The d-Diastereomer of ShK Toxin Selectively Blocks Voltage-gated K⁺ Channels and Inhibits T Lymphocyte Proliferation*\(^\text{1,2}\)

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The polypeptide toxin ShK is a potent blocker of Kv1.3 potassium channels, which are crucial in the activation of human effector memory T cells (T\(_{\text{EM}}\)); selective blockers constitute valuable therapeutic leads for the treatment of autoimmune diseases mediated by T\(_{\text{EM}}\) cells, such as multiple sclerosis, rheumatoid arthritis, and type-1 diabetes. The critical motif on the toxin for potassium channel blockade consists of neighboring lysine and tyrosine residues. Because this motif is sufficient for activity, an ShK analogue was designed based on d-amino acids. d-Allo-ShK has a structure essentially identical with that of ShK and is resistant to proteolysis. It blocked Kv1.3 with \(K_d\) 36 nM (2,800-fold lower affinity than ShK), was 2-fold selective for Kv1.3 over Kv1.1, and was inactive against other K⁺ channels tested. d-Allo-ShK inhibited human T\(_{\text{EM}}\) cell proliferation at 100-fold higher concentration than ShK. Its circulating half-life was only slightly longer than that of ShK, implying that renal clearance is the major determinant of its plasma levels. d-Allo-ShK did not bind to the closed state of the channel, unlike ShK. Models of d-allo-ShK bound to Kv1.3 show that it can block the pore as effectively as ShK but makes different interactions with the vestibule, some of which are less favorable than for native ShK. The finding that an all-d analogue of a polypeptide toxin retains biological activity and selectivity is highly unusual. Being resistant to proteolysis and nonantigenic, this analogue should be useful in K⁺ channel studies; all-d analogues with improved Kv1.3 potency and specificity may have therapeutic advantages.

Sea anemones contain a family of polypeptide toxins that block potassium channels, the first representative of which to be isolated and characterized was ShK toxin, from Stichodactyla helianthus (1, 2). Its solution structure, determined by NMR spectroscopy (3, 4), consists of two short \(\alpha\)-helices encompassing residues 14–19 and 21–24, and an N terminus with an extended conformation up to residue 8, followed by a pair of interlocking turns that resembles a 3\(_{10}\)-helix. It contains no \(\beta\)-sheet and is thus distinct from the \(\alpha/\beta\) fold found in scorpion K⁺ channel blockers such as charybdotoxin (5) and margatoxin (6) but is similar to BgK toxin (7).

The surface of ShK involved in binding to voltage-activated (Kv) channels has been mapped using alanine scanning and selected toxin analogues (8, 9). Two residues, Lys\(^{22}\) and Tyr\(^{24}\), are crucial for activity. Other residues in ShK also contribute to its affinity and selectivity for K⁺ channels, including Ile\(^7\), Arg\(^{11}\), Ser\(^{20}\), and Phe\(^{27}\) (9) and His\(^{19}\) and Arg\(^{24}\) (10), although the role of His\(^{19}\) may be at least partly structural (4).

ShK blocks K⁺ channels by binding to a shallow vestibule at the outer entrance to the ion conduction pathway and occluding the entrance to the pore. Mutational strategies similar to those employed for the scorpion toxins agitoxin and kaliotoxin (11, 12) were utilized to understand the molecular basis for channel blockade by ShK, although the availability of the KcsA crystal structure (13) allowed a more reliable model of the pore vestibule region of Kv1.3 to be constructed. ShK was docked initially with a crude model using restrained molecular dynamics simulations guided by data from mutant cycle analyses (14). In this configuration, Lys\(^{22}\) of ShK projected into the ion conduction pathway, and Arg\(^{11}\) of ShK lay in the vicinity of His\(^{409}\) in one Kv1.3 subunit. Subsequently, the channel model was refined (15, 16), and ShK was docked using a larger number of restraints from complementary mutational analyses.

ShK blocks not only Kv1.3 (\(K_d = 11\) pm) but also Kv1.1 (\(K_d = 16\) pm), Kv1.6 (\(K_d = 165\) pm) (14), and Kv3.2 (17, 18). More selective analogues have been created, such as ShK-Dap\(^{22}\), in which the critical Lys\(^{22}\) was replaced by the shorter, positively charged, non-natural residue 1,3-diaminopropionic acid (Dap) (14), ShK-F6CA, a fluorescein-labeled analogue of ShK (19), and ShK(L5), in which a Tyr(P) residue is attached through a hydrophilic linker to Arg\(^{17}\) (17).

