

Cn11, the first example of a scorpion toxin that is a true blocker of Na⁺ currents in crayfish neurons

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Summary

A novel crustacean toxin (Cn11) was isolated and characterized from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann. It contains 63 amino acid residues and is stabilized by four disulphide bridges. It is lethal to crustaceans (*Cambarellus montezumae*), less toxic to insects (crickets) and non-toxic to mammals (mice) at the doses assayed. In neurons isolated from the X organ–sinus gland system of the crayfish *Procambarus clarkii*, it blocks the Na⁺ currents with an estimated K_m of 320 nmol l⁻¹, without affecting the Ca²⁺ and K⁺ currents. The voltage-gated tetrodotoxin-sensitive Na⁺ current was recorded from X organ neurons in culture 24 h after plating using the whole-cell clamp configuration. The Na⁺

current was isolated by blocking Ca²⁺ currents with Cd²⁺ and Cs⁺ and K⁺ currents with tetraethylammonium and 4-aminopyridine. Under control conditions, the Na⁺ currents were activated at -40 mV with a maximum amplitude at 0 mV. In the presence of 1 μmol l⁻¹ Cn11, the Na⁺ current amplitude was reduced by 75% without apparent modifications to the gating mechanism. These findings suggest that Cn11 selectively blocks a Na⁺ channel. It is the first representative of a new group of scorpion toxins specific for this molecular target.

Key words: amino acid sequence, *Centruroides noxius*, crayfish, neuron, Na⁺ channel, *Procambarus clarkii*, scorpion toxin.

Introduction

The best-studied components of scorpion venom are the polypeptides that recognise ion channels and receptors in excitable membranes. They are harmful to a variety of organisms including man (for reviews, see Possani et al., 1999a, 2000). The molecules responsible for the toxicity of the scorpion venom are polypeptides of 4–8 kDa that have been classified according to their receptor targets. Four different families of toxin have been found to interact specifically with ion channels: Na⁺ channels (for a review, see Catterall, 1996), K⁺ channels (for a review, see Garcia et al., 1997), Cl⁻ channels (Debin et al., 1993) and Ca²⁺ channels (for a review, see Valdivia and Possani, 1998). Scorpion toxins that affect Na⁺ channels are basic proteins with 60–76 amino acid residues, are stabilized by four disulphide bridges and are capable of specifically recognizing the Na⁺ channels of mammals, insects and crustaceans (Possani et al., 1999a). Their molecular mechanism of action has been described as being one in which the gating function of the channel (opening and closing kinetics) is modified. In contrast to these long-chain peptides, the short-chain peptides

(23–41 amino acid residues) are specific blockers of K⁺ channels (Batista et al., 2000) (for reviews, see Tytgat et al., 1999; Possani et al., 1999b).

Tetrodotoxin (TTX) and saxitoxin (STX) are small molecules with one and two guanidinium groups, respectively, and are known to block Na⁺ currents by interacting with the outer entrance of the ion channel pathway. Since TTX and STX are small molecules, it is conceivable that their binding site within the channel protein is very close to the narrowest part of the pore, which is believed to be the selectivity filter (Terlau and Stühmer, 1998). These neurotoxins bind to receptor site 1 of the Na⁺ channel, where the blocker toxins interact (Catterall, 1996). To date, the only peptide described as a blocker of Na⁺ channels is μ-conotoxin GIII, a 22-amino-acid peptide isolated from the venom of the snail *Conus geographus*. This toxin acts in a similar manner to TTX and STX (McIntosh et al., 1999; Dudley et al., 1995).

Apart from the functional classification, there is also specificity in the toxins affecting Na⁺ channels, in either mammals or arthropods (mostly insects and/or crustaceans).

However, binding studies, correlated with toxicity towards mammals and insects, have revealed that even toxins known as mammal-specific can be toxic to insects and *vice versa* (Gordon et al., 1996). Therefore, the animal group specificity is only relative, and definite cross-reactivity exists (Selisko et al., 1996).

Scorpion toxins are classified according to their structure, mode of action and binding site on different channels or channel subtypes (Martin-Eauclaire and Couraud, 1995; Gordon et al., 1996). Each class consists of several peptides isolated from the venom of different species of scorpion. The long-chain toxins affecting Na^+ channels have been subdivided primarily into two major sub-types, α - and β -toxins (Jover et al., 1980; Wheeler et al., 1983). The α -toxins bind to receptor site 3 of the voltage-gated Na^+ channels of vertebrates in a membrane-dependent manner (Catterall, 1986). For these toxins, Tugarinov et al. (1997) and Zilberberg et al. (1992) have described the area of the surface (active site) of the toxin that is capable of interacting directly with the receptor. The β -toxins, initially isolated from American scorpions, bind to receptor site 4 on vertebrate Na^+ channels, producing a shift to a more negative potential. Binding is independent of the membrane potential (Catterall, 1986; Couraud and Jover, 1984).

Two other distinct classes of toxin showing specificity for arthropods, termed depressant and excitatory, have been described (Zlotkin, 1987). These four classes of long-chain toxin share only 20–40% similarity of amino acid sequences (Dufton and Rochat, 1984; Martin-Eauclaire and Couraud, 1995).

