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MECHANISM OF ACTION OF THE PRESYNAPTIC NEUROTOXIN, TETANUS TOXIN

ANNUAL REPORT

Terry B. Rogers, Ph.D

April 30, 1987

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The major goal of this contract is to identify fundamental mechanisms involved in the action of the Clostridial neurotoxins, in particular tetanus toxin. During the past year we have developed a cultured cell line, PC12 cells, that are very sensitive to tetanus toxin, i.e. the toxin inhibits depolarization evoked release of acetylcholine. This model cell system has been very valuable in the characterization of a binding-internalization-function process for tetanus toxin. The toxin binds with nanomolar affinity and inhibits ACh release at doses as low as 0.1 nM. An important discovery is that we have found that analogues of cyclic guanosine monophosphate are capable of reversing the effects to tetanus toxin once they have been established in these cells. This is important for two reasons: (1) the potential for development of therapeutic agents directed against Clostridial intoxication is very real; and (2) these results suggest that there is an important role for cGMP in the underlying mechanism of action of tetanus toxin.

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on the Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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### Tetanus Toxin - Significance

Neurotoxins are invaluable tools in the study of neurotransmission (Ceccarelli & Clementi, 1979). For example, our understanding of the ACh receptor was greatly enhanced by studies using the snake venom toxin,  $\alpha$ -bungarotoxin (Lee, 1972). Tetrodotoxin and saxitoxin were instrumental in characterizing the sodium channel.

Tetanus toxin is an ideal toxin to study for four reasons. First, it is a potent inhibitor of neurotransmitter release. In fact, it is one of the most potent neurotoxins known to man (LD<sub>50</sub> in rodents is 1 ng/kg) (Wellhoner, 1982). Its potency suggests that the toxin acts selectively at specific recognition sites in the central nervous system (CNS) that are critical for neuronal function. Tetanus toxin may produce its effects via bioamplification; that is, it may act as an enzyme. In this way, it may be analogous to the well characterized cholera, pertussis and diphtheria toxins (Gill, 1976; Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Uchida, 1983).

Secondly, an unusual characteristic of this toxin is that its mechanism of action is presynaptic. Exposure of primary cultured neurons, brain slices, isolated neuromuscular preparations and synaptosomes to tetanus toxin results in inhibition of neurotransmitter release from presynaptic terminals (Collingridge et al., 1980; Bigalke et al., 1978; Pearce et al., 1983; Osborne & Bradford, 1973). Electrophysiological studies have demonstrated that tetanus decreases the spontaneous and evoked release of neurotransmitter while leaving postsynaptic membranes intact and responsive to agonists (Curtis & DeGroat, 1968; Davies & Tongroach, 1979; Bergey et al., 1983).

Third, tetanus toxin preferentially binds to neurons. Mirsky (1978) using immunofluorescence demonstrated that tetanus toxin can be used to distinguish neurons from non-neuronal cells in a wide variety of dissociated

cell cultures. These studies involving rat and chick preparations, extended the findings that  $^{125}\text{I}$ -tetanus toxin binds selectively to neurons in cultures of mouse brain and spinal cord (Dimpfel et al., 1975).

Fourth, tetanus toxin is selectively internalized and translocated into neurons. When the toxin is injected into the neuromuscular junction, it enters the CNS by first becoming internalized into synaptic terminals of motoneurons (Schwab & Thoenen, 1976). It is then translocated by a retrograde intra-axonal transport mechanism into the dorsal horn of the spinal cord (Schwab & Thoenen, 1977; Schwab et al., 1979). In the dorsal horn, it undergoes retrograde trans-synaptic transfer through at least two neurons (Habermann, et al., 1977) and, it remains intact through this process (Dumas et al., 1979a,b).

In this research project we have decided to use cultured cell lines of neuronal origin to study the mechanism of action of tetanus toxin. The rat adrenal pheochromocytoma PC12 cell line (Greene & Tischler, 1982), offers a unique model system for such studies for several reasons. First, cultured neuronal cells have several distinct advantages over intact neural tissues. In animals, the amount of toxin that will reach the ventral horn after peripheral injection is difficult to quantitate. With direct spinal injection of toxin, the amounts of radiolabeled toxin necessary to yield detectable counts are rapidly fatal (Wellhoner, 1982). Cell cultures, however, provide a system in which known concentrations of toxin can be added and assayed in the medium. The cultures can be maintained indefinitely at concentrations of toxin that would be lethal in vivo.

The second reason is that intact cell systems have advantages over other subcellular preparations. In an intact cell system, the functional effects of tetanus (blockade of neurotransmitter release) can be correlated with both

toxin binding and internalization; this cannot be done with other types of preparations. The use of intact, stable cells is particularly important in tetanus toxin studies since there is a characteristic lag phase observed for the effects of tetanus to develop.

Third, cell lines lend themselves to biochemical studies because of their abundance and homogeneity. Studies using primary cultures have restricted cell numbers, and heterogeneity is unavoidable. For example, cholera toxin's mechanism of action was greatly facilitated by studies with transformed cell lines (Joseph et al., 1979; Fishman, 1982). Fourth, PC12 cells have a highly differentiated neurotransmitter release process that is analogous to neurotransmitter release in vivo. These cells synthesize, store, and release both ACh (Schubert & Klier, 1979) and catecholamines (Greene & Tischler, 1976). Calcium-dependent neurotransmitter release is evoked by depolarization conditions (60 mM KCl; 200  $\mu$ M veratridine) and by stimulation of cholinergic receptors. These properties permit correlative biochemical and functional studies of tetanus toxin's mechanism of action in PC12 cells. The principal investigator's laboratory has discovered that another cell line, N18-RE-105, binds tetanus toxin in a manner similar to mammalian brain (Staub et al., 1986). However, the neurotransmitter system in this cell line has been difficult to determine (Malouf et al., 1985). Thus, although the N18-RE105 cells have been very valuable in the study of the binding and internalization of tetanus toxin, these cells are less useful for the study of the effects of tetanus on neurosecretion.

During the past year of this contract the principal investigator's laboratory has been very active in characterizing the effects of tetanus toxin on PC12 cells. We report evidence that trisialogangliosides are a potential high affinity receptor for tetanus toxin on these cultured cells. Further, we

have found that the toxin is rapidly internalized by these cells in a temperature and metabolic energy-dependent manner. We have found that tetanus toxin is a potent inhibitor of neurotransmitter release in this system. Finally we have documented a method for rapidly reversing the effects of intoxication once established in these cells. This is the first report of a method for reversing the intoxication of the *Clostridial* neurotoxins. This discovery provides us with several important insights into the mechanism of action of the *Clostridial* neurotoxins and suggests novel approaches for unraveling this complex story.

