the functions specified in the text using the computer program Origin 4.0 (Microcal Inc., Northampton, MA).

2.3. Amino acid analysis and microsequencing of IpTx$_a$

Amino acid analysis of IpTx$_a$ was performed on samples hydrolyzed in 6 N HCl with 0.5% phenol at 110°C in evacuated, sealed tubes as described [12]. Reduction of IpTx$_a$ with dithiothreitol, and alkylation with iodoacetic acid was performed as described [12]. The sequence of the intact native and reduced/carboxymethylated IpTx$_a$ was determined using a model 6400/6600 automatic liquid-phase protein sequencer (Milligen/BioSearch Prosequencer) employing standard Edman degradation programs and CD immobilon membranes. To confirm the correctness of the carboxy-terminal sequence, 20 μg of IpTx$_a$ was hydrolyzed with S. aureus V8 in 100 mM ammonium bicarbonate (pH 7.8). The peptide fragments were purified as described and directly sequenced as described for native IpTx$_a$.

2.4. Synthesis of IpTx$_a$

A linear analog of IpTx$_a$ was synthesized by the solid-phase methodology with Fmoc-amino acids in an Applied Byosystems peptide synthesizer (model 432A). After cleavage with 90% trifluoroacetic acid

for 4 h at room temperature, the crude linear peptide was extracted with 5% acetic acid and dried by vacuum centrifugation. The cyclization reaction to make disulfide bridges in the molecule was carried out in 0.1 M NaCl, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 20 mM NaHPO$_4$ (pH 8.0) and 30 μM of synthetic IpTx$_a$. The crude cyclized product was purified in a C$_8$ reverse-phase HPLC column using the conditions described in the text. The structure and the purity of the synthetic toxin were confirmed by analytical HPLC, amino acid analysis and mass spectrometry. For amino acid analysis, synthetic toxin was hydrolyzed in 350 μl of 6 N HCl, 15 μl of phenol at 165°C for 50 min, then analyzed in an Applied Biosystems analyzer (model 421). Mass spectrometry was carried out in a Bruker Reflex II MALDI-TOF spectrometer.

2.5. Sequence comparisons

The amino acid sequence of IpTx$_a$ was compared with those of other proteins deposited in the protein database of GenBank (Los Alamos National Laboratory, Los Alamos, NM) by computer analysis using the program Blitz version 1.5 (Biocomputing Research Unit, University of Edinburgh, UK).

3. Results and discussion

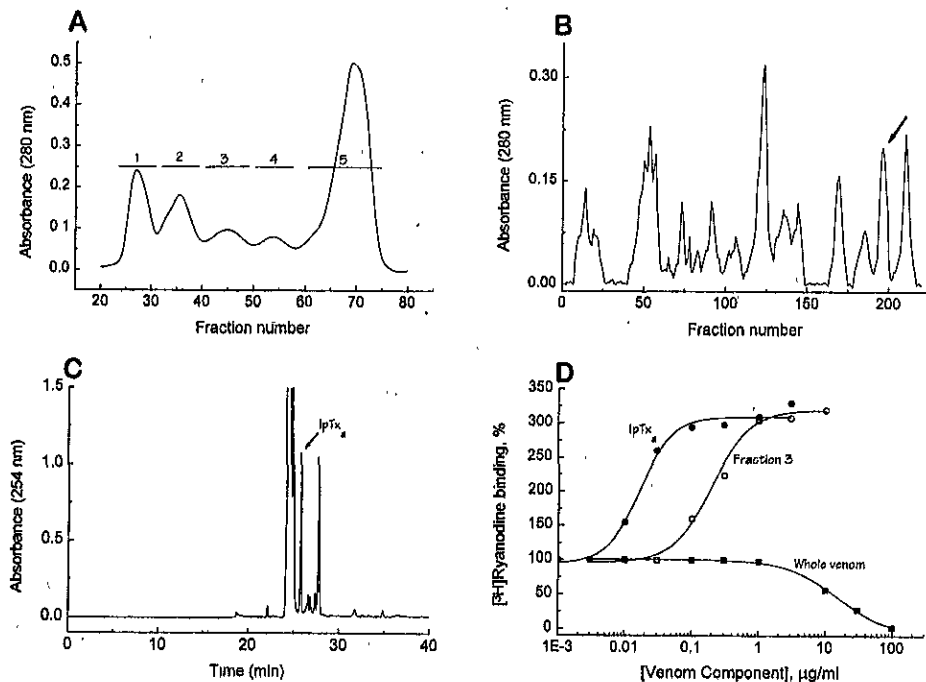Whole P. imperator scorpion venom completely inhibited

Fig. 1. Purification of IpTx$_a$. A: P. imperator soluble venom (120 mg protein) was applied to a Sephadex G-50 column (0.9 × 190 cm) equilibrated and run with 20 mM NH$_4$AcOH (pH 4.7). Samples (5 ml) were collected and tested for their capacity to modify [^{3}H]ryanodine binding. B: Fraction 3 was further separated through a CM-Cellulose column (0.9 × 30 cm), equilibrated and run with 20 mM NH$_4$AcOH (pH 4.7). A linear gradient of sodium chloride resolved multiple sub-fractions, one of which (labeled with arrow) contained IpTx$_a$. C: The sub-fraction from the CM-cellulose column containing IpTx$_a$ was lyophilized and injected into a C$_8$ reverse-phase HPLC column and eluted with a 0-100% linear gradient of acetonitrile containing 0.075% trifluoroacetic acid. D: Dose-response curve for whole venom and purified components. [^{3}H]Ryanodine (7 nM) was incubated with skeletal SR protein in 0.2 M KCl, 10 μM CaCl$_2$, 10 mM Na-HEPES pH 7.2, in the absence (control, 100%) and the presence of indicated concentrations of venom components. Nonspecific binding was determined in the presence of 20 μM ryanodine and has been subtracted from this and subsequent results.
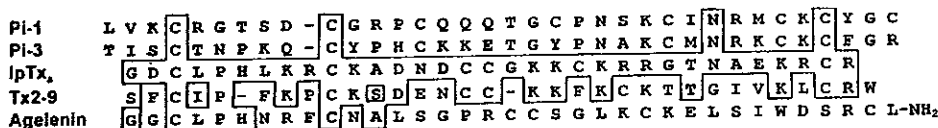
Pi-1	L V K	C	R G T S D -	C	G R P C Q Q Q T G C P N S K C I	N	R M C K	C	Y G C
Pi-3	T I S	C	T N P K Q -	C	Y P H C K K E T G Y P N A K C M	N	R K C K	C	F G R
IpTxₐ	G	D	C L P H L K R	C	K A D N D C C G K K C K R R G T	N	A E K R	C	R
Tx2-9	S F	C	I P - F K P	C	K S D E N C C - K K F K C K T T G I V	K	L	C	R W
Agelenin	G	G	C L P H N R F	C	N A L S G P R C C S G L K C K E L S I W D S R C L-NH₂

Fig. 2 Amino acid sequence of IpTxₐ and comparison with other scorpion and spider toxin sequences. The amino acid sequence of IpTxₐ was obtained by direct Edman degradation of the reduced and alkylated IpTxₐ. Gaps (−) in the amino acid sequence of Pi-1 and Pi-3, two K^+ channel blockers from the scorpion *P. imperator* [13,14], as well as in Tx2-9, a P-type Ca^{2+} channel blocker from the spider *P. nigriventer* [16], have been introduced to maximize homology. Agelenin, also a spider toxin that blocks P-type Ca^{2+} channel [17], is presented without gaps

[³H]ryanodine binding to skeletal SR with a concentration of 15 µg/ml yielding the half-maximal effect (ED_{50}) (Fig 1D, see also [8]). The presence of components that stimulate RyRs is only apparent after separation of the stimulatory and inhibitory components. The purification of IpTxₐ, an activator of RyRs, was performed in three chromatographic steps as described in the Section 2 and shown in Fig. 1. After fractionation of whole venom in Sephadex G-50 (Fig. 1A), five fractions were collected and assayed for effects on [³H]ryanodine binding. Fraction 2 contained peptides in the range of 10-20 kDa that were responsible for the inhibitory effect exhibited by the whole venom. Fraction 3 *increased* [³H]ryanodine binding with an $ED_{50} = 0.19$ µg/ml (Fig. 1D), but since it accounts for ≤5% of the total venom, its effect is probably masked in the whole venom by that of Fraction 2. Fraction 3 was subsequently applied to a carboxymethyl-cellulose and eluted with a gradient of NaCl (Fig. 1B). The peak containing IpTxₐ (marked by an arrow) eluted late in the run, suggesting that IpTxₐ was a strongly basic peptide. Fig. 1C shows the chromatographic profile of IpTxₐ (marked with an arrow) after elution from a reverse-phase C_8 HPLC column. IpTxₐ increased [³H]ryanodine binding with an $ED_{50} = 0.016$ µg/ml Given this value and the molecular weight of IpTxₐ (3.7 kDa, see below), the apparent dissociation constant (K_d) was −5 nM. The proportion of IpTxₐ in the whole venom is very small, about 0.03%, but since it displays a high potency to stimulate RyRs, the [³H]ryanodine binding assay offers a sensitive method to track its functional activity.

