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Research Article

Cytolytic and K^+ channel blocking activities of β-KTx and scorpine-like peptides purified from scorpion venoms

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Abstract. Among the scorpion venom components whose function are poorly known or even show contrasting pharmacological results are those called "orphan peptides". The most widely distributed are named β-KTx or scorpine-like peptides. They contain three disulfide bridges with two recognizable domains: a freely moving N-terminal amino acid sequence and a tightly folded C-terminal region with a cysteine-stabilized α/β (CS-αβ) motif. Four such peptides and three cloned genes are reported here. They were assayed for their cytolytic, antimicrobial and K^+ channel-blocking activities. Two main characteristics were found: the existence of an unusual structural and functional diversity, whereby the full-length peptide can lyse cells or kill microorganisms, and a C-terminal domain containing the CS-αβ motif that can block K^+ channels. Furthermore, sequence analyses and phylogenetic reconstructions are used to discuss the evolution of this type of peptide and to highlight the versatility of the CS-αβ structures.

Keywords. Cysteine-stabilized α/β-motif, cytolytic peptides, DNA cloning, gene topology, K^+ channel blocker, scorpion toxin, sequence analysis.

Introduction

Scorpion venoms are composed of a large repertoire of polypeptides presenting a variety of pharmacological actions; most of them, however, are not well characterized [1]. Scorpion toxins having a cysteine-stabilized α/β (CS-αβ) structural motif [2, 3] are the best-known venom components; among which are the molecules responsible for the main neurotoxic symptoms observed during scorpion envenomation. These are also the most prevalent components of the venoms thus far studied and reported in the literature. Most neurotoxic symptoms are associated with abnormal cellular depolarization due to the fact that scorpion toxins interfere with the ion conductance pathways of excitable membranes (reviewed in [1]). According to their size, these neurotoxins can be classified into two main categories: (1) long-chain toxins of 53 – 78 amino acid residues, stabilized by three or four disulfide

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bridges, most of which modulate sodium (Na^+) channels function, and (2) short-chain toxins of 23–42 amino acid residues, stabilized by three or four disulfide bridges, usually acting as blockers of potassium (K^+) channels. There are, however, several scorpion venom components that do not fit within this general classification [4–6].

A distinct type of CS-αβ motif was found in TsTX-Kβ, a K^+ channel blocker first identified in the venom of the Brazilian scorpion *Tityus serrulatus* [7]. This toxin is a long-chain peptide with three disulfide bridges and two structural domains: a putative α-helical N terminus and a cysteine-rich C terminus, with the consensus signature of the CS-αβ motif [8, 9]. Subsequently, transcripts encoding for putative homologs of TsTX-Kβ were identified in the North African scorpion *Androctonus australis* (AaTXKβ [8]), the Asian scorpion *Mesobuthus martensii* (BmTXKβ and BmTXKβ2 [10]) and the South American scorpions: *T. costatus* (Tco 42.14 and TcoKIK [6, 11]), *T. discrepans* and *T. trivittatus* (TdiβKTx, TdiKIK, TtrβKTx and TtrKIK [6]). Additionally, a peptide identical to TsTX-Kβ was isolated and chemically characterized from the venom of a closely related scorpion *T. stigmurus* [6, 12]. All these scorpion species belong to the medically important Buthidae family. More recently, a putative homolog, HgeβKTx, was identified in the non-medically important scorpion *Hadrurus gertschi* from the Caraboctonidae family [6]. Structurally, β-KTxs are polypeptides with 45–68 amino acid residues, including 6 cysteines and the two structural domains described above. When a systematic nomenclature for scorpion toxins that recognize K^+ channels was proposed by Tytgat et al. [13], TsTX-Kβ and AaTX-Kβ were classified as a novel family called β-KTx. However, the actual function of the peptides belonging to this family has been verified only for TsTX-Kβ [7] and for a recombinant version of the putative homolog BmTXKβ [14]. The possible function of the remaining peptides of this family is poorly characterized.

Scorpine is another long-chain peptide with CS-αβ motif that contains three disulfide bridges and shows high sequence similarity with the β-KTxs family (above 50%) and also is composed of a putative α-helical N terminus and a CS-αβ motif-containing C terminus. Scorpine was isolated from the venom of the Central African scorpion *Pandinus imperator* of the Scorpionidae family [15]. Scorpine homologs have been identified in the South African and South Asian scorpionids *Opistophthalmus carinatus* (Opiscorpines 1–4 [9]) and *Heterometrus laoticus* (HS-1 [16]), respectively, and in the North American caraboctonid *Hadrurus gertschi* (HgeScplp 1 and 2 [6, 17]). Scorpine exhibits relatively high antimicrobial activity against sexual stages of *Plasmodium berghei* and to some bacterial strains [15]. Similarly, a synthetic peptide based on the N-terminal sequence of Opiscorpine 1 was shown to inhibit the growth of some bacteria and fungi [9]. Apart from these two pieces of evidence, no other scorpine-like peptide has been functionally assayed, hence leaving this group of scorpion venom components poorly characterized.

Several authors have suggested that, on the basis of sequence similarity, β-KTxs and scorpine-like peptides are closer to invertebrate defensins – a widely spread group of antimicrobial peptides, which also adopts the CS-αβ motif [18] – than to common scorpion toxins [8, 9]. In fact, scorpions do have recognizable invertebrate defensins [19–21]; even, the conserved gene topology of scorpion defensins and common scorpion toxins suggest that both groups are paralogs in scorpions [22, 23]. Genes encoding scorpion defensins and most scorpion toxins share the same gene topology, in which a single phase I intron interrupts the sequence encoding the signal peptide. The reported gene organization of BmTXKβ (classified as β-KTx [24]), Opiscorpine 1 and HS-1 (two scorpine-like peptides [9, 16]) is quite distinct, with large phase I introns interrupting the sequence encoding the mature protein, instead of the signal peptide. At least for the case of the scorpine-like peptide-encoding genes, there is another intron upstream of the initiation codon [9, 16]. Other scorpion toxin-encoding genes also include large introns at the 5' untranslated sequences [25, 26], raising the question of whether or not this is a common topology for genes encoding scorpion toxins. Unfortunately, since the number of fully characterized genes (full transcripts and full genomic sequences) is rather limited, this question cannot be solved at this moment.

The structural characteristics and the wide species distribution of β-KTxs and scorpine-like peptides make them unique examples within scorpion venom components. Previously, we have defined β-KTxs and scorpine-like peptides as "orphan peptides", because their biological activities were not satisfactorily characterized [6], despite the fact that their content in the whole venom is significant [15].

In this report, we present data on the K^+ channel blockade, antimicrobial and cytolytic activities of one β-KTx (TstβKTx from *T. stigmurus* [6, 12]) and one scorpine-like peptide (HgeScplp1 from *H. gertschi*). We also show that two full-length caraboctonid peptides (HgeβKTx and HgeScplp1) lyse prokaryotic and eukaryotic cells, whereas the CS-αβ domain of HgeScplp1 obtained by chemical hydrolysis, corresponding to the most C-terminal region of the molecule, is capable of specifically blocking three different Kv1 channels. The genomic DNA encoding

the precursors of TtrβKTx, TtrβKIK and HgeScplp1 are also reported here. Finally, we describe a novel, naturally occurring, truncated form of HgeScplp1 (here named Hge36), which lacks cytolytic activity but is able to block Kv1.1 channels. In summary, this communication identifies functions for the earlier called "orphan peptides".

