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CHARACTERIZATION OF THE P. BREVIS POLYETHER NEUROTOXIN BINDING COMPONENT IN EXCITABLE MEMBRANES

ANNUAL SUMMARY REPORT

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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°C 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 pmoles toxin bound per mg synaptosomal protein. Labeled toxin 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-sensitive 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.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) have adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHFW Publication No. (NIH) 78-23, revised 1978).

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I. List of Appendices, Illustrations, Tables

II. Statement of the Problem

The research described herein is aimed at characterization of the binding site(s) for Ptychodiscus brevis neurotoxin PbTx-3 (formerly known as T17) in nerve tissue, specifically in rat brain synaptosomes. To achieve this objective, we have:

(1) prepared synaptosomes accorrding to established procedures;

(2) determined the degree of toxin PbTx-3 binding to synaptosomes using ^{3}H-labeled PbTx-3, including determination of apparent K_D;

(3) delineated some of the classes of compounds which do not displace labeled PbTx-3 from its site of binding;

(4) determined the degree of displacement of labeled PbTx-3 by other brevetoxins and chemical derivatives of brevetoxins.

III. Background

A. History

We have previously succeeded in purifying to homogeneity two toxic compounds from the 1953 isolate of P. brevis (1). Extraction of toxic principles from red tides has led to identification of the same principles. Following extensive purification, each toxin migrates uniformly in the solvent systems listed in Table 1. In addition, both toxins are now routinely available to us in crystalline form (2,3).

TABLE 1. MIGRATION OF TOXINS IN SEVERAL DIFFERENT SOLVENT SYSTEMS

Solvent system	Thin-layer plate	R_f T17	R_f T34
2-Butanone/light petroleum			
(15/85)	Silica gel	0.10	0.29
(20/80)	Silica gel	0.17	0.30
Acetone/light petroleum			
(40/60)	Silica gel	0.21	0.42
(60/40)	Silica gel	0.35	0.60
Ethanol/water			
(95/5)	Silica gel	0.42	0.90
Acetone	Alumina	0.57	0.85
Ethyl acetate/light petroleum			
(50/50)	Silica gel	0.08	0.16
(70/30)	Silica gel	0.17	0.30

Several different solvent systems were used to ascertain homogeneity of toxic fractions. Analytical thin-layer plates, 5 × 20 cm, were used as absorbent. Plates were examined under short-wave ultraviolet light. Each fraction was scraped from the plate and lethality was assessed using the mosquito fish assay (BADEN *et al.*, 1979).

The two toxins we have isolated, PbTx-2 and PbTx-3 (formerly T34 and T17, respectively), differ in quantity produced and

potency. PbTx-3 is a more potent ichthyotoxic agent (700 fish units per mg) than PbTx-2 (130 fish units per mg), but is produced in smaller quantities than PbTx-2 (largely dependent upon culture conditions). Consequently, in situ ichthyotoxicity is a function of at least these two toxins, but is not synergistic. Signs and symptoms are in animals are dose-dependent and the active agents are lethal regardless of route of administration. Potencies in Swiss white mice are illustated in Table 2 (3).

TABLE 2. ACUTE LETHALITY OF T17 AND T34 BY DIFFERENT ROUTES OF ADMINISTRATION

Route of administration	Acute 24 hr LD_{50} (mg/kg) T17	T34
Intravenous	0.094 (0.075–0.116)	0.20 (0.17–0.25)
Intraperitoneal	0.17 (0.14–0.21)	0.20 (0.15–0.27)
Oral	0.52 (0.37–0.73)	6.6 (2.9–14.8)

Results are shown as means (95% confidence limits).

PbTx-3 produces bronchoconstriction in anesthetized artificially-ventilated guinea pigs (4). The pulmonary response to PbTx-3 differed slightly from those of histamine and acetylcholine in its longer persistence at peak levels. Rate of onset was equivalent, however. When normalized against authentic ACh, the response to 0.02 mg/kg PbTx-3 was equivalent to 0.05 ug/kg acetylcholine (Fig 1).

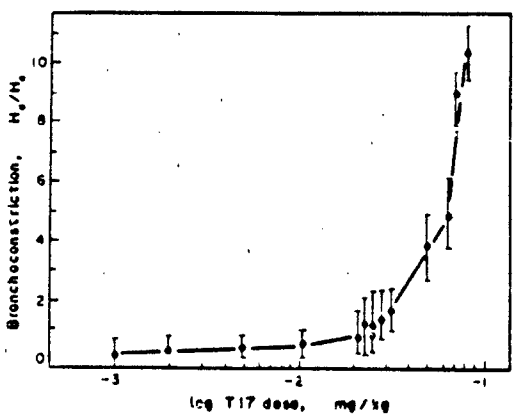


FIG. 1. DOSE–RESPONSE CORRELATION OF BRONCHOCONSTRICTION RESPONSE *in vivo* IN ANESTHETIZED GUINEA-PIGS WITH INCREASING T17 DOSAGE.

The resistance to artificial inflation in guinea-pigs following increasing doses of *P. brevis* toxin T17 was determined by measuring the height of the recorded response, and was normalized against the response obtained following administration of 0.05 μg/kg acetylcholine (ACh). The ratio of the recorded bronchoconstriction response of T17 (H_d) to the height of the recorded response to ACh (H_s) is an index of bronchoconstriction. Normalization was necessary to correlate responses obtained from different animals. Multiple (greater than 5–6) doses of T17 given to a single animal often resulted in "desentization"; a reduction in the recorded response. The curve shown is derived from data obtained using 13 animals.

In most cases, the response was accompanied by rhinorrhea, salivation, urination, and defecation, all of which were relieved by atropine administration. Refractoriness often occured when PbTx-3 was administered at regular 10 min intervals.

