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^{3}H-Batrachotoxinin-A Benzoate Binding to Voltage-Sensitive Sodium Channels: Inhibition by the Channel Blockers Tetrodotoxin and Saxitoxin

George B. Brown

The Neurosciences Program and the Department of Psychiatry, The University of Alabama at Birmingham, Birmingham, Alabama 35294

The sodium channel blockers tetrodotoxin (TTX) and saxitoxin (STX) and the channel activator batrachotoxin (BTX) produce their effects by binding to separate and distinct sites on the channel protein. The fact that TTX- and STX-modified sodium channels are blocked to sodium flux has precluded drawing any direct conclusions regarding the effect of TTX/STX on BTX binding based on electrophysiological or ^{22}Na flux measurements. Nevertheless, these sites have been presumed to be noninteracting. In this study, ^{3}H-batrachotoxinin-A benzoate (BTX-B), a tritiated congener of BTX, has been used to provide a direct assessment of these binding interactions. Equilibrium specific binding of ^{3}H-BTX-B to sodium channels in vesicular preparations of mouse brain in the presence of scorpion toxin was measured using a filtration assay procedure. At 25°C both TTX and STX inhibit ^{3}H-BTX-B binding in a concentration-dependent and noncompetitive manner. This inhibition is markedly temperature-dependent, being negligible at 37°C and maximal at 18°C, the lowest temperature investigated. Scatchard analysis of BTX-B binding isotherms at 25°C in the presence and absence of 1 μM TTX revealed that inhibition is due to a 3-fold decrease in the affinity of BTX-B binding with no change in the number of binding sites sites (B_{max}). The concentration dependence for TTX inhibition of both specific ^{3}H-STX and ^{3}H-BTX-B binding is identical, suggesting that inhibition of ^{3}H-BTX-B binding is due to a direct effect of TTX/STX binding at their specific sodium channel site. The channel blockers did not alter the binding of scorpion toxin under these assay conditions, nor did BTX-B affect the binding of ^{3}H-STX. These findings support the conclusion that occupancy of the sodium channel TTX/STX binding site by the channel blockers can be accompanied by a conformational perturbation of the BTX site resulting in decreased BTX-B binding affinity.

The voltage-sensitive sodium channel is responsible for the rapid rising phase of the action potential in a variety of excitable membranes. In order to mediate this transient process, it is clear that the ionophore must be conformationally mobile, able to assume different states. The conformational dynamics of the sodium channel are directly reflected in the voltage-dependent gating process by which, in response to a depolarizing pulse, the channel protein progresses through multiple closed, nonconducting states to an open, conducting conformation that is maintained only briefly before closing to a distinct inactivated state, different from the resting conformation (Armstrong, 1981). The conformational state of the voltage-sensitive sodium channel may also be affected by several specifically acting neurotoxins and, conversely, the binding characteristics of certain toxins are a function of sodium channel conformation. Because of this relationship, biochemical and pharmacological study of these ligand–channel interactions can provide additional insight into the structure and function of this important membrane-bound protein (Albuquerque and Daly, 1976; Cahalan, 1980; Khodorov, 1985; Narahashi, 1974, 1982; Rogart, 1981).

At least three classes of specifically acting neurotoxins have been used extensively in the study of voltage-sensitive sodium channels. These include the heterocyclic guanidines tetrodotoxin (TTX) and saxitoxin (STX), the "lipid-soluble" toxins batrachotoxin (BTX), veratridine, aconitine and grayanotoxin, and the alpha-polypeptide neurotoxins from scorpion and sea anemone. In the formalism of Catterall (1980), TTX and STX bind at type I sites at or near the mouth of the channel, causing blockade of ion flux. The lipid-soluble toxins, of which BTX is the most potent, bind at site II, resulting in stabilization of an open conformation of the channel. The third site, type III, binds the polypeptide α-neurotoxins, which act by inhibition of sodium channel inactivation. The binding of toxins at type II and III sites has been shown to be positively cooperative, and an allosteric model describing this interaction has been presented (Catterall, 1977b). Type I and type II sites, however, have been considered to be distinct and noninteracting based primarily on the inability of BTX to affect specific binding of radiolabeled TTX or STX (Catterall and Morrow, 1978; Colquhoun et al., 1972). The complementary question, that of the effect of the channel blockers on the binding of BTX, has not been directly investigated, since a suitable labeled BTX analog has not been available and the actions of TTX (channel blocker) and BTX (channel activator) are antagonistic, thus rendering assessments based on electrophysiological or flux measurements problematical.

Recently, Brown et al. (1981) have synthesized a radiolabeled BTX derivative that retains full biological activity. The availability of ^{3}H-batrachotoxinin-A 20-α-benzoate (^{3}H-BTX-B) has permitted a direct measurement of the effect of the channel blockers on toxin binding at site II. The experiments presented here show that TTX and STX inhibit BTX-B binding to voltage-sensitive sodium channels at temperatures less than 37°C and suggest that sites I and II are conformationally coupled.

A preliminary report of these findings has appeared (Brown and Johnston, 1983).

