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Brevetoxins, Unique Activators of Voltage-Sensitive Sodium Channels, Bind to Specific Sites in Rat Brain Synaptosomes

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SUMMARY

The polyether lipid-soluble toxins isolated from the marine dinoflagellate *Ptychodiscus brevis* (formerly *Gymnodinium breve*) have been determined to bind to a unique site associated with rat brain synaptosomes. Using [^{3}H]brevetoxin PbTx-3 as a specific probe, binding was determined at 4° in rat brain synaptosomes using a rapid centrifugation technique. Rosenthal analysis yields a K_D of 2.9 nM and a B_{max} of 6.8 pmol of toxin/mg of protein. Labeled probe can be displaced by unlabeled PbTx-3, PbTx-2, or synthetic PbTx-3 (reduced PbTx-2) but not by a nontoxic, synthetic oxidized derivative of PbTx-2. Competition experiments using natural toxin probes specific for sites 1–4 of the voltage-dependent sodium channel have illustrated that PbTx-3 does not bind to any of the previously described sites associated with the channel. A fifth site is proposed. In addition, because of the varied nomenclature associated with the brevetoxins, a new classification system is proposed.

The marine dinoflagellate *Ptychodiscus brevis* is the planktonic organism responsible for Florida's toxic red tides (1, 2). Since the discovery of *P. brevis* as the toxigenic organism, the toxins have been known by many names including Gymnodin, GBTX, T17 and T34, and GB series, and the brevetoxins. Several of the names have fallen into disfavor since the reclassification of *Gymnodinium breve* to *Ptychodiscus brevis* (2). In addition, many of the designations are redundant now that structural cross-correlation of potent fractions from several laboratories has been completed. We propose a new notation system that correlates the toxins isolated from all laboratories. In this notation system, the numbering system proposed by Shimizu (3) is used, and it is preceded by the letters PbTx, denoting *P*tychodiscus *b*revis *tox*in. A summary of the proposed system is given in Table 1.

From our laboratory cultures of the organism, we have purified and crystallized two toxins with nearly identical polyether structures (see Fig. 1) (4–7). PbTx-2 (Fig. 1a) is the predominant toxin and has been isolated by a number of groups (8, 9). The second toxin we isolated, PbTx-3 (Fig. 1a) (6, 10) is present at about one-third the concentration of PbTx-2 in log-phase cultures. To date, six other potent polyether materials have been isolated and purified from *P. brevis* (11).

The potency of toxins isolated from *P. brevis* is well documented. Several recent review articles and symposia detail the chemistry/pharmacology of the brevetoxins and also detail the effects caused by red tides which liberate these potent materials into the environment (3, 8, 13–17).

The neurotoxins isolated from *P. brevis* exert their effects primarily by altering the membrane properties of excitable cell types in ways that activate sodium channels at normal membrane resting potential (13–16). The neurotoxic effect of PbTx-3 on membrane permeability in crayfish giant axon has been reported previously (18). External or internal application of PbTx-3 caused a dose-dependent depolarization which was half-maximal at about 1 nM and was reversed by application of tetrodotoxin or by bathing the preparation in a low Na^+ solution. Because brevetoxins depolarize excitable membranes in a dose-dependent manner (17–19), and because it has been shown that depolarization is sodium specific (19, 20), a site associated with the voltage-dependent sodium channel has been postulated (19–22).

In preliminary studies conducted with PbTx-1, the first experimental evidence was provided that brevetoxins could enhance ^{22}Na influx rates caused by veratridine in neuroblastoma cells. The effective concentration range for brevetoxin enhancement was in the ng/ml to μg/ml range, maximally stimulating sodium ion influx some 9-fold over veratridine alone, at 1 μg/ml brevetoxin (21). The first binding experiments

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ABBREVIATIONS: BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE 1
Nomenclature for the brevetoxins

Abbreviation	Synonyms	Reference	Ring system[a]	Substituent	
				R_1	R_2
PbTX-1[b]	Brevetoxin-A	(9, 10)	b	CHO	
	GB-1	(11)			
PbTx-2	Brevetoxin-B	(9, 10)	a	H	$CH_2C(=CH_2)CHO$
	GB-2	(3)			
	T34	(5, 6)			
PbTx-3	GB-3	(3, 10)	a	H	$CH_2C(=CH_2)CH_2OH$
	T17	(6)			
PbTx-4	GB-4	(11)		no structural information	
PbTx-5	GB-5	(11)	a	Ac	$CH_2C(=CH_2)CHO$
PbTx-6	GB-6	(11)		27,28 epoxide of PbTx-2	
PbTx-7	GB-7	(11)	b	CH_2OH	
PbTx-8	Brevetoxin-C	(12)	a	H	CH_2COCH_2Cl

[a] Refer to Fig. 1 for ring configurations.
[b] Structural correlation of brevetoxin-A and GB-1 is uncertain.

