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Charybdotoxin and noxiustoxin, two homologous peptide inhibitors of the $K^+(Ca^{2+})$ channel

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We show that noxiustoxin (NTX), like charybdotoxin (CTX) described by others, affects Ca^{2+}-activated K^+ channels of skeletal muscle ($K^+(Ca^{2+})$ channels). Chemical characterization of CTX shows that it is similar to NTX. Although the amino-terminal amino acid of CTX is not readily available, the molecule was partially sequenced after CNBr cleavage. A decapeptide corresponding to the C-terminal region of NTX shows 60% homology to that of CTX, maintaining the cysteine residues at the same positions. While CTX blocks the $K^+(Ca^{2+})$ channels with a K_d of 1–3 nM, for NTX it is approx. 450 nM. Both peptides can interact simultaneously with the same channel. NTX and CTX promise to be good tools for channel isolation.

Noxiustoxin; Charybdotoxin; Amino acid sequence; K^+ channel; Single-channel recording; (Skeletal muscle)

1. INTRODUCTION

In recent years, the understanding of the structure and function of membrane-bound receptor proteins, including ion channels, has been greatly increased, due to the discovery of specific natural peptide inhibitors used as probes at molecular and cellular levels [1–6]. Although many different types of K^+ channels have been extensively studied electrophysiologically, few biochemical data are available [7,8], primarily due to the lack of specific biochemical ligands for isolating channels in pure form. From a variety of substances described as blockers of K^+ channels (Cs^+, tetraethylammonium, 4-aminopyridine, etc., see review [9]), none have proven to be useful tools for channel isolation, because of the high concentration needed (millimolar range) and lack of specificity.

Recently, CTX, a protein isolated from the venom of the scorpion *Leiurus quinquestriatus quinquestriatus* was shown to block $K^+(Ca^{2+})$ channels from rat skeletal muscle [10]. In single-channel recordings, CTX induced long-lived non-conducting states characteristic of a high-affinity channel blockade. The effect was reversible and was shown to be attributable to a basic peptide with a molecular mass in the range 7–10 kDa [11]. NTX, a peptide with 39 amino acid residues, was isolated from the venom of the scorpion *Centruroides noxius* [12] and was shown to block the voltage-dependent K^+ channel from squid axon [13,14], and changed K^+ permeability in brain synaptosomes [15]. Since neither the primary structure of CTX nor the effect of NTX on

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Abbreviations: CTX, charybdotoxin; CM-cellulose, carboxymethylcellulose; $K^+(Ca^{2+})$ channels, Ca^{2+}-activated K^+ channels; NTX, noxiustoxin

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$K^+(Ca^{2+})$ channels was known, we have decided to verify the structure of CTX and compare the effect of both peptides in skeletal muscle preparation by means of single-channel recording techniques, which is the subject of this communication.

2. MATERIALS AND METHODS

2.1. *Materials*

Only analytical grade reagents were used throughout the purification and characterization procedures; sources have been previously indicated [16].

Venom from the scorpion *C. noxius* was obtained by electrical stimulation of anesthetized animals [16], while venom from *L. q. quinquestriatus* was purchased from Sigma (St. Louis, MO).

2.2. *Purification of toxins*

NTX was purified as in [17]. CTX was purified by a three-step procedure. The soluble venom (100 mg) from *L. q. quinquestriatus* was gel filtered on a Sephadex G-50 column (1.8 × 200 cm), equilibrated in 20 mM ammonium acetate buffer, pH 4.7. The blocking activity of CTX-containing fractions was followed by monitoring their effect on channels incorporated into bilayers, as described below. Fraction III eluting from the Sephadex column displayed CTX activity and was further separated in a CM-cellulose column (0.9 × 30 cm) equilibrated in 20 mM ammonium acetate buffer, pH 4.7, and eluted with a salt gradient (from 0 to 0.5 M NaCl in the same buffer). Fraction 13 contained CTX activity and was finally purified by high-performance liquid chromatography (HPLC) using a reverse-phase column similarly to the work of Smith et al. [11]. Homogeneity of samples was confirmed by polyacrylamide gel electrophoresis in two different systems [18,19]. The first system contained acetate-urea buffers. The second contained SDS and the sample was reduced by boiling in the presence of 5% β-mercaptoethanol, and applied to the gel for determination of the molecular mass. A small aliquot of CTX (50 μg) was isotopically labeled with ^{125}I by the lactoperoxidase method of Morrison and Bayse [20] and also run in parallel to verify that the protein-staining band in the gel corresponded to the radiolabeled CTX.