Results from the Principal Investigator's Laboratory  
During the Past Year

The main focus during the past year of this contract was to establish a cell line that would be a useful system to study the effects of tetanus intoxication at the molecular level. The results reported here describe our progress in this area. We report that PC12 cells bind tetanus toxin with high affinity and internalization the toxin in a physiologically relevant manner. We also report on functional studies where tetanus toxin potently inhibits neurotransmitter release from these cells. Finally we report that analogues of cGMP are effective in reversing the effects of tetanus on neurotransmitter release in these cells.

Binding and Internalization of Tetanus Toxin by PC 12 Cells

The saturability of tetanus toxin binding to PC12 cells was assessed both by quantitating the amount of specific toxin bound in the presence of increasing amounts of  $^{125}\text{I}$ -tetanus toxin (Figure 1) and by examining the reduction of a fixed amount of  $^{125}\text{I}$ -tetanus toxin bound in the presence of increasing amounts of non-radioactive tetanus toxin (Figure 2). When the data was transformed into Scatchard plots, similar values for the binding parameters were obtained using either method. There is a single population of binding sites with a dissociation constant ( $K_d$ ) of  $1.25 \pm 0.2$  nM. The maximum number of binding sites is  $56.5 \pm 4.8$  pmol/million cells ( $= 238$  pmol/mg of membrane protein). Since either method gave the same quantitative results, the other saturation binding isotherms described below were performed using the competition-binding method, which was technically more easy to perform. As shown in Figure 2, the competition binding curves obtained with PC12 membranes were similar to those from rat SPM ( $K_d = 0.83 \pm 0.07$ ;  $B_{\text{max}} = 482 \pm$

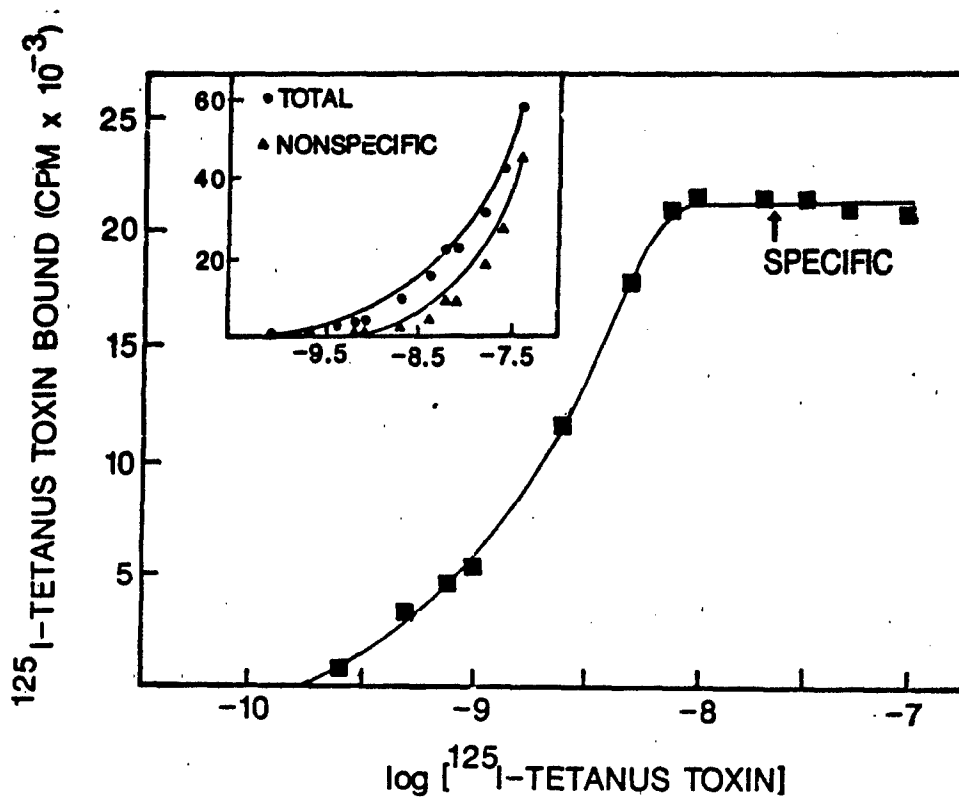


Figure 1. Binding of <sup>125</sup>I-tetanus toxin to PC12 cell membranes. PC12 microsomes (250 ng protein) from 14 day NGF treated cells were incubated with increasing amounts of <sup>125</sup>I-tetanus toxin in 0.2 ml "Binding buffer" at 0°C for 3 hours. The specific binding was quantitated and was expressed as the mean of sextuplet cultures ± s.e.m.

