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TOXINAS DE ALACRÁN QUE AFECTAN CANALES DE POTASIO DEL TIPO hERG1

T E S I S

QUE PARA OBTENER EL GRADO DE

D O C T O R E N C I E N C I A S

P R E S E N T A :

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ÍNDICE

ABREVIATURAS

(φ80)	Célula acarreadora del profago lamba φ80.
ΔU169	Deleción del operón *lac* del cromosoma.
Amp^r	Gen de resistencia a ampilicilina.
Ecol ori	Origen de replicación de *E. coli.*
AgTx2	Agitoxina.
Kir	Canales de potasio entrantes.
EAG	*Ether-à-go-go.*
HUGO	Human Genome Organisation = Organización del Genoma Humano.
HGNC	HUGO Gene Nomenclature Comitte = Comité de Nomenclatura de los Genes HUGO.
LQT2	Síndrome QT largo del tipo 2.
IUPHAR	International Union of Pharmacology = Unión Internacional de Farmacología.
ChTx	Caribdotoxina.
deoR	Gen regulador que sigue la expresión constitutiva de deoxirribosa en la síntesis de genes.
DNA	Ácido desoxirribonucleico.
DEAE	Dietil-amino-etil-celulosa.
EDTA	Ácido etilendiaminotetraacético.
EGTA	Ácido etilenglicol -bis(β-aminoetileter) N,N,N',N' tetraacético.
endA1	Mutación en el gen endonucleasa I.
F'	La cepa contiene el plásmido F.
glnV	Supresión del codón de paro ámbar (UAG).
gyrA96	Mutación en el gen DNA girasa.
HEPES	N-[2-Hydroxietil] piperazina N'-[ácido 2 etanosulfónico].
erg	*ether-á-go-go related gene.*
RP-HPLC	Cromatografía Líquida de Alta Presión en Fase Reversa.
IPTG	Isopropil β-tiogalactósido.
KDa	Kilo Daltons**.**

ABREVIATURAS

Kv	Canal de potasio dependiente de voltaje.
F^-	La cepa no contiene el plásmido F.
lacIq	Sobreproducción del gen que codifica para la proteína represora lac.
lacY	Mutación en el gen galactosido permeasa. Inhibe la utilización de lactosa.
NaCl	Cloruro de Sodio.
KTx	Toxinas de alacrán que afectan a canales de potasio.
nm	nanómetros.
RMN	Resonancia Magnética Nuclear.
nupG	Gen regulador que sigue la expresión constitutiva de deoxirribosa en la síntesis de genes.
PAGE	Electrofóresis en Gel de Poliacrilamida.
PBS	Buffer de fosfatos salino.
PCR	Reacción en Cadena de la Polimerasa.
PDB	Protein Data BanK (Banco de datos de Proteínas).
recA	Abolición de la recombinación homóloga.
relA	Fenotipo relajado, permite la síntesis de RNA en ausencia de síntesis de proteínas.
rErgtx1	Ergtoxin1 recombinante.
rpm	revoluciones por minuto.
SDS	Dodecil Sulfato de Sodio.
supE	Supresor del codón de paro ámbar (UAG).
TFA	Ácido Trifluroacético.
thi-1	Mutación en el gen del metabolismo de la tiamina. La cepa requiere de tiamina para crecer en medio mínimo.
Tris	Tris (hidroximetil) aminometano.
(lacZ)M15	Deleción parcial del gen ß-galactosidasa.

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

ABSTRACT

Ergtoxin 1 (ErgTx1) is a 42 amino acid peptide purified from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann, capable of blocking specifically human potassium channels of the *ether-á-go-go*-related gene family (hERG). This peptide binds to a partially overlapping site on the channel outer mouth, in which residues of the S5-P linker are critically involved. Naturally oxidized Met35 decreases by three orders of magnitude the affinity of the peptide for the hERG1 channels. This result is quite relevant, because it shows two possible situations: either Met35 is involved in the proper folding of the molecule or it plays a direct role in the interaction with the channel, i.e., constitutes part of the interacting surfaces. These two situations were evaluated by preparing heterologously expressed ErgTx1 gene and a mutant containing alanine in position 35. Similarly other recombinant mutants of the gene coding for ErgTx1 were expressed heterologously in *E. coli*, properly folded and their affinities and interactions with hERG1 channels were determined by patch-clamp techniques. Residues in position Y14, Y17, M35A and F37 of the solvent exposed hydrophobic surface, and charged residues at position K13 and K38 of ErgTx1 were shown to cause a decrement of the affinity from 20 folds to 3 orders of magnitude, thus suggesting that they are certainly participating on the binding surface of this toxin towards the hERG1 channels. Double mutants at positions K13 and F37, Y14 and F37, Y17 and F37 and K13 and K38 were also expressed and assayed, but the findings obtained are not much different from the single point mutants of ErgTx1. The findings of the present work indicate the most probable surface area of ErgTx1 that makes contact with the hERG channels.

RESUMEN

ErgTx1 es un péptido de 42 aminoácidos aislado del veneno del alacrán mexicano *Centruroides noxius* Hoffmann, capaz de actuar específicamente sobre la familia de canales de potasio humano *ether-á-go-go* (hERG). Este péptido se une parcialmente sobre la boca externa del vestíbulo del canal, en donde los residuos del asa S5-P están principalmente involucrados. La oxidación natural del residuo Met35 reduce tres órdenes de magnitud la afinidad del péptido por el canal hERG1. Estos resultados revelan dos posibles situaciones: la metionina está involucrada en el plegamiento de la molécula o bien, interacciona directamente con el canal, formando parte de la superficie de interacción. Estas dos situaciones se evaluaron expresando de manera heteróloga el gen que codifica para el péptido ErgTx1 y una mutante que contiene alanina en la posición 35. Similarmente, otras variantes del gen que codifica para la ErgTx1 se expresaron en *E. coli*, y sus afinidades con el canal hERG1 se determinaron mediante la técnica de fijación de voltaje en un parche de membrana (*Patch-clamp)*. La sustitución por alanina de los aminoácidos Y14, Y17, M35 y F37, que forman parte del parche hidrofóbico, así como, el residuo cargado K13 de la ErgTx1 muestran un decremento en la afinidad de 20 veces a 3 órdenes de magnitud. Lo anterior, sugiere que ciertamente estos residuos participan en la superficie de unión de la toxina con los canales hERG1. Las dobles mutantes en las posiciones K13A-F37A, Y14A-F37A, Y17A-F37A y K13A-K38A se expresaron y ensayaron, pero los resultados obtenidos no son muy diferentes a los de las mutantes puntuales de la ErgTx1. Los resultados del presente trabajo indican la probable superficie de la ErgTx1 que hace contacto con los canales hERG1.

I. INTRODUCCIÓN

El transporte de moléculas polares hacia dentro y fuera de la célula o entre los diferentes compartimentos intracelulares, se lleva acabo, por proteínas de membrana como son: bombas, transportadores y canales iónicos (Alberts y cols., 1996).

Los canales iónicos son proteínas transmembranales, que forman en su interior la estructura que permite que un gran número de iones puedan pasar a través de ellos. El flujo de iones a través de estos canales es a favor de su gradiente de concentración química, pero al estar cargados eléctricamente, también deben hacerlo a favor del gradiente eléctrico; el punto en que la fuerza química de arraste de un ion se equlibra exactamente con la fuerza eléctrica de arrastre de ese ion se llama potencial electroquímico o de Nernst.

Los canales iónicos se pueden clasificar en función del ion para el que muestran permeabilidad selectiva (Na^+, K^+, Ca^{2+}, etc.) o bien en función de las circunstancias o elementos que provocan la apertura del canal. Un canal puede estar abierto de forma permanente, de manera que el gradiente electroquímico es la única circunstancia que en un momento determinado condiciona el paso de un ion específico, pero con extraordinaria frecuencia los canales están cerrados y son abiertos (activados) e inactivados en respuesta a señales específicas, entre las cuales se encuentran: a) despolarización o modificación del potencial transmembrana (canales dependientes de voltaje); b) activación por ligandos extracelulares que interactúan con dominios específicos de la molécula que conforma el canal, el cual se comporta como aunténtico transportador; c) elementos intracelulares generados previamente por modificaciones causadas tras el paso del ion (fijación de Ca^{2+} al canal, desfosforilación de la proteína provocada por Ca^{2+}, etc.).

La selectividad del canal implica varios conceptos: en primer lugar, el tipo de estímulo que lo activa (si es un ligando, un cambio de voltaje, etc.) y dentro de un determinado estímulo, que tipo de ligandos o que intensidad de voltaje lo abren; y en segundo lugar, el tipo de iones que deja pasar a través del canal, lo cual dependerá tanto de las características del ion (carga eléctrica, tamaño y grado de hidratación) como de las del canal (estructura, tamaño y composición que condiciona la carga eléctrica de las paredes del poro; Alberts y cols., 1996).

Hasta ahora se conocen más de 400 genes que codifican para proteínas relacionadas con canales iónicos (Yu y cols., 2005) y entre sus diversas funciones, son responsables del mantenimiento del potencial de membrana, de la contracción muscular, permiten la regulación del volumen celular, participan en diversas vías de señalización y son los encargados de la generación y propagación del impulso nervioso.

1. Canales de potasio

Los canales de potasio permiten el paso de iones K^+ a través de la membrana celular a favor de su gradiente electroquímico y participan en diferentes procesos celulares, incluyendo la regulación del volumen celular, la secreción hormonal y participan en la repolarización del impulso eléctrico en células excitables (Hille, 2001).

En el genoma humano se han encontrado alrededor de 78 genes que codifican para canales de K^+, con base en su modo de activación y número de segmentos transmembranales se han clasificado en tres familias: (1). Familia 2TM o también conocida como familia de canales de K^+ rectificadores entrantes (Kir) e incluye a los activados por proteínas G y a los sensibles a ATP. Su estructura funcional es tetramérica formada por subunidades-α y cada una constituida por dos segmentos transmembranales (TM); (2). Familia 4TM, su estructura funcional es dimérica y cada subunidad esta formada dos segmentos transmembranales y un dominio formador del poro. En esta familia se encuentran los canales de fuga de potasio o "leak", los cuales son regulados por una amplia gama de mediadores bioquímicos y neurotransmisores; (3). Familia de 6TM o 7TM, su estructura funcional es tetramérica y cada subunidad-α está constituida por seis o siete segmentos transmembranales. En esta familia se encuentran los canales de potasio dependientes de voltaje (Kv) y los activados por calcio (K_{Ca2+}; Coetzee y cols., 1999; Korn y Trapani 2005; Gutman y cols., 2005).

Los canales Kv están ampliamente distribuidos en diversos tejidos, se conocen alrededor de 40 genes que codifican para este tipo de canales y se clasifican en 12 subfamilias, Kv1-Kv12 (Gutman y cols., 2005; ver Figura 1).

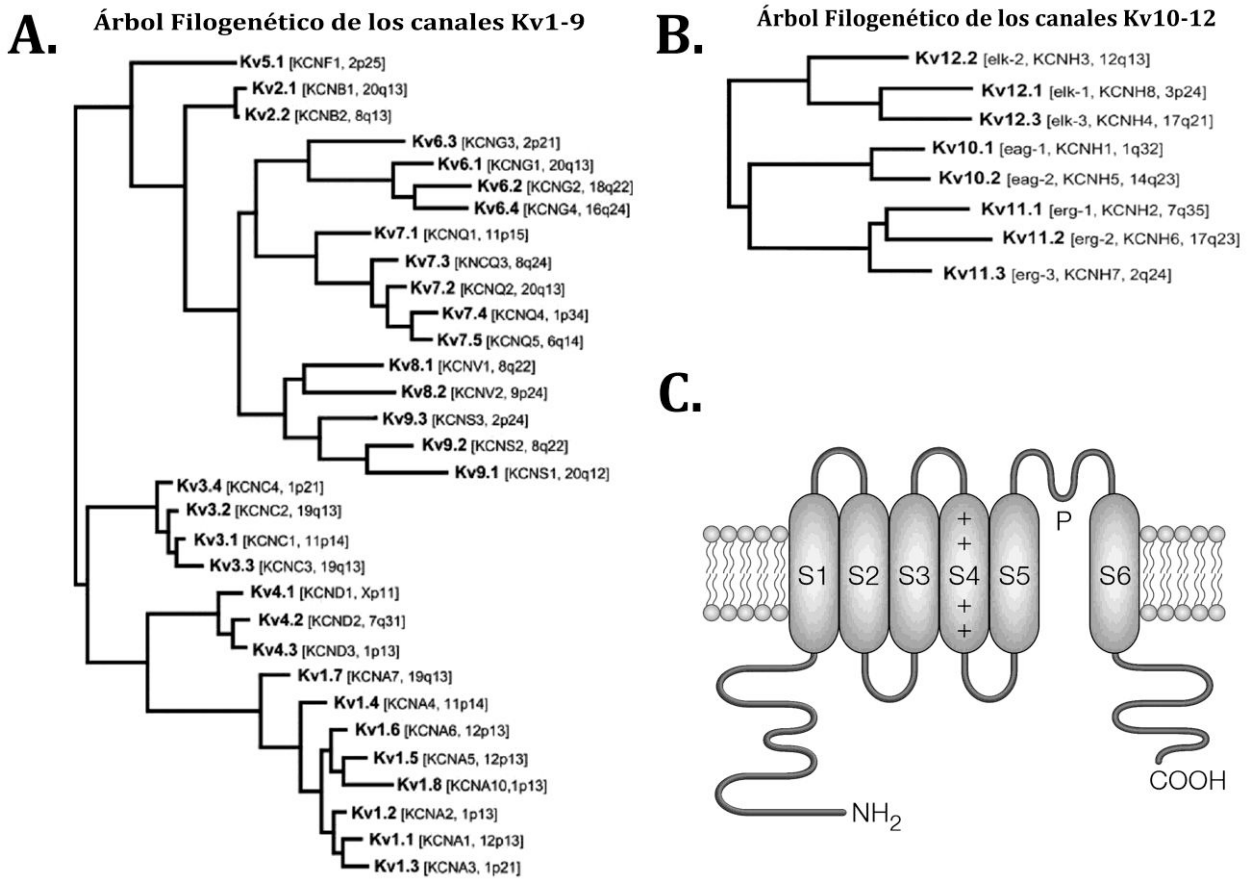

Figura 1. Familia de los canales de potasio dependientes de voltaje (Kv) y representación esquemática de la subunidad principal. A. Árbol filogenético de la familia de canales Kv1-9. La reconstrucción del árbol se realizó alineando secuencias de proteínas de los canales Kv humanos usando el programa CLUSTALW y un análisis de máximo parsimonia con el programa PAUP, resultando árboles sin raíces que comprenden a las familias de canales Kv1 al Kv9. Para el análisis se utilizó solo la región hidrofóbica (S1-S6). En paréntesis se muestra el nombre alternativo asignado por el HGNC (*HUGO Gene Nomenclature Committee* = Comité de Nomenclatura de los Genes *HUGO (Human Genome Organisation* = Organización del Genoma Humano)), seguido por la localización cromosómica de cada uno de los genes que codifican para su respectivo canal. **B.** Árbol filogenético de la familia de canales Kv10-12 (construido con los mismos críterios que en el panel A). **C.** Representación esquemática de la subunidad principal (α) de los canales Kv**.** Esta familia de canales forma parte de la familia 6TMD, constituida por seis hélices transmembranales (representadas por cilindros) y una asa-P entre las hélices S5 y S6 que forma parte esencial del poro del canal (Tomado de Gutman y cols., 2005; Choe, 2002).

2. Canal de K^+ hERG1

La subfamilia *EAG* (*ether-à-go-go*) pertenece a la familia de canales Kv que a su vez consiste de tres subfamilias: *eag, elk* ("*eag*-like") y *erg* ("*eag*-related gene"; Warmke y Ganetzky, 1994; Ganetzky y cols., 1999). De esta última se han encontrado tres isoformas caracterizadas principalmente en humanos y ratas: *erg1, erg2, erg3* (Bauer y cols., 1998; Shi y cols., 1997). El canal ERG1 de humano (hERG1) ha sido especialmente relevante para el área médica y la industria farmaceútica, debido a su implicación en distintos eventos celulares, los cuales serán abordados más adelante.

2.2.1. Bases Estructurales del canal hERG1

El canal hERG1, de acuerdo a la nomenclatura establecida por la IUPHAR *(International Union of Pharmacology=* Unión Internacional de Farmacología) pertenece a la familia Kv 11.1 (Figura 1; *K*: canal de potasio; *v*: dependiente de voltaje; *11*: miembro de la tercera subfamilia de canales de mamíferos *erg*; *1*: primer miembro descrito del grupo).

La estructura funcional del canal hERG1 la constituyen cuatro subunidades principales (α), cada una flanqueada por subunidades accesorias en el extremo amino (dominio PAS: *Per-Arnt-Sim*; 1-135 aa) y carboxilo-terminal (sitio de unión a nucleótidos cíclicos; cNBC; ver Figura 2A, B; Morais-Cabral y cols., 1998). Las subunidades α tiene seis segmentos transmembranales con una estructura secundaria de α-hélice (denotados como S1-S6 y representados como cilindros en la Figura 2A, B). Los primeros cuatro segmentos transmembranales (S1-S4) de cada subunidad forman el sensor de voltaje (Zhang y cols., 2004; Trudeau y cols., 1995; Seoh y cols., 1996; Liu y cols., 2003). La secuencia de aminoácidos que se extiende desde el inicio del S5 y al final del S6 en cada una de las subunidades que forman el tetrámero constituyeel poro y el filtro de selectividad (TVGYG; Doyle y cols., 1998) del canal. En esta región los canales hERG1 son similares a otros miembros de la familia de canales de potasio (Warmke y Ganetzky, 1994; Liu y cols., 2002; Pardo-López y cols., 2002a); sin embargo, el asa extracelular que conecta a la hélice del poro con el inicio del S5 (S5-P *loop*) es más larga en el hERG1, tiene 43 aminoácidos

comparado con 14-23 del resto de los canales Kv. Y además es una región involucrada en la interacción con diversas toxinas de animales (ver Figura 2C; Liu y cols., 2002; Pardo-López y cols., 2002a, b; Zhang y cols., 2003).

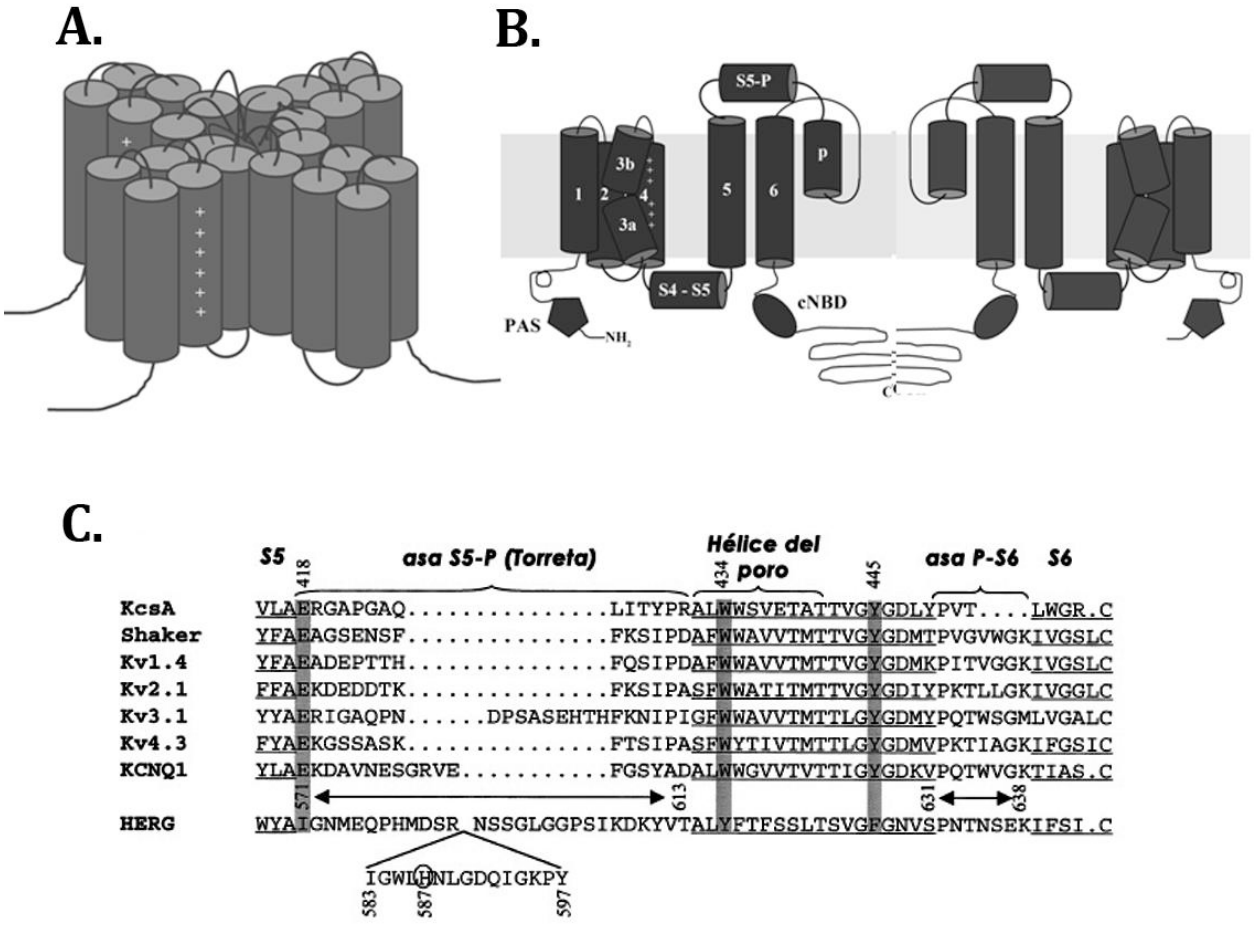

Figura 2. Representación estructural del canal de K^+ hERG1 y comparación de la secuencia de aminoácidos de los segmentos S5 al S6 con otros canales Kv. A. Representación tetramérica del canal formado por subunidades-α que al unirse forman en el centro el poro del canal. Cada subunidad-α está representada por seis estructuras cilíndricas (S1-S6) y en el cuarto cilindro (S4) de cada dominio se muestran las 6 cargas positivas importantes para el sensado del voltaje. **B.** Ilustra el arreglo de dos subunidades α, en donde cada una se constituye de: un dominio PAS (*Per-Art-Sim;* ⬟; 1-135), región N-terminal proximal (138-406), sensor de voltaje (S1-S4; 407-545), región de la Torreta (hélice S5-P; 583-631), dominio del poro (P; 545-665), hélice S6 (666-742), dominio de unión a nucleótidos cíclicos (cNBD; ⬬; 743-844), región C-terminal distal (845-1159). **C.** Alineamiento de secuencias del extremo C-terminal del segmento S5 al N-terminal del S6 del canal hERG1 y otros canales de K^+ dependientes de voltaje. Se ilustra mediante puntos (…) que la región del asa S5-P es más larga en el canal hERG1 (Liu y cols., 2002; Perrin y cols., 2008).

2.2.2. Propiedades biofísicas del canal hERG1

Los canales de potasio hERG1 son proteínas dinámicas que pueden oscilar entre distintas conformaciones, presentando un mecanismo de compuerta o "gating" que responde a cambios en el potencial de membrana. La variación hacia valores menos negativos del potencial de transmembrana interno desplaza el equilibrio conformacional del canal desde un estado cerrado no conductor, hacia el estado abierto (activación) que permite el flujo de K^+ hacia el exterior de la célula impulsado por el equilibrio electroquímico del ión. A este cambio hacia potenciales menos negativos se le conoce como despolarización. Si la despolarización se mantiene, los canales se desplazan a un estado conformacional inactivo no conductor (inactivación). Esta secuencia de eventos es revertida con la repolarización de la membrana, desplazando el estado conformacional del canal de nuevo a través del estado abierto hacia el estado cerrado (desactivación; ~ -80 mV; Tristani-Firouzi y cols., 2002; Figura 3A).

El canal hERG1 presenta una cinética de compuerta inusual en comparación con otros canales Kv, tiene una cinética de activación y desactivación lenta con una duración de cientos de ms a s, a diferencia de la inactivación que ocurre en un lapso de ms a décimas de ms (Wang y cols., 1998; Zhou y cols., 1998). Estas propiedades biofísicas hacen que el tiempo de residencia del hERG1 en el estado abierto sea muy breve durante la despolarización de la membrana y la corriente de K^+ transportada por el canal sea reducida. Por el contario, cuando el potencial de membrana regresa a su valor negativo ocurre una rápida recuperación de la inactivación seguida de una desactivación lenta, lo que mantiene a los canales en su conformación abierta de forma prolongada, observándose una gran corriente entrante de K^+ conocida como "corriente de cola" (Figura 3B; Bauer y Schwarz, 2001; Vandenberg y cols., 2001).

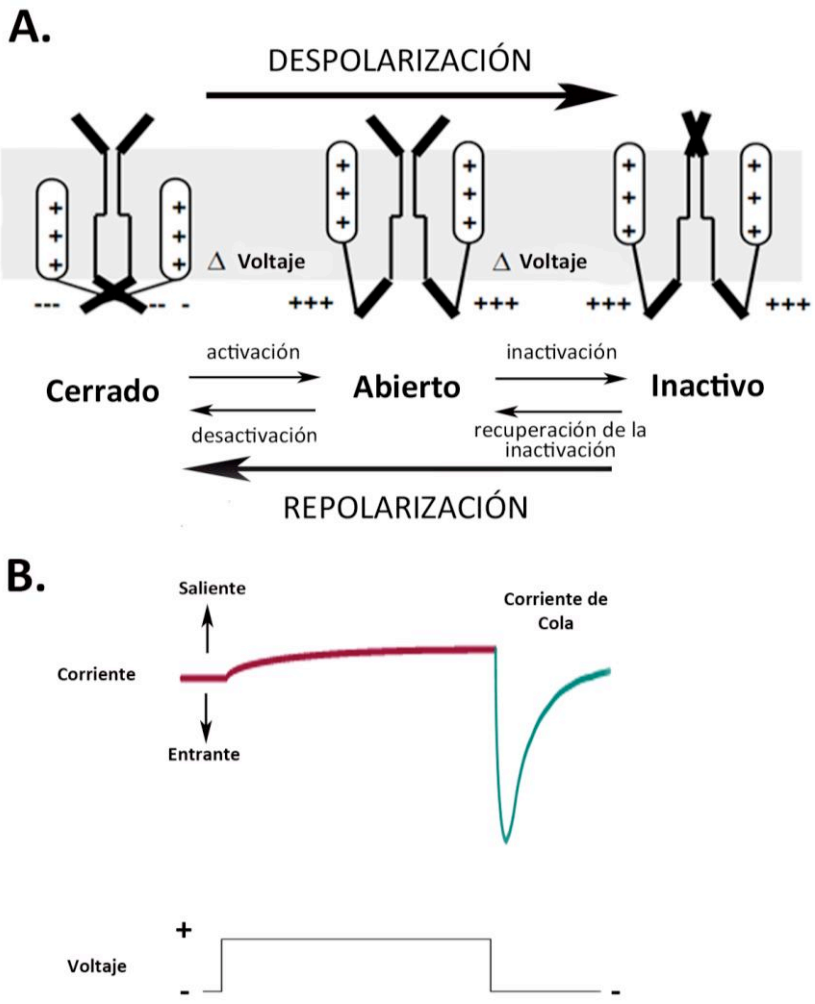

Figura 3. Electrofisiología de los canales de K^+ hERG1. **A.** Representación de los tres estados conformacionales del hERG1: cerrado (izquierda), abierto (centro) e inactivado (derecha), representados con dos de las cuatro subunidades que forman el poro del canal. La transición entre el estado cerrado al abierto se conoce como activación e involucra la apertura de la compuerta intracelular que ocurre lentamente (cientos de ms a s). En cambio, la inactivación es rápida (ms a décimas de ms) y consiste en la transición del estado abierto al inactivo, caracterizada por la constricción del filtro de selectividad del canal (Perrin y cols., 2008). **B.** Esquematización de la cinética de activación e inactivación del canal hERG1. En el potencial de membrana en reposo (~ -80 mV) los canales de potasio hERG1 están cerrados y desactivados. El desplazamiento del potencial hacia valores menos negativos (~-70 a 0 mV) causa la activación del canal y simultáneamente su inactivación resultando una pequeña corriente saliente de K^+ (linea roja). Por el contrario, cuando el potencial de membrana regresa a su valor negativo (- 120 mV), ocurre una rápida recuperación de la inactivación seguida de una desactivación lenta, lo que mantiene a los canales en su conformación abierta de forma prolongada, observándose una gran corriente entrante de K^+ conocida como "corriente de cola" (línea verde; Tomado de Bauer y Schwarz, 2001; Vandenberg y cols., 2001).

Trabajos previos sugieren que la activación lenta del canal hERG1 se debe al lento movimiento del segmento S4 (Smith y Yener, 2002) en respuesta a cambios en el potencial de membrana. Cuando el canal esta cerrado, los residuos cargados negativamente de los segmentos S1 y S2 pueden formar puentes salinos con los residuos básicos de los dos extremos del segmento S4 (D411-K538 y D456-K525). Estos puentes salinos estabilizan al canal en la conformación cerrada, retardando su activación (Figura 4A; Zhang y cols., 2004)

Por otro lado, en la inactivación del hERG1 se propone que el movimiento del segmento S4 hacia lado extracelular del canal causado por la despolarización de la membrana, interviene con el desplazamiento de las hélices S5-P y del poro. Esto hace que el poro externo del canal se cierre (ver Figura 4; Fan y cols., 1999; Ficker y cols., 1998; Liu y cols., 1996; Schönherr y Heinemann, 1996; Smith y cols., 1996). Este mecanismo es análogo a la inactivación tipo C descrita para los canales Kv1 o *Shaker* (Smith y cols., 1996, Hoshi y cols., 1991). Sin embargo, la inactivación en el canal hERG1 es más rápida, por ejemplo a +60 mV tiene una duración de 0.71 ± 0.14 ms y a -60 mV 2.93 ± 0.17 ms (Lu y cols., 2001), mientras que en los canales *Shaker* es mayor a 1 segundo (Meyer y Heinemann, 1997). Una de las principales diferencias entre el canal hERG1 y el *Shaker* es la narturaleza del residuo aromático del filtro de selectividad (Fan y cols., 1999). En el *Shaker*, y en otros canales de potasio, la secuencia de aminoácidos es GYG (Doyle y cols., 1998), en cambio, en el hERG1 la secuencia es GFG. Se ha descrito que la tirosina de los aminoácidos GYG forma puentes de hidrógeno con los residuos W434, W435 y Y445 (localizados en la hélice del poro) que estabilizan al vestíbulo externo del canal en el estado abierto, retardando la inactivación del *Shaker* (Yang y cols., 1997; Doyle y cols., 1998). La ausencia de estos puentes de hidrógeno en el canal hERG1 hace que la hélice del poro sea más flexible y por lo tanto la inactivación más rápida (Fan y cols., 1999). En estudios previos se determinó que residuos localizados en la hélice S5-P (W585, I586, H587, L589, G590, D591, I593, G594; Fan y cols., 1999; Pardo-López y cols., 2002a; Liu y cols., 2002) y en la hélice del poro (G628, S620, S630 y S641; Ficker y cols., 1998; Schönherr y Heinemann, 1996; Herzberg y cols., 1998) contribuyen en la cinética de inactivación del hERG1, ya que mutaciones puntuales de estos residuos pueden retardarla o anularla.

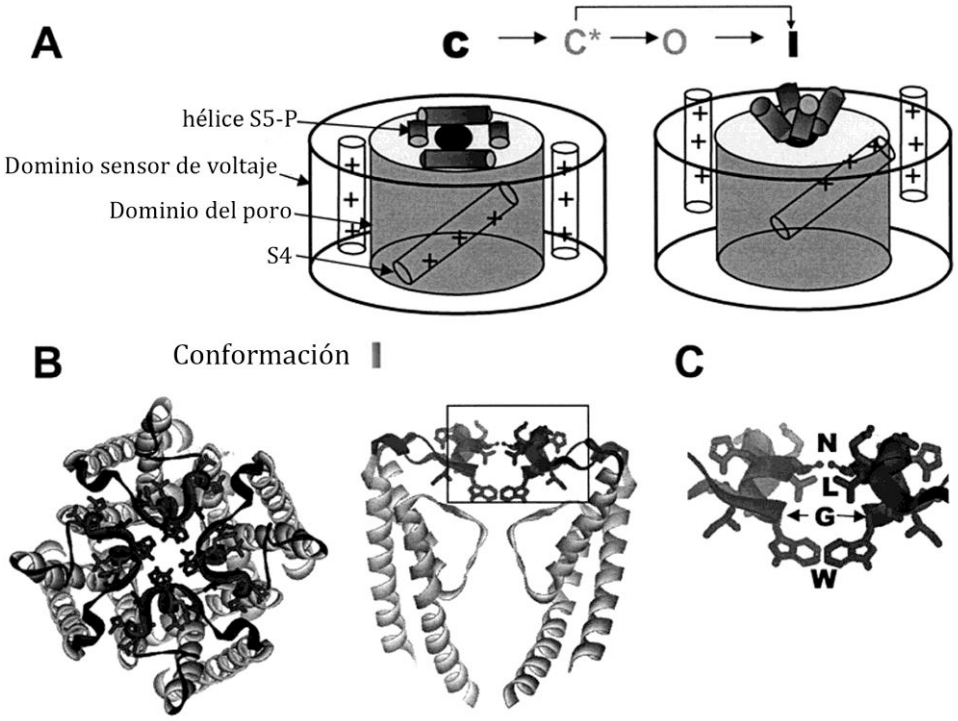

Figura 4. Representación de los cambios conformacionales en el canal hERG1 durante la despolarización de la membrana. A. Cambios conformaciones del canal hERG1: C (cerrado), C* (preabierto), O (abierto) e I (inactivado). A la izquierda, se muestra la conformación del estado cerrado. El canal está representado por un cilindro, en el centro se ilustra el poro rodeado por las cuatro hélices S5-P. Los cilindros con los signos positivos representan a los segmentos S4. A la derecha, se ilustra la conformación inactiva del canal, la cual se desencadena por el movimiento del S4 durante la depolarización de la membrana, permitiendo la interacción de los residuos de los extremos extracelulares del S4 y S5. Esto interrumpe la formación de puentes de hidrógeno entre los residuos del vestíbulo externo del canal causando un colapso del poro externo (interacción de las cuatro hélices del S5-P están representadas como cilindros en la parte externa del poro). **B.** Representación de la estructura secundaria del estado inactivo del canal hERG1. El modelo se construyó usando como templado el dominio del poro del canal KvAP. A la izquierda, se muestra una vista del canal del lado extracelular, en negro se representan las cuatro hélices S5-P y en gris el resto de las hélices que constituyen el tetrámero del canal. A la derecha, se ilustra una vista lateral del canal mostrando solo dos subunidades y en cada una se muestran: en gris las hélices de los segmentos S5, P y S6 y en negro las hélices S5-P. Esta última región se resalta y se describe en el siguiente panel. **C.** Estructura secundaria de dos de las cuatro regiones S5-P involucradas en el colapso del poro externo del canal hERG1 durante la inactivación. La interacción entre las cadenas laterales de los residuos G584, N588, W585, L589 de las subunidades adyascentes de las hélices S5-P cierran el eje central del poro, impidiendo el flujo de K^+ (Tomado de Tseng y Guy, 2005).

