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CARACTERIZACIÓN DE ANTIBIÓTICOS PEPTÍDICOS INVOLUCRADOS EN LA RESPUESTA INMUNE INNATA DEL ALACRÁN *Centruroides limpidus limpidus*

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HCD

Dedicatorias

A mi Familia: Ma. Teresa, Héctor Ricardo, Héctor Rafael, Mario Stalin, Teresa Azucena, Nora Adela, Ma. Camila, Martha Cecilia, Massimo.

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RICARDO C. RODRÍGUEZ DE LA VEGA CUÉLLAR

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PREFACIO

El cuerpo de este documento constituye la disertación final sobre mi proyecto doctoral. La exposición comienza con una breve introducción a los tópicos de la inmunidad innata y los péptidos antimicrobianos, prosigue con los antecedentes directos al proyecto, el conjunto de resultados publicados formalmente, algunos datos adicionales no incluidos en dicho reporte y culmina con una discusión de los resultados —someramente más amplia que la presentada en el artículo mencionado, cuya referencia es:

> Rodríguez de la Vega RC, García BI, D'Ambrosio C, Diego-García E, Scaloni A y Possani LD. Antimicrobial peptide induction in the haemolymph of the Mexican scorpion *Centruroides limpidus limpidus* in response to septic injury. *Cell. Mol. Life Sci.* 61:1507-1519.

Al final de la exposición incluí un documento anexo que ahonda sobre un tópico colateral al objeto de esta tesis, los análisis realizados en este rubro han sido publicados, o aceptados, como a continuación se refiere:

> Batista CVF, Gómez-Lagunas F, Rodríguez de la Vega RC, Hajdu P, Pany G, Gaspar R y Possani LD (2002). Two novel toxins from the Amazonian scorpion Tityus cambridgei that block Kv1.3 and Shaker channels with distinctly different affinities. *Biochim. Biophys. Acta* 1601:123-131.
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> Rodríguez de la Vega RC, Merino E, Becerril B y Possani LD (2003). Novel interactions between K+ channels and scorpion toxins. *Trends Pharmacol. Sci.* 24:222-227.
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> Possani LD y Rodríguez de la Vega RC (2003). Response to Xu et al.: Hypothesis-driven science paves the way to new discoveries. *Trends Pharmacol. Sci.* 24:448-449.
>
> Rodríguez de la Vega RC y Possani LD. Current views on scorpion toxins specific for K+ channels. *Toxicon* (aceptado).
>
> del Río-Portilla F, Hernández-Marín E, Pimienta G, Coronas FIV, Zamudio FZ, Rodríguez de la Vega RC, Wanke E, y Possani LD. NMR solution structure of Cn12, a novel peptide isolated from Mexican scorpion Centruroides noxius having a typical β sequence but with a α-like effect. *Eur. J. Biochem.* (aceptado).

ABREVIATURAS

α/β-EC	Motivo estructural α/β Estabilizado por Cisteínas
α-KTx	Toxina de alacrán especifica para canales de K^+
Cll-dlp	*Centruroides limpidus limpidus* defensin like peptide
Na-ScTx	Toxina de alacrán especifica para canales de Na^+
PAM	Péptido AntiMicrobiano
PMNP	Patrones Moleculares asociados a lo No-Propio
RRP	Receptores de Reconocimiento a PMNP

Otras abreviaturas de referencia frecuente en la literatura científica se usan sin mayor explicación.

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Resumen

Los organismos invertebrados han proporcionado un panorama general de la activación inmune que involucra Péptidos AntiMicrobianos (PAM's), a partir del cual emergen tres estrategias alternativas: una es definida por la inducción inmediata de genes que codifican para PAM's; otra está caracterizada por la liberación sistémica inducible de PAM's a partir de reservorios celulares, y; la tercera es la producción constitutiva de PAM's. Estos tres sistemas alternativos de defensa han sido documentados en organismos quelicerados. En el presente trabajo se usó una estrategia de despliegue diferencial evaluado por Cromatografía Líquida de Alta Resolución y Espectrometría de Masas para mostrar que el reto séptico induce una respuesta inmune en el alacrán Mexicano *Centruroides limpidus limpidus*. Se aislaron varios componentes de la hemolinfa de este alacrán, de los cuales el denominado Cll-dlp (*C. limpidus limpidus* defensin like peptide) fue caracterizado *in extenso*. Este componente se acumula en la hemolinfa en respuesta al reto séptico en un periodo de 24 h, regresando a niveles basales a las 48 h después de la infección. Experimentos de RT-PCR demostraron que esta acumulación sistémica es independiente de regulación transcripcional. La secuencia de Cll-dlp contiene 32 residuos de aminoácidos, estrechamente empaquetados por tres puentes disulfuro formados entre los residuos Cis2-Cis21, Cis7-Cis29 y Cis11-Cis31. El gen que codifica para Cll-dlp fue clonado y secuenciado a partir de ADNc y ADNg, mostrando la presencia de un péptido señal de 24 aminoácidos, el cual se interrumpe con un intrón de 128 pb, seguido por la secuencia madura que finaliza en un codón de término. Este péptido constituye un nuevo miembro de la familia de las defensinas de invertebrados. Adicionalmente, se registró un efecto antibacteriano cooperativo entre Cll-dlp y dos componentes constitutivos de la hemolinfa.

SUMMARY

Invertebrate models have provided a general picture of innate immune activation involving AntiMicrobial Peptides (AMPs), from which three alternative strategies emerged: one is defined by the immediate up-regulation of genes encoding for AMPs; another is characterized by the inducible systemic release of AMPs from cellular reservoirs; the third alternative is the constitutive production of AMPs. In Chelicerate organisms the three alternative defensive systems have been documented. In this work a differential High Performance Liquid Chromatography / Mass Spectrometry approach was used to show that septic injury elicits an immune response in the haemolymph of the Mexican scorpion *Centruroides limpidus limpidus*. Several haemolymph components were isolated, from which one named Cll-dlp (*C. limpidus limpidus* defensin-like peptide) was comprehensively characterised. This component accumulates in the haemolymph in response to septic injury within 24 hours, returning to basal levels after 48 hours post-infection. The systemic accumulation of Cll-dlp is independent of transcriptional regulation as demonstrated by RT-PCR assays. The Cll-dlp sequence comprises 32 amino acid residues, closely packed by three disulfide bridges formed between Cys2-Cys21, Cys7-Cys29 and Cys11-Cys31. The gene encoding Cll-dlp was cloned from cDNA and genomic sources, showing the presence of a signal peptide containing 24 amino acid residues, interrupted by an intron with 128 bp, and a mature peptide ending with a stop codon. This peptide constitutes a novel member of the invertebrate defensin family of AMPs. Additionally, a cooperative antibacterial effect was registered between Cll-dlp and two other constitutive haemolymph polypeptides.

INTRODUCCIÓN

Unidad por unidad, el mundo está habitado por una abrumadora mayoría de organismos unicelulares (microorganismos): Archeas, Bacterias y varios tipos de Eucarias, incluyendo protistas, hongos y algas [1]. Si bien la generalidad de los microorganismos se desarrolla en vida libre, para algunos de éstos los sistemas multicelulares constituyen su hábitat, estableciendo relaciones simbióticas con sus hospederos —durante alguna o el total de las etapas de sus ciclos de vida—, ya sea en forma de mutualismo, comensalismo, amensalismo o parasitismo. La definición ecológica de parasitismo refiere a la interacción entre dos organismos cualquiera, donde uno de ellos (el parásito) afecta la homeostasis del otro (el hospedero), generando, por tanto, una patología [2].

Las ventajas adaptativas que ofrece la multicelularidad se intuyen al reconocer que varios linajes eucariotes y procariotes la han desarrollado de forma independiente, incluso en más de una ocasión dentro de una misma línea evolutiva [3,4]; sin embargo, los sistemas multicelulares se encuentran en desventaja numérica y cinética con respecto a los microorganismos, mismos que —así sea sólo por esta diferencia ecológica— les son potencialmente peligrosos [5]. Los hospederos multicelulares han desarrollado estrategias de defensa que limitan la posibilidad de establecer infecciones por parte de los parásitos potenciales. Dichas estrategias incluyen barreras físicas (*v.g.* las condiciones extremas de humedad, salinidad y pH en los epitelios), eliminación inespecífica (*v.g.* el recambio de matrices extracelulares del tipo de las mucosas) y una batería de respuestas celulares y humorales que, en conjunto, constituyen el sistema inmune del organismo [6,7]. Se ha reconocido que la presión evolutiva que significa el parasitismo es una de las principales fuerzas motrices de la diversificación de los sistemas biológicos [2]. De hecho, se ha propuesto que dos de los fenómenos biológicos más complejos que se conocen —la reproducción sexual y, obviamente, las estrategias de defensa conocidas como sistema inmune— podrían haber surgido como consecuencia de la presión evolutiva ejercida por los parásitos [5,8].

Discriminación inmune de lo no-propio

En los organismos multicelulares el paradigma de la inmunidad concierne al reconocimiento de lo no-propio, es decir: cómo puede el hospedero distinguir entre una célula que le es ajena y su propia diversidad de tipos celulares, y al mismo tiempo discernir qué tipo de respuesta requiere para evitar ser dañado por el potencial parásito [9-11]. En el caso específico de los metazoas, los mecanismos de inmunidad han sido tradicionalmente diferenciados en innatos (o naturales: todos los miembros de una misma especie los poseen) y adaptativos (o adquiridos: en cada individuo son diferentes como resultado de las distintas ontogenias) [6,9,12,13]¶. Paradójicamente, esta distinción de mecanismos sólo es

¶ La presencia de mecanismos adaptativos de defensa fue rápidamente identificada (Tucidides por ejemplo, hace 2434 años, reconoce que "...sin embargo, fue en aquellos que se habían recuperado de la enfermedad en quienes la postración y la muerte encontró mayor compasión"..."porque el mismo hombre jamás fue atacado dos veces, al menos no fatalmente" [Historia de la guerra del Peloponeso. Porrúa. México]). Sin embargo, fue hasta principios del siglo XX que las características básicas de este sistema fueron establecidas, en particular su capacidad de memoria, su propiedad de responder específicamente ante distintos antígenos y la función efectora de los anticuerpos (si bien éstos no fueron identificados como entidades moleculares hasta la segunda mitad del siglo) [14]. La respuesta celular de los mecanismos innatos fue observada por primera vez en la misma época, con la descripción del fenómeno de fagocitosis, presentando también evidencia de su ubicuidad en los metazoas [Metchikoff E. Immunity in the infectious diseases. Macmillan. Nueva York], pero su análisis a detalle ha sido realizado hasta muy recientemente.

válida para los vertebrados con mandíbula, un subphylum de los cordados; en el resto de los metazoas (~90 % de las especies existentes) el sistema inmune reside exclusivamente en las respuestas innatas [10,15]. Los mecanismos adaptativos están centrados en el sistema antígeno-específico de producción de opsonizadores y/o receptores —anticuerpos y receptores de células T— llevado a cabo por leucocitos mediante rearreglos génicos y expansión clonal. El sistema inmune adaptativo tiene, además, la propiedad de guardar memoria de retos previos, mediante la conservación de linfocitos diferenciados que mantienen los receptores seleccionados en contra de las entidades responsables de los retos anteriores [6,13].

En contraposición, mecanismos innatos están presentes en todos los metazoas [10,11,15]. La variedad de respuestas celulares y humorales que constituyen el sistema inmune innato están centradas en el reconocimiento de lo no-propio en forma de Patrones Moleculares (PMNP), esto es: estructuras químicas presentes en los parásitos potenciales y ausentes en el hospedero. Dicho reconocimiento está mediado por un número limitado de receptores codificados en la línea germinal del organismo, denominados Receptores de Reconocimiento a PMNP (RRP) [9,11]. Al censar el reto de lo no-propio, el sistema inmune innato responde mediante la activación rápida de mecanismos efectores, los cuales culminan con la eliminación de la entidad invasora, así como, en el caso de los vertebrados, la activación del sistema inmune adaptativo [12,13,16-18]. Los mecanismos que participan de manera temprana en la eliminación incluyen: fagocitosis por células especializadas (*v.g.* diversos tipos de hemocitos), cascadas proteolíticas (*v.g.* coagulación) y la síntesis y/o liberación de moléculas con actividad antiparasitaria directa (*v.g.* Péptidos AntiMicrobianos (PAM's)) [11,13,15,17]. Esta descripción general del sistema inmune innato aplica para todos los modelos metazoas estudiados a detalle [13,17-25], e incluso, en su acepción más amplia, para la respuesta inmune de todos los sistemas multicelulares [26]¶. De hecho, buena parte del renovado interés en la inmunidad innata deviene del reconocimiento de paralelismos entre los sistemas antimicrobianos inducibles de *Drosophila melanogaster* (ver más adelante) y las respuestas tempranas del sistema inmune de mamíferos (*v.g.* la respuesta de fase aguda) [15-17,27] (**Recuadro 1**). En ambos modelos, el reconocimiento de PMNP activa las respuestas tempranas a la infección, mediadas éstas por genes regulados por factores transcripcionales de la familia Rel/NF-κB [28], los cuales son liberados de su represor citoplásmico, IκB, como respuesta a cascadas de señalización iniciadas por receptores transmembranales de la familia Toll/IL-1R [29].

Los paralelismos mostrados en el **Recuadro 1** son, indudablemente, llamativos en el contexto de la evolución del los mecanismos innatos de defensa presentes en metazoas. En ambos modelos, mamíferos e insectos, están presentes proteínas homólogas, aunque no siempre con las mismas funciones [26]. Es importante recalcar que ni la respuesta mediada por Toll ni la vía IMD son, en *sensu stricto*, equivalentes a las cascadas activadas por, respectivamente, TLRs/IL-1R o el receptor a TNF-α. Lo anterior es reafirmado por el estudio de las respuestas inmunes de otros metazoas, así como de plantas que poseen homólogos de las proteínas involucradas, fundamentalmente especies peptídicas similares a Toll (*v.g.*

¶ En todos los sistemas eucariotes multicelulares existen paralelismos importantes en los módulos utilizados para discernir lo no-propio, sugiriendo un origen común de los sistemas inmunes innatos, por lo que se ha propuesto que precedería a la separación de los linajes eucariotes principales; sin embargo, las diferencias entre los distintos sistemas son considerables (ver más adelante en el texto). Una evaluación integral de las presiones evolutivas que han dado origen a los distintos sistemas multicelulares, antepone serias consideraciones a esta interpretación.

[35,36]). El estudio del nemátodo inmunocompetente *Caenorhabditis elegans* es relevante en este sentido, toda vez que posee homólogos de varias de las proteínas que participan en la vía mediada por Toll en *D. melanogaster*; sin embargo, ninguna de éstas parece estar involucrada en los mecanismos de defensa que este organismo despliega ante el reto microbiano. El sistema inmune de *C. elegans* es mediado por MAP cinasas de la familia p38 y proteínas receptoras de factores de crecimiento [24,25]. MAP cinasas de las familias JNK y p38 se han involucrado en la vía IMD de la respuesta inmune de *D. melanogaster* y la inducción de citocinas proinflamatorias en mamíferos [37]. Éstas y otras analogías (*v.g.* las familias de RRP conocidas como NOD, proteínas intracelulares encontradas en plantas y mamíferos que activan factores transcripcionales de la familia Rel/NF-κB [38], y las proteínas de unión a peptidoglicanos presentes en insectos y mamíferos [39]), sugieren que los sistemas inmunes innatos de los organismos multicelulares, si bien no son homólogos, habrían evolucionado mediante la cooptación diferencial de un número limitado de módulos proteicos, involucrados éstos en el reconocimiento de lo no-propio y la activación de las respuestas tempranas a la infección [11,18,20,26].

Recuadro 1. Activación inmune innata

Desde un punto de vista evolutivo, las dos entidades moleculares que más llaman la atención son los receptores transmembranales de la familia Toll/IL-1R y los factores transcripcionales Rel/NF-κB, toda vez que están conservadas en los dos linajes principales de metazoas: protostomas (la mayoría de los invertebrados) y deuterostomas (cordados, equinodermos y algunos tipos de gusanos). En *D. melanogaster*, el modelo protostoma mejor conocido, proteínas circulantes en la hemolinfa del organismo censan la presencia de hongos y bacterias Gram(+), por lo cual son consideradas RRP, activando cascadas proteolíticas, llevadas a cabo en la hemolinfa, que generan el ligando de Toll-1 (Spätzle). La unión de Spätzle a Toll-1 es transducida intracelularmente a través de dMyd88, Tube, Pelle y otras proteínas aún no identificadas, culminando en la fosforilación del inhibidor Cactus. La fosforilación de éste condiciona la liberación de DIF, el cual es un factor transcripcional de la familia Rel/NF-κB y el principal activador de la respuesta antimicótica de *D. melanogaster*. El efector final de esta ruta es el PAM Drosomicina [20,21]. En mamíferos, los deuterostomas más estudiados, los receptores homólogos de Toll (TLR 1-10) se encuentran expresados en células del sistema inmune innato, en las cuales censan directamente la presencia de PMNP, es decir: a diferencia de lo que sucede en el modelo de *D. melanogaster*, en mamíferos los TLR fungen como RRP. La señalización intracelular en este caso depende de las proteínas adaptadoras Myd88 e IRAK, homóloga de Pelle, y la activación del complejo IKK dependiente de las proteínas TRAF 6 y TAK 1. El complejo IKK fosforila a IκB, originando la liberación de proteínas de la familia Rel/NF-κB, las cuales a su vez son los factores transcripcionales responsables de la activación de la expresión de citocinas pro-inflamatorias y proteínas coestimuladoras de linfocitos T [12,18]. Notablemente, la expresión de defensinas β —PAM's encontrados en diversos epitelios de vertebrados— es transcripcionalmente regulada por proteínas de la familia Rel/NF-κB, dependiente ésta de la activación directa o indirecta de TLR [30-32]. En el genoma de *D. melanogaster* hay homólogos de TRAF 6, TAK 1 y las proteínas del complejo IKK; sin embargo ninguna de ellas parece estar involucrada en la cascada activada por Toll. Por el contrario, las dos últimas participan en un mecanismo de activación inmune independiente, llamado la vía IMD, el cual define la respuesta de la mosca ante bacterias Gram(-) [33]. El producto del gen *imd* es homólogo a la proteína efectora de mamíferos RIP, la cual participa en la cascada activada por el receptor a TNF-α. En la vía IMD actúan, además, la proteína efectora dFADD y la caspasa DREDD, ambas con homólogos claros en la vía activada por TNF-α en mamíferos (FADD y caspasa-8, respectivamente). Tanto la vía IMD como la del TNF-α pueden culminar en la activación de factores transcripcionales de la familia Rel/NF-κB, Relish en el caso de *D. melanogaster*, dependiente del complejo IKK [20,21]. En *D. melanogaster* las vías Toll e IMD regulan la mayor parte de los procesos asociados a la respuesta inmune de estos insectos [34].

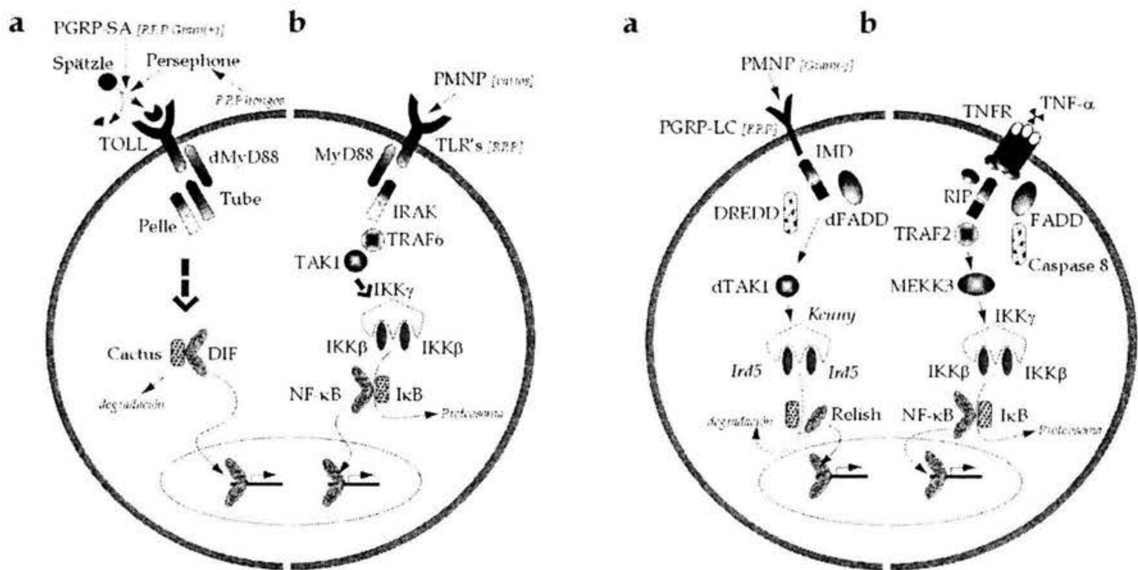

Figura R-1 | Izquierda: Transducción del reconocimiento de lo no-propio en *D. melanogaster* (**a**) y mamíferos (**b**). Derecha: Similitudes entre la vía IMD (**a**) y la señalización inducida por TNF-α (**b**).

Ahora bien, como se ha visto, no existe una relación directa entre los mecanismos de defensa innata seguidos por los dos linajes metazoas principales. Para comprender las relaciones que subyacen a los paralelismos documentados, en particular el reclutamiento de módulos proteicos homólogos y la conservación de algunos efectores, es necesario analizar la respuesta inmune de otros organismos, preponderantemente de aquellos que pertenecen a clases distintas de las ya estudiadas (Insecta, Mamalia y Chromadorea).

Péptidos antimicrobianos

Los mecanismos efectores activados por los sistemas de defensa innatos incluyen una batería de respuestas celulares y humorales, parte fundamental de las cuales reside en la producción y/o liberación de moléculas con actividad antiparasitaria directa. Uno de los elementos más sobresalientes de la respuesta humoral es la acción efectora de polipéptidos con función antibiótica¶, codificados en el genoma del hospedero y sintetizados en ribosomas, los cuales son conocidos nominalmente como PAM's. Moléculas dentro de esta categoría se han involucrado en la respuesta innata de todos los sistemas multicelulares en los cuales se han buscado [13,15,17,27,31,42,48-50]. De hecho, se estima que cada individuo del reino metazoa produce entre 5 y más de 40 PAM's distintos [42,49,51]. Los PAM's constituyen una categoría funcional y estructuralmente heterogénea, si bien comparten algunas características como la abundancia de residuos básicos y la anfipaticidad de la conformación activa. La mayoría de los PAM's se han clasificado en cuatro categorías

¶ Los PAM's en concentraciones micromolares son capaces de esterilizar poblaciones de 10^6 microorganismos en periodos cortos de tiempo (1 -10 minutos). Estas moléculas presentan, distintivamente, una alta especificidad por membranas de microorganismos, en particular de origen procariote; sin embargo, el mecanismo mediante el cual los PAM's actúan no ha sido del todo esclarecido. De hecho, la diversidad de estructuras moleculares que presentan los péptidos clasificados en esta categoría, sugiere que es poco probable que todos sigan un mismo patrón. En general el único modelo aceptado es el que describe la actividad de los PAM's α-helicoidales [40-42]; si bien, evidencia se acumula para el caso del los ricos en cisteína (v.g. [30,43-47]). Cabe resaltar que, en ambos casos, se ha descartado que el proceso de eliminación microbiana dependa exclusivamente de la actividad membranolítica de los PAM's, misma que fue vista durante muchos años como el mecanismo subyacente al efecto biológico de éstos.

estructurales [27,52]¶, a saber: péptidos lineales capaces de adoptar estructuras α-helicoidales al entrar en contacto con sistemas membranosos [53]; péptidos ricos en ciertos aminoácidos (*v.g.* Pro [54] o Trp [55,56]); péptidos con asas cerradas por un puente disulfuro [57,58], y; péptidos ricos en cisteínas, subdivididos a su vez en aquellos con estructuras netamente β (defensinas de vertebrados [30], protegrinas [45,59], taquiplesinas [60,61], adroctonina [44,62,63] y gomesina [64-66]), y moléculas peptídicas que adoptan el motivo estructural α/β Estabilizado por Cisteínas (α/β-EC), las cuales conforman el grupos más ampliamente distribuido de PAM's, encontrándose en una variedad de plantas (defensinas y tioninas γ [67]) e invertebrados (defensinas [58,68])§. En la **Tabla I-1** se presentan algunas secuencias representativas de cada una de estas categorías, mientras que en la **Figura I-1** se muestran estructuras tridimensionales representativas de cada grupo.

