A four-disulphide-bridged toxin, with high affinity towards voltage-gated K⁺ channels, isolated from *Heterometrus spinnifer* (Scorpionidae) venom

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A new toxin, named HsTX1, has been identified in the venom of *Heterometrus spinnifer* (Scorpionidae), on the basis of its ability to block the rat Kv1.3 channels expressed in *Xenopus* oocytes. HsTX1 has been purified and characterized as a 34-residue peptide reticulated by four disulphide bridges. HsTX1 shares 53 % and 59 % sequence identity with *Pandinus imperator* toxin1 (Pi1) and maurotoxin, two recently isolated four-disulphide-bridged toxins, whereas it is only 32–47 % identical with the other scorpion K⁺ channel toxins, reticulated by three disulphide bridges. The amidated and carboxylated forms of HsTX1 were synthesized chemically, and identity between the natural and the synthetic amidated peptides was proved by mass spectrometry, co-elution on C₁₈ HPLC and blocking activity on the rat Kv1.3 channels. The disulphide bridge pattern was studied by (1)

limited reduction–alkylation at acidic pH and (2) enzymic cleavage on an immobilized trypsin cartridge, both followed by mass and sequence analyses. Three of the disulphide bonds are connected as in the three-disulphide-bridged scorpion toxins, and the two extra half-cystine residues of HsTX1 are cross-linked, as in Pi1. These results, together with those of CD analysis, suggest that HsTX1 probably adopts the same general folding as all scorpion K⁺ channel toxins. HsTX1 is a potent inhibitor of the rat Kv1.3 channels (IC₅₀ approx. 12 pM). HsTX1 does not compete with ¹²⁵I-apamin for binding to its receptor site on rat brain synaptosomal membranes, but competes efficiently with ¹²⁵I-kalioxin for binding to the voltage-gated K⁺ channels on the same preparation (IC₅₀ approx. 1 pM).

INTRODUCTION

A number of scorpion K⁺ channel inhibitors have been previously characterized that target primarily the *Shaker*-related subfamily of voltage-gated K⁺ channels and/or the Ca²⁺-dependent K⁺ channels [1–4]. Those toxins are composed of a single polypeptide chain of 31–39 amino acid residues, reticulated by three conserved disulphide bridges. It has been shown that they share a common overall folding comprising a β -sheet linked to an α -helix by two disulphide bridges, and to an extended fragment by the third disulphide bridge [5,6]. However, besides the conserved cysteine residues, those peptides show a high variability in their sequence that is thought to be responsible for their differential affinities on each subtype of K⁺ channel.

Structure–activity relationships of some scorpion K⁺ channel inhibitors have been studied extensively, by using mainly monosubstituted mutants [7–11] or synthetic chimaeras of already known toxins [12]. Nevertheless such mutational studies are necessarily confined to small variations around the original structure under investigation. To extend the exploration of the structure–activity relationships of this toxin family, more substantial jumps in structure have to be accomplished. The discovery of new natural toxins might give access to active structures displaying multipoint mutations compared with already known toxins.

In this study we have screened, by means of electrophysiological recordings, 16 commercially available scorpion venoms for their ability to inhibit the rat Kv1.3 K⁺ channels expressed in *Xenopus* oocytes. The venom from *Heterometrus spinnifer* (Scor-

pionidae) was shown to contain a probably very active K⁺ channel inhibitor, which was further purified and sequenced. This new toxin, called HsTX1, is one of the most active peptidic inhibitors of rat Kv1.3 channels ever described, with an IC₅₀ of approx. 12 pM. It also competes with ¹²⁵I-kalioxin (KTX) (known to be a high-affinity ligand of the Kv1.3 channels [13]) for binding to its receptor site on rat brain synaptosomes, with an IC₅₀ of approx. 1 pM, whereas it is unable to compete with ¹²⁵I-apamin (a specific ligand of the small-conductance Ca²⁺-activated K⁺ channels [14]) for binding in the same preparation. HsTX1 belongs to a new structural class of scorpion K⁺ channel inhibitors discovered only recently [3,4,15], which includes *Pandinus imperator* toxin 1 (Pi1) [3,16] and maurotoxin (MTX) [4], two toxins containing 35 residues and four disulphide bridges. However, these two latter toxins display a much lower affinity towards voltage-gated K⁺ channels than does HsTX1. We have determined the complete primary structure of HsTX1, including the assignment of its four disulphide bridges, and we discuss structure–activity relationships.

EXPERIMENTAL

Materials

All scorpion venoms were obtained from Latoxan (Rosans, France). Trypsin and apamin were from Sigma Chem. Co. (St. Louis, MO, U.S.A.). Trypsin was also purchased from Takara (Kyoto, Japan), together with AspN-endorpeptidase. Pronase

Abbreviations used: AgTX, agitoxin; ChTX, charybdotoxin; Fmoc, fluorenylmethoxycarbonyl; HsTX1, *Heterometrus spinnifer* toxin 1; KTX, kalioxin; MALDI-TOF-MS, matrix-assisted laser desorption ionization–time-of-flight MS; MgTX, margatoxin; MTX, maurotoxin; Pi1, *Pandinus imperator* toxin 1; sHsTX1, synthetic *Heterometrus spinnifer* toxin 1; TCEP, tris-(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

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was from Calbiochem (La Jolla, CA, U.S.A.). Tris-(2-carboxyethyl)phosphine (TCEP) hydrochloride was from Molecular Probes (Pittsburgh, OR, U.S.A.). The poroszyme®-immobilized trypsin cartridge was purchased from PerSeptive Biosystems (Framingham, MA, U.S.A.).

Expression of K⁺ channels

The Kv1.3 (also called RCK3 [17]) channel gene from rat brain was in a pAS18 vector. The plasmid was linearized with *EcoRI* and the clone was transcribed *in vitro* with SP6 RNA polymerase with a transcription kit (Ambion Inc., Austin, TX, U.S.A.). Capped cRNA was injected into *Xenopus* oocytes 16–72 h before electrophysiological recordings were made.

Electrophysiological recordings

Oocytes were impaled by two 3 M KCl-filled microelectrodes (resistance 0.5–2 M Ω) and voltage-clamped with an Axoclamp-2B two-electrode voltage-clamp amplifier in bath-clamp mode, interfaced to a personal computer running the Pclamp 6.0 software, with a Digidata analogue/digital board (Axon Instruments). Currents were low-pass filtered at 1 kHz with an eight-pole Bessel filter (CyberAmp 320; Axon Instruments) and digitized at 10 kHz. The holding potential was –80 mV with less than –40 nA holding current. Pulses were delivered at a rate of one every 30 or 60 s, to allow full recovery from inactivation. Oocytes were bathed in ND96 solution [0.3 mM CaCl₂/1 mM MgCl₂/2 mM KCl/96 mM NaCl/5 mM Hepes/NaOH (pH 7.6)] supplemented with 50 mg/l BSA. The 100 μ l recording chamber was continuously perfused with ND96, with or without toxins at various concentrations, flowing from one of several reservoirs at a rate of 4 ml per min. Switching from one reservoir to another was done with isolatch Teflon valves (General Valve Corporation). All measurements were made at room temperature (approx. 22 °C). The toxin concentration that blocked half of the K⁺ current (IC₅₀) was determined by least-squares fitting of the equation $I_x = 1/[1 + (X/IC_{50})]$, where I_x is the normalized K⁺ current amplitude measured during the perfusion of the toxin at concentration X , to data points.

Purification of the toxin

Freeze-dried *Heterometrus spinifer* scorpion venom (25 mg) was diluted in 0.5 M acetic acid. Insoluble material was removed by centrifugation at 3000 *g* for 20 min. After three washes of the pellet, the supernatants were pooled and the extract was passed through Millex-GV filters (0.45 μ m pore size). The extract was fractionated by HPLC procedures with a Shimadzu 10A HPLC system, equipped with a variable-wavelength detector. At each step the fractions collected were tested for their inhibitory activity on rat Kv1.3 expressed in *Xenopus* oocyte. The purified peptide was freeze-dried before physicochemical characterization.

Amino acid composition, molecular mass and sequence determination

The amino acid composition of natural and synthetic HsTX1 (sHsTX1) (carboxylated and amidated forms) was determined after acid hydrolysis followed by phenyl isothiocyanate derivatization of amino acids, under the same conditions and apparatus as described previously [2].

MS analysis of natural and synthetic HsTX1 (carboxylated and amidated forms), as well as of proteolytic fragments of sHsTX1, was performed on a Voyager Elite matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass

spectrometer (PerSeptive Biosystems), either in linear mode or in reflector mode, as described previously [2].

The sequences of reduced and pyridylethylated HsTX1 (starting from 2 nmol of natural peptide), of partly reduced and alkylated HsTX1 and sHsTX1, and of purified tryptic fragments of sHsTX1 were determined on an automatic Shimadzu PPSQ10 gas-phase sequencer as described previously [2].

