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The Batrachotoxin Receptor on the Voltage-Gated Sodium Channel is Guarded by the Channel Activation Gate

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ABSTRACT

Batrachotoxin (BTX), from South American frogs of the genus *Phyllobates*, irreversibly activates voltage-gated sodium channels. Previous work demonstrated that a phenylalanine residue approximately halfway through pore-lining transmembrane segment IVS6 is a critical determinant of channel sensitivity to BTX. In this study, we introduced a series of mutations at this site in the $Na_v1.3$ sodium channel, expressed wild-type and mutant channels in *Xenopus laevis* oocytes, and examined their sensitivity to BTX using voltage clamp recording. We found that substitution of either alanine or isoleucine strongly reduced channel sensitivity to toxin, whereas cysteine, tyrosine, or tryptophan decreased toxin action only modestly. These data suggest an electrostatic ligand-receptor interaction at this site, possibly involving a charged tertiary amine on BTX. We then used a mutant channel (mutant F1710C) with intermediate toxin sensitivity to examine the properties of the toxin-receptor reaction in more detail. In contrast to wild-type channels, which bind BTX almost irreversibly, toxin dissociation from mutant channels was rapid, but only when the channels were open, not when they were closed. These data suggest the closed activation gate trapped bound toxin. Although BTX dissociation required channel activation, it was, paradoxically, slowed by strong membrane depolarization, suggesting additional state-dependent and/or electrostatic influences on the toxin binding reaction. We propose that BTX moves to and from its receptor through the cytoplasmic end of the open ion-conducting pore, in a manner similar to that of quaternary local anesthetics like QX314.

Natives of the Choco rain forest of Colombia have traditionally used secretions from the skin of frogs of the genus *Phyllobates* to make poisoned darts (Albuquerque et al., 1971). Batrachotoxin (BTX), the main active ingredient from these skin secretions, is an extraordinarily potent neurotoxin. Its toxic effects are caused by sustained, irreversible opening of voltage-gated sodium channels of nerve and muscle (for reviews, see Brown, 1988; Cestèle and Catterall, 2000). BTX profoundly alters various aspects of sodium channel behavior. Voltage-dependent activation is shifted to more negative potentials, inactivation is disabled, and pore conductance and selectivity are altered (Khodorov and Revenko, 1979; Khodorov et al., 1981; Quandt and Narahashi, 1982; Tanguy and Yeh, 1991; Wang and Wang, 1994). Modification of sodium channels by BTX requires channel activation. In whole-cell voltage-clamp experiments, little channel modification occurs at hyperpolarized holding potentials. However, repeated channel opening by trains of depolarizing stimulus pulses causes a progressive buildup of modified channels. These characteristics have been explained by an allosteric model in which BTX binds preferentially to the open channel conformation, thus shifting the conformational equilibrium between closed open and inactivated channel states strongly toward the open state (Catterall, 1977).

The main structural component of sodium channels is the 260-kDa α subunit. The α subunit consists of four domains (I–IV), each containing six α-helical transmembrane segments (S1–S6) (Catterall, 2000). The regions between S5 and S6 in each of the four domains form pore loops that dip into the membrane to create a narrow selectivity filter at the external end of the ion-conducting pore. The remainder of the pore is formed by the four S6 segments (Doyle et al., 1998; Lipkind and Fozzard, 2000). Recent site-directed mutagenesis studies have shown that specific amino acid residues within each of the four S6 segments are important determinants of BTX action (Linford et al., 1998; Wang and Wang, 1998, 1999; Wang et al., 2000, 2001). Interestingly, some of these residues are also critical for the action of local anesthetics and other related sodium channel inhibitors. For example, substitution of alanine for a phenylalanine residue located approximately halfway through transmembrane segments IVS6 profoundly reduces sodium channel sensitivity to both BTX (Linford et al., 1998) and local anesthetics (Ragsdale et al., 1994). Based on these results, Linford et al. (1998) proposed that this site represents a point of overlap between the receptors for BTX and local anesthetics.

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ABBREVIATIONS: BTX, batrachotoxin.

In a previous study, we examined how the critical phenylalanine in IVS6 stabilizes local anesthetic binding by making a series of amino acid substitutions that systematically altered the size, hydrophobicity, and aromaticity of the side chain at this site (Li et al., 1999). These data showed that low affinity binding of local anesthetics to resting sodium channels depends on hydrophobicity at this site, whereas higher affinity interaction with the inactivated channel state requires an aromatic residue at this position. In the study presented here, we investigated how these same mutations affect modification of sodium channels by BTX. We found that mutant channels with nonpolar substitutions at this site were virtually insensitive to BTX, whereas channels with polar or aromatic substitutions exhibited moderate to strong modification by BTX. We then took advantage of a mutant channel that showed intermediate sensitivity to BTX to examine the biophysical properties of toxin interaction with its receptor. For the mutant channel, we found that BTX can rapidly leave the receptor when the channel is open, but not when it is closed, as if bound toxin were trapped by the closed activation gate, and that toxin dissociation from open channels is faster at negative potentials than at positive potentials, perhaps because the binding reaction is intrinsically voltage-dependent. In these respects, BTX binding to the sodium channel resembles the action of quaternary amine local anesthetics like QX314. Thus, the notion of a hydrophilic access pathway through the cytoplasmic end of the open pore, originally proposed for QX compounds (Hille, 1977), may apply to BTX as well.

