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

ANNUAL REPORT

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The polyether lipid-soluble toxins isolated from the marine dinoflagellate Ptychodiscus brevis (formerly Gymnodinium breve) bind to a unique site, Site V, associated with the voltage-sensitiv sodium channel(VSSC) in brain synaptosomes. Using tritiated PbTx-3 as a specific probe for binding at Site V, dissociation constants and binding equilibri have been determined in rat, fish, and turtle brain synaptosomes. Dissociatio constants approximate nM values in each system, and binding maxima are all in the pmol/mg synaptosomal protein range. In all systems, labeled brevetoxin can be displaced by unlabeled brevetoxin in a concentration-dependent manner. More detailed srudies have been carried out using the rat system Binding of labeled brevetoxin is membrane potential independent, and both Kd and Bmax appear unchanged in intact versus depolarized synaptosomes, in membrane fragments, or in solubilized sodium channels; inhibition constants for tritiated brevetoxin binding nave been determined for each of the naturally-occurring

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Summary

The polyether lipid-soluble toxins isolated from the marine dinoflagellate *Ptychodiscus brevis* (formerly *Gymnodinium breve*) bind to a unique site, Site V, associated with the voltage-sensitive sodium channel (VSSC) in brain synaptosomes. Using tritiated PbTx-3 as a specific probe for binding at Site V, dissociation constants and binding maxima have been determined in rat, fish, and turtle brain synaptosomes. Dissociation constants approximate nM values in each system, and binding maxima are all in the pmol/mg syanptosomal protein range. In all systems, labeled brevetoxin can be displaced by unlabeled brevetoxin in a concentration-dependent manner. More detailed studies have been carried out using the rat system: Binding of labeled brevetoxin is membrane-potential independent, and both K_d and B_{max} appear unchanged in intact versus depolarized synaptosomes, in membrane fragements, or in solubilized sodium channels derived from synaptosomes. Inhibition constants for tritiated brevetoxin binding have been determined for each of the naturally-occurring derivative brevetoxins. All of the type-1 brevetoxins displace in a purely competitive manner, whereas the type-2 brevetoxins inhibit tritiated brevetoxin binding in a mixed competition pattern, implying a partial common binding component for type-1 and type-2 toxins and a partial unique binding character for the type-2 toxins. Photoaffinity probes synthesized using brevetoxin PbTx-3 inhibit tritiated brevetoxin binding with inhibition constants approximating inhibition constants derived for other type-1 brevetoxins. The probes have been stabilized and the syntheses confirmed and optimized. Preliminary studies have been conducted to produce a brevetoxin-linked affinity column, and using solubilized α-subunit from rat brain sodium channels preliminary purification of the brevetoxin binding site has been undertaken.

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 Institue of LAboratory Animal Resources, National Research Council (DHEW Publication Number (NIH) 86-23, revised 1985)).

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II. Statement of the Problem

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

[1] prepared synaptosomes from rats, turtles, and fish according to established procedures,

[2] determined the degree of toxin PbTx-3 binding to each species synaptosomes using tritiated PbTx-3, and have determined apparent dissociation constants (K_d) and binding maxima (B_{max});

[3] determined the degree of displacement of labeled PbTx-3 by other naturally-occurring and synthetic derivatives of brevetoxins in each species synaptosomes;

[4] determined the degree of displacement between brevetoxin and synthetic photoaffinity derivative of PbTx-3 for the specific site in rat synaptosomes;

[5] determined the inhibition type for each natural derivative brevetoxin and synthetic photoaffinity labels by using iterative classical competition studies. Comparisons of inhibition constants (Ki) derived from these methods were compared with inhibition constants derived from the Cheng-Prusoff equation;

[6] determined the dissociation constants and binding maxima for three new brevetoxin probes.

III. Background

A. Brevetoxin Binding Site

Florida's red tide dinoflagellate *Ptychodiscus brevis* produces at least eight different sodium channel neurotoxins (1), designated PbTx 1-8. The toxins are multi-ring polyether compounds; six of which are based on a single backbone of type-1 (2), and the remaining two of which are based on a carbon backbone of type-2 (1,3), as illustrated in Figure 1.

The toxins bind at Site 5 which is associated with the voltage-sensitive sodium channel (4-6) and is allosterically linked to Sites 2 and 4 (7). Binding at Site 5 by brevetoxins results in a shift of the activation voltage in mouse neuroblastoma cells (4) or in crayfish axon (8) to more negative values, and also inhibits inactivation of the channel (7). We previously demonstrated specific binding of tritiated brevetoxin PbTx-3 to rat brain synaptosomes, and illustrated that PbTx-3 binds to both intact and lysed synaptosomes. The effect of decreasing membrane potential on specific binding of tritiated PbTx-3 to Site 5 has been explored in greater detail and quantified in this communication.

Those brevetoxins which are derived from structural backbone type-2 are 10-50 times more potent than are the toxins based on structural backbone type-1 (1-5), implying that the former toxins may interact at Site 5 with higher affinity than do the latter ones. We previously showed that [3H] PbTx-3 could be specifically displaced by unlabeled PbTx-2 or PbTx-3, but not by a nontoxic oxidized derivative of PbTx-2 (5), illustrating a potency/binding affinity relationship for these type-1 brevetoxins. Insufficient amounts of type-1 competitor toxins

and no type-2 toxins were available at that time for the determination of inhibition constants. This report also describes [^{3}H] PbTx-3 competition studies utilizing naturally occurring brevetoxins PbTx-1, -2, -3, and -7, and illustrates that derived inhibition constants (K_i) reflect the literature reports of individual potency for each brevetoxin quantified in *Gambusia* fish bioassay.

In order to investigate the primary structure characteristics of the brevetoxin binding site in synaptosomes, two photoaffinity probes linked to brevetoxin PbTx-3 have been synthesized. Specific displacement experiments involving brevetoxin PbTx-3 covalently-linked to these two experimental photoaffinity probes illustrate that these derivatized toxins still bind with high affinity to Site 5 located on the channel. Hence, these derivatized toxins are of potential use as specific covalent probes of the binding site.