In the in vitro phrenic nerve hemidiaphragm preparation of rat, PbTx-3 is a potent inhibitor of neuromuscular transmission, with an ED_{50} of 0.11 + 0.04 nM and an ED_{90} of 0.12 + 0.03 nM. Inhibition of nerve-stimulated muscular contraction is preceded by a dose-related contracture. TTX or d-tubocurarine effectively blocks the contracture, as does bathing the preparation in a low Na^+ solution. Within 25 min of application, PbTx-3 can be washed from the preparation (Fig. 2).

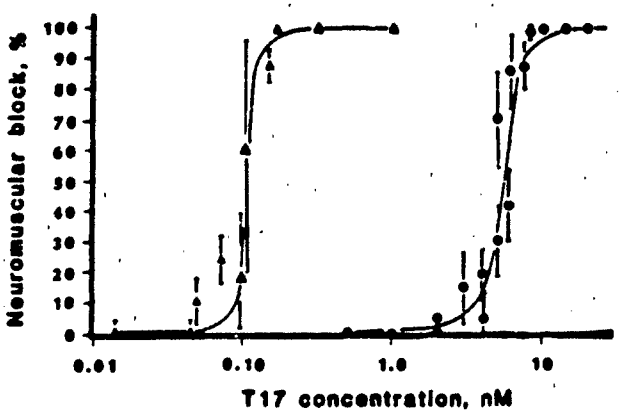


FIG. 2. THE LOG-CONCENTRATION – RESPONSE CURVE OF T17 ON NEUROMUSCULAR TRANSMISSION *IN VITRO*.

Each point was determined by a single exposure of individual diaphragms to concentrations of T17 in modified Krebs' solution. Per cent neuromuscular block is defined in the text. The neuromuscular potency of T17 was greater during stimulation with short trains of tetani (triangles) than during stimulation with single impulses (circles). The IC_{50} and IC_{90} values were derived from data on 64 diaphragms. Per cent neuromuscular block is indicated ± S.E.

Electron microscopy of toxin-inhibited neuromuscular junction show a complete complement of clear-core vesicles, regardless of toxin concentration or time to preparation fixation following block, up to a limit of 15 minutes.

Intracellular recording at the rat neuromuscular junction illustrated that brevetoxins increased miniature endplate potential frequency and at times, amplitude. PbTx-3, at concentrations ranging from 3-20 nM, caused a dose-dependent increase in m.e.p.p. frequency without altering amplitude (5). Voltage clamp experiments using squid giant axon, or crayfish axon (6), illustrated that the brevetoxins act primarily on voltage dependent sodium channels. External application of toxin caused a dose-dependent depolarization, transient repetitive discharges, followed by depression of the action potential leading to a complete block of excitability.

The reversibility of the depolarizing action upon washing decreased as the time of toxin treatment was increased. PbTx-3 induced depolarization was effectively inhibited by 0.3 uM tetrodotoxin, or 1 mM external Na solution. Voltage clamp experiments specifically indicate that PbTx-3 toxin affected sodium currents only. The activation voltage for sodium current

was shifted in the hyperpolarizing direction by more than 35 mV. PbTx-3 also greatly depressed the fast inactivation of sodium current. These results indicate that PbTx-3, and likely all the brevetoxins, depolarizes the membrane by selectively opening sodium channels at fairly negative potentials and by inhibiting the fast sodium inactivation. We also infer that the binding site for PbTx-3 is different from that of tetrodotoxin (Fig. 3).

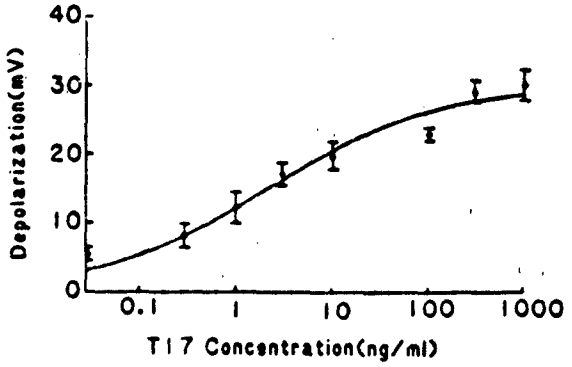


Fig. 3. Dose-response curve of membrane depolarization as a function of T17 concentration. Data from a total of 22 axons were pooled; each axon received only one dose. Data are plotted as means and S.E.s of depolarization amplitudes. The solid line is a theoretical curve calculated with an ED_{50} of 1.5 ng/ml, maximum depolarization 30 mV and a Hill's coefficient of 2.

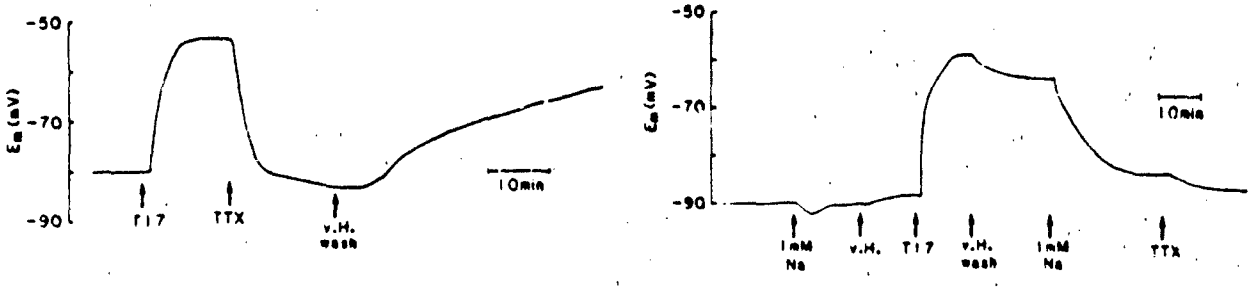


Fig. 4. Reversal of the depolarization by the low sodium external solution, 1 mM Na van Harreveld's solution, with equimolar substitution of 204 mM Na by tetramethylammonium ions. T17 toxin concentration, 1 μg/ml, and TTX, 300 nM.

