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MECHANISM OF ACTION OF PRESYNAPTIC NEUROTOXINS

FINAL REPORT

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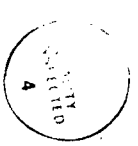

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~~The major goal of this contract was to identify~~ the fundamental mechanisms involved in the action of the Clostridial neurotoxins. ~~We have identified~~ a neuroblastoma cell line, N18 RE105 that has a large number of high affinity tetanus toxin receptors analogous to those found in normal brain. Complex glycolipids, known as ganliosides, were found in these cells and most likely function as the physiological recptor for tetanus toxin. These cels were also used to identify a rapid tempoerature dependent uptake of toxin into the cells. This high affinity uptake mechanism is dependent upon the metabolic energy of neuronal cells. This internalization is almost certainly involved in the intoxication process and explains why once exposed to toxin it is not possible to save patients with antibody treatment. A number of compounds have been screened to try to inhibit this internalization, and one, cytochalasin B is very promising. In further studies we have identified another cell line, PC12, that also binds tetanus toxin with high affinity. We have found that it is necessary to differentiate these cells with nerve growth factor to get significant binding. These cells should be a valuable system to use ~~in screening assays for Clostridial neurotoxins.~~

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Tetanus Toxin- Background and Significance

A. Why is it Important to Study Tetanus Toxin?

Tetanus neurotoxin is a protein (M_r = 150,000) produced by the anaerobic bacterium *Clostridium tetani* and is wholly responsible for the symptoms of clinical tetanus. Several recent reviews summarize earlier studies on this interesting toxin. (Wellhoner, 1982; Mellanby and Green, 1981). The most striking property of the toxin is its enormous potency, acting in rodents in doses as low as 1 ng/kg. This suggests that the toxin is acting selectively at specific recognition sites in the central nervous system that are critical for neuronal function. A second unique characteristic of this toxin is that its mode of action is clearly a presynaptic one. Exposure of synapses in the CNS to very low doses of tetanus toxin results in the complete inhibition of the release of neurotransmitter from the presynaptic terminal. From these earlier studies a general scheme has emerged that describes the intoxication process: (1) specific, high affinity binding of toxin to nervous tissue; (2) uptake of toxin by neurons; (3) translocation of tetanus toxin in the CNS; and finally (4) expression of toxic effect, inhibition of neurotransmitter release.

(1) *Binding of tetanus toxin to nervous tissue and cells.* It has been recognized for some time that tetanus toxin binds selectively to nervous tissue (Mellanby and Whittaker, 1968; Habermann, 1973; Price et al., 1977; Dimpfel et al., 1977). These results have led to the conclusion that tetanus toxin is a valid marker for neurons in the CNS and neuronal cells when grown in culture (Mirsky et al., 1978). Recently, the binding interactions have been characterized and quantitated using ^{125}I-tetanus toxin and brain membranes (Lee et al., 1979; Rogers and Snyder, 1981; Goldberg et al., 1981).

^{125}I-Tetanus toxin binds to a homogeneous class of sites on synaptic membranes with dissociation constants in the nanomolar range. The specificity and distribution of the binding sites, as well as the affinity of these receptors for toxin, provide strong circumstantial evidence that biologically relevant binding determinants have been measured.

One approach to study the interactions of tetanus toxin with the membrane receptor is to characterize the interactions of tetanus toxin with intact cultured cells. It is well documented that tetanus toxin binds to primary cultured neuronal cells (Mirsky et al., 1978; Yavin et al., 1981; Dimpfel and Habermann, 1977; Critchley et al., 1985). However these cultures are not an ideal system for biochemical experiments due to the low yields of cells and cell heterogeneity in the cultures. For these reasons cell lines of neuronal origin would be very valuable. Unfortunately, most cell lines do not contain complex polysialogangliosides (Rebel et al., 1980) and do not have the capacity to bind tetanus toxin (Dimpfel et al., 1977; Yavin and Habig, 1984). Recently, the principal investigator has characterized a cell line which has a high capacity to bind tetanus toxin with high affinity (Staub et al., 1986). These cells have proven to be a very useful system to characterize toxin-neuron interactions (see next section).

