AD_____

MECHANISMS OF ACTION OF CLOSTRIDIAL NEUROTOXINS ON DISSOCIATED
MOUSE SPINAL CORD NEURONS IN CELL CULTURE

FINAL REPORT

GREGORY K. BERGEY

SEPTEMBER 30, 1991

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-86-C-6056

University of Maryland at Baltimore
Baltimore, Maryland 21201

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden. to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 30, 1991	Final Report 1 Jan 86 - 31 Dec 89

4. TITLE AND SUBTITLE	5. FUNDING NUMBERS
Mechanisms of Action of Clostridial Neurotoxins on Dissociated Mouse Spinal Core Neurons in Cell Culture	DAMD17-86-C-6056

6. AUTHOR(S)

Gregory K. Bergey

62787A
3M162787A871 AA
DA309151

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Maryland at Baltimore
Baltimore, Maryland 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

14. SUBJECT TERMS

RA 1; Neurotoxin; BD; Mice; Cultured Cells; Neurobiology

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-230-5500

Standard Form 298 (Rev. 2-89)

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 Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

Accesion For	
NTIS CRA&I	☑
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

TABLE OF CONTENTS

STATEMENT OF PROBLEM UNDER STUDY

The focus of this proposal has been to further investigate the actions of the clostridial neurotoxins (tetanus toxin) to better understand mechanisms and characteristics of toxin action using the in vitro system of dissociated mouse spinal cord neurons in culture.

The specific aims of this proposal included:

1. Investigations of structure function relationships of tetanus toxin. These investigations focused upon whether the nicking of tetanus toxin into a two chain structure was important for maximal toxin activity. Monoclonal antibodies directed against various epitopes of the toxin molecule were used to determine which regions of the molecule were the active subunits.

2. Further investigations on the mechanisms of action of the clostridial neurotoxins were designed to study specificity of action on inhibitory synapses.

3. The ability to block and recover from toxin action, using both selected agents and newly developed monoclonal antibody conjugates.

BACKGROUND

Although the clostridial neurotoxin tetanus toxin is one of the most potent biological substances, little is known about its specific mechanism of action. The toxin is a 150,000 mw. bacterial protein comprised of two chains (Matsuda and Yoneda, 1975; Helting and Zwisler, 1977; Robinson and Hash, 1982); the amino acid structure has been elucidated (Eisel et al., 1986). A binding subunit is located on the carboxyterminal of the 96,000 m.w. heavy chain. The papain produced fragment (fragment C) containing this binding subunit is not toxic, but binding is necessary for the toxin to gain access to the intracellular compartment where the toxin is active. In the intact animal tetanus toxin in the periphery binds to local and remote nerve terminals and then is transported retrograde to the spinal cord (Price et al., 1975). Once in the motoneurons, the toxin then moves transsynaptically to the presynaptic terminals synapsing on the motoneurons (Schwab et al., 1976, 1979) . Although a number of substances have been demonstrated to move retrograde in axons, tetanus toxin is unique in its ability to move transsynaptically.

The clinical picture of tetanus is one of disinhibition. It has been speculated for some time that tetanus toxin might preferentially affect inhibitory neurons, with perhaps a specificity for glycinergic synapses. It should be remembered, however, that in the intact ventral spinal cord inhibitory synapses are frequently glycineric and tend to cluster in the perisomatic regions, in contrast to excitatory inputs which can be quite dispersed along the dendritic tree (Burke et al., 1971, Price et al., 1976). Therefore strictly on anatomical grounds inhibitory synapses might appear to be more sensitive to the toxin in intact animals.

Using the in vitro system of dissociated spinal cord neurons, however, this preferential action of the toxin on inhibitory synapses was confirmed in a system where the toxin had equal access to all synapses (Bergey et al., 1983, 1987). Despite this relative sensitivity, it is clear that tetanus toxin can affect all synapses, excitatory as well as inhibitory. Indeed the toxin can inhibit exocytosis from adrenal chromaffin cells as well (Penner et al., 1986). It may well be that tetanus toxin can block many type of vesicular exocytosis.

It has been postulated that the action of tetanus toxin requires three steps, binding, translocattion and subsequent internalization (Schmitt et al., 1981, Critchley et al., 1985). There the toxin acts at some, as yet unidentified site to block presynaptic neurotransmitter release. It appears that the action of the toxin is at a time after calcium ingress, although the locus of action may be a calcium sensitive one. Indeed the elegant studies by Penner et al. (1986) using adrenal chromaffin cells have shown that intracellularly injected toxin produces a blockade of exocytosis without affecting calcium ingress. Toxin applied outside the adrenal cells does not bind and therefore is not active.

The molecular mechanism of action of tetanus toxin remains to be elucidated. While it has be speculated that tetanus might be acting as an enzyme, no definite evidence exists to establish this. Lysosomotropic agents such as ammonium chloride and methylamine hydrochloride have delayed the onset of action of the clostridial neurotoxins (Simpson, 1983) and diphtheria toxin is also antagonized by these agents (Sandvig and Olsnes, 1980; Draper and Simon, 1980). The role of lysosomes in the action of tetanus toxin, however, is not yet clear.

Probes are needed to allow localization of the tetanus toxin at its subcellular locus of action. Monoclonal antibodies are potentially such probes. Various groups have produced antibodies to various regions of the toxin molecule and the Kenimer and Habig (Kenimer et al., 1983) have characterized some of these antibodies; these were subsequently utilized in the spinal cord culture system here. In addition the recent availability of nontoxic fragment C conjugates with monoclonal antibodies allowed for extension of these studies.

The system of dissociated fetal mouse spinal cord neurons in tissue culture offers a number of advantages in the investigations of toxin action. Specific amounts of toxin can be added to the cultures without the requirements of axoplasmic transport or diffusion through dense neuropil. The toxin is accessible to all neuronal membranes and synaptic structures. In this system the toxin has been shown to first produce reduce inhibition, producing convulsant activity manifest by paroxysmal depolarizing events (PDE), abrupt depolarizing shifts of membrane potential with associated triggered action potentials. These cultures are quite sensitive to the toxin; less than 0.1 mouse lethal dose (MLD) can produce the convulsant activity. Subsequently excitatory transmission is also reduced and then blocked. Unlike intact animal systems, long term studies are readily performed (Habig et al., 1986); the toxin is not cytotoxic. While the phenomena of toxin action have been well characterized (Bergey et al., 1981, 1983, 1987; Habig et al., 1986), to better characterize the

effects of various conditions and agents on toxin action and recovery better means for quantification of the action of the toxin are needed. Later investigations reported here focused on the application of a system for continuous detection of postsynaptic potentials to this experimental system to provide quantification of synaptic effects of the toxin.

MATERIALS AND METHODS

Culture Techniques

Cultures of fetal mouse spinal cord neurons were prepared as described in detail previously by Ransom et al. (1977). Spinal cords were removed from 13-14 day old fetal mice and then pretreated with trypsin before mechanical dissociation. The cells were then plated on collagen-coated 35 mm plastic culture dishes. The culture medium was Eagle's minimal essential medium (MEM) supplemented with glucose (final concentration 30 mM and bicarbonate (final concentration 44 mM). Cultures were grown and maintained at 35 C in 10% CO_2. During the first 24 hours both 10% fetal calf serum and 10% horse serum (HS) were included in the culture medium. After this time only 5% HS was included and 1% N3 solution (Romijn, 1982) was added. The antimetabolite 5-fluoro-2-deoxyuridine was used for a 24-h period after day 6 to limit the growth of nonneuronal cells. Cultures were maintained with biweekly subtotal changes of medium for 4-8 weeks at which time they were used for experiments. Hippocampal cell cultures from neonatal rats were prepared in a similar fashion.