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Correspondence should be addressed to Dr. Brown, Neurosciences Program, University of Alabama at Birmingham, University Station, Birmingham, AL 35294.

Materials and Methods

Materials

Leiurus quinquestriatus quinquestriatus scorpion venom and TTX were obtained in lyophilized form from Sigma Chemical Co. (St. Louis, MO). STX was kindly provided by Dr. John Daly, National Institutes of Health. Tritiated STX was prepared by tritium exchange and purified by high-voltage paper electrophoresis as described by Ritchie et al. (1976). The preparation used in these studies had a specific activity of 3 Ci/mmol and a radiochemical purity of 70%. Veratridine was purified from a mixture of veratrum alkaloids (Sigma Chemical Co.) essentially as described by Blount (1935). BTX-B and ^{3}H-BTX-B were prepared as previously reported (Brown et al., 1981). ^{3}H-BTX-B had a specific activity of 14 Ci/mmol and a radiochemical purity of 90%. [^{3}H-BTX-B is currently available from New England Nuclear (Boston, MA) at a specific activity of 30–50 Ci/mmol.] All other reagents were of the highest grade commercially available.

Vesicular preparations of rodent brain

Synaptosomal membrane vesicles were prepared from the brains of 6-month-old male Sprague-Dawley rats by a combination of differential and sucrose density-gradient centrifugation as described by Catterall et al. (1979). The material sedimenting at the 1.0/1.2 M interface was aspirated and collected by centrifugation following dilution of the suspension in 0.32 M sucrose. The pellet was resuspended in standard incubation buffer (see below) at a final protein concentration of 3–4 mg/ml and incubated at 37°C for 20 min prior to use in the binding assays. In most of the experiments reported here, however, another vesicular preparation described as "microsacs" or "synaptoneurosomes" was used as a source of neuronal sodium channels. The basic biochemical and morphological properties of this preparation have been previously characterized (Creveling et al., 1980; Daly et al., 1980). The predominant vesicular elements of the preparation appear to consist of resealed postsynaptic membrane with attached, resealed presynaptic endings. Previous studies have shown that the microsac preparation behaves similarly to synaptosomal preparations with respect to sodium channel neurotoxin binding (e.g., Angelides and Brown, 1984). Microsacs were prepared from Swiss Webster mouse brain cerebral cortical slices by a modification (Creveling et al., 1983; Daly et al., 1980) of the original procedure of Chasin et al. (1974). Briefly, the freshly dissected tissue was homogenized in 2 volumes (wt/vol) of ice-cold standard incubation buffer using 10 strokes of a loose-fitting glass–glass homogenizer. The homogenate was diluted with 2 additional volumes of cold buffer and centrifuged at 1000 × *g* for 15 min at 5°C. The supernatant was discarded, and the pellet was resuspended in standard incubation buffer by repetitive pipetting using a 9 in. Pasteur pipette. The protein concentration was adjusted to approximately 3 mg/ml. All protein determinations were performed using the procedure of Peterson (1977) with BSA as a standard.

Measurement of tritiated toxin binding

Specific binding of ^{3}H-BTX-B was measured using minor modifications of the previously described procedure (Catterall et al., 1981). Binding reactions were initiated by addition of 150 μl of vesicular preparation containing 150–500 μg protein to a solution in standard incubation buffer of ^{3}H-BTX-B, 50 μg *L. quinquestriatus quinquestriatus* scorpion venom and various unlabeled effectors as indicated. The concentration of ^{3}H-BTX-B was generally 20–25 nM, and the total assay volume was 335 μl. Standard incubation buffer contained 130 mM choline chloride, 50 mM HEPES buffer adjusted to pH 7.4 with Tris base, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 1 mg/ml BSA. Incubations were carried out for 60 min at the indicated temperature and were then terminated by addition of 3 ml ice-cold wash buffer. The tissue was immediately collected on Whatman GF/C glass fiber filters and washed 3 more times with 3 ml cold wash buffer. The wash buffer contained 163 mM choline chloride, 5 mM HEPES (pH 7.4), 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mg/ml BSA. Radioactivity associated with the tissue was determined by liquid-scintillation spectroscopy of the filters suspended in 10 ml scintillation cocktail (3a70B; RPI). Nonspecific binding was determined from parallel incubations containing 250 μM veratridine and has been subtracted from the data. Specific binding measured in this way was nominally 75% of the total binding.

Binding of ^{3}H-STX was measured in a manner analogous to that described for ^{3}H-BTX-B. In these assays, the concentration of ^{3}H-STX was adjusted to approximately 1 nM, and unlabeled TTX or STX at a concentration of 1 μM was included in parallel assays for the determination of nonspecific binding. Under these conditions, specific ^{3}H-STX binding accounted for 95% of the total binding.

