Mutant cycle analysis with modified saxitoxins reveals specific interactions critical to attaining high-affinity inhibition of hNaV1.7

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Improper function of voltage-gated sodium channels (NaVs), obligatory membrane proteins for bioelectrical signaling, has been linked to a number of human pathologies. Small-molecule agents that target NaVs hold considerable promise for treatment of chronic disease. Absent a comprehensive understanding of channel structure, the challenge of designing selective agents to modulate the activity of NaV subtypes is formidable. We have endeavored to gain insight into the 3D architecture of the outer vestibule of NaVs through a systematic structure–activity relationship (SAR) study involving the bis-guanidinium toxin saxitoxin (STX), modified saxitoxins, and protein mutagenesis. Mutant cycle analysis has led to the identification of an acetylated variant of STX with unprecedented, low-nanomolar affinity for human NaV1.7 (hNaV1.7), a channel subtype that has been implicated in pain perception. A revised toxin-receptor binding model is presented, which is consistent with the large body of SAR data that we have obtained. This new model is expected to facilitate subsequent efforts to design isoform-selective NaV inhibitors.

M odulation of action potentials in electrically excitable cells is controlled by tight regulation of ion channel expression and distribution. Voltage-gated sodium ion channels (NaVs) constitute one such family of essential membrane proteins, encoded in 10 unique genes (NaV1.1–NaV1.9, NaV9) and further processed through RNA splicing, editing, and posttranslational modification. Sodium channels are comprised of a large (∼260 kDa) pore-forming α-subunit coexpressed with ancillary β-subunits. Misregulation and/or mutation of NaVs have been ascribed to a number of human diseases including neuropathic pain, epilepsy, and cardiac arrhythmias. A desire to understand the role of individual NaV subtypes in normal and aberrant signaling motivates the development of small-molecule probes for regulating the function of specific channel isoforms (1–4).

Nature has provided a collection of small-molecule toxins, including (+)-saxitoxin (STX, I) and (-)-tetrodotoxin (TTX), which bind to a subset of mammalian NaV isoforms with nanomolar affinity (5–7). Guanidinium toxins inhibit Na+ influx through NaVs by occluding the outer pore above the ion selectivity filter (site 1). This proposed mechanism for toxin block follows from a large body of electrophysiological and site-directed mutagenesis studies (Fig. L4 and refs. 8–10). The detailed view of toxin binding, however, is unsupported by structural biology, as no high-resolution structure of a eukaryotic NaV has been solved to date (11–16). NaV homology models, constructed based on X-ray analyses of prokaryotic NaV and K+ voltage-gated channels, do not sufficiently account for experimental structure–activity relationship (SAR) data (6, 17–20), and the molecular details underlying distinct differences in toxin potencies toward individual NaV subtypes remain undefined (5, 6, 21–23). The lack of structural information motivates a comprehensive, systematic study of toxin–protein interactions.

Double-mutant cycle analysis has proven an invaluable experimental method for assessing protein–protein, protein–peptide, and protein–small-molecule interactions in the absence of crystallographic data (Fig. 1B and Fig. S1 and refs. 9, 10, and 24–31). Herein, we describe mutant cycle analysis with NaVs using STX and synthetically modified forms thereof. Our results are suggestive of a toxin–NaV binding pose distinct from previously published views. Our studies have resulted in the identification of a natural variant of STX that is potent against the STX-resistant human NaV1.7 isoform (hNaV1.7). Structural insights gained from these studies provide a foundation for engineering guanidinium toxins with NaV isoform selectivity.

Results

Methylated STX Analogs. Double-mutant cycle analysis of the STX binding site necessitates both ligand and protein modification. A collection of isomeric toxins bearing a methyl substituent group was targeted to ensure that relative differences in solvation energies between these compounds would be minimal. Varies of ligand affinity could thus be attributed to steric factors governing toxin–NaV interactions (32, 33). Previous bis-guanidinium toxin–NaV mutant cycle analyses have been limited to naturally occurring derivatives (9, 10). Accordingly, five monomethylated analogs (2–5 and 8, Table 1), along with two dimethylated variants (6 and 9) and decarbamoyl-STX (dcSTX. 7) were prepared from L-serine methyl ester using routes adapted from our previously disclosed studies (34). Although naturally occurring STX derivatives with substitution at N7 or C13 appear in nature, to our knowledge no N9- or C10- natural product congeners have been identified (35–37).