All human T lymphocytes express two types of K⁺ channels, Kv1.3 and KCa3.1, which play crucial roles in human T cell activation (20–22). The number of channels expressed by a given cell depends on its state of activation and differentiation (23). Kv1.3 channels dominate in terminally differentiated effector memory (T\(_{\text{EM}}\)) cells, and Kv1.3 blockers inhibit the activation of these cells, whereas KCa3.1 blockers are ineffec-

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and supplemental Fig. S1.

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EXPERIMENTAL PROCEDURES

Peptide Synthesis—Fmoc-d-amino acids (Bachem Feinchemikalien) included D-Arg(Pmc), D-Asp(OtBu), D-Cys(-Trr), D-Gln(Trr), D-His(Trr), D-Lys(Boc), D-Ser(tBu), and D-Thr(tBu). All other non-side chain protected derivatives used in the comparison with d-allo-ShK were single coupled. At this point, half of the resin was removed to effect better mixing. The remainder of the peptide sequence was double coupled to the remaining resin aliquot. All of the couplings were mediated by dicyclohexylcarbodiimide in the presence of 2 eq of 1-hydroxybenzotriazole. Following final removal of the Fmoc group, the peptide resin (2.42 g) was cleaved from the resin and simultaneously deprotected using reagent K(27) for 2 h at room temperature. Following cleavage, the peptide was filtered to remove the spent resin beads and precipitated with ice-cold diethyl ether. The peptide was collected on a fine filter funnel, washed with ice-cold ether, and finally extracted with 20% AcOH in H2O. The peptide extract was subsequently diluted into 2 liters of H2O, the pH adjusted to 8.0 with NH4OH and allowed to air oxidize at room temperature for 36 h. Following oxidation of the disulfide bonds, the peptide solution was acidified to pH 2.5 and pumped onto a Rainin Dynamax C18 column (5.0 × 30 cm). The sample was eluted with a linear gradient from 5 to 30% acetonitrile into H2O containing 0.1% trifluoroacetic acid. The resulting fractions were analyzed using two analytical RP-HPLC systems: trifluoroacetic acid and triethylammonium phosphate (28). Pure fractions were pooled and lyophilized. Upon lyophilization, 120 mg of d-allo-ShK toxin amide was obtained, representing a theoretical yield of 21% (from the starting resin).

Peptide Analysis—Synthetic peptide samples were hydrolyzed in 6 N HCl at 110 °C for 22 h in vacuo. Amino acid analysis was performed on a Beckman 126AA System Gold amino acid analyzer. Matrix-assisted laser desorption ionization time-of-flight mass spectroscopic analysis was performed on a Kratos Kompact mass spectrometer using α-cyano-4-hydroxycinnamic acid as a matrix. Amino acid analysis of the purified d-allo-ShK showed the following average amino acid ratios: Asx (1) 1.02, Thr (4) 3.89, Ser (4) 4.04, Glx (1) 0.98, Pro (1) 0.93, Gly (1) 1.02, Ala (1) 1.00, Met (1) 0.88, Ile (2) 1.78, Leu (1) 1.01, Tyr (1) 0.99, Phe (2) 2.00, Lys (4) 3.99, His (1) 0.95, Arg (4) 3.89, and Cys (< 6) 5.26.

NMR Spectroscopy—The spectra were recorded on a sample of d-allo-ShK in 95% H2O, 5% 2H2O at pH 4.9. Two-dimensional homonuclear total correlation spectra with a spin-lock time of 60 ms and double quantum filtered correlation NMR spectra were acquired at 500 MHz on a Bruker AMX-500 spectrometer. A two-dimensional NOESY spectrum with a mixing time of 200 ms was also acquired on a Bruker AMX-500 spectrometer. NOESY spectra for ShK were acquired at 500 and 600 MHz as described previously (3, 4); the 600-MHz NOESY was used in the comparison with d-allo-ShK. Water was suppressed using the WATERGATE pulse sequence (29). All of the spectra were collected at 20 °C unless otherwise stated and were referenced to an impurity peak at 0.15 ppm or to the water resonance.

Diffusion measurements were performed using a pulsed field gradient longitudinal eddy current delay pulse sequence (30, 31) as implemented by Yao et al. (32). The spectra were processed using XWINNMR (Version 3.5), Bruker Biospin) and analyzed using XEASY (Version 1.3.13) (33). Structural figures were prepared using VMD (34).

