Title
Mechanism of action of a presynaptic neurotoxin, beta-bungarotoxin

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MECHANISM OF ACTION OF A PRESYNAPTIC NEUROTOXIN, BETA-BUNGAROTOXIN

by

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ABSTRACT

MECHANISM OF ACTION OF A PRESYNAPTIC NEUROTOXIN, BETA-BUNGAROTOXIN

Beta-bungarotoxin is a protein neurotoxin isolated from the venom of the snake Bungarus multicinctus. Brief exposure of neuromuscular preparations to this toxin is known to disrupt transmitter release irreversibly. Within 30 min after adding the toxin to a rat diaphragm-phrenic nerve preparation the quantal content increases ten-fold and the frequency of miniature endplate potentials, four-fold. No increase in miniature endplate potential frequency is observed in the absence of extracellular calcium. Since mitochondria may be involved in regulating intracellular calcium levels, and since beta-bungarotoxin is known to inhibit mitochondrial calcium uptake, the rate at which transmitter release is turned off was studied by measuring delayed release in the presence and absence of toxin. Delayed release is elevated about eight-fold by the toxin. If delayed release is due to residual calcium, as has been hypothesized, these data may be explained if the toxin does not alter the amount of calcium which enters the terminal, but rather the rate at which calcium is removed. Alternatively, a calcium-dependent modification of the release process itself might be produced.

Beta-bungarotoxin was recently shown to be a phospholipase A2 with activity comparable to that of highly purified enzymes from other snake venoms. In addition this activity has been found to require calcium both for its enzymatic action and to initiate its effects on transmitter release. However, despite the calcium dependence of toxin action, we could not be certain that the enzymatic function of the toxin is required for its physiological effects since transmitter release is also calcium dependent.

To decide whether the phospholipase activity is required for modification of transmitter release requires a method of inhibiting the enzymatic activity of the toxin without disrupting the release process itself. The finding that strontium inhibits the toxin's phospholipase activity whereas strontium can substitute for calcium in transmitter release, provides us with just such an inhibitor. In calcium the toxin causes an increase and subsequent decrease in miniature endplate potential frequency relative to controls. In contrast, only small changes are observed in strontium-containing solutions. This suggests that the toxin's ability to modify release is a function of its phospholipase activity.

The observation that beta-bungarotoxin has phospholipase A2 activity made us wonder whether phospholipase A2's from other sources were also toxic. Peter Strong of our laboratory found that injection of four-fold higher activities of Naja naja and Vipera russellii phospholipase A2's are not toxic to mice and likewise have no detectable effect on transmitter release. This means that although the phospholipase A2 activity of beta-bungarotoxin may be required for modification of transmitter release, this property in itself is not sufficient to account for the toxicity of beta-bungarotoxin.

To determine if the toxicity of this molecule is due to specific binding to neural tissue, an analysis of high affinity, saturable binding of ^{125}I labeled toxin to cell membranes is attempted. At low membrane protein concentration ^{125}I-toxin binding is directly proportional to the amount of membrane; at fixed membrane concentration ^{125}I-toxin shows saturable binding. Competition experiments indicate that iodination does not modify the binding affinity of the toxin. Comparison of toxin binding to brain, liver and red blood cell membranes shows that all have high affinity binding sites with dissociation constants of about one nanomolar. However, the density of these sites shows marked variation (e.g., 13.0 pmoles/mg for brain, 2.4 pmoles/mg for liver and 0.25 pmoles/mg for red blood cells). I also demonstrate that ^{125}I-toxin does not appear to cross the synaptosomal plasma membrane, suggesting that mitochondria may not be the toxin's site of action in vivo. A model able to account for the electrophysiological and biochemical observations is proposed.

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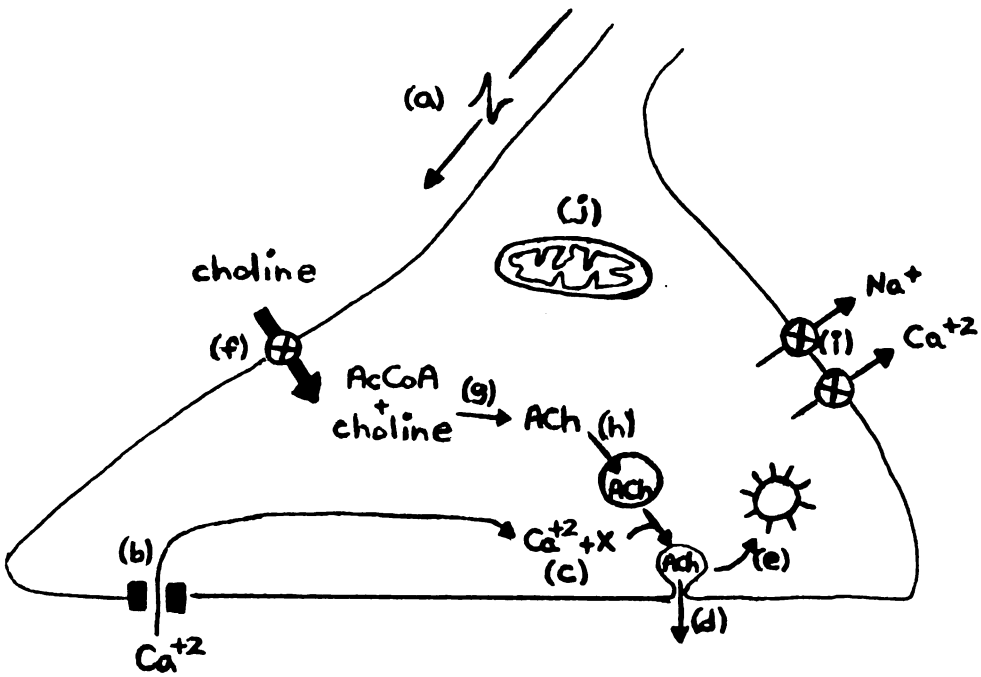
SECTION I - INTRODUCTION

Synaptic transmission, especially at the neuromuscular junction, has been extensively studied using electrophysical and anatomical techniques since before the turn of the century. It has been shown that at most synapses the presynaptic and postsynaptic cells are separated by a narrow gap (200 - 600 Å) filled with extracellular fluid which prevents direct electrical coupling between the cells. The transmission of electrical signals across such synapses occurs by means of release from the presynaptic nerve terminal of small molecules called neurotransmitters, hence the term chemical synaptic transmission. During the process of signal transmission, an electrical signal on the presynaptic cell causes the release of its appropriate transmitter into the extracellular gap, where it diffuses to specific sites on the postsynaptic cell causing a change in membrana permeability.

Events involved in chemical synpatic transmission have been studied most extensively at the vertebrate neuromuscular junction since the accessibility and anatomical simplicity of these synapses permit more direct analysis to be made than is possible at other chemical synapses. At the chemical synapse, transmission can be conveniently divided into presynaptic and postsynaptic events. In this report I will be concerned with the presynaptic events, in particular the mechanism of transmitter release. The major events now thought to be involved in this process are outlined in Figure 1.

Figure 1 MAJOR EVENTS IN NEUROMUSCULAR TRANSMISSION



(a) Conduction of action potential to the nerve terminal

(b) Calcium entry through voltage dependent channels

(c) Interaction of intra-terminal calcium with an "effector" molecule increasing the probability of vesicle fusion

(d) Exocytosis of vesicles containing acetylcholine

(e) Re-cycling of membrane

(f) Choline uptake

(g) Acetylcholine synthesis

(h) Packaging of acetylcholine into vesicles

(i) Re-establishing electrochemical gradients
i.e. extrusion of intraterminal calcium and sodium

(j) Energy supply and calcium buffering capacity of the mitochondria

Certain of the steps outlined in Figure 1 can be specifically inhibited by pharmacological agents. For example, transmitter release evoked by nerve stimulation (evoked release) can be prevented by blocking nerve conduction (a) with tetrodotoxin (1); by filling the calcium channels (b) with manganese or other metals (2); by preventing sodium extrusion (i) using cardiac blycosides (3); or by modifying the energy supply of the terminal (j) with anoxia (4).

Although pharmacological findings such as these have proved useful in describing transmitter release, there are two major difficulties hindering further progress. One is the lack of inhibitors for some of the more crucial steps initiating transmitter release, such as the interaction of Ca^{+2} with the effector molecule and the exocytosis process itself. The second is that, with the exception of tetrodotoxin and ouabain, pharmacological agents are of questionable specificity. For example, hemicholinium -3, a pharmacological agent widely used to inhibit choline uptake, affects four additional steps in synaptic transmission (5) and the well known postsynaptic acetylcholine antagonist, curare, has been reported to act presynaptically (6).

In recent years, a number of protein neurotoxins have been purified and shown to block neuromuscular transmission by irreversibly binding with specific macromolecules of the synapse. For example, alpha-bungarotoxin, isolated from the venom of krait Bungarus multicinctus, binds specifically and irreversibly to the acetylcholine receptors of the postsynaptic membrane, rendering the muscle cell

unresponsive to its chemical transmitter. (7) The ability of this toxin to bind with the acetylcholine receptor, combined with the facts that this toxin is easily isolated and radioactively labeled (8) has provided the biochemist with the first specific label for a macromolecule involved in synaptic function. In view of the specificity of protein toxins like alpha-bungarotoxin, the lack of specificity of presynaptic pharmacological agents might be solved by using protein neurotoxins which act presynaptically.

Several presynaptic neurotoxins are now known. Two familiar examples of presynaptic toxins are those produced by the anaerobic bacteria Clostridium botulinum and Clostridium tetani. However, the awesome toxicity of both botulinum (9) and tetanus toxin (10) has severely hindered proper biochemical fractionation work even with the modest mg quantities of material required for structural analysis with present "nanomole" scale techniques.

Presynaptic neurotoxins exhibiting functional similarity to these bacterial toxins have been isolated from three different elapid venoms. The venom neurotoxins are many orders of magnitude less potent than botulinium toxin. The elapid neurotoxins include notexin from the Australian tiger snake (Notechis scutatus scutatus) (11), taipoxin from the Australian snake, Taipan (Oxyuranus scutellatus) (12), and beta-bungarotoxin from the Formosan Krait (Bungarus multicinctus) (13).

Presynaptic neurotoxicity is not confined to only elapid snake venoms. Crotoxin, from the venom of the Brazilian rattlesnake, Crotalus durissus terrificus is predominately presynaptic in action (14).

Also, the venom of the black widow spider, Latrodectus mactans tridecimguttatus, causes bursts of spontaneous release that deplete motor nerve terminals of acetylcholine and synaptic vesicles (15). The toxicity of this spider venom seems to be associated with several high molecular weight proteins although only a little characterization data is now available (16).

Despite the varied sources of these toxins, all are defined as presynaptic because they block neuromuscular transmission by disrupting transmitter release while leaving the sensitivity of the endplate to acetylcholine unaffected (17). This implies that these toxins act by blocking one or more of the events outlined in Figure 1.

Some characteristics that these presynaptic neurotoxins have in common are: (a) following short incubation with these molecules, repeated washes are unable to prevent subsequent physiological effects, (b) inhibition of acetylcholine output from motor nerve terminals only occurs after a latent period of usually more than one hour (c) this latent period is not shortened by increasing the dose, however, as was found with botulinum (18), and beta-bungarotoxin (13), the higher the rate of indirect stimulation, the more rapid the blockage, (d) this latent period can also be shortened by increasing the temperature (e) pre-treatment of neuromuscular preparations with d-tubocurarine does not protect against its neuromuscular blocking action.

Explanations that have been evoked to account for the common features of these toxins are that they are irreversibly bound to nerve terminal sites, with the number bound being proportional to

toxin dose. This would account for the irreversible character of these agents. Once bound, some sort of time consuming, dose independent mechanism occurs, for instance pinocytosis of the toxin molecule to the inside of the terminal where it might interfere with some aspect of the release process. This sort of step has been used to explain the dose independent latency, temperature dependence and increased rate of onset of paralysis during indirect stimulation. To test some of these proposals concerning the mechanism of action of these presynaptic agents require a biochemical analysis of these agents. However, a complete examination of both electrophysiological and biochemical aspects has not been made for any of these toxins.

This work is concerned with the mechanism of action of one of these presynaptic neurotoxins, beta-bungarotoxin. Section I reports on the changes in transmitter release induced by the toxin when applied to a rat neuromuscular preparation in vitro. These modifications are analyzed in terms of the events listed in Figure 1 and possible mechanisms of actions are proposed. This electrophysiological analysis of toxin action is in press and that paper (19) comprised the first section of the report.

Beta-bungarotoxin was recently shown to a phospholipase A_2 with specific activity comparable to that of highly purified enzymes from other sources (20). This finding leads us to question whether the activity plays some role in toxin modification of transmitter release. To answer the question, Section II of the report includes

experimental evidence linking the hydrolytic activity of the toxin with the electrophysiological effects of Section I. The results indicate that beta-bungarotoxin's phospholipase A_2 activity is essential for toxin induced modification of the release process.

Although the phospholipase A_2 activity of beta-bungarotoxin is required for initiation of its presynaptic effects, this activity by itself may not be able to account for the high toxicity of the molecule. This was suggested by the finding that much higher activities of Crotalus adamateus, Naja naja, and Vipera russellii phospholipase activity of beta-bungarotoxin may be required for its action, the activity by itself is not able to account for its toxic effects.

In the third section, the interaction of radioactively labeled toxin with cell membranes is studied. ^{125}I-labeled toxin was found to bind with high affinity to a limited number of membrane sites, which had a much higher concentration in membranes of neural origin than in liver or red blood cell membranes.

The observations made in this study fit with a molecular model which accounts for the toxicity of beta-bungarotoxin by postulating selective binding to neural membrane sites followed by modification of the membrane by its phospholipase activity. The biochemical analysis of Section III is in press (22).

To summarize this work on the mechanism of action of beta-bungarotoxin, the electrophysiological data of Section I, along with the biochemical characterization of the last two sections are contrasted

to what is known about the action of other presynaptic neurotoxins. These findings are then analyzed in terms of current concepts of release (Figure 1) and in the final section a model able to account for these observations is proposed.