Recently, Possani et al. (1999a) proposed a new classification, comprising 10 different groups, on the basis of structural and physiological effects and species-specificity. Toxins specific for mammals, insects and crustaceans and toxins with more than one specificity were placed in distinct groups. Below, we show that the toxin described here does not fit into any of the previously described groups.

The molecular basis of toxin specificity has been widely studied for various reasons: (i) to understand the toxic effects of scorpion venom as a pre-requisite for the development of more effective and safer antidotes or vaccines; (ii) to study ion channels, the target molecules of scorpion toxins, in order to understand their molecular structure and function; and (iii) to develop insect-specific toxins that can be used as insecticides. However, the existing information is still limited. Thus far, small differences, i.e. single amino acid modifications within or near the critical binding region between the toxin and its receptor, have been considered. Also, a significant number of natural ligands that recognize these receptor molecules are still to be identified and studied from the venom of scorpions, among other organisms (Possani et al., 1999a).

This paper describes the mechanism of action of a new Na^+ channel toxin, relatively specific for crustaceans. Cn11 blocks the Na^+ current in cultured neurons from the X organ-sinus gland of crayfish without modifying the gating mechanism, and it is the first example of a new group (the eleventh) of scorpion toxins with such a molecular effect.

Materials and methods

Scorpions and venom

Scorpions, *Centruroides noxius* Hoffmann, were collected in the State of Nayarit (México). The venom was recovered by electrical stimulation, as described previously (Possani et al., 1981). The crude venom was dissolved in double-distilled water and centrifuged at 15 000g for 15 min. The soluble venom (supernatant) was freeze-dried and kept at -20°C until use. Approximately 20 000 scorpions were needed to obtain 1 g of soluble venom.

Purification procedures and lethality tests

Gel-filtration chromatography on Sephadex G-50 columns was used as a first step to separate the components, followed by ion-exchange chromatography (Possani et al., 1981). Further purification of subfractions by high-performance liquid chromatography (HPLC) was conducted as described previously (Selisko et al., 1996; García et al., 1997; Possani et al., 1999a). Briefly, 100 mg of soluble venom was applied to a Sephadex G-50 column (0.9 cm \times 200 cm) in the presence of 20 mmol l $^{-1}$ ammonium acetate, pH 4.7, at a flow rate of 20 ml h $^{-1}$. Toxic fractions were tested in animal models (mice, crickets and crayfish) and further separated on a carboxymethyl cellulose column (CM-Cellulose; 0.9 cm \times 30 cm) equilibrated and developed in the presence of 20 mmol l $^{-1}$ ammonium acetate buffer, pH 4.7, with a continuous salt gradient from 0 mol l $^{-1}$ to 0.5 mol l $^{-1}$ NaCl. The third purification step was conducted by HPLC, with a Waters (Millipore Co., Milford, MA, USA) system, using a C4 or a C18 reverse-phase column (Vydac, Hesperia, CA, USA).

For lethality tests, three designations were used: non-toxic means that the animals showed no symptoms of intoxication within 24 h after injection, and the effect was similar to that of injecting phosphate-buffered saline or water alone. The toxic effect consisted of a host of symptoms such as salivation, lacrimation, dyspnoea, temporary paralysis of the limbs (mice) or paralysis (crickets and crayfishes). The toxic effect was termed lethal if at least one of the test animals died after injection.

Determination of the primary structure of Cn11

The amino acid sequence of the toxin (SWISS-PROT accession no. P58296) was obtained (i) by direct sequencing of the native toxin, (ii) by sequencing the reduced and carboxymethylated toxin (RC-toxin) and (iii) by sequencing fragments generated by enzymatic cleavage of RC-toxin. Reduction of the toxin with dithiothreitol, alkylation with iodoacetic acid, enzymatic cleavage with trypsin, chymotrypsin and endoproteinase V8 and separation by HPLC of the sub-peptides were performed as described previously (Possani et al., 1999a). Microsequence determination was carried out by automatic Edman degradation using either a Millipore apparatus (ProSequencer model 6600) or a Beckman (LF 3000 Protein Sequencer) machine, as described previously (García et al., 1997). For the most C-terminal amino acid residues, mass spectrometry analysis (Scaloni et

al., 2000) or a new strategy developed in our laboratory (G. B. Gurrola, T. Olamendi-Portugal, C. Balderas and L. D. Possani, unpublished observations) was used. For the latter, a synthetic pentapeptide containing the sequence *N*-acetyl-Lys-Cys-Lys-Tyr-Lys was prepared to make a disulphide bridge with the terminal cysteine residue of the unknown peptide for sequencing. Since the *N*-terminal lysine residue of the synthetic peptide was blocked, only the unknown peptide containing a free thiol group would be sequenced, after preparing a heterodimer by mixed disulphide bridge formation, with the synthetic carrier pentapeptide.