Tabla I-1 | *Secuencias de PAM's*

i. PAM's α-helicoidales			
Cecropin A	*Hyalophora cecropia*	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK*	P01507
Moricin	*Bombix mori*	PWNIFKEIERAVARTRDAVISAGPAVRTVAAATSVAS*	P48821
Cecropin A1	*D. melanogaster*	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATAR*	P14954
Sarcotoxin ID	*Sarcophaga peregrina*	GWIRDFGKRIERVGQHTRDATIQTIAVAQQAANVAATLK*	P18312
Cecropin C	*Anopheles gambiae*	RRFKKFLKKEGAGRRVANAAQKGLPLAAGVKGLV*	AAM82612
Cecropin A	*Aedes albopictus*	GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKAL*	P81417
Cecropin P1	*Ascaris suum*	SWLSKTAKKLENSAKKRISEGIAIAIXGGXR*	P14661/AW165880
Hadrurin	*Hadrurus gerstchi*	GILDTIKSIASKVWNSKTVQDLKRKGINWVANKLGVSPQAA	P82656
Pandinin 2	*Pandinus imperator*	FWGALAKGALKLIPSLFSSFSKKD	P83240
Magainin 2	*Xenopus laevis*	GIGKFLHSAKKFGKAFVGEIMNS	P11006
PGLa	*Xenopus leavis*	GMASKAGAIAGKIAKVALKAL*	Q99134
Dermaseptin S1	*Phyllomedusa sauvagei*	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	P24302
Dermaseptin B5	*Phy. Bicolor*	GLWNKIKEAASKAAGKAALGFVNEMV	P81487
Dermadistinctin K	*Phy. Distincta*	GLWSKIKAAGKEAAKAAAKAAGKAALNAVSEAV	P83638
LL37	*Homo sapiens*	FALLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	P49913
CRAMP	*Mus musculus*	ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	P51437
ii. PAM's ricos en ciertos aminoácidos			
Apidaecin IA	*Apis mellifera*	GNNRPVYIPQPRPPHPRI	P35581
Apidaecin Bp	*Bombus pasuorum*	GNRPVYIPPPRPPHPRL	P81464
Abaecin	*Apis mekkufera*	YVPLPNVPQPGRRPFPTFPGQGPFNPKIKWPQGY	P15450
Abaecin Bp	*Bomb. pasuorum*	FVPYNPPRPGQSKPFPSFPGHGPFNPKIQWPYPLPNPGH	P81463
Metchnikowin A	*D. melanogaster*	HRHQGPIFDTRPSPFNPNQPRPGPIY	Q24395
Penaedin-1	*Litopenaeus vannamei*	YRGGYTGPIPRPPPIGRPPLRLVVCACYRLSVSDARNCCIKFGSCCHLVK	P81056
Penaedin-3	*Litopenaeus vannamei*	QVYKGGYARPIPRPPPFVRPLPGGPIGPYN... ...GCPVSCRGISFSQARSCCSRLGRCCHVGKGYSG	P81060
PR-39	*Sus scrofa*	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFPGKR	P80054
Bac-5	*Bos taurus*	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFPGRR	P19660
Indolicidin	*Bos taurus*	ILPWKWPWWPWRRG	P33046
Tritrp	*Sus scrofa*	VRRFPWWWPFLRR	1D6X
iii. PAM's con asas cerradas			
Thanatin	*Podisus maculiventris*	GSKKPVPIIYCNRRTGKCQRM	P55788
Brevinin 1	*Rana Brevipoda*	FLPVLAGIAAKVVPALFC-KITKKC	P32423
Brevinin 2	*R. brevipoda*	GLLDSLKGFAATAGKGVLQSLLSTASC-KLAKTC	P32424
Brevinin 2Ed	*R. esculenta*	GILDSLKNLAKNAGQILLNKASC-KLSGQC	P40840
Esculentin 1	*R. esculenta*	GIFSKLGRKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGC-KIKGEC	P32414
Gaegurin 4	*R. rugosa*	GILDTLKQFAKGVGKDLVKGAAQGVLSTVSC-KLAKTC	P80398
Rugosin B	*R. rugosa*	SLFSLIKAGAKFLGKNLLKQGAQYAAC-KVSKEC	P80955
Ranalexin	*R. catesbiana*	FLGGLIKIVPAMIC-AVTKKC	P39084
iv. PAM's ricos en cisteínas			
2 puentes disulfuro* 1-4,2-3			
Tachyplesin I	*Tachypleus tridentatus*	---KWCF--RVCYR-GICYRRCR----	P14213
Tachyplesin III	*Tach. Gigas*	---KWCF--RVCYR-GICYRKCR----	P18252

¶ La base de datos más completa puede consultarse en: http://www.bbcm.univ.trieste.it/~tossi/pag1.htm. En ella se enlistan más de 750 PAM's.

§ Péptidos que adoptan el motivo estructural α/β-EC se han encontrado en plantas e invertebrados, en los cuales llevan a cabo funciones muy diversas. Un análisis detallado sobre los péptidos que adoptan este motivo estructural se presenta como anexo a esta disertación.

Péptido	Secuencia	Acceso
Polyphemusin I|*Limulus polyphemus*	--RRWCF--RVCYR-GFCYRKCR----	P14215
Androctonin|*Androctonus australis*	----QCR--RLCYK-QRCVTYCRGR--	P56684
Gomesin|*Acanthoscurria gomesiana*	--RSVCRQIKICRRRGGCYYKCTNRPY	P82358
Protegrin I|*Sus scrofa*	RGGRLC----YCRR-RFCV--CVGRG-	P32194
Defensinas α[a] 1-6,2-4,3-5		
HNP-1|*Homo sapiens*	--------AC-----YCRIPACIAGERRYGTCIYQGRLWAFCC----	P59665
HNP-3|*Homo sapiens*	--------DC-----YCRIPACIAGERRYGTCIYQGRLWAFCC----	P59666
HNP-4|*Homo sapiens*	VSGSTRGMVC-----SCRLVFCRRTELRVGNCLIGGVSFTYCCTRVD	P12838
HAD-5|*Homo sapiens*	-------ATC-----YCRTGRCATRESLSGVCEISGRLYRLCCR---	Q01523
HAD-6|*Homo sapiens*	------AFTC-----HCR-RSCYSTEYSYGTCTVMGINHRFCCL---	Q01524
RhAD-1|*Macaca mulata*	--------AC-----YCRIPACLAGERRYGTCFYLGRVWAFCC----	P60030
Cryptidin-1|*Mus musculus*	----LRDLVC-----YCRSRGCKGRERMNGTCRKGHLLYTLCCR---	P11477
Cryptidin-5|*Mus musculus*	----SKKLIC-----YCRIRGCKRRERVFGTCRNLFLTFVFCCS---	P28312
MCP-1|*Oryctolagus cuniculus*	-------VVC-----ACRRALCLPRERRAGFCRIRGRIHPLCCRR--	P01376
RK-1|*Oryctolagus cuniculus*	-------MPC-----SCK-KYCDPWEVIDGSCGLFNSKY-ICCREK-	P81655
GNCP-1|*Cavia porcellus*	-------RRC-----ICTTRTCRFPYRRLGTCIFQNRVYTFCC----	P11478
Gallinacin alpha|*Gallus gallus*	----GRKSDCFRKNGFCAFLKCPYLTLISGKCS----RFHLCCKRIW	P46157
Defensinas β[a] 1-5,2-4,3-6		
HBD-1|*Homo sapiens*	-----------DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK-------------	P60022
HBD-2|*Homo sapiens*	--------GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP-----------	O15263
HBD-3|*Homo sapiens*	-----GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK----------	P81534
HBD-4|*Homo sapiens*	--------EFELDRICGYGTARCRKK-CRSQEYRIGRCP-NTYACCLRKWDESLLNRTKP	Q8WTQ1
RhBD-1|*Macaca mulatta*	-----------DHYNCVRSGGQCLYSACPIYTRIQGTCYHGKAKCCK-------------	O18794
mBD-1|*Mus musculus*	-GILTSLGRRTDQYKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCKS------------	P56386
mBD-2|*Mus musculus*	VGSLKSIGYEAELDHCHTNGGYCVRAICPPSARRPGSCFPEKNPCCKYMK----------	P82020
RBD-2|*Rattus norvegicus*	-------QSINNPITCLTKGGVCWGP-CTGGFRQIGTCGLPRVRCCKKK-----------	O88514
gBD-1|*Capra hircus*	-----------SRRSCHRNKGVCALTRCPRNMRQIGTCFGPPVKCCRKK-----------	O97946
sBD-1|*Ovis aries*	-------QGVRNRLSCHRNKGVCVPSRCPRHMRQIGTCRGPPVKCCRKK-----------	O19038
TAP|*Bos taurus*	-----------NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK-----------	P25068
LAP|*Bos taurus*	----GFTQGVRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK-----------	Q28880
BNBD-5|*Bos taurus*	-------QVVRNPQSCRWNMGVCIPISCPGNMRQIGTCFGPRVPCCRRW-----------	P46163
BNBD-11|*Bos taurus*	-----------GPLSCRRNGGVCIPIRCPGPMRQIGTCFGRPVKCCRSW-----------	P46169
THP-1|*Meleagris gallopavo*	-----G-----KREKCLRRNGFCAFLKCPTLSVISGTCS-RFQVCCKTLLG---------	P80391
Gallinacin 2|*Gallus gallus*	-------------LFCK--GGSCHFGGCPSHLIKVGSCF-GFRSCCKWPWNA--------	P46158
Defensinas α/β-EC[a,b] 1-4,2-5,3-6,(N-C)		
DefA_PROTE|	----ATCDLLS----GTGINHSACAAHCLLRG---NR-GGYCNGKG---VCVCRN------------	P10891
Sapecin_SARPE|	----ATCDLLS----GTGINHSACAAHCLLRG---NR-GGYCNGKA---VCVCRN------------	P18313
SapecinB_SARPE|	----LTCEIDR----------SLCLLHCRLKG---YL-RAYCSQQK---VCRCVQ------------	P31529
AlCRP_ACALU|	----AICYPLS------------CNQICFPR-------LGSCY---Y-NTCRCN-------------	BAC78809
Tenecin_TENMO|	----VTCDILSVEAKGVKLNDAACAAHCLFRG---RS-GGYCNGKR---VCVCR-------------	Q27023
Royalisin_APIME|	----VTCDLLS---FKGQVNDSACAANCLSLG---KA-GGHCEKV----GCICRKTSFKDLWDKRFG	P17722
Def_PALPR|	----ATCDALSFSSKWLTVNHSACAIHCLTKG---YK-GGRCVNT----ICNCRN------------	P80407
Galleremycin_GALME|	----PGCVFYE------------CIANCRSRG---YKNGGYCTIN----GCQCLR------------	AAM46728
DefA_MAMBR|	----ASCYLLD----GYAAGRDDCRAHCIAPR---NR-RLYCASYQ---VCVCRY------------	AAL69980
Heliomicin_HELVI|	DKLIGSCVWG------AVNYTSDCNGECKRRG---YK-GGHCGSFAN-VNCWCET------------	P81544
Termicin_PSEUS|	-----ACNFQS------------CWATCQAQHSIYFR-RAFCDRS----QCKCVFVRG---------	P82321
Def_AESCY|	---GFGCPL----------DQMQCHRHCQTIT---GRSGGYCSGPLK-LTCTCYR------------	P80154
Def_LEIQU|Scorpiones	---GFGCPL----------NQGACHRHCRSIR---RR-GGYCAGFFK-QTCTCYRN-----------	P41965
MGD-1_MYTGA|Mollusks	---GFGCP-----------NNYQCHRHCKSIP---GRCGGYCGGWHR-LRCTCYRCG----------	P80571
ASABFe_ASCSU|Nematode	----ATCGYDD---AKLNRPTIGCILSCKVQG---CE-TGACYLRDSRPICVCKRC-----------	BAC57591
Drosomycin|*D. melanogaster*	-DC---LSGRYKGPCAVWDNETCRRVCK-EEGRSSGHCSP---SLKCWCEG-C-----	P41964
gamma1-P|Plant	KIC-RRRSAGFKGPCM--SNKNCAQVCQ-QEGWGGGNCDG--PFRRCKCIRQC-----	P20158
gamma1-H|Plant	RIC-RRRSAGFKGPCV--SNKNCAQVCM-QEGWGGGNCDG--PLRRCKCMRQC-----	1gpt
Rs-AFP1|Plant	KLC-ERPSGTWSGVCG--NNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC-----	AAA69541
Ah-AMP1|Plant	-LCNERPSQTWSGNCG--NTAHCDKQCQDWEKASHGACHKRENHWKCFCYFNC-----	1bk8
Psd1|Plant	KTC-EHLADTYRGVCF--TNASCDDHCKNKAHLISGTCHN----WKCFCTQNC-----	P81929
PhD1|Plant	-----------DSVCI--NKKPCVACCK-KAKFSDGHCSK--ILRRCLCTKECVFEKT	AAN64750

[a] La secuencia de los péptidos de estos grupos se encuentran alineadas con el programa CLUSTAL_X [69].

[b] Consultar el anexo a esta disertación para una lista más extensiva de secuencias dentro de este grupo estructural.

El papel crítico que desempeñan los PAM's en la defensa del hospedero ha sido establecido sólidamente a partir de diversas líneas y modelos de estudio. Cronológicamente, la primera evidencia sobre la importancia de los PAM's fue la identificación de las entidades moleculares responsables de la actividad microbicida inducible en la hemolinfa de insectos [71], un fenómeno que había sido registrado por primera vez 75 años antes [7,14]. Poco tiempo después, los componentes principales de los gránulos primarios de neutrófilos —células del sistema inmune innato de vertebrados [72]— fueron identificados como PAM's

ricos en cisteínas [73]. Evidencia más directa devino del estudio de mutaciones asociadas a fenotipos inmunodeficientes de *D. melanogaster*, estableciéndose que las respuestas antibacterianas [33] y antimicóticas [74] dependían, en última instancia, de la producción de PAM's (ver **Recuadro 1**). Más recientemente, organismos genéticamente manipulados para anular la expresión de uno o varios PAM's, han demostrado que su presencia es indispensable para evitar la colonización de bacterias patógenas, e incluso para mantener el equilibrio entre el hospedero y sus simbiontes (*v.g.* [75-77]; en contraposición, la expresión transgénica de un sólo PAM puede aumentar (*v.g.* [78-82]) o restituir, en modelos deficientes en PAM's [83,84], la capacidad del hospedero para combatir infecciones microbianas.

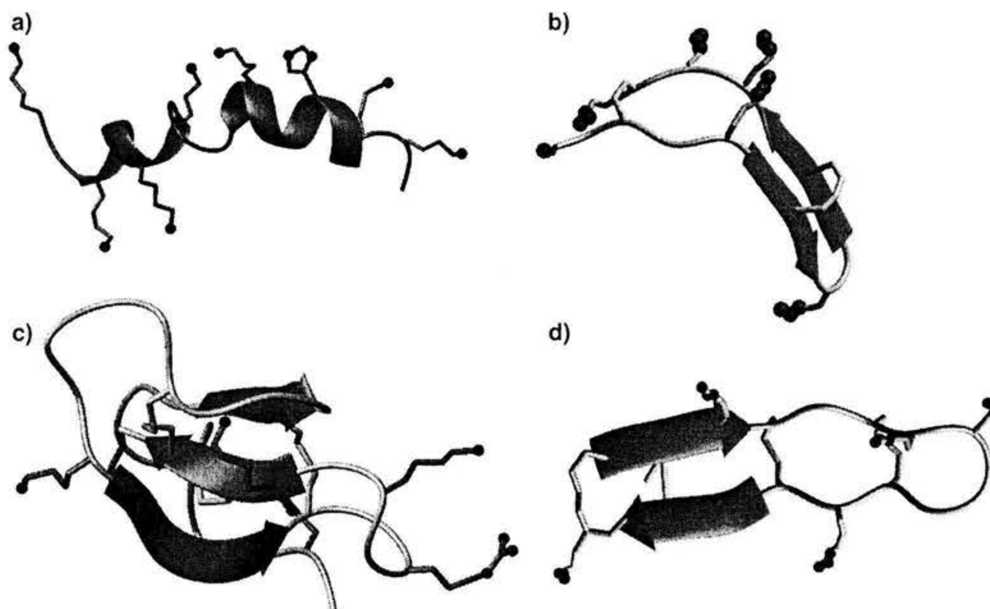

Figura I-1 | *Estructuras tridimensionales de algunos PAM's.* Estructuras tridimensionales del PAM α-helicoidal híbrido $CE_{1\text{-}8}MA_{1\text{-}12}$ (**a**), la taquiplesina I de *T. tridentatus* (**b**), la defensina β-2 de humano (**c**) y la defensina θ de mono rhesus (**d**), representadas con el programa MOLMOL [70]. Los códigos de acceso son, respectivamente: 1f0d, 1ma2, 1fd3 y 1hvz. Los residuos básicos (Arg, His y Lis) y los puentes disulfuro se muestran en detalle.

ANTECEDENTES

PAM's involucrados en el componente humoral del sistema inmune de los invertebrados

La vasta batería de técnicas genéticas disponibles para el estudio del díptero *D. melanogaster* (Neóptera, Insecta), ha permitido que esta mosca sea el modelo arquetípico para la disección de los mecanismos inmunes innatos de invertebrados del linaje protostoma. Dos vías principales regulan la respuesta sistémica de este organismo ante bacterias Gram(-) (la vía IMD) y Gram(+) y hongos (la vía que depende de Toll) [33,34,74,85]. En ambos casos, la expresión de PAM's específicos [86] es inducida en el cuerpo graso —aunque también participan hemocitos [87-89] y células epiteliales en las respuestas locales [89,90]— por la acción directa de factores transcripcionales de la familia Rel/NF-κB —Relish y DIF respectivamente—, para después ser liberados al torrente (hemolinfa), en donde participan

en forma crítica en el proceso de eliminación de las entidades invasoras [20,21]. Este mecanismo de activación inmune ha sido verificado en otros insectos de los órdenes Díptera [22,23,91,92] y Lepidóptera [93,94], así como en arácnidos del orden Ixodida [95-98], anélidos de la clase Hirudinida [99] y nemátodos de la clase Chromadorea [36,100]. Como se mencionó anteriormente, algunas defensinas β de mamíferos son, de forma análoga, transcripcionalmente inducidas en las respuestas locales de epitelios por proteínas de la familia Rel/NF-κB, las cuales son activadas como respuesta al reconocimiento de lo no-propio mediado por receptores de la familia TLR/IL-1R [30-32]. La amplia distribución filogenética de los organismos que recurren a esta estrategia de inducción de PAM's, ha constituido una de las fuerzas motrices del renovado interés en la inmunidad innata; sin embargo, como en el caso de las cascadas transduccionales, el estudio del contexto en el cual participan los PAM's en otros organismos ha revelado una alta diversidad en las estrategias de activación de PAM's.

En varios invertebrados del linaje protostoma la activación de PAM's sigue un camino alternativo que reside en el almacenamiento de éstos en células especializadas, las cuales liberan su contenido a la hemolinfa en respuesta al reto de los potenciales parásitos. El modelo mejor estudiado son quelicerados del orden Xiphosura (Merostomata, Chelicerata), cuyos hemocitos contienen gránulos ricos en PAM's y otras moléculas involucradas en la defensa del hospedero, los cuales son vaciados hacia el torrente al censar PMNP¶ [19]. Esta estrategia de almacenamiento de PAM's en hemocitos y su liberación sistémica ha sido descrita en otros quelicerados (el arácnido *Ac. gomesiana* [66,102]), así como en crustáceos de la clase Malacostraca (el camarón *Litopenaeus vennamei* [103-105]) e incluso moluscos de la clase Bivalvia (los mejillones *Mytulis edulis* y *M. galloprovinsialis* [106]). Este escenario se asemeja al descrito para defensinas α y cathelicidinas en mamíferos, las cuales son almacenadas en los gránulos de neutrófilos y liberados al fagosoma o el medio circundante en respuesta al reconocimiento de lo no propio [30,31]. En este caso, como en el anterior, la diversidad de organismos que recurren a esta estrategia ha servido para argumentar que ésta constituiría una de las respuestas ancestrales de los hospederos ante el reto parásito. El panorama se diversifica al reconocer una tercera alternativa, que ha sido propuesta para el caso de la termita *Pseudocanthotermes spiniger* (Isóptera, Insecta) [107] y los alacranes *Leiurus quinquiestratus habraeus* [108] y *Androctonus australis* [62]. En estos organismos la producción y/o liberación de PAM's sería independiente de la activación inmune.

Con base en lo expuesto en los párrafos anteriores, la participación de PAM's en las respuestas humorales de invertebrados parecería ser más o menos homogénea en cada clase de invertebrados: en insectos y nemátodos Chromadorea, fundamentalmente mediante la activación transcripcional de éstos; en crustáceos Malacostraca y moluscos Bivalvia por vía de su liberación a partir de hemocitos. Sin embargo, la regulación a nivel transcripcional de PAM's registrada en arácnidos, así como los organismos que parecen no responder activamente al reto de los potenciales parásitos (termitas y alacranes), resaltan la necesidad de contar con una mayor diversidad de modelos biológicos, con el fin de esclarecer las relaciones evolutivas que subyacen al desarrollo del sistema inmune de invertebrados. De particular interés resulta el caso de los arácnidos, toda vez que sus PAM's parecen estar participando en las tres estrategias alternativas en distintos organismos de esta clase.

¶ La cascada de coagulación de estos organismos ha sido uno de los sistemas más utilizados para determinar la presencia del LPS, el principal componente de la membrana externa de bacterias Gram(-). Los hemocitos granulares de estos quelicerados liberan su contenido ante concentraciones de LPS en el orden de 10^{-10} M [101].

PAM's en arácnidos

Los arácnidos constituyen la segunda clase más diversa de metazoas con ~100000 especies descritas, la mayoría de ellas pertenecientes a los órdenes Aranae o Acari [109,110]. Estos artrópodos habitan en casi todos los ecosistemas terrestres actuales; de hecho constituyen una de las clases terrestres más antiguas —fósiles de alacranes se encuentran en sedimentos con 410 millones de años de antigüedad (MA) [109,111]. Los nichos ecológicos ocupados por los arácnidos son diversos: algunas especies son parásitos obligados (*v.g.* garrapatas), mientras que otras son depredadores estrictos (*v.g.* arañas y alacranes). Los organismos de esta clase han sido estudiados, fundamentalmente, por su importancia médica; ya sea porque son vectores de patógenos humanos (*v.g.* varios tipos de garrapatas [112-114]) o por el efecto neurotóxico de sus venenos, que en ocasiones causan accidentes fatales [115]. El estudio de los arácnidos ponzoñosos —arañas y alacranes— ha sido también estimulado por la utilidad que las entidades moleculares responsables de la acción tóxica significan para el campo de la fisiología [116,117]¶.

Ahora bien, PAM's involucrados —de forma análoga a otros grupos de invertebrados— en la defensa sistémica de estos organismos han sido aislados a partir de arácnidos de los órdenes Scorpionidae [62,108], Aranae [64,102] e Ixodida [98,118-120]. Todas estas moléculas, excepto una encontrada en la araña *Ac. gomesiana* [102], se ubican en la categoría de los PAM's ricos en cisteínas, tanto del grupo con estructuras netamente β [61-64], como del correspondiente a las defensinas de invertebrados [62,98,108,118-120]. En todos los casos estos PAM's se encuentran en la hemolinfa de los organismos respectivos, ya sea constitutivamente [62,108] o liberados en respuesta a reto inmune, proceso que puede ser [95-98] o no [66,102] dependiente de activación transcripcional. Adicionalmente, péptidos con actividad antibiótica se han encontrado formando parte del veneno de varias especies de arañas y alacranes [121]; la mayoría de éstos parecen fungir como agentes citolíticos irrestrictos, esto es: a diferencia de la mayoría de los PAM's, rompen con la misma efectividad membranas biológicas de origen diverso. Estos péptidos asemejan al bien conocido modelo de la melitina de abeja, un péptido con características estructurales similares a los PAM's α-helicoidales, la cual actúa facilitando la acción de los componentes neurotóxicos del veneno [122].

JUSTIFICACIÓN Y OBJETIVOS

El estudio de invertebrados protostoma ha revelado un panorama —en creciente expansión— de la diversidad de mecanismos involucrados en el sistema inmune innato de los hospederos, que aplica, incluso, al estudio de los miembros del otro linaje principal de metazoas (deuterostomas); se han registrado tanto analogías notables, como substanciales diferencias entre los distintos modelos caracterizados. Sin embargo, no han sido esclarecidos aspectos fundamentales de los paralelismos descritos (*v.g.* los mecanismos moleculares de la regulación del proceso de liberación de PAM's a partir de hemocitos), así como las determinantes evolutivas de la diversificación de estos sistemas (*v.g.* la cooptación de módulos proteicos homólogos para funciones diferenciadas). El análisis de los sistemas innatos de defensa de otros modelos biológicos, ciertamente ayudaría a establecer las relaciones evolutivas que subyacen a la diversidad de mecanismos de defensa empleados

¶ Una discusión más amplia sobre las toxinas de alacrán se presenta como anexo a esta disertación.

por los organismos multicelulares en contra del reto de los potenciales parásitos. En este escenario se inserta el trabajo objeto de esta exposición; en éste se analiza la respuesta al reto séptico del alacrán Mexicano *Centruroides limpidus limpidus* (Buthidae, Scorpiones, Arachnida, Arthropoda), con especial énfasis en la caracterización de especies peptídicas que pudiesen estar involucrados en ésta.

Adicionalmente, como se explica en el anexo a esta disertación, el veneno de los alacranes está compuesto mayoritariamente por una batería de neurotoxinas que adoptan el motivo estructural α/β-EC. Como se ha visto, PAM's con el mismo motivo estructural se han encontrado en la hemolinfa de dos alacranes Buthidae, sugiriendo que ambos tipos de proteínas constituirían un grupo monofilético —siendo parálogos en los alacranes— (lo cual no es evidente a partir del uso de las técnicas de evaluación de filogenia molecular). Un análisis integral de los péptidos y los genes que los codifican ayudaría a esclarecer las relaciones evolutivas en esta familia estructural, en particular podría sugerir elementos estructurales que definirían el destino funcional de los péptidos que adoptan el motivo α/β-EC. La identificación y caracterización de péptidos similares a defensinas en otros alacranes sustentaría el origen parálogo de ambos tipos de proteínas, añadiendo información relevante en el contexto evolutivo que habría dado origen a la enorme variedad de toxinas de alacrán conocidas.