(2) Possible mechanisms of action of tetanus toxin. Studies on the molecular mechanism of action of this potent neurotoxin are important since this information will provide valuable insight into the mechanism of neurotransmission. The mode of action of tetanus toxin is the result of the blockade of central inhibitory mechanisms in the spinal cord, thereby leaving excitatory activity of motorneurons unopposed (Curtis and Degroat, 1968). Electrophysiological studies have confirmed that the effects of the toxin are presynaptic. Tetanus decreases the spontaneous and evoked release of

neurotransmitter while leaving postsynaptic membranes still responsive to agonists (Curtis and DeGroat, 1968; Davies and Tongroach, 1979; Bergey et al., 1983). A number of neurochemical studies with primary cultured neurons, brain slices, isolated neuromuscular preparations, and synaptosomes indicate that tetanus toxin inhibits the release of neurotransmitter (Dreyer and Schmitt, 1981; Collingridge et al., 1980; Bigalke et al., 1978; Pearce et al., 1983; Osborne and Bradford, 1973; Schmitt et al., 1981).

In this regard, tetanus toxin and botulinum toxin are analogous neurotoxins. They are both proteins of the same molecular weight and subunit structure produced by closely related bacteria. They both bind to nervous tissue, and to gangliosides, and inhibit the release of neurotransmitter from the same systems *in vitro*, such as the neuromuscular junction (Simpson, 1981; Mellanby, 1984). Although the synaptic mechanisms have not been identified, it is most likely that the fundamental toxic mechanisms involved with all of these *Clostridial* neurotoxins are the same at the molecular level (Mellanby, 1984).

There are many possible mechanisms that could account for the presynaptic effects of tetanus toxin and a number of these possibilites have been excluded. (1) Tetanus toxin does not cause cell death or disrupt the ultrastructure of the presynaptic terminal (Schwab and Thoenen, 1976; Mellanby and Green, 1981). (2) There are no consistent effects of the toxin on neurotransmitter synthesis, storage, degradation, or uptake (Collingridge et al., 1980; Osborne and Bradford, 1973). (3) The toxin does not inhibit the transmission of the action potential into the fine nerve termnials (Gundersen et al., 1982). Finally, (4) the voltage-dependent entry of Ca^{2+} into the presynaptic terminal is not inhibited (Gundersen et al., 1982). There are now indications that the toxin alters the neurotransmitter process triggered

by calcium. Agents which enhance intracellular Ca^{2+} in the presynaptic terminal, such as A23187, 4-aminopyridine, and ouabain, normally stimulate the release of transmitter. However, these agents have no effect on toxin-infected synapses (Habermann et al., 1980; Thesleff and Lundh, 1979).

Since the approaches and systems that have been used to study the mechanism of tetanus toxin at the biochemical level have been distinctly different from those used to study some of its functional effects, it is difficult to identify the specific molecular mechanisms involved. Another difficulty in interpreting the results is that there is very little information on the molecular events involved in the neurotransmitter release processs itself. However, taken together, these results do suggest that tetanus toxin inhibits some Ca-dependent events that occur after Ca^{2+} enters the presynaptic terminal. In order to study the effects of tetanus toxin at the biochemical level, an *in vitro* system needs to be developed where toxin binding, internalization, and release inhibition can be studied in a single system. One of the important goals of this research program has been to develop such a system.

B. *Significance of the Contract Research for the U.S. Army*

The research goals contained in the recently completed contract have considerable significance for the U.S. Army in the area of Military Disease Hazards (RA I) with particular relevance with the mission related to the medical biological warfare defense program. The specific areas of significance are described below.

