

PHYSIOLOGY

A designer pore-blocking peptide reveals an electrostatic complementation mechanism for the inhibition-resistant Kv1.5 channel

Ruiming Zhao^{1*†}, Maha Nayak^{1†}, Rong Shen^{2†}, Hui Dai¹, Punyanuch Hua¹, Eduardo Perozo², Steve A. N. Goldstein^{1*}

Most voltage-gated potassium (Kv) channels are inhibited by peptide neurotoxins that occlude the pore with a positively-charged lysine. Kv1.5 repels these toxins electrostatically via four arginines (R487), one on each pore-forming subunit. Here, we describe chimera toxin (CmTx), a potent and selective blocker that prefers the slow-inactivated Kv1.5 conformation promoted by rapid firing and acidosis ($K_i = 127$ nM), conditions associated with atrial fibrillation and ischemia. CmTx was isolated by cell-based, phage-display library panning of de novo peptides engineered on a SAK1 scaffold and shows minimal inhibition of seven other Kv subtypes. Scanning mutagenesis identified CmTx and Kv1.5 residues essential to binding. AlphaFold modeling shows how acidic CmTx residues can neutralize the channel arginines, enabling one peptide to plug the Kv1.5 pore. These findings present an electrostatic pore-blocking mechanism driven by complementary charge interactions and offer CmTx as a tool to probe Kv1.5 physiology and a potential therapeutic lead.

INTRODUCTION

Toxin peptides that modulate ion channels serve as powerful tools for biophysical research, diagnosis, and therapeutic development (1–4). However, some ion channels with key physiological roles have proven refractory to the identification of peptide binders with high affinity and specificity (5). In this study, we describe the isolation of a de novo peptide blocker for the human Kv1.5 voltage-gated potassium channel, an inhibition-resistant Kv channel with basic pore residues known to repel pore-directed toxins. We present a cell-based panning strategy for sorting phage display libraries that will have utility for other receptors that are difficult to purify in their native conformation but are well expressed in cultured cells.

Kv1.5 is expressed in the heart, smooth muscle, brain, pancreatic β cells, and immune cells and is considered to have untapped potential as a therapeutic target (6, 7). In the heart, Kv1.5 underlies the ultrarapid delayed rectifier potassium current (IKur), which plays a critical role in early atrial repolarization and has been implicated in the pathogenesis of atrial fibrillation. Gain-of-function mutations in Kv1.5 shorten the atrial action potential, thereby increasing cellular excitability. Loss-of-function mutations prolong the refractory period and enhance susceptibility to early afterdepolarizations (8). In addition to cardiac pathophysiology, Kv1.5 dysregulation has been linked to pulmonary hypertension, hypothyroidism, hypoxic pulmonary vasoconstriction, and several cancers (5, 7).

Kv1.5 channels are composed of four identical subunits that assemble into tetrameric complexes (Fig. 1A) (5, 9). Each Kv1.5 subunit contains six transmembrane segments (S1 to S6), where the S1 to S4 segments form the voltage-sensing domains, and the S5 to S6

segments from each subunit collectively assemble a central ion-conducting pore (5, 10). Upon membrane depolarization, this pore opens to permit transmembrane flux of K^+ ions. Notably, each Kv1.5 subunit contributes an arginine residue at position 487 (R487) into the outer pore vestibule region adjacent to the selectivity filter (Fig. 1, A to C). This is a key feature that distinguishes Kv1.5 from other Kv channels and plays a critical role in its distinct pharmacological properties.

Classical Kv channel peptide pore blockers inhibit ion flux by inserting a positively charged basic residue, most commonly a lysine, into the selectivity filter, thereby physically occluding the permeation pathway (11–14). In Kv1.5, the four basic R487 residues in the pore vestibule are thought to repel these classical blockers electrostatically (5, 15). This idea is supported by the observation that substitution of R487 with tyrosine or valine (R487Y/V), residues present in peptide-sensitive Kv channels (Fig. 1C), renders Kv1.5 susceptible to inhibition by the sea anemone peptide toxin BgK at nanomolar concentrations (15).

Previously, we constructed a phage display library with ~1.5 million variants through restricted combinatorial permutations of 150 known and predicted peptides with sea anemone type I (SAK1) neurotoxin scaffolds. Screening this library against purified KcsA channels yielded Hui1, a high-affinity and specific peptide blocker (16). Here, we applied the library to Kv1.5 channels expressed in mammalian cells, leading to the isolation of chimera toxin (CmTx). CmTx inhibits Kv1.5 but does not significantly block Kv1.1, Kv1.2, Kv1.3, hERG, nor three other channels with basic residues in their outer pore vestibules, Kv1.4, Kv7.1, and Kv2.1.

To identify Kv1.5 residues critical for CmTx binding, we used point mutagenesis and found five residues in the pore whose substitution reduced toxin sensitivity by more than 10-fold (estimated $\Delta\Delta G$ change >1.4 kcal/mol): N459, Q460, G461, H463, and R487. Using our membrane-tethered peptide approach (12, 17) to scan mutants of the 30 non-cysteine residues in tethered CmTx (T-CmTx), we identified seven positions whose mutation decreased binding affinity over 10-fold ($\Delta\Delta G > 1.4$ kcal/mol): K11, E17, Y22, E23, K24, E28, and P30. AlphaFold modeling of the CmTx-Kv1.5 complex predicts interaction

¹Departments of Pediatrics and Physiology & Biophysics, School of Medicine and Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, Susan and Henry Samueli College of Health Sciences, University of California, Irvine, CA 92697, USA. ²Department of Biochemistry and Molecular Biology, Gordon Center for Integrative Science, University of Chicago, Chicago, IL 60637, USA.

*Corresponding author. Email: ruimiz3@uci.edu (R.Z.); sgoldst2@uci.edu, sg24@unm.edu (S.A.N.G.)

†These authors contributed equally to this work.

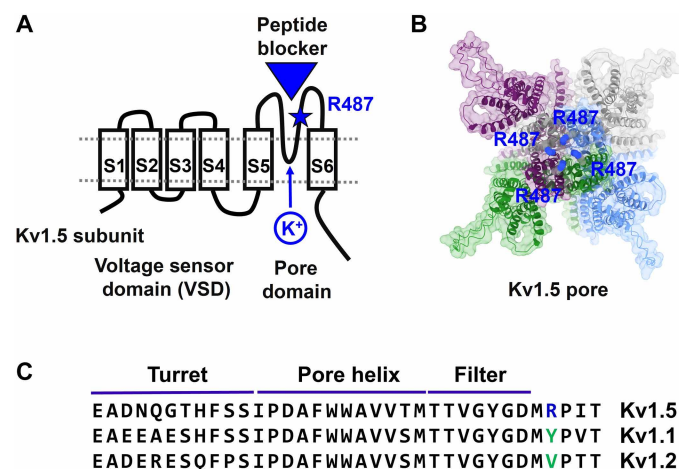


Fig. 1. Human Kv1.5 cannot be blocked by classical venom peptide blockers. (A) Cartoon depicting the architecture of the Kv1.5 channel and toxin peptide blocker in the pore of Kv1.5. (B) Top view of the Kv1.5 channel predicted using the AlphaFold Server, with the four subunits shown in different colors. The four R487 residues, highlighted as stick representations, are located within the outer pore vestibule. (C) Sequence alignment of Kv1.5, Kv1.1, and Kv1.2 channels. The R487 residue immediately after the channel selective filter can repel the binding of classical peptide blockers that are usually highly positively charged.

of the basic R487 residues and three acidic CmTx residues (E23, E27, and E28) that are absent in classical Kv channel blockers (12, 16). Suggesting that it may prove useful as a therapeutic lead, CmTx associates more rapidly with the slow-inactivated state of Kv1.5, a conformation favored by elevated pulse rates and mild acidosis.

RESULTS

Library sorting on cells expressing Kv1.5 yields CmTx

The phage display library was constructed on the SAK1 toxin scaffold (16), which has six Cys residues forming three disulfide bonds with a CysI–CysVI, CysII–CysIV, and CysIII–CysV pattern (Fig. 2, A and C). A panel of 150 natural and predicted parental peptides was aligned on the conserved Cys residues, and CysIII and CysIV were selected as boundaries for three combinatorial domains (Fig. 2C). The 150 sequences containing 125, 94, and 133 distinct A, B, and C domains, respectively, were designed into oligonucleotides for unidirectional ligation to restrict permutations to genes encoding peptides with segments in the order A-B-C; this yielded a diversity of 1,562,600 de novo peptides and the 150 parental toxins. Peptides were encoded in-frame with phage particle coat protein pIII, thereby exposing the peptides on the phage surface, allowing phage display library selection.

We constructed a Kv1.5-RFP plasmid in which the C terminus of the human Kv1.5 channel was fused to the monomeric red fluorescent protein TagRFP-T. The plasmid was transiently transfected into either HEK293T or Chinese hamster ovary (CHO) cells for expression and subsequent phage sorting (Fig. 2A). We found that >90% of cells expressed the channel based on the RFP signal. High expression of functional Kv1.5 channels was demonstrated using whole-cell patch clamp to measure over 10-nA K^+ currents at +40 mV in both cell types, consistent with expression of more than 30,000 channels on the surface of each cell 24 hours after transfection, when the cells were used for library sorting. Stably bound phages were eluted with 0.1 M

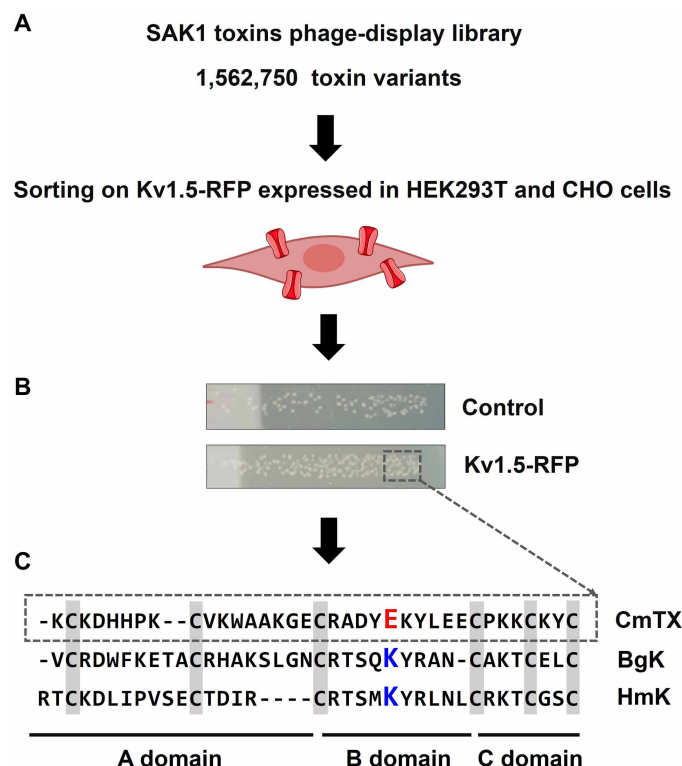


Fig. 2. Kv1.5 peptide blockers are isolated from the SAK1 toxin phage display library by cell panning. (A) SAK1 toxin library contains ~1.5 million natural and novel toxin peptides (16). Library phages were sorted on HEK293T and CHO cells expressing Kv1.5-RFP to select ligands for Kv1.5 channels. (B) After five rounds of selection on cells expressing Kv1.5-RFP, phage enrichment was observed compared with control cells without Kv1.5 expression. (C) Sequence alignment of CmTx, BgK, and HmK. A, B, and C domains that were used for SAK1 toxin library construction are underlined. Classical pore occluding residues in BgK (K25) and HmK (K22) were labeled blue, and the corresponding residues in CmTx (E23) were labeled red. The sequences and origins of the three parental peptides that contributed segments to CmTx are shown in fig. S1.

triethylamine, amplified, and subjected to another round of selective sorting. After five rounds of panning, DNA sequencing of 25 phage-mids revealed that CmTx was enriched ~180,000-fold relative to its initial representation in the library and accounted for 12% of the isolated phages. (Fig. 2, B and C). The A, B, and C domains in CmTx are sequences in three predicted peptides in the sea anemone *Nematostella vectensis* genome that have no reported targets (fig. S1) (16).

CmTx block favors the slow-inactivated state

We first examined CmTx using whole cell patch clamp recording with HEK293T cells expressing Kv1.5 depolarized from a holding potential of −80 to 0 mV every 1 s for 0.2 s with extracellular and intracellular pH values of 7.4 and 7.2, respectively, these conditions approximate physiological pH and a heart rate of ~60 beats per min. The subsequent application of 2 μ M CmTx reduced Kv1.5 currents by ~40%, yielding an estimated half-maximal equilibrium inhibition constant (K_i) of only ~2.9 μ M (Fig. 3, A and C), far lower than the nanomolar affinity that has previously allowed isolation of phage by toxin-mediated binding to purified targets (16, 18).

We surmised that CmTx might bind more avidly to channels in states that accumulate in resting cells in culture conditions such as

