AD_____

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

ANNUAL REPORT

GREGORY K. BERGEY

SEPTEMBER 26, 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

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	26 September 1991	Annual Report 1 Jan 89 - 31 Dec 89

4. TITLE AND SUBTITLE

Mechanisms of Action of Clostridial Neurotoxins on Dissociated Mouse Spinal Core Neurons in Cell Culture

5. FUNDING NUMBERS

DAMD17-86-C-6056

62787A
3M162787A871 AA
DA309151

6. AUTHOR(S)

Gregory K. Bergey

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

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.

TABLE OF CONTENTS

INTRODUCTION

This period of contract time represents a no cost extension of the original three year contract. During this time period the research effort was enhanced by the collaboration of Dr. Piotr Franaszczuk, the Jeffrey I. Bennett Fellow in Neurophysiology who worked full time on the various projects described, while receiving this external support. Because of Dr. Franaszczuk's expertise in computer systems and modeling we devoted much of our time to application of his program for post-synaptic potential detection to the system of action of tetanus toxin. Much of the early portion of the research period was hampered by the limitations of our microcomputer (a 80286 based CPU); the later addition of a 25 Mhz 80386 unit greatly facilitated data analysis.

Experiments with tetanus toxin in the dissociated spinal cord neuronal system have thus far revealed that the toxin binds to the neurons and after a dose-dependent latent period produces convulsant activity manifest by paroxysmal depolarizing events (PDE), abrupt depolarizing shifts (typically 5-20 mV) of membrane potential associated with triggered action potentials lasting 200-2000mS. This convulsant period is produced by the reduction and ultimate block of synaptic inhibition by the toxin. Tetanus toxin has been demonstrated to block the release of neurotransmitter at a presynaptic locus (Bergey et al., 1983, 1987).

After the convulsant period produced by the toxin, the frequency of the PDE is reduced and ultimately these events disappear. This parallels the apparent disappearance of all spontaneous postsynaptic potentials, excitatory as well as inhibitory. Tetanus toxin has been demonstrated by quantal analysis to block the presynaptic release at excitatory synapses (Bergey et al, 1987). Therefore, while tetanus toxin has a preferential effect on inhibitory neurotransmission, all synaptic transmission appears to be blocked by the toxin.

To date, our observations of the effects of the toxin have been limited to observations of discrete synaptically connected cell pairs or to qualitative observations of intracellular recordings. It became apparent that if one wished to quantitatively study the effects of the toxin a means for analysis of postsynaptic potentials would be necessary. Such an analysis is particularly important in the experiments on reversal or recovery from the toxin, where changes may be manifest in changes in postsynaptic potential (PSP) patterns or amplitudes rather than the more dramatic paroxysmal depolarizing events. Previous experiments on reversal of the effects of the toxin have been hampered by the lack of the means for such analysis.

Preliminary applications of an algorithm to detect continuous spontaneous PSPs are reported here with analyses of various patterns of PSPs following the application of tetanus 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.

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 2×10^7 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.

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).

A dilution of purified tetanus toxin (400-4000 ng/ml) was added to the cultures in 500 ul 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

Figure 1 illustrates a sample of the PSP detection capabilities of the algorithm, showing a plot of relative frequency versus amplitude. Frequency versus time plots can also be generated. The changes in EPSP pattern from the control situation to that produced by tetanus toxin are shown in Figure 2. Although the neuron illustrated in the control situation (Figure 2A) has a greater frequency of EPSPs, bursting (i.e. PDE) is not present, whereas in the experimental situation, despite a lower frequency of EPSPs, the greater network synchrony results in PDE (not shown). Figure 3 illustrates samples of continuous analysis of both inhibitory and excitatory potentials as well as action potentials early in the convulsant period. The PSPs and action potentials cluster at the time of the paroxysmal depolarizing events. This clustering in fact becomes even more evident with time (not shown). The patterns of both IPSPs and EPSPs are similar suggesting that they are both triggered by the network synchrony produced by the tetanus toxin. Later IPSPs become much less apparent. Action potentials can be sampled by adjustments in threshold detection and exclusion. It is important to sample action potentials because in some instances of increased excitation, EPSP frequency could decrease if more action potentials are triggered. About 25 neurons have been sampled during the early convulsant period (operationally defined as the first 90 minutes after the addition of toxin). In addition to the clustering of PSPs that occurs at the time of each PDE (frequency approximately 3-30/min) there also appears to be a clustering

