The rapid delayed rectifier (I_{Kr}) is an important repolarizing current in many regions of the heart (1). The major subunit that forms the I_{Kr} channel in human heart is HERG (2). Inherited mutations in HERG have been identified and linked to congenital long QT syndrome (LQT2) (2, 3). Furthermore, the C-type inactivation process in the I_{Kr}/HERG channel appears to be intimately related to channel sensitivity to many different drugs (4–6). C-type inactivation results from conformational changes in the outer mouth region of the channel, which prevent current flow through the pore (7). Therefore, an important target for structural analysis is the outer mouth region of the I_{Kr}/HERG channel.

There is a general consensus that the outer vestibules of various potassium channels share a common architecture (8–10). Indeed, homology modeling of outer vestibules of mammalian voltage-gated K^+ (Kv) channels based on the crystal structure of KcsA (9), a bacterial proton-gated potassium channel, has been successful in several cases (10). However, such a strategy may not be applicable to the HERG channel. First, the C-type inactivation in the I_{Kr}/HERG channel is unique among all potassium channels that have this gating process: C-type inactivation in I_{Kr}/HERG is extremely fast in onset and in reversal and is strongly voltage-dependent (11). These unique kinetic features suggest that conformational changes in the outer mouth region of HERG during membrane depolarization may be different from those of the other channels. Second, HERG has an unusually long S5-P linker (43 amino acids), which may be different from those of the other channels. The rapid delayed rectifier in I_{Kr} is an important repolarizing current in many regions of the heart (1). The major subunit that forms the I_{Kr} channel in human heart is HERG (2). Inherited mutations in HERG have been identified and linked to congenital long QT syndrome (LQT2) (2, 3). Furthermore, drugs that suppress I_{Kr} have been linked to "acquired LQT" (3).
lated to the channel’s sensitivity to various drugs.

Peptide toxins have been very useful tools in structural analysis of potassium channels, well before the data of KscA crystal structure were available (13, 14). Many short peptide scorpion toxins (H9251-KTx) are available that can block various potassium channels with differences in specificity and potency (13–15).

Many H9251-KTx toxins have six cysteines, forming three disulfide bridges (15). They have a well-conserved positive residue (Lys-27 in ChTx) that serves to plug the channel pore (Fig. 1B) (16). ErgTx is purified from Centruroides noxius Hoffmann (GenBank™ accession number CnErg1). It has 42 amino acids and 4 disulfide bonds (17). Its amino acid sequence and disulfide bond pattern are not homologous to those of representative H9251-KTx toxins shown in Fig. 1B. Previous work has shown that ErgTx is a potent blocker of HERG expressed in mammalian cells and native IKr in guinea pig ventricular myocytes (IC50 in the low nanomolar range) (18). It does not block IK,S, IK,A, ATP, IK,R, or EAG at 1 μM (18). Recently, we showed that ErgTx sensitivity may be determined by the S5-P linker of the HERG channel (19).

Due to the four disulfide bonds, ErgTx should have a compact and rigid structure, amenable to NMR analysis of its solution...
briefly, crude venom was dissolved in water and centrifuged at 10,000 \times g for 15 min. The supernatant was lyophilized and kept at 
\(-20^\circ\text{C}\) until final purification. After Sephadex G-50 gel filtration, fraction II was directly applied to a semi-preparative C18 reverse-phase column (Vydac, Hesperia, CA) and eluted with a linear gradient from 5% solvent A (0.12% trifluoroacetic acid in water) to 60% solvent B (0.10% trifluoroacetic acid in acetonitrile) over 90 min. The component eluted at \(-30\) min was further chromatographed by high performance liquid chromatography using an analytical C18 reverse-phase column. This gave a major pure component (ErgTx), whose primary structure was obtained by direct amino acid sequencing and by mass spectrometry (Fig. 1B) (17).

