IDENTIFICATION OF NEW BATRACHOTOXIN-SENSING RESIDUES IN SEGMENT IIIS6 OF 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 IIIS6 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 the BTX action. Using these data along with published data as distance constrains, 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 group is engaged in cation-pi interactions with Phe_3i16 and BTX moieties interact with known BTX-sensing residues in all four repeats. Oxygen atoms at the horseshoe inner surface constitute a transient binding site for permeation cations, while 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, each repeat having six transmembrane helices (S1-S6). The S1-S4 helices form the voltage sensor domain. Positively charged S4 helices move outward in response to membrane depolarization. The S5 and S6 helices contribute to the pore-forming domain. The extracellular linkers connecting S5 and S6 helices form the four reentrant P-loops, which contain the selectivity-filter residues. In the absence of x-ray structures of NaV channels, their homology models, which are based on x-ray structures of potassium channels, are used to explain structure-activity relationships of various sodium-channel ligands including local anesthetics (1-4), steroidal activators (5-8), and pyrethroid insecticides (9,10). Recent reinterpretation of data on substituted cysteine accessibility of CaV2.1 channel (11) in view of a the channel homology model, which is based on the x-ray structure of the open voltage-gated potassium channel Kv1.2 (12), further supports general similarity of the
inner-pore architecture in different voltage-gated cationic channels (13).

Sodium channels are targets for numerous drugs and naturally occurring toxins. Batrachotoxin (BTX) is a steroidal sodium-channel agonist, which was first isolated from the skin of the Colombian frog *Phyllobates bicolour* (14). BTX binds preferentially to the open NaV channels (15-17) and alters several channel properties. First, it shifts the voltage-dependence of activation in the hyperpolarizing direction thus causing the channel to open at more negative membrane potentials. Second, BTX inhibits inactivation. Third, BTX-modified channels demonstrate reduced selectivity to sodium ions and reduced conductance (18-21). Due to their high affinity and specificity to NaV channels, BTX and other steroidal agonists are useful tools to probe the channel functions, including the gating mechanisms.

Earlier studies demonstrated that point mutations in the inner helices IS6 and IVS6 (L^{119}K, N^{120}K, L^{123}K, F^{413}K, and N^{420}K) make the NaV1.4 channel BTX-resistant (22,23), leading to the idea that BTX binds in the interface between repeats I and IV (24). Subsequent mutational studies identified BTX-sensing residues in the inner helices of all four repeats including residues S^{3i15} and L^{3i19} in repeat III (8,10,25). Architecture of voltage-gated potassium channels is inconsistent with a scenario that a lipid-exposed ligand simultaneously binds to more than two inner helices. A unitary Hill coefficient of BTX action (17) and largely different amino acid sequences of the four repeats rule out a possibility that the channel contains more 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 is that BTX is directly exposed to the permeation pathway (5,30).

In a structural model of 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 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 S^{3i15} in the octapeptide segment IFSFTFLL (S^{3i15} and L^{3i19} are underlined) of a cockroach sodium channel BgNa1-1a (31) and explored effects of mutations on the channel gating in the presence and absence of BTX. We identified F^{3i16} and a putative gating-hinge glycine G^{3i14} as new BTX-sensing residues. Using these and published data as distance constraints, we explored different possible binding models of BTX in the Kv1.2-based model of the open BgNa channel and arrived to 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. pClamp 8.2 software (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/Gmax = [1 + exp (V - V1/2)/k]^{-1}, or with the sum of two such expressions, in which V is the potential of the voltage pulse, V1/2 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 pre-pulses 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 pre-pulse potential. Data were fitted with a two-state Boltzmann equation of the form I/Imax = [1 + (exp(V - V1/2)/k)]^{-1}, in which I is the peak sodium current, Imax is the maximal current evoked, V is the potential of the voltage prepulse, V1/2 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). Stock solution of BTX (1 mM), was dissolved in dimethyl sulfoxide (DMSO). The working concentration was prepared in ND96 recording solution just prior to the experiments. The concentration of DMSO 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 channel, have been built based on the crystal structure of the closed potassium channel KcsA (36). The ZMM molecular modeling program (www.zmmsoft.com) has been used in all calculations. The 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 in ref. (38). The Monte Carlo-energy minimization (MCM) method (39) was used to optimize the channel model and dock BTX. The energy was calculated using the AMBER force field (40,41) and solvent exposure- and distance-dependent dielectric function (42). Atomic charges at 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 (Supplementary Table S1). A constraint is a flat-bottom parabolic penalty function added to the energy expression. When a 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. Ten thousands BTX conformations were generated and the ten 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 were sampled 200,000 times by assigning random values to six rigid-body degrees of freedom of the ligand. The energy of the BTX-receptor complexes (including the distance-constraint penalties) was calculated without energy minimization and the ten lowest energy complexes were collected. In the third stage, the ten collected complexes were refined by a 1,000 step MC-minimization and the lowest energy structure was used as a BTX binding model consistent with the given combination of distance constraints. At this stage, the torsion angles in the protein side chains and in BTX were 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 constraints-imposed binding mode, the latter was excluded from further analysis.