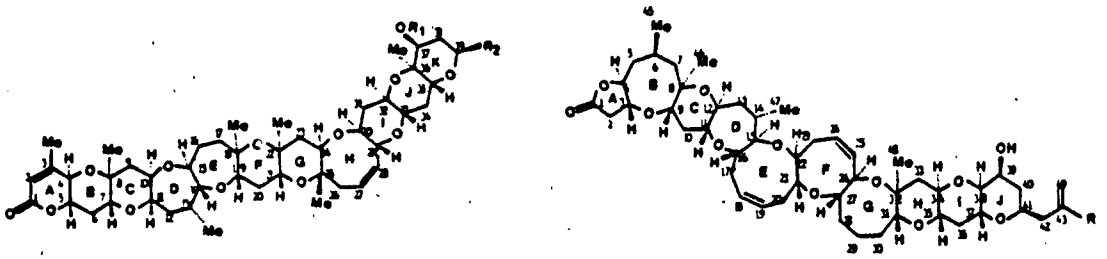

Figure 1. The brevetoxins are based on two different backbone structures, as indicated. Type 1 toxins (left) include: PbTx-2 [R1=H, R2=CH2CH(=CH2)CHO)]; PbTx-3 [R1=H, R2=CH2CH(=CH2)CH2OH)]; PbTx-5 [R1=Ac, R2=CH2(=CH2)CHO]; PbTx-6 [R1=H, R2=CH2C(=CH2)CHO, 27,28 epoxide]; PbTx-8 [R1=H, R2=CH2COCH2Cl]. Type 2 toxins (right) include: PbTx-1 [R=CHO]; PbTx-7 [R=CH2OH]. No structual information is available on PbTx-4.

B. Previous Work *In Vitro*

The toxicological consequences of *P. brevis* red tides are: mass mortality of fishes exposed to the red tide; toxic shellfish which, if consumed, result in human Neurotoxic Shellfish Poisoning; and, an irritating aerosol which results from contact with *P. brevis* cell particles entrapped in seaspray. In all cases, the threshhold levels for intoxication are in the picomolar to nanomolar concentration ranges, implying a specific locus or loci of action for brevetoxins (reviewed in 6).

Electrophysiological protocols utilizing crayfish and squid giant axons revealed that external application of brevetoxin caused a concentration-dependent depolarization, repetitive discharges, and a depression of the action potential leading to a block of excitability (Figure 2). Voltage clamp experiments illustrated that only sodium currents were affected (7). Early experiments utilizing neuroblastoma cells illustrated that application of Brevetoxin-A (PbTx-1) (8) induced

an influx of $^{22}Na^{+}$ in a dose-dependent manner. This work was followed by experiments using brevetoxin PbTx-3 and rat brain synaptosomes, once again illustrating a dose-dependent uptake of $^{22}Na^{+}$ following toxin application (Figure 3) (4).

Catterall and Risk (8) demonstrated that brevetoxins did not interfere with binding of sodium channel-specific neurotoxins which bind at Sites 1-3, and Catterall and Gainer illustrated the lack of brevetoxin interaction at Site 4 (9). That brevetoxins bind at a unique site associated with voltage-sensitive sodium channels (VSSC) was suggested by this data (8,9). Specific binding of brevetoxins to synaptosomes was first demonstrated by Poli *et al.* (Figure 4) (4), by utilizing brevetoxin PbTx-2 synthetically reduced with sodium borotritiide to yield tritiated PbTx-3 with specific activities approaching 20 Ci/mmole (10). Poli demonstrated saturability, competition for specific binding sites by nonradioactive brevetoxin agonists , binding maxima in the pmole/mg protein concentration range, reversibility of radioactive toxin binding, $t_{1/2}$ times for association and dissociation consistent with specific binding, distinct brain regional and subcellular distribution, the presence of a pharmacological response at appropriate concentrations for binding, tissue linearity, and temperature dependence (11). Dissociation constants, binding maxima, and competitive displacement curves for brevetoxin at site 5 parallel those constants derived for saxitoxin binding at site 1 (Figure 5).

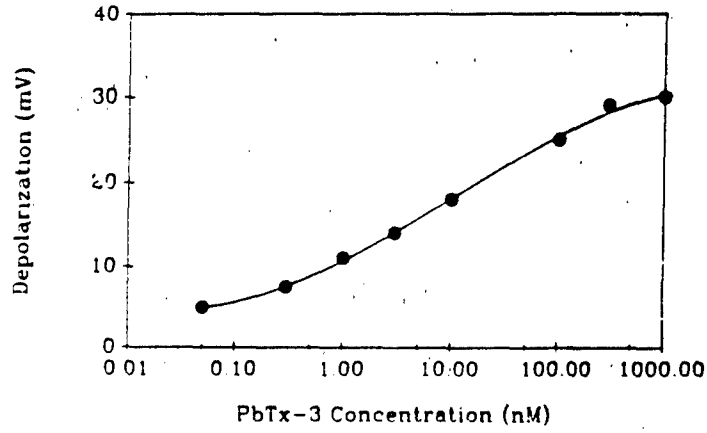

Figure 2. Dose-response curve of membrane depolarization as a function of PbTx-3 concentration. Data from a total of 22 axons were pooled; each axon received only one dose. Data are plotted as means of depolarization amplitudes. The solid line is a theoretical 3rd order fit with an IC_{50} of 1.5 nM, maximum observed depolarization of 30 mV and a Hill's coefficient of 2 (7).

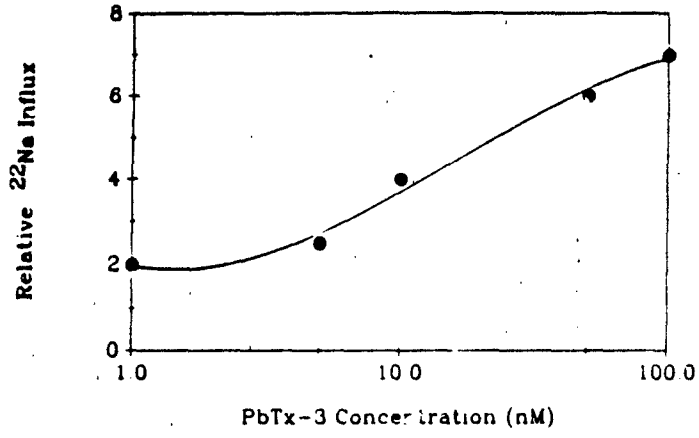

Figure 3. Concentration dependence of the stimulation of $^{22}Na^{+}$ influx by PbTx-3. Synaptosomes were pre-incubated for 30 min with indicated concentrations of PbTx-3 in the presence of aconitine. Influx is plotted as specific influx, points representing means of triplicate determinations (4).

