Biochimica et Biophysica Acta, 732 (1983) 160–169
Elsevier

BBA 71727

STRUCTURE AND ACTION OF HETERONEMERTINE POLYPEPTIDE TOXINS

BINDING OF *CEREBRATULUS LACTEUS* TOXIN B-IV TO AXON MEMBRANE VESICLES

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(Received October 7th, 1982)
(Revised manuscript received April 13th, 1983)

Key words: Neurotoxin; Membrane-toxin interaction; Toxin B-IV; (Axon membrane)

The binding of the crustacean selective protein neurotoxin, toxin B-IV, from the nemertine *Cerebratulus lacteus* to lobster axonal vesicles has been studied. A highly radioactive, pharmacologically active derivative of toxin B-IV has been prepared by reaction with Bolton-Hunter reagent. Saturation binding and competition of ^{125}I-labeled toxin B-IV by native toxin B-IV have shown specific binding of ^{125}I-labeled toxin B-IV to a single class of binding sites with a dissociation constant of 5–20 nM and a binding site capacity, corrected for vesicle sidedness, of 6–9 pmol per mg membrane protein. This compares to a value of 3.8 pmol [^{3}H]saxitoxin bound per mg in the same tissue. Analysis of the kinetics of toxin B-IV association ($k_{+1} = 7.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$) and dissociation ($k_{-1} = 2 \cdot 10^{-3}$ s^{-1}) shows a nearly identical K_d of about 3 nM. There is no competition of toxin B-IV binding by purified toxin from *Leiurus quinquestriatus* venom while *Centruroides sculpturatus* Ewing toxin I appears to cause a small enhancement of toxin B-IV binding.

Introduction

Cerebratulus lacteus, a large heteronemertine found along the northeast coast of the United States, produces a family of four structurally homologous, crustacean-selective, polypeptide neurotoxins which induced spontaneous, repetitive action potentials in crab and lobster walking leg nerves. At higher concentrations, these toxins cause crustacean axons to become inexcitable (Refs. 1–3 and Kem, W.R., personal communication). These activities suggest that the *Cerebratulus* B-toxins interact with some portion of the action potential-generating ion permeability control system. The B-toxins are highly basic miniproteins of known covalent structure [4] having molecular weights of about 6000. Previous studies of toxin B-IV have resulted in assignment of essential tyrosine [5] and tryptophan [6] residues in the amino acid sequence [4] of the toxin. Our recent interest has been to investigate the binding interaction of toxin B-IV with its receptor in crustacean nerves.

Vesicles of the plasma membrane of lobster nerve have been shown to exhibit Na^+ fluxes that are activated by veratridine and are blocked by tetrodotoxin [7,8]. Saturable, high affinity binding of tritium-labeled tetrodotoxin to crab [9] and lobster [10] axonal membrane has also been reported. However, only limited study of the binding of polypeptide neurotoxins to crustacean nerve membranes has as yet been done [11]. In this paper, we describe preparation of a labeled derivative of *Cerebratulus lacteus* toxin B-IV and char-

Abbreviations: Hepes, 4-(2-hydroxymethyl)-1-piperazine-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

acterization of its binding to lobster axonal membrane vesicles.

A preliminary report of a portion of these data has appeared [12]. Also included in this article are data from a thesis by G.P.T. submitted in partial fulfillment of the requirement for the degree of Ph.D., University of Cincinnati.

Experimental procedures

Materials. Chemicals were obtained from the following sources: ^{125}I-Bolton-Hunter reagent (2200 Ci/mmol) was purchased from Amersham Corp. *N*-Succinimidyl-3-(4-hydroxyphenyl)propionate was obtained from Pierce. Batrachotoxin was generously provided by Dr. John Daly (Natl. Inst. of Arth., Metab., and Digestive Diseases, NIH). Purified toxin from *Leiurus quinquestriatus* venom was a generous gift of Dr. W.A. Catterall (University of Washington, Seattle, WA). [^{3}H]Saxitoxin, prepared by the specific ^3H_2O exchange technique of Ritchie et al. [32] was obtained from Dr. Raymond Bahor (Toxicology Study Section, NIH). Unlabeled saxitoxin was provided by the FDA. Live lobsters were obtained locally. *Cerebratulus* toxin B-IV was isolated as described by Kem [1]. *Centruroides sculpturatas* Ewing toxin I was purified as described by Babin et al. [13].

Chemical modification and ^{125}I-labeling of Cerebratulus toxin B-IV. The reaction of *N*-succinimidyl-3-(4-hydroxyphenyl)propionate with toxin B-IV was carried out in 0.1 M sodium borate over the pH range 6.0 to 8.5 and at a reagent to toxin excess of 5 : 1. After 30 min at 4°C, the reaction was terminated by addition of a 10-fold excess of glycine. Incorporation of reagent was monitored by the increase in absorbance of the protein at 280 nm ($\epsilon = 1200$) after exhaustive dialysis.

