Hg1, Novel Peptide Inhibitor Specific for Kv1.3 Channels from First Scorpion Kunitz-type Potassium Channel Toxin Family*[

Zong-Yun Chen1,2, You-Tian Hu1,2, Wei-Shan Yang1, Ya-Wen He1,2, Jing Feng1, Bin Wang1, Rui-Ming Zhao1,2, Jiu-Ping Ding3,4, Zhi-Jian Cao1,2, Wen-Xin Li1,2, and Ying-Liang Wu1,2

From the 1State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, China and the 2Key Laboratory of Molecular Biophysics, Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

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The potassium channel Kv1.3 is an attractive pharmacological target for autoimmune diseases. Specific peptide inhibitors are key prospects for diagnosing and treating these diseases. Here, we identified the first scorpion Kunitz-type potassium channel toxin family with three groups and seven members. In addition to their function as trypsin inhibitors with dissociation constants of 140 nm for recombinant LmKTT-1a, 160 nm for LmKTT-1b, 124 nm for LmKTT-1c, 136 nm for BmKTT-1, 420 nm for BmKTT-2, 760 nm for BmKTT-3, and 107 nm for Hg1, all seven recombinant scorpion Kunitz-type toxins could block the Kv1.3 channel. Electrophysiological experiments showed that six of seven scorpion toxins inhibited ~50–80% of Kv1.3 channel currents at a concentration of 1 μM. The exception was rBmKTT-3, which had weak activity. The IC50 values of rBmKTT-1, rBmKTT-2, and rHg1 for Kv1.3 channels were ~129.7, 371.3, and 6.2 nm, respectively. Further pharmacological experiments indicated that rHg1 was a highly selective Kv1.3 channel inhibitor with weak affinity for other potassium channels. Different from classical Kunitz-type potassium channel toxins with N-terminal regions as the channel-interacting interfaces, the channel-interacting interface of Hg1 was in the C-terminal region. In conclusion, these findings describe the first scorpion Kunitz-type potassium channel toxin family, of which a novel inhibitor, Hg1, is specific for Kv1.3 channels. Their structural and functional diversity strongly suggest that Kunitz-type toxins are a new source to screen and design potential peptides for diagnosing and treating Kv1.3-mediated autoimmune diseases.

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‡ Both authors contributed equally to this work.

1 To whom correspondence may be addressed: State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, China. Tel.: 86-0-27-68752831; Fax: 86-0-27-68752146; E-mail: liwxb@whu.edu.cn.

‡1 To whom correspondence may be addressed: State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, China. Tel.: 86-0-27-68752831; Fax: 86-0-27-68752146; E-mail: ylwu@whu.edu.cn.

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Scorpion Kunitz-type Inhibitor Specific for Kv1.3 Channels

cDNA cloning, bioinformatic analyses, and functional evaluations, we identified the first scorpion Kunitz-type potassium channel toxin family composed of four new members (LmKTT-1c, BmKTT-1, BmKTT-2, and BmKTT-3) and three known members (LmKTT-1a, LmKTT-1b, and Hg1) (26, 27). In addition to their functions as trypsin inhibitors, six of the recombinant scorpion Kunitz-type toxins also block 50–80% of Kv1.3 currents at a concentration of 1 μM. The exception was rBmKTT-3, which had weak activity. Among these peptides, a specific Kv1.3 inhibitor Hg1 was discovered with an IC₅₀ value of ~6.2 ± 1.2 nM. Significantly different from classical Kunitz-type potassium channel toxins with the N-terminal region as the channel-interacting interface, Hg1 adopted the C-terminal region as the main channel-interacting interface. Our results describe the first scorpion Kunitz-type potassium channel toxin family, and the identification of the specific Kv1.3 inhibitor Hg1. Kunitz-type toxins are a new group of toxins that can be used to screen and design potential peptides for diagnosing and treating Kv1.3-mediated autoimmune diseases.