Materials and Methods

Site-directed mutations were introduced into the rat $Na_v1.3$ subtype of the sodium channel α subunit (Kayano et al., 1988) in vector pSP64t using either the Altered States mutagenesis kit (Promega, Madison WI) or polymerase chain reaction-based mutagenesis, as described in detail elsewhere (Li et al., 1999). RNA was transcribed from wild-type and mutant $Na_v1.3$ constructs using the mMessage mMachine RNA synthesis kit (Ambion, Austin TX), and resuspended in RNAase free 0.1 mM EDTA and 5 mM HEPES, pH 7.5

Oocytes were isolated from female *Xenopus laevis* frogs (Boreal, St. Catherine, Ontario) anesthetized with 3-aminobenzoic acid ethyl ester, as described previously (Li et al., 1999). On the day after isolation, oocytes were microinjected with 50 nl of wild-type or mutant $Na_v1.3$ RNA. Sodium currents were examined 2 to 5 days later by two-electrode voltage clamp (Li et al., 1999). All recordings were performed at room temperature in a bath chamber with a volume of 100 μl. BTX (a generous gift from Dr. John Daly, National Institutes of Health, Bethesda, MD) was dissolved in ethanol to make a 0.5 mM stock solution. This stock solution was pipetted directly into the bath to give the appropriate concentration (10 μM in most experiments) and then mixed thoroughly into the bath solution with a pipetter. In some experiments, BTX was subsequently washed out by superfusion.

Results

BTX Alters Gating of Wild-Type $Na_v1.3$ Sodium Channels Expressed in *X. laevis* Oocytes. To investigate the molecular mechanisms of BTX action, we expressed wild-type or mutant $Na_v1.3$ sodium channels in *X. laevis* oocytes and examined their responses to BTX applied to the bath. The effects of BTX on wild-type $Na_v1.3$ channels expressed in oocytes are summarized in Fig. 1. Activation of wild-type channels in control conditions was just detectable at −35 mV (Fig. 1A, middle control trace), whereas depolarization to 0 mV resulted in a rapidly activating whole-cell current that decayed to baseline by the end of the 70-ms test pulse (Fig. 1A, bottom control trace). BTX had little affect on closed $Na_v1.3$ channels at the hyperpolarized holding potential (−90 mV in this and subsequent experiments). However, repeated opening of sodium channels by application of 2400 pulses to 0 mV at a frequency of 2 Hz resulted in a number of profound changes in channel behavior. First, channel activation was shifted negative compared with control. Thus, after BTX modification, whole-cell currents were clearly present at −50 mV, a potential at which no current was present in control (Fig. 1A, top traces), and were maximal at −35 mV, a potential at which current was barely detectable in control (Fig. 1A, middle traces). Overall, activation of BTX modified channels was shifted approximately 30 mV negative compared with control (Fig. 1B). A second effect of BTX was to strongly disrupt inactivation, resulting in a large sustained current at the end of a 70-ms depolarization to 0 mV (Fig. 1A, bottom BTX trace). Mean sustained currents (scaled with respect to peak currents) for control and BTX are shown in Fig. 1C. Finally, after BTX modification, a large tail current was observed when the membrane potential was returned to −90 mV at the end of the depolarizing test pulse (Fig. 1A, bottom BTX trace). The tail currents were caused by increased driving force through BTX modified channels that were open at the end of the test pulse and closed slowly when

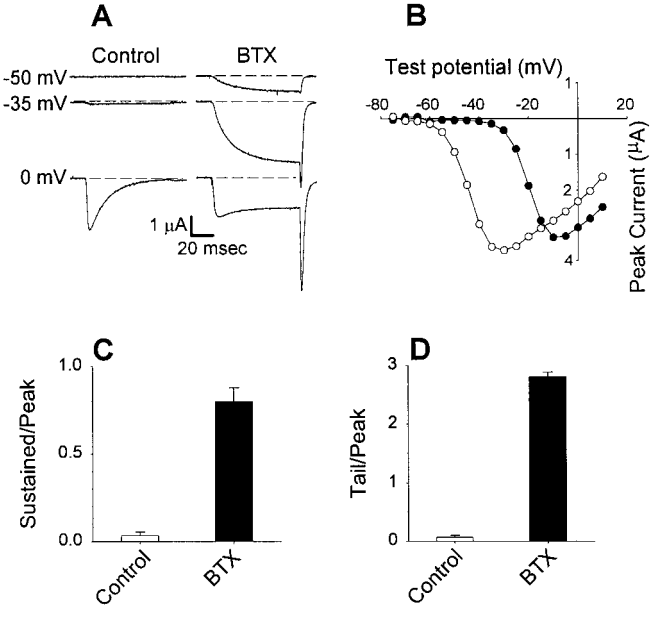

Fig. 1. Batrachotoxin modifies the properties of cloned Nav1.3 sodium channels expressed in *X. laevis* oocytes. A, typical whole-cell sodium currents elicited in control conditions (left traces) and after 2400 35-ms pulses to 0 mV in the presence of BTX (right traces). The example traces show currents evoked by 70-ms depolarizations to −50, −35, or 0 mV. In this and subsequent figures, the holding potential was −90 mV and the concentration of BTX was 10 μM. B, complete current-voltage relationships for the experiment shown in A in control (●) and with BTX (○). The data points show peak amplitudes of currents elicited by depolarizations to test potentials from −80 to +10 mV, plotted as a function of test potential. C, the ratios of the amplitudes of sustained currents/peak currents elicited by depolarization to 0 mV, in control and with BTX. Sustained currents were measured near the end of the 70-ms test pulse. Error bars indicate S.E.M. D, ratios of the amplitudes of the tail currents/peak currents evoked by depolarization to 0 mV in control and with BTX.

the membrane was returned to −90 mV. Mean tail/peak currents are shown in Fig. 1D.

