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CARACTERIZACION FUNCIONAL Y ESTRUCTURAL DE LA NOXIUSTOXINA

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN INVESTIGACION BIOMEDICA BASICA

P R E S E N T A

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- ¿Quisiera usted decirme que
camino debo tomar para irme
de aquí?

- Eso depende, en mucho, del lugar
a donde quiera ir -respondió el gato.

- No me preocupa mayormente el
lugar ... - dijo Alicia.

- En tal caso poco importa el camino
- respondió el gato.

- ... con tal de llegar a alguna parte
añadió Alicia, a manera de explicación

- ¡Oh! -dijo el gato - puede usted
estar segura de llegar, con tal de que
camine durante un tiempo bastante largo.

Lewis Carroll (Alicia en el
país de las Maravillas)

CONTENIDO

Prefacio

En años recientes se han purificado, a partir del veneno de alacranes, pequeños péptidos tóxicos, que tienen efecto sobre diferentes tipos de canales de potasio en diversos tejidos (Carbone *et al.*, 1982; Possani *et al.*, 1982; revisado por: Castle *et al.*, 1989; Blaustein *et al.*, 1991).

Los estudios realizados sobre estas toxinas han sido muy importantes no sólo para identificar y comprender las propiedades biofísicas de dichos canales, sino que también han resultado ser instrumentos idóneos para el aislamiento y caracterización de las mismas proteínas que conforman los mencionados canales (Prestipino *et al.*, 1989; García-Calvo *et al.*, 1994).

Es importante mencionar que la estructura primaria de estos péptidos es muy semejante (entre ellos) y, probablemente, tienen una configuración tridimensional con algún "motivo" estructural conservado (Ménez *et al.*, 1992), aunque el mecanismo molecular de acción, en términos de afinidad y especificidad, varía considerablemente.

De acuerdo con lo antes mencionado, nuestro trabajo pretende incrementar el conocimiento de la relación 'estructura-función' de toxinas sobre canales de potasio, tomando en cuenta las siguientes consideraciones:

a) Buscamos caracterizar la región de la molécula de noxiustoxina que interacciona con su receptor en membranas de cerebro de rata;
b) nuestras investigaciones procuran describir el efecto de modificaciones químicas de la toxina en esta interacción;
c) también buscamos determinar la posición de los puentes disulfuro en esta molécula para delinear en forma más completa su estructura;
d) por último, emprendimos la tarea de comparar la estructura y sitio activo de nuestra toxina con otra

análoga: en este caso, la caribdotoxina. De este modo completamos la investigación que nos hemos impuesto realizar.

Por lo que respecta a la presentación de la investigación y sus resultados, la hemos dividido en cinco grandes apartados, como hemos señalado en el índice-contenido. En la introducción describimos el cuadro completo de los elementos estudiados, como una referencia. A continuación presentamos los objetivos propuestos a realizar, los cuales estan avalados en los resultados y que son presentados aquí en formato de artículos. Estos se han dividido en dos secciones. La primera contiene un solo artículo, el cual se encuentra en proceso de dictaminación para ser publicado, antecedido por su ficha bibliográfica. En la segunda sección se incluyen tres artículos ya publicados. También llevan antepuesta su respectiva ficha bibliográfica.

En las últimas secciones se presenta una discusión en la que se incluyen los aspectos más relevantes del trabajo de tesis, cuyos detalles se localizan en los artículos incluídos en el punto III.

Abreviaturas

Aa H I	Toxina I del veneno del alacrán *Androctonus australis*
Aa H II	Toxina II del veneno del alacrán *Androctonus australis*
Cll 1	Toxina 1 del veneno del alacrán *Centruroides limpidus limpidus*
Cll 2	Toxina 2 del veneno del alacrán *Centruroides limpidus limpidus*
Cn 2	Toxina 2 del veneno del alacrán *Centruroides noxius*
Cs E v 3	Toxina variante 3 del veneno del alacrán *Centruroides sculpturatus*
CTX	Caribdotoxina
DR K	Canal de potasio rectificador tardío
DTX	Dendrotoxina
IbTX	Iberotoxina
IC_{50}	Concentración de inhibición al 50%
^{125}I-CTX	CTX marcada con iodo radiactivo
^{125}I-DTX	DTX marcada con iodo radiactivo
^{125}I-NTX	NTX marcada con iodo radiactivo
IR K	Canal de potasio rectificador entrante
K_{Ca}	Canal de potasio dependiente de calcio
Kd	Constante de afinidad
Ki	Constante de inhibición
KTX	Kaliotoxina
MgTX	Margatoxina
NMR	Espectroscopía de resonancia magnética nuclear
NTX	Noxiustoxina
T K	Canal de potasio de corriente transitoria
TsTX Kα	Tityustoxina Kα

Resumen

El tema de esta tesis se centra en la identificación de la región de la molécula de noxiustoxina (NTX) que interacciona con su receptor en membranas de cerebro de rata.

La NTX es un péptido formado por 39 aminoácidos (Possani *et al.*, 1982) estabilizado por tres puentes disulfuro, que bloquea canales de potasio en diferentes tejidos.

Por medio de digestión enzimática con la endopeptidasa Lisina-C, se logró identificar la posición de los puentes disulfuro: cis7-cis29, cis13-cis34 y cis17-cis36. Por otro lado, utilizando péptidos sintéticos se mostró que los fragmentos correspondientes a la secuencia amino terminal de esta toxina, son capaces de competir con ^{125}I-NTX en la unión a membranas del cerebro de rata, no así los fragmentos de la región carboxilo terminal.

A su vez, se probó el efecto que ciertas modificaciones químicas pueden tener sobre la capacidad de unión de la toxina al receptor. Se encontró, por ejemplo, que la hidrólisis con proteasa V8 (en la posición 19 que contiene ácido glutámico) modifica la constante de afinidad de esta toxina por el receptor, no así la ruptura en la metionina 30, con bromuro de cianógeno.

Por medio del modelado molecular, por computación, a partir de la estructura obtenida por NMR para la caribdotoxina, se obtuvo un modelo tridimensional de la NTX. Se pudo observar que la primera región de configuración beta plegada y la región de alfa hélice se encuentran en el mismo lado de la molécula, abarcando del aminoácido 1 al 20. Este segmento de la molécula parece estar involucrado en el reconocimiento de los canales de potasio de membranas de cerebro de rata y en el caso de la caribdotoxina la región más relevante para su interacción con el mismo receptor se encuentra hacia el extremo carboxilo terminal.

Summary.

The principal objective of this thesis was to identify the molecular region of noxiustoxin (NTX) that binds to rat brain membranes.

Noxiustoxin is a basic peptide isolated from the venom of the scorpion *Centruroides noxius*. Its primary structure and effect on K^+ channels are known (Possani *et al.*, 1982; Carbone *et al.*, 1982).

Selective enzymatic digestion of NTX by exposure to Lys-C endoproteinase produced fragments capable of defining the disulfide bond arrangements of NTX: cys7-cys29, cys13-cys33 and cys17-cys36.

We studied the effect of several synthetic peptides corresponding to various segments of the N-terminal and C-terminal regions of noxiustoxin on ^{125}I-NTX binding to rat brain synaptosomes. Only the N-terminal peptides inhibited ^{125}I-NTX binding. These findings suggest that the N-terminal domain of NTX is involved in the toxin-channel interaction.

Computer modeling using the three dimensional structure obtain from ^{1}H-NMR spectroscopy of Charybdotoxin and chemical and enzymatic rupture of Noxiustoxin with cyanogen bromide and protease V8 from *staphylococcus aureus* confirms that the N-terminal segment, including an alpha-helix from residues 10-20 are important for channel recognition. A synthetic peptide corresponding to the C-terminal sequence (24-37) of Charybdotoxin, however, showed that it is the important part for the binding of this molecule to rat brain synaptosomal membranes.

"Parikuté, el dueño de los animales, tenía temor de que el alacrán hiciera daño a la gente y pidió al zopilote que acarreara un canasto de los que usan los chamanes. Debía colocarlo en una repisa alta. No le dijo que contenía el más peligroso de los alacranes y le ordenó que no mirara, pues lo que estaba aprisionado podía escapar. Pero el zopilote era curioso y abrió el canasto; el alacrán salió y se escondió"

Mito Huichol.

(P. Furst y S. Najad, 1972)

I. Introducción

1.1 Las toxinas como herramientas en el estudio de receptores

Una gran variedad de organismos terrestres y marinos producen toxinas. Estos las utilizan como medio de defensa contra depredadores o como arma inmovilizadora para atrapar a las presas, que son su sustento. Esta es una realidad, que por otra parte el hombre ha podido constatar a través de la observación empírica. Pero esta situación no ha quedado ahí. También, a través del tiempo y de las diversas culturas en el mundo, el hombre ha sido capaz de utilizar las toxinas en prácticas medicinales o en prácticas ceremoniales. Recientemente muchas toxinas se han adaptado para uso terapéutico o para diagnóstico o como agentes insecticidas. E incluso, han servido como herramientas que revelan mecanismos fisiológico, celulares y moleculares de varios receptores (Adams y Olivera, 1994).

Con el advenimiento de la clonación molecular y el incremento en el número de canales y receptores identificados, la necesidad de estudiar y de utilizar, las toxinas selectivas, como herramientas farmacológicas se hace indispensable en nuestros días. (Castle *et al.*, 1989; Rehm, 1991).

De este modo los venenos de los animales ponzoñosos se han convertido en una valiosa fuente de compuestos de este tipo. Pues estos animales producen una diversidad importante de toxinas que actúan sobre diferentes tipos de receptores que afectan el sistema nervioso o muscular de sus presas. Algunas de estas toxinas se han estudiado ampliamente respecto a su estructura, modo de acción y localización de su sitio funcional. Tal es el caso de las toxinas que actúan sobre el receptor de acetil colina, en particular las alfa-conotoxinas y toxinas de veneno de serpientes (alfa bungarotoxina y cobratoxina) que contribuyeron exitosamente al aislamiento del receptor nicotínico de acetil colina (Silveira y Daja, 1994).

Dentro de la subunidad alfa del canal de sodio se han podido identificar dominios funcionales por medio de experimentos fisiológicos y bioquímicos usando diferentes toxinas como ligandos específicos. Hasta la fecha se han identificado más de cinco sitios distintos de unión de toxinas, algunos de ellos se han asociado con activación, inactivación y propiedades de permeación del canal (Trainer *et al*, 1993).

Algunas toxinas con diferentes especificidades se han utilizado en la purificación de receptores, por ejemplo: el receptor de glicina, usando estricnina (Betz, 1991); y para el canal de calcio tipo N se usó omega-conotoxina GVIA (McEnery *et al.*, 1991). Algunas de las proteínas de las terminales nerviosas involucradas en la liberación de neurotransmisor vesicular se purificaron utilizando a las toxinas tetánica y botulínica y a la latrotoxina (Schiavo et al., 1992; Blasi et al., 1993; Petrenko et al., 1991).

Tanto en la purificación como en la clonación de canales y receptores, las toxinas de los venenos son útiles para determinar la presencia y la integridad funcional de la(s) proteína(s).

1.2. Canales de potasio

Los canales iónicos son moléculas que forman poros en las membranas celulares que permiten el flujo de iones. Estas moléculas permiten principalmente el paso de un tipo de ión por lo cual se les ha clasificado de acuerdo con su selectividad en canales de sodio, potasio, calcio y cloro.

Estos canales están ampliamente distribuidos en la células eucariotas y controlan la excitación, secreción y movilidad (Jan y Jan, 1989).

En el caso del ión potasio, se han caracterizado varios canales, mostrándose que éstos están involucrados en distintos procesos fisiológicos, como la actividad eléctrica neuronal, contracción muscular, procesos secretorios, proliferación y regulación del volumen celular (Rudy B., 1988).

Desde el punto de vista funcional, los canales de

potasio, se han clasificado en: 1. Dependientes de voltaje que incluyen tres clases, a) rectificadores tardíos (DR K), b) los de corriente transitoria (T K) y c) los rectificadores entrantes (IR K); 2. Los canales de potasio que, además del voltaje, requieren de ligandos, se les ha denominado canales de potasio activados por ligandos (L K). Los canales de esta clase tienen propiedades de DR, T o IR; pero además del potencial de membrana, requieren de iones como calcio o sodio o un nucleótido como ATP o una proteína G para su activación (Brown, 1993).

Los canales de potasio dependientes de voltaje están formados por subunidades que fluctuan en un rango de 400 a 700 aminoácidos y cuatro de estas subunidades se ensamblan como homo o heterotetrámeros. Por último un péptido adicional parece estar asociado con el canal de potasio, pero su función aún es desconocida. (Parcej *et al.*, 1992; Kanaus *et al.*, 1994).

En virtud de la ausencia de datos estructurales de los canales iónicos, los estudios funcionales han proporcionado gran información sobre la activación, corrientes de "disparo", permeación iónica y transición entre los estados cerrado, abierto e inactivado de los canales. A los elementos responsables de estas funciones, se les ha dado nombres tales como los de: sensor de voltaje, "disparo", poro y filtro de selectividad y se han ordenado en un modelo electromecánico.

Recientemente, el enfoque de estos estudios ha cambiado de estrictamente funcional, al de utilización de análisis mutacional y mediciones electrofisiológicas, debido a que muchos de los genes que codifican para estos canales se han clonado.

Estructuralmente, cada subunidad de los canales de potasio tiene 6 segmentos transmembranales (excepto los de tipo IR que sólo tienen dos de estos segmentos); por su parte los dominios amino y carboxilo terminales se encuentran situados en el lado citoplasmático.

Asimismo entre las zonas transmembranales 5 y 6, llamadas S5 y S6, se encuentra el poro (es decir, la zona formada por

las 4 subunidades) por donde pasan los iones. La región que se piensa es el sensor de voltaje, se encuentra en el dominio S4. De la misma manera se han asociado tres zonas de la secuencia de aminoácidos de los canales de potasio con diferentes tipos de inactivación: la región amino terminal, la sexta región transmembranal, S6, y la región del poro (Miller, 1991).

Hasta el momento se han clonado genes para una serie de canales de potasio de diversas especies y tejidos, habiéndolos clasificado en cuatro grupos de acuerdo a sus semejanzas estructurales (Jan y Jan, 1990a; Chandy, 1991).

Por expresión de mensajeros de diferentes canales de potasio en oocitos se ha demostrado que éstos pueden formar heterotetrámeros. Lo anterior puede explicar la diversidad que estos canales muestran en la naturaleza (Jan y Jan, 1990b; Stühmer *et al.*, 1989).

El grupo de Dolly demostró la presencia de estructuras heterotetraméricas, "in vivo", (Scott *et al.*, 1994), utilizando anticuerpos monoclonales contra diferentes tipos de canales de potasio, encontrando que varios de estos anticuerpos reconocían a un solo receptor purificado.

Se ha demostrado, utilizando anticuerpos monoclonales, una distribución específica de canales de potasio en diferentes partes del cerebro de la rata (McNamara *et al.*, 1993; Beckh y Pongs, 1990).

1.3. Toxinas de alacrán y canales de potasio

El veneno de los alacranes es una fuente de péptidos de bajo peso molecular que son tóxicos a una variedad de organismos, incluyendo al hombre (Zlotkin *et al.*, 1978; Possani, 1984). La mayoría de los venenos estudiados contienen dos clases de toxinas; péptidos de cadena larga (60-70 aminoácidos) que bloquean canales de sodio de membranas excitables (Catterall, 1977; Couraud *et al.*, 1982) y péptidos de cadena corta (30 a 40 aminoácidos) que afectan diferentes clases de canales de potasio (Possani, 1984; Moczydlowski *et al.*, 1988; Castle *et al.*, 1989).

Las toxinas que actúan sobre canales de potasio tienen características comunes, como: a) baja concentración relativa en los venenos, b) ser péptidos de cadena corta, c) ser básicos, d) estructuralmente compactos y e) poder soportar condiciones drásticas de temperatura y acidez (Possani, 1984).

Examinando su estructura primaria se puede notar que éstas se pueden alinear de acuerdo a la posición de las cisteínas, lo que indica que posiblemente la posición de los enlaces disulfuro esté conservada (Ménez *et al.*, 1992).

Se ha investigado la estructura tridimensional de algunas de estas toxinas (caribdotoxina e iberotoxina) utilizando espectroscopía de resonancia magnética nuclear. Esta técnica revela que dichas toxinas son moléculas globulares, con una región de triple lámina beta plegada, unida a una de alfa hélice a través de dos de los tres enlaces disulfuro presentes en estas moléculas (Bontems *et al.*, 1992; Johnson y Sugg, 1992).

Cuando hacen una comparación de la estructura de las toxinas de cadena larga (variante 3 de *Centruroides sculpturatus* y toxina II de *Androctonus australis* Hector) con la de las toxinas de cadena corta (caribdotoxina), Ménez *et al* (1992) observan que existen semejanzas estructurales. Los principales elementos de estructura secundaria y los enlaces disulfuro se sobreponen en ambas clases de toxinas y se encuentra un núcleo estructural conservado para estas toxinas. Por esta razón se propone que muchas de las toxinas de alacrán tienen una estructura secundaria conservada y por lo tanto, un "motivo" estructural común (Menez *et al.*, 1992).

El núcleo estructural puede aceptar numerosas mutaciones de un solo sitio; la región de alfa hélice y las dos regiones beta plegada del extremo C-terminal, tienen pocos cambios de una toxina a otra en términos de longitud. En contraste, los segmentos que unen a los elementos conservados pueden acomodar asas, que se caracterizan por el gran número de mutaciones y por ser de longitud variable. Por ejemplo, las dos regiones de beta plegada que se localizan en la parte C-terminal de la

molécula, están unidas una a la otra por dos residuos de aminoácidos en caribdotoxina, variante 3 y toxina I de *Androctonus australis*, y por siete residuos en la toxina II de *Androctonus australis*. En estas toxinas se encuentra una secuencia invariable :
-cis7-[...]-cis13-aa-aa-aa-cis17-[...]-cis28-[...]-cis33-aa-cis35-[..]- (tomando la posición en la secuencia de la caribdotoxina) (ver figs. 1 y 2). Lo anterior nos hace suponer que los pequeños cambios en secuencia entre las toxinas que actúan sobre canales de potasio confieren un reconocimiento mayor o menor hacia un receptor dado.

Así existe una serie de toxinas conocidas que tienen acción sobre canales de potasio:

La primera toxina, con efecto sobre canales de potasio, descrita en la literatura fue la noxiustoxina (NTX) (Carbone *et al.*, 1982; Possani *et al.*, 1982). En vista de que la NTX es el principal objeto de este trabajo se describirá en forma más detallada al final de esta sección.

La caribdotoxina (CTX) es un péptido de 37 aminoácidos básico que se aisla del veneno del alacrán *Leiurus quinquestriatus hebraus* (Miller *et al.*, 1985). Esta toxina bloquea canales de K^+ dependientes de Ca^{2+} de alta conductancia del músculo esquelético, con una Kd=1-3 nM (Valdivia *et al.*, 1988). También sabemos que se une con alta afinidad a membranas de cerebro (Kd=30 pM) (Vázquez *et al.*, 1990) y también bloquea un canal de K^+ dependiente de voltaje de linfocitos (Kd=14 pM) (Deutsch *et al.*, 1991). En este veneno encontramos también a la leiurotoxina I (scyllatoxina), un péptido de 31 aminoácidos, que presenta una similitud de acción con la apamina (péptido de 18 aminoácidos proveniente del veneno de la abeja *Apis melifera*), ya que ambas toxinas bloquean el canal de K^+ dependiente de calcio de pequeña conductancia (Auguste *et al.*, 1990).

