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FACULTAD DE MEDICINA

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DETERMINACION DE LAS CONSTANTES DE AFINIDAD DE TOXINAS DEL VENENO DE ALACRANES MEXICANOS A TEJIDOS EXCITABLES: CEREBRO Y MUSCULO

T E S I S

QUE PARA OBTENER EL TITULO DE

DOCTOR EN CIENCIAS BIOMEDICAS

P R E S E N T A

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SOLO ASI HE DE IRME?
COMO LAS FLORES QUE PERECIERON?
NADA QUEDARA EN MI NOMBRE?
NADA DE MI PASO AQUI EN LA TIERRA?
AL MENOS FLORES, AL MENOS CANTOS...

Cantos de Huexotzingo.

"To every thing there is a season, and a
time to every purpose under the heaven:
A time to born, and a time to died,
A time to plant and a time to pluck up
that which is planted;
A time to kill, and a time to heal
A time to break down, and a time to buil up"

ABREVIACIONES:

aa	Aminoácido
ADNc	Acido desoxiribonucleico complementario
ARN	Acido ribonucleico
ARNm	Acido ribonucleico mensajero
BTx	Batracotoxina
CLAP	Cromatografía líquida de alta presión
cm	Centímetros
CMC-32	Carboximetilcelulosa
kD	KiloDaltones
K_D	Constante de disociación
min	Minutos
mg	Microgramos
mM	Micromolar
NaCl	Cloruro de sodio
nM	Nanomolar
PM	Peso molecular
STx	Saxitoxina
α ScTx	Alfa escorpiotoxinas o toxinas de alacrán de tipo α
β ScTx	Beta escorpiotoxinas o toxinas de alacrán de tipo β
TFA	Acido trifluroacético
TTx	Tetrodotoxina
TRIS	Tris-(hidroximetil)-amino-metano
UA	Unidades de absorbencia

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LISTA DE ANEXOS:

Anexo 1: Publicación como primer autor:

Ramírez, A.N., Martin, B.M., Gurrola, G.B. and Possani, L.D. " Amino acid sequence of a novel toxin from the venom of the scorpion *Centruroides limpidus limpidus Karsch.*" Toxicon, Vol. 32, No. 4, pp 479-490, (1994)

Anexo 2: Publicaciones como co-autor.

A) Lourival D. Possani, Hector H. Valdivia, Angelina N. Ramírez, Georgina B. Gurrola and Brian M. Martin. "K+ channel blocking peptides isolated from the venom of scorpions". In: Recent Advances in Toxinology Research (Gopalakrisnakone, P. and Tan C.K. eds.) vol. 1, pag. 39-58, National University of Singapore Press, Singapore (1992)

B) Prestipino, G., Valdivia, H.H., Lievano, A., Darzon, A., Ramírez, A.N. y Possani, L.D. "Purification and reconstitution of potassium channel proteins from squid axon membranes". FEBS Letters 250: 570-574 (1989).

C) Alagón, A.C., Guzmán, S.H., Martin, B.M., Ramírez, A.N., Carbone, E. and Possani, L.D. "Isolation and characterization of two toxins from the Mexican scorpion *Centruroides limpidus limpidus* Karsch". Comp. Biochem. Physiol. Vol. 89B, No. 1 pp 153-161 (1988).

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PRESENTACION DE LA TESIS

La presente tesis describe los resultados experimentales obtenidos durante mi entrenamiento de posgrado como alumna de la UNAM (Facultad de Medicina, área Bioquímica).

El trabajo está dividido en 3 partes. En la primera se engloba una serie de experimentos todavía no publicados, la segunda parte está constituida por un artículo como primer autor y en la tercera sección se anexan 3 publicaciones como coautor todas ellas publicadas en revistas internacionales.

Además de los datos sobre la purificación y caracterización química de una nueva toxina aislada del veneno del alacrán *C. limpidus limpidus* se incluye una introducción sobre canales iónicos, específicamente de canales de Na^+ voltaje-dependientes y sobre toxinas de diferentes fuentes naturales que los bloquean, asi como la determinación de las constantes de afinidad de una serie de péptidos aislados de dos venenos de alacranes mexicanos en cerebro y músculo, título de esta tesis.

También, se describen en formato de artículo científico los procedimientos para la purificación y caracterización de algunos péptidos nuevos bloqueadores de canales de K^+ a partir del veneno del *C. limpidus limpidus*, los cuales son semejantes a Noxiustoxina. También se describe el uso de esta toxina en el aislamiento de un canal de K^+ de axón gigante de calamar.

RESUMEN

Las toxinas que constituyen los venenos de alacrán se han clasificado en dos familias, una de cadena larga y otra de cadena corta. Las primeras se encuentran en mayor abundancia en el veneno y ejercen su acción fisiológica sobre canales de Na+ dependientes de voltaje. Por el ensayo biológico utilizado para su purificación se han clasificado en toxinas contra mamíferos, insectos y crustáceos. Las toxinas a mamíferos se han clasificado a su vez en toxinas α y toxinas β dependiendo de los efectos farmacológicos y del sitio específico de pegado en el canal de Na+. La mayoría de ellas comparten una alta homología estructural, no asi toxicidad y/o efectos fisiológicos. Con la idea de profundizar en el conocimiento de la relación de la estructura-función de las toxinas que involucran a canales de Na+ , en esta tesis se reporta la purificación y caracterización bioquímica de varios péptidos aislados del veneno de los alacranes mexicanos *Centruroides noxius y Centruroides limpidus limpidus*, algunos de los cuales podrían ser buenos candidatos en el estudio de la interacción existente entre el péptido y su receptor. Estos péptidos son de cadena larga y ejercen su acción biológica sobre el canal de Na+. Las toxinas aisladas del *Centruroides noxius* fueron, la II.13.3 y II.13.4 mientras que del veneno del *Centruroides limpidus limpidus* fue la II.8.4.3 (toxina 1 de esta tesis). De este veneno, se purificaron parcial y totalmente otra serie de componentes, algunos con acción bloqueadora sobre canales de K+ (anexo 2). Las toxinas II.13.3,toxina 2 y toxina 3 de *Centruroides noxius* se marcaron con ^{125}I y se determinó sus constantes de afinidad en diferentes tejidos excitables. En sinaptosomas de cerebro de rata la Kd determinada para la toxina 2 fue de 10 nM, para la toxina 3 en sinaptosomas de cerebro de conejo fue de 63 nM y para la toxina II.13.3 en el mismo sistema fue de 1.94 nM. En músculo esquelético la toxina 3 tiene una constante de afinidad de 55 nM, mientras que para la toxina II.13.3 es de 0.97 nM. Posteriormente la toxina ^{125}I-II.13.3 fue desplasada por una serie de peptidos puros y se encontró que la mayoría de ellos tienen una constante de afinidad similar a la de la toxina marcada, a excepción de la toxina II.131.4 de *Centruroides noxius* cuya constante de afinidad es alrededor de 50-100 pM. Todas estas toxinas por competir por el mismo sitio receptor se clasificaron dentro de la familia de toxinas de tipo β.

SUMMARY

The venom of scorpions contain several families of toxic peptides. The most thoroughly studied are those that modify Na^+ permeability. These peptides are the most abundant in the venom, and also the most variable ones: there are toxins specific for mammals, insects or crustaceans. The Na^+-channel blocking peptides were classified in α and ß toxins. The α-toxins slow the inactivation process of the channel and bind to site 3, while the ß-toxins modify the activation mechanism of the channel and bind to the site 4. Additionally, in the venom of the scorpions there are at least two types of toxic peptides concerning their size: the long chain peptides recognize Na^+-channels, the short chain are specific for K^+ channels.

This thesis reports the purification and characterization of several toxins from the venoms of two Mexican scorpions *Centruroides noxius* and *Centruroides limpidus limpidus*. Most of the peptides described in this thesis belong to the class of long-chain toxins, that modify Na^+ permeability of excitable cells. They were called toxin II.13.3 and toxin II.13.4, isolated from *Centruroides noxius*, and toxin 1 (component II.8.4.3) from *Centruroides limpidus limpidus*. Toxin II.13.3 and toxins 2 and 3 from *Centruroides noxius* (purified by others in our group) were all labeled with ^{125}Iodine and used for binding experiments in different types of excitable tissues. The K_D for toxin 2 was estimated to be 10 nM, using membranes from rat brain synaptosomes. In rabbit brain synaptosomal membranes toxins 3 and II.13.3 showed a K_D of 63 nM and 2 nM, respectively. Using sarcolemma vesicles of rabbit skeletal muscle the K_D determined for toxin 3 was 55 nM, while that of toxin II.13.3 was about 1 nM. Additional experiments were conducted with iodinated toxin II.13.3 in various tissues by means of binding and displacement experiments with cold toxins from *Centruroides noxius*: toxins 2, 3, II.13.3 and II.13.4, and from *Centruroides limpidus limpidus*: toxin 1, toxins II.8.4.(45:7) and II.10.11. The values found for the K_D were all in the range of 10 nM, except for toxin II.13.4 of *Centruroides noxius*, whose K_D was about 50-100 pM. All these toxins seems to be ß-scorpion toxins, because they compete for the same binding site as that of toxin 2 from *C. noxius*, previously shown by direct electrophysiological measurements to affect the activation mechanism of the channel.

Finally, in annex-2 we present data obtained in the purification and characterization of short-chain peptides, from the venom of *Centruroides limpidus limpidus*, whose activity on K^+ channels was also demonstrated.

Vo. Bo.

INTRODUCCION

A) GENERALIDADES:

El amor a la vida, quiza tanto, como el temor a la muerte han obligado al hombre a tratar de entender todos aquellos fenómenos que le son adversos. Y es así como los animales ponzoñosos, han formado parte de su cultura y religión desde tiempos inmemoriables.

Cuando comparamos el tamaño que tiene un alacrán con la sintomatología tan aparatosa que su picadura produce en las víctimas, nos damos una idea de la potencia de sus componentes y nos explicamos el interés despertado por los venenos de estos arácnidos en los diferentes campos de la ciencia. La temprana investigación desde el punto de vista médico, al paso del tiempo, fué substituida por una investigación básica ya que las moléculas aisladas, al ser probadas en ensayos biológicos presentaban una alta afinidad por su receptor e incluso algunas de ellas podían discernir entre receptores de una misma familia, característica que las hizo herramientas valiosas en el estudio de proteínas de membrana. Hasta el momento, todas las toxinas reportadas tienen una actividad biológica sobre canales iónicos, principalmente de Na^+ y de K^+ y en menor proporción sobre canales de Ca^{2+}.

A pesar de que existen más de 700 especies de alacranes distribuidas en todo el mundo, pocas de ellas son peligrosas para el ser humano. Y así, en el Viejo Mundo, los géneros *Androctonus, Leiurus, Buthus, Parabuthus, Tamulus* y *Scorpio* engloban todas las especies de interés médico (Shulov y Levy, 1978), mientras que en el Nuevo Mundo, sólo el *Centruroides* y el

Tityus son de importancia médica (Mazzotti y Bravo-Becherelli, 1963; Bucherl, 1971).

Las moléculas que integran a estos venenos son: aminoácidos libres, sales inorgánicas, nucleótidos, lípidos, proteínas y péptidos de diferentes pesos moleculares que pueden ser tóxicos a vertebrados y/o invertebrados (Ramírez, A.N., 1987).

Estos péptidos han sido clasificados en dos grandes familias, una de ellas denominada de cadena larga, está formada por 61-70 aminoácidos de longitud, con un peso molecular (PM) aproximado de 7000 daltones y cuya acción fisiológica es sobre canales de Na^+ (Miranda y col., 1970; Grishin y col., 1980; Darbon y col., 1982; Possani y col., 1983). La otra familia es llamada de cadena corta y esta formada por 36-39 aminoácidos de largo, con un PM aproximado a 4000 daltones y cuya acción biológica es sobre canales de K^+ (Zhdanova y col., 1978; Possani y col., 1982). Ambas familias son de naturaleza básica y conservan su estructura tridimensional por 3 o 4 puentes de disulfuro (Kopeyan y col., 1974; Fontecilla-Camps y col., 1980; Possani y col., 1982), cualidad que las hace moléculas altamente empacadas y de estructura rígida, proporcionandoles resistencia a agentes desnaturalizantes, tales como cambios de pH, calentamiento, enzimas proteolíticas, etc. Su actividad biológica se pierde cuando se reducen los puentes disulfuro.

Desde el punto de vista de la estructura tridimensional, existen pocos ejemplos en la literatura de estudios de difracción de rayos X de toxinas de alacrán de cadena larga; dos de ellos son el estudio de los cristales obtenidos de la variante 3 del *Centruroides sculpturatus* (Fontecilla-Camps y col., 1980) y de la toxina II del *Androctonus australis* Hector (Fontecilla-Camps y col., 1988). Posteriormente, dada la alta homología que existía en

la estructura primaria con estas toxinas, se predijo el plegamiento de la toxina excitatoria I, contra insectos, del *Androctonus australis* Hector, utlizando gráficos computarizados y procedimientos de minimización de energía (Fontecilla-Camps, J.C.,1989) y para 1991 Darbon y col. determinaron su estructura secundaria por RMN. De todos estos reportes, se concluye que las toxinas de alacrán son proteínas globulares que semejan el puño cerrado de una mano derecha, con rizos α-hélice formando los nudillos y una región β plegada entre el segundo y el tercer dedo formando una región densa con tres puentes de disulfuro (Fig. 1). Tres de los 4 puentes disulfuro parecen estar comunmente apareados en todas las toxinas mientras que el cuarto no se conserva. En la α-toxina II del *Androctonus australis* Hector la cisteina del C-terminal se une a la Cys del N-terminal (Fontecilla-Camps, J.C., 1989), mientras que en la toxina contra insectos AaH It del mismo alacrán, la Cys del carboxilo terminal se aparea con otra Cys adicional ubicada en el cuerpo de la molécula (Darbon y col. 1991). Esta diferencia, sin embargo, parece no tener un efecto substancial sobre el plegado total de la toxina. Los trabajos de El Ayeb y col. (1986) y Kharrat y col. (1989, 1990) mostraron que la parte de la muñeca corresponde al C-terminal y que casi toda la molécula es antigénica a excepción de una región altamente conservada cercana al amino terminal la cual parece estar involucrada en el reconocimiento del receptor (Fig. 1). Poca duda existe, pues, en el hecho de que las toxinas de cadena larga adoptan un replegamiento conservado en la evolución, mas sin embargo, una investigación detallada de la estructura muestra que las diferentes moléculas pueden acomodar inserciones, deleciones y mutaciones, lo cual presumiblemente proporcionaría a la toxina diferentes especificidades

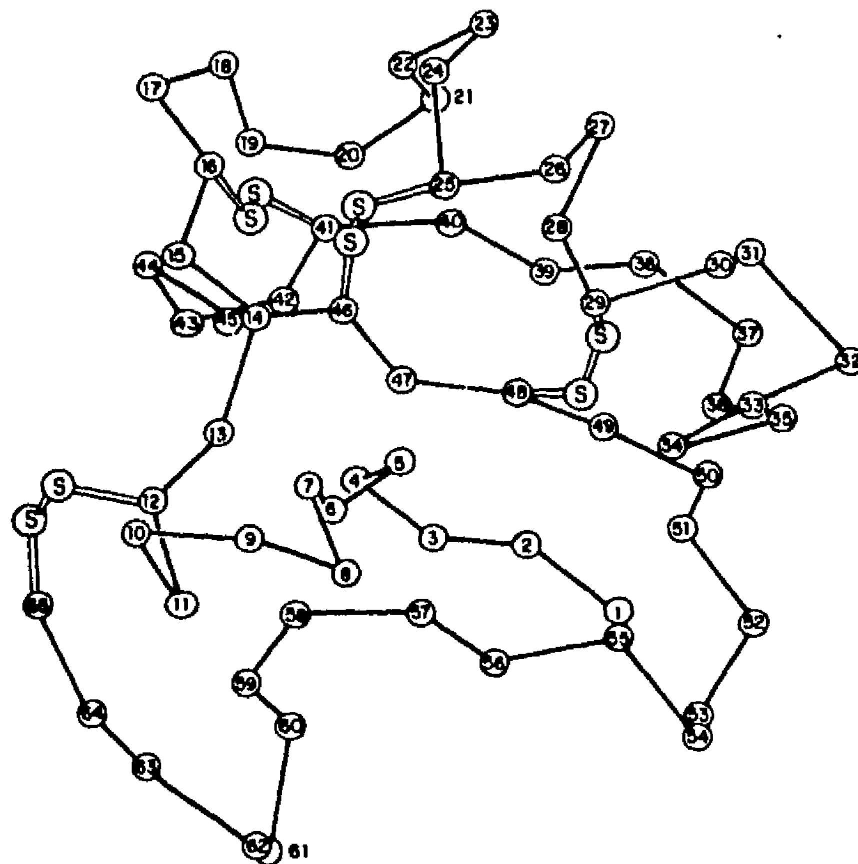


Fig. 1: Estructura de Unión de los Carbonos α de la Variante 3 del *Centruroides sculpturatus.*

Los círculos numerados coinciden con los átomos de carbono α . Los átomos de azufre de los residuos de cisteína se muestran a lo largo con uniones a los átomos de carbono α (Tomado de Fontecilla-Camps, J.C.,1980).

(Menez, A., 1992).

Como ya se mencionó, la alta afinidad que estas moléculas muestran por su receptor las ha hecho herramientas valiosas para el estudio de proteínas de membrana, específicamente canales iónicos sensibles a voltaje. La caracterización del canal de Na^+ dependiente de voltaje por Catterall y col. (1988, 1991), se llevó a cabo utilizando toxinas de diferentes fuentes naturales. Catterall determinó cinco sitios de pegado para las toxinas y Jover y col. en 1980 clasificaron a las toxinas de alacrán en toxinas α (α-escorpiotoxinas) y toxinas β (β-escorpiotoxinas) por el sitio de pegado al canal (Tabla 1).

Además de las toxinas de alacrán, existe una serie de toxinas cuya actividad biológica es, también, sobre el canal de Na^+ dependiente de voltaje. Estas toxinas han sido aisladas de diferentes fuentes naturales y algunas de ellas están bien caracterizadas mientras que de otras sólo se tiene un conocimiento vago. Brevemente serán descritas algunas de ellas en el siguiente inciso.

B) TOXINAS QUE ACTUAN A NIVEL DE CANALES IONICOS:

En los últimos años, las toxinas obtenidas de microorganismos, arañas, alacranes, viboras y animales marinos hán sido herramientas invaluables en neurobiología por la alta afinidad que estas moléculas presentan por su receptor. Muchos de los avances en esta área, así como en neurofisiología se deben a la satisfactoria utilización de las toxinas como instrumentos disectivos tanto de estructuras como de funciones fisiológicas en sistemas neuronales, razón por la cual el término NEUROTOXINA se acuñó para aquellos compuestos biológicamente activos, cuya acción es sobre estructuras nerviosas (sistema nervioso central, perifiérico, fibras

TABLA # 1

SITIO DE PEGADO DE NEUROTOXINAS EN EL CANAL DE SODIO

Sitio Receptor	Ligandos	Efectos Fisiológicos
1	Tetrodotoxina Saxitoxina μ-conotoxina	Inhibe el transporte iónico
2	Veratridina Batracotoxina Aconitina Grayanotoxina	Activación Persistente
3	α-escorpiotoxinas Toxinas de Anemona de mar.	Inhibe la inactivación Incrementa la activación persistente.
4	β-escorpiotoxinas	Corto Circuito en la activación.
5	Ciguatoxinas Brevetoxinas	Disparos repetitivos Activación persistente
No Identifacados	Insecticidas	Disparos repetitivos activación persistente
	Toxina Gonoipora Toxina Conus striatus	Inhibición de la inactivación
	Anestésicos locales Antiarritmicos Anticonvulsivantes	Inhibición de la frecuencia y de la dependencia de voltaje

Tomado de Catterall, W.A., 1991.

nerviosas, sinapsis, etc.).

La alta especificidad de las neurotoxinas ha permitido agruparlas en dos categorías: aquellas que actúan sobre canales iónicos (canales de Na^+, K^+ y Ca^{2+}), y las que se unen a receptores en sinapsis (Mebs y Hucho, 1989). Asi pués, sólo nos referiremos a toxinas que actúan a nivel de canales iónicos involucrados en el impulso nervioso, específicamente canales de Na^+ dependientes de voltaje.

1.) TETRODOTOXINA (TTx)

La Tetrodotoxina (TTx), es un heterocíclico de guanidina con un grupo funcional hemiacetálico que une a los dos anillos de la molécula (Fig. 2), que fue aislada por primera vez en 1909 del pez globo *Spheroides (fugu) rubripes* por Tahara, pero no fue hasta 40 años más tarde que su estructura química fue determinada. A pesar de su estructura única, la TTx ha sido encontrada en un número increible de vertebrados e invertebrados, los cuales en muchos casos no están filogenéticamente relacionados, sólo otra especie de pez contiene TTx, el *Gobius criniger* (Hashimoto y col., 1979). Una gran variedad de moluscos gastrópodos tales como *Charonia sauliae* y *Babylonia japonica*, los peces estrella de los géneros *Astropecten*, así como los cangrejos venenosos *Atergatis floridus* contienen TTx, pero los especímenes del pacífico tropical poseén este compuesto en mayor cantidad que los que habitan las aguas alrededor del Japón. La Chiriquitoxina, molécula aislada de la piel de la rana *Atelopus chiriquiensis* (Kim y col., 1975), es un análogo natural de TTx, es equipotente en el bloqueo de los canales de Na^+, pero además tiene acción en canales de K^+.

La biogénesis de la TTx es todavía obscura, compuestos radiactivos

tales como acetato, arginina y glucosa no se incorporan a la molécula. Estudios recientes en cangrejos venenosos muestran que una especie de *Vibrion* aislada del *Atergatis floridus* produce TTx anhidra (Noguchi y col., 1986). Por otro lado, el hecho que el pez globo mantenido en cautiverio es esencialmente no tóxico, apoya la hipótesis que la TTx es de orígen microbiano y es incorporada por contacto o detritus de comida o sedimentos en el estado silvestre (Saito y col., 1984). De la piel del pez globo se han aislado varias cepas bacterianas de las cuales una especie de *Pseudomona* fue identificada como productora de TTx y sus derivados en cultivo (Yotsu, Y., 1987).

La TTx es una molécula estable a temperatura ambiente, soluble en agua acidificada donde adquiere la naturaleza de zwitterion. La síntesis total de la molécula dá un producto el cual es idéntico a la toxina nativa en sus propiedades químicas, así como en su toxicidad. Los estudios de Kao (1964) y Narahashi y col. (1964) establecieron la alta afinidad de la toxina por su receptor. El mecanismo molecular es el bloqueo de los canales de Na^+ de la membrana, impidiendo la entrada de este ión. El canal de Na^+ se inhibe sin alterar la relación de la dependencia de voltaje de su activación e inactivación. Su pegado es independiente del estado de excitabilidad del canal (reposo, activado o inactivado). TTx comparte el mismo sitio receptor que la Saxitoxina (STx) y junto con ella, ha jugado un papel importante en el aislamiento y caracterización del canal de Na^+, de la topología de sus subunidades en la membrana y su biosíntesis (Catterall, W.A., 1980, 1986; Evans, M.H., 1972; Narahashi, T., 1974; Kao y col., 1975).

Los estudios de relación de estructura-función revelan que parte de la guanidina (la porción donde se encuentra el nitrógeno) y los grupos hidroxilos ácidos son esenciales para la actividad biológica de la toxina y

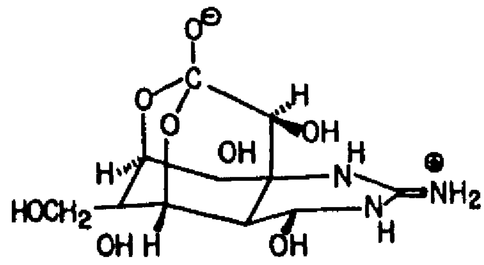
están relacionados con el pegado al receptor.

2.) SAXITOXINA (STx)

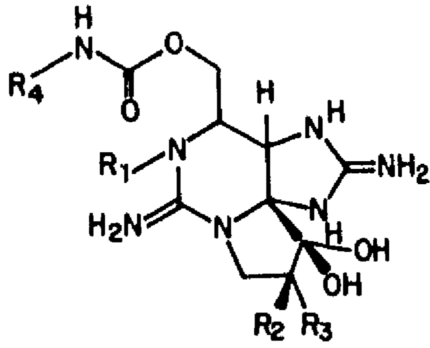
Esta toxina aislada de la almeja *Saxidomus giganteus*, es una tetrahidropurina compuesta de dos guanidinas unidas por un enlace azoacetal (Fig. 2) (Schantz y col., 1975; Bordner y col., 1975). En la actualidad se conocen 11 derivados de STx aislados de varias cepas de dinoflagelados, todos son altamente polares y solubles en agua y se han clasificado en 4 grupos dependiendo del lugar de la substitución química (Tanino y col., 1977; Taguchi y col., 1977). La acción de la STx en tejidos excitables es altamente específica y esencialmente idéntica a la de TTx; se une reversiblemente al receptor y bloquea la entrada de iones Na+ sin afectar el potencial de reposo de la membrana. Este bloqueo de los canales de Na+ parece estar acompañado del pegado a la cara externa de la membrana en un tercer punto de interacción, sugiriendo que la toxina actúa como una tapa más que como un tapón del canal de sodio (Shimizu, Y., 1982).

3.) ACONITINA

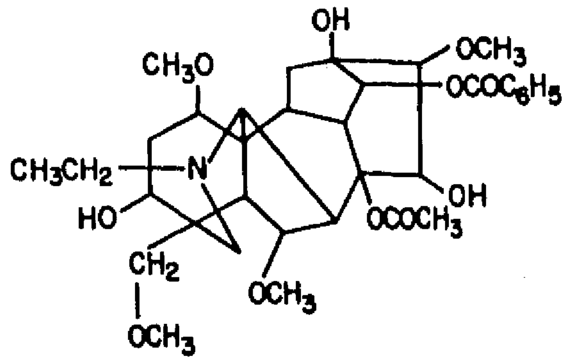
Es un alcaloide extraido de la planta *Aconitum nepellus* (Fig. 2), cuya acción en preparaciones nerviosas es una depolarización prolongada, evitando la repolarización de la membrana. Este efecto es bloqueado por TTx, por lo que su acción es, probablemente, sobre canales de Na+ dependientes de voltaje. Los efectos fisiológicos son similares a los inducidos por Batracotoxina (BTx), esto es, inhibe la inactivacion del canal de Na+ (Catterall, W.A., 1980; Benn y col., 1983), y se une al sitio 2 del mismo.



Tetrodotoxina



Saxitoxina



Aconitina

Figura # 2

4.) GRAYANOTOXINA

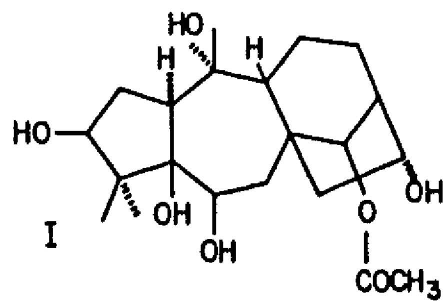
La Grayanotoxina es un diterpeno tóxico (Fig. 3) aislado de las hojas de varias especies de plantas de los géneros *Rhododendron, Kalmia, Leucothoe* y *Ericaceae*. Su acción es depolarizar la membrana nerviosa y muscular por un incremento en la permeabilidad al Na+, este efecto es reversible por lavado y puede ser abolido por TTx de manera no competitiva. Estos resultados indican que las dos toxinas se unen a diferentes sitios receptores, y como BTx y Veratridina, Grayanotoxina actúa en el canal de Na+ dependiente de voltaje en su conformación abierta, inhibiendo su inactivación. El pegado parece ser en el sitio 2 del canal (Khodorov, B.I., 1985; Catterall, W.A., 1980).

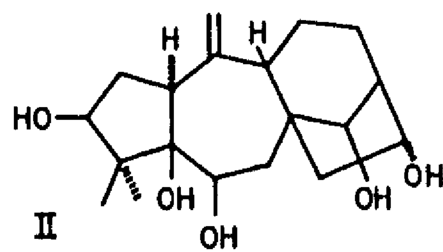
5.) VERATRIDINA

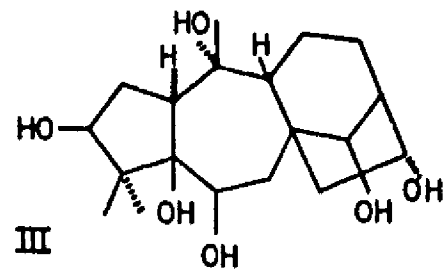
Este es un compuesto esteroidal (Fig. 3), extraido de las plantas de los géneros *Veratrum Zygadenus* y *Schoenocaulon* (Liliaceae). La veratridina interactúa con los canales de Na+ dependientes de voltaje durante su apertura (activación), e inhibe su cerrado (inactivación). Sin embargo la modificación del canal es inestable y el canal puede regresar a su estado de reposo normal cuando la membrana es repolarizada. El efecto de la Veratridina en membranas excitables es abolido por la TTx de manera no competitiva, lo cual indica que están actuando en dos sitios diferentes. Se ha demostrado que la Veratridina actúa en el sitio 2 del canal de Na+ (Catterall, W.A., 1980).

6.) BATRACOTOXINA (BTX).

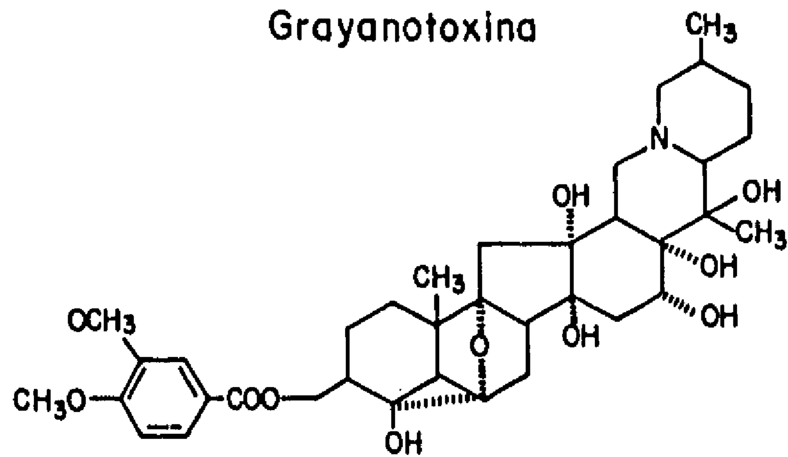
El principio activo preponderante (un alcaloide esteroidal, Fig. 4) de la secreción de la piel de las ranas venenosas *Phyllobates aurotaenias, P.*







Grayanotoxina



Veratridina

Figura # 3

bicolor, P. vittatus, P. lugubris y *P. terribilis* ha sido llamado Batracotoxina (BTx), uno de los productos naturales más tóxicos que a la fecha se conocen. De una manera similar a la TTx, las ranas en cautiverio pierden totalmente los alcaloides tóxicos de la piel en la segunda generación, sugiriendo la implicación de microorganismos en simbiosis u otros factores ambientales. Esta molécula había sido encontrada anteriormente en plantas superiores. La BTx produce una depolarización cuando se aplica en preparaciones nerviosas, la cual se debe a la prevención de la inactivación del canal de Na^+. Este efecto parece ser casi irreversible en muchas preparaciones, pero es reversible en células de neuroblastoma. La STx y la TTx, bloqueadores del canal de Na^+ dependiente de voltaje, previenen y revierten el efecto de la BTx, sin embargo su acción inhibitoria es no competitiva, indicando que se unen a un sitio receptor diferente en el canal. Cuando se mapearon los sitios receptores se encontró que el sitio 2 es el ocupado por BTx. Grayanotoxina, Veratridina y Aconitina, también actúan en el mismo sitio, aunque son menos potentes en su acción, esto es, parecen ser agonistas parciales. Las interacciones con polipéptidos tóxicos de alacrán y de anémona de mar incrementan la afinidad de BTx por su sitio receptor, llamandosele a esto una unión de cooperatividad (Khodorov, B.I., 1985).

7.) TOXINAS DE ANEMONA DE MAR O ANEMONATOXINAS.

Varias toxinas que actúan a nivel de membranas excitables han sido aisladas de anémonas de mar, tales como, *Anemonia sulcata, Anthopleura elegantissima, A. xanthogrammica, A. fuscoviridis, Actinodendron plumosum, Parasicyonis actinostoloides* y *Radianthus paumotensis*. Estas moléculas tienen PM entre 2,000-3,000 y 4,000-6,000 daltons

respectivamente y las obtenidas de *Condylactis aurantiaca* y *C. gigantea* tienen un PM de 10,000 daltons. El mecanismo de acción de estas toxinas es inhibir el paso de inactivación del canal de Na+ sin alterar su activación. Estas moléculas ocupan el mismo sitio receptor de las α-ScTx, esto es, el sitio 3 del canal de Na+. Sin embargo, las toxinas de anémona de mar parecen ser más eficientes para detectar diferencias entre los canales de Na+ de algunos tejidos excitables. Por ejemplo, las toxinas de anemona o anemonatoxinas (ATx) interactúan preferencialmente con canales resistentes a TTx, que con canales de Na+ sensibles a TTx como los de miotubulos, células de neuroblastoma y fibroblastos, apoyando la hipótesis de que existen formas múltiples del canal de Na+ (Frelin y col., 1984).

8.) BREVETOXINA

La brevetoxina es una molécula liposoluble sintetizada por el dinoflagelado *Ptychodiscus brevis*, (Fig. 4). Provoca una serie de efectos fisiológicos tales como depolarización de la membrana de músculo esquelético, liberación de acetilcolina de las terminaciones nerviosas e incremento de la frecuencia de disparo del potencial de acción en cordón nervioso de cangrejo (Wu y col., 1985; Catterall y col., 1985). La TTx y bajas concentraciones de Na+ pueden bloquear estos efectos. Brevetoxina no bloquea el pegado de STx ni de α−ScTx, pero incrementa la activación del canal de Na+ cuando se aplica junto con Veratridina, Aconitina y BTx. Se postula que la Brevetoxina actúa en un sitio receptor nuevo, el sitio 5 del canal de Na+.

9.) CIGUATOXINA (CIGUATERA)

Ciguatera es una enfermedad que se presenta después de la ingesta de peces que habitan en regiones tropicales y subtropicales. La molécula no ha sido totalmente caracterizada, pero es de naturaleza lipídica, altamente oxigenada y de peso molecular de 1111.7 daltones. Se piensa que la ciguatoxina es un poliester complejo similar al ácido ocádico o a brevetoxina (Bidard y col., 1984) (Fig. 4). Su acción fisiológica es sobre canales de Na^+, pero no se conoce su sitio de pegado.