1. Through studies on the mechanism of action of tetanus toxin, it will be possible to identify and develop specific therapeutic agents that prevent or minimize the toxic sequelae of exposure to *Clostridial* neurotoxins. These agents would act on the fundamental processes of toxic action, i.e. on its internalization or biochemical activity. A significant advantage is that these agents should be effective against all types of related *Clostridial* neurotoxins, including botulinum toxin, for which there may be no

immunization protection available.

2. A major goal of this program is to develop *in vitro* bioassays for tetanus toxin. This technology will have great potential for it can be used to develop simple, standardized laboratory assays to identify *Clostridial* neurotoxins in collected samples.

3. Information obtained on the unique mechanism of internalization and sequestration of tetanus toxin into neuronal cells will help in the development of pharmacological tools for the delivery of therapeutic agents to nerve endings.

4. Successful completion of this project will provide valuable fundamental information on the mechanism of neurotransmitter release and is modulation by endogenous and exogenous toxic agents. This information will be useful in the development of new therapeutic agents effective against presynaptic neurotoxins.

Results from the Principal Investigator's Laboratory During the Contract Period

The primary goal during the contract period has been to develop cultured neuronal cell systems which would allow for a detailed examination of the mode of action of tetanus toxin. At the time this work was initiated there were virtually no cell systems available that were appropriate for the biochemical approaches that we had proposed. As mentioned in the previous section, most cell lines of neuronal origin do not synthesize complex gangliosides nor do they have the capacity to bind tetanus toxin. However, the principa' investigator's laboratory was able to identify a neuroblastoma hybrid cell line, N18-RE-105, that has a ganglioside composition similar to that found in brain.

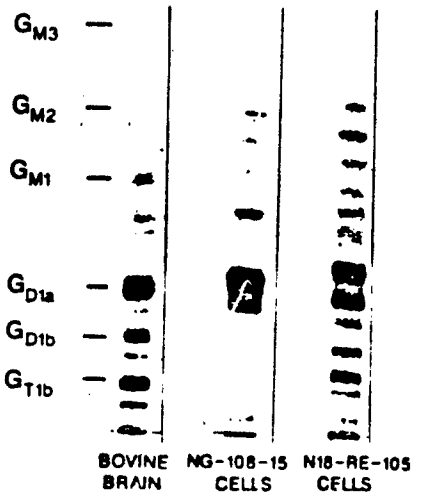

FIGURE 1. TLC chromatogram showing the ganglioside pattern found for two neuroblastoma cell lines compared to mammalian brain.

As shown in Figure 1, N18-RE-105 cells contain material that co-chromatographs with normal GT_{1b} and GD_{1b}. The gangliosides have been purified from the N18-RE-105 cells and their structures were verified by partial hydrolysis studies performed by the principal investigator in collaboration with others during the past year (see Staub et al., 1986 for details).

Consistent with the hypothesis that gangliosides are toxin receptors,

the N18-RE-105 cells have a high capacity to bind tetanus toxin. The binding properties of these receptors were nearly identical to those found in brain. First, the binding was sensitive to pH, ionic strength, and temperature in an identical manner compared to rat brain membranes (Staub et al., 1986). Secondly, bioassays and SDS gel analyses of the bound toxin revealed that the cells were binding authentic tetanus toxin. Finally, the specificity of the receptor was consistent with a biologically relevant binding determinant (Table 1).

Table 1. Specificity of ^{125}I-tetanus toxin binding to N18-RE-105 cells

Compound added	Total ^{125}I-tetanus toxin bound (B/B_0) (%)
Control	100
Unlabeled tetanus toxin (10 μM)	0
Unlabeled tetanus toxin (10 nM)	53
Tetanus toxoid (10 μM)	90
Tetanus antitoxin (2 units)	5
Mixed gangliosides (20 μM)	25

The binding is inhibited by tetanus toxin and gangliosides but not by the biologically inactive toxoid.

Tetanus toxin binding parameters were characterized in competition binding studies as shown in Figure 2.