En cuanto a un péptido de 37 aminoácidos, llamado iberiotoxina (IbTX) tenemos referencia de que se encuentra en

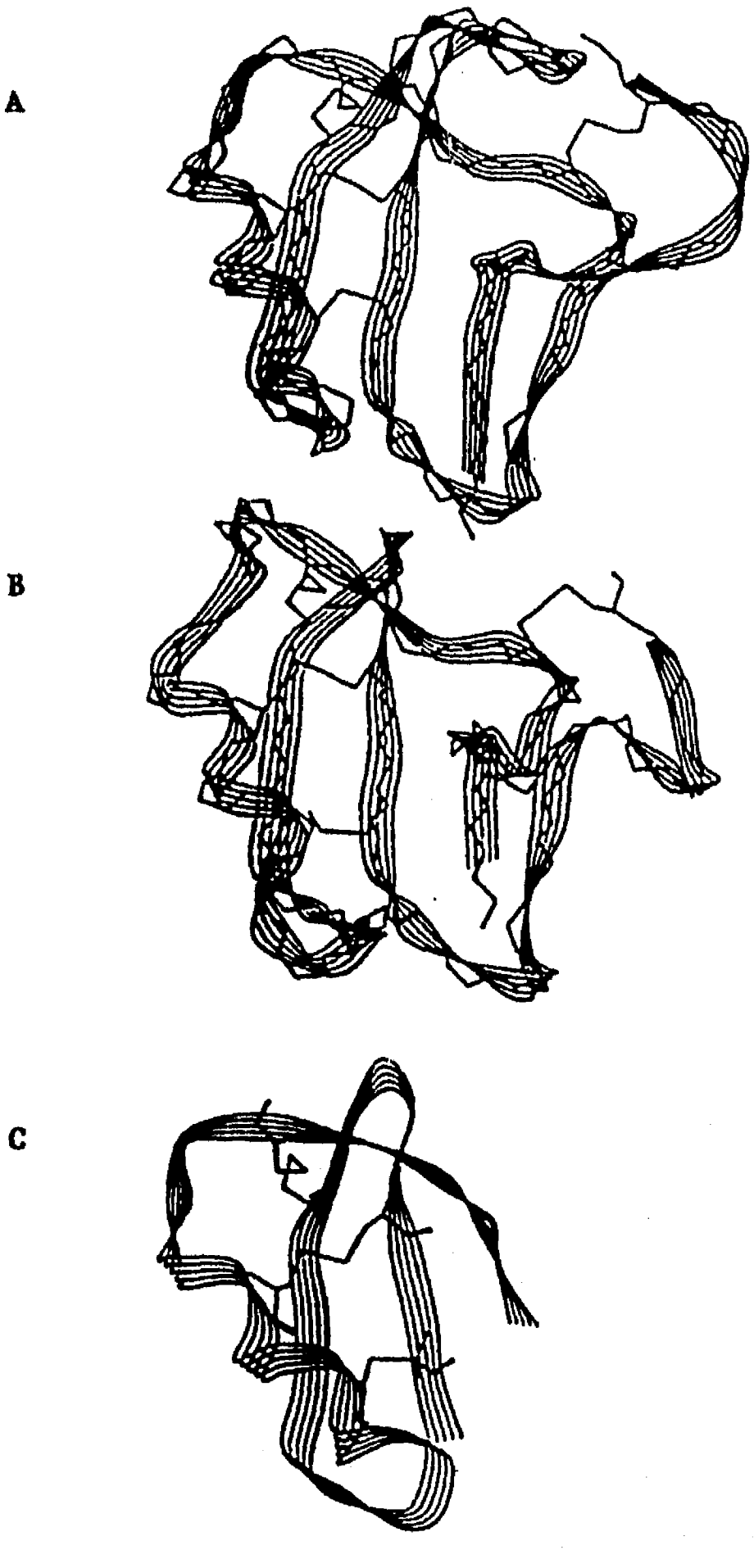

Figura 1. Estructura tridimensional de toxinas de alacrán A. toxina II de *A. australis*, **B.** variante-3 de *C. sculpturatus*, **C.** caribdotoxina de *L. quinquestriatus*. En todas las estructuras se observa el "motivo" estructural formado por tres regiones con estructura beta plegada (formando una lámina beta), dos de las cuales están unidas a la región de alfa hélice por dos puentes disulfuro. Las regiones que unen a las zonas conservadas forman asas de longitud variable (tomado de Ménez *et al.* 1992).

Figura 2. Comparación de secuencias de aminoácidos de toxinas de alacrán de cadena corta y de cadena larga.

```
                ßßß                 αααααααααα       ßßßßß        ßßßßßß
AaH I      KKNGYAVDSSGKAPECL-L--SNYCNNQCTKVH-YADKGYCCL-----LSCYCFGLNDDKKVLEISDTRKSYCDTTIIN
CsE v3     KEGYLVKKSDGCKYGCLKLGENEGCDTECKAKNQGGSYGYCYA-----FACWCEGLPESTPTY-PLPNKS--C
AaH II     VKDGYIVDDVNCTYFCGR---NAYCNEECTKL--KGESGYCQWASPYGNACYCYKLPDHVRTK--GPGR---CH
CTX           ZFT------NVSCTT---SKECWSVCQRL-HNTSRGKCMN-----KKCRCYS
           ---------------C--------CXXXC-----------C---------CXC-------------------------
```

Las secuencias de aminoácidos están alineadas de acuerdo a la posición de las cisteínas.
Los simbólos α y ß se refieren a las conformaciones de alfa-hélice y beta-plegada.
La secuencia consenso se indica al final de las secuencias.
(Tomado de Ménez *et al.*, 1992).

el veneno del alacrán *Buthus tamulus*. Este péptido bloquea con alta afinidad al canal de K^+ activado por calcio de alta conductancia por oclusión del poro. Esta interacción está influída fuertemente por interacciones electrostáticas (Giangiacomo *et al.*, 1992).

Otra toxina que bloquea específicamente al canal de K^+ activado por calcio de alta conductancia, es la kaliotoxina (KTX). Este péptido se purifica del veneno del alacrán del norte de Africa *Androctonus mauretanicus mauretanicus* (Crest *et al.*, 1992). Otra toxina llamada P05, que encontramos también en este veneno, es estructural y funcionalmente muy semejante a la leiurotoxina I, pero aparentemente su unión al receptor es irreversible a diferencia de esta última (Sabatier *et al.*, 1993).

En el veneno de los alacranes llamados del Nuevo Mundo también se encuentra este tipo de toxinas. Así tenemos a la margatoxina, un péptido de 39 aminoácidos, que se aisló del veneno del alacrán *Centruroides margaritatus*. Parece que bloquea sólo el canal de potasio dependiente de voltaje (Kv 1.3) de linfocitos T humanos (Garcia-Calvo *et al.*, 1993). También existe la tityustoxina Kα (TsTx-Kα), que se encuentra en el veneno del alacrán *Tityus serrulatus*. Está compuesta por 37 aminoácidos y bloquea canales de potasio dependientes de voltaje (Blaustein *et al.*, 1991; Ragowski *et al.*, 1994).

Noxiustoxina

La noxiustoxina es un péptido de 39 aminoácidos. El grupo de Possani lo aisló, por primera vez en 1982, del veneno del alacrán *Centruroides noxius* (Possani *et al.*, 1982).

La primera evidencia de su efecto sobre canales de potasio dependientes de voltaje fue en axón gigante de calamar, en experimentos de fijación de voltaje. Ahí se observó una disminución del pico de corriente de potasio, sin afectar la cinética de apertura y cierre del canal, presentando una Kd=300 nM (Carbone et al., 1982, 1987).

En sinaptosomas de cerebro de ratón estímula la

liberación de 4-amino[^{3}H]butírico e inhibe el eflujo de ^{86}Rb, por bloqueo de canales de potasio con una IC_{50}=2.9nM (Sitges *et al.*, 1986).

En otro experimento se observó que en canales de potasio dependientes de calcio de alta conductancia de membranas de músculo esquelético, la NTX induce estados breves de bloqueo, aunque con baja afinidad (Kd=450 nM) (Valdivia *et al.*, 1988).

Esta toxina también bloquea canales de potasio dependientes de voltaje en linfocitos T, con alta afinidad, Kd=0.2 nM (Sands *et al.*, 1989). Se ha demostrado que NTX inhibe alostéricamente la unión de ^{125}I-CTX a membranas sinaptosomales de cerebro de rata (Ki=8 pM). En este sistema se cree que CTX se une a canales de potasio dependientes de voltaje y no a los activados por calcio (Vazquez *et al.*, 1990). También, NTX inhibe la unión de ^{125}I-DTX* a membranas de cerebro de rata, facilita la liberación de acetil colina en uniones neuromusculares de manera semejante a la dendrotoxina (Harvey *et al.*, 1992).

Algunos péptidos sintéticos correspondientes a la secuencia N-terminal de NTX muestran efectos tóxicos que se parecen a los presentados por la toxina nativa (Gurrola *et al.*, 1989). Los mismos péptidos inducen diversos grados de bloqueo en canales de potasio dependientes de calcio de pequeña conductancia, en endotelio (Vaca *et al.*, 1993).

En el veneno del alacrán *Centruroides noxius* se encuentran péptidos que comparten determinantes antigénicos y características funcionales con la NTX, por ejemplo: la NTX 2. Esta tiene una similitud del 64% con NTX, comparte algún determinante antigénico ya que es reconocido por un suero anti-NTX, y es capaz de competir con ^{125}I-NTX en la unión a membranas de cerebro de rata (Nieto, 1994). Existen además, aproximadamente 6 toxinas semejantes a NTX dentro de este

*Dendrotoxina (DTX), péptido de 59 aminoácidos, purificado del veneno de la serpiente, *Dendroaspis angusticeps*, que afecta canales de potasio dependientes de voltaje (Dolly *et al.*, 1984).

veneno, pero sus secuencias no se han terminado.

En el veneno de otros alacranes se ha encontrado la presencia de péptidos capaces de competir con ^{125}I-NTX en la unión a membranas de cerebro de rata. Algunos ejemplos son: *Centruroides limpidus limpidus*, *Centruroides elegans* y *Pandinus imperator*. Las secuencias de estos péptidos son semejantes a NTX (datos no publicados).

II. Objetivos

El objetivo propuesto en esta tesis es caracterizar las zonas de interacción de la noxiustoxina con su receptor en membranas de cerebro de rata y comparar con otras toxinas semejantes en estructura, abarcando los siguientes aspectos:

1. Determinar la posición de los puentes disulfuro de la noxiustoxina para terminar su caracterización estructural.
2. Valorar los posibles cambios funcionales de la noxiustoxina (unión a membranas de cerebro de rata) por modificaciones específicas de ésta, utilizando reactivos químicos y enzimas hidrolíticas.
3. Obtener mediante síntesis química, fragmentos de la NTX y ensayar su capacidad de mimetizar el efecto de la toxina nativa en membranas de cerebro de rata y en células endoteliales, mapear regiones importantes.
4. Comparar el efecto de toxinas de otros venenos de alacrán que afectan canales de potasio, sobre la unión de la noxiustoxina en membranas de cerebro de rata.

Finalmente, elaborar un modelo de la estructura tridimensional de la noxiustoxina basado en las coordenadas obtenidas por estudios de espectroscopía de resonancia magnética nuclear, para una toxina semejante a la NTX: la caribdotoxina.

Esta información es importante para entender los mecanismos de selectividad de las diferentes toxinas por cierto tipo de canales de potasio, para determinar qué aminoácidos modulan la afinidad por el receptor, lo que eventualmente permitirá el diseño de nuevas variantes de toxinas teniendo una única especificidad o función.

III. Resultados

En esta sección presentamos los resultados producto de investigación del tiempo en que se ha realizado nuestra tesis. Se presentan en forma de artículos terminados en vista de que el programa mismo de doctorado así lo requiere; pero también obedece a la razón de no repetirme, como al hecho de dejar constancia de la participación de mis compañeros de equipo y de mi jefe de investigación. Su trabajo está reconocido con la aparición de su nombre, el lado mío, en estas publicaciones.

He decidido presentar estos resultados mediante la ficha técnica bibliográfica, en primer lugar, acompañada del resumen del artículo. A continuacción se presentan los artículos completos y divididos en dos secciones pertinentes para el caso. 3.1 Para un artículo en proceso de publicación. Al momento de la redacción del presente trabajo, no tenía en mi poder el artículo impreso. 3.2 Artículos publicados, tres en total.

Finalmente debo añadir que estos artículos evidencian la mayor parte de las investigaciones y dan un panorama bastante cabal de los propósitos que nos impusimos investigar desde la primera hora en que iniciamos nuestro trabajo, bajo la dirección del Dr. L.D. Possani.

3.1 Artículo sometido a publicación

Gurrola, G. B. and Possani, L.D. "Structural and functional features of Noxiustoxin: a K+ channel blocker", sometido en *FEBS Letters*

En este trabajo se determinó la posición de los puentes disulfuro en la molécula de NTX, por medio de digestión enzimática utilizando la endopeptidasa lisina-C e identificando por secuencia de aminoácidos los péptidos obtenidos, lo que permitió asignar las siguientes posiciones: cis7-cis29, cis13-cis34 y cis17-cis36.

Basándose en las coordenadas obtenidas por estudios de espectroscopía de resonancia magnética nuclear, para una toxina semejante a la NTX: la caribdotoxina, se presenta un modelo de la estructura tridimensional de la molécula de NTX.

Se probó la capacidad de péptidos sintéticos, correspondientes a secuencias de aminoácidos de la noxiustoxina, de competir con ^{125}I-NTX en la unión a membranas de cerebro de rata. Los péptidos sintéticos que fueron capaces de inhibir la unión de ^{125}I-NTX a membranas de cerebro de rata fueron los correspodientes a las secuencias 1-14, 1-21 y 11-39, con una IC_{50} semejante (160, 800 y 250 nM respectivamente); no así los péptidos correspondientes a las secuencias 1-9 y 30-39 con las concentraciones probadas (100 μM).

Se probaron también péptidos correspondientes a secuencias de aminoácidos de caribdotoxina (1-10 y 24-37) ya que la NTX es capaz de inhibir no competitivamente la unión de ^{125}I-CTX a membranas de cerebro de rata (Vazquez *et al.*, 1990) encontrando que en este caso sólo el péptido correspondiente al segmento carboxilo terminal fue capaz de inhibir la unión de ^{125}I-NTX.

Al observar el modelo tridimensional de la NTX, vemos que la región del aminoácido 1 al 20 se encuentran en el mismo plano. Esta zona parece ser una parte importante del sitio de unión con el receptor, ya que cuando a la toxina nativa se le quitan los primeros seis aminoácidos (queda NTX7-39), disminuye su afinidad por el receptor, y disminuye aún más cuando no tiene los primeros diez aminoácidos (NTX11-39).

Cuando la NTX es tratada con proteasa V8 el enlace

peptídico entre el ácido glutámico 19 y la leucina 20 se rompe, lo que produce una disminución en la afinidad por el receptor, no encontrando ninguna modificación de ésta cuando la toxina es tratada con bromuro de cianógeno y el enlace peptídico hidrolizado está entre la metionina 30 y asparagina 31. En el modelo tridimensional de la molécula de NTX, se puede observar que, el ácido glutámico de la posición 19 se encuentra en una estructura de alfa hélice. Al romper este enlace peptídico se pudo haber desestabilizado la estructura de la molécula (perder parte de la estructura alfa hélice) y por lo tanto no mantener en la posición adecuada los aminoácidos que interaccionan con el receptor (cargas positivas). Debido a que la metionina 30 se encuentra en el asa que une a las dos últimas regiones de beta plegada, la hidrólisis de este enlace peptídico, no tiene efectos significativos sobre la unión de la toxina al receptor.

De estos resultados concluimos, que para la NTX la región amino terminal forma parte importante del sitio de unión a su receptor y que para la CTX es la región carboxilo terminal la más relevante.

Structural and functional features of Noxiustoxin: a K^+channel blocker

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Running Title: Active-site of Noxiustoxin

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SUMMARY

The disulfide bridges of Noxiustoxin (NTX) were found to be: Cys7-Cys29, Cys13-Cys34 and Cys17-Cys36. A three-dimensional model of NTX is presented. Binding of ^{125}[I]-NTX to rat brain synaptosome membranes and displacement with synthetic peptides corresponding to the amino acid sequence of NTX, show that the N-terminal segment, including part of the alpha-helix, of this toxin is implicated in the recognition of brain K^+-channels. Cleavage of NTX with cyanogen bromide or protease V8 support this findings. On the contrary, a synthetic C-terminal tetradecapeptide of charybdotoxin (ChTX), show that in this K^+-channel blocker, the C-terminal region, rather than the N-terminal is capable of displacing ^{125}[I]-NTX binding to brain membranes.

1. INTRODUCTION

NTX is a 39 amino acid polypeptide with three disulfide bonds isolated from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann [1], originally described to block voltage-activated delayed rectifier K^+ channels in the giant axon of squid [2]. It was subsequently shown that it also blocks other classes of voltage and Ca^{2+}-dependent K^+channels from various excitable [3-6] and non excitable tissues [7,8], with highly variable affinities, Kds ranging from μM to pM (a million fold difference!), depending on the tissue under study. Charybdotoxin (ChTX), another K^+channel blocker was also widely studied [9-12], and shown to share a considerably degree of similarity with NTX in terms of primary

structure (44% identity). Both toxins, recognize several distinct classes of K^+-channels, and apparently bind to distinct sites of brain synaptosome membranes [6, 13]. In this preparation, ^{125}[I]-ChTX binds to a voltage-dependent K^+ channel with an affinity of 25-30 pM, and is effectively displaced by NTX [6]. Synthetic peptides corresponding to the amino acid sequence of NTX (nonapetide and eicosapeptide at the N-terminal part) were shown to be toxic to mice, presumably by blocking K^+-channels [14], while a decapeptide at the C-terminal segment was inactive. Similar results were obtained with these synthetic peptides in another preparation, where electrophysiological measurements at the level of single channels (Ca^{2+} activated K^+ from aortic endothelial cells) were conducted with membrane fragments incorporated in planar bilayers [15]. On the contrary, the C-terminal region of ChTX seems to be essential for its activity [9, 12]. Modification of Lys27 by genetic engineered ChTX abolishes its activity on the Ca^{2+}-activated K^+ channel from skeletal muscle. Similarly, the results of experiments with chimeric synthetic peptides of ChTX [13] suggest that the presence of charged amino acid residues at the C-terminus are important for binding to a strongly inactivating voltage-gated K^+-channel ($K_{v1.3}$). Thus, NTX and ChTX have similar structural features, recognize similar K^+channels with distinct affinities, and the region of the molecule that binds to the channels seems to be the N-terminal part for NTX and the C-terminal for ChTX, when assayed in the brain preparation (presumably binding to the $K_{v1.3}$ channels).

Here we report the synthesis of peptides corresponding to the N-terminal and C-terminal regions of both NTX and ChTX, and conducted chemical modifications of NTX in order to test their effect on the K^+-channels of rat brain synaptosome membranes.
The disulfide bridges of NTX were also determined and their positions discussed in view of the recent results obtained by NMR spectroscopy, for the homologous charybdotoxin [16].

2. MATERIALS AND METHODS

2.1 Disulfide determination

The disulfide bridges of NTX were found by sequencing heterodimeric peptides separated by HPLC of NTX hydrolyzed by mild enzymatic cleavage. Briefly, 50 μg of NTX was dissolved in 0.1 M Tris-HCl, 1 mM EDTA buffer pH 7.2 (250 μl total volume), and hydrolyzed with 0.5 μg of Lys-C endoprotease (Boehring-Manheim, Germany), for 18 h at 37 °C. Separation was obtained in a C18 reverse-phase column with a linear gradient of acetonitrile (0 to 60%) in 0.1% aqueous trifluoracetic acid. Eight fractions were collected and sequenced.

2.2 Three-dimensional modeling

The stereo-image of the three dimensional model of NTX was obtained using the NMR parameters determined for ChTX (See acknowledgements). The suit of programs for homology modeling from Biosym Technologies (San Diego, USA) was used for model generation, including minimization, and molecular dynamics at 400 °K for 5 pS and then 5 ps at 300 °K, as earlier described [17].

2.3 Chemical synthesis

Chemical synthesis of peptides were conducted according to Merrifield's method [18] of solid phase peptide synthesis, using tert-butyl-oxy-carbonyl amino acids, as described [14]. Peptides were purified by high performance liquid chromatography (HPLC), and the synthesis confirmed by amino acid analysis and, when necessary, by microsequencing, as described [19].

2.4 Cleavage of NTX

Native NTX was purified by chromatographic procedures [1], with an additional separation through a C4 reverse column, using the HPLC system described [19]. The cleavage of NTX (50 μg) with cyanogen bromide [1], and the hydrolysis of 50 μg of NTX by protease V8, from *Staphylococcus aureus* was also conducted as described for other toxins [19]. Cleaved products of NTX were separated by HPLC, and the resulting peptides were used for binding assays, and chemical analysis.

2.5 Binding assays

Rat brain synaptosome membranes (fraction P3) were prepared essentially by the procedure of Catterall [20].

