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A CORE FACILITY FOR THE STUDY OF NEUROTOXINS OF BIOLOGICAL ORIGIN

Annual Report

June 15, 1989

Lance L. Simpson

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Studies have been done on a variety of toxins that affect the nervous system. The toxins of major interest have been dendrotoxin, tetrodotoxin, saxitoxin, botulinum neurotoxin, tetanus toxin and crotoxin. Experiments have been conducted mainly on three types of tissue preparations: i.) brain synaptosomes, ii.) phrenic nerve-hemidiaphragm and iii.) cells grown in tissue culture, including neuroblastoma cells and adrenal medullary tumor cells. The major accomplishments have been: i.) isolation of homogeneous preparations of dendrotoxin, ii.) partial characterization of dendrotoxin binding sites, iii.) partial characterization of tetrodotoxin and saxitoxin binding properties, using channels inserted into lipid membranes, iv.) the discovery of an intracellular effect mediated by tetanus toxin, this being impaired mobilization of protein kinase C, v.) a study of raiolabeled crotoxin binding to brain slices, and vi.) a pharmacological characterization of crotoxin binding to cholinergic nerve endings.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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INTRODUCTION

The Core Facility for the Study of Neurotoxins is composed of several scientific projects, and these are supported by a central administrative staff. The scientific projects conduct research that falls into three broad categories: i.) the development of *in vitro* systems to assay toxin activity, ii.) exploratory and definitive studies to determine mechanism of toxin action, and iii.) screening efforts to identify agents that will protect against or reverse the effects of poisoning.

During the past year, a number of new projects have been initiated, as follows:

- Dr. Roger Sorensen has developed techniques for isolating and purifying several of the dendrotoxins. He has also developed methods for extraction, solubilization and partial characterization of dendrotoxin receptors.

- Dr. Lee Chabala has constructed an apparatus for patch clamp analysis of sodium and potassium channels in nerve tissue that is injected into lipid membranes. He has utilized this technique to study the actions of tetrodotoxin and saxitoxin.

The past year has also seen the continuation of several projects, as follows:

- Dr. Alexander has continued his work on toxin action in the central nervous system. He has prepared an iodinated derivative of crotoxin and studied its binding characteristics to brain slices.

• Dr. Sherwin has continued his research on clostridial neurotoxins and intracelluar messengers. He has made the discovery that certain of the toxins alter the disposition of protein kinase C.

• Dr. Simpson has continued his work on toxins that affect the cholinergic nerve ending, including clostridial toxins and PLA_2 toxins. He has completed a study that compares these two toxins in terms of the sequence of events that are involved in blocking exocytosis.

During the coming year the various investigators will pursue work that is related to that mentioned above. Thus, effort will be focused on dendrotoxins, guanidinium toxins (e.g., tetrodotoxin and saxitoxin), and toxins that paralyze neuromuscular transmission.

Guillermo M. Alexander, Ph.D.
Assistant Professor of Neurology

Scientific Progress During Year

A. Specific Aims

Our aims during this year were:

1) To complete the study of the interaction of antibodies raised to gyroxin with a variety of other thrombin-like enzymes.

2) To purify crotoxin from the venom of Crotalus durissus terrificus.

3) To establish procedures for the dissociation and separation of the subunits of crotoxin.

4) To calibrate plastic ^{14}C standards for tissue ^{125}I levels.

5) To radiolabel crotoxin and the basic subunit of crotoxin with ^{125}I.

6) To reassociate the radiolabelled basic subunit with unlabelled acidic subunits.

7) To investigate the binding properties of crotoxin and its subunits on rat brain slices.

8) To begin our study on the binding of crotoxin and its subunits to primate brain.

9) To study the binding of dendrotoxin to rat brain.

B. Methods

Rabbit serum containing antibodies to purified gyroxin was obtained from Dr. Simpson's laboratory. Samples of gyroxin, as well as equal concentrations of other thrombin-like enzymes, were incubated with both the gyroxin antibody containing serum and non-antibody containing serum from control rabbits. Thrombin-like activity and the ability of the protein-antibody mixtures to elicit barrel rotation in mice were tested.

Thrombin-like activity was measured by the time to reach a 10% change in the transmittance at 425 nm of a 0.5% sheep fibrinogen solution in 5 mM Tris-HCl, 75 mM NaCl, pH 8.0. The time to clot of the solution was inversely proportional to the number of thrombin-like units.

Crotoxin is isolated from the venom of Crotalus durissus terrificus by a two column procedure. Venom is dissolved in low pH buffer (20 mM glycine, 150 mM NaCl, pH 1.9), centrifuged and the supernatant applied to a Sephadex G-75-(120) column (2.5 x 90 cm) equilibrated with the same buffer. Crotoxin containing fractions are pooled and given to the laboratory of Dr. Lance Simpson for further purification. There the sample is dialyzed against buffer to allow for complete reassociation of the crotoxin. The sample is then exhaustively dialyzed against deionized H_2O and lyophilized. The lyophilized protein is resuspended and subjected to gradient anion-exchange chromatography.

Crotoxin subunits are isolated by modification of a procedure described by Faure and Bon (1988). The lyophilized purified crotoxin is dissolved and allowed to sit for at least 2 hours in a buffer containing 6 M urea and 50 mM sodium phosphate (NaPi) at a pH of 6.5. The sample is then applied to a DEAE-Sephacel column (2.5 x 15 cm) equilibrated with this buffer. The basic, PLA_2 containing, subunit is not retained by the anion exchange column under these conditions. The acidic subunit is retained and is eluted with 6 M urea, 50 mM NaPi, 500 mM NaCl, pH 6.5. The pooled fractions are exhaustively dialyzed

against deionized H_2O and then lyophilized.

Protein concentrations of crotoxin and its subunits were determined spectrophotometrically using the extinction coefficients reported by Faure and Bon (1988).

Phospholipase A_2 activity was measured by the method of Lobo de Araujo and Radvanyi (1987). This assay uses a pH indicator (phenol red) to quantitate spectrophotometrically the H^+ released when fatty acids are cleaved from phosphatidylcholine.

Crotoxin was radiolabelled using a modification of the chloramine T iodination method of Bon et al. (1979). The lyophilized crotoxin is dissolved in 10 mM NaPi (pH 6.0) to a concentration of 5 mg/ml. One millicurie of $Na^{125}I$ is added to 200 ul of the crotoxin solution and the reaction started by the addition of 5 ul of 4 mM chloramine T. The reaction is allowed to proceed for 10 seconds and then is terminated by dilution with 1 ml of H_2O. Radiolabelled crotoxin is isolated from unreacted $Na^{125}I$ by size exclusion chromatography. The reaction mixture is applied to a Sephadex G-25-(80) column (0.9 x 30 cm) equilibrated with 10 mM sodium acetate buffer (pH 4.5). Fractions (0.5 ml) are collected and those fractions corresponding to the elution position of crotoxin are pooled.

The radioiodinated crotoxin was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) after incubation for 1 hour at 37° C in denaturation buffer (1% SDS, 1 mM

dithiothreitol). After completion of the electrophoresis, the lane containing the ^{125}I-crotoxin was cut out of the gel and immediately put on a glass plate placed on top of crushed ice. The gel was cut into 2.5 mm slices with a razor blade and the sequential slices were placed into test tubes for ^{125}I quantitation in a gamma counter. The remaining lanes of the gel, containing standards and purified crotoxin subunits, were stained for protein with coomassie blue.

Crotoxin was dissociated into its component acidic and basic subunits by the methods already described. The basic subunit of crotoxin was radiolabelled by the same method as that used for the radioiodination of the native toxin described above.

Primate brains were obtained from monkeys (Macaca fascicularis) weighing between 3 and 6 kilograms that were being sacrificed for other studies not involving the central nervous system. The brains were removed within 15 minutes of sacrifice and frozen in isopentane cooled to -40°C with dry ice. The frozen brains were sectioned into 20 micron sections, picked up on glass slides, dried for 10 minutes on a warming plate at 40°C, and stored at -20°C.

Binding of ^{125}I-crotoxin was done on 20 micron monkey brain frozen sections. The sections were cut in the coronal plane at 6 mm anterior to the interaural plane. The assay buffer was 10 mM phosphate (pH 7.4), 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mg/ml bovine serum albumin. The brain sections were pre-washed for 5 min. at 4°C, incubated while in the presence of labelled crotoxin

for one hour at room temperature (21°C), post-washed twice in 4°C buffer for 5 min. and then dipped twice in distilled H_2O. The sections were dried with cold, dry nitrogen, placed on a warming plate at 40°C for one hour and then plated on x-ray film along with plastic ^{14}C-standards calibrated for ^{125}I equivalent concentration. The concentrations of crotoxin used were 100 nM, 30 nM and 10 nM. Non-specific binding was determined in the presence of 250 fold excess unlabelled crotoxin.

After the ligand binding study was performed the tissue sections, along with plastic standards, were placed in light-tight cassettes opposing x-ray film. Following autoradiography, the tissue isotope concentration were determined from the autoradiographs with computer assisted densitometry (Alexander and Schwartzman, 1984).

Alpha and beta-dendrotoxin (DTX) were purified and radioiodinated by Dr. Roger Sorensen. The specific activities were 4.78 x 10^5 CPM/nMol for alpha-DTX, and 0.8 x 10^5 CPM/nMol for beta-DTX.

Binding of ^{125}I-dendrotoxin (^{125}I-DTX) was done on 20 micron rat brain frozen sections. The sections were cut on the horizontal plane at approximately interaural 2.0. The assay buffer was 10 mM phosphate (pH 7.3) with 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ and 1 mg/ml bovine serum albumin. The brain sections were pre-washed in buffer at 4°C for 5 min., incubated in the presence of labelled DTX at room temperature (21°C) for 30 min. and post-washed in buffer at 4°C for

5 min. The sections were dried and plated on x-ray film for 2 days along with plastic ^{14}C-standards calibrated for ^{125}I equivalent concentration. The concentrations of alpha-DTX used were 2,1,0.5,0.25 and 0.125 nM and for beta-DTX 6,3,1.5,0.75 and 0.375 nM. Non-specific binding was determined in the presence of 250 fold excess of cold toxin for alpha and 80 fold excess for beta-DTX.

Tissue ^{125}I standards were made by homogenizing rat brains to a paste. To 250 mg of this brain paste was added various concentrations of ^{125}I-crotoxin. The paste was frozen into cylinders and cut at 20 micron thickness. The paste sections were plated along with ^{14}C-plastic standards on x-ray film. The optical density of the brain paste sections were read with computer assisted densitometry and a ^{125}I versus optical density table was generated. The ^{14}C-plastic standards were read and assigned equivalent ^{125}I-tissue values.

C. Results

Rabbit antibody added to gyroxin completely neutralized the thrombin-like and gyroxin syndrome producing activities of the enzyme. Gyroxin antibody also inhibited the thrombin-like and gyroxin-like activities of crotalase and the thrombin-like enzyme from the Central American rattlesnake, Crotalus durissus durissus. Serum from a control rabbit completely inhibited the ability of thrombin to clip fibrinogen, and inhibited the thrombin-like activity of ancrod (Malayan pit viper) by 50%. Antibody-containing serum did

not further inhibit the activity of ancrod. Control rabbit serum had no effect on the thrombin-like or gyroxin activities of gyroxin, crotalase or the thrombin-like enzyme of C. d. durissus.

The first step of the purification of crotoxin was size exclusion chromatography at pH 1.9. At this pH crotoxin is dissociated into its two smaller subunits, thus facilitating its separation from the monomeric esterases of similar molecular weight. After reassociation and concentration, crotoxin was further purified by G-75-40 chromatography at pH 7.5. This step removes small molecular weight contaminates.

Purified crotoxin was successfully fractionated into its two subunits by anion exchange chromatography (Figure 1). As previously reported, the basic subunit was found to posess the PLA_2 activity. The acidic subunit was totally devoid of this activity, and as reported by Canziani et al. (1983) inhibits the PLA_2 activity of the basic subunit. Intraperitoneal injection of 0.25 nanomoles of crotoxin into 25 gram mice was fatal, whereas 0.25 nanomoles of the basic (PLA_2 containing) subunit was not fatal. When 0.25 nanomoles of of the acidic subunit was added to 0.25 nanomoles of the basic subunit the mixture was fatal to mice. These observations are consistent with those of Hendon and Fraenkel-Conrat (1971).

The brains of 12 monkeys were sectioned at the level of the anterior commissure and at the level of the substantia nigra. The

sections were dried and are stored at -20°C. The advantage of using ligand autoradiography on brain sections is that it will allow us to determine whether crotoxin and/or its two subunits bind to the same anatomical sites. This will also enable us to correlate the distribution of toxin binding with known distributions of neurotransmitters and neuromodulators.

Crotoxin was radioiodinated with a resulting specific activity of 1.68 x 10^6 CPM per microgram of protein. The results of SDS-PAGE of the radiolabelled crotoxin are plotted in the form of a histogram (Fig.2). The total recovery of radioactivity from the gel was 87%. The radioactive peak migrating to 57 mm corresponds to the migration of the basic subunit of crotoxin, as demonstrated by coomassie staining of other lanes of the gel containing purified basic subunit. The radioactive peak migrating to 100 mm corresponds to the acidic subunit. The basic subunit contains 74% and the acidic subunit contains 20% of the recovered radioactivity. The remaining counts were smeared throughout the gel. The ratio of the radioactivity of the basic to the acidic subunit (3.7) corresponds to the ratio of the number of tyrosine residues (average of 9.2 for the basic subunit and 2.5 tyrosine residues per acidic subunit, Hendon and Fraenkel-Conrat, 1971). This would seem to indicate that all of the tyrosines in the crotoxin molecule are easily iodinated.

The iodinated crotoxin bound throughout the monkey brain sections. ^{125}I-crotoxin bound to all the structures observed on the sections, the highest binding was seen in the hippocampus and

cortical areas (Fig. 3B). White matter structures demonstrated the lowest binding. The non-specific binding, as determined with a 250 fold excess of unlabelled crotoxin, was extremely high (60-80% of the total binding). The high apparent non-specific binding could be the result of the crotoxin undergoing a structural change on iodination, thus binding tighter to receptors than the unlabelled toxin itself. It has been shown that iodinated acidic subunit loses 40% of its efficiency in activating native basic subunit (Bon et al., 1979). It is also possible that crotoxin, as well as interacting with receptors, may be absorbed non-specifically into membranes. At high concentrations of labelled crotoxin this would lead to a situation where the non-specific binding is a large proportion of the total binding.

The iodinated alpha-DTX bound throughout the rat brain sections, the highest binding was seen in the hippocampus and in white matter areas (Fig. 3A). Our preliminary results (n=1) demonstrate a dissociation constant of 3-4 nM with a B_{max} varying between 160 uMol/gm (hippocampus) and 90 uMol/gm (caudoputamen). The iodinated beta-DTX also bound throughout the rat brain, but the 80 fold excess of unlabelled beta-DTX was insufficient to calculate the non-specific binding. The resulting data was not suitable for Scatchard analysis.

The basic subunit of crotoxin was radioiodinated with a resulting specific activity of 7.82×10^7 CPM per milligram of protein. After reconstitution with unlabelled acidic subunit, the holotoxin had a specific activity of 4.15×10^7 CPM per milligram of

protein.

The iodinated crotoxin (^{125}I labelled basic subunit) bound throughout the brain sections. ^{125}I-crotoxin bound to all the structures observed on the sections. The non-specific binding, as determined with a 250 fold excess of unlabelled crotoxin, was extremely high (70-85% of the total binding). This is approximately the same as was previously seen with iodinated holotoxin (radiolabelled acidic and basic subunits).