2.3. *Chemical characterization of CTX*

A sample of CTX was hydrolyzed in 6 N HCl [16] and the composition was determined in a Beckman 6300 amino acid analyzer. Another aliquot of CTX (50 μg) was reduced and pyridylethylated as in [12]. Part of this material was directly loaded in the spinning cup of a Beckman 890M microsequencer for automatic Edman degradation [21]; another aliquot of the reduced and alkylated toxin was cleaved with CNBr [16], and separated by HPLC for further sequence analysis.

2.4. *Preparation of transverse tubule-enriched membranes, and planar bilayer methods*

Membranes derived from the transverse tubule of skeletal muscle were prepared by a slight modification of the procedure of Meissner [22]. Membrane fractions from the 10/20 and 20/25% sucrose interfaces routinely show high PN 200-110-binding activity and were therefore used for incorporation of transverse tubule ion channels into the planar bilayers of a Mueller-Rudin type of chamber. Single-channel currents were measured via a List EPC-7 patch clamp electrometer (List Electronic, Darmstadt) and filtered through an 8-pole Bessel low-pass filter. Analog signals from the patch clamp were digitized and stored on 10 Mbyte hard disks for later analysis with software run on an IBM-AT computer.

3. RESULTS AND DISCUSSION

Gel electrophoresis analysis of CTX purified by our procedure shows that it migrates as a single component of apparent molecular mass 4.8 kDa (see fig.1). The electrophoretic mobility of CTX is between that of γ-toxin (7 kDa) and NTX (4.2 kDa), which were used as molecular mass markers. Furthermore, ^{125}I-CTX migrates as a single component and corresponds to the protein band stained with Coomassie blue in the gel. The final recovery of CTX obtained by this procedure is of the order of 0.5%, assuming a molar extinction coefficient of 17000 $M^{-1} \cdot cm^{-1}$, as suggested [11]. The amino acid composition of CTX reported by Smith et al. [11] is close to the values we have found, except for their assumption of a molecular mass of 9.2 kDa, twice of what we have