Radio-labelling of NTX with ^{125}[Iodine] [21] and binding to P3 membranes were performed as described [5], with minor modifications. Briefly, 30 μg of membrane proteins were incubated with 50 pM ^{125}[I]-NTX in 200 μl of incubation medium (50 mM NaCl, 20 mM Tris-HCl buffer pH 7.8 and 0.1% bovine serum albumin) in

absence or presence of increasing concentrations of either toxin or peptides, at room temperature for 60 min. The reaction was stopped by the addition of 5 ml of 100 mM NaCl, 20 mM Tris-HCl buffer, pH 7.8, followed by rapid filtration through GF/B filters (Whatman) presoaked in 1% polyethyleneimine, washing twice with the same buffer.

3. RESULTS AND DISCUSSION

Since the disulfide bridges of native NTX were not known, we have cleaved native NTX with Lys-C protease, separated the peptides by HPLC (data not shown), and sequenced. The disulfide bridges are made between: Cys7-Cys29, Cys13-Cys34 and Cys17-Cys36 (Table 1). These results were obtained after many trials, since NTX is resistent to tryptic hydrolysis, and only small amounts (about 10% each time) are ruptured, in our experimental conditions using LysC endoprotease. The pair Cys13-Cys34 was found after sequencing a heterotrimer, in which the peptide-bond of the lysil residue at position 15 was not cleaved (Table 1), resulting three distinct amino acid for each position during microsequencing (corresponding to peptides Gln12 to Lys18, Cys34 to Lys35 and Cys36 to Asn39). Since the disulfide pairing of Cys17-Cys36 and Cys7-Cys29 were unequivocally determined (Table 1), the only other combination compatible with the sequence cf the heterotrimer was the pair Cys13-Cys34. It is worth mentioning that cyanogen bromide cleavage of methionine at position 30, shows that the other possible pair Cys34-Cys36 does not exist.

These results show that the disulfide bridges of NTX and ChTX occurs at equivalent positions of the primary structure. We have prepared a stereo model of the three-dimensional structure of NTX, using the NMR-coordinates obtained for ChTX [16], generously provided to us by Dr. Andre Menez (CEA, Gif-Sur-Yvette, France). Figure 1 shows the model obtained, including the positions of the disulfide bridges. The same general structural features obtained for ChTX [16] is present in NTX, despite the possibility discussed elsewhere [22], that the Ala at position 27 instead of Gly present in ChTX and other K^+ channel blockers [22], would probably generate steric hindrance for the appropriate folding of NTX. The fact that NTX has an extra amino acid (Gly before Ala) in this stretch of the primary structure (amino acids 20 to 30, Table 1) when compared to ChTX is the rational explanation for not having the hindrance previously foreseen.

In order to gain more information on structural and functional characteristics of these toxins, we have synthesized a series of peptides corresponding to the primary structure of both, NTX and ChTX, and assayed their effect on K^+channels of rat brain synaptosome, demonstrated [5,6], to be a good model to study binding and displacement properties of these peptides. In this preparation, as already mentioned, there is a voltage dependent K^+ channel, for which the affinity of both toxins, NTX and ChTX is in the order of pM [6]. The following peptides, corresponding to the amino acid sequence of NTX or ChTX were synthesized, purified by HPLC and confirmed by amino acid analysis and microsequencing:

NTX1-9, (numbers correspond to the amino acids position on the primary structure of the toxin), NTX1-14, NTX1-20, NTX11-39, NTX30-39, NTX1-39, ChTX1-10, ChTX24-37 (bottom part of Table 1). The presence of several free sulphydryl groups, in cysteine-containing peptides always poses an important problem for interpretation of the data, when the cross-linking through disulfide pairing is not known. For these reason NTX1-21 was also synthesized with alanines, replacing cysteines. In this manner, even without knowing the correct disulfide bridges of segments containing mixed thiol groups, we could surmise the participation of that given segment of the primary structure of the ligand in recognizing the receptor molecule. The results of the binding-displacement of the various synthetic segments of NTX and that of native NTX are shown in Fig.2a, and will be discussed shortly. Before accessing the effect of synthetic toxin and their fragments we started by simple chemical modifications of native NTX, such as reduction and alkylation. When NTX is reduced and alkylated, either with iodomethane or iodoacetic acid the toxin becomes completely inactive. The modified native peptides are incapable of displacing the binding of radio-labelled NTX to brain membranes (data not shown). However, when NTX is reduced but not alkylated, and is purified from reactants (gel filtered through Biogel P-30 in the same conditions used for the alkylated samples), the native conformation is partially regained, or respected. The IC_{50} value for binding displacement of native NTX versus iodinated-NTX is 200 pM (Fig.2a), while the refolded NTX has an IC_{50} value of about 800

pM (data not shown), 4-fold reduced in affinity. Concerning the synthetic NTX and the corresponding segments, we have found that the full span synthetic NTX (residues 1 to 39) was obtained in pure form after air oxidation [11] and HPLC purification, given essentially the same results as native toxin (Fig.2a, black squares and white squares, respectively). This data confirms our original reports on the purification and sequencing of native NTX [1].

The possibility that native NTX, at the C-terminal residue (Asn) is amidated [23], is not dismissed by the present results. The method used by us for the primary structure determination [1] of NTX did not allowed the determination of a possible amidated form of Asn39. The synthetic peptides NTX1-9 (extreme of the N-terminal region) and NTX30-39 (the C-terminal region) do not displace the binding of ^{125}[I]NTX to brain synaptosome membranes, even at the relatively high concentration tested (100 μM). However, when the synthetic segments are larger, like NTX1-14, the displacement is evident (Fig. 2a) with an IC_{50} of 160 nM. Similar results were obtained with synthetic NTX1-20 (data not shown). More important yet, are the results with the synthetic peptide NTX1-21, in which alanines replace cysteines (Fig.2a). This peptide does not have possible constrains of disulfide bridges, and is capable of displacing the binding with an IC_{50} of 800 nM. Equally effective is the fragment NTX11-39, with an IC_{50} of 250 nM (Fig. 2a). Our interpretation of these results is consistent with the conclusion that the N-terminal part of the molecule, including amino acids spanning over residue 9 are important for binding to the receptor ($K_{v1.3}$-channel). The

fact that NTX1-9, recognizes the channel [14,15] but does not displace the binding is not surprising since the affinity of native toxin for this specific type of channel is in the range of 200 pM. It is worth recalling that previous experiments [14,15] also indicate that the C-terminal part of NTX does not recognizes the channels.

By observation of the three-dimensional structure of ChTX [16] and our Fig.1 of NTX, we can conclude that residues Lys11 to Leu20 are forming a three turns of an alpha-helix, while segments Thr1-Asn4, Ala25-Cys29 and Lys33 to Tyr37 are probably forming three anti-parallel beta-sheet structures. Methionine of position 30 is situated in a turn, between two beta-sheets structures. Thus, to test the need of an intact alpha-helix in the N-terminal portion of the NTX for the binding, we have hydrolyzed the glutamyl-bond in position 19 (see Fig.2 b) with protease V8. The resulting NTX was purified by HPLC, and the cleaved toxin was sequenced to proof that in fact the glutamyl residue was hydrolyzed. This cleaved NTX decreases significantly the affinity for the channels (1.5 order of magnitude less) with an IC50 of about 12 nM, showing that the presence of an intact alpha-helix region is important for binding. Digestion of NTX with LysC-endopeptidase produced a segment lacking the N-terminal hexapeptide (positions 1 to 6). The resulting native NTX7-39 is also less active by one order of magnitude (Fig. 2b), showing that the N-terminal is important for recognition of the channels. However, the cyanogen bromide cleaved Met30 does not change the affinity for the channel, having essentially the same

affinity as native NTX (Fig. 2b), compatible with the idea that the C-terminal segment is not important for binding to the membranes. The endopeptidase-cleaved NTX, and the cyanogen bromide ruptured NTX were also confirmed, by purification of the resulting peptides and by amino acid sequence determination (data not shown).

Finally, in order to test the results obtained by others with ChTX [10-13], we have synthesized, purified and assayed two peptides with the amino acid sequence corresponding to charybdotoxin: first, a decapeptide at the N-terminus (ChTx1-10) and second, a tetradecapeptide at the C-terminus (ChT24-37). Our results of Fig.2c clearly shows that is the C-terminal fragment of ChTX the one important for binding, in our assay of synaptosome membranes, and not the N-terminal (not shown), which was completely ineffective.

In conclusion, our data indicate that the N-terminal segment of NTX, including the alpha-helix region, is important for recognition and binding to the channels of the brain membrane preparations, while the C-terminal part seems not to be that important. These are in agreement with our previous data [14-15]. ChTX, however, probably interacts with the channels of these membranes by the C-terminal region, confirming also data available in the literature [10-13]. These findings are important for the understanding of the immense diversity of toxic peptides, evolved by scorpions, with similar structural motifs, but with exquisite specificities for various different types of ion channels, displaying a tremendous variation in affinity, which can be as great as 1 million fold

difference.

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LEGEND FOR FIGURES

Fig. 1. Stereo-image of the three dimensional model of NTX. Coordinates for ChTX (See Acknowledgements) were used for modeling the NTX structure, using a Silicon-Graphics IRIS 4D/35; with programs from Biosym Technologies, Inc. Energy minimization, and molecular dynamics at 400 °K for 5 ps and then 5 ps at 300 °K were employed to refine the model, a water shell 5 A thick was included. NH2- and COOH- termini are signaled in the ribbon model. The disulfide bridges (numbers correspond to the cysteine positions in the primary structure) are represented as sticks linking the main peptidic backbone structure of NTX. E19 and M30 indicate the positions of Glu19 and Met30, respectively. The complete primary structure of NTX is shown in Table 1.

Fig. 2. Displacement of ^{125}[I]-NTX binding to P3 membranes, by NTX and synthetic peptides.

Brain synaptosomal membranes (fraction P3) were incubated with 50 pM ^{125}[I]-NTX plus increasing concentrations of a) native NTX (open squares), full-size synthetic NTX1-39 (closed squares), NTX 1-9 (crosses), NTX1-14 (open circles), Ala containing NTX1-21 (open triangles), NTX11-39 (open diamonds), NTX30-39 (closed triangles). b) native NTX (open squares), NTX cleaved by BrCN (open triangles), NTX digested by protease V8 (closed triangles), NTX digested with Lys-C endopeptidase (closed diamonds) and c) native NTX (open squares), synthetic fragment of Charybdotoxin, ChTX24-37 (closed circles), for 60 min at room temperature, before filtration. Non specific binding has been subtracted.

Table 1: AMINO ACID SEQUENCE OF HETERODIMERIC AND HETEROTRIMERIC PEPTIDES CORRESPONDING TO THE DISULFIDE BRIDGES OF NTX

Elution time HPLC	Amino acid sequence	Corresponding disulfide
	1 2 3 4	
17.81	Pro-CYS-Lys	
	Xxx-Tyr-Asn-Asn	Cys17-Cys36
	1 2 3 4 5 6 7	
20.91	Gln-CYS-Ser-Lys-Pro-CYS-Lys	
	Xxx-Lys	Cys13-Cys34
	Xxx-Tyr-Asn-Asn	Cys17-Cys36
	1 2 3 4	
21.66	CYS-Thr-Ser-Pro	
	Xxx-Met-Asn-Gly	Cys7-Cys29
	1 10 20 30 39	
NTX	TIINVKCTSP KQCSKPCKEL YGSSAGAKCM NGKCKCYNN	
ChTX	pEFTNVSCTTS KECWSVCQRL HNTSRG-KCM NKKCRCYS	

Amino acid sequences were obtained by microsequencing peptides purified by HPLC, after cleavage with Lys-C endopeptidase. Numbers on top of the amino acid means the positions in the sequence, Xxx, means a blank position corresponding to a 1/2 Cys residue (cysteine), while CYS the position of a cystine residue (usually in our machine we can identify a component that absorbs at 330 nm which corresponds to this amino acid).
The bottom lines contain the complete structure of NTX, as determined by Possani *et al* [1], and ChTX, as reported by Gimenez-Gallego *et al* [24]. A gap (-) was introduced in the sequence of ChTX to enhance similarities. Cysteines are in bold.

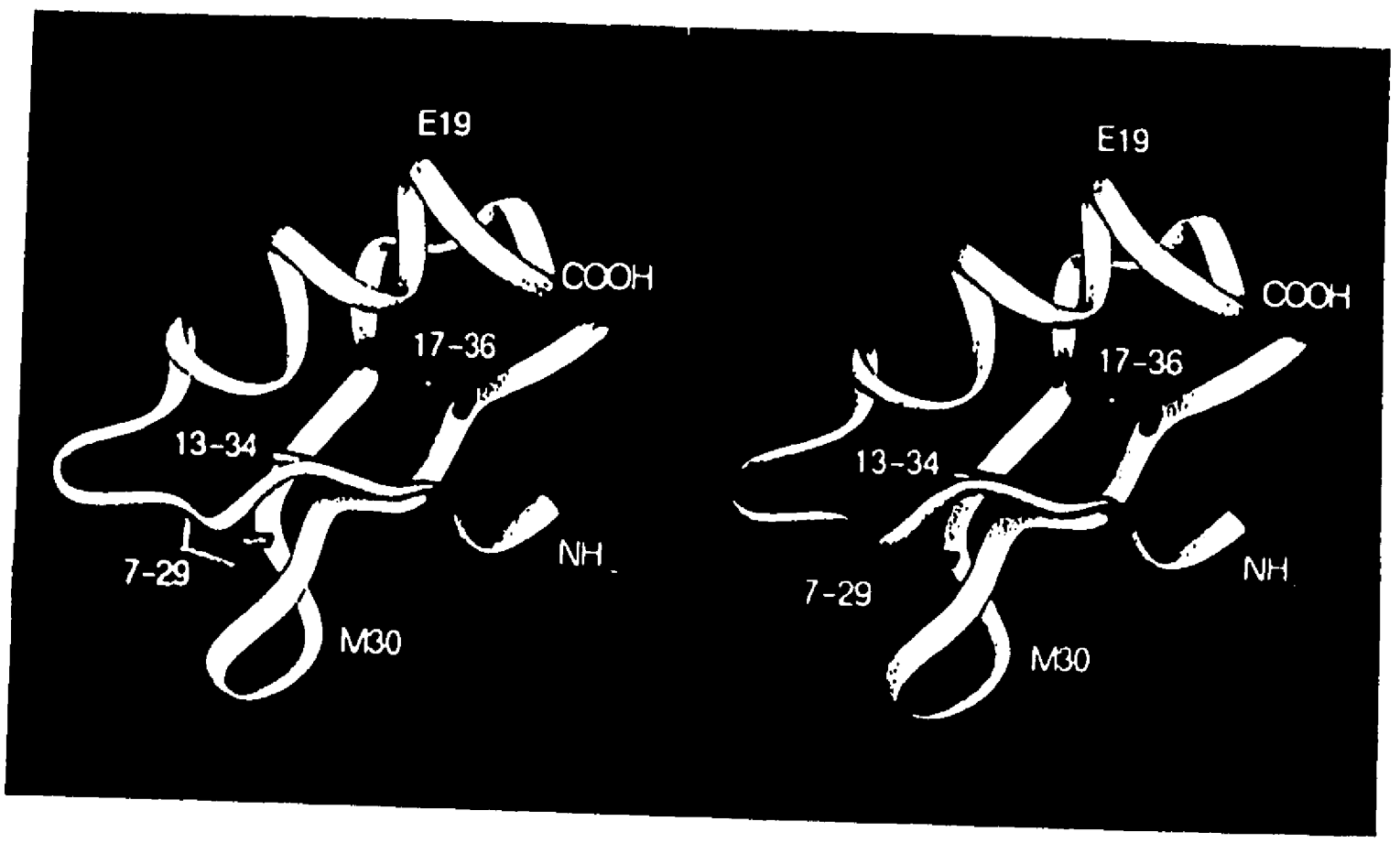
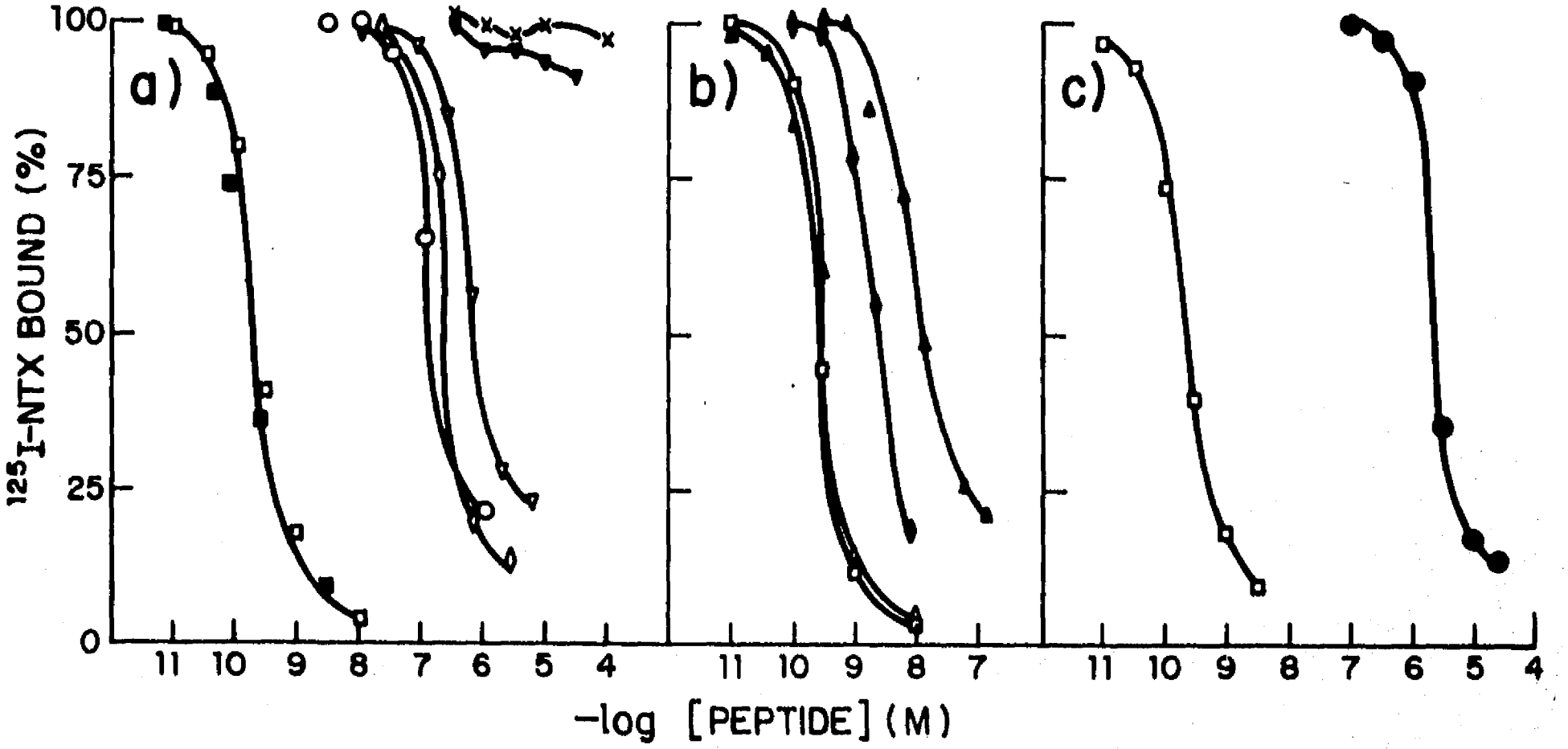


FIGURE 1


a)
b)
c)
100
75
50
25
0
^{125}I-NTX BOUND (%)
11 10 9 8 7 6 5 4
11 10 9 8 7
11 10 9 8 7 6 5 4
-log [PEPTIDE] (M)

3.2 Artículos publicados

3.2.1 Gurrola, G.B., G. Moreno-Hagelsieb, F.Z. Zamudio, M. García, X. Soberón, L.D. Possani, (1994)."The disulfide bridges of toxin 2 from the scorpion *Centruroides noxius* Hoffmann and its three-dimensional structure calculated using the coordinates of variant 3 from *Centruroides sculpturatus*", *en FEBS Letters*, **347.** *59-62p*.

En este trabajo se determinó la posición de los puentes disulfuro de una toxina de cadena larga purificada del veneno del alacrán *Centruroides noxius*, llamada Cn 2. Esta toxina es un péptido de 66 aminoácidos con cuatro puentes disulfuro que bloquea canales de sodio de varios tejidos y pertenece a las toxinas de tipo beta.

