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MECHANISM OF ACTION OF PRESYNAPTIC NEUROTOXINS

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During the past year we have identified a cell line of neural origin, N18 RE105 that binds tetanus toxin in a manner identical to that found in normal brain tissue. Analysis of the ganglioside content of these cells reveals that it has the same composition as brain. These cells were found to internalize tetanus toxin very rapidly at 37 °C. The rapid internalization was found to be dependent on temperature and ATP within the cell. Therefore this cell line has provided a valuable model system to characterize tetanus toxin interactions and internalization by brain cells. However, these cells do not release neurotransmitter and therefore functional responses cannot be measured. We have found that another cell line that does have a neurotransmitter release system, the PC12 cells does bind tetanus toxin with high affinity as well. Differentiation of these cells with nerve growth factor leads to an five fold increase in tetanus toxin receptors on these cells. Studies are now underway using this cell line in order to correlate binding, internalization, and biochemical responses of cell with inhibition of neurotransmitter release.

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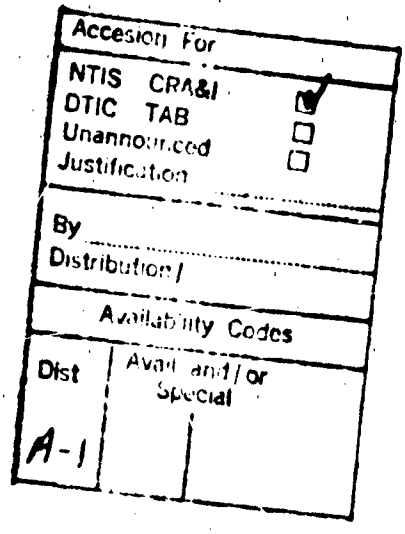

Tetanus Toxin- Background and Significance

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 at specific recognition sites in the central nervous system that are critical for neuronal function. 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.

There is considerable evidence that the chemical components in neuronal membranes involved in the recognition of tetanus toxin are gangliosides (Van Heyningen, 1963; Weigandt, 1979; Dimpfel et al.,1977). More recent studies have shown that the polysialogangliosides of the b series (with two sialic acid residues on the internal galactose moiety) bind tetanus toxin with high affinity (Holmgren et al., 1980; Rogers and Snyder 1981). Yavin and Habig (1984) and Yavin (1984) reported that ^{125}I-tetanus toxin binding to cultured neuronal cells was greatly enhanced after gangliosides had been inserted into the plasma membranes.

Despite all of the evidence, it is important to realize that there is no direct evidence that tetanus toxin binding to gangliosides or other membrane components is required for the toxic effect. All of the conclusions are by extrapolation. One of the main limitations in tetanus toxin studies at the present time is the lack of an appropriate *in vitro* model system.

One approach to develop such an *in vitro* system is to study the interaction 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 du 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., 1985). These cells have proven to be a very useful system to characterize toxin-

neuron interactions (see next section).

(2) Internalization and Translocation of Tetanus Toxin in the Nervous System. Internalization of tetanus toxin by neuronal cells is a process considered fundamental to its mode of action (Mellanby and Green, 1981). Yavin et al. (1981), using primary cultured neurons, presented some evidence that about 50% of cell associated ^{125}I-tetanus toxin was internalized into some cellular compartment. Recently, Critchley et al. (1985) have used immunofluorescent methods to show that tetanