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SATURABLE BINDING TO CELL MEMBRANES OF THE PRESYNAPTIC NEUROTOXIN, β-BUNGAROTOXIN

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SUMMARY

Brief exposure to the protein neurotoxin, β-bungarotoxin, is known to disrupt neuromuscular transmission irreversibly by blocking the release of transmitter from the nerve terminal. This neurotoxin also has a phospholipase A2 activity, although phospholipases in general are not very toxic. To determine if the toxicity of this molecule might result from specific binding to neural tissue, we have looked for high affinity, saturable binding using ^{125}I-labeled toxin. At low membrane protein concentration ^{125}I-labeled toxin binding was directly proportional to the amount of membrane; at fixed membrane concentration ^{125}I-labeled toxin showed saturable binding. It was unlikely that iodination markedly changed the toxin's properties since the iodinated toxin had a comparable binding affinity to that of native toxin as judged by competition experiments. Comparison of toxin binding to brain, liver and red blood cell membranes showed that all had high affinity binding sites with dissociation constants between one and two nanomolar. This is comparable to the concentrations previously shown to inhibit mitochondrial function. However, the density of these sites showed marked variation such that the density of sites was 13.0 pmol/mg protein for a brain membrane preparation, 2.4 pmol/mg for liver and 0.25 pmol/mg for red blood cell membranes.

In earlier work we had shown that calcium uptake by brain mitochondria is inhibited at much lower toxin concentrations than is liver mitochondrial uptake. Both liver and brain mitochondria bind toxin specifically, but the density of ^{125}I-labeled toxin binding sites on brain mitochondrial preparations (3.3±0.3 pmol/mg) exceeded by a factor of ten the density on liver mitochondrial preparations (0.3±0.05 pmol/mg). It is also shown that labeled toxin does not cross synaptosomal membranes, suggesting that mitochondria may not be the site of action of the toxin in vivo. We conclude that β-bungarotoxin is an enzyme which can bind specifically with high affinity to cell membranes.

INTRODUCTION

β-Bungarotoxin is a presynaptic neurotoxin isolated from the venom of the krait, *Bungarus multicinctus*. Electrophysiological analysis of its effects on the rat

phrenic nerve diaphragm preparation indicate that brief exposure to the toxin results in irreversible disruption of transmitter release [1]. Because of the potential value of a presynaptic marker whose site of action is known, we and others have studied the electrophysiological changes produced by this toxin [1–3] and its biochemical properties [4–6]. In this paper we use radioactively labeled toxin in an attempt to identify toxin binding sites, with two aims in mind: (a) to determine if labeled toxin is a specific probe for neural tissue and (b) to discover whether the inhibition of mitochondrial calcium uptake by the toxin could account for its physiological effects.

It was especially important in this work to minimize non-specific binding, due, presumably, to the highly charged nature of the toxin at neutral pH [2]. Since early attempts to measure binding to nerve terminal regions of nerve-muscle preparations were discouraging, we developed other conditions under which we can demonstrate high affinity, saturable binding using brain membrane preparations. Using a gel filtration technique to separate membrane-bound and free toxin we show that high affinity sites are present in many membrane preparations, but are more dense in the plasma membranes of neural tissue. In addition, we find that the toxin cannot cross the plasma membranes of isolated nerve terminals. We propose that the diverse action of the toxin on synaptosomal transmitter uptake [4] and calcium uptake by mitochondria and sarcoplasmic reticulum [5, 6] may result from its phospholipase A2 activity; whereas its presynaptic action is also due to an ability to bind avidly to specific plasma membrane sites.

METHODS

Materials. Crude venom from *Bungarus multicinctus* was obtained from Ross Allen Reptile Institute, Silver Spring, Florida. The following compounds were obtained from Sigma, St. Louis, Missouri: ouabain octahydrate, disodium ethylenediaminetetraacetic acid (EDTA), trypsin inhibitor (soybean) bovine albumin (crystallized and lyophilized) and bovine albumin (essentially fatty acid free). Fischer supplied sodium metabisulfite and chloramine T. Calbiochem provided the adenine nucleotides. $Na^{125}I$ (pH 8–10, carrier-free) was obtained from New England Nuclear.