Sometiendo la toxina Cn 2 a digestión enzimática con las endopeptidasas tripsina y quimotripsina, se obtuvieron varios fragmentos de los que se logró identificar cuatro péptidos que mostraban doble secuencia. Dado que la estructura primaria de esta toxina se conoce fue fácil identificar dentro de ésta los péptidos obtenidos y asignar los correspondientes enlaces disulfuro (cis12-cis65, cis16-cis41, cis25-cis46 y cis29-cis48), los cuales tienen posiciones equivalentes a los puentes disulfuro de otras toxinas de alacrán que bloquean canales de sodio (Variante 3 de *C. sculpturatus*, toxina II de *A. australis*).

Basándose en las coordenadas obtenidas por difracción de rayos X por Fontecilla-Camps, para la variante 3 de *C. sculpturatus*, se presenta un modelo tridimensional de la molécula de Cn 2, mostrando que estas toxinas tienen una estructura semejante.

Se puede observar la presencia de tres regiones con estructura beta-plegada, dos de las cuales estan unidas a una región de alfa-hélice por dos de los cuatro puentes disulfuro. Las tres regiones de beta-plegada forman una región de lámina beta. Este "motivo" estructural se encuentra presente también en las toxinas de alacrán de cadena corta que afectan canales de potasio (ver trabajo anterior).

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FEBS 14121

The disulfide bridges of toxin 2 from the scorpion *Centruroides noxius* Hoffmann and its three-dimensional structure calculated using the coordinates of variant 3 from *Centruroides sculpturatus*

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Abstract

The disulfide bridges of toxin 2 from the venom of the scorpion *Centruroides noxius* Hoffmann were found by amino acid sequence determination of fragments of native toxin, produced by enzymatic cleavage and separated by high-performance liquid chromatography (HPLC). They are: Cys^{12}-Cys^{65}, Cys^{16}-Cys^{41}, Cys^{25}-Cys^{46} and Cys^{29}-Cys^{48}. The coordinates of the X-ray diffraction structure of toxin variant 3 of *C. sculpturatus* [(1980) Proc. Natl. Acad. Sci. USA 77, 6496–6500] were used to construct a three-dimensional model of toxin 2. All the amino acid replacements were easily accommodated, and the modeled structure reveals a clustered pattern of sequence variation, which may help to identify residues responsible for functional differences among toxins of mammals and insects.

Key words: Scorpion toxin; Disulfide bridge; Homology modeling; *Centruroides noxius*; *Centruroides sculpturatus*

1. Introduction

To elucidate the structure–function relationships of scorpion toxins and their targeted ion channels [2] more information is needed, not only on their primary structure, of which many are known [3,4], but also on their three-dimensional folding [1,5]. One of the structural features of the Na^+ channel-directed toxins is the high content of disulfide bridges, but few disulfide pairs have been directly determined [1,5,6]. Here we describe the experimental determination of the disulfide bridges of toxin 2, a mammalian-specific toxin. We also present a model of its three-dimensional structure, based on the crystal structure of its insect-specific homologue, variant 3 of *C. sculpturatus* [1]. Insights into possible structure–function relationships are discussed.

2. Material and methods

Toxin 2 was purified as described by Zamudio et al. [7]. This peptide (1.2 mg, equivalent to 170 nmol) was hydrolysed by chymotrypsin and trypsin, and separated by HPLC, following the procedure of Sugg et al. [8]. Heterodimeric peptides containing the native disulfide bridges were identified by microsequencing in a Millipore ProSequencer [7]. Since the primary structure of this toxin is known it was easy to assign its disulfide pairing.

Computational analysis made use of the coordinates of variant 3 of the related scorpion *C. sculpturatus* [1]. The suit of programs for homology modeling from Biosym Technologies (San Diego, USA) was used for model generation (including minimization, and molecular dynamics at 400 K for 5 ps and then 5 ps at 300 K). A water shell was included.

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3. Results and discussion

The few experimentally determined disulfide bridge patterns of Na^+ channel-specific scorpion toxins are those of variant 3 from *C. sculpturatus* [1], toxin M9 from *Buthus eupeus* [5], toxin III and toxin II [5,6], from *Androctonus australis*, a typical α-scorpion toxin [9]. The disulfide bridges of these toxins all correspond to equivalent positions on the primary structure (Cys^{12}-Cys^{65}, Cys^{16}-Cys^{41}, Cys^{25}-Cys^{46} and Cys^{29}-Cys^{48}), except for the insect toxin I from *A. australis* [10], which has the pairing Cys^{16}-Cys^{37}, Cys^{22}-Cys^{42}, Cys^{26}-Cys^{44}, and Cys^{38}-Cys^{64}. Toxin 2 from *C. noxius* [7], initially called component II-9.2.2 [11], was shown to recognize Na^+ channels of several tissues [12–14]. This toxin is a typical β-scorpion toxin [15].

Since the species specificity and the fine mechanism of action of insect, and mammalian α- and β-scorpion toxins are different, we decided to determine the relative positions of the disulfides of toxin 2 from *C. noxius*. Separation of enzymatically cleaved peptides of native toxin 2 gave rise to approximately 30 components when applied to a C_{18} reverse-phase column on HPLC (data not shown). Under sequence analysis, peptides that eluted at 47.15 min (step-gradient 9.82 min), 59.28 (8.51), 64.36 (8.28) and 68.76 min gave two amino acids each at every step of the microsequencing process. The values in parenthesis after the time (min) mean that the same component before sequencing was further applied to HPLC and eluted at the time indicated, when using a continuous gradient mode (step-gradient). Since the primary structure of toxin 2 was known [7], this allowed us to assign the the disulfide pairing unequivocally (Table 1). The

SSDI 0014-5793(94)00507-R

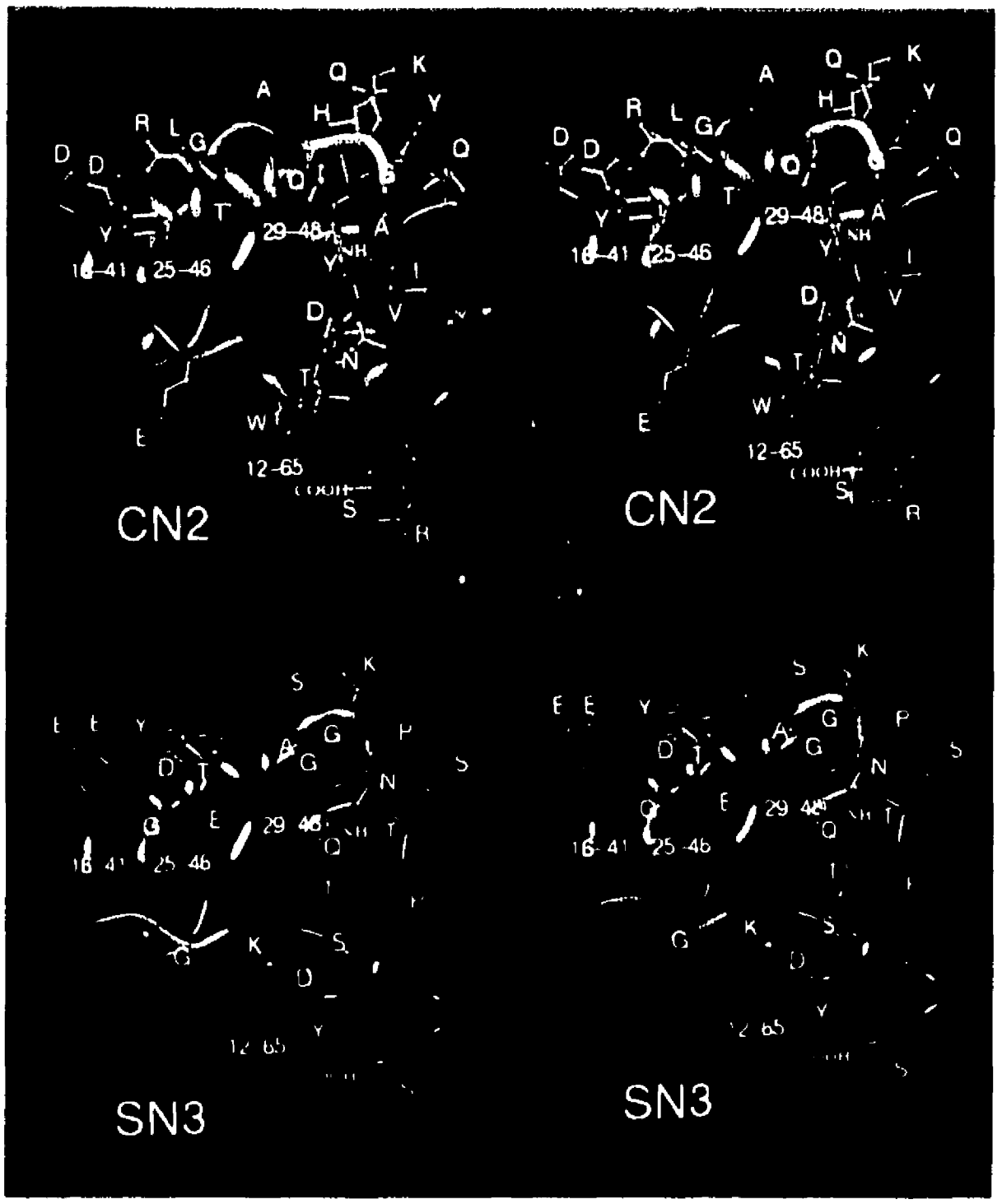

Fig. 1. Stereo-image of the three dimensional models of toxin 2 from *C. noxius* (CN2), and variant 3 of *C. sculpturatus* (SN3). Coordinates for variant 3 of *C. sculpturatus* (Brookaven data bank 1sn3) were used for modeling the Cn2 structure, using a Silicon-Graphics IRIS 4D/35 and a Cray Y-MP4/464; with programs from Biosym Technologies Inc. Energy minimization, and molecular dynamics at 400 K for 5 ps and then 5 ps at 300 K were employed to refine the model: a water shell 5 Å thick was included. NH_2- and COOH-termini are indicated. Variant amino acids (one letter code) are represented as ball and stick side chains, protruding from the main chain represented as a solid ribbon. Disulfide bridge-forming atoms, used as a reference, are represented by dark sticks, and labeled with numbers corresponding to the cysteines involved. Other identical amino acids are not shown. The complete primary structure of these two proteins is shown in Table 1.

relative positions of the disulfide bridges of toxin 2 correspond to equivalent positions in variant 3 of *C. sculpturatus* [1] and toxin II from *A. australis* [6].

We next modelled the structure of toxin 2 of *C. noxius*, using the available coordinates of the three-dimensional structure of variant 3 (Brookhaven entry 1SN3, from the original reference of Fontecilla-Camps et. al. [1]). The alignment of the two sequences was straightforward and the amino acid residues of toxin 2 were substituted on the SN3 structure. A simple energy minimization protocol revealed very good compatibility of the toxin 2 sequence with that of SN3 structure. In order to have a slightly better sampling of conformational space we also conducted limited molecular dynamics simulations which further adjusted the model to a sterically plausible one. Inspection of the model in Fig. 1 shows the preservation of a face rich in aromatic residues (corresponding to the front of the 'hand model' proposed by Fontecilla-Camps et al. [1], shown here facing away from the viewer). The figure uses a ribbon representation, in which only the side chain corresponding to variable amino acids are shown as stick representations. Numerous amino acid differences between the two toxins occur mainly towards the opposite face (back of the hand model, facing towards the viewer in the figure). For example, in CN2 there are charged amino acids, such as glutamic acid at position 15 (Glu^{15}), and Arg^{64}, facing this side, which replace, respectively, Gly^{15} and Ser^{64}, present in SN3. Also noteworthy is the substitution of Asp^{7} in CN2 for Lys^{7} of SN3, which faces the front of the hand, at the

Table 1
Amino acid sequence of heterodimeric peptides corresponding to the disulphide bridges of toxin 2

Elution time HPLC	Amino Acid Sequence	Corresponding disulfide
47.15 (9.82) min	1 2 3 4 5 6 Asn-Thr-Gly-CYS-Lys-Tyr Xxx-Ser	Cys12-Cys65
59.28 (8.51) min	1 2 3 4 5 Glu-CYS-Lys Xxx-Thr-His-Leu-Tyr	Cys29-Cys48
64.36 (8.28) min	1 2 3 Glu-CYS-Leu Xxx-Tyr	Cys16-Cys41
68.67 min	1 2 3 Xxx-Leu-Arg Ala-CYS-Trp	Cys25-Cys46

```
     1         10        20        30        40        50        60
CN2  KEGYLVDKNTGCKYECLKLGDNDYCLRECKQQGYKGAGGYCYAFACWCTHLYEQAIVWPLPNKRCS
SN3  KEGYLVKKSDGCKYGCLKLGENEGCDTECKAKNQGGSYGYCYAFACWCEGLPESTPTYPLPNKSC
```

Amino acid sequences were obtained by microsequencing peptides purified by HPLC after cleavage with chymotrypsin and trypsin. Numbers on top of the amino acid mean the positions in the sequence; Xxx, blank position corresponding to a 1/2 Cys residue (cysteine); CYS, position of a cysteine residue (usually in our machine we can identify a component that absorbs at 330 nm which corresponds to this amino acid). The two bottom lines contain the complete primary structure of CN2, as determined by Zamudio et al. [7], and SN3, as corrected by Fontecilla-Camps et al. [1]. Cysteinyl residues are printed in bold.

bottom center of Fig. 1. From observation of Fig. 1 it seems that the portion facing forward at the back of the hand of these two molecules may play an important role in defining species specificity. On the other hand, the analysis made earlier by Kobayashi et al. [16] shows another important structural feature of the scorpion toxins. The spatial arrangement of these toxins seems to follow the same pattern: they all have the same general motif, around which many variants have been constructed by natural selection [17]; the only highly conserved residues are the cysteines [16]. Thus the substitutions of amino acids in key positions of the toxin, at the protruding J- and B-loops, located, respectively, at relative positions 15–25 and 40–45 [18]), and at the confluent N- and C-terminal regions of the molecules [1,5,10], seem to be important structural determinants of the species specificity and/or fine mechanism of action of these toxins (eg. α- vs. β-scorpion toxins), as already noted [4]. Loret et al. [19] have proposed that the species specificity might reside in the C-terminal region of the molecule. Toxins having proline-rich segments at the C-terminus are thought to be more rigid, so they would not fit well into the binding sites of the Na^+ channels of mammals, while those having a more flexible C-terminus, like γ-toxin (toxin VII) from *Tityus serrulatus* [19], can recognize the Na^+ channel of either mammals or insects.

Although chemical modification of lysine at position 56 of toxin II from *A. australis* [20] showed it to be crucial for toxicity, the study of the structure–function relationship of Na^+ channel scorpion toxins is less advanced than in other toxins, such as charybdotoxin, a K^+ channel toxin [21]. Park and Miller [21] showed by genetic engineering that a single lysine residue at position 27 was fundamental to toxin activity. Also, Gurrola et al. [22] and Vaca et al. [23] have found that the N-terminal segment of noxiustoxin, another K^+ channel scorpion toxin, was important for its toxicity. The latter authors showed that a synthetically prepared nonapeptide of noxiustoxin was sufficient for channel recognition and modulation.

Thus, concerning the species specificity of Na^+ channel scorpion toxins, chemical or genetic modifications of the residues discussed above should still be conducted before a clear-cut picture can emerge. The construction and expression of artificially mutated toxins, by genetic engineering of the cloned cDNA genes [24–26], should allow the hypotheses discussed above to be tested.

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3.2.2 Vaca, L., G.B. Gurrola, L.D. Possani, D.L. Kunze, (1993) "Blockade of a K_{Ca} channel with synthetic peptides from noxiustoxin: a K^+ channel blocker". en *The Journal Membrane Biology*, 134, 123-129p.

En este trabajo se muestra el efecto de péptidos sintéticos correspondientes a secuencias de aminoácidos de la noxiustoxina sobre un canal de potasio activado por calcio de pequeña conductancia, obtenido de cultivo de células endoteliales de aorta de bovino.

La NTX induce una reducción de la probabilidad de apertura (Po) del canal de potasio dependiente de calcio de pequeña conductancia. Este bloqueo es dosis dependiente, teniendo una IC_{50} de 310 nM. Los péptidos sintéticos correspondientes a la región N-terminal de la NTX son capaces de reducir la probabilidad de apertura del canal de forma semejante a la NTX, con diferente afinidad (NTX1-20, IC_{50}=5μM; NTX1-9, IC_{50}=40μM; NTX1-6 IC_{50}=500μM). Los péptidos correspondientes a secuencias del extremo C-terminal (NTX35-39, NTX30-39) en concentraciones hasta de 1 mM, no tienen efecto sobre este canal. Dado que las curvas dosis respuesta de la NTX y los péptidos sintéticos son paralelas, se sugiere que la diferencia de acción entre estos péptidos y la toxina nativa reside en la afinidad aparente por el canal.

El principal efecto de la NTX y los péptidos sintéticos es sobre la reducción de la constante de tiempo del estado abierto (conductor) sin tener efecto en la distribución de tiempo del estado cerrado.

La región mínima para observar bloqueo de este canal fue NTX1-6.

Estos resultados sugieren que en la región amino terminal está localizada parte de la secuencia que reconoce al canal de potasio dependiente de calcio de pequeña conductancia, lo cual concuerda con trabajos anteriores realizados "in vivo" (Gurrola *et al.*, 1989) y con los trabajos relizados en membranas de cerebro de rata. (primera parte de este trabajo de tesis).

J. Membrane Biol. 134, 123–129 (1993)

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Membrane
Biology

Blockade of a K_{Ca} Channel with Synthetic Peptides from Noxiustoxin: A K^+ Channel Blocker

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Abstract. Using the outside-out configuration of the patch-clamp method, we studied the effect of several synthetic peptides corresponding to various segments from the N-terminal region of noxiustoxin (NTX) on single Ca^{2+}-activated K^+ (K_{Ca}) channels of small conductance obtained from cultured bovine aortic endothelial cells. These peptides induced diverse degrees of fast blockade in the endothelial K_{Ca} channel. The most effective blockers were the peptides NTX_{1-39} (IC_{50} = 0.5 μM) and NTX_{1-20} comprising the first 20 amino acids from the native toxin ($IC_{50} \approx 5$ μM), while less effective was the hexapeptide NTX_{1-6}, from the first six amino acid residues of NTX (IC_{50} = 500 μM). This was the minimum sequence required to block the channel.

By testing overlapping sequences from the entire molecule, specially those corresponding to the N-terminal region of NTX, we have been able to determine their different apparent affinities for the K_{Ca} channel. Synthetic peptides from the C-terminal region produced no effect on the K_{Ca} channel at the concentrations tested (up to 1 mM). These results confirm that in the N-terminal region of the NTX is located part of the sequence that may recognize K^+ channels, as we have suggested previously from in vivo experiments. The blockade induced by native NTX was poorly affected by changes in membrane potential; however, the blockage induced by synthetic peptides lacking the C-terminal region was partially released by depolarization.

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Key words: Noxiustoxin — Synthetic peptides — Patch clamp — K^+ channels

Introduction

Noxiustoxin (NTX) is a 39 amino acid peptide purified from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann [14]. This was the first animal toxin described as a specific K^+ channel blocker [3]. NTX can reversibly block several types of K^+ channels, including the delayed rectifier [3], voltage-gated K^+ channels from human T lymphocytes [15], Ca^{2+}-activated K^+ (K_{Ca}) channels from skeletal muscle [18] and whole-cell K_{Ca} currents from bovine aortic endothelial cells [4]. However, NTX has no effect on the inward rectifier K^+ channel [4].

In previous studies we showed that the synthetic peptides corresponding to the amino acid sequence 1–9 (NTX_{1-9}) 1–20 (NTX_{1-20}) and 1–39 (NTX_{1-39}) of NTX are toxic to mice, inducing symptomatology similar to that produced by native NTX [9]. We have shown also that these synthetic peptides can induce neurotransmitter release mediated through K^+ channels, suggesting that the peptides are capable of blocking K^+ channels [9]. However, a direct measurement of the effect of these synthetic peptides on a K^+ channel has not been provided, thus far.

We show that several synthetic peptides corresponding to the N-terminal region of NTX can induce diverse degrees of blockade on a K_{Ca} channel from bovine aortic endothelial cells (BAECs), confirming an earlier suggestion [9] that part of the sequence that may recognize K^+ channels is located in this region.

