Mapping the Interaction Site for a \(\beta\)-Scorpion Toxin in the Pore Module of Domain III of Voltage-gated Na\(^+\) Channels*

Joel Z. Zhang‡, Vladimir Yarov-Yarovoy†1, Todd Scheuer‡, Izhar Karbat†, Lior Cohen§, Dalia Gordon†, Michael Gurevitz‡, and William A. Catterall‡2

From the ‡Department of Pharmacology, University of Washington, Seattle, Washington 98195-7280 and the §Department of Molecular Biology and Ecology of Plants, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel

Background: \(\beta\)-Scorpion toxins enhance activation of voltage-gated sodium (Na\(_v\)) channels.

Results: Four amino acid residues in the IIIS2-S6 extracellular loop contribute to toxin binding and efficacy.

Conclusion: The pore module of domain III and the voltage-sensing module of domain II form the receptor site.

Significance: Scorpion toxins make a three-point interaction with Na\(_v\) channels and alter voltage sensor function.

Activation of voltage-gated sodium (Na\(_v\)) channels initiates and propagates action potentials in electrically excitable cells. \(\beta\)-Scorpion toxins, including toxin IV from Centruroides suffusus suffusus (CssIV), enhance activation of Na\(_v\) channels. CssIV stabilizes the voltage sensor in domain II in its activated state via a voltage-sensor trapping mechanism. Amino acid residues required for the action of CssIV have been identified in the S1-S2 and S3-S4 extracellular loops of domain II. The extracellular loops of domain III are also involved in toxin action, but individual amino acid residues have not been identified. We used site-directed mutagenesis and voltage clamp recording to investigate amino acid residues of domain III that are involved in CssIV action. In the IIIS2-S6 loop, five substitutions at four positions altered voltage-sensor trapping by CssIV\(^{1\,\text{E15A}}\). Three substitutions (E1438A, D1445A, and D1445Y) markedly decreased voltage-sensor trapping, whereas the other two substitutions (N1436G and L1439A) increased voltage-sensor trapping. These bidirectional effects suggest that residues in IIIS2-S6 make both positive and negative interactions with CssIV. N1436G enhanced voltage-sensor trapping via increased binding affinity to the resting state, whereas L1439A increased voltage-sensor trapping efficacy. Based on these results, a three-dimensional model of the toxin-channel interaction was developed using the Rosetta modeling method. These data provide additional molecular insight into the voltage-sensor trapping mechanism of toxin action and define a three-point interaction site for \(\beta\)-scorpion toxins on Na\(_v\) channels. Binding of \(\alpha\)- and \(\beta\)-scorpion toxins to two distinct, pseudo-symmetrically organized receptor sites on Na\(_v\) channels acts synergistically to modify channel gating and paralyze prey.

Voltage-gated sodium (Na\(_v\)) channels initiate action potentials in nerve and muscle (1). They activate and then inactivate on the millisecond time scale to generate a pulse of inward sodium current that underlies the rising phase of the action potential. The \(\alpha\) subunit of Na\(_v\) channels has four homologous domains (I–IV), and each domain is composed of six transmembrane segments, S1–S6 (2). The first four segments, S1–S4, form the voltage-sensing module, and 4–8 positively charged arginine or lysine residues at every third position in the S4 segment serve as gating charges (2). The S5–S6 segments form the pore module, and the short SS1 and SS2 segments between them form the vestibule and narrow selectivity filter at the extracellular end of the pore (2). The three-dimensional arrangement of these segments has been defined by x-ray crystallography of mammalian K\(_{1.2}\) channels (3), a K\(_{1.2}\)-K\(_{2.1}\) chimera (4), and the bacterial Na\(_v\)_channel, Na\(_v\)_Ab (5).

Several classes of neurotoxins act at distinct receptor sites on Na\(_v\) channels (6, 7). The \(\alpha\)-scorpion toxins bind at neurotoxin receptor site 3 and slow Na\(_v\) inactivation, whereas the \(\beta\)-scorpion toxins bind at neurotoxin receptor site 4 and enhance activation (7). These effects work together to depolarize nerve and muscle fibers and cause conduction block. \(\beta\)-Scorpion toxins are thought to act via a voltage-sensor trapping mechanism in which the bound toxin binds and stabilizes the voltage sensor in domain II in its activated conformation (8, 9). Toxin derivatives can act as agonists or competitive antagonists of voltage-sensor trapping (10). Previous ligand binding (8, 9) and electrophysiological studies (11) defined a molecular map of the receptor site for \(\beta\)-scorpion toxin IV of Centruroides suffusus suffusus (CssIV)\(^3\) in the S1-S2 (IIIS1-S2) and S3-S4 (IIIS3-S4) extracellular loops of the voltage-sensing module in domain II. Structural studies of homotetrameric Na\(_v\)_ and K\(_v\)_ channels show that the S5–S6 loop in each subunit is in close proximity to the S1-S2 and S3-S4 loops of the adjacent subunit (3–5). Therefore, the conformational change of the voltage-sensing module of one subunit in response to depolarization can be transmitted to the pore module of the neighboring subunit in clockwise direction

† Present address: Dept. of Physiology and Membrane Biology, University of California, Davis, CA 95616.
‡ To whom correspondence should be addressed. E-mail: wcatt@uw.edu.

* This work was supported, in whole or in part, by National Institutes of Health Research Grants U01 NS058639 (to W. A. C. and M. G.) and R01 NS15751 (to W. A. C.), and a grant from the Binational Agricultural Research and Development Fund of the United States and Israel (to M. G. and W. A. C.).

1 The abbreviations used are: CssIV, IV of Centruroides suffusus suffusus; \(\nu_{\text{SSP}}\), voltage-sensor trapping current.
to open the central ion conduction pore. Analysis of channel chimeras showed that the IIIS2-S6 region is important in binding of β-scorpion toxins (8) and in determining the specificity for binding of a β-scorpion toxin to individual Na$_v$ channel subtypes (12). These results led us to hypothesize that the single receptor site for the β-scorpion toxin CssIV in Na$_v$ channels (8) is formed by IIIS1-S2, IIIS3-S4, and IIIS2-S6 extracellular loops. In the present work, we mapped the individual amino acid residues that contribute to the receptor site for the β-scorpion toxin derivative CssIV$^{E15A}$. Five substitutions at four positions in the region between Asn$^{1436}$ and Asp$^{1445}$ altered toxin binding and/or efficacy for voltage-sensor trapping. Three of them markedly decreased voltage-sensor trapping, whereas two increased voltage-sensor trapping. These bidirectional effects suggest that residues in IIIS2-S6 make both positive and negative interactions with bound toxin. Our data show that IIIS3-S4 plays a primary role, whereas IIIS1-S2 and IIIS2-S6 play important secondary roles, in determining binding affinity and efficacy at a single β-scorpion toxin receptor site. They also indicate that the SS2-S6 loop in domain III is in close proximity to the voltage-sensing module of domain II in mammalian Na$_v$ channels, as suggested by the structure of bacterial Na$_v$ channels (5).

**EXPERIMENTAL PROCEDURES**

*Scorpion Toxin*—A high-affinity mutant derivative of toxin IV from *C. suffusus suffusus*, CssIV$^{E15A}$ (11), was used for all experiments. It was prepared as described previously (11).