These results are not compatible with the hypothesis that crotoxin specifically binds to brain sections in the same distribution as one of the known neurotransmitters. Crotoxin seems to bind non-specifically to all brain structures as evidenced by the inability of a 250 fold excess of unlabelled crotoxin to displace a significant portion of the labeled crotoxin from its binding sites. The use of frozen brain sections may be unsuitable for the study of the distribution of crotoxin binding.

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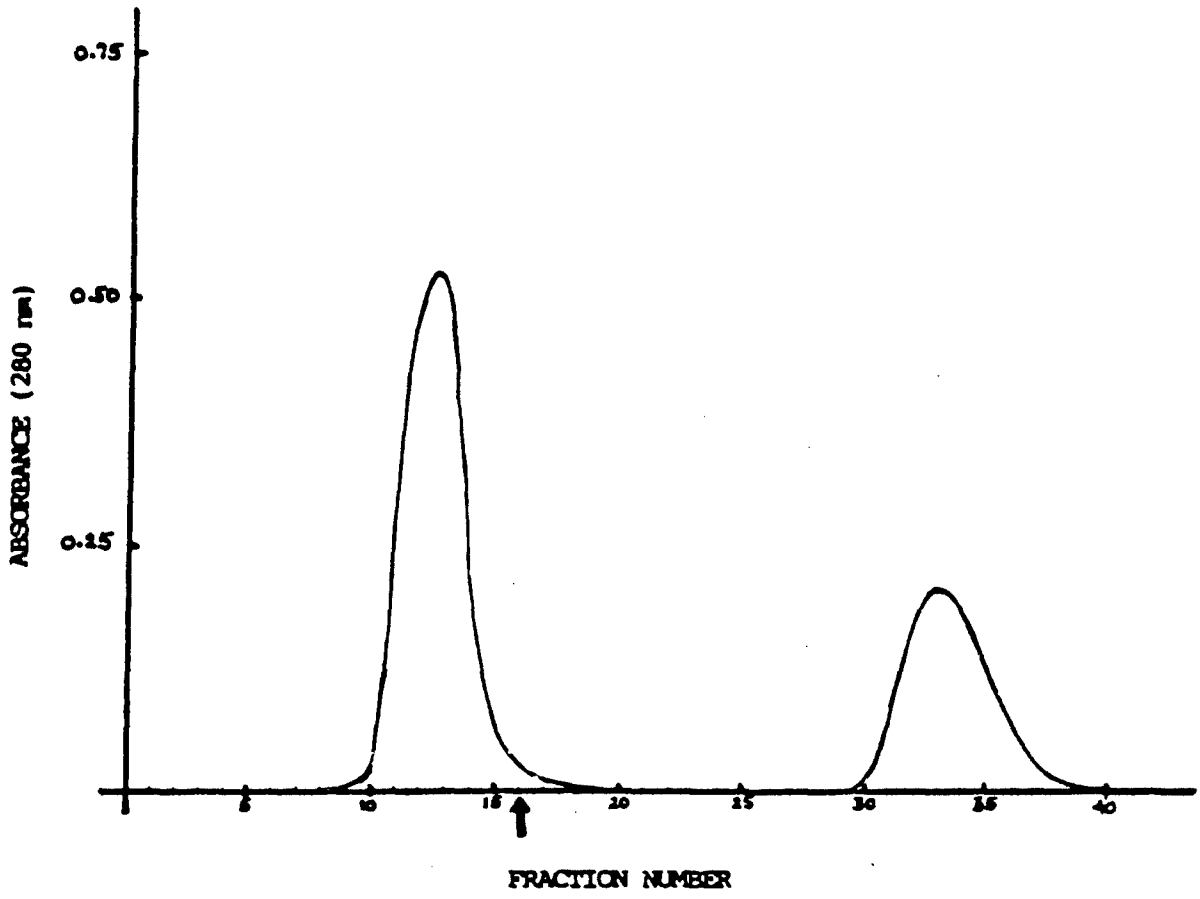

Figure 1. DEAE-Sephacel chromatography of purified crotoxin run under dissociating conditions. The sample was applied and the basic, PLA_2 containing, subunit was eluted in the equilibration buffer (6 M urea, 50 mM NaPi, pH 6.5). High salt buffer (6 M urea, 50 mM NaPi, 500 mM NaCl, pH 6.5) was applied at the point indicated by the arrow. The acidic subunit of crotoxin, which was retained by the column, was then eluted.

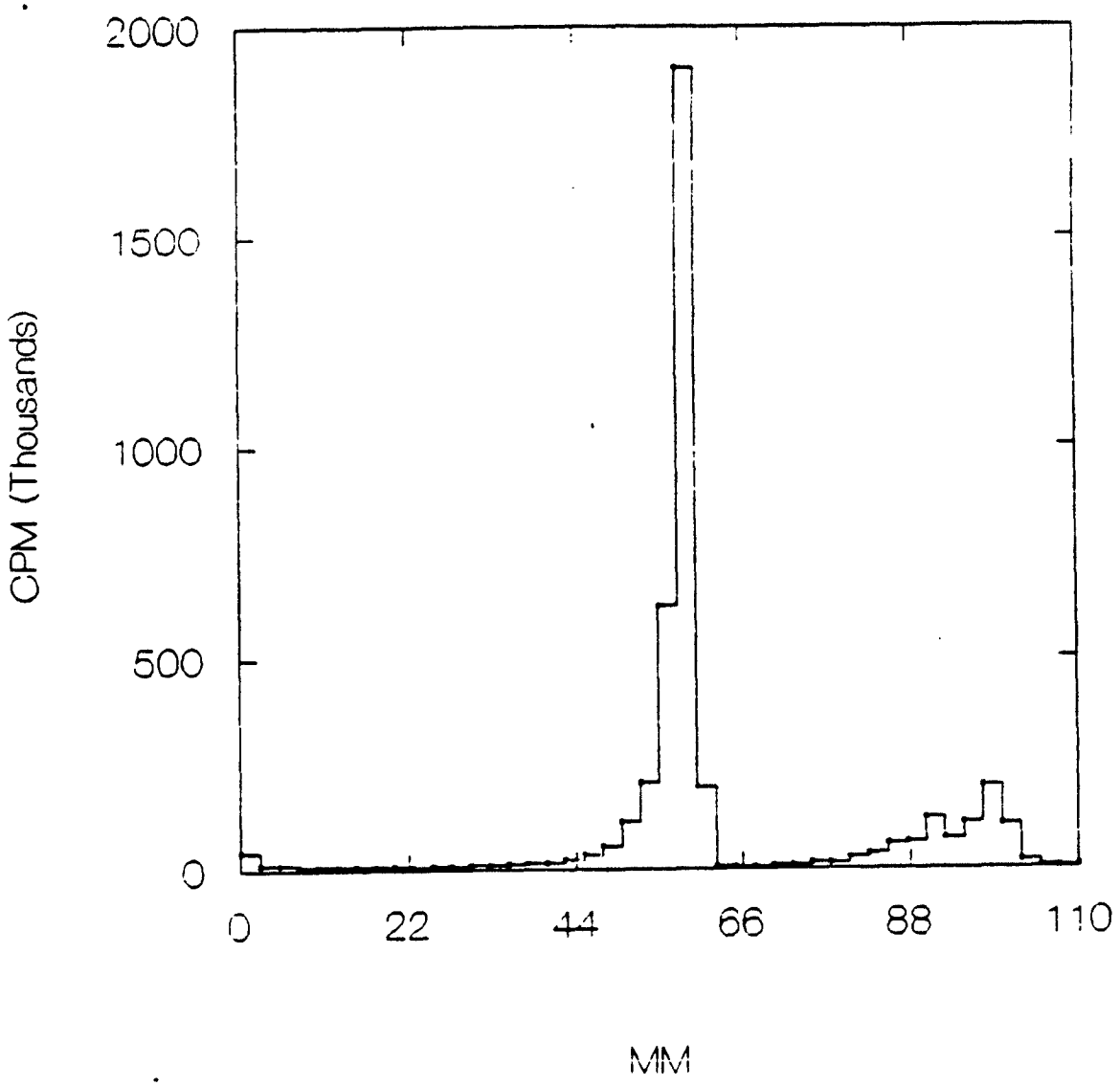

Figure 2. SDS-PAGE (16% polyacrylamide gel) of ^{125}I-crotoxin. The gel was cut into 2.5 mm slices starting at the top of the running gel. The peak of radioactivity at 57 mm corresponds in migration to the purified basic subunit (B subunit) of crotoxin. The radioactive peak at 100 mm corresponds in migration to purified acidic subunit (A subunit).

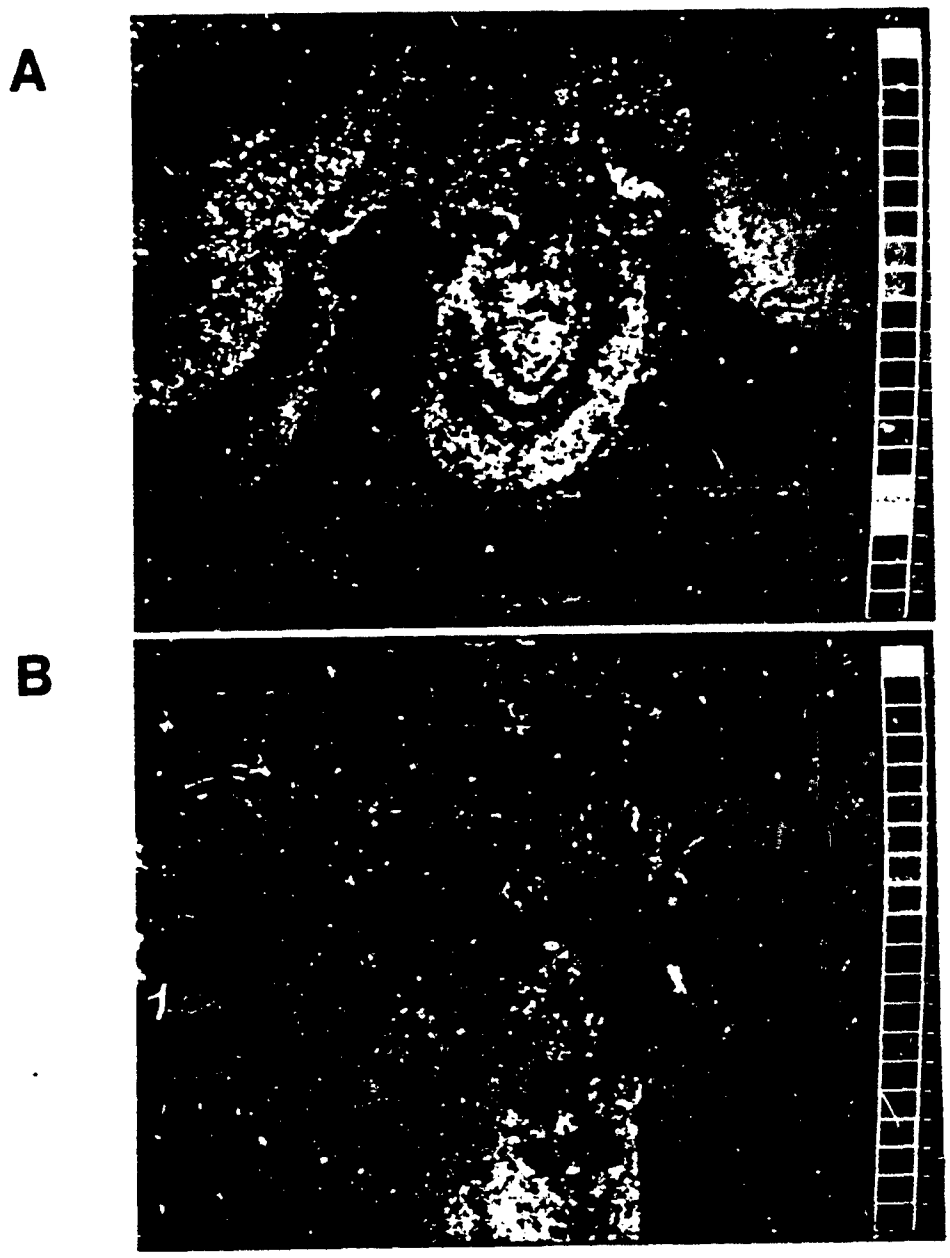

Figure 3. (A) Pseudocolor image of alpha-DTX binding to rat brain. The toxin bound to all brain structures analyzed with the hippocampus (1) and white matter (2) demonstrating the highest binding. (B) Pseudocolor image of crotoxin binding to monkey brain. The toxin bound to all brain structures analyzed with the hippocampus (1) and cortical areas (2) demonstrating the highest binding.

Roger G. Sorensen, Ph.D.
Assistant Professor of Medicine

SUMMARY of SCIENTIFIC PROGRESS for YEAR 88-89

A. SPECIFIC AIMS

This section of the core facility projects concerns the use of several neurotoxins, dendrotoxins, isolated from the venom of the green mamba, Dendroaspis angusticeps, to study and purify mammalian brain potassium (K) channels. There are three primary goals of the proposed experiments in this section.

1) Characterization of Toxin Receptor Binding and Purification of the Receptor Proteins. Conventional pharmacological and protein purification methodologies are used to describe the binding properties of several dendrotoxins to brain membrane receptors, and to isolate and purify the receptor proteins.

2) Functional Reconstitution of Dendrotoxin Receptors/K Channels. Tracer flux methods are used to assay for the predicted K channel activity of the soluble and purified receptor protein(s) after their reconstitution into phospholipid vesicles.

3) Structural Characterization of Dendrotoxin Receptors/K Channels. Biochemical, molecular biological and immunochemical methodologies are employed to determine the primary sequence of the purified receptor/channel protein, and to deduce other structural features that give the protein its ion channel activity.

During the past year (88-89), significant progress has been made towards completion of the first goal.

B. BACKGROUND

DaTX is a small polypeptide, M_r = 7000, found in the venom of the green mamba, D. angusticeps. Although originally shown to produce synaptic facilitation (1), this neurotoxin has subsequently been shown by electrophysiological measurements to exert its effect through its ability to block several types of voltage-dependent K channels in a variety of tissue preparations (2-5).

Using a tracer (^{86}Rb) efflux assay to measure K channel activities in a rat brain nerve terminal (synaptosome) preparation, Benishin et. al. (6) identified dendrotoxin (alpha-DaTX) and three additional components (beta-DaTX, gamma-DaTX and delta-DaTX) of D. angusticeps venom that block K channels. Interestingly, these polypeptides have selective effects. Two of the toxins, alpha-DaTX and delta-DaTX, are selective blockers of voltage-gated, rapidly-inactivating (within 1 sec of K-stimulated depolarization) K channels. The other two, beta-DaTX and gamma-DaTX, are selective blockers of voltage-gated, non-inactivating (lasting for 2-5 sec after depolarization) K channels.

The focus of this project has been on alpha-DaTX and beta-DaTX. These two dendrotoxins were chosen because they had the highest potencies for block of brain K channels, were selective blockers of different voltage-gated K channels, and in preliminary experiments, had the highest affinities for binding sites located on rat brain synaptic membranes. These results suggested that the dendrotoxins can be used as specific ligands for the study and purification of voltage-dependent K channels.

C. METHODS

Radioiodination of dendrotoxins

Alpha-DaTX and beta-DaTX were radioiodinated essentially as described by Black et. al. (7). The polypeptides (10 ug) were incubated in 150 mM sodium phosphate, pH 7.0, containing 1 mCi $Na^{125}I$ in a total volume of 30 ul. Radiolabelling was catalyzed by the addition of chloramine T (two 5 ul aliquots of a 0.25 mg/ml solution for 30 sec each). The mixtures were diluted with 0.5 ml SOLUTION A (20 mM sodium phosphate, pH 7.0, 200 mM NaCl) containing 2 mg/ml NaI, and applied to a 2-5 ml CM-Sephadex C-25 ion exchange column. Unincorporated ^{125}I was eluted with SOLUTION A, and the radiolabelled toxins eluted with SOLUTION A containing 700 mM NaCl.