10.) CONOTOXINAS

Las conotoxinas son aisladas de los caracoles del género *Conus* (Olivera, B., 1985). El veneno de estos gastrópodos esta formado por una gran variedad de péptidos biológicamente activos y proteínas, las cuales parecen alcanzar sus órganos blanco rápidamente, provocando parálisis en su presa. Los venenos de *Conus geographus* y *Conus magus* son los mejor estudiados y por lo menos tres tipos de toxinas peptídicas que producen parálisis se han aislado y se han clasificado de la siguiente manera:

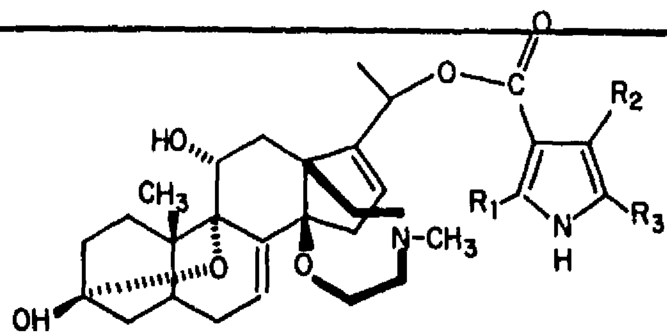
A) α-Conotoxinas: Acción sobre receptores de acetilcolina.

B) ω-Conotoxinas: Bloqueo de los canales de Ca^{2+} en membranas presinápticas.

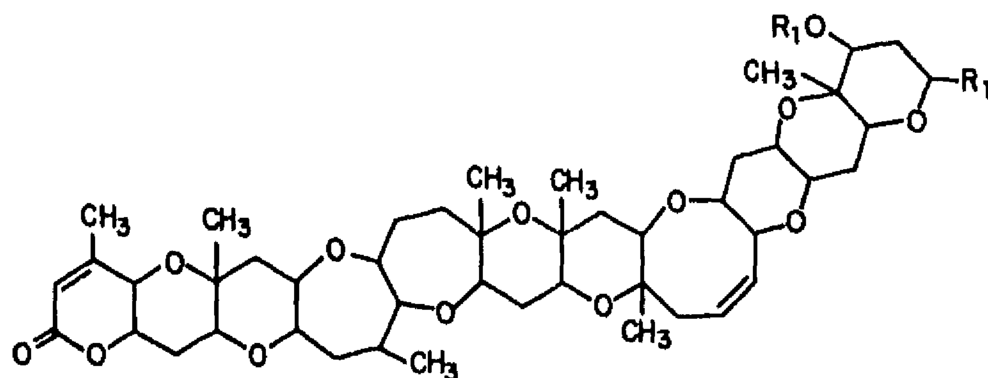
C) μ-Conotoxinas: Bloqueo de los canales de Na^+.

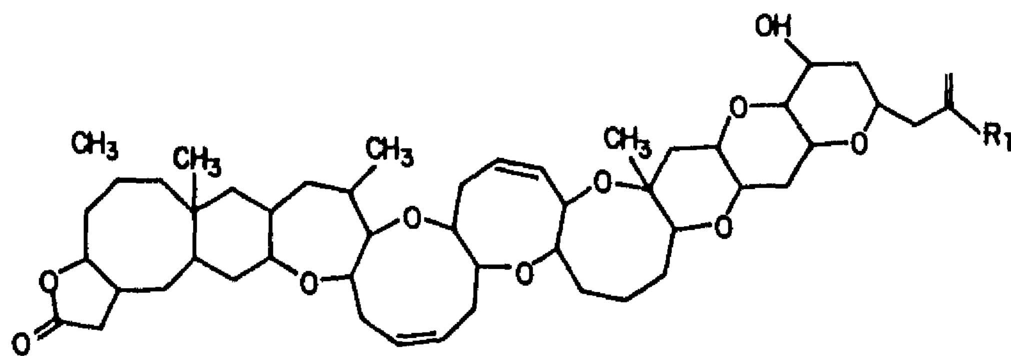
Todos estos péptidos son de naturaleza básica, de 13 a 27 residuos de aminoácidos, ricos en cisteínas y por ende con una gran cantidad de puentes disulfuro que hacen del péptido una molécula altamente empacada (Olivera, B., 1985).

Los estudios electrofisiológicos en canal unitario indican que la μ-Conotoxina GIIIA bloquea los canales de Na^+ de músculo esquelético,

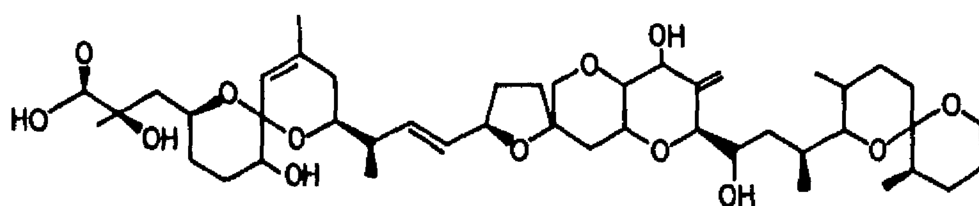


Batracotoxina





Brevetoxina



Posible estructura homologa a ciguatoxina

F I G U R A # 4

mientras que los canales de Na+ de nervio y de cerebro no son esencialmente afectados (Cruz y col., 1985). Este efecto bloqueador es dependiente de voltaje y similar al de la TTx y STx. TTx y GIIIA tienen afinidades similares por las membranas de electroplaca, sin embargo, la TTx tiene una afinidad 100 veces mayor por la fracción P_2 de cerebro de rata que la GIIIA (Yanagawa y col., 1986). Esto apoya la hipótesis de que existen diferencias estructurales entre los canales de Na+ de membranas de músculo y nervio.

Otro de los rubros en los cuales se hará enfasis es, sobre canales iónicos, por estar estrechamente relacionados con el tema de tesis.

C) CANALES IONICOS DEPENDIENTES DE VOLTAJE:

Los canales iónicos son proteínas intrínsicas de membrana que tienen dominios en contacto con las interfases acuosas limitadas por la membrana. El papel de ellos es formar un poro acuoso, a través del cual ocurre el flujo trasmembranal pasivo de iones esenciales para el funcionamiento celular (Liévano, A., 1989).

De una manera esquemática se podría decir que todos los canales conocidos a la fecha, de los cuales se tiene un cierto conocimiento bioquímico, parecen compartir un mismo patrón arquitectónico, esto es, están formados por unidades polipeptídicas homólogas (4, 5 ó 6 subunidades) las cuales son ensambladas simétricamente dentro de la membrana, formando un poro en su centro, y que los canales tienen dos estados escenciales; *abierto y cerrado.* Sin embargo, esta es una manera muy simplista de ver a estas moléculas, ya que la mayoría de ellas tienen una cinética compleja, que se traduce en la presencia de más de un estado de apertura o cierre, o bien de más de un estado conductor. Por otro lado,

a nivel molecular la estructura detallada del canal no ha sido todavía resuelta, y preguntas tan básicas como; ¿cuál es el diseño que hace a los canales tan eficientes facilitando el transporte selectivo, a través de la membrana, de cientos de iones en un segundo ? ó ¿Cuáles son los principios fisicoquímicos de la selectividad iónica que hace que los canales permitan el paso de algunos iones, e impidan el paso de otros? ó bien ¿Cuales son los cambios conformacionales que están involucrados en el "disparo", la rápida transición entre el estado cerrado y abierto? no hán podido ser contestadas (Unwin, N. 1989).

Mucha de la información bioquímica que se tiene sobre los canales iónicos, ha sido generada en las últimas dos decadas por el estudio de estas proteínas como receptores de *toxinas, drogas y hormonas,* ya que aunque los canales iónicos no son regulados por ligandos fisiológicos conocidos, son los blancos de numerosas moléculas de ese tipo (Catterall W.A., 1991).

Existen diversos tipos de canales iónicos cuya actividad puede ser controlada por la célula. De acuerdo con esto, los canales iónicos se pueden clasificar como operados por:

A) Ligando,

B) El potencial transmembranal y por ligando,

C) Segundos mensajeros y

D) Tensión (stretch-activated y stretch-inactivated) (ver tabla 2)(Lievano, A., 1989).

En esta tesis sólo nos referiremos al canal de Na^+ dependiente de voltaje, que pertenece a los canales operados por potencial trasmembranal de tipo catiónico.

Los canales de Na^+ sensibles a voltaje, son las proteínas de membrana responsables del influjo de Na^+ durante la fase rápida del

TABLA # 2

TIPO DE CANALES IONICOS

Canales operados por ligando:

a) Catiónicos:

Receptores muscarínicos a acetilcolina (1)
Receptores a glutamato (excitatorios, 2)
Receptor a NMDA (2)
Receptor a ATP (3)

b) Aniónicos:

Receptor a GABA (acido gamma amino butírico) (4)
Receptor a Glicina (inhibitorios, 4)

Canales operados por el potencial transmembranal:

a) Catiónicos:

Canales de Na^+ (5)
Canales de K^+ (6)
Canales de Ca^{2+} (7)

b) Aniónicos:

Canales de Cl^- (8)
Porinas (9)
VDAC (canales aniónicos dependientes de voltaje) (10)

Canales operados por potencial y por unión de ligando:

Canales de K^+ activados por Ca^{2+} (11)
Canales de K^+ activados por ATP (12)
Canales de Cl^- activados por Ca^{2+} (13)

Canales operados por segundo mensajero:

Canales catiónicos del fotoreceptor (14)
Canales catiónicos del receptor olfatorio (14)

Canales operados por presión (15).

Tabla 1. Tipos de canales iónicos de acuerdo con la regulación de su actividad. Para cada tipo se tomo sólo un ejemplo. (1) Montal y col. 1986. (2)- Cull-Candy y Usowicks, 1987. (3) Benham y Tsien, 1987. (4) Hamill y col., 1984. (5) Catterall, 1986. (6) Rudy, 1988. (7) Tsien y col., 1987. (8) Miller y White, 1984. (9) Benz, 1986. (10) Colombini, 1986. (11) Latorre y Miller, 1984. (12) Ashcroft, 1988. (13) Owen y col., 1984. (14) Gold y Nakamura, 1987. (15) Yang y Sachs, 1989. Tomado de Lievano, A., 1989.

potencial de acción de células excitables (Hille, B., 1984). Estos canales tienen al menos cinco sitios receptores separados para neurotoxinas, cada uno de los cuales tiene diferente efecto en la función del canal (tabla 1) (Catterall , W.A, 1991; Narahashi, T., 1984).

Los componentes protéicos del canal fueron identificados utilizando α-toxinas de alacrán fotoreactivas las cuales se unían covalentemente a dos polipéptidos de PM aproximado a 260 kD y 36 kD, en sinaptosomas de cerebro de rata. Estas subunidades denominadas α y β_1 respectivamente, son también marcadas por β-toxinas de alacrán fotoreactivas, las cuales actúan en el sitio 4 del canal de Na^+. Esto sugiere que los sitios 3 y 4 del canal se encuentran localizados cerca de las regiones de contacto de estas dos subunidades.

Utilizando el pegado de alta afinidad de STx como marcador específico se determinó que los canales de Na^+ de cerebro de mamíferos son un complejo heterotrimérico de subunidades α (260 kD), β_1 (36 kD) y β_2 (33kD) en una relación 1:1:1, formando un complejo de 320 kD (Fig. 5). La subunidad β_2 se encuentra unida a la subunidad α por puentes disulfuro, mientras que la subunidad β_1 no se encuentra covalentemente unida (Hartshorne y col., 1982). Los canales de Na^+ de electroplaca de anguila y corazón de pollo están constituidos por una sola subunidad α (Agnew y col., 1980; Miller y col., 1983; Lombet y Lazdunski, 1984), mientras que los canales de Na^+ en músculo esquelético y corazón de rata son heterodímeros, compuestos por subunidades α y β_1 (Kraner y col., 1985; Gordon y col., 1988).

También se ha reportado que las tres subunidades están altamente

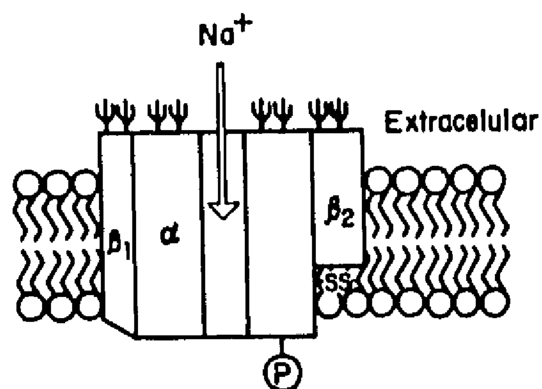


Fig. 5: Posible Estructuración de las Subunidades que Conforman el Canal de Na+ de Cerebro de Mamífero.

P Sitios de Fosforilación
Ψ Sitios de Glicosilación

(Tomado de Catterall, W.A., 1991)

glicosiladas, aproximadamente un 30% de su masa aparente son carbohidratos (Agnew y col., 1980; Miller y col., 1983; Casadei y col., 1986; Messner y Catterall, 1985; Wollner y col., 1987), lo cual indica que parte de las moléculas están expuestas a la superficie extracelular.

Por otro lado la subunidad α es fosforilada por adenosin 3,5-monofosfato proteincinasa dependiente de AMPciclico en células intactas y en sinaptosomas (Costa y col., 1982; Coombs y col., 1988; Gordon y col., 1988), indicando que esta cadena peptídica está expuesta a la superficie intracelular y por lo tanto es una proteína trasmembranal. Las subunidades β_1 y β_2 son proteínas intrínsecas de membrana que tienen un dominio hidrofóbico substancial, marcado por pruebas hidrofóbicas de fotoafinidad y son preferencialmente extraidas en la fase hidrofóbica de detergentes (Reber y Catterall, 1987).

Cuatro isoformas de la subunidad α del canal de Na^+ de cerebro de rata se han identificado por clonación y secuenciación (Noda y col., 1986; Kayano y col., 1988; Auld y col., 1988) y existen también multiples subtipos de β_1 expresados en cerebro de rata, nervio ciático, músculo esquelético y corazón (Sutkowski y Catterall, 1990). La expresión de la subunidad alfa del canal de Na^+ de cerebro de rata en ovocitos de *Xenopus* (Goldin y col., 1986; Noda y col., 1986; Susuki y col., 1988; Joho y col. 1990) o en células de mamíferos (Scheuer y col., 1990; West y col., 1992) producen canales de Na^+ funcionales que se activan e inactivan en respuesta a depolarización, pero su inactivación es más lenta comparada con los canales de Na^+ de neuronas. Si se coinyecta con RNAs mensajeros (mRNAs) de cerebro, de bajo PM, se incrementa el tamaño de la corriente de Na^+, se inhibe la dependencia de voltaje de la inactivación y se restaura

la inactivación rápida normal (Auld y col., 1988; Krafte y col., 1990), sugiriendo un posible requerimiento de la subunidad β_1 y β_2, para la expresión funcional exacta y eficiente de los canales de Na^+ de cerebro. Y por lo menos para la subunidad β_1 se ha demostrado que es crucial en el ensamble, expresión y modulación funcional del complejo heterotrimérico del canal de Na^+ de cerebro de rata (Isom y col., 1992).

La estructura primaria completa de la subunidad α del canal de Na^+ de electroplaca, cerebro de rata, músculo esquelético y corazón se dedujeron de secuencias de DNA complementario (cDNA). Esta subunidad consiste de 4 dominios internos homólogos cada uno de los cuales tiene 6 segmentos alfa-helicoidales trasmembranales (S1-S6) basados en análisis computacionales de hidrofobicidad (Fig. 6). La transfección de cDNA que codifica para la subunidad α en células de mamíferos resulta en la expresión de canales de Na^+ con propiedades fisiológicas normales y respuesta normal a neurotoxinas que actúan en los sitios 1-3 (Scheuer y col., 1990). Evidentemente, la subunidad α del canal de Na^+ es funcionalmente autónoma en la mediación de la conductancia iónica dependiente de voltaje que es modulado por drogas y neurotoxinas.

La meta principal del estudio de las corrientes en los canales iónicos dependientes de voltaje es definir los componentes estructurales que son responsables de aspectos específicos en la función del canal. Para esto se han utilizado dos herramientas experimentales: la primera de ellas es el uso de Anticuerpos (Ac) levantados en contra de segmentos peptídicos cortos (aprox. 20 residuos) de la subunidad α del canal, para verificar los dominios que se requieren, o se encuentran involucrados en las funciones específicas del mismo (activación, inactivación, sensor de voltaje, etc.), o

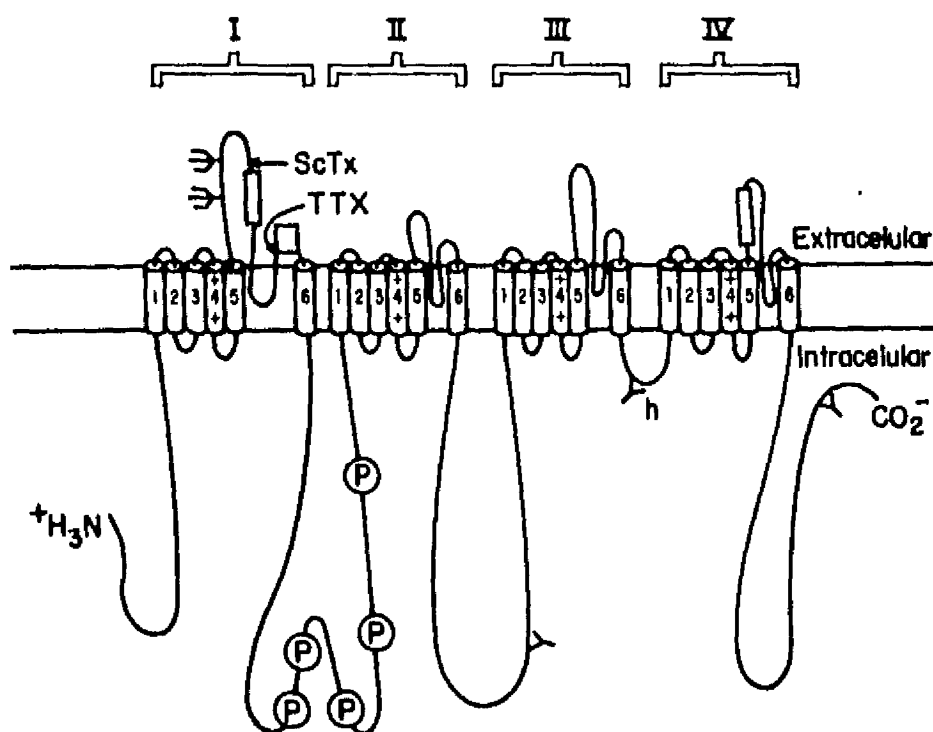


Fig. 6: Plegamiento Trasmembranal Propuesto para la Subunidad α del Canal de Na^+.

Las α hélices trasmembranales se ilustran como cilíndros. El resto de la cadena polipeptídica se ilustra como una línea sólida con una longitud aproximadamente proporcional a la secuencia de aminoácidos de cada segmento polipeptídico.

(P)	Sitios de fosforilación determinados experimentalmente.
(ψ)	Sitios de glicosilación.
(ScTx)	Sitios de pegado covalente para α-escorpiotoxinas.
(TTx)	Sitio de pegado para TTx.
Y	Sitios de pegado para Anticuerpos antipéptido los cuales inhiben el pegado de las α-escorpiotoxinas
h	Sitio de bloqueo de la inactivación rápida del canal de Na^+

(Tomado de Catterall, W.A. 1991).

que pueden ser covalentemente unidos por drogas, neurotoxinas o fosforilación. La segunda herramienta, es la mutación dirigida de los cDNA que codifican para las subunidades α, y expresarlos en células recipientes y analizarlos por registros electrofisiológicos.

La activación del canal de Na^+ dependiente de voltaje requiere dentro de su estructura de un sensor de voltaje, el cual deberá tener cargas localizadas en la región trasmembranal ya que los cambios de potencial eléctrico de la membrana deben ejercen una fuerza sobre estas cargas y causar un cambio conformacional del canal hacia el estado conductor activo. El movimiento de estas cargas durante la activación del canal ha sido medido directamente y es equivalente a 4 o 6 cargas positivas moviendose hacia afuera através del campo eléctrico total de la membrana. El segmento trasmembranal S4 es tanto hidrofóbico como cargado positivamente (Fig. 6) y además es altamente conservado en la mayoría de las subunidades α de los diferentes canales iónicos dependientes de voltaje. Este segmento se ha propuesto para servir de sensor de voltaje en los modelos de hélice deslizante ("sliding helix" y "propagating helix"), los cuales proponen que la región S4, bajo la influencia de un campo eléctrico, se desliza hacia afuera formando una espiral o se proyecta hacia afuera por un cambio estructural de una α-hélice por una β-plegada. Los experimentos de mutagénesis dirigida en esta subunidad muestran que la neutralización de las cargas positivas, disminuye el número de cargas de disparo del canal y que la alteración de los residuos hidrofóbicos en el segmento S4 del dominio II abolen la dependencia de voltaje de la activación del canal (Stuhmer y col., 1989; Auld y col.,1990).

El segmento del canal de Na^+ responsable de la inactivación no ha

sido identificado. Una estrategia para su identificación fue el uso de Ac formados contra fracciones cortas de la subunidad α. Los Ac se aplicaron a la superficie intracelular del canal, através de la pipeta, en experimentos de voltaje sostenido de célula completa o en la solución del baño en experimentos de registro de canal unitario. En ambos casos sólo un Ac dirigido contra el segmento intracelular corto que conecta los dominios homólogos III y IV inhiben la inactivación del canal de Na^+. El pegado y el efecto del pegado de estos Acs fue voltaje dependiente; a potenciales de membrana negativos, donde los canales de Na^+ no estan inactivados, el Ac se une rápidamente e inhibe la inactivación del canal. A potenciales de membrana a los cuales los canales de Na^+ estan inactivados, el pegado de los Acs se mostró disminuido ó abolido. Estos experimentos permiten proponer que esta región se encuentra involucrada en el cambio conformacional que permite la inactivación del canal. Durante este cambio conformacional, el segmento se doblaría dentro de la estructura del canal, y ocluiría el poro trasmembranal haciendolo inaccesible al pegado de los Acs. Un modelo similar es apoyado por experimentos de mutagénesis dirigida (Stuhmer y col., 1989) en los cuales la expresión de la subunidad α del canal de Na^+ en ovocitos de *Xenopus* en 2 partes, correspondientes a los tres primeros dominios y el cuarto, da como resultado canales que activan normalmente, pero que carecen de inactivación rápida. La característica fisiológica de estos "canales cortados" son necesariamente idénticas a aquellas de canales de Na^+ con bloqueo de la inactivación por Ac dirigidos contra este sitio.

La inactivación del canal de Na^+ también es retardada por numerosas neurotoxinas, incluidas las toxinas α de alacrán las cuales como ya se

mencionó actúan en el sitio 3 del receptor en la cara extracelular del canal de Na^+. El pegado de este tipo de toxinas a su sitio receptor es altamente dependiente de voltaje, sugiriendo que los segmentos del canal involucrados en la activación e inactivación, también contribuyen a la formación del sitio receptor de las α-toxinas (Catterall, W.A., 1988 y Strichartz y col., 1987). Este sitio se encuentra localizado en el asa extracelular propuesta entre el segmento trasmembranal S5-S6 y en el dominio I de la subunidad α (Fig. 6) (Tejedor y Catterall, 1989). Para identificar si otro segmento del canal se encuentra involucrado en la formación del receptor, se estudió la efectividad de varios Acs dirigidos en contra de las asas extracelulares propuestas para la subunidad α del canal para inhibir el pegado de las α-toxinas (Thomsen y col., 1989). Los Acs que inhibieron el pegado fueron aquellos que reconocían los segmentos S5 y S6 del dominio I. Sorpresivamente, un Ac dirigido contra el asa extracelular correspondiente al dominio IV también inhibió el pegado de la toxina. Estos segmentos se encuentran retirados en la estructura primaria del canal pero muy cercanos en la estructura terciaria. Actualmente, se cree que los cuatro dominios homólogos del canal de Na^+ forman un cuadrado, rearreglado en la membrana con un poro trasmembranal en el centro (Catterall, W.A., 1988). La posición cercana de segmentos del asa extracelular entre los segmentos S5-S6 del dominio I y IV pueden formar el sitio receptor para las α-toxinas.

Aunque se han reportado diferentes tipos de canales de Na^+ la variedad no parece ser tan extensa como es el caso de los canales de K^+; sin embargo, puede afirmarse que existe diversidad en estas proteínas, ya que una serie de evidencias muestran que un grupo de toxinas competitivas

entre sí, incluyendo TTx y STx y sus congéneres, pueden distinguir entre canales de Na+ en células en diferentes estados de desarrollo y en diferentes tejidos (Trimer y Agnew, 1989). TTx y STx son con mucho las más específicas de las neurotoxinas de canal de Na+ y en la mayoría de los casos el pegado es de alta afinidad (Kd=1-10 nM) a un solo sitio extracelular en la molécula; y revertido rápidamente (segs ó minutos). La inhibición de la corriente de sodio resultante puede deberse al pegado en la superficie de la proteína (canal) para obstruir la conductancia o debido a un cambio conformacional de la molécula (Green y col., 1987).

Por otro lado, las corrientes de Na+ de células cardíacas son típicamente insensibles a TTx ($K_{1/2}$ = 0.6 -10 μM o mayor) y STx ($K_{1/2}$ = 50-1000 nM) y en estudios de pegado se ha encontrado que los sitios de baja afinidad son cuatro veces más que los de alta afinidad y que los segundos parecen ser más abundantes en terminaciones nerviosas simpáticas (Baer y col., 1976; Catterall y col., 1981; Doyle y col., 1985; Renaud y col., 1983). En músculo esquelético, la sensibilidad a TTx y STx depende de la inervación del músculo. Los canales de Na+ de músculo neonatal son resistentes a ambas toxinas (Frelin y col., 1984; Pappone, P., 1980) pero después de la inervación el pegado de alta afinidad se expresa. Otro ejemplo sería la presencia de canales de Na+ en células que no son consideradas clásicamente excitables, demostrado por el pegado de alta afinidad de STx a canales de Na+ dependientes de voltaje en células de Schwann de nervios periféricos de gato y de conejo, en los cuales se ha inducido degeneración por cirugía (Shrager y col., 1985; Pellegrino y col., 1986; Ritchie y col., 1983).

Dentro de este marco teórico y con las interrogantes anteriormente planteadas, sí se acepta que la capacidad de una toxina con alta afinidad

para reconocer diferentes tipos de canales iónicos se da por la interacción química entre algún(nos) de los grupos funcionales de la toxina y su proteína receptora o canal, se puede asumir que las toxinas pueden ser utilizadas como marcadores especificos o "sondas" para la identificación de sitios importantes en el funcionamiento del canal, como también que, la farmacología de una toxina dada es de suma importancia en la identificación de la contribución fisiológica de un canal determinado cuando se analizan corrientes macroscópicas que serían la suma de varios tipos de canales actuando en sincronía. Además, con el conocimiento molecular que se tiene de los canales iónicos, sería posible correlacionar diferencias farmacológicas entre diferentes tipos de canales con diferencias estructurales en los sitios de pegado de las toxinas. Asi pués, uno de los objetivos de esta tesis es la contribución en la caracterización bioquímica de nuevos ligandos, cuya estructura primaria nos ayudaría en la identificación de sus "sitios activos" y el grado de mutación permitida antes de la abolición de su efecto. Indudablemente, esto constituye otra forma de abordar el estudio del conocimiento molecular de los canales de Na^+ que redituará en el mejor conocimiento de su función.

D) TOXINAS DE ALACRAN.

En México existen 6 especies de alacranes de importancia médica, todas ellas pertenecientes al género *Centruroides*. En los últimos 20 años el grupo del Dr. Possani se ha dedicado al estudio de los componentes de algunos de estos venenos, y ha reportado la purificación y caracterización de 40 toxinas para canales de sodio y 5 para canales de potasio: 11 toxinas de *C. noxius* (Possani y col., 1981a; Possani y col., 1981b; Dent, M.A.R., 1982; Possani y col., 1982; Zamudio y col., 1992), 5 de *Centruroides*

limpidus tecomanus (Possani y col., 1980; Ramírez y col., 1988; Martin y col., 1988), 1 de *Centruroides elegans* (Possani y col., 1978; Ramírez y col., 1981), 6 de *Tityus serrulatus* (Possani y col., 1977, 1981c, 1982, 1985, 1991) 10 de *Tityus bahiensis* (Possani y col., 1992), 5 de *Centruroides limpidus limpidus* y 2 más de *Centruroides infamatus infamatus* (Dehesa-Davila, M., 1987; Possani y col., 1991). Mientras que de los alacranes del viejo mundo, cinco venenos de la familia *Buthidae* se han estudiado: *Androctonus australis* Hector (AaH), *Androctonus mauretanicus mauretanicus* (Amm), *Buthus occitanus tunetanus* (Bot), *Buthus occitanus Paris* (Bop) y *Leiurus quinquestriatus quinquestriatus* (Lqq), y de ellos se han aislado 31 toxinas activas en mamíferos, 2 activas en insectos y 1 activa en crustáceos (Rochat y col., 1979; Zlotkin y col., 1978).

De la comparación de la secuencia de aminoácidos de más de 20 toxinas, se puede concluir que las toxinas de alacrán forman un grupo de polipéptidos homólogos que muestran varias substituciones conservativas y pocas substituciones radicales en ciertas partes de la molécula. Además de la alta homología, las toxinas de alacrán del viejo mundo *(Androctonus, Buthus, Leiurus)* y las del nuevo mundo (*Centruroides*) tienen peculiaridades estructurales las cuales indican una temprana dicotomia en la evolución. A pesar de que la homología secuencial sugiere la misma conformación general, los datos de dicroismo circular revelan que las toxinas forman una serie de variantes conformacionales. Esto podría ser importante en la correlación de la estructura con las propiedades farmacológicas de la molécula, por ejemplo, el reconocimiento del sitio receptor.

Por otro lado, los estudios de estructura tridimensional de la variante 3 de *Centruroides sculpturatus* Ewing (CsV3), indican que la proteína tiene

una larga superficie aplanada, en la cual se encuentran muchos de los aminoácidos conservados y una alta concentración de residuos hidrofóbicos (2 leucinas, 1 alanina, 2 prolinas), los cuales pueden interactuar con receptores específicos en membranas exciables (Fontecilla-Camps y col., 1981). Esto parece sugerir que muchas toxinas de alacrán tienen esta forma estructural con una superficie hidrofóbica, sin embargo, en experimentos de modificación química de las toxinas I y II de *Androctonus australis* Hector, se ha demostrado que la lisina 56 (residuo cargado positivamente) parece ser esencial para la actividad biológica (Habersetzer-Rochat y col., 1981). Las toxinas de alacrán difieren considerablemente en toxicidad y su clasificación como toxinas para mamíferos, crustáceos e insectos puede ser engañosa. Es evidente que algunas toxinas ejercen relativa especificidad de especie, pero esto puede ser resultado de la grán variabilidad en la toxicidad, la cual puede ser explicada por las características de la estructura primaria de las moléculas y/o por la conservación del receptor a través de la evolución.

En estudios electrofisiológicos las toxinas puras dan diferentes tipos de efectos y esto permite agrupar a las toxinas en varias clases (Watt y col., 1984):

A) Toxinas de las Especies del Viejo Mundo: Disminuyen la inactivación del canal de Na^+ sin alterar su activación. Este efecto es dependiente de voltaje y es disminuido por la depolarización de la membrana. Las toxinas de este tipo han sido llamadas Toxinas α (α-ScTx).

B) Toxinas Aisladas de Especies del Nuevo Mundo: Producen una depolarización transitoria del canal de Na^+ induciendo un corto circuito en la dependencia de voltaje de la activación. Las toxinas de este tipo han sido

llamadas de tipo β (β-ScTx). Ejemplo de este tipo de moléculas son las toxinas I, II y IV de *Centruroides sculpturatus*.

C) Toxina gamma de Tityus serrulatus (TiTx γ): Modifica tanto la activación como la inactivación del canal de Na^+ y tiene la constante de afinidad más pequeña hasta ahora reportada (Jonas y col., 1986).

D) Toxinas con Acción Sobre Canales de K^+: ejemplos de este grupo son la Noxiustoxina (NTx), Caribdotoxina (CTx), Iberotoxina (IBTx), Leiurotoxina (LeTx), kaliotoxina (KTx) (Carbone y col., 1987; Miller y col., 1985; Valdivia y col., 1988; Auguste y col., 1990; Galvez y col., 1990).

ANTECEDENTES

En cada veneno de alacrán existe un conjunto de toxinas cuyas características fisicoquímicas son muy similares y algunas de ellas comparten homología estructural, no así toxicidad. Si comparamos las toxinas conocidas de un veneno con las del veneno de otra especie, encontramos toxinas que son equivalentes, sin llegar a ser idénticas, y aún las toxinas dentro de un mismo veneno, a pesar de la alta homología que comparten, pueden tener efectos fisiológicos diferentes en los sistemas biológicos donde se han probado. Tal es el caso de la toxina II.10 (Cn 4) de *C. noxius* la cual afecta el mecanismo de activación de los canales de sodio de axón de calamar, pertenece a las toxinas de tipo β, es muy tóxica a ratón y rata y se une a sinaptosomas de cerebro de rata con una afinidad de 2 a 10 nM (Ramírez y col., 1992). Por otro lado la toxina II.9.2.2 (Cn 2) de *C. noxius* afecta muy pobremente a los canales de sodio de axón de calamar, pero es muy tóxica a ratón, uniendose a sinaptosomas de cerebro de rata con una afinidad de 10 nM (anexo 1), y dado que afecta el mecanismo de

activación del canal de sodio es también una toxina de tipo β. La homología estructural existente entre estas dos toxinas es del 83% (Vázques y col., 1993). Por otro lado, existen toxinas que parecen tener una estructura primaria similar pero cuyo plegamiento terciario no es homólogo ya que pueden reconocer canales de Na^+ en especies filogenéticamente distantes, por ejemplo las toxinas Cs EV 1, 2 y 3 aisladas del veneno del *C. sculpturatus* Ewing tienen efectos fisiológicos característicos de las toxinas α y son muy tóxicos a insectos y crustaceos, pero tienen un pobre efecto en mamíferos. La otra cara de la moneda sería la toxina gamma (γ) (toxina VII) aislada del veneno del alacrán sudamericano *Tityus serrulatus* (Possani y col., 1977), la cual es una toxina β clásica, pero que es tanto tóxica en mamíferos como en insectos (K_D 0.2 nM en membranas sinaptosomales de cabeza de mosca y $K_{0.5}$ 0.08 nM en sinaptosomas de cerebro de rata) (De Lima y col., 1986), lo que parece indicar que esta toxina, estructuralente hablando, sería una intermedia entre las toxinas de mamíferos y las toxinas contra insectos. Otro punto que hace importante el estudio de las toxinas de alacrán por unión específica a diferentes tejidos es determinar si aquellas toxinas que no presentan toxicidad en un ensayo biológico simple, como sería la administración de ellas en una especie biológica determinada, realmente no se pegan a un sitio receptor o bien lo hacen sin lograr provocar la activación o bloqueo del canal. Esto es, si algunas de estas toxinas no pudieran tener un efecto de tipo antagonista.

Todo esto nos hace suponer que los pequeños cambios estructurales que hay entre molécula y molécula dan un reconocimiento mayor o menor por un receptor dado, y que estos cambios estructurales pueden hacer que el doblamiento de la molécula sea otro y su carga neta cambie, o que los

aminoácidos involucrados con el reconocimiento del receptor sean más expuestos, permitiendo la discriminación entre diferentes tipos de receptores, lo cual también apoya la posibilidad de utilizar a las toxinas de alacrán como herramientas para la identificación de subtipos de canales de sodio, sentando bases para un estudio mas profundo de relación de estructura-función.

