Eur Biophys J (2001) 29: 569–573
DOI 10.1007/s002490000101

ARTICLE

A. Frau · M. Pisciotta · G.B. Gurrola
L.D. Possani · G. Prestipino

Synthetic undecapeptide (NTX10–20) of noxiustoxin blocks completely the I_A potassium currents of cerebellum granular cells

Received: 28 March 2000 / Revised version: 3 August 2000 / Accepted: 3 August 2000 / Published online: 14 November 2000

Abstract Native noxiustoxin (NTX) and synthetic peptides corresponding to its primary sequence, from positions 1–9, 1–14, 1–20, 10–20, 21–39 and 30–39, were prepared and assayed on the K^+ currents of cerebellum granular cells, using the patch-clamp technique in the whole-cell configuration system. Native toxin has a reversible inhibitory effect ($IC_{50} = 360$ nM), whereas synthetic peptides NTX1–20 and NTX1–9 had a half-effective dose IC_{50} of approximately 2 and 10 μM, respectively, which correlates with their biological effects in vivo. Synthetic peptide NTX10–20 was quite remarkable in having a preference for the I_A current, which was completely inhibited at high peptide concentration. The effects of the other peptides (NTX1–14, NTX21–39 and NTX30–39), although positive and reversible, required higher concentrations (50–200 μM) to block both currents, suggesting no affinity or, at least, much lower specificity for the channels responsible for the potassium currents in the granular cells studied.

Key words Granular cells · Noxiustoxin · Synthetic peptides · Scorpion toxin · Potassium ion channels

A. Frau · M. Pisciotta · G. Prestipino (✉)
Istituto di Cibernetica e Biofisica, CNR,
Via De Marini 6, 16149 Genoa, Italy
E-mail: presti@icb.ge.cnr.it
Tel.: +39-10-6475575
Fax: +39-10-6475500

M. Pisciotta
N.B.T. Group, Department of Biophysical and
Electronic Engineering, University of Genoa,
16145 Genoa, Italy

G.B. Gurrola · L.D. Possani
Instituto de Biotecnologia,
National Autonomous University of Mexico,
Avenida Universidad 2001,
Cuernavaca 62210, Mexico

Introduction

Natural ligands capable of affecting K^+ ionic currents in biological membranes comprise an important group of peptides, extracted from scorpions, spiders, snakes and marine animals (for review, see Garcia et al. 1997). The most widely known are peptides obtained from scorpion venoms, among which is noxiustoxin (here abbreviated NTX), the first such peptide described in the literature (Carbone et al. 1982; Possani et al. 1982). NTX is a 39 amino acid residue peptide, isolated from the Mexican scorpion *Centruroides noxius*, which affects K^+ channels of various tissues (for review, see Possani et al. 1999b). Several peptides corresponding to the primary structure of noxiustoxin were synthesized and assayed for effects in different biological preparations (Gurrola et al. 1989; Gurrola and Possani 1995). A synthetic gene containing the nucleotide sequence encoding NTX and various mutants were also prepared and assayed in synaptosomal membranes and on K^+ channels of the Kv1.1 sub-type, expressed in *Xenopus laevis* oocytes (Martinez et al. 1998). The work, performed with synthetic peptides corresponding to the amino acid sequences of NTX and mutants prepared by genetic engineering and expression in *Escherichia coli*, showed that several segments corresponding to the primary structure of NTX are important for channel recognition, binding or toxicity in vivo (Gurrola et al. 1989; Martinez et al. 1998).

In this paper we report the effect of several synthetic peptides corresponding to the amino acid sequence of NTX1–9, 1–14, 1–20, 10–20, 21–39 and 30–39 on K^+ currents of granular cells from the cerebellum (Stuart et al. 1989). The most remarkable finding is that the constants for current inhibition in granular cells showed some correlations with toxicity in vivo, and that the segment corresponding to the α-helix region of NTX (Dauplais et al. 1995), from residue 10 to 20, is capable of completely inhibiting the I_A currents of granular cells, whereas much less effect was observed on the delayed rectifier (I_d) section of the K^+ currents.

