Peptides 32 (2011) 1509–1517

Peptides

journal homepage: www.elsevier.com/locate/peptides

The new kappa-KTx 2.5 from the scorpion *Opisthacanthus cayaporum*

Thalita Soares Camargos[a], Rita Restano-Cassulini[b], Lourival Domingos Possani[b], Steve Peigneur[c], Jan Tytgat[c], Carlos Alberto Schwartz[a], Erica Maria C Alves[a], Sonia Maria de Freitas[d], Elisabeth Ferroni Schwartz[a,*]

[a] *Laboratório de Toxinologia, Universidade de Brasília, Brasília, DF 70910-900, Brazil*
[b] *Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, National Autonomous University of Mexico, Cuernavaca 62210, Mexico*
[c] *Laboratory of Toxicology, University of Leuven, B-3000 Leuven, Belgium*
[d] *Laboratório de Biofísica, Universidade de Brasília, Brasília, DF 70910-900, Brazil*

ARTICLE INFO

Article history:
Received 14 March 2011
Received in revised form 13 May 2011
Accepted 14 May 2011
Available online 23 May 2011

Keywords:
kappa-KTx
Liochelidae
Potassium channel blocker
Scorpion
Venom

ABSTRACT

The kappa-KTx family of peptides, which is the newest K^+-channel blocker family from scorpion venom, is present in scorpions from the families Scorpionidae and Liochelidae. Differently from the other scorpion KTx families, the three-dimensional structure of the known kappa-KTxs toxins is formed by two parallel α-helices linked by two disulfide bridges. Here, the characterization of a new kappa-KTx peptide, designated kappa-KTx 2.5, derived from the Liochelidae scorpion *Opisthacanthus cayaporum*, is described. This peptide was purified by HPLC and found to be identical to OcyC8, a predicted mature sequence precursor (UniProtKB C5J89) previously described by our group. The peptide was chemically synthesized and the circular dichroism (CD) spectra of both, native and synthetic, conducted at different temperatures in water and water/trifluoroethanol (TFE), showed a predominance of α-helices. The kappa-KTx 2.5 is heat stable and was shown to be a blocker of K^+-currents on hKv1.1, and hKv1.4, with higher affinity for Kv1.4 channels (IC_{50} = 71 μM). Similarly to the other kappa-KTxs, the blockade of K^+-channels occurred at micromolar concentrations, leading to uncertainness about their proper molecular target, and consequently their pharmacologic effect. In order to test other targets, kappa-KTx2.5 was tested on other K^+-channels, on Na^+-channels, on bacterial growth and on smooth muscle tissue, a known assay to identify possible bradykinin-potentiating peptides, due to the presence of two contiguous prolines at the C-terminal sequence. It has no effect on the targets used except on hKv1.1, and hKv1.4 expressed in Chinese hamster ovary cells. Since the only plausible function found for kappa-KTx2.5 seems to be the blockade of K^+-channels, a discussion regarding the analysis of structure–function relationships is included in this communication, based on sequence alignments of members of the kappa-KTx toxin family, and on computational simulation of a structural model of the kappa-KTx2.5-Kv1.2 complex.

1. Introduction

Ion channels are important targets for treatment of many diseases or clinical abnormalities. Among them, K^+-channels have received much attention as they are widely spread in almost any tissue and also due to the high diversity of K^+-channels expressed in mammalian cells. Some organic compounds and peptides derived from animal venoms affect K^+-channel function preventing current flow, and they are functionally named potassium-channels blockers. Among the venomous animals, scorpions [9,29,35,37] are the main source of potassium-channels toxins (KTxs), followed by spiders [7,34], snakes [12], cone-snail [11,36] and sea anemone [1,6] peptides. These KTxs show different arrangements of their three-dimensional (3D) structures. The folding types earlier found are: αα, α ββ and βαββ [14,22,23]. Despite the conformation differences, most of these peptides have common residues which promote the binding with the potassium-channel vestibule, such as a lysine residue distant from an aromatic residue for 6.6 ± 1.0 Å [3]. The scorpion KTxs are formed by 20–95 amino acid residues stabilized by two, three or four disulfide bonds, making this structure relatively stable. The scorpion KTxs were originally classified into three families named α, β and γ [37], all of them have the highly conserved secondary structural arrangement α/β stabilized by cysteines (CSα/β). More recently, scorpion KTxs presenting a different structural arrangement, with only two α-helices stabilized by two disulfide bonds, CSα/α, were described, and these peptides were named κ-KTxs [2,32]. By possessing the functional dyad for KTxs – the two amino acid residues (Y5 and K19) – their pharmacolog-

* Corresponding author at: Laboratorio de Toxinologia, Departamento de Ciencias Fisiologicas, Instituto de Ciencias Biologicas, Universidade de Brasilia, Brasilia, 70910-900 DF, Brazil. Tel.: +55 61 31073106; fax: +55 61 31073123.
E-mail address: efschwa@unb.br (E.F. Schwartz).

0196-9781
doi:10.1016/j.peptides.2011.05.017

ical targets are thought to be potassium channels. The first κ-KTx described was κ-Hefutoxin1 (systematically named κ-KTx1.1), isolated from the Scorpionidae *Heterometrus fulvipes*, and that blocks Kv1.2 and Kv1.3 channels at μM concentrations [32]. The κ-KTx1.3, which shows 60% identity with the κ-KTx1.1, was isolated from *Heterometrus spinifer*, and had blocking activity on Kv1.1, 1.2, and 1.3 channels [24]. The Om-toxins, isolated from *Opisthacanthus madagascariensis* [2], had lower identities (about 20%) with the κ-KTx1.1, 1.2 and 1.3, and have been classified as κ-KTx2.1, 2.2, 2.3 and 2.4. These peptides also have the CSα/α conformation and the presence of the functional dyad – Y5 (or Y4) and K15 residues, but as the κ-KTx1.1 and 1.2, have low affinity to K^+-channels. The κ-KTx2.3 caused 70% reduction of K^+ currents in Kv1.3 channels, but the effects were obtained at very high concentrations (500 μM) [2].

Using transcriptome approach, we identified in the venom gland of *Opisthacanthus cayaporum*, two sequences showing high identity to the Omtoxins, OcyC8 and OcyC9 [31]. Here we describe the purification and functional characterization of the mature peptide coded by OcyC8 (GenBank ID: FM998750). This novel κ-KTx is a 28 amino acid long peptide with two disulfide bridges, to which, due to its structural characteristics, it was given the systematic name κ-KTx2.5. As the other κ-KTxs, κ-KTx2.5 was capable of blocking reversibly K^+-channels with a Kd at μM concentrations. Due to its low affinity on K^+-channels tested, we evaluated the effect of κ-KTx2.5 on other molecular targets. The results are presented and discussed here with an analysis of structure–function relationship considering the amino acid sequences and a computational simulation of the structural model of the κ-KTx2.5-Kv1.2 complex.

