Identification of New Batrachotoxin-sensing Residues in Segment III S6 of the Sodium Channel*§

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Ion permeation through voltage-gated sodium channels is modulated by various drugs and toxins. The atomistic mechanisms of action of many toxins are poorly understood. A steroidal alkaloid batrachotoxin (BTX) causes persistent channel activation by inhibiting inactivation and shifting the voltage dependence of activation to more negative potentials. Traditionally, BTX is considered to bind at the channel-lipid interface and allosterically modulate the ion permeation. However, amino acid residues critical for BTX action are found in the inner helices of all four repeats, suggesting that BTX binds in the pore. In the octapeptide segment IFGSFFTL in III S6 of a cockroach sodium channel BgNaV, besides Ser 3i15 and Leu 3i19, which correspond to known BTX-sensing residues of mammalian sodium channels, we found that Gly 3i14 and Phe 3i16 are critical for BTX action. Using these data along with published data as distance constraints, we docked BTX in the Kv1.2-based homology model of the open BgNaV channel. We arrived at a model in which BTX adopts a horseshoe conformation with the horseshoe plane normal to the pore axis. The BTX ammonium moiety interacts with known BTX-sensing residues in all four repeats. Oxygen atoms at the horseshoe inner surface constitute a transient binding site for permeating cations, whereas the bulky BTX molecule would resist the pore closure, thus causing persistent channel activation. Our study reinforces the concept that steroidal sodium channel agonists bind in the inner pore of sodium channels and elaborates the atomistic mechanism of BTX action.

Voltage-gated sodium channels (NaV) are responsible for the rapid rising phase of the action potential in nerve and muscle cells. The pore-forming α-subunit of NaV channels contains four repeats, and each repeat has six transmembrane helices (S1–S6). The S1–S4 helices form the voltage sensor domain.

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The abbreviations used are: BTX, batrachotoxin; MC, Monte Carlo.

We designate residues using labels that are universal for P-loop channels (Table 1). A residue label includes the repeat number (1–4), segment type (p, P-loop; i, the inner helix; and o, the outer helix) and the relative number of the residue in the segment.

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Identification of BTX-sensing Residues in Segment IIS6

than one BTX receptor (17, 26–28). Although allosteric effects could explain the modifications of channel conductance, ion selectivity, and gating by BTX (29), another possibility could be that BTX is directly exposed to the permeation pathway (5, 30).

In a structural model of the sodium channel with BTX exposed to the permeation pathway, the pore should accommodate a large BTX molecule. Furthermore, the model should suggest how ions permeate through the pore in which a bulky steroidal agonist is bound. Homology models of sodium channels, which are based on x-ray structures of distantly related potassium channel templates, are not expected to be precise enough to unambiguously predict a ligand-binding model based solely on the computed toxin-channel binding energy. Additional experimental constraints are desirable to elaborate details of BTX binding inside of the pore. Systematic mutations of residues around known BTX-sensing residues may reveal additional amino acids involved in BTX binding and thus provide further experimental constraints to dock BTX in the sodium channel model.

In this study, we first mutated six residues flanking the BTX-sensing Ser3115 in the octapeptide segment IFGSFTTL (Ser3115 and Leu3119 underlined) of a cockroach sodium channel BgNa1-1a (31) and explored the effects of mutations on the channel gating in the presence and absence of BTX. We identified Phe3116 and a putative gating-hinge glycine Gly3114 as new BTX-sensing residues. Using these and published data as distance constraints, we explored different possible binding modes of BTX in the Kv1.2-based model of the open BgNa channel and arrived at a new model, which is consistent with most of the available experimental data on BTX actions on sodium channels. We further tested the new model by generating seven additional mutations in the four pore-forming repeats and found that most of the mutations exhibited the BTX sensitivity in agreement with the model.

EXPERIMENTAL PROCEDURES

Expression of BgNa, Sodium Channels in Xenopus Oocytes—The procedures for oocyte preparation and cRNA injection are identical to those described previously (32). For robust expression of the BgNa, sodium channels, cRNA was coinjected into oocytes with Drosophila melanogaster tipE cRNA (1:1 ratio), which enhances the expression of insect sodium channels in oocytes (33, 34).