N-Succinimidyl-3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionate (specific radioactivity 2200 Ci/mmol) was dried from benzene under a stream of nitrogen in a Duoseal vial. Purified toxin B-IV, dissolved in 200 μl 0.1 M sodium borate, pH 6.5, was added to the vial and incubated on ice for 30 min. After this time 15 μl of 10 mM glycine, pH 6.5, was added to consume unreacted reagent. The reaction mixture was then diluted to 5 ml with 50 mM ammonium acetate at 4°C and dialyzed exhaustively against the same buffer. The concentration of the labeled protein was determined by amino acid analysis.

Purification of lobster axonal vesicles and membrane fragments. The method used was similar to the rat brain synaptosome preparation of Gray and Whittaker [14] as modified by Ray et al. [15]. Nervous tissue was dissected from walking legs, claws, tail, and mid-body of the lobster *Homarus americnus* and homogenized in ice-cold 0.3 M sucrose, 10 mM Tris, 135 mM KCl, pH 7.5 using 8 ml buffer per gram wet weight with 10 strokes of a motor-driven Teflon/glass homogenizer; all subsequent steps were as described by Ray et al. [15].

Solubilized lobster axonal vesicles were prepared as described by Hartshorne et al. [16]. The pellet obtained from the 1.0–1.2 M interface was resuspended in 0.5 ml of 100 mM choline chloride, 20 mM Hepes (adjusted to pH 7.4 with Tris base), 0.1 mM phenylmethylsulfonyl fluoride. An equal volume of 4% Triton X-100 was added in 100-μl aliquots with stirring and the resulting solution incubated for 30 min at 4°C. Following centrifugation at $165\,000 \times g$ for 30 min the supernatant was removed and adjusted to 10 mM $CaCl_2$. This preparation was used in subsequent binding studies.

Preparation of electron micrographs. Isolated membrane preparations were initially fixed with 4.0% glutaraldehyde in phosphate buffered saline for 2 h at 4°C. Following primary fixation all samples were pelleted by centrifugation at $1000 \times g$ for 30 min. The resultant pellets were washed three times with phosphate-buffered saline, post-fixed overnight in 2.0% OsO_4, and dehydrated through a graded series of ethanol/water dilutions. During the dehydration all samples were treated with 0.5% uranyl acetate in 70% ethanol to enhance contrast. After dehydration each sample was infiltrated and embedded in Epon 812 (Electron Microscope Science, Ft. Washington, PA) according to Luft [17]. Ultrathin sections (100 nm) were cut with a diamond knife (Electron Microscope Sciences), picked up on uncoated copper grids, and stained with 2% (aqueous) uranyl acetate and Reynold's lead citrate. All samples were viewed on a JEOL-100B electron microscope operating at 80 kV.

Measurement of ^{125}I-labeled toxin B-IV binding. Binding of ^{125}I-labeled toxin B-IV was measured

using a rapid filtration assay described by Ray et al. [15]. Lobster axonal vesicles or membrane fragment pellets were resuspended in binding medium consisting of 130 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.4 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mg/ml bovine serum albumin to give 1–2 mg membrane protein per ml. Binding reactions were initiated by addition of 400 μl of resuspended vesicles or membrane fragments to 1.0 ml of the binding medium containing the appropriate concentration of ^{125}I-labeled toxin B-IV. Nonspecific binding of ^{125}I-labeled toxin B-IV was determined by the inclusion of micromolar concentrations of 8 μM native toxin B-IV and subsequent samples were mixed and incubated for 15 min at 4°C. Aliquots (200 μl) were then collected on glass fiber filters (Whatman GF/C) under vacuum, washed with 20 ml of wash medium consisting of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mg/ml bovine serum albumin, and counted. Prior to filtration, the filters were prewashed with 10 ml of wash medium and 1 ml of 7.4 μM unlabeled toxin B-IV to diminish nonspecific adsorption of ^{125}I-labeled toxin B-IV to diminish nonspecific adsorption of ^{125}I-labeled toxin B-IV to the filters.

In experiments aimed at determination of the membrane potential dependence of toxin B-IV binding, the above procedure was followed exactly except that binding was measured in high K^+ (135 mM) binding medium. Competition of toxin B-IV binding by either *Leiurus* toxin or *Centruroides sculpturatus* Ewing toxin I was measured using the binding assay described above at both 4 and 37°C. The binding medium contained 8 nM ^{125}I-labeled toxin B-IV and the appropriate concentration of *Leiurus* or *Centruroides* toxin.

Measurement of [^{3}H]saxitoxin binding. The concentration of [^{3}H]saxitoxin was determined by its ability to inhibit batrachotoxin-stimulated $^{22}Na^+$ uptake by neuroblastoma cells relative to that of FDA standard saxitoxin as described previously [18]. The specific radioactivity was determined according to a modified procedure of Baumgold [19] to be 4.4 dpm per fmol (23% radiochemical purity). Binding of [^{3}H]saxitoxin to lobster axonal vesicles was measured using a rapid filtration assay. Following addition of vesicles to binding medium containing [^{3}H]saxitoxin, the mixture was incubated at 4°C for 20 min followed by filtration of aliquots on glass fiber filters under vacuum. Nonspecific biding of [^{3}H]saxitoxin was measured in the presence of 2 μM unlabeled saxitoxin.

