Site directed mutants of Noxiustoxin reveal specific interactions with potassium channels

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Abstract Several site directed mutations were introduced into a synthetic Noxiustoxin (NTX) gene. Alanine scanning of the nonapeptide at the N-terminal segment of NTX (threonine 1 (T1) to serine 9 (S9)) was constructed and the recombinant products were obtained in pure form. Additionally, lysine 28 (K28) was changed to arginine (R) or glutamic acid (E), cysteine 29 was changed to alanine, and residues 37–39 (Tyr-Asn-Asn) of the carboxyl end were deleted. The recombinant mutants were tested for their ability to displace ^{125}I-NTX from rat brain synaptosome membranes, as well as for their efficiency in blocking the activity of $K_v1.1$ K^+ channels expressed in *Xenopus laevis* oocytes. The main results indicate that residues K6, T8 at the amino end, and K28 and the tripeptide YNN at the carboxyl end are involved in specific interactions of NTX with rat brain and/or $K_v1.1$ K^+ channels.

Key words: *Centruroides noxius*; K^+ channel; Mutagenesis; Oocyte expression; Noxiustoxin; Scorpion toxin

1. Introduction

Scorpion toxins are very useful tools for the study of ion channels [1]. NTX, a 39 amino acid peptide purified from the Mexican scorpion *Centruroides noxius* Hoffmann [2], was the first reported animal toxin described as a specific K^+ channel blocker [3]. NTX can reversibly block several types of K^+ channels [3–5]. K^+ channels are tetrameric membrane proteins involved in many biological phenomena [6–8], with a large variety of gating mechanisms and kinetics, but with a high selectivity for K^+ ions [6]. Each monomer is composed of six transmembrane segments connected by loops. The loops located between segments S5 and S6, of each monomer, were proposed to form the pore of the channel and are responsible for its ion selectivity [9,10]. Apart from the pore itself, it has been proposed that loops S5-S6 conform a vestibule which has been demonstrated to be the target site for many toxins that have in common the capacity of occluding the pore of the channel [11]. The specific interaction of toxins from different sources with defined residues of the vestibule and the pore is under intensive study [7–12]. Scorpion toxins that affect K^+ channels are short peptides (31–40 amino acid residues), with a well conserved three-dimensional structure stabilized by 3–4 disulfide bridges [13]. In spite of this conserved structure and small size, differences in sequence are involved in their specificity toward different K^+ channels. Scorpion toxins whose three-dimensional structures are known, have been key tools for revealing the putative architecture of the ion-channel pore in the absence of direct evidence [10–12]. Cyclic mutagenesis of channel and toxin amino acid residues has revealed those residues that are critical for the specific interaction channel-toxin [9–11]. In this communication we report an analysis of the interaction of a recombinant NTX and a series of mutants, with K^+ channels present in rat brain synaptosome membranes and/or $K_v1.1$ K^+ channels expressed in *Xenopus laevis* oocytes, in order to identify the interacting epitope of NTX towards these channels.

2. Materials and methods

2.1. Synthesis of oligonucleotides and generation of NTX mutants

Mutant oligonucleotides were synthesized on a PerSeptive Biosystems Expedite Model 8905 nucleic acid synthesis system using standard cyanoethyl phosphoramidite chemistry. A previously designed synthetic NTX gene [14], was used as template to generate the mutants here analyzed. Mutants were obtained by means of PCR using a series of mutagenic oligonucleotides as described [14]. Residues 1–9 from the amino terminus were changed to alanine (alanine scanning), using oligonucleotides that contained nine nucleotides at the 3′ side of the corresponding mutant codon (GCT, alanine) and a variable size at the 5′ side. All these mutagenic oligonucleotides of the amino terminus of NTX started at the sequence CGGATTC..., which is located five nucleotides downstream from the 5′ end of oligonucleotide No. 1 used previously for the synthesis of NTX gene [14]. The second primer for these PCR reactions was oligonucleotide No. 6 from the same work [14]. Amino terminus mutants were rescued in a second PCR with oligonucleotides Nos. 1 and 6 [14]. Deletion mutant (ΔYNN, residues 37–39), was generated with oligonucleotide No. 4 [14], from which the first three nucleotides of 5′ end were deleted. The second primer for this PCR was oligonucleotide No. 1 [14]. Rescuing of this mutant was done with oligonucleotide 1 and modified oligonucleotide 6 from which the last two nucleotides of the 3′ end were deleted and substituted by nine nucleotides complementary to codons for residues 34–36. In the case of mutants of residue K28 (K28R and K28E) and C29A, the recursive PCR technique [15] was used as previously described [14], in which mutant codons were carried by oligonucleotide No. 3 [14]. The substitution of the cysteines at positions 7 and 29 was done as a double mutation, in order to eliminate one of the disulfide bridges and avoid having free thiol groups that could make polymeric NTX. All mutations were verified by nucleotide sequencing as described [14]. Amino acid sequence of the mutants was determined by automated Edman degradation. Recombinant NTX and derived mutants were expressed and purified as described [14]. The vector pCSP105 used for expression of recombinant and mutant NTXs was generously given to us by Dr. Christopher Miller, Brandeis University, Walthman Massachusetts, USA.

