**κ-Hefutoxin1, a Novel Toxin from the Scorpion Heterometrus fulvipes with Unique Structure and Function**

**IMPORTANCE OF THE FUNCTIONAL DIAD IN POTASSIUM CHANNEL SELECTIVITY**


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An important and exciting challenge in the post-genomic era is to understand the functions of newly discovered proteins based on their structures. The main thrust is to find the common structural motifs that contribute to specific functions. Using this premise, here we report the purification, solution NMR, and functional characterization of a novel class of weak potassium channel toxins from the venom of the scorpion *Heterometrus fulvipes*. These toxins, κ-hefutoxin1 and κ-hefutoxin2, exhibit no homology to any known toxins. NMR studies indicate that κ-hefutoxin1 adopts a unique three-dimensional fold of two parallel helices linked by two disulfide bridges without any β-sheets. Based on the presence of the functional diad (Tyr5/Lys19) at a distance (6.0 ± 1.0 Å) comparable with other potassium channel toxins, we hypothesized its function as a potassium channel toxin. κ-Hefutoxin 1 not only blocks the voltage-gated K⁺-channels, Kv1.3 and Kv1.2, but also slows the activation kinetics of Kv1.3 currents, a novel feature of κ-hefutoxin 1, unlike other scorpion toxins, which are considered pore blockers. Alanine mutants (Y5A, K19A, and Y5A/K19A) failed to block the channels, indicating the importance of the functional diad.

Scorpions, the largest arachnids, are among the oldest (more than 450 million years) living groups of animals (1). There are ~1500 distinct species of scorpions around the world, and their venoms are rich sources of toxins that affect the ion channel functions of excitable and nonexcitable cells (2–4). Of about 100,000 different peptides surmised to exist in all of these species, less than 0.02% are well characterized. Scorpion toxins are classified into four groups according to their effects on specific ion channels, such as sodium (Na⁺), potassium (K⁺), calcium (Ca²⁺), and chloride (Cl⁻) channels. Voltage-sensitive Na⁺ channel toxins are long-chain toxins containing 60–70 amino acid residues with four disulfide bridges (4). A subset of short-chain scorpion toxins has been found to interact with the voltage-dependent or Ca²⁺-activated K⁺ channels or Cl⁻ channels. Potassium toxins typically contain 31–38 amino acids with three or four disulfide bridges (2, 3). Recently, a 23-residue toxin closely packed by three disulfide bridges was purified from the scorpion *Tityus canitioides* and was reported to block reversibly the Shaker B K⁺ channels (7). Two scorpion peptides have been reported to modify the binding of ryanoctine to Ca²⁺ channels. Imperatoxin A, which is 33 amino acids long, increases the binding of ryanoctine (8), resulting in continuous Ca²⁺ channel stimulation. Imperatoxin I is a heterodimer (104 and 27 amino acids, respectively) that blocks Ca²⁺ channel by inhibition of ryanoctine binding (9). Scorpion toxins that inhibit Cl⁻ channels are ~36 amino acids long with four disulfide bridges (10, 11). Scorpion toxins possess a highly conserved secondary structure arrangement comprising double- or triple-stranded antiparallel β-sheet and a stretch of α-helix maintained by a couple of disulfide bridges (4, 12).

*Heterometrus fulvipes*, also known as the Asian forest black scorpion, belongs to the family *Scorpionidae*, members of which are reported to be less lethal than the scorpions belonging to the *Buthidae* family, which include the Indian red scorpion *Mesobuthus tamulus*, the Chinese scorpion *Buthus martensi Karsh*, and the Israeli scorpion *Leirus quinquestratiatus* (13). *H. fulvipes* is widely distributed in South India, Indonesia, and Malaysia. Much of the research attention on the venom has been directed toward isolation of enzymes and to study the effect of the crude venom in animals (14–16). Characterization of neurotoxins that interacts with ion channel function has not been reported so far. Here we report for the first time purification, solution NMR, and functional characterization of a novel toxin, κ-hefutoxin 1, from the venom of *H. fulvipes*. κ-Hefutoxin 1 is a 22-residue peptide with two disulfide bridges, the shortest scorpion toxin reported so far, and possesses a unique three-dimensional structure and biological ac-


