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Allosteric Modulation of Neurotoxin Binding to Voltage-Sensitive Sodium Channels by *Ptychodiscus brevis* Toxin 2

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SUMMARY

The effects of *Ptychodiscus brevis* toxin 2 (PbTx-2) on the binding of neurotoxins at four different neurotoxin receptor sites on voltage-sensitive sodium channels in rat brain synaptosomes were examined. Binding of saxitoxin at neurotoxin receptor site 1 and *Leiurus quinquestriatus* α-scorpion toxin (LqTx) at neurotoxin receptor site 3 was unaffected. PbTx-2 enhanced binding of batrachotoxinin A 20-α-benzoate (BTX-B) to neurotoxin receptor site 2 and *Centruroides suffusus suffusus* β-scorpion toxin (CsTx II) to site 4 on sodium channels. These results support the proposal that PbTx-2 and related toxins act at a new receptor site (site 5) that has not been previously analyzed in binding experiments. Half-maximal effects of PbTx-2 were observed in the range of 20–50 nM PbTx-2. The enhancement of BTX-B binding was reduced by depolarization. Saturating concentrations of PbTx-2 reduced K_D values for binding of BTX-B and CsTx-II 2.9-fold and 2.6-fold, respectively. The effects of PbTx-2 and LqTx in enhancing BTX-B binding were synergistic. A model involving both preferential binding of BTX-B, PbTx-2, LqTx, and CsTx II to active states of sodium channels and allosteric interactions among the four receptor sites at which these toxins act accommodates these and previous results.

The Florida red tide dinoflagellate *Ptychodiscus brevis* produces multiple nonprotein toxins that have been purified to homogeneity including PbTx-1 (also designated T_{46}, GB-1, or brevetoxin A. Refs. 1–3), PbTx-2 (also designated T_{47}, GB-2, T34, or brevetoxin B; Refs. 1, 4, and 5), and PbTx-3 (also designated GB-3 or T17; Refs. 2 and 6). PbTx-1 (3) and PbTx-2 (5) have been determined by X-ray crystallography to be distinct, ladderlike structures of 10 or 11 fused ether rings. PbTx-3 can be produced by reduction of the aldehyde moiety of PbTx-2 to a primary alcohol.

Pure preparations of PbTx-1 enhance persistent activation of sodium channels in mouse neuroblastoma cells at concentrations consistent with its toxic actions on fish and mice (7, 8). PbTx-2 and -3 each depolarized squid and crayfish axons under voltage clamp by causing prolonged activation of sodium channels (9). The voltage dependence of channel activation is shifted 35 mV to more negative membrane potentials and inactivation is blocked (9). These results establish the sodium channel as an important receptor site for the toxic actions of the pure brevetoxins.

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The sodium channel has multiple receptor sites for neurotoxins (reviewed in Refs. 10–12). Tetrodotoxin and saxitoxin bind at neurotoxin receptor site 1 and inhibit ion conductance. Grayanotoxin and the alkaloids veratridine, batrachotoxin, and aconitine bind to neurotoxin receptor site 2 and cause persistent activation of sodium channels. Polypeptide α-scorpion toxins and sea anemone toxins bind at neurotoxin receptor site 3, slow sodium channel inactivation, and enhance persistent activation of sodium channels by toxins acting at site 2 via an allosteric mechanism. β-Scorpion toxins bind at neurotoxin receptor site 4 and alter sodium channel activation. In this report, we analyze the effects of PbTx-2 on toxin binding at each of these four neurotoxin receptor sites and establish that PbTx-2 enhances toxin binding at sites 2 and 4 via all via an allosteric mechanism.

Experimental Procedures

Materials. Chemicals were obtained from the following sources: veratridine from Aldrich; tetrodotoxin from Calbiochem; and scorpion venom (*Leiurus quinquestriatus*) from Sigma. BTX-B and [^{3}H]BTX-B were prepared and purified as described by Brown *et al.* (13) and were a gift from Dr. George Brown, Neuroscience Program, University of Alabama. Batrachotoxin was a gift from Dr. John Daly, Laboratory