OBJETIVOS

Para llevar a cabo este estudio se plantearon los siguientes objetivos:

A) Obtención de toxinas homogeneas para los experimentos de pegado.

B) Determinación de la composición química y de la composición de aminoácidos de las toxinas que ya fueron reportadas (Toxinas 2 y 3 de *C. noxius*).

C) Caracterización bioquímica de las nuevas toxinas aisladas de los venenos de *Centruroides noxius* y *Centruroides limpidus limpidus.*

D) Preparación de membranas de cerebro de rata, de cerebro y músculo esquelético de conejo y músculo cardiaco de conejo.

E) Marcado de las toxinas con ^{125}I y determinación de las constantes de afinidad de aquellas que incorporen bien el Iodo radiactivo y que reconozcan su receptor en alguno de los tejidos excitables.

F) Experimentos de desplazamiento por competencia de las toxinas entre sí, para determinar si están actuando en el mismo sitio receptor y con que afinidad lo hacen.

MATERIAL Y METODOS.

No se detallará la metodología porque ésta se encuentra descrita tanto en el anexo 1 como en el anexo 2.

RESULTADOS Y DISCUSION

Los péptidos tóxicos más abundantes en el veneno de alacrán son aquellos que tienen actividad sobre canales de sodio. La proporción entre los componentes de las familias de cadena larga y cadena corta es de 20% y 2% respectivamente (Valdivia, H.H., 1991).

En 1992, Zamudio y col. reportaron la secuencia completa de dos toxinas aisladas del veneno del *C. noxius*, una de ellas, la toxina 2 anteriormente habia sido estudiada como F-II.9.2.2 de *C. noxius.* Esta toxina había sido probada en sinaptosomas de cerebro de rata y se reportó que incrementaba la liberación de GABA y que no tenía acción bloqueadora en canales de sodio de axón de calamar (Carbone, E., 1984). Con la nueva técnica de purificación se logró obtener de forma homogénea esta toxina de manera más rápida y eficiente, además de que se purificaba simultáneamente otro de los componentes, al cual se le llamó toxina 3, la cual es muy similar a la toxina 2 pero cuya concentración es menor en el veneno. Ambas fueron caracterizadas bioquímicamente y posteriormente inmunoquímicamente con la ayuda de 6 anticuerpos monoclonales levantados contra la toxina 2 de *C. noxius* (Zamudio y col., 1992).

De manera paralela, se estudiaba el veneno de *C.l.limpidus*, y en

1988, Alagón y col. reportaron el N-terminal de 2 toxinas aisladas de este veneno, una contra mamíferos (toxina II-6) y otra contra crustáceos (Toxina II-9) (ver anexo 2). Continuando con el estudio de este veneno, se empezó a trabajar con la fracción II.8, la cual a pesar de encontrarse en menor cantidad en el veneno tiene un efecto tóxico mayor que la fracción II.6 reportada por Alagón y col. La purificación y caracterización bioquímica de esta toxina (toxina 1 de C.l.l) es el tema del artículo del anexo 1.

Con la intención de continuar con la busqueda de toxinas análogas se empezó a trabajar con uno de los componentes minoritarios del veneno de *C. noxius*, la fracción II-13, y dado que las toxinas 2 y 3 ya habían sido reportadas en seguida se describe el aislamiento y caracterización bioquímica de los componentes de esta fracción. Posteriormente, con la intención de detallar más la función de la toxina 2 de *C. noxius* y poderla comparar con las nuevas toxinas aisladas de ambos venenos, se hicieron experimentos de unión y desplazamiento de ^{125}I-toxina 2 a sinaptosomas de cerebro de rata.

Cuando esta fracción (F-II.13) se inyecta intraperitonealmente en un ratón, la sintomatología que provoca es muy similar a aquellas toxinas bloqueadoras de canales de sodio. Para el aislamiento del(os) componente(s) responsables de estos efectos se siguió el siguiente patrón de purificación: un primer paso cromatográfico por filtración en gel, seguido de 2 más en intercambio iónico y un último paso de purificación por CLAP.

I. Separación por Peso Molecular del Veneno de *C. noxius*.

Un total de 956 mg (1,219 UA) del veneno soluble fueron fraccionados en 3 separaciones cromatográficas independientes. El veneno se disolvió en amortiguador de acetato de amonio 20 mM, pH 4.7 y se aplicó en alicuotas de 6 ml a una columna (3 cm x 200 cm) de Sephadex G-50 medio equlibrada y eluida en el mismo amortiguador. El veneno se separó en 4 fracciones (I, II, III y IV) de las cuales, la fracción II es la única tóxica a mamíferos. En la Fig. 7 se muestra el perfil cromatográfico de una de las separaciones y los porcentajes de recuperación se muestran en la tabla # 3. Tanto el patrón cromatográfico, como el rendimiento de cada una de las fracciones en las diferentes aplicaciones cromatográficas fueron altamente reproducibles y concordantes con los datos reportados en la literatura (Dent, M.A.R., 1982).

II. Separación por Intercambio Iónico.

Siguiendo el esquema utilizado en el laboratorio para la separación de los componentes básicos de los venenos de alacrán, el siguiente paso de purificación fue una columna de intercambio iónico. La Fracción II obtenida por filtración en gel, se aplicó a una columna (0.9 cm x 33 cm) de CMC-32 equilibrada con amortiguador de acetato de amonio 20 mM (pH 4.7) y se eluyó con un gradiente salino de 0 a 0.5 M de NaCl en el mismo amortiguador. La cantidad de proteína fraccionada fue de 943 mg (por absorbencia) en un total de 6 separaciones cromatográficas procesadas en las mismas condiciones experimentales. Nuevamente, los datos fueron altamente reproducibles entre sí y concordantes con lo ya reportado. Para fines ilustrativos se muestra un ejemplo en la Fig. 8 y los datos de recuperación se reportan en la tabla # 4. Como también ya había sido

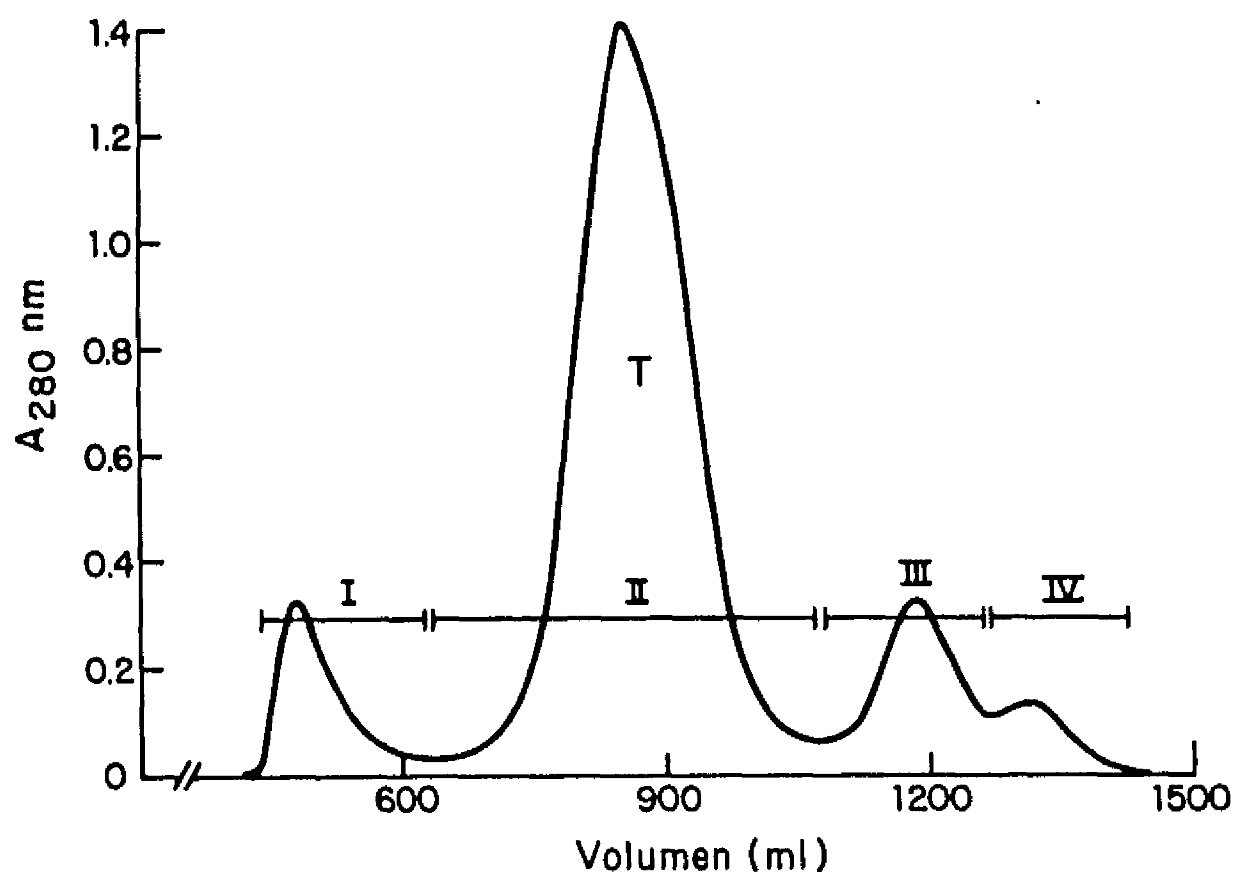


Fig. 7: Filtración en Gel del Veneno Total de *Centruroides noxius*:

En una columna (3cm x 200 cm) de Sephadex G-50 medio, equilibrada con amortiguador de acetato de amonio 20 mM, pH 4.7, se aplicaron 6 ml (296 U.A) del veneno total de *C. noxius*. La columna se eluyó con el mismo amortiguador a una velocidad de flujo de 60 ml/hr, colectandose fracciones de 6 ml cada una. La proteína se monitoréo a 280 nm de absorbencia. La toxicidad de las fracciones fue rastreada utilizando ratones albinos como ensayo biológico. La líneas horizontales indican los cortes que se hicieron (ver anexo 1 para mayores detalles sobre material y métodos).

TABLA # 3

RECUPERACION Y LETALIDAD DE LOS COMPONENTES CROMATOGRAFICOS OBTENIDOS DE LA COLUMNA DE SEPHADEX G-50 (Fig. 7)

Componente proteíco	Cantidad Recuperada	% Recuperación[a]	Letalidad[b]
Veneno souble 296 mg	269.4	91.0	Letal
Fracción I	23.0	7.8	No tóxico
II	201.2	68.0	Tóxico
III	32.7	11.0	No tóxico
IV	12.5	4.2	No tóxico

a) Los valores reportados son porcentajes calculados del número de unidades de absorbencia a 280 nm recuperadas, asumiendo que 1 unidad de absorbencia a 280 nm es igual a 1 mg/ml.

b) La letalidad de la proteína fue determinada administrando las diferentes fracciones a ratones albinos CD1 de 20 g de peso, generalmente un ratón por fracción cromatográfica. El término "No tóxico" significa que los ratones tienen un comportamiento similar a los ratones que fueron tratados con solución salina; "Letal"significa que los ratones murieron con las dosis administradas.

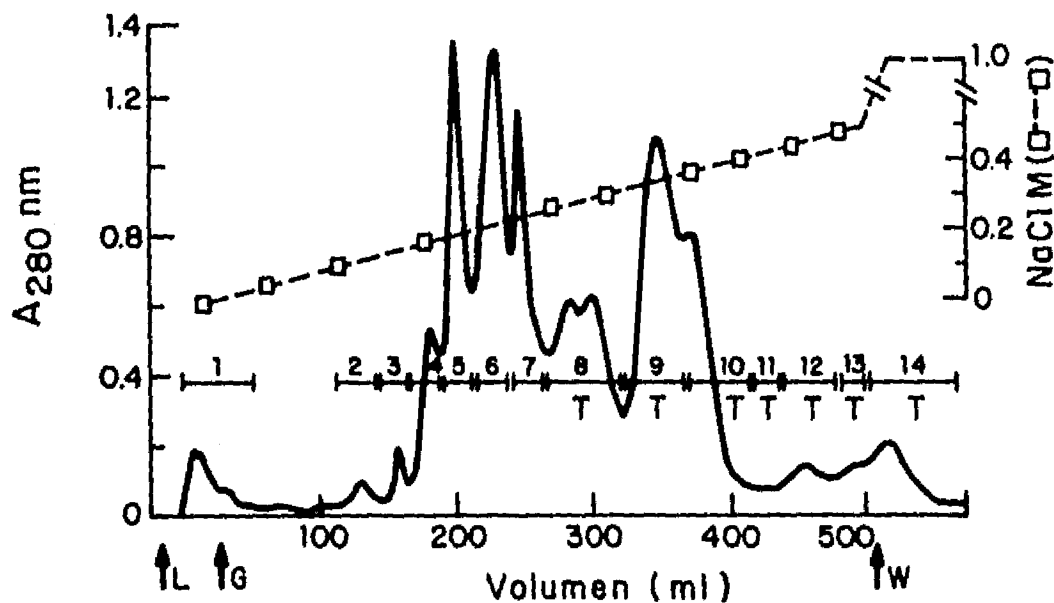


Fig. 8: Cromatografía de Intercambio Iónico de la FII de *Centruroides noxius*.

A) 168.9 mg (A_{280}) de la fracción II de *C. noxius* se aplicaron en una columna (0.9 cm x 33 cm) de CMC-32 equilibrada con acetato de amonio 0.02 M, pH 4.7. La columna se eluyó con un gradiente salino de 0 (250 ml)-0.5 (250 ml) M de NaCl en el mismo amortiguador, la velocidad de flujo fue de 30 ml/hr y las fracciones colectadas fueron de 2 ml cada una. Al final del gradiente se lavó la columna con alta fuerza iónica (100 ml de NaCl 1 M en acetato de amonio 20 mM, pH 4.7). (L) es el cargado de la columna, (G) es el inicio del gradiente, (W) muestra el inicio del lavado de la columna con alta fuerza iónica y (T) denota cual de los picos es tóxico a mamíferos.

TABLA # 4

RECUPERACION Y LETALIDAD DE LOS COMPONENTES PROTEICOS DE LA FRACCION II

Columna usada	Componente Protéico	% Recuperación [a]	letalidad [b]
CMC-32	Fracción II (168.9 mg)	100	L
	Fracción II.1	2.7	NT
	II.2	0.9	NT
	II.3	1.2	NT
	II.4	4.3	NT
	II.5	11.2	NT
	II.6	13.6	NT
	II.7	8.6	NT
	II.8	15.5	L
	II.9	12.7	L
	II.10	15.3	L
	II.11	0.8	L
	II.12	2.7	L
	II.13	1.1	L
	II.14	3.6	L
Proteína recuperada	159.1 mg	94.2	

a y b tienen el mismo significado que en la tabla anterior.
(L) Significa letal, (NT) Significa no tóxico.

reportado anteriormente, el patrón cromatográfico varía en la posición donde se encuentran localizadas las fracciones no tóxicas o sea, toxinas a insectos y crustáceos, pero se mantiene el patrón de elución de las toxinas a mamíferos, las cuales empiezan a eluir alrededor de 0.3 M de NaCl.

En este paso de purificación la fracción II.13 mostraba 2 componentes en geles cilíndricos de poliacrilamida en acetato urea (Reissfeld y col., 1981) (datos no mostrados).

III. Cromatografía de Intercambio Iónico de la F II.13 de *C. noxius*.

Esta fracción, corresponde a uno de los componentes tóxicos minoritários del veneno y había sido estudiado parcialmente por Valdivia y col. (comunicación personal). Así pues, con la idea de completar ese trabajo y terminar de caracterizar bioquímicamente los componentes de esta fracción se continúo con la purificación de las toxinas que la constituyen. Para ello, se tuvo que recurrir a 2 pasos cromatográficos adicionales, uno de intercambio iónico y otro por CLAP.

La mezcla de la Fracción II.13 obtenida del segundo paso cromatográfico (Fig. 8) se dializó contra agua (cuatro cambios de 30 min cada uno), y un último cambio contra el amortiguador de corrida del siguiente paso de purificación. La proteína dializada se aplicó a una columna de intercambio catiónico (CMC) equilibrada con amortiguador de fosfatos de sodio 50 mM, pH 6.0, y se eluyó con un gradiente salino de 0 a 0.5 M de NaCl en el mismo amortiguador (ver Fig. 9 y Tabla # 5). De los 6 componentes separados por el gradiente salino sólo la fracción II.13.3 y II.13.4 fueron tóxicos a mamíferos. Estas fracciones mostraban un solo componente en geles cilíndricos de acetato urea, por lo cual se procedió a

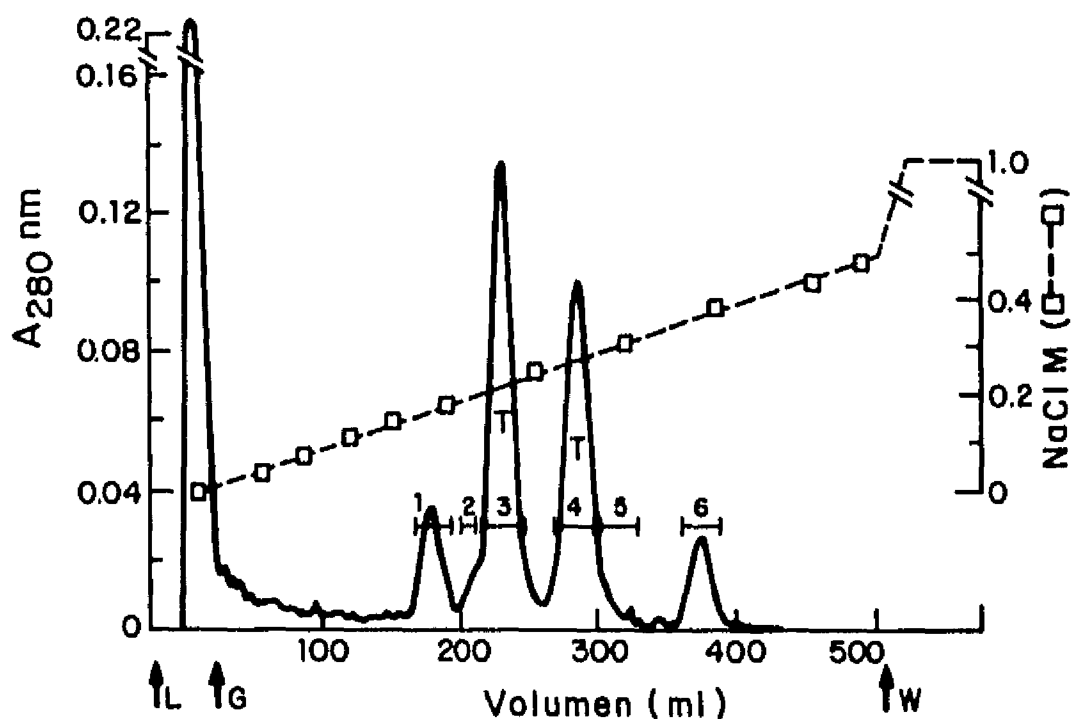


Fig. 9: Cromatografía de Intercambio Iónico de la Fracción II.13 de *Centruroides noxius*.

La fracción II.13 (10 mg) obtenida del cromatograma anterior se mezcló, dializó y aplicó a una columna de CMC-32 (0.9 cm x 33 cm) equilibrada con amortiguador de fosfatos de Na^{+} 50 mM, pH 6.0. La columna se eluyó con un gradiente salino de 0-0.5 M de NaCl (250 ml por vaso) en el mismo amortiguador. La velocidad de flujo fue de 24 ml/hr y se colectaron fracciones de 2.5 ml por tubo. Al finalizar el gradiente se lavó la columna con 1 M de NaCl en el mismo amortiguador (100 ml). L, G, W y T tienen el mismo significado que en la fig. 8.

TABLA # 5

RECUPERACION Y TOXICIDAD DE LOS COMPONENTES PROTEICOS DE LA FRACCION II.13 DE *C. noxius* OBTENIDOS POR INTERCAMBIO IONICO EN FOSFATOS DE POTASIO

Componente protéico	Cantidad recuperada mg	Recuperación %	Letalidad
Fracción II.13	11.0	100	Letal
Fracción II.13.1	0.7	6.4	No Tóxico
II.13.2	0.3	2.7	No Tóxico
II.13.3	2.6	23.6	Letal
II.13.4	2.4	21.8	Letal
II.13.5	0.4	3.6	No Tóxico
II.13.6	0.7	6.4	No Tóxico
Cargado	3.0	27.3	No tóxico
Total recuperado	10.1	91.8	

verificar su homogeneidad por CLAP. Hasta este punto, la F-II.13.3 y F-II.13.4 corresponden al 0.2% del veneno total.

IV. Purificación de las Fracciones II.13.3 y II.13.4 por CLAP.

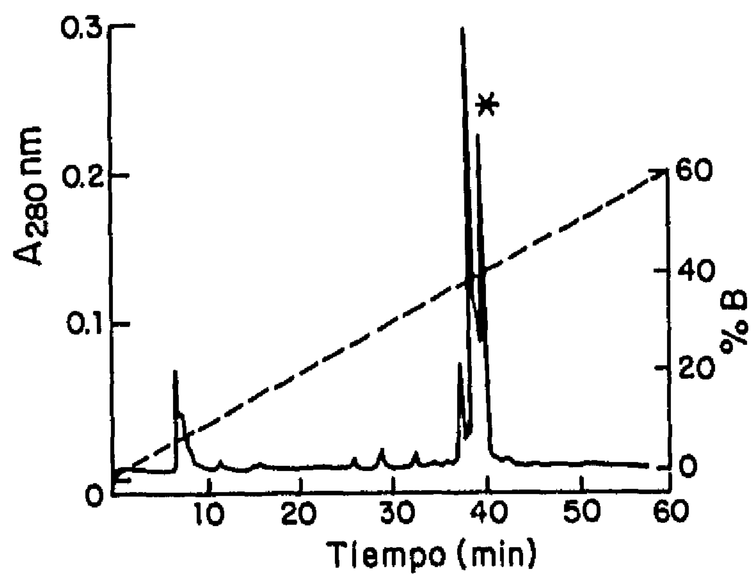
Tanto la F-II.13.3 como la F-II.13.4 se purificaron por cromatografía líquida de alta presión. Para esto se utilizó una columna Vydac C18 de fase reversa, la cual se eluyó con un gradiente lineal de 0 a 60 % de solución B en 60 min., a una velocidad de flujo de 2 ml/min y una sensibilidad de 1. La fase movil A consistió de TFA 0.12 % y la fase movil B fue acetonitrilo con 1 % de TFA. Para cada péptido se aplicaron 2 mg y se recuperaron:

F-II.13.3		F-II.13.4	
Pico 1 (39:47)	423 μg	Pico 1 (36:80)	422 μg
Pico 2 (40:99)	221 μg	Pico 2 (38:94)	200 μg
Valle entre 1 y 2	209 μg		

(ver Fig. 10: (A) F-II.13.3 y (B) F-II.13.4).

V. Caracterización Bioquímica de las Toxinas II.13.3 y II.13.4 de *C. noxius.*

Los componentes así obtenidos fueron caracterizados químicamente. Iniciamos dicha caracterización con los dos componentes mayoritarios de la F.II.13.3 obtenidos en CLAP. Ambos componentes fueron secados para eliminar el TFA y posteriormente resuspendidos en agua, se determinó la concentración de proteína por absorbencia a 280 nm, y se aplicaron de 5 a 15 μg de las muestras puras intraperitonealmente a ratones albinos de 18 a 20 gr de peso corporal. Se encontró que ambos componentes son letales y parte de la muestra se usó para la determinación de la estructura primaria.



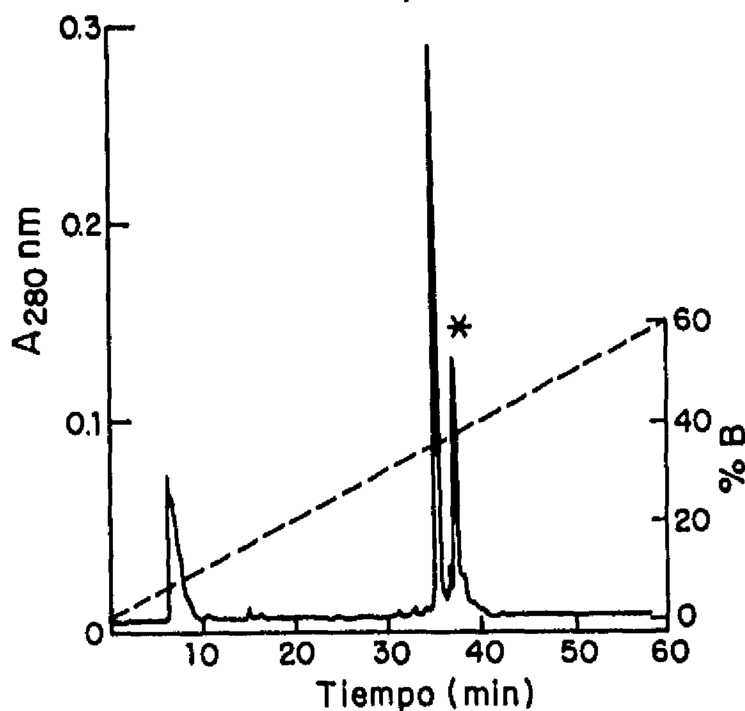


Fig. 10: Purificación de las Fracciones II.13.3 y II.13.4 de *Centruroides noxius* por Cromatografía Líquida de Alta Presión (CLAP).

Las fracciones II.13.3 y II.13.4 fueron purificadas en una columna de fase reversa C18 Vidac equilibrada con una solución de TFA al 0.12 % en agua. La columna se eluyó con un gradiente lineal de 0-60 % de B (TFA 0.1 % en acetonitrilo) en 60 min a una velocidad de flujo de 2 ml/min. (A) F-II.13.3, (B) F-II.13.4, Los asteriscos indican la fracción que corresponde al péptido de interés, confirmado por secuencia.

Esta muestra se redujo y carboximetiló y se puso en un secuenciador Milligen utilizando una membrana Sequelon para anclar la toxina y poder ser sometida a una degradación automática de Edman. Los derivados fenil-tio-hidantoinados de los aminoácidos (PTH-aminoácidos) fueron determinados por CLAP. Para poder determinar la totalidad de la molécula fue necesario someter a digestion enzimática otra parte de la muestra, utilizando proteasa V8 de *Staphilococcus aureus*. Lo mismo se hizo con la F-II.13.4 y los dos componentes obtenidos por CLAP se utilizaron para secuencia, pero éstos sólo fueron sometidos a secuenciación directa de Edman. De esta manera se logró determinar la secuencia casi completa de la F-II.13.3 y los N-terminal de los tres componentes restantes. Comparando las secuencias obtenidas se observó que la fracción minoritaria de la CLAP de la F.II.13.4 (componente 2), es la fracción minoritaria de la CLAP de la F.II.13.3 (componente 2), ya que por lo menos hasta el aminoácido No. 30 no existe diferencia entre uno y otro. Esto sugiere que las F.II.13.3 y II.13.4 de la cromatografía de intercambio iónico a pH 6 se encuentran contaminadas la una con la otra, pero no será hasta tener las secuencias completas de los 3 componentes restantes que esto se podrá afirmar categóricamente ó discernir si se trata de 4 toxinas diferentes con una alta homología.

En la tabla No. 6 se presentan las secuencias de aminoácidos de las toxinas II.13.3 y II.13.4 de *C. noxius*, comparandas con las secuencias de las toxinas 2 y 3 también de *C. noxius*, así como la de la toxina 1 de *Centruroides limpidus limpidus*.

	1 10 20 30 40 50 60	%
C.l.l 1	KEGYIVNLSTG C KYE C YKLGDNDY C LRE C KQQYGKGAGGY C YAFG C W C THLYEQAVVWPLPKKT C T	100
Cn 2	KEGYLVDKNTG C KYE C LKLGDNDY C LRE C KQQGYKGAGGY C YAFA C W C THLYEQAIVWPLPNKR C T	82
Cn 3	KEGYLVELGTG C KYE C FKLGDNDY C LRE C KARYGKGAGGY C YAFG C W C TQLYEQAVVWPLKNKT C R	85
Cn II.13.3	KEGYIVNYHDG C KYE C YKLGDNDY Y LRE C K	
Cn II.13.4	KEGYIVNYYTG C KFA	

TABLA No. 6: Comparación de Secuencias de Aminoácidos de la Toxina 1 con otras Toxinas de Alacrán.
En esta tabla se compara la estructura primaria de la toxina 1 de C.l.limpidus con las toxinas 2 y 3 de C. noxius (Zamudio y col.,1992) y con los amino terminales de las toxinas II.13.3 y II.13.4 de C. noxius, caracterizadas en esta tesis. El porcentaje de similitud se anota en el lado derecho de la tabla y se separaron las cisteinas para mejor visualización de su posición.

VI. Marcaje a Alta Radioactividad Especifica de las Toxinas 2 , 3 y II.13.13 de *C. noxius.*

En experimentos independientes se marcaron las toxinas 2, 3, II.13.3 y II.13.4 de *C. noxius* y la toxinal de *C.l.limpidus.* Las toxinas 1 y II.13.4 no incorporaron bien el Iodo, razón por la cual se determinaron sus constantes de afinidad por competencia con las otras toxinas marcadas.

Para marcar las toxinas se utilizó el método descrito por Morrison y Bayse (1970) que se basa en la oxidación del anillo fenólico de la tirosina por lactoperoxidasa en presencia de H_2O_2 o glucosa oxidasa. Las condiciones se detallan en el anexo 1, aquí brevemente se describirá el experimento de marcado de la toxina II.13.3. Un total de 28 μg (4 nmoles) de la toxina II.13.3 se iodinaron por el método de la lactoperosidasa. Después de completada la reacción, la mezcla fue aplicada en una columna de Sephadex G-10 (0.8 x 13 cm) equilibrada y eluida con 100 mM de NaCl en fosfatos de sodio 0.1 M, pH 7.4 a un flujo de 10 ml/hr, colectándose fracciones de 0.7 ml cada una. Los tubos 8, 9, 10 y 11 contenían toda la toxina marcada. La actividad biológica de la F.II.13.3 no se elimina, ya que lotes de ^{125}I-II.13.3 con una radiactividad específica de 50 Ci/mmol inyectados intraperitonealmente a un ratón en dosis comparables a la II.13.3 nativa, matan al animal experimental con síntomas y en un lapso similar a los registrados para dicha toxina. Este procedimiento se aplicó a todas las toxinas marcadas con ^{125}I.

El primer tejido biológico que se utilizó para determinación de pegado fue cerebro tanto de rata como de conejo. Los sinaptosomas se prepararon siguiendo el método descrito por Catterall y col. (1989), y la concentración de proteína se determinó por el método de Lowry. En

general, la reacción se inicia al adicionar los sinaptosomas (66 µg) a una mezcla de reacción que conteniene la toxina marcada y otros efectores, como se indica en los pies de figura de cada experimento, el volumen final de reacción para cada condición experimental fue de 500 µl de medio de incubación. Las muestras fueron mezcladas e incubadas por 30 min a temperatura ambiente, y la reacción se detuvo por adición de 5 ml del medio de incubación frio. Los sinaptosomas fueron inmeditamente colectados en filtros de fibra de vidrio (Whatman GF/B) con presión negativa y lavados 3 veces con el mismo medio frio. Los filtros se colocaron en tubos de ensaye y la readioactividad se determinó en un contador-gamma. El tiempo que toma detener la reacción es cerca de 10-20 segs, y por experimentos hechos por Valdivia y col. (comunicación personal) sabíamos que la reacción se completa antes de los 30 min, a temperatura ambiente, a las concentraciones mas bajas de toxina estudiada y que el complejo toxina-receptor se disociaba con un tiempo medio de 1 a 2 min (Ray y col. 1978), estas condiciones permiten realizar los experimentos de pegado al equilibrio.

La toxina marcada con iodo radiactivo que se utilizó como control fue la toxina 2 de *C. noxius* (anexo 1), ya que por los reportes previos sabíamos que tenía acción sobre canales de sodio. Los primeros experimentos que se hicieron, después de determinar la constante de afinidad (K_D) de la toxina 2, fue un rastreo de aquellas toxinas que tuvieran una actividad en el mismo sitio receptor que el de la toxina 2, no importando que dichas toxinas no estuviesen totalmente purificadas. Para lo cual los sinaptosomas fueron incubados con 10 nM de toxina marcada y se agregó una concentración de 1000 veces más de toxina fria. Si las

toxinas utilizadas como competidores comparten el mismo sitio receptor que el de la toxina marcada estos sitios deben ser ocupados por dichas toxinas, resultando en un decremento del pegado de la toxina marcada. En la tabla No. 7 se resume el porcentaje (%) de inhibición de las diferentes toxinas aisladas de los venenos de *Centruroides limpidus limpidus* y *Centrurides noxius*. Las toxinas C.l.l II.10.9 -12 (marcadas con un asterisco), son toxinas cuya acción biológica es sobre canales de K^+, ya que todas ellas dán reacción cruzada con los anticuerpos monoclonales (mAb) anti NTX, y algunas de ellas ya han sido caracterizadas bioquímicamente (anexo 2A). Las toxinas C.l.l-II.11.4.2, C.l.l-II.11.5 y C.l.l II.12 dán reacción cruzada con mAb anti NTX, pero también inhiben el pegado de la toxina 2 a sinaptosomas de cerebro de rata. Esto podría explicarse de dos maneras, o se encuentra una mezcla de toxinas de ambas familias, o existe un epítope conservado en ambos tipos de moléculas. La toxina C.l.l II.13.4 es una toxina contra crustaceos, cuya secuencia primaria es homóloga a las toxinas de tipo β o bloqueadoras de canales de Na^+, la cual muestra un reconocimiento pobre del receptor. Con estos antecedentes se trató de obtener el mayor número de toxinas en forma homogenea para poder continuar con el estudio de pegado. La siguiente toxina que se marcó fue la toxina 3 de *Centruroides noxius*, y se determinó su curva de saturación variando la concentración de ^{125}I- toxina 3 en el rango de 3 a 100 nM. El pegado inespecífico se determinó incubando concentraciones crecientes de toxina marcada en presencia de un exceso de toxina fria (10 µM) y el total se midió incubando concentraciones crecientes de toxina marcada. El pegado específico se determinó como la diferencia entre las dos curvas. En la Fig. 11 se muestra la curva de saturación para esta toxina y la K_D

TABLA # 7

PEGADO ESPECIFICO DE ^{125}I-TOXINA 2 EN PRESENCIA DE OTRAS TOXINAS DE ALACRAN.

Toxina	Concentración (M)	Unión de ^{125}I-Toxina 2 (%)
Buffer		100
Toxina 2	5×10^{-6}	22
C.l.l II.8.4 (1)	10×10^{-6}	21
C.l.l II.8.4 (2)	"	23
C.l.l II.8.4 (3)	"	41
C.l.l II.9.4.2 (1)	"	21
C.l.l II.9.4.2 (2)	"	19
C.l.l II.9.4.2 (3)	"	18
C.l.l II.10.5	"	17
C.l.l II.10.9	"	67
C.l.l II.10.10	"	74
C.l.l II.10.11	"	72
C.l.l II.10.12	"	58
C.l.l II.11.4.2	"	23
C.l.l II.11.5	"	25
C.l.l II.12		24
C.l.l II.13.2.4	"	42
C.n II.13.3 (1)	"	17
C.n II.13.3 (2	"	8

La concentración de receptor (sinaptosomas de cerebro de rata) fue de 66 μg/tubo.
El control representa el pegado total de 10 nM ^{125}I-toxina 2 a sinaptosomas.