Fig. 2 shows the complete amino acid sequence of IpTxₐ as determined by direct automated microsequencing. IpTxₐ is composed of 33 amino acids with a calculated $M_r = 3765$. As most short scorpion toxins, IpTxₐ is a basic peptide containing three pairs of cysteine residues that stabilize the three-dimensional conformation by forming disulfide bridges. Fig. 2 also shows a comparison of the amino acid sequence of IpTxₐ with those of Pi-1 and Pi-3, two peptide blockers of K^+ channels from the same *P. imperator* venom [13,14], reportedly the smallest ionic channel-targeted scorpion peptides. Pi-3 is representative of a group of homologous toxins with three disulfide bridges such as noxiustoxin, charybdotoxin, iberiotoxin, etc. (for a review, see [15]); Pi-1 forms a K^+ toxin subgroup on its own by having the remarkable characteristic of possessing four disulfide bridges [13]. No significant similarity is observed between IpTxₐ and any of these toxins, even when gaps (-) are introduced at the level of Cys^{10} to maximize homology. Even less homology is found when the comparison involves the larger Na^+ channel-selective peptides from scorpion venoms (not shown). Thus, IpTxₐ constitutes a novel class, the smallest yet, of scorpion toxins targeted against ionic channels.

Albeit not dramatic, a higher sequence homology was found when the comparison was made with Tx2-9 of the spider *P. nigriventer* [16], and agelenin of the spider *A. asperta* [17], two peptide blockers of neuronal P-type calcium channels (Fig. 2). If gaps are introduced to maximize homology, Tx2-9 is the most similar with 45% sequence identity. Agelenin has only limited resemblance (21% sequence identity), although higher homology may be ascribed if two-residue gaps are introduced. Regardless of the extent of similarity, it is clear that IpTxₐ is more related to these two spider toxins than to other toxins present in scorpion venoms. Thus, IpTxₐ adds to the emerging notion that peptide toxins found in
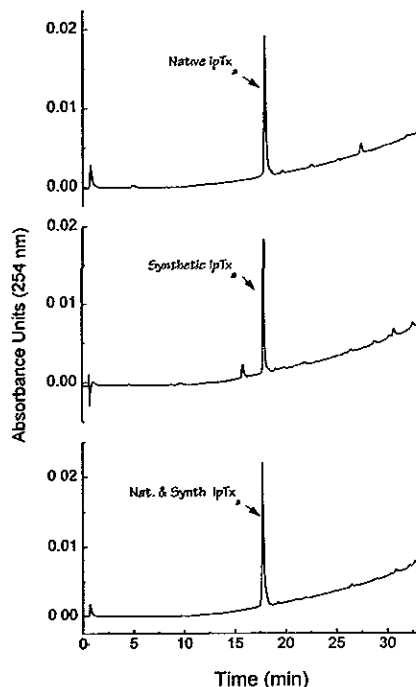

Fig. 3. HPLC comparison of native and synthetic IpTxₐ. Native and Synthetic IpTxₐ (−1.5 nmol each), as well as an equimolar mixture (−1 nmol each) were chromatographed on a Pierce C_8 analytical column. The peptides were eluted using 0.075% trifluoroacetic acid as solvent, applying a linear gradient from 0 to 100% (v/v) of acetonitrile at a flow rate of 1 ml/min.

venomous animals of different phyla contain similar structural motifs [18].

We used the IpTx$_a$ amino acid sequence to synthesize a linear analog of IpTx$_a$. After purification and cyclization of the synthetic analog as described in Section 2, synthetic IpTx$_a$

was analyzed by reverse-phase HPLC for comparison with native IpTx$_a$ (Fig. 3). The elution time of synthetic IpTx$_a$ was identical to that of native IpTx$_a$, and a single sharp peak was observed upon mixing synthetic and native IpTx$_a$.

We also tested the functional activity of synthetic IpTx$_a$. Fig. 4A shows that synthetic IpTx$_a$ increased [^{3}H]ryanodine binding to *skeletal* SR with the same potency and affinity as native IpTx$_a$. Both sets of data points were overlapping and could be fitted with the same line. We used the function: $B = B_{max}/1+(K_d+[IpTx_a])^n$ to fit the data, where B is the specific binding of [^{3}H]ryanodine, B_{max} is the maximal activation of [^{3}H]ryanodine binding evoked by IpTx$_a$ (325%), K_d is the apparent dissociation constant of IpTx$_a$ (5 nM), and n is the Hill number (1.58). Unlike this dramatic effect on skeletal RyR, no significant activation of [^{3}H]ryanodine binding to *cardiac* SR was observed with synthetic IpTx$_a$ (results not shown)). Hence, synthetic IpTx$_a$ also displays selectivity for skeletal-type RyRs, just as native IpTx$_a$ does.

In a previous study [9], we reported that native IpTx$_a$ increases [^{3}H]ryanodine binding by sensitizing RyRs to Ca^{2+}. Fig. 4B shows the Ca^{2+}-dependence of [^{3}H]ryanodine binding to skeletal SR and the effect of synthetic IpTx$_a$. Specific binding in the absence of the peptide (Fig. 4B, Control, ○) had a threshold for detection at pCa 7-6 and was maximal at ~50 μM [Ca^{2+}]. In the presence of 1 μM synthetic IpTx$_a$ (Fig. 4B, +Synthetic IpTx$_a$, ●), the threshold for detection of [^{3}H]ryanodine binding decreased to pCa 8 and the binding curve was dramatically augmented in absolute values. The EC$_{50}$ for the activation of [^{3}H]ryanodine binding by Ca^{2+} (ascending limb of the curve) was ~5 μM and 0.8 μM for control and synthetic IpTx$_a$, respectively. Thus, synthetic IpTx$_a$ also activates [^{3}H]ryanodine binding by sensitizing RyRs to Ca^{2+}.

Another functional attribute of native IpTx$_a$ was its ability to potentiate its effect with caffeine [9]. Fig. 4C shows the interaction of caffeine and synthetic IpTx$_a$. At pCa 7, binding was 0.02 pmol/mg in the absence of synthetic IpTx$_a$ and caffeine and increased to 0.39 pmol/mg in the presence of 20 mM caffeine (Fig. 4C, ○). In the presence of 1 μM synthetic IpTx$_a$ (Fig. 4C, ●), binding was 1.52 pmol/mg in the absence of caffeine and increased to 4.51 in the presence of 20 mM caffeine, i.e., a net gain of ~3 pmol/mg. Since the binding increment evoked by the combined addition of synthetic IpTx$_a$ and caffeine was larger than that evoked by caffeine or synthetic IpTx$_a$ alone, this suggested a cooperative interaction between the synthetic IpTx$_a$- and caffeine-binding sites.

A mass spectrometry analysis of native and synthetic IpTx$_a$ yielded the expected molecular mass (3765.8 Da) based on amino acid composition and sequence. Thus, by all structural and functional criteria applied, synthetic IpTx$_a$ is identical to native IpTx$_a$. The design of a synthetic analog of IpTx$_a$ with functional attributes identical to the native IpTx$_a$ verifies the correctness of the amino acid sequence, since even single amino acid substitutions in analogous peptides result in large changes of affinity towards their acceptor site [13]. The availability of relatively large amounts of synthetic IpTx$_a$ should accelerate its use as a peptide probe of RyR function.
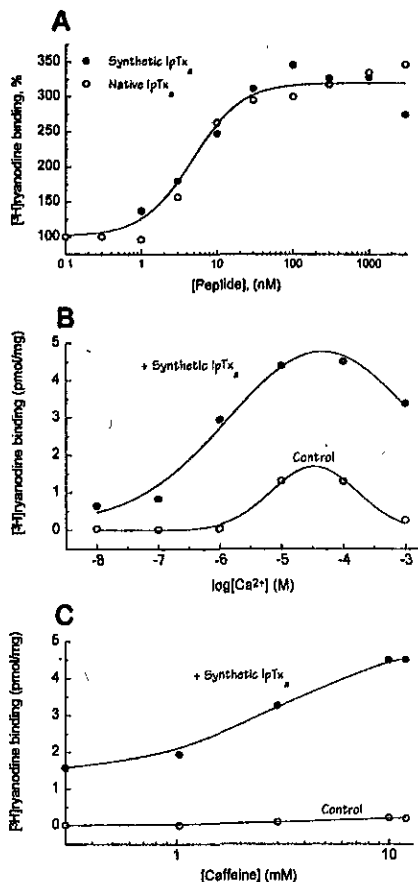
Fig. 4. Functional properties of synthetic IpTx$_a$. A: Dose-response relation for native (○) and synthetic IpTx$_a$ (●). [^{3}H]Ryanodine (7 nM) was incubated with skeletal SR in the standard incubation medium described in Section 2 in the presence of the indicated concentrations of IpTx$_a$. Both sets of data points were fitted with the same line. K_d was 5 nM and B_{max} was 325%, which corresponded to 2.36 pmol/mg protein. B: Effect of synthetic IpTx$_a$ on the Ca^{2+}-dependence of [^{3}H]ryanodine binding. The standard binding medium contained 1 mM EGTA and several CaCl$_2$ concentrations to yield the desired [free Ca^{2+}]. Synthetic IpTx$_a$ (1 μM) was present throughout the incubation period. Smooth lines linking data points have no theoretical meaning. C: Potentiation of synthetic IpTx$_a$ effect by caffeine. The binding of [^{3}H]ryanodine was determined in incubation medium containing 100 nM free Ca^{2+} (1 mM EGTA and 385 μM CaCl$_2$). Caffeine was added at the beginning of the incubation as 10-μl aliquots from 100-fold stocks for concentrations up to 10 mM, and as powder form to reach 20 mM.

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IV.- DISCUSION Y CONCLUSIONES.