Experimentación y Resultados

Esta sección está dividida en tres partes: en la primera se exponen las estrategias experimentales empleadas, ubicándolas dentro del contexto de las disciplinas en las que se integran; en la segunda se reproduce el artículo publicado, el cual contempla el grueso de los resultados obtenidos durante el desarrollo de este proyecto, y; en la última se presenta una breve exposición de resultados dentro del mismo proyecto, pero que no fueron incluidos en el manuscrito referido.

Estrategias experimentales y metodología general

Organismos: Durante el desarrollo del proyecto se manejaron miles de individuos de la especie *C. limpidus limpidus*, la cual, junto con las subespecies *C. l. tecomanus*, *C. infamatus infamatus* y *C. sufussus sufussus*, son las responsables de la mayoría de los accidentes ocasionados por alacranes en México. El estudio de los mecanismos de inmunidad en condiciones de laboratorio requiere que la población sea suficientemente homogénea en su interacción con el medio, con el propósito de minimizar las posibles influencias que las condiciones de manutención pudiesen ejercer sobre el sistema de defensa del hospedero [123,124]. En consecuencia, todos los experimentos comparativos (ver más adelante) se realizaron con hembras del mismo peso (~ 1 g), mantenidas sin alimentación, pero con agua, por dos semanas y en densidades de 7 a 10 individuos por dm^2. Para evaluar el efecto de la infección aguda, los organismos fueron inoculados con una mezcla de bacterias con ~ 10^3 unidades formadoras de colonia (u.f.c), mediante punción directa a la hemolinfa. Dos grupos control fueron usados, uno es el de alacranes sin reto inmune (organismos *naive*) y el otro es el de organismos con el mismo daño mecánico, pero en condiciones asépticas. La infección experimental tiene un ligero efecto sobre la mortalidad de los organismos, consistentemente entre 5 y 6 % de los organismos infectados murió en menos de 48 hrs, mientras que este valor en grupos control por daño mecánico no fue mayor al 2 %.

Despliegue diferencial: La identificación de moléculas involucradas en la respuesta al reto séptico se evaluó mediante tres metodologías. En las técnicas de "peptidómica" —i.e. identificación de péptidos presentes en una condición dada, ya sea mediante mapeo sistemático o comparativo [125], estas técnicas también son llamadas "despliegue diferencial de péptidos" [126]— se emplearon dos tipos de muestras: una con hemolinfa libre de células y la otra una subfracción de ésta obtenida a partir de HPLC, en ambos las muestras comparadas fueron normalizadas para contener la misma cantidad de proteína total. En la primera estrategia utilizada se analizaron los perfiles cromatográficos obtenidos mediante HPLC, evaluando la prevalencia de las subfracciones con respecto a la proteína total (*v.g.* [102,127-133]); en la segunda, las muestras se analizaron por medio de Espectrometría de Masas (EM) tipo MALDI-TOF, identificando las diferencias de intensidad de las señales asociadas al reto séptico (*v.g.* [130,132-137]); en la tercera metodología se analizó la expresión de un componente, previamente identificado en las otras dos, mediante RT-PCR semicuantitativo [138], usando como control el gen de la subunidad ribosomal 18S.

Miscelánea de métodos experimentales: Los componentes con actividad antimicrobiana se identificaron mediante ensayos microbiológicos típicos: inhibición de crecimiento bacteriano en medio sólido (placas de LB/agar) y líquido (microdiluciones en medio Müeller-Hinton). Dado que las muestras probadas fueron de naturaleza peptídica, se emplearon algunas modificaciones a los ensayos tradicionales [139,140], con el propósito de considerar los efectos de difusión (para los ensayos en medio sólido) [141-143] e inactivación por absorción en las placas (para los ensayos en medio líquido) [124,143-145]¶. Los procedimientos bioquímicos incluyeron, entre otros: separaciones cromatográficas en fase reversa; electroforesis tipo Schägger - von Jagow [146]; cuantificación cromatográfica de aminoácidos mediante hidrólisis ácida [147,148]; determinación de los coeficientes de absortividad molar para los componentes aislados [149]; cuantificación de proteína total por el método de Bradford [150], y; los procedimientos bioquímicos y de biología molecular descritos en los siguientes incisos de esta sección.

Resultados publicados

Notas aclaratorias: i) La evaluación de las respuestas asociadas al reto séptico y la caracterización extensiva de un componente involucrado en la respuesta a éste constituyeron el cuerpo principal del manuscrito que a continuación se reproduce, mismo que ha sido publicado en la revista *Cellular and Molecular Life Sciences.* ii) Algunos datos que aparecen como resultados no mostrados, se presentan en la **Figura E-1**: a) perfiles cromatográficos de la hemolinfa a diferentes tiempos posteriores a la infección experimental. b) ensayos de RT-PCR para el gen que codifica para Cll-dlp. iii) Las referencias [151-170] comprenden artículos no referidos hasta aquí, respetando la numeración secuencial que contempla el trabajo publicado.

¶ El proceso modificado se encuentra en vías de aprobación por el National Committee of Clinical Laboratory Standards (NCCLS) y puede consultarse en: www.cmdr.ubc.ca/bobh/methods.php.

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Research Article

Antimicrobial peptide induction in the haemolymph of the Mexican scorpion *Centruroides limpidus limpidus* in response to septic injury

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Abstract. Antimicrobial peptides (AMPs) are essential components of host defences against infectious microrganisms. In chelicerate organisms they have been implicated in three alternative defensive systems: one is defined by the immediate up-regulation of genes encoding AMPs, another is characterized by the inducible systemic release of AMPs from cellular reservoirs and the third alternative is the systemic constitutive production of AMPs. In this work we used a differential high-performance liquid chromatography and mass spectrometry approach to show that septic injury elicits an immune response in the haemolymph of the Mexican scorpion *Centruroides limpidus limpidus*. We isolated several haemolymph components, one of which one was characterized extensively (amino acid sequence, disulphide pairing, cDNA and genomic clones) and demonstrated to be a novel member of the invertebrate defensin family and consequently named *C. limpidus limpidus* defensin-like peptide (Cll-dlp). This peptide accumulates in the haemolymph in response to septic injury, independently of transcriptional regulation.

Key words. Antimicrobial peptide; cysteine-stabilized α/β motif; defensin; haemolymph; innate immunity; scorpion.

Arthropods are the mostly widely spread phylum among living animals, comprising at least 75% of all extant metazoas and inhabiting an outstanding variety of ecological niches, where they cohabit with a huge diversity of potentially harmful microorganisms. This evolutionary success can be associated, in part, with their relatively simple but highly effective innate immune system [1, 2]. Their effectiveness relies primarily in the recognition of infectious non-self organisms and consequent activation of cellular and humoral responses leading to the clearance of foreign invaders [3–5]. Analysis of invertebrate immunity reveals striking similarities between their strategies of non-self recognition and the early immune responses of vertebrate organisms [6–8].

Antimicrobial peptides (AMPs) are a highly diverse category of gene-encoded molecules that critically participate in the clearance phenomenon by humoral processes, mainly due to their antibiotic properties, but also as effectors that can drive the whole process [9–12]. Apart from their diversity, AMPs share a few important structural characteristics, like the presence of several basic residues and the amphipathic character of the functional conformation of their polypeptide chains. Four families of AMPs have been recognized on the basis of structural

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similarities [9], namely: linear peptides that can adopt amphipathic α-helical structures in membrane environments; cysteine-rich peptides, closely packed by disulphide bridges; linear peptides with an unusually high content of certain amino acids (e.g. Pro, His or Trp), and relatively long polypeptides rich in Gly residues (a comprehensive list is available in the Antimicrobial Peptides Database at www.bbcm.univ.trieste.it/~tossi/pag1.htm).

Among cysteine-rich AMPs, the largest family corresponds to the invertebrate and plant defensins with the cysteine-stabilized α/β (CS-$\alpha\beta$) motif, which is defined by the presence of two disulphide bridges that join the consensus sequence...Cys$_i$-Xaa-Xaa-Xaa-Cys$_{i+4}$...Cys$_j$-Xaa-Cys$_{j+2}$..., through a Cys$_i$-Cys$_j$ and Cys$_{i+4}$-Cys$_{j+2}$ pairing [13, 14]. The CS-$\alpha\beta$ motif is a remarkably versatile framework shared by a variety of peptides with different functions, including the antimicrobial defensins from invertebrates [1, 15] and plants [16], the scorpion toxins, which are ion channel modulators [17], the sweet-tasting protein brazzein [18] and the rapeseed class of plant serine protease inhibitors [19]. Defensins containing the CS-$\alpha\beta$ motif have been found in three different phyla of invertebrates: arthropods [15], molluscs [20, 21] and nematodes [22–24]. At present, the most representative group comes from arthropods of the insect class, particularly from different orders of the subclass Neoptera.

From the best-characterized models of invertebrate immunity, in particular from the Diptera (Insecta) order [4, 5] and the Xiphosura (Merostomata) [3], two alternative mechanisms of systemic immune activation have been found. One is characterized by the inducible transcription of genes encoding AMPs in response to an immune challenge. The other is defined by the storage of AMPs in haemocyte granules which are either fused with phagocyted microbes or delivered to the haemocell after nonself recognition. Many insect orders appear to follow the first strategy [4, 5, 25–27], whereas some arachnids (the spider *Acanthoscurria gomesiana* [28, 29]), merostomatans (the horseshoe crab *Tachypleus tridentatus* [3]), malacostracans (the shrimp *Litopenaeus vannamei* [30, 31]) and molluscs (the edible mussels *Mytilus* spp. [32]) seem to adopt solely the second mechanism. However, at least in termites [33] and scorpions [34, 35], a third mechanism seems to be present, by which the AMPs are systemically constitutive and independent of an exogenous immune challenge. Intriguingly, the production of AMPs in the Ixodida (Arachnida) [36–39] and the relatively distant class Chromadorea (Nematoda) [40] are inducible by immune challenge.

In the subphylum Chelicerata the three different mechanisms of AMPs production were shown to exist [3, 28, 29, 34–39, 41]. Specifically regarding AMPs from scorpions, 9 different peptides have been documented, 5 were isolated from the venom of 4 different species of the Scorpionoidea superfamily [41] and 4 were found in the haemolymph of 2 Buthoidea species [34, 35]. In all the cases, AMPs appear as constitutive components. Four of such peptides present the consensus sequence of CS-$\alpha\beta$ motif.

In the present work, we describe the immune response caused by septic injury in the Mexican scorpion *Centruroides limpidus limpidus* (Buthidae, Scorpiones, Arachnida, Chelicerata). We isolated and characterized several components from the haemolymph of control and experimentally challenged organisms, using a combined differential high-performance liquid chromatography (HPLC) and mass spectrometry (MS) approach, as well as a semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). One of the inducible components was studied in detail, and shown to be a novel member of the invertebrate defensin family.

Material and methods

Animals, septic injury and haemolymph collection

Adult scorpions of the species *C. limpidus limpidus* were collected in the field (state of Guerrero, Mexico) and maintained in the laboratory with water and food (*Tenebrio* larvae and crickets) for several months. Experimental and control groups consisted of female animals (approximately 1 g body weight each), which were deprived of food (water ad libitum) for 2 weeks before experimentation. Scorpions can live perfectly well and healthily in captivity for up to month without solid food, but with water.

Individuals from test groups were pricked with a fine needle soaked in a mixture of bacteria (*Escherichia coli* DH5α and *Bacillus subtilis*). Two control groups were either kept untreated (naïve scorpions) or pricked with a sterile needle. Haemolymph (25–35 µl per scorpion) was collected by a puncture between the second and third segments of the scorpion metasoma, and collected into ice-cooled plastic tubes. Haemolymph was extracted from control and infected groups (50 specimens each) at 6, 12, 24 and 48 h. To obtain sufficient polypeptide amounts for further biochemical characterization, haemolymph was collected in a separate experiment from 1000 scorpions (male and female individuals) 24 h after bacterial inoculation.

Haemolymph fractionation and peptide purification

The following procedure was adapted from Hetru and Bulet [42]. After collection, haemolymph was immediately centrifuged (Beckman OptimaTL ultracentrifuge) for 30 min at 4°C and 30,000 g to remove cellular content. The supernatant was then diluted 1:1 with 0.10% trifluoroacetic acid (TFA) (v/v), kept in an ice-bath with agitation for 30 min to improve cationic extraction, and again centrifuged for 30 min at 4°C and 30,000 g. This

acidic extract (indicated here as HCII) was loaded onto Sep-Pack Vac C18 cartridges (Waters Associates) equilibrated with 0.10% TFA (v/v). The first fraction collected was the washing with 0.10% TFA (v/v). Thereafter, cartridge elution was performed stepwise with solutions containing 50 and 100% acetonitrile in 0.10% TFA (v/v). The fraction eluted at 50% acetonitrile is indicated here as HCII-II. All fractions were freeze dried under vacuum (Speed Vac; Savant Instruments), except for those derived from the large infected group, which due to their quantity were lyophilized.

The three Sep-Pack fractions (washing, 50% and 100% acetonitrile) were reconstituted in MilliQ water, and applied on a semi-preparative reverse phase (RP) C18 column (10 × 250 mm, Vydac TM) equilibrated with 0.12% TFA (v/v) (solution A) and eluted with acetonitrile containing 0.10% TFA (v/v) (solution B). Elution was performed with a triphasic acetonitrile gradient (0–30% solution B for 15 min, 30–50% solution B for 20 min and 50–100% solution B for 15 min), at a flow rate of 2 ml/min. Active subfractions (see below) were further loaded onto an analytical C18 column (4.6 × 250 mm, Vydac TM) equilibrated with solution A, and eluted with a triphasic solution B gradient (0–25% for 12.5 min, 25–40% for 22.5 min and 40–100% for 15 min), at a flow rate of 1 ml/min. Final peptide purifications were performed on the same analytical C18 column, equilibrated with 15% solution B and eluted with a linear 15–40% gradient of solution B for 45 min, at a flow rate of 0.75 ml/min. All experiments were carried out using a Waters HPLC system model 600E coupled with a tuneable absorbance detector model 486. The column effluent was monitored by absorbance at 230 nm.

Antibacterial and haemolytic assays

Two Gram-positive (*B. subtilis* and *Staphylococcus aureus* ATCC 25923) and two Gram-negative (*E. coli* DH5$\alpha\beta$ and *Klebsiella pneumoniae* ATCC 13883) strains were used for antibacterial assays. The HCII extract and fractions from Sep-Pak and RP-HPLC steps were qualitatively proven according to the classical growth inhibition assay in solid medium [43]. Briefly, circa 10 µg of total proteins resuspended in 10 µl of sterile ultra-pure water were placed in dishes over plates of Luria Bertiani (LB) medium with agar containing 5×10^5 bacteria. Antibacterial activity was visualized as clear zones (no bacterial growth) after overnight incubation at 37 °C.

To determine minimal inhibitory concentration (MIC) values, pure peptides and reconstituted HPLC subfractions were tested in a liquid growth inhibition assay following an adapted microdilution protocol of the National Committee of Clinical and Laboratory Standards [44] performed in polypropylene plates with 96 wells (Corning). Briefly, 50 µl of sample (50 µg/ml final concentration) was mixed with 50 µl of Mueller-Hinton medium (Becton Dickinson), serial dilutions were performed and each well was inoculated with 5 µl of saline buffer containing 5×10^4 bacteria. The plates were incubated overnight at 37 °C.

Haemolytic activity was assayed as described in Torres-Larios et al. [45]. Freshly collected human blood was washed several times and aliquots of resuspended cells in phosphate buffer saline (PBS) were incubated with a final concentration of 100 µg/ml of total protein from HCII-II subfractions or pure peptides. Haemolysis was estimated by absorbance at 541 nm of the supernatant after 1 h incubation at 37 °C. Positive and negative controls were obtained using 1% Triton X-100 (v/v) and cells without treatment kept in the appropriate buffer, respectively.

Structural characterization

Amino acid composition of the native peptide was obtained after acid hydrolysis using a Beckman 6300 analyser, essentially as previously described [45]. Sequence analysis was performed using a Beckman L300 instrument on in situ alkylated native peptide samples (propionamide-cysteine derivatives obtained as described by the manufacturer) or a Procise 491 instrument (Applied Biosystems) on peptides generated from enzymatic hydrolysis of carboxamidomethylated or native species. Cystine-containing peptides were analysed according to Haniu et al. [46]. Mass analysis of native peptide or enzymatic digests was obtained using a Thermo Finnigan LCQ DUO electrospray ionisation-ion trap (ESI-IT) mass spectrometer or a MALDI-TOF mass spectrometer, as reported above.

Purified peptide samples were eventually treated with 10 mM dithiothreitol in 0.25 M Tris-HCl, pH 8, for 45 min at 55 °C, before alkylation, or directly alkylated with 55 mM iodoacetamide in 0.25 M Tris-HCl, 1.25 mM EDTA, 6 M guanidinium chloride, pH 7, for 30 min at 25 °C. Peptide products were freed from salt and reagent excess by passing the reaction mixture through an analytical C18 column (4.6 × 250 mm, Vydac TM), as previously reported [47], manually collected and dried for further characterization. Native or carboxamidomethylated peptide samples were digested with trypsin in 50 mM ammonium bicarbonate, pH 6.5, overnight. Tryptic digests were directly analysed by MALDI-TOF MS or resolved on a microbore C18 column (1 × 250 mm, Vydac TM) equilibrated with 5% solution B and eluted with a linear 5–40% gradient of solution B for 45 min, at a flow rate of 0.06 ml/min. Peptide fractions were manually collected for further characterization.

Differential MALDI-TOFMS analysis

Samples of cell-free haemolymph and the HCII-II fraction were obtained as described above and used for MS analysis following the addition of 200 pmol fragment

(1–17) from adreonocorticotropic hormone (ACTH), used as internal standard. The analysis of cell-free haemolymph (30 µl) was conducted on six independent samples, each obtained from a single individual. Three control (sterile injured) and three 24-h post-infection samples were investigated, all presenting a similar protein content as determined with the Bio-Rad Protein Assay. These samples were filtered through sterilized 0.45-µm filters before mass spectrometric analysis. The HCll-II fractions were obtained from a pool of haemolymph from about 30 scorpions in each sample (three control and three infected), normalized to contain the same amount of protein.

Differential MALDI-TOF MS analyses were performed using a Voyager-DE PRO mass spectrometer (Applied Biosystems). Samples (1 µl) were loaded on the instrument target, using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid [5 mg/ml in 50% acetonitrile, 5% formic acid (v/v)] as matrix, as previously reported [47]. Spectra were acquired either in reflectron or linear mode with delayed extraction. Spectra were calibrated either by external or internal calibration using the molecular ions from angiotensin I, ACTH (18–39), ACTH (7–38) and bovine insulin. Data are reported as average masses.

Genomic and cDNA cloning

To prevent excessive cuticle debris and to avoid venom gland contamination, total RNA was obtained only from the first six tergites of the scorpion opisthosoma. Total RNA was isolated from infected *C. limpidus limpidus* scorpions (females of same weight), after 12 h of infection, using the Promega TotalRNA isolation system. First-strand synthesis was achieved with SuperScriptII Moloney murine leukaemia virus (M-MLV) RT (Gibco-BRL) in the presence of RNase inhibitor (Roche Diagnostics), using poly$(T)_{22}$NN as primer. The PCR was performed with Vent DNA polymerase (New England Biolabs), using first-strand cDNA as template and 25-mer degenerated oligonucleotide (5′-GCNTGYCARTTYTGGWSYTGYAAY-3′) and poly$(T)_{22}$NN as primers. The cloning and sequencing were obtained using PCR products purified in a Centricon 100 column (Millipore), following the manufacturer's instructions and then ligated into the *Eco*RV site of the pKS plasmid (Stratagene). This construct was used to transform *E. coli* DH5α cells. Plasmid DNA was isolated from clones carrying the insert and sequenced from both strands in an Applied Biosystems 3100 apparatus, as described by the manufacturer. The 5′ cDNA sequence was obtained by the rapid amplification of cDNA ends (RACE) method using 5′RACE system version 2.0 (Invitrogen). Briefly, first-strand cDNA was synthesized from total RNA using a gene-specific primer (5′-TTATTGACACTGGCAATATTT-3′) and M-MLV RT. A homopolymeric tail was then added to the 3′ end of the cDNA using TdT and dCTP. PCR amplification was accomplished using Taq DNA polymerase, an anchor oligonucleotide provided in the kit as sense primer and a nested gene-specific antisense primer (5′-TTTATACTGTATTCCCCAGCAATA-3′). The 5′RACE products were cloned into an appropriate vector for subsequent characterization as described above.

The genomic DNA was obtained from scorpion females with the DNeasy mini kit (Qiagen). Two specific oligonucleotides were used as primers for PCR amplification (sense, 5′-ATGAAAGCAATCGTTGTTCTT-3′; antisense, 5′-GGCAATATTTATACTGTATTC-3′), using Taq DNA polymerase. Products visualized in agarose gel electrophoresis were purified with a QIAquick gel extraction kit (Qiagen) and cloned into the pGEM vector (Promega). Sequencing was determined as described above.

Semiquantitative RT-PCR analysis

Gene expression was analysed by RT-PCR. Total RNA extraction and first-strand cDNA synthesis from two naïve and two bacteria-challenged scorpions were performed as described above, except that random decamers (Ambion) were used as primers. Equal amounts of each cDNA were used in the subsequent PCR, carried out with Taq DNA polymerase and two specific primers (sense, 5′-CATGACAACCGTGGAAGG-3′; antisense, 5′-TTATTGACACTGGCAATATTT-3′), which resulted in an expected 117-base pair product. The primers for the housekeeping gene of 18S rRNA were used according to the QuantumRNA Universal 18S Internal Standards Kit (Ambion). PCR samples were performed for 26, 29, 32 and 35 cycles; the products stained with ethidium bromide were analysed in 2% agarose gel electrophoresis.

Similarity searches and sequence analysis

The sequences obtained in this work were deposited at SWISS-PROT (P83738) and GenBank (AY520534) databases. Similarity searches were performed using BLAST (www.ncbi.nlm.nih.gov/BLAST) and FASTA 3 (www.ebi.ac.uk/fasta33) against non-redundant databases, using BLOSUM 62, PAM 70 or PAM 120 weight matrices. Sequence alignments were performed with CLUSTAL_X [48]. The signal peptide was predicted using the SignalP server (www.cbs.dtu.dk/services/SignalP).

Results

Antibacterial activity in fractions of *C. limpidus limpidus* haemolymph

Earlier work conducted with haemolymph of two Old World scorpions, *Leiurus quinquestriatus hebraeus* [34] and *Androctonus australis* [35] showed the presence of constitutive antibacterial peptides. This motivated our re-

search with haemolymph of the New World species *C. limpidus limpidus*. At the starting point of this work, 500 naive individuals were used for haemolymph extraction and analysis. The animals were kept and fed in captivity for several months. Five groups of individuals were separated containing 50–250 individuals each. The haemolymph from each group was treated independently, as reported in Materials and methods. Each fraction from the C18 cartridge extraction was tested for antibacterial activity, using a solid-medium assay [43]. In all cases, only the HCll-II fraction contained antibacterial activity. These fractions were further separated on a semi-preparative RP-HPLC column, from which three positive subfractions were found and called HCll-II.10, -II.11 and -II.12 (fig. 1, table 1). The chromatographic profiles obtained with haemolymph of the different scorpion groups was quite reproducible, except for the subfaction labelled with an asterisk in figure 1 (subfraction II.11). The relative concentration of this subfraction was not constant when compared with various haemolymph samples from other scorpion groups (data not shown).

Components of *C. limpidus limpidus* haemolymph involved in the septic injury response

The unexpected finding that the subfraction indicated with an asterisk in figure 1 was variable within different haemolymph batches prompted us to examine if these differences could be due to extrinsic factors, rather than to

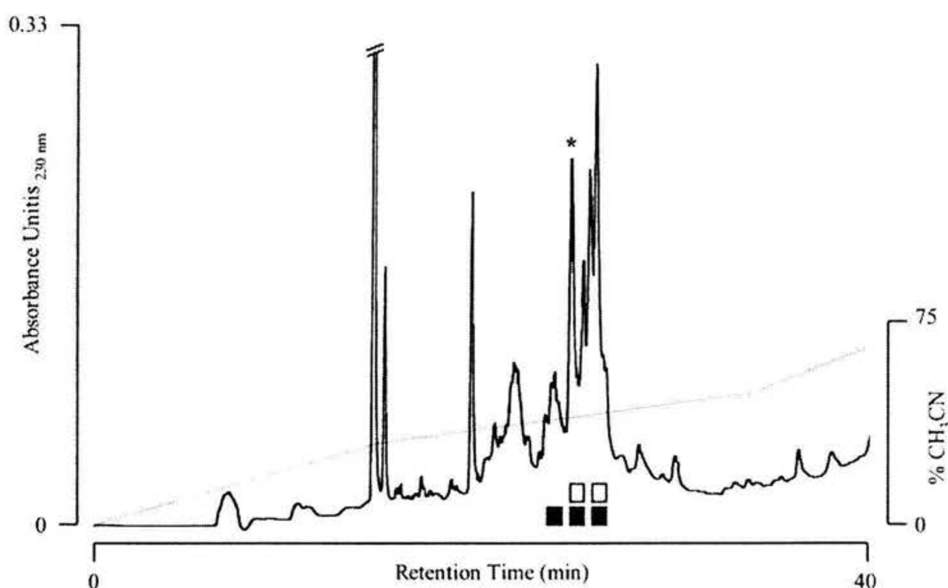

Figure 1. Chromatographic separation of HCll-II. One milligram of total protein from the HCll-II fraction was loaded on a C18 semi-preparative column and eluted with the acetonitrile gradient displayed (broken line). Squares under the chromatogram profile indicate subfractions with antibacterial activity in a solid-medium assay against *E. coli* (empty) or *B. subtilis* (closed). Asterisk indicates a variable subfraction called HCll-II.11 (see text for details).