Materials and Methods

Reagents

All salts, solvents and chemicals used were analytical grade, obtained as previously described [9]. Reagents used for peptide synthesis were HPLC grade. Protected amino acids (t-BOC-amino acids) and resins containing the first amino acid bound were purchased from Peninsula Laboratories. Solvents used for peptide synthesis were obtained from Aldrich and Pierce Chemical.

Noxiustoxin

Purification of NTX from whole *C. noxius* venom was achieved as previously described [14], using a Sephadex G-50 gel filtration, followed by ion exchange chromatography in carboxymethyl-cellulose resins with 20 mM ammonium acetate pH 4.7, and re-chromatography with the same resin in 50 mM phosphate buffer, pH 6.0.

Peptide Synthesis and Characterization

All synthetic peptides were synthesized using the solid phase method [11] as previously described [9]. The yield of each newly incorporated amino acid in the growing polypeptidic chain was ascertained by the ninhidrin reaction [16]. At the end of the synthesis, the peptides were liberated from the resin by cleavage with fluorhydric acid [11]. All peptides were purified by high performance liquid chromatography (HPLC) using a C18 reverse phase column eluted with a linear gradient of acetonitrile from 0 to 60% in presence of 0.1% trifluoroacetic acid. The resulting peptides were hydrolyzed by HCl 6 N, 110°C, and their compositions were determined by amino acid analysis [9]. When needed, an additional separation using an isocratic gradient was applied to the peptides. Some peptides were confirmed by direct amino acid sequence using an automatic Beckman 890M micro-sequencer. Only highly purified peptides were used for the experiments described here.

Solutions

The HiK solution contained in mM: 150 K aspartate, 10 HEPES, 2 $CaCl_2$, 2.2 EGTA. pH adjusted to 7.2 with H_2SO_4. The free Ca^{2+} concentration was 1 μM [7]. All peptides were applied to the membrane patch with a perfusion system modified from Carbone and Lux [1] driven by gravity.

Cell Culture

BAECs were obtained as previously described [6]. Cells were kept in culture and used from passages 10 to 20 [4]. Confluent monolayers were mechanically dispersed with a plastic pipette and replated on a petri dish allowing cell reattachment for 10–20 min. With this procedure single cells were obtained and used for patch-clamp experiments.

Single Channel Recording

All experiments were performed at room temperature. The outside-out configuration of the patch clamp [10] was used to study single channels obtained from excised patches from single endothelial cells. Pipettes were fabricated from thick-walled glass (8161, Garner) using a two-stage pipette puller (Narishige), and fire-polished with a microforge (Narishige). Pipette resistances ranged from 5–12 MΩ when filled with the HiK solution. The reference electrode used was a Ag-AgCl plug connected to the bath solution via a 150 mM KCl agar bridge. The extracellular face of the patch was used to report voltages. The amplifier was the Axopatch 1C from Axon Instruments. Single channel fluctuations were initially stored on FM tape (Racal) and digitized later for computer analysis using an analog-to-digital interface (Axon Instruments) connected to an IBM 386 clone. The signal was filtered with a low-pass 8-pole Bessel filter (Frequency Devices) at 5 KHz and digitized at 10 KHz (100 μsec/sample). All the records with single channel activity were filtered at 1 kHz for illustrative purposes.

Single Channel Analysis

Fetchan and Pstat (Axon Instruments) were used for data analysis. The half-amplitude criterion was used to distinguish between the open and the closed states of the channel [5]. P_o was calculated from 30–60 sec records using the equation P_o = (open time/total time). Time distributions have been binned logarithmically to improve the resolution of multiple exponential components [17]. The routine used to fit the data consisted of a generalized nonlinear least-squares procedure based on the Levenberg-Marquadt algorithm, which fit up to four exponentials to raw data. For previously binned data (distributions), the method used for fitting was the maximum likelihood. Fitting iterations proceeded until convergence was reached, as defined when successive improvements in parameters produce a change in the chi-square value less than 2.5×10^{-7}.

Results

Synthesis of Peptides

Figure 1 shows the amino acid sequence of noxiustoxin with the peptides synthesized for this work underlined. Eight overlapping hexapeptides, corresponding to the full amino acid sequence of NTX were synthesized. A nonapeptide and an eicosapeptide from the N-terminal region and a pentapeptide and decapeptide from the C-terminal were also synthesized. Figure 2 represents an example of HPLC separation of a synthetic peptide. The main peak from the chromatogram was identified as the hexapeptide NTX_{1-6} by amino acid sequence. A similar procedure was followed to identify all the synthetic peptides used in this work.

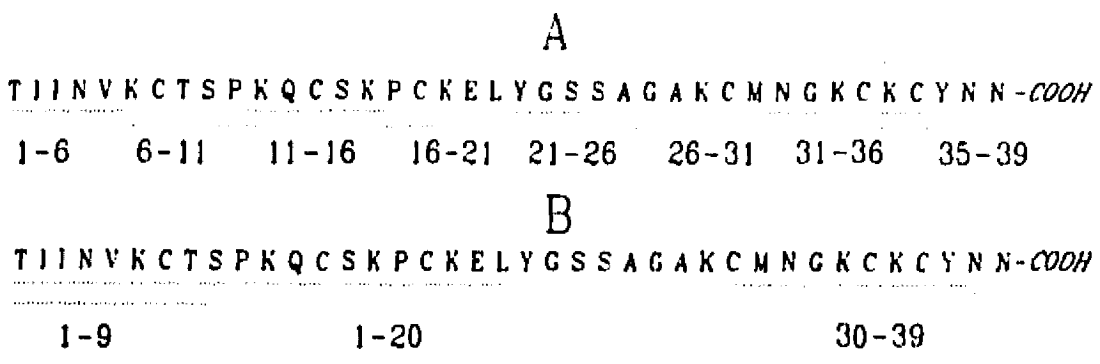

Fig. 1. Peptides synthesized for this work. *Panel A:* Small peptides (hexapeptides). *Panel B:* larger peptides. All peptides are aligned by their N-terminal region with NTX sequence.

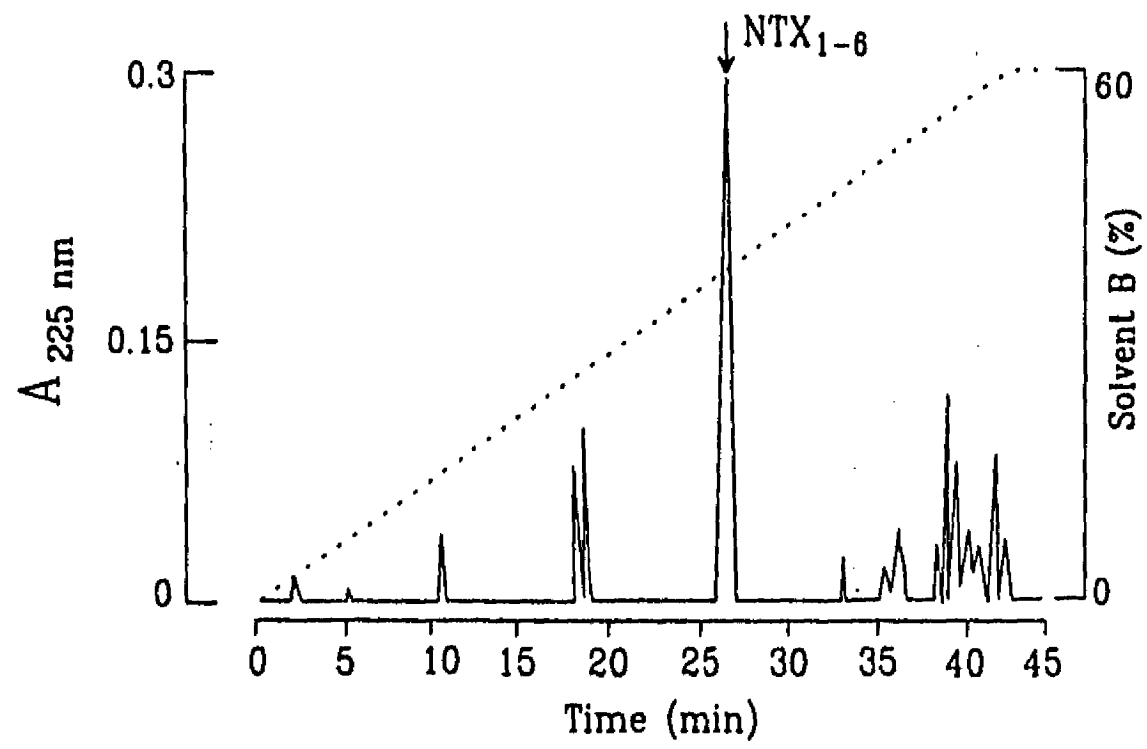

Fig. 2. HPLC separation of synthetic peptide NTX_{1-6}. The peptide (228 μg) was injected at time zero in a Beckman chromatographic system. An Altex C_{18} reverse phase column was used to separate the peptides. An isochratic gradient from solvent A (0.12% trifluoroacetic acid in distilled water) to solvent B (0.1% trifluoroacetic acid in acetronitrile) was used to separate the components. Absorbance was measured at 225 nm. The largest peak in the chromatogram (indicated by the arrow) corresponds to NTX_{1-6} according to amino acid analysis and sequence. Chromatographic separations like this one were used to purify the synthetic peptides in this study.

NTX Blocks Single K_{Ca} Channels

The effect of various concentrations of NTX on K_{Ca} channel activity is shown in Fig. 3. NTX induced a concentration-dependent reduction of channel open probability (P_o) with an apparent IC_{50} of ≈310 nM ($n = 4$). NTX blocked this channel only when used in the extracellular solution. When 1 μM NTX was applied to the intracellular face of the channel, no effect on channel P_o was observed ($n = 3$, *data not shown*).

Blockage Induced by Synthetic Peptides

Synthetic peptides corresponding to overlapping regions from the primary structure of NTX (Fig. 1) were used at different concentrations to identify the region in the NTX sequence responsible for binding and blocking this K_{Ca} channel. Only sequences corresponding to the N-terminal region of NTX were capable of inducing a concentration-dependent reduction of channel P_o. Figure 4 shows the concentration-response curve for those peptides that affected channel P_o. In general, we found that larger peptides were more effective in reducing channel P_o. The most effective channel blockers were the peptides NTX_{1-39} ($IC_{50} \approx 0.5$ μM) and NTX_{1-20} ($IC_{50} \approx 5$ μM) which comprise the first 20 amino acids from NTX. Less effective were the nonapeptide NTX_{1-9} ($IC_{50} \approx 40$ μM) and the hexapeptide NTX_{1-6} ($IC_{50} \approx 500$ μM). The pentapeptide NTX_{35-39} and the decapeptide NTX_{30-39} corresponding to the C-terminal region of NTX had no effect on channel P_o at the concentrations tested (up to 1 mM, $n = 3$, *data not shown*). The hexapeptides NTX_{6-11} and NTX_{11-16}, which are contained in the peptide NTX_{1-20}, produced no effect on channel P_o at the concentrations tested (up to 1 mM, $n = 4$, *data not shown*). The hexapeptides NTX_{16-21} and NTX_{21-26} were also unable to modify channel P_o (1 mM, $n = 3$, *data not shown*), just like the other peptides from the C-terminal region of NTX. These results indicate that the first 1–20 amino acids of NTX are essential for recognizing this K_{Ca} channel but only the peptides containing the region 1–6 can block the channel. This was the minimum region required to block the channel.

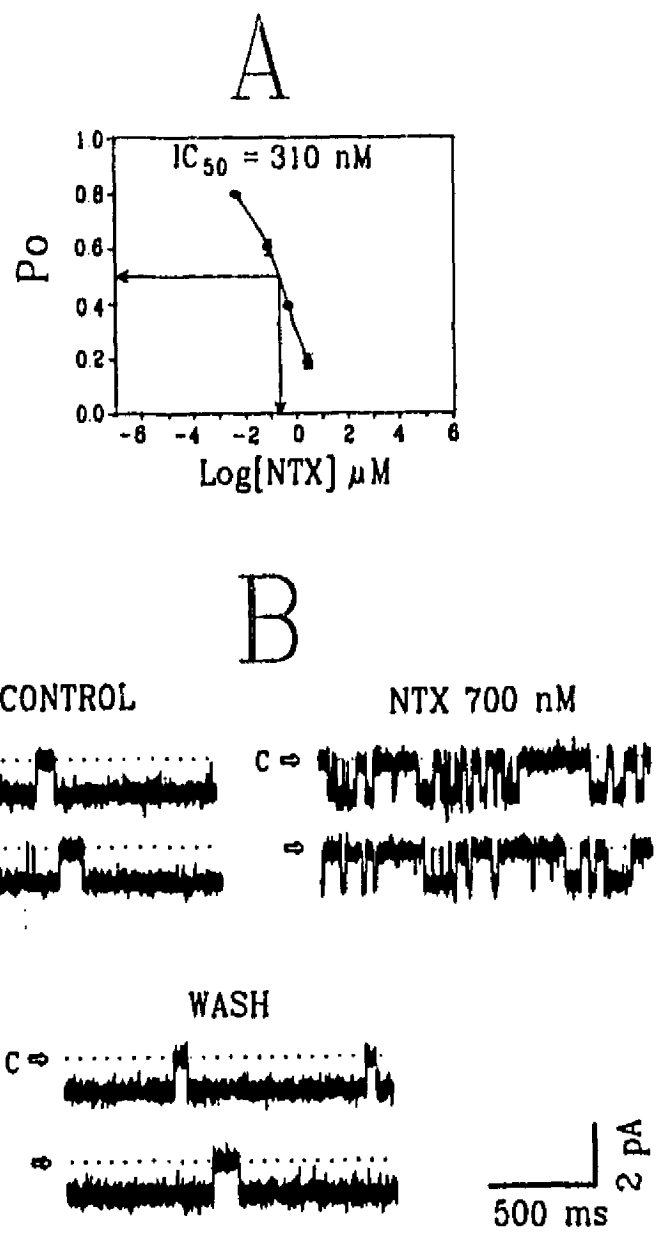

Fig. 3. Noxiustoxin blocks single K_{Ca} channels in BAECs. (*A*) Concentration-response curve for NTX obtained at −40 mV. Unbroken line was used to connect data points. The half inhibitory concentration (IC_{50}) = 310 nM (n = 4). (*B*) Examples with channel activity at −40 mV from an outside-out patch containing one K_{Ca} channel. Solutions used symmetrical HiK. The dotted line indicates the zero current level (baseline). Arrows point to the closed level (*C*). Channel activity was monitored under control conditions and after addition of several concentrations of NTX (only 700 nM shown). Full recovery was achieved after replacing the bath solution with toxin-free buffer.

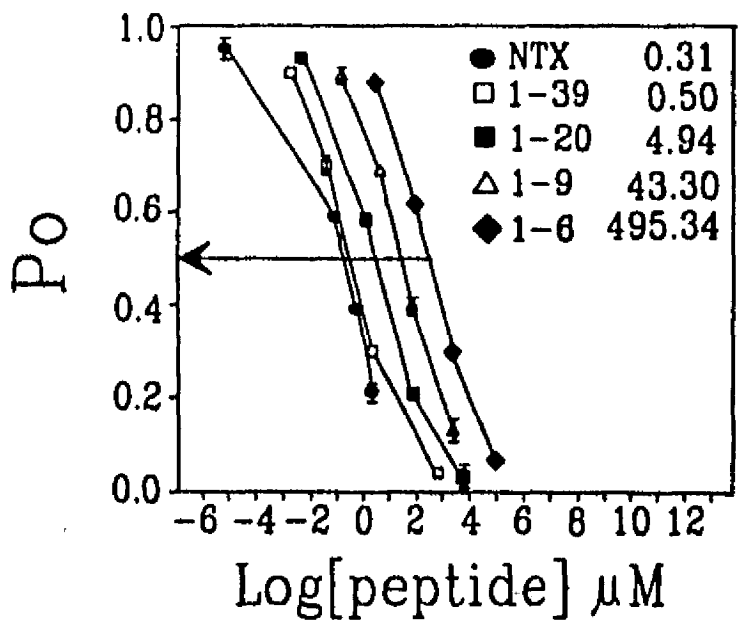

Fig. 4. Concentration-response curves for synthetic peptides. Concentration-response curve for the peptides that affected channel P_o. Half inhibitory concentrations for native NTX (●, n = 4), NTX_{1-39} (□, n = 3), NTX_{1-20} (■, n = 5), NTX_{1-9} (Δ, n = 4) and NTX_{1-6} (◆, n = 6). Unbroken lines were used to connect data points. The holding potential is −40 mV. Solutions used symmetrical HiK.

Modulation of Open and Closed Times

Figure 5 illustrates the effect of NTX on channel open and closed time distributions. Under control conditions the channel displayed two mean open and two mean closed times when measured at −40 mV. The time constants for the open time distributions were ≈150 and ≈6 msec. The time constants for the closed state were ≈0.6 and ≈8 msec. At the IC_{50} of the NTX the long-lived open time was reduced from 147 ± 6 msec (control) to 5.1 ± 2 msec (300 nM NTX). The short-lived open time was also affected by NTX. At the IC_{50} this time constant was reduced from 6.15 msec (control) to 0.38 msec (300 nM NTX).

Figure 6 shows the effect of the N-terminal synthetic peptides on the time distributions. NTX and the synthetic peptides affected in a similar way the time distributions of the channel. All the effective peptides reduced the long-lived open time of the channel ≈25–30 times and the short open time ≈7–13 times with little effect on the closed time distributions.

Effect of Voltage on K_{Ca} Channel Block

Previous studies reporting the effect of NTX on voltage-gated K^+ currents from squid axon indicated that at low concentrations the blockade induced by NTX was voltage insensitive but at larger concentrations (≳1.5 μM) the blockade was voltage sensitive [2]. Contrary to this report, we found that in this K_{Ca} channel the blockade induced by NTX was poorly affected by changes in the membrane potential; however, voltages outside the range ±60 mV were not explored. Figure 7 illustrates the effect of two different voltages (+60 and −60 mV) on the IC_{50} for NTX and the synthetic peptides. Positive voltages released more effectively the blockade produced by the peptides NTX_{1-6} and NTX_{1-9} with little or no effect on the blockade produced by NTX, NTX_{1-39} or NTX_{1-20}. This result indicates that the blockage by small peptides lacking the region NTX_{10-20} can be slightly attenuated by membrane depolarization.

Discussion

Blockade by NTX and Synthetic Peptides

The parallel shift in the concentration-response curves of NTX and the synthetic peptides suggests that the difference between the native toxin and the peptide fragments resides in their apparent affinities for the channel. We found that larger peptides were more effective channel blockers. However, large peptides lacking the sequence NTX_{1-6} produced no effect on channel P_o (e.g., peptide NTX_{30-39}). The minimum sequence capable of blocking the channel was the hexapeptide NTX_{1-6}; however, the most effective channel blockers were the peptides NTX_{1-39}, NTX_{1-20} and NTX_{1-9} (in that order). When measuring in previous studies the ability of these peptides to induce neurotransmitter release in mouse synaptosomes mediated through voltage-gated K^+ channels, we found the same potency sequence [9]. This result suggests that the binding site recognized by NTX and the synthetic peptides is conserved among various types of K^+ channels. However, the affinity of NTX for different types of K^+channels is variable. In this study we found an apparent affinity of ≈300 nM for native NTX. Valdivia et al. [18] reported that NTX blocks K_{Ca} channels of large conductance with an apparent affinity of 450 nM. Carbone et al. [2] reported an apparent affinity of 290 nM for the delayed rectifier while Sands et al. [15] found that NTX blocks voltage gated K^+ channels from T lymphocytes with an apparent affinity of 0.20 nM. The apparent affinity reported here is within the range of previously published values obtained in whole-cell experiments with BAECs [4].

Modulation of Open Time by the Toxins

The major effect of NTX and synthetic peptides was the reduction of the open time constants with little or no effect on the closed time distributions. The affinity of the toxins (NTX and synthetic peptides) for the channel is proportional to the ability of the toxins to reduce the association constant. At the IC_{50} of the toxins the long mean open time was reduced ≈30 times while the short-lived open time was reduced ≈7–13 from the control values. Interestingly, a third nonconducting state (presumably the blocked state) could not be identified. This suggests that the mean lifetime of the blocked state is similar to that of the nonconducting (closed) states. The relative occurrence of the blocked state is difficult to calculate since the blocked and the closed states are nonconducting (they have the same amplitudes) and no significant difference was observed on either closed time constants at any of the toxin concentrations tested.