SECTION II - MODIFICATION OF TRANSMITTER RELEASE AT THE NEUROMUSCULAR JUNCTION

SUMMARY

The protein, beta-bungarotoxin, a pre-synaptic neurotoxin isolated from the venom of the snake Bungarus multicinctus, is known to inhibit mitochondrial function. Within 30 minutes after adding the toxin to a rat diaphragm-phrenic nerve preparation the quantal content increased ten-fold and the frequency of miniature endplate potentials, four-fold. No increase in miniature endplate potential frequency was seen in the absence of extracellular calcium. Since mitochondria may be involved in regulating intracellular calcium levels, the rate at which transmitter release is turned off was studied by measuring delayed release in the presence and absence of toxin. Delayed release is elevated about eight-fold by the toxin. If delayed release is due to residual calcium, as has been hypothesized, these data may be explained if the toxin does not alter the amount of calcium which enters the terminal, but rather the rate at which that calcium is removed. Alternatively, a calcium-dependent modification of the release process itself might be produced.

INTRODUCTION

In several cases the task of describing the molecular basis of nerve cell function has been simplified by the availability of highly specific neurotoxins. For example, identification and partial purification of the voltage-dependent sodium channel and the nicotinic acetylcholine receptor have been made possible by the availability of the neurotoxins tetrodotoxin (23, 24, 25) and alpha-bungarotoxin (8, 26). It might be hoped that a similar approach using toxins

specific for presynaptic sites might yield information, for example, on the nature of the voltage-dependent calcium channels or the transmitter release mechanism. Several protein neurotoxins are available which act presynaptically to modify both spontaneous release of transmitter and release evoked by nerve stimulation. These include botulinum toxin (27, 28, 29), tetanus toxin (30), Black Widow spider venom (31, 32), beta-bungarotoxin (33, 34) and Australian Tiger snake venom (35, 36). Unfortunately, for none of these toxins has the site or sites of action been precisely localized.

This paper presents electrophysiological data which help to identify the site of action of one of these presynaptic neurotoxins, beta-bungarotoxin. Earlier electrophysiological observations of the toxin's effect on synaptic transmission have shown that, in the period preceding synaptic failure, beta-bungarotoxin produces a small increase in the frequency of miniature endplate potentials (m.e.p.p.'s) and alters both the quantal content and the delayed release of transmitter, although no quantitative data were reported for the latter two (33, 34). Delayed or late release, the enhanced m.e.p.p. frequency in the few millisecond period following evoked release, has been associated with the removal of calcium from the intra-terminal cytoplasm (37, 38), and is modified in the presence of polyvalent cations, praseodymium (39) and strontium (40). Modification of delayed release by beta-bungarotoxin appeared significant to us since calcium uptake into mitochondria and sarcoplasmic reticulum are reduced in the presence of the toxin (41, 42). The implication that the toxin might affect calcium metabolism in the nerve terminals

led us to re-examine in more detail the electrophysiological response to beta-bungarotoxin, particularly with regard to delayed release and the requirement for extracellular calcium. The data presented here suggest that the toxin either reduces the rate at which calcium is removed from the nerve terminal, or alters the release mechanism itself.

METHODS

Purification of Toxin. Crude Bungarus multicinctus venom was obtained from Ross Allen Reptile Institute, Silver Springs, Florida. Purification of the beta-bungarotoxin component to homogeneity was according to the procedure of Kelly and Brown (34).

Electrophysiological Recording. Synaptic events were recorded intracellularly from muscle fibers of the rat diaphragm using standard electrophysiological techniques. Except during exposure to toxin (see below) the 5 ml bath containing the preparation was perfused at flow rates of 10 to 15 ml/min with Krebs solution containing 134 mM NaCl, 1 mM KH_2PO_4, 11 mM glucose, 12 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. Where different calcium, potassium and magnesium concentrations were used, these are given in the text. Iso-osmolarity was maintained by adjusting the NaCl concentration. Rapid perfusion rates were used since in earlier experiments (Kelly and Brown, unpublished observations), toxin-treated preparations were unusually susceptible to hypoxia. The Krebs solution was aerated with 95% O_2, 5% CO_2. The phrenic nerve was stimulated using square pulses of 0.5 msec duration, 0.5 to 1.0 volts magnitude, at frequences of 10 Hz for 10 sec intervals. All measurements were made at room temperature (22-25^{o}C).

The data from all cells with resting potentials below -60mV and with detectable miniature endplate potentials (m.e.p.p.'s) were used without further selection in calculating average values and standard errors. Values of the quantal content, estimated either from the number of failures or by the ratio of the average endplate potential amplitude to the average m.e.p.p. amplitude (43) were in good agreement.

To measure delayed release the time after each stimulus was divided into 10 msec intervals and the probability of detecting a quantal event in each 10 msec interval was measured.

Exposure to Toxin. Because of the limited supplies of pure beta-bungarotoxin it was not possible to perfuse the preparation with solutions containing beta-bungarotoxin. Since, from previous measurements (34), we knew that exposure of a preparation to beta-bungarotoxin for 15 min at 20 μg/ml was sufficient to get the maximum response (minimum time to fail), these conditions were employed throughout these experiments. At the end of the exposure period the perfusion rate was returned to normal

RESULTS

When an isolated rat diaphragm phrenic-nerve preparation was exposed to beta-bungarotoxin at 25°C, the tension produced by indirect stimulation was not affected for the first 100 minutes, and then began to fall to undetectable levels over the next several hours. Intracellular recording during the period of declining tension showed that sub-threshold e.p.p.'s of reduced quantal content could be detected accompanied with a decline in m.e.p.p. frequency (34).

The second part of this paper examines the possible causes of the decline in m.e.p.p. frequency, associated with the failure of neuromuscular transmission. The first part is concerned with the early changes in spontaneous, evoked and delayed release which occur immediately after adding the toxin. During this early time period the toxin acts to enhance the probability of release.

Modification of Spontaneous and Evoked Release by Beta-bungarotoxin

Beta-bungarotoxin has been reported to cause a small 4- to 5-fold transient increase in the frequency of m.e.p.p.'s in rat diaphragm muscle bathed in normal Krebs solution (33, 34). In contrast, other inhibitors such as Black Widow spider venom (32), cardiac glycosides (40), mitochondrial uncouplers (45) and lanthanum (46), produce a several hundred-fold stimulation in m.e.p.p. frequency before inhibiting release. These latter poisons also resemble each other in that the stimulation of m.e.p.p. frequency does not require extracellular calcium. Beta-bungarotoxin, however, does not raise the frequency of m.e.p.p.'s when added to a preparation in the absence of calcium (Figure 2).

If the peak m.e.p.p. frequency observed in toxin-treated preparations was compared to that for control preparations as a function of external calcium concentration, the slopes observed in a log-log plot (Figure 3) were the same, and close to that observed by Hubbard et al. (47) under similar conditions. To obtain a linear relationship it is necessary to subtract from the total m.e.p.p. frequency that measured in the absence of extracellular calcium to get the calcium-dependent rate of release. Thus, the calcium-dependent

Figure 2. Modification of spontaneous and evoked release in rat diaphragm-phrenic nerve preparations by beta-bungarotoxin. Using Krebs solution modified to contain 0.1 mM calcium and 1.5 mM magnesium, quantal content (—o—o—) was estimated either from the number of failures or by dividing the average endplate potential amplitude by that of the average endplate potential. Spontaneous release was measured on the same fibers (—Δ—Δ—). In a separate experiment, spontaneous release after toxin addition was measured in calcium-free solutions containing 1 mM EGTA (—▲—▲—). Preparations were exposed to beta-bungarotoxin at a final concentration of 20 μg/ml for 20 minutes, conditions known to produce neuromuscular block in a minimum time. Except for the first four points of the quantal content curve, all points represent the average of from 4 to 12 consecutive impalements and include the standard error of the means.

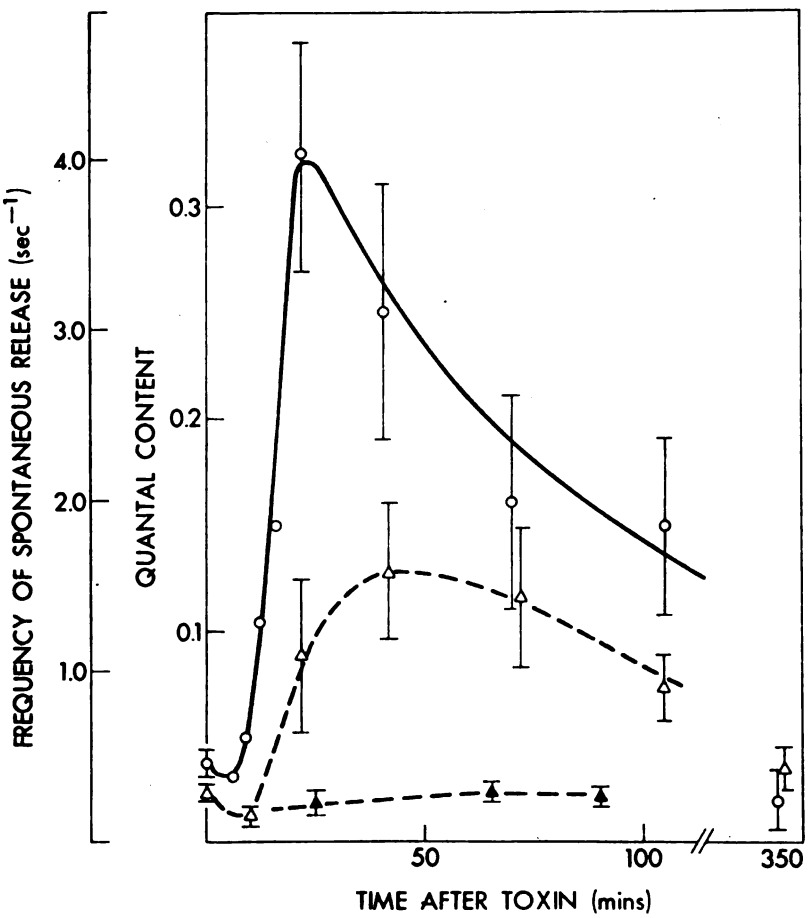
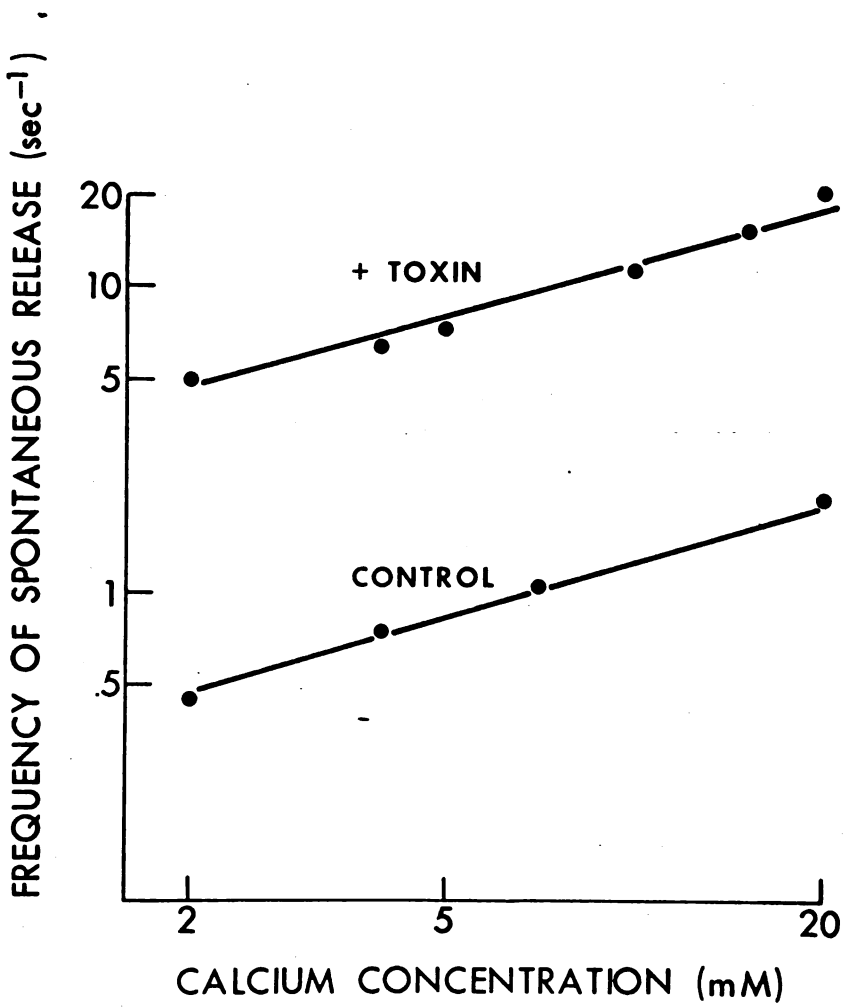

FREQUENCY OF SPONTANEOUS RELEASE (sec⁻¹)
4.0
3.0
2.0
1.0
QUANTAL CONTENT
0.3
0.2
0.1
50
100
350
TIME AFTER TOXIN (mins)

Figure 3. Maximum spontaneous release rate as a function of the extracellular calcium concentration in the presence and absence of toxin. To begin the experiment both control and toxin treated preparations were in Krebs containing 2 mM calcium, 4 mM KCl and 1 mM $MgCl_2$. At t=0 appropriate preparations were exposed to toxin (25 μg/ml) for 15 min and then perfused with Krebs medium containing the given concentrations of $CaCl_2$. All points represent the average of 6 or more consecutive impalements during a 5 to 10 min interval during which maximum release was observed. The release rate in the absence of added calcium (0.3 sec^{-1}) has been subtracted from all m.e.p.p. frequencies.


FREQUENCY OF SPONTANEOUS RELEASE (sec⁻¹)
20
10
5
1
.5
+ TOXIN
CONTROL
2
5
20
CALCIUM CONCENTRATION (mM)

rate of spontaneous release is increased about ten-fold by the toxin over the range of calcium concentrations studied.