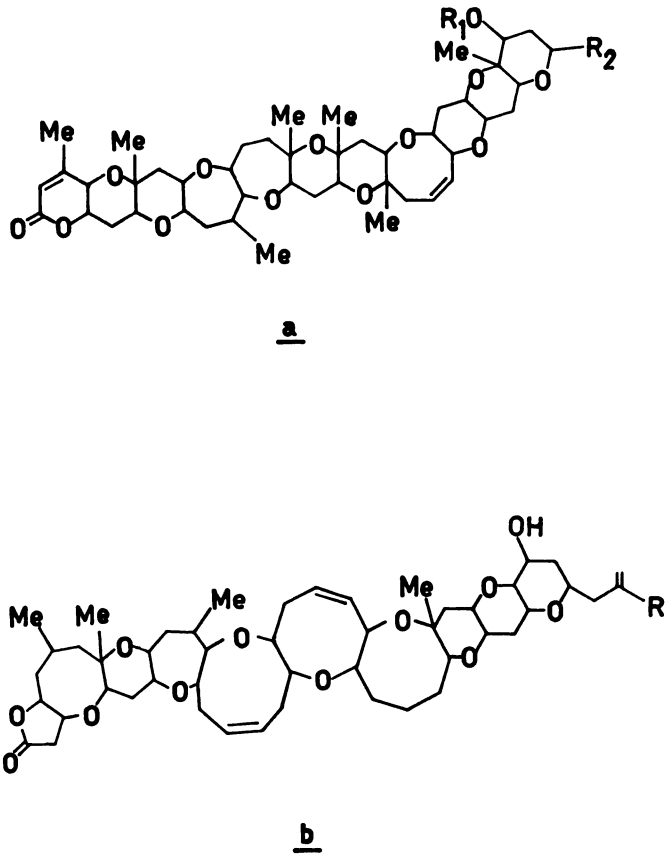

Fig. 1. The brevetoxins. a. PbTx-2 [R_1 = H, R_2 = $CH_2C(=CH_2)CHO$]; PbTx-3 [R_1 = H, R_2 = $CH_2C(=CH_2)CH_2OH$]; PbTx-5 [R_1 = Ac, R_2 = $CH_2C(=CH_2)CHO$]; PbTx-6 [R_1 = H, R_2 = $CH_2C(=CH_2)CHO$], 27,28 epoxide; PbTx-8 [R_1 = H, R_2 = CH_2COCH_2Cl]. b. PbTx-1 [R = CHO]; PbTx-7 [R = CH_2OH]. No structural information is available on PbTx-4.

which illustrated that PbTx-1 did not displace toxins which bind specifically at sites 1–3 located on, or proximal to, the voltage-dependent sodium channel were performed by Catterall and Gainer (22) using radioactive toxins specific for voltage-sensitive sodium channel sites 1–3. Evidence that the brevetoxins did not bind at site 4 was presented by Catterall (23), illustrating the enhancement of ^{125}I-labeled *Centruroides suffusus suffusus* toxin II to neurotoxin receptor site 4 by the brevetoxins. Catterall and Gainer (22) suggested that the brevetoxins bind at a new site, site 5; a site probably located on a region of the sodium channel involved in voltage-dependent gating.

Until recently, there have been no radioactively labeled brevetoxin probes for a direct investigation of specific binding. We have produced tritium-labeled PbTx-3 with a maximum specific activity of 15 Ci/mmol, in response to our needs for developing radioimmunoassays for toxin detection in food sources (24). This probe has become our most important tool for investigation of the specific binding characteristics of brevetoxins to excitable membranes. We believe the labeling technique described below could be utilized to produce other labeled brevetoxins including tritiated PbTx-7 from PbTx-1, and both reduced PbTx-5 and PbTx-6. Of particular interest would be the production of labeled toxins which possess the structural backbone illustrated in Fig. 1b.

An abbreviated report illustrating that labeled PbTx-3 binds with high affinity at sites other than sites 1–2 in rat brain synaptosomal preparations has appeared (25).

Materials and Methods

Excitable tissue preparations. Synaptosomes were prepared fresh daily from rat brain using the techniques described by Dodd *et al.* (26). Synaptosome integrity was evaluated by using electron microscopy (27) and by ^{22}Na influx experiments as described below. To prepare lysed synaptosomal fragments, the synaptosomal pellet was resuspended in 5 mM sodium phosphate (pH 7.4) and incubated with occasional stirring for 30 min in an ice bath. An equal volume of double-strength binding medium (minus BSA) was then added and this solution was used for binding experiments. Protein was measured on resuspended intact synaptosome or lysed synaptosome pellets just prior to binding experiments using the protein determination technique described by Bradford (28).

Toxin preparations. Brevetoxins PbTx-2 and PbTx-3 were prepared from laboratory cultures of *P. brevis* as we have previously described using solvent-solvent partitioning, and silica gel column and thin layer chromatography (5–7). Both synthetic ^{3}H-labeled and unlabeled PbTx-3 were prepared by chemical reduction of PbTx-2 using NaB^3H_4 or $NaBH_4$, respectively. In the case of syntheses using tritium, precautions were taken to trap all volatile compounds arising from the reduction reaction. Typically, 3–4 mg of toxin PbTx-2 were dissolved in 0.5 ml of acetonitrile and stirred in a 5-ml Wheaton reaction vial using a magnetic stirrer. An equimolar amount of $NaBH_4$ or NaB^3H_4, dissolved as a saturated solution in acetonitrile, was added with stirring. The reaction was allowed to proceed for 3.5 min at 24°, after which it

was terminated by the addition of 1 ml of acetone. The solvent and propanol (arising from the reduction of acetone) were evaporated. In the case of tritiated syntheses, the labeled reduction product was exchanged by repeated dissolution in 0.5 ml of ethanol followed by evaporation under nitrogen and subsequent passage of the volatiles through dry ice-cold fingers and sulfuric acid traps. The crude reduction mixture was then subjected to silica gel thin layer chromatography in ethyl acetate/petroleum ether (70:30) followed by C-18 reverse phase HPLC (15 cm column, 850 psi) of the active fraction utilizing an isocratic elution (1.4 ml/min) solvent of 85% methanol/15% water and monitoring absorbance at 215 nm (27).