Proteolytic Digestion—The stability of d-allo-ShK to proteolytic digestion was investigated under the same conditions used to determine the disulfide bridges of ShK toxin (35). d-Allo-ShK (15 μg) was dissolved in 0.05 M HEPES, pH 6.5, containing 10

3 The abbreviations used are: Fmoc, N-(9-fluorenyl)methoxy carbonyl; NOESY, nuclear Overhauser enhancement spectrometry; RP, reversed phase; HPLC, high pressure liquid chromatography; MD, molecular dynamics; DTH, delayed-type hypersensitivity; T_CM, central memory T; T_EM, effector memory T.
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mm CaCl₂ (30 μl) and trypsin, chymotrypsin, or a mixture of trypsin and chymotrypsin (enzymesubstrate1:50, w/w, 30 °C, 6 h). The digestion was terminated by acidification with 10% aqueous trifluoroacetic acid (3 μl), the solution was centrifuged (13,000 × g, 5 min), and the supernatant was analyzed directly by RP-HPLC.

**Modeling and Docking**—An initial model of d allo-ShK was created by inverting the structure of ShK derived by NMR (3) (Protein Data Base code 1ROO, structure 1) and correcting the side chains of threonine and isoleucine residues for the appropriate stereochirality. Both the d allo-model and NMR-derived structures were subjected to molecular dynamics (MD) simulation using the GROMACS (v3.3.1) package of programs (36). All of the simulations consisted of an initial minimization of water molecules followed by 100 ps of MD with the peptide fixed. Following positional restraints MD, the restraints on the peptide were removed, and MD continued for a further 10 ns.

MD simulations of both diastereomers of ShK were performed using the OPLS-aa force field (37). Ionizable residues were assumed to be in their standard state at neutral pH. Each peptide was placed in a 50 × 50 × 50 Å³ water box with no pressure coupling. The total charge on the system was made neutral by replacing water molecules with chloride ions using the Genion program. Peptide, water, and ions were coupled separately to a thermal bath at 300 K using a Berendsen thermostat (38) applied with a coupling time of 0.1 ps. All of the simulations were performed with a single nonbonded cut-off of 10 Å, applying a neighbor list update frequency of 10 steps (20 fs). The particle mesh Ewald method was used to account for long range electrostatics, applying a grid width of 1.2 Å, and a fourth-order spline interpolation. Bond lengths were constrained using the LINCS algorithm (39). All of the simulations consisted of an initial minimization of water molecules followed by 100 ps of MD with the peptide fixed. Following positional restraints MD, the restraints on the peptide were removed, and MD continued for a further 10 ns.

Comparative models of the trans-membrane region (only) of the murine Kv1.3 channel were constructed using the x-ray structure of the K⁺ channel from Streptomyces lividans (KcsA, Protein Data Base code 1BL8) as a template. The MODELLER (6v2) program (40) was used to create nine models based on the sequence alignment shown in supplemental Table S2. mKv1.3 has good sequence similarity with KcsA over the entire pore domain (32% identity), whereas there is 91% sequence identity with Kv1.2. Despite the greater sequence similarity with Kv1.2, the structure of KcsA was chosen as the template for model building because the structure of the two loops comprising the extracellular face of KcsA, the site of toxin binding, has been well characterized (41), whereas those in the more closely related channel, Kv1.2, are disordered in the electron density (42), and are therefore less suitable for model building.

Complexes of the d allo- and l forms of ShK with mKv1.3 were modeled using the ZDOCK program (43). This program uses a fast Fourier transform to explore all of the possible binding modes of the two proteins; docking was restricted to residues on the extracellular surface of the channel to ensure the exclusion of unphysical binding predictions. The interaction is evaluated using shape complementarity, desolvation energy, and electrostatics.

Models of each form of the toxin were extracted at 1-ns intervals during the MD simulation. Including the initial model, we considered 11 models of each form of the toxin. Each model of the toxin was docked with one of the nine models of the channel; thus, we considered all 99 possible combinations of toxin with channel for both d allo and l forms of the toxin. The top 2,000 scoring predictions from each combination were then refined using the RDOCK program (44), in which the binding interface was refined using molecular mechanics minimization. The final docking predictions from all 198,000 complexes (for both forms of the toxin) were ranked according to the RDOCK scoring function.

The highest ranked complexes of both d allo- and l forms of ShK with the channel were subjected to a short 100-ns MD simulation to permit further relaxation of the atoms at the interface. The extracellular face of the complex was capped in a sphere of water molecules, with the molecules at the surface of the sphere fixed at their originally minimized positions (to prevent evaporation). Atoms of residues of the channel more than 8 Å from the ShK peptide were held fixed during these MD calculations. The structures of the complexes were minimized without restraints at the completion of the MD simulation.