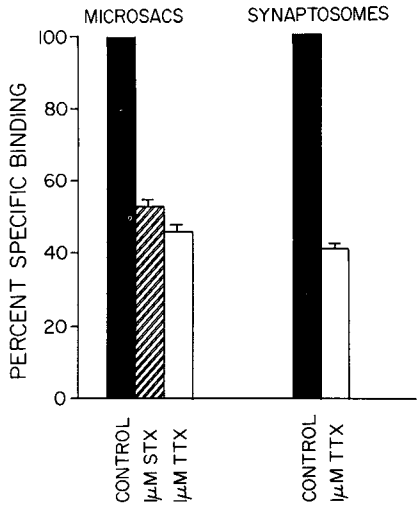

Figure 1. Inhibition of ^{3}H-BTX-B binding by TTX and STX. Microsacs or synaptosomes were equilibrated with 20 nM ^{3}H-BTX-B and 150 μg/ml *L. quinquestriatus* scorpion venom at 25°C in the presence and absence (control) of 1 μM TTX or STX. Specifically bound label for each case, determined as the difference between total binding and binding in the presence of 250 μM veratridine, is plotted as the percentage of specific binding normalized to control binding, defined as 100%. The data are the means ± SD of 3 experiments run in triplicate.

Data analysis

All data points were determined in triplicate and are presented as the mean of these determinations. Unless otherwise indicated, the figures show the results of 1 experiment that is representative of 2 or more separate determinations. Curvilinear fits to the data have been approximated by eye, whereas linear fits were determined by regression analysis.

Results

Inhibition of BTX-B binding by channel blockers

An initial assessment of the effect of the channel blockers TTX and STX on ^{3}H-BTX-B binding to neuronal sodium channels was made by inclusion of these toxins in standard binding assays using mouse microsacs incubated at a temperature of 25°C. Figure 1 graphically shows that 1 μM TTX or STX reduced specific ^{3}H-BTX-B binding by 50–60% relative to control binding in the absence of the channel blockers. This degree of inhibition remained unchanged when the concentration of the blocker was increased to 5 μM or when the incubation time was extended from 60 to 120 min. Thus, under equilibrium conditions, maximal inhibition of BTX-B binding by TTX or STX was substantial, but incomplete, suggesting a noncompetitive mechanism for the observed inhibition. The results of a similar experiment using rat brain synaptosomes confirmed that the TTX- or STX-induced inhibition was not peculiar to the microsac preparation (Fig. 1).

The effect of TTX and STX on BTX-B binding was readily reversible. In one series of experiments, microsacs were pre-equilibrated with 1 μM TTX or STX in standard incubation buffer for 30 min at 25°C in the absence of ^{3}H-BTX-B. Subsequent addition of ^{3}H-BTX-B and assay of specific binding produced the expected inhibition relative to control tubes pre-equilibrated without TTX or STX. If, on the other hand, microsacs that had been pre-equilibrated with blocking agent were washed

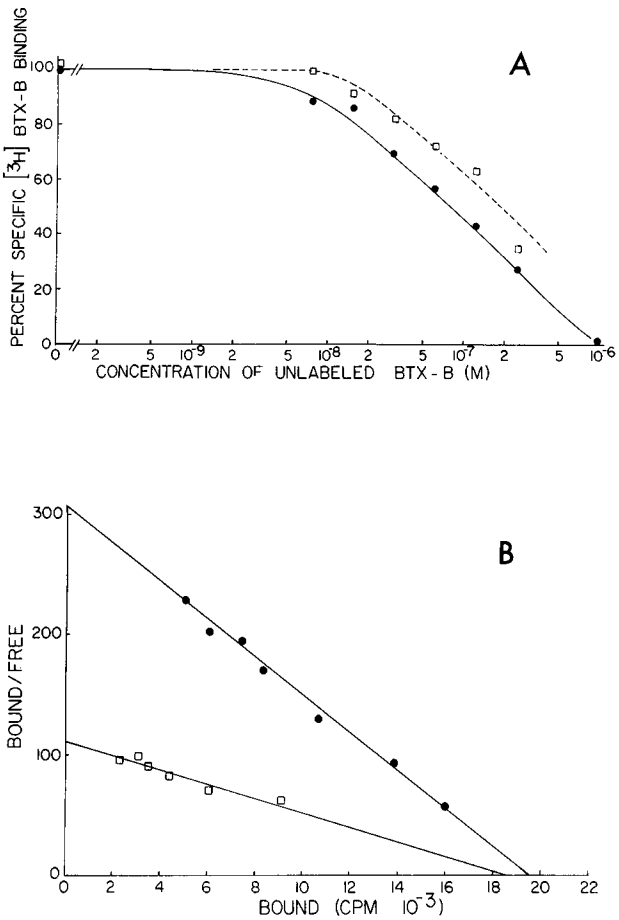

Figure 2. Displacement of specific ^{3}H-BTX-B binding in mouse brain cortical microsacs by BTX-B in the presence and absence of TTX. *A*, Microsacs were incubated with 22 nM ^{3}H-BTX-B and the indicated concentrations of unlabeled BTX-B in the presence (□) or absence (●) of 1 μM TTX for 1 hr at 25°C. Specifically bound label was measured by filtration assay as described under Materials and Methods. The displacement curve for BTX-B binding is shifted to the right in the presence of TTX. *B*, The data in *A* presented in the form of a Scatchard plot. There is little change in B_{max}, but the K_D for BTX-B binding, determined from the slopes of the lines, is increased from 65 nM in the absence of TTX to 180 nM in the presence of TTX.

by 3 repetitions of centrifugation and resuspension in standard incubation buffer prior to assay of ^{3}H-BTX-B binding, 87% of control binding was recovered.