MATERIALS AND METHODS

cDNA Library Construction and Screening—Venom gland cDNA libraries of scorpion Buthus martensii, Isometrus maculates, Lychas mucronatus, Heterometrus spinifer, Scorpiops tibetanus, and Scorpioesper jendeke were constructed as described in our previous work (26). Some new randomly selected colonies were sequenced using the ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA). Sequences were identified for open reading frames using the ORF finder program (http://www.ncbi.nlm.nih.gov/projects/orffinder/). After excluding the signal peptides, the similarity was annotated by searching against the GenBank™ NCBI database (http://www.ncbi.nlm.nih.gov/blast) using BLAST algorithms. Three known genes encoding Kunitz-type toxins Hg1, SdPI, and SdPI-2, and four new genes encoding Kunitz-type toxins, BmKTT-1, BmKTT-2, BmKTT-3, and LmKTT-1c were chosen. According to the nomenclature proposed recently for all peptide toxins (41), BmKTT-1, BmKTT-2, and LmKTT-1c would be named BmKTT-1, BmKTT-2, and LmKTT-1c respectively, but the simple names will be used throughout this paper.

Expression and Purification of Scorpion Kunitz-type Toxins—We used the cDNA sequences of LmKTT-1a, LmKTT-1b, and LmKTT-1c from Lychas mucronatus venom gland cDNA libraries and BmKTT-1, BmKTT-2, and BmKTT-3 from scorpion B. martensii cDNA libraries as the templates for PCR to generate respective fragments (27). The PCR product of Hg1 was generated by overlapping PCR. All PCR products were digested with NdeI and XhoI and inserted into expression vector pET-28a. After confirmation by sequencing, the plasmids were transformed into competent Escherichia coli BL21(DE3) cells for expression. QuikChange site-directed mutagenesis kits (Stratagene, Santa Clara, CA) were used for generating the mutants based on the wild-type plasmid pET-28a-Hg1. All mutant plasmids were verified by DNA sequencing before expression. Kunitz-type toxins and mutants were expressed according to our previous protocol (26). For example, the recombinant LmKTT-1a was found to accumulate exclusively in inclusion bodies and was refolded in vitro. Renatured protein was finally purified by HPLC on a C₁₈ column (10 mm × 250 mm, 5 μm Dalian Elite). Peaks were detected at 230 nm. The fraction containing recombinant LmKTT-1a was eluted at 20–21 min and further analyzed by MALDI-TOF-MS (Voyager-DESTR, Applied Biosystems).

Determination and Modeling of Scorpion Kunitz-type Toxin Structures—The secondary structures of scorpion Kunitz-type toxins and mutants with a control peptide BPTI were analyzed by circular dichroism (CD) spectroscopy. All samples were dissolved in water at a concentration of 0.2 mg/ml. Spectra were recorded at 25 °C from 250 to 190 nm with a scan rate of 50 nm/min, on a Jasco-810 spectropolarimeter (Jasco Analytical Instruments, Easton, MD). The CD spectra were collected from averaging three scans after subtracting the blank spectrum of water. The three-dimensional structure of Hg1 was modeled using BPTI (PDB4 code 6PTI) as a template through the SWISS-MODEL server as we have described previously (28).

Molecular Docking—Molecular docking of Hg1 interacting with the Kv1.3 channel was carried out as previous computational approaches (13, 29). First, the structure of the Kv1.3 channel was modeled using KcsA (PDB code 1K4C) as a template. Second, molecular docking was performed on the modeled Hg1 peptide and Kv1.3 channels using the ZDOCK program (30), and the docking results were then filtered by scoring combined with detailed mutagenesis information; Finally, a reasonable Hg1-Kv1.3 complex that was consistent with the experimental alanine-scanning mutagenesis was screened out.

Serine Protease Inhibitory Activity Assay—The inhibitory activities of seven Kunitz-type toxins were tested in the presence of serine proteases as described previously (26). Trypsin (bovine pancreatic trypsin; EC 3.4.21.4), α-chymotrypsin (bovine pancreatic α-chymotrypsin; EC 3.4.21.1), elastase (porcine pancreatic elastase; EC 3.4.21.36), and their respective chromogenic substrates, Na-benzoyl-L-arginine, 4-nitroanilide hydrochloride, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, N-succinyl-Ala-Ala-Ala-p-nitroanilide, were purchased from Sigma (Sigma-Aldrich). Trypsin was incubated with various amounts of rHg1 (100 to 400 nM) for 30 min at a final concentration of 400 nM. The reactions were initiated by adding varying concentrations of substrate, ranging from 0.1 to 0.8 mM. The initial rate of p-nitroanilide formation was monitored continuously at 405 nm for 5 min at 25 °C. The inhibitory activity of rHg1 was estimated by setting the initial velocity obtained with only protease as 100%. The inhibitory constant (Ki) of the trypsin-inhibitor complex was determined by Lineweaver-Burk plots and further slope replotting. The methods for chymotrypsin and elastase assay were the same, except the final concentration of chymotrypsin was 100 nM.