Materials and methods

Venom sources, purification procedures and amino acid sequence determinations

Venom was obtained by mild electrical stimulation of CO_2-anesthetized scorpions. The material was dissolved in water and centrifuged 10 000 *g* for 10 min. The soluble supernatant was stored at –20°C and when needed was separated by high performance liquid chromatography (HPLC) as described previously for: *T. trivittatus* [27], *H. gerstchi*, [28] and *T. stigmurus* [12]. The fractions of interest were pooled and further purified by HPLC to obtain β-KTxs or scorpine-like peptides in homogeneous form. The amino acid sequences of pure peptides were obtained by automatic Edman degradation in a Beckman LF 3000 Protein Sequencer (Palo Alto, CA, USA) and mass spectrometry (MS) analysis, following protocols earlier described [12, 27, 28].

Mass spectrometry determination

Usually, for mass spectrometry analysis, 5 µl of each sample was injected into an LC/MS system composed of a Finnigan LCQ^{Duo} ion trap mass spectrometer (San Jose, CA, USA) with a nanoelectrospray ionization (ESI) source using a Surveyor MS syringe pump delivery system and a C_{18} PicoFrit column/needle (75 µm×10.1 cm), which was set on an *xyz* multi-axis translational stage for optimizing the ESI signal. The LC/ESI-MS/MS data acquisition program was set up to collect ion signals from the eluted peptides using an automatic, data-dependent scan procedure, in which a cyclic series of three different scan modes (full scan, zoom scan and MS/MS scan) were performed. First, the most abundant peptide ion peak in a full scan (*m/z* 300–2000) was selected as the precursor ion. Second, a zoom scan was performed to determine the charged state of the precursor ion. Finally, an MS/MS scan was used to determine the sequence of the precursor ion using collisionally induced dissociation with relative collision energy of 40%. The data acquisition and automatic analysis were performed with the Xcalibur software on a Windows NT PC system. The MS/MS spectra from peptides enzymatically generated were analyzed manually and by the Sequest software. Peptides and toxins previously separated by HPLC were direct injected into the mass spectrometer.

Cleavage of HgScplp1 with cyanogen bromide

Cleavage of HgScplp1 at methionines (positions 3 and 32) was performed with cyanogen bromide (CNBr) from Aldrich Chemical Co. (Milwaukee, WI), according to the protocol earlier described [29, 30]. After overnight incubation at room temperature the reaction product was separated by HPLC. Individual fragments had their molecular masses determined as described above.

Genomic DNA cloning and sequencing

The total genomic DNA was isolated as described before [21]. Based on the information obtained from cDNA of the β-KTxs and scorpine-like peptides from *H. gerstchi* and *T. trivittatus* (GenBank accession numbers: DQ465345, DQ465346 and DQ465351), several oligonucleotides were designed and synthesized. For HgScplp1 gene the primers were HgScor5': 5'-ATG AAT ACC AAA TTA ACT GTT CTT TGT TTT CTG GG -3'; HgScor3': 5'-GCC ATC TTG TGT ACA ATT GCT TTT GCT GCG -3'; HgScorInt6: 5'-GCT GAT TAG ATG ATG AGC AAC-3' and HgScorIn3: 5'-CTA CAG TTA ACG ATA GAT TAT GAG CC-3'. For TtrβKTx gene the primers were TriBetaFor2: 5'-ATG GAA AGG AAA TGG GCT CTT CTC-3'; TriBetaintron1: 5'-CCA GTT ATT CCC CAA CCA ATT C-3'; TriBetaRev: 5'-CCG CCT TCA GAA TGG ATC TC-3' and TriBetaRe2: 5'-CCG TCT TTT CGT TTT ATG TC-3'. For TtrKIK gene the primers were TriKIKfor: 5'-ATG GTG GCC ACG AAT CGT TGC TGT G-3'; TriKDKIKrev: 5'-CCA TCC AGA CTT AAT CTT GTC TTT CGC-3' and TriKIKstop: GGA TTT ACA GAG ATT ACA ATT TGA TGC-3'. The DNA Synthesizer model 391 of Applied Biosystems (Foster city, CA, USA) was used. The genomic DNA from *H. gerstchi* and *T. trivittatus* were used as template for the PCR reaction. The PCR reaction was performed as described [6] with the following protocol: hot start with 4 min denaturation at 94°C, pre-cycle 1 min at 94°C, 3 min at 45°C and 3 min at 72°C, after the mixtures were incubated 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, for 35 cycles, followed by a 7-min final step at 72°C. PCR products were purified, ligated into the pGEM-plasmid (Promega, Madison, WI, UA) and used to transform competent *E. coli* DH5-α cells. Positive clones were sequenced from both ends using an automatic machine Model 3100 (Applied Biosystems). The nucleotide sequences obtained in this work are deposited in GenBank (accession numbers: EF613115, EF613116 and EU089740).

Sequence analyses and phylogenetic reconstruction

Intron sequences were analyzed in ASD-Alternative Splicing web server of the European Bioinformatics

Institute ((http://www.ebi.ac.uk/asd-srv/wb.cgi). Similarity searches were performed with PSI-BLAST (www.ncbi.nlm.nih.gov/blast) protocol [31]. Multiple sequence alignments (MSA) were obtained using MUSCLE [32, 33]. The MSA of 21 β-KTxs and scorpine-like peptides were used to build a Hidden Markov Model (HMM), and then used to perform an hmmsearch [34] against UniProt [35] to identify any possibly existing homologs amongst proteins identified to date. Another MSA of 142 scorpion peptides with the CS-αβ motif signature (87 α-KTxs; 21 β-KTxs and scorpine-like peptides; 27 γ-KTxs; 4 unclassified peptides and 3 defensins) was used to estimate the tree topology by Bayesian inference with MrBayes 3.1.2 [36, 37], essentially as described in [38]. This MSA is available on request from the corresponding author. The Bayesian block used was: rates=invgamma; aamodelpr=mixed; ngen=5000000 printfreq=100 samplefreq=100 nchains=4 savebrlens=yes startingtree=random. The 1000 trees with best posterior probabilities, after coalescence, were merged to calculate a 50% consensus tree. This reconstruction supported well all major partitions between scorpion CS-αβ peptides (even for the three groups of β-KTxs and scorpine-like peptides, see below). The low-order relationships within the clades are not satisfactorily resolved. To exclude any artificial splitting due to the long N-terminal extension of β-KTxs and scorpine-like peptides, as compared with defensins and short-chain scorpion toxins, the same procedure was performed with a clipped alignment in which the N-terminal extension was not taken into consideration. Finally, with the aim of achieving a better resolution for β-KTxs and scorpine-like peptides, another Bayesian reconstruction was run with an MSA including 21 β-KTxs and scorpine-like peptides plus the sequence of the putative paralog *A. australis* defensin (P56686), used to root the tree, with the same parameters as above.

Electrophysiological experiments

Expression in oocytes: Kv1.1–1.6, Shaker IR and hERG channels were studied. cRNA for Kv1.1–1.3 channels were prepared as previously described [27]. Next the cRNAs were synthesized from the linearized plasmids using the large-scale SP6 mMESSAGE mMACHINE transcription kit (Ambion, Foster City, CA, USA). Stage-V and -VI *Xenopus laevis* oocytes were harvested by partial ovariectomy under anesthesia (3-aminobenzoic acid ethyl ester methanesulfonate salt, 0.5 g/l from Sigma, Bornem, Belgium). Anesthetized animals were kept on ice during dissection. The oocytes were defolliculated by treatment with 2 mg/ml collagenase (Sigma) in Ca^{2+}-free ND-96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES adjusted to pH 7.5). Between 1 and 24 h after defolliculation, oocytes were injected with 50 nl of 50–100 ng/μl cRNA. The oocytes were then incubated in ND-96 solution (supplemented with 50 mg/l gentamycin sulfate) at 16°C for 1–5 days.

Electrophysiological measurements: The following experiments were performed in ND-96 medium containing 1.8 mM Ca^{2+}. Two-electrode voltage-clamp recordings were performed at room temperature using a GeneClamp 500 amplifier (Molecular devices, Sunnyvale, CA, USA) controlled by a pClamp data acquisition system (Molecular devices). Whole-cell currents from oocytes were recorded 1–5 days after injection. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept low (<0.5 MΩ). Currents were sampled at 500, 250 and 1 kHz and filtered using a four-pole low-pass Bessel filter, at 200, 100 and 500 Hz for Kv1.1–Kv1.5 and hERG, respectively. The pClamp program was used for data acquisition, and data files (Molecular devices) were directly imported, analyzed and visualized with Origin software. The percentage reduced current was calculated using the equation:

% reduction = (reduced current amplitude/control current amplitude)×100

Current percentages were used for the calculation of concentration–response curves, using the Hill equation:

$$I=I_{max}/[1+(IC_{50}/A)^{n}{}_{H}]$$

where, I represents the current percentage, I_{max} the maximal current percentage, IC_{50} the concentration of the toxin that evokes the half-maximal response, A the concentration of toxin, and n_H the Hill coefficient. Averaged data are indicated as mean ± SEM. Statistical analysis of differences between groups was carried out with Student's *t*-test and a probability of 0.05 was taken as the level of statistical significance.

Hemolytic assay

The hemolytic activity of the peptides was determined using human red blood cells with modifications of a reported protocol [39]. Fresh red blood cells, collected with heparin, were washed three times (10 min at 5000 *g*) with buffer (0.81% NaCl with 20 mM HEPES pH 7.4) and suspended in the same buffer. The final concentration of blood cells for testing the peptides was $10^{6-}10^{7}$ cells/ml solution. Usually to 50 μl solution containing red cells were added 50 μl toxin dissolved in buffer, to complete a final volume of 100 μl per assay. The peptide concentrations tested were 200 and 500 nM. The samples were incubated in triplicates at

37°C for 10 and 30 min. After centrifugation, hemolysis was determined by measuring absorbance of the supernatant at 570 nm. Controls for zero and 100% hemolysis consisted of human red blood cells suspended in buffer and 0.5% Triton X-100 in buffer solution, respectively.

Antibacterial activity assay

Purity of peptides was verified by mass spectrometry and the protein content of HgeScplp1, HgeβKTx and N-terminal fragment of HgeScplp1 were estimated by measuring the absorbance at 280 nm. It was assumed that one unit of absorbance in a 1-cm cell pathway is equal to 1 mg/ml concentration of protein. Their activities towards Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* (ATCC 29213), were tested by liquid growth inhibition test based on the protocol described in [40]. Bacterial colonies were inoculated in Luria Bertani (LB) sterile liquid medium and were left to grow at 37°C overnight. An aliquot was transferred to a fresh medium and incubated for 6 h to obtain the cells at mid-logarithmic phase. The initial inoculum was approximately 1.5×10^5 colony-forming units/ml. The experiments were performed in triplicates. Positive and negative controls were carried out with the inoculum plus LB medium and medium only, respectively. The spectrophotometric readings (595 nm) were performed after 8-h incubation. Pandinin [41] was used for comparative purposes.

Results

Purification and characterization of peptides

The venom from *H. gerstchi* was separated by HPLC, as described previously [28]. Three fractions of this chromatogram were further characterized and are reported in this communication (Fig. 1a). The fractions eluting at 43.20 and 48.00 min (labeled 2 and 3 in Fig. 1a) were further purified using an analytical C_{18} reverse-phase column, from which two pure peptides were obtained (Fig. 1b, c). The major component of Figure 1b (labeled with asterisk) was sequenced by automatic Edman degradation allowing the identification of the first 45 amino acid residues of the N-terminal region of the peptide. This sequence was shown to be identical to the sequence of *Hadrurus gertschi* scorpine-like peptide 1 (HgeScplp1), earlier deduced from cDNA sequencing of a gene cloned by our group [6]. Further identification of the full primary structure of the peptide was obtained after cleaving HgeScplp1 with CNBr. Figure 1d shows the profile of HPLC separation of the chemically treated sample. The molecular masses of the major components were determined by MALDI-TOF MS analysis. Component labeled b in Fig. 1d corresponded to residues 4–32 from the N-terminal region of HgeScplp1 (here named N-HgeScplpDig). The molecular weight experimentally found for this fragment was 3166.8 $(M+H)^+$ atomic mass units (a.m.u), which corresponds well with the theoretically expected mass of 3166.87 a.m.u. The other major fragment (letter a in Fig. 1d) corresponds to residues 33–76 of the most C-terminal sequence of HgeScplp1 (here named C-HgeScplpDig). Its experimentally determined mass was 4797.0 $(M+H)^+$ a.m.u., which also corresponds well with the theoretical expected mass of 4796.57 a.m.u. for HgeScplp1(33–76). The theoretical mass calculated for the native HgeScplp1 was 8369.27 a.m.u., in good agreement with the experimentally determined value of 8370.0 $(M+H)^+$. In this manner, the complete identification of the scorpine-like peptide of *H. gertschi* is confirmed and corresponds to the gene cloned and sequenced from this scorpion as earlier described [6].

The component eluted at 48.00 min (labeled 3 in Fig. 1a) was further separated as shown in Figure 1c (component labeled with asterisk), and the Edman degradation analysis allowed the unequivocal identification of the first 38 amino acid residues (Fig. 2). It corresponds exactly to the N-terminal amino acid sequence deduced for the β-KTx-like peptide (HgeβKTx) earlier cloned and described [6].

Component 1 of Figure 1a, eluted at 36.12 min, was further separated by HPLC (see Fig. 1e). The major peptide labeled with an asterisk in Figure 1e was submitted to Edman degradation, which provided the identification of 24 amino acid residues that were identical to the amino acid sequence from position 29 to 53 of the scorpine-like peptide (see second line sequence of Fig. 2). The molecular mass of this peptide was determined by MS, given a value of 5294.95 $(M+H)^+$. This is the theoretical molecular mass expected for the 48 most C-terminal situated amino acid residues of HgeScplp1 (5294.53 a.m.u.). These data support the interpretation that in the soluble venom of *H. gertschi* there are two *bona fide* peptides sharing the same 48-amino acid sequences, one corresponding to the entire sequence of HgeScplp1 and another to its C-terminal amino acid sequence from position Val29 to Leu76, here named Hge36.

Finally, it is worth mentioning that the isolation and chemical characterization of the other peptides analyzed in this communication were previously reported (TstβKTx [6, 12]) or will be reported elsewhere (TtrKIK; manuscript in preparation).

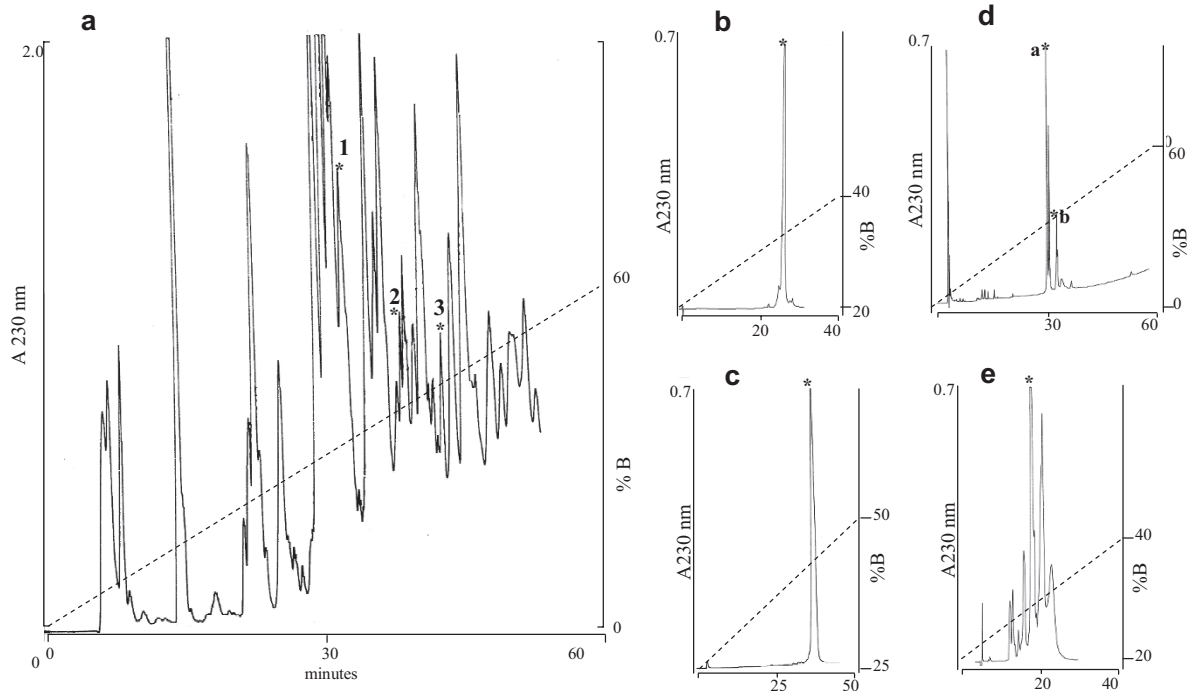

Figure 1. HPLC separation of scorpion venom components from *Hadrurus gertschi.* (*a*) Soluble venom of *H. gertschi* was separated in a C18 reverse-phase column, as described in [26]. The elution was obtained by application of a linear gradient from solution A [0.12% trifluoroacetic acid (TFA) in water] to solution B (0.10% TFA in acetonitrile), as indicated by the dashed lines. Labels 1–3 indicate the components further separated in (*b, c, e*). (*b*) Final purification of component 2 (HgeScplp1). (*c*) Final HPLC separation of component 3 (HgeβKTx). (*d*) HPLC profile of separation of the CNBr cleaved component 2. Letter 'a' corresponds to the C-terminal segment (C-HgeScplpDig) and letter 'b' to the N-terminal segment (N-HgeScplpDig). (*e*) Separation of component 1 by HPLC. The asterisk showed pure component Hge36.

	1 10 20 30 40 50 60 70	Molecular Mass (a.m.u.)	
		Expected	Experimental
HgeScplp1	GWMSEKKVQGILDKKLPEGIIRNAAKAIVHKMAKNQFGCFANVDVKGDCKRHCKAEDKEGICHGTKCKCGVPISYL	8369.27	
component2|43.20	GWMSEKKVQGILDKKLPEGIIRNAAKAIVHKMAKNQFGCFANVDV...		8370.0
C-HgeScplpDig(Da)	[----------]	4796.57	4797.0
N-HgeScplpDig(Db)	[----------]	3166.87	3166.8
HgeScplp1(29-76)	VHKMAKNQFGCFANVDVKGDCKRHCKAEDKEGICHGTKCKCGVPISYL	5294.53	
component1|Hge36	VHKMAKNQFGCFANVDVKGDCKRHC...		5294.95
HgeβKTx	KSTVGQKLKKKLNQAVDKVKEVLNKSEYMCPVVSSFCKQHCARLGKSGQCDLLECIC	6426.6	
component3|48.0	KSTVGQKLKKKLNQAVDKVKEVLNKSEYMCPVVSSFCK...		6427.0

Figure 2. Amino acid sequences of *H. gertschi* peptides. The deduced sequences and expected molecular masses from cDNA clones of HgeScplp1 and HgeβKTx [6] are compared with the sequences and masses of peptides isolated from *H. gertschi* venom (component1|Hge36, component2|43.20 and component3|48.0) or chemically digested component HgeScplp1 [C-HgeScplpDig(Da) and N-HgeScplpDig(Db)]. The numbers on top indicate the relative amino acid positions of the corresponding peptides, where the segments were aligned accordingly. The dashes in lines 3 and 4 means corresponding molecular masses of the peptides in question (see molecular masses on the columns on the right).

Electrophysiological analyses

HgeβKTx and HgeScplp1 showed cytolytic activity on the oocytes, inducing disruption of the cell membrane and resulting in leak currents and cell death (Fig. 3a, b). Due to the damage caused to the oocytes, it was not possible to evaluate the blocking effect, if any, of HgeβKTx and HgeScplp1 on K^+ channels expressed. TstβKTx and the fragment C-HgeScplpDig inhibited ion currents in Kv1.1 (Fig. 3c, e), Kv1.2 and Kv1.3 (not shown) channels. Dose-response curves on Kv1.1 channel showed IC_{50} values of 96±0.9 nM for TstβKTx, 185±34 nM for Hge36 and 88±14 nM for C- HgeScplpDig (Fig. 3f–h). The blockage effect was also verified in Kv1.2 and 1.3 channels for the three peptides (Table 1). Of note, the Hge36 peptide blocked Kv1.1 channels (Fig. 3d), but did not block

Table 1. Antibacterial and cytolytic activities of βKTx and scorpine-like peptides.

Peptide	Conc. (nM)	Antibacterial[a]		Eukaryotic cell disruption[b]	Kv1.1	Kv1.2	Kv1.3
		Bs	Sa				
HgeβKTx	200–500	++[c]	+	+++	Cell lysis	Cell lysis	Cell lysis
HgeScplp1	200–500	++	–	+++	Cell lysis	Cell lysis	Cell lysis
Hge36	200–500	n.t.	n.t.	+	+	–	–
C-HgeScplpDig	200	n.t.	n.t.	+	++	++	++
N-HgeScplpDig	500	–	–	n.t.	n.t.	–	–
TstβKTx	200	n.t.		–	++	++	++
Pandinin	100 μM [d]	++	++	++			

Relative potency is indicated with increasing plus signs as follows: + incomplete inhibition at the highest concentration tested; ++ complete inhibition at high concentration, and; +++ complete inhibition at any range of concentration tested. Concentration in nM except otherwise indicated.
[a] Qualitative assay; Bs: *B. subtilis*; Sa: *S. aureus*; n.t.: not tested.
[b] Hemolytic assays and disruption of oocytes membrane.
[c] +, –: positive or lack of activity, respectively.
[d] Effective dose according to [41].

