ShK-Dap$^{22}$, a Potent Kv1.3-specific Immunosuppressive Polypeptide*

Katalin Kalman, Michael W. Pennington‡, Mark D. Lanigan§, Angela Nguyen, Heiko Rauer¶, Vladimir Mahnir§, Kathy Paschetto**, William R. Kem, Stephan Grissmer*, George A. Gutman, Edward P. Christian**, Michael D. Cahalan, Raymond S. Norton§, and K. George Chandy‡‡

From the Departments of Physiology & Biophysics, and Microbiology and Molecular Genetics, University of California, Irvine, California 92697-4560, ‡Bachem Bioscience, Incorporated, King of Prussia, Pennsylvania 19406, ¶Biomolecular Research Institute, Parkville 3052, Victoria, Australia, the **Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, Florida 32610, the ***Department of Respiratory, Inflammatory and Neurological Disorders, Zeneca Pharmaceuticals, Wilmington, Delaware 19850, and the ‡‡Department of Applied Physiology, University of Ulm, 89081 Ulm, Germany.

The voltage-gated potassium channel in T lymphocytes, Kv1.3, is an important molecular target for immunosuppressive agents. A structurally defined polypeptide, ShK, from the sea anemone Stichodactyla helianthus inhibited Kv1.3 potently and also blocked Kv1.1, Kv1.4, and Kv1.6 at subnanomolar concentrations. Using mutant cycle analysis in conjunction with complementary mutagenesis of ShK and Kv1.3, and utilizing the structure of ShK, we determined a likely docking configuration for this peptide in the channel. Based upon this topological information, we replaced the critical Lys22 in ShK with the positively charged, non-natural amino acid diamino propionic acid (ShK-Dap$^{22}$) and generated a highly selective and potent blocker of the T-lymphocyte channel. ShK-Dap$^{22}$, at subnanomolar concentrations, suppressed anti-CD3 induced human T-lymphocyte $[^{3}H]$thy midine incorporation in vitro. Toxicity with this mutant peptide was low in a rodent model, with a median paralytic dose of $\sim 200$ mg/kg body weight following intravenous administration. The overall structure of ShK-Dap$^{22}$ in solution, as determined from NMR data, is similar to that of native ShK toxin, but there are some differences in the residues involved in potassium channel binding. Based on these results, we propose that ShK-Dap$^{22}$ or a structural analogue may have use as an immunosuppressant for the prevention of graft rejection and for the treatment of autoimmune diseases.

Human T lymphocytes express a unique voltage-gated potassium (Kv)$^{1}$ channel encoded by the Kv1.3 gene (1). A homotetramer of Kv1.3 subunits forms the functional channel in T lymphocytes (1). Earlier studies showed that structurally dissimilar blockers of this channel suppressed mitogen-induced $[^{3}H]$thymidine incorporation and interleukin-2 production by T lymphocytes (1–3). More specific, high affinity blockers discovered in recent years have demonstrated convincingly that Kv1.3 blockers depolarize the T-cell membrane and attenuate the calcium signaling pathway that is vital for lymphocyte activation (1, 4–9). Although Kv1.3 is found in B lymphocytes, macrophages, osteoclasts, platelets, and the brain, only in T lymphocytes does Kv1.3 channel activity seem to dominate the membrane potential (1, 8). The critical role of Kv1.3 during T-cell activation, coupled with its functionally restricted tissue distribution, has stimulated a search for potent and selective Kv1.3 antagonists for potential use as immunosuppressants (e.g. see Refs. 8 and 9).

Many potent polypeptide inhibitors of Kv1.3 have been isolated from scorpion venom. These polypeptides adopt well defined conformations constrained by 3 or 4 disulfide bonds and bind with extremely high affinity to a shallow vestibule at the external entrance to the Kv1.3 pore (10, 11). The most selective of these, margatoxin (MgTX), suppresses T-lymphocyte activation in vitro and is immunosuppressive in vivo (9), suggesting the possibility of using MgTX as an injectable immunosuppressant. However, MgTX potently blocks the closely related Kv1.1 and Kv1.2 channels (12, 13), which are expressed in the brain, peripheral nerves, and heart (14), raising concerns about potential cardiac and neuronal toxic side effects. Extensive efforts are therefore ongoing to identify other more selective and potent peptide and non-peptide inhibitors of Kv1.3.

Recently, a 35-amino acid-residue polypeptide (ShK) from the sea anemone Stichodactyla helianthus was shown to block the Kv1.3 channel at low picomolar concentrations (15, 16). Like scorpion toxins, ShK has a well defined conformation constrained by three disulfide bonds, minimizing possible structural changes upon its binding to the channel. However, the structure of ShK is significantly different from those of scorpion toxins (17, 18). Using alanine-scanning mutagenesis, the channel-binding surfaces of ShK (15, 16) and its closely related homologue, BgK (19), have been determined. Despite differences in the scaffolds, the sea anemone and scorpion toxins share a conserved diad of residues that is essential for block of potassium channels (16, 19). This diad consists of a critical lysine (Lys$^{27}$ in the scorpion toxins and Lys$^{22}$ and Lys$^{25}$ in ShK and BgK) and a neighboring aromatic residue (Tyr$^{36}$ in CtX, Tyr$^{35}$ in ShK, Tyr$^{38}$ in BgK) separated by $\sim 7$ Å (19). Lys$^{27}$, in scorpion toxins, couples with the tyrosine (Tyr$^{400}$ in Kv1.3, Tyr$^{375}$ in Shaker) in the potassium channel selectivity filter (11, 20). A better understanding of the interactions between ShK and the Kv1.3 channel may guide the design of specific ShK mutants with the potential to be used clinically as immunosuppressants. Here, we describe a mutant polypeptide that shows selectivity for Kv1.3, inhibits T-cell activation in vitro, and is minimally toxic in vivo.

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Fax: 949-824-3143; E-mail: gchandy@uci.edu.

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Peptide Synthesis—Fmoc-amino acid derivatives were obtained from Bachem A.G. (CH-4416 Bubendorf, Switzerland). Solid-phase assembly was initiated with Fmoc-Cys(Troc)-2-chlorotrityl resin to minimize potential racemization of the C-terminal Cys residue (21). Automated stepwise assembly was carried out entirely on an ABI-431A peptide synthesizer (Applied Biosystems, Foster City, CA). Fmoc-Dap/β-butyrolactone was the NCA protector in place of Lys(Trt) in the assembly of the polypeptide. The Dap22-substituted polypeptide was cleaved and deprotected with reagent K (22) containing 5% trisopropylsilane. The ShK-Dap22 analogue was solubilized, oxidized, and purified by reverse phase-high pressure liquid chromatography using the same method described previously for other ShK analogues (15). High pressure liquid chromatography-pure fractions were pooled and lyophilized. The structure and purity of the peptides were confirmed by reverse phase-high pressure liquid chromatography, amino acid analysis, and electrospray ionization-mass spectroscopy analysis. All other ShK analogues were synthesized, purified, and characterized as reported previously (15, 16).

Results—Cell lines stably expressing mKv1.1, mKv1.2, mKv1.3, hKv1.5, and hKv1.3 (7, 12) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and G418 (1 mg/ml). Human IKCa channels were studied in activated human T cells as described previously (7). All the mKv1.3 mutants and mKv1.4 used in this study have been described previously (7, 10–12). mKv1.6 and rKv3.3 were gifts from Dr. Olaf Pongs (ZMNH, Hamburg, Germany). 125I-ChTX was purchased from NEN Life Science Products. Fetal calf serum, penicillin, streptomycin, and citrate were purchased from Sigma. The isolated MNCs were incubated (37 °C, 5% CO2) for 24 h in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The assay was conducted in a 96-well plate by first adding monoclonal anti-CD3 and various polypeptide concentrations to wells in triplicate. Cold anti-CD3 was titrated to dilutions that produced a 4–25-fold increase in [3H]thymidine incorporation. The ShKpeptide concentrations were added to wells at a final concentration of 0.3 μg/ml. The cRNA/fluorescein isothiocyanate solution was filled into glass fiber filters (Packard GF/C unifilters) using a multi-well har-}

Mammalian Cells—Each construct was linearized with EcoRI and transcribed in vitro (8, 10, 11). cRNA was diluted with fluorescent fluorescein isothiocyanate-dye (0.5% fluorescein isothiocyanate-Dex-}

Materials and Methods

Activation of Human T Cells by Anti-CD3 Antibody—Mononuclear Cells (MNCs) were isolated over a Ficoll-Hypaque density gradient (Sigma). The isolated MNCs were incubated (37 °C, 5% CO2) for ≥2 days in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The assay was conducted in a 96-well plate by first adding monoclonal anti-CD3 and various polypeptide concentrations to wells in triplicate. Anti-CD3 was titrated to dilutions that produced a 4–25-fold increase in [3H]thymidine incorporation. MNCs were resuspended in fresh med-}

Similar cycles were constructed to measure coupling interactions between Asp602 (Asp602 → Lys602), Asp602 (Asp602 → Asp602), Tyr600 (Tyr600 → Val600, Tyr600), and polypeptide positions 9, 11, and 22.