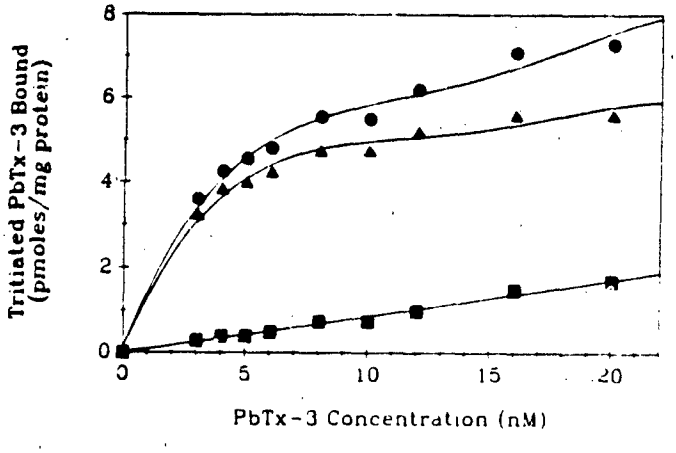

Figure 4. Binding was measured in rat brain synaptosomes using a rapid centrifugation technique. Total (circles), and nonspecific (squares) binding of tritiated PbTx-3 were measured, their difference representing specific binding (triangles). Rosenthal analysis yields a K_d of 2.6 nM and a B_{max} of 6.0 pmoles toxin bound/mg protein (5).

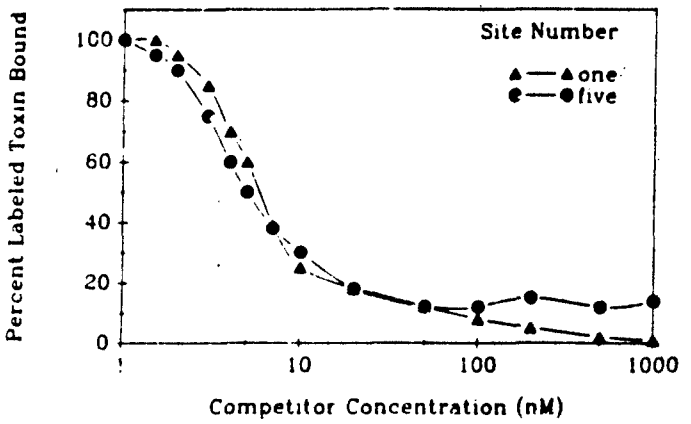

Figure 5. Comparison of specific displacement of 10 nM tritiated saxitoxin (triangles) or 10 nM tritiated PbTx-3 by unlabeled competitor saxitoxin or brevetoxin, respectively in rat brain synaptosomes. ED_{50} in each case is 5-10 nM.

IV. Technical Approach

A. Synaptosome Binding Assays

Excitable tissue preparations were obtained fresh daily from live animals using the technique described by Dodd *et al.* (12). Protein was measured on each synaptosome preparation using the Coomassie Brilliant Blue dye technique described by Bradford (13); results were expressed as "toxin bound per mg synaptosome protein".

Binding of tritiated PbTx-3 was measured using a rapid centrifugation technique. All binding experiments were conducted 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 (4). In addition, 370 mM sucrose was added to fish synaptosome experiments to maintain iso-osmolarity.

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 polypropylene microfuge tubes. After mixing and incubating at the desired temperatures for 1 hr, samples were centrifuged at 15,000 x *g* for 2 min. Supernatant solutions were sampled for the measurement of free toxin concentrations, and the remainder was aspirated in each case. Pelleted synaptosomes were rapidly washed with 4 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. Pellets were transferred to liquid scintillation vials containing 3 mL of liquid scintillant, and the bound radioactivity was measured using

liquid scintillation techniques. Nonspecific binding was measured in the presence of a saturating concentration of unlabeled PbTx-3 and was subtracted from total binding to yield specific binding.

B. Photoaffinity Probes

As we described in the Year 2 Annual Report, we have succeeded in producing two photoaffinity probes which can be covalently attached to toxin PbTx-3. These two compounds are: PbTx-3-*p*-azidobenzoyl ester (figure 6, left), and PbTx-3-*p*-hydroxyphenyl-3-*p*-azidophenyl propionyl ethylene diamine monoamide (figure 6, right). The former compound should be amenable to tritiation of the PbTx-3 moiety prior to coupling; the latter compound is amenable to pre-tritium labeling of the toxin moiety or post-radioactive iodine labeling in the ring of the photoaffinity portion of the coupled probe. Both compounds possess inhibition constants for displacement of tritium labeled PbTx-3 from its specific binding site in rat brain synaptosomes of 3.09 and 10.3 nM, respectively. Thus, we conclude they bind to Site 5 with sufficient affinity to be of use to us in identifying the brevetoxin binding component (14,15). Our principal task this year has been to stabilize the preparations.

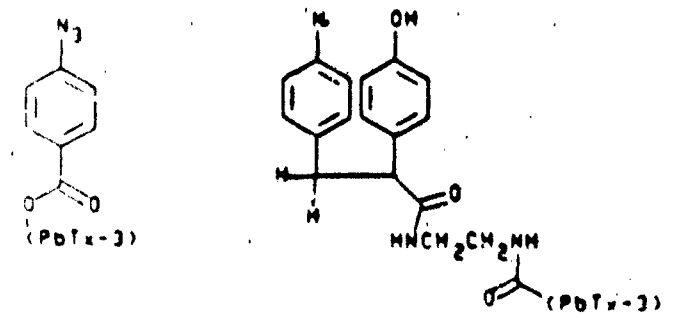

Figure 6. Photoaffinity Probes Constructed Using Brevetoxin PbTx-3. PbTx-3-*p*-azidobenzoyl ester (left) and PbTx-3-*p*-azidophenyl propionyl ethylene diamine monoamide (right).