Electrophysiological Recording and Analysis—The voltage dependence of activation and inactivation was measured using the two-electrode voltage clamp technique. Methods for two-electrode recording and data analysis were similar to those described previously (35). Sodium currents were measured with a Warner OC725C oocyte clamp (Warner Instrument, Hamden, CT) and processed with a Digidata 1322A interface (Axon Instruments, Inc., Foster City, CA). Data were sampled at 50 kHz and filtered at 2 kHz. Leak currents were corrected by p/4 subtraction, a technique commonly used to correct for the passive membrane current of a cell. pCLAMP software (version 8.2; Axon Instruments, Inc., CA) was used for data acquisition and analysis. The maximal peak sodium current was limited to <2.0 μA to achieve optimal voltage control by adjusting the amount of cRNA and the incubation time after injection.

The voltage dependence of sodium channel conductance (G) was calculated by measuring the peak current at test potentials ranging from −80 mV to +65 mV in 5-mV increments and divided by (V − Vrev), where V is the test potential, and Vrev is the reversal potential for sodium ion. Peak conductance values were normalized to the maximal peak conductance (Gmax) and fitted with a two-state Boltzmann equation of the form $G/G_{max} = (1 + \exp(V - V_h)/k)^{-1}$, or with the sum of two such expressions, in which V is the potential of the voltage pulse, V_h is the voltage for half-maximal activation, and k is the slope factor.

The voltage dependence of sodium channel inactivation was determined by using 100-ms inactivating prepulses ranging from −120 mV to 0 mV in 5-mV increments from a holding potential of −120 mV, followed by test pulses to −10 mV for 20 ms. The peak current amplitude during the test depolarization was normalized to the maximum current amplitude and plotted as a function of the prepulse potential. Data were fitted with a two-state Boltzmann equation of the form $I/I_{max} = (1 + \exp(V - V_o)/k)^{-1}$, in which I is the peak sodium current, $I_{max}$ is the maximal current evoked, V is the potential of the voltage prepulse, V_o is the half-maximal voltage for inactivation, and k is the slope factor.

BTX was a generous gift from John Daly (National Institutes of Health, Bethesda, MD). A stock solution of BTX (1 mM) was dissolved in dimethyl sulfoxide. The working concentration was prepared in ND96 recording solution just prior to the experiments. The concentration of dimethyl sulfoxide in the final solution was <0.5%, which had no effect on the function of sodium channels in the experiments. The method for application of chemicals in the recording system was identical to that described by Tan et al. (35). The effects of BTX were measured 10 min after toxin application.

Homology Model—We have built a homology model of the open BgNa1-1a channel based on the crystal structure of the open potassium channel Kv1.2 (12). A model of the closed BgNa1-1a, Kv1.2, and KcsA sequences were aligned (Table 1) as before (3, 37). The extracellular loops, which are far from BTX-sensing residues, were not included in the model. The P-loops were modeled as described in Ref. 38. The Monte Carlo-energy minimization method (39) was used to optimize the channel model and dock BTX. The energy was calculated using the AMBER force field (40, 41) and a solvent exposure- and distance-dependent dielectric function (42). Atomic charges at the BTX molecule were calculated using the AM1 method (43) realized in MOPAC. The energy was minimized in the space of generalized coordinates (44, 45). Bond angles were varied in BTX but not in the protein. Program SCWRL3 (46) was used to assign starting conformations of the channel side chains.