The change in coupling energy, ΔG, for a given pair of ShK-Kv1.3 residues and their mutants was calculated using the formula ΔG = kTlnθ, where θ is a dimensionless value given by the formula θ = [Kd] (WT ShK-Wt Kv1.3) × [Kd] (mut ShK-mut Kv1.3) / ([Kd] (WT ShK-mut Kv1.3) + [Kd] (mut ShK-Wt Kv1.3)). For θ values <1 the inverse was used (11). Schreiber and Fersht (25) reported that ΔG values of ≥0.5 kcal mol−1 were of practical use to indicate that a pair of ShK and channel residues are physically close to each other. These interactions, therefore, may not be detected by this method (26).
All of the peptide-mapping studies were performed on channels expressed in Xenopus oocytes, whereas the studies described in Fig. 1 were performed on channels expressed in mammalian cells. In general, there was good correspondence between the $K_v$ values measured on channels expressed in mammalian cells and oocytes, although ShK-Dap22 blocked the channels four-fold more potently in the oocyte system ($K_v = 3.3 \pm 1.9$ pm, $n = 12$) compared with mammalian cells (see Fig. 1).

Structure Determination—Two-dimensional $^1$H NMR spectra were recorded at 600 MHz on a ~2 mM solution of synthetic ShK-Dap22 in 90% H$_2$O, 10% $^2$H$_2$O (d2o) or 100% $^2$H$_2$O at pH 4.9 and 293 K, as described (17, 27), but with water suppression using the Watergate scheme and a 3-9-19 selective pulse (28). Spectra were also recorded at 278 K in an attempt to sharpen backbone amide resonances from Ser19, Cys2, Met41, Dap22, and Tyr23. Chemical shifts for Dap resonances in the synthetic peptide GlyGlyDapGlyGly-OH were measured from one-dimensional and total correlation spectroscopy spectra at 293 and 298 K in 90% H$_2$O, 10% $^2$H$_2$O at pH 5.0, using 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard.

A figure (Fig. S1) included in the “Appendix” summarizes the sequential assignments, slowly exchanging amides, backbone coupling constants, and medium-range NOEs for ShK-Dap22, together with a table of $^1$H chemical shifts (Table S1). Methods for obtaining distance and angle restraints, generating structures in DYANA (29), and refining the structures by restrained simulated annealing and restrained energy minimization in X-PLOR (30) were as described previously (17, 27). The final NMR restraint list (from which values redundant with the conformation) was generated from ShK mutants, ShK-Dap22 and ShK-Nle22, also displaced $^{125}$I-hKv1.3 currents with 1:1 stoichiometry (Fig. 1) compared with mammalian cells (see Fig. 1).

Model of Kv1.3 and ShK Docking—To create a model of the pore and vestibule of Kv1.3 (residues 380–410), we relied heavily on the recent crystal structure of the bacterial K channel, KcsA (33, 34), and on a molecular model of the Shaker channel (35). Residues Phe125, Lys463, Thr549, Glu550, Pro551, and Phe552 from the Shaker model (35) were changed to the corresponding Kv1.3 residues Gly386, Asn388, His404, Thr407, Ile408, and Gly409, respectively, using Insight II. Modifications to the backbone and side chain dihedral angles were then made so that the local and global structure of the channel model better resembled the corresponding region of the KcsA channel (33). Following conjugate gradient minimization of the model using Discover (MSI), the closest-approach structure (17) was juxtaposed with the channel so as to preclude steric contact between the two. The backbone atoms (N, C, and C) of Kv1.3 were fixed in space during the simulation, whereas the backbone fold of ShK was maintained by 16 medium-range and 3 long-range distance constraints. Inter-molecular distance constraints were added to the peptide-channel complex in conjunction with a 50 kcal mol$^{-1}$ force constant in Discover so as to reflect data from NMR distance cycle analyses (see “Results”), with Lys463$^N$ (ShK) being kept within 5 Å of Tyr549$^C$ from each of the four Kv1.3 subunits and Arg$^{11}C$ being kept within 5 Å of a single His$^{404}N^5$. A lower limit of 6 Å was maintained between Arg$^{11}C$ and Asp$^{492}C$ to restrict any interaction between these two residues, which show no coupling (see “Results”). The complex was energy minimized using 10,000 steps of conjugate-gradient minimization, and then a 260-ps molecular dynamics simulation was performed in vacuo at 300 K with a 1-fs time step, a distance-dependent dielectric, and a 15-Å non-bonded interaction cut-off. After equilibration of the complex, the configuration with the lowest van der Waals repulsive energy was chosen for further energy minimization, carried out as above.

RESULTS

ShK, A Potent Blocker of the Kv1.3 Channel in T Lymphocytes—The polypeptide ShK blocks mKv1.3 currents with a $K_v$ of 11 ± 1.4 pm (n = 4, mean ± S.E.; Fig. IA, Table I) and with 1:1 stoichiometry (Fig. 1B). Similar results were obtained for block of Kv1.3 channels in human peripheral blood T cells (data not shown).