Modification of Sodium Channel Function by BTX Depends on the Properties of the Amino Acid at Position 1710. It was previously shown that substitution of alanine for a critical phenylalanine residue located approximately halfway through segment IVS6 of the sodium channel α subunit (F1710 in the $Na_v1.3$ channel) virtually eliminates channel sensitivity to BTX (Linford et al., 1998). Phenylananine is a hydrophobic aromatic residue. Thus, substitution of alanine could in principle disrupt BTX binding to the sodium channel by reducing hydrophobicity or aromaticity at this position. To investigate how this residue influences BTX binding, we examined the affects of various amino acid substitutions at this site. Figure 2A shows typical currents, elicited by 70-ms test pulses to 0 mV in control and after 2400 pulses in the presence of BTX, for oocytes expressing wild-type or mutant channels. Figure 2, B and C, summarize the effects of these mutations on sustained currents and tail currents, respectively. As described previously (Linford et al., 1998), alanine substitution at position 1710 (mutant F1710A) virtually eliminated sodium channel modification by BTX. Similarly, isoleucine (F1710I), a large hydrophobic residue, strongly disrupted BTX action. The properties of the F1710I mutant indicate that hydrophobicity alone was not sufficient to preserve high-affinity toxin binding. In contrast, mutant channels with polar (cysteine, F1710C), or aromatic (tryptophan, F1710W; tyrosine, F1710Y) substitutions remained sensitive to BTX, with only moderate reductions in sensitivity to toxin, compared with wild-type. As discussed in more detail below, both polar and aromatic residues can interact electrostatically with ligands. Thus, one possible interpretation of these findings is that BTX binding to the sodium channel normally involves an electrostatic interaction with the phenylalanine at position 1710, an interaction that is partially preserved by polar or aromatic substitutions at this site.

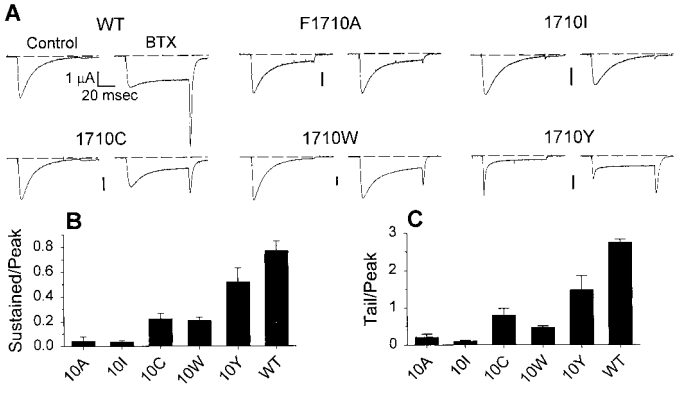

Fig. 2. Site-directed mutations at position 1710 alter sodium channel sensitivity to BTX. A, typical sodium currents in control and with 10 μM BTX recorded in oocytes expressing wild-type or mutant Nav1.3 sodium channels. Currents were evoked by 70-ms depolarizations to 0 mV. In each case, the left-hand trace was in control and the right-hand trace was with BTX. The vertical scale bars indicate 1 μA. B, mean sustained current/peak current ratios for oocytes expressing wild-type or mutant channels. For simplicity, the sustained/peak currents in control have been subtracted off. Thus, the bars indicate the increase in sustained current caused by BTX. For wild-type, F1710I, F1710C, and F1710W channels, sustained current near the end of the test pulse was negligible in control, whereas the F1710A and F1710W mutations exhibited small sustained currents even in control conditions (see example traces). C, mean tail current/peak current ratios for wild-type or mutant channels. As in B, tail/peak currents in control have been subtracted off. Error bars indicate S.E.M.

BTX Dissociates Rapidly from F1710C Channels, but Only When They Are Open. BTX binding to wild-type sodium channels is virtually irreversible over the time course of a typical voltage clamp experiment. This property places limitations on the types of questions one can ask about the biophysical mechanisms of the toxin-receptor reaction. In our initial screen of mutations at position 1710, we found that several mutations significantly reduced but did not eliminate sodium channel modification by BTX. We wondered whether it might be possible to use these mutant channels to examine the dynamics of toxin binding and unbinding. Mutant F1710C expressed well in oocytes and showed the appropriate intermediate sensitivity to BTX. Therefore, we examined this mutation in more detail.

First, we compared the time course of development of BTX modification in oocytes expressing wild-type or F1710C channels. Fig. 3A shows the progressive buildup of BTX-modified channels over the course of 2-Hz trains of pulses to 0 mV for typical wild-type and F1710C experiments. For the wild-type experiment, the level of channel modification built up progressively throughout the entire pulse train, so that even after 2400 pulses, tail current amplitude had not reached a clear steady state. This observation is consistent with the idea that a small fraction of open unmodified channels irreversibly bound BTX during each depolarization.

