Structure-guided Transformation of Charybdotoxin Yields an Analog That Selectively Targets Ca$^{2+}$-activated over Voltage-gated K$^+$ Channels*

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We have used a structure-based design strategy to transform the polypeptide toxin charybdotoxin, which blocks several voltage-gated and Ca$^{2+}$-activated K$^+$ channels, into a selective inhibitor. As a model system, we chose two channels in T-lymphocytes, the voltage-gated channel Kv1.3 and the Ca$^{2+}$-activated channel IKCa1. Homology models of both channels were generated based on the crystal structure of the bacterial channel KcsA. Initial docking of charybdotoxin was undertaken with both models, and the accuracy of these docking configurations was tested by mutant cycle analyses, establishing that charybdotoxin has a similar docking configuration in the external vestibules of IKCa1 and Kv1.3. Comparison of the refined models revealed a unique cluster of negatively charged residues in the turret of Kv1.3, not present in IKCa1. To exploit this difference, three novel charybdotoxin analogs were designed by introducing negatively charged residues in place of charybdotoxin Lys32, which lies in close proximity to this cluster. These analogs block IKCa1 with ~20-fold higher affinity than Kv1.3. The other charybdotoxin-sensitive Kv channels, Kv1.2 and Kv1.6, contain the negative cluster and are predictably insensitive to the charybdotoxin position 32 analogs, whereas the maxi-Kv channel, hSlo, lacking the cluster, is sensitive to the analogs. This provides strong evidence for topological similarity of the external vestibules of diverse K$^+$ channels and demonstrates the feasibility of using structure-based strategies to design selective inhibitors for mammalian K$^+$ channels. The availability of potent and selective inhibitors of IKCa1 will help to elucidate the role of this channel in T-lymphocytes during the immune response as well as in erythrocytes and colonic epithelia.

Potassium channels are a diverse superfamily of ~80 integral membrane proteins that play crucial roles in many different physiological processes and are widely recognized as therapeutic targets. Venoms from spiders, scorpions, snakes, bees, and marine extracts have yielded polypeptide inhibitors of mammalian K$^+$ channels, many of which bind with high affinity to a vestibule at the external entrance of the channel pore. Some of these polypeptide toxins have been used as molecular calipers to estimate the dimensions of K$^+$ channel vestibules (1–7). Recently, the structure of the bacterial K$^+$ channel KcsA from Streptomyces lividans has been determined by x-ray crystallography (8). The turret region and the external pore of this channel correspond to the external toxin-binding vestibule in eukaryotic K$^+$ channels, and the crystallographic dimensions of the KcsA channel vestibule are remarkably similar to those estimated by toxin-mapping methods for eukaryotic K$^+$ channels (9). The convergence of these two approaches raises the possibility of exploiting structure-based strategies to design specific inhibitors that target pharmacologically relevant K$^+$ channel targets.

To test the feasibility of this approach, we have used charybdotoxin (ChTX), a polypeptide that potently blocks the voltage-gated channel Kv1.3 and the Ca$^{2+}$-activated channel IKCa1, both present in human T-lymphocytes (10, 11) to design an analog that selectively targets IKCa1. These two channels have been chosen as our model system since they are widely regarded as therapeutic targets. Both channels regulate the membrane potential of resting and activated T-cells and modulate the calcium signaling response that is essential for their activation (12). Inhibitors of these channels block the activation of human T-lymphocytes (13–15). Several potent and selective peptide and non-peptide inhibitors are available for Kv1.3. However, there is a dearth of selective blockers of the Ca$^{2+}$-activated IKCa1 channel, and the most selective inhibitor of this channel, clotrimazole, also inhibits cytochrome P450-dependent enzymes (16–19). The IKCa1 gene also encodes the “Gardos” channel in erythrocytes and is thought to encode the I$^*_K(Ca)$ channel in colonic epithelial cells, platelets, and pancreatic islets (20–23). IKCa1 inhibitors are currently being evaluated for prevention of chloride and water loss in diarrhea and for the treatment of erythrocyte dehydration in sickle cell disease (19, 24, 25). Highly specific blockers of the IKCa1 channel may therefore have clinical use in both these ailments as well as a potential use as immunosuppressants.

In this study, we have constructed homology models of IKCa1 and Kv1.3 based on the crystal structure of the KcsA channel and performed preliminary docking of ChTX for heuristic purposes. The accuracy of these docking configurations

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† The abbreviations used are: ChTX, charybdotoxin; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography; Dap, diaminopropionic acid; Cpa, p-carboxyphenylalanine.
was tested by mutant cycle analysis that measures the strength of coupling between interactive pairs of toxin and channel residues (26). Using this approach, we have determined the ChTX docking configuration in both channels and identified a structural feature unique to the ChTX-IKCa1 interaction surface. This paper describes the guided design and electrophysiological characterization of three novel ChTX analogs that specifically target this unique IKCa1 motif.