La participación del canal hERG1 en eventos celulares ha sido caracterizada principalmente en el corazón, es el responsable junto con otros canales de K^+ de aumentar la corriente de K^+ hacia el exterior de la célula, causando la repolarización de la membrana y marcando el fin del ciclo de la excitación y contracción cardiaca (Sanguinetti y cols., 1995; Nerbonne, 2000; Tseng y cols., 2001).

Es importante comentar que mutaciones en el gen *herg1* o el bloqueo del canal hERG1 pueden causar un retraso en la repolarización, limitando la aparición de las corrientes despolarizantes de Na^{+} y Ca^{+2}, prolongando así el período entre potenciales de acción sucesivos y causando lo que se conoce como síndrome QT largo del tipo 2 (LQT2) que se caracteriza por una prolongación en el intervalo QT en el electrocardiograma causando arritmias ventriculares malignas, como taquicardia ventricular y arritmia ventricular polimórfica (*Torsades de pointes* = Torcimiento de puntas) o muerte súbita (Curran y cols., 1995; Kiehn y cols., 1996; Sanguinetti y cols., 1996). El bloqueo del hERG1 es causado como un efecto colateral de diversos fármacos como: antihistamínicos, antiarrítmicos, hipolipemiantes, diuréticos, procinéticos, antidepresivos, antibióticos, antipalúdicos y neurolépticos (Roden, 1998).

La expresión del canal ERG1 no solo se restringe al corazón, también se ha descrito en diversos tejidos en los cuales la excitabilidad es una propiedad esencial, tales como en: músculo liso (Akbarali y cols., 1999; Ohya y cols., 2002; Shoeb y cols., 2003; Parr y cols., 2003), cuerpos carotideos (Overholt y cols., 2000), células lactotropas (Corrette y cols., 1995; Schäfer y cols., 1999; Bauer, 1998), células β pancreáticas (Rosati y cols., 2000), células cromafines (Gullo y cols., 2003), arteriola glomerular y vasos renales (Carrisoza y cols., 2010), células de Purkinje (Sacco y cols., 2003), núcleos de rafé (Hirdes y cols., 2005), interneuronas (Furlan y cols., 2005, 2007).

3. Interacción de las toxinas de alacrán sobre canales de potasio

Con el fin de conocer la relación estructura-función de los canales de potasio se han empleado herramientas farmacológicas que interfieran con su actividad, como las aminas cuaternarias (tetraetilamonio = TEA), sin embargo no son muy específicos para un subtipo de canal y son de baja afinidad. Por otro lado, las toxinas naturales aisladas del veneno de diversas especies de animales, tales como: alacranes, serpientes, arañas, abejas, anémonas y caracoles de mar (García y cols., 1997) se han usado para evaluar la actividad celular de varios canales de potasio, así como, para realizar estudios de estructura-función.

Las toxinas de alacrán se han utilizado para caracterizar la estructura y función de los canales de potasio (Aiyar y cols., 1996; Doyle y cols., 1998). En 1982, Possani y cols., identificó la primera toxina de alacrán (Noxiustoxina de *Centruroides noxius*) capaz de bloquear la permeabilidad del potasio en el axón gigante de calamar (Possani y cols., 1982; Carbone y cols., 1982). Tres años después, Miller y cols., purificó la Caribdotoxina (ChTx), una toxina aislada del veneno del alacrán *Leiurus quinquestriatus hebraeus* (Miller y cols., 1985; Miller, 1995), con la cual se realizó un análisis mecanístico del funcionamiento y de las dimensiones relativas del vestíbulo del poro de los canales de potasio (Park y cols., 1991; Bontems y cols., 1992; Giangiacomo y cols., 1992). Hasta ahora, se han caracterizado más de 140 toxinas de alacrán que afectan a los canales de potasio (KTx) y se han clasificado en cuatro familias: alfa, beta, gamma y kappa, que se abrevian como α-KTx, β-KTx, γ-KTx y κ-KTxs (Tytgat y cols., 1999). Estas toxinas son péptidos de 22 a 48 aminoácidos, con un motivo estructural α/β estabilizado por tres o cuatro puentes disulfuro (Rodríguez de la Vega y cols., 2003; Rodríguez de la Vega y cols., 2004).

Dentro de la familia α-KTx se encuentran la ChTx que actúa sobre los canales K_{Ca} 1.1, K_{Ca} 3.1, *Shaker* y Kv1.3 con una afinidad de 0.17 a 120 nM (Anderson y cols., 1988; Rauer y cols., 2000; Stocker y Miller, 1994; Rodríguez de la Vega y cols., 2003), así como la Agitoxina (AgTx2) que inhibe las corrientes de los canales *Shaker,* Kv1.1 y Kv1.3 con una afinidad de 0.004-0.16 nM (Miller, 1995; Krezel y cols., 1995; Rodríguez de la Vega y cols., 2003). Este tipo de toxinas bloquea al canal de potasio por oclusión física de la región del poro con una relación estequiométrica 1:1 (toxina: canal), impidiendo el paso de los iones K^+ a través del canal (Miller y cols., 1985; MacKinnon y Miller, 1998; 1989; Miller, 1988; Giangicomo y cols., 1992).

La superficie de contacto de este tipo de toxinas con los canales de potasio la constituye principalmente un par de aminoácidos localizados en la segunda hoja-β plegada. Este par esta definido por un residuo básico (Lys) y un aromático (Tyr o Phe) y se refiere a ellos como a la "diada funcional" o "diada catalítica". De este modo, se sabe que una vez que se une la toxina en la región del vestíbulo del canal, la presencia de residuos específicos mantienen a la toxina unida al canal (ChTx: R25, M29, N30, R34 y Y36; AgTx2: R25, R34, S22, F25, M29 y N30), mientras que un residuo conservado de lisina en la posición 27 (para el caso de la ChTx, AgTx2 y

otras toxinas α-KTx), apunta y ocluye el poro, bloqueando el paso del ion K^+ (Ver Figura 5; Goldstein y cols., 1994; Naranjo y Miller, 1996; Naini y Miller, 1996; Hidalgo y cols., 1995; Gross y cols., 1996).

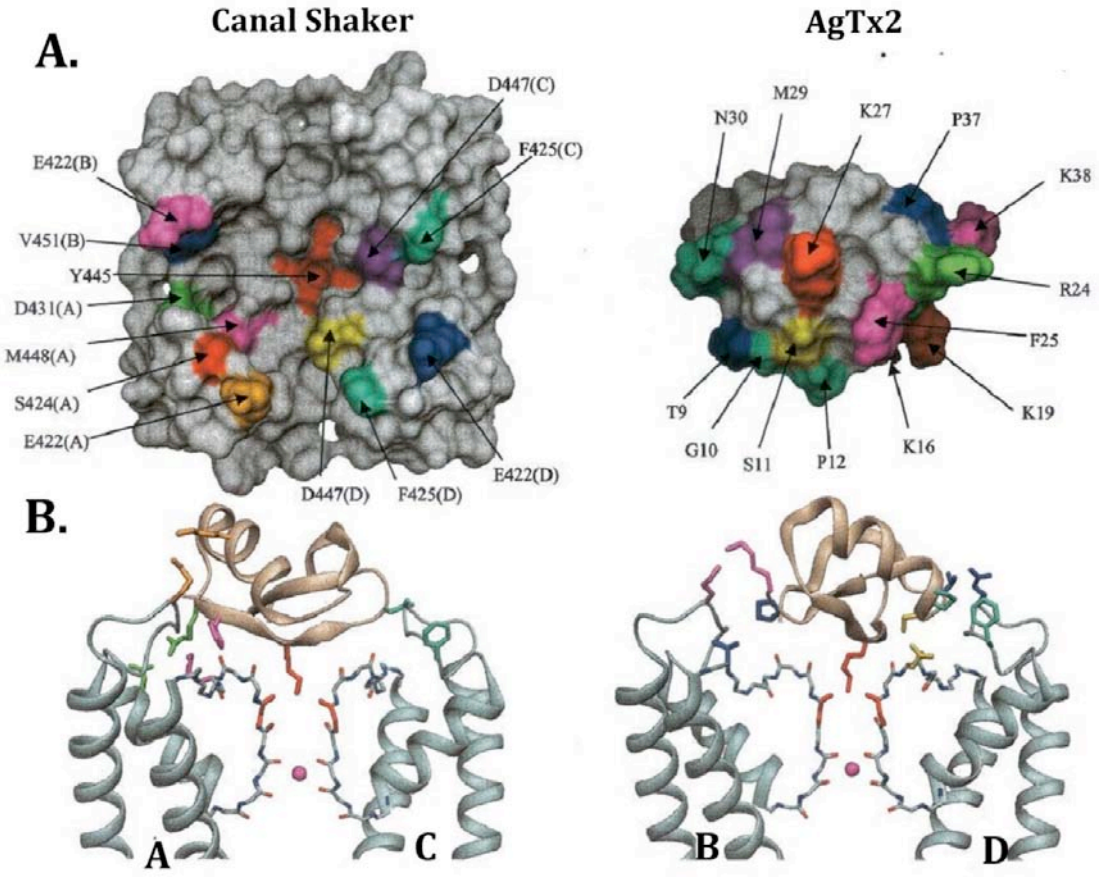

Figura 5. Modelo estructural que muestra la superficie de contacto de la toxina AgTx2 con el canal de K^+ *Shaker*. **A.** Ilustra los residuos importantes en la unión entre la AgTx2 y el canal *Shaker* (la letra en el paréntesis se refiere a los monómeros del canal, recordando que este es tetramérico). La estructura del canal *Shaker* fue modelada usando como templado la región formadora del poro de la estructura cristalográfica del canal KcsA de *Streptomyces lividans* con una similitud del 49% (Doyle y cols., 1998; Fiser y cols., 2000, Sali y cols., 1995) y el programa MODELLER v.4 (Zhou y cols., 2001). El modelado del complejo toxina-canal, se construyó usando la estructura de la AgTx2 determinada por RMN (Krezel y cols., 1995) y programas de dinámica molecular como CHARMM (Eriksson y Roux, 2002). **B.** Muestra dos vistas del complejo toxina-canal rotadas con un giro de 90°, usando como eje el poro del canal. El monómero del fondo y el del frente se removieron para una mejor visualización. El ion K^+ en el filtro de selectividad se muestra como una esfera de color magenta y los colores de las cadenas laterales corresponden a los mismos residuos del panel A, que ilustra los residuos apareados en el complejo, donde: (Izquierda) rojo, K27-Y445; verde, R24-D431(A); magenta, F25-M448(A); naranja, K19-E422(A) y cian, N30-F425(C). (Derecha) Rojo, K27-Y445, azul, P37-V451(B) y T9-E422(B); magenta, K38-E422(B); amarillo, S11-D447(D) y cian P12-F425(D) (Tomado de Eriksson y Roux, 2002).

Para realizar los estudios de estructura-función se ha recurrido tanto a la síntesis química de péptidos y al uso de sistemas de expresión en bacterias, ya que la cantidad de toxina purificada a partir del veneno es limitada y además es necesario generar variantes que aporten información sobre la función de cada aminoácido en dichas toxinas.

Aunque los primeros estudios de toxinas sobre canales iónicos se realizaron exitosamente gracias a la síntesis química de dichos péptidos, existen limitaciones en la síntesis en fase sólida, en las que se encuentran: la insolubilidad de la forma reducida de los péptidos en los sistemas oxidativos de síntesis, el plegamiento incorrecto que da formas oxidadas inactivas y sobretodo, el bajo rendimiento de síntesis proporcional al tamaño de la molécula (Lecomte y cols., 1998).

Por otro lado, desde 1989, se ha reportado la expresión de toxinas de alacrán en diferentes sistemas incluyendo, *Escherichia coli*, levaduras, células de insectos, células de mamíferos, etc. (Shao y cols., 1999). Desafortunadamente, no existe un sistema de expresión heterólogo general para la obtención de péptidos estructural y funcionalmente semejantes a las toxinas nativas, en cantidades suficientes y plegadas correctamente. A pesar de ello, se han logrado expresar con éxito toxinas recombinantes en *E. coli*. Las características principales de los sistemas utilizados son: la expresión de genes sintéticos bajo el control de un promotor inducible, la expresión del péptido de interés como proteína de fusión, la purificación del producto por cromatografía de afinidad, el uso de cepas bacterianas modificadas, como la cepa Origami, que favorecen la formación de puentes disulfuro en el citoplasma (Bessette y cols., 1999), la expresión de la proteína fusionada a un péptido señal exportándola al espacio periplásmico, en donde el ambiente oxidante facilita la formación de puentes disulfuro.

II. ANTECEDENTES

En el presente trabajo se aborda el estudio de la relación estructura-función de la toxina ErgTx1 aislada del veneno del alacrán *Centruroides noxius* Hoffmann que afecta a canales de K^+ hERG1.

En el laboratorio del Dr. Possani se estudia la composición y actividad del veneno de diversas especies de alacranes. La caracterización de estos venenos ha demostrado la presencia de sales, componentes proteicos con actividad enzimática, y aquellos con propiedades bloqueadoras o modificadoras de la actividad de distintos canales iónicos.

La ErgTx1 o también conocida como γ-KTx1.1 (Tytgat y cols., 1999), fue caracterizada por Gurrola y cols., (1999) como la primera toxina de alacrán que actúa sobre esta subfamilia de canales con una afinidad en el rango de nanomolar. Es un péptido de 42 aminoácidos, con 4 puentes disulfuro (Gurrola y cols., 1999; Scaloni y cols., 2000). Su estructura tridimensional fue resulta por RMN, muestra un arreglo típico de las toxinas bloqueadoras de los canales de K^+, definido por la presencia de cisteínas que estabilizan un motivo α/β (Figura 6; Torres y cols., 2003; Frénal y cols., 2004).

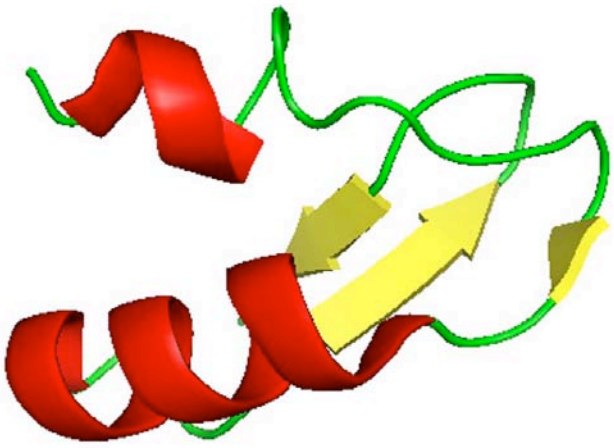

Figura 6. Representación de la estructura secundaria de la toxina ErgTx1. En el diagrama de listón se muestra la estructura secundaria de la toxina formada por dos α-hélices en rojo (3-7 y 18-27) y tres hojas β-antiparalelas en amarillo (14-15, 32-35 y 39-41; Torres y cols., 2003; Frénal y cols., 2004).

En el 2002, nuestro grupo en colaboración con el grupo de la Dra. G.A. Robertson de la Universidad de Wisconsin y la Dra. G.N. Tseng de la Universidad de Virginia Commonwealth (USA) elucidó la región de interacción del canal hERG1 con la ErgTx1, mediante la construcción de quimeras del canal hERG1 con el canal murino EAG (*ether-á-go-go*). Se observó que la interacción de la toxina se daba con la región S5-P-S6 (Figura 7; Pardo-López y cols., 2002a). Posteriormente, se realizaron cambios puntuales de los residuos que constituyen el asa S5-P (posiciones 571-613, 43 aa) y el asa P-S6 (posiciones 631-638, 8 aa) por cisteína (Pardo-López y cols., 2002b). Los resultados de estos experimentos sugieren que los cambios por cisteína de los residuos W585C, G590C y I593C, modifican la estructura de α-hélice anfipática de la región S5-P y afectan la interacción de la ErgTx1 sobre el canal (Pardo-López y cols., 2002b).

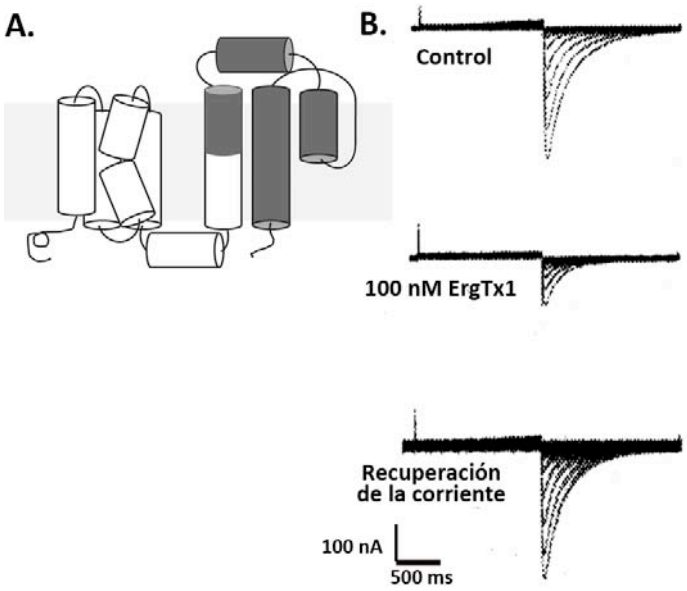

Figura 7. Efecto de la toxina ErgTx1 sobre la quimera del canal hERG1 expresado en ovocitos de *Xenopus laevis*. A. Esquema que muestra la construcción de la quimera, del segmento S1 hasta la T556 del segmento S5 corresponden al canal mEAG (blanco) y la secuencia restante al hERG1 (gris). **B.** Registros electrofisiológicos que muestran las corrientes entrantes obtenidas después de una repolarización a -100 mV, para el control (registro superior), después de la aplicación de 100 nM de la ErgTx1 (centro) y después del lavado (panel inferior). En los registros realizados con la toxina se observa una reducción de la corriente en comparación al control y que su efecto es reversible, esto demuesta que la interacción de la ErgTx1 se da con parte del segmento S5-P-S6 (Tomado de Pardo-López y cols., 2002a).

Se sabe que el mecanismo de interacción típico de las toxinas bloqueadoras del poro específicas para canales de K^+, como la ChTx, kaliotoxina, iberotoxina, AgTx2, entre otras; es

unirse al vestíbulo externo del canal y bloquear la conducción iónica por oclusión física del poro. Considerando lo anterior y las características estructurales de estas toxinas se pueden encontrar algunas diferencias y/o semenjanzas con la ErgTx1 que ayuden a explicar como esta interaccionando esta última con el canal hERG1, las cuales se detallarán a continuación:

1. En la AgTx2, los aminoácidos involucrados en la interacción con la hélice del poro y el filtro de selectividad del *Shaker*, se encuentran en la hoja-β de la toxina (Ranganathan y cols. 1996). El residuo básico K27 y el residuo aromático F25, distanciados por 6.6 ± 1 Å, forman la llamada diada funcional y ambos residuos estan involucrados en la interacción con el canal; sin embargo, la lisina entra en el poro, se une con los aminoácidos del filtro de selectividad y bloquea el flujo iónico. Por lo tanto, si se aumenta la ocupancia del ion K^+ en el interior del poro o se eleva su concentración alrededor del vestíbulo externo la unión de la AgTx2 con el canal *Shaker* y otros canales Kv se desestabliza (Figura 8; Goldstein y cols., 1994; Ranganathan y cols., 1996; Dauplais y cols., 1997).

2. En el caso de la ErgTx1, no se tiene definida una diada funcional, sin embargo se ha propuesto que la K25 y Y17 que están localizados en la parte helicoidal de la molécula pueden formar la diada funcional. Por otro lado, la K38 situada al final de la primera hoja-β a una distancia de 7 Å de la F36 fácilmente podría satisfacer el criterio de la diada funcional. Adicionalmente, la K13 se encuentra a una distancia de 7 Å de la Y14, F36 y F37 también podrían formar la diada. De esta manera, se tienen varias propuestas de los posibles residuos que pueden formar la diada funcional en ErgTx1; sin embargo hasta no realizar estudios de mutagénesis de la toxina se podría definir la función de estos residuos en su interacción con el canal hERG1 (Figura 8; Torres y cols., 2003; Frénal y cols., 2004).

3. Por otro lado, se sabe que en la unión de las toxinas de alacrán con los canales de K^+ están involucradas interacciones electrostáticas, hidrofóbicas y de puentes de hidrógeno (MacKinnon y Miller, 1989; Eriksson y Roux, 2002; Ranganathan y cols., 1996). Por ensayos electrofisiológicos se sabe que la unión de ErgTx1 al canal hERG1 no está influenciada por la concentración de K^+ extracelular, por lo que las interacciones electrostáticas no intervienen

en esta interacción (Pardo-López y cols. 2002b). Además, la estructura de la ErgTx1 se caracteriza por presentar un gran parche hidrofóbico formado por los residuos Y14, Y17, F36 y F37 rodeando a un residuo básico (K13), esto puede sugerir que las interacciones hidrofóbicas pueden ser una fuerza importante en la unión de la toxina con el canal hERG1 (Figura 8; Torres y cols., 2003; Frénal y cols., 2004).

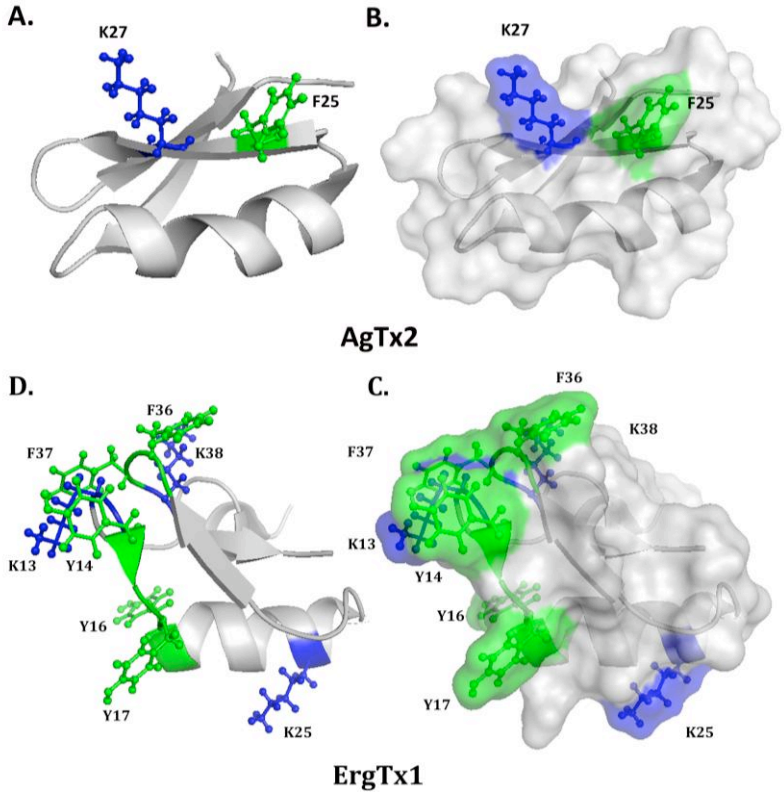

Figura 8. Representación de las estructuras de las toxinas AgTx2 y ErgTx1. A. Diagrama de listón que muestra la estructura secundaria de la AgTx2 resuelta por resonancia magnética nuclear (RMN; con código de acceso al PDB 1AGT), se resaltan las cadenas laterales de los aminoácidos importantes para la interacción con el canal *Shaker* y otros canales Kv (verde, F25; azul, K27), localizados en la segunda hoja β antiparalela de la toxina. **B.** Superficie molecular de la AgTx2 que muestra que los aminoácidos que constituyen a la diada funcional se encuentran expuestos al solvente, favoreciendo su interacción con el receptor. **C.** Diagrama de listón que muestra la estructura secundaria de la ErgTx1 determinada por RMN (PDB 1PX9) con un motivo α/β al igual que la AgTx2, pero en lugar de presentar la típica hoja-β en el N-terminal tiene una α-hélice corta, además se enfatizan las cadenas laterales de los aminoácidos que probablemente estén involucrados en formar parte de una diada funcional (K38-Y17, K25-F36, K13-Y14, F36 o F37). **D.** Superficie molecular de la ErgTx1 que muestra el parche hidrofóbico formado por la Y14, Y17, F36 y F37 rodeando a la K13, mostrando que son residuos con cadenas laterales expuestas al solvente aumentando la probabilidad de que estas puedan interaccionar con el canal hERG1. La región hidrofóbica se muestra de color verde, mientras que los residuos básicos se muestran en azul. Las figuras se generaron usando el programa Pymol (Tomado de Krezel y cols., 1995; Torres y cols., 2003; Frénal y cols., 2004).

Lo anterior, sugiere que la ErgTx1 tiene un modo de interacción diferente al ya descrito para las toxinas bloqueadoras del poro, como la AgTx2 y la ChTx sobre los canales Kv.

Por otro lado, datos no publicados en nuestro laboratorio indican que la toxina ErgTx1 es susceptible de oxidarse durante el almacenamiento en solución. Resultados de espectrometría de masas señalan que el péptido oxidado tiene una diferencia de 16 Da más en comparación a la masa de la ErgTx1 nativa (4746 contra 4730 Da), equivalente a la oxidación de la metionina en la posición 35 en la toxina. Esto se verificó por secuenciación automática de Edman de una muestra de ErgTx1 oxidada. Además, resultados de electrofisiología muestran que la afinidad del péptido oxidado por el canal hERG1 decrece tres órdenes de magnitud con respecto a la toxina nativa (15 µM contra 7 nM; Figura 9). Esto hace pensar en dos situaciones: 1) la metionina esta formando puentes de hidrógeno con los residuos vecinos, estabilizando parte de la estructura terciaria de la toxina, o bien; 2) la cadena lateral de la metionina forma parte de la superficie de interacción con el canal hERG1.

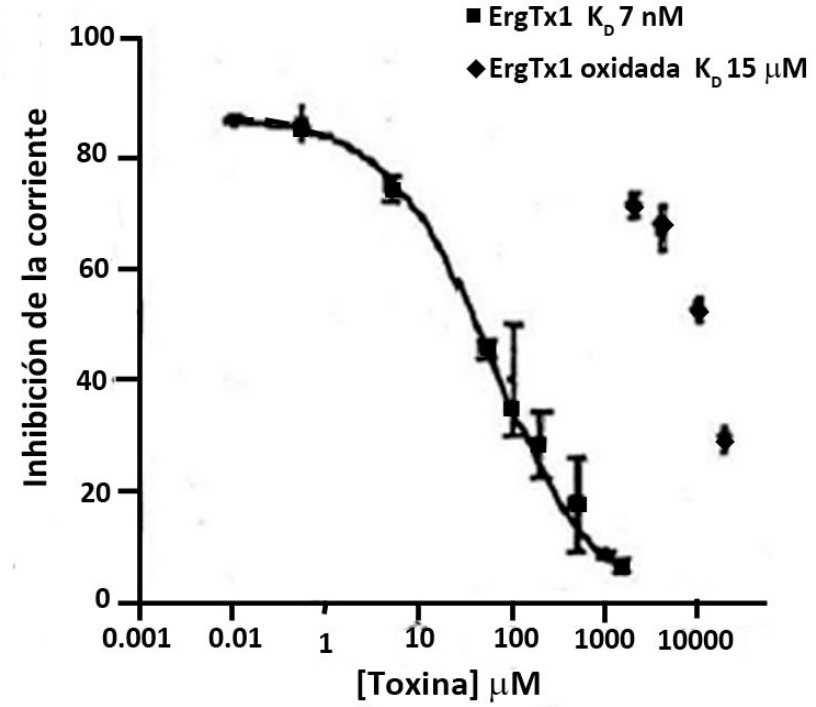

Figura 9. Curva dosis-respuesta de la toxina ErgTx1 nativa e isoforma oxidada. La actividad electrofisiológica de ambos péptidos se ensayó a diferentes concentraciones, mediante la técnica de *voltage-clamp* (fijación de voltaje) en ovocitos de la rana *Xenopus laevis* transfectados con el canal de potasio hERG1. La concentración de toxina ensayada se graficó contra la fracción de corriente inhibida por el péptido probado. La curva de la izquierda corresponde a la aplicación de la toxina nativa ErgTx1 (■) y la de la derecha al péptido oxidado en la posición 35 (♦). Las K_D estimadas fueron de 7 nM para ErgTx1 y 15.8 µM para la forma oxidada.

III. HIPÓTESIS

Usando sistemas de expresión bacterianos, es posible obtener toxinas recombinantes con estructura y función igual al de la toxina nativa ErgTx1, en cantidades suficiente para identificar que los residuos que constituyen el parche hidrofóbico (K13, Y14, Y17, F37) son importantes para la actividad y/o afinidad de la toxina hacia el canal hERG1.

La metionina 35 en la ErgTx1 es un aminoácido susceptible a oxidarse ocasionando una disminución en la afinidad por el canal hERG1, indicando que este residuo forma parte importante de la superficie de interacción con el canal.

IV. OBJETIVO GENERAL

Determinar que residuos de la toxina ErgTx1 están involucrados en la interacción y bloqueo del canal de potasio del hERG1.

4.1. OBJETIVOS PARTICULARES

- Generar variantes de la toxina ErgTx1, mediante mutagénesis sitio dirigido.
- Expresar las variantes, empleando la cepa TG1 de *Escherichia coli*.
- Purificar y caracterizar las toxinas recombinantes.
- Determinar la afinidad de cada una de las variantes sobre el canal hERG1 expresado en la línea celular CHO ("*Chinese Hamster Ovary*").
- Determinar posibles modificaciones de la estructura secundaria de las variantes de la toxina, mediante dicroísmo circular.
- Determinar si hay diferencias en la afinidad de cada una de las variantes por el canal hERG1 y compararlas con la de la toxina nativa.
- Identificar que residuos de la toxina ErgTx1 hacen contacto con el canal hERG1.

V. MATERIALES Y METODOS

5.1. Reactivos, plásmidos y cepas de E. coli

Para las amplificaciones por PCR se empleó la Vent Polimerasa (New England Biolabs). Las enzimas de restricción y la T4 DNA ligasa se obtuvieron de Boehringer o New England Biolabs. Para la amplificación de los plásmidos se empleó la cepa DH5α (F^-*gyr*A96 (NAI^r) *recA relA1 endA1 thi-1 hsdR17* ($r_K^- m_K^+$) *glnV44 deoR Δ(lacZYA-argF)U169*[ϕ80dΔ(*lacZ*)M15]) y para la expresión de las toxinas recombinantes la cepa TG1 (*supE thi1Δ(lac/proAB) Δ(mcrB/hsdSM)5(*rK-mK-) [F´*traD36proAB lacI*q*ZΔM15*]) de *Escherichia coli*. Los plásmidos pThioC (Invitrogen) y pSyn1 se usaron para clonar y expresar las toxinas recombinantes fusionadas a la proteína acarreadora Thioredoxina, respectivamente.

5.2. Purificación de la toxina nativa ErgTx1

La colecta de alacranes de la especie *Centruroides noxius* Hoffmann se realizó en las costas de Nayarit. El veneno se extrajó por estimulación eléctrica del telson (último segmento de la cola del alacrán). El fluído extraído se resuspendió en agua tetradestilada hasta quedar totalmente disuelto y se centrifugó a 14000 rpm durante 15 minutos a 4°C. La purificación del veneno se realizó utilizando exclusión molecular, intercambio catiónico (Sephadex G-50 y carboximetil celulosa, respectivamente) y cromatografía líquida de alta presión (HPLC), como se describió previamente en Gurrola y cols., 1999. El componente activo sobre el canal hERG1 se purificó usando una columna analítica C_{18} y un gradiente lineal de 5-40% de solución B (acetonitrilo y 0.1% de ácido trifluoroacético) por 50 minutos. El peso molecular se confirmó por espectrometría de masas usando un espectrómetro con trampa de iones Finningan LCQ^{DUO} (San Jose, CA, USA).

5.3. Construcción de vectores de expresión

Para el diseño de las construcciones se empleó como templado de DNA el plásmido **pKS^{+}-ErgTx1** (construido por la Dra. Blanca García con número de acceso en el GeneBank AAG38523) y oligonucleótidos específicos (ver Tabla 1).