Binding of [^{3}H]saxitoxin to solubilized axonal vesicles was measured by a rapid ion-exchange chromatography assay since free saxitoxin (a divalent cation) is bound to a cation exchange column, whereas the saxitoxin-receptor complex is not retained [20]. One milliliter columns of Dowex 50 × 2-200 were washed successively with 2 ml of deionized water and 2 ml of 10 mM Hepes (adjusted to pH 7.4 with Tris base), containing 50 mM choline chloride 0.1% Triton X-100, 0.02% egg phosphatidylcholine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM $CaCl_2$. Solubilized vesicles (400 μl) prepared as described above were incubated for 20 min at 4°C in 1.6 ml binding medium consisting of 50 mM choline chloride, 10 mM Hepes (adjusted to pH 7.4 with Tris base), 0.1 mM PMSF, 0.04% egg phosphatidylcholine, 10 mM $CaCl_2$ and 20 nM [^{3}H]saxitoxin. Aliquots (300 μl) of this mixture were then loaded onto Dowex columns and the sample eluted by forcing 1 ml of the above column equilibration buffer through the column with a syringe. The collected eluate was measured by radioactivity.

Protein and acetylcholinesterase assays. Proteins were determined by the method of Lowry et al. [21] and acetylcholinesterase activity (EC 3.1.1.7) as described by Ellman et al. [22].

Results

Chemical modification of toxin B-IV

In order to study the interaction of *Cerebratulus* toxin B-IV with nervous tissue from the lobster *Homarus americanus*, preparation of a highly radioactive, pharmacologically active derivative was necessary. Due to the known involvement of a toxin B-IV tyrosine residue in biological activity [5], an alternative site of iodination was sought. We ultimately chose the iodinated derivative of *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (Bolton-Hunter reagent), a reagent known to react selectively with amino groups [23] and which has been successfully employed in receptor binding

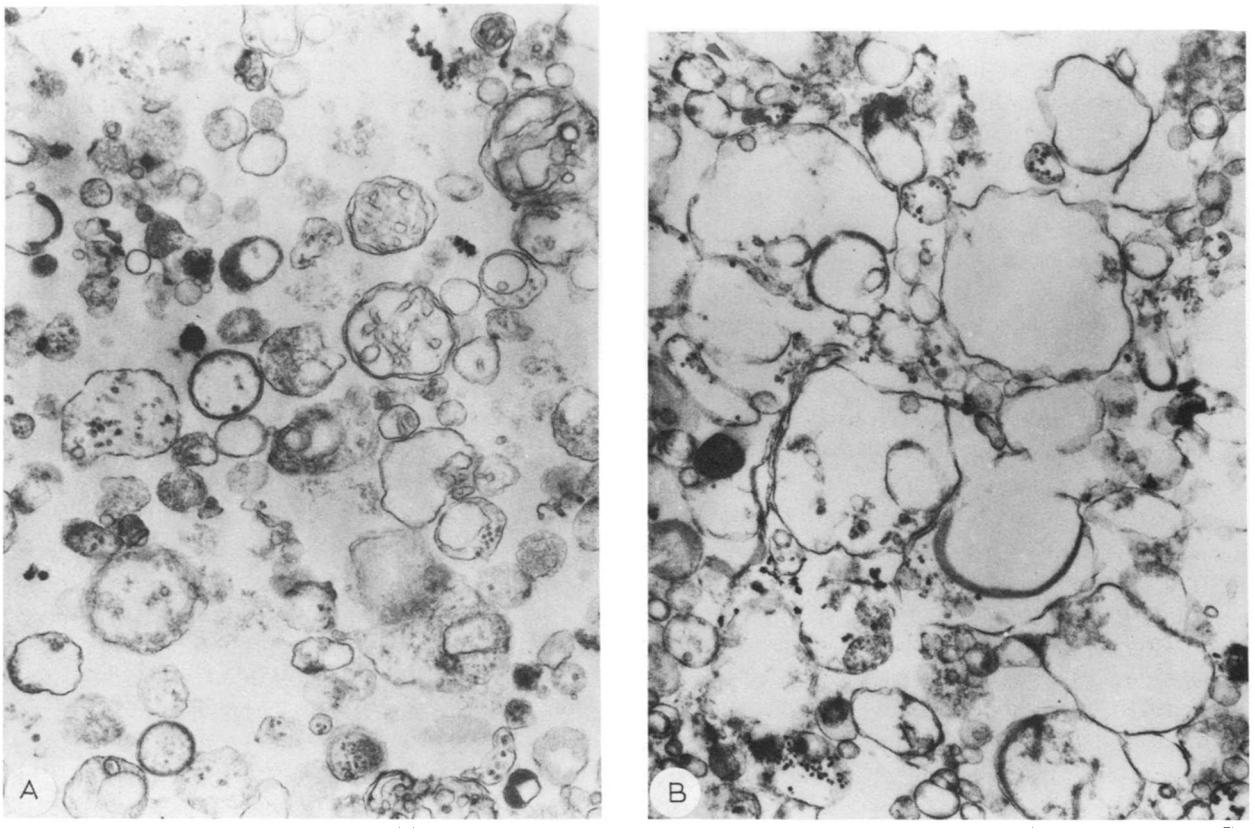

Fig. 1. Electron micrograph of membranes from lobster nerves. Membranes isolated from sucrose gradient interfacial bands were pelleted at $40000 \times g$ and prepared for electron microscopy as described in Experimental Procedures. Magnification: ×45000. (A) Membranes from 1.0–1.2 M sucrose gradient interfacial band. (B) Membranes from 0.6–0.8 M sucrose gradient interfacial band.

studies with scorpion toxin [24] and calmodulin [25].

