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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 microsequencer. 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.

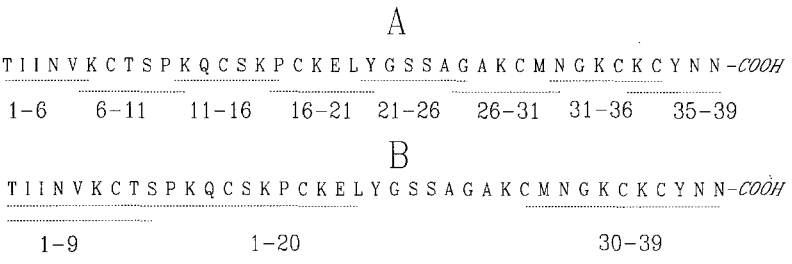

Fig. 1. Peptides synthesized for this work. *Panel A:* Small peptides (hexapeptides). *Panel B:* larger peptides. All peptides are aligned by their N-terminal region with NTX sequence.

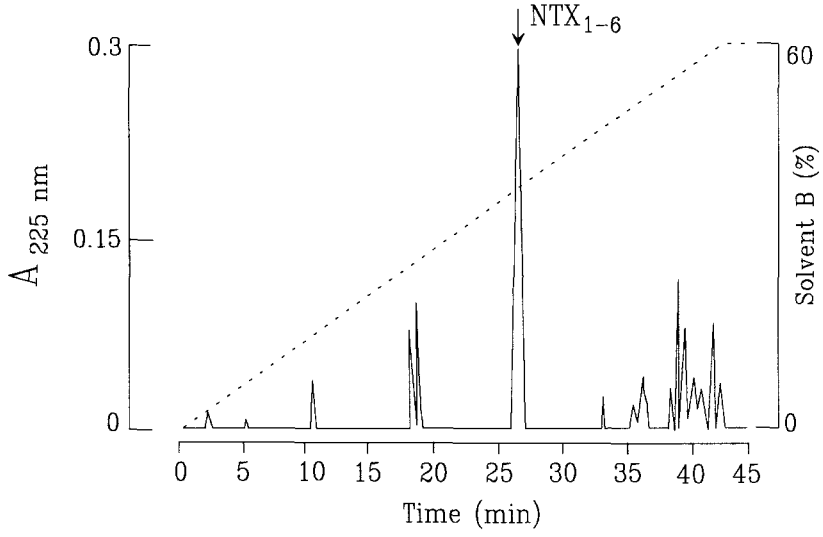

Fig. 2. HPLC separation of synthetic peptide NTX_{1-6}. The peptide (228 μg) was injected at time zero in a Beckman chromatographic system. An Altex C_{18} reverse phase column was used to separate the peptides. An isochratic gradient from solvent A (0.12% trifluoroacetic acid in distilled water) to 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 $\approx$310 nM ($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.