B. Toxins

Since the discovery of *P. brevis* as the toxigenic organism, the toxins have been known by many names including Gymnodin, GBTX, T17 and T34, the GB series, and the brevetoxins. Several of these names have fallen into disfavor since the reclassification of *Gymnodinium breve* to *Ptychodiscus brevis*. In addition, many of the names are redundant how 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 (7) is used, and is preceded by the letters PbTx denoting *Ptychodiscus brevis* toxin. A summary of the proposed system is given in Table 3.

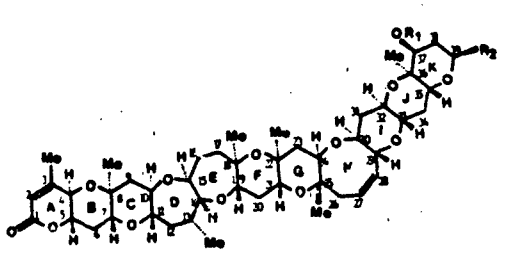
TABLE 3. NOMENCLATURE FOR THE BREVETOXINS*

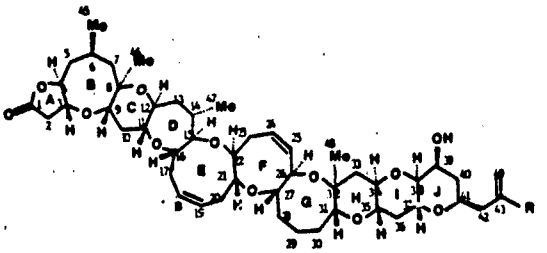
Notation	Synonyms	Reference
PbTx-1	Brevetoxin-A GB-1	
PbTx-2	Brevetoxin-B GB-2 T34	
PbTx-3	GB-3 T17	
PbTx-4	GB-4	
PbTx-5	GB-5	
PbTx-6	GB-6	
PbTx-7	GB-7	
PbTx-8	Brevetoxin-C	

*refer to figure 5 for structures

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

(7) (Figure 5).





	R_1	R_2	R	
PbTx-2	H	$CH_2C(=CH_2)CHO$		
PbTx-3	H	$CH_2C(=CH_2)CH_2OH$	PbTx-1	CHO
PbTx-5	Ac	$CH_2C(=CH_2)CHO$	PbTx-7	CH_2OH
PbTx-6	H	$CH_2C(=CH_2)CHO$ (27,28 epoxide)		
PbTx-8	H	CH_2COCH_2Cl		

No structural information available on PbTx-4

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 (10-14).

C. Molecular Pharmacology

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 of influx was in the ng/mL to ug/mLx range, maximally stimulating sodium ion influx some 9-fold over veratridine alone, at 1 ug/mL brevetoxin (15). The first binding experiments 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, using radioactive toxins specific for voltage-sensitive sodium channel sites 1-3 (16). Evidence that the brevetoxins did not bind at Site 4 was presented by Sharkey et al. (17), illustrating the enhancement of ^{125}I-labeled Centruroides suffusus suffusus toxin II to neurotoxin receptor Site 4 by the brevetoxins. Catterall and Gainer (16) suggested that the brevetoxins bind at a new site, Site 5; a site likely located on a region of the sodium channel involved in voltage-dependent gating.

Until recently, there have been no radioactive labels in the brevetoxins; a necessity for the direct investigation of brevetoxin binding. We have produced tritium-labeled PbTx-3 with a maximum specific activity of 15 Ci/mmole in response to our needs for developing radioimmunoassays for toxin detection in

food sources (18). This probe has become our most important tool for investigation of the specific binding characteristics of brevetoxins to excitable membranes.

IV. Technical Approach

A. Cultures of the Toxigenic Organism

Unialgal cultures of P. brevis were grown in the laboratory in 10 liter quantities in glass carboys. Carboys were kept at a constant temperature of 24°C in plexiglass controlled temperature tanks. Typically, 80 liters of culture could be brought to maximum density per week, 450 liters of continuous culture being constantly maintained. Cultures at peak density, if they were not to be extracted, were diluted 50/50 with autoclaved NH-15 artificial seawater medium (19). Twenty-one days after inoculation, cultures reach maximum density. More frequent dilutions, or dilutions of greater than 50/50 routinely resulted in cultures which failed to thrive.

B. Extraction and Purification of Toxins

Toxin was extracted from whole 10 liter cultures by the addition of 1 liter of chloroform. Cells were disrupted and cultures mixed by use of a vibrating liquid homogenizer. Following flash-evaporation of the chloroform fraction, the residue was dissolved in 90% aqueous methanol and was extracted thrice with equal portion of petroleum ether to remove nontoxic pigments. The residue which remained after evaporation of the methanol fraction was extracted with acetone and non-toxic insoluble materials were removed by centrifugation. The semi-purified toxin was next subjected to three silica gel chromatographic procedures. The first silica gel step, a flash-column, was performed using $CHCl_3$/methanol/acetic acid (100/10/1) as developing solvent. Two column volumes of solvent were passed through the column, collecting all eluent for flash-evaporation. This step is necessary prior to thin-layer chromatography. The entire purification procedure takes two days; the first day progressing through the acetone solubilization and centrifugation step. The column is used to remove decomposition impurities which occur overnight during storage.