The ShK polypeptide inhibited $^{125}$I-ChTX binding to its receptor in the external vestibule of hKv1.3. Fig. 2 shows the concentration-dependent displacement of specifically bound $^{125}$I-ChTX by ShK. Fitting the concentration-response curve to a Hill equation yields an IC$_{50}$ value for ShK of 115 ± 20 pm (n = 5; mean ± S.E.) and 1:1 peptide:channel stoichiometry. Two ShK mutants, ShK-Dap22 and ShK-Nle22, also displaced $^{125}$I-ChTX binding to hKv1.3 with 1:1 stoichiometry and IC$_{50}$ values of 102 ± 17 pm (n = 8) and 663 ± 172 pm (n = 6), respectively. MgTX had an IC$_{50}$ value of 78 ± 10 pm (n = 6) in the same binding assay. Collectively, the electrophysiology and binding data indicate that ShK and ShK-Dap22 are potent blockers of the Kv1.3 channel, and these sea anemone polypeptides inter-
ShK-Kv1.3 interactions. Guided by the solution structure of the ShK polypeptide (17), we undertook double mutant cycle analysis. Collectively, these results of mutant cycle experiments on several additional peptide-channel interactions. Peptide binding studies. Data for the mutant cycle Kv1.3(Asp386→Arg29)-ShK(Dap22) are as follows: Asp386→Arg29, K<sub>50</sub> = 21 pM; Lys386→Arg29, K<sub>50</sub> = 660 pM; Asp386→Ala29, K<sub>50</sub> = 13 pM; Lys386→Ala29, K<sub>50</sub> = 1920 pM; ω = 4.7. Examples of two mutant cycles are presented in Fig. 3A. Replacing His404 in Kv1.3 with the hydrophobic valine (Val404) significantly altered the interaction of ShK-Dap22 with the channel (ΔΔG = 2.0 kcal·mol<sup>-1</sup>) but not that of the longer Lys22 analogue (ΔΔG = 0.19 kcal·mol<sup>-1</sup>). Fig. 3B summarizes the results of mutant cycle experiments on several additional peptide-channel pairwise interactions. Collectively, these results identified seven pairs of significant ShK-Kv1.3 interactions involving four channel positions as follows: Arg<sup>11</sup>-His404, Lys<sup>22</sup>-Tyr<sup>400</sup>, Orn<sup>22</sup>-Tyr<sup>400</sup>, Orn<sup>22</sup>-Asp<sup>402</sup>, Dap<sup>22</sup>-Asp<sup>402</sup>, Dap<sup>22</sup>-His<sup>404</sup>, and Dap<sup>22</sup>-Asp<sup>386</sup>. Using a molecular model of Kv1.3 based on the known crystal structure of the KcsA channel (33), we used restrained molecular dynamics simulations to dock the ShK peptide into the channel (Fig. 4). Linear correlation of the C-terminal residues (His<sup>404</sup>, Asp<sup>402</sup>, Tyr<sup>400</sup>, and Arg<sup>386</sup>) that have been shown previously to be important for scorpion toxin binding (10, 11) is still observed when these four C-terminal residues (His<sup>404</sup>, Asp<sup>402</sup>, Tyr<sup>400</sup>, and Arg<sup>386</sup>) are positioned at the outer entrance to the ion conduction pathway (10, 33). The ring of four His<sup>404</sup> residues is unique to Kv1.3, and compounds that target this ring might be selective for the lymphocyte channel (1, 7, 8). The highly conserved Tyr<sup>400</sup> (KcsA-Tyr<sup>78</sup>) and Asp<sup>402</sup> (KcsA-Asp<sup>80</sup>) in the critical signature sequence (GYGD) form part of the ion selectivity filter and couple with Lys<sup>22</sup> in the scorpion toxins (11, 20, 36). Asp<sup>386</sup> (KcsA-Arg<sup>11</sup>) lies ∼10–14 Å from the center of the pore and interacts with Arg<sup>29</sup> in kalitoxin and agitoxin-2 and with Arg<sup>29</sup> in charybdotoxin (10, 36). Three residues in ShK were chosen for mutagenesis: Arg<sup>11</sup> and Lys<sup>22</sup> on the surface, thought to interact with Kv1.3, and Lys<sup>29</sup> on the opposite surface (15–18). We focused on four channel residues (His<sup>404</sup>, Asp<sup>402</sup>, Tyr<sup>400</sup>, and Arg<sup>386</sup>) that have been shown previously to be important for scorpion toxin binding (10, 11). His<sup>404</sup> (KcsA-Tyr<sup>78</sup>) lies at the outer entrance to the ion conduction pathway (10, 33). The ring of four His<sup>404</sup> residues is unique to Kv1.3, and compounds that target this ring might be selective for the lymphocyte channel (1, 7, 8). The highly conserved Tyr<sup>400</sup> (KcsA-Tyr<sup>78</sup>) and Asp<sup>402</sup> (KcsA-Asp<sup>80</sup>) in the critical signature sequence (GYGD) form part of the ion.
This docking configuration, which resembles that of agitoxin-2 docked in the KcsA channel (36), was used to guide the identification of ShK mutants that exhibit Kv1.3 specificity. For example, the ShK-Dap22 mutant that couples strongly with the ring of four His404 residues unique to Kv1.3 and makes contact with Asp386 might be selective for the lymphocyte channel. To test this idea, we evaluated the ShK-Dap22 mutant in our selectivity screen.