Material and methods

Peptide synthesis and characterization

Peptides containing the amino acid sequence of various segments of NTX (the numbers correspond to the position of the amino acid sequence selected for synthesis) were prepared: NTX1–9, 1–14, 1–20, 21–39 and 30–39, by the solid phase peptide synthesis method of Merrifield (1963), using *tert*-butoxycarbonyl (t-BOC) protected amino acids, as described (Gurrola et al. 1989). For NTX10–20, Fmoc-amino acids (9-fluorenylmethoxycarbonyl derivatives) were used for the synthesis. This last peptide was prepared with its C-terminal amidated, in order to mimic the amide bond of the peptide in the native toxin. For control purposes a synthetic peptide corresponding to the amino acid sequence from position 1 to 11 of toxin Cn4, a sodium-channel-specific toxin not related to K^+ currents, was also prepared. After synthesis the peptides were purified by high performance liquid chromatography (HPLC), using a C18 reverse phase column, eluted with a gradient containing 0.12% trifluoroacetic acid (TFA) in water to various concentrations of acetonitrile (Gurrola et al. 1989; Gurrola and Possani 1995). The acidic medium helps maintain the thiol groups reduced, thus decreasing the possibility of disulfide bridge formation. The correct sequence was confirmed by automatic Edman degradation methodology, using a Beckman-Porton LF3000 Protein Sequencer (Fullerton, Calif., USA).

Cell culture

Experiments were performed on cerebellum granular cells in primary culture obtained from 8-day-old Wistar rats. Dissociated cell cultures were prepared by trypsin digestion and mechanical trituration, following the procedure of Levi et al. (1984). Cells were plated at a density of 2.5×10^6 per dish, on 35 mm plastic dishes or on glass coverslips, coated with 10 mg/mL poly-L-lysine and kept at 37 °C in humidified 95% air/5% CO_2 atmosphere. Experiments were performed 5–10 days after plating.

Patch-clamp measurements

Ionic currents were recorded in whole-cell patch-clamp technique configuration (Hamill et al. 1981), as described earlier by our group (Pisciotta et al. 1998). Patch pipettes were made from borosilicate glass (Clark Electromedical Instruments) and fire polished to obtain resistances between 2 and 4 MΩ. Cell responses were amplified and filtered at 2 kHz by an AxoPatch-1D (Axon Instruments). The whole-cell currents, elicited by 150–200 ms-long voltage steps between −60 to 80 mV from −50 and −80 mV holding potentials (HP), were acquired at a sampling time of 200 μs. The capacitive transient component and the series resistance (7 ± 2 MΩ) of the recorded currents were analog-compensated. P/4 leakage subtraction was performed on-line. Data were stored on a hard disk for subsequent analysis. The composition of the pipette filling solution was the following (in mM): 90 KF, 30 KCl, 2 $MgCl_2$, 2 EGTA, 5 NaCl, 10 HEPES, 30 glucose, pH 7.35. The external standard solution, designed to suppress Na^+ and Ca^{2+} currents, was (in mM): 135 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 0.3 tetrodotoxin, 0.2 $CdCl_2$, 10 HEPES, 10 glucose, pH 7.35. Purified peptide was directly added in the chamber containing 200 μL of external solution. All the experiments were carried out at room temperature (23 ± 2 °C).

Pharmacology

The two main components of the outward K^+ currents, present on new-born rat cerebellar granule cells, were separated by holding potential and by the classical K^+ channel blockers at over-concentration. Inhibition of the transient component was obtained at the HP of −50 mV in the presence of 10 mM 4-aminopyridine (4-AP), while the inhibition of the non-inactivating current occurred at the HP of −80 mV and 20 mM of tetraethylammonium ion (TEA) in the bath.

