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# Interaction of Pumiliotoxin B with an "Alkaloid-Binding Domain" on the Voltage-Dependent Sodium Channel

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## SUMMARY

The alkaloid pumiliotoxin B (PTX-B) "activates" voltage-dependent sodium channels in synaptoneurosomes and neuroblastoma cells. It appears that PTX-B activates sodium channels by interacting with a site that is allosterically coupled to other sites on the sodium channel, namely two scorpion toxin sites and the brevetoxin site. In guinea pig cortical synaptoneurosomes, α-scorpion toxin, β-scorpion toxin, and brevetoxin induce a dose-dependent potentiation of PTX-B-induced $^{22}Na^+$ influx. The synergism with β-scorpion toxin differentiates PTX-B from the alkaloid veratridine, which induces an activation of sodium channels that is not affected by β-scorpion toxin. PTX-B does not inhibit [$^3$H]batrachotoxinin-A benzoate ([$^3$H]BTX-B) binding to the alkaloid site on sodium channels. On the other hand, aconitine, which activates sodium channels and inhibits [$^3$H]BTX-B binding, induces a $^{22}Na^+$ influx that, like PTX-B-induced $^{22}Na^+$ influx, is potentiated by α-scorpion toxin, β-scorpion toxin, and brevetoxin. Inhibition of [$^3$H]BTX-B binding by aconitine is reduced in the presence of PTX-B. Both a type I pyrethroid (allethrin) and a type II pyrethroid (fenvalerate) inhibit PTX-B- and PTX-B/α-scorpion toxin-mediated $^{22}Na^+$ influx. Allethrin and fenvalerate also inhibit aconitine-mediated $^{22}Na^+$ flux but not BTX-mediated $^{22}Na^+$ influx. It is proposed that on the sodium channel there is an "alkaloid-binding domain" at which alkaloids exert stimulatory actions. However, depending on the region on the domain to which the binding occurs, different allosteric interactions with other sites can be observed. PTX-B is proposed to interact with a part of the alkaloid-binding domain that is shared by aconitine but not by batrachotoxin or veratridine, whereas aconitine interacts with a part of the domain shared by PTX-B and by batrachotoxin/veratridine.

The binding of neurotoxins to voltage-dependent sodium channels has revealed the presence of several sites that modulate channel function (1, 2). The alkaloids BTX, veratridine, and aconitine bind to site 2 on the sodium channel. Binding of BTX at site 2 results in persistent activation of the channel. Peptide toxins that bind to site 3 on the sodium channel, such as α-ScTx and sea anemone toxin, slow the inactivation of the channel and potentiate the action of alkaloids that bind to site 2. Peptide toxins that bind to site 4, such as β-ScTx, affect the activation of the channel but do not appear to affect the action of alkaloids that bind to site 2 (1, 2). The alkaloid PTX-B activates sodium channels by binding to a site that appeared to be different from alkaloid binding site 2 (3). PTX-B induces a sodium influx in neuroblastoma cells and synaptoneurosomes that is potentiated in the presence of both α-ScTx and β-ScTx. Indeed, in neuroblastoma cells, PTX-B-mediated sodium flux has only been observed in the presence of α-ScTx. The actions of PTX-B are reminiscent of those of aconitine (2). However, in contrast to aconitine, PTX-B does not inhibit [$^3$H]BTX-B binding in synaptoneurosomes, suggesting that PTX-B interacts with a site different from site 2 (BTX binding site) (3).

We have now characterized more fully the interactions between the PTX-B site and other modulatory sites on the sodium channel, including the scorpion toxin sites, the brevetoxin site, and pyrethroid site(s) in guinea pig synaptoneurosomes. The effects of aconitine are compared with those of PTX-B. We propose that all of the presently known alkaloids interact with an "alkaloid-binding domain," corresponding to site 2 on the sodium channel, resulting in activation of the channel, but that different alkaloids interact with different subdomains of the alkaloid-binding region.

