A novel K$^+$ channel blocking toxin from *Tityus discrepans* scorpion venom

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Abstract **A novel toxin (TdK1) was purified from the venom of the scorpion *Tityus discrepans*, sequenced and functionally characterized. It contains 37 amino acid residues and blocks reversible the *shaker*B K$^+$ channel expressed in SF9 cells with a K_d in the order of 280 nM. The proposed systematic nomenclature for this peptide is α-KTx4.3.**
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Key words: Amino acid sequence; K$^+$ channel; *Shaker*B; Scorpion venom; *Tityus discrepans*

1. Introduction

Scorpion venoms are the most widely known source of peptides with action on Na$^+$, K$^+$, Cl$^-$ and Ca^{2+} channels (reviews [1,2]). Among all agents that block K$^+$ selective channels, the scorpion K-toxins are particularly useful since most of them affect only K$^+$ channel permeability, with high affinity. For notation purposes, the peptides specific for K$^+$ channels will be called simply K-toxins. Different K$^+$ channels are affected by these toxins: voltage-gated, high- and low-conductance Ca^{2+}-activated potassium channels and Ca^{2+} dependent apamin sensitive K$^+$ channels [3–6]. These toxins occur in minute amounts, usually less than 1% of the venom. They are short, 32–39 amino acid residues, basic peptides cross-linked by three or four disulfide bridges [2,6]. The three-dimensional structure presents a common motif formed by a short segment of α-helix and two or three strands of β-sheet structure [6]. Except for the constant relative positions of the cysteines, most of the amino acids of the primary structure are variable. This variability could be an evolutionary attempt to meet with the diversity of ion channels and with the affinity variations of these toxins towards the members of their corresponding families of channels. Therefore, the discovery and determination of the structure of new specific toxins adds an important tool for physiological studies, where ion channels play a role. This paper reports the complete amino acid sequence, the molecular mass and the physiological effect of a novel peptide isolated from the venom of the South American scorpion *Tityus discrepans* on a voltage dependent K$^+$ channel, expressed in Sf9 cells. It is called TdK1, meaning *T. discrepans* K channel toxin 1, but according to reference [6] the proposed systematic name should be α-KTx4.3.

2. Materials and methods

2.1. Venom source and toxin purification

T. discrepans scorpions kept alive in the laboratory were anaesthetized once a month with CO$_2$ and milked for venom by means of electrical stimulation. The venom was dissolved in double distilled water and centrifuged at $15\,000 \times g$ for 15 min. The supernatant was freeze-dried and stored at $-80°C$ until use. Soluble venom in the amounts of 1 mg each time, was applied to a HPLC system (Waters 600, with a dual wavelength detector model 2487, from Waters, Milford, MA, USA) equipped with a C18 reverse-phase analytical column (Vydac, Hysperia, CA, USA) and eluted at a flow rate of 1 ml/min during 70 min. A linear gradient made of 0.12% trifluoroacetic acid (TFA) in water (solvent A) to 0.10% TFA in acetonitrile (solvent B) was applied to the column. Individual fractions were collected, freeze-dried and stored at $-20°C$ until use. Pure peptides were obtained by applying the sub-fractions to a microbore C18 reverse-phase column using a Waters 625 chromatographer, equipped with a photodiode array detector, model 996, from Waters, Milford, MA, USA.

2.2. Toxin microsequencing and molecular weight determination

Amino acid sequence of pure native peptide TdK1 was determined using an automatic ProSequencer (Beckman LF3000, Fullerton, CA, USA), employing standard Edman degradation programs with the peptide attached to CD-immobilon membranes. To complete the carboxyl-terminal sequence of TdK1, the native peptide was blocked with acetic anhydride at the amino-terminal, cleaved with cyanogen bromide at the methionine in position 29 and sequenced again, using standard procedures. Cysteines were identified using an independent sample carboxymethylated in the Sequencer, using the protocols described by the manufacturer. The exact molecular mass, which permitted the completion of the primary structure, was obtained in a MALDI-TOF [7] mass spectrometer, using a cyano-4-hydroxyconamic acid as the matrix.