Dendrotoxin binding to synaptic membranes

The binding of [^{125}I]alpha-DaTX to rat forebrain synaptic membranes was assayed by a rapid centrifugation technique. The membranes (1 mg protein/ml) were incubated at 37^{o} in BUFFER A, (145 mM NaCl, 5 mM KCl, 1.4 mM $MgCl_2$ and 20 mM HEPES, pH 7.0) containing usually 1 nM [^{125}I]alpha-DaTX and increasing amounts of non-radiolabelled alpha-DaTX. After 30 min, 0.2 ml of the binding mixtures were layered on top of 0.2 ml of dinonyl phthalate:silicone oil (65:35) in microcentrifuge tubes. The membranes and bound iodinated toxin were collected by centrifugation through the oil mixture at 8700 x g for 4 min. The membrane pellets were washed once with BUFFER A and counted for gamma radiation.

Receptor solubilization and assay

The dendrotoxin receptor was solubilized by the following procedure. Synaptic membranes (4 mg protein/ml) were incubated on ice for 15 min in BUFFER B [150 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.0], containing 0.25% lecithin, and 0.8% zwittergent 3-12. The solubilized proteins were subsequently collected by centrifugation of the mixtures for 30 min at 4°C and 171,000 x g. To assay for receptor binding, [^{125}I]alpha-DaTX (usually 1 nM) and various amounts of non-radiolabelled alpha-DaTX were added to 0.6 ml aliquots of the undiluted solubilized proteins, (final total volume = 0.7 ml). The binding mixtures were incubated at 37° for 30 min. Bound toxin was separated from unbound toxin by a spun column method: 0.3 ml aliquots of the binding mixtures were applied to a 2 ml (0.4 x 5 cm) Sephadex G-50 column equilibrated with BUFFER B containing 0.025% lecithin and 0.08% zwittergent 3-12. The columns were centrifuged at 800 x g for 90 s at room temperature, and the eluate (containing the receptor and bound toxin) was counted for gamma radiation. Under these conditions, the columns retain 93-95% of the unbound toxin, and 88% of the total applied protein is recovered in the eluate.

D. RESULTS

Binding to membrane receptors

[^{125}I]alpha-DaTX and [^{125}I]beta-DaTX each labelled a single class of binding sites on brain synaptic membranes. Scatchard analysis gave apparent dissociation constants (K_D) of 0.67 ± 0.09 nM (N = 13) and 35.9 ± 7.1 nM (N = 7) for [^{125}I]alpha-DaTX and

[^{125}I]beta-DaTX, respectively, and the respective binding site densities (B_{max}) were 1.8 $\pm$ 0.2 and 8.6 $\pm$ 1.7 pmol ligand bound/mg protein.

pH dependence

The binding of [^{125}I]alpha-DaTX and [^{125}I]beta-DaTX showed similar pH dependencies. Specific binding was maximal at pH 6-7, and decreased with increasing pH. The latter decrease in dendrotoxin binding resulted from both a decrease in the total binding of the respective toxins to the membranes, and an increase in the non-specific binding (measured in the presence of excess unlabelled toxin). Because the dendrotoxins are basic proteins, these results suggest that it is the charged form of the dendrotoxins that are recognized by specific membrane receptor sites, presumably involving ionic interactions with exposed lysine residues on the toxin molecules (3).

Displacement by unlabelled dendrotoxins

The four K channel blockers (dendrotoxins) from D. angusticeps venom [alpha, beta, gamma, and delta-DaTX] inhibited the binding of [^{125}I]alpha-DaTX (fig. 1A) and [^{125}I]beta-DaTX (fig. 1B) to synaptic membranes. The displacement profiles for the two iodinated dendrotoxins were similar: alpha-DaTX > beta-DaTX > gamma-DaTX. The displacement curves for delta-DaTX did not parallel the others; this may indicate that delta-DaTX has more complex interactions at the toxin binding sites.

·Solubilization of dendrotoxin receptor

The alpha-DaTX receptor has been solubilized from brain membranes in the presence of 0.8% zwittergent 3-12 and 0.25% lecithin. Scatchard analysis showed that 30% of the membrane binding sites (B_{max} = 0.55 ± 0.13 pmol/mg protein) were recovered after receptor solubilization, and that there was a 10-fold decrease in the apparent affinity of [^{125}I]alpha-DaTX for the soluble receptor, (K_D = 6.5 ± 1.5 nM, N = 5).

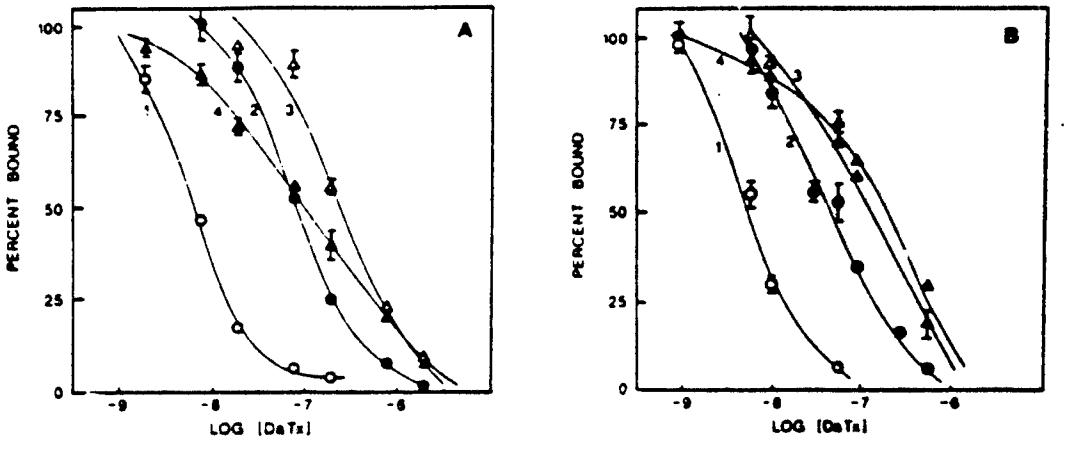

Fig. 2. Dendrotoxins block binding to synaptic membranes Shown are competitive displacement curves for the inhibition of [^{125}I]alpha-DaTX (A) and [^{125}I]beta-DaTX (B) binding by each of the four K channel blockers (dendrotoxins) isolated from D. angusticeps venom. Error bars are ± SE of triplicate determinations. (1) alpha-DaTX, (2) beta-DaTX, (3) gamma-DaTX, (4) delta-DaTX.

E. PLANS FOR YEAR 89-90

Current progress is being made towards the characterization of the binding of two snake neurotoxins, dendrotoxins, to an enriched synaptic membrane preparation. Furthermore, towards the goal of receptor purification, these receptors have been successfully solubilized from brain membranes. The goals for the coming year include:

1) The continued characterization of dendrotoxin binding to rat brain receptors. Some of these experiments will examine the effect of membrane potential gradients for dendrotoxin binding to synaptosomes (to determine differences for toxin binding to K channels in the open or closed state). Other experiments will use group modifying reagents to determine the structural requirements for dendrotoxin binding.
2) The functional reconstitution of the solubilized dendrotoxin receptors and assay for K channel activity. These experiments will measure tracer (^{86}Rb) efflux from proteoliposomes formed in the presence of the solubilized dendrotoxin receptors.
3) The continued purification of the dendrotoxin receptors/K channels. These experiments will employ various protein separation techniques for purification of the solubilized receptors.

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12 June 89

Annual Report for Core Facility

A. Specific Aims.

The objective of the work outlined below is to provide an understanding of the molecular properties of voltage-dependent sodium channels from brain and cardiac tissue. Ion channels (like all integral membrane proteins) carry net negative charges in the form of protein-linked carboxyl groups, and those charges appear to modulate crucial functions. Many neurotoxins and therapeutic reagents by virtue of their net positive charge, are attracted to spatially separate regions of the sodium channel. The resulting electrostatic interactions are of crucial importance to understanding how the sodium channel works. Hence a major focus of the work summarized and proposed below is to uncover the role played by these negative surface charges in regulation ion permeation, neurotoxin binding, and gating behavior of brain and cardiac sodium channels.

B. Background & Methods.

My laboratory currently uses planar lipid bilayer techniques to study voltage-dependent sodium channels from dog and rat brain tissue; those methods were previously reported in detail. This work is currently divided into four areas: the pharmacological effects of various neurotoxins that bind to sodium channels; the impact of protein-linked negative charges on neurotoxin binding and open-channel conductance; the effects of monovalent and divalent cations on conductance and gating (voltage activation) behavior, and the effects of pH on guanidinium toxin potency.

C. Results.

i) Carbodiimide-Induced Modification of Canine Brain Sodium Channels. The prevailing hypothesis for the mechanism of action of guanidinium toxins like tetrodotoxin (TTX) or saxitoxin (STX) is that they inhibit current flow through sodium channels by binding to a negatively charged site in the channel entryway near the selectivity filter where they physically occlude ion passage; in this toxin-plug model, toxin

binding and channel closure are synonymous. Despite wide acceptance of this model or its surface receptor occlusion variation, it has no direct experimental support; moreover, recent studies on the voltage-dependent action of differentially charged guanidinium toxins on batrachotoxin (BTX)-modified sodium channels in planar lipid bilayers raise problems for the model. We investigated the relation between the channel entrance and the toxin binding site by chemical modification of carboxyl groups at the extracellular surface of bilayer-incorporated BTX-modified dog brain sodium channels with an impermeable carbodiimide in the absence or presence of exogenous nucleophiles. There are four possible reaction sequences for modification of protein-linked carboxyl groups after formation of the unstable O-acylisourea (i.e. hydroysis to reform carboxyl group, formation of N-acylurea, formation of peptide bond with exogenous nucleophile, internal cross-linking with endogenous nucleophile). We have seen two real-time reversible decreases in the current (i.e. hydrolysis to reform carboxyl group can occur). Those are always the same, and are different than the substates that show up occasionally. That suggests that there are two potentially modifiable carboxyl groups, and that turned out to be the case. The activated carboxyl groups can also react with exogenous nucleophiles to set up different stable conductances. We found that the two activated carboxyl groups could be modified such that the single-channel conductance is irreversibly decreased while TTX binding is unaffected. Because the final conductance level depend on the exogenous nucleophile, formation of the peptide bond must be occurring. A third carboxyl group can be modified such that TTX affinity is <u>increased</u>, while ion permeation is essentially unaffected. These effects can occur independently. In contrast to the toxin-plug model, the results suggest that the channel entrance and the TTX binding site are spatially separate; furthermore, the mechanism by which guanidinium toxins close sodium channels appears to involve a conformational change in a step subsequent to toxin binding.

ii) <u>Evidence for Negative Charges that Modulate Conductance and Toxin Binding in Rat Brain Sodium Channels</u>. To examine whether negative charges near the channel

entrance aid sodium flow through sodium channels, BTX-modified sodium channels from rat brain were incorporated into neutral phospholipid bilayers. Single-channel currents were measured in symmetrical NaCl solutions. The current-voltage relations were linear, but the conductance-concentration relationship could not be described by a simple saturating (Langmuir) relation; instead, it is consistent with the presence of a net negative charge near the channel entrance, which serves to concentrate cations and increase the conductance over that of a neutral channel. The results can be described by combining a Langmuir isotherm with a local accumulation of sodium ions at the channel entrance as described by the Gouy-Chapman theory of the diffuse double layer. The relative channel-closing potencies of STX and TTX vary as a function of the sodium concentration, which indicates that there is also a net negative charge at the STX/TTX binding site. The toxins have similar binding affinity at high salt, but due to electrostatics, STX, which is divalent, binds much tighter at low salt where the Debye length is longer. The active toxin species are concentrated by the surface potential according to the Boltzmann distribution. As a first approximation, the Gouy-Chapman equation can be used to estimate the apparent negative charge distribution in the vicinity of the toxin binding site. When analyzing the conductance data, the apparent charge density is about 0.26 e/nm^2, the maximal conductance is about 35 pS, and the sodium dissociation constant is approximately 0.5 M. These findings are similar to our findings for dog brain sodium channels, which suggests that a net negative charge at the STX/TTX binding site and at the channel entrance is a conserved feature of sodium channels.

iii) Steady-State Activation of Dog Brain Sodium Channels. BTX-modified sodium channels from dog brain were incorporated into lipid bilayers. The steady-state voltage-activation (gating) characteristics were found to be sigmoidal functions of membrane potential. The midpoint potential of the activation curve varied as a function of mono- and divalent cation concentration, while the apparent effective gating charge (3.4-3.9 elementary charges) showed no systematic variation. The midpoint potential also varied among channels under identical ionic conditions.

That is, the midpoint potential was found to vary by as much as 20 mV from one gating determination to the next. Sodium channels can also show spontaneous shifts in gating behavior; that generally caused a shift in the activation curve without any change in the apparent gating charge. The voltage shifts in steady-state activation as a function of ionic composition were similar to those reported in the literature for macroscopic voltage-clamp experiments on sodium channels. We interpreted the shifts in terms of screening of negative charges. The extracellular surface of the sodium channel protein carries the larger apparent charge density (approx. -0.38 e/nm^2); because the lipids are neutral and the Debye length is comparatively short, those charges are probably protein-linked carboxyl groups and are thus integral characteristics of the sodium channel. Experiments in symmetrical NaCl before and after modest additions of the divalent cation barium resulted in a positive shift of the midpoint potential along the voltage axis. Larger shifts were seen when barium was added asymmetrically to the extracellular solution. These results are as expected if barium is screening negative charges and if the apparent charge density is larger on the extracellular surface. But there is a binding effect as well; barium also reduced the apparent gating charge. Gating-state changes and binding effects of divalent cations have not been reported by others.

iv) Effects of pH on the Binding of Guanidinium Toxins.

Guanidinium toxins such as TTX and STX bind to and close voltage-dependent sodium channels, and it is generally recognized that positively charged guanidinium moieties play a key role in the mechanism of toxin action. The channel-closing potency of guanidinium toxins decreases at alkaline pH as the toxin molecules become deprotonated. A straight forward interpretation of the pH profile of toxin potency is complicated, however, by the presence of a net negative charge near the toxin binding site that concentrates the local toxin and cation species. The rational is being developed for discussing the pH dependence of guanidinium toxin binding in the presence of a surface potential in terms of the valence of the active molecular species of each toxin molecule. Some of the problems associated with assigning an

average or effective toxin valence to guanidinium toxins when discussing toxin binding and the pH dependence of toxin binding are outlined.

D. Near-Term Plans.

I plan to continue working on voltage-dependent sodium channels from dog and rat brain; that work will also be extended to cardiac sodium channels. An effort will be made to finish writing several papers that are in preparation. Particular new projects involve studying the relative STX/TTX binding potency in brain sodium channels at alkaline pH where those toxins begin to loose potency (cf. iv above). A second project involves characterization of cardiac sodium channels with respect to conductance, STX/TTX binding potency, and gating behavior. I will also begin to study voltage-dependent potassium channels from brain using similar planar lipid bilayer techniques.

E. Publications Related to Above Work.

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Scientific Progress During The Year

A. Specific Aims

The overall objective of this project is to screen toxins for actions mediated through cyclic AMP, cyclic GMP or other second messenger systems. As part of this objective toxins are tested on either untreated cells, cell fractions or cells treated with various hormonal or pharmacological agents. In this manner we hope to obtain cells or cell systems that respond to the neurotoxins so as to be able to determine their mechanism of action.

