A family of orthologous proteins from centipede venoms inhibit the hKir6.2 channel

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Inhibitors targeting ion channels are useful tools for studying their functions. Given the selectivity of any inhibitor for a channel is relative, more than one inhibitor of different affinities may be used to help identify the channel in a biological preparation. Here, we describe a family of small proteins in centipede venoms that inhibit the pore (hKir6.2) of a human ATP-sensitive K⁺ channel (hKATP). While the traditional peptide-sequencing service gradually vanishes from academic institutions, we tried to identify the sequences of inhibitory proteins purified from venoms by searching the sequences of the corresponding transcriptomes, a search guided by the key features of a known hKir6.2 inhibitor (SpTx1). The candidate sequences were cross-checked against the masses of purified proteins, and validated by testing the activity of recombinant proteins against hKir6.2. The four identified proteins (SsdTx1-3 and SsTx) inhibit hKATP channels with a Kᵩ of <300 nM, compared to 15 nM for SpTx1. SsTx has previously been discovered to block human voltage-gated KCNQ K⁺ channels with a 2.5 μM Kᵩ. Given that SsTx inhibits hKir6.2 with >10-fold lower Kᵩ than it inhibits hKCNQ, SsTx may not be suitable for probing KCNQ channels in a biological preparation that also contains more-SsTx-sensitive KATP channels.

Results
Searching for hKir6.2-inhibiting activity and biochemical purification. The interaction between SUR1 and Kir6.2 is normally necessary for Kir6.2 to reach the cell membrane. However, if the C-terminal 26 residues in the Kir6.2 polypeptide chain are deleted, the resulting mutant Kir6.2 channel (dubbed Kir6.2-ΔC26) alone can reach the membrane without the co-expression of SUR1. This mutant channel has a markedly reduced ATP sensitivity. Also, to a varying degree, many PNDM-causing point mutations lower Kir6.2’s sensitivity to ATP, among which the V59G mutation nearly abolishes the sensitivity. Thus, we combined the two mutations, ΔC26 and V59G, to create a mutant channel that not only expresses as a functional channel on its own but also conducts
robust current even in the presence of millimolar concentrations of intracellular ATP in an intact cell. The channel with this background double-mutation ΔC26-V59G, dubbed Kir6.2^{R11A)', served as a convenient preparation for us to search for and study the blocker of the Kir6.2 pore here in a heterologous expression system.

Figure 1a shows that the venom of the centipede Scolopendra subspinipes dehaani (Ssd; a species that inhabits Asia) suppressed the current through hK_{ATP} channels, formed by hKir6.2 co-expressed with hSUR1, in Xenopus oocytes. To biochemically identify the underlying inhibitory proteins, we purified the venom proteins through sequential steps of HPLC chromatography and tested the activities of the resulting fractions (Figs 1b and 2). The crude venom was first fractionated on a semi-preparative reversed-phase C18 column (Fig. 2a). Two of the resulting fractions, indicated by the magenta and blue arrows, contained inhibitory activity against hK_{ATP} channels and were further fractionated on an analytical C18 column (Fig. 2b,c).

In this second round of HPLC purification, the fraction, indicated by the blue arrow in Fig. 2a, was fractioned to three major peaks, among which the middle, smallest peak contained the inhibitory activity, labeled as SsdTx1 for latter reference (Fig. 2b). An adjacent peak also contained weaker inhibitory activity, called SsdTx2. The fraction, which is indicated by the magenta arrow in Fig. 2a, exhibited two major peaks, between which the second, smaller peak contained the inhibitory activity, dubbed SsdTx3 (Fig. 2c). Like SpTx1, the materials that underlie these active peaks have molecular masses ~6 kDa determined on mass spectrometry.

On the basis of our present experience in purifying a minimal amount of these active materials for mass analysis and previous experience in purifying and identifying SpTx1, it would be practically unfeasible for us to determine the full sequence of any of the three potential inhibitors by the Edman degradation method. For SsdTx2 and SsdTx3, we might not even be able to obtain an adequate amount of purified materials to obtain partial sequences.