Tritiated toxin was quantitated employing ultraviolet HPLC detector tracings, and standards curves were developed using unlabeled toxin PbTx-3. Radioactivity was determined using liquid scintillation techniques and appropriate quenched tritium standards. HPLC-purified ^{3}H-PbTx-3 had a specific activity of 10–15 Ci/mmol, or one-fourth the specific activity of the chemical reductant (24, 27). Aliquots of tritiated toxin were stored under nitrogen atmosphere at −20° in ethyl alcohol solution. Labeled toxin so stored is stable for about 4 months and is repurified by HPLC as necessary to eliminate radiochemical contaminants.

The aldehyde function in PbTx-2 (Fig. 1a) was oxidized to the corresponding carboxylic acid employing the method of Corey *et al.* (29), using argentic oxide in methanol solution in the presence of NaCN at room temperature for 12.5 hr. Purification was achieved using silica gel thin layer chromatography and a solvent system consisting of chloroform/methanol/trifluoroacetic acid (100:10:1). Saxitoxin was obtained from the Food and Drug Administration, batrachotoxin was from the National Institutes of Health, and sea anemone and scorpion toxins were from Sigma Chemical Co. Competitor toxins were used as obtained, without further purification except for a preliminary centrifugation step to remove insoluble materials. Intraperitoneal mouse bioassays and ^{22}Na influx measurements using synaptosomes were performed using a small quantity of each toxin to ensure potency.

Binding assay. Binding of ^{3}H-PbTx-3 was measured using a rapid centrifugation technique. All binding experiments were performed in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/ml BSA, and 0.01% Emulphor-EL 620 (a nonionic detergent used as an emulsifier). Emulsifier was required to solubilize the high concentration of unlabeled PbTx-3 used in the measurement of nonspecific binding (see below). Synaptosomes (40–80 μg total protein) or osmotically lysed synaptosomes, suspended in 0.1 ml of binding medium minus BSA, were added to a reaction mixture containing ^{3}H-PbTx-3 and other effectors in 0.9 ml of binding medium in 1.5 ml of polypropylene Microfuge tubes. After mixing and incubating at the desired temperature for 1 hr, samples were centrifuged for 2 min at 15,000 × *g*. Supernatant solutions were aspirated and the pellets were rapidly washed with several drops of a wash medium consisting of 5 mM HEPES (pH 7.4), 163 mM choline chloride, 1.8 mM calcium chloride, 0.8 mM magnesium sulfate, and 1 mg/ml BSA. The pellets were then transferred to liquid scintillation vials containing 10 ml of Aquasol, and the bound radioactivity was estimated using liquid scintillation techniques. Nonspecific binding was measured in the presence of a saturating concentration of unlabeled PbTx-3 (10 μM) and was subtracted from total binding to yield specific binding. Free ^{3}H-PbTx-3 was determined by counting directly an aliquot of the supernatant solutions prior to aspiration.

Sodium influx. $^{22}Na^{+}$ influx was measured by a modification of the method of Tamkun and Catterall (30). Synaptosomes containing 150–200 μg total protein in 50 μl of binding medium were pre-incubated with 100 μM aconitine and PbTx-3 concentrations of 1, 5, 10, 50, or 100 nM, or 1 μM for 30 min at room temperature. Following pre-incubation, 150 μl of $^{22}Na^{+}$ influx solution were added to each tube with stirring. This solution contained the same toxin concentrations used in the pre-incubations plus 5.4 mM potassium chloride, 0.8 mM magnesium sulfate, 55 mM glucose, 50 mM HEPES (pH 7.4), 128 mM choline chloride, 2.66 mM sodium chloride, 5 mM ouabain, 1 mg/ml BSA, and 1.3 μCi/ml carrier-free ^{22}NaCl.

Synaptosomes were incubated for 5, 10, or 15 sec, and influx was halted by the addition of 2 ml of ice-cold wash medium. Synaptosomes were then rapidly filtered through Millipore HVLP filters (0.45-μm pore size) and were washed twice with 2-ml aliquots of ice-cold wash medium. Nonspecific $^{22}Na^{+}$ influx was measured in the presence of 1 μM saxitoxin and was subtracted from total influx to yield specific influx. Initial rates of sodium ion influx were determined in each case.