Electrophysiological Recordings—The cDNAs encoding mKv1.1, hKv1.2, mKv1.3, and hSKCa3 were provided generously by professor Stephan Grissmer (University of Ulm, Ulm, Germany).

4 The abbreviation used is: PDB, Protein Data Bank.
Germany) and professor George Chandy (University of California, Irvine, CA). We saved the mBKCa channel. All of the channels were subcloned into the pIREs2-EGFP vector (Clontech, Mountain View, CA) and transformed into HEK293 cells. The whole-cell patch clamp was used to measure and record the channel currents according to a previously described procedure (13, 31). Peptides were dissolved in stock solutions containing 1% BSA and diluted into solutions containing 0.01% BSA for toxin application in electrophysiological experiments.

Results are shown as mean ± S.E., with n being the number of experiments. The significance between two means was calculated with the Student’s t test using Origin software (version 6.0, Microcal Software, Northampton, MA). Differences in the mean values were considered significant at probability < 0.05.

Using IGOR software (WaveMetrics, Lake Oswego, OR), concentration versus response relationships were fitted according to the modified Hill equation:

\[
\frac{I_{\text{toxin}}}{I_{\text{control}}} = \left(1 + \left(\frac{[\text{toxin}]}{\text{IC}_{50}}\right)^n\right)^{-\frac{1}{n}}
\]

where \(I\) is the peak current, and \([\text{toxin}]\) is the concentration of toxin. The parameters to be fitted were concentration at half-maximal effect (IC\(_{50}\)).

RESULTS

Primary Structures of Scorpion Kunitz-type Toxins—On the basis of our cDNA libraries and further random sequencing, four new genes encoding Kunitz-type toxins were obtained. Three were isolated from scorpion \textit{B. martensii}, which were named BmKTT-1, BmKTT-2, and BmKTT-3, and one was isolated from scorpion \textit{L. mucronatus} and named LmKTT-1c. Combined with the three known Kunitz-type toxins, Hg1, LmKTT-1a, and LmKTT-1b (26, 27), seven scorpion Kunitz-type toxins can be classified into three groups according to their disulfide bridge patterns (Fig. 1). Hg1 and BmKTT-3 belong to the first group, which adopted classical disulfide pairings similar to HWTX-XI toxin from spider (20), dendrotoxin K from snake (32), and APEKTx1 from sea anemone (23). LmKTT-1a, LmKTT-1b, and LmKTT-1c, and BmKTT-1 belong to the second group, which adopted a unique cystine framework that we described in our previous work (26). These toxins lack the normal CysII–CysIV disulfide bonds present in BmKTT-3 from group I but contain two new cysteine residues near the C terminus of the mature peptide. Different from all known Kunitz-type toxins from various animals, BmKTT-2 in the third group has eight cysteine residues, which may adopt novel disulfide bonds (17). Our findings demonstrate the molecular diversity of scorpion Kunitz-type toxins.

Preparation and Structural Analysis of Scorpion Kunitz-type Toxins—To evaluate the function of scorpion Kunitz-type toxins, we obtained seven recombinant toxins as described previously (26, 27). Expression and purification of LmKTT-1a toxin was as follows. A His\(_6\) tag and a thrombin cleavage site were fused to LmKTT-1a at the N terminus. Inclusion bodies of LmKTT-1a fusion peptide were suspended in LB medium and refolded successfully in vitro. The soluble protein was further separated by RP-HPLC and SDS-PAGE (supplemental Fig. S1, A and B). By MALDI-TOF MS, the molecular weight was 8657.8 Da, which is in good agreement with the calculated molecular mass of 8658.6 Da (supplemental Fig. S1C).

BPTI is a classical Kunitz-type peptide (33). By circular dichroism spectroscopy, all seven recombinant scorpion Kunitz-type toxins were found to have similar secondary structures to BPTI (supplemental Fig. S2), which suggests the conserved structures of Kunitz-type peptides.