Table 1. Antibacterial assays.

	E. coli		*K. pneumoniae*		*B. subtilis*		*S. aureus*	
	solid	liquid	solid	liquid	solid	liquid	solid	liquid
HCll-II	+	NT	+	NT	+	NT	+	NT
Cll-dlp	–	> 50	–	> 50	–	> 50	–	> 50
HCll-II.12a	+	50–25	NT	NT	+	NT	NT	> 50
HCll-II.12b	+	50–25	NT	NT	+	NT	NT	> 50
12a + 12b	+	50–25	NT	NT	+	NT	NT	NT
Cll-dlp + 12a + 12b	NT	20*	NT	20*	+	20*	NT	20*

Solid-medium assay [43]: +, presence of inhibition zones; –, no inhibition.
Liquid growth inhibition assay [44], concentrations are given in µg/ml: MICs are expressed as intervals between the minimum concentration at which no growth was observed and the maximum at which bacterial growth was still present.
NT, not tested.
* No serial dilutions were performed.

constitutive determinants. The idea was that a mechanism of innate defence could be associated with these variations, depending on the environmental conditions used to maintain and feed the scorpions. To test this hypothesis, we selected three independent groups of 350 female scorpions of the same weight (around 1 g), kept starved for 2 weeks to avoid feeding interferences, and injected with the bacteria described in Materials and methods. Haemolymph collected from control and test groups was analysed using the same protocol described above. Samples were separated by a stepwise elution on the C18 cartridge and the respective HCll-II fractions (normalized to contain 1 mg of total protein) were loaded onto a C18 semi-preparative column. The results indicated that the animals challenged with bacteria injections showed a higher relative content of the subfraction eluting at 24 min (data not shown). This increment was visible from 6 to 24 h after injection, but returned to the basal level after 48 h. The highest value was obtained at 24 h after injection. This suggested that septic injury could elicit an increment of the relative abundance of this subfraction in the haemolymph. The remaining components showed comparable relative concentrations, independent of the treatment applied. The control groups did not show the inducible increment of this specific subfaction in the same time intervals. These experiments were performed on three independent groups, with duplicate analysis of each.

The corresponding subfractions of HCll-II from the control and bacterial-challenged group of scorpions were separated by analytical RP-HPLC. The chromatographic profile of the injured animals showed comparable subcomponents, except for the one shown with an asterisk in figure 2A, which increased from 6 to 12 and to 24 h after infection (highest level), decreasing to normal levels at 48 h after injury. This component was purified to homogeneity after a third RP-HPLC step (inset in fig. 2A), and its molecular mass was estimated by ESI-IT MS, showing a monoisotopic signal at 3816.5 atomic mass units. This peptide corresponded to 0.018% of the total protein content for haemolymph extracts of challenged scorpions. On the basis of our chromatographic and MS determinations we estimated that induction by septic injury would double or triple the relative concentration of this component in the circulating haemolymph. To proceed with its chemical characterization, the haemolymph of 1000 infected scorpions was obtained and the corresponding peptide isolated as described above. About 180 µg was used for final characterization. This peptide was named Cll-dlp, meaning *C. limpidus limpidus* defensin-like peptide.

Amino acid sequence and disulphide bridges of Cll-dlp

The polypeptide sequence was obtained by direct Edman degradation of the native species (first 31 amino acids) and mass mapping measurement and sequencing of overlapping fragments generated following tryptic digestion of a reduced and alkylated peptide sample (fig. 2C). The sequence obtained is consistent with the amino acid composition determined by acid hydrolysis (data not shown). The peptide presented six cysteine residues, all involved in disulphide bridges, as determined by ESI-IT MS and MALDI-TOF MS analysis of native and carboxamidomethylated peptide samples, following alkylation in reducing or non-reducing denaturing conditions (data not shown).

Cll-dlp disulphide bridge pairing was investigated by a combined mass spectrometric-Edman degradation approach. Native Cll-dlp was digested with trypsin and the resulting peptide mixture was directly analysed by MALDI-TOF MS, producing the spectrum shown in figure 2B. A series of signals were assigned to S-S-bridged peptides on the basis of their unique mass values and their disappearance following incubation with dithiothreitol. The signal at 3475.6 m/z was associated with a three-peptide cluster involving fragments (1–14), (18–27) and (28–32) (theoretical value: 3475.9 m/z) linked by three disulphides. The remaining peaks were assigned to S-S-containing peptides originating from the mentioned above one, following non-specific hydrolysis at Trp5. In fact, the signal at 1897.6 m/z was interpreted as arising from peptides (1–5) and (18–27) linked by the disulphide Cys2-Cys21 (theoretical value: 1897.4 m/z). Similarly, the signal at 1595.7 m/z was associated with a complementary peptide cluster involving fragments (6–14) and (28–32) (theoretical value: 1596.1 m/z) linked by two disulphides. The final S-S assignment was obtained by direct Edman degradation of these latter Cys-containing peptides, following chromatographic purification and identification of the PTH-cystine at the expected degradation cycles [46]. This analysis led to the expected sequence in the case of the component with $(M+H)^+$ at 1897.6 m/z, with a straightforward identification of the disulphide Cys2-Cys21. Similarly, the peptide at 1595.7 m/z, associated with the peptide cluster (6–14) + (28–32) showed the presence of PTH-cystine at the second and the sixth cycle during Edman degradation, thus demonstrating the occurrence of the disulphides Cys7-Cys29 and Cys11-Cys31. On the basis of these results, the determined Cll-dlp disulphide pairing (fig. 2C) was equivalent to that already reported for other invertebrate defensins (CS-$\alpha\beta$ motif signature pairing). Moreover, BLAST and FASTA 3 searches grouped the sequence of Cll-dlp with other invertebrate defensins and CS-$\alpha\beta$ motif-containing peptides (see below).

Cll-dlp systemic liberation in response to septic injury

To present an additional semi-quantitave measurement of this phenomenon, haemolymph samples from control animals and septic-injured scorpions were also analysed by

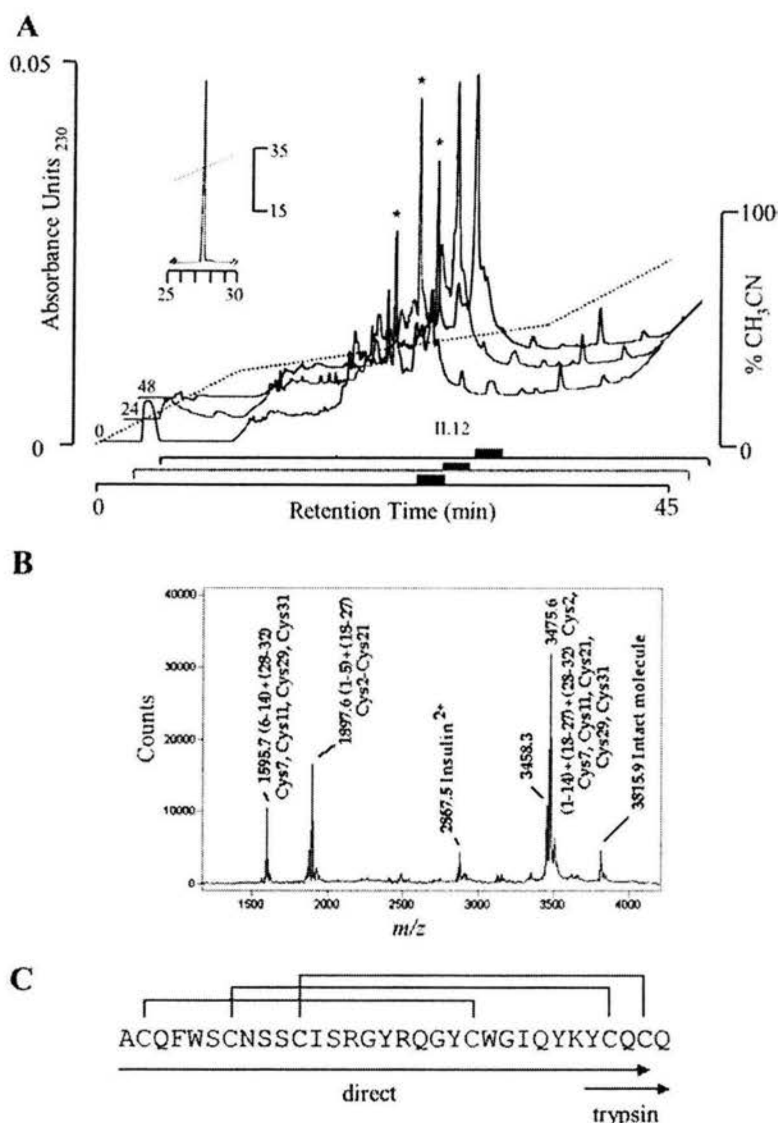

Figure 2. Purification and characterization of CII-dlp. (*A*) The variable subfraction (HCII-II.11) in semi-preparative HPLC from naive (0) and bacterial-challenged scorpions after 24 (24) and 48 (48) h were further fractionated on an analytical C18 column eluted with the acetonitrile gradient displayed (broken line). The retention times over which the components HCII-II.12a and HCII-II.12b elute are indicated by solid rectangles. The inset shows the final purification of CII-dlp from pooled peaks indicated with an asterisk in the main figure. (*B*) MALDI-TOF mass spectrum of native CII-dlp following tryptic hydrolysis. Signals corresponding to the disulphide-bridged peptides are reported. Each signal was assigned to the corresponding peptide pair or triplet on the basis of its mass values, peptide sequence and enzyme specificity. The cysteine residues involved in S-S bonds are indicated. (*C*) Amino acid sequence of CII-dlp as determined by Edman degradation and MS analysis. The fragments considered to assemble the full sequence are indicated below. Disulphide pairing is also displayed. The CII-dlp sequence is in the SWISSPROT database under accession number P83738.

MALDI-TOF MS procedures (fig. 3). Two kinds of samples were used in these experiments: cell-free haemolymph extracts (fig. 3A) and fraction HCII-II (fig. 3B). Consistently, the MS spectra of cell-free haemolymph showed the same set of components in samples from both control and bacterial-challenged groups; however, a clear induction of at least four components with a molecular mass ranging from 3100 to 4100 m/z was registered in experimentally infected scorpions (compare lower and upper panels in fig. 3A). As a result of a stepwise purification, fractions HCII-II from control and septic-injured animals seemed to be enriched in the component with the molecular mass at 3816.9 m/z. This species showed a clear apparent increase in samples from septic-injured scorpions (fig. 3B). The weaker signals occurring in the spectra were not considered to be statistically significant (experiments done in triplicate). Important to not is that the component CII-dlp with an $(M+H)^+$ signal at 3816.9 m/z was the most conspicuously inducible.

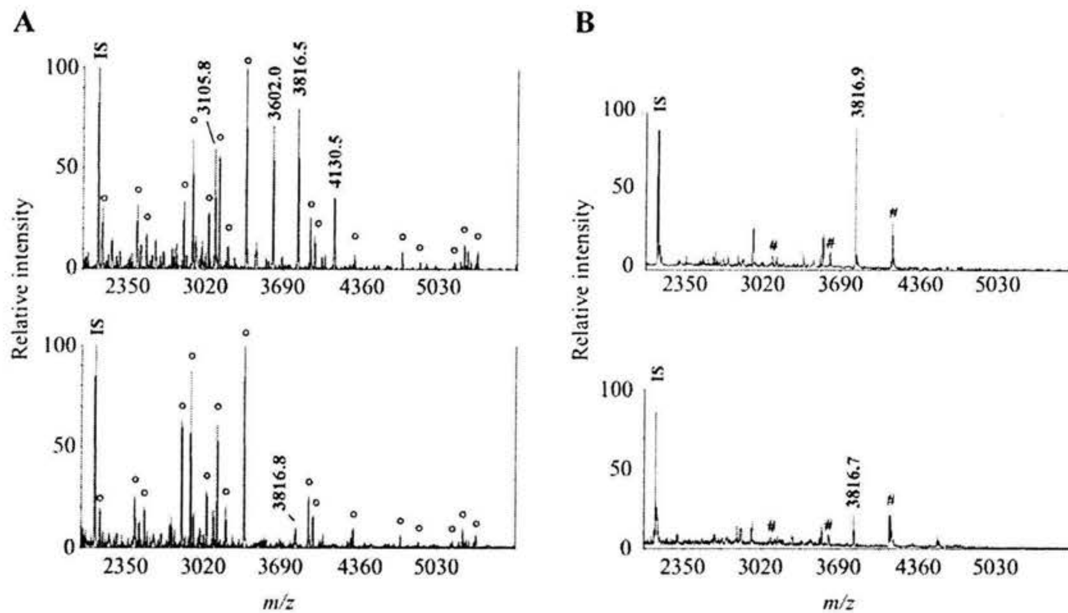

Figure 3. Differential MALDI-TOF MS analysis. (*A*) The cell-free haemolymph was collected from a single-bacterial challenged scorpion (upper panel) or sterile injured (lower panel), to which with the ACTH (1 – 17) internal standard was added and analysed as reported in Materials and methods. Circles indicate molecular components common to both samples, whereas indicated m/z values correspond to the signals variable between both kinds of samples. (*B*) The fraction HCll-II was collected from the stepwise purification of challenged (upper panel) or sterile-injured (lower panel) scorpions, to which with the internal standard was added, and analysed as mentioned above. Indicated with # are the peptides with $(M+H)^+$ at 3105.8, 3602.0 and 4130.5 m/z, already reported in (*A*). All analyses were performed in duplicate for three independent sterile-injured or bacterial-challenged scorpion samples; all samples were prepared in parallel under the same experimental conditions. IS means the internal standard.

Cll-dlp gene structure and mRNA expression after septic injury

The apparent increase in Cll-dlp in response to septic injury could be associated with its de novo synthesis (as in some insects, nematodes and arachnids [4, 5, 25–27, 36–40]), or to the release of pre-synthesized and stored peptide in special reservoirs, which could liberate the peptide upon stimulation (as in mussels, shrimps and some chelicerates [3, 28–32]). To solve this question, we cloned the gene from cDNA and studied the expression of mRNA in response to septic injury.

The cDNA sequence encoding mature Cll-dlp was obtained by classical 3′ and 5′RACE protocols. This clone consisted of 363 base pairs (bp), comprising 61 at the 5′ untranslated region, a putative 24-residue-long signal peptide, the full mature sequence for Cll-dlp followed by a stop codon and 131 bp at the 3′ untranslated region, including a putative polyadenylation signal (fig. 4). The genomic sequence was determined by PCR and cloning. The sequence overlaps with cDNA from position –72 to position +88. This clone included a single-phase I intron of 128 bp with canonical splicing sites splitting an Ala codon within the putative signal peptide region (fig. 4). Determination of the nucleotide sequence provided the information needed to synthesize the appropriate oligonucleotides for mRNA expression analysis. RT-PCR assays were performed with mRNA isolated from naive and bacterial-challenged scorpions (10 h post-infection), using specific primers for Cll-dlp and the housekeeping rRNA of ribosomal subunit 18S (see Materials and methods). No significant differences in relative abundance of the Cll-dlp transcript were observed between these two conditions (data not shown).

Cooperative antibacterial effect of Cll-dlp

The HCll-II subfraction from which Cll-dlp was isolated (asterisk in fig. 1), showed antibacterial activity against *E. coli* and *B. subtilis* in solid-medium qualitative assays (table 1). However, for the pure peptide Cll-dlp, only marginal activity was observed at relatively high concentrations (maximum concentration tested was 70 μg/ml, due to scarcity of sample). Nevertheless, almost one-half of the Cll-dlp-containing HCll-II subfraction consisted of two other major components: peptides HCll-II.12a and HCll-II.12b, which coelute in the HPLC conditions used (labelled HCll-II.12 in fig. 2A). These two peptides were isolated in homogeneous form after a third HPLC step and are now being analysed. These components were tested for their antibacterial activity: the MIC values against *E. coli* were 50–25 μg/ml (table 1). When a mixture of HCll-II.12a and HCll-II.12b, at a ratio of 1 : 1, was prepared, the measured MIC was practically the same. Thus, apparently no cooperative antibacterial effect existed for these peptide species. This situation was quite different when a pep-

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                                                              ******
ACACTGAAGTTTCGGATAGAAGACCTGTTGGTTTGATAAATTTGATTAAAAATCGTTAAAAATGAAA      -67
                                                              M  K       -23

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GCAATCGTTGTTCTTCTTATCTTGGCTCTCATCTTATGCCTTTATGgtaaggacatttttgaattt      -21
A  I  V  V  L  L  I  L  A  L  I  L  C  L  Y  A--------------------      - 7

Taaaaactttctatctgtaaatgtgcattgtttaagaaataatacatttgtggtattgaaaaatata
-------------------------------------------------------------------

                                        ***************************
atttaaatgtctaaaaaagagaaataatttttccgaacagCCATGACAACCGTGGAAGGTGCTTGCC        7
---------------------------------------- M  T  T  V  E  G  A  C  Q        3

*******************************************************************
AATTTTGGAGTTGCAACAGTAGTTGTATTTCGAGAGGATATAGACAAGGGTATTGCTGGGGAATACA       74
 F  W  S  C  N  S  S  C  I  S  R  G  Y  R  Q  G  Y  C  W  G  I  Q        25

**************
GTATAAATATTGCCAGTGTCAATAAAATCTTTCTTAATATCTCTGTAGTTGATTACCACTGAAATTT      141
 Y  K  Y  C  Q  C  Q  end                                                 32

GTTTAAGTAACTCAATGTTGTAACGTCTTGCAGATATGTATAACCCTGAATAATAGTTTGTAATTAA      208

TAAAGAAATATTCTTTAAACTTA                                                  231
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Figure 4. Nucleotide sequences encoding Cll-dlp. The cDNA encoding Cll-dlp was obtained with standard 3' and 5'RACE protocols, molecular cloning and sequencing. Specific primers flanking the cDNA of the mature region (indicated by forward and reverse arrows) were used to amplify a partial genomic clone. Asterisks above the sequence indicate the overlap between the cDNA and genomic clones. Exons are in uppercase letters. Splice donor and acceptor sites are grey shaded. The polyadenylation signal is double underlined. The translated amino acid sequence is below the nucleotide sequence and the putative signal peptide is on italics. Numbers on the right correspond to the full the cDNA clone taking the first nucleotide of mature sequence as position +1. The sequence from the cDNA clone is available in the GenBank database under accession number AY520534.

tide mixture was prepared including Cll-dlp. Equal amounts of pure components (Cll-dlp, HCll-II.12a and HCll-II.12b) were mixed and assayed in a liquid growth inhibition assay. The rationale for choosing this proportion of components was based on the fact that it resembles the one present in the semi-preparative HPLC subfraction from which the Cll-dlp was obtained. When a total peptide content of 20 μg/ml was tested, the *E. coli* growth was null. We extended these results by testing the same mixture with other bacterial strains and found that this preparation was active at the same concentration against *B. subtilis*, *K. pneumoniae* and *S. aureus*. Thus, an apparently cooperative antibacterial activity existed between constitutive (HCll-II.12a and 12b peptides) and inducible (Cll-dlp) haemolymph components.

Because other AMPs isolated from scorpion [41] have been reported to have an intrinsic haemolytic activity, we needed to verify if any of the peptides we isolated could have similar activities on erythrocytes. The effect of all the fractions purified, including the cell-free haemolymph, did not show any haemolytic activity in human erythrocytes, assayed in the conditions described in Materials and methods.

Discussion

As described in the Materials and methods and results, this work required the use of several thousand scorpions. The handling of live dangerous animals to obtain the haemolymph was not a trivial task. Nevertheless, the purification of the haemolymph soluble peptide fractions was relatively simple, comprising a centrifugation followed by a reverse-phase extraction step on Sep-Pack cartridges and a couple of HPLC separations. The complete covalent structure of Cll-dlp was successfully obtained. Regarding its biological activity, Cll-dlp seemed to be a poor antibacterial agent; however, we were able to demonstrate a cooperative effect between this peptide and the constitutive components HCll-II.12a and 12b. After incubation with the mixture, no bacterial growth was recovered for four bacterial strains (table 1). This situation is similar to that already reported for mammalian antibacterial peptides (some of which are immune inducible) and lysozyme (which is constitutive) [49]. Cll-dlp and the other peptides shown to be present in subfraction HCll-II.11 (fig.1, labelled with asterisk) were also assayed for possible membrane lytic activity on hu-

man erythrocytes and ovarian insect cells obtained from *Spodoptera frugiperda* (cell line Sf9); in neither cases was disruptive activity recorded (data not shown). Taking together these results suggest that Cll-dlp is preferentially active against bacterial cells, but whether Cll-dlp acts like other invertebrate defensins, which display a membrane lytic activity [50–52], remains unclear. Further experiments are needed to explore if Cll-dlp follows a similar mode of action.

Comparison of the amino acid sequence of Cll-dlp with known invertebrate defensins

The Cll-dlp sequence resembles the known scaffold of the invertebrate defensins and most scorpion toxins [18]. Several authors have proposed a close relationship between the primary structure of some scorpion toxins and invertebrate defensins [53, 54]. The results of BLAST and FASTA 3 searches grouped the Cll-dlp sequence with both kinds of peptide, although with low scores. When all available sequences of short chain scorpion toxins (121 to date) and invertebrate defensins (a total of 60) were taken into consideration for analysis, the alignment obtained with CLUSTAL_X [48] confidently clustered the Cll-dlp sequence within defensins, whereas all the scorpion-venom-derived peptides were grouped with toxins (not shown). In figure 5, the Cll-dlp sequence is aligned with other invertebrate defensins; this figure clearly shows that the only fully conserved residues are cysteines. Two main defensin groups can be identified on the basis of this alignment. The largest one includes defensins from different orders of the subclass Neoptera (Diptera, Coleoptera, Hemiptera and Hymenoptera), although the defensins from lepidoterans are more diverse. A second group, often referred to as ancestral, contains defensins from phylogenetically distant invertebrates (mussels, arachnids and the dragonfly *Aeschnea cyanea*) [1, 15, 20, 21, 34–36, 39]. This analysis reveals that the Cll-dlp sequence is quite distant from both groups, but remains closer to the defensins than to the scorpion toxins.

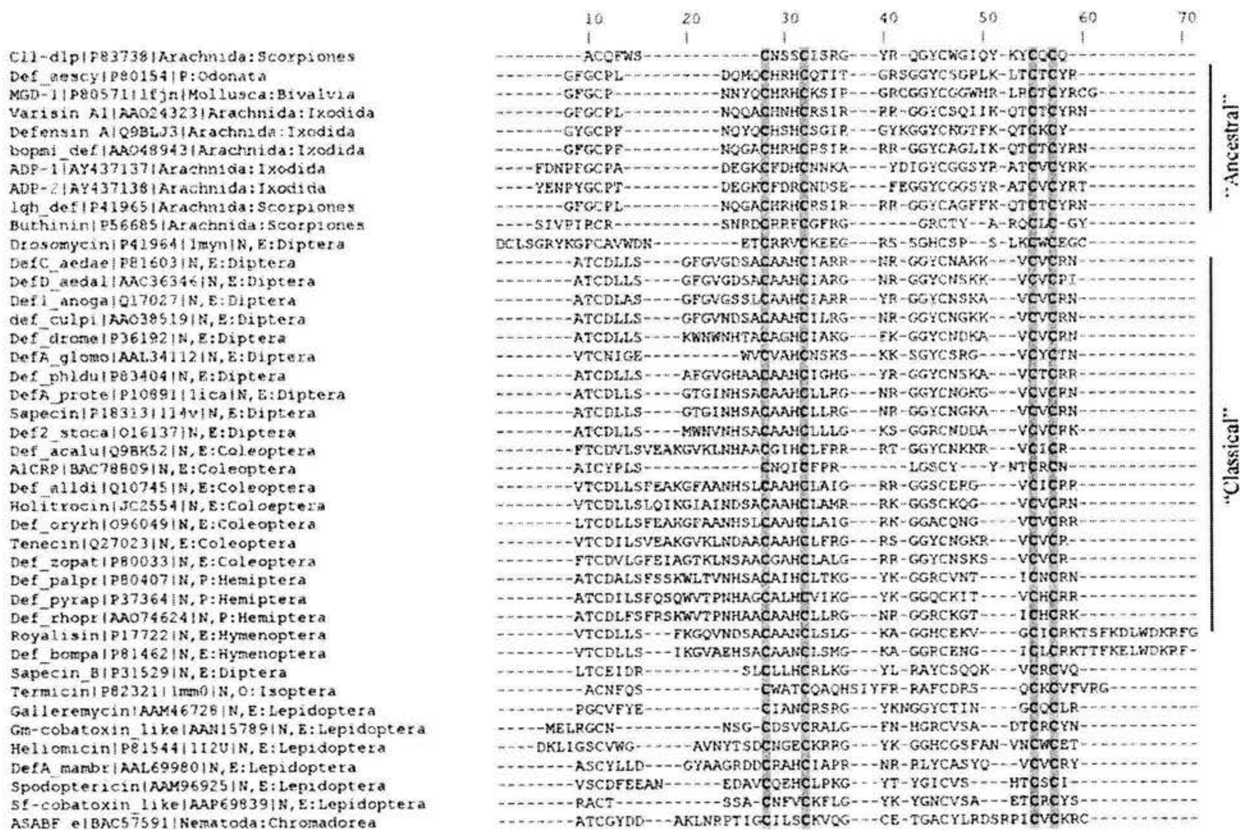

Figure 5. Multiple sequence alignment of some invertebrate defensins. Amino acid sequences of invertebrate defensins were retrieved from public databases and aligned with CLUSTAL_X [48]. Fully conserved amino acid residues are indicated by asterisks (*) below the alignment. Cys residues of the CS-$\alpha\beta$ motif signature sequence are grey shaded. Names, accession numbers and sources are indicated in the left column. In defensins from the class Insecta, the subclass (N, Neoptera; P, Palaeoptera), the infraclass (E, Endopterygota; O, Orthopteroidea; P, Paraneoptera) and the order are indicated. In defensins from other arthropods, only the class and order are indicated. For defensins from organisms of other phyla (molluscs and nematodes), the phylum and the class are indicated. Vertical lines on the right indicate the two main groups of invertebrate defensins: ancestral and classical.