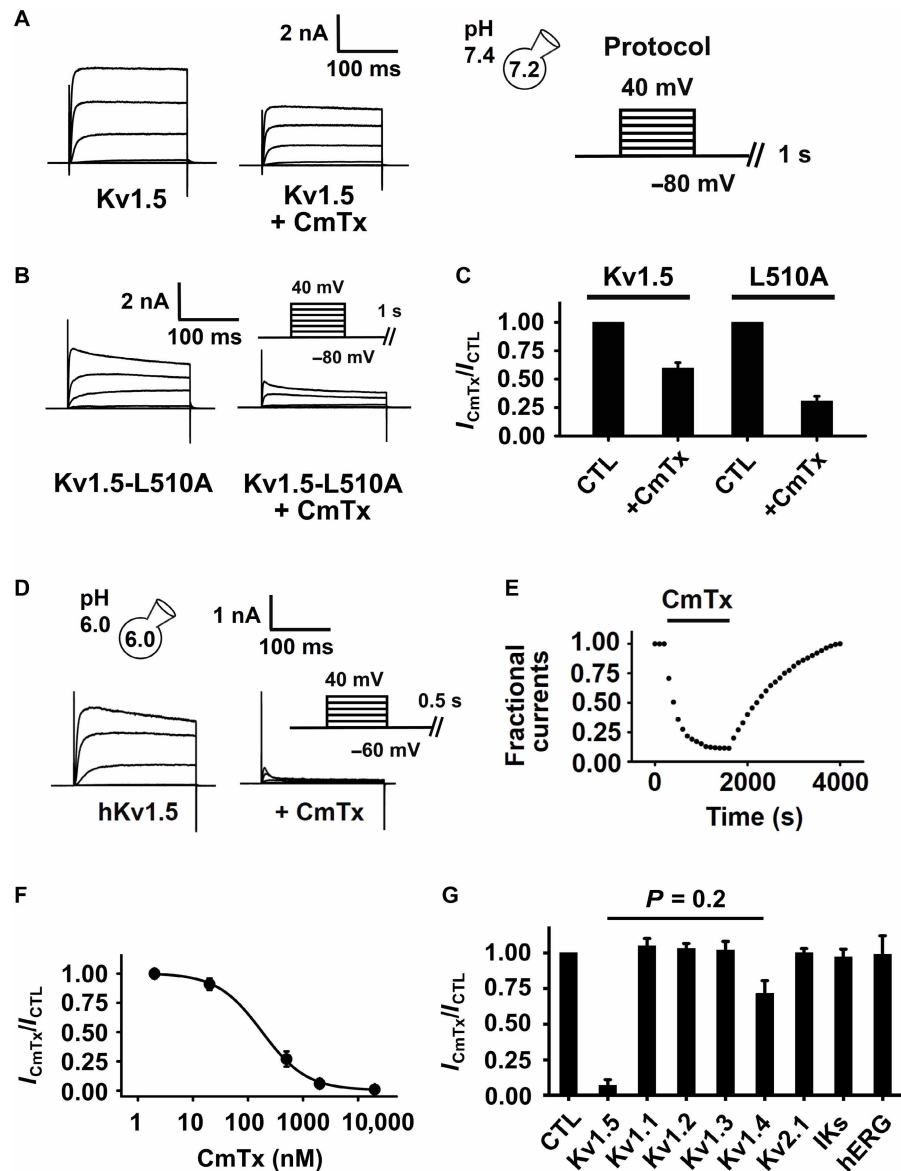


Fig. 3. Block of Kv1.5 channels by CmTx. Kv1.5, Kv1.5-L510A, and other Kv channels expressed in HEK293T cells and recorded using the whole-cell patch clamp to evaluate equilibrium inhibition and kinetic blocking parameters. Two protocols were employed. Physiological conditions (A to C) mimicked the firing frequency and pH of healthy cardiac tissue: cells were depolarized to +0 mV for 0.2 s from a holding potential of −80 mV at 1 s intervals, with bath and intracellular pH of 7.4 and 7.2, respectively. Pathological conditions (D to G) used increased firing frequency and acidosis: Cells were depolarized to +20 mV for 0.2 s from a holding potential of −60 mV at 0.5-s intervals, with bath and intracellular pH maintained at 6.0. Values are means $\pm$ SEM. Some error bars are smaller than symbols. (A) Representative current traces for Kv1.5 channels at steady state before and in the presence of 2 μ M CmTx. Currents were elicited with 0.2-s depolarizing steps in 20 mV increments from −80 to +40 mV at 1 s intervals ($pH_i = 7.2$, $pH_o = 7.4$). (B) Representative current traces for Kv1.5-L510A channels at steady state before and in the presence of 2 μ M CmTx using the conditions in (A). (C) Inhibition of Kv1.5 and Kv1.5-L510A channels at 0 mV by 2 μ M CmTx in (A) and (B). $n = 5$ cells for each condition. (D) Representative current traces for Kv1.5 channels at steady state before and with 2 μ M CmTx. Currents were elicited with 0.2-s depolarizing steps in 20-mV increments from −60 mV to +40 mV at 0.5 s intervals ($pH_i = pH_o = 6$). At +40 mV, Kv1.5 exhibited a slow-inactivation time constant ($\tau_{inactivation}$) of 216 ± 27 ms. In the presence of 2 μ M CmTx, $\tau_{inactivation}$ was accelerated 11-fold to 19 ± 2 ms (table S1). (E) The time course for block and unblock of Kv1.5 on acute application (bar) and washout of 2 μ M CmTx at pH 6. Currents were elicited with 0.2 s depolarizing steps from −60 to +20 mV at 0.5-s intervals. Fractional currents (I_{CmTx}/I_{CTL}) were plotted. The inhibition constant, calculated as K_{off}/K_{on} from single-exponential fits to the toxin association and dissociation kinetics, was 63 ± 7 nM. (F) Dose-response relationships for CmTx inhibition of Kv1.5 studied as in (D) at pH 6 using 0.2-s steps of 20 mV from −60 to +40 mV at 0.5-s intervals. Inhibition at five CmTx concentrations was determined and fitted to the Hill equation (Materials and Methods). The inhibition constant K_i of CmTx at +20 mV was estimated to be 127 ± 5 nM (Hill coefficient $h = 1.2 \pm 0.07$). This value is comparable to the K_i estimated from the association and dissociation kinetics (E). $n = 9$ cells for the 2 μ M CmTx group and $n = 5$ cells for each of the other four concentrations. (G) Inhibition by 2 μ M CmTx of Kv1.5, Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv2.1, cardiac IKs channels (Kv7.1 + KCNE1), and hERG channels (Kv11.1). Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv2.1, and IKs channels studied as in (D) at pH 6. Given its rapid inactivation, hERG channels were studied using 3-s steps in 20-mV increments from −80 to +40 mV, followed by a 2-s step to −40 mV at 5-s intervals ($pH_i = pH_o = 6$). CmTx reduced Kv1.4 currents by 28%; however, this inhibition was not statistically significant based on Student's t test ($P = 0.2$). $n = 9$ cells for Kv1.5, $n = 5$ cells for Kv1.4, and $n = 3$ cells each for Kv1.1, Kv1.2, Kv1.3, Kv2.1, IKs, and hERG.

those used for phage selection. To assess whether CmTx bound preferentially to the closed state, we extended the interpulse interval from 1 to 10 s. Under these conditions, CmTx blocked Kv1.5 even less effectively, with an estimated $K_i \sim 27 \mu\text{M}$ (fig. S2A). Conversely, increasing the holding potential from -80 mV to -60 mV and extending the depolarizing pulse duration from 0.2 to 2 s to favor inactivation improved blockade by $2 \mu\text{M}$ CmTx to $\sim 75\%$, yielding an improved estimated K_i to $\sim 0.67 \mu\text{M}$ (fig. S2B). Increasing the pulse rate and lowering the pH, are also reported to promote Kv1.5 slow inactivation (19–21) and using steps from a holding voltage of -60 to $+20 \text{ mV}$ for 0.2 s every 0.5 s (120 beats per min) and lowering pH bilaterally to 6, yielded an $\sim 94\%$ decrease in Kv1.5 current with $2 \mu\text{M}$ CmTx and an estimated K_i of $\sim 0.13 \mu\text{M}$ (Fig. 3D).

Further, CmTx blocks Kv1.5 with higher affinity at voltages that favor inactivation, in contrast to most classical peptide blockers of Kv channels, which typically exhibit reduced affinity at more depolarized voltages. The analysis of the current-voltage (I - V) relationships before and after application of $2 \mu\text{M}$ CmTx shows that the extent of current inhibition increases from 92 to 96% as the test potential is raised from 0 to $+40 \text{ mV}$, corresponding to an ~ 2.1 -fold increase in apparent affinity (fig. S3).

Last, demonstrating energetic coupling between peptide binding and channel state, $2 \mu\text{M}$ CmTx accelerated Kv1.5 C-type inactivation 11-fold, from a time constant ($\tau_{\text{inactivation}}$) of $216 \pm 27 \text{ ms}$ at $+40 \text{ mV}$ to $19 \pm 2 \text{ ms}$ (Fig. 3D and table S1). In addition, the analysis of the inactivation-voltage relationship showed that $2 \mu\text{M}$ CmTx shifted the half-inactivation voltage ($V_{1/2 \text{ inactivation}}$) of Kv1.5 from $9 \pm 1 \text{ mV}$ to $-1.3 \pm 0.2 \text{ mV}$ (fig. S4).

Under conditions that favored inactivation, CmTx blocked Kv1.5 reversibly with association and dissociation constants of $K_{\text{on}} = 4.1 \times 10^3 \pm 0.3 \times 10^3 / \text{M} \cdot \text{s}$ and $K_{\text{off}} = 2.6 \times 10^{-4} \pm 0.3 \times 10^{-4} / \text{s}$; these rates are 12-fold faster and 4-fold slower, respectively, compared to control conditions (Fig. 3E and Table 1). Blocking kinetics of CmTx at 500 nM, $2 \mu\text{M}$, and $20 \mu\text{M}$ showed that association was linearly dependent on concentration, whereas dissociation was concentration independent, consistent with a simple bimolecular interaction, as observed for other SAK1 toxin blockers (fig. S5). CmTx inhibited the channel in a dose-dependent manner, such that a fit of the dose-response relationship at $+20 \text{ mV}$ with Hill equation (Materials and Methods) yielded a $K_i = 127 \pm 5 \text{ nM}$ and a Hill coefficient of 1.2 ± 0.07 (Fig. 3F). To further validate that CmTx binding favored inactivated channels, we studied Kv1.5-L510A (Fig. 3B) (19), a channel mutant in the S6 span reported to enhance inactivation (22). CmTx inhibited the mutant channel threefold more potently than the

wild-type (WT) channel chiefly due to accelerated association (Fig. 3C and Table 1).

