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Assignment of voltage-gated potassium channel blocking activity to κ-KTx1.3, a non-toxic homologue of κ-hefutoxin-1, from *Heterometrus spinifer* venom

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Abstract

A new family of weak K^+ channel toxins (designated κ-KTx) with a novel "bi-helical" scaffold has recently been characterized from *Heterometrus fulvipes* (Scorpionidae) venom. Based on the presence of the minimum functional dyad (Y5 and K19), κ-hefutoxin-1 (κ-KTx1.1) was investigated and found to block Kv 1.2 (IC_{50} ~40 μM) and Kv 1.3 (IC_{50} ~150 μM) channels. In the present study, κ-KTx1.3, that shares ~60% identity with κ-hefutoxin 1, has been isolated from *Heterometrus spinifer* venom. Interestingly, despite the presence of the functional dyad (Y5 and K19), κ-KTx1.3 failed to reproduce the K^+ channel blocking activity of κ-hefutoxin-1. Since the dyad lysine in κ-KTx1.3 was flanked by another lysine (K20), it was hypothesized that this additional positive charge could hinder the critical electrostatic interactions known to occur between the dyad lysine and the Kv 1 channel selectivity filter. Hence, mutants of κ-KTx1.3, substituting K20 with a neutral (K20A) or a negatively (K20E) or another positively (K20R) charged amino acid were synthesized. κ-KTx1.3 K20E, in congruence with κ-hefutoxin 1 with respect to subtype selectivity and affinity, produced blockade of Kv 1.2 (IC_{50} = 36.8 ± 4.9 μM) and Kv 1.3 (IC_{50} = 53.7 ± 6.7 μM) but not Kv 1.1 channels. κ-KTx1.3 K20A produced blockade of both Kv 1.2 (IC_{50} = 36.9 ± 4.9 μM) and Kv 1.3 (IC_{50} = 115.7 ± 7.3 μM) and in addition, acquired affinity for Kv 1.1 channels (IC_{50} = 110.7 ± 7.7 μM). κ-KTx1.3 K20R failed to produce any blockade on the channel subtypes tested. These data suggest that the presence of an additional charged residue in a position adjacent to the dyad lysine impedes the functional block of Kv 1 channels produced by κ-KTx1.3.

Keywords: Scorpion toxin; Voltage-gated potassium channel; Functional dyad; κ-Hefutoxin 1; κ-KTx1.3; *Heterometrus spinifer*

The venoms of the black scorpion *Heterometrus* spp. (Scorpionidae) including the Malaysian black scorpion (*Heterometrus spinifer*) have been reported to be of a lower order of toxicity in comparison to those from the Buthidae scorpions [1] from which highly potent neurotoxins that target Na^+ as well as various subtypes of K^+ channels have been isolated [2]. In contrast, mostly neurotoxins which modify K^+ channel activity, the role of which is presumed to be of minor consequence in lethal envenomation, have been found to be present in low quantities in the venoms of some Scorpionidae members [3–5]. Scorpion toxins that target K^+ channels are compact peptides that typically contain 23–43 amino acid residues and three or four disulfide bridges [2,6,7]. Moreover, almost all of these toxins adopt a highly conserved

Abbreviation: K^+, potassium

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secondary structure, the cysteine-stabilized α/β-fold, which consists of a segment of an α-helix and a double- or triple-stranded β-sheet that are held together in a stable conformation by two or more disulfide bridges [2,7–10]. These toxins have been classified into three-subfamilies, called α-, β- and γ-KTxs [10–12].

We have recently reported the isolation and characterization of a 22-residue long peptide with weak K^+ channel blocking activity (IC_{50} ~40 to 150 μM), κ-hefutoxin 1, from *Heterometrus fulvipes* venom that adopts a novel fold comprising of two parallel α-helices cross-linked by two disulfide bridges [13]. Interestingly, the K^+ channel blocking activity of κ-hefutoxin 1 was deduced following its structural characterization that revealed the presence of two key residues, tyrosine and lysine at positions 5 and 19, respectively, positioned at a distance of~6.0 Å between the lysine's α-carbon and the center of the aromatic face of the tyrosine [13]. Such a critically positioned dyad, composed of a positively charged amino acid and a hydrophobic residue, is well-known to constitute the minimum functional requirement for K^+ channel blocking activity of a variety of toxins isolated from venoms across different phyla [13–15].

We have now isolated a new 23-residue peptide from the venom of *H. spinifer* that shares ~60% identity with κ-hefutoxin 1, and based on sequence similarity, was identified as the third member of the κ-KTx subfamily and hence designated as κ-KTx1.3. However, despite the presence of the putative functional dyad (Y5 and K19) in identical positions in its sequence, κ-KTx1.3 failed to reproduce the blocking activity of κ-hefutoxin 1 on Kv 1.2 and Kv 1.3 channels. On analyzing the primary structure of κ-KTx1.3, it was found that the critical lysine (K19) was flanked by another lysine (K20) and it was postulated that this additional positive charge may hinder critical electrostatic interactions reported to occur between the dyad lysine extremity and the carbonyl oxygen atoms of conserved residues in the Kv 1 channel selectivity filter. We have therefore, chemically synthesized mutants of κ-KTx1.3 substituting the flanking lysine with a neutral amino acid (K20A) or a negatively charged glutamic acid (K20E) as found in κ-hefutoxin 1. Interestingly, by these single-residue substitutions, we were able to assign K^+ channel blocking activity to a scorpion venom-derived peptide that was otherwise inactive on Kv channels.

1. Materials and methods

1.1. Materials

H. spinifer venom was extracted from live scorpions maintained in captivity in the Venom and Toxin Research Laboratory, National University of Singapore as described previously [16]. Pre-packed chromatography columns were purchased from Pharmacia Biotech. All drugs and chemicals were purchased from Sigma Chemicals with the exception of the following, which were obtained from the sources indicated: reagents for N-terminal sequencing, acetonitrile (Fisher Scientific) and trifluoroacetic acid (Fluka Chemika-Biochemika). HPLC-grade water was obtained by using a Milli-Q purification system (Millipore).

1.2. Purification of κ-KTx1.3

Pooled scorpion venom was subjected to ultra-filtration using Mr = 5000 micro-concentrators (Amicon). The venom (500 μl) was transferred to the sample reservoir of the microconcentrator and centrifuged at 4500 × *g* for 90 min at 4 °C. The filtrate of Mr < 5000 was then subjected to a single-step reverse phase HPLC using a Sephasil C8 (0.21 cm × 10 cm) column using a Vision Biocad Workstation (Bio-Rad Laboratories). The column was equilibrated with 0.1% trifluoroacetic acid and the proteins were eluted with a linear gradient (20–50% over 80 min) of eluant (80% acetonitrile in 0.1% trifluoroacetic acid). Elution of proteins was monitored at 215 nm.