Diferentes a otros ligandos exógenos, las imperatoxinas (IpTxa, IpTxi) constituyen el primero de un grupo de péptidos que sirven como herramientas para el estudio del receptor de Rianodina. Parecidos a la rianodina, estos péptidos regulan al RyR a concentraciones nanomolares, al menos 300 a 1000 veces menos de la que se necesita para observar efectos con otros ligandos exógenos.

En este trabajo, reportamos algunos de las características funcionales y estructurales de IpTxa e IpTxi y discutimos su utilidad en la elucidación de la relación estructura función del RyR.

Imperatoxina A

Debido a que la rianodina se une preferentemente al canal liberador de calcio (RyR) cuando este se encuentra en estado abierto y los ligandos que activan al canal también aumentan la unión de [^{3}H]rianodina y los que bloquean o cierran el canal también disminuyen la unión de rianodina marcada, se utilizó el sistema de unión de rianodina a su receptor para buscar y caracterizar compuestos del veneno de alacrán *Pandinus imperator* que tuvieran efecto sobre el RyR. Asumiendo que los cambios producidos en la unión reflejan activación o inhibición sobre la actividad del canal.

Se determinó la secuencia de aminoácidos completa de la toxina IpTxa por microsecuenciación automatizada. La IpTxa esta compuesta de 33 aminoácidos con un peso molecular calculado de 3765. Como muchas de las toxinas de cadena corta que se encuentran en el veneno de los alacranes, es un péptido básico que contiene tres pares de cisteínas que estabilizan su conformación tridimensional formando puentes disulfuro. Sin embargo, la IpTxa no muestra una semejanza significativa con otras toxinas de alacrán bloqueadoras de canales de K^+ ó Cl^- (conocidas como toxinas de cadena corta) ni con las toxinas de cadena larga (70 aminoácidos) que actúan sobre los canales de Na^+ dependientes de voltaje (Possani 1984, 1998). Así, Iptxa constituye una nueva clase de toxinas de alacrán que actúa sobre canales iónicos

Las toxinas peptídicas cuya estructura tridimensional se conoce se han agrupado en solo tres estructuras básicas (fig. 6) de acuerdo a su plegamiento global (Omecinsky y col. 1996). El criterio de clasificación de las toxinas peptídicas dentro de estos grupos es independiente de su origen animal, especificidad sobre un canal iónico o secuencia de aminoácidos. El criterio de clasificación es el de aparear los residuos de cisteína que son críticos para mantener el arreglo tridimensional. Con base en la estructura tridimensional ya conocida de dos toxinas que presentan un arreglo de cisteínas similar a la IpTxa, µ-agatoxina IVA y ω-conotoxina GVIA, se ha propuesto un modelo tridimensional de la IpTxa, formado por tres hojas beta unidas por asas, que corresponde al tipo I de acuerdo a la clasificación de Omecinsky (fig. 6) En el modelo, dentro de la asa mayor que va del residuo glicina 18 a la alanina 28 existe un aminoácido hidroxilado (Tre 26) precedido de un grupo de cinco aminoácidos básicos (Lis 19-Arg 24). Este arreglo de aminoácidos es un rasgo único de IpTxa, que puede participar en la unión de la toxina al RyR porque otras
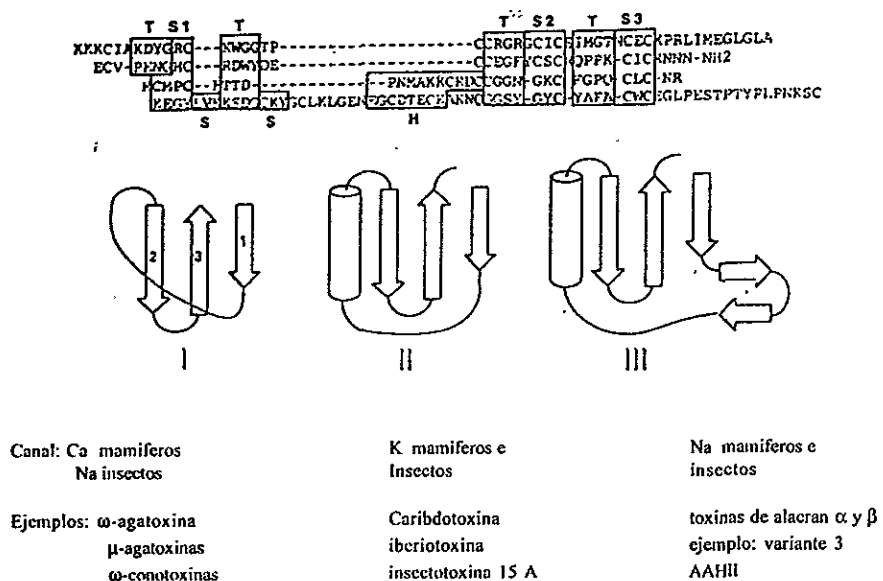
FIGURA 6. Comparación de elementos de estructura primaria y secundaria en péptidos encontrados en venenos de araña, caracoles y alacranes que actúan sobre canales iónicos. Las secuencias corresponden (de arriba a abajo) ω-Aga-IVA (1IVA), μ-Aga-I que actúan sobre canales de calcio tipo P que presentan una estructura de tipo I; toxina para insectos 15A (ISIS) que tiene acción sobre canales de potasio (ejemplo de estructura del tipo II) y una neurotoxina de alacrán variante 3 (2SN3) que afecta canales de sodio (corresponde a la estructura de tipo III) En el alineamiento de las secuencias, los elementos de estructura secundaria son designados como sigue: S1-S3 = Segmentos de hoja-β conservados; S = segmentos de otras hojas-β; T = asa; H = α-hélice. (Omecinsky y col 1996).

toxinas relacionadas a IpTXa con una asa igual de grande, pero diferente secuencia de aminoácidos (como ω-conotoxina GVIA) no tiene ningún efecto sobre el RyR. Alineando las secuencias de aminoácidos de la IpTxa y la secuencia de aminoácidos de la región llamada II-III loop del receptor de dihidropiridina (región que se ha postulado interacciona directamente con el RyR en el mecanismo excitación-contracción) se observa que no tiene una identidad significativa respecto a la secuencia de aminoácidos pero presentan un rasgo característico que es la presencia de un aminoácido hidroxilado precedido por un grupo de cinco aminoácidos básicos. Utilizando la IpTxa sintética (uno de los logros de este trabajo de tesis), Valdivia y Possani, (1998) reportan la importancia del grupo de residuos básicos y la treonina 26 para la unión de Iptxa a su receptor; además de una semejanza funcional importante con la región II-III loop del receptor de dihidropiridina.

Así, esta toxina resulta ser una herramienta interesante para estudiar el mecanismo de activación del RyR y poder relacionarlo con el mecanismo fisiológico involucrado.

Morrisett y col. (1996), purificaron la rianotoxina, un péptido de 7 kDa del veneno del alacrán *Buthotus judaicus* que induce, al igual que la IpTxa, la aparición de estados de subconductancia en el receptor de rianodina esquelético. La estabilización de aperturas del canal en un estado de subconductancia parece ser un rasgo común en los péptidos de veneno de alacrán moduladores del RyR.

Imperatoxina I

La Imperatoxina I (IpTxi) es otra proteína aislada del veneno del alacrán *P. imperator* que afecta la actividad del RyR con una afinidad nanomolar, pero con un mecanismo de acción radicalmente diferente del descrito para la IpTxa. Se preparó una biblioteca de cDNA a partir de las glándulas venenosas del alacrán y se uso para clonar el gene que codifica para la IpTxi. De esta forma, se confirmó la secuencia de aminoácidos determinada por microsecuenciación automatizada directa de la proteína. La IpTxi consiste de una subunidad grande (104 residuos de aminoácidos) con una secuencia de aminoácidos que tiene un 38% de identidad con fosfolipasas del tipo A2 del veneno de abejas (*Apis mellifera*) y 35% con la fosfolipasa A2 del veneno del heloderma (*Heloderma horridum*). Esta subunidad esta unida covalentemente por un puente disulfuro a una subunidad pequeña (27 aminoácidos) estructuralmente no relacionada. Entonces, la IpTxi es una proteína heterodimérica con acción lipolítica, propiedad que sólo es compartida por las β-bungarotoxinas, un grupo de neurotoxinas de venenos de serpiente (Kwong y col 1995) que inhiben la liberación de neurotrasmisores.

La inhibición de la actividad fosfolipasa de la IpTxi, ya sea por pérdida de su estructura (reducción y carboximetilación) o por tratamiento con reactivos específicos como el bromuro de p-bromofenacilo, inhibe también su efecto sobre el receptor de rianodina. Esto sugiere que un producto de la actividad fosfolipásica, más que una interacción directa con el receptor, es la responsable de la inhibición de la unión de rianodina a su receptor. La

incubación de RyR con el sobrenadante de vesículas de RS preincubadas con IpTxi, mostró un efecto inhibitorio de la unión de [^{3}H]rianodina al receptor, lo que refuerza la hipótesis de que algún producto de hidrólisis y no la IpTxi *per se* afectan al RyR. Pensando en la posibilidad de que el efecto enzimático de la toxina pudiera afectar la integridad del receptor convirtiendo el efecto en irreversible, se incubaron las vesículas de RS con una concentración 1 μM de IpTxi y después de centrifugar y lavar el precipitado, se probó la capacidad de unión de [^{3}H]rianodina, de estas vesículas mostrando básicamente la misma unión que en las membranas control, no tratadas con toxina. Confirmamos así, que el efecto de IpTxi es reversible y que no existe alguna alteración sobre la estructura del canal o del medio que lo rodea que afecte su capacidad de unión de rianodina.

Sí la interacción de los productos de la actividad enzimática de la IpTxi y el receptor de rianodina resultan en una inhibición del canal liberador de calcio, esto se reflejaría en una disminución de la probabilidad de apertura del canal. Para confirmar esta suposición, se probó el efecto de la toxina y de ácidos grasos sobre el RyR incorporado en bicapas. Encontramos que IpTxi bloquea al RyR en una forma dependiente de la concentración. A baja concentración (<200nM) IpTxi disminuye el tiempo medio de apertura, haciendo las aperturas del canal más breves, al aumentar la concentración de toxina las aperturas del canal desaparecen. La disminución en la frecuencia y duración de los eventos de apertura del canal contribuyen a la disminución de la probabilidad de apertura. La IC$_{50}$ de la IpTxi es de 140 nM.

Estos resultados nos indican que el producto de la actividad fosfolipasa de la IpTxi es capaz de interaccionar con el RyR o con alguna proteína asociada que regulan el mecanismo de apertura y cierre del canal.

Los productos de la acción enzimática de las fosfolipasas A2 son ácidos grasos libres y lisofosfolípidos, para discernir sí alguno de estos productos son los que tienen efecto sobre el RyR, probamos tanto ácidos grasos libres como lisofosfolípidos separadamente tanto en la unión de rianodina marcada como en canal unitario. Encontramos que los lisofosfolípidos probados hasta una concentración 300 µM no tienen efecto sobre el RyR. Por otro lado no todos los ácidos grasos probados tuvieron el efecto inhibidor sobre el RyR. Los ácidos araquidónico, linoleico y oleico (ácidos grasos insaturados) que se encuentran en cantidades abundantes en las membranas de retículo sarcoplásmico de músculo esquelético y cardiaco inhiben totalmente al RyR, no así los ácidos grasos saturados como el ácido palmítico.

Estos resultados indican que son los ácidos grasos producto de la actividad fosfolipasa tipo A_2 de la IpTxi los que causan la inhibición del RyR.

Papel de los ácidos grasos en la liberación de calcio

Se sabe que los lípidos, los ácidos grasos y metabolitos relacionados, participan en señales de transducción que estimulan la liberación de Ca^{2+} de los almacenes intracelulares en diversos tejidos. Una cascada bien conocida, es la iniciada por el rompimiento del fosfatidil inositol bifosfato en inositol trifosfato y diacilglicerol (Berridge e Irvine, 1989). Otros metabolitos que movilizan Ca^{2+} incluyen el ácido araquidónico, ácidos grasos insaturados o derivados de esfingosina, que movilizan Ca^{2+} de almacenes intracelulares pero independiente de la vía del inositol trifosfato. (Wolf y col. 1986; Chow y Jondal, 1990; Ghosh y col. 1990)

El-Hayek y col. (1993) muestran que la palmitoil carnitina es un activador del canal liberador de calcio de músculo esquelético de diferentes especies por un efecto directo sobre el mecanismo de apertura y cierre del RyR y proponen que esta interacción puede representar una vía por la cual el metabolismo del músculo esquelético podría influir en el Ca^{2+} intracelular y ser responsable de las manifestaciones fisiopatológicas musculares presentadas en la enfermedad genética conocida como deficiencia de palmitoil transferasa II.

Los ácidos grasos (particularmente el ácido araquidónico) son conocidos reguladores de varios eventos fisiológicos en diferentes tipos de células. Se conoce también que los ácidos grasos producen una variedad de efectos en vesículas de retículo sarcoplásmico; estos dependen de la concentración, la longitud de la cadena, el grado de saturación y las condiciones experimentales usadas. Los efectos observados parecen ser promovidos por interacción de los ácidos grasos con los fosfolípidos de la membrana que rodea a la ATPasa de calcio del RS. Cardoso y De-Meis (1993) usando una preparación de vesículas de retículo sarcoplásmico que no presenta RyR reportan que los ácidos grasos saturados (C_{16}, C_{18}, y C_{20}) y el ácido araquidónico inhiben la acumulación de Ca^{2+} en el RS, estimulan el eflujo de Ca^{2+} de vesículas de retículo sarcoplásmico y activan a la ATPasa de calcio. Estos efectos son antagonizados por tapsigargina un inhibidor específico la Ca^{2+}-ATPasa, sugiriendo un mecanismo de acción común. Ellos encontraron que los ácidos grasos insaturados son más efectivos que los ácidos grasos saturados, proponiendo dos hipótesis: 1. Los ácidos grasos saturados e insaturados tienen sitios de acción diferentes; 2. Actúan sobre el mismo sitio, pero la afinidad de los ácidos grasos saturados es menor que la afinidad de los ácidos grasos insaturados.

El transporte de calcio en el retículo sarcoplasmico y la actividad de la ATPasa de Ca^{2+} se pueden modificar por manipulación del medio ambiente lipídico que rodea a la enzima (Johammsson y col 1981). La hidrólisis parcial de los fosfolípidos de membrana por fosfolipasas, la remoción de los productos de hidrólisis con albúmina y la adición de ácidos grasos o de lisofosfolípidos a las membranas depletadas de lípidos, pueden alterar de forma diferente la actividad de la ATPasa de calcio y el transporte de calcio de las vesículas de retículo sarcoplásmico (Messineo y col, 1984).

Los mecanismos por los cuales los ácidos grasos producen estos efectos complejos sobre el retículo sarcoplásmico aún no se entienden, aunque la exposición de vesículas de retículo sarcoplásmico a concentraciones micromolares de ácidos palmitico y oleico puede alterar la estructura de la membrana. (Herbette y col, 1984). Messineo y col (1984), estudiaron los mecanismos por los cuales los ácidos oleico y palmítico modifican el secuestro de calcio por el retículo sarcoplásmico examinando los efectos de estos ácidos grasos sobre la actividad de la ATPasa de Ca^{2+} y la permeabilidad pasiva del calcio a través de la membrana. Ellos proponen que el ácido oleico inhibe el secuestro de calcio por el RS por un incremento de la permeabilidad de la membrana al calcio mientras que el ácido palmítico parece estimular el secuestro de calcio al intercalarse en la membrana lipídica y formar un complejo calcio-ácido graso.

Cheah (1981) mostró que el transporte de calcio de retículo sarcoplasmico de músculo esquelético se inhibe por los ácidos grasos araquidónico, oleico y linoleico. La actividad de la ATPasa de Ca^{2+} del retículo sarcoplásmico se estimula a bajas concentraciones de ácido araquidónico y oleico, pero la enzima se inhibe por altas concentraciones de estos ácidos.

La inhibición del transporte de calcio por estos ácidos grasos no puede por lo tanto atribuirse a la inhibición de la ATPasa, y probablemente se deba a un incremento de la permeabilidad a calcio del retículo sarcoplásmico.

El ácido araquidónico es uno de los ácidos grasos liberados de la membrana plasmática por activación de fosfolipasas A2. El ácido araquidónico y sus metabolitos se han implicado como segundos mensajeros. Se ha sugerido que están involucrados en la movilización de calcio del retículo sarcoplásmico de células cardiacas. Damron y Bond (1993) mostraron que el ácido araquidónico incrementa las concentraciones de Ca^{2+} de miocitos cardiacos cargados con Fura-2. Detbarn y Palade (1993) reportan que el ácido araquidónico acelera la liberación de Ca^{2+} de vesículas de membranas de retículo sarcoplásmico cardiaco. Varios de los efectos del ácido araquidónico se han reportado utilizando diferentes materiales y métodos experimentales y se han discutido una variedad de posibles mecanismos. Sin embargo, el blanco intracelular de las moléculas de ácido araquidónico aún no se ha identificado, y el mecanismo de interacción entre el ácido araquidonico y su molécula blanco no se conoce.

Los Acidos grasos y el RyR

En cuanto al efecto de los ácidos grasos sobre el receptor de rianodina, Urehara y col. (1996) presentan un estudio de la interacción del ácido araquidónico, con los canales liberadores de calcio de retículo sarcoplásmico cardiaco, uno de los canales intracelulares más importantes para la movilización de calcio. El ácido araquidónico ejerce un efecto inhibitorio en la unión de [^{3}H]rianodina a vesículas de retículo sarcoplásmico cardiaco, tanto en las vesículas como en el receptor solubilizado. Ellos demuestran que a bajas dosis

(menos de la concentración micelar critica alrededor de 100 µM) de ácido araquidónico modifica la unión de [³H]rianodina a vesículas de retículo sarcoplásmico reduciendo el valor de Bmax e incrementando el valor de Kd. Los resultados obtenidos utilizando el receptor solubilizado sugieren una unión directa del ácido araquidónico con la molécula del canal. También, encuentran dos tipos de inhibición: competitiva y no competitiva, sugiriendo así la existencia de dos sitios de unión para el ácido araquidónico.

En un trabajo previo a éste, Valdivia y col. (1992) encontraron que la IpTxi inhibía alrededor del 40% la unión de [³H]PN200-110 a túbulos-T de músculo esquelético. El [³H]PN200-110 es una droga selectiva para un sitio de alta afinidad sobre el receptor de dihidropiridina. IpTxi tiene efectos sobre el transiente de Ca^{2+} resaltando la contracción de miocitos de corazón. A tiempos cortos, 1 minuto, la contracción y los transientes de Ca^{2+} son muy similares en las células control y en las células tratadas con IpTxi; sin embargo, a tiempos largos 5-9 minutos (presumiblemente cuando ya se han liberado los ácidos grasos por la acción fosfolipasa de la toxina) la contracción disminuye a menos del 10 % y el transiente de calcio también se reduce significativamente con respecto al control bajo las mismas condiciones Estas observaciones sugieren que la IpTxi desacopla la excitación y contracción en miocitos cardiacos por una inhibición del receptor de rianodina.