The buried surface areas were calculated from the difference in surface areas of channel and toxin from the complex. The surface areas were calculated using the NACCESS program (45).

**Cells and Cell Lines**—L929, B82, and MEL cells stably expressing mKv1.1, rKv1.2, mKv1.3, and hKv1.5 have been described previously (46) and were maintained in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 4 mM l-glutamine, 1 mM sodium pyruvate, and 500 μg/ml G418 (Calbiochem). LTK cells expressing hKv1.4 were obtained from M. Tamkun (University of Colorado, Boulder, CO), CHL cells expressing mKv1.7 were from Vertex Pharmaceutical Inc (San Diego, CA), and HEK293 cells stably expressing hKCa3.1 were a kind gift from Dr. Khaled Houamed (Chicago, IL). PAS T cells, a major histocompatibility complex class II-restricted myelin basic protein-specific encephalitogenic CD4⁺ rat T cell line (47), were a kind gift from Dr. Evelyne Béraud (Marseille, France). Mononuclear cells were isolated from Lewis rat (Harlan-Sprague-Dawley, Indianapolis, IN) spleens using Histopaque-1083™ gradients (Sigma).

**Electrophysiology**—The cells were studied in the whole-cell configuration of the patch clamp technique. The holding potential in all experiments was −80 mV. Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, and Kv1.7 currents were recorded in normal Ringer solution with a calcium-free pipette solution containing 145 mM KF, 10 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 7.2, 300 mM osm, as described previously (46). KCa3.1 currents were recorded as described previously (48). The Kᵥ values were determined from dose-response curves shown using Microcal Origin software.

[^3H]Thymidine Incorporation Assays—Rat splenocytes seeded at 2 × 10⁵ cells/well in RPMI culture medium in flat-bottomed 96-well plates (final volume, 200 μl) were preincubated with increasing concentrations of ShK or d allo-ShK for...
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from rats were obtained from the saphenous vein (50) at various times after a single subcutaneous injection of 1 mg/kg δ-allo-ShK in phosphate-buffered saline + 2% rat serum. In another series of experiments, the rats received daily subcutaneous injections of 1 mg/kg δ-allo-ShK, and blood was drawn 24 h after each injection. The serum samples were tested for Kv1.3 blocking activity by patch clamp, and the levels of δ-allo-ShK were determined from the standard curve as described (25).

For the DTH experiments, the rats were immunized with an emulsion of ovalbumin in complete Freund’s adjuvant (Difco, Detroit, MI) (51). Seven days later, they received an injection of ovalbumin dissolved in saline in the pinna of one ear and saline in the other ear (52). The rats then received a subcutaneous injection of δ-allo-ShK (1 mg/kg) or vehicle (phosphate-buffered saline + 2% rat serum). Ear swelling was measured 24 h later using a spring-loaded micrometer (Mitutoyo, Spokane, WA).

RESULTS

Synthesis of δ-Allo-ShK Amide—ShK amide consists of 35 residues with a C-terminal amide (1). Synthesis of δ-allo-ShK with a C-terminal amide was initiated on Ramage amide resin using an automated protocol where the first 12 residues

were single-coupled, and the remaining 22 residues were double-coupled. The biologically active form of ShK toxin requires proper folding of three disulfide bonds (53). Following cleavage and deprotection, 36 h was allowed for peptide folding and disulfide bond formation under alkaline conditions in the presence of air under the same conditions used for ShK amide (2); after 36 h there was no further change in the HPLC profile (Fig. 1). Folding proceeded smoothly to a unique, earlier eluting, major product with the same profile as ShK amide. The purified product using δ-cyano-4-hydroxycinnamic acid as the matrix. Theoretical mass is 4054.1 observed M+H = 4055.2. D, RP-HPLC profile of δ-allo-ShK following 6 h of treatment with chymotrypsin and trypsin. Gradient conditions were identical with those used in A. C, matrix-assisted laser desorption ionization time-of-flight mass spectral analysis of the purified product using α-cyano-4-hydroxycinnamic acid as the matrix. Theoretical mass is 4054.1 observed M+H = 4055.2. D, RP-HPLC profile of δ-allo-ShK following 6 h of treatment with chymotrypsin and trypsin. Gradient conditions were identical with those used in A. E, amino acid sequence of ShK toxin showing location of three disulfide bridges.