Tetanus Toxin

Homogeneous tetanus toxin was prepared from sterile filtrates of Clostridium tetani cultures as previously described by Ledley et al. (1977). The toxin has about 2 X 10^7 mouse lethal doses (MLD) per milligram of toxin protein. An MLD is defined as the least amount of toxin that will kill a 15-18 g mouse within 96 h following subcutaneous injection into the inguinal fold region. The toxin was kindly provided by W.H. Habig, FDA.

Homogenous nicked tetanus toxin was prepared from sterile culture filtrates of the Massachusetts C_2 strain of Clostridium tetani cultures. Unnicked toxin was extracted from washed C. tetani cells with 1M NaCl, 0.1 M sodium citrate at pH 8.7 (Raynaud, 1951). The extraction solution also contained 1 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl floride) and 10 mM benzamidine as potential protease inhibitors. Both toxin preparations were purified by $(NH_4)_2SO_4$ precipitation with 60% saturated ammonium sulfate, and gel filtration on Sephadex G-100. The toxin fractions were pooled, dialyzed against 5 mM potassium phosphate, pH 6.8, and were applied to a hydroxyapatite column and eluted with a gradient of 5 mM to 250 mM KPO_4, pH 6.8. After concentration by ultrafiltration, protein was measured by the Lowry protein assay using BSA as a standard (Lowry et al., 1951).

Electrophoretic analysis of the toxin preparations on SDS gels in the presence and absence of mercaptoethanol determined the proportion of unnicked, single-chain toxin. Reduction of disulfide bonds in nicked toxin results in complete dissociation into heavy and light chains whereas the unnicked, single-chain toxin is not dissociable. Gells stained with Coomassie Brillant Blue R-250 (Bio Rad Laboratories) were used for qualitative indications of the compositions, but for quantification of the amounts of unnicked toxin in the various preparations, toxin was labeled with ^{125}I using the Bolton-Hunter reagent (Bolton and Hunter, 1973). SDS gel electrophoresis was then performed in the presence of 2-mercaptoethanol. Gels (10 cm length) were sliced into 2 mm fractions using an Aliquogel Fractionator (Gilson Medical Electronics) and the gel fractions counted. The percentage of nicked toxin was calculated from the total counts in the unnicked toxin, heavy chain and light chain regions of the reduced SDS gels and compared to gels obtained with stock (100% nicked, two-chain) toxin. For all physiological experiments a preparation that was 10% nicked and 90% unnicked was used. This was the preparation most enriched for unnicked toxin. For experiments assaying for endogenous protease activity in the cultures another preparation that was 22% nicked, 78% unnicked was also used. Previous experiments have demonstrated preservation of bioactivity of tetanus toxin following labeling with the Bolton-Hunter reagent (Habig et al., 1986).

^{125}I-labeled toxin was added for 10 minutes or 14 hours to spinal cord cultures that had been washed free of horse serum and placed in MEM. The cultures were then washed and the structure of the cell-associated toxin was analyzed by SDS gel electrophoresis in the presence of 2-mercapto-ethanol. A total of 3 cultures were pooled for each gel analysis.

For experiments to test whether nicking of the 90% single-chain toxin preparation increased activity, radio- labeled unnicked tetanus toxin (90% unnicked) was treated with partially purified nicking protease from a toxin minus strain of C. tetani (strain BT 101, Laird et al., 1980 and Finn et al., 1984). After treatment both the control and protease treated toxin samples were passed over a Sephadex G-100 column primarily to remove any contaminating clostridial hemolysin (tetanolysin) present in the protease preparation which could damage cultured neuronal cells. SDS gels of ^{125}I-labeled untreated and protease treated toxins were obtained after the respective toxin preparations were reduced with 1% 2-mercaptoethanol. These labeled toxin preparations were used in both neuronal cell physiologic assays and mouse toxicity studies. In neuronal cell physiologic assays 50,000 counts of either the unnicked preparation or the protease treated toxin preparation were added to each culture. Intracellular recordings (described below) determined the onset of convulsant activity. To check for residual hemolysin activity additional cultures were incubated with 50,000 counts of the protease-treated toxin for 72 hours and examined under phase optics for gross morphological changes.

Bioassays of the unnicked and protease nicked toxins in mice were performed. Dilutions of 1.5 fold of the labeled toxin preparations were counted and injected into groups of 4 mice per dilution. The percentages of mortality were calculated after 96 hours.

Antitetanus Antibodies

Monoclonal antibodies specific for epitopes on fragment B (light and heavy chain regions) and Fragment C were prepared by fusion of P3X63Ag8 BALB/c myeloma cells with spleen cells from BALB/c mice immunized with tetanus toxoid or fragment B as described in detail previously (Kenimer et al., 1983). From the library of 14 monoclonal antibodies produced against various toxin epitopes, 4 monoclonal antibodies were selected for experimentation based on neutralization studies in whole animals (Kenimer et al., 1983). In this paper the monoclonal antibodies will be identified using the same nomenclature as in the original report. Two monoclonal antibodies directed against the C fragment of tetanus toxin were used, one (18.1.7) shown to have a neutralization titer of 0.3 U/ml, the other (18.2.12.6) having no detectable neutralization titer (<0.001). Two monoclonal antibodies directed against the B fragment of tetanus toxin were utilized. One (21.76.10) was directed against the heavy chain portion of the B fragment and had a high neutralization titer of 3.0 U/ml. The other (21.18.1) was directed against a site on the light chain of the B fragment and had no detectable neutralization titer (<0.001). Figure 1 illustrates the various monoclonal antibodies and their binding domains.

Horse serum (Gibco Laboratories) and mouse serum (supplied by W. Habig) were used as sources of polyclonal antibodies.

Toxin Binding Studies

Experiments were performed to determine the effects of respective monoclonal antibodies on the binding of tetanus toxin to spinal cord neurons. To determine the specificity of toxin binding, experiments were done where a 10-fold excess (relative to labeled toxin) of unlabeled toxin was applied to cultures prior to the addition of ^{125}I-labelled tetanus toxin.

[125]I-tetanus toxin was preincubated in culture media with excess monoclonal antibody or polyclonal antibody for one hour prior to the addition to spinal cord cultures. The number of counts added was determined by an assay of equal volume (2cc) of the antibody-toxin mixture. Cultures were incubated with the various antibody-toxin combinations for 1 hour at 35 C. Cultures were then washed 3 times in MEM with 1% FCS (which contains proteins but no antitetanus toxin antibodies). For each assay, 5 cultures were used. Following the washes, cultures were harvested and counts of culture associated ^{125}I-tetanus toxin were made.

Monoclonal Antibody Conjugates

As discussed above in the introduction previous experiments with selected monoclonal antibodies have identified a single monoclonal antibody 21.76.10 directed against the

nonbinding B subunit. Conjugates (kindly supplied by Dr. Jane Halpern, FDA) of the 21.76.10 antibody and purified fragment C (0.6 mg in phosphate buffered saline pH 7.2) were obtained following incubation with Traut's reagent (1.0mM) for 30 minutes at room temperature. Each protein was then desalted into PBS on a small G-25 column in order to remove free cross-linking reagents. After mixing the sample was chromatographed on a TSK-3000 gel filtration column in PBS. Fractions were analyzed by SDS-PAGE, and the ganglioside binding assay. SDS-PAGE revealed a high molecular weight band which was the appropriate size for an antibody-fragment C complex.

To assay (assays by J. Halpern) whether the conjugate of fragment C and the 21.76.10 antibody was bifunctional , microtiter plates were coated with 0.1 ug of GT1b in methanol and allowed to dry. The wells were blocked with PBS containing 5 mg.ml BSA. 21.76.10-Fragment C was diluted into PBS plus 1 mg/ml BSA and various dilutions were added to individual wells and incubated for 2 hours. Fragment C (100 ug/ml) to block binding to tetanus toxin to GT1b plus 100,000 cpm of ^{125}I-tetanus toxin were added to each well and incubated for 1.5 hours. The plates were washed and individual wells were counted in a gamma counter.