TTX decreases BTX-B binding affinity without affecting the number of binding sites

At the first level of analysis, the decrease in ^{3}H-BTX-B binding in the presence of the channel blockers may be due to either a decrease in the affinity of BTX-B for its binding site, a loss of the available number of binding sites, or both. In order to distinguish between these possibilities the equilibrium binding isotherms for ^{3}H-BTX-B binding to mouse brain microsacs at 25°C were determined in the presence and absence of 1 μM TTX by displacement assay. Specific binding for a constant concentration of ^{3}H-BTX-B was measured as a function of increasing concentrations of unlabeled BTX-B. The resulting inhibition curves (Fig. 2*A*) reveal a substantial shift to the right for ^{3}H-BTX-B displacement in the presence of 1 μM TTX. The apparent $K_{0.5}$ values are 85 and 200 nM in the absence and presence of TTX, respectively. For further analysis, bound and free amounts of BTX-B were calculated for each concentration point, and the values used to construct Scatchard plots (Fig. 2*B*). The linearity

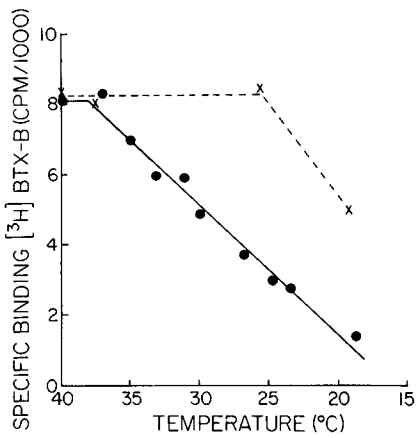

Figure 3. Temperature dependence of TTX inhibition of ^{3}H-BTX-B binding. Specific binding of ^{3}H-BTX-B in mouse brain cortical microsacs in the presence (●) or absence (×) of 1 μM TTX is presented as a function of incubation temperature. All incubations were carried out for 60 min using the same tissue preparation and a constant concentration of ^{3}H-BTX-B. Temperature was controlled by immersion of the assay tubes in thermostatted water baths, and the tubes were capped to prevent loss due to evaporation.

of the plots, both in the absence and presence of TTX, is consonant with BTX-B binding to a single class of noninteracting sites. From the slopes of the lines, the apparent dissociation constants for BTX-B binding in the absence and presence of TTX were calculated to be 65 and 180 nM, respectively. As can be seen from the intercepts on the abscissa, TTX does not appreciably affect the number of binding sites, so that inhibition of ^{3}H-BTX-B binding may be solely ascribed to the approximate 3-fold reduction in BTX-B binding affinity.

Similar experiments were carried out using veratridine as the displacing ligand in place of unlabeled BTX-B. In this case, the apparent K_D for veratridine binding was increased 4-fold, from 3.5 μM in the absence of TTX to 14 μM in the presence of TTX (data not shown). Thus, the effect of TTX is not specific for BTX-B binding, but extends as well to other ligands that bind at type II sites on the sodium channel.

Temperature dependence of TTX inhibition

In a previous study, Creveling et al. (1983) reported that inclusion of TTX at a concentration of 1 μM in ^{3}H-BTX-B binding assays to guinea pig microsacs at 37°C in the presence of scorpion toxin *increased* specific binding by approximately 7%. Since high-affinity ^{3}H-BTX-B binding is dependent on the positively cooperative effect of scorpion toxin binding (Catterall et al., 1981) and the binding of α-scorpion toxins is membrane-potential-dependent (Catterall, 1977a, b), addition of TTX was presumed to increase BTX-B binding indirectly by stabilization of the membrane potential, and hence scorpion toxin binding, against depolarization mediated by any residual sodium ions present in the vesicular preparation. The reported lack of TTX inhibition of ^{3}H-BTX-B binding at 37°C prompted a more detailed examination of the effect of temperature on TTX/BTX-B binding interactions.

Specific binding of ^{3}H-BTX-B to mouse microsacs in the presence and absence of 1 μM TTX was measured as a function of temperature in the range 18–40°C. The data in Figure 3 indicate that, as previously reported, TTX does not inhibit ^{3}H-BTX-B binding at 37°C. However, inhibition is already marked at 35°C and increases approximately linearly as the temperature is lowered to 18°C. The slope corresponds to 5% reduction in specific ^{3}H-BTX-B binding/°C. Specific binding in the absence of TTX is, in contrast, temperature-insensitive in the range 25–40°C.