CmTx peptide block of Kv1.5 channels is specific

To assess the specificity of CmTx for Kv1.5, we evaluated its effects on a panel of Kv channels (Fig. 3G and fig. S6). CmTx at $2 \mu\text{M}$ did not block Kv1.1, Kv1.2, Kv1.3 or hERG, channels without a basic residue in the pore. CmTx also failed to inhibit both Kv2.1 and I_{Ks} (formed by Kv7.1 + KCNE1), channels bearing a Lys in the outer pore vestibule at positions nonequivalent to Kv1.5-R487. Kv1.4 mediates the slow transient outward potassium current ($I_{\text{to,s}}$) in cardiac myocytes, and its pore region shares 86% sequence identity with that of Kv1.5 and carries a Lys at the position corresponding to Kv1.5-R487 (fig. S6). CmTx was ~ 39 -fold less potent on Kv1.4 than on Kv1.5; thus, the application of $2 \mu\text{M}$ reduced the current by $\sim 28\%$, yielding an estimated K_i of $5 \mu\text{M}$ ($P = 0.2$) (Fig. 3G).

Identification of Kv1.5 pore residues important for CmTx binding

Classical SAK1 toxins block Kv channels from the external side of the membrane by occluding the conduction pore with a positively charged residue (Fig. 1A), such as Lys²² in HmK (Fig. 2C) (12). Pore residues in the external regions before and after the reentrant loop that carries the selectivity filter are hot spots for SAK1 toxin interaction (16, 23). To explore the role of these residues in CmTx binding, 10 sites were individually mutated to Ala, and CmTx block of the mutant Kv1.5 channels was studied at $+20 \text{ mV}$ using whole-cell patch clamp (Fig. 4A). Of the 10 mutant channels, Kv1.5-N459A, Kv1.5-Q460A, Kv1.5-G461A, and Kv1.5-H463A attenuated CmTx affinity >10 -fold ($\Delta\Delta G > 1.4 \text{ kcal/mol}$; Fig. 4A). Alanine substitutions of Kv1.5 residues immediately following the selectivity filter, M486A, R487A, and I489A resulted in nonfunctional channels or channels with K^+ currents so small they were insufficient for peptide blockade study. Moreover, we studied Kv1.5-R487V channels and found that the native basic residue was required for CmTx blockade (Fig. 4A). The large affinity changes in CmTx binding caused by the Kv1.5 mutants R487V, N459A, Q460A, G461A, and H463A, which resulted in ~ 3921 -, 509 -, 31 -, 11 -, and 1559 -fold attenuation in affinity, respectively, are posited to result mostly from their direct interaction with CmTx (Fig. 4B), as among these mutants only R487V was found to slow $\tau_{\text{inactivation}}$ by more than two-fold, whereas the other four mutants had little (<2 -fold) or no effect on the kinetics of slow inactivation under the conditions used for testing (table S1).

Table 1. CmTx blockade of Kv1.5 and Kv1.5-L510A. CmTx peptide was synthesized and its inhibition of the indicated channels at equilibrium ($K_i \pm \text{SEM}$) was determined by whole-cell patch clamp. The pathological condition (with elevated pulse rates and mild acidosis) was mimicked using a protocol with depolarization of membrane potential to $+20 \text{ mV}$ for 0.2 s from a holding voltage of -60 mV every 0.5 s, with both extracellular and intracellular pH = 6. The physiological condition (reminiscent of healthy heart tissue rate and pH) was mimicked using a protocol with depolarization of membrane potential to $+0 \text{ mV}$ for 0.2 s from a holding voltage of -80 mV every 1 s with an extracellular pH = 7.4 and intracellular pH = 7.2. Values are means $\pm \text{SEM}$; $n = 9$ cells for Kv1.5 under pathological conditions and $n = 5$ cells each for Kv1.5 and Kv1.5-L510A under physiological conditions.

	Condition	K_{on} ($\text{M}^{-1} \text{s}^{-1}$)	K_{off} (s^{-1})	K_i (nM)
Kv1.5	Pathological	$4.1 \times 10^3 \pm 0.3 \times 10^3$	$2.6 \times 10^{-4} \pm 0.3 \times 10^{-4}$	127 ± 5
Kv1.5	Physiological	$3.3 \times 10^2 \pm 0.2 \times 10^2$	$1.1 \times 10^{-3} \pm 0.1 \times 10^{-3}$	2944 ± 246
Kv1.5-L510A	Physiological	$1.2 \times 10^3 \pm 0.2 \times 10^3$	$1.1 \times 10^{-3} \pm 0.1 \times 10^{-3}$	892 ± 36

Identification and mapping of CmTx interaction residues

To facilitate the characterization of CmTx by mutational screening, CmTx and its variants were expressed from a gene in tethered form on the extracellular surface of cells via a glycosyl phosphatidyl inositol (GPI)-anchored membrane-embedded leash, as we have done before with other toxins (12, 17, 24). The construct includes a signal peptide sequence, CmTx variants, and a 46-residue flexible linker followed by a GPI targeting sequence (Fig. 5A). This

reduces the dimensionality of the binding equilibrium and greatly facilitates the study of the inhibition of Kv1.5 by T-CmTx variants in *Xenopus* oocytes using two-electrode voltage clamp (TEVC). Complementary RNA (cRNA) for T-CmTx variants were synthesized in vitro and coinjected with cRNA encoding Kv1.5 into oocytes. The result was T-CmTx variants expressed on the extracellular leaflet of the oocyte membrane via the GPI anchor after natural cleavage of the signal peptide, together with the Kv1.5 channels (Fig. 5A).

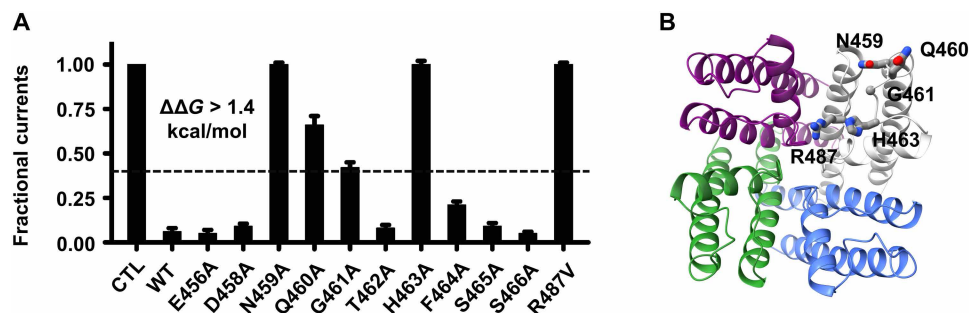


Fig. 4. Effects of Kv1.5 pore mutations on CmTx affinity. CmTx inhibition of Kv1.5 and point mutant channels expressed in HEK293T cells was determined as in Fig. 3D, using 0.2 s depolarizing steps in 20 mV increments from -60 to $+40$ mV at 0.5 s intervals ($pH_i = pH_o = 6$). (A) K_i of CmTx for 11 channel mutants estimated using the Hill equation and assuming the same Hill coefficient as in the WT channel (Fig. 3F). Fractional currents ($I_{\text{CmTx}}/I_{\text{WT}}$ or mutant) for WT or mutant channels in the presence of $2 \mu\text{M}$ CmTx were plotted. Changes of free energy of CmTx blockade ($\Delta\Delta G$) were calculated as described in the Materials and Methods. Values are means $\pm$ SEM; $n = 9$ cells for WT Kv1.5; $n = 5$ cells each for the N459A, Q460A, G461A, H463A, and R487V mutants; and $n = 3$ cells each for the remaining six mutants. (B) AlphaFold-predicted tetrameric structure of Kv1.5. Individual subunits are shown in different colors, and five pore residues essential for CmTx binding are highlighted in stick representation on a single subunit.