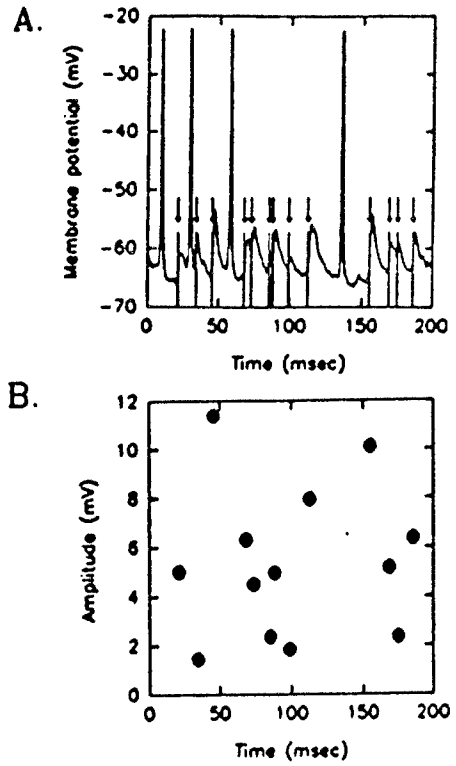

Figure 1. PSP detection. A. A sample data record from an intracellular recording from a spinal cord neuron in culture chosen to illustrate the detection of potentials. The arrows and vertical lines mark the onsets of detected EPSPs. B. The amplitudes of the potentials detected in record A can be determined and are shown. C. The relative frequencies of various amplitudes of EPSPs can be displayed. This histogram was generated from an extended 25 minute recording from the same neurons as in panels A and B. The threshold for detection was set a 0.5 mV; bins are 0.1 mV wide.

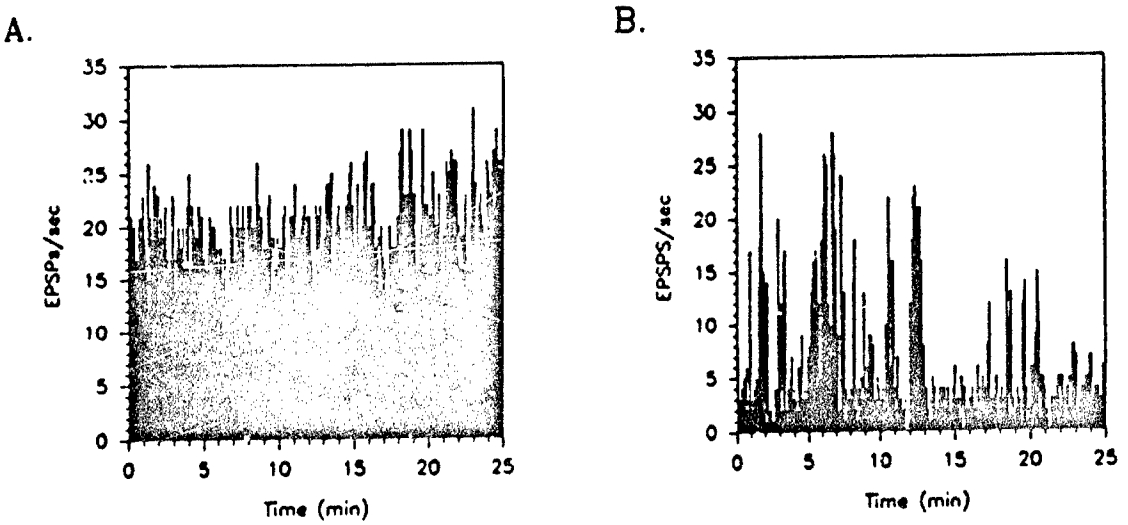

Figure 2. PSP clustering. Changes in EPSP patterns and frequency are seen. A recording from a spinal cord neuron under control conditions often reveals frequent EPSPs with little clustering (A). A recording from a neuron 90 minutes after the addition of tetanus toxin reveals generally fewer EPSPs and clustering of the remaining potentials (B). The histograms are generated from potentials greater than 1 mV in amplitude; bins are 1 second wide.