Conociendo ahora el mecanismo por el cual la IpTxi actúa sobre el receptor de rianodina sugerimos que los resultados observados por Valdivia y col (1992) se deben al efecto de los ácidos grasos liberados por el efecto fosfolipasa de la IpTxi y presumiblemente debido principalmente al ácido araquidónico. El efecto observado en este mismo trabajo sobre el

DHPR puede ser debido también por los ácidos grasos liberados, ya que se ha reportado que estos deprimen las corrientes de calcio tipo L (Shimada y Somlyo, 1992).

Se sabe que el ácido araquidónico y otros ácidos grasos se acumulan en isquemia del músculo de corazón (Corr y col. 1984; Prinzen y col. 1984; Engels y col. 1990), en distrofia de músculo esquelético (Kawamoto y Baskin, 1986) y en hipertermia maligna (Cheah y Cheah, 1981; Fletcher y col.1990). También al ácido araquidónico y otros ácidos grasos suprimen la función cardíaca tanto en corazón normal como isquémico (Liedtke y col. 1978; Piper, 1990), e inhiben la fuerza de contracción en músculo esquelético y liso (Kossler y col. 1986). Estos efectos inhibitorios del ácido araquidónico pueden estar relacionados con nuestros resultados en cuanto al efecto de IpTxi o de los productos de su actividad de fosfolipasa, sobre el receptor de rianodina.

V.-PERSPECTIVAS.

De acuerdo a los resultados hasta ahora obtenidos, se puede decir que dentro de las perspectivas más importantes serian la de realizar, para el caso de la IpTxa un estudio en el que la toxina sea marcada radioactivamente y por medio de entrecruzadores químicos unirse al receptor con el objeto de mapear la región de pegado y así poder llegar a entender un poco mas la relación estructura-función del RyR en vesículas del retículo sarcoplásmico o del receptor purificado. A este respecto podría destacarse la limitante de que estos péptidos no atraviesan la membrana plasmática celular por lo cual el experimento no podría hacerse en células intactas. En relación a la proteína IpTxi, es el primer ejemplo reportado en que un péptido con actividad fosfolipásica es acompañado por otro péptido pequeño, aparentemente no relacionado con esta actividad, por lo que sería interesante tratar de encontrar cual es la función de esta subunidad pequeña y cual es su molécula blanco. ¿Existe alguna relación con el mecanismo molecular de la toxicidad?. ¿Involucra esto anormalidades en la movilización de Ca^{2+} intracelular debido al bloqueo de receptores de rianodina?

Estas y otras preguntas relacionadas todavía quedan como perspectivas futuras de este trabajo.

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VII.- APENDICE

F. Gómez-Lagunas, T. Olamendi, F. Z. Zamudio, L. D. Possani(1996). "Two novel toxins from the venom of the scorpion *Pandinus imperator* show that the N-terminal amino acid sequence is important for their affinities towards *shaker* B K^+ channels" J. Membrane Biol. 152, 49-56

El veneno del alacrán *Pandinus imperator* resultó ser una valiosa fuente de péptidos que tienen efectos sobre canales iónicos.

En el presente trabajo se muestran los resultados obtenidos con dos péptidos de cadena corta (35 aminoácidos) que bloquean canales de potasio.

Siguiendo los mismos pasos de purificación descritos en los artículos anteriores, se logró obtener a homogeneidad dos péptidos que comparten la misma secuencia de aminoácidos, con una sola diferencia en la posición 7, Prolina en el péptido Pi2 y Glutámico en el péptido Pi3. Este solo cambio resultó ser muy importante en su interacción con el canal de potasio tipo shaker B, modificando la Kd de 8.2 nM a 140 nM.

Two Novel Toxins from the Venom of the Scorpion *Pandinus imperator* Show that the N-terminal Amino Acid Sequence is Important for their Affinities towards *Shaker* B K⁺ Channels

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Abstract. Two novel peptides were purified from the venom of the scorpion *Pandinus imperator*, and were named Pi2 and Pi3. Their complete primary structures were determined and their blocking effects on *Shaker* B K⁺ channels were studied. Both peptides contain 35 amino acids residues, compacted by three disulfide bridges, and reversibly block the *Shaker* B K⁺ channels. They have only one amino acid changed in their sequence, at position 7 (a proline for a glutamic acid). Whereas peptide Pi2, containing the Pro7, binds the *Shaker* B K⁺ channels with a K_d of 8.2 nM, peptide Pi3 containing the Glu7 residue has a much lower affinity of 140 nM. Both peptides are capable of displacing the binding of ^{125}I-noxiustoxin to brain synaptosome membranes. Since these two novel peptides are about 50% identical to noxiustoxin, the present results support previous data published by our group showing that the amino-terminal region of noxiustoxin, and also the amino-terminal sequence of the newly purified homologues: Pi2, and Pi3, are important for the recognition of potassium channels.

Key words: K⁺ channel — Scorpion toxin, — *Pandinus imperator* — *Shaker* B expression — Sf9 cells

Introduction

Blocking agents against ion channels have been instrumental in defining both structural and mechanistic properties of the channels. They also allow to discriminate, hence to study, the various different conductances normally present in most cells [12].

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It is well known that among blocking agents of K⁺ channels, the toxins from scorpion venoms are a particularly useful group. Most scorpion toxins against K⁺ channels (K⁺ toxins) are short peptides, composed of 31–39 amino acid residues, with a highly variable amino acid sequence [reviews 7,17]. Regardless of their great variability on the primary structure, the three-dimensional structure of K⁺ toxins share a common motif: a short alpha helix and three strands of beta sheet structures, stabilized by three disulfide bridges [15]. Concerning their mode of action, previous work conducted with noxiustoxin (NTX), the first K⁺ toxin ever described [3,18], suggested that part of the sequence that recognizes K⁺ channels in NTX is located towards the amino terminal region. In fact, synthetic peptides with only nine and/or twenty amino acid residues corresponding to the amino terminal sequence of NTX are toxic to mice [9], and effectively block Ca²⁺-activated K⁺ channels [20]. Furthermore, while NTX needs the N-terminal segment for channel recognition, another K⁺ toxin: charybdotoxin (ChTX) has the C-terminal segment as the most important segment for channel recognition [10,16], despite the fact that Ser10 and Trp14, at the N-terminal region are also considered to be "crucial residues" [19].

Since the affinity of the synthetically prepared N-terminal peptides, for the K⁺ channels studied [10], are considerably lower than that of native NTX, it is quite possible that the C-terminal part is also necessary for full recognition and binding to the channels, either because the C-terminal segment also interacts with the receptors, or it is important for structural reasons, positioning the N-terminal region in its best conformation.

In this work, we describe for the first time two new K⁺ toxins purified from the venom of the scorpion *P. imperator*. The toxins have identical primary structures except for position 7, at the N-terminal region, where a

Pro substitute for a Glu. While Pi2 (Pro7) has a 8.2 nM K_d to *Shaker* B K⁺ channels, expressed in Sf9 cells, Pi3 (Glu7) has a *seventeenfold lower affinity (K_d) of about* 140 nM). The differential affinity of these toxins, supports our previous suggestion that the amino terminal residues of NTX-like K⁺ toxins are part of the domain that recognizes K⁺ channels.

Materials and Methods

SOURCE OF VENOM

Scorpions of the species *P. imperator* (Gabon, Africa) maintained alive in the laboratory were anesthetized monthly with CO_2 and milked for venom by electrical stimulation. The venom was placed in double distilled water and centrifuged at 15,000 × g for 15 min. The supernatant was freeze-dried and stored at −20°C until use.

SEPARATION AND BIOASSAYS

The soluble venom was initially fractionated in a Sephadex G-50 column and their subfractions were further separated by HPLC, using a C18 reverse-phase column (Vydac, Hysperia, CA), of a Waters 600E high performance liquid chromographer (HPLC), equipped with a variable wavelength detector, and a WIPS 712 automatic sample injector.

The homogeneity of the purified peptides was confirmed by step-gradient HPLC and by direct Edman degradation using an automatic sequencer [14], Amino acid analysis of peptides confirmed the molecular mass and the sequences found.

The bioassay used to identify these peptides was through binding and displacement experiments from brain synaptosome membranes of previously bounded ¹²⁵[I]-NTX [21]. With this procedure two peptides eluting initially in the fraction III of the Sephadex G-50 column, and subsequently in the peak 2 and 3 of the HPLC, respectively, were isolated in homogeneous form and used for chemical characterization and electrophysiological experiments,

AMINO ACID ANALYSIS AND SEQUENCING

Samples (about one nmol each) of the pure native peptides Pi2 and Pi3, and their fragments generated by enzymatic digestion, were analyzed in a Beckman 6300E amino acid analyzer, after acid hydrolysis for 20 hr in 6 N HCl at 110°C. An aliquot of each one of the pure peptides (100 μg) was reduced and alkylated with iodoacetic acid as described [14]. An automatic ProSequencer (Millipore model 6400/6600) was used to determine the amino acid sequence of: (i) the individual native peptides, (ii) their reduced and carboxy-methylated derivatives (RC-peptides), and (iii) their HPLC-purified fragments produced by protease V8 endopeptidase (Bohering, Manheim, Germany) digestion. The cleavage of RC-peptides with protease V8 was performed with samples containing 50 μg peptide in 200 μl, buffered with 100 mM ammonium bicarbonate, pH 7.8; incubated for 4 hr at 37°C, in the presence of 2 μg digestive enzyme. For Pi2, an additional hydrolysis was performed with cyanogen bromide using a 25 μg sample in 400 μl of 10% formic acid at room temperature overnight. The corresponding fragments were also separated by HPLC, and used for sequence and amino acid analysis.

