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A CORE FACILITY FOR THE STUDY OF NEUROTOXINS OF BIOLOGICAL ORIGIN

Annual Report

June 15, 1990

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Studies have been done on the variety of toxins that affect the nervous system. The toxins of major interest have been dendrotoxin, tetrotoxin, 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 tetrotoxin and saxitoxin binding properties using channels inserted into lipid membranes, iv) further characterization of the intracellular actions of clostridial toxins, v) development of binding assays for clostridial toxins, and vi) study of structure-function relationships in clostridial neurotoxins and snake neurotoxins.

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TABLE OF CONTENTS

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. Nabil Bakry has developed techniques for labeling clostridial toxins and for studying their binding to brain membrane preparations. He has found that several previously published and widely used methods for measuring toxin binding are flawed, and he has proposed revised methods that measure binding to authentic receptors.

- Dr. Roger Sorensen has isolated and characterized several dendrotoxins, as well as structurally and functionally similar toxins from other sources. He has used iodinated preparations of these toxins to extract and solubilize receptors, which appear to be potassium channels.

- Dr. Lee Chabala has used the lipid bilayer technique to study the interaction between small molecular weight toxins and ion channels. He has found that several of the physical

properties of channels that account for their gating behavior also account for their affinity for neurotoxins.

- Dr. Joseph Sherwin has continued to study the intracellular effects of clostridial toxins, and in the process has developed an improved method for measuring protein kinase activity. His work indiciates that toxins alter intracellular disposition of the kinase, although the underlying mechanism remains obscure.

- Dr. Lance Simpson has studied the structure of clostridial toxins, including the native and nicked molecules. He has discovered that there is a structural distinction between activation and aging of the neurotoxins. He has also employed a new method for measuring hydrophobicity of proteins and used this technique to characterize pH-induced changes in toxin structure.

Nabil Bakry, Ph.D.
Research Associate in Medicine

Scientific Progress During the Year

A. Specific Aims

The aim of this project is to characterize the binding of toxins to the nervous system. Work in the past has been done on crotoxin, but this substance has proved difficult to study. Various attempts are being made to deduce why the non-specific binding of crotoxin is so high. In the meantime, work will be done to characterize the binding of clostridial toxins.

The majority of research on clostridial toxin binding has been done with tetanus toxin. There are several reasons for this, including the fact that tetanus toxin binds to brain synaptosomes, a tissue that is abundantly available. Thus, tetanus toxin binding studies greatly exceed in number and to some extent serve as a prototype for botulinum neurotoxin binding studies. This is somewhat unfortunate. In the opinion of Dr. Simpson as well as the present author, the work on tetanus toxin is seriously flawed. A methodology has been introduced and widely utilized for studying toxin binding under unphysiological conditions. There is strong reason to believe that this binding is not biologically relevant.

The purpose of the work described here is to develop a rational and scientifically sound method for studying tetanus toxin binding. If this effort is successful, it may then serve as an authentic model for studies on botulinum neurotoxin binding.

B. Methods

Materials. Tetanus toxin was purchased from Calbiochem and from the Massachusetts Public Health Biological Laboratories. The material that was used had an in vivo toxicity of 5×10^{-8} MLD/mg protein. Sodium ^{125} Iodine and the Bolton-Hunter Reagent - ^{125}Iodine were purchased from Amersham. Chloramine-T, glycine, tyrosine and all salts and buffers were from Sigma Chemical Co. and Fisher.

Iodination procedures. The chloramine-T method was used essentially as previously described. Tetanus toxin (100 lg) in sodium phosphate buffer (100 mM, pH 7.4) was mixed with chloramine-T (0.5 mM) and Na 125I (1 mCi) for 30 sec. The reaction was terminated by adding tyrosine (50 lg), and the mixture was applied to a minicolumn of Sephadex G-50. 125I-Tetanus toxin was eluted with sodium phosphate buffer (100 mM, pH 7.4) containing sodium chloride. The protein concentration, specific activity and residual toxicity of the preparation were determined (see Results).

The Iodobead method was used as suggested by the manufacturer (Pierce Co.). Tetanus toxin (100 μg) in sodium phosphate buffer (100 mM, pH 6.5) that contained sodium chloride (200 mM) was mixed with 2 beads that had been presoaked in the same buffer containing Na ^{125}I (1 mCi). The reaction proceeded for 15 min at room temperature, after which the reaction mixture was eluted on a minicolumn of Sephadex G-

50 (see above). The physical and biological characteristics of the labelled protein were determined (see Results).

The Bolton Hunter method was used as described. Tetanus toxin (100 μg) in sodium borate buffer (100mM, pH 7.5) was mixed with ^{125}I-Bolton Hunter reagent at room temperature. At the end of 30 min, the reaction was terminated by adding glycine (200 mM) and the mixture was applied to a Sephadex G-50 column. The various characteristics of the labelled preparation were compared with those of the toxin labelled by other procedures.

Toxicity. The in vivo potency of native and labelled tetanus toxin was determined by methods that have been described in previous reports.

Tissues. Experiments were done on rat brain membrane preparations. Adult Sprague-Dawley rats were decapitated, after which brains were removed, washed and homogenized with a Polytron in iced buffer (Tris-HCl, 50 mM, pH 7.4). The homogenate was centrifuged for 10 min at 1000 x g, and the resulting supernatant was recentrifuged for 45 min at 40,000 x g. The pellets were suspended in Tris-HCl buffer (50 mM, pH 7.4).

Binding assay. The binding of tetanus toxin to nerve membrane preparations was determined by using a centrifugation assay. Labelled toxin with or without unlabelled toxin was incubated with tissue for 30 min at room temperature. The reaction volume was 1 ml; the buffer, ionic strength and pH of the medium was varied as described under Results. The

reaction was terminated by centrifugation. The bottom of the microtube was cut and the amount of ^{125}I was quantified in a Packard gamma counter. The data were corrected for non-specific counts.

The association rate constant was measured by incubating labelled toxin and membrane for various times, then terminating the reaction by centrifugation. The dissociation rate constant was determined by first incubating labelled toxin and tissue for 30 min. Dissociation was initiated by adding a large molar excess of unlabelled toxin (2×10^{-7} M), and at various times thereafter the reaction was terminated by centrifugation. As before, results were corrected for non-specific binding.

Analysis of binding studies. The data were evaluated by using an interactive linear regression method, i.e., the equilibrium binding data analysis (EBDA) program. When more than on binding affinity was apparent, the SCAFIT program was used to do non-linear regression analysis. This provided a best fit analysis for toxin-receptor interactions in which there were two or more binding sites.

C. Results

General properties of labelled toxin. The specific activity of the various preparations of labelled toxin was as follows: chloramine-T, 110-130 Ci/mmole; Iodobead, 109 Ci/mmole; Bolton-Hunter, 600-750 Ci/mmole.

The preparations were also assayed for neurotoxicity, using an in vivo lethality test. The results, expressed as residual toxicity after labelling, were: chloramine-T, ~10%; Iodobead, < 10%; Bolton-Hunter, ~50%.

Finally, the preparations were assayed for binding activity using rat brain membrane preparations. Tetanus toxin (1×10^{-10} M labelled material) was incubated with tissue [100 μg/ml; Tris-HCl (50 mM, pH 7.4, plus 100 mM NaCl) with BSA (1 mg/ml). The reaction mixture (1 ml) was incubated for 30 min at room temperature. The results, expressed as pmole toxin bound/mg protein, were: chloramine-T, 0.197 pmole/mg protein, Iodobead, 0.050 pmole/mg protein, and Bolton-Hunter, 0.197 pmole/mg protein.

Binding to rat brain membrane. Toxin preparations labelled by chloramine-T and Bolton-Hunter methods were examined for their binding characteristics. When toxin (2×10^{-10} M) was incubated (Tris-HCl buffer, 50 mM, pH 7.4, plus 100 mM NaCl in a final volume of 1 mL) with a fixed amount of brain membrane (100 μg/assay), there was time-dependent binding. An apparent equilibrium was obtained within 15 to 20 min for both types of labelled toxin. When toxin (1.5×10^{-10} M) was incubated (30 min; buffer as before) with increasing amounts of brain membrane, there were increases in the amount of bound toxin.

When varying concentrations of labelled toxin were incubated (30 min; buffer as before) with a fixed amount of brain membrane preparation (100 μg/assay), there were

increasing amounts of toxin bound to the tissue. When these data were re-examined by Scathard analysis, they yielded the following constants: chloramine-T labelled toxin, K_d ~ 0.063 nM and Bmax ~ 0.36 pmole/mg protein for high affinity site; K_d ~ 1.0 nM and Bmax ~ 1.5 pmole/mg protein or low affinity site. The values for the Bolton-Hunter labelled material were: K_d ~ 0.042 nM and Bmax ~ 0.3 pm/mg protein for high affinity site; K_d ~ 0.89 nM and Bmax ~ 1.55 pmole/mg protein for low affinity site.

A second strategy was used to confirm the binding constants. Varying concentrations of unlabelled toxin were mixed with a fixed concentration of labelled toxin, and the amount of labelled material bound to membranes was determined. The results were corrected for the dilution in specific activity. The displacement technique yielded the following constants: chloramine-T, K_d ~ 0.07 nM and Bmax ~ 0.4 pmole/mg protein for the high affinity site; K_d ~ 2.56 nM and Bmax ~ 3.0 pmole/mg protein for the low affinity site. The corresponding values for the Bolton-Hunter material were: K_d ~ 0.07 nM and Bmax ~ 0.34 pmole/mg protein for the high affinity site and K_d 3.2 nM and Bmax ~ 3.0 pmole/mg protein for the low affinity site.

Effect of Buffer Composition. A series of studies were performed in Tris-HCl and Tris-Acetate. The Tris-Acetate buffer was similar to that used in most earlier studies (25 mM; pH 6.0); the Tris-HCl buffer was more physiologically relevant (50 mM; pH 7.4; plus 100 mM NaCl). When labelled

tetanus toxin was incubated with varying amounts of rat brain membrane, the amount of binding was significantly affected by buffer composition (Figure 1). In Tris-Acetate buffer the labelled toxin appeared to associate with membrane at low protein concentration (i.e., 10 - 100 ng), and there was an apparent plateau at about 100 μg. In the Tris-HCl buffer, labelled toxin bound to membrane at higher concentrations of protein.

An effort was made to determine the extent to which pH was a factor that influenced binding. Experiments were done with Tris-Acetate (25 mM) and Tris-HCl (25 mM) buffers at varying pH values. The results showed that binding was enhanced as pH was lowered from 8.0 to 6.0. A similar approach was used to determine the effects of ionic strength, but the results showed that when NaCl concentration was varied from 25 to 150 mM, the effect on binding was minimal.

Although the amount of binding at low ionic strength and pH was greater than that under physiological conditions, there is reason to question its significance. To begin with, Scathard analysis (Figure 2) of the binding showed that the phenomenon at unphysiologic conditions (Figure 2b) yielded values indicating low affinity and unusually high capacity. This is quite unlike what one would expect for a very potent toxin. By contrast, the binding in physiological medium (Figure 2c) indicated higher affinity and lower capacity. This is the expected outcome.

Neuromuscular junction experiments. In a parallel series of experiments, isolated phrenic nerve-hemidiaphragms from mice were incubated with tetanus toxin or botulinum neurotoxin under conditions that permitted binding but retarded internalization (e.g., 4^{o} c; low calcium; high magnesium). This was done in Tris-Acetate buffer of low pH and ionic strength or in Tris-HCl buffer of normal pH and ionic strength. Equiactive concentrations of tetanus toxin and botulinum neurotoxin were tested.

The results of the neuromuscular junction epxeriments can be summarized as follows (n=5 or more per group).

	Paralysis Times	
	Physiological Medium	Non-Physiological Medium
Botulinum neurotoxin	129 ± 13	211 ± 18
Tetanus toxin	121 ± 9	130 ± 11

The data show that unphysiological medium, which increases the amount of tetanus toxin buding, did not increase toxicity. And in addition, the unphysiological medium actually depressed the toxicity of botulinum toxin.

Antibody experiments. There are at least two types of known binding sites for clostridial toxins: one is the true receptor in nerve tissue, and the other is the class of monoclonal antibody that attaches to the toxin's binding domain. A human monoclonal antibody that recognizes the binding domain in tetanus toxin has been described in previous

Quarterly Reports. It has been utilized here in the study of tetanus toxin binding.

Iodinated tetanus toxin was allowed to bind to brain membrane in physiological (o) or non-physiological (o) medium, as illustrated in Figure 3. This binding was done in the presence of increasing concentraitons of the monoclonal antibody. As shown in the figure, the antibody was effective in diminishing binding under both conditions, but it was clearly more effective under physiological conditions.

Iodination of Botulinum Neurotoxin. Four methods are being explored as possible ways to iodinate botulinum neurotoxin. In this series of experiments the techniques are being applied to serotype B. The methods are: Bolton-Hunter reagent, Chloramine-T, Iodobead and Enzymobead. Results have been obtained with the first two methods.

Previous work by Dolly and his associates indicate that the chloramine-T method is usable with botulinum neurotoxin; work in our laboratory and in others suggests that the Bolton-Hunter reagent is usable with tetanus toxin. We have decided to try both with botulinum neurotoxin type B.

