Tc1, from *Tityus cambridgei*, is the first member of a new subfamily of scorpion toxin that blocks K^+-channels

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Abstract A new peptide, Tc1, containing only 23 amino acids closely packed by three disulfide bridges was isolated from the Amazonian scorpion *Tityus cambridgei*. It blocks reversibly the *Shaker* B K^+-channels with a K_d of 65 nM and displaces binding of noxiustoxin to mouse brain synaptosome membranes. It is the shortest known peptide from scorpion venom that recognizes K^+-channels and constitutes a new structural subfamily of toxin, classified as alphaKTx 13.1.

Key words: Scorpion toxin; K^+-channel; *Tityus cambridgei*

1. Introduction

Scorpion venoms are rich sources of peptides with a variety of pharmacological functions, especially those that affect membrane permeability for Na^+, K^+, Ca^{2+} and Cl^- of excitable and non-excitable cells (review [1]). They have been excellent tools to study ion-channel structure and function [2]. About 50 different peptides from scorpion venoms were recently revised and classified within 12 subfamilies, based mainly on structural similarities [3]. All these peptides have between 30 and 40 amino acid residues and are compacted by either three or four disulfide bridges, having in common a segment of α-helix and two or three anti-parallel β-sheet secondary foldings, maintained by two disulfide bridges established between a stretch of amino acid sequence CXC and CXXXC (C, stands for cysteine and X for any amino acid) that maintain the three-dimensional structure and are essential for channel recognition [4]. The venom of the Amazonian scorpion *Tityus cambridgei* is practically unknown, except for a short abstract of our group [5]. Here we describe the first complete amino acid sequence of a toxin from this scorpion, that turned out to be a novel structural class of peptide, containing only 23 amino acids, but effective in displacing the binding of noxiustoxin (NTX) to mouse brain synaptosomal membranes and was shown to be an excellent blocker of the *Shaker* B K^+-channel.

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2. Materials and methods

2.1. Chemicals, venom and purification procedure

All chemicals were analytical grade reagents, obtained from sources already described [6,7]. Scorpions of the species *T. cambridgei* were collected in four different regions of the Amazonas in Brazil (Balbina, Amazonas state; Ilha Para, Para state; Samuel, Roraima state; Santana, Amapa state). Fresh venom was collected from these animals, by electrical stimulation, at the Butantan Institute (São Paulo, Brazil) and air-dried. The crude venom was dissolved in double distilled water and spun at $10\,000 \times g$ for 15 min. The soluble part of the venom was separated by high performance liquid chromatography (HPLC), using a semi-preparative C18 reverse-phase column (Vydac, Hisperia, CA, USA). Several independent runs with 2 mg each of soluble venom were separated and the corresponding components were mixed. Pool of subfractions were further separated in an analytical C18 reverse-phase column (same company) using a different gradient, as indicated in the figure legend.

2.2. Amino acid sequence

The amino acid sequence was performed by automatic Edman degradation in a Beckman LF 3000 protein sequencer (Palo Alto, CA, USA), using the chemicals and procedure previously described [6]. Samples of approximately 1 nmol each, both of native toxin and reduced and alkylated peptide were applied to the sequencer.

2.3. Mass spectrometry determination

The molecular masses were obtained on a Voyager DE-PRO (PerSeptive Biosystems, Framingham, MA, USA) equipped with a nitrogen laser (337 nm), as described elsewhere [8].

2.4. Binding assays

NTX, a well known K^+-channel blocker isolated from the venom of the scorpion *Centruroides noxius* (see review [9] was radiolabeled with ^{125}Iodine [10], and used for binding experiments. Mouse brain synaptosome membranes were prepared and used for binding assays as also described [10].