1.3. Mass spectrometry

Purified κ-KTx1.3 was subjected to electrospray ionization mass spectrometry as described previously [16] using a Perkin-Elmer Sciex API 300 triple quadrupole instrument (Sciex) equipped with an ion-spray interface. The ion-spray and orifice voltages were set to 4600 and 30 V, respectively. Nitrogen was used as curtain gas with a flow rate of 0.6 l/min while compressed air was used as a nebulizer gas. The sample was infused by flow injection at a flow rate of 50 μl/min using Shimadzu 10 AD pumps as the solvent delivery system. Matrix-assisted laser desorption ionisation–time of flight (MALDI–TOF) mass spectrometry was performed on a Voyager DE-STR Biospectrometry Workstation (Applied Biosystems). Saturated sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (10 mg/ml) in 1:1 acetonitrile:water containing 0.3% trifluoroacetic acid was used as the matrix. The sample (~5 pmol in 1 μl) was spotted onto a stainless steel sample plate with 1 μl of matrix solution and dried off. Molecular ions were generated using a nitrogen laser (337 nm) at an intensity of 1800–2200 and extraction of ions was delayed by 800 ns. The accelerating voltage was set at 25,000 V and the grid and guide wire voltages at 93.0 and 0.3%, respectively. The spectrum was calibrated using external standards.

1.4. Determination of the N-terminal amino acid sequence

Amino terminal sequencing of the native and pyridylethylated protein was done by automated Edman degrada-

tion using a Perkin-Elmer Applied Biosystems 494 pulsed-liquid phase protein sequencer (Procise) with an on-line 785A phenylthiohydantoin-derivative analyzer. For pyridylethylation, native κ-KTx1.3 was re-suspended in 100 μl of denaturant buffer (6.0 M guanidinium hydrochloride, 0.13 M Tris, 1 mM EDTA, pH 8.0) containing 0.07 M β-mercaptoethanol and heated at 37 °C for 2 h. Subsequently, 1.5-fold molar excess (over sulfhydryl groups) of 4-vinylpyridine was added and incubated at room temperature for 2 h, after which the sample was desalted by reverse phase HPLC.

1.5. Peptide synthesis

Linear precursors of κ-KTx1.3 with free (κ-KTx1.3(OH)) and amidated (κ-KTx1.3(NH_2)) carboxy terminals as well their analogues (κ-KTx1.3 K20A, κ-KTx1.3 K20E and κ-KTx1.3 K20R) were synthesized by solid phase methodology with Fmoc chemistry on an Applied Biosystems (model 433A) peptide synthesizer, oxidized by air oxidation and purified by HPLC as described previously [13]. The structures and purity of the synthetic peptides and native κ-KTx1.3 were confirmed by HPLC co-injection analysis and MALDI–TOF mass spectrometry measurements. In order to determine the disulfide pairings, synthetic κ-KTx1.3(OH) (0.4 mg) was dissolved in 0.4 ml of 0.1 M phosphate buffer pH 6.5) and digested with trypsin (0.1 mg at 37 °C for 3 h). The digested peptide fragments were separated by HPLC and subjected to MALDI–TOF mass spectrometry measurements.

1.6. Molecular modeling

The NMR structure of κ-hefutoxin 1 (PDB accession number 1HP9; first out of the ensemble of 20 lowest energy structures) from *H. fulvipes* was used as the template for comparative molecular modeling of κ-KTX1.3. The molecular model was constructed using InsightII (Molecular Simulations Inc., USA) as described previously [17].

1.7. Expression in Xenopus oocytes

Kv 1.1 (rat), Kv 1.2 (rat) and Kv 1.3 (human) channels were studied. Plasmids containing Kv 1.1 were first linearized with PstI (New England Biolabs) 3′ to the 3′ non-translated β-globin sequence in a custom-made high expression vector for oocytes, pGEM-HE [18] and then transcribed using Ambion's mMESSAGE mMachine T7 transcription kit (Ambion). The cDNA encoding Kv 1.2 in its original vector, pAKS2, was first subcloned into pGEM-HE [18]. The insert was released by double restriction digest with *Bgl*II and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of pGEM-HE. For in vitro transcription, the cDNA was linearized with *Sph*I and transcribed using the large-scale T7 mMESSAGE mMACHINE transcription kit (Ambion). The plasmids pCI.neo containing the gene for Kv 1.3 were linearized with *Not*I (Promega) and transcribed like Kv 1.2. Stage-V and -VI *Xenopus laevis* oocytes were harvested by partial ovariectomy under anaesthesia (3-aminobenzoic acid ethyl ester methanesulfonate salt, 0.5 g/l, Sigma). Anaesthetized 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 (in mM: NaCl 96, KCl 2, $MgCl_2$ 1, HEPES 5 adjusted to pH 7.5). Between 1 and 24 h after defolliculation, oocytes were injected with 10 nl of 50–100 ng/μl cRNA. The oocytes were then incubated in ND-96 solution (supplemented with 50 mg/ml gentamycin sulphate) at 16 °C for one day.

1.8. Electrophysiological measurements

Two-electrode voltage-clamp recordings were performed at room temperature using a GeneClamp 500 amplifier (Axon Instruments) controlled by a pClamp data acquisition system (Axon Instruments). Whole-cell currents from oocytes were recorded 1 day after injection. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept as low as possible (<0.5 MΩ). Bath solution composition was (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4). Using a four-pole low-pass Bessel filter, currents were filtered at 1 kHz and sampled at 2 kHz. Current traces were evoked in an oocyte expressing Kv channels by depolarizations to 0 mV from a holding potential of −90 mV. Statistical analysis between groups of data was carried out using the Student's *t*-test and a probability of <0.05 was considered to be statistically significant.

2. Results

2.1. Isolation and purification of κ-KTx1.3

Ultracentrifugation of *H. spinifer* resulted in a filtrate of Mr < 5000, which would likely contain the neurotoxins directed against K^+ channels. The venom ultrafiltrate was then directly subjected to reverse-phase HPLC which resulted in the separation of over 30 small peptides (Fig. 1A). The most prominent peak (identified by arrow) was subjected to mass spectrometry and found to contain a near-homogenous (∼97% purity) peptide with a molecular mass of 2620.58 ± 0.55 Da (electrospray ionization mass spectrometry) (Fig. 1B) and 2620.71 Da (MALDI–TOF mass spectrometry) (data not shown). This peptide was subsequently identified as κ-KTx1.3.