Neuron culture techniques

Adult crayfish, *Procambarus clarkii*, were collected from Río Conchos, Chihuahua, México, and adapted to laboratory conditions for 1 week, under a 12h:12h light:dark photoperiod. Eyestalks were excised and placed in chilled crayfish saline solution (in mmol l⁻¹): 205 NaCl, 5.4 KCl, 13.5 CaCl₂, 2.6 MgCl₂ and 10 Hepes, adjusted to pH 7.4 with NaOH. The exoskeleton, muscles and connective tissue surrounding the neural structures were carefully removed under a dissecting microscope to expose the neuronal somata. Isolated X organ neurons were incubated in collagenase/dispase (200 µl ml⁻¹, Boehringer Mannheim) dissolved in modified Leibovitz L-15 (Gibco) culture medium for 60 min. The enzyme was washed out, and the X organ neurons were dissociated by gentle suction through fire-polished micropipettes, as described previously (García et al., 1990), and plated onto a 200 µl recording chamber precoated with Concanavalin A (Type III, Sigma). The ionic composition of the culture medium was increased to match that of the crayfish saline solution. In addition, 5.5 mmol l⁻¹ glucose, 2 mmol l⁻¹ L-glutamine, 16 µg ml⁻¹ gentamycin (Shering Plough) and 5 µg ml⁻¹ streptomycin (Sigma) were added. Cultured cells were kept in darkness for 24 h before the experiments were conducted.

Electrophysiology

Voltage-clamp experiments in the whole-cell configuration were performed using cultured X organ neurons. Na⁺ current recordings were obtained using an Axopatch-200A amplifier (Axon Instruments). Pipettes were constructed from borosilicate capillaries (Sutter Instruments; CA, USA) using a horizontal puller (P-87 Flaming Brown, Sutter Instruments, USA). Pipettes were filled with a solution containing (in mmol l⁻¹): 20 NaCl, 2 CaCl₂, 2 MgCl₂, 210 CsCH₃SO₄, 5 EGTA-K and 10 Hepes, adjusted to pH 7.4 with CsOH. After being filled, the pipette tip resistance was 3.3±0.2 MΩ. Series resistance was compensated at 65–80%. Na⁺ currents were filtered at a frequency of 5 kHz and stored on hard disk using the DigiData 1200 hardware acquisition system and its software (pClamp6, Axon Instruments, USA). Transient capacitive and leak currents were subtracted using the P/4 protocol.

To isolate the Na⁺ current, the neurons were superfused in a solution containing (in mmol l⁻¹): 190 NaCl, 5 KCl, 10

CaCl₂, 2 MgCl₂, 20 tetraethylammonium chloride (TEA-Cl), 5 4-amino pyridine (4-AP), 2 CdCl₂ and 10 Hepes at pH 7.4. The toxin samples were kept lyophilized and freshly prepared for each experiment by dissolving in saline solution at the desired concentration and using the solution immediately. The solution containing the toxin (10 ml) was continuously superfused in the experimental chamber (200 µl). The toxin assays were made in neurons with stable Na⁺ currents.

Results

Purification and sequencing of Cn11

As reported previously (Possani et al., 1981), when applied to a Sephadex G-50 column, the venom of the scorpion *Centruroides noxius* resolves into three components, the second of which contains most of the peptides toxic to mice, crickets and crayfishes (García et al., 1997). This fraction, after ion-exchange column chromatography (Possani et al., 1981), gives rise to approximately 14 different components. Fraction II-5 (i.e. fraction II from Sephadex, sub-fraction 5 from CM-Cellulose) is not toxic to mice at doses up to 180 µg per 20 g mouse mass (see García et al., 1997), but is toxic to arthropods. After HPLC separation, as shown in Fig. 1, it resolves into approximately nine main sub-components.

The component numbered 4 contained the peptide under study and was finally obtained in homogeneous form after HPLC separation in a C18 reverse-phase column, set at 25% solvent B, as shown in the inset of Fig. 1. The fraction labelled with an asterisk in the inset was homogeneous, as determined by amino acid sequence and mass spectrometry analyses (relative molecular mass experimentally determined to be 6972). This peptide corresponds to 1.8% of the whole soluble venom. It is lethal to the crayfish *Cambarellus montezumae* (a freshwater species native to the ponds in the area of Cuernavaca, México) at doses of 15 µg per adult animal. It is also toxic, but not lethal, to crickets (*Acheta* spp.) at doses of approximately 20 µg per animal.

In Fig. 2, we summarize the results necessary to align the complete amino acid sequence of this novel crustacean toxin, termed Cn11 (Cn from the abbreviation for the species *Centruroides noxius* and 11 because it was the eleventh peptide specific for Na⁺ channels purified to homogeneity from this venom).