BTX Docking—BTX binding modes, which are consistent with mutational data, were imposed by distance constraints (supplemental Table S1). A constraint is a flat-bottom parabolic penalty function added to the energy expression. When the distance between a given BTX atom and a given atom in the BTX-sensing residue exceeds the upper limit of the constraint (5 Å in this study), the penalty contribution to the energy...
increases sharply, with the force constant of 100 kcal-mol$^{-1}$-Å$^{-1}$. A flat-bottom constraint ensures proximity between two atoms but does not impose specific contacts between them (e.g. an H-bond or a cation-π contact). To search for low energy binding modes of BTX, we employed our three-stage flexible docking protocol (42). In the first stage, a library of BTX conformers was generated by randomly sampling BTX torsions, followed by energy minimizations to ensure that all the rings were closed. Thousands of BTX conformations were generated, and the 10 lowest energy conformations were collected for docking. The lowest energy conformer in the library corresponds to the x-ray structure of BTX. In the second stage, the position and orientation of each BTX conformer in the library was searched for low energy binding modes of BTX, employing a three-stage flexible docking protocol (42). In the first stage, a library of BTX conformers was generated by randomly sampling BTX torsions, followed by energy minimizations to ensure that all the rings were closed. Thousands of BTX conformations were generated, and the 10 lowest energy conformations were collected for docking. The lowest energy conformer in the library corresponds to the x-ray structure of BTX. In the second stage, the position and orientation of each BTX conformer in the library was sampled 200,000 times by assigning random values to six rotation angles in the protein side chains and in BTX were torsionally sampled. Finally, all distance constraints were removed and the model was MC-minimized to check its intrinsic stability. If during the final MC minimization BTX moved away from the model was used as a BTX binding model consistent with the constraints-imposed binding mode, this mode was excluded from further analysis.

RESULTS

G$^{314}$A and F$^{316}$A/K Substitutions Reduce Action of BTX on BgNa$_{1-1a}$ Channel—Previously, we reported that two amino acid residues, Ser$^{315}$ and Leu$^{319}$, are critical for the action of BTX on the cockroach sodium channel (10). To determine whether other residues flanking these two BTX-sensing residues in the IFGSFTTL segment (Ser$^{315}$ and Leu$^{319}$ are underlined) are also involved in the action of BTX, we examined the effect of BTX on six mutant BgNa$_{1-1a}$ channels, I$^{312}$A, F$^{313}$A, G$^{314}$A, F$^{316}$A, F$^{316}$A, and T$^{318}$A, that were made previously for another study (10). None of the substitutions alter channel gating except for G$^{314}$A, which shifted the voltage dependence of activation in the depolarizing direction by $\pm$12 mV (10).

In agreement with results reported for mammalian sodium channels, e.g. (16, 24), BTX inhibited inactivation inducing a non-inactivating current and a tail current upon repolarization and shifted the voltage dependence of activation to more negative membrane potentials (Fig. 1, A and B). The BTX effects on BgNa$_{1-1a}$ channels were incomplete because two voltage-dependent components of activation were observed: one with a voltage dependence similar to unmodified channels and the other with the negatively shifted voltage dependence of BTX-modified channels (Fig. 1B). At 500 nm, 44% of BgNa$_{1-1a}$ channels were modified by BTX, and BTX did not alter the amplitude of peak current. The BTX effect on inactivation is also evident in the voltage dependence of steady-state inactivation where the foot of the inactivation curve at the depolarizing potentials was lifted in the presence of BTX (Fig. 1C).

Alanine substitutions of Gly$^{314}$ and Phe$^{316}$ significantly reduced the percentage of the BTX-induced tail current and non-inactivating current (Fig. 1, D and E). In contrast, F$^{312}$A, F$^{313}$A, and T$^{318}$A did not (Fig. 1, D and E). Furthermore, a lysine substitution of Phe$^{316}$ almost completely abolished the action of BTX (Fig. 1, D and E). Similarly, the BTX effect was not observed in the voltage dependence of inactivation of G$^{314}$A and F$^{316}$A/K channels (Fig. 1, F–H). (Note a slightly lifted foot of the inactivation curve in the G$^{314}$A and F$^{316}$A channels.) Consistent with these results, substitutions G$^{314}$A and F$^{316}$A, but not T$^{318}$A, F$^{313}$A, and T$^{318}$A, significantly reduced the percentage of BTX-modified channels (Fig. 1B). No F$^{316}$A/K channels were modified by BTX (500 nm) (Fig. 1I). These results collectively demonstrated that G$^{314}$A and F$^{316}$A/K substitutions significantly reduced the effects of BTX on the BgNa$_{1-1a}$ channel.

BTX-bound Model of Sodium Channel—The BTX molecule has a hydrophobic and hydrophilic faces (Fig. 2) (5). Previously published data on BTX-sensing residues were rationalized in a model in which BTX extends along the pore axis, its hydrophobic face interacts with hydrophobic residues that line the inner pore, and the hydrophilic face contributes in the ion permeation pathway along with the hydrophilic residues of the channel (5, 6). Our present finding that phenylalanine Phe$^{316}$ is essential for BTX binding is inconsistent with the previously proposed orientation of BTX. This motivated us to elaborate an updated model, which would be consistent with all currently available experimental data on BTX action.