To observe changes in quantal content after the addition of toxin the extracellular calcium concentration was reduced, to give sub-threshold e.p.p.'s. Multiple penetrations were made at intervals after the addition of toxin to the preparation and both m.e.p.p. frequency and quantal content were determined for each fiber. The data (Figure 2) show that after the addition of toxin the quantal content increased transiently to a maximum which was ten-fold greater than before toxin, while the maximum m.e.p.p. frequency was only four times greater than the control value.

An increase in quantal content to supra-threshold levels after exposure to beta-bungarotoxin has already been reported by Chang et. al. (33). While following the response to toxin of a single muscle fiber they also noticed complete failure of evoked release 80 min after toxin addition. We do not confirm this finding, but find that both the quantal content and the m.e.p.p. frequency increase and decline with a generally similar time-course (Figure 2).

It was possible to measure the dependence of quantal content on extracellular calcium only over a very small range of calcium concentrations (0.05 to 0.3 mM). In this range, a plot of log (quantal content) against log (extracellular calcium concentration) gave a slope of 1.9±.4 for the control and 1.7±.3 for the toxin-treated preparation.

Delayed Release in Toxin-Treated Preparations

It was noted by Kelly and Brown (34) that the frequency of m.e.p.p.'s increased markedly during stimulation of a toxin-treated preparation and that the frequency seemed to be highest just after an evoked response. In those experiments no attempt was made to quantitate the observations on what is commonly called delayed or late release. In the present set of experiments delayed release was systematically recorded in every fiber which had a measurable quantal content by determining the probability of a quantal event occurring in each 10 msec interval after nerve stimulation. In untreated preparations with 0.2 mM calcium the probability that a m.e.p.p. will occur in each interval after stimulation was small and relatively constant (Figure 4A, triangles). Addition of beta-bungarotoxin caused a large increase in the delayed release, as shown in Figure 4A. Delayed release was maximal 30 min after toxin addition and then slowly dimished with a time course that paralleled the changes in quantal content and spontaneous release. At 10 mM calcium the toxin again enhanced delayed release up to 8-fold (Figure 4B). Thus the extent of delayed release is increased about 8-fold, independent of extracellular calcium concentration, as was found for calcium-dependent spontaneous release (Figure 3).

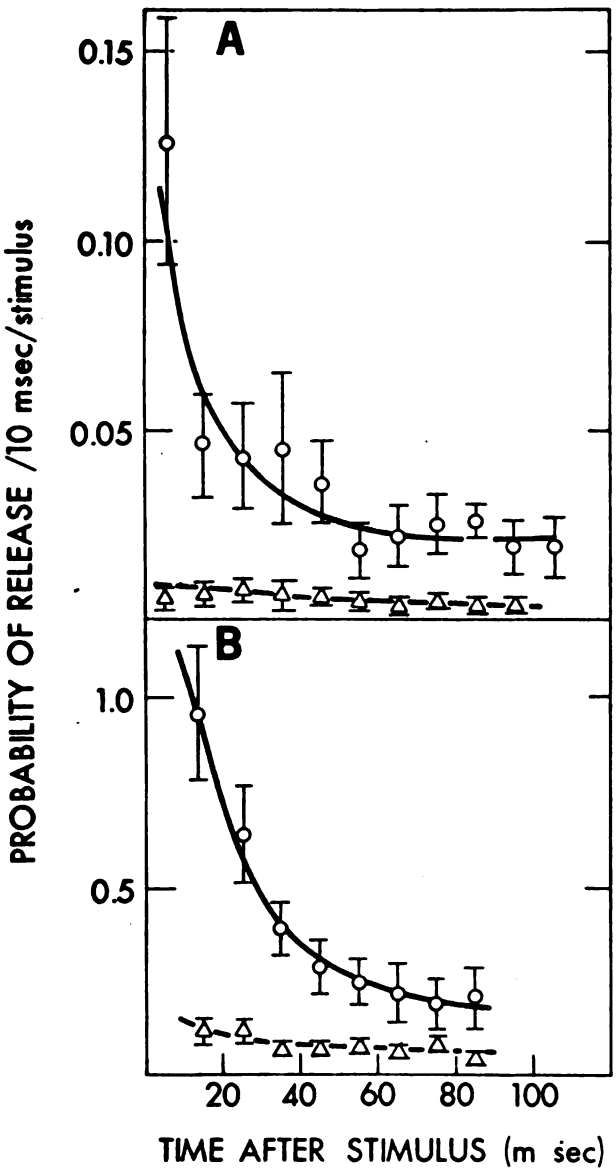
Delayed Release and Quantal Content

The toxin causes a parallel increase in delayed release and in quantal content. In their studies using the frog neuromuscular junction, Rahamimoff and Yaari (37) showed that raising the extracellular calcium also causes both quantal content and delayed release

Figure 4.

A. Modification of delayed release by beta-bungarotoxin. Experimental conditions as in Figure 1 except 0.2 mM calcium was used. The lower curve ( ) represents the average probability of detecting a quantal event in each 10 msec interval in 8 fibers measured before the addition of toxin; the upper curve (0), the equivalent values form 7 fibers measured 50 to 60 minutes after toxin were 0.5 ± 0.15 sec^{-1}, and 0,34 ± 0.07, respectively; 50 to 60 minutes after toxin addition, the corresponding values were 2.0 ± 0.5 sec^{-1} and 2.5 ± 0.5.

B. As in A except the calcium concentration was 10 mM, the magnesium 1 mM and the sodium concentration reduced to preserve osomolarity. Measurements were made on 8 fibers before toxin (spontaneous release rate = 2.7 ± 0.6 sec^{-1}) and 11 fibers, 30 to 80 minutes after toxin (spontaneous release rate 6.5 ± 0.8 sec^{-1}). Measurements in the first 10 msec interval were not possible because of the action potentials.


A
B
0.15
0.10
0.05
1.0
0.5
20
40
60
80
100
PROBABILITY OF RELEASE /10 msec/stimulus
TIME AFTER STIMULUS (m sec)

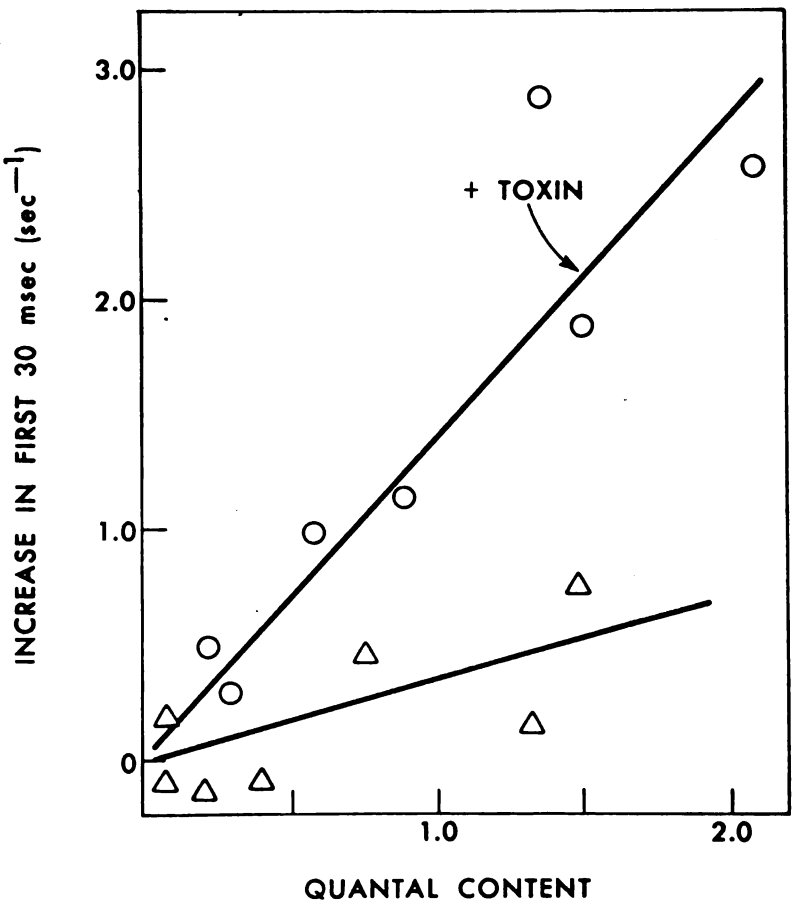
to increase. If raising quantal content of necessity raises delayed release then the findings described above might provide no new information on the toxin's action. It was important therefore to compare delayed release in control and toxin-treated preparations at the same quantal content, rather than at the same extracellular calcium concentration. Quantal content and delayed release were measured over a range of calcium concentrations in the presence and absence of toxin (Figure 5). Although the delayed release of control preparations does increase as the quantal content is increased, at any given quantal content it is higher in the toxin-treated preparation than in the control. Enhancement of delayed release by the toxin therefore is not a direct consequence of the increased quantal content.

Possible explanations of early enhancement of spontaneous, evoked and delayed release will be discussed later. The question of what causes the decline of transmitter release leading to failure of neuromuscular transmission will now be discussed.

Reduction of Transmitter Release by Toxin

The response of the neuromuscular preparation to beta-bungarotoxin is a two-fold one, consisting of the initial stimulation of quantal content, delayed release and m.e.p.p. frequency, which we have characterized above; and then a gradual decline in the release rate over a several hour period (Figure 2). In preparations exposed to Black Widow spider venom a similar response is seen and in this case the reduction in transmitter release has been attributed to depletion of the nerve terminals of releasable acetylcholine. If the reduction

Figure 5. Delayed release as a function of quantal content in the presence (0) and absence (Δ) of toxin. The experimental procedure was identical to that in Figure 1, but, for convenience in measuring delayed release is here expressed as the difference between the average rate of release per second during the first 30 msec after a stimulus, and the rate in the next 50 msec. Each point is the average of readings from 8 to 12 cells. Quantal content was altered by varying calcium concentrations between 0.05 and 0.3 mM.


3.0
2.0
1.0
0
1.0
2.0
+ TOXIN
INCREASE IN FIRST 30 msec (sec^{-1})
QUANTAL CONTENT

observed with beta-bungarotoxin-treated preparations was caused by vesicle depletion then it should depend on the rate of release of transmitter from the nerve terminal. In our experiments we chose to vary the rate of release of transmitter not by nerve stimulation since conduction block is a problem in energy depleted preparations (48) but by potassium depolarization of the terminals. Whereas control preparations could sustain high m.e.p.p. frequencies for several hours (Figure 6), potassium depolarization of toxin-treated preparations gave an initially higher rate of release, but this rate rapidly declined to very low levels. The response of these preparations to potassium depolarization, in fact, showed a general similarity to the changes in quantal content (Figure 2), namely an early stimulation followed by a slow reduction. The time course of changes in the m.e.p.p. frequency of toxin-treated preparations which had been bathed in different potassium concentrations is shown in Figure 7. Since the total number of quanta released could be varied more than 30-fold by changing the potassium from 4 to 27.5 mM, it is unlikely that depletion of releasable quanta caused the reduction in release rate.

The maximum frequency of spontaneous release in potassium depolarized preparations was shown to be linearly proportional to the external calcium concentration in a log-log plot, with a slope in the presence of toxin of $1.5 \pm .6$ and in its absence of $2.1 \pm .3$. It was also possible from data such as those in Figures 6 and 7 to compare maximum frequencies in different K^+ concentrations (4 to 27.5 mM), keeping extracellular

Figure 6. Spontaneous release in a potassium depolarized preparation in the presence (●---●) and absence (0--0) of toxin. To start the experiment, two hemidiaphragms from the same rat were exposed to Krebs containing no calcium, 4 mM KCl,1 mM $MgCl_2$ and 0.2 mM EGTA. At t=0 one preparation (●---●) was exposed to toxin (25 μg/ml) for 20 minutes and then both preparations were washed with Krebs containing 2 mM $CaCl_2$, 25 mM KCl and 1 mM $MgCl_2$. Release was alternately measured from both preparations throughout the experiment and data was represented as described in Figure 1.