El gen que codifica para la toxina ErgTx1 se clonó dentro del vector pThioC, se fusionó a la proteína acarreadora Thioredoxina y a la secuencia de aminoácidos (DDDDK) reconocida por la enzima enterocinasa. El gen se amplificó por PCR usando como templado el vector pThio-EK-ErgTx1, el producto se digirío con las enzimas de restricción correspondientes y clonados en el vector de expresión pSyn1.

El vector pSyn1 está construido de tal manera que permite insertar cualquier secuencia y fusionarla al gen que codifica para el péptido señal, PelB. La expresión de la proteína es inducida con IPTG y su regulación se encuentra bajo el promotor *lac.* Además, contiene genes que le confieren al huésped resistencia a ampicilina y a kanamicina, pero esta última es eliminada al insertar una secuencia dentro del sitio múltiple de clonación en el vector (Figura 10).

La primera construcción fue nombrada pSyn1Thio-EK-ErgTx1 y para su diseño se emplearon los oligonucleótidos *Sfi-Thio y Not-Erg* (Tabla 1). Las condiciones de la reacción de PCR fueron: 5 min a 94 °C, 30 ciclos de 1 min a 94 °C, 1 min a 55 °C, 1 min a 72 °C y finalmente 7 min a 72 °C de extensión. El producto de PCR y el plásmido pSyn1 se digieren con las enzimas de restricción *Not*I y *Sfi*I a 37 °C y 50 °C, respectivamente, los fragmentos se purificaron por columna (QIAquick) antes de la ligación. La reacción de ligación (20 µl) se realizó con T4 ADN ligasa en una relación de inserto con respecto al plásmido de 10:1 a 16 °C durante 16 h. Cinco µl de la reacción de ligación se usó para transformar células competentes DH5-α de *E.coli*. Las clonas positivas con el inserto esperado se crecieron en medio LB adicionado con ampicilina (200 µg/ml). La construcción fue secuenciada para comprobar que el marco de lectura se encontrara en fase.

Para el diseño de cada variante se utilizaron oligonucleótidos específicos que insertaron las mutaciones puntales propuestas (Tabla 1). El templado utilizado para la amplificación de cada variante fue pSyn1Thio-EK-ErgTx1. Los genes de las variantes K13A, Y14A, Y17A y M35A se sintetizaron utilizando un protocolo de dos etapas, descrito por Kanoksilapatham y cols. (2007), así como los oligonucleótido *BamHI-EntERG, K13A, Y14, Y17A, M35Arev y Not-Erg*. Para la síntesis de las variantes F37A y K38A se emplearon los oligonucleótidos *BamHI-EntERG, F37Arev* y *K38Arev*. Para la amplificación por PCR de las variantes F37A y K38A se emplearon las mismas condiciones que la toxina nativa. Los productos de PCR de cada una de las variantes se digieren con las enzimas de restricción *BamH*I *y NotI* y ligados al vector pSyn1. Las construcciones se secuenciaron para comprobar que el marco de lectura este en la fase correcta.

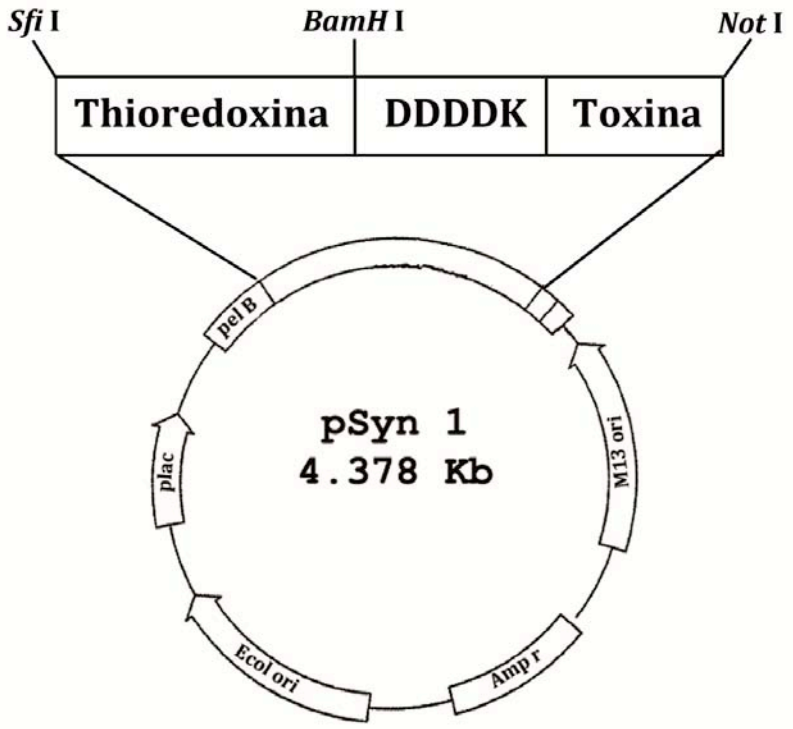

Figura 10. Diseño y construcción del sistema de expresión para *E. coli.* La construcción del vector de expresión se realizó insertando el gen que codifica para la toxina fusionado al gen que codifica para la proteína acarreadora Thioredoxina separados por cinco codones que codifican para los aminoácidos DDDDK, secuencia reconocida por la proteasa enterocinasa. Los genes están flanqueados por las enzimas de restricción (*Sfi* I, *BamH*I y *Not*I) que se utilizaron para la inserción de los genes en el vector. El vector pSyn1 contiene el origen de replicación para *E. coli* (Ecol ori) y para el fago M13 (M13 ori), un gen que le confiere al huésped resistencia a ampicilina (Amp r), un gen que codifica para el péptido señal (pelB) que se fusionará a la proteínay la transportará al periplasma.

Tabla 1. Oligonucleótidos empleados para la construcción de las toxinas recombinantes	
Nombre	***Secuencias***
Sfi-Thio	5´-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAC AGA GAT AGC TGC GTC GAC-3´
Not-Erg	5´-GAG TCA TTC TCG ACT TGC GGC CGC ACG TGA TTA CGC ACA TTT ACA CTT GAA AAA-3´
BamHI-EntERG	5´-GGC TCT GGA TCC GGT GAT GAC GAT GAC AAG GAC AGA GAT AGC TGC GTC-3´
K13A	5´-CA CGA TGC GCA ***GCA*** TAT GGA TAC-3´
Y14A	5´-GA TGC GCA AAA ***GCT*** GGA TAC TAC-3´
Y17A	5´-CA AAA TAT ***GGA*** TAC GCC CAA GAG TG-3´
M35Arev	5´-CGC ACA TTT ACA CTT GAA AAA ***CGC*** ACA GGT TCC TCC ATT GTG-3´
F37Arev	5´-CG ACT TGC GGC CGC ACG TGA TTA CGC ACA TTT ACA CTT ***GGC*** AAA CAT ACA GGT TCC-3’
K38Arev	5´-CG ACT TGC GGC CGC ACG TGA TTA CGC ACA TTT ACA ***CGC*** GAA AAA CAT ACA GGT TCC-3’
Los nucleótidos subrayados corresponden a la secuencia reconocida por la enzima de restricción y los resaltados en negro corresponden al codón del aminoácido que se cambió por alanina.	

5.4. Expresión y purificación de las proteínas recombinantes

La cepa TG1 de *Escherichia coli* se transformó de manera individual con cada una de las construcciones generadas (pSyn1Thioredoxina-EK-Toxina). Las transformantes se crecieron en medio YT2X suplementado con ampicilina (200 μg/ml) y glucosa (0.1%) a 37 °C, una vez alcanzada una densidad óptica de 1.0 a una longitud de onda de 600 nm, la expresión de la proteína se indujó con 1 mM de IPTG. Se tomaron muestras antes y después de la inducción para determinar el nivel de expresión en un gel de Tricine-SDS-PAGE siguiendo el protocolo descrito por Schagger y Jagow, 1987. La inducción se realizó a 30 °C durante 16 h. Después de la cosecha las células se centrifugaron a 8000 rpm por 15 min. Las células bacterianas se resuspenden en 12.5 ml de Sacarosa (20 mg/ml), Tris-HCl 20 mM pH 8.0 y se incuban a 4 °C por 20 min, posteriormente se centrifugan a 8000 rpm por 20 min. El sobrenadante es removido y las células se resuspenden en 12.5 ml de agua destilada a 4 °C (la diferencia en la concentración de solutos generó un choque osmótico) y se incuban en agitación lenta a 4 °C por 20 min. Las células se remueven por centrifugación (8000 rpm a 4 °C por 20 min) y el sobrenadante

corresponde a la fracción periplásmica, en donde se encuentra la proteína de fusión de interés. Esta fracción se dializó contra Tris-HCl 20 mM pH 8.0 y se carga en una columna de intercambio aniónico con una capacidad de 64 ml que contiene la resina dietil-amino-etil-celulosa (DEAE), la cual previamente se equilibró con la misma composición de buffer empleado en la diálisis. Después de la aplicación de la proteína, la columna se lava por 30 min con el mismo amortiguador ("buffer") y las proteínas se eluyen con un gradiente de NaCl de 0-0.3 M. Los productos se purificaron posteriormente por cromatografía de líquidos de alta presión en fase reversa (RP-HPLC) empleando una columna semi preparativa C_{18} con un gradiente lineal de buffer A (Agua tetra-destilada con 012% de TFA v/v) a buffer B (Acetonitrilo con 0.1% de TFA) de 0-65% de B en 65 min. Las proteínas de fusión purificadas se digieren con enterocinasa (Sigma Aldrich), utilizando 1 mg de proteína para 0.02 U de enzima resuspendidas en el buffer Tris-HCl 500mM, $CaCl_2$ 2mM, Tween-20 10%, pH 8.0, e incubadas por 16 h a 25 °C. Los péptidos recombinantes se purificaron por RP-HPLC usando una columna analítica C_{18} y un gradiente de 0-65% de buffer B por 65 min. Los péptidos recombinantes se analizaron por "Western blotting", su masa se confirmó por espectrometría de masas (ESI-MS) y la secuencia de aminoácidos por degradación de Edman.

5.5. Determinación de la estructura secundaria por dicroísmo circular

Los espectros de dicroísmo circular se obtienen en un espectropolarímetro Jasco J-725 (Jasco, Japón). La lectura de los espectros se realizó en un rango de 250 a 190 nm en 20mM de Tris-HCl a pH 8.0 a 20°C con una celda de camino óptico de 1mm. Los datos se colectaron a 1 nm con una velocidad de exploración de 20 nm/min y un tiempo constante de 0.5 segundos. La concentración del péptido fue de 0.2-0.25 mg/ml. Los resultados se expresaron en elipticidad molar, $[\theta]_{MRW\lambda} = (\theta_{obs}/10)$ (M/cl), donde θ_{obs} es la elipticidad observada a determinada longitud de onda, *M* es el peso molecular residual medio, *I* es la longitud del camino óptico recorrido en centímetros, y *c* la concentración de proteína expresada en mg/ml. Las unidades de $[\theta]$ son $deg.cm^2.dmol^{-1}$ (Pain, 2004).

5.6. Ensayos electrofisiológicos

Con el fin de registrar las corrientes de potasio se utilizó la técnica "*Patch-clamp"* (fijación de voltaje en un parche de membrana) en su configuración de célula completa "*whole-cell"*. La técnica de "*patch clamp"* fue desarrollada por Neher y Sakmann a principios de los ochenta, es una variación de la técnica de fijación de voltaje y consiste en mantener fija la diferencia de voltaje de un pequeño trozo de membrana; de esta forma, midiendo la corriente necesaria para mantener dicha diferencia de potencial, se puede cuantificar el flujo de iones a través de este trozo de membrana (Hamill y cols., 1981). Esta técnica permite el registro de corrientes a nivel macroscopico, del orden de pA. El primer paso en la ejecución de la técnica es la formación de un sello de alta resistencia entre la membrana y la punta de la pipeta. Para ello se utiliza una pipeta de cristal cuya punta tiene un diámetro de 1 µm. La pipeta se pone sobre la superficie de la célula, se aplica una presión negativa mediante una ligera succión de tal forma que la porción de la membrana localizada en la luz de la pipeta se invagina formando un sello de alta resistencia, en condiciones ideales se pueden conseguir sellos con resistencias de 10-100 GΩ. A partir de este punto, para conseguir la configuración de célula completa "*whole-cell"* se aplica una succión adicional rompiendo la porción de la membrana invaginada, lo que permite la comunicación entre el interior de la pipeta y el medio intracelular, con lo que la pipeta de "patch" ahora registra la actividad de la totalidad de canales iónicos presentes en la membrana celular. Inmediatamente tras la ruptura del parche de membrana, la solución intracelular de la célula se equilibra con la solución del interior de la pipeta. En estas condiciones se fija el potencial de membrana al potencial deseado y mediante distintos protocolos de voltaje se registra la corriente deseada. La corriente resultante es la suma de todos los canales presentes en la membrana activables en ciertas condiciones o también llamadas corrientes macroscópicas. El equipo, las soluciones empleadas y el protocolo utilizado para los distintos registros se describen más adelante en esta sección.

5.6.1. Cultivo Celular

El ADNc que codifica para el canal de potasio *hERG1,* se expresó en células CHO ("*Chinese Hamster Ovary")* y se cultivaron en el medio Eagle modificado por Dulbecco (DMEM) suplementado con 4.5 g/l de glucosa, geneticina (450 µg/ml) y suero fetal bovino al 10%. Las células se incubaron a 37 °C en una atmósfera con 95% de húmedad y 5% de CO_2.

5.6.2. Soluciones de Registro

Durante los registros las células se mantuvieron en una solución extracelular estándar, que contenía: 130 mM NaCl, 5 mM KCl, 2mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM buffer HEPES-NaOH, 5 mM D-Glucosa, pH 7.29. Para los ensayos biofísicos, las células se perfundieron con una solución extracelular en alto contenido de K^+ ($[K^+]$= 40 mM), en la cual el NaCl se remplazó por cantidades equimolares de KCl. La solución intracelular (micropipeta) contenía: 130 mM Aspartato-K^+, 10 mM NaCl, 1.3 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA-KOH y 10 mM buffer HEPES-NaOH, pH 7.3.

5.6.3. Registros de "Patch-Clamp" y Análisis de los Datos

La medición de las corrientes se hizo a temperatura ambiente utilizando un amplificador MultiClamp 700B y el software pClamp10 (Molecular Device, USA). La resistencia de la micropipeta fue de 1.5–2.2 MΩ; los errores de capacitancia celular y resistencia en serie se compensaron antes de cada protocolo de fijación de voltaje con el objeto de reducir los errores de voltaje a menos del 5%. El protocolo de pulsos consistió de una repolarización a –120 mV por 500 ms, precedida por una despolarización a 60 mV por 500 ms a una frecuencia de 0.1 Hz; el potencial de mantenimiento fue a –80 mV. La adquisición y procesamiento de los datos se realizó utilizando los programas pClamp (versión 10.0, Axon Instruments Co., U. S. A.) y Origin (versión 7.0, Micrococal software, MA). Los datos se mostraron como la media ± SEM.

5.6.4. Determinación de las constantes de disociación (IC_{50s})

La curvas dosis-respuesta para los canales hERG1 se construyó graficando el pico de cada corriente medida tras la aplicación de la toxina nativa o de las variantes después de 100 segundos sobre las células CHO que expresan el canal hERG1. La corriente medida se normalizó con la corriente obtenida en condiciones control. Los datos experimentales (media de 4-9 células $\pm$ SEM) se ajustaron utilizando la ecuación de Hill:

$$I_T/I_{max} = A2 + (A1-A2)/(1 + ([T]/KD)p)$$

Donde $\mathbf{I_{max}}$ es la corriente máxima; **[T]** es la concentración de toxina; $\mathbf{I_T}$ es la corriente a cierta **[T]**; KD es la constante de disociación correspondiente a la **[T]** en la cual se reduce el 50% de la corriente; **p** es el coeficiente de Hill; **A1** y **A2** son las asíntotas inferior y superior, respectivamente. Tomando en consideración que el bloqueo de la corriente en el canal hERG1 por la toxina ErgTx1 no es completo, independientemente de las altas concentraciones aplicadas. El ajuste de los datos se realizó con un coeficiente de Hill de 1, la asíntota superior a 0 y dejando libre la asíntota inferior. Para algunas mutantes fue necesario dejar el coeficiente de Hill libre o fijar el valor de la asíntota inferior a 0.

VI. RESULTADOS

Los resultados obtenidos en el desarrollo de esta tesis doctoral se publicaron en dos artículos científicos de revistas internacionales indexadas y hacen parte de los anexos que están incluidos al final de la tesis (Jiménez-Vargas y cols., 2011; Jiménez-Vargas y cols., 2012a). El tercer anexo corresponde a una revisión sobre toxinas moduladoras o bloqueadoras de canales de potasio ERG (Jiménez-Vargas y cols., 2012b).

6.1. Expresión y purificación de las proteínas de fusión

Para obtener cantidades significativas de los péptidos recombinantes se empleó el sistema de expresión de *E. coli*. Expresamos la toxina silvestre ErgTx1 y 10 mutantes (K13A, Y14A, Y17A, M35A, F37A, K38A, K13A-F37A, Y14A-F37A, Y17A-F37A y K13A-K38A) como proteínas fusionadas a la proteína acarreadora Thioredoxina. Se estima que la cantidad de proteína de fusión producida es de 2 a 6 mg por litro de cultivo dependiendo de la variante. En el gel de la figura 11 se ilustra el nivel de expresión de cada toxina como proteína de fusión y su reconocimiento en el "*western blot*" por anticuerpos policlonales anti-ErgTx1 levantados en conejo, observando una banda alrededor de los 17,000 Da.

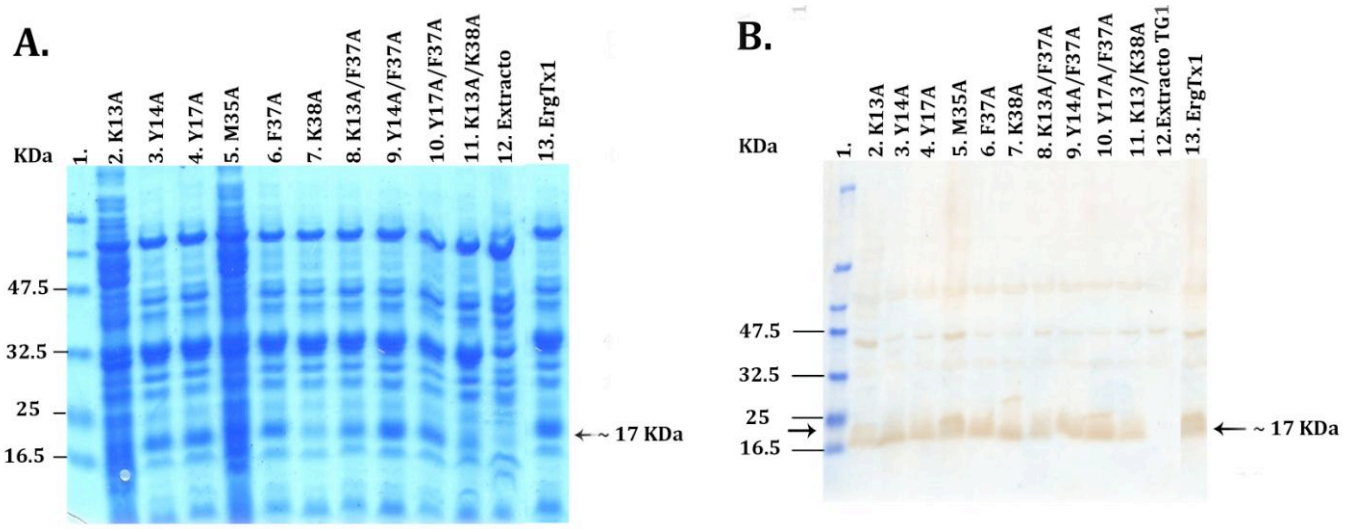

Figura 11. Gel Tricine-SDS-PAGE y "Western blot" de los productos de expresión de las variantes fusionadas a la Thioredoxina. A. Gel Tricine-SDS-PAGE. En el carril 1). Marcador de peso molecular; 2). K13A; 3). Y14A; 4). Y17A; 5). M35A; 6). F37A; 7). K38A; 8). K13A-F37A; 9). Y14A-F37A; 10). Y17A-F37A; 11). K13A-K38A; 12). Extracto periplásmico de TG1; 13). ErgTx1r **B.** Western blot. Las proteínas se cargaron en el mismo orden que en el panel A. Para el revelado se utilizó un primer anticuerpo policlonal de conejo anti-ErgTx1 y un segundo anticuerpo cabra anti-conejo acoplado a peroxidasa. Las proteínas de fusión migran alrededor de los 17 KDa, indicadas por flechas.

En la Figura 12 se muestra un ejemplo de como se purificaron cada una de las toxinas recombinantes desde la proteína de fusión de extracto periplásmico hasta obtener la toxinas recombinante pura. Se determinó el peso molecular por espectrometría de masas de las variantes previamente purificadas: K13A (4,673.2 Da), Y14A (4,638 Da), Y17A (4,638 Da), M35A (4,670 Da), F37A (4,654 Da), K38A (4,673 Da), K13A-F37A (4,597 Da) Y14A-F37A (4,562 Da),

Y17A-F37A (4,562 Da), K13A-K38A (4,616 Da), y se realizaron los ensayos electrofisiológicos correspondientes.

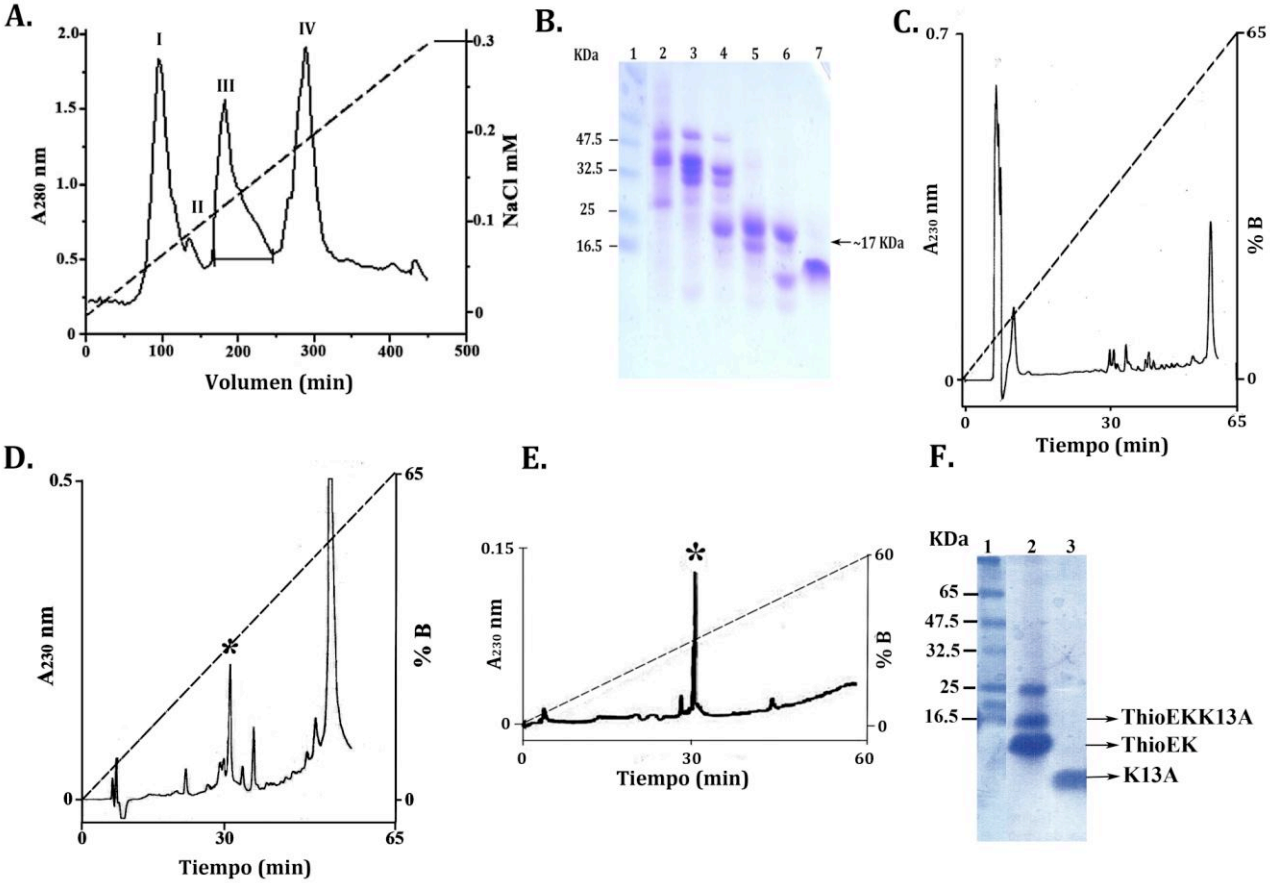

Figura 12. Purificación de la variante K13A. A. La proteína obtenida en la fracción periplásmica se aplicó en una columna de intercambio aniónico, la elución se realizó como se describe en la sección de materiales y métodos, obteniéndose 4 fracciones, en donde la fracción III contiene la proteína de fusión ThioEKK13A. **B.** Gel SDS-PAGE de las fracciones obtenidas de la cromatografía de intercambio iónico. En el carril 1). Marcador de peso molecular (KDa); 2). Fracción I; 3). Fracción II; 4-6). Fracción III, que contiene la proteína de fusión ThioEKK13A, la cual migra alrededor de los 17 KDa, señalada por una flecha y 7). Fracción IV. **C.** De la Fracción III se puificó la proteína de fusión por cromatografía de líquidos fase reversa (RP-HPLC) utilizando una columna semipreparativa C_{18} con un gradiente lineal de 0 – 65 % de buffer B (Acetonitrilo, 0.1% TFA), la proteína ThioEKK13A es eluída alrededor de los 58 min. **D.** ThioEKK13A es digerida con enterocinasa y los productos de la digestión son purificados por RP-HPLC con una columna analítica C_{18} aplicando un gradiente lineal de 0 – 65 % de buffer B. La toxina recombinante K13A es eluída alrededor de los 30 min (*), mientras que la proteína de fusión no digerida, así como la proteína acarreadora Thioredoxina son eluídas después de los 55 min. **E.** Purificación de K13A. El componente marcado con astérisco, corresponde a la K13A, el cual es recromatrografíado en las mismas condiciones anteriores (Panel D). **F.** Gel Tricine- SDS-PAGE. En el carril 1). Marcador de peso molecular de proteínas (KDa); 2). Contiene 100 µg del producto de la digestión con enterocinasa, en donde la proteína ThioEKK13A no digerida migra alrededor de los 17 KDa en tanto que la Thioredoxina migra alrededor de los 12 KDa, la banda que migra alrededor de los 25 KDa corresponde a la enzima enterocinas y 3). La toxina recombinante K13A obtenida en el último paso de purificación de RP-HPLC (Panel E), la cual migra alrededor de los 4 KDa.

La purificación de todas las demás variantes de la ErgTx1 se obtuvo básicamente como se muestra en la Figura 12, para el caso de la variante K13A, con pequeñas variaciones de elución en los últimos pasos de purificación por cromatografía de RP-HPLC (representados en la Figura 12D y E). Sin embargo, se puede identificar fácilmente a las mutantes.

6.2. Espectros de dicroísmo circular

Para demostrar cuales son los residuos de la toxina que están interaccionando con el canal, primero fue necesario determinar que las sustituciones realizadas por alanina no causaron una modificación en la estructura secundaria de la toxina que pudiera explicar un cambio en la afinidad de la variante por el canal. Para tal fin, se realizaron ensayos de dicroísmo circular de la toxina nativa ErgTx1, de la recombinante y de las variantes, los cuales se ilustran en la figura 13. Las lecturas se realizaron en un rango de longitud de onda entre 190 nm y 250 nm correspondiente al UV lejano, resultando el típico espectro de péptidos que tienen una mezcla de dos dominios α y β separados, presentando una banda negativa mínima a 222 nm y una transición electrónica de $n \rightarrow \pi^*$, otra banda negativa mínima entre 208-210 nm y una banda positiva máxima a 190-195 nm que corresponden a una transición $\pi \rightarrow \pi^*$ paralelo y perpendicular al eje de la hélice, respectivamente.

El programa informático K2d (http://coot.embl.de/~andrade/k2d/k2d.pl) permite estimar a partir de los datos experimentales de dicroísmo circular el contenido de estructura α-hélice, hoja-β y aleatorizada, resultando un 37%, 36% y 38%, respectivamente.

Los espectros obtenidos de las toxinas recombinantes son similares al espectro de la toxina ErgTx1 nativa, por lo tanto el aminoácido sustituido por alanina en cada una de las variantes de la toxina, no generó una modificación de la estructura secundaria.

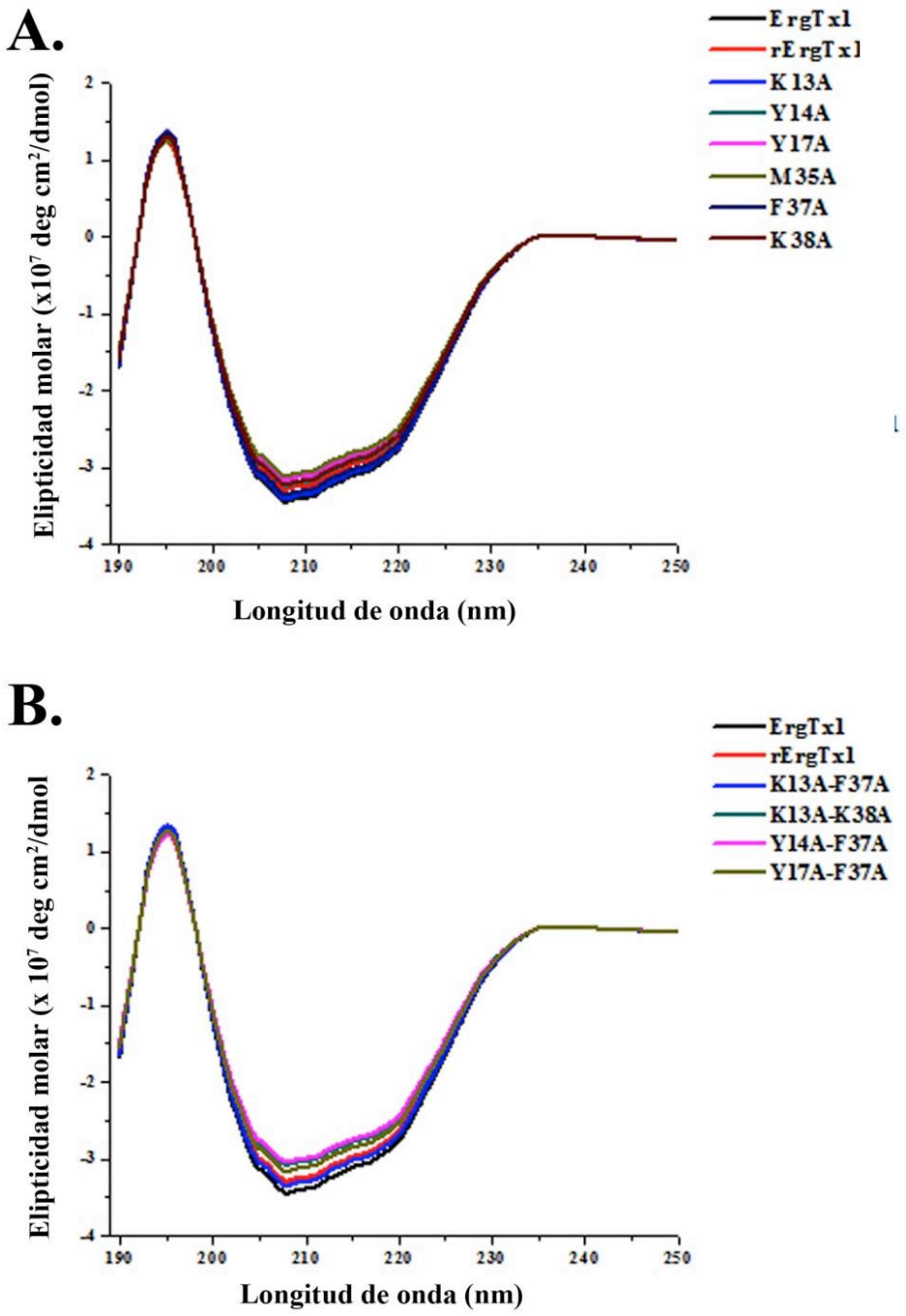

Figura 13. Espectros de dicroísmo circular de la toxina nativa ErgTx1 y de las toxinas recombinantes. Las lecturas se realizaron en el rango de longitud de onda de UV lejano entre los 190 y 250 nm, los datos se expresaron en elipticidad molar. **A.** Los trazos corresponden a las toxinas nativa, recombinante y a las mutantes puntuales de ErgTx1, mostrando que son superponibles con dos mínimos a 208-210 y 222 nm y un máximo a 190-195 nm, típicos trazos de péptidos con motivos α/β. **B.** Para las dobles mutantes se muestran los mismos espectros en comparación a la toxina nativa y recombinante ErgTx1.

6.3. Caracterización de la toxina recombinante ErgTx1

El efecto de la recombinante ErgTx1 sobre la corriente del canal de potasio hERG1 se determinó utilizando células establemente transfectadas con el canal, se aplicaron 100 nM de la toxina disuelta en la solución extracelular durante 100 s. En la Figura 14A se muestran los registros de corriente en situación control y en presencia de la toxina a un pulso de -120 mV después de un potencial de mantenimiento de -80 mV y un pulso de preacondicionamiento de +60 mV. Se observa que la toxina recombinante tiene un comportamiento semejante a la toxina nativa (Gurrola y cols., 1999; Pardo-López y cols., 2002a), ambas reducen la corriente de K^+ un 90% con respecto a su control (n=4-9, ver Figura 14B). Posteriormente, se determinó la afinidad de la toxina recombinante, para ello se utilizaron diferentes concentraciones en un rango de 2-200 nM. La constante de afinidad obtenida fue de 11.7±0.2 nM (como se ilustra en la Figura 15) manteniéndose en el mismo rango que la toxina nativa (K_D 7-10 nM; Gurrola y cols., 1999; Pardo-López y cols., 2002a).