Preparation of ^{125}I-labeled toxin. Toxin, isolated according to the procedure of Kelly and Brown [2], was chromatographed on a 10 ml G-50 Sephadex column equilibrated with 0.25 M Tris · HCl buffer (pH 7.5) prior to its iodination. Iodination was performed by a modification of the procedures described by Banerjee et al. [7]. 10 μl of chloramine T (2.5 mg/ml) was added to 0.10 ml of 0.25 M Tris · HCl (pH 7.5) containing about 200 μg of toxin and 5 or 10 mCi of $Na^{125}I$. After a 30-s incubation at 25 °C, 0.1 ml of a 1 % albumin (w/v), in 0.25 M Tris · HCl (pH 7.5) was added and the sample was layered on a 10 ml column of Sephadex G-25 (fine) equilibrated with 0.25 M Tris · HCl (pH 7.5) containing 0.1 % (w/v) albumin. The radioactive fractions in the excluded volume of this column were pooled then applied to a 50 ml column of Bio-Gel P-60 (100–200 mesh) and was eluted with 0.05 M Tris · HCl (pH 7.6) containing 0.1 % trypsin inhibitor. This column separated the iodinated toxin from the iodinated albumin which also was produced during the labeling procedure. To determine the specific activity of the iodotoxin a 5 μl aliquot of the reaction mixture (before albumin addition) was added to 4 ml of 0.25 M Tris · HCl (pH 7.6) containing 0.5 % (w/v) albumin plus 0.04 % (w/v) sodium metabisulfite

and aliquots of this mixture were added to 10 % trichloroacetic acid and the percent precipitable counts determined. The specific activity of the iodinated toxin (^{125}I-labeled toxin) varied between 10 and 28 μCi/μg depending on the concentrations of toxin and Na^{125}I. If a molecular weight of 21 800 is assumed [2] for the toxin, this represents a specific activity of 220–610 Ci/mmol, or about 0.1 to 0.28 mol of ^{125}I per mol of toxin.

When freshly prepared ^{125}I-labeled toxin was combined with native toxin both protein and radioactivity co-migrated as a single discrete band during sodium dodecyl sulfate gel electrophoresis in the absence of reducing agents. When a mixture of native and labeled toxin was dissociated into two dissimilar subunits (approximately 12 000 and 8000 daltons) by reduction with 2-mercaptoethanol [2], the protein and ^{125}I comigrated as two discrete bands of the appropriate subunit molecular weights, implying that both toxin subunits were labeled by this procedure. ^{125}I-labeled toxin was stored at −20 °C for 2–3 weeks with less than a 5 % loss in specific binding activity.

β-Bungarotoxin is a potent phospholipase A2 with activity comparable to other highly purified enzymes isolated from *Naja naja* and *Vipera russellii* snake venoms. Since this enzyme activity is also required for the presynaptic action of this molecule [8], an assay of the enzyme activity provides a test of the effect on the toxin of exposure to chloramine T. Toxin was labeled with non-radioactive iodine according to the above procedures. The phospholipase activity of native and iodinated toxin were determined using a pH-stat according to the procedures of Strong et al. [8]. The iodinated derivative retained at least 85 % of the native enzymatic activity.

Assay of ^{125}I-labeled toxin binding to membranes. The procedure used to assay ^{125}I-labeled toxin binding to membrane preparations was similar to that described by Almon et al. [9]. Membrane fractions were incubated for 20 min at 25 °C with given concentrations of ^{125}I-labeled toxin in 0.2 ml of isolation medium (pH 7.6) containing 0.05 % (w/v) trypsin inhibitor. These conditions were shown to be sufficient for binding equilibrium to be reached. Bound toxin was separated from unbound by fractionating 0.1 ml samples at 4 °C on 4.5 ml G-200 Sephadex columns (0.7 × 10 cm) with 50 mM Tris · HCl (pH 7.6), 1 mM EDTA and 0.05 % (w/v) trypsin inhibitor as shown in Fig. 1. The entire excluded volume (approximately 2 ml) from each of these columns was collected and counted to give the total ^{125}I-labeled toxin excluded. In the absence of membranes as much as 10 % of the total ^{125}I-labeled toxin applied to the G-200 column was eluted in the first 0.25 ml fraction (Fig. 1) due to wall effects. Consequently all values given for bound ^{125}I-labeled toxin were corrected for this unbound fraction excluded in the absence of membrane protein. The entire column separation process was completed in less than 25 min at 4 °C. The fraction bound was calculated from the fraction of radioactivity in the excluded volume, correcting for an 85 % recovery of both membrane protein and ^{125}I-labeled toxin from these columns.