2.5. Electrophysiological measurements

Insect Sf9 cells were grown at 27°C in Grace's media (Gibco BRL). The cells were infected with a recombinant baculovirus containing the cDNA of *Shaker* B K^+-channels [11]. Electrophysiological recordings were done 48–72 h after the infection, as previously reported [12]. Macroscopic currents were recorded with the whole cell configuration of the patch-clamp technique [13], with an Axopatch 1D (Axon Instruments, Inc.). The currents were filtered at 5 kHz and sampled every 100 μs with a TL1 interface (Axon Instruments, Inc.). The electrodes were pulled from borosilicate glass (KIMAX 51) to a 1.5 MOhm resistance, and 80% of the series resistance was electronically compensated. The holding potential used throughout the work was −90 mV. The solutions used were: external bath (in mM): 145 NaCl, 10 Ca_2Cl, 10 HEPES–Na at pH 7.2; pipet internal solution (in mM): 90 KF, 30 KCl, 10 EGTA, 10 HEPES–K at pH 7.2.

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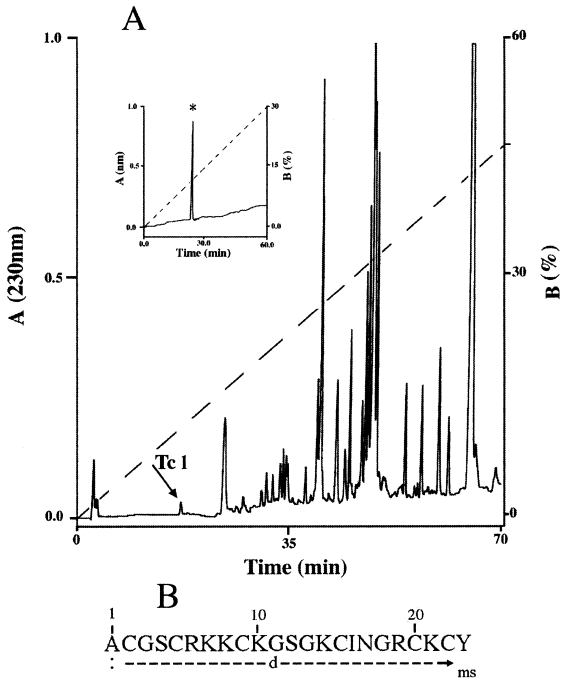
3. Results and discussion

Fig. 1 shows the chromatographic profile of *T. cambridgei* venom separated in a semi-preparative column. More than 60 different components are partially distinguishable in this chromatogram (Fig. 1a). In order to easily assess possible functions of these components, the binding and displacement experiments using ^{125}I-NTX and mouse brain synaptosome preparations [10] were used as a starting assay. The first visible component, eluting at 21 min in the linear gradient from 5 to 60% acetonitrile for 90 min, corresponded to a peptide that displaced the binding of ^{125}I-NTX to brain synaptosomal membranes. The samples corresponding to this elution time from 10 independent runs were pooled and further applied to an analytical C18 reverse-phase column, given a major component, labeled with an asterisk in the inset of Fig. 1a. This peptide was used for sequence determination and additional functional analysis. The pure component corresponds to about 0.5% of the soluble venom. The automatic amino acid sequence of the native peptide gave an unique sequence up to residue 22, with blank spaces, possibly corresponding to cysteinyl residues. A sample of the peptide was reduced and alkylated in situ with *N*-buthylphosphine and acrylamide, following the Beckman manufacturer instructions, and sequenced. The alkylated peptide gave unequivocal results for the first 22 amino acid residues, as indicated in Fig. 1B. The last residue Tyr23 was surmised based on molecular mass determination. The experimental value obtained for the native peptide was 2446.4, very closely to the theoretical monoisotopic mass of 2446.2 for the sequence shown in Fig. 1B. The sum of the masses of the first 22 amino acids was 2283.06, thus the missing amino acid should have a mass of 163, corresponding exactly to the mass of tyrosine. In the event that a dipeptide could be missing, instead of tyrosine, the closest approximation would be glycine plus cysteine, with a mass of 160, much farther apart from the experimental value found. Furthermore, the molecular mass of the native peptide (2446.4), as obtained from MALDI-TOF, was found to be 2791 after reduction and alkylation of the sample with iodoacetamide (mass +57 per residue), showing that the sample contains six half-cystines, already found by direct Edman degradation analysis. Thus, the peptide Tc1 (from *T. cambridgei*, toxin 1), is a 23 amino acid residue peptide, that contains three disulfide bridges. Comparative analysis of the amino acid sequence of this peptide, with those from the literature, shows that it has a very low similarity to all the other known [3], and requires the designation of a new subfamily of K^+-channel blocking scorpion peptides. The introduction of many gaps in Fig. 1C is needed in order to approach Tc1 to a consensus sequence, when compared to the other 12 pre-existing subfamilies of peptides. Additional structural considerations that support our new classification (Fig. 1C) will be discussed at the end of this section.