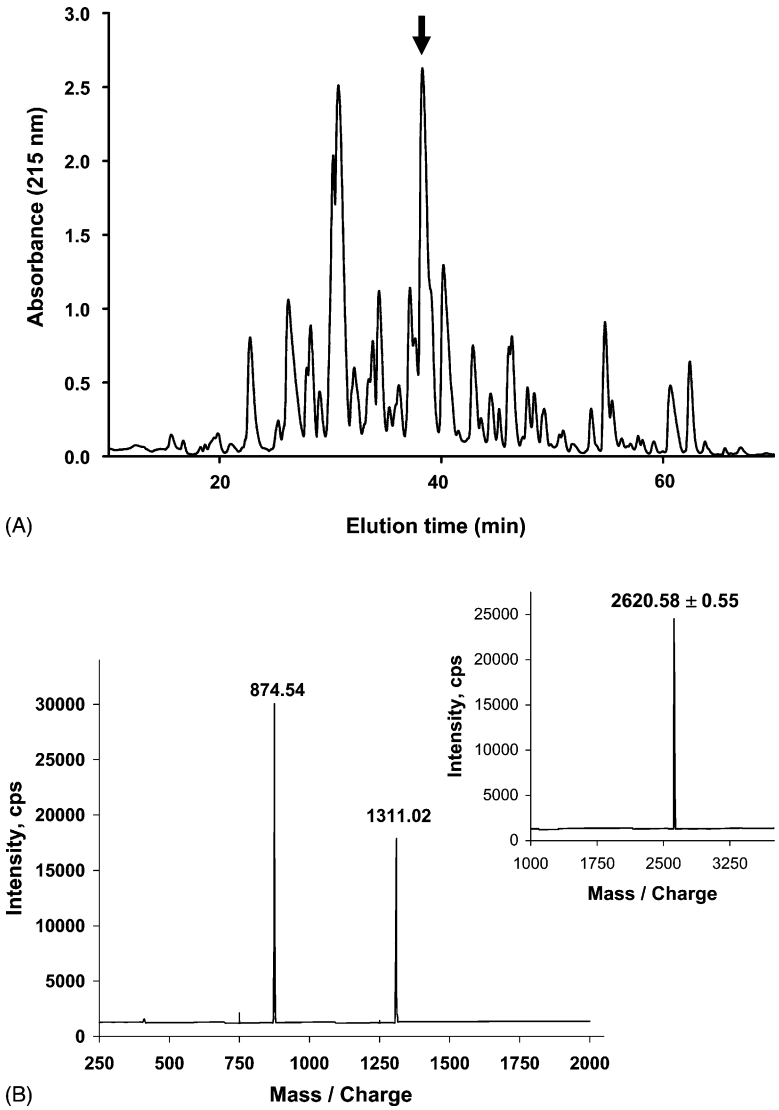


Fig. 1. Isolation and purification of κ-KTx1.3: (A) reverse-phase HPLC of *H. spinifer* venom ultrafiltrate (Mr < 5000) on a Sephasil C8 (0.21 cm × 10 cm) column equilibrated with 0.1% trifluoroacetic acid. Proteins were eluted with a linear gradient (20–50% over 80 min) of eluent (80% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. The elution of proteins was monitored at 215 nm. The peak indicated by arrow was identified as κ-KTx1.3 and subjected to mass spectroscopy, (B) electrospray ionization mass spectrum of κ-KTx1.3 shows a series of multiply charged ions, corresponding to a single, homogenous peptide of molecular mass 2620.58 ± 0.55 Da (inset, reconstructed spectrum).

2.2. Determination of the amino acid sequence of κ-KTx1.3

We were able to unequivocally identify all the residues and determine the complete amino acid sequence of both native (blank cycles where cysteine residues are found) and pyridylethylated κ-KTx1.3. κ-KTx1.3 has 23 amino acid residues including four cysteine residues. Its calculated mass, with the expected two disulfide bridges, was 2620.91, which coincides well with the estimated molecular mass. It shared ∼60% identity to κ-hefutoxin 1 (κ-KTx1.1) and κ-hefutoxin 2 (κ-KTx1.2) [13] and almost no sequence similarity to any other known scorpion toxins. Hence, κ-KTx1.3 was identified as the third member of the recently identified κ-KTx subfamily of weak K^+ channel toxins and was designated as κ-KTx1.3 (Fig. 2). The amino acid sequence of κ-KTx1.3 is deposited in the SWISS-PROT protein database (accession number P83655).

2.3. Solid phase synthesis of κ-KTx1.3

Due to the low yield (∼0.1%) of native κ-KTx1.3, it was chemically synthesized for further characterization. Since κ-hefutoxin 1 and κ-hefutoxin 2 had amidated and free-carboxy termini, respectively, linear precursors of κ-KTx1.3 with both, amidated and free carboxy-termini, were synthesized by Fmoc solid phase method. Random air oxidation of the linear precursors of κ-KTx1.3 afforded a major product which was purified until it migrated as a single peak on analytical HPLC. The purity of synthetic

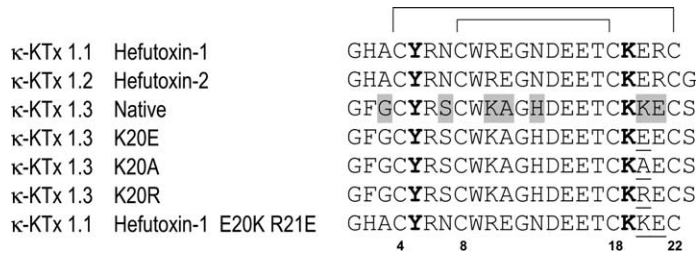


Fig. 2. Amino acid sequences of the members of the κ-KTx subfamily. The cysteines are numbered and the disulfide linkages outlined. The residues contributing to the functional dyad are in bold. The residues by which κ-KTx1.3 differs from κ-hefutoxin 1 are shaded in grey and the mutated residues in κ-KTx1.3 and κ-hefutoxin 1 are underlined. The Swiss-Prot (Swiss Institute for Bioinformatics) accession numbers are P82852, P82851 and P83655, respectively, for κ-hefutoxin 1 and 2 (*Heterometrus fulvipes*) and κ-KTx1.3 (*Heterometrus spinifer*). The International Union of Pure and Applied Chemistry one-letter notation for amino acids is used (*J Biol Chem* 1968;243:3557–9).

peptides was confirmed by analytical HPLC and MALDI–TOF mass spectrometry. Synthetic κ-KTx1.3 with either amidated or free carboxy-termini were co-injected with native κ-KTx1.3 on to an analytical HPLC which resulted in κ-KTx1.3 (OH), but not κ-KTx1.3 (NH_2), eluting together with the native peptide as a single peak (Fig. 3). This confirmed that κ-KTx1.3 has a free carboxyl group at its carboxy-terminal end.