The initial rate of BTX modification of F1710C channels was similar to wild-type (Fig. 3A), suggesting that the BTX on-rate for the mutant channels was approximately the same as for wild-type channels. However, in contrast to wild-type, BTX modification of F1710C channels rapidly approached a lower steady-state level. In Fig. 3B, we have normalized the two sets of data with respect to the maximum tail currents in each experiment to more clearly illustrate how much more rapidly modification of F1710C reached a steady state compared with wild-type. To quantify this difference, we fit time course of modification of wild-type and mutant currents with single exponential functions. Mean time constants from these fits were 13.4 ± 4.1 min (n = 5) for wild-type versus 2.0 ± 0.4 min (n = 4) for F1710C. Thus, modification of mutant channels approached steady state approximately 6.5 times faster than modification of wild-type channels.

Both the faster equilibration and the lower steady-state level of F1710C modification can be accounted for by faster dissociation of BTX from mutant channels than from wild-type channels. To test this hypothesis, we performed washout experiments to examine directly the rates of BTX dissociation. Figure 4A shows an experiment in an oocyte expressing wild-type channels. After establishing BTX modification with a 2-Hz pulse train, the toxin was washed out and the rate of toxin dissociation was subsequently monitored by applying pulses to 0 mV, once every 30 s, from a holding potential of −90 mV. As expected, no detectable toxin dissociation occurred over approximately 30 min. Figure 4B shows a similar washout experiment for an oocyte expressing F1710C channels. Initially, the washout pulses were applied once every 30 s, just as in the wild-type experiment. According to the modulated receptor model for BTX action (Catterall, 1977), toxin binds with low affinity to resting sodium channels. Thus, toxin dissociation should be favored at hyperpolarized holding potentials, where most channels are in

the resting state. However, to our surprise, using this protocol, there was little dissociation of BTX over more than 10 min. At the time point designated by the arrow in Fig. 4B, the rate of test pulse application was increased to 1 Hz. With these more rapid pulses, tail currents rapidly returned to their control levels, indicating rapid toxin dissociation from the channels. These data suggest that toxin dissociation from F1710C channels was frequency dependent, presumably because dissociation was rapid during depolarizing test pulses, but much slower or nonexistent between the pulses. Figure 4C shows a different experiment, illustrating this striking frequency dependence. In this experiment, we repeatedly induced BTX modification of F1710C channels by 2 Hz pulses in the presence of toxin and then washed the BTX out of the bath and pulsed the toxin off the channels using pulse trains of 2, 0.33, or 0.033 Hz. This experiment clearly demonstrates that the faster the pulse rate, the faster the rate of toxin dissociation. Because BTX-modified channels were open throughout depolarizing test pulses and closed between pulses, these data suggest that rapid toxin dissociation from F1710C channels requires the open state. We can estimate the rate constant for toxin dissociation from open F1710C channels by assuming that toxin dissociation from open channels occurred throughout each 35-ms depolarization, but that no dissociation from closed channels occurred between depolarizations. Using this approach, we obtained a time constant (τ) for dissociation from open channels of 12.0 $\pm$ 1.8 s (n = 4) and a dissociation rate constant ($1/\tau$) of 0.084/s.

We next asked whether we could pulse BTX off of wild-type channels using a similar experimental protocol. Figure 5 shows an experiment addressing this question. Interestingly, we observed modest BTX dissociation from wild-type channels over approximately 20 min with application of a 2-Hz pulse train. This observation suggests that BTX can also dissociate from open wild-type channels, although the rate of dissociation is slow compared with F1710C. In summary,