Various experimental paradigms were used involving preincubation of the neurons with the conjugate (4 ng/ml), washes and subsequent addition of whole tetanus toxin. To allow for internalization of any conjugate that might still be on the neuronal surface, a 6 hour period in MEM was allowed after exposure to the conjugate for 24 hrs. To further control for any possible remaining C fragment exposed on the cell surface, some experiments included exposures to tetanus toxoid (10 ug/ml, Sclavo).

Tests of Potential Antagonists of Tetanus Toxin

Experiments were performed to determine whether ammonium chloride (5-20 mM final concentration), chloroquine (0.01-0.1 uM final concentration) or methylamine HCl (10 mM final concentration) reduced the time to onset of convulsant activity produced by tetanus toxin.

Initial experiment were performed by mixing the respective agents in culture media, changing the culture media to include the agent. Control intracellular recordings were made from 10-20 spinal neurons at 30 minutes to 6 hours after the addition of the new media to determine whether the agent itself had any affect on the neuronal behavior. Cultures were also observed under a phase microscope to assay for any obvious morphological changes.

If no deleterious morphological changes were seen (i.e. cytotoxicity) and no overt changes in the physiological behavior of the neurons was observed then comparisions were made to see if the selected agent retarded or prevented the onset of action of tetanus toxin as measured by the appearance of paroxysmal depolarizing events. Spinal cord cultures

were preincubated for hours in the experimental media and the time to onset of convulsant activity following addition of tetanus toxin (final concentration) was noted. This was compared with the time to onset of convulsant activity produced in control cultures. Five to 10 assays of onset of PDE were performed for each experimental condition.

Experiments with Concanavalin A were conducted in a similar fashion to those above using spinal cord cultures preincubated in 50-400 ug/ml Concanavalin A (Sigma) for 1-6 hours. Experiments with FITC-labeled Con A have shown that binding occurs to both neuronal and nonneuronal cells in this system.

To assay whether the cGMP analogue, 8 bromoguanasine 3'-5'-cyclic monophosphate (Sigma, 8-bromo GMP), could reverse the tetanus toxin produced reductions in neurotransmitter release, 8 bromo cGMP was added (final concentration 0.1-1.0 mM) to spinal cord cultures previously incubated for 24 hours in 100 ng/ml tetanus toxin. Single neurons were sampled using intracellular electrodes to assay for spontaneous electrical activity. In addition pair of potentially connected neurons were sampled to determine whether any functional synaptic connections were present. Other experiments were performed during the onset of convulsant activity produced by tetanus toxin. Following addition of tetanus toxin (100 ng/ml final concentration), pairs of monosynaptically (excitatory) connected neurons were identified (a monosynaptic connection was operationally defined as a connection with less than 5 mS latency and low failure rate). Recordings of synaptic cell pairs were performed in media containing 6 mM Ca^{2+} and 6 mM Mg^{2+} to reduce spontaneous synaptic activity yet allow evoked synaptic activity. Only after a stable recording period had been established with no drift in resting membrane potential and the effects of tetanus toxin began to be evident as manifested by the gradual onset of the reduction in the monosynaptic postsynaptic potential (stimulating the presynaptic neuron at 1/sec) was the 8 bromo-cGMP added. The size of the evoked PSP was monitored continuously.

Electrophysiology

Cultures were selected for electrophysiological studies after growing for 4 to 12 weeks. Cultures were washed 3X in MEM with 1% fetal calf serum to remove all horse serum (horse serum contains antitetanus antibodies at high titers). After washing no detectable horse serum is present (i.e. <0.001 U). Cultures were then placed in HEPES-buffered MEM solution on a heated (28-33 C.), stage of an inverted phase-contrast microscope. Intracellular recordings were made under direct vision using microelectrodes filled with 4 M potassium acetate at neutral pH and pulled to yield resistances of 30-50 MOhm. A conventional bridge circuit was used for recordings in conjunction with a storage oscilloscope and continuous chart recorder and storage on video tape following digitalization of the signal by a pulse code modulator (A.R. Vetter).

Dilution of purified tetanus toxin (400-4000 ng/ml) were added to the cultures in 500

mcl aliquots to yield a final concentration of toxin of 100-1000 ng/ml. This amount was chosen to allow fairly rapid evolution to convulsant activity (typically over 45-90 minutes at 32-33 C) and further evolution to synaptic blockade after 6-8 hours. Intracellular recordings were begun after the addition of the toxin preparations and at various times throughout; continuous recordings were made from single neurons or pairs of neurons for 30 to 180 minutes.

Detection of Potentials

The recorded signals (either on-line analog or digitally stored) were sampled by a Labmaster-DMA 12 bit board with 10 kHz sampling rate and stored on the magnetic disc of a PC AT compatible microcomputer with 80386-25 MHz processor. To allow for analysis of recordings longer that the capacity for data on the hard disk (25 minutes of recording requires approximately 30 MB of storage) the electrophysiologic signal was recorded on VCR magnetic tapes by a digital pulse modulator. Stored data could be played back and analyzed identically to data recorded on-line. The procedure for acquisition of data was coded in assembler language to take advantage of transmitting data from the Labmaster board to disk by DMA channels.

An algorithm for detection of postsynaptic potentials was utilized (Franaszczuk, unpublished data) based on assumptions of PSP morphology and computations of approximations of first and second derivatives of the signals. The time to peak, amplitude, rise time, half decay time and the shape parameter can be calculated for each PSP.

RESULTS

Following a dose-dependent latent period, tetanus toxin produces convulsant activity in the cultured spinal cord neurons as manifest by the appearance of paroxysmal depolarizing events (PDE). The appearance of these PDE coincides with the reduction and disappearance of inhibitory transmission. Typically following addition of 1 mcg/ml of toxin at 32-33 C. the latency to appearance of convulsant action is 30-50 minutes; the latency is prolonged at lower temperatures. This appearance of PDE allows for measurement of toxin action. Figure 1 illustrates a typical transistion from the latent period to convulsant activity.

Actions of Nicked and Unnicked Toxin Preparations

After considerable effort (Lin and Habig) toxin preparations enriched to 90% unnicked toxin were obtained as demonstrated by SDS gel electrophoresis of radiolabled toxin (see annual report 1986). No pure unnicked preparation could be obtained, probably because even pure intracellular preparations have some nicked toxin. The spinal cord

A.

Time after toxin

75 min

B.

85 min

C.