## Experimental Procedures

**Materials.** $^{22}NaCl$ (25 Ci/mmol, carrier free) was from Amersham (Arlington Heights, IL). [$^3$H]BTX-B was from New England Nuclear (Boston, MA). Tetrodotoxin, ScV (from *Leiurus quinquestriatus*), veratridine, and aconitine were from Sigma (St. Louis, MO). Allethrin and fenvalerate were provided by Dr. D. Rossignol (E. I. DuPont, Wilmington, DE). BTX was isolated from the Colombian poison dart frog

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**ABBREVIATIONS:** BTX, batrachotoxin; PTX-B, pumiliotoxin B; α-ScTx, α-scorpion toxin; β-ScTx, β-scorpion toxin; BTX-B, batrachotoxinin-A benzoate; ScV, scorpion venom; PTX-A, pumiliotoxin A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

*Phyllobates terribilis* (4). Hydrofluor, Betafluor, and Filtron X were from National Diagnostics (Sommerville, NJ). PTX-A and -B were isolated from the Panamanian poison frog *Dendrobates pumilio* (5). *erythro*-PTX-B was synthesized (6) and provided to us by Dr. L. Overman (University of California, Irvine). β-ScTx from *Centruroides suffussus suffussus* was provided by Dr. F. Couroud (Marseille, France). Brevetoxin (GB-3) was provided by Dr. K. Nakanishi (Columbia University, New York, NY). α-ScTx (from *L. quinquestriatus*) was provided by Dr. D. Rossignol (Eisai, Andover, MA).

**Synaptoneurosome preparation.** Guinea pig cerebral cortical synaptoneurosomes were obtained essentially as described by Hollingsworth *et al.* (7). Briefly, the cortex of one brain was homogenized in 7–10 volumes of sodium-free buffer (pH 7.4) in a glass-glass homogenizer (five strokes). Sodium-free buffer composition was as follows (in mM): choline chloride, 130; HEPES (adjusted to pH 7.4 with 50 mM Tris), 50; glucose, 5.5; $MgSO_4$, 0.8; KCl, 5.4. The suspension was centrifuged at 1000 × *g* for 10 min, the supernatant was decanted, and the pellet was reconstituted in 35 ml of buffer. The suspension was filtered first through two layers of nylon material (100 mesh) and then through 10-μm-pore Millipore filters (LCWP-047). After centrifugation for 10 min at 1000 × *g*, the new pellet was reconstituted in an appropriate volume of fresh sodium-free buffer, as indicated in each case.

**Sodium influx into synaptoneurosomes.** Sodium flux studies were carried out essentially by the method of Tamkun and Catterall (8), as modified for synaptoneurosomes (9). Aliquots of synaptoneurosome suspensions containing approximately 200–300 μg of protein were preincubated for 10 min at 37° in a volume of 100 μl of an incubation buffer containing various test agents. The incubation buffer consisted of fresh sodium-free buffer (see above for composition) to which 1 mg/ml bovine serum albumin had been added. The $^{22}NaCl$ (1.3 μCi/ml) was added in a volume of 150 μl of influx buffer containing 2.66 mM NaCl, 50 mM HEPES-Tris (pH 7.4), 128 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1 mg/ml bovine serum albumin, and 5 mM ouabain. The final volume was 250 μl. The influx buffer contained test agents in the same concentration as was present in the preincubated samples. Influx of $^{22}Na^+$ was stopped after 10 sec by addition of 3 ml of cold washing buffer. Samples were immediately collected on Gelman filters (GN-6, 0.45-μm pore size) and further washed with 2 × 3 ml of buffer. Washing buffer contained 5 mM HEPES-Tris, 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 1 mg/ml bovine serum albumin (pH 7.4). Filters were dissolved in Filtron-X (National Diagnostics) for liquid scintillation counting (efficiency, approximately 99.1%). The specific uptake of $^{22}Na^+$ was determined by subtracting nonspecific uptake obtained in the presence of 5 μM tetrodotoxin from the total uptake.