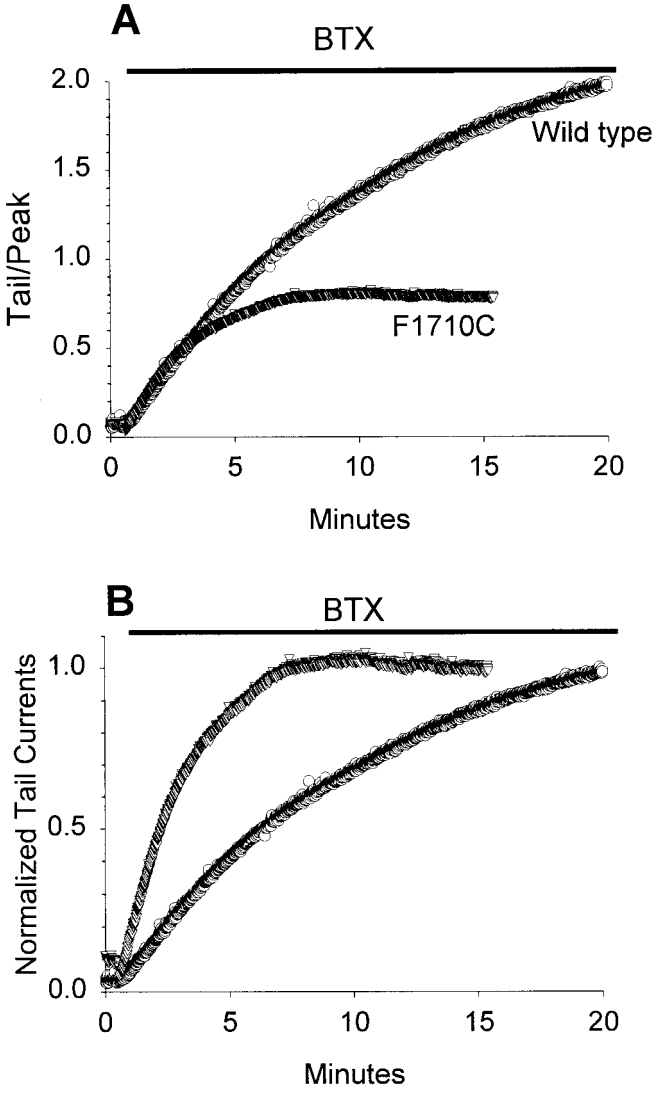

Fig. 3. BTX modification of whole-cell currents approaches steady state more rapidly in oocytes expressing mutant F1710C than in oocytes expressing wild-type channels. A, typical wild-type (○) and F1710C (▽) experiments showing the progressive increase in tail current amplitudes caused by BTX modification of channels during 2 Hz trains of 35-ms depolarizations to 0 mV. In this and subsequent figures, we measured the level of BTX modification by amplitudes of tail currents. We found this to be a convenient and reliable way to assess changes in the levels of channel modification throughout the course of rapid pulse trains. Unless otherwise indicated, we scaled tail amplitudes with respect to peak currents, so that we could compare one experiment with another. BTX was present in the bath for the time indicated by the bar. B, the data from A normalized with respect to the maximum tail currents in each experiment.

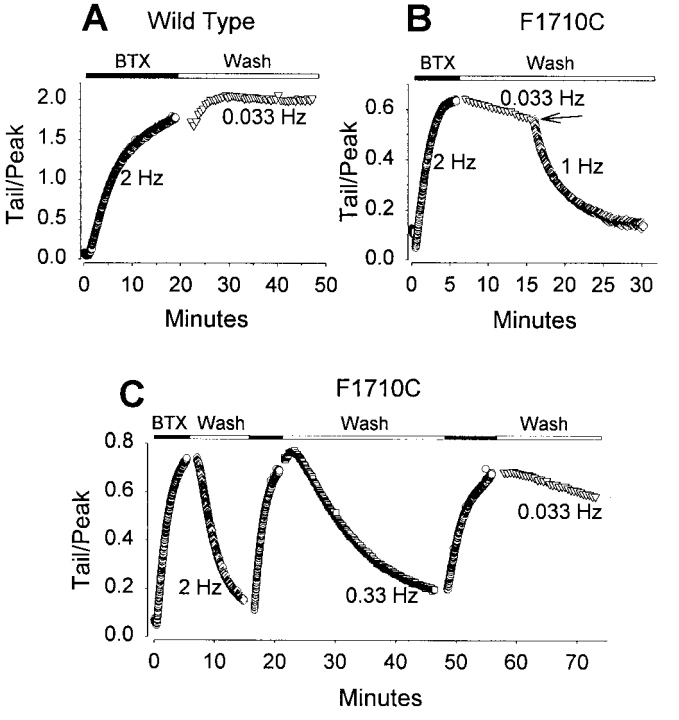

Fig. 4. The rate of BTX dissociation from F1710C channels depends on stimulus frequency. A, BTX did not dissociate from wild-type channels. In this experiment, we first induced BTX modification with a 2-Hz pulse (○). We then washed BTX out of the bath and monitored toxin dissociation by applying depolarizations to 0 mV, once every 30 s (▽). B, BTX dissociation from F1710C mutant channels was accelerated by frequent channel activation. After building up BTX modification of mutant channels by a 2-Hz pulse train (○), we washed toxin out of the bath and then monitored the rate of toxin dissociation by applying depolarizing test pulses to 0 mV, initially at a rate of once very 30 sec (▽). At the arrow, we increased the stimulus frequency to 2 Hz (◇), which caused the toxin to dissociate much more rapidly from the channels. C, the rate of toxin dissociation from F1710C channels is a function of stimulus frequency. BTX was repeatedly pulsed on with 2 Hz pulse trains to 0 mV (○) and then washed out of the bath and pulsed off the channels with trains of 2 Hz (◇), 0.33 Hz (□), or 0.033 Hz (▽).

these data suggest that the pathway by which BTX dissociates from its receptor is only available when the sodium channel is open. Because this pathway is a two-way street, we suggest that toxin access to the unbound receptor also requires the open channel conformation. In other words, the channel activation gate guards the BTX receptor.

BTX Dissociation from F1710C Channels Is Voltage-Dependent. If, as predicted, BTX dissociation requires the open channel conformation, then dissociation should be rapid at test potentials where most channels are open and slower at test potentials where the probability of channel opening is low. To test this prediction, we examined the rate of toxin dissociation from F1710C channels over a range of test potentials. The results of these experiments are summarized in Fig. 6. As expected, the dissociation rate drops off rapidly between −30 and −60 mV (Fig. 6, A and B), a potential range over which the proportion of activated BTX-modified channels goes from ~0.5 to near 0 (Fig. 6C). Interestingly, however, the rate of BTX dissociation showed the reverse voltage-dependence over a more positive range of test potentials. In other words, dissociation was faster at −30 mV than at 0 mV, and faster at 0 mV than at +60 mV (Fig. 6, A and B). The clear voltage-dependence over a range of test potentials (0 mV to +60 mV) in which channel activation is maximal suggests that this voltage dependence is not related to channel activation but instead reflects voltage-dependent dissociation of toxin from open sodium channels. Possible mechanisms for this voltage-dependent dissociation are discussed below.

