Common Molecular Determinants of Tarantula Huwentoxin-IV Inhibition of Na\(^+\) Channel Voltage Sensors in Domains II and IV\(^*$\)

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The voltage sensors of domains II and IV of sodium channels are important determinants of activation and inactivation, respectively. Animal toxins that alter electrophysiological excitability of muscles and neurons often modify sodium channel activation by selectively interacting with domain II and inactivation by selectively interacting with domain IV. This suggests that there may be substantial differences between the toxin-binding sites in these two important domains. Here we explore the ability of the tarantula huwentoxin-IV (HWTX-IV) to inhibit the activity of the domain II and IV voltage sensors. HWTX-IV is specific for domain II, and we identify five residues in the S1–S2 (Glu-753) and S3–S4 (Glu-811, Leu-814, Asp-816, and Glu-818) regions of domain II that are crucial for inhibition of activation by HWTX-IV. These data indicate that a single residue in the S3–S4 linker (Glu-818 in hNav1.7) is crucial for allowing HWTX-IV to interact with the other key residues and trap the voltage sensor in the closed configuration. Mutagenesis analysis indicates that the five corresponding residues in domain IV are all critical for endowing HWTX-IV with the ability to inhibit fast inactivation. Our data suggest that the toxin-binding motif in domain II is conserved in domain IV. Increasing our understanding of the molecular determinants of toxin interactions with voltage-gated sodium channels may permit development of enhanced isoform-specific voltage-gating modifiers.

Voltage-gated sodium channels (VGSCs)\(^3\) play critical roles in the generation and propagation of action potentials. Nine VGSC α subunit subtypes (Nav1.1–1.9) have been cloned and characterized from mammals (1). These subtypes are expressed in different excitable tissues and are involved in distinct physiological functions such as neurotransmitter release, muscle contraction, secretion, and pain sensation (2–4). The nine α subunits share a common four-domain (DI–DIV) structure, in which each domain has six transmembrane segments (S1–S6) (1). Based on distinct functional behaviors during channel activity, the six transmembrane segments are generally separated into two structural components (5). The central pore module is the basis for the ion conduction pathway and is formed by the S5–S6 region of the channel. The voltage sensor modules are formed by the S1–S4 regions and are essentially independent structures that directly respond to changes in the transmembrane potential with conformational alterations that are coupled to opening and closing of the pore module. VGSCs undergo voltage-dependent activation subsequently followed by fast inactivation through successive activities of the voltage sensors in the four domains. The S4 segments in the voltage sensors of DI, DII, and DIII are determinants of channel activation, whereas that of DIV is predominantly involved in channel inactivation (5–8). The four membrane-spanning segments (S1–S4) of the voltage sensors of mammal VGSCs exhibit high sequence similarity in the four domains, but the amino acids and sequence length of the intracellular and extracellular linkers are quite divergent. Because crystal structures of sodium channels are lacking, it remains unknown whether the voltage sensor differences result in altered conformations of the membrane-spanning segments or whether the orientations of amino acid side chains are structurally conserved at corresponding positions in the four different voltage sensors.

Because of their high binding affinity and subtype-specific selectivity, animal toxins are powerful tools for investigation of the structure-function relationship of VGSCs (9). The voltage sensors of DII and DIV are the two most common toxin-binding sites in VGSCs (see Fig. 1A). Scorpion β-toxins bind to the DII voltage sensor to enhance voltage-dependent activation by trapping DIIS4 in the activated state (10–12). Scorpion α-toxins bind to the DIV voltage sensor and inhibit fast inactivation by trapping DIVS4 in the closed state (13, 14). These findings seem to suggest that there are substantial differences between the toxin-binding sites on the voltage sensors of DII and DIV. However, we recently demonstrated that ProTx-II, another tarantula toxin, can both inhibit hNav1.7 activation by interact-

\(^*\) This work was supported, in whole or in part, by National Institutes of Health Grants NS054642 and NS053422 (to T. R. C.). This work was also supported by the 973 Research Program of China under Contract 2010CB529800 (to S. L.); the Scientific Research Fund of Hunan Provincial Education Department (07A035) and the Program for New Century Excellent Talents in University under Contract NCET-07-0279 (to Y. X.).

\(^{1}\) The online version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S4 and Figs. S1 and S2.