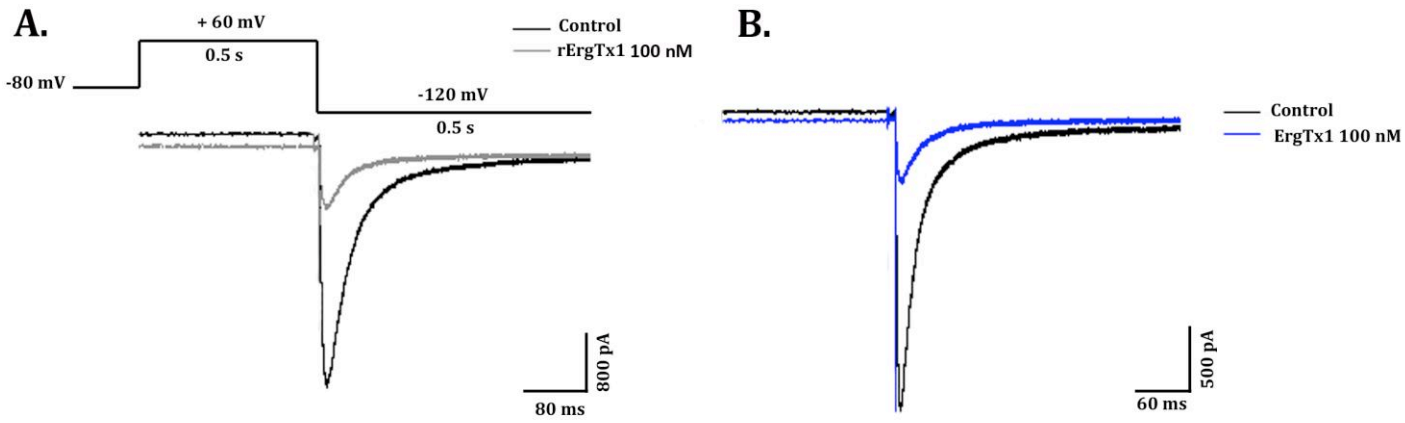

Figura 14. Reducción de la corriente de K^* de los canales hERG1 por la presencia de la toxina ErgTx1. A. Se muestra en la línea negra el registro de las corrientes de K^+ obtenidas por un pulso de prueba de +60 mV con una duración de 0.5 s, precedido por una repolarización a -120 mV. La aplicación de la rErgTx1 (100 nM) produce una disminución de la corriente (línea gris). El efecto de la toxina recombinante es comparable con la toxina nativa, como se observa en el panel. **B.** Se empleó el mismo protocolo de estimulación y se aplicó una concentración de 100 nM de la nativa (línea azul) observándose también una reducción en la corriente de potasio.

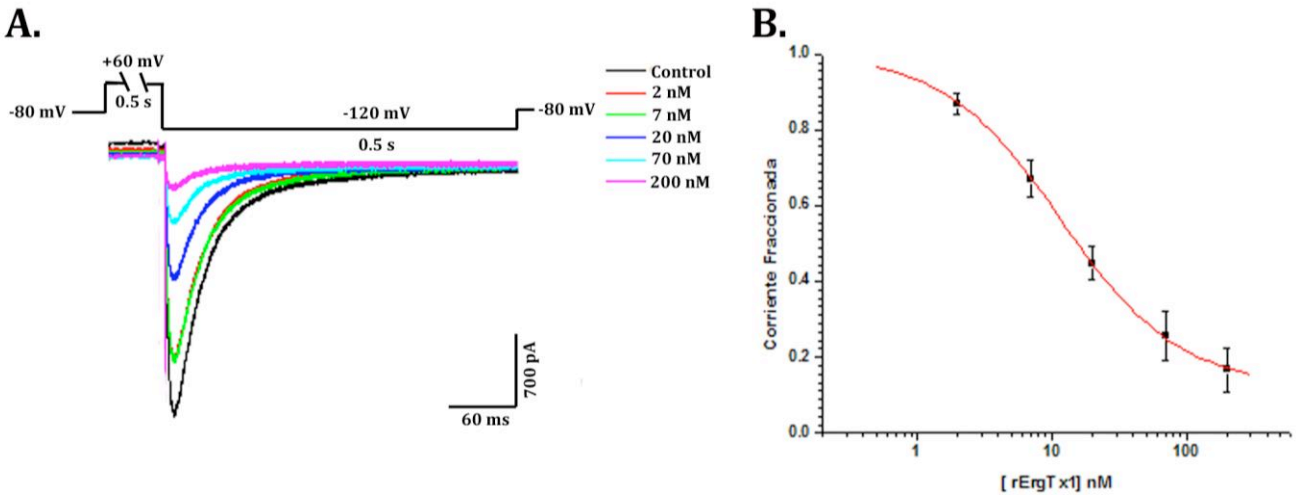

Figura 15. Efecto de la toxina recombinante ErgTx1 sobre el canal hERG1 y curva dosis-respuesta. A. Ilustra la reducción de la corriente de K^+ en respuesta a diferentes concentraciones de toxina recombinante. **B.** Para la construcción de la curva dosis-respuesta de la toxina rErgTx1, los datos se normalizaron con respecto a su propio control. Los datos se graficaron en función a la concentración de la toxina, obteniéndose un valor de K_D 11.7 nM, con un coeficiente de Hill de 1 (n=9).

6.4. Caracterización de las variantes de la ErgTx1

Los efectos electrofisiológicos de las variantes de la ErgTx1 sobre el canal hERG1, se registraron las corrientes de K^+, aplicandoun pulso de estimulación de -120 mV con una duración de 0.5 s, precedido de un pulso de mantenimiento de -80 mV y un pulso de preacondicionamiento de +60 mV de 0.5 s. Para la determinación de las constantes de afinidad de las variantes se aplicaron diferentes concentraciones durante 100 seg (0.1 – 35000 nM). Los picos de corriente residual se normalizaron con respecto a su propio control. Los datos se graficaron en función a la concentración de toxina y se ajustaron con la ecuación de Hill, obteniéndose los valores de K_D, coeficiente de Hill y la variación en la energía libre de Gibbs (ver Tabla 2) de cada variante.

En el caso de las mutantes M35A y K38A su efecto sobre el canal se determinó realizando una curva dosis-respuesta con concentraciones de las toxinas en un rango de 1 nM a 2.4 μM disueltas en la solución extracelular durante 100 seg (ver Figura 16 y 17), resultando valores de K_D de 342.2±31 y 14.5±1.8 nM, respectivamente.

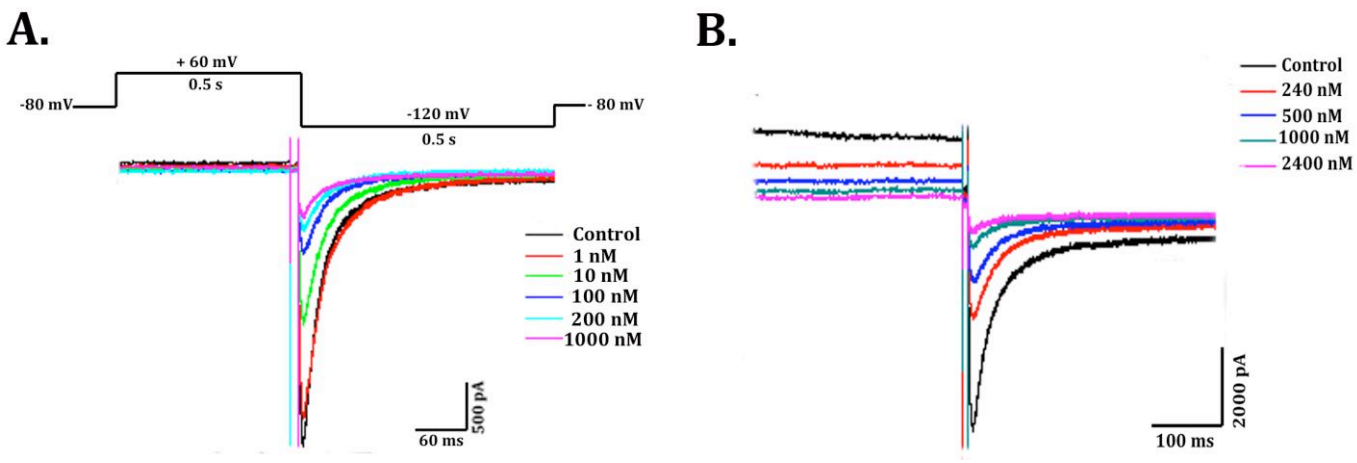

Figura 16. Efecto de las variantes K38A y M35A sobre la corriente de K^+ de los canales hERG1. A. Arriba de los registros de corriente se muestra el protocolo de estimulación utilizado para medir el efecto de la variante K38A sobre el canal hERG1, observándose que a medida que aumenta la concentración de la toxina hay una disminución de la corriente del canal, pero no llega inhibirla completamente a la concentración máxima de 1 µM. La variante K38A tiene un comportamiento similar a la toxina recombinante ErgTx1. **B.** Muestra los registros de corriente obtenidos después de aplicar diferentes concentraciones de la variante M35A sobre el canal. Por las concentraciones utilizadas se puede observar que la variante tiene una menor afinidad al canal hERG1 en comparación a la toxina nativa y/o recombinante.

Para la variante K13A se determinó su IC_{50}, utilizando un rango de concentración de 2 µM – 10 µM sobre las células CHO transfectadas establemente con el canal hERG1, observándose una pérdida de afinidad por el canal de 292 veces con respecto a la toxina nativa. La K_D para K13A fue de 3.5±0.08 µM (n=4; ver Figura 16). En el caso de la variante Y17A las concentraciones empleadas 1.6 a 17 µM, obteniendo una K_D de 1.7±0.09 µM, 142 veces menos afín que la toxina silvestre. Con la variante Y14A se utilizó una concentración de 22 µM (Figura 17), sin embargo no se observó ningún efecto sobre la corriente del canal. En la variante F37A se emplearon de 1.5 a 25 µM para la determinación de la K_D, resultando un valor de 5.3±0.5 µM, es decir, cerca de 442 veces menos afín que la toxina nativa.

Además de la síntesis de las mutantes puntuales se decidió sintetizar mutantes dobles esperando obtener un efecto sinérgico al cambiar residuos que forman parte del parche hidrofóbico, así como, los residuos positivos que rodean a este parche (Figura 8B). En efecto se se observarón pequeñas diferencias en los valores de K_{Ds} en comparación a los obtenidos con las mutantes puntuales (ver Figura 17 y Tabla 2) sobretodo cuando se sustituyen por alanina los pares de aminoácidos K13-F37 y Y17-F37 que son diez veces menos afines al canal en

comparación a su correspondiente mutante puntual. En este caso se puede afirmar que si se obtuvó un efecto sinérgico al cambiar ambos aminoácidos.

Se ha reportado previamente que la relación estequiométrica de interacción ente la toxina ErgTx1 y el canal hERG1 es de 1:1 con un bloqueo incompleto, independientemente de las altas concentraciones empleadas de toxina (Pardo-López y cols., 2002; Hill y cols., 2007). Sin embargo, el coeficiente de Hill calculado para las variantes K13A y Y17A es mayor a 1 (ver Tabla 2) sugiriendo que más de una molécula puede interactuar con un canal. Interesantemente, para estas mutantes y también en el caso de la M35A, los datos obtenidos sugieren un bloqueo completo de la corriente del canal hERG1, similarmente al bloqueo de producido por la ErgTx1 sobre la isoforma hERG3 (Restano-Cassulini y col., 2006). Las dobles mutantes Y14A-F37A y K13A-K38A muestran un comportamiento similar al de las mutantes puntuales con un coeficiente de Hill más alto de 1 y con tendencia a suprimir completamente la corriente, como se observa en la Figura 17B. De acuerdo a lo anterior, se puede afirmar que la superficie hidrofóbica formada por los residuos Y14, Y17, M35 y F37, así como, el residuo cargado K13 son importantes en la interface de interacción con el canal hERG1.

En la tabla siguiente se resumen los valores del coeficiente de Hill, la K_{Ds} (IC_{50}) y la diferencia de energía libre de Gibbs ($\Delta\Delta G = -RT \ln (K_D^{mut}/K_D^{wt})$). El valor de la corriente fraccional se determinó para cinco diferentes concentraciones de toxina en cuatro a nueve (n) células para cada concentración (Ver Figura 17A y 17B).

Tabla 2. Perturbaciones en la interacción entre ErgTx1 y los canales hERG1, resultado de mutaciones en la toxina.

	Hill	***K_D***	***K_D^{mut}/K_D^{wt}***	***$\|\Delta\Delta G$ Kcal/mol$\|$***	***n***
ErgTx1	1	7 – 10 nM	-	-	4
ErgTx1 oxidada	1	15.8 µM	1350	4.4	9
ErgTx1 recombinante	1	11.7 ± 0.08 nM	-	-	4
K13A	2.5 ± 0.1	3.5 ± 0.08 µM	292	3.4	4
Y14A	-	>22 µM	1833	>4.5	6
Y17A	2.4 ± 0.3	1.7 ± 0.09 µM	142	3	6
M35A	1	342 ± 31 nM	29	2	6
F37A	1	5.3 ± 0.1 µM	442	3.7	6
K38A	1	14.5 ± 1.8 nM	1.2	0.2	6
K13A/F37A	3.6 ± 0.4	15.3 ± 0.5 µM	1275	4.3	4
Y14A/F37A	-	> 30 µM	> 2500	> 4.7	4
Y17A/F37A	2.6 ± 0.3	18.7 ± 0.8 µM	1558	4.4	4
K13A/K38A	1.6 ± 0.08	8 ± 0.2 µM	667	3.9	4

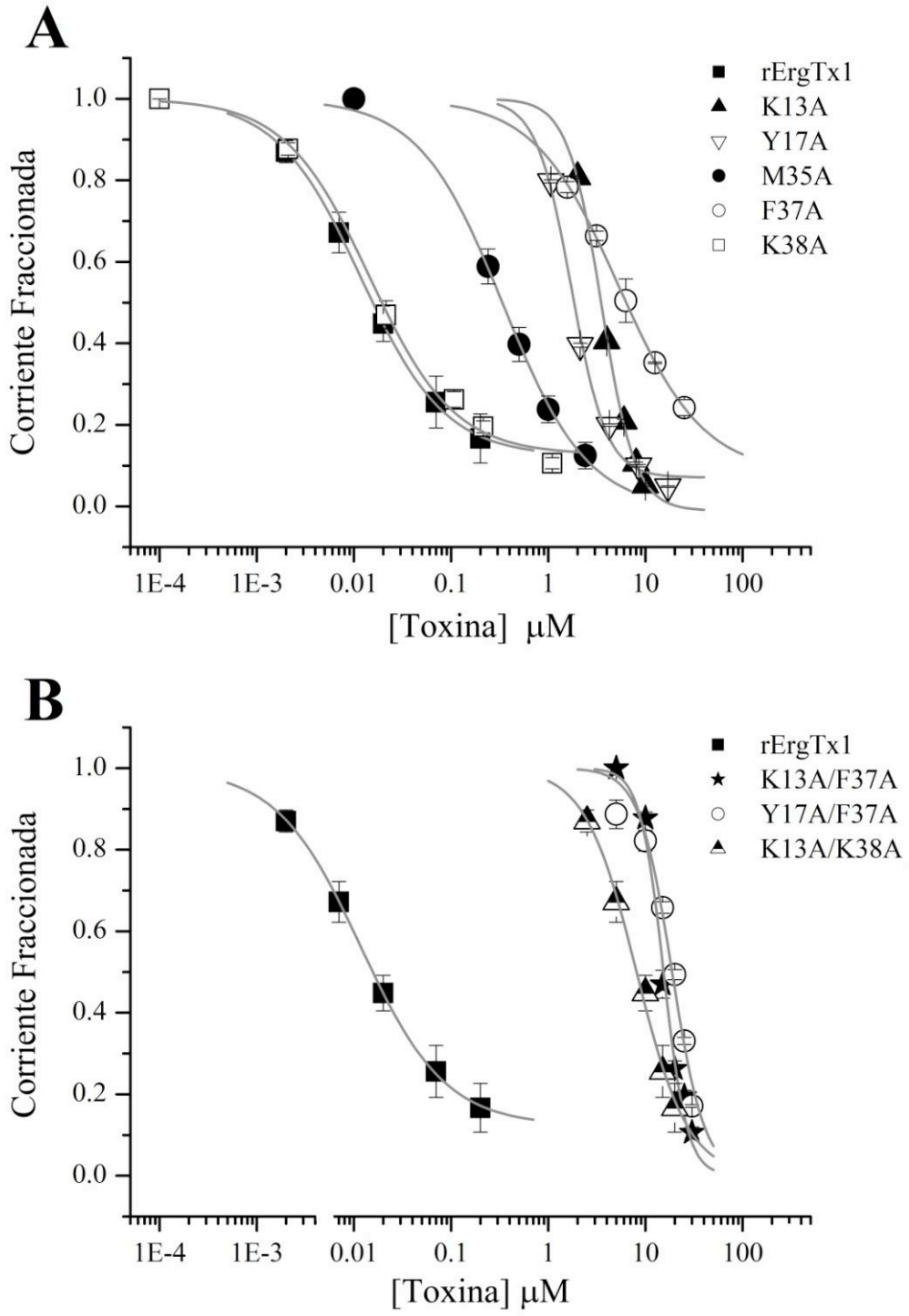

Figura 17. Relación dosis-respuesta de la inhibición de las corrientes K^+ del canal hERG1 afectadas por las toxinas recombinantes. A y B. Muestran las curvas dosis-respuesta obtenidas de experimentos realizados a diferentes concentraciones de las toxinas recombinantes, en un rango de 0.1 – 35000 nM. Los datos de cada registro se normalizaron con respecto a su propio control, se analizaron utilizando la ecuación de Hill y la gráfica se construyó en función a la concentración de la toxina contra la corriente normalizada en condiciones control. Los valores de la constante de afinidad para cada variante se reportaron en la Tabla 1.

VII. DISCUSION

La habilidad de producir toxinas ricas en puentes disulfuro con un plegamiento similar al nativo a través de métodos de DNA recombinante ha sido una herramienta útil para realizar estudios de estructura-función. *Escherichia coli* es uno de los modelos mejor caracterizados para la expresión heteróloga de proteínas. Todas las toxinas de alacrán contienen puentes disulfuro que son esenciales para su conformación estructural, por lo que para su expresión de forma recombinante se requiere un ambiente oxidante. Por lo tanto, la secreción de este tipo de péptidos dentro del periplasma de *E. coli* aumenta la probabilidad de obtener un plegamiento debido a las condiciones oxidantes de este compartimento celular. Sin embargo, también se requiere de un péptido señal (PelB) que transloque la proteína al periplasma y la presencia de una proteína acarreadora que aumente la solubilidad del péptido, que aumenten la eficiencia de la secreción al periplasma y la probabilidad de obtener una proteína soluble. En este trabajo, usamos la proteína Thioredoxina como proteína acarreadora porque además de aumentar la solubilidad del péptido que normalmente se produce en forma insoluble (LaVallie y cols., 1993), también puede catalizar la formación de puentes disulfuro al ser exportada al periplasma tiene una actividad similar a la chaperona DsbA, promoviendo la formación de puentes disulfuro (Huber y cols., 2005). Gracias a este sistema híbrido fue posible obtener toxinas recombinantes que muestran un arreglo de cisteínas igual a la toxina nativa.

El objetivo de este trabajo fue determinar la superficie de interacción de la ErgTx1 sobre el canal hERG1. Los antecedentes sugieren que el principal tipo de interacción involucrada en esta interface es hidrofóbica, lo que indica que posiblemente los residuos que constituyen el parche hidrofóbico en la ErgTx1 y aminoácidos básicos pueden estar implicados en la superficie de interacción con el canal hERG1 (ver Figura 6B). Se propone que esta región hidrofóbica de la toxina pudiera estar interaccionando con los residuos de la α-hélice anfipática del canal. Por lo que se decidió sustituir por alanina a los cinco residuos (K13, Y14, Y17, M35, F37, K38) que forman parte del parche hidrofóbico e hidrofílico de la ErgTx1, diseñando tanto mutantes puntuales como dobles, esperando con estas últimas obtener un efecto sinérgico con la

sustitución de dos aminoácidos importantes para la interacción. El aminoácido de alanina se eligió para remplazar estos residuos porque este solo elimina la cadena lateral del carbono-β sin alterar la conformación de la cadena principal ni impone efectos electrostáticos o impedimentos estéricos (Ashkernazi y cols., 1990).

Otro dato importante obtenido en nuestro grupo, es que al oxidarse el residuo M35A de la ErgTx1 nativa la afinidad por el canal decrece 3 órdenes de magnitud. Por lo que se plantearon 2 situaciones: 1) la pérdida de afinidad se debe a que este residuo es importante en la superficie de interacción con el canal o 2) la oxidación de este residuo a sulfóxido de metionina modifica la estructura secundaria de la toxina y por lo tanto se produce la pérdida de afinidad. Por esta razón, se decidió también sustituir por alanina este residuo para determinar la participación de la cadena lateral de la M35 en la unión de la toxina con el canal o bien en la estabilización de la estructura secundaria de la toxina.

Analizando las dos estructuras tridimensionales resueltas por RMN de la toxina ErgTx1 (Torres y col., 2003; Frénal y col., 2004) se muestra que todos los residuos que se decidieron mutar forman parte de las asas en la estructura espacial de la toxina, están altamente expuestos al solvente (Figura 8B) y ninguna cadena lateral de residuos sustituidos por alanina están formando interacciones que estabilicen la estructura tridimensional de la toxina, como puentes de hidrógeno. Por lo que teóricamente la conformación estructural de las variables recombinantes no debe cambiar. Esto se confirmó con los resultados de dicroísmo circular en donde los espectros presentan una proporción similar de α-hélice y hojas-β en comparación a la toxina nativa (Figura 13). Para cada variante se determinaron los valores de K_D (ver Tabla 2) y la diferencia de energía libre de unión y se comparó con la toxina nativa, los cuales revelan que la sustitución por alanina de los residuos K13A, Y14A, Y17A, M35A y F37A hace que las variantes tengan una menor afinidad al canal hERG1 de 292, >1833, 142, 29 y 442 veces menos que la toxina silvestre ErgTx1. Para las dobles mutantes K13-F37A, K13A-K38A, Y14A-F37A, Y17A-F37A la afinidad por el canal se reduce 1275, 667, 2500 y 1558 veces, respectivamente (ver Tabla 2).

El valor de la K_D de la variante M35A (342 nM) en comparación con la isoforma oxidada de la ErgTx (15.8 µM) muestran diferencias términos de afinidad al canal hERG1. Se sabe que los residuos de metionina son notablemente susceptibles a la oxidación en proteínas y por lo tanto este cambio a sulfóxido de metionina puede afectar la función algunas proteínas y para otras la oxidación de este residuo se usa como un mecanismo de regulación (Hoshi y Heinemann, 2001; Nabuchi y cols., 1995; Liu y cols., 1998; Van Patten y cols., 1999; Yin y cols., 1999). En el análisis *in sílico* de la estructura tridimensional de la ErgTx1 (Torres y cols., 2003; Frénal y cols., 2004) se muestra que la M35 está localizada sobre la cara hidrofóbica de la tercera hoja-β antiparalela (ver Figura 18B) y que su cadena principal está formando cinco puentes de hidrógeno con la cadena principal de los residuos K38 y F37. Por lo tanto, la sustitución de la metionina por otro residuo o la oxidación de la misma no cambian la conformación estructural de la toxina, pero si se modifica la cadena lateral involucrada en la interface de interacción con el canal, tal vez el átomo de azufre de la metionina este formando un puente de hidrógeno con esta superficie, lo que explicaría la diferencia de afinidad entre la isoforma oxidada de ErgTx1 y la mutante M35A.

Interesantemente, con estos resultados se asume que la principal superficie de interacción de la toxina sobre el canal está orientada en una cara hidrofóbica de la molécula, la cual esta formada por los residuos Y14, Y17, M35 y F37. Esta área hidrofóbica rodea al residuo básico K13, que también forma parte de la superficie de contacto en la unión de ErgTx1 con el canal hERG1 (Torres y cols., 2003; Frénal y cols., 2004; Jiménez-Vargas y cols., 2012a).

En estudios previos realizados con la toxina BeKm-1 del alacrán *Buthus eupeus* (Koralkova y cols., 2001, 2002; Milnes y cols., 2003; Zhang y cols., 2003), toxina específica para el canal de potasio hERG1, muestran que la superficie de la toxina que hace contacto con el canal está situada en la α-hélice. Comparando los datos obtenidos en esta tesis con los obtenidos para BeKm-1 (Korolkova y cols., 2002; Zhang y cols., 2003; Korolkova y cols., 2004) confirman que la superficie de interacción entre las toxinas ErgTx1 y BeKm1 sobre el canal son diferentes, ya que en la primera los residuos se encuentran ubicadas en las asas de la toxina, mientras que en la segunda se encuentran en la superficie de la α-hélice (ver Figura 18; Zhang y cols., 2003; Torres y cols., 2003; Frénal y cols., 2004). Ambas toxinas se unen a la región de la torreta del canal

causando una oclusión parcial del poro, es decir que ninguna es capaz de suprimir completamente la corriente del canal hERG1, aún en presencia de 1 µM de toxina (equivalente a 100 veces el valor de sus K_{DS}; Pardo-López y cols., 2002a; Hill y cols., 2007; Tseng y cols., 2007). A diferencia de las toxinas bloqueadoras del poro (ejemplo: AgTx2, ChTx) que inhiben completamente la corriente de K^+ de otros canales Kv.

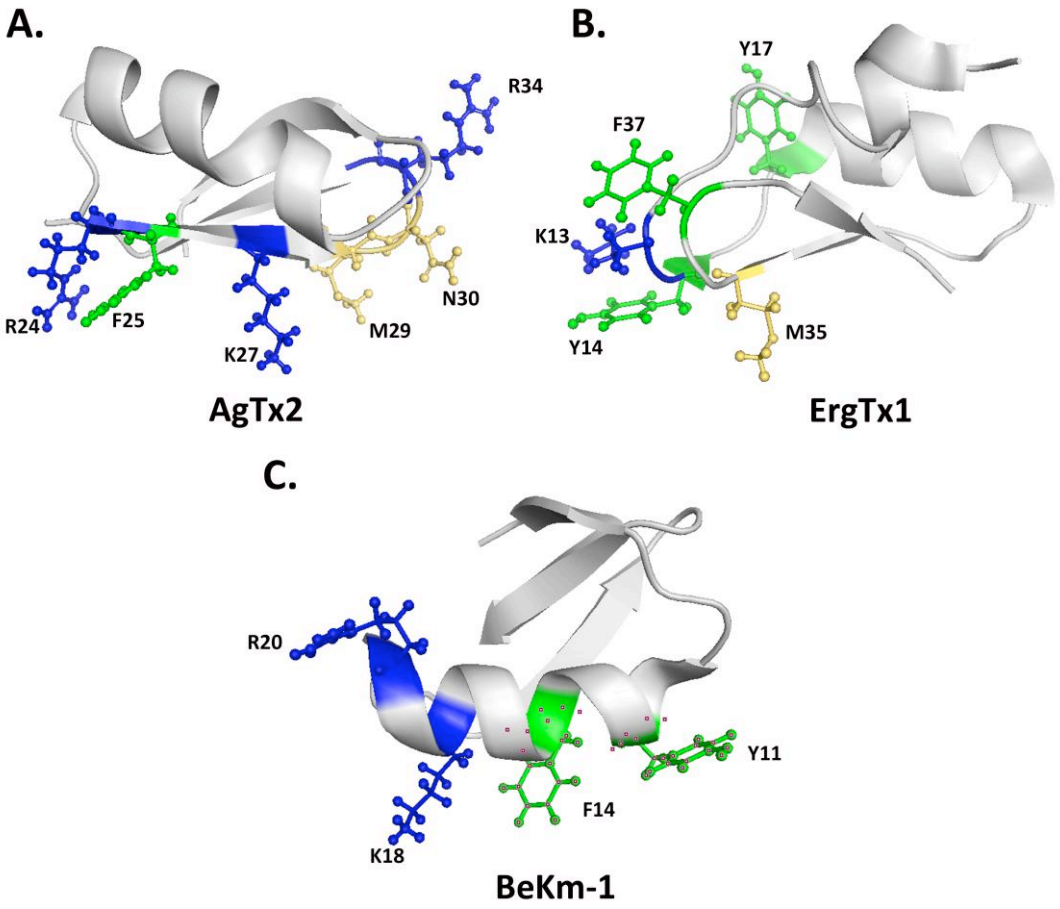

Figura 18. Superficies de interacción de las toxinas AgTx2, BeKm1 y ErgTx1 sobre ciertos canales Kv. A. Diagrama de listón que muestra la estructura secundaria de la AgTx2 resuelta por resonancia magnética nuclear (RMN; con código de acceso al PDB 1AGT), se resaltan las cadenas laterales de los aminoácidos importantes para la interacción con el canal Shaker y otros canales Kv (verde, F25; azul, R24, R34 y K27; amarillo, M29, N30), localizados en la segunda hoja β antiparalela de la toxina. **B.** Diagrama de listón que muestra la estructura secundaria de la ErgTx1 determinada por RMN (PDB 1PX9), se enfatizan las cadenas laterales de los aminoácidos involucrados en la interacción con el canal hERG1 (verde, Y14, Y17 y F37; azul, K13; amarillo, M35). **C.** Diagrama de listón que muestra la estructura secundaria de la BeKm-1 (PDB 1LGL) y las cadenas laterales de los aminoácidos importantes para la interacción con el canal hERG1 (verde, Y11 y F14; azul, K18 y R20) localizados en la α-hélice de la toxina. Las figuras se generaron usando el programa Pymol (Tomado de Krezler y cols., 1995; Korolkova y cols., 2002; Torres y cols., 2003; Frénal y cols., 2004).

Tseng y cols., 2007 propusieron un modelo de la interacción de BeKm-1/hERG1 en el que se muestra que la toxina es capaz de cruzar el vestíbulo externo e interactúar con el poro del canal, de tal manera que la hélice anfipática S5-P del hERG1 hace contacto con los residuos Y11 y F14 localizados en un extremo de la α-hélice en la BeKm-1, mientras que el otro extremo de la α-helice entra al poro, en donde las cadenas laterales de los residuos K18 y R20 hacen contacto con las cadenas laterales de los residuos S631 de las subunidades adyascentes en el canal. La BeKm-1 no penetra el poro con profundadidad, lo que puede explicar porque la unión BeKm-1–hERG1 no es sensible a concentraciones altas de potasio al interior del poro. En contraste con la unión de AgTx2 o ChTx con el canal *Shaker,* la cadena lateral de un residuo de lisina (K27) sobresale de la superficie de la toxina, entra al poro y se une al filtro de selectividad. En este caso, launión de las toxinas con el canal *Shaker* se desestabiliza al incrementar la ocupación del ión potasio en el interior del poro o elevando la concentración de potasio alrededor del vestíbulo externo del canal (Ranganathan y cols., 1996). En la BeKm-1, las cargas positivas de las cadenas laterales de los residuos K18 y R20, apuntan hacia la entrada externa del poro del canal, ocluyéndolo y bloqueando el flujo de iones K^+ (ver Figura 19).

Como se ha mencionado, tanto la AgTx2, así como otras toxinas tienen un motivo conocido como diada funcional el cual esta formado por una Lisina y un residuo aromático (K27, F25), estos residuos interactúan con el filtro de selectividad del receptor suprimiendo completamente la corriente de potasio en los canales Kv. En el caso de las toxinas BeKm-1 y ErgTx1 no quiere decir que no exista este motivo, si no que las características del receptor son diferentes, es decir la región de la torreta es más larga en los canales hERG1 que en el resto de canales Kv, por lo que esto puede ser el impedimento para que residuos básicos de las toxinas BeKm-1 y ErgTx1 puedan entrar al poro e interactuar con el filtro de selectividad y suprimir la corriente de K^+.