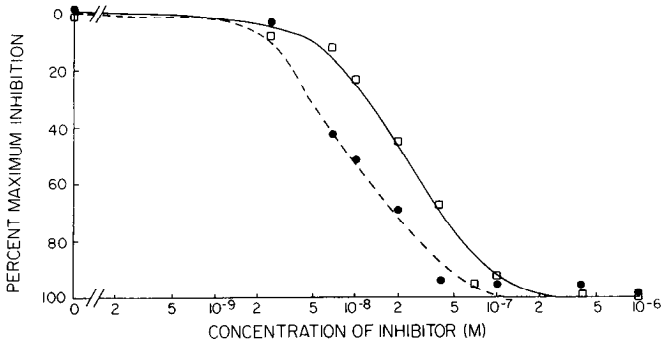

Figure 4. Dependence of TTX and STX inhibition of specific ^{3}H-BTX-B binding on concentration. Mouse brain cortical microsacs were incubated with a constant concentration of ^{3}H-BTX-B (20 nM) and the indicated concentrations of either TTX (□) or STX (●) for 1 hr at 25°C. Specifically bound label was then assayed as described under Materials and Methods. The effect of TTX or STX at a concentration of 1 μM was defined as the maximal inhibition, and the data are plotted as the percentage of this maximal inhibition vs concentration of the inhibitor.

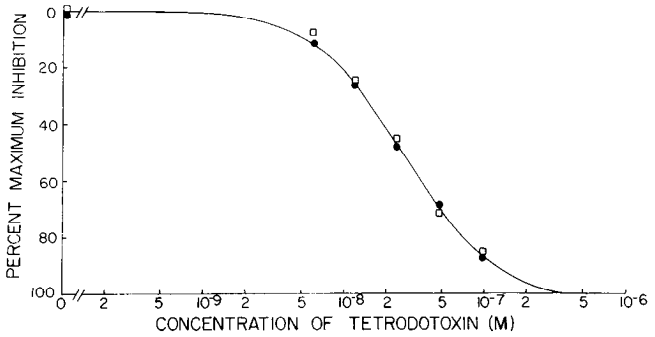

Figure 5. Comparison of the concentration dependence of TTX inhibition of ^{3}H-STX and ^{3}H-BTX-B binding to mouse brain cortical microsacs. Microsacs from the same tissue preparation were incubated with a constant concentration of either ^{3}H-STX (1 nM, ●) or ^{3}H-BTX-B (20 nM, □) and the indicated concentrations of unlabeled TTX for 1 hr at 25°C. Specific binding was determined by filtration assay as described under Materials and Methods. The results are expressed as percentage of maximal inhibition, defined as the inhibition in the presence of 1 μM unlabeled TTX. The resulting displacement curves are superposable, giving a $K_{0.5}$ value of approximately 20 μM for TTX inhibition of both ^{3}H-STX and ^{3}H-BTX-B binding.

The decrease in specific binding of ^{3}H-BTX-B between 25 and 18°C in the absence of TTX is expected as a consequence of decreased scorpion toxin binding affinity at lower temperatures, with a concomitant reduction of the positively cooperative effect on ^{3}H-BTX-B binding (Brown et al., 1981; Catterall, 1977a, b).

Concentration dependence of TTX and STX inhibition

If inhibition of ^{3}H-BTX-B binding by TTX and STX is mediated by interaction of the channel blockers at type I sites on the sodium channel, the concentration dependence of this inhibition should follow the appropriate Langmuir absorption isotherm for that binding. In order to examine this possibility, specific ^{3}H-BTX-B binding to mouse cerebral cortex microsacs was measured at 25°C as a function of both STX and TTX concentration in the range of 3–1000 nM. The data are presented in Figure 4 in the form of inhibition curves from which estimates can be made of the concentration displacing 50% of the total displaceable binding (IC_{50}). The apparent IC_{50} values are 10 and 20 nM for STX and TTX, respectively. For comparison, the apparent equilibrium dissociation constants for STX and TTX binding to rat brain sodium channels are approximately 2 and 15 nM, respectively (Catterall et al., 1979). Thus, the concentration dependence of STX and TTX inhibition of ^{3}H-BTX-B binding is in the range appropriate for binding of these ligands at their known sodium channel site.

In these experiments it was helpful to use a concentration of tissue (1.5 mg protein/ml) such that an adequate specific to nonspecific binding ratio for ^{3}H-BTX-B could be maintained even when 60% of specific binding was blocked. Under these conditions the concentration of ^{3}H-BTX-B binding sites in the assay was found to be 3.6 nM. Although the site density for ^{3}H-STX binding was not determined for this tissue preparation, previous studies (Catterall et al., 1981) using rat brain synaptosomes suggest that there may be 2–3 times as many STX sites as BTX-B sites. Thus, the concentration of STX sites in the present experiments is at least as high as, and perhaps several times higher than, the dissociation constant for STX binding. As discussed by Chang et al. (1975), this circumstance leads to artifactually high values for the apparent dissociation constant (in this case, the IC_{50}) when the data are treated as in Figure 4. In order to circumvent this problem, 2 displacement binding assays were carried out in parallel at 25°C using the same tissue preparation and identical conditions for both, except that in one assay ^{3}H-STX (1 nM) replaced ^{3}H-BTX-B (20 nM) as the labeled ligand. The specific binding of both ^{3}H-STX and ^{3}H-BTX-B was then measured as a function of the concentration of unlabeled TTX. The data, normalized to the percentage of maximum inhibition to facilitate comparison, are presented as displacement curves in Figure 5. The 2 curves are essentially superposable, consistent with the conclusion that TTX inhibition of ^{3}H-BTX-B binding is a result of TTX binding to type I sites on the voltage-sensitive sodium channel.