The British group has employed the Chloramine-T technique with both types A and B botulinum toxin. They report obtaining specific activity that is acceptably high to do ligand binding studies while still retaining substantial biological activity (60 to 80%). One curious aspect of this work is the amount of

iodine used in labelling experiments (i.e. 5 mCi). We have attempted to reproduce their work, but we have not obtained the same results. When using high amounts of iodine, we do obtain very high specific activity (1000 Ci/m mole and greater). However, there are marked losses in toxicity.

We have also tested the Bolton-Hunter reagent, and the methods used were exactly as described in earlier reports. Using this approach, we have obtained a toxin preparation with acceptably high specific activity (250-300 Ci/m mole) and with a substantial amount of residual toxicity (60% or greater).

The material that was labelled with the Bolton-Hunter reagent was submitted to SDS-PAGE and autoradiography. The native and the reduced forms of the labelled toxin migrated identically to the corresponding forms of the unlabelled toxin. When examined by autoradiography, the two chains of the toxin were differtially labelled. Approximately 70% of the iodine was found in the heavy chain and the balance was in the light chain.

Binding Experiments. Some initial binding experiments have been done with the type B toxin labelled by the Bolton-Hunter reagent. The data are internally consistant, but are at variance with the findings of the British group.

A determination has been made of the on-rate and off-rate constants, and from these data an apparent Kd has been calculated. The graphically determined constants were as

follows: K_{-1}, 0.05 x $moles^{-1}$; K_1, 0.042 x $moles^{-1}$. These data yield an apparent Kd of 1.2 nM.

A closely similar result was obtained by doing a Scathard analysis. The results gave an apparent Kd of 2.08 nM and an apparent Bmax of 2.6 x 10^{-12} moles/mg. It is here that the distinction between our findings and those of the British group are most pronounced. The latter reported two binding sites with Kd's of about 0.5 nM and 20 nM. There may be an explanation for the contradictory findings, as proposed below.

In a third approach to the problem, a displacement technique was used to quantify the apparent Kd. The value (Figure 3) was about 2.0 nM. Thus, the calculated Kd from the on- and off-rate constants and the apparent Kd obtained by Scathard analysis were all virtually the same.

The labelled toxin showed time-dependent binding to synaptosomes, and a plateau was observed within 60 to 90 minutes. The amount of binding was proportional to the amount of membrane protein. Binding was also influenced by pH and ionic strength, and the results were qualitatively similar to those obtained with tetanus toxin (see last Report), but further work is needed to quantify the pH and salt effects.

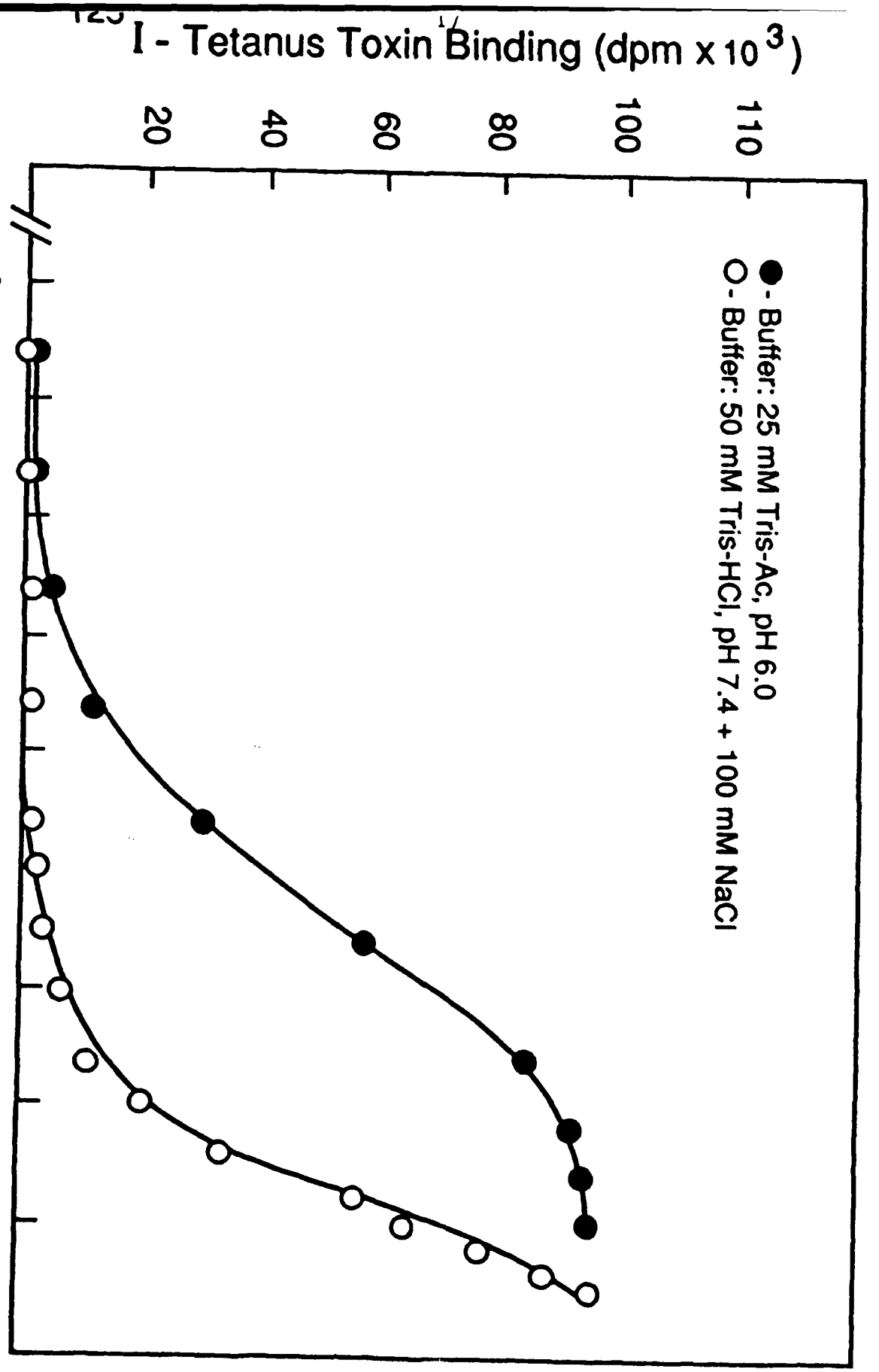
^{125}I - Tetanus Toxin Binding (dpm x 10^3)
20
40
60
80
100
110
●- Buffer: 25 mM Tris-Ac, pH 6.0
○- Buffer: 50 mM Tris-HCl, pH 7.4 + 100 mM NaCl
8
7
6
5
4
-log membrane protein concentration (g/ml)

FIGURE #1

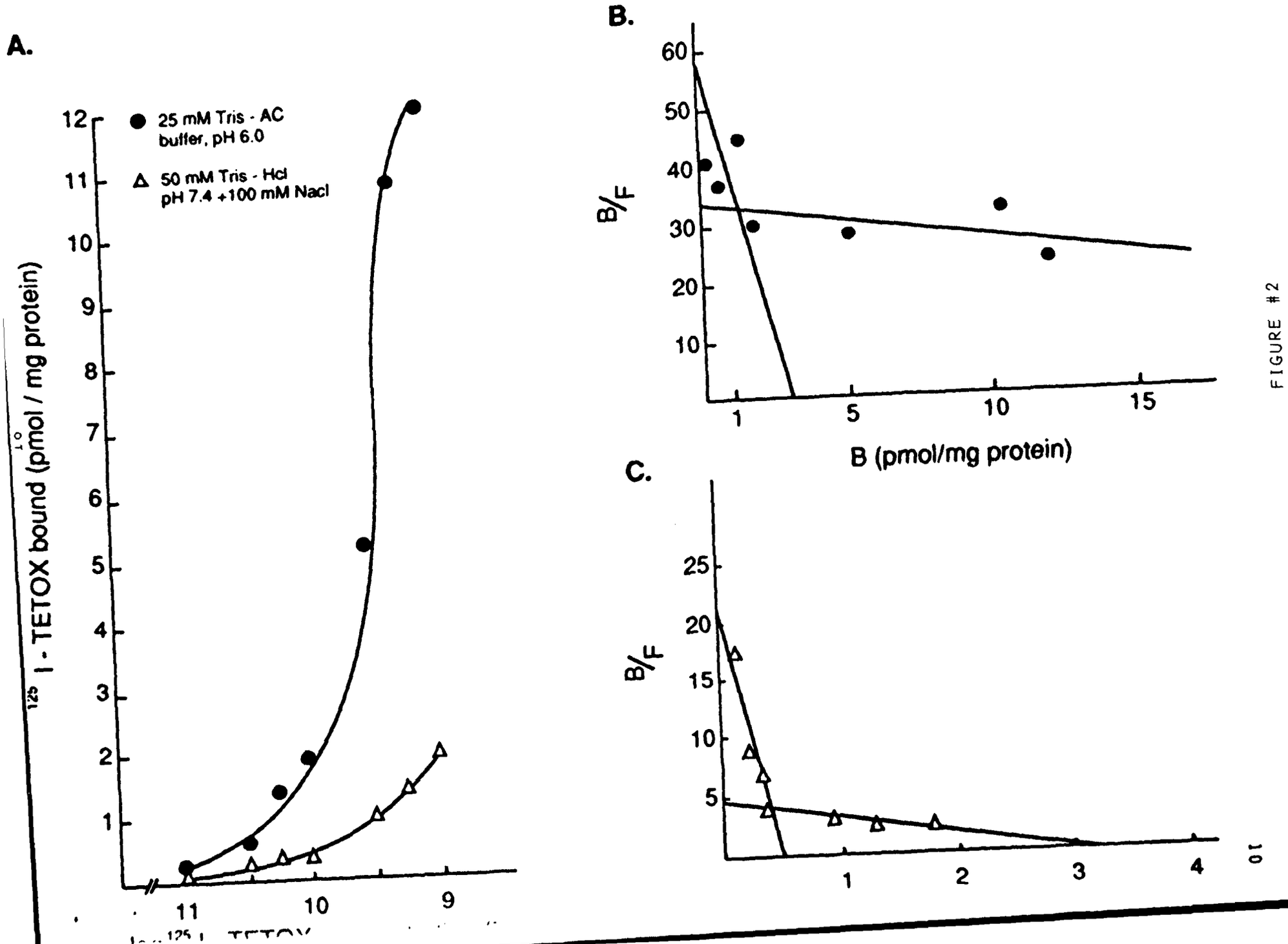
A.
25 mM Tris - AC buffer, pH 6.0
50 mM Tris - Hcl pH 7.4 +100 mM Nacl
125 I - TETOX bound (pmol / mg protein)
B.
B/F
B (pmol/mg protein)
C.
B/F

FIGURE #2

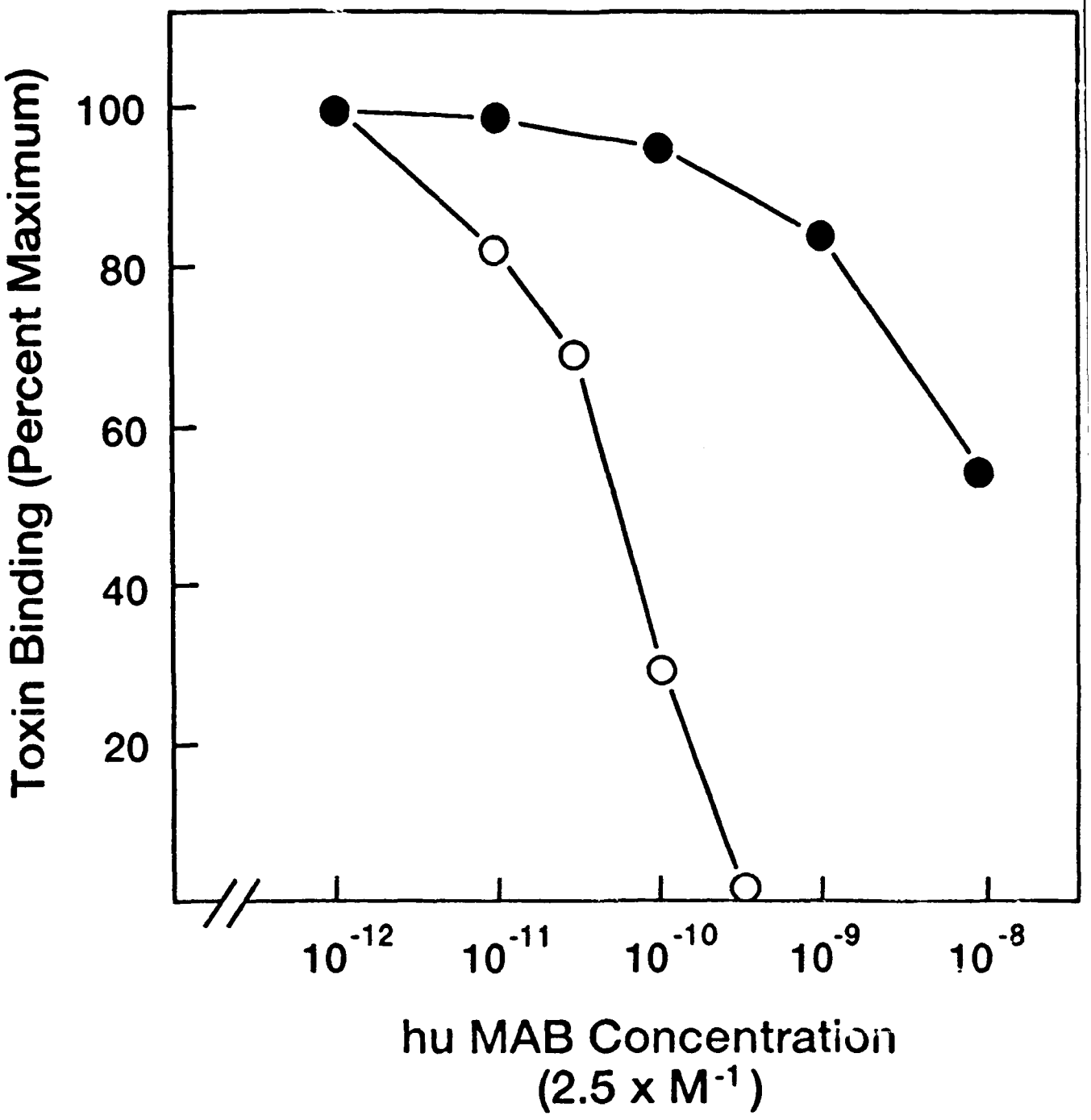
Toxin Binding (Percent Maximum)
100
80
60
40
20
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
hu MAB Concentration
(2.5 x M^{-1})

FIGURE #3

ROGER G. SORENSEN, PH.D.
ASSISTANT PROFESSOR of MEDICINE

SUMMARY of SCIENTIFIC PROGRESS for YEAR 89-90

A. SPECIFIC AIMS

This project concerns the characterization and purification of rat brain dendrotoxin (DaTX) receptors. Because these toxins block K channel activities, it is expected that the binding sites for the dendrotoxins are located on the K channel proteins. Experiments during the past year have addressed the following:

a) characterization and solubilization of dendrotoxin receptors. These experiments described additional properties of dendrotoxin binding to their rat brain membrane receptor sites, and conditions for the detergent solubilization of the receptors;

b) voltage-dependency of dendrotoxin binding. K channels are sensitive to changes in the voltage potential (regulated by ionic gradients) across the cell membrane. Changes in the membrane potential gradients induce conformational changes to the channel molecules, thereby, regulating the opening and closing of the channels and, hence, ion permeability. It has been shown for some compounds that binding to ion channel proteins is dependent on their conformational state. These experiments addressed the voltage-sensitivity for the binding of [^{125}I]alpha-DaTX to rat brain synaptosomes, i.e., whether the conformational state (open or closed configuration) of the channel molecule influences toxin binding;

c) re-evaluation of D. angusticeps venom neurotoxins that block K channels. An initial report identified four polypeptide components of D. angusticeps venom that block brain K channels. By modifying the original isolation procedure, an additional polypeptide

component present in D. angusticeps venom was found that blocks brain K channels. The present studies were undertaken to re-identify and characterize those polypeptides found in D. angusticeps venom that block K channels.

B. BACKGROUND

Several venoms have been shown to contain polypeptides that block K channels. This would include the venom from the green mamba, Dendroaspis angusticeps, which has been shown to contain polypeptides (dendrotoxins, DaTX) that block different brain K channels; alpha-DaTX that selectively blocks voltage-dependent, rapidly-inactivating K channels, and beta-DaTX that selectively blocks voltage-dependent, non (or slowly)-inactivating K channels (1). Both dendrotoxins have been radioiodinated and shown to label a single class of binding sites in rat brain.

Very little is known about the structural properties between different K channels. The receptor for the dendrotoxin homolog, DTX_1, isolated from D. polylepis venom, has been purified (2,3). Yet, these results only suggest a putative subunit structure for this receptor, which has been reconstituted in lipid bilayers and shown to possess voltage-dependent K channel activity (4).

Voltage-dependent K channels have been cloned from brain cDNA libraries (6,7). Models of the putative amino acid sequences deduced from these clones may predict possible tertiary structures for the channel proteins, but again do not provide information concerning those structural properties between K channels having different gating kinetics. It has recently been suggested that alternative subunit associations may be responsible for the differences between K channels (8).

By identifying toxins that selectively recognize different K channels, it may be possible to use these toxins to obtain and compare structural information concerning those properties of the channel proteins that make the channel unique. With this hypothesis in mind, experiments were done to compare the binding of the two dendrotoxins described above, alpha-DaTX and beta-DaTX, to their brain receptor sites, which should reside on different K channels.

Recently, it was necessary to isolate additional dendrotoxins to be used for K channel characterization and purification. The toxin isolation procedure was modified to include an additional ion exchange chromatographic step. It was noted that the beta-DaTX fraction could be resolved into two peptides, designated $beta_1$-DaTX and $beta_2$-DaTX. Therefore, experiments were undertaken to re-identify those polypeptide components (dendrotoxins, DaTX) of D. angusticeps venom that block rat brain K channels, and to determine the selectivities of the polypeptides for the block of the channels.

The dendrotoxins apparently bind to receptor sites located on K channels. Ion channels respond to changes in membrane potential (their voltage-sensitivity) which is responsible for the regulation of their opening and closing. This sensitivity is associated with conformational changes to the protein molecule. Thus, some compounds that interact with channel proteins may preferentially recognize certain conformational (open or closed) states of the proteins. Although the dendrotoxins have been shown to block K channels in a variety of preparations, none of these studies have been able to identify any voltage-dependency for channel block by these toxins. Synaptosomes, "pinched-off" and re-sealed nerve terminal preparations, maintain normal ionic concentration gradients. This enables the synaptosome to retain membrane potential gradients. Therefore, [^{125}I]alpha-DaTX binding was measured to synaptosomes both kept

at resting membrane potential levels, or after membrane depolarization, to compare the voltage-sensitivity of alpha-DaTX binding to its brain receptor, a voltage-dependent, rapidly-inactivating K channel. Voltage sensitivity has not been shown for the block of K channels by the dendrotoxins.

C. METHODS

Toxin binding to synaptosomes. Synaptosomes were prepared from rat brain forebrain and subsequently equilibrated in 5K solution (5 mM KCl, 145 mM NaCl, 1.4 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4). Aliquots of the equilibrated synaptosomes were then taken to assay for [^{125}I]alpha-DaTX binding under the following conditions:

a) resting membrane potential: an equal volume of 5K solution was added to the synaptosome preparation and toxin binding measured;

b) K-stimulated depolarization: 2 - 3 volumes of 145K solution (as above but containing 145 mM KCl and 5 mM NaCl) to bring the K^+ concentration to 75 mM or 100 mM were added to depolarize the synaptosomal membrane prior to assaying for toxin binding;

c) veratridine-stimulated depolarization: an equal volume of 5K solution containing 20 uM veratridine was added prior to assaying for toxin binding.

Toxin was added simultaneously with the addition of the above solutions, and the incubation was allowed to proceed for usually 10 min at 37°. Toxin bound to protein was separated from unbound toxin by centrifugation for 4 min at 12,000 rpm. The pellet was washed once with 5K buffer and counted for gamma radiation.

Toxin binding to brain membranes. Brain membranes, obtained after lysis of synaptosomes, were incubated in Na-HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$) containing radiolabelled toxin and other test compounds. After incubation for

30 min at 37°, bound toxin was separated from unbound toxin by centrifugation as described above.

Isolation of D. angusticeps toxins. The separation of the D. angusticeps venom polypeptides was as previously described (1) with the following modifications. The fraction collected from the Sephadex G-50 column that exhibited K channel blocking activity was applied to a 2.0 x 18 cm S Sepharose Fast Flow cation exchange column equilibrated in 50 mM ammonium acetate, pH 6.8. Adsorbed protein was eluted with a 0.05 to 1.4 M ammonium acetate, pH 6.8, continuous gradient at a flow rate of 0.5 ml/min. Four fractions, eluting with concentrations of ammonium acetate greater than 0.6M, were found to inhibit [^{125}I]alpha-DaTX binding. These fractions, tentatively identified as alpha-DaTX, beta-DaTX, gamma-DaTX and delta-DaTX as previously described (1), were collected individually and re-chromatographed by cation exchange HPLC.

Cation exchange HPLC resolved the fraction corresponding to beta-DaTX into two components, designated beta$_1$-DaTX and beta$_2$-DaTX. Each of these two toxins are able to inhibit [^{125}I]alpha-DaTX binding to rat brain membranes. Some of the toxins have been lyophilized, and are presently being tested for both their potency towards the [^{125}I]alpha-DaTX receptor site, and their abilities to selectively block brain K channels.

D. RESULTS

Original Characterization of Dendrotoxin Receptors

Effects of monovalent and divalent cations. It was originally described (1) that D. angusticeps venom contains four polypeptides that block rat brain synaptosomal K

channels. The binding of two of the polypeptides, alpha-DaTX, which blocks voltage-dependent, rapidly-inactivating K channels, and beta-DaTX, which blocks voltage-dependent, slowly (or non-) inactivating K channels, to their receptors was studied. Because these two toxins selectively block different K channels, it was proposed that these binding studies could add to the description of the structural differences between the two channel proteins.

It was previously established that both [^{125}I]alpha-DaTX and [^{125}I]beta-DaTX label a single class of binding sites on a rat brain membrane preparation. It was noted that toxin binding could be measured only in solutions containing high salt concentrations. By measuring binding in the presence of various monovalent cations, it was found that toxin binding was optimally supported in the presence of 150 mM Na. The amounts of the two radioiodinated dendrotoxins bound to the brain membranes decreased, relative to the levels bound in the presence of Na, as Na was replaced isoosmotically by (in order of increasing loss of toxin binding): K > Li > Rb > Cs. Cs (IC_{50} = 9-13 mM) was the only monovalent cation that completely prevented the binding of the two radiolabelled toxins.

The divalent cations, Ba and Ca, were also tested. Ba (IC_{50} = 4-6 mM) was more effective than Ca (IC_{50} = 9-13 mM) in inhibiting dendrotoxin binding. Scatchard analysis showed that both Cs and Ba are allosteric inhibitors at the alpha-DaTX receptor.

Receptor solubilization. The recovery of toxin binding from detergent extracts of brain membranes was low and variable in solutions containing 150 mM Na. Therefore, monovalent cations were again tested for their ability to support toxin binding after detergent solubilization of the dendrotoxin receptor sites. It was found that optimal toxin

binding was supported after detergent extraction in the presence of 150 mM K. Other monovalent cations were less effective of decreasing order: K > Rb > Cs > Li > Na.

From sucrose density gradient centrifugation of the solubilized receptor, it was estimated that the molecular weight for the alpha-DaTX receptor was 270,000.

Voltage-dependence of Dendrotoxin Binding

For the following experiments, [^{125}I]alpha-DaTX binding was measured to synaptosomes in solutions containing low (5 mM) KCl concentrations to keep the synaptosomes under resting membrane potential conditions, and in solutions containing either high (100 mM) KCl concentrations, or veratridine (10 uM in 5 mM KCl), for K-stimulated and veratridine-evoked depolarization, respectively, of the synaptosomes.

Time dependence of toxin binding. [^{125}I]alpha-DaTX binding to non-depolarized (in 5K solution) and K-stimulated depolarized (in 75K solution) synaptosomes was measured. Toxin binding in both 5K and 75K solutions was substantial after 5 min at 37°, and only slightly increased upon further incubation up to 30 min. There was a larger amount of toxin specifically bound to the synaptosomes when assayed in 5K solution. After 5 min incubation, the difference in the levels of toxin binding measured in 5K and 75K solutions remained relatively constant.

Similar experiments were also done in which [^{125}I]alpha-DaTX binding was measured to non-depolarized (5K solution) or veratridine-stimulated depolarized (10 uM vertradine in 5K solution) synaptosomes (data not shown). Again, some experiments showed less toxin binding after depolarization (veratridine-stimulated in this case.) However, this was not a consistent observation.

Displacement binding curves. Synaptosomes were incubated in either 5K and 75K solutions in the presence of [^{125}I]alpha-DaTX for various lengths of time. From Scatchard analysis, it was found that there were differences both in the affinities of the toxin for the receptor (K_D = 5.9 nM in 5K solution, 4.0 nM in 75K solution), and in the binding site densities (B_{max} = 4.3 pmol ligand bound/mg protein in 5K; 2.0 pmol/mg in 75K) for toxin binding measured in low K and high K solutions, respectively.

[^{125}I]alpha-DaTX binding was also similarly measured in 5K solution in the absence and presence of 10 uM veratridine (data not shown). Unlike K^+-stimulated depolarization of the synaptosomes, veratridine-stimulated depolarization had little effect in the apparent binding constants for [^{125}I]alpha-DaTX binding.

Finally, [^{125}I]alpha-DaTX binding was measured in low and high K solutions in the absence and presence of 0.2 mg/ml saponin. This detergent will "poke holes" in the synaptosomal membrane resulting in the collapse of the ionic gradient across the membrane. As expected, the presence of saponin had no effect on [^{125}I]alpha-DaTX binding to synaptosomes when assayed in 5K (non-depolarized) solution. However, the presence of saponin also had no effect on [^{125}I]alpha-DaTX binding to synaptosomes when assayed in 75K or 100K (containing 100 mM KCl and 50 mM NaCl) solutions to depolarize the synaptosomal membrane.