MATERIALS AND METHODS

Reagents—Cell lines stably expressing mouse Kv1.1, rat Kv1.2, mouse Kv1.3, human Kv1.4, human Kv1.5, and mouse Kv3.1 (27) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and G418 (1 mg/ml). The rat Kv1.6 expression construct was a kind gift from Dr. O. Pongs (ZMNH, Hamburg, Germany). The human IKCa1 expression construct (11) and the PCR-generated (28) IKCa1-Asn239 and IKCa1-Lys239 mutants (7) have been described. The hSlo (BKv1.1) expression construct was a kind gift from Dr. L. Toro (UCLA). Endogenously expressed IKCa and Kv1.3 currents were studied in phthahemagglutinin-activated human T-lymphocytes, and endogenous SKCa currents were studied in the human Jurkat T-cell line (29, 30). Fetal calf serum, L-glutamine, penicillin, and streptomycin were obtained from Life Technologies, Inc.

Polypeptide Synthesis—Fmoc-derivatives were obtained from Bachem AG (Bubendorf, Switzerland). Solid-phase assembly was initiated with t-buty1-Fmoc-Ser resin. Automated stepwise assembly was carried out entirely on an ABI 431A peptide synthesizer (Applied Biosystems, Foster City, CA). The ChTX position 32 analogs were solubilized, oxidized, and purified by reversed-phase HPLC, and fractions were pooled and lyophilized. The structure and purity of the peptides were confirmed by reversed-phase HPLC, amino acid analysis, and electrospray ionization mass spectrometry analysis. Samples were weighed and adjusted to account for peptide content prior to bioassay. The ChTX-Asp25 analog has been described (4). Recombinant ChTX-Gln31, ChTX-Glu31, and ChTX-Orn27 were kind gifts from Dr. C. Miller (Brandeis University).

Homology Models of Kv1.3 and IKCa1—Fig. 1 shows the amino acid sequence of the turret region, pore, and parts of the inner helix of the KcsA channel aligned with the corresponding regions of IKCa1 and Kv1.3. These channel regions interact with polytoxin peptide inhibitors, and mapping studies with these toxins have shown that the external vestibules of the Kv1.3 and IKCa1 channels are topologically similar to that of KcsA (7, 9, 31). Based on the alignment, a homology model (homology modeling, homology) and published structural data for the KcsA channel (8), homology models of the IKCa1 and Kv1.3 vestibules were constructed and energy-minimized. Coordinates for KcsA (Protein Data Bank code 1BL8) were kindly supplied by Dr. MacKinnon (Rockefeller University). Residues in each subunit that were not defined in the crystal structure (Arg251, Leu252, Arg256) were inserted according to the procedure of Insight98 (Molecular Simulations Inc., San Diego, CA). Models of Kv1.3 and IKCa1 were generated from the corrected structure by mutating appropriate KcsA residues (between positions 23 and 119) in Biopolymer, thus simulating the S5-P-S6 regions of the two larger mammalian channels (see Fig. 1). These models were energy-minimized in the CVFF force field of the Discover module of Insight98. 10,000 iterations were performed using the conjugate gradient algorithm with a 25-Å cutoff for non-bonded atoms and a distance-dependent dielectric in place of explicit water molecules (10,000 iterations were sufficient to bring each model to steady state).

K+ channels possess a conserved selectivity filter, Gly-Tyr-Gly-Asp, located in the channel pore. In KcsA, the backbone carbonyl groups of this selectivity filter are arranged in the pore lumen so as to form a series of oxygen rings, the dimensions of which allow the coordination of divalent K+ ions. The Tyr side chains are oriented away from the pore and form hydrogen bonds with surrounding Trp side chains, suggesting the possibility that this acts like a spring to hold the pore open to the appropriate dimensions (8). In KcsA, this hydrogen bonding is observed between Tyr232 and Trp350. Although a Trp232-Trp350 diad is conserved in Kv1.3 and Shaker, only the equivalent of Trp350 is found in IKCa1 (Trp344). Assuming that the spring mechanism exists in IKCa1 as it does in KcsA, it is presumably mediated by Tyr232-Trp344 hydrogen bonding. In an attempt to preserve the overall architecture of each channel model while allowing any mutated residues to find their respective local energy minima, the Cα atoms of all channel residues were fixed in Cartesian space during the course of energy minimization (and during subsequent molecular dynamics simulations). Our Kv1.3 model is similar to that described previously (31), although the earlier model was constructed without access to the KcsA coordinates.