**Molecular Biology—**HERG in a vector pGPH19 was a kind gift of Dr. Gail Robertson (University of Wisconsin-Madison). We subcloned the HERG cDNA sequence (GenBank\textsuperscript{TM} accession number U04270) into the KpnI/XbaI site of a vector, pAlterMax, which was required for the oligonucleotide-directed mutagenesis procedure with a commercial kit (Altered Site II in vitro mutagenesis system, Promega). Cysteine substitution mutations were confirmed by direct DNA sequencing around the mutation sites. In most cases, two separate colonies from each mutant were used for cRNA transcription and oocyte expression. No differences were seen in the phenotype of channels translated from the two cRNAs. For transcription, wild-type (WT) and mutant HERG sequences in the pAlterMax vector were linearized with NotI and transcribed using the T7 RNA polymerase and a commercial kit (SAGE mMACHINE, Ambion, Austin, TX). All cRNA samples were quantified by denaturing RNA gels using densitometry (Chemilumager model 4400, A-Innotech Corp.).

**Oocyte Preparation and Injection—**Oocyte isolation and cRNA injection were as described previously (25). Briefly, Stage V oocytes were isolated from follicular cell layer after mild collagenase digestion and injected with cRNA solution using a Drummond digital microdispenser. The injection volume was \(-40\) nl/oocyte, equivalent to cRNA of 12–18 ng/oocyte.

**Electrophysiological Experiments—**Three to five days after cRNA injection, channel function was studied using the two-microelectrode voltage clamp method as previously described (26). During recordings, oocytes were superfused with a low-Cl\textsuperscript{−} ND96 solution at room temperature. For experiments shown in Fig. 5, oocytes were not superfused but were placed in the bath solution of a fixed volume (1 ml). ErgTx was dissolved in sterile bovine serum albumin (0.1 mg/ml) solution at 2 \(\mu\)M and frozen in small aliquots. An aliquot was thawed and used for experiments in \(-2\) days without refreezing. After control data were obtained, \(5\) \(\mu\)l of the ErgTx stock solution was added to the bath solution (1 ml) to reach a final concentration of 10 nm. Repetite pipetting was needed to ensure complete equilibration of ErgTx in the bath solution. Toxin effects were evaluated when steady state was reached (4–10 min). Voltage clamp protocol generation and data acquisition were controlled by pClamp 5.5 (Axon Instruments). Data analysis was performed with pClamp 6 or 8, Excel (Microsoft) and PeakFit (Jandel Scientific). Specific protocols and methods of data analysis are described in the figure legends. Where appropriate, data are presented as mean \(\pm\) S.E. Statistical analysis was performed with one-way analysis of variance, followed by Dunn’s test (SigmaStat 2.0, SPSS).

**RESULTS**

**Effects of Mutations in the Extracellular Linkers Suggest a Unique Outer Vestibule Structure in the HERG Channel—**Fig. 2A (panels a and c) shows the hallmark of wild-type (WT) HERG currents: strong inward rectification due to rapid onset and reversal of C-type inactivation in a voltage-dependent manner. A depolarization pulse to +60 mV elicited little outward current because of C-type inactivation. Subsequent repolarization to +40 to –60 mV induced outward tail currents with a distinct rising phase (recovery from C-type inactivation). This led to a prominent negative slope in the tail I–V relationship (+60 to –60 mV, Fig. 2A, panel c). At +40 mV, tail currents became less outward and reversed at \(-100\) mV (reversal potential \(E_{rev}\)), close to the Nernst \(K^+\) equilibrium potential \(E_{rev} = \text{RT} / F \ln [K/2][K^+]\).