**[$^3$H]BTX-B binding assay with synaptoneurosomes.** Incubations contained 10 nM [$^3$H]BTX-B, 1 μM tetrodotoxin, 60 μg/ml ScV, about 400 μg of synaptoneurosome protein, and various agents, in a final volume of 250 μl of sodium-free buffer (see composition above). After 30 min at 37° the incubations were terminated by dilution of the reaction mixture with 3 ml of cold washing buffer (see composition above) and filtration through Whatman GF/C filters. Filters were washed three times with 3 ml of cold washing buffer. Filters were placed into scintillation vials. Hydrofluor was added (4 ml) and radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 300 μM veratridine.

## Results

PTX-B induced a sodium influx in synaptoneurosomes that was augmented in the presence of brevetoxin, α-ScTx, and β-ScTx (Fig. 1). The $EC_{50}$ value for PTX-B-induced sodium influx in the presence of ScV, which contains α-ScTx, was 29.5 μM, similar to previous results (3). Brevetoxin, α-ScTx, and β-ScTx induced a dose-dependent increase of sodium uptake in synaptoneurosomes in the presence of PTX-B (100 μM) (Fig. 2). The analogs PTX-A and *erythro*-PTX-B gave similar, albeit smaller, responses (Fig. 3). The alkaloid aconitine, which binds to the BTX binding site (10), also generated a synergistic response in sodium flux in combination with brevetoxin and α-ScTx (Fig. 4). Combinations of aconitine and β-ScTx in one set of experiments had greater than additive effects on sodium influx, as follows: aconitine (100 μM), 0.36 ± 0.3 nmol/assay; aconitine plus β-ScTx (100 nM), 0.67 ± 0.2 nmol/assay. A limited supply of β-ScTx precluded further studies on interactions with aconitine, BTX, and veratridine. In synaptosomes, β-ScTx does not potentiate veratridine-induced sodium influx (11). BTX- and veratridine-induced $^{22}Na^+$ fluxes were potentiated with α-ScTx and brevetoxin (Fig. 4).

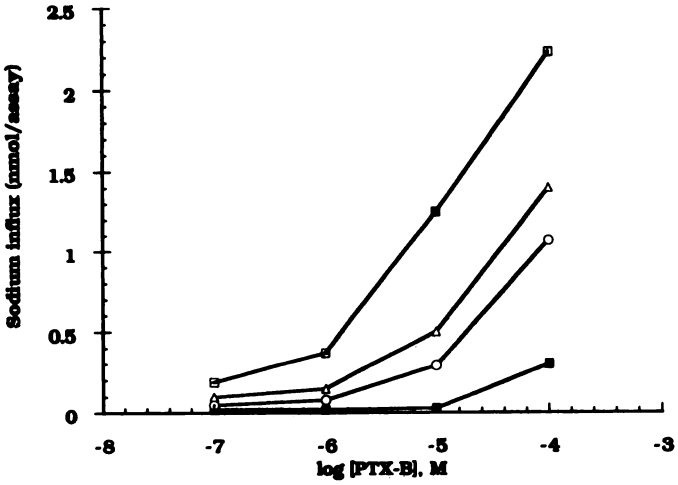


**Fig. 1.** Dose-dependent stimulation of $^{22}Na^+$ influx in guinea pig synaptoneurosomes by PTX-B. Synaptoneurosomes were incubated in the presence of the indicated concentrations of PTX-B alone (■) or in combination with 100 nM α-ScTx (□), 1 μM brevetoxin (○), or 100 nM β-ScTx (△). Influx of $^{22}Na^+$ was determined as described in Experimental Procedures. Values correspond to the means of three independent experiments performed in triplicate. Standard error values were smaller than the size of the symbols. In the absence of PTX-B, sodium influx with α-ScTx, brevetoxin, and β-ScTx was 0.36 ± 0.03, 0.09 ± 0.04, and 0.11 ± 0.01 nmol of sodium/assay, respectively.