2. Materials and methods

2.1. Purification of the κ-KTx2.5 and peptide synthesis

The crude venom was submitted to chromatography according to [30]. Briefly, the crude venom was obtained by electrical stimulation, freeze-dried, and then dissolved in water and centrifuged at 10,000 × *g* for 10 min. The soluble supernatant was separated by high performance liquid chromatography (HPLC) in a C18 reverse-phase (RP) analytical column (Phenomenex, Inc., USA), using a linear gradient from 0% solvent A (0.12% trifluoroacetic acid, TFA, in water) to 60% solvent B (0.10% TFA in acetonitrile) run for 60 min, at a flow rate of 1 mL/min. The fraction corresponding to the κ-KTx2.5 was further purified in the same column, in a gradient of 20–40% of acetonitrile in 40 min, at 1 mL/min. κ-KTx2.5 was synthesized by solid phase methodology using Fmoc chemistry by GenWay Biotech, Inc. (San Diego, CA). Synthetic peptide was purified by reversed-phase high performance liquid chromatography and characterized by mass spectroscopy and amino acid analysis by GenWay Biotech, Inc. Considering the same disulfide bridge pattern of κ-KTx peptides, the disulfide pairings Cys1–Cys4 and Cys2–Cys3 were adopted for the chemical synthesis of κ-KTx2.5. The purity of synthetic peptide was verified by HPLC analysis and the correctness of the sequence was assessed by MALDI-TOF mass spectrometry measurements. Native and synthetic κ-KTx2.5 were mixed and submitted to HPLC separation using the same conditions used for purification of the peptide. The structural identity between the native and synthetic peptides was verified by RP-HPLC coelution.

2.2. Mass spectrometry

The peptide molecular mass was determined in an UltraFlex II MALDI-TOF/TOF Mass Spectrometer (Bruker Daltonics, Billerica, MA). The peptide was dissolved in an α-cyano-4-hydroxycinnamic acid matrix solution (1:3, v:v), spotted onto a MALDI target plate and dried at room temperature for 15 min. The monoisotopic masses were obtained in reflector mode with external calibration, using the Peptide Calibration Standard for Mass Spectrometry calibration mixture (up to 4000 Da mass range, Bruker Daltonics).

2.3. Circular dichroism of native and synthetic κ-KTx2.5

CD spectra were recorded on a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier type temperature controller. The Far-UV spectra of the peptides in H_2O and 10, 30 and 50% TFE (v/v) at 25 °C were recorded using 0.1 cm pathlength quartz cuvette. Thermal denaturation assays were performed raising the temperature at 0.5 °C/min, from 20 °C to 95 °C. The observed ellipticities were converted into molar ellipticity ($[\theta]$) based on molecular mass per residue of 112 Da. The α-helix secondary structure content was estimated evaluating the signal at 208 nm using the following equation [21]:

$$f_H = \frac{[\theta]_{208} - 4,000}{-33,000 - 4,000}.$$

2.4. Electrophysiological measurements

2.4.1. K^+-channels

Cell culture. Chinese hamster ovary cells (CHO) were routinely cultured in DMEM (Gibco) containing 10% fetal calf serum (Gibco) and maintained at 37 °C in humidified atmosphere with 5% CO_2. Approximately 2×10^4 cells were transfected with 1–1.5 μg of human Kv1.1 or Kv1.4 pcDNA3.1 vector along with 0.2 μg of green fluorescent protein (GFP) in pEGFP-C1 (Clontech, USA) using lipofectamine reagent kit (Invitrogen) following the instructions of the manufacturer. Currents were recorded 24–72 h following transfection.

Solutions. Standard extracellular solution contained (in mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES-NaOH 10, D-glucose 5, adjusted at pH 7.40.

Standard pipette solution contained (in mM): K^+-aspartate 130, NaCl 10, $MgCl_2$ 2, EGTA-KOH 10, HEPES-KOH 10, at pH 7.30 and nominal $[Ca^{2+}]i$ of ~50 nM. Peptides were added to the extracellular solution from stock in distilled water.

2.4.2. Expression of voltage gated ion channels in Xenopus laevis oocytes and electrophysiological recordings

For the expression of the VGPCs ($rK_V1.1$, $rK_V1.2$, $hK_V1.3$, $rK_V1.4$, $rK_V1.5$, $rK_V1.6$, Shaker IR, $rK_V2.1$, $rK_V3.1$, $rK_V4.2$, $rK_V4.3$, hERG) and the VGSCs ($rNa_V1.2$, $rNa_V1.3$, $rNa_V1.4$, $rNa_V1.8$, DmNaV1) in *Xenopus* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMESSAGE-mMACHINE transcription kit (Ambion). The harvesting of stage V–VI oocytes from anaesthetized female *Xenopus laevis* frog was previously described [24]. Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng/nL using a microinjector (Drummond Scientific, USA). The oocytes were incubated in a solution containing (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4), supplemented with 50 mg/l gentamycin sulfate.

Two-electrode voltage-clamp recordings were performed at room temperature (18–22 °C) using a Geneclamp 500 amplifier (Axon Instruments, USA) controlled by a pClamp data acquisition system (Axon Instruments, USA). Whole cell currents from oocytes were recorded 1–4 days after injection. Bath solution composition was (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.7 and 1.5 MΩ. The elicited currents were filtered at 1 kHz and sampled at 500 Hz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. For Na_V channels, representative whole-cell currents were elicited by a 100 ms voltage pulse to 0 mV, from a holding potential of −90 mV. For K_V channels, currents were evoked

by 500 ms depolarizations to 0 mV followed by a 500 ms pulse to −50 mV, from a holding potential of −90 mV.

2.5. Guinea pig ileum

Adult male guinea pigs (*Cavia porcellus*) (180–250 g) were kept fast for 24 h, and then were deeply anesthetized using 100 mg/kg of thionembutal i.p. and euthanized by exsanguination. The ileum was collected and rinsed with Tyrode solution (138 mM NaCl; 2.7 mM KCl; 1 mM $MgCl_2$; 0.36 mM NaH_2PO_4; 12 mM $NaHCO_3$; 5.5 mM D-glucose, pH 7.4). Pieces measuring 2 cm length were cut and kept on aerated Tyrode solution. The segment was tied at one end to an iron stick and submerged in a 10 mL total volume bath, kept at 35 °C. The other end was coupled to an isomeric transducer F-60 connected to a polygraph, both from NARCO BioSystems. The preparation was stabilized for 30 min, ventilated with carbogen (5% CO_2 and 95 O_2) and changing solution each 10 min. After stabilized, bradykinin at concentrations 16×10^3 to 4×10^3 μM was applied into the system, and the effects registered for 1 min. After that, the preparation was rinsed with Tyrode solution for five times. The bradykinin potentiating activity of kappa-KTx2.5 was evaluated by adding the synthetic peptide at concentrations of 3.19, 6.38 or 9.58 μM to the bath 3 min before the application of 4×10^3 μM bradykinin to the bath. The experiment was done in triplicate. The experimental protocol was approved by the University of Brasilia Animal Care and Use Committee (number 46594/2009).

2.6. Antimicrobial assay

The activity of kappa-KTx2.5 toward Gram-positive (*Staphylococcus aureus* ATCC 29213) and Gram-negative (*Escherichia coli* ATCC 25922) bacteria was tested by the broth microdilution assay. The bacteria were grown in Luria-Bertani (LB) medium to the optical density of 0.5 at 600 nm. The highest concentration of the peptide used was 256 μM. Positive and negative controls were carried out with the inoculums plus LB medium and medium only, respectively. The spectrophotometric reading (630 nm) was performed after 12 h incubation time at 37 °C.