ShK-Dap22 Is a Potent and Selective Blocker of Kv1.3—The ShK-Dap22 mutant blocked mKv1.3 currents with a $K_d$ of $23 \pm 3$ pM ($n = 4$, mean \pm S.E.; Fig. 5A) and a Hill coefficient close to unity (Fig. 5B). Human Kv1.3 channels are blocked with a similar potency (data not shown). ShK-Dap22 displaced $^{125}$I-ChTX binding to hKv1.3 with an IC$_{50}$ of $102 \pm 17$ pM ($n = 8$; Fig. 2) and with 1:1 stoichiometry, indicating that the peptide binds in the external vestibule in a site overlapping the ChTX receptor. These results corroborate the mutant cycle data presented in Fig. 3.

In a selectivity screen, ShK-Dap22 was found to be a highly selective inhibitor of Kv1.3. ShK-Dap22 blocked mKv1.1, mKv1.4, rKv1.6, and other potassium channel targets with significantly less potency than Kv1.3 (Fig. 5, C and D; Table I).

ShK, ShK-Dap22, and MgTX Inhibit Human T Cell Activation with Similar Potency—We compared the ability of ShK, ShK-Dap22, and MgTX to suppress anti-CD3-stimulated $[^{3}H]$thymidine incorporation by human peripheral blood T cells. All three polypeptides inhibited mitogen-stimulated $[^{3}H]$thymidine incorporation to a maximum level of $50–60\%$ (Fig. 6). However, the midpoint of inhibition (IC$_{50}$) for each toxin was below 500 pM, in keeping with their affinity for the Kv1.3 channel. Consistent with our results, an earlier study reported that peripheral blood T cells isolated from mini-pigs during intravenous MgTX infusion never showed more than a $60\%$ inhibition of mitogen-stimulated $[^{3}H]$thymidine incorporation in an ex vivo proliferation assay (9).

ShK-Dap22 Does Not Exhibit Acute Toxicity following Intravenous Injection into Rodents—As an initial evaluation of the toxicity of ShK and ShK-Dap22, mice ($n = 5$ in each case) were injected intravenously with each polypeptide. ShK toxin displayed a remarkably low toxicity when injected into mice, the median paralytic dose being approximately 0.5 mg per 20 g mouse, or 25 mg/kg body weight. ShK-Dap22 was even less toxic; a 1.0-mg dose failed to cause any symptoms (hyperactivity or seizures) or mortality, and the median paralytic dose was $200$ mg/kg body weight.

**Solution Structure of ShK-Dap22 and Comparison with the**
Structural statistics for the 20 energy-minimized structures of ShK-Dap22 from X-PLOR

| r.m.s. deviations from experimental distance restraints (Å) (343) | 0.028 ± 0.001 |
| r.m.s. deviations from experimental dihedral restraints (deg) (367) | 0.47 ± 0.15 |
| r.m.s. deviations from idealized geometry | 0.53% |

Bonds (Å) 0.0107 ± 0.0006
Angles (deg) 2.66 ± 0.05
Impropers (deg) 0.37 ± 0.02
Energies (kcal/mol) -1

E\text{Ecore} 14.1 ± 1.1
E\text{dih} 0.53 ± 0.28
E\text{improp} -126 ± 7
E\text{bond} + E\text{angle} + E\text{improper} 111 ± 4
E\text{acc} -513 ± 29

Mean pairwise r.m.s. difference (Å):
Residues 1–35 0.63 ± 0.15
Residues 21–23–35 0.51 ± 0.13
0.14 ± 0.14

* The numbers of restraints are shown in parentheses. None of the structures had distance violations > 0.3 Å or dihedral angle violations > 5°.
* Backbone heavy atoms.
* All heavy atoms.

Comparison with ShK—The overall structures of ShK and ShK-Dap22 are quite similar, as shown in Fig. 8B. Pairwise r.m.s. differences over the backbone heavy atoms N, C\text{\textalpha}, and C between the closest-to-average structures for ShK and ShK-Dap22 are 1.82 Å over residues 1–35, 1.70 Å over residues 21–23–35 (the well defined region of the analogue), and 1.38 Å over the well defined region of ShK (residues 3–33). The main secondary structure elements of the two molecules are the same, but ShK-Dap22 also has a recognizable helix near the C terminus involving residues 29–32. In ShK, this region has a similar structure but does not satisfy the criteria for a helix. The only appreciable differences between the backbone dihedral angles of the two structures occur at Pro\text{\textgamma}(\phi), Thr\text{\textgamma}(\phi), and the three C-terminal residues (\phi).