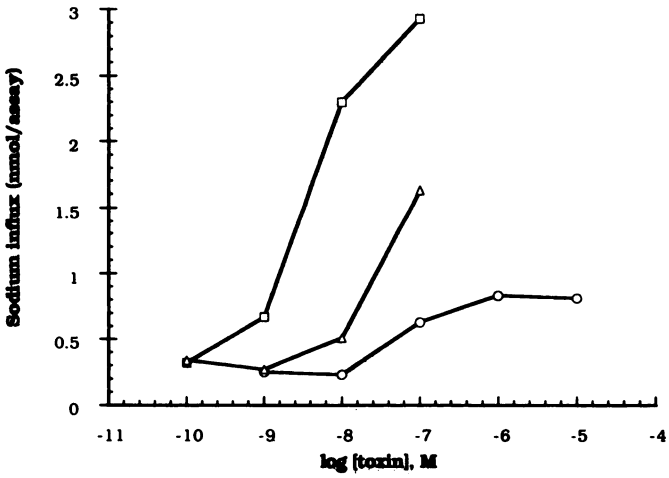


**Fig. 2.** Dose-dependent stimulation of $^{22}Na^+$ influx in guinea pig synaptoneurosomes by α-ScTx (□), β-ScTx (△), and brevetoxin (○). Synaptoneurosomes were incubated in the presence of the indicated concentrations of the toxins in combination with 100 μM PTX-B. Influx of $^{22}Na^+$ was determined as described in Experimental Procedures. Values correspond to a representative experiment performed in triplicate. The experiment was repeated three times with similar results.

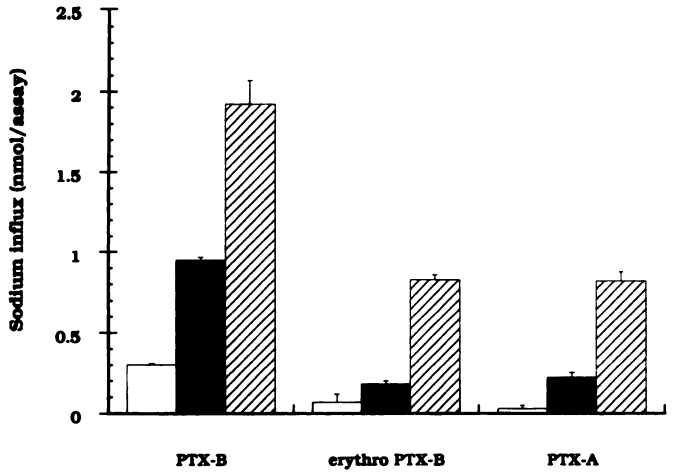


**Fig. 3.** Potentiation of PTX-B analog-induced $^{22}Na^+$ influx by α-ScTx and brevetoxin. Synaptoneurosomes were incubated in the presence of PTX-B, *erythro*-PTX-B, or PTX-A at 100 μM either alone (□) or in combination with 1 μM brevetoxin (■) or 100 nM α-ScTx (▨). Influx of $^{22}Na^+$ was determined as described in Experimental Procedures. Values correspond to the means ± standard errors of three independent experiments performed in triplicate.