2.2. Binding and displacement assays

Rat brain synaptosome membranes were prepared as described [14]. Fraction P3 of this preparation was used for binding assays. ^{125}I-NTX was labeled with the peroxidase method as described [16]. Native and recombinant NTXs and all the NTX mutants generated in this study were tested, except the double mutant C7A-C29A, which was not obtained in properly folded and purified form.

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2.3. Electrophysiological recording

pGEMHE vector (kindly provided by Dr. Mauricio Montal, University of California, San Diego, CA, USA), containing the cDNA that encodes mouse brain $K_v1.1$ channel was transcribed in vitro, as described [17]. Oocytes obtained from adult *Xenopus laevis* (Nasco, Fort Atkinson, USA), were injected with $K_v1.1$ RNA as reported by Ferrer-Montiel and Montal [17], and maintained at 17°C for 48 h in the recording solution (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 sodium pyruvate pH 7.6), containing 50 μg/ml gentamicin. Channels were expressed to a level of 5–10 μA outward current, elicited by 50 ms depolarizations from a holding potential of −70 mV to potentials between −30 mV and +30 mV in steps of 15 mV. The time between pulses was 15 s and all currents were recorded at 18–20°C. Oocyte membrane potential was controlled and current recorded by means of a two electrode voltage clamp amplifier (model CA1a, DAGAN Co., USA). Electrodes were filled with a solution containing: 1 M KCl, 10 mM EGTA and 10 mM HEPES pH 7.4, which had resistances from 0.3 to 1 mΩ. Currents were filtered at 2 kHz and sampled every 100 μs (10 kHz), using the Digidata 1200 interface (Axon Instruments, Burlingame, USA). Linear capacitive and leakage currents were removed on line with a P/-4 pulse protocol. Currents were recorded in the absence and presence of toxins. The recording reservoir was washed with 30 ml recording solution during 3 min, thereafter, currents were recorded periodically to verify recovery from toxin blockade. Native, recombinant and mutant K6A, T8A, K28R, K28E and ΔYNN37–39 NTXs were tested in this assay.

3. Results and discussion

3.1. Design of mutated recombinants of NTX

The rationale guiding the design of the mutants prepared and used in the present work was based on previous results:

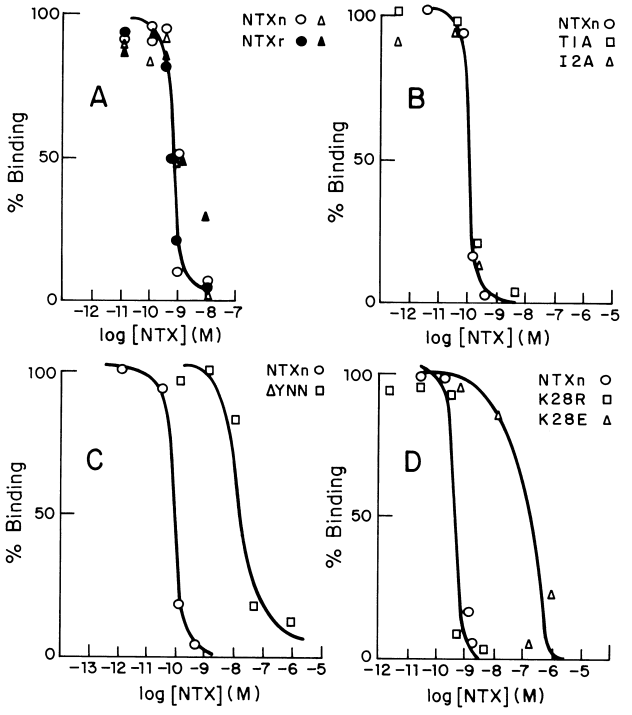

Fig. 1. Binding inhibition of ^{125}I native NTX by non-labeled native, recombinant and mutant NTXs to synaptosomal membranes. Rat brain synaptosomes were incubated with ^{125}I-NTX at 100 pM in the absence of native, recombinant or mutant NTXs (100% binding). Binding inhibition of ^{125}I-NTX to synaptosomes by increasing concentrations of cold native, recombinant or mutant NTXs is represented by the curves shown. Values are the mean of experiments carried out in triplicate. Double symbols in panel A refer to two independent experiments by triplicate.

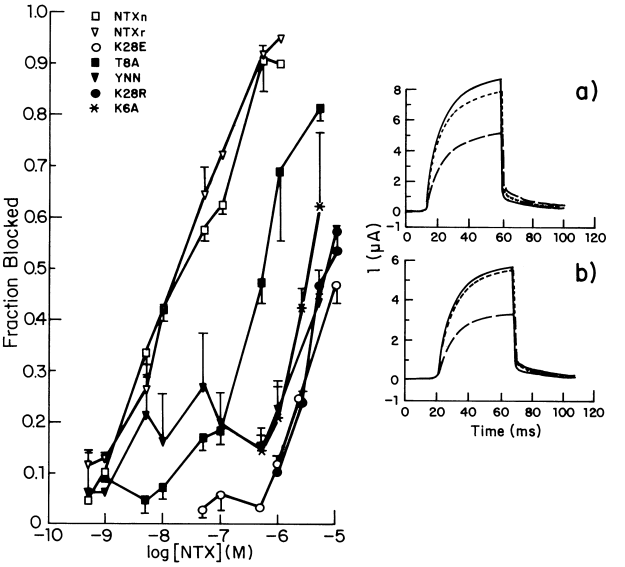

Fig. 2. Blocking assays on $K_v1.1$ channels of native, recombinant and mutant NTXs. $K_v1.1$ channels were expressed in *X. laevis* oocytes (for details see Section 2.3). Left part of this figure shows the fraction of current blocked with different concentrations of native, recombinant or mutant NTXs. Values are the means of 3–7 measurements. Standard deviations are also shown (only upper or lower values are drawn for clarity). Inset: K^+ blockade currents by native (a) or recombinant (b) NTXs at a concentration of 10 nM. Continuous lines represent control currents, broken lines show currents in the presence of the toxin, dotted lines indicate the current recovered after toxin washing. These recordings correspond to a potential of 0 mV from a holding potential of −70 mV.

(a) our laboratory showed that the N-terminal nonapeptide of NTX was toxic per se to mice [18,19] or was able to recognize Ca^{2+}-dependent K^+ channels from aortic tissue [20]; (b) several laboratories have shown that similar toxins, i.e. Charybdotoxin (ChTX) [11] and Agitoxin 2 (AgTX) [12] have a crucial lysine residue (K27 for Charybdotoxin), in equivalent positions as that of K28 for NTX, shown to be very important for channel blockade, and (c) the fact that amidation of asparagine at the C-terminal region of NTX makes no difference on binding of radiolabeled NTX to brain synaptosome membranes [14], as well as that a synthetic decapeptide from the C-terminal segment of NTX had no effect on binding displacement experiments [19]. All the peptides were obtained in pure form and in enough quantities to conduct the experiments described below, except as mentioned before, the double mutant (C7A-C29A), which eliminated one of the three disulfides of NTX.