Hands-free docking of semiflexible BTX in the channel with flexible side chains is possible (42), but the lowest energy binding mode may or may not correspond to the native ligand-channel conformation. Indeed, even when high resolution x-ray structures of proteins are used to dock flexible ligands, the
Probability that the ligand conformation and orientation in the apparent global minimum would match those in the x-ray structure does not exceed 70% (47–49). The homology model of the sodium channel is obviously less precise than high resolution x-ray structures. Therefore, we sought various low energy binding modes of BTX, which are consistent with mutational data on BTX-channel interactions, by applying various combinations of distance constraints that bring different BTX moieties to BTX-sensing residues of the channel (see “Experimental Procedures”). Among many possibilities, we focused on those that satisfy the following criteria. First, the model should be stable after all distance constraints are removed and refining unconstrained MC minimization is performed. Second, the energy of the specific BTX binding mode should not exceed the energy of the apparent global minimum by more than 7 kcal/mol. Third, as many as possible experimentally known BTX-sensing residues should directly interact with the ligand. Fourth, in the model, BTX should not block the pore but should allow ion permeation through the BTX-bound channel. Fifth, the model should explain why a BTX-bound channel resists the activation gate closure (15). For each binding mode, which was initially imposed by the distance constraints, the 10 lowest energy complexes were collected and then refined without any constraints. Several constraint-imposed BTX binding modes, in which only part of the known BTX-sensing residues directly interact with BTX, are described in the supplemental data.
The binding mode in which many of the currently known BTX-sensing residues are in direct contact with BTX is shown in Fig. 3. This lowest energy BTX-channel complex was obtained by flipping BTX in the constraint-imposed model (supplemental Fig. S1F) by 180° around the vertical axis and by MC-minimizing the complex. In this binding mode, BTX adopts a horseshoe conformation with the horseshoe plane normal to the pore axis. The ligand ammonium group is engaged in cation-π interactions with the BTX-sensing residue Phe3i16, which was identified in the current study. We tested the axial and equatorial orientations of the ammonium hydrogen. In both orientations, the cation-π interactions are possible. The advantage of the axial orientation is that the BTX ammonium group donates an H-bond to Ser3i15, a known BTX-sensing residue (50). The carbonyl oxygen and pyrrole nitrogen of BTX accept H-bonds from Asn2i15 and Ser1i15, respectively, in agreement with the data that mutations of these amino acids affect BTX action (7, 8). Hydrophobic groups in the outer surface of the horseshoe interacts with the hydrophobic BTX-sensing residues Leu2i19 (25), Leu3i19 (50), and Phe4i15 (22). In addition to binding to BTX-sensing residues in the inner helices, BTX strongly interacts with Phe3p49 by its oxazepane ring. The latter BTX-sensing residue was initially predicted in the modeling study (5), and later, lysine and arginine substitutions of Phe3p49 were demonstrated to dramatically decrease BTX action (6). Most importantly, four oxygen atoms of BTX as well as pyrrole...
nitrogen atom and \( \pi \)-electrons of carbon atoms in the pyrrole ring line the inner surface of the horseshoe, which bends over the pore axis. In other words, the inner surface of the BTX horseshoe forms a hydrophilic arch inside the inner pore of the channel. Na\(^+\) ions would permeate through this arch and thus through the BTX-bound channel (Fig. 4, A and B).

In our model, BTX adopts the horseshoe conformation, which is about 2 kcal/mol less preferable than the global minimum found by MC minimization of BTX in vacuum. (The global minimum corresponds to the BTX conformation in the crystal.) The cause of the intramolecular BTX strain is electrostatic repulsion between the BTX ether oxygen in the linker between the pyrrole ring and steroidal core and the oxygen atom of the hydroxy substituent in the steroidal ring C. The BTX strain is compensated by strong attraction of BTX to BTX-sensing residues (Table 2) so that the BTX-channel complex shown in Figs. 3 and 4 remains stable upon MC minimization in the absence of distance constraints. This result suggests that the strained BTX may slightly widen the pore. Our homology model is not expected to be precise enough to simulate possible conformational rearrangements of the open channel upon BTX binding. But in

![Figure 4](image)
view of these data, the slow onset of the BTX effect may be due to BTX binding to low populated states of the channel in which the inner pore is wider than in the most populated open states.