Potassium Channel Binding Residues in ShK-Dap22—In Fig. 8C, the structures of ShK-Dap22 and ShK are aligned over N, C\text{\textalpha}, C, and C\text{\textgamma} of residues 11–23, which includes the most important residues for potassium channel binding (15, 16) (Figs. 3 and 4). In this view, the side chains of Arg\text{\textgamma} and Tyr\text{\textbeta} have similar orientations, although they have moved closer together. The distances from Tyr\text{\textgamma} C\text{\textalpha} to Arg\text{\textgamma} C\text{\textalpha} are 3.9 ± 0.2 and 7.4 ± 0.7 Å, respectively, in ShK-Dap22 and ShK. The functionally more important distances from the centroid and phenolic oxygen of Tyr\text{\textgamma} to Arg\text{\textgamma} C\text{\textalpha} are, respectively, 4.9 ± 0.2 and 3.3 ± 0.2 Å in ShK-Dap22 and 6.7 ± 1.1 and 4.7 ± 1.4 Å in ShK. In ShK, the Lys\text{\textgamma} side chain is not as well defined as other side chains in this region. The shorter Dap22 side chain of ShK-Dap22 is better defined and in most structures is oriented toward the Tyr\text{\textgamma} ring (there may be a weak hydrogen-bonding interaction with the positively charged NH\text{\textgamma} group and the aromatic ring). Distances from the centroid and phenolic oxygen of Tyr\text{\textgamma} to Arg\text{\textgamma} C\text{\textalpha} or C\text{\textbeta} of residue 22 are, respectively, 4.5 ± 1.1 and 4.9 ± 1.0 Å in ShK-Dap22 and 6.6 ± 0.8 and 8.1 ± 0.8 Å in ShK. Corresponding distances from C\text{\textalpha} of Arg\text{\textgamma} to N\textalpha or C\textbeta of residue 22 are, respectively, 8.0 ± 1.1 in ShK-Dap22 and 11.7 ± 1.5 Å in ShK. Thus, it seems that these three functionally important residues (11, 22, and 23) have moved closer together in ShK-Dap22.

There has been an associated shift in the positions of the side chains of Ile\text{\textgamma} and Phe\text{\textgamma}. The centroid of the aromatic ring of Tyr\text{\textgamma} is 6.3 ± 0.2 Å from the centroid of the phenyl ring of Phe\text{\textgamma} in ShK-Dap22, compared with 4.5 ± 0.4 Å in ShK, and is
6.8 ± 0.2 Å from C\textsubscript{b} of Ile\textsuperscript{7}, compared with 7.9 ± 0.7 Å in ShK. Distances from N\textsubscript{g} or C\textsubscript{g} of residue 22 to the centroid of the Phe\textsuperscript{27} ring, however, are unchanged at about 6.2 Å. The shorter side chain of Dap\textsuperscript{22} (compared with that of Lys\textsuperscript{22} in ShK) might be expected to increase the solvent accessibility of nearby residues. The largest increase in ShK-Dap\textsuperscript{22} (1.4-fold) was for His\textsuperscript{19}, with the flanking residues showing little deviation.

**DISCUSSION**

In this study, we pursued three overlapping goals. First, using the ShK peptide as a structural template and applying thermodynamic mutant cycle analysis, we determined the spatial proximity of eight pairs of ShK and Kv1.3 residues. These data, along with those obtained from earlier mapping studies with scorpion toxins (10, 11), guided our docking of ShK into the channel. This docking configuration might provide insights into the interaction of other members of this novel structural class of sea anemone peptides (e.g. BgK) and potassium channels.

Second, we used the docking model to identify the Kv1.3-specific ShK mutant, ShK-Dap\textsuperscript{22}. ShK-Dap\textsuperscript{22} inhibited mitogen-stimulated human T-cell activation \textit{in vitro} (4–9). The most potent and selective of these, MgTX, has also been shown to effectively suppress delayed-type hypersensitivity and alloimmune responses \textit{in vivo} in micro- and mini-pigs, despite its inability to completely suppress T-lymphocyte activation \textit{in vitro} (9). However, MgTX also potently blocks the closely related channels Kv1.1 and Kv1.2 (12, 13), which are expressed in the brain and peripheral neurons (14), and is therefore potentially toxic. An equally potent but more selective peptide blocker of Kv1.3 might not exhibit these side effects. The structurally defined peptidic inhibitor, ShK-Dap\textsuperscript{22}, exhibits the requisite potency and specificity for the Kv1.3 channel target.