PROTEIN CONTENT

The concentration of peptides used for binding and electrophysiological measurements were estimated based on amino acid analysis. A solution containing 1.0 mg/ml peptide, for both toxins, absorbs 0.65 units at 280 nm, using a cuvette with 1 cm pathway.

BINDING AND DISPLACEMENT ASSAYS

NTX was radiolabeled with ¹²⁵Iodine and used for binding and displacement assays as described [21]. Rat brain synaptosomal membranes (fraction P3) were prepared by the method of Catterall, Morrow and Harfshorne [4], and used for assessing the capability of Pi2 and Pi3 to displace the binding of ¹²⁵I-NTX.

SF9 INSECT CELLS CULTURE, AND *SHAKER* B K⁺ CHANNELS EXPRESSION

The culture of the insect cell line Sf9 from the army-worm caterpillar *Spodoptera frugiperda*, and the expression of *Shaker* B K⁺ channels were done as previously reported [8]. In brief: the cells were kept at 27°C in Grace's media, and infected with the recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus, containing the *Shaker* B K⁺ channel cDNA [13]. The recombinant baculovirus was a kind gift of Dr. C. Armstrong

ELECTRODES, ELECTRICAL RECORDINGS AND SOLUTIONS

The electrodes for patch-clamp recordings were pulled from borosilicate glass (KIMAX 51), to a resistance of 1 to 1.8 MΩ, and used without further treatment. Macroscopic currents through *Shaker* B K⁺ channels were recorded under whole-cell patch-clamp [11], two days after the infection of the cells, with an Axopatch ID (Axon Instruments). The delivery of the pulses and the acquisition of the data were done through a TL-2 interface (Axon Instruments) connected to a PC computer, with the pClamp 5.5 software Currents were sampled at 100 μsec/point. Between 70-75% of the series resistance were electronically compensated.

The holding potential was −80 mV. Pulses were delivered at a rate of one each 15 sec to allow full recovery from inactivation.

The internal solution was (in mM) 90 KF, 30 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES-K, pH 7.2. The external solution was: 145 NaCl, 10 $CaCl_2$, 10 MES-Na, pH 6.4.

Results and Discussion

PEPTIDE PURIFICATION AND AMINO ACID SEQUENCE

Two unknown K⁺- channel-blocking peptides (Pi2, and Pi3) were isolated from the venom of the scorpion *P. imperator*, by chromatographic procedures. Briefly, the soluble venom was initially separated by gel-filtration chromatography in Sephadex G-50 (Fig. 1a). The fraction III, which contained the peptides capable of displacing the binding of ¹²⁵I-NTX to synaptosome membranes, was subsequently applied to an HPLC column (Fig. 1b). *Subfractions 2 and 3 from this column were rechromatographed in a step gradient, giving a homogeneous com-*
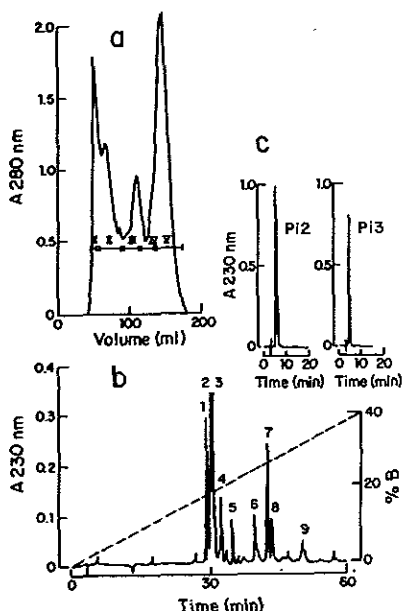
Fig. 1. Sephadex G-50 and HPLC purification of Pi2 and Pi3. (*a*) *P. imperator* soluble venom (120 mg) was applied to a Sephadex G-50 column (0.9 × 200 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 30 ml per hr. Five subfractions (horizontal bars *I-V*) were obtained. (*b*) Subfraction III was further separated in a C18 reverse-phase column of an HPLC system, using a linear gradient from solution A (0.12% trifluoroacetic acid in water) to 40% solution B (0.1% trifluoroacetic acid in acetonitrile), for 60 min (*c*) Fractions 2 and 3 were further separated by a step gradient (12% solution B for Pi2, and 13% B for Pi3) given pure toxins Pi2 and Pi3, as demonstrated by Edman degradation and amino acid analysis.

ponent (Fig. 1*c*). These peptides were named Pi2 and Pi3, which are the abbreviations of *P. imperator* toxin 2 and 3, respectively (the word toxin is not included). The total amount of these peptides, based on recovery values from the chromatographic separations, showed that Pi2 is about 2.1%, while Pi3 is 2.5% of the whole soluble venom.

Amino acid analysis (Table 1) of both peptides showed that they are composed of 35 amino acid residues each, with a calculated molecular weight of 4,036 for Pi2 and 4,068 for Pi3, respectively. To confirm the initial amino acid composition and to determine the full amino acid sequence of these toxins, samples of native peptides, RC-toxins and the endopeptidase V8 digestion products of RC-toxins, after HPLC fractionation (Fig. 2), were sequenced. The HPLC-separated fragments for both Pi2 and Pi3 (Fig. 2), were analyzed *de novo*, for

their amino acid content (Table 1). Both peptides have confirmed the amino acid composition initially determined for the native peptides and also provided evidence for the presence of two arginines in both peptides.

In Fig. 3, we show the full amino acid sequence of Pi2 and Pi3, indicating the corresponding overlapping segments. The sequence of the C-terminal segment of Pi2 was confirmed after cyanogen bromide rupture of Met at position 26. Since the composition of Pi2 and Pi3 differs by only one amino acid, the sequence determination of Pi3 was conducted directly with RC-toxin. Pi2 and Pi3 are naturally occurring single amino acid mutants of each other. Their three dimensional structures are assumed to be closely compacted by three disulfide bridges (6 cysteines each), as demonstrated for the case of NTX and ChTx [7,10].

THE NOVEL PEPTIDES, PI2 AND PI3, BLOCK VOLTAGE-DEPENDENT K⁺ CHANNELS

Two assays were used to determine the possible function of these peptides. By binding and displacement experiments, using radiolabeled noxiustoxin [21] it was found that both Pi2 and Pi3 were effective in competing for similar binding sites (K⁺ channels) of membranes obtained from rat brain synaptosomes (*data not shown*). To confirm these data, we decided to verify their effects on the activity of well-defined K⁺ channels. For this purpose we expressed *Shaker* B K⁺ channels in the insect cell line Sf9, by infection with a recombinant baculovirus (*see Materials and Methods*). This system has the advantage that the *Shaker* B channels are the only voltage-dependent macroscopic conductance in the membrane of the cells, thus making the results unambiguous [13].

Addition of Pi2 to the external solution effectively reduces the current through *Shaker* B K⁺ channels, in a reversible manner. This is shown in Fig. 4. The upper panel shows macroscopic currents through *Shaker* B K⁺channels under whole-cell patch clamp. The channels were opened by depolarizing the membrane to the indicated voltages, from a holding potential of −80 mV (upper traces, control). Addition of 150 nM of Pi2 to the external solution, produced an almost 100% reduction of the current at all voltages (middle traces). The effect was easily reverted by washing the cells with the control external solution (lower traces, recovery). This is best seen by looking at the current *vs.* voltage curve (*IV*) in the bottom panel.

Using lower concentrations of Pi2, it is readily seen that the reduction in the current is not voltage dependent (*see below*), and does not change the kinetics of the macroscopic currents; therefore the simplest interpretation of the reduction of the current induced by Pi2, is that the peptide is blocking the K⁺ channels.