To explore whether BTX would resist the inner pore closing, we have built a KcsA-based model of the closed BgNav1-1a and open states. N.A., not applicable.

We further generated alanine substitutions of three residues, which contribute energy to BTX binding in our model, and evaluated their BTX sensitivity (Table 2 and Fig. 5). In agreement with the model, mutant T3p48A has demonstrated a substantially decreased BTX sensitivity. However, point mutations Q1p49A and L1i18A in repeat I did not change BTX sensitivity. It should be noted that repeat I is diagonally opposed to repeat III. The latter contains five BTX-sensing residues, more than any other repeat (Table 2), suggesting that BTX binds tightly to repeat III. The BTX sensitivity of mutants Q1p49A and L1i18A may indicate that the distance between repeats I and III in the BgNav1-1a sodium channel is somehow larger than in its Kv1.2-based model and thus repeat III-bound BTX is farther from the pore lumen than that of the Kv1.2-based model does not affect conclusions of our study due to two reasons. First, the proposed BTX-channel model remains consistent with most of the mutational data shown in Table 2, including BTX sensitivity of five of the seven mutants, which have been designed and generated to test the model. Second, ligand-channel contacts may be maintained upon some shift of the backbone due to the ligand and side chain flexibility. Indeed, ligand docking to homology models of the L-type calcium channel demonstrated that contacts between specific ligand moieties and channel residues are much less sensitive to the choice of

Identify: BTX-sensing residues in segment III S6

| Residue | Energy contribution (E), kcal/mol<sup>a</sup> | Point mutation | BTX sensitivity of the mutant | Agreement with the model | Ref. |
|---------|---------------------------------------------|----------------|-------------------------------|-------------------------|------|
| Phe<sup>3i49</sup> | −3.6 | K | Decrease | Yes | Refs. 5 and 6 |
| Phe<sup>4i15</sup> | −2.8 | K | Decrease | Yes | Ref. 22 |
| Phe<sup>2i16</sup> | −2.5 | A | Decrease | Yes | This study |
| Asp<sup>2i15</sup> | −1.9 | K | Decrease | Yes | Ref. 25 |
| Leu<sup>3i19</sup> | −1.7 | K | Decrease | Yes | Ref. 50 |
| Ser<sup>1i15</sup> | −1.7 | K | Decrease | No change<sup>c</sup> | This study |
| Leu<sup>1i18</sup> | −1.7 | A | No change<sup>c</sup> | No | No | This study |
| Ser<sup>1i15</sup> | −1.6 | K | Decrease | No change<sup>c</sup> | Yes | This study |
| Q<sup>1p49</sup> | −1.4 | A | No change | No change | Yes | This study |
| Thr<sup>3p48</sup> | −1.0 | A | Decrease | Yes | This study |
| Leu<sup>2i19</sup> | −0.8 | K | Decrease | No change | Yes | This study |
| Gly<sup>2i14</sup> | [E] < 0.1 | A | Decrease | No change | Yes | This study |
| Ile<sup>1i18</sup> | [E] < 0.1 | A | Decrease | No change | Yes | This study |
| Leu<sup>1i19</sup> | [E] < 0.1 | F | No change | No change | No change | Yes | This study |
| Leu<sup>2i16</sup> | [E] < 0.1 | A | No change | Yes | Yes | This study |
| Leu<sup>2i16</sup> | [E] < 0.1 | A | No change | Yes | Yes | This study |

<sup>a</sup> Side chain contributions are shown.  
<sup>b</sup> Energy contributions were calculated for the horseshoe binding model proposed in this work.  
<sup>c</sup> Mutant L1118K of rNav1.4 did not express (23).  
<sup>d</sup> The gating hinge mutation can modify the activation gating, but structural interpretation of this effect on BTX action is hardly possible in our "static" model of the open channel.
Identification of BTX-sensing Residues in Segment III-S6

**FIGURE 5. BTX sensitivity of seven mutants generated to test the horseshoe model of BTX binding.** A and B, effects of amino acid substitutions on BTX-induced tail current (A) and nonactivating current (B); C, percentage of channels modified by BTX. The recording protocols and data analysis are the same as those described in the legend to Fig. 1. The data are presented as mean ± S.D. An asterisk indicates a significant difference from the wild-type channel as determined by t tests (p < 0.05).