2.4. Assignment of disulfide pairings in κ-KTx1.3

To determine the disulfide bond pairings, synthetic κ-KTx1.3 was digested with trypsin which yielded three digest products of MW 1639.60, 1511.53 and 1039.08 that agreed with the calculated MW for the digested fragments SCWKAGHDEETCKK + H_2O, SCWKAGHDEETCK + H_2O and GFGCYRECS + H_2O, respectively (Fig. 4). These data confirmed that κ-KTx1.3, like κ-hefutoxin-1 and 2 [13], also has disulfide combinations of C1–C4 and C2–C3 (Fig. 5A and 5B). The circular dichroism spectra of synthetic κ-KTx1.3 (OH) revealed that it has a conformation typical of α-helical structure (data not shown).

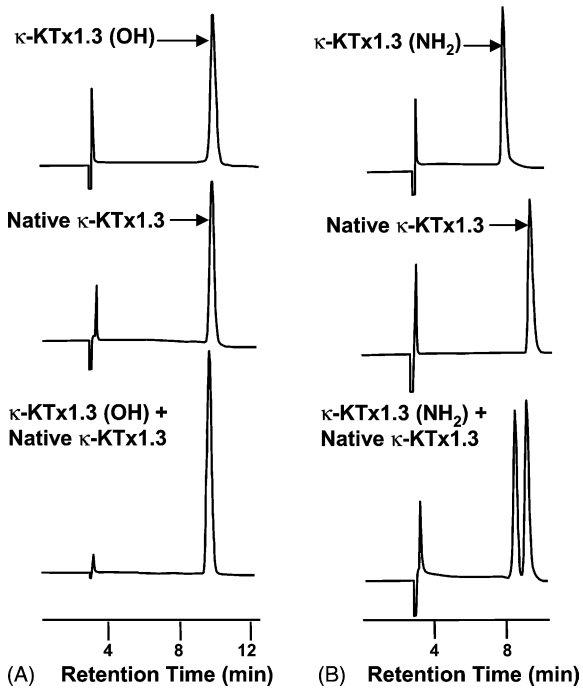
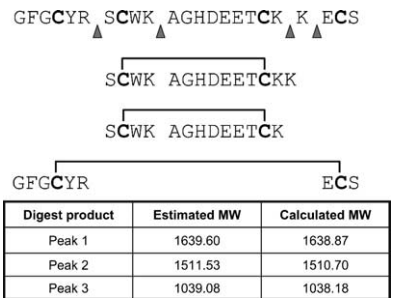


Fig. 3. Synthesis of κ-KTx1.3. Linear precursors of κ-KTx1.3 with free (κ-KTx1.3(OH)) and amidated (κ-KTx1.3(NH_2)) carboxy termini were synthesized by solid phase methodology with Fmoc chemistry. Synthetic κ-KTx1.3(OH) and κ-KTx1.3(NH_2) were co-injected with native κ-KTx1.3 on to an analytical HPLC which resulted in κ-KTx1.3 (OH) (A), but not κ-KTx1.3 (NH_2) (B), eluting together with the native peptide as a single peak. This confirmed that κ-KTx1.3 has a free carboxyl group at its carboxy-terminal end.

2.5. Molecular modeling of κ-KTx1.3

Since κ-KTx1.3 shared ∼60% identity in primary sequence including disulfide pairing with κ-hefutoxin 1, the NMR structure of the latter (PDB accession number 1HP9) was used as a template to create a molecular model of κ-KTx1.3. Like κ-hefutoxin 1, the model of κ-KTx1.3 revealed a compact structure consisting of two parallel α-helices that are held together by the two disulfide bridges (C4–C22 and C8–C18) (Fig. 5A and 5B). The K^+ channel blocking activity of κ-hefutoxin was deduced based on the presence of the minimum functional dyad, K19 and Y5, positioned at a distance of ∼6.01 Å between the α-carbon of the lysine and the center of the aromatic face of the tyrosine (Fig. 5C) [13]. The model of κ-KTx1.3 also



Digest product	Estimated MW	Calculated MW
Peak 1	1639.60	1638.87
Peak 2	1511.53	1510.70
Peak 3	1039.08	1038.18

Fig. 4. Assignment of disulfide pairings in κ-KTx1.3. To determine the disulfide bond pairings, synthetic κ-KTx1.3 was digested with trypsin which yielded three digest products of MW 1639.60, 1511.53 and 1039.08 that agreed with the calculated MW for the digested fragments SCWKAGHDEETCKK + H_2O, SCWKAGHDEETCK + H_2O and GFGCYRECS + H_2O, respectively. These data confirmed that κ-KTx1.3 has disulfide combinations of C1–C4 and C2–C3.