Data analysis and fit

Voltage-clamp protocols and data acquisition were controlled by a 486 computer interfaced to a 16-bit AD/DA converter (DigiData 1200 using pClamp 6.0, Axon Instruments). Data analysis and curve fitting were conducted using IGOR Pro v. 3 software (WaveMetrics). The experimental points of dose-response curves versus peptide concentrations were fitted to the Michaelis-Menten (MM) and to the Hill equations. The MM equation, $I/I_{max} = 1/(1 + IC_{50}/C)$, where I is the peak of the current, I_{max} is the peak of the saturating peptide current, C is the concentration of peptide and IC_{50} is the half effective dose, has been used to fit the experimental points of peptides affecting the channels with a simple mechanism, by which a single molecule interacts with the channel. The MM equation represents the particular case of the Hill equation, $I/I_{max} = C^n/(C^n + IC_{50}{}^n)$, with $n = 1$. In the case of the NTX 10–20 peptide described in the text, the Hill coefficient, n, represents the number of molecules interacting with the channel.

Results and discussion

As earlier discussed (Gurrola and Possani 1995), the synthesis of peptides containing free thiol groups always poses problems of correct folding. In the present work the peptides were purified in acidic conditions to avoid prompt thiol oxidation and were immediately freeze-dried. We have no direct evidence for the presence of mixed disulfide bridges or dimerization of samples. Additionally, the HPLC profile was compatible with the presence of a homogenous sample, and, at least concerning the chemical characterization, the amino acid sequence obtained in the microsequencer indicated that the sequences were those expected. Figure 1 shows the fragments of the primary structure corresponding to each of the peptides used for this work. The rationale used for selecting some of these peptides is based on previous results obtained with other systems, by our group (Gurrola et al. 1989; Martinez et al. 1998). The peptide NTX10–20 corresponds to the α-helix segment, as can be verified by inspection of the three-dimensional model of NTX (Dauplais et al. 1995). This structural

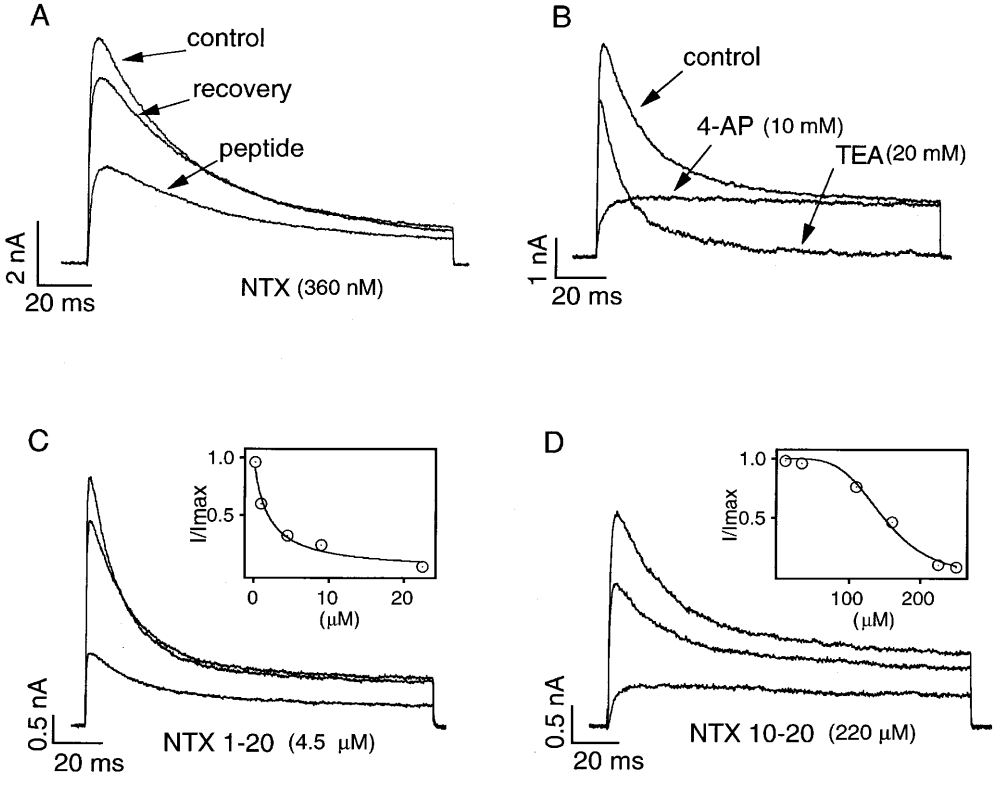
```
          1         10         20         30
NTX       TIINVKCTSP KQCSKPCKEL YGSSAGAKCM NGKCKCYNN
NTX1-9    TIINVKCTS
NTX1-14   TIINVKCTSP KQCS
NTX1-20   TIINVKCTSP KQCSKPCKEL
NTX10-20            P KQCSKPCKEL*
NTX21-39                       YGSSAGAKCM NGKCKCYNN
NTX30-39                                M NGKCKCYNN
Cn4       KEGYLVNSYT G
```