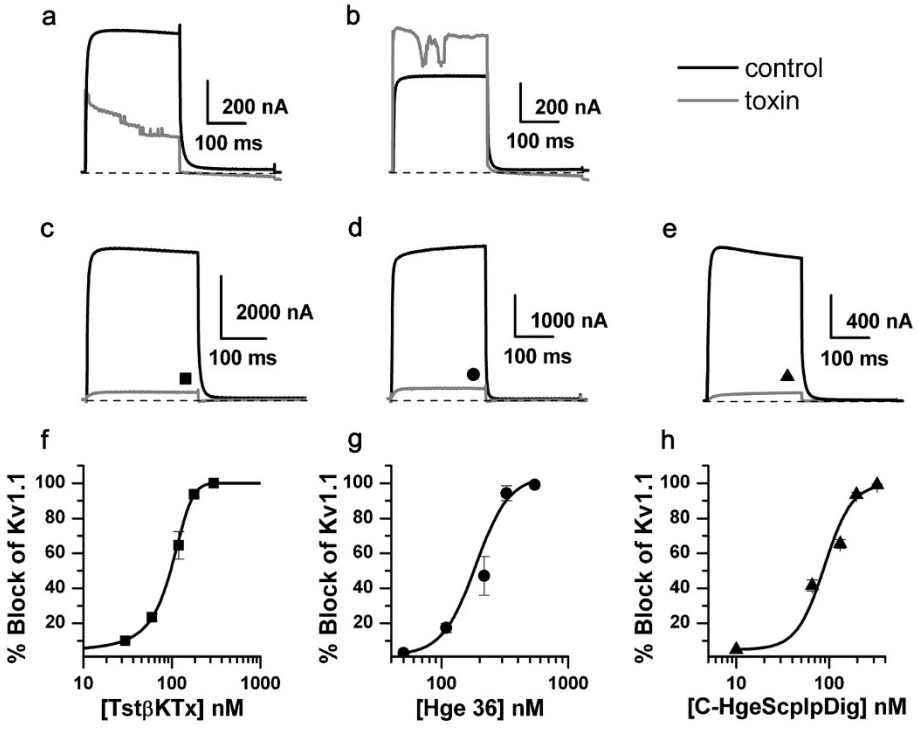

Figure 3. Electrophysiological experiments on Kv1.1 channel. Selected current traces showing the effects of HgeβKTx toxin (*a*) and Hge-scorpine-like (*b*). Both toxins induce disruption of the cell membrane, which causes leak currents and cell death. Current inhibition by peptides TstβKTx, Hge36 and fragment C-HgeScplpDig (*c–e*, respectively). The effects were induced upon addition of 300 nM peptides. Gray lines show toxin effects, normal lines control conditions. Dose-response curves of the current inhibition of Kv1.1 channels upon the application of the peptides (*f–h*). IC_{50} values were 96±0.9 nM for TstβKTx, 185±34 nM for Hge36 and 88±14 nM for fragment C-HgeScplpDig. Currents were evoked by a depolarization to 0 mV from a holding potential of –90 mV with –50 mV tail potential. Data are the mean ± SEM of at least three experiments at each concentration.

Kv1.2 and Kv1.3 channels, in sharp contrast to C-HgeScplpDig, which is an effective blocker of the three channels reported. TstβKTx was also tested on Kv 1.4–1.6, *Shaker* IR and hERG channels but it did not show any blocking activity in these channels (data not shown). Preliminary studies of TtrKIK tested in cells expressing hKv1.1, hKv1.4, hKv11.1, hKv11.2, hKv11.3, Nav1.2, Nav1.5 and Nav1.6 show no blocking activity at 1 μM concentration.

Antibacterial and hemolytic activities

Scorpine, HS-1 and a synthetic fragment of Opiscorpine 1 have been reported to be antimicrobial agents [9, 15, 16]. Although no satisfactory description of their mechanism of action is available, it is likely that their antimicrobial activities are related with cell lysis [9, 15, 16], similarly to other analogous antimicrobial peptides. Table 1 summarizes the cytolytic activity of HgeScplp1 and HgeβKTx observed in eukaryotic oocytes and red blood cells. These peptides also inhibit bacterial growth at 2 μM concentration. Hge36 and C-

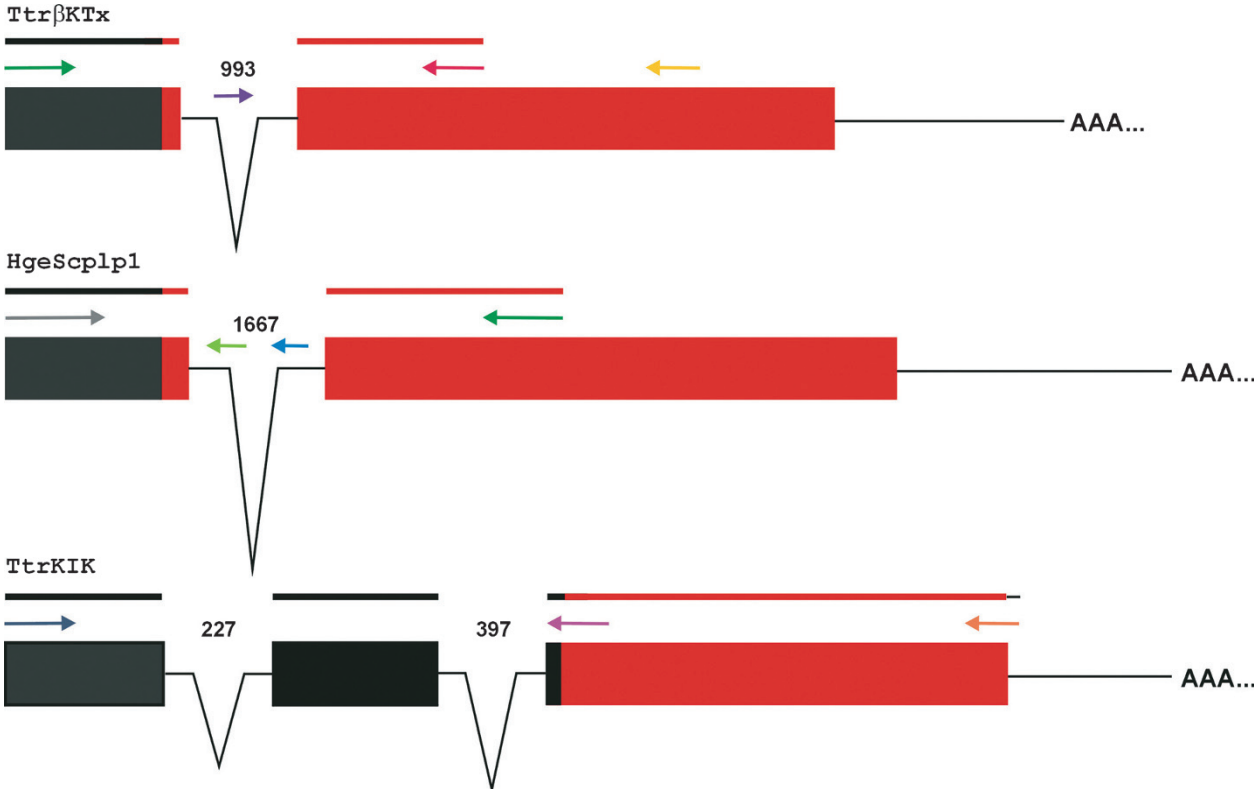

Figure 4. Schematic representation of gene structures. The cartoon shows the gene topology of TtrβKTx (top), HgeScplp1 (middle) and TtrKIK (bottom). Large boxes are the translated sequences (signal peptide in black and mature peptide in red), whereas thin lines above boxes match the deduced sequences from cDNA clones. Introns and 3' untranslated sequences are represented as thin lines. Arrows indicate the relative position of the primers used to obtain the full sequences (dark green, TriBetaFor2; purple, TriBetaintron1; red, TriBetaRev; yellow, TriBetaRe2; gray, HgScor5'; light green, HgScorInt6; blue, HgScorIn3; green, HgScor3'; dark blue, TriKIK; violet, TriKDKIKrev; orange, TriKIKstop). Numbers above the introns indicate the corresponding size. Further details are given in the GenBank entries.

HgScplpDig fragment did not lyse oocytes and their hemolytic activity was severely reduced. TstβKTx at 200 nM did not show cytolytic activity against any of the eukaryotic cells tested (Table 1).