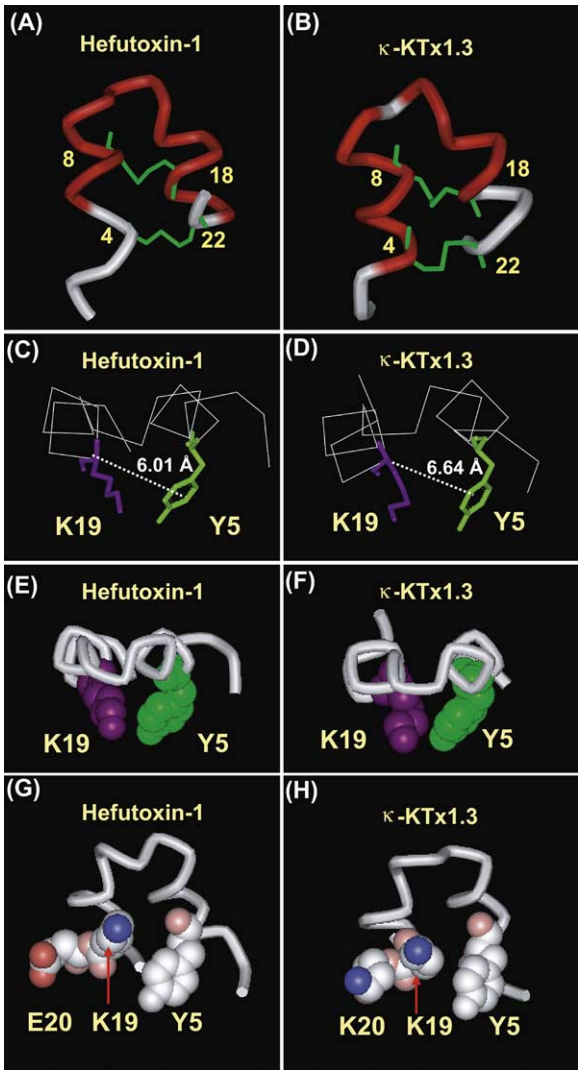


Fig. 5. Molecular model of κ-KTx 1.3. The NMR structure of κ-hefutoxin 1 (A) (PDB accession number 1HP9; first out of the ensemble of 20 lowest energy structures) was used as a template to construct a molecular model of κ-KTX1.3 (B) using InsightII (Molecular Simulations Inc., USA). Like κ-hefutoxin 1, the model of κ-KTx1.3 revealed a compact structure consisting of two parallel α-helices that are held together by the two disulfide bridges (C4–C22 and C8–C18) (shown in green). The K^+ blocking activity of κ-hefutoxin 1 was deduced based on the presence of the functional dyad, Y5 and K19, positioned at a distance of 6.01 Å between the α-carbon of the lysine and the center of the aromatic face of the tyrosine (C). The molecular model of κ-KTX1.3 also showed the presence of this dyad (Y5, K19) positioned at a distance of 6.64 Å (D). The α-carbon backbone structure is presented in C and D. In both, κ-hefutoxin 1 (E) and κ-KTX1.3 (F), the dyad residues (shown in Corey–Pauling–Koltun presentation) K19 (violet) and Y5 (green) protrude out of a flat surface formed by the edges of the two parallel α-helices, in conformity with the general architecture of other pore-blocking K^+ channel toxins. The amino acid residue at position 20, Glu20 in κ-hefutoxin 1 (G) and Lys20 in κ-KTX1.3 (H) is also shown in relation to the functional dyad (K19, Y5) in the respective toxins. In (G) and (H), both toxins are shown in similar orientations along the long-axis of the side chain of K19 with the selected residues in Corey–Pauling–Koltun representation. The amino acid residues are coloured according to their charge, with blue and red depicting positive and negative charge, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

revealed the presence of this functional dyad (K19/Y5) which were positioned at a comparable distance of 6.64 Å between them (Fig. 5D). As observed in κ-hefutoxin 1 (Fig. 5E), both K19 and Y5 in κ-KTx1.3 also protrude out of a flat surface formed by the edges of the two parallel α-helices (Fig. 5F). These data strongly suggested that the molecular targets of κ-KTx1.3 could also be voltage-gated K^+ channels.

2.6. Biological activity of κ-KTx1.3

Synthetic κ-KTx1.3 was screened for K^+ channel blocking activity in *X. laevis* oocytes expressing a single type of voltage-gated K^+ channel (Kv 1.1, Kv 1.2 or Kv 1.3). κ-KTx1.3 did not inhibit currents through Kv 1.1, Kv 1.2 or Kv 1.3 channels even at high concentrations of 1 mM (see Fig. 6) suggesting that it was functionally inert on Kv 1 channels.

2.7. Rationale for the synthesis of mutants of κ-KTx1.3

On analyzing the primary sequences of κ-KTx1.3 and κ-hefutoxins 1 and 2, it was found that the functional dyad lysine (K19) in κ-KTx1.3 was flanked by another lysine (K20) (Figs. 2 and 5). Experimental and modeling data have previously shown the dyad lysine to be a key player in binding to K^+ channels principally via electrostatic interactions between its positively charged extremity and carbonyl oxygen atoms of the channel selectivity filter [9,10,19]. It was thus hypothesized that the presence of an additional positive charge in a position adjacent to the putative dyad lysine (Fig. 5) could impair the electrostatic interactions necessary for toxin binding. Thus, mutants of κ-KTx1.3, with the lysine at position 20 mutated to a neutral (κ-KTx1.3 K20A) or another positively charged (κ-KTx1.3 K20R) amino acid or subjected to charge-reversal (κ-KTx1.3 K20E), were chemically synthesized for further electrophysiological characterization.

2.8. Functional characterization of κ-KTx1.3 mutants

The effects of the κ-KTx1.3 mutants on oocyte-expressed Kv 1.1, Kv 1.2 or Kv 1.3 channels were studied. The application of 200 μM of κ-KTx1.3 K20E produced 80.3 and 38.2% blockade of Kv 1.2 and Kv 1.3 channels, respectively, whereas only a small effect (10.7% blockade) was observed on Kv 1.1 channels (Fig. 6). The addition of 200 μM κ-KTx1.3 K20A resulted in a 44.6% blockade of Kv 1.1 channels as well as 35.0 and 44.1% blockade of Kv 1.2 and Kv 1.3 channels, respectively (Fig. 6). κ-KTx1.3 K20R did not produce any blockade of the three Kv 1 channels.

The blockade induced by κ-KTx1.3 K20A on Kv 1.1, Kv 1.2 and Kv 1.3 (Fig. 7A–C), and by κ-KTx1.3 K20E on Kv 1.2 and Kv 1.3 (Fig. 7D and 7E), were concentration-dependent. The IC_{50} values for κ-KTx1.3 K20A deter-