In Vivo Experiments—Female inbred Lewis rats 9–11 weeks old were purchased from Harlan-Sprague-Dawley (Indianapolis, IN) and housed under barrier conditions with irradiated rodent chow and acidified water ad libitum. All of the experiments were in accordance with National Institutes of Health guidelines and approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

To determine the circulating half-life of δ-allo-ShK, known amounts of δ-allo-ShK were added to Lewis rat serum, and the blocking activity on Kv1.3 channels was tested by patch clamp to establish a standard dose-response curve. Blood samples

were single-coupled, and the remaining 22 residues were double-coupled. The biologically active form of ShK toxin requires proper folding of three disulfide bonds (53). Following cleavage and deprotection, 36 h was allowed for peptide folding and disulfide bond formation under alkaline conditions in the presence of air under the same conditions used for ShK amide (2); after 36 h there was no further change in the HPLC profile (Fig. 1). Folding proceeded smoothly to a unique, earlier eluting, major product with the same profile as ShK amide. The purified δ-allo-ShK was homogeneous by analytical RP-HPLC in two different solvent systems (trifluoroacetic acid and triethylammonium phosphate) (Fig. 1). Matrix-assisted laser desorption ionization time-of-flight analysis yielded a (M+H) of 4054, consistent with the theoretical value following formation of three disulfide bonds.

Susceptibility of δ-Allo-ShK to Proteolysis—Neither trypsin nor chymotrypsin nor a mixture of both proteases had any effect on δ-allo-ShK, as assessed by RP-HPLC (Fig. 1D). Cleavage at basic or aromatic residues was prevented by the δ-stereo-
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chemistry at \( C^\circ \). This result was not unexpected, because Milton et al. had shown previously that only an all-\( d \) enzyme was capable of cleaving an all-\( d \) peptide substrate, but it could not digest an all-\( l \) peptide substrate (54).

NMR Spectroscopy—The backbone conformation of \( d \)-allo-ShK is a mirror image of that of naturally occurring \( (L-) \) ShK, but chiral side chains such as those of Ile and Thr retained their native configurations. Selected regions of the NOESY spectra of ShK and \( d \)-allo-ShK are shown in supplemental Fig. S1. One difference between the two molecules is that \( d \)-allo-ShK is C-terminally amidated, whereas native ShK is not. In fact, the C-terminally amidated analogue of native ShK is fully active (see below). The C-terminal amide gives rise to additional peaks in the spectrum (from the amide group), and amidation affects the chemical shifts of residues near the C terminus, which, at the pH used for NMR (4.9), is negatively charged. The chemical shifts for \( d \)-allo-ShK are tabulated in supplemental Table S1, and differences of more than 0.03 ppm from those of ShK (3, 4) are highlighted.

A potentially more discriminating comparison of the two structures is afforded by a comparison of NOESY cross-peak intensities. This is compromised somewhat by the higher quality of the \( d \)-allo-ShK NOESY spectrum compared with that for ShK, but some differences are noted here. A \( d_{on} \) NOE between Thr\(^6\) and Ile\(^7\) is missing in \( d \)-allo-ShK, probably reflecting an alteration in the backbone in this region. A long range NOE from Ile\(^7\) to Arg\(^{29}\) is also missing in \( d \)-allo-ShK, but two new NOEs between these residues are present. There are \( \sim 20 \) additional NOEs that are observed only in the \( d \)-allo-ShK spectrum, including sequential medium range and long range NOEs; about half of these involve the side chains or backbone of Thr\(^6\), Ile\(^7\), Thr\(^{13}\), Thr\(^{31}\), and Thr\(^{34}\), reflecting the different side chain conformations relative to the backbone. Nonetheless, the overall similarities of the chemical shifts and NOE patterns for the ShK and \( d \)-allo-ShK indicate that their conformations are essentially mirror images of one another and that the different stereochemistry of the Thr and Ile side chains in \( d \)-allo-ShK relative to the backbone does not cause significant perturbations beyond the resolution of the solution structures.

Our findings for ShK versus \( d \)-allo-ShK are consistent with previous studies of all-\( d \) analogues of other structured polypeptides. Crystals of racemic rubredoxin, prepared by independent chemical synthesis of the \( l \)- and \( d \)-enantiomers, were centrosymmetric, and the 2 \( \AA \) resolution structures of the two forms were mirror images of one another (55). NMR spectra of the all-\( d \) and all-\( l \) versions of the plant trypsin inhibitor EETI-II were identical with each other (56).

\( K^+ \) Channel Blocking Activity—We tested ShK, ShK-amide, and \( d \)-allo-ShK on Kv1.3 and Kv1.1 channels stably expressed in L929 cells. Fig. 2A shows the effects of ShK and \( d \)-allo-ShK on Kv1.3 (left panels) and Kv1.1 (left panels) currents elicited by 200-ms depolarizing pulses from a holding potential of \(-80 \) to 40 mV. Both peptides reversibly blocked Kv1.3 and Kv1.1 in a dose-dependent manner with Hill coefficients of 1 (Fig. 2B).