Since toxin B-IV contains eleven amino groups per molecule, a major problem was to limit the extent of reaction. Thus, the effects of pH and reagent excess on the extent of incorporation of Bolton-Hunter reagent into toxin B-IV were studied. Reaction was carried out in 0.1 M sodium borate buffer at a range of pH from 6.0 to 8.5 and a reagent to toxin excess of 5 : 1. Incorporation of reagent was monitored by the increase in absorbance at 280 nm ($\epsilon = 1200$) after desalting. The incorporation of the 4-hydroxyphenylpropionate moiety into B-IV varied as a function of pH. At pH 6.4, 1.0–1.3 moles were incorporated per mole of toxin. Virtually all of this incorporation occurs at the α-amino group of toxin B-IV since the modified toxin is refractory to N-terminal analysis by dansylation or Edman degradation. Edman degradation of the modified toxin resulted in less than 0.1 mole Ala per mole toxin B-IV. Therefore, greater than 90% of the toxin was modified at the amino terminus.

When a similar modification was carried out, but under conditions of toxin excess and using ^{125}I-Bolton-Hunter reagent for the purpose of trace labeling the toxin, specific radioactivities in the range of $(2–5) \cdot 10^6$ cpm per μg toxin were routinely obtained. Following extensive dialysis of the iodinated toxin B-IV, ion-exchange chromatography of the modified toxin on CM-cellulose showed that 97% of the ^{125}I counts eluted from the resin near the same ionic strength as native toxin B-IV. This chromatographic procedure confirmed that the iodinated toxin B-IV had been freed of excess iodinated reagent.

To determine whether mono-Bolton-Hunter derivatized toxin B-IV retained pharmacologic activ-

ity, activity of the modified toxin prepared as described above under conditions of reagent excess was compared to that of native toxin using the crayfish paralytic assay described by Kem [1]. Modified toxin B-IV was 75% as active (PD_{50} = 2.5 ng per g body weight) as the native toxin (PD_{50} = 2.0 ng per g body weight). The same specific toxicity was obtained when the reagent was iodinated prior to reaction with toxin. This iodinated derivative of toxin B-IV was used for subsequent binding studies to lobster nervous tissue. It was assumed from the above bioassay data that trace labeled toxin B-IV would contain molecules of approximately equal activity.

Purification of lobster axonal membrane vesicles

Binding studies with polypeptide axonal toxins from *Leiurus* [26], *Androctonus* [27] and *Anemonia* [28] have indicated that binding of these molecules to their receptors is dependent upon membrane potential. Since membrane vesicles have been shown to be capable of maintaining a membrane potential [29], we decided to prepare axonal membrane vesicles from lobster nerves. Ultimately, a procedure similar to that described by Ray et al. [15] for the purification of rat brain synaptosomes was followed. The material at the 1.0–1.2 M interface of the discontinuous sucrose density gradient was isolated since it corresponded to the position of synaptosomes in sucrose gradient fractionation of homogenized rat brain [15]. The material in this band is enriched approximately 4-fold for acetylcholinesterase activity as compared to the original homogenate.

To confirm that the 1.0–1.2 M sucrose interfacial band did indeed contain vesicular structures, the pooled material was studied by electron microscopy. Fig. 1A, an electron micrograph of this fraction at a magnification of 45 000 ×, shows primarily sealed membrane structures. Moreover, electron micrographs of the 0.6–0.8 M interfacial band, Fig. 1b, show primarily open membranous fragments; the corresponding band in the synaptosome preparation of Ray et al. [15] is likewise largely composed of open structures. Vesicles formed in the presence of ^{125}I-labeled bovine serum albumin, prepared by lactoperoxidase -catalyzed iodination [30], retained the labeled bovine serum albumin, allowing the calculation of an intravesicular volume of 2.9 μl per mg protein (the internal volume of neuroblastoma cells is 3.3 μl per mg [26]). Also, results from subsequent toxin B-IV binding experiments with these vesicles suggested that the vesicular forms were capable of maintaining a K^+-derived membrane potential. The isolated vesicles were used for all subsequent toxin binding studies.

Binding of ^{125}I-labeled toxin B-IV

Incubation of increasing concentrations of iodinated toxin B-IV with axon membrane vesicles followed by rapid filtration allowed detection of a specific toxin B-IV binding component. The concentration dependence of this binding under steady-state conditions is shown in Fig. 2 over a wide range of ^{125}I-labeled toxin B-IV concentrations from 1.8 to 75 nM. Nonspecific binding was measured in the presence of 8 μM unlabeled toxin B-IV and has been subtracted to yield the data shown. Specific, saturable toxin B-IV binding exhibits an apparent dissociation constant of about 15 nM and a binding capacity of 1.6 pmol per mg membrane protein. Analysis of this data according to Scatchard [31], using linear regression analysis, shows a dissociation constant of 21 nM and a binding site density of 1.6 pmol per mg (Fig. 3); the linearity of the Scatchard plot indicates a single class of non-interacting binding sites.