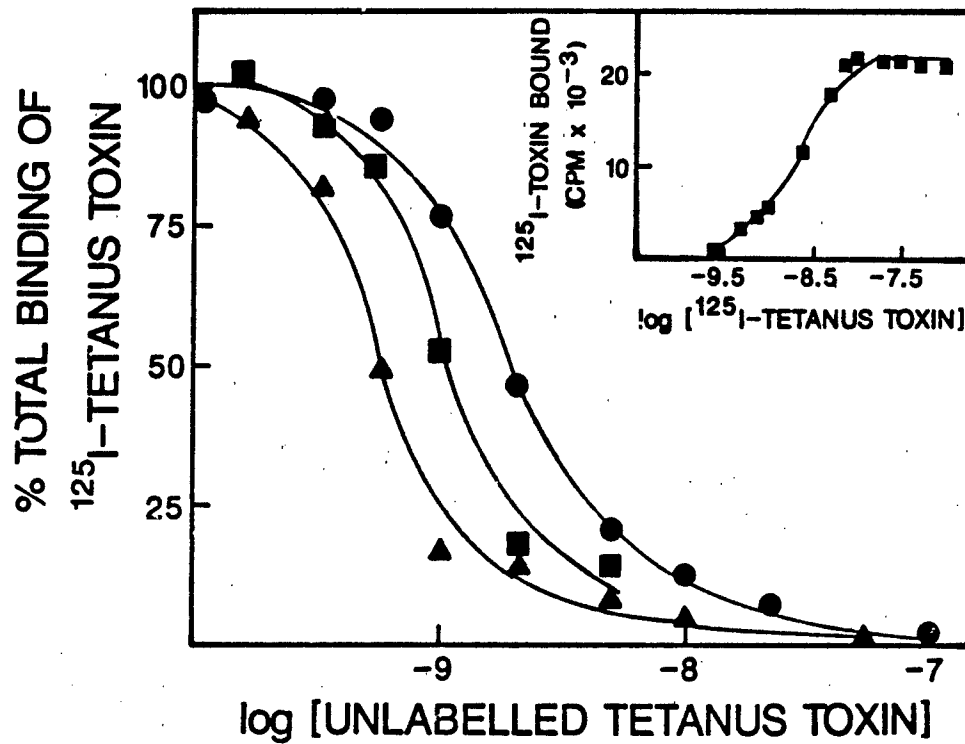


FIGURE 2. Competition of  $^{125}\text{I}$ -Tetanus Toxin Binding with Unlabelled Toxin to Rat SPM, PC12 Membranes and Intact PC12 Cells at  $0^\circ\text{C}$ . PC12 microsomes (250 ng protein) were prepared from 14 day NGF-treated cells. Intact PC12 cells (50-100  $\mu\text{g}$  cell protein/ $10\text{ cm}^2$ ) were grown in the presence of NGF for 14 days.  $^{125}\text{I}$ -Tetanus toxin (0.2 nM) was incubated with rat SPM ( $\blacktriangle$ ) or PC12 membranes ( $\blacksquare$ ) and Intact PC12 cells were incubated with  $^{125}\text{I}$ -tetanus toxin (0.5 nM) ( $\bullet$ ). All toxin incubations were for 3 h at  $0^\circ\text{C}$  in the presence of increasing amounts of unlabeled tetanus toxin.

35 pmol/mg of SPH protein). Taken together, these results provide evidence that the toxin receptor on the PC12 cells is similar to the receptor found in mammalian brain.

Previous studies suggest that the tetanus toxin receptor is composed of complex gangliosides (Rogers & Snyder, 1981; Holmgren et al., 1980). Furthermore, it has recently been demonstrated that differentiation can stimulate ganglioside expression in PC12 cells (Walton et al., 1987). This finding prompted the examination of toxin binding to PC12 membranes prepared from cells grown under various differentiating conditions.

PC12 cells were grown for 14 days in the presence of:  $1 \times 10^{-6}$  M dexamethasone (DEX); 100 ng/ml NGF; or for 1 week, at high density ( $8 \times 10^5$  cells/cm<sup>2</sup>). Figure 3 and Table 1 summarize the Scatchard binding results from the binding studies. Both NGF treatment ( $B_{max} = 56.5 \pm 4.8$  pmol/million cells) and high density plating ( $B_{max} = 31.7 \pm 3.6$  pmol/million cells) increased the number of toxin binding sites when compared to control cells ( $B_{max} = 11.1 \pm 1.4$  pmol/million cells) grown under non-differentiating conditions ( $10^4$  cells/cm<sup>2</sup>). Dexamethasone had little effect on the number of toxin binding sites ( $B_{max} = 18.5 \pm 2.1$ ) when compared to control levels. No significant differences were detected in binding affinities between the four different PC12 membrane preparations.

When trisialoganglioside expression was compared to tetanus toxin binding to PC12 cells grown under various conditions described above, the correlation is remarkable (Figure 4). The ratio of binding sites to trisialoganglioside was approximately 7 in all of these preparations. These results strongly support the contention that complex gangliosides act as tetanus toxin receptors and further emphasize the findings that differentiated

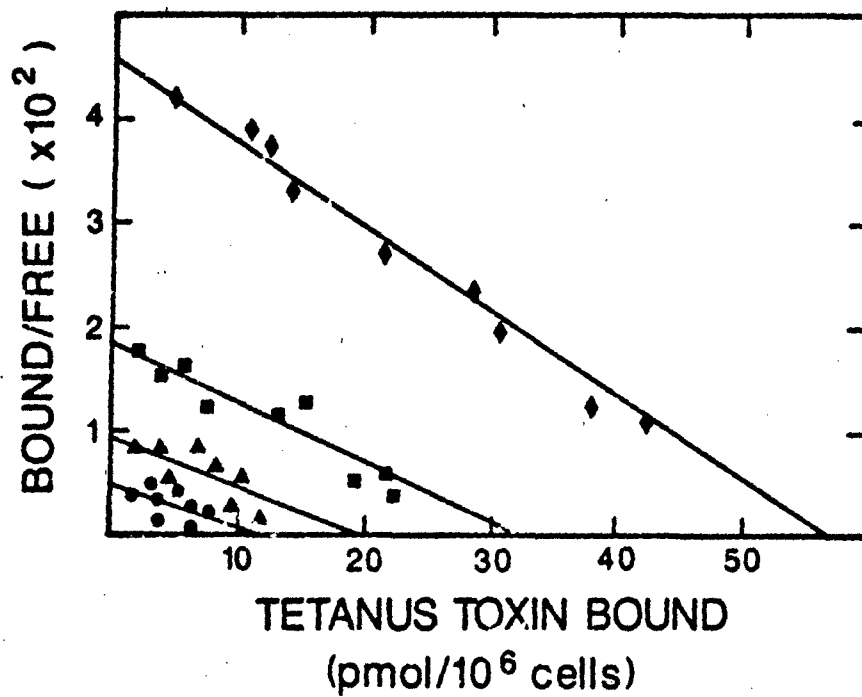


FIGURE 3. Effects of Differentiation on <sup>125</sup>I-Tetanus Toxin Binding to PC12 C Membranes. Microsomes (250 ng protein) were prepared from PC12 cells plated at 10<sup>5</sup> cells/cm<sup>2</sup> and grown for 14 days in the presence of NGF (100 ng/ml) (◆) or Dexamethasone (Dex) (▲). After 7 days in culture, microsomes were prepared from sparse (cells plated at 10<sup>4</sup>/cm<sup>2</sup>) (●) and dense (cells seeded at 8 x 10<sup>5</sup>/cm<sup>2</sup>) PC12 cells. <sup>125</sup>I-Tetanus toxin competition curves were performed and the data recalculated to fit a Scatchard plot.