Fig. 1 Amino acid sequence of NTX and synthetic peptides. The *numbers* on top of the sequences correspond to the position of the amino acids in the primary structure of NTX. The various synthetic peptides corresponding to segments of NTX are indicated by the corresponding numbers. The *asterisk* on peptide NTX10–20 means that the leucine amino acid was amidated, in order to mimic the amide bond of this peptide in NTX. The undecapeptide Cn4 corresponds to toxin 4 from *Centruroides noxius*, a sodium-channel-specific toxin used as control

feature of NTX seems to be important for binding to synaptosome membranes, as shown by a decrement of affinity by a factor of 1.5 order of magnitude when the glutamyl bond situated at position 19 was cleaved with protease V8 (Gurrola and Possani 1995). Thus, it was important to test if a synthetic peptide corresponding to the α-helix alone was capable of recognizing the channels. Additionally, the work performed earlier with synthetic peptides of NTX showed that the peptides corresponding to the N-terminal region, from amino acids at positions 1 to 9 and 1 to 20, per se, were toxic to mice (Gurrola et al. 1989). However, in the NTX mutants (Martinez et al. 1998) the presence of Lys28, equivalent to Lys27 in charybdotoxin (see reviews by Hidalgo and McKinnon 1995; Garcia et al. 1997; Possani et al. 1999b) was crucial for recognition of Kv1.1 channels, but this position mutated to Arg28 had no apparent effect on binding and displacement experiments using radiolabeled ^{125}I-NTX in mouse brain synaptosomal membranes (Martinez et al. 1998). Also, the last three residues of the molecule (Tyr-Asn-Asn) are very important for channel recognition and function in both preparations: synaptosomes and Kv1.1 channels (Martinez et al. 1998). Thus, the recognition, affinity and specificity of the mutants and synthetic peptides depends on the type of channel assayed.

Here, we report the results obtained with the synthetic peptides shown in Fig. 1, on the K^+ currents from cerebellum granular cells. In the granular cells system used for this work there are several types of K^+ channels with different kinetics and pharmacology, as previously described (Pisciotta et al. 1998). Using the patch-clamp technique in the whole-cell configuration system, two outward potassium currents were characterized (see traces labeled control in Fig. 2A and B). The first is a fast transient, low-voltage activated current (I_A) blocked by 4-AP at 10 mM concentration, which decays to a steady-state current level in about 100 ms owing to the second component (I_d), which is completely blocked by 20 mM TEA in Fig. 2B. The latter component has electrophysiological and pharmacological properties similar to the classical squid axon K^+ channels (Robello et al. 1989). The inhibition of K^+ currents on cerebellum granular cells is shown in Fig. 2A, for the native toxin, Fig. 2B for 4-AP and TEA, Fig. 2C for peptide NTX1–20 and Fig. 2D for NTX10–20. As can be observed, NTX blocks both currents, but the first one