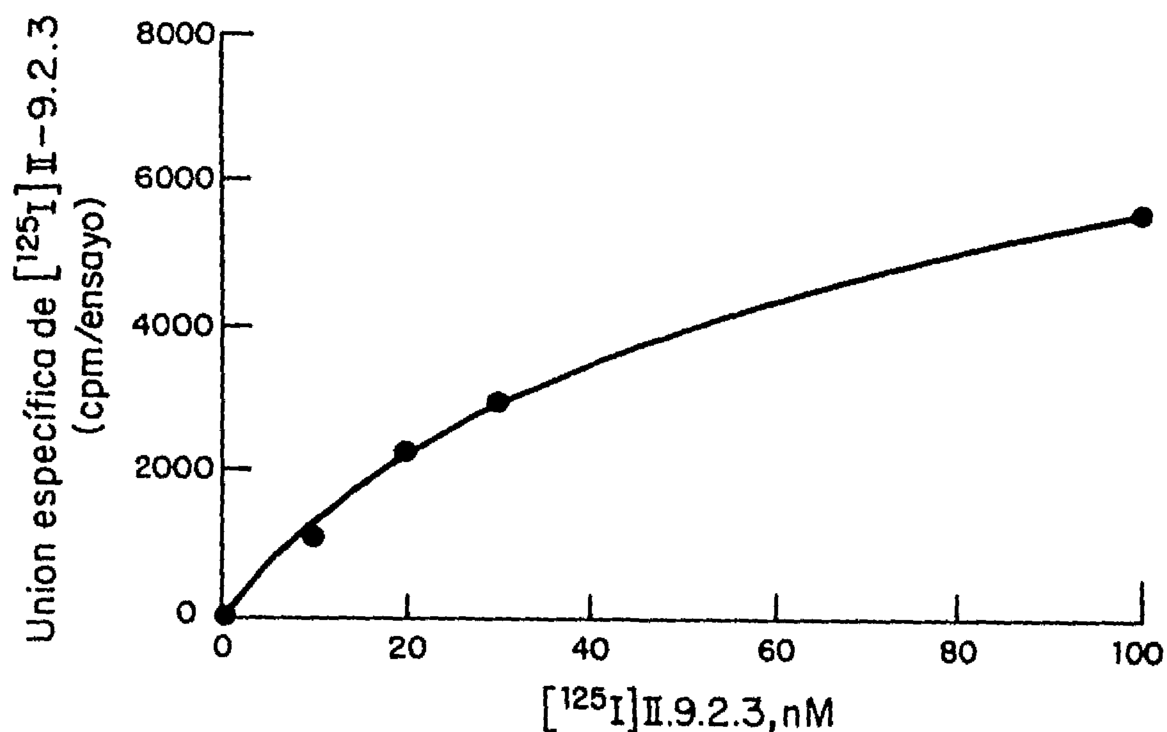


Fig. 11: Pegado Específico de la ^{125}I-Toxina 3 a Sinaptosomas de Cerebro de Conejo.

60 µg de sinaptosomas se incubaron en presencia de concentraciones crecientes de toxina 3 de *C. noxius* marcada con ^{125}I (0.5 ml volumen final). El medio de incubación fue: NaCL 140 mM, KCl 5 mM, Cl_2Ca 1.5 mM, $MgCl_2$ 0.8 mM, Tris/HCl 20 mM, pH 7.4 y albumina sérica bovina 0.1 %. La reacción se llevo a cabo a temperatura ambiente por 60 min y al final de este tiempo las mezclas se filtraron con presión negativa a través de filtros Whatman GF/B, los cuales se leyeron en un contador gamma. La K_D determinada para la ^{125}I-toxina 3 en sinaptosomas de cerebro de conejo es de 63 nM.

determinada en sinaptosomas de cerebro de rata fue de 63 nM. La otra toxina que logró incorporar cantidades adecuadas de ^{125}I fue la toxina II.13.3 de *C. noxius*. La curva de saturación para esta toxina en sinaptosomas de cerebro de rata se muestra en la fig. 12 y la K_D determinada fue de 2.0 nM.

Otro de los tejidos en los cuales se probó si existian sitios de pegado para las toxinas fue en músculo esquelético. Estas mebranas se prepararon siguiendo el método de Valdivia y col. (1991). Las K_D determinadas para las toxinas II.13.3 y toxina 3 de *C. noxius* fue de 55 nM y 1.0 nM respectivamente (Figs. 13 y 14). Dado que la toxina II.13.3 de *C. noxius* mostraba una afinidad mayor por su receptor se decidió probar un conjunto de toxinas totalmente purificadas para determinar si competían por el mismo sitio y con que afinidad, y se encontró que sólo la toxina II.13.4 de *C. noxius* presenta una afinidad mayor por el receptor y que el resto de las toxinas tienen una afinidad similar a la de la toxina II.113.3.

Las toxinas ^{125}I-2, ^{125}I-3 y ^{125}I-II.13.3 de *Centruroides noxius* fueron probadas, por último, en membranas de corazón. Los datos obtenidos con este tejido son preliminares ya que no se contaba con toxina suficiente para terminar el estudio de pegado. Estos datos mostraron que las toxinas ^{125}I-3 y ^{125}I-II.13.3 reconocen un sitio de pegado en estas membranas pero de baja afinidad, ya que el número de cuentas es muy bajo, mientras que la toxina ^{125}I-2 no mostró datos claros con respecto a una curva de saturación en este tejido. Asi pués, para poder afirmar que las toxinas reconocen al canal de Na^+ insensible a TTx cardiaco con una afinidad menor, o que están reconociendo otro tipo de canal de sodio que se encuentre en menor proporción en este tejido son necesarios un mayor

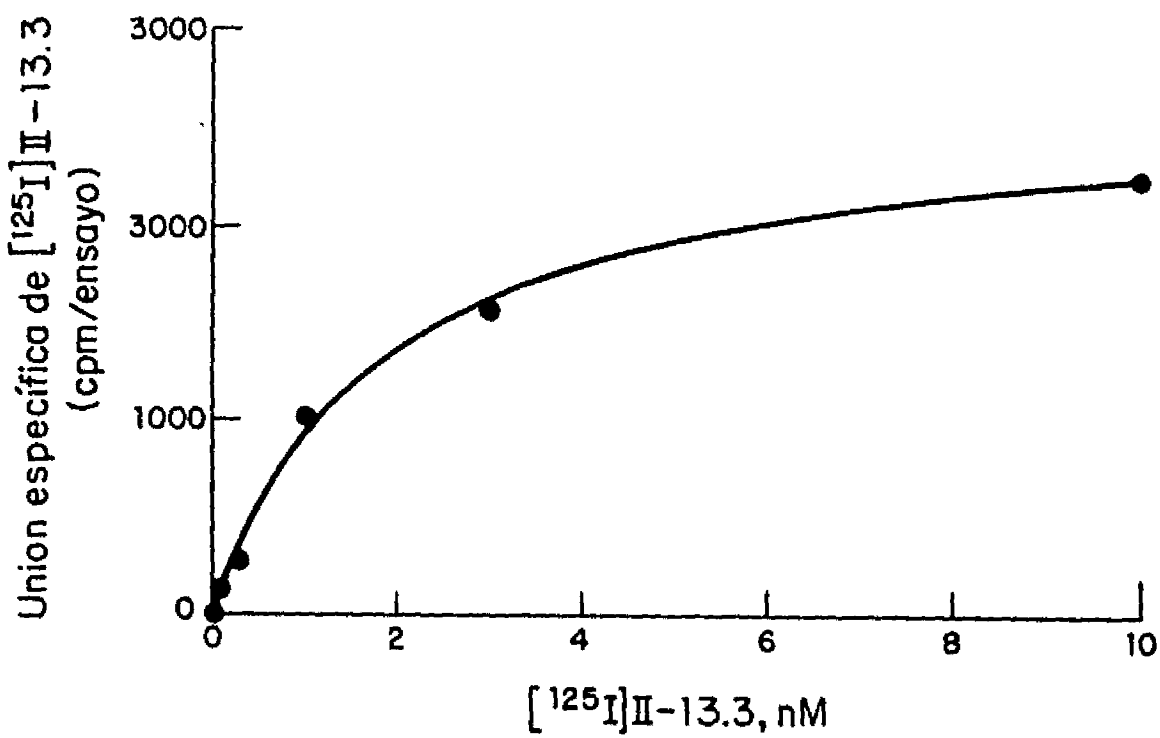


Fig. 12: Pegado Especifíco de la ^{125}I-II.13.3 de *C. noxius* a Sinaptosomas de Cerebro de Conejo.

60 µg de sinaptosomas de cerebro de conejo se utilizaron en los experimentos para la determinación de la curva de saturación de la toxina ^{125}I-II.13.3. Las condiciones experimentales fueron las mismas que en la Fig. 11 y la K_D calculada para la toxina ^{125}I-II.13.3 fue de 1.94 nM.

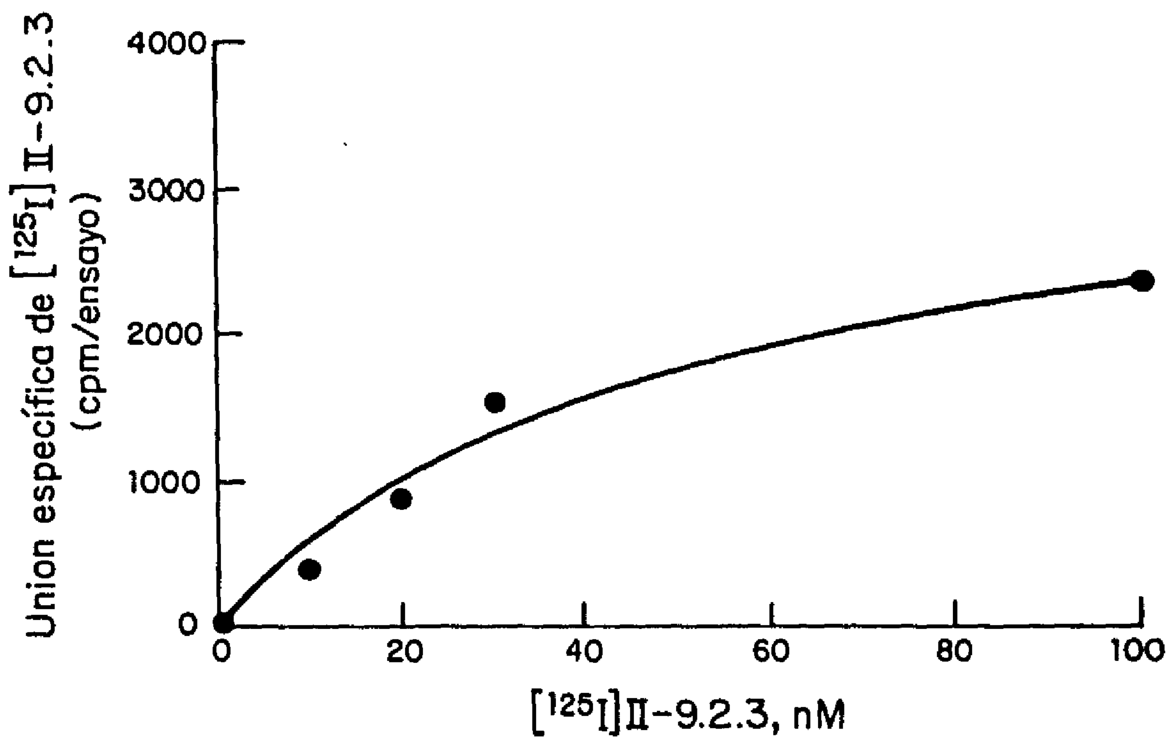


Fig. 13: Pegado Específico de la ^{125}I-Toxina 3 a Vesículas de Sarcolema de Músculo Esquelético de Conejo.

La ^{125}I-toxina 3 se incubó con vesículas de sarcolema de músculo esquelético (60 μg) por 1 hr a temperatura ambiente. El medio de incubación, las condiciones y el procedimiento experimental fué el mismo que en la Fig. 11. La K_D determinada para esta toxina en músculo esquelético fue de 55 nM.

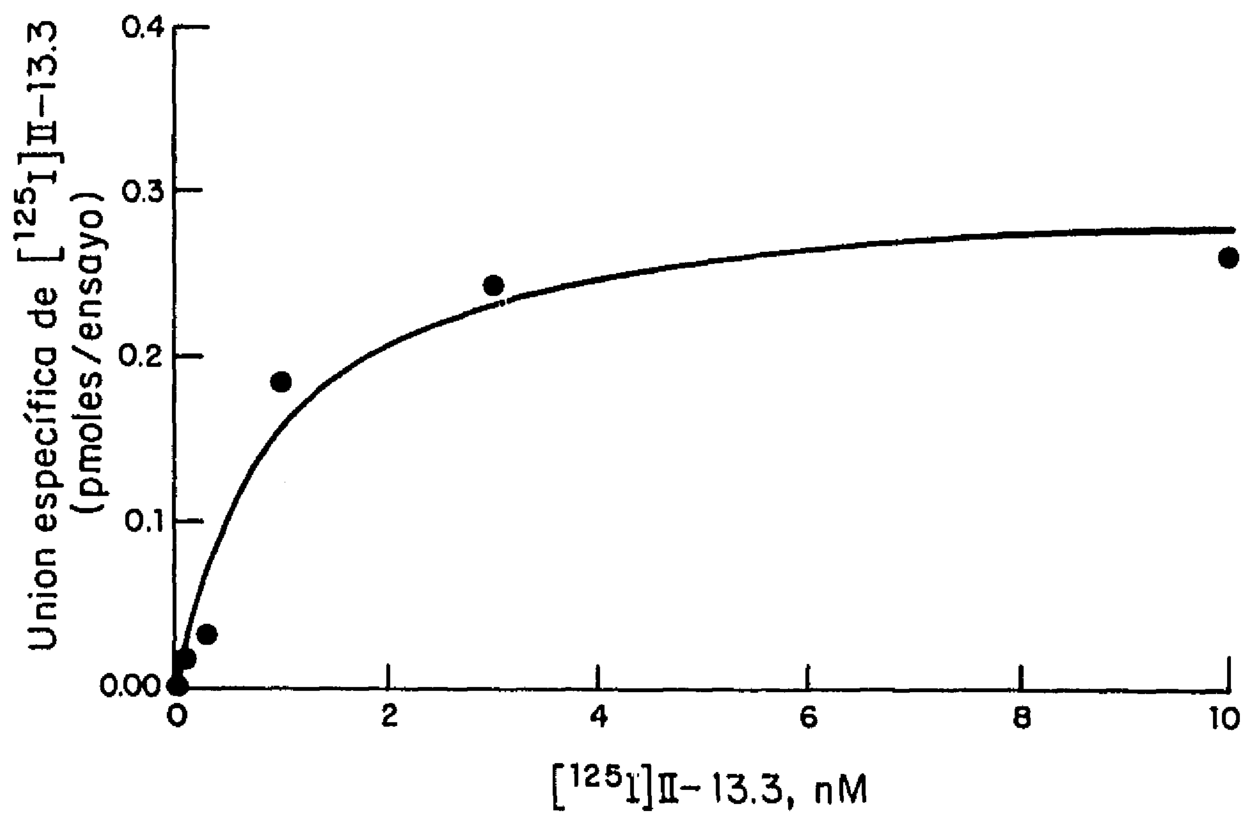
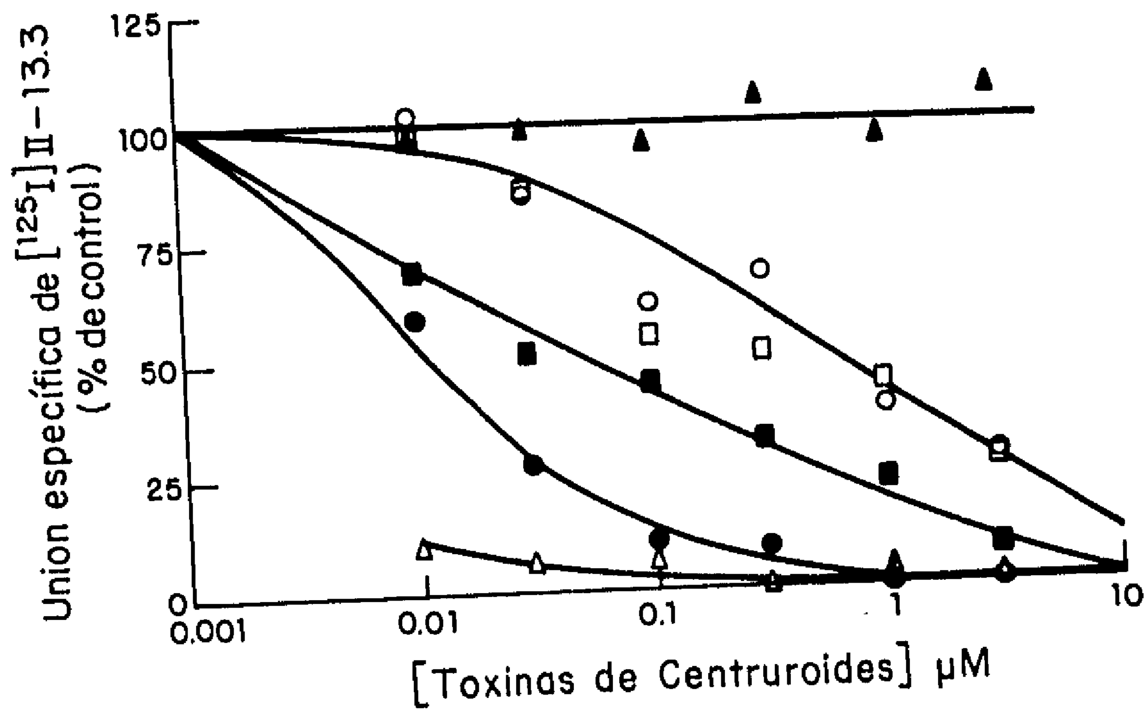
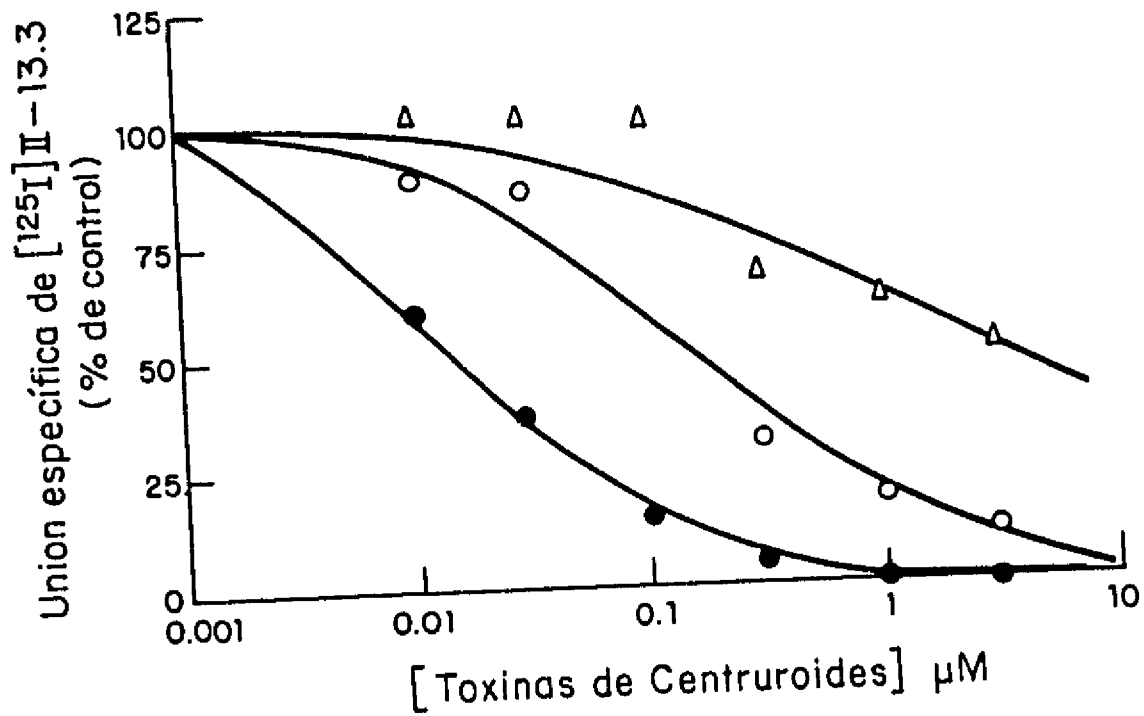


Fig. 14: Pegado Específico de la ^{125}I-II.13.3 de *C. noxius* a Vesículas de Sarcolema de Músculo Esquelético de Conejo.

La misma concentración de membranas de músculo esquelético, asi como las mismas condiciones experimentales que fueron utilizadas en la Fig. 13 se utilizaron para llevar a cabo el cálculo de la K_D de la toxina ^{125}I-II.13.3. La constante de afinidad determinada para esta toxina fue de 0.97 nM para músculo esquelético.



Union específica de [125I]II−13.3
(% de control)
125
100
75
50
25
0
0.001
0.01
0.1
1
10
[Toxinas de Centruroides] μM



Union específica de [125I]II−13.3
(% de control)
125
100
75
50
25
0
0.001
0.01
0.1
1
10
[Toxinas de Centruroides] μM

Fig. 15: Desplazamiento de ^{125}I-13.3 por Diferentes Toxinas Aisladas de los Venenos de *C. noxius y C. limpidus limpidus* en Vesículas de Sarcolema de Músculo Esquelético de conejo.

Las vesículas de sarcolema de músculo esquelético (60 μg) fueron incubados con 3 nM ^{125}I-II.13.3 más las concentraciones indicadas de las siguientes toxinas:

A) ● *C. noxius* II.13.3 (tesis)
- ■ *C. noxius* II.13.3 (39:47), primer componente de CLAP
- △ *C. noxius* II.13.4 (38:94), segundo componente de CLAP
- □ *C. noxius* II.9.2.2 (Toxina 2 de *C. noxius*)
- ○ *C.l.limpidus* II.8.4 (anexo 1)
- ▲ *C.l.limpidus* II.10.11 (anexo 2A)

B) ○ *C.l. limpidus* II.8.4 (45:76)
- △ *C.l. limpidus* II.9.2.3
- ● *C. noxius* II.13.4 (tesis)

por 60 min a temeperatura ambiente. La toxina II.10.11 de *C.l.limpidus* (ver anexo 2A) es una toxina bloqueadora de canales de K^+, que fue usada como control.

número de experimentos de pegado.

CONCLUSIONES

Tomando en cuenta los resultados de este trabajo y dentro del marco teórico existente, se puede concluir que:

- Se aislaron 2 toxinas a partir del veneno del *Centruroides noxius* y una del veneno de *Centruroides limpidus limpidus*, las tres con actividad sobre la función de los canales de Na^+, y se determinó la existencia de por lo menos 5 toxinas con acción biológica sobre los canales de K^+.
- Se determinó la constante de afinidad (K_D) de la toxina 2 de *C. noxius* en sinaptosomas de cerebro de rata (10 nM).
- Se determinó la K_D de las toxinas 3 y II.13.3 de *C. noxius* en sinaptosomas de cerebro de conejo (63 nM y 2 nM respectivamente), mientras que la K_D de estas mismas toxinas en membranas de músculo esquelético fué de 55 nM y 1.0 nM respectivamente. Como puede observarse la afinidad que cada una estas toxinas tienen por los receptores de músculo esquelético y cerebro es muy similar, y comparando las K_D entre ellas, la diferencia es un orden de magnitud, lo cual no es una diferencia substancial.
- Este par de toxinas reconocen un sitio de pegado en membranas de músculo cardiaco, pero no se pudo determinar si es un sitio de baja afinidad o un receptor que se encuentra en baja concentración en estas membranas.
- Los experimentos de desplazamiento por competencia de las toxinas de *Centruroides* en membranas de músculo esquelético mostraron que, a excepción de la toxina II.13.4 de *C. noxius*, el resto de ellas tienen una afinidad similar a la de la toxina II.13.3 de *C. noxius*. Dado que estas

toxinas compiten por el mismo sitio receptor que la toxina 2 de *C. noxius* pude considerarse que todas ellas pertenecen a la familia de las β-toxinas.
- La toxina II.13.4 de *C. noxius* cuya afinidad en músculo esquelético es en el rango de picomolar, es un buen candidato para ser utilizada en el estudio de la determinación de la región o regiones del canal involucrada(s) en la formación del sitio receptor para las toxinas de tipo β, la(s) cual(es) tambien debe(n) estar involucrada(s) en el disparo o activación del canal.

PERSPECTIVAS FUTURAS

Dentro de las perspectivas futuras que se abren con la terminación de esta fase del proyecto y algunas de las cuales ya se estan llevando a cabo son:

1. La terminación de las secuencias faltantes de las toxinas reportadas en esta tesis, tanto de las que tienen acción biológica en canales de Na^+ como de las que tienen actividad sobre canales de K^+.
2. Verificación por Resonancia Magnética Nuclear (RMN) de la estructura tridimencional de estas toxinas, o utilización de los parametros de difracción de rayos X de la V3 del *Centruroides sculpturatus* para comparar el plegamiento de aquellas toxinas vs esta y definir diferencias estructurales entre ellas.
3. La clonación de los genes que codifican para estas toxinas (Becerril y col., 1992) de tal forma que el estudio de relación de estructura-función pueda llevarse a cabo por mutaciones puntuales de las moléculas.
4. La utilización de la toxina II.13.4 de *C. noxius* en la determinación del sitio de pegado de las β-toxinas en el canal de sodio dependiente de voltaje.

5. La expresión de canales iónicos bien caracterizados, en ovocitos de *Xenopus laevis* , para determinar la acción biológica de estas toxinas en una sola población de canales por la técnica de fijación voltaje por 2 electrodos.

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ANEXO 1

"ISOLATION AND CHARACTERIZATION OF A NOVEL TOXIN FROM THE VENOM OF THE SCORPION *CENTRUROIDES LIMPIDUS LIMPIDUS* KARSCH".

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ISOLATION AND CHARACTERIZATION OF A NOVEL TOXIN FROM THE VENOM OF THE SCORPION *CENTRUROIDES LIMPIDUS LIMPIDUS* KARSCH

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A. N. RAMÍREZ, B. M. MARTIN, G. B. GURROLA and L. D. POSSANI. Isolation and characterization of a novel toxin from the venom of the scorpion *Centruroides limpidus limpidus* Karsch. *Toxicon* **32**, 479–490, 1994. — A novel peptide, toxic to mice, was purified from the venom of the Mexican scorpion *Centruroides limpidus limpidus*, by means of gel filtration and ion exchange chromatography, followed by high performance liquid chromatography (HPLC). The complete amino acid sequence was determined by automatic Edman degradation of reduced and alkylated toxin, and by overlapping sequences of fragments of the toxin, generated by cleavage with proteinase V8 separated by HPLC. This toxin is composed of 66 amino acid residues, contains eight half-cystine residues, and is highly similar (91%) to the amino acid sequence deduced for toxin 1 of *C. limpidus tecomanus* and toxin 4 from *C. noxius* venom (89%). This peptide displaces the binding of radiolabeled toxin 2 of *C. noxius* from synaptosomal membranes of rat brain with superimposable kinetics, supporting the conclusion that it belongs to the β-scorpion toxin class. Further characterization of *C. l. limpidus* toxin 1, as we have named it, was performed by means of competition experiments with monoclonal antibodies and various purified scorpion toxins, using an ELISA assay. A panel of six distinct monoclonal antibodies (mAB) against toxin 2 and 3 of *C. noxius* was used. From these, only three clones, originally named BCF1, BCF8 and BCF9, were able to recognize toxin 1 from *C. l. limpidus*.

INTRODUCTION

TOXINS isolated from natural sources such as plants, sea anemones, spiders, gastropods (*Conus geographus*) and scorpion venoms have proven to be useful tools for studying the structural differences among various types of sodium channels, and evaluating their physiological contribution to cellular function (CATTERALL, 1980; OLIVERA *et al.*, 1985; MOCZYDLOWSKI *et al.*, 1988; CASTLE *et al.*, 1989). From the above-mentioned sources, scorpion toxins have been one of the most valuable tools (CATTERALL, 1976; ROCHAT *et al.*,

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1979; MEVES et al., 1986). The scorpion Na^+ channel toxins have been classified into two groups, those that affect the inactivation mechanism of the channels (α-scorpion toxins) and those that modify the activation mechanism (β-scorpion toxins) (JOVER et al., 1980; MEVES et al., 1986). Furthermore, it has been shown that among scorpion toxins that bind Na^+ channels there is a marked species specificity. There are toxins specific for mammals, insects or crustaceans (BABIN et al., 1974; ZLOTKIN et al., 1975, 1991; DE LIMA et al., 1986; LORET et al., 1990). There are toxins that cause depression or excitation on experimental animals (ZLOTKIN et al., 1991). Comparison of the primary structure (POSSANI, 1984), physiological data (MEVES et al., 1986) and three-dimensional structure either by X-ray diffraction (FONTECILLA-CAMPS et al., 1988) or by computational analysis (MENEZ et al., 1992) have shown interesting results. The general molecular architecture of the various classes of scorpion toxin seems to follow the same pattern, showing a well-defined and conserved motif for alpha-helix and three-stranded beta sheet arrangement, which forms a dense core maintained by three or four well-conserved disulfide bridges. A general characteristic is also the presence of two highly variable protruding loops and relatively loose N- and C-terminal regions (MENEZ et al., 1992). Thus, fractional differences among these toxic polypeptides, such as α- and β-scorpion toxins, or species specificity, must reside on structural variations observed either in their amino acid sequences (highly variable regions) or in the total length of the various peptides. For these reasons, in our opinion, it is worth studying the entire panel of scorpion toxins available, in order to understand the full span of their variability and diversity. In this communication we describe the isolation and the primary structure determination of a novel toxin from the venom of *C. l. limpidus*. We also include results obtained with binding studies of radiolabeled toxins to synaptosomes and some comparative properties using monoclonal antibodies.

MATERIALS AND METHODS

Source of venom

Crude venom was obtained by electrically stimulating the telsons of scorpions collected in the state of Guerrero (Mexico). The animals were anesthetized with carbon dioxide before venom extraction (DENT et al., 1980). The venom was recovered in double-distilled water and centrifuged for 20 min at 15,000 rpm (27,200 × g) in a refrigerated Beckman centrifuge equipped with a JA20 rotor. The supernatant was freeze-dried and stored at −20°C until used.

Materials

Sephadex G-50 (medium), Tris, o-phenylenediamine, Tween 20, bovine serum albumin, choline chloride and lactoperoxidase were obtained from Sigma (St. Louis, MO, U.S.A.). Carboxymethyl-cellulose (CM-32) was from Whatman (Clifton, NJ, U.S.A.). All other chemicals and reagents were analytical grade as described (RAMIREZ et al., 1988). Double distilled water was used throughout this work.

Lethality test

The mouse lethality of various protein fractions was observed after intraperitoneal injection of different amounts of protein (usually from 5 to 100 μg) in 0.1–0.3 ml buffer solutions, into adult 20–25 g mice (strain CD1). Three designations were used to define lethality of the various protein components of the venom. 'Non-toxic' means that the mouse injected did not show symptoms of intoxications, similar to injections of saline or buffer alone. 'Toxic' means that the animal injected showed any of the following symptoms: excitability, salivation, lacrimation, dyspnea, temporary paralysis of limbs, but recovered within 20 hr after injection. 'Lethal' means the animal died after showing some or all of the above symptoms.

Purification procedures

The soluble venom (705 mg) was divided into two aliquots and applied independently to a Sephadex G-50 (medium) column. The toxin containing tubes were pooled (fraction number II) and divided into seven aliquots

and chromatographed separately on a CM-cellulose column, equilibrated and run in 20 mM ammonium acetate buffer at pH 4.7. The toxic fraction II.8 was dialyzed, loaded in a CM-32 column and eluted using a linear gradient of salt from 0 to 0.5 M NaCl in 50 mM potassium phosphate buffer, pH 6.0. Further separation of fraction II.8.4 was performed by high performance liquid chromatography (HPLC). Chromatographic conditions are indicated in the figure legends. Columns were run at room temperature (25°C).

Chemical characterization

A sample of toxin *C. l. limpidus* II.8.4.3, containing 400 μg of peptide was reduced and carboxymethylated (RC-toxin) using the same protocol previously described (MARTIN *et al.*, 1988). A small aliquot (1.0 nmol) of RC-toxin was attached to a Sequelon-AA membrane and sequenced using a Millipore 6625 ProSequencer (Bioanalytical Division).

Enzymatic digestion and peptide separation

Enzymatic cleavage of RC-toxin was conducted by digestion with *Staphylococcus aureus* V8 protease (Miles laboratories, Stoke Poges, Bucks, U.K.) as described previously (POSSANI *et al.*, 1985). The resulting peptides were separated by HPLC and attached to Sequelon-AA membrane for sequence determination. Peptide V8-2 was sequenced after attachment to sequelon-AA on the prosequencer as well as from solution on an Applied Biosytem 470 A gas-phase sequencer.

Preparation of synaptosomes

Synaptosomes were prepared using a similar procedure to that described by CATTERALL *et al.* (1979). Briefly, the brains of six Wistar rats were removed and homogenized in ice-cold 0.32 M sucrose at 10% (w/v) with ten strokes of a motor driven Teflon/glass homogenizer. The resulting homogenate was sedimented at 2500 rev/min (1000 × *g*) for 10 min. The supernatant was saved and the sediment was resuspended in 0.32 M sucrose, homogenized and again sedimented at 2500 rev/min (1000 × *g*) for 10 min. Both supernatants were mixed and centrifuged at 11,500 rev/min (17000 × *g*) for 1 hr and the pellets resuspended in a preparation buffer made of 5 mM Tris-HCl, pH 7.4, with 0.32 M of sucrose. Protein concentration in the synaptosomal preparation was measured by the method of LOWRY *et al.* (1951).

Binding measurements of scorpion toxin

Scorpion toxin 2 from *C. noxius* (ZAMUDIO *et al.*, 1992) was iodinated using the lactoperoxidase method of MORRISON and BAYSE (1970). Scorpion toxin binding was measured using a rapid filtration assay. Synaptosomes were incubated with ^{125}I-labeled scorpion toxin in the binding medium consisting of 140 mM choline chloride, 5 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin. The binding reaction was initiated by addition of brain membranes to a reaction mixture containing scorpion ^{125}I-toxin with or without unlabeled toxin. The samples were mixed and incubated for 60 min at room temperature. The reactions were then stopped by addition of 5 ml of cold binding medium. The membranes were immediately collected on glass fiber filters (Whatman GF/B) under vacuum and washed two times with cold binding medium. The filters were dried and counted in a gamma counter. All values are an average of at least triplicate experiments (see figures).