Isolation of human red blood cells. Red blood cells were isolated by a slight modification of the method of Dodge et al. [10]. 1.5 ml of whole heparinized blood was suspended in 15 ml of 0.9 % NaCl, 5 mM Tris · HCl (pH 7.6) and was centrifuged at 1100 × *g* for 10 min at 0 °C. The supernatant and white cell layer were removed by suction. This protocol was repeated four additional times and the final cell pellet was suspended in 1 ml 50 mM Tris · HCl (pH 7.6) containing 0.05 % trypsin inhibitor.

Preparations of brain and liver membranes. Brain membranes (crude mitochondrial fraction) were prepared by homogenization and differential centrifugation in isolation medium containing 0.32 M sucrose, 5 mM Tris · HCl (pH 7.6) and 1 mM EDTA according to the procedure of Morgan et al. [11]. Liver membranes were prepared using the same procedures. Membrane protein concentrations were determined by the method of Hartree [12] with bovine albumin used as a standard.

Preparation of brain and liver mitochondria. Both brain and liver mitochondria were prepared from white male rats by the procedure of Clark and Nicklas [13].

Further fractionation of brain membranes. Brain membranes were sub-fractionated into "myelin", "synaptosomal" and "mitochondrial" enriched membrane fractions on discontinuous, isotonic Ficoll-sucrose density gradients as described by Morgan et al. [11]. Synaptosomal fractions from this Ficoll density gradient step were fractionated further by hypoosmotic lysis in 5 mM Tris · HCl (pH 7.6), 0.1 mM EDTA and centrifugation through a discontinuous sucrose density gradient (0.4 M, 0.6 M, 0.8 M, 1.0 M and 1.2 M) used for the isolation of synaptosomal plasma membrane [11]. All membranes fractions were characterized by assay for specific enzyme activities (lactate dehydrogenase, cytochrome *c* oxidase, and $(Na^+ + K^+)$-ATPase).

Enzyme assays. Lactate dehydrogenase, a cytoplasmic marker, was assayed according to procedure of Johnson [14]. Cytochrome *c* oxidase, a marker for the inner mitochondrial membrane, was assayed by the procedure of Lu et al. [15], $(Na^+ + K^+)$-ATPase, a plasma membrane marker, was assayed according to Morgan et al. [11].

Analytical techniques. Inorganic phosphate was selectively precipitated as phosphomolybdic acid [16], collected on glass fiber filters and assayed using a Nuclear Chicago Model 470 Gas-Flow Detector. All spectrophotometric assays were performed using a Cary Recording Spectrophotometer. ^{125}I-labeled toxin was counted at 41 % efficiency with a Beckman Gamma 300 counter for sufficient time to obtain greater than 10^5 counts. [γ-^{32}P]ATP was prepared by the procedure of Chamberlin and Ring [17] and was further purified by paper chromatography in order to minimize inorganic phosphate contamination.

RESULTS

Gel filtration binding assay

Binding of small molecules to cell membranes is often assayed by Millipore filtration or differential centrifugation [18]. Preliminary experiments showed that use of these techniques to study the binding of β-bungarotoxin was not feasible because of large amounts of non-specific binding to filters and centrifuge tubes. Consequently, we used a more laborious technique of gel filtration on G-200 Sephadex columns to separate bound from free toxin. Membrane protein and bound ^{125}I-labeled toxin was excluded by such columns, whereas the peak of unbound ^{125}I-labeled toxin appeared at 0.9 times the column volume (Fig. 1). A 3-fold increase in the time of incubation of toxin with the membrane fractions did not alter the amount bound. This procedure has been used to ask if saturable binding to membranes occurs and to measure the affinity of binding.

Effect of brain and liver membrane concentration on ^{125}I-labeled toxin binding

