Structure, Molecular Modeling, and Function of the Novel Potassium Channel Blocker Urotoxin Isolated from the Venom of the Australian Scorpion *Urodacus yaschenkoi*

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ABSTRACT

This communication reports the structural and functional characterization of urotoxin, the first K⁺ channel toxin isolated from the venom of the Australian scorpion *Urodacus yaschenkoi*. It is a basic peptide consisting of 37 amino acids with an amidated C-terminal residue. Urotoxin contains eight cysteines forming four disulfide bridges with sequence similarities resembling the α-potassium channel toxin 6 (α-KTx-6) subfamily of peptides; it was assigned the systematic number of α-KTx-6.21. Urotoxin is a potent blocker of human voltage-gated potassium channel (Kᵥ)1.2 channels, with an IC₅₀ of 160 pM, whereas its affinity for other channels tested was in the nanomolar range (hKᵥ1.1, IC₅₀ 91 nM; hKᵥ1.3, IC₅₀ 70 nM). The toxin had no effect on hKᵥ1.4, hKᵥ1.5, human ether-à-go-go-related gene type 1 (hERG1), or human ether-à-go-go-like (hELK2) channels. Multiple sequence alignments from the venom gland transcriptome showed the existence of four other new peptides similar to urotoxin. Computer modeling of urotoxin’s three-dimensional structure suggests the presence of the α/β-scaffold characteristic of other scorpion toxins, although very likely forming an uncommon disulfide pairing pattern. Using molecular dynamics, a model for the binding of this peptide to human Kᵥ1.2 and hKᵥ1.1 channels is presented, along with the binding of an in silico mutant urotoxin (Lys25Ala) to both channels. Urotoxin enriches our knowledge of K⁺ channel toxins and, due to its high affinity for hKᵥ1.2 channels, it may be a good candidate for the development of pharmacologic tools to study the physiologic functions of K⁺ channels or related channelopathies and for restoring axonal conduction in demyelinated axons.

Introduction

Scorpion venoms provide a rich source of neurotoxins that bind to ion channels. These toxins have proven to be excellent tools for the identification and classification of Na⁺, K⁺, Ca²⁺, and Cl⁻ channels and their families and subfamilies, for the characterization of the tissue distribution of the ion channels and for the understanding of the role of ion channels in certain types of pathologies (reviewed in Possani et al., 1999; Gati et al., 2012).

Potassium channels are ubiquitous membrane proteins found in both excitable and nonexcitable cells (Shieh et al., 2000). Ninety-two K⁺ channel genes have been identified in the human genome (http://www.genenames.org/genefamilies/KCN), which makes K⁺ channels the most diverse of all ion channel families regarding primary structure and physiologic function. They are involved in the regulation of various cellular processes, including cell proliferation (Wonderlin and Strobl, 1996), apoptosis (Burg et al., 2006), hormone secretion, K⁺ homeostasis, neurotransmitter release, and

ABBRévIATiONS: α-KTx, α-potassium channel toxin; 3D, three dimensional; GFP, green fluorescent protein; HEK, human embryonic kidney cells; hELK2, human ether-à-go-go-like; hERG1, human ether-à-go-go-related gene type 1; HPLC, high-performance liquid chromatography; MD, molecular dynamics; Kᵥ, voltage-gated potassium channel; NGS, Next Generation Sequencing; PDB, Protein Data Bank; RF, remaining current fraction; TFA, trifluoroacetic acid.
modulation of the action potential (Bauer and Schwarz, 2001). Consequently, changes in the activity of K⁺ channels due to K⁺ channel gene mutations, drug actions, and/or regulation of channel functions by hormones and neurotransmitters lead to diseases of the central nervous system, heart, kidney, and pancreas (Shieh et al., 2000; Restrepo-Angulo et al., 2010).

Toxins that block K⁺ channels are minor components of scorpion venom, but they are also found in venoms of other organisms (Legros et al., 1996). Based on to the alignment of the cysteine residues and other highly conserved amino acids, the K⁺ channel-blocking scorpion toxins have been classified into four families: α-potassium channel toxin (α-KTX), β-KTX, γ-KTx, and α-KTx (Tytgat et al., 1999; Rodríguez de la Vega and Possani, 2004). The α-KTX is the best studied family; its members are small basic peptides (up to 4 kDa) consisting of 23 to 40 amino acids with the structure stabilized by three to four disulfide bridges. To date, the α-KTX family is classified into 27 subfamilies (http://www.uniprot.org/docs/scorpktx) (Tytgat et al., 1999; Goudet et al., 2002; Huys et al., 2004; Rodríguez de la Vega and Possani, 2004; Tan et al., 2006; Zhijian et al., 2006; Gurrola et al., 2012) with K⁺ channel affinities varying in the micromolar to picomolar range. The α-KTX toxins target mainly the voltage-gated potassium (Kₐ) channels, especially the K₁ family members and some Ca²⁺-activated K⁺ channels (Tytgat et al., 1999; Rodríguez de la Vega and Possani, 2004). The α-KTX toxin structures are similar, and their specificity and affinity depend on the amino acid side chains situated on the external surface and those pointing toward the contact surface with the channel (Rodríguez de la Vega et al., 2003; Jouirou et al., 2004). There are currently 131 known α-K⁺-channel toxins from scorpion venom (Martin-Eauclaire and Bougis, 2012; http://www.uniprot.org/docs/scorpktx).

This work describes the first K⁺-channel toxin from an Urodacidae scorpion. The peptide was isolated from the venom of the Urodacus yaschenkoi scorpion, and its primary structure was determined by automatic Edman degradation and nucleotide sequencing, using a Next Generation Sequencing approach. The peptide is a very potent blocker of the hK₁.2 channel, although it also inhibits hK₁.1, hK₁.3, and hKCa₃.1 channels with lower potency, but does not block other channels included in this study: hK₄.4, hK₄.5, human ether-à-go-go–related gene type 1 (hERG1), and human ether-a-go-go–like (hELK2). The computer-modeled (Maestro software; Schrödinger, Portland, OR) three-dimensional (3D) structure of the toxin revealed an αβ-scaffold characteristic of the α-KTx scorpion toxins. Molecular dynamics simulation showed that the interaction of urotoxin with hK₁.2 relies on the hydrogen bonds between lysines and an arginine on the peptide and acidic residues in the turret and the pore helix of the channel. Additionally, a simulation with mutant-urotoxin (Lys25Ala) indicated that Lys25 does not have the same role in the binding of urotoxin to hK₁.2 as in other α-KTx-6 toxins. Our results suggest that urotoxin may be useful as a pharmacological tool to reveal the physiologic function of hK₁.2 channels in in vitro and in vivo experiments, as a lead for peptidomimetics for the targeting of hK₁.2 channels, and as an experimental therapeutic tool to restore axonal conduction in demyelinated axons, where inhibition of K⁺ channels has beneficial effects (Beraud et al., 2006; Shi and Sun, 2011).