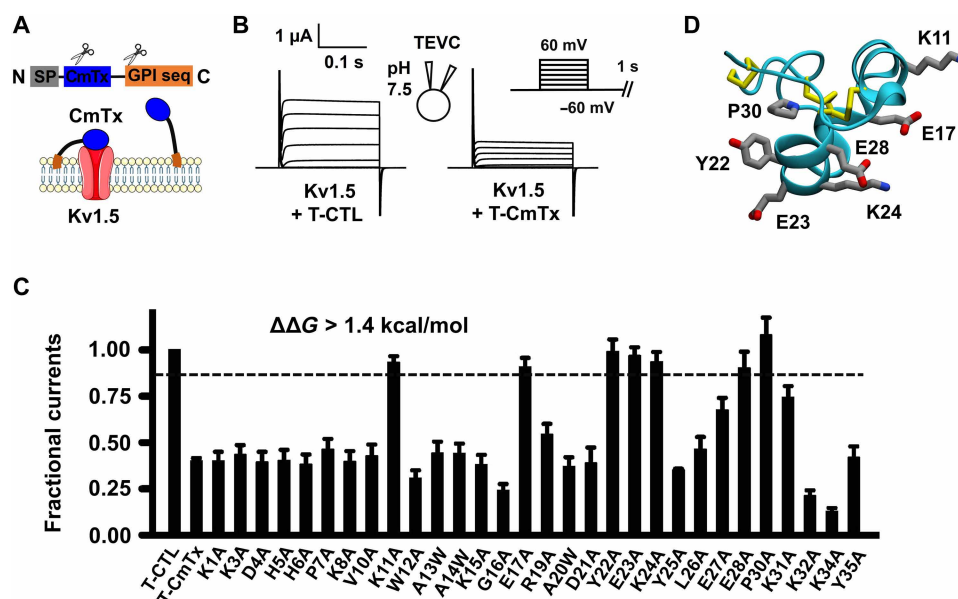


Fig. 5. Screening mutagenesis of T-CmTx. Kv1.5 was expressed in oocytes and inhibition by T-CmTx and its variants were studied by TEVC after coinjection of Kv1.5 and T-CmTx variant cRNAs. (A) T-CmTx was constructed as chimeric fusion protein with an N-terminal secretory signal sequence (SP; gray), the CmTx sequence (blue), a hydrophilic flexible linker (46 residues), and a C-terminal GPI membrane anchor targeting sequence (orange). The colors in the schematic mark T-CmTx components. (B) Representative current traces (0.2-s depolarizing steps from -60 to $+60$ mV at 0.5-s intervals, $pH_o = 7.5$) for Kv1.5 channels with coinjection of 2 ng of T-CmTx cRNA or 2 ng of T-CTL, which is the T-toxin construct without the CmTx sequence (Materials and Methods). (C) Kv1.5 currents at $+20$ mV with T-CmTx variants coinjection were measured and normalized to unblocked Kv1.5 currents with T-CTL coinjection ($I_{\text{T-CmTx}}/I_{\text{T-CTL}}$), recorded within the same injection batch and day. Values are means $\pm$ SEM; $n = 10$ cells for each variant. (D) Ribbon representations of the AlphaFold-predicted structure of CmTx. Side chains of key residues identified by screening mutagenesis are shown in stick representation. Disulfide bonds are colored yellow.

When Kv1.5 currents were measured 2 days after injecting 0.4-ng channel cRNA and 2-ng T-CmTx cRNA, blockade was ~60% of the outward K^+ current at +20 mV (Fig. 5B).

CmTx has 36 residues with three disulfide bonds maintaining the SAK1 toxin scaffold. To identify CmTx residues that might interact with Kv1.5, we generated T-CmTx point mutations at all non-Cys residues (Fig. 5C). The flexible linker of T-CmTx carries a c-Myc epitope, allowing comparison of the surface levels of T-CmTx and six critical variants by enzyme-linked immunosorbent assay (ELISA), as we have described (12, 17). None of the variants showed a notable change in surface expression, ruling out altered expression as the cause for lowered blockade (fig. S7). Thus, compared to WT T-CmTx, seven of the T-CmTx variants exhibited less than 10% blockade, corresponding to a change of affinity >10-fold ($\Delta\Delta G > 1.4$ kcal/mol, Fig. 5C). The mutations with high impact were CmTx-K11A, E17A, Y22A, E23A, K24A, E28A, and P30A. Reflecting the stability of the three-disulfide scaffold of SAK1 peptides, point mutations at these sites are expected to have only local effects, although, the possibility that they alter peptide folding cannot be excluded.

We predicted the 3D structure of CmTx using AlphaFold and observed a model that retains a SAK1 scaffold, showing a helix-kink-helix motif similar to HmK (12). Mapping the sites identified as critical to Kv1.5 blockade on the structure shows the likely interaction surface with the Kv1.5 pore (Fig. 5D). CmTx residues Y22, E23, K24, and E28 are located on one face of the molecule, forming a negatively charged electrostatic surface that likely mediates interactions with the positively charged extracellular region of the Kv1.5 pore (Fig. 6, A and B). In contrast, the R487V mutation in Kv1.5 reverses the local electrostatic potential from positive to negative, thereby abolishing CmTx-mediated channel blockade (Fig. 6B). On the opposite face of CmTx, K11 and adjacent residues, including K15, offset the negative electrostatic potential of E17, forming a region of positive surface potential. Residue P30 is buried within the toxin core. These three residues (K11, E17, and P30) are likely to contribute to proper folding, molecular orientation, and solvation of the peptide.

A proposed model of CmTx binding to Kv1.5

Based on the toxin and channel residues identified as critical for binding (Figs. 4 and 5), a structural model of the CmTx-Kv1.5 complex was generated using AlphaFold (Fig. 6C and fig. S8A). For complex modeling, amino acid sequence of the Kv1.5 pore region (residues M431-Y521) was used. The predicted CmTx-Kv1.5 complex displays residue-residue interactions consistent with experimental observations from scanning mutagenesis of both the channel and CmTx.

As shown in Fig. 6A, residues E23 and E28 form a negatively charged region on one face of CmTx that allows complementary interaction with the strong positive potential near R487 in the extracellular pore vestibule (Fig. 6B). In the modeled complex, CmTx is oriented such that its negatively charged face aligns directly above the Kv1.5 pore, establishing multiple electrostatic interactions between CmTx residues Y22, E23, K24, E27, and E28 and the four R487 residues, as well as one H463 residue in the pore (Fig. 6C). These interactions stabilize the toxin in a pore-blocking position and explain its high selectivity for Kv1.5. In contrast, the R487V mutation reverses the local surface potential from positive to negative (Fig. 6B), disrupting these electrostatic contacts and abolishing CmTx-mediated inhibition (Fig. 4A). Notably, the E27A mutation reduced CmTx affinity by ~3-fold ($\Delta\Delta G = 0.7$ kcal/mol),

consistent with a moderate but specific contribution to toxin binding (Fig. 5C).

To further investigate the potential molecular mechanism underlying state- and pH-dependent blockade of Kv1.5 by CmTx, we performed additional structural predictions of the CmTx-Kv1.5 complex using the cryo-EM structure of apo Kv1.3 as a template (25). In the apo Kv1.3 structure, the selectivity filter adopts a putative inactivated conformation, similar to those observed in NaK (26, 27), Shaker-W434F (28), and Kv1.2-2.1-3m (29) channels. The pK_a (where K_a is the acid dissociation constant) value of D485, located at the entrance to the selectivity filter of Kv1.5, was markedly shifted from 4.47 ± 0.90 in the conductive state to 6.57 ± 0.20 in the inactivated state due to disruption of the hydrogen-bonding interaction between D485 and W472 (Fig. 6D). This shift results in a more positively charged outer vestibule of Kv1.5 at lower pH, attributable to the protonation of D485 (Fig. 6E). Actual interface predicted template modeling (actifpTM) scores for CmTx bound to conductive and inactivated Kv1.5 were comparable (fig. S9, A and B), suggesting similar confidence in the predicted binding interfaces. CmTx adopted a slightly altered orientation in the inactivated state, inserting E23 into the selectivity filter to establish extensive interactions with D485 (Fig. 6F).