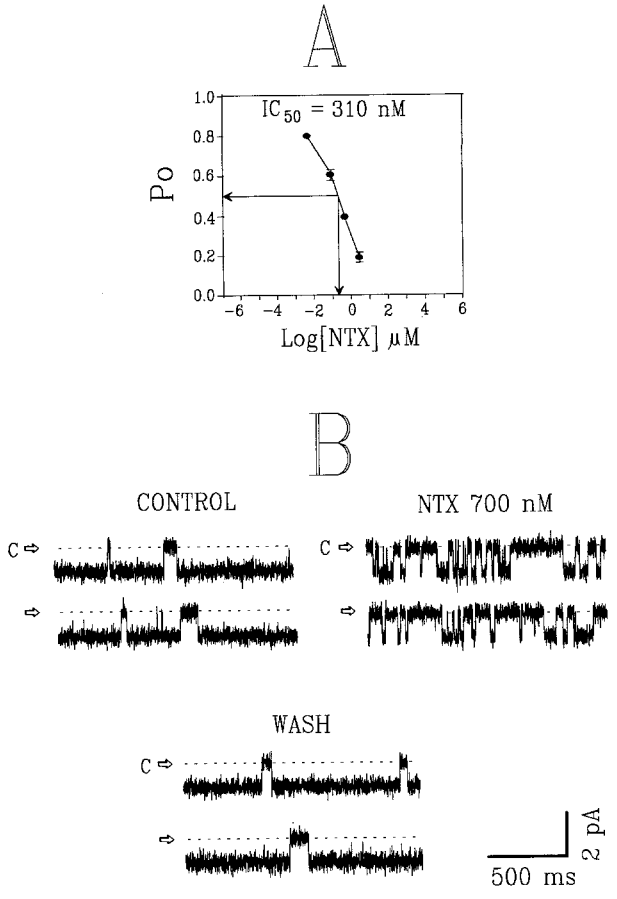

Fig. 3. Noxiustoxin blocks single K_{Ca} channels in BAECs. (*A*) Concentration-response curve for NTX obtained at −40 mV. Unbroken line was used to connect data points. The half inhibitory concentration (IC_{50}) = 310 nM (n = 4). (*B*) Examples with channel activity at −40 mV from an outside-out patch 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.

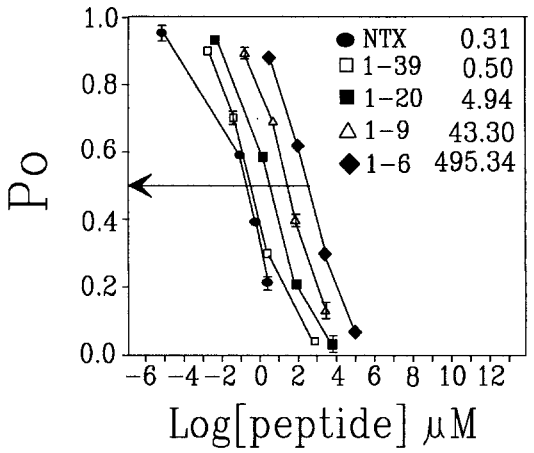

Fig. 4. Concentration-response curves for synthetic peptides. Concentration-response curve for the peptides that affected channel P_o. Half inhibitory concentrations for native NTX (●, n = 4), NTX_{1-39} (□, n = 3), NTX_{1-20} (■, n = 5), NTX_{1-9} (△, n = 4) and NTX_{1-6} (◆, n = 6). Unbroken lines 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.