Materials and Methods

Specimen Collection and Venom Extraction

U. yaschenkoi scorpions were collected in the semiarid and arid regions of Australia (near Broken Hill, New South Wales, Australia) by setting pitfalls along red sand dunes. The captured animals were maintained in plastic boxes with water ad libitum and were fed fortnightly with crickets.

Venom was obtained in the laboratory by electrical stimulation in the articulation of the telson, as previously described elsewhere (Luna-Ramírez et al., 2013). The venom was collected in Eppendorf Lo-bind tubes (Eppendorf AG, Hamburg, Germany), and then centrifuged at 14,000g for 15 minutes at 4°C. The supernatant was pooled and finally freeze-dried and stored at −20°C until use. The protein concentration was determined by spectrophotometer Nanodrop (Thermo Scientific, Wilmington, DE) using the default program for proteins at λ = 280 nm.

Venom Separation

Initially, the soluble U. yaschenkoi venom was separated by reverse-phase high-performance liquid chromatography (HPLC). The stored venom was solubilized in water and spun at 10,000g for 5 minutes, then 100 μl containing 3.0 mg of the soluble venom was directly submitted to an analytical C18 reverse-phase column (250 mm × 10 mm) obtained from Vydac (Hesperia, CA). Elution of the venom was made with a linear gradient of solution A [0.12% trifluoroacetic acid (TFA) in water] to 60% solution B (0.10% TFA in acetonitrile), run for 60 minutes, using a Waters 625 LC System coupled with a Waters 996 Photodiode Array Detector at 230 nm of absorbance with 0.5 U sensitivity and eluted at 1 ml/min flow-rate (Waters Corporation, Milford, MA). Fractions were collected manually every 5 minutes and finally freeze-dried using a Speed-Vac Savant drier from ThermoFisher (San Jose, CA).

Based on our previous work (Luna-Ramírez et al., 2013), the fractions containing mainly components with molecular mass ranging from 4–7 kDa were assayed for their K⁺ channels inhibition properties using cellular electrophysiology (patch-clamp technique). The effect of fractions 4 and 5 corresponding to retention times of 15:20:36 and 20:37:20:36 (Fig. 1) were screened against four potassium channels expressed heterologously: hK₁.1, hK₁.4, hERG1 (hK₁.11.1), and hELK2 (hK₁.12.2). Because fraction 4 contained a putative K⁺ channel-blocking peptide, it was further purified (as described later) and screened against eight K⁺ channels.

Repurification of Active Fraction

Fraction 4 was repurified using reverse-phase HPLC with a linear gradient of 5–30% of solution B (0.10% TFA in acetonitrile), run for 60 minutes using the same conditions as described previously. Each peak was collected manually and then assayed once more for the K⁺ channel blocking potency using patch-clamp. This strategy permitted the selection of highly purified peptides for further characterization concerning molecular weight and sequence determination.

Amino Acid Sequence Determination and Mass Spectrometry Analysis

Amino acid sequence determination of the N-terminal segment of the purified peptide was obtained by automatic Edman degradation into a protein sequencer PSQ-31A Shimadzu (Kyoto, Japan) using the chemicals and procedures recommended by the provider.

The molecular weight determination of pure urotoxin was performed by liquid chromatography-electrospray ionization-trap mass spectrometry with a Thermo Electron/Finnigan LCQ Ion Trap Mass spectrometer (San Jose, CA). The sample (0.1–0.5 μg/μl) was dissolved in 50% acetonitrile with 0.1% acetic acid and directly applied into the liquid chromatography-mass spectrometry system as described earlier by our group (Batista et al., 2007).
Sequence Elucidation by Whole Transcriptome Using Next Generation Sequencing

An mRNA-Seq library was generated from the venom gland of a carefully identified specimen of *U. yaschenkoi* according to Illumina’s sample preparation instructions (http://supportres.illumina.com/documents/documentation/chemistry_documentation/samplepreps_truseq/truseq_rna_sampleprep_guide_15008136_a.pdf). The purified cDNA library was used for cluster generation on Illumina’s Cluster Station and then sequenced on Illumina HiSeq 2000 following the vendor’s instructions. Typically, a paired-end sequencing run with a 101 nucleotides (nt) read length was used. For each run, RNA-Seq read was performed. Raw sequencing intensities were then extracted, and the bases were called using Illumina’s RTA software, followed by sequence quality filtering. The extracted sequencing reads were saved as a pair of fastq files for the first and second read, respectively. All raw reads generated from the sequencer were de novo assembled into contigs using the Trinity program (Grabherr et al., 2011). The information concerning proteins of scorpions was collected from the US National Center for Biotechnology Information nonredundant (nr) database. These annotated proteins were aligned to the assembled contigs to identify the homologous genes in *U. yaschenkoi* using TBLASTN (E-value £ 0.1). The nucleotide sequence of urotoxin was obtained from this library by comparison with the amino acid sequence obtained by Edman degradation and the theoretical molecular weight of this sequence with that directly measured by mass spectrometry.

Electrophysiology

Cell Culture. Cell lines were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and maintained at 37°C in 5% CO₂ atmosphere and 95% humidity atmosphere. For transient expression of hK₅.1 and hK₅.4, Chinese hamster ovary cells were cotransfected with plasmids of interest along with plasmid for green fluorescent protein (GFP) by using Lipofectamine (Life Technologies, Carlsbad, CA). Transient expression of hK₅.2: Cos7 cells were transfected with pCMV6 vector containing the GFP-tagged hK₅.2 gene (OriGene Technologies, Rockville, MD) using Lipofectamine 2000 reagent (Life Technologies). Human embryonic kidney (HEK) tsA201 cells were transiently transfected with the hK₅.3.1 gene in pEGFP-C1 (gift of Dr. Heike Wulff, University of California, Davis, CA) or with the hK₅.1.5 gene in pYFP-C1 plasmid (gift of Dr. Antonio Felipe, University of Barcelona, Barcelona, Spain) using Lipofectamine 2000. Cells were cultured under standard conditions. Currents were measured 1 to 2 days after transfection.