Native ShK and ShK-amide blocked Kv1.3 with essentially identical affinities, and both displayed a 2-fold selectivity for Kv1.3 over Kv1.1 (ShK: \( K_d \) for Kv1.3 \( 13 \pm 4 \) \( n \)M and for Kv1.1 \( 29 \pm 3 \) \( n \)M; ShK-amide: \( K_d \) for Kv1.3 \( 14 \pm 3 \) \( n \)M and for Kv1.1

\( 31 \pm 4 \) \( n \)M), as expected (Fig. 2). \( d \)-Allo-ShK blocked Kv1.3 with a 2,800-fold lower affinity than ShK (\( K_d \) 36 \( \pm 3 \) \( n \)M) but displayed the same 2-fold selectivity for Kv1.3 over Kv1.1 as ShK (\( K_d \) on Kv1.3 \( 83 \pm 9 \) \( n \)M) (Fig. 2). \( d \)-Allo-ShK had no effect on Kv1.2, Kv1.4, Kv1.5, Kv1.7, or KCa3.1 at concentrations up to 1 \( \mu \)M (Fig. 2C). The activation time constant (\( \tau_a \)) and the inactivation time constant (\( \tau_i \)) of the Kv1.3 current (\( \tau_i = 6.2 \pm 1.3 \) ms; \( n = 13 \); \( \tau_i = 174 \pm 6 \) ms) (57) were not altered by ShK (\( \tau_i = 7.4 \pm 1.6 \) ms; \( n = 6 \); \( \tau_i = 161 \pm 6 \) ms), and \( d \)-allo-ShK (\( \tau_i = 5.1 \pm 2 \) ms; \( n = 6 \); \( \tau_i = 165 \pm 27 \) ms) did not alter these parameters at concentrations sufficient to induce 60% block (data not shown).

To further characterize the blocking activity of ShK and \( d \)-allo-ShK on Kv1.3, we tested their ability to bind to the closed state of the channel. We allowed equilibration of the internal solution for 5 min before applying a 200-ms pulse from \(-80 \) to 40 mV to elicit a control Kv1.3 current (Fig. 3). We then perfused ShK (20 \( \mu \)M) or \( d \)-allo-ShK (50 \( n \)M) into the bath, whereas the channel was closed for 5 min before pulsing again at 30-s intervals. ShK blocked 60% of the Kv1.3 current at the first pulse after peptide incubation, and this blockade did not increase after applying a further 10 depolarizing pulses, indicating that ShK binds to the closed state of the Kv1.3 channel. In contrast, \( d \)-allo-ShK had no effect on the current at the first pulse after peptide incubation, and steady-state block was only reached after several depolarizing pulses, a phenomenon termed “use-dependent block,” indicating that \( d \)-allo-ShK binds to an open or inactivated conformation of the channel.

The activities of both ShK and \( d \)-allo-ShK on the proliferation of rat Kv1.3\(^{\text{high}}\)KCa3.1\(^{\text{low}}\) T\(_{EM}\) and Kv1.3\(^{\text{low}}\)KCa3.1\(^{\text{high}}\) naïve/T\(_{EM}\) lymphocytes were also tested. In keeping with its loss of affinity for Kv1.3 channels, \( d \)-allo-ShK was significantly less potent than ShK in inhibiting T\(_{EM}\) cells (\( p < 0.05 \) at \( 10^{-1} \))
nM and p < 0.01 at all other concentrations) (Fig. 4A), and both peptides were less effective in suppressing the proliferation of naïve/TcM lymphocytes (Fig. 4B).

Circulating Half-life and Plasma Stability—A patch clamp bioassay was used to determine the circulating half-life of d-allo-ShK. Known amounts of d-allo-ShK were added to normal Lewis rat serum, and the samples were tested on Kv1.3 channels stably expressed in L929 cells. Normal rat serum did not exhibit detectable blocking activity, indicating an absence of endogenous channel blockers. The spiked serum samples blocked Kv1.3 currents in a dose-dependent fashion ($K_d = 38 \pm 4 \, \text{nM}$), similar to the effect of d-allo-ShK observed in the absence of serum (not shown). Levels of d-allo-ShK in rats following a single subcutaneous injection of 1 mg/kg were determined by comparison with the standard curve. d-Allo-ShK was detectable in serum 25 min after injection (Fig. 5A). Peak levels (950 nM) were reached within 50 min, and the level then fell to 15 nM within 24 h (Fig. 5A). The disappearance of d-allo-ShK from the serum could be fitted by a single exponential (Fig. 5B), and its circulating half-life was estimated to be 40 min.