Trypsin Inhibitory Activities of Scorpion Kunitz-type Toxins—Based on the conserved structures of scorpion Kunitz-type toxins, they were assayed for inhibitory activity against trypsin.

![FIGURE 1. Primary structures of scorpion Kunitz-type toxins.](image)

![FIGURE 2. Inhibitory effects of scorpion Kunitz-type toxins on trypsin. A, inhibitory effects of rHg1 peptide on trypsin with BPTI and BSA as controls. B, inhibitory effects of seven scorpion Kunitz-type toxins at different concentrations on trypsin using the same conditions. Data represent the mean ± S.E. of at least three experiments.](image)
chymotrypsin, and elastase. All seven recombinant toxins exhibited apparent inhibitory effects on trypsin (Fig. 2), but no inhibitory effect on chymotrypsin or elastase, even at higher concentrations. Recombinant LmKTT-1a, LmKTT-1b, LmKTT-1c, BmKTT-1, and Hg1 could completely inhibit the trypsin activity at the ratio of 1:1 with a dissociation constant of 140, 160, 124, 136, and 107 nM, respectively (supplemental Fig. S3). The rBmKTT-2 could completely inhibit the trypsin activity at a ratio of ~1.5:1, with a dissociation constant of 420 nM, and rBmKTT-3 could inhibit ~85% of the trypsin activity at a ratio of ~4:1, with a dissociation constant of 760 nM (Table 1).

**TABLE 1**

| Name   | Source      | Trypsin activity | Channel activity | Ref.          |
|--------|-------------|------------------|------------------|---------------|
| Hg1    | Scorpion    | 107 nM           | 6.2 (Kv1.3)     | Ref. 26 and this work<sup>a</sup> |
| LmKTT-1a | Scorpion    | >1000 nM         | >1000 (Kv1.3)   | Ref. 25 and this work<sup>a</sup> |
| LmKTT-1b | Scorpion    | >1000 nM         | >1000 (Kv1.3)   | Ref. 25 and this work<sup>a</sup> |
| LmKTT-1c | Scorpion    | 124 nM           | >1000 (Kv1.3)   | This work     |
| BmKTT-1 | Scorpion    | 136 nM           | 129.7 (Kv1.3)   | This work     |
| BmKTT-2 | Scorpion    | 420 nM           | 371.3 (Kv1.3)   | This work     |
| BmKTT-3 | Scorpion    | 760 nM           | >1000 (Kv1.3)   | This work     |
| α-DTX  | Snake       | 0.004 nM         | 0.004 (Kv1.1)   | Ref. 20       |
| δ-DTX  | Snake       | <0.01 nM         | <0.01 (Kv1.1)   |              |
| HWTX-X1 | Spider      | 23 nM            | 2570 (Kv1.1)    | Ref. 19       |
| Conk-S1 | Conus       | 124 nM           | 502 (Shaker)    | Ref. 24       |
| APEKTx1 | Sea anemone | <30 nM           | 2800 (Kv1.2)    | Ref. 23       |
| AKC1   | Sea anemone | <30 nM           | 1100 (Kv1.2)    |              |
| AKC2   | Sea anemone | <30 nM           | 1300 (Kv1.2)    |              |

<sup>a</sup> First function characterization in this work.

<sup>b</sup> First characterization of potassium channel inhibitory function in this work.

<sup>c</sup> --, lack of activity.

**Inhibitory Activities of Scorpion Kunitz-type Toxins on Kv1.3 Potassium Channel**—The pharmacological activities of the scorpion Kunitz-type toxins on Kv1.3 channels were evaluated. Fig. 3, A–C–H, show the inhibitory effects on Kv1.3 currents at a concentration of 1 μM. rLmKTT-1a, rLmKTT-1b, and rLmKTT-1c could inhibit ~50% of Kv1.3 channels currents, whereas rHg1, rBmKTT-1, and rBmKTT-2 could inhibit ~60–80% of Kv1.3 channels currents. The rBmKTT-3 had a weak effect on Kv1.3 channel currents. However, rHg1 at a decreased concentration of 100 nM still had a significant inhibitory effect on Kv1.3 channels (Fig. 3B). Concentration-dependent experiments further showed that rBmKTT-1, rBmKTT-2, and rHg1 inhibited Kv1.3 channel currents with IC₅₀ values of 129.7 ± 31.3 nM, 371.3 ± 82.1, and 6.2 ± 1.2 nM, respectively (Fig. 3, I–K). These results provide the first characterization of a scorpion Kunitz-type potassium channel toxin family, which contains seven new members and can inhibit both potassium channels and trypsin.

