Characterization of a new peptide from *Tityus serrulatus* scorpion venom which is a ligand of the apamin-binding site

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Abstract A new ligand (Ts κ) of the apamin binding site on rat brain synaptosomes ($K_{0.5}$ = 300 pM) was purified and characterized from the venom of *Tityus serrulatus*. It is a polypeptide toxin of 35 amino acid residues, with three disulfide bridges. Its cDNA was amplified from a venom gland cDNA library and the nucleotide sequence determined. A model of Ts κ was constructed by amino acid replacement using charybdotoxin structure as determined by ^{1}H nuclear magnetic resonance as starting model.

Key words: Scorpion toxin; cDNA; Amino acid sequence; Apamin; Potassium channels

1. Introduction

Scorpion venoms contain potent neurotoxins which specifically modify ionic channel functions. Electrophysiological, radioactive ion flux and ligand binding studies show that some neurotoxins recognize different binding sites on the voltage-sensitive Na^+ channels of mammals and insects and others recognize various K^+ channels [1]. Toxins active on K^+ channels are minor components of venoms. They are single chain polypeptides of 30–40 amino acid residues, with three disulfide bridges [2]. They are useful tools for investigating the mechanisms of ion conduction, channel selectivity and architecture of the pore region of the K^+ channels [3–5]. In this group, those able to compete with apamin (a neurotoxin from bee venom, 18 residues, two disulfide bridges) to small conductance calcium activated K^+ channels (SKCa) [6] are the smallest described scorpion toxins. Leiurotoxin I (LTX 1) [7,8] and P05 [9] are 31 residues long, with differences at only three positions. ^{1}H nuclear magnetic resonance (NMR) analysis of P05 [10] has demonstrated that these molecules are composed of an α-helix (residues 5–14) connected at a tight turn to a two-stranded antiparallel β-sheet (sequences 17–22 and 25–29). Chemical synthesis of analogs [9,11] and modifications of charged residues [8] shows that three Arg residues, in the solvent-exposed side of the α-helix, constitute a highly positively charged surface involved in the pharmacological activity of P05 or LTX 1. A multipoint interaction with the receptor is likely. A pair of Arg residues is also crucial for the pharmacological activity of apamin [6]. We report the purification from the venom of the South american scorpion *Tityus serrulatus* (Ts) of a new inhibitor of [^{125}I]apamin binding to rat brain synaptosomes. It is a peptide dissimilar to LTX 1 and P05. The corresponding cDNA sequence was determined.

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2. Materials and methods

The Ts venom (0.98 g), obtained from scorpions collected near Santa Barbara, was a gift from Dr. C.R. Diniz, Instituto E. Diaz, Belo Horizonte, M.G., Brazil. Apamin was purchased from Sigma. Kaliotoxin (KTX) was synthesized in the laboratory. Other solvents and reagents were analytical grade commercial products from Sigma and Merck. Na[^{125}I] was from Amersham Corp.

2.1. Purification of Ts κ

The venom was dialysed against distilled water to eliminate salts and small peptides (24 h, 4°C, 1 liter × 5, using Spectrapor 3 membrane with a mol. wt. cut-off < 1000). The HPLC experiments were performed as previously described [12] with a Millipore/Waters Associates system. After gel filtration through Sephadex G-50, the sample was loaded onto a Merck 4 × 250 mm analytical high pressure liquid chromatography (HPLC) column (Lichrospher, 5 μm, 100 RP-18). Solvent A was 0.1% TFA (v/v) in water and solvent B was 0.1% TFA (v/v) in acetonitrile.

2.2. Competition assays using rat brain synaptosomes

Each HPLC fraction was tested in competition assays for binding to rat brain synaptosomal fraction (P_2) with mono[^{125}I]apamin according to [13] or [^{125}I]KTX [12]. To study only ligands exhibiting the highest affinity for the apamin receptor, purified fractions were screened at a final dilution of 5×10^{-5}.

2.3. Chemical characterization

Amino acid composition determinations and automatic sequencing were as described [12].

2.4. Construction of the cDNA library

Standard recombinant DNA techniques were used [14]. Enzymes were from Biolabs. The construction of the venom gland cDNA library λgt 10 (Promega) has been described in detail [15]. The library contained 2.5×10^6 independent phage clones.