RESULTS

\[ G^{3i14}A \text{ and } F^{3i16}A/K \text{ substitutions reduce the action of BTX on the } BgNa_{1-1a} \text{ channel.} \]

Previously we reported that two amino acid residues, \( S^{3i15} \text{ and } L^{3i19} \) 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 IFGSFFTL segment (\( S^{3i15} \text{ and } L^{3i19} \) are underlined) are also involved in the action of BTX, we examined the effect of BTX on six mutant BgNa\(_{1-1a}\) channels, \( I^{3i12}A, F^{3i13}A, G^{3i14}A, F^{3i16}A, F^{3i17}A \text{ and } T^{3i18}A \), that were made previously for another study (10). None of the substitutions alter channel gating except for \( G^{3i14}A \) which shifted the voltage-dependence of activation in the depolarizing direction by ca. 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. 1A and 1B). The BTX effects on BgNa\(_{1-1a}\) channels were incomplete because two voltage dependent components of activation were observed: one with the 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 \( G^{3i14} \text{ and } F^{3i16} \) significantly reduced the percentage of the BTX-induced tail current and non-inactivating current (Fig. 1D and 1E). In contrast, \( I^{3i12}A, F^{3i13}A, F^{3i17}A \text{ and } T^{3i18}A \) did not (Fig. 1D and 1E). Furthermore, a lysine substitution of \( F^{3i16} \) almost completely abolished the action of BTX (Fig. 1D and 1E). Similarly, the BTX effect was not observed in the voltage-dependence of inactivation of \( G^{3i14}A \text{ and } F^{3i16}A/K \) channels (Fig. 1F-1H) (note a slightly lifted foot of the inactivation curve in the \( G^{3i14}A \text{ and } F^{3i16}A \) channels). Consistent with these results, substitutions \( G^{3i14}A \text{ and } F^{3i16}A, \) but not \( I^{3i12}A, F^{3i13}A, F^{3i17}A \text{ and } T^{3i18}A, \) significantly reduced the percentage of BTX-modified channels (Fig. 1B and 1I). No \( F^{3i16}K \) channels were modified by BTX (500 nM) (Fig. 1I). These results collectively demonstrated that \( G^{3i14}A \text{ and } F^{3i16}A/K \) 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 channel hydrophilic residues (5,6). Our present finding that phenylalanine \( F^{3i16} \) is essential for BTX binding is inconsistent with the previously proposed orientation of BTX. This motivated elaboration of
an updated model, which would be consistent with all currently available experimental data on BTX action.

Hands-free docking of semi-flexible BTX in the channel with flexible side chains is possible (42), but 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 Methods). 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 BTX. Fourth, BTX in the model should not block the pore, but allow the ion permeation through the BTX-bound channel. Fifth, the model should explain why BTX-bound channel resists the activation-gate closure (15). For each binding mode, which was initially imposed by the distance constraints, ten 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 Supplementary Data.