Peptide synthesis

HsTX1 was synthesized by the solid-phase method on an Applied Biosystems 433 peptide synthesizer, with the fluoren-9-yl-methoxycarbonyl (Fmoc) methodology as described previously [2]. The peptide chain was assembled stepwise on (1) 0.25 m-equiv. of Fmoc-amide resin (0.5 m-equiv. amino group/g; Watanabe), to give the amidated form, and on (2) 0.1 m-equiv. of Fmoc-Cys(trityl) (0.5 m-equiv. amino group/g; Applied Biosystems), to give the carboxylated form. Procedures for elongation of the peptidyl chain and cleavage of the peptide from the resin were performed as described previously [2]. Crude reduced peptides were oxidized by air exposure for 30 h at room temperature, at a final concentration of 0.5 μ M, in 0.1 M Tris/HCl, pH 8.2, containing 1 mM oxidized glutathione and 1 mM reduced glutathione. Oxidized peptides were first purified on a semi-preparative reverse-phase C₁₈ HPLC column (Waters; 25 cm $\times$ 1 cm) equilibrated at 32 °C in solvent A [0.1 % trifluoroacetic acid (TFA) in water], with a 100 min linear gradient of solvent B [0.1 % TFA in acetonitrile/water (1:1, v/v)], from 10 % to 60 %, at a flow rate of 4 ml/min, monitored by the absorbance at 230 nm. Further purification allowed us to obtain peptides at 98 % homogeneity.

The carboxylated and amidated forms of sHsTX1 were compared with the natural HsTX1 by (1) analytical reverse-phase C₁₈ HPLC [Lichrospher (Merck); 12.5 cm $\times$ 0.4 cm; gradient from 5 % to 20 % of 0.1 % TFA/acetonitrile in 0.1 % TFA/water, over 30 min, after 3 min at initial conditions; flow rate 1 ml/min; monitored by absorbance at 230 nm] (2) MALDI–TOF MS analysis and (3) amino acid analysis. The amidated form of sHsTX1 was further compared with the natural HsTX1 by limited reduction–alkylation and sequencing, as described below.

Determination of the disulphide bridge pairing

By partial reduction–alkylation and sequencing

Partial reduction–alkylation of HsTX1 was performed as described previously [18], with slight modifications. Briefly, 2–3 nmol of HsTX1 [in 25 μ l of 0.1 M ammonium acetate/MeCN (90:10, v/v) (pH 4)] was incubated with TCEP (2 molar excess) at 50 °C for 10 min. A 100-fold molar excess of *N*-phenylmaleimide was then added and reacted at 50 °C for 30 min. TFA (0.1 % in water; 200 μ l) was added and the mixture was purified by reverse-phase C₁₈ HPLC (Lichrospher, 12.5 cm $\times$ 0.4 cm; Merck), with a linear gradient of solvent B (0.1 % TFA in acetonitrile) in solvent A (0.1 % TFA in water), from 5 % to 45 % in 80 min (after 3 min at initial conditions). MALDI–TOF MS analysis allowed us to identify the mono-reduced, dialkylated peptide. Sequencing was then performed as described above, without a further reduction–alkylation procedure.

After proteolytic cleavage

Tryptic cleavage of HsTX1 was performed by using the Poroszyme®-immobilized trypsin cartridge from PerSeptive Biosystems. Briefly, the cartridge was mounted on an HPLC system in an oven set at 40 °C, and equilibrated in digestion buffer [10 mM CaCl₂/50 mM Tris/HCl (pH 8)]/acetonitrile (95:5,

v/v), at a flow rate of 1 ml/min. sHsTX1 (1 nmol) was dissolved in 50 μ l of digestion buffer and injected on the column; the flow rate was 50 μ l/min. A stop-flow allowed a reaction time of 20 min. The reaction mixture was then eluted with 3 column vol. (3 $\times$ 100 μ l) of digestion buffer/acetonitrile (95:5, v/v) at a flow rate of 50 μ l/min, and collected in a polypropylene tube containing 6 μ l of 6 M HCl (final pH approx. 2) for immediate storage at -80°C . The reaction mixture was desalted by reverse-phase C₁₈ HPLC (Merck; 12.5 cm $\times$ 0.4 cm), with a linear gradient of 0.1 % TFA/acetonitrile in 0.1 % TFA/water from 0 % to 40 % in 80 min, at a flow rate of 1 ml/min after 3 min at initial conditions, monitored by absorbance at 230 nm. Peaks were freeze-dried before physicochemical characterization (molecular mass analysis and sequencing). The tryptic fragment eluted at 24 min was further cleaved by AspN-endopeptidase [15 min at 37 $^{\circ}\text{C}$ in 1 μ l of 10 mM Tris/HCl, pH 8.1; approx. 40 pmol of peptide; protease-to-peptide ratio approx. 4 % (w/w)], directly on the microplate of the MALDI-TOF mass spectrometer. The molecular masses of the subsequent fragments were analysed as described above.