Increasing evidence suggests that BTX binds in the inner pore of voltage-gated sodium channels. However, atomistic details of BTX binding are poorly understood. In this study, we found that mutations of the putative gating hinge glycine Gly314 and phenylalanine Phe316 in the inner helix III-S6 significantly decreased BTX action. These newly discovered BTX-sensing residues do not face the inner pore. This finding motivated us to revise the previously proposed binding model in which BTX contacted only the pore-facing residues, and the agonist ammonium group was rather close to the permeation pathway (5, 6). We used possible interactions of Phe316 with BTX as new distance constraints to dock BTX. In combination with the distance constraints implied from previous experimental studies by Ging-Kuo Wang and coauthors on BTX-sensing residues in mammalian channels (6, 8, 22, 25, 50), the new distance constraints have driven our computations to predict the horseshoe binding model described under “Results.”

In our model, BTX binds to BTX-sensing residues in all four repeats. These residues are top contributors to BTX binding energy (Table 2). Phe316 plays a significant role by stabilizing the ammonium group of BTX via cation–π interactions. The mutation G314A affects BTX action (Fig. 1), but Gly314 does not interact directly with BTX in our model. We suggest that the alanine substitution of this gating hinge glycine affects the channel gating and therefore has an allosteric effect on BTX action. Most of the currently known BTX-sensing residues directly interact with BTX in our model (Figs. 3 and 4 and Table 2). Besides Gly314, other exceptions are Asn42 and Asn420, whose lysine substitutions affect BTX action (22, 23). These asparagines do not face the pore in our models of sodium channels. In a homology model of the CaV2.1 channel, respective asparagines are engaged in strong inter-repeat interactions (13). Mutations of analogous asparagines in the sodium channels may affect open pore stability and/or geometry and therefore allosterically affect BTX action. Mutation L123K makes the channel BTX-resistant (23). Leu123 does not interact with BTX in our model, but it is exposed to the pore lumen at the cytoplasmic entry to the pore; the lysine substitution can repel the charged BTX approaching its binding site from the cytoplasm.

A permanently charged BTX derivative activates the sodium channel albeit in a much higher concentration (53). An advantage of the horseshoe model of BTX binding is the location of the ligand ammonium group in the repeat interface where it interacts with Phe316 and Ser315 (Fig. 4A). In this location, the BTX cationic group would not strongly repel permeating ions. The low potency of the permanently charged BTX derivative may be due to its inability to donate an H-bond to Ser315. The BTX ammonium group would facilitate the BTX approach to the open cationphilic pore and then establish favorable interactions with the aromatic and H-bonding residues in the repeat interface.

The pyrrole ring is essential for BTX activity (54, 55). In the horseshoe binding model, the pyrrole ring approaches Ser315, and the BTX carbonyl oxygen is within H-bonding distance from the amide group of Asn215. Mutations N215K and N215R cause BTX to block rather than activate hNav1.5 (7). In the latter mutants, the lysine or arginine residues are too long to donate an H-bond to the carbonyl oxygen of BTX and may repel the pyrrole group toward the pore axis, where it would block the ion permeation.

The reduced conductance of BTX-modified channels (56) is readily explained by our model in which the binding site for a Na+ ion within the BTX horseshoe is much narrower than the channel cross-section at the level iiS5. This level contains highly conserved serine, asparagine, and phenylalanine residues, which are likely to form a binding site for a hydrated Na+ ion in the inner pore.