The binding mode in which many of the currently known BTX-sensing residues are in direct contacts with BTX is shown in Fig. 3. This lowest-energy BTX-channel complex was obtained by flipping BTX in the constraints-imposed model (Fig. S1F) by 180° around the vertical axis and 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 F<sup>3i16</sup>, 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 BTX ammonium group donates an H-bond to S<sup>3i15</sup>, a known BTX-sensing residue (50). The carbonyl oxygen and pyrrole nitrogen of BTX accept H-bonds from N<sup>2i15</sup> and S<sup>1i15</sup>, 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 L<sup>2i19</sup> (25), L<sup>3i19</sup> (50) and F<sup>4i15</sup> (22). Besides binding to BTX-sensing residues in the inner helices, BTX strongly interacts with F<sup>3p49</sup> by its oxazepane ring. The latter BTX-sensing residue was initially predicted in the modeling study (5) and later lysine and arginine substitutions of F<sup>3p49</sup> were demonstrated to dramatically decrease BTX action (6). Most importantly, four oxygen atoms of BTX as well as pyrrole nitrogen atom and π-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<sup>+</sup> ions would permeate through this arch and thus through the BTX-bound channel (Fig. 4A and 4B).

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 Figures 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 view of these data the slow onset of 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 imposed distance constrains between BTX and BTX-sensing residues to maintain the horseshoe-like conformation of the agonist at the same level of the pore as in the open channel. After intensive MC-minimization in the presence of the constraints numerous clashes were found between BTX and the channel, including BTX sensing residues (not shown). The channel closure substantially narrowed the pore lumen at the level of BTX and also decreased the “diameter” of the BTX horseshoe (cf. Figs. 4A and 4B). In the constrained complex (Fig. 4B) the BTX-channel interaction energy was 18.7 kcal/mol higher than in the unconstrained complex (Fig. 4A). When we removed the BTX-channel constraints and further MC-minimized the complex, BTX shifted from level 115 towards the selectivity filter, changed its conformation, and fit in the central cavity of the closed channels (Fig. 4D and 4F). These results are consistent with the data that BTX can be trapped in the closed channel (51).

Testing the BTX binding model. An anonymous reviewer has suggested to test our BTX binding model by mutating residues that do not contribute to the BTX-channel energy, yet are in close proximity to the residues participating in BTX binding. Following up this suggestion, we have generated four mutants: L2i16A, L2i16F, L4i16A, and I4i19A. In agreement with the model, none of these mutants demonstrated a decreased BTX sensitivity (Fig. 5, Table 2). The lack of effect of mutations L2i16A, L2i16F, and L4i16A on BTX action is of special interest because positions 2i16 and 4i16 are symmetric to position 3i16, which contains phenylalanine residue involved in π-cation interactions with BTX. The presumed BTX contact with F3i16 served as a critical constraint to build our model in which the positively charged ammonium group of BTX binds in the repeat interface, rather far from the pore lumen where Na⁺ ions move through BTX. If mutation F3i16A were affecting the BTX binding indirectly (e.g. by changing inter-repeat contacts) one could expect analogous effects of mutations in symmetric positions 2i16 and 4i16. The fact that these mutants occurred as BTX sensitive as the WT channel reinforces our conclusion on cation-π interactions of BTX with F3i16 and thus supports our model.

We further generated alanine substitutions of three residues, which contribute energy to BTX binding in our model, and evaluated their BTX sensitivity (Table 2; 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 the 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 repeat I than in our model. (Reduced BTX sensitivity of the S1i15K mutant does not rule out this possibility because the long, pore-facing sidechain of lysine can repel the positively charged BTX without contacting it, whereas an alanine substitution is expected to weaken BTX attraction to the channel.) The possibility that the sodium channel pore is wider 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 maintain 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 the x-ray template than to the alignment between K⁺ and Ca²⁺ channels (52).
DISCUSSION