Veratridine-dependence. [^{125}I]alpha-DaTX binding to synaptosomes was measured in 5K solution containing increasing amounts of veratridine (data not shown). These experiments were unable to show any concentration dependence of veratridine (0.1 to 10 uM) on [^{125}I]alpha-DaTX binding.

K^+-dependence. Lastly, the effect of K^+ on [^{125}I]alpha-DaTX binding to intact synaptosomes was compared with toxin binding to a lysed synaptosomal membrane preparation. The intact synaptosomes will maintain an ionic gradient across the membrane, whereas, the synaptosomal membranes, obtained after lysis of the synaptosomes, will not have a membrane potential gradient. The results from these experiments showed that K^+ had a similar inhibitory effect (IC_{50} > 100 mM) on [^{125}I]alpha-DaTX binding to both preparations.

Re-identification of K Channel Toxins of D. angusticeps Venom

Inhibition of [^{125}I]alpha-DaTX binding. By adding an additional ion exchange chromatography step to the original toxin isolation procedure (1), beta-DaTX was resolved into two components, now termed $beta_1$-DaTX and $beta_2$-DaTX. Each of the five polypeptides inhibited [^{125}I]alpha-DaTX binding. The displacement curves for alpha-DaTX and delta-DaTX are similar to that which had been originally reported. Gamma-DaTX shows a higher (approximately 2-fold) affinity for the alpha-DaTX receptor after its isolation by the modified procedure. However, whereas in the original report beta-DaTX inhibited [^{125}I]alpha-DaTX binding with an apparent IC_{50} of 25 nM, after isolation by the modified procedure, both $beta_1$-DaTX and $beta_2$-DaTX inhibit binding with apparent IC_{50} greater than 10-fold lower (300 to 500 nM). Furthermore, although beta-DaTX was of higher affinity for the alpha-DaTX receptor than both gamma-DaTX and delta-DaTX in the previous reports, both $beta_1$-DaTX and $beta_2$-DaTX are of lower affinity than both of those two toxins.

A second difference between a previous report (5) and this report concerns the ability to measure the binding of the beta toxins to brain membranes. In the former (5), beta-DaTX was iodinated and shown to label a single class of binding sites on brain

membranes. In the present experiments, $beta_1$-DaTX was iodinated, but no measurable specific binding to brain membranes by the radiolabelled toxin has been found using unlabelled toxin to measure non-specific binding. $Beta_2$-DaTX has not been radioiodinated as yet.

Block of brain synaptosome K channels. Each of the five toxins has been tested for the ability to block brain K channels using the ^{86}Rb efflux assay. Preliminary results have shown that alpha-DaTX, gamma-DaTX, and delta-DaTX selectively block the same voltage-dependent K channels as previously reported (1). Like that reported for beta-DaTX (1), $beta_1$-DaTX is also a selective blocker of the voltage-dependent, non-inactivating K channels. However, $beta_2$-DaTX is a selective blocker of the voltage-dependent, rapidly-inactivating K channels.

Summary. The effects of monovalent and divalent cations on the binding of two dendrotoxins, alpha-DaTX and beta-DaTX, to rat brain membranes were similar. This is despite the fact that these two toxins selectively block different synaptosomal voltage-dependent K channels. These results suggest that cations have similar effects on voltage-dependent K channels.

Alternatively, a recent isolation of beta-DaTX from a different lot of venom, and using a modification to the original toxin isolation procedure, resolved this component into two polypeptides that have different selectivities for the block of brain K channels. The polypeptides of D. angusticeps venom that block K channels are currently being re-identified and characterized for the future use in the study and purification of K channels.

Finally, experiments described in this report were not able to show any voltage-

dependency for alpha-DaTX binding to its brain membrane receptor.

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E. PLANS for YEAR 90-91

1. Identification of toxins towards K channels

The recent studies described above will be completed, thereby characterizing and re-identifying those polypeptide components of D. angusticeps venom that block brain K channels. Also in progress is the screening of D. polylepis polylepis and Leiurus quinquestriatus venoms for additional polypeptides that block K channels. The goals are to identify possible toxins that selectively block different K channels. This will be useful for the structural characterization and purification of different brain K channels. However, these experiments have been temporarily halted because of the loss of access to a lyophilizer. Other arrangements for a lyophilizer are being made at this time.

2. Purification of dendrotoxin receptors

The purification of the rat brain dendrotoxin receptors will continue to be pursued using standard biochemical techniques. The goals are to obtain a highly purified

dendrotoxin binding protein preparation, to determine the subunit composition by gel electrophoresis, and to isolate the individual subunits comprising the receptor proteins. Afterwards, partial amino acid sequence information of the subunits will be obtained to prepare oliginucleotide probes, or polyclonal antibodies will be prepared, either of which can be used to screen cDNA libraries for clones of the receptors. Toxin binding characterization, combined with site directed mutagenesis, will be used for the structural mapping of the toxin binding sites on the receptor proteins.

3. Molecular cloning of toxin receptors

The long-term goal of this project is the structural characterization of the dendrotoxin receptor as a K channel. A cloned K channel that is sensitive to dendrotoxin has been expressed in oocytes (3). Thus, this K channel can be classified as one type of dendrotoxin receptor. I will begin the structural studies of dendrotoxin receptors/K channels by obtaining the cDNA clone of the dendrotoxin-sensitive K channel, either by isolation from brain cDNA libraries using oligonucleotide probes derived from published base sequences of the K channels, or otherwise obtained. Site-directed mutagenesis will be used to identify the dendrotoxin binding regions of the K channel protein. Work will also continue towards: a) the purification of brain toxin receptors/K channels as described above; and b) the molecular cloning of K channels and their functional expression in oocytes.

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21 June 90

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. Ion channels are integral membrane proteins that 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 sodium channels.

B. Methods.

My laboratory uses lipid bilayer techniques to study batrachotoxin (BTX)-modified sodium channels from brain tissue; those methods were previously reported in detail.

C. Results.

i) Steady-State Activation of BTX-Modified Dog-Brain Sodium Channels. BTX-modified sodium channels from canine forebrain were incorporated into neutral planar lipid bilayers. The steady-state activation curve was a sigmoidal function of membrane potential in symmetrical NaCl solutions. The midpoint potential of the activation curve (V_a) varied as a function of [NaCl] (-109 mV in 0.1 M NaCl, -88 mV in 0.5 M NaCl and -75 mV in 1.0 M NaCl), whereas the apparent gating charge (z_a = 3.4-3.7 elementary charges) showed no systematic variation. The observed voltage shifts in the activation curve with increasing [NaCl] were similar to those reported in the literature for macroscopic voltage-clamp experiments on sodium channels. The shifts

were interpreted in terms of screening of negative charges near the sodium channel gating machinery according to the Gouy-Chapman theory of the diffuse double layer. The extracellular surface of the channel carries the larger apparent negative charge density, and the magnitude of the charge density was quantified by experiments in symmetrical and asymmetrical NaCl solutions. Because the lipids are neutral and the Debye length is short, those negative charges are probably protein-linked carboxyl groups associated with the primary sequence of the sodium channel protein.

We observed several qualitative types of gating mode changes during these experiments. Individual channels could undergo spontaneous shifts in their gating behavior; that could shift V_a without any change in z_a, or it could modify both gating parameters. Under identical ionic conditions and pulse protocols, the mid-point potential varied by up to 40 mV in the same sodium channel. In fact, the most basic observation that we had was the presence of parallel or non-parallel shifts in the gating curves of the same sodium channel. This is more variability than one would expect from statistical fluctuations or from fitting the data to gating curves. The gating diversity that we observed must be an intrinsic property of BTX-modified (and perhaps native) sodium channels.

The apparent gating charge is steeper for single channels (by about one elementary charge) than it is for macroscopic gating data averaged across channels. That is a new finding, although it is more or less what one would really expect: single-channel gating curves must be less steep than multi-channel gating curves. Such a finding has implications for computer models of sodium channels.

Experiments in symmetrical 0.1 M NaCl solutions before and after asymmetrical additions of 0.005 M barium to the extracellular solution resulted in a positive shift of V_a and a reduction in z_a. When the gating curves were corrected for the nonlinear current-voltage (I-V) relationship following the asymmetrical addition of barium, V_a was shifted in the hyperpolarizing direction by a few millivolts and the

reduction in z_a was completely eliminated; the resulting gating curves were parallel to control gating curves with ΔV_a = 16.8 mV. When 0.005 mM barium was then added to the inside, V_a was shifted about 4 mV in the hyperpolarizing direction such that ΔV_a = 12.7 mV. These effects are expected if barium is screening negative charges and if the apparent negative charge density is larger on the extracellular surface. The addition of barium to the intracellular solution, however, appears to have a binding effect as well, because it caused a reduction in z_a that was not changed by correcting the gating curves for the largely linear I-V relationship in symmetrical barium. As in the control experiments, there was substantial kinetic diversity observed.

The parallel shifts reported above could occur if the rate constants controlling the simple two-state gating model were not constant, but shifted back and forth between stable values. The Markovian assumption of constant rate constants might need to be relaxed if there were, for instance, protonatable groups located near the gating machinery. The large changes in the apparent gating charge might mean that, for reasons not yet understood, the amount of charge that moves to open the sodium channel is also not constant. Two, three, or four gating charges may move at different times to open the sodium channel. A full-length manuscript including 10 figures and 5 tables is in preparation and should soon be submitted for publication.

ii) Gating Diversity in Single Rat-Brain Sodium Channels. As a natural extension of the above work, I have more recently been carrying out gating experiments in BTX-modified rat-brain sodium channels. These experiments are designed to explore gating diversity in more detail than was possible in the experiments described above. We are directly testing the possibility that the parallel shifts observed in symmetrical sodium solutions are due to the presence of protonatable groups located near the gating machinery. We are thus measuring the gating behavior in symmetrical 0.1 M NaCl solutions before and after altering the pH in a well-defined manner. The signal-to-noise ratio has been improved through several technical modifications by a

factor of 4-5 in these experiments, so we can now look for transitions between stable conductance levels in the gating region. That is, there are intermediate stable conductance levels between fully open and fully closed levels.

iii) On the Location of the Sodium Channel Entrance and the TTX Binding Site. The description of this work is similar to the one I provided last year, although the manuscript, which now includes 10 figures and 4 tables, has undergone extensive revision and includes much more data; it should be submitted in the near future.

The relationship between the sodium channel entrance and the tetrodotoxin (TTX) binding site was investigated by chemical modification at the extracellular surface of bilayer-incorporated BTX-modified sodium channels using an impermeant carbodiimide in the presence or absence of exogenous nucleophiles. Two (carboxyl) groups can be modified such that the open-channel conductance is decreased while TTX binding is unaffected, and TTX does not protect against modification of the conductance. Because the final conductance level depends on the exogenous nucleophile, the covalent modification appears to involve the formation of a peptide bond. In contrast to previous work, TTX binding affinity was neither eliminated nor reduced. That result may be a consequence of mild reaction conditions or of BTX modification, which may produce a less accessible TTX binding site. A third carboxyl group can be modified, however, such that TTX binding affinity is increased. The latter modification may involve the formation of an intramolecular cross link (an isopeptide bond). In contrast to the toxin-plug model of TTX action, 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 that is subsequent to toxin binding.

iv) Modulation of Conductance and Toxin binding by Negative Charges in Rat Brain Sodium Channels. The work described in this section is still in progress.

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 $\underline{e}/nm^2$, 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 the presence of a net negative charge at the STX/TTX binding site and at the channel entrance is a conserved feature of sodium channels.

v) Effects of pH on the Binding of Guanidinium Toxins. The manuscript describing this work has been reviewed by the journal editors and is presently under revision. Some difficult theoretical considerations deep within Debye-Huckel theory have delayed this work. The issue (not pointed out by the reviewers) is whether monovalent TTX and divalent STX "feel" the same surface potential. There is no problem

when the Poisson-Boltzmann equation is developed with respect to the potential of mean force, but when one jumps to the electrostatic potential energy, the problem becomes manifest. The summary given below is essentially the same as that given last year.

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. Future Plans.

i) Brain Sodium Channels. I plan to continue working on brain sodium channels and may extend that work to cardiac sodium channels. 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. above).

ii) Channels ormed by Clostridial Toxins. We are also just beginning to study the channel forming properties of tetanus and botulinum toxins in lipid bilayers. The large channels that are formed by these clostridial toxins appear to be the means by which the tissue-poisoning fragments are translocated in situ across the membrane into the cytosol. The whole toxins and the specific channel-forming fragments (the heavy chains) are routinely purified in Professor Simpson's laboratory. Thus, we

can take advantage of the existing bilayer techniques and the availability of toxins to further characterize the mechanism of toxin translocation. In preliminary work, it does look like we will be able to incorporate the purified clostridial toxins and toxin fragments into decane-containing bilayers and characterize the toxin channels with respect conductance, kinetics, ion selectivity, and pH dependence.

iii) Molecular Cloning and Expression of Voltage-Dependent Potassium Channels. We are also starting a series of projects involving the molecular cloning and expression of voltage-dependent brain potassium channels. One joint project that we (Dr. Roger Sorensen and I) are working on involves cloning, sequencing, site-directed mutagenesis, and functional expression of brain potassium channels in Xenopus oocytes. We are using oligonucleotide probes based on known sequence data and PCR amplification techniques to pull out cDNAs that code for voltage-sensitive potassium channels from a mouse cDNA library. Toward that end, we recently purchased the Perkin-Elmer-Cetus PCR machine.