ELISA assays

ELISA 96-well vinyl-plates (Costar, Cambridge, MA, U.S.A.) were coated by incubating overnight, at 4°C, with *C. l. limpidus* toxin II.8.4.3 (100 μl of a 3 mg/litre solution in 20 mM $NaHCO_3$, pH 9.2). After saturation of the remaining sites with 1% ovalbumin in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.8 (NaCl/Pi), at room temperature for 2 hr, the antibodies were added in the dilution buffer consisting of NaCl/Pi, 0.05% Tween 20, 10% (by volume) horse serum, 1% bovine serum albumin, and incubated in the wells overnight at 4°C. After extensive washing with NaCl/Pi and 0.05% Tween 20, bound antibodies were revealed using peroxidase-labeled rabbit anti-mouse IgG antibodies (4 hr, at room temperature) and the chromogenic substrate (*o*-phenylenediamine 0.4 mg/ml and urea hydroperoxide 0.2 mg/ml in 0.1 M NaH_2PO_4, pH 5.0).

Competition experiments

Centruroides noxius toxin 2 coated plates were prepared as described above. The wells were then filled with 50 μl of inhibitor peptide or venom (at various dilutions) and 50 μl of a suitable concentration of mAb (ZAMUDIO *et al.*, 1992). After overnight incubation at 4°C, bound antibodies were revealed as described above.

RESULTS

Repeated chromatographic separations of *C. l. limpidus* soluble venom in a Sephadex G-50 column, followed by ion exchange chromatography, gave reproducible results

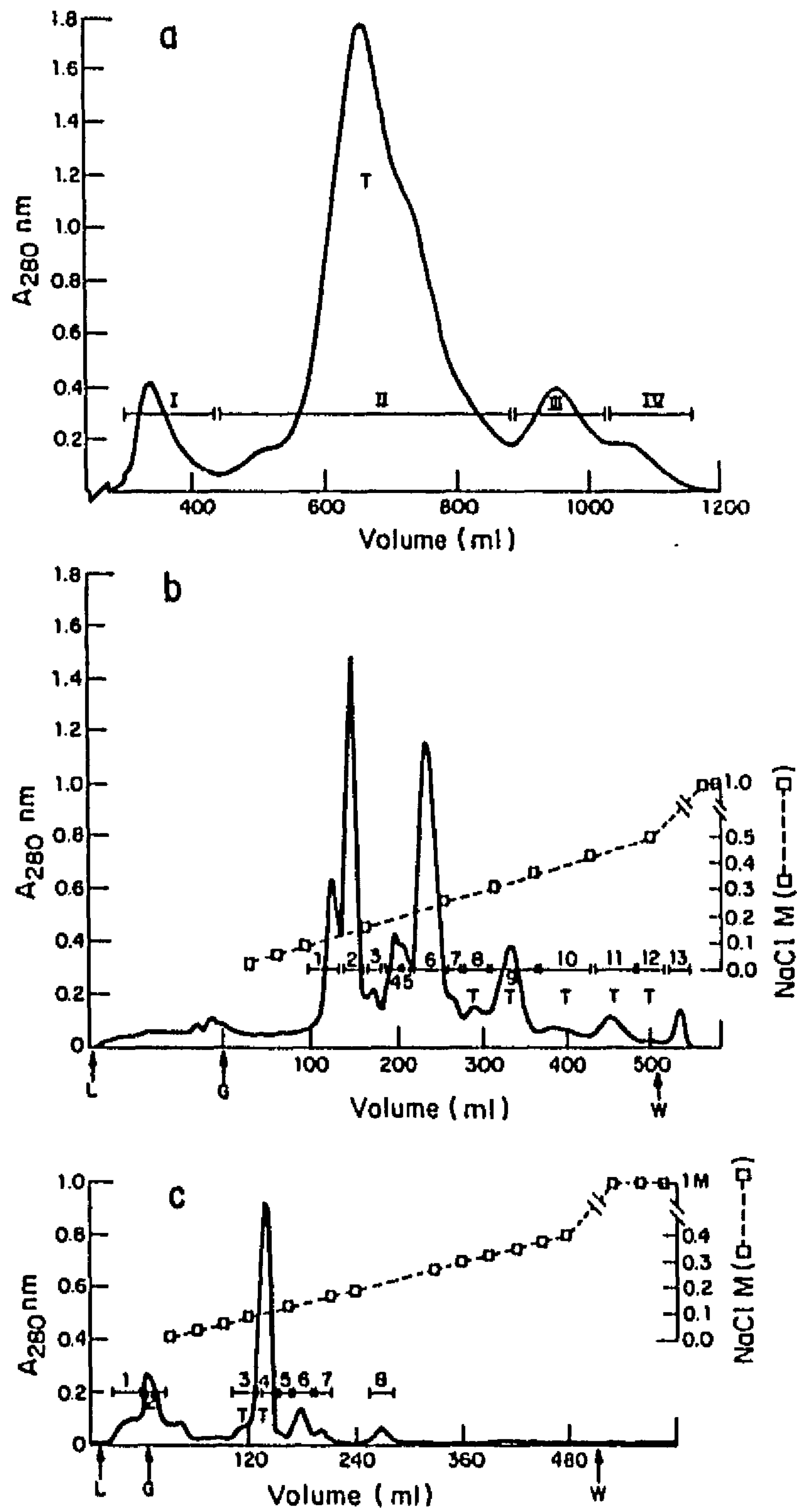

Fig. 1. *Caption on opposite page.*

TABLE I. RECOVERY AND LETHALITY OF CHROMATOGRAPHIC FRACTIONS

Column	Fraction	Protein content (mg)*	Recovery (%)	Lethality†
Sephadex G-50 (Fig. 1a)	soluble venom	394	100.0	lethal
	fraction I	29	7.4	non-toxic
	fraction II	301	76.4	lethal
	fraction III	45	11.4	non-toxic
	fraction IV	19	4.8	non-toxic
	protein recovered		100.0	
CM-cellulose (Fig. 1b)	fraction II	90.2	100.0	lethal
	sub-fraction II.1	9.0	9.9	non-toxic
	sub-fraction II.2	16.2	17.9	non-toxic
	sub-fraction II.3	1.9	2.1	non-toxic
	sub-fraction II.4	6.0	6.7	non-toxic
	sub-fraction II.5	4.1	4.5	non-toxic
	sub-fraction II.6	22.4	24.8	non-toxic
	sub-fraction II.7	2.4	2.7	toxic
	sub-fraction II.8	3.5	3.9	lethal
	sub-fraction II.9	10.8	12.0	lethal
	sub-fraction II.10	3.7	4.1	lethal
	sub-fraction II.11	3.7	4.1	lethal
	sub-fraction II.12	1.4	1.6	lethal
	sub-fraction II.13	2.0	2.2	non-toxic
	protein recovered		96.5	

* The values reported are percentages obtained from the number of absorbancy units at 280 nm, as described in Material and Methods.

† Non-toxic fractions were assayed with samples containing up to 100 μg protein; toxic fractions with protein content about 80 μg per mouse and lethal with as little as 10 μg per animal.

(Fig. 1a, b). The mean value for the total protein recovery in the column of Fig. 1a was quantitative, that of Fig. 1b was 96.5%. In Fig. 1a tubes corresponding to four fractions were pooled as indicated by the horizontal bars. The only toxic fraction (number II), subjected to CM-cellulose, in 20 mM ammonium acetate buffer at pH 4.7, provided 12

FIG. 1. PURIFICATION OF TOXIN II.8.4.

(a) Sephadex G-50 gel filtration of soluble venom. The column (3 × 200 cm) equilibrated with 0.02 M ammonium acetate buffer, pH 4.7, was loaded with 394 mg of *C. l. limpidus* venom and eluted with the same buffer at a flow rate of 88 ml/hr. Tubes containing 5 ml were pooled as shown by the horizontal bars (I to IV) according to the absorbance at 280 nm. Only fraction II was toxic. Fraction I corresponds to 7.4%, fraction II to 76.4%, fraction III to 11.4% and fraction IV to 19% of the material recovered. T means toxic. (b) Ion-exchange separation of fraction II. The toxic fraction from Sephadex G-50 was separated in a CM-cellulose column (0.9 × 33 cm) equilibrated and run with 20 mM acetate buffer, pH 4.7. Fraction II (90.2 mg) was applied to the column and eluted with salt at a flow rate of 30 ml/hr, a linear gradient was formed by mixing 250 ml of buffer in 0 M NaCl with 250 ml of buffer in 0.5 M NaCl, and resolved 12 components. Recovery of the non-toxic fractions was about 68%, while fractions II.7 to II.12 correspond, respectively, to: 2.7%, 3.9%, 12%, 4.1%, 4.1% and 1.6% of the material recovered. Washing (w) the column with 1 M NaCl resolved an additional component. L and G are, respectively, loading the sample and starting point of the gradient. T denotes toxic or lethal fraction. (c) Ion-exchange separation of fraction II.8. Fraction II.8 from Fig. 1b (31.7 mg) was applied to a CM-cellulose column (0.9 × 33 cm) equilibrated in 50 mM potassium phosphate buffer, pH 6.0, and run at a flow rate of 30 ml/hr. A linear gradient of salt from 0 M (250 ml) to 0.38 M NaCl (250 ml) in the equilibration buffer was applied to the column. Fractions of 2.5 ml per tube were collected. The overall recovery was 94.9% and toxin II.8.4 corresponded to 20.2% of total material absorbing at 280 nm. L, G, W and T: as in Fig. 1b.

TABLE 2. RECOVERY AND LETHALITY OF CHROMATOGRAPHIC SUB-FRACTIONS

Column	Sub-fractions	Protein content (mg)*	Recovery (%)	Lethality†
CM-cellulose (Fig. 1c)	F-II.8	31.7	100.0	lethal
	F-II.8.1	3.0	9.5	non-toxic
	F-II.8.2	8.0	25.2	non-toxic
	F-II.8.3	4.2	13.2	lethal
	F-II.8.4	6.4	20.2	lethal
	F-II.8.5	1.4	4.4	non-toxic
	F-II.8.6	3.3	10.4	non-toxic
	F-II.8.7	2.4	7.6	non-toxic
	F-II.8.8	1.4	4.4	non-toxic
	protein recovered		94.9	

* The values reported are percentages obtained from the number of absorbancy units at 280 nm, as described in Material and Methods.

† Non-toxic fractions were assayed with samples containing up to 100 μg protein per animal, while lethal fractions were effective with less than 10 μg per mouse.

distinct components (Fig. 1b), from which II.8 to II.12 were all toxic to mice. Fraction II.8 (3.9% of fraction II) was further chromatographed in CM-cellulose column equilibrated in 50 mM sodium-phosphate buffer, pH 6.0 (Fig. 1c). Tables 1 and 2 summarize the results of recovery and lethality tests obtained with the various chromatographic

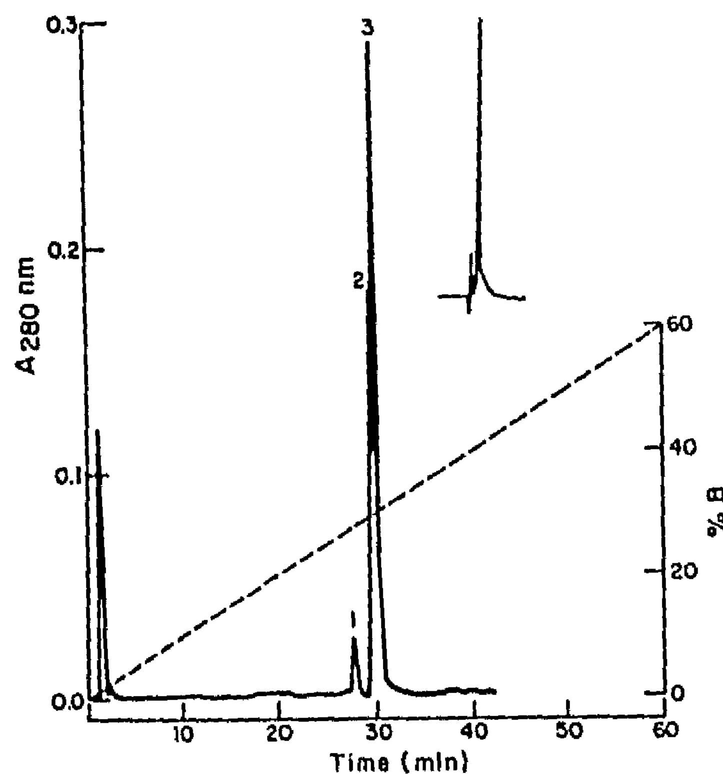

FIG. 2. HPLC SEPARATION OF FRACTION II.8.4.
Peptide II.8.4 from Fig. 1(c) (100 μg) was fractionated in a C8 reverse phase column, with a linear gradient of solution A (0.12% TFA in water) to 60% solution B (0.1% TFA in acetonitrile) during 1 hr. Component number 3 (II.8.4.3) was the only toxic peptide. It elutes as a single component in an isocratic gradient (see inset) and was shown to be homogeneous as demonstrated by polyacrylamide gel electrophoresis and amino acid sequence.

fractions of Fig. 1a–c. Toxic component II.8.4 from Fig. 1c was dialyzed, lyophilized and finally applied to a C8 reverse-phase column for HPLC separation (Fig. 2). The major component eluting at 31.58 min was shown to be homogeneous both by polyacrylamide gel electrophoresis and by rechromatography on reverse-phase HPLC using a step gradient (insert, Fig. 2).

The purified toxin was reduced and alkylated (data not shown). An aliquot (1.0 nmol) of the RC-toxin was loaded and sequenced in a microsequencer (ProSequencer-Millipore) and provided results consistent with the presence of a unique peptide, the sequence of which is shown in Fig. 3. The results of direct Edman degradation allowed unequivocal identification of the first 61 residues, labeled D in Fig. 3. Digestion of 29 nmoles of RC-toxin II.8.4.3 with protease V8 from *Staphylococcus aureus* was conducted in order to obtain fragments of the toxin for complete determination of the primary structure. HPLC separation of the hydrolyzed RC-toxin gave six peptides. The fraction eluted at 35.66 min, when subjected to amino acid sequence analysis gave two sequences, one corresponding to the N-terminal part of the molecule and the other to the C-terminal part. Rechromatography on a narrow-bore C18 (Vydac) provided homogeneous C-terminal peptide (V8-2 in Fig. 3), corresponding to residues 53 to 66. Amino acid analysis of peptide V8-2 gave the following composition: 1 Glu, 0.5 CM-Cys, 2 Thr, 1 Ala, 2 Pro, 1.3 Val, 1 Leu, 2 Lys. Additionally, Trp was not determined by this acid hydrolysis procedure; however, the sequence revealed the tryptophan. Furthermore, the sequence analysis showed the presence of two valines at position 55 and 56, thus the value of 1.3 moles of Val (per peptide) obtained by amino acid analysis confirms the difficulty of hydrolyzing two consecutive bulky amino acids with a 20 hr hydrolyzate. In this manner, the amino acid analysis of the C-terminal fragment revealed the presence of 13 amino acid residues, consistent with the results of Fig. 3. This novel toxin (component II.8.4.3) was named toxin I from *C. l. limpidus*, because it is the first peptide from this scorpion for which the total primary structure is determined. The remaining fractions (II.7 to II.12) of Fig. 1b, toxic to mice, were also rechromatographed in order to characterize their toxins (unpublished). For example, fraction II.9 although a major lethal component, contains several toxins difficult

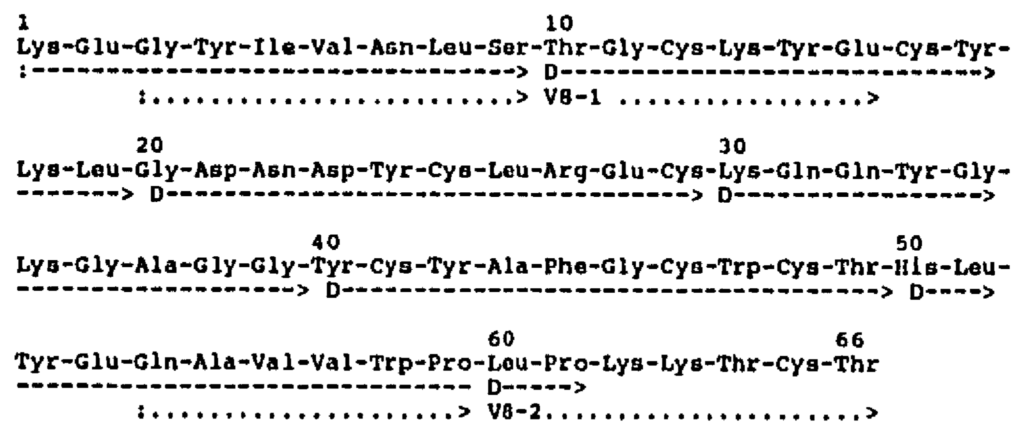

Fig. 3. Amino acid sequence of toxin II.8.4.3 from *C. l. limpidus*.
Residues at positions 1 to 61 were identified by direct sequence of RC-toxin (labeled with :--D-- >). Two additional peptides were sequenced after cleavage with proteinase V8 of *Staphylococcus aureus*, and separation by HPLC. They are labeled :..V8-1.. > and :..V8-2.. >, and correspond to residues numbers 3–15 and 54–66, respectively.

to obtain in pure form, except for a mammalian toxin already described by our group (ALAGON *et al.*, 1988).

Comparison of the primary structure of *C. l. limpidus* toxin 1 with other scorpions of the genus *Centruroides* shows a high degree of similarity with toxin 1 of *C. l. tecomanus* (91%) and toxins 2 and 3 from *C. noxius* (82% and 85%, respectively). Since *C. noxius* toxin 2 (originally called toxin II.9.2.2, ZAMUDIO *et al.*, 1992) was shown to recognize Na^+ channels of rat brain (SITGES *et al.*, 1987), we have decided to test both toxins: *C. noxius* 2 and *C. l. limpidus* 1 in this preparation. We first obtained and labeled *C. noxius* 2 with iodine (^{125}I) because it was the best known toxin, and determined its affinity for rat brain synaptosomes (done for the first time). As shown in Fig. 4a, ^{125}I-*C. noxius* toxin 2 binds specifically to rat brain synaptosomal membranes in a saturable manner. This peptide binds with high affinity (K_D 10 nM) to a single class of relatively low density sites (13 pmoles/mg of membrane protein), as indicated by a linear Scatchard plot (Fig. 4b). In Fig. 5 it can be observed that both *C. noxius* toxin 2 and *C. l. limpidus* toxin 1 can displace, in an overlapping manner, the binding of ^{125}I-*C. noxius* toxin 2 to brain synaptosome membranes, from which we conclude that both toxins must recognize the same site on the Na^+ channels, with very similar affinities.

Futher characterization of *C. l. limpidus* toxin 1 was performed by means of competition experiments with monoclonal antibodies and various purified scorpion toxins, using an ELISA assay. A panel of six distinct monoclonal antibodies against toxin 2 and 3 of

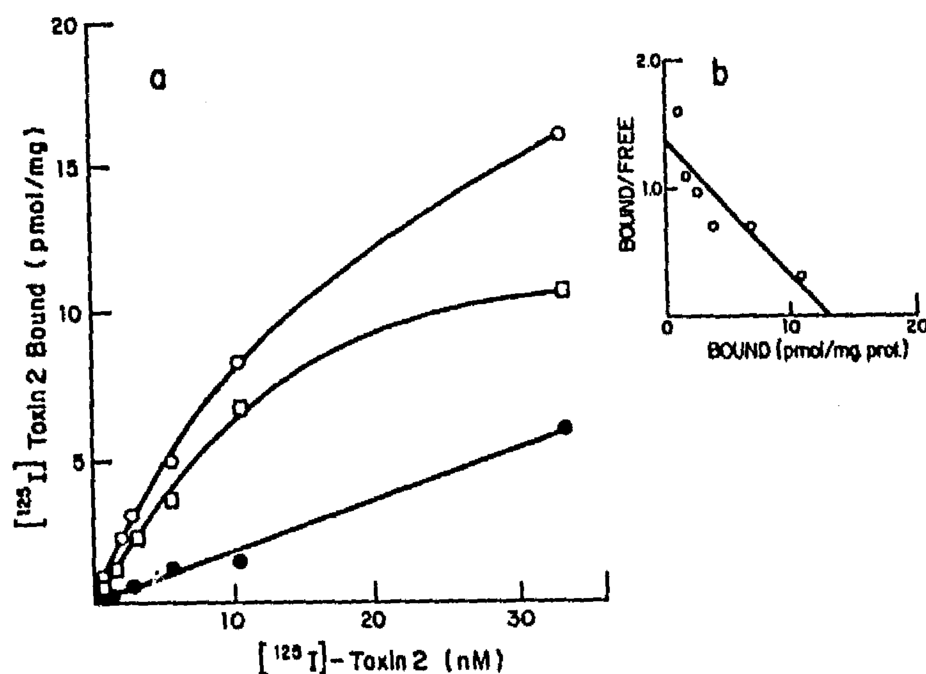

FIG. 4. BINDING OF ^{125}I-TOXIN 2 TO RAT BRAIN SYNAPTOSOMES.
(a) Saturation binding analysis. Synaptosomes (66 μg/ml protein) were incubated with increasing concentrations of ^{125}I-toxin 2 (0.5 ml final volume). The incubation medium consisted of (in mM): 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 0.8 $MgCl_2$, 20 Tris-HCl, pH 7.4, and 0.1% bovine serum albumin. The reaction was carried out at room temperature for 60 min. Total binding (open circles) and nonspecific binding determined in the presence of 1 μM of toxin 2 (filled circles) are represented. Specific binding (open squares) was assessed from the difference between total and nonspecific ligand binding. (b) Analysis of ^{125}I-toxin 2 binding at equilibrium. Specific binding data from (a) are presented in a Scatchard plot.

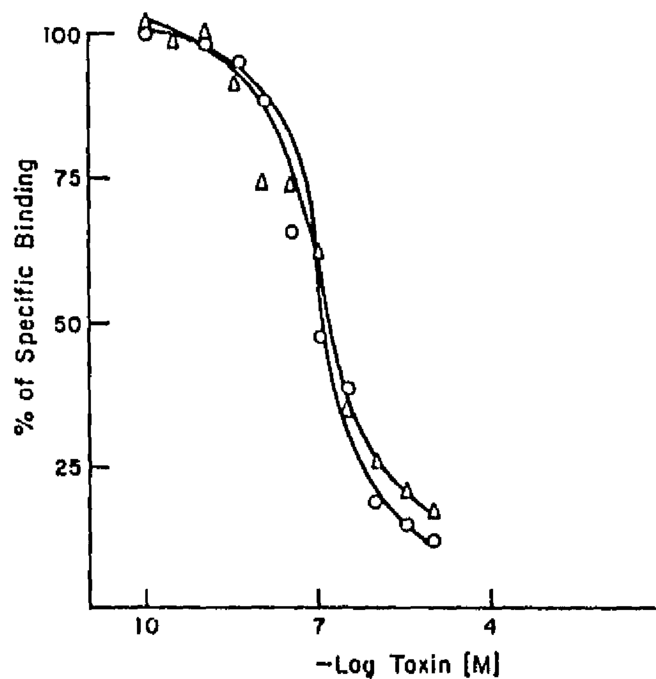

FIG. 5. DISPLACEMENT OF ^{125}I-TOXIN 2 BINDING TO SYNAPTOSOMES.
Displacement experiments were conducted using synaptosomal membranes from rat brain (60 µg protein/ml) incubated with 10 nM of *C. noxius* ^{125}I-toxin 2 at room temperature for 60 min (final volume of 500 µl), in the presence of increasing concentrations of native *C. noxius* toxin 2 (circles) or toxin 1 from *C. limpidus limpidus* (triangles), filtered and counted, as described in Materials and Methods.

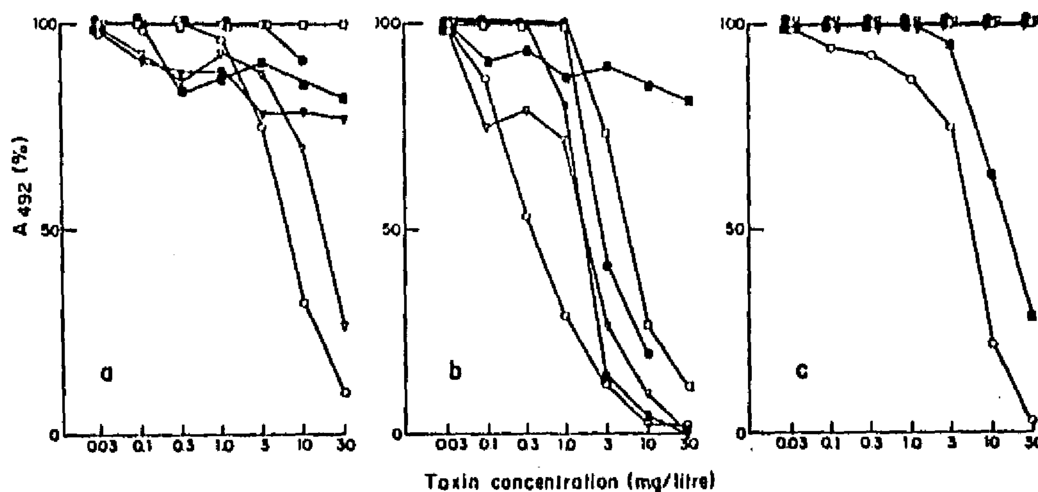

FIG. 6. CROSS-REACTIVITY OF MONOCLONAL ANTIBODIES WITH SCORPION TOXINS.
(a) The binding of mAB (cell line BCF1, at 3 mg/litre concentration), to *C. noxius* toxin 2 coated in ELISA plates, was assayed in the presence of serial dilutions of possible displacing toxins: *C. noxius* 2 (open circles), *C. noxius* II.13.4 (closed triangles), *C. noxius* II.13.3 (closed circles), *C. l. limpidus* I (open triangles), *C. l. limpidus* II.13.2.4 (open squares), and *L. q. quinquestriatus* V (closed squares). Bound antibodies were revealed using rabbit anti-mouse-IgG conjugated with peroxidase in the presence of the chromogenic substrate, as described in Materials and Methods. The 100% binding (control) was in the range of 1.4 units of absorption at 492 nm. Results are means of duplicates. (b) Same as (a), using mAB (cell line BCF8). The control binding (100%) in absence of competing toxins was about 1.0 unit at 492 nm. (c) Same as (a), using BCF 9, for which 100% binding was in the range of 1.0 unit at 492 nm.

C. noxius was used (ZAMUDIO *et al.*, 1992). From these, only three clones, originally named BCF1, BCF8 and BCF9, were able to recognize toxin 1 from *C. l. limpidus*. Figure 6 shows the results obtained using these mAB and six different scorpion toxins: from *C. l. limpidus* (toxin 1 and II.13.2.4); from *C. noxius* (toxin 2, II.13.3 and II.13.4) and from an unrelated species *Leiurus quinquestriatus quinquestriatus* (toxin V). *Centruroides noxius* toxin 2 was bound to the ELISA plates and the binding of the various mAB to this toxin was displaced by toxin 1 of *C. l. limpidus* in Fig. 6a (with mAB BCF1), by all toxins in Fig. 6b (with mAB BCF8) and only by *L. q. quinquestriatus* in Fig. 6c (with mAB BCF9). In all cases the homologous toxin (*C. noxius* 2) was capable of self-displacement, as expected (open circles).

DISCUSSION

In Mexico there are six species of scorpion from the genus *Centruroides* that are important for human health (HOFFMANN, 1936; HOFFMANN and NIETO, 1939). Over 200,000 people are stung every year by these arachnids (DEHESA-DAVILA, 1989). About half of the accidents are due to the species *C. limpidus limpidus* and *C. l. tecomanus*, for which limited information is available on the structure and function relationship of their toxins (RAMIREZ *et al.*, 1988; MARTIN *et al.*, 1988). For *C. l. limpidus* venom there are two publications describing partial characterization of toxic peptides (TATO *et al.*, 1978; ALAGON *et al.*, 1988). We have decided to extend this work by purifying and determining the first complete amino acid sequence for a toxin isolated from *C. l. limpidus*, following the procedure described in the Results. The strategy reported earlier by ALAGON *et al.* (1988) permitted the separation of nine fractions in the second step (chromatography on ion exchange in CM-cellulose), while the slight modifications introduced here allow us to obtain 12 different fractions, of which six are toxic or lethal to mice (Table 1). Furthermore, toxin 1 is different from the mammalian and crustacean toxin already described by ALAGON *et al.* (1988). In our fraction II.9 co-elutes the mammalian toxin (component II-6) purified and partially sequenced by ALAGON *et al.* (1988).

Comparative analysis of this toxin with other related peptides purified from the venoms of *Centruroides* scorpions (Fig. 7) shows that it is 91% similar to toxin 1 from *C. limpidus tecomanus*, and 89% to that of toxin 4 from *C. noxius*. From 82 to 86% similarities were found when compared to other toxins from *C. noxius* and *C. suffusus suffusus* and about 55% with toxins sequenced from *C. sculpturatus* venom. Toxin V, from the Israeli scorpion *L. q. quinquestriatus* displayed only 26% similarity with this peptide. Concerning the functional characteristics of *C. l. limpidus* toxin 1 in brain synaptosomes (Fig. 5), our data suggest that it is a Na^+ channel blocker, which belongs to the β-scorpion toxin class, just like that of toxin 2 from *C. noxius*. Furthermore, the results of Fig. 6 confirm previous work by our group (ZAMUDIO *et al.*, 1992), showing that six different mAB raised against toxins of the scorpion *C. noxius* recognize at least four distinct epitopes on toxins from the genus *Centruroides*. Three of the six mAB did not react with *C. l. limpidus* toxin 1, at the concentrations tested, and the remaining mAB had a distinct affinity for this toxin. Binding of BCF1 to the ELISA plates containing bound *C. noxius* 2 was displaced at similar concentrations of *C. l. limpidus* toxin 1 and the homologous *C. noxius* toxin 2 (Fig. 6a), while the clone BCF9 was not displaced by toxin 1 of *C. l. limpidus* (Fig. 6c). Binding of BCF8 was displaced by all toxintested (Fig. 6b), except *L. q. quinquestriatus* toxin V, an unrelated genus of scorpion. Surprisingly, however, this unrelated toxin V was able to displace the binding of BCF9 to the ELISA plates (Fig. 6c). In our opinion, when more

```
           1        10        20        30        40         50        60     %
Cll 1    KEGYIVNLSTGCKYECYKLGDNDYCLRECKQQYGKGAGGYC+YAFGCWCTHLYEQAVVWPLPKKTCT 100
Clt 1    KEGYLVNHSTGCKYECFKLGDNDYCLRECRQQYGKGAGGYC+YAFGCWCTHLYEQAVVWPLPNKTCS  91
Cn 4     KEGYLVNSYTGCKYECFKLGDNDYCLRECKQQYGKGAGGYC+YAFGCWCTHLYEQAVVWPLKNKTCN  89
Css II   KEGYLVSKSTGCKYECLKLGDNDYCLRECKQQYGKSSGGYC+YAFACWCTHLYEQAVVWPLPNKTCN  86
Cn  3    KEGYLVELGTGCKYECFKLGDHDYCLRECKARYGKGAGGYC+YAFGCWCTQLYEQAVVWPLKNKTCR  85
Cn  2    KEGYLVDKNTGCKYECLKLGDNDYCLRECKQQGYKGAGGYC+YAFACWCTHLYEQAIVWPLPNKRCS  82
CsE V2   KEGYLVNKSTGCKYGCLKLGENEGCDKECKAKNQGGSYGYC+YAFACWCEGLPESTPTYPLPNK-CSS 57
CsE I    KDGYLVEK-TGCKKTCYKLGENDFCNRECKWKHIGGSYGYC+YGFGCYCEGLPDSTQTWPLPNK-CT  56
CsE V3   KEGYLVKKSDGCKYGCLKLGENEGCDTECKAKNQGGSYGYC+YAFACWCEGLPESTPTYPLPNKSC-  55
CsE V1   KEGYLVKKSDGCKYDCFWLGKNEHCNTECKAKNQGGSYGYC+YAFACWCEGLPESTPTYPLPNK-CS  55
Cn  1    KDGYLVDA-KGCKKNCYKLGKNDYCNRECRMKHRGGSYGYC+YGFGCYCEGLSDSTPTWPLTNKTC-  53
Lqq V   LKDGYIVDD-KNCTFFC---GRNAYCNDECKKK-G-GESGYC+YGNACWCYKLPDR-VSIKEKG-RCN  26
                                                QWASP
         K-GY-V---K-C---C---G-N--C--ECK-----G--GYC+Y---CWC--L-------------C-
```

FIG. 7. AMINO ACID SEQUENCE COMPARISON OF TOXIN I WITH OTHER SCORPION TOXINS.
The one-letter code was used to compare the primary structure of toxin I from *C. l. limpidus* (Cll 1) with *C. l. tecomanus* toxin I (Clt 1) from MARTIN *et al.* (1988), *C. noxius* toxin I (Cn 1) from POSSANI *et al.* (1985), *C. noxius* 2 and 3 (Cn 2 and 3) from ZAMUDIO *et al.* (1992), *C. noxius* 4 (Cn 4) from VAZQUEZ *et al.* (1993), *C. suffusus suffusus* II (Css II) from ROCHAT *et al.* (1979). *C. sculpturatus* Ewing (CsE I, VI to V3) revised by BABIN *et al.* (1975) and *L. q. quinquestriatus* (Lqq V) revised by ROCHAT *et al.* (1979). Percentages of similarities are listed on the right of the figure. Artificial gaps (-) were introduced in order to align the cysteinyl amino acids and a plus sign (+) shows the place corresponding to the B-loop of the Old World scorpion toxins (sequence QWASP) which has no corresponding sequence on the New World scorpion toxins (revised by MEVES *et al.*, 1986). The bottom sequence represents the amino acids identical in all the sequences compared, in which the dashes (-) correspond to positions of variable amino acids or gaps.

toxins with different primary structures become available, this approach will be important to unravel the relevant epitopes of *Centruroides* scorpion toxins, and also will certainly contribute to the study of the structure-function relationship of these toxic peptides.

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ANEXO 2

"K+ CHANNEL BLOCKING PEPTIDES ISOLATED FROM THE VENOM OF SCORPIONS".
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K^+ channel blocking peptides isolated from the venom of scorpions

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ABSTRACT

In this communication we report the binding properties of Noxiustoxin (NTX) to brain synaptosome membranes. Scatchard plot of specific binding revealed a single class of non-interacting binding sites with K_d of 300 nM and a Bmax of 2.2 pmoles/mg protein. Apamin, a neurotoxic peptide isolated from honeybee venom, which recognizes K-channels from brain, does not displace the binding of NTX to synaptosomal membranes, but isotopically labelled NTX was displaced 100% by a novel peptide, Leiurotoxin III (LeTX), which we have purified from the venom of *L. quinquestriatus*. The N-terminal amino acid sequence of LeTX III: Gly-Leu-Ile-Asp-Val-Arg-Cys-Tyr-Asp-Ser-Ser-Gln-Cys-Glu, was shown to be similar to that of NTX and Charybdotoxin (ChTX). From the venom of the Brazilian scorpion *Tityus serrulatus* three new low molecular mass peptides (Serrulatoxins) were isolated and shown to affect the Ca-dependent K-channels of skeletal muscle. Using monoclonal antibodies two new NTX-like peptides were purified from the venom of the scorpion *Centruroides limpidus limpidus*. Amino acid sequence of these peptides revealed a significant degree of similarities to that of NTX and ChTX. A comparative figure containing the amino acid sequence of eight peptides purified from different scorpion venoms is presented, showing that the K^+channel blocking toxins belong to a low molecular weight peptide family, with exquisite properties towards K-channels, opening the possibility to dissect the intrincate molecular mechanism of action of one of the most diverse receptors present in excitable membranes: the K^+-channels (Castle *et al.*, 1989; Miller, 1991).