A preparative thin-layer chromatography step using silica gel plates (500 u thickness) utilizes acetone/petroleum ether (30/70) as solvent and resolved two toxic fractions PbTx-2 and PbTx-3 (Rf= 0.34 and 0.17, respectively). A final thin-layer chromatography step (500 u thickness, silica) utilized ethyl acetate/petroleum ether (50/50) for PbTx-2 (Rf=0.36); a similar step utilizing a solvent system of 70/30 ethyl acetate/petroleum ether and affords PbTx-3 in purified form (Rf=0.30). The toxins can be visualized under short-wave ultraviolet light. Toxins were eluted from the silica gel using acetone or methanol. PbTx-2 was recrystallized from acetone by the addition of ethanol. PbTx-3 was subjected to C-18 reverse phase high pressure liquid chromatography (1.4 mL/min, 85% methanol/15% water, isocratic, uv detection at 215 nm).

C. Rat Brain Synaptosomal Binding Assay

Biological preparation. Synaptosomes were prepared fresh daily from rat brain using the techniques described by Dodd et al. (20). Synaptosome integrity was evaluated using electron microscopy, and by ^{22}Na influx experiments. 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 minutes in an ice bath. Protein was measured on resuspended intact synaptosomes or lysed synaptosomes just prior to binding experiments using the technique described by Bradford (21).

Toxin probe preparation. Natural toxins were used as obtained, purified from cultures. Synthetic [^3H]-labeled PbTx-3 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 volatiles arising from the reduction reaction. In either case, toxin PbTx-2 was mixed with equimolar reductant, each present in saturated solution. Under stirring conditions, the reactants are mixed and allowed to react for 3.5 minutes, after which excess acetone is added as sacrificial substrate (reduces to propanol). The solvent and propanol was evaporated, and residue was redissolved in minimal acetone. Acetone-soluble material was thin-layer chromatographed on silica gel plates using ethyl acetate/petroleum ether 70/30 as solvent, followed by high pressure liquid chromatography utilizing an isocratic elution (1.4 ml/min) solvent of 85% methanol/15% water and monitoring absorbance at 215 nm.

Tritiated toxin was quantitated employing uv HPLC detector tracings and standard curves were developed using unlabeled toxin PbTx-3. Radioactivity was determined using liquid scintillation techniques and appropriate quenched tritium standards. HPLC-purified radioactive PbTx-3 has a specific activity of 10-15 Ci/mmole, or one-fourth the specific activity of the chemical reductant. Aliquots of tritiated toxin are stored under nitrogen atmosphere at -20°C in ethyl alcohol solution. Labeled toxin is stable for 4-6 months, repurification by HPLC being performed as necessary.

Other toxins. The aldehyde function in PbTx-2 was oxidized to the corresponding acid employing the method of Corey et al. (22), using argenic oxide in methanol solution in the presence of NaCN at room temperature for 12.5 hrs. Purification was achieved using silica gel thin-layer chromatography and a solvent system consisting of $CHCl_3$/MeOH/trifluoroacetic acid (100/10/1). Saxitoxin was obtained from the FDA, batrachotoxin from NIH, and sea anemone, and scorpion toxins 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/or ^{22}Na influx measurements using synaptosomes were performed using a small quantity of each toxin to ensure potency.

Binding assay. Binding of tritiated toxin was measured using a rapid centrifugation technique (23). 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 bovine serum albumin, and 0.01% Emulphor-EL 620 as an emulsifier; the latter necessary to solubilize the high concentration of unlabeled PbTx-3 used in measurement of nonspecific binding. Synaptosomes (40-80 ug total protein), or osmotically-lysed synaptosomes, suspended in 0.1 mL binding medium minus BSA were added to a reaction mixture containing tritiated PbTx-3 and other effectors in 0.9mL binding medium in 1.5 mL polypropylene microcentrifuge tubes. After mixing and incubating at the desired temperature for 1 hour, samples were centrifuged for 2 min at 15000 g. Supernatant solutions were aspirated and the pellets were rapidly washed with several drops of a wash medium consisting of 5 mM HEPES (pH7.4), 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 liquid scintillant and the bound radioactivity estimated using liquid scintillation techniques. Nonspecific binding was measured in the presence of a saturating concentration of unlabeled PbTx-3 (10 uM) and was subtracted from total binding to yield specific binding. Free tritiated probe 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 Tankum and Catterall (24). Synaptosomes containing 150-200 ug total protein in 50 uL binding medium were pre-incubated with 100 uM aconitine and PbTx-3 concentrations of 1,5,10,50,100 nM, or 1 uM for 30 minutes at room temperature. Following pre-incubation, 150 uL of $^{22}Na+$ influx solution was 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 uCi/mL carrier-free $^{22}NaCl$..

Synaptosomes were incubated for 5,10,or 15 seconds, followed by addition of 2 mL ice-cold wash medium. Synaptosomes were then rapidly filtered through 0.45 u filters and were washed twice with 2 mL aliquots of ice-cold wash medium. Non-specific influx was measured in the presence of 1 uM saxitoxin and was subtracted from the results to yield specific influx. Initial rates of influx were determined in each case.

V. Results and Discussion

A. Toxins

Number. Since 1973, we have been able to detect and purify only two of the now seven described brevetoxins. Our PbTx-2 (formerly T34) and PbTx-3 (Formerly T17) were present at approximately a 3:1 mg ratio. During the time period 1973-1985, cultures were maintained as described in IV A at the Medical Campus of the University of Miami. Beginning late in 1985, we began establishing a culture facility at the Marine Campus of the University of Miami, and gradually phased out cultures at the Medical Campus.

Abundance. During the transition period (same cultures, physically moved), we began to recognize a gradual disappearance of PbTx-3 from cultures, with no apparent mass loss in PbTx-2 amounts. In addition, three new toxins made their appearance (Fig 6). The shift in toxin profile has little effect on the contract as written, as we are able to produce chemically the necessary PbTx-3 from PbTx-2 to carry out the experiments. The appearance of the new toxins gives us the potential of examining other brevetoxins for their ability to displace the radioactive probe from its site of action.