*Mutagenesis*—PCR-based site-directed mutagenesis was employed to generate single point mutations in WT rNa$_v$.2 cDNA (9, 11). Alanine-scanning mutagenesis was performed on the majority of the residues in IIIS2-S6 region. Chimeras were constructed for residues that are not conserved between β-scorpion toxin-sensitive rNa$_v$.2 and β-scorpion toxin-resistant hNa$_v$.1.5 channels. For some charged residues, charge-reversal mutants were also generated to explore the role of charged amino acid side chains in voltage-sensor trapping of the β-scorpion toxin. All the WT and mutant cDNAs were subcloned into the pCDM8 vector (13).

*cDNA Transfection*—Detailed cDNA transfection procedures have been described elsewhere (9, 11). cDNAs encoding WT or mutant rNa$_v$.2a channels were co-transfected into tsA-201 cells with the marker protein pEBO-pCD8-leu2 using the calcium phosphate method. Transfected cells were subcloned 12–18 h after transfection, and electrophysiological recordings were carried out 1–24 h later. WT and mutant rNa$_v$.2a cDNAs were always transfected in parallel to ensure that differences from WT were truly caused by mutations. Polystyrene microspheres conjugated with anti-CD8 antibody were added in the extracellular recording solution to identify the cell surface CD8 protein as a marker of cells expressing Na$_v$.2 channels.

*Electrophysiological Recording and Data Analysis*—The whole cell patch clamp configuration was utilized to record Na$^+$ current. The extracellular recording solution contained (in mM) NaCl (150), Cs-HEPES (10), MgCl$_2$ (1), KCl (2), CaCl$_2$ (1.5), and 0.1% BSA, pH 7.4. The intracellular recording solution contained (in mM) NaCl (150), Cs-HEPES (10), MgCl$_2$ (10), NaCl (10), and EGTA (5), pH 7.4. Linear leak and capacitance currents were subtracted using an online P/-4 subtraction paradigm. To assess the extent of negative shift of the voltage dependence of activation caused by CssIV$^{E15A}$, tsA-201 cells were held at −100 mV and test depolarizations were applied to potentials from −100 to +20 mV in 5-mV increments. Current-voltage (I-V) plots were generated from peak currents elicited at each test potential. The test depolarization was either applied alone or was preceded by a 1-ms prepulse to +50 mV followed by a 60-ms interval at the holding potential. We tested the functional properties of each mutant rNa$_v$.2a construct in the absence of toxin to examine the effect of the mutant residue, followed by recordings in the presence of CssIV$^{E15A}$. The voltage dependence and kinetics of each mutant channel were initially screened with 500 nM CssIV$^{E15A}$ to detect differences from WT. All data were analyzed with Igor Pro (WaveMetrics, Lake Oswego, OR). Normalized I-V curves were fit with a function including a one-component Boltzmann equation of the form $(V - V_{\text{rev}}) \times G_{\text{max}}/(1 + \exp[(V_{\text{1/2}} - V)/k])$, where $V_{\text{1/2}}$ is the half-activation voltage in mV, and $k$ is a slope factor in mV. In the presence of both CssIV$^{E15A}$ and a prepulse, I-V plots were fit with a two-component Boltzmann equation. All data are presented as mean ± S.E. The mean values for voltage dependence of activation and inactivation for all mutants are presented in Tables 1 and 2.

*Structural Modeling of the CssIV-Na$_v$.2 Complex*—Homology and de novo modeling of the voltage-sensing module of domain II and pore-forming module of domain III of the rat Na$_v$.1.2 channel was performed using the Rosetta-membrane method (14–16). The voltage-sensing module (residues Val$^{148}$–Gly$^{579}$) and pore-forming module (residues Ser$^{1336}$–Phe$^{1476}$) from Na$_v$.1.2 were aligned with the voltage-sensing module (residues Met$^{1}$–Pro$^{128}$) and pore-forming module (residues Gly$^{129}$–Met$^{221}$) from the NavAb channel (5), respectively, using ClustalX software (17). 5,000 models were generated in the first round of modeling using cyclic coordinate descent loop modeling (18) in the Rosetta-membrane method followed by model clustering (19). The 20 largest clusters of models from the first round of modeling were then used as input for the second round of modeling using the kinematic loop modeling protocol (20) in the Rosetta-membrane method followed by model clustering (19). The centers of the largest clusters of models and the lowest energy models were visually examined, and the best Na$_v$.1.2 model was chosen based on a fit with available experimental data on key residues contributing to interaction between the β-scorpion CssIV toxin and the Na$_v$.1.2 channel (8, 9, 11, 21). Modeling of the β-scorpion toxin was performed using the Rosetta method for modeling of soluble proteins (22) and the structure of neurotoxin 2 (X8, PDB 1JA2) using ClustalX software (17). The β-scorpion CssIV toxin model was then docked with the best Na$_v$.1.2 model in the same starting orientation as in a recently published model of β-scorpion CssIV complex with the Na$_v$.1.2 voltage-sensing module of domain II (11). The docked model of β-scorpion CssIV toxin bound to the voltage-sensing module of domain II and pore-forming module of domain III of Na$_v$.1.2 was then subjected to Rosetta full atom relax protocol (22) using 1000 independent simulations and the lowest energy model was chosen as the best model.
RESULTS

Modification of Voltage-dependent Activation of WT rNav1.2a Channels by CssIVE15A—As in our previous work (11), we used the toxin mutant CssIVE15A, which has increased affinity but an identical mechanism of action compared with CssIV itself. We measured voltage-sensor trapping using a three-pulse protocol in the whole cell patch clamp configuration. Following equilibration of the toxin with its receptor sites, cells were depolarized to 50 mV for 1 ms to activate the voltage sensor and allow voltage-sensor trapping, repolarized to the holding potential of −100 mV for 60 ms to allow recovery of the channels from fast inactivation, and finally depolarized to a range of test pulse potentials to record Na+ current (Fig. 1A). As reported previously (11), CssIVE15A shifted the voltage dependence of activation of WT rNav1.2a channels to more negative membrane potentials, but only following the strong depolarizing prepulse (11). In the presence of 500 nM CssIVE15A but with no prepulse, WT rNav1.2a channels did not conduct Na+ current at a test potential of −60 mV (Fig. 1B). In contrast, when a 1-ms prepulse to 50 mV was applied 60 ms before each test pulse, marked Na+ current was observed at −60 mV (Fig. 1B), and a substantial component of the Na+ current was activated with a negatively shifted voltage dependence (Fig. 1C). With 500 nM CssIVE15A, a mean of 16.2% of the peak Na+ current was activated at −60 mV (Fig. 1, B and C, and Table 3). The additional current generated by depolarization to −60 mV following a strong, short-duration prepulse was termed “I_VST,” for voltage-sensor trapping current (11). Fitting a function with two Boltzmann components to the +Pre I-V curve (Fig. 1C) yielded the following fitting parameters: V_{a1} = −34.2 ± 0.9 mV, k_{1} = −6.4 ± 0.2 mV, V_{a2} = −76.1 ± 1.3 mV, k_{2} = −6.4 ± 0.4 mV. In this equation, V_{a1} and k_{1} are the voltage of half-maximal activation and the slope factor of the first Boltzmann component, respectively, and V_{a2} and k_{2} are the voltage of half-maximal activation and the slope factor of the second, more negatively shifted component, respectively (Fig. 1C). The negatively shifted component represented 9.4 ± 0.02% of the total conductance of the + Pre curve. Fitting the concentration-response curve (I_VST versus toxin concentration) with a first-order Hill equation resulted in an EC_{50} of 1100 nM for toxin binding to WT rNav1.2a channels in the resting state (Fig. 1D). These data suggest that CssIVE15A binds to WT rNav1.2a channels in the resting state and, in response to strong, transient depolarization, traps the voltage sensor in its activated state. In the following sections, we first describe the functional properties of several informative mutants, followed by presentation of a twodimensional molecular map and three-dimensional model of the toxin-receptor interaction in the IIIIS2-S6 region.