The functional expression involves the microinjection of transcribed mRNA obtained from the cDNA into Xenopus oocytes. In this case, the mRNA codes for specific voltage-sensitive potassium channels that are not expressed in the native oocyte. The expressed potassium channel activity is then measured several days later by recording ionic currents from the oocytes under voltage-clamp conditions. I have a (Drummond) microinjection system set up to carry out the mRNA injections into the oocytes. The macroscopic voltage-clamp work will be carried out using my (Dagan) two-microelectrode voltage clamp, while the single-channel currents will be recorded using one of my patch clamp setups.

The objective of these experiments is to characterize neurotoxin receptor sites associated with voltage-dependent potassium channels. Dr. Sorensen has purified several neurotoxins that bind to voltage-dependent potassium channels; thus, we have in mind the study of toxin-host interactions in cloned potassium channels.

iv) Potassium Channels as PLA_2 Receptors. There is increasing evidence that some venom phospholipase A two toxins (PLA_2s) may be targeting voltage-dependent potassium channels. We will test this hypothesis using the oocyte expression system. Brain poly (A) mRNA, which codes for (among other things) potassium channels, will be extracted in the usual way (i.e. using guanidinium thiocyanate extraction followed by cesium chloride centrifugation) and purified on an oligo(dT)-cellulose column. The mRNA will then be injected into the oocytes, which will process the poly (A) mRNA into functional potassium channels. Macroscopic and single-channel currents will be recorded from injected oocytes to see if different PLA_2s do indeed bind to potassium channels. This work can, of course, also be carried out in cloned potassium channels. The advantage of the poly (A) injections experiments, however, is that they do not necessarily depend on isolation of a cloned potassium channel.

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. Further characterize the time and dose of tetanus toxin required to attenuate the reduction in NG-108 cytosolic protein kinase C (PKC) activity induced by a 30 min treatment with 10 μM neurotensin. Specifically, 10^{-12} and 10^{-13}M tetanus toxin were tested in 4 hr pretreatment protocols.

2. Determine the minimum tetanus toxin (10^{-10}M) pretreatment time required for significant attenuation of neurotensin-induced mobilization of cytosolic PKC.

3. Examine the effect of a 30 and 60 sec potassium (44 mM) depolarization on the mobilization of cytosolic PKC in NG-108 cells.

4. Study the effect of tetanus toxin pretreatment of NG-108 cells on the subsequent mobilization of cytosolic PKC induced by depolarization with 44 mM potassium.

5. Characterize the synthetic peptide (KRTLRR) phosphorylation assay for the measurement of membrane associated protein kinase C activity.

6. Examine the effect of tetanus toxin pretreatment on the activation of membrane associated PKC induced by exposure to 10 μM neurotensin.

7. Evaluate the ability of tetanus toxin pretreatment to attenuate the increase in membrane associated PKC induced by exposure to 0.1 μM phorbol myristate acetate (PMA).

B. Methods

The culture and treatment of cells as well as the assay for cytosolic PKC activity have been described in the previous annual report.

Membrane PKC activity was measured by the synthetic peptide assay developed by Heasley and Johnson (1989a). Briefly, the peptide (KRTLRR) was synthesized using a solid phase SAM TWO 9500 automated peptide synthesizer (Biosearch) using Fmoc chemistry. The peptide sequence is based on a major PKC phosphorylation site within the EGF receptor and has been demonstrated to be PKC specific (Heasley and Johnson, 1989b). For the determination of membrane PKC activity differentiated NG-108 cells were pretreated with tetanus toxin for 2 h. Following toxin pretreatment the cells were stimulated with PMA or neurotensin either by addition of the substances in fresh culture medium, or by addition of a concentrated solution directly into the toxin containing pretreatment medium. After a 5 or 15 min incubation the medium was removed and replaced with a salt solution containing 137 mM NaCl, 5.4 mM

KCl, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 1 mg/ml dextrose, and 20 mM HEPES (pH 7.2) supplemented with 40 μM digitonin, 10 mM $MgCl_2$, 25 mM beta-glycerophosphate, 100 μM gamma ^{32}P-ATP (500 cpm/pmol), 5 mM EGTA, 2.5 mM $CaCl_2$, and 300 μM KRTLRR peptide. Following a 10 min incubation at 30°C the reaction was terminated by addition of 25% trichloroacetic acid (TCA) and the phosphorylated peptide was blotted onto phosphocellulose paper. The phosphocellulose paper squares were washed in 75 mM phosphoric acid (3x) and 75 mM NaH_2PO_4, pH 7.5 (1x). Phosphorylation of the peptide was quantified by scintillation spectrometry.

C. Results

Tetanus toxin effect on neurotensin-mobilized protein kinase C
Dose Response

In our previous annual report we presented evidence suggesting that a 4 hr pretreatment of differentiated NG-108 cells with tetanus toxin (10^{-10}-10^{-11}M) resulted in a significant attenuation of neurotensin-induced mobilization of cytosolic PKC. These observations lead us to explore 10^{-12} and 10^{-13}M tetanus toxin in an attempt to define the lower limit of sensitivity of this system.

As shown in Table 1 neurotensin mobilized 12±3 pmol/mg/min of the cytosolic PKC in this series of experiments. A 4 hr pretreatment with 10^{-12}M tetanus toxin completely abolished the ability of neurotensin to mobilize PKC. This attenuation of neurotensin mobilized PKC was statistically significant in 5 experiments (Student's paired t-test).

Exposure of differentiated cells to 10^{-13}M toxin for 4 hr also attenuated neurotensin-mobilized cytosolic PKC. Toxin pretreatment significantly reduced the mobilization of cytosolic PKC to -6±5 pmol/mg/min. This effect was not significant when the data were expressed as a % of control. However, only 3 experiments have been performed at this low dose and additional experiments are needed to confirm this observation. These experiments lend further support to the notion that the action of tetanus toxin is time, as well as dose-dependent and that this system is extraordinarily sensitive to the toxin.

Tetanus toxin effect on neurotensin-mobilized protein kinase C Time Response

In addition to defining the lowest effective dose of toxin we performed experiments to determine the minimum pretreatment time necessary for tetanus toxin to significantly reduce cytosolic PKC mobilization. As depicted in Figure 1 pretreatment with 10^{-10}M tetanus toxin for 4, 2 and 1 hr significantly attenuated the ability of neurotensin to mobilize cytosolic PKC. However, a 30 min pretreatment with 10^{-10}M toxin did not significantly attenuate the ability of neurotensin to mobilize cytosolic PKC. In other experiments at 10^{-11}M tetanus toxin, pretreatment for 2 hr, but not for 1 hr, was sufficient to signficantly reduce neurotensin-mobilized cytosolic PKC activity (data not shown). Sandberg *et al.* (1989) have documented in the PC12 neurosecretory cell line that the internalization of tetanus toxin is rapid. In these studies 75% of the toxin was internalized within 5 min. Assuming the internalization of tetanus toxin into NG-108 cells occurs on a time scale similar to that observed by Sandberg et al. (1989), the results above would suggest that internalization is not the rate-limiting step. Two possibilities for rate-limiting steps could be the internal processing of the toxin or the actual modification of the putative toxin substrate.

Effect of potassium on cytosolic PKC

As previously reported NG-108 cells prelabeled for 24-36 hrs with ^{14}C-choline and subsequently exposed to 44 mM potassium for 10 min, demonstrate an increased secretion of ^{14}C-acetylcholine. Since tetanus toxin has been shown to inhibit this K^+-induced secretion, it was of interest to examine the effect of potassium on mobilization of cytosolic PKC in NG-108 cells. A 30 min exposure to 44 mM potassium did not result in a detectable mobilization of cytosolic PKC (data not shown). It is most likely that within this time frame the effect of K^+ on the distribution of PKC may have reversed as observed in permeablized adrenal chromaffin cells (Terbush et al., 1988). Moreover, our own studies have suggested that K^+-depolarization-induced ^{14}C-acetylcholine secretion returns to basal

levels within 10 min despite continued stimulation. For this reason we studied 30 and 60 sec K^+ depolarization. As shown in Table 2 exposure of NG-108 cells to 44 mM K^+ for either 30 or 60 sec results in a mobilization of cytosolic PKC. A 30 sec treatment with K^+ mobilized 11±5 pmol/mg/min of cytosolic PKC, an effect on the distribution of PKC comparable to that induced by 10 μM neurotensin. Exposure to K^+ for 60 sec mobilized 19±12 pmol/mg/min of the cytosolic PKC activity. Although a 60 sec exposure appears to mobilize more cytosolic activity, there was no difference between the two treatments when the mobilized activity is expressed as a % of the control activity in cells not exposed to 44 mM K^+.

Effect of tetanus toxin pretreatment on K^+-induced mobilization of cytosolic PKC

As described above, depolarization of differentiated NG-108 cells with 44 mM K^+ caused the release of acetylcholine as well as the mobilization of cytosolic PKC. Since tetanus toxin pretreatment inhibits K^+-induced acetylcholine release we investigated the effect of the toxin on the ability of 44 mM K^+ to mobilize cytosolic PKC. As shown in Table 3, a 30 sec exposure to 44 mM K^+ resulted in a significant mobilization of cytosolic PKC ($p<0.0005$; Student's paired t-test). Surprisingly, a 2 hr pretreatment with 10^{-10}M tetanus toxin caused a significant reduction in the basal level of cytosolic PKC. This is the first time we have observed a direct effect of toxin pretreatment on the basal level of cytosolic PKC.

Since the basal cytosolic PKC activity is lower in these experiments, assessment of the mobilization of cytosolic PKC induced by depolarization is difficult. While it appears that K^+ is unable to mobilize cytosolic PKC in tetanus toxin pretreated cells immediately following removal of the toxin, the lowered basal cytosolic activity complicates this interpretation of the data.

Characterization of synthetic peptide phosphorylation

As shown in Figure 2, phosphorylation of the peptide in digitonin-permeabilized non-stimulated NG-108 cells was linear with reaction time up to 20 min. A reaction time of 10 min was chosen for further experimentation. Please note that the cpm incorporated/mg protein in this figure have not been normalized with respect to background and therefore, the extent of phosphorylation appears greater than that reported in the following tables. Background represents phosphorylation of other substrates within the permeabilized cells that bind to the phosphocellulose strips as well as nonspecific binding of radioactivity. Filters bound less than 0.075% of the total radioactivity when the reaction mixture was blotted directly on the paper. Background due to phosphorylation of other cellular components or non-specific binding of radioactive phosphate to protein was less than 20% of the total phosphorylation measured in control cells.

The use of digitonin to permeabilize cells has been criticized since this detergent can make large holes in the cell membrane. The observation that the rate of phosphorylation has not begun to decline by 20 min of digitonin treatment suggests that the constituents necessary for PKC activation have not diffused from the cells. In addition, this concentration of digitonin, while permeabilizing the cells almost instantaneously as determined by trypan blue treatment, does not cause gross morphological alterations or loss of cells from the plating surface.

The effect of neurotensin on membrane associated PKC activity

As illustrated in Table 4, a 5 min exposure to 10 μM neurotensin significantly increased the membrane associated PKC activity 151±17 % of control ($p<0.025$; Student's paired t-test). It should be noted that the elevation in membrane PKC activity observed in our experiments is comparable to that observed by Heasley and Johnson (1989b) following exposure of PC12 cells to nerve growth factor. Pretreatment with 10^{-10}M tetanus toxin for 2 h significantly elevated the basal PKC activity over control ($p<0.05$; Student's paired t-test). Neurotensin treatment elevated

the membrane PKC activity in toxin-treated cells to approximately the same level as that observed in the control cells. However, since the PKC activity in toxin treated cells is elevated, the ability of neurotensin to activate membrane PKC activity appears to be blocked.

This is the second instance in which we have observed a change in the unstimulated level of PKC activity in toxin-treated cells (see above discussion of K^+-induced PKC mobilization). We believe that this difference is most likely due to an effect of the addition of fresh medium on PKC activity. In several experiments control cells were exposed to fresh medium for either 15 min or for 5 min. Statistical comparison of these paired controls indicates that the extent of phosphorylation at 5 min is significantly less than that measured at 15 min ($p<0.05$; Student's paired t-test, $n=12$). Further experiments are planned to investigate the effect of medium change on PKC activity.

PMA-induced activation of membrane associated PKC activity and the effect of tetanus toxin pretreatment on this activity

In initial experiments we observed a PMA-induced increase in digitonin-permeabilized cell membrane PKC activity of 135% of the basal value. Although this increase was statistically significant by Student's paired t-test comparison, in order to better assess the effect of toxin, we investigated ways of enhancing the stimulation of membrane PKC activity by PMA. Based on our previous observation that the basal level of membrane PKC activity appeared to fluctuate as a function of medium changes (see above), we performed a series of experiments in which the PMA was added directly to the pretreatment medium rather than in fresh medium. The PMA was added in a volume of 10 µl to 1 ml of culture medium. As shown in Table 5, using this direct addition protocol the basal level of membrane PKC activity was 3407±913 cpm/mg/10 min. A 15 min exposure to 0.1 µM PMA significantly increased the membrane PKC activity to 6136±1351 cpm/mg/10 min ($p<0.005$; Student's paired t-test). The increase in membrane PKC induced by direct addition of PMA represented 222±35% of the basal level activity by paired comparison. This increase in activity is

comparable to that observed by Heasly and Johnson (1989) in PMA-stimulated PC12 cells.