**Inhibitory activity of recombinant SsdTx 1-3 proteins against hKir6.2.** To determine whether SsdTx1-3 were indeed the inhibitors against hK_{ATP} channels, we produced the recombinant proteins based on the sequences that have both four cysteines and a P-P segment harboring positively charged residues (Fig. 4a; experimentally determined partial sequence of SsdTx1 is shown in Fig. 4b). One of them has the predicted mass of 6031.98 Daltons, which is about four Daltons greater than the experimentally determined value of 6027.74 for SsdTx1 (blue arrow-pointed peak, Fig. 2b). This difference would be consistent with the formation of two disulfide bonds among the four cysteine residues, as is the case of SpTx1. Another transcript has the same sequence as the putative SsdTx1, except for position 43 where it is leucine instead of phenylalanine in SpTx1. This second transcript most likely corresponds to SsdTx2, the activity associated with the peak preceding that of SsdTx1 (Fig. 2b). The predicted mass of the third transcript (6101.15 Daltons) is about four Daltons greater than the observed mass (6105.08 Daltons) of SsdTx3 (magenta arrow-pointed peak, Fig. 2c), again a difference that is consistent with the formation of two pairs of disulfide bonds.

**Determination of critical basic residues in SpTx1.** To circumvent the problem, we proceeded to determine the critical residues in SpTx1 (Fig. 3a) underlying its potent inhibition of Kir6.2, so as to use this information to help identify SsdTx1-3 among the several hundreds of transcripts in the venom transcriptome of Scolopendra subspinipes dehaani, whose sequences have been determined. As positively charged residues in the venom proteins generally play critical roles in their block of the K^+ pores, we replaced each of such residues with alanine (Fig. 3a) and tested the resulting mutant SpTx1 against hKir6.2bgd. Among the mutations, R11A and K15A had the largest effects on lowering the potency of SpTx1 (Fig. 3b). Analysis of the dose-inhibition curves yielded the K_I values of 1.6 μM and 4.5 μM for the channels to interact with SpTx1 carrying the R11A and K15A mutation, compared to a K_I value of 8.5 nM for wild-type SpTx1 (Fig. 3c).

Residues R11 and K15 are located in a segment delineated by two proline residues, here dubbed the P-P segment (Fig. 3a). We thus used this positively charged residue-containing segment and the feature of four cysteines to identify SsdTx1-3.

**Identification of inhibitors from the venom transcriptome.** Going through the published transcript sequences of the venom transcriptome of Scolopendra subspinipes dehaani one by one, we found three transcripts that have both four cysteines and a P-P segment harboring positively charged residues (Fig. 4a). One of them has the predicted mass of 6031.98 Daltons, which is about four Daltons greater than the experimentally determined value of 6027.74 for SpTx1. Another transcript has the same sequence as the putative SsdTx1, except for position 43 where it is leucine instead of phenylalanine in SpTx1. This second transcript most likely corresponds to SsdTx2, the activity associated with the peak preceding that of SsdTx1 (Fig. 2b). The predicted mass of the third transcript (6105.08 Daltons) is about four Daltons greater than the observed mass (6101.15 Daltons) of SsdTx3 (magenta arrow-pointed peak, Fig. 2c), again a difference that is consistent with the formation of two pairs of disulfide bonds.

Figure 1. Inhibition of hK_{ATP} channels by Scolopendra subspinipes dehaani venom and a purified venom protein. (a,b) Currents of hK_{ATP} activated by adding 3 mM azide to a 100 mM K^+ containing bath solution and recorded in the absence (black traces) or presence (violet traces) of 1:500 dilution of crude venom (a) or the HPLC fraction indicated by asterisk in Fig. 2d (b). The currents were recorded by stepping voltages from the holding potential 0 mV to −80 mV. The dotted lines indicate zero current levels.
identified sequences. An analysis of the dose-inhibition curves against hKATP channels revealed a $K_d$ of 278 nM, 260 nM, or 281 nM for SsdTx1, SsdTx2 or SsdTx3 (Fig. 5a). We have thus identified SsdTx1-3 as inhibitors against hKATP channels, with $K_d$ values differing from SpTx1. Furthermore, we tested the three inhibitors against the current of Kir6.2bgd (without co-expression with hSUR1). All of them caused comparable levels of inhibition and had similar $K_d$ values (Fig. 5b). Thus, like SpTx1, these inhibitors also target hKATP channels by blocking hKir6.2.