Loss of Voltage-sensor Trapping by CssIVE15A with NaV1.2a Mutants E1438A, D1445A, and D1445Y—In the absence of toxin, mutation E1438A in NaV1.2a channels did not affect the I-V relationship compared with WT (Fig. 2A, left, Table 1). These results indicate that this mutation does not alter the voltage-dependent activation of Na+ channels. As for WT channels, 500 nM CssIVE15A did not induce detectable Na+ current when cells expressing E1438A channels were depolarized to −60 mV without a preceding depolarizing prepulse (Fig. 2A, right, Table 2). However, the I-V relationship of NaV1.2a/E1438A was altered only slightly by a 1-ms depolarizing prepulse to 50 mV in the presence of CssIVE15A (Fig. 2A, right), and I_VST was only 3.67 ± 0.54% at 500 nM (Table 3). These results show that CssIVE15A had reduced voltage-sensor trapping activity on the NaV1.2a/E1438A mutant channel. This lack of effect on NaV1.2a/E1438A could be caused by decreased binding affinity to the resting state, decreased voltage-sensor trapping efficacy, or a combination of both effects.

The mutations NaV1.2a/D1445A and NaV1.2a/D1445Y also impaired voltage-sensor trapping by CssIVE15A (Fig. 2, B and C, Table 3). Neither NaV1.2a/D1445A nor NaV1.2a/D1445Y altered the I-V relationships in the absence of toxin (Fig. 2, B and C, left, Table 1). These results indicate that neither mutation altered the voltage dependence of activation of NaV channels. For NaV1.2a/D1445A, I_VST was decreased to 4.8 ± 0.7% at 500 nM CssIVE15A compared with 16.2 ± 2.6% for WT. Interestingly, a single residue chimera between β-toxin-sensitive rNav1.2 and β-toxin-insensitive hNav1.5 at the same locus, D1445Y, completely abolished voltage-sensor trapping by CssIVE15A at 500 nM (Fig. 2C, right, Table 3). These results suggest that Asp1445 is crucial for modification of voltage dependence of activation of NaV channels by CssIVE15A. The mutations NaV1.2a/D1445A and NaV1.2a/D1445Y might reduce voltage-sensor trapping activity by CssIVE15A via reduced toxin binding affinity for the resting state, decreased voltage-sensor trapping efficacy, or a combination of both effects.

Increased Voltage-sensor Trapping with NaV1.2a Mutants N1436G and L1439A—In our previous studies, we identified three consecutive mutations in the IIS3-S4 extracellular linker

FIGURE 1. Voltage-sensor trapping activity of CssIVE15A on WT rNav1.2a channels. The cell membrane was depolarized to a series of potentials ranging from −100 to +20 mV in 5-mV increments with or without a +50 mV, 1-ms conditioning prepulse. When used, the prepulse was applied 60 ms earlier than the I-V pulse protocols. A, pulse protocol with a +50 mV, 1-ms prepulse. B, voltage-sensor trapping current, I_VST, in response to 15-ms test pulse depolarizations to −60 mV. The traces were acquired with (+Pre) or without (−Pre) the prepulse. C, I-V plots obtained for WT rNav1.2a channels with (filled circles, + Pre) or without (open circles, − Pre) the prepulse. The solid lines are global fits of a function with 2 Boltzmann components to the I-V curves without and with prepulses. D, I_VST concentration plot with the prepulse (open circles). I_VST was normalized to the maximal peak current of the I-V plot in the presence of the prepulse. I_VST concentration data were fit with first-order Hill equations (n ≥ 4). Error bars represent S.E.
that markedly enhanced voltage-sensor trapping by CςIV^{E15A} (11). This enhancement resulted from substantially increased binding affinity of CςIV^{E15A} to the resting state of the mutant channels (11). In our present study of the IIISS2-S6 region, we identified two mutations (N1436G and L1439A) that greatly enhance voltage-sensor trapping by CςIV^{E15A}.

Mutation NaV1.2a/N1436G did not affect the I-V relationship compared with WT in the absence of toxin, indicating that voltage-dependent activation of the mutant channel remained unchanged (Fig. 3A, Table 1). Similarly, in the presence of 500 nM CςIV^{E15A} but with no prepulse, the voltage-dependent activation of mutant NaV1.2a/N1436G was no different from WT (Fig. 3B, Table 2). No $I_{VST}$ was detectable at $-60 \text{ mV}$ without the prepulse (Fig. 3B, inset). However, following a 1-ms prepulse to 50 mV in the presence of 500 nM CςIV^{E15A}, $I_{VST}$ increased to $38.2 \pm 3.8\%$ (Fig. 3B), which was 2.4-fold greater than WT. Fitting the concentration-response curve for this mutant with a first-order Hill equation yielded an efficacy for voltage-sensor trapping by bound CςIV that was similar to WT, but an EC$_{50}$ that was lower than WT (Fig. 3E, top). Re-plotting these data in a Scatchard-like plot reveals the decrease in EC$_{50}$ for voltage-sensor trapping by Na$_{\alpha}$.1.2/N1436G as an increase in slope (Fig. 3E, bottom). In our voltage-sensor trapping protocol, we must repolarize to $-100 \text{ mV}$ to allow for

**TABLE 1**

Voltage dependence of activation

The current-voltage relationship of WT and mutant channels was measured as described under “Experimental Procedures” under control conditions without toxin. The voltage of half-activation ($V_{0.5}$) and slope factor of each channel were derived from fitting the corresponding voltage-dependent activation curve with a single Boltzmann equation. Data are presented as mean ± S.E.

| Channel | $V_{0.5}$ | Slope | $n$ |
|---------|-----------|--------|-----|
| WT      | $-30.8 \pm 0.8$ | $-5.4 \pm 0.3$ | 7   |
| N1436G  | $-31.5 \pm 0.7$ | $-5.1 \pm 0.2$ | 5   |
| E1438A  | $-33.9 \pm 1.0$ | $-4.8 \pm 0.2$ | 5   |
| L1439A  | $-29.2 \pm 0.9$ | $-6.1 \pm 0.1$ | 5   |
| D1445A  | $-32.5 \pm 0.4$ | $-4.9 \pm 0.3$ | 5   |
| D1445Y  | $-32.4 \pm 1.1$ | $-4.8 \pm 0.2$ | 5   |

**TABLE 2**

Voltage dependence of activation with CςIV^{E15A}

The voltage dependence of activation was measured as described under “Experimental Procedures” in the presence of 500 nM CςIV^{E15A} but without the prepulse. The voltage of half-activation ($V_{0.5}$) and slope factor of each channel were derived from fitting the corresponding voltage-dependent activation curve with a single Boltzmann equation. Data are presented as mean ± S.E.