Direct sequencing of native peptide resolved the first 20 amino acid residues (Fig. 2). This sequence was confirmed, including the location of cysteine residues, when the toxin was reduced and carboxymethylated before sequencing. The first 34 residues were unequivocally identified (under-labelled RCM in Fig. 2). Additional sequence was obtained by overlapping the sequences obtained from a series of peptides, after aspartic-*N*-endopeptidase digestion (labelled Asp-N), from residues 20–43, endopeptidase V8 digestion (corresponding to an elution time of 36.62 min in a column similar to that in the inset in Fig. 1; data not shown), which gave the sequence of amino acids in positions 48–59, and two tryptic peptides obtained after trypsin digestion. The tryptic

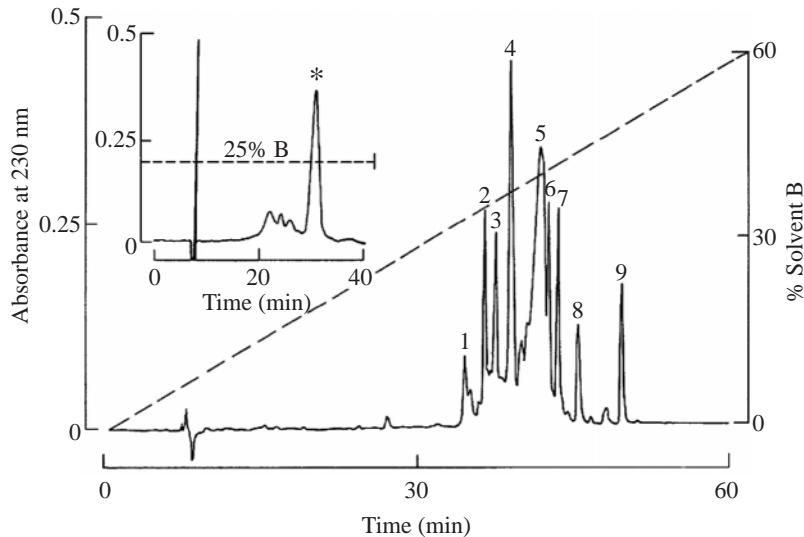


Fig. 1. Purification of toxin Cn11. Sub-fraction II-5 (1 mg), obtained by previous separation of soluble venom of the scorpion *Centruroides noxius*, was applied to an HPLC C4 reverse-phase column (Vydac, Hisperia, CA, USA) and eluted with a linear gradient of solvent A (0.12% trifluoroacetic acid in water) to 60% solvent B (0.1% trifluoroacetic acid in acetonitrile) over 60 min. Component 4 (140 μ g) was further separated by HPLC using a C18 reverse-phase column (Vydac) by application of 25% solvent B (inset). The asterisk indicates the elution position of the pure toxin.

peptides are under-labelled Tryp1 and Tryp2 (Fig. 2). The first corresponds to an elution time of 29.37 min and gave the position of residues 39–52, and the second (Tryp2) gave the sequence from Val at position 55 to the C terminus.

To complete the full sequence, two situations were also considered; the first concerns the peptide Tryp1, whose sequence starts with Thr at position 39, indicating that this enzyme had some residual chemotryptic activity contaminant because it cleaved at Tyr38. The second observation is that the second tryptic peptide, from residue Val55, gave unequivocal identification only up to residue Asn61. The molecular mass, experimentally determined to be 6972 Da, suggested that two additional residues were lacking, compatible with the presence of a Thr and a Cys. The latter was needed to complete the four disulphide bridges, since one cysteine was still missing. However, the exact positions of the missing Thr and Cys were not known. For this reason, a pentapeptide (described in Materials and methods) was synthesized and used to make a mixed disulphide heterodimer with Tryp2. The microsequencer results showed unequivocally that the amino acid in position 62 was Thr, leaving a cysteine at position 63. The theoretical relative molecular mass calculated for native Cn11 was 6973, close to the value determined experimentally. Thus, Cn11 is a peptide that contains 63 amino acid residues,

folded compactly by four disulphide bridges, similar to other Na^+ -channel-specific scorpion toxins (for a review, see Possani et al., 1999a).

Effects of Cn11 toxin on crayfish neuronal Na^+ channels

To obtain better voltage-clamp control, the effect of Cn11 on the Na^+ current was tested in neurons after 24 h in culture; at this time, the cells show discrete axonal regeneration, but the mean current amplitude at 0 mV was approximately 2 nA.

Fig. 3A shows a set of superimposed traces of inward Na^+ currents obtained before and during Cn11 toxin ($1 \mu\text{mol l}^{-1}$) superfusion evoked in response to 0 mV depolarising voltage steps applied every 15 s from a holding potential of -60 mV. The first three traces under control conditions were averaged (lowest trace, labelled control). In the presence of the toxin, the current amplitude was gradually reduced, the maximum blocking effect being reached 3.5 min later (75% reduction in current amplitude). This effect was only partially reversible (not shown).

Cn11 does not modify Na^+ channel gating

The Na^+ current elicited at 0 mV under control conditions reached a peak amplitude 1.0 ± 0.13 ms after the onset of the depolarizing step and then decayed with a half-decay time of

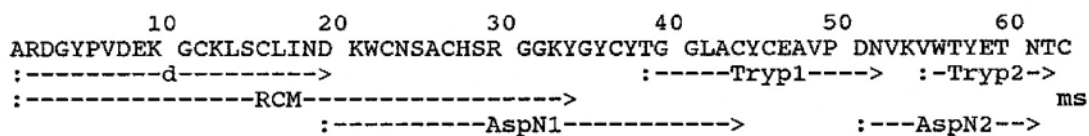


Fig. 2. Complete amino acid sequence of Cn11. The N-terminal amino acid sequence up to residue 20 (underlined -d-) was obtained by direct sequencing of a sample of native peptide, whose sequence was confirmed up to residue 34 with a reduced and carboxymethylated sample (underlined -RCM-). Four additional sub-peptides obtained by enzymatic cleavage of alkylated toxin, followed by HPLC separation (as in Fig. 1, data not shown), were sequenced. They are under-labelled Tryp1 (positions 39–52) and Tryp2 (positions 55–62), for trypsin digestion and AspN1 (positions 20–44) and AspN2 (positions 51–61), for aspartic-N endopeptidase digestion. The last residue in position 63 was determined by mass spectrometry (under-labelled ms).