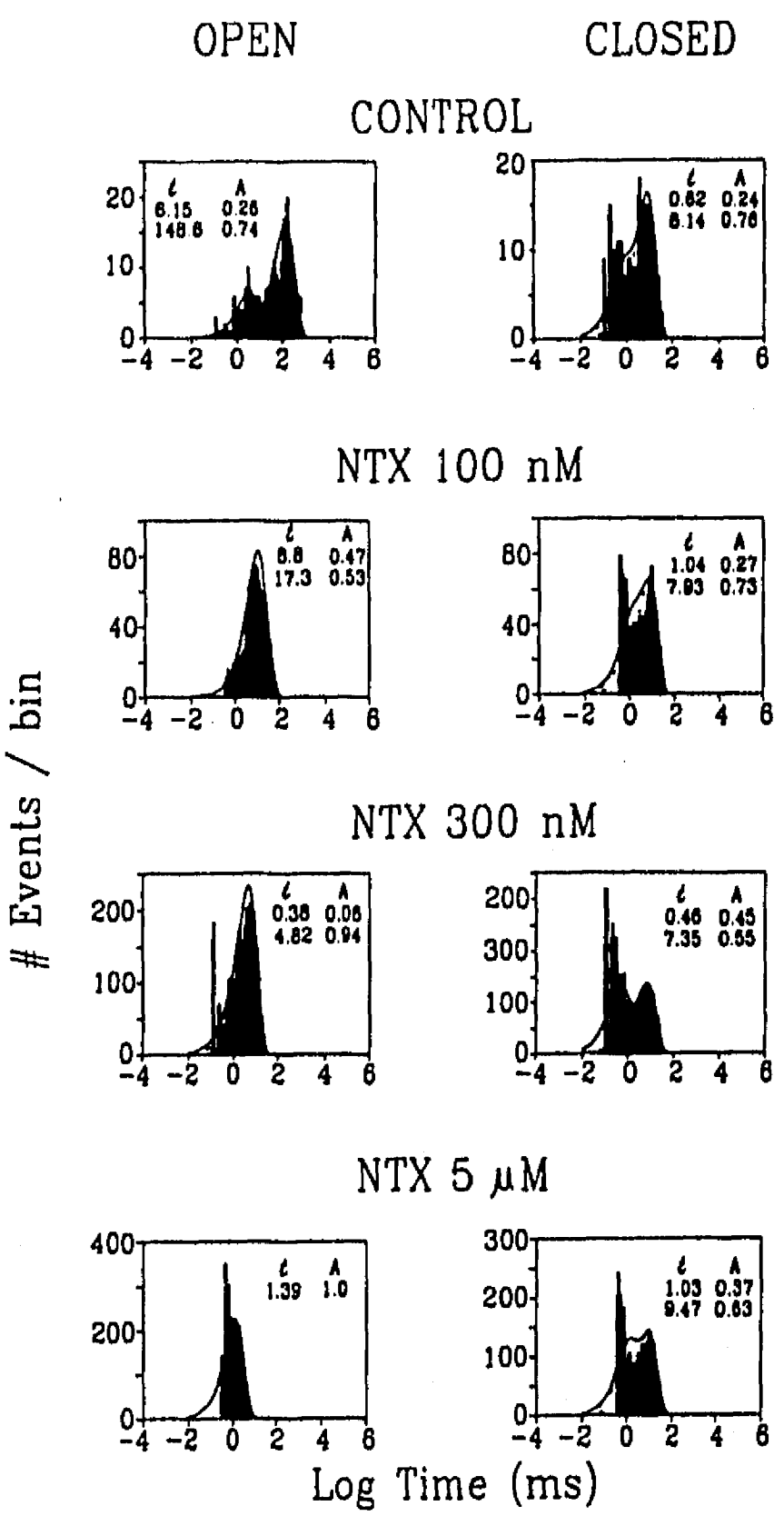

Fig. 5. Effect of NTX on channel open and closed time distributions. Dwell-time distributions obtained from one outside-out patch under control conditions and after addition of 100 nM, 300 nM and 5 μM NTX to the bath (extracellular) solution. Open and closed time distributions were binned logarithmically from records containing 50–60 sec of continuous channel activity for each experimental condition. The binwidth used was 0.1 msec. Time constant (τ) in milliseconds and amplitude (A) for each exponential component are shown in the inset. The P_o obtained for each experimental condition was 0.95 (control), 0.68 (100 nM), 0.52 (300 nM) and 0.14 (5 μM). The dotted line indicates the individual exponential and the unbroken line represents the fit to a double exponential function. Holding potential for all measurements was −40 mV. Symmetrical HiK solution.

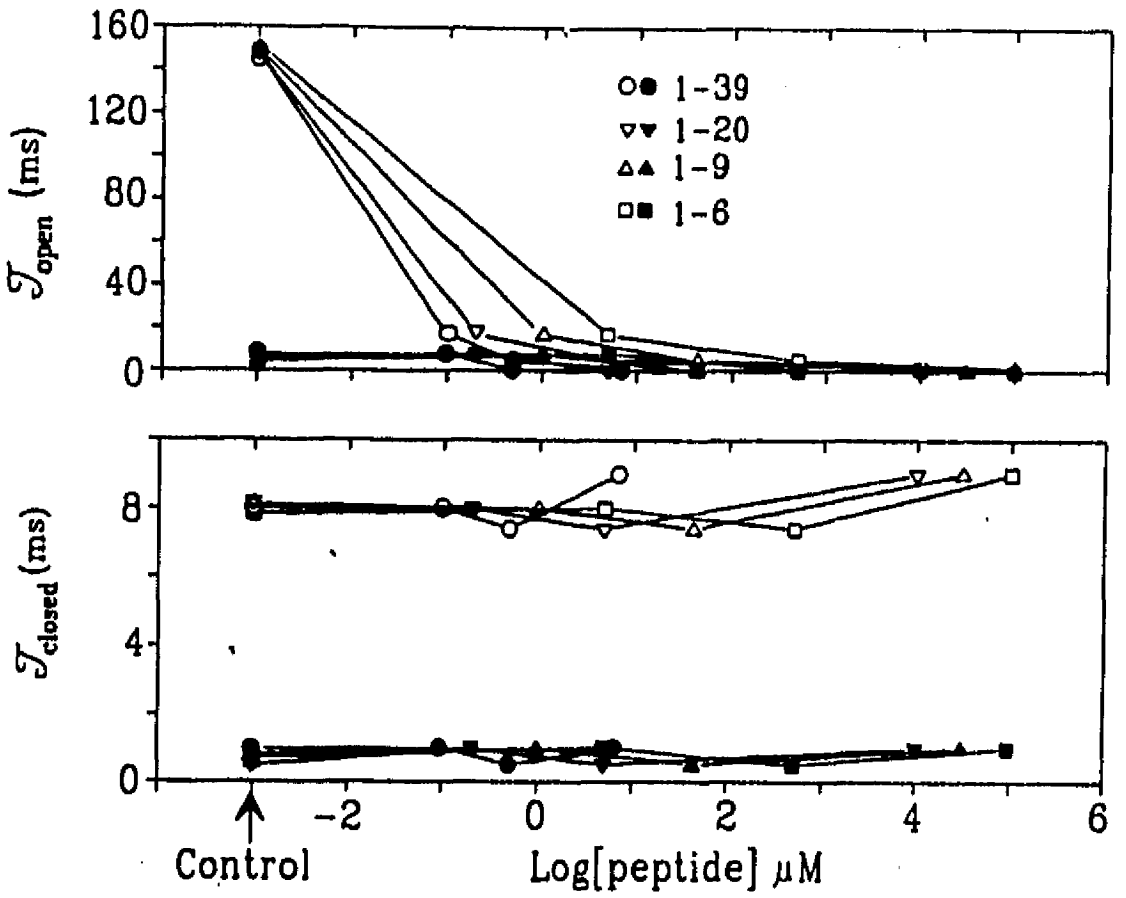

Fig. 6. Effect of synthetic peptides on channel open and closed time distributions. Mean open and closed times obtained at different concentrations of the synthetic peptides. These mean times were obtained after fitting with a double exponential function experiments like the one described in Fig. 5. Open symbols represent long-lived events while close symbols indicate short-lived events for NTX_{1-39} (○●, n= 3), NTX_{1-20} (▽▼, n = 4), NTX_{1-9} (△▲, n = 3) and NTX_{1-6} (□■, n = 2). The unbroken lines through the data represent a linear least-squares fit. Holding potential for all measurements was −40 mV. Channel activity was obtained from at least one minute of continuous recording in symmetrical HiK solution.

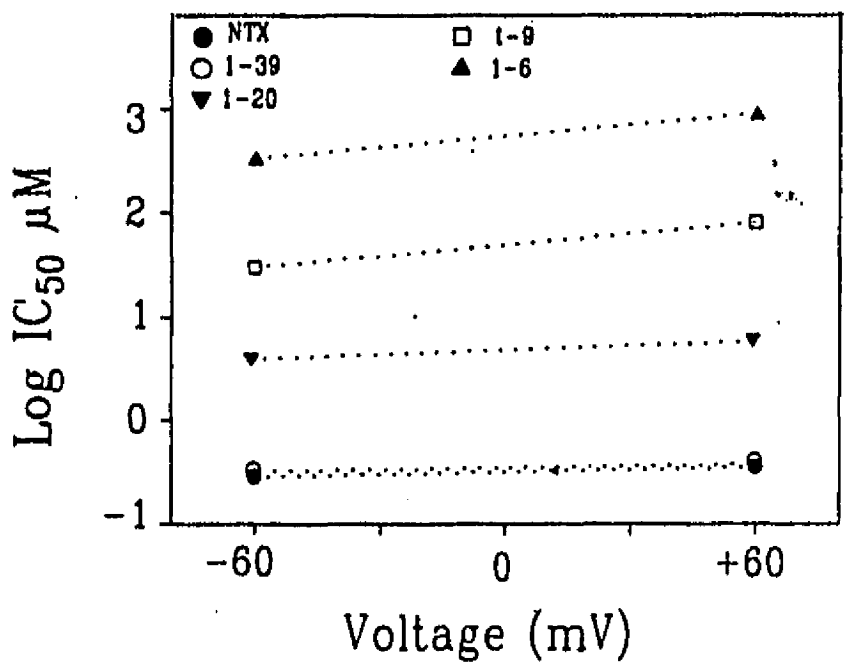

Fig. 7. Effect of voltage on channel blockade. The effect of two different voltages (−60 and +60 mV) on the IC_{50} of NTX (●, n = 3) and the synthetic peptides NTX_{1-39} (○, n = 3), NTX_{1-20} (▼, n = 4), NTX_{1-9} (○, n = 4) and NTX_{1-6} (▲, n = 3) explored in outside-out patches. The IC_{50} used here were obtained from concentration-response curves as those illustrated in Fig. 4. Solutions used symmetrical HiK.

If we assume a simple bimolecular binding model to approach the mechanism of block by the toxins, we can express it as follows:

$$\text{TOXIN} + \text{CHANNEL} \underset{K_{off}}{\overset{K_{on}}{\longleftrightarrow}} \text{TOXIN-CHANNEL}$$

Where K_{on} = association constant and K_{off} = dissociation constant. The equilibrium dissociation constant should be $K_d = K_{off}/K_{on}$. For this model to be valid the following criteria need to be met. (i) The time constants of the blocked state ($1/K_{off}$) should be independent of the toxin concentration, (ii) the time constant of the unblocked state ($1/K_{on}$) should decrease proportionally with increasing toxin concentrations. Our results are consistent with this model.

Effect of Voltage on Channel Blockade

The blockade induced by native NTX is insensitive to changes in membrane potential in the range ±60 mV with symmetrical K^+. However, the blockade produced by synthetic peptides lacking the C-terminal region of NTX can be partially released by depolarization. This result suggests that in the region 10–20 of the primary structure of NTX there is a specific sequence which prevents the release of blockade by depolarization. This sequence may stabilize the binding of the toxin to its receptor in the channel or prevent the toxin from sensing the transmembrane potential (or both).

Structure-Function Relationships

NTX belongs to a family of small peptides targeting K^+ channels. This family of toxins is composed of NTX, charybdotoxin (CTX) a toxin isolated from a European scorpion [12] and the recently isolated iberiotoxin (IBX) [8]. CTX and IBX share 68% sequence homology between them and about 50% ho-

mology with NTX. CTX and IBX block K_{Ca} channels in a similar way—both toxins induce long-lasting nonconducting periods of minutes in duration. The effect of NTX on the K_{Ca} channel is clearly different. NTX induces a fast flickering block in K_{Ca} channels ([17] and this study). We have shown here that the amino acid sequence that recognizes K_{Ca} channels is located in the N-terminal region of NTX. A recent report indicates that the C-terminal region of CTX appears to be involved in recognizing the K_{Ca} channel [13]. Point mutations of CTX at Arg25, Lys27 and Arg34 decreased the toxin affinity for the channel. In that study the affinity change was produced by an increased dissociation rate. In our study, changes in the toxin affinity for the K_{Ca} channel were related to a decrease in the association rate. Interestingly, the higher homology among these toxins (CTX, IBX and NTX) occurs at the C-terminal region. Amino acids 25, 27 and 34 are identical between CTX and IBX; however, in NTX the only amino acid conserved is Lys27, the other two amino acids are replaced by an Ala at position 25 and a Lys at position 34.

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3.2.3 Martin, B.M., A.N. Ramírez, G.B. Gurrola, M. Nobile, G. Prestipino, L.D. Possani (1994). "Novel K^+ channel blocking toxins from the venom of the scorpion *Centruroides limpidus limpidus* Karsch". *The Biochemical Journal*, (en prensa)

En este trabajo se describe la purificación, a partir del veneno del alacrán *Centruroides limpidus limpidus*, de dos péptidos (Cll-1 y Cll-2) que bloquean canales de potasio dependientes de voltaje de corriente transitoria (T) en células granulares de cerebelo de rata, con una IC_{50} de 1.5 μM.

Estos péptidos se identificaron en el veneno utilizando anticuerpos monoclonales anti-Noxiustoxina, suponiendo que toxinas semejantes pueden compartir determinantes antigénicos.

Estas nuevas toxinas son péptidos de 38 aminoácidos teniendo una similitud del 64% con NTX; son capaces de competir con ^{125}I-NTX en la unión a membranas de cerebro de rata, mostrando una IC_{50} de 100pM.

La toxina Cll-1 tiene en la posición 30 una isoleucina en lugar de una metionina como presentan otras toxinas que actúan sobre canales de potasio (ver discusión al final de este trabajo de tesis). En estudios realizados con caribdotoxina, se ha observado que el cambio de esta metionina por isoleucina modifica de forma importante la afinidad (1000 veces menor) de esta toxina por el canal de potasio dependiente de calcio de alta conductancia (Stampe *et al.*, 1994). En este caso podemos decir que en la toxina Cll-1 este aminoácido no es tan importante en la interacción con el receptor.

Novel K^+-channel blocking toxins from the venom of the scorpion *Centruroides limpidus limpidus* Karsch

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Two novel toxins were purified from the venom of the Mexican scorpion *Centruroides limpidus limpidus*, using an immunoassay based on antibodies raised against noxiustoxin (NTX), a known K^+ channel-blocker-peptide. The primary structure of *C. l. limpidus* toxin 1 was obtained by Edman degradation and was shown to be composed of 38 amino acid residues, containing six half-cystines. The first 36 residues of C.l.l toxin 2 were also determined. Both toxins are capable of displacing the binding of radiolabelled NTX to rat brain synaptosomes with high affinity (about 100 pM). These toxins are capable of inhibiting transient K^+-currents (resembling I_A-type currents), in cultured rat cerebellar granule cells. About 50% of the peak currents are reduced by application of a 1.5 μM solution of toxins 1 and 2. The K^+ current reduction is partially reversible, under washing, but not voltage-dependent. Comparison of the primary structure of C.l.l. toxin 1 with other known toxins shows 74% identity with margatoxin, 64% with NTX, 51% with kaliotoxin, 39% with iberiotoxin, 37% with charybdotoxin and Lq2, and 29% with leirutoxin 1. The only invariant amino acids in all these toxins are the six cysteines, a glycine in position 26 and two lysines at positions 28 and 33, respectively. The relevance of these differences in terms of possible structure-function relationships is discussed.

INTRODUCTION

Scorpion venoms contain a variety of peptides toxic to man [1], insects [2] and crustaceans [3,4]. Several families of peptides which target the ion channels of excitable membranes have been described (Na^+ [5,6], K^+ [7,8], Ca^{++} [9] or Cl^--channels [10]. Our group has contributed to this field by isolating and characterizing the first K^+-channel blocking peptide: noxiustoxin (NTX) [7,11], and by showing the structural similarities of NTX and Charybdotoxin [12]. NTX, purified from the venom of the Mexican scorpion *Centruroides noxius* was shown to block several K^+-channels from a variety of tissues, with different affinities: squid axon [7], brain synaptosomes [13], skeletal muscle [12], aortic endothelial cells [14] and T-cell lymphocytes [15,16,17]. Recently, the number of known peptides from scorpion venom which interfere with excitable and non-excitable cells has increased considerably [18,29,20,21,22,23,24,13,25]. The primary structure of these peptides is similar and they possibly have a three-dimensional structure with conserved motifs [26], but the molecular mechanism of action in terms of affinity and specificity varies considerably [27]. Thus, in order to increase our knowledge on the structure–function relationship of toxins *versus* ion-channels there is need for a continuing effort in the direction of isolating and characterizing all peptides available in scorpion venoms. It is worth mentioning the pressure of selection to which these animals have been subjected for millions of years, during which period scorpions have evolved interesting peptides to help them prey on or defend themselves against other animals [4,1,2].

In this paper we describe two novel K^+-channel toxins purified from the Mexican scorpion *Centruroides limpidus limpidus*, which affect K^+ permeability of rat cerebellar granule cells and displace binding of Noxiustoxin to rat brain synaptosomes.

MATERIALS AND METHODS

Source of venom and separation procedures

Crude venom from *C. l. limpidus* was obtained in the laboratory, by electrically stimulating the telsons of scorpions collected in the state of Guerrero. The animals were anaesthetized with carbon dioxide before venom extraction. The venom was recovered in doubly distilled water and centrifuged for 10 min at 4°C and 18000 *g* ($r_{max.}$ 10.8 cm). The supernatant was lyophilized and stored at −20 °C before use.

The purification of toxic peptides from the soluble venom was carried out by several chromatographic steps starting with gel-filtration on a Sephadex G-50 (medium grade, Pharmacia Fine Chemicals, Uppsala, Sweden) column. Freeze-dried venom was dissolved in 20 mM ammonium acetate buffer, pH 4.7, and applied directly to the column. Subsequently, two chromatographic separations on carobxymethyl-cellulose (CM-cellulose) ion-exchange resin (CM-32 from Whatman Inc., Clifton, New Jersey, U.S.A.) run at pH 4.7 and pH 6.0 respectively, were necessary in order to separate the toxic components of the venom. The final purification step consisted of h.p.l.c.

Detailed information about these procedures is given in the figure legends. Recovery was calculated based on absorbance at 280 nm. Electrophoresis was performed by the method of Reisfeld et al. [28]. Columns were run at room temperature (25 °C). All chemicals and solvents were of analytical-reagent grade.

Chemical characterization

Chemical characterization was performed by amino acid analysis, mass spectrometry and microsequencing, as described earlier [23,29]. Briefly, amino acid composition was obtained after acid

Abbreviations used: CM-cellulose, carboxymethyl-cellulose; NTX, noxiustoxin; TFA, trifluoroacetic acid

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hydrolysis of peptides, using a Beckman 6300 analyser. Molecular masses of toxins were confirmed by mass spectrometry of electrospray-ionized samples and the Millipore 6225 Pro-sequencer (Bioanalytical Division) was used for sequence determination of reduced and carboxymethylated toxins [29].

Lethality test

Three designations were used to define the lethality of the various protein components of the venom. 'Nontoxic' means that the mouse injected did not show symptoms of intoxication, similar to injection of saline or buffer alone. 'Toxic' means that the animal showed any of the following symptoms: excitability, salivation, lacrimation, dyspnea, temporary paralysis of limbs, but recovered within 20 h after injection. 'Lethal' means the animal died after showing some or all of the above symptoms. Since these effects are dose- and mouse-strain-dependent we always injected less than 100 μg of the protein per animal (assuming that an absorbance of 1 at 280 nm is equal to 1 mg/ml). The same strain (CD1) of albino mice (18–20 g weight) was used throughout the experiments. The number of mice were kept at the minimum strictly necessary to define the different fractions (1–2 animals), in accordance with protocols approved by the animal care committee of our Institute.