Discussion

In this article, we report three novel findings concerning modification of voltage-gated sodium channels by BTX: 1) nonpolar substitutions for a critical phenylalanine residue in transmembrane segment IVS6 strongly reduce sodium channel sensitivity to BTX modification, whereas polar or aromatic substitutions at the same site disrupt toxin action only

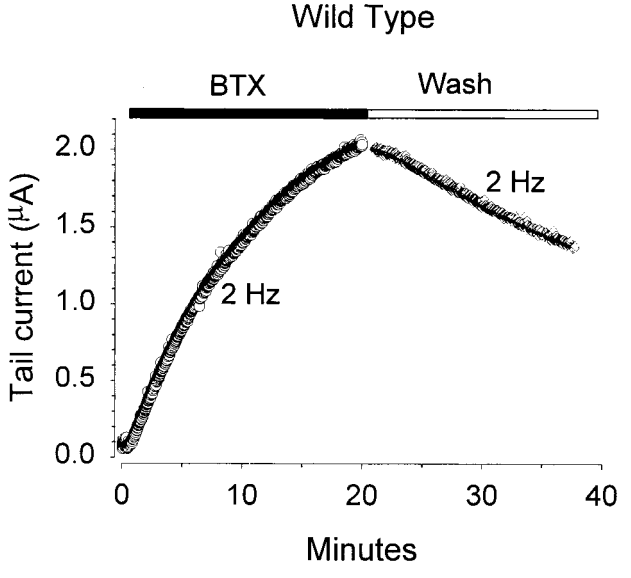

Fig. 5. BTX dissociation from wild-type channels is accelerated by rapid trains of depolarizing test pulses. BTX was first pulsed on using a 2 Hz pulse train (○), then washed out of the bath and pulsed off using a 2 Hz train (◇).