Replacing histidine at position 587 to lysine (H587K, Fig. 2, top), disrupted both the C-type inactivation process and the \(K^+\) selectivity of the pore. Current traces from H587K were elicited by the same voltage clamp protocol as that used for WT HERG (Fig. 2A, panel b). The step to +60 mV induced a prominent

**EXPERIMENTAL PROCEDURES**

**ErgTx Purification—**Venom was purified from scorpions Centruroides noxius as described previously (17,18) with minor modifications.
outward current. Subsequent repolarization steps elicited smaller outward currents that reversed between −10 and −20 mV. The I–V relationship of H587K was almost linear in the voltage range between −140 and 0 mV, with an upward turn at more positive voltages (Fig. 2A, panel c). Therefore, the C-type inactivation process was disrupted in H587K. Furthermore, removing extracellular Na\(^+\) ions shifted the \(E_{\text{rev}}\) of H587K in the negative direction to about −60 mV (Fig. 2A, panel d), indicating that external Na\(^+\) ions contributed significantly to currents through the H587K channel pore. The calculated K\(^+\) to Na\(^+\) permeability ratio (\(P_{\text{K}}/P_{\text{Na}}\)) for H587K was 1.5 ± 0.1, much lower than that of the WT HERG (191 ± 91). These changes in H587K were not due to the added permanent positive charge, because increasing the protonation of H587 in WT HERG (by changing the extracellular pH from 8.5 to 6.5) did not affect the C-type inactivation process or the K/Na selectivity of the pore (27). Furthermore, replacing His-587 with proline creates the same phenotype as H587K (12). These observations indicate that the extracellular S5-P linker of HERG, or at least the middle of this linker including position 587, participates in conformational changes that determine the channel’s outer mouth properties. There has been no report on similar effects of mutations made in the S5-P linker of the Shaker channel on the channel’s outer mouth properties (7). Surprisingly, replacing Ser-631 with an aromatic residue (S631Y) reduced the sensitivity to external TEA (Fig. 2B, panel d). These data suggest that the outer mouth configuration in the HERG channel differs significantly from that of the Shaker channel so that the side chain at position 631 is shielded from bound TEA.

**ErgTx Selectivity**—Fig. 3 shows that ErgTx potently suppressed the HERG current amplitude. The suppressing effect could be detected at 1 nM ErgTx. However, ErgTx did not affect Kv1.4, Kv2.1, or KvLQT1 even at 50 nM. This apparent selectivity, by itself, is not conclusive evidence for a unique outer vestibule structure in the HERG channel among the potassium channels examined here. Therefore, we further investigated the mechanism by which ErgTx suppresses HERG current, and positions in HERG that are important for ErgTx binding. These features are compared with those of CbTx (or its analogs) blockade of the Shaker (or Shaker-like) channel. In the latter cases, the mechanism and site of action of the toxins
Mechanism and Site of Action of a HERG-specific Toxin, ErgTx

Concentration Dependence of ErgTx Suppression of HERG—Fig. 4A illustrates a representative time course of changes in HERG current amplitude when the oocyte was exposed to increasing concentrations of ErgTx (1–100 nM) and after wash out of the toxin. Currents were elicited by repetitive depolarization pulses from -80 to +20 mV for 1 s applied once every minute. The peak tail current amplitudes before ErgTx application (Ic) and that at the steady state of ErgTx effect (Itx) were used to measure the fraction of unblocked channels (Itx/Ic). ErgTx suppressed HERG current in a concentration-dependent manner. The effect was totally reversible. The data points can be well fit with Equation 1,

$$ I_{c} / I_{t} = A_{max} / (1 + [ErgTx] / K_d) + (1 - A_{max}) $$

(Eq. 1)

where $A_{max}$ is the fraction of current sensitive to ErgTx (93 ± 3%), and $K_d$ is the dissociation constant (6.45 ± 1.03 nM).

It is important to note that there is a residual current (on average −10% of control) not suppressed by high concentrations of ErgTx. This is clearly shown in Fig. 4A: increasing [ErgTx] from 50 to 100 nM induced little further suppression of the current. In the presence of 100 nM ErgTx, the current's waveform resembled that of the control current (showing C-type inactivation and a high K⁺ selectivity, trace d of Fig. 4A). Therefore, this cannot be due to an ErgTx-insensitive background or “leak” conductance. Instead, it suggests that ErgTx did not totally occlude the HERG pore. This is different from ChTx blockade of the Shaker channel: Lys-27 of ChTx binds and plugs the channel pore completely (22, 23). The difference between ErgTx suppression of HERG and ChTx blockade of Shaker is consistent with the lack of a “Lys-27-equivalent” in the ErgTx sequence (Fig. 1B).