\(^{2}\) The abbreviations used are: VGSC, voltage-gated sodium channel; DI, DII, DIII, and DIV, domains I, II, III and IV; HWTX-IV, huwentoxin-IV; Kv, voltage gated potassium channel; VSD, voltage sensing domain; Sn, transmembrane segment n.
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ing with DII and impair hNav1.7 inactivation by interacting with DIV (15). This raises the possibility that there might be conserved molecular determinants of toxin binding in the DII and DIV voltage sensors. Here we used electrophysiology and point mutagenesis to extensively explore the molecular determinants involved in huwentoxin-IV (HWTX-IV) interactions with hNav1.7. HWTX-IV is a voltage sensor modifier from the tarantula *Ornithoctonus huwena* that interacts with the DII voltage sensor. However, in contrast to scorpion β-toxins, HWTX-IV traps DIIS4 in the closed state and specifically inhibits channel activation (16, 17). Our results indicate that HWTX-IV partially DIIS4 in the closed state and specifically inhibits channel activation. Positively charged residues (typically Arg) embedded within the S4 segment are primary contributors to gating charge and voltage sensitivity of ion channels.

**EXPERIMENTAL PROCEDURES**

Molecular Biology—All of the hNav1.7 mutations were constructed using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer’s instruction. The constructs were sequenced to confirm that the appropriate mutations were made.

Electrophysiological Recording—WT and mutant hNav1.7 channels were transiently transfected into HEK293 cells using the standard calcium phosphate precipitation method as previously described (15).

Whole cell patch clamp recordings were carried out at room temperature (−21 °C) using an EPC-10 amplifier (HEKA, Lambrecht, Germany). Fire-polished electrodes were fabricated from 1.7-mm capillary glass (VWR, West Chester, PA) using a P-97 puller (Sutter, Novato, CA). The standard pipette solution contained 140 mM CsF, 1 mM EGTA, 10 mM NaCl, and 10 mM HEPES, pH 7.3. The standard bathing solution was 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES, pH 7.3. After filling with pipette solution, the access resistance of electrode pipette ranged from 0.7 to 1.3 MΩ. The liquid junction potential for these solutions was <8 mV; data were not corrected to account for this offset. Voltage errors were minimized using 80% series resistance compensation, and the capacitance artifact was canceled using the computer-controlled circuitry of the patch clamp amplifier. Linear leak subtraction, based on resistance estimates from four to five hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage clamp recordings. Membrane currents were usually filtered at 5 kHz and sampled at 20 kHz.

The stock solutions for 50 μM CssIV and 1 mM HWTX-IV were made using bathing solution containing 1 mg/ml BSA, and aliquots were stored at −20 °C. Before use, the solution was diluted to the concentrations of interest with fresh bathing solution. Toxin was diluted into the recording chamber (volume of 300 μl) and mixed by repeatedly pipetting 30 μl to achieve the specified final concentration. The extent of the inhibitory effect of the toxin was typically assessed around 20 min after toxin treatment.

Homology Modeling—Three-dimensional models of hNav1.7 DII-VSD (Phe-728 to Lys-846) were created based on the complete closed state and open state structures of the mammalian Kv1.2 (Ser-158 to Ile-316) provided by Pathak et al. (18). Sequence alignment was conducted using the ClusterW1.8 program, and the alignment of the four transmembrane segments (S1–S4) was then refined manually. Homology modeling was performed using Accelrys Discovery Studio 1.6 software (Accelrys, San Diego, CA). The models were further refined by energy “minimization” protocol using a CHARMM force field, in which both the minimization algorithm “steepest descent” and “conjugated gradient” were set to 500 steps.

Data Analysis—The data were analyzed using the Pulsfit (HEKA) and GraphPad Prism 4 (GraphPad Software) programs. All of the data points are shown as the means ± S.E. n is presented as the number of experimental cells. Statistical analysis was carried out by Student’s t test, and p < 0.05 indicated a significant difference. Steady-state activation and inactivation curves were fitted using the Boltzmann equation: 

\[
y = \frac{1}{1 + \exp\left(\frac{V - V_{1/2}}{k}\right)}
\]

where X is the toxin dose, V_{1/2} is the Hill coefficient, and IC50 is the half-maximal inhibitory concentration. In this study, the IC50 was set to 1 because our mutagenesis data have shown that the toxin had a single high affinity binding site in sodium channels. For HWTX-IV action on fast inactivation of WT and mutant Nav1.7, the IC50 was also set to 1 because only sodium channel DIV is involved in channel inactivation gating.