ABBREVIATIONS: PbTx-1, -2, or -3, *Ptychodiscus brevis* toxin 1, 2, or 3; BTX-B, batrachotoxinin A 20-α-benzoate; LqTx, *Leiurus quinquestriatus* α-scorpion toxin; [^{125}I]$_1$LqTx, scorpion mono[^{125}I]iodo toxin; CsTx II, *Centruroides suffusus suffusus* β-scorpion toxin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. Saxitoxin was obtained from the Toxicology Study Section, National Institutes of Health, and was radiolabeled with ^3H by the specific ^3H_2O exchange procedure of Ritchie *et al.* (14) and purified and characterized as described previously (15). The α-scorpion toxin from *L. quinquestriatus* (LqTx) was purified, labeled with ^{125}I by lactoperoxidase-catalyzed iodination, and repurified by ion exchange chromatography as described previously (16). $[^{125}I]_1$LqTx was used in all experiments. The β-scorpion toxin from *Centruroides suffusus suffusus* (CsTx II) was purified and radiolabeled by lactoperoxidase-catalyzed iodination as previously described (17). PbTx-2 was purified from laboratory cultures of *P. brevis* as described by Baden *et al.* (4). Solutions of BTX-B and PbTx-2 were prepared in methanol or acetone, respectively. Stock solutions were diluted so that less than 0.5% of organic solvent was present in the assay solutions. Control experiments established that this solvent level had no effect on neurotoxin binding. Synaptosomes were prepared from rat brain by previously described methods (18).

Neurotoxin binding assays. Specific binding of [^{3}H]saxitoxin to neurotoxin receptor site 1 on sodium channels in synaptosomes was measured as described previously (19). Synaptosomes (75 μg) in 25 μl of standard binding medium consisting of 130 mM choline chloride, 50 mM Hepes (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 bovine serum albumin were added to a reaction mixture containing [^{3}H]saxitoxin and other toxins as noted in the figure legends in 175 μl of standard binding medium. Samples were mixed and incubated 30 min at 37°. The reactions were stopped by addition of 3.0 ml of wash medium at 0° consisting of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$, and the membranes were collected by filtration on Whatman GF/C filters and washed twice within 10 sec. The filters were then placed in counting vials and radioactivity was determined. Nonspecific binding was measured in the presence of a saturating concentration of tetrodotoxin (1 μM) and was subtracted from all results (19).

Specific binding of [^{3}H]BTX-B to neurotoxin receptor site 2 on sodium channels was measured as described previously (20). Synaptosomes (200 μg of protein) in 100 μl of standard binding medium containing 1 μM tetrodotoxin were mixed with 7–10 nM [^{3}H]BTX-B and other neurotoxins as noted in the figure legends in 150 μl of standard binding medium at 37°. After incubation for 30 min, reactions were stopped by addition of 3 ml of wash medium containing 1 mg/ml bovine serum albumin and synaptosomes were collected by filtration on GF/C filters and washed rapidly three times with wash medium at 0°. The filters were placed in counting vials and bound radioactivity was determined. Nonspecific binding was determined in the presence of 300 μM veratridine and was subtracted from all results.

Specific binding of $[^{125}I]_1$LqTx to neurotoxin receptor site 3 on sodium channels was measured as described previously (21). The assay is exactly as described for [^{3}H]BTX-B except that washes were performed at 37°, and nonspecific binding was determined in the presence of 200 nM LqTx.

Specific binding of [^{125}I]CsTx II to neurotoxin receptor site 4 was measured by a modification of previously described procedures (17). The assay was identical to that described for $[^{125}I]_1$LqTx except that nonspecific binding was determined in the presence of 500 nM CsTx II.

Other methods. Protein concentrations were determined by the method of Peterson (22). Except where specifically mentioned, the data presented are the results of a single experiment which are representative of two or more similar experiments. All data points are the mean of two to five replicate determinations. Smooth curves describing data points were drawn by eye. Straight lines on Scatchard plots were computed by linear regression.

Results

Neurotoxin receptor site 1. The interaction of the sodium channel blockers tetrodotoxin, saxitoxin, and geographutoxin II, a toxin from cone snails, with neurotoxin receptor site 1 on sodium channels in rat brain synaptosomes can be monitored by measurement of specific binding of [^{3}H]saxitoxin (18). Fig. 1 illustrates the effect of PbTx-2 on specific [^{3}H]saxitoxin binding. No effect is observed at concentrations between 1 nM and 1 μM. Since PbTx-2 modifies sodium channel properties in squid giant axon in this concentration range (9), the results indicate that it has no effect on ligand interaction with neurotoxin receptor site 1.