TABLE 1

Effects of Differentiation on Tetanus Toxin Binding to PC12 Cells

Differentiation Inducer		Enzyme Levels		Scatchard Analysis	
Conditions		CAT (pmol/mg/min)	TH (nmol/mg/min)	Kd (nM)	Bmax
Control	Sparse	140 ± 12	0.25 ± 0.01	2.41 ± 0.3	11.1 ± 1.4 <sup>a</sup>
Adrenergic	Dex	156 ± 16	3.81 ± 0.25	2.05 ± 0.3	18.5 ± 2.1 <sup>a</sup>
Cholinergic	NGF	988 ± 86	0.37 ± 0.02	1.25 ± 0.2	56.5 ± 4.8 <sup>a</sup>
Both	Auto	802 ± 69	2.25 ± 0.19	1.72 ± 0.3	31.7 ± 3.6 <sup>a</sup>
Rat synaptic plasma membranes				0.83 ± 0.07	482 ± 35 <sup>b</sup>

<sup>a</sup> pmol/10<sup>6</sup> cells; <sup>b</sup> pmol/mg protein.

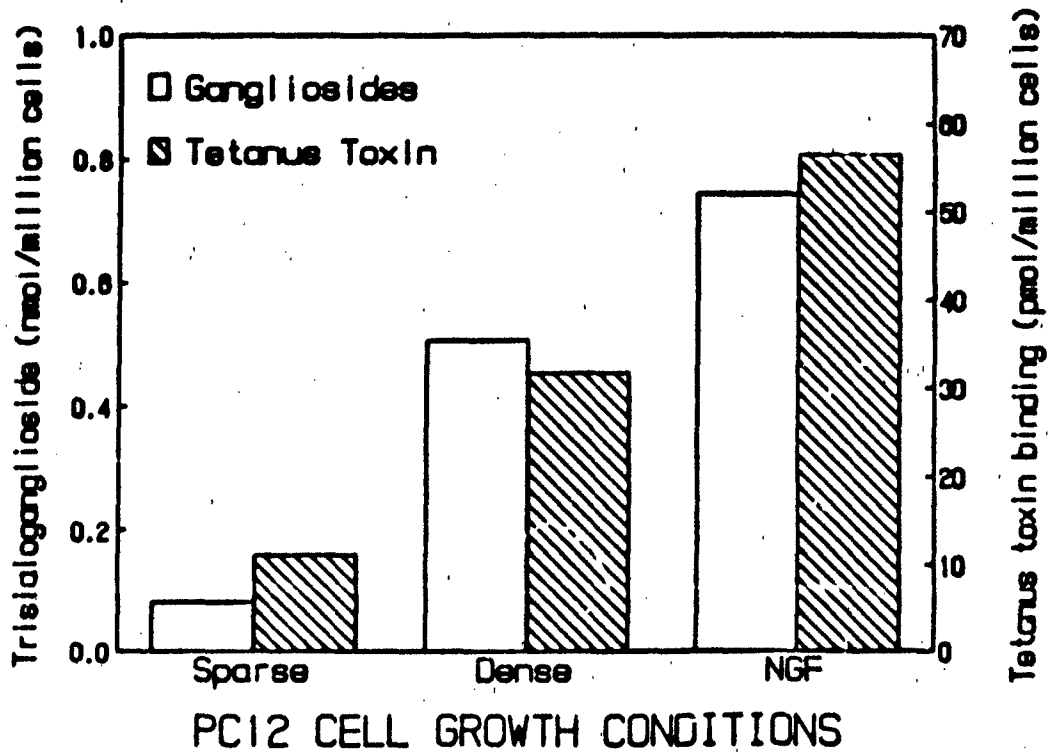


FIGURE 4. Comparison of Trisialoganglioside Expression and Tetanus toxin Binding in PC12 Cells. Trisialoganglioside expression (Walton et al., 1987) in PC12 cells grown under the indicated conditions is compared with the amount of tetanus toxin binding sites on cells grown under the same conditions.

PC12 cells express relatively high concentrations of both trisialogangliosides and tetanus toxin receptors.

Although tetanus toxin binding and toxin-induced paralysis have been studied in detail (Mellanby & Greene, 1981), much less is known about toxin translocation. Receptor mediated ligand internalization commonly occurs through a clathrin coated pit endocytotic pathway (Steinman et al., 1983). Previous electron microscopy studies using colloidal gold labeled-tetanus toxin suggest that toxin internalization is not mediated through this pathway (Goldstein et al., 1979; Steinman et al., 1983). Upon injection into the rat anterior eye chamber, most toxin gold particles in the axon are found within smooth membranous elements rather than clathrin coated pits (Schwab & Thoenen, 1978). No evidence for coated pits was found when the internalization of colloidal gold labeled-toxin was followed in liver cells (Montesano et al., 1982). Therefore internalization of tetanus toxin is of considerable interest because of its unusual endocytotic process. Instead of the conventional clathrin coated pit receptor mediated endocytotic pathway, it appears that tetanus toxin uptake is mediated through noncoated surface microinvaginations.

Studies to illuminate this process have been done on cerebral neuron cultures (Yavin et al., 1981) and mouse spinal cord neurons (Critchley et al., 1985). These studies have provided qualitative evidence that neurons internalize toxin rapidly (in minutes) and in a temperature dependent manner. Recently, the principal investigator's laboratory (Staub et al., 1986) designed a protease assay that has permitted quantification of the toxin internalization process in the N18-RE-105 neuroblastoma cell line. Using this protease assay, toxin was found to be internalized by the N18-RE-105 cells with a  $t_{1/2}$  of 5 min at 37°C. In contrast, low temperature prevented toxin internalization.

To further characterize the toxin translocation process, the fate of tetanus toxin after binding to PC12 cells was investigated. The binding of  $^{125}\text{I}$ -tetanus toxin to intact PC12 cells was examined in competition binding assays. Results in Figure 3 demonstrate that unlabelled toxin is a potent inhibitor of  $^{125}\text{I}$ -toxin binding to PC12 membranes and intact cells at  $0^\circ\text{C}$ . In contrast, at  $37^\circ\text{C}$ , little competition between  $^{125}\text{I}$ -toxin and unlabeled toxin was seen even at 100 nM unlabeled tetanus toxin (100 x Kd) concentrations (Figure 5, inset). The lack of binding inhibition at  $37^\circ\text{C}$  was further investigated in experiments in which increasing amounts of  $^{125}\text{I}$ -toxin were added to intact cells at  $0^\circ\text{C}$  and  $37^\circ\text{C}$  (Figure 5). These binding studies confirm that tetanus toxin binds to a saturable number of receptor sites on intact PC12 cells at  $0^\circ\text{C}$ , but the binding is non-saturable at  $37^\circ\text{C}$ .