**RESULTS**

Electrophysiological Properties of WT and Mutant Nav1.7 Channels—To explore hNav1.7-HWTX-IV interactions and identify the crucial residues in hNav1.7, we mutated each residue in the extracellular portions of the DII S1–S2 and S3–S4 regions. Mutations were principally designed using the following rules: 1) charged residues were mutated to be neutral, 2) unique residues in the spider VGSC (19), which is presumably resistant to HWTX-IV, were introduced into hNav1.7, and 3) if an uncharged residue is conserved in hNav1.7 and the spider VGSC, it was substituted by a smaller side chain residue, either Ala or Cys (Fig. 1).

We first characterized the electrophysiological properties of WT and mutant Nav1.7 channels, expressed in HEK293 cells, using the whole cell recording configuration and compared the voltage-dependent properties of steady-state activation and inactivation to WT Nav1.7. Consistent with previous reports that the voltage sensor in DII is an important determinant of channel activation (7, 20), mutations of most amino acid residues significantly altered channel activation (Fig. 2A and supplemental Table S1). In particular, both hydrophilic and hydrophobic residues in the DIIS4 segment are involved in favoring hNav1.7 in the closed state. Positively charged residues (typically Arg) embedded within the S4 segment are primary contributors to gating charge and voltage sensitivity of ion channels.
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Critical Residues in hNav1.7 DII Interacting with HWTX-IV—
In our previous work, we demonstrated that an acidic residue in the extracellular DIIS3–S4 region (Glu-818) is critical for inter-
acting with HWTX-IV. The mutation of Glu-818 to Gln and Cys reduced toxin binding affinity for hNav1.7 by 63- and 400-
fold, respectively (15, 16). Because the DII voltage sensor in VGSCs is a mobile structure, we hypothesized that interaction
with only Glu-818 would not be sufficient for HWTX-IV to trap the DII voltage sensor in the closed state and that other residues
must be involved in the toxin-channel interaction that under-
lies channel inhibition. In the current study, we found that
mutation of four additional amino acid residues (Glu-753 in the
DIIS1-S2 region and Glu-811, Leu-814, and Asp-816 in the
DIIS3–S4 region) produced substantial decreases in HWTX-IV
inhibition of hNav1.7 activation (Fig. 3). Fig. 3A shows typical
current traces of WT and these four mutant Nav1.7 channels
elicited by a 20-ms depolarization of −10 mV from a holding
potential of −100 mV. As reported previously, 1 μM HWTX-IV
completely blocks WT Nav1.7 (16). However, in the presence of
1 μM HWTX-IV, the amount of current that still could be acti-
vated ranged from 6.2 ± 1.3 to 31.1 ± 2.1% of control for these
four mutant channels (n = 4–8). The IC₅₀ values for
HWTX-IV blocking WT and all 39 mutant hNav1.7 channels
tested are summarized in Fig. 3B. These data indicate that
the E753Q, E811Q, L814C, and D816A mutations reduced toxin
binding affinity by 5-, 18-, 9-, and 13-fold, respectively (15, 16).
Although three mutations (V817A, G819P, and S825A) slightly
increased HWTX-IV sensitivity for hNav1.7, the IC₅₀ values for
these mutations were only ~2-fold smaller than that for WT
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Seven of the eight mutations that altered HWTX-IV inhibi-
tion (with V817A being the exception) significantly shifted the
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potentials (Fig. 2B). However, it seems unlikely that changes in
HWTX-IV binding affinity result from altering the voltage-dependent gating of the mutant channels, because there was no direct correlation between changes in the voltage dependence of activation and changes in sensitivity to HWTX-IV when all of the mutations are considered. We also examined whether exposure to HWTX-IV had obvious effects on the voltage-dependent properties of the various mutant channels. For WT Nav1.7 and the majority of mutant channels, in the presence of 0.1 (or 1) μM HWTX-IV, there was no apparent change in the voltage dependence of activation and inactivation (supplemental Fig. S1 and Tables S2 and S3). This lack of an effect most likely reflects that in the presence of HWTX-IV, we are mainly measuring the activity of residual currents from channels that did not interact with HWTX-IV. This is consistent with our previous data showing that in the presence of saturating concentrations of HWTX-IV, no Nav1.7 current can be detected unless the channels are depolarized beyond +100 mV, reflecting a depolarizing shift of ∼200 mV in the voltage dependence of activation of hNav1.7 by HWTX-IV (16). However, for three mutations (R827Q/C and L828A) in DII54, 0.1 μM HWTX-IV not only shifted the voltage dependence of channel activation (by +7.2, +8.7, and +6.2 mV, respectively), but it also doubled the slope factors for the voltage dependence of channel activation (Fig. 4 and supplemental Table S2). HWTX-IV did not affect inactivation properties of either WT or these mutant channels (supplemental Table S3). None of these mutations (R827Q/C or L828A) altered the IC₅₀ for HWTX-IV (Fig. 3B), indicating that neither Arg-827 nor Leu-828 is directly involved in the toxin-channel interaction. Therefore, these three mutations most likely enhance the ability of hNav1.7 channels to activate with HWTX-IV bound by altering the stability of the closed configuration of the DI5 voltage sensor.