Results and Discussion

The chemical reduction of PbTx-2 to synthetic PbTx-3, either labeled or unlabeled, results in a mixture of two potent compounds which are resolved by HPLC. Peak I elutes in 6.03 min under the given conditions (Fig. 2, *top*) and is synthetic PbTx-3 based on proton magnetic resonance spectrometry (6), equipotency in fish and mouse bioassays (7), and comigration in HPLC with native PbTx-3 (Fig. 2, *bottom*). The structure of peak II (elution time = 6.6 min) has not been determined

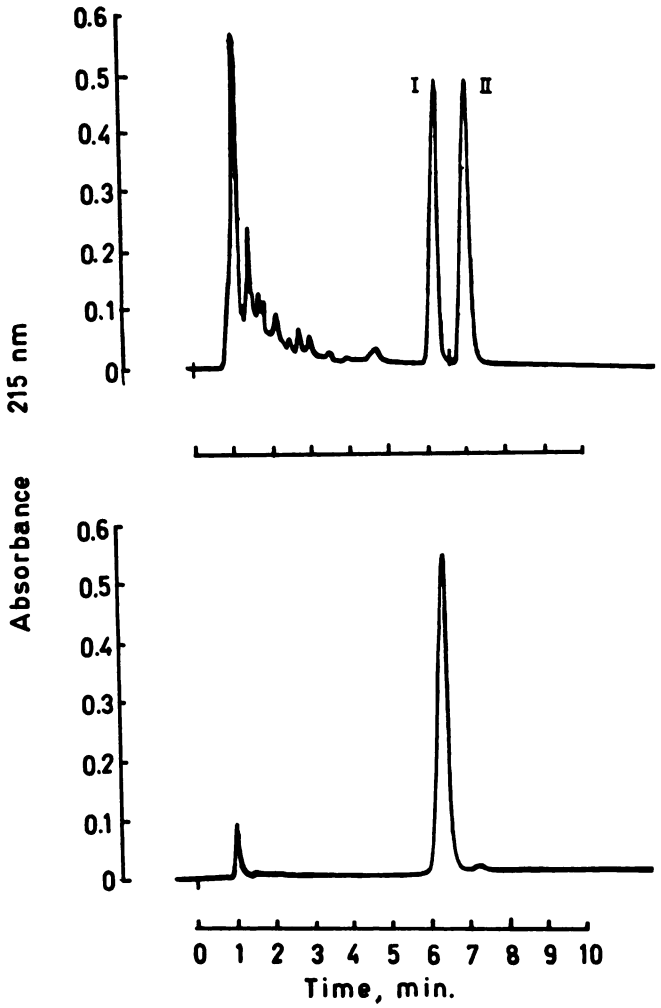

Fig. 2. Reverse phase HPLC of reduced PbTx-2 and authentic PbTx-3. Brevetoxin PbTx-2 was reduced using sodium borohydride (or borotritiide for production of labeled material) in acetonitrile solution according to the method described in the text. Subsequent HPLC of the reduced material yielded a trace as shown in the *upper panel*. Peak I was pooled from multiple injections and its identity was confirmed by rechromatography together with an equimolar amount of authentic PbTx-3 (*lower panel*). HPLC conditions: solvent 85% methanol/15% water; flow rate 1.4 ml/min; 15 cm C-18 reverse phase column; detection at 215 nm.

unequivocally but is believed to be the C_{41} methylene-reduced derivative of PbTx-3. This conclusion is based empirically on its specific activity (twice that of ^{3}H-PbTx-3) and probable mechanism of reduction. Yields for peak I vary from 60 to 75% of total reduction product. Following HPLC, the radiochemical purity of ^{3}H-PbTx-3 was determined by isotope dilution in the synaptosomal binding assay and was found to be in excess of 99%.

Tritiated PbTx-3 binds with high affinity and specificity to rat brain synaptosomes. Binding is linear with increasing tissue concentration up to 250 μg of protein/ml of binding medium. Equilibrium is reached in 20–30 min ($t_{½}$ = 1–2 min), and ^{3}H-PbTx-3 is stable at 4° during the 1-hr incubation in the presence of synaptosomes (Fig. 3). Specific binding is reversible during the 60-min incubation period by the addition of 10 μM unlabeled PbTx-3 ($t_{½}$ = 1–2 min). The microcentrifugation technique was preferred to the more familiar filtration assay, the latter technique resulting in a high degree of nonspecific binding to the filters. In addition, centrifugation permits the concentration of free ligand to be determined directly by counting an aliquot of the supernatant solution.

At 4° and a label concentration corresponding to half-maximal binding, specific binding comprises approximately 90% of total binding in synaptosomes (Fig. 4a) or in lysed synaptosomes. Rosenthal analysis of specific binding (Fig. 4b) suggests a single class of non-interacting binding sites with a mean apparent dissociation constant of 2.9 nM in synaptosomes (n = 5) and 3.2 nM in lysed synaptosomes (n = 3), and with a binding maximum of 6.8 pmol of toxin bound/mg of protein and 4.5 pmol/mg of protein in lysed synaptosomes.

The experimentally derived K_D for PbTx-3 binding to rat brain synaptosomes is in reasonable agreement with its half-maximal depolarizing activity in squid axons of 1.7 nM (20) and neuromuscular blocking action in rat phrenic nerve hemidiaphragm preparations of 5 nM (31). The affinity in the synaptosomal binding assay is in the range reported for other potent marine toxins like tetrodotoxin and saxitoxin (1.7–2.3 nM), α-scorpion toxin (1.9 nM), and batrachotoxin benzoate (50–70 nM).