Bioassay with monoclonal antibodies

The bioassay used during purification was based on monoclonal antibodies generated against NTX. Since the isolation of K^+-channel blocking peptides, using direct electrophysiological measurements as a routine assay, is very cumbersome and time-consuming we decided to use an immunological approach by means of specific antibodies. The rationale behind this approach was that if K^+-channel peptides share similar functions they must share structural similarities. Monoclonal antibodies were prepared essentially by the same method described previously by our group for Na^+-channel blocking peptides [30]. Briefly, after immunization of Balb/c mice with NTX, spleen cells were fused with a selected subclone of the SP2/0-Ag14 myeloma line [31], plated in selection medium in 96-well microplates (at a density corresponding to 10^5 spleen cells per well) on a feeder layer consisting of 5×10^3 mouse peritoneal macrophages. Hybridomas were cloned in soft agar and immunoglobulin-secreting clones were detected *in situ*, as previously described [32]. Two different monoclonal antibody-producing lines (named BNTX-14 and BNTX-16) were selected for screening procedures.

Enzymic immunoassays

Enzymic immunoassay (e.l.i.s.a.) was performed on 96-well vinyl plates (Costar, Cambridge, MA, U.S.A.) coated with NTX (100 μl/well of a 3 mg/l solution in 20 mM $NaHCO_3$, pH 9.2) and incubated overnight at 4 °C. Unsaturated sites were masked by incubation with 1% ovalbumin in 0.15 M NaCl/0.02 M phosphate buffer (NaCl/Pi buffer), pH 7.8, at room temperature for 4 h.

A mixture of increasing concentration of purified peptides (50 μl solution) plus a fixed concentration of monoclonal antibodies (in 50 μl BNTX-14 at 3 μg/ml or 50 μl BNTX-16 at 0.3 μg/ml) were added to each well of the plate in NaCl/Pi buffer, pH 7.4, containing 1% BSA and 0.1% Tween. Appropriate washing was conducted at each required step and the final reaction was revealed by means of peroxidase-labelled rabbit anti-(mouse IgG) antibodies (4 h at room temperature) using 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).

Binding experiments

NTX was iodinated by the lactoperoxidase method of Morrison and Bayse [33]), as previously described by our group [13]. Rat-brain synaptosomes were prepared by a similar procedure as that described by Catterall [34]. The rapid filtration assay described originally [29,13] was used to determine binding and competition experiments of NTX and the novel toxins described here. Briefly, binding experiments were initiated by addition of synaptosome membranes to a reaction mixture containing ^{125}I-NTX plus unlabelled NTX or unlabelled new component at increasing concentrations, and incubated at room temperature for 1 h. The reactions were stopped by addition of 5 ml of cold binding medium (140 mM choline chloride/5 mM KCl/1.5 mM $CaCl_2$/0.8 mM $MgCl_2$/20 mM Tris/HCl (pH 7.4/0.1% BSA) and immediately filtered through glass-fibre filters (Whatman GF/G) 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 measurements.

Electrophysiological measurements

Cerebellar granule cells were obtained from newborn rats 8–10 days old. The neurons were prepared as described by Levi et al. [47] and plated on 33 mm Petri dishes coated with 10 μg/ml poly-L-lysine at a density of 2.5×10^6. Cells were maintained in Basal Medium Eagle (BME) with 10% fetal calf serum/100 μg/ml gentamicin/25 mM KCl/2 mM L-glutamine. Cytosine arabinoside (50 μM) was added 20 h after plating in order to prevent the replication of non-neuronal cells. Dishes were maintained at 37 °C, in a 5% CO_2 incubator. Experiments conducted at room temperature (20–22 °C), were performed 4 days after plating. External solution was as follows (mM): 120 NaCl/3 KCl/2 $CaCl_2$/2 $MgCl_2$/20 glucose/10 Hepes. Tetrodotoxin (3×10^{-7} M) was added to the external solution to abolish Na^+ currents, while Ca^{2+} currents were blocked by 1 mM Cd^{2+}. Pipette-filling solution was (mM): 120 KCl/5 EGTA/0.24 $CaCl_2$/30 glucose/10 Hepes. The pH was adjusted to 7.3 with NaOH and KOH for the external and the internal solutions respectively, osmolarity was set to 290 ± 10 mosmol with Manitol. Currents were recorded in patch-clamp whole-cell configuration [20]. Glass electrodes were pulled with a programmable puller (Sachs-Flamming PC-84) to give a tip resistance of about 8 MΩ; holding potential was held at −80 mV. Voltage-pulses and data acquisition were performed on-line with a PDP 11–23 mini-computer. Voltage-pulses 200 ms long were applied from −40 mV to +60 mV in steps of 20 mV. Data were filtered at 3 kHz and leak-subtracted with the P/4 technique from a holding potential of −100 mV.

RESULTS AND DISCUSSION

Separation of soluble venom was performed initially by gel-filtration chromatography in Sephadex G-50 and gave rise to at least four sub-fractions (I–IV), of which number II was lethal to mice. This fraction was subsequently separated on a CM-cellulose ion-exchange column equilibrated and run in 20 mM ammonium acetate buffer, pH 4.7, from which 13 components were derived. Results for Sephadex- and CM-cellulose-chromatography were essentially equal to those previously described [29] and for this

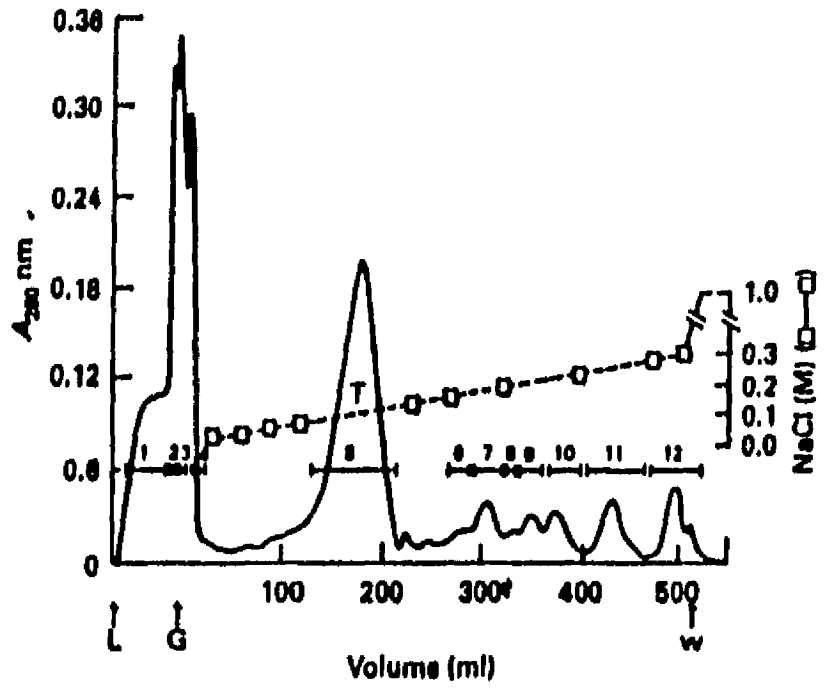

Figure 1 Purification of toxin II.10.9

Toxic fraction II.10 (26.5 mg in 30 ml solution) from reference 37 was applied to a CM-cellulose column (0.9 cm × 33 cm) equilibrated and run in 50 mM potassium phosphate buffer, pH 6.0, at a flow rate of 30 ml/h. A linear NaCl gradient (250 ml 0 M–250 ml 0.3 M) in the equilibration buffer was applied to the column. Fractions of 2.5 ml per tube were collected. The overall recovery was 99.1% (Table 1) and toxin II.10.9 corresponds to 5.2% of the total material absorbing at 280 nm. L, G, W and T mean: loading the sample, starting the gradient, washing with 1 M NaCl and toxic component, respectively.

Table 1 Recovery and lethality of chromatographic fractions

The values reported are percentages calculated from the absorbance at 280 nm, as described in Materials and methods.

Column	Fractions	Protein content (mg)	Recovery (%)	Bioassay
CM-32	II.10	26.5	100.0	Lethal
(Fig. 1)	II.10.1	4.0	14.0	Non-toxic
	II.10.2	1.0	3.5	Non-toxic
	II.10.3	2.5	8.7	Non-toxic
	II.10.4	2.5	8.7	Non-toxic
	II.10.5	9.8	34.3	Lethal
	II.10.6	0.8	2.8	Non-toxic
	II.10.7	1.2	4.2	Non-toxic
	II.10.8	0.5	1.7	Non-toxic
	II.10.9	1.5	5.2	Lethal
	II.10.10	1.1	3.8	Non-toxic
	II.10.11	1.9	6.6	Non-toxic
	II.10.12	1.6	5.6	Non-toxic
Protein recovered			99.1	

reason are not included in Figure 1. In this figure we show further separation of toxic component II-10, after dialysis and lyophilization. Another CM-cellulose column was equilibrated and run with sub-fraction II-10 in 50 mM phosphate buffer, pH 6.0, and after elution with a salt (NaCl) gradient afforded 12 sub-components (Table 1). Two of those were lethal to mice (Figure 1). In Table 1 we show the results of recovery and lethality tests conducted. All fractions obtained with the ion-exchange chromatographic separation at pH 4.7 and pH 6.0 were assayed by e.l.i.s.a., as described in Materials and methods,

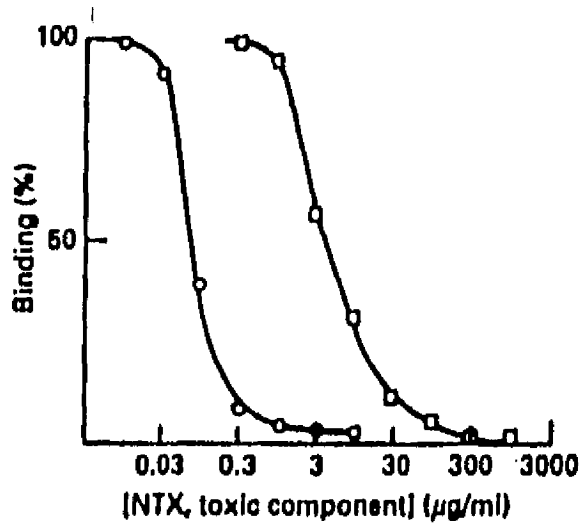

Figure 2 Cross-reactivity of toxins with monoclonal antibody BNTX-14

E.l.i.s.a. trays were coated with NTX. Purified mAB BNTX-14 was incubated in the wells together with increasing concentrations of NTX (○) and toxic component II.10.9 (□). Antibodies bound to the solid phase were then revealed using rabbit anti-(mouse antibody) conjugated with peroxidase. Absorbance at 492 nm was considered to indicate 100% binding when free NTX was absent (competing with buffer alone). Monoclonal antibody BNTX-16 gave essentially the same results.

for the presence of NTX-like peptides. Figure 2 shows the results obtained from binding-displacement experiments in which NTX bound to the plate competed for the binding of monoclonal NTXB12, in the presence of increasing amounts of free NTX and free component II-10-9, respectively. Essentially the same results were obtained when assayed with monoclonal antibody NTX16 (data not shown). Free NTX displaced, with comparable affinity, the binding of both monoclonals, while component II-10.9 displayed lower affinity. This was expected, since the monoclonal antibodies were raised against native NTX. PAGE performed with component II-10.9, according to the method of Reisfeld et al. [28] showed one band (data not shown). These results suggested that we might have purified a NTX-like toxin from *C. l. limpidus* venom. We decided to assay this fraction, using the electrophysiological test described in Materials and methods, with rat cerebellar granule cells in culture.

These cells display a transient K^+ outward current when depolarizing voltage pulses of between −40 to +60 mV are applied, from a holding potential of −80 mV as shown in Figure 3. The elicited outward current rapidly activated and inactivated transiently, reaching a steady-state level. In these cells the application of toxic-component II-10.9 at 1.5 μM reduced the peak currents by about 50%, but not the steady-state level, in a voltage independent way (see Figure 3a). In Figure 3b current–voltage (I–V) relationships relative to peak K^+ currents are shown for control conditions, perfusion in the presence of toxin and recovery after washing. Incomplete recovery was obtained after 2 min of washing. The experimental data are representative of seven other cells.

In summary, Figure 3 indicated that fraction II-10.9 was capable of inhibiting the transient I_a-like K^+-current present in these cells, in a partially reversible manner, confirming our conclusions that we had purified a novel K^+-channel peptide. However, when this peptide was subjected to Edman degradation in the Millipore microsequencer it became clear that two very similar peptides were mixed at a ratio of about 3:1. This conclusion was confirmed by narrow-bore h.p.l.c. separation (Figure 4) which provided homogeneous toxin II-10-9.1, called toxin 1, and homogeneous toxin II-10.9.2, called toxin 2. The full amino acid sequence of toxin 1 was determined (Figure 5). Amino acid analysis (data not shown) and mass spectrometry

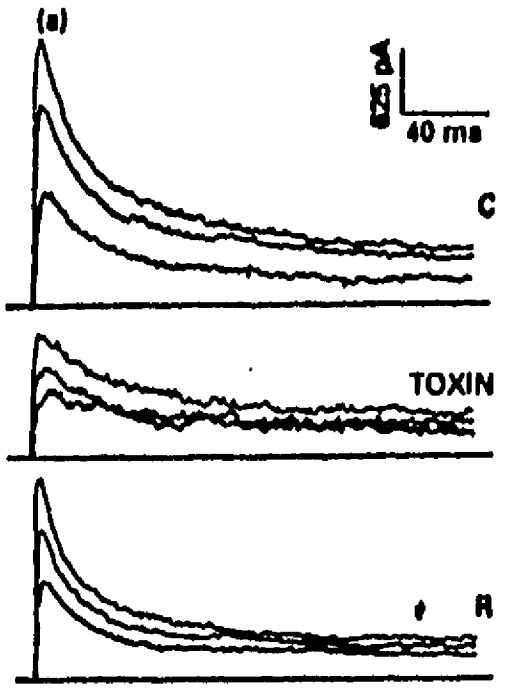

Figure 3 K^+-currents in cerebellum granule cells

(a) Outward currents elicited by depolarizing voltage pulses of 20, 40 and 60 mV from a holding potential of −80 mV (C means control). Outward currents after application of 1.5 μM component II-10.9 (toxin), and finally recovery (R) after removing the toxin from the bathing solution. (b) *I–V* relationship of the peak K^+ currents. Data were obtained from the same cell of Figure 3a. The current value before (■), during perfusion with toxin II-10.9 (●) after a rapid wash-out of toxin (□).

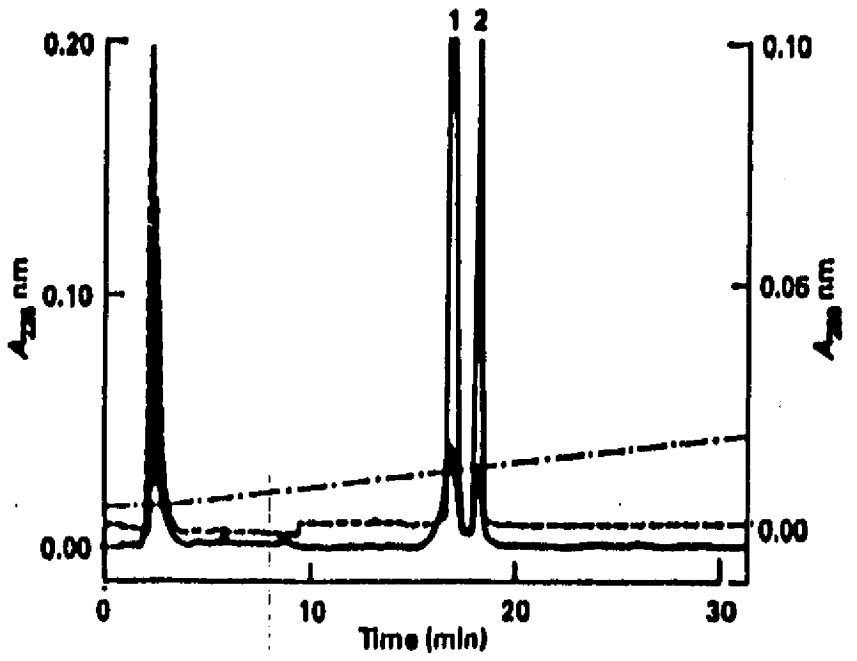

Figure 4 H.p.l.c. separation on C_{18} reverse phase

Re-chromatography of toxin fraction II-10.9 on narrow-bore reverse phase. Toxin applied in 12% solvent B and eluted with a linear gradient from 12% B to 35% B over 50 min. The column was a Vydac C-18 (2.1 × 250 mm) and the flow-rate was 200 μl/min. Solvent A: 0.12% trifluoroacetic acid (TFA) in water, while solvent B is 0.1% TFA in acetonitrile. Absorbance was monitored at 225 and 280 nm, as indicated.

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 1                5                10                15
Ile-Thr-Ile-Asn-Val-Lys-Cys-Thr-Ser-Pro-Gln-Gln-Cys-Leu-Arg-

16               20                25                30
Pro-Cys-Lys-Asp-Arg-Phe-Gly-Gln-His-Ala-Gly-Lys-Gly-Cys-Ile-

31               35        38
Asn-Gly-Lys-Cys-Lys-Cys-Tyr-Pro.
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Figure 5 Amino acid sequence of toxin 1

Amino acid sequence determined on carboxy-methylated toxin 1 using the Milligen 6600 Prosequencer. The toxin was coupled either to (1) DITC-Sequelon (Millipore Co.), or (2) AA-sequelon (Millipore Co.) and subsequently reduced and carboxy-methylated prior to sequence determination. The sequence was identified to residue 34 using (1) and to residue 37 using (2). Proline was assigned as residue 38 by mass analysis and amino acid composition.

confirmed the results shown in Figure 5, giving a relative molecular mass of 4191 (disulphide bridged) or 4197 (reduced disulphides) for toxin 1, compatible with the sequence obtained. Toxin 1 is a peptide with 38 amino acid residues and has six half-cystines, most likely linked by three disulphide bridges, like NTX [11]. Toxin 2 was also sequenced, showing the following amino acid sequence, starting from the N-terminal region: Thr-Val-Ile-Asp-Val-Lys-Cys-Thr-Ser-Pro-Lys-Gln-Cys-Leu-Pro-Pro-Cys-Lys-Glu-Ile-Tyr-Gly-Arg-His-Ala-Gly-Ala-Lys-Cys-Met-Asn-Gly-Lys-Cys-Lys-Cys-...A couple of amino acids are still unidentified at the C-terminal to complete its primary structure.

With these data, an important additional question arose: which of these two peptides was responsible for the results in Fig. 3? In order to clarify this question we chose a direct-binding experiment using brain synaptosome membranes [13]. Fig. 6 shows that both toxins 1 and 2 compete for the binding of ^{125}I-

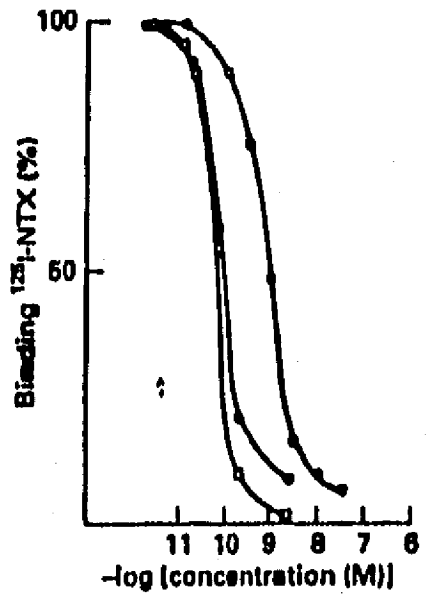

Figure 6 Binding inhibition of ^{125}I-NTX by purified toxins to synaptosome membranes

Rat-brain synaptosome membranes (20 μg protein) were incubated with ^{125}I-NTX at 100 pM, in 500 μl final volume of a 20 mM Tris/HCl buffer, pH 7.6, plus 50 mM NaCl and 0.1% BSA albumin in the absence and presence of increasing concentrations of cold NTX (○), toxin 1 (●) and toxin 2 (■), for 1 h at room temperature. The reaction mixtures were diluted and filtered immediately through glass-fibre filters and the radioactivity measured as described in the section of Material and methods. Values are the mean of triplicates.