2.3. Channel expression, electrophysiology and solutions

Insect Sf9 cells from *Spodoptera frugiperda* were kept in culture at 27°C in Grace's media (GIBCO BRL). The cells were infected with a recombinant baculovirus having the cDNA of *shaker*B K$^+$ channels [8] and were used for the experiments 48 h after the infection, as previously reported [3]. Macroscopic currents were recorded under whole cell patch-clamp [9] with an Axopatch 1D (Axon Instruments, Inc.). The currents were filtered on-line at 5 kHz and sampled every 100 μs. Borosilicate glass electrodes (KIMAX 51) were pulled to a 1.5 MOhms resistance. Eighty percent of the series resistance was electronically compensated. The external control solution was composed of (mM): 145 NaCl, 10 Ca$_2$Cl, 10 HEPES-Na at pH 7.2. The internal solutions was composed of (mM): 90 KF, 30 KCl, 10 EGTA, 10 HEPES-K at pH 7.2.

3. Results and discussion

In a previous publication [10], we showed that the venom of *T. discrepans* contains a K$^+$ channel specific toxin. In this communication, following results obtained with the venom of other scorpions, we selected the HPLC fractions with similar elution time and assayed them for an effect on the *shaker*B

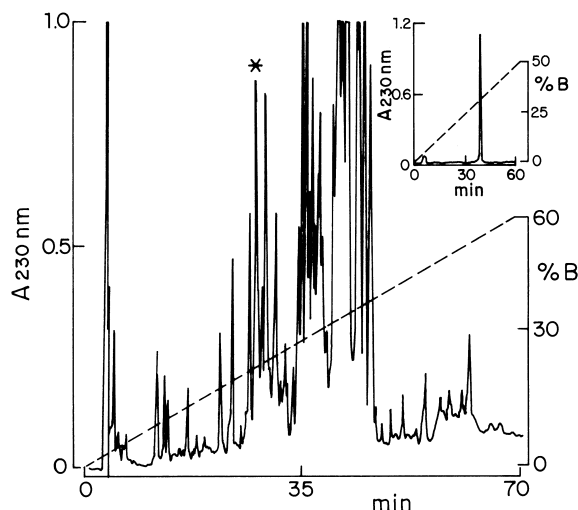*Corresponding author. Fax: (58) (2) 5041093.
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Fig. 1. HPLC separation of soluble venom. Soluble venom of *T. discrepans* (1 mg) was applied to a C18 reverse-phase column and eluted at the flow rate of 1 ml/min for 70 min with a linear gradient of solvent A (0.12% TFA) in water to 60% solvent B (0.10% TFA) in acetonitrile. Component labeled with star corresponds to elution time 27.1 min and was used for further separation in a C18 micro-bore column from 0 to 50% B, for 60 min, as indicated in the inset.

K$^+$ channels. Over 50 distinct components were obtained from the first HPLC separation (Fig. 1). The component eluting at 27.1 min (labeled with asterisk) showed N-terminal amino acid sequence similar to other K-toxins, and when assayed in Sf9 cells proved to be a bona-fide K$^+$ channel blocker. This fraction was then further separated in a micro-bore column, as described in Section 2, from which the main component (see inset Fig. 1) was used for full sequence determination and for the physiological experiments reported.

The homogeneity of the purified peptide was confirmed by the sharp HPLC profile, by amino acid sequencing (only one amino acid residue per cycle), and by mass spectrometry data. The position of the first 33 amino acid residues of TdK1 was determined by direct sequencing of an aliquot of native peptide, and confirmed by sequencing another sample of reduced and carboxymethylated toxin. Residues 34 to 36 were obtained by sequencing the peptide resulted from cyanogen bromide cleavage of an N-terminal acetylated TdK1. Since this cleavage occurred at methionine in position 29, an overlapping peptide (positions 29 to 33) was obtained by this procedure. The last amino acid residue in position 37 could not be determined directly on the Sequencer, but was surmised based on the results of mass spectroscopy. The molecular mass experimentally determined was 3816.8. Evaluation of the mass contributed by the first 36 amino acid residues directly determined gave a value of 3721.79, thus the missing residue should have a mass of 101, corresponding exactly with that