In initial experiments in which the toxin pretreatment medium was replaced with fresh medium (3/90 quarterly report), a 2 hr tetanus toxin pretreatment (10-10M) resulted in a significantly lower unstimulated membrane PKC activity. In addition we did not detect any toxin effect on the PMA-induced increase in membrane PKC activity. Subsequently, as described above, a direct addition protocol was developed which maximized the effect of PMA and minimized the non-specific effect of medium change on PKC activity. Using the direct addition protocol, a 2 h pretreatment with 10^{-10}M tetanus toxin had no effect on the basal level of membrane PKC (Table 5). Exposure of tetanus toxin pretreated cells to 0.1 μM PMA resulted in a significant increase in membrane asssociated PKC activity ($p<0.05$; Student's paired t-test). However, the ability of PMA to increase membrane associated PKC activity was significantly attenuated by toxin pretreatment. PMA mobilized 2729±587 cpm/mg/10min of PKC activity in control cells and 1890±539 cpm/mg/10min of activity in toxin pretreated cells ($p<0.05$; Student's paired t-test). The attenuation of PMA-induced mobilization is apparent in this study for two reasons. First, there is no effect of medium change on the basal membrane activity and second, the PMA-induced mobilization is significantly greater using the direct addition protocol. Indeed, if the previously reported tetanus toxin/PMA data (3/90 quarterly report) are reevaluated by imposing the requirement for a minimum PMA-induced activation of PKC, the interpretation is different. If only experiments in which PMA increased the membrane associated PKC activity greater than 40% over the basal are included, tetanus toxin pretreatment results in a significant attenuation of the ability of PMA to mobilize PKC (2113±409 versus 680±210 cpm/mg/10min of activity in control and toxin pretreated cells respectively; n=8). For these reasons we plan to reevaluate by the direct addition protocol the effect of neurotensin on membrane PKC activity as well as the effect of tetanus toxin pretreatment.

D. Future Plans

The main thrust of the experiments to be conducted over the next year will be designed to clarify the effect of tetanus toxin pretreatment on PMA or hormone stimulated PKC mobilization. The development of the permeabilized cell/synthetic peptide assay provides an opportunity to rapidly screen pharmacological agents that activate PKC and to test the effect of tetanus toxin pretreatment on the ability of these agents to mobilize PKC. Experiments to date strongly suggest that 30-60 min following addition of tetanus toxin to the culture medium, the cells are modified in some way that results in a block of PMA, neurotensin and possibly potassium induced localization of PKC. Given the current understanding of the phosphoinositol-diacylglycerol-PKC pathway, tetanus toxin may act at any one of three possible sites. First, the toxin may directly modify protein kinase C in such a manner as to prevent its binding to membrane components necessary for activation. Second, it is possible that tetanus toxin pretreatment alters the distribution of a specific intracellular calcium pool associated with the cell membrane. Since PKC activation is calcium dependent, a reduction in the local calcium concentration at the membrane might be associated with an attenuation of hormone- or PMA-induced PKC activation. A final possibility, since activation of PKC requires specific phospholipid components in addition to calcium and diacylglycerol, is that the toxin enzymatically changes the membrane mileau such that a PKC binding and activation is less efficient.

Experiments designed to clarify these three possible hypothesis are easily conducted utilizing the specific PKC peptide substrate. The first possibility discussed above was previously tested by directly treating cytosol containing PKC with tetanus toxin for 10 min (see annual report 6/89). These initial experiments suggested that direct addition of the toxin had no affect on PKC activity. Subseqently, our observation that at least a thirty minute lag period was required before tetanus toxin had an effect in intact cells suggests that the direct toxin/PKC interaction experiments need to be repeated using longer treatment times. Utilizing the peptide substrate in place of non-specific histone will allow us to accurately measure only PKC activity. Specific addition/deletion of phospholipids, diacylglycerol and calcium is also now possible since the peptide assay

does not rely on the difference between kinase activity measured in the presence and absence of these cofactors.

The role of intracellular calcium is to be investigated in two ways. Since the inositol phosphates influence intracellular calcium concentration, as well as PKC activity, we will monitor the generation of inositol polyphosphates in response to hormonal stimulation. Radiolabeling of the cells followed by separation of the various inositol polyphosphates will be performed by the method of Downes et al. (1986). This technique provides a rapid and convenient measure of the production of the major inositol polyphosphates. Tetanus toxin may alter the inositol phosphate cascade which could result in a change in the intracellular calcium concentration and as a result, alter PKC metabolism. A direct measure of intracellular calcium concentration in response to stimulation will be accomplished by fluorescent dye techniques. Tetanus toxin pretreated cells will be labeled with fura 2 or quin 2 and loaded into a spectrophotometric cuvette. Hormones will be added directly to the cuvette and the rise in intracellular calcium concentration monitored. This technique will allow us to determine if calcium release from the endoplasmic reticulum is altered by toxin pretreatment.

The use of permeabilized cells allows us to conduct reconstitution experiments to determine if a membrane constituent is altered by the toxin. Specific phospholipids required for PKC activation are added to permeabilized toxin-pretreated cells to determine whether these substances are capable of reversing the tetanus toxin attenuation of PKC activation. In addition, cytosol from non-toxin treated cells could be added to toxin treated cells in an attempt to return PKC activity, as well as secretory capacity, to normal. One final reconstitution would be the cytosol from toxin treated cells into permeabilized control cells. If PKC activity in the control cells is attenuated following the addition of toxin-treated cytosol, it would suggest that the toxin substrate is within the cytosol.

It is known that the botulinum toxins are in many ways similar to tetanus toxin. The experiments outlined above represent a protocol that can be used to screen the botulinum toxins for effects on PKC and inositol polyphosphate metabalism. We have begun preliminary screening of the botulinum toxins for their ability to inhibit secretion from differentiated

NG-108 cells. This line of investigation will continue in a manner similar to that utilized for tetanus toxin as we screen for mechanisms of action that involve second messenger systems.

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F. Publications

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Considine RV, Simpson LL, Sherwin JR The effect of botulinum C2 toxin on steroid release from adrenal Y-1 cells: Evidence for a role for *microfilaments in the regulation of steroid secretion.* (In preparation)

Considine RV and Simpson LL Molecular pharmacology of binary toxins possessing ADP-ribosyltransferase activity. (In preparation)

TABLE 1. 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)
Control	5	39 ±5	27 ±6	12 ±3	33 ±8
Tetanus toxin 10^{-12}M		35 ±4	38 ±5	-3[a] ±4	-10[a] ±13
Control	3	35 ±9	23 ±8	13 ±5	38 ±12
Tetanus toxin 10^{-13}M		31 ±3	37 ±2	-6[a] ±5	-23[b] ±18

Cells were pretreated 4 hr in the presence or absence of tetanus toxin (10^{-13} or 10^{-12}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.

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

b=$p<0.1$ by Students paired t-test comparison to cells not exposed to tetanus toxin.

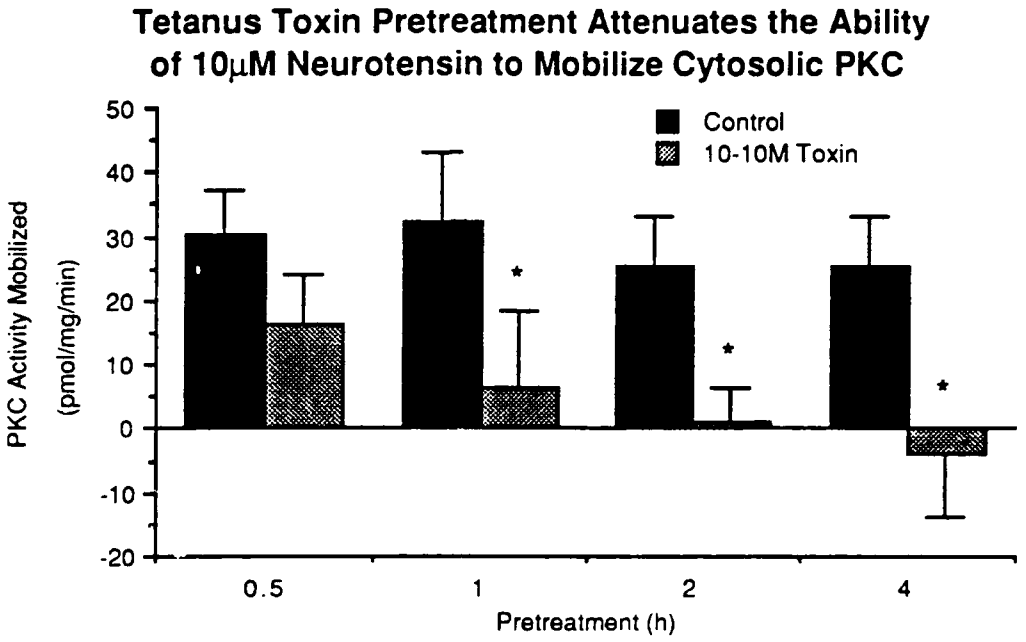

Figure 1. NG-108 cells were pretreated with 10^{-10}M tetanus toxin for the times indicated. The toxin-containing medium was then removed and the cells challenged with neurotensin in fresh culture medium for 30 min. The data is expressed as the cytosolic PKC activity mobilized by exposure to neurotensin in untreated and toxin pretreated cells. Values represent the mean ±SEM of at least 6 experiments.
*$p<0.05$; students paired t-test

TABLE 2. MOBILIZATION OF CYTOSOLIC PKC ACTIVITY BY 44 mM POTASSIUM IN DIFFERENTIATED NG-108 CELLS

K^+ Exposure	(n)	Cytosolic PKC Activity (pmol/mg/min) $-K^+$	Cytosolic PKC Activity (pmol/mg/min) $+K^+$	K^+-Mobilized PKC Activity (pmol/mg/min)	K^+-Mobilized PKC Activity (% Control PKC Activity)
30 seconds	8	93 ±15	82[a] ±12	11 ±5	11 ±4
60 seconds		119 ±23	101[a] ±15	19 ±12	11 ±6

Cells were exposed to 44 mM potassium for 30 and 60 seconds. Following potassium treatment the cytosolic PKC activity was determined as previously described. 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 44 mM potassium.

TABLE 3. Mobilization of cytosolic PKC activity by depolarization with 44 mM potassium in untreated and tetanus toxin pretreated NG-108 cells

Group	(n)	Cytosolic PKC Activity (pmol/mg/min) -K^+	Cytosolic PKC Activity (pmol/mg/min) +K^+	K^+-Mobilized PKC Activity (pmol/mg/min)	K^+-Mobilized PKC Activity (% Control PKC Activity)
Untreated	14	131 ±17	97[a] ±15	34 ±8	26 ±5
10^{-10}M toxin 2 h		109[a] ±12	109[a] ±17	0 ±13	-3 ±11

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 44 mM potassium.

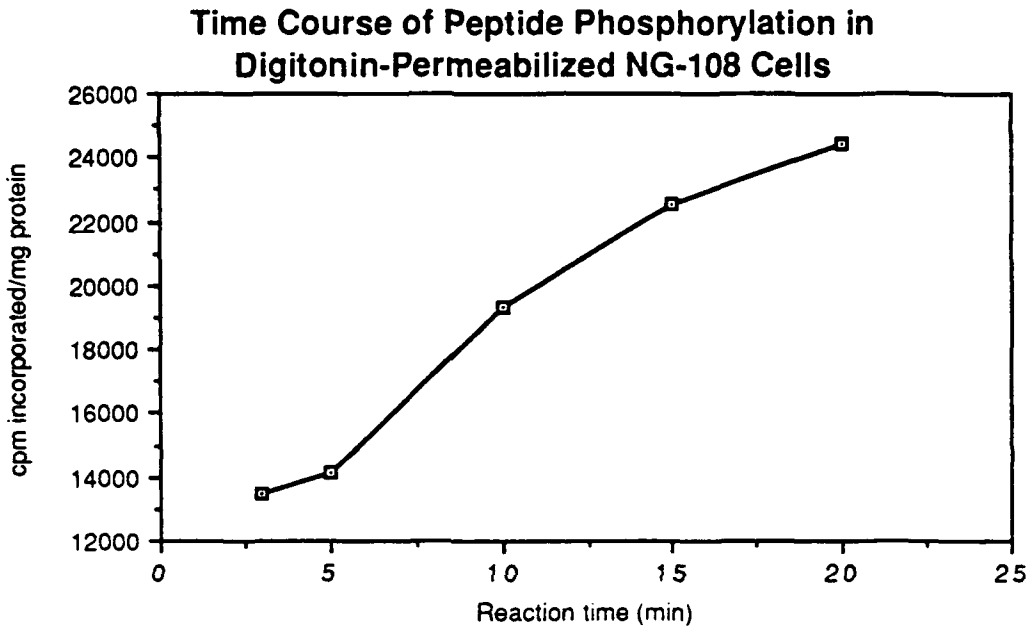

Figure 2. Characterization of the time course of KRTLRR peptide phosphorylation. Peptide phosphorylation in the presence of calcium-containing reaction mixture was monitored over a 20 min period. Each point represents the result of two separate experiments. Background phosphorylation has not been subtracted from the above values and therefore the incorporation appears higher than in the subsequent tables.