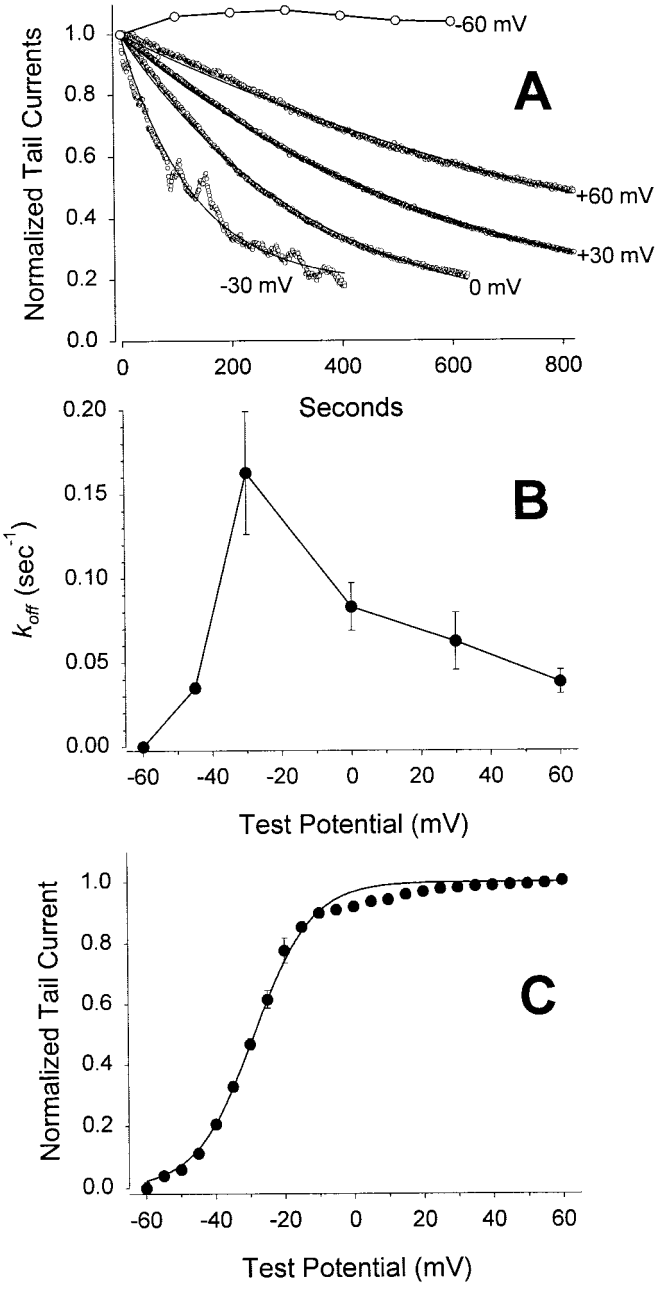

Fig. 6. The rate of BTX dissociation from F1710C channels is voltage-dependent. A, typical experiments showing normalized amplitudes of tail currents elicited by toxin washout pulses applied at different test potentials. In each case, pulses were 35 ms and were applied at a frequency of 1 Hz from a holding voltage of −90 mV. At −60 mV and −45 mV (see B), currents evoked at the test potential were too small to accurately measure. Therefore, at these test potentials, dissociation was assessed by the amplitude of the tail evoked by a single pulse to 0 mV, applied once every 100 pulses. The smooth lines are exponential fits of the data. B, rate constants for toxin dissociation, plotted as a function of test potential. Each symbol shows mean ± S.E.M. for two (−60 mV, −45 mV), three (−30 mV, +30 mV), or four (0 mV, +60 mV) individual experiments. For each experiment, the time course of dissociation, as assessed from the amplitudes of tail currents, was fit by single exponential functions, as in A. The time constants from these fits were multiplied by 0.035, based on the assumption that dissociation occurred only during each 35-ms depolarization. The inverse of this adjusted time constant gave the dissociation rate constant k_{off}. C, the voltage-dependence of activation for BTX modified F1710C channels. Activation was determined, for the experiments in B, from the normalized tail currents evoked, after BTX modification, by 70 msec-long depolarizations to a range of test potentials. Error bars indicate S.E.M.

partially; 2) BTX dissociates rapidly from F1710C mutant channels, but only when the channels are open, not when they are closed, as if the toxin were trapped in the closed channel by the activation gate; 3) toxin dissociation from open F1710C channels is voltage-dependent, with dissociation at negative potentials faster than at positive potentials. In the following sections, we discuss each of these observations.

Sodium Channel Sensitivity to BTX Depends on the Properties of the Residue at Position 1710 in Segment IVS6. The native phenylalanine residue at position 1710 contains a hydrophobic, aromatic side chain that in principle is capable of binding ligands through hydrophobic or electrostatic interactions. At least two types of electrostatic interactions involving aromatic groups are physiologically important: cation-π interactions between a positively charged moiety on the ligand and the negatively charged π orbitals of the aromatic ring (Dougherty, 1996) and aromatic-aromatic interactions between the π face of one aromatic ring and the partially positively charged hydrogen atoms on the edge of another ring (Burley and Petsko, 1985). The striking loss of BTX-mediated modification with substitution of isoleucine, a hydrophobic nonaromatic residue, suggests that hydrophobic interactions at this site are not important for stabilizing BTX binding. Furthermore, the observation that channel modification is preserved with substitution of cysteine, a polar nonaromatic residue, indicates that aromatic-aromatic interactions are not involved. This leaves the possibility that cation-π (for the native Phe or Tyr and Trp substitutions) or ion-dipole (for Cys substitution) interactions are important for stabilizing BTX binding.

How could BTX contribute to these electrostatic interactions? One possibility is through the tertiary amine on its homomorpholino ring (Fig. 7A), which has a p*K*a of ~8.2 (Brown and Daly, 1981) and thus will be almost fully protonated, and positively charged, at physiological pH. This positive charge could interact electrostatically with the native phenylalanine residue as well as with aromatic or polar substitutions at this site. Thus, we propose that an electrostatic interaction between the positively charged tertiary amine on BTX and the aromatic side chain of the phenylalanine residue in transmembrane segment IVS6 is important for stabilizing BTX binding to its receptor. This would leave the pyrrole moiety on BTX free to interact with residues in the IS6 transmembrane segment, as is suggested by a previous photoaffinity labeling study (Trainer et al., 1996) and subsequent site-directed mutagenesis data (Wang and Wang, 1998).

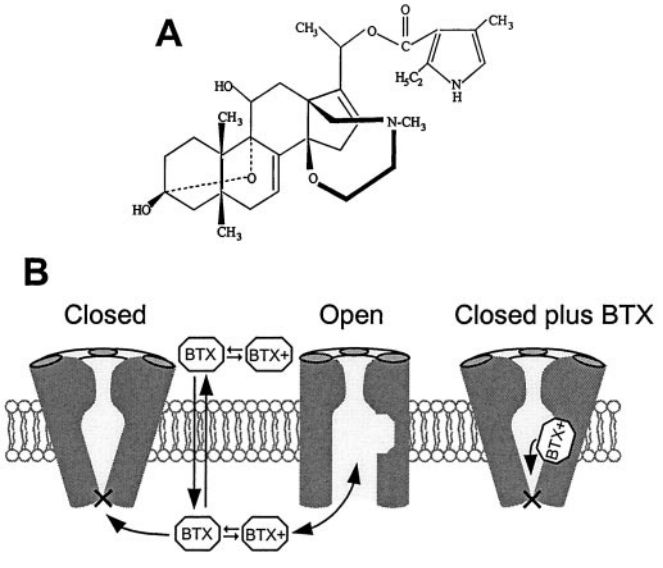

Fig. 7. A model for gated access to the BTX receptor within the pore of the voltage-gated sodium channel. A, the structure of BTX. B, BTX passes through the membrane in its uncharged form, but at physiological pH it is predominantly positively charged within the cell cytoplasm. The toxin reaches its receptor only from the cytoplasmic side of the membrane. The closed activation gate blocks BTX access to the receptor and traps bound toxin within the pore.