Conformational analysis by CD

Short-wavelength UV spectra of HsTX1 (natural and synthetic) and charybdotoxin (ChTX) (Peptide Institute, Osaka, Japan) were recorded on a Jasco J-600 dichrograph (Tokyo, Japan) calibrated with (+)-camphor 10-sulphonic acid. The spectra were recorded from 260 to 190 nm in 20 mM sodium phosphate buffer, pH 7.1, at room temperature, with a 1 mm path-length cell. Data were collected at 0.1 nm intervals with a scan rate of 100 nm/min and a time constant of 500 ms. The concentration of all peptides, as determined by amino acid analysis, was 80 $\mu\text{g/ml}$. Results are expressed as molar ellipticity ($[\theta]_{\text{M}}$) per residue and were analysed by the method of Compton and Curtis Johnson [19].

Binding assays

Rat brain synaptic-nerve-ending particles (synaptosomes, P2 fraction) were prepared by the method of Gray and Whittaker [20]. Protein content was assayed by a modified Lowry method. KTX and apamin were radiolabelled with the iodogen method, as described previously for KTX [21,22] and for apamin [23]. A specific radioactivity of 2000 Ci/mmol was routinely obtained for a monoiodo derivative. Competition assays were performed as described previously [4,21,22].

RESULTS AND DISCUSSION

Sixteen commercially available scorpion venoms have been screened for their ability to inhibit the rat Kv1.3 K⁺ channels expressed in *Xenopus* oocytes. The potency of the venoms was compared on the basis of the measured IC₅₀ values on K⁺ currents (expressed in weight of crude venom per litre) and time constant(s) (τ_{off}) for recovery from blocking. One of the most active venoms tested was from the scorpion *Heterometrus spinifer*. It showed an estimated IC₅₀ of 6 $\mu\text{g/l}$ and the recovery from blocking could be fitted by two exponentials with τ_{off} values of 116 and 1013 s (results not shown). The latter τ_{off} value indicated the presence of a probably very active K⁺ channel inhibitor.

Bioassay-guided purification of HsTX1 and amino acid sequence determination

The new K⁺ channel inhibitor peptide (HsTX1) has been purified from the venom of *Heterometrus spinifer* by chromatographic

procedures. Throughout the purification procedure the fractions were tested for their ability to inhibit the rat Kv1.3 K⁺ currents expressed in *Xenopus* oocytes. The soluble venom was initially fractionated by reverse-phase C₁₈ HPLC (Figure 1A). The most active fraction (shaded in Figure 1A) was further purified by ion-exchange chromatography (Figure 1B). The active material was eluted by 590–640 mM NaCl (shaded in Figure 1B). This latter fraction was desalted and resolved into two components by reverse-phase C₁₈ HPLC (Figure 1C); only the major component was active.

The purified peptide displayed a monoisotopic molecular mass of 3815.63 Da, as determined by MALDI-TOF-MS analysis in reflector mode. Amino acid analysis of HsTX1 gave the following ratios: 3.6 Asx (4); 1.1 Glx (1); 1.0 Ser (1); 2.3 Gly (2); 2.0 Thr (2); 2.1 Ala (2); 3.0 Pro (3); 3.8 Arg (4); 0.8 Tyr (1); 0.9 Met (1); 5.0 Lys (5) (the nearest integer is given in parentheses). On the basis of amino acid analysis, 17 nmol was recovered from 25 mg of freeze-dried crude venom; thus HsTX1 represents 0.26 % (w/w) of the starting material.

The sequence of HsTX1 was determined by Edman degradation of the reduced and pyridylethylated derivative, in a single run. It consists of a single polypeptide chain of 34 amino acid residues, including eight cysteine residues; its one-letter code sequence is ASCRTPKDCADPCRKETGCPYGKCMNRKC-KCNRC. The monoisotopic molecular mass calculated from this sequence, including four disulphide bridges, is 3816.65 Da, 1 Da higher than measured (3815.63 Da). This discrepancy between theoretical and experimental masses could be due to a C-terminal amidation in the natural toxin, as has been previously demonstrated for other scorpion K⁺ channel inhibitors (noxiustoxin [24], leiurotoxin [25], BmP05 [2], MTX [4] and Pi1 [26]). To test this hypothesis we chemically synthesized HsTX1 on a solid phase, both in the carboxylated and amidated forms, and compared the reoxidized synthetic compounds with the natural HsTX1.