TABLE 4. KRTLRR Peptide Phosphorylation Induced by Exposure to 10 μM Neurotensin and the Effect of a 2 Hour Tetanus Toxin Pretreatment on this Phosphorylation

Group	(n)	Membrane PKC Activity (cpm/mg/10 min) - hormone	+ hormone	Hormone-Activated membrane PKC (cpm/mg/10 min)
Control	14	2601 ±406	3867[a] ±752	1266 ±513
Tetanus toxin 10^{-10}M		3580[b] ±565	4021 ±700	441 ±445

Cells were exposed to 10 μM neurotensin for 5 min. Values represent the mean ±SEM of the indicated number of experiments.

[a] $p<0.025$ by Student's paired t-test comparison to corresponding control cells not exposed to neurotensin.

[b] $p<0.05$ by Student's paired t-test comparison to cells not exposed to tetanus toxin.

TABLE 5. KRTLRR Peptide Phosphorylation Induced by Direct Addition of 0.1 μM PMA and the Effect of a 2 Hour Tetanus Toxin Pretreatment on this Phosphorylation

Group	(n)	Membrane PKC Activity (cpm/mg/10 min) - PMA	+ PMA	PMA-Activated membrane PKC (cpm/mg/10 min)
Control	9	3407 ±913	6136[a] ±1351	2729 ±587
Tetanus toxin 10^{-10}M		3191 ±561	5082[a] ±914	1890[b] ±539

Cells were exposed to 0.1 μM PMA for 15 min. Values represent the mean ±SEM of the indicated number of experiments.

[a] $p<0.05$ by Student's paired t-test comparison to corresponding control cells not exposed to PMA.

[b] $p<0.05$ by Student's paired t-test comparison PMA-induced mobilization in cells not exposed to tetanus toxin.

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Scientific Progress During the Year

Work has been conducted in two general areas: the use of antibodies to study mechanism of toxin action, and an analysis of structure-function relationships. Progress had been made in both areas.

I. Antibody Research

A. Background

A number of investigators have described monoclonal antibodies that interact with botulinum neurotoxin. Antibodies have been reported that recognize epitopes in the light chain and heavy chain, and in addition there are antibodies that differentiate between the aminoterminus and carboxyterminus of the heavy chain. These findings suggest that appropriately selected antibodies might serve as tools for probing structure-function relationships within the toxin molecule.

In spite of the value that monoclonals have as potential research tools, they have only rarely been utilized (Murayama et al., 1987). In fact, monoclonal antibodies have not previously been employed to study the interaction between botulinum neurotoxin and the cholinergic neuromuscular junction. The present study represents an initial attempt to remedy this deficiency. A small library of monoclonal antibodies has been used to help identify sites on the toxin molecule that remain exposed after binding as well as to

clarify the general conformation of the toxin after association with its receptor.

B. Methods

The techniques used during the Year have been described in earlier reports.

C. Results

Characterization of antibodies. Four mouse monoclonal antibodies were chosen for study; their designations and sites of binding are as follows: E3 (light chain), E14 (heavy chain), E17 (heavy chain) and E32 (light chain).

Each of these antibodies was examined in an ELISA assay to quantify interaction with the toxin. Experiments were done with three different states of the antigen, as follows: i.) single chain toxin (i.e., the immediate post-translocational product that is not fully activated), ii.) dichain toxin (i.e., the fully activated molecule that has undergone proteolytic cleavage with trypsin to yield a heavy chain and a light chain linked by a disulfide bond), and iii.) denatured toxin. The latter was produced by two different means: exposure (60 min) to urea (2 M) in the presence of dithiothreitol (50 mM), and exposure (60 min) to heat (80^{0}C) in the presence of dithiothreitol (50 mM).

Each of the antibodies interacted with the single chain and dichain forms of the toxin, and the apparent binding characteristics as revealed by ELISA were essentially the same. By contrast, the antibodies showed less affinity for

denatured toxin. Monoclonals E17 and E32 showed almost no binding, and monoclonals E3 and E14 showed diminished binding.

Phrenic nerve-hemidiaphragm preparations were used to bioassay the activity of the single chain unactivated molecule, the dichain activated molecule and the denatured molecule. The dose-response data for the unactivated and activated molecules will be presented shortly.

Denatured toxin was examined at a single concentration (1×10^{-11}M). Phrenic nerve hemidiaphragms exposed to protein that had been treated with urea (n=4) or with heat (n=4) did not paralyze within 250 minutes. Preparations exposed to activated toxin at the same concentration paralyzed in approximately 120 minutes (see below). These data indicate that denaturation caused the biological activity of the toxin to be reduced by more than 90%.

Pharmacologic properties of antibodies. Various properties of the antibodies have been partially described in the past (Kozaki et al., 1986). For the purposes of the present study, each antibody was assayed for its ability to diminish toxicity in vivo and in vitro. A molar excess of antibody (5-fold) was mixed with activated toxin (2.7×10^4 LD50) in 0.05 M phosphate buffer, pH 7.2, that contained 0.15 M NaCl. The mixture was incubated at 37^{o} C for 30 minutes and then administered to mice intravenously in a volume of 0.1 ml. The results (Table 1) showed that E3 failed to diminish toxicity, but E14, E17 and E32 were active. Increasing the amount of antibody did not produce further

decreases in toxicity. Thus, none of the antibodies completely neutralized the test dose of toxin.

Similar types of experiments were done in vitro. Activated neurotoxin (1×10^{-11} M) was incubated with E14, E17 or E32 at various ratios for 30 minutes at 37^{o} C. The mixtures were added to phrenic nerve-hemidiaphragm preparations and paralysis times were monitored. Again, all three antibodies demonstrated an ability to diminish toxicity, but none produced complete neutralization.

E14, E17 and E32 were tested in combinations of two or three. Antibodies were incubated with toxin as described above and then added to neuromuscular preparations. As expected, the combinations of antibody were more effective in diminishing toxicity than were individual antibodies. One implication of the data in is that the three antibodies do not have a common binding site, a conclusion that reinforces earlier work (Kozaki et al., 1986).

Interaction with unactivated toxin. The neurotoxin was isolated and purified to homogeneity in the unactivated form, and portions of this material were converted to the activated form by exposure to trypsin. The toxicity of the unactivated and activated material was then assayed on the phrenic nerve-hemidiaphragm preparation. The data showed that proteolytic cleavage of the molecule was associated with an approximately 30-fold increase in toxicity.

E14, E17 and E32 were tested for their abilities to diminish toxicity of the unactivated form of the toxin. The

neurotoxin (3×10^{-10} M) was incubated with antibody (8-fold molar excess) as described above. When the mixtures were added to isolated neuromuscular preparations, the results showed that the three antibodies diminished toxicity of the unactivated molecule, and the magnitude of effect was comparable to that obtained with the activated molecule

Interaction with heterologous toxins. Types A and B botulinum neurotoxin, as well as tetanus toxin, were examined for their potential reactivity with E3, E14, E17 and E32. The results were universally negative. When examined by ELISA, none of the monoclonal antibodies demonstrated significant affinity for heterologous toxin.

The apparent absence of reactivity was verified in tissue experiments. When individual antibodies were incubated with heterologous toxin (antibody:toxin ratio, 5:1; 37°C, 30 min) and then added to phrenic nerve-hemidiaphragm preparations, there was no significant loss in toxicity.

Susceptibility of bound toxin. Activated type E botulinum neurotoxin (1×10^{-11} M) was incubated with tissues for 60 min at 4° C. Under these conditions the toxin can bind but, due to low temperature, it cannot be internalized. Tissues were then washed with repeated changes of physiological solution and incubated in medium without added toxin but with antibody (10-fold molar excess) for 60 min at 4° C. At the end of incubation, tissues were again washed and transferred to tissue baths (35 - 35° C). The paralysis times of these tissues were compared with those of tissues

that had been exposed to toxin and washing but not to antibody.

The results (Table 3) showed that antibodies E14, E17 and E32 continued to exert effects, even when toxin was already bound to nerve endings. Interestingly, the magnitude of effect was about comparable to that when antibody and toxin were mixed prior to addition to neuromuscular preparations.

In the next series of experiments, activated neurotoxin (1×10^{-11} M) was incubated with tissue for 60 min at 4^{o} C. Tissues were vigorously washed and then transferred to tissue baths at 37^{o} C. Phrenic nerves were stimulated at 0.2 Hz for 30 min, after which tissues were returned to incubation tubes. They were exposed to antibodies for 60 minute at 4^{o} C, then washed and again transferred to tissue baths. The paralysis times of these tissues were compared with those of tissues that had been similarly manipulated, except that there had been no exposure to antibody.

The paralysis times of tissues that had not been exposed to antibody was 129 ± 13 (n = 6). The paralysis times of experimental tissues were as follows: E14, 142 ± 14 (n=6); E17, 133 ± 15 (n=4); E32, 134 ± 9 (n=5). None of the experimental values is significantly different ($p > 0.01$) from the control value.

The effectiveness of antibody in diminishing the potency of bound toxin was compared before (Table 3) and after (above) application of nerve stimulation. For each of the antibodies there was a statistically significant difference ($p < 0.01$).

The magnitude of the induced loss in toxicity was greater before rather than after nerve stimulation.

D. Comment

Relatively little work has been done to characterize the structure-function relationships of the botulinum neurotoxin molecule. There is suggestive evidence that the heavy chain possesses a tissue-targeting domain (Black and Dolly, 1986 a,b; Bandyopadhyay et al., 1987) and stronger evidence that the light chain can act inside cells to block exocytosis (Bittner et al., 1989). In addition, there are experimental findings that implicate the aminoterminus of the heavy chain as being the portion of the toxin molecule that promotes internalization (Hoch et al., 1985).

Data from various sources have been interpreted to mean that internalization of the toxin molecule is associated with marked conformational changes. The model that has drawn widest attention can be articulated as follows (Simpson, 1986). Botulinum toxin enters cholinergic nerve endings by receptor-mediated endocytosis. As the proton pump in the endosome membrane lowers pH, the toxin is induced to insert into the lipid environment and form channels. It is not clear whether these channels are the route the toxin uses to traverse the membrane or whether they are phenomena that are secondary to some other mechanism for penetration. Nevertheless, the fact that the toxin can be induced to insert

into the membrane and form channels almost certainly means there have been notable conformational changes.

In contrast to the work on internalization, there are no experimental findings or arguments by deduction that allow one to determine whether the initial binding step or the later poisoning step is associated with conformational changes. The present study represents the first attempt to address these issues, especially as they pertain to binding. The method of approach has been to use monoclonal antibodies as probes of structure-function relationships. Interestingly, this also represents the first attempt to use monoclonal antibodies as research tools to study the interaction between botulinum toxin and the cholinergic neuromuscular junction.

Properties of the monoclonal antibodies. Four monoclonal antibodies were selected for study; the general location of the epitopes recognized by these antibodies is as follows: E3, light chain; E14, carboxyterminus of the heavy chain; E17, aminoterminus of the heavy chain; and E32, light chain. One of these antibodies (E3) did not produce significant loss of botulinum toxin activity, though it did associate with toxin in ELISA assays. The remaining three antibodies produced loss of botulinum toxin activity in vivo and in vitro, and they also reacted with the antigen in ELISA assays.

The properties of three of the monoclonals (E14, E17,E32) were studied in detail, and a number of their characteristics were determined. The data show that each of the antibodies reacted only with type E toxin. Several laboratories have

shown sequence homology among the botulinum toxin serotypes and tetanus toxin (Eisel et al., 1986; DasGupta and Datta, 1988), and other laboratories have shown that individual monoclonal antibodies can react with several clostridial toxins (see Tsuzuki et al., 1988, for references). However, the monoclonal antibodies used in the present study were highly selective.

E14, E17 and E32 were shown to diminish toxicity in vivo and in vitro, but they did not possess neutralizing activity. When used in combination they produced substantially greater effects than when used individually. These data, as well as those on epitope mapping (Kozaki et al., 1986), show that the three antibodies do not share a common binding site.

All four antibodies shared the property of interacting with unactivated and activated toxin. In each case the apparent affinity before and after proteolytic activation was comparable. The activation process is due, in part, to cleavage of an arginine or lysine residue in the single chain protein, resulting in a dichain molecule with an interchain disulfide bond. At the very least, the antibody data mean that proteolytic activation does not cause the domains recognized by E3, E14, E17 or E32 to become occult. The data further suggest that activation is not associated with marked changes in the domains recognized by E17 and E32, though one must be more cautious about interpreting the possibility of changes in the regions recognized by E3 and E14 (see below). In spite of these findings, it would be incorrect to assume

that activation does not involve molecular rearrangements within the toxin molecule. Work currently in progress in the author's laboratory indicates that activation causes one occult domain to become exposed (or more fully exposed) after trypsin-induced proteolysis.