Binding of iodinated toxin B-IV was completed by unlabeled toxin, as shown in Fig. 4. Vesicles were incubated with 8 nM ^{125}I-labeled toxin B-IV

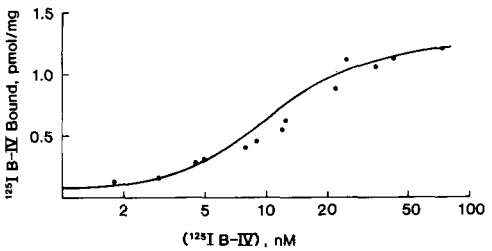

Fig. 2. Binding of ^{125}I-labeled toxin B-IV to lobster axonal vesicles. Axonal vesicles were incubated with increasing concentrations of ^{125}I-labeled B-IV from 1.8 to 75 nM for 15 min at 4°C and bound toxin B-IV was measured by the rapid filtration procedure described under Experimental Procedures. Nonspecific binding of ^{125}I-labeled toxin B-IV in the presence of 8 μM native toxin B-IV has been substracted from these results. Binding measurements at each concentration were done in sextuplicate.

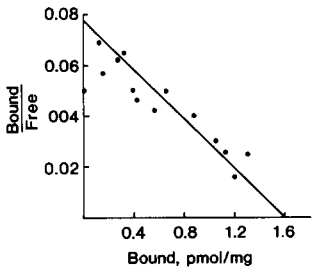

Fig. 3. Scatchard plot of specific ^{125}I-labeled toxin B-IV binding. The units for the ordinate are pmol per mg per nM.

and increasing concentrations of native toxin B-IV over the range from 0.5 to 1000 nM. A specific, saturable toxin B-IV binding component is again detected. The midpoint of the competition curve gives an apparent dissociation constant for the toxin-receptor complex of 5 nM, very similar to the value derived from the saturation binding analysis. Use of the Michaelis-Menten equation adapted for ligand binding, $b_o = B_{max}[S]/([S] + K_d)$, allowed calculation of the site density from the total binding capacity of vesicles incubated in the subsaturating concentration of ^{125}I-labeled toxin B-IV (8 nM) alone; the value obtained, 1.6 pmol per mg membrane protein, is nearly identical to that from the saturation binding experiment.

The association and dissociation kinetics of

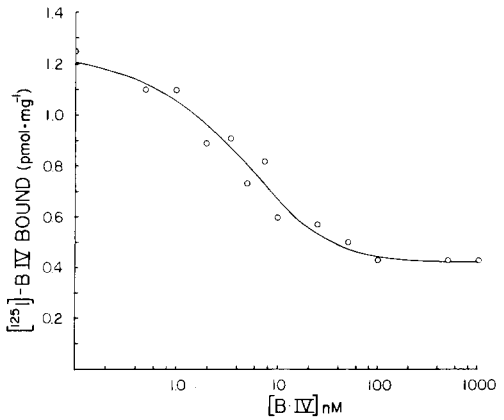

Fig. 4. Competition of ^{125}I-labeled toxin B-IV binding by unlabeled toxin B-IV. Axonal vesicles were incubated with 8 nM ^{125}I-labeled toxin B-IV and increasing concentrations of native toxin B-IV. Bound ^{125}I-labeled toxin B-IV was measured by the rapid filtration procedure described under Experimental Procedures. Binding measurements at each concentration were done in sextuplicate.

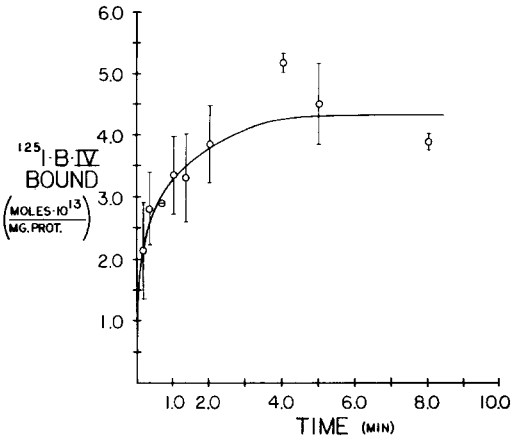

Fig. 5. Time course of association of ^{125}I-labeled toxin B-IV to lobster axonal vesicles. Following suspension of axonal vesicles in 30 nM ^{125}I-labeled toxin B-IV at time zero, in the presence and absence of excess native toxin B-IV, aliquots were filtered at the indicated times. Specific binding was determined by subtraction of nonspecific binding from total binding. Binding measurements at each time point were done in triplicate.