The hERG1 and hELK2 channels were stably expressed in Chinese hamster ovary and HEK cells, respectively (Redaelli et al., 2010).
Characterization of the Potassium Channel Blocker Urotoxin

hK1,1, hK1,4, HERG1 plasmids and hELK2 cell line were a gift from E. Wanke, Università di Milano Bicocca, Italy.

K1.3 currents were measured in human peripheral T lymphocytes isolated from healthy male volunteers. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Collected cells were washed twice with Ca²⁺- and Mg²⁺-free Hank's balanced salt solution containing 25 mM HEPES buffer, pH 7.4. Cells were cultured in a 5% CO₂ incubator at 37°C in 24-well culture plates in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 0.5 × 10⁶/ml density for 2 to 6 days. Lymphocytes were activated with 2.5, 5, or 10 μg/ml phytohemagglutinin A (Sigma-Aldrich Kft, Budapest, Hungary) in the culturing medium.

**Solutions.** For recording hK1,1 and hK1,4 currents, the standard extracellular solution contained (mM): 130 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 5 n-glucose, pH 7.40, whereas the pipette solution contained (in mM): 130 K⁺-aspartate, 10 NaCl, 2 MgCl₂, 10 EGTA-KOH, 10 HEPES-KOH at pH 7.4 and nominal free Ca²⁺ concentration of 50 nM (Redaelli et al., 2010). For recording hK1,2, hK1,3, hK1,5, and hKCα3.1, the extracellular (bath) solution contained (in mM): 145 NaCl, 5 KCl, 2.5 CaCl₂, 5.5 glucose, 10 HEPES, and 0.1 mg/ml bovine serum albumin, pH 7.35. For the recordings of hK1.2, hK1.3, hK1.5, and hKCα3.1 currents, the pipette-filling solution contained (in mM) 140 KF, 2 MgCl₂, 1 CaCl₂, 10 HEPES, and 11 EGTA, pH 7.2; for the recording of hKCα3.1 currents, it contained 150 K⁻-aspartate, 5 HEPES, 10 EGTA, 8.7 CaCl₂, 2 MgCl₂, pH 7.2. This latter pipette-filling solution contained 1 μM free Ca²⁺ concentration to fully activate the KCα3.1 current (Grissem et al., 1993).

The hERG1 and hELK2 currents were recorded in a high K⁺ extracellular solution ([K⁺]₀ = 40 mM) where NaCl was replaced by an equimolar amount of KCl and using the K⁺-aspartate-based pipette-filling solution (see earlier). This experimental condition provided the best signal-to-noise relation for hERG1 and hELK2 channels. Due to the low amount of material in fraction 4 of the venom, the high K⁺-extracellular solution was also used to analyze the effect of the venom fractions on the hKv1.1 and hKv1.4 currents. Toxin fractions from concentrated stocks in distilled water were added, giving an overall cation concentration to fully activate the KCa3.1 current (Grissmer et al., 1993).

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**Patch-Clamp Recordings and Data Analysis.** Currents were measured using whole-cell or outside-out patch configuration in voltage clamp mode using Axopatch 200A and MultiClamp 700B amplifiers and Digidata 1200/1440A digitizers. For data analysis, the pClamp9/10 software package was used. For recording of hK1.2, hK1.3, hK1.4, currents, the cells were held at −100 mV holding potential and depolarized to +50 mV for 15 milliseconds (hK1.3, 100 milliseconds (hK1.2), or 40 milliseconds (hK1.5) every 15 seconds to fully activate the currents. For recording of hK1.1 and hK1.4 currents, cells were held at −90 mV holding potential and depolarized to +60 mV for 200 or 300 milliseconds followed by a repolarizing step at −50 mV (100 milliseconds). The time between pulses was 2.7 seconds. For recording of hERG1 tail currents, the cells were held at −80 mV, depolarized at +60 mV for 500 milliseconds to activate the current, and the membrane potential was stepped back at −120 mV to record the inward tail current, pulses were delivered every 5 seconds. The hELK2 currents were evoked by voltage steps to −120 mV for 500 milliseconds from a holding potential of +30 mV. The hKCα3.1 currents were evoked by 150-millisecond voltage ramps from −120 to +40 mV every 10 seconds from a holding potential of −100 mV. Glass micropipettes were pulled from GC 150 F-15 borosilicate capillaries with a resistance of 3–5 MΩ in the bath solution. When necessary, 80–90% of cell capacitance and series resistance errors were compensated for prior to each voltage clamp protocol to decrease the voltage errors to less than 5% of the protocol pulse. The effect of the toxin in a given concentration is displayed as remaining current fraction (RF = I/IO, where I is the current amplitude measured in the presence of the toxin upon reaching block equilibrium, IO is the current amplitude measured in the toxin-free control bath solution). Data points of the concentration-response curves are averages of three to five independent measurements where the error bars represent the S.E.M. The two parameter Hill equation was fitted on the points

\[ RF = \frac{IC_{50}^H}{IC_{50}^H + [Tx]^H} \]

where IC₅₀ is the half-maximum inhibition dose, H is the Hill coefficient, and [Tx] is the toxin concentration. The limited amount of natural urotoxin did not allow determination of the IC₅₀ in the nanomolar range for K1.3 and KC₃.1, so we used the Lineweaver–Burk analysis, where I/RF calculated from three to five independent measurements was plotted as a function of toxin concentration and a straight line was fitted to the points, where IC₅₀ = 1/slope.

**Modeling**

**Homology Model of Urotoxin.** The primary structure of urotoxin shows high sequence similarity with α-KTx-6 family members containing the toxin 2 superfamily region (according to BlastP) and the eight cysteine-binding motif. To model its 3D structure, we used a template based on the most similar toxin to urotoxin that has a crystal or solution nuclear magnetic resonance structure. A search for similar sequences using the BLAST program against the Protein Data Bank Proteins (PDB) database revealed 57.9% identity with the sequence of spinoxin (PDB ID 1V56), whose 3D structure has been solved. The homology model was made with Maestro software (version 9.3; Schrödinger, New York, NY), and the amidation of the last residue was made using the VMD psfgen plugin (http://www.ks.uiuc.edu/Research/vmd/).