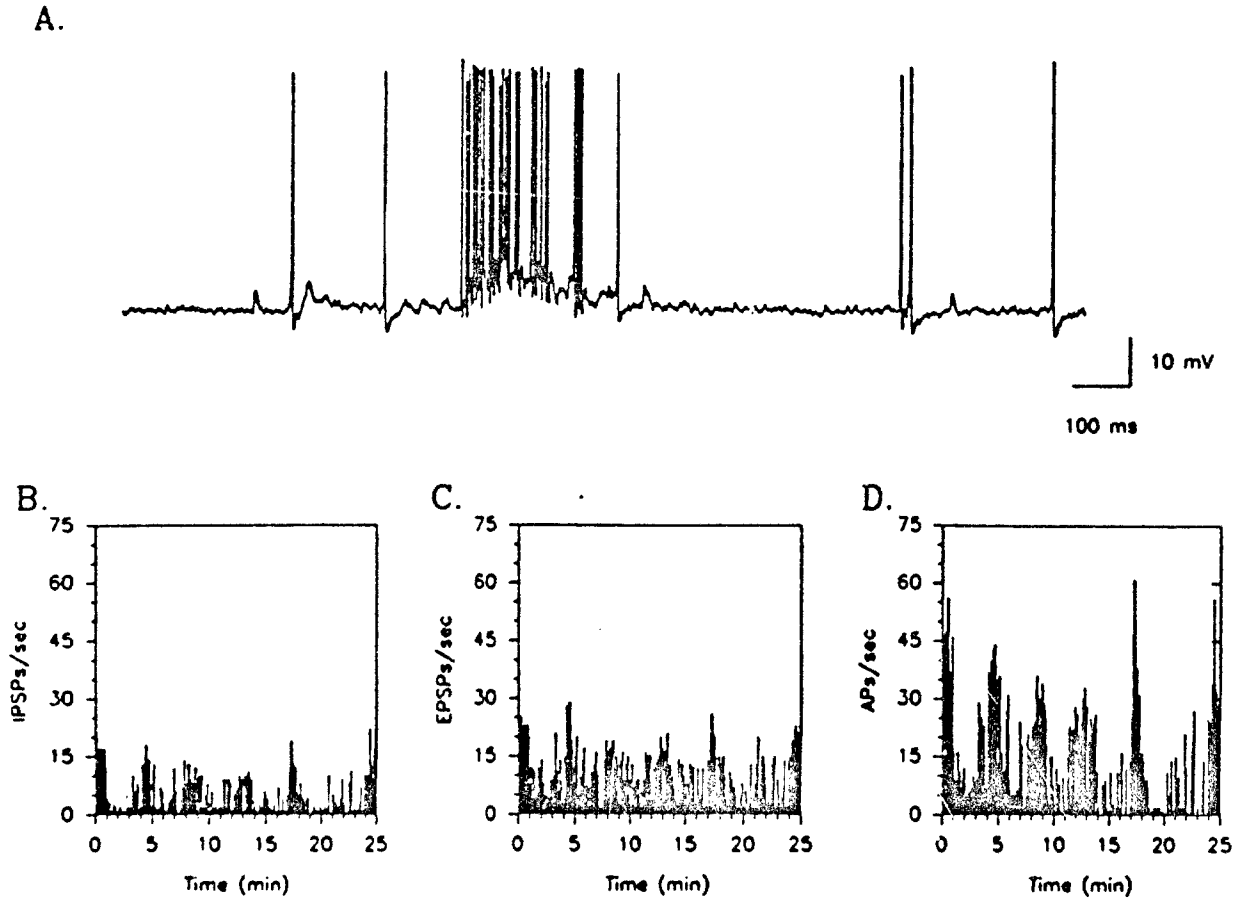

Figure 3. PSP detection during early convulsant action. A representative portion of the digitized intracellular recording from a spinal cord neuron 45 minutes after the addition of tetanus toxin: action potentials, postsynaptic potentials and a typical early paroxysmal depolarizing event (PDE) are seen (A). The detection algorithm was applied to a 25 minute period of the recording beginning 30 minutes after the addition of toxin. Frequency histograms were generated for IPSPs (B), EPSPs (C) and action potentials (APs, D) greater than 1 mV in amplitude for this interval; bins are 1 sec wide.

that has a much longer periodicity (i.e. 5-10 minutes).

When amplitudes of spontaneous IPSPs and EPSPs were compared during the early convulsant period, scatter plots of inhibitory potentials reveal that inhibition is clearly preferentially affected when compared to excitation (Figure 4). Dramatic reductions in the amplitudes of continuously sampled IPSPs are seen at a time when the amplitudes of simultaneously sampled EPSPs are not affected. The illustrated analysis is during the early

convulsant period when PDE are just beginning to be seen. This occurs somewhat later in the recording shown because the stage temperature was 28 C. and the action of tetanus toxin is temperature dependent (Schmitt et al., 1981).