C. Affinity Columns

For purposes of producing immobilized brevetoxin for future Site 5 purification, AH-Sepharose 4B (Pharmacia LKB) was chosen as affinity matrix. AH-Sepharose 4B has free amino groups at the end of a 6-carbon spacer arm for coupling ligands containing free carboxyl groups. For purpose of coupling, PbTx-3 succinate was prepared using succinic anhydride and PbTx-3 as previously described (16).

PbTx-3-succinate and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, hydrochloride were mixed together at a 1:10 molar ratio in minimal 50% aqueous pyridine and the solution was stirred at room temperature for 2 hours. The toxin-succinate ligand solution was then added in a 10-fold molar excess (with respect to the spacer arms on the gel) to an acidic (pH 4.5-6.0) suspension of AH-Sepharose 4B. The suspension was allowed to react at room temperature for 36 hrs (with gentle swirling to prevent damage to the sepharose bead solid support), after which time the matrix was loaded into a small column. Twelve column volumes of 0.1 M phosphate buffer was used to wash the column and remove unreacted

toxin succinate, carbodiimide and pyridine. The column was stored in phosphate buffered saline containing 0.1% sodium azide.

ification of Binding Site

.pilization. For purposes of Site 5 purification from rat brain synaptosomes, it was necessary to solubilize the membrane bound protein with detergent. Frozen synaptosomes in 5 mL synaptosome binding medium (15)(-80°C) from a single rat brain were thawed and centrifuged at 130,000 x *g* for 35 minutes. The pellet was resuspended in 2.5 mL of a solubilization medium consisting of: 100 mM choline chloride, 20 mM HEPES (pH 7.4), 0.05% egg phosphatidyl choline, 0.1 mM PMSF, 1 mM iodoacetamide, 0.001 mM pepstatin A, and 1 mM o-phenanthroline.Over a period of 20 minutes, 0.25 mL aliquots of 4% Triton X-100 were added until 2.5 mL total detergent had been added. The resulting suspension containing solubilized membrane components was centrifuged at 150,000 x *g* for 40 minutes, and the supernatant solution was saved. A portion was examined for specific binding activity using tritiated PbTx-3. To the remainder of the supernatant solution was added 10mM final concentration of calcium chloride.

Column Chromatography. Sephacryl S-300 was packed in a 1 cm x 37 cm column and was washed with two bed volumes of mobile phase consisting of: 0.1% Triton X-100, 0.02% egg phosphatidyl choline, 50 mM choline chloride, 10 mM HEPES, 10 mM calcium chloride, 0.02% sodium azide, and the protease inhibitors at concentrations used for solubilization. The flow rate was adjusted to 2 psi, and the column was standardized. Solubilized synaptosomes were loaded on the column and fractions were collected from void volume to total volume. Bradford protein and specific brevetoxin binding activity was assessed in each fraction using the binding protocol established for brevetoxin radioimmunoassays (16), another solubilized specific brevetoxin binding component. The fractions which bind tritiated PbTx-3 in a specific manner will be pooled and subjected to purification by brevetoxin affinity column chromatography using the column matrix described in the previous section. This work will likely occur in the time span between the submission of this annual report and the termination date of the contract, 15 November 1988.

E. New Toxin Probes

Three new tritiated brevetoxin probes have been synthesized using procedures employed for the reductive tritiation of PbTx-3 from PbTx-2. Both PbTx-1 and PbTx-2 are utilized as precursor toxins, and each is reduced using sodium borotritiide under identical reaction conditions. This procedure was described in our annual report last year. Following reduction in acetonitrile solution, excess acetone was added to degrade unused borotritiide. The solvent and propanol were evaporated, and the residue subjected to thin-layer chromatgraphy on silica gel utilizing ethyl acetate/petroleum ether (70/30) as solvent system, followed by high pressure liquid chromatogrpahy using an isocratic elution (1.4 mL/min) solvent of 85% aqueous methanol and monitoring absorbance at 215 nm. Single and double reduction products are generated from each of the precursor toxins. PbTx-1 reduction

results in PbTx-7 (see figure 1, specific activity 25% of the borotritiide employed) and PbTx-7 peak II (α-methylene reduced in addition to the aldehyde function, specific activity 50% of the borotritiide employed). PbTx-2 reduction results in PbTx-3 (see figure 1, specific activity 25% of the borotritiide employed) and PbTx-9 (α-methylene reduced in addition to the aldehyde function, specific activity 50% of the borotritiide employed). Both labeled PbTx-3 and PbTx-9 have long shelf lives (3-6 months) in 85% aqueous methanol in the freezer; PbTx-1 reduction products decompose with a half-life of days-weeks.

V. Results

A. Synaptosome Preparations

Synaptosomes from rats, turtles, or fish are prepared in approximately the same manner with some minor exceptions. The method of Dodd *et al.* (12) works well for each species. Turtle synaptosomes are slightly more dense than are rat synaptosomes, and are collected at a 0.32 M/1.2 M sucrose interface following ultracentrifugation. Fish synaptosomes require the addition of 370 mM sucrose during ultracentrifugation and during experiments to maintain iso-osmolarity with fish serum. In contrast to turtle and rat synaptosomes, fish synaptosomes do not demonstrate increased specific binding beyond the P2 step during purification.

B. Dissociation Constants and Binding Maxima

Comparison of Rats, Turtles, and Fish. Brevetoxins bind with high specificity to synaptosomes of fish (*Tilapia* sp.), turtles (*P. scripta*), and rats (Table I). In all cases, the K_d was in the nanomolar concentration range, and B_{max} was in the pMol/mg protein range. Specific binding was 80-90% at the dissociation constant concentration in each case. These findings illustrate the general phylogenetic topographic homology of the brevetoxin binding site, and also illustrates that binding maxima increase from fish to turtles and rats.

Derivative brevetoxins. In rat synaptosomes, six of the eight known brevetoxins displace tritiated PbTx-3 from its specific binding site (Figure 7). At 10 nM tritiated PbTx-3, IC_{50} values for the brevetoxins are range from 3.5 to 20 nM in the rat system (Table II). Only four brevetoxins could be isolated in sufficient quantities from laboratory cultures, or through chemical modification of another natural toxin, to permit classical competition binding studies (Figure 8). These four toxins are PbTx-2 and PbTx-3, each based on type-1 structural brevetoxin backbone (Figure 1); and PbTx-1 and PbTx-7, toxins based on type-2 structural brevetoxin backbone (Figure 1) with equivalent substituent derivatization to PbTx-2 and PbTx-3 distal to the lactone functionality. Tritiated PbTx-3 is competitively displaced by unlabeled brevetoxins, with K_i values determined by classical graphical methods, ranging from 1.4 to 9.9 nM (5) (Figure 9).