During the last year various experiments were conducted to:

1. Characterize the tetanus toxin attenuation of phorbol myristate acetate (PMA)-induced translocation of protein kinase C (PKC). Specifically, studies were done to:
    a. Compare the dose response relationship of this effect of tetanus toxin in both differentiated and undifferentiated NG-108 cells

b. Characterize the time dependency of this tetanus toxin response in both differentiated and undifferentiated NG-108 cells

2. Examine the possible direct effect of tetanus toxin on partially purified protein kinase C

3. Develop a method for quantifying acetylcholine release from differentiated NG-108 cells

4. Confirm the inhibitory effect of tetanus toxin on potassium (44 mM)-stimulated acetylcholine release from NG-108 cells

5. Characterize the dose and time dependency of neurotensin-induced mobilization of cytosolic PKC in differentiated NG-108 cells

6. Characterize the tetanus toxin attenuation of neurotensin-induced translocation of PKC with specific studies on:
   a. The dose response relationship of toxin and attenuation of PKC mobilization
   b. The time dependency of the toxin response

7. Investigate the effect of neurotensin on acetylcholine secretion from NG-108 cells

8. Investigate the effect of tetanus toxin on 48/80-induced histamine secretion from a rat mast cell preparation

B. Methods

The culture and treatment of cells and assays for PKC activity have been described in the previous annual report.

Acetylcholine release was measured by modification of the method of McGee *et.al.*(1978). NG-108 cells were grown in 60 mm dishes and differentiated as described above. 24-36 h prior to release studies, cells were equilibrium labelled with $1.5x10^{-6}$M [methyl ^{14}C]-choline (50mCi/mmol, NEN) in differentiation medium containing dbcAMP. Tetanus toxin was added at the end of the labelling period prior to removal of unincorporated choline. The cells were then washed with DMEM over a 30 min period (medium changed every 5 min) till a baseline was reached. Medium was then changed to DMEM with 0.1% BSA, 0.1 mM eserine sulfate, and 44 mM $NaHCO_3$ (unstimulated cells) or 44 mM $KHCO_3$ (stimulated cells). Medium was removed and fresh medium added every 3 min for a 21 min period. Fractions were counted for ^{14}C and the cells harvested to determine DNA content.

Rat mast cells were isolated by peritoneal lavage of male Sprague Dawley rats (200-400 g) followed by Percoll gradient centrifugation (Nemeth and Rohlich, 1980). The isolated mast cells were incubated up to 6 h in Krebs-Ringer-HEPES (10 mM) buffer, with or without toxin, and then stimulated to release histamine with 10 µg/ml Compound 48/80. Histamine release into the medium was measured by the fluorometric method of Shore *et al.* (1971).

C. Results

Dose-response characteristics of the tetanus toxin-induced attenuation of PMA-mediated reduction in cytosolic PKC activity in undifferentiated NG-108 cells

As noted in our previous annual report, a 4 h pretreatment of undifferentiated NG-108 cells with tetanus toxin (10^{-8}M) was associated with a significant reduction in the ability of PMA to induce a translocation of PKC to the cell membrane, as measured by a reduction in cytosolic PKC activity. During the present year further studies were done to characterize this response with lower concentrations of tetanus toxin. Table 1 presents the cytoplasmic protein kinase activity obtained in the presence or absence of added phospholipids and the difference in these activities (the PKC activity) for cells pretreated 4 h with tetanus toxin (10^{-8} and 10^{-10}M) and then challenged for 1 h with 0.1 μM PMA. As can be seen, tetanus toxin pretreatment has no effect on the basal PKC activity of NG-108 cells. Treatment of the cells with PMA elicited an expected fall in cytoplasmic PKC activity. However, pretreatment of the cells with 10^{-8}M tetanus toxin attenuated the action of PMA to mobilize cytosolic PKC. This tetanus toxin attenuation of PMA-induced mobilization of PKC is shown more clearly in Table 2. In this table the cytosolic PKC activity mobilized by 0.1 μM PMA is expressed as a % of the control PKC activity from non-PMA treated cells. As shown, pretreatment for 4 h with 10^{-8}M tetanus toxin significantly attenuated the ability of PMA to mobilize cytosolic

PKC activity. Pretreatment of undifferentiated cells with 10^{-10}M tetanus toxin for 4 h did not attenuate the ability of PMA to mobilize cytosolic PKC.

Tetanus toxin inhibition of the PMA-induced reduction in cytosolic PKC activity in differentiated NG-108 cells.

In agreement with our observations in undifferentiated NG-108 cells, tetanus toxin also attenuated the ability of PMA to mobilize cytosolic PKC activity in differentiated NG-108 cells. Two differences between the undifferentiated and differentiated cell state are readily apparent in Table 2. The first is that the degree of PMA-induced mobilization of cytosolic PKC was much smaller in the differentiated state, however the differentiated cell is more sensitive to tetanus toxin than the undifferentiated cell. In undifferentiated NG-108, cells addition of 0.l μM PMA for l h resulted in a 50±12% reduction in subsequently measured cytosolic PKC. In differentiated cells PMA mobilized only 35±12% of the cytosolic PKC. Treatment of differentiated NG-108 cells for 4 hr with $l0^{-10}$M tetanus toxin completely eliminated PMA-induced mobilization of cytosolic PKC. As shown in Figure 1 pretreatment with tetanus toxin ($l0^{-10}$M) for l or 2 h did not significantly alter the ability of PMA to mobilize cytosolic PKC due to the large errors in measuring the reductions in cytosolic PKC compared to the small changes observed in controls.

As shown in Figure 2, tetanus toxin was active in differentiated NG-108 cells at concentrations as low as 10^{-12}M. This is in contrast to undifferentiated cells which required at 10^{-8}M toxin to elicit a significant response. These results represent only 3 experiments and additional experiments are underway to explore lower concentrations of tetanus toxin in this preparation.

Failure of tetanus toxin to directly affect PKC activity

Tetanus toxin had no direct effect on cytosolic PKC. As shown in Figure 3, addition of up to 10^{-7}M tetanus toxin directly to partially purified cytosolic PKC had no effect on the subsequently determined phospholipid-dependent incorporation of ^{32}P-phosphate into histone protein.

Potassium-induced acetylcholine release from isotopically labeled differentiated NG-108 cells

Wellhoner and Neville (1987) have reported that tetanus toxin binds to and inhibits acetylcholine release from differentiated NG-108 cells. In order to correlate our observed changes in cytosolic PKC activity with inhibited acetylcholine release, we conducted experiments to 1.) establish a method for measuring acetylcholine release from NG-108 cells and 2.) confirm and extend the initial observations of Wellhoner and Neville. Our initial experiments explored the time course of acetylcholine release from cells

equilibrium (24-36 h) labeled with [methyl ^{14}C] choline. As shown in Figure 4A labeled acetylcholine (confirmed by an organic extraction technique) was released from prelabeled cells in a characteristic, monoexponential function. When the medium was changed to one in which 44 mM Na^+ was replaced by K^+, there was an increase in acetylcholine release observed within the first 3 min. In spite of continued high K^+, acetylcholine release returned to a basal rate exemplified by the return to a monoexponential release curve. Figure 4B presents the plot of the natural log (ln) of the counts remaining in the cells as a function the total counts originally present versus time of incubation. This shows the observed rapid K^+-induced release. Since the K^+-induced release occurred quickly, we subsequently selected a single 10 min collection of K^+-released radioactivity to represent depolarization-induced release.

The data in Table 3 shows that pretreatment of NG-108 cells with tetanus toxin does result in an inhibition of K^+ stimulated acetylcholine release compared to cells not subject to tetanus. This finding confirms the observation made by Wellhoner and Neville (1987). A 4 h pretreatment with 10^{-10} M toxin resulted in a complete inhibition of the stimulated release. Pretreatment with 10^{-11}M toxin blocked 81% of the stimulated release. 10^{-12} M tetanus was not as effective at blocking acetylcholine release, a result which also is in agreement with the data published by Wellhoner and Neville.

Effect of Neurotensin on cytosolic PKC in differentiated NG-108 cells

Neurotensin addition to differentiated NG-108 cells mobilized cytosolic PKC. As shown in Table 4, cytosolic PKC levels were reduced by a 30 min, but not 15 or 60 min, treatment of NG-108 cells with 10 μM neurotensin.

Neurotensin dose response

As shown in Figure 5 exposure of NG-108 cells to 0.6-30 μM neurotensin for 30 min results in a dose-dependent mobilization of cytosolic PKC. In 5 experiments mobilization is apparent at a dose as low as 3 μM and appears maximal between 10 and 30 μM. Additional experiments will be completed to characterize this relationship.

Tetanus toxin attentuation of neurotensin-mobilized PKC activity in differentiated NG-108 cells

In order to determine whether tetanus toxin would attenuate neurotensin-induced mobilization of PKC in a dose- and time-dependent fashion, two experiments were performed. Differentiated NG-108 cells were pretreated for 1,2 or 4 h with either 10^{-10}M tetanus toxin (Experiment 1) or 10^{-11}M toxin (Experiment 2).

Thereafter, the cells were challenged for 30 min with 10 μM neurotensin. As shown in Table 5, in experiment 1, neurotensin mobilized 25 pmol/mg/min of cytoplasmic PKC, 23±5% of the control activity. Tetanus toxin at 10^{-10}M totally abolished this mobilization in pretreatment periods as short as 1 h. In experiment 2 shown in Table 5, neurotensin mobilized 11±3 pmol/mg/min of the cytosolic PKC in untreated cells. A 4 h pretreatment with tetanus toxin (10^{-11}M) reduced mobilization of PKC to -2±5 pmol/mg/min. This attenuation of neurotensin mobilized PKC was statistically significant in 7 experiments (student's paired t-test). Pretreatment with 10^{-11}M toxin for 2 h also appeared to attenuate neurotensin-mobilized cytosolic PKC but this effect was not significant. Additional experiments are needed to delineate the significance of the toxin effect with pretreatment times of less than 4 h. However, these experiments lend further support to the notion that the action of tetanus toxin is time, as well as dose-dependent in this system.

Neurotensin effect on acetylcholine release

The effect of neurotensin on secretion of ^{14}C-acetylcholine from NG-108 cells was investigated. Neurotensin was assessed for its ability to directly stimulate secretion. As shown in Table 6 stimulation of prelabeled cells with 44 mM potassium for 10 min results in a statistically significant increase in the release of ^{14}C-acetylcholine over that observed from unstimulated cells. However, neither a 10 min or 30 min exposure to 10 μM neurotensin resulted

in any release of ^{14}C-acetylcholine that was different from that observed from control cells. Phorbol myristate acetate (PMA), another agent shown to mobilize cytosolic PKC in these cells, was also ineffective in directly stimulating ^{14}C-acetylcholine release. These observations are in keeping with the proposal that protein kinase C may not directly stimulate secretion but may act more to enhance the secretory process (Terbush *et al.*, 1988; Malhtra *et al.*, 1988). Moreover, in this cell line potassium induces the release of more ^{14}C-acetylcholine than any other secretagogue tested therefore it is possible that potassium-induced secretion can not be further enhanced by mobilization of PKC. An additional explanation is that the effect of translocated PKC on secretion may not become apparent in 30 min. We are currently pursuing experiments with other secretagogues to more fully characterize the effect of agonist-induced PKC mobilization and secretion in this cell system.

Effect of potassium on cytosolic PKC

In NG-108 cells prelabeled for 24-36 hrs with with ^{14}C-choline, addition of 44 mM potassium induces a secretion of ^{14}C-acetylcholine. Since tetanus toxin has been shown to inhibit this K^+-induced secretion, the effect of potassium on mobilization of cytosolic PKC in NG-108 cells was examined. Treatment of the cells for 30 min with 44 mM potassium did not result in a detectable mobilization of cytosolic PKC (data not shown). This finding is in agreement with the observation of Terbush et al. (1988) in

permeablized adrenal chromaffin cells. These investigators found a rapid mobilization (within 2 sec) of PKC to the membrane in their preparation, which was reversed by 30 sec. If potassium has an effect on cytosolic PKC in NG-108 cells it may occur more rapidly or is reversible despite continued exposure to high potassium. More experiments with shorter exposure times are needed to characterize an effect of high potassium on cytosolic PKC, and the effect of toxin on this response.

Failure of tetanus toxin to modify Compound 48/80-induced histamine release from primary rat mast cells

In order to determine whether tetanus toxin might inhibit the secretory response of a formed blood element, isolated rat peritoneal mast cells were pretreated 4 h with 10^{-8}M toxin. The cells were subsequently challenged with 10 µg/ml Compound 48/80 to elicit secretion of histamine. As shown in Table 7, tetanus toxin pretreatment did not result in any inhibition of histamine release induced by this agent.

D. Future Plans

A manuscript describing our initial findings on tetanus toxin-induced attenuation of phorbol ester mobilization of protein kinase C has been accepted in Toxicon. A second manuscript on neurotensin stimulation of NG-108 cell is in preparation, as is a more complete

follow-up paper to that accepted in Toxicon. During the next quarter we will be expending a major effort to complete sufficient experiments for detailed statistical anaiysis of many of the areas discussed in this and previous quarterly reports. A major new area that will be addressed is the screening on potential secretagogues for stimulation of ^{14}C-acetylcholine release from prelabeled NG-108 cells. While the degree of stimulation achieved in the present studies is similar to that reported by earlier workers it is not of the magnitude for analysis of the modulatory effect of other hormones or agents. The results of our studies indicate that the relation between the effect of tetanus toxin on PKC mobilization and the subsequent inhibition of secretion is not straight forward. Tetanus clearly blocks K^+-stimulated secretion but we are not convinced that PKC mobilization is a component of this secretory response. Neurotensin-induced PKC mobilization is blocked by tetanus toxin but neurotensin is not an effective stimulator of acetylcholine release. Other secretagogues to be tested for their effects on both secretion and PKC-mobilization include PGE_1, epinephrine, serotonin, bradykinin and barium. These studies will have a significant impact on our analysis of potential sites of tetanus action.

We have also begun experiments to determine whether tetanus toxin inhibits the phosphorylation of an 80 kDa protein reported to be a substrate for PKC (Wolfman *et al.*, 1987). It is anticipated that this will be a better measure of PKC activity in this system.

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Considine RV, Bielicki JK, Simpson LL, Sherwin JR Tetanus toxin attenuates the ability of phorbol myristate acetate to mobilize cytosolic protein kinase C in NG-108 cells.
Accepted for publication in Toxicon

TABLE 1. DOSE RESPONSE EFFECT OF TETANUS TOXIN PRETREATMENT ON THE ACTIVITY OF CYTOSOLIC PROTEIN KINASES FROM UNDIFFERENTIATED NG-108 NEUROBLASTOMA CELLS

		^{32}P Incorporation into histone protein (pmol/mg min)					
		Control Cytosol			PMA Cytosol		
	(n)	Lipid + Ca^{+2}	EGTA	PKC[a]	Lipid + Ca^{+2}	EGTA	PKC[a]
Non-toxin treated	7	172 ±20	94 ±17	78 ±17	140 ±18	97 ±6	43 ±14
Tetanus Toxin 10^{-8}M		178 ±16	99 ±8	79 ±10	159 ±14	101 ±9	59 ±11
Non-toxin treated	8	168 ±20	87 ±9	81 ±13	139 ±23	89 ±12	50 ±13
Tetanus toxin 10^{-10}M		174 ±22	91 ±12	84 ±12	145 ±18	88 ±11	57 ±8

Effect of tetanus toxin pretreatment on the activity of cytosolic protein kinase C isolated from undifferentiated NG-108 cells. Cells were pretreated with decreasing conconcentrations of tetanus toxin followed by 1 h exposure to PMA (0.1μM). Cytosolic protein kinase C was isolated and its activity measured as described in the Methods.