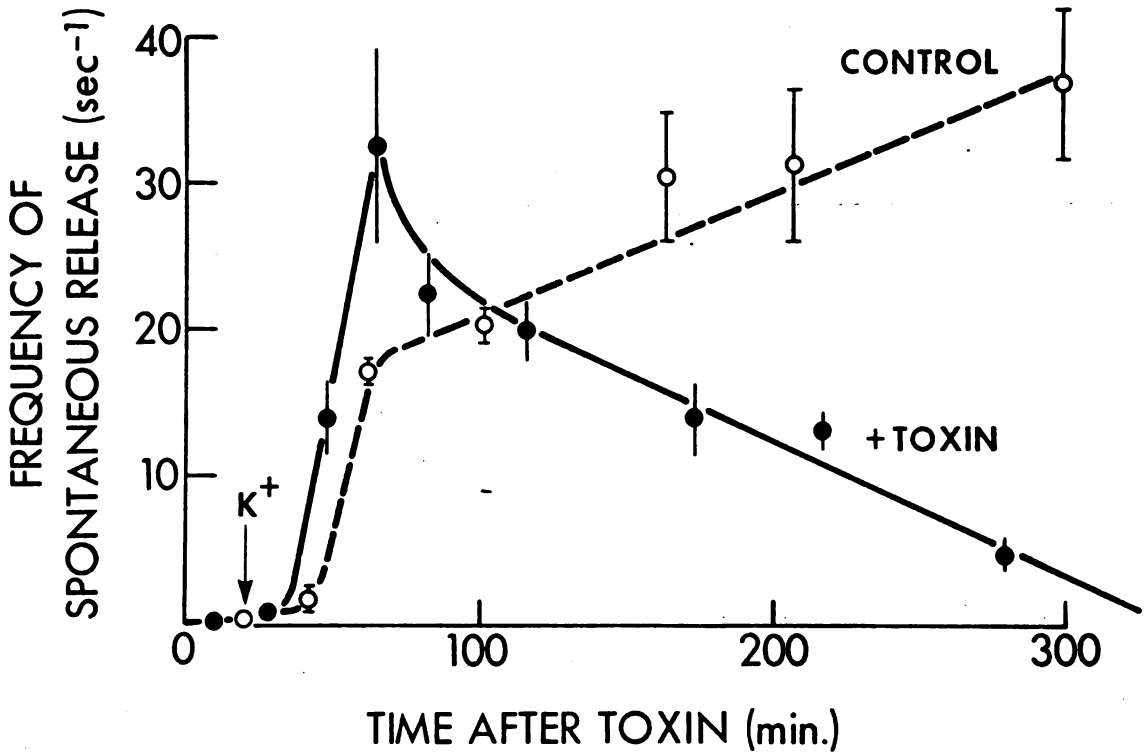
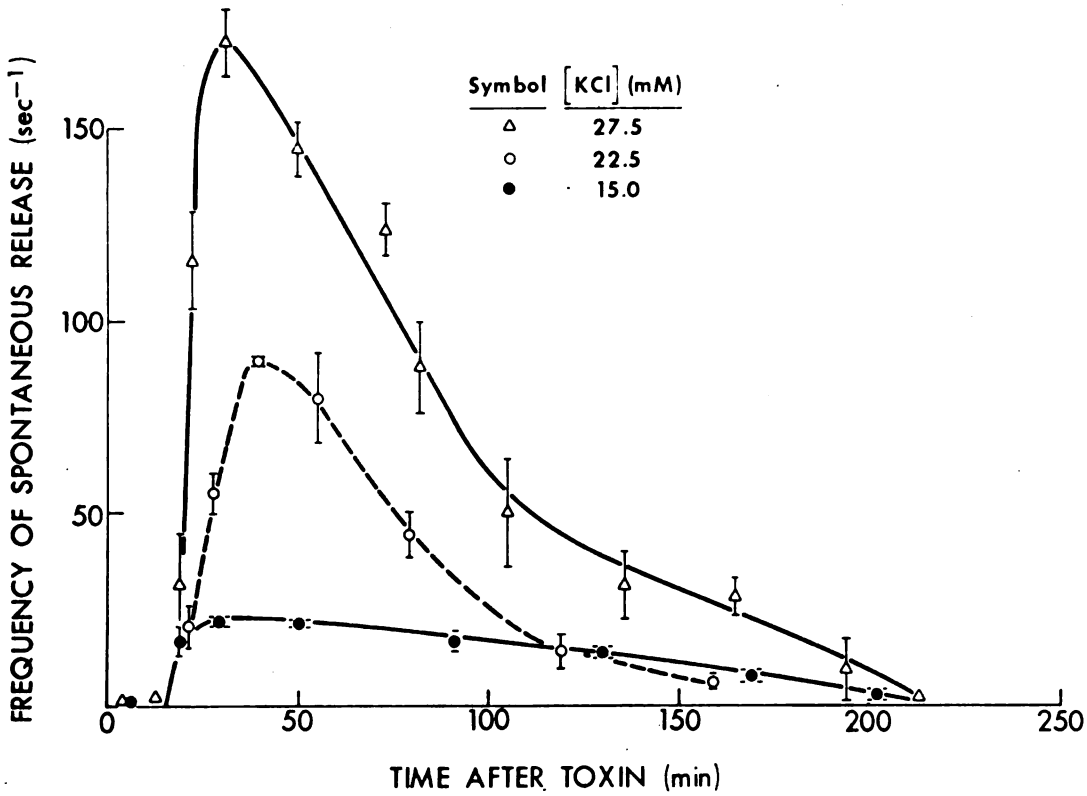

FREQUENCY OF SPONTANEOUS RELEASE (sec⁻¹)
40
30
20
10
0
100
200
300
CONTROL
+TOXIN
K+
TIME AFTER TOXIN (min.)

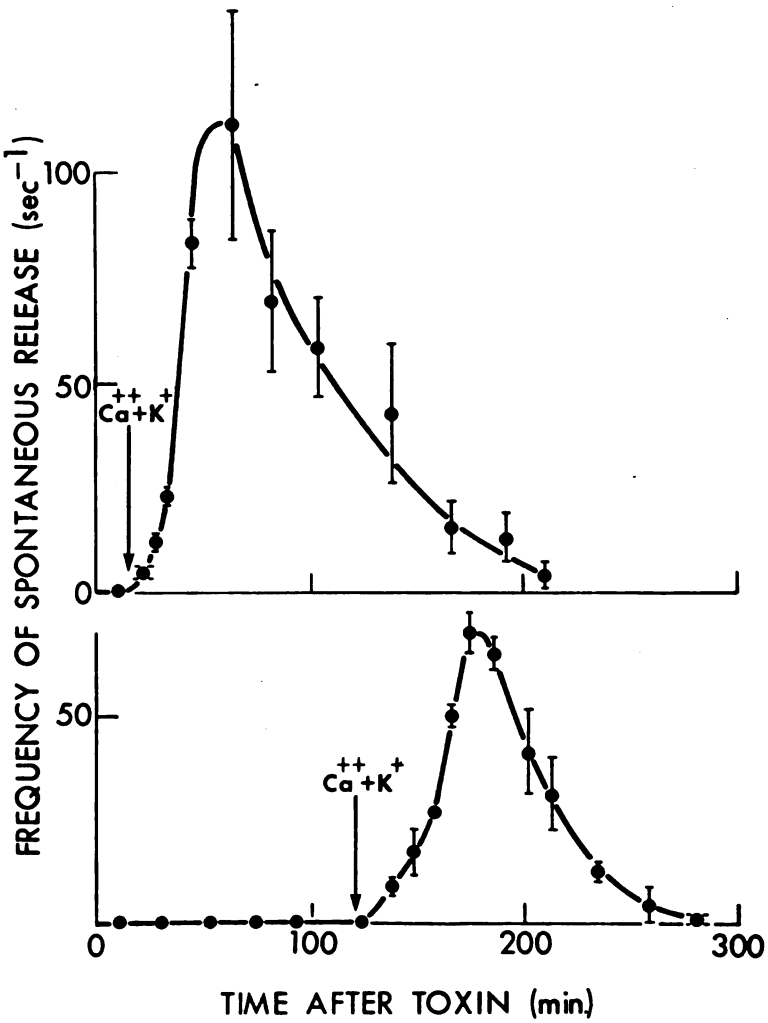
Figure 7. Modification of spontaneous release by toxin as a function of extracellular potassium. Experimental conditions as in Figure 5, except that toxin treated preparations were washed with Krebs containing 0.2 mM $CaCl_2$, 1 mM $MgCl_2$ and specified concentrations of KCl (15 mM, ●; 22.5 mM, 0; 27.5 mM, Δ). Mean frequencies were determined and presented as described in Figure 1.


Symbol
[KCl] (mM)
27.5
22.5
15.0
FREQUENCY OF SPONTANEOUS RELEASE (sec⁻¹)
150
100
50
0
50
100
150
200
250
TIME AFTER TOXIN (min)

Ca^{++} constant (2mM). Again the toxin produced no significant change in slope. These results suggest that the toxin does little to modify these aspects of the secretory process.

We have observed one other indication of the importance of extracellular calcium in toxin action. Earlier the requirement for calcium in the stimulation of m.e.p.p. frequency was demonstrated (Figure 2). If the drop in transmitter release is caused by a different mechanism than the early increase, extracellular calcium might not be required. In such a case extended exposure to beta-bungarotoxin in the absence of calcium should markedly reduce the total number of quanta released when calcium is returned to the bathing medium. In Figure 8 the results of an experiment are shown in which calcium was added back at 20 min, or after a 120 min delay. No marked reduction was observed after the 120 min delay, which led us to conclude that calcium is required not only for the initial stimulation of transmitter release but also for the events leading to eventual failure of release.

Figure 8. A specific calcium requirement for beta-bungarotoxin modification of spontaneous release. Both hemi-diaphragms from the same rat were equilibrated in Krebs modified to contain no calcium, and 0.2 mM EGTA (Ca^{++} chelating agent) Following 15 min incubation in this media one of the preparations was washed with Krebs containing 0.2 mM Ca^{++}, and 25 mM K^{+}. The second preparation was maintained for 2 hours in the toxin medium before it was washed with the same modified Krebs medium. Both preparations went through the same sequence of changes in spontaneous release following the addition of Ca^{++} and a depolarizing concentration of K^{+}. Data collected and presented as in Figure 1.


FREQUENCY OF SPONTANEOUS RELEASE (sec^{-1})
100
50
0
$Ca^{++}+K^{+}$
50
$Ca^{++}+K^{+}$
0
100
200
300
TIME AFTER TOXIN (min)

DISCUSSION

In these experiments we have studied several forms of transmitter release, spontaneous release in the absence of stimulation, release evoked by nerve stimulation and by potassium depolarization, and delayed release. After toxin addition all of these forms of release are stimulated and then decline with similar time courses. At all extracellular calcium concentrations studied, the maximum increase in quantal content and delayed release was about 10-fold. The maximum increase in spontaneous release is more difficult to assess. The maximum enhancement of spontaneous release was about half that of quantal content (Figure 2). However, if only calcium-dependent spontaneous release is measured the maximum increase after toxin-addition is once again about 10-fold (Figure 3). We conclude that toxin modifies all forms of calcium-dependent transmitter release to about the same extent, and with about the same time course.

When a nerve impulse invades a synaptic terminal, release of neurotransmitter results from an influx of calcium which briefly raises the calcium concentration in the terminal (49). After release is complete, the intra-terminal calcium concentration does not return immediately to its original level. It has been hypothesized that this "residual calcium" may be responsible for the phenomenon of facilitation (50) and delayed release (37). When extracellular calcium is raised the increased influx of calcium during stimulation should raise the quantal content and also delayed release, because of the time needed to reduce the elevated intra-terminal calcium to background

levels. Calcium-dependent spontaneous release is thought to depend on the steady-state level of intra-terminal calcium. Using this model of transmitter release, what sorts of modification of calcium metabolism might the toxin produce to give the observed effects? Possible modifications can be divided into three classes; increased calcium leakiness into the terminal; increased calcium influx through voltage-dependent channels; or a reduced rate of calcium removal from the terminal, either by transport sites on the plasma membrane, or intra-terminal organelles (51).

Let us consider first the possibility of a passive calcium leak into the terminal. If spontaneous and evoked release occur by the same mechanism, and specifically are dependent on the same power of the internal calcium concentration, it is difficult to imagine how the relatively small increase in internal calcium necessary to account for the small increase in m.e.p.p. frequency would not be completely swamped by the calcium which enters during nerve stimulation. Yet the toxin causes a 10-fold increase in quantal content (Figure 2). With the present set of assumptions, it is difficult to see how a small passive calcium leak could explain this result. The second possibility is that the voltage-dependent channel is modified allowing greater calcium influx during nerve stimulation. However, if we cause an increased calcium influx by raising the extracellular calcium we do not mimic the effect of the toxin and in particular, we cannot cause as large increases in delayed release as are seen in the presence of toxin (Figure 5). It is, therefore, unlikely that the toxin increases calcium influx. The third possibility, that the rate of calcium removal is reduced, is attractive since it could give rise to an increased

steady-state concentration of calcium, increasing the m.e.p.p. frequency; it could explain the quantal-content increase by extending the time course of elevated calcium in the terminal; and, of course, the latter might also produce the large amounts of delayed release which we observe. Finally, such a hypothesis is consistent with the biochemical findings that the toxin inhibits calcium uptake by mitochondria (41). If the toxin affects calcium metabolism in the nerve, then the most likely site of action is not the entry of calcium, but the calcium removal system. Such a model resembles that proposed by Alnaes and Rahaminoff (39) to account for the increase in delayed release which they observed in the presence of praseodymium. A major problem in this interpretation is that the toxin, a highly basic protein, must penetrate the plasma membrane of the nerve terminal to reach the mitochondria.

It may be misleading, however, to explain the data on the basis of the residual calcium hypothesis, to the exclusion of other alternatives. The rate of transmitter release depends not only on the intraterminal calcium concentration but on the release mechanism itself which is as yet undefined. The toxin may have no direct effect on the calcium metabolism of the nerve terminal, but instead might modify the terminal to alter the probability of a vesicle fusing with the presynaptic membrane. In some ways, this is a more attractive hypothesis, since three forms of transmitter release, spontaneous, evoked and delayed, which differ dramatically in their dependence on extracellular calcium, all increase to approximately the same extent, and with similar time courses. Since calcium is needed for toxin action, direct modifi-

cation of the release process would have to involve extracellular calcium. One possible molecular mechanism for a generalized stimulation of release might be an increase in the lysophospholipid concentration of the presynaptic terminal (52). In support of such a mechanism, we have recently noted that purified beta-bungarotoxin contains a very active calcium-dependent phospholipase A_2 activity. The enzyme activity of the toxin, and its modification of transmitter release are both inhibited when calcium is replaced by strontium (21).

The ultimate cause of transmission failure is puzzling. The decline of transmitter release is not hastened either by increasing the release rate or by elevating the calcium, although calcium is required for failure. Neither a gradual depolarization of the nerve terminal nor a failure of mitochondrial oxidative phosphorylation with concomitant release of calcium stores (45) would cause the m.e.p.p. frequency to decline.

SECTION III. ROLE OF PHOSPHOLIPASE A_2 ACTIVITY IN MODIFICATION OF TRANSMITTER RELEASE

The enzyme, phospholipase A, is one of the most abundant of a wide variety of hydrolytic enzymes found in snake venoms (53). A few early studies claimed that these phospholipase activities were responsible for the neurotoxicity of certain venoms, (54,55), only to have further purification separate phospholipase and neurotoxic components.