Docking of IKCa1 and Kv1.3 with ChTX—Energy-minimized channel models were juxtaposed with the closest-to-average conformation of ChTX in such a way as to preclude steric contact (typically this resulted in an overlap of the closest ligand and receptor being 1 Å). ChTX was then positioned manually so that Lys22 was facing the pore and Arg29 was oriented toward channel residues, consistent with earlier studies (4). Further mutant cycle analyses were performed to test the ChTX-IKCa1 interactions predicted by the docking model. To preserve the overall architecture of ChTX and the channel models during docking, various constraints were placed on both molecules prior to performing molecular dynamics. The Cα atoms of all channel residues were fixed in Cartesian space, and distances measured from the structure of ChTX (33 d11(i,i+1) distances along the backbone of ChTX and 3 d11 distances, representing the disulfide bonds at positions 7–28, 13–33, and 15–35 were applied as constraints in Discover with a tolerance of ±0.1 Å and a force constant of 1000 kcal-mol−1. Docking simulations were performed by first energy-minimizing the restrained ligand-receptor complex as outlined above, followed by 250 ps of molecular dynamics at 300 K with a 1-0 fs time step, a 25-Å cutoff for non-bonded atoms, and a distance-dependent dielectric. A 25-Å cutoff distance was used even though it lengthens the computation time because initial docking simulations with cutoff distances of 15 and 20 Å did not reproduce some observed interactions with charged side chains of the toxin and channel. After allowing ~ 50 ps for equilibration, the lowest van der Waals energy conformation was further energy-minimized as described above. Models of ChTX docked to Kv1.3 and IKCa1 were analyzed using Insight98. An alternative docking simulation was tried in which the turret region of the Kv1.3 model (residues 373–379 of each subunit) was left unrestrained to determine what effect this might have on potential electrostatic interactions between it and the Lys22 region of ChTX. In this simulation, the turret moved outward (further away from ChTX) relative to the simulation in which it was restrained.

Electrophysiological Analysis—Each K+ channel expression construct was specifically linearized and transcribed in vitro. As described earlier, the cRNA, together with a fluorescent fluorescein isothiocyanate-labeled dye, was injected into rat basophilic leukemia cells. Fluorescent cells were visualized after 2–6 h of incubation, and specific currents were measured using the patch-clamp technique (32, 33). Cells measured in the whole-cell configuration were bathed in mammalian Ring er’s solution containing 160 mM NaCl, 4.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES (adjusted to pH 7.4 with NaOH) with an osmolality of 270–320 mosmol. For the internal pipette solution for Kv currents, recordings contained 134 mM potassium fluoride, 1 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, and 10 mM EGTA (adjusted to pH 7.2 with KOH) with an osmolality of 290–310 mosmol, and these currents were measured following 200-ms depolarizing pulses to 40 mV from the holding potential every 30 s. The internal pipette solution for IKCa and SKCa recordings contained 135 mM potassium aspartate, 2 mM MgCl2, 10 mM HEPES, and 10 mM EGTA, and the internal solution for KOH (adjusted to pH 7.2) with an osmolality of 290–310 mosmol (free [Ca2+]i = 10−6 M). Kv currents were activated by a 200-ms voltage step from a holding potential of −80 to 40 mV every 30 s. KvCa currents were activated with 1 mM free Ca2+, and 200-ms voltage ramps from −150 to 50 mV applied every 5 s (holding potential = −80 mV). For hSlo expression, 25–50 ng of cRNA was injected into Xenopus laevis oocytes 3 to 6 days prior to recording. The outside-out patch-clamp configuration was used to record macroscopic hSlo currents. For these experiments, the external solution contained 140 mM Na2SO4, 10 mM HEPES, 2 mM MgCl2, and 2 mM KCl (adjusted to pH 7.4 with NaOH), whereas the internal solution contained 140 mM K2SO4, 10 mM HEPES, 2 mM MgCl2, 2 mM KCl, and 1 mM H-EDTA, and CaCl2 was added to obtain a free [Ca2+]i of 5 × 10−6 M (adjusted to pH 7.2 with KOH). hSlo currents were activated with 50 μM free Ca2+, and 200-ms voltage ramps from −80 to 80 mV applied every 5 s. Series-resistance compensation (80%) was used if the current exceeded 2 nA. Leak currents were subtracted using the P/4 procedure for Kv currents. Kv currents were measured using the equation $K_v = (\frac{I_{\text{peak}}}{I_{\text{ss}}}) - 1$ (y = unblocked fraction of current) and are shown as mean ± SEM. n ≥ 4.