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

C11TX 1, toxin 1 from this work; MgTX, margatoxin [16]; NTX, noxiustoxin [35]; K1TX, kaliotoxin [12]; IbTX, iberiotoxin [15]; ChTX, charybdotoxin [18]; Lq2, Leiurus quinquestriatus toxin 2 [27]; LeTX I, leirutoxin 1 [2,10]. Consensus means only positions in which amino acids are conserved in all sequences. Gaps (–) were introduced to enhance similarities

Toxin	Amino acid sequence	Similarity (%)
	1 10 20 30 39	
C11TX 1	ITINVKCTSP QQCLRPCKDR FGQHAGGKCI NGKCKCYP	100
MgTX	TIINVKCTSP KQCLPPCKAQ FGQSAGAKCM NGKCKCYPH	74
NTX	TIINVKCTSP KQCSKPCKEL YGSSAGAKCM NGKCKCYNN	64
K1TX	GVEINVKCSGS PQCLKPCKDA GMRF-G-KCM NRKCHCTP	51
IbTX	pEFTDVDCSVS KECWSVCKDL FGVDRG-KCM GKKCRCYQ	39
ChTX	pEFTNVSCTTS KECWSVCQRL HNTSRG-KCM NKKCRCYS	37
Lq2	pEFTQESCTAS NQCWSICKRL HNTNRG-KCM NKKCRCYS	37
LeTX I	AF----CNL- RMCQLSCRSL -GL-LG-KCI GDKCECVKH	29
Consensus	-------C--- --C---C--- -----G-KC- --KC-C---	

NTX to K^+-channels of synaptosomes, with an indistinguishable affinity of about 10^{-10} M. This result is not surprising since they share a high degree of similarity in their amino acid sequences. Actually, both toxins had a higher affinity for synaptosomal membrane K^+-channels than NTX. For comparative purposes, in Figure 7 we have included the amino acid sequence of several known K^+-channel toxins, selecting only those from which the total primary structure is available. *C. l. limpidus* toxin 1 is 74% identical to margatoxin, a new K^+-channel blocking peptide purified from the scorpion *C. margaritatus* [35], and 64% similar to NTX [11] from *C. noxius*, while kaliotoxin from *Androctonus mauretanicus mauretanicus* [21] has 51% similarity. Iberiotoxin from the venom of the scorpion *Buthus tamulus* [36] displays 39% identity. The sequence of charybdotoxin [37], Lq2 [38] and leirutoxin 1 (scyllatoxin) [18,39] from the venom of *Leiurus quinquestriatus* have only 37% (equal for both Lq2 and charybdotoxin) and 29% similar sequences, respectively. Among the amino acid residues rigorously conserved (consensus in Table 2) are the cysteines, because they are important in stabilizing the three-dimensional structure of the toxins, as demonstrated by n.m.r. studies of charybdotoxin [40], iberiotoxin [41] and leirutoxin 1 [42]. Lysines at position 28 and 33 of Figure 7 are also highly conserved. The lysine at position 28, that corresponds to Lys 27 in charybdotoxin, has been shown by site-directed mutagenesis to be critical for blocking the voltage-dependent Maxi-K^+ channel [43]. glycine at position 26 has been suggested to be important for appropriate formation of the disulphide pairing [44], and is also conserved throughout these sequences. The N-terminal sequences of the K^+-channel blocking peptides from the genus *Centruroides* (New World scorpions) are very much conserved. A synthetic nonapeptide corresponding to this segment of NTX was shown to be toxic to mice [45] and to recognize single Ca^{2+}-activated K^+ channels of small conductance, obtained from cultured bovine aortic endothelial cells [24]. Thus, it seems that the general motif of the three-dimensional structure of these peptides is important in targeting K^+-channels, however, the sequences (Figure 7) do show a high degree of variability, and this could explain the diversity of function and affinity for the various classes of K^+-channels, as extensively discussed by others [46,35,41,43].

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IV. Discusión y conclusiones

Considerando los datos mostrados proponemos que la región amino terminal de la noxiustoxina forma una parte importante del sitio de unión a su receptor. Esta zona involucra la primera región de beta plegada y la región de alfa hélice que se encuentran del mismo lado de la molécula, como se muestra en la estructura tridimensional.

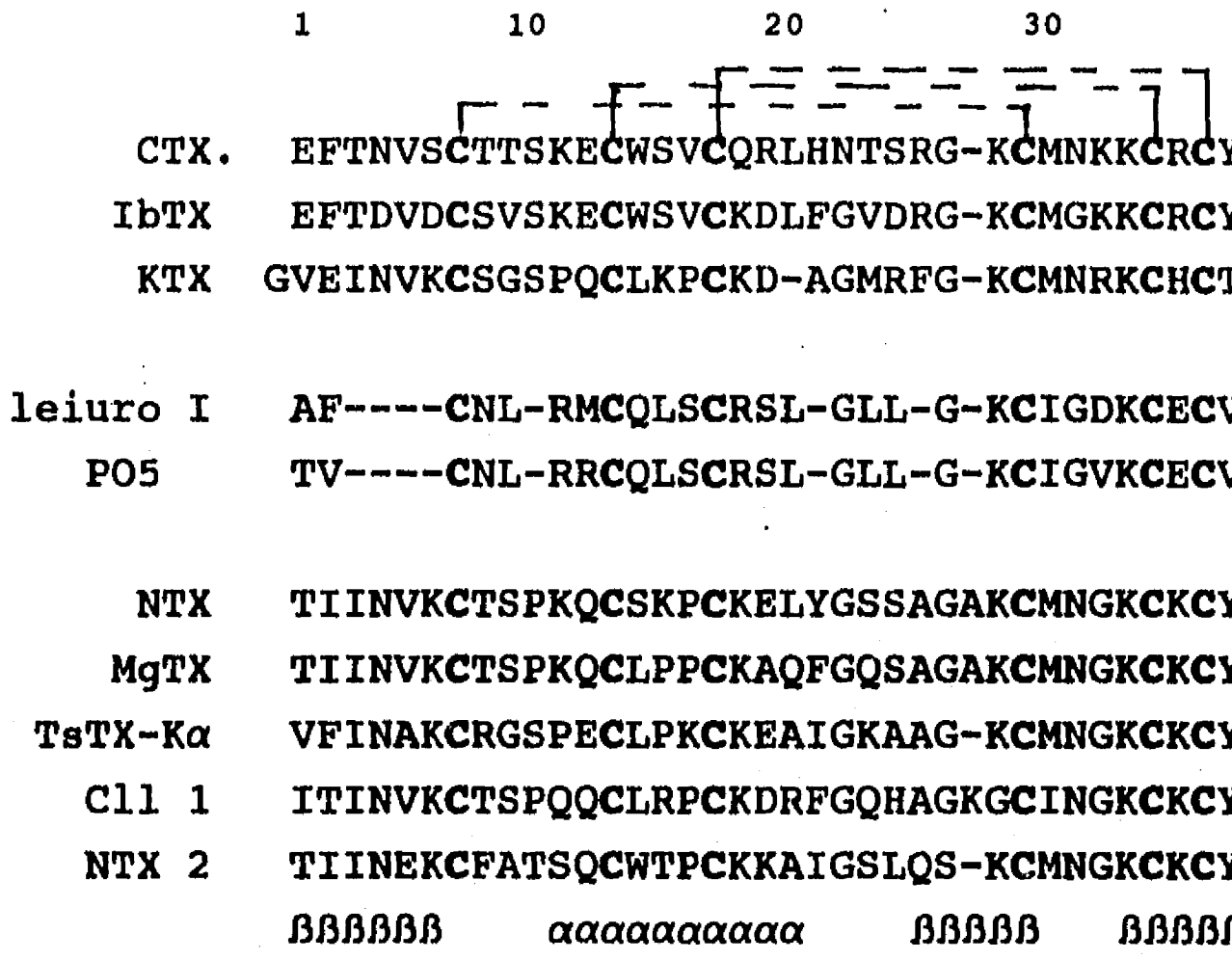
Un reporte reciente indica que el segmento carboxilo terminal de la caribdotoxina parece participar en el reconocimiento del canal de potasio dependiente de calcio. Mutaciones puntuales de CTX en las posiciones Arg26, Lis27 y Arg34, disminuyen la afinidad de la toxina por el canal. En este estudio el cambio de afinidad se produjo por un incremento en la velocidad de disociación.

De acuerdo con lo que propone Ménez (1992), las toxinas de alacrán presentan un plegamiento conservado ("motivo" estructural conservado), existiendo regiones que permiten inserciones, deleciones y mutaciones, lo que posiblemente proporciona a las toxinas diferente especificidad.

Así por ejemplo, tenemos a las toxinas CTX e IbTX, las cuales actúan sobre el canal de potasio dependiente de calcio de alta conductancia; a pesar de su similitud en secuencia (ver tablas 1 y 2) presentan una diferencia notable en carga neta (+1 y +5 respectivamente) y diferente cinética de interacción con el receptor. La KTX, otra toxina que actúa sobre el mismo receptor, presenta una semejanza del 37 y 43% con CTX e IbTX, respectivamente, existiendo algunas diferencias en el mecanismo de bloqueo.

El mayor grado de similitud entre estas toxinas se encuentra en el extremo carboxilo terminal, por lo que algunos autores (Crest *et al.*, 1992) proponen que la secuencia del aminoácido 26 al 33 está implicada en el reconocimiento del receptor. Lo anterior está de acuerdo con los estudios

Tabla 1. Comparación de secuencias de algunas toxinas de alacrán con actividad en canales de potasio.



los símbolos α y ß se refieren a las conformaciones de alfa-hélice y beta-plegada, repectivamente, las secuencias se han alineado, conservando la posición de las cisteínas, los puentes disulfuro se indican con lineas punteada.

Tabla 2 Porcentaje de identidad que presentan entre si diferentes toxinas de alacrán con efecto sobre canales de potasio.

% Identidad.	CTX	IbTX	KTX	leiuro I	P05	NTX	MgTX	TsTX-Kα	Cll 1	NTX 2
CTX		67	37	24	27	48	46	43	34	33
IbTX			43	32	32	43	43	40	36	31
KTX				27	27	54	56	59	52	44
leiuro I				87	35	35	32	26	26	
P05					35	35	32	26	28	
NTX						79	53	64	63	
MgTX							56	73	57	
TsTX-Kα								52	52	
Cll 1									47	
NTX 2										

realizados por el grupo de Miller (Park y Miller, 1992) quienes mostraron la importancia de la Arg 25, Lis 27 y Arg 34 para esta interacción. También con los trabajos de Giangiacomo y su equipo, en los que usando péptidos sintéticos de CTX se observó que uno de ellos, sin los primeros 6 aminoácidos (CTX7-37) causa una inhibición completa de la unión de ^{125}I-CTX a membranas de cerebro de rata, con la misma potencia que la CTX (Ki=20 pM). Estos resultados indican que los primeros seis aminoácidos no son importantes en la unión al receptor (Giangiacomo *et al.*, 1993). Estos autores sugieren que el extremo carboxilo terminal es importante en la interacción con el canal de potasio dependiente de voltaje y que la porción amino terminal también esta involucrada en el reconocimiento del canal de potasio dependiente de calcio.

En nuestro trabajo observamos que, en el caso de la NTX, los primeros seis aminoácidos participan en la unión con el receptor, ya que la NTX7-39 desplaza a la ^{125}I-NTX de las membranas de cerebro de rata, aunque con menor afinidad.

Asimismo tenemos que un péptido correspondiente al extremo carboxilo terminal de la CTX (CTX24-37) es capaz de inhibir la unión de ^{125}I-NTX a membranas de cerebro de rata, no así los péptidos CTX1-10 (amino terminal de CTX) y NTX30-39 (carboxilo terminal de NTX), como se confirma con los resultados anteriormente expuestos.

Por otro lado, tenemos toxinas como MgTx, TsTX-Ka y NTX (motivo de este estudio), entre las cuales existe mayor similitud, y menor con las anteriores (ver tablas 1 y 2). Curiosamente, se ha reportado que estas toxinas son bloqueadoras de canales de potasio dependientes de voltaje y no de los dependientes de calcio (ver tabla 3). Aunque se ha reportado que NTX actúa sobre canales de potasio dependientes de calcio, la afinidad que tiene por este receptor es muy baja (400 nM) comparada con la que tiene por los dependientes de voltaje (20 pM).

En el caso de CTX, se ha reportado que actúa tanto sobre canales dependientes de calcio como en los dependientes de

Tabla 3. Características funcionales y selectividad de toxinas de alacrán con acción sobre canales de potasio.

	Canales de Potasio					
	Dependientes de Voltaje		Dependientes de Ca^{++}			Región importante para
Toxinas	DR	T	alta	Med	peq	la unión al receptor
CTX	Ki=10pM	IC_{50}=40nM	Kd=1nM	–	–	K27,R25,R34,W14 M29,Y36,S10,N30
IbTX	–	–	Kd=1nM	–	–	L19-Q37
KTX	–	–	Kd=20nM	–	–	G26-T36
leiuro 1	–	–	–	–	Kd=80pM	
P05	–	–	–	–	$K_{0.5}$=20pM	R6,R7,H31
NTX	Ki=8pM	IC_{50}>>300nM	Kd=450nM	–	IC_{50}=310nM	T1-L20
MgTX	IC_{50}=36pM	ND	–	–	–	ND
TsTX-Kα	IC_{50}=8nM	–	–	–	–	ND
Cll 1	IC_{50}=0.1nM	IC_{50}=1.5μM	ND	ND	ND	ND
NTX 2	IC_{50}=100nM	ND	ND	ND	ND	ND

Los guiones significa que no tiene actividad sobre este canal y ND no determinado.

voltaje en diferentes tejidos, siempre con el mismo rango de afinidad, por lo que parece ser que comparte rasgos estructurales con ambos tipos de toxinas. Con excepción de IbTX, CTX presenta un porcentaje de identidad semejante con las demás toxinas. Algunos autores (Rogowski *et al.*, 1994) han hecho notar que el aminoácido de la posición 25, para CTX, IbTX y KTX es una arginina y para MgTX, TsTX-Ka, Cl1 1 y NTX es una alanina. Lo anterior nos induce a pensar que esa diferencia podría contribuir a la selectividad. Parece ser que la carga de la Arg 25 es crítica para la unión de CTX con el canal de potasio dependiente de calcio (Park y Miller, 1992).

Recientemente, haciendo mutaciones puntuales de la CTX recombinante se han identificado los residuos que interaccionan directamente con el canal de potasio activado por calcio (Stampe et al 1994). Así tenemos a los residuos cargados: Lis27, Arg25, y Arg34, los hidrofóbicos Trp14, Met29 y Tir36 y a los residuos con capacidad para formar puentes de hidrógeno, Ser10 y Asn30.

Como ya se mencionó, la toxina Cl1-1 inhibe la unión de la ^{125}-NTX a membranas de cerebro de rata con alta afinidad. Esta toxina se puede considerar como una mutante natural de la NTX. La tabla 1 muestra que esta toxina presenta una isoleucina en la posición 30 en lugar de una metionina. Este cambio en la CTX induce una disminución de tres órdenes de magnitud en afinidad por el receptor (Stampe *et al.*, 1994), por lo que podemos concluir que en el caso de la NTX este aminoácido (Met30) no es importante en la interacción con el receptor.

La leiurotoxina I y la toxina P05 formarían el grupo de toxinas semejantes a apamina, con la cual comparten características estructurales y funcionales. Para estas toxinas se propone que la región de estrucura alfa hélice es importante para su función, ya que esta estructura mantiene las cargas positivas de los residuos de arginina en una posición adecuada para su actividad. Lo anterior apoya nuestros resultados de que en NTX la estructura de alfa hélice es importante para su interacción con el receptor, ya que son tres cargas positivas

las que se encuentran en esta región (Lis 11, Lis 15, Lis 18).

Analizando las secuencias de las toxinas de alacrán que se conocen hasta el momento y la información vertida en la literatura, podemos concluir que para IbTX, KTX y CTX el segmento carboxilo terminal forma parte importante de la región de reconocimiento al receptor. En cambio para las toxinas de venenos de alacranes del Nuevo Mundo MgTX, NTX, Cl1-1, NTX-2 y TsTX-Kα la región importante se sitúa en el extremo amino terminal y, posiblemente, estas regiones también determinen la especificidad de las toxinas (tabla 3).

Como ya se mencionó, se ha reportado que NTX interacciona con diferentes tipos de canales de potasio con distintas afinidades. Analizando ahora al receptor, se podría pensar que los canales de potasio comparten una región que reconoce la toxina, pero que las diferencias entre ellos explican las distintas afinidades que muestran por la toxina. Ya que se ha demostrado que los canales de potasio pueden estar formados como homo o heterotetrámeros, posiblemente la proporción que exista de una subunidad dada contribuya a determinar la afinidad de la toxina (siempre y cuando la toxina tenga una sola región de reconocimiento al receptor y que reconozca siempre la misma zona en los canales).

En resumen podemos decir que:

1. La región amino terminal de la noxiustoxina es una parte muy importante de la zona que interacciona con los canales de potasio dependientes de voltaje en membranas de cerebro de rata.

2. La región de alfa hélice (Lis11-Leu20) es importante para el reconocimiento del receptor, probablemente por la presencia de las tres cargas positivas (Lis11, Lis15, Lis18), ya que el péptido NTX11-39 a pesar de no tener los primeros 10 aminoácidos es capaz de reconocer al receptor, no así solo la región carboxilo terminal (NTX30-39).

3. Para una toxina análoga, la caribdotoxina, la región carboxilo terminal juega un papel más crítico en la interacción con su receptor que en el caso de la NTX.

4. La metionina de la posición 30 de la NTX no es crucial para su unión con el receptor, como lo es para la CTX, lo que prueba que estas toxinas no se unen de igual forma al receptor a pesar de ser estructuralmente semejantes.

5. Posiblemente esta diferencia en zonas clave para el reconocimiento con el receptor esté relacionada con la selectividad que presentan estas toxinas sobre las diferentes clases de canales de potasio.

Por último, quisiéramos enfatizar que la búsqueda y caracterización de nuevas toxinas que actúan sobre canales iónicos, en particular canales de potasio, es importante por la utilidad que representan en varios aspectos del estudio de los canales. Así por ejemplo, su uso como ligandos en la purificación de los diferentes tipos de canales de potasio, complementado con los métodos de biología molécular ha permitido tener más información respecto a la estructura y función de éstos. Las toxinas puras pueden usarse para determinar algunas características estructurales del canal.

Dado que estas toxinas son pequeñas y que se conoce la estructura de varias de ellas, se pueden sintetizar o clonar para poder realizar modificaciones selectivas en ellas.

Estas toxinas pueden, también, ser prototipos de ligandos endógenos con actividades similares (Fosset *et al.*, 1984).

V. Perspectivas

Como perspectivas podemos mencionar, que debido a que la NTX se ha podido expresar en E. coli en el laboratorio (Baltazar Becerril y Fernando Martinez) será más fácil obtener toxinas con modificaciones que nos permitan delimitar más estrictamente la región importante en la interacción con canales de potasio, principalmente los dependientes de voltaje que se encuentran en cerebro, por los cuales tiene mayor afinidad.

Uno de los aspectos más importantes que debemos probar es la relevancia de la Lis27. Aunque en la toxina Cl1 1 en esta posición se encuentra una glicina, la lisina se encuentra en la posición 26 que podría ser equivalente en términos funcionales.

Por otro lado es necesario quitar algunos aminoácidos del extremo carboxilo terminal, para poder delimitar hasta cual residuo es importante para la interacción con el receptor. Como la NTX tiene el extremo carboxilo terminal amidado es resistente a la acción de las carboxipeptidasas, por lo que no se ha podido mapear esta región de la molécula. La NTX recombinante no tiene el extremo carboxilo terminal amidado y se ha probado que en el sistema de unión a membranas de cerebro de rata no se modifica su acción (Baltazar Becerril y Fernando Martínez comunicación personal), por lo que resulta un buen candidato para el uso de carboxipeptidasas.

Por otro lado, se cuenta ya con canales de potasio, shaker B, expresados en células COS (células de riñón de mono), lo que nos permitirá observar el comportamiento de la NTX y sus variantes en una población homogénea de canales de potasio.

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