D-Allo-ShK Inhibits DTH Response in Rats—As an assessment of the immunosuppressive activity of d-allo-ShK in vivo, we tested its ability to inhibit a DTH reaction to ovalbumin mediated predominantly by skin-homing TEM cells (58). All vehicle-treated control rats developed ear swelling 24 h after ovalbumin challenge in the ear, but the DTH reaction was significantly milder in animals treated with 1 mg/kg d-allo-ShK at the time of challenge in the ear (Fig. 5C). Thus, d-allo-ShK inhibited the TEM-mediated DTH response.

Docking of D-Allo-ShK with Kv1.3—To examine how d-allo-ShK could block Kv1.3, we constructed models of the complexes of both d-allo-ShK and ShK with the pore vestibule region and inner helices of the channel. The models of the highest ranked predictions from the docking of ShK and the chiral analogue, d-allo-ShK, with the channel are presented in Fig. 6. In both models, Lys22 is located in the ion selectivity filter, blocking passage of K$^+$ ions through the channel; its ammonium group forms hydrogen bonds with the backbone carbonyl oxygen atoms of the tetrad of Gly399 residues. Phe27 packs alongside Lys22, in the space created by two Gly401 residues of the ion selectivity filter from neighboring channel monomers (monomers A and B for ShK, and monomers C and D for d-allo-ShK, in Fig. 6), and the side chains of Asp402 and His404. The side chain of Met21 occupies the equivalent pocket in the space diametrically opposite the pocket filled by Phe27.

The interactions between residues at the interface between ShK and the channel were assessed in relation to earlier experimental mutant cycle analysis (16). Mutant cycle analysis had indicated that ShK was strongly coupled with His404 of the channel. In their model of ShK complexed to Kv1.3, Lanigan et al. (16) noted that the distance of closest approach of Arg11 of ShK with His404 of Kv1.3 was 11 Å. In the current model this separation is 4.3 Å (Arg11 N' with HisB404 N'). The channel residues Asp386 and Ser379 were also implicated in coupling
with Arg\textsuperscript{11} of ShK in the complex. In the present model the distance between the N\textsuperscript{e} of Arg\textsuperscript{11} and the O\textsuperscript{a} of Asp\textsubscript{386} is 2.6 Å. Notably, the Asp\textsubscript{386} and Ser\textsubscript{379} that contact Arg\textsuperscript{11} are on a different channel monomer from the His\textsubscript{404} also close to Arg\textsuperscript{11}.

Mutant cycle analysis also suggested that Ser\textsubscript{379} and His\textsubscript{404} of the channel were close to Arg\textsuperscript{29} of ShK. In the current model the separations are 8.3 Å (between Ser\textsubscript{379} O\textsuperscript{a} and Arg\textsuperscript{29} N\textsuperscript{e}) and 4.4 Å (between His\textsubscript{404} N\textsuperscript{e} and Arg\textsuperscript{29} N\textsuperscript{e}); these residues on the channel are on different monomers from those that interact with Arg\textsuperscript{11}. The mutant cycle analysis indicated cooperativity between Asp\textsubscript{386} of the channel and Arg\textsuperscript{29} of ShK. In the model, these residues are ~9 Å apart. Although mutant cycle analysis did not support significant energetic coupling between His\textsubscript{404} of the channel and Phe\textsubscript{27} of ShK, these residues are in close contact in the current model (His\textsubscript{404} N\textsuperscript{e} is separated by 4.4 Å from the centroid of the aromatic ring of Phe\textsubscript{27} of ShK). In contrast, Asp\textsubscript{386} of the channel and Ser\textsubscript{379} of ShK are separated by almost 10 Å in the model, consistent with only modest coupling observed in the mutant cycle analysis. In the Lanigan \textit{et al.} (16) model His\textsubscript{404} and Ser\textsubscript{20} were separated by 11.6 Å, whereas in the current model they are separated by 3.9 Å.

Although the side chains of Tyr\textsuperscript{400} of the channel are not in close contact with the N\textsuperscript{e} of Lys\textsubscript{22}, these groups help maintain the shape of the selectivity filter of the channel, in particular ensuring close contact with N\textsuperscript{e} of Lys\textsubscript{22} and the backbone carbonyl of Gly\textsuperscript{399}. The cooperativity observed in the mutant cycle analysis between Tyr\textsuperscript{400} and Lys\textsubscript{22} reflects the structural role played by the side chains of Tyr\textsuperscript{400}. Similarly, the backbone atoms of Asp\textsubscript{402} form part of the selectivity filter; the side chains of these residues contribute to the structure of the filter. A summary of several internuclear distances for the two diastereomeric models of ShK complexed with the channel is presented in supplemental Table S3.
Thus, the models suggest that D-allo-ShK and ShK bury roughly the same surface area upon channel binding.