Synthesis of HsTX1

The carboxylated form of HsTX1 was assembled with a yield of 87 % and cleaved with a yield of approx. 94 % to give 650 mg of crude synthetic peptide. The amidated form of HsTX1 was assembled and cleaved with slightly lower yields (82 % and approx. 60 % respectively) to give 842 mg of crude synthetic peptide.

During oxidation, the formation of native-like peptides was monitored by C₁₈ HPLC. The eight cysteine residues of sHsTX1 oxidized readily within 30 h, in a basic pH buffer, in the presence of reduced and oxidized glutathione. The overall yield of chromatographic purification and oxidation of synthetic amidated and carboxylated peptides was approx. 4 %. Amino acid ratios for purified peptides were in agreement with those expected (results not shown). sHsTX1 did not react with iodoacetate or 4,4'-dithiodipyridine, confirming that the eight cysteine residues were cross-linked in four disulphide bridges.

HsTX1 has an amidated C-terminal

MALDI-TOF-MS in reflector mode revealed no significant difference between the molecular masses of the amidated sHsTX1 and the natural HsTX1 (3815.67 and 3815.63 Da respectively). These values are in accordance with the monoisotopic mass deduced from the sequence with an amidated C-terminus (3815.67 Da). The synthetic carboxylated HsTX1 displayed an experimental monoisotopic mass of 3816.61 Da, 1 Da higher than that of the natural HsTX1.

Retention time (min)	Mass (MH ⁺)		Sequence	Corresponding disulphide
	Exp.	Calc.		
42.8	4167.7	4167.8		Cys ₁₉ – Cys ₃₄
23.5	956.3	957.1		Cys ₃ – Cys ₂₄
25.3	972.4	973.1		Cys ₁₉ – Cys ₃₄
28.5	787.3	787.8		Cys ₁₉ – Cys ₃₄
24*	1415.2	1416.6		Cys _{9,13} – Cys _{29,31}
—	879.9	880		Cys ₁₃ – Cys ₃₁
—	—	555.6		Cys ₉ – Cys ₂₉

When co-injected into C₁₈ HPLC, the natural toxin and the amidated synthetic peptide were co-eluted in a single sharp peak (26.6 min; results not shown). Under the same HPLC conditions and in separate injections, the amidated sHsTX1 was eluted at 26.6 min, whereas the carboxylated sHsTX1 was eluted nearly 1 min later (27.4 min) (results not shown).

We conclude that HsTX1 has an amidated C-terminus. Therefore the amidated form of the synthetic toxin can be denoted sHsTX1.

Disulphide bridge pairing determination

The recently described sequences of Pi1 [3] and MTX [4] both have eight cysteine residues in conserved positions compared with the HsTX1 sequence. Pi1 displays the three conserved disulphide bridges found in classical scorpion K⁺ channel inhibitors, the two extra half-cystine residues being cross-linked [3], whereas in MTX only two of the three classical bridges are conserved, the members of the third being paired with each of the two extra half-cystine residues [4]. Therefore it was interesting to investigate the disulphide bridge pairing in HsTX1. We first planned to do so by enzymic cleavage, as described for Pi1 [3] and MTX [4]. Surprisingly, HsTX1 and sHsTX1 proved to be very resistant to the proteolysis by several proteases (trypsin, pronase, pepsin), under classical conditions. In contrast, lysine-C endoprotease from *Achromobacter* cleaved sHsTX1 and HsTX1 very efficiently, but mass analysis of the resulting mixture of peptides demonstrated that a scrambling of disulphide bonds had occurred. Such intramolecular disulphide bond rearrangement had already been observed during proteolysis by the same enzyme of an antifungal protein of 51 residues reticulated by four disulphide bonds [27]. We therefore studied the disulphide bridges of sHsTX1 and HsTX1 by limited reduction-alkylation under acidic conditions to limit any scrambling process (TCEP and *N*-phenylmaleimide; see the Experimental section) and sequencing. The partly reduced and alkylated peptide was purified from the unreacted native one by C₁₈ HPLC; MALDI-TOF-MS analysis confirmed that only one disulphide bridge had been reduced per molecule of toxin and that the liberated cysteine residues had been alkylated. The phenylthiohydantoin derivatives of alkylated cysteine residues were identified by HPLC only at steps 19 and 34 of the Edman degradation. These results demonstrate that Cys¹⁹ and Cys³⁴ are linked by a disulphide bond (see Table 1, top row). The same result was obtained for both HsTX1 and sHsTX1. However, this method proved to be inefficient for studying the other bridges of HsTX1.