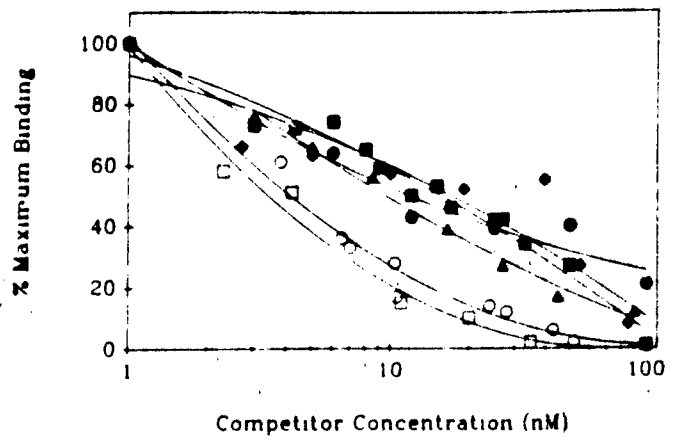

Figure 7. Specific displacement of tritiated PbTx-3 by unlabeled brevetoxins. Incubations, in the presence of 50 ug synaptosomal protein and 10 nM tritiated PbTx-3 with increasing amounts of unlabeled PbTx-1 (□), PbTx-2 (■), PbTx-3 (●), PbTx-5 (▲), PbTx-6 (◆) or PbTx-7 (○), were for one hour at 4°C. Each point represents the mean of three triplicates.

Regardless of the organism used for synaptosomal preparations, it is apparent to us that the topographic characteristics of the brevetoxin binding site on the VSSC are comparable. Using brevetoxins PbTx-1, -2, and -3, IC_{50} data for specific displacement of tritiated PbTx-3 shows comparable data in each case. The more hydrophobic type-2 brevetoxins are most efficacious in their ability to compete for site 5 binding. It is of interest to note that ciguatoxin is thought to resemble brevetoxin-A (14).

Table I. Comparison of Dissociation Constant (K_d) and Binding Maximum (B_{max}) in Fish, Turtles, and Rats*

Species	K_d (nM)	B_{max} (pMol/mg Protein)	Temp. Optimum (°C)	Specific Binding at K_d
Fish	6.1	1.40	23	80%
Turtle	1.5	2.25	4	80%
Rat	2.6	6.80	4	90%

*mean values for K_d and B_{max}, n=9, 4, 6 for fish, turtles, and rats respectively.

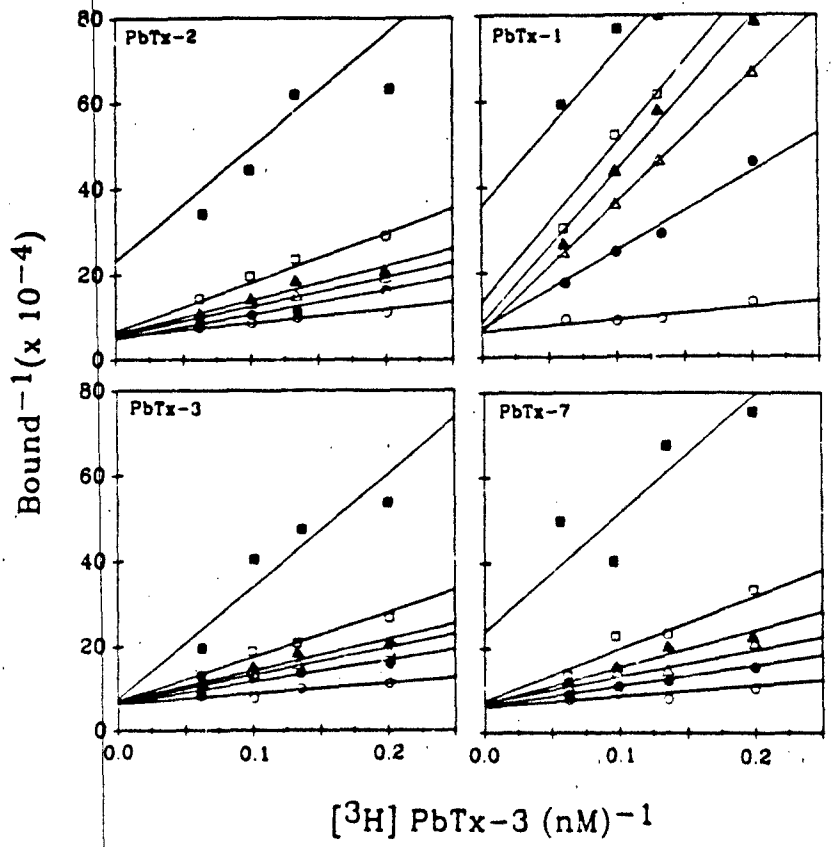

Figure 8. Inhibition of [^{3}H] PbTx-3 specific binding by unlabeled brevetoxins. Specific binding of tritiated brevetoxin PbTx-3 was measured to intact rat brain synaptosomes at 4°C in standard binding medium at four different concentrations of labeled toxins-- 5.0, 7.5, 10.0, and 15.0 nM-- in the presence of unlabeled brevetoxins at 0 (○), 5.0 (●), 7.5 (△), 10.0 (▲), 25.0 (□), 50.0 (■), or 100.0 (data not shown) nM. Each concentration point was determined in triplicate. The results are representative of three separate experiments. All competition experiments were performed on a single pooled brain syraptosome preparation, three separate preparations utilized to obtain the three experimental replicates.