To find the appropriate range of membrane concentrations for affinity mea-

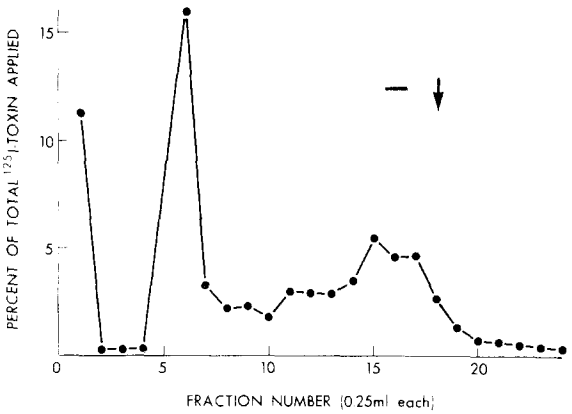

Fig. 1. Separation of free from membrane bound ^{125}I-labeled toxin by Sephadex G-200 gel filtration. 100 μg of brain membrane was incubated for 20 min at 25 °C in 0.2 ml of 50 mM Tris · HCl (pH 7.6), 0.05 % trypsin inhibitor containing 1.0 pmol ^{125}I-labeled toxin (610 Ci/mg). 0.1 ml of this reaction mix was layered on top of 4.5 ml Sephadex G-200 columns and was eluted with the 50 mM Tris · HCl (pH 7.6) 0.05 % trypsin inhibitor 0.1 % sodium azide. 0.25 ml fractions were collected and assayed for ^{125}I. If brain membrane is omitted, ^{125}I-labeled toxin elutes as a single peak at position indicated by a horizontal bar. The arrow indicates the total column volume.

surements the approximate density of sites was measured by incubating increasing amounts of membrane with a constant toxin concentration. Also, in hope of demonstrating neural specificity, toxin binding to brain membranes was compared to binding to liver membranes (Fig. 2). At low membrane concentrations, the binding of ^{125}I-labeled toxin was directly proportional to the concentration of brain and liver membrane protein but a marked preference for brain membranes was noticed. It would

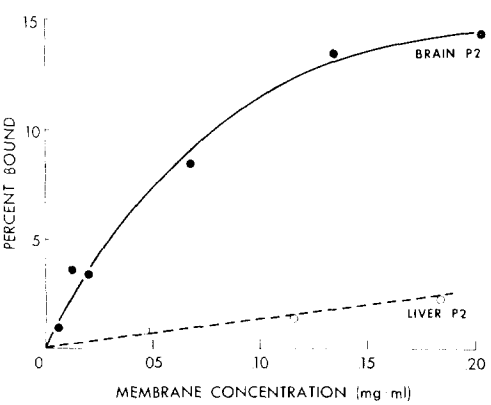

Fig. 2. Percent of ^{125}I-toxin bound to brain (● - ●) and liver (○ - - - ○) membrane as a function of membrane protein concentration. The indicated concentration of membrane protein was incubated for 20 min at 25 °C in 0.2 ml of 50 mM Tris · HCl (pH 7.6), 0.05 % trypsin inhibitor containing 0.5 pmol ^{125}I-labeled toxin (610 Ci/mmol). Percent ^{125}I-labeled toxin bound was determined by Sephadex G-200 filtration as described in Methods section. All points in this and subsequent figures represent individual determinations.

appear that brain membranes have a much greater density of high affinity sites than liver membranes. Even at very high concentrations of both brain and liver membranes, a maximum of 15 % of the ^{125}I-labeled toxin could be bound at 2.5 nM (Fig. 2). This may mean that only about 15 % of the iodinated toxin was capable of binding. However, when the experiment was repeated using toxin which had not bound the first time, once again, a maximum of about 20 % bound to brain membranes (300 μg/ml). The reason for this effect is not known, but might involve a high concentration of low affinity membrane binding sites which dissociate during gel filtration.

Since calcium and strontium are both known to modify the physiological and biochemical [8] properties of β-bungarotoxin, we wondered if the presence or absence of these ions might modify toxin binding. To test this, we compared ^{125}I-labeled toxin binding as described in Fig. 2, with or without 2 mM concentrations of either calcium or strontium. Neither ion had an effect on ^{125}I-labeled toxin binding relative to controls lacking divalent cations.

Affinity of ^{125}I-labeled toxin binding to brain and liver membranes

To measure the affinity of ^{125}I-labeled toxin for membranes, a concentration of membrane was chosen such as to be in the linear range in Fig. 2, and the amount of ^{125}I-labeled toxin bound to brain or liver membranes was measured as a function of free ^{125}I-labeled toxin (Fig. 3). Assuming that the included toxin represented unbound ^{125}I-labeled toxin and that binding equilibrium was not altered by column chromatography, a Scatchard analysis of this data [19] was used to determine the dissociation constant (K_d) and the amount of ^{125}I-labeled toxin binding sites per mg membrane protein (R_T) (Table I). Both brain and liver membranes showed comparable dissociation constants of 1.7±0.5 and 2.2±0.6 nM, respectively, but different densities of binding sites (13.0±6.0 pmol/mg for brain and 2.4±0.8 pmol/mg for liver). Thus specific binding occurs in the same concentration range that is required to inhibit calcium accumulation by brain mitochondria (50 % inhibition at 0.7 nM toxin and 8 pmol/mg protein) [5].