[a] Protein kinase C (PKC) activity was obtained as the difference between Lipid + Ca^{+2} and EGTA treated cytosolic activity.

TABLE 2. TETANUS TOXIN ATTENUATION OF 0.1 μM PMA-MOBILIZABLE PKC ACTIVITY IN UNDIFFERENTIATED AND DIFFERENTIATED NG-108 CELLS

Group	(n)	Cytosolic PKC Activity (pmol/mg/min) - PMA	Cytosolic PKC Activity (pmol/mg/min) + PMA	PMA-Mobilized PKC Activity (pmol/mg/min)	PMA-Mobilized PKC Activity (% Control PKC Activity)
Undifferentiated	7				
Control		78 ±17	43 ±14	35 ±14	50 ±12
Tetanus toxin 10^{-8}M		79 ±10	59 ±11	20 ±14	18[a] ±19
Undifferentiated	8				
Control		81 ±13	50 ±13	31 ±5	45 ±9
Tetanus toxin 10^{-10}M		84 ±12	57 ±8	27 ±5	33 ±4
Differentiated	7				
Control		42 ±13	31 ±11	10 ±3	35 ±12
Tetanus toxin 10^{-10}M		42 ±12	43 ±14	-1 ±7	-13[a] ±18

Cells were pretreated 4 hr in the presence or absence of tetanus toxin (10^{-8} or 10^{-10}M) followed by incubation in the presence or absence of 0.1 μM PMA for 1 hr. PKC activity and PMA-mobilized PKC activity as percent of control were calculated for each experiment as described in the Methods. Values represent the mean ±SEM of the indicated number of experiments.

a=$p<0.05$ by Students paired t-test comparison to cells not exposed to tetanus toxin.

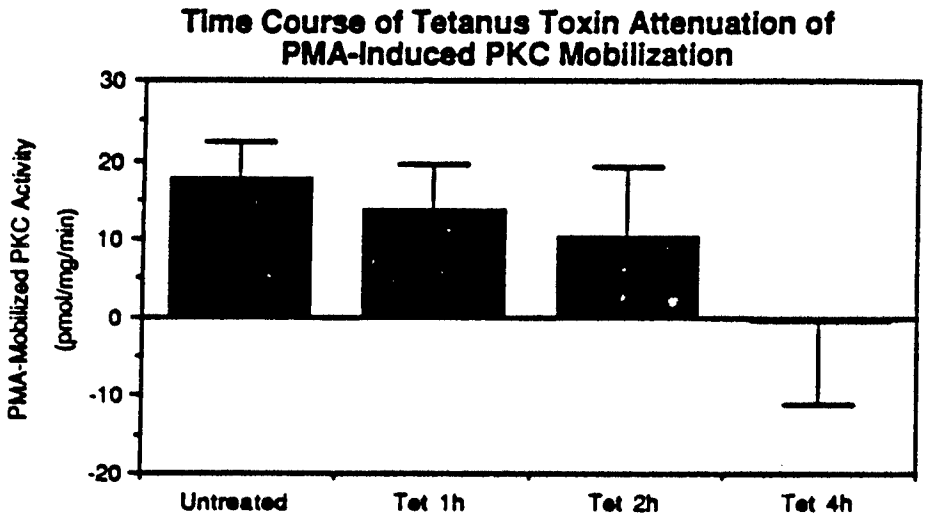

Figure 1. Differentiated NG-108 cells were pretreated with 10^{-10}M tetanus toxin for 1, 2 and 4 h , and then exposed to 0.1 μM PMA for 1 h. Data is expressed as the PKC activity moliblized by PMA compared to unstimulated cells. Values are the mean ±SEM of at least 3 experiments.

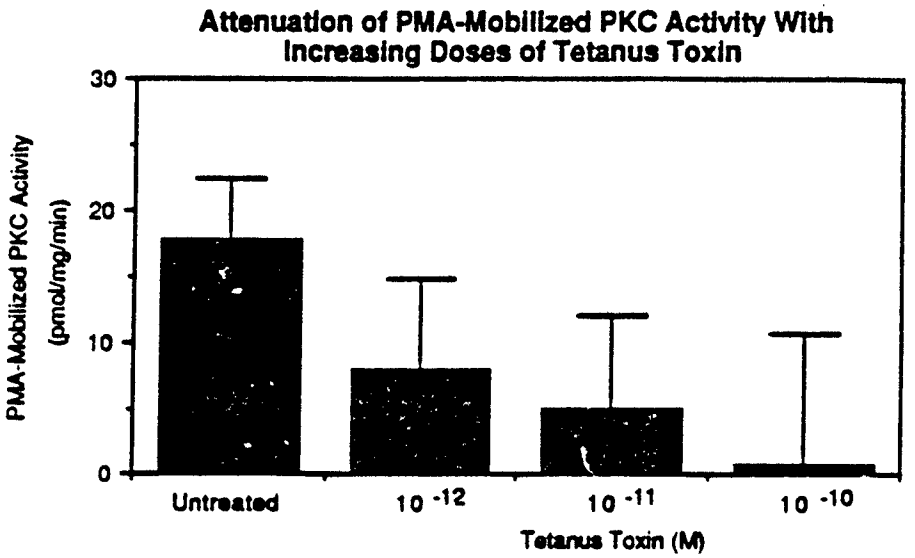

Figure 2. Differentiated NG-108 cells were pretreated with various doses of tetanus toxin for 4 h and then challenged for 1 h with 0.1 μM PMA. Data is expressed as the PKC activity mobilized by PMA compared to unstimulated cells. Values represent the mean ±SEM of 3 experiments.

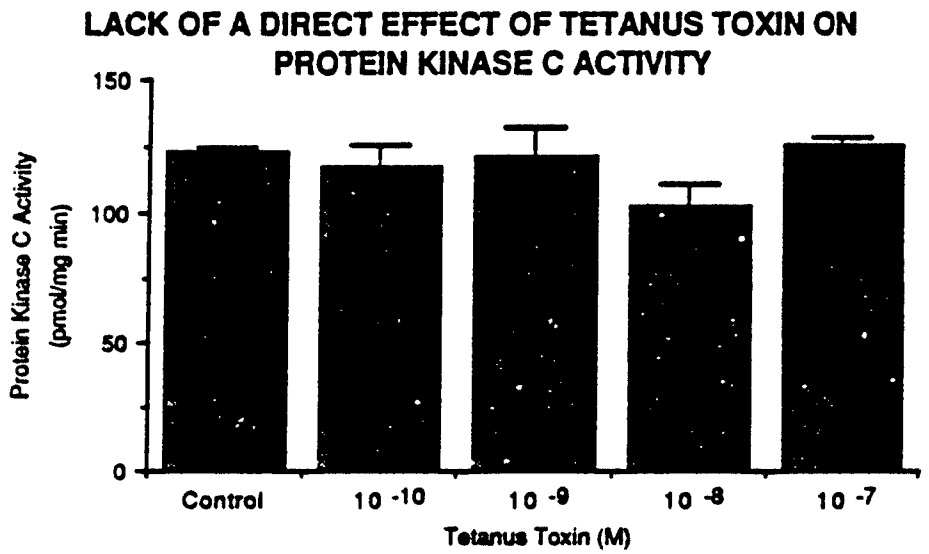

Figure 3. Lack of a direct effect of tetanus toxin on PKC activity. The activity of cytosolic PKC from untreated NG-108 cells was measured in the presence of increasing concentrations of tetanus toxin (10^{-10}-10^{-7}M). Addition of toxin had no effect on the phospholipid-dependent incorporation of ^{32}P-phosphate into histone protein.

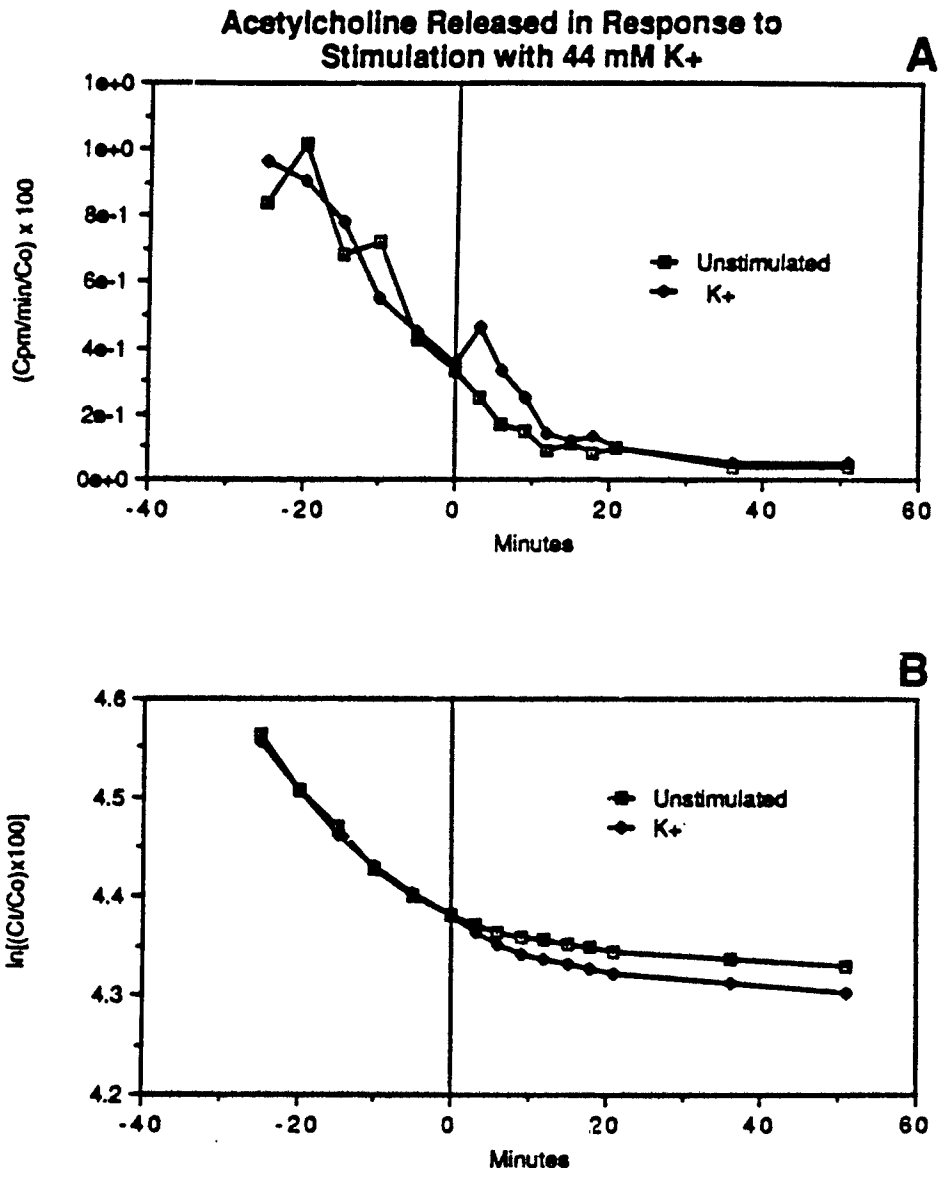

Figure 4. Differentiated NG-108 cells were equilibrium labeled with ^{14}C-choline for 24-36 h as described in the Methods. Cells were washed with fresh medium over a 30 min period and then stimulated with 44 mM K^+. Panel A shows washout of radioactivity is a monoexponential function. K^+-stimulated acetylcholine release occurs in the first 3 min and returns to the exponential baseline by 10 min. Panel B represents the natural log transform of the release in panel A.

TABLE 3. EFFECT OF 4 HOUR TETANUS TOXIN PRETREATMENT ON K^+ STIMULATED ACETYLCHOLINE RELEASE FROM DIFFERENTIATED NG-108 CELLS

Group	K^+ Stimulated Acetylcholine Release (% of Control)	Inhibition by Tetanus Toxin
Untreated Control	132±6	----
10^{-12}M toxin	115±7	53%
10^{-11}M toxin	106±9	81%
10^{-10}M toxin	96±2	100%

Differentiated NG-108 cells were equilibrium labeled with ^{14}C-choline for 24-36 h prior to release studies. The cells were washed to remove unincorporated choline and release measured over a 10 min collection period. K^+-stimulated release is normalized to the release measured in cells exposed to neither K^+ nor toxin. Values represent the mean ±SEM of at least 2 experiments at each dose of tetanus toxin.

TABLE 4. NEUROTENSIN MOBILIZATION OF CYTOSOLIC PROTEIN KINASE C IN DIFFERENTIATED NG-108 CELLS

Duration of Neurotensin Exposure (min)	Reduction in Cytosolic PKC (pmol/mg/min)	% of Control
15	-3.3±10.5	121±26
30	17.8±5.6	47±15
60	-10.2±6.5	125±22

Differentiated NG-108 cells were exposed to 10 μM neurotensin for the times indicated followed by measurement of cytosolic PKC activity as previously described. The data is expressed both as the amount of PKC activity that neurotensin could mobilize and as a % of the cytosolic activity in untreated cells. Values represent the mean ±SEM for 3 experiments.

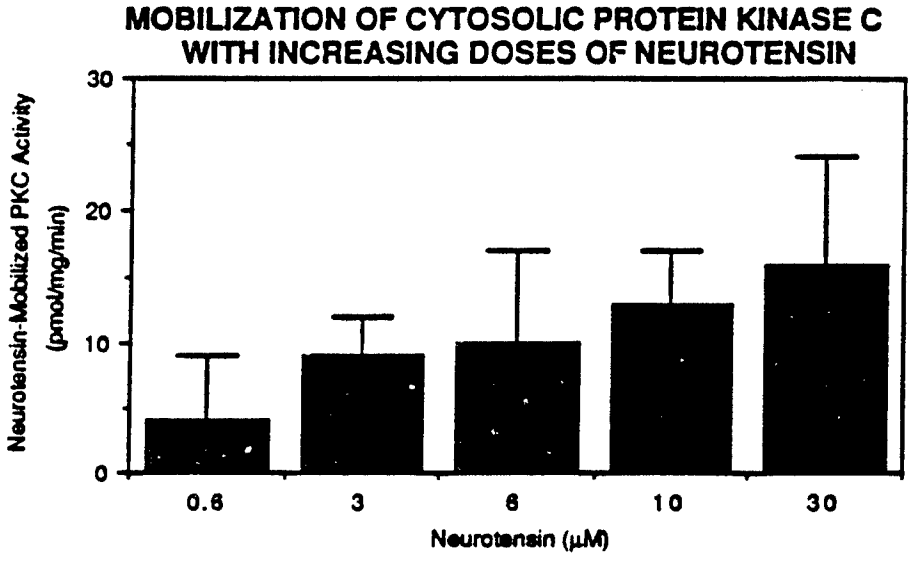

Figure 5. Differentiated NG-108 cells were treated with neurotensin (0.6-30 μM) for 30 min to mobilize cytosolic PKC. Values are expressed as the pmol/mg/min mobilized by neurotensin and represent the mean ±SEM of 5 experiments.