95 min
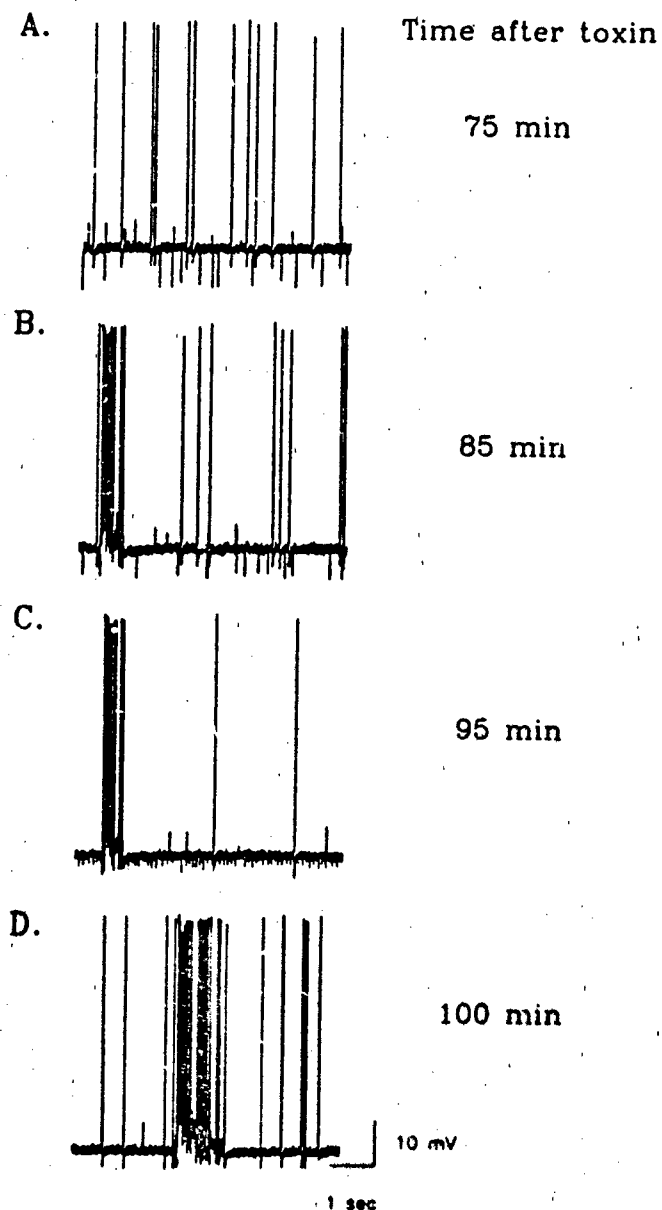
D.

100 min

10 mV

1 sec

Figure 1. Onset of convulsant activity following addition of tetanus toxin (1 mcg/ml) at 28 C. Illustrated are selections from a continuous intracellular recording from a spinal cord neuron in culture. The transistion from the late latent period (A, 75 minutes) to subsequent convulsant activity (B,C,D) is seen. EPSPs and IPSPs are evident prior to (A) and shortly after the onset of PDE, but IPSPs are markedly diminished in amplitude (C) and number (D) as the convulsant activity becomes established.

cultures did not contain proteases that produced nicking; unnicked preparations were not affected by incubation on the cultures for up to 14 hours.

Measurements of the time of onset of PDE in the spinal cord neurons following the addition of pure nicked toxin preparations and 90% unnicked preparations revealed a delay in the onset of PDE in the unnicked preparations (Figures 2,3). A dose response plot for three concentrations of the two preparations revealed significantly increased activity of the

pure nicked toxin (Figure 3). The average times of PDE onset for the 90% unnicked preparation at 10^{-6} and 10^{-7} (53.5 and 75.0 minutes respectively) were similar to the average times to PDE onset for the pure nicked toxin preparation at 10^{-7} and 10^{-8} (54.8 and 75.8 minutes).

100% NICKED TOXIN PREPARATION   **90% UNNICKED/10% NICKED TOXIN PREPARATION**
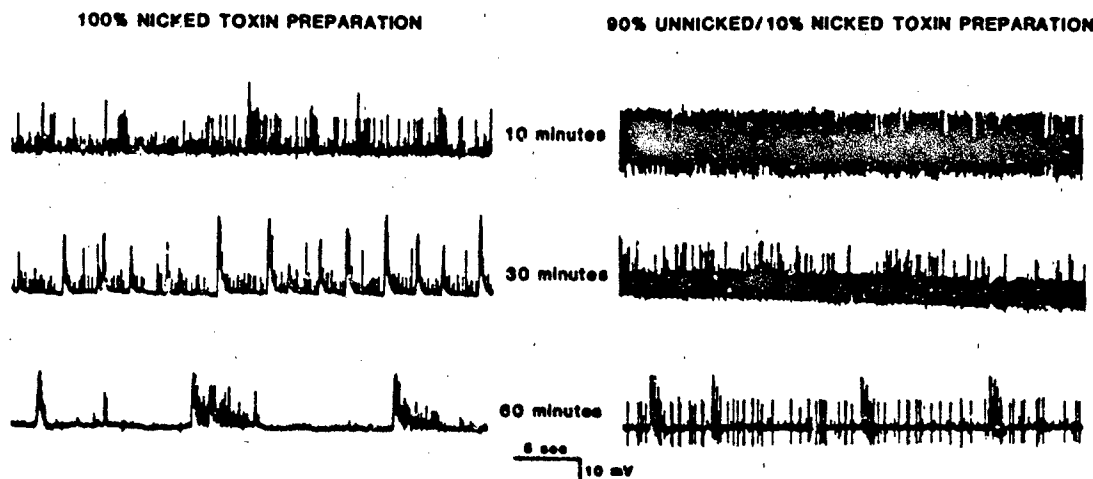

Figure 2. Representative penwriter records of intracellular recordings from spinal cord neurons at 10, 30 and 60 minutes following the addition of pure nicked, double-chain toxin and 90% unnicked toxin (each 10^{-6} g/ml) reveals the earlier onset of action with the pure nicked preparation. In each instance intracellular recordings at 10 minutes reveal a combination of spontaneous action potentials and post-synaptic potentials (inhibitory and excitatory). After 30 minutes, the culture exposure to pure nicked toxin shows the early onset of paroxysmal activity that is even more prominent at 60 minutes. Intracellular recordings from neurons in a culture exposed to the toxin enriched for the unnicked form continues to show essentially a control pattern of activity; recordings at 60 minutes reveal paroxysmal activity (tetanus-PDE).
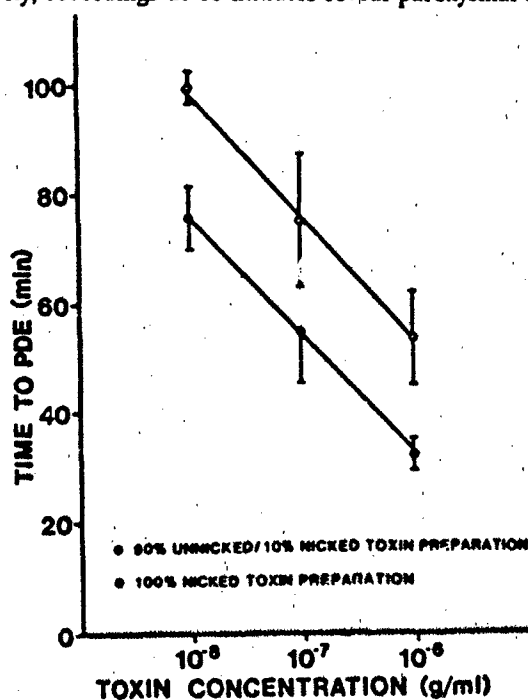

Figure 3. Average times to the onset of paroxysmal depolarizing events (PDE) produced by three concentrations of the 100% nicked toxin and 90% unnicked toxin preparations. Averages shown represent 3-5 experiments per point (+ S.E.M.). Analysis of covariance produced a nicked-unnicked difference of 21.8 + 5.5 (p < 0.001)

Treatment of the 90% unnicked toxin preparation with a protease derived from a non-toxin producing strain of C. tetani significantly shortened the time to onset of tetanus produced PDE (Figure 4).
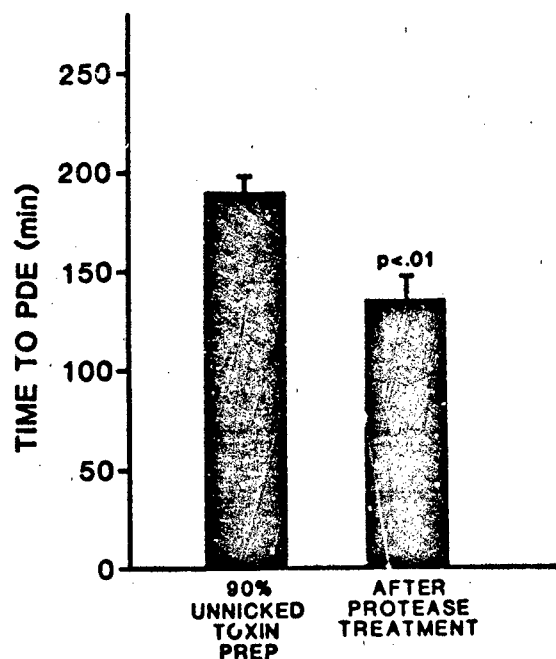

Figure 4. Treatment of the 90% labeled, unnicked toxin preparation (shown at left) with protease from a toxin-minus strain of Clostridium tetani increased the convulsant activity of the toxin in vitro. Average times to onset of PDE are shown + S.E.M. for the 90% unnicked toxin preparation and the preparation following protease treatment.