**EXPERIMENTAL PROCEDURES**

**Materials**

Venom from *H. fulvipes* was milked by electrical stimulation as described previously (17). Prepacked Superdex 30 and Sephasil C18 columns were from Amersham Biosciences. Acetonitrile and trifluoroacetic acid were purchased from Fisher and Fluka Chemika (Buchs, Switzerland), respectively. α-Cyano-4-hydroxyccinamic acid and all reagents for the peptide synthesis and sequencing were purchased from Applied Biosystems (Chiba, Japan). Chemicals used for electrophysiological studies were of analytical grade and from Sigma.

**Purification of κ-Hefutoxins**

κ-Hefutos were purified from the crude venom by two-step purification procedures of gel filtration followed by reverse phase HPLC.1 The lyophilized crude venom was applied on to a Superdex 30 Hiloal (16/60) column equilibrated with 50 mM Tris buffer, pH 7.5. Proteins eluted were monitored at 280 nm. The biologically active fraction (fraction 5) was then applied upon a Sephasil C18 reverse phase column, equilibrated with trifluoroacetic acid. The bound peptides were eluted using a linear gradient of acetonitrile. Elution was monitored at 215 nm.

**Electrospray Ionization Mass Spectrometry**

Samples were analyzed using a PerkinElmer Life Sciences Sciex API 300 triple quadrupole mass spectrometer equipped with an ion spray interface (Sciex, Ontario, Canada). The ion spray voltage was set to 5500 V, and the orifice voltage was set at 30 V. The mass was determined by flow injection analysis at a flow rate of 50 μl/min using Shimadzu 10 AD pumps as the solvent delivery system.

**N-terminal Amino Acid Sequence**

The N-terminal amino acid sequence of the peptides were determined by automated Edman degradation using an Applied Biosystems 494 pulsed liquid phase sequencer equipped with an on-line 785A phenylthiohydantoin-derivative analyzer.

**Peptide Synthesis**

κ-Hefutoxin 1 and the three alanine mutants (Y5A κ-hefutoxin 1, K19A κ-hefutoxin 1, and Y5A/K19A κ-hefutoxin 1) were synthesized by solid phase methodology using Fmoc chemistry on an Applied Biosystems 435A peptide synthesizer and were purified by HPLC. The purified peptides were characterized by MALDI-TOF mass spectrometry measurements.

**Circular Dichroism of Wild-type and Mutant κ-Hefutoxin 1**

CD spectra were recorded on a Jasco J-600 spectropolarimeter in H2O solution (10 mM sodium phosphate, pH 7.0) at 20 °C, with a quartz cell of 1-mm path length. The results are expressed as molar ellipticity (θ).

**NMR Spectroscopy and Structure Calculation**

NMR spectra of the κ-hefutoxin 1 (5 μM) were recorded on a Bruker DMX 600-MHz spectrometer in 90% H2O, 10% D2O at pH 4.0 and a temperature of 25 °C. The two-dimensional experiments included TOCSY (18), NOESY (18), DQF-COSY (18), and E-COSY (19) experiments with 4094 data points in the f2 dimension and 512 increments in the f1 dimension over a spectral width corresponding to 12 ppm. Slow exchanging amide protons were identified in a series of one-dimensional and TOCSY spectra following the dissolution of the toxin in D2O at 25 °C (pH 4.0).

The structure of κ-hefutoxin 1 was calculated based on distance restraints derived from the NOESY spectra collected in 90% H2O, 10% D2O with a mixing time of 200 ms using X-PLOR version 3.85. The backbone dihedral angle restraints derived from 3JHH, 3JHT, and 3JHT coupling constants (20) and the ψ, χ dihedral angles derived from E-COSY spectra (21) were used as additional constraints for the structure calculation. The structures were calculated using the standard simulated annealing and energy minimization protocols.

**Oocyte Expression**

The three Kv1 type channels were studied respectively from rats (Kv1.1 and Kv1.2 channels) and humans (Kv1.3 channels).