Table II. Specific Displacement of [^{3}H] PbTx-3 from Synaptosome Binding by Unlabeled Brevetoxins, Comparison with LD_{50}

Toxin	Competitor Toxin Concentration (nM)			
	Turtle (1) (IC_{50})	Fish (2) (IC_{50})	Rat (3) (IC_{50})	Fish (LD_{50})
PbTx-1	3.0	30	3.5	4.4
PbTx-2	10.3	70	17.0	21.8
PbTx-3	15.0	110	12.0	10.9
PbTx-5	----	---	13.0	42.5
PbTx-6	----	---	32.0	35.0
PbTx-7	----	---	4.1	4.9

Tritiated toxin concentrations were (1) 10.0 nM, (2) 12.0 nM, and (3) 10.0 nM.

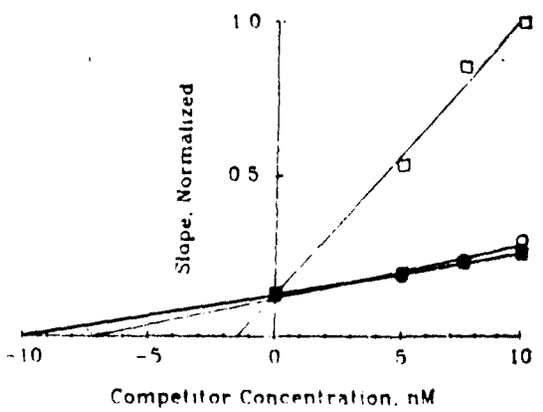

Figure 9. The inhibition of [^{3}H]PbTx-3 binding by four brevetoxins: K_i determinations. Inhibition constants for each of brevetoxins -1 (□), -2 (■), -3 (●), and -7 (○) were determined by utilizing tritium-labeled PbTx-3 concentrations of 5.0, 7.5, 10.0, and 15.0 nM, and competitor brevetoxin concentrations ranging from 0, 5.0, 7.5, and 10 nM at each labeled toxin concentration. The slopes of the appropriate lines in Figure 8 were determined, and each slope was normalized to the slope of PbTx-1 inhibition at 10.0 nM.

Implication of binding experiments. In rat synaptosomes with normal membrane potential, we investigated, using a graphical method, the individual abilities of brevetoxin-1, -2, -3, and -7 to specifically displace tritiated PbTx-3 from its binding site. Brevetoxins -1, -2, -

3, and -7 each displace tritiated PbTx-3 from its specific binding site in a competitive manner within concentration constraints of their physiological action (Fig 8); double reciprocal plots of competitor concentration versus specific binding reveal families of lines which intersect at the inverse-bound axis (11). Depending on the specific toxin used in competition studies, inhibition character became non-competitive in nature beyond 25-100 nM competitor concentration. This noncompetitive data is presented only to illustrate that regressed lines do not intersect at the inverse bound toxin axis, and the derived slopes of these regressed lines have considerable uncertainty associated with them.

Regression analysis and calculation of inhibition constants for the competitive portions of each displacement experiment confirm our original hypothesis that the more potent brevetoxins PbTx-1 (K_i= 1.4 nM) and PbTx-7 (K_i= 7.1 nM) must bind with higher affinity to site 5 than do either PbTx-2 (K_i= 9.6 nM) or PbTx-3 (K_i= 9.9 nM) (Fig 9).

Analysis of IC_{50} values revealed no marked differences in the displacing abilities between any of the type-1 toxins, and similarly there was no apparent difference between displacing abilities of PbTx-1 or -7, both type-2 toxins. Although some specific details require correlation, a gross comparison indicates that sodium channels in brain are similar in the systems examined. In the system studied most extensively, the rat brain synaptosome, t-test analysis revealed no significant differences between PbTx-2 and PbTx-3 IC_{50}, or between PbTx-1 and PbTx-7 IC_{50}, but statistically significant differences were found between the two classes ($P<0.01$) (5). If the Cheng-Prusoff equation (15) is applied:

$$K_i = IC_{50} / (1+C/K_d)$$

where K_i = the inhibition constant, IC_{50} = the inhibitory concentration of competitor toxin required for 50% specific displacement of radioactive toxin, C = the concentration of radioactive toxin, and K_d = the dissociation constant of the radioactive toxin, then relative affinities of the toxins for the various species receptors can be determined (Table III). Determination of inhibition constants by this equation, however, requires that the compounds of interest interact with only a single receptor subclass. In other words, the inhibition must be clearly and solely competitive in nature. Also implicit is the use of radioactive toxin concentrations at or near the K_d, which necessitates use of high specific activity radioactive toxin (at or above 10 Ci/mmole). Work in progress indicates that all type-1 brevetoxins inhibit tritiated PbTx-3 binding in a purely competitive manner, whereas the type-2 brevetoxins inhibit in a mixed competition manner at higher concentrations (Figure 8).

The comparison of fish bioassays (6) with the calculated effective doses indicate that the two most potent brevetoxin, i.e. PbTx-1 and PbTx-7, also are most effective at displacing tritiated probe from its specific site of action. The considerably lower potency of brevetoxins PbTx-5 and PbTx-6 in the rat system suggest that these two toxins may bind with lesser affinity to Site 5. In a general sense, this is indicated in Table II, and is summarized in Table III.

Table III. Inhibition Constants for Derivative Brevetoxins Derived from the Cheng-Prusoff* Equation

Toxin	K_i (nM) Turtle	Fish	Rat
PbTx-1	0.39	10.10	0.72
PbTx-2	1.34	23.57	3.51
PbTx-3	1.96	37.04	2.47
PbTx-5	----	----	2.68
PbTx-6	----	----	6.60
PbTx-7	----	----	0.85

*see discussion concerning the limits of applicability for this treatment.

The affinities of each toxin, in each test system, are presumably based on structural considerations: the portion of the toxin molecule which binds to the specific site on the sodium channel must retain sufficient structural integrity to permit binding. Obviously those toxins which are altered sufficiently so they no longer bind to the site, are no longer toxic. This conjecture is supported by past work which indicated that oxidation of the C-42 aldehyde of PbTx-2 to the corresponding carboxylic acid reduced both potency and affinity for the site (11), and that opening of the lactone in ring A destroys all activity (6). Thus, detailed studies of derivative brevetoxins based on the type-1 backbone may lead to increased understanding of the three-dimensional site specificity of brevetoxin binding. Of significance is the demonstrated increased potency of the type-2 toxins PbTx-1 and PbTx-7 (2,5,8) and their increased affinity for the site associated with sodium channels (5). The larger 8- and 9-membered rings (D,E,F) of these toxins may confer a greater binding affinity to synaptosomes. This increased affinity may be a function of increased flexibility of the type-2 backbone (about a 40o bending capability, 14) over the type-1 backbone. The added flexibility and its enhanced propensity to conform to the topography of the channel is an area of potential importance (Figure 1).