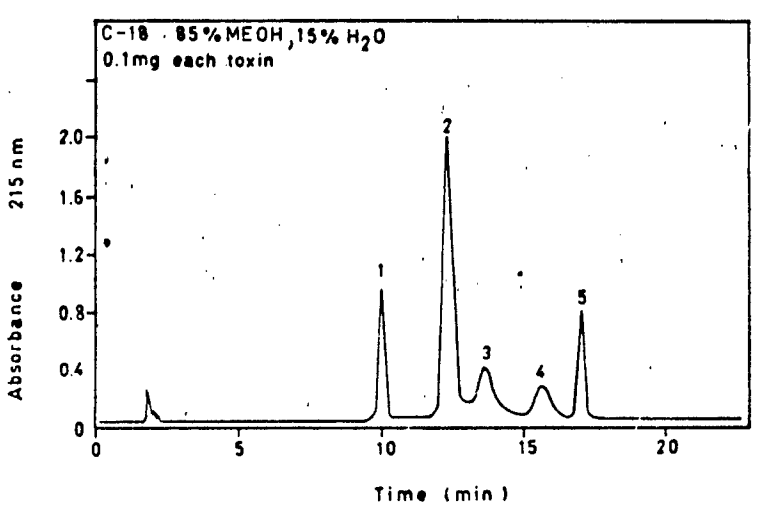


FIG. 6. UV detector (215 nm) tracing of 100 ug remixed purified toxins from laboratory cultures. Mixed sample run on C-18 reverse phase high pressure liquid chromatography at 1.4 mL/min 85% methanol/15% water isocratic elution. (1) PbTx-3, (2) PbTx-2, (3) PbTx-6, (4) unknown, (5) likely PbTx-1.

Effects of culture conditions. It has been hypothesized by others for some 18 years that the profile of brevetoxins produced in laboratory culture is largely dependent upon culture conditions. Owing to our past invariable toxin profile with respect to PbTx-2 and PbTx-3, we were staunch opponents of this hypothesis. However, we have now accepted the conjecture, although we are at a loss to explain the sudden shift in toxin profile in our cultures. We can ascertain no obvious difference in the culture conditions we maintain at the Medical versus the Marine Campus.

We believe it may be important to our understanding of how and why the dinoflagellates coordinate synthesis of these potent materials, to investigate potential culture condition perturbations. Our preliminary results indicate that time of harvest in the cell cycle plays a major role in the toxin profile present; PbTx-3 being present in largest proportion during mid-logrithmic phase, and nearly absent during stationary phase. Similarly, the "new" toxins appear during stationary phase.

However, these studies are not central to the theme of the contract, and so will be studied aside from the stated effort spent on the contract tasks.

B. Molecular Pharmacology

Radioactive probe.The chemical reduction of PbTx-2 to synthetic PbTx-3 (Fig 7), either labeled or unlabeled, results in a mixture of two potent compounds. Peak I and Peak II are resolved by HPLC in less than 10 minutes under the given conditions.

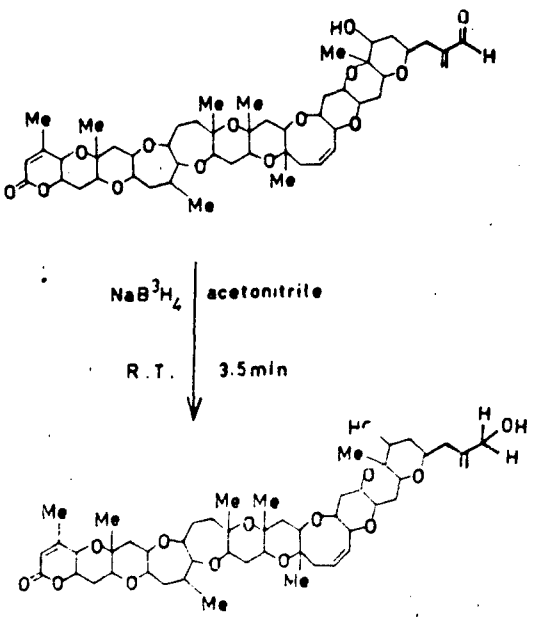


FIG. 7. The chemical reduction of PbTx-2 to PbTx-3.

Peak I is synthetic PbTx-3 based on pmr spectrometry, equipotency in fish and mouse bioassay, comigration in HPLC with native PbTx-3, and by isotope dilution in the molecular pharmacology receptor binding assay. The structure of Peak II has not been unequivocally determined 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 PbTx-3) and probable mechanism of reduction. Yields of Peak I vary from 60-75% of total reduction product (Fig 8). Following HPLC, the radiochemical purity of tritiated PbTx-3 was determined to be in excess of 99% by isotope dilution.