Kv1.1—For *in vitro* transcription, plasmids were first linearized with PstI (New England Biolabs) 3’ to the non-translated β-globin sequence in our custom made high expression vector for oocytes, pGEMHE (22), and then transcribed using T7 RNA polymerase and a cap analogue, diguanosine triphosphate (Promega, Madison, WI).

Kv1.2—The cDNA encoding Kv1.2 (originally termed RCK5) in its original vector, pAKS2, was first subcloned into pGEM-HE (22). The insert was released by double restriction digest with BglII and EcoRI. The cDNA was loaded onto an agarose gel, fragments of interest were cut, cleaned (Qiagen, CA), and ligated into the BanHI and EcoRI sites of pGEM-HE. For *in vitro* transcription, the cDNA was linearized with NotI and transcribed using the large scale 37 nMMESSAGE mMACHINE transcription kit (Ambion).

Kv1.3—The plasmid pcDNA containing the gene for Kv1.3 was linearized with NotI (New England Biolabs) and transcribed like Kv1.2. Stage V-VI *Xenopus laevis* oocytes were isolated by partial ovariectomy under anesthesia (tricaine; 1 g/liter). Anesthetized animals were then cut, cleaned (Qiagen, CA), and ligated into the BanHI and EcoRI sites of pGEM-HE. Steady-state blockade of the toxin was measured at the end of the test pulse at different test potentials by stepping from a holding potential of –90 mV. Fitted Kc values were obtained after calculating the percentage of current left over after application of several toxin concentrations in different oocyte experiments (mean ± S.E., n).

**RESULTS**

**Purification and N-terminal Sequencing of κ-Hefutoxins**

During our search of novel low molecular weight peptides from different scorpion venoms, we isolated two novel peptides from the venom of the scorpion *H. fulvipes* by gel filtration followed by reversed-phase HPLC (Fig. 1, A and B). Peak 5b contained two peptides with molecular weights of 2655.43 ± 0.30 and 2713.33 ± 0.73, respectively (Fig. 1C), and the complete sequences of these peptides were determined by automated Edman degradation with a reproducible yield of 95%. During sequencing, blank cycles were found at positions 4, 8, 19, and 22. With cysteine at these positions, the calculated molecular weights of the peptides were 2655.84 and 2712.89, respectively, which matched the observed molecular weight data. The peptides were named κ-hefutoxin 1 (22 residues with an amidated C terminus) and κ-hefutoxin 2 (23 residues with a free C terminus) (Fig. 1D), and they show no sequence homology with any known scorpion toxis.

**Solid-phase Synthesis of κ-Hefutoxin 1**—Since κ-hefutoxin 1 was the shortest scorpion toxin isolated, it was synthesized...
chemically for structural and functional characterization. Linear precursor of \(\text{H9260}\)-hefutoxin 1 was chemically synthesized by Fmoc methodology and oxidized effectively by air oxidation. The main product had the same retention time as the native toxin, and they co-eluted on a reverse phase HPLC column (Fig. 2A). The formation of other disulfide bond isomers was negligible. The disulfide bond pairings were determined by successive enzymatic digestion of \(\text{H9260}\)-hefutoxin 1 with lysyl endopeptidase and thermolysin (which hydrolyzes the N-terminal peptide bond of hydrophobic amino acids like Trp and Tyr) (Fig. 2B), and the molecular weight of the digest was 1919.41 with lysyl endopeptidase (0.05 mg) at 37 °C for 2 days. The major product was separated with an ODS column and further digested with thermolysin (0.05 mg) in 0.2 ml of 0.1 M ammonium formate buffer (pH 6.5) containing 1 mM calcium chloride at 37 °C for 24 h.

**Solution NMR of \(\text{H9260}\)-Hefutoxin 1**—Peak assignments of \(\text{H9260}\)-hefutoxin 1 were acquired at 25 °C using a well established sequential assignment procedure. Ambiguities in the assignments due to peak overlap were resolved by comparison of NOESY and TOCSY spectra. The presence of a significant number of \(d_{\text{SN}}\) NOEs (Fig. 3A) in the NOESY spectrum indicates that the backbone of the toxin is well structured. Information on short and medium range NOEs, coupling constants, chemical shift index, and slowly exchanging amide protons shown in Fig. 3B reveals that the peptide has two helices but no
β-sheet elements. This conclusion is strongly supported by the chemical shift index plot, which shows residues in two stretches of the toxin (positions 5–10 and 15–21) having negative chemical shift index values (23). Similarly, most coupling constants in these two stretches measured from the DQF-COSY spectrum are less than 6 Hz, implying that these regions of the peptide backbone adopt helix conformation.