2.7. Docking

The docking of the κ-KTx2.5 to the Kv1.2 was performed by AutoDock 4 (http://autodock.scripps.edu/). The κ-KTx2.5 was modeled by Modeller9v6, using the template PDB ID: 1WQD [31]. The Kv1.2 potassium channel coordinates were obtained from its crystal structure PDB ID: 2A79 in its open conformation, and for the docking only the S5 and S6 helices were selected. The interacting portion channel-peptide of Kv1.1, 1.2 and 1.4 are similar. The Kv1.2 channel has a crystal structure, which explains our choice to modeling with the Kv1.2 channel, despite the biological assays done in different in Kv1.1 and 1.4. Both molecules were submitted to atomic charges calculation according to Gasteiger method [10]. The affinity grid maps were built with X-126, Y-126 and Z-126 dimensions, spacing by 0.6 Å. The channel was remained rigid while the peptide flexible, so the docking was carried out through the Lamarkian Genetic Algorithm [20]. For each run were used 15 million evaluations, and the other parameters in default. The results were analyzed with Pymol (http://www.pymol.org/) and the contact maps by the server Sting (http://www.nbi.cnptia.embrapa.br).

3. Results

3.1. Peptide purification and sequencing

The fractionation of the crude soluble venom of *O. cayaporum* by RP-HPLC yielded more than 80 fractions [30]. The component that eluted at 25.9% acetonitrile/0.1% TFA was further purified by analytical RP-HPLC as shown in Fig. 1. The component eluting at retention time of 12.58 min (see inset Fig. 1) was found to be the pure peptide here named κ-KTx 2.5, which corresponded to the predicted mature sequence for OcyC8, a precursor (UniProt ID: C5J89) previously described by our group [31]. This peptide had its N-terminal sequence determined by Edman degradation in earlier study [30]. Monoisotopic molecular mass of this peptide is 3132.26 Da, as expected based on its amino acid sequence.

Sequence similarity searches showed 17–62% identities (see Fig. 2) of κ-KTx2.5 to peptides from κ-KTx family, such as κ-KTx1.1-1.2 (UniProt ID: P82850 and P82851, respectively) isolated from *Heterometrus fulvipes* [32], κ-KTx1.3 from *Heterometrus spinifer* (UniProt ID: P83655) [24], and κ-KTx2.1 and 2.3 (UniProt ID: P0C1Z3 and P0C1Z4, respectively) from *Opisthacanthus madagascariensis* [2].

On the basis of sequence similarities, number of disulfide bridges, CSα/α conformation, and adopting the criteria first defined in [37], implemented by the Swiss-Prot toxin annotation program [15], this peptide constitutes the 8th member of the κ-KTx family, subfamily κ-KTx2 (systematic number: κ-KTx2.5).

3.2. Circular dichroism

The presence of α-helices in both native and synthetic κ-KTx2.5 was confirmed by CD measurements, as indicated by the negative dichroic bands at 208 and 222 nm. Low differences in the CD spectra obtained for synthetic and native κ-KTx2.5κ-KTx2.5 in H_2O or TFE in different concentrations were observed (Fig. 3A and 3B). The fractional helicity, f_H, calculated considering the molar residue ellipticity at 208 nm, $[\theta]_{208}$ [18] were consequently similar for native (60% and 79%) and synthetic (51% and 77%) peptides in water and 50% TFE, respectively. These results indicate that native and synthetic κ-KTx2.5 are most likely to adopt a similar folding pattern in α-helices secondary structure. The thermal stability was also evaluated, and both native and synthetic remained predominantly α-helix in the temperature ranging from 25 to 95 °C.

3.3. Electrophysiology

The native κ-KTx2.5 was tested on hKv1.1 and hKv1.4 channels, transiently expressed in CHO cells (see Section 2.4). At 16 μM concentration, the peptide reduced approximately 20% of the hKv1.1 currents, whereas in hKv1.4 channels the reduction was about 50% of the current (Fig. 4).

Since we noticed that the synthetic peptide had the same activity as that of native one, further experiments were performed using only the synthetic peptide (labeled κ-KTx2.5s). Another reason to use the synthetic peptide was due to the fact that the amount of native peptide available was not enough for conducting the experiments at higher peptide concentration. The concentration–response curves for the κ-KTx2.5s were obtained using hKv1.1 and hKv1.4 channels and the IC_{50} values obtained were 217 ± 46 μM and 71 ± 8.9 μM, respectively (Fig. 5A and C). Fig. 5B shows an example of the Kv1.4 currents obtained in a protocol using 10 mV increment steps from −80 mV to 80 mV, in control (black) and after application of 64 μM of toxin (gray). The I/V ratio in control (black squares) and after application of κ-KTx2.5s (gray circles) show the non-dependence of voltage for the blockage.

The left panel of Fig. 5D illustrates the superimposed traces obtained with hKv1.1 channels recorded with a single 50 mV step depolarization from and holding potential of −80 mV, in control (black) and in the presence of 145 μM of toxin (gray line). An additional trace was obtained by scaling up the toxin current to the control level as shown by the dotted light gray trace. At 50 mV, the running time of the activation process was fitted with a double

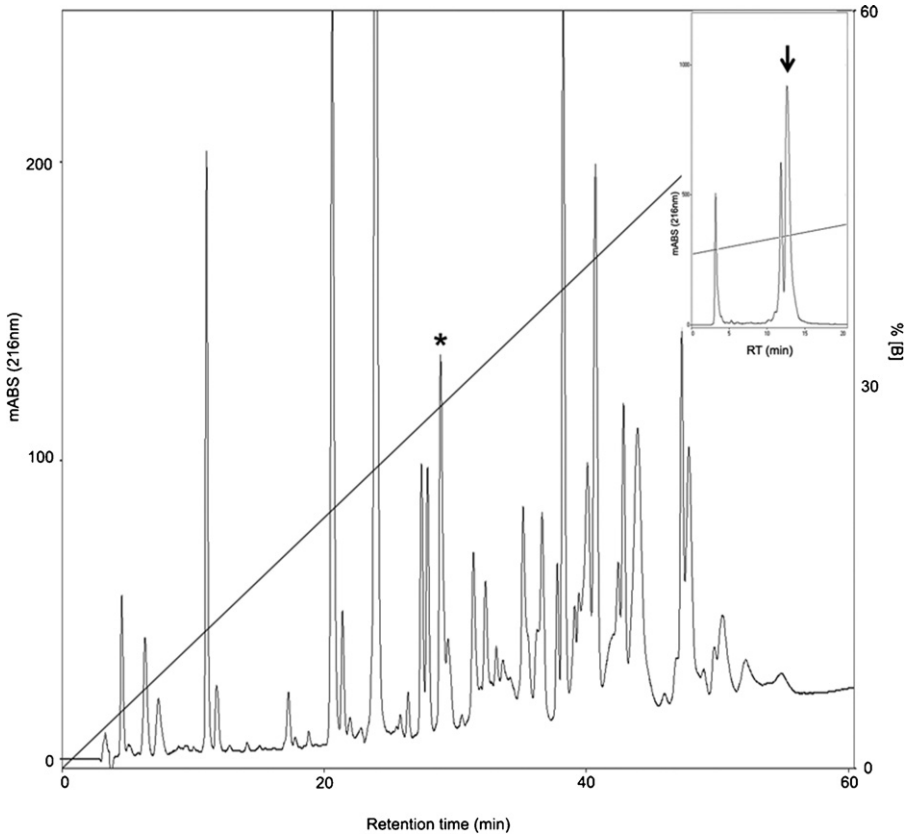

Fig. 1. Purification of κ-KTx2.5 by HPLC. Soluble venom from *O. cayaporum* (1 mg protein content) was separated by RP-HPLC as described in Section 2. The fraction labeled with asterisk (*) contained peptide κ-KTx2.5, which was further separated as shown in the inset and marked with an arrow.