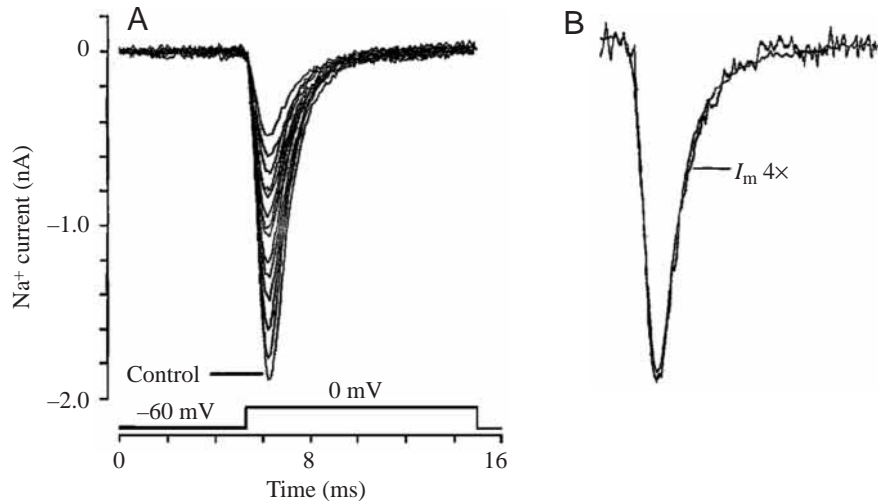


Fig. 3. Blockade of Na^+ currents by Cn11 in crayfish neurons. (A) After 3 min of establishing the whole-cell configuration, inward Na^+ currents were elicited by test depolarizations to 0 mV from -60 mV, each for 15 s. The first three traces were averaged and taken as the control value in the absence of toxin. Subsequent traces were obtained during perfusion of the preparation with $1 \mu\text{mol l}^{-1}$ Cn11. The traces show how the current decreases over time. (B) Cn11 blocks the current without affecting its kinetics, as shown by superimposition of the time course of control Na^+ currents and those in the presence of toxin (scaled up fourfold), which blocked 75 % of the control current (labelled $I_m 4\times$).

0.86 ± 0.05 ms (means \pm S.E.M., $N=6$). The traces obtained at 75 % blockade of the Na^+ currents were analysed and compared with those obtained under control conditions by scaling up the current traces in the presence of the toxin fourfold, as shown in Fig. 3B. The traces with and without Cn11 were very similar, showing that neither the time to peak nor the half-decay time was significantly modified. Thus, the toxin seems to be a *bona fide* blocker of the currents.

The effect of Cn11 is time- and concentration-dependent

To explore the concentration-dependence and the time course of the blockade induced by Cn11, the Na^+ current amplitude was monitored every 10 s using the pulse protocol described in Fig. 3A. Three current traces were obtained under control conditions; the cells were then superfused with the same external solution but with Cn11 added for 160 s. The toxin was then washed from the recording chamber and the current was recorded for a further 160 ms. As is shown in Fig. 4A, the current amplitude was normalized with respect to the mean value obtained under control conditions. In the absence of the toxin, the Na^+ current decreased by 10 % during the first 160 s; this rundown was taken into account in the subsequent estimation of the Na^+ current amplitude in the presence of the toxin. After removal of the toxin from the recording chamber, the Na^+ current recovered slowly for the lowest concentration tested (50 nmol l^{-1}).

To determine the K_m for the effect of Cn11 on X organ neurons, the amplitude of the Na^+ current was measured 70 s after exposure to the toxin. The percentage of current blockade was plotted as a function of the toxin concentration, assuming a rundown of 5 %. The data were fitted to a Boltzman's function, giving a calculated K_m of 320 nmol l^{-1} (Fig. 4B).

Cn11 does not modify the voltage-dependent activity of the neuronal Na^+ channels

The voltage-dependence of the blockage exerted by Cn11 on the Na^+ current was explored using command pulses to voltages between -50 and 50 mV from a holding potential of

-60 mV. Under control conditions, the apparent activation threshold of the current was always -40 mV, and the maximal current was consistently near 0 mV (Fig. 5, open circles). During superfusion of $1 \mu\text{mol l}^{-1}$ (filled squares) or $3 \mu\text{mol l}^{-1}$ (open squares) Cn11, neither the apparent activation threshold nor the potential giving the maximal current was modified after 3 min of exposure to toxin, suggesting that the mechanism of action of Cn11 is quite similar to those of TTX and STX.