HWTX-IV Does Not Bind to WT Nav1.4—Our previous studies have shown that WT rNav1.4 (the skeletal muscle VGSC) is resistant to HWTX-IV and that mutation of Gln-657 in rNav1.4 (which corresponds to Glu-818 in hNav1.7; see Fig. 1) greatly increases toxin sensitivity of rNav1.4 (16). Because three of the other crucial residues that we identified in this study are highly conserved in rNav1.4 and hNav1.7 (Fig. 1), we next asked whether HWTX-IV still binds to WT rNav1.4, even though inhibition of current amplitude is not observed. To test this hypothesis, we designed a simple competitive binding experiment with CssIV (11), a typical scorpion β-toxin. CssIV partially shares the binding site on the DI5 voltage sensor of VGSCs with HWTX-IV because mutations of three residues in rNav1.2a (Glu-779, Glu-837, and Leu-840, corresponding to Glu-753, Glu-811, and Leu-814 in hNav1.7, respectively; Fig. 1) have previously been shown to additively reduce CssIV binding affinity for rNav1.2a by 175-fold (10). However, in contrast to HWTX-IV, CssIV traps the DI5 voltage sensor in the activated state, thereby shifting the voltage dependence of VGSC activation to more negative potentials. Therefore, we reasoned that if HWTX-IV was able to “silently” bind to WT Nav1.4, HWTX-IV pretreatment would be expected to substantially interfere with CssIV binding to rNav1.4. To test this, a triple-pulse protocol (described in the legend of Fig. 5) was used to assess the fraction of rNav1.4 channels modified by CssIV. In the absence of CssIV and HWTX-IV, the rNav1.4 channels were closed at potentials less than −60 mV, and no current was recorded by the 20-ms depolarizing pulse to −65 mV from a holding potential of −100 mV (Fig. 5A; n = 6). In the presence of 0.5 μM CssIV, inward rNav1.4 current, resulting from the negative shift of channel activation, was evidently induced at the subthreshold potential (−65 mV), and the ratio of the cur-
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**FIGURE 4. Effects of HWTX-IV on the voltage-dependent activation of four hNav1.7 mutants expressed in Hek293 cells.** Four mutations of interest (R824Q (A), R827Q (B), L828A (C), and R830Q (D)) were located in the DIVS4 segment. The cells were held at \(-100\) mV. Families of currents before and after 100 nM HWTX-IV treatment (left panels) were induced by 50-ms depolarizing steps to various potential ranging from \(-80\) to \(+100\) mV. Channel conductance values before and after 100 nM HWTX-IV treatment (right panels) were calculated with the equation as described for Fig. 2A.

current elicited at \(-65\) mV (\(I_{-65}\)) to that elicited at \(-10\) mV (\(I_{-10}\)) was \(0.035 \pm 0.003\) (\(n = 3\)). This ratio was not altered by additional application of 1 \(\mu M\) HWTX-IV (Fig. 5A, upper panels), the concentration that blocks \(77.8 \pm 2.0\%\) of the Q657E mutant rNav1.4 channels in our previous work (16). In Fig. 5A (lower panels), 1 \(\mu M\) HWTX-IV by itself did not induce rNav1.4 current at \(-65\) mV, consistent with our previous observation on hNav1.7 (16). Additional application of 0.5 \(\mu M\) CssIV in the presence of HWTX-IV still induced inward current at \(-65\) mV, and the ratio of \(I_{-65}\) to \(I_{-10}\) (control) was \(0.035 \pm 0.004\) (\(n = 3\)), which is identical to the value caused by CssIV without HWTX-IV (Fig. 5B). These results demonstrate that HWTX-IV did not affect CssIV binding to WT rNav1.4, indicating that HWTX-IV does not bind silently to rNav1.4 in DIV. Together with our previous results on specific point mutations in rNav1.4 (Q657E) and in hNav1.7 (E818Q/C) (15, 16), these results suggest that interaction with the acidic residue Glu at position 818 in the DIVS3–S4 linker of hNav1.7 is not only the key determinant of voltage sensor trapping and channel inhibition but is also the key factor for allowing HWTX-IV to access and interact with the other crucial residues.