Peter Strong of our laboratory has recently detected phospholipase A activity in our beta-bungarotoxin preparation (21). Concerned that this activity might be due to a minor contaminant in our toxin preparation, the toxin was subjected to another ion-exchange purification step. Although a minor peak (approx. 10%) was resolved in this step, most of the toxicity and all of the phospholipase activity was associated with the major protein peak. This major component subsequently shown to be homogeneous by isoelectric focusing and SDS gel electrophoresis is known to have phospholipase A_2 specificity and a specific activity twice that of the highly purified Naja naja phospholipase A_2 isoenzyme ( ). Thus, the beta-bungarotoxin is a phospholipase A_2 with activity comparable to that of highly purified enzymes from other sources (21).

The finding of a phospholipase activity in beta-bungarotoxin led to the obvious question of whether this activity might be responsible for the toxin's effects on transmitter release (as outlined in Section I). In support of such a mechanism of action, beta-bungarotoxin has been found to require calcium both for its enzymatic action and to initiate its effects on transmitter release (Figure 7). However,

despite the calcium dependence of toxin action, we could not be certain that the enzymatic function of the toxin is required for its physiological effects since transmitter release is also calcium dependent (56). It is still possible that transmitter release itself is required for the initiation of its toxic effects. Such a proposal is supported by the finding of Lee et al (13) that transmission failure induced by the toxin can be facilitated by nerve stimulation. In other words, the calcium needed for the toxin's presynaptic action could be due to a requirement for an actively releasing nerve terminal, rather than any direct calcium requirement of the toxin itself.

To decide whether the phospholipase activity is required for modification of transmitter release requires a method of inhibiting the enzymatic activity of the toxin without disrupting the release process itself. The finding that strontium inhibits the toxin's phospholipase activity (21), whereas strontium can replace the calcium required for transmitter release (57), provided us with just such an inhibitor.

The ability of strontium to substitute for calcium in transmitter release was demonstrated in a frog neuromuscular preparation (57). Consequently, before we could look for strontium inhibition of toxin action we needed evidence that strontium can substitute for calcium at the rat neuromuscular junction. A comparison of the effects of spontaneous release rate in calcium and strontium containing Krebs are given as control experiments in Figure 9. The comparable response in both media indicate that strontium substitutes very effectively for calcium at the rat neuromuscular junction.

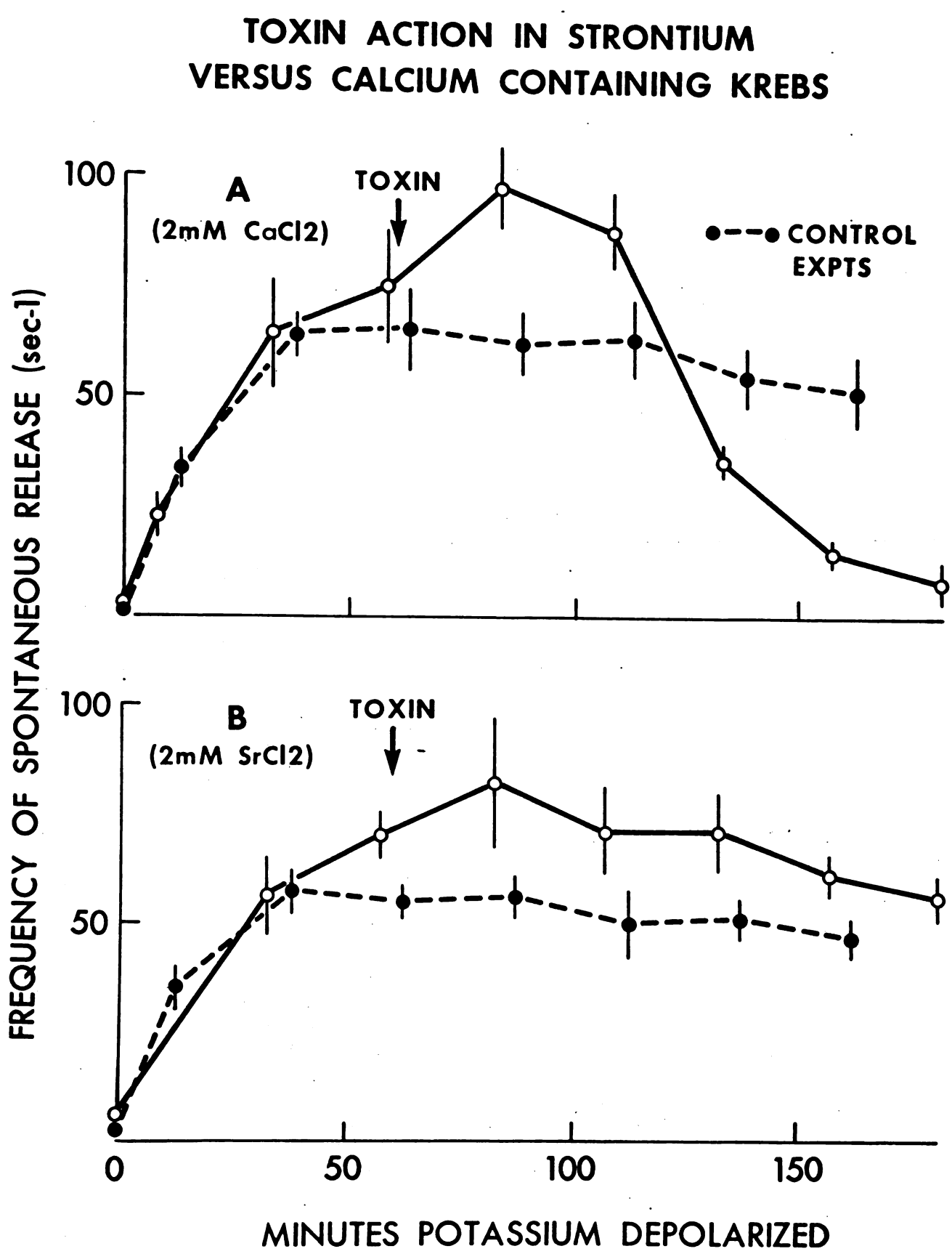
Figure 9.

A) Beta-bungarotoxin's modification of spontaneous transmitter release in Krebs media containing 2 mM $CaCl_2$.

A rat hemidiaphragm was dissected and placed in Krebs containing no calcium and 0.75 mM EGTA for 30 minutes at room temperature. The hemidiaphragms were then dluted for 10 minutes in this same media plus 2 mM $CaCl_2$. Next, 15 mM KCl was added to the elution media to depolarize the preparation. Following perfusion at 5 ml/minute for one hour, the flow was stopped and 125 mg toxin was added to the 5 ml bath. Following 15 minute exposures to toxin, flow was resumed.

The control preparation consisted of another hemidiaphragm exposed to the same procedures minus toxin. All recordings were made by multiple punctures in different areas of the diaphragm and are averaged over 25 minute intervals. All points are mean values from seven or more endplates. ($\pm$ standard error of mean).

B) This data was obtained by the procedures described in Part A except that 2 mM $SrCl_2$ was substituted for calcium in all of the media.


TOXIN ACTION IN STRONTIUM
VERSUS CALCIUM CONTAINING KREBS
100
50
A
(2mM CaCl2)
TOXIN
CONTROL
EXPTS
100
50
B
(2mM SrCl2)
TOXIN
FREQUENCY OF SPONTANEOUS RELEASE (sec-1)
0
50
100
150
MINUTES POTASSIUM DEPOLARIZED

The ability of strontium to inhibit beta-bungarotoxin's action on spontaneous release is also illustrated in Figure 9. Hemidiaphragms in either calcium (A) or strontium (B) were depolarized by increasing the potassium concentration in order to increase the m.e.p.p. frequency to steady state levels that allow rapid determinations. In calcium, the toxin caused a stimulation and subsequent decrease in frequency relative to the control preparation. In contrast, only small changes were observed in strontium-containing solutions. This suggests that beta-bungarotoxin's ability to modify is a function of its phospholipase activity.

The phospholipase activity of beta-bungarotoxin made us wonder whether phospholipases A_2's from other sources were also toxic agents. It was found that injection of greater than 100 fold higher activities of Vipera russellii and Naja naja phospholipase A_2's were not toxic to mice (17). In addition, exposure of rat neuro-muscular preparations to five times as many phospholipase units of Russell's viper phospholipase as are contained in the usual doses of beta-bungarotoxin had no detectable effect on m.e.p.p. frequency. This means that although the phospholipase A_2 activity of beta-bungarotoxin may be required for modification of transmitter release, this property in itself is not sufficient to account for the toxicity of beta-bungarotoxin. Consequently, beta-bungarotoxin has some unusual property that other phospholipases lack. Section IV of this report involves the analysis of a specific property which might account for the unusual toxicity of this molecule.

SECTION IV. HIGH-AFFINITY BINDING OF TOXIN TO CELL MEMBRANES

SUMMARY

Brief exposure to the protein neurotoxin, beta-bungarotoxin, is known to disrupt neuromuscular transmission irreversibly by blocking the release of transmitter from the nerve terminal. This neurotoxin also has a phospholipase A_2 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-toxin binding was directly proportional to the amount of membrane; at fixed membrane concentration ^{125}I-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 pmoles/mg protein for a brain membrane preparation, 2.4 pmole/mg for liver and 0.25 pmole/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-toxin binding sites on brain

mitochondrial preparations (3.3 ± 0.3 pmoles/mg) exceeded by a factor of ten the density on liver mitochondrial preparations (0.3 ± 0.05 pmoles/mg). It is also shown that labeled toxin does not cross synaptosomal membranes, suggesting that mitochondria may not be the site of action of toxin *in vivo*. We conclude that beta-bungarotoxin is an enzyme which can bind specifically with high affinity to cell membranes.

INTRODUCTION

Beta-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 (13). 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 (13,34,19) and its biochemical properties (41,42,58). 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 (34). Since early attempts to measure binding to 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 (58) and calcium uptake by mitochondria and sarcoplasmic reticulum (4142) may result from its phospholipase A_2 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 ethylene diamine-tetraacetic 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 (34), 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 Banarjee et al. (59). Ten 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 sec incubation at 25°, 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% trichloracetic acid and the percent precipitable counts determined. The specific activity of the iodinated toxin (^{125}I-toxin) varied between 10-28 μCi/μg depending on the concentrations of toxin and $Na^{125}I$. If a molecular weight of 21,800 is assumed (34) for the toxin, this represents a specific activity of from 220 to 610 Ci/mmole, or about 0.1 to 0.28 moles of ^{125}I per mole of toxin.

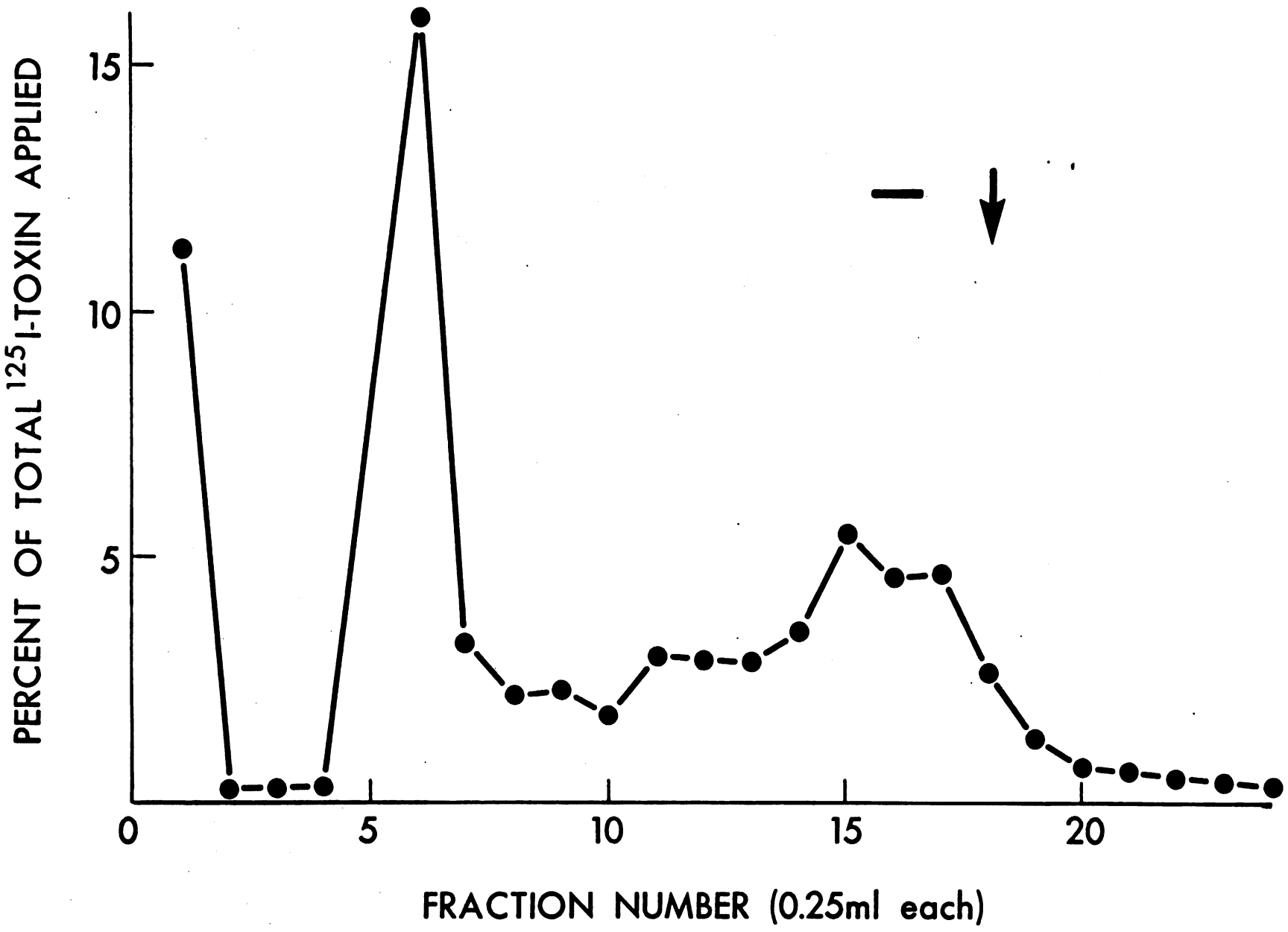
When freshly prepared ^{125}I-toxin was combined with native toxin both protein and radioactivity co-migrated as a single discrete band during sodium dodecyl sulfate gel electrophoresis in the absence of reducing agents. When a mixture of native and labeled toxin was dissociated into two dissimilar subunits (approximately 12,000 and 8,000

daltons) by reduction with 2-mercaptoethanol (34), the protein and ^{125}I co-migrated as two discrete bands of the appropriate subunit molecular weights, implying that both toxin subunits were labeled by this procedure. ^{125}I-toxin was stored at -20° for 2 to 3 weeks with less than a 5% loss in specific binding activity.