By using the Poroszyme®-immobilized Trypsin (PerSeptive Biosystems) which allows a high enzyme excess (approx. 500:1 sHsTX1, w/w) and rapid reaction (20 min), we cleaved sHsTX1 into four fragments, with almost 100% cleavage yield and without scrambling of disulphide bonds. The pairings were determined, by mass spectrometry and sequencing, from the heterodimers obtained, as shown in Table 1. The peptide from the tryptic digest that was eluted at 24 min contained two disulphide bonds, which included half-cystine residues from positions 9, 13, 29 and 31. An aliquot of this fragment (approx. 40 pmol) was further digested by AspN-endopeptidase, directly on a MALDI-TOF microplate. The mass analysis of the two subsequent fragments gave an unequivocal identification of the disulphide bridges: Cys¹³-Cys³¹ and Cys⁹-Cys²⁹ (Table 1).

Therefore HsTX1 displays the same disulphide bridge pairings as in Pi1, including the three conserved S-S bonds observed in classical short K⁺ channel toxins from scorpions, and also the extra disulphide bond Cys¹⁹-Cys³⁴.

Figure 2 CD spectra of synthetic (■) and natural (○) HsTX1

A CD spectrum of ChTX (◆) is shown for comparison. The concentration of all peptides was 80 µg/ml in 20 mM sodium phosphate buffer, pH 7.1. Spectra were recorded with a 1-mm-pathlength cell.

Figure 3 HsTX1 is a high-affinity inhibitor of rat Kv1.3 channels

(A) Oocytes expressing rat Kv1.3 channels were recorded under two-electrode voltage clamping. K⁺ currents were obtained by depolarization from a holding potential of -80 mV to various voltages (from -70 to 50 mV, in 10 mV steps). The upper panel shows recordings obtained in control solution. Superfusion with 10 pM HsTX1 (lower panel) caused an almost 50% inhibition of K⁺ currents at all voltages. (B) Transient perfusion with HsTX1 at a concentration of 100 pM caused a more than 85% reversible decrease in K⁺ currents. (C) Dose-dependent inhibition curves of rat Kv1.3 currents by HsTX1 (○), sHsTX1 (■) and sHsTX1-CO₂H (▲). Data points are means ± S.D. for at least four independent experiments. The solid lines through data are from the equation $I_K = 1/[1 + (X/IC_{50})]$, with IC₅₀ values of 12.5, 13.3 and 68.7 pM for HsTX1, sHsTX1 and sHsTX1-CO₂H respectively.

Conformational analysis

The CD spectra of sHsTX1 and sHsTX1-CO₂H (results not shown) are nearly identical with that of natural HsTX1 (Figure 2). The CD spectrum of ChTX is displayed to permit a comparison of the secondary structure content with that of

Figure 4 Competition binding assays to rat brain synaptosomes

Competition for ^{125}I -KTX binding by KTX ($\square$), sHsTX1 ($\blacksquare$) and sHsTX1- CO_2H ($\blacktriangle$). Non-specific binding was determined in the presence of an excess ($0.1\ \mu\text{M}$) of unlabelled KTX and was less than 25% of total binding. Data points correspond to means $\pm$ S.D. for three to eight independent experiments, performed in triplicate. Solid lines through data points are from the equation $B/B_0 = 1/[1 + (X/IC_{50})]$, where X is the concentration of toxin. The corresponding IC_{50} values are 1 pM for sHsTX1, 11 pM for KTX and 306 pM for sHsTX1- CO_2H .

HsTX1. Although the CD spectrum of ChTX is slightly shifted when compared with that of HsTX1, with a minimum at 218 nm and maximum at 195 nm (compared with 220 nm and 190 nm respectively for HsTX1), the secondary structure contents are comparable. The results show the presence of α -helix (36%, 29%, 34% and 28%), β -sheet (18%, 21%, 20% and 20%) and β -turn (19%, 14%, 12% and 14%) for ChTX, sHsTX1- CO_2H , sHsTX1 and natural HsTX1 respectively. These preliminary structural data allow us to suggest that HsTX1 probably adopts the same overall folding as ChTX. Pi1 [3,15,26] and MTX [4] have also been shown to possess a similar general folding, although MTX displayed an unusual disulphide bridging pattern [4]. Molecular modelling of HsTX1, using the parameters obtained for ChTX [5] (not shown), confirmed this result.