toxin B-IV binding were investigated to further confirm the dissociation constant determined by the above methods. Association of ^{125}I-labeled toxin B-IV was initiated by suspension of freshly isolated vesicles in 30 nM iodinated toxin B-IV at time zero in the presence and absence of micromolar concentrations of unlabeled toxin, followed by rapid filtration of aliquots of this mixture at short time intervals. Fig. 5 shows that specific association of ^{125}I-labeled toxin B-IV is complete after 5 min. To obtain the association rate constant, k_{+1}, the integrated rate equation for a reversible second-order reaction was used:

$$k_{+1}t = k_\infty \left(\frac{1}{-a_o b_o + p_\infty^2} \right) \ln \left[\frac{a_o b_o (p_\infty - p)}{p_\infty (a_o b_o - p p_\infty)} \right]$$

where a_o = initial concentration of ^{125}I-labeled toxin B-IV, b_o = initial number of binding sites calculated from $b_o = B_{max}[S]/(K_d + [S])$, $p = {}^{125}$I-labeled toxin B-IV bound at time t, $p_\infty = {}^{125}$I-labeled toxin B-IV bound at equilibrium. The slope of the plot of ω versus t yields k_{+1}. The slope of the curve in Fig. 6 gives a value for the association rate constant, k_{+1}, of $7.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$.

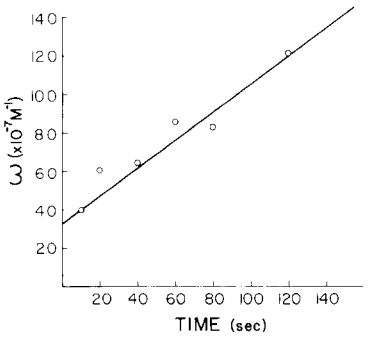

Fig. 6. Plot of the integrated rate equation for a reversible second order reaction. Data from the association of ^{125}I-labeled toxin B-IV with axonal vesicles were plotted as (as described in Results) versus time yielding a slope equal to k_{+1}.

Dissociation kinetics of the binding of ^{125}I-labeled toxin B-IV were studied by incubation of axonal vesicles with 30 nM iodinated toxin B-IV, in the presence and absence of micromolar concentrations of unlabeled toxin, followed by centrifugation of this mixture and resuspension of the vesicles in medium without labeled toxin at time zero. Aliquots were subsequently filtered at short time intervals to detect loss of toxin binding. Initial study of toxin dissociation revealed essentially full retention of the toxin binding following resuspension of the vesicles. Subsequently, dissociation was studied in the presence of micromolar unlabeled toxin B-IV. This allowed measurement of toxin B-IV dissociation with the kinetics shown in Fig. 7. The dissociation rate constant, k_{-1}, for this process is is approx. $2 \cdot 10^{-3}$ s^{-1}. The calculated binding dissociation constant, $K_d = k_{-1}/k_{+1}$ is approx. $3 \cdot 10^{-9}$ M, nearly identical to that obtained in the competition experiment. The off-rate (k_{-1}) for ^{125}I-labeled toxin B-IV binding was determined using data from the initial linear portion of the curve. A similar rate constant can be calculated from the linear curve obtained by subtracting the 40% residual binding observed at 80 min from the data in Fig. 7. These data are further treated in the Discussion.

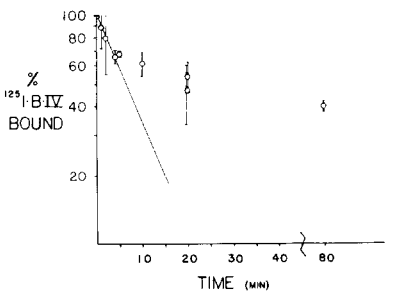

Fig. 7. Time course of dissociation of ^{125}I-labeled toxin B-IV from axonal vesicles. Vesicles were incubated with 30 nM ^{125}I-labeled toxin B-IV for 30 min at 4°C before centtrifugation and resuspension in binding medium containing micromolar unlabeled B-IV. Binding assays were done in triplicate. The data are corrected for nonspecific binding.

The binding of polypeptide axonal toxins from scorpion [26] and sea anemone [28] to sodium channels in both cultured neuroblastoma cells and rat brain synaptosomes has been shown to be dependent upon the membrane potential. Since the pharmacologic activity of the polypeptide neurotoxin B-IV suggests involvement with the ion permeability apparatus of crustacean axon and since we were unable to detect toxin binding to axon membrane fragments, we were interested in determining whether toxin B-IV binding might likewise be dependent upon membrane potential. Accordingly, we measured the binding of ^{125}I-labeled toxin B-IV to our axonal vesicles in the presence of 135 mM K^+ to eliminate the electrogenic K^+ gradient from the inside of the vesicles (135 mM K^+) to the external medium (5 mM K^+ in binding medium). Toxin B-IV binding was decreased by 60% as a result of this treatment.

[^{3}H]Saxitoxin binding

Since the *Cerebratulus* polypeptide toxin B-IV appears to interact with a macromolecule involved with either inactivation of voltage-sensitive sodium or activation of potassium channels and since the binding of [^{3}H]tetrodotoxin has already been reported for membrane fragments from lobster walking-leg nerves as a probe of sodium channel density [10], we were interested in comparing the binding capacity of our lobster axonal vesicle preparation for ^{125}I-labeled toxin B-IV and for [^{3}H]saxitoxin.