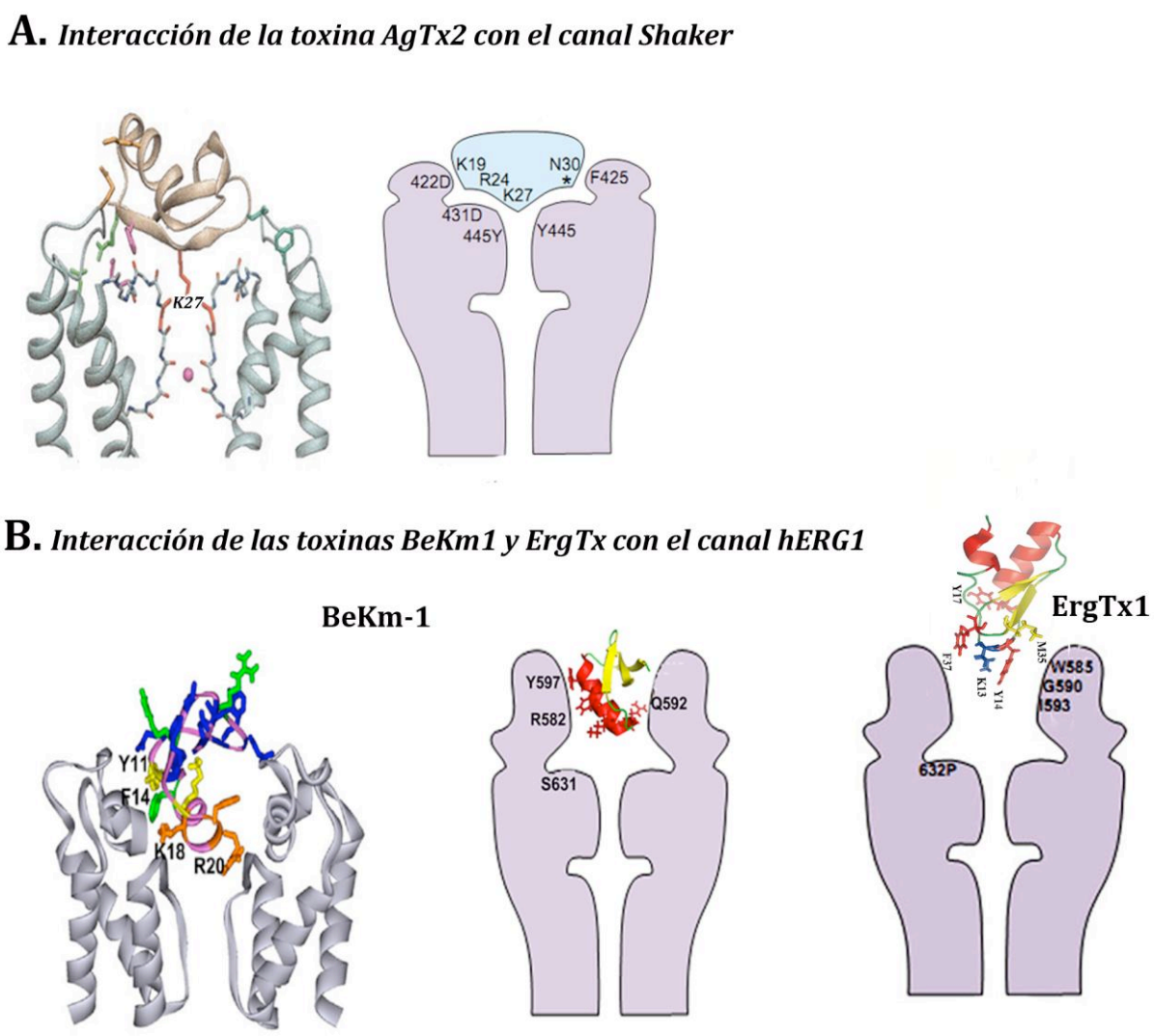

Figura 19. Representación en diagramas de listón y cartón de la superficie de contacto de toxinas de alacrán que interactúan con los canales Kv. A. Interacción de la toxina AgTx2 con el canal Shaker. (Izquierda) Se muestra un modelo en diagrama de listón en el que se ilustra la interacción de los residuos de la toxina (K27) con el filtro de selectividad del canal *Shaker,* se muestra solo dos subunidades del canal para que sea más fácil la visualizar las interacciones. La superficie de interacción se determinó mediante ciclos de dobles mutantes (Eriksson y Roux, 2002). *(*Derecha) Modelo de cartón que ilustra los residuos de la toxina que están interactuando con el poro del canal: K27-Y445; R24-D431; K19-D422. **B.** Interacción de las toxinas BeKm-1 y ErgTx1 sobre el canal hERG1. (Izquierda) Vista lateral de la región formadora del poro del canal hERG1 en donde los dominios se ilustran de color gris. La BeKm-1 esta ubicada al centro del poro y en color se muestran los residuos que interactúan con el vestíbulo del canal, naranja, K18 y R20; verde, F14 y amarillo, Y11. (Centro) Ilustra la región formadora del poro del canal hERG1y a los aminoácidos R585, Q592, Y597, S632 que interactúan con Y11, F14, K18 y R20 de la BeKm-1. (Derecha) Ilustra la región formadora del poro del canal hERG1 en donde los aminoácidos de la torreta (W585, I593, G590, P632; Pardo-López y cols., 2002b) están interaccionando con los residuos que forman el parche hidrofóbico en la ErgTx1 (rojo, Y14, Y17, F37; amarillo, M35; tomado de Rodríguez de la Vega y cols., 2003).

VIII. CONCLUSIONES

- La cadena lateral de la M35 en la toxina ErgTx1 forma parte de la superficie de interacción con el canal hERG1.

- La sustitución por alanina del residuo K13 reduce 350 veces la afinidad de la ErgTx1 por el hERG1 con respecto a la toxina nativa, resultando una diferencia de energía libre de unión de 3.5 kcal/mol.

- La sustitución por alanina de los residuos que forman parte del parche hidrofóbico en la ErgTx1 (Y14, Y17 y F37) causa una reducción en la afinidad de >1800, 140 y 450 veces, respectivamente, por el canal hERG1.

- El parche hidrofóbico formado por los residuos Y14, Y17, M35, F37 representa una superficie importante de la ErgTx1 para interaccionar probablemente con la α-hélice anfipática que forma el vestíbulo externo del canal hERG1. De la misma forma, el residuo cargado en la posición 13 posiblemente está interaccionando con esta región del canal.

- Con las mutantes dobles de la ErgTx1 se confirmó que en realidad los residuos inicialmente considerados probables (parte del parche hidrofóbico) están involucrados en la superficie de interacción toxina-canal.

IX. PERSPECTIVAS

- Los residuos que resultaron ser importantes en la capacidad de la ErgTx1 de unirse a los canales de potasio hERG1 ahora son candidatos para caracterizar la interacción de esta toxina sobre las isoformas ERG2 y ERG3.

- Como tenemos las toxinas mutantes sería deseable en colaboración con la Dra. G. N. Tseng, con la cual nuestro laboratorio colaboró en el pasado y quien tiene los mutantes del canal ERG1, realizar experimentos con dobles mutantes, afin de confirmar los resultados obtenidos por electrofisiología reportados en esta tesis.

- De misma forma, como el sistema de expresión de estas toxinas fue exitoso, se podría ampliar el número de mutantes en otras posiciones distintas de las hechas en esta tesis, para comprobar que las modificaciones efectuadas realmente corresponden a la superficie más probable de interacción con el canal ERG1.

X. REFERENCIAS

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XI. ANEXOS

Artículos Indexados

ANEXO 1: Jiménez-Vargas JM, Restano-Cassulini R, Quintero-Hernández V, Gurrola GB, Possani LD. Recombinant expression of the toxin peptide ErgTx1 and role of Met35 on its stability and function. Peptides 2011; 32: 560-567.

ANEXO-2: Jiménez-Vargas JM, Restano-Cassulini R, Possani LD. Interacting sites of scorpion toxin ErgTx1 with hERG1 K^+ channels. Toxicon 2012; 59: 633-641.

ANEXO-3: Jiménez-Vargas JM, Restano-Cassulini R, Possani LD. Toxins modulators and blockers of hERG K^+ channels., Toxicon 2012 (en prensa).

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Recombinant expression of the toxic peptide ErgTx1 and role of Met35 on its stability and function

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ABSTRACT

Ergtoxin 1 (ErgTx1) is a 42 amino acid peptide purified from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann, capable of blocking specifically human potassium channels of the *ether-á-go-go*-related gene family (hERG). This peptide binds to a partially overlapping site on the channel outer mouth, in which residues of the S5-P linker are critically involved. Here we describe results of site directed mutagenesis of the ErgTx1 gene and its heterologous expression in *Escherichia coli*. The recombinant products show the fundamental role played by methionine in position 35 (Met35) of the primary structure. Naturally oxidized Met35 decreases by three orders of magnitude the affinity of the peptide for the hERG1 channels. This result is quite relevant, because it shows two possible situations: either Met35 is involved in the proper folding of the molecule or it plays a direct role in the interaction with the channel, i.e., constitutes part of the interacting surfaces. These two situations were evaluated by preparing heterologously expressed ErgTx1 gene and a mutant containing alanine in position 35. Additionally circular dichroism measurements of both native and recombinant peptides were performed. The electrophysiological recordings and the structural values obtained by optical measurements, strongly support the idea that Met35 is indeed a key residue on the interacting surfaces of the toxin with the channels.

1. Introduction

The human *ether-á-go-go* related gene (*hERG*) is a member of the *eag* (*ether-á-go-go*) potassium channel family. It was initially isolated by Warmke and Ganeztky [41]. Unlike most other potassium channels, hERG1 displays unique properties which are important in the repolarization phase of the cardiac action potential [33,38]. Mutations of hERG1 are a common cause of inherited long QT syndrome, a disorder of cardiac repolarization that predisposes affected individuals to "torsade de pointes" arrhythmias and sudden death [3,16]. Acquired long QT syndrome is far more common than inherited long QT syndrome often caused by blockade of hERG channels as a side effect of treatment with commonly used medications including antiarrhythmic, antihistaminic, antibiotic, psychoactive drugs, prokinetic agents and gastrointestinal drugs [23,30,40]. Likewise, the physiological role of the channel has been characterized in different types of tissues in which excitability is an essential property, such as smooth muscles [1], carotid bodies [25], in the regulation of prolactin secretion in lactotrops [2], pancreatic β-cells [32] and chromaffin cells [8].

This channel has a tetrameric structure formed by co-assembly of four identical α-subunits, each composed by six α-helical transmembranal domain (denoted S1–S6), with the S4 domain containing six positive charges, typical for voltage gated potassium channels [41]. The S5 and S6 domains in each of the subunit together form the pore domain which contains the pore helix, the selectivity filter (extracellular end of the pore) and a lengthy S5-P linker ("turret"; 43 amino acid) with an amphipathic helix (the turret helix). The charged S4 domain is the "voltage sensor" responding to changes in membrane potential [17,27,38,43]. However, hERG1 has an unusually longer S5-P linker compared with other potassium channels (43 amino acid versus 14–23 amino acid residues, respectively); and considerable conformation flexibility of this S5-P linker region has been found recently by circular dichroism spectropolarimetry and NMR spectroscopy studies [14,17,37]. Mutations in this loop affect the rapid voltage dependent inactivation of the hERG1K^+ channel that is critical for its normal function [17]. Therefore, the discovery of natural substances that can specifically recognize and reversibly block the channel at this site is essential for the research and development of new drugs for the treatment of certain diseases and malfunctions associated with the channel hERG1 [35]. The search for high affinity probes specific for potassium channels has been a wide and important subject of

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research. Soon, it became clear that venom from various species of animals, such as scorpions, snakes, spiders, marine snails of the genus *Conus* and sea anemone represent a rich source of components that show a direct activity over different families of K^+ channels [7]. The scorpion toxins that interact with the voltage-dependent potassium channels have provided us with powerful tools to define different aspects of structure–function relationships and has been reported that this interaction can be performed through two distinct mechanisms. In the first mechanism, the toxin binds to the outer vestibule of the ion conduction pore and inhibits the flow of ions. The best studied examples of this mechanism are the scorpion toxins charybodotoxin and agitoxin [22], two particularly interesting voltage-gated K^+ channels inhibitors that were used to define the pore forming region of potassium channels [20]. In the second type of mechanism the toxin binds to a region of the channel that changes conformation and influences the gating mechanism by altering the relative stability of closed, open or inactivated states [7].

One of the toxins isolated and characterized in our laboratory is ErgTx1 purified from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann. This peptide has 42 amino acids stabilized by four disulfide bridges [10,34]. ErgTx1 acts specifically on the hERG potassium channels and through studies of site directed mutagenesis, the toxin binding site was identified to occur at the S5-P loop of the channel. The interacting amino acid residues of this channel with the toxin were identified [27,28]. Another important data obtained by our group, which is described in detail for the first time in this communication, is that upon oxidation, residue methionine in position 35 of the primary structure of ErgTx1 causes a decrement of affinity towards the channel by three orders of magnitude. There are two distinct possibilities to explain such huge variation on affinity: 1) either Met35 is one of the principal residues making direct contact with the channel, or 2) it plays a fundamental role in maintaining intact the three-dimensional (3D) structure of ErgTx1, which is known to be important for function. It is documented that destabilization of the 3D-structure of K^+-channel toxins causes lost of function [9]. In order to test the two main hypotheses for such variations, here we report the results of experiments conducted with heterologously expressed native and modified ErgTx1. The results of site-directed mutagenesis, electrophysiological recordings and circular dichroism measurements support the conclusion that in fact, Met35 is an important residue that directly interacts with Erg1 channels.

2. Materials and methods

2.1. Bacterial strains, enzymes and plasmids

Restriction enzymes, vent polymerase, enterokinase and T4 DNA ligase were purchased from New England Biolabs and Sigma Research. *Escherichia coli* DH5-α (F^-*gyr*A96 (NAI^r) *recA relA1 endA1 thi-1 hsdR17* ($r_K^-m_K^+$) *glnV44 deoR Δ(lacZYA-argF)U169*[ϕ80dΔ(*lacZ*)M15]) was used for plasmid propagation and TG1 strain (*supE thi1Δ(lac/proAB) Δ(mcrB/hsdSM)5(*rK-mK-) [F′*traD36proAB lacIqZΔM15*]) was used for the expression of the toxin-fusion proteins. Plasmid pThioC (Invitrogen) and pSyn1 were used for cloning and expression of the fusion protein with thioredoxin, respectively.

2.2. Purification native ErgTx1

Soluble venom from the scorpion *C. noxius* was separated by Sephadex G-50 and carboxymethyl-cellulose (CM-cellulose) columns and two additional HPLC separations, a earlier described [10]. Component active on hERG1 channel was finally separated, using a C18 analytical column run with linear gradient from 5% to 40% solution B for 50 min. This strategy produced a highly pure peptide, whose primary structure was confirmed by de novo sequencing using an automatic LF3000 Protein Sequencer (Beckman, CA, USA). The correct molecular weight was confirmed by electro spray ionization mass spectrometry using a Finningan LCQ^{DUO} ion trap mass spectrometer (San Jose, CA, USA).

2.3. Construction of the expression plasmid

From a cDNA library of venomous gland of *C. noxius* Hoffmann scorpion, the gene coding for the sequence of ErgTx1 was obtained (GeneBank, accession number AAG38523, work performed by Blanca Ines García-Gómez of our laboratory). This gene was cloned into the pThioC vector (Invitrogen), which contains the sequence that code for the carrier protein thioredoxin and a stretch of amino acid sequence that is the site of cleavage of the enzyme enterokinase.

Then, this construction was amplified by PCR from vector pThio-EK-ErgTx1 and cloned into the expression vector pSyn1, which is designed in such way to permit insertion of a specific sequence and to merge the gene encoding the signal peptide PelB (in this case the fusion protein: thioredoxin-EK-toxin). Thus, the expression plasmid contained: the sequence coding for the thioredoxin, the sequence coding for the amino acid recognized by the protease (EK) and the gene of ErgTx1. This construction was named pSyn1Thio-EK-ErgTx1, and its product is here abbreviated: Thio-EK-ErgTx1. The PCR amplifications were carried our using the following oligonucleotides: *Sfi-Thio* corresponds to 5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAC AGA GAT AGC TGC GTC GAC-3′. This contained the *Sfi*I restriction site (underlined) and the nine NH2-terminal residues of the protein thioredoxin; reverse oligonucleotide Not-Erg: 5′-GAG TCA TTC TCG ACT TGC GGC CGC ACG TGA TTA CGC ACA TTT ACA CTT GAA AAA-3′. This primer includes *Not*I restriction site (underlined), the two stop codons and the sequence that codes for amino acids 36–42 of native ErgTx1. The PCR conditions were: 5 min at 94 °C, 30 cycles for 1 min 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final extension of 5 min at 72 °C. The PCR products and the plasmid pSyn1 were digested with the corresponding restriction enzymes at 37 °C and 50 °C for *Not*I and *Sfi*I, respectively and purified by column (QIAquick) before ligation. The ligation reaction (20 μL) was carried out with T4 DNA ligase with a 10 folds insert excess over plasmid for 16 h at 16 °C. Ten microliters of the ligation reaction were used to transform competent *E. coli* DH5-α cells. Positive clones with the expected insert were grown in LB ampicillin medium. The plasmids of positive colonies were purified by means of the High Pure Plasmid Isolation Kit (Roche). Plasmid constructs were verified by sequencing from both sites, the insert boundaries to conform the reading frame and conservation of restriction sites. TG1 strains were transformed with the corresponding plasmid during 2 min at 42 °C, followed by 5 min in ice and 30 min recovery at 37 °C in LB plates contained 200 μg/mL of ampicillin.

To generate the mutation in the Met35 an oligonucleotide was design for the insertion of alanine instead of methionine in this same position, abbreviated here M35A. The recombinant M35A was generated with the following oligonucleotides: *BamH*I-EntThio-Fwd and M35Arev. The first one is: 5′-GGC TCT GGA TCC GGT GAT GAC GAT GAC AAG GAC AGA GAT AGC TGC GTC-3′. It corresponds to *BamH*I restriction site (underlined), the sequence coding for the amino acids recognized by the protease (EK) and the six NH2-terminal residues of the gene mutant M35A. The second oligonucleotide (M35Arev) is: 5′-CGC ACA TTT ACA CTT GAA AAA CGC ACA GGT TCC TCC ATTT GTG-3′. For the site directed mutagenesis the construction pSyn-Thio-EK-ErgTx1 was used as template and the PCR employed the oligonucleotides *BamH*I-EntThio-Fwd

and M35rev using the same conditions of PCR previously described. The PCR product was digested with *Bam*HI and *Eco*RI enzyme and ligated to pSyn1 and transformed into strain *E. coli DH5α* for plasmid propagation. Plasmid constructs were verified by DNA sequencing from both sites, to confirm the reading frame and conservation of restriction sites.

2.4. Overproduction and purification of rErgTx1 and mutant M35A

The rErgTx1 and mutant M35A were expressed as protein fused to thioredoxin (*Thioredoxin-EK-toxin)* and expressed in *E. coli* strain TG1. One colony was used to inoculate a 10 mL YT2X media supplemented with 200 μg/mL ampicillin and 0.1% glucose and grown overnight at 37 °C in a shaking incubator. The overnight culture was added to 500 mL YT2X media supplemented with ampicillin and glucose (200 μg/mL and 0.1%, respectively). The culture was grown at 37 °C, and when the OD_{600} reached 1.0, the inductor isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was harvested 16 h later and centrifuged at 8000 rpm for 15 min. The bacterial pellet was resuspended in 12.5 mL of 20 mg/mL sucrose, 20 mM Tris–HCl, pH 8.0 and after standing at 4 °C for 15 min, the cells were harvested by centrifugation at 8000 rpm for 20 min. The supernatant was removed and the cell pellet resuspended in 12.5 mL distilled H_2O (4 °C) (which causes an osmotic shock), and left incubating with gentle agitation at 4 °C for 20 min. The resulting spheroplasts were harvested (13,000 × *g* at 4 °C for 20 min) and the supernatant, referred to as the periplasm fraction was recovered. This fraction containing the fusion protein, which was dialyzed against 20 mM Tris–HCl buffer pH 8.0 and loaded onto a 64 mL capacity ion exchange column, containing resins of diethyl-amino-ethyl-cellulose (DEAE), which had been equilibrated previously with 20 mM Tris–HCl buffer pH 8.0, washed for 30 min with the same buffer and eluted with 0–0.3 M NaCl gradient. This product was purified further by RP-HPLC with a C18 semi-preparative column. The fusion protein or hybrid protein, as we called it, was eluted with a linear gradient, from buffer A (0.12% (v/v) TFA in water) to buffer B (0.1% TFA in acetonitrile). The gradient used was 0–65% buffer B, run for 65 min.

Cleavage of rErgTx1 and the mutant M35A from the fusion protein was carried out by incubating with enterokinase in buffer (500 mM Tris–HCl, 2 mM $CaCl_2$, 10% Tween 20, pH 8.0). One milligram of the fusion protein was treated with 0.02 units of enterokinase for 16 h at 25 °C. The peptide was purified by RP-HPLC using a C18 analytical column. The recombinant toxins were eluted with a linear A–B gradient of 0–65% buffer B, for 65 min.

The identity of peptides was confirmed by ESI mass spectrometry (ESI-MS) and direct sequencing through automatic Edman degradation. The tertiary structure of the peptides was confirmed by far-UV circular dichroism (CD) measurements, as described in the next section.

2.5. Circular dichroism (CD) measurements

CD spectra were obtained on a Jasco J-720 spectropolarimeter (Jasco, Japan). The spectra were measured from 250 to 190 nm in 20 mM Tris–HCl, pH 8.0 at 20 °C, with a 1 mm path-length cell. Data were collected at 0.1 mm with a scan rate of 20 nm/min and a time constant of 0.5 s. The concentration of the toxins rErgTx1, native ErgTx1 and M35A variant was 0.2–0.25 mg/mL. Results are expressed as the mean residue ellipticity $[\theta]_{MRW,\lambda} = (\theta_{obs}/10)$ (M/c l), where θ_{obs} is the observed ellipticity at a give wavelength, *M* is the mean residue mass, *l* is the cuvette path-length in centimeters, and *c* the protein concentration expressed as mg/mL. The units of $[\theta]$ are° cm^2 $dmol^{-1}$ [26]. The mean residue molecular mass for ErgTx1, taken as 112.6 Da and 111 Da for M35A, were calculated from its amino acid composition [10]. The far ultraviolet CD spectra between 190 and 250 nm were recorded at 20 °C in a 1 mm path-length quartz cell at a protein concentration of 0.2–0.25 mg/mL. All CD spectra resulted from averaging four scans and the final spectrum was corrected by subtracting the corresponding base line spectrum under identical conditions. All measurements were performed in 20 mM Tris–HCl, pH 8.0.

2.6. Electrophysiological experiments

2.6.1. Channel expression in oocytes

The oocytes were prepared following the technique described earlier [11]. In brief, female *Xenopus laevis* frogs were anesthetized by 15 min exposure to 0.15% of 3aminobenzoic acid ethyl ester. The oocytes were surgically removed from the ovary, after which the frog was closed by suturing and place in water to allow recovery from anesthesia. Defolliculation was performed by incubating for 1 h in 1.5 mg/mL collagenase in Ca^{2+}-free oocyte ringer solution (mM): NaCl 92.5, KCl 2.5, $MgCl_2$ 1, Na_2HPO_4 and HEPES 5 with gentle agitation. Oocytes were stored in physiologic solution ND96 (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 0.03 $CaCl_2$, and 5 HEPES buffer adjusted to pH 7.4 with NaOH. Oocytes were injected with 12–18 ng/oocyte of cRNA of hERG1 channel by using a micro-dispenser and a micropipette. Injected oocytes were incubated at 18 °C for 24–48 h in ND96, before analysis. Channels were expressed to a level where 0.5–2.0 μA of current was recorded during a depolarizing step from a holding potential of −80 mV to potentials between −80 and +60 mV, and repolarizing at −100 mV. Currents were recorded using the two-electrode voltage clamp method. Control records were taken prior to the addition of toxin. On the addition of toxin the perfusion medium was stopped to allow homogeneous dispersion of the toxin. In most experiments toxin was removed from the bath to demonstrate recovery.

2.6.2. Cell culture

Chinese hamster ovary cells (CHO) line stably expressing hERG1 was culture in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and maintained at 37 °C in 5% CO_2 atmosphere.

2.6.3. Solutions and drugs

Standard extracellular solution contained (in mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES-NaOH 10, D-glucose 5, adjusted at pH 7.4. During ERG current registrations a high potassium extracellular solution was used: ($[K^+]_o$ = 40 mM) where NaCl was replaced by an equimolar amount of KCl. This experimental condition allowed obtaining the best relation of current versus noise. The standard pipette solution contained (in nM): K^+-aspartate 130, NaCl 10, $MgCl_2$ 2, EGTA-KOH 10, HEPES-KOH 10 at pH 7.3 and nominal $[Ca^{2+}]$ of ∼50 nM. Toxins were added to the extracellular solution from stocks in distilled water. When appropriate, 2 μM of specific ERG blocker WAY 123,398 [36] was applied at the end of each experiment and by subtraction, the WAY 123,398 sensitive current was obtained. The extracellular solutions were delivered through a 9-hole (0.6 mm) remote-controlled linear positioned with an average time of 1 s.

2.6.4. Patch-clamp recordings and data analysis

The ERG currents were elicited under conditions of relatively high $[K^+]_o$ (40 mM). The currents were recorded at room temperature (around 25 °C) using MultiClamp 700B amplifier and pClamp10 software (Molecular Device, USA). Pipette resistance was about 1–5 2.2 MΩ; cell capacitance and series resistance errors were carefully compensated for (85–90%) before each voltage clamp protocol run in order to reduce the voltage errors to less than 5% of the protocol pulse. Currents were elicited by stimulation at −120 mV for

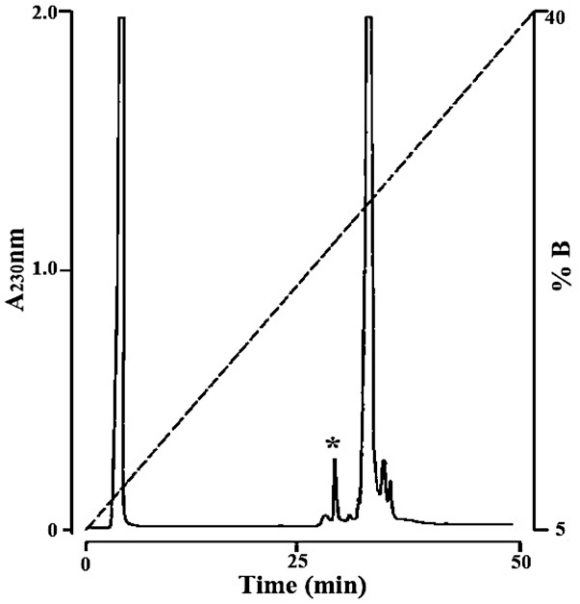

Fig. 1. Purification of toxins. The purified component of the last step of HPLC (see Ref. [10]) after storage was re-chromatographed again in order to separate the oxidized form of ErgTx1. The separation was obtained using a C_{18} analytical reverse-phase column in a Waters HPLC system, run from 5 to 40% B during 50 min. The major component eluting at 32 min is ErgTx1 and the minor component labeled with an asterisk is the oxidized isoform of ErgTx1. A considerable artefactural peak is seeing at the beginning of the gradient, due to the buffer change from water to 5% B. An aliquot of 350 μg of protein was applied to column and the recovery were 304 μg for the native ErgTx1 and 11.4 μg of the oxidized isoform.

500 ms, preceded by a 500 ms at 60 mV at frequency of 0.1 Hz; holding potential was clamped at −80 mV. The software pClamp 10.0 (Axon Instruments, CO, USA) and Origin 7 (Micrococal Inc., USA) were routinely used during off line analysis. Data are shown as mean ± SEM.

2.6.5. Dissociation constant determination

The dose–response curve for hERG1 channels was obtained by plotting peak currents measured after 100 s application of indicated toxin concentration and normalized to the current obtained in control conditions. The experimental data (mean of 6–9 cells ± SEM) were fitted with a Hill equation:

$$\frac{T_T}{I_{\max}} = \{1 + ([T]/K_D)^n\}^{-1}$$

where $I_{\max}$ is the maximal current; [*T*] is the toxin concentration; I_T is the current at a certain [*T*]; K_D is the dissociation constant, corresponding at the [*T*] which leads 50% of the current; *n* is the Hill coefficient.

3. Results

3.1. Purification of native ErgTx1 and its oxidized form, and IC_{50}s

Purification of native ErgTx1 and its oxidized isoform were obtained by a combination of chromatographic steps as described in Section 2. Fig. 1 shows the results of the second HPLC separation (see Fig. 1, Ref. [10]), after storage of the purified sample for a couple of months at −20 °C. During this time the oxidation of methionine takes place. The small component eluting earlier in Fig. 1, labeled with asterisk, corresponds to the oxidized form of ErgTx1. Amino acid sequence determination and mass spectrometry analysis of this minor component showed that it corresponds to the same toxin with methionine in position 35 oxidized [34]. This peptide contained only one methionine residue and it was

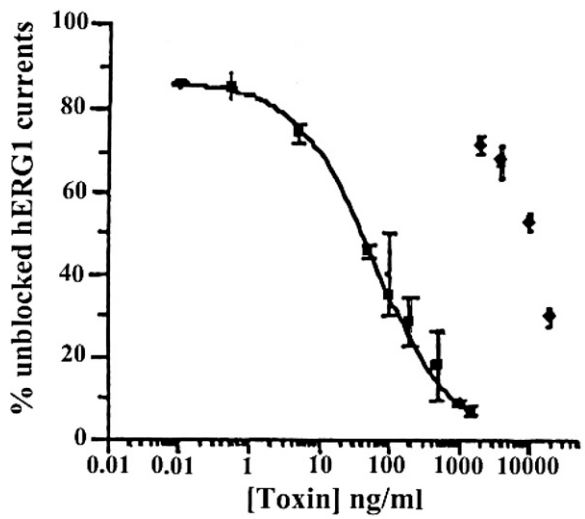

Fig. 2. Determination of IC_{50}s. The IC_{50} of native and oxidized forms of ErgTx1 were obtained by plotting the percentage of unblocked currents as a function of peptide concentrations. The curve to the left corresponds to the application of native ErgTx1, whereas the discontinuous points (right side) were obtained with the oxidized Met35 isoform. The estimated IC_{50}s, were 7 nM for ErgTx1 and 15.8 μM for oxidized isoform.

oxidized to methionine sulfoxide. The molecular weight of the oxidized isoform contained 16 mass units more than the unmodified ErgTx1. Its experimentally determined molecular weight was 4730 Da, as expected. The native ErgTx1 was assayed in the oocyte system described and was shown to be a potent inhibitor of the channel activity. ErgTx1 suppresses the hERG1 current amplitude and this effect is practically fully reversible ([10], see also below). A dose–response curve for the effect of ErgTx1 (Fig. 2, left trace) shows an effective dose 50% (IC_{50}) value for the native toxin of 7 nM. The IC_{50} estimated for the oxidized sample (Met35) was in the order of 15 μM (Fig. 2, discontinuous points in the right side), i.e., about three orders of magnitude less efficient. This result is quite relevant as indicated in the introductory section, because it means that either Met35 plays a direct role at the interface of channel–toxin interaction, or it is important for proper 3D-folding of the molecule.

3.2. Over expression and purification of fusion proteins

The nucleotide coding sequences of rErgTx1 and the mutant M35A were cloned into the pSyn1 expression vector and the resulting constructs were used for transformation of *E. coli* strain TG1. This vector contains the sequence of the signal peptide that codifies for the gene of the pectate enzyme liase (pelB) of *Erwinia carotovora*. The signal peptide pelB is translated fused to the extreme N-terminal of the fusion protein for its translocation to the periplasmic space of *E. coli*. As indicated in Section 2, the plasmids pSyn1-ThioEKErgTx1 and pSyn1-ThioEKM35A were constructed introducing additional amino acid residues in the gene for facilitating folding and increasing yields of the rErgTx1 and M35A mutant. Therefore, these plasmids contained the information for expressing the thioredoxin protein followed by five amino acids (DDDDK) that corresponds to the cleaving site for the enzyme enterokinase, and the 42 amino acid residues corresponding to the toxin, respectively for rErgTx1 or M35A.