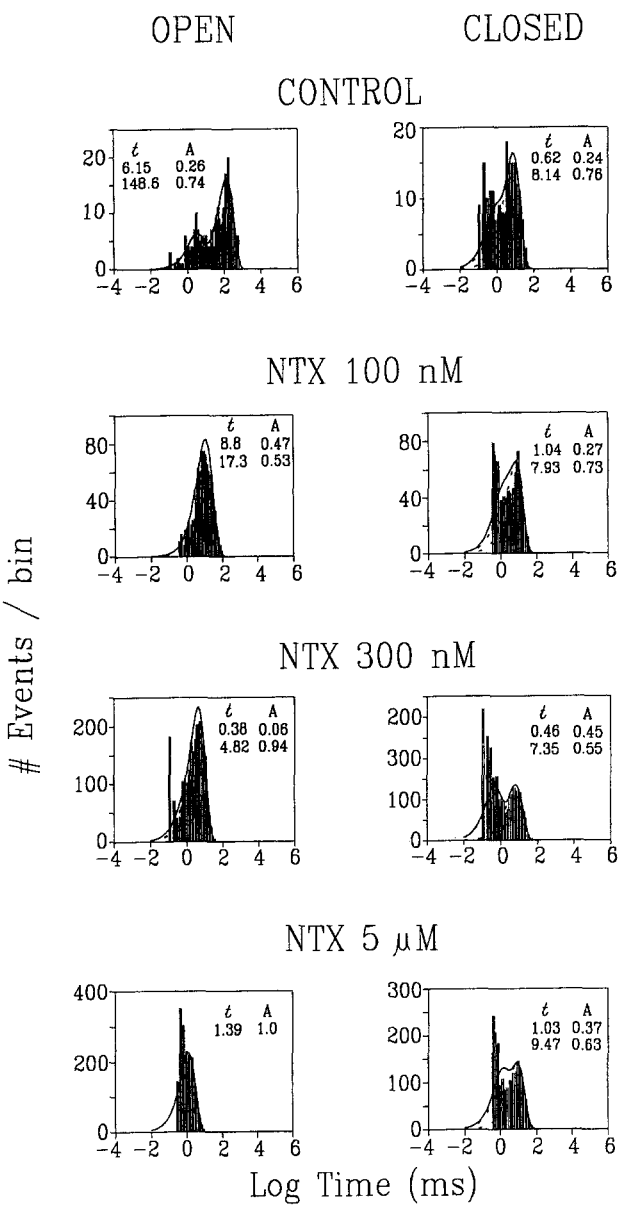

Fig. 5. Effect of NTX on channel open and closed time distributions. Dwell-time distributions obtained from one outside-out patch under control conditions and after addition of 100 nM, 300 nM and 5 μM NTX to the bath (extracellular) solution. Open and closed time distributions were binned logarithmically from records containing 50–60 sec of continuous channel activity for each experimental condition. The binwidth used was 0.1 msec. Time constant (t) 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.

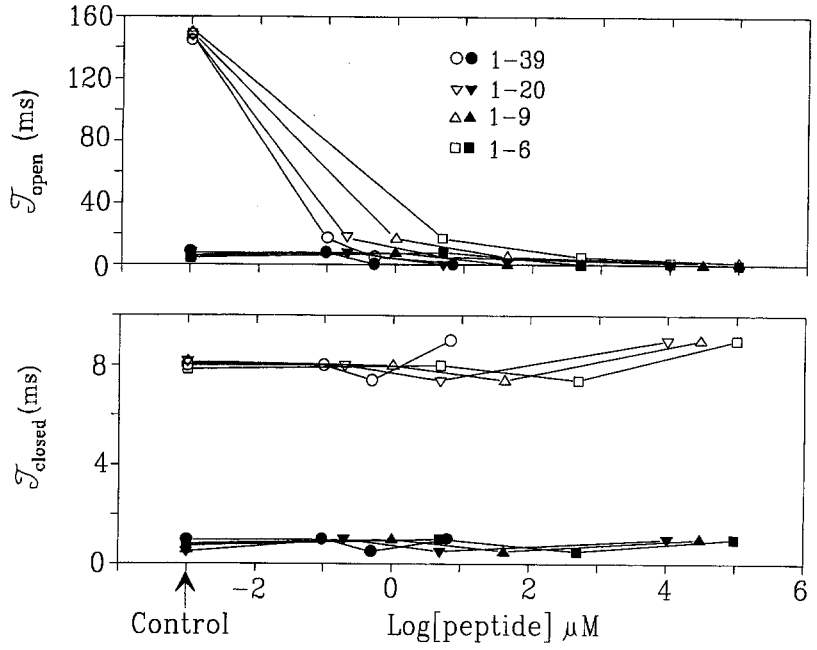

Fig. 6. Effect of synthetic peptides on channel open and closed time distributions. Mean open and closed times obtained at different concentrations of the synthetic peptides. These mean times were obtained after fitting with a double exponential function experiments like the one described in Fig. 5. 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.

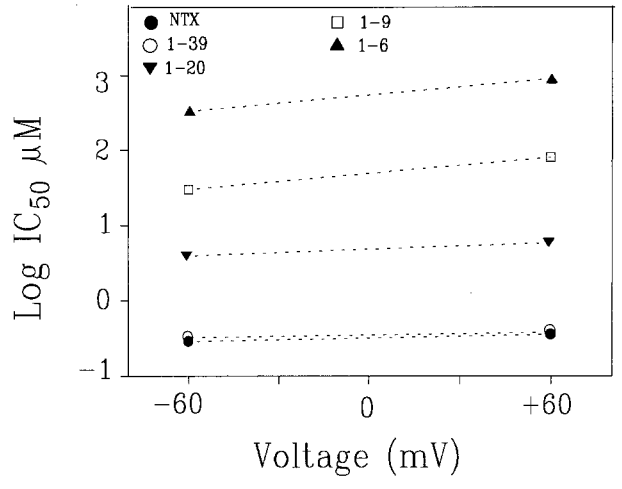

Fig. 7. Effect of voltage on channel blockade. The effect of two different voltages (−60 and +60 mV) on the IC_{50} of NTX (●, n = 3) and the synthetic peptides NTX_{1-39} (○, n = 3), NTX_{1-20} (▼, n = 4), NTX_{1-9} (○, n = 4) and NTX_{1-6} (▲, n = 3) explored in outside-out patches. The IC_{50} used 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:

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

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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