Genomic organization of HgeScplp1, TtrβKTx and TtrKIK

The cDNA sequences of the genes encoding HgeScplp1 and the β-KTx-like sequences of *T. trivittatus* (TtrβKTx and TtrKIK) were reported earlier [6]. Based on these sequences, various oligonucleotides were designed for PCR amplifications of genomic DNA clones. Several complete clones were obtained and sequenced. Figure 4 shows a cartoon of the topology of genes encoding HgeScplp1, TtrβKTx and TtrKIK (see GenBank accession nos. EF613115, EF613116 and EU089740, respectively, for further sequence details). The HgeScplp1 and TtrβKTx genes contain two exons disrupted by a single phase I intron of 1617 (HgeScplp1) and 933 (TtrβKTx) base pairs. These introns are located at the beginning of the mature peptide-encoding regions, similar to the introns of other β-KTxs and scorpine-like peptides reported earlier [9, 16, 24]. Unexpectedly, the gene structure of TtrKIK is different, with two phase I introns in the coding region of the non-mature sequence, which in turn is rather large (44 amino acids long). The A/T contents of the introns were 63 % for TtrβKTx, 73 % for HgeScplp1 and 64 % for TtrKIK. These values are comparable with other introns of scorpion genes [9, 16, 21, 24]. Donor and acceptor sites, U2-type branch points and polypyrimidine tracts of the three genes (see GenBank entries) corresponded well to spliceosome-dependent canonical eukaryotic introns, as verified by intron analysis in the ASD-Alternative Splicing web server of the European Bioinformatics Institute.

Sequence analyses and phylogenetic reconstruction

Three groups of β-KTxs and scorpine-like peptides have been recognized on the basis of sequences relatedness (Fig. 5a), namely, peptides similar to TsTX-Kβ, peptides similar to scorpine and peptides similar to BmTXKβ [6]. Indeed, all the β-KTxs and scorpine-like peptides deposited in databanks are retrieved within the first three iterations of PSI-

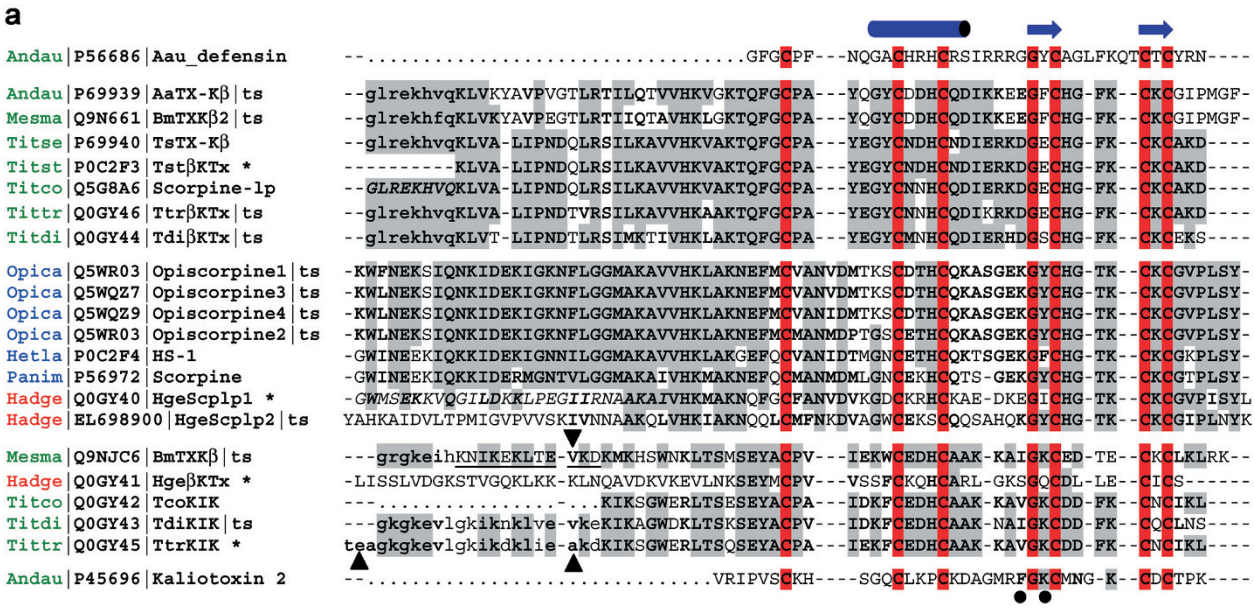

Figure 5. Multiple sequence alignment and tree topology. (*a*) All known βKTxs and scorpine-like peptides were aligned with MUSCLE. Barrel and arrows above the alignment represent the secondary structure elements, α-helix and β-strands, identified in α-KTxs. Lower case amino acids represent putative pro-sequences, aligned with the N terminus of native Tco42.14 [6]. The underlined sequence of Mesma|Q9NJC6 refers to the possibly processed extension of the recombinant peptide tested in [14]. Italicized sequences of Titco|Q5G8A6 and Hadge|Q0GY40 correspond to N-terminal extensions, possibly processed to generate the shorter peptides actually identified in the corresponding venoms ([6] and this work). Also included are the sequences of the putative paralogs from *A. australis:* 4-kDa defensin (P56686) and Kaliotoxin 2 (α-KTx 3.5, P45696). Arrowheads indicate the amino acids whose encoding codons are split by the single introns of the BmTXKβ precursor and both introns of TtrKIK precursor. Dots below Kaliotoxin 2 sequence indicate residues identified as part of the pharmacophore of α-KTxs [45]. Conserved residues on all sequences are on red background; conserved residues within each of the three groups are shaded in gray, and conservative substitutions within each group are in bold. (*b*) Simplified phylogenetic tree showing high order relationships among scorpion CS-αβ peptides. The tree is drawn on the basis of Bayesian tree topology reconstruction with 142 sequences and displayed with iTOL [50]. Colors indicate the four major groups of scorpion CS-αβ peptides, whereas the prevalence (×100) of major partitions on the final tree set are indicated by red dots at the corresponding nodes (see Materials and methods for details). Branches' names were removed for clarity. Full tree is available from corresponding author upon request. (*c*) The MSA shown in (*a*) was used to reconstruct tree topology with MrBayes 3.1.2. The prevalence of a given partition (×100) in the final tree set (1000) is depicted under the corresponding node. Arrows indicate the partitions that distinguish between Old World and New World homologs. In all panels each sequence is indicated by the abbreviated names of the corresponding genus and species; the Swiss-Prot accession number (except for Hadge|EL698900, from GenBank) and by common names. Genus and species abbreviations are: Andau, *A. australis*; Hadge, *H. gertshi*; Hetla, *H. lauticus*; Leiqh, *Leiurus quinquiestratus hebraeus*; Mesma, *Mesobuthus martensii*; Titco, *T. costatus*; Titdi, *T. discrepans*; Titse, *T. serrulatus*; Titst, *T. stigmurus*; Tittr, *T. trivittatus.* Colors highlight the family to which each species belong to: green, Buthidae; blue, Scorpionidae; red, Caraboctonidae. Peptides analyzed in this work are labeled with asterisks. Translated sequences are marked with ts at the end of their names.

BLAST searches. Similar identification is obtained independently of which sequence from the tree groups is used as query. More remote putative homologs can be identified only with the fifth iteration (starting with any sequence from the three groups), although with very poor expectance values (e >0.01). The only putative paralog identified was a defensin isolated from the North African scorpion *A. australis.* Noteworthy is the fact that no scorpion toxin sequences, apart from the deposited β-KTxs and scorpine-like peptides, were retrieved during the PSI-BLAST or hmmsearch searches. To clarify phylogenetic relationships amongst β-KTxs and scorpine-like peptides, as well as their relationships with the two putative paralog families (scorpions' typical K^+ channel blocking toxins and defensins), the tree topology of β-KTxs and scorpine-like peptides was reconstructed by Bayesian inference, on the basis of an MSA including 142 sequences of scorpion's CS-αβ peptides. This procedure convincingly supports the monophyly of TsTX-Kβ and scorpine-related peptides (prevalence of the partition in the merged set: 100%), whereas BmTXKβ-related peptides are segregated into a different monophyletic clade (Fig. 5b). Interestingly, the BmTXKβ clade appears as the sister group of short-chain scorpion toxins (Fig. 5b, see below). Moreover, this phylogenetic reconstruction also supports the basal character of scorpion defensins, validating their use for rooting the trees of highly diversified scorpion toxins.