2.5. Polymerase chain reaction (PCR)

PCR was used for amplification from the library of the cDNA encoding the Ts κ as previously described [16]. The primers used for PCR amplifications were the following: for the first amplification, reverse primer 1, 5′-CAA/G TCA/G CAC/T CTT/A CCG TTA/C GTA/G CAT TT-3′; forward primer 2, 5′-CT TTT GAG CAA GTT CAG CCT GGT TAA GT-3′; forward primer 3, 5′-T CAA TTC TGA TAA TAC GAA AAC ATG-3′; reverse primer 4, 5′-GAG GTG GCT TAT GAG TAT TTC TTC CAG GGT A-3′. The product with the appropriate size was recovered, purified using the Wizard PCR Prep DNA Purification System (Promega) and reamplified by a second PCR reaction in the same conditions.

2.6. Sequence analysis of PCR products

The purified PCR products were filled in with dNTPs using the Klenow fragment according to standard methods [14], and ligated into the vector pBlueScript SK$^+$ (Stratagene). The recombinant clones were analyzed using standard techniques [14] and sequenced using sequenase (US Biochemicals) and the Sanger dideoxynucleotide chain termination method [14].

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2.7. Modelling

The TURBO-FRODO [17] and X-PLOR 3.1 [18] programs were used for construction and refinement of the model, respectively. The model of Ts κ was deduced from the structure of charybdotoxin (ChTX) in solution previously determined by ^{1}H-NMR [19]. After amino acid residue replacement, the starting model was refined by 10 000 steps of Powell minimizer to correct geometry and Van der Waals as well as electrostatic interactions.

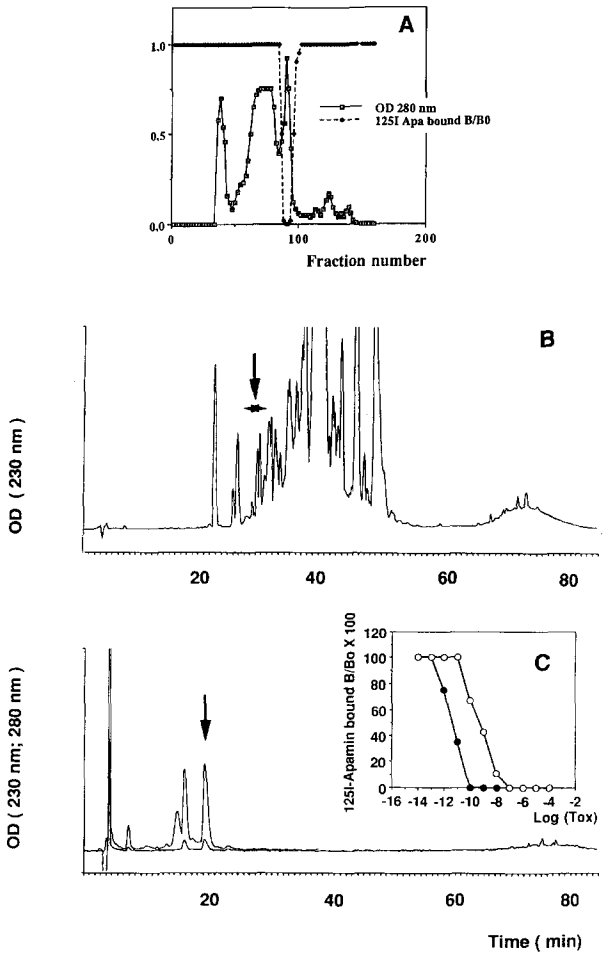

Fig. 1. Purification of Ts κ. (A) Gel filtration through three columns (2.5×100 cm) in series; Sephadex G-50 fine in 30% acetic acid, flow rate: 40 ml/h. The fraction able to compete with [^{125}I]apamin specifically bound to its receptor site on rat brain synaptosomal membranes is indicated with a dotted line. (B) Reverse-phase chromatography of the previous fraction (1/80% of the optical density obtained at 280 nm) in a linear gradient of 5–50% B in A for 60 min and 50–80% B in A for 5 min; flow rate 1 ml/min. 1 optical density unit full scale (OD_UFS) at 230 nm. Arrow indicates the fraction (RT = 31 min) able to compete for [^{125}I]apamin binding. (C) Reverse-phase of the entire fraction RT = 31 min, by isocratic HPLC with solvent B (18%) in solvent A for 60 min. 1 OD_UFS at 230 nm and 280 nm. A component with inhibitory binding activity was recovered in the peak with a RT = 20 min and was called Ts κ. Inset: Inhibition of [^{125}I]apamin binding to rat brain synaptosomal fraction. In the competition assay, 0.1 nM [^{125}I]apamin (50 μl) and synaptosomes (400 μl at 0.4 mg of protein/ml) were incubated for 1 h at 0°C with 50 ml of each of a series of concentrations of native apamin or Ts κ. The incubation buffer was 25 mM Tris-HCl and 10 mM KCl, pH 7.2. The experiment was stopped by centrifugation at 11 000×g for 3 min. The membrane pellets were washed twice with 1 ml of cold washing buffer and the radioactivity bound to the membrane was counted. B_0 is the binding of [^{125}I]apamin in the absence of competitor and B is the binding in the presence of the indicated concentrations of native apamin (●) or Ts κ (○). Non-specific binding was less than 10%. Data points correspond to duplicates from which non-specific binding has been subtracted. The standard error of the mean of the data was around 5%.