TABLE 5. TETANUS TOXIN ATTENUATION OF 10 μM NEUROTENSIN-MOBILIZABLE PKC ACTIVITY IN DIFFERENTIATED NG-108 CELLS

Group	(n)	Cytosolic PKC Activity (pmol/mg/min) - Hormone	Cytosolic PKC Activity (pmol/mg/min) + Hormone	Neurotensin-Mobilized PKC Activity (pmol/mg/min)	Neurotensin-Mobilized PKC Activity (% Control PKC Activity)
Untreated	11	98 ±20	73 ±14	25 ±8	23 ±5
10^{-10}M toxin					
1 h	8	96 ±24	89 ±20	6 ±12	-1[b] ±11
2 h	11	87 ±17	86 ±18	1 ±5	-4[b] ±7
4 h	6	88 ±17	91 ±19	-4 ±10	-8[b] ±9
Untreated	7	83 ±27	72 ±25	11 ±3	17 ±7
10^{-11}M toxin					
1 h	7	84 ±25	86 ±36	3 ±11	10 ±11
2 h	7	84 ±30	93 ±39	-9 ±11	-8 ±10
4 h	7	83 ±29	85 ±33	-2 ±5	1[b] ±4

Cells were pretreated 1, 2 and 4 h in the presence or absence of tetanus toxin (10^{-10} and 10^{-11}M) followed by incubation in the presence or absence of 10 μM neurotensin for 30 min. Values represent the mean ±SEM of the indicated number of experiments.

b=$p<0.05$ by Students paired t-test comparison to cells not exposed to tetanus toxin.

TABLE 6. LACK OF STIMULATION OF ACETYLCHOLINE RELEASE FROM DIFFERENTIATED NG-108 CELLS WITH AGONISTS OF PROTEIN KINASE C

Pretreatment	Treatment	Acetylcholine Release (cpm/mg /10 min)	(% control)
None	Control	645±97	----
None	44 mM K^+	813±135	127±4*
None	0.1 μM PMA	559±127	103±15
None	10 μM Neurotensin	524±126	93±12
10μM Neurotensin	None	735±164	109±7

Cells were pretreated 30 min with either no additions or 10 μM neurotensin, then challenged in a 10 min treatment incubation with the indicated secretagogue. Acetylcholine release was expressed as cpm released per mg of protein during the 10 min release incubation and as percent of control values obtained in cells receiving no pretreatment or incubation additions. Values are the mean ±SEM of at least six experiments.
*$p<0.05$ by student's paired t-test

TABLE 7. LACK OF EFFECT OF TETANUS TOXIN ON COMPOUND 48/80 INDUCED HISTAMINE SECRETION FROM RAT PERITONEAL MAST CELLS

	Untreated		Tetanus toxin (10^{-8}M)	
Exp.	Control	48/80	Control	48/80
1.	30.3%	71.7%	28.4%	69.6%
2.	9.5%	54.2%	11.8%	47.0%

Lack of an effect of tetanus toxin (10^{-8}M) on Compound. 48/80 induced histamine release from rat peritoneal mast cells. Mast cells ($1x10^5$ cells/ml) were incubated with or without toxin for 4 h and then challenged with 10 µg/ml 48/80 for 30 min. Histamine release is expressed as the percentage of total cell histamine content.

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Professor of Medicine

Scientific Progress During the Year

A. Specific Aims

The major goal during the past year has been to study the structure-function relationships of toxins that block exocytosis. The major accomplishment has been to compare the neuromuscular blocking properties of clostridial toxins and phospholipase A2 toxins.

B. Background

There are two major groups of potent protein toxins that act presynaptically to inhibit transmitter release. One group is the clostridial neurotoxins, composed of botulinum neurotoxin and tetanus toxin (Simpson, 1986; Habermann and Dreyer, 1986), and the other group is the phospholipase A2 neurotoxins of snake venom origin, composed of more than two dozen representatives (Chang, 1985; Rosenberg, 1986). Although a considerable amount of work has been done on both groups of protein toxins, the complete mechanisms of action have not been determined.

For the clostridial neurotoxins there is evidence that three sequential steps are involved in toxin-induced blockade of exocytosis. There is a membrane binding step, an internalization step, and an intracellular poisoning step (Simpson, 1980, 1981). This scheme of events was deduced from a series of pharmacologic and immunologic experiments

(Simpson, 1980), and it was later confirmed by morphologic (Black et al., 1986a,b) and other means (Penner et al., 1986).

The sequence of events in phospholipase A2 toxin-induced blockade of transmitter release is less clear. There is an initial binding step, and this appears to be distinct from the poisoning step (Chang et al., 1977). However, there is no compelling evidence to indicate whether additional steps are interposed between binding and paralysis, nor is there agreement on whether the toxin must be internalized to express its poisoning effect.

A paradigm has been developed for studying the several steps in clostridial neurotoxin action (see Methods and Discussion), and this paradigm has been employed successfully in a variety of contexts (Simpson, 1980; Schmitt et al., 1981). Interestingly, this paradigm has not been applied to the study of any of the phospholipase A2 neurotoxins. Therefore, we have applied this method of investigation to the study of crotoxin and its subunits.

Crotoxin is a 24,000 dalton protein obtained from the venom of Crotalus durissus terrificus (Habermann et al., 1978; Bon et al., 1986). It is composed of two non-covalently associated subunits, one of which is basic and possesses phospholipase A2 activity (15,000 daltons), and the other of which is acidic and may play a role in facilitating receptor binding (9,000 daltons). Individually the two subunits have little neuropharmacological activity,

but in combination they have a potent neuromuscular blocking action (Chang and Su, 1978).

In the present study we have added the holotoxin or the individual subunits to the isolated phrenic nerve-hemidiaphragm preparation. This was done in the presence or absence of drugs or procedures known to alter the progression of clostridial neurotoxin-induced paralysis. The results of these experiments have helped to clarify the mechanism of action of phospholipase A2 neurotoxins.

C. Methods

Crotoxin. The neurotoxin was isolated from the crude venom of Crotalus durissus terrificus. Approximately 300 mg of venom were applied to a Sephadex G-75 column (100 x 2.5 cm) equilibrated with 20 mM glycine buffer containing 150 mM NaCl (pH 1.9); the flow rate was 20 ml/hr. Various fractions were collected, and the one that possessed neuromuscular blocking activity was dialyzed against 10 mM sodium phosphate buffer (pH 7.2) and then concentrated. This material was further purified by ion exchange chromatography on a DEAE Sephadex A-50 column (1.5 x 30 cm) equilibrated with 50 mM potassium acetate (pH 6.0) containing 75 mM KCl. After the neurotoxin was applied, the column was washed with one void volume of the acetate buffer containing KCl. The neurotoxin was then eluted in a KCl gradient of 75 to 300 mM; the material appeared at approximately 180 mM KCl. The identity of crotoxin was

confirmed on the basis of its presynaptic site of action and its apparent molecular weight (24,000 daltons) on SDS-PAGE.

The basic (phospholipase A2) and acidic (crotapotin) subunits of crotoxin were separated and isolated by the method of Hendon and Frankel-Conrat (1971). The neurotoxin was applied to a DEAE Sephadex A-50 column (1.5 x 30 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.3) containing 100 mM KCl and 6 M urea. After the toxin had been added, a KCl gradient was applied to elute the subunits. The basic subunit was eluted immediately at a KCl gradient of 112 to 130 mM; the acidic subunit was eluted in a gradient of 600 to 760 mM. The identity of the subunits was confirmed on the basis of their ability to form reconstituted and biologically active toxin and on the basis of their apparent molecular weights (basic ~ 15,000 daltons; acidic ~ 9,000 daltons).

Antibodies. A polyclonal preparation and a monoclonal antibody were used to analyze the neuromuscular blocking action of crotoxin. Rabbit polyclonal antisera were raised against the basic subunit of crotoxoin precisely as previously described (Kaiser et al., 1986), and murine monoclonal antibodies were produced against the basic subunit, also as previously described (Kaiser and Middlebrook, 1988). For the purpose of the present study, the neutralizing monoclonal antibody is referred to as Mab 1.

Rabbit antiserum was quantified in terms of its neutralizing titer. One ml of the preparation neutralized 460 LD50 of intact crotoxin. Mab 1, crotoxin and its subunits were quantified in terms of protein concentration. This was done spectrophotometrically using silica cuvets, a 1 cm light path, and the following $E_{280\ nm}$ values: Mab 1, 13.5; crotoxin, 15.7; acidic subunit, 9.6; and basic subunit 17.0.

Chemical modification of crotoxin. A single histidine residue was modified with p-bromophenacyl bromide according to the procedure of Abe et al. (1977). The crotoxin complex was dissociated, and the basic subunit was isolated. The protein (5 mg) was dissolved in 5 ml of 100 mM cacodylate buffer (pH 6.0) containing 100 mM NaCl. To this solution was added p-bromophenacyl bromide (1 mg in 0.05 ml acetone). The reaction mixture was incubated at 30° C for 12 hr. The reaction was terminated by applying the mixture to a Sephadex G-25 column (25 x 0.9 cm) that was equilibrated with 5 mM Tris- HCl (pH 7.4) that contained 100 mM NaCl. The modified basic subunit was eluted from the column and reassociated with acidic subunit. This was accomplished by mixing and stirring the two subunits in phosphate buffer (0.5 M; pH 7.2) for 30 minutes at room temperature. The unreacted subunits were separated from the reconstituted toxin by gel chromatography (Sephadex G-50; 45 x 0.9 cm; phosphate buffer, as above).

Amino acid analysis. The native basic subunit and the modified protein derivative were subjected to hydrolysis with constant boiling in 6 N HCl containing 1% phenol (150°C; 1 hr) in a sealed, evacuated vial. The hydrolysates were first treated with redrying solution consisting of ethanol: water: triethylamine (2 : 2 : 1, vol/vol) and then the samples and an amino acid standard were derivatized with phenylthiohydantoin. Amino acid analyses were performed on a Waters Pico-Tag Amino Acid Analysis System. The results showed that amino acid composition of unmodified basic subunit was identical to that reported in the literature (Breithaupt et al., 1974). The amino acid analysis of the modified basic subunit showed that only one histidine was modified of the two histidines that are present.

Neuromuscular preparation. Phrenic nerve hemidiaphragms were isolated from female mice weighing 20-30 g. These preparations were suspended in tissue baths (~ 20 ml) containing Tyrode solution of the following composition mM; NaCl, 137; KCl, 2.8; $CaCl_2$, 1.8; $MgCl_2$, 1.1; NaH_2PO_4, 0.33; $NaHCO_3$, 11.9; and glucose, 11.2. In some experiments, calcium (1.8 mM) was replaced by strontium (3.6 mM). The solution was bubbled with a mixture of 95% O_2 and 5% CO_2. Temperature was maintained at 37° C.

Phrenic nerves were stimulated supramaximally with bipolar electrodes. The parameters of nerve stimulation were 0.2 Hz of 0.1 msec duration. Muscle twitch was recorded via a strain-gauge transducer. Toxin-induced

neuromuscular blockade was defined as a 90% reduction in muscle response to nerve stimulation.

Incubation conditions. Experiments were done in an effort to identify and characterize the steps in crotoxin-induced neuromuscular blockade. The methods were similar to those developed for the study of botulinum neurotoxin (Simpson, 1980). Tissues were incubated with toxin at low temperature (10° C, 60 min, 8 ml) and in the absence of nerve stimulation. This was intended to allow toxin to bind, but at the same time it retarded any energy-dependent mechanism inherent in the nerve ending (viz., receptor-mediated endocytosis) or in the toxin molecule (viz., phopholipase activity). Tissues were then washed three or more times in a large volume of solution to remove unbound or loosely associated toxin, and tissues were resuspended in toxin-free solution. In some cases temperature was raised, nerve stimulation was applied, and paralysis times were monitored. In other cases (see Results), drugs or antibodies were added at various times after the original incubation. The purpose was to determine whether bound toxin remained at the cell surface or was internalized.

D. Results

Establishing experimental conditions. Preliminary experiments were done to determine the dose-response characteristics for crotoxin-induced neuromuscular blockade. This was done under two conditions: i.) toxin was added to

tissue baths at 37° C, nerves were stimulated, and paralysis times were measured, or ii.) toxin was incubated with tissues at 10° C for 60 minutes, after which tissues were washed and suspended at 37° C with nerve stimulation, and paralysis was measured. These preliminary experiments revealed two unexpected outcomes. Firstly, the variability in paralysis times for low concentrations of toxin was rather high, especially when the incubation paradigm was used. Secondly, tissues that had been incubated with low doses of crotoxin would occasionally begin to paralyze but not become fully paralyzed (Figure 1).

Given the past literature on protein toxins in general and crotoxin in particular, one can propose at least two explanations for these findings. To begin with, crotoxin is very basic, and like other highly charged proteins it may bind to nonspecific sites. This would be particularly evident at low toxin concentrations. In addition, Chang et al. (1977) have reported that crotoxin can be inactivated by muscle tissue. Again, this would be more evident at low toxin concentrations.

The accepted mechanism for diminishing nonspecific binding is to add an auxiliary protein. In the studies described below, crotoxin was added to tissues in the presence of 0.02% gelatin. This did reduce variability, and it reduced - but did not completely eliminate - the phenomenon of incomplete blockade. No explanation has been

provided for the proposed tissue inactivation of crotoxin, and thus there are no obvious mechanisms for preventing it.

Dose-response experiments. Various concentrations of crotoxin were added to phrenic nerve-hemidiaphragms under the two conditions described above. The results are illustrated in Figure 2. Within the concentration range of 0.3 to 10.0 μg/ml (0.125 to 4.16 x 10^{-7} M), crotoxin produced dose-dependent paralysis when added directly to stimulated muscles. The same concentrations caused paralysis when incubated with unstimulated tissues at 10° C, but the amount of time needed for development of paralysis was greater at the lower concentrations.

In contrast to the holotoxin, the individual subunits possessed little toxicity. When tested at concentrations of 10^{-6} M, neither subunit produced observable effects on transmission within 200 minutes. This result was obtained irrespective of whether subunits were added to tissue baths with stimulated nerves or to incubation tubes with unstimulated nerves.

Factors that influence binding. Tissues were incubated with toxin (2 μg/ml) at 10° C and for varying lengths of time (see Figure 3). At the end of incubation, tissues were washed and resuspended in solution without added toxin (37° C). Nerves were stimulated and paralysis times were monitored. As expected, increases in incubation time produced decreases in eventual paralysis time, until a steady-state was attained. Assuming that the steady state

condition reflects saturation or near-saturation of binding, the apparent half-time for tissue association was approximately 22 minutes.

In the next experiment, tissues were incubated with toxin (2 μg/ml) for 60 minutes and temperature was varied (7 to 37° C). The results (see Figure 4) showed that at 7° C and 17° C toxin activity was not affected by temperature. However, as temperature was raised (e.g., 27° C and 37° C), the eventual paralysis was shortened. This finding may reflect that there is a temperature-dependent step in paralysis; at low temperatures this step is arrested but at higher temperatures it is initiated (see Discussion).

When tissues were incubated with toxin at low temperature (10° C) and for sufficient time to allow apparent saturation of binding (60 min), the eventual development of paralysis was not affected by nerve stimulation. Tissues became paralyzed in the same amount of time, regardless of whether nerves were stimulated or unstimulated (Table 1).

The binding of the holotoxin to the neuromuscular junction was not significantly reversible, assuming that toxin was incubated with tissue under conditions that allowed binding to approach saturation (10° C, 60 minutes). Washing tissues for 60 minutes and with numerous changes of medium did not prolong eventual paralysis. However, this was not true when toxin was incubated with tissue for less

time than was needed to reach steady-state. For example, when tissues were incubated with toxin for 20 minutes (10° C), a subsequent wash (60 minutes) almost doubled the eventual paralysis time (Table 1).

Finally, the replacement of calcium by strontium during the incubation period did not delay the eventual onset of paralysis. Tissues incubated in either of the two divalent cations had approximately the same paralysis times (Table 1). On the other hand, replacement of calcium by strontium after incubation (i.e., after tissues had been washed and returned to medium at 37° C and with nerve stimulation) had a marked effect. Tissues suspended in strontium-containing medium required more than twice as long to develop paralysis (Table 1).