Genomic organization of the Cll-dlp gene and the evolution of the CS-$\alpha\beta$ structural family

The structure of the gene coding for Cll-dlp, found in this work, seems similar to those of scorpion toxins. There is a signal peptide, interrupted by a short intron of variable length, followed by the sequence coding for the mature peptide and ending with a stop codon. Our results are consistent with the suggestion given by Froy and Gurevitz [55] for the gene encoding a defensin obtained from the North African scorpion *L. quinquestriatus hebraeus*, although these authors did not report any sequence or give any database reference useful to compare experimental results.

When comparing scorpion toxins and defensins with the CS-$\alpha\beta$ motif, due to their low sequence similarity (only cysteines were conserved at identical positions), making any valuable evolutionary predictions about the two types of peptide is difficult. However, taking into account the Cll-dlp gene organization and the common conservation of the CS-$\alpha\beta$ signature among the two peptide groups, there does seem to be an evolutionary relationship between these two classes of peptide. The very wide phylogenetic distribution of defensins suggests that they might be the ancestors of the CS-$\alpha\beta$ motif-containing peptide family.

Defensin gene organization is highly variable both at the genomic and transcript levels. Apart from scorpion defensins, all the other cloned transcripts for defensins contain a pro-sequence [55, 56]. The pro-segment of the sequence in other arthropods is situated at the N-terminal side of the mature peptide, whereas in molluscs and nematodes the pro-segment is at the C-terminal side. Unfortunately, there is a reduced number of known genomic clones available for defensins, and yet they show great variability [55–58]. The tick defensin genes contain two introns, one in the signal peptide, the other at the pro-sequence; mussels have a single intron interrupting the signal peptide, and for some dipterans and lepidopterans an intron is situated at the pro-sequence. For *Drosophila* and other insects there are no introns. Such variations are hard to reconcile with a divergent evolutionary process, although they could be achieved as a result of exon-shuffling of the mature region, as recently proposed by Froy and Gurevitz [55].

Inducible liberation of Cll-dlp in the context of invertebrate immune systems

Two previous reports on scorpion defensins [34, 35] showed that there is no difference in the level of these peptides when comparing the control with challenged animals. In both cases, the time elapsed between infection and haemolymph extraction was 1 week. These results could be contradictory to our report here. However, in our opinion this is not the case because different time intervals were used for the experiments. We performed a kinetic investigation with shorter time intervals. At 24 h, the amount of Cll-dlp was maximal, and this value decreased after 48 h (figs. 2A, 3). Thus, if measured 1 week later we would expect to find the same basal peptide level, as already reported for the other scorpion defensins. Our results on the relative amount of Cll-ldp transcripts (mRNA measured by RT-PCR) also support the idea that this peptide is not synthesized in response to septic injury, but is, rather, liberated from a still uncharacterized cell reservoir. In this way, the systemic accumulation of Cll-dlp is similar to that already reported for AMPs from horseshoe crab [3], mussels [32], shrimps [30, 31] and spiders [28, 29]. The phylogenetic variety of organisms whose innate immune responses depend on the systemic release of AMPs, independently of transcriptional regulation, suggests that this strategy is ancestral to that followed by recent insect orders [3, 5, 32]. In the latter, the AMPs are often transcriptionally inducible. However, there are other arachnids of the order Ixodida [36–39] as well as the distant nematodes Chromadorea [40], in which the response is also via transcriptional activation of AMP genes. Thus, the documented cases show two distinct strategies for immune activation in invertebrates, on the one hand, the liberation of AMPs, prestored in haemocytes, and on the other, the transcriptional activation of AMP genes. However, still not clear is whether an evolutionary relationship exists between the two strategies. Additional studies with phylogenetically diverse organisms are needed before a better and clearer idea of the evolutionary history of innate immune systems.

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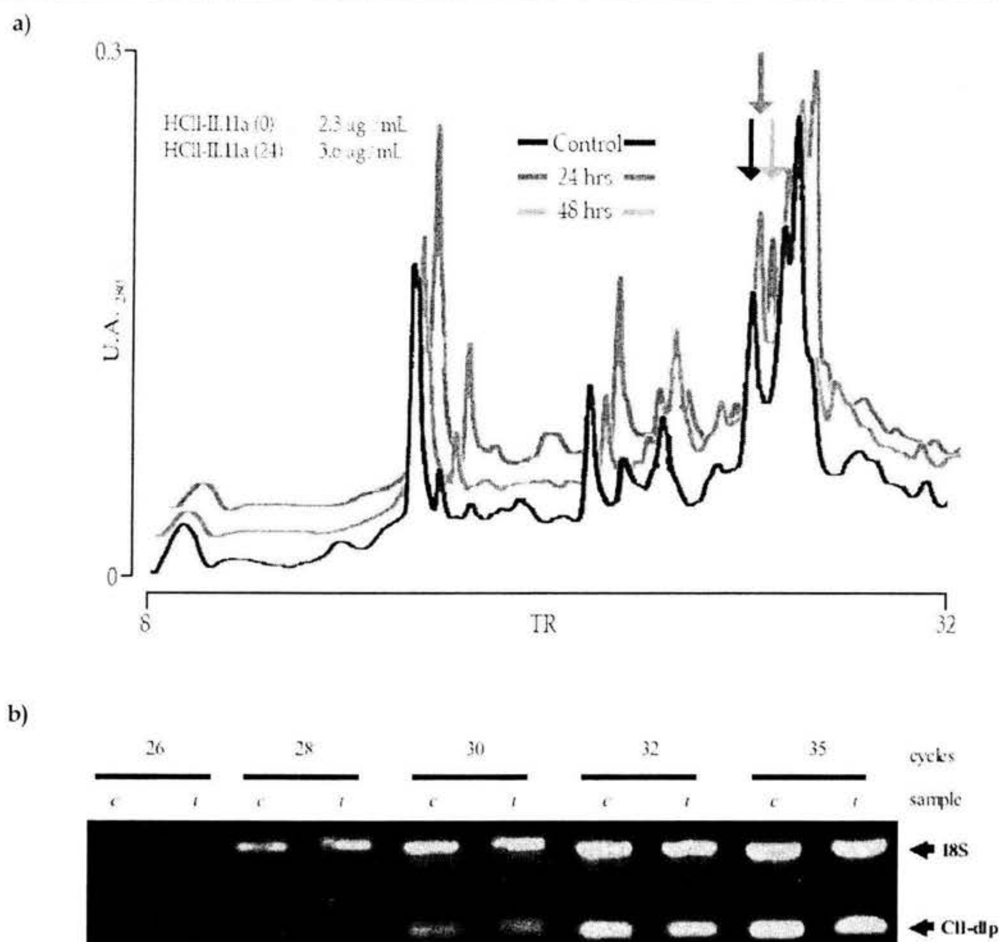

Figura E-1 | *Análisis diferencial de la respuesta inducida por el reto séptico.* a) Perfiles cromatográficos de la fracción HCll-II de alacranes naïve (control) y 24 o 48 hrs después del reto séptico. b) RT-PCR del gen que codifica para el componente Cll-dlp obtenido a partir de ARN total de alacranes naïve (c) o 12 hrs (i) después de la infección experimental.

Resultados no publicados

La subfracción de la hemolinfa de *C. limpidus limpidus* a partir de la cual se aisló el péptido similar a defensinas (HCll-II.11 [*Resultados publicados*: **Fig. 3A**]), contiene además otros dos componentes mayoritarios, los cuales corresponden a los componentes de la subfracción HCll-II.12, que a su vez eluye inmediatamente después en las condiciones de la primera cromatografía [*Resultados publicados*: **Fig. 1**]. El total de esta subfracción y sus dos componentes principales (HCll-II.12a y -II.12b) se identificaron como activos en los ensayos de inhibición de crecimiento, con Concentraciones Mínimas Inhibitorias (CIM) idénticas [*Resultados publicados*: **Fig. 1** y **Table 1**]. La secuencia N-terminal y los pesos moleculares de ambos componentes fueron determinados mediante degradación Edman y EM con ionización por electrospray, respectivamente (**Figura E-2a**). En los primeros 38 residuos de ambos componentes se observa un solo cambio (Glu22Leu con respecto a HCll-II.12a) y sus pesos moleculares son similares (14059 y 14026 para -II.12a y -II.12b respectivamente). Estos polipéptidos difirieron en su susceptibilidad al tratamiento reductor con β-mercaptoetanol;

la muestra de HCll-II.12b incubada con este agente reveló una banda de ~7 kDa en electroforesis tipo Schägger - von Jagow (**Figura E-2b**), lo cual indica que éste es un dímero unido por enlaces disulfuro intermoleculares. Cabe señalar que la concentración relativa de estos componentes en la hemolinfa no se modifica en respuesta al reto séptico.

a)

Componente	Secuencia	PM
HCl1-II.12a	DTAxQRSRARELEKDKPFVIHExDEx...	14059
HCl1-II.12b	DTACQRSRARELEKDKPFVIHLCDECGKYMPLPNVGTP...	14026

b)

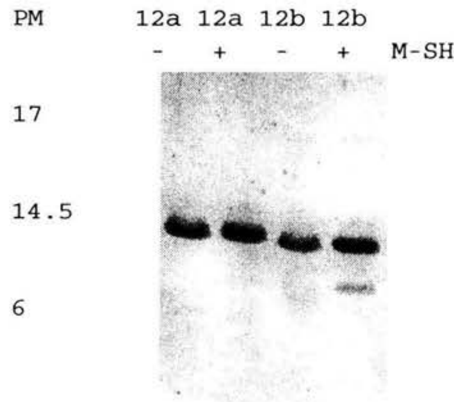

Figura E-2 | *Caracterización parcial de los componentes HCll-II.12a y -12b.* a) Secuencias N-terminales y pesos moleculares. b) Electroforesis desnaturalizante tipo Schägger - von Jagow en presencia (+) o no (-) de β-mercaptoetanol (M-SH).

NOTAS ADICIONALES PARA LA DISCUSIÓN Y PERSPECTIVAS

En este trabajo se demuestra que el alacrán Mexicano *C. limpidus limpidus* es capaz de montar una respuesta al reto séptico en forma análoga a otros organismos invertebrados, en particular ésta es similar a la reportada para los quelicerados Xiphosura *Tachypleus tridentatus* [19] y Aranae *Ac. gomesiana* [66,102], el crustáceo *Lp. Vannamei* [103-105] y los moluscos *Mytilus* sp. [106], esto es: PAM's son liberados a la hemolinfa en respuesta al reto séptico en ausencia de regulación transcripcional. En este sentido, es relevante recalcar que el protocolo experimental empleado permitió definir la participación de PAM's en el contexto de la activación inmune de *C. limpidus limpidus,* demostrándose que la liberación sistémica ocurre en un periodo de horas, retornando a niveles basales en dos días. De hecho, en todos los casos reportados, la liberación sistémica inducible independiente de activación transcripcional es consistentemente transitoria (*v.g.* [103,171]); en contraste, la inducción de mensajeros es más heterogénea en su intervalo de duración, iniciando en minutos (*v.g.* [33,94]) y extendiéndose hasta semanas (*v.g.* [95,130]). Los resultados obtenidos establecen, también, que las observaciones previas acerca de la presencia constitutiva de PAM's en la hemolinfa de los alacranes *L. quinquiestratus* [108] y *A. australis* [62] estuvieron sesgadas por la diferencia de tiempos entre los cuales se comparó (en ambos casos 1 semana), y restringe la participación no inducible de PAM's en el contexto de la inmunidad innata al caso de la termita *Ps. spiniger* (Termitidae, Isóptera, Insecta) [103], lo cual es relevante para el estudio

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de la evolución de los sistemas inmunes de invertebrados. La identificación del reservorio celular propuesto para Cll-dlp, la definición de los PMNP a los cuales responde y la caracterización de los posibles RRP, contribuiría a esclarecer la relación entre las similitudes observadas con los modelos mencionados, toda vez que el mecanismo molecular que regula este proceso se ha definido únicamente para el caso de los quelicerados Xiphosura [19].

Ahora bien, aunque no se registró actividad antibacteriana para preparaciones homogéneas del componente Cll-dlp, este péptido se aisló a partir de una subfracción activa en ensayos cualitativos de inhibición de crecimiento y la combinación de éste con los otros dos componentes principales de la subfracción (HCll-II.12a y -II.12b) presentó un efecto cooperativo en la capacidad de inhibir el crecimiento de cuatro cepas bacterianas. En conjunto, este fenómeno asemeja al sinergismo reportado en el caso de PAM's liberados de neutrófilos y la lisozima constitutiva en torrente [164,172]. En este sentido es relevante analizar sistemáticamente dicho efecto, para lo cual es necesario completar la caracterización bioquímica de los componentes antibacterianos constitutivos.

Por último, la distribución filogenética de las defensinas de invertebrados y la conservación de la estructura génica entre toxinas y defensinas de alacrán sugiere que los PAM's que adoptan el motivo α/β-EC preceden, evolutivamente, a las toxinas. De hecho, la correspondencia entre las organizaciones génicas de las toxinas y Cll-dlp —también la anunciada para la defensina de *L. quinquiestartus hebraeus* [167]— ofrece evidencia sobre un origen parálogo de ambas familias de proteínas en alacranes. La ruta evolutiva seguida por los posibles homólogos de Cll-dlp —defensinas α/β-EC de otros invertebrados— es menos clara, toda vez que¶: i) las secuencias están pobremente conservadas; ii) la organización de los mensajeros precursores y los genes correspondientes es notoriamente variable [165-168], y; iii) la diversidad filogenética de los organismos en los cuales están presentes es muy extensa —artrópodos de las clases Arachnida e Insecta y moluscos de la clase Bivalvia—, sin encontrarse ejemplos en otros organismos dentro de los Phyla correspondientes (en particular no se han reportado en ninguna clase de crustáceos, ni en otra clase de moluscos además de la Bivalvia). Cabe sañalar que la hipótesis que sostiene que la evolución de las defensinas de invertebrados habría estado mediada por recombinación de exones de la región madura [165] no parece ser satisfactoria, en particular considerando la falta de evidencia que sustente que ésta habría estado flanqueada por intrones de la misma fase —con lo cual calificaría como un "protomódulo" de acuerdo a la definición de L. Patthy [173]. De cualquier forma, la caracterización del componente Cll-dlp ofrece un panorama más completo para estudiar el proceso de diversificación funcional que debió haber originado la enorme batería de toxinas que se encuentran presentes en los venenos de los alacranes, contribuyendo así a elucidar las determinantes moleculares del destino funcional de la familia estructural que adopta el motivo α/β-EC [ver anexo].

¶ Esta breve discusión se restringe a invertebrados del linaje protostoma, por lo cual excluímos a las defensinas que adoptan este patrón estructural provenientes de nemátodos y plantas.

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ANEXO: ESTUDIOS DE CASO DENTRO DE LA FAMILIA ESTRUCTURAL α/β-EC

La gran diversidad de funciones llevadas a cabo por las proteínas ha evolucionado sobre un número limitado de andamiajes estructurales [1a]¶, esto se debe a que, en rangos de tiempo evolutivo, la secuencia de una proteína se modifica varias veces más rápido que su estructura. Consecuentemente, las relaciones evolutivas de las proteínas pueden inferirse mejor a partir del análisis de su estructura, es por ello que se utilizan criterios basados en la conservación estructural para la identificación de homólogos distantes (*v.g.* las bases de datos SCOP [3a,4a] y CATH [5a,6a]). Considerando que el número de secuencias de proteínas disponibles en las bases de datos, inferidas o determinadas, rebasa en varios órdenes de magnitud al número de proteínas funcional y/o estructuralmente caracterizadas [7a], se desprende que la mayoría de las anotaciones en las bases de datos estén basadas en la identificación de homólogos ya caracterizados; sin embargo, en el caso de homólogos remotos, las relaciones evolutivas pueden encubrirse por la ausencia de similitudes significativas entre las secuencias comparadas, derivado esto por procesos de diversificación extensos [8a]. En ausencia de protocolos experimentales que operativamente acorten este desfase, el análisis exhaustivo de las bases de datos mediante búsquedas basadas en perfiles de secuencia autogenerados (*v.g.* PSI-BLAST [9a]) o en "cadenas ocultas de Markov" (*v.g.* SAM-T99 [10a] y Superfamily [11a]) constituye la estrategia más accesible para delimitar estructural y funcionalmente el universo proteico [8a]. Cabe resaltar que conociendo el camino evolutivo de una familia de proteínas pueden inferirse las determinantes moleculares que subyacen a la diversificación de las funciones desempeñadas por sus miembros, ayudando, por tanto, a depurar el mapa de las relaciones estructura-función dentro de un espacio de secuencia dado [12a].

Ahora bien, como se ha mencionado las proteínas que poseen la firma consenso del motivo estructural α/β-EC (definida por los disulfuros C_i-C_j y C_{i+4}-C_{j+2}) adoptan un mismo tipo de plegamiento tridimensional y, en consecuencia, están ubicadas dentro de una misma superfamilia, tanto en la clasificación SCOP (Scorpion toxin-like) como en la implementada en CATH (3.30.30.10). En la versión actual de la base de datos SCOP (v1.65) se contemplan ~60 estructuras, de las cuales 44 corresponden a toxinas de alacrán (18 de cadena corta y 26 de cadena larga), 6 a defensinas de invertebrados y 7 son derivadas de plantas (incluyendo al edulcorante brazeína y al inhibidor de proteasas ATTP). En general, todos los miembros de una superfamilia de las clasificaciones implementadas en SCOP y CATH corresponden a proteínas monofiléticas [8a,13a], es decir: provienen de un ancestro común; sin embargo la baja representación de la extensa variabilidad en las secuencias de las proteínas que poseen la firma consenso del motivo estructural α/β-EC —el total de secuencias disponibles en las bases de datos es mayor a 300, mientras que las identidades promedio menores al 15%— no permite evaluar los eventos evolutivos que relacionarían a los miembros de esta superfamilia. En este sentido, la implementación de otros algoritmos§ (*v.g.* basados en el reconocimiento ponderado de los patrones de cisteínas [14a,15a]) y el análisis detallado de los grupos de secuencias más estrechamente emparentadas dentro de esta superfamilia, permitirá definir y validar, o refutar, los procesos evolutivos que habrían dado origen a la sofisticada diversidad de funciones que desempeñan sus miembros.

¶ Se ha estimado que el número de secuencias únicas de proteínas está en el orden de 5×10^{10} [2a], mientras que el número de plegamientos monomodulares ronda 1×10^3 [1a].

§ Cabe señalar que la clasificación jerárquica de la base de datos SCOP está basada en inspección manual, mientras que la correspondiente a CATH deriva de alineamientos de secuencia basados en criterios estructurales.

Caso 1. Toxinas de alacrán que bloquean canales de K^{+}¶

El veneno de los alacranes se compone principalmente de dos tipos de toxinas que adoptan el motivo estructural α/β-EC, diferenciadas por la longitud de su cadena polipeptídica y sus blancos celulares. En general, las "toxinas de cadena corta" (23-42 residuos) actúan bloqueando canales de K^{+} —nomenclatura sistemática de la forma α-KTx *x.y*— mientras que las "toxinas de cadena larga" (59-76 residuos) ejercen su función modificando las propiedades de apertura y cierre de los canales de Na^{+}. Estas toxinas han servido para elucidar las propiedades bioquímicas, farmacológicas, biofísicas e incluso estructurales de los canales iónicos que les fungen como receptores. En los trabajos que a continuación se discuten se abordan dos temas comunes al estudio de las relaciones estructura-función de las proteínas en un contexto evolutivo, a saber: tomando en cuenta la notable conservación del plegamiento tridimensional, i) se enfatiza la estrecha relación entre las estrucuturas primarias y las especificidades de función que despliegan, y; ii) se especula sobre las distintas alternativas que el proceso evolutivo ha generado en estos péptidos en el contexto de la adecuación de las superficies que interactúan con sus receptores.

Evidencias sobre la diversidad de modos de interacción entre α-KTx's y canales de K^{+}: Las toxinas de alacrán han sido extensivamente utilizadas en el estudio de los canales de K^{+}. En particular, la disponibilidad de mutantes sitio dirigidas de varias toxinas y distintos tipos de canales, permitió que la interacción de los pares ligando - receptor correspondientes se definiera en gran detalle, aún en ausencia de información estructural del receptor. La evidencia acumulada a partir del estudio de toxinas de las familias α-KTx 1, 2 y 3 resaltó un par de residuos conservados en las toxinas que desempeñan un papel crítico en el efecto de bloqueo a los canales $K_V1.x$ y $K_{Ca}1.1$. La identificación de residuos análogamente importantes para otros bloqueadores de canales de K^{+} fundamentó la propuesta de que éstos constituirían un farmacóforo mínimo —denominado "diada funcional", constituida por un residuo básico y uno hidrofóbico ubicados a ~ 7 Å de distancia— a los péptidos capaces de bloquear la conducción a través del poro de los canales de K^{+}. Ahora bien, la toxina Tc32, aislada del alacrán *Tityus cambridgei*, es el único miembro caracterizado de la subfamilia α-KTx 18. Su estructura primaria es altamente divergente con respecto a las demás α-KTx's, en particular no posee el par de residuos repetidamente señalados como indispensables para el efecto bloqueador de éstas (**Figura A-1**); aún así, es capaz de inhibir totalmente la corriente asociada a los canales $K_V1.3$ de linfocitos humanos. A partir de estas observaciones resulta claro que no todas las α-KTx's interactúan de la misma manera con los canales de K^{+}.

---

a)

```
                                             o       o
18.1|Tc32       -TGPQTTCQAA-MCEAGCKGL-GKSME-SCQGDTCKCKA-
1.1|Chtx        -QFTNVSCTTSKECWSVCQRLHNTSRG-KCMNKKCRCYS-
2.1|Ntx         -TIINVKCTSPKQCSKPCKELTGSSAGAKCMNGKCKCYNN
3.2|Agtx2       GVPINVSCTGSPQCIKPCKDA-GMRFG-KCMNRKCHCTPK
4.1|TsTXKα      -VFINAKCRGSPECLPKCKEAIGKAAG-KCMNGKCKCYP-
6.2|Mautx       -----VSCTGSKDCYAPCRKQTGCPNA-KCINKSCKCYGC
```

¶ Las referencias relevantes para esta discusión se encuentrán en los artículos incluidos, por lo cual no se reproducen en esta sección.

b)

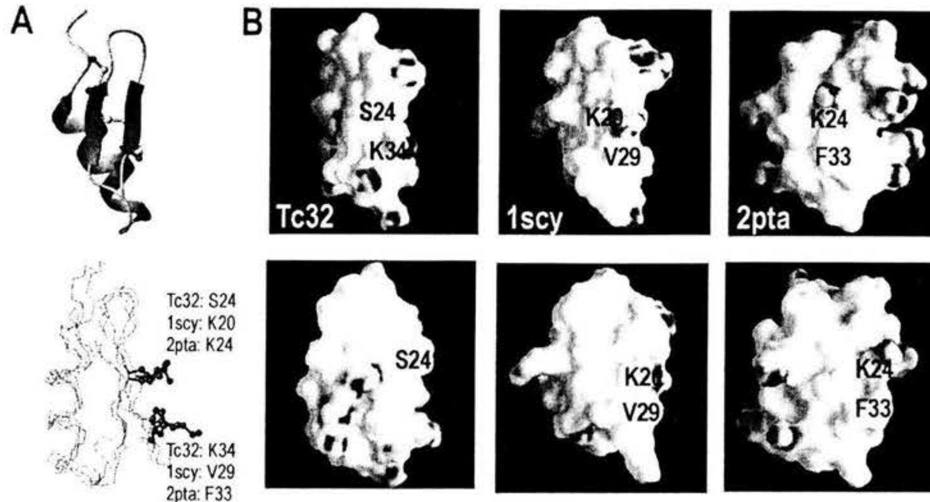

Figura A-1 | *Secuencia y modelo tridimensional de la toxina Tc32 (α-KTx 18.1).* a) La secuencia de Tc32 se alineó con miembros representativos de las familias α-KTx 1 (Chtx), 2 (Ntx), 3 (Agtx2), 4 (TsTXKα) y 6 (Mautx), para las cuales estudios con mutantes puntuales han señalado a los residuos marcados en negritas como determinantes en el efecto de bloqueo. El alineamiento se obtuvo con el programa CLUSTAL_X [16a]. bA) Arriba: Representación del modelo de Tc32 generado por el programa SWISS-MODEL disponible en la suite ExPASy (www.expasy.org) y minimizado en el módulo DISCOVER del programa Insight II. Abajo: Sobreimposición del modelo de Tc32 con las estructuras de las toxinas Scytx (1scy) y Pi2 (2pta), mostrando los residuos propuestos como esenciales en la función de bloqueo. La figura fue preparada con el programa MOLMOL [17a]. bB) Superficies electrostáticas del modelo de Tc32 (izquierda) y las toxinas Scytx (en medio) y Pi2 (derecha), calculadas con el programa GRASP. Las imágenes arriba y debajo de ambos páneles se encuentran en la misma orientación relativa.