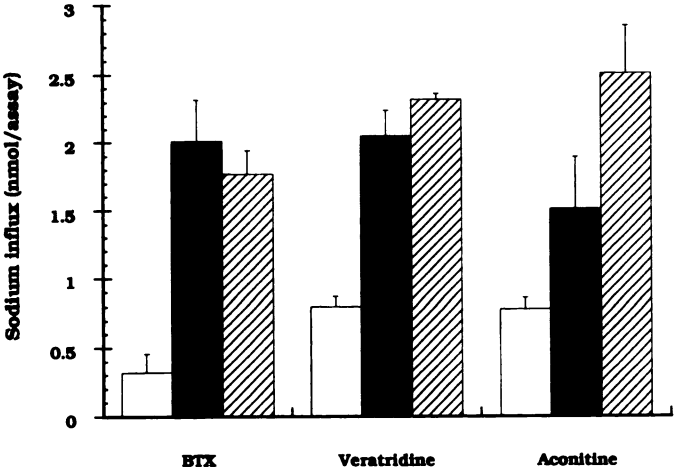


**Fig. 4.** Potentiation of BTX-, veratridine-, and aconitine-induced $^{22}Na^+$ influx in synaptoneurosomes by α-ScTx and brevetoxin. Synaptoneurosomes were incubated in the presence of 100 nM BTX, 10 μM veratridine, or 100 μM aconitine alone (□) or in the presence of 1 μM brevetoxin (■) or 100 nM α-ScTx (▨). Influx of $^{22}Na^+$ was determined as described in Experimental Procedures. Values correspond to the means ± standard errors of three independent experiments performed in triplicate.

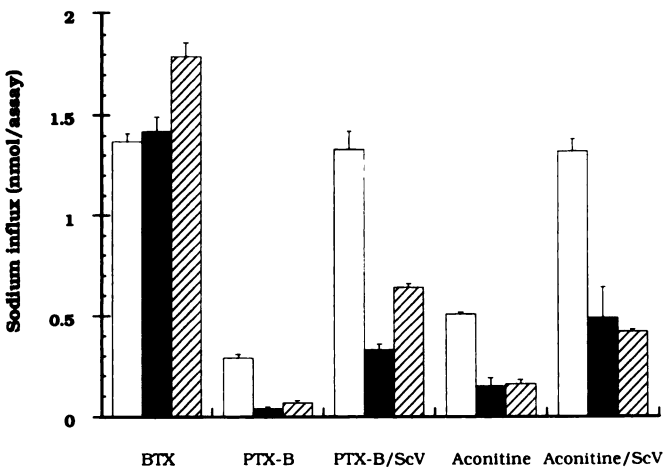


**Fig. 5.** Effect of pyrethroids on BTX-, PTX-B-, and aconitine-induced $^{22}Na^+$ influx in synaptoneurosomes. Synaptoneurosomes were incubated in the presence of BTX (1 μM), PTX-B (100 μM), PTX-B with ScV (1 μg/ml), aconitine (100 μM), or aconitine with ScV either alone (□) or in combination with 100 μM allethrin (■) or 100 μM fenvalerate (▨). Influx of $^{22}Na^+$ was determined as described in Experimental Procedures. Values correspond to the means ± standard errors of three independent experiments performed in triplicate.

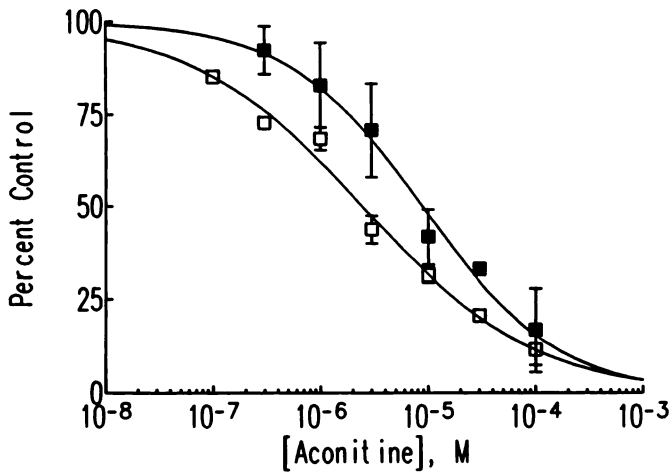


**Fig. 6.** Effect of PTX-B on the inhibition of [$^3$H]BTX-B binding by aconitine. Synaptoneurosomes were incubated with 10 nM [$^3$H]BTX-B and varying concentrations of aconitine alone (□) or in combination with 100 μM PTX-B (■). Values are means ± standard errors of three independent experiments. Standard error values smaller than the size of the symbol are not shown. *Curves* were fit using the program GraphPad.