Beta-bungarotoxin is a potent phospholipase A_2 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 (20), an assay of the enzyme activity provides a sensitive test of the effect of iodination of the toxin. 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 (21). The iodinated derivative retained at least 80% of the native activity.

Assay of ^{125}I-Toxin Binding to Membranes. The procedure used to assay ^{125}I-toxin binding to membrane preparations was similar to that described by Almon et al (60). Membrane fractions were incubated for 20 min at 25° with given concentrations of ^{125}I-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° on 4.5 ml G-200 Sephadex columns (0.7 x 10 cm) with 50 mM Tris-HCl (pH 7.6), 1 mM EDTA and 0.05% (w/v) trypsin inhibitor as shown in Figure 10. The entire excluded volume (approximately 2 ml) from each of these

Figure 10. Separation of free from membrane bound ^{125}I-toxin by Sephadex G-200 gel filtration. 100 µg of brain membrane was incubated for 20 min at 25° in 0.2 ml of 50 mM Tris-HCl (pH 7.6), 0.05% trypsin inhibitor containing 1.0 pmole ^{125}I-toxin (610 Ci/mmole). 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-toxin elutes as a single peak at position indicated by a horizontal bar. The arrow indicates the total column volume.


PERCENT OF TOTAL ^{125}I-TOXIN APPLIED
15
10
5
0
5
10
15
20
FRACTION NUMBER (0.25ml each)

columns was collected and counted to give the total ^{125}I-toxin excluded. In the absence of membranes as much as 10% of the total ^{125}I-toxin applied to the G-200 column was eluted in the first 0.25 ml fraction (Figure 10) due to wall effects. Consequently all values given for bound ^{125}I-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°. 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-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 (61). 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°. 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 (62) 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 Clarke and Nicklas (64).

Further Fractionation of Brain Membranes. Brain membranes were subfractionated into "myelin", "synaptosomal" and "mitochondrial" enriched membrane fractions on discontinuous, isotonic Ficoll-sucrose density gradients as described by Morgan et al. (62) 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 membranes (62). All membrane 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 (65). Cytochrome C oxidase, a marker for the inner mitochondrial membrane, was assayed by the procedure of Lu et al. (66), (Na^+-K^+)-ATPase, a plasma membrane marker, was assayed according to Morgan et al. (62).

Analytical Techniques. Inorganic phosphate was selectively precipitated as phosphomolybdic acid (67), 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 Spectrophometer. ^{125}I-toxin was counted at 41% efficiency with a Beckman Gamma 300 counter for sufficient time to obtain greater than 10^5 counts. Gamma-^{32}P-labeled ATP was prepared by the procedure of Chamberlin and Ring (68) and was further purified by paper chromatography in order to minimize inorganic phosphate contamination.

RESULTS

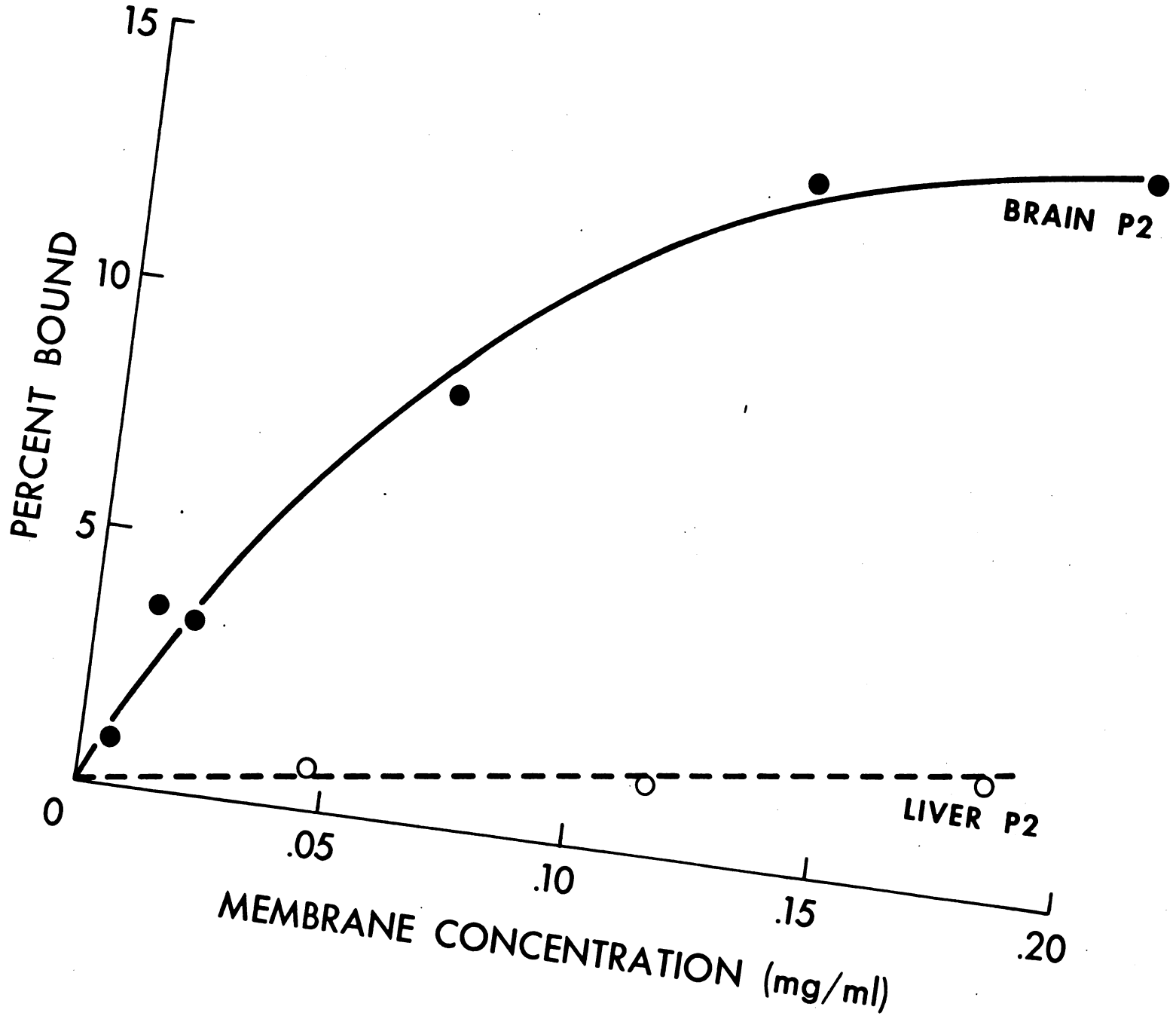
Gel Filtration Binding Assay

Binding of small molecules to cell membranes is often assayed by Millipore filtration or differential centrifugation (69). Preliminary experiments showed that use of these techniques to study the binding of beta-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-toxin appeared at 0.9 times the column volume (Figure 10). Increasing 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-Toxin Binding

To find the appropriate range of membrane concentrations for affinity measurements 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 (Figure 11). At low membrane concentrations, the binding of ^{125}I-toxin was directly proportional to the concentration of brain and liver membrane protein but a marked preference for brain membranes was noticed. It would appear that brain membranes have a much greater density of high affinity sites than liver membranes. Even at very high concentra-

Figure 11. Percent of ^{125}I-toxin bound to brain (●---●) and liver (0---0) membrane as a function of membrane protein concentration. The indicated concentration of membrane protein was incubated for 20 min at 25° in 0.2 ml of 50 mM Tris-HCl (pH 7.6), 0.05 trypsin inhibitor containing 0.5 pmoles ^{125}I-toxin (610 Ci/mmole). Percent ^{125}I-toxin bound was determined by Sephadex G-200 filtration as described in Methods section.


15
10
5
0
PERCENT BOUND
BRAIN P2
LIVER P2
.05
.10
.15
.20
MEMBRANE CONCENTRATION (mg/ml)

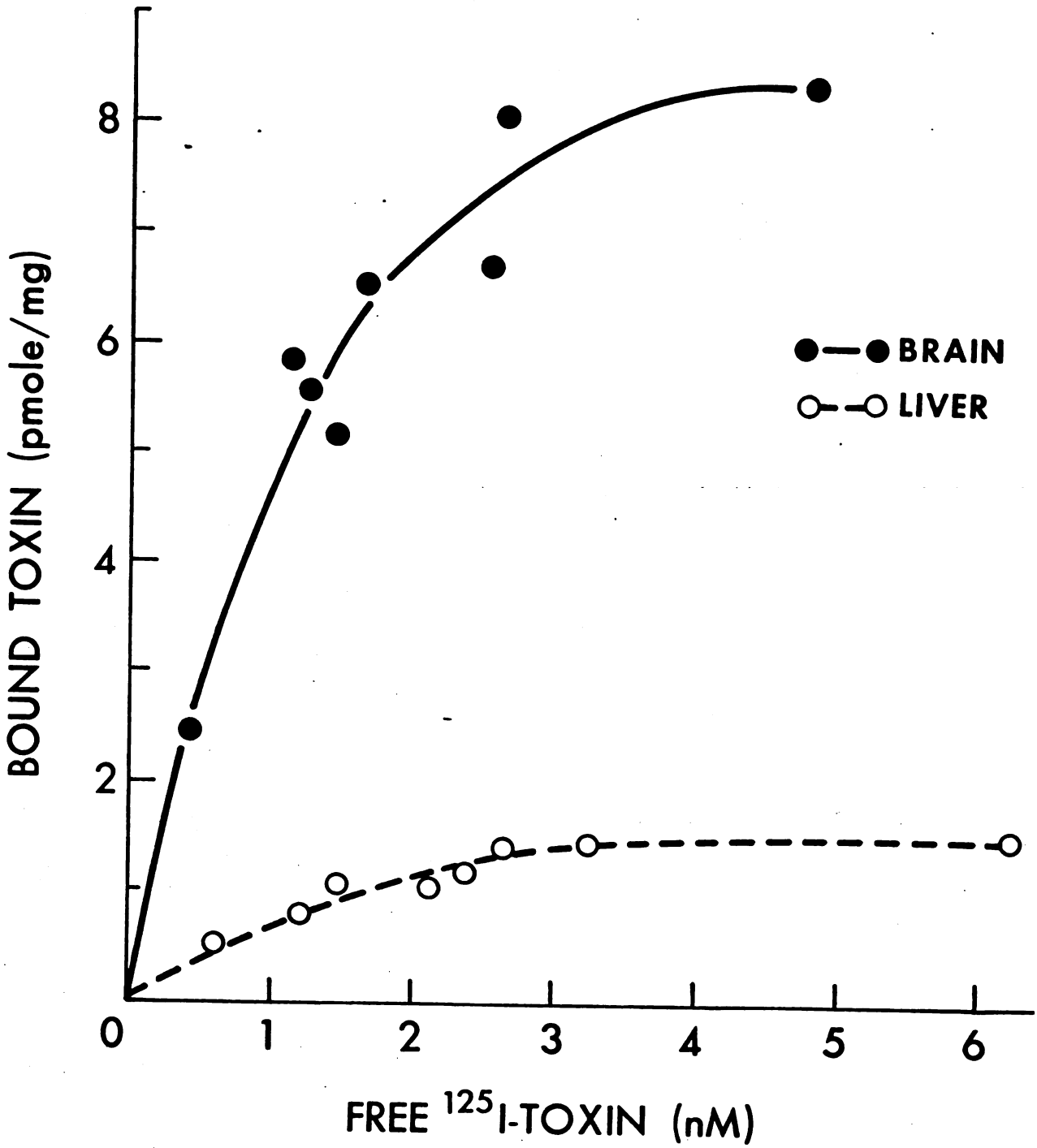
tions of both brain and liver membranes, a maximum of 20% of the ^{125}I-toxin could be bound at 2 nM (Figure 11). This may mean that only about 20% of the iodinated toxin was capable of binding. However, when the experiment was repeated using toxin which had not bound the first time, once again, a maximum of 22% bound to brain membranes (300μg/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 (21) properties of beta-bungarotoxin, we wondered if the presence or absence of these ions might modify toxin binding. To test this, we compared ^{125}I-toxin binding as described in Figure 11, with or without 2 mM concentrations of either calcium or strontium. Neither ion had an effect on ^{125}I-toxin binding relative to controls lacking divalent cations.

Affinity of ^{125}I-Toxin Binding to Brain and Liver Membranes

To measure the affinity of ^{125}I-toxin for membranes, a concentration of membrane was chosen such as to be in the linear range in Figure 11, and the amount of ^{125}I-toxin bound to brain or liver membranes was measured as a function of free ^{125}I-toxin (Figure 12). Assuming that the included toxin represented unbound ^{125}I-toxin and that binding equilibrium was not altered by column chromatography, a Scatchard analysis of this data (70) was used to determine

Figure 12. Effect of ^{125}I-toxin concentration on its binding to brain and liver membranes. Assay tubes contained 12.8 µg brain or 63.7 µg liver membrane protein and indicated concentration of ^{125}I-toxin (610 Ci/mmole) in 0.2 ml of 50 mM Tris-HCl (pH 7.6), 0.05% trypsin inhibitor. Binding was assayed as described in Methods.


BOUND TOXIN (pmole/mg)
8
6
4
2
0
1
2
3
4
5
6
FREE 125I-TOXIN (nM)
BRAIN
LIVER

dissociation constant (Kd) and the amount of ^{125}I-toxin binding sites per mg membrane protein (Rt) (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 pmole/mg for brain and 2.4 ± 0.8 pmole/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 pmoles/mg protein) (41).