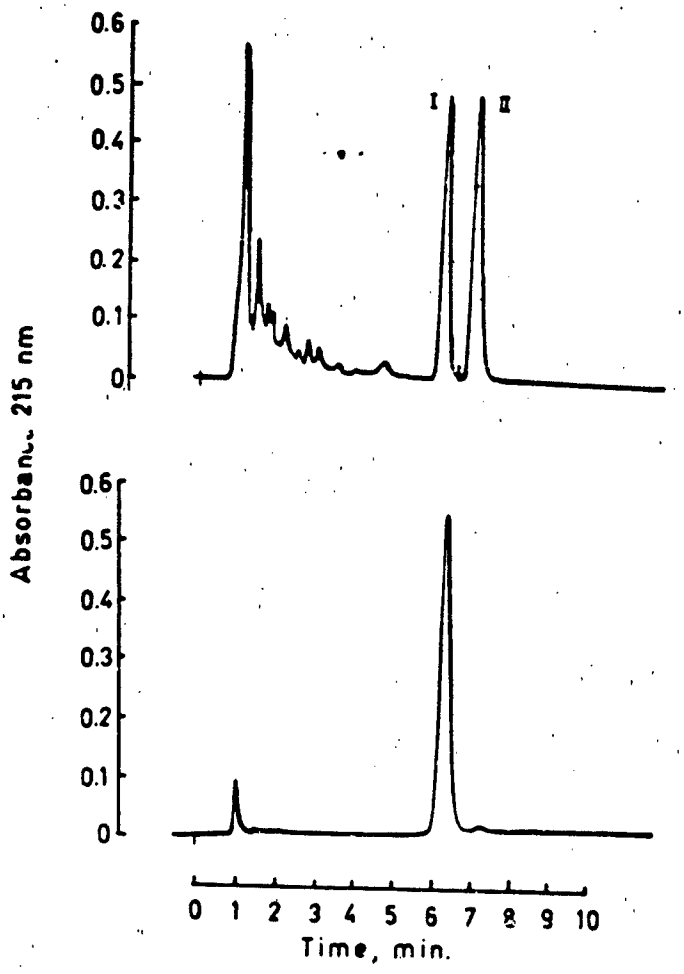


FIG. 8. HPLC of chemically-reduced PbTx-2. PbTx-2 from cultures was reduced according to the scheme outlined in Fig. 7. The reduction products were separated by reverse phase C-18 high pressure liquid chromatography using an isocratic elution of 85% methanol/15% water at a flow rate of 1.4 mL/min, detection at 215 nm. The top panel illustrates the two reduction products, peak I corresponds to authentic PbTx-3 from cultures as illustrated by a mixed comigration of synthetic and authentic (lower panel) toxin. Peak II is likely doubly-reduced PbTx-2 (methylene-reduced PbTx-3).

Brevetoxin binding characteristics. Tritiated PbTx-3 binds with high affinity and specificity to rat brain synaptosomes. Binding is linear with increasing tissue concentration up to 250 ugrams protein/mL binding medium. Equililbrium is reached in 20-30 min ($t_{1/2}$=1-2 min) and ^{3}H PbTx-3 is stable at 4°C during the 1 hour incubation in the presence of synaptosomes (Fig 9).

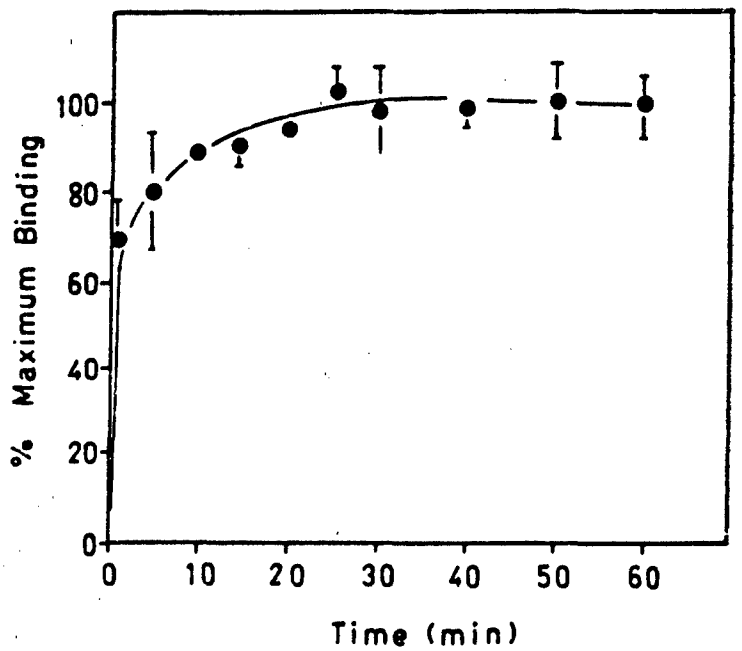


FIG. 9. Time course of tritiated PbTx-3 binding to rat brain synaptosomes. Synaptosomes were incubated with 25 nM toxin at 4°C. Aliquots were taken at timed intervals and total bound and non-specific radioactivity was determined as described. Binding plateaus in approximately 20 min. Specific binding is reversible by addition of unlabeled toxin (data not shown) at kinetics comparable to on-rates. Each point is the mean of duplicate measurements.

At 4°C and a label concentration corresponding to half-maximal binding, specific bindng comprises approximately 90% of total binding in synaptosomes (Fig10a), or in lysed synaptosomes. Rosenthal analysis of specific binding (Fig10b), 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 capacity of 6.8 pmoles toxin bound/mg protein. There was a 34% loss of binding sites upon depolarization.