Specific binding is saturable and is relatively membrane potential independent, there being a comparable affinity for sodium channels in synaptosomes with normal resting potential, or in osmotically lysed synaptosomes (Fig. 4b). In this regard, the brevetoxins bind as do saxitoxin and tetrodotoxin at site 1, whereas osmotic lysis and subsequent depolarization results in an inhibition of *Leiurus quinquestriatus* binding at

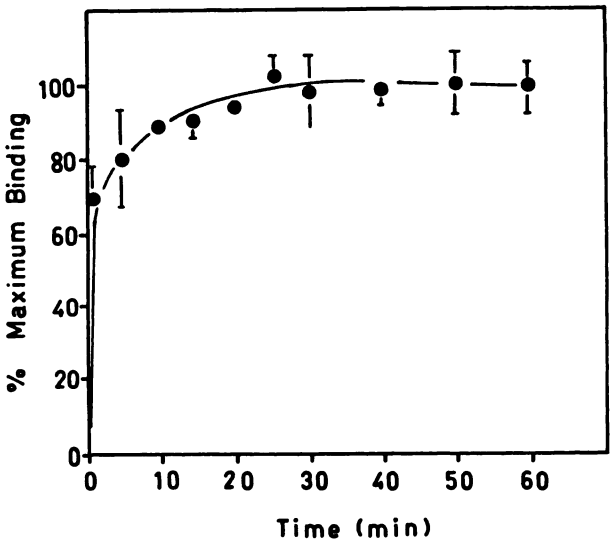

Fig. 3. Time course of ^{3}H-PbTx-3 binding to rat brain synaptosomes. Synaptosomes were incubated with 25 nM ^{3}H-PbTx-3 at 4°. Aliquots were taken at timed intervals and total bound and nonspecific radioactivity was determined as described in the text. Binding plateaus in approximately 20–30 min ($t_{½}$ = 1–2 min) and remains stable for at least 1 hr under these conditions. Specific binding is reversible by addition of 10 μM unlabeled PbTx-3 (data not shown, $t_{½}$ = 1–2 min). Each *point* represents the mean of duplicate determinations. *Error bars* span the range of individual measurements.

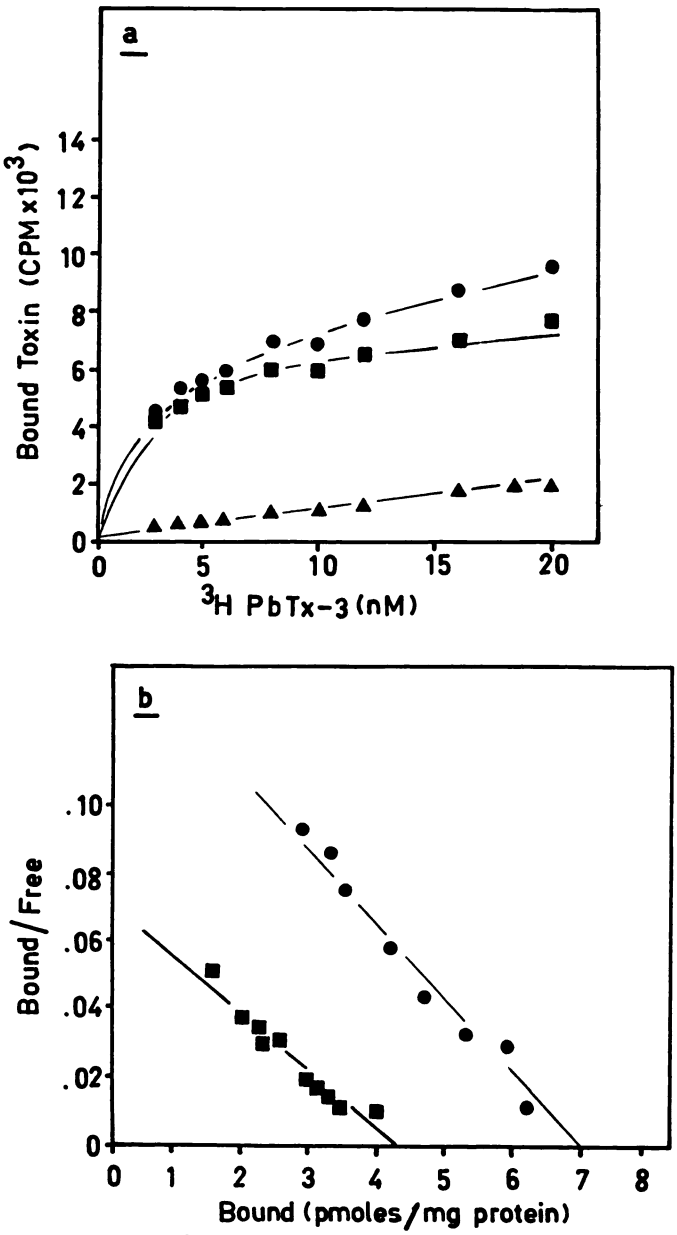

Fig. 4. Binding of ^{3}H-PbTx-3 to rat brain synaptosomes. Binding was measured in rat brain synaptosomes using a rapid centrifugation technique at 4°, as described in the text. a. Total (●) and nonspecific (▲) binding were measured by liquid scintillation techniques, their difference representing specific (■) binding. Results are representative of five replicates at each probe concentration. b. Rosenthal analysis of binding yields a K_d of 2.9 nM and a B_{max} of 6.8 pmol of toxin bound per mg of synaptosomal protein (●). For comparison, Rosenthal analysis of toxin binding to lysed synaptosomes is included (■).