Preliminary electrophysiological recordings to assess analog potencies were conducted with wild-type (WT) rat skeletal muscle α-subunit (rNaV1.4) heterologously expressed in CHO cells. Double-mutant cycle analysis was used to assess binding of analogs to rNaV1.4 (Fig. 1A and Table S1). Analog potency was assessed using a reporter construct of hNaV1.7 with the N9 region replaced with the rNaV1.4 N9 region (hNaV1.7-N9rNaV1.4) and the N10 region replaced with the rNaV1.4 N10 region (hNaV1.7-N10rNaV1.4). Potential bis-guanidinium toxin analogs were tested in two assays: (1) a whole-cell voltage-clamp assay in which potassium influx through NaVs was measured (22) and (2) a filtration assay in which relative differences in solvation energies between these compounds would be minimal. Varies of ligand affinity could thus be attributed to steric factors governing toxin–NaV interactions (32, 33). Previous bis-guanidinium toxin–NaV mutant cycle analyses have been limited to naturally occurring derivatives (9, 10). Accordingly, five monomethylated analogs (2–5 and 8, Table 1), along with two dimethylated variants (6 and 9) and decarbamoyl-STX (dcSTX. 7) were prepared from L-serine methyl ester using routes adapted from our previously disclosed studies (34). Although naturally occurring STX derivatives with substitution at N7 or C13 appear in nature, to our knowledge no N9- or C10- natural product congeners have been identified (35–37).

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Significance

Chronic pain plagues at least 50 million Americans and has an estimated annual cost in excess of $200 billion. Consequently, there exists significant interest in developing effective, nonnarcotic analgesics. Drugs that target individual voltage-gated sodium channel (NaV) subtypes have appreciable therapeutic potential as pain medicines. Our work uses small-molecule design and protein mutagenesis to gain insight into the molecular architecture of the ion conduction pore of NaVs. These studies have revealed structural differences in the outer mouth of the channel that potentiate the binding of guanidinium toxins. Such findings are helping advance the preparation of isoform-selective NaN antagonists.

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cells (Table 1 and Fig. S2). Consistent with previous observations, N21 mono- and dimethylcarbamoylated derivatives 5 and 6 retain high affinity for nNav1.4, with IC50 values of 1.9 ± 0.1 nM and 4.5 ± 0.2 nM, respectively (38–41). In contrast, the introduction of a single methyl group on the tricyclic bisguanidinium core of STX significantly reduces potency, between 74- and 300-fold for N7, N9, and C13 substitution (2–4). Concentration-response measurements for dcSTX 7 and C13-OAc STX 8 give intermediate IC50 values (35 ± 1.4 nM and 83 ± 3.9 nM, respectively), whereas the potency of isobutyrate STX is substantially diminished (338 ± 9.3 nM). The 12-fold reduction in affinity of dcSTX 7 compared with STX is consistent with previous literature reports (7, 35). These data motivate further studies to interrogate the binding pose of guanidinium toxins in the outer vestibule of the conductance pore.

**Mutant Cycle Analysis with Site 1 Mutants.** To localize precise interactions responsible for high-affinity STX block of the channel, nine single-point Nav1.4 mutants were initially prepared and characterized (Fig. 2, SI Discussion, and Fig. S1). Alanine residues were systematically introduced into the pore-loop region, above the Na+ selectivity filter (i.e., DEKA loop), in all four domains (DI-DIV) of the channel. Given the large perturbations to STX binding previously reported when key carbohydrate residues, E403 and E758, are neutralized, only aspartate mutations were introduced at these positions (8, 10). Single-point mutants generated measurable macroscopic currents (1–6 nA) and exhibited typical NaV gating kinetics when expressed in CHO cells. For each toxin-channel pair, concentration-response curves for INa block were obtained (Figs. S2 and S3). All amino acid mutations reduced the affinities of STX and methylated derivatives (2–6 and 8) compared with WT rNav1.4 (Table S1). Values of ΔΔEΩ obtained for methylated toxins are represented in Fig. 2. In select cases, IC50 values were estimated to be >50 μM and, consequently, ΔΔEΩ values were not determined.