Fig. 2A–D Inhibition of K^+ currents of granular cells by NTX and its synthetic peptides. Whole cell currents of individual granular cells, elicited by a voltage step of +40 mV from a holding potential of −80 mV, were registered under the effect of native NTX (**A**), classical K^+ channel blockers (**B**) and synthetic peptides (**C** and **D**). **A** A control record is indicated where the immediate uprise of the current corresponds to the I_A currents (see Results and discussion). In the presence of 360 nM native NTX the current is depressed by half (approximate IC_{50} value), which is almost entirely recovered after washing the preparation with buffer free of toxin. **B** Blocking effect of 10 mM 4-AP and 20 mM TEA. **C** Effect of 4.5 μM NTX1–20 on the same currents. Both currents are inhibited (see IC_{50} values in Table 1). The *inset* shows a good Michaelis-Menten curve fit when plotting the fraction blocked as a function of peptide concentration. **D** Similar recordings in the presence of 220 μM NTX10–20. Here the entire I_A currents are inhibited, but the *inset* shows a graph which follows the Hill equation, with a Hill coefficient n calculated as 4 ± 1. The blockage is partially reversible



(I_A) much better, with a k_d of 360 nM (see Table 1). The blockage is reversible. The synthetic NTX1–20 also blocks both K^+ currents in a reversible manner, and furthermore the k_d is relatively low, compared with the others (Table 1). Also, for this peptide, as shown in the inset of Fig. 2C, the graphics fit well to a MM-type of curve, suggesting that only one chemical form of NTX1–20 is binding to the channel. This is a pertinent result because, as already mentioned, the possibility of disulfide formation among the cysteine residues of the synthetic peptide could lead to the formation of inhomogeneous samples.

Gurrola and Possani (1995) showed that a synthetic peptide NTX1–21, containing alanines instead of cysteines, did not modify the capability of the peptide to displace the binding of radiolabeled NTX to brain synaptosome membranes. However, in the present work, one of the most interesting results is the blocking effect of the synthetic peptide NTX10–20 (Fig. 2D), which at high concentrations, with a k_d of 148 μM, seems to be highly efficient and relatively specific for the blockage of the I_A type of K^+ currents in granular cells. Even more interesting is the finding that the blockage fits a Hill equation with a Hill coefficient of 4 ± 1 (see inset of Fig. 2D). This would suggest that either four different chemical forms of the peptide are binding to one channel molecule, or that four peptides are binding to each of the four subunits of the K^+ channel molecule. Several possibilities are now being investigated by means of novel synthetic peptides, designed to solve these questions. One of them consists in the synthesis of NTX10–20 with alanines replacing the cysteines, and another the synthesis of a covalent tetrameric form of the peptide.

The blockade of the I_A currents by addition of peptide NTX10–20 (Fig. 1D) shows that it is a better blocking agent than 4-AP, which is needed in at least one order of magnitude more concentrated for the same biological effect. Furthermore, as can be observed in Table 1, the k_d values for both the synthetic peptides NTX1–9 and NTX1–20 are much lower (which means they have higher affinity) than the others (NTX1–14, NTX21–39 and NTX30–39). This correlates with the toxicity results earlier found for these two peptides (NTX1–9 and NTX1–20) in the in vivo experiments with mice (Gurrola et al. 1989) and with the Ca^{2+}-dependent K^+ channels of low conductance from aortic membranes (Vaca et al. 1993). The unrelated undecapeptide $Cn4_{1–11}$, prepared according to the sequence of toxin Cn4, specific for sodium channels (Possani et al. 1999a) and used at high concentration (over 100 μM) as an internal control, had no effect on the K^+ current of granular cells (not shown).

Table 1 Inhibition constants (IC_{50}) of synthetic peptides on granular cells. Each figure represents the mean value (± SD) of 4–8 experiments

Peptide	I_A (μM)	I_d (μM)
Native NTX	0.36 ± 0.05	1.6 ± 0.4
NTX1–9	11 ± 3	4 ± 1
NTX1–14	61 ± 7	53 ± 24
NTX1–20	1.8 ± 0.5	2.0 ± 0.8
NTX10–20	148 ± 4	–[a]
NTX21–39	44 ± 2	40 ± 10
NTX30–39	55 ± 19	59 ± 18
$Cn4_{1–11}$	No effect	No effect

[a] Value not detectable owing to the high peptide concentration required to reach full inhibition

However, what is the real significance of the present results? What does it mean in terms of channel recognition? It is obvious that not only Lys28 and its surroundings (Martinez et al. 1998) of NTX are important for recognition and binding to K^+ channels. It is clear that other crucial residues are implicated in the affinity of NTX to K^+ channels, and that residues situated in different segments of the three-dimensional space of the molecule are important for channel recognition, and can interact with different segments of the K^+ channels. Definitively, the use of synthetic peptides looks promising to obtain a better view of the toxic peptide-channel interactions.