Interaction of HsTX1 with the rat Kv1.3 channel

We characterized the affinity of natural HsTX1 towards the rat Kv1.3 channels expressed in the *Xenopus* oocyte system by measuring the dose-dependent inhibition of the voltage-gated outward current. Figure 3(A) shows the outward currents from an oocyte expressing rat Kv1.3 channels and depolarized to various voltages, in control solution (Figure 3A, top panel) and in the presence of 10 pM HsTX1 (Figure 3A, bottom panel). HsTX1 inhibited the outward current by nearly 50% at all voltages. The reversibility of inhibition is demonstrated in Figure 3(B), after transient perfusion with HsTX1 at a concentration of 100 pM had caused a more than 85% decrease in the current. The dose-dependent inhibition curve of rat Kv1.3 currents by HsTX1 (Figure 3C, $\circ$) allows us to estimate the IC_{50} at approx. 12 pM. sHsTX1 displayed a similar inhibitory activity towards rat Kv1.3 (IC_{50} approx. 13 pM; Figure 3C, $\blacksquare$), whereas sHsTX1- CO_2H was approx. one-fifth as potent, with an IC_{50} of approx. 69 pM (Figure 3C, $\blacktriangle$).

Binding assays

sHsTX1 and sHsTX1- CO_2H were tested in competition experiments with ^{125}I -KTX and ^{125}I -apamin for binding to rat brain synaptosomes. sHsTX1 and sHsTX1- CO_2H competed with ^{125}I -

		1	10	20	30	40	Similarity (%)
HsTX1	-----ASCRT	PKDCADPCRK	ETGCPYG-KC	MNRKCKCNRC*			100
maurotoxin	-----VSCGT	SKDCYAPCRK	QTGCPN-AKC	INKSKCYGC*			59
Pi1	---L-VKCRG	TSDCGRPCQQ	QTGCPNS-KC	INRMCKCYGC*			53
ClITX 1	-ITINVKCTS	PQQCLRPCKD	RFGQHAGGKC	INGKCKCYP-			41
MgTX	-TIINVKCTS	PKQCLPPCKA	QFGQSAGAKC	MNGKCKCYPH			47
NTX	-TIINVKCTS	PKQCSKPCKE	LYGSSAGAKC	MNGKCKCYNN*			47
KTX	GVEINVKCSG	SPQCLRPCKD	AGMRF-G-KC	MNRKCHCTPK			38
AgTX1	GVPINVKCTG	SPQCLRPCKD	AGMRF-G-KC	INGKCHCTPK			32
AgTX2	GVPINVSCGT	SPQCIKPCKD	AGMRF-G-KC	MNRKCHCTPK			41
AgTX3	GVPINVPCTG	SPQCIKPCKD	AGMRF-G-KC	MNRKCHCTPK			38
ChTX	-ZFTNVSCSTT	SKECWSVCQR	LHNTSRG-KC	MNKKCRCCYS-			41
IbTX	-ZFTDVCDSV	SKECWSVCCKD	LFGVDRG-KC	MGKKCRCCYQ-			35
LQ2	-ZFTQESCTA	SNQCWSICKR	LHNTNRG-KC	MNKKCRCCYS-			35

Figure 5 Sequence alignment of scorpion K^+ channel inhibitors

The sequences shown are *Heterometrus spinifer* toxin 1 (HsTX1, this study), MTX [4], Pi1 [3], *Centruroides limpidus limpidus* toxin 1 (ClITX1 [32]), MgTX [33], noxiustoxin (NTX [34]), KTX [35], AgTX1,2 and 3 [31], ChTX [36], iberiotoxin (IbTX [37]) and *Leiurus quinquestriatus* toxin 2 (Lq2 [38]). Cys residues are indicated in bold type. Asterisks indicate an amidated C-terminal. Percentages of sequence identity are given relative to HsTX1. Gaps (—) have been introduced to enhance similarities.

KTX with IC_{50} values of 1 and 306 pM respectively (Figure 4), whereas they were unable to compete with ^{125}I -apamin at a concentration as high as $1\ \mu\text{M}$ (results not shown).