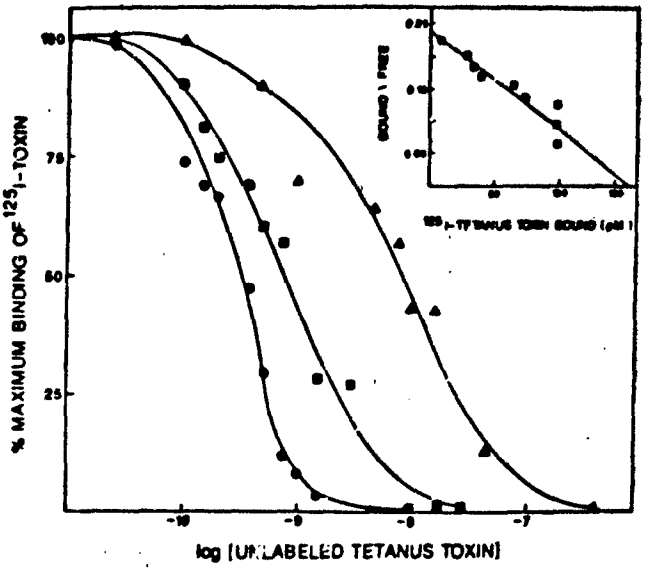

FIGURE 2. Competition binding of ^{125}I-tetanus toxin with unlabeled tetanus toxin to membranes from rat brain(●), N18-RE-105 cells(■), and intact N18-RE-105 cells (▲) at 0°C.

^{125}I-Tetanus toxin binding to membranes prepared from N18-RE-105 cells was saturable and of high affinity, and displayed nearly the same potency when compared to rat brain membranes (Figure 2). The binding parameters were calculated from a Scatchard analysis of the displacement curves (Inset) and were: K_D= 0.62 ± 0.05 nM, B_{max}= 196 ± 45 pmol/ mg protein. With intact cells at 0°C, the binding was more complex (Figure 2) and Scatchard curves generated from these data were nonlinear. The difference was even more striking with intact cells at 37°C where the binding was nonsaturable and no displacement was seen with 1 μM unlabeled toxin. Control experiments revealed that no ^{125}I-tetanus toxin metabolism occured during the course of the experiment that could account for the lack of saturable binding at 37°C.

These results are analogous to earlier reports with primary neuronal cells in culture (Yavin et al., 1981) and suggested that tetanus toxin was being internalized by N18-RE-105 cells in a temperature-dependent manner. To explore this possibility in detail, we developed an assay that would effectively distinguish surface bound from internalized toxin. We reasoned

that ^{125}I-tetanus toxin that had been transferred from the cell surface to another cellular compartment should become resistant to proteolysis. In the next series of experiments, we found that pronase could degrade all of the ^{125}I-tetanus toxin that was bound to membranes at 0°C or 37°C or that was bound to intact cells at 0°C (Table 2).

Table 2. Quantitation of releasable ^{125}I-tetanus toxin bound to membranes and N18-RE-105 cells

Preparation	Concentration of pronase (μg/ml)	Incubation temperature (°C)	^{125}I-tetanus toxin released (%)
Microsomes	5	0	65
		37	73
Microsomes	20	0	98
		37	98
N18-RE-105 cells	5	0	49
		37	34
N18-RE-105 cells	20	0	94
		37	55

However, under the same conditions with intact cells at 37°C, about 50% of the bound toxin was resistant to proteolysis. We operationally defined this pronase-resistant fraction as "internalized" toxin.

The rate of formation of the protease resistant toxin was characterized as shown in Figure 3.