The protease treatment also increased toxicity in whole animal studies. An approximate 3-fold increase in toxicity was observed with nicking of the 90% unnicked preparation. The protease treatment had no effect on toxicity of already nicked toxin.

Monoclonal Antibody Studies

Of the available monoclonal antibodies directed against various epitopes on the toxin molecule, four were selected for study (Figure 5). The antibodies were assayed with regard to their ability to block binding of labeled toxin. One of the monoclonal antibodies directed against the C fragment (18.1.7) was effective in preventing toxin binding. Only 1.5% of counts were bound (versus 16% in control cultures). The other antibody directed against the C fragment (18.2.12.6) did not reduce the amount of toxin bound. One of the antibodies directed against an epitope on the B-fragment (21.76.10) produced a small reduction (25% compared to control) of toxin binding; the other antibody directed against a B-fragment epitope did not affect binding.

The ability of the various monoclonal antibodies to inhibit the convulsant activity (i.e. PDE) produced by tetanus toxin was assayed following preincubation of the respective antibodies with 100 ng/ml of toxin. Intracellular recordings from spinal cord neurons were

INTACT TOXIN
180,000 MW

LIGHT CHAIN S ———— S   HEAVY CHAIN

PAPAIN

FRAGMENT B
98,000 MW

FRAGMENT C
47,000 MW

S ———— S

21.18.1   21.76.10   18.1.7
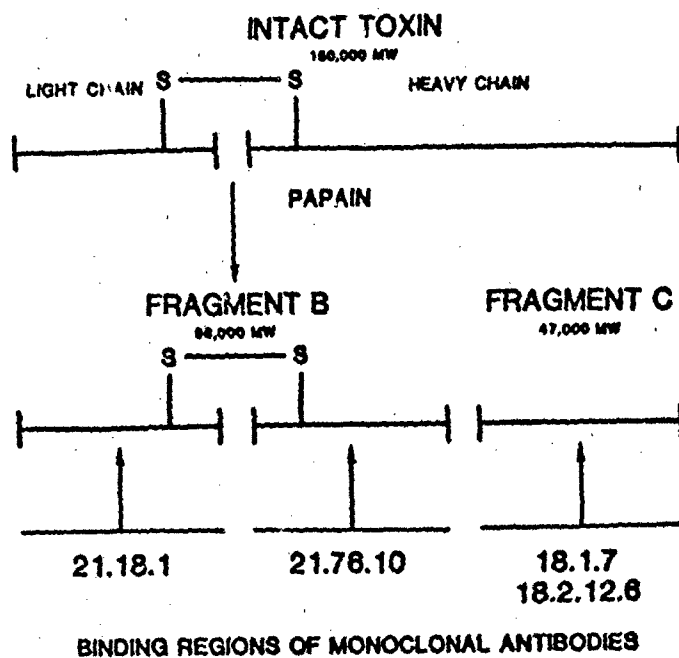18.2.12.6

BINDING REGIONS OF MONOCLONAL ANTIBODIES

Figure 5. The binding domains of the four monoclonal antibodies used for experiments are shown, as determined previously (Kenimer et al., 1983) by ELISA reactivity.

TOXIN ALONE

TOXIN + POLYCLONAL AB   NEUTRALIZING

TOXIN + 18.2.12.6 (ANTI-C)   NONNEUTRALIZING

TOXIN + 18.1.7 (ANTI-C)   NEUTRALIZING

TOXIN + 21.18.1 (ANTI-B)   NONNEUTRALIZING

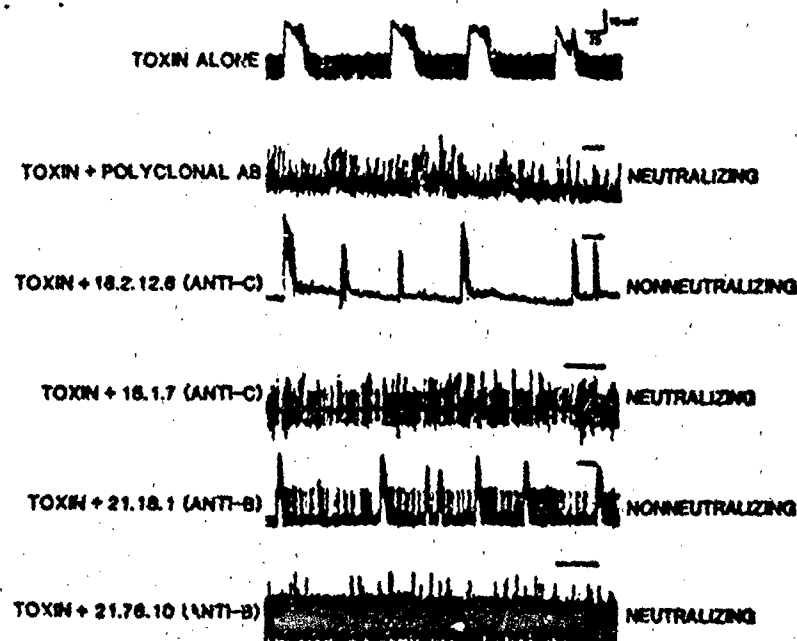TOXIN + 21.76.10 (ANTI-B)   NEUTRALIZING

Figure 6. Representative chart records from spinal cord neurons 1 1/2 to 3 hours after exposure to 100 ng/ml of tetanus toxin preincubated with selected antibodies. Neutralization is the ability of the antibody to prevent the development of the convulsant activity produced by tetanus toxin as manifest by the prominent, often rhythmic paroxysmal depolarizing events (PDE).

were made during the latent period after addition of toxin and for up to 3 hours after toxin addition (Figure 6). Polyclonal antibody and tetanus antitoxin (not shown) produced effective neutralization of the action of the toxin, that is no convulsant action resulted. The monoclonal antibody directed against fragment C that blocked toxin binding (18.1.7) also neutralized the action of tetanus toxin. The other monoclonal antibody directed against the C fragment did not prevent the production of tetanus-PDE (this antibody did not prevent toxin binding). One of the monoclonal antibodies directed against the B fragment (21.76.10) was neutralizing; the other (21.18.1) was not.

Fragment C-Antibody Conjugates

Conjugates of Fragment C and the neutralizing antibody 21.76.10 (Figure 7) were tested to determine whether they could block or reverse the actions of tetanus toxin.