Effects of Membrane Integrity. Binding maxima for intact synaptosomes reported herein are slightly lower than the binding maximum of 6.9 pMol/mg protein reported earlier, a difference attributable to different preparations of synaptosomes and individual variations in purity (5). Within our present studies, there appears to be no significant change in K_d or B_{max} for [^{3}H] PbTx-3 binding to intact, lysed, or depolarized synaptosomes (Fig 10). The derived K_d's are 2.6 (intact), 2.9 (lysed), and 3.3 (depolarized) nM, and B_{max}'s are 6.01, 5.83, and 5.75 pMol bound/mg synaptosomal protein, respectively. At fixed 12.5 nM [^{3}H] PbTx-3 concentration, step-wise depolarization experiments utilizing intact synaptosomes revealed no significant change in apparent specific binding with increasing potassium chloride concentration (Fig 11). These results indicate that the binding of PbTx-3 to its specific site is essentially membrane potential independent.

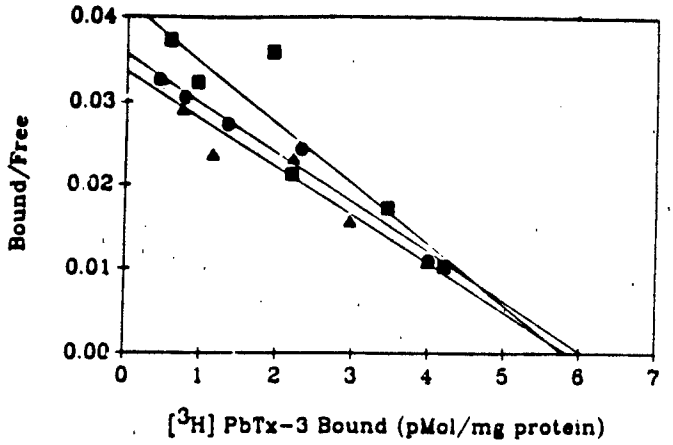

Figure 10. Rosnethal analysis of specific [^{3}H] PbTx-3 binding to intact (●), lysed (▲), and depolarized (■) synaptosomes. Binding was measured at 4°C in rat brain synaptosomes or lysed synaptosomes according to protocols explained in the text. These results are representative of four separate experiments, each done in triplicate.

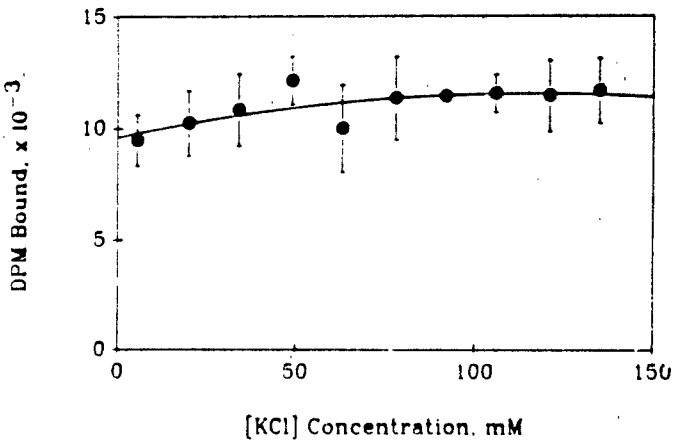

Figure 11. Voltage dependence of binding of [^{3}H]PbTx-3 to synaptosomes was measured at 4°C at the indicated concentrations of potassium ion, as described in exerimental. KCl was exchanged for choline chloride to maintain iso-osmolarity. The membrane potential varies from -55 mV in stnadard binding medium to 0 mV at 135 mM KCl. The figure represents a composite of four experiments, triplicate determination in each. Error bars represent 95% confidence limits.

Photoaffinity Probes. As mentioned earlier, each PbTx-3 photoaffinity probe possesses an inhibition constant in the nanomolar concentration range, 3.09 nM for the *p*-azidobenzoyl derivative, and 10.3 nM for the *p*-azidophenyl propionyl ehtylene diamine monoamide derivative (see Figure 6). In the latter case, we believe we have produced a toxin derivative with solubility characteristics close to unaltered PbTx-3, and hence will be of greater use in identifying the binding component in intact synaptosomes. A minor problem was encountered in producing high yield products from the toxin coupling to affinity probe, however, in subsequent experiments. This was solved by protecting the phenolic group on the photoaffinity portion of the molecule to prevent self-condensation (the coupling function on the toxin PbTx-3 being an alcohol, also). Protecting with a cyclohxyl functionality, with subsequent cleavage after brevetoxin condensation, appears to provide the solution. These reactions are currently being evaluated, and products are being spectroscopically analyzed by FT-IR to provide the evidence for structure integrity and indentity. We expect those studies to be complete by 15 Novmeber 1988, the conclusion of this contract.

C. New Radioactive Brevetoxins

Preliminary work reported herein indicates that tritium labeled PbTx-7 (reduced PbTx-1), PbTx-9 (doubly reduced PbTx-2), and PbTx-10 (doubly reduced PbTx-1) also interact with Site 5 associated with the VSSC, with dissociation constants and binding maxima consistent with the data presented for tritiated PbTx-3 (Table 4). Huang *et al.* (7) suggested that the brevetoxin binding site lies in the hydrophobic portion of the channel, and since PbTx-1 and PbTx-7 are also the most hydrophobic of the toxins, their potency may also be in part due to solubility considerations. In general, the more hydrophobic the toxin, the higher is its potency and ability to displace tritiated PbTx-3 from its specific binding site. It is our contention that substituent character on each toxin's K (type-1) or J (type-2) ring determines solubility and hence access to the site. The A-ring in each toxin backbone carries the active portion of the toxin. It is clear to us that both solubility and flexibility characteristics confer differential potency and binding abilities to individual toxins. We feel that naturally-occurring toxins, as well as synthetic derivatives, will aid in our understanding of the site-specific potency of toxin from P. brevis. Our principal aim is to synthesize derivative toxins with discrete alterations at specific sites without changing their lipid solubiities and hence their access to site 5 associated with voltage-sensitive sodium channels. A current problem pertains to the PbTx-1 reduction products, and their apparent short half-lives in aqueous solution. According to Shimizu (personal communication) these compounds decompose by an internal rearrangement and elimination reaction of the side group linked to ring J (see Figure 1). This results in a detoxification, and a loss of the tritium atom introduced. The toxins can be stabilized in aprotic solvent. These latter two materials (i.e. PbTx-7 and PbTx-10) are still under development. PbTx-9 however, is as stable as PbTx-3 and is currently available at twice the maximum specific activity of PbTx-3.