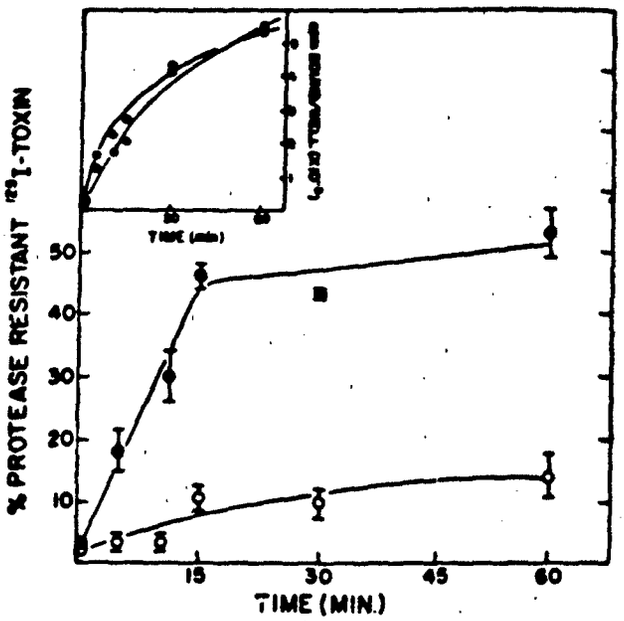

FIGURE 3. Characterization of ^{125}I-tetanus toxin internalization with NI8-RE-105 cells incubated at either 0°C (O) or 37°C (●).

Within 5 min, a significant fraction of nonreleasible ^{125}I-tetanus toxin was detected when incubations were done at 37°C, in comparison to controls that were at 0°C. After 15 min, about 45% of the total cell associated ^{125}I-tetanus toxin was resistant to pronase.

Temperature pulse studies were performed to distinguish the internalization step from the binding step. In these experiments the cells were incubated with ^{125}I-tetanus toxin at 0°C to label the surface with toxin, the unbound ligand was removed, and the cells were warmed to 37°C. The results are shown in Figure 4.

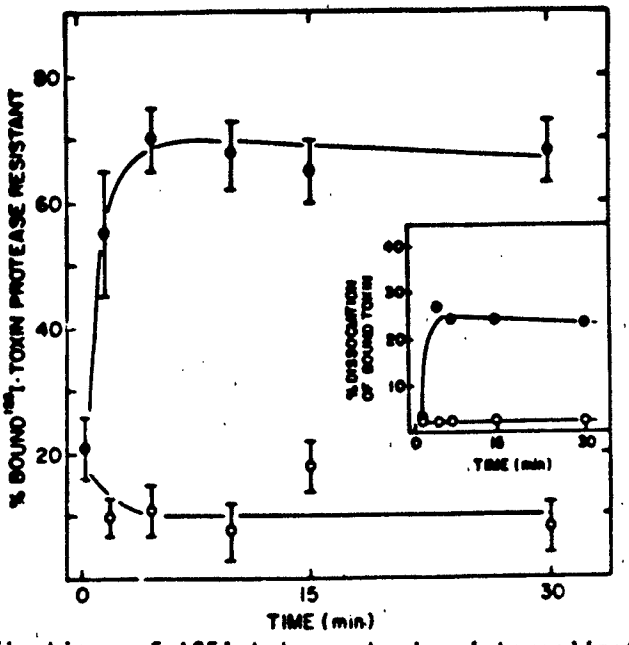

FIGURE 4. Kinetics of 125I-tetanus toxin internalization. After preincubating the N18-RE-105 cells with toxin at 0°C, the labeled cells were either warmed to 37°C (●) or maintained at 0°C (○).

As expected, in the control experiments when the 125I-tetanus toxin surface-labeled cells were maintained at 0°C, most of the toxin was degraded. In contrast, when the cells were warmed, 125I-tetanus toxin rapidly disappeared from the cell surface and within 10 min about 70% of the bound 125I-tetanus toxin was pronase-resistant (Staub et al., 1986).

Recent experiments have focused on characterizing the internalization process using the N18-RE-105 cells and the internalization assay. Apparently 125I-tetanus toxin is not rapidly delivered to lysosomes since: (1) all of the internalized radioactivity can be precipitated by acid even after 4 hr at 37°C; and (2) internalized 125I-tetanus toxin migrates with authentic toxin on SDS gels (Staub et al., 1986). This is consistent with recent reports that tetanus toxin is stable in primary cultured neurons for many hours (Critchley et al., 1985).