**Comparison of Structures of ShK and ShK-Dap\textsuperscript{22}**—The overall structure of ShK-Dap\textsuperscript{22} is similar to that of native ShK toxin, but there are some differences in the side chains involved.
FIG. 8. **Solution structure of ShK-Dap**

**A**, stereo view of the best 20 structures of ShK-Dap, superimposed over the backbone heavy atoms N, Cα, and C of residues 2–21 and 23–35. Only the backbone heavy atoms are shown, except for the three disulfide bonds (3–35, 12–28, and 17–32), which are shown in color. **B**, ribbon diagrams of the closest-to-average structures for ShK-Dap (red) and ShK (blue) superimposed over the backbone heavy atoms of residues 3–33, excluding residue 22. **C**, ribbon diagrams of ShK-Dap and ShK showing key residues for potassium channel binding (15, 16) as follows: Arg11 (cyan), Dap22/Lys22 (dark blue), Tyr23 (red), Ile7 (pink), Ser20 (purple), and Phe27 (orange). The structures were aligned over N, Cα, C, and Cβ of residues 11–23. The ribbon of the closest-to-average structure is shown in each case, together with the relevant side chains of all 20 structures. This diagram was generated using Insight II.
in Kv1.3 binding (Fig. 8). Are these differences significant, or do they reflect differences between the number and distribution of NMR-based restraints in key regions in the structure (Fig. 7A)? The \(^1\)H chemical shifts of the two molecules are very similar, the only differences >0.1 ppm being for Met\(^{21}\) NH (\(\Delta\delta\) 0.25 ppm), Dap\(^{22}\), and residues 26–28 (Table SI and Fig. S2 in “Appendix”). The \(J_{\text{HNCO-H}}\) coupling constants, which are dependent on backbone \(\phi\) angles, also differed by >1Hz for residues 26, 27, and 29 (other residues in this category were 9, 10, 16, and 35). The backbone amide resonance of Dap\(^{22}\) was not observed, and those of Met\(^{21}\) and Tyr\(^{23}\) in ShK-Dap\(^{22}\) were broader than in ShK. As a result, there were fewer NOEs to these protons (Fig. S3 in “Appendix”), and this region of the structure is not as well defined in ShK-Dap\(^{22}\). Part of the reason for the broader Dap\(^{22}\) NH resonance is that the intrinsic line width is greater, as found in the pentapeptide GlyGly-DapGlyGly; this presumably reflects the proximity of the side chain ammonium group of Dap to the backbone. However, this is unlikely to be the explanation for the flanking residues, suggesting that this region has greater conformational flexibility in ShK-Dap\(^{22}\). To confirm the difference between ShK-Dap\(^{22}\) and ShK, we recorded a 2D NOE spectrum on a mixture of the two at pH 4.7 and 293 K. Resonance overlap prevented any comparison for Tyr\(^{23}\), but it was quite clear that the cross-peaks from Met\(^{21}\) of ShK-Dap\(^{22}\) were broader and weaker than those of ShK. As the chemical shift of Met\(^{21}\) NH was also perturbed, it seems that there are some genuine differences in the local structure and dynamics of ShK-Dap\(^{22}\) around the substituted residue. The backbone amides of ShK-Dap\(^{22}\) also show slightly faster exchange than those of ShK (although respective rate constants are within a factor of 2), suggesting that the overall structure of ShK-Dap\(^{22}\) may be slightly more flexible than that of ShK.

In other regions, particularly the N and C termini, the apparent structural differences (Fig. 8) stem partly from the presence of a few NOE unique to one of the restraint sets. The ShK-Dap\(^{22}\) structures are better defined than those of ShK at both termini, but it is important to note that there is some flexibility in these regions of both structures and that both may change when bound to Kv1.3. Finally, the close similarity between the structures of ShK-Dap\(^{22}\) and ShK confirms that the change when bound to Kv1.3. The parent structural differences (Fig. 8) stem partly from the flexibility in these regions of both structures and that both may show slightly faster exchange than those of ShK. As the chemical shift of Met\(^{21}\) NH was also perturbed, it seems that there are some genuine differences in the local structure and dynamics of ShK-Dap\(^{22}\) around the substituted residue. The backbone amides of ShK-Dap\(^{22}\) also show slightly faster exchange than those of ShK (although respective rate constants are within a factor of 2), suggesting that the overall structure of ShK-Dap\(^{22}\) may be slightly more flexible than that of ShK.