Double-mutant Cycle Analysis—Toxin-channel interactions, predicted from the docking configuration of ChTX in IKCa1, were tested experimentally by mutant cycle analysis. This method evaluates the strength of the interaction between any given pair of channel and toxin residues. For each mutant cycle, we measured the potency ($K'_v$) of ChTX and its analogs on IKCa1 and its mutants. The change in coupling energy (ΔΔG) for a given pair of ChTX-IKCa1 residues and their mu-
FIG. 1. Amino acid sequence alignment of Kcα, IKCa1, Kv1.3, Kv1.2, Kv1.6, and hSlo showing sequence similarities >50%. The positions of the turret region, pore, and inner helix are indicated, and critical amino acids are shown in boldface. The negatively charged cluster present in Kv channels (shaded), and homologous residues in Kcα channels are boxed.

Guided Design of Selective K_{Ca} Channel Inhibitors

**RESULTS AND DISCUSSION**

**Overall Strategy**

Our approach to design a selective inhibitor relies on the identification of a unique feature in the ChTX-IKCa1 interface that is absent from the ChTX-Kcα1.3 binding surface. Therefore, the success of our comparative structure-based design strategy depends on understanding how ChTX interacts with the IKCa1 and Kcα1.3 vestibules. To accomplish this, we developed homology models of the Kcα1.3 and IKCa1 pore and vestibule regions (SS-P-S6 segments) based on the amino acid sequence alignment shown in Fig. 1 and the crystal structure of Kcα (see “Materials and Methods”). Initial docking of ChTX was performed based on published data for Kcα1.3 and then carried out for IKCa1 under the assumption that ChTX may interact in a similar way. Both docking models were then tested by mutant cycle analyses, and these results were used to generate final refined docking models (Fig. 2). Comparison of the two docking models revealed a unique motif in the ChTX-Kcα1.3 interface that is absent in the ChTX-IKCa1 binding surface, and three related ChTX analogs were designed to target this difference.

**Docking of ChTX in Kcα1.3**

Several well characterized ChTX-Kv1.3 interactions, previously identified by mutant cycle analyses and electrostatic-compliance experiments (4), were used to dock ChTX in the vestibule of the Kcα1.3 model. In this configuration, Lys57 of ChTX protrudes into the pore in close proximity to Kcα1.3-Tyr400 in the selectivity filter. ChTX-Arg25 interacts with Kcα1.3-Asp386 (in one subunit) and Kcα1.3-His304 (from two adjacent subunits). The docking model based on these data also predicts an interaction between ChTX-Lys31 and Kcα1.3-Asp386 in the subunit diagnostically opposite to that interacting with ChTX-Arg25. Mutation cycles were therefore performed with ChTX(XLys31 → Gln)-Kcα1.3(Asp386 → Lys) and ChTX(XLys31 → Gln)-Kcα1.3(Asp386 → Lys); these yielded ΔΔG values of 1.35 and 0.74 kcal mol⁻¹, respectively, indicating that Kcα1.3-Asp386 and ChTX-Lys31 lie within 5 Å of each other (26). Consistent with our results, Lys31 in agitoxin has been reported to couple to the residue in the Shaker channel (Asp431) that is homologous to Kcα1.3-Asp386 (3, 6).

**Docking ChTX in IKCa1 Based on Its Docking with Kcα1.3**

The polypeptide toxin ChTX blocks Kcα1.3 (Kₐ = 2 nM) and IKCa1 (Kₐ = 5 nM) with almost identical potency (10, 11, 30), and another toxin, Stichodactyla helianthus toxin, utilizes a similar core-binding domain to bind to both channels (7), albeit with very different potencies (16 pm versus 30 nM). Hence, ChTX might be expected to sit in the IKCa1 vestibule with a similar geometry to that in Kcα1.3. Guided by the ChTX-Kv1.3 docking model, a docking simulation of ChTX in the IKCa1 vestibule was performed for heuristic reasons. This docking model predicts that IKCa1-Asp239, the residue homologous to Kcα1.3-Asp386 (Fig. 1), lies in close proximity to ChTX-Arg25 and ChTX-Lys31, but not to ChTX-Lys32. In this model, the critical ChTX residue, Lys27, protrudes into the channel pore and lies in the vicinity of Tyr253 in the selectivity filter. These docking models were tested experimentally by measuring the coupling energy of specific residues predicted to be in close proximity and then refined. Specifically, we determined the coupling energies for the following pairs of specific toxin/channel residues using mutant cycle analyses: ChTX-Arg25/IKCa1-Asp239, ChTX-Lys31/IKCa1-Asp239, ChTX-Lys32/IKCa1-Asp239, and ChTX-Lys27/IKCa1-pore.