Con datos de: Batista CVF, Gómez-Lagunas F, Rodríguez de la Vega RC, Hajdu P, Pany G, Gaspar R y Possani LD (2002). Two novel toxins from the Amazonian scorpion Tityus cambridgei that block Kv1.3 and Shaker channels with distinctly different affinities. *Biochim. Biophys. Acta* 1601:123-131.

Expandiendo los modos de interacción toxina - canal: Tres elementos han propiciado un renovado interés en el estudio de este par ligando - receptor, a saber: i) la determinación de estructuras tridimensionales de varios canales de K^+; ii) el desarrollo de programas computacionales que permiten evaluar interacciones bimolecualres con "verosimilitudes" cada vez más altas¶, y; iii) la caracterización acelerada de nuevos ligandos y mutaciones puntuales de los mismos que permiten evaluar, directa o indirectamente —a través de análisis evolutivos—, la relevancia de ciertos residuos en el contexto de la complementariedad de las superficies interactuantes. En el primero de los artículos que a continuación se reproducen se señalan las limitaciones que, de alguna manera, han definido el campo de estudio de los pares α-KTx's - canales de K^+, se compendian los modos de interacción descritos hasta esa fecha (1er. Semestre del 2003), en un contexto evolutivo se hace hincapié sobre las relaciones estructura - función de las toxinas y se hipotetiza sobre los modos de interacción aún no descritos, aunque previstos por la evidencia experimental. En el segundo se describen los avances más recientes en el campo de las α-KTx's, los cuales, en conjunto, soportan y expanden las predicciones señaladas en el primero.

¶ El par α-KTx - canal de K^+ es particularmente accesible para este tipo de estudios, toda vez que ambas moléculas son relativamente rígidas —los canales por estar embebidos en la membrana y la toxinas en virtud de la abundancia de disulfuros que la entrecruzan—, las atracciones primarias son fudamentalmente de naturaleza electrostática y, en general, presentan una notable complementaridad geométrica.

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A-4

Novel interactions between K⁺ channels and scorpion toxins

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K⁺ channels are macromolecules embedded in biological membranes, where they play a key role in cellular excitability and signal transduction pathways. Knowledge of their structure should help improve our understanding of their function and lead to the design of therapeutic compounds. Most pharmacological and structural characteristics of these channels have been elucidated by using high-affinity channel blockers isolated from scorpion venoms. Recent data on the three-dimensional structures of K⁺ channels and novel scorpion toxins suggest a variety of novel interacting modes of these channels and toxins, which should help increase our understanding of the K⁺ channel structure–function relationship.

Recently, knowledge of transmembrane ion channels has greatly advanced, thanks to the discovery of specific ligand inhibitors and to the determination of the three-dimensional (3D) structure of some channels by X-ray diffraction [1–3]. Among these specific ligands are peptides extracted from venomous animals such as scorpions, snails, spiders, snakes and sea anemone, among others [4]. Of the ion channels, the most thoroughly studied are the K⁺ channels. Virtually all living cells possess K⁺ channels, which are involved in a variety of biological functions, ranging from the control of membrane excitability to the regulation of signal transduction pathways. K⁺ channels comprise homo- or hetero-tetrameric peptidic subunits that are responsible for specific K⁺ ion conduction [5]. The structural and functional variability of all known K⁺ channels and the increasing number of human pathologies associated with these channels (e.g. episodic ataxia type 1, long-QT syndrome and benign familial neonatal epilepsy) make them attractive therapeutic targets [6].

Scorpion toxins specific for K⁺ channels, here abbreviated as KTx, are short-chain peptides comprising 23–43 amino acid residues that are cross-linked by three or four disulfide bridges; these toxins have recently been divided into three subfamilies, called α-, β- and γ-KTxs [7,8]. The α-KTxs have proven to be powerful tools for testing the pharmacological, physiological, biochemical, biophysical and even structural characteristics of K⁺ channels and their associated ionic currents [9–11].

In this article, a representative list of various subfamilies of scorpion toxins with known affinities for various types and subtypes of K⁺ channels is provided. A phylogenetic tree that contains information enabling correlations to be made between the primary structure of toxins and their specificities for K⁺ channels is described. Finally, the different interacting modes already described for the KTx–K⁺-channel pair are discussed. In summary, we stress that, because a huge biodiversity of toxins and channels exists, novel emerging modes of interaction, yet to be described, will add fresh information to the field.

Structural characteristics of scorpion toxins specific for K⁺ channels

Most known scorpion toxins are structurally related, consisting of a cysteine-stabilized α/β scaffold (CS–α/β) that comprises an α-helix connected to a double- or triple-stranded β-sheet by two highly conserved disulfide bridges, formed between the consensus sequences CXXXC and CXC, where X is any amino acid residue [10,12]. The CS–α/β fold is a remarkably versatile framework shared by a variety of peptides with different functions, including some antimicrobial peptides, such as defensins, from invertebrates [13] and plants [14], the sweet-tasting brazzein [15] and the rapeseed class of plant serine protease inhibitors [16]. Thus, the 3D folding, although important for the high stability of the peptide and allowing sequence variability, does not correlate directly with a specific target molecule. Furthermore, in all these peptides with the CS–α/β fold, ~80–90% of the amino acids are exposed to the peptide surface. Thus, the type and position of these different surface amino acids are crucial in determining the biological function of the protein.

Channel affinities of toxins

Table 1 summarizes representative information available in the field, where the known K_d values (affinities) for the interaction of scorpion toxins with K⁺ channels were reported. The information in the table emphasizes the huge variability in the affinities of α-KTxs for a wide variety of K⁺ channels, and provides a comprehensive list of the experimentally determined K_d values for each toxin–channel pair. Although the 3D folding of these α-KTx peptides is similar, it is important to note that their physiological actions, specificity and affinities depend on the amino acids situated at their external surface and, of course, the surface contact with the channel molecules (see below).

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http://tips.trends.com 0165-6147/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved. doi:10.1016/S0165-6147(03)00080-4

Table 1. Activities of scorpion toxins specific for K^+ channels

Toxin[a]	Channels tested[b]	K_d (nM)[c]	Refs	Toxin[a]	Channels tested[b]	K_d (nM)[c]	Refs
Charybdotoxin/α-KTx 1.1	*Shaker* (d)	120	[48][d]	Maurotoxin/α-KTx 6.2	*Shaker* (d)	2.4	[51]
	K_V1.3 (r)	0.17	[48]		K_V1.2 (m)	0.12	[41]
	K_{Ca}1.1 (r)	2.1	[48]		K_V1.3 (h)	150	[48]
	K_{Ca}3.1 (h)	5	[35]		K_{Ca}2.2 (h)	High[e]	[29]
Iberiotoxin/α-KTx 1.3	K_{Ca}1.1 (b)	1.7	[48]		K_{Ca}2.3 (h)	High	[29]
Noxiustoxin/α-KTx 2.1	*Shaker* (d)	160	[48]		K_{Ca}3.1 (h)	1.1	[41]
	K_V1.1 (m)	24	[48]	Pi2/α-KTx 7.1	K_V1.1 (d)	8.3	[48]
	K_V1.3 (r)	0.31	[48]		K_V1.3 (h)	0.044	[52]
	K_{Ca}1.1 (r)	450	[48]	P01/α-KTx 8.1	K_{Ca}2.2 (h)	High	[29]
Margatoxin/α-KTx 2.2	*Shaker* (d)	160	[48]		K_{Ca}2.3 (h)	High	[29]
	K_V1.3 (h)	0.03	[48]	BmP02/α-KTx 9.1	I_{TO} (r)	NA[f]	[46]
Kaliotoxin/α-KTx 3.1	K_V1.3 (r)	0.41	[48]	Cobatoxin/α-KTx 10.1	*Shaker* (d)	700	[48]
Agitoxin 2/α-KTx 3.2	*Shaker* (d)	0.16	[48]		K_V1.1 (h)	500	[48]
	K_V1.1 (r)	0.044	[48]	PbTx1/α-KTx 11.1	NA[f]	–	–
	K_V1.3 (r)	0.004	[48]	Butantoxin/α-KTx 12.1	*Shaker* (d)	660	[53]
TsKα/α-KTx 4.1	K_V1.2 (m)	0.2	[48]	Tc1/α-KTx 13.1	*Shaker* (d)	65	[54]
Tsκ/α-KTx 4.2	K_{Ca}2.2 (h)	80	[29]	OsK2/α-KTx 13.2	K_V1.1 (r)	High	[55]
	K_{Ca}2.3 (h)	197	[29]		K_V1.2 (r)	97	[55]
Scyllatoxin/α-KTx 5.1	K_{Ca}2.2 (h)	0.3	[49]	BmKK1/α-KTx 14.1	NA[f]	–	–
	K_{Ca}2.3 (h)	1.1	[29]	Aa1/α-KTx 15.1	*Shaker* (d)	High	[56]
P05/α-KTx 5.2	K_{Ca}2.2 (h)	22	[29]		I_A (r)	150	[56]
	K_{Ca}2.3 (h)	25	[29]	Martentoxin/α-KTx 16.2	K_{Ca}1.1 (r)	21	[57]
Tamapin/α-KTx 5.4	K_{Ca}2.1 (h)	42	[30]	TXKs4/α-KTx 17.1	NA[f]	–	–
	K_{Ca}2.2 (r)	0.02	[30]	Tc32/α-KTx 18.1	*Shaker* (d)	High	[47]
	K_{Ca}2.3 (r)	1.7	[30]		K_V1.3 (h)	10	[47]
Pi1/α-KTx 6.1	*Shaker* (d)	32	[48]	Ergtoxin/γ-KTx 1.1	I_{ERGs} (m)	16	[58]
	K_V1.3 (h)	11	[50]		K_V11.1 (h)	6.5	[22]
	K_{Ca}2.2 (h)	100	[29]	BemK-1/γ-KTx 2.1	K_V11.1 (h)	6.2	[30]
	K_{Ca}2.3 (h)	250	[29]	CnErg2/γ-KTx 3.1	I_{ERGs} (r)	418	[59]
				CsEKerg1/γ-KTx 4.12	I_{ERGs} (m)	230	[60]
				CsErg5/γ-KTx 5.1	NA[f]	–	–

[a]The common names followed by the systematic names as outlined in [7,8] are shown.
[b]For comparative purposes only K_V1.x, K_{Ca}1.1, K_{Ca} 2.x, K_{Ca}3.1 and K_V11.x channels are considered. Sources are as follows: b, cattle; d, fruit fly; h, human; m, mouse; r, rat.
[c]Only data from electrophysiological experiments in which the molecular identity of the channel involved is clearly established were considered, except if otherwise indicated. Dissociation constants are derived either from the kinetic k_{off}/k_{on} ratio or solution of Hill's equation in dose–response experiments.
[d]A review reference was used for the sake of space; the authors apologize for the exclusion of the original references.
[e]The affinity is either too low or undetectable.
[f]Abbreviations: I_A, fast, transient, low-voltage-activated current; I_{ERGs}, *ether-a-go-go*-related currents; I_{TO}, transient outward current; NA, not available.

Phylogenetic tree of scorpion toxins specific for K^+ channels

Figure 1 shows the results of a phylogenetic tree constructed using the data available in the literature. Grossly, the dispersion of sequences of α-KTxs throughout the tree support the proposed classification of these toxins [8,9]. The peptide toxins that are specific for the Ca^{2+}-activated K^+ channels of large-(K_{Ca}1.1) and small- (K_{Ca}2.x) conductance are branched in distantly related clusters, whereas those specific for the *Shaker*-related voltage-dependent K^+ (K_V1.x) channels are more disperse but fall on the same side of the tree, opposite to the toxins specific for the *ether-a-go-go*-related K^+ (K_V11.x) channels, which are all at the right side of the tree. In general, there is a good correlation between pharmacological findings and the position of the toxins in the phylogenetic tree. However, toxin BeKm-1 (γ-KTx 2.1), although specific for K_V11.x channels, is not clustered together with the remaining γ-KTxs, but rather with the α-KTx 14 subfamily, which thus far have not been tested for their affinities for K^+ channels.

At least two different groups of 3D structures of KTxs exist. The first and better described group contains a triple-stranded β-sheet (or a double-stranded β-sheet complemented with a third pseudo-strand at the N-terminal section) with either three or four disulfide bridges. This group includes α-KTx subfamilies 1–4, 6, 7 and 12 and the only member of γ-KTx subfamily 2 (BeKm-1). The second group is characterized by N-terminal deletion with respect to the other group, which results in the lack of the first strand of the β-sheet. This group includes α-KTx subfamilies 5, 8, 9 and 13. A third group (not shown in Fig. 1) has a triple-stranded β-sheet and four disulfide bridges. This group includes chlorotoxin, insectotoxin and similar peptides and, very likely, the remaining γ-KTx subfamilies.

Interacting surfaces of scorpion toxins and K^+ channels

Following earlier studies of α-KTxs, it was suggested that the architecture of the pore region of K^+ channels should be highly conserved [9,11]. Recently, these data received strong experimental support from the analysis of: (1) the 3D structure of K^+ channels; and (2) K^+ channel mutants from *Streptomyces lividans* (KcsA) that are capable of being blocked by α-KTxs, but are normally insensitive to α-KTxs [17–20]. Despite the fact that there is a homologous structural folding of the α-KTxs and similar architecture of the vestibule of the channel pore, binding of α-KTxs is characteristic for each type of K^+ channel (Table 1). Thus, channels and/or toxins should have subtle

A-5

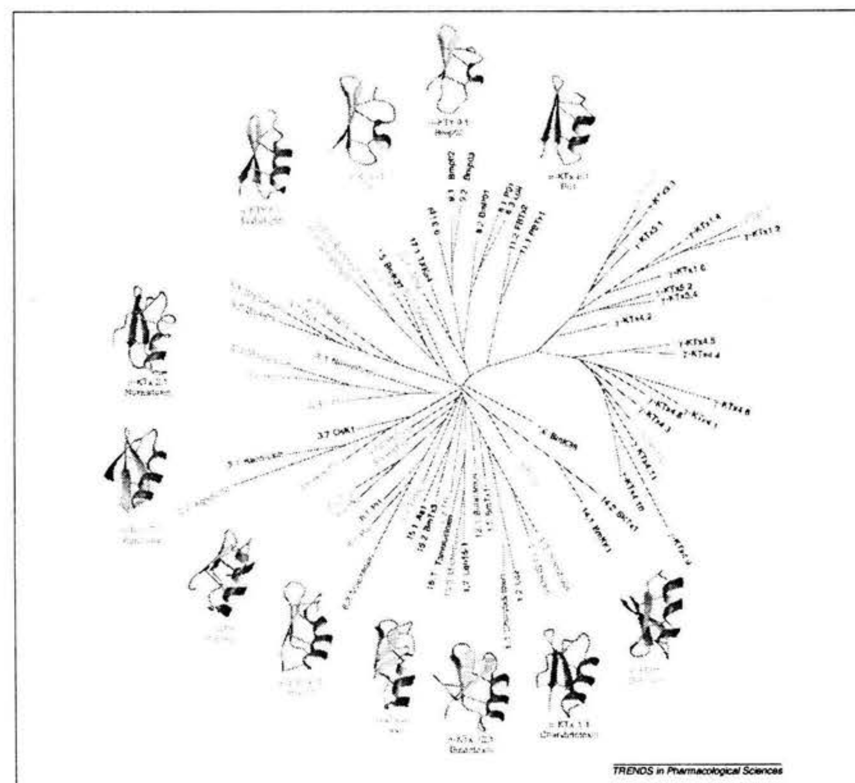

Fig. 1. Unrooted phylogenetic tree of scorpion toxins that are specific for K^+ channels (KTxs). A multiple alignment of 105 KTx sequences was used to calculate a matrix with the genetic distances for each pair of the sequences. Based on this matrix, successive clustering of lineages were used to construct the tree with the neighbor-joining algorithm, as described in [12]. Each branch of the tree ends with the abbreviated name of the representative KTxs and their number according to the systematic nomenclature, except for the general term α-KTx. All the γ-KTxs are indicated. Representative examples of the three-dimensional structures of KTxs are also included with both the systematic and the trivial names. These show the two different structural groups of toxins. One group, which includes α-KTx subfamilies 1–4, 6, 7 and 12 and the γ-KTx subfamily 2 toxin BeKm-1, contains a triple-stranded β-sheet (blue arrows) (or a double-stranded β-sheet complemented with a third pseudo-strand at the N-terminal section) and either three or four disulfide bridges (yellow lines). The second group, which includes α-KTx subfamilies 5, 8, 9 and 13, is characterized by an N-terminal deletion with respect to the first group, which results in the lack of the first strand of the β-sheet. The coordinates were obtained from the Protein DataBank (http://www.rcsb.org/pdb/). The colors of the toxins were assigned according to the type of channel for which they are specific: blue, K_{Ca}1.1; brown, K_V1.x and K_{Ca}2.x; green, K_V1.x; red, K_{Ca}2.x; purple, K_V1.x and K_{Ca}1.1; pink, K_V11.x; black, those toxins that have not as yet been tested. Asterisks denote sequences deposited in the Protein DataBank that, as yet, have not been classified or functionally analyzed.

differences that would explain the specific interactions found for each channel–toxin pair [21,22].

Work in various laboratories [23–26] has clearly established that the interaction of the K_{Ca}1.1 and K_V1.x channels with α-KTxs from the subfamilies 1–3 is characterized by the interaction of amino acid residues situated at the β-hairpin face of the toxins with amino acids located at the vicinity of the selectivity filter of the respective K^+ channel. However, in agreement with earlier proposals [27], it has been demonstrated recently that for other toxins (e.g. α-KTx 4.2 and the entire α-KTx subfamily 5) the interacting residues of the K_{Ca}2.x channels are situated at the 'turret' (i.e. the extracellular loop between the transmembrane segments S5 and S6 of the channel) and the bottom of the vestibule, whereas the amino acids of the toxins are located at the α-helix, instead of the β-hairpin [28–31]. In the case of the K_V11.x channels, for which an entirely new family of specific

inhibitors (γ-KTxs) has been described [8], the architecture of the S5-P (pore) segment seems to contain an extra α-helix region [22,32]. The interaction of γ-KTxs with the mouth of the channel must be situated more externally [22,33,34].

Finally, it is worth noting that small changes, either in the K^+ channels or in the KTxs, might drastically change their affinities for each other. For example, recent reports show that point mutations at the β-turn of charybdotoxin (α-KTx 1.1) and iberiotoxin (α-KTx 1.3) render analogs with severely affected specificities for $K_{Ca}1.1$ and $K_{Ca}3.1$ channels but no change in affinities for $K_V1.x$ channels [35,36]. A chimeric construct involving residues of the α/β loop of iberiotoxin grafted into noxiustoxin (α-KTx 2.1) loses the ability to block $K_V1.3$ channels, but has a greatly augmented ability to block $K_{Ca}1.1$ channels [37]. Grafting of three residues commonly present in specific blockers of $K_V1.x$ channels into BmP05 (α-KTx 5.3) confers to the chimera the ability to block a fraction of the Ca^{2+}-independent currents [38]. Incorporation into BeKm-1 (γ-KTx 2.1) of two highly conserved residues present in specific blockers of $K_{Ca}1.1$ and $K_{Ca}3.1$ channels results in a mutant capable of blocking both $K_V11.1$ and $K_{Ca}1.1$ channels with high affinity [34].

Summary of known surface contacts

From the data described above, we propose an interpretative model for the known contact surfaces of KTxs (Fig. 2). In Fig. 2a a ribbon molecular model of α-KTx 1.1 manually docked into a KcsA-based *Shaker* model, as previously reported [10,17,35,39], is shown taking account of a pair of interactions derived from double-mutant cycles (which consist of preparing pairwise mutations of residues that correspond to the two contacting surfaces) [40]. In Fig. 2b a two-dimensional cartoon model indicating the interactions of α-KTx 3.2 with pore-forming segments of the *Shaker* channel is shown. Interestingly, this model holds for all the data obtained, both experimentally [23–26,35,37,39,41] and model derived [17,42–44], for KcsA, $K_V1.x$, $K_{Ca}1.1$ and $K_{Ca}3.1$ channel interactions with α-KTxs from subfamilies 1–4 and 6. The amino acids that are clearly known to make contacts are indicated. Figure 2c illustrates the case in which the α-helix segment of the toxin replaces the β-hairpin of the model shown in Fig. 2a. In this case, double-mutant cyclic analysis was not performed; however, the data obtained with toxins from the subfamily 5 and α-KTx 4.2 interacting with the $K_{Ca}2.x$ channel isoforms support the interpretation of the cartoon [28–30] and also satisfies Brownian dynamics-derived interactions [31]. Finally, Fig. 2d shows the cartoon representing the $K_V11.1$ channel, where an additional α-helix segment was suggested to protrude from the mouth of the pore-forming region of the channel, between the S5 segment and the P site [22,32]. The interactions with two toxins (ergtoxin-1 and BeKm-1) were studied but the double-mutant cyclic analysis was also not performed. Thus, in Fig. 2d some amino acid residues known to be important for recognition are indicated for both the toxin and the channel [22,34], without specification of the contact pairs.

Thus, Fig. 2 summarizes the documented cases for the surface areas of scorpion toxins that interact with known K^+ channels. Three different modes of interactions are clearly discernible: (1) internal, involving residues at the 'turret' region, the pore helix and the selectivity filter of the channel, which interacts with the β-hairpin of the toxins; (2) intermediate, involving residues at the 'turret' region and the bottom of the vestibule of the channel; and (3) external, involving residues distant from the selectivity filter of the channel. The latter two cases seem to involve the α-helix segment of the toxins, rather than the β-hairpin.

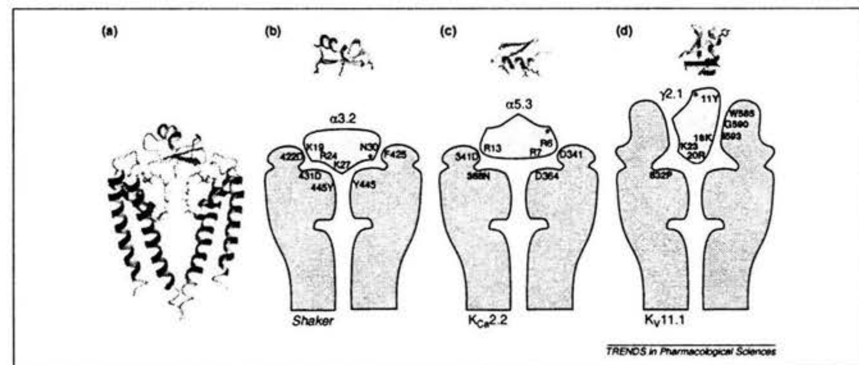

Fig. 2. Two-dimensional cartoon model of scorpion toxins that are specific for K^+ channels (KTxs), and K^+ channel interactions. (a) Ribbon docking model of the interaction between α-KTx 1.1 and two of the four segments forming the pore region of the KcsA-based *Shaker* model [10,17,35,39]. (b) Cartoon model of the interaction between toxin α-KTx 3.2 and a model of *Shaker* [43]. Relevant contact amino acid residues obtained from double-mutant cycles are indicated. (c) Interactions between toxin α-KTx 5.3 and the $K_{Ca}2.2$ channel [31]. Four amino acids in the channel and three on the toxin were directly determined by data obtained independently [29,30]. The toxin position was assumed based on the best fit Brownian dynamics-derived calculations [31]. (d) The $K_V11.1$ channel showing amino acids important for the blocking activity of γ-KTx 1.1 [22]. The model of γ-KTx 2.1 (note that the three-dimensional structure of γ-KTx 1.1 is not known) was arbitrarily situated on top of the vestibule. Indicated amino acids were chosen based on experiments with site-directed mutants of the toxin assayed electrophysiologically with the channel [34]. At the top of parts b–d, a ribbon molecular model of the corresponding KTxs is shown. In all panels, amino acids shown in bold are those crucial for function, but there is no direct demonstration of contact. Asterisks indicate the approximate position of the β-turn region of the toxins.