TABLE I

COMPARISON OF ^{125}I-LABELED TOXIN BINDING TO VARIOUS MEMBRANE PREPARATIONS

The data presented in this table were obtained by making Scatchard plots [19] of the binding data presented in Figs. 3 and 5. In such plots, the slope is equal to $-1/K_d$ and the extrapolated intercept on the abscissa is equal to the number of binding sites (R_T) present in the incubation mixture. A least squares analysis was performed on this data. All values are presented ± standard deviations. Six or more individual determinations of ^{125}I-labeled toxin binding (obtained as described in Methods) were used for the calculation of each binding constant and its standard deviation.

Membrane preparation	K_d (nM)	R_T (pmol/mg)	R_T/R_T (brain)
Brain	1.7 ±0.5	13.0±6.0	1.0
Liver	2.2 ±0.6	2.4 ±0.8	0.19
Brain mitochondria	0.7 ±0.1	3.3 ± 0.3	0.25
Liver mitochondria	—	0.3 ±0.05	0.023
Red blood cells	0.71±0.05	0.25 ±0.03	0.019

Binding of native versus iodinated toxin

In order to test whether the binding of ^{125}I-labeled toxin (Fig. 3) was similar to that of native toxin, brain membranes were incubated with nanomolar concentrations of ^{125}I-labeled toxin diluted with various concentrations of native toxin. The amount bound as a function of free toxin was then calculated for both the diluted and undiluted toxin (Fig. 4). If the binding properties of the labeled and native toxin were

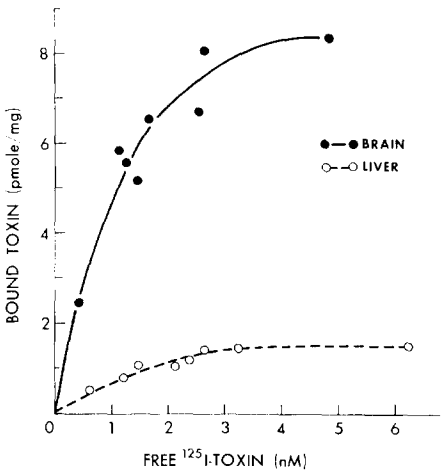

Fig. 3. Effect of ^{125}I-labeled toxin concentration on its binding to brain and liver membranes. Assay tubes contained 12.8 μg brain or 73.6 μg liver membrane protein and indicated concentration of ^{125}I-labeled toxin (610 Ci/mmol) in 0.2 ml of 50 mM Tris · HCl (pH 7.6), 0.05 % trypsin inhibitor. Binding was assayed as described in Methods.

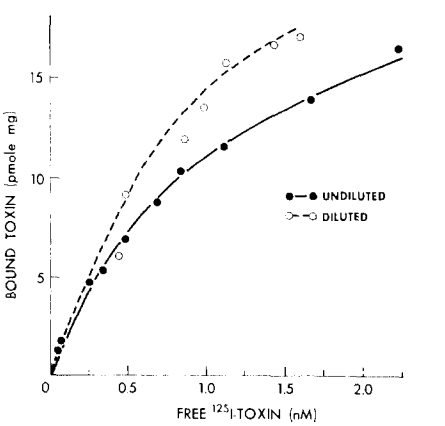

Fig. 4. Binding of ^{125}I-labeled toxin of different specific activities to brain membranes. 0.2 ml samples containing 10 μg membrane protein in 50 mM Tris · HCl (pH 7.6) and 0.05 % trypsin inhibitor were incubated for 20 min at 25 °C with different concentrations of the undiluted ^{125}I-labeled toxin preparation (550 Ci/mmol) (●) or with diluted preparations (○) containing 0.5 nM ^{125}I-labeled toxin plus different amounts of native toxin to give the concentrations specified. (The difference in the extent of binding here and in Fig. 3 probably represents differences in the purity of the membrane fractions used).

identical, the reduction in specific activity should not change the amount of toxin bound at any given free toxin concentration. The amount of toxin bound at various concentrations of free toxin did not differ significantly even though the specific activity was reduced up to 6.5-fold and the dissociation constants determined from these data were within experimental error (2.0±0.4 nM for low specific activity versus 1.5±0.2 nM for high specific activity determination). Since the binding characteristics are the same for native and labeled toxin, we conclude that iodination has not altered binding significantly, and used the binding of iodinated toxin as a measure of native toxin binding without correction.