**Mutant Cycle Analyses of Predicted Docking Configuration**

**ChTX-Arg25 and ChTX-Lys31 Interactions with IKCa1**—Replacement of the negatively charged residue Asp239 in IKCa1 with the neutral asparagine (Asn239) reduced the Kₐ for native ChTX by ~14-fold. A more substantial reduction in affinity of ~180-fold was observed when a positively charged lysine (IKCa1-Lys239) was introduced at this channel position (Fig. 3, A and B). Neither mutation altered the biophysical properties of the channel. Charge reversal mutations at ChTX positions 25 (Arg25 → Asp) and 31 (Lys31 → Glu) also dramatically decreased toxin affinity by ~1325- and ~47-fold, respectively, for the wild-type IKCa1 channel (Fig. 3, A–C). In contrast, a similar mutation at ChTX position 32 (Lys32 → Asp) decreased affinity by only 9-fold (Fig. 3D).

Thermodynamic double-mutant cycle analyses were performed to measure the change in coupling energy between IKCa1-Asp239 and each of the three ChTX residues Arg25, Lys31, and Lys32. The ΔΔG values for the mutant cycles shown in Fig. 3B are 2.25 and 3.1 kcal mol⁻¹, respectively, indicating that IKCa1-Asp239 and ChTX-Arg25 lie within 5 Å of each other. Strong coupling was also seen between IKCa1-Asp239 and ChTX-Lys31, with the coupling energies being 1.9 and 2.4 kcal mol⁻¹ for the two cycles shown in Fig. 3C. In contrast, the ΔΔG of 0.02 kcal mol⁻¹ for the cycle in Fig. 3D indicates that IKCa1-Asp239 and ChTX-Lys32 are not energetically coupled.

**CtTX-Lys27 Interactions with the Pore of IKCa1**—Two approaches were used to test the proximity of ChTX-Lys27 to the IKCa1 pore, the first showing that it lies close to a K⁺-binding site and the second demonstrating that a shorter analog at this position (ChTX-Orn27) interacts with a residue at the outer mouth of the pore.

All K⁺ channel toxin inhibitors contain a critical lysine residue (in this case, ChTX-Lys27) that projects into the channel pore (34) and lies close to a K⁺-binding site near the conserved tyrosine (Tyr400 in Kcα1.3) in the selectivity filter (5–7, 9, 31). Occupancy of this site by K⁺ ions destabilizes toxin interactions with Kcα1.3 via electrostatic repulsion of this critical lysine (5–7). If ChTX-Lys27 lies close to a K⁺-binding site near Tyr253 in the selectivity filter of the IKCa1 pore, then occupancy of this site by K⁺ ions should reduce the affinity of the
toxin for the channel. We tested this by examining the effect of increasing the external $K^+$ concentration from 4.5 to 164 mM on the affinity of the $IK_{Ca1}$ channel for ChTX-Lys27. As a control, we also performed this experiment using the ChTX analog ChTX-Dap27, in which Lys27 was replaced by the shorter (2.5 Å) positively charged non-natural amino acid diaminopropionic acid. Earlier studies on $Kv1.3$ have shown that Dap27 interacts with His404 at the entrance of the pore (5), rather than with the $K^+$-binding site in the selectivity filter, and the same may be true for $IK_{Ca1}$. Therefore, the interaction of Dap27 with the $IK_{Ca1}$ pore should be insensitive to changes in the external $K^+$ concentration. Consistent with our prediction, the mutant cycle shown in Fig. 4A yields a strong coupling energy ($\Delta G = 0.76$ kcal/mol), indicating that ChTX-Lys27 lies within 5 Å of a $K^+$-binding site located in the $IK_{Ca1}$ pore. Interestingly, the critical lysine at position 22 in the sea anemone $S. helianthus$ toxin also interacts with a $K^+$-binding site within the pore of the $IK_{Ca1}$ channel, and the affinity of $S. helianthus$ toxin for $IK_{Ca1}$ is reduced as the external $K^+$ concentration is increased (7).

We also examined the sensitivity of $Kv1.3$ and $IK_{Ca1}$ to the analog ChTX-Orn27, in which the shorter positively charged ornithine (side chain length of 5.0 Å) was substituted for the critical Lys27 residue (chain length of 6.3 Å). As shown in Fig. 4B, this analog blocked $Kv1.3$ in the nanomolar range ($K_d = 196$ nM), but was significantly less effective against $IK_{Ca1}$ ($K_d = 3300$ nM). Since ChTX-Lys27 protrudes into the pores of both $IK_{Ca1}$ and $Kv1.3$, why does ChTX-Orn27 have lower affinity for $IK_{Ca1}$ than for $Kv1.3$? The outer pore regions of both channels are almost identical (Fig. 1), except for the presence of a histidine (His404) at the entrance to the $Kv1.3$ pore in place of valine (Val257) in $IK_{Ca1}$. To determine whether this difference might contribute to the differential sensitivity to ChTX-Orn27, we replaced $Kv1.3$-His404 with valine and measured the affinity of ChTX-Orn27 for this mutant channel, which more closely resembles the $IK_{Ca1}$ pore. In keeping with our hypothesis, ChTX-Orn27 blocked $Kv1.3$-Val404 with an affinity similar to that for $IK_{Ca1}$ and significantly less than that for wild-type $Kv1.3$ (Fig. 4B). We were unable to determine the effect of the reverse mutation (Val257 $\rightarrow$ His) on the sensitivity of $IK_{Ca1}$ to ChTX-Orn27 since this mutant channel is nonfunctional.