No significant coupling interactions were measured between N7-Me STX 2 and N9-Me STX 3 and the pore-lining amino acids highlighted in Fig. 2 (ΔΔEΩ < 0.4 kcal/mol). By contrast, the C10-Me compound 4 displayed ΔΔEΩ values of 1.7 ± 0.1 and 1.3 ± 0.1 kcal/mol with DI residues Y401 and E403, respectively. These interaction energies were the largest recorded among toxin derivatives 2–8 with any of the nine Ala mutants. Conversely, the relative affinities of N-methylcarbamate–modified toxins 5 and 6 against Nav1.4 mutants were similar to STX (ΔΔEΩ < 0.5 kcal/mol).

Two toxin derivatives, dcSTX 7 and C13-OAc STX 8, displayed notable coupling interactions with DIII residues. In experiments with the former compound, W1239A and Y401A afforded the largest ΔΔEΩ values (ΔΔEΩ of 0.9 ± 0.1 and 0.8 ± 0.1 kcal/mol, respectively), whereas ester 8 exhibited the most substantial coupling energies with W1239A and D1241A (ΔΔEΩ = 0.6 ± 0.1 and 0.6 ± 0.1 kcal/mol, respectively).

Additional Mutagenesis Reveals Localized Interactions Between C13-Modified Toxins and DIII. Amino acid substitution of DIII p-loop residues M1240 and D1241 has been found to significantly influence guanidinium toxin potency (6, 8). In previous work from our laboratory, STX resistance in primate Nav1.7 was noted and ascribed to a natural variation of two pore-forming amino acids at positions 1240 and 1241. Given the modest coupling observed between C13-modified toxins 7 and 8 with DIII alanine mutants, as well as an interest in restoring bis-guanidinium toxin affinity to hNav1.7, nine additional single-point 1240 and 1241 NaV1.4 mutants with amino acids of varying steric size and polarity were expressed (Fig. 3, Fig. S4, and Table S2). Position 1239 was left unperturbed, because substitution of this residue considerably destabilizes STX and STX derivative binding (all IC50 < 1.9 μM with W1239A).

Within the series of single-point M1240 mutants, threonine, serine, and isoleucine residues had the most demonstrable influence on dcSTX 7 and C13-OAc STX 8 affinity (Fig. 3A). For the former two amino acids, the magnitude of ΔΔEΩ was markedly greater for acetylated toxin 8 than for decarbamoylated toxin 7 (M1240T, ΔΔEΩ = 2.7 ± 0.1 kcal/mol versus 0.9 ± 0.1 kcal/mol; M1240S, ΔΔEΩ = 1.4 ± 0.1 kcal/mol versus 0.6 ± 0.1 kcal/mol). Mutation of M1240 to an H-bond donor did not adversely influence the affinity of derivatives 7 and 8 to the same extent as STX, which displayed 29- and 63-fold reduced potency against Thr and Ser mutants, respectively (Fig. S4 and Table S2). For all other M1240 mutants, binding of compound 7 was destabilized relative to STX, as revealed by negative interaction energies (ΔΔEΩ < −0.8 to −0.2 kcal/mol; Table S2). Conversely, mutant cycle analysis with acetyl ester derivative 8 gave coupling values ranging from −0.1 to 1.0 kcal/mol for all other M1240 single-point mutants. The increasing magnitude of ΔΔEΩ for analog 8 follows the trend Asn < Ala < Val < Ile < Ser < Thr.

At position 1241, the most favorable DIII interactions were recorded for isoleucine, threonine, and asparagine mutants with compound 8 (ΔΔEΩ of 1.5 ± 0.1, 1.0 ± 0.1, and 0.9 ± 0.1 kcal/mol, respectively; Fig. 3A). Replacement of D1241 with a neutral

### Table 1. Affinities of STX and analogs 2–8 for nNav1.4 determined by whole-cell voltage-clamp electrophysiology

<table>
<thead>
<tr>
<th>Compound</th>
<th>R²</th>
<th>R³</th>
<th>R¹⁰</th>
<th>R¹³</th>
<th>WT rNav1.4 IC₅₀, nM</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C(O)NH₂</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>C(O)NH₂</td>
<td>370 ± 50</td>
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<tr>
<td>3</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>C(O)NH₂</td>
<td>879 ± 53</td>
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<tr>
<td>4</td>
<td>H</td>
<td>H</td>
<td>Me</td>
<td>C(O)NH₂</td>
<td>215 ± 16</td>
</tr>
<tr>
<td>5</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C(O)NH₂</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C(O)NM₂</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>35 ± 1.4</td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C(O)Me</td>
<td>83 ± 3.9</td>
</tr>
<tr>
<td>9</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C(O)CH₂Me</td>
<td>338 ± 9.3</td>
</tr>
</tbody>
</table>
studies were performed using the corresponding double-point calculations are given in Fig. S2 and Table S1.