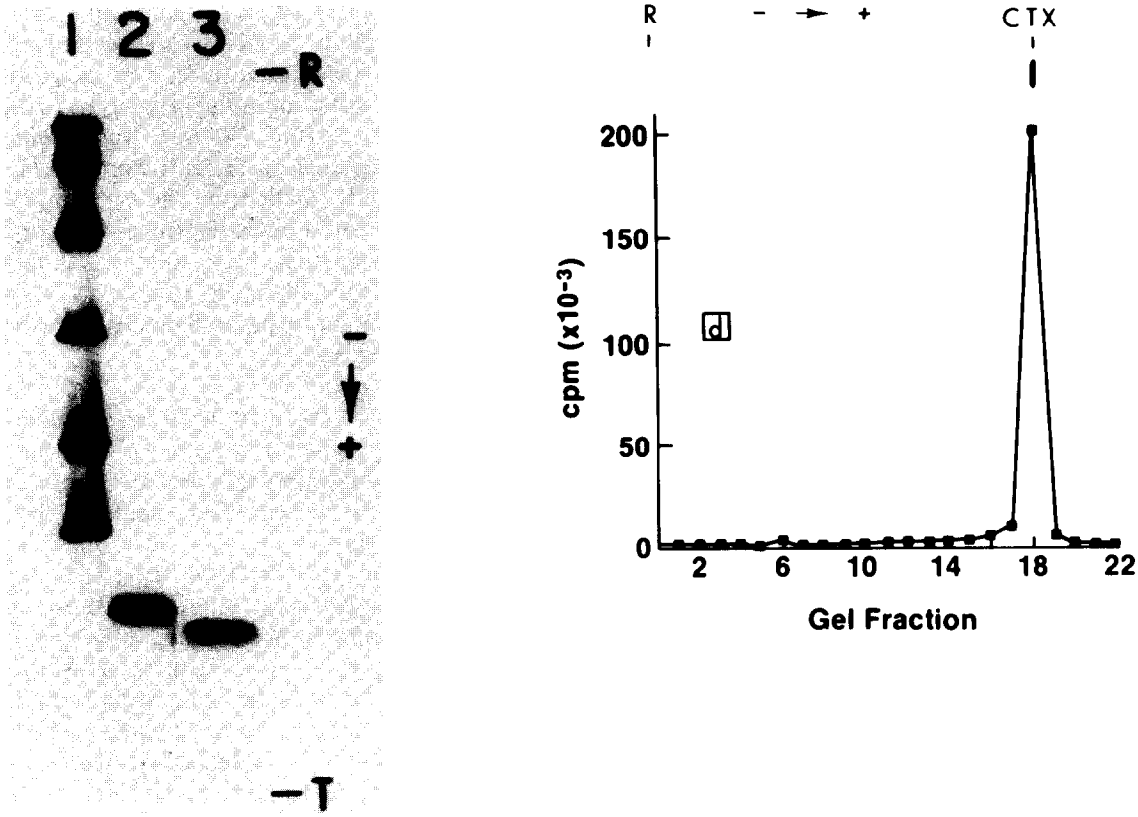

Fig.1. SDS gel electrophoresis of native and iodinated CTX. The gel was sliced into 0.5-cm portions and ^{125}I-CTX was counted in a gamma-counter. The peak of radioactivity co-migrates with the protein band on the gel (see slab-gel strip horizontally placed). A mixture of molecular mass markers was applied in the same gel, in parallel, in order to determine the apparent molecular mass of CTX. A linear plot (log molecular mass vs R_f of markers) showed the molecular mass of CTX to be 4.8 kDa. The following markers (in kDa) were used: phosphorylase *b* (92.5), bovine serum albumin (62.5), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21.5), lysozyme (14) (see lane 1). Lane 2 contains 4 μg γ-toxin from *T. serrulatus* (7 kDa); and lane 3 is CTX (2 μg).

found. For example, they have reported 6 Arg, 2 His, 2 Tyr and 2 Phe; we have found exactly half of these values. However, we do agree on the values of Ile (0) and Met (1). Values for the composition of the amino acid Met are usually underestimated because of partial oxidation. It is worth mentioning that cysteines were measured as cysteic acid, after oxidation with performic acid [11], and we do obtain the same number of residues (6) per molecule of CTX. Except for the discrepancy in molecular mass we conclude that we are studying the same CTX molecule, because both are basic peptides, are extracted from the same source of scorpion venom, have very similar affinity (K_d) for the same type of channels measured via the same method and display the same kinetic behavior towards the $K^+(Ca^{2+})$ channels.

For the first time it has been shown that NTX affects the $K^+(Ca^{2+})$ channels of skeletal muscle (see fig.2), suggesting the possible existence of a related family of toxic peptides in scorpion venom, which affect these channels, and that an important stretch of the CTX primary structure is homologous with NTX. We were unable to obtain direct sequencing data with the intact CTX molecule, probably because the N-terminal amino acid is blocked. For this reason we have treated CTX with CNBr. Two peptides were generated, one corresponding to the blocked N-terminal peptide and another with the following sequence: Met-