site 3 (31), and in an enhancement of binding at site 2 by the alkaloid toxins (22). However, there was a 34% decrease in the number of binding sites upon lysis, a decrease we cannot fully explain at this time.

Possible explanations for the observed decrease in B_{max} include: 1) a partial solubilization of the binding site in lysed synaptosomes; 2) an incomplete sedimentation of lysed membrane fragments during microcentrifugation; or 3) a membrane potential-sensitive subpopulation of the binding site component. We rule out the first explanation for, if solubilization were to occur, we would expect it to be time dependent and it is not. Specific binding remains constant and, once plateaued, does not change for 6 hr from the time of lysis, including the time necessary to centrifuge the membranes and resuspend. The second explanation seems unlikely for, in order for B_{max} to decrease 34%, we would have to fail to sediment 34% of the membranes. There is no evidence to indicate incomplete sedimentation. A membrane potential-sensitive subpopulation of brevetoxin-binding component, as suggested in the third explanation, would require at least two different binding sites, with a roughly equivalent binding affinity for each. A reduction in binding at one of the types of sites upon depolarization could result in a reduction in B_{max} with little observed change in K_D. This possibility cannot be decided until very carefully designed depolarization experiments using intact synaptosomes can be carried out.

K_D, B_{max}, and percentage of specific binding are temperature dependent (Table 2). We believe the temperature dependence of B_{max} is in part dependent on membrane fluidity and accessibility of the lipid-soluble brevetoxins to lipid "solvents." An increase in temperature is reflected in an increase in toxin solubility in membrane lipids. This conjecture, we believe, is reinforced by the observed reduction in percentage of specific binding observed with increasing temperature. The increase in K_D observed with increasing temperature could also be a result of increased nonspecific brevetoxin binding to (or solubilization in) lipids, the actual concentration of brevetoxin in solution being progressively reduced by partitioning in lipid. It would therefore require a higher concentration of added brevetoxin to achieve half-saturation. Therefore, unless otherwise indicated, all experiments were performed at 4° to minimize nonspecific binding and to increase the stability of the synaptosome preparation.

Incubation of synaptosomes with PbTx-3 alone does not stimulate $^{22}Na^+$ influx but greatly enhances the influx elicited by 100 μM aconitine (Fig. 5). The effective concentration range for PbTx-3 action agrees quite well with the experimentally derived K_D. The ability of PbTx-3 to enhance Na^+ channel activation by aconitine is concentration dependent in the range of 1–100 nM and is reminiscent of the toxin T46-veratridine synergistic effect on sodium influx rate observed by Catterall and Risk (21). Influx is blocked by 1 μM saxitoxin, indicating the likely participation of voltage-dependent sodium channels in the brevetoxin-induced enhancement of Na^+ influx.

The synergistic effect on sodium ion influx by the brevetoxins in the presence of aconitine is expected since PbTx-3 has been shown to depolarize excitable membranes by shifting the voltage dependence of activation to more negative membrane potentials, resulting in a greater number of channels being open at normal resting potential (18). The site 2 alkaloid neurotoxins bind with greater affinity to the active states of the channel (22) and, thus, an enhancement of their activity by sodium channel activators would be expected. In fact, tritiated batrachotoxin benzoate binding is enhanced up to 5-fold in the presence of PbTx-1 (22). The enhancement of either binding or ^{22}Na influx is an indication that the brevetoxins do not bind at the veratridine site on excitable membranes.

PbTx-2, native PbTx-3, and synthetic PbTx-3 all exhibited equivalent abilities in displacing tritiated probe from its specific binding site (Fig. 6). Inhibition was competitive, as determined by double reciprocal plots of increasing concentrations of potential inhibitors at several ^3H-PbTx-3 concentrations (data not shown). PbTx-2, which had been oxidized to the corresponding carboxylic acid, did not displace tritiated probe, nor was it potent by either mouse or fish bioassay.

Brevetoxins appear to bind to synaptosomes at sites other than sites 1–4, there being no displacement of tritiated brevetoxin binding by tetrodotoxin or saxitoxin (site 1), batrachotoxin or aconitine (site 2), sea anemone toxin II or *L. quinquestriatus* venom (site 3), or *Centruroides sculpturatus* venom (site 4) (Fig. 7).