One of the most important characteristics of the antibodies is that they have affinity for conformational rather than linear epitopes. The antibodies interacted with the toxin in its native state, but they showed reduced (E3, E14) or virtually no (E17, E32) affinity for the denatured toxin. The fact that the antibodies react mainly or exclusively with conformational rather than linear epitopes is essential to their use as structure-function probes. A conformational epitope will cease to exist with any significant change in the structure of the holotoxin, whereas a linear epitope may continue to be exposed even with marked changes in conformation. It is this simple principle that allows one to make the deductions proposed above about the state of the toxin molecule before and after activation. The same arguments apply when using the antibodies as probes for toxin interaction with the cholinergic nerve ending.

Structure-function relationships that govern toxin binding to the plasma membrane. The proposed model for botulinum toxin action envisions an initial binding step that is followed by receptor-mediated endocytosis. There are many questions that remain to be answered about binding and internalization, two of which can be tentatively addressed by

the data in this study. Firstly, it would be desirable to know whether the binding step causes detectable changes in the toxin molecule. And secondly, it would be important to establish whether the entire toxin molecule is endocytosed. Monoclonals E14, E17 and E32 are well suited to answering these questions, because their binding sites are distributed among what are thought to be the three functional domains in the toxin molecule (see Introduction).

Experiments were done with phrenic nerve-hemidiaphragm preparations under conditions that allow for separation of the binding and internalization steps. After the toxin had become bound to nerves but before it had become internalized, it remained accessible to E14, E17 and E32. This type of phenomenon has previously been demonstrated with polyclonal antibodies (Simpson, 1980; Simpson and DasGupta, 1983), but this is the first such demonstration with monoclonals. It is interesting that domains in the light chain as well as both halves of the heavy chain continued to expose antigenic determinants. In fact, the antibodies were about equivalent in their abilities to diminish toxicity whether reacted with antigen before or after the latter became bound to nerve endings. In keeping with the reasoning proposed above, these data suggest that those portions of the toxin molecule recognized by E14, E17 and E32 do not undergo marked rearrangements after binding. They also indicate that the sites of attachment of these antibodies are not the sites in the toxin molecule that govern binding to the nerve membrane.

Previous experiments have shown that nerve stimulation and physiological temperature cause bound toxin to become internalized within 15-30 minutes (Simpson, 1980). The present work with monoclonal antibodies has demonstrated a similar outcome. Within 30 minutes or less, bound toxin could escape from the effects of monoclonal antibodies. There are two possible explanations for this finding: either the entire molecule is endocytosed, or the molecule dissociates and sends an active fragment into the nerve while leaving the remainder on the cell surface. Further work is needed to show conclusively which of these two possibilities is correct, though it seems reasonable to suggest that the former is more likely.

The studies reported here begin to shed light on events that surround toxin binding to the nerve ending. It is worth noting that there are at least two directions in which the work could be pursued. The various serotypes of botulinum toxin have molecular masses of about 150,000. The size of these molecules, when combined with existing knowledge about their immunologic properties, argues that there may be a dozen or more antigenic sites in each serotype. This suggests that it would be appropriate to do studies similar to those described here but with an exhaustive library of monoclonals. In a similar vein, it would be worthwhile to use monoclonals in a study designed to characterize the internalization process. Given the existing model for penetration, which hypothesizes pH-induced insertion of the toxin molecule into

membranes, one would predict there should be conformational changes that are associated with alterations in antigenic domains. These and related predictions are now being tested.

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Table 1

The Effects of Various Antibodies on the In Vivo Toxicity of Activated Botulinum Neurotoxin Type E

Antibody	Initial Toxicity	Residual Toxicity	Percent of Control
None	2.7×10^4	---	100
E3	2.7×10^4	3.0×10^4	111
E14	2.7×10^4	1.5×10^2	0.6
E17	2.7×10^4	4.6×10^3	17
E32	2.7×10^4	2.4×10^3	8.8

The methods for incubating toxin and antibody, administration of the mixture, and bioassay for toxicity are described in the text.

Table 2

The Individual and Combined Effects of Monoclonal Antibodies Directed Against Botulinum Neurotoxin Type E[a]

Group	Monoclonal Antibody [b] E14	E17	E32	Paralysis Time [c]
1	-	-	-	127 ± 14
2	+	-	-	264 ± 21
3	-	+	-	165 ± 9
4	-	-	+	201 ± 13
5	+	+	-	300 ± 19
6	+	-	+	420 ± 16
7	-	+	+	255 ± 24
8	+	+	+	> 450

[a] The final concentration of toxin added to phrenic nerve-hemidiaphragm preparations was 1×10^{-11} M.

[b] Antibody was mixed with toxin at a 10-fold molar excess and allowed to incubate at 30°C for 30 minutes.

[c] Each value is the mean ± SEM of at least five observations.

Table 3

The Effects of Various Antibodies on the Biological Activity of Botulinum Neurotoxin Bound to Cholinergic Nerve Endings[a]

Group	Antibody [b] E14	E17	E32	Paralysis Time [c]
1	-	-	-	131 ± 14
2	+	-	-	255 ± 22
3	-	+	-	161 ± 13
4	-	-	+	214 ± 18
5	+	+	+	> 300

[a] Tissues were incubated with 1×10^{-11} M botulinum neurotoxin Type E for 60 minutes at 4^{o} C.

[b] A molar excess of antibody was added to tissues for 60 minutes at 4^{o} C. See text for additional details.

[c] Each value is the mean ± SEM of at least five observations.

A. Specific Aims

An effort is underway to identify structural changes in toxin molecules that are related to the process of poisoning. There are two types of structural changes of interest: 1.) those that underlie the phenomenon of activation, and 2.) those that pertain to the three step model of toxin action. In the current report both of these issues are explored. Data are presented that bear on the phenomenon of activation, but which lead to the conclusion that there are other structural changes that have a different timecourse. Data are also presented that relate to pH-induced changes in toxin structure.

B. Methods

The techniques for isolation and characterization of the botulinum neurotoxin molecule have been described previously. The current study deals with nicked and unnicked type B toxin as well as type A toxin. The methods for structural characterization are given below.

C. Results

Nicking and activation. Limited proteolysis of the single chain molecule to give a light chain and a heavy chain is referred to as nicking. The process of nicking is thought to be necessary but not sufficient to produce full activation.

Type B neurotoxin was isolated from young cultures in the unnicked condition. The aminoterminus of the nicked

molecule [B(n)] and the unnicked molecule [B(un)] were then determined. An analysis of the first 15 residues shows that the original aminoterminus is identical before and after nicking (Figure 1).

Nicking of the molecule leads to the presence of two aminotermini: the original amino end of the native molecule, and the amino end of the newly generated heavy chain. The aminoterminus of the heavy chain was determined, and it is illustrated in Figure 1.

Work is currently in progress to sequence the carboxyterminus of the nicked and unnicked toxin. Until this is done, one cannot know whether proteolysis has affected the carboxy end of the molecule. However, if there are such changes they must be minimal in nature. The apparent molecular weights determined before and after nicking do not differ significantly. Thus, proteolysis - if it were to affect the carboxyterminus - could not involve more than a small number of residues.

Activation versus aging. As part of the sequencing work, additional steps were taken to characterize the toxin molecule. These involved measuring the in vivo toxicity of the native and activated molecule and performing carboxymethylation.

The type B toxin was activated with trypsin, and at various times after trypsin exposure the toxin was administered to laboratory mice. The toxicity of the unnicked material was approximately 4.5×10^5 LD50 (Figure

2). Following proteolysis with trypsin, the toxin increased in potency to about 2.9 x 10^7 LD50. This increase in toxicity is comparable to that reported by other investigators. The timecourse of activation was very rapid. Within 30 to 60 minutes of trypsin treatment, the toxin was near to or at the point of maximal activation.

In related experiments, the toxin was exposed to iodoacetic acid in order to produce carboxymethylation. This was done with the nicked and unnicked molecules, and the results were quite interesting. The data indicated that the unicked molecule could not be fully carboxymethylated. There were approximately 6 -SH residues that appeared not to be accessible in the unnicked molecule but accessible in the nicked molecule. In experiments to determine the timecourse of transition, the accessibility of -SH groups was monitored at various times after nicking. The results (Figure 2) showed that 2 to 4 days were necessary for the -SH groups to become exposed and carboxymethylated.

The data tend to support the hypothesis that nicking is associated with two molecular changes. The first occurs within minutes and is associated with activation; the second occurs over days and might be referred to as "aging". Proteolysis between the heavy and light chains may lead to slow occuring rearrangements in the macrostructure of the toxin.

Induced conformational changes. An intracellular fall in pH is thought to be necessary for toxin action. When the

pH inside endosomes falls to 5 or lower, this may promote movement of the toxin from the endosome to the cytosol.

Experiments are being done to identify and quantify the pH-induced changes. This could ultimately help to identify that portion of the toxin molecule that promotes internalization. An example of such an approach is shown in Figure 3. Type A botulinum toxin was exposed to 2-p-toluidine-naphthelene sulfonate (TNS). This compound has the valuable property that it has minimal fluoresence by itself but it emits when associated with hydrophobic domains of proteins. This is illustrated in the figure. TNS alone (o) provided only a minimal response by itself, but when mixed with type A toxin (o) there was a strong signal.

Figure 3 shows the results at pH 7.0. An identical experiment was done at pH 4.0, and there was a striking increase in the fluorescence signal. By comparing the respective curves, one can calculate that the increase in intesity was approximately 10-fold.

Translocation. We have applied a strategy that has been used successfully by others to study the partioning of molecules between aqueous and organic phases. The technique utilizes Triton X-114, a substance that has temperature-dependent miscibility properties. At room temperature (~25^{o}C) and lower, the detergent and water form a mixture, but at high temperatures the two separate into distinct phases. Therefore, at 37^{o}C, the detergent and the water

phase are clearly separate and one can be physically removed (e.g., aspiration) from the other.

The properties of the detergent allow one to study the pH-induced changes in the conformation of protein toxins. In a typical experiment, toxin is added to a mixture of Triton X-114 and a physiological buffer. The pH of the mixture is adjusted by the addition of acid or base. When the desired pH is attained, the solution is warmed to 37°C. The fraction of the protein of interest in the two phases is quantified, and the results serve as a reflection of the hydrophobicity of the molecule. By comparing the partition characteristics at normal pH and at acidic pH, one can monitor the induced conformational changes.

The technique has value not only because it allows for study of toxin partioning, but also because an investigator can make slight modifications to determine how other factors affect the pH-induced changes. These range from composition of the aqueous phase to the use of ancillary molecules that might alter the toxin (i.e., antibody). It is also a rapid assay technique for gauging whether site-directed reagents that modify specific amino acid residues will also modify partioning.

The technique can be utilized in two ways. In one, the native toxin is added to the mixture and later an aliquot is bioassayed for toxicity. Alternatively, the toxin can be iodinated and the distribution of label can be monitored. Both approaches have been used here.

Time- and pH-dependence of partioning. Botulinum neurotoxin type E was added to a detergent-aqueous mixture, and at the end of 30 minutes the pH was altered (4, 5, or 6) or left at 7. After an additional 30 minutes the temperature of the mixture was raised from 25°C to 37°C. At various times thereafter the two phases were sampled, and the fraction of the toxin in each was measured.

The results were closely in keeping with the expectations that arose from previous electrophysiological experiments, including those done in the author's laboratory. When the pH of the solution was lowered, a progressively greater fraction of the toxin migrated to the organic phase. For example, at pH 7.0 less than 15% of the toxin was in the Triton phase, but at pH 4.0 more than 80% was in this phase. The "transition" point was around pH 5.0, which is in reasonable agreement with the electrophysiology data.

The timecourse of transition was very rapid. Within two minutes (and possibly less), the partitioning of toxin had reached steady-state. This was confirmed both by measuring the movement of labelled toxin and by doing bioassays.

Reversibility. In a parellel set of experiments, an attempt was made to induce the backward partioning of toxin by raising pH. This proved to be only minimally successful. Less than 25% of the toxin in the organic phase could be routed back to the aqueous phase. This phenomenon was more time-dependent than partioning into Triton. By lengthening

the experiment one could increase the amount of material in physiological buffer, but extrapolation of the data suggested that full reversal, assuming it occurred, would have taken days.

These results could be interpreted in two ways: 1.) the pH-induced changes in conformation are irreversible, or 2.) the Triton binds to the pH-induced hydrophobic domains, and this alters the ability of the toxin to re-assume its native state, or the detergent itself confers so much hydrophobicity that reverse partioning is not possible.

Antibody Experiments. Monoclonal antibodies are available that interact with the three presumed functional domains of the toxin molecule; the light chain, the aminoterminus of the heavy chain and the carboxyterminus of the heavy chain. These antibodies have been used to determine whether occulsion of an epitope would impair the ability of the toxin to partition. Interestingly, none of the monoclonal antibodies had a significant effect. This could mean: 1.) the antibodies do not react with the hydrophobic domain, or 2.) they react with the domain at neutral pH, but they are conformationally displaced when pH-induced changes occur. To help resolve this, we will capture toxin in the organic phase and determine whether antibody is still attached.

Work will continue in an effort to determine molecular changes that relate to biological activity. Two principal goals will be: 1.) to determine whether the pH-induced

changes have a timecourse that is comatible with the internalization process, and 2.) to determine whether pH-induced changes are cpmparable in nicked and unnicked preparations.

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