Pharmacologic actions of the subunits. As indicated above, neither subunit alone was appreciably neurotoxic. However, the reconstituted molecule blocked neuromuscular transmission. When the acidic and basic subunits were mixed at a molar ratio of one-to-one, paralytic activity was restored (Table 2). This could be demonstrated either by mixing the subunits in a reaction vessel (25° C, 30 minutes) and adding the product to tissues, or by adding the subunits to a bath with a phrenic nerve-hemidiaphragm preparation.

Three types of experiments were done to characterize the interaction between the subunits or between individual subunits and the holotoxin. In the first, the acidic subunit was incubated with tissue (10° C, 60 minutes).

After washing, tissues were incubated with an equimolar concentration of the basic subunit (10° C, 60 minutes). Tissues were again washed, then suspended in physiologic medium at 37° C and with nerve stimulation. The results showed that the apparent toxicity of this sequence of additions of subunits was no more than 10% of that of the subunits added together or that of the holotoxin (Table 2).

The second experiment involved the same protocol, except that the order of addition of the subunits was reversed. The results were essentially the same as before. The individually added subunits possessed only about 10% of the toxicity of the holotoxin (Table 2).

In the final experiment, the subunits were examined for their potential interaction with the parent molecule. Either the acidic or basic subunit of the toxin was incubated with tissue (10° C, 60 minutes), and after washing there was an equivalent incubation with the holotoxin. Tissues were then washed and suspended in physiologic medium (37° C) with nerve stimulation. In both sequences of additions, the subunits were added at a 10-fold molar excess to the holotoxin. The results (Table 2) showed that prior addition of subunit did not alter the eventual course of paralysis caused by the holotoxin.

Pharmacologic actions of the modified basic subunit. The basic subunit was separated from the holotoxin and covalently modified at a histidine residue as described under Methods. The modified polypeptide was tested for

biologic activity either alone or when reconstituted with the acidic subunit.

When added to stimulated tissues at 37° C, the modified subunit (10^{-6} M) had no obvious effect on neuromuscular transmission. This is in keeping with earlier results on unmodified basic subunit (see above). When the modified subunit was reconstituted with the acidic subunit, a small measure of neuromuscular blocking activity was obtained. A comparison of the dose-response characteristics of modified, reconstituted toxin and the unmodified toxin (e.g., Fig 1) showed that the former possessed 10% or less of the activity of the latter.

The weakly active, reconstituted molecule could not be used as an effective antagonist of the native toxin. When the modified toxin (8 μg/ml) was incubated with the tissues along with native crotoxin (2 μg/ml) for 60 min at 10° C followed by washing and stimulation at 37° C in physiologic medium, it did not protect tissues from the paralytic action of the native toxin (2 μg/ml; Table 2).

Antibody experiments. Antiserum containing polyclonal antibody was titrated for its ability to neutralize a fixed concentration of crotoxin (2 μg/ml). Substantial neutralization was obtained when 0.2 ml of antiserum was incubated with toxin for 30 min at 37° C (Table 3; Groups 1 and 2). The ability of polyclonal antibody to neutralize tissue-bound toxin was studied in two ways. In the first, tissues were incubated (10° C, 60 minutes, no stimulation)

with toxin (2 μg/ml), then washed free of unbound toxin and incubated (10° C, 60 minutes, no stimulation) with antibody (0.2 ml). The tissues were again washed, then suspended at 37° C with stimulation. The results showed that tissue-bound toxin was still accessible to the neutralizing effect of antibody (Table 3; Group 3).

In the second experiment, tissues were incubated with toxin (2 μg/ml, 10° C 60 min, no stimulation), then washed and suspended in a tissue bath (37° C, with stimulation). After 30 minutes the tissues were returned to incubation tubes that contained antibody (0.2 ml, 10° C, 60 min, no stimulation). The tissues were later returned to the tissue bath (37° C, with stimulation), and paralysis times were monitored. The results revealed an interesting outcome. Even after tissues had been warmed and stimulated for 30 min, the toxin remained at least partially accessible to the neutralizing effects of antiserum (Table 3; Group 4).

An identical series of experiments was performed with a monoclonal antibody (Mab 1). Titration experiments revealed that crotoxin (2 μg/ml) could be neutralized by an excess of the antibody (32 μg/ml) when the two were mixed and incubated (37° C, 30 min) prior to addition to tissues. However, unlike the polyclonal preparation, the monoclonal antibody did not neutralize tissue-bound toxin (results not illustrated).

Drugs that antagonize endocytosis. Ammonium chloride and methylamine hydrochloride antagonize the actions of many

substances that must be endocytosed, including clostridial neurotoxins (Simpson, 1981). A series of experiments was done to determine whether these drugs would also antagonize crotoxin. Phrenic nerve-hemidiaphragms were pretreated (20 min) with ammonium chloride (10 mM) or methylamine hydrochloride (20 mM) and then exposed to toxin (2.0 μg/ml). The paralysis times of these tissues were not significantly different from those of controls (results not illustrated).

E. Conclusions

Comparison of protein toxins. Crotoxin is a 24,000 dalton protein that acts at the neuromuscular junction to block exocytosis. The molecule is composed of two subunits: a 9,000 dalton acidic polypeptide that reportedly plays a role in receptor binding, and a 15,000 dalton basic polypeptide that possesses phospholipase A2 activity.

At least superficially, crotoxin appears to be similar to a host of potent protein toxins, such as diphtheria toxin, abrin and ricin, and botulinum and tetanus neurotoxins (Gill, 1978; Olsnes and Pihl, 1982; Hayaishi and Ueda, 1982; Eidels et al., 1983; Alouf et al., 1984; Foster and Kinney, 1984-85; Simpson, 1986; Habermann, 1986). Each of these potent toxins has two polypeptide chains, one of which acts as a ligand that binds to receptors on the cell surface and the other of which acts as an enzyme to modify an intracellular substrate. In addition, these toxins progress through the same sequence of events in expressing their effects. There is an initial binding step, a membrane

translocation step, and an intracellular poisoning step. The translocation step is normally due to receptor-mediated endocytosis.

Although crotoxin possesses characteristics that appear to make it a member of this class of potent protein toxins, a close inspection of the literature and of the data in the present study raise questions. To begin with, neither subunit of the toxin behaves like a true tissue-targeting component when separated from the parent molecule (and see below). Furthermore, there is no compelling evidence to indicate whether the toxin is internalized. Chang and his colleagues (Chang et al., 1985; Su and Chang, 1984;) have provided anecdotal comments suggesting that phospholipase A2 neurotoxins are internalized, but this has not been supported by experimental evidence. And finally, the actual mechanism of poisoning, whether expressed on the outside or the inside of the nerve membrane, has not been definitively established. The toxin does have phospholipase A2 activity, but the role this plays in neurotoxicity has been questioned (Ghassemi et al., 1988; Rosenberg, 1986).

In spite of these uncertainties, there is one point on which virtually all investigators agree. The ligand binding property of the intact toxin is not synonymous with the neurotoxic property. One or more steps intervene between binding and eventual expression of toxicity. The present study accepts this premise and uses it as a basis for addressing two questions. Firstly, what are the

characteristics that govern tissue association by the toxin? And secondly, is the binding step followed by a receptor-mediated endocytosis step?

Characterization of the binding step. The binding of crotoxin has been studied by using a paradigm that has worked well with other toxins, including clostridial neurotoxins (Simpson, 1980; Schmitt et al. 1981). Tissues have been incubated with toxin at a low temperature, which allows diffusion-mediated processes but which arrests energy-dependent processes. Thus, diffusion of the toxin into the neuromuscular junction and association with receptors can proceed, but active mechanisms such as endocytosis by the nerve or phospholipid cleavage by the toxin cannot. After binding is complete, the tissue is washed free of unbound toxin, then suspended in tissue baths under conditions that permit endocytosis and/or catalysis (i.e., 37^{o} C, nerve stimulation).

This simple paradigm can provide useful information about toxin action, because the two principal steps in toxin action, i.e., binding and paralysis, do not overlap. When combined with the use of neutralizing antibodies or drugs, it can also provide important clues about the possible existence of a receptor-mediated endocytosis step.

When tested in the binding paradigm and in the presence of an auxiliary protein, crotoxin produced concentration-dependent paralysis of transmission. Tissue association by the toxin, which likely reflects receptor association, had

an apparent half-time of about 22 minutes. When tissue association was allowed to go to completion, it appeared to be essentially irreversible. However, when incubation times were shortened, extensive washing prolonged the eventual paralysis times.

In experiments in which the binding step was allowed to approach completion, several observations were made. Binding was not affected by low temperature, it was not enhanced by nerve stimulation, and it was not retarded by the presence of strontium. Deductions based on previous studies in which the binding step was not separated from later steps support these conclusions (Chang et al., 1977; Su and Chang, 1984).

The isolated subunits of crotoxin did not possess significant neurotoxicity compared to the parent molecule. In addition, prior exposure of tissues to the individual subunits did not enhance or inhibit the subsequent toxicity of the intact molecule. There are two deductions that stem from these data. Firstly, it would appear that neither subunit alone possesses the properties of a ligand that can bind to the crotoxin receptor. This deduction is based on the observation that molar excesses of the subunits do not antagonize the intact molecule. Secondly, the ability of the toxin to bind to specific receptors may be due to an interaction between the two subunits. One possibility is that the binding domain is distributed between the two

units; another possibility is that one subunit allosterically modifies the other to induce a binding site.

The apparent absence of specific binding by the individual subunits may be reflected in a finding from reconstitution experiments. When the acidic and basic polypeptides were mixed in solution, they reformed a biologically active toxin. However, when either subunit was added to tissues, followed by washing and subsequent addition of the other subunit, there was negligible toxicity. This means that the individual subunits bind poorly to receptors, or the individual subunits bind in a conformation that does not allow reconstitution.

Further insights into the mechanisms of tissue association by crotoxin and its subunits will have to await authentic ligand binding studies, which are now being done. In the meantime, it is worth noting that experiments similar to those just discussed have been done with other toxins. It has been shown that the individual components of toxins such as ricin (Houston, 1982), botulinum neurotoxin (Bandyopadhyay et al., 1987), and the botulinum binary toxin (Simpson, in press) can reconstitute on the membranes of vulnerable cells.

Evaluation of a possible endocytosis step. The data thus far support the widely held belief that crotoxin binds to receptors on the nerve terminal and that the binding step is not synonymous with the paralytic step. Work is in progress to better characterize the binding site, which may

be a potassium channel. Beyond this, work has been done to try to determine whether a receptor-mediated endocytosis step is interposed between binding and toxicity. There are many toxins that are known to utilize an endocytosis step, and thus numerous techniques have evolved to demonstrate this phenomenon. The four techniques that have been used most widely are: 1.) antagonism by drugs, 2.) antibody escape, 3.) substrate localization, and 4.) ultrastructural analysis.

The first two approaches have been used in this study, and the data do not support the concept of receptor-mediated endocytosis. Drugs that inhibit this process, such as ammonium chloride and methylamine hydrochloride, did not delay onset of toxin action. Furthermore, there did not appear to be the phenomenon of antibody escape that is customarily seen with endocytosed ligands. Endocytosis normally occurs very rapidly (3 to 15 min), and thus an antigen should escape from accessibility to antibody within a short time. Complete escape should occur before there is onset of poisoning, but this was not the result with crotoxin. The toxin remained at least partially accessible to antibody for a length of time that was too long to be compatible with endocytosis.

In conclusion, the data indicate that crotoxin - but not the isolated subunits - binds avidly to the mammalian neuromuscular junction. The binding step is followed by a poisoning step, and there may be intermediate events as

well. However, the data do not support the concept of a receptor-mediated endocytosis step that is interposed between binding and paralysis.

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TABLE 1

Effects of various manipulations on crotoxin activity[a]

Preparation	Paralysis Time[b]
No nerve stimulation [c]	120 ± 11
Nerve stimulation (1 Hz)[c]	117 ± 17
Incubation, 60 min; Wash, 5 min [d]	120 ± 11
Incubation, 60 min; Wash, 60 min[d]	124 ± 10
Incubation, 20 min; Wash, 60 min[d]	288 ± 20
Binding in 1.8 mM Ca^{2+} Tyrode [e]	115 ± 28
Binding in 3.6 mM Sr^{2+} Tyrode [e]	120 ± 28
Stimulation in 1.8 mM Ca^{2+} Tyrode[f]	146 ± 37
Stimulation in 3.6 mM Sr^{2+} Tyrode[f]	287 ± 22

[a] Unless otherwise indicated, tissues were incubated with toxin (2μg/ml, 60 min, 10°C), then washed and suspended in tissue baths (37°C) without added toxin. Phrenic nerves were stimulated and paralysis times were monitored.

[b] Time (minutes; mean ± S.D.) for 90% blockade of neuromuscular transmission. N=4 or more per data point.

[c] Tissues were unstimulated or stimulated during incubation. After washing, all tissues were stimulated at 1 Hz.

[d] Tissues were incubated with toxin and later washed for the times indicated. After washing, all tissues were treated identically (37°C, nerve stimulation at 1 Hz).

[e] Tissues were incubated in the indicated cation. After washing, all tissues were suspended in normal Tyrode solution.

[f] Tissues were incubated in normal Tyrode solution. After washing, tissues were bathed and stimulated in the indicated cation.

TABLE 2

Effect of Crotoxin and its Subunits on Neuromuscular Transmission

Protein	Concentration μg/ml	Paralysis Time[a]
Crotoxin[b]	3.0	94 ± 14
Acidic Subunit [b]	10.0	>310
Basic Subunit [b]	10.0	>290
Acidic Subunit + Basic Subunit [b]	0.32 0.50	82 ± 7
Crotoxin[c]	2.0	169 ± 22
Acidic Subunit [c]	10.0	>390
Basic Subunit [c]	10.0	>374
Acidic Subunit + Basic Subunit [c]	1.17 2.0	148 ± 25
Acidic Subunit; Basic Subunit [d]	1.17 2.0	346 ± 15
Basic Subunit; Acidic Subunit [d]	2.0 1.17	322 ± 15
Acidic Subunit; Crotoxin[d]	7.47 2.0	185 ± 10
Basic Subunit; Crotoxin[d]	12.45 2.0	183 ± 29
Unmodified Crotoxin [c]	2.0	167 ± 8
Unmodified Crotoxin + Modified Crotoxin [c,e]	2.0 8.0	170 ± 6

[a] Time (minutes; mean ± S.D.) for 90% blockade of neuromuscular transmission. N=4 or more per data point.

[b] Proteins were added to tisues that were stimulated (37° C; 1 Hz), and paralysis times were measured.

[c] Proteins were incubated with tissues (10° C; 60 min). Tissues were then washed and suspended in baths (37° C) with nerve stimulation (1 Hz), and paralysis times were measured.

[d] Tissues were incubated with one protein (10° C; 60 min), then washed and similarly incubated with the other protein. Tissues were washed again and suspended in baths (37°C) with nerve stimulation (1 Hz). Paralysis was monitored.

[e] Crotoxin was modified with p-bromophenacyl bromide (see text).

TABLE 3

Effect of Antibodies on Crotoxin Activity

roup	Delay	Paralysis Time [e]
Control[a]	--	123 ± 14
Antiserum[b]	--	>300
Antiserum[c]	0 min	>300
Antiserum[d]	30 min	226 ± 20

[a] Crotoxin (2 μg/ml) was incubated with tissues (10° C; 60 min), er which the tissues were washed and then suspended in baths (37° C) h nerve stimulation (1 Hz).

[b] Antiserum (0.2 ml) was reacted with Crotoxin for 30 min at 37° C then incubated with tissues.

[c] After incubation with toxin and washing, tissues were incubated h antiserum (0.2 ml) for another 60 min at 10° C. Tissues were then hed and suspended at 37° C with stimulation.