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TTTTATATTCAATTCTGATAATACGAAAAAC ATG AAA GTT TTA   5TsA13
        TCAATTCTGATAATACGAAAAAC ATG AAA GTT TTA   3TsII.8
                                M   K   V   L
                                        -20

TAT GGT ATT CTG ATC ATA TTT ATC TTA TGT TCA ATG   5TsA13
TAT GGT ATT CTG ATC ATA TTT ATC TTA TGT TCA ATG   3TsII.8
 Y   G   I   L   I   I   F   I   L   C   S   M
           -15                     -10

TTT TAT TTA AGC CAA GAA GTT GTT ATA GGT CAA AGA   5TsA13
TTT TAT TTA AGC CAA GAA GTT GTT ATA GGT CAA AGA   3TsII.8
 F   Y   L   S   Q   E   V   V   I   G   Q   R
    -5                  +1                  +5

TGT TAC AGA TCG CCT GAC TGT TAT TCA GCG TGT AAG   5TsA13
TGT TAC AGA TCG CCT GAC TGT TAT TCA GCG TGT AAG   3TsII.8
 C   Y   R   S   P   D   C   Y   S   A   C   K
           +10                 +15
AAA CTT GTA GGA AAG GCT ACA GGC AAA TGT ACT AAC   5TsA13
AAA CTT GTA GGA AAG GCT ACA GGC AAA TGT ACA AAT   3TsII.8
 K   L   V   G   K   A   T   G   K   C   T   N
   +20                 +25                 +30

GGA AGA TGC GAC TG                               5TsA13
GGC AGA TGC GAC TGT TAA ACATAACTCTACTGTTTGCGTAA   3TsII.8
 G   R   C   D   C  End
               +35

AAGAGTAATACATGTATTTGATTATTGTATACTAAGCACTGAATCAT   3TsII.8

GTAATTTTCGCAAGCTAACGTGTTAATTGTAAAATATTTTACACAGT   3TsII.8

TTCATATTCAACAGATTCTACAGTGTAAGTATGTTCAGTTAAAATAG   3TsII.8

TTGTCAAAATTTAAACATGATTTGATGTTATGCATGTAATGAATTCG   3TsII.8

ACACAAATGCCTCCCGCTTTTGATAAAAAGTAACTTAAGATTCTAAT   3TsII.8

GACCAGATTCTGTATTGCTTTCTT                          3TsII.8
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Fig. 2. Nucleotide sequence of the cDNA encoding the Ts κ precursor. The predicted protein sequence is given below the nucleotide sequence and is numbered starting from the NH_2-terminal amino acid residue of the toxin; primers 1 and 3 are underlined. 5TsA13 and 3TsII.8 are the sequences obtained after the first and the second PCR respectively.