Name	Sequence	%Identity
κ-KTx1.1	GHA**C**YRN**C**WREGNDEET**C**KER**C**------	27
κ-KTx1.2	GHA**C**YRN**C**WREGNDEET**C**KER**C**G-----	26
κ-KTx1.3	GFG**C**YRS**C**WKAGHDEET**C**KKE**C**S-----	17
κ-KTx2.1	-DP**C**YEV**C**LQQHGNVKE**C**EEA**C**KHPVE-	61
κ-KTx2.2	-DP**C**YEV**C**LQQHGNVKE**C**EEA**C**KHPVEY	59
κ-KTx2.3	NDP**C**YEV**C**LQHTGNVKA**C**EEA**C**Q-----	52
κ-KTx2.4	-DP**C**YEV**C**LQQHGNVKE**C**EEA**C**KHP---	62
κ-KTx2.5	YDA**C**VNA**C**LEHHPNVRE**C**EEA**C**KNPVPP	100

Fig. 2. Sequence alignment of known κ-KTX peptides. This figure shows the amino acid sequences and systematic names of all known members of the κ-KTx family. The identity percentage with respect to κ-KTx2.5 is listed on the last column.

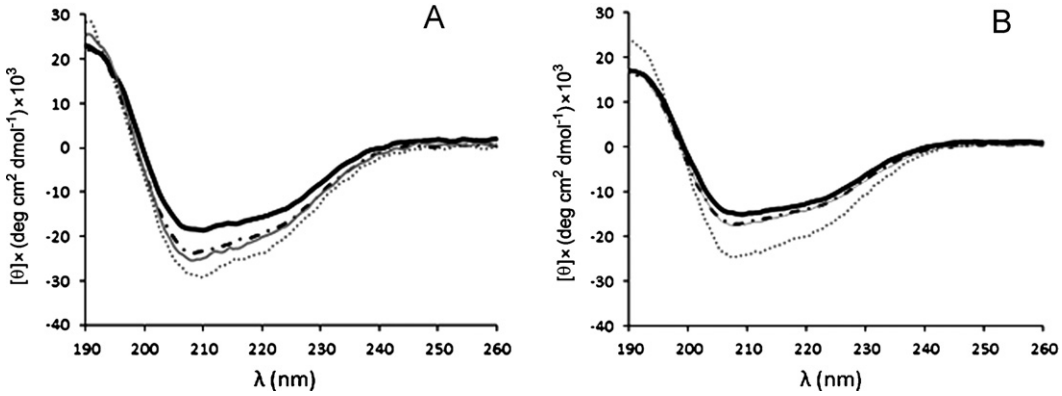

Fig. 3. CD spectra of the κ-KTx peptides. Letter A shows the circular dichroism spectra of native κ-KTX2.5, whereas B shows the spectra of the synthetic peptide, in water (solid black line), 10% TFE (black trace), 30% TFE (solid gray) and 50% TFE (dotted gray). The peptides showed a pattern of ellipticity characteristic of α-helices. θ: ellipticity, λ: wavelength.

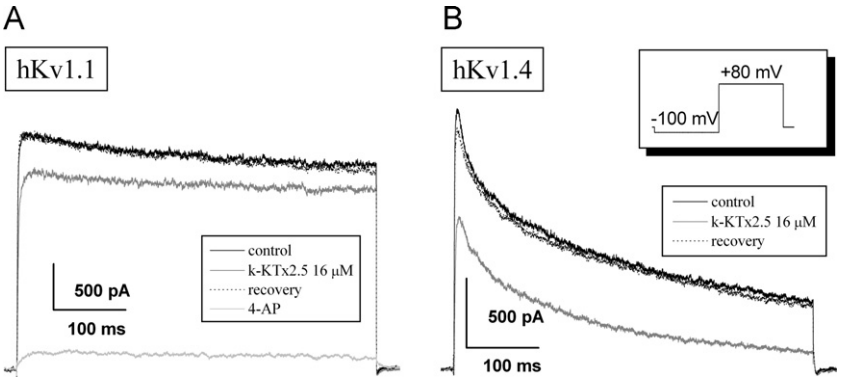

Fig. 4. Blockade of K^+-ion-channels by κ-KTx2.5. Effects of native κ-KTx2.5 on hKv1.1 and hKv1.4 channels. At 16 μM concentration κ-KTx2.5 was able to reversibly reduce the K^+-currents in hKv1.1 (A), and hKv1.4 (B) ion-channels expressed in CHO cells.

exponential equation. For the control, the fast time constant (τ_{fast}) found was 1.8 ± 0.3 ms and the slow time constant (τ_{slow}) was 34 ± 0.4 ms. During toxin application no significant change was noticed for τ_{fast} (3.3 ± 1.2 ms), whereas the toxin was able to increase significantly the τ_{slow} (76 ± 8 ms), as it can be seeing on the bar diagram of Fig. 5D (right panel). A similar effect was previously reported for κ-KTx1.1 in K^+ currents of the type Kv1.3 [32].

Interestingly, in the electrophysiology bioassays done by heterologous expression of ion channels in *Xenopus laevis* oocytes, the synthetic κ-KTx2.5 did not show any blocking activity at a concentration of 250 μM in rKv1.1, rKv1.2, rKv1.3, rKv1.4, rKv1.5, rKv1.6, hERG, Shaker, rKv2.1, rKv3.1, rKv4.2, and rKv4.3 potassium channels, nether in Nav1.2, Nav1.3, Nav1.4, Nav1.8, and DmNav1, sodium channels (at concentration of 2.5 μM).