[d] After incubation with toxin and washing, tissues were transferred baths at 37° C with stimulation. After 30 min of stimulation tissues e incubated with antiserum (0.2 ml) for 60 min at 10° C with no mulation. Tissues were then washed and returned to tissue baths at C with stimulation.

[e] Time (minutes; mean ± S.D.) for 90% blockade of neuromuscular nsmission. N=4 or more per experiment.

Figure 1 The effect of a low concentration of crotoxin on neuromuscular transmission. The figure illustrates a representative result obtained with a tissue exposed to 1 μg/ml of crotoxin. Lower concentrations produced a lesser effect on transmission; higher concentrations produced complete paralysis (see Tables 1 to 3).

Figure 2 Effect of various concentrations of crotoxin on paralysis times of neuromuscular preparations (□, crotoxin added directly to stimulated preparations; ●, crotoxin incubated with unstimulated preparations then added to stimulated preparations). Experimental details are provided in the text. Each data point is the mean response ± S.D. of four or more experiments.

Figure 3 Effect of incubation time on blockade of neuromuscular transmission caused by crotoxin (2μg/ml). Experimental details are provided in the text. Each data point is the mean response ± S.D. of four or more experiments.

Figure 4 Effect of temperature on 90% blockade of neuromuscular transmission caused by crotoxin (2μg/ml). Experimental details are provided in the text. Each data point is the mean response ± S.D. of four or more experiments.

FIGURE 1

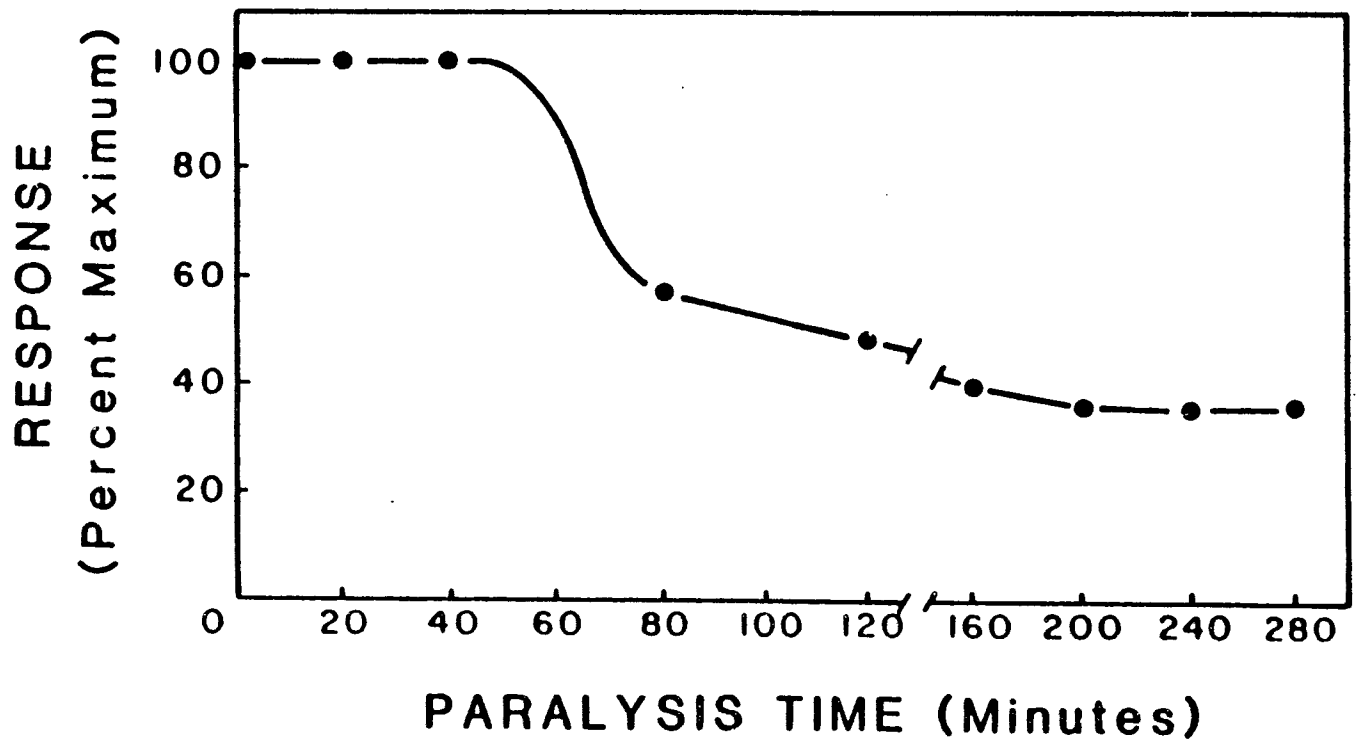
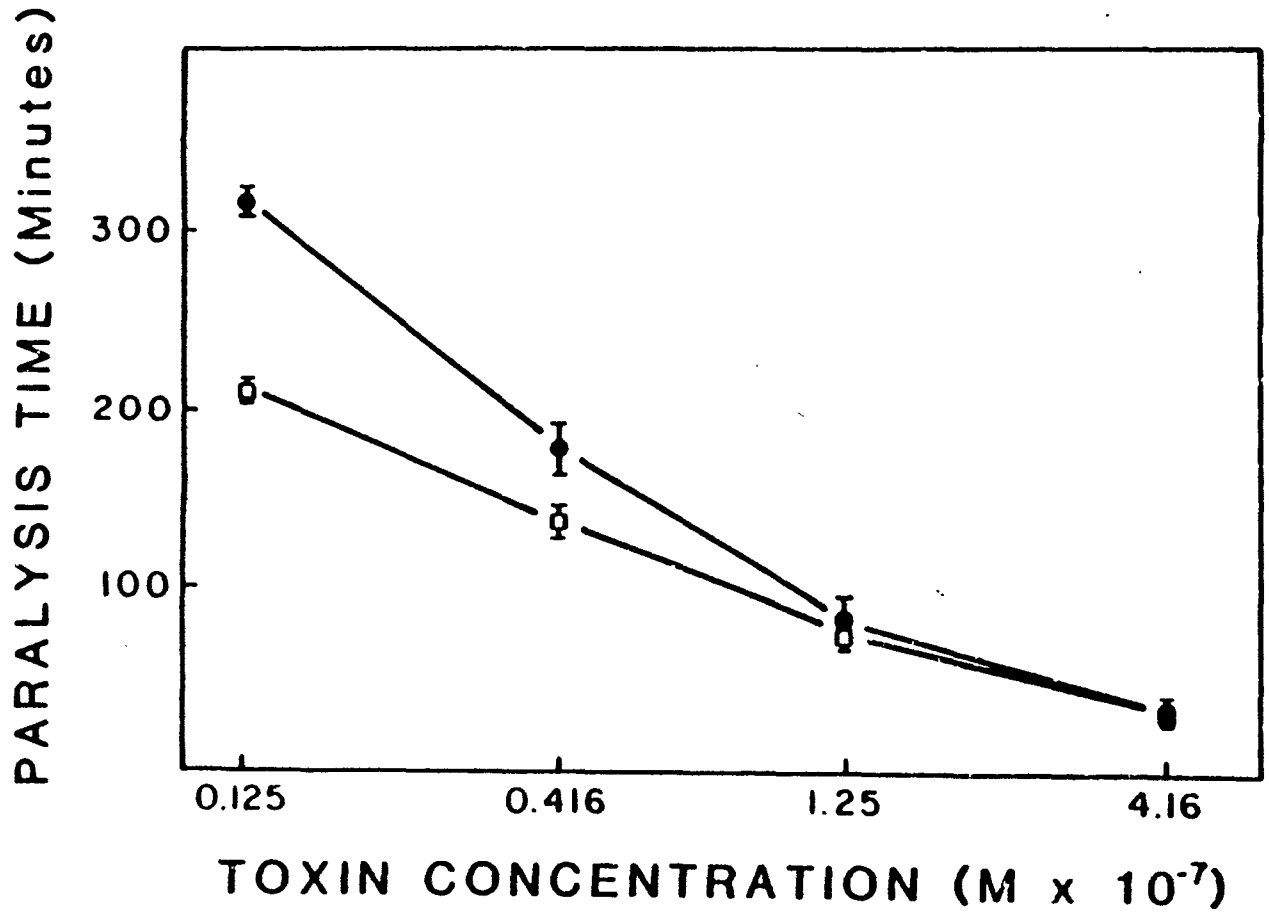
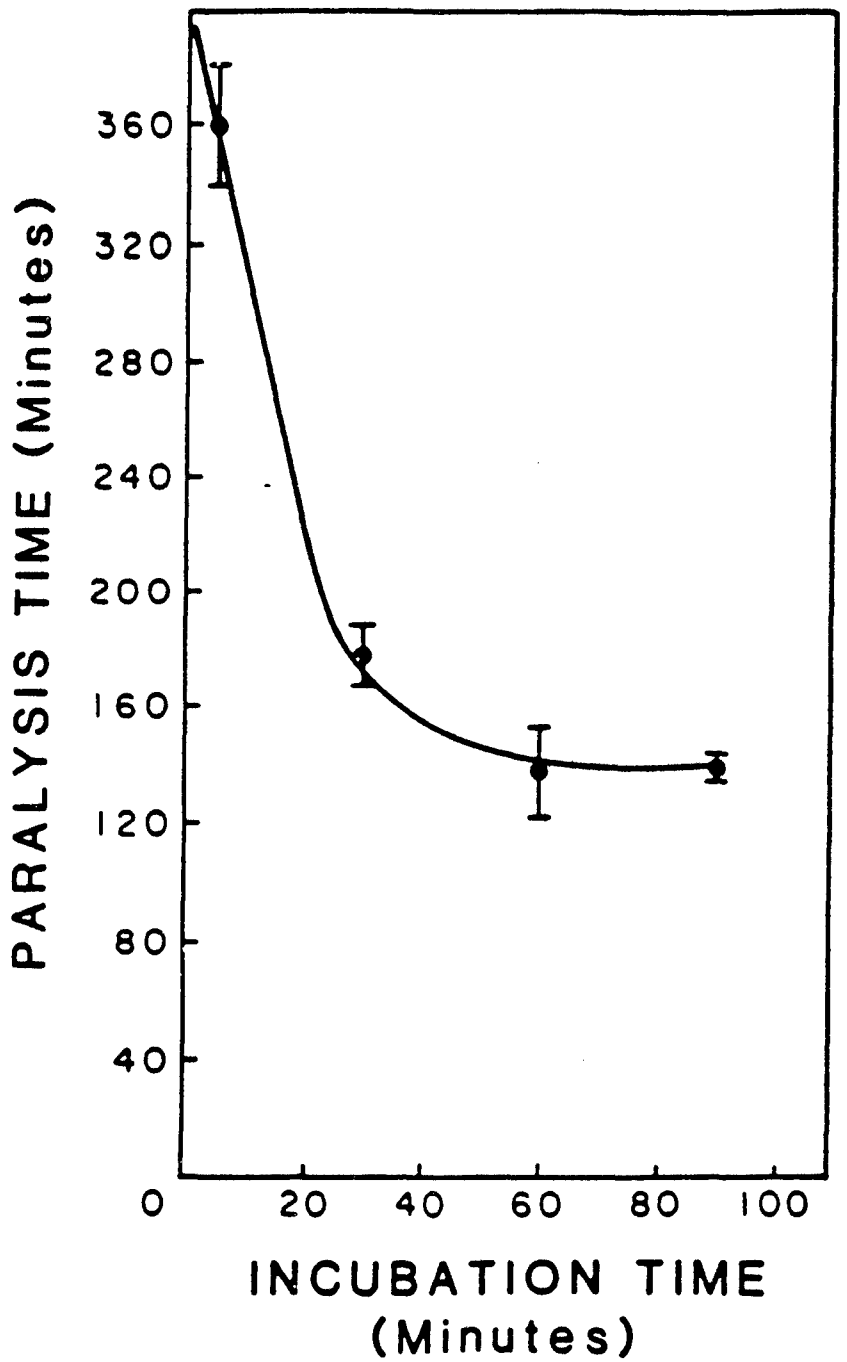
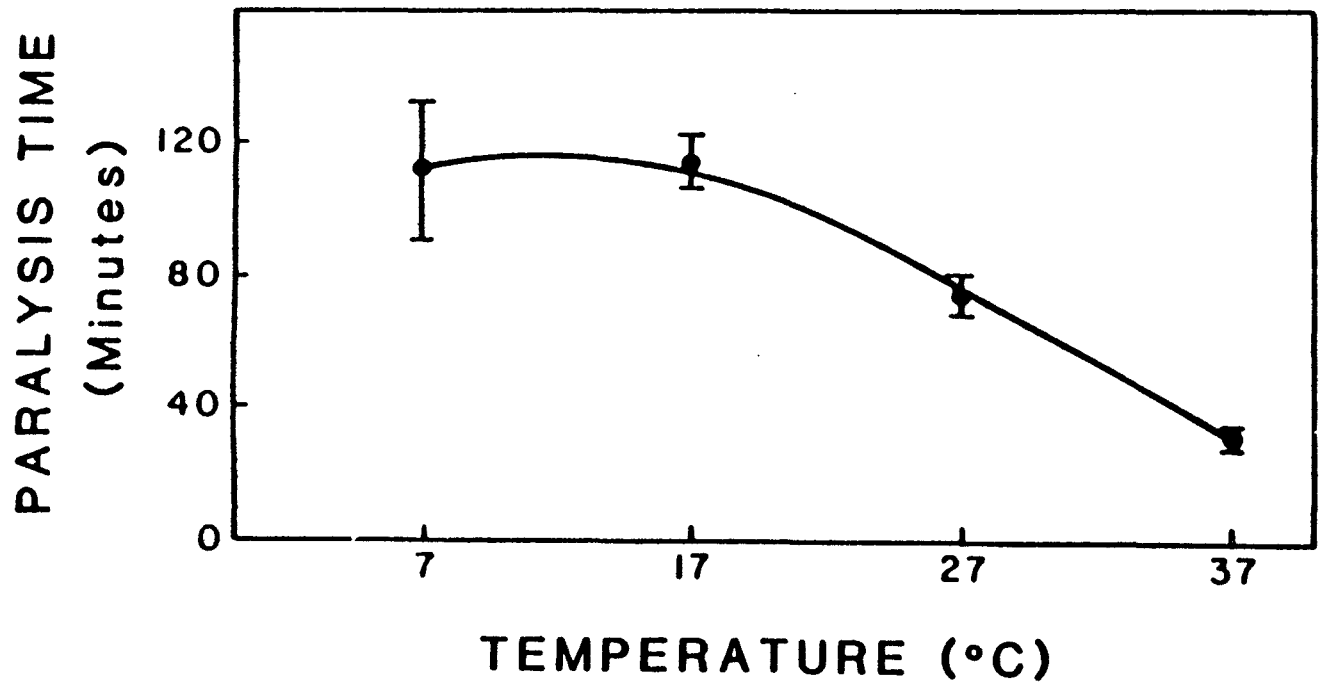

RESPONSE
(Percent Maximum)
100
80
60
40
20
0
20
40
60
80
100
120
160
200
240
280
PARALYSIS TIME (Minutes)

FIGURE 2


PARALYSIS TIME (Minutes)
300
200
100
0.125
0.416
1.25
4.16
TOXIN CONCENTRATION (M x 10⁻⁷)


PARALYSIS TIME (Minutes)
360
320
280
240
200
160
120
80
40
0
20
40
60
80
100
INCUBATION TIME
(Minutes)


PARALYSIS TIME
(Minutes)
120
80
40
0
7
17
27
37
TEMPERATURE (°C)

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