Increasing evidence suggest 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 G3i14 and phenylalanine F3i16 in the inner helix IIIS6 significantly decreased the 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 F3i16 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, 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). F3i16 plays a significant role by stabilizing the ammonium group of BTX via cation-π interactions. Mutation G3i14A affects BTX action (Fig. 1), but G3i14 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, 4 and Table 2). Besides G3i14, exceptions are N1i20 and N4i20 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 the open-pore stability and/or geometry and therefore allosterically effect the BTX action. Mutation L1i23K makes the channel BTX-resistant (23). L1i23 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 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 F3i16 and S3i15 (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 S3i15. The BTX ammonium group would facilitate the BTX approach to the open cationophilic 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 S1i15 and the BTX carbonyl oxygen is within H-bonding distance from the amide group of N2i15. Mutations N2i15K and N2i15R cause BTX to block rather than activate hNa1.5 (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 towards 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 i15. 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 70s (57,58). More recent studies show that BTX reduces 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 the channel with a wider selectivity filter would 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 and leaves unobstructed the ion side, which is exposed to the hydrophobic interface between IS6 and IVS6 (Fig. 4C). The altered ion selectivity of BTX-modified channels may be also related to the reduced sensitivity of these channels to proton block (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 pKa values of titrable residues in the DEKA locus, in particular pKa of $K^{3p49}$, a critical determinant of the sodium channel selectivity (60).

It is difficult to perform biophysical analysis of BTX-modified sodium channels in Xenopus oocytes since only about 40% of BgNa 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 as was thought before. 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 $F^{3i16}$ 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 sidechain hydroxyl of $S^{i15}$. (The previous model suggested that permeating ions bind the sidechain oxygen of $N^{2i5}$ and an oxygen atom of BTX.)

Limitations of our model should be spelled out. 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 direct 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 out of 16 point mutations of the sodium channel (Table 2) changed the BTX sensitivity in agreement with the predicted BTX-channel contacts (or lack of such contacts) strongly supports the current model.

**REFERENCE**


**FOOTNOTES**

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**FIGURE LEGENDS**

FIGURE 1. Residues G314 and F316 in IIIS6 are critical for the action of BTX. A. Sodium currents before and after the application of 500 nM BTX. BTX-induced non-inactivating current and tail current were elicited by a 20-ms test pulse to -10 mV from a holding potential of -120 mV after 3000 repetitive pulses to -10 mV at a frequency of 10 Hz in the presence of 500 nM BTX. B and C. Voltage-dependence of activation (B) and inactivation (C). Data from a previous study (10) and the current study were pooled to generate activation and inactivation curves in B and C. D and E. Effects of amino acid substitutions on BTX-induced tail current (D) and non-inactivating current (E). The amplitude of tail current and non-inactivating current induced by BTX was normalized to the peak current after toxin application. F - H. Voltage dependence of inactivation of G314A (F), F316A (G) and F316K (H) channels before and after the application of BTX. I. Percentage of channels modified by BTX for BgNa,1-1a and mutants. The voltage-dependence of activation (conductance curves) in the presence of BTX was fitted with the sum of
two Boltzmann relationships to determine the percentage of channels that were modified by BTX. The asterisks indicate significant differences from the wild-type channel as determined by t-test (p<0.05).

FIGURE 2. Structural formulae of BTX.

FIGURE 3. Predicted binding mode of BTX in BgNa,1-1. The pore-forming domain of the channels is shown with the inner helices (thick rods), outer helices (thin rods), P-helices (ribbons) and ascending limbs (thin rods). Repeats I, II, III, and IV are colored orange, cyan, green and blue respectively. BTX sensing residues are spaced filled. BTX is shown by sticks with yellow carbons, red oxygens, and blue nitrogens. BTX adopts a horseshoe conformation with its exterior predominantly hydrophobic side interacting with the inner helices and interior hydrophilic side exposed to the pore axis. A and B. Top views. C and D. Side views with the front repeat removed for clarity. E. Schematic view of BTX-channel interactions.