Hg1, Selective Inhibitor for Kv1.3 Channels—Based on the pharmacological properties of rHg1, the first potent Kv1.3 channel inhibitor with a Kunitz-type fold, we further investigated its effects on different types of potassium channels. As shown in Fig. 4, rHg1 could inhibit <50% of the Kv1.1 and Kv1.2 channel currents at a concentration of 1 μM (Fig. 4, A and B), and had little effect on SKCa3 and BKCa channel currents at identical concentrations (Fig. 4, C and D). These data indicate that Hg1 is a selective peptide inhibitor specific for Kv1.3 channels.

**FIGURE 3. Effects of seven scorpion Kunitz-type toxins on mKv1.3 channel currents.** A and B, blocking effects of Hg1 on mKv1.3 K⁺ currents. C, blocking effects of rBmKTT-3 on mKv1.3 currents. D, blocking effects of rLmKTT-1a on mKv1.3 currents. E, blocking effects of rLmKTT-1b on mKv1.3 currents. F, blocking effects of rLmKTT-1c on mKv1.3 currents. G, blocking effects of rBmKTT-1 on mKv1.3 currents. H, blocking effects of rBmKTT-2 on mKv1.3 currents. I, concentration-dependent inhibition of mKv1.3 channels by rBmKTT-1. J, concentration-dependent inhibition of mKv1.3 channels by rBmKTT-2. K, concentration-dependent inhibition of mKv1.3 channels by rHg1. Data represent the mean ± S.D. of at least three experiments.
Unique Molecular Mechanism of Hg1 Blocking Kv1.3 Channels—By using the alanine-scanning strategy, we investigated the molecular mechanism of Hg1 toxin blocking Kv1.3 channels. As shown in Fig. 5, A–H, there were no apparent effects of His-2, His-3, Asn-4, Arg-5, Leu-9, Leu-10, and Lys-13 residues on the toxin pharmacological activities, whereas there were less conformational changes for toxin mutants (Fig. 5, I and J). These data indicate that Hg1 toxin did not use N-terminal res-
idues to inhibit the Kv1.3 channel, which was different from the known molecular mechanism of Kunitz-type toxins such as δ-dendrotoxin and HWTX-XI. These toxins mainly use N-terminal residues to block Kv1.1 channels (20, 34). Among classical animal toxins affecting potassium channels, basic residues as critical residues are common features (35–37). We then focused the second cluster of basic residues, located at the C terminus of Hg1 toxin (Fig. 1) and found the dominant effects of Lys-56, Arg-57, Phe-61, and Lys-63 residues on the toxin affinities when using identical 100 nM concentration of wild-type and mutant Hg1 toxin with less conformational changes (Fig. 6, A–E). The IC50 values were 582.0 ± 184.5 nM for Hg1-K56A, 305.0 ± 93.7 nM for Hg1-R57A, 360.1 ± 116.5 nM for Hg1-F61A, and 457.9 ± 187.3 nM for Hg1-K63A mutants. Replacement by alanine reduced the ability of the toxin to inhibit Kv1.3 channels by 94-, 49-, 58-, and 74-fold, respectively (Fig. 6F). These structure and function relationships demonstrate that Hg1 toxin mainly uses the C-terminal region as the channel-interacting interface to inhibit Kv1.3 channels.

To further reveal the recognition mechanism of Hg1 peptide toward Kv1.3 channels, a structural model of the Hg1-Kv1.3 complex was obtained through our previous computational approaches (13, 34). The importance of four Lys-56, Arg-57, Phe-61, and Lys-63 residues on the toxin affinities when using identical 100 nM concentration of wild-type and mutant Hg1 toxin with less conformational changes (Fig. 6, A–E). The IC50 values were 582.0 ± 184.5 nM for Hg1-K56A, 305.0 ± 93.7 nM for Hg1-R57A, 360.1 ± 116.5 nM for Hg1-F61A, and 457.9 ± 187.3 nM for Hg1-K63A mutants. Replacement by alanine reduced the ability of the toxin to inhibit Kv1.3 channels by 94-, 49-, 58-, and 74-fold, respectively (Fig. 6F). These structure and function relationships demonstrate that Hg1 toxin mainly uses the C-terminal region as the channel-interacting interface to inhibit Kv1.3 channels.