Acknowledgements The authors are indebted to Dr. Fernando Zamudio, Mr. Fredy Coronas and Mr. Gaggero Giacomo for technical assistance. This work was partially supported by grants: CNR/CONACyT for international cooperation to G.P. and L.D.P., and grant 75197-527107 from the Howard Hughes Medical Institute to L.D.P.

References

Carbone E, Wanke E, Prestipino G, Possani LD, Maelicke A (1982) Selective blockage of voltage-dependent K^+ channels by a novel scorpion toxin. Nature 296: 90–91

Dauplais M, Gilquin B, Possani LD, Gurrola-Briones G, Roumestand C, Menez A (1995) Determination of the three-dimensional solution structure of noxiustoxin: analysis of structural differences with related short-chain scorpion toxins. Biochemistry 34: 16563–16573

Garcia ML, Hanner M, Knaus HG, Koch R, Schamalhofer W, Slaughter RS, Kaczorowski GJ (1997) Pharmacology of potassium channel. Adv Pharmacol 39: 425–471

Gurrola GB, Possani LD (1995) Structural and functional features of noxiustoxin: a K^+ channel blocker. Biochem Mol Biol Int 37: 527–535

Gurrola GB, Molinar-Rode R, Sitges M, Bayon A, Possani LD (1989) Synthetic peptides corresponding to the sequence of noxiustoxin indicate that the active site of this K^+ channel blocker is located on its amino-terminal portion. J Neural Transm 77: 11–20

Hamill OP, Marty A, Sakmann B, Neher E, Sigurth FT (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391: 85–100

Hidalgo P, McKinnon R (1995) Revealing the architecture of a K^+ channel pore through mutant cycles with a peptide inhibitor. Science 268: 307–310

Levi G, Aloisi M, Ciotti M, Gallo V (1984) Autoradiographic localization and depolarization-induced release of acidic amino acids in differentiating granule cells cultures. Brain Res 290: 77–86

Martinez F, Munoz-Garay C, Gurrola GB, Darszon A, Possani LD, Becerril B (1998) Site directed mutants of noxiustoxin reveal specific interactions with potassium channels. FEBS Lett 429: 381–384

Merrifield BR (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 85: 2144–2154

Pisciotta M, Coronas ID, Possani LD, Prestipino G (1998) The *Androctonus australis* garzoni scorpion venom contains toxins that selectively affect voltage-dependent K^+ channels in cerebellum granular cells. Eur Biophys J 27: 69–73

Possani LD, Martin BM, Svendsen I (1982) The primary structure of noxiustoxin: a K^+ channel blocking peptide from the venom of the scorpion *Centruroides noxius* Hoffmann. Carlsberg Res Commun 47: 285–289

Possani LD, Becerril B, Delepierre M, Tytgat J (1999a) Scorpion toxins specific for Na^+ channels. Eur J Biochem 264: 287–300

Possani LD, Selisko B, Gurrola GB (1999b) Structure and function of scorpion toxins affecting K^+-channels. Perspect Drug Discovery Des 15/16: 15–40

Robello M, Carignani C, Marchetti C (1989) A transient voltage-dependent outward current in cultured cerebellar granules. Biosci Rep 9: 451–457

Stuart GC-C, Marshall CG, Ogden D (1989) Voltage-activated membrane currents in rat cerebellar granule neurones. J Physiol (London) 414: 179–199

Vaca L, Gurrola GB, Possani LD, Kunze D (1993) Blockade of a K_{Ca} channel with synthetic peptides from noxiustoxin: a K^+ channel blocker. J Membr Biol 134: 123–129