3. Results and discussion

Ts venom was subjected to chromatography: a gel filtration through Sephadex G-50. One fraction competed for the apamin binding site (Fig. 1A). Further separation of this fraction using reverse-phase HPLC led to an heterogeneous diagram (Fig. 1B). One fraction with a retention time (RT) of 31 min specifically bound to the apamin binding site. This fraction was loaded onto a RP-C18 column for isocratic purification (Fig. 1C). One peak (RT = 20 min) competed with [^{125}I]apamin binding. The corresponding protein constituted 0.25% of the weight of the crude venom. It was a peptide of 35 residues, named Ts κ (from Ts Kapa, i.e. K for K^+ channels and apa for apamin) with the following amino acid composition: Asx, 2.97 (3), Thr, 2.13 (2), Ser 2.20 (2), Pro, 1.10 (1), Glx, 1.31 (1), Gly, 4.65 (5), Ala, 2.02, (2), Cys, 5.34 (6), Val, 1.65 (2), Ile, 0.88 (1), Leu, 1.20 (1), Tyr, 1.52 (2), Lys, 3.82 (4), Arg, 2.75 (3), no Met, Phe or His. Its activity was assayed on rat brain synaptosomes (Fig. 1C, inset). It inhibited [^{125}I]apamin binding with a $K_{0.5}$ value of 300 pM ($K_{0.5}$ values of: apamin = 8 pM, P05 = 20 pM, LTX 1 = 400 pM). To determine the sequence of Ts κ, 1 nmol of native toxin was subjected to Edman degradation. The initial Edman degradation yield was 46.1% and the repetitive yield was 92.8%. Identification of the amino acid was possible up to the 34th residue, except positions 7, 13, 17, 28, 33, which, by homology to

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Apamin        CNCKAPETALCARRCQQH*

LTX           -----AFC-NLRMCQLSCRSL-GLL-G-KCIGDKCECVKH*
P05           -----TVC-NLRRCQLSCRSL-GLL-G-KCIGVKCECVKH

              1        10        20        30        40

Ts κ          -VVIGQRCYRSPDCYSACKKLVGKATG-KCTNGRCDC

TsTX-Kα       -VFINAKCRGSPECLPKCKEAIGKAAG-KCMNGKCKCYP
MgTX          -TIINVKCTSPKQCLPPCKAQFGQSAGAKCMNGKCKCYPH
NTX           -TIINVKCTSPKQCSKPCKELYGSSAGAKCMNGKCKCYNN*

KTX           GVEINVKCSGSPQCLKPCKDA-GMRFG-KCMNRKCHCTPK
KTX2          -VRIPVSCKHSGQCLKPCKDA-GMRFG-KCMNGKCDCTPK
AgTX1         GVPINVKCTGSPQCLKPCKDA-GMRFG-KCINGKCHCTPK
AgTX2         GVPINVSCTGSPQCIKPCKDA-GMRFG-KCMNRKCHCTPK
AgTX3         GVPINVPCTGSPQCIKPCKDA-GMRFG-KCMNRKCHCTPK

IbTX          -ZFTDVDCSVSKECWSVCKDLFGVDRG-KCMGKKCRCYQ
ChTX          -ZFTNVSCTTSKECWSVCQRLHNTSRG-KCMNKKCRCYS
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Fig. 3. Sequence identities between Ts κ and other scorpion toxins acting on K^+ channels. Sequence comparison of Ts κ (this work) and other scorpion toxins acting on different types of K^+ channels: toxins specific for the small conductance Ca^{2+} activated K^+ channels: apamin from bee venom [6]; LTX1 [7] or P05 [9]. See references for the other toxins in Miller [2]: ChTX, charybdotoxin from *Leiurus quiquestriatus hebraeus*; IbTX, iberiotoxin from *Buthus tamulus*; NTX, noxiustoxin from *Centruroides noxius*; MgTX, margatoxin from *Centruroides margaritus*; KTX, kaliotoxin from *Androctonus mauretanicus mauretanicus*; AgTx1, 2, 3, agitoxin 1, 2 and 3 from *Leiurus quiquestriatus hebraeus*; KTX2, kaliotoxin 2 from *Androctonus australis*, Ts Kα, tityus toxin Kα from *Tityus serrulatus*; identical residues are in bold; *, C-amidated residue.

other small scorpion toxins, should be Cys. The sequence was the following: VVIGQRXYRSPDXYSAXKKLVGKATGK-XTNGRXD. To confirm the amino acid sequence and to help predict the structure of the toxin precursor, the sequence of the cDNA encoding Ts κ was determined.