3.4. Antimicrobial and guinea pig ileum assays

At the concentration of 128 μM, the κ-KTx2.5s had no activity against *E. coli* and *S. aureus*. In the guinea-pig ileum assay, the addition of bradykinin promoted a dose-dependent contraction (data not shown). The κ-KTx2.5s did not induce any effect on segments of guinea-pig ileum by itself. When the ileum segment was pre-incubated with the κ-KTx2.5s, the response to bradykinin was not

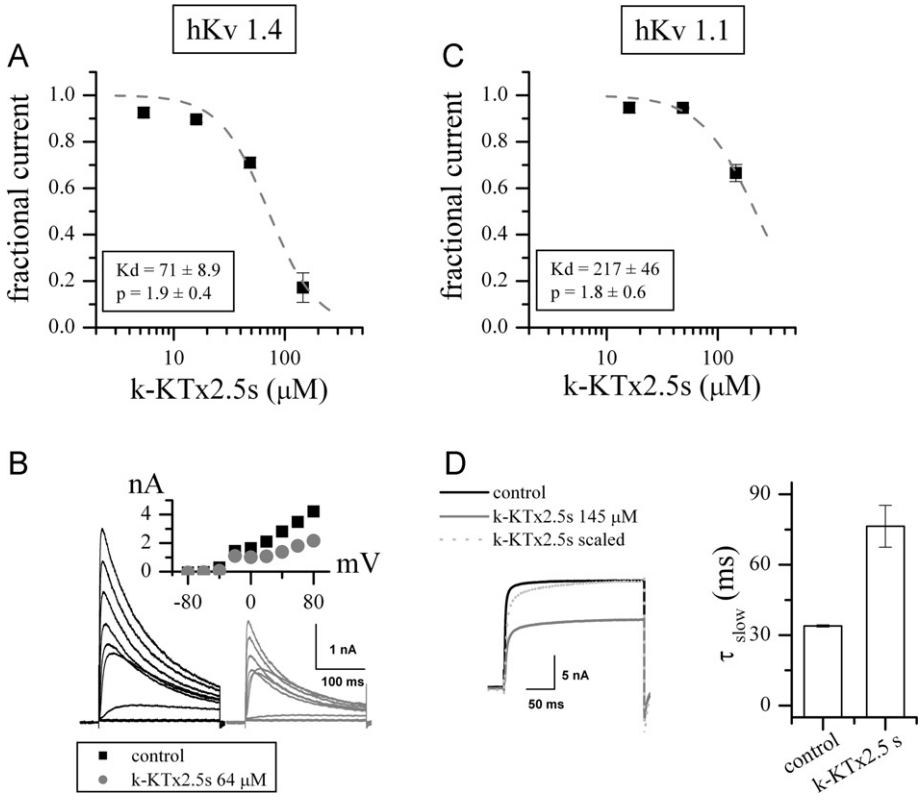

Fig. 5. Dose–response curves of blockade. (A) Concentration dependence for κ-KTx2.5 s inhibition of hKv1.4. Data were fitted with a logistic equation (dash gray curve) giving a IC_{50} of 71 ± 1.9 μM and Hill coefficient of 1.9 ± 0.4. (B) Traces of hKv1.4 currents elicited with a 10 mV increment protocol from −80 mV to 60 mV, with holding potential of −80 mV, in control (black traces) and after 64 μM toxin application (gray traces). The inset shows the *I/V* relation in control (black square) and after toxin application (gray circle). The blocking effect is not voltage dependent. (C) Concentration dependence for κ-KTx2.5s inhibition of hKv1.1. Data were fitted with a logistic equation (gray curve) giving a IC_{50} of 217 ± 46 μM and Hill coefficient of 1.8 ± 0.6. (D) Superimposed hKv1.1 current traces in control (black trace), in 145 μM toxin (gray trace) and toxin trace normalized to the control (dotted light gray). Current were elicited with a single depolarization step at 50 mV. At this potential, the time course of current activation is well fitted with a double exponential equations being τ_{fast} (1.8 ± 0.3 ms) and τ_{slow} (34 ± 0.4 ms) the time constants of the process. During toxin application there is no significant changes on the τ_{fast} (3.3 ± 1.2), but the toxin is able to slow the τ_{slow} (76 ± 8 ms), as shown in the right panel.

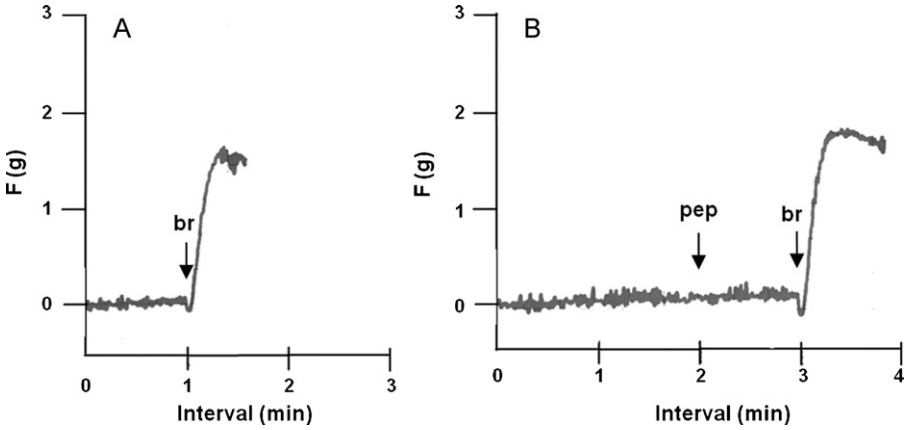

Fig. 6. Guinea pig ileum assay. The graphic (A) shows the record after the addition of bradykinin (br) at 4×10^3 μM; whereas in (B) is the trace obtained after application of κ-KTx2.5 s at 6.39 μM (pep), and application of bradykinin 4×10^3 μM after pre-incubation with κ-KTx2.5s 6.39 μM.

Table 1
Putative interactions between κ-KTx2.5 and Kv1.2 channel obtained by docking method.

k-KTx2.5		Kv1.2		Distance (Å)
Residue	Atom	Residue	Atom	
Ala-7	CB	Gln-326	CB	3.6
Asn-24	CG	Asp-348	CG	3.7
Pro-25	CD	Asp-348	CA	3.1
Pro-27	CD	Met-349	CB	3.5
Lys-23	NZ	Glu-319	OE2	2.9
Lys-23	NZ	Asp-348	OD2	5.1
Lys-23	NZ	Thr-352	OG1	3.0

CA, Alpha Carbon; CB, Beta Carbon; CG, Gamma Carbon; CD, Delta Carbon; NZ, Z Nitrogen; OG1, Gamma Oxygen 1; OD2, Delta Oxygen 2; OE2, Epsilon Oxygen 2.

altered significantly (Fig. 6), based on the average of three experiments.

3.5. Docking

As shown in Fig. 7, docking of κ-KTx2.5 to the vestibule of Kv1.2 suggests that the interacting residues of the channel are situated at the extracellular loop between the transmembrane segments S5 and S6 of the channel (the P-region of the pore of the channel), whereas the amino acids of the scorpion peptide are mostly located at the C-terminal part of the toxin, which lacks structural restraints and may present a higher mobility in solution. The N24 residue of κ-KTx2.5 seems to interact with the D348 residue of the K-channel Kv1.2, with a distance of 3.7 Å, and it happens with only one subunit, leaving the other subunits and the pore free. The toxin K23 residue probably helps the recognition and anchoring to the K^+-channel. The docking shows an interaction between the peptide K23 residue and the channel D348 residue, with the distance of 5.1 Å. Additional interaction suggestions are presented in Table 1.