E. coli strain TG1 cells expressed the fusion proteins ThioEKr-ErgTx1 and ThioEKM35A. Fig. 3 shows the main steps on the purification and processing of the hybrid protein for production of recombinant ErgTx1. Essentially the same results were obtained

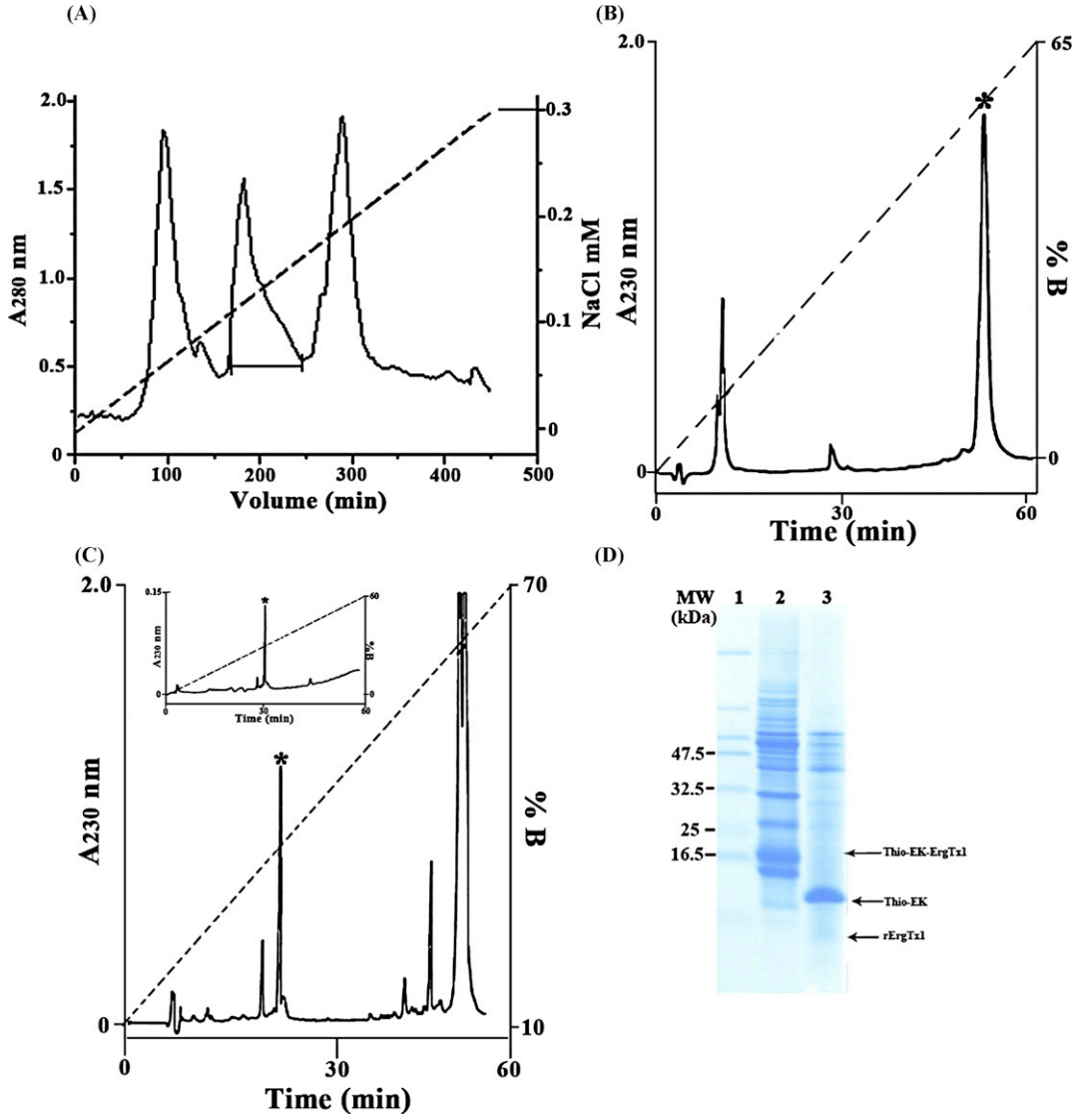

Fig. 3. Purification of rErgTx1. (A) The protein obtained from the periplasmic fraction (300 mg) was applied to the column and separated by DEAE-cellulose chromatography as described in Section 2. The middle component (indicated by the horizontal bar) contained the Thio-EK-ErgTx1 fusion protein (about 89 mg). (B) An aliquot containing 1 mg protein of Thio-EK-ErTx1 (from letter A above) was applied to a semi-preparative C18 reverse-phase-column and separated by HPLC, as indicated. The major peak labeled with asterisk contained the Thio-EK-ErgTx1, which corresponds to approximately 80% of the material applied. (C) To an analytical C18 reverse-phase column a sample of 1 mg protein was applied and separated after cleaving with enterokinase. The component eluting at about 30% buffer B corresponds to rErgTx1 (approximately 90 μg). The peak at 59% buffer B corresponds to Thio-EK. The component labeled with asterisk corresponds to the rErgTx1. It was further separated into a C18 reverse-phase analytical column as indicated by the inserted figure. (D) Polyacrylamide gel electrophoresis separation of the products after expression and cleavage of the fused protein, using the Tricine-SDS-PAGE system. Lane 1, protein standard with molecular weight markers (in kDa); lane 2, An aliquot containing 100 μg of periplasm fraction; lane 3, 20 μg of the product after cleavage by enzyme and HPLC separation. The labeled "Thio-EK-ErgTx1" is indicating the protein in lane 2 that corresponds approximately to 17,000 Da, whereas "Thio-EK" shows the cleaved protein of lane 3, which has a molecular weight of about 12,000 Da. The faint band at about 4700 Da in lane 3 corresponds to the rErgTx1.

with the hybrid protein that contained the mutated M35A (data not shown). After the osmotic shock, the protein recovered from the periplasmic fraction was dialyzed and purified by anion exchange chromatography as shown in Fig. 3A. The tube fractions indicated by the horizontal bar (second main peak) were pooled and further separated into a HPLC C18 reverse-phase column as shown in Fig. 3B. The major component indicated by the asterisk contained the hybrid protein, which was identified by Tricine-SDS-PAGE gel (Fig. 3D). The molecular weight determined by mass spectrometry of this component was 17,338 Da which corresponds to the hybrid protein ThioEKrErgTx1. For the case of ThioEKM35A mutant the molecular weight determined was 17,277 Da, as theoretical expected based on its the primary structure. At this stage the recovery amount of protein was usually in the order of 6–10 mg/L of culture. In order to obtain the pure recombinant ErgTx1 the hybrid protein was digested with enterokinase and the products separated by HPLC as indicated in Fig. 3C. The component labeled with asterisk corresponds to the rErgTx1. It was further separated into a C18 reverse-phase analytical column as indicated by the inserted figure in Fig. 3C. The molecular masses experimentally determined were 4730.4 Da for rEgTx1 and 4671 Da for M35A. These values confirm the theoretical molecular mass values expected for both peptides. The Edman degradation of the two recombinant toxins confirmed that the first 36 amino acid residues correspond to the expected sequences of the bona fide toxins rErgTx1 and M35A.

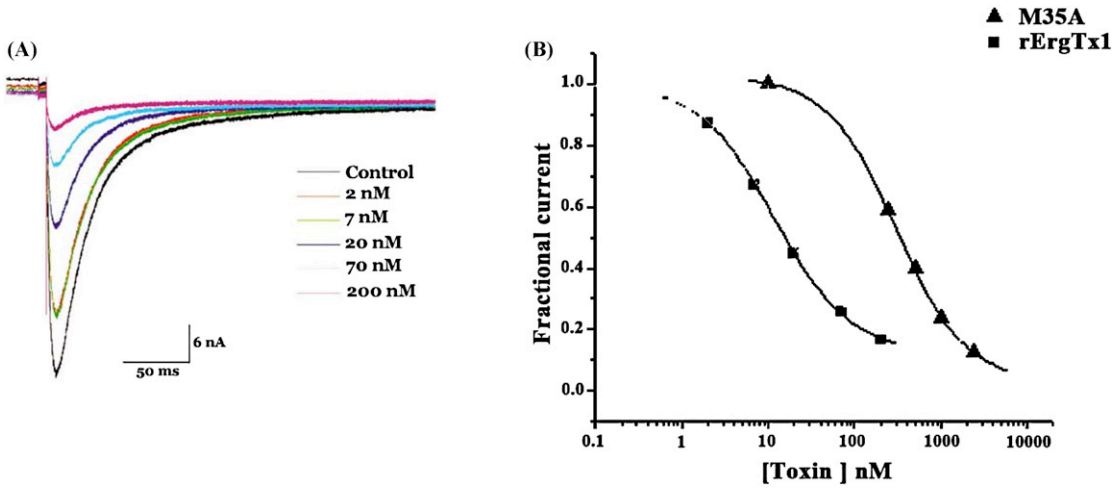

Fig. 4. Effect of rErgTx1 and mutant M35A on the hERG1 channel. (A) Traces of the electrophysiological recordings before and after application with 2, 7, 20, 70 and 200 nM of rErgTx1. (B) Doses–response curve of the fractional I_{hERG1} blocked as a function of rErgTx1 and mutant M35A concentrations. The data were fitted by using a Hill equation, giving IC_{50} of 11.7 ± 0.2 nM for rErgTx1 (quadrate; $n = 9$) and 303.4 ± 8.9 nM for M35A (triangle; $n = 6$).

3.3. Electrophysiological effects of rErgTx1 and mutant M35A on hERG1 channel expressed in CHO cells

The electrophysiological effects of recombinant ErgTx1 were determined using human ERG1 channels. Fig. 4A shows the electrophysiological recordings obtained by the application of various concentration of rErgTx1 and Fig. 4B shows the IC_{50} obtained for this peptide, which was practically the same result obtained with native ErgTx1 [10]. Similar results were obtained with the mutant ErgTx1M35A, but with a lower IC_{50} value. The doses–response relationship for hERG1 was calculated by applying different concentrations of rErgTx1 and M35A mutant in a range of 2–200 nM for 90 s. Peak of residual currents, normalized to the maximal current, were plotted versus concentration (Fig. 4B) and experimental data ($n = 6$–9) were fitted with a Hill equation, giving a Kd values of 11.7 ± 0.2 nM for rErgTx1 and 303.4 ± 8.9 nM for M35A.

3.4. Circular dichroism spectra

The far-UV CD spectrum of the native toxin, recombinant and mutant M35A reflects the mixed α-helix and β-sheet structure of the folded toxin. The spectra obtained at 20 °C are very similar, indicating that the secondary structures of the recombinant toxins are unaffected in comparison with the native toxin. The CD spectra shown in Fig. 5 exhibit a shape typical for peptides with a high content of α/β structure, characterized by three peaks: a negative peak at ~222 nm has been assigned to the peptide $n \rightarrow \pi^*$ transition. Another negative peak of similar intensity between 208 and 210 nm and a stronger positive peak between 190 and 195 nm are assigned to the $\pi \rightarrow \pi^*$ exition splitting polarized parallel and perpendicular to the helix axis, respectively. K2d, and online server for the estimation of the percentages of protein secondary structure from UV circular dichroism spectra (http://www.embl.de/~andrade/k2d/), gave the α-helical, β-sheet and random content of these three peptides being of 37%, 26% and 38%, respectively.

4. Discussion

The ability to produce disulfide rich toxins in a natively folded form using recombinant DNA methods provides a powerful tool for studying structure–function relationships as well as folding pathways in this class of peptides [20,22]. *Escherichia coli* is one of the best characterized models for heterologous expression of proteins. It is widely used because it can combines many different expression vectors. Most scorpion toxins contain essential disulfide bonds [29] that are required for structure formation in the extracellular oxidizing environment. Secretion of these peptides into the *E. coli* periplasm gives a better chance of proper folding due to the more oxidizing conditions in this extracellular compartment. However it also requires a signal peptide in order to translocate the proteins across the cytoplasmic membrane. The presence of this signal sequence does not always ensure efficient protein translocation. In this case, linking of the target molecule to a larger fusion partner can be a solution, and may lead to efficient secretion and an increment of the final proper folded protein [21]. In this work, we used thioredoxin as the hybrid protein, because it does not only tend to enhance the solubility of the peptides normally produced in an insoluble form [15], but also appears to catalyze the formation of disulfide bonds because when it is exported to the periplasm can partially replace the activity of the DsbA chaperone promoting the formation of disulfide bonds [13]. When we first started this project

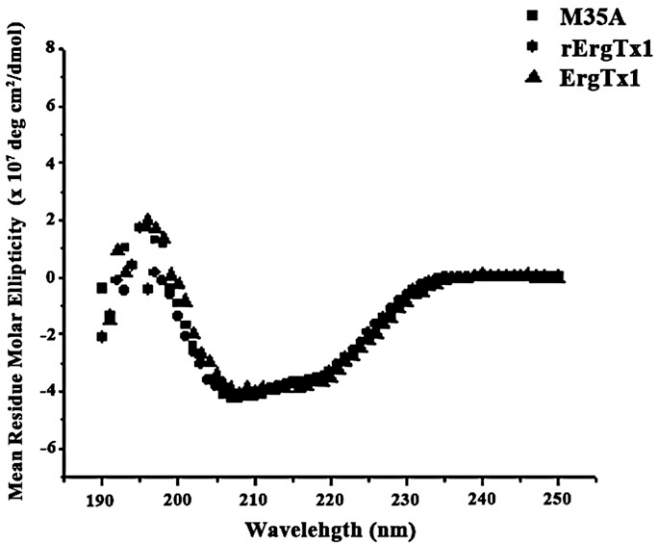

Fig. 5. Circular dichroism spectra of toxins. The graphic shows the far-UV (190–250 nm) circular dichroism spectra of ErgTx1 (triangles), rErgTx1 (stars) and M35A mutant (squares). Peptides were dissolved in 10 mM Tris–HCl buffer, pH 8.0. The traces for the three toxins are superimposable. The CD spectra from 190 to 250 show two minima at 208–210 and 222 nm and maximum at 190–195, typical for peptides α/β.

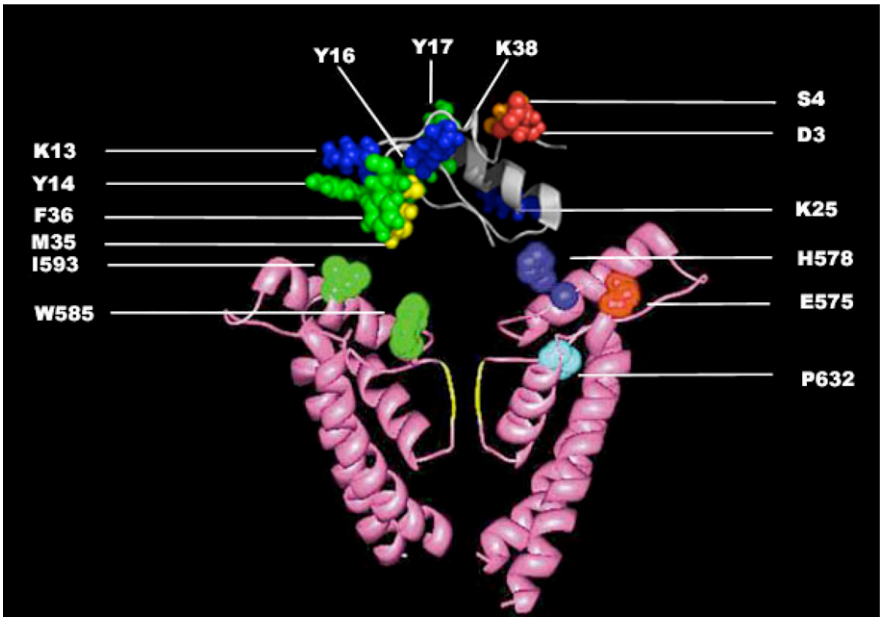

Fig. 6. Docking models of channel-toxin. The figure shows a proposed docking model between ErgTx1 and hERG1 channel. The residues Tyr14, Tyr16, Tyr17, Met35 and Phe36 can tightly bind to Trp585 and Ile593 on the amphipathic α-helix via the hydrophobic interaction. Lys38 and Lys13 can form the cation–π interaction with Trp585. Electrostatic repulsion may occur between Glu575, His578 on the channel and Asp3, Lys25 on ErgTx1, respectively. Pro632 may form some hydrogen bonds with the hydrophilic patch.

several attempts were performed trying to use direct expression of the cloned genes, without success (data not shown). The problem was finally solved when the constructions were performed in the format of hybrid expression with thioredoxin. Thanks to this system, the rErgTx1 and the mutant M35A were cloned and expressed as fusion proteins (ThioredoxinEKToxin). Starting with 1 L of cell culture, the amount of hybrid protein produced was in the order of 6–10 mg of protein. After purification, cleavage with enterokinase and the final HPLC purification step (Fig. 3C), the amount of rErgTx1 and M35A mutant obtained were in between 1 and 2 mg/L. Both analogs when subjected to Edman degradation gave the expected amino acid sequences and their molecular weights determined by mass spectrometry, showed the presence of only one component with the expected masses 4730.4 Da for rEgTx1 and 4671 Da for M35A. This implies that the four disulfide bridges were formed. The circular dichroism of ErgTx1, rErgTx1 and M35A showed that the three peptides adopt the same typical α/β motif, common to most K^+ channel scorpion toxins (Fig. 5, see also [29,31]). These results and the fact that the IC_{50}s of the three toxins (native, recombinant and mutant ErgTx1) are considerably different (Figs. 3 and 4) are taken as good evidence that Met35 plays an important role on the interacting surfaces of hERG1-channel and ErgTx1. Methionine residues are remarkable for their susceptibility to oxidation in proteins. It is expected that its oxidation have profound consequences for protein function and might constitute a mechanism for protein regulation [12]. The side chain of methionine sulfoxide with the extra oxygen atom is stiffer and more polar than that of the methionine side chain. However, the oxidation of methionine residues in proteins does not necessarily cause either structural changes or loss of biological activity, indicating that oxidation of the methionine side chain is a physiologically relevant phenomenon [42]. There are numerous examples of sulfoxide formation of specific methionine residues in proteins, some leading to complete inactivation and others having little or no effect [6]. Oxidation of methionine residues has been reported for proteins such as parathyroid hormone [24], antithrombin [39], leptin [18], vascular endothelial growth factor [4], and human granulocyte colony stimulating factor GCSF [19]. In this paper, the analyses *in silico* of the 3D-structure of the toxins, solved by NMR spectroscopy, both for the native [5] and synthetic [37] ErgTx1, show that the methionine 35 is located on the hydrophobic face of the triple stranded antiparallel β-sheet, stabilized by five hydrogen bonds, two of which involved the main chain of the methionine 35 and the lysine denoted by HN35–O38 and HN38–O35, and one with the principal chain of the amino acid F37 (HN37–O35). It shows also the establishment of hydrophobic interactions with the residues F36 and Y14. Therefore the substitution for other residue hydrophobic or the oxidative modification of this methionine does not disrupt aspects of the secondary structure and other noncovalent interactions that stabilize the tridimensional structure of the toxin. These observations were consistent with the circular dichroism spectra, where the substitution of methionine 35 by alanine does not induce a conformational change of the secondary structure compared with the native toxin. However, this substitution causes a 30 folds reduction in the affinity of the toxin for the channel, suggesting that the loss of affinity for the channel is due to this residue, which is important for the interaction with the channel and not to a conformational change of the structure of the toxin. The prediction of toxin-channel complex structures by computational approaches, such as docking and molecular dynamic simulations, has become a good alternative way to understand the interacting mechanisms between K^+ channels and peptide inhibitors. Cysteine scanning mutagenesis studies of the S5-P and P-S6 linker regions of the channel demonstrated that the residues that contribute most to the tight interaction of the complex hERG1-ErgTx1 are, in the channel: W585, G590, I593 and P632 (the changes of free energy of association, $\Delta\Delta G_{mut\text{-}wt}$, are close to 2 kcal/mol for each of these mutated residues) and in ErgTx1 we demonstrated that the residue Met35 is contributing with about 2.0 kcal/mol to $\Delta\Delta G_{mut\text{-}wt}$. Another important point is that the three positions in the S5-P linker (Trp-585, Gly-590 and Ile-593) all fall within the putative amphipathic α-helix [27,28]. Additionally, it has been demonstrated that the binding of K^+ channel specific scorpion toxins to their receptors on the outer vestibule of the channel pore involves a combination of hydrophobic, hydrogen bonding and electrostatic interactions. However, in the binding of ErgTx1 to hERG1 the electrostatic interactions should not be the main force for toxin binding. The authors proposed that the hydrophobic interactions should be the driving force, due to the

presence of a large hydrophobic patch in the toxin defined by Tyr14, Tyr16, Met35, Phe36, and Phe37, which as proposed, would be making contacts with residues Trp585 and lle593 of the hERG1 channel [5]. In this way, the hydrophobic patch composed of 4 aromatic residues (Tyr14, Tyr16, Tyr17 and Phe36) in ErgTx1 can tightly bind to Trp585 and Ile593 on the amphipathic α-helix. In the cases of Met35 residue it is very close to this patch demonstrating once again that it is an important residue in the interaction of ErgTx1 with hERG1 channel. In Fig. 6 we show a diagram of the 3D-model of the possible interacting residues of ErgTx1 with a simplify cartoon of two of the four subunits of the K^+-channel, modified after the original publication of [5]. The residue Met35 (in yellow) is directly facing the segment of the extra loop of the S5-P region of the channel, as earlier demonstrated by work performed in collaboration with our group [28]. Additional mutants are in preparation (including the Lys13, Tyr14, Tyr17, Lys25, Phe36, Phe37, Lys 38 in order) to add more information on the interacting surface existing between ErgTx1 and the ERG-channel.

Acknowledgements

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Interacting sites of scorpion toxin ErgTx1 with hERG1 K^+ channels

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ABSTRACT

Peptides purified from scorpion venoms were shown to interact with specific amino acid residues present in the outer vestibule of various sub-types of potassium channels, occluding the pore and causing a decrement of K^+ permeability through the membrane of excitable and non excitable cells. This communication describes the identification of several interacting sites of toxin ErgTx1, a toxin purified from the venom of the scorpion *Centruroides noxius*, with the human ERG1 K^+ channels, by means of site-directed mutagenesis of specific residues of the toxin. Recombinant mutants of the gene coding for ErgTx1 were expressed heterologously in *Escherichia coli*, properly folded and their affinities and interactions with hERG1 channels were determined by patch–clamp techniques. Residues in position Y14, Y17 and F37 of the solvent exposed hydrophobic surface, and charged residues at the position K13 and K38 of ErgTx1 were shown to cause a decrement of the affinity from 20 folds to 3 orders of magnitude, thus suggesting that they are certainly participating on the binding surface of this toxin towards the hERG1 channels. Double mutants at positions K13 and F37, Y14 and F37, Y17 and F37 and K13 and K38 were also prepared and assayed, but the results obtained are not much different from the single point mutants of ErgTx1. The results of the present work indicate the most probable surface area of ErgTx1 that makes contact with the hERG channels.

1. Introduction

Peptide toxins from venomous animals have been widely used as structural probes for studying membrane proteins, particularly ion channels. In the case of scorpion toxins specific for potassium channels (here abbreviated KTxs) three distinct subfamilies: α, β, and γ were identified based on sequence similarities and channel specificities (Tytgat et al., 1999; Rodríguez de la Vega et al., 2003). Toxins that recognize K^+ channels of the family *ether-á-go-go* related gene (ERG) were classified as γ-KTxs. Thus far, 27 different peptides of this kind were identified (Corona et al., 2002; Korolkova et al., 2001; Coronas et al., 2005; Restano-Cassulini et al., 2008). Among the sub-family α-KTxs, there is also one peptide (α-KTx 15) described to affect the ERG channels (Abdel-Mottaleb et al., 2008). Two of the γ-KTxs have had their three-dimensional (3D) structure determined: a chemically synthesized ErgTx1 (Torres et al., 2003) as well as its native counterpart (Frenál et al., 2004) and a recombinant format of toxin BeKm-1 (Korolkova et al., 2002). These γ-KTxs have different disulfide bridge patterns and do not share extended primary sequence similarities, but both peptides interact with the human ERG K^+ channels (hERG), (see Gurrola et al., 1999; Zhang et al., 2003; Pardo-López et al., 2002a, b). The hERG channels play an important role in different tissues. For example, in heart they constitute an important component of the delayed rectifier channels, contributing to the repolarization phases of cardiac action potential (Roden et al., 2002; Tseng, 2001). This channel has an unusual loop S5-P linker, much longer than others Kv channels (43 versus 12–23 amino acid residues) and form

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an amphipathic α-helix that together with the P-S6 linker are binding sites for scorpion toxin interaction (Pardo-López et al., 2002a, b; Liu et al., 2002). Interestingly, the two peptides (BeKm1 and ErgTx1) interact with the receptor sites through two different regions of hERG channels. Studies of alanine scanning mutagenesis of BeKm1 has shown that the residues Y11 located on the N-terminal part of the helix, K18 and R20 located at the end of the α-helix, and K23 located after the helix, are important for the activity on the hERG channel (Korolkova et al., 2002). Furthermore, analysis of double mutants cyclic demonstrated that various pairs of BeKm1/hERG residues are probably interacting closely, among which are: Y11 of toxin with I583 and Y597 of the channel, and similarly F14 with R582, Y597 and D609; K18 with S631; and R20 with and S631 (Korolkova et al., 2003).

Concerning ErgTx1, experiments were performed after cysteine–scanning mutagenesis of the hERG channels, by replacing all residues in the S5-P linker (positions 571–613, 43 residues) and the P-S6 linker (positions 631–638, 8 residues) of hERG, one at a time, and studying the resulting effects of the toxin on current suppression. Results of these experiments suggested that three positions in the S5-P linker (W585, G590 and I593) and one position in the P-S6 linker (P632) when are mutated to cysteine disrupt the amphipathic helix structure; indicate that the S5-P linker α-helix is critical for the bind of the ErgTx1. These results provided supporting evidence for the possible 3D-arrangement of the contacting structure of ErgTx1 with residues of the outer face of vestibule of this channel. In addition it was demonstrated that the interacting surface of ErgTx1 with hERG channels is not influenced by the extracellular potassium concentration ($[K^+]$) and that the electrostatic interaction is not the main force defining the contact between the toxin and the channel. Therefore, it was proposed that hydrophobic interactions should be playing a more important role in the interaction ErgTx1-hERG (Pardo-López et al., 2002a, b). Evolutionary and structural analyses demonstrated the presence of two important functional residues in the ErgTx1 that are mainly located in two patches: one hydrophobic centered around an aromatic cluster formed by Y14, Y16, Y17, F36 and F37 and the other hydrophilic located at the two opposite heads of the toxin molecule. Furthermore, in the middle of this hydrophobic patch lies K13. It has been suggested that ErgTx1 binds to the hydrophobic surface of an amphipathic helix in the outer vestibule of the hERG K^+ channel (Torres et al., 2003; Xu et al., 2003; Frenál et al., 2004). An oxidized form of ErgTx1 at M35 was shown to decrease by 3 orders of magnitude its binding properties towards the channel, being the first residue clearly shown to form part of the interacting surface (Jiménez-Vargas et al., 2011). Our ultimate goal is to map the ErgTx1 binding site on hERG and to identify amino acid pairs that interact with each other across the toxin-channel interface. To elucidate these interacting sites we performed site-directed mutagenesis of the residues that formed the hydrophobic patch, changing for alanine the K13, Y14, Y17, F37 and K38. Similarly, four distinct double mutants of ErgTx1 were prepared and their binding properties to hERG channels were analyzed. Since the 3D-structure of ErgTx1 is known, we can surmise based on our experimental data, which is the most probable surface of the toxin that makes contact with the hERG1 channels. In this communication the results found are presented and discussed.

2. Materials and methods

2.1. Bacterial strain

All molecular biological techniques and large-scale preparation of plasmid DNA were performed with *Escherichia coli* strain DH5α (F^- *gyr*A96 (NAI^r) *recA relA1 endA1 thi-1 hsdR17* ($r_K^- m_K^+$) *glnV44 deoR* Δ(*lacZYA-argF*)*U169* [φ80dΔ(*lacZ*)M15]). Expression constructs were transformed into TG1 strain (*supE thi1*Δ(*lac/proAB*) Δ(*mcrB/hsdSM*)*5*(rK-mK-) [F'*traD36proAB lacIqZ*Δ*M15*]). Restriction enzymes, Vent polymerase, Enterokinase and T4 DNA ligase were purchased from New England Biolabs (Pickering, Ontario, Canada) and Sigma Aldrich (St. Louis, Missouri, USA). Plasmid pSyn1 was used for cloning and expression of the fusion protein of toxins with Thioredoxin.

2.2. Production of recombinant toxins and site-directed mutagenesis

For expression in *E. coli*, purification and folding of the recombinant ErgTx1 (rErgTx1) and their mutants, the protocols and experimental conditions described previously by our group (Jiménez-Vargas et al., 2011) were adopted.

For the production of each mutant, the corresponding genes were engineered by means of specific primers designed to overlap the areas in which point-mutated amino acids were selected for modification. The general template used for production was pSyn1Thio–EK–ErgTx1, where pSyn1 is the vector, Thio–Ek is the sequence that codes for the protein thioredoxin, used as carrier, plus a segment of amino acid sequence specific for cleavage with enterokinase (EK), which would allow producing free recombinant peptides. The recombinant genes of the mutants K13A, Y14A and Y17A were synthesized by a two-steps protocol, using the technique described by Ke and Madison (1997). The nucleotide sequence of the forward primer (BamH1-EntERG) was: 5′-GGC TCT GGA TCC GGT GAT GAC GAT GAC AAG GAC AGA GAT AGC TGC GTC-3′, which contains the *BamH*I restriction enzyme site and the corresponding sequence that codes for five amino acids of enterokinase cleavage site, plus the amino-terminal residues in positions 1–6 of ErgTx1. The reverse primer (Not-Erg) was: 5′-TTT TTC AAG TGT AAA TGT GCG TAA TCA CGT GCG GCC GCA AGT CGA GAA TGA CTC-3′. The nucleotide sequences of the three mutagenic primers were: K13A: 5′-CA CGA TGC GCA GCA TAT GGA TAC-3′; Y14A: 5′-GA TGC GCA AAA GCT GGA TAC TAC-3′; and Y17A: 5′-CA AAA TAT GGA TAC GCC CAA GAG TG-3′. For the synthesis of the mutants F37A and K38A, primers corresponding to the amino acid sequence from positions 33–42 were synthesized. The forward primer BamHI-EntERG and the mutagenic primer F37A (5′-CG ACT TGC GGC CGC ACG TGA TTA CGC ACA TTT ACA CTT GGC AAA CAT ACA GGT TCC-3′) and the primer for K38A (5′-CG ACT TGC GGC CGC ACG TGA TTA

CGC ACA TTT ACA CGC GAA AAA CAT ACA GGT TCC-3′) were used. A *Not*I restriction site and stop codons are included. Mutagenesis of F37 and K38 were achieved using two sequential polymerase chain reactions as earlier described (Jiménez-Vargas et al., 2011). The same strategy and primers were used for the double mutants. The PCR products and the plasmid pSyn1Thio–EK–ErgTx1 were digested with the corresponding restriction enzymes at 37 °C for *BamH*I and *Not*I, respectively and purified by column chromatography (QIAquick, Roche, city, state, USA) before ligation.

2.3. Expression and purification of mutants of rErgTx1

The mutants of the ErgTx1 were expressed as protein fused to thioredoxin (*Thioredoxin–EK–toxin)* in *E. coli* strain TG1 as previously described (Jiménez-Vargas et al., 2011). Briefly, overnight culture from a single colony was induced to produce recombinant toxin by the use of isopropyl-β-D-thiogalactopyranoside (IPTG). After several steps (see description in Jiménez-Vargas et al., 2011), the supernatant of the various cell cultures, referred to as the periplasm fraction, were recovered. This fraction containing the fusion protein was dialyzed and the product was purified further by RP–HPLC with a C18 semi-preparative column. The fusion protein was eluted with a linear gradient, from buffer A (0.12% (v/v) TFA in water) to buffer B (0.1% TFA in acetonitrile), whereas the rErgTx1 and the mutants were released from the fusion protein using the enzyme enterokinase, and further purified from the cleaved mixtures by RP–HPLC on a C18 analytical column. Molecular weights of purified toxins were confirmed by ESI mass spectrometry (ESI-MS) and direct sequencing through automatic Edman degradation. The tertiary structure of the peptides was confirmed by Far-UV circular dichroism (CD) measurements, as described in the next section. The identity of peptides was confirmed.

2.4. Circular dichroism (CD) measurements

CD spectra were obtained on a Jasco J-720 spectropolarimeter (Jasco, Japan). Wavelength scans from 250 to 190 nm in 20 mM Tris–HCl, pH 8.0 were taken at 25 °C in a 1 mm path-length quartz cuvette. Molar ellipticity per residue on the buffer-subtracted CD spectrum using the following relationship: $[\theta]_{MRW,\lambda} = (\theta_{obs}/10)$ (M/c l), where θ_{obs} is the observed ellipticity at a give wavelength, *M* is the mean residue mass, *l* is the cuvette path-length in centimeters, and *c* the protein concentration expressed as mg/mL. The units of [θ] are deg.cm^2. dmol^{-1} (Pain, 2004). All Data were collected at 0.1 mm with a scan rate of 20 nm/min and a time constant of 0.5 s. The protein concentration of rErgTx1, native ErgTx1, K13A, Y14A, Y17A, F37A and K38A mutants were 0.2–0.25 mg/mL.

2.5. Electrophysiological experiments

2.5.1. Cell culture

Chinese hamster ovary cells (CHO) line stably expressing hERG1 was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and maintained at 37 °C in 5% CO_2 and 95% humidity atmosphere.

2.5.2. Solutions and drugs

Standard extracellular solution contained (in mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES–NaOH 10 buffer, D-glucose 5, adjusted at pH 7.29. During ERG current registrations a high potassium extracellular solution was used: ($[K^+]_o$ = 40 mM) where NaCl was replaced by an equimolar amount of KCl. This experimental condition allowed obtaining the best relation of current versus noise. The standard pipette solution contained (in mM): K^+–aspartate 130, NaCl 10, $MgCl_2$ 2, EGTA-KOH 10, HEPES-KOH 10 buffer, pH 7.3 and nominal [Ca^{2+}] of ~50 nM. Toxins from concentrated stocks in water were added to the extracellular solution, after the proper dilution, according to the required experimental protocol. When appropriate, 2 μM of specific ERG blocker WAY 123,398 (Overholt, 2000) was applied at the end of each experiment and by subtraction, the WAY 123,398 sensitive current was obtained. The extracellular solutions were delivered through a 9-hole (0.6 mm) remote-controlled linear positioned with an average time of 1 s.

2.5.3. Patch–Clamp recordings and data analysis

The currents were recorded at room temperature using the MultiClamp 700B Amplifier (Axon Instruments, USA) as previously described (Jiménez-Vargas et al., 2011); pipette resistance was about 1.5–2.2 MΩ. The cell capacitance and series resistance were carefully (85–90%) compensated before each run of the voltage clamp protocol in order to reduce voltage errors to less that 5% the of protocol pulse. The pClamp10 (Axon Instruments, U.S.A) and Origin 7 (Microcal Inc. U.S.A) softwares were used during data acquisition and analysis. All data recording were obtained using stimulation at –120 mV for 500 ms, preceded by a 500 ms at 60 mV at frequency of 0.1 Hz; holding potential was clamped at −80 mV. Data are shown as mean ± SEM.

2.5.4. Dissociation constant determination

The dose–response curve for hERG1 channels was obtained by plotting peak currents measured after 100 s application of indicated toxin concentration and normalized to the current obtained in control conditions. The experimental data (mean of 6–9 cells ±SEM) were fitted with a Hill equation:

$$I_T/I_{max} = A2 + (A1\text{-}A2)/(1 + ([T]/K_D)^{\rho})$$

Where I_{max} is the maximal current; [*T*] is the toxin concentration; I_T is the current at a certain [*T*]; K_D is the dissociation constant, corresponding at the [*T*] which leads 50% of the current; ρ is the Hill coefficient; *A1* and *A2* are top and bottom asymptotes, respectively. It is known that ErgTx1 does not block completely the ERG1 current, even at high concentrations. For this reason the fitting of data were performed with Hill coefficient set at 1; the top asymptote to 0 and leaving free the bottom asymptote. For some mutants it was necessary either to leave the Hill coefficient free or fixed the bottom asymptotes to 0.