[^{3}H]Saxitoxin was obtained from the National Institute of Health and was characterized both by its ability to inhibit the uptake of ^{22}NaCl by neuroblastoma cells (to determine its concentration) and by its binding to homogenized rat brain as described in Experimental Procedures (to determine its specific radioactivity).

Saturation binding was measured by incubation of 20 nM [^{3}H]saxitoxin with axonal vesicles followed by rapid filtration. 20 nM saxitoxin has been shown to saturate its receptor in lobster axonal membranes [10]. Specific binding of [^{3}H]saxitoxin was determined by subtraction of the nonspecific binding measured in the presence of 2 μM unlabled saxitoxin from the total binding. Using vesicles isolated from the 1.0–1.2 M sucrose interface of the discontinuous density gradient, an average binding capacity of 1.0 pmol per mg membrane protein was found. We then characterized [^{3}H]saxitoxin binding to lobster axonal vesicles that had been solubilized with 2% Triton X-100 as described in Experimental Procedures. Binding was measured by incubation of [^{3}H]saxitoxin with the 165 000 $\times$ g supernatant of the solubilized mixture followed by rapid ion exchange chromatography of aliquots of the binding reaction mixture on Dowex X-50. The binding capacity of 3.8 pmol per mg membrane protein which resulted was very similar to values previously reported for lobster nervous tissue [9,10,19].

Vesicle sidedness was investigated in order to ascertain a true value for the total number of toxin B-IV binding sites in this tissue. This could then be compared to the site density for saxitoxin obtained in the solubilized vesicle studies above to determine the stoichiometry of these two classes of neurotoxin binding sites.

Tetrodotoxin and saxitoxin have been shown to bind only to the outside of axonal membranes [33] and not to exhibit binding potential dependence [34]. Therefore, [^{3}H]saxitoxin could be used as an independent marker to compare vesicle binding capacity with that of fully exposed axonal membrane. Since solubilized membrane, by definition, lacks sidedness, the binding capacity of 3.8 pmol per mg protein obtained was taken as the total saxitoxin binding to lobster axonal membrane. Comparison of this value for total binding to lobster axonal membrane with the [^{3}Hsaxitoxin binding capacity of the vesicles indicates that our vesicle preparation is 74% inside-out. Thus, the total toxin B-IV binding capacity of these vesicles would be approx. 6.1 pmol toxin per mg membrane protein. Comparison of the binding capacities of axonal vesicles for toxin B-IV and saxitoxin reveals that the receptors for these toxins are present in very similar concentrations. This will be further treated in the Discussion.

Competition of toxin B-IV binding by Leiurus and Centruroides toxins

One way to ascertain whether toxin B-IV mediates its pharmacologic activity by interacting at the voltage sensitive sodium channel would be to determine whether binding of ^{125}I-labeled toxin B-IV to our vesicles was competed by toxins from the venoms of *Leiurus* or *Centruroides sculpturatus*. Both of these toxins have been shown to associate specifically with sites on voltage-sensitive sodium channels [35,36]. Competition binding studies using 8 nM ^{125}I-labeled toxin B-IV were carried out as described previously, at both 4 and 37°C. Neither *Leiurus* nor *Centruroides* toxin, at concentrations up to 1 μM, inhibited the binding of toxin B-IV. At higher concentrations *Centruroides* toxin stimulated the binding of *Cerebratulus* toxin by as much as 48%.

Discussion

In this paper we have extensively characterized the binding of *Cerebratulus* toxin B-IV to its receptor on vesicles formed from lobster axonal membrane. We have detected specific, saturable binding of toxin B-IV to a single class of binding sites with a dissociation constant of 5–20 nM and a binding site capacity, corrected for vesicle sidedness, of about 6 pmol per mg membrane protein. Multiple measurements were done to determine the binding dissociation constant. Analysis of equilibrium binding, competition studies, and binding kinetics are all consistent with a receptor, present at 6 pmol per mg membrane protein, having a dissociation constant of 5–21 nM. There is no competition of toxin B-IV binding by either purified toxin from *Leiurus quinquestriatus* venom or by *Centruroides sculpturatus* Ewing toxin I at concentrations up to 1 μM. The significance of the small increase in toxin B-IV binding in the presence of 1 μM *Centruroides* toxin is not clear at this time.

Throughout the course of these studies, a high degree of nonspecific binding of ^{125}I-labeled toxin B-IV to our vesicles has been observed. We feel that this non-specific binding, as well as retention

of specific binding of toxin to vesicles in the dissociation experiment are very likely due to hydrophobic interactions of the modifying 3-(4-hydroxy-3-[^{125}I]iodophenyl)propionyl moiety with the hydrophobic milieu of the vesicular membrane. Ray and Catterall [24] have found a similarly high degree of nonspecific binding using *Leiurus* toxin modified with Bolton-Hunter reagent. However, when *Leiurus* toxin was iodinated by the lactoperoxidase method, much lower values of nonspecific binding were observed. We have been precluded from using this latter type of iodination due to the presence of an essential tyrosine residue in toxin B-IV [5].