DISCUSSION

Peptide toxin phage display for receptors expressed on living cells

Identifying natural toxins able to block Kv1.5 with high affinity has been a major challenge in K^+ channel pharmacology (5). In our hands, Osu1 and Ts6 (5), two toxins reported to block Kv1.5, showed negligible inhibition on Kv1.5 at μM level (15). Other K^+ channels that carry a positively charged residue in their outer vestibule—such as Kv7.1, Kv2.1 and Kv1.4—also lack high affinity peptide pore blockers (fig. S6). BgK, identified from the sea anemone *Bunodosoma granulifera*, can block Kv1.1, Kv1.2, and Kv1.3 channels with a K_i of 18 to 777 pM but is unable to block WT Kv1.5 channels (15). However, mutating Kv1.5-R487 to Val (the corresponding residue in Kv1.2) or Tyr (the corresponding residue in Kv1.1) enables BgK blockade with a K_i of 191 and 765 pM, respectively.

Sea anemones are a rich source of peptide toxins able to block K^+ channels; the peptides have been used to study the structure and function of K^+ channels and are being explored as therapeutic agents for diseases including multiple sclerosis, psoriasis, and rheumatoid arthritis (1, 30). Previously, we identified natural and designer blockers of the bacterial KcsA K^+ channel by screening a SAK1-derived phage display library with purified channel protein (16). KcsA has high sequence and structural homology to the S5-S6 domain of Kv1 channels (fig. S6) (31). Screening with purified KcsA, we isolated HmK, a natural toxin in the library isolated from the sea anemone *Heterotactica magnifica* and observed it to be potent but promiscuous, blocking KcsA, Kv1.1, Kv1.2 and Kv1.3 channels with K_i of 0.08 nM to 3 nM, whereas the affinity for Kv1.5 was weak, exhibiting a K_i of ~10,000 nM (16, 32).

In addition to pore-directed libraries such as SAK1, we have applied phage display to other purified ion channels (18, 33). For example, we developed a combinatorial library of ~1 million peptides based on the inhibitor cystine knot (ICK) scaffold of gating-modifier toxins that target voltage-sensing domains (18). Panning this library against the voltage-gated proton channel Hv1 yielded C6 peptide,

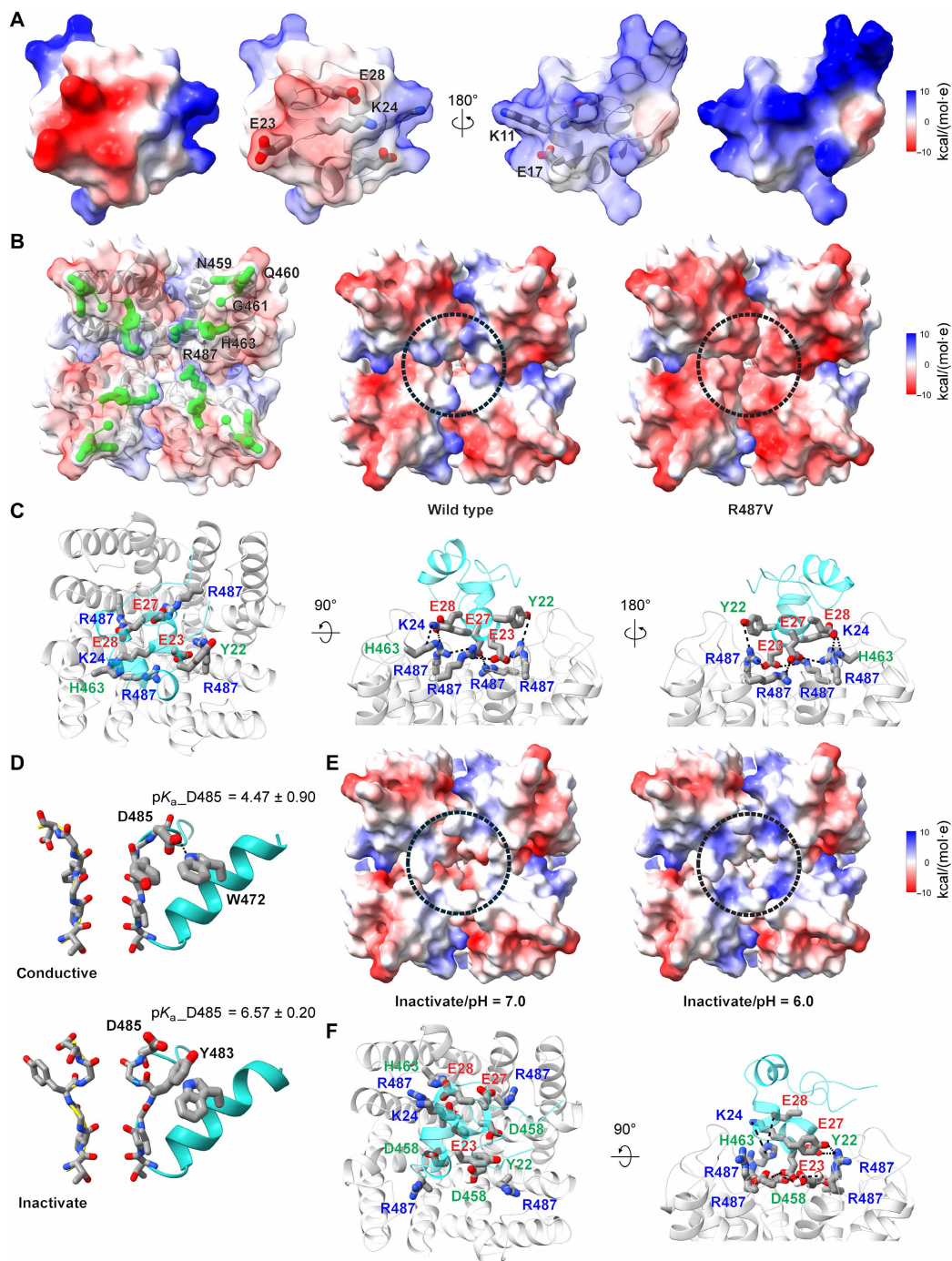


Fig. 6. Structural models of the Kv1.5-CmTx complex predicted using AlphaFold. (A) Surface and ribbon representations of the predicted structure of CmTx. The α atoms and side-chain heavy atoms of key charged residues identified through mutagenesis screening (Fig. 5C) are shown as sticks. The electrostatic potential surface is shown alongside the structure. (B) Top view of the conductive Kv1.5 channel with key residues (Fig. 4A) highlighted as sticks (left). Molecular surfaces of WT Kv1.5 (middle) and the R487V mutant (right) are colored according to electrostatic potential, as calculated using ChimeraX (40). The R487V mutation reverses the local electrostatic potential from positive to negative (circled), thereby abolishing CmTx block. (C) Close-up view of the interaction interface between CmTx and conductive Kv1.5 in the structural model. Residues Y22, E23, K24, E27, and E28 of CmTx form extensive electrostatic interactions with R487 and H463 at the extracellular entrance of the Kv1.5 selectivity filter, highlighted by dashed lines. (D) Structural comparison of the conductive and inactivated selectivity filters of Kv1.5. Outward flipping of Y483 in the inactivated state disrupts the hydrogen-bonding interaction between D485 and W472, resulting in a marked shift in the pK_a of D485. pK_a values are presented as means $\pm$ SD for the four subunits. Two opposing subunits are shown for clarity. (E) Electrostatic potential surfaces of inactivated WT Kv1.5 at neutral (left) and acidic pH (right), calculated using UCSF ChimeraX. At pH 6.0, the D485 residues were protonated in ChimeraX before electrostatic potential calculation. (F) Close-up view of the interaction interface between CmTx and inactivated Kv1.5 in our structural model. Residue E23 of CmTx is predicted to insert into the entrance of the inactivated selectivity filter and form extensive interactions with protonated D485 residues of Kv1.5.

the first specific, high-affinity inhibitor of Hv1, which has enabled elucidation of Hv1's physiological roles in human sperm, neutrophils, and microglia (2–4, 24, 34).

Cell-based phage panning has rarely been reported, presumably because the abundance of competing nontarget proteins on the surfaces present a challenge to selection that is avoided with purified protein targets (35). Here, we overcame the challenge by optimizing target expression and cycling between two cell types to isolate CmTx, a required approach because Kv1.5 has resisted purification as intact tetrameric complexes. This approach of live-cell panning, combined with scaffold-based toxin libraries, now offers a generalizable platform for the discovery of selective peptide ligands for disease-relevant ion channels that cannot be purified in a manner that recapitulates their native structure.