Both a type I pyrethroid, allethrin, and a type II pyrethroid, fenvalerate, at 100 μM inhibited sodium flux elicited by PTX-B, the PTX-B/α-Sctx combination, aconitine, and the aconitine/α-Sctx combination but not that elicited by 1 μM BTX (Fig. 5). Pyrethroids also failed to inhibit sodium flux induced by a submaximal concentration of BTX (100 nM) (data not shown). Similarly, preincubation with 100 μM allethrin or 100 μM fenvalerate (37°, 10 min) did not result in an inhibition of the sodium flux elicited by BTX (data not shown). Fenvalerate slightly potentiated BTX-induced sodium flux (Fig. 5).

PTX-B at 100 μM did not inhibit [$^3$H]BTX-B binding in guinea pig synaptoneurosomes (data not shown; also see Ref. 3). However, in the presence of 100 μM PTX-B, the dose-dependent inhibition of [$^3$H]BTX-B binding by aconitine was significantly reduced (Fig. 6). The $IC_{50}$ for aconitine-induced displacement of [$^3$H]BTX-B binding was 1.8 μM in the absence and 24 μM in the presence of 100 μM PTX-B. Higher concentrations of PTX-B did not seem to induce any further change in the $IC_{50}$ for aconitine-induced displacement of [$^3$H]BTX-B binding. However, at those concentrations (300–1000 μM) PTX-B has an inhibitory action of its own on [$^3$H]BTX-B binding, most likely unrelated to its activating properties at the sodium channel (data not shown). Allethrin or fenvalerate (100 μM) had no significant effect on the potency of aconitine as an inhibitor of [$^3$H]BTX-B binding (Fig. 7).

BTX (1 μM)-induced sodium flux (1.77 ± 0.09 nmol/assay) was significantly inhibited in the presence of 200 μM aconitine (0.65 ± 0.12 nmol/assay). Such inhibition was partially reversed by 300 μM PTX-B (0.91 ± 0.13 nmol/assay).