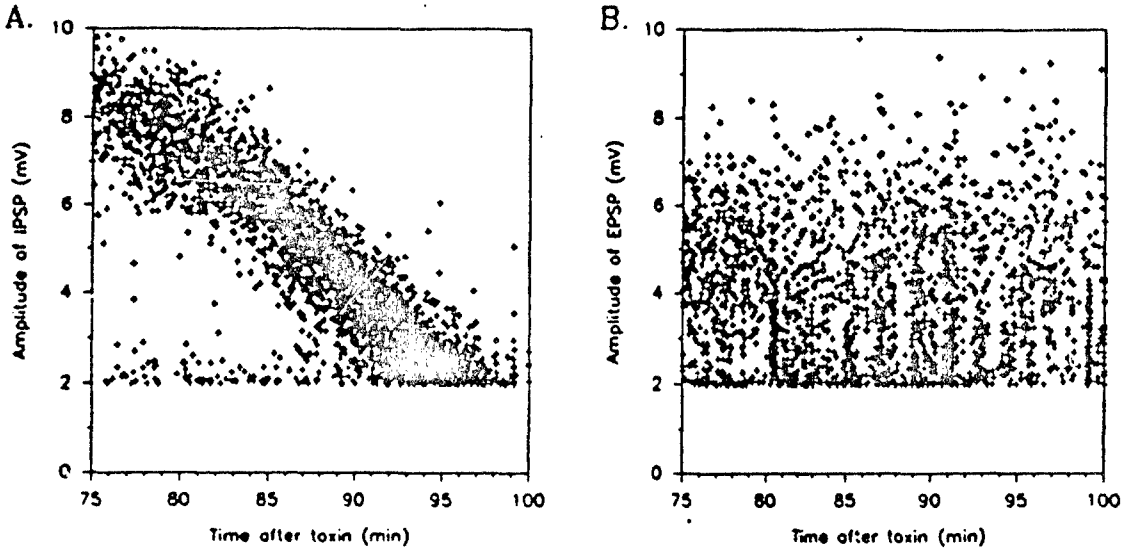

Figure 4. Scatterplots of continuously detected PSPs detected over a twenty-five minute period beginning 75 minutes after the addition of tetanus toxin at 28 C. Paroxysmal depolarizing events began to evolve at about 85-95 minutes after toxin addition. Amplitudes of IPSPs were dramatically reduced (A) while EPSP amplitudes were relatively unaffected. 3570 IPSPs were detected and plotted; 2364 EPSPs were detected and plotted.

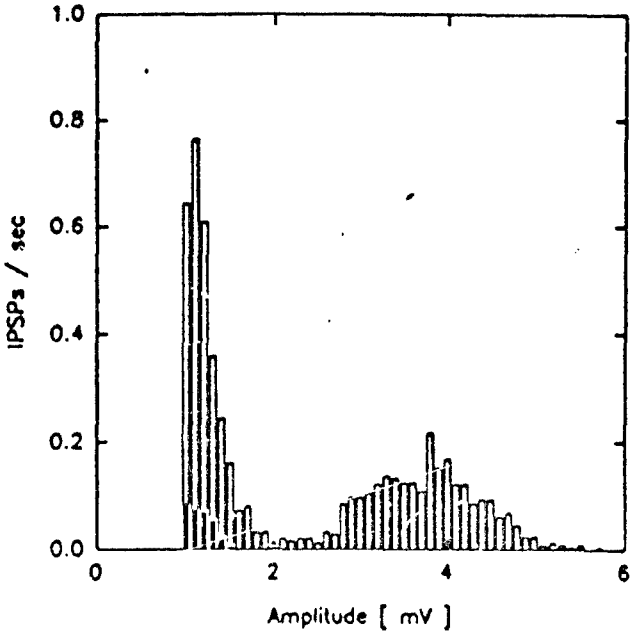

Figure 5. Frequency versus amplitude plot of spontaneous IPSPs. The open bars are from a 25 minute recording from a control neuron. The closed bars are from a neuron exposed to tetanus toxin (1 mcg/ml) for eight hours. Review of the low amplitude events detected as IPSPs suggests that these were membrane oscillations and not true IPSPs.