The data from the dissociation of ^{125}I-labeled toxin B-IV from the axonal vesicles presented difficulties. 40% of the specifically bound toxin was either irreversibly bound or dissociated extremely slowly. The possibility of internalization of the toxin was eliminated since binding experiments were done at 4°C, a temperature at which internalization processes are inhibited. Since dissociation only occurred in the presence of excess unlabeled toxin we felt that access of the dissociated toxins to the bulk solution was barred. Consequently, we used the initial, linear rate of toxin dissociation to determine a dissociation rate constant. The resulting dissociating constant, calculated from the ratio k_{-1}/k_{+1}, agreed with the dissociation constants from the ^{125}I-labeled toxin B-IV saturation and competition binding experiments. Results similar to these have recently been obtained in studies of β-bungarotoxin dissociation from membrane receptors in chick brain [37].

Our results suggest the possibility that the binding of ^{125}I-labeled toxin B-IV may be dependent upon membrane potential. This conclusion is based on the following criteria.

(1) The axonal vesicles were in fact vesicular structures.

(2) Catterall [30] has observed a 10-fold increase in the dissociation constant for *Leiurus* toxin binding to neuroblastoma cells in the presence of a depolarizing concentration of K^+ (135 mM). Incubation of 30 nM ^{125}I-labeled toxin B-IV with axonal vesicles in 135 mM K^+ causes a 60% decrease in specific binding; in 5 mM K^+ this toxin B-IV concentration yields saturation of specific sites (Fig. 2). If we assume that vesicle depolarization causes a 10-fold increase in the K_d for toxin B-IV, then the percentage of specifically bound toxin B-IV remaining in high K^+, calculated from the Michaelis-Menton equation, would be 38%. This is in excellent agreement with the experimentally determined value, 40%.

(3) Binding of toxin B-IV to lobster axon membrane fragments, prepared as described by Denburg [38], is at most 10% of that to vesicles at equivalent toxin concentrations.

Comparison of the binding of labeled saxitoxin to axonal vesicles and to solubilized vesicles allowed us to determine a sidedness ratio of the vesicles of 74% inside-out. This value allowed for a corrected binding capacity of 6 pmol toxin B-IV bound per mg membrane protein. Comparison of this value to that of total saxitoxin binding gave a ratio of about two toxin B-IV receptor sites for each saxitoxin receptor site. Thus, the number of toxin B-IV binding sites is very similar to the number of Na^+ channels.

The data in the literature do not permit a functional identification of the toxin B-IV binding site. However, the results of Bacq [2,3] and Kem [1] indicate that the *Cerebratulus* B-toxins induce spontaneous trains of action potentials and prolong the repolarization phase of the action potential when applied to crustacean neurons. These suggest that the pharmacologic activity of the toxins is mediated by ionic channels in the nerve membrane. Recent unpublished data, obtained by voltage clamp analysis, show that B toxins cause a small (5 mV) depolarization of the resting potential in nerves (Kem, W.R., personal communication). The above data would be most easily explained by an interaction of the toxin with the voltage-regulated Na^+ channel. It is therefore interesting that toxin B-IV binding is not competed by either *Leiurus* and *Centruroides* toxins, both of which are known to interact with Na^+ channels in nerve. However, lack of competition of ^{125}I-labeled toxin B-IV binding by polypeptide neurotoxins from *Leiurus* or *Centruroides* does not absolutely preclude the interaction of toxin B-IV with the voltage-sensitive sodium channel since several noncompetitive neurotoxin binding sites have been discovered [39] and the precise organization of the above Na^+ channel polypeptide neurotoxin binding sites remains unresolved.

Preliminary ion flux studies in our laboratory have shown that toxin B-IV stimulates an influx of ^{22}Na into depolarized phosphatidylcholine liposomes reconstituted with lobster axon membrane fragments. This toxin B-IV stimulated ^{22}Na influx is not blocked by tetrodotoxin but is blocked by the potassium channel blockers tetraethylammonium and 3,4-diaminopyridine. These results suggested that toxin B-IV might alter the ion selectivity of potassium channels to allow sodium flux through them much the way that scorpion and anemone toxins alter sodium channels to allow K^+ and Rb^+ fluxes [40].

A major objective of future experiments will be to further develop a system for studying the effect of toxin B-IV on channel regulated ion fluxes in lobster nervous tissue and to prepare chemically-modified toxin B-IV capable of covalent cross linking to its receptor in order to isolate the receptor for further characterization. Only upon complete purification of this macromolecule will it be possible to accurately determine the site density ratios for the various neurotoxins and to begin to construct a model describing ion channel function.

Acknowledgments

We are indebted to Ms. Catherine Azar and Nancy Kreider for assistance in some of these experiments, to Mr. Dale Blankenship for the amino acid analyses, and to Ms. Marcia McLachlan for expert typing of the manuscript. Special thanks are due to Dr. Randall Morris who kindly provided the electron micrographs. Aided by a grant (NS-14368) from the National Institutes of Health. G.P.T. is a Fellow of the Albert J. Ryan Foundation. K.M.B. is a Research Career Development Awardee (NS-00304) of the National Institutes of Health, U.S. Public Health Service.

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