Neurotoxin receptor site 2. The interaction of the sodium channel activators batrachotoxin, veratridine, aconitine, and grayanotoxin with neurotoxin receptor site 2 on sodium channels in rat brain synaptosomes can be monitored by measurement of specific binding of [^{3}H]BTX-B (20). In the absence of other neurotoxins, specific binding of [^{3}H]BTX-B is relatively low (13, 20). However, it can be markedly enhanced by LqTx through allosteric interactions at neurotoxin receptor site 3 (20). Fig. 2 shows that PbTx-2 increases specific binding of [^{3}H]BTX-B 2.9-fold with half-maximal effect at 20 nM. Evidently, PbTx-2, like LqTx, can enhance [^{3}H]BTX-B binding through allosteric interactions.

The effects of PbTx-2 and LqTx on [^{3}H]BTX-B binding are synergistic. Fig. 3 illustrates the time course of [^{3}H]BTX-B binding in the presence of saturating concentrations of PbTx-2 (1 μM) alone or PbTx-2 and LqTx (1 μM). The time course of binding is similar, approaching equilibrium in 60 min at 36°. However, the equilibrium binding of [^{3}H]BTX-B is increased 3.1-fold over that observed with PbTx alone. Table 1 summarizes the specific binding of 10 nM [^{3}H]BTX-B observed in the absence of other neurotoxins or in the presence of saturating concentrations of PbTx-2, LqTx, or a combination of the two toxins. The two toxins, when present together, cause an enhancement of [^{3}H]BTX-B binding that is more than the sum of the effects of PbTx-2 and LqTx when present separately. This synergistic interaction suggests that PbTx-2 and LqTx can simultaneously bind at different receptor sites associated

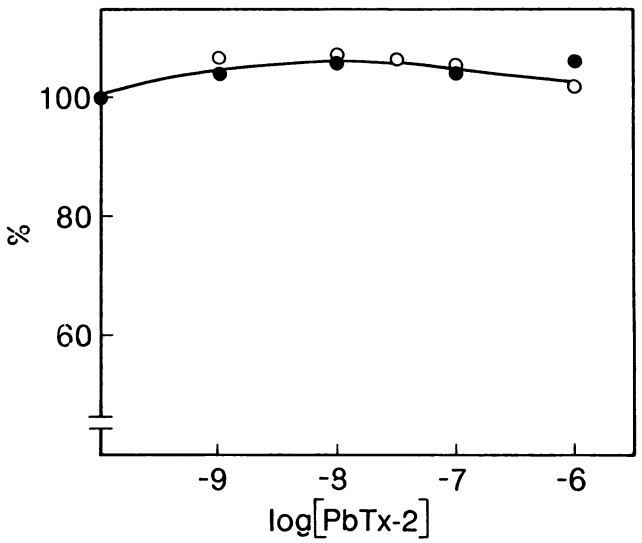

Fig. 1. Effect of PbTx-2 on specific binding of [^{3}H]saxitoxin and $[^{125}I]_1$LqTx to synaptosomes. Specific binding of 3 nM [^{3}H]saxitoxin (●) or 0.1 nM $[^{125}I]_1$LqTx (○) to synaptosomes was measured as described under Experimental Procedures in the presence of the indicated concentrations of PbTx-2.

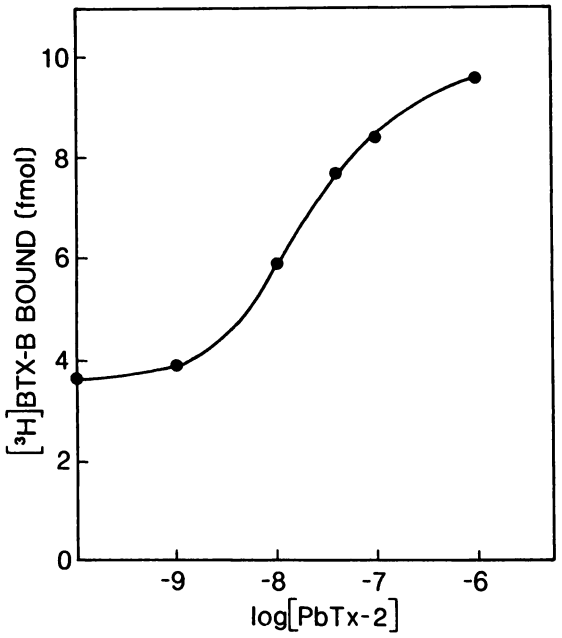

Fig. 2. Enhancement of specific binding of [³H]BTX-B to synaptosomes by PbTx-2. Specific binding of 10 nM [³H]BTX-B to synaptosomes was measured as described under Experimental Procedures in the presence of the indicated concentrations of PbTx-2. The increase in [³H]BTX-B binding is closely fitted by a hyperbolic titration curve with $K_{0.5}$ = 20 nM and B_{max} = 6.1 fmol. At 1 μM PbTx-2, 98% occupancy is predicted.