In order to characterize the uptake system in more detail, compounds have been tested that might inhibit the process. Toxin internalization is dependent on metabolic energy as shown in Figure 5.

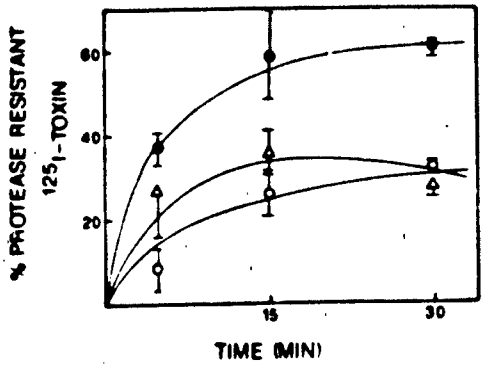

FIGURE 5. Effect of metabolic inhibitors on ^{125}I-tetanus toxin internalization. Pronase resistant toxin was monitored in cells at 0°C (○), cells at 37°C (●), or cells pretreated with oligomycin-rotenone at 37°C (△).

When the cells were pretreated with oligomycin-rotenone, under conditions that reduced ATP levels by 90%, most of the internalization was inhibited. In preliminary results, we have identified another compound that inhibits uptake. As shown in Figure 6, cytochalasin B, which is known to disrupt microfilament structure (Tanenbaum, 1978), does inhibit uptake of ^{125}I-tetanus toxin.

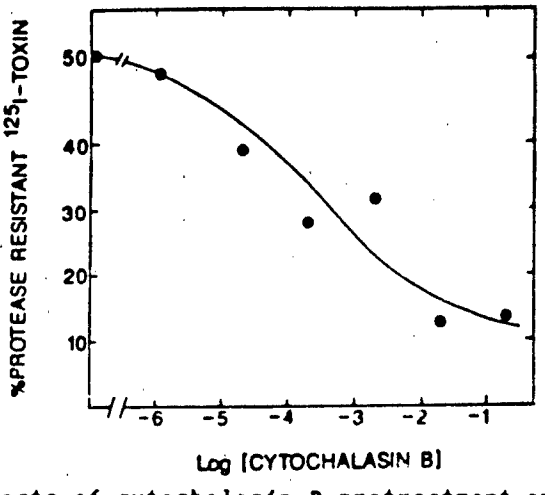

FIGURE 6. Effects of cytochalasin B pretreatment on tetanus toxin uptake into N18-RE-105 cells.

Interpretations of these results should be made with caution since more controls are needed to verify that microfilaments are directly involved. The

experimental strategy for these experiments is described in the next section. It is clear that more studies are needed to further characterize this internalization process and to determine the fate of tetanus toxin after it has been translocated from the cell surface. This is an important goal for the immediate future.

It has not been possible to directly study the effects of tetanus toxin on N18-RE-105 cells since the neurotransmitter characteristics of the hybrid cell line have eluded identification (Malouf and Schnaar, 1984). Therefore it would be very useful to have a cell line that binds tetanus toxin and has a well defined neurotransmitter release system. For this crucial reason recent efforts in the laboratory have been devoted to develop such a system. We have found that ^{125}I-tetanus toxin binds to a pheochromocytoma cell line, PC12, with high affinity (Sandberg and Rogers, 1985). Further, these cells are known to be very responsive to nerve growth factor and other stimuli that promote differentiation. We have found that differentiation can have a profound effect of the expression of tetanus toxin receptors.