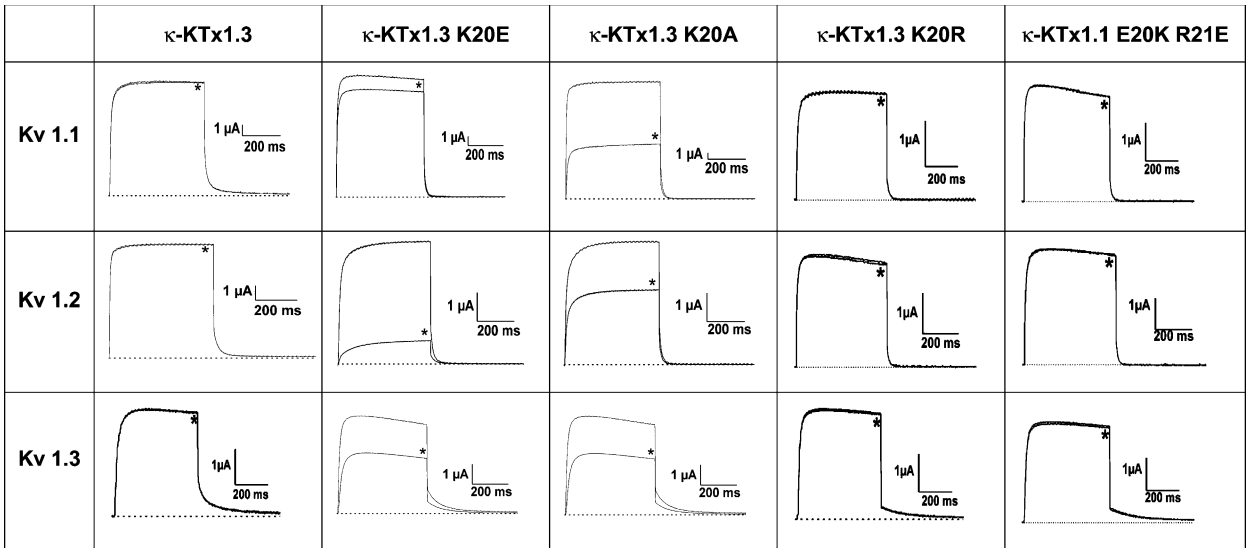


Fig. 6. Effects of κ-KTx1.3, κ-KTx1.3 K20E, κ-KTx1.3 K20A, κ-KTx1.3 K20R and the double mutant κ-KTx1.1 E20K R21E on Kv 1.1, Kv 1.2 and Kv 1.3 channels expressed in *X. laevis* oocytes. Current traces were evoked in oocytes expressing Kv channels by depolarizations to 0 mV from a holding potential of −90 mV and then clamped back to −50 mV. Asterisk (*) indicates the comparison of control condition to the presence of the toxin. After application of 200 μM of κ-KTx1.3 K20E, a small effect was observed on Kv 1.1 channels (10.7 ± 2.4% block) whereas 80.3 ± 6.9% and 38.2 ± 4.1% blockade were obtained on Kv 1.2 and Kv 1.3 channels, respectively. After application of 200 μM of κ-KTx1.3 K20A, we observed 44.6 ± 3.1% block on Kv 1.1 channels, 35.0 ± 3.8% on Kv 1.2 channels and 44.1 ± 4.2% block on Kv 1.3 channels. Native κ-KTx1.3 as well as κ-KTx1.3 K20R and κ-KTx1.1 E20K R21E did not produce any block of the Kv 1 channels tested. Data are the mean ± S.E.M. of at least four experiments.

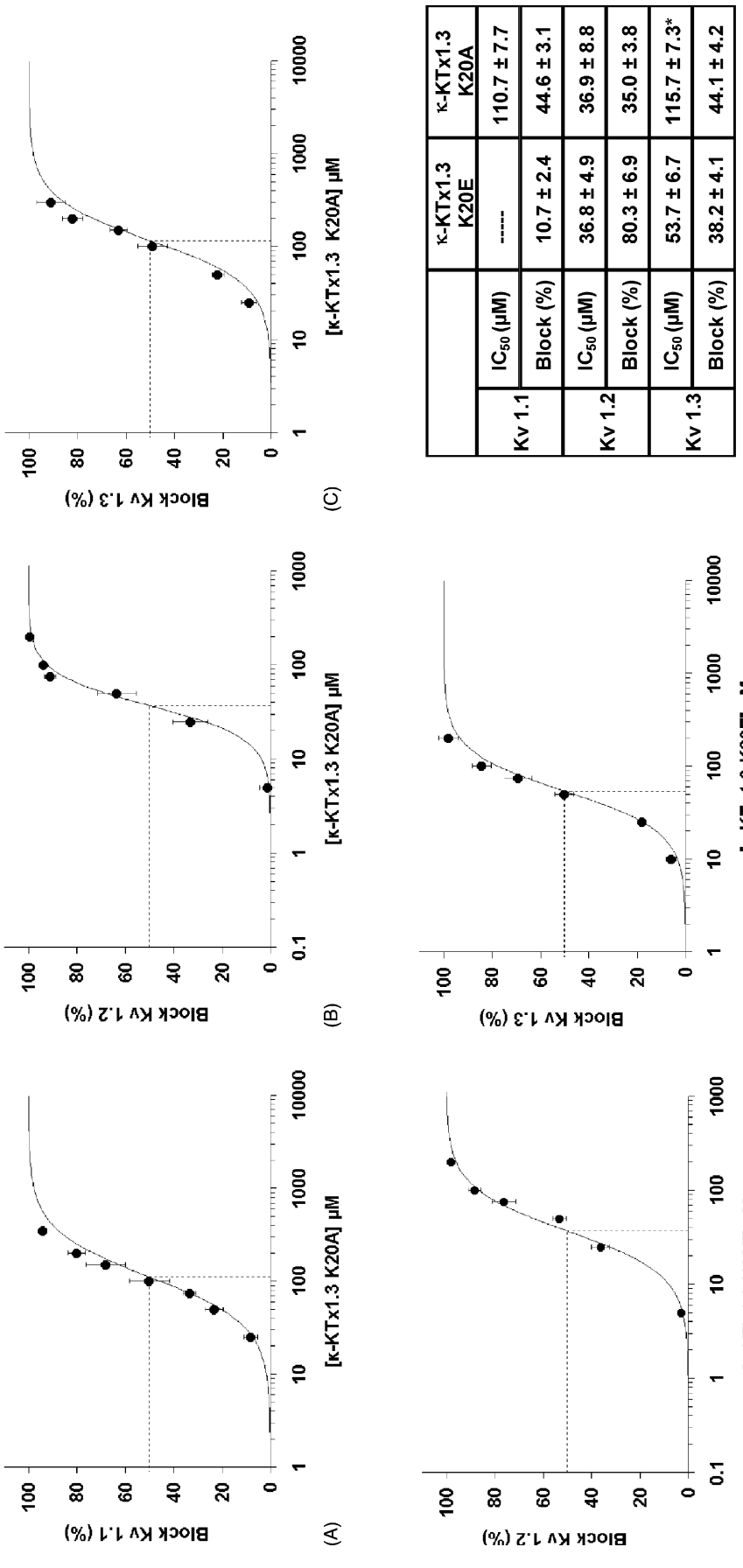
mined by a sigmoidal fit were 110.7 ± 7.7 μM for Kv 1.1, 36.9 ± 4.9 μM for Kv 1.2 and 115.7 ± 7.3 μM for Kv 1.3 (Fig. 6F). The IC_{50} values for κ-KTx1.3 K20E were 36.8 ± 4.9 μM for Kv 1.2 and 53.7 ± 6.7 μM for Kv 1.3 (Fig. 6F). The block by both mutants was reversible upon washing-out and also shown to be voltage-independent (data not shown).

3. Discussion

Molecular models based on comparative analysis of the protein or peptide under investigation can pave the way for key experimental work to determine their biological activity more rapidly and in greater detail [20]. In this context, the significant structural homology between κ-KTx1.3 and κ-hefutoxin 1, including the presence of the functional dyad residues Y5 and K19 in identical positions, strongly suggested that the biological activity of κ-KTx1.3 would correspond to that of κ-hefutoxin 1. Previously, it was on the basis of the secondary structure of κ-hefutoxin 1, specifically the presence of the functional dyad (Y5, K19), that its K^+ channel blocking activity was predicted [13]. This was experimentally confirmed by the demonstration that κ-hefutoxin 1 produced concentration-dependent, voltage-independent and reversible blockade of currents through oocyte-expressed Kv 1.2 and Kv 1.3 channels with IC_{50} values of 40 and 150 μM, respectively [13]. Kv 1.1 channels were not significantly affected by κ-hefutoxin, suggesting that its affinity for Kv 1-type channels was Kv 1.2 > Kv 1.3 ⋙ Kv 1.1. Furthermore, mutational analysis identified Y5 and K19 as the key residues for bioactivity since their mutation to alanine, singly or together, resulted in a total loss of K^+ channel blockade [13].