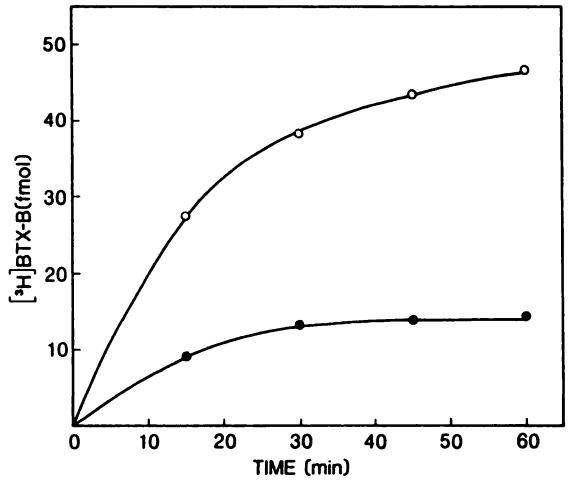

Fig. 3. Time course of specific binding of [³H]BTX in the presence of PbTx-2 or PbTx-2 and LqTx. Specific binding of 10 nM [³H]BTX-B to synaptosomes was measured after incubation for 60 min in the presence of 1 μM PbTx-2 (●) or 1 μM PbTx-2 plus 1 μM LqTx (○) after incubation for the indicated time at 37° as described under Experimental Procedures.

with sodium channels and can each enhance [³H]BTX-B binding.

The effects of combinations of LqTx and PbTx-2 on K_D and B_{max} values for specific binding of [³H]BTX-B were studied by Scatchard analysis of the concentration dependence of toxin binding (Fig. 4). No significant changes in B_{max} were observed. The K_D in the presence of LqTx alone is 116 nM, in reasonable agreement with earlier work (20). The K_D in the presence of PbTx-2 (270 nM) is higher than in the presence of LqTx, as expected from the lower levels of binding observed at a fixed concentration (10 nM) of [³H]BTX-B (Table 1). In the presence

TABLE 1

Synergistic effects of PbTx-2 and LqTx on BTX-B binding to sodium channels

Specific binding of [³H]BTX-B (10 nM) to sodium channels in synaptosomes was measured as described under Experimental Procedures in the presence of the indicated concentrations of PbTx-2 and LqTx.

Condition	Specifically bound [³H]BTX-B
	fmol
Control	3.0
1.0 μM PbTx-2	9.6
1.0 μM LqTx	19.7
1.0 μM PbTx-2 + 1.0 μM LqTx	35.3

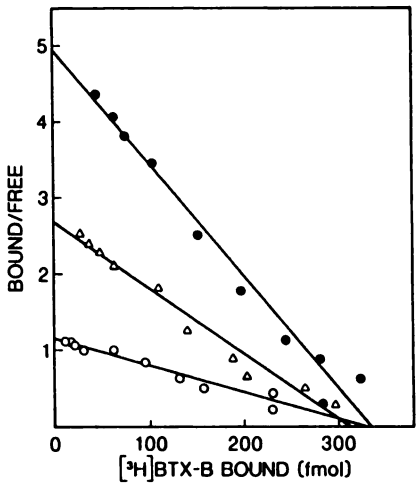

Fig. 4. Scatchard analysis of specific binding of [³H]BTX-B in the presence of LqTx and PbTx. Specific binding of 10 nM to 1.0 μM [³H]BTX-B to synaptosomes was measured after incubation for 60 min in the presence of 1 μM PbTx-2 (○), 1 μM LqTx, (△), or 1 μM PbTx-2 plus 1 μM LqTx (●) as described under Experimental Procedures. *Ordinate*, fmol/nM.

of both agents, the K_D for BTX-B is reduced to 68 nM. These changes in K_D are consistent with the synergistic effects of PbTx-2 and LqTx on BTX-B binding at 10 nM [³H]BTX-B, although a quantitative comparison is not possible because the low level of specific binding observed in the absence of LqTx and PbTx-2 prevents an accurate measurement of K_D.