Dissociation of BTX from F1710C Sodium Channels Requires the Open Channel Conformation. BTX binding to wild-type sodium channels is virtually irreversible over the time course of a typical electrophysiology experiment; however, BTX rapidly dissociated from F1710C mutant channels. Perhaps the most surprising finding of this study is that this rapid dissociation only occurred during depolarizing stimulus pulses, when BTX-modified channels were open. This observation was unexpected, because the open channel state is thought to put the BTX receptor in a high affinity conformation, whereas resting channels are thought to have a much lower affinity for BTX. Based on the modulated receptor model for BTX action (Catterall, 1977), one might have predicted that toxin dissociation should be faster at hyperpolarized potentials, where most channels are in low-affinity resting states, and slowed by depolarizing pulse trains, which repeatedly put channels in the high-affinity open state. Yet we observed just the opposite effects.

The requirement of the open channel state for both binding and unbinding of BTX is strikingly similar to the behavior of quaternary amine local anesthetics like QX314 and other related channel blockers (Strichartz, 1973; Hille, 1977; Cahalan, 1978). These compounds are permanently positively charged, and they act only from the cytoplasmic side of the membrane. Their receptor is within the cytoplasmic vestibule of the ion-conducting pore. They can reach this receptor only when the channel is open, probably by passing directly through the cytoplasmic end of the open pore. The closed channel conformation blocks access to the receptor. Bound drug can rapidly leave the receptor when the channel is open, by the same open pore pathway; however, closing of the activation gate traps the drug, preventing its dissociation (Yeh and Tanguy, 1985). We propose that BTX moves to and from its receptor by the same open pore pathway. Our model is summarized in Fig. 7B. The positively charged and uncharged forms of BTX are in dynamic equilibrium in aqueous solution. At physiological pH, most BTX molecules are charged, whereas only a small fraction of BTX molecules are uncharged. The uncharged form of BTX is hydrophobic and readily dissolves in and passes through the cell plasma membrane. Once inside the cell, the tertiary amine of the BTX molecule picks up a proton and becomes positively charged. The charged form of BTX, which at physiological pH predominates within the cell, can reach its receptor only when the channel is open, through the cytoplasmic end of the open pore. Closing of the activation gate prevents toxin in the intracellular space from reaching the free receptor and conversely prevents bound toxin from escaping back into the intracellular space. The toxin can dissociate from the open channel, with a rate that depends on the affinity of the open

state, which is quite high for wild-type channels but lower for F1710C mutant channels.

This hypothesis is different from the conventional modulated receptor model of BTX action; however, it does not exclude that model. Indeed, we think that the receptor for BTX is both modulated by channel state and guarded by the channel activation gate. A modulated receptor provides the best explanation for the striking affects of BTX on channel gating. According to this allosteric model (Catterall, 1977), channel opening converts the BTX receptor from a low-affinity conformation to a high-affinity conformation. Binding of BTX to this high-affinity form of the receptor shifts the conformational equilibrium between resting open and inactivated channel states strongly in favor of the open state. We propose that channel opening, in addition to forming the high-affinity BTX receptor, also creates the access pathway through which the toxin reaches this receptor.

BTX Dissociation Is Voltage Dependent. BTX dissociation showed a surprising voltage-dependence over test potentials ranging from −30 mV to +60 mV, with progressively slower dissociation at progressively more depolarized test potentials. The fact that this voltage dependence was observed over a range of potentials at which channel activation was maximal suggests that it was not caused by the closed channel conformation's having a lower affinity for toxin than the open channel conformation. (Indeed, the data indicate that dissociation from closed channels at hyperpolarized potentials is extremely slow.) However, other state-dependent mechanisms are conceivable. For example, one possibility is that there are at least two open states (for example, see Correa et al., 1992; Correa and Bezanilla, 1994), one which predominates at moderately depolarized test potentials and has a relatively low affinity for BTX and a second that predominates at strongly depolarized test potentials and has a higher affinity for BTX. An alternative possibility is that the toxin binding reaction is intrinsically voltage-dependent, because the positive charge on the BTX molecule moves through part of the membrane electric field as the toxin enters its binding site (Woodhull, 1973). This idea is consistent with the location of a critical binding determinant approximately halfway through transmembrane segment IVS6 and with the hypothesis that the toxin binding site is close to the receptor for quaternary local anesthetics, which also exhibit voltage-dependent binding (Strichartz, 1973; Cahalan, 1978; Gingrich et al., 1993; Zamponi and French, 1994). In addition, if the toxin binds at least partially within the ion-conducting pore, it could be "knocked off" by electrostatic interactions with sodium ions entering the channel from the extracellular end of the pore. This knock off effect would be greater at hyperpolarized potentials, where single channel ion flux is high, than at more depolarized potentials, where single channel current is lower.

Summary

In summary, we propose that the pathway to and from the BTX receptor is through the cytoplasmic end of the ion-conducting pore. This pathway is available when the channel is open but is occluded by the closed activation gate. Toxin dissociation from open channels is voltage dependent, due either to state-dependent or to electrostatic mechanisms. Finally, we suggest that the phenylalanine residue at position 1710 within transmembrane segment IVS6 stabilizes toxin binding by an electrostatic interaction, perhaps with the positively charged tertiary amine of the toxin. As long as you are not a sodium channel pharmacologist, your chances of being poisoned by the secretions of *Phyllobates* frogs are exceedingly low. Nevertheless, the actions of batrachotoxin are fascinating, and a clearer picture of how this toxin modifies sodium channel function will probably give new insights into the molecular mechanism of channel gating.

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