CmTx blocks Kv1.5 with a unique mechanism

Unlike classical pore-blocking toxins, CmTx uses a fundamentally distinct mechanism to occlude ion conduction. Members of the classical SAK1 toxin family, including BgK (15) and HmK (12), use a central lysine residue (e.g., K25 in BgK and K22 in HmK) to physically occlude ion conduction by inserting its positively charged side chain into the selectivity filter to block the K^+ pathway within the pore. However, the Kv1.5 outer vestibule is uniquely characterized by a ring of four R487 residues, generating a strong positive electrostatic potential that putatively repels basic toxin residues (Fig. 6B). Consequently, while HmK and BgK potently inhibit other Kv1 channels with K_i values in the picomolar to nanomolar range, they exhibit negligible activity toward WT Kv1.5. Substituting R487 with neutral or aromatic residues (e.g., valine or tyrosine) produces sensitivity to BgK, highlighting the critical role of the R487 ring as an electrostatic barrier to classical pore-blocking toxins (15).

CmTx overcomes the electrostatic barrier through a novel blocking mechanism. Rather than using a basic residue as a central “plug,” CmTx presents a cluster of acidic side chains that form complementary interactions with the positively charged Kv1.5 vestibule (Fig. 6A). In the predicted CmTx-Kv1.5 complex, three acidic residues on CmTx-E23, E27, and E28 are oriented toward the channel pore, forming salt bridges with the four R487 residues that decorate the Kv1.5 outer vestibule (Fig. 6C). These ion pairs stabilize CmTx in a pore-occluding position without the need for the canonical lysine insertion observed in traditional Kv blockers. This electrostatic complementarity defines a previously unrecognized mechanism of peptide-mediated pore blockade. It suggests that engineering toxins with acidic binding surfaces may provide a novel and broadly applicable strategy to target Kv channels containing positively charged outer-pore residues, such as Kv1.4, Kv2.1, and Kv7.1, targets that have historically resisted inhibition by classical pore-blocking toxins. Thus, CmTx establishes a structural and mechanistic paradigm for Kv channel inhibition, demonstrating how charge complementarity can be exploited to design selective peptide ligands for ion channels previously considered “toxin-resistant.”

Notably, CmTx exhibits state- and pH-dependent blockade of Kv1.5. Previous studies of Kv1.2, Kv1.3, and Shaker channels have suggested that C-type inactivation involves conformational rearrangements of the selectivity filter that propagate to the extracellular mouth/turret region of the pore, thereby altering outer-pore geometry and ion coordination. Although the structure of Kv1.5 has not been resolved experimentally, the high sequence similarity of its pore region to those of Kv1.2 and Kv1.3 supports the use of these

channels as structural templates. The predicted model of the CmTx-Kv1.5 complex in the inactivated state, generated using the inactivated cryo-EM structure of apo-Kv1.3 (fig. S8B), revealed substantial reorientation of CmTx and a marked pK_a shift of D485 at the extracellular entrance to the selectivity filter of Kv1.5 (Fig. 6D). This shift results in a more positively charged outer vestibule under acidic conditions. Accordingly, CmTx appears capable of adjusting its orientation to insert residue E23 into the selectivity filter, where it interacts with D485 and occludes the pore, reminiscent of the classical lysine-insertion mechanism proposed for traditional Kv channel blockers (Fig. 6F). Although AlphaFold-based models are in close agreement with our electrophysiological data, these findings remain hypothetical. The proposed structure of Kv1.5 in the inactivated state, as well as the binding conformation and residue level interactions of CmTx with conductive and inactivated Kv1.5, will require validation through future experimental structural studies.

Compared with other well-characterized SAK1 toxin blockers, such as HmK and ShK, the apparent association of CmTx with its target is markedly slower. HmK and ShK block Kv1.3 channels with affinities of 3 nM and 0.05 nM and K_{on} of 1×10^6 and $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively (table S2) (12, 16). These rates are ~250- and 7500-fold faster than that of CmTx under pathological conditions. This slow apparent association rate of CmTx is likely the result of limited access to the Kv1.5 pore binding site revealed by channel inactivation.

Both electrophysiological studies (fig. S5) and computational modeling (Fig. 6 and fig. S8) support a 1:1 stoichiometry for CmTx blockade of Kv1.5 channels. However, the Hill coefficient derived from the dose-response analysis ($h = 1.2$; Fig. 3F) is greater than 1. This discrepancy may reflect heterogeneity in channel state occupancy, rather than cooperative binding or the presence of multiple toxin molecules per channel.

CmTx is a potential drug lead

Blocking Kv1.5 channels is considered to be a potential treatment for atrial fibrillation and other life-threatening arrhythmias (6). Kv1.5 is also associated with other diseases, such as pulmonary hypertension, hypothyroidism, hypoxic pulmonary vasoconstriction, and cancer (5, 7). However, small molecules that inhibit Kv1.5 have weak affinities and are poorly specific (36). For example, 4-aminopyridine inhibits Kv1.5 with an affinity of ~50 μM and is promiscuous, blocking Kv1.1 and Kv1.2 with a similar affinity. Diphenyl phosphine oxide-1 blocks Kv1.5 with an affinity of ~0.3 μM , but its selectivity over Kv4.2/Kv4.3 and Kv3.1 is only 8-fold and 15-fold, respectively (36). Small-molecule blockers of Kv1.5 developed to treat atrial fibrillation, such as AVE0118, XEN-D0101, and vernakalant also have only micromolar affinity and cross-react with other Kv channels (5, 36). This may explain why none have proven successful in clinical trials.

CmTx has advantages as a drug lead. First, it appears to be specific to Kv1.5, making it less likely to manifest off-target effects. Second, the state-dependent inhibition of CmTx makes it less potent under normal physiological conditions, $K_i \sim 2.9 \mu\text{M}$ at 60 beats per min and physiological pH, whereas under pathological conditions involving elevated pulse rates (120 beats per min and acidosis), the K_i is ~127 nM, a much higher affinity than other Kv1.5 blockers (5). Under pathological conditions such as atrial fibrillation or ischemia, Kv1.5 channels are more likely to occupy inactivated states, thereby enhancing CmTx binding and inhibition. Conversely, under normal conditions, fewer inactivated channels would result in a lower effective potency for CmTx. This combination is likely

to expand CmTx therapeutic window, allowing for a lower dosage and fewer side effects.

To our knowledge, we report the first specific peptide inhibitor of Kv1.5, revealing an electrostatic pore-occlusion mechanism mediated by complementary charge interactions, demonstrate cell-based phage display as a powerful strategy for discovering ligands to previously intractable ion channels that are difficult to purify, and present CmTx for regulation of Kv1.5 in health and disease.

MATERIALS AND METHODS

Molecular biology

Human Kv1.5 (NM_002234) in-frame with TagRFP-T (EU582019), Kv1.1 (NM_000217), Kv1.2 (NM_004974), Kv1.3 (NM_002232), Kv1.4 (NM_002233), Kv7.1 (NM_000218) and hERG (NM_000238) were cloned in pMAX+ vector with the 5' and 3' portions of the *Xenopus laevis* β -globin gene, and a T7-promoter for in vitro transcription. Per our prior reports (12, 17), T-CmTx was constructed by replacing the sequence of lynx1, a natural tethered nicotinic acetylcholine receptor peptide modulator, with the sequence of CmTx (with N terminus at front) in frame between the trypsin secretory signal sequence and a 46-residue flexible linker containing Gly-Asn repeat and a c-Myc epitope. The whole sequence of T-CmTx was cloned into the pCS2⁺ plasmid vector that has an SP6 promoter for in vitro transcription of cRNA. T-CTL was constructed by removing the CmTx sequence from the T-CmTx plasmid. Point mutation for the Kv1.5 channel and T-CmTx was introduced using the QuikChange Site-Directed Mutagenesis Kit (Agilent). The sequences of all constructs were confirmed by DNA sequencing. cRNAs for the Kv1.5 and T-CmTx were synthesized in vitro. The plasmids were linearized using PmeI (New England BioLabs) and purified using QIAquick PCR Purification Kit (QIAGEN), and cRNAs were synthesized using the mMESSAGE mMACHINE kit for T7 (Invitrogen). cRNAs were diluted with RNase-free water, and concentrations were measured using NanoDrop 2000 (Thermo Fisher Scientific) and stored at -80°C until use.