Lack of [K]o Sensitivity in ErgTx/HERG Interaction—Fig. 4B also shows that ErgTx potency was not affected by elevating [K]o, from 2 to 98 mM. This is distinctly different from the situation of ChTx blockade of the Shaker channel. In this case, Lys-27 of ChTx is critical for $K_o$ sensitivity (16, 22). It is suggested that Lys-27 of ChTx plugs the potassium channel pore by binding to a site close to the $K_o$-sensing binding site within the pore (16, 22). Elevating [K]o increases K⁺ occupancy inside the pore, and the resulting electrostatic repulsion between K⁺ ions and Lys-27 of ChTx destabilizes toxin binding. The lack of $K_o$ sensitivity in ErgTx suppression of HERG is again consistent with the lack of a Lys-27-equivalent residue in ErgTx and with the suggestion that ErgTx does not physically plug the HERG pore.

Cysteine Scanning Mutagenesis Detects Positions Critical for ErgTx Binding to HERG—Studies of toxin binding to potassium channels have implicated the SS-P linker (turret of KcsA, Fig. 1A) and P-S6 linker as important components of toxin binding site (23, 24). Therefore, we performed cysteine-scan mutagenesis by replacing all residues in the SS-P linker (631–638, 8 residues) and the P-S6 linker (659–663, 4 residues) of HERG with cysteine one at a time and measured. The fraction of remaining current in the presence of 10 nM ErgTx ($I_{t} / I_{c}$) was used to estimate the dissociation constant ($K_d$) based on modified Equation 1 (assuming that the maximal toxin effect is 90% reduction of the current, see Fig. 4): $I_{t} / I_{c} = 0.90(1 + [ErgTx] / K_d) + 0.1$. The mutation-induced changes in free energy of toxin binding are calculated based on Equation 2: $\Delta G = RT \ln(K_d^{mut}/K_d^{wt})$, where $K_d^{mut}$ and $K_d^{wt}$ are the $K_d$ values of mutant and WT HERG, respectively ($K_d^{mut} = 7.2 ± 1.1$ nM, $n = 7$). Plotted are $\Delta G$ values (means and S.E. bars, $n = 3–7$ each) against channel types along the abscissa. The asterisks denote mutants whose expression level was too low for measuring ErgTx effects (N573C, K595C, P605C, N633C, E637C, and K638C). White bars represent data not different from WT. Gray and black bars represent data different from WT at $p < 0.05$ and $p < 0.01$, respectively. W585C, G590C, and P632C were not suppressed even in the presence of 100 nM ErgTx (estimated $\Delta G > 3$ kcal/mol). Shown at the top is alignment of partial amino acid sequences of Shaker and HERG. The putative amphipathic $\alpha$-helix in the SS-P linker of HERG (583–585) is shown as an insert and boxed. The corresponding sequence along the Shaker is also boxed. Shaded residues in the Shaker sequence are those important for binding of ChTx or analog. Shaded residues in the HERG sequence are those important for ErgTx binding ($\Delta G > 2$ kcal/mol).
K638C). Of the remaining 45 mutants, the ErgTx potency was evaluated in 98 mM [K], using the same voltage clamp protocol as shown in Fig. 4A. One ErgTx concentration (10 nM) was used in all measurements, and the $K_0$ values were calculated using the following modified Equation 2,

$$I_o/I = 0.9/(1 + [\text{ErgTx}]/K_0) + 0.1 \quad \text{(Eq. 2)}$$

The assumption is that the maximal effects in all cases were a 90% suppression (Fig. 4). This is valid because WT HERG has an $IC_{50}$ of 7.2 ± 1.1 nM when estimated using Equation 2 based on data obtained with 10 nM ErgTx, very close to the $IC_{50}$ determined from the complete concentration-response relationship (6.5 ± 1.0 nM, Fig. 4).