APPENDIX

**TABLE SI**

Proton chemical shifts of ShK-Dap\(^{22}\) at 20 °C and pH 4.9

| Residue | NH | \(\alpha\)H | \(\phi\)H | \(\gamma\)H | \(\delta\)H | Other |
|---------|----|--------|--------|--------|--------|-------|
| Arg\(^2\) | —  | 4.10   | 1.95   | 1.69   | 3.23   | N'\(H\) 7.30 |
| Ser\(^6\) | 8.92 | 4.48   | 3.80   |        |        |       |
| Cys\(^3\) | 9.03 | 4.85   | 2.98   |        |        |       |
| Ile\(^4\) | 7.78 | 4.66   | 1.95   |        |        |       |
| Asp\(^5\) | 8.63 | 5.31   | 3.26, 2.70 |        |        |       |
| Thr\(^6\) | 9.49 | 4.48   | 4.57   | 1.25   |        |       |
| Ile\(^7\) | 7.27 | 4.79   | 1.90   |        |        |       |
| Pro\(^8\) | —  | 4.27   | 2.42, 1.76 | 1.95, 2.07 | 3.40, 3.82 |       |
| Lys\(^9\) | 8.36 | 3.89   | 2.03, 1.86 | 1.52   | 1.75   | C'\(H2\) 3.07 |
| Ser\(^10\) | 8.46 | 4.10   | 3.91   |        |        |       |
| Arg\(^11\) | 8.15 | 4.44   | 1.94, 2.29 | 1.76   | 3.28, 3.14 | N'\(H\) 7.47 |
| Cys\(^12\) | 7.99 | 5.04   | 3.28, 2.91 |        |        |       |
| Thr\(^13\) | 7.28 | 4.38   | 4.78   | 1.31   |        | O'\(H\) 5.84 |
| Ala\(^14\) | 8.88 | 3.98   | 1.47   |        |        |       |
| Phe\(^15\) | 8.53 | 4.15   | 3.24, 2.88 |        |        | C(2,6) 7.09; C(3,5) 7.06 C(4) 6.86 |

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TABLE SI—continued

| Residue | NH  | αH  | βH  | γH  | δH  | Other          |
|---------|-----|-----|-----|-----|-----|----------------|
| Gln16   | 7.80| 4.18| 1.95| 1.49|     | 2.28, 2.35    |
| Cys17   | 8.52| 4.22| 3.20| 2.94|     | C²Hegend. 2.85|
| Lys18   | 7.52| 4.01| 1.57| 1.41|     | 0.94          |
| His19   | 7.79| 4.46| 3.07| 2.35|     | C(4)H 6.50    |
| Ser20   | 8.35| 5.05| 4.10| 3.90|     | C(2)H 8.34    |
| Met21   | 9.40| 4.13| 2.17| 2.67| 2.56| C¹H 2.06      |
| Dap22   | —   | 4.19|     |     |     | N³CH₁ 7.15    |
| Tyr23   | 8.10| 3.97| 3.38| 2.61|     | C(2,6) 7.50;  |
| Arg24   | 8.06| 3.94| 2.25| 1.77| 1.70| 3.36, 3.22    |
| Leu25   | 8.20| 4.43| 1.77| 1.49|     | 0.89, 0.85    |
| Ser26   | 7.21| 4.74| 3.55| 3.38|     | C(2,6) 6.20;  |
| Phe27   | 7.48| 5.30| 3.27| 2.50|     | C(3,5) 7.22;  |
| Cys28   | 8.60| 5.83| 3.28| 3.13|     | C(4) 7.15     |
| Arg29   | 8.43| 3.91| 1.63| 1.83| 1.46| N²H 7.15, N²H₂|
| Lys30   | 7.21| 4.17| 1.84|     |     | 1.63          |
| Thr31   | 10.87| 3.87| 4.08|     |     | C¹H₆ 3.09     |
| Cys32   | 9.16| 4.78| 3.34| 2.91|     |               |
| Gly33   | 7.87| 4.08| 4.08|     |     |               |
| Thr34   | 8.73| 4.16| 4.42|     |     |               |
| Cys35   | 7.79| 4.33| 3.34| 2.94|     |               |

*a* This resonance could not be assigned due to fast exchange with water.

**FIG. S1.** Summary of NOE connectivities and other NMR data for ShK-Dap²² at pH 4.9 and 293 K. Dap²² is represented by δ. The intensities of dαN, dαN, and dαN connectivities are represented as strong, medium, or weak by the height of the bars. The shaded bar indicates a dα connectivity to Pro°. Medium range connectivities are also shown but with no indication of their relative strength. Values of JNHCαH < 6 Hz are indicated by †, those >8 Hz by ‡; blanks indicate values that could not be measured due to overlap or were between 6 and 8 Hz. Slowly exchanging amide protons (present up to 11.5 h after dissolution in ²H₂O) are indicated by filled circles and those with intermediate exchange rates (present up to 6 h after dissolution in ²H₂O) with open circles.

**FIG. S2.** Plots of deviations from random coil chemical shifts (Δδ) (38) for NH and C¹H resonances of ShK (left) and ShK-Dap²² (center). Differences between the NH and C¹H chemical shifts of ShK-Dap²² are shown on the right. Asterisks for ShK-Dap²² indicate NH resonances that were broad at 293 K compared with those of ShK.
**Fig. S3.** Unique medium and long range NOEs in ShK and ShK-Dap22 plotted as a function of residue number. Filled bars indicate medium range NOEs, open bars long range NOEs. A, NOEs in ShK-Dap22 that were not present in native ShK; B, NOEs observed in ShK but not the analogue.

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