^{125}I-labeled toxin binding to different brain membrane fractions

We sought to determine if the site of toxin binding in brain membranes could be localized more precisely by measuring binding to various subfractions of brain. This was of special interest, since earlier work has shown that brain mitochondrial function was inhibited by the toxin [5]. Brain membranes were fractionated into low density membrane fragments, synaptosomes and mitochondria [11], as defined by morphology and specific membrane markers and the binding of ^{125}I-labeled toxin to these fractions, was determined as described in Fig. 2. The initial slopes of such binding curves provide a relative measure of the density of binding sites in each of these fractions (Table II). As shown, the relative densities of binding sites in low density membrane fragments, synaptosomes and mitochondria-enriched fractions were 1, 0.7 and 0.3, respectively.

TABLE II

^{125}I-TOXIN BINDING TO FICOLL DENSITY FRACTIONS OF BRAIN

Brain membranes were fractionated into low density membrane fragments, synaptosomes and mitochondria by sedimentation through a discontinuous Ficoll density gradient as described in Methods. Different concentrations of these membrane fractions resuspended in 0.32 M sucrose, 5 mM Tris · HCl (pH 7.6) and 1 mM EDTA, were incubated with 2.2 nM ^{125}I-labeled toxin and a binding curve was constructed for each as shown in Fig. 2. The initial linear slope of each binding curve was used to estimate the density of binding sites in each of these brain membrane fractions.

Brain membrane fraction	Initial binding slope (% bound/μg per ml)	Density relative to fragments
Low density fragments	2	1.0
Synaptosomes	1.4	0.7
Mitochondria	0.6	0.3

Relative density of ^{125}I-labeled toxin binding sites in different membrane preparations

Since nanomolar concentrations of β-bungarotoxin are known to inhibit calcium uptake into brain mitochondria but not into liver mitochondria [20], we compared saturable binding of ^{125}I-labeled toxin to these preparations. Brain mitochondria were prepared according to procedures used in these uptake studies and ^{125}I-labeled toxin binding was assayed at a fixed concentration of mitochondrial protein. As illustrated in Fig. 5, high affinity (K_d = 0.7±0.1 nM) saturable binding was observed. However, the number of sites in brain mitochondria (3.3±0.3 pmol/mg) was 10-fold greater than the number found in an equivalent experiment using

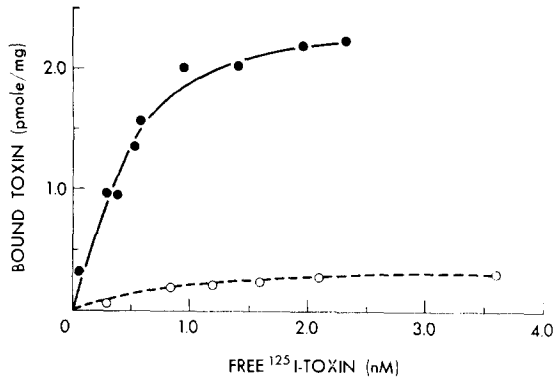

Fig. 5. ^{125}I-labeled toxin binding to brain mitochondria (●) and red blood cell (○) membranes. Assay tubes contained 12.8 μg of brain mitochondria or 280 μg red blood cell membranes and the indicated concentrations of ^{125}I-labeled toxin (610 Ci/mmol) in 0.2 ml of 50 mM Tris · HCl (pH 7.6), 0.05 % trypsin inhibitor. All tubes were incubated for 20 min at 25 °C. Binding was assayed as described in Methods section.

purified liver mitochondria (0.3±0.05 pmol/mg), which suggests that the increased toxin sensitivity of brain mitochondria might result from preferential binding of the toxin.

^{125}I-labeled toxin also binds with high affinity to human blood cells (K_d = 0.7±0.05 nM). The number of binding sites is around 0.25 pmol per mg of blood cell protein (Fig. 5). This is equivalent to about 4000 ^{125}I-labeled toxin-binding sites per cell. The density of binding sites in different membrane preparations is summarized in Table I. Although high affinity binding sites were found in all of these preparations, the density was highest in brain.