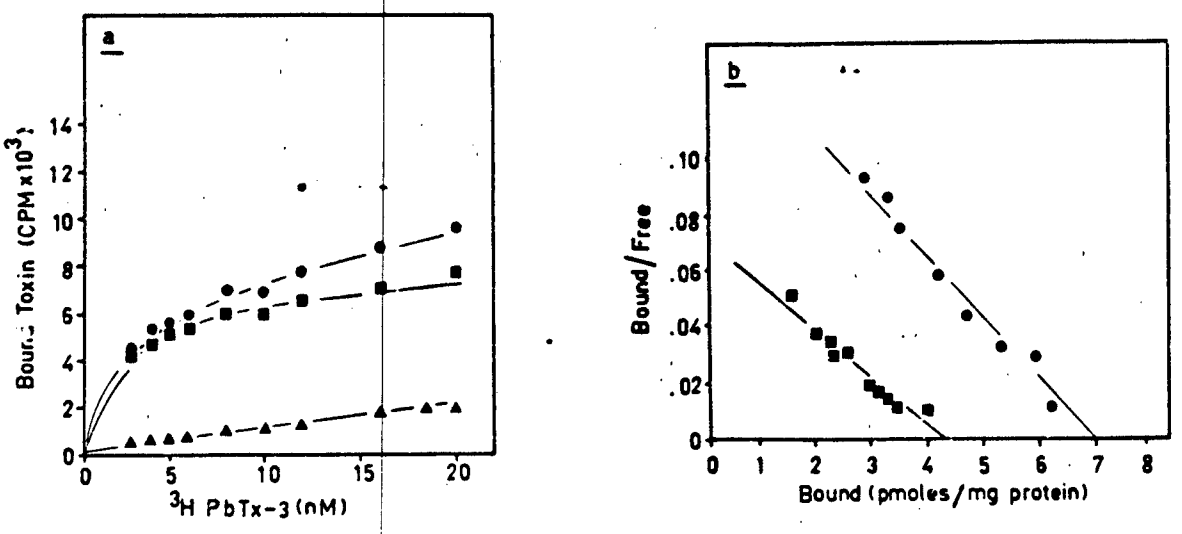


FIG. 10. Binding of tritiated PbTx-3 to rat brain synaptosomes. Binding was measaured using a rapid centrifugation technique at 4°C, as described in the text. Panel a Total(●) and non-specific (▲) binding were measured by liquid scintillation techniques, their difference representing specific (■) binding. Results are representative of five replicates at each probe concentration. Panel b Rosenthal analysis of binding yields a dissociation constant of 2.9 nM and a binding maximum of 6.8 pmoles of toxin bound per mg synaptosomal protein (●). For comparison, Rosenthal analysis of toxin binding to lysed synaptosomes is included (■).

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 (6) and neuromuscular blocking action in rat phrenic-nerve hemidiaphragm preparations of 5 nM (25). 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), alpha-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 (as shown in Fig. 4b). In this regard, the brevetoxins bind as do STX and TTX at Site 1, whereas osmotic lysis and subsequent depolarization results in an inhibition of L. quinquistriatus toxin binding at Site 3 (26), and in an enhancement of binding at Site 2 by the alkaloid toxins(16). However, the decrease of 34% in the number of binding sites which resultls upon osmotic lysis cannot be fully explained at this time.

Possible explanations for the observed decrease in binding maximum 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 (1) 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 six hours from the time of lysis, including the time necessary to centrifuge the membranes and resuspend. Number (2) seems unlikely for in order for binding maximum 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 sub-population of brevetoxin binding component, as suggested in (3) 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 site upon depolarization could result in a reduction in B_{max} with little effect on 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 % specific binding are temperature-dependent (Table 4). 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 % specific binding observed with increasing temperature. The increasae 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°C to minimize non-specific binding and to increase the stability of the synaptosome preparation.

TABLE 4. Effect of Temperature and Membrane Potential on Brevetoxin Binding to Rat Brain Sodium Channels

Tissue	Temp. (C)	K_D (nM)	B_{max} (pmoles/mg protein)	Specific Binding
Intact Vesicles	4(5)	2.9	6.8	88-93 %
	22(3)	5.6	13.0	75-80 %
	37(3)	7.7	12.9	57-62 %
Lysed Vesicles	4(3)	3.2	4.5	85-92 %

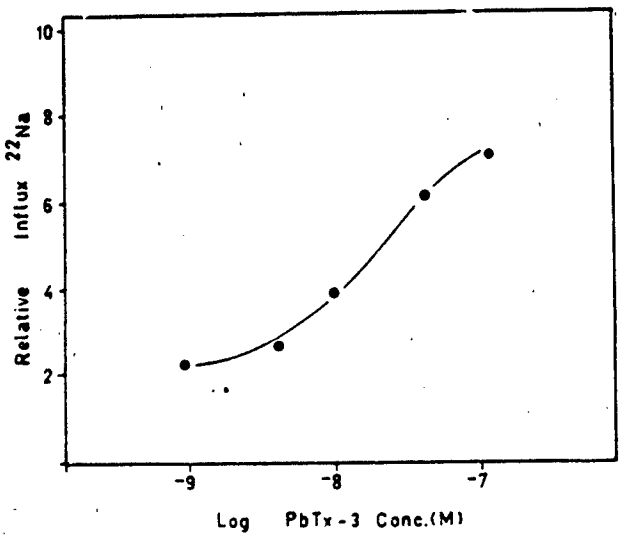


FIG 11. Concentration dependence on the stimulation of aconitine induced sodium ion influx by brevetoxins. Synaptosomes were preincubated for 30 minutes with increasing concentrations of synthetic PbTx-3 in the presence of 100 uM aconitine. ^{22}Na was added and specific influx was measured as described. Influx is plotted as relative initial specific influx rate, with that occurring in the presence of 100 uM aconitine alone set equal to 1.0. Each point represents the mean of triplicates.

Incubation of synaptosomes with PbTx-3 alone does not stimulate $^{22}Na^+$ influx, but greatly enhances the influx elicited by 100 uM aconitine (Fig 11). 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 reminicent of the toxin T_{46}-veratridine synergistic effect on sodium influx rate observed by Catterall and Risk (15). Influx is blocked by 1 uM 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 (6). The site 2 alkaloid neurotoxins bind with greater affinity to the active states of the channel (16) 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 (16). 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 12). 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 *Leiurus quinquestraitus* venom (site 3), or *Centruroides sculpturatus* venom (site 4) (Fig 13).