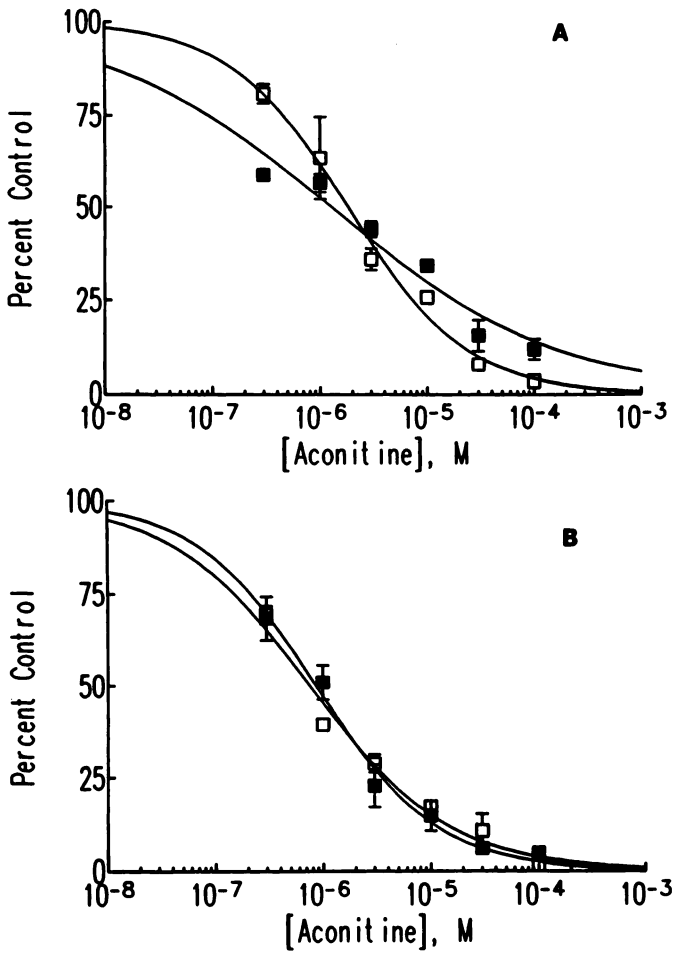


**Fig. 7.** Effect of pyrethroids on the inhibition of [$^3$H]BTX-B binding by aconitine. Synaptoneurosomes were incubated with 10 nM [$^3$H]BTX-B and varying concentrations of aconitine alone (□) or in combination with 100 μM allethrin (■) (A) or 100 μM fenvalerate (■) (B). Values are means ± standard errors for three independent experiments. Standard error values smaller than the size of the symbol are not shown. *Curves* were fit using the program GraphPad.

## Discussion

The alkaloid PTX-B interacts with voltage-dependent sodium channels, causing sodium influx in guinea pig synaptoneurosomes and neuroblastoma cells (3, 12). It has been proposed that PTX-B delays inactivation of sodium channels and thereby leads to repetitive firing in neuromuscular preparations (13). However, in hippocampal neurons PTX-B appeared to increase both the rate of opening and the rate of closing of sodium channels (14), leading to spontaneous repetitive firing.

PTX-B does not inhibit the binding of the alkaloid [$^3$H] BTX-B to site 2 on the channel (3) and, therefore, it appeared that PTX-B interacts with a different site on the sodium channel. Allosteric interactions among the toxin binding sites on the sodium channel have been described. The peptide toxin α-ScTx binds to site 3 and enhances the binding and action of alkaloids, such as BTX, veratridine, and aconitine, that bind to site 2 (2). In contrast, β-ScTx, which binds to site 4, has no effect on the action of veratridine in synaptosomes (11). The polyether toxins brevetoxin and ciguatoxin bind to site 5 and enhance the binding and action of site 2 toxins and of peptide toxins that act at site 4 but not of peptide toxins that act at site 3 (15). PTX-B, which appeared to bind to a novel site, interacts allosterically not only with peptide toxins that bind at site 3 (α-ScTx) but also with peptide toxins that bind at site 4 (β-ScTx) and with brevetoxin, which binds to site 5 (3). Thus, PTX-B, which like BTX interacts with toxins that bind to site 3 and site 5, shows additional allosteric interactions with toxins that interact with site 4.

The alkaloid aconitine also induces sodium influx in synaptoneurosomes (Fig. 4). Similar to PTX-B, aconitine-mediated sodium influx is enhanced in the presence of brevetoxin and α-ScTx. In contrast to PTX-B, aconitine does inhibit [$^3$H]BTX-B binding (Fig. 6), indicating that aconitine binds to site 2 on the sodium channel. However, aconitine, unlike veratridine, does show allosteric interactions with β-ScTx (see Results). Thus, aconitine appears closely related to PTX-B in some respects but differs from PTX-B in inhibiting binding of [$^3$H] BTX-B to site 2 on the sodium channel. Aconitine-mediated inhibition of [$^3$H]BTX-B binding is shifted significantly rightward by the presence of PTX-B (Fig. 6), indicating that, in spite of the fact that PTX-B does not affect site 2 directly, it can render an alkaloid (aconitine) that does interact with site 2 less effective. It seems, therefore, that the site of interaction for aconitine on the sodium channel consists of two apparently nonoverlapping regions, one region for PTX-B and the other for BTX/veratridine. Higher concentrations of PTX-B did not induce a further change in aconitine-mediated inhibition of [$^3$H]BTX-B binding, but at concentrations of PTX-B of 300 μM and higher there was some direct inhibition of [$^3$H]BTX-B binding (see Results). Nevertheless, the inability of higher concentrations of PTX-B to cause further rightward shifts in the aconitine inhibition curves argues against a pure competitive interaction. Instead, it is possible that PTX-B exerts a negative allosteric action on the aconitine binding site, which is fully manifest at 100 μM PTX-B, the same concentration of PTX-B that has near-maximal effects on sodium flux. If that is the case, the aconitine and PTX-B sites may not necessarily be located in overlapping subdomains.