^{125}I-labeled toxin binding to intra-synaptosomal mitochondria. If the toxin acts in vivo to inactivate mitochondria it must first pass through the plasma membrane of the nerve terminal. Assuming that brain synaptosomes are a reasonable model for a nerve terminal, it is possible to test whether the toxin can penetrate the synaptosomal plasma membranes and bind to cytoplasmic mitochondria. Intact synaptosomes shown to contain less than 10 % free mitochondria (Wagner, G. M. and Kelly, R. B., manuscript in preparation) were prepared by density sedimentation, labeled with ^{125}I-toxin (1 nM) and centrifuged to separate bound from unbound ^{125}I-labeled toxin. The labeled synaptosomes were then exposed to hypo-osmotic conditions resulting in greater than 90 % release of cytoplasmic constituents (measured as release of lactic dehydrogenase activity). The lysed synaptosomes were then fractionated on a discontinuous sucrose gradient. Fractions were collected and assayed for ^{125}I-labeled toxin and membrane specific enzymes. As shown in Fig. 6, the peak specific activity of ^{125}I-labeled toxin co-migrates with the peak of ($Na^+ + K^+$)-ATPase activity, a specific plasma membrane marker. Moreover, the distribution of cytochrome *c* oxidase activity, a specific mitochondrial marker, did not correlate at all with the distribution of ^{125}I-labeled toxin. It thus appears that ^{125}I-labeled toxin was unable to cross the plasma membrane and bind to intrasynaptosomal mitochondria under these assay conditions. This experiment does not eliminate the possibility that the toxin enters and forms some low affinity complex with the mitochondria.

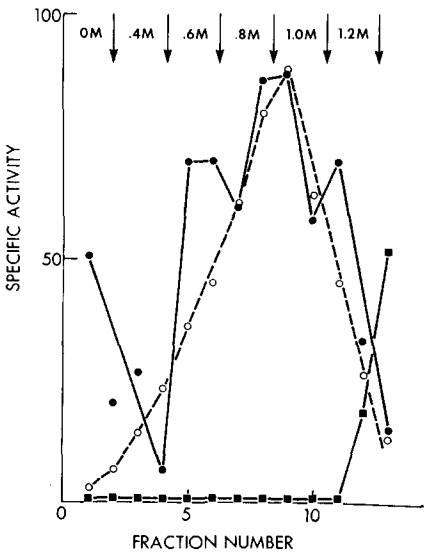

Fig. 6. Toxin binding to the plasma membrane fraction of brain synaptosomes. Synaptosomal membranes (from 4 rats) following Ficoll density gradient step [11] were resuspended in 2 ml (50 μg protein/ml of isolation media) and incubated with 8 pmol of ^{125}I-labeled toxin (200 Ci/mmol) for 20 min at 0 °C. The suspension was then centrifuged for 20 min at 27500 $\times g$ and the supernatant was discarded. The membrane pellet was resuspended in 2.2 ml 1 mM Tris · HCl (pH 7.6) 0.1 mM EDTA and incubated for 15 min at 25 °C causing hypo-osmotic lysis (greater than 90 % of total lactate dehydrogenase activity was released). 2 ml of this suspension was then layered on a 10 ml discontinuous density gradient composed of 2 ml each of 0.4, 0.6, 0.8, 1.0 and 1.2 M sucrose and was spun at 58 500 $\times g$ for 2 h. 1-ml fractions were collected and assayed for ^{125}I-labeled toxin binding, $(Na^+ + K^+)$-ATPase activity, cytochrome *c* oxidase activity and protein as described in Methods. ^{125}I-labeled toxin specific activity (○) co-sedimented with the peak of $(Na^+ + K^+)$-ATPase specific activity (●) but not with the mitochondrial marker, cytochrome *c* oxidase (■). 100 units of specific activity is equivalent to 100 pmol P_i released · h^{-1} · μg $protein^{-1}$ for $(Na^+ + K^+)$-ATPase (●), 500 cpm ^{125}I-labeled toxin bound/mg protein (○), and 1 μg cytochrome *c* oxidized · min^{-1} · μg $protein^{-1}$ for cytochrome *c* oxidase (■).