Neurotoxin receptor site 3. The interaction of polypeptide neurotoxins from scorpion and sea anemone venoms with receptor site 3 on sodium channels can be monitored by measurement of specific binding of [¹²⁵I]$_1$LqTx (18). In order to test directly whether PbTx-2 affects toxin binding and action at site 3, its effects on specific binding of [¹²⁵I]$_1$LqTx were studied. The results of Fig. 1 show that PbTx has no effect on neurotoxin binding at site 3. Evidently, its effects on binding of [³H]BTX-B arise from interaction at a receptor site different from that for LqTx.

Neurotoxin receptor site 4. The interaction of β-scorpion toxins with neurotoxin receptor site 4 on sodium channels can be studied by measurement of specific binding of [¹²⁵I]CsTx II (17). Fig. 5 illustrates the effect of PbTx-2 on CsTx II binding. Specific binding is increased 2.7-fold with a half-maximal effect at approximately 30 nM PbTx-2. This effect is similar in magnitude and concentration dependence to the enhancement of [³H]BTX-B binding by PbTx-2 (Fig. 2).

Analysis of the effects of PbTx-2 on B_{max} and K_D for [¹²⁵I]CsTx II is illustrated in the form of a Scatchard plot in Fig. 6. The K_D is decreased 2.5-fold from 5.2 nM to 2.3 nM

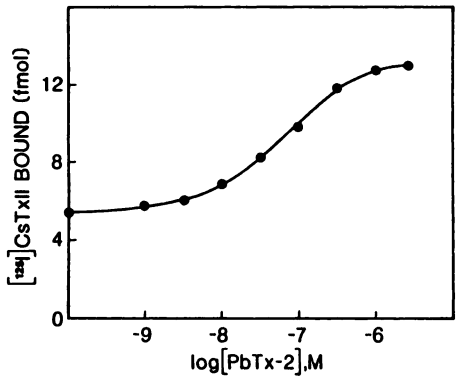

Fig. 5. Enhancement of specific binding of 0.25 nM [^{125}I]CsTx II to synaptosomes was measured in the presence of the indicated concentrations of PbTx-2 as described under Experimental Procedures.

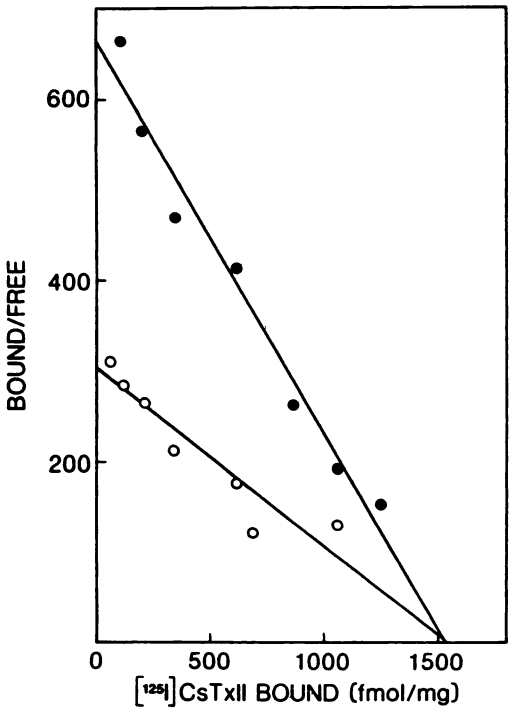

Fig. 6. Scatchard analysis of specific binding of [^{125}I]CsTx II to synaptosomes in the presence of PbTx-2. Specific binding of 0.25–8.0 nM [^{125}I] CsTx II to synaptosomes was measured in the absence (○) or presence (●) of 1 μM PbTx-2 as described under Experimental Procedures. *Ordinate*, fmol/nM.

without a significant change in B_{max}. Thus, the effect of PbTx to enhance CsTx II binding results from an effect on K_D with no change in B_{max}, like its effect on BTX-B binding.