| Channel | $V_{0.5}$ | Slope | $n$ |
|---------|-----------|--------|-----|
| WT      | $-30.5 \pm 0.5$ | $-6.0 \pm 0.1$ | 33  |
| N1436G  | $-29.1 \pm 1.3$ | $-5.4 \pm 0.4$ | 7   |
| E1438A  | $-30.3 \pm 0.8$ | $-5.7 \pm 0.2$ | 7   |
| L1439A  | $-32.3 \pm 2.4$ | $-6.5 \pm 0.3$ | 5   |
| D1445A  | $-29.4 \pm 0.9$ | $-5.1 \pm 0.3$ | 4   |
| D1445Y  | $-32.0 \pm 1.0$ | $-5.1 \pm 0.3$ | 5   |
recovery from fast inactivation after the prepulse to 50 mV induces voltage-sensor trapping (Fig. 1A). Inevitably, there is a partial reversal of voltage-sensor trapping during this repolarization. Correction for this loss of \( \text{VST} \) during the 60-ms repolarization in our voltage-sensor trapping protocol further illustrates the shift of the EC_{50} for voltage-sensor trapping by Na\(_{\alpha,1.2a}/\text{N1436G} \) to lower concentrations in both concentration-response curve and Scatchard-like formats (Fig. 3F). These data suggest that the Na\(_{\alpha,1.2a}/\text{N1436G} \) mutation enhances voltage-sensor trapping by CsslIV\(_{E15A} \) by increasing toxin binding affinity to Na\(_{\alpha,1.2a}/\text{N1436G} \) channels in the resting state. The values for EC_{50} derived from these results obtained with preincubation of toxin at \(-100 \text{ mV} \) are essentially equivalent to the \( K_d \) for binding of CsslIV\(_{E15A} \) to the resting state of the voltage sensor, according to the biophysical model of voltage-sensor trapping developed previously (11).

As we observed for Na\(_{\alpha,1.2a}/\text{N1436G} \), the voltage dependence of activation of the L1439A mutant was similar to WT in the absence of CsslIV\(_{E15A} \) (Fig. 3C, Table 1). In the presence of 500 nM CsslIV\(_{E15A} \) but with no prepulse, the voltage-dependent activation of L1439A was not altered either (Fig. 3D, Table 2). However, following a 1-ms prepulse to 50 mV in the presence of 500 nM CsslIV\(_{E15A} \), \( \text{VST} \) was increased to 31.3 ± 6.6% (Fig. 3D, Table 3). A 15-fold greater than WT. Fitting the concentration-response curve for this mutant with a first-order Hill equation revealed greater efficacy than for WT (Fig. 3E). On the other hand, the EC_{50} was similar to WT after correction for loss of \( \text{VST} \) during the 60-ms repolarization step in our stimulus protocol (Fig. 3F). These data suggest that the L1439A mutation enhanced voltage-sensor trapping by CsslIV\(_{E15A} \) in a different manner than for Na\(_{\alpha,1.2a}/\text{N1436G} \), increasing the efficacy of voltage-sensor trapping by bound CsslIV\(_{E15A} \) rather than increasing binding affinity to resting Na\(_{\alpha} \) channels. The effect of the L1439A mutation on efficacy was diminished when the

### TABLE 3

| Channel | Concentration (nM) | \( \text{VST} \) (+Pre) | \( n \) | EC_{50} (mV) |
|---------|-------------------|-----------------|-----|------------|
| WT      | 50                | 3.2 ± 1.7%      | 3   | 1100       |
|         | 100               | 5.0 ± 0.9%      | 8   |            |
|         | 200               | 7.9 ± 1.0%      | 6   |            |
|         | 500               | 16.2 ± 2.6%     | 5   |            |
|         | 1,000             | 28.0 ± 4.3%     | 4   |            |
|         | 2,000             | 49.8 ± 10.0%    | 5   |            |
|         | 5,000             | 48.9 ± 3.9%     | 10  |            |
| N1436G  | 500               | 54.2 ± 5.5%     | 3   |            |
|         | 1,000             | 42.8 ± 3.8%     | 7   |            |
|         | 2,000             | 58.2 ± 3.8%     | 12  |            |
| E1438A  | 500               | 40.7 ± 0.5%     | NA^a|            |
| L1439A  | 500               | 6.6 ± 1.8%      | 7   | 1017.5     |
|         | 200               | 17.4 ± 2.1%     | 5   |            |
|         | 500               | 31.3 ± 6.6%     | 5   |            |
|         | 1,000             | 38.8 ± 4.6%     | 8   |            |
|         | 2,000             | 59.2 ± 3.9%     | 4   |            |
|         | 5,000             | 71.6 ± 6.7%     | 4   |            |
| D1445A  | 500               | 4.8 ± 0.7%      | 4   | NA         |
|         | 1,000             | 5.0 ± 0.8%      | NA  |            |
| D1445Y  | 500               | 0.4 ± 0.3%      | 5   | NA         |

*a NA, not applicable.

### FIGURE 3

**Voltage-sensor trapping activity of CsslIV\(_{E15A} \) on N1436G and L1439A mutant Na\(_{\alpha,1.2a} \) channels.** A, normalized I-V plots obtained in the absence of toxin for WT (open circles) and N1436G mutant channels (open triangles). B, normalized I-V plots for N1436G mutant channels in the presence of 500 nM CsslIV\(_{E15A} \) with (filled triangles, +Pre) or without (open triangles, −Pre) a ±50 mV, 1-ms prepulse. Inset, \( \text{VST} \) traces recorded in the presence of 500 nM CsslIV\(_{E15A} \) with a 15-ms test pulse to −60 mV in the absence (open triangle) or presence (filled triangle) of the prepulse. The solid lines are global fits to I-V curves with and without a prepulse with parameters \( \text{V}_{\text{p}} = -29.6 \text{ mV}, k_1 = -5.5 \text{ mV}, k_2 = -56.5 \text{ mV}, k_3 = -9.6 \text{ mV} \). 0% conductance was activated with the more negative voltage dependence without the prepulse, whereas 56.4% of the total conductance was activated with the more negative voltage dependence with the prepulse. C, I-V plots obtained in the absence of toxin for WT Na\(_{\alpha,1.2a} \) channels (circles) and L1439A mutant channels (squares). D, I-V plots for L1439A mutant channels in the presence of 500 nM CsslIV\(_{E15A} \) with (filled squares, +Pre) or without (open squares, −Pre) the prepulse. Inset, \( \text{VST} \) traces recorded in the presence of 500 nM CsslIV\(_{E15A} \) with a 15-ms depolarizing test pulse to −60 mV in the absence (open square) or presence (filled squares) of a ±50-mV, 1-ms prepulse 60 ms earlier. The solid lines are global fits to I-V curves with and without prepulses with parameters \( \text{V}_{\text{p}} = -32.6 \text{ mV}, k_1 = -6.4 \text{ mV}, k_2 = -70.2 \text{ mV}, k_3 = -10.3 \text{ mV} \). 0% conductance was activated with the more negative voltage dependence without the prepulse, whereas 24.5% of the total conductance was activated with the more negative voltage dependence with the prepulse. E, top, concentration-response curves for normalized \( \text{VST} \) of WT, N1436G (open triangles), and L1439A (open squares) mutant channels induced by CsslIV\(_{E15A} \). Bottom, the same results are re-plotted as a Scatchard-like plot, in which the slope is proportional to \( K_d \) and the intercept is maximum at \( I_{\text{VST}} \). Note the reduced apparent \( K_d \) (i.e., increased slope) for N1436G compared with the increased in maximum \( I_{\text{VST}} \) for L1439A. F, the results from panel E were re-plotted after correction for the loss of \( \text{VST} \) during the 60-ms repolarization to −100 mV.
results were corrected for loss of $I_{\text{VST}}$ during repolarization (compare Fig. 3, E and F). This comparison suggests that the primary effect of the L1439A mutation is to slow the reversal of voltage-sensor trapping upon repolarization, and this effect is compensated for by the correction for loss of voltage-sensor trapping upon repolarization to $-100 \text{ mV}$ applied in Fig. 3F. This mechanism derives further support from kinetic analysis of the rates of onset and reversal of voltage-sensor trapping below.