INTRODUCTION

Almost 50 years ago, Del Pozo *et al.* (1944, 1945), working with mexican scorpions reported the action of the venom from the genus *Centruroides* on perypheral nervous system and muscle preparations. In 1968 Koppenhoefer and Schmidt described a drastic decrease on the ionic permeability properties of Na and K channels of the node of Ranvier, caused by application of scorpion venoms. These observations were further confirmed and extended by many other investigators (Cahalan, 1975; Nonner, 1979; Catterall, 1976; Catterall *et al.*, 1979; Couraud *et al.*, 1980). Most of the work was devoted to the Na-channel blocking peptides, major components of scorpion venoms, assumed to be the principal cause of human envenomation due to stings by these arachnids (see reviews by Zlotkin *et*

al., 1978; Rochat et al., 1979; Catterall, 1980; Meves et al., 1986; Lazdunski et al., 1986). The K⁺channel blockade was not studied until 1982, when Carbone et al., described the effect of a purified peptide from the venom of the scorpion *Centruroides noxius* on the squid giant axon. Noxiustoxin (NTX), was the first peptide purified and sequenced (Possani et al., 1982), which was shown to affect the voltage-dependent K-channel of axons. The ever-increasing knowledge on the electrical events of membrane depolarization and the advent of more sophisticated and sensitive electrophysiological techniques to dissect K^+ currents facilitated the isolation and characterization of minor components of several species of scorpion venom, that were responsible for the blockade of K channels. Charybdotoxin (ChTx), a basic polypeptide from the asian scorpion *Leiurus quinquestriatus*, originally described as a blocker of the Ca-dependent K channel (Miller et al. 1985), Leiurotoxin (LeTX), a low-conductance, apamin-sensitive K^+ channel blocker from the same venom (Castle and Strong, 1986, Chicchi et al., 1988, Auguste et al., 1990) and Iberiotoxin (IbTX), a high-conductance Ca-activated K channel from the venom of the scorpion *Buthus tamulus* (Galvez et al., 1990) are additional examples of low molecular weight peptides specific for K^+channels of various excitable tissues.

The purpose of this communication is to report the binding properties of NTX to membranes of brain synaptosomes, to describe new toxic peptides from the venom of the scorpion *L. quinquestriatus* and *Centruroides limpidus limpidus*; and finally ,to describe the isolation of three new small molecular weight components from the venom of the scorpion *Tityus serrulatus*.

MATERIAL AND METHODS

Source of Venoms.

Scorpions from the species *Centruroides noxius* Hoffmann and *Centruroides limpidus limpidus* Karsch were collected in the field (State of Nayarit and Guerrero, Mexico, respectively) and their venoms were obtained in the laboratory by electrical stimulation of anesthetized animals, as described (Dent et al., 1980). Venom from the scorpion *Tityus serrulatus* Lutz and Mello was obtained from the Instituto Butantan (São Paulo, Brazil). Venom from the scorpion *Leiurus quinquestriatus quinquestriatus* was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Prior to fractionation the dried or freeze-dried venoms were resuspended in water and centrifuged for 15 min, at 15,000 x g in a refrigerated Sorvall centrifuge, with an SS34 rotor, in order to remove cellular debris and the opaque and viscous material associated with the venoms.

Chemicals, Solvents and Resins.

Only analytical grade reagents were used throughout the purification and

characterization procedures. Columns were run at room temperature (20 °C). Sephadex G-50 (medium) was from Pharmacia Fine Chemicals (Uppsala, Sweden); CM-cellulose microgranular (CM-32) was from Whatman (Clifton, NJ, USA). The C18 reverse phase column was from Vydac (Hesperia, CA, USA). Reagents for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories, (Richmond, CA, USA). Sequencer-grade reagents were purchased from Beckman Instruments (Palo Alto, CA, USA).

Chromatographic Separations.

From three to five chromatographic steps were usually required to obtain these peptides in homogeneous form. Chromatographic profiles were obtained by UV-monitoring (Uvicord S) on LKB-fraction collector system or by reading at 280 nm in a Beckman DU-50 spectrophotometer. Protein content was calculated by absorbance at 280 nm, assuming an extinction coefficient of $E^{1\%}_{280}$ of 10.0, unless otherwise specified.

a. *Centruroides noxius* venom. This venom was fractionated as described before (Possani et al., 1981a, see review Possani, 1984). Briefly, the soluble venom applied to a Sephadex G-50 column gives three main fractions; only fraction II was toxic to mice. This was further separated by two chromatographic columns in CM-cellulose resins at pH 4.7 and 6.0, respectively. Recoveries were better than 93% (Possani et al., 1981c). Noxiustoxin and component II-10.2 were obtained in pure form by this method.

b. *Centruroides limpidus limpidus* venom. This venom was fractionated by a Sephadex G-50 column, followed by two CM-cellulose chromatographic separations at pH 4.7 and 6.0, respectively, as described for the venom of *C. noxius*. An additional step of high performance liquid chromatography (HPLC) was needed to obtain three peptides similar to NTX (Martin BM, Ramirez AN, Gurrola GB, Nobile M, Magnelli V and Possani LD, paper in preparation).

c. *Tityus serrulatus* venom. This venom was separated through a Sephadex G-50 column, followed by ion-exchange chromatography as described (Possani et al, 1981b). By this procedure a toxic peptide (Ts II-9A) was obtained (Possani et al. 1982). Two additional and independent columns containing CM-cellulose resins, equilibrated and run at pH 6.0, were used to separate three new peptides as described in the section of results below.

d. *Leiurus q. quinquestriatus* venom. This venom was initially separated by Sephadex G-50 gel filtration and two CM-cellulose columns in the same

conditions described for the other venoms, at pH 4.7 and 6.0, respectively. Two additional chromatographic steps, one in Sephadex G-50 with high salt (1M NaCl) and the other by HPLC were necessary to produce toxin Leirutoxin III in pure form, as described below.

Criteria for Purity and Molecular Weight Determination.
The homogeneity of the peptides was confirmed by polyacrylamide gel electrophoresis in two systems: Reisfeld et al. (1962) and Laemmli (1970). The second system contains sodium dodecyl sulfate (SDS) and was used for molecular weight determination. Most peptides were subjected to automatic Edman degradation (Edman & Begg, 1967) in a Beckman 890M microsequencer. Only one band in gel electrophoresis, and a unique amino acid sequence, with high yield, at the N-terminal part of the molecule were taken as minimum requirements for homogeneity. When needed, a small amount of peptide was hydrolyzed in 6 N HCl and analyzed in a Durrum D500 amino acid analyzer, as described (Possani et al.,1985).

Radioisotopic-Labeling of NTX.
Noxiustoxin was isotopically labeled with ^{125}I by the lactoperoxidase method of Morrison & Bayse (1970). The reaction products were separated by Sephadex G-10 column (0.7 x 20 cm) equilibrated and run with 0.1 M NaCl in 0.1M Na-phosphate buffer, pH 7.4. The fractions containing radiolabeled toxins eluted in the void volume and free iodine was included into the gel matrix. Specific radioactivity was determined by counting small aliquots (2 µl) of each tube in a Gamma 5500 counter (Beckman Instruments Co., Palo Alto, CA, USA) assuming a protein recovery of 85%. Native and radiolabeled-toxin were run in SDS-gel electrophoresis. The polyacrylamide gels either were stained for protein or cut in small pieces (5 mm) and the radioactivity measured for colocalization of toxic-peptide and radioactivity.

Preparation of transverse tubule enriched membranes.
Membranes derived from the transverse tubule of skeletal muscle were prepared by slight modification of the procedure of Meissner (1984). Membrane fractions from the 10/20 and 20/25% sucrose interfaces routinely show high PN 200-110-binding activity and were therefore used for incorporation of transverse tubule ion channels into the planar bilayers of Mueller-Rudin type of chamber. The channel we are using as assay is the

large-unitary-conductance Ca^{++}-activated K^{+} channel also abbreviated K(Ca)-channel.

Planar Bilayer Methods.

For the measurement of K(Ca)-channel activity skeletal muscle T-tubule prepared as above were added to one chamber adjacent to a Mueller-Rudin bilayer membrane. This chamber, designated cis, routinely contained 250 mM KCl, 10 mM HEPES TRIS or KOH, pH 7.4, and 0.1 mM Ca-EGTA (2 µM free Ca^{++}). The opposite chamber (trans-ground side) contained 50 mM KCl, 10 mM HEPES TRIS or KOH, pH 7.4. Normally, 200 mM NaCl was added to the trans chamber following channel incorporation to inhibit the further insertion of channels. K(Ca)-channels were easily recognized under these conditions as upward current deflections with an amplitude of 7 pA at 0 mV holding potential. Single channel currents were measured via a List EPC-7 patch clamp electrometer (List Electronic, Darmstadt, FDR) and filtered through as 8 pole Bessel low-pass filter. Analog signals from the patch clamp were digitized and stored on 10 Mbyte hard disks for later analysis with software run on an IMB-AT computer.

Toxins were screened in the bilayer assay for activity against native K(Ca)-channels recorded in the presence of 2 µM free Ca^{++}. Whole venoms, toxic fractions or purified peptides were added to the trans bilayer chámber which normally corresponds to the extracellular face of the ion channel.

RESULTS

Radio-labeling NTX.

Fig.1a represents the profile of Sephadex G-10 column used for separation of ^{125}Iodine-labeled-NTX from side products. Since NTX contains 2 tyrosines which are potential sites for iodination, the first peak eluted from the column possibly represents a mixture of monoiodo-, diiodo and unlabeled NTX. The specific activity of labeled-NTX obtained under these conditions was 90 Ci/mmol, because we have isotopically diluted the ^{125}INa used for labeling (the maximum theoretical value for incorporation of 1 iodine/peptide molecule is in the order of 2100 Ci/mmol). Fig.1b shows the colocalization of NTX, run in gel electrophoresis (horizontally placed on top of Fig.1b) and radioactivity measurements of fractions (lower profile). The position corresponding to the electric mobility of native

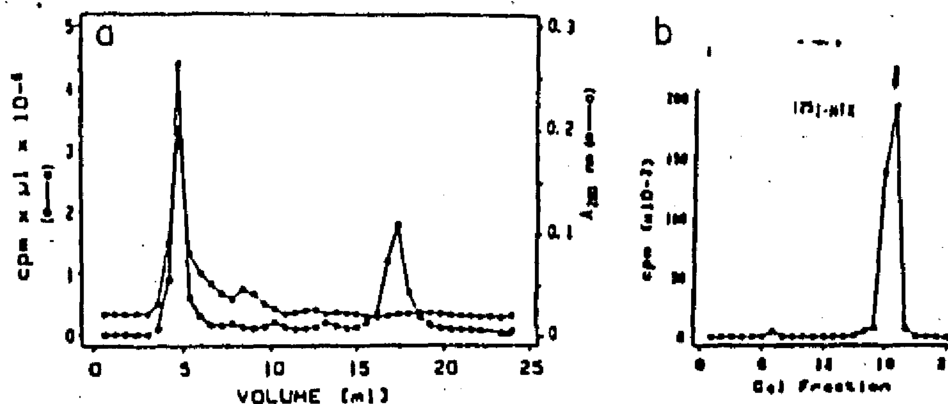

Figure 1. Labeling of NTX and SDS-PAGE analysis of [^{125}I]-NTX.
(a) NTX (200 μg, 46 nmol) was iodinated by the lactoperoxidase method. After completion of the reaction the mixture was loaded on a Sephadex G-10 column (0.8 x 13 cm) equilibrated and eluted with 100 mM NaCl, 100 mM sodium phosphate buffer, pH 7.4 at a flow rate of 10 ml/h. Fraction containing 0.5 ml were collected and absorbance at 280 nm (circles), and radioactivity (squares) were monitored.
(b) SDS-gels (15% acrylamide) were run according to Laemmli (1970). Small aliquots of iodinated toxin (5 μl) and native toxin (3 μg) were applied in separated lanes of the gel, run in parallel and stained with Coomassie-Blue (see photo of the gel placed horizontally in the upper part of the figure). The lane of the polyacrylamide gel containing the labeled toxin was cut 5 mm apart along the axis of electrophoretic migration, and the corresponding radioactivity was measured (squares).

NTX coincides with the radioactivity found in the gel. As minimum criteria to determine whether radioiodinated NTX could be used as a ligand of K^+channels in synaptosomes, it was necessary to demonstrate that the sample was radiopure and that it retained the ability to be recognized by antibodies raised against native NTX. The latter was demonstrated by radioimmunoassays, where polyvalent antibodies against NTX coprecipitated up to 95% of ^{125}I-NTX (Gurrola, 1986).

Specific binding of [^{125}I]-NTX to synaptosomes.

Fig. 2a shows the results of binding experiments in which increasing concentrations of [^{125}I]-NTX were added to a fixed concentration of synaptosomes, in the presence (non specific binding) or absence (total binding) of 100-200 fold excess of unlabeled NTX. Suitable concentrations of receptor were selected on the basis of a receptor dilution curve (not shown) and were determined to be 0.2-0.4 mg/ml of synaptosomal protein. The specific binding (not shown) was defined as the difference between total and non specific binding. The dissociation constant (K_d) of the

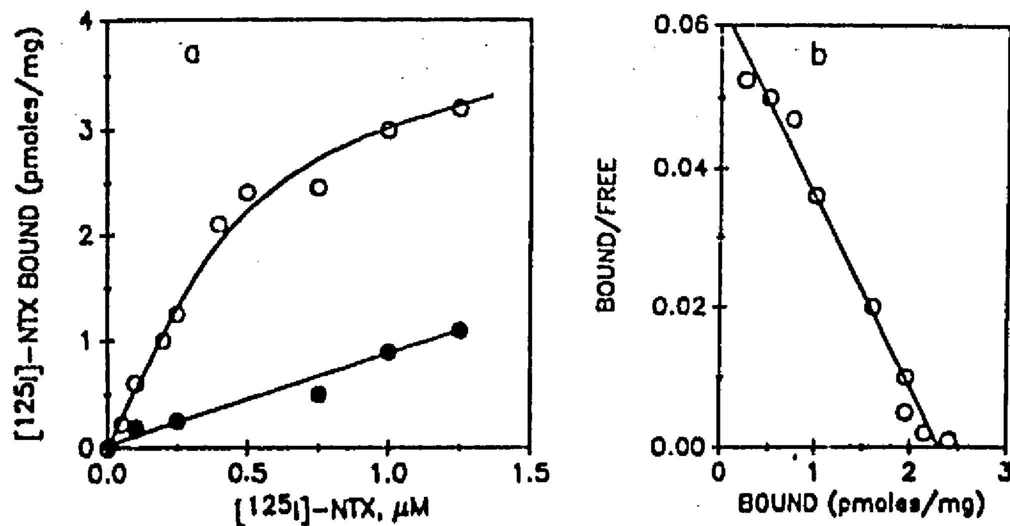

Figure 2. Binding of [125I]-NTX to synaptosomes. Synaptosomes were incubated with increasing concentration of [125I]-NTX from 0.05 to 1.5 µM for 60 min at 25 C and bound NTX was measured using the rapid filtration assay described in Materials and Methods. (a) Total binding (open circles) and non specific binding (filled circles) measured in the presence of 100-200-fold concentration of unlabeled NTX, plotted versus free [125I]-NTX concentration. (b) Scatchard plot of the specific binding, calculated from the difference between the experimental values for total binding and the fitted curve for the non specific binding.

complex formed between [125I]-NTX and synaptosomes was determined by the slope of the Scatchard plot: 200-400 nM (4 separate experiments), in good agreement with kinetic data experiments (Valdivia HH, Gurrola GB, Escobar L, Martin BM and Possani LD, manuscript in preparation). The linearity of this plot (Fig.2b) demonstrated that, at least in this range, [125I]-NTX binds to a single class of noninteracting binding sites with a maximal binding capacity of 1.9-2.2 pmoles/mg of protein. Further experiments were conducted to verify the inhibition of [125I]-NTX binding to synaptosomes by other peptides and drugs (Valdivia HH et al., in preparation, as mentioned above). Among the relevant data obtained by this displacement experiments are: long-chain scorpion toxins, known to recognize Na^+ channels and apamin a 18-residues long neurotoxic peptide purified from the honeybee venom do not displace the binding of NTX. However, Leiurotoxin III, purified as described below, was capable of displacing 100% the binding of NTX to synaptosomes.

Purification of novel scorpion toxins from *C. limpidus limpidus* venom.
Several monoclonal antibodies were obtained against NTX (Gurrola GB, Herion P, Sanchez MR, Saavedra R and Possani LD, unpublished) and were used as probes in immunoenzymatic assays (ELISA) for identification of peptides from other scorpion venoms, which share the same or similar epitopes as those found in NTX. One of the venoms studied was *C. l. limpidus*, from which we have purified three different peptides with similar amino acid sequences as that of NTX (Martin BM, Ramirez AN, Gurrola GB, Nobile M, Magnelli V and Possani LD, paper in preparation). The N-terminal amino acid sequence of one of such peptides (II-10.11.4) is shown in a figure below. Additionally, these peptides were studied in patch-clamp experiments using cerebellum granular cells, and were shown to block the outward K^{+} currents (Martin BM, et al., as above).

Purification of toxins from *Tityus serrulatus* venom.
In earlier studies we have reported the purification and N-terminal amino acid sequence of toxin II-9A from the venom of the Brazilian scorpion *T. serrulatus* (Possani et al., 1981b). This toxin was shown to block the delayed rectifier K^{+} channel from squid axon, fraction 1, in Carbone et al., (1983).
Three novel components from the venom of *T. serrulatus* were purified to homogeneity in a three steps purification procedure. Initially, the venom separation in Sephadex G-50 gives four distinct fractions (Possani et al., 1981 b), from which fraction number III contains peptides with fast blocking effects on the K(Ca)channel preparation described in Material and Methods. This fraction applied to CM-cellulose in 20 mM ammonium acetate buffer, pH 4.7, separates 10 different fractions (Possani et al., 1981b). Fractions III-8 and III-10 demonstrated rapid blocking activity in the bilayer assay and so were further sub-fractionated on CM-cellulose columns at pH 6.0, as shown in Fig.3. Three peptides with similar blocking effects (Fig.4, $SrTX_i$) were obtained and were called Serrulatoxins (SrTX), after the name of the scorpion species. SrTX1 and SrTX2 correspond to fraction III-8.4 and III-8.6 respectively, in Fig. 3b. SrTX3 is fraction III-10.2 (Fig. 3a). SrTX1 to 3 were shown to be homogeneous (Fig. 5) by the ß-alanine-acetate-urea gel of Reisfeld et al. (1962), and correspond aproximately to 0.4%, 0.7%, 1.3% of the soluble venom, respectively. They are basic peptides with an electrophoretic mobility similar to that of pyronin Y (tracking dye). By SDS-gel electrophoresis in 20 % acrylamide,

Laemmli (1970), SrTX1 to 3 were shown to be homogeneous, with an apparent molecular weight in the range of 1,000 (data not shown). The peptides are retained in a dialysis bag (M.W. cutoff 1,000) when desalting against water for short periods of times (4 changes for 15 min each), and they are included in Sephadex G-10 columns (M.W. exclusion 1,000) when gel

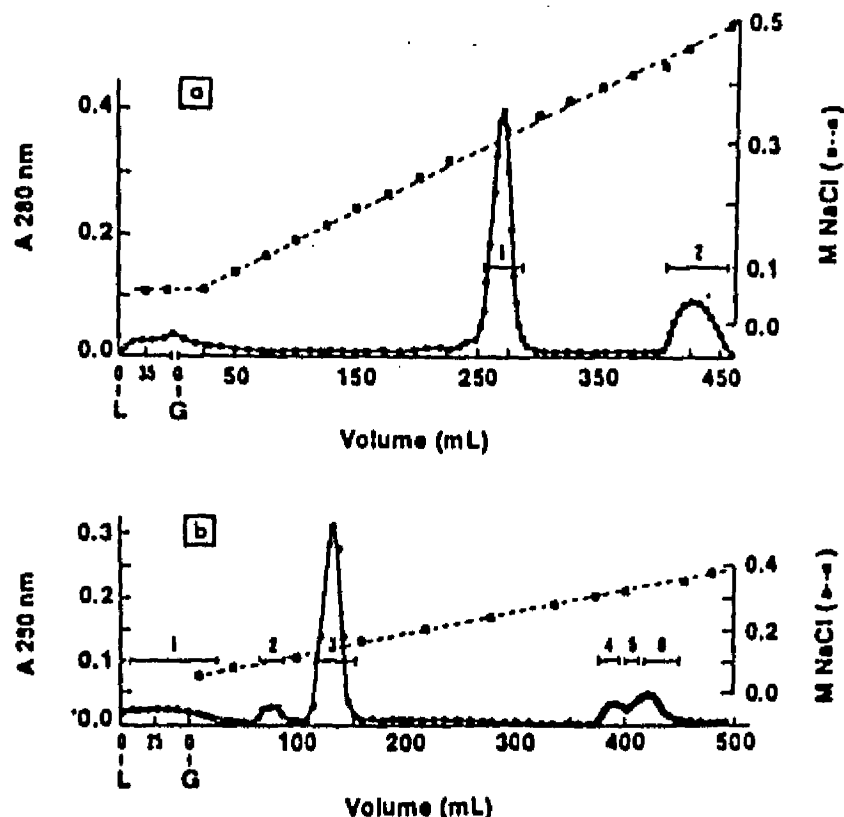

Figure 3. Final purification of Serrulatoxin-peptides.
SrTX-1 to 3 were finally purified in CM-cellulose columns (0.9 x 27 cm) equilibrated and run with 50 mM sodium phosphate buffer, pH 6.0, at a flow rate of 20 ml/h.
(a) Fraction III-8 (10.5 mg of protein) from previous chromatography (Possani et al., 1981b) was applied to the column (L) and eluted with a linear gradient containing 250 ml each of equilibration buffer and this buffer with 0.38 M NaCl. Fractions 4 to 6 contain SrTX-like activity. Since overlapping occurs in these fractions, three pools were separated, as indicated by horizontal bars. Fraction III-8.4 and III-8.6 were homogeneous, while fraction III-8.5 contained a mixture of SrTX-1 and SrTX-2. Recovery was in the order of 98% of applied material.
(b) Fraction III-10 (12 mg protein) was loaded in the column. A linear gradient containing 250 ml each of equilibration buffer (Na-phosphate, poH 6.0) and this buffer plus 0.5 M NaCl was started as indicated (G). The fractions were pooled as shown by horizontal bars. Recovery was 98%. Fraction III-10.1 is toxin gamma and fraction III-10.2 is SrTX-3.

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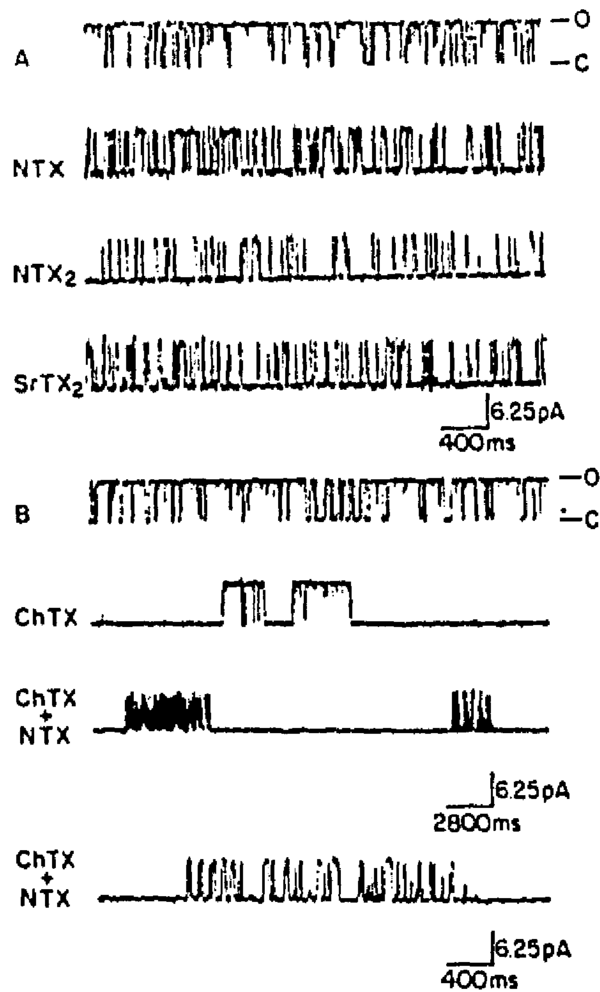

Figure 4. Effect of toxins on single channel currents of Ca^{++}-activated K^{+} channels from skeletal muscle T-tubles.

Single K(Ca) channels were recorded in 250/50 mM KCl, 200 mM NaCl (trans) as described in Methods. O means open channel, C means closed channel. The figure contains 8 traces, the upper trace (A) is control activity at 0 mV, 500 Hz filter, 2 KHz sampling. Open probability (Po) was 0.85. Second trace shows the effect of 800 nM NTX added trans. Po was reduced to 0.44. Third trace shws blocking effect of 300 nM NTX_2 trans. Po was 0.32. Fourth trace was recorded in the presence of 300 nM SrTX-2 (Po=0.37). Control activity and third trace were recorded from the same experiment. NTX and $SrTX_2$ effects were recorded from separate experiments with similar control activity.
The last four traces represent the blockade produced by CTX and NTX. B is control, with Po=0.80. Sixth trace was recorded with 20 nM ChTX trans. Po from entire file (10 min recording time) was 0.12. Seventh trace shows the effect produced by 20 nM ChTX plus 2 µM NTX trans. Lowermost recording is an expansion of the right hand burst of activity shown in the seventh trace. Recordings were taken at 0 mV, 500 Hz filter and 2 KHz sampling.

filtered. UV-absorption spectra of SrTX1-3 show maxima at 210, 224, and 280 nm, consistent with the peptide nature of the toxins. Tentative N-terminal amino acid sequence determination of SrTX3 gave no identifiable amino acid residue, maybe because it is blocked. Whether these three peptides have similar amino acid sequences still remains to be established. All three SrTX peptides produce a fast blockade similar to

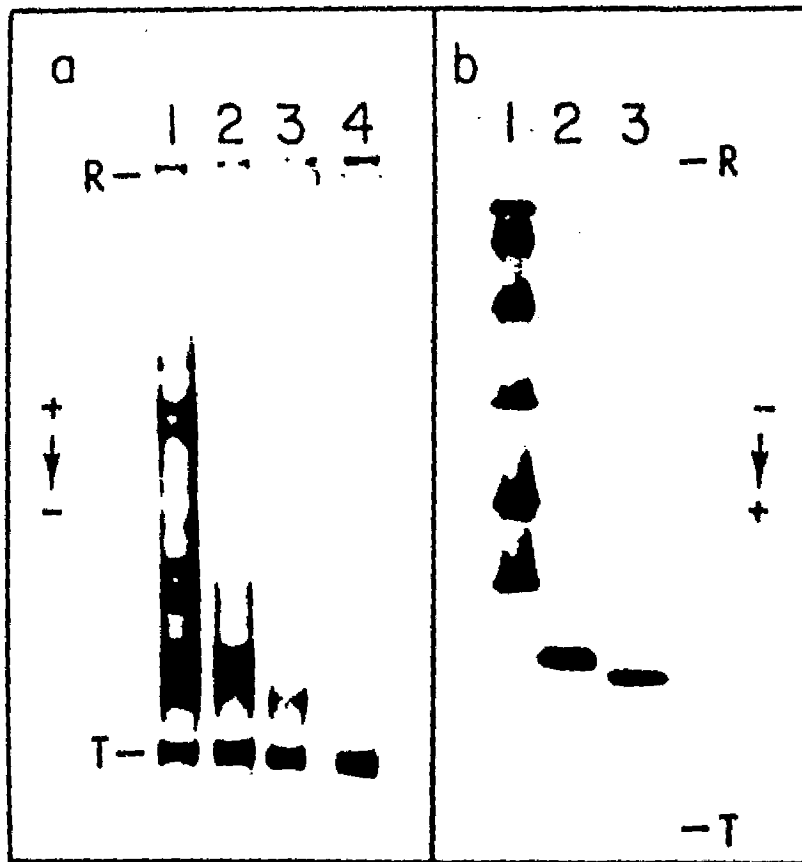

Figure 5. Gel electrophoresis of purified peptides.
Cylindrical gels (Reisfeld et al., 1962) and SDS-gels (Laemmli, 1970) were run with venom and purified fractions. R means running gel, T means tracking dye.
(a) ß-alanine-acetate urea gels from *T. serrulatus*: lane 1 (100 µg) of soluble venom; 2 (30 µg) of fraction III; 3 (30 µg) of fraction III-10; 4 (30 µg) of SrTX-3. Similar results were obtained with SrTX-1 and 2. Protein migrates to the cathode.
(b) SDS-gels of purified components: lane 1 (20 µg) of following molecular weight markers: Phosphorylase B (92,500), Bovine serum albumin (62,500), Ovalbumin (45,000), Carbonic anhydrase (31,000), Soybean trypsin inhibitor (21,500), Lysozyme (14,400). Lane 2 (4 µg) of toxin gamma from *T. serrulatus*, (mol. weight of 7,000). Lane 3 (2 µg) of ChTX purified from *L. quinquestriatus* as described here. Protein migrates to the anode.

that seen with *C. noxius* peptides NTX and NTX_2 (Fig. 4). The inhibition constant (K_i) for blockade with Serrulatoxins is on the order of 200-300 nM.

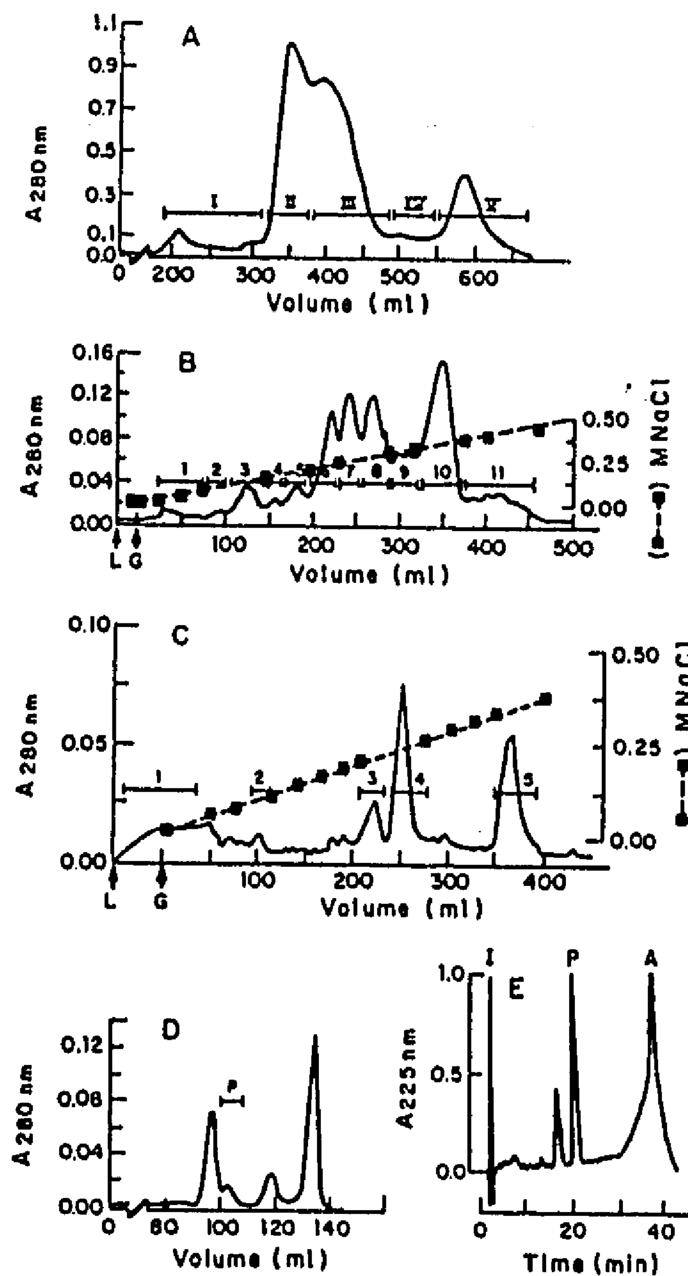

Figure 6. Purification of Leirutoxin III from *L. quinquestriatus* venom. (A) Soluble venom of *Leiurus quinquestriatus quinquestriatus* (100 mg, with a total of 133 units of absorbance at 280 nm, in 5 ml) was applied to a

Sephadex G-50 column (1.8 x 200 cm) equilibrated and run in 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 50 ml/h. Fractions containing 6 ml each were collected and the absorbance at 280 nm was read. Five pools were prepared (I-V) corresponding to major fractions, as indicated by horizontal bars. Recovery was 83% of the material applied to the column.
(B) Fraction III contained toxic material to mouse and also most of the ChTX and LeTX activities. This fraction (48 mg) was applied to a CM-cellulose column (0.9 x 27 cm) equilibrated and run in presence of 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 30 ml/h. A salt gradient from 0 to 0.5 M NaCl in the same buffer, contained in 250 ml each, eluted 11 sub-fractions. Fraction III-11 contained ChTX and LeTX activities. Recovery from this column was in the order of 97%. Tubes (2.5 ml each) corresponding to this fraction were pooled and dyalized against water to prepare the next column. The last 15 min were dyalized against the buffer of the letter C.
(C) After dyalisis fraction III-11, was loaded to another CM-cellulose column (0.9 x 27 cm) equilibrated and run in 50 mM Na-phosphate, pH 6.0, at flow rate of 20 ml/h. A linear gradient (started in G) containing 250 ml each of equilibration buffer and this buffer with 0.38 M NaCl was applied to the column to elute 5 sub-fractions, indicated by horizontal lines. Recovery was quantitative.
(D) Fraction III-11.5 was dyalized, freeze-dryed and applied to a Sephadex G-50 column (0.9 x 190 cm) equilibrated in 0.1 M Tris-HCl buffer, pH 8.2, containing 1.0 M NaCl. The column was run at a flow rate of 30 ml/h and tube-fractions of 1 ml each were collected. P shows the chromatographic peak containing LeTX activity. Recovery was also quantitative.
(E) HPLC separation of fraction III-11-P, after dyalisis. This figure shows the profile of 50 µg of peptide P (from letter D) fractionated in a C18 reverse column, with a linear gradient of solution A (0.12% TFA in water) to 60% solution B (0.10% TFA in acetonitrile) during 1 h. Peptide P was homogeneous as demonstrated by SDS-gel electrophoresis and amino-terminal sequence, and was named Leirutoxin III. I means injection artefact and A possible contaminants plus elution artefacts.

Purification of peptides from *Leiurus q. quinquestriatus* venom.