Two experiments suggest that the lack of binding inhibition at  $37^\circ\text{C}$  was not the result of increased  $^{125}\text{I}$ -toxin metabolism. First, supernatant radioactivity co-migrated with authentic  $^{125}\text{I}$ -toxin on SDS gels (data not shown). Second, supernatant  $^{125}\text{I}$ -toxin bound when exposed to fresh cells at  $0^\circ\text{C}$  (data not shown). Previously  $^{125}\text{I}$ -tetanus toxin was demonstrated to retain 91% of its biological activity in mice after a 4 h incubation.

The lack of saturability of  $^{125}\text{I}$ -tetanus toxin binding to intact PC12 cells at  $37^\circ\text{C}$  is consistent with previous data demonstrating internalization of tetanus toxin in primary cultures (Critchley et al., 1985) and in the N18-RE-105 cell line (Staub et al., 1986). In order to characterize this internalization process further, a modified version of a previously reported (Staub et al., 1986) protease assay was designed. "Internalized" toxin was defined operationally as the fraction of cell associated  $^{125}\text{I}$ -tetanus toxin which was resistant to pronase degradation. Accordingly, surface bound toxin

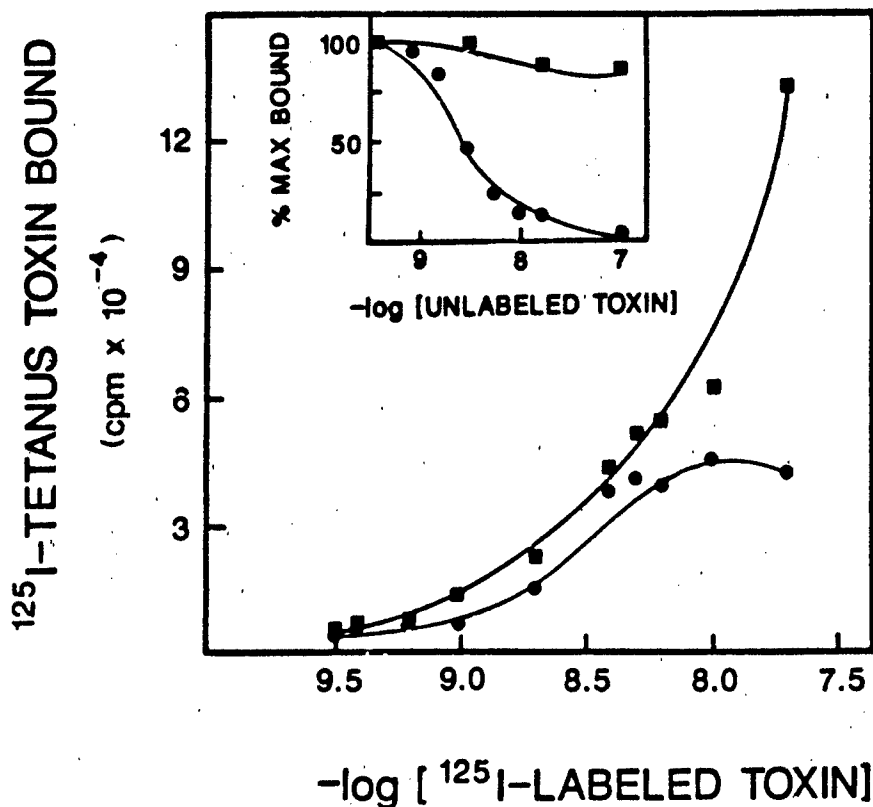


FIGURE 5. Saturation Isotherm of  $^{125}\text{I}$ -Tetanus Toxin Binding to Intact PC12 Cells at 0°C and 37°C. PC12 cells (50-100  $\mu\text{g}$  protein/ 10  $\text{cm}^2$ ) previously treated with NGF for 10 days were incubated with various amounts of  $^{125}\text{I}$ -tetanus toxin in 0.5 ml "Binding buffer" at 0°C or 37°C. **Inset.** Competition of  $^{125}\text{I}$ -Tetanus Toxin Binding with Unlabeled Toxin to PC12 Microsomes and Intact cells at 37°C. PC12 microsomes (250 ng protein) were prepared from 14 day NGF-treated cells. Intact PC12 cells (50-100  $\mu\text{g}$  cell protein/10  $\text{cm}^2$ ) were grown in the presence of NGF for 14 days.  $^{125}\text{I}$ -Tetanus toxin (0.2 nM) was incubated with PC12 membranes ( $\bullet$ ) or  $^{125}\text{I}$ -tetanus toxin (0.5 nM) was incubated with intact PC12 cells ( $\blacksquare$ ) for 3 hours at 37°C in the presence of increasing amounts of unlabeled tetanus toxin.

was defined as cell-associated toxin that was susceptible to proteolytic degradation.

Temperature pulse studies were performed to distinguish the toxin internalization step from the binding step. In these experiments, cells were incubated with  $^{125}\text{I}$ -tetanus toxin at  $0^\circ\text{C}$  in order to label the surface with toxin. After the unbound toxin was removed by washing, the cells were warmed to  $37^\circ\text{C}$  for varying times before the addition of pronase. Within 2 min, a significant fraction (45%) of non-releasable  $^{125}\text{I}$ -tetanus appeared in the cells. After 15 min, the pronase resistant fraction reached a maximum of 78% (Figure 6). Most of the toxin (>75%) associated with cells which had been incubated at  $0^\circ\text{C}$ , remained pronase degradable for the duration of the experiment. These results indicate that once toxin is bound to PC12 cells, it is rapidly internalized in a temperature-dependent manner.