We have shown that Pi2 is a highly effective blocker

Table 1. Amino acid composition of peptides

Amino acid	Pi2 nmol (i)[a]	Pi3 nmol (i)	Pi2N nmol (i)	Pi2C nmol (i)	Pi3N nmol (i)	Pi3M nmol (i)	Pi3C nmol (i)
Asp	3.4 (3)	3.2 (3)	1.2 (1)	2.0 (2)	1.1 (1)	t.a (0)[b]	2.0 (2)
Thr	2.6 (3)	2.6 (3)	1.7 (2)	1.0 (1)	1.7 (1)	t.a (0)	0.8 (1)
Ser	0.9 (1)	1.0 (1)	1.0 (1)	t.a (0)	0.7 (1)	t.a (0)	t.a (0)
Glu	2.4 (2)	3.2 (3)	2.1 (2)	t.a (0)	1.1 (1)	2.2 (2)	t.a (0)
Pro	2.6 (3)	1.8 (2)	1.7 (2)	0.9 (1)	t.a (0)	0.9 (1)	0.8 (1)
Gly	2.4 (2)	2.3 (2)	t.a (0)	2.0 (2)	t.a (0)	t.a (0)	2.1 (2)
Ala	1.1 (1)	1.1 (1)	t.a (0)	1.0 (1)	t.a (0)	t.a (0)	1.0 (1)
Val	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)
Met	1.1 (1)	0.9 (1)	t.a (0)	0.6 (1)	t.a (0)	t.a (0)	0.4 (1)
Ile	0.6 (1)	0.7 (1)	0.9 (1)	t a (0)	1.0 (1)	t.a (0)	t.a (0)
Leu	t a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)
Tyr	1.8 (2)	1.8 (2)	0.9 (1)	1.0 (1)	t.a (0)	0.7 (1)	0.8 (1)
Phe	1.0 (1)	1.0 (1)	t.a (0)	1.1 (1)	t.a (0)	t.a (0)	0.8 (1)
His	1.0 (1)	1.2 (1)	0.9 (1)	t.a (0)	t.a (0)	1.0 (1)	t.a (0)
Lys	6.2 (6)	6.3 (6)	2.9 (3)	3.1 (3)	t.a (0)	2.9 (3)	2.7 (3)
Arg	1.2 (1)[c]	1.2 (1)[c]	t.a (0)	1.9 (2)	t.a (0)	t.a (0)	2.1 (2)
Trp[d]	– (0)	– (0)	– (0)	– (0)	– (0)	– (0)	– (0)
½ Cys[d]	– (6)	– (6)	– (3)	– (3)	– (1)	– (2)	– (3)
Total	35	35	17	18	7	10	18

Pi2 and Pi3 are toxins 2 and 3, respectively. Pi2N and Pi3N, and Pi2C and Pi3C are N-terminal and C-terminal fragments generated by endopeptidase v8 hydrolysis of Pi2 and Pi3, respectively, while Pi3M means intermediate peptide from v8 cleavage of Pi3.

[a] (i), means nearest integer number of residues found per mol

[b] t.a, means trace amounts or none

[c] Arg were confirmed to be two per molecule

[d] –Trp and ½ Cys number determined by sequencing

of *Shaker* B K⁺ channels. It was therefore of major interest to examine the effect of Pi3, a peptide considered to be a natural point mutation of Pi2, as mentioned before. Figure 5 shows the effect of 150 nM Pi3 over the activity of *Shaker* B. It is clear from Fig. 5 that Pi3 also blocks the channels, but whereas the addition of Pi2 caused an almost 100% reduction in the amplitude of the current, the addition of Pi3, at the same concentration, caused only approx. 48% reduction of the current. Therefore, the affinity of Pi3 for the channels seems to be significantly smaller than that of Pi2.

Neither the block by Pi2, nor that by Pi3 have a noticeable voltage dependence. This is shown in Fig. 6, where the fraction of blocked channels is plotted against the voltage. It is interesting to observe that even 7.5 nM of Pi2 produces a stronger block than 150 nM Pi3.

The above results clearly show that the change of Pro7 in Pi2, for Glu7 in Pi3 significantly reduces the affinity of Pi3 towards the *Shaker* B K⁺ channels. To quantitate this point we measured the extent of the block produced by several concentrations of the peptides. The results in Fig. 7 show that; (i) the concentration dependence of the block, by both Pi2 and Pi3, follows a Michaelis-Menten saturation curve, meaning that each channel is blocked by a single peptide, (ii) with zero K⁺ (*not added*) in the external solution, the K_d of Pi2 is 8.2 nM, and (iii) the change Pro7->Glu7 dramatically reduces the affinity of the peptide for the channels, K_d over 140 nM.

These results are not pH dependent in the range of 5.5 to 8.1 (*data not shown*). Since the binding of Pi2 and Pi3 is dependent on the concentration of external K⁺, and for comparative purposes in identical conditions, these experiments were performed in absence of potassium. It is worth mentioning that K⁺ is an effective competitor of the toxin binding after 5 mM. There is practically no difference between 0 and 5 mM external K⁺ concentration (*data not shown*).

STRUCTURE-FUNCTION RELATIONSHIP

Scorpion toxins against K⁺ channels are short peptides of about 31 to 39 amino acids [7,17], whereas the toxins against Na⁺ channels are longer peptides containing 61 to 70 amino acids residues [17,18]. Concerning their primary structure, both families of toxins are highly variable. Except for the constant relative positions of the cysteines, and a couple of the other residues, most of the amino acids of the primary structure are variable. This variability certainly explains the differential affinities of these peptides for their various subtypes of ion channels. For example, noxiustoxin (NTX) binds with 100 pM affinity to the K⁺ channels present in rat brain synaptosomes [10], whereas it binds with 450 nM affinity to Ca²⁺-dependent K⁺ channels of rabbit skeletal muscle [22], and with 310 nM to Ca²⁺-dependent K⁺ channels of epithelial cells [20]. Its homologous peptide (about 50%
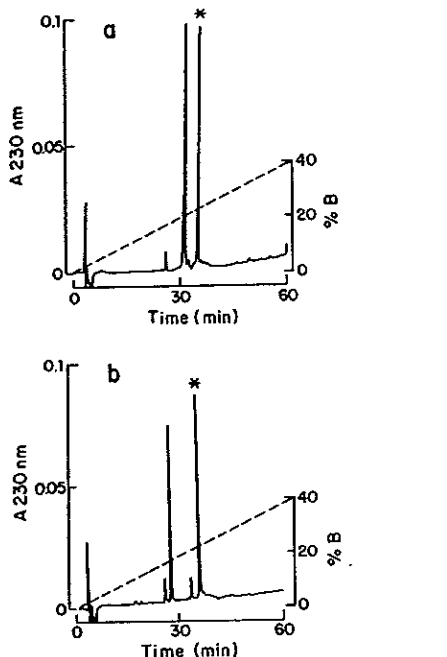
Fig. 2. HPLC separation of peptides from Pi2 and Pi3. Samples containing 50 μg each of Pi2 (Fig. 2a) and Pi3 (Fig. 2b) were hydrolyzed with endopeptidase v8 and separated in a C18 analytical reverse-phase column, using the same system as Fig. 1b. The stars in each chromatogram indicate the elution time of the C-terminal fragment.
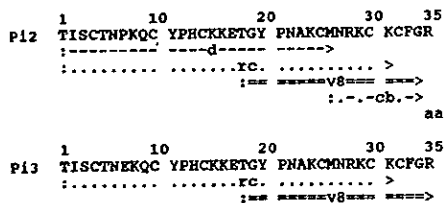
```
        1         10        20        30    35
Pi2   TISCTNPKQC YPHCKKETGY PNAKCMNRKC KCFGR
      :--------- ----d----- ----->
      :.......... ........rc. ..........  >
                 :=== ===x=v8=== ===x>
                        :.-.-cb.->
                                    aa

        1         10        20        30    35
Pi3   TISCTNEKQC YPHCKKETGY PNAKCMNRKC KCFGR
      :.......... ........rc. ..........  >
                 :=== ===x=v8=== ===x>
```

Fig. 3. Amino acid sequence of Pi2 and Pi3. Numbers on top of the sequences indicate the positions of the amino acids after ordering the final sequence, according to overlapping segments obtained by direct sequencing the native toxin (-d-), RC-toxin (.rc.), v8 endopeptidase cleavage (= v8 =), cyanogen bromide (- cb.-) and amino acid analysis (aa).

identical), charybdotoxin (ChTX) has a K_d of 25–30 pM to plasma K⁺ channels of membranes prepared from rat brain synaptosomes [reviewed in 7] and 1.8 nM to Ca²⁺-dependent K⁺ channels from rabbit skeletal muscle [22].
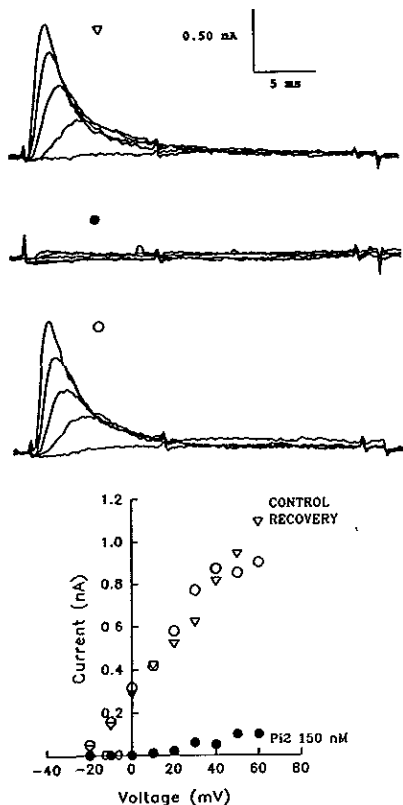

Fig. 4. Pi2 added to the external solution blocks *Shaker* B K⁺ channels The upper traces shows macroscopic currents under whole cell patch clamp, in the control external solution (see Materials and Methods) The channels were opened by 30 msec depolarizations from −10 to +60 mV in 20 mV increments, from the holding potential of −80 mV Addition of 150 nM of Pi2 to the external solution produced an almost 100% reduction of the current (middle traces). The effect of Pi2 is easily and totally reversed by washing the cells with the control external solution (lower traces). These effects are best shown by the complete peak current *vs.* voltage curve (*IV*), at the bottom of the figure