**Molecular Dynamics Simulation of Urotoxin Binding to hK1,1 and hK1,2 Channels.** Homology models of the pore domains of hK1.1 and hK1.2 channels from crystal structure of rat K1.2 (PDB 3LUTT) (Chen et al., 2010) were constructed using the methods of Chen et al. (2011). For hK1.1, sequence gi|119395748 was used and for hK1.2 gi|4826782. Molecular dynamics simulation of urotoxin binding to the hK1.1 and hK1.2 homology models was made using Maestro software in the super computer AVOCA (IBM Blue Gene/Q, University of Melbourne). Channel-toxin complexes were refined with molecular dynamics (MD) simulations to identify the interacting residues of urotoxin with hK1.1 and hK1.2 channels. Each channel was embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer (∼80 lipids/leaflet) and a box of explicit TIP3P water (∼87,000 molecules). Approximately 12 K⁺, 88 Cl⁻, and 74 Na⁺ ions were added, giving an overall cation concentration of 0.2 M. First, the system was equilibrated for 25 nanoseconds where the C-α atoms in the toxin and the whole channel were kept rigid to ensure that the membrane had good contact with the protein channel. Subsequently, a 5-nanosecond equilibration with no restraints was performed. Finally, two sets of 10 × 50-nanosecond brute force MD simulations were performed at 310 K using NAMD v2.9 (http://www.ks.uiuc.edu/Research/namd/). Recognition residues and interaction contacts for the binding were identified during this time of simulation. The CHARMM27 force field was used. Visualizations of molecules are in VMD software.

**In Silico Generation of the Lys25Ala Mutant Urotoxin and MD Simulation.** Residue lysine-25 was mutated in silico to alanine (Lys25Ala) with the Mutator plugin from VMD and the 500-nanosecond brute force dynamics simulation was performed as described earlier.

**Results**

**Isolation of Urotoxin and Molecular Weight Determination**

A sample containing 3.0 mg of protein from the soluble venom of the scorpion *U. yaschenkovi* was routinely separated by HPLC, as previously shown elsewhere (Luna-Ramírez...
et al., 2013). The fraction collection from the HPLC was performed every 5 minutes of elution time to reduce the number of fractions to be screened in electrophysiology. Figure 1 shows the HPLC separation of the soluble venom, where the peak at minute 18 (within fraction 4) indicates the elution of the peptide described below. The main component of fraction 4 (labeled with an arrow) was collected separately and finally obtained in pure form (see Fig. 1 inset), using a gradient of 5–30% solution B [0.10% TFA in acetonitrile], over 60 minutes. The pure component eluted at 27.3 minutes (see Fig. 1 inset) and was homogeneous and shown by electrospray ionization-mass spectrometry to contain a peptide with molecular mass of 4012.75 Da, corresponding adequately to the mass fingerprinting made previously by our group (Luna-Ramírez et al., 2013). This peptide is referred to as urotoxin throughout the paper.

**Sequence Elucidation**

Urotoxin was sequenced by automatic Edman degradation giving a partial N-terminal amino acid sequence from residues number 1 up to 19 \((\text{GDIKCSGTRQCGWGPCKKQT})\). Because the peptide was not previously reduced and alkylated for Edman degradation, the X residues were assumed to be cysteines, which was later confirmed using nucleotide sequence analysis. Using Next Generation Sequencing (NGS) analysis, we unveiled the full sequence of this toxin (Supplemental Fig. 1). From the NGS study, four additional new peptides similar to urotoxin were detected (isotigs), which may be isoforms or bona fide similar peptides present in the venom. Alternatively, different genes may encode the same peptide, which has to be confirmed a posteriori. These five similar transcripts were analyzed as previously described elsewhere (Luna-Ramírez et al., 2013) to obtain the mature peptide (Fig. 2). Briefly, with the sequences provided by NGS analysis, having hits with K⁺ channel toxins, the ExPASy Translate tool (http://web.expasy.org/translate/) was used to find the open reading frame; later Blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm that the translated sequence shares homology with K⁺ channel toxins, and finally, the mature peptide was found with SignalP (http://www.cbs.dtu.dk/services/SignalP/).

The experimental molecular weight determined by mass spectrometry of the purified peptide, showed a molecular mass of 4012.75 Da. The translated sequence gave a theoretical expected molecular mass of 4070.7 Da for the folded peptide, meaning there was a difference of 57.95 Da, which corresponds to the molecular mass of glycine. This is consistent with the C-terminal amidation of urotoxin. According to the known processes that occur during expression of scorpion toxins (Becerril et al., 1993), the last residue of the peptide (glycine) is eliminated during the process of maturation, and the amino group of the glycine is used for amidation of the previous residue (resulting in this case in valinamide). Thus, we conclude that the complete amino acid sequence of mature urotoxin is \(\text{GDIKCSGTRQCGWGPCKKQTTCNTSNKCMNGKCKCYGCVC(G)}\), where * means that valine (V) is amidated. The sequence analysis indicates that urotoxin belongs to the \(\alpha\)-KTX-6 subfamily. Toxins from this subfamily share important features in their primary structure such as a central lysine (K25 or K23 in other toxins) that is critical for inhibition of Shaker channels, an essential dyad KC-N at positions 25–28, and the presence of eight conserved cysteines to stabilize the 3D structure of the toxins by disulfide bonds.

**Pharmacologic Effects of Urotoxin**

The in vitro pharmacologic effects of urotoxin were determined on eight K⁺ channels using electrophysiology. Among these, five channels were members of the voltage-gated Shaker family (hK1.1, hK1.2, hK1.3, hK1.4, and hK1.5); other channels included in the study were hERG1 and hELK2 and the intermediate conductance calcium-activated potassium channel hKca3.1. The initial assays were conducted using 10 nM toxin concentrations. When positive results were found, additional concentrations were assayed ideally up to 1 \(\mu\)M. Lack of available material limited the use of higher concentrations in several assays as well as the broadening of the selectivity studies over a wider range of ion channels.

First, fraction 4 (elution time 15 to 20 minutes; Fig. 1) was tested against hK1.1, hK1.4, hERG1, and hELK2. Figure 3 shows the electrophysiologic recordings obtained upon application of 50 \(\mu\)g/ml protein to hK1.1, hK1.4, hERG1, and hELK2 channels. Because fraction 4 contains several components, we cannot specify the exact molar concentration used. This fraction induced an almost complete inhibition of the hK1.1 current, whereas no effect was observed at identical toxin concentrations on other potassium channel types included in the screening (Fig. 3).