Differences in the efficacy of voltage-sensor trapping by CssIV$^{E15A}$ for mutants Na$_v$1.2a/N1436G and Na$_v$1.2a/L1429A might arise from differences in the rate of onset of voltage-sensor trapping at the prepulse potential and/or in the rate of reversal of voltage-sensor trapping upon repolarization. To measure the rate of onset of voltage-sensor trapping by CssIV$^{E15A}$, we varied the duration of the prepulse to $50 \text{ mV}$ from 0 to 5 ms, followed by a 60-ms interval of repolarization at the holding potential and a 15-ms test pulse to $-60 \text{ mV}$ (Fig. 4A, inset) and recorded the increase in $I_{\text{VST}}$ with increasing prepulse duration (Fig. 4A). These values for $I_{\text{VST}}$ after prepulses of increasing duration were normalized to the maximal peak current of a preceding I-V plot measured with no prepulse (Fig. 4B). In this experimental protocol, $I_{\text{VST}}$ increased with prepulse duration and reached a maximal effect at $\sim 1 \text{ ms}$ for Na$_v$1.2a/L1439A and $\sim 4 \text{ ms}$ for Na$_v$1.2a/N1436G (Fig. 4B).

The extent of voltage-sensor trapping also increased with toxin concentration for both mutants as expected for a bimolecular binding reaction between CssIV and its receptor site in the resting state of Na$^+$ channels (Fig. 4C, top). The increase was greatest for the N1436G mutant because of its higher toxin-binding affinity. The rate constants for onset of voltage-sensor trapping did not increase with toxin concentration (Fig. 4C, bottom). These results are consistent with our three-step model for voltage-sensor trapping in which the final trapping step is concentration independent (8, 9, 11). However, the rate constant for the onset of voltage-sensor trapping decreased significantly with toxin concentration for N1436G, consistent with slowed activation of toxin-bound channels (see “Discussion”).

To measure the rate of reversal of voltage-sensor trapping, we depolarized the cell to $50 \text{ mV}$ for 1 ms, followed by repolarization to the resting membrane potential for 0–3,000 ms, and depolarization to $-60 \text{ mV}$ for 15 ms to measure $I_{\text{VST}}$ (Fig. 4D, inset), and we measured the peak values for $I_{\text{VST}}$ during repetitive application of this pulse protocol with increasing repolarization times (Fig. 4D). These values for $I_{\text{VST}}$ were normalized to the maximal peak current from a preceding I-V plot with no prepulse. The rate of reversal of voltage-sensor trapping at $-100 \text{ mV}$ for Na$_v$1.2a/N1436G was comparable with WT (Fig. 4, E and F, bottom). In contrast, the rate of reversal of voltage-sensor trapping at $-100 \text{ mV}$ was substantially decelerated for Na$_v$1.2a/L1439A (Fig. 4, E, and F, bottom). On the other hand, the extent of voltage-sensor trapping was greatest for N1436G because of its higher toxin-binding affinity (Fig. 4F, top). The striking differences in the kinetics of onset and reversal of voltage-sensor trapping for N1436G and L1439A mutants are considered further under “Discussion.”

FIGURE 4. Rates of the development and recovery of voltage-sensor trapping by CssIV$^{E15A}$ on N1436G and L1439A mutant channels. To measure the rates of the development of voltage-sensor trapping (A–C), the cell membrane was depolarized to $+50 \text{ mV}$ for durations that varied from 0 to 5 ms, followed by repolarization to the holding potential for 20–3000 ms and by a test pulse to $-60 \text{ mV}$ (A, upper panel inset). $I_{\text{VST}}$ traces at $-60 \text{ mV}$ for N1436G (upper panel) and L1439A (lower panel) mutant channels. Some traces were omitted for clarity. B, plots of normalized $I_{\text{VST}}$ versus prepulse duration for WT Na$_v$1.2a (circles), N1436G (squares), and L1439A (triangles) channels. C, magnitude of $k_{\text{on}}$ (upper panel) and the time constants (lower panel) of the development of voltage-sensor trapping by CssIV$^{E15A}$ in WT Na$_v$1.2a, N1436G, and L1439A channels at a series of CssIV$^{E15A}$ concentrations. To measure the rates of recovery from voltage-sensor trapping (D–F), the cell membrane was depolarized to $-50 \text{ mV}$ for 1 ms followed by a repolarization to the resting potential for 20–3000 ms and by a test pulse to $-60 \text{ mV}$ for 15 ms (D, inset). D, $I_{\text{VST}}$ traces for N1436G (upper panel) and L1439A mutant channels (lower panel). E, plots of peak $I_{\text{VST}}$ in D versus recovery time for WT Na$_v$1.2a (open circles), N1436G (open squares), and L1439A (open triangles) channels. F, intercept extrapolated to $t=0$ (upper panel) and time constants (lower panel) of the recovery of the voltage-sensor trapping by CssIV$^{E15A}$ in WT Na$_v$1.2a, N1436G and L1439A mutant channels.

A Molecular Map of the $\beta$-Scorpion Toxin Receptor Site in the I1152-S6 Loop—Our previous studies of Na$_v$1.2a channel chimeras implicated the extracellular I1152-S2 and I1153-S4 loops of Na$_v$ channels in formation of the receptor site for $\beta$-scorpion toxins and defined several amino acid residues in the I1152-S2 and I1153-S4 linkers that contribute to the receptor site for $\beta$-scorpion toxin CssIV$^{E15}$ (8, 9, 11). To complete the mapping of the receptor site for $\beta$-scorpion toxin on Na$_v$ channels, we
constructed 32 mutants by substitutions for 25 residues in IIISS2-S6. Unfortunately, 11 of those mutants did not conduct detectable Na\(^{+}\)/H\(^{+}\) current when expressed in tsA-201 cells. A linear map of the functional effects of the remaining 21 mutants is illustrated in Fig. 5 in terms of the \(I_{\text{VST}}\) ratio (mutant/WT). This partial scan of amino acid residues in IIISS2-S6 revealed several positions of interest (Fig. 5). Four residues in the IIIS2-S6 loop (Asn1436, Glu1438, Leu1439, and Asp1445) are important for CssIV voltage-sensor trapping, but they are not as closely co-localized in the linear amino acid sequence as the hot spot of consecutive residues observed in IIS3-S4 (11). Mutations of these amino acid residues can either increase or decrease voltage-sensor trapping by CssIV\(^{E15A}\). These results uncover a substantial role for single amino acid residues in the IIIS2-S6 loop in voltage-sensor trapping of CssIV toxin. However, compared with our previous experiments (11), these effects are weaker than those in the IIS3-S4 loop. Therefore, our results as a whole suggest that the IIS3-S4 loop plays the primary role in binding and voltage-sensor trapping by CssIV toxin and controls the functional effects of the toxin, whereas IIS1-S2 and IIIS2-S6 segments play important, but secondary roles.