The data are summarized in Fig. 5. Most mutants (30 out of 45) showed little or no change in ErgTx binding (changes in binding free energy, $\Delta G$, less than 0.5 kcal/mol). Of the remaining 15 mutants, three in the S5-P linker (W585C, G590C, and I593C), and one in the P-S6 linker (P632C) caused outstanding changes in the binding free energy (>2 kcal/mol). For the remaining 11 mutants, the changes in binding free energy were modest although statistically significant (0.5–1 kcal/mol).

Fig. 5, top, shows a sequence alignment between Shaker and HERG in the outer vestibule region. We compare the positions known to be important for ChTx (or its analog) binding to Shaker or Shaker-like channels (20, 22–24, 30, 31), with those important for ErgTx binding to HERG. There are two differences. First, in the Shaker channel, the positions important for ChTx binding cluster close to the P-loop. In the HERG channel, the positions in the S5-P linker important for ErgTx binding are farther away from the P-loop. Second, charge neutralization in the Shaker channel can have profound effects on ChTx binding (32). However, this is not the case for ErgTx binding to HERG. Therefore, neutralizing positively charge residues in the S5-P linker (R582C, K608C, and K610C) did not enhance ErgTx binding, and neutralizing negatively charged residues here (E575C, D580C, D591C, and D609C) had little or only modest effects. Residues that are critical for ErgTx interaction are all uncharged. Another important point is that the three positions in the S5-P linker (Trp-585, Gly-590, and Ile-593) all fall within the putative amphipathic $\alpha$-helix (boxed in both the HERG sequence and the abscissa of Fig. 5). The implications of these findings will be addressed under “Discussion.”

Do Cysteine Mutations Disrupt ErgTx Binding by Inducing Global Changes in the HERG Outer Vestibule?—Some of the cysteine mutations caused a disruption of the C-type inactivation process and the $K^+$ selectivity of the pore, similar to the phenotype of H587K shown in Fig. 2A. One such example is shown in Fig. 6B. G590C did not cause C-type inactivation or select $K^+$ over $Na^+$ ($E_{rev} = -15$ mV) (left of Fig. 6B). It could not be suppressed by 10 (or even 100) nM ErgTx (Fig. 6B, right). This is in sharp contrast to the behavior of WT HERG, which showed a strong C-type inactivation and $K^+$ selectivity (Fig. 6A, left) and was strongly suppressed by ErgTx (10 nM suppressed current by >50%, Fig. 6A, right). These observations call into question whether the changes in ErgTx binding resulted from global changes in the outer vestibule conformation. Fig. 6 (C and D) suggests that this is not the case. Although D591C could not cause C-type inactivation or select $K^+$ over $Na^+$ ions ($E_{rev} = -20$ mV), it retained a high ErgTx sensitivity (Fig. 6C). On the other hand, Q592C retained C-type inactivation and a high $K^+$ selectivity ($E_{rev} = -100$ mV) but had a significantly lowered ErgTx sensitivity than WT (Fig. 6D).

ErgTx Binds to the Outer Mouth of HERG and Electrostatic Forces Are Involved in Toxin Binding—We suggested above that ErgTx does not plug the HERG channel pore, as is the case for ChTx blockade of the Shaker channel. This, in conjunction with the pattern of positions involved in ErgTx binding to HERG, raises the following question: Does ErgTx suppress HERG current by binding to the outer mouth region and hindering current flow through the pore or by binding away from the pore and modifying other channel function, such as gating? In Shaker or Shaker-like channels, the external TEA binding site overlaps with that of $\alpha$-KTx (16). Therefore, TEA binding and $\alpha$-KTx binding are mutually exclusive. Fig. 7A shows that application of 50 mM TEA ($IC_{50}$, Fig. 2B) reduced the degree of WT HERG current suppression by ErgTx markedly (p < 0.001). This observation supports an outer mouth binding site for ErgTx.