The Ts κ cDNA was obtained by PCR. A degenerate oligonucleotide based on the C-terminal region (from Lys^{27} to Cys^{35}) was used as a reverse primer with a forward primer corresponding to the phage λgt10 sequence (primers 1 and 2). A total cDNA library transcribed from venom gland mRNA was used as template. A 280 bp product was obtained. It encompassed an open reading frame corresponding to the N-terminal part of Ts κ (residues 1–27) and an upstream sequence encoding a putative signal peptide. Based on this sequence, primer 3, corresponding to the 5′ non-coding part of the clone, was used in a new PCR with the reverse primer 4 corresponding to the phage λgt10. A 550 bp PCR-amplified product was obtained. This second PCR product encoded an open reading frame corresponding to a Ts κ precursor of 57 amino acid residues (Fig. 2). Its organization is similar to that of the precursors of 'long' and 'short' scorpion toxins [12,20]. The deduced sequence of the mature Ts κ was identical to that determined by Edman degradation, with 22 residues encoded upstream from the NH_2-terminal Val. As far as we know, signal peptides never terminate with Glu [21]. So, the predicted precursor could be composed of a signal peptide probably terminated by a Ser (−3), followed by a short pro-region, Gln-Glu. The cDNA sequence confirms the position of the Cys residues and indicates that Ts κ is not amidated: the codon encoding the last amino acid residue is followed by a stop codon, and not a codon encoding a Gly which is necessary for C-terminal amidation [15,20].

Ts κ was compared to other scorpion peptidyl blockers of K^+ channels. The positions of the Cys residues are the same in all these molecules. Ts κ shows 29% of sequence identity (Fig. 3) with the two other peptidyl blockers of the SKCa channel able to compete for the apamin binding site, i.e. LTX1 and P05, both of 31 amino acid residues long. Gly^{26} and Lys^{27} are present in all these toxins. Ts κ is more similar to the 37–38 residues long scorpion toxins able to block non-apamin-sensitive K^+ channels: for example, 46% identity between Ts κ and Ts TX-Kα [22], purified from the same venom, 32% of identity with ChTX and 38% with KTX. The largest differences are in the C-terminal part: (1) Ts κ is shorter than these other toxins; (2) in a highly conserved region (i.e. residues 26–32), involved in channel recognition [3–5,12,23]. Ts κ contains Thr^{29} instead of Met and Arg^{32} instead of Lys. This could explain why Ts κ did not compete (up to 0.1 μM) with [^{125}I]KTX bound to its receptor in rat brain synaptosomes (not shown).

A conformational model of Ts κ was designed from sequence alignments with ChTX [19]. The positions of the six half-cystines were used as topological references (Fig. 4). The conformation of Ts κ could be similar to that of ChTX: the α-helix runs from residue 11 to 18 and the three-strand antiparallel β-sheet is made of sequences 2–4, 25–29 and 32–36. The final model has a total energy of −769 kcal/mol and is geometrically correct. Recognition by apamin, P05 and LTX 1 of their receptor depends primarily on electrostatic interactions involving the positive charge carried by the side chains of the α-helix. They are: Arg^{13}–Arg^{14} for apamin [6], Arg^{6}–Arg^{7} for P05 [9], Arg^{7}–Arg^{13} for LTX 1 [8,11]. Thus Arg^{6} and Arg^{9} of Ts κ may be involved in recognition of the apamin binding site. Even if Arg^{6} and Arg^{9}, as well as Lys^{18} and Lys^{19}, are located on either side of the α-helix, they would nevertheless provide a highly basic exposed surface, possibly able to recognize the apamin binding site. Analogs obtained

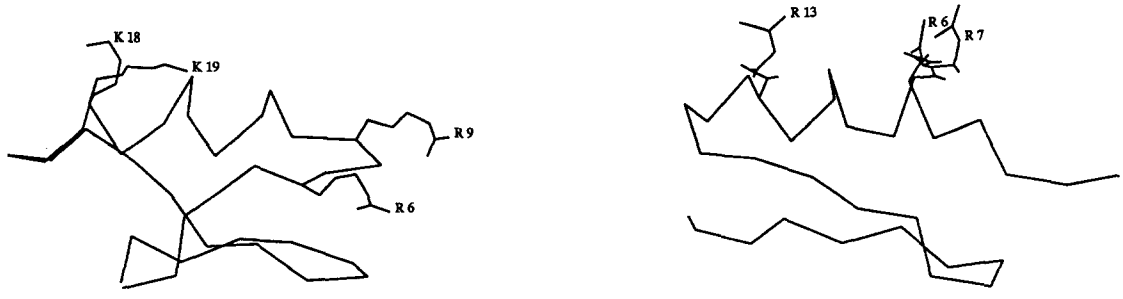

Fig. 4. Modelling of Ts κ by molecular dynamics. Comparison of the structure of P05 (right side) and the Ts κ model (left side). Cα atoms and side chains of interest (see text) are displayed.

by heterologous expression or chemical synthesis would be useful for investigating these possibilities.

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