A Structural Model for the \(\beta\)-Scorpion Toxin-Na\(\_\) Channel Complex—We used the Rosetta-membrane program to develop a molecular model of the CssIV toxin receptor site based on the crystal structure of the bacterial Na\(\_\) channel NavAb (5). Because they are not present in the NavAb structure, the extracellular loops of the Na\(\_\), 1.2 channel were built \textit{ab initio} using the kinematic loop protocol in the Rosetta-Membrane algorithm. The CssIV toxin was then docked in the putative receptor site formed by the cleft among the IIS1-S2, IIS3-S4, and IIIS2-S6 loops of the Na\(\_\),1.2 channel as described under “Experimental Procedures” (Fig. 6). CssIV docks in the center of these three extracellular loops, and nearly all of the amino acid residues in the Na\(\_\),1.2 channel that were most important for CssIV binding point toward the bound toxin. In IIIS2-S6, Asn1436, Glu1438, and Leu1439 are well positioned to interact with the bound CssIV toxin (Fig. 6). In contrast, Asp1445 is located on the opposite side of the IIIS2-S6 loop; therefore, mutations in this position likely affect toxin binding indirectly through interactions with Asn1436, Glu1438, and/or Leu1439 positioned across the IIIS2-S6 loop from Asp1445. It is also possible that the prediction of the fold of the IIIS2-S6 loop in our Rosetta model is inaccurate with respect to placement of Asp1445. However, models that allow Asn1436, Glu1438, Leu1439, and Asp1445 to all interact directly with bound CssIV would require distortions of the conformation of this region of the Na\(\_\) channel that seem incompatible with the NavAb crystal structure. Therefore, we favor the conclusion that Asp1445 interacts with bound CssIV indirectly, perhaps by influencing the conformation of Asn1436, Glu1438, and Leu1439, which are just across the S5-S6 helical bundle from it.

As in our previous models of the CssIV-Na\(\_\) channel complex (11), the wedge-shaped core domain of CssIV is bound between the two faces of the IIS1-S2 and IIS3-S4 loops. This orientation of CssIV places its amino acid residues whose side chains are most important for determining toxin affinity and
Receptor Site for β-Scorpion Toxins on Na\textsubscript{V} Channels
efficacy in position to interact with these two extracellular loops. In contrast, the N- and C-terminal segments of CsslIV point toward the IIISS2-S6 loop, but the only amino acid residue that is required for toxin binding in this part of the toxin, Trp\textsuperscript{568}, interacts with the channel indirectly through its neighboring residues in this structural model. Thus, in this region of the toxin-receptor complex, the identity of the side chains of the amino acid residues on the Na\textsubscript{V},1.2 channel are important determinants of toxin binding and efficacy, but the overall shape of the CsslIV toxin and its backbone carbonyls may be sufficient to sustain essentially normal interaction with the Na\textsubscript{V} channel, even when one amino acid residue has been mutated to alter the interactions of its side chain. The IIISS2-S6 loop of the Na\textsubscript{V},1.2 channel may provide a stable wall against which the toxin leans to exert force on the gating movements of the IIS3-S4 segment and thereby trap the voltage sensor in its activated conformation.

DISCUSSION

The Receptor Site for β-Scorpion Toxins Includes Amino Acid Residues in the IIISS2-S6 Loop—In our previous experiments, we mapped the molecular determinants of voltage-sensor trapping in domain II (8, 9, 11). These results demonstrated that both IIS1-S2 and IIS3-S4 loops are required for normal binding and voltage-sensor trapping by β-scorpion toxins, but the IIS3-S4 loop plays a dominant role as mutations in it have larger effects on binding affinity and unique effects on toxin efficacy (11). Based on the domain-swapped organization of the voltage-sensing module and pore module of Kv\textsubscript{A} and Na\textsubscript{V} channels revealed by x-ray crystallography (4, 5), we hypothesized that amino acid residues in the IIISS2-S6 loop would also play an important role in toxin binding and action. In the experiments described here, we have identified key amino acid residues in IIISS2-S6 that are critical for voltage-sensor trapping by CsslIV. These data confirm the involvement of the IIISS2-S6 loop in voltage-sensor trapping by β-scorpion toxins. This conclusion is in agreement with our previous finding that transferring the entire IIISS2-S6 region of a β-scorpion toxin-insensitive Na\textsubscript{V} channel (Na\textsubscript{V},1.5) to a β-scorpion toxin-sensitive Na\textsubscript{V} channel (Na\textsubscript{V},1.2) markedly decreased the binding affinity of β-scorpion toxins in Na\textsubscript{V} channels (8). Similarly, our results agree with previous evidence that this segment of Na\textsubscript{V} channels determines the specificity for β-scorpion toxin interaction with different channel subtypes (12, 23).

Hydrophilic Pathway for Access to the CsslIV Receptor Site—In the crystal structures of the K\textsubscript{C},1.2-K\textsubscript{C},2.1 chimera (4) and Na\textsubscript{Ab} (5), several phospholipid molecules were observed tightly bound to each subunit of the channel proteins. However, no phospholipid molecules were observed in the extracellular aqueous cleft formed by the S1-S2 and S3-S4 loops of one subunit and the S5-S6 loop of the neighboring subunit. This observation is consistent with the conclusion that this surface is hydrophilic and water accessible. Therefore, it is likely that the large, highly charged β-scorpion toxins approach their receptor site on Na\textsubscript{V} channels from the extracellular medium, rather than from the membrane phase. This inference agrees with previous findings that CsslIV toxin does not partition into the cell membrane (24).

Mutations in the IIISS2-S6 Region Can Strengthen or Weaken Voltage-sensor Trapping by CsslIV<sup>E135A</sup>—Unlike the IIS1-S2 loop where only two mutants reduced the binding affinity of β-scorpion toxins, we identified both mutants that enhance and mutants that weaken voltage-sensor trapping in IIISS2-S6. The unidirectional effects of mutations in IIS1-S2 suggest that key residues in this loop form only positive interactions with CsslIV, whereas the bidirectional mutational effects in IIISS2-S6 suggest that residues in IIISS2-S6 form both positive and negative interactions with toxin residues. These differential positive and negative binding interactions of amino acid residues may contribute to the efficacy of the bound toxin in voltage-sensor trapping.

Eight high-impact residues in IIS3-S4 were identified in our previous experiments (11). Five of those residues strongly reduced or even abolished voltage-sensor trapping, whereas the other three greatly enhanced voltage-sensor trapping. Similar to IIS3-S4 mutations, we identified two mutants in IIISS2-S6 that enhanced voltage-sensor trapping and three mutations at two residues markedly decreased voltage-sensor trapping. However, these high-impact residues are not in consecutive positions, in contrast to the hot spot of critical amino acid residues in the IIS3-S4 loop, and they have lesser effects on toxin action.

Na\textsubscript{V},1.2a/N1436G is a single residue chimera between Na\textsubscript{V},1.2a, on which β-toxins have strong voltage-sensor trapping action, and Na\textsubscript{V},1.5, on which β-toxins have very weak voltage-sensor trapping action (8). Interestingly, the N1436G mutation strongly enhanced voltage-sensor trapping by β-toxins. In this respect, it is similar to the N842R mutant in the IIS3-S4 loop, which is also a single residue chimera between rNa\textsubscript{V},1.2a and hNa\textsubscript{V},1.5 (8, 11). CsslIV can trap the voltage-sensor of N842R at the resting membrane potential without a depolarizing prepulse, whereas a prepulse is required to observe increased voltage-sensor trapping with N1436G. CsslIV has similar efficacy for voltage-sensor trapping Na\textsubscript{V},1.2a/N1436G as for WT, but it has a lower EC<sub>50</sub> that indicates higher affinity binding for CsslIV. In contrast, CsslIV has a higher efficacy for voltage-sensor trapping of Na\textsubscript{V},1.2a/L1439A than WT, but binds to Na\textsubscript{V},1.2a/L1439A with an EC<sub>50</sub> that is similar to WT. Therefore, mutation N1436G increased voltage-sensor trapping by CsslIV via increasing its binding affinity to channels in the resting state, similar to N842R, V843A, and E844N described previously (11). In contrast, mutation L1439A increased voltage-sensor trapping via increasing the efficacy of voltage-sensor trapping by CsslIV, primarily by slowing the reversal of voltage-sensor trapping. It is likely that Leu<sup>1439</sup> is not required for toxin binding to channels in resting state, but stabilizes voltage-sensor trapping by enhancing CsslIV binding to activated Na\textsubscript{V} channels.