Upon purification of urotoxin to homogeneity (Fig. 1), it was assayed against the hK1.1, hK1.2, hK1.3, hK1.5, and hKca3.1 channels to provide more information about its selectivity. Electrophysiologic recordings (Fig. 4A) show that 1 \(\mu\)M of pure urotoxin inhibited hK1.1 channels. The concentration-response curve (remaining current fraction recorded after application of different toxin concentrations) is plotted in Fig. 4B, where the solid line is the best fit resulting in an IC₅₀ and Hill coefficient (H) of 253 nM and 1.1, respectively. The inhibition of the hK1.1 current in the presence of 1 \(\mu\)M urotoxin was fast and reversible (Fig. 4C).

As the hK1.1 channel is closely related to hK1.2 and hK1.3 (Wang et al., 1994; Xie et al., 2010; Robbins and Tempel, 2012), urotoxin was additionally tested in the latter two channels. Urotoxin, at 10 nM, fully blocked the whole cell hK1.2 current (Fig. 5A), and the block was fully reversible.
when the recording chamber was washed using toxin-free solution (Fig. 5C). The concentration-response relationship of the block of hKv1.2 channels gave an IC_{50} of 160 pM (H = 1.1) (Fig. 5E). Figure 5, B and D, shows that 10 nM urotoxin quickly and reversibly inhibits the hKv1.3 current with an IC_{50} of 91 nM (Fig. 5F). These data mean that urotoxin is \( \sim 560 \)-fold selective for hKv1.2 over hKv1.3 and \( \sim 1600 \)-fold when compared with hKv1.1. Based on the classification of Giangiacomo et al. (2004), urotoxin is selective for the hKv1.2 over the hKv1.1 and hKv1.3 channels. Due to its unique binding geometry to K^+ channels (see below), whether urotoxin inhibits the toxin-resistant hKv1.5 channel was also tested. Figure 6D shows that 10 nM urotoxin does not inhibit the hKv1.5 channel (RF = 1.0, S.E.M. = 0.003, N = 5).

Finally, the blocking potency of urotoxin was tested on hKCa3.1 channels for two reasons. First, several peptides that inhibit K_{v1.2} also block this channel, such as maurotoxin (Regaya et al., 2004; Wulff and Castle, 2010) and charybdotoxin (Grissmer et al., 1994). Second, because urotoxin inhibits hKv1.3, and the selectivity of a peptide for hKv1.3 over the other K^+ channel of human T cells, hKCa3.1, is of great interest due to the potential therapeutic application of these peptides.

Figure 6A shows that urotoxin inhibits the hKCa3.1 current with a lower potency than charybdotoxin; the latter peptide was used as a positive control during the recording of the hKCa3.1 current in HEK cells by use of a pipette-filling solution allowing full activation of the channels (see Materials and Methods). Figure 6B shows that 10 nM urotoxin reversibly inhibits the hKCa3.1 current. The Lineweaver–Burk analysis (Fig. 6C) resulted in an IC_{90} = 70 nM, which is two orders of magnitude higher
than the IC50 value for hKv1.2, confirming the preference of urotoxin for the hKv1.2 channel and indicating similar potential of the toxin for inhibiting hKv1.3 and hKCa3.1 channels.

Analyzing the kinetics of the development and the relief of the inhibition equilibrium, we could determine the Kd values of urotoxin for hKv1.1, hKv1.2, and hKCa3.1 channels according to the following formula: $K_d = k_{off}/k_{on}$, where $k_{off}$ is $(T_{OFF})^{-1}$, $k_{on}$ is $((T_{ON})^{-1} - (T_{OFF})^{-1})/|T_x|$, $|T_x|$ is the toxin concentration, and $T_{OFF}$ and $T_{ON}$ are the time constants for the development and relief of the inhibition at a given toxin concentration (Goldstein and Miller, 1993), respectively. For further details see Supplemental Fig. 7. The calculated $K_d$ values were 468 nM, 190 pM, and 109 nM for the hKv1.1, hKv1.2, and hKCa3.1 channels, respectively, in good agreement with the IC50 values obtained from the inhibition equilibrium. For K1.3, the kinetics of association and dissociation of urotoxin were too fast to be resolved due to the inherent properties of K1.3 (i.e., cumulative inactivation).

**Modeling of the 3D Structure of Urotoxin**

Urotoxin shares high sequence similarity with the α-KTx-6 subfamily of scorpion toxins. Maurotoxin (α-KTx-6.2) and spinoxin (α-KTx-6.13) are known members of this subfamily with which urotoxin shares 50 and 57.9% identity, respectively (Fig. 7). The 3D solution structures of maurotoxin and spinoxin are known (Kharrat et al., 1996; Kobayashi et al., 2003; http://www.ebi.ac.uk/pdbe-srv/view/entry/1v56/summary.html); of these, spinoxin was chosen to make the homology modeling of urotoxin. Schrödinger’s Maestro software was used for this purpose. Urotoxin is 37 residues long, although it contains three more amino acid residues than spinoxin (two at the beginning and one more at the end), but the cysteines are conserved in corresponding positions (Fig. 7). Figure 8 shows the modeled tertiary
Fig. 5. Reversible block of hKv1.2 and hKv1.3 currents by 10 nM urotoxin. Urotoxin inhibits hKv1.2, but not hKv1.3, with high affinity. (A) The hKv1.2 currents were measured in a voltage-clamped COS7 cell transiently expressing the channel. Currents were evoked by 200-millisecond depolarizing pulses from a holding potential of −100 to +50 mV every 15 seconds. (B) hKv1.3 currents were recorded in a voltage-clamped activated human peripheral lymphocyte expressing endogenous hKv1.3 channels. The channels were activated by 15-millisecond depolarization pulses from a holding potential of −100 to +50 mV every 15 seconds. Representative traces show the K⁺ currents in the absence (control and wash) and in the presence of 10 nM urotoxin applied in the extracellular bath solution, upon equilibration of the block. (C and D) Time course of the development and the relief of the block of macroscopic hKv1.2 (C) and hKv1.3 (D) currents by 10 nM urotoxin. Gray bars indicate the perfusion of the recording chamber with 10 nM urotoxin in the bath solution. Peak currents were determined from traces shown in A and B during repeated depolarizations (see details in A and B) and plotted as a function of time. (E) Concentration-response of the inhibition of hKv1.2 channels. Remaining current fraction (RF = I/I₀, where I is the peak current in the presence of the toxin in a given concentration and I₀ is the peak current measured in the control solution) is plotted as a function of urotoxin concentration.
structure of urotoxin. It has one α-helix (residue T8-T19) and two β-sheets (residues S24-C26 and Y31-C33, respectively). Residues G1-I3 are random coil (gray); K4 is an isolated bridge (ochre); C5-G7 turn (blue-green); T8-T19 α-helix (purple); T20 coil (gray); C21-N23 turn (blue-green); S24-C26 β-sheet (yellow); M27-G29 turn (blue-green); K30 isolated bridge (ochre); C31-C33 β-sheet (yellow); Y34-C36 3-10-helix (blue), and V37 unarranged or coil (gray). The best fitting for the homology model was obtained with disulfide bridges between C5-C26, C11-C31, C15-C21, and C33-C36, which resembles the cysteine pairing pattern of maurotoxin (C3-C24; C9-C29; C13-C19; C31-C34; Kharrat et al., 1996, 1997; Rochat et al., 1998).