Do electrostatic forces play any role in ErgTx binding to the HERG channel? ErgTx has a pI of 7.88 and carries <2 positive charges at pH 7. Fig. 5 further shows that positions in the HERG channel important for ErgTx binding are all uncharged. Charge neutralization in the neighboring region had modest or no effect on ErgTx binding. To explore whether electrostatic forces play any role in ErgTx/HERG interaction, we altered the net charge on ErgTx by changing the extracellular solution pH from 7.5 to 8.5 or to 6.5 and examined the resulting changes in toxin potency. Fig. 7B shows that, for WT HERG, changing pH, from 7.5 to 8.5 reduced ErgTx potency. This suggests that net negative charges on ErgTx hindered ErgTx/HERG binding. Shifting pH, from 7.5 to 6.5 did not affect ErgTx binding to WT.
HERG. However, removing either of the two histidines in the S5-P linker (H578C or H587C) made ErgTx more potent at pH \(6.5\). This indicates that protonation of these histidine residues in the WT HERG channel negated the increased binding affinity of positively charged ErgTx at pH \(6.5\) (probably by increasing the protonation of H29 of ErgTx, Fig. 1B). On the other hand, putting a permanent positive charge at position 578 (H578K) reduced ErgTx potency at pH \(6.5\) and 7.5 (when ErgTx should be positively charged) but not at pH \(8.5\) (when ErgTx should be negatively charged). This is consistent with an electrostatic repulsion between positive charge at 578 and positive charge on ErgTx at the lower pH range that hindered toxin binding. Therefore, ErgTx-HERG interaction can be influenced by electrostatic forces. However, the degree of influence is much less for ErgTx-HERG binding than for ChTx-Shaker binding (32).

**Voltage Sensitivity of ErgTx Binding to the HERG Channel**—

Binding of ChTx to the Shaker channel is voltage-sensitive: Membrane depolarization destabilizes ChTx binding (16). This voltage sensitivity is exclusively mediated by Lys-27 of ChTx (16). Although ErgTx does not have a Lys-27-equivalent, previous work has shown that depolarization also reduces ErgTx binding to the HERG channel (18). It was suggested that this is due to the C-type inactivation process of the HERG channel at depolarized voltages, which reduces ErgTx binding. We have confirmed this finding (Fig. 8) and further explored the mechanism. Fig. 8B summarizes the data and compares it to the voltage dependence of HERG activation. The steep slope of \(I_{\text{Ca}}/I_{\text{L}}\) data points in the voltage range of \(-40\) to \(0\) mV coincides with the voltage range of a steep increase in channel activation. This suggests that HERG channel activation hinders or destabilizes ErgTx binding. However, stronger depolarization at the plateau of the activation curve (\(>0\) mV) further reduced ErgTx binding; although the slope was less steep than in the negative voltage range. This decrease in ErgTx binding was not related to C-type inactivation, because in a mutant that did not C-type inactivate but maintained a high ErgTx sensitivity (G572C) membrane depolarization still reduced toxin potency in this voltage range (data not shown). The decrease in ErgTx binding could not be due to an increase in \(K^+\) ion efflux, because strong depolarization reduced outward current due to C-type inactivation (Fig. 8A). Finally, this could not be due to effects of voltage on the binding of a charged ErgTx molecule within the transmembrane electrical field. As shown in Fig. 8B, the volt-