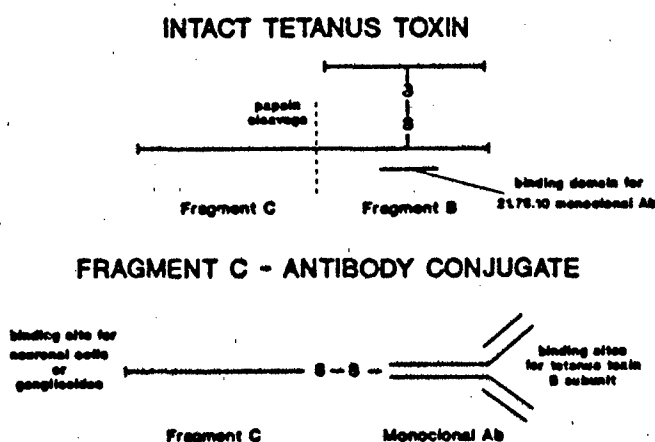INTACT TETANUS TOXIN

FRAGMENT C - ANTIBODY CONJUGATE

Figure 7. The native intact tetanus toxin is a two chain structure of approximately 150,000 molecular weight, comprised of a light chain (approx. 47,000 daltons) and a heavy chain (approx. 96,000 daltons). Papain cleavage can separate the toxin into a fragment C portion of the heavy chain that can bind to neurons but is nontoxic. The neutralizing monoclonal antibody 21.76.10 binds to the nonbinding region of the heavy chain of fragment B. The fragment C-antibody conjugate incorporates the monoclonal antibody and the nontoxic binding fragment C subunit.

The conjugates were demonstrated to bind to ganglioside coated plates. The conjugates themselves produced no change in neuronal activity. Preincubation of spinal cord neurons with the conjugates protected the neurons from subsequently added tetanus toxin (Figure 8). Experimental paradigms were designed to allow the conjugates to move from the cell surface into the neurons (Figure 8B) and to bind any antibody that may have remained exposed (Figure 8C). In each instance the conjugate prevented the development of PDE. When added after tetanus toxin (even as little as 10 pg/ml) the conjugate was not able to reverse the effects of toxin even after 6 days.

Specificity of Tetanus Toxin

Attempts were made to try and determine whether indeed tetanus toxin was specific for glycinergic synapses. First experiments were made to determine whether there appeared
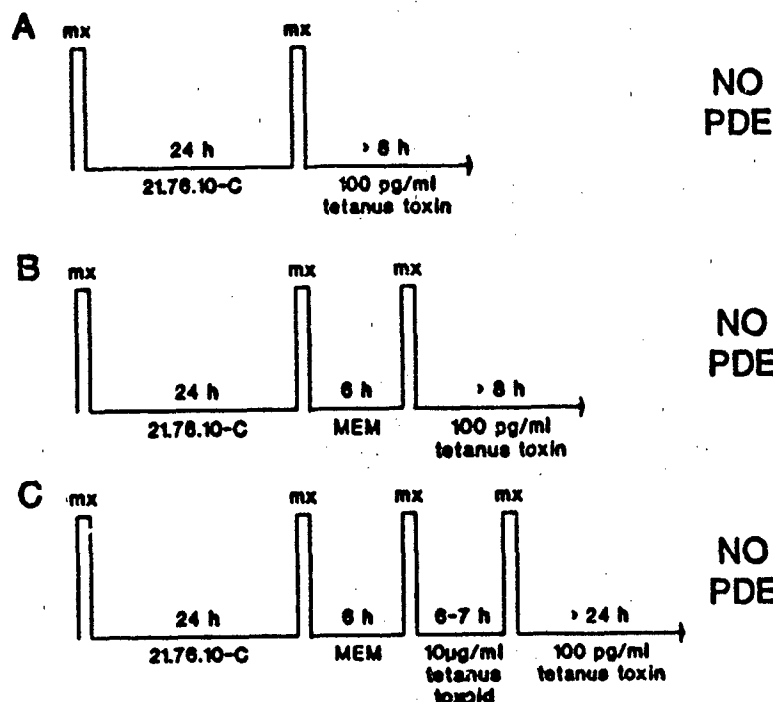
Figure 8. Preincubation of spinal cord neurons with the antibody-fragment C conjugate protected against subsequent effects of tetanus toxin. In A the toxin was added after the cells were washed (mx) in MEM X 2, in B a 6 hour incubation in MEM separated the preincubation with conjugate and the addition of toxin and in C a 6 hour incubation in MEM was followed by incubation with excess tetanus toxoid prior to washing and addition of tetanus toxin. In each instance the preincubation protected against the development of convulsant activity (PDE) produced by tetanus toxin.

to be two types of IPSPs in the spinal cord cultures. The hope was that long IPSPs might be the least affected by tetanus toxin and be GABAergic. However measurements of the t 1/2 of IPSPs (n = 32) did not reveal two distinct populations of IPSPs under control situations. The IPSPs seen after the onset of PDE (i.e. Figure 1), presumably the more resistant IPSPs, were not longer than control PSPs. Therefore no conclusions could be made regarding specificity of tetanus for any specific inhibitory transmitter. Cultures of hippocampal neurons were grown with the hope that these culture, which have predominantly GABAergic inhibition, could be assayed for time of onset of PDE and compared with the spinal cord neurons which have both glycinergic and GABAergic inhibition. The hippocampal culture, however, showed few IPSPs and frequently had PDE under control situations, making it difficult to assay for action of tetanus toxin.

Detecti of Spontaneous and Evoked Potentials

Ap_ ution of the computer based program for continuous detection of synaptic potentials was made after considerable modification. The program revealed the ability to reliably continuously sample spontaneous PSPs above 2 mV in amplitude and to generate both scatterplots (Figure 9) and amplitude histograms (not shown). Such analyses revealed the preferential effects on inhibitory transmission; while clearly demonstrating that excitatory transmission was also blocked by tetanus toxin (Figure 10). Observations on over 30 neuronal cell pairs confirmed the effects of tetanus toxin on reducing and ultimately blocking detectable excitatory transmission.
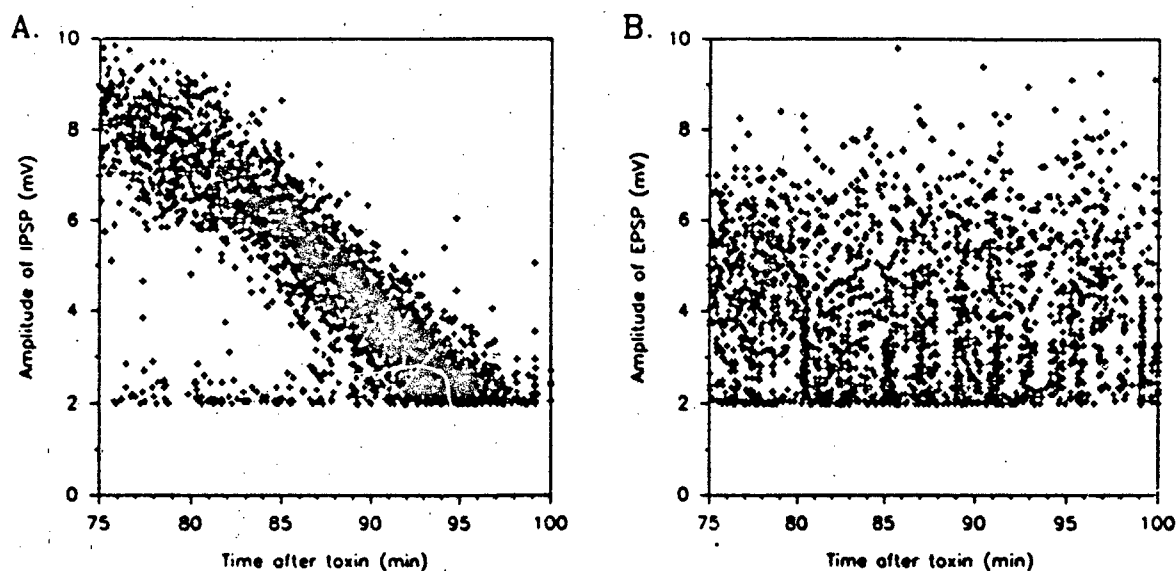
Figure 9. Scatterplots of continuously detected PSPs detected over a twenty-five minute period beginning 75 minutes after the addition of tetanus toxin at 28 C. Paroxysmal depolarizing events began to evolve at about 85-95 minutes after toxin addition. Amplitudes of IPSPs were dramatically reduced (A) while EPSP amplitudes were relatively unaffected. 3570 IPSPs were detected and plotted; 2364 EPSPs were detected and plotted.
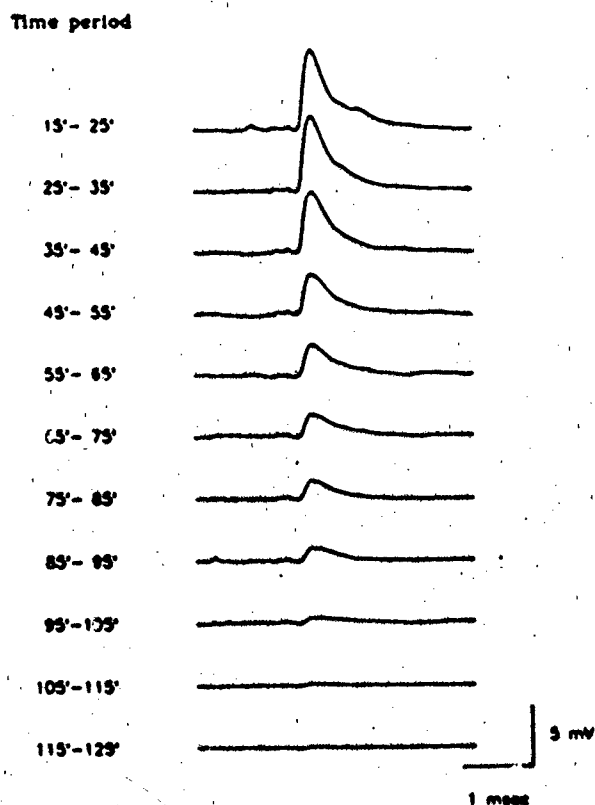