The distance and angular constraints used for the structural calculation of κ-hefutoxin 1 using the X-PLOR (24) software included 192 interresidual distance constraints (71 sequential, 67 medium range, and 24 long range restraints). In addition, slow amide exchange and coupling constant data allowed the introduction of 10 restraints for hydrogen bonds, nine backbone dihedral angles, and 11 side chain dihedral angles. A family of 20 thermally stable structures obtained from a set of 50 structures was chosen to represent the solution structure of κ-hefutoxin 1 (Fig. 3C). The structure of κ-hefutoxin 1 is well defined except in the N and C termini, which showed few NOE constraints. The final structures are in good agreement with the experimental restraints (Fig. 3D). Mean r.m.s. deviation calculated over the backbone heavy atoms (N, Cα, C) and all heavy atoms of the whole molecule were 0.56 ± 0.07 and 1.2 ± 0.01 Å.
residues at the ends of helix I (Tyr 5 and Arg10) show 3

residues 10 and 13. The presence of the

resdide bonds (Cys4–15–21 (helix II) that are held together in position by two disulfide bonds (helix I) and

two parallel helices encompassing residues 5–15

The solution structure of \( \kappa \)-hefutoxin 1 (Fig. 3D) consists of two parallel helices encompassing residues 5–10 (helix I) and 15–21 (helix II) that are held together in position by two disulfide bonds (Cys4–Cys22 and Cys8–Cys18). The centers of the two helices are separated by a spatial distance of 6.1 Å, and the loop connecting the two helices contains a type 1 \( \beta \)-turn between residues 10 and 13. The presence of the \( \beta \)-turn is supported by the presence of hydrogen-deuterium exchange support the presence of the hydrogen bond (Fig. 3D). Helix I is a classical \( \alpha \)-helix (with 3.6 residues/turn), whereas helix II appears distorted and presents features of a \( \beta \)-helix. The residues at the ends of helix I (Tyr2 and Arg10) show \( ^3J_{\text{H,HH}} \) values greater than 7 Hz. A striking feature of the structure of \( \kappa \)-hefutoxin 1 is the presence of a nonclassical turn formed due to a hydrogen bond between Ala4 NH and Arg6 CO. Observation of a strong i to i + 3 NOE between Ala3 and Arg6 and the protection of the Ala4 NH against hydrogen-deuterium exchange support the presence of the hydrogen bond (Fig. 3B). Overall, \( \kappa \)-hefutoxin 1 (Protein Data Bank accession code 1HP9) has a unique three-dimensional fold.

**Structural Alignment of \( \kappa \)-hefutoxin 1 with Heat-stable Enterotoxin B and \( \alpha \)-Conotoxin—DALI and VAST searches to the structural classification of proteins database**

*No NOE distance and dihedral angle restraint was violated by more than 0.2 Å or 3°, respectively, in any of the structures.

**Mean coordinates were obtained by averaging coordinates of the 20 calculated structures.

\*Ramachandran plots were obtained by averaging coordinates of the 20 calculated structures.

**Table 1**

<table>
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<th>Parameters</th>
<th>Values</th>
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<td>r.m.s. deviation from experimental restraints*</td>
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<tr>
<td>NOE distance restraints (Å)</td>
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<tr>
<td>Dihedral angle restraints (Å)</td>
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<td>r.m.s. deviation from idealized geometry</td>
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<td>Bonds (Å)</td>
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<td>Angles (deg)</td>
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<td>r.m.s. deviation from mean coordinates*</td>
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<tr>
<td>Backbone atoms (N, Ca, C) (Å)</td>
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<tr>
<td>Heavy atoms (Å)</td>
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<tr>
<td>Disallowed regions (%)</td>
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</table>

Asterisk (*) refers to published sequence alignments of large proteins, it represents 41% of the proteins.