Following chromatographic procedures similar to those used for *C. noxius* and *T. serrulatus* venoms, we have isolated from the venom of *L. q. quinquestriatus* a peptide with high affinity for the K(Ca) channels (Fig.4, labeled CTX). This peptide was shown to be Charybdotoxin (Valdivia et al., 1988). However, we have used a different method than that originaly described by Miller et al (1985). According to our procedure (Fig. 6A), after gel filtration on Sephadex G-50, fraction III showed ChTX-like blocking activity. When this was applied to a CM-cellulose (Fig. 6B) it separates a large chromatographic fraction III-11, which actually can be subdivided in three small sub-fractions. In one of the preparations we have selected the last peak, corresponding to fraction III-13 and rechromatographed by HPLC in a C18 reverse phase column, using a linear

gradient of 0.12% trifluoroacetic acid (TFA) from 0 to 60 % acetonitrile in 0.10% TFA (data not shown). ChTX was eluted with a retention time of approximately 18 min. SDS-gel electrophoresis of this component (ChTX) shows a single band corresponding to an apparent molecular weight in the order of 4,800 (Fig.5b). Another minor component of this venom was purified to homogeneity following the steps shown in Fig. 6C to 6E). Component III-11 contained a strong inhibitory effect on the K(Ca) channel bilayer system of Fig.4, and because it was rich in low molecular weight peptides a second Sephadex G-50 column was equilibrated and run with high salt (1M NaCl) in which the lyophylized component III-11 was applied. Fraction labeled P in Fig. 6D was further separated by HPLC in the same system described above for ChTX. Amino acid composition, SDS-gel electrophoresis and N-terminal amino acid sequence of the purified peptide shows that it has approximately 40 amino acid residues with a molecular weight in the order of 4,800, and N-terminal sequence similar to that of NTX and ChTX (Fig.7, labeled LeTxIII), and we have decided to call it Leirutoxin III (Valdivia HH, Gurrola GB, Escobar L, Martin BM and Possani LD, manuscript in preparation).

Fig.7: SUMMARY OF AMINO ACID SEQUENCES OF K^+-CHANNEL BLOCKING PEPTIDES.

Toxins	Amino acid sequences	References*
	1 10 20 30 39	
Cn NTX	TIINVKCTSP KQCSKPCKEL YGSSAGAKCM NGKCKCYNN	(1)
Cn II-10.2	TFIDVKCGSS KECXP...	(1)
Cll II-10.11.4	TVINVKCTSP KQCLLPCKQI...	(2)
Ts II-9.A	VFINAKCRGS PECLPKCKEA XGKAAGKCXN...	(1)
Ts SrTX 3	blocked ?	(3)
	1 10 20 30 37	
Lq ChTX	pEFTNVSCTTS KECWSVCQRL HNTSRGKCMN KKCRCYS	(4)
Lq LeTXIII	GLIDVRCYDS SQCE...	(3)
Bt IbTX	pEFTDVDCSVS KECWSVCKDL FGVDRGKCMG KKCRCYQ	(5)
Lqh LeTX I	AF----CNL- RHCQLSCRSL -GL-LGKCIG DKCECVKH	(6)

*References: (1) Possani et al., 1982); (2) Martin, BM., Ramirez AN., Gurrola, GB., Nobile, M., Magnelli, V. and Possani, LD., manuscript in preparation; (3) This communication; (4) Gimenez-Gallego et al., 1988; (5) Galvez et al., 1990; (6) Chicchi et al., 1988.

DISCUSSION

The discovery of NTX (Carbone et al., 1982) as a K^+ channel blocker peptide immediately motivated the search for similar toxins in other venoms. Possani et al. (1982) reported the primary structure of NTX and the presence of peptides with similar amino acid sequences in the venom of the same scorpion (*C. noxius*) as well as in *T. serrulatus* venom (see also Fig.7). One year latter Carbone et al., (1983) demonstrated by voltage-clamp techniques that the K+ currents of squid axons were blocked by venoms from the scorpions *C. elegans, C. limpidus limpidus*, and *C. limpidus tecomanus*; and Prestipino et al., (1983) showed the same with venoms from *C. suffusus suffusus* and *C. limpidus acatlanensis*. During the following years the emphasis was placed in the characterization of NTX, which was shown to induce transmitter release from synaptosomes, by blocking K^+ permeability (Sitges et al., 1986). A complete physiological characterization of the action of NTX on squid axon was reported (Carbone et al., 1987). Gurrola et al., (1989) using synthetic peptides was able to show that the nonapeptide of NTX retained some features of the native NTX, suggesting that the active site of this toxin is probably located at the N-terminal part of the molecule. While this work was being carried out with NTX, Miller et al., (1985) and Smith et al., (1986) reported a new peptide from the venom of the scorpion *Leiurus quinquestriatus*, named charybdotoxin, that was able to block the K^+(Ca)-currents of T-tubule membranes from skeletal muscle. In 1988, our research group (Valdivia et al., 1988) showed that charybdotoxin was half the size reported by Smith et al., (1986) and was indeed a similar peptide as the originally described NTX. Howver, the mechanism of action of both toxins were not identical in terms of the affinity for the different types of K^+ channels recognized by both toxins, as also shown in our Fig.4. Further characterization of either NTX (Prestipino et al., 1989, Colden-Stanfield et al., 1990) or ChTX continued. Some of the latest publication on ChTX are: Massefski et al., 1990, Schneider et al., 1989, Vazquez et al., 1989, MacKinnon and Miller, 1989, Sugg et al., 1990, Bontems et al., (1991) and Deutsch et al (1991). Recently two additional new peptides: Leirutoxin I

(Chicchi et al., 1988, Auguste et al., 1990) from *L. quinquestriatus* venom and Iberiotoxin, from the venom of the scorpion *Buthus tamulus* (Galvez et al., 1990) were purified and studied. Most of these work compare the effects of the newly purified peptides with that of NTX and/or ChTX.

In the present communication we describe additional characteristics of NTX and the procedure for purification of similar peptides from the venom of *C. limpidus limpidus*, *L. quinquestriatus* and *T. serrulatus* (Fig.s 3,5 and 6). Labeled NTX (Fig. 1) retains biological activity, and direct binding experiments to synaptosomes (Fig. 2) yield a K_d* of 200-400 nM, in good agreement with k_d* determined electrophysiologically (Carbone et al., 1982) and kinetically (Valdivia HH, Gurrola GB, Escobar L, Martin BM and Possani LD, unpublished). ChTX purified by our procedure, yield a final recovery of about 1% from the initial venom, assuming one absorbance unit at 280 nm equals to 1 mg/ml peptide (see Material and Methods), or 0.5% assuming a molar extinction coefficient of 17,500 M^{-1} cm^{-1}, as suggested by Smith et al., (1986). Solubilization of the venom in water might have increased the final yield of ChTX. Smith et al. (1986) have reported a recovery of only 0.1% of this peptide from the total venom, they have initially dissolved the venom in 10 mM Na-borate, plus 10 mM Na2-carbonate buffer, at pH 9.0; hence, near the isoelectric point. This might explain the low recevery yield obtained.

Figure 4 (ChTX) shows the characteristics slow blockade of K(Ca) channels produced by 20 nM charybdotoxin added to the external face of the channel. Blocked stated were typically 10-15 s duration at room temperature. 2 μM NTX added in the presence of ChTX had an additional inhibitory effect. In the presence of both toxins channels displayed blocked states of long and short duration characteristic of those produced by each toxin added separately (Fig.4, ChTX+NTX). When bursts of activity in the presence of both toxins were examined on an expanded time scale, the open and closed events appeared kinetically similar to those seen in the presence of NTX alone (compare trace NTX and ChTX + NTX expanded scale, botton trace in Fig.4). Thus apparently, both CTX and NTX can interact simultaneously with the same ion channel. At this point, however, it is not clear whether the toxin binding sites are the same for CTX and NTX. The difference in the blocking kinetics τ_s =10 s for CTX and τ_s=50 ms for NTX is most easily explained by the different apparent affinities of the two toxins for inhibitory sites on the channel. The effects of both NTX_3 and $SrTX_3$ (Fig. 4) in the same channels are comparable to that of NTX.

As shown in Fig.7, there are extense amino acid sequence similarities among the eight peptides thus far sequenced, except for the short peptide LeTX I, where gaps have to be introduced (dashes) in order to align the cysteinyl residues, important for disulfide bridge formation, hence for stabilization of the tridimensinal structure of these toxins. Based in the considerations and data presented here it can be predicted that many more such peptides will be purified and characterized from the scorpion venoms, in the coming years. It is possible to predict that this new family of basic, low molecular weight peptides, K^+ channel blockers will play a very important role in the isolation and characterization of K^+-channels, the most diverse and certainly a very important ion channel, that presides the membrane excitability (Miller, 1991).

ACKNOWLEDGEMENTS

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Purification and reconstitution of potassium channel proteins from squid axon membranes

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Voltage-dependent K^+ channels are responsible for repolarization of the cell membrane during the late phase of the action potential. Here we report the purification of proteins from squid axon membranes which bind the K^+-channel blocker noxiustoxin (NTX), and their subsequent functional reconstitution in planar bilayers. The NXT-affinity purified proteins had M_r values of 60000 ± 6000, 160000 ± 15000 and 220000 ± 20000. Their incorporation into bilayers resulted in single-channel currents with three conductances, the most frequent one of 11 pS in 300/100 mM KCl (*cis/trans*). The voltage dependence, reversal potential and bursting behavior suggest that these are the K^+ channels involved in the squid axon action potential.

Ion channel reconstitution; Squid axon; Delayed rectifier; Planar bilayer

1. INTRODUCTION

The squid axon delayed rectifier responsible for the repolarization of the cell membrane during the late phase of the action potential was the first K^+ channel described [1,2]. However, its purification, as well as the purification of other types of K^+ channels, which are the most diversified [18], has not been achieved due to the lack of high affinity compounds. Previously, it was shown that noxiustoxin (NTX), a 4.3 kDa peptide toxin obtained from the venom of the Mexican scorpion *Centruroides noxius*, is a potent blocker of the delayed rectifier in the squid axon [4]. In the present work NTX was used to purify NTX-binding proteins from a detergent extract of squid axon membranes. The purified proteins were reconstituted in a functional form into planar lipid bilayers.

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2. MATERIALS AND METHODS

2.1. *Solubilization of membranes and affinity column*

Batches of approximately 10 g of squid axons were routinely used to prepare the rich membrane fraction I, as described [6]. The membranes from fraction I were solubilized in a buffer containing the following composition: 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in 10 mM Hepes-Na buffer, pH 7.4, 150 mM NaCl, 2.5 $CaCl_2$, 0.02% phosphatidylcholine, plus the following protease inhibitors: phenylmethylsulfonyl fluoride (0.1 mM), iodoacetamide (1 mM), 1:10 phenanthroline (1 mM) and pepstatin A (1 μM). The preparation was incubated for 1 h at 4°C and spinned down (60000 × g) for 20 min. The soluble material (3.2 mg in 6 ml) was incubated with 5 ml of NTX-affinity beads (capacity: 1 mg of NTX/5 ml resin) for 1 h at 25°C and loaded into the column. The column was washed with 30 ml of buffer A (same as solubilization buffer, but decreasing CHAPS concentration to 0.1%) and the bound protein was eluted with buffer A containing 1 mM 4-aminopyridine (4-AP). Approximately 30 μg of protein was recovered.

2.2. *Binding of [^{125}I]NTX to crude axonal membranes*

Membranes of squid axons obtained by centrifugation in 0.33 M sucrose, after disruption (Polytron) and homogenization (teflon pestle), display a B_{max} of 0.81 pmol/mg protein when assayed with 60 nM iodinated NTX. Incubation was performed in 5 mM Tris-HCl buffer, pH 8.0, in artificial sea

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water. Membranes were incubated 30 min at 25°C and free [^{125}I]NTX separated by centrifugation.

2.3. *Cross-linking of [^{125}I]NTX to purified proteins*

Protein eluted from the affinity column with 4-AP was dialyzed and incubated for 20 min with 300 nM [^{125}I]NTX, either in the absence or the presence of 20 μM cold NTX. After incubation, the protein content of each tube was cross-linked by the addition of disuccinimidyl suberimidate (final concentration 1 mM) in dimethylsulfoxide. Samples were run in the SDS-PAGE system as described above, stained, dried and placed in the Kodak holder with appropriate films for autoradiography.

2.4. *Planar lipid bilayers*

Bilayers were made according to the Mueller-Rudin [13] technique using diphytanoylphosphatidylcholine (20 mg of lipid per ml of *n*-decane, Avanti). They were formed over a 250 μm diameter aperture in a 50 μm thick plastic septum that separated two 400 μl teflon chambers. The hole was pretreated with the same lipid solution used for bilayer formation and allowed to dry under a N_2 stream. A patch-clamp amplifier (Biologic RK-300) was used to record the current and to control the voltage across the bilayer. Ag/AgCl electrodes in series with 1 M KCl agar bridges were used. The voltage was applied to the *cis* side of the bilayer and the *trans* side was held at virtual ground. In all the records, channel opening is shown as an upward deflection if the applied potential is positive. The membrane currents were recorded on a modified digital audio processor connected to a VCR [17], and the data analyzed off line. Ion channel reconstitution was performed using the fusion strategy [14], as follows: either liposomes containing the purified receptor, or the purified soluble receptor (<1 μg/ml), were added to the *cis* side compartment. Liposomes were formed by dialysing the purified protein at 4°C for 3 days against 50 mM KCl, Hepes-K 10 mM, pH 7.4. Thereafter, they were sonicated for 2 min in the presence of 0.6 M sucrose. Experiments were performed at 20 ± 2°C.

3. RESULTS AND DISCUSSION

Axon membranes from the squid *Loligo vulgaris* were used in this study since NTX, a toxin purified from the Mexican scorpion *Centruroides noxius*, selectively affects their delayed rectifier [4,5]. After isolating axonal membranes as described in section 2, they were solubilized in a buffer containing 2% CHAPS. The solubilized material was incubated with NTX-Affigel P-10 beads and the protein bound to the NTX-Affigel P-10 (~30 μg) was eluted from the column with 1 mM of 4-AP and 0.1% of CHAPS. Assuming that all the recovered protein corresponds to channel proteins, the maximum purification in this step would be 100-fold (see fig.1). This purification procedure was repeated three times with comparable results.

Gel electrophoresis of purified NTX-receptor

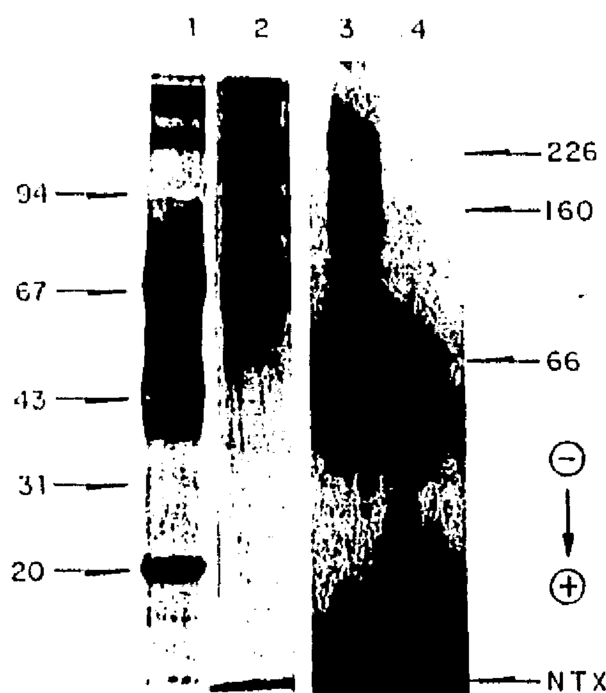

Fig.1. Electrophoresis of purified NTX-binding proteins. Lanes 1 and 2 are silver stained SDS/polyacrylamide (10%) gel electrophoresis (PAGE) [7]. Lane 1 shows molecular weight markers (10 μg each; in kDa): phosphorylase b (94); bovine serum albumin (67); ovalbumin (43); carbonic anhydrase (31); trypsin inhibitor (20). Lane 2 shows the proteins (2 μg) eluted with 4-AP from the NTX-affinity column. Lanes 3 and 4 are autoradiographic pictures of the proteins (0.5 μg) eluted from the affinity column and cross-linked with [^{125}I]NTX ran on a 4-12% gradient SDS-PAGE, thus their electrophoretic mobility is different from lanes 1 and 2. Lane 4 is the same as 3 but with a 75-fold excess cold-NTX added prior to cross-linkage. A parallel lane (not shown) was used to run molecular weight markers (200 kDa myosin, 116 kDa beta-galactosidase, and others similar to lane 1). Arrows on the right indicate approximate M_r of radiolabeled proteins.

showed mainly four peptides (lane 2, fig.1). Two strong bands with apparent M_r values of 53 ± 3 and 60 ± 6, and two additional faint bands corresponding to higher M_r forms. The protein pattern obtained with the purified preparation is in good agreement with data obtained by cross-linking the purified material with [^{125}I]NTX (lane 3, fig.1). The NTX binding sites are found in proteins with M_r of 66 ± 6 (broad doublet), 160 ± 15 and 220 ± 20. The 66 kDa band protein is within the same range as that proposed for the K^+ channel of the *Shaker* locus of *Drosophila* [8] and those of the mouse and rat brain K^+ channels [9–11]. As shown in lane 4, fig.1, the lower molecular mass

peptide (53 kDa) binds NTX non-specifically. The presence of four bands before and after NTX cross-linking, clearly shows that the 160 and 220 kDa polypeptides are not a polymerization artifact of the 66 kDa polypeptide; however, their M_r values (in lane 3) are overestimated due to the covalent attachment of NTX molecule(s). These results show the presence of three distinct peptides in squid axon membranes, which might correspond to three different types of NTX-sensitive K^+ channels. In fact, single-channel currents from patch-clamp of the inside surface of cut-open squid axons, revealed three distinct types of K^+-channel activity [12].

The protein recovered from the affinity column with 4-AP was extensively dialyzed against buffer and used for reconstitution experiments. Fusion with planar lipid bilayers, of either purified soluble receptor or liposomes containing purified receptor gave similar results, indicating that ion channels are present in the purified fractions. Fig.2a shows a long time record of single-channel current fluctuations across a planar membrane following addition of purified receptor protein (<1 μl ml^{-1}) to the *cis* side. In the presence of a 5-fold KCl concentration gradient, at an applied membrane voltage of 100 mV, the channels display a bursting behavior: grouping in time of opening events separated by relatively long segments of inactivity [2,15]. The mean current of the predominant channel was 1.5 pA (fig.2b), corresponding to a chord conductance of 11 pS (at 100 mV). This value is similar to that of 9–11 pS reported for K^+ channels of squid axon by Conti and Neher [2] and for the smaller K^+ channel reported by Llano et al. [12] in cut-open squid axon. A single-channel conductance of 15 pS was also reported for the delayed rectifier K^+ channels from skeletal muscle [16]. The single-channel conductances have been expressed as chord conductances, $\gamma = i/(E_m - E_k)$, where E_m is the voltage applied to the membrane and E_k is the K^+ reversal potential, because the

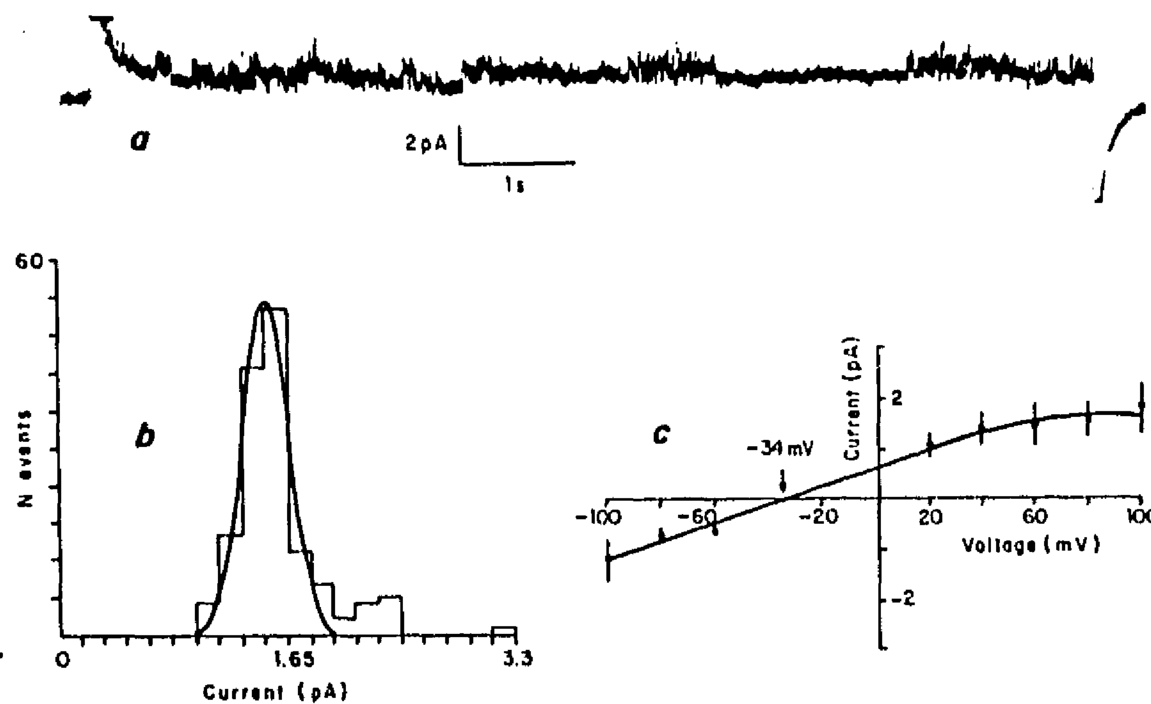

Fig.2. Reconstitution of the purified NTX-binding protein channels into lipid bilayers. (a) Occurrence of single-channel bursts recorded with a membrane potential held at 100 mV in asymmetric solutions. The record was filtered at 100 Hz with an eight-pole Bessel filter and displayed on a Gould chart recorder. The first and last parts of the record show the capacitative transients corresponding to the application and removal of E_m. The solutions bathing the bilayers were asymmetric and contained 0.5 M KCl, 0.1 mM $CaCl_2$, 10 mM Hepes at pH 8.0 in *cis* side, and the same but 0.1 M KCl in *trans*. (b) Amplitude histogram based on single-channel current transitions. The number of analyzed events was 152. The mean amplitude was 1.5 ± 0.2 pA at a membrane potential of 100 mV in asymmetric KCl solutions. (c) Open-channel current voltage relationship. The mean single-channel current at each point was obtained from five different bilayers. The arrow indicates the zero-current voltage. The vertical lines indicate the standard deviation of each point.

single-channel current-voltage relation shows rectification at high positive voltages (fig.2c). This figure also illustrates the selectivity of the single channels. In these experiments we measured the current passing through a single channel in the presence of 5-fold KCl concentration gradient. The reversal potential for the single channel was −34 mV, in agreement with that predicted by the Nernst equation for a cationic selective channel.

Fig.3a shows recordings of single-channel current at different membrane potentials of the more frequently observed unitary current. For large positive voltages, the open probability is higher than for negative ones. In a few records we have encountered two other types of channels (fig.3b). These channels opened infrequently and their single-channel conductances were around 22 and 32 pS (at 100 mV). We have not studied these channels in detail; however, it is possible that they also contribute to the total macroscopic K^+ current elicited by a depolarizing pulse in the squid giant axon membrane. The voltage-dependence of the channel can be seen in the current versus time histograms (fig.3c). The time that the channels spent in the closed current level was less at more positive potentials. In the 100 mV histogram the main current peak (1.5 pA) and two larger ones are evident (c = closed channel current). At 100 mV the open time constants were 0.4 and 2.3 ms (606 events) and the closed ones 17.6 and 89 ms (667

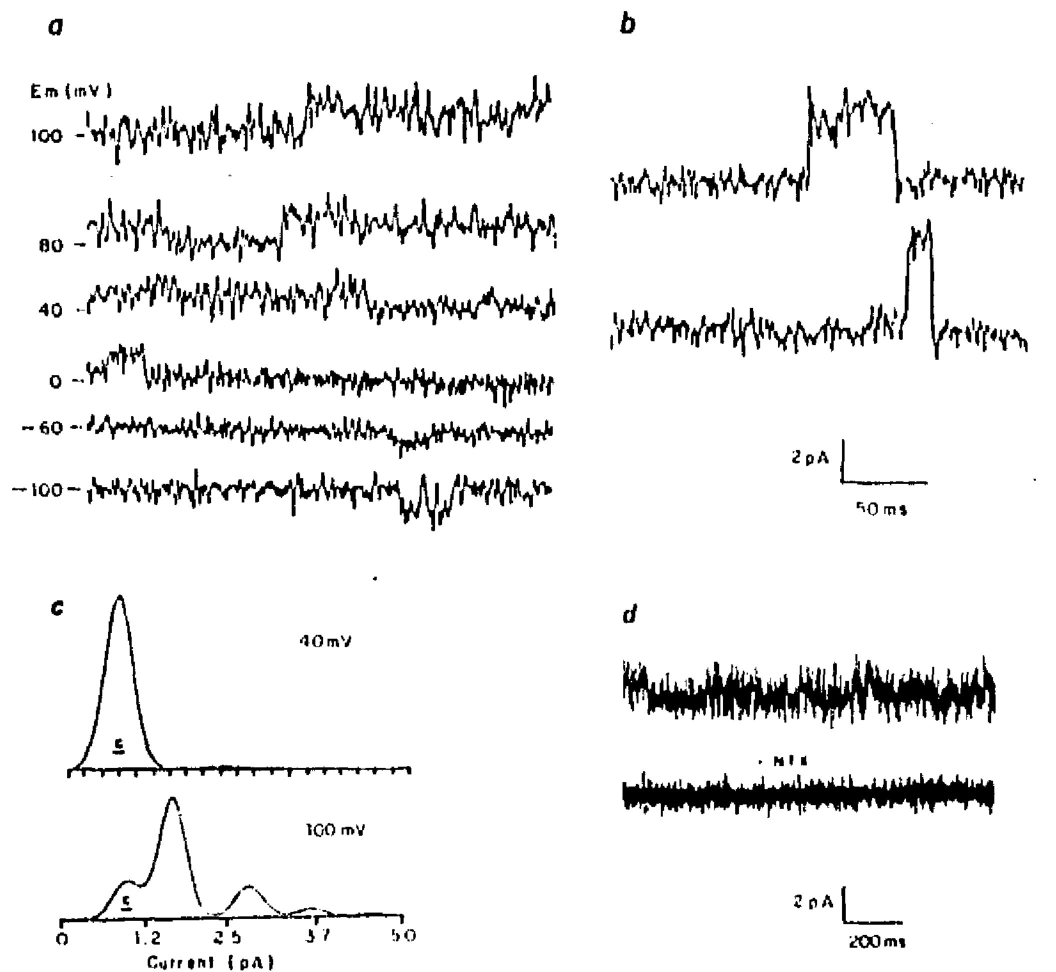

Fig.3. Properties of the main channel. (a) Representative single-channel records at the indicated voltages (E_m), of the predominant channel filtered at 300 Hz, digitized at 2 kHz sampling interval and plotted by computer. (b) Current records of the two less frequent K^+-channels incorporated into bilayers. (c) Current level histograms from a bilayer. Records were filtered at 100 Hz and each histogram was constructed with 28 s of records at the indicated potentials. Each peak represents the percent of time that the current spent in each level. c indicates the basal bilayer current (closed state of the channel). (d) NTX block of the channel. Addition of 2 μM NTX to the *cis* compartment blocked the current fluctuations. Upper record is the control before NTX addition. Membrane potential: 100 mV.

events). The biexponential fit of the open and closed dwell histograms agrees with the complex bursting kinetics seen in fig.2a. Preliminary results indicate that the K^+-channel activity is blocked by NTX (fig.3d) and tetraethylammonium (TEA^+). However, in some cases, after seeing blockage, we have observed increases in the current which could be due to further channel incorporation since the NTX receptor protein loaded liposomes are very fusogenic.

The results demonstrate that the purified NTX binding protein displays single-channel activity when incorporated into planar lipid bilayers. A main 11 pS conductance and two larger ones were observed. The reversal potential, the voltage dependence and the preliminary results with NTX and TEA^+ suggest that the purified fraction contains the delayed K^+ channel from the squid giant axon. To our knowledge, this is the first report in the literature showing both reconstitution and activity of a K^+ ion channel isolated by affinity column from a solubilized membrane preparation, and the used strategy could be useful for the purification of K^+ channels from other membrane preparations.

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ISOLATION AND CHARACTERIZATION OF TWO TOXINS FROM THE MEXICAN SCORPION *CENTRUROIDES LIMPIDUS LIMPIDUS* KARSCH§

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Abstract—1. Several toxic polypetides were found in the venom of the scorpion *Centruroides limpidus limpidus*. Comparative studies of the potency of the venom in different strains of mice were conducted.

2. A new type of toxin (component II.9), specific for crustaceans (crayfish and isopods), was isolated from this scorpion and was shown to have the following *N*-terminal amino acid sequence: Lys-Lys-Asp-Gly-Tyr-Leu-Val-Asn-Lys-Tyr-Thr-Gly-Cys-Lys-Val-Asn-Cys-Tyr-Lys-Leu-Gly-Glu-Asn-Lys-Phe-Cys-Asn-Arg-Glu-.

3. A polypeptide toxic to mice (component II.6) from this venom was shown to have the following *N*-terminal sequence: Lys-Glu-Gly-Tyr-Leu-Val-Asn-His-Ser-Thr-Gly-Cys-Lys-Tyr-Glu-Cys-Tyr-Lys-Leu-Gly-Asp-Asn-Asp-Tyr-Cys-Leu-Arg-Glu-Cys-Lys-.

4. In cultured chick dorsal root ganglion cells, 1 μM of toxin II.6 was shown to reduce the size of sodium currents and to slow-down their activation inactivation kinetics. The toxin had also a depressive action on the classical Ca^{2+} current activated at high membrane potentials (>0 mV).

INTRODUCTION

On the American Continent there are two genera of scorpions from the family Buthidae: *Centruroides* and *Tityus* which represent a medical problem and a life hazard to humans. From the genus *Centruroides* one of the most dangerous species is *Centruroides limpidus limpidus* from the state of Morelos and Guerrero (Mexico) and its venom is one of the least studied so far. A preliminary report concerning some toxic peptides from this scorpion has been published (Talo *et al*., 1978), but no further biochemical or physiological characterization has since been presented (see review by Possani, 1984).

In the present communication we describe the chemical, biological and electrophysiological characterization of several components from this venom. A comparative study of the venom potency on different strains of mice was conducted. In addition a new toxin (component II.9) was discovered, which specifically affects crustaceans and not mice. Another, component II.6 is toxic to mice and chick (vertebrates) but had no effect on crustaceans. In avian sensory neurons, toxin II.6 acts upon the Na^+ and Ca^{2+} channels which are responsible for the action potential electrogenesis in these cells. The activation of both channels was strongly depressed by application of 1 μM of the toxin to the external bath.

MATERIALS AND METHODS

Source of venom

The scorpions were collected by us in the field and venom obtained by electrical stimulation of anesthetized animals as previously described (Dent *et al*., 1980). Water solubilized venom was centrifuged at 15,000 *g* for 15 min in a Sorvall SS-34 rotor. The supernatant was freeze-dried and stored at −20°C until used.

Materials

All chemicals and reagents were analytical grade and were purchased from the companies indicated previously (Possani *et al*., 1985). Sephadex G-50 (medium) was from

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Abbreviations used—AaH, *Androctonus australis* Hector; Cll, *Centruroides limpidus limpidus* Karsch; Clt, *Centruroides limpidus tecomanus* Hoffmann; CM-cellulose, carboxymethyl-cellulose; Cn, *Centruroides noxius* Hoffmann; CsE, *Centruroides sculpturatus* Ewing; Css, *Centruroides suffusus suffusus*; DRG, dorsal root ganglion; EGTA, ethylene glycol, Bis-(2-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Lqq, *Leiurus quinquestriatus quinquestriatus*; mol-wt, molecular weight; *n*., *noxius*; *s*., *serrulatus*; SDS, sodium dodecyl sulfate; *T*., *Tityus*; TTX, tetrodotoxin; Ts, *Tityus serrulatus* Lutz and Mello

Pharmacia Fine Chemicals (Uppsala, Sweden); Carboxymethyl-cellulose (CM-32) was from Whatman (Clifton, NJ, USA) and Bio-Rex 70 was from Bio-Rad Laboratories (Rockville Centre, NY, USA).

Lethality tests

The mouse lethality of various protein fractions was observed after intraperitoneal injection of different amounts of protein (usually from 5 to 100 μg) in 0.1–0.3 ml saline or buffer solutions, into adult 20–25 g mice (strain CD_1). To define the toxicity of various chromatographic components, three designations were used (Possani et al., 1977). "Lethal" means that the component at the dose injected was enough to kill the tested mouse within 20 hr of injection. "Toxin" means that the mouse shows any of the following symptoms: excitability, salivation, temporary paralysis of rear limbs, dyspnoea, but recovered within 20 hr after injection. "Non-toxic" means normal behaviour similar to injection of 0.9% NaCl or buffer solutions.

Mice from the strains BALB/c, BALB/k, CD_1, C_{57}, DBA, NIH and Mexican SSA were used for the comparative LD_{50} determinations.

The method of Reed and Muench (1938) was applied to calculate the values of LD_{50} in the different mice strains for the soluble venom and sub-fractions of *C. l. limpidus* venom. Systematically we have used 10 mice for each dose of venom or fraction of venom. Several different doses were used for each LD_{50} at decreasing amount of protein (by a factor of two).

Two species of crustaceans were also used for toxicity tests: *Procambarus bouvieri* (crayfish) and *Oniscus* sp. ("cochinilla") a very common isopod found in gardens of Mexico City. This last animal was used based on the original report by Zlotkin et al. (1975) who described this technique. "Cochinillas" were injected in the dorsal part of the body between the cephalothorax and abdomen with a Hamilton microsyringe (10 μl capacity) not exceeding 5 μl total volume of injection. Toxicity or lethality was observed through the following symptoms; irregular movements and curvature of the body, paralysis of legs, immobilization in an upside-down position and finally death. The initiation of the symptoms was doses dependent, and the final results were taken after 12 hr of injection. Rare mechanical damage due to injection could clearly be differentiated from actual intoxication symptoms.

The crayfish were injected with a Hamilton microsyringe (50 μl capacity) in the dorsal cavity (heart). In crayfish the main symptoms of intoxication observed were interpreted as a generalized contraction (mainly the legs), followed by a half-turn over the body, sometimes in a complete up-side-down position and finally death within 20 hr of injection.

Crayfishes were used to corroborate the "cochinillas" results, but due to the difficulty of obtaining crayfish fewer animals were used.

Purification procedures

The soluble venom (835 mg) was divided into seven aliquots and applied independently to a Sephadex G-50 (medium) column. The toxin containing tubes were pooled (fraction number II), divided into five aliquots and chromatographed individually on a CM-cellulose column, equilibrated and run in 20 mM ammonium acetate buffer at pH 4.7. The most toxic fraction obtained (II.6) was subsequently purified by three further steps of ion exchange chromatography employing CM-cellulose and Bio-Rex-70 columns at different pHs. The conditions for the CM-cellulose columns are indicated below, in the footnotes of the figures. For the Bio-Rex-70 column (0.9 × 30 cm) a linear gradient of 250 ml of 50 mM sodium phosphate pH 6.4 and 250 ml of the same buffer containing 250 mM NaCl was applied. The column was previously equilibrated with the initial buffer. All columns were run at a flow rate of 40 ml/hr and 4 ml fractions were collected. Due to the heat-stability of the toxins and the absence of proteinases in this venom the columns were run at room temperature (20°C).