Because both C13 derivatives 7 and 8 displayed ΔΔEΩ absolute values are shown in units of kilocalories per mole. Dose–response curves, relative inhibition constants (IC50), and SEM for calculations are given in Fig. S2 and Table S1.

mutant residue (Ala and Ile) resulted in an eight- and 22-fold decrease in STX IC50 values, compared with a ninefold and 1.5-fold reduction in the affinity of ester 8. With the exception of the D1241I mutant, the relative potency of dcSTX was measured, a value similar to that obtained from experiments with mutant cycle analysis with N21-dimethyl STX and Tables S2 and S3). The largest ΔΔEΩ was obtained from experiments with two additional NaV1.4 double mutants, M1240A/D1241A and M1240T/D1241I, and compounds 6–9 followed analogous trends (Fig. S4 and Tables S2 and S3).

To further define toxin–DIII interactions, additional mutant cycles with isobutyrate ester 9 were analyzed (Table S3). Binding energies for toxin derivatives 6 and 8 with rNaV1.4 were used as starting values for these cycles to directly probe steric constraints within DIII. Coupling of the branched ester 9 to M1240T/D1241I was modest compared with dimethylcarbamate 6 (ΔΔEΩ = 0.7 ± 0.1 kcal/mol) but significantly diminished (ΔΔEΩ = −2.2 ± 0.1 kcal/mol) relative to C13-acetate derivative 8.

Previously reported docking models for STX have positioned C10 and C11 proximal to the pore loop of DIII (6, 17–20). Accordingly, C10-Me STX was tested against NaV1.4 M1240T/D1241I in addition to other DIII mutant constructs (Fig. 3B, Fig. S4, and Table S2). The largest ΔΔEΩ of 0.8 kcal/mol was obtained for 4 against the 1240T/1241I double mutant, a value that is decidedly smaller than coupling energies calculated for 6, 7, and 8 (Fig. 3B).

C13-OAc STX 8 Retains High Affinity for hNaV1.7. Given the potency of C13-OAc STX 8 for the NaV1.4 M1240T/D1241I double mutant (IC50 = 26 ± 2.3 nM), this compound was tested against human nociceptive NaV1.7 (hNaV1.7) stably expressed in HEK cells. An IC50 of 19 ± 1.7 nM (Fig. 3C and D and Fig. S5) was measured, a value similar to that obtained from experiments

**Fig. 2.** Mutant cycle analysis with compounds 2–8 and NaV single-point mutants. ΔΔEΩ absolute values are shown in units of kilocalories per mole. Dose–response curves, relative inhibition constants (IC50), and SEM for calculations are given in Fig. S2 and Table S1.

**Fig. 3.** Additional mutagenesis of M1240 and D1241 reveals interactions responsible for coupling of C13-substituents to DIII. (A) Absolute values of ΔΔEΩ (kilocalories per mole) for compounds 7 and 8 with M1240 and D1241 single-point mutants. (B) Absolute values of ΔΔEΩ (kilocalories per mole) for compounds 4, 6, 7, and 8 with the rNaV1.4 M1240T/D1241I double-point mutant. (C) Concentration response curves for current inhibition of rNaV1.4 (solid line) and hNaV1.7 (dotted line) by 8 determined by whole-cell voltage-clamp electrophysiology. (D) Current recordings elicited by a 10-ms voltage step from −100 to 0 mV before (black) and following (red) application of 25 nM 8 to CHO cells expressing rNaV1.4 (Top) and HEK cells expressing hNaV1.7 (Bottom). WT rNaV1.4 and STX were used as references in ΔΔEΩ calculations.
with Na\textsubscript{V}1.4 M1240T/D1241I. By comparison with binding data recorded with other WT isoforms (rNa\textsubscript{V}1.2, rNa\textsubscript{V}1.4, and hNa\textsubscript{V}1.5), C13-OAc STX is two- to 240-fold more selective for the 1.7 channel (Fig. S5).