IIISS2-S6 Plays a Secondary Role in Voltage Sensor Trapping—Our previous mapping of the key amino acid residues for CsslIV binding to the IIS1-S2 and IIS3-S4 loops and our new data described here emphasize the dominant role of IIS3-S4 in determining β-scorpion toxin binding affinity and voltage-sensor trapping. Altogether, the results suggest that β-scorpion toxins interact with short segments of IIS1-S2 and IIISS2-S6 and a broader region of IIS3-S4. Evidently, these three distant
regions of the primary structure of Na$_v$ channels are close to each other in the three-dimensional structure of Na$_v$ channels and form a single toxin-binding site that interacts with the bound toxin on three sides (8, 9, 11).

Our voltage sensor-trapping model predicts that β-scorpion toxins bind to their receptor site in the resting state of Na$_v$ channels and then, upon activation of the voltage sensor, trap the voltage sensor in domain II in an activated conformation. This effect causes the channel to activate at more negative membrane potentials because one of the voltage sensors is already activated. This three-step model predicts that the rate of toxin binding would be concentration-dependent, the activation of the voltage sensor would be voltage-dependent, and the trapping process would be both concentration- and voltage-independent. Our studies of the kinetics of onset and reversal of CsslIV action agree with this model: toxin binding increases with concentration, as assessed from the maximal effect of CsslIV, but the rate of voltage sensor trapping does not increase with concentration (Fig. 4). Unexpectedly, however, the rate of voltage sensor trapping for mutant N1436G decreased significantly with concentration (Fig. 4C). This effect is predicted by the voltage sensor-trapping model if bound CsslIV both enhances the extent of voltage sensor trapping and also slows the transition into the activated or trapped states. This would occur if binding favors the trapped state but the bound toxin also presents a kinetic barrier to the conformation change into the activated or trapped states. Further development of our voltage sensor-trapping model and additional kinetic data under a wider range of conditions will be required to explore this idea further.

**Spatial Arrangement of the Four Domains of Na$_v$ Channels**—
The crystal structures of K$_v$1.2, K$_v$1.2-K$_v$2.1, and Na$_v$Ab channels (3–5) have a domain-swapped arrangement of their voltage-sensing modules and pore modules in which the voltage sensor of one subunit intersects noncovalently with the pore domain of the neighboring subunit in clockwise order as viewed from the extracellular solution. Vertebrate Na$_v$ channels are composed of four homologous, but nonidentical domains in a single polypeptide. Our studies of amino acid residues required for toxin binding and voltage-sensor trapping in IIS1–S2, IIS3–S4, and IIIS2–S6 (see Refs. 8, 9, and 11, and this paper) show that the IIIS2–S6 loop is in close proximity to the voltage-sensing module of domain II. Therefore, the domain-swapped arrangement of bacterial Na$_v$ channels is present in mammalian Na$_v$.1.2 channels, and the four domains of mammalian Na$_v$ channels must also be arranged in a clockwise manner as viewed from the extracellular side.

**Single Receptor Sites for α- and β-Scorpion Toxins on Na$_v$ Channels**—Vertebrate Na$_v$ channels have four homologous domains connected by large intracellular linkers (2). The short, highly conserved intracellular linker connecting domains III and IV serves as the fast inactivation gate, and α-scorpion toxins slow fast inactivation specifically. Identification of the amino acid residues in the IVS3–S4 loop that are required for high affinity binding of α-scorpion toxins led to the proposal that the voltage sensor in domain IV is primarily involved in fast inactivation (13). Consistent with that model, mutations of the gating charges in the IVS4 segment completely occlude the effects of α-scorpion toxins on gating current (27). Fluorescent labeling studies show that the four voltage sensors in the different domains are functionally specialized. The voltage sensors in domains I and II activate most rapidly and drive the rapid activation of Na$^+$ conductance, whereas the voltage sensor in domain IV activates slowly with a time course similar to fast inactivation (28). Detailed mapping of the amino acid residues in the ISS2–S6, IVS1–S2, and IVS3–S4 segments that are required for high-affinity binding of α-scorpion toxins has led to the conclusion that these three extracellular loops form the receptor site for α-scorpion toxins with a similar conformation as we have proposed here for the receptor site for β-scorpion toxins (29). Moreover, single amino acid mutations in the IVS3–S4 loop reduce binding of α-scorpion toxins by nearly 100-fold (13, 29, 30). These results argue strongly that the high-affinity receptor site for α-scorpion toxins on the voltage sensor in domain IV is unique and cannot be substituted by the other three voltage sensors in Na$_v$ channels.

Our results on β-scorpion toxins also point to a single unique receptor site on Na$_v$ channels. The single amino acid mutation G845N in IIIS3–S4 completely blocks voltage-sensor trapping by β-scorpion toxins (8), indicating that binding to the toxin receptor site in domain II is required for toxin action. Similarly, other mutations clustered near Gly$^{845}$ have dramatic effects on toxin binding and efficacy (9, 11), indicating that toxin binding to the other three voltage-sensing modules cannot substitute for domain II. Thus, it seems most likely that both α- and β-scorpion toxins exert their toxic effects by interaction with a single receptor site on one voltage sensor.

Placing the α- and β-scorpion toxins onto a projection of their binding sites on Na$_v$ channels illustrates the pseudo 2-fold symmetric relationship of these two distinct scorpion toxin receptor sites, with the α-scorpion toxin receptor site formed by the voltage sensor in domain IV interacting with the pore module of domain I and the β-scorpion toxin receptor formed by the voltage sensor of domain II interacting with the pore domain of domain III (Fig. 7). Evidently, these two toxins bind to voltage-sensor domains having different physiological functions in vertebrate Na$_v$ channels and in that way impose different functional modifications, slowed inactivation for the
α-scorpion toxins and negatively shifted activation for the β-scorpion toxins. These two functional effects are synergistic and act together to yield depolarization block of action potential conduction in nerve and muscle fibers. To our knowledge, this is the first example of two distinct toxins from a single type of venom that bind to pseudo-symmetric receptor sites and act synergistically to alter neuromuscular function and immobilize prey.

In contrast to this model of scorpion toxin action, homotrameric Kv channels bind hanatoxin and other cysteine-knot gating modifier toxins to receptor sites on all four voltage sensors (31). Transplanting voltage sensors among different Kv channels or from Naᵥ channels onto Kv channels transfers toxin sensitivity, although the form and affinity of toxin action are often substantially altered (25, 32). These studies raised the possibility that gating-modifier toxins in general are promiscuous and can alter the function of any voltage sensor. Our studies show that this is not the case for scorpion toxins acting on four-domain mammalian Naᵥ channels. The α- and β-scorpion toxins each have a specific receptor site on Naᵥ channels, which are related by a pseudo 2-fold axis of symmetry in the channel structure. The high-affinity, synergistic actions of these two toxin types enable potent paralysis of prey by scorpion venoms. Evidently, evolution of the scorpion toxins has allowed them to act selectively yet synergistically on two different vertebrate Naᵥ voltage sensors and thereby enhance the potency of venoms containing both toxin types.