Voltage dependence of PbTx action. The binding and action of neurotoxins at receptor site 3 on sodium channels are highly voltage dependent (11, 12), increasing progressively with membrane depolarization. In order to examine possible voltage dependence of PbTx action in enhancing specific binding of [^{3}H]BTX-B, synaptosomes were incubated with [^{3}H]BTX-B and a concentration of PbTx-2 (30 nM) near its apparent K_D, in the presence of varying concentrations of K^+ to alter the resting membrane potential. Fig. 7 compares the effect of increased external K^+ on the binding of [^{125}I]$_1$LqTx and on the enhancement of [^{3}H]BTX-B binding. Between the resting membrane potential of synaptosomes at 5 mM K^+ [approxi-

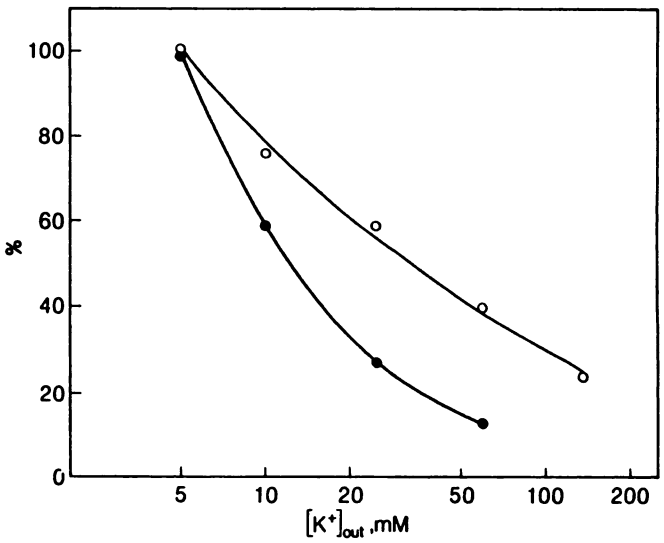

Fig. 7. Voltage dependence of enhancement of [^{3}H]BTX-B binding by PbTx-2. Specific binding of 10 nM [^{3}H]BTX-B to synaptosomes was measured in the presence of 30 nM PbTx-2 and the indicated concentrations of K^+ as described under Experimental Procedures (○). KCl was exchanged for an equal concentration of choline chloride in standard binding medium. Binding of [^{3}H]BTX-B binding by PbTx-2. Specific binding of 10 nM [^{3}H]BTX-B in the absence of PbTx-2 was subtracted from the data. For comparison, specific binding of 0.2 nM [^{125}I]$_1$LqTx to synaptosomes was measured under the same conditions as described under Experimental Procedures (●).

mately −55 mV (23)] and the membrane potential at 135 mM K^+ [approximately 0 mV (23)], binding of [^{125}I]$_1$LqTx is reduced 8-fold (Fig. 7, *solid circles*). The enhancement of [^{3}H]BTX-B binding by PbTx-2 is also reduced by depolarization of synaptosomes with elevated external K^+ (Fig. 7, *open circles*). The dependence of K^+ concentration is less steep than is observed for binding of [^{125}I]$_1$LqTx with a 4-fold change between 5 mM K^+ and 135 mM K^+. The binding of [^{3}H]BTX-B itself is not strongly affected across this voltage range. The results suggest a significant voltage dependence of PbTx-2 binding and action.

Discussion

Our results provide clear evidence that PbTx-2 exerts its effects on sodium channels by interaction with a new receptor site not previously described in direct radioligand binding studies. Table 2 summarizes the sites of neurotoxin binding on sodium channels that have been defined by radioligand binding (reviewed in Refs. 11 and 12). We show here that PbTx-2 has no effect on neurotoxin binding at sites 1 and 3 and enhances neurotoxin binding at sites 2 and 4. Thus, it must act at a new neurotoxin receptor site, site 5, associated with sodium channels. This new receptor site has recently been detected in direct binding studies using [^{3}H]PbTx-3 produced by reductive tritiation of PbTx-2 (24). As expected from the results described here, toxins acting at sites 1–4 do not block specific binding of [^{3}H]PbTx-3, providing further evidence of action at a new receptor site.