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**Fig. 7.** A, effects of an outer mouth blocker, TEA, on ErgTx (10 nm) blockade of WT HERG. \(I_{\text{Ca}}/I_{\text{L}}\) was measured as described in Fig. 4 in 2 mM [K\(_o\)], before and after addition of TEA (50 mM) to the bath solution (with equimolar reduction of [Na\(_o\)], \(n = \) 5 and 4 each). B, effects of changing pH\(_o\), on ErgTx blockade of WT and mutant HERG channels. All oocytes expressing cysteine mutants were treated with dithiothreitol (5 mM, 0.25 h) before recordings. Recordings were conducted in 2 mM [K\(_o\)], [WT and H578C] or in 98 mM [K\(_o\)], [H587C, H578K]. \(I_{\text{Ca}}/I_{\text{L}}\) in ErgTx (10 nm) was measured as described in Fig. 4, and plotted against pH\(_o\), (\(n = \) 3–6 each). In most cases, each oocyte was tested for two or all three pH\(_o\) conditions in a random order. Shown at the top is partial HERG amino acid sequence in the outer vestibule region, with shaded areas highlighting positions important for ErgTx binding (see Fig. 5). His-578 and His-587 are marked.

**Fig. 8.** Voltage dependence of ErgTx blockade of WT HERG at pH\(_o\) \(6.5\) and \(8.5\). A, representative WT HERG current traces recorded at specified pH\(_o\) (in 98 mM [K\(_o\)]) using the protocol shown in the inset. The peak amplitudes of tail currents were used to construct the activation curve and to calculate the fraction of remaining current in the presence of 10 nm ErgTx (\(I_{\text{Ca}}/I_{\text{L}}\)). B, comparison of voltage dependence of WT HERG activation and ErgTx blockade. The relationship between test pulse voltage (\(V\_t\)) and tail current amplitudes (normalized by the peak current amplitudes) is fit with a simple Boltzmann function to estimate the half-maximum activation voltage (\(V_{1/2}\)) and a slope factor (\(k\)), as shown in the following equation: normalized tail = \(1/[1 + \exp((V_{1/2} - V_t)/k)]\). The data are shown as solid symbols based on the right ordinate. Superimposed curves are calculated using the above equation with parameter values: pH \(6.5, V_{1/2} = -13.3 \pm 0.8\) mV, \(k = 8.8 \pm 0.2\) mV (\(n = 4\); pH \(8.5, V_{1/2} = -16.0 \pm 1.1\) mV, \(k = 9.5 \pm 0.4\) mV (\(n = 5\)). Data of \(I_{\text{Ca}}/I_{\text{L}}\) are shown as open symbols based on the left ordinate. The superimposed lines are calculated based on modified Equation 1 (given in Fig. 5 legend) and the following equation: \(K_{\text{f}}(V) = K_{\text{f}(0)} \exp(\delta V/RT)\), where \(\delta\) denotes the ‘effective valence’ of the voltage-sensing domain that affects ErgTx binding. For pH \(6.5, K_{\text{f}(0)} = 9.7\) nm and \(\delta = 0.2\); for pH \(8.5, K_{\text{f}(0)} = 11.5\) nm and \(\delta = 0.36\).
age effect was more prominent at pH 8.5 than at pH 6.5 ("effective valence" of voltage-sensing domain 0.36 and 0.2 at pH 8.5 and 6.5, respectively). This is opposite to the expected effect (e.g. ErgTx should be negatively charged at pH 8.5 and thus membrane depolarization should enhance ErgTx binding).

**DISCUSSION**

Our data suggest that ErgTx binds to the HERG channel with a 1:1 stoichiometry, leading to a suppression of current through the channel pore. Cysteine-scanning mutagenesis experiments suggest that three positions in the S5-P linker (Trp-585, Gly-590, and Ile-593) and one position in the P-S6 linker (Pro-632) are critical determinants of ErgTx binding. Below we compare the behavior of ErgTx binding to the HERG channel with that of ChTx (or an analog) to the Shaker or Shaker-like channels. Based on the comparison and what is known about the mechanism and site of action of ChTx, we further deduce the mechanism by which ErgTx suppresses HERG currents and the possible location of ErgTx receptor site on the HERG channel.