TTX does not affect scorpion toxin enhancement of ^{3}H-BTX-B binding

In all of the experiments reported here, *L. quinquestiatus quinquestriatus* scorpion venom at a concentration of 150 μg/ml was included in the assays to enhance the binding affinity of ^{3}H-BTX-B. To assess the possibility that the TTX-induced decrease in BTX-B binding affinity is secondary to a direct effect on scorpion toxin binding, dose–response curves for scorpion toxin enhancement of ^{3}H-BTX-B binding in the presence and absence of 1 μM TTX were compared. The results demonstrate that the concentration dependence of scorpion toxin enhancement of ^{3}H-BTX-B binding is not altered by TTX (Fig. 6). It is unlikely, therefore, that the mechanism of TTX inhibition involves modification of the cooperative interaction between BTX-B and scorpion toxin binding. This conclusion is further supported by the finding that, in a variety of tissues, binding of the purified ^{125}I-labeled α-scorpion toxin from *L. quinquestriatus quinquestriatus* to voltage-sensitive sodium channels is unaffected by the channel blockers (Catterall, 1977a; Ray and Catterall, 1978; Ray et al., 1978). It should be noted, however, that Sieman and Vogel (1983) recently reported on the ability of TTX to block effects of purified α-scorpion toxins from *Buthus tamulus* on sodium channel inactivation in single nerve fibers of *Xenopus laevis*. This blocking action was observed only if TTX was added simultaneously with or prior to addition of scorpion toxin to the incubation medium. Similar findings have been described earlier for the effect of TTX on the actions of scorpion toxin I from *Androctonus australis* Hector on crayfish neuromuscular junction and rat brain synaptosomes (Romey et al., 1976).

Discussion

Characterization of the binding to voltage-sensitive sodium channels for a growing number of specifically acting neurotoxins has provided a valuable perspective contributing to the development of a molecular understanding of the structure and func-

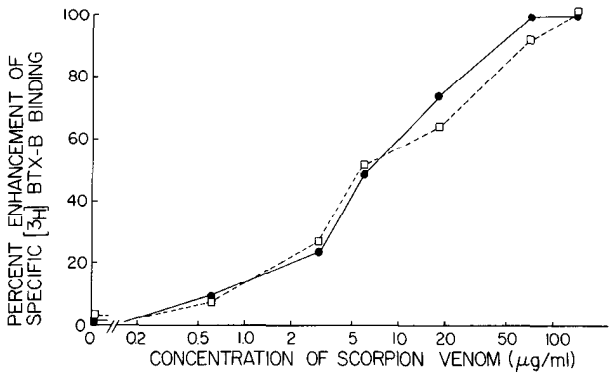

Figure 6. Effect of TTX on the concentration dependence for scorpion toxin enhancement of ^{3}H-BTX-B binding. Mouse brain cortical microsacs were incubated with ^{3}H-BTX-B (25 nM) and the indicated concentrations of *L. quinquestriatus* scorpion venom in the presence (□) or absence (●) of 1 μM TTX for 1 hr at 25°C. Scorpion venom was added from a concentrated stock solution prepared by gentle stirring of the lyophilized venom in distilled water (2 mg/ml) at 5°C for 60 min, followed by high-speed centrifugation to remove insoluble material. Specific binding of ^{3}H-BTX-B was measured by filtration assay as described under Materials and Methods. Since maximal binding of ^{3}H-BTX-B in the presence of TTX is less than that in the absence of TTX, the data have been normalized to a percentage of the maximum enhancement produced by scorpion venom in either the presence or absence of 1 μM TTX. In each case, maximal enhancement was defined as the difference between specific ^{3}H-BTX-B binding in the absence of scorpion venom (≈0) and that in the presence of scorpion venom at a concentration of 150 μg/ml.

tion of the sodium channel. The demonstration that ^{3}H-BTX-B, an active congener of batrachotoxin, interacts selectively at the batrachotoxin binding site, or site II, of the sodium channel (Brown et al., 1981; Catterall et al., 1981) provided a mechanism to make a direct assessment of effects of other sodium channel neurotoxins and ligands on toxin binding at site II. A noteworthy observation from these studies is that a surprisingly large number of unrelated compounds, binding at distinct sites on the voltage-sensitive sodium channel, are capable of modifying the binding of ^{3}H-BTX-B. A list of these compounds (or class of compounds) and their effect on ^{3}H-BTX-B binding is presented in Table 1. One of these interactions, the positively cooperative effect of α-scorpion toxins and anemonetoxin, has had the practical impact of allowing convenient measurement of ^{3}H-BTX-B binding by virtue of the toxin-induced increase in affinity (Catterall et al., 1981).