Figure 10. Tetanus toxin blocks evoked excitatory synapses. The EPSP from a stable monosynaptic cell pair is shown at various time windows after exposure to 1 mcg/ml tetanus toxin at 32 C. Each response is the average response for all evoked PSPs (stimulation frequency 1 Hz) in each 10 minute window.

Effects of Potential Antagonists of Tetanus Toxin Activity

Experiments with methylamine HCl (10 mM) produced no* changes in the average time to onset of tetanus-PDE (54.8 + 18.4 min [sd] control and 61 + 17.8 min with methylamine). Chloroquine was nonspecifically toxic to the neurons even after several hours. Ammonium chloride (5-10 mM) produced PDE itself making assay of tetanus -PDE impossible. Concanavalin A blocked spontaneous synaptic activity and subsequent convulsant activity could not be produced by tetanus toxin.

The experiments with 8-bromo cGMP did not reveal the ability to reverse the action of tetanus toxin (42 cell; 32 potential cell pairs) after 24 hours. To assay for smaller changes monosynaptically connected neurons were monitored following the addition of toxin. At the time when PSP amplitude began to be reduced, cGMP did not reverse the progressive diminution of PSP amplitude.

DISCUSSION

The experiments performed demonstrate the utility of the in vitro system of dissociated spinal cord neurons for investigations of action of tetanus toxin. The studies utilizing an enriched unnicked toxin preparation reveal that nicking of toxin is necessary for maximal toxin activity. Tetanus toxin therefore shares this enhancement with other bacterial toxins such as diphtheria, cholera, pseudomonas, Shigella and botulinum toxins (Drazin et al., 1971; Gill, 1976; Olsnes et al., 1981; Simpson, 1981; Vasil et al., 1977). Whether the single chain unnicked form is nontoxic or merely less toxic is not clear. Because of the inability to prepare a pure unnicked preparation this question remains unanswered. The fact that the dose response curve for the unnicked preparation was shift over by one log unit indicates that all of the activity observed could be explained by the 10% nicked toxin present in the preparation. Therefore the possibility that the unnicked single chain form is nontoxic is a distinct possibility.

The experiments with monoclonal antibodies indicate that toxin activity can be blocked by antibodies that interfere with binding of the toxin to neurons, as would be expected. More importantly, an antibody directed against the heavy chain portion of the B fragment was able to neutralize activity. This data suggests that the B subunit is the toxic subunit. The toxicity may reside on the heavy chain portion. Alternatively the antibody could be interfering with light chain function. The ability of this antibody (21.76.10, anti-B) conjugated with nontoxic fragment C provides further evidence. Both the antibody and the conjugate can now be used as probes for toxin activity. Using labelled whole toxin, comparisons can be made between the localization of toxin alone, toxin antibody complexes, and toxin applied to cells preincubated with the fragment C complex. Correlating the physiology of the cells with the ultrastructural localization of the toxin may provide important insights into the site of action of the toxin.

Unfortunately no delineation of specificity of toxin for specific inhibitory synapses can yet be made. We were not able to delineate GABAergic and glycinergic synapses in this culture system. The specificity of tetanus toxin for certain synapses (i.e. inhibitory) is due to different sensitivities of release mechanisms. Alternatively, sensitivity could reflect more bind sites for tetanus on the presynaptic terminals.

In our hands methylamine hydrochloride did not retard the action of tetanus toxin on the spinal cord neurons. Other lysosomotropic agents were either toxic (chloroquine) or produced paroxysmal activity (ammonium chloride). No evidence at this time exists in this system to implicate lysosomal processes. In addition 8-bromo cGMP did not reverse the action of the toxin.

The program for continuous detection of spontaneous synaptic potentials provided the best demonstrations to date of the relative sensitivity of inhibitory synapses to tetanus toxin by allowing examination of spontaneous synaptic events. Evoked potentials can also be examined with this program. This program will be an invaluable tool in the examination of reversal or recovery from toxin actions since it will allow quantification of spontaneous events at a time when connected cell pairs may be difficult to find.

REFERENCES

Bergey G.K., Nelson P.G., Macdcnald R.L., Habig W.H. (1981) Tetanus ioxin produces blockade of synaptic transmission in mouse spinal cord neurons in culture. Soc. Neuroscie. Abstr. 7,439.

Berge; G. K., Macdonald R. L., Habig W. H., Hardegree M. C., and Nelson P. G. (1983) Tetanus toxin: convulsant action on mouse spinal cord neurons in culture. J. Neurosci. 3, 2310-2323.

Bergey G. K., Bigalke H., and Nelson P. G. (1987) Diffnrential effects of tetanus toxin on inhibitory and excitatory synaptic transmission in mammaïian spinal cord neurons in culture: a presynaptic locus of action for tetanus toxin. J. Neurophysiol. 57, 121-131.

Bolton A. E. and Hunter W. M. (1973) The labelling of proteins to high specific activities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 133, 529-539.

Critchley D. R., Nelson P. G., Habig W. H., and Fishman P. H. (1985) Fate of tetanus toxin bound to the surface of primary neurons in culture: evidence for rapid internalization. J. Cell Biol. 100, 1499-1507.

Draper R.K. and Simon M.I. (1980) The entry of diptheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. J. Cell Biol. 87, 849-854.

Drazin R., Kandel J., and Collier R. J. (1971) Struture and activity of diptheria toxin. II. Attack by trypsin at a specific site within the intact toxin molecule. J. Biol. Chem. 246, 1504-1510.

Eisel U., Jarausch W., Goretski K., Henschen A., Engels J., Weller U., Hudel M., Habermann E., and Niemann H. (1986) Tetanus toxin: primary structure, expression in E. coli, and homology with botulinum toxins. EMBO J. 5, 2495-2502.

Finn C. W., Silver R. P., Habig W. H., Hardegree M. C., Zon G., and Garon C. F. (1984) The structural gene for tetanus neurotoxin is on a plasmid. Science 224, 881-884.

Gill D. M. (1976) The arrangment of subunits in cholera toxin. Biochem. 15, 1242-1248.