Pyrethroid insecticides have neurotoxic properties that seem to reflect effects on sodium channels (16). Effects of pyrethroids are reminiscent of PTX-B in that type II pyrethroids also cause a delay in sodium channel closing (17), which can result in repetitive firing, increases in sodium flux, and even depolarization (17). Type I pyrethroids cause only a slight delay in sodium channel closing (18). Type II pyrethroids enhance [$^3$H] BTX-B binding in synaptoneurosomes, and the enhancement can be blocked by type I pyrethroids (19). Thus, under certain conditions, type I pyrethroids have inhibitory actions on sodium channels. Both allethrin (type I) and fenvalerate (type II) stimulate phosphoinositide breakdown in synaptoneurosomes (20). The response to fenvalerate was antagonized by tetrodotoxin, indicative of an involvement of "activated" sodium channels, whereas the response to allethrin was not (20). Neither allethrin nor fenvalerate alone has significant effects on sodium influx in synaptoneurosomes (data not shown). However, both allethrin and fenvalerate inhibit both PTX-B- and aconitine-mediated sodium influx (Fig. 5). Allethrin and fenvalerate did not inhibit BTX-mediated sodium influx (Fig. 5), providing another distinction between PTX-B- and aconitine-elicited responses and those elicited by BTX. Indeed, fenvalerate slightly enhanced the response to BTX (Fig. 5).

Pyrethroids did not affect aconitine-mediated inhibition of [$^3$H] BTX-B binding (Fig. 7).

In summary, it appears that the alkaloids BTX, veratridine, aconitine, and PTX-B may activate sodium channels by binding to a topographically common region on the protein, to be referred to as the alkaloid-binding domain. If so, then the alkaloid-binding domain must contain subdomains for the binding of and channel activation by the different alkaloids. On one hand, the subdomain for BTX binding can elicit full channel activation, shows allosteric interactions with $\alpha$-ScTx, as evidenced by an increase in potency of BTX in the presence of $\alpha$-ScTx, is not affected by a type I pyrethroid, and is potentiated by a type II pyrethroid. The subdomain for aconitine binding can elicit partial activation and shows allosteric interactions with $\alpha$-ScTx, $\beta$-ScTx, and brevetoxin. The subdomains for BTX and aconitine binding overlap to some extent, because aconitine blocks the binding of [$^3$H]BTX-B. On the other hand, the PTX-B-binding subdomain can elicit partial channel activation and shows allosteric interactions with $\alpha$-ScTx, as evidenced by an increase in both potency and efficacy of PTX-B, and with $\beta$-ScTx and brevetoxin. The subdomain for PTX-B may overlap to some extent with the aconitine subdomain, because PTX-B reduces the inhibition of [$^3$H] BTX-B binding caused by aconitine. However, because very high concentrations of PTX-B cannot completely prevent aconitine effects, the interaction between these two toxin sites may be allosteric rather than competitive. Both type I and type II pyrethroids appear to inhibit the action of alkaloids acting at the PTX-B subdomain or the aconitine subdomain but not those acting at the BTX subdomain. Although pyrethroids inhibited sodium influx elicited by aconitine (Fig. 5), they had no effect on the ability of aconitine to inhibit [$^3$H]BTX-B binding (Fig. 7). Thus, the relationship of pyrethroid binding sites on the sodium channel to the alkaloid-binding domain requires further study. It is possible that the inhibition of PTX-B- and aconitine-elicited responses by pyrethroids is allosteric in nature. Interacting subdomains for modulatory sites have been proposed previously for the GABA receptor/channel complex (21). The present model for subdomains of an alkaloid-binding modulatory site on voltage-dependent sodium channels provides the basis for further electrophysiological studies on interactions of sodium channel toxins.

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