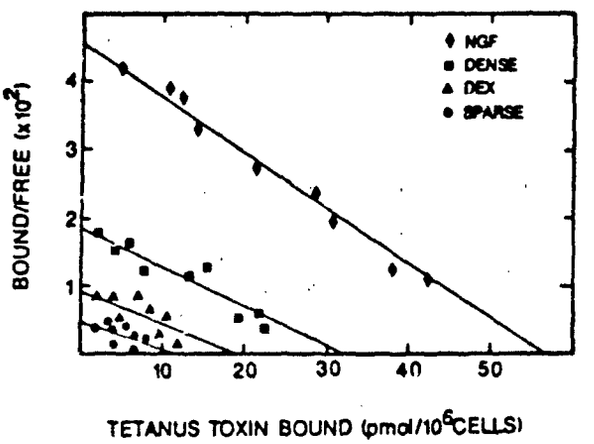

FIGURE 7. Effects of differentiation on tetanus toxin binding to PC12 cells. PC12 cells were grown under a variety of distinct culturing conditions as indicated in the figure. Tetanus toxin binding was analyzed by the use of Scatchard plots.

It is clear from these results that treatment of the cells with NGF results in a 5-fold increase in tetanus toxin receptor number compared to cells grown under sperse conditions. This increase in binding is consistent with the effect of NGF on the phenoptypic properties of PC12 cells, i.e. they become more "neuron-like". These preliminary results are very encouraging since these cells have a well characterized neurotransmitter release system (Greene and Tischler, 1982) and have been extensively used as a model system in neurobiology. A major goal for future work will be to exploit this promising system to study the actions of tetanus toxin at the molecular level. The results to date suggest that it should be possible to correlate biochemical changes with functional responses in the PC12 cell line.

Conclusions and Recommendations

The major thrust of the research program sponsored under this contract was to develop an *in vitro* model system to study the mechanism of action of tetanus toxin. We decided that a powerful approach would be to use cultured cell lines of neural origin. They had clear advantages for biochemical studies compared to previously used systems such as synaptosomes or primary cultured neurons. We have obtained a number of important results during the course of our studies that indicate that our approach is a valid one. We have identified two cell lines, N18 RE105 and PC12, that bind tetanus toxin in a manner identical to that seen in normal neural tissue. Further, these intact cell systems have proved to be a powerful tool in elucidating a specific high affinity uptake mechanism for the toxin.

It is important to know if this internalization process is related to the toxic mechanism of tetanus toxin in biological target tissues. The high affinity binding-internalization reaction of tetanus toxin with N18-RE-105 cells is complimentary to and expands upon the previous results from other analogous systems. Several reports have provided qualitative evidence that primary cultured neurons appear to internalize tetanus toxin in a tempertaure dependent manner (Yavin et al, 1981). Schmitt et al. (1981) have postulated a temperature mediated internalization step preceedes tetanus toxin induced blockade of neurotransmission in neuromuscular junctions. A rapid internalization, on the order of minutes, of tetanus toxin into primary cultured neurons has been reported (Critchley et al., 1985). Botulinum toxin becomes inaccessible to anti-toxin with a half time of 5 min (Simpson, 1980). Finally, Dolly et al. (1984) used autoradiographic methods to show qualitatively that metabolic inhibitors prevented the uptake of botulinum neurotoxin into neuromuscular junctions. Taken together, these results document the biological relevance of the toxin entry process that we have

identified on N18-RE-105 cells. This provides further support for the cultured cell approach that we have initiated.

The specific recommendations for the future are summarized below. A major focus should be to attempt to correlate biochmeical mechanisms with functional responses of the cells.

(1). The internalization process should be further characterized by identifying specific inhibitors of tetanus toxin uptake into N18-RE-105 cells. Further studies will examine the specificity of the cytochalasin inhibitory effects.

(2). Internalization should also be studied by immunocytochemical techniques. It should be possible to identify the subcellular location of tetanus toxin at the light microscopic level.

(3). A major effort should be devoted to examining the effects of tetanus toxin on the release of neurotransmitter from PC12 cells. The preliminary results that we have obtained indicate that this cell system should become a very valuable system in both the study of the mechanism of action of the toxin as well as provide a convenient and sensitive bioassay for all of the *Clostridial* neurotoxins.

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