The crustacean toxin (most basic component of the venom, fraction II.9) was further purified in a CM-cellulose column (see Results).

Whenever it was necessary the fractions were dialysed against the appropriate buffers prior to rechromatography, using a Spectrapor 3M dialysis membrane (Spectrum Medical Industries, Los Angeles, CA). The purity of the fractions was followed by electrophoresis in polyacrylamide gel containing urea as described by Reisfeld et al. (1962), or in the presence of sodium dodecyl sulfate (SDS) according to Laemmli (1979).

Hyaluronidase activity was measured by the turbidimetric method of Tolksdorf et al. (1949), assuming that 1 unit of enzymatic activity is equal to the amount of enzyme required to reduce the concentration of hyaluronic acid (Sigma Chemical Co., St Louis, MO, grade I or grade III-P) from 0.2 to 0.1 mg/ml in 20 min at 25°C.

Chemical characterization of the toxins

Amino acid analysis was conducted using (after acid hydrolysis), a Durrum D-500 analyser. Duplicate samples were hydrolyzed *in vacuo* for 24 and 48 hr at 110°C in 6 N HCl. The values for serine and threonine were obtained by extrapolation to zero time, whereas the values for valine and leucine were obtained after 48 hr hydrolysis.

The *N*-terminal amino acid sequence of both toxins were determined by automatic Edman degradation (Edman and Beggs, 1967) in a Beckman 890M liquid-phase sequencer (Possani et al., 1985), following reduction and pyridylethylation (Possani et al., 1981a).

Electrophysiological experiments

The experiments were performed on primary cultures of dorsal root ganglion cells obtained from a 10 days old chick (*Gallus domesticus*). DRG cells were prepared as already described (Carbone and Lux, 1986) and used 6–12 hr following plating. During this period of incubation cells are of spherical shape, with no visible processes, and stick to the substrate. The experimental set-up, fabrication of patch electrodes and the I-V converter were similar to those described elsewhere (Carbone and Lux, 1984). Whole-cell clamp currents were measured according to the method of Hamill et al. (1981). Data were stored on a FM magnetic tape with a band width of 5 KHz. Analog recordings were digitized at a frequency of 12 KHz by a 12 bit A/D converter and analyzed by a LSI 11/23 minicomputer. Leakage and capacitative currents were reduced by analogue circuitry and the residual minimized by subtracting a scaled signal which was the average of ten hyperpolarizing pulses of appropriate amplitude.

Solutions were filtered through 0.22 μm Millipore filters before use. The external bath contained (mM): 120 NaCl, 3 KCl, 2 $CaCl_2$ and 10 Na-HEPES buffer solution (pH 7.3). The pipette filling solution was (mM): 130 CsCl, 10 tetraethylammonium chloride, 10 EGTA, 2 $MgCl_2$ and 10 Cs-HEPES buffer (pH 7.3). Stock solution of toxin II.6 was prepared by dissolving the lyophilized peptide in the external Ringer solution. Microliter amounts of the stock solution were then added directly to the bath via a Gilson pipette to the desired concentration. Since experiments were done only to test the effects of the toxins on cultured neurons, no attempts were made to verify recovery by washing the poisoned cells with appropriate buffers, as done previously (Carbone et al., 1982, 1984).

RESULTS AND DISCUSSION

Characterization of the soluble venom

Electrical stimulation of the telsons of 3922 scor-

Table 1. LD_{50} values of the venom in different strains of mice

Mouse strain	LD_{50} (mg/kg)
BALB/c (white)	2.78
BALB/k (white)	3.31
CD_1 (white)	3.30
C_{57} (black)	1.20
DBA (brown)	1.20
NIH (white)	1.32
SSA (white)	0.61

The LD_{50} were calculated by the method of Reed–Meunch (1938).

pions gave 1364 mg of soluble venom (calculated by absorbance at 280 nm and assuming that 1 absorbance unit, in a quartz cell with 1 cm pathway, is equal to mg/ml protein), with a mean value of 348 μg of venom protein per scorpion.

The lethality tests conducted with the soluble venom from this scorpion showed a considerable variation according to the mice strain used for LD_{50} determination (Table 1). The electrophoretic behaviour of soluble venom from *C. l. limpidus* is shown in Fig. 1. At least ten components were discriminated on the basis of charges (Fig. 1A, left lane) and many more different components (approx. 20) were shown to be present, under denaturating conditions, with sodium dodecyl sulfate (SDS) polyacrylamide gels (Fig. 1B, left lane). It is worth mentioning the broad band at the mol. wt corresponding to scorpion toxins (number 4 in Fig. 1B), suggesting the presence of a heterogeneous population of low mol. wt peptides in this position of the gel (mol. wt = 7000).

The specific activity of the hyaluronidase present in the soluble venom was 43.5 ± 5.6 U/mg protein ($N = 6$).

Chromatographic purification of two toxins from this venom

Seven chromatographic separations of soluble venom in Sephadex G-50 columns gave essentially the same results (Fig. 2). The mean value for the total protein recovery in the columns of Fig. 2 was 85.4%. Tubes corresponding to four fractions were pooled as indicated by the horizontal bars. Only fraction II was toxic. Recoveries, enzymatic activity and lethality tests are indicated in Table 2. The hyaluronidase activity was eluted only in Fraction I of Fig. 2, and corresponded to 95.6% of the total activity applied to the column. Fraction II from Sephadex G-50 applied to a CM-cellulose column gave reproducible results (Fig. 3) and the final recovery was 90% (Table 2). Components II.4 to II.8 were all toxic to mice, but not toxic to crustaceans. Polyacrylamide gel electrophoresis of the toxic components showed from two to four bands in each fraction (data not shown). Component II.9 was obtained by washing the column with 1 M NaCl (Fig. 3) and was shown to be toxic only to crustaceans. The only other toxic component to crustaceans was fraction II-3 (Fig. 3), which at a dose of 80 to 200 μg was toxic to crayfish. The most potent toxin to mouse (component II.6) and the fraction

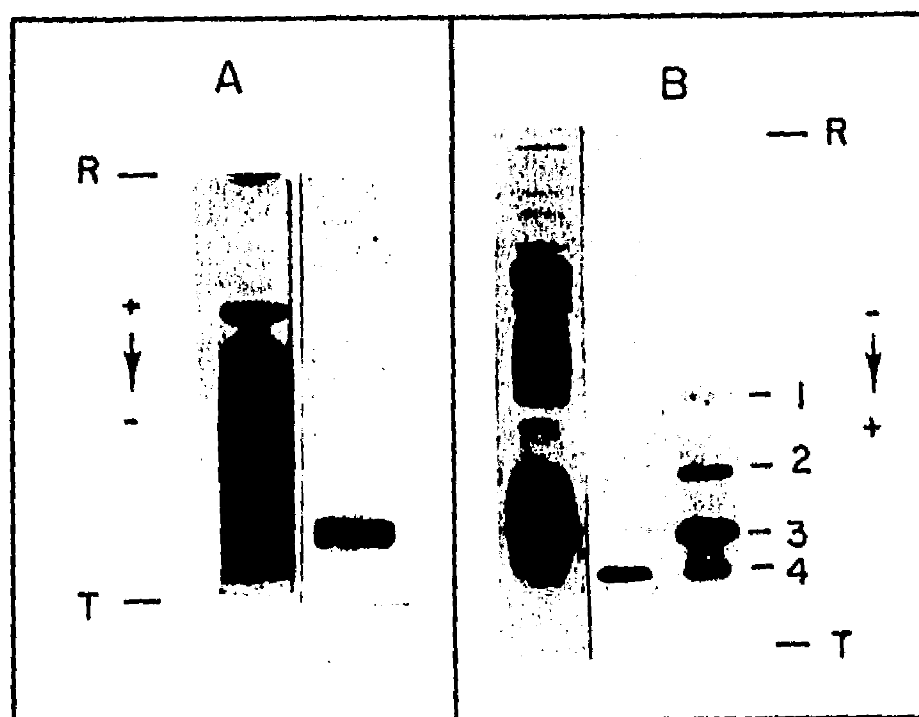

Fig. 1. Polyacrylamide gel electrophoresis separation of soluble venom and purified toxins from *C. l. limpidus*. A. Gel-electrophoresis separation in the β-alanine-acetate-urea system described by Reisfeld *et al* (1962). Left lane: 180 μg of soluble venom. The protein runs toward the cathode; approximately 10 bands were separated. Right lane: 13 μg of purified toxin II.6. B. Gel-electrophoresis in slab gel (15% acrylamide), containing SDS according to the technique of Laemmli (1970). Left lane: 140 μg of soluble venom. The proteins run toward the anode and at least 20 components were resolved. Middle lane: 10 μg of the crustacean toxin (II.9). Right lane: molecular weight markers: 1 (soy bean trypsin inhibitor, 21,500); 2 (lactoglobulin, 18,400); 3 (ribonuclease, 13,500); 4 (toxin II.9.2.2 from *C. noxius*, 7000).

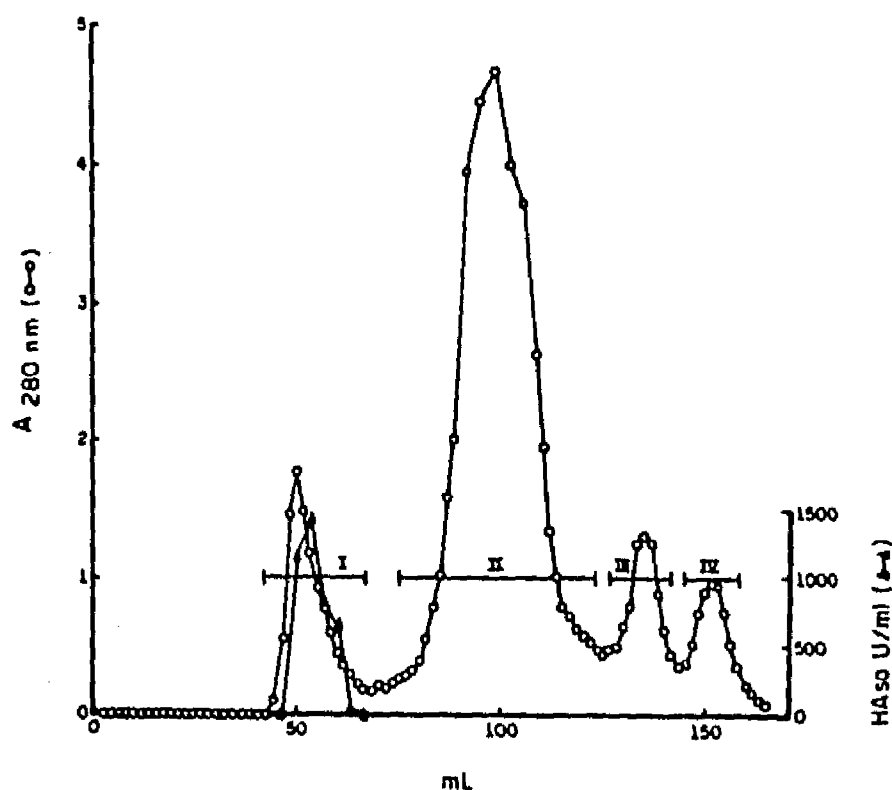

Fig. 2. Sephadex G-50 gel filtration of soluble venom. The column (0.9 × 200 cm) equilibrated with 0.02 M ammonium acetate buffer, pH 4.7, was loaded with 115.5 mg of *C. l. limpidus* venom and eluted with the same buffer at a flow rate of 20 ml/hr. Tubes containing 1.66 ml were pooled as shown by the horizontal bars (I to IV) according to the absorbance at 280 nm. Hyaluronidase activity is indicated in the figure.

II.9, toxic to crustaceans were further purified to homogeneity. For fraction II.6 three successive columns were used, whereas for fraction II.9 only one more steps was necesary (Fig. 4). The profile of the column shown on Fig. 4A was obtained in 50 mM ammonium acetate buffer, pH 5.6. Several components were separated; the main peak (II.6.2) was further chromatographed in a Bio-Rex 70 column (data not shown). The major component (II.6.2.2) corresponding to 66% of the material applied to the Bio-Rex 70 column, was finally separated on a CM-cellulose column as shown (Fig. 4B and right lane of Fig. 1A). Pure toxin II.6.2.2.2 represents approximately 1.1% of the protein present in the soluble venom and had a LD_{50} of 0.333 mg/kg mouse of the strain CD_1. This component injected at a dose of

Table 2. Recovery and lethality of chromatographic fractions

Column	Protein content (mg)	% Recovery*	Lethality LD_{50}†
Sephadex G-50 (Fig. 2)	Soluble venom (835.0)	100	3.3
	Fraction I (36.0)	4.3	Not toxic‡
	II (555.0)	66.5	2.9
	III (90.3)	10.8	Not toxic
	IV (32.0)	3.8	Not toxic
	Protein recovered	85.4	
CM-cellulose (Fig. 3)	Fraction II (490.0)	100.0	2.9
	II.1 (48.8)	9.9	Not toxic
	II.2 (53.6)	10.9	Not toxic
	II.3 (28.7)	5.8	Not toxic
	II.4 (169.6)	34.6	13.58
	II.5 (22.9)	4.7	0.89
	II.6 (56.4)	11.5	0.53
	II.7 (18.8)	3.8	0.66
	II.8 (23.8)	4.8	0.60
	II.9 (14.7)	3.0	Not toxic
	Protein recovered	90.0	

*The values reported are percentages calculated from the number of absorbancy units at 280 nm, as described in Materials and Methods.
†LD_{50} were determined by the method of Reed–Muench (1938).
‡Not toxic means that doses of 150 μg protein of the fraction per 20 g mouse weight was uneffective.

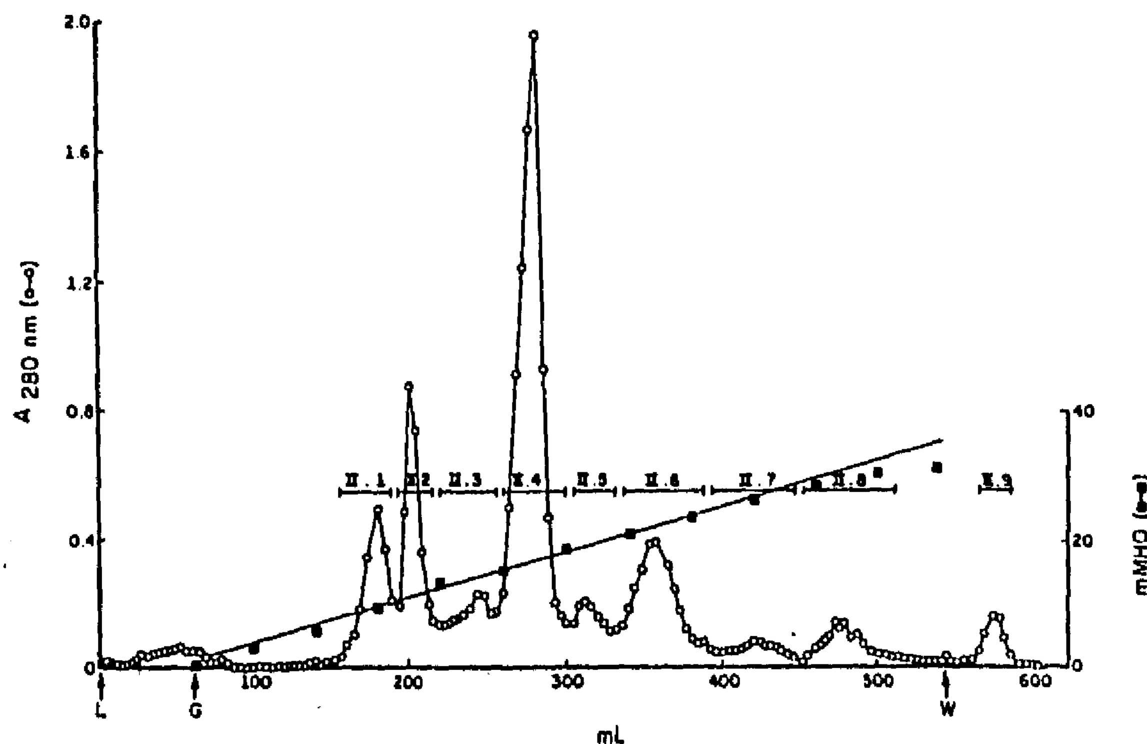

Fig. 3. Ion-exchange chromatography of fraction II. The toxic fraction from Sephadex G-50 was separated in a CM-cellulose column (0.9 × 30 cm) equilibrated and run with 0.02 M ammonium acetate buffer, pH 4.7. Fraction II (100 mg) was applied to the column and eluted with salt at a flow rate of 40 ml/hr; a linear gradient was formed by mixing 250 ml of buffer in 0 M NaCl with 250 ml of buffer in 0.55 M NaCl. Fractions of 3.9 ml were collected and pooled according to the absorbance at 280 nm (horizontal bars). The arrow with L means loading sample; G means starting point of the gradient; W means washing with 1 M NaCl.

0.75 μg per "cochinilla" was not toxic, giving essentially the same results as the control animal injected with saline. The toxin at a dose of 73 μg per crayfish was also not toxic.

The crustacean toxin was obtained in pure form after the CM-cellulose column shown (Fig. 4C and middle lane of Fig. 1B). Component II.9.2 corresponds to approximately 0.9% of the initial soluble venom. Eight out of ten "cochinillas" injected with 0.50 μg of toxin II.9.2 died within the first 12 hr after injection. Three crayfish with 25 g body weight injected with 25, 50 and 125 μg of toxin II.9.2 died with all the symptoms of intoxication, within 12 hr of injection. The same component II.9.2 injected at a dose of 150 μg/20 g mouse weight was not toxic. In order to simplify notation toxic components II.6.2.2.2 and II.9.2 will be called simply toxin II.6 and II.9 respectively.

Chemical charaterization of toxins II.6 and II.9

The apparent mol. wt of both toxins by SDS polyacrylamide gel was near 7000 (see Fig. 1B). The amino acid composition of the toxins shown in Table 3 revealed the lack of isoleucine and methionine in either toxin. The latter amino acid (Met) is absent in most scorpion toxins (Possani *et al.*, 1977). The crustacean toxin II.9 is a slightly larger molecule than most mammalian scorpion toxins, generally having 60–65 amino acid residues. Toxin II.9 has approx. 70 amino acid residues, similar to the insect toxin from *Androctonus australis* Hector which has 71 amino acids (Darbon *et al.*, 1982).

The *N*-terminal amino acid sequence of toxins II.6 and II.9 are compared in Table 4 with some representative toxins from other known scorpion species. Toxins II.6 and II.9 are different not only with respect to the biological activity: mammalian and crustacean specificity respectively, but also with regard to their amino acid composition and number of residues (Table 3) as well as differences in the amino acid sequence (Table 4). However, both toxins have 65% of the positions occupied by identical amino acid residues at the *N*-terminal sequences including the cysteine residues, which are important for disulfide bridge formation and hence for stabilization of the three dimensional structure of the molecules.

If we compare the mammalian toxin II.6 from *C. l. limpidus* with similar toxins from other *Centruroides* scorpions (Table 4) the similarity is even greater. Toxin *C. s. suffusus* II shows 90% identity, toxin *C. sculpturatus* I shows 70% identity and toxin *C. noxius* II-14 has 65% identity. All the cysteine residues are conserved at equivalent positions. If we compare toxin Cll II.6 with toxin γ from the Brazilian scorpion *T. serrulatus* we find less similarities (50% identity); but if the comparison is made with mammalian toxins from the North African scorpion from the genus *Androctonus* and *Leiurus* the difference is greater, and also some of the cysteine residues are not conserved at the same position. The insect toxin (IT)

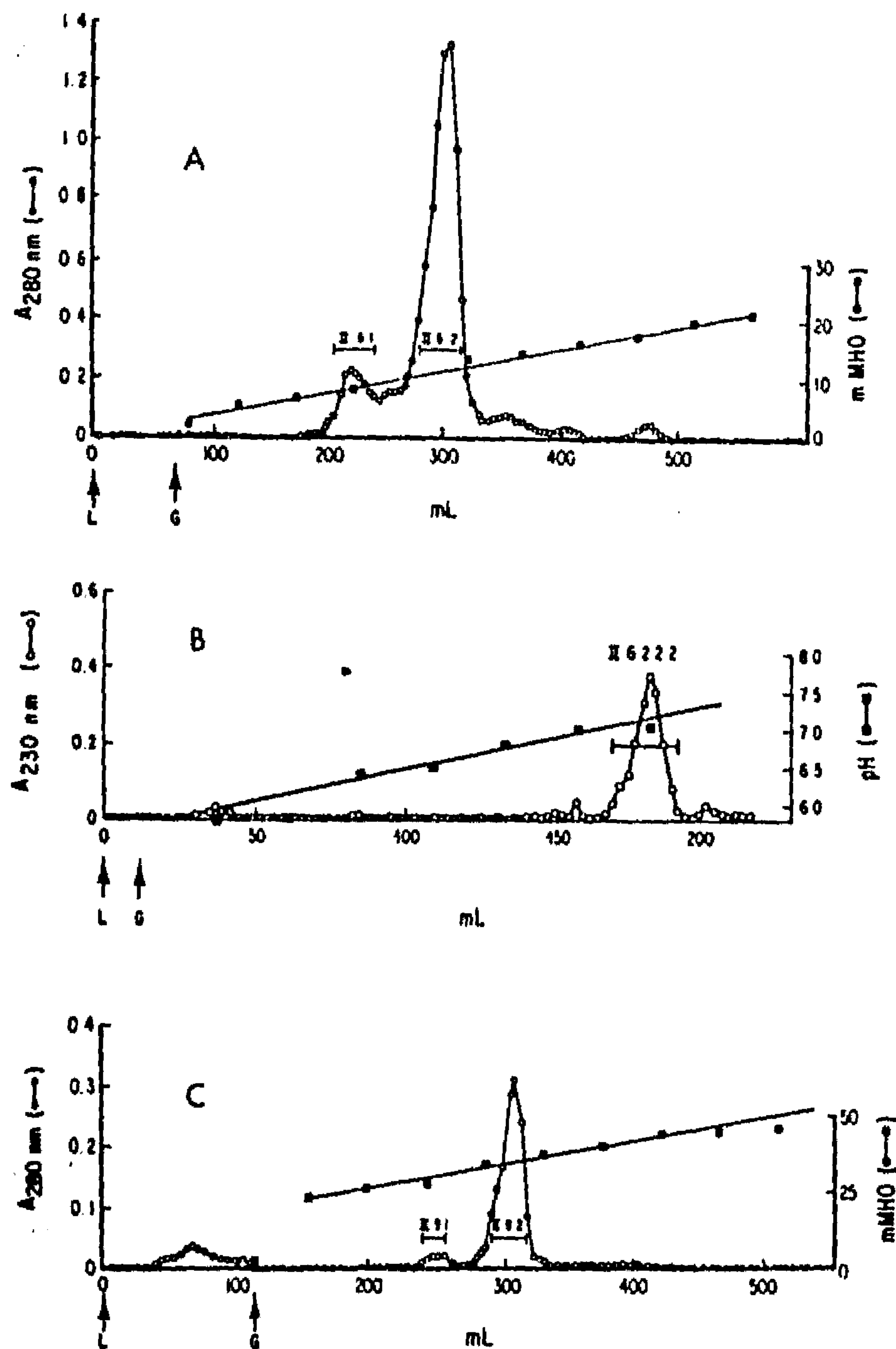

Fig. 4. Ion-exchange chromatographic separation of toxic sub-fractions. A. Fraction II.6 from Fig. 3 (51.5 mg) was applied to a CM-cellulose column (0.9 × 30 cm) equilibrated and run in 0.05 M ammonium acetate buffer, pH 5.6, at a flow rate of 40 ml/h with a salt gradient from 0-0.4 M NaCl (250 ml each). Tube fractions of 4.6 ml volume each were collected and pooled according to the horizontal bars. Toxin II.6.2 corresponded to 25 mg protein, 48.5% of the material applied to the column. B. Final purification of fraction II.6.2.2 from Bio-Rex 70 (see Results). A CM-cellulose column (0.9 × 30 cm) was equilibrated in 0.075 M ammonium acetate buffer, pH 5.8. Toxin (5.5 mg) was applied to the column and eluted at flow rate of 10 ml/h with a linear pH gradient (total volume 200 ml) from 5.8 to 8.0, in 2.42 ml fractions. The absorbance was read at 230 nm and the fractions corresponding to toxin II.6.2.2.2 were pooled, as indicated by the horizontal bar. Purity of sample is shown in Fig. 1A. C. Final purification of the crustacean toxin (II.9.2) in a CM-cellulose column (0.9 × 30 cm) equilibrated and run with 0.02 M ammonium acetate buffer, pH 4.7 at a flow rate of 40 ml/hr. Fraction II.9 (11.7 mg) was applied to the column and eluted with a linear gradient from 250 ml buffer containing 0.4 M NaCl to 250 ml of the same buffer containing 1.0 M NaCl. Fractions of 4 ml each were collected and pooled as indicated by the horizontal bars. Purity of sample is indicated in Fig. 1B.

Table 3. Comparison of amino acid composition of pure toxins II.6 and II.9 with other American scorpion toxins*

Amino acid	Cll† II.6	Cll† II.9	Clt II.9.3	Cn II.9.2.2	Cn II.14	Ts gamma	CsE I	Css II
Asp	5	7	5	6	8	4	6	5
Thr	3	3	3	2	4	1	5	3
Ser	2	4	2	1	3	4	2	4
Glu	7	5	7	7	3	3	5	7
Pro	1	2	2	2	2	3	3	2
Gly	8	10	8	7	9	8	9	6
Ala	3	3	3	4	1	3	0	3
1/2Cys	nd‡	nd	8	8	8	8	8	8
Val	2	2	3	2	1	2	1	3
Met	0	0	0	0	1	1	0	0
Ile	0	0	1	1	0	2	1	0
Leu	5	3	4	6	3	3	4	6
Tyr	6	5	6	6	7	5	6	7
Phe	1	2	2	1	1	1	2	1
His	3	2	2	1	1	1	1	1
Lys	5	7	6	8	8	6	8	7
Trp	nd	nd	1	1	1	3	2	2
Arg	1	3	2	2	3	3	1	1
Total	nd	nd	65	65	64	61	64	66

*Cll II.6 and II.9 are from *C. l. limpidus*, this work; Clt II.9.3 from *C. l. tecomanus*, Possani *et al.* (1980); Cn II.9.2.2 and II.14 from *C. noxius*, and Ts gamma from *T. serrulatus*, Possani *et al.* (1985); CsE I from *C. sculpturatus*, Babin *et al.* (1975); Css II from *C. s. suffusus*, Garcia (1976).

†Cll, nearest integer number of amino acid, obtained by acid hydrolysis at 24 and 48 hr, as described in Materials and Methods.

‡nd means not determined.

from *A. australis* has 40% identity with Cll II.6, while *L. q. quinquestriatus* IV has 27%, and toxin *A. australis* I has only 20% identity (Table 4). These results indicate that the mammalian toxins from the North American scorpions are more closely related to each other than the South American or North African scorpion toxins. They also show that the similarity between the North American and the Brazilian scorpion toxins are greater than to the North African, which confirms results previously found when comparing sequence homologies of scorpion toxins from these three distinct geographical localizations by the more rigorous method of metric analysis (see Possani *et al.*, 1985, Erickson and Sellers, 1983). If we compare the crustacean toxin Cll II.9 with toxin γ from *T. serrulatus* the number of identical positions is 38% (data not shown) with conserved cysteine residues, but it is only 27% identical when compared with both North African AaH I and Lqq IV, without conservation of one of the cysteine positions. The insect toxin IT from *A. australis* showed more homology to crustacean toxin Cll II.9 (38%), but also with one residue out of register.

Electrophysiological studies of toxin II.6

Figure 5a shows a family of membrane currents recorded from a whole cell clamped chick DRG bathed in a Ringer solution containing 120 mM NaCl, 2 mM $CaCl_2$ and internally perfused with 130 mM CsCl, 10 mM tetraethylammonium chloride. Under these conditions, outward potassium currents are minimized and the major portion of membrane currents flow inward through open Na and Ca channels (see Fig. 3 in Carbone and Lux, 1986). At positive potentials, Na currents (I_{Na}) activate quickly and inactivate within 8 ms (at temperature of 18°C). Calcium currents (I_{Ca}) activate also rather quickly but inactivate very slowly in the presence of a strong intracellular Ca^{2+} – buffer (10 mM EGTA). Thus, for pulses lasting 12 ms, I_{Na} can be roughly identified

Table 4. Comparison of the *N*-terminal amino acid sequence of toxins II.6 and II.9 of *C.l.limpidus* with other known scorpion toxins*

	1 5 10 15 20 25 30	(%)†
Cll II.6	- - K E G Y L V N H S T G C K Y E C Y K L G D N D Y C L R E C K...	100
Css II	- - K E G Y L V S K S T G C K Y E C L K L G D N D Y C L R E C K...	90
CsE I	- - K D G Y L V E - K T G C K K T C Y K L G E N D F C N R E C K...	70
Cn II.14	- - K D G Y L V D A K - G C K K N C Y K L G K N D Y C N R E C R...	66
Cll II.9	- K K D G Y L V N K Y T G C K V N C Y K L G E N K P C N R E ...	65
Ts γ	- - K E G Y L M D H - E G C K L S C F I R P S G - Y C G R E C G...	50
AaH IT	- K K N G Y A V D - S S G K A P E C L - L - S N - Y C N N Q C T...	40*
AaH I	- K R D G Y I V Y P N N - C V Y H C V P P C D - G L C K K N G G...	20
Lqq IV	G V R D A Y I A D D K N - C V Y T C - - - G S N S Y C N T E C T...	27

*Cll II.6 and Cll II.9 toxins from *C.l. limpidus*, this work; Css II is a toxin from *C.s.suffusus*, Garcia (1976); CsE I is from *C.scupturatus*, Babin *et al.* (1975); Cn II.14 from *C. noxius* and toxin γ from *T.serrulatus* (Possani *et al.* (1985); AaH IT and AaH I are toxins from *A. australis*, Darbon *et al.* (1982) and Rochat *et al.* (1970), respectively; Lqq IV is from *L.q.quinquestriatus*, Kopeyan *et al.* (1982).

†Percentage of identity was calculated by taking the number of identical amino acids in the same equivalent position and dividing by the number of total amino acids under comparison. Note that blank spaces were included "–" in order to increase homology. Alignments of cysteines were taken as guide-lines for the introduction of the blank spaces.

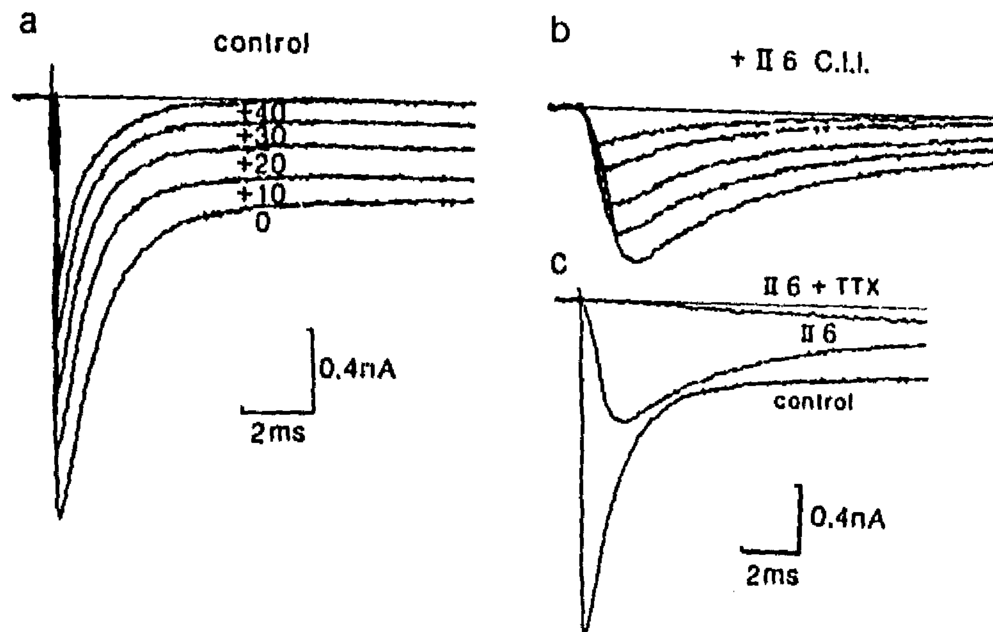

Fig. 5. Electrophysiological effects of pure toxins on DRG cells. Action of toxin II.6 on Na^+ and Ca^{2+} currents recorded from a chick dorsal root ganglion cell at the membrane potentials indicated. (a), (b): inward current records obtained before (control) and after 8 min addition of 1 μM CII II.6 to the bath. In (c) are shown the current traces recorded at 0 mV before (control) and during application of 1 μM of toxin II.6 superimposed with the membrane current remaining after addition of 0.3 μM Tetrodotoxin (TTX) to the bath. V_h: −80 mV. Ionic solutions as described in Materials and Methods. Temp. 18°C.

with the transient phase of the current and I_{Ca} with the steady-state level reached near the end of the pulse.

As shown in Fig. 5b, application of 1 μM of toxin II-6 to the external bath caused a drastic modification of the time course of both Na and Ca currents. In the potential range examined, the following features were consistently observed: (i) the peak current amplitude (I_{Na}) decreased by about 60% (58 ± 12% in five cells), (ii) the steady state level of the current (I_{Ca}) nearly halved, (iii) the time to peak and the time constant of inactivation increased by a factor of 3 and 6, respectively, (iv) the toxin-modified membrane currents were almost fully blocked by addition of 0.3 μM of Tetrodotoxin (TTX) to the external bath (Fig. 5c). All this suggested that toxin II-6 exerts a depressive action on both Na and Ca currents. Ca currents activated at positive potentials (Carbone and Lux, 1984) appear to be almost completely blocked by toxin II-6 while Na currents are only partially reduced. The time course of the toxin-modified Na current show strong prolongations.

Our data do not allow more quantitative conclusions, but it is interesting to point out that the action of toxin II-6 on the membrane currents of chick DRG has some similarity with those of other scorpion toxins with close amino acid sequence. Thus, toxin *C. s. suffusus* II (which has 90% identity with our toxin II-6, has been shown to reduce the size of Na currents in skeletal muscle at concentrations below 1 μM (Jaimovich *et al.*, 1982). Toxin *C. sculpturatus* I and toxin γ from *T. serrulatus* (which show 70 and 50% identity, respectively) have been reported to affect the voltage-dependent probability of Na channel opening in various preparations (Meves *et al.*, 1982; Vijverberg, 1984). Finally, we found that 1 μM of toxin *C. noxius* II-14 (66% identity) causes also a marked reduction of Na and Ca currents in chick DRG neurons (Carbone and Possani, unpublished).

In conclusion, the structural identities outlined here might support close biological and physiological actions reported among various toxins of different scorpions.

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