Habig W. H., Bigalke H., Bergey G. K., Neale E. A., Hardegree M. C., and Nelson P. G. (1986) Tetanus toxin in dissociated spinal cord cultures: long-term characterization of form and action. J. Neurochem. 47, 930-937.

Helting T. B. and Zwisler O. (1977) Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. J. Biol. Chem. 252, 187-193.

Kenimer J.G., Habig W.H., and Hardegree M.C. (1983) Monoclonal antibodies as probes of tetanus toxin structure and function. Infect. Immun. **42**, 942-948.

Laird W. J., Aaronson W., Silver R. P., Habig W. H., Hardegree M. C. (1980) Plasmid-associated toxigenicity in Clostridium tetani. J. Inf. Dis. **142**, 623

Ledley F.D., Lee G., Kohn L.D., Habig W.H., Hardegree M.C. (1977) Tetanus toxin interactions with thyroid plasma membranes: implications for structure and function of tetanus toxin receptors and potential pathophysiological significance. J. Biol. Chem. **252**, 4049-4055.

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951) Protein determination with the Folin phenol reagent. J. Biol. Chem. **193**, 265-275.

Matsuda M. and Yoneda M. (1974) Dissociation of tetanus neurotoxin into two polypeptide fragments. Biochem. Biophys. Res. Comm. **57**, 1257-1262.

Matsuda M. and Yoneda M. (1975) Isolation and purification of two antigenically active "complementary" polypeptide fragments of tetanus neurotoxin. Infect. Immun. **12**, 1147-1153.

Matsuda M. and Yoneda M. (1977) Antigenic substructure of tetanus neurotoxin. Biochem. Biophys. Res. Comm. **77**, 268-274.

Middlebrook J. L. and Dorland R. B. (1984) Bacterial toxins: cellular mechanisms of action. Microbiol Rev. **48**, 199-221.

Olsnes S., Reisbig R., and Eiklid K. (1981) Subunit structure of Shigella cytotoxin. J. Biol. Chem. **256**, 8732-8738.

Penner R., Neher E., and Dreyer F. (1986) Intracellular injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. Nature **324**, 76-78.

Price D. L., Griffin J. W., Young, A., Peck K., and Stocks A. (1975) Tetanus toxin: direct evidence for retrograde intraaxonal transport. Science **188**, 945-947.

Price D.L., Griffin J.W., and Peck K. (1977) Tetanus toxin: Evidence for binding at presynatpic nerve endings. Brain Res. **121**, 379-384.

Ransom B. R., Neale E. A., Henkert M., Bullock P. N., and Nelson P. G. (1977) Mouse spinal cord in cell culture. I. Morphology and intrinsic neuronal electrophysiologic properties. J. Neurophysiol. **40**, 1132-1150.

Raynaud M. (1951) Extraction de la toxine tetanique a partir des corps microbiens. Ann. de l'Inst. Pasteur **80**, 356-377.

Robinson J. P. and Hash J. H. (1982) A review of the molecular structure of tetanus toxin. Mol. Cell. Biochem. 48, 33-44.

Romijn H. J., Habets A. M. M. C., Mud M. T., and Wolters P. S. (1982) Nerve outgrowth, synaptogenesis and bioelectric activity in fetal rat cerebral cortex tissue cultured in serum-free, chemically defined medium. Dev. Brain Res. 2, 583-589.

Sandvig K. and Olsnes S. (1980) Diptheria toxin entry into cells is facilitated by low pH. J. Cell. Biol. 87, 828-832.

Schmitt A., Dreyer F., and John C. (1981) At least three sequential steps are involved in the tetanus toxin-induced block of neuromuscular transmission. Nauyn-Schmiedeberg's Arch. Pharmacol. 317, 326-330.

Schwab M. E., Suda K., and Thonen H. (1979) Selective retrograde trans-synaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. J. Cell Biol. 82, 798-810.

Schwab M. E., Suda K., and Thonen H. (1979) Selective retrograde trans-synaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. J. Cell Biol. 82, 798-810.

Simpson L. (1981) The origin, structure and pharmacologic activity of botulinum toxin. Pharmacol. Rev. 33, 155-188.

Simpson L.L. (1983) Ammonium chloride and methylamine hydrochloride antagonize clostridial neurotoxins. J. Pharmacol. Exp. Ther. 225. 546-552.

Vasil M. L., Kabat D., and Iglewski B. H. (1977) Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16, 353-361.

PUBLICATIONS SUPPORTED BY CONTRACT

Bergey GK, Habig WH, Lin C, Bennett JI: Nicking of tetanus toxin increases activity. J Neurochem 53:15.-161, 1989.

Bergey GK, Halpern JL, Franaszczuk P, Habig WH: A novel fragment C immunotoxin conjugate prevents the action of tetanus toxin on spinal cord neurons in culture. Neurology 40 (Suppl 1):211, 1990.

Bergey GK, Franaszczuk PJ: Spontaneous postsynaptic potentials in cultured spinal cord neurons during development of early convulsant activity produced by tetanus toxin. In press, Soc. Neurosci Abstr., 1991.

Franaszczuk PJ, Bergey GK: Detection of spontaneous synaptic potentials associated with paroxysmal depolarizing events produced by tetanus toxin in cultured neurons. Presented at the Focus on Epilepsy Symposium, Montreal, Que., August, 1991.

Franaszczuk PJ, Bergey GK: Patterns of spontaneous postsynaptic potentials during organized convulsant activity produced by tetanus toxin in cultured mouse spinal cord neurons. To be presented at 1991 American Epilepsy Society Meeting.

In Preparation:

Bergey GK and Franaszczuk PJ: Spontaneous postsynaptic potentials during the evolution of convulsant activity produced by tetanus toxin in mouse spinal cord neurons. In preparation.

Bergey GK, Nelson PG, Macdonald RL, Habig WH, Neale EA, Franaszczuk PJ: Tetanus toxin produces blockade of synaptic transmission in spinal cord cultures. In preparation.

Bergey GK, Franaszczuk PJ, Fishman PF, Bigalke H,: Blockade of excitatory synaptic transmission by tetanus toxin: an explanation for the presentation of cephalic tetanus. In preparation.

PERSONNEL RECEIVING CONTRACT SUPPORT

Yvonne Logan, B.S., Laboratory Scientist II, 1986-1989

Jeffrey I. Bennett, M.D., Pcstdoctoral Fellow, 1986-87

Piotr Franaszczuk, Ph.D., Postdoctoral Fellow, 1989
> Note: Dr. Franaszczuk received no salary support from contract, but was primarily involved in execution of contract research while supported by other (i.e. departmental) funding sources.

PUBLICATIONS SUPPORTED BY CONTRACT

Bergey BK, Habig WH, Lin C, Bennett JI: Nicking of tetanus toxin increases activity.
    J Neurochem 53:155-161, 1989.

Bergey GK, Halpern JL, Franaszczuk P, Habig WH: A novel fragment C immunotoxin conjugate prevents
    the action of tetanus toxin on spinal cord neurons in culture. Neurology 40 (Suppl 1):211,
    1990.

Bergey GK, Franaszczuk PJ: Spontaneous postsynaptic potentials in cultured spinal cord neurons during
    development of early convulsant activity produced by tetanus toxin. In press, Soc. Neurosci
    Abstr., 1991.

Franaszczuk PJ, Bergey GK: Detection of spontaneous synaptic potentials associated with paroxysmal
    depolarizing events produced by tetanus toxin in cultured neurons. Presented at the Focus on
    Epilepsy Symposium, Montreal, Que., August, 1991.

Franaszczuk PJ, Bergey GK: Patterns of spontaneous postsynaptic potentials
    during organized convulsant activity produced by tetanus toxin in cultured mouse spinal cord
    neurons. To be presented at 1991 American Epilepsy Society Meeting.

END

FILMED

DATE:

1-92

DTIC