Despite their unrelated structures and sources, toxins derived from animal venoms across different phyla, contain a key "dyad" composed of a positively charged residue (usually a lysine) and a hydrophobic residue (usually an aromatic amino acid) that constitute a minimal functional core for these toxins to bind to Kv 1 channels [8,13–15,21–23]. Additional residues may confer each toxin with a specific binding profile, the determination of subtype specificity for instance [21,24]. Interestingly, recent studies have also suggested that the functional dyad per se is not a prerequisite for toxin binding to Kv channels, in which case, other residues may act to form "multipoint interactions" with these channels [25–27].

This diverse array of toxins, however, all bind to a highly conserved region among K+ channels located at the pore helix and selectivity filter [9,10,15,22,27]. Generally, the binding of toxins to Kv 1 channels involves a combination of electrostatic, hydrophobic and hydrogen bonding interactions [27]. The major determinant of toxin binding to Kv 1 channels is likely to be the electrostatic interactions between the extremity of the dyad lysine, which protrudes into the ion-channel pore, and carbonyl oxygen atoms of residues from the highly conserved region of the channel selectivity filter [9,10,15,19,22,27,28]. Hydrophobic interactions between the dyad hydrophobic residue and aromatic residues protruding in the channel vestibule are believed to reinforce the critical electrostatic interaction



		κ-KTx1.3 K20E	κ-KTx1.3 K20A
Kv 1.1	IC_{50} (μM)	-----	110.7 ± 7.7
	Block (%)	10.7 ± 2.4	44.6 ± 3.1
Kv 1.2	IC_{50} (μM)	36.8 ± 4.9	36.9 ± 8.8
	Block (%)	80.3 ± 6.9	35.0 ± 3.8
Kv 1.3	IC_{50} (μM)	53.7 ± 6.7	115.7 ± 7.3*
	Block (%)	38.2 ± 4.1	44.1 ± 4.2

(F)

Fig. 7. Concentration dependence of the block induced by κ-KTx1.3 K20A on Kv 1.1, Kv 1.2 and Kv 1.3 (A–C) and κ-KTx1.3 K20E on Kv 1.2 and Kv 1.3 (D and E) channels. Currents were normalized as a function of the maximal block percentage set at 100%. Currents were evoked by a depolarization to 0 mV from a holding potential of −90 mV. The IC_{50} values and the maximal percentage block are shown (F). Data are the mean ± S.E.M. of at least four experiments at each concentration. $^{*}p < 0.05$, statistically significant when compared to κ-KTx1.3K20E.

by surrounding the lysine side-chain and allowing its exclusion from solvent [22]. Thus, it appears that the conserved functional dyad residues from scorpion toxins bind via conserved molecular interactions to Kv 1 channels.

However, on screening κ-KTx1.3 for K^+ channel blocking activity on oocyte-expressed Kv 1-type channels, it was found that the toxin did not show the expected electrophysiological effects even at concentrations as high as 1 mM. On analyzing the primary structure of κ-KTx1.3, it was hypothesized that the presence of an additional positively charged residue (K20) that was found flanking the putative key lysine (K19) in its primary sequence could hinder the proposed electrostatic interaction of κ-KTx1.3 with the K^+ channel. Interestingly, the single mutation of K20 in κ-KTx1.3 to a negatively charged glutamic acid, as found in κ-hefutoxin 1, resulted in the mutant κ-KTx1.3 K20E acquiring biological activity almost identical to that of κ-hefutoxin 1 with respect to subtype selectivity and affinity. Accordingly, κ-KTx1.3 K20E produced blockade of Kv 1.2 (IC_{50} ~37 μM) and Kv 1.3 (IC_{50} ~54 μM) but not Kv 1.1 channels. In contrast, the mutant κ-KTx1.3 K20A produced blockade of both Kv 1.2 (IC_{50} ~37 μM) and Kv 1.3 (IC_{50} ~116 μM) and in addition, also acquired affinity for Kv 1.1 channels (IC_{50} ~111 μM). Although, both κ-hefutoxin 1 and the κ-KTx1.3 mutants produced partial blockade of Kv 1 channels, such partial blocks of these channels have also been reported before for several scorpion toxins, possibly due to imperfect ion channel pore occlusion [28–30]. To provide conclusive evidence of our hypothesis, two other mutants were synthesized: κ-KTx1.3 K20R, wherein lysine 20 was mutated to arginine, and κ-KTx1.1 E20K R21E, a double mutant of κ-hefutoxin 1, with the residues at positions 20 and 21 mutated to lysine and glutamic acid, respectively, as found in κ-KTx1.3. Both caused no significant block on the three Kv channels.

These data suggest that the presence of an additional positive charge in a position adjacent to the dyad lysine in κ-KTx1.3 is sufficient, presumably by electrostatic repulsion, to prevent its ability to produce functional blockade of Kv 1.2 and Kv 1.3 channels. On the other hand, the functional blockade of Kv 1.1 channels by κ-KTx1.3 was impeded if the dyad lysine was flanked by either a positively or negatively charged amino acid. Together, these data support previous reports [13–15,22–24] that Kv 1 channel toxins establish high affinity interactions principally or in part via electrostatic interactions involving the key dyad lysine and in addition suggest that the toxin-induced functional blockade of Kv 1 channels may be easily compromised by changes in the charge environment of the dyad region.

While the κ-KTx subfamily of scorpion toxins, which interact only weakly with Kv 1 channel subtypes, are yet to be investigated for biological activity against other K^+ channels, it must also be remembered that despite the remarkable achievements made in the recent past with respect to the biology of K^+ channels, many K^+ channel currents remain elusive and await the discovery of novel ligands for their identification and characterization [10,12]. Hence it is possible that scorpion toxins such as κ-hefutoxin 1 and κ-KTx1.3 may have been selected by natural evolution for other high-affinity molecular targets that are still currently unknown.