4. Discussion

The present study reports the purification and some structural and functional characteristics of a new scorpion peptide of the family κ-KTx, named κ-KTx2.5. Using whole soluble venom, this peptide elutes from the HPLC column at the retention time 25.93 min (25.9% acetonitrile/0.1% TFA), and it is obtained in homogeneous form after purification by RP-HPLC in a different gradient, as described. The amino acid sequence of the first 22 amino acid residues of this peptide was previously reported in the manuscript describing the mass spectrometry analysis of *O. cayaporum* venom [30]. However, the full amino acid sequence was identified after sequencing the gene OcyC8 from a cDNA library of the same scorpion, where a precursor (UniProt ID: C5J89) with the same sequence was found [31]. The molecular mass determined for native κ-KTx2.5 (3132.26 Da) was consistent with the expected amino acid sequence identified by DNA sequencing, but was also consistent with the fact that this peptide has four cysteines forming two disulfide bonds. In the two publications previously reported by our group [30,31] the full sequence was not directly verified and no functional activity whatever was described for this peptide. The present communication describes for the first time the full structural features and functional characteristics of κ-KTx2.5. Based on sequence similarities (Fig. 2) a strong suggestion supported the idea that this peptide could be a K^+-channel blocker, belonging to the new κ-KTx family, which was confirmed, as discussed below. Additional confirmation of similarity between native and synthetic peptides came from CD analysis, which indicated similar folding pattern for both molecules (Fig. 3). The secondary structure contents of native and synthetic κ-KTx2.5 peptide, evaluated by CD in water and water/TFE, are similar, presenting high content of α-helices at 50% TFE concentration. The thermal stability of native and synthetic κ-KTx2.5 was tested in temperature ranged from 25 to 95 °C at 10 °C intervals. The CD spectra and unfolding curves (data not shown) revealed no secondary structure variation neither unfolding pattern in the whole temperature range, as indicative of high structural stability of both peptides.

Both native and synthetic κ-KTx2.5 showed blocking activity of K^+-channels (expressed in CHO cells) at micromolar concentrations. The IC_{50} for the synthetic κ-KTx2.5 was about 71 μM on Kv1.4 channels and 217 μM on Kv1.1 channels. This high concentration required for channel blockade suggests that the real biological targets of κ-KTxs could be other subtypes of K^+-channels or even more distinct molecular targets. Attempts to clarify this situation were conducted with κ-KTx2.5s, using the following ion-channels heterologously expressed in *Xenopus* oocytes: rKv1.1, rKv1.2, rKv1.3, rKv1.4, rKv1.5, rKv1.6, hERG, Shaker, rKv2.1, rKv3.1, rKv4.2, and rKv4.3 potassium channels, and in Nav1.2, Nav1.3, Nav1.4, Nav1.8, and DmNav1, sodium channels. At the concentrations assayed no important modifications where found for none of the above channels, using this system. We will come back to this point latter.

All the κ-KTx peptides previously described possess the functional dyad commonly described for the K^+-channel blockers [2,24,32]. This dyad is usually composed of a Lys residue and an aromatic residue (Y, F) [3] distant to each other by 6–7 Å. They are thought to take part in the interaction with the channel, being determinant to the affinity of the peptide, and contributing to the blockage of the potassium flux across the channel [14]. As observed

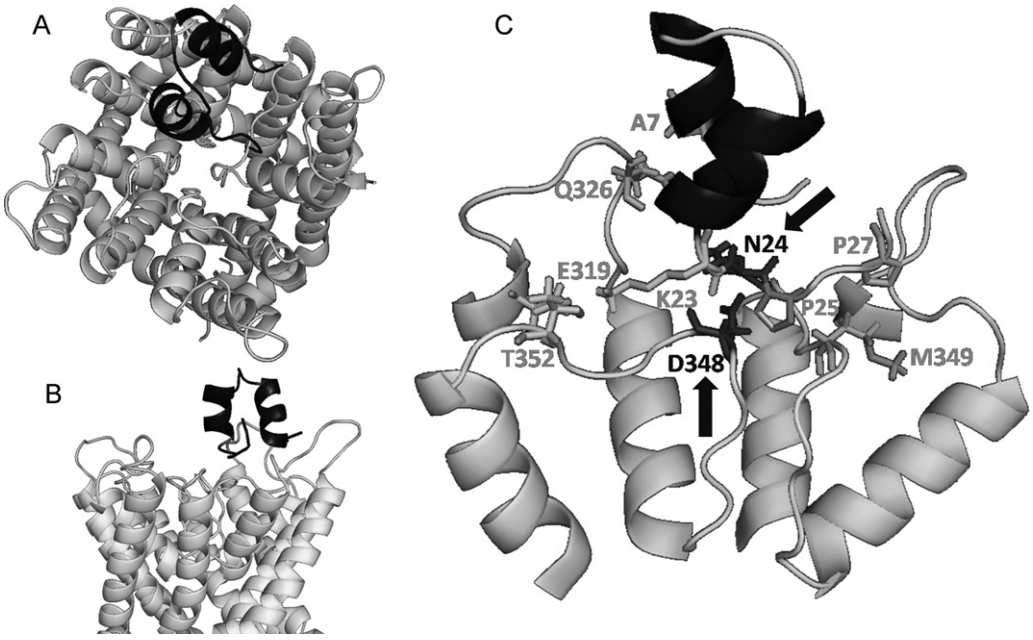

Fig. 7. Modeling of the possible surface contacts between κ-KTX2.5 and the Kv1.2 ion-channel. This cartoon represents the results of docking the toxin in three distinct areas of the channel: the top view in (A) which is the most external surface of the channel, the side view (B) which is the vertical cross-section of the channel and the (C) view, which is a sliced segment of the channel showing only two enlarged subunits of the channel. In the (C) picture, the most probable amino acid residues making contacts between the channel and toxins are indicated, using the stick model The arrows show the contact position of N24 and D348), which correspond to the conserved interaction between a scorpion toxin and a Shaker potassium channel described before (N30/D386) by [17]. Additional amino acids are also indicated, as putative contacts originated by the docking procedure used.

in the alignment with the others κ-KTxs (Fig. 2), the κ-KTx2.5 possesses the amino acid residue V in the position corresponding to the Y, and in the place of the Lys residue, it possess another amino acid in which the side chain has positive charge at pH 7.0, the R15, which could comply with the chemical characteristics required for the binding, as it has been proposed for the K15/K19 present in the others κ-KTxs. Interestingly, from a cone snail species some peptides were described with a tertiary structure that resembles the Csα/α scorpion toxins, and where the functional dyad is absent, with indicative K^+-channel blocking activity [19].

Despite these differences in the amino acid composition between κ-KTx2.5 and the others κ-KTxs, the native κ-KTx2.5 (16 μM) reduced K^+ currents through Kv1.4 and Kv1.1 by 50 and 20%, respectively. The absence of the functional dyad, the K15/K19 and the aromatic residue (Y5 in κ-KTxs), in this case, did not caused the affinity loss for voltage-gated K^+-channels, and is not necessarily essential for the Kv1.1, and Kv1.4 blockage as shown for the κ-KTx2.5 data. For this reason a simulation of the interaction between κ-KTx2.5 and the Kv1.2 channel, whose structure has been solved, was done by an in silico docking. The docking suggests an interaction between the K^+-channel D348 residue and peptide N24 residue, with the distance of 3.7 Å, but it happens only with one channel subunit, and left the remainder subunits free. The peptide stands up one subunit, leaving the channel pore unbarred. A second κ-KTx2.5 added to the docking simulation interacted to another channel subunit (data not shown), and could then clogged the pore mainly by toxin-toxin interactions. This could be sustained by the Hill coefficients experimentally obtained of almost 2. Assuming this is a reasonable mode of interaction between κ-KTx2.5 and K^+-channels, it could explain the great amount of toxin needed to reduce K^+-currents through the channels. It is worth mentioning that the recognition sites of Kv1.x (the loop between S5 and S6 segments) are highly conserved in Kv1.1, Kv1.2, Kv1.3, and Kv1.4 (Fig. 8), particularly the D348 residue, allowing us to extrapolate the experimental data obtained with Kv1.1 and Kv1.4 to the in silico studies.