**Molecular Determinants of HWTX-IV Binding Are Conserved on DII and DIV—**Scorpion toxins that target VGSCs are typically classified as either \(\alpha\)-toxins that specifically inhibit inactivation by selectively binding to the extracellular portion of the DIVS3–S4 region or \(\beta\)-toxins that specifically enhance activation by selectively binding to the extracellular portion of the DIIS3–S4 region (10–14). This indicates that the extracellular portion of the S3–S4 region of the DII and DIV voltage sensors, also referred to as the voltage sensor paddles, may be structurally distinct. However, the tarantula toxin ProTx-II can reportedly interact with multiple voltage sensor paddles (23), and we have recently demonstrated that ProTx-II can inhibit both activation and inactivation of Nav1.7 (15). This suggests that either ProTx-II is relatively promiscuous in its interactions with the voltage sensors of sodium channels or that the DII and DIV voltage sensors are more similar than might be predicted based on the specificity of scorpion toxins. Nav1.7 DIV is resistant to HWTX-IV because the toxin does not inhibit fast inactivation of either WT or E818C mutant channels (15, 16, 24) (Fig. 3A). However, sequence alignment indicates that three of the five critical residues in Nav1.7 DIV are conserved in DIV (Fig. 6A). Although the other two critical residues, Glu-811 and Glu-818, are correspondingly replaced by Gly-1581 and Ile-1588, respectively (Fig. 6A), the four membrane-spanning segments (S1–S4) in DII show relatively high sequence similarity (~66%) with those in DIV. Therefore, we next asked whether the molecular determinants of toxin binding on the Nav1.7 DIV voltage sensor might be conserved in the DIV voltage sensor. To address this question, we constructed six Nav1.7 DIV con-
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A.  

![Diagram](image)

B.  

![Diagram](image)

FIGURE 6. Functional effects of mutations of amino acid residues in hNav1.7 DIV-VSD. A, amino acid sequence alignment of S1–S2 and S3–S4 regions of Nav1.7 DII and DIV. Compared with DII, the identical residues are marked with a dot (.) in DIV. Five crucial residues in DIV and DII are shaded in gray. B, effects of mutations of amino acid residues in DIV-VSD on steady-state activation (left) and inactivation (right) of the E818C mutant hNav1.7 channel. The cells were held at −100 mV. Channel conductance and steady-state inactivation were determined using the method as described for Fig. 2. The V_{1/2} values and slope factor are summarized in supplemental Table S4. Each DIV construct is identified by the five-letter code representing the amino acid residue in DII.

structures, containing systemic substitutions of the five residues in DIV that correspond to the five crucial residues in DII (Fig. 6). These channel constructs were generated in the Nav1.7-E818C channel variant because this construct is substantially resistant to inhibition of activation by HWTX-IV, allowing investigation of the effect of HWTX-IV on the DIV mutants.

Of the five corresponding residues in DIV (Glu-1524, Gly-1581, Leu-1584, Asp-1586, and Ile-1588), only the second and last amino acids differ from those in DII (Fig. 6A). To simplify the description of the various mutant channels, we will refer to each construct by the five-letter code for the five key residues. In addition, we will capitalize the amino acid residue if it is identical to that found in DII. Thus, the native DIV construct is EgLDi, and the construct where all five match those in DII is EELDE.

The electrophysiological properties of these mutant channels, expressed in Hek293 cells, were determined (Fig. 6B and supplemental Table S4). Compared with E818C, additional single mutations shifted the voltage dependence of activation by less than +7 mV, but the voltage dependence of inactivation was more substantially affected (ranging from −13.9 to +12.2 mV). This result is consistent with the general principle that the DIV voltage sensor is predominantly involved in channel inactivation (5, 6, 8).

Studies on scorpion α-toxins have demonstrated that stabilizing the DIV voltage sensor in the closed state impairs fast inactivation of VGSCs (13). To estimate HWTX-IV efficacy for inhibiting fast inactivation, we measured the I_{max}/I_{peak} ratio, providing an estimate of the probability that the channel is not inactivated after 5 ms. The EELDi construct did not exhibit altered sensitivity to inhibition of fast inactivation by HWTX-IV (Fig. 7A), despite the fact that in this construct four of the five crucial residues identified in DII are now present in DIV. In contrast, the introduction of Glu at the last position (creating the EgLDE construct) made Nav1.7 DIV substantially more sensitive to HWTX-IV. This is consistent with our previous observation that the corresponding Glu residue in the DIIIs of Nav1.4 and Nav1.7 is absolutely crucial for high affinity interaction of the voltage sensor with HWTX-IV (16). In the EgLDE channels, 10 μM HWTX-IV inhibited fast inactivation by 56.1 ± 7.3% (n = 4; Fig. 7A). This effect on inactivation was almost doubled by addition of the final difference, producing the EELDE construct. In this channel construct, where all five critical residues in DIV are present at the corresponding positions in DIV, HWTX-IV is able to substantially inhibit inactivation of Nav1.7 channels (Fig. 7, A and B).