3.2. Binding and displacement of native, recombinant and mutant NTXs

Recombinant NTX (NTXr) displaced labeled native NTX (^{125}I-NTX) from synaptosome membranes in a very similar manner as cold native NTX (NTXn) did (Fig. 1, panel A). The same results were obtained for mutants T1A, I2A, I3A, N4A, V5A, K6A, T8A and S9A. Only data for mutants T1A and I2A are shown in panel B of Fig. 1. Variants ΔYNN (Fig. 1, panel C) and K28E (Fig. 1, panel D) displayed shifts to lower displacements of approximately three orders of magnitude, whereas the mutant K28R had no significant effect. Also, in contrast to our previous work with synthetic peptides on the binding assay to brain synaptosome membranes [19]

and to Ca^{2+}-dependent K^+ channels from bovine aorta [20], no significant differences were found between binding displacement assays of native NTX and those of the N-terminal segment of the mutated NTXs. Alanine scanning has been considered a reliable approach to evaluate the importance of defined amino acid side chains on the function of a protein [21,22]. Thus, based on the results presented in Fig. 1, it can be concluded that K28 and the tripeptide Y37-N39 are important residues involved in the binding capacity of NTX to populations of K^+ channels present in rat brain synaptosome membranes.

3.3. Electrophysiological assays

Recombinant NTX blocked $K_v1.1$ channel in a comparable manner as native NTX (Fig. 2). Washing of the corresponding toxin bound to the channels allowed a recovery of more than 90% of the control current (Fig. 2, inset), indicating that toxin binding is reversible. Mutants K6A, T8A, K28R, K28E and ΔYNN showed a lower blocking capacity compared to native or recombinant NTX (Fig. 2). Some mutants behaved differently in the electrophysiological assay with $K_v1.1$ channel, and in the synaptosome system. Mutants K6 and T8 were less potent blockers of $K_v1.1$ than the native or recombinant NTXs, whereas in the synaptosome binding assay they showed no difference. The T8 mutant displayed a higher blocking activity than K6 (Fig. 2). This result suggests that position K6 represents one of the critical interactions of NTX with $K_v1.1$ channel, which is consistent with our previous work [19,20].

The mutant K28R, on the oocyte expressed K^+ channels, displayed a lower blocking capacity, whereas in the synaptosome system there was no difference with regard to native NTX, indicating that the side chain of K28 is critical for blocking channels of the $K_v1.1$ type. However, the apparent contradiction of the results obtained with the K28R mutant and the N-terminal mutants of NTX1-9 on synaptosome membranes, is solely confirmatory that for valid comparisons we need to have defined channels with defined toxins. Much work still remains to be done, using both natural and recombinant mutant toxins with respect to different channels, to obtain information about the structure of the channels using toxin-channel interactions. Mutants K28E and ΔYNN showed equivalent effects in both systems, indicating that K28 and the carboxyl end of NTX are important for binding to both rat brain K^+ channels and mouse $K_v1.1$ channels. Similar results were obtained for this critical lysine residue in ChTX and AgTX [11,12]. In conclusion, residues K6, T8, K28 and tripeptide YNN37–39 are important for binding to $K_v1.1$ channel. Equivalent K28 residues in other scorpion toxins have been shown to be one of the most critical residues for the interaction of these toxins with their respective channels [11,23]. The carboxyl end (YNN) deletion mutant also showed an important reduction in its blocking capacity towards $K_v1.1$ channel, whereas the amidated or non-amidated forms of N39 are irrelevant for the effect in both brain and oocyte assays. A synthetic homolog toxin (Margatoxin, MgTX, 79% homology), in which the last three residues of the carboxyl terminus were deleted, exhibited a 7500-fold decrease in its relative affinity to Jurkat cell membranes, which are enriched in $K_v1.3$ channels [24]. It has been shown that the carboxyl terminus is important in Charybdotoxin for channel binding [25]. These results indicate that K28 and the carboxyl end (YNN) in NTX are important both for binding to synaptosome membranes and for blocking $K_v1.1$ channel. When the three-dimensional models of ChTX and AgTX are compared to NTX (data not shown), it can be observed that residues at equivalent positions to Y37 and N39 of NTX, are present in ChTX and AgTX. These residues have been shown to be critical for channel blocking [11,12]. If the three-dimensional models of scorpion toxins that block K^+ channels are compared to anemone K^+ channel toxins whose three-dimensional structure are known (data not shown), it also can be observed that functionally equivalent residues to K28 and carboxyl end residues of NTX (in particular Y37), are found in the anemone toxins at approximately the same spacial location, despite the fact that the three-dimensional foldings of scorpion and anemone toxins are quite different [26]. These observations indicate a functional convergence between scorpion toxins and anemone toxins that block K^+ channels and probably a recurrent theme in other animal K^+ channel blockers.

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