of threonine. Hence, TdK1 is a single polypeptide composed of 37 amino acids, as shown in Fig. 2. The molecular mass is typical of a K-toxin from Buthidae scorpions [6]. Analysis of the amino acid sequences (Fig. 2) shows a 78% identity with TsTxKα [12] (systematic name α-KTx4.1 [6]), a K$^+$ channel blocking toxin from the parent species of the Brazil scorpion *Tityus serrulatus* [12]. Actually, the latter peptide was the first K$^+$ channel specific peptide ever purified from scorpions of the genus *Tityus*, under the name of toxin TsII-9 [13], subsequently renamed TsTxKα, by Rogowski et al. [12]. Only 51% identity was observed when compared to TsK or α-KTx4.2 [6], another K-toxin that competes for the binding of apamin to K$^+$ channels of rat brain synaptosomes [11]. The six cysteines are assumed to be forming three disulfide bonds as the related toxins.

TdK1 added at nanomolar concentration to the extracellular solution reversibly blocks *shaker*B K$^+$ channels, as shown in Fig. 3. The upper frame of Fig. 3A presents a family of control K$^+$ currents recorded by stepping the membrane from −30 to +50 mV in 10 mV increments from the holding potential of −80 mV. Addition of 250 nM toxin to the external solution caused a reduction of the current at all voltages (middle frame traces), that was reversed by perfusing the cell with the control external solution (bottom frame traces).

Toxin addition did not affect the kinetics of the remaining current, this is shown in Fig. 3B where the current in the control at +50 mV in A is shown superimposed with that recorded at the same voltage in the presence of the toxin; the latter multiplied by a factor of 2.32. The reversible, constant, reduction of the current at all voltages caused by the toxin addition is best seen in Fig. 3C, that presents the current versus voltage curve of the traces in A. The above features are characteristic of scorpion toxin block of the pore of voltage dependent K$^+$ channels, and thus indicate that the toxin here reported acts as a typical K-toxin. Fig. 3C shows that the concentration dependence of block follows a Michaelis–Menten relation with a K_d of 282 nM. Thus, TdK1 is a new member of the sub-family 4 of scorpion K-toxins, for which the systematic name should be α-KTx4.3, following suggestions of a recent classification [6].