Binding of Native Versus Iodinated Toxin

In order to test whether the binding of ^{125}I-toxin (Figure 12) was similar to that of native toxin, brain membranes were incubated with nanomolar concentrations of ^{125}I-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 (Figure 13). If the binding properties of the labeled and native toxin were 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 this 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.

^{125}I-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

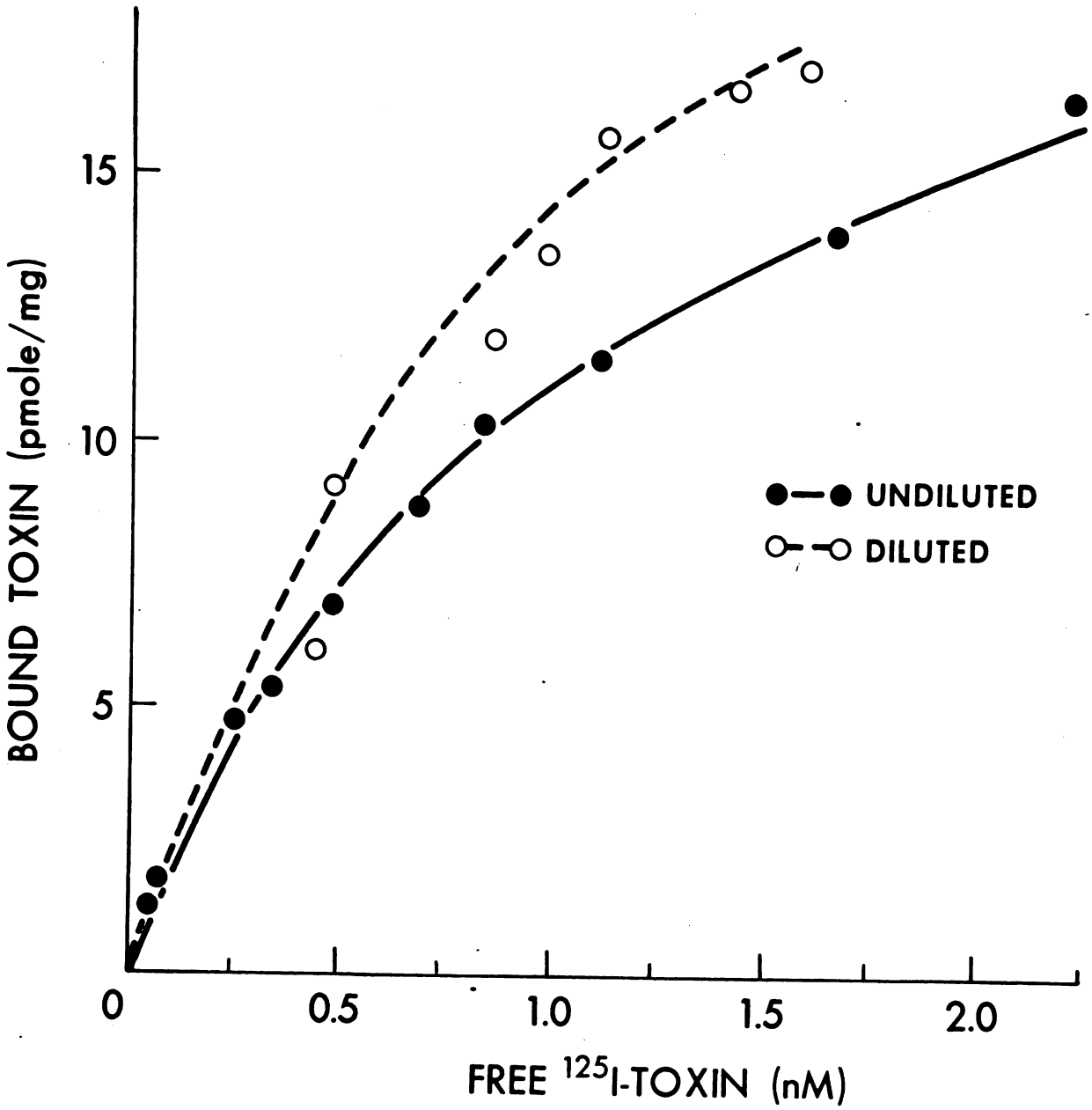
TABLE I

Comparison of ^{125}I-Toxin Binding to Various Membrane Preparations

Membrane Preparation	Kd (nM)	R_T (pmole/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 ± .05	0.023
Red Blood Cells	0.71 ± .05	0.25 ± 0.03	0.019

The data presented in this table was obtained by making Scatchard plots (19) of the binding data presented in Figures 2 and 4. In such plots, the slope is equal to -1/Kd and the extrapolated intercept on the abscissa is equal to the number of binding sites (R_T) present in the incubation mixture. A least square analysis was performed on this data. All values are presented ± standard deviations.

Figure 13. Binding of ^{125}I-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° with different concentrations of the undiluted ^{125}I-toxin preparation (550 Ci/mmole) (●) or with diluted preparations (0) containing 0.5 nM ^{125}I-toxin plus different amounts of native toxin to give the concentrations specified.


BOUND TOXIN (pmole/mg)
15
10
5
0
0.5
1.0
1.5
2.0
FREE 125I-TOXIN (nM)
UNDILUTED
DILUTED

various sub-fractions of brain. This was of special interest since earlier work had 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-toxin to these fractions was determined as described in Figure 10. 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 density of binding sites in low density membrane fragments, synaptosomes and mitochondria enriched fractions were 1 to 0.7 to 0.3 respectively.

Relative Density of ^{125}I-Toxin Binding Sites in Different Membrane Preparations

Since nanomolar concentrations of beta-bungarotoxin are known to inhibit calcium uptake into brain mitochondria but not into liver mitochondria (20), we compared saturable binding of ^{125}I-toxin to these preparations. Brain mitochondria were prepared according to procedures used in these uptake studies and ^{125}I-toxin binding was assayed at a fixed concentration of mitochondrial protein. As illustrated in Figure 5, high affinity (Kd = 0.7 $\pm$ 0.1 nM) saturable binding was observed. However, the number of sites in brain mitochindria (3.3 $\pm$ 0.3 pmole/mg) was ten-fold greater than the number found in an equivalent experiment using purified liver mitochondria (0.3 $\pm$ 0.05 pmole/mg) which suggests that the increased toxin sensitivity of brain mitochondria might result from preferential binding of the toxin.

^{125}I-toxin also binds with high affinity to human blood cells (Kd = 0.7 $\pm$ 0.05 nM). The number of binding sites is around 0.25 pmoles per mg of blood cell protein (Figure 14). This is equivalent to about 4000 ^{125}I-toxin binding sites per cell. The density of binding sites in different membrane preparations is summarized in Table I. Although

TABLE II

^{125}I-Toxin Binding to Ficoll Density Fractions of Brain

Brain Membrane Fraction	Initial Binding Slope (% bound/μg/ml)	Density Relative to fragments
Low density fragments	2	1.0
Synaptosomes	1.4	0.7
Mitochondria	0.6	0.3

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-toxin and a binding curve was constructed for each as shown in Figure 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.

high affinity binding sites were found in all of these preparations, the density was highest in brain.

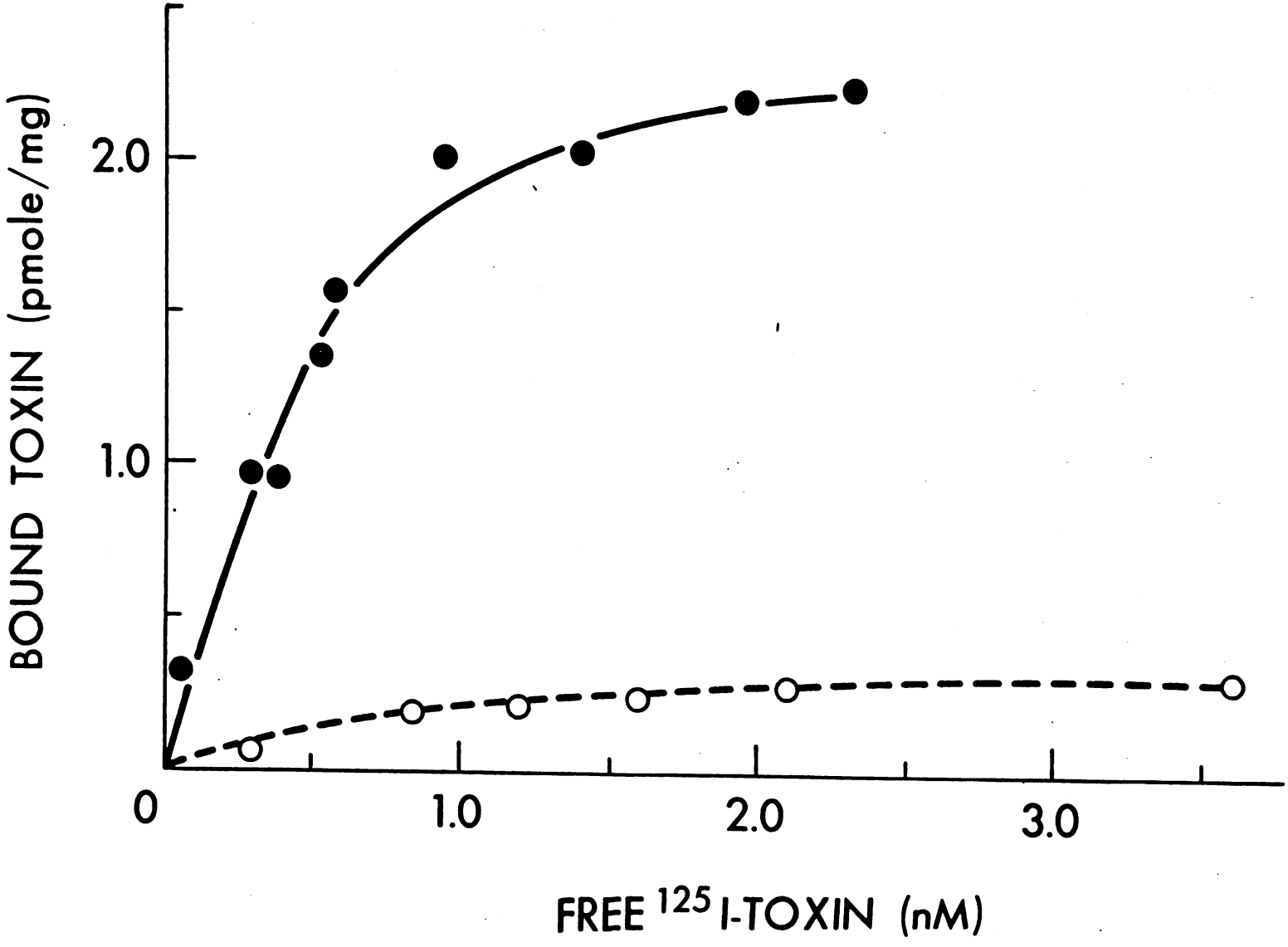
^{125}I-Toxin Binding in Intra-Synaptosomal Mitochondria

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 (71), were prepared by density sedimentation, labeled with ^{125}I-toxin (1nM) and centrifuged to separate bound from unbound ^{125}I-toxin. The labeled synaptosomes were then exposed to hypoosmotic 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-toxin and membrane specific enzymes. As shown in Figure 15, the peak specific activity of ^{125}I-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-toxin. It thus appears that ^{125}I-toxin was unable to cross the plasma membrane and bind to intra-synaptosomal mitochondria under these assay conditions.

DISCUSSION

In this report we have shown high affinity binding of ^{125}I-toxin to a limited number of sites in brain membrane preparations using gel

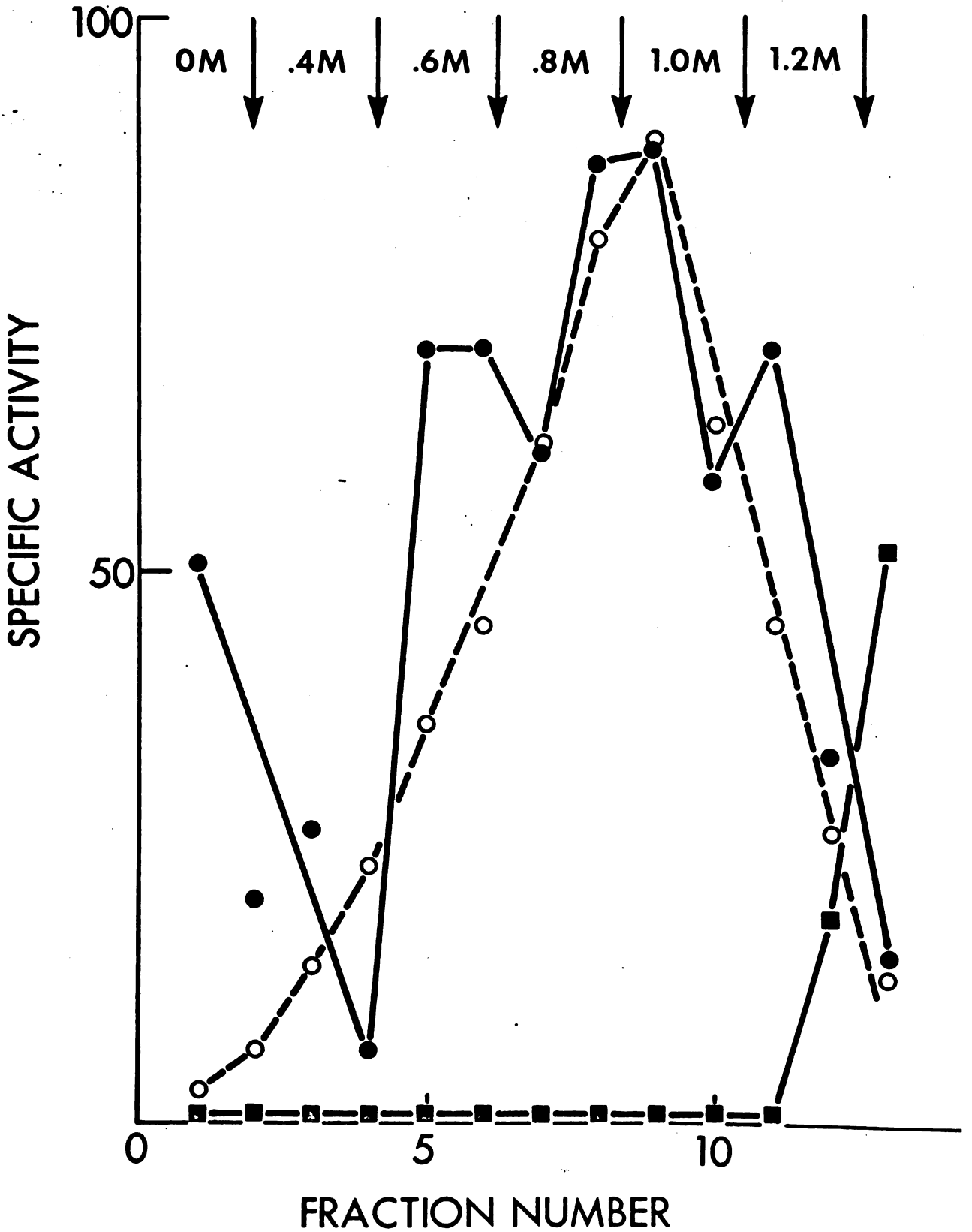
Figure 14. ^{125}I-toxin binding to brain mitochondria (●) and red blood cell (○) membranes. Assay tubes contained 12.8 g of brain mitochondria or 280 g red blood cell membranes and the indicated concentrations of ^{125}I-toxin (610 Ci/mmole) in 0.2 ml of 50 mM Tris-HCl (pH 7.6), 0.05% trypsin inhibitor. All tubes were incubated for 20 min at 25°. Binding was assayed as described in Methods section.