Tritiated brevetoxin binding is slightly enhanced (5–10%) in the presence of higher concentrations of saxitoxin (or tetrodotoxin) but is not concentration dependent above 10 nM added saxitoxin. Catterall and Risk (21) previously described a similar enhancement of tritiated saxitoxin binding by PbTx-1. This enhancement of binding also occurs in the nM concentration range. Since saxitoxin is thought to bind equally well to resting, active, or inactivated sodium channels, an allosteric interaction between site 1 and the brevetoxin-binding site is possible. However, we have no information on the nature of the interaction with respect to K_D or B_{max}, and further studies are indicated in order to pursue this issue.

Likewise, scorpion toxins which bind at sites 3–4 also slightly enhance tritiated PbTx-3 binding, with maximum effects occurring in the nM–μM concentration range. It is known that impure α-scorpion toxins (site 3) frequently contain some β-scorpion toxin (site 4) activity.[2] This fact, together with the

[2] W. A. Catterall, personal communication.

TABLE 2

Temperature dependence of K_D, B_{max}, and percentage of specific binding in intact and lysed synaptosomes

Tissue	Temperature	N	K_D	B_{max}	Percentage of specific binding[a]
			nM	*pmol/mg protein*	
Intact synaptosomes	4°	5	2.9 (2.0–3.9)	6.8 (6.2–7.2)	88–93
	22°	3	5.6 (4.9–6.2)	13.0 (11.5–15.7)	75–80
	37°	3	7.7 (7.2–8.6)	12.9 (12.5–13.3)	57–62
Lysed synaptosomes	4°	3	3.2 (2.4–3.7)	4.5 (4.3–5.0)	85–92

[a] Specific binding as a percentage of total binding, measured at the ^3H-PbTx-3 concentration corresponding to half-maximal binding.

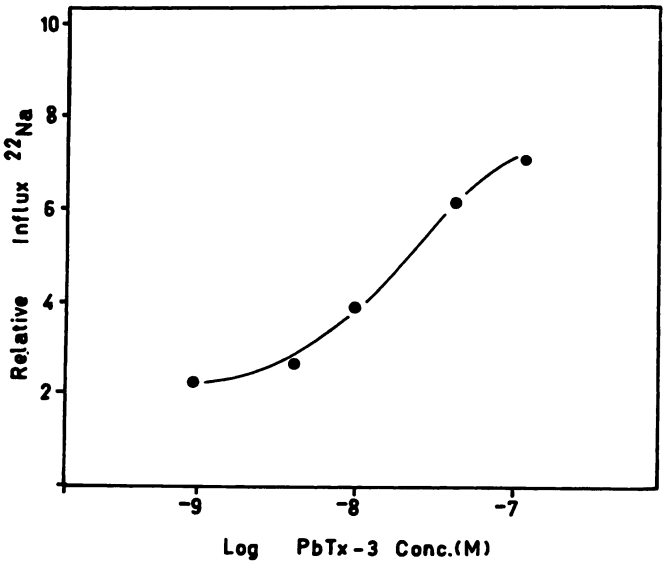

Fig. 5. Concentration dependence of the stimulation of $^{22}Na^+$ influx by synthetic PbTx-3. Synaptosomes were pre-incubated for 30 min with increasing concentrations of synthetic PbTx-3 in the presence of 100 μM aconitine. $^{22}Na^+$ was added and specific influx was measured as described in the text. Influx is plotted as relative initial specific influx rate, with that occurring in the presence of 100 μM aconitine alone set equal to 1.0. Each *point* represents the mean of triplicate determinations.

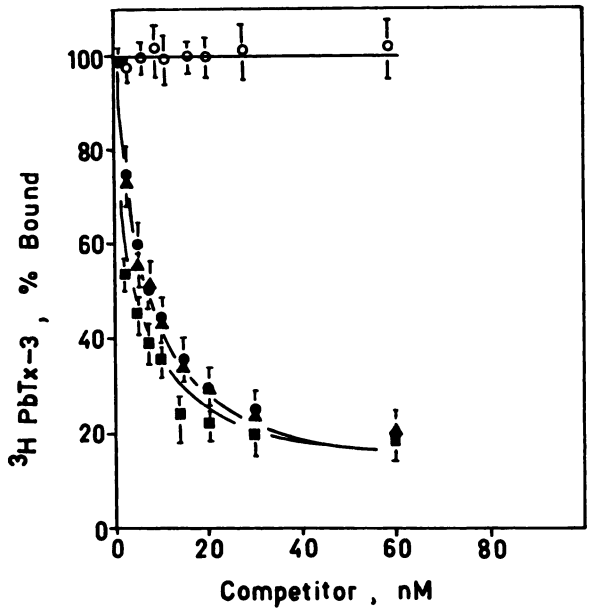

Fig. 6. Effect of brevetoxin analogs on tritiated PbTx-3 binding. Synaptosomes were incubated for 1 hr at 4° in the presence of 10 nM ^3H-PbTx-3 and increasing concentrations of native PbTx-3 (▲), reduced PbTx-2 (●), PbTx-2 (■), or oxidized PbTx-2 (○). Total and nonspecific binding were then measured as described. Native PbTx-3, reduced PbTx-2, and PbTx-2 were all equipotent in their ability to inhibit specific probe binding. Subsequent analysis revealed each analog to inhibit in a competitive manner. Oxidized PbTx-2, which is no longer potent in either the fish or mouse bioassay, failed to decrease tritiated probe binding. *Errors bars* span the range of individual measurements.