**Refinement of the Docking Models**

Our mutant cycle studies demonstrate that ChTX-Arg25 and ChTX-Lys31 interact with $IK_{Ca1}$-Asp239 in different subunits, whereas ChTX-Lys27 lies close to a $K^+$-binding site in the pore.
The docking configuration of ChTX with both models was re-calculated using these experimental data as restraints in molecular dynamics simulations (Fig. 2). Where significant coupling was observed between toxin and channel residues ($\Delta G > 0.7 \text{ kcal/mol}$), a target distance (as noted below) was applied between specific atoms from each residue with a 50 kcal/mol force constant (31). Thus, docking constraints were applied between the following pairs of atoms: ChTX-Kv1.3 docking, Lys27 N to Tyr400 C$_{\beta}$ (from all four subunits), Arg25 C to His404 N$_{\text{d1}}$ (from two adjacent subunits, target distance of 8.0 Å), and Lys31 N to Asp386 C$_{\text{g}}$ (from a single subunit, target distance of 6.0 Å); and ChTX-IKCa1 docking, Lys27 N to Tyr253 C$_{\beta}$ (from all four subunits), Arg25 C to Asp239 C$_{\text{g}}$ (from a single subunit), and Lys31 N to Asp386 C$_{\text{g}}$ (from a single subunit, target distance of 6.0 Å).

The final docking configurations of ChTX with Kv1.3 and IKCa1 (Fig. 2) place the toxin in similar orientations in the two channels, highlighting the usefulness of homology modeling approaches in defining toxin-channel interactions. In the case of Kv1.3, the orientation of the toxin about the pore axis is guided by the proximity of Arg25 to His404. Coupling of Arg25 to two His404 side chains in adjacent subunits brings the former in closer proximity to Asp386, an interaction alluded to by Aiyar et al. (5), than if Arg25 is coupled to only one His404 side chain. This docking configuration of ChTX with Kv1.3 matches closely the ChTX docking configuration with IKCa1 (Fig. 2). Thus, ChTX utilizes the same three key residues, Arg25, Lys27, and Lys31, to interact with homologous residues in the external vestibules of Kv1.3 and IKCa1. ChTX-Arg25 and ChTX-Lys31, positioned at opposite ends of the toxin, interact with Kv1.3-Asp386 and IKCa1-Asp239, respectively, in diametrically opposite subunits of these channel tetramers. ChTX-Lys27, located at the center of the channel-binding surface in the toxin, projects into the pores and lies close to the selectivity filter of both channels. A similar toxin-channel interaction has also been shown for S. helianthus toxin, which utilizes a conserved core domain to interact with Kv1.3 and IKCa1 (7) despite having a structural fold that bears no resemblance to ChTX (35).
three negatively charged residues, Glu373, Asp376, and Asp376, in the turret region of Kv1.3 (shaded) that are not present in IKCa1. The Kv1.3 model (Fig. 2, A and B) shows the locations of these three acidic residues within the vestibule of Kv1.3. Glu373 and Asp376 are oriented toward the center of the channel pore, whereas Asp375 is at the outer edge of the turret. The docking configuration of ChTX in Kv1.3 indicates that ChTX-Lys32 lies in the vicinity of Glu373 and Asp376, although the terminal ammonium group of this Lys32 is ~10 Å away. No intermolecular constraints were applied among any of these residues during the docking, but if a weak constraint was included between Asp376 C' and Lys32 N, the distance from Lys32 to this part of the turret decreased to ~8 Å, without any significant change elsewhere. Based on this observation, the introduction of a negatively charged residue at ChTX position 32 might therefore significantly reduce the affinity of such an analog for Kv1.3 via electrostatic repulsion.

IKCa1 contains two neutral residues (Ala226 and Glu226) and one basic residue (Arg228) (Figs. 1 and 2, C and D) in place of the acidic residues in the turret of Kv1.3. The only negatively charged residue in the turret region of IKCa1 (Glu227) is located at the outer edge of the turret, pointing away from the center of the vestibule (Fig. 2, C and D), and is therefore unlikely to interact directly with any ChTX residues. In contrast to Kv1.3, a ChTX analog containing a negatively charged residue at position 32 would be expected to retain most of its potency against the IKCa1 channel.