**Fig. 4. Structural alignment of \( \kappa \)-hefutoxin 1, heat-stable enterotoxin B, and \( \alpha \)-conotoxin.** Shown are MolScript (41) C-atom trace schematic diagrams colored by protein chain: \( \kappa \)-hefutoxin 1 (green), enterotoxin B (c&ollor), \( \alpha \)-conotoxin (yellow). N-terminal positions are shown in CPK. Structurally aligned regions are shown as thick ribbons. A, view perpendicular to helical axis; B, view along the helical axis; C, sequence alignment of \( \kappa \)-hefutoxin 1 with enterotoxin B and \( \alpha \)-conotoxin, derived from structural alignment. Colored residues in uppermost part represent the structurally aligned positions between 1EHS/1HP9 and 1AOM/1HP9. Asterisk, fully conserved; colon, conservatively substituted; dot, chemically similar.

**Functional Characterization of \( \kappa \)-Hefutoxin 1—**The biological effect of \( \kappa \)-hefutoxin 1 was investigated in the X. laevis oocyte system heterologously expressing a single type of voltage-gated \( K^+ \) channel. \( \kappa \)-Hefutoxin 1 (40 \( \mu \)M) reduced \( K^+ \) currents through Kv1.3 and Kv1.2 by 50% and 17%, respectively, but did not inhibit Kv1.1 currents (Fig. 5, A, B (a and b), and C (a and b)). The dose-dependent reduction in the \( K^+ \) current was voltage-independent, since the degree of block was not different in the range of test potentials from \(-30 \) to \(+40 \) mV. The \( K_d \) values for Kv1.3 and Kv1.2 obtained from the dose-response curves were 40 and 150 \( \mu \)M, respectively. Since \( \kappa \)-hefutoxin 1 block was reversible, we investigated whether blockade followed a kinetic behavior of a simple bimolecular reaction. Inhibition of currents upon \( \kappa \)-hefutoxin 1 application and recovery upon washing followed a single exponential time course, compatible with a bimolecular reaction scheme. For Kv1.3, \( k_{\text{on}} \) was 736.1 s\(^{-1}\) M\(^{-1}\), and \( k_{\text{off}} \) was 0.031 s\(^{-1}\). These values yield a \( K_J \) of 42 \( \mu \)M, which is in perfect harmony with the \( K_J \) value from the dose-response curve (40 \( \mu \)M). For Kv1.2, \( k_{\text{on}} \) was 282.7 s\(^{-1}\).
A depolarizing steps were applied from 0.98 mV, and 200-ms depolarizing steps were applied from /H11002 or /H11002 of /H9260 to 40 mV every 5 s, in the absence and presence of 100 /H9260 (Kv1.3). Mean ± S.E. values for the ratio of current by 40 /H9260, comparison of the effect of /H9260, the oocyte was clamped at /H9260 3); Kv1.2, 0.83 /H11006 A 