observed lack of effect on brevetoxin binding by purified sea anemone toxin II (site 3) leads us to postulate that the brevetoxin site and site 4 are allosterically linked. In a similar manner, brevetoxins enhance binding of ^{125}I-*C. suffusus suffusus* toxin II on neurotoxin receptor site 4. Purification of both

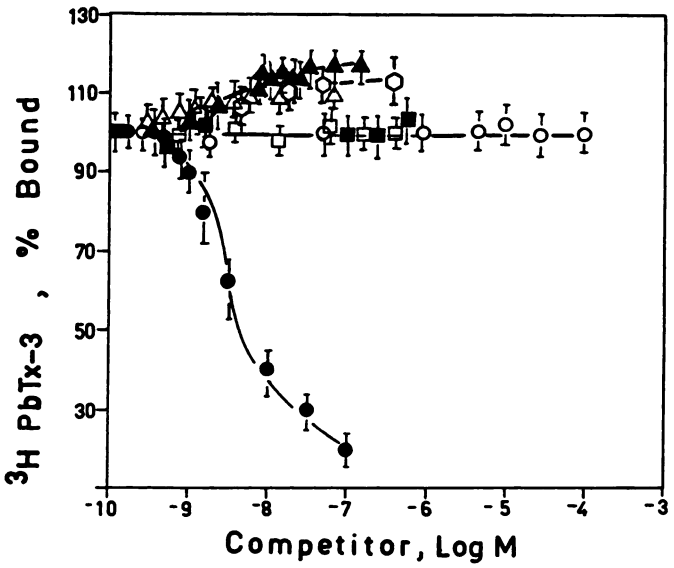

Fig. 7. Effect of toxins specific for sites 1–4 on brevetoxin binding. Synaptosomes were incubated for 1 hr at 4° together with 5 nM tritiated PbTx-3 and increasing concentrations of saxitoxin (△), batrachotoxin (■), aconitine (○), sea anemone toxin II (□), *L. quinquestriatus* venom (▲), *C. sculpturatus* venom (○), or native PbTx-3 (●). With the exception of saxitoxin experiments, 1 μM saxitoxin was added to each sample during incubation to maintain synaptosomal membrane polarization in the presence of the depolarizing competitors. In addition, 550 nM batrachotoxin was added to site 3–4 experiments to allosterically activate polypeptide toxin binding. Each *point* represents the mean of triplicate determinations. *Error bars* span the range of individual measurements.

α- and β-scorpion toxins is currently proceeding, to further clarify potential allosteric interactions.

We observe no effect on brevetoxin binding in the presence of batrachotoxin, in deference to the remarkable enhancement of batrachotoxin binding by PbTx-1 (21). In our studies, even nearly μM concentrations of batrachotoxin had no demonstrable effect on PbTx-3 binding. Catterall and Gainer (22) classify the brevetoxins as heterotropic allosteric modulators of sodium channel activation. Using the allosteric model of neurotoxin action proposed by Catterall in 1977 (33), the brevetoxins presumably act to reduce the value of M_{RT} (the energy of activation) required to open a channel (2). To put the concept in electrophysiological terms, the brevetoxins act to shift the activation voltage for sodium current in the hyperpolarizing direction (18). The reduction in activation energy results in an increase in the fraction of sodium channels in the open or active state at any particular time. Site 2 neurotoxins, which bind with orders of magnitude higher affinity to active channels than to inactive ones (22), bind to the now greater preponderance of active channels. Thus, site 2 binding (as well as the pharmacological effects elicited by them) is enhanced. Since we have shown that brevetoxin PbTx-3 toxin binding is essentially membrane potential independent, that is, it binds with equal affinity to polarized or depolarized membranes, an enhancement of brevetoxin binding by batrachotoxin (or any other site 2 toxin for that matter) should not occur.

Thus, the brevetoxins would appear to be the first ligands described for a new neurotoxin receptor site associated with excitable membranes. Brevetoxins PbTx-2 and PbTx-3 bind with equal affinity to a receptor site associated with rat brain synaptosomes. Binding of toxin to this receptor site is saturable, temperature-dependent, and strongly enhances aconitine-stim-

ulated sodium ion influx. Osmotic lysis results in a reduction of the number of available receptor sites but does not alter the apparent affinity of toxin for the remaining sites. Competition experiments conducted with sodium channel neurotoxins clearly demonstrate that the brevetoxins bind to a previously undescribed site which modulates the normal flux of sodium ions.

We are continuing competition studies, utilizing the other brevetoxins as they become available. Although we have no reason to expect that PbTx-1 and PbTx-7 (Fig. 1b) will bind to a site other than that occupied by the other known brevetoxins (Fig. 1a), their slightly different structure is worthy of investigation. The radiosynthetic reduction of PbTx-1 to yield tritiated PbTx-7 for binding studies would be quite interesting from a comparative point of view. A detailed investigation of toxin binding in intact synaptosomes under conditions of increasing K^+ depolarization is proceeding as well.

We are also preparing a PbTx-3-linked photoaffinity label with which to covalently link the specific binding component, a component we believe is a fifth neurotoxin binding site associated with voltage-sensitive sodium channels.

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