The venom of *T. cambridgei* obtained in four different regions of the Amazonian rain forest of Brazil gave a similar eluting component and were also used for sequence and mass spectrometry determination. They all showed the same N-terminal sequence and same molecular mass (data not shown), suggesting that the presence of exactly the same peptide in the venom is constant, regardless of the distance (1000 km apart from each other) in which the *T. cambridgei* were collected.

The physiological effect of Tc1 was further investigated in the Sf9 cell culture system, expressing the *Shaker* B K^+-channel, as shown in Fig. 2A–E. In Fig. 2A a series of control macroscopic K^+-currents through *Shaker* B K^+-channels are shown. The channels were activated by stepping the membrane from −30 to +50 mV in 10 mV increments from the holding potential of −90 mV. The subsequent addition of 0.4 μM Tc1 to the external solution completely abolished the K^+-



C

Subfamily	Consensus
1	---------C--S--C---C---------GKC-----C-C----
2	-----I--KC----QC---CK---G-----KC-NG-KCKCY---
3	---V-I-V-C--S-QC--PCK-A-GMR-FGKC-N--KC-CTP--
4	---V-I---C--SP-C---CK---G-KA-GKC-NG--C-C----
5	---------C-NL--CQLSCRSL-GL--LGKCIG--KCECVKH-
6	---------C----DC--PC---TGC-----C-N---CKC--C-
7	---TI---SCTN-KQCYPHCKKETGYPNA-KCMNR-KCKCFGR-
8	---------C---EDCP-HC-TQ----A-AKCDND-KCVCEP--
9	-------VGC---EECPMHCKGK---NA-PTCD--GVCNCNV--
10	---------C-VYRTCDKDC--R--YR-SGKCINN-ACKCYPY-
11	--DEEPKE-C-SDEMCVIYCKGE-EYS-TGVCDGPQKCKCSD--
12	WCSTCLDLACGASRECYDPCFKAFG-RAHGKCMNN-KCRCYTN-
13	--------ACG-S--CRKKCK---G---SGKCIN-GRCKCY---

Fig. 1. Purification and sequence of Tc1. A: Soluble venom of *T. cambridgei* (2 mg each time) were separated in a preparative C18 reverse-phase column of a HPLC system [12], using a linear gradient, run from 5% buffer A (0.12% trifluoroacetic acid (TFA) in water) to 60% buffer B (acetonitrile with 0.1% TFA) for 90 min, at a flow rate of 2 ml/min. The arrow indicates elution of the active component, rechromatographed in an analytical column (inset) that corresponded to pure Tc1 peptide (labeled with an asterisk). B: Amino acid sequence of Tc1 determined from a reduced and alkylated sample (1 nmol) using the Beckman LF 3000 protein sequencer. The direct sequence was obtained for the first 22 amino acids (underlabeled with: –d–>), and the last residue Tyr23 as determined by mass spectrometry analysis (underlabeled with ms). C: Redrawn consensus comparative analysis taking data of Fig. 2 from our previous publication [3], into which the amino acid sequence of Tc1 was included, to show that it corresponds to a new subfamily, number 13. Gaps (–) were introduced in order to increase identity.