Introduction of Negatively Charged Residues at Position 32 in ChTX Results in Analogs Selective for IKCa1 over Kv1.3

To test these predictions, we replaced Lys32 in ChTX with glutamate and tested the affinities of this novel analog (ChTX-Glu32) and native ChTX for the cloned IKCa1 and Kv1.3 channels. Native ChTX blocked both IKCa1 and Kv1.3 in the low nanomolar range with \( K_d \) values of 5 and 2 nm, respectively (Figs. 5, A and B, and 6). As anticipated from our model, introduction of a negatively charged residue at ChTX position 32 reduced the affinity of this analog for Kv1.3 by ~30-fold while only minimally affecting (~6-fold) its affinity for IKCa1. Thus, ChTX-Glu32 exhibits a ~20-fold higher affinity for IKCa1 than for Kv1.3.

Since both channels are expressed endogenously in activated human T-lymphocytes, we also examined the effect of the ChTX-Glu32 analog on native IKCa1 and Kv1.3 currents. Fig. 5C shows a ramp protocol eliciting \( K^+ \) currents in activated human T-lymphocytes. IKCa1 was the main carrier of \( K^+ \) currents at potentials more negative than ~40 mV, whereas at depolarized potentials, \( K^+ \) currents were carried by a combination of IKCa1 and Kv1.3 channels. Consistent with the results on the cloned channels, the voltage-dependent Kv1.3 current in activated T-lymphocytes was affected only minimally by 250 nM ChTX-Glu32 (Fig. 5C), a concentration that blocked ~25% of the cloned Kv1.3 current, whereas this concentration of ChTX-Glu32 almost completely inhibited IKCa1 currents. In contrast, native ChTX blocked both channels equally in the low nanomolar range (Fig. 5C). These results show that ChTX-Glu32 is a selective and potent inhibitor of the cloned IKCa1 channel and its native counterpart in human T-lymphocytes compared with Kv1.3.

Encouraged by the selective properties of ChTX-Glu32, we generated two additional ChTX analogs. The negatively charged residues aspartate (ChTX-Asp32) and p-carboxyphenylalanine (ChTX-Cpa32) were substituted for Lys32 in ChTX to investigate the influence of the side chain length on potency and selectivity. The aspartate side chain is shorter (3.1 Å) than in glutamate (4.6 Å), whereas the p-carboxyphenylalanine side chain is longer (7.3 Å). ChTX-Asp32 and ChTX-Cpa32 blocked IKCa1 channels significantly more potently than Kv1.3 (Fig. 6), although ChTX-Glu32, the analog with the intermediate-sized side chain, was the most selective because it exhibited the greatest difference in affinity between IKCa1 and Kv1.3. Thus, using a structure-based homology modeling strategy, we predicted a novel toxin-channel interaction that was exploited in the design and engineering of three novel ChTX analogs (ChTX-Asp32, ChTX-Glu32, and ChTX-Cpa32). Each of them contains a negatively charged residue at position 32 and selectively blocks IKCa1 channels while being significantly less effective against Kv1.3.

Selectivity Profile of the ChTX Position 32 Analogs

ChTX is reported to block three other \( K^+ \) channels potently besides IKCa1 and Kv1.3, including the voltage-gated \( K^+ \) channels Kv1.2 (27) and Kv1.6 (36) and the large-conductance \( Ca^{2+} \)-activated \( K^+ \) channel hSlo (37, 38). In an attempt to predict the behavior of the ChTX position 32 analogs on these channels, we
Guided Design of Selective $K_{Ca}$ Channel Inhibitors

Fig. 6. Selectivity profile of the novel ChTX position 32 analogs on all ChTX-sensitive K$^+$ channels. Shown is a $K_d$ value comparison of native ChTX (ChTX-wt) and the ChTX position 32 analogs ChTX-Asp$^{32}$ (side chain length of 3.1 Å), ChTX-Glu$^{32}$ (4.6 Å), and ChTX-Cpa$^{32}$ (7.3 Å) on the Kv$_{1.3}$ channel IKCa1 and hSlo and the Kv channels Ko.1.3 and Ko.1.2. ChTX-Glu$^{32}$ was most potent and selective for IKCa1 and hSlo. ChTX-Asp$^{32}$ and ChTX-Cpa$^{32}$ were less selective than ChTX-Glu$^{32}$. Ko.1.3 and Ko.1.2 were weakly blocked with a similar profile by all three analogs.