We next asked whether substitutions of the three residues in DIV that were originally conserved compared with DII could modulate the ability of HWTX-IV to inhibit inactivation of channels containing the critical I1588E mutation. We found that substitutions at all three of these conserved residues produced a decrease in the toxin sensitivity of the DIV voltage sensor. In comparison with the EELDE construct, 10 μM HWTX-IV inhibited fast inactivation of the EgLDE construct by only 16.9 ± 1.9% (n = 3). In comparison with the EELDE construct, 10 μM HWTX-IV inhibited fast inactivation of the qELDE and EELaE constructs by only 46.3 ± 4.8% (n = 4) and 76.6 ± 1.9% (n = 5), respectively. In Fig. 7C, the I_{50} values for HWTX-IV inhibition of inactivation were estimated from the data shown in Fig. 7C and are reported in Table 1. From these data, it is clear that DIV substitutions that mimicked critical
residues in DII (G1581E and I1588E) enhanced the ability of HWTX-IV to inhibit inactivation and, conversely, DIV mutations that mimic disruptive mutations of critical residues in DII (E1524Q, L1584C, and D1586A) decreased the ability of HWTX-IV to inhibit inactivation. Furthermore, as with HWTX-IV inhibition of activation (16), inhibition of fast inactivation of the DIV construct containing all five crucial residues was voltage-dependent (Fig. 7 B and supplemental Fig. S2 A). The inhibition was reversible but required substantial depolarizations (> +100 mV), suggesting that interaction of HWTX-IV with the DIV voltage sensor is relatively strong. However, 10 μM HWTX-IV did not significantly shift the steady-state activation and inactivation for the unblocked DIV mutant channels (supplemental Fig. S2 B).

In our previous work (15, 16), the IC_{50} value for HWTX-IV is estimated to be 26 nM for WT Nav1.7 DII, which is ~100-fold smaller than that for modulation of inactivation with the EELDE channel, the construct having all five of the critical residues conserved in the VSDs of both DII and DIV. The identity of the amino acid residues at the five key positions in DIV are shown.

| Identity of five key residues in DIV | Inhibition of inactivation (10 μM) | Estimated IC_{50} |
|-------------------------------------|-----------------------------------|------------------|
| EgLDi                               | None detected                     |                  |
| EELDi                               | None detected                     |                  |
| EgLDE                               | 56.1 ± 7.3%                       |                  |
| EELDE                               | 80.6 ± 1.1%                       | 7.2              |
| EgdE                                | 16.9 ± 1.9%                       | 47.4             |
| qELDE                               | 46.3 ± 4.8%                       | 9.7              |
| EELae                               | 7.6 ± 1.9%                        | 115.7            |

Inhibition of inactivation of the E818C mutant hNav1.7 channels by HWTX-IV

The identity of the amino acid residues at the five key positions in DIV are shown. Those that differ from the key residues in DII are shown as lowercase letters.

| TABLE 1                                                                 |
|------------------------------------------------------------------------|
| Inhibition of inactivation of the E818C mutant hNav1.7 channels by      |
| HWTX-IV                                                                |
| The identity of the amino acid residues at the five key positions in    |
| DIV are shown. Those that differ from the key residues in DII are shown |
| as lowercase letters.                                                   |

| Identity of five key residues in DIV | Inhibition of inactivation (10 μM) | Estimated IC_{50} |
|-------------------------------------|-----------------------------------|------------------|
| EgLDi                               | None detected                     |                  |
| EELDi                               | None detected                     |                  |
| EgLDE                               | 56.1 ± 7.3%                       |                  |
| EELDE                               | 80.6 ± 1.1%                       | 7.2              |
| EgdE                                | 16.9 ± 1.9%                       | 47.4             |
| qELDE                               | 46.3 ± 4.8%                       | 9.7              |
| EELae                               | 7.6 ± 1.9%                        | 115.7            |

Inhibition of inactivation of the E818C mutant hNav1.7 channels by HWTX-IV

The identity of the amino acid residues at the five key positions in DIV are shown. Those that differ from the key residues in DII are shown as lowercase letters.