**DISCUSSION**

We have created a mirror image analogue of ShK consisting entirely of D-amino acids. D-Allo-ShK folded in good yield and gave a high resolution NMR spectrum essentially identical to the native (all-L) polypeptide. The most significant NMR spectral changes could be attributed to the presence of a C-terminal amide on D-allo-ShK, compared with a free C-terminal carboxylate on naturally occurring ShK. D-Allo-ShK is not a perfect mirror image of ShK because the stereochemistry of the side chains was not changed in concert with the change in the backbone, but this is only significant for the two Ile and four Thr side chains, and our NMR data show that the local structure in the vicinity of these residues is essentially identical to that of ShK.

Polypeptides that are mirror images of their naturally occurring (all-L) homologues are expected to show no activity against their natural targets when those targets are other proteins. However, D-allo-ShK blocked the Kv1.3 channel with nM affinity, making it a weaker blocker than the native toxin but nonetheless still a potent inhibitor. It also maintained the selectivity profile of the native peptide but interestingly did not block the closed state of the channel, unlike ShK. D-Allo-ShK was also able to block T cell proliferation. It is highly unusual for an all-D analogue of a folded polypeptide or protein that acts at a specific binding site on a target protein to retain activity. Kent and co-workers (54) found that an all-D analogue of human immunodeficiency virus, type 1 protease was capable of cleaving an all-D peptide substrate but was inactive against the natural all-L peptide substrate. Similarly, Nielsen et al. (56) found that an all-D version of the plant trypsin inhibitor EETI-II was inactive against trypsin, even though its conformation as judged by NMR was identical to that of the native all-L polypeptide. Recently, an intriguing exception to the lack of activity of all-D polypeptides was found in the case of the tarantula toxin GsMTx4, a selective inhibitor of stretch-activated cation channels (59). Both GsMTx4 and its enantiomer modified channel gating of stretch-activated cation channels, a result interpreted as indicating that this polypeptide acted at the channel-lipid interface rather than at a specific binding site exclusively associated with the channel protein.

We had originally proposed that the homotetrameric Kv1.3 channel could accommodate an enantiomer or diastereomer of the native toxin, and its ability to block the channel with nM affinity affirms our underlying hypothesis. However, the lack of a center of inversion within the 4-fold symmetric channel precludes D-allo-ShK from binding in a manner that is the mirror
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(or inverse) of its native enantiomeric partner. It is not surprising then that d-allo-ShK is not equipotent with native ShK in its ability to block Kv1.3. The overlay of the polypeptide trace of the two peptides in Fig. 6 illustrates how the two peptides engage the channel differently and without any formal symmetry relationship between them. Nonetheless, the two enantiomeric peptides make many similar interactions with the channel in the two models (for example Lys22 fills the ion selectivity filter, and Arg24 and Arg29 of the peptides interact with His404 of the channel), although these interactions are not identical in the two models. Because symmetry plays no role in determining the binding of d-allo-ShK to Kv1.3, and d-allo-ShK showed the same selectivity for Kv1.3 over Kv1.1 and other K+ channels as Shk, it is likely to be active against heterotetrameric channels consisting of mixtures of various K+ channel subunits.

ShK has a circulating half-life of ∼30 min (25). Such a rapid clearance from the blood could be due to renal elimination and/or proteolysis. The finding that d-allo-ShK has a similar half-life (∼40 min) implies that the disappearance of ShK and d-allo-ShK is due to rapid renal clearance because endogenous proteases can only cleave t. experimental situations where its resistance to proteolysis prolongs its lifetime. Moreover, it is expected not to be recognized by the immune system because it is not likely to be antigenic in vivo. Further modification of the all-d polypeptide to enhance Kv1.3 selectivity, as achieved for the native toxin (14, 17, 19) should yield a selective, protease-resistant, and nonimmunogenic reagent for studies of channel structure and function. More generally, our results demonstrate that mirror image polypeptides can retain pot ent activity and may have broader applications in both in vitro and in vivo studies than hitherto appreciated.

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The D-Diastereomer of ShK Toxin Selectively Blocks Voltage-gated K+ Channels and Inhibits T Lymphocyte Proliferation

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