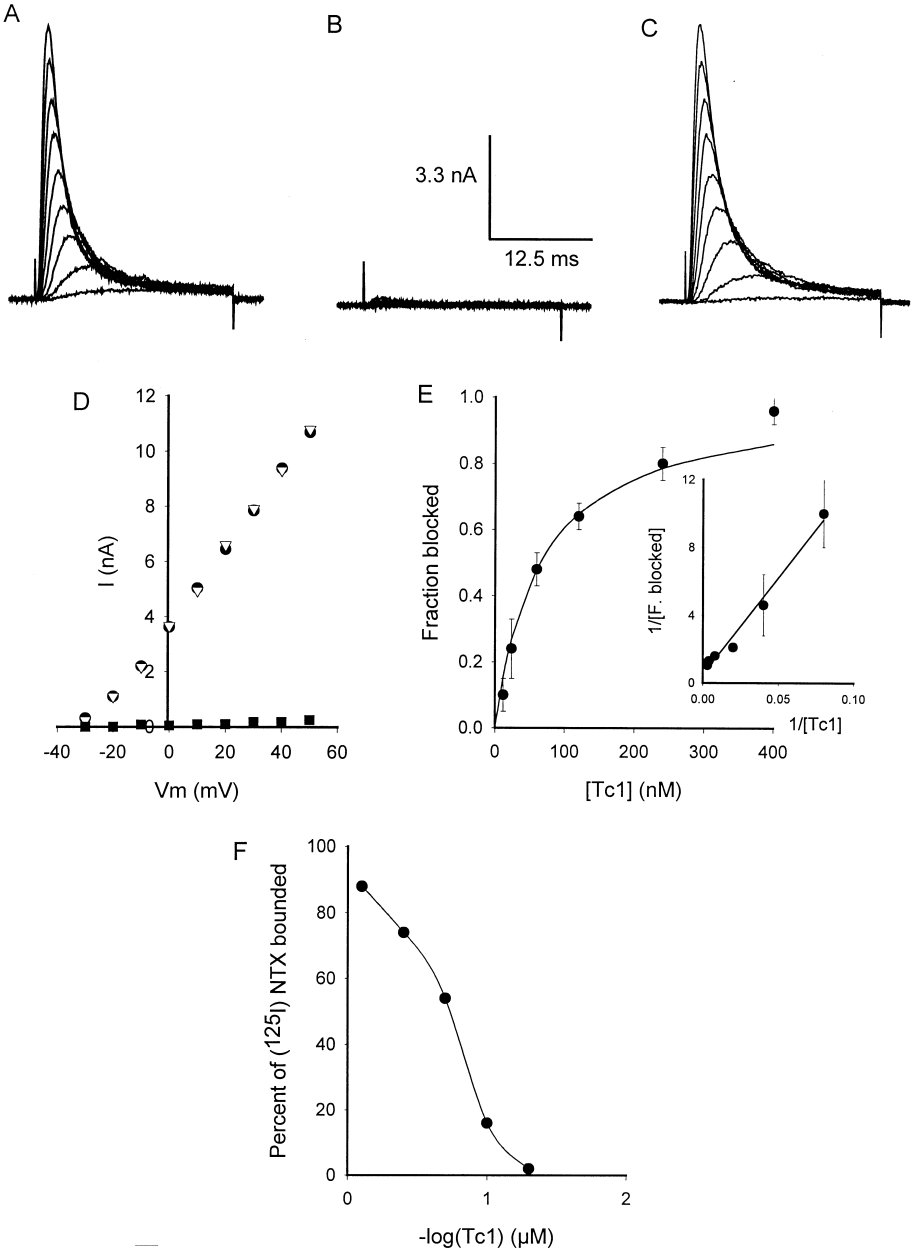


Fig. 2. *Shaker* B K^+channel blockage and NTX displacement. A: Shows the control traces of macroscopic K^+-currents through *Shaker* B K^+-channels. The channels were activated by 30 ms pulses from −30 to +50 mV in 10 mV increments, delivered every 20 s from the holding potential of −90 mV. B: K^+-currents left after the addition of 0.4 μM Tc1. C: Complete current recovery after perfusing the cell with the control external solution. D: Peak-current versus voltage relationship of the traces in A–C; closed circles for control, open triangles after washing and closed squares for toxin effect. E: Fraction blocked = $1-(I/I_0)$, where I_0 is the control peak current, and I is the peak current in the presence of the indicated toxin concentration. Each point is the mean ± S.D. (for three independent experiments). The line is the fit of the points with a Michaelis–Menten equation, $K_d = 65$ nM. The inset is the double reciprocal plot of the points, $r = 0.991$. F: Shows the binding and displacement experiments to mouse brain synaptosome membranes as described [10], using ^{125}I-NTX for binding and cold Tc1, at the concentrations indicated, as competitor. The calculated IC_{50} was in the order of 200 nM.