As reviewed by [28], at least three modes of interaction of K^+-channels and scorpion toxins have been proposed: (1) amino acids residues located in the β-hairpin of the toxin interacting with residues near to the selectivity filter of the channels; (2) amino acid residues of the peptide α-helix interact with residues of the loop between S5 and S6 segments; (3) amino acids residues of both β-hairpin and α-helix of scorpion γ-KTxs interact with the external mouth of the pore region of the Kv11.1 channel, leaving the peptide setting up in the channel. Although the docking data presented here are preliminary, we could suppose that there are new possibilities for interaction and recognition of K^+-channels by scorpion toxins.

Considering the low affinity of κ-KTxs to Kv1 channels, other molecular targets were tested in the present work. The synthetic κ-KTx2.5 was not able to affect K^+-currents through rKv2.1, rKv3.1, rKv4.2, or rKv4.3 potassium channels, nether to alter the function of Nav1.2, Nav1.3, Nav1.4, Nav1.8, and DmNav1, sodium channels using ion-channels heterologously expressed in *Xenopus* oocytes. As the toxin did not blocked rKv1.1 and rKv1.4 expressed in *Xenopus* oocytes even though it blocked human Kv1.1 or Kv1.4 expressed in CHO cells, we suppose the toxin is not a true pore blocker like TTX or several α-KTxs, nor a turret blocker like γ-KTxs, but that it interacts with the phospholipid(s) of the cell membrane surround-

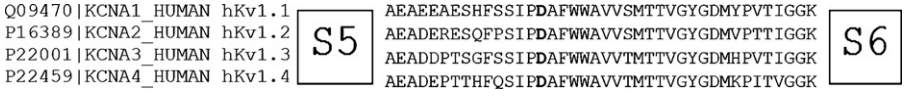

Fig. 8. Amino acid sequence alignment of the pore region of the Kv1.1, Kv1.2, Kv1.3, and Kv1.4 K^+-channels (S5–S6 linker).

ing the K^+-channel protein. Even in the absence of a clear crystal structure of the toxin bound on the channel, or in the absence of mutagenesis data, it can be speculated that κ-KTx2.5 interacts to the outer region of the channel. It can be seen from the top view in Fig. 7A and the docking, the toxin is not located that far away from the lipid environment. Given the fact that the composition of the cell membrane in oocytes is different, it is possible that oocytes represent not the ideal cell system for proper pharmacology of these toxins. In fact, they may be 'absorbed' in the vast surface of cell membrane in oocytes, precluding any block as seen in the case of CHO.

The κ-KTx folding pattern is unusual in scorpion toxins, but it was described for cytotoxic thionin proteins purified from plants, such as the viscotoxins [25]. For this reason, κ-KTx2.5 was tested against bacterial growth. The κ-KTx2.5 has a net negative charge and pI of 4.92, and although most antimicrobial peptides are usually cationic so that the interaction between the helix and negatively charged membrane of bacteria is facilitated, there are some anionic peptides capable of acting as bactericidal [5,33]. We tested the effect of κ-KTx2.5 on *E. coli* and *S. aureus*, but up to the concentration of 128 μM, it did not inhibit growth of both types of bacteria.

The presence of two prolines in the C-terminus of κ-KTx2.5 is characteristic of bradykinin potentiating peptides, such as those from snakes [8,13], and from the scorpion *Tityus serrulatus* [38]. Despite the presence of proline-proline at the C-terminal, the κ-KTx2.5, in micromolar concentrations, did not show any direct effect in segments of guinea-pig ileum, neither potentiated the bradykinin-stimulated contraction. It is known that bradykynin contracts the ileum by a direct action [4] and part of this effect occurs through production of IP3 [26] which by in turn reduces calcium intracellular levels. The classical potassium blockers -TEA and 4-AP – stimulated the tonic activity of intestine slices by blocking Ca^{2+}-dependent K^+-channels [16]. The lack of direct effect on the smooth muscle could also evidence that κ-KTx2.5 does not have activity on Ca^{2+}-dependent K^+-channels.

In conclusion this communication describes structural and functional characteristics of a new member of the κ-KTx scorpion toxins purified from the venom of a scorpion of the family Liochelidae, whose only function found thus far is the blockade, at micromolar concentration, of Kv1.1 and Kv1.4 ion channels. Based on our docking models, it could be that they represent a novel manner by which these peptides interact with ion-channel, although the possibility that there is a different target for the action of these peptides is not discarded. It is known that scorpion and spider peptides are promiscuous in their action [27]. However, a better target candidate is not known yet.

Acknowledgements

Financial support: CNPq/CONACyT (EFS and LDP), CNPq (306281/2006-6; 472731/2008-4 to EFS), CAPES (TSC), FINEP (SMF), F.W.O.-Vlaanderen (G.0257.08 and G.0330.06 to JT), K.U. Leuven (OT-05-64 to JT) and 'Universitaire Attractiepool' of the Federal Government of Belgium (P6/31, UAP to JT). The authors greatly acknowledge Dr Carlos Bloch from Mass Spectrometry Laboratory, EMBRAPA, Brazil, Dr Werner Treptow from Biophysics Laboratory, University of Brasilia, Brazil, and "Laboratório Exame" (Brasília – DF, Brazil) for the kind gift of the bacteria strains used in this work.