compared the amino acid sequences of the turret regions of these channels with those of Kv.1.3 and IKCa1.1, Kv.1.2 and Kv.1.6 channels both contain a negatively charged cluster at the position homologous to that in Kv.1.3 (Fig. 1). If the vestibules of these channels have a similar architecture to that of Kv.1.3, they would exhibit a lower affinity for the ChTX position 32 analogs compared with wild-type ChTX. In contrast, hSlo has only two negatively charged residues in the turret region, Glu$^{205}$ and Asp$^{202}$ (Fig. 1). If its vestibule has a comparable topology to that of IKCa1, hSlo-Glu$^{205}$ and hSlo-Asp$^{202}$ would be at the same positions as IKCa1-Glu$^{222}$ and IKCa1-Asp$^{229}$, respectively (Fig. 2C and D), and neither residue would lie in close proximity to ChTX-Lys$^{22}$. Therefore, the introduction of negatively charged residues at this toxin position should have little effect on toxin potency for hSlo. In keeping with our predictions, the introduction of negatively charged residues at position 32 in ChTX significantly reduced the affinity of the three analogs for Ko.1.2 in a similar fashion to Ko.1.3 while only minimally affecting their affinity for hSlo. We found that the Ko.1.6 channel was insensitive to ChTX, in contradiction to some published data (36), but in confirmation of other studies (39). Consequently, Ko.1.6 was also resistant to the ChTX-Glu$^{32}$ analog (Fig. 7). Since our comparative modeling approach accurately predicted the sensitivity of the Ko.1.2 and hSlo channels to the three ChTX position 32 analogs, it is likely that the external vestibules of Ko.1.2, Ko.1.3, IKCa1, and hSlo are structurally similar to that of the KcsA channel.

To further evaluate the selectivity profile, we tested the most selective analog, ChTX-Glu$^{32}$, on a panel of four ChTX-resistant voltage-gated K$^+$ channels (Ko.1.1, Ko.1.4, Ko.1.5, and Ko.3.1) as well as on the apamin-sensitive, small-conductance Ca$^{2+}$-activated K$^+$ channel (SK$_{Ca}$) endogenously expressed in human Jurkat T-cells (27, 29). All five channels were resistant ($K_d \geq 5 \mu M$) to native ChTX as well as to ChTX-Glu$^{32}$ (Fig. 7).

Fig. 7. Selectivity profile of ChTX and ChTX-Glu$^{32}$. Shown are the $K_d$ values for Ko.1.1, Ko.1.4, Ko.1.5, Ko.1.6, and Ko.3.1 as well as the SK$_{Ca}$ channel endogenously present in human Jurkat T-cells (JK-SK$Ca$) with native ChTX and ChTX-Glu$^{32}$. The $K_d$ values for the cloned Ko.1.3 and IKCa1 channels and for the endogenous IKCa1 (native T-cells) and Ko.1.3 (Jurkat T-cells; JK-Ko.1.3) channels are shown for comparison.

Concluding Remarks

In this study, we generated homology models of the pore regions of two prototypical mammalian K$^+$ channels, the voltage-gated Kv.1.3 channel and the Ca$^{2+}$-activated IKCa1 channel, both present in human T-lymphocytes, based on their known structural similarity to the KcsA channel. Our Ko.1.3 model differs in detail from the previous models (4, 5), for example, in the orientation of the key residue Tyr$^{403}$ in the selectivity filter. Guided by established ChTX-Ko.1.3 interactions, we docked this toxin in the Ko.1.3 model and used mutant cycle analysis to confirm the proximity of a pair of ChTX (Lys$^{31}$) and Ko.1.3 (Asp$^{426}$) residues predicted to be close. Since ChTX blocks IKCa1 with roughly equivalent potency compared with Ko.1.3, we hypothesized that ChTX might sit in the IKCa1 vestibule with a similar geometry to that in Ko.1.3. We therefore performed a docking simulation of ChTX in IKCa1 for heuristic purposes. Multiple sets of predicted interactions were confirmed by mutant cycle analyses and then used to generate refined models of the docking configurations. Comparison of the two toxin-channel interfaces suggested a unique structural motif, a cluster of negatively charged residues present only in the Kv channel, that was exploited in the design and generation of three novel ChTX analogs. These analogs, containing negatively charged residues at toxin position 32, exhibit specificity for the IKCa1 channel over Ko.1.3. They do not block other voltage-gated and small-conductance Ca$^{2+}$-activated K$^+$ channels that lack this unique feature, but they inhibit hSlo channels, which resemble IKCa1 in the turret region.

Our results strongly suggest that a polypeptide toxin with comparable affinities for different K$^+$ channels, even those belonging to widely divergent subfamilies (e.g. Ca$^{2+}$-activated IKCa1 and voltage-gated Kv.1.3 channels), interacts with a topologically similar toxin-binding site in the external vestibule of these channels (Fig. 2). This has allowed us to start with the SK$_{Ca}$ channel to guide the design of selective and potent inhibitors for a large variety of mammalian K$^+$ channels.
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