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 (15) 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-uM concentration range. It is known that impure alpha-scorpion toxins (site 3) frequently contain some beta-scorpion toxin (site 4) activity (personal communication,

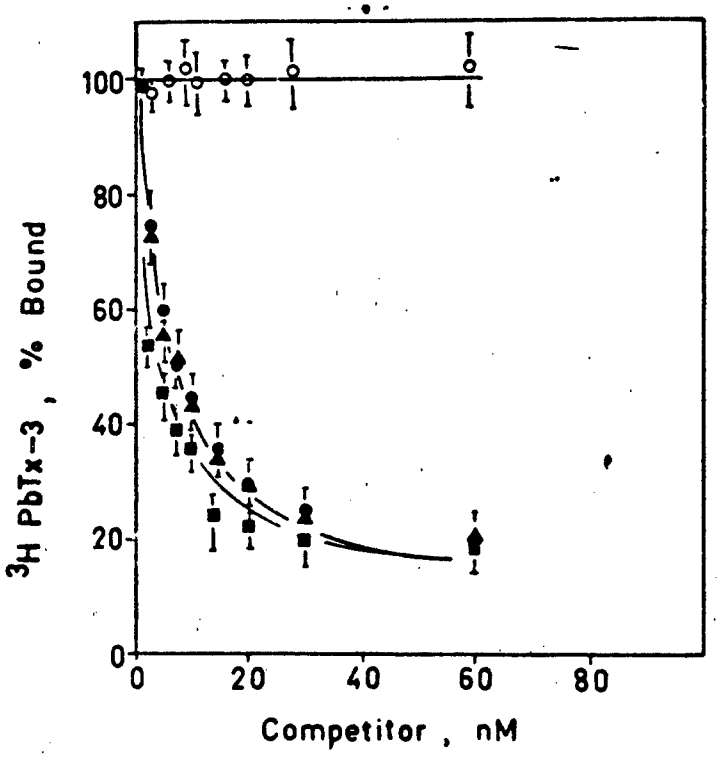


FIG 12. Effect of brevetoxin analogs on tritiated PbTx-3 binding. Synaptosomes were incubabted for one hour at four degrees in the presence of 10 nM ^{3}H-PbTx-3 and increasing concentrations of native PbTx-3 (closed triangles), reduced PbTx-2 (closed circles), PbTx-2 (closed squares), or oxidized PbTx-2 (open circles). Total and nonspecific binding were then measured as described in the text. Native PbTx-3, reduced PbTx-2, and PbTx-2 were all equipotent in their abilities to inhibit specific probe binding. Subsequent analysis revealed each anolog inihibted in a competitive manner. Oxidized PbTx-2, which is no longer potent in either fish or mouse bioassay, failed to decrease tritiated probe binding. Error bars span the range of individual measurements.

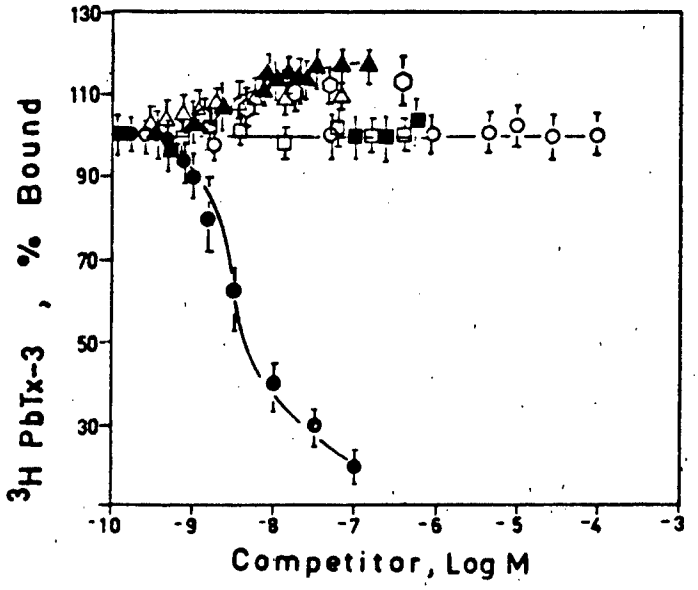


FIG 13. Effect of toxins specific for Sites 1-4 on brevetoxin binding. Synaptosomes were incuabted for 1 hour at 4 degrees together with 5 nM tritiated PbTx-3 and increasing concentrations of saxitoxin (open triangles), batrachotoxin (closed squares), aconitine (open circles), sea anemone toxin II (open squares), alpha-scorpion venom (closed triangles), beta-scorpion toxin (open hexagon), and native PbTx-3 (closed circles). With the exception of saxitoxin experiments, 1 uM 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.

W.A. Catterall). This fact, together with the 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 Centruroides suffusus suffusus toxin II on neurotoxin receptor site 4. Purification of both alpha- and beta-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 (15). In our studies, even nearly uM concentrations of batrachotoxin had no demonstrable effect on PbTx-3 binding. Catterall and Gainer classify the brevetoxins as heterotropic allosteric modulators of sodium channel activation (16). Using the allosteric model of neurotoxin action proposed by Catterall in 1977 (27), the brevetoxins presumably act to reduce the value of M_{RT} (the energy of activation) required to open a channel (15). To put the concept in electrophysiological terms, the brevetoxins act to shift the activation voltage for sodium current in the hyperpolarizing direction (6). 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 (16), 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 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.

VI. Conclusions

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-stimulated 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. While we have no reason to expect that PbTx-1 and PbTx-7 (Fig 5b) will bind to a site other than the site occupied by the other known brevetoxins (Fig 5a), 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.

VII. Recommendations

Our work on the molecular binding pharmacology for the brevetoxins has progressed quite well during the past year. For the coming year, accomplishment of the following tasks is expected:

(1) examine the binding of brevetoxin PbTx-3 under conditions of potassium ion depolarization;
(2) fully explore synthesis of brevetoxin photoaffinity labels;
(3) examine the potential that brevetoxins of type shown in Fig 5b compete/or don't compete for the site involved in PbTx-3 bindng;
(4) attempt preparation of reduced PbTx-1 (to yield a radioactive PbTx-7) and thereby provide us with a labeled probe for the new structural backbone illustrated in Fig 5b;
(5) if photoaffinity label synthesis is successful as in (2), begin initial characterization of the brevetoxin binding component once covalently linked.

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