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                    1        10        20        30        37 References
α-KTx4.3    VFINVKCTGS KQCLPACKAA VGKAAGKCMN GKCKCYT  This work
α-KTx4.2    VVIGQRCYRS PDCYSACKKL VGKATGKCTN GRCDC    [11]
α-KTx4.1    VFINAKCRGS PECLPKCKEA IGKAAGKCMN GKCKCYP  [12]
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Fig. 2. Primary structure of TdK1 (α-KTx4.3). Amino acid sequence of TdK1 obtained by direct Edman degradation of native and alkylated toxin, plus an overlapping segment obtained from cyanogen bromide cleavage and mass spectrometry data. The sequences of TsK (α-KTx4.2) [11] and TsTxKα (α-KTx4.1) [12], isolated from *Tityus serrulatus* scorpion venom are included for comparative purposes. Numbers on top indicate amino acid position in the sequence, and asterisks on the bottom, indicate identical amino acid in all three toxins of this sub-family.
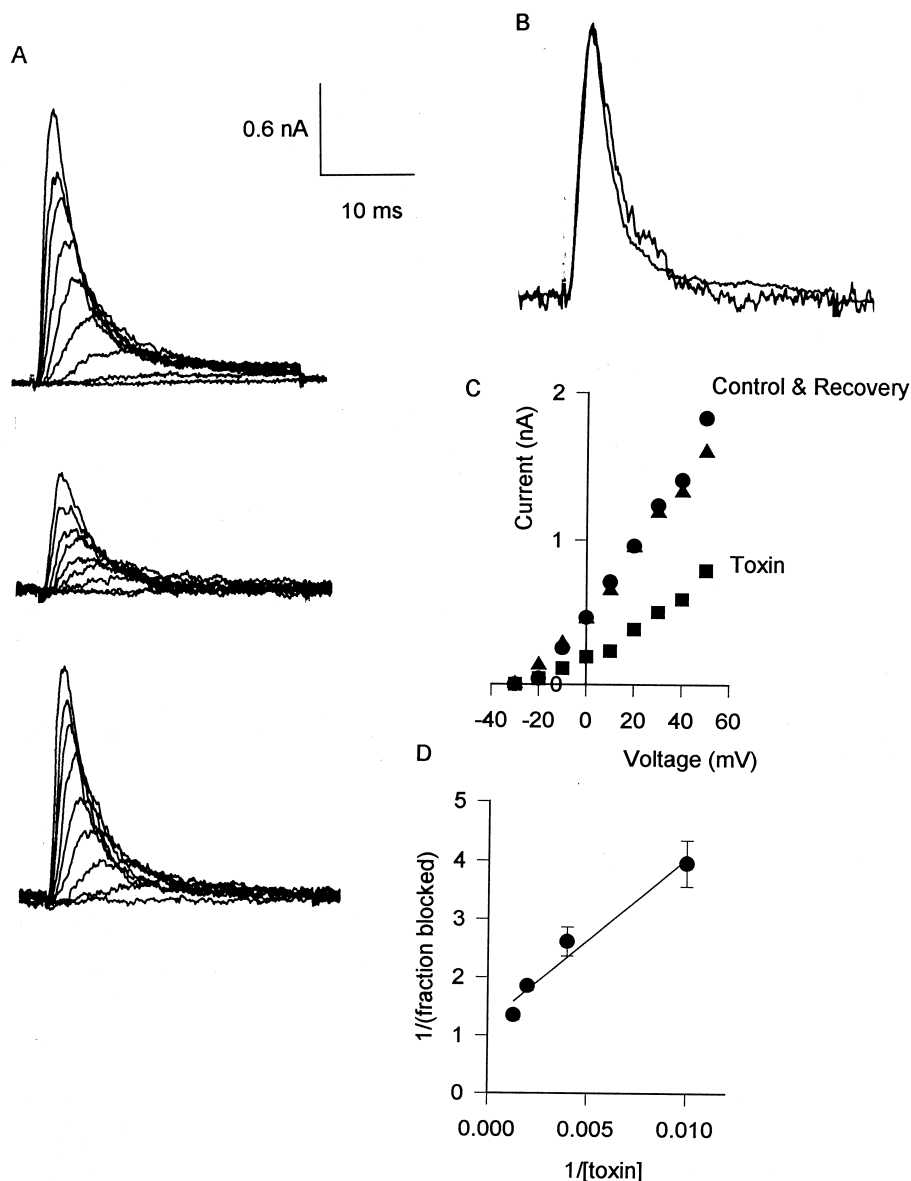
Fig. 3. Effect of TdK1 on the *shaker*B K$^+$ channels. Toxin reversibly blocks *shaker*B K$^+$ channels at nanomolar concentrations. A: The upper traces are control macroscopic K$^+$ currents through *shaker*B K$^+$ channels activated by 30 ms pulses of −30 to +50 mV in 10 mV increments delivered every 20 s from the holding potential of −80 mV. The middle traces are the currents after the addition of 250 nM TdK1 to the external solution. Notice the reduction of the current. The bottom traces are the currents recorded after extensively perfusing the cell with the control external solution. Notice the recovery of the current. B: Current at +50 mV in the control in A superimposed with that at the same voltage recorded in the presence of the toxin in A, scaled by a factor of 2.32. C: Peak current as a function of the pulse voltages of the traces in A. D: Double reciprocal plot of the fraction of blocked channels (fraction blocked = $1 - I/I_0$, where I is the peak current in the presence of toxin and I_0 is the control peak current) against the TdK1. The points are the mean ± S.E.M. of three experiments at each concentration. The line is the least squares fit to the points, $K_d = 282$ nM, $r = 0.982$.

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