BOUND TOXIN (pmole/mg)
2.0
1.0
0
1.0
2.0
3.0
FREE 125I-TOXIN (nM)

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 if either 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-toxin (Figure 13). 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 (Figure 15). 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 20% observed in Figure 11. 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-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 ten times greater density of sites than liver. Since we have found that brain mitochondria are activated at twenty times lower concentration of toxin than are liver mitochondria

Figure 15. 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 pmoles of ^{125}I-toxin (200 Ci/mmole) for 20 min at 0°. The suspension was then centrifuged for 20 min at 27,500 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° causing hypoosomotic lysis (greater than 90% of total LDH activity was released). Two milliliters 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 g for 2 hr. One ml fractions were collected and assayed for ^{125}I-toxin binding, Na^+-K^+ ATPase activity, cytochrome C oxidase activity and protein as described in Methods. ^{125}I-toxin specific activity (0) 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 pmoles Pi released hr^{-1} x (μg protein)$^{-1}$ for (Na^+-K^+)-ATPase (0), 500 cpm ^{125}I-toxin bound/mg protein (0), and 1 μg cytochrome C oxidized $(min)^{-1}$ x (μg protein)$^{-1}$ for cytochrome C oxidase. (■).


100
50
0
5
10
0M
.4M
.6M
.8M
1.0M
1.2M
SPECIFIC ACTIVITY
FRACTION NUMBER

(71), it is possible that the higher sensitivity of brain mitochondria results from more extensive binding. In addition, the dissociation constant for ^{125}I-toxin binding to brain mitochondria (0.7 $\pm$ 0.1 nM) equals the concentration of native toxin required for half-maximal inhibition of calcium uptake (41). Since the density of mitochondrial toxin binding sites is much less than the number of respiratory sites (3nmoles/mg protein) and calcium uptake sites (2nmoles/mg) (72), 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 (73), 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 (74). 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 II) 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 (41), and since inhibition of mitochondrial function could give rise to its electrophysiological effects

(19), 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 beta-bungarotoxin can be reversed by subsequent addition of antiserum against the toxin (75). Also, the inability of ^{125}I-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 A_2 activity (21), since mitochondrial function if known to be highly sensitive to phospholipases (76). Evidence has been presented that the toxin makes use of its phospholipase activity to inactivate synaptic transmission (21). Whether or not this inactivation involves direct modification of the plasma membrane by the phospholipase, or release of toxic components into cytoplasm, remains to be determined.

The surprising aspect of this study is that a toxin which shows binding characteristics very similar to cholera toxin (77) and nerve growth factor (78) 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 (21), we suggest that beta-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.

SECTION V: MODEL OF BETA-BUNGAROTOXINS MECHANISM OF ACTION

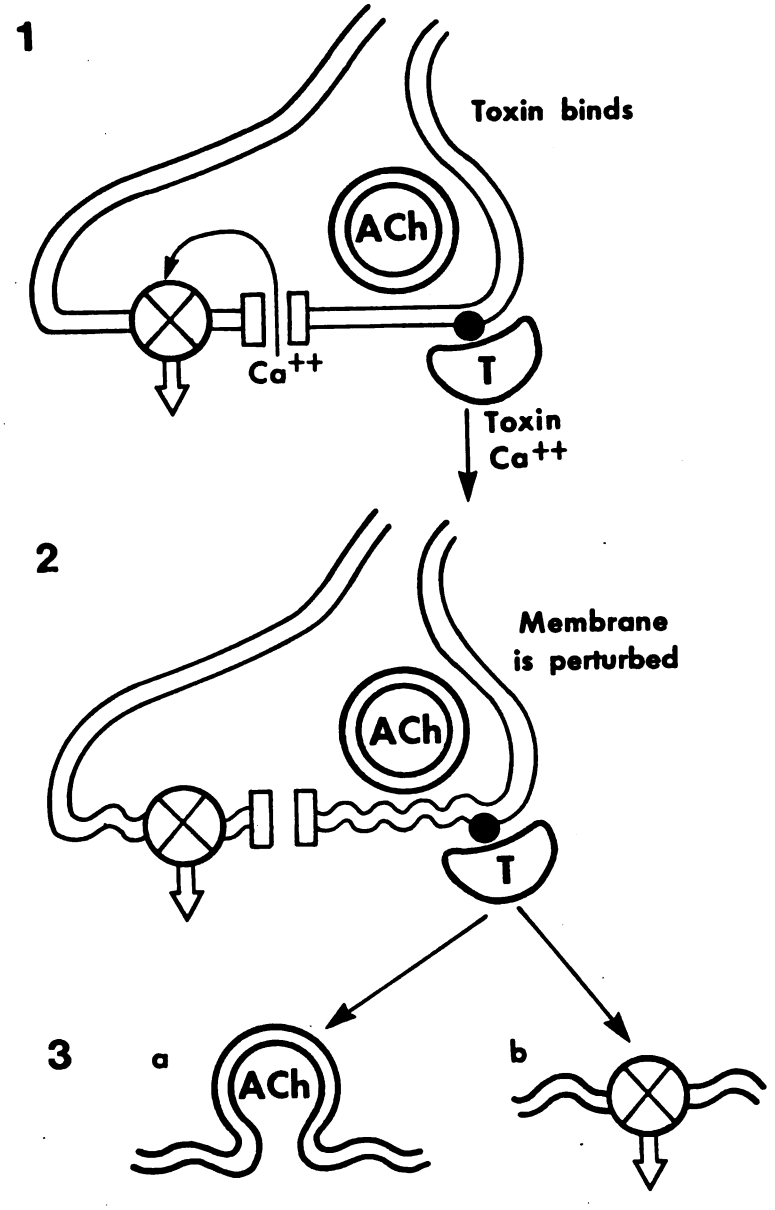
SUMMARY

A model able to account for the physiological and biochemical observations of this report is summarized in Figure 16. In this model, the toxin's presynaptic action is divided into four sequential events. (1) the toxin binds to the motor nerve terminal (2) the terminal is 'perturbed' in a calcium dependent manner (3) either the calcium removal mechanism and/or the transmitter release mechanism is modified resulting in enhancement of release and (4) transmitter release is inhibited and eventually fails. The data present in this report provide an adequate basis for the first three steps of this model, whereas an explanation of the later inhibitory effects await further analysis.

In the introduction we reported on a few characteristics shared by many presynaptic neurotoxins. Of these, the one characteristic that all seemed to have was that once applied, repeated washes were unable to prevent subsequent toxic effects. The high affinity binding described for beta-bungarotoxin and comparable demonstrations made for other presynaptic toxins (e.g. tetanus (79), and botulinum (80)) seem to account for the "irreversible" nature of these molecules.

The initial effect of beta-bungarotoxin is an increase in spontaneous release that begins very soon after application (Figure 2). Two alternate proposals able to account for these electrophysiological findings were that the toxin either modifies the intraterminal levels of calcium or some aspect of the release mechanism itself. The observations that spontaneous, evoked and delayed release, all known to have markedly different dependence on extracellular calcium, were modified

MODEL OF β-BUNGAROTOXIN ACTION



Release is enhanced by

a) Increasing the probability of fusion

b) Inhibiting calcium removal

to approximately the same extent and with similar time courses implied that a modification of the release process seemed more plausible than a change in calcium metabolism. Since calcium is needed for toxin action (Figure 8) the modification of the release process appeared to involve extracellular calcium in some manner. Subsequent findings that calcium is essential for the phospholipase A_2 activity of beta-bungarotoxin and that this enzyme activity is required for initiation of the presynaptic effect (Section III), provided us with a ready explanation of the requirement for extracellular calcium.

As proposed in Section III, although beta-bungarotoxin's neurotoxicity appears to involve hydrolysis of phospholipids, its phospholipase activity must be an unusual one since much higher activities of other phospholipases A_2's are not toxic (20). The demonstration that this enzyme binds very tightly to cell membranes (Section III) provided one explanation of this phenomena. We propose that the neurotoxic nature of this phospholipase could be due to its ability to avidly bind to nerve terminal membrane sites thereby increasing the effective enzyme concentration at such sites, which in turn causes hydrolysis of phospholipids in the neighborhood of the binding site.

Beta-bungarotoxin is not the only presynaptic neurotoxin known to have phospholipase A activity. For example taipoxin (11), notexin (12), and crotoxin (81), are all highly purified presynaptic toxins having such activity. This raises the possibility that the neurotoxic action of all these molecules might invovle their hydrolytic activity as was found for beta-bungarotoxin.

It was recently proposed that in addition to the catalytic site, active phospholipase A_2's from pig, sheep and ox pancreas contain a so-called "recognition site", not present in their zymogens, that allow these enzymes to interact with organized lipid-water interfaces. This led to a suggestion by Eaker et al (82) that such a recognition site, along with some modification conferring high specificity toward some unique feature the nerve terminal membrane, could be responsible for the blocking action of presynaptic toxins possessing phospholipase activity. Likewise, our model proposes just such a mechanism to explain beta-bungarotoxin's presynaptic action. With these facts in mind, it might prove interesting to look for saturable binding of these other "phospholipase" type toxins and for an absence of binding by more conventional phospholipases.

One of the most peculiar findings that emerged from this study was that a molecule with enzymatic activity was also capable of strongly binding to its biological substrate. At first sight, these findings seem mutually exclusive because an enzyme incapable of rapidly dissociating from its substrate would not be catalytic. However, when one considers the cmplexity of cell membranes which are this enzyme's normal substrate and the other enzymes which are known to have distinct catalytic substrate and cofactor binding sites, these apparently contradictary properties can be reconciled. This can be accomplished by making two simple assumptions. The first is that the catalytic and binding sites are located in different parts of the molecule, possibly in different subunits. The second is that the membrane binding site is not phospholipids, this enzyme's normal substrate, but some other

membrane component, e.g. protein, ganglioside, etc. This second proprosal is supported by the density of binding sites present in brain membranes (13 pmole/mg) which is orders of magnitude lower than the density of phospholipids commonly found in plasma membranes (83). Once bound to membrane sites, the toxin then perturbs the membrane leading to modification of the transmitter release mechanism. A mechanism by which a phospholipase A might modify release is by increasing the probability of vesicles fusing with the terminal membrane. Such a role is suggested by demonstrations of an increased probability of cell fusion in the presence of lysophospholipids (84) and an increased rate of fusion of synthetic vesicles containing fatty acids (85).

APPENDIX

The following are experimental observations made during the binding experiments yet not included in the final draft of the paper.

1. Commonly, chloramine T-stimulated iodination reactions are stopped by addition of sodium metabisulfite, a strong reducing agent. However, when this reducing step was employed during beta-bungarotoxin iodination, a large proportion of the ^{125}I-toxin was excluded from Sephadex G-200 columns and numerous high molecular weight (>9) SDS gel bands were observed. However, when metabisulfite was omitted, as described in Methods, ^{125}I-toxin was no longer excluded and only one 22,000 dalton gel band was found. This implies that addition of sodium metabisulfite reduces ^{125}I-toxin and results in the formation of large toxin complexes ($>200,000$ daltons).

2. ^{125}I-toxin seems to form high molecular weight complexes with bovine serum albumin (BSA). When binding was done in assay and elution media containing 0.1% BSA, greater than 44% of the toxin was excluded from Sephadex G-200 columns. However, when BSA was replaced with comparable concentrations of trypsin inhibitor, less than 2% was excluded. Thus it appears that the highly basic ^{125}I-toxin molecule (pI $>$ 10) forms large complexes ($>200,000$ daltons) with the acidic BSA (pI) that result in exclusion of a large proportion of it during gel filtration. Consequently, trypsin inhibitor was used rather than BSA to maintain high levels of protein in incubation mixes while ^{125}I-toxin binding was assayed.

3. The ionic strength of incubation media and column elution buffers might be important when elutions of membrane preparations from gel filtration columns are attempted. In a few gel filtration experiments, poor recovery of membrane protein was observed when these preparations were eluted with normal Krebs media rather than the low ionic strength buffer described in Methods. This effect might be due to the salting-out of highly charged membranes as the ionic strength is increased.

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