Previous studies of the actions of a related sodium channel-specific neurotoxin, PbTx-1, on sodium channels in mouse neuroblastoma cells and rat brain synaptosomes reached the conclusion that PbTx-1 must also act at a new sodium channel receptor site (7, 8). This toxin enhances activation of sodium channels by veratridine, aconitine, and batrachotoxin acting at

TABLE 2

Neurotoxin receptor sites associated with the sodium channel

Receptor site[a]	Ligands	Physiological effects
1	Tetrodotoxin Saxitoxin Geographutoxin II and other μ-conotoxins[b]	Inhibit ion conductance
2	Veratridine Batrachotoxin Aconitine Grayanotoxin	Persistent activation
3	α-Scorpion toxin Sea anemone toxin	Inhibit inactivation
4	β-Scorpion toxins	Shift activation
5	Brevetoxins	Shift activation Inhibit inactivation

[a] The sodium channel also has additional receptor sites for local anesthetics, certain anticonvulsants, pyrethroid insecticides, and coral toxins. However, since these receptor sites have not been directly identified in ligand binding studies, they are not listed here.
[b] Shown to act at neurotoxin receptor site 1 in Refs. 26–28.

neurotoxin receptor site 2 without inhibition of binding of saxitoxin or [^{125}I]$_1$LqTx at sites 1 and 3, respectively. Interactions at neurotoxin receptor site 4 have not been studied. PbTx-2 blocks specific binding of PbTx-3 to neurotoxin receptor site 5 on sodium channels, consistent with the conclusion that it shares this receptor site for toxin action (24). We expect that PbTx-1 also shares this receptor site although direct experimental evidence for this conclusion is not yet available.

The allosteric modulation of neurotoxin binding and action at sites 2 and 4 by PbTx-1 and PbTx-2 acting at site 5 indicate that there are conformational interactions among these three sites mediated through the sodium channel structure. Allosteric interactions between neurotoxin receptor sites 2 and 3 have been described previously in terms of a model in which neurotoxin action on sodium channels results from selective high affinity binding to individual functional states of sodium channels (25). Neurotoxins acting at receptor sites 2, 4, and 5 have some similar effects on sodium channel function: each of these classes of toxins shifts the voltage dependence of sodium channel activation to more negative membrane potentials, and toxins acting at sites 2 and 5 slow or block sodium channel inactivation (reviewed in Refs. 9, 11, and 12). These actions suggest that all three of these classes of neurotoxins may act, at least in part, by binding with high affinity to active states of sodium channels and stabilizing them according to the law of mass action, as has been previously shown for the site 2 toxins (25). The actions of LqTx and PbTx-2 to enhance BTX-B binding are easily understood in terms of this model. Successive binding of LqTx and PbTx-2 to their separate receptor sites increasingly favors active channel states having a high affinity for [^{3}H]BTX-B by reducing the equilibrium constant [designated M_{RT} in the original formulation of this model (25)] for the conformational change between the low and high affinity states. This stabilization is due to the interaction energy derived from preferential binding of LqTx and PbTx-2 to these same states. At saturating concentrations of PbTx-2 and LqTx, the sum of the interaction energies defines the new value of M_{RT} according to:

$$-RT\ln M'_{RT} = \Delta G_{RT} + \Delta G_{\mathrm{LqTx}} + \Delta G_{\mathrm{PbTx}}$$

$$= -RT\ln M_{RT} \frac{K_R^{\mathrm{LqTx}}}{K_T^{\mathrm{LqTx}}} \frac{K_R^{\mathrm{PbTx}}}{K_T^{\mathrm{PbTx}}}$$

$$M'_{RT} = M_{RT} \frac{K_R^{\mathrm{LqTx}}}{K_T^{\mathrm{LqTx}}} \frac{K_R^{\mathrm{PbTx}}}{K_T^{\mathrm{PbTx}}}$$

where ΔG_{RT} is the change in conformational free energy accompanying the transition from inactive (T) to active (R) states, ΔG_{LqTx} and ΔG_{PbTx} are the differences in free energy of binding to the R and T states, and K_R and K_T are the equilibrium dissociation constants for binding to those two states (see Eqs. 6 and 8 of Ref. 25). This relationship predicts a synergistic (greater than additive) effect of the presence of both PbTx-2 and LqTx on [^{3}H]BTX-B binding as observed in the data of Table 1 and Fig. 4. Thus, a simple model for allosteric interaction of LqTx binding at site 3 with batrachotoxin in binding at site 2 can be extended to the present results indicating allosteric interactions between PbTx binding at site 5 and [^{3}H]BTX-B binding at site 2. Similar mechanisms can also accommodate our results indicating allosteric interactions between PbTx-2 binding at site 5 and CsTx II binding at site 4. As the structure of the sodium channel is becoming known in some detail, we are optimistic that the location and structure of these toxin-binding sites and the pathways of interaction between them may be elucidated in the future.

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