Intensive work was performed for the experimental determination of the disulfide pairing of urotoxin. Unfortunately the native toxin was extremely resistant to proteolytic digestion, a condition necessary to open the folded structure and produce peptide fragments that would facilitate identification of the disulfide arrangement. Several aliquots of native urotoxin were separately treated for digestion with trypsin, a mixture of trypsin and chymotrypsin, and both enzymes in the presence of low amounts of solvent (acetoni-trile) for 24 hours at 37°C. Selective reduction was also performed before proteolytic digestion, but then the fragments obtained were not sufficiently clear to obtain the disulfide pairing.

Molecular Dynamics Simulations

Binding of Urotoxin to the hKv1.2 Channel. From MD simulation, the key residues required for binding to the hKv1.2 channel were identified (Supplemental Data: hKv1.2). The average position of the toxin throughout the simulation is above the pore, possibly preventing the ion conduction (Fig. 9). The toxin binds to the channel mainly in the turret region and on fewer occasions in the pore domain through lysines (K) and arginine (R) interacting strongly with the acidic residues aspartate (D) and glutamate (E) of the channel.

Supplemental Figure 2 shows the bound state of the toxin-channel complex. Some of the most recurrent toxin-channel residue pairs that form H-bonds are shown in Supplemental Fig. 3. In detail, Supplemental Fig. 3A shows urotoxin bound to the channel through K17, forming H-bonds with D363 and D379 at the same time; in fact, it is forming a hydrogen network with one residue of the pore region and the other one from the extended turret region. Supplemental Figure 3B shows another example of binding throughout the simulation where K17-E355 form an H-bond; on the other end of urotoxin’s α-helix, at the same time, R9 forms an H-network with D363 and D379. These bindings occur on one side, in the turret region and on the other side, in the pore helix of the channel. The interaction of K17 and E355 is dynamic: the H bond forms and breaks during the whole simulation, thereby allowing K17 to form an H-network with D363 and D379 as well. Another frequent residue pair that was observed is K30-E353 and K16-D379. These H-bonds also form and break during the simulation, indicating the active nature of the toxin-channel interaction. In all cases, the toxin residues were found to act as hydrogen donors.

The results suggest that the toxin maximizes its electrostatic interactions with the peripheral acidic residues of the channel. Data are in agreement with the surface representation of the toxin and channel, showing the toxin in blue (positive) and the channel in red (negative) electrostatically predisposed to interact (Supplemental Fig. 4). The net charge of the hKv1.2 channel surface is −16 whereas that of urotoxin is +7. It is important to remember that this MD simulation uses brute force dynamics instead of docking, so the toxin and the channel conserve their flexibility and are free to move during the simulation.

Analysis of the toxin-channel interactions shown above reveals that the toxin binds to the channel primarily, but not exclusively, via two types of basic residues, lysines (positions 16, 17, and 30) and one arginine (position 9), to glutamate and aspartate in the channel.

The lysine residue at position 25 in urotoxin (or position 23 in spinoxin and maurotoxin, as well as in certain other toxins) is conserved across all α-KTx-6 scorpion toxins. Several theoretical docking studies have suggested that this lysine residue protrudes into the channel selectivity filter (Yi et al., 2008; Chen et al., 2011; Chen and Chung, 2012), but in this case K16, K17, and R9 were the most active residues, followed by K30, K25, K32, and K4, in forming hydrogen bonds with E355, E353, and D379, and D363 in the turret and pore helix region, respectively (Supplemental Table 1). A hydrogen bond is considered formed if the donor and acceptor atoms (nitrogen or oxygen) are within 3.2 Å of each other.

All the lysines and arginine-9 in the toxin form salt bridges with D363, D379, E353, and E355. K25 forms a salt bridge only with E355 or E353 (Supplemental Table 2). A salt bridge is considered formed if the distance is less than 3.2 Å between a side chain oxygen atom from an acidic residue and a nitrogen atom from a basic residue.

MD Simulation of the Binding of Urotoxin to the hKv1.1 Channel. During the 500-nanosecond simulation (Supplemental Data hKv1.1), K25 and K30 from urotoxin were the most common residues involved in forming hydrogen bonds and salt bridges with D377, E351, and E353 of hKv1.1; none of these residues are in the pore helix domain. Other residues involved in the binding were K4, K16, and R9 from the toxin and D377, Y375, and Y379 from the channel (Supplemental Fig. 5; Supplemental Tables 3 and 4). These bonds alternate during the simulation. Based on these data, an apparently weaker interaction of urotoxin is predicted with the hKv1.1 channel as compared with the hKv1.2 channel (5 times fewer bonds), which agrees well with the experimental data where a higher affinity binding of urotoxin to hKv1.2 was observed (see Figs. 4 and 5).

Binding of Mutant Urotoxin (Lys25Ala) to hKv1.1 and hKv1.2. As mentioned before, within the α-KTx subfamily, K23 has been pointed out as a key residue for the binding of concentration. Peak currents were determined at equilibrium block from experiments shown in A. Fitting the Hill equation to the data points yielded IC50 = 160 µM and H = 1.1. The error bars indicate the S.E.M. of three to five independent measurements. (F) The IC50 for the hKv1.3 channel was determined from Lineweaver–Burk analysis, where 1/RF was plotted as a function of toxin concentration and fitting a line to the points and IC50 = 1/slope, and H = 1 was used for the Hill equation, resulting in IC50 = 81 nM on hKv1.3.
the toxin to Shaker channels. Because the MD results presented here indicate that Lys25 of urotoxin (equivalent to Lys23 in other toxins) is not the key residue for binding to the channel, we decided to run an MD simulation with a mutant urotoxin that lacks K25 (urotoxin-K25A) (Supplemental Data: hKv1.2_Ala and hKv1.1_Ala) to assess the validity of our brute force dynamic simulation and the role of K25 in the binding.

