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. limpidus* toxin 2 were also determined. Both toxins are capable of displacing the binding of radio-labelled 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. limpidus* 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], Ca²⁺ [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 [13,18–25]. 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 [1,2,4].

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 carboxymethyl-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

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 Prosequencer (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 was 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 10⁵ spleen cells per well) on a feeder layer consisting of 5 × 10³ 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.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 NaHCO₃, 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 NaH₂PO₄, 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 to that described by Catterall [34]. The rapid filtration assay described originally [13,29] 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 ¹²⁵I-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 CaCl₂/0.8 mM MgCl₂/20 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. [35] and plated on 33 mm Petri dishes coated with 10 µg/ml poly-L-lysine at a density of 2.5 × 10⁶. 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% CO₂ 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 CaCl₂/2 MgCl₂/20 glucose/10 Hepes. Tetrodotoxin (3 × 10⁷ M) was added to the external solution to abolish Na⁺ currents, while Ca²⁺ currents were blocked by 1 mM Cd²⁺. Pipette-filling solution was (mM): 120 KCl/5 EGTA/0.24 CaCl₂/30 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 mannitol. Currents were recorded in patch-clamp whole-cell configuration [36]. 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 (28.5 mg in 30 ml solution) from reference 29 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 (Fig. 1)	II.10	28.5	100.0	Lethal
	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, for the presence of NTX-like peptides. Figure 2 shows the results

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.

obtained from binding-displacement experiments in which NTX bound to the plate competed for the binding of monoclonal BNTX-14, 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 BNTX-16 (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 confirmed the results shown in Figure 5, giving a relative

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 $1.5 \mu\text{M}$ 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 (●) and after a rapid wash-out of toxin (□).

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 -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.

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 $200 \mu\text{l}/\text{min}$. 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.

1 Ile-Thr-Ile-Asn-Val-Lys-Cys-Thr-Ser-Pro-Gln-Gln-Cys-Leu-Arg-
 5
 10
 15
 16 Pro-Cys-Lys-Asp-Arg-Phe-Gly-Gln-His-Ala-Gly-Lys-Gly-Cys-Ile-
 20
 25
 30
 31 Asn-Gly-Lys-Cys-Lys-Cys-Tyr-Pro
 35
 38

Figure 5 Amino acid sequence of toxin 1

Amino acid sequence determined on carboxymethylated 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 carboxymethylated 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.

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

Rat-brain synaptosome membranes ($20 \mu\text{g}$ protein) were incubated with ^{125}I -NTX at 100 pM , in $500 \mu\text{l}$ final volume of a 20 mM Tris/HCl buffer, pH 7.8, 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 Material and methods section. 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 [37]; NTX, noxiustoxin [11]; K1TX, kaliotoxin [21]; IbTX, iberiotoxin [38]; ChTX, charybdotoxin [39]; Lq2, *Leirus quinquestriatus* toxin 2 [40]; LeTX 1, leirutoxin 1 [18,41]. 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	pEFTNVSCCTS	KECWSVCQRL	HNTSRG-KCM	NKKCRCS		37
Lq2	pEFTQESCTAS	NQCWSICKRL	HNTNRG-KCM	NKKCRCS		37
LeTX 1	AF-----CNL-	RMCQLSCRSL	-GL-LG-KCI	GDKCECVKH		29
Consensus	-----C---	--C---C---	-----G-KC-	--KC-C---		

Actually, both toxins had a higher affinity for synaptosomal membrane K⁺-channels than NTX. For comparative purposes, in Table 2 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* [37], 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* [38] displays 39 % identity. The sequences of charybdotoxin [39], Lq2 [40] and leirutoxin 1 (scyllatoxin) [18,41] from the venom of *Leirus quinquestriatus* have only 37 % (equal for both Lq2 and charybdotoxin) and 29 % similarity, 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 [42], iberiotoxin [43] and leirutoxin 1 [44]. Lysines at position 28 and 33 of Table 2 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 [45]. Glycine at position 26 has been suggested to be important for appropriate formation of the disulphide pairing [46], 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 [47] and to recognize single Ca²⁺-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 (Table 2) 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 [37,45,46,48].

We thank M.Sc. Fernando Zamudio, Mr. Fredy Coronas and M.Sc. Timoteo Olamendi for technical assistance. We are also indebted to Dr. Mark Duncan of the Biomedical Mass Spectrometry Unit, University of New South Wales, Australia, for mass spectrometer analysis and W. K. Eliason of NIMH for helpful discussions. This research was partially supported by grants from Howard Hughes Medical Institute, (75191-527104), CONACyT-Mexico (0018-N9105), and DGAPA-UNAM (IN205893) to L. D. P.

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