Following the positive identification of the inhibitors against hKir6.2, we went back to biochemistry, solely for additional confirmation of the validity of the present approach. We further purified SsdTx1 through a third round of HPLC and were able to accumulate a sufficient amount of the active material for performing the Edman sequencing, through total 28 runs of repeated, sequential HPLC purification of samples (Figs 1b, 2d). This analysis revealed the sequence of N-terminal 45 residues of SsdTx1 (Fig. 4b), which indeed corresponds to that of the transcript identified as SsdTx1 (Fig. 4a).

hKir6.2-inhibiting activity of SsTx previously known to inhibit hKCNQ channels. Using the sequences of those Kir6.2 inhibitors as baits, we fished out SsTx from the sequence deposited in the gene bank. SsTx is a 53-residue protein naturally present in the venom of Scolopendra subspinipes mutilans, and has previously been identified as an inhibitor against various hKCNQ isoforms, with practically the same micromolar affinity ($K_d$ of ~2.5 μM)26. SsTx has four cysteine residues and a P-P segment that contains several positively charged residues (Fig. 4a). These features predict that SsTx would inhibit the current through the hKir6.2 pore. An analysis of the dose-inhibition relation yields a $K_d$ value of 180 nM for SsTx, compared to 8.5 nM for SpTx1 (Fig. 6). Despite the fact that it blocked the Kir6.2 pore with lower affinity than SpTx1 did, SsTx still blocked the Kir6.2 pore with much higher affinity than it blocked the hKCNQ pore.

We also tested SpTx1 against the hKCNQ1 channels, co-expressed with hKCNE1, in Xenopus oocytes. Less inhibition by SpTx1 than SsTx occurred at all positive voltages where meaningful currents were elicited (Fig. 7). In any case, both inhibitors blocked the pore of hKir6.2 much more potently than they blocked hKCNQ1 pore.
Discussion

In the present study, we have shown that the venoms of multiple centipede species contain one or more proteins that target hKATP channel by blocking the hKir6.2 pore. This family of five proteins, thus far identified, has the conserved features of four cysteine residues and a P-P segment that contains positively charged residues important for blocking the K⁺ pore (Fig. 4). As always, the selectivity of inhibitors among different types of K⁺ channel is relative. SsTx has previously been shown to block the KCNQ pore with a Kᵩ of 2.5 μM. We have now found that SpTx1 blocks the pore with a slightly lower affinity, but both of them block the hKir6.2 pore with much higher affinities (a Kᵩ of 15 nM for SpTx1 or 180 nM for SsTx).
Judging from the relevant $K_d$ values, SsTx would profoundly block the hKir6.2 channel at a dose that begins to produce any meaningful inhibitory effect on hKCNQ channels. Thus, caution must be exercised when using SsTx as a tool to probe hKCNQ channels in a preparation that contains SsTx-sensitive hKir6.2 channels. However, SpTx1 is not expected to have any meaningful impact on hKCNQ channels at a dose that would cause marked

**Figure 5.** Inhibition of hKATP and hKir6.2bgd channels by SsdTx1, SsdTx2 and SsdTx3. (a) Fractions of hKATP currents plotted against the concentration of SsdTx1, SsdTx2, or SsdTx3. The curves superimposed on data correspond to the fits of an equation for a bimolecular reaction, yielding $K_d$ values of $2.78 \times (\pm 0.62) \times 10^{-7}$ M for SsdTx1, $2.60 \times (\pm 0.52) \times 10^{-7}$ M for SpTx2, and $2.83 \times (\pm 0.46) \times 10^{-7}$ M for SsdTx3. (b) Fractions of hKir6.2bgd currents plotted against the concentration of SsdTx1, SsdTx2, or SsdTx3. The curves superimposed on data correspond to the fits of an equation for a bimolecular reaction, yielding $K_d$ values of $1.26 \times (\pm 0.35) \times 10^{-7}$ M for SsdTx1, $1.23 \times (\pm 0.10) \times 10^{-7}$ M for SpTx2, and $1.18 \times (\pm 0.07) \times 10^{-7}$ M for SsdTx3. All data are plotted as mean $\pm$ s.e.m. ($n = 5–7$).