The results after 500 nanoseconds of simulation showed that urotoxin-K25A still binds to hKv1.2 and hKv1.1 channels. Even more, the binding seems to be stronger with this mutant-urotoxin than with native urotoxin (based on the number of bonds made during the simulation). In addition, the binding of the mutant urotoxin involved the same residues as with native urotoxin (all the remaining Ks and R9 from urotoxin binding with the acidic residues in the channel). Therefore, K25 in urotoxin is not the key residue for its binding to hKv1.1 and hKv1.2 channels, and our simulations can be considered accurate (see Supplemental Fig. 6).

**Discussion**

Our work describes the isolation and characterization of the K^+ channel blocker toxin urotoxin from the venom of the Australian scorpion *U. yaschenkoi*. Urotoxin is a selective and potent blocker of the hKv1.2 channel (IC$_{50} = 160$ pM); it also blocks hKv1.1, hKv1.3, and hKCa3.1 channels with at least 400-fold lower potency, but it does not inhibit other channels examined in this study.

Comparison of its sequence—37 amino acids including 8 cysteine residues and C-terminal amidation—with others in the literature shows it belongs to the $\alpha$-KTx-6 family (Tytgat et al., 1999). The systematic number proposed for urotoxin is $\alpha$-KTx 6.21, and its assigned Genbank accession number is
KC818423. This subfamily consists of a cysteine-stabilized α/β-scaffold that comprises an α-helix connected to a double-stranded β-sheet. So far, the α-KTx-6 subfamily is represented by four different scorpion families but has not yet been found in the venom of Buthidae scorpions; urotoxin is the first example from an Urodacidae scorpion.

Based on sequence identity, the closest peptides to urotoxin in the α-KTx-6 subfamily are OcKTx5, OcKTx4, and spinoxin. The OcKTx peptides from Opistophthalmus carinatus (OcKTx) have not been electrophysiologically characterized; however, the predicted structure for these toxins shows the last residue amidated as with urotoxin. OcKTx4 is the only member of the OcKTx family that does not have an amidated C-terminal, probably because it lacks the G at the last position, which is important for amidation of the previous residue (Zhu et al., 2004).

Peptides with ~50–60% identity to urotoxin—Pi-1, Pi-4, hemitoxin, and maurotoxin—all inhibit rat K_1.2 channels with high affinity, ranging from IC50 58 pM to 16 nM (Rochat et al., 1998; M'Barek et al., 2003; Mouhat et al., 2004; Srairi-Abid et al., 2008). Pi-4 has no effect on rat K_1.1 and K_1.3 channels, but a small effect is observed in rat Ca^2+^-activated (SK channel) K_1 channels though the highest affinity for Pi-4 was toward rat K_1.2 (M’Barek et al., 2003). Pi-1 inhibits the voltage-gated K_1 channel of human T lymphocytes K_1.3, with nanomolar affinity (IC50 11 nM) (Peter et al., 1998) and competes with 125I-apamin binding to the SK channels in rat brain synaptosomes with an IC50 of 50 pM (Mouhat et al., 2004). Maurotoxin, similarly to Pi-1, also inhibits K_1.3 but also blocks rat K_1.1 and the Ca^2+^-activated K_1 channels IKCa1 (K_1.3.1) of human T cells (Rochat et al., 1998). Another peptide in the α-KTx-6 subfamily that inhibits K_1.2 with
nanomolar affinity is anuroctoxin (IC_{50} = 5 nM) (Bagdany et al., 2005). Anurotoxin has the lowest identity score to urotoxin and also inhibits K_{1.3} with subnanomolar affinity. Thus, considering the pharmacologic profile of α-KTx-6 toxins, urotoxin and maurotoxin share a similar pattern of ion channel inhibition, except that urotoxin is particularly selective for K_{1.2} channel.

The 3D structure of urotoxin was predicted based on homology modeling using the solution structure of spinoxin. The 3D model of urotoxin shows the α/β scaffold with one α-helix and two β-sheets bound by four disulfide bridges with the following proposed connectivity: 5-26, 11-31, 15-21, 33-36. The β-sheets in urotoxin, spinoxin, and maurotoxin are highly conserved (Table 1), and each has the K_{23} or K_{25} positioned properly to form part of the functional dyad required for bioactivity, complemented with the CYGC at the C termini to provide the aromatic residue for dyad formation. Furthermore, these three toxins have the C termini amidated. Three amino acids (R14, K15, and G33) were reported to be responsible for the nonconventional pairing of the disulfide bridges in maurotoxin (Kharrat et al., 1997). The amino acids in equivalent positions in urotoxin are K16, K17, and G35, which support the validity of the 3D model and the cysteine pairing of urotoxin proposed in this paper. Nonetheless, it would be desirable to confirm experimentally the disulfide bridge pattern of urotoxin. The need for experimental confirmation is underlined by the discrepancy of the 3D model of hemitoxin, which suggested an unusual disulfide bridging pattern, compared with the experimental data that showed a conventional one (Srairi-Abid et al., 2008). The nonconventional disulfide pairing seems to be important for high-affinity binding of maurotoxin to rat K_{1.2} channels. Rendering the disulfide pattern to a conventional one (C1-C5; C2-C6; C3-C7; C4-C8, Pi-1-like) reduced its affinity for K_{1.2} by a factor of ~46 and simultaneously increased its affinity for Shaker B channels by one order of magnitude (M’Barek et al., 2003).

The 3D folding, although important for the high stability of the peptide, does not correlate directly with a specific target molecule (Rodríguez de la Vega et al., 2003). Even though maurotoxin, spinoxin, and urotoxin share high sequence and 3D folding similarities, their affinities appear to be different toward ion channels (Table 2). For example, maurotoxin and urotoxin both inhibit K_{1.1}, K_{1.2}, and K_{1.3} channels, but the affinity of urotoxin for K_{1.2} is at least two orders of magnitude higher than for the other two channels. In contrast, maurotoxin inhibits K_{1.1}, K_{1.2}, and K_{1.3} channels with a smaller degree of selectivity. Specificity for a given channel requires a minimum a 100-fold difference in IC_{50} (Giangiacomo et al., 2004). This criterion is only fulfilled by urotoxin (ratio IC_{50} K_{1.3}/IC_{50} K_{1.2} = 562, for IC_{50} K_{1.1}/IC_{50} K_{1.2} = 1579 and for IC_{50} K_{Ca3.1}/IC_{50} K_{1.2} = 435). In contrast, for maurotoxin the ratio is IC_{50} K_{1.1}/IC_{50} K_{1.2} = 56, and IC_{50} K_{1.3}/IC_{50} K_{1.2} = 225, whereas for spinoxin IC_{50} K_{Ca3.1}/IC_{50} K_{1.2} = 25 (Prof. P. Gopalakrishnakone, National University of Singapore, personal communication).