![FIGURE 8. Effects of HWTX-IV on hNav1.7 DII/DIV chimeras expressed in Hek293 cells. A, amino acid sequence alignment of S3–S4 regions of hNav1.7 DII (red) and DIV (black). The S3 segment and S3–S4 linker in DII that was transplanted into DIV is highlighted in red in the four chimeras. Positively charged residues (Arg) in S4 segment are shaded in black bars. B, characterization of functional properties of the DII/DIV chimeras. The cells were held at −100 mV. Channel conductance (left) and steady-state inactivation (right) were determined using the method as described in Fig. 2. C, typical current traces from four hNav1.7 DII/DIV chimeras before and after application of 10 μM HWTX-IV. D, dose-response curves of HWTX-IV slowing fast inactivation of four Nav1.7 DII/DIV chimeras. The data points (means ± S.E.) from three to four cells are fit to a Hill equation. Note that all DII/DIV chimeras were constructed based on the E818C mutant hNav1.7 channel.](image-url)
**Conserved Toxin-binding Motif in Na\(^+\) Channel Voltage Sensors**

**DISCUSSION**

In this study, we investigated the molecular determinants of VGSC voltage sensor trapping by the tarantula toxin HWTX-IV. We first identified the specific residues in the extracellular portions of the S1–S2 and S3–S4 regions of DII from hNav1.7 that are crucial for the action HWTX-IV. Based on these results, we determined that some of the essential molecular determinants of the HWTX-IV receptor in DII are conserved in DIV. These results contribute to our fundamental understanding of how tarantula toxins interact with VGSCs and modify the activity of specific voltage sensors.

Previously we showed that HWTX-IV is a VGSC gating modifier that inhibits activation by trapping DIIS4 in the closed state (16). Interestingly, our current data indicate that none of the amino acid residues in the N-terminal part (extracellular half) of DIIS4 directly interact with HWTX-IV (Fig. 3A). The S4 segment is a mobile amphipathic \( \alpha \)-helical structure composed of positive, polar, and hydrophobic residues. Consistent with the proposal that positive residues serve as the primary originator of voltage sensitivity, charge-neutralizing mutations of the three outermost Arg residues in hNav1.7 DIIS4 segment shift the voltage dependence of activation to more positive potentials. Other residues in DIIS4 are also involved in voltage sensor activation (Fig. 2A). Among them, Leu-828 is unique because the Ala mutation negatively shifts voltage dependence of activation, suggesting that Leu-828 favors DIIS4 in the closed state. In our previous work, HWTX-IV action greatly increases the energy required for DIIS4 activation. Only toxin-unbound WT Nav1.7 channels can activate within the physiological voltage range (16). Of all the mutants examined, HWTX-IV only significantly shifted the voltage-dependent activation of the R827Q/C and L828A mutants to more positive potentials. This behavior can be explained by either activation of toxin-bound VGSCs or dissociation of toxin-VGSCs at depolarized voltages within the physiological range for these three mutants. This result indicates that interaction of Arg-827 and Leu-828 with other residues in the voltage sensor may provide a higher energy barrier for DIIS4 activation than with other residues in DIIS4.

Our data, together with earlier work (15, 16), identify five residues that are critically involved in the interaction of HWTX-IV with hNav1.7. One residue, Glu-753, is in the DIIS1-S2 linker, and the others, Glu-811, Leu-814, Asp-816, and Glu-818, are located in the extracellular portion of DIIS3 or the DIIS3-S4 linker. Glu-818 is the most crucial because the E818C mutation eliminated the sensitivity of hNav1.7 to HWTX-IV (15, 16). However, interaction of HWTX-IV with only Glu-818 is not sufficient to stabilize DIIS4 in the closed state. Mutations of the other four residues are predicted to collectively reduce toxin binding affinity by 10,530-fold (assuming their effects are additive). Their cumulative effects would theoretically increase the IC\(_{50}\) value from 26 \( \mu \text{M} \) to 274 \( \mu \text{M} \).