In the face of such extensive interactions between the binding of ^{3}H-BTX-B and other sodium channel ligands, it seems reasonable to suggest that binding site II is in a domain that exhibits considerable conformational flexibility and that is conformationally coupled, directly or allosterically, with other more remote regions of the channel protein(s). A similar impression might be reached by considering the effects of BTX on sodium channel function. Virtually all parameters constituting an electrophysiological description of the channel are altered in the presence of BTX. Inactivation is blocked, the voltage dependence of activation is shifted to more negative potentials, the conductance of the channel is decreased, and the selectivity filter becomes less discriminating to positively charged ions (see Khodorov, 1985, for review).

Results of the experiments presented here provide the first evidence for an interaction between STX or TTX binding at site I and ^{3}H-BTX-B binding at site II on the voltage-sensitive sodium channel. These 2 sites have been considered to be noninteracting based primarily on the inability of BTX to affect the binding of labeled STX or TTX. Indeed, in the present studies,

Table 1. Modifiers of BTX-B binding

Compound (or class)	Effect	Reference
α-Scorpion toxin, sea anemone toxin	Increase affinity	Catterall et al. (1981)
Local anesthetics	Decrease affinity	Creveling et al. (1983), Postma and Catterall (1984)
Diphenylhydantoin and carbamazepine (anticonvulsants)	Decrease affinity	Willow and Catterall (1982)
α-Cyano pyrethroid insecticides	Increase affinity	Brown and Olsen (1984)
TTX and STX	Decrease affinity	This study

under no condition was any effect of BTX-B on ^{3}H-STX binding discernible, even using concentrations of BTX-B as high as 5 μM (data not shown). On the other hand, both TTX and STX inhibit ^{3}H-BTX-B binding to neuronal sodium channels in a concentration- and temperature-dependent manner. Since the concentration dependence of TTX inhibition of ^{3}H-STX binding parallels exactly the concentration dependence of inhibition of ^{3}H-BTX-B binding (Fig. 5), it appears that inhibition is mediated by interaction of the channel blockers with their normal type I site on the sodium channel.

At saturating concentrations of STX or TTX at 25°C, the binding data indicate the presence of a single class of BTX-B sites characterized by a 3-fold lower affinity for BTX-B binding (Fig. 2*B*). There is no evidence for a population of sodium channels that are not so modified. This observation requires some comment, since the vesicular preparations used in these studies could possibly contain a percentage of "inside out" vesicles. Sodium channels in these vesicles could still bind the lipid-soluble BTX derivative but not TTX or STX, since type I sites are externally located and the channel blockers, being highly charged, cannot penetrate the membrane (Narahashi et al., 1966). Experimentally, however, BTX-B binding to such "inside out" vesicles would not be measured with the filtration assay used in these studies. Sodium channels in "inside out" vesicles would also be inaccessible to binding of α-scorpion toxin and, as a consequence, would exhibit 10- to 15-fold lower affinity for BTX-B binding than in "right side out" vesicles, where the positively cooperative interaction between BTX-B and scorpion toxin binding is manifest. Further, the off-rate for BTX-B binding in the absence of scorpion toxin is quite rapid and specifically bound ligand is lost from the tissue during filtration and washing (Brown et al., 1981). These 2 factors combine to ensure that specific ^{3}H-BTX-B binding measured in these studies essentially reflects binding only to those sodium channels that are also accessible to TTX and STX.

As mentioned above, TTX and STX are positively charged at physiological pH by virtue of the highly basic guanidinium moiety common to both structures. BTX and its derivatives exist as mixtures of positively charged and neutral species at pH 7.4, representing titration of the homomorpholino ring nitrogen. Recent evidence suggests that it is the charged species of BTX-B that binds preferentially at the sodium channel site (Angelides and Brown, 1984; Brown and Daly, 1981). If binding of these ligands were to occur such that the positive charges of BTX-B and the channel blocker were situated within the Debye length, then electrostatic repulsion might be a possible mechanism for TTX/STX inhibition of BTX-B binding. However, since BTX-B has no effect on TTX/STX binding, this possibility may be safely discarded.

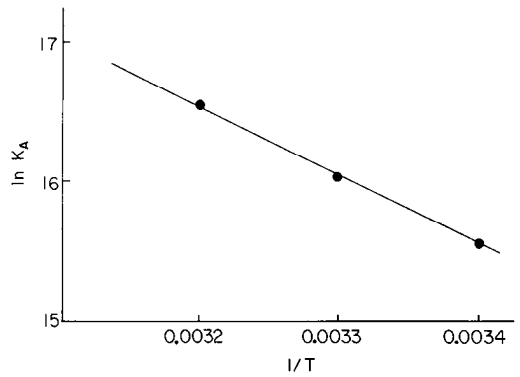

Figure 7. Van't Hoff analysis of the temperature dependence for ^{3}H-BTX-B binding in the presence of 1 μM TTX. The logarithms of the association constants, K_A, for specific ^{3}H-BTX-B binding to mouse brain cortical microsacs in the presence of 1 μM TTX at representative temperatures of 25, 30, and 37°C plotted against the inverse of the temperature in degrees Kelvin. The linearity of the plot suggests the absence of a membrane phase transition or significant differences in the heat capacities of reactants and products in this temperature range. The standard enthalpy change for the binding reaction can therefore be calculated from the slope of the line ($-\Delta H^0/R$), yielding $\Delta H^0 = 9.95$ kcal/mol.