Altered ionic selectivity of BTX-modified sodium channels was described in 1970s (57, 58). More recent studies show that BTX reduces the selectivity of Na+ over K+, Rb+, and Cs+ by less than 3-fold but does not change selectivity for Na+ over Li+ (26, 27) or NH4+ (56). The authors of the latter study suggest that the ion selectivity measured through the reversal potential for different ions is highly sensitive to experimental conditions. The selectivity filter in the BTX-modified channels is estimated to be wider than in the native channels (57, 58). Why would the channel with a wider selectivity filter show a reduced conductance? In our model BTX does not directly interact with the...
DEKA locus but may widen the pore (see “Results”) and thus the selectivity filter. Furthermore, the binding site for permeating ions within BTX, besides decreasing the channel permeability (and thus attenuating the ion selectivity of the DEKA locus), may poorly discriminate the permeating ions. In particular, BTX-modified channels show increased permeability for methylammonium. This organic cation would readily permeate through the BTX horseshoe, which in our model partially embraces an ion, whereas a part of the ion is exposed to the hydrophobic interface of IS6 and IVS6 (Fig. 4C). The altered ion selectivity of BTX-modified channels may also be related to the reduced sensitivity of these channels to block proteins (59). In our model, BTX would displace water molecules from hydrophilic residues at level i15, which is proximal to the selectivity filter. The deficiency of water molecules in the inner pore of BTX-modified channels may shift $K_p$ values of titratable residues in the DEKA locus, in particular $K_p$ of Lys$^{3(p+9)}$, a critical determinant of sodium channel selectivity (60).

It is difficult to perform biophysical analysis of BTX-modified sodium channels in Xenopus oocytes as only ~40% of BgNav channels were affected by BTX at 500 nM concentration. Future studies using whole cell Na$^+$ current recordings in cell lines with patch clamp techniques could determine the effects of BTX on conductance and ion selectivity.

Our study reinforces the concept that BTX binds in the inner pore (5) rather than at the protein-lipid interfaces. A common feature of the previous and current structural models is that BTX binds in the inner pore and permeating ions bind between oxygen atom(s) in the BTX molecule and a pore-facing polar residue. The major peculiarity of the new model is that the BTX ammonium group is engaged in cation–π interactions with Phe$^{311}$ in the repeat interface, rather far from the ion permeation pathway, and thus only weakly repels the permeating ions by electrostatic interactions. Another peculiarity of the new model is that BTX adopts the horseshoe conformation and permeating ions bind between polar BTX atoms in the horseshoe inner surface and the side chain hydroxyl of Ser$^{311}$.5. (The previous model suggested that permeating ions bind the side chain oxygen of Asn$^{210}$ and an oxygen atom of BTX.)

The limitations of our model should be detailed clearly. Building homology models and docking ligands require energy optimizations. However, a homology model, which includes only a part of a large transmembrane protein and lacks explicit water molecules and membrane lipids, is not expected to correspond to the global energy minimum. The touchstone of a model is its consistency with experimental observations and ability to produce experimentally testable predictions. Although we described in Table 2 some aspects of the ligand-channel energetics, the concrete numbers should be treated with caution. On the other hand the fact that 13 of 16 point mutations of the sodium channel (Table 2) changed BTX sensitivity in agreement with the predicted BTX channel contacts (or lack of such contacts) strongly supports the current model.