Most IPSPs are reduced in amplitude early after the addition of tetanus toxin. At 32-33 C. it unusual to find identifiable IPSPs after 60-70 minutes of exposure (at 1 mcg/ml). Figure 5 illustrates the absence of IPSPs late (> 8 hours) after toxin exposure. In contrast to IPSPs, EPSPs are much less affected early (Figure 4B), but after additional time excitatory connections are also affected. Figure 6 illustrates the averaged EPSP from a monosynaptic excitatory connection after exposure to tetanus toxin. Some reduction begins early, after 35-45 minutes; after 2 hours the PSP was not detectable. Figure 7 illustrates the spontaneous EPSPs recorded from the postsynaptic neuron, subtracting out the evoked response. The amplitudes of the spontaneous PSPs also dramatically diminish. The intrinsic excitability (i.e. action potential threshold, amplitude) of the pre and postsynaptic neurons did not change significantly over this time period; resting membrane potential was also not affected. The results from this prolonged recording, an unusually stable monosynaptic pair, are similar to the observations from > 30 shorter records in single cells and cell pairs. Some EPSPs appear to be less affected that the illustrated pair, with reductions in amplitude beginning later.

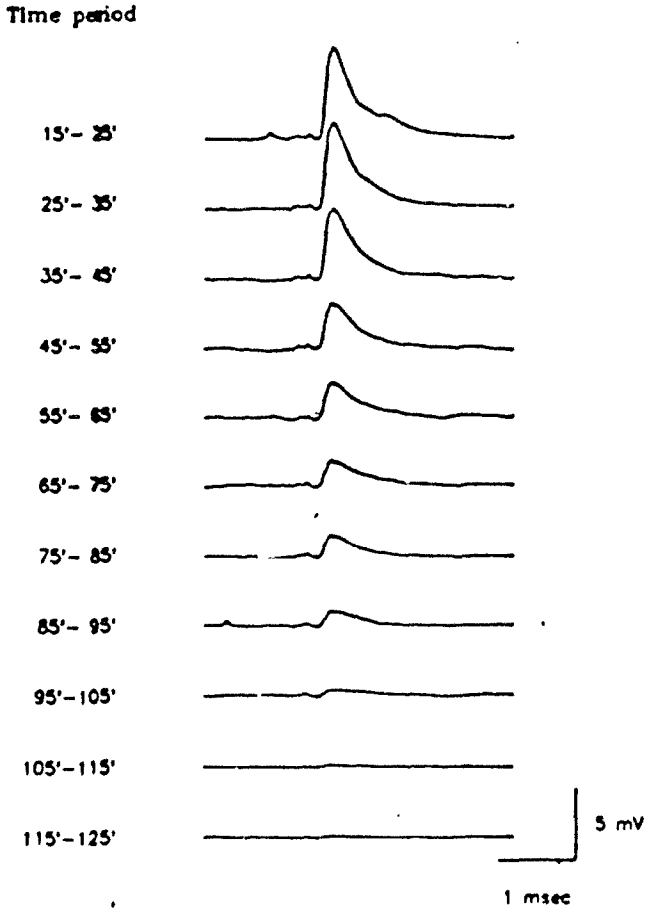

Figure 6. Tetanus toxin blocks evoked excitatory synapses. The EPSP from a stable monosynaptic cell pair is shown at various time windows after exposure to 1 mcg/ml tetanus toxin at 32 C. Each response is the average response for all evoked PSPs (stimulation frequency 1 Hz) in each 10 minute window.