References

[1] Castañeda O, Harvey AL. Discovery and characterization of cnidarian peptide toxins that affect neuronal potassium ion channels. Toxicon 2009;54:1119–24.
[2] Chagot B, Pimentel C, Dai L, Pil J, Tytgat J, Nakajima T, et al. An unusual fold for potassium channel blockers: NMR structure of three toxins from the scorpion *Opisthacanthus madagascariensis*. Biochem J 2005;388:263–71.
[3] Dauplais M, Lecoq A, Song J, Cotton J, Jamin N, Gilquin B, et al. On the convergent evolution of animal toxins. Conservation of a dyad of functional residues in potassium channel-blocking toxins with unrelated structures. J Biol Chem 1997;272:4302–9.
[4] Day M, Vane JR. An analysis of the direct and indirect actions of drugs on the isolated guinea-pig ileum. Br J Pharmacol Chemother 1963;20:150–70.
[5] Dennison SR, Howe J, Morton LH, Brandenburg K, Harris F, Phoenix DA. Interactions of an anionic antimicrobial peptide with *Staphylococcus aureus* membranes. Biochem Biophys Res Commun 2006;347(4):1006–10.
[6] Diochot S, Lazdunski M. Sea anemone toxins affecting potassium channels. Prog Mol Subcell Biol 2009;46:99–122.
[7] Estrada G, Villegas E, Corzo G. Spider venoms: a rich source of acylpolyamines and peptides as new leads for CNS drugs. Nat Prod Rep 2007;24(1):145–61.
[8] Ferreira SH, Bartelt DC, Greene LJ. Isolation of bradykinin potentiating peptides from *Bothrops jararaca* venom. Biochemistry 1970;9:2583–93.
[9] Garcia ML, Hanner M, Kaczorowski GJ. Scorpion toxins: tools for studying K^+ channels. Toxicon 1998;36:1641–50.
[10] Gasteiger J, Marsili M. Iterative partial equalization of orbital electronegativity – a rapid access to atomic charges. Tetrahedron 1980;36(22):3219–28.
[11] Han TS, Teichert RW, Olivera BM, Bulaj G. *Conus* venoms – a rich source of peptide-based therapeutics. Curr Pharm Des 2008;14(24):2462–79.
[12] Harvey AL. Recent studies on dendrotoxins and potassium ion channels. Gen Pharmacol 1997;28(1):7–12.
[13] Ianzer D, Konno K, Marques-Porto R, Portaro FCV, Stöcklin R, Camargo ACM, et al. Identification of five new bradykinin potentiating peptides (BPPs) from *Bothrops jararaca* crude venom by using electrospray ionization tandem mass spectrometry after a two-step liquid chromatography. Peptides 2004;25:1085–92.
[14] Jouirou B, Mouhat S, Andreotti N, De Waard M, Sabatier JM. Toxin determinants required for interaction with voltage-gated K-channels. Toxicon 2004;43:909–14.
[15] Jungo F, Bairoch A. Tox-Prot, the toxin protein annotation program of the Swiss-Prot protein knowledgebase. Toxicon 2005;45(3):293–301.
[16] Lamarca V, Grasa L, Fagundes DS, Arruebo MP, Plaza MA, Murillo MD. K^+ channels involved in contractility of rabbit small intestine. J Physiol Biochem 2006;62(4):227–36.
[17] Lipkind GM, Fozzard HA. A model of scorpion toxin binding to voltage-gated K^+ channels. J Membr Biol 1997;158(3):187–96.
[18] Marqusee S, Baldwin RL. Helix stabilization by Glu-.Lys+ salt bridges in short peptides of de novo design. Proc Natl Acad Sci USA 1987;84:8898–902.
[19] Möller C, Rahmankhah S, Lauer-Fields J, Bubis J, Fields GB, Marí F. A novel conotoxin framework with a helix–loop–helix (Cs alpha/alpha) fold. Biochemistry 2005;44(49):15986–96.
[20] Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, et al. Automated docking using a lamarckian genetic algorithm and empirical binding free energy function. J Comput Chem 1998;19:1639–62.
[21] Morrisett JD, David JS, Pownall HJ, Gotto Jr AM. Interaction of an apolipoprotein (apoLP-alanine) with phosphatidylcholine. Biochemistry 1973;12:1290–9.
[22] Mouhat S, Andreotti N, Jouirou B, Sabatier JM. Animal toxins acting on voltage-gated potassium channels. Curr Pharm Des 2008;14(24):2503–18.
[23] Mouhat S, Jouirou B, Mosbah A, De Waard M, Sabatier JM. Diversity of folds in animal toxins acting on ion channels. Biochem J 2004;378:717–26.
[24] Nirthanan S, Pil J, Abdel-Mottaleb Y, Sugahara Y, Gopalakrishnakone P, Joseph JS, et al. Assignment of voltage-gated potassium channel blocking activity to kappa-KTx1.3, a non-toxic homologue of kappa-hefutoxin-1, from *Heterometrus spinifer* venom. Biochem Pharmacol 2005;69:669–78.
[25] Pal A, Debreczeni JE, Sevvana M, Gruene T, Kahle B, Zeeck A, et al. Structures of viscotoxins A1 and B2 from European mistletoe solved using native data alone. Acta Crystallogr D Biol Crystallogr 2008;64(Pt 9):985–92.
[26] Ransom RW, Goodman CB, Young GS. Bradykinin stimulation of phosphoinositide hydrolysis in guinea-pig ileum longitudinal muscle. Br J Pharmacol 1992;105(4):919–24.
[27] Redaelli E, Restano-Cassulini R, Fuentes-Silva D, Clement H, Schiavon E, et al. Target promiscuity and heterogeneous effects of tarantula venom peptides affecting Na^+ and K^+ ion channels. J Biol Chem 2010;285:4130–42.
[28] Rodríguez de la Vega RC, Merino E, Becerril B, Possani LD. Novel interactions between K^+ channels and scorpion toxins. Trends Pharmacol Sci 2003;24:222–7.
[29] Rodríguez de la Vega RC, Possani LD. Current views on scorpion toxins specific for K^+-channels. Toxicon 2004;43:865–75.
[30] Schwartz EF, Camargos TS, Zamudio FZ, Silva LP, Bloch Jr C, Caixeta F, et al. Mass spectrometry analysis, amino acid sequence and biological activity of venom components from the Brazilian scorpion *Opisthacanthus cayaporum*. Toxicon 2008;51(8):1499–508.
[31] Silva EC, Camargos TS, Maranhão AQ, Silva-Pereira I, Silva LP, Possani LD, et al. Cloning and characterization of cDNA sequences encoding for new venom peptides of the Brazilian scorpion *Opisthacanthus cayaporum*. Toxicon 2009;54(3):252–61.
[32] Srinivasan KN, Sivaraja V, Huys I, Sasaki T, Cheng B, Kumar TKS, et al. Kappa-hefutoxin1, a novel toxin from the scorpion *Heterometrus fulvipes* with unique

structure and function. Importance of the functional diad in potassium channel selectivity. J Biol Chem 2002;277:30040–7.
[33] Steffen H, Rieg S, Wiedemann I, Kallbacher H, Peschel A, Garbe C, et al. Structural requirements and mechanism of antimicrobial activity of dermcidin-derived peptides. Exp Dermatol 2006;15:204.
[34] Swartz KJ. Tarantula toxins interacting with voltage sensors in potassium channels. Toxicon 2007;49:213–30.
[35] Tenenholz TC, Klenk KC, Matteson DR, Blaustein MP, Weber DJ. Structural determinants of scorpion toxins: tools for studying K^+ channels. Toxicon 2000;140:135–85.
[36] Twede VD, Miljanich G, Olivera BM, Bulaj G. Neuroprotective and cardioprotective conopeptides: an emerging class of drug leads. Curr Opin Drug Discov Dev 2009;12(2):231–9.
[37] Tytgat J, Chandy KG, Garcia ML, Gutman GA, Martin-Eauclaire MF, van der Walt JJ, et al. A unified nomenclature for short-chain peptides isolated from scorpion venoms: alpha-KTx molecular subfamilies. Trends Pharmacol Sci 1999;20:444–7.
[38] Verano-Braga T, Rocha-Resende C, Silva DM, Ianzer D, Martin-Eauclaire MF, Bougis PE, et al. *Tityus serrulatus* hypotensins: a new family of peptides from scorpion venom. Biochem Biophys Res Commun 2008;371:515–20.