REFERENCES

1. Hille, B. (2001) Ionic Channels of Excitable Membranes, 3rd Ed., Sinauer Associates Inc., Sunderland, MA
2. Catterall, W. A. (2000) From ionic currents to molecular mechanisms. The structure and function of voltage-gated sodium channels. Neuron 26, 13–25
3. Long, S. B., Campbell, E. B., and MacKinnon, R. (2005) Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. Science 309, 897–903
4. Long, S. B., Tao, X., Campbell, E. B., and MacKinnon, R. (2007) Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment. Nature 450, 376–382
5. Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W. A. (2011) The crystal structure of a voltage-gated sodium channel. Nature 475, 353–358
6. Catterall, W. A. (1980) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu. Rev. Pharmacol. Toxicol. 20, 15–43
7. Catterall, W. A., Castèl, S., Yarow-Yarovoy, V., Yu, F. H., Konoki, K., and Scheuer, T. (2007) Voltage-gated ion channels and gating modifier toxins. Toxicon 49, 124–141
8. Castèl, S., Qu, Y., Rogers, J. C., Rochat, H., Scheuer, T., and Catterall, W. A. (1998) Voltage-sensor trapping. Enhanced activation of sodium channels by β-scorpion toxin bound to the S3–S4 loop in domain II. Neuron 21, 919–931
9. Castèl, S., Yarow-Yarovoy, V., Qu, Y., Sampieri, F., Scheuer, T., and Catterall, W. A. (2006) Structure and function of the voltage sensor of sodium channels probed by a β-scorpion toxin. J. Biol. Chem. 281, 21332–21344
10. Karbat, I., Ilan, N., Zhang, J. Z., Cohen, L., Kahn, R., Benveniste, M., Scheuer, T., Catterall, W. A., Gordon, D., and Gurevitz, M. (2010) Partial agonist and antagonist activities of a mutant scorpion β-toxin on sodium channels. J. Biol. Chem. 285, 30531–30538
11. Zhang, J. Z., Yarow-Yarovoy, V., Scheuer, T., Karbat, I., Cohen, L., Gordon, D., Gurevitz, M., and Catterall, W. A. (2011) Structure-function map of the receptor site for β-scorpion toxins in domain II of voltage-gated sodium channels. J. Biol. Chem. 286, 33641–33651
12. Leipold, E., Hansel, A., Borges, A., and Heinemann, S. H. (2006) Subtype specificity of scorpion β-toxin Tu1 interaction with voltage-gated sodium channels is determined by the pore loop of domain 3. Mol. Pharmacol. 70, 340–347
13. Rogers, J. C., Qu, Y., Tanada, T. N., Scheuer, T., and Catterall, W. A. (1996) Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3–S4 extracellular loop in domain IV of the Na+ channel a subunit. J. Biol. Chem. 271, 15950–15962
14. Yarov-Yarovoy, V., Baker, D., and Catterall, W. A. (2006) Voltage sensor conformations in the open and closed states in ROSETTA structural models of K+ channels. Proc. Natl. Acad. Sci. U.S.A. 103, 7929–7929
15. Yarov-Yarovoy, V., Schonbrun, J., and Baker, D. (2006) Multipass membrane protein structure prediction using Rosetta. Proteins 62, 1010–1025
16. Barth, P., Schonbrun, J., and Baker, D. (2007) Toward high-resolution prediction and design of transmembrane helical protein structures. Proc. Natl. Acad. Sci. U.S.A. 104, 15682–15687
17. Jeanninouin, F., Thompson, J. D., Gouy, M., Higgins, D. G., and Gibson, T. J. (1998) Multiple sequence alignment with Clustal X. Trends Biochem. Sci. 23, 403–405
18. Wang, C., Bradley, P., and Baker, D. (2007) Protein-protein docking with backbone flexibility. J. Mol. Biol. 373, 503–519
19. Bonneau, R., Strauss, C. E., Rohl, C. A., Chivian, D., Bradley, P., Malmström, L., Robertson, T., and Baker, D. (2002) De novo prediction of three-dimensional structures for major protein families. J. Mol. Biol. 322, 65–78
20. Mandell, D. J., Coutias, E. A., and Korremte, T. (2009) Sub-angstrom accuracy in protein loop reconstruction by robotics-inspired conformational sampling. Nat. Methods 6, 551–552
21. Cohen, L., Karbat, I., Gilles, N., Ilan, N., Benveniste, M., Gordon, D., and Gurevitz, M. (2005) Common features in the functional surface of scorpion β-toxins and elements that confer specificity for insect and mammalian voltage-gated sodium channels. J. Biol. Chem. 280, 5045–5053
22. Rohl, C. A., Strauss, C. E., Misura, K. M., and Baker, D. (2004) Protein structure prediction using Rosetta. Methods Enzymol. 383, 66–93
23. Gurevitz, M., Karbat, I., Cohen, L., Ilan, N., Kahn, R., Turkov, M., Stankiewicz, M., Stühmer, W., Dong, K., and Gordon, D. (2007) The insecticidal potential of scorpion β-toxins. Toxicon 49, 473–489
24. Cohen, L., Gilles, N., Karbat, I., Ilan, N., Gordon, D., and Gurevitz, M. (2006) Direct evidence that receptor site-4 of sodium channel gating modifiers is not dipped in the phospholipid bilayer of neuronal membranes. J. Biol. Chem. 281, 20673–20679
25. Bosmans, F., and Swartz, K. J. (2010) Targeting voltage sensors in sodium channels with spider toxins. Trends Pharmacol. Sci. 31, 175–182
26. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF chimera, a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612
27. Sheets, M. F., Kyle, J. W., Kallen, R. G., and Hanck, D. A. (1999) The Na channel voltage sensor associated with inactivation is localized to the external charged residues of domain IV, S4. Biophys. J. 77, 747–757
28. Chanda, B., and Bezanilla, F. (2002) Tracking voltage-dependent conformational changes in skeletal muscle sodium channel during activation. J. Gen. Physiol. 120, 629–645
29. Wang, J., Yarov-Yarovoy, V., Kahn, R., Gordon, D., Gurevitz, M., Scheuer, T., and Catterall, W. A. (2011) Mapping the receptor site for α-scorpion toxins on a Na+ channel voltage sensor. Proc. Natl. Acad. Sci. U.S.A. 108, 15426–15431
30. Gur, M., Kahn, R., Karbat, I., Regov, N., Wang, J., Catterall, W. A., Gordon, D., and Gurevitz M. (2011) Elucidation of the molecular basis of selective recognition uncovers the interaction site for the core domain of scorpion α-toxins on sodium channels. J. Biol. Chem. 286, 35209–35217
31. Li-Smerin, Y., and Swartz, K. J. (2000) Localization and molecular determinants of the hanatoxin receptors on the voltage-sensing domains of a K+ channel. J. Gen. Physiol. 115, 673–684
32. Bosmans, F., Martin-Eauclaire, M. F., and Swartz, K. J. (2008) Deconstructing voltage sensor function and pharmacology in sodium channels. Nature 456, 202–208