**Figure 6.** Comparison of inhibition of hKir6.2bgd by SpTx1 and SsTx. Fractions of channel currents plotted against the concentration of SpTx1 or SsTx. The curves superimposed on data correspond to the fits of an equation for a bimolecular reaction, yielding $K_d$ values of $8.52 \times (\pm 0.37) \times 10^{-9}$ M for SpTx1, and $1.80 \times (\pm 0.42) \times 10^{-7}$ M for SsTx. All data are plotted as mean $\pm$ s.e.m. ($n = 5$).
inhibition of hKir6.2 channels. Furthermore, one can use the high-affinity SpTx1 in conjunction with another inhibitory of lower affinity (SsdTx1-3), instead of SpTx1 alone, to identify hKir6.2 in a biological preparation, on the basis of the expected different Kd values of the two inhibitors.

The difficulty in discovering a new toxin targeting a given channel has been twofold. First, the probability to find a venom that contains the inhibitory activity is often very low. Second, it can be a very challenging process to biochemically identify the inhibitor. Such identification requires all individual inhibitory proteins be purified to near homogeneity and then their sequences be determined typically by the Edman degradation methods27, which may not be practically achievable, especially for those containing more than 50 residues. Unfortunately, the Edman degradation method is abandoned by more and more academic service facilities. Over the years, we have migrated from Harvard Medical School’s facility to Yale University’s, and then to Stanford University’s for sequencing service, all of which have sequentially ceased their Edman degradation sequencing services.

As we are losing high-quality sequencing services at academic institutions (staffed with expert consulting scientists), we had to become more resourceful in the process of identifying toxins targeting ion channels. Here, we tested the feasibility of using the information learned from a known inhibitor to guide our discovery of new inhibitors by searching the sequences of a transcriptome, and cross-checked the identified candidates using experimental molecular masses of purified active venom components. Determination of the mass of a protein generally requires a test sample merely in pmole quantity, which does not need to be highly pure because the masses of individual proteins in a mixture can usually be determined by mass spectrometry. Finally, we confirmed the positive identification of useful inhibitors by producing active recombinant proteins. The present study illustrates a successful example of identifying inhibitors in a venom against a chosen channel without relying on protein peptide sequence information.

Methods

Mutagenesis and electrophysiological recordings. The hKir6.2 and hSUR1 cDNAs were subcloned in pGEMHE, whereas hKCNQ1 and hKCNE1 were in the pSP64 vector. All mutant cDNAs were produced through PCR-based mutagenesis and confirmed by DNA sequencing. cRNAs were synthesized with T7 polymerase or SP-6 polymerase using the corresponding linearized cDNAs as templates. A two-electrode voltage clamp amplifier

Figure 7. Comparison of inhibition of hKCNQ1 channel currents by SpTx1 and SsTx. (a,b) I-V curves of hKCNQ1 co-expressed with hKCNE1 obtained in the absence (control) or presence of 5 μM SsTx (a) or 5 μM SpTx1 (b). All data are plotted as mean ± s.e.m. (n = 6–8). The bath solution contained 5 mM KCl and 95 mM NaCl.
Biochemical purification of inhibitors from the Scolopendra subspinipes dehaani venom. In each of 28 runs, 40 μL of Scolopendra subspinipes dehaani crude venom (Spider Pharm) was loaded onto a Semi-preparative (Beckman) reverse-phase C18 column and the fractions were eluted with a linear water-to-acetonitrile gradient at a changing rate of 1% per minute; the flow rate of the mobile phase was 1 mL per minute. All the peaks were collected, dried and re-suspended before assaying them for activity. Active fractions were further purified in two sequential HPLC steps with an analytical C18 column, where samples were eluted with a linear water-to-methanol gradient, or a gradient of water to 1:2 mixture of methanol and isopropanol, each of which had a changing rate of 1% per minute; the flow rate of the mobile phase was 1 mL per minute. The masses of purified materials were determined on MALDI-TOF (Wistar Institute, Proteomics facility). The material purified as shown in Fig. 2d was sequenced by Edman degradation (Stanford University, Protein and Nucleic acid (PAN) facility), which yielded the sequence of N-terminal 45 residues (Fig. 4b).

Data Availability

The datasets used in the present study are available from the corresponding author on reasonable request.

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**Author Contributions**

Y. Ramu and Z. Lu designed research. Y. Ramu performed experiments and analyzed data. Z. Lu and Y. Ramu wrote the manuscript.

**Additional Information**

**Competing Interests:** The authors declare no competing interests.

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