If we compare the primary structures of Pi2 and Pi3 with those of other known peptides that recognize K⁺ channels, it is evident that they are quite different. As shown on Table 2, the toxins from scorpions of the New World, genus *Centruroides*, are most closely related to Pi2 and Pi3 (54 to 42% identity), than those of the Old World scorpions of the genera *Leiurus*, *Buthus* and *Androctonus* (only 38 to 29% identity). Apart from the six common cysteinyl residues, the only other amino acid
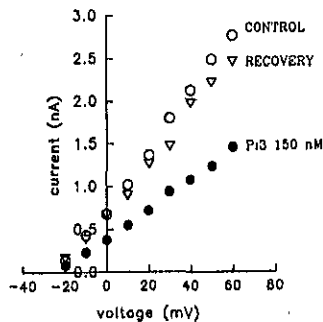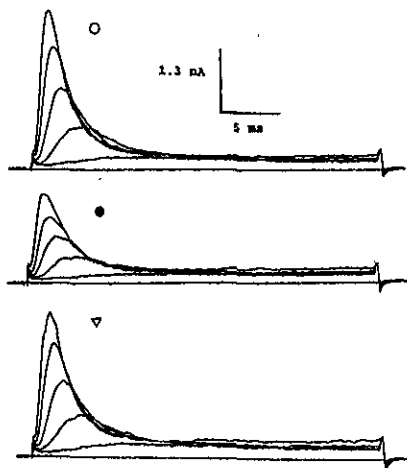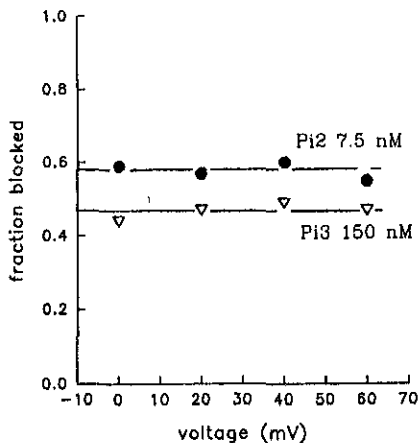
Fig. 6. Block by both Pi2 and Pi3 is not voltage dependent. The fraction of the channels blocked is plotted against the voltage, for two different cells. 7.5 nM of Pi2 caused an average 58% of block at all voltages, whereas 150 nM of Pi3 caused an average 49% of block. Thus, the extent of block by both toxins is not appreciably voltage dependent (the extent of block also does not depend on the holding potential—*not shown*). The fraction blocked at each voltage was calculated as $1-(It/Ic)$, where Ic is the peak current in the control and It is the peak current after the addition of the toxin.

Fig. 5. Pi3 added to the external solution blocks *Shaker* B K⁺ channels. The upper traces shows macroscopic currents under whole cell patch clamp, in the control external solution (*see* Materials and Methods). The channels were opened as indicated in Fig. 4. Addition of 150 nM of Pi3 to the external solution produced a moderate ~48% reduction of the current (middle traces). The effect of Pi3 is easily and totally reversed by washing the cells with the control external solution (lower traces). These effects are best shown by the complete *IV* at the bottom of the figure.

absolutely conserved in all these sequences is lysine in position 28 of Table 2, which corresponds to Lys27 in charybdotoxin, shown to be essential for channel binding [7,16]. However, the most relevant information obtained by comparing these K⁺-toxins (Table 2) is that Pi2 and Pi3 differs in only one position (number 7), and that is enough to change their affinities for the same type of *Shaker* B K⁺ channels by at least seventeenfold.

This constitutes a clear indication that the N-terminal sequence of both toxins are important for channel recognition and/or affinity, in a fashion similar to that described for NTX [9,10]. The presence of a negatively charged amino acid (Glu) in position 7, substituting for the neutral proline, probably interacts unfavorably with the *Shaker* B K⁺ channels. Nevertheless, the fact that the C-terminal regions have several cysteinyl residues clustered together in a highly conserved manner, including the constant Lys at position 28, is also suggestive that this part of the molecule must be important for maintaining the three-dimensional structure of the molecule and/or for channel recognition.

Additionally, the fact that in Table 2, at the N-terminal segment of the two newly purified toxins we added several gaps to enhance similarities between Pi2, Pi3 and NTX, it would suggest that the N-terminal region of these two newly purified peptides do admit some structural variations in their structures without loss of channel-binding capacity. These facts could again suggest an evolution of structural features to fit more properly with variations at the channel level.

In this same direction are the observations related to the amount of these peptides present in the various scorpion venoms. For example, the amount of Pi2 plus Pi3 (2.1% and 2.5, respectively) and Pi1 (also a K⁺ toxin) which is approx. 1.5% [*unpublished results*], all together
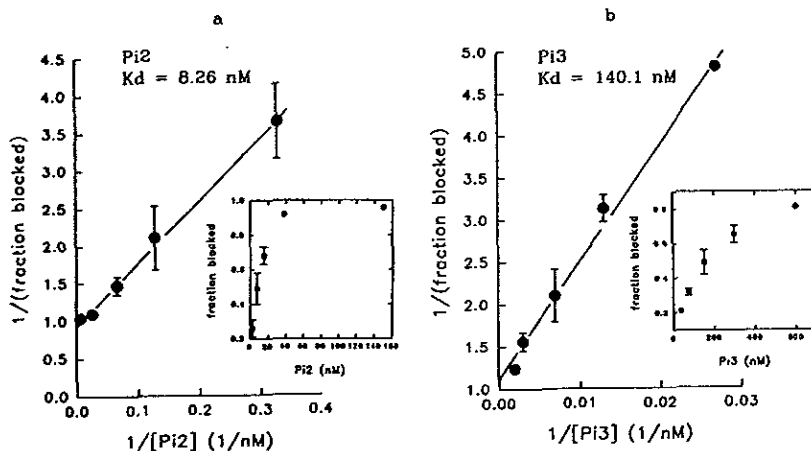
Fig. 7. The extent of block is a function of the toxin concentration. (*a*) The fraction blocked by Pi2 is shown in a double-reciprocal plot, each point represents the mean ± SD of at least three different cells. The block by Pi2 follows a Michaelis-Menten saturation curve with a K_d of only 8.2 nM ($r = 0.998$). The inset shows the direct (fraction blocked *vs.* concentration) curve. (*b*) Double-reciprocal plot of the fraction of channels blocked by Pi3. The block follows a Michaelis-Menten saturation curve with a K_d of 140 nM ($r = 0.995$). The inset shows the direct plot. The points represent the mean ± SD of at least 3 different cells.

Table 2. Comparative amino acid sequence of K⁺ channel toxins

Toxin	Amino acid sequence					Identity (%)
	1	10	20	30	39	
Pi2	-TI---SCTNP	KQCYPHCKKE	TGYPN-AKCM	NRKCKCFGR		100
Pi3	-TI---SCTNE	KQCYPHCKKE	TGYPN-AKCM	NRKCKCFGR		97
MgTX	-TIINVKCTSP	KQCLPPCKAQ	FGQSAGAKCM	NGKCKCYPH		54
NTX	-TIINVKCTSP	KQCSKPCKEL	YGSSAGAKCM	NGKCKCYNH		51
Pi1	---L-VKCRGT	SDCGRPCQQQ	TGCPN-SKCI	NRMCKCYGC		43
c11TX 1	ITI-NVKCTSP	QQCLRPCKDR	FGQHAGGKCI	NGKCKCYP-		42
Lg2	pEFTQESCTAS	NQCWSICKRL	HNTNRG-KCH	NKKCRCYS-		38
AgTX2	GVPINVSCTGS	PQCIKPCKDA	GHRF-G-KCM	NRKCHCTPK		38
AgTX3	GVPINVPCTGS	PQCIKPCKDA	GHRF-G-KCM	NRKCHCTPK		36
ChTX	pEFTNVSCTTS	KECWSVCQRL	HNTSRG-KCM	NKKCRCYS-		33
KlTX	GVEINVKCSGS	PQCLKPCKDA	GHRF-G-KCM	NRKCHCTPK		33
IbTX	pEFTDVDCSVS	KECWSVCKDL	FGVDRG-KCM	GKKCRCYQ-		32
AgTX1	GVPINVKCTGS	PQCLKPCKDA	GHRF-G-KCI	NGKCHCTPK		30
LeTX I	AF----C-NL	RMCQLSCRSL	-GL-LG-KCI	GDKCECVKH		29
Consensus	-----C---	--C---C---	-----KC-	---C-C---		

Pi2 and Pi3, from this work; Pi1 from T. Olamendi-Portugal et al., *unpublished results*; c11TX 1, toxin 1 from [14]; NTX, noxiustoxin from [18]; MgTX (margatoxin), ChTX (charybdotoxin), IbTX (iberio-toxin), Lq2 (*L. quinquestriatus* toxin 2) and AgTX1 to AgTX3 from *L. quinquestriatus* var. hebraeus [reviewed in 7] LeTX I, leirutoxin 1 [1,5]. KlTX, kaliotoxin [6] Consensus means only positions in which amino acids are conserved in all sequences Gaps (-) were introduced to enhance similarities

comprises 6.1% of the whole soluble venom, which is a much greater amount in the venom of *P. imperator* than the related toxins, charybdotoxin and noxiustoxin, isolated respectively from *Leiurus quinquestriatus* and *Centruroides noxius*. The latter two occur in less than 0.5%

in each of the corresponding venoms [2,17]. This fact probably indicates some evolutionary trait. While the first (*Pandinus*) is nontoxic to mammals, the two other species of scorpions *Centruroides* and *Leiurus* are Thus, we can hypothesize that scorpions of the genus

Pandinus have compensated for the lack of typical Na⁺-channel blocking toxins (rich in the two other genera) by producing a larger amount of K⁺-channel specific toxins. Since *P. imperator* venom is toxic to insects and crustaceans (*unpublished results*), we are tempted to speculate that maybe these K⁺ toxins are rather species specific. Hence, this variability could be, again, an evolutionary occurrence that meets with the diversity of ion channel molecules.

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