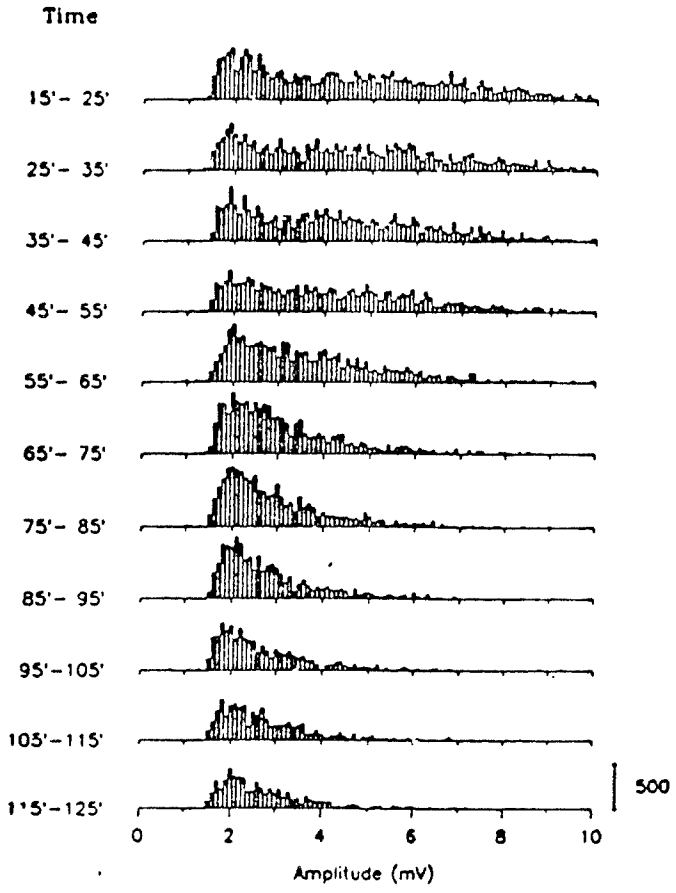

Figure 7. Spontaneous EPSPs recorded from the postsynaptic neuron in Figure 6. All EPSPs recorded during each ten minute epoch. The evoked EPSPs were selected out. A progressive reduction in the amplitudes of the spontaneous EPSPs is seen that mirrors the changes seen with evoked EPSPs.

Amplitude histograms of spontaneous EPSPs

DISCUSSION

Tetanus toxin has been demonstrated to block and reduce both inhibitory and excitatory synaptic transmission. While inhibition is affected first, excitation is also blocked at the concentrations of toxin used here. At much lower concentrations (1-10 pg/ml) excitation does not appear to be blocked (at least in several days). These qualitative differences have been previously noted (Bergey et al., 1983, 1987) but in order to quantitatively assess effects of toxin and recovery new methods needed to be developed. As described above application of a continuous PSPs detection program provides such quantification. This allows for both analysis of evoked and spontaneous postsynaptic

potentials (although the program is better suited for the latter). In the longer experiments it is not always possible to maintain stable recordings from a given cell pair. The ability to assess spontaneous PSPs from a single cell is therefore a definite asset. Obviously the spontaneous potentials reflect all synaptic inputs, and as such may be from multiple neurons. One can separate out different populations when present (not shown); most neurons appear to have one or two excitatory or inhibitory inputs.

Utilization of this program has allowed for quantitative confirmation of the blockade of inhibitory and excitatory synaptic transmission by tetanus toxin. The reason for the relative sensitivity of inhibitory transmission is not known. It could reflect more receptors for the toxin on the presynaptic bouton or a greater sensitivity of the transmitter release mechanisms.

Interestingly an especially severe form of tetanus, cephalic tetanus exists that follows severe wounds to the face and neck (Dastur et al., 1977, Vieira et al., 1986). This type presents with paralysis of the face or (less commonly) the eyes. Since neuromuscular transmission can be preserved there has been controversy as to how tetanus could produce this picture. The ability to block excitatory synapses centrally provides an answer.

The implementation of this PSP program for use with the tetanus model has been a long process (in part because of the specialized nature of the program and the system). We now, however, have a way to quantitatively assess recovery from toxin. The half-life of the toxin has been determined (Habig et al., 1986), recovery from lower doses of toxin should begin in days to weeks if toxin degradation is an important mode of recovery (versus sprouting). This program will allow us to determine whether recovery is gradual (i.e. small PSPs in many neurons) or more abrupt (larger PSPs). Indeed all perturbations that affect toxin action can now be more exactly expressed and examined.

REFERENCES

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

Bergey 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., Nelson P.G., Macdonald R.L., Habig W.H. (1981) Tetanus toxin produces blockade of synaptic transmission in mouse spinal cord neurons in culture. Soc. Neurosci. Abstr. **7**,439.

Dastur F.D., Shabani M.T., Dastur, D.H., Kohiyar, F.N., Bharucha, E.P., Mondkar, V.P., Kashyap, G.H., and Nair K.G. (1977) Cephalic tetanus: Demonstration of a dual lesion. J. Neurol. Neurosurg. Psychiatry. **40**, 782-786.

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

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.

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.

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.

Schmitt A., Dreyer F., 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.

Vieira B.I., Dunne J.W., and Summers, Q. (1986) Cephalic tetanus in an immunized patient. Clinical and electromyographic findings. Med. J. Aust. **145**, 156-157.