### Fig. 5. Electrophysiology.
A, effect of wild-type /H9260 1 (k-Hftx1) on Kv1.1 (a), Kv1.2 (b), and Kv1.3 channels (c). All channels were first activated by depolarizing to 0 mV from a holding potential (V_hold) of −90 mV and then clamped back to either −50 mV (Kv1.1 and Kv1.2) or −90 mV (Kv1.3). Mean ± S.E. values for the ratio of current by 40 /H9260 k-Hftx1 to current in control conditions are as follows (n = 4): Kv1.1, 0.98 ± 0.02 (n = 3); Kv1.2, 0.83 ± 0.05 (n = 3); Kv1.3, 0.51 ± 0.07 (n = 4). B, effect of k-Hftx1 on Kv1.2. a, the oocyte was clamped at V_hold = −90 mV, and 200-ms depolarizing steps were applied from V_test = −70 to 40 mV every 5 s, in the absence and presence of 100 /H9260 k-Hftx1. B, steady-state current-voltage relationship (n = 3–5). C, effect of k-Hftx1 on Kv1.3. a, the oocyte was clamped at V_hold = −90 mV, and 500-ms depolarizing steps were applied from V_test = −70 to 20 mV every 5 s, in the absence and presence of 40 /H9260 k-Hftx1. b, steady-state current-voltage relationship (n = 3–5). D, comparison of the effect of k-Hftx1 on Kv1.3 channels in HK solution at −30 mV (a) and 40 mV (b). Inward and outward currents were evoked by depolarizing the oocyte to 500 ms to −30 and 40 mV, respectively, from a holding potential of −90 mV. The cells were clamped back to −50 mV (n = 4). Application of 40 /H9260 k-Hftx1 reduced the inward current by 32.1 ± 5.6% (n = 4) and the outward current by 11.0 ± 7.1% (n = 4). For each experiment, currents were normalized as a function of the maximal current percentage set at 100%: c, the values were statistically different (p < 0.05). E, k-Hftx1 induced slowing of the activation kinetics of Kv1.3. The oocyte was clamped at V_hold = −90 mV, and a 500-ms depolarizing step was applied to V_test = 0 mV. Both current traces have been superimposed after scaling of the trace in the presence of k-Hftx1. The toxin-induced slowing of the activation kinetics is characterized by τ-values of 11.2 ± 1.4 ms in control (n = 13) and 12.8 ± 0.8 (n = 3); 17.5 ± 0.45 (n = 5), and 18.1 ± 0.9 ms (n = 5) in the presence of 25, 50, and 100 μM toxin, respectively. The time course of activation was fitted using a Hodgkin-Huxley type model with a 4th power function of the following form: I = A(1 – exp(–τ/τ)) + C, where A represents the macroscopic and time-dependent current, τ is the time constant, and C is a constant. b, steady state activation curve in control and 40 μM k-Hftx1 obtained after fitting with a Boltzmann function (I = I_0(1 + exp(–(V_c–V_0)/V_m)))**1, V_m = −26 mV, is not shifted by k-Hftx1 WT as illustrated by the dashed lines. Slope values (m) for the control and the toxin curves are 10 and 11 ms, respectively. c, competitive binding studies between AgTx2 and k-Hefutoxin1. 10 pm agonist 2 was added to produce a 50% block of the Kv1.3 channel, followed by 40 μM hefutoxin 1 (which by itself reduces the current by ~50%) in the presence of AgTx2. Very little hefutoxin 1-sensitive current was observed after the preceding AgTx2 application.
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30046 (Accelyrs Inc.): Lys 3/Tyr4 of dendrotoxin K (aromatic ring.

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kinetics. Interestingly, very little 
toxin 1, respectively.

From this, it is concluded that 

proteins that bind to the same site of a receptor/acceptor pro-

Furthermore, to show that \( \kappa \)-hefutoxin 1 can be considered as a pore blocker, which at the same time modifies the gating kinetics, the following series of experiments was conducted. First 10 pM AgTx2, a potent pore blocker of voltage-gated 

K-channel (28), was added to produce 50% block of Kv1.3 channels, followed by 40 \( \mu \)M \( \kappa \)-hefutoxin 1 in the presence of 10 pM AgTx2. The rationale was that if AgTx2 and \( \kappa \)-hefutoxin 1 possess different, nonoverlapping binding sites, the co-application of \( \kappa \)-hefutoxin 1 would induce a supplementary block of the remaining current together with a slowing of the activation kinetics. Interestingly, very little \( \kappa \)-hefutoxin 1-sensitive current was observed after preceding AgTx2 application (Fig. 5F).

From this, it is concluded that \( \kappa \)-hefutoxin 1 and AgTx2 share the same, or at least partially the same, binding pocket in the pore.

DISCUSSION

Scorpion venom contains a mixture of polypeptide toxins that affects the physiology of various ion channels. \( \kappa \)-Hefutoxin 1 purified from \( H. \) fulvipes venom is 22 amino acids long packed by two disulfide bridges. The toxin bears no sequence homology to any known toxins reported so far, which interested us in studying its structure and function. Solution NMR data revealed that \( \kappa \)-hefutoxin 1 adopts a unique three-dimensional fold of two parallel \( \alpha \)-helices held together by two disulfide bridges and separated by a loop with no evidence of \( \beta \)-sheets. All known scorpion toxins possess a highly conserved three-dimensional arrangement comprising double- or triple-stranded antiparallel \( \beta \)-sheet and a stretch of \( \alpha \)-helix maintained by a couple of disulfide bridges (4, 12).