Although these major partitions are well supported, phylogenetic relationships within the clades were not satisfactorily resolved by the described procedure, due to the high sequence diversity of the whole dataset. To

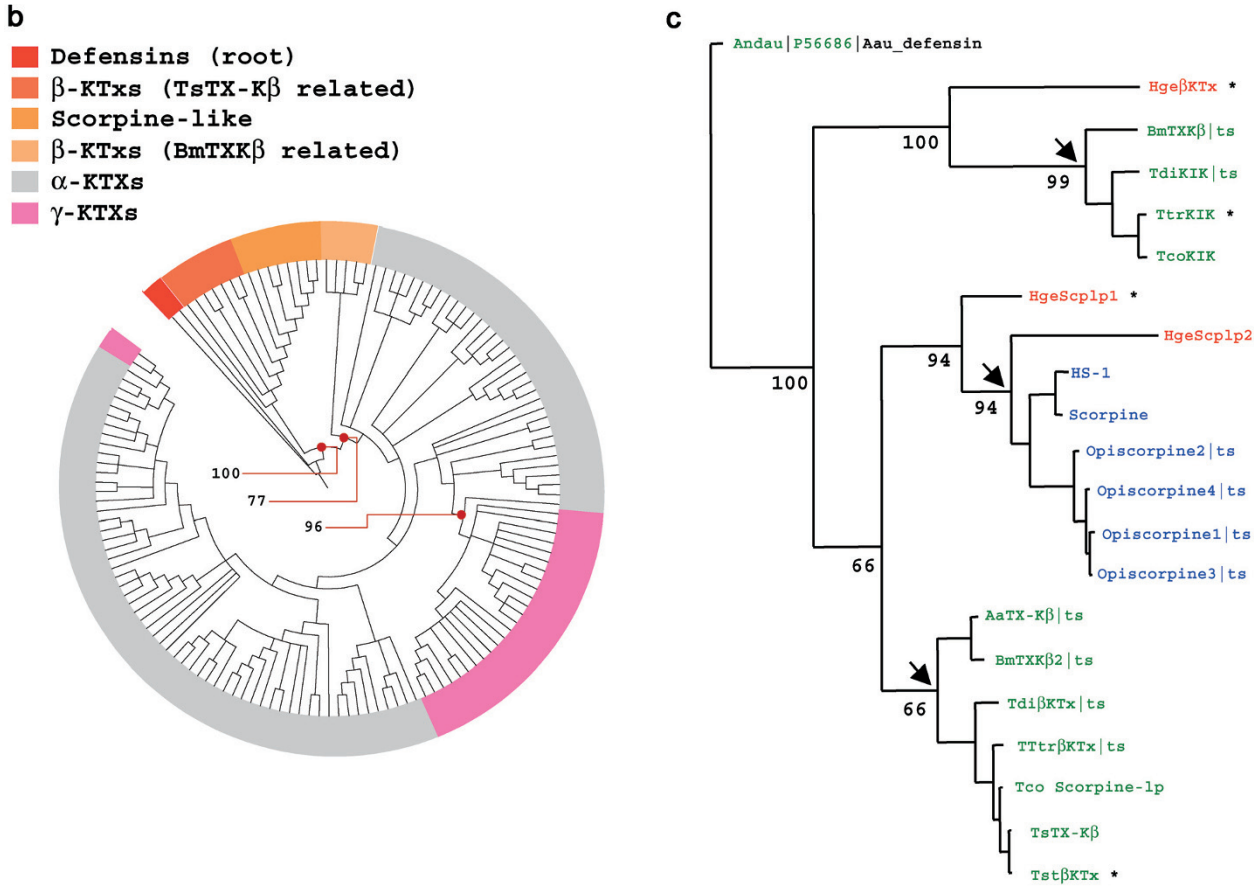

Figure 5. (continued)

attain a better resolution of the phylogenetic relationships of β-KTx and scorpine-like peptides, another reconstruction was performed with an MSA of 21 β-KTx and scorpine-like peptides, plus two scorpion defensins – previously validated as paralogs. The consensus tree shown in Figure 5c strongly supports the earlier distinction of three groups of β-KTxs and scorpine-like peptides: peptides similar to TsTX-Kβ and scorpine clustered into two sister branches, whereas BmTXKβ-related peptides clustered in a separate clade, with the caraboctonid HgeβKTx as the sister branch of the buthid members. Within each of the three clades, the procedure also distinguished between peptides from Old World (Africa and Asia) scorpions and their New World (American) homologs (see arrows in Fig. 5c).

Discussion

An unusual group of scorpion venom components is represented by "chimeric" peptides with relatively long N-terminal sequence (cysteine-free) and a C terminus with a recognizable CS-αβ motif. The β-KTxs or scorpine-like peptides are found in the venoms from at least three different taxonomic families of scorpions and have been classified into three groups based on sequence similarities [6] as shown in Figure 5a. This rather wide distribution and their relative abundance in the venoms, up to 1 % of total protein content [15], suggest that these peptides could play an important role in the function of scorpion venoms. There is, however, only fragmentary and even contrasting evidence about their putative function(s) [7, 9, 14, 15]; hence, these peptides have been dubbed as "orphan peptides". Here we provide the first comprehensive functional, biochemical and phylogenetic analyses of β-KTxs and scorpine-like peptides.

Bioactivities of β-KTxs and scorpine-like peptides

Since both K^+ channel blockade [7, 14] and antimicrobial activities [15] have been reported for β-KTxs and scorpine-like peptides, we tested both functions for each one of the three groups. Two caraboctonid peptides, HgeScplp1 and HgeβKTx, are efficient cytolytic agents of two eukaryotic cell types (oocytes and erythrocytes) and inhibit the growth of *B. subtilis* at low or submicromolar concentrations (Fig. 3 and

Table 1). The low amount of TstβKTx available was only enough for the assays on eukaryotic cells, on which no lysis were observed at 200 nM. In contrast, at the same concentration TstβKTx is an effective blocker of Kv1.1, 1.2 and 1.3 channels expressed in oocytes, with an IC_{50} of 96 nM on Kv1.1 channels. In agreement, TsTX-Kβ (which is chemically identical to TstβTx) was first identified as a blocker of the delayed-rectifying K^+ currents in rat brain synaptosomes [7], a current later shown to be mainly conducted through Kv1.1 channels [42]. In sharp contrast, BmTXKβ blocks I_{to} currents in rabbit atrial myocytes [14], which are fast-inactivating currents, associated with heteromultimeric channels with Kv4.2 and Kv4.3 subunits [40]. We were unable to evaluate the possible K^+ inhibitory activity of HgeβKTx, the putative caraboctonid ortholog of BmTXKβ, due to its high cytolytic activity (Fig. 3 and Table 1), but we did test the *T. trivittatus* ortholog, TtrKIK, on five different K^+ channels and three different Na^+ channels, and no blocking activity was recorded at 1 μM (not shown).