It is important to note that most scorpion toxins still need to be screened for affinities towards the immense variety of existing K^+ channels. Furthermore, the 3D structures of most K^+ channels are still not determined. Thus, it is reasonable to assume that the surface areas of contact between scorpion toxins and K^+ channels are still an open question. This is supported by recent data obtained with KTxs of several subfamilies, in which the dyad residues (i.e. two important residues for recognition) [45] or the RXCQ motif (found in toxins specific for the $K_{Ca}2.x$ channel) [10] are absent in toxins of the subfamilies α-KTx 9, 11, 14 and 18. However, at least α-KTx 9 and 18 toxins are *bona fide* blockers of the channel activity [46,47].

Concluding remarks

Although an increasing amount of molecular data is being published on this subject, many K^+ channel currents remain elusive. Specific ligands are needed to study K^+ channels, and this is a major area of current interest. Despite the fact that toxin–channel interactions have been important tools to characterize channels, it seems that some of the available data still need to be re-evaluated, taking into account not only the diversity of ligands but also the diversity of the K^+ channels.

Extensive studies have been performed with α-KTxs of the subfamilies 1–6. Regarding the ion channels, only *Shaker*, $K_V1.x$, $K_{Ca}1.1$, $K_{Ca}2.x$ and $K_V11.x$ channels have been characterized in depth with respect to their interactions with scorpion toxins. Moreover, the faces of the α/β scaffold of the scorpion toxins that interact with K^+ channels are not unique, and as a result of the great diversity that exists in nature we believe that this field is still under-studied.

Finally, knowledge of the interacting surfaces between the channels and toxins, and the modification of ligands, constitute a hot research topic that should be directed towards the rational design of more specific drugs and the control of pathologies associated with K^+ channels.

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Mini-review

Current views on scorpion toxins specific for K^+-channels

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Abstract

Much of our knowledge on K^+-channels was elucidated using specific peptide ligands isolated from a number of venomous organisms. Recently, this field received a strong support and increased interest due to the solution of the three-dimensional structure of a couple of K^+-channels. At the same time, several new subfamilies of specific toxins for K^+-channels were isolated from scorpion venoms, enhancing the availability and diversity of such useful molecular tools. It opened new lines of research for the better understanding of K^+-channel biophysics and pharmacology. In this review, we listed 120 amino acid sequences of peptides isolated from scorpion venoms. They were demonstrated or assumed to be specific for K^+-channels. These sequences were aligned and used to generate a rooted phylogenetic tree. The evolutionary tree indicates that several clusters of divergent peptides show preference for specific subtypes of channels. The three-dimensional structures of representative examples of these peptides were drawn and analysed concerning the molecular fitness of their interactions with the channel targets. Four different interacting modes were identified to exist between scorpion toxins and the various subtypes of K^+-channels.

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Keywords: Amino acid sequence; Evolution; K^+-channel; Scorpion toxin

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1. Introduction

A little bit over 20 years have elapsed since the first short chain scorpion venom peptide (Noxiustoxin) was isolated from *Centruroides noxius* (Possani et al., 1982) and shown to affect potassium permeability in squid giant axon (Carbone et al., 1982). The discovery of Charybdotoxin (Miller et al., 1985), initially thought to be unique on its genre, prompted a significant increase of research in this area, since the peptide was shown to be an excellent ligand model for studying K^+-channel function and structure (Miller, 1995). By the years 1999, the review of Tytgat et al., grouped the K^+-channel acting peptides into three families (the α-, β- and γ-scorpion toxins, abbreviated KTx's) and a systematic numbering system was proposed for the three families. The α-KTx family, the largest one, by that time contained 49 different peptides, comprising 12 subfamilies. Since then, the number of known peptides, extracted from scorpion venoms that block or modify potassium permeability in excitable and non-excitable cells have increased drastically to the point that now about 120 peptides are known, object of this communication.

Here we present a comprehensive list of K^+-channel specific scorpion toxins known to date, we analyse their

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three-dimensional-structures in reference to the interface contacts with distinct subtypes of channels and we present a extended, rooted phylogenetic tree, in which all known K^+-channel specific scorpion toxins and a few other related structures have been included.

2.-How many different structurally related toxins are known?

Except for the κ-hefutoxins, all the K^+-channel specific peptides isolated from scorpion venoms are structurally related and were taken into consideration for the present analysis. The structural signature of these peptides is defined by the presence of the Cysteine-Stabilized α/β motif (CS-αβ), in which two disulfide bridges, C_i-C_j and $C_{i+4}-C_{j+2}$, covalently link a segment of α-helix with one strand of the β-sheet structure (see below). From 120 sequences known (Table 1), isolated peptides or inferred from cDNA sequences, 75 are classified as α-KTx's, comprising 18 different subfamilies (Goudet et al., 2002; Batista et al., 2002), 4 are β-KTx's, 26 are γ-KTx's, 2 are κ-hefutoxins, 5 are chlorotoxin/insectotoxin-like peptides and 8 are unclassified CS-αβ motif-containing short chain peptides. One hundred and three sequences are deposited in data-banks, the remaining were obtained from the literature (see references in Table 1). Seventy-five are from scorpions of the Old-World and 45 are from the New World scorpions. Seven genus of the Buthidae family contributed with 109 known peptides on this list, whereas the remaining came from 4 genus belonging to the Scorpionidae family. The peptides listed in Table 1 have from 23 to 64 amino acid residues in length and are well packed by either three or four disulfide bridges. Tc1 from *Tityus cambridgei* is the shortest one described, with only 23 residues (Batista et al., 2000), and the β-KTx's, AaTXKβ and BmTXKβ2 are the longest ones, containing 64 amino acid residues each (Legros et al., 1998; Zhu et al., 1999).

3. How many different target types and subtypes of K^+-channels are recognized by these toxins?

There are more than 100 subtypes of known K^+-channels (Miller, 2000) and only for a relatively small number of them was ever a peptide found to affect their corresponding function (Table 1). About half of the 120 toxins listed in Table 1 have been tested directly against some K^+-channels and/or associated currents. For 59 of the peptides reported, there is no direct evidence of function described. One of them (Chlorotoxin) was shown to be specific for Cl^- channels (DeBin et al., 1993), instead of K^+-channels. The majority of the known functions were determined on *Shaker*-related channels (subfamily $K_V1.x$), although some peptides were shown to act on the Ca^{2+}-activated K^+-channels of large ($K_{Ca}1.1$), intermediate ($K_{Ca}3.1$) or small conductance ($K_{Ca}2.x$). Recently a full new family of toxins (γ-KTx's) was described to be specific for the *ether-a-go-go* related family of K^+-channels ($K_V11.x$) (Gurrola et al., 1999; Korolkova et al., 2001; Lecchi et al., 2002; Nastainczyk et al., 2002; Corona et al., 2002).

4. Structural features of scorpion toxins implicated in K^+-channel recognition

The interacting surfaces of scorpion toxins with K^+-channels were thought to depend on several side-chain residues mainly located at the beta-sheet face of the toxins (Miller, 1995; Menez, 1998). The discovery of peptides from *Dendroaspis* snake venom (Harvey, 2001) and sea anemone toxins (reviewed in Garcia et al., 1997) with similar functions, was used to highlight the requirement for two amino acid residues as a minimum for channel blockade, called 'the functional dyad theory' (Dauplais et al., 1997). The solution of the three-dimensional structure of three different K^+-channels by the Nobel laureate MacKinnon's group (MacKinnon, 2004), and the discovery of truly blocker toxins for which the dyad theory concerning the molecular mechanism of binding to the channel, did not seem to fit exactly to the predicted model (Tong et al., 2000; Batista et al., 2002; Dhawan et al., 2003), prompted the discovery of novel interface contact points between scorpion toxins and the various types and subtypes of K^+-channels (Rodríguez de la Vega, 2003; Zhang et al., 2003; Xu et al., 2003; Zhu et al., 2004).

Several authors, starting with the work performed with Scyllatoxin (Auguste et al., 1992) suggested that the α-helix segment of this toxin was implicated in the functional blockade of Ca^{2+}-activated K^+ currents in neuroblastome cells. Further to this work, it was demonstrated that toxins affecting the $K_{Ca}2.x$ channels (Lecomte et al., 1999; Shakkottai et al., 2001; Pedarzani et al., 2002; Cui et al., 2002) and the ergtoxins were capable of interacting with the channels in a different manner than that earlier proposed by the functional dyad theory (Korolkova et al., 2002; Xu et al., 2003; Huys et al., 2004b; Zhu et al., 2004). Additionally, three-dimensional models of toxin-channel interactions generated with Pi4 (M'Barek et al., 2003b), Cobatoxin 1 (Jouirou et al., 2004), Lq2, Agitoxin2, Charybdotoxin and Iberiotoxin (MacKinnon et al., 1998; Cui et al., 2001; Eriksson and Roux, 2002; Gao and Garcia, 2003; Takeuchi et al., 2003) and Maurotoxin (Fu et al., 2002; Castle et al., 2003; M'Barek et al., 2003a) have all suggested that other amino acid residues surrounding the functional dyad make important contacts with specific residues at the turret regions of the KcsA, $K_V1.x$, $K_{Ca}1.1$ and $K_{Ca}3.1$ channels. The amino acid residues of the toxins implicated in this recognition site of the K^+-channels was defined as the 'basic ring' residues (Mouhat et al., 2004).

All the above-mentioned toxins fit well to the binding surface of the various subtypes of K^+-channels, except for the toxins that bind to the ERG-channels, because the mouth structure of the latter seems to be quite different, including the presence of an extra α-helix within the pore loop (Liu et al.,

Table 1
Scorpion toxins specific for K^+-channels and related peptides

Toxin[a]	Targets[b]	Sequence	Reference[c]
α-1.1|Lqh Charybdotoxin 1	1,2,3	QFTNVSCTTSKECWSVCQRLHNTSRGKCMNKKCRCYS	P13487 (2CRD)
α-1.2|Lqh2	1,2	QFTQESCTASNQCWSICKRLHNTNRGKCMNKKCRCYS	P45628 (1LIR)
α-1.3|Bt Iberiotoxin	2	QFTDVDCSVSKECWSVCKDLFGVDRGKCMGKKCRCYQ	P24663
α-1.4|Clb Limbatotoxin	2	VFIDVSCSVSKECWAPCKAAVGTDRGKCMGKKCRCYX	Tytgat et al., 1999
α-1.5|BmTx1	1,2	QFTDVKCTGSKQCWPVCKQMFGKPNGKCMNGKCRCYS	Q9NII6 (1BIG)
α-1.6|BmTx2	1,2	QFTNVSCSASSQCWPVCKKLFGTYRGKCMNSKCRCYS	Q9NII5 (2BMT)
α-1.7|Lqh15-1 (Chtx2)	n.d.	GLIDVRCYDSRQCWIACKKVTGSTQGKCQNKQCRCY	P45660
α-1.9|Clb Hongotoxin 2	1	HFIDVKCTTSKECWPPCKAATGKAAGKCMNKKCKCQX	P59848
α-1.10|Psp PbTx3	1	EVDMRCKSSKECLVKCKQATGRPNGKCMNRKCKCYPR	P83112
α-1.11|Cn Slotoxin	2	TFIDVDCTVSKECWAPCKAAFGVDRGKCMGKKCKCYV	García-Valdes et al., 2001
α-2.1|Cn Noxiustoxin	1,2	TIINVKCTSPKQCSKPCKELTGSSAGAKCMNGKCKCYNN[d]	P08815 (1SXM)
α-2.2|Cm Margatoxin	1,3	TIINVKCTSPKQCLPPCKAQFGQSAGAKCMNGKCKCYPH	P40755 (1MTX)
α-2.3|CllTx1 (II.10.9.1)	6	ITINVKCTSPQQCLRPCKDRFGQHAGGKCINGKCKCYP	P45629
α-2.4|Cn Ntx2	2	TIINEKCFATSQCWTPCKKAIGSLQSKCMNGKCKCYNG	AAB50864
α-2.5|Clb Hongotoxin 1	1	TVIDVKCTSPKQCLPPCKAQFGIRAGAKCMNGKCKCYPH	P59847 (1HLY)
α-2.7|CllTx2 (II.10.9.2)	6	TVIDVKCTSPKQCLPPCKEIYGRHAGAKCMNGKCKC	P45630
α-3.1|Am Kaliotoxin	1	GVEINVKCSGSPQCLKPCKDAGMRFGKCMNRKCHCTP[d]	P24662 (2KTX)
α-3.2|Lqh Agitoxin2	1	GVPINVSCTGSPQCIKPCKDAGMRFGKCMNRKCHCTPK	P46111 (1AGT)
α-3.3|Lqh Agitoxin3	1	GVPINVPCTGSPQCIKPCKDAGMRFGKCMNRKCHCTPK	P46112
α-3.4|Lqh Agitoxin1	1	GVPINVKCTGSPQCLKPCKDAGMRFGKCINGKCHCTPK	P46110
α-3.5|Am Kaliotoxin 2	1	VRIPVSCKHSGQCLKPCKDAGMRFGKCMNGKCDCTPK	P45696
α-3.6|BmKTX	1	VGINVKCKHSGQCLKPCKDAGMRFGKCINGKCDCTPK[d]	Q9NII7 (1BKT)
α-3.7|OsK1	4	GVIINVKCKISRQCLEPCKKAGMRFGKCMNGKCHCTPK	P55896 (1SCO)
α-3.8|Bt Bs6	n.d.	GVPINVKCRGSPQCIQPCRDAGMRFGKCMNGKCHCTPQ	P59886
α-3.9|Bo KTx3	n.d.	VGIPVSCKHSGQCIKPCKDAGMRFGKCMNRKCDCTPK	P59290
α-4.1|TsTXKα (TSII-9/TsK4)	1	VFINAKCRGSPECLPKCKEAIGKAAGKCMNGKCKCYP	P46114 (1HP2)
α-4.2|Tsκ|23-57|SCal	4	VVIGQRCYRSPDCYSACKKLVGKATGKCTNGRCDC	P56219 (1TSK)
α-4.3|TdK1|Td1	1	VFINVKCTGSKQCLPACKAAVGKAAGKCMNGKCKCYT	P59925
α-4.4|Tc30|Tc1	1	VFINVKCRGSKECLPKCKAAVGKAAGKCMNGKCKCYP	Batista et al., 2002
α-5.1|Lqh Scyllatoxin(LeTx1)	4	AFCNLRMCQLSCRSLGLLGKCIGDKCECVKH[d]	P16341 (1SCY)
α-5.2|Am PO5	4	TVCNLRRCQLSCRSLGLLGKCIGVKCECVKH	P31719 (1PNH)
α-5.3|BmPO5	4	AVCNLKRCQLSCRSLGLLGKCIGDKCECVKH[d]	AAF03044
α-5.4|Bt Tamapin	4	AFCNLRRCELSCRSLGLLGKCIGEECKCVPY	P59869
α-5.5|Bt Tamapin-2	4	AFCNLRRCELSCRSLGLLGKCIGEECKCVPH	P59870
α-6.1|Pi1 (PiTx-Kγ)	1,4	LVKCRGTSDCGRPCQQQTGCPNSKCINRMCKCYGC	Q10726
α-6.2|Sm Maurotoxin	1,3,4	VSCTGSKDCYAPCRKQTGCPNAKCINKSCKCYGC[d]	P80719 (1TXM)
α-6.3|HsTx1	1	ASCRTPKDCADPCRKETGCPYGKCMNRKCKCNRC[d]	P59867 (1QUZ)
α-6.4|Pi4	1	IEAIRCGGSRDCYRPCQKRTGCPNAKCINKTCKCYGCS	P58498 (1N8M)
α-6.5|Pi7	n.d.	DEAIRCTGTKDCYIPCRYITGCFNSRCINKSCKCYGCT	P58490 (1QKY)
α-6.6|OcKTx1	n.d	AEVIKCRTPKDCAGPCRKQTGCPHGKCMNRTCRCNRC[d]	AAP73817
α-6.7|OcKTx2	n.d.	AEVIKCRTPKDCADPCRKQTGCPHGKCMNRTCRCNRC[d]	AAP73818
α-6.8|OcKTx3	n.d.	AEVIKCRTPKDCAGPCRKQTGCPHAKCMNKTCRCHRC[d]	AAP73819
α-6.9|OcKTx4	n.d.	AEIIRCSGTRECYAPCQKLTGCLNAKCMNKACKCYGCV	AAP73820
α-6.10|OcKTx5	n.d.	AEVIRCSGSKQCYGPCKQQTGCTNSKCMNKVCKCYGC[d]	AAP73821
α-7.1|Pi2 (PiTx-Kα)	1	TISCTNPKQCYPHCKKETGYPNAKCMNRKCKCFGR	P55927 (2PTA)
α-7.2|Pi3 (PiTx-Kβ)	1	TISCTNEKQCYPHCKKETGYPNAKCMNRKCKCFGR	P55928 (1C49)
α-8.1|Am P01	*4*	VSCEDCPEHCSTQKAQAKCDNDKCVCEPI	P56215 (1ACW)
α-8.2|BmP01	n.d.	ATCEDCPEHCATQNARAKCDNDKCVCEPK	AAF03045
α-8.3|Lqh LpII	n.d.	VSCEDCPDHCSTQKARAKCDNDKCVCEPI	P80670
α-8.4|Lqh LpIII	n.d.	VSCEDCPDHCSTQKARAKCDNDKCVCEPK	P80671
α-9.1|BmP02	6	VGCEECPMHCKGKNAKPTCDDGVCNCNV	Q9NJP7 (1DU9)
α-9.2|BmP03	n.d.	VGCEECPMHCKGKNANPTCDDGVCNCNV	Q9U8D1
α-9.3|Lqh LpI	n.d.	VGCEECPMHCKGKNAKPTCDNGVCNCNV	P80669
α-9.4|Bt BTK-2	*1*	VGCAECPMHCKGKMAKPTCENEVCKCNIG	Dahwan et al., 2003

(continued on next page)

Table 1 (*continued*)

Toxin[a]	Targets[b]	Sequence	Reference[c]
α-10.1|Cn Cobatoxin1	1,3	AVCVYRTCDKDCKRRGYRSGKCINNACKCYPY[d]	O46028 (1PJV)
α-10.2|Cn Cobatoxin2	*1*	VACVYRTCDKDCTSRKYRSGKCINNACKCYPY	P58504
α-11.1|Psp PBTx1	*1*	DEEPKESCSDEMCVIYCKGEEYSTGVCDGPQKCKCSD	Huys and Tytgat, 2003
α-11.2|Psp PBTx2	n.d	DEEPKETCSDEMCVIYCKGEEFSTGVCDGPQKCKCSD	Huys and Tytgat, 2003
α-11.3|Psp PBTx10	n.d.	DEEPKETCSDDMCVIYCKGEEYSTGVCDGPQKCKCS	Huys and Tytgat, 2003
α-12.1|Ts Butantoxin (TsTXIV)	1,*2*	WCSTCLDLACGASRECYDPCFKAFGRAHGKC MNNKCRCYT	P59936 (1C55)
α-13.1|Tc1	1	ACGSCRKKCKGSGKCINGRCKCY	P83243 (1JLZ)
α-13.2|OsK2	1	ACGPGCSGSCRQKGDRIKCINGSCHCYP	P83244
α-14.1|Bmkk1	n.d.	TPFAIKCATDADCSRKCPGNPSCRNGFCACT	Q967F9
α-14.2|Bmkk2 (BmP07)	n.d.	TPFAIKCATDADCSRKCPGNPPCRNGFCACT	Q95NK7 (1PVZ)
α-14.3|Bmkk3	n.d.	TPFEVRCATDADCSRKCPGNPPCRNGFCACT	Q9BJX2
α-14.4|Bm SKTx1	n.d.	TPFAIKCATNADCSRKCPGNPPCRNGFCACT	AAK31986
α-15.1|Aa1	*6*	QNETNKKCQGGSCASVCRRVIGVAAGKCINGRCVCYP	Pisciotta et al., 2000
α-15.2|BmTx3A	*5,6*	QVETNVKCQGGSCASVCRKAIGVAAGKCINGRCVCYP	AAN34656
α-15.3|AaTx1 (AmmTX3)	*6*	QIETNKKCQGGSCASVCRRVIGVAAGKCINGRCVCYP	CAD20742
α-15.4|AaTx2	n.d.	QVETNKKCQGGSCASVCRRVIGVAAGKCINGRCVCYP	CAD20743
α-16.1|Bt TmTX	n.d.	DLIDVKCISSQECWIACKKVTGRFEGKCQNRQCRCY	Possani et al., 2000
α-16.2|Bm Martentoxin (BmTx3B)	2,6	FGLIDVKCFASSECWTACKKVTGSGQGKCQNNQCRCY	AAF87224 (Q9NBG9)
α-16.3|Bm Kchtx1	n.d.	FGLIDVKCFASSECWIACKKVTGSVQGKCQNNQCRCY	Q8MQL0
α-17.1|Bm TXKs4 (Bmkk4/BmP06)	*6*	QTQCQSVRDCQQYCLTPDRCSYGTCYCKTT[d]	Q95NJ8 (1KLH)
α-18.1|Tc32	1	TGPQTTCQAAMCEAGCKGLGKSMESCQGDTCKCKA	Batista et al., 2002
β-1|TsTXKβ	n.d.	KLVALIPNDQLRSILKAVVHKVAKTQFGCPAYEGYCNDH CNDIERKDGECHGFKCKCAKD	(Legros et al., 1998)
β-2|AaTXKβ	n.d.	KLVKYAVPVGTLRTILQTVVHKVGKTQFGCPAYQGYC DDHCQDIKKEEGFCHGFKCKCGIPMGF	(Legros et al., 1998)
β-3|BmTXKβ AAF31480	*6*	KNIKEKLTEVKDKMKHSWNKLTSMSEYACPVIEKWCEDHC AAKKAIGKCEDTECKCLKLRK	
β-4|BmTXKβ2 AAF31479	n.d.	KLVKYAVPEGTLRTIIQTAVHKLGKTQFGCPAYQGYCDD HCQDIKKEEGFCHGFKCKCGIPMGF	
γ-1.1|CnErg1	5	DRDSCVDKSRCAKYGYYQECQDCCKNAGHN GGTCMFFKCKCA	AAO22234 (1NE5)
γ-1.2|CeErg1	n.d	RDSCVDKSRCAKYGYYQECTDCCKKYGHNG GTCMFFKCKCA	AAO22215
γ-1.3|CgErg1	n.d.	RDSCVDKSRCAKYGHYQECTDCCKKYGHNGG TCMFFKCKCA	AAO22218
γ-1.4|CsErg1	n.d.	RDSCVDKSRCAKYGYYQECQDCCKKAGHNG GTCMFFKCKCA	AAO22225
γ-1.5|CllErg1	n.d.	RDSCVDKSRCSKYGYYQECQDCCKKAGHNG GTCMFFKCKCA	AAO22221
γ-1.6|CexErg1	n.d.	RDSCVDKSRCAKYGYYQECQDCCKKAGHSG GTCMFFKCKCA	AAO22230
γ-2.1|BeKm-1	5	PTDIKCSESYQCFPVCKSRFGKTNGRCVNGFCDCF	AAK28021 (1LGL)
γ-3.1|CnErg2	5	RDSCVNKSRCAKYGYYSQCEVCCKKAGHKG GTCDFFKCKCKV	P59939
γ-3.2|CeErg2	n.d.	RDSCVDKSRCAKYGYYQQCEICCKKAGHRG GTCEFFKCKCKV	AAO22216
γ-3.3|CsEErg2	n.d.	RDSCVDKSRCAKYGYYGQCEVCCKKAGHRG GTCDFFKCKCKV	AAO22226
γ-3.4|CgErg2	n.d.	RDSCVDKSRCQKYGNYAQCTACCKKAGHNK GTCDFFKCKCT	AAO22219
γ-4.1|CllErg3	n.d.	RDSCVDKSKCSKYGYYGQCDECCKKAGDRA GNCVYFKCKCNP	AAO22222

(*continued on next page*)

Table 1 (*continued*)