FIGURE 4. BTX in the open-state (A,C,E) and closed-state (B,D,F) models of the sodium channel. For clarity, only parts of the outer and inner helices around level i15 are shown at the top (A - D) and side (E, F) views. The front helix IS6 is removed at E (except for BTX-sensing residue S115) and repeats I and IV are removed at F. In the open state, the central inner-pore cavity at level i15 is wide enough to accommodate BTX in the horseshoe conformation. The van der Waals shape of the BTX horseshoe conformation approximately fits the central inner cavity (C). A Na⁺ ion (orange sphere) binds to the inner surface of the BTX horseshoe (A, C). B, KcsA-based model of the closed channel with BTX constrained at the same level as in A. Due to the small lumen of the closed pore, this orientation of BTX is unstable. D, F. Upon removal of the BTX-channel distance constraints, BTX extend along the pore axis of the closed pore. This model shows how BTX could be trapped in the closed channel.

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 non-inactivating current (B). C. Percentage of channels modified by BTX. The recording protocols and data analysis are the same as described in legend to Figure 1.
### TABLE 1
Sequence alignment and BTX-sensing residues

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<tr>
<th>Channel</th>
<th>Helix</th>
<th>First residue #</th>
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<th>11</th>
<th>21</th>
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<td>RAGA</td>
<td>AAT</td>
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<td>322</td>
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<td>LLI</td>
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</table>

Experimentally determined BTX sensing residues are bold-typed and underlined. **Repeat I.** rNav1.4 point mutants I$^{1i19}$K, N$^{1i20}$K, and L$^{1i23}$K are BTX-resistant (23). Mutations S$^{1i15}$K and S$^{1i15}$R make hNav1.5 channel completely insensitive to 5 μM BTX (8). **Repeat II.** Point mutations N$^{2i15}$K and L$^{2i19}$K render rNav1.4 completely insensitive to 5 μM BTX (25). **Repeat III.** Point mutations S$^{3i15}$K and L$^{3i19}$K render rNav1.4 completely insensitive to 5 μM BTX (50). Mutations F$^{3p49}$K and F$^{3p49}$R make hNav1.5 resistant to BTX (6). Effects of BTX on BgNa, mutants G$^{3i14}$A and F$^{3i16}$A are described in the current study. **Repeat IV.** Point mutations F$^{4i15}$K and N$^{4i20}$K render rNav1.4 completely insensitive to 5 μM BTX (22). Mutation V$^{4i19}$C protects rNav1.4 from modification by BTX (61).
TABLE 2

Residues that provide the largest contributions to the BTX-channel energy, selected residues that do not contribute significant energy, and effects of mutations of respective residues on the sodium channel BTX sensitivity

<table>
<thead>
<tr>
<th>Residue</th>
<th>Energy contribution (E), kcal/mol a,b</th>
<th>Point mutation</th>
<th>BTX sensitivity of the mutant</th>
<th>Agreement with the model</th>
<th>Reference</th>
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<tr>
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<tr>
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<td>(50)</td>
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<td>&lt; 0.1</td>
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a Side chain contribution.
b For the horseshoe binding model proposed in this work.
c Mutant L118K of rNav1.4 did not express (23).
d 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.
Figure 1

A: Record showing a comparison between Control and BTX 500 nM conditions.

B: Graph showing Normalized $g_{Na}$ with voltage ($V_p$) plotted against $I_{Na}$ as a function of $V_p$.

C: Graph showing Normalized $I_{Na}$ with voltage ($V_p$) plotted against percentage modification.

D: Bar graph for $I_{tail}$ (%). WT and mutants are compared with Control and BTX.

E: Bar graph for Nonactivating $I_{Na}$ (%). Similar comparison as in D.

F: Graph showing Normalized $I_{Na}$ with voltage ($V_p$) plotted against percentage modification.

G: Graph showing Normalized $I_{Na}$ for $F^{3i16}A$.

H: Graph showing Normalized $I_{Na}$ for $F^{3i16}K$.

I: Bar graph showing % Modification for WT and mutants with Control and BTX.
Figure 4
Identification of new batrachotoxin-sensing residues in segment IIIS6 of sodium channel
Yuzhe Du, Daniel Garden, Lingxin Wang, Boris S. Zhorov and Ke Dong

J. Biol. Chem. published online February 8, 2011

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