**Discussion**

Small molecules that functionally “knock out” specific Na\textsubscript{V} isoforms hold promise as tools for exploring the role of individual channel subtypes in modulating compound action potentials. The development of such inhibitors through rational design, however, is challenged by the absence of crystallographic data for eukaryotic Na\textsubscript{V}s. To obtain structural insights into the molecular determinants that govern high-affinity Na\textsubscript{V} block by bis-guanidinium toxins, mutant cycle analysis was performed with six, nonnatural methylated saxitoxin derivatives, as well as dcSTX and C13-OAc STX, 18 single-point and 3 double-point Na\textsubscript{V}1.4 mutants. Significant coupling energies (≥1 kcal/mol) were calculated for multiple toxin–mutant channel pairs (Figs. 2 and 3). These data have led us to propose a new toxin–receptor docking model.

An initial screen of p-loop mutants (Fig. 2) with modified STX analogs showed evident coupling interactions between residues in D1 (Y401A and E405D) and the C10-Me derivative 4. Additionally, compounds altered at C13, dcSTX 7 and C13-OAc STX 8, displayed modest coupling with alanine mutants of DIII residues W1239, M1240, and D1241. These results, together with a previous report by our laboratory detailing STX–hNa\textsubscript{V}1.7 binding and the importance of DIII residues in defining guanidinium toxin affinity (6), prompted further study of compounds 7 and 8 against a number of M1240 and D1241 single-point mutants (Fig. 3C). In these experiments, significant interaction energies (≥1.4 kcal/mol) were computed for C13-OAc STX 7 with M1240T and M1240S for both dcSTX 7 and C13-OAcSTX 8 with D1241I (≥1.3 kcal/mol).

Differences in the polarity and steric size of C13-modified toxins 7 and 8 compared with STX, as well as within DIII mutant channels, provide rationale for the relative potencies of the three ligands. Low-nanomolar block of Na\textsubscript{V}s by STX requires a large, hydrophobic amino acid residue at position 1240 and an acidic residue at position 1241. Replacing M1240 with smaller, polar groups such as Thr and Ser substantially perturbs STX binding, decreasing respective IC\textsubscript{50} values by 29-fold and 63-fold. Conversely, C13-OAc STX 7 binds with threefold increased potency to the M1240T mutant channel. In the case of position 1241, neutralization of the Asp residue to Ile, Asn, or Glu has a more profound influence on STX binding than on the acetate ester analog.

The striking results with dcSTX 7 and C13-OAc STX 8 and DIII single-point mutants compelled us to perform subsequent investigations of M1240/D1241 double-point mutant channels (Fig. 3B). Ester 7 binds to rNa\textsubscript{V}1.4 M1240T/D1241I with high affinity, exhibiting threefold greater potency toward this double mutant than WT rNa\textsubscript{V}1.4. Compared with STX, acetate 8 is 44 times more potent against this same mutant channel. These observed differences in affinity are recapitulated against native hNa\textsubscript{V}1.7 (Fig. 3 C and D), which contains threonine and isoleucine at structurally equivalent positions to 1240 and 1241 in rNa\textsubscript{V}1.4. To the best of our knowledge, compound 8 represents the first example of an STX variant with low-nanomolar potency to hNa\textsubscript{V}1.7.

Previous homology models of the Na\textsubscript{V} pore with STX bound have posited three key electrostatic interactions between (i) the five-membered ring guanidinium and E755, (ii) the six-membered ring guanidinium and D1532, and (iii) the C12-hydrated ketone and E758. Thus, all three principal contacts between toxin and receptor are maintained.