MD simulation is a powerful tool at the molecular level for understanding the electrophysiologic experiments we performed. The MD simulation shows that residue lysines 16, 17, and 30 and arginine-9 are critical for the blocking of the hK_{1.2} channel, and the residues involved from the channel are the acidic residues aspartate (363 and 379) and glutamate (355 and 353). Urotoxin forms more favorable electrostatic interactions with the outer vestibule of hK_{1.2} over hK_{1.1}, consistent with the selectivity observed experimentally. Our brute force MD simulation results are in agreement with the previous docking models that have been made with α-KTx-6 toxins and Shaker channels, in which “toxins have two to three amino acids that are essential for binding usually arginines or lysines” (Visan et al., 2004; Chen et al., 2011; Chen and Chung, 2012). To our knowledge, this is the first nondocking simulation made with Shaker channels and the longest simulation so far.

Based on in silico docking studies, the general inhibitory mechanism of K_{1.2}-specific toxins consists of two steps.

### Table 1: Secondary structure of urotoxin, spinoxin, and maurotoxin

<table>
<thead>
<tr>
<th>Toxin</th>
<th>α-Helix</th>
<th>β1-Sheet</th>
<th>β2-Sheet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urotoxin</td>
<td>8-19 T-T</td>
<td>24-26 SKC</td>
<td>31-33 SKC</td>
</tr>
<tr>
<td>Spinoxin</td>
<td>10-17 Y-T</td>
<td>22-24 AKC</td>
<td>29-31 CKC</td>
</tr>
<tr>
<td>Maurotoxin</td>
<td>6-17 S-T</td>
<td>22-25 AKC</td>
<td>28-31 SKCK</td>
</tr>
</tbody>
</table>

### Table 2: Bioactivity comparison of maurotoxin, spinoxin, and urotoxin showing IC_{50} values at nM concentrations

<table>
<thead>
<tr>
<th>Toxin/Channel</th>
<th>K_{1.1}</th>
<th>K_{1.2}</th>
<th>K_{1.3}</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maurotoxin</td>
<td>45</td>
<td>0.8</td>
<td>180</td>
<td>SK/KCNN and K_{Ca3.1} (1 nM)</td>
</tr>
<tr>
<td>Spinoxin</td>
<td>NA</td>
<td>2.5</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Urotoxin</td>
<td>253</td>
<td>0.16</td>
<td>91</td>
<td>K_{Ca3.1} (70 nM)</td>
</tr>
</tbody>
</table>

K_{Ca3.1}/K_{Ca3.1}, intermediate conductance calcium-activated potassium channels; NA, not active; SK/KCNN, apamin-sensitive small conductance calcium-activated potassium channels.
During the first step, a ring of positively charged amino acids (R10, R19, K30, and K33 in Pi4) guide the recognition and correct positioning of the toxin by means of electrostatic interactions with acidic residues in the channel. The subsequent high-affinity binding is mediated by hydrophobic forces and hydrogen bonding between the dyad (Y35 in Pi4) and the aromatic cluster of amino acids of the channel (W366, W367, Y377), whereas the dyad lysines protrude into the selectivity filter and is stabilized there by carboxyl oxygens of the selectivity filter aspartate-379 (M'Barek et al., 2003). Our MD simulation supports strongly the existence of this first step, where electrostatic interactions guide urotoxin to the pore of hK1.2. In addition, the formation of H-bonds takes place involving D379 from the channel and the lysines in the toxin, emphasizing a similar mechanism to other in silico docking simulations; however, the final position of the toxin in the pore was beyond the limits of the simulation.

Although the dyad hypothesis (Dauplais et al., 1997) is suitable to explain the binding of many toxins to the channel pore, high-affinity binding of Tc32 to K1.1.3 was also described in the absence of these critical residues in the toxin (Batista et al., 2002). Furthermore, the presence of the functional dyad itself is not a prerequisite for the binding of Pi-1 to K1.2 (Mouhat et al., 2004), where mutant toxins with substituted dyad residues (IA24, A33, PI-1) still displayed affinity for K1.2. These results allow alternative explanations for the final step of toxin-channel interaction, which may evolve from the position of urotoxin in the pore predicted by the MD simulation (Fig. 9). Nevertheless, the MD simulation with the mutant urotoxin (K25A) supports the accuracy of our simulations as the binding still happens in the absence of K25. This result might suggest that urotoxin would not be able to block the hK1.5 channel as K25 does not make unfavorable interactions with the charged amino acid at the entrance of the pore. The experiment (Fig. 6D) did not confirm this hypothesis. It is important to consider that E355 is missing in K1.5 (it is G461 in the equivalent position of K1.5), which may explain the lack of inhibition of the hK1.5 current by urotoxin (please note E355 in K1.2 and K1.1 is important in urotoxin binding).

Overall, these results demonstrate the potential for urotoxin to become a pharmacologic tool to characterize the hK1.2 channel. Knowledge of the interacting surfaces between the channels and toxins may allow the design of specific drugs to control pathologies associated with K+ channels, such as demyelinating diseases (e.g., Guillain-Barré syndrome, multiple sclerosis) and Lambert-Eaton myasthenic syndrome (Judge and Bever, 2006; Shi and Sun, 2011).

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**Conducted experiments:** Luna-Ramírez, Bartok, Restano-Cassulini, Coronas, Quintero-Hernández, Christensen.

**Contributed new reagents or analytic tools:** Luna-Ramírez, Possani, Quintero-Hernández.

**Performed data analysis:** Luna-Ramírez, Restano-Cassulini, Bartok, Quintero-Hernández, Coronas, Christensen, Panyi, Possani.

**Wrote or contributed to the writing of the manuscript:** Luna-Ramírez, Quintero-Hernández, Restano-Cassulini, Bartok, Wright, Panyi, Possani.

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