Discussion

The purification of Cn11 followed a procedure similar to those used for Cn5, a crustacean toxin (García et al., 1997), and Cn10, an insect toxin (Selisko et al., 1996), purified from the same venom. The third major peak shown in Fig. 1 (labelled 3) corresponds to Cn10, the fourth peak to Cn11, and the fifth peak to Cn5 (see Selisko et al., 1996). An additional step was necessary to obtain Cn11 in homogeneous form (component labelled with an asterisk in the inset of Fig. 1). None of the three toxins thus far isolated, sequenced and studied from fraction II-5 of *C. noxius* is considered to be toxic to mammals. They are all arthropod-specific toxins. Furthermore, they are all long-chain peptides that, as shown by sequence similarities and functional analysis, correspond to the family of Na^+ -channel-specific peptides (see Possani et al., 1999a). However, the determination of the primary structure of Cn11, reported here, was not straightforward.

Several cleavages with different enzymes followed by HPLC separation of the corresponding fragments and individual sequencing were required to obtain the full amino acid sequence. The last two residues were identified only by using a synthetic carrier pentapeptide, as described in Materials and methods.

Before analyzing in detail the results presented here, it is important to discuss briefly the current state of knowledge of Na^+ channel blockade and permeability properties in the presence of different ligands including scorpion toxins. The

specificity of Cn11 for the Na^+ channel was assayed using cultured neurons from the crayfish eyestalk. As mentioned above, Cn11 did not affect the kinetic properties of isolated Ca^{2+} currents or outward K^+ currents. Surprisingly, a small Na^+ current (5–15%) remained after blockade by Cn11 at high concentration and for a prolonged incubation time, suggesting that more than one type of Na^+ channel is present in these cells. In a previous communication (Onetti et al., 1990), we showed that, there are at least two types of Na^+ channel in these cells,

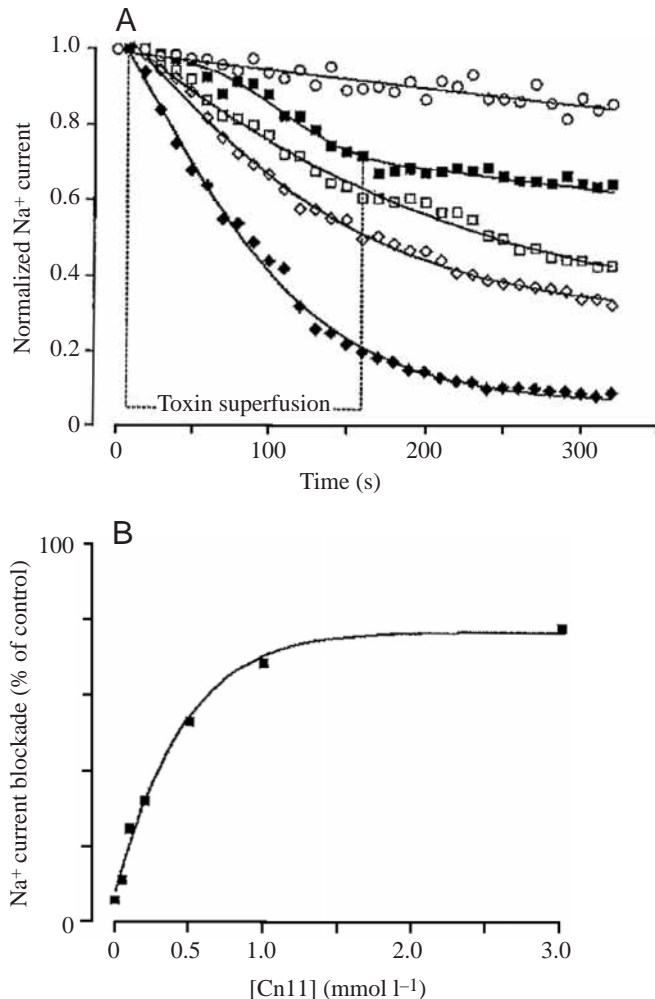


Fig. 4. Time course of the blockade of Na^+ currents by Cn11 and determination of K_m . (A) Under control conditions, the current amplitude decreases steadily by approximately 10% during the first 160 s, and this is taken as a normal rundown (open circles). Subsequent curves were obtained after superfusion with the toxin at various concentrations: filled squares, 50 nmol l^{-1} ; open squares, 100 nmol l^{-1} ; open diamonds, 200 nmol l^{-1} ; filled diamonds, 500 nmol l^{-1} . Normalized current (I_m) was obtained as I/I_m . After 160 s, the preparation was superfused with the external solution. The current amplitude decreased in a concentration-dependent manner, with no apparent recovery. (B) The percentage of Na^+ current blockade plotted against Cn11 concentration, 70 s after application of the toxin, corrected for 5% rundown. The points were fitted to a Boltzmann's equation, giving a K_m of approximately 320 nmol l^{-1} .

one sensitive and the other insensitive to TTX. Assuming that this is the case, Cn11, like TTX, can discriminate between these two types of Na^+ current.