currents at all voltages (Fig. 2B). The effect is completely reversed by perfusing the cell with the control external solution (Fig. 2C). In Fig. 2D the peak-current versus voltage relationship of the traces is shown. Tc1 blockage is not appreciably voltage dependent, and it does not change the kinetics of the currents (not shown). Fig. 2E shows that Tc1 blockage of *Shaker* follows a Michaelis–Menten saturation relationship with a K_d of 65 nM. Finally in Fig. 2F, a dose–response curve of binding and displacement experiments using radiolabeled NTX (^{125}I-NTX) shows that Tc1 competes with NTX for the binding to the synaptosomal membranes. The IC_{50} value is in the order of 200 nM, which can be considered a low competing affinity for the same channels. Taking together the results of Fig. 2, they show unequivocally that Tc1 is a bona fide K^+-channel blocker. It recognizes not only the *Shaker* B K^+-channel, but also the voltage dependent K^+-channels present in brain, despite its short length.

It should be noted that Tc1 is a very charged molecule,

composed of 30% positively charged residues, 30% hydrophobic amino acids, 26% cysteines and no acidic residues (Asp or Glu).

Finally, it is worth mentioning some additional features that warrant the classification of Tc1 in a new special subfamily of scorpion K^+-toxins, here denominated alphaKTx 13.1, because it is the first member of the subfamily 13. To begin with, it is the shortest of all known sequences of scorpion K^+-channels blockers, that although having the signature sequence CXC, CXXXC (C for cysteine and X for any amino acid) segments tighten with two conserved disulfide bridges, it lacks entirely the N-terminal segment and also lacks a substantial part of the α-helix forming region of other K^+-channel toxins. For example, the three-dimensional structure of NTX (the competing toxin for the assay in Fig. 2F) shows that residues Lys11 to Leu20 are forming three turns of α-helix, whereas Thr1–Asn4, Ala25–Cys29 and Lys33–Tyr37 are involved in anti-parallel β-sheet formation [14]. Between the third and fourth cysteinyl residue of NTX there are 11 amino acids, whereas in Tc1 there are only five. However, the last segment of Tc1, residues Lys14–Tyr23 do correspond considerably to other K-toxins, with overall identities in the order of 30–40%. If the sequences are manually compared (data not shown), introducing gaps to maximize identity and aligning the cysteines in equivalent positions, the percentage of identity with NTX of subfamily 2 (see [2]), gave 41% identity, which is one of the highest scores obtained. For Agitoxin 1 of subfamily 3 the identity was 37%; for toxin TsTxIV of *Tityus serrulatus* (related species), belonging to subfamily 12, it was 36%; for Pi1 of subfamily 6 it was 34% and for BmPO1 of subfamily 8 it was 31%. The identity with all other scorpion toxins is lower than 50%. The essential residue Lys27 in charybdotoxin [2] that corresponds to Lys28 in NTX [2,9] would correspond to Lys14 in Tc1. Nevertheless, Tc1 with half the size of the other peptides has a similar function. It has been shown by side-directed mutagenesis [2,15] that the recognition site of most scorpion toxins blockers specific for K^+-channels reside in the C-terminal segment of the molecules, despite the fact that for some toxins, such as NTX [10], Pi2 and Pi3 from *Pandinus imperator* [16], some residues of the N-terminal region also play a critical role in the recognition and binding to K^+-channels. Here, the structural analysis of Tc1 supports the idea that the C-terminal segment of the peptide is the one showing the highest sequence identity to other K^+-channel blockers and that the N-terminal segment is probably less important. Thus, Tc1 is an excellent candidate for three-dimensional structure determination and site-directed mutagenesis in order to confirm its recognition site towards K^+-channels.

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