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References

[1] Gwee MCE, Nirthanan S, Khoo HE, Gopalakrishnakone P, Kini RM, Cheah LS. Autonomic effects of some scorpion venoms and toxins. Clin Exp Pharmacol Physiol 2002;29:795–801.
[2] Martin-Euclaire M-F. Neurotoxins from scorpion venoms. In: Massaro EJ, editor. Handbook of neurotoxicology, vol. 1. Totowa, NJ: Humana; 2001. p. 503–8.
[3] Olamendi-Portugal T, Gomez-Lagunas F, Gurrola GB, Possani LD. A novel structural class of K^+ channel blocking toxin from the scorpion *Pandinus imperator*. Biochem J 1996;315:977–81.
[4] Kharrat R, Mansuelle P, Sampieri F, Crest M, Oughideni R, Van Rietschoten J. Maurotoxin, a four disulfide bridge toxin from *Scorpio maurus* venom: purification, structure and action on potassium channels. FEBS Lett 1997;406:284–90.
[5] Lebrun B, Romi-Lebrun R, Martin-Eauclaire MF, Yasuda A, Ishiguro M, Oyama Y, et al. A four-disulphide toxin, with high affinity towards voltage-gated K+ channels, isolated from *Heterometrus spinifer* (Scorpionidae) venom. Biochem J 1997;328:321–7.
[6] Strong PN. Potassium channel toxins. Pharmacol Ther 1990;46:137–62.
[7] Possani LD, Merino E, Corona M, Becerril B. Scorpion genes and peptides specific for potassium channels: structure, function and evolution. In: Menez A, editor. Perspectives in molecular toxinology. Chichester, England: John Wiley & Sons; 2002. p. 201–11.
[8] Darbon H, Blanc E, Sabatier J-M. Three-dimensional structure of scorpion toxins: towards a new model of interaction with potassium channels. Perspect Drug Discov Des 1999;15/16:41–60.
[9] Garcia ML, Gao Y, McManus OB, Kaczorowski GJ. Potassium channels: from scorpion venoms to high-resolution structure. Toxicon 2001;39:739–48.
[10] Rodriguez 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.
[11] Tytgat J, Chandy KG, Garcia ML, Gutman GA, Martin-Euclaire M-F, Walt JJ, et al. A unified nomenclature for short-chain peptides isolated from scorpion venoms: α-KTx molecular subfamilies. Trends Pharmacol Sci 1999;20:444–7.
[12] Possani LD, Selisko B, Gurrola GB. Structure and function of scorpion toxins affecting K+-channels. Perspect Drug Discov Des 1999;15/16:15–40.
[13] Srinivasan KN, Sivaraja V, Huys I, Sasaki T, Cheng B, Kumar TKS, et al. κ-Hefutoxin 1, a novel toxin from *Heterometrus fulvipes* with unique structure and function. Importance of the functional dyad in potassium channel selectivity. J Biol Chem 2002;277:30040–7.
[14] 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.

[15] Menez A, Servent D, Gasparini S. The binding sites of animal toxins involve two components: a clue for selectivity, evolution and design of proteins? In: Menez A, editor. Perspectives in molecular toxinology. Chichester, England: John Wiley & Sons; 2002. p. 175–200.
[16] Nirthanan S, Joseph JS, Gopalakrishnakone P, Khoo HE, Cheah LS, Gwee MC... Biochemical and pharmacological characterization of the venom of the black scorpion (*Heterometrus spinifer*). Biochem Pharmacol 2002;63:49–55.
[17] Srinivasan KN, Nirthanan S, Sasaki T, Sato K, Cheng B, Gwee MCE, et al. Functional site of bukatoxin, a K-type sodium channel neurotoxin from the Chinese scorpion (*Buthus Martensi* Karsch) venom: probable role of the 52 PDKVP 56 loop. FEBS Lett 2001;494:145–9.
[18] Liman ER, Tytgat J, Hess P. Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. Neuron 1992;9:861–71.
[19] Miller C. The charybdotoxin family of K^+ channel-blocking peptides. Neuron 1995;15:5–10.
[20] Possani LD, Rodriguez de la Vega RC. Response to Xu et al.: Hypothesis-driven science paves the way for new discoveries. Trends Pharmacol Sci 2003;24:448–9.
[21] Gasparini S, Danse J-M, Lecoq A, Pinkasfeld S, Zinn-Justin S, Young LC, et al. Delineation of the functional site of α-dendrotoxin. The functional topographies of dendrotoxins are different but share a conserved core with those of other Kv 1 potassium channel-blocking toxins. J Biol Chem 1998;273:25393–403.
[22] Gilquin B, Racape J, Wrisch A, Visan V, Lecoq A, Grissmer S, et al. Structure of the BgK-Kv 1.1 complex based on distance restraints identified by double mutant cycles. Molecular basis for convergent evolution of Kv 1 channel blockers. J Biol Chem 2002;277:37406–13.
[23] Huys I, Tytgat J. Evidence for a function-specific mutation in the neurotoxin, parabutoxin 3. Eur J Neurosci 2003;17:1786–92.
[24] Alessandri-Haber N, Lecoq A, Gasparini S, Grangier-Macmath G, Jacquet G, Harvey AL, et al. Mapping the functional anatomy of BgK on Kv 1.1, Kv 1. 2 and Kv 1. 3. Clues to design analogs with enhanced selectivity. J Biol Chem 1999;274:35653–61.
[25] Batista CV, Gomez-Lagunas F, Rodriguez de la Vega RC, Hajdu P, Panyi G, Gaspar R, et al. Two novel toxins from the Amazonian scorpion *Tityus cambridgei* that block Kv 1.3 and *Shaker* B K^+ channels with distinctly different affinities. Biochem Biophys Acta 2002;1601:123–31.
[26] Mouhat S, Mosbah A, Visan V, Wulff H, Delepierre M, Darbon H, et al. The ''functional'' dyad of scorpion toxin Pi1 is not itself a prerequisite for toxin binding to the voltage-gated Kv 1.2 potassium channels. Biochem J 2004;377:25–36.
[27] Xu C-Q, Zhu S-Y, Chi C-W, Tytgat J. Turret and pore block of K^+ channels: what is the difference?. Trends Pharmacol Sci 2003;24: 446–68.
[28] M'Barek S, Mosbah A, Sandoz G, Fajloun Z, Olamendi-Portugal T, Rochat H, et al. Synthesis and characterization of Pi4, a scorpion toxin from *Pandinus imperator* that acts on K^+ channels. Eur J Biochem 2003;270:3583–92.
[29] Fajloun Z, Carlier E, Lecomte C, Geib S, Di Luccio E, Bichet D, et al. Chemical synthesis and characterization of Pi1, a scorpion toxin from *Pandinus imperator* active on K^+ channels. Eur J Biochem 2000;267:5149–55.
[30] Huys I, Dyason K, Waelkens E, Verdonck F, van Zyl J, du Plessis J, et al. Purification, characterization and biosynthesis of parabutoxin 3, a component of *Parabuthus transvaalicus* venom. Eur J Biochem 2002;269:1854–65.