Voltage-gated Na^+ channels are the molecular target for a broad range of neurotoxins that act at six or more distinct receptor sites on the channel protein. There are hydrophilic polypeptides, of either low or high molecular mass, that physically block the pore and prevent the conductance of Na^+ . There also exist alkaloid toxins and related lipid-soluble toxins that alter the voltage-dependent gating of Na^+ channels *via* an allosteric mechanism by binding to intramembranous receptor sites. In contrast, polypeptide toxins alter channel gating by voltage sensor trapping through binding to extracellular receptor sites. Studies defining the receptor sites and mechanisms of action of these diverse toxins were recently reviewed by Cestele and Catterall (2000).

If we analyze this review and that of Possani et al. (1999a), it is clear that none of the scorpion toxins studied thus far has a mechanism of action identical to that described here for Cn11. In Fig. 6, we show a comparative analysis of the identity found when comparing representative examples of each of the 10 proposed groups (Possani et al., 1999a). Pairwise comparisons with Cn11 were made separately between each of the 10 groups described. Gaps were introduced to enhance similarities and to place cysteine residues in equivalent positions. Only group 10, which is an insect toxin (AaHIT), has a disulphide pairing that does not fit exactly with the others, and it has been reported that the disulphide arrangement of this toxin is different from that of the others (for reviews, see Gordon et al., 1998; Possani et al., 1999a). The most closely

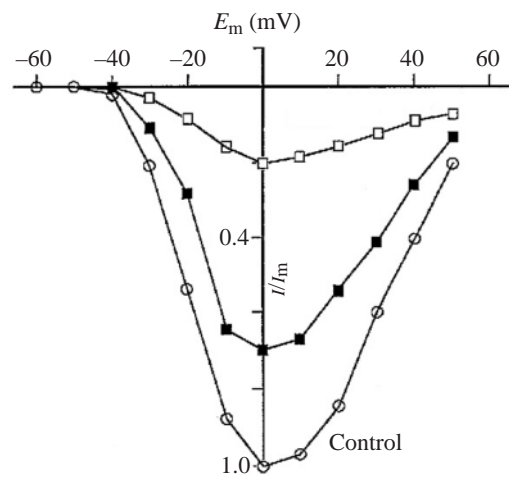


Fig. 5. The blockade of the Na^+ current by Cn11 is not voltage-dependent. Current/voltage relationships were obtained under control conditions (open circles) and 3 min after the application of 1 $\mu\text{mol l}^{-1}$ (filled squares) or 3 $\mu\text{mol l}^{-1}$ (open squares) Cn11. The inward currents were elicited by depolarizing from a holding potential of -60 mV to $+50$ mV, in increments of 10 mV, with pulses of 10 ms duration. Currents start at approximately -40 mV and reach a maximum value of approximately 0 mV. I/I_m , normalized Na^+ current.

Gp	Toxin	Amino acid sequence										Percentage identity																																																																	
		1	10	20	30	40	50	60	70																																																																				
1	AaHII	V	K	D	G	Y	I	V	-	D	D	V	N	C	T	Y	F	C	G	R	-	-	N	A	Y	C	N	E	E	C	T	K	L	K	G	E	-	-	S	G	Y	C	Q	W	A	S	P	Y	G	N	A	C	Y	C	Y	K	L	P	D	H	V	R	-	-	T	K	G	P	G	R	C	H	36				
2	CssII	-	K	E	G	Y	L	V	S	K	S	T	G	C	K	Y	E	C	L	K	L	G	D	N	Y	C	L	R	E	C	Q	Y	G	K	S	S	G	G	Y	C	-	A	-	-	-	F	A	C	W	C	T	H	L	Y	E	Q	A	V	V	W	P	L	P	N	K	T	C	N	32								
3	Tsgamma	-	K	E	G	Y	L	M	D	H	E	-	G	C	K	L	S	C	F	I	-	R	P	S	G	Y	C	G	R	E	C	G	I	K	K	-	-	S	S	G	Y	C	A	W	-	P	-	-	-	A	C	Y	C	G	L	P	N	W	V	K	V	W	D	R	A	T	N	K	C	-	46						
4	LqhIT2	-	-	D	G	Y	I	K	R	R	D	-	G	C	K	V	A	C	L	I	G	-	N	-	E	G	C	D	K	E	C	K	A	-	Y	G	G	-	S	Y	G	C	W	T	-	-	G	L	A	C	W	C	E	G	L	P	D	D	-	K	T	W	K	S	E	T	N	T	C	G	56						
5	LqqIV	G	V	R	D	A	I	A	D	D	K	N	C	V	T	C	G	S	-	-	N	-	S	Y	C	N	T	E	C	T	-	K	N	-	G	A	E	S	G	Y	C	Q	W	L	G	K	Y	G	N	A	C	W	C	I	K	L	P	D	K	V	-	-	P	I	R	I	P	G	K	C	R	34					
6	LqqIII	V	R	D	A	I	A	K	N	Y	-	N	C	V	E	C	F	R	-	-	D	S	Y	C	N	D	L	C	T	-	K	N	-	G	A	S	S	G	Y	C	Q	W	A	G	K	Y	G	N	A	C	W	C	Y	A	L	P	D	N	V	P	I	-	-	R	V	P	G	K	C	H	37						
7	AaHIT4	-	E	H	G	Y	L	L	N	K	Y	T	G	C	K	V	W	C	V	I	-	-	N	N	E	E	C	G	Y	L	C	N	-	K	R	R	G	Y	Y	G	Y	C	F	W	K	L	-	-	-	A	C	Y	C	Q	G	A	R	K	S	-	E	L	W	N	Y	K	T	N	K	C	D	L	42				
8	CsEv3	-	K	E	G	Y	L	V	K	K	S	D	G	C	K	Y	G	C	L	K	L	G	E	N	E	G	C	D	T	E	C	K	A	N	Q	G	S	Y	G	Y	C	Y	A	-	F	-	-	-	A	C	W	C	E	G	L	P	E	S	T	P	T	Y	P	L	P	N	K	S	-	35							
9	Cn10	-	K	E	G	Y	L	V	N	K	S	T	G	C	K	Y	N	C	L	I	L	G	E	N	K	N	C	D	M	E	C	K	A	N	Q	G	S	Y	G	Y	C	Y	K	-	L	-	-	-	A	C	W	C	E	G	L	P	E	S	T	P	T	Y	P	I	P	G	K	T	C	R	T	39					
10	AaHIT	K	K	N	G	Y	A	V	D	S	-	G	K	A	P	E	C	L	L	S	-	N	Y	-	C	Y	N	N	E	C	T	K	V	H	Y	A	D	K	-	G	Y	C	L	-	L	-	-	-	S	C	Y	C	F	G	L	N	D	D	K	V	L	E	I	S	D	T	R	K	S	Y	C	D	T	T	I	N	28
11	Cn11	A	R	D	G	Y	P	V	D	E	K	-	G	C	K	L	S	C	L	I	-	-	N	D	K	W	C	N	S	A	C	H	S	R	-	G	G	K	Y	G	Y	C	T	-	G	-	-	G	L	A	C	Y	C	E	A	V	P	D	N	V	K	V	W	T	Y	E	T	N	T	C	-	100					