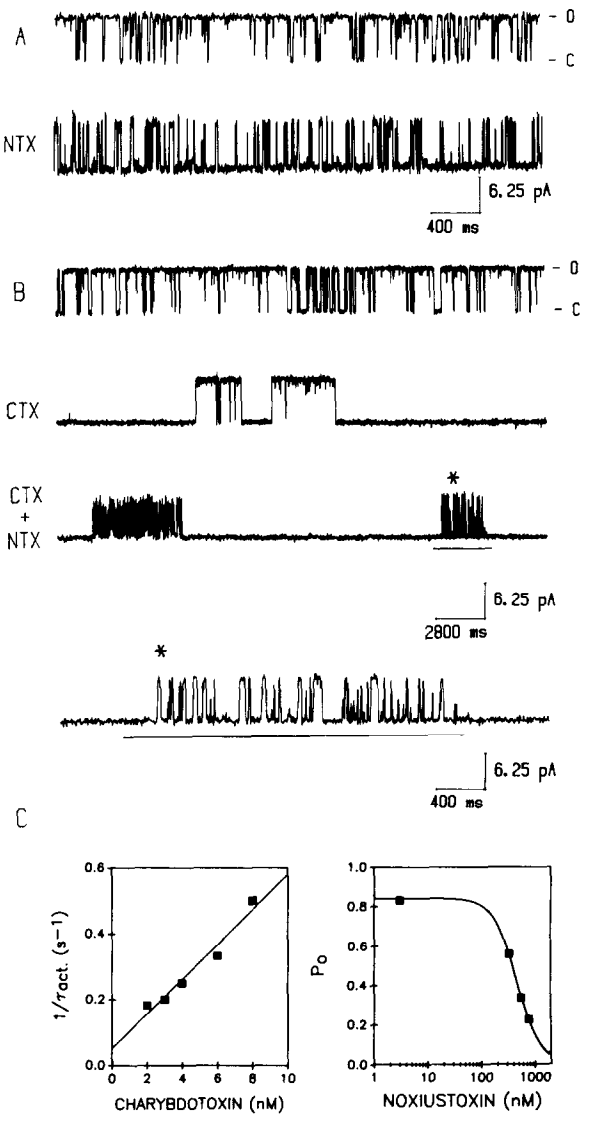

Fig.2. Effect of toxins on single-channel currents of Ca^{2+}-activated K^+ channels from skeletal muscle T-tubules. (A) Single $K^+(Ca^{2+})$ channels were recorded in 250 mM KCl (internal side), 50 mM KCl, 200 mM NaCl (external side). Upper trace, control activity at 0 mV, 500 Hz filter, 2 kHz sampling. Open probability (P_o) was 0.85. Second trace shows the effect of 800 nM NTX added *trans*. P_o was reduced to 0.44. (B) Blockade produced by CTX and NTX. Upper trace, control (P_o = 0.80). Second trace was recorded with 20 nM CTX added to the external side of the channel (*trans*). P_o from entire file (10 min recording time) was 0.12. Third trace shows the effect produced by 20 nM CTX plus 2 μM NTX *trans*. Lowermost recording is an expansion of the right-hand burst of activity shown in the third trace. Recordings were taken at 0 mV, 500 Hz filter and 2 kHz sampling. (C) Dose-response curves for CTX and NTX blockade of $K^+(Ca^{2+})$ channels. (Left) Reciprocal of the mean active time [10] vs CTX concentration. The slope gives a second-order rate constant of block, $K_{on} = 1/[CTX]\tau_{act} = 0.053 \times 10^9\ M^{-1} \cdot s^{-1}$. The apparent $K_d = K_{off}/K_{on} \simeq 1.8$ nM. (Right) Plot of P_o (fraction open time) vs [NTX]. The apparent K_d is 450 nM.

Asn-Lys-Lys-Cys-Arg-Cys-Tyr-Ser-Ser. As shown in table 1 this sequence corresponds to the decapeptide at the C-terminal amino acid sequence of NTX with 60% identity and with perfect alignment of the cysteinyl residues. The blocking effects of both peptides on $K^+(Ca^{2+})$ channels are shown in fig.2. NTX reduces open probability by driving the channel into a brief blocked state. The CTX trace shows the characteristic long-term blockade of $K^+(Ca^{2+})$ channels produced by 20 nM CTX. Both toxins are only effective when added to the external face of the channel. Blocked states in-

Table 1

Comparison of the C-terminal amino acid sequence of noxiustoxin with that of a CNBr-cleaved peptide from charybdotoxin

Scorpion toxin	Amino acid sequence
C. noxius noxiustoxin	1 30 35 Thr-Ile-Ile-.....Met-Asn-Gly-Lys-Cys-Lys-Cys-Tyr-Asn-Asn
L. q. quinquestriatus charybdotoxin	blocked (?).....Met-Asn-Lys-Lys-Cys-Arg-Cys-Tyr-Ser-Ser

duced by CTX were typically 10–15 s in duration at room temperature. 2 μM NTX added in the presence of CTX had an additional inhibitory effect. In the presence of both toxins channels displayed blocked states of long and short duration characteristic of those produced by each toxin added separately (trace CTX + NTX). When bursts of activity in the presence of both toxins were examined on an expanded time scale, the open and closed events appeared kinetically similar to those seen in the presence of NTX alone (cf. final and NTX traces). Thus, apparently, both CTX and NTX can interact simultaneously with the same ion channel. At this point, however, it is not clear whether the toxin-binding sites are the same for CTX and NTX. The difference in mean duration of toxin-induced blocked states ($\tau \simeq 10$ s for CTX and $\tau \simeq 50$ ms for NTX) is most easily explained by the different apparent affinities of the two toxins for inhibitory sites on the channel. The K_d values obtained in a series of experiments conducted with different concentrations of both toxins indicated that CTX has a K_d of 1–3 nM, while for NTX it is 450 nM (see fig.2C). Similar differences in affinity were found when comparing the K_d of NTX for the delayed rectifier of squid axon (of the order of 290 nM [13]) with the ED_{50} (approx. 2 nM) in the release of γ-aminobutyric acid from brain synaptosomes [15]. Although the specificity of NTX seems to be rather broad concerning the type of K^+ channels affected, it seems to be related exclusively to K^+ channels [13–15]. It does not modify Na^+ or Ca^{2+} currents [13–15]. Our results support the idea that CTX and NTX are candidates for use as tools for K^+ channel isolation.

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