Toxin[a]	Targets[b]	Sequence	Reference[c]
γ-4.2|CnErg5	n.d.	RDSCVDKSKCGKYGYYQECQDCCKNAGHNG GTCVYYKCKCNP	AAO22214
γ-4.3|CexErg2	n.d.	RDSCVDKSKCGKYGYYGQCDECCKKAGDRA GICEYYKCKCNP	AAO22231
γ-4.4|CexErg3	n.d.	RDSCVDKSKCAKYGYYYQCDECCKKAGDRA GTCEYFKCKCNP	AAO22232
γ-4.5|CexErg4	n.d.	RDSCVDKSQCAKYGYYYQCDECCKKAGDRA GTCEYFKCKCNP	AAO22233
γ-4.6|CllErg3	n.d.	RDSCVDKSKCSKYGYYGQCDKCCKKAGDRAG NCVYFKCKCNQ	AAO22223
γ-4.7|CllErg4	n.d.	RDSCVDKSKCAKYGYYGQCDECCKKAGDRAG NCVYLKCKCNQ	AAO22224
γ-4.8|CeErg3	n.d.	RDSCVDKSKCGKYGYYHQCDECCKKAGDRA GNCVYYKCKCNP	AAO22217
γ-4.9|CsEErg3	n.d.	RDSCVDKSRCGKYGYYGQCDDCCKKAGDRA GTCVYYKCKCNP	AAO22227
γ-4.10|CsEErg4	n.d.	RDSCVDKSRCGKYGYYGQCDECCKKAGDRA GTCVYYKCKCNP	AAO22228
γ-4.11|CnErg4	n.d.	RDSCVDKSQCGKYGYYGQCDECCKKAGERV GTCVYYKCKCNP	AAO22213
γ-4.12|CsEKerg1	5	RDSCVEKSKCGKYGYYGQCDECCKKAGDRA GTCVYYKCKCNP	Nastainczyk et al., 2002
γ-4.13|CnErg3	n.d.	RDSCVDKSKCGKYGYYGQCDECCKKAGDRA GTCVYYKCKCNP	AAO22212
γ-5.1|CsEErg5	n.d.	RDSCVDKSRCAKYGYYGQCEVCCKKAGHNGGTCM FFKCMCVNSKMN	AAO22229
γ-5.2|CgErg3	n.d.	RDSCVDKSRCQKYGPYGQCTDCCKKAGHTGGT CIYFKCKCGAESGR	AAO22220
κ-HfTx1|Hefutoxin 1	*1*	GHACYRNCWREGNDEETCKERC[d]	P82850 (1HP9)
κ-HfTx2|Hefutoxin 2	n.d.	GHACYRNCWREGNDEETCKERCG	P82851
a.1|Bt Tamulustoxin 1[e]	*1*	RCHFVVCTTDCRRNSPGTYGECVKKEKGKECVCKS	Q9BN12
a.2|Bt Tamulustoxin 2	n.d.	RCHFVICTTDCRRNSPGTYGECVKKEKGKECVCKS	Q9BN11
a.3|BmK37 (BmBKTx1)	n.d.	ACYSSDCRVKCVAMGFSSGKCINSKCKCYK	P83407 (1Q2K)
a.4|BmK38	n.d.	KTATFCTQSICQESCKRQNKNGRCVIEAEGSLIYHLCKCY	AAM94410
a.5|Bsp Neurotoxin	n.d.	VSIGIKCDPSIDLCEGQCRIRYFTGYCSGDTCHCS	P83108
1|Tspep1| |	n.d	KPKCGLCRYRCCSGGCSSGKCVNGACDCS	Pimenta et al., 2003
2|Tspep2|27-55| |	n.d.	TVKCGGCNRKCCAGGCRSGKCINGKCQCY	Pimenta et al., 2003
3|Tspep3| |	n.d.	TVKCGGCNRKCCPGGCRSGKCINGKCQCY	Pimenta et al., 2003
ClTx|Lqq Chlorotoxin 1	Cl^-	MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR	P45639 (1CHL)
ClTx-like|Lqh-8/6 (Bs8)	n.d.	RCSPCFTTDQQMTKKCYDCCGGKGKGKCYGPQCICAPY	P55966 (P15229)
IT.1|Be Insectotoxin I1	n.d.	MCMPCFTTRPDMAQQCRACCKGRGKCFGPQCLCGYD	P15220
IT.2|Be Insectotoxin I5A	n.d.	MCMPCFTTDPNMAKKCRDCCGGNGKCFGPQCLCNR[d]	P15222 (1SIS)
IT.3|Bt ButaIT	n.d.	RCGPCFTTDPQTQAKCSECCGRKGGVCKGPQCICGIQ	P81761

[a] Systematic nomenclature are followed by common names with the abbreviated name from the species from which the peptides were isolated as a prefix (Aa, *Androctonus australis*; Am, *A. mauretanicus*; Be, *Mesobuthus eupeus*; Bm, *M. martensii*; Bo, *Bothus occitanus*; Bt, *M. tamulus*; Cex, *Centruroides exilicauda*; Cg, *C. gracilis*; Clb, *C. lmbatus*; Cll, *C. limpidus limpidus*; Cm, *C. margaritatus*; Cn, *C. noxius*; Cs, *C. sculpturatus*; Hf, *Heterometrus fulvipes*; Hs, *H. spinifer*; Lqh, *Leiurus quinquiestratus hebraeus*; Lqh, *Leiurus quinquiestratus quinquiestratrus*; Oc, *Opistophthjalmus carinatus*; Os, *Orthochirus scrobiculosus*; Pi, *Pandinus imperator*; Psp, *Parabuthus* species; Sm, *Scorpio maurus*; Tc, *Tityus cambridgei*; Td, *T. discrepans*; Ts, *T. serrulatus*).

[b] Electophysiologicaly tested pharmacological targets: 1, Shaker related K^+-channels ($K_V1.x$ family); 2, Ca^{2+}-activated K^+-channel of high conductance ($K_{Ca}1.1$); 3, Ca^{2+}-activated K^+-channel of intermediate conductance ($K_{Ca}3.1$); 4, Ca^{2+}-activated K^+-channel of small conductance ($K_{Ca}2.x$ family); 5, *ether-a-go-go* related K^+-channel ($K_V11.x$ family); 6 other, molecular uncharacterized, K^+ currents. Numbers in italics means low activity (Kd > 1 μM).

[c] Sequence references are databank accession numbers (either SwissProt, TREMBL or GenBank) as retrieved from www.ncbi.nih.gov/entrez (PDB codes, where available, are beetwen parenthesis) or literature references.

[d] C-terminal amidation.

[e] Peptides a.1 to a.5 are clearly grouped within alpha-KTx family although no systematic numbering has been proposed. Peptides Tspep1, Tspep2 and Tspep3 isolated from T. Serrulatus contains the CS-alphabeta signature but are not currently classified as alpha-KTx's because of the lack of functional data.

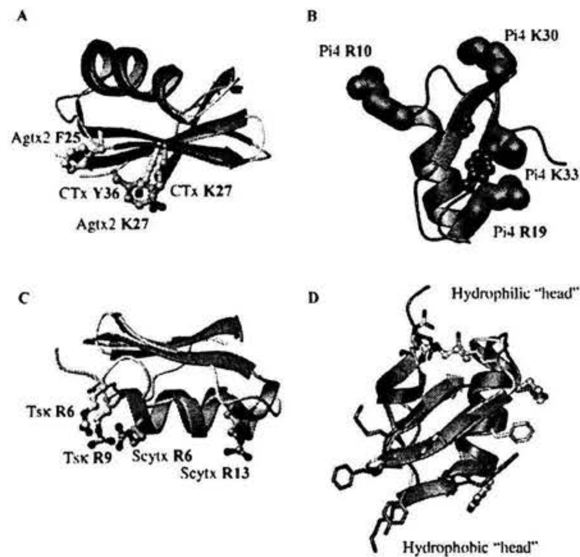
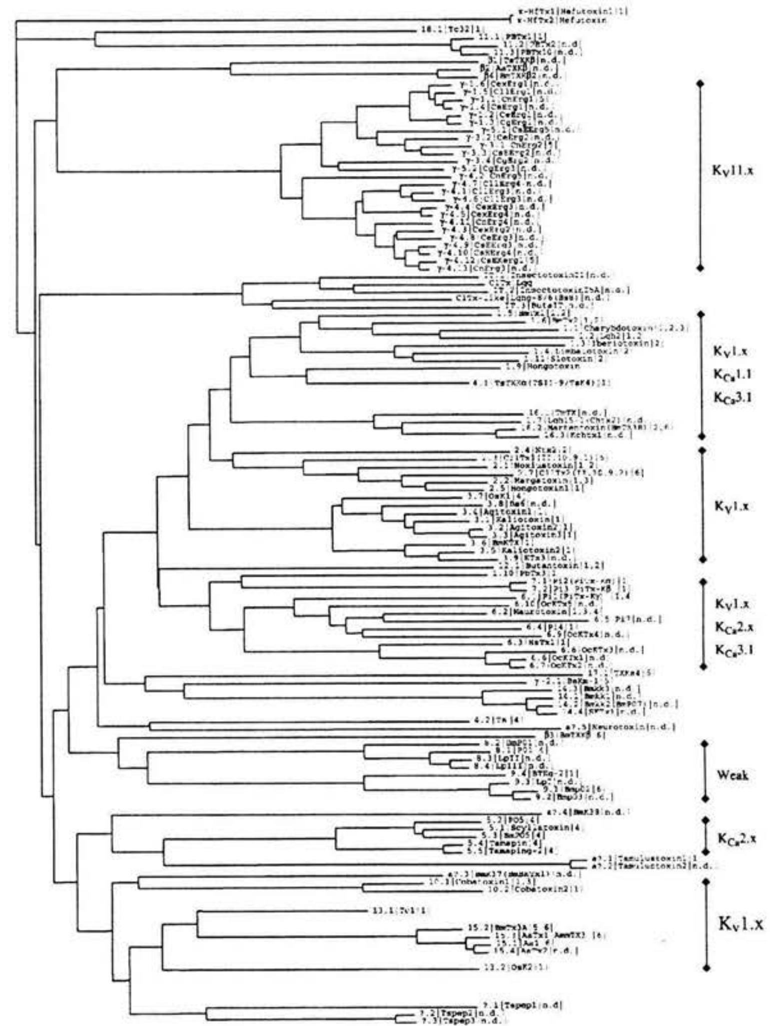


Fig. 1. Functional epitopes of scorpion toxins specific for K$^+$-channels. (A) Functional dyad in two variants: similar to Charybdotoxin (purple), residues K27 and Y36, and to Agitoxin 2 (green), residues K27 and F25. (B) The basic ring of Pi4 (violet) as defined by M'Barek et al. (2003b) and Guijarro et al. (2003). Residues R10, R19, K30 and K33 are displayed as space filing models, whereas K26 and Y35 (functional dyad residues) are in ball and chain representation. (C) Basic residues of the apamin-like motif of the $K_{Ca}2.x$ specific toxins Tsκ (yellow), residues R6 and R9, and Scyallotoxin (red), residues R6 and R13. (D) Superimposition of CnERG1 (blue) and BeKm-1 (grey) showing the residues important in BeKm-1 binding to the ERG channel (Korolkova et al., 2002) and some residues of CnERG proposed to be important for its binding are also indicated (Xu et al., 2003; Frenal et al., 2004). The 'two heads' of this interacting mode proposed by Frenal et al. (2004) are indicated. The important residues identified by mutagenesis in BeKm-1 and confirmed by the ET analysis of γ-KTxs are displayed using ball and chain models. Other residues identified by only one of these methods are shown using the stick model. Ribbon models of averaged and minimized structures were displayed with MOLMOL software (Koradi et al., 1996). PDB entries: Charybdotoxin, 2CRD; Agitoxin 2, 1AGT; Tsκ, 1TSK; Scyallotoxin, 1SCY; Pi4, 1QKY; BeKm-1, 1LGL; CnERG1, 1NE5.

Fig. 2. Phylogenetic tree of scorpion toxin specific for K$^+$-channels. A total of 131 sequences were retrieved from public databases, literature or unpublished results from our laboratory. Only 120 branches are shown. The tree was calculated from a distance matrix obtained with a CLUSTAL X (Thompson et al., 1997) alignment, encompassing the full set of sequences. The tree was rooted to the structurally unrelated, but functionally similar K$^+$-channel blocking peptides κ-hefutoxins. The systematic names are indicated at the tip of the branches, followed by the abbreviated common names. The vertical lines at the right side of the tree are indicating the main pharmacological targets of the toxins, according to the data of Table 1. The α-KTx term was omitted for clarity.



2002; Pardo-López et al., 2002). It is clear that the presence of this extra α-helix of the ERG-channels might play a role as additional binding sites for scorpion toxin interaction, as shown for the CnErg1 and BmK-1 toxins (Pardo-López et al., 2002; Zhang et al., 2003). Indeed, those toxins, as well BmTX3A, seems to interact with the ERG-channel with a different functional epitope (Korolkova et al., 2002; Huys et al., 2004b). This epitope was proposed to be formed by one hydrophilic and one hydrophobic patch in separated regions of the toxins. They would interact with both, the extra α-helix and the vestibule of the ERG-channel. This would form a 'two heads' mode of interaction (Xu et al., 2003; Frenal et al., 2004).

Several lines of evidence support the notion that the presence of a functional dyad is sufficient to confer the ability to block $K_V1.x$ or $K_{Ca}1.x$ channels (see BeKm-1 and PhTx's by Korolkova et al., 2002; Huys and Tytgat, 2003; Huys et al., 2004a). However, as recently shown the activity of toxins such as Tc32 (Batista et al., 2002), Pi1 (Mouhat et al., 2004) and α-KTx's 9.x (Tong et al., 2000; Dhawan et al., 2003) do not require the integrity of the dyad into the toxin for channel blockade. Noteworthy, work earlier performed with Noxiustoxin, suggested that not only the functional dyad (Lys28 and Tyr37) were needed for blockade of KV1.x channels (Martinez et al., 1998), but other segments of the toxin were involved in differently blocking certain subtypes of K^+-channels, as demonstrated with a synthetic segment of Noxiustoxin corresponding to the α-helix structure (Frau et al., 2001). This portion of the toxin was shown to be capable of blocking I_A K^+ currents of cerebellum granular cells. Thus, from this analysis, it seems that apart from the β-sheet segment of these toxins, the α-helix segment was also playing a fundamental role on channel binding and blockage of the currents. These findings were expanded during the last two years, by several independent groups, working with toxins: BeKm-1 (Korolkova et al., 2002), BmP05 (Wu et al., 2002), Pi1 (Mouhat et al., 2004), PBTx1 (Huys et al., 2004a) and BmTx3A (Huys et al., 2004b). The results of all these publications clearly show that the presence of the functional dyad in these toxins are not essential for channel recognition and blockage.

All these new contributions support the concept that some structural characteristics of the primary structure, rather than the three-dimensional folding, are important determinants for the degree of recognition and binding to the various subtypes of channels. In this way, although the overall three-dimensional structure of different peptides were shown to have the same folding, the actual recognition of the K^+-channel subtypes depend on certain amino acid residues, positioned in specific points of the toxin molecule. The current view of our knowledge is drawn in Fig. 1. In this figure we superimposed several three-dimensional structures of scorpion toxins, highlighting the main residues implicated in their interaction with different subtypes of K^+-channels. Four pictures are shown, the first one corresponds to the 'functional dyad theory' (K,Y/F), the second corresponds to the 'basic ring' (four or five non-identical basic residues that might stabilize the interaction with the channels), the third is the 'apamin-like' mode (basic residues in the α-helix) and the last one represents the case of ERG-channels specific toxins, highlighting the 'two heads' model.

5. Evolutive considerations of scorpion toxins specific for K^+-channels

Since scorpions are very ancient organisms, showing an enormous variability of different peptides related by their function (blocking of K^+-channels), some attempts have been made to analyse their evolutionary pattern. Starting with the earlier unrooted phylogenetic tree (Possani et al., 2000) suggesting that similar primary structures could be correlated with some degree of specificity towards certain subtypes of channels, other tree topologies were proposed using a different method, the 'evolutionary trace analysis' (Zhu et al., 2004). Due to the recent increase on the total number of K^+-channel specific toxins, we decided to analyse the entire known universe of peptides in a more inclusive manner, and in Fig. 2 we show the results of this analysis. This phylogenetic tree was rooted to the κ-hefutoxin, peptides capable of blocking K^+-channels but with a three-dimensional structure quite different from all the other ones (Srinivasan et al., 2002). The general distribution of the toxins in this novel tree is not notoriously different from the previous data in the literature (Possani et al., 2000; Rodríguez de la Vega et al., 2003; Zhu et al., 2004; Huys et al., 2004b), except for the significant increment on the total number of sequences analysed.

The cluster of the γ-toxins (ergtoxins) is well defined and is quite distant from the others. The largest truthfully segregated cluster includes subfamilies α-KTx's 1–4 and 16. On the basis of this alignment, toxins α-KTx 1.7 and 1.10 seems not to be properly assigned, because the first (Lqh15-1) falls in the internal cluster of subfamily 16, whereas the second (PiTx3) appears to be quite distant. Toxins in this cluster present high specificity for $K_V1.x$ (subfamilies 2–4), $K_{Ca}1.1$ (subfamily 16 and some peptides from subfamily 1) or both subtypes of K^+-channels (subfamily 1). Toxin α-KTx 4.2 falls very distant from the remaining peptides in the same subfamily and could be better referred as the only example of its class. Another well-defined cluster includes toxins from subfamilies 6.x and 7.x. They recognize a wide variety of pharmacological targets, affecting $K_V1.x$, $K_{Ca}2.x$ and $K_{Ca}3.1$ channels with high affinity. For example, see the case of α-KTx 6.1 (Pi1) and 6.2 (maurotoxin). In this cluster is located α-KTx 6.5 (Pi7), a peptide for which no pharmacological action was found, thus far (Delepierre et al., 1999). Toxins specific for $K_{Ca}2.x$ often belongs to the α-KTx subfamily 5, which also defines a well-resolved branch in the tree. Weak toxins from subfamilies α-KTx 8 and 9 are segregated from the remaining sequences in a single cluster. Finally, chlorotoxin/insectotoxin-like peptides are all in a well-defined branch, separated from the K^+-channels specific toxins. The β-KTx's are in a separated cluster from short chain toxins, except BmTXKβ, which are the most divergent β-KTx described to date. In this analysis, the most distant sequences are the toxins of the subfamilies α-KTx 11 (acidic toxins) and α-KTx 18, (represented by Tc32). Thus, in general there is a good agreement between phylogeny and functionality for most of the peptides in this figure. Interestingly, two ERG-channel blocking peptides, BeKm-1 and BmTX3A, are well clustered with two different α-KTx's subfamilies, α-KTx 14 and 15, respectively. It is worth noting that both peptides can also block other K^+ currents (Korolkova et al., 2001; Huys et al., 2004b).

This phylogenetic tree could help designing experiments aiming at verifying putative functions of the uncharacterised peptides. It is also important to note that the tree was generated using 131 similar peptides, including all the known K^+-channel specific toxins, but containing additional related peptides whose structure adopts the CS-αβ motif (see legend in Fig. 2).

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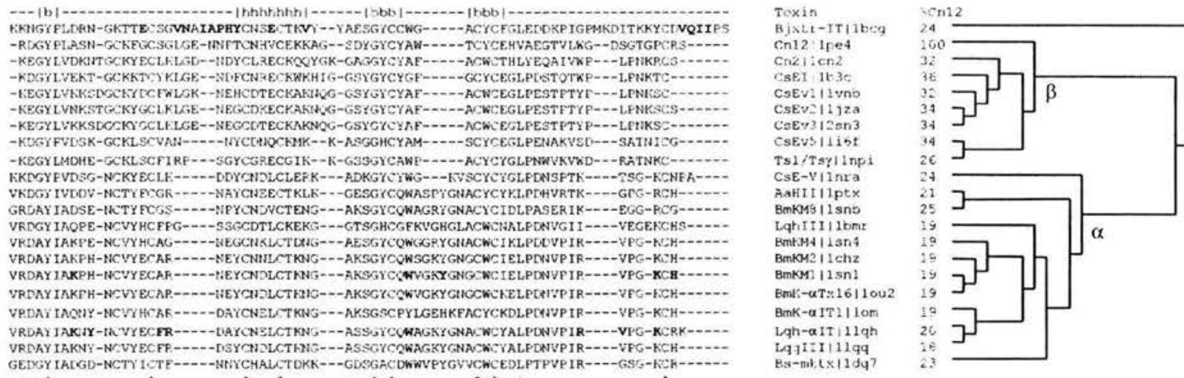
Caso 2. Toxinas de alacrán que modulan la apertura y cierre de los canales de Na^+

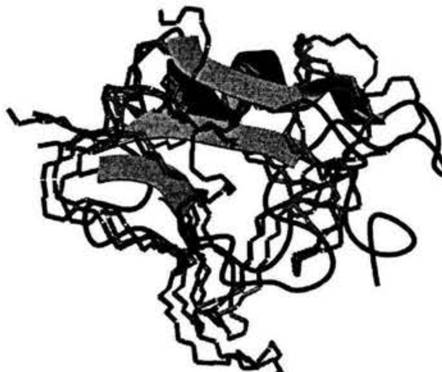
Las toxinas de alacrán de cadena larga suelen ser el componente mayoritario de los venenos de las especies peligrosas para mamíferos, su estudio ha estado impulsado porque son las responsables del efecto letal que pueden producir los venenos de aquellos organismos [18a]; paradójicamente la interacción con sus receptores —canales de Na^+— ha sido menos estudiada que su contraparte en el par α-KTx - canal de K^+, como consecuencia de la dificultad que ofrecen los canales de Na^+ para su estudio electrofisiológico y la escasez de mutantes, tanto de los ligandos como de los receptores, que ayudarían a definir los aminoácidos directamente involucrados en la interacción entre ambas moléculas. Ahora bien, tradicionalmente las toxinas de alacrán específicas para canales de Na^+ (Na-ScTx) se diferencian farmacológicamente por el efecto que producen en el proceso de apertura y cierre de los canales: las toxinas α inhiben el proceso de cierre uniéndose al sitio receptor 3 (definido por las asas extracelulares que unen los segmentos S5-S6 del dominio I y S3-S4 del dominio IV), mientras que las toxinas β recorren el potencial de activación de los canales uniéndose al sitio receptor 4 (S3-S4 del dominio II) [19a,20a]. La relación entre las secuencias de Na-ScTx's y su efecto farmacológico es directa en la mayoría de los casos [21a,22a]; sin embargo, la toxina Cn12 posee una secuencia típicamente β (**Figura A-2a**), pero ejerce un efecto tipo α. La comparación de las estructuras tridimensionales o las superficies electrostáticas para ambos tipos de toxinas tampoco refleja patrones que pudiesen estar asociados a la definición de los efectos farmacológicos (**Figura A-2b**). Lo anterior resalta la plasticidad del motivo estructural α/β-EC, sobre el cual se parecen haber desarrollado dos soluciones independientes que derivan en un mismo tipo de función.

a)



b)

A) Face B Superposition



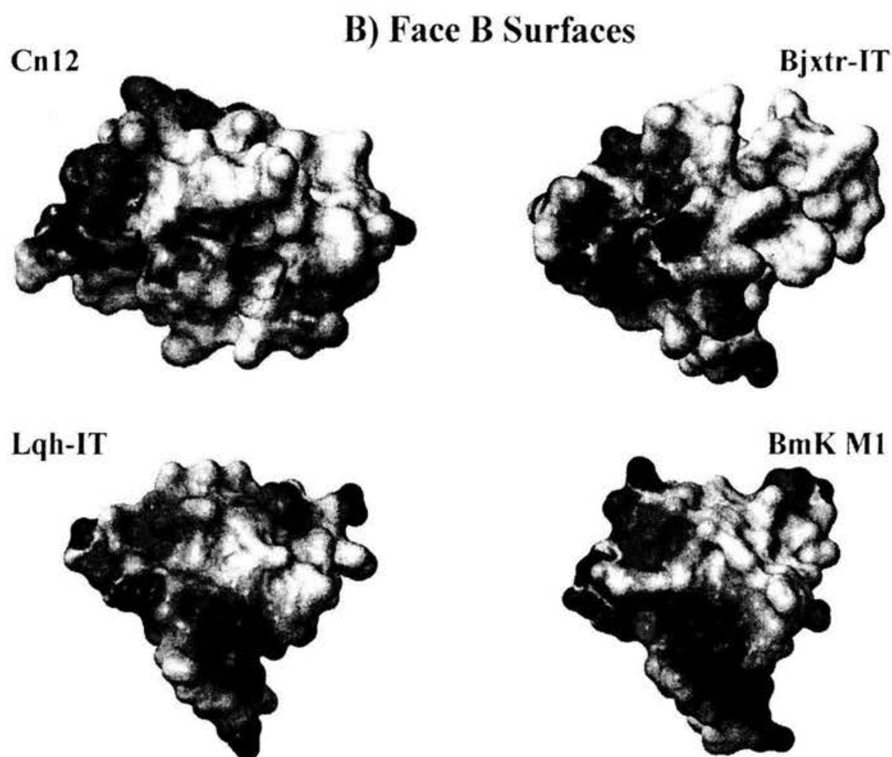


Figura A-2 | *Comparación de secuencia y estructura de la toxina Cn12 con otras Na-ScTx's.* a) La secuencia de Cn12 se alineó con otras Na-ScTx's cuya estructura tridimensional es conocida, usando el programa CLUSTAL_X [16a]. En la parte derecha de la figura se muestran los porcentajes de identidad con respecto al Cn12 y un arbol filogenético simplificado, el cual está enraizado con Bjxtr-IT. Omitiendo a Cn12, el árbol segrega adecuadamente a los dos grupos farmacológicos descritos (nodos marcados como α y β). b) comparación de la estructura de Cn12 obtenida por RMN con otras Na-ScTx's. bA) La toxina Cn12 se muestra en diagrama de listones, sobrepuesta sobre las estructuras de la toxina α específica para insecto Lqh-IT, la toxina tipo α BmK M1 y la toxina exitatoria Bjxtr-IT, cuyo efecto farmacológico es tipo β. bB) Superfcies electrostáticas de las toxinas sobrepuestas en bA. La única similitud evidente se da entre Lqh-IT y BmK M1. Las estructuras depositadas en el PDB se desplegaron con el programa MOLMOL [17a].

Con datos de: del Río-Portilla F, Hernández-Marín E, Pimienta G, Coronas FIV, Zamudio FZ, Rodríguez de la Vega RC, Wanke E, y Possani LD (2004). NMR solution structure of Cn12, a novel peptide isolated from Mexican scorpion Centruroides noxius having a typical beta sequence but with a alpha-like effect. *Eur. J. Biochem.* Aceptado.

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