Fig. 6. Pairwise comparison of identity between Cn11 and the other 10 representative groups of scorpion toxin specific for Na⁺ channels. The amino acid sequence of the 10 representative examples of each sub-group of Na⁺ channel scorpion toxin was compared with the amino acid sequence of Cn11 (the eleventh group proposed here). AaHII is toxin II from *Androctonus australis* Hector, the prototype of an α -scorpion toxin; CssII is toxin II from *Centruroides suffusus suffusus*, the prototype of a β -scorpion toxin; Tsgamma is toxin gamma from *Tityus serrulatus*; LqhIT2 is insect toxin 2 from *Leiurus quinquestriatus hebraeus*; LqqIV is toxin IV from *Leiurus quinquestriatus quinquestriatus*; LqqIII is toxin III from the same scorpion; AaHIT4, toxin 4, is an insect toxin from *Androctonus australis* Hector; CsEv3 is variant 3 from *Centruroides sculpturatus* Ewing; Cn10, toxin 10, is an insect toxin from *Centruroides noxius* Hoffmann; AaHIT is an insect toxin from *Androctonus australis* Hector; and Cn11 is from this study (data from Possani et al., 1999a, 2000, 2001). Gaps (dashes) were introduced to enhance similarities. Cysteine residues are in bold type. The right-hand column indicates the percentage identity of the pairwise comparison.

related group (group 4) has only 56% identity with Cn11. All the other toxins have identities ranging from only 28 to 46% (see Fig. 6). The two consensus (signature) sequences CXXXC and CXC, where C stands for cysteine and X for any residue, that serve to stabilize the three-dimensional structure by linking the α -helix stretch with the β -sheet segments are conserved, except in AaHIT, as discussed. However, the most important argument in classifying Cn11 as a distinct group resides in its function. Cn11 blocks the Na⁺ currents of crayfish neurons in culture without apparently affecting the gating mechanism of the channels, an effect known to occur for the other groups of scorpion toxins.

Indeed, the blocking effect is time- and concentration-dependent, with a K_m of 320 nmol l⁻¹. The effect of Cn11 is similar to the blocking effects of TTX and STX, which physically block the passage of ions by binding to the channels, acting on the channel like a cork in a bottle, with the guanidinium group of the toxin being the cork. The only other peptide described as a potent Na⁺ channels blocker is the 22-amino-acid peptide μ -conotoxin from a marine snail (McIntosh et al., 1999; Dudley et al., 1995).

Cn11 is quite specific for crustaceans, having no detectable effect on mammals at the relatively high concentration used (more than 500 μ g per 20 g mouse) (S. F. Serrano-Hernández and T. Olamendi-Portugal, unpublished). Cn11 could be a valuable tool in the investigation of the shape and structure of the outer vestibule of Na⁺ channels, as has been reported for other systems (Frech and Dudley, 1999; Marban et al., 1998; Chahaine et al., 1998; Dudley et al., 1995; Li et al., 1997; Fozzard and Lipkind, 1996).

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