Table 4. Comparison of K_d and B_{max} for Four Different Tritiated Brevetoxin Probes

Toxin	K_d (nM)	B_{max} (pmoles/mg protein)
PbTx-3	2.13	6.99
PbTx-9	8.76	6.75
PbTx-7	1.91	6.38
PbTx-10	1.56	6.46

D. Brevetoxin Affinity Columns

The results of coupling reactions indicate that we were successful in coupling PbTx-3 to AH Sepharose 4B. Using a small amount of tritiated PbTx-3 in the reaction mix as tracer, we calculate about a 25% efficiency in coupling. We are currently attempting to improve on efficiency, and expect to have most of the shortcomings worked out by 15 November 1988.

Preliminary results employing the afifnity column deal solely with brevetoxin specific antibodies as specific binding model system. The results indicate that we can pass crude IgG solutions through the column, wash with excess phosphate buffer (until no protein is detected in eluent) and then simply strip brevetoxin-specific antibodies from the column with 3 M NaCl solution. We feel the coupling is a success and that the affinity column feasibility has been demonstrated.

E. Purification of the Brevetoxin Binding Site

We have successed in demonstrating that solubilized membrane protein from rat brain synaptosomes can be separated on Sephacryl A-300 columns (size fractionation). The results of a representative separation are illustrated in Figure 12. Of the fractions collected, significant specific binding of tritiated brevetoxin PbTx-3 was seen in fractions 30-37. Similarly, significant amounts of protein were demonstrated in the same fractions. The size to which this large protein peak corresponds is in the 230,000 to 350,000 dalton molecular weight range. This suggests that the brevetoxin binding component interacts with the α-subunit of voltage-sensitive sodium channels in rat brain synaptosomes. There is little specific brevetoxin binding in any other smaller molecular weight range, further illustrating that brevetoxins do not likely interact with either of the β-subunits. This finding, coupled with our already known facts about specific brevetoxin binding being retained in solubilized sodium channel, gives us an excellent potential for isolating and identifying the brevetoxin binding component in excitable tissues.

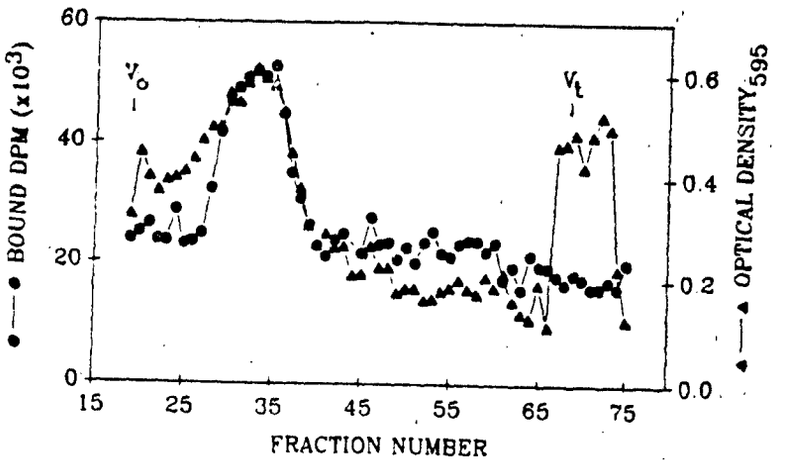

Figure 12. Demonstration of specific tritiated brevetoxin binding in Sephacryl A-300 fractions of solubilized rat brain synaptosomes. Membrane proteins were solubilized using detergents in the presence of protease inhibitors. Solubilized proteins were chromatographed on a Sephacryl A-300 size partition column. Eluted fractions were assayed for both specific brevetoxin binding (circles) and protein (triangles).

VI. Conclusions

All of the toxins isolated from the marine dinoflagellate *Ptychodiscus brevis* interact with Site 5 associated with the voltage-sensitive sodium channel. The binding characteristics of the site are present and similar in rats, fish, and turtles. Binding initiates a concentration-dependent membrane depolarization, which can be measured by microelectrode or $^{22}Na^{+}$ influx. The stoichiometry of binding appears to be 1:1 with saxitoxin; with K_d in the 2-3 nM concentration range and B_{max} in the 6-7 pmole/mg protein range. Binding is membrane potential independent. All naturally occurring brevetoxins, derivatized brevetoxins, and photoaffinity probe brevetoxins displace tritiated brevetoxin, with K_i in the 0.5-50 nM concentration range. More potent brevetoxins displace with greater efficiency. Both tritiated reduction products of PbTx-2 (i.e. PbTx-3 and PbTx-9) and PbTx-1 (i.e. PbTx-7 and PbTx-10) bind with nearly identical B_{max}, and PbTx-1 reduction products possess a greater affinity for the binding site. The brevetoxin binding site retains its specific binding capabilities upon detergent solubilization, can be purified using a Sephacryl S-300 size fractionation column, and is a protein of approximate molecular weight comparable to that of the α-subunit of the sodium channel. Solid-support linked brevetoxin PbTx-3 is capable of binding with high avidity, and releasing upon high salt denaturation, specific brevetoxin binding components.

VII. Recommendations

This current contract expires on 15 November 1988. Certain unfinished experiments are being completed:

(1) complete the evaluation of the PbTx-3 affinity column including specific binding capacity;

(2) complete examination of photoaffinity probe PbTx-3 synthesis;

(3) stabilize PbTx-1 radioactive reduction products;

(4) investigate PbTx-1 photoaffinity probes.

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