Protein toxins bind to receptors/acceptors with high specificity. In general, only a small number of critical residues contribute to these protein-protein interactions (30–32). In addition, proteins that bind to the same site of a receptor/acceptor protein share similar structural features. Such a structural convergence in functional sites is well exemplified among toxins that interact with voltage-gated potassium channels. As shown in Fig. 6A, these potassium channel toxins isolated from the venoms of snake, scorpion, spider, sea anemone, and cone snail have distinctly different three-dimensional structures. However, these toxins possess a diad, consisting of a lysine and a hydrophobic residue (mostly Phe or Tyr), that is fully exposed from a flat surface and plays a critical role in their interaction with voltage-gated 

K-channels (31, 33, 34). Despite their differences in the protein folding, the conserved diads are superimposable, and the average distance between the lysine \( \alpha \)-carbon and the center of the aromatic ring of the hydrophobic
residue was ~6.52 ± 0.62 Å (Fig. 6, B and C). Site-directed mutagenesis has shown that the diad is critical for toxin-channel interaction (33, 35, 36), and substitution of lysine or tyrosine residues results in a loss of 80–100- or 20-fold activity, respectively (33). Interestingly, in k-hefutoxin 1, Tyr5 and Lys19 protrude out of the flat surface formed by the edges of the two parallel α-helices. The average distance between the lysine α-carbon and the center of the aromatic ring of tyrosine in the 20 conformers was 6.0 ± 1.0 Å. This diad was superimposable with the critical diads of other potassium channel toxins (Fig. 6C). Therefore, we predicted that k-hefutoxin 1 might be a new class of potassium channel toxin with different topography.

k-Hefutoxin 1 was found to block the Kv1.3 voltage-gated K⁺-channel, and it also modifies the gating kinetics. Indeed, all known potassium channel toxins, except for hanatoxin from the tarantula Grammostola spatulata (37) and to some extent dndrotoxin K from the elapid Dendroaspis polyelegpis (38), merely block these channels by physically plugging the pore without affecting the kinetics of channel gating. In the case of hanatoxin, channel inhibition and gating modification seem to result exclusively from the interaction with the voltage-sensing domains of the potassium channel. As a consequence, the mid-point (V½) of the Boltzmann steady-state activation curve is shifted to more depolarized potentials (39). With k-hefutoxin 1, a voltage-independent modification of the gating kinetics was favored (in a final, voltage-independent step before opening of the channel).

**Mutational Analysis**—To determine the structure-function relationships and the role of critical diad, three mutants were synthesized in which lysine and tyrosine were substituted by alanine: Y5A, K19A, and Y5A/K19A. Although there may be local structural difference around replaced residues, we consider that the mutation did not affect the overall conformation of k-hefutoxin 1 (Fig. 7A). Minor differences observed in the CD spectra of k-hefutoxin 1 and its mutants (Y5A and Y5A/K19A) could be attributed to the contribution of the phenolic side chain of Tyr. It is well documented that losses of aromatic side chains affect significantly the CD spectra of peptides/proteins (40). All three mutants, despite having similar conformation failed to reduce the K⁺ current of Kv1.3 and Kv1.2 channels (Fig. 7B and C). These results support the importance of Tyr5 and Lys19 in the mechanism of action of k-hefutoxin 1 in its potassium channel activity. Except for the lack of affinity on two-pore background K⁺ channels (TASK-1), the pacemaker channel (HCN2) and hH1 Na⁺ channels, further studies are needed to reveal the specificity on other channels.

In conclusion, the biological activity of k-hefutoxin 1 was predicted based on its novel and unique three-dimensional structure and the presence of the functional diad. These results provide a strong impetus to structure-based recognition of biophysiological function of newly discovered proteins in the post-genomic era.
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