Previous studies with the N18-RE-105 cell line (Staub et al., 1986) showed that the internalization of tetanus toxin is dependent upon metabolic energy. Similarly, metabolic energy inhibitors markedly inhibited the  $^{125}\text{I}$ -tetanus toxin uptake process in PC12 cells. In these experiments, PC12 cells (grown in the presence of NGF for 14 days) were pretreated with metabolic inhibitors for 30 min under conditions that consistently reduced ATP levels by 85%. The cells were then incubated with  $^{125}\text{I}$ -toxin, and the amount of internalized toxin was quantified. After 30 min at  $37^\circ\text{C}$ , the degree of toxin internalization was similar to that observed in control experiments (untreated cells at  $0^\circ\text{C}$ ). These findings were demonstrated in the presence of sodium azide ( $1\mu\text{M}$ ), dinitrophenol ( $25\mu\text{M}$ ) and a mixture of oligomycin ( $0.2\mu\text{g/ml}$ ) and rotenone ( $0.2\mu\text{M}$ ). These results were not attributable to differences in  $^{125}\text{I}$ -toxin binding, since the total amount of cell associated  $^{125}\text{I}$ -toxin between cells treated with metabolic inhibitors and untreated controls was

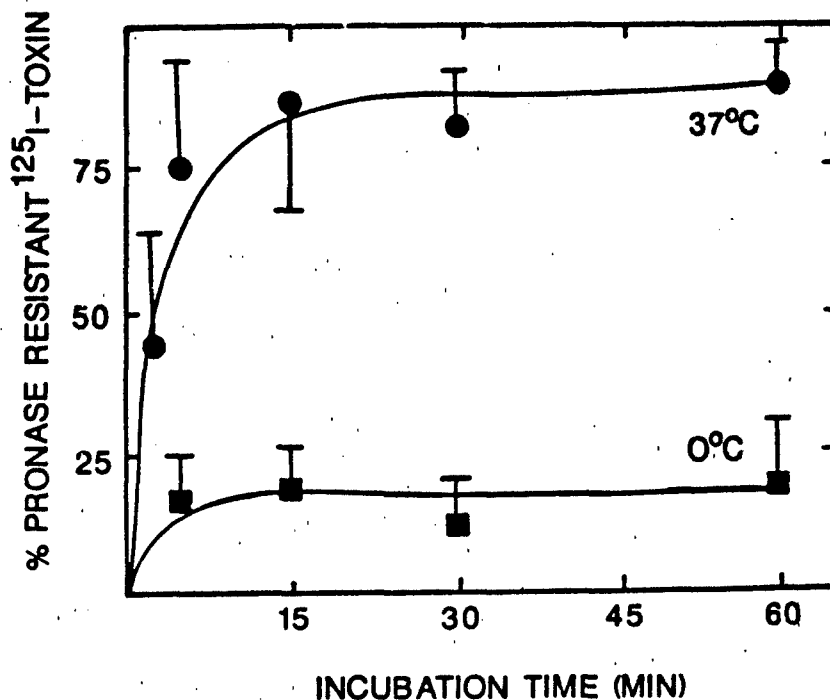


FIGURE 6 Kinetics of <sup>125</sup>I-Tetanus Toxin Internalization into PC12 Cells. PC12 cells ( $5 \times 10^5/10 \text{ cm}^2$ ) were incubated with 0.5 nM <sup>125</sup>I-tetanus toxin in 1 ml of incubation buffer for 30 min at 0°C. At the end of this time, the dishes were rinsed with 2 ml of ice cold rinse buffer and then 1 ml of rinse buffer at 0°C was added to each dish. The dishes were either rapidly warmed to 37°C or were maintained at 0°C (This is the zero time value on the figure). The cells were incubated for the indicated time period and then exposed to pronase. Each data point is expressed as the percentage of bound toxin that is resistant to pronase digestion relative to controls which were treated in an identical manner except that pronase was not added. Therefore the 37°C data are corrected for dissociation that occurs during the incubations. Each point is the mean of 6 different experiments each performed in triplicate  $\pm$  s.e.m.

similar.

## Functional Studies Inhibition of Neurotransmitter Release

### Toxin blocks stimulus-evoked ACh release

The rise in free calcium necessary to elicit secretion from PC12 cells is generated by calcium entering the cell from the extracellular medium through calcium channels which are voltage sensitive. The increased calcium permeability is triggered by depolarization of the plasma membrane. Depolarization can be induced in PC12 cells by ACh agonists and veratridine (an alkaloid that opens sodium channels). In addition, the voltage sensitive secretory pathway can be bypassed by barium ions. Barium is thought to trigger ACh release by inhibiting the time- and voltage-dependent outward potassium current, the M current in a manner analogous to muscarinic depolarization (Constanti, 1981). Figure 7 shows that all three depolarization protocols leads to a marked enhancement of [<sup>3</sup>H]ACh release. Further, under all three conditions, the stimulus-evoked secretory response in NGF-treated PC12 cells is inhibited by tetanus toxin. The toxin had no detectable effect on spontaneous levels of ACh release. These data suggest that tetanus toxin inhibits a common step in cholinergic stimulus-evoked secretion.

The tetanus toxin induced inhibition of ACh release from PC12 cells was specific. Preincubating 4 nM tetanus with anti-tetanus antibody (3 units/ml) for 30 min at 0°C neutralized toxin action. Tetanus toxoid (1 µM), a biologically inactive derivative of tetanus toxin, and heat inactivated toxin (10 min at 100°C) both failed to depress [<sup>3</sup>H]ACh release from PC12 cells. Furthermore, the observed inhibition was not due to depressed levels of [<sup>3</sup>H]choline uptake or differences in intracellular [<sup>3</sup>H]ACh pools (data not