Comparison of HsTX1 with other scorpion K^+ channel inhibitors

The sequence of HsTX1 is significantly shorter and only 32–47% identical with that of the three-disulphide-bridged scorpion toxins acting on voltage-gated K^+ channels, whereas it is similar in length and 53–59% identical with that of the four-disulphide-bridged Pi1 and MTX (Figure 5). On the basis of chain length, number of disulphide bridges and sequence similarity, HsTX1, MTX and Pi1 therefore constitute a new structural class of scorpion K^+ channel inhibitors. It is noteworthy that this structural type has never been found in the venom from Buthidae scorpions. Indeed, Pi1, HsTX1 and MTX have been isolated from the venom of Scorpionidae.

As it seems that this novel structural class of K^+ channel inhibitors adopts the same overall folding as the classical scorpion K^+ channel toxins, a comparison of aligned sequences of all these toxins, together with the set of data available on their biological activities, might help to highlight some key amino acid residues for the activity of HsTX1.

HsTX1 differs strikingly from MTX in its pharmacological activity: first, it is 12500-fold more active on the rat Kv1.3 channels (12 pM compared with 150 nM [4]); secondly, it does not bind to the apamin-sensitive Ca^{2+} -dependent K^+ channels. MTX has been proved to display an unusual broader specificity towards K^+ channels, being able to block both the voltage-gated and apamin-sensitive Ca^{2+} -dependent K^+ channels [4]. It has been hypothesized that two positively charged residues (Arg¹⁴ and Lys¹⁵) protruding from the α -helix in MTX could be functionally equivalent to the crucial Arg¹³-Arg¹⁴ in apamin [28,29], and Arg⁶-Arg⁷ in P05 [30]. It is noteworthy that HsTX1 also displays this pair of basic residues, but flanked by two acidic residues (Asp¹¹, Glu¹⁶), not found in MTX, that could be responsible for the lack of activity of HsTX1 towards the apamin-sensitive K^+ channels.

HsTX1 is one of the three most active peptidic inhibitors of rat Kv1.3 channels ever described. It is about five times more active than margatoxin (MgTX) [7] and one-third as active than agitoxin2 (AgTX2) [31]. Although the primary structure of HsTX1 is only 47% and 41% identical with those of the two

latter toxins, it displays most of the amino acid residues described as important for interacting with voltage-gated K⁺ channels. Energetically important residues in AgTX2 for the binding to the *Shaker* channels were determined as Lys²⁷, Asn³⁰, Arg²⁴, Ser¹¹, Thr³⁶, Met²⁹ and Phe²⁵ [11]. The highly similar KTX (89% sequence identity with AgTX2) was studied for its binding to Kv1.3 channels, and the residues Lys²⁷, Asn³⁰, Arg²⁴, Met²⁹, Phe²⁵ and Thr³⁶ were pointed out as being important [7]. HsTX1 displays some similar residues in conserved positions (see Figure 5 for alignments): Lys²³ (Lys²⁷), Asn²⁶ (Asn³⁰), Met²⁵ (Met²⁹) and Tyr²¹ (Phe²⁵). However, HsTX1, like MgTX, lacks an equivalent of the Arg²⁴ in AgTX2 and KTX. The absence of this residue should be compensated for in some way so that HsTX1 and MgTX have affinities on Kv1.3 comparable to that of AgTX2.

Most scorpion toxins acting on voltage-gated K⁺ channels have a carboxylated C-terminus. It is noteworthy that the three four-disulphide-bridged K⁺ channel toxins described so far all have an amidated C-terminus. On the basis of molecular modelling, Rogowski et al. [15] pointed out the particular orientation of the C-terminus of Pi1, compared with classical three-disulphide-bridged scorpion toxins. It seemed that the supplementary disulphide bridge in Pi1 brings the C-terminus to the face of the toxin that holds the conserved amino acid residues thought to be important for binding to voltage-gated K⁺ channels. This particular orientation was confirmed recently by NMR studies of Pi1 [26]. Our molecular model of HsTX1 indicates that the C-terminus of this new toxin is similarly oriented to that in Pi1. Our pharmacological results indicate that the amidated form of sHsTX1 is 5-fold more potent than the carboxylic form in electrophysiological experiments, and up to 300-fold more active in binding experiments. It is tempting to speculate that a carboxylated C-terminus in HsTX1 could destabilize the toxin-K⁺ channel complex by electrostatic repulsion. The greater effect observed in binding experiments than in electrophysiological tests could then be attributed, at least in part, to the conditions of lower ionic strength used in the former.

HsTX1 constitutes a new tool to investigate voltage-gated K⁺ channels. Experiments are under way to define its three-dimensional structure as well as its selectivity towards various subtypes of voltage-gated K⁺ channels.

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