3. Results and discussion

3.1. Expression and purification of the ErgTx1 mutants

Earlier work performed with ErgTx1, in which the hERG1 channels were mutated by cysteine scanning of 51 amino acid residues situated between the S5 and S6 segment of the channel showed the most important residues of the channel for binding to ErgTx1 (Pardo-López et al., 2002a, b). The hERG1 channel have three positions in S5-P linker (W585C, G590C and I593C) and one position in P-S6 linker (P632C) that when mutated to cysteine induce an important changes in ErgTx1 binding affinity ($\Delta\Delta G > 2$ kcal/mol). In the present work, our aim was to identify the amino acids of the ErgTx1 that are important for interaction with hERG1 channel. To achieve this objective five amino acids that form part of hydrophobic patch in ErgTx1 where replaced by alanine. Similarly double mutants of these residues were also mutated to alanine. The amino acid alanine was chosen as a replacement residue because it eliminates the side chain beyond the β-carbon and yet, does not alter the main chain conformation, neither does it imposes important electrostatic or steric hindrance effects. Furthermore, alanine is the most abundant amino acid in proteins and it may be found in both buried and exposed positions of proteins, and in all varieties of secondary structures (Ashkenazi et al., 1990).

In a previous communication (Jiménez-Vargas et al., 2011) it was shown that the recombinant ErgTx1 was properly folded and displayed the same activity as the native ErgTx1, as purified from fresh scorpion venom. Using the same strategy various mutants were prepared and the results of their interactions with the hERG1 channel are here reported for the first time. The mutants prepared were: K13A, Y14A, Y17A, F37A, K38A and four pairs of double mutants: K13A–F37A, Y14A–F37A, Y17A–F37A, and K13A–K38A. The rationale used for selection of these amino acids was based on earlier suggestions by Torres et al. (2003) and Frenál et al., 2004. However, no direct experiments confirming these propositions were conducted until now. It is also important to note that not all residues of ErgTx1 were mutated to alanine. The previous results obtained by our group, and others, showing that hydrophobic residues and positively charged residues of ErgTx1 and BeKm1 were the most probable residues implicated into the interaction toxin-channel, were selected for mutagenesis studies, simplifying the otherwise painstaking work of producing all the possible mutants of the toxin. Here we describe the experimental results obtained with the mutants. These variants were expressed in the strand TG1 of *E. coli* as hybrid proteins together with thioredoxin. The gene coding for the carrier was followed by the five residues (DDDDK) that corresponds to the cleavage site of the enzyme enterokinase and then by the 42 amino acids residues of the toxins. The levels of expression of each mutant were analyzed by Tricine–SDS–PAGE gel and Western blot (Fig. 1). In this figure the samples used were from the periplasmic extracts of the cultures, but showed a protein band corresponding to the expected molecular weight of the fusion protein (see arrow), which were confirmed by Western blot using rabbit polyclonal antibodies rose against ErgTx1. The amount of fusion protein obtained for each variant was around 2–6 mg per liter of culture. The periplasmic extracts were separated by anion exchange chromatography followed by HPLC C18 semi-preparative reverse-phase column (Fig. 2). The elution patterns of the various proteins are quite reproducible and essentially the same as those obtained for the rErgTx1 earlier described (Jiménez-Vargas et al., 2011). Fig. 2 shows an example obtained when purifying the mutant K13A and, as mentioned, all the mutants elute in quite the same manner. Tricine–SDS–PAGE gel and mass spectrometry determination showed a molecular weight of 17,280 Da for the hybrids proteins ThioEKK13A and ThioEKK38A, 17,245 Da for ThioEKY14A and ThioEKY17A and 17,261 Da for ThioEKF37A (data not shown). The molecular masses obtained for the double mutants were: 17,204 Da for ThioEKK13A–F37A, 17,223.6 Da for ThioEKK13A–K38A and 17,169.6 Da for ThioEKY14A–F37A and ThioEKY17A–F37A. The fusion proteins were digested with enterokinase and the products separated by HPLC (see example of Fig. 2). The molecular masses experimentally determined were 4673 Da for K13A and K38A, 4638 Da Y14A and Y17A and 4654 Da for F37A. For the double mutants the molecular masses were: 4597 Da for K13A–F37A, 4616 for K13A–K38A and 4562 Da

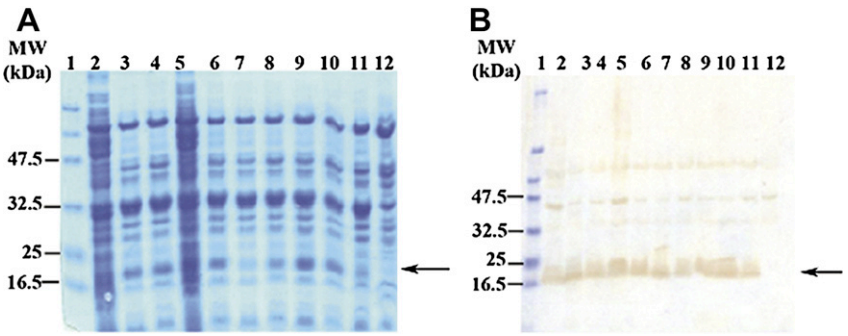

Fig. 1. *Expression of the ErgTx1 variants.* (A). Tricine–SDS–PAGE results of periplasmic extract (100 μg each) for the various mutants. Lane 1 contains protein standard with molecular weight markers (in kDa), lane 2 is ThioEKK13A; lane 3, ThioEKY14A; lane 4, ThioEKY17A; lane 5, ThioEKF37A; lane 6, ThioEKK38A; lane 7, ThioEKK13A-F37A; lane 8, ThioEKY14-F37A; lane 9, ThioEKY17-F37A; lane 10, ThioEKK13A-K38A; lane 11 ThioEKErgTx1 and lane 12, periplasmic extract of TG1 strain without plasmid. The arrow indicates position of the hybrid (fusion protein) corresponding to a molecular weight around 17 kDa (B) Western blot analysis. Lane 1–12; the same as in Fig. 1(A).

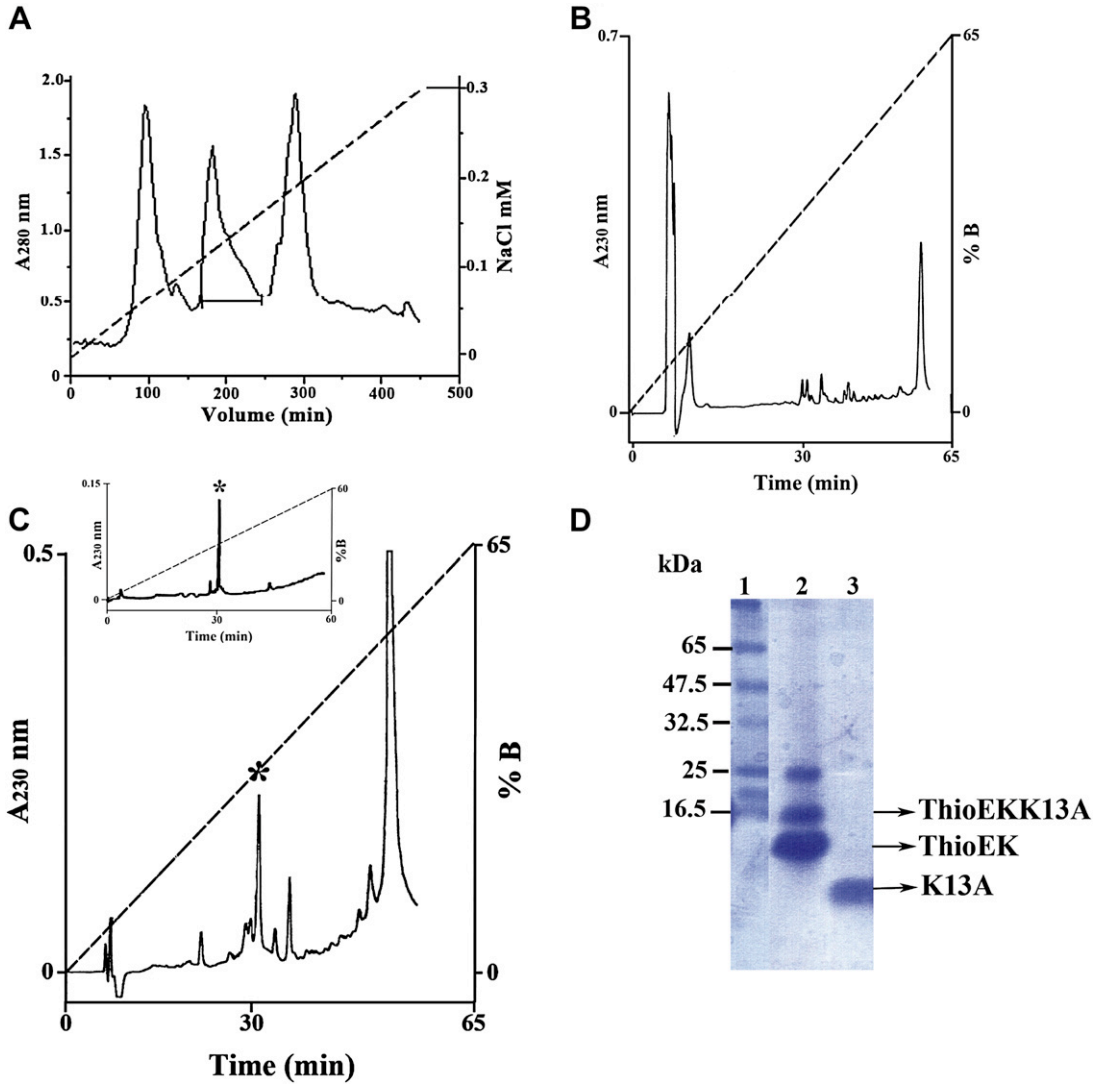

Fig. 2. *Purification of the toxin mutated K13A.* (A) The protein obtained from the periplasmic fraction was applied to the column and separated by DEAE-cellulose chromatography as described in material and methods. The fraction indicated by the horizontal bar contained the ThioEKK13A fusion protein. (B) The fraction indicated was further separated into a RP–HPLC C18 semi-preparative column, as indicated. The major peak eluting around 52 min corresponds to ThioEKK13A. (C) Separation of this fraction onto an analytical C18 reverse-phase column, after cleavage with enterokinase. The component eluting at about 32% buffer B corresponds to K13A (labeled with asterisk). It was further separated into a C18 reverse-phase analytical column as indicated by the insert. (D) Polyacrilamide gel electrophoresis separation of the products after cleavage of the fused protein and the purification of the K13A, using the Tricine–SDS–PAGE system. Lane 1, protein standard with molecular weigth markers (in kDa); lane 2, an aliquot containing 20 μg of the product after enzymatic cleavage; lane 3, K13A purified by RP-HPLC. The labeled "ThioEKK13A" is indicating the protein in lane 2 that corresponds approximately to 17,000 Da, whereas "ThioEK" shows the cleaved protein of lane 2, which has molecular weight of about 12,000 Da. The faint band at about 4600 Da in lane 3 corresponds to free peptide K13A.

for Y14A–F37A and Y17A–F37A. These values confirm the theoretical molecular mass value expected for all peptides.

3.2. Circular dichroism spectra

The far-UV CD spectrum of the mutants in comparison with the native toxin and recombinant reflects the mixed α-helix and β-sheet structure of the folded toxin. Fig. 3A and B shows the CD spectra obtained from the single point mutation and for the double mutants, respectively. The shape of the spectra are of typical peptides with a high content of α/β structure, characterized by three CD bands: a positive and negative contribution at 190–195 nm and 208–210 nm, respectively, caused by $\pi \rightarrow \pi^*$ transitions, and a negative contribution at 222 nm caused by $n \rightarrow \pi^*$ transitions are assigned to the excitation splitting polarized parallel and perpendicular to the helix axis, respectively. These results suggest that all the mutants studied here do not exhibit substantial changes in secondary structure in comparison with native toxin. This fact allows assuming that different toxin-channel interactions should be due primarily to different activities of the mutated amino acids and not to a change in secondary structure of the toxin.

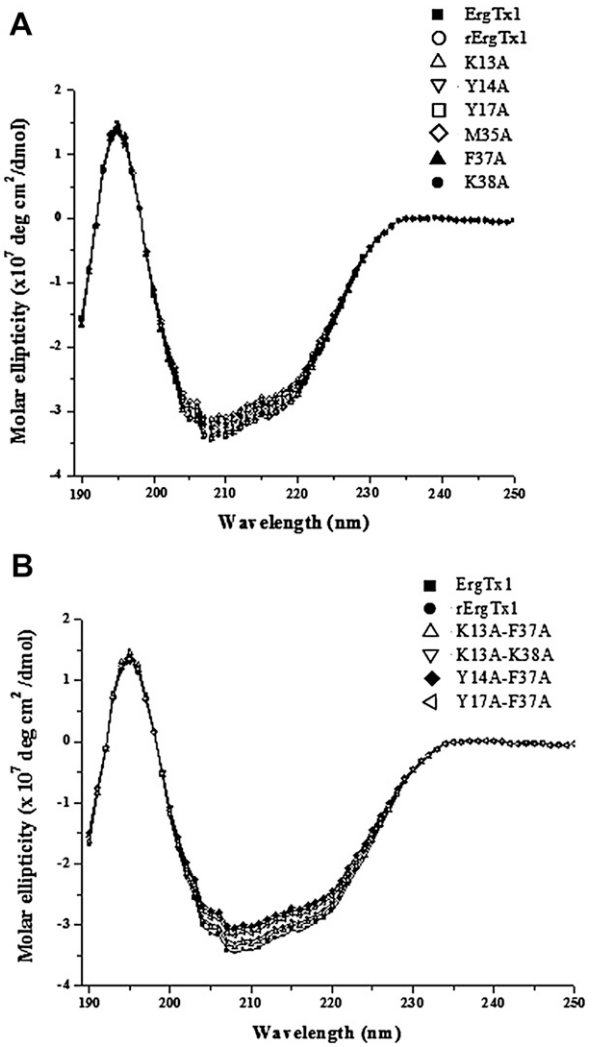

Fig. 3. *Circular dichroism spectra of recombinant toxins.* The CD spectra were measured from 190 to 250 nm and data are expressed in $[\theta]_{MRM\lambda}$. (A) The traces for the rErgTx1 and of the points mutants: K13A, Y14A, Y17A, M35A, F37A and K38A showed that are superimposable with a two minima at 208–210 and 222 nm and maximum at 190–195, typical of peptides folded as α/β secondary structure arrangements. (B) The graphic shows the same spectra for the double mutants: K13A–F37A, Y14A–F37A, Y17A–F37A and K13A–K38A.

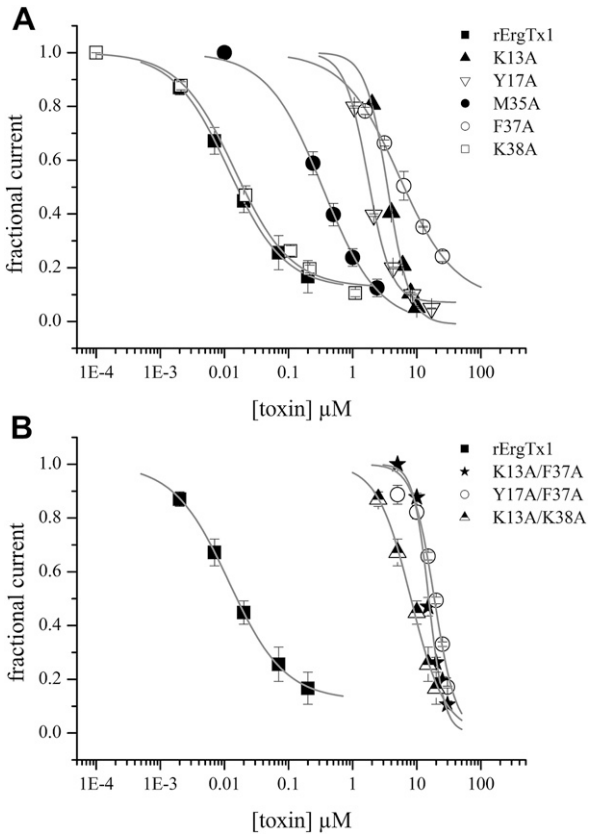

Fig. 4. *Dose–response relationship and dissociation constant determination of the variants of ErgTx1 on the hERG1 channel.* Dose-response curve were obtained using different concentrations in the range from 1 nM to 25 μM. The data were fitted using the Hill equation and the K_D values found are reported in Table 1; each data points are mean ± SEM from 4 to 6 cells at every toxin concentration. (A) K38A is a mutant that does not show significant K_D alteration, contrary to the variants K13A, Y14A, Y17A and F37A that show an important difference in K_D, when compared to the native peptide. (B) The doubles mutants showed a behavior similar to the points mutants.

3.3. Characterization of ErgTx1 mutants

The electrophysiological effects of the mutants of ErgTx1 were determined using human ERG1 channel. Currents were elicited by pulse stimulation at frequency 0.1 Hz (see Material and Methods section). The dose–response relationship for ERG1 was calculated by applying different concentrations of K13A, Y14A, Y17A, F37A and K38A for 100 s (Fig. 4A). For each mutant we determined the K_D, obtaining a concentration dependent curve of the toxin current inhibition. Similarly, Fig. 4B shows the inhibition curve obtained with the double mutants. Table 1 gives both K_D and perturbation energy (ΔΔG) values for each mutant. As it can be observed in Fig. 4A and Table 1, the mutant K38A exhibits the same wild-type activity, with K_D of 14.5 nM and

Table 1
Perturbations in the interaction between ErgTx1 and hERG1 channels, resulting from mutations in the toxin.

	Hill	K_D	K_D^{mut}/K_D^{wt}	|ΔΔG| Kcal/mol	n
Wild-type	1	11.7 ± 0.3 nM	–	–	4
K13A	2.5 ± 0.1	3.5 ± 0.08 μM	292	3.4	4
Y14A	–	>22	1833	>4.5	6
Y17A	2.4 ± 0.3	1.7 ± 0.09 μM	142	3	6
M35A	1	342 ± 31 nM	29	2	6
F37A	1	5.3 ± 0.1 μM	442	3.7	6
K38A	1	14.5 ± 1.8 nM	1.2	0.2	6
K13A/F37A	3.6 ± 0.4	15.3 ± 0.5 μM	1275	4.3	4
Y14A/F37A	–	>30 μM	>2500	>4.7	4
Y17A/F37A	2.6 ± 0.3	18.7 ± 0.8 μM	1558	4.4	4
K13A/K38A	1.6 ± 0.08	8 ± 0.2 μM	667	3.9	4

The values of fractional current were determined for 5 different toxin concentrations in 4–6 cells at each concentration (see Fig. 4A and B). K_Ds and Hill coefficient are indicated. Free energy changes were calculated as: $\Delta\Delta G = -RT\ln(K_D^{mut}/K_D^{wt})$. The number of experiments is indicated (n).

|ΔΔG| values <1 kcal/mol. When alanine was substituted for K13, the affinity of ErgTx1 for hERG1 was reduced by over 290 folds. In accordance with this, the calculated binding free energy for K13A gives a maximum value of 3.5 kcal/mol. Replacement with alanine of the critical ErgTx1 residues Y14A, Y17A and F37A, reduces by >1830, 140 and 440-folds respectively, the toxin inhibitory potency on hERG1 channels. For the double mutants K13A–K38A, K13A–F37A, Y14A–F37A and Y17A–F37A the affinity for the channel were reduced 660, 1270, 2500 and 1550 folds, respectively. The substitution of alanine for methionine in position 35 resulted in only moderate decreases in binding affinity (Jiménez-Vargas et al., 2011), as shown by the small changes of the calculated ΔΔG (about 2 kcal/mol) when compared to the other mutants. The only methionine of ErgTx1 is located on the hydrophobic face of the third strand β (Fig. 5) and its side chain does not formed interactions that stabilize the secondary structure of the toxin (Torres et al., 2003; Frenál et al., 2004). Therefore, the substitution of the methionine by another residue with similar properties (the side chain of alanine displays hydrophobic characteristics) would not be expected to change substantially the affinity for the channel, assuming that this is a crucial site of interaction with the channel as earlier demonstrated (Jiménez-Vargas et al., 2011). However the oxidation of the sulfur atom of methionine35, would cause a dramatic modification of the hydrophobic environment of the toxin, supporting the data obtained, where the oxidized methionine looses almost three order of magnitude of its affinity for the channel, whereas the alanine mutant changes only 30 folds. Although the K_D value for Y14A cannot be determined accurately because even the highest concentrations of toxin gives relatively high fraction unbound, the mutants K13A, Y14A, Y17A and F37A clearly exhibit profound perturbations in their interaction with the channel. Interestingly, these results reveal that one face of ErgTx1 stands out as containing a large number of residues (K13, Y14, Y17, M35 and F37) where mutations to alanine display dramatic perturbations of the binding affinity.

It is well documented that ErgTx1 binds to hERG channel in a stoichiometric relation 1:1, and the lock is incomplete, even at high concentration of toxin (Pardo-López et al., 2002a, b; Hill et al., 2007). However the Hill coefficient calculated for mutatnts K13A and Y17A is higher that 1 (see Table 1) suggesting that more than one molecule might interact with one channel. Interestingly, for these mutants and also in the case M35A, the data obtained suggest a complete block of the hERG1 current, similarly to what was earlier demonstrated for the blockade of ErgTx1 on the isoform hERG3 (Restano-Cassulini et al., 2008). Doble-mutants K13A/F37A, Y14A/F37A and K13A/K38A showed a behavior similar to the correspondent point mutants with a Hill coefficient higher than 1 and the tendency to complete suppress the current.

Comparison of the amino acid sequence between ErgTx1 and BeKm-1 shown about 8.4% identity. The binding of both toxins to the channel occurs preferentially in the closed state, having a 1:1 binding stoichiometry with a maximum of 90% suppression of the current amplitude, thought to be due to a similar mechanism. However, the contact points of BeKm-1 to hERG channels are suggested to be located in the α-helix (Y11, F14, K18 and R20), whereas the surface contact of ErgTx1 is very likely situated in the loops formed by the hydrophobic patch (Y14, Y17, M35, F37) according to Pardo-López et al., (2002a), (b), Milnes et al. (2003), Zhang et al. (2003) and Jiménez-Vargas et al. (2011). It has been proposed that for the case of BeKm-1 the amino acid K18 (Zhang et al., 2003) plays the equivalent role of that described for K27 of the Charybdotoxin (ChTx) and AgTx2 toxins (Mackinnon and Miller, 1989). For the late toxins the interactions with the channel are sensitive to potassium concentration, where the epsilon amine of K27 occupies the inside position of the pore causing electrostatic repulsions between K^+ ions and K27. Contrary to this, ErgTx1 does not contain the equivalente K27 of ChTx and thus cannot physically plug the channel pore (Pardo-López et al., 2002a, b). The surface area contacts between ErgTx1 and the channel, and BeKm-1 and the channel are thus not the same. This is not new or unexpected, as earlier demonstrated in the review by Rodríguez de la Vega et al., 2003. Different toxins might use different surfaces of the 3D-structure to interact with various sub-types of different K^+-channels.

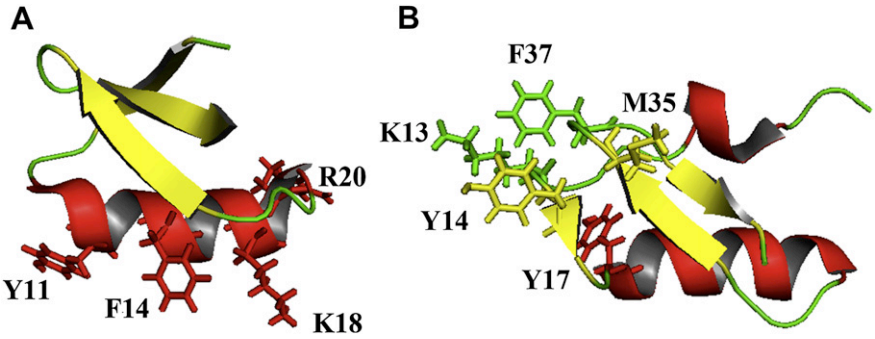

Fig. 5. *Comparison of the sites of interaction of the scorpion toxins BeKm-1 and ErgTx1 on the hERG1 channel.* Cartoon diagrams of the NMR structures: (A) BeKm-1 (1LGL, Korolkova et al., 2001) showing functionally important residues K18 and R20, which are located at the last turn of the helix, as well as, the aromatic site chains of Y11 and F14 (red) that cover most of the helix surface of the toxin. (B) ErgTx1 (1px9, Frenál et al., 2004) showing that the interaction surface with hERG1 channel is oriented in one side of the toxin formed hydrophobic patch center around an aromatic cluster defined by: Y14 (yellow), Y17 (red), F37 (green), K13 (green). Furthermore, in the middle of this hydrophobic patch lies K13 (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5 shows the comparison of the functional surfaces of the toxins BeKm-1 and ErgTx1 assumed to be the most important face of these toxins that interacts with the hERG1 channels.

The binding of other potassium channel specific scorpion toxins to their receptors on the outer vestibule of the channel pore involves a combination of hydrophobic, hydrogen bonding, and electrostatic interactions (Eriksson and Roux, 2002; Mackinnon and Miller, 1989; Ranganathan et al., 1996). Studies previously conducted with ErgTx1 suggested that the hydrophobic interactions are the main force involved in the binding to the hERG1 channel (Pardo-López et al., 2002a, b). Additionally it was proposed that the hydrophobic patch (Y14, F36 and F37) with a central basic residue K13 was also important for channel recognition (Torres et al., 2003). In the same publication and conducting a computational evolutionary trace analysis these authors have predicted two amino acid clusters, one hydrophobic made by Y14, Y16, Y17, F37 and one hydrophilic made by K13 and K38, which acts as cation-π interacting residues. However, Frenál et al. (2004) also mentioned the possibility that a cluster made by D3, S4 and K25 could be implicated in channel recognition. The results reported here, demonstrate for the first time experimentally, that K13, Y14, Y17, and F37 are all critical residues for the interaction of ErgTx1 with the channels, apart from the M35 already described (Jiménez-Vargas et al., 2011).

4. Conclusions

This communication shows experimentally, for the first time, that the hydrophobic patch composed by Y14, Y17, M35 and F37 of ErgTx1 probably can bind to the amphipathic α-helix of the hERG1 channel, as well as, the basic residue K13 could be making contact with this region of the channel.

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Ethical statement

The authors declare that there is no ethical statement in this manuscript.

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Conflict of interest

None declared.

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Toxin modulators and blockers of hERG K^+ channels

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ABSTRACT

The K^+ channel encoded by the *Ether-á-go-go-Related Gene* (ERG) is expressed in different tissues of different animal species. There are at least three subtypes of this channel, being the sub-type 1 (ERG1) crucial in the repolarization phase of the cardiac action potential. Mutations in this gene can affect the properties of the channel producing the type II long QT syndrome (LQTS2) and many drugs are also known to affect this channel with a similar side effect. Various scorpion, spider and sea anemone toxins affect the ERG currents by blocking the ion-conducting pore from the external side or by modulating channel gating through binding to the voltage-sensor domain. By doing so, these toxins become very useful tools for better understanding the structural and functional characteristics of these ion channels. This review discusses the interaction between the ERG channels and the peptides isolated from venoms of these animals. Special emphasis is placed on scorpion toxins, although the effects of several spider venom toxins and anemone toxins will be also revised.

1. Introduction

The human *ether-a-go-go-related gene* (hERG) 1 was first cloned in 1994 (Warmke and Ganeztky et al., 1994) and characterized as a voltage-gated inwardly rectifying potassium channel (Trudeau et al., 1995). These inward rectifying properties reside in the unique structure of the pore domain, making the channel crucial in the repolarization phase of the cardiac action potential (Warmke and Ganeztky, 1994; Sanguinetti et al., 1995) and in different types of tissues in which excitability is an essential property, such as: smooth muscles (Akbarali et al., 1999), carotid bodies (Overholt et al., 2000), lactotrops (Corrette et al., 1996), pancreatic β-cells (Rosati et al., 2000) and chromaffin cells (Gullo et al., 2003). It is well characterized that mutations in the *erg1* channel gene were associated to the long QT syndrome type 2 (LQTS2), an inherited cardiac disorder that causes syncope, seizures, and sudden death in otherwise healthy individuals (reviewed in Thomas et al., 2003; Anderson et al., 2006). The gene responsible for this phenotype has been mapped in the chromosome 7q35-36 (Jiang et al., 1994; Curran et al., 1995; Sanguinetti et al., 1995). In mammals other two members of this channel subfamily ERG2 and ERG3 have been cloned from a rat cDNA library and characterized showing electrophysiological properties similar to those of *erg1* (Lees-Miller et al., 1997; London et al., 1997; Bauer et al., 1998; Shi et al., 1997) and subsequently it was found that these genes are well expressed in the rodent central nervous system (Saganich et al., 2001; Papa et al., 2003; Guasti et al., 2005). It has also been demonstrated that, where co-expressed, the ERG subtype are able to form hetero-tetramers, making difficult the functional characterization of each channel subtype (Wimmers et al., 2001).

Natural toxins are useful probes for evaluating the involvement of K^+ channels in cell activity, and for investigating K^+ channel structure and localization. Recently, toxins able to specifically recognize ERG channels were

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isolated from different venoms (scorpion, spider and sea anemone) and shown to affect the ERG channels through two distinct targeting mechanisms. In the first mechanism the toxins bind to the extracellular region on the N-terminal side of the re-entrant pore loop, spanning between transmembrane domains S5 and S6 (Pardo-López et al., 2002a,b; Zhang et al., 2003; Korolokova et al., 2004) and inhibit the function of the channel by physically occluding the ion-conducting pathway. In the second type, the toxins interact with defined regions within the voltage sensor, affecting the gating motions and altering the energy of the voltage-dependent gating process (Ruta and MacKinnon, 2004). The objective of this manuscript is to revise mainly the mechanism of the interaction and surface contact areas of the different peptides from animal venoms with ERG channels.

2. Structural basis of hERG channel

The hERG channel is a tetramer of four α-subunits arranged circumferentially around a central pore (Fig. 1) as homo- or hetero-tetramer. Each subunit includes six transmembrane α-helical segments S1–S6 and a membrane-reentering P-loop (P) consisting of an extracellular S5-P linker (called turret), a pore helix (P-helix), an ascending limb containing the potassium selectivity signature sequence TVGYG, and the extracellular linker P-S6 (Doyle et al., 1998). In the N-terminal region, every α-subunit is flanked by "PAS" (Per-Arnt-Sim; aa 2-138) domain that consists of a helix-loop-helix structure and might interact with accessory proteins. This is important for gating transition in modulating the channel function; alternatively it is considered critical for the slow deactivation of ERG channels, presumably by binding to the S4–S5 linker (Schönherr and Heinemann, 1996; Morais-Cabral et al., 1998; Chen et al., 1999; Viloria et al., 2000; Tristani-Firouzi and Sanguinetti, 2003; Al-Owais et al., 2009). Also, in the C-terminal region it contains a cyclic nucleotide-binding domain (cNBD) that may contribute to the current modulation (Morais-Cabral et al., 1998). The first four domains of each subunit (S1–S4) comprise the voltage sensor which controls the channel gating during membrane potential transitions. In comparison with other voltage-dependent potassium channels, transmembrane helices S2 and S3 contain more negative charges (D460, D466, D501 and D509) (Liu et al., 2003) that may be responsible for slowing down the activation/deactivation process, characteristic in ERG channels kinetic (Planelis-Cases et al., 1995; Seoh et al., 1996), as well as, the positive charges (K525, R528, R531, R534, R537 and K538) of the S4 segment which is thought to play a key role in stabilizing the channel in the closed state (Smith and Yellen, 2002; Piper et al., 2003, 2005; Liu et al., 2003; Zhang et al., 2004; Subbiah et al., 2004, 2005). The very fast transition from open to inactivated state (C-type inactivation) involves conformational changes in the outer pore region of the channel that causes collapse of the pore and prevents ion fluxes, or alters the ion selectivity in such a way that the channel cannot conduct K^+ currents (Choi et al., 1991; Hoshi et al., 1991; Yellen et al., 1994; Liu et al., 1996; Smith et al., 1996; Schönherr and Heinemann, 1996). In these channels the inactivation is not only very fast, occurring in milliseconds, but it is also intrinsically voltage-sensitive (Sanguinetti et al., 1995; Rasmusson et al., 1998). This process is associated with the unique turret region of ERG channels, which is much longer than other Kv channels (43 aa vs 14-23aa; see Fig. 1), due to the presence of an amphipathic α-helix that play a unique role in the inactivation gating process (Liu et al., 2002) and in the toxin-channel interactions (Pardo-López et al., 2002b; Zhang et al., 2003; Huys et al., 2004). Extensive studies on the voltage sensor in Kv channels have identified a specific S3b-S4 helix-turn-helix structural motif (also known as the "voltage-sensor paddle") which moves in contact with the surrounding lipid membrane in response to changes in membrane voltage. It was recently shown that paddle motifs are modular units and can be transferred between ion channels and proteins with a voltage-sensing domain without losing their functional properties, suggesting that this motif resides in relatively unconstrained environment (Alabi et al., 2007; Bosmans et al., 2008). This paddle region in the hERG1 channel is highly homologous to the Kv2.1 channel (Fig. 1) and it is an important pharmacological target for ion channels function, because various sea anemone and spider toxins were shown to interact with this region (Phillips et al., 2005; Alabi et al., 2007; Milescu et al., 2009).