DISCUSSION

In this report we have shown high-affinity binding of ^{125}I-labeled toxin to a limited number of sites in brain membrane preparations using gel filtration to separate included from excluded toxin. To obtain an apparent dissociation constant and the density of binding sites we assumed that the included toxin represents free toxin and the excluded represents bound toxin. This assumption is invalid either if the "free" toxin is composed of a significant amount of inactive toxin or if the toxin-membrane complex dissociates appreciably during gel filtration. It is unlikely that a significant fraction of the free toxin is inactive, since previously unbound toxin was able to bind just as effectively as the original toxin to added membranes. In addition, unlabeled toxin competes equally for binding sites with ^{125}I-labeled toxin (Fig. 4). Nor is it likely that the toxin dissociates rapidly from its high-affinity binding sites since, when labeled synaptosomes are separated from free toxin, lysed and then centrifuged, a large fraction of the toxin remains bound to these membranes (Fig. 6). It is possible

however that there is a high density of low-affinity sites, which dissociate during gel filtration, giving rise to the maximum binding of 15 % observed in Fig. 2. However, the existence of low-affinity sites, toxin dissociation from high-affinity sites, and partial inactivation of toxin by iodination would all lead to an underestimate of both the affinity and density of high-affinity sites.

Comparison of ^{125}I-labeled toxin binding to several membrane preparations showed high affinity binding to all membranes tested, but variation in the density of binding sites. In particular, brain mitochondrial preparations were shown to have a 10 times greater density of sites than liver. Since we have found that brain mitochondria are inactivated at twenty times lower concentration of toxin than are liver mitochondria (Wagner, G. M. and Kelly, R. B., manuscript in preparation), it is possible that the higher sensitivity of brain mitochondria results from more extensive binding. In addition, the dissociation constant for ^{125}I-labeled toxin binding to brain mitochondria (0.7 ± 0.1 nM) equals the concentration of native toxin required for half-maximal inhibition of calcium uptake [5]. Since the density of mitochondrial toxin binding sites is much less than the number of respiratory sites (3 nmol/mg protein) and calcium uptake sites (2 nmol/mg) [21], it is unlikely that the toxin inhibits mitochondria by a direct block of the ATP synthesizing sites or calcium channels.

The number of toxin binding sites per red blood cell is 125-fold less than the density of glycoprotein molecules in the membrane [22], which carries the majority of the polysaccharide groups on the cell surface, and is about 20 times more than the number of $(Na^+ + K^+)$-ATPase molecules [23]. As yet, attempts to solubilize a toxin-binding site from brain membranes have been unsuccessful. Thus it is as yet unresolved whether the saturable binding is to a protein, or carbohydrate or even some membrane lipid.

Comparison of the density of binding sites between different brain organelles (Table I) is of limited value if, as we have found, the toxin does not permeate plasma membranes. For example, the number of binding sites/mg protein might be quite different for intact and for lysed synaptosomes. Consequently, quantitative determination of the purity of such fractions using appropriate enzyme markers is needed before more meaningful comparisons can be made.

Since the toxin has been shown by us and others to exhibit a powerful inhibitory effect on mitochondria [5], and since inhibition of mitochondrial function could give rise to its electrophysiological effects [3], it is important to know if the toxin can penetrate the plasma membrane of the nerve terminal. The results here indicate that the toxin does not readily cross synaptosomal plasma membranes. This lack of permeation is consistent with the observation that the activity of β-bungarotoxin can be reversed by subsequent addition of antiserum against the toxin [24]. Also the inability of ^{125}I-labeled toxin to partition into organic solvents (unpublished observations) argues against membrane permeation due to lipid solubility.

If mitochondria are not the primary targets of toxin action in vivo how are we to explain the in vitro sensitivity? This paradox was resolved with the recent discovery that the toxin has phospholipase A2 activity [8], since mitochondrial function is known to be highly sensitive to phospholipases [25]. Evidence has been presented that the toxin makes use of its phospholipase activity to inactivate synaptic transmission [8]. Whether or not the inactivation involves direct modification of the plasma membrane by the phospholipase, or release of toxic components into the cytoplasm,

remains to be determined.

The surprising aspect of this study is that a toxin which shows binding characteristics very similar to cholera toxin [26] and nerve growth factor [27] should at the same time have a potent enzymatic activity which appears to be involved in its biological function. Since other phospholipases are not very toxic [8], we suggest that β-bungarotoxin has two sites, one a phospholipase site and another which, by permitting high-affinity binding to appropriate membranes, increases the local concentration of the toxin in the vicinity of its substrate. In the presence of calcium, preferential hydrolysis of phospholipids at the nerve terminal might then result. Whether or not the specific mechanisms we postulate are correct, the observations reported here make us wonder whether other protein molecules that bind to membrane sites with high affinity might also have enzymatic activity.

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