Scorpion \( \beta \)-toxin CssIV is another DII voltage sensor modifier that has been extensively characterized (10, 11). CssIV partially shares the binding site on the DII voltage sensor with HWTX-IV. Three of the critical residues involved in HWTX-IV action have been identified as molecular determinants of CssIV activity. This finding is surprising because in contrast to HWTX-IV, which stabilizes the DII voltage sensor in the closed state, CssIV traps the DII voltage sensor in the activated state. However, Asn-842 and Glu-844 in rNav1.2a, which correspond to Asp-816 and Glu-818 in hNav1.7, are not involved in forming the CssIV-binding receptor (11). Conversely, mutation of rNav1.2a Gly-845 to Asn decreases CssIV-binding affinity by 13-fold in rNav1.2a, whereas the corresponding mutation in hNav1.7 (G819N) does not significantly change HWTX-IV sensitivity. Thus, although the interaction sites of CssIV and HWTX-IV overlap, the distinct confirmations of the DII voltage sensor in the closed and activated states are likely to be important in the formation of the divergent toxin-binding motifs for these two voltage-gating modifiers.

Three of the critical residues in hNav1.7 are conserved in rNav1.4 (the exceptions being Asp-816 and Glu-818). Our data show that HWTX-IV does not interact with wild-type rNav1.4. However, mutation of rNav1.4 Gln-657 (corresponding to Glu-818 in hNav1.7) to Glu makes rNav1.4 sensitive to HWTX-IV (16), consistent with the seemingly crucial role that Glu-818 in hNav1.7 plays in trapping the voltage sensor in the closed configuration. We constructed closed state and activated state models of the Nav1.7 DII voltage sensor based on three-dimensional structures of the Kv1.2 channel in the closed and activated states (18, 24) to gain some additional insight into the role of Glu-818 (Fig. 9). In the closed state homology model, the side chain of Glu-818 is located at the extracellular surface of the voltage sensor with a vertical orientation, but the side chains of the other crucial residues (Glu-753, Glu-811, Leu-814, and Asp-816) are predicted to be embedded within the voltage sensor structure. Therefore, we propose that the action of HWTX-IV trapping hNav1.7 DIIS4 in the closed state might be separated into two successive steps: HWTX-IV first interacts with Glu-818 and then interacts with the other four residues deeper in the voltage sensor structure. Interestingly, Glu-818 in Nav1.7 corresponds to Glu-276 in Kv1.2 and Asn-283 in Kv2.1, respectively (18, 25), and based on the proposed orientation of these potassium channel residues in the open configuration, the side chain of hNav1.7 Glu-818 rotates into the protein interior of the DII voltage sensor (or possibly into the lipid membrane) in the model of the activated state. Therefore, our interaction model predicts that HWTX-IV would not be able to favorably...
interact with Glu-818 in the activated state and would be likely to dissociate from sodium channels following activation of the voltage sensor. This prediction from homology modeling is consistent with our previous experimental work, in which the Nav1.7-HWTX-IV complex could be dissociated by strong depolarization, after which reinhibition of hNav1.7 occurred with the same time course as the initial inhibition (16).

The S3b-S4 region in the voltage sensors of voltage-gated ion channels, referred to as the voltage sensor paddle, has been deemed to be a major determinant of toxin sensitivity (23, 26–28). The voltage sensor paddle regions of domains II and IV are only 30% identical (Fig. 6A). One possibility is that toxins that interact with multiple paddles do so through distinct toxin-paddle interfaces, perhaps with different toxin residues interacting with distinct motifs on the different paddles (28). However, our data show that the ability of HWTX-IV to interact with DIV can be substantially modified by simply modifying the five residues in DIV that correspond to the five residues identified as critical molecular determinants of HWTX-IV binding to DII. Each of the five critical residues in the DII voltage sensor and the corresponding residues in DIV modulate the interaction of their respective voltage sensor with HWTX-IV in an identical manner. Moreover, consistent with the observation for the DII voltage sensor, the amino acid at position 1588, corresponding to Glu-818 in DII, directly determines the “all-or-none” toxin sensitivity of the DIV voltage sensor. As can be seen in Fig. 6, only two of the five critical residues (Glu-811 and Glu-818) are not conserved in hNav1.7 DIV. The first corresponds to Gly-1588. However, the mutation G1588E by itself failed to significantly alter the resistance of hNav1.7 DIV to HWTX-IV (Fig. 7) and only is able to impact the effects of HWTX-IV if the crucial glutamine corresponding to Glu-818 is present in DIV. These results suggest that the HWTX-IV-binding motif of the DII voltage sensor, possibly generating a Nav1.7 isoform-specific modifier that selectively inhibits activation of Nav1.7 channels. Our increased understanding of the molecular determinants of toxin interactions with Nav1.7 and other VGSCs should aid the rational design of enhanced voltage-gating modifiers.

**Acknowledgment**—We thank Dr. Marie-France Martin-Eauclaire for generously providing the scorpion β-toxin CssIV.

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