Phage display library screening

SAK1 toxin library was constructed as described before (16). HEK293T and CHO cells were purchased from American Type Culture Collection (ATCC) and maintained in DMEM (Gibco) and F12K (Gibco) supplemented with 10% FBS and 1% penicillin and streptomycin. Kv1.5-RFP plasmids were transfected into cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Twenty-four hours posttransfection, cells were used for phage library screening. Transfected cells (10^7) were resuspended and blocked with tris-buffered saline (TBS) [25 mM tris-HCl, 140 mM NaCl, and 3 mM KCl (pH 7.4)] containing 2% bovine serum albumin (BSA). After 1 hour incubation at 4°C , cells were centrifuged and washed once with TBS containing 0.5% BSA (TBSB). Cells were then mixed with 10^{10} library phages in 1 ml of TBSB. After 1 hour incubation at 4°C , cells were centrifuged and washed twice with TBS containing 0.1% Tween 20 and 0.5% BSA. Poorly adherent phage particles were removed, and bound phage particles were eluted with 0.1 M triethylamine by incubation for 10 min. The pH of the eluate was adjusted to 7.5 with 1 M tris-HCl (pH 8.0) and used to infect *Escherichia coli* XL1-Blue for phage amplification and the next round of selection. To suppress the isolation of HEK293T-specific phages, two rounds of sorting performed on transfected HEK293T cells were followed by one round on transfected CHO cells and then two more rounds of sorting on transfected

HEK293T cells. Phage particles were quantified by titrating before and after selective library sorting and genotyped by DNA sequencing.

Peptide toxin synthesis and recombinant expression

CmTx was purchased as synthetic peptide (CSBio); oxidative folding reactions were quenched by acidification and products purified by reverse-phase HPLC before evaluation by mass spectrometry, as before (16, 18). Representative HPLC chromatograms of the folded peptide, along with mass spectrometry confirmation of the formation of three disulfide bonds, are shown in fig. S9. CmTx had greater than 90% purity and was lyophilized for storage at -20°C . It was dissolved in the recording solution for whole-cell patch clamp before use.

Whole-cell patch clamp

K^+ currents passed by Kv1.5 and mutants were recorded in whole-cell mode using an Axopatch 200B amplifier. Stimulation and data collection were done with a Digidata1322A and pCLAMP 10 software (Molecular Devices). Cells were perfused with an external solution composed of 4 mM KCl, 120 mM NaCl, 5 mM glucose, 10 mM MES, 0.5 mM CaCl_2 , 1.2 mM MgCl_2 at pH 6 (pathological condition) or 4 mM KCl, 120 mM NaCl, 5 mM glucose, 10 mM Hepes, 0.5 mM CaCl_2 , and 1.2 mM MgCl_2 at pH 7.4 (physiological condition). Pipettes with resistances between 3 to 5 M Ω were filled with 136 mM KCl, 2 mM K_2ATP , 5 mM EGTA, 10 mM MES, 1 mM MgCl_2 at pH 6 (pathological condition) or 136 mM KCl, 2 mM K_2ATP , 5 mM EGTA, 10 mM HEPES, and 1 mM MgCl_2 at pH 7.2 (physiological condition). Capacitance was subtracted online. Sampling frequency was 10 kHz with filtering at 1 kHz. The equilibrium inhibition of CmTx was determined by fitting the concentration-response curve. K_{on} and K_{off} were estimated from fits of the kinetics of toxins wash-in and wash-off. Thus, according to a bimolecular scheme with a single toxin-bound state and changes in toxin concentration compared to the speed of block and unblock, one can assess steady-state and kinetic parameters of blockade. The unblocked fractional current at equilibrium (F_{un}) with a toxin concentration [Tx] relates to the concentration dependence of inhibition according to Hill equation: $F_{\text{un}} = (1 + [\text{Tx}]/K_i)^{-1}$, where F_{un} is related to the second-order association rate constant K_{on} (1/Ms) and first-order dissociation rate constant K_{off} (1/s) according to equation $F_{\text{un}} = K_{\text{off}}/(K_{\text{on}}[\text{Tx}] + K_{\text{off}})$, and the equilibrium inhibition constant for half-maximal blockade K_i is given by the equation $K_i = K_{\text{off}}/K_{\text{on}}$. Time constants of block and unblock (τ_{on} and τ_{off}) were determined by single exponential fits of the kinetics of toxin wash-in and wash-off, and these parameters are related to K_{on} and K_{off} by equations: $\tau_{\text{on}} = (K_{\text{on}}[\text{Tx}] + K_{\text{off}})^{-1}$ and $\tau_{\text{off}} = (K_{\text{off}})^{-1}$ (16, 32). Free-energy changes were calculated using $\Delta\Delta G = RT \ln(K_{i \text{ mutation}}/K_{i \text{ WT}})$, where R is the gas constant and T is the temperature in Kelvin.

Two electrode voltage clamp

X. laevis stage VI oocytes were selected and injected with 0.4 ng of cRNA encoding Kv1.5 in the laboratory pMAX⁺ vector, as before (16). To study the blocking effect of T-CmTx (or variants), cRNAs for T-CmTx (or variants, 2 ng) and Kv1.5 were mixed and coinjected into the oocytes. The recording solution was 4 mM KCl, 100 mM NaCl, 1 mM MgCl_2 , 0.3 mM CaCl_2 , 10 mM Hepes (pH 7.5). Recordings were performed with a constant gravity flow of solution at 2 ml/min, yielding chamber exchange in 5 s. Currents were recorded 2 days after cRNA injection using an Oocyte clamp amplifier OC-725C (Warner Instruments) and electrodes filled with 3 M KCl with a resistance of

0.3 to 1 M Ω . Data were filtered at 1 kHz and digitized at 20 kHz using pClamp software and assessed with Clampfit 9 and Origin 8.

Enzyme-linked immunosorbent assay

Surface expression of T-CmTx and variants were quantitated using ELISA as previously described (17). Oocytes injected with cRNAs of T-CmTx variants bearing the c-Myc tag were blocked with BSA and then bound with c-Myc-Tag monoclonal antibody HRP (1 μ g/ml) (Invitrogen). Oocytes were washed and incubated with 50 μ l of 1-Step Ultra TMB-ELISA solution (Thermo Fisher Scientific). The reaction was stopped by adding 50 μ l of 2 M H₂SO₄. Surface ELISA signals were quantified at 450 nm.

AlphaFold protein structure prediction

We used ColabFold v1.6.1 to generate structural models of CmTx, WT Kv1.5 pore domain (residues 431 to 521), its R487V mutant, and the CmTx-Kv1.5 pore domain complex. Sequence alignment was performed using MMseqs2, and complex prediction was conducted with AlphaFold2_multimer_v3 using 48 recycling steps (37, 38). For structural prediction of the inactivated state of Kv1.5, the cryo-EM structure of Kv1.3 [Protein Data Bank (PDB): 7WF3] was used as a template (25). Five models were generated for each prediction, and the top-ranked model based on the interface predicted template modeling score was selected as the representative structure. The pK_a values of residues in the predicted structures were calculated using PDB: 2PQR (39).

Statistical analysis

Data were analyzed using a two-sided unpaired Student's *t* test. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). A *P* value $\leq$ 0.05 was considered statistically significant.

Supplementary Materials

This PDF file includes:

Figs. S1 to S9

Tables S1 and S2

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A designer pore-blocking peptide reveals an electrostatic complementation mechanism for the inhibition-resistant Kv1.5 channel

Ruiming Zhao, Maha Nayak, Rong Shen, Hui Dai, Punyanuch Hua, Eduardo Perozo, and Steve A. N. Goldstein

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