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**DISCUSSION**

The Kv1.3 potassium channel is an attractive pharmacological target for autoimmune diseases, and specific peptide inhibitors are useful tools for diagnosing and treating these diseases (38, 39). To screen and design the potent and selective peptide inhibitors, efforts to improve peptide specificity are continuing (11–13). Kunitz-type toxins are a kind of ancient toxin family that has been identified in many animal venoms, such as those of snake, cone snail, spider, sea anemone, and scorpion (14–18). Several Kunitz-type toxins have been found to inhibit potassium channels (Table 1).

In this work, we adopted a new strategy to screen novel peptide inhibitor specific for Kv1.3 channels from scorpion Kunitz-type toxins. We identified the first scorpion Kunitz-type potassium channel toxin family with three groups and seven members, from which a novel peptide inhibitor, Hg1, specific for Kv1.3 channels, was obtained. Overall, our work provided following unique structural and functional features of the Kunitz-type potassium channel toxin family.

**Molecular Diversity of Kunitz-type Potassium Channel Toxins**—Combined with the known Kunitz-type toxins affecting potassium channels (Table 1) (21), 17 toxins were found to block potassium channels. Among these, there were significant differences in toxin sequences, sequence lengths, and number and distribution of cysteine residues (20). Most notably, BmKTT-2 toxin from scorpion was found to form four disulfide bridges, which is different from all known Kunitz-type animal toxins (17). The finding of seven addi-
tional scorpion Kunitz-type toxins strongly enriched the molecular diversity of Kunitz-type toxins inhibiting potassium channels.

Conserved Structures of Kunitz-type Toxins—Studies have shown that the backbone of dendrotoxin I, a potassium channel blocker from snake venom, superimposes on BPTI with a root mean square deviation of <1.7 Å (33). Using circular dichroism spectroscopy analyses, we also found structural similarity between BPTI peptide and seven scorpion Kunitz-type toxins, especially with different disulfide bridges (supplemental Fig. S2). The structural similarity of Hg1 and BPTI was shown by the BPTI structure (PDB code 6PTI) and the Hg1 structural model.
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(supplemental Fig. S4). This indicates that structures were conserved for Kunitz-type toxins.

Functional Diversity of Kunitz-type Potassium Channel Toxins—Previous reports showed several Kunitz-type toxins could affect Kv1.1, Kv1.2, and Shaker channels (Table 1). In this work, we found that scorpion Kunitz-type toxins could inhibit Kv1.3 channels. Except for the scorpion Kunitz-type toxin rBmKTT-3, the other six toxins inhibited Kv1.3 currents at a concentration of 1 μM (Fig. 3). The IC_{50} value of rHg1 for Kv1.3 channels was 6.2 ± 1.2 nM. The functional diversity of Kunitz-type potassium channel toxins will hopefully encourage a functional evaluation of this kind of toxins on additional potassium channels.

Unique Inhibitory Mechanism of Kunitz-type Potassium Channel Toxins—Kunitz-type toxins δ-dendrotoxin and HWTX-XI mainly use N-terminal residues to block Kv1.1 channels (Fig. 8, A–C) (20, 40). In contrast, Hg1 toxin mainly uses C-terminal residues as a channel-interacting interface to inhibit Kv1.3 channels (Fig. 8D).

In conclusion, we have characterized the first scorpion Kunitz-type potassium channel toxin family with the unique pharmacological property of blocking Kv1.1 channels. From this toxin family, a potent and selective Kv1.3 channel inhibitor, Hg1, was identified. Hg1 is the first Kunitz-type toxin identified that interacts with potassium channels by its C-terminal region as the main channel interacting interface. The structural and functional diversity of these Kunitz-type potassium channel toxins may provide a new source of potassium channel inhibitors used for the diagnosis and treatment of autoimmune disorders.

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