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REFERENCES

1. Lipkind, G. M., and Fozzard, H. A. (2005) Mol. Pharmacol. 68, 1611–1622
2. Tikhonov, D. B., and Zhorov, B. S. (2007) Biophys. J. 93, 1557–1570
3. Bruhova, I., Tikhonov, D. B., and Zhorov, B. S. (2008) Mol. Pharmacol. 74, 1033–1045
4. Tikhonov, D. B., Bruhova, I., and Zhorov, B. S. (2006) FEBS Lett. 580, 6027–6032
5. Tikhonov, D. B., and Zhorov, B. S. (2005) FEBS Lett. 579, 4207–4212
6. Wang, S. Y., Mitchell, J., Tikhonov, D. B., Zhorov, B. S., and Wang, G. K. (2006) Mol. Pharmacol. 69, 788–795
7. Wang, S. Y., Tikhonov, D. B., Mitchell, J., Zhorov, B. S., and Wang, G. K. (2007) Channels 1, 179–188
8. Wang, S. Y., Tikhonov, D. B., Zhorov, B. S., Mitchell, J., and Wang, G. K. (2007) Pflugers Arch. 454, 277–287
9. O’Reilly, A. O., Khambay, B. P., Williamson, M. S., Field, L. M., Wallace, B. A., and Davies, T. G. (2006) Biochem. J. 396, 255–263
10. Du, Y., Lee, J. E., Nomura, Y., Zhang, T., Zhorov, B. S., and Dong, K. (2009) Biochem. J. 419, 377–385
11. Zhen, X. G., Xie, C., Fitzmaurice, A., Schoonover, C. E., Orenstein, E. T., and Yang, J. (2005) J. Gen. Physiol. 126, 193–204
12. Long, S. B., Campbell, E. B., and Mackinnon, R. (2005) Science 309, 897–903
13. Bruhova, I., and Zhorov, B. S. (2010) J. Gen. Physiol. 135, 261–274
14. Daly, J. W., Wirtkop, B., Bommer, P., and Biemann, K. (1965) J. Am. Chem. Soc. 87, 124–126
15. Hille, B. (2001) Ion Channels of Excitable membranes, Sinauer Associates Inc., Sunderland, MA, U.S.A.
16. Wang, S. Y., and Wang, G. K. (2003) Cell. Signal. 15, 151–159
17. Khodorov, B. I., and Revenko, S. V. (1979) Neuroscience 4, 1315–1330
18. Ulbricht, W. (1969) Ergeb. Physiol. 61, 18–71
19. Zlotkin, E. (1999) Annu. Rev. Entomol. 44, 429–465
20. Narahashi, T. (2000) J. Pharmacol. Exp. Ther. 294, 1–26
21. Quandt, F. N., and Narahashi, T. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6732–6736
22. Wang, S. Y., and Wang, G. K. (1999) Biophys. J. 76, 3141–3149
23. Wang, S. Y., and Wang, G. K. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 2653–2658
24. Linford, N. I., Cantrell, A. R., Qu, Y., Scheuer, T., and Catterall, W. A. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 13947–13952
25. Wang, S. Y., Barile, M., and Wang, G. K. (2001) Mol. Pharmacol. 59, 1100–1107
26. Behrens, M. I., Oberhauser, A., Bezanilla, F., and Latorre, R. (1989) J. Gen. Physiol. 93, 23–41
27. Garber, S. S., and Miller, C. (1987) J. Gen. Physiol. 89, 459–480
28. Huang, L. Y., Moran, N., and Ehrenstein, G. (1984) Biophys. J. 45, 313–322
29. Catterall, W. A. (1977) J. Biol. Chem. 252, 6869–8867
30. Zhorov, B. S., Folkman, E. V., and Anantharayanan, V. S. (2001) Arch. Biochem. Biophys. 393, 22–41
31. Song, W., Liu, Z., Tan, J., Nomura, Y., and Dong, K. (2004) J. Biol. Chem. 279, 32554–32561
32. Tan, J., Liu, Z., Nomura, Y., Goldin, A. L., and Dong, K. (2002) J. Neurosci. 22, 5300–5309
33. Feng, G., Deck, P., Chopra, M., and Hall, L. M. (1995) Cell 82, 1001–1011
34. Warnke, J. W., Reenan, R. A., Wang, P., Qiao, S., Arena, J. P., Wang, J., Wunderler, D., Liu, K., Kaczorowski, G. J., Van der Ploeg, L. H., Ganetzky, B., and Cohen, C. J. (1997) J. Gen. Physiol. 110, 119–133
35. Tan, J., Liu, Z., Wang, R., Huang, Z. Y., Chen, A. C., Gurvitz, M., and Dong, K. (2005) Mol. Pharmacol. 67, 513–522
36. Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
37. Zhorov, B. S., and Tikhonov, D. B. (2004) J. Neurochem. 88, 782–799
38. Tikhonov, D. B., and Zhorov, B. S. (2005) Biophys. J. 88, 184–197
39. Li, Z., and Scheraga, H. A. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 6611–6615
40. Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Chio, C., Alagona, G., Profeta, S., and Weiner, P. K. (1984) J. Am. Chem. Soc. 106, 765–784
41. Weiner, S. J., Kollman, P. A., Nguyen, D. T., and Case, D. A. (1986) J. Com-
Identification of BTX-sensing Residues in Segment III S6