Diversifying function by posttranslational processing

Between TsTX-Kβ-like and scorpine-like groups, the most conserved region is the CS-αβ motif-containing C terminus (Fig. 5a). Due to the fact that α-KTxs also adopt this structural motif but are devoid of the linear N-terminal extension, we tested whether or not the C terminus of HgeScplp1 is capable of blocking K^+ channels. The C-terminal fragment obtained by chemical digestion (C-HgeScplpDig, spanning from residues 33 to 76 of HgeScplp1), is an effective blocker of Kv1.1, 1.2 and 1.3 channels at 200 nM concentrations. The IC_{50} value against Kv1.1 was 86 nM (Fig. 3 and Table 1). The cytolytic action of C-HgeScplpDig was severely reduced as compared to the full-sized precursor (Table 1). Therefore, we have demonstrated that the CS-αβ motif-containing region of scorpine-like peptides could act as K^+ channel blockers, but that this activity is masked by the cytolytic action of the full-sized peptides. Additionally, the cytolytic action of this group of peptides might be dependent on the N-terminal extended sequence, similar to the reported antibiotic activity of a synthetic peptide based on the N-terminal sequence of Opiscorpine-1 [9].

Unexpectedly, we found a naturally occurring peptide (Hge36) in the venom of *H. gertschi* whose first 25 N-terminal amino acids are identical to HgeScplp1 residues 29–53 (Figs 1, 2). Similar to C-HgeScplpDig, the cytolytic action of Hge36 is quite poor, but it is a Kv1.1 blocker with an IC_{50} of 185 nM (Fig. 3 and Table 1). Compared with C-HgeScplpDig, Hge36 contains a four residues extension in the N-terminal region. This relative minor difference produces a substantial change in the K^+ channel blocking activity of Hge36. In our opinion these four residues are very important for the correct fitting of both peptides into the vestibule of the different sub-types of K^+ channels, but this deserves further analysis. It is worth remembering that the presence of two naturally occurring peptides sharing the same C-terminal region in scorpion venoms have been previously documented in *T. costatus* [6, 11]; here, we provide evidence that such modifications might have functionally relevant consequences.

Taken together, the evidence shows that, after all, the so-called "orphan peptides" probably play a very important function as biologically active venom components. A single longer peptide (HgeScplp1) is cytolytic *per se*, but can be processed to give smaller fragment with specific functions. A recent publication dealing with the transcriptome analysis of the venom of *H. gertschi* [17] has shown that the venomous gland of this scorpion does transcribe genes that encode for putative proteases. In this manner, the results reported here are supportive of the idea that all venom components of scorpion are there because they have a specific activity to fulfill, although for many of them we still need to identify the corresponding functions.

Different organization of the genes encoding βKTxs and scorpine-like peptides

Previously, the gene structures of the precursors encoding BmTXKβ [24] and two scorpine-like peptides (Opiscorpine-3 [9] and HS-1 [16]) were revealed to have an unusual topology for toxin-encoding scorpion genes, with a large intron interrupting the sequence that encodes the mature peptide. Here, we confirm that the genes encoding for the TsTx-Kβ-like peptide TtrβKTx and the scorpine-like peptide HgeScplp1 display the same topology, but the gene encoding the BmTXKβ-related peptide called TtrKIK is completely different (Fig. 4) concerning the precursor organization and its exon/intron structure. Based on sequence comparisons and phylogenetic reconstruction (see below) TtrKIK is a clear ortholog of BmTXKβ (54% amino acid sequence identity over the full-length precursors and 61% nucleotide identity over all the coding exons); even the position of the single intron of BmTXKβ gene aligns well with the second intron of the TtrKIK gene, whereas the starting point of the first intron of TtrKIK also align well with the beginning of TtrβKTx and HgeScplp1 single introns, and with the starting point of the second intron of Opiscorpine 1 and HS-1.

Although the biological consequences of such unusual organization are not clear at this moment, both TtrKIK introns are on the same phase and the intermediate exon encodes for a putative pro-seg-

ment. Therefore the gene could encode for two transcripts while still being able to translate into the same full mature sequence. Although Zhijian et al. [43] have previously shown that the precursor encoding BmKK2 can be "cryptically" spliced in two messengers when transiently transfected into HEK cells; it is worth recalling that alternative splicing has never been described directly in the venom gland. We have cloned the full mRNA encoding TtrKIK (DQ465346) with specific primers, but never observed shorter products during the PCR reactions, suggesting that even if taking place, alternative splicing of TtrKIK mRNA generates only minimal amounts of the shorter version.

The intermediate exon of TtrKIK is the first protomodule (*sensu* Patthy [44]) identified within a scorpion toxin gene. Protomodules are exons flanked by symmetrical introns, which can be inserted into other introns on the same phase to generate multidomain proteins in a process called exon-shuffling, a mechanism proposed to be a major source of evolutionary novelty in the metazoan linage [44]. Independently of the significance of exon-shuffling on scorpion toxin evolution (if any), which is still unclear, it certainly constitutes a subject that deserves further attention. Finally, it is worth remembering that BmTXKβ has never been isolated directly from its natural source (*M. martensii* venom), hence its mature form remains unknown. Actually, the evidence from its putative orthologs in *Tityus* scorpions suggests that the real peptide encoded by the BmTXKβ gene could be shorter than initially predicted [6]. The results presented in this communication leave little doubt that the gene encoding HgeScplp1 expresses a peptide that is finally processed to give two mature peptides. It needs to be confirmed whether the same process occurs with the gene encoding BmTXKβ from *M. martensii* scorpion.

The CS-αβ motif is a versatile framework for K^+ channel recognition and blockade

Phylogenetic reconstruction strongly supports that TsTx-Kβ-like and scorpine-like peptides are sister clades (Fig. 5b, c), quite distant from the α-KTxs but closer to some scorpion defensins. Conversely, BmTXKβ-related peptides clustered apart from the TsTx-Kβ-like and scorpine-like groups; even within the α-KTxs clade if these toxins are taken into consideration during the reconstruction procedure (Fig. 5b).

The molecular determinants of K^+ channel recognition and blockade by α-KTxs have been extensively studied [45]; most of these toxins block the ion conduction pathway of Kv1.x, KCa1.1 or KCa3.1 channels. The available data indicate that the pharmacophore of these toxins are quite similar, all of them having a conserved lysine that plugs into the selectivity filter of the K^+ channels. There are, however, at least three other modes of K^+ channel recognition by short-chain scorpion toxins [46]. Sequence analyses reveal that buthid BmTXKβ-related peptides indeed have a conserved lysine in a position homologous to those of most α-KTxs (see alignment in the lower part of Fig. 5a), therefore we hypothesized that BmTXKβ-related peptides might bind to K^+ channels in an analogous fashion to that of α-KTxs. These shared molecular determinants could be derived from common ancestry of BmTXKβ-related peptides and α-KTxs (*i.e.*, symplesiomorphic characters of both families), or alternatively could be the result of functional convergence driven by structurally similar receptors (a homoplasy). Although the available data do not permit solving this ambiguity, it is worth recalling that BmTXKβ blocks fast-inactivating K^+ currents in rabbit myocytes, whereas only toxins from the α-KTxs subfamily 15 have been reported to block this particular current [47, 48].

In contrast, apart from the CS-αβ motif, in TsTx-Kβ-like and scorpine-like peptides none of the conserved features of typical α-KTxs are evident. Nonetheless, TsTX-Kβ, TstβKTx and the chemically digested C-terminal part of HgeScplp1 (C-HgeScplpDig) are all good blockers of K^+ channels. The molecular determinants of this effect were not identified in this work, but it is clear that these will define a novel pharmacophore for K^+ channel recognition and blockade, different from the ones identified within the α-KTxs family. The sequence just at the N terminus of the CS-αβ motif-containing region might be involved in such a novel pharmacophore, due to the fact that a four amino acid extension at the N terminus of the Hge36, comparing with C-HgeScplpDig, abolishes its blocking effect on Kv1.2 and Kv1.3 channels and reduced by twofold its affinity for Kv1.1 channels. Taken together, the evidences presented here reinforce the versatility of the CS-αβ motif as a scaffold to support different molecular determinants for K^+ channel recognition and blockade, a characteristic that could be exploited to design custom-made pharmacological tools [49].

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