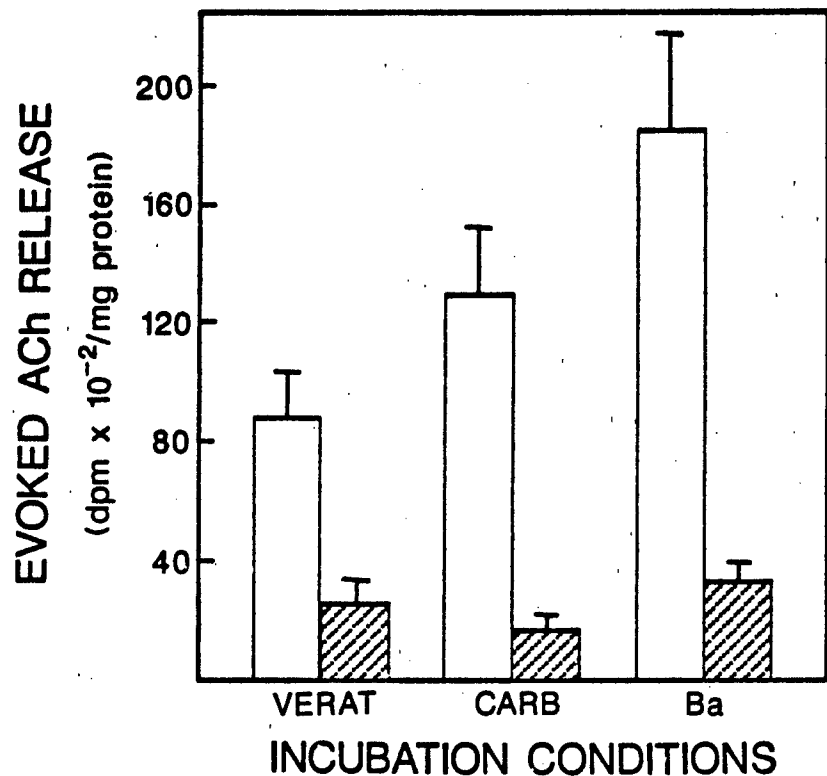


Figure 7. Inhibition of Acetylcholine Release from PC12 Cells by Tetanus Toxin. PC12 cells were incubated overnight with [<sup>3</sup>H]choline followed by 4 nM tetanus toxin for 3 hr. The cells were depolarized and the amount of [<sup>3</sup>H]acetylcholine released into the medium was measured. Shown are the amount of [<sup>3</sup>H]ACh released when the cells were depolarized with veratridine, carbachol, or 2 mM barium chloride in the absence (open bars) and in the presence (stippled bars) of toxin.

shown).

We have been searching for methods that would reverse the intoxication process in the PC12 cells. The identification of such methods should give valuable insight into the mechanism of action of the toxin. We report here that we have for the first time identified specific compounds that can reverse the intoxication once established in PC12 cells. When toxin-treated cells were exposed to 100  $\mu$ M 8-Br-cGMP during the last 15 min of the toxin incubation before assaying for neurotransmitter release, the evoked release of [3H]ACh was restored to 65% of control (toxin free) levels (Figure 8). However, the fact that 8-Br-cGMP treatment in the absence of depolarizing stimulus does not cause ACh release, indicates that elevation of cGMP alone is not sufficient to elicit neurotransmitter release (Figure 8). Control experiments revealed that these results can not be explained by increased cell lysis. The amount of lactate dehydrogenase, a cytosolic marker, released into the media in the presence of 8-Br-cGMP was nearly identical to that released in control cultures (data not shown). Figure 8 (inset) shows that the effect of this nonhydrolyzable cGMP analog was dose dependent. Significant effects were seen at 10  $\mu$ M and the half maximal effect was observed at approximately 50  $\mu$ M.

Examination of the nucleotide specificity demonstrated that nonhydrolyzable analogs of cGMP specifically reverse the toxin-mediated inhibition of [3H]ACh release (Figure 9). In contrast, analogs of cAMP and other nucleotides, at concentrations as high as 1 mM, were not effective in reversing the action of tetanus toxin (Fig. 8). Furthermore, the binding of  $^{125}$ I-tetanus toxin (15) to these cells was not affected by nucleotide addition (data not shown). These findings reveal that the ability to reverse the blockade of neurotransmitter release induced by tetanus toxin is specific to

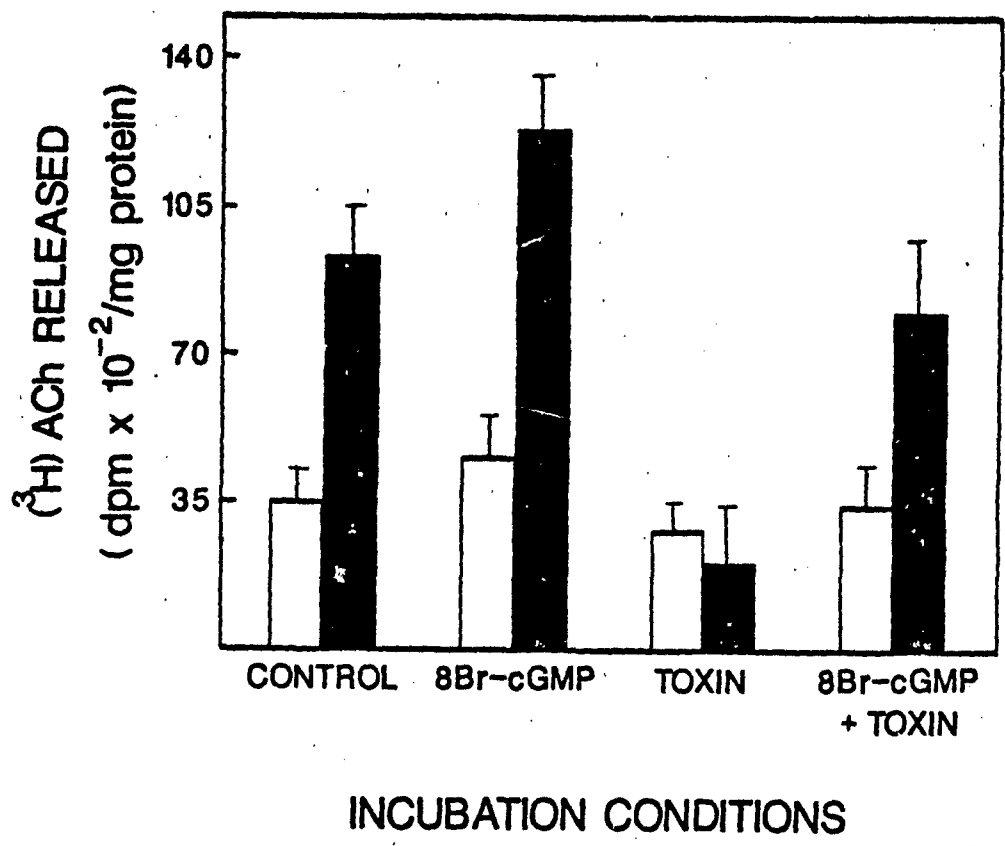


Figure 8. Reversal of the Effects of Tetanus Toxin by 8-Br-cGMP. Neurotransmitter release was measured as described in Figure 7, legend. The amount of acetylcholine released spontaneously (open bars) and evoked in the presence of veratridine (solid bars). 4 nM tetanus toxin inhibited release and these effects were reversed by a 15 min incubation of the infected cells with 100  $\mu\text{M}$  8Br-cGMP.