**Pharmacology**

**Chemistry**

**Fig. 4.** Docking of STX at site 1 of rNa\textsubscript{V}1.4 in the proposed (A) and proposed (B) orientations. The four domains are shown in cartoon representations and colored orange (DI), red (DII), gray (DIII), and teal (DIV). Images highlighting the differences between electrostatic potential surfaces of STX (C) and C13-OAcSTX (D) docked to the rNa\textsubscript{V}1.4 outer pore in the proposed orientation. Equivalent images showing STX (E) and C13-OAc STX (F) in the binding pocket of the M1240T/D1241I double mutant. Depicted potential range is −20 (red) to +5 kT (blue). Toxin structures generated using OMEGA version 2.5.1; docking performed with OEDocking version 3.0.1 (OpenEye Scientific Software, www.eyesopen.com).

Shape complementarity and hydrogen bonding between the C13-carbamate in STX and DIII D1241 favor close positioning of these groups. In this same orientation, binding of C13-OAc STX 8 to WT Na\textsubscript{V}1.4 is destabilized relative to STX owing to the less polar nature of the ester group (Fig. 4D and Dataset S1). Conversely, in the M1240T/D1241I mutant channel, the strength of the C13-carbamate interaction with DIII residues is mitigated (Fig. 4E). The ester derivative 8 displays higher affinity for this double mutant (threefold relative to rNa\textsubscript{V}1.4), presumably due to the increased hydrophobicity of the binding pocket and the presence of a hydrogen bond donor in Thr (Fig. 4F and Dataset S2). In this model, differences in binding affinity between the acetate 8 and isobutyrate 9 may be ascribed to the small volume cleft between DIII and DIV, which does not easily accommodate the sterically large, branched isopropyl group. On the other hand, the perturbation to binding caused by the planar N21-dimethylated carbamate (i.e., 6) is minimal.

More than one explanation may account for differences between our revised STX docking model and those previously published. In prior work, specific constraints imposed on bis-guanidinium contacts with carbonylate residues E755, E758, and D1532 limited the sampling of STX poses (6, 20). By relaxing these constraints, we have identified an alternative ligand binding model that accounts for our SAR data as well as previous results of this type (9, 10). It is possible, however, that STX and
STX analogs adopt more than one high-affinity binding arrangement within the mouth of the channel. In native rNaV1.4, the canonical STX binding model may indeed be preferred, but in certain mutant NaVαs or with specific STX derivatives the docking orientation suggested by our findings could be favored (42–44).

The potential of bis-guanidinium toxins to occlude site 1 through DI and DIII p-loop amino acids that are not explained by existing models of STX lodged in the channel pore. These data have led us to propose an alternative bis-guanidinium toxin binding model, which could aid future efforts to design subtype-selective inhibitors of NaV function around this chemotype.

Access to STX derivatives through chemical synthesis has facilitated a systematic investigation of toxin–receptor interactions that govern potnet NaV block. Mutant cycle analysis has revealed unexpected nearest-neighbor contacts between modified toxins and DI and DIII p-loop amino acids that are not explained by existing models of STX lodged in the channel pore. These data have led us to propose an alternative bis-guanidinium toxin binding model, which could aid future efforts to design subtype-selective inhibitors of NaV function around this chemotype.

Materials and Methods

Toxin Synthesis. Toxins were prepared from L-serine methyl ester according to a previously reported route (34). All reagents used were obtained commercially unless otherwise noted. Final compounds were purified using semipreparative HPLC performed on a Varian ProStar model 210. Characterization information for modified toxins can be found in SI Materials and Methods.

Cell Culture and Electrophysiology. Electrophysiology experiments were conducted using CHO cells transiently expressing the α-subunit of rat NaV1.4 (rNaV1.4,1.4) or mutant thereof (45), human NaV1.5 (hNaV1.5), rat NaV1.2 (rNaV1.2), or HEK cells stably expressing the α-subunit of human NaV1.7 (hNaV1.7). Cell culture was performed as described previously (38). Additional information can be found in SI Materials and Methods.

Sodium currents were measured using the patch-clamp technique in the whole-cell configuration with an Axopatch-200b amplifier (Axon Instruments), as previously described by Moran et al. (46). Borosilicate glass micropipettes (Sutter Instruments) were fire-polished to a tip diameter yielding a resistance of 1.2–3.0 MΩ in the working solutions. For CHO cells, the micropipette was filled with 40 mM NaF, 1 mM EDTA, 20 mM Heps, and 125 mM CsCl. The external solution had the following composition: 140 mM NaCl, 3 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM Hepes. The pH of all solutions was adjusted to 7.4 with 50 wt % aqueous CsOH.