There are some apparent similarities in the behavior of binding of ErgTx and ChTx to their target channels: 1) TEA, an outer mouth blocker, can antagonize toxin binding in both cases. This supports the notion that ErgTx binds to the outer mouth of HERG, and the binding site overlaps with that of TEA. 2) Electrostatic forces are involved in toxin binding in both cases, although to different degrees. In the case of ChTx binding to the Shaker channel, electrostatic forces play a major role in toxin binding and pore blockade. ChTx has a pI value of 9.03 and carries about six positive charges at pH 7. Negative charges around the receptor site help orient the toxin in binding to the receptor (32). Furthermore, the lysine at position 27 of ChTx (Lys-27) binds within the pore and occludes current through the channel (16, 22). The pH experiments shown in Fig. 7B suggest that charge-charge interactions also matter in ErgTx binding to the HERG channel. However, such charge-charge interactions are not a major factor in ErgTx binding to HERG. This is not surprising, because ErgTx has a pI value of 7.88 and carries less than two positive charges at pH 7. Furthermore, ErgTx does not have a positive charge equivalent to Lys-27 in ChTx. 3) Membrane depolarization reduces toxin binding in both cases but likely by different mechanisms. Membrane depolarization destabilizes ChTx binding by two mechanisms, both of which are mediated by Lys-27. First, depolarization enhances K+ ion occupancy inside the pore by promoting K+ ion efflux. This can dislodge ChTx bound to the pore through electrostatic repulsion between K+ ions and Lys-27. Second, Lys-27 of bound ChTx senses 10% suppression of the HERG current, not 100%. Again, this is consistent with the notion that ErgTx is not a "molecular plug" of the HERG pore. This situation is similar to δ-dendrotoxin suppression of Shka1.1: δ-Dendrotoxin does not physically plug the pore but binds in an "off-center" position in the outer vestibule. This leads to a reduction, but not a total occlusion, of current through the pore (33).

What can we conclude about the mechanism by which ErgTx suppresses the HERG current? We can conclude that ErgTx binds to the outer vestibule of HERG, but it probably does not plug the pore with a positive charge as is the case for Lys-27 in ChTx. The maximal effect of ErgTx is ~90% suppression of the HERG current, not 100%. Again, this is consistent with the notion that ErgTx is not a "molecular plug" of the HERG pore. The pattern of positions important for toxin-channel interaction differs between the two (Fig. 5, top). Positions important for ChTx binding to the Shaker channel are those flanking the pore loop, with positions farther away from the pore loop having decreasing importance in influencing toxin binding (22, 23). For ErgTx:HERG, the positions in the S5-P linker important for toxin binding are far away from the pore loop in one-dimensional sequence. Charge mutations in Shaker have marked effects on ChTx binding (31, 32) but little or no effects on ErgTx binding to the HERG channel.

What can we learn about the ErgTx receptor site on the HERG channel? The cysteine-scanning mutagenesis data suggest that S5-P and P-S6 linkers are both involved. Although these two domains are not contiguous in one-dimensional sequence, in three-dimensional space, the long (43 amino acids) S5-P linker of HERG may come close to the P-S6 linker and thus to the channel pore. Our working hypothesis is illustrated by the schematic in Fig. 9A. An analysis of possible secondary structures in the S5-P linker using the program, Protein, in LaserGene (34) suggests that positions 583–594 may form an amphipathic α-helix. The helical wheel plot in Fig. 9B shows that hydrophobic residues is this region cluster to one face of the α-helix, among which Trp-585, Gly-590, and Ile-593 may form contact points with bound ErgTx. The other face of the α-helix may involve hydrophilic residues. This face of the α-helix is not involved in ErgTx binding, because neutralizing the negative charge here, D591C, has no effects on toxin binding (Figs. 5 and 6).

In summary, ErgTx binds to the HERG channel contributes importantly to the outer mouth properties of this channel, supporting the conclusion from a previous report (19). We propose that this linker can engage in intimate interactions with the pore’s entryway and participates in conformational changes important for the C-type inactivation.
process and for K:Na selectivity of the pore. Future work will be focused on identifying toxin and channel residues interacting across the toxin-channel interface. This, in conjunction with ErgTx’s solution structure obtained by the NMR technique, will yield a three-dimensional structure of the outer vestibule of the HERG channel.

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