3. Pore-blocking peptide toxin

As earlier mentioned, in recent years, several peptides that bind to the outer vestibule of the ERG channels were described; thus are potentially very useful tools for studying and understanding the structure and function of these types of channels. The first peptide inhibitor was isolated from the venom of the scorpion *Centruroides noxius* Hoffmann and early named ErgTx1 (Gurrola et al., 1999) or CnErg1 (Corona et al., 2002). BeKm-1 was the second peptide described to have an inhibitory action on this type of channel. It was isolated from the venom of the Central Asian scorpion *Buthus eupeus* (Korolkova et al., 2001, 2002). The amino acid sequence of BeKm-1 shows only 8.3% identity to that of ErgTx1. These toxins adopt a compact fold made up of α-helix and three β-stands arranged in a strongly twisted antiparallel β-sheet and has been referred to as a member of the γ-KTx subfamily of scorpion toxins (Milnes et al., 2003; Rodríguez de la Vega et al., 2003; Torres et al., 2003; Frenál et al., 2004). Following these two initially discovered peptides, 25 additional members of this subfamily (Ergtoxin-like peptides) were identified on tissues from venomous glands of scorpions of the genus *Centruroides*. Most of their primary structures were deduced from cDNA cloning, although a few of them have been directly determined from isolates of the scorpion venoms. The percentage of identity among this subfamily of peptides (Fig. 2) ranges between 67 and 97% (Lecchi et al., 2002; Nastainczyk et al., 2002; Corona et al., 2002; Coronas et al., 2005; Restano-Cassulini et al., 2008). Interestingly, toxins BmTx3 and AmmTx3 isolated from the venoms of the scorpions *Mesobuthus martensii* and *Androctonus mauritanicus mauritanicus* respectively, are members of the voltage-dependent K^+ channels (Kv), of the

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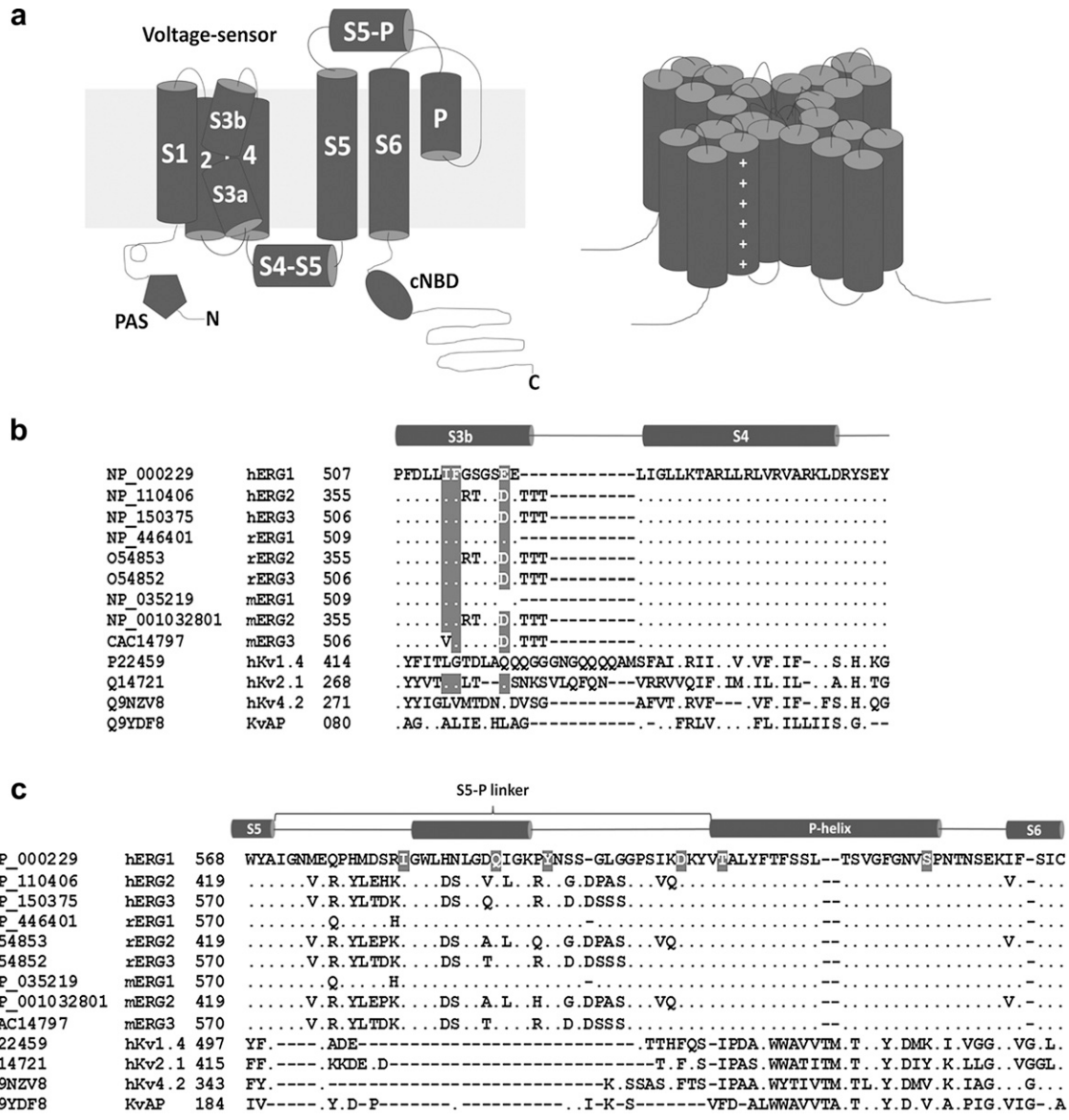

Fig. 1. Structure and assembly of hERG1 K^+ channel pore-forming α-subunits. (a) Topology of a single ERG channel subunit, consisting of six hydrophobic segments (S1–S6). The S1–S4 segments form the voltage-sensor domain, and the S5–S6 segments contribute to the pore. Four subunits or repeats assemble to form the central ion-conducting pore surrounded by four voltage-sensor domains. (b). Sequence comparison of the interacting sites for voltage-sensor toxins in the voltage-sensor domain. Cylinders (S3b and S4) represent approximate positions of predicted α-helices (Ruta and MacKinnon et al., 2004; Zhang et al., 2007). Multiple sequence alignment of S3b-S4 segments of ERG channels are from human, rat and mouse sequences of hKv1.4, hKv1.2 and KvAP channels. Residues in S3b, highlighted gray, alter the affinity of the channel for voltage-sensor toxins when mutated. Mutations of I273, F274 and E277 of Kv2.1 decrease the affinity of the channel for tarantula toxins (Swartz and MacKinnon, 1997; Li-Smerin et al., 2000); the equivalent residues on hERG1 are I512, F513, and E518 (Zhang et al., 2007). (c). Multiple sequences alignment of pore-forming domain of ERG channels from different species with hKv1.4, hKv2.1, hKv4.2 and KvAP channels. Cylinders indicate the approximate positions of predicted α-helices of S5, S5-P linker, P-helix and S6 (Liu et al., 2002; Tseng et al., 2007). Residues known to make contact with peptide toxins are highlighted gray. When mutated, changes the interaction mode of the ErgTx1 and BeKm-1 toxins with hERG1 channel (Pardo-López et al., 2002a,b; Zhang et al., 2003; Tseng et al., 2007). Identical amino acids of hERG1 are indicated by points (.).

subfamily α-KTx-15, but are also capable of blocking ERG potassium currents. Huys et al., 2004 and Abdel-Mottaleb et al., 2008 found that these two peptides have a functional epitope formed by basic residues (R18 and K19) of the α-helix side of the peptide that could induce a hERG1 channel blocking without alteration of the gating kinetics, although with lower efficiency, working at μM concentrations (see Table 1; Huys et al., 2004; Abdel-Mottaleb et al., 2008). Not all these toxins have been tested on the three sub-types of ERG channels. These peptides can be selective for certain members of the various families of channels (Table 1). In the case of ErgTx1, BeKm-1, ErgTx2, CeErg4 and CeErg5 their activities have been characterized in the three isoforms of ERG channels of

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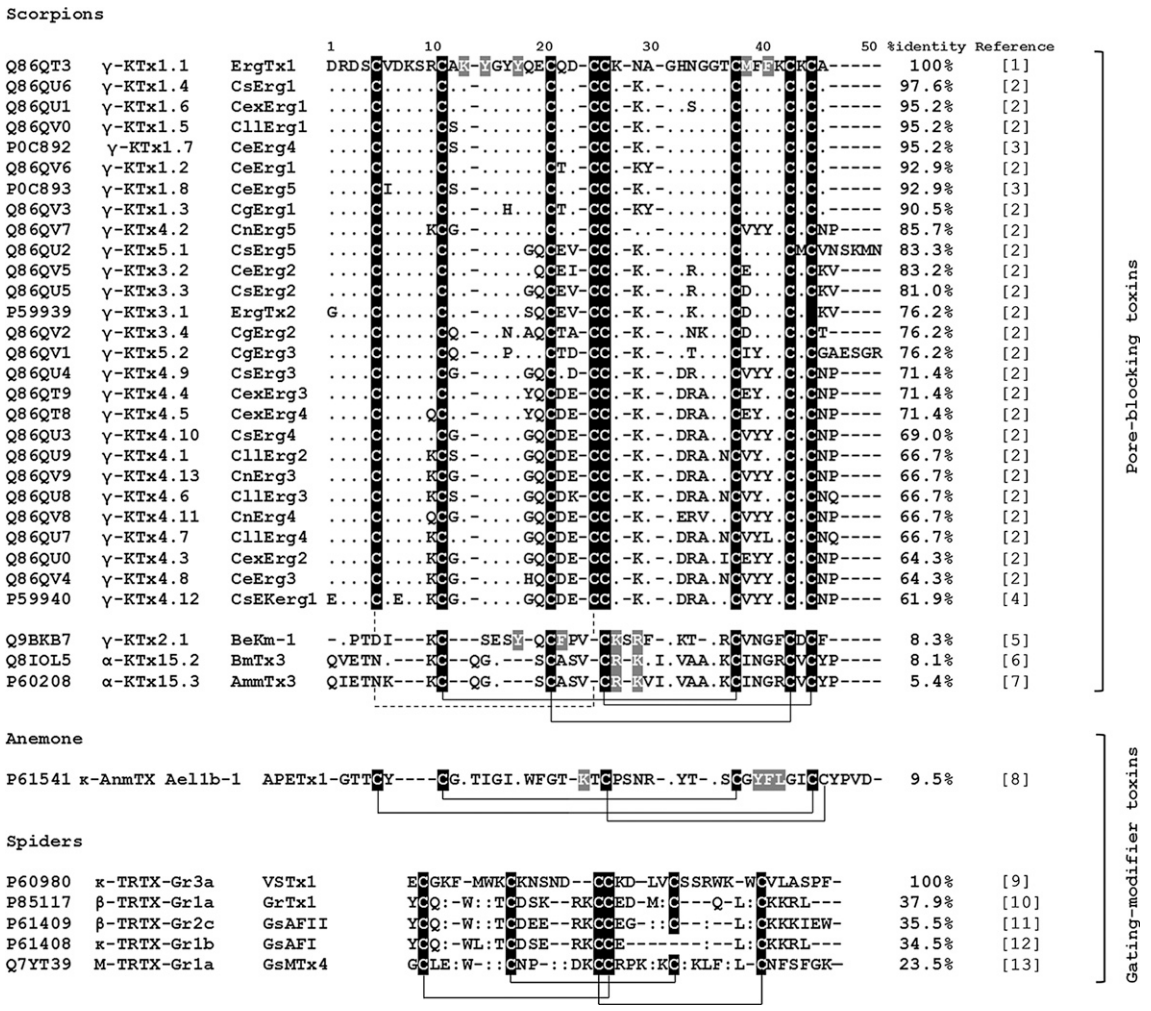

Fig. 2. Multiple sequence alignment of scorpion, anemone and spider toxins that target ERG channels. The Swiss-Prot accession numbers are followed by the systematic names as outlined in (Tytgat et al., 1999; Rodríguez de la Vega and Possani, 2004; Kozlov and Grishin, 2012; King et al., 2008) and abbreviated names. The fourth column gives the full amino acid sequences aligned according to equivalent topological structure. Identical residues to ErgTx1 are indicated by points (.), whereas those identical to VSTx1 by colons (:). The important residues involved in the interaction with ERG channels are highlighted in gray, which were identified based on mutagenesis studies. Cysteines are highlighted black and paired according to the specific disulfide pairing. The fifth column gives identity score based on pairwise comparison against ErgTx1 for the scorpion and anemone toxins sequence and VSTx1 for the spider toxins sequences. The last column provides the references: [1]. Gurrola et al., 1999; [2]. Corona et al., 2002; [3]. Restano-Cassulini et al., 2008; [4]. Nastainczyk et al., 2002; [5]. Korolkova et al., 2001; [6]. Vacher et al., 2001; [7]. Vacher et al., 2002; [8]. Diochot et al., 2003; [9]. Jiang et al., 2003; [10]. Clement et al., 2007; [11]. Lampe and Sanchs, 1999; [12]. Lampe, 1999; [13]. Suchyna et al., 2000. Physiological effects are indicated at the most right-hand side.

human and rat. The data reveal that ErgTx2 eliminates the slow component of the rERG2 current without affecting rERG3, and weakly blocking rERG1 (see Table 1; Lecchi et al., 2002; Restano-Cassulini et al., 2006). ErgTx1 inhibits human and rat ERG1 in a similar manner, however, it was unable to affect human ERG2 current while completely blocking the rat ERG2 channel with poor recuperation. Interestingly hERG2 and rERG2 differ in only three residues in the surface of interaction with the toxin (see Fig. 1c residues H432P, V442A and R447Q), suggesting that these residues can be essential for the recognition process. For ERG3, this toxin showed preference for the human channel, eliminating the current completely and without recuperation, whereas for rat ERG3 channel weak blockade occurred. In this case channels differ by one amino acid residue in the pore-forming region at position 593 (glutamine versus threonine). This natural mutation in rERG3 channel (Q593T) decreases the affinity of ErgTx1 by ~200 fold (see Fig. 1c and Table 1 in Restano-Cassulini et al., 2006). The blocking effects of CeErg4 and CeErg5 on ERG channels of human and rat are similar to ErgTx1 (Table 1; Restano-Cassulini et al., 2008). BeKm-1 discriminates well the three rat channels, with K_D values in the range of 1.5–757 nM. The effects on human ERG1 and ERG3 are similar, whereas in ERG2 channels the effect is weaker (Restano-Cassulini et al., 2006).

The interacting sites of ErgTx1 and BeKm-1 on the hERG1 channel were determined by cysteine scanning

Table 1
Effects of scorpion, anemone and spider toxins on ERG channels.

	Specie	Cells	ERG1	ERG2	ERG3	Reference
Scorpions						
ErgTx1	Human	CHO	4.5–7.3 nM	n.e	0.18 ± 0.03 nM	Restano-Cassulini et al., 2006; Hill et al., 2007a,b.
		Xenopus laevis oocytes	11.4 nM	–	–	Pardo-López et al., 2002a,b
	Rat	CHO	6.8 ± 0.21 nM	1.16 ± 0.09 nM	38.1 ± 4.1 nM	Restano-Cassulini et al., 2006.
		Fll-neuroblastoma	16 ± 0.4 nM			Gurrola et al., 1999
ErgTx2	Rat	MMQ	0.4 μM			Lecchi et al., 2002
CllErg1	Human	*X. laevis* oocytes	10 nM	–	–	Coronas et al., 2005
CeErg4	Human	CHO	12.8 ± 0.9 nM	n.e	0.88 ± 0.06 nM	Restano-Cassulini et al., 2008
	Rat	CHO	9.9 ± 1.2 nM	1.45 ± 0.04 nM	> 210 nM	
CsEKErg1	Mouse	NG108-15	0.232 μM			Nastainczyk et al., 2002
BeKm-1	Human	CHO	7.7 ± 0.6 nM	77 ± 16 nM	11.5 ± 1.1 nM	Restano-Cassulini et al., 2006
		HEK-293	3.3 nM	–	–	Korolkova et al., 2001
		X. laevis oocytes	4.4–8.1 nM	–	–	Zhang et al., 2003; Tseng et al., 2007
	Rat	CHO	19 ± 1.6 nM	1.51 ± 0.12 nM	747 ± 58 nM	Restano-Cassulini et al., 2006
BmTx3	Human	*X. laevis* oocytes	1.9 ± 0.14 μM	–	–	Huys et al., 2004
AmmTx3			7.9 ± 1.4 μM	–	–	Abdel-Mottaleb et al., 2008
Anemone						
APETx1	Human	COS-7	34 nM	n.e	n.e	Diochot et al., 2003
	Mouse	NG108-15	>30 nM			
Spiders						
VSTx1	Human	CHO	7.4 ± 0.3 μM	45 ± 3 μM	55 ± 4 μM	Redaelli et al., 2010
GrTx1			4.2 ± 0.5 μM	>200 μM	>200 μM	
GsAFI			4.8 ± 0.4 μM	>200 μM	>200 μM	
GsAFII			4.7 ± 0.3 μM	>200 μM	>200 μM	
GsMTx4			11 ± 0.26 μM	10.9 ± 0.3 μM	53 ± 5 μM	

This table summarizes the blocking properties of all reported venom toxins know to inhibit ERG channels expressed in different cells lines. n.e, no effect, (−) no tested. The assays realized on the series of tumor cells intrinsically express ERG currents (*erg1, erg2 and erg3*).

mutagenesis of fifty one residues of S5-P and P-S6 linker regions of the hERG1 channel (Pardo-López et al., 2002a,b; Liu et al., 2002; Zhang et al., 2003; Korolokova et al., 2004). Based on the results found, it was proposed that ErgTx1 preferentially binds to closed channels, whereas BeKm-1 is able to bind to both closed and inactivated channels, but even at high concentrations these toxins are unable of entirely suppressing the ERG1 currents, suggesting that they never block completely the entrance of the pore (Milnes et al., 2003; Tseng et al., 2007). However, there are also important differences between BeKm-1 and ErgTx1 in terms of their contact points with the channel and the binding site environment. First, the impact of protonated histidine at position 578 at acidic pH on toxin binding differs between BeKm-1 (no impact) and ErgTx1 (reduced binding). Second, cysteine substitutions at the following positions: I571, L589, D591, P596, P605, and S631 affect BeKm-1 binding, but not that of ErgTx1. In particular, the difference in the impact of S631C (a mutation right at the edge of the pore entrance) suggests that BeKm-1 may bind deeper into the pore than ErgTx1, and thus senses the effects of mutating the 631 side chain residue (Pardo-López et al., 2002b; Zhang et al., 2003). Additional experiments in which several residues of the toxins were substituted by alanine (alanine scanning) permitted identifying the active surfaces of interaction between the toxins and the hERG1 channel. The functionally important residues K18 and R20 of BeKm-1, which are located at the last turn of the helix, together with R1, K23, and possibly R27 form a surface with the strongest positive electrostatic potential in the whole molecule (Fig. 3), which is essential for interaction with the negatively charged outer vestibule of the channel. The aromatic side chains of Y11 and F14 are situated in the hydrophobic patch, which cover most of the helix surface of BeKm-1 (Zhang et al., 2003). However, the main interacting surface of the ErgTx1 is located in the loops that form a hydrophobic patch (Fig. 3; Y14, Y17, M35, F36 and F37) surrounding K13, located near the β-hairpin between the second and third strands of the β-sheet (Torres et al., 2003; Frenál et al., 2004; Jiménez-Vargas et al., 2012). In both cases, the results of mutagenesis suggest that eight pairs of BeKm-1/hERG1 residues potentially interact: Y11 with I583 and Y597; F14 with R582, Y597 and D609; K18 with S631; R20 with Q592 and S631 (Zhang et al., 2003; Korolkova et al., 2002, 2004). For ErgTx1, the hydrophobic patch comprises the residues: Y14, Y17, M35 and F37 that can tightly bind to the hERG1 channel, very likely making contacts with residues of the amphipathic α-helix side of the channel. It also suggests that K13 could be interacting with this region of the hERG1 channel (Pardo-López et al., 2002a,b; Torres et al., 2003; Frenál et al., 2004; Jiménez-Vargas et al., 2011; Jiménez–Vargas et al., 2012). Recently, two kinetic models have been proposed for explaining the unique mechanism of hERG channels' blockage by toxins ErgTx1 and BeKm-1. To explain the well-known phenomena of ErgTx1 incomplete blockage even at high concentration, Hill et al., 2007a proposed a kinetics model where first the amphipathic α-helix binds to channel sufficiently peripheral, in such way that the toxin molecule does not occlude the ion conduction pathway. Therefore, there is a subsequent conformational rearrangement that brings the toxin close to the central axis of the pore where it can block ion conduction. However, these interactions that stabilize the blocked conformation must be relatively weak

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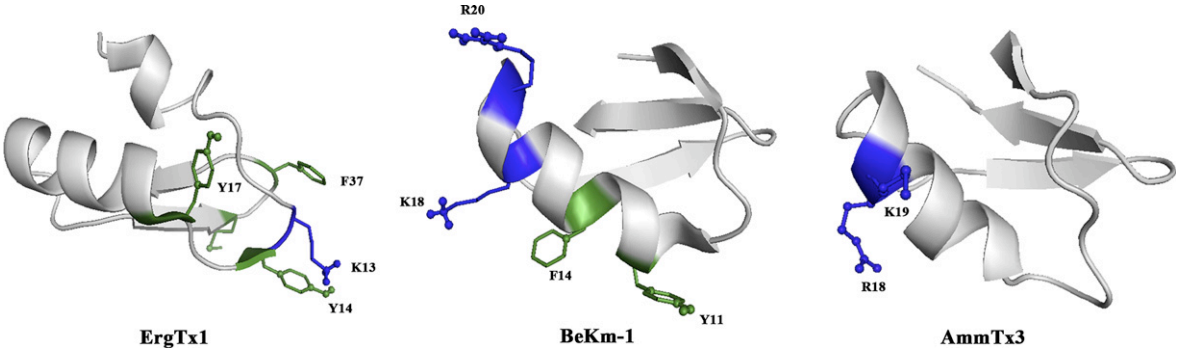

Fig. 3. Three-dimensional models. This figure shows functional maps of the 3D-structure models of ErgTx1 from *Centruroides noxius* (1px9; Gurrola et al., 1999; Torres et al., 2003; Frenál et al., 2004; Jiménez-Vargas et al., 2011; Jiménez-Vargas et al., 2012), BeKm-1 from *Buthus eupeus* scorpion (1lgl; Korolkova et al., 2001, 2002; Milnes et al., 2003; Zhang et al., 2003) and AmmTx3 from *Androctonus mauritanicus mauritanicus*, obtained by means of the program CPHMODELS-3.0, using as template the structure registered under the accession number 1hp2, toward ERG channels. Toxin structures are shown in cartoon representation and the residues hydrophobic are shown in green and the basic residues in blue. The functional maps highlight the multi-point interactions of the toxins with the hERG1 channel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and hence have a rapid dissociation rate, relative to the association rate, and the equilibrium between the bound and the un-bound states gives rise to the incomplete blockage of the hERG current (Hill et al., 2007a). In another hand, Tseng et al. (2007) proposed a state-depending binding model where the BeKm-1 preferentially binds to a closed state of the channel which induces channel deactivation, as earlier reported by Tseng et al. (2007) and Zhang et al. (2003). Rodriguez-de-la-Vega et al. (2009) pointed out that the two models are not mutually exclusive, but taken together may explain all major properties of scorpion toxin ERG1 channel blockade. On the other hand, the binding site of ErgTx1 on hERG1 is also thought to be critically involved in voltage-dependent inactivation of the channel (Clarke et al., 2006), in which mutations in the position 631 (S631A) disrupted distinct aspect of the inactivation pathway and caused a ~20-fold increase in the affinity of ErgTx1 for hERG with complete block of the current (Hill et al., 2007b). This shows that the S631A mutation appears to switch the ErgTx1 block mechanism to a pore plug, resembling all the characteristics defined by the classical K^+ channel blockers like charybdotoxin, kaliotoxin, and iberiotoxin. These toxins are known to bind to the outer vestibule of the channel causing blockage of the ion conduction by physically occluding the pore (García et al., 2001; Rodriguez de la Vega et al., 2003). It has been suggested that the residue K13 of ErgTx1 is a candidate for plugging the selectivity filter in S31A hERG channels (Hill et al., 2007b).

4. Gating-modifier toxins

The gating-modifier toxins bind to a region of the channel that changes conformation during gating and influence this mechanism by altering the relative stability of closed, open or inactivated states. Gating-modifier toxins differ from pore-blocking toxins in two additional aspects: the stoichiometry of the toxin–channel interaction and the location of the binding site. While pore-blocking toxins bind with a 1:1 stoichiometry to the receptor site involving multiple amino acid residues in the outer vestibule of the channel, experiments with saturating concentrations of gating-modifier toxins suggest that 3–4 toxin molecules bind to the four voltage-sensor domains of a single channel molecule (Swartz and MacKinnon, 1997; Phillips et al., 2005). Peptides with this mechanism of interaction on ERG channels have been isolated from sea anemone and spider venoms.

APETx1 is a peptide of 42 amino acid residues purified from sea anemone *Anthopleura elegantissima*; has 20% of identity to ErgTx1 and is a selective blocker of hERG1 channel (Diochot et al., 2003; Restano-Cassulini et al., 2006). Their tridimensional structures in comparison with the ErgTx1 and BeKm-1 show an all β-strand folding pattern. The structural analysis suggests that positive charges along with neighboring aromatic/hydrophobic residues (K18 with Y5, and K18 with Y32/F33; Chagot et al., 2005) may be involved in APETx1 binding to the ERG channels. Electrophysiological recordings suggest that the APETx1 binding site is distinctly different from that of pore-blocking peptide toxins: BeKm-1 and ErgTx1. APETx1 is capable of shifting the activation curve of the hERG channel in the positive direction and reduces the current amplitude elicited by strong depolarizing pulses that reach the maximum level of channel activation without interference with the inactivation of the channel. Competition studies carried out with APETx1 and BeKm-1 indicated that there is no overlapping between the binding sites of both toxins on the hERG channel. BeKm-1 occupies the central position of the hERG pore domain (Zhang et al., 2007; Tseng et al., 2007), whereas APETx1 binding site is probably in the voltage-sensing domain. Mutations introduced into the S3b region of hERG1 channel, in which the residues at positions 514–519 were substituted by cysteines (G514C and E518C) and submitted to the effect of toxin, showed that these modifications cause a dramatic impact on the toxin–channel interaction, abolishing the effect of APETx1. These data suggest that APETx1 is a gating-modifier toxin of the hERG1 channel, which shares characteristics with those of gating-modifier toxin-binding sites on other Kv channels

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(Zhang et al., 2007). The interacting surface of APETx1 has not been determined, but an inspection of its structure in comparison with other gating-modifier toxins suggests that K18 and the residues that form the hydrophobic patch L34/F33/Y32 are likely to be involved in the interacting surface with the hERG1, but not with hERG2 or hERG3 channels. Probably the presence of three extra threonine residues in their S3b (see Fig. 1b), absent in hERG1, may perturb the conformation of the S3b helix, disrupting APETx1 binding. On the other hand, hERG2 has a positively charged arginine at position 362 (R362), whereas the equivalent position of hERG1 has a glycine residue (G514), (see Fig. 1b and Zhang et al., 2007).

On the other hand, five peptides purified from the venom of the tarantula *Grammostola rosea* were assayed on hERG channels (Redaelli et al., 2010). These peptides were defined as promiscuous, because they are not very specific. The peptides GrTx1 (Clement et al., 2007), GsAFI, GsAFII (Lampe, 1999; Lampe and Sanchs, 1999), VSTx1 (Jiang et al., 2003; Lee et al., 2004) and GsMTx4 (Suchyna et al., 2000; Jung et al., 2006) were purified and characterized regarding their physiological actions against the three hERG isoform channels (Fig. 4). The structural studies show that the toxins GsMTx4 and VSTX1 are composed by 34 amino acid residues, packed by three disulfide bridges, adopting a "cysteine knot peptide" conformation (Suchyna et al., 2000; Oswald et al., 2002; Jiang et al., 2003; Lee and Mackinnon, 2004; Jung et al., 2006). Although the overall fold and amphipathic nature of the toxins that recognize the voltage-sensor segment of the channels are quite similar, there are detailed differences, in particular for the residues that compose the hydrophobic surface. The toxin binds equally well to the isolated S1–S4 voltage-sensing domain compared to the full-length channel (Ruta and MacKinnon, 2004), suggesting that the receptor site is situated within the voltage sensor. Also, experiments with chimeric channels show that moving the S3–S4 paddle motif from KvAP into Kv2.1 transfers sensitivity to VSTx1. In this case, the amino acid sequence of hERG's S3b-S4 region is highly homologous to KvAP channel (Fig. 1b; Zhang et al., 2007; Alabi and Swartz, 2006). Therefore, it suggests that the sites of interaction of these toxins with both types of channels are the same. The negatively charged residues of the channels establish electrostatic interactions with the positively charged residues of the toxins (Li-Smerin et al., 2000). In addition, a large cluster of solvent-exposed hydrophobic residues that are surrounded by basic and acidic residues in the toxins may be involved at the surface of interaction of the hERG channels (Fig. 4; Phillips et al., 2005; Lee et al., 2004). For VSTx1 a large cluster of hydrophobic residues is formed by F5, M6, W7, L19, W25, W27, V29 and L30, as well as, charged residues K4, K8, K17, D18, K26, which can be important for binding with the pore domain of the hERG channels. Likewise the hydrophobic surface (F5, W6, W7, F27, F32 and F34) and charged residues (E4, K8, R18) exposed to solve the GsMTx4 might be the expected sites of interaction with the hERG channel (Jung et al., 2006). In the case of the toxins GrTx1, GsFAI and

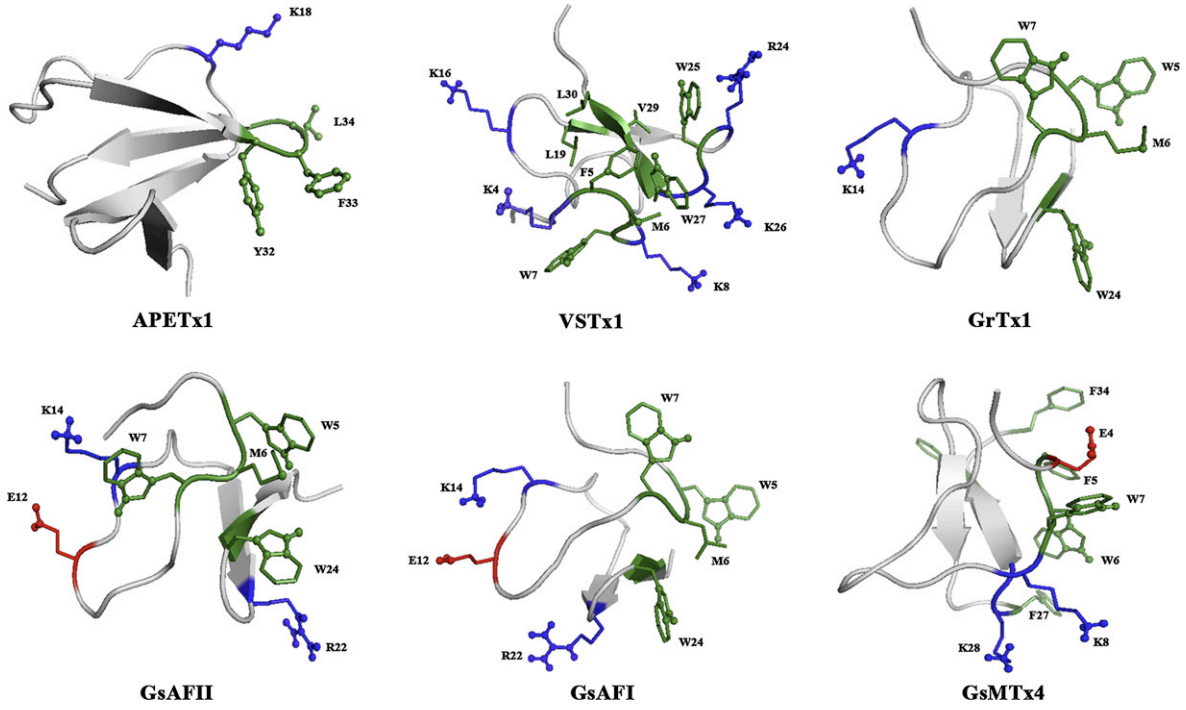

Fig. 4. Cartoon models of the gating-modifiers toxins of the ERG channels. This figure shows the putative interacting surfaces of the toxins APETx1, VSTx1, GrTx1, GsAFII, GsAFI and GsMTX4 with ERG channels. Residues' colors are: basic residues (blue), acidic residues (red), hydrophobic amino acids (green). Backbone fold is shown on top in gray. Images of GrTx1, GsAFI and GsAFII were created using as template the models of the peptides with accession numbers 1v7f and 1lup, with program PyMOL and server CPHMODELS-3.0. Protein Data Bank accession IDs: 1s6x for VSTx1 (Jiang et al., 2003; Lee et al., 2004) and 1lu8 for GsMTx4 (Jung et al., 2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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GsFAII their three-dimensional structures are still not determined, but results of alignment of these amino acid sequences support the idea that they share a common structural motif "cysteine knot peptide" and as a consequence of such folding display a hydrophobic patch surrounded by a unique "charge belt" in their interacting surface with hERG1 channel (Fig. 4; Huang et al., 2007). The specificities of these toxins for the three human ERG channels were tested (Table 1), showing that GrTx1, GsAFI and GsAFII block in a voltage-dependent manner the hERG1 isoform only, whereas VSTx1 and GsMTx4 block all three isoforms. The highest affinity is exhibited toward hERG1 (Redaelli et al., 2010).

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Conflict of interest

There is no conflict of interest.

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