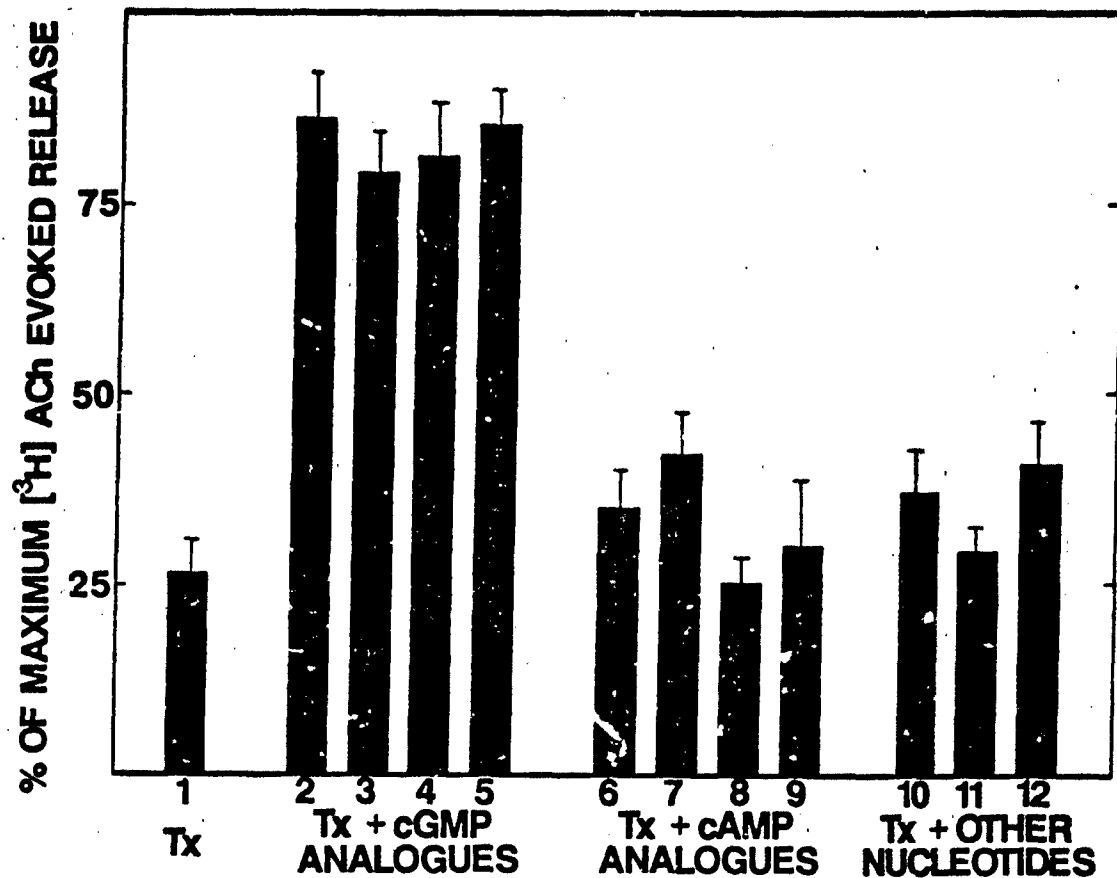


Figure 9. Specificity of cGMP Analogues for Reversing the Inhibitory Actions of Tetanus Toxin on Neurotransmitter Release. During the last 15 min of the 4 nM tetanus toxin 4 h incubation at 37°C, various nucleotides were added to the PC12 cells: 1, no nucleotides; 2, 100 nM 8-Br-cGMP; 3, 1 mM N<sup>6</sup>,2'-O-dibutyryl-cGMP; 4, 1 mM N<sup>2</sup>-monobutyryl-cGMP; 5, 1 mM O<sup>2</sup>-monobutyryl-cGMP; 6, 100 nM 8-Br-cAMP; 7, 1 mM N<sup>6</sup>,2'-O-dibutyryl-cAMP; 8, 1 mM N<sup>2</sup>-monobutyryl-cAMP; 9, 1 mM O<sup>2</sup>-monobutyryl-cAMP; 10, 100 nM 8-Br-Inosine-cyclic monophosphate; 11, 1 mM 8-Br-guanosine monophosphate; 12, 1 mM 8-Br-guanosine. [<sup>3</sup>H]ACh release is expressed as percent of the maximum evoked [<sup>3</sup>H]ACh release under control (individual nucleotides were present without the addition of tetanus toxin) conditions.

cGMP analogs.

## CONCLUSIONS

One of the major goals of this research program has been to identify underlying biochemical mechanisms that are responsible for the presynaptic actions of *Clostridial* neurotoxins on neurotransmitter release. Therefore, it has been very important to devise a model cell system that will enable us to study these events at the molecular level. During the past year considerable progress has been made on developing such a system. We have shown that a transformed cell line of neural origin, the PC12 cell line, has become a valuable system for studies on the binding and internalization of tetanus toxin. Further, these cells have been shown to be an excellent model system to study the mechanism of action of the toxin. We have demonstrated that tetanus toxin is very potent in inhibiting the release of [<sup>3</sup>H]acetylcholine from these cells.

An important discovery during the past year is that analogues of the cyclic nucleotide, cGMP, can reverse the effects of tetanus toxin once it has been fully expressed in intact cells. This discovery is important for several reasons. First, to our knowledge, this is the first report that the actions of tetanus toxin can be specifically reversed once they have been clearly expressed in intact tissue. These results, although preliminary in nature, strongly suggest that the mechanism of action of toxin is in part due to an interference in the metabolism of cGMP within neuronal cells. Further, these studies suggest that cGMP plays an important role in neurotransmitter release and that tetanus toxin may be an excellent probe to study the role of this nucleotide in the release process. Although the fact that cGMP itself does not evoke release underscores the complex role of cGMP in neurosecretion. Therefore research during the next year will focus on the site of action of tetanus toxin in the cGMP metabolism process and will continue to use tetanus

as a probe to study the role of cGMP in neurotransmitter release.

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PERSONNEL INVOLVED IN CONTRACT WORK

1. Kathryn Sandberg, Graduate Student, 80% Time
2. Cathy Berry, Research Assistant, 100% time

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