As shown in Figure 3, binding of ^{3}H-BTX-B is highly temperature-dependent in the presence of TTX, whereas in the absence of TTX the K_D for ^{3}H-BTX-B binding is constant in the range 25 to 37°C. Analysis of the equilibrium thermodynamic parameters underlying these differing behaviors can provide additional insight into the mechanism of TTX/BTX-B binding interactions. The standard free energy of association is related to the equilibrium association constant by the expression

$$\Delta G^0 = -RT \ln K_A \quad (1)$$

where R is the gas constant and T is the temperature in degrees Kelvin. Further, the standard free energy defines the differences in standard enthalpy, ΔH^0, and entropy, ΔS^0, between reactants and products by the relationship

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (2)$$

The standard enthalpy can be calculated from the temperature dependence of the association constant expressed as a van't Hoff plot of ln K_A vs $1/T$. The slope of the resultant line is $-\Delta H^0/R$. (For more complete discussion of these considerations, see Weiland and Molinoff, 1981.) To assess the standard enthalpy change for BTX-B binding in the presence of TTX, data from Figure 3 have been used to construct the van't Hoff plot in Figure 7, yielding $\Delta H^0 = 9.95$ kcal/mol. Thus, in the presence of TTX, BTX-B binding is strongly endothermic. In the absence of TTX, however, $\Delta H^0 = 0$, since BTX-B binding is temperature-insensitive in the temperature range under discussion. In this case, high-affinity binding is driven solely by a large positive entropy change. Now, knowing the association constants for BTX-B binding at 25°C in the absence and presence of TTX (15.2×10^6 and 5.6×10^6 M^{-1}, respectively, from Fig. 2) and the standard enthalpies calculated above, all relevant thermodynamic parameters comparing BTX-B binding at 25°C in the absence and presence of TTX can be determined from appropriate substitutions in equations (1) and (2). These parameters are collected in Table 2. Brief inspection shows that, in the presence of TTX, the positive enthalpy change is largely counteracted by an increase in the entropy. Relative to binding in the absence of TTX, the entropy change is approximately doubled. The large positive ΔH^0 suggests that in the presence of TTX there is a net loss of favorable dipole–dipole, hydrogen bond, or ionic interactions on binding of BTX-B, whereas the gain in ΔS^0 indicates an increase in the number of equivalent energy states available to products relative to reactants. The magnitude of these changes induced by inclusion of TTX is striking. To place this in perspective, consider that, were it not for the compensatory increase in ΔS^0, the unfavorable enthalpy change would reduce BTX-B binding affinity at 25°C to zero. Considering the substantial changes in both binding interactions and "randomness" characterizing BTX-B binding in the presence of TTX, significant conformational differences are likely to be involved. The most straightforward hypothesis is that binding of the channel blockers at type I sites of the sodium channel is accompanied by a conformational perturbation in the type II binding domain, reflected in decreased binding affinity for the "lipid-soluble" neurotoxins. Whatever the mechanism, it is clear that type I and type II sites can no longer be considered to be noninteracting.

Table 2. Thermodynamic parameters for BTX-B binding in the presence and absence of TTX at 25°C

1 μM TTX	ΔG^0 (kcal/mol)	ΔH^0 (kcal/mol)	ΔS^0 (cal/mol deg)
Absent	−9.8	0	32.9
Present	−9.2	9.95	64.3

The interesting relationship between TTX/STX and BTX-B binding disclosed by the experiments presented here underscores the view that further information regarding the spatial relationship between type I and type II sites on the voltage-sensitive sodium channel will be very useful in developing a molecular understanding of structure–function relationships for this protein. Two relevant parameters are (1) the distance separating type I and type II sites, and (2) identification of the BTX-B binding protein(s). With regard to the first parameter, Angelides and his colleagues have applied techniques of fluorescence resonance energy transfer to assess the distance between a number of specifically bound sodium channel neurotoxins (Angelides and Brown, 1984; Angelides and Nutter, 1983, 1984; Darbon and Angelides, 1984). There is currently no information regarding the distance separating STX/TTX and BTX or BTX derivatives bound to the sodium channel. Concerning point (2), isolation and characterization of the TTX/STX and α-scorpion toxin binding proteins of neuronal sodium channels have been achieved (reviewed in Catterall, 1984), but no comparable data concerning the BTX binding protein(s) are available. Since solubilization of the sodium channel results in loss of BTX-B binding, progress on this front will require covalent labeling with a suitable derivative *in situ* prior to solubilization. Current experiments in this laboratory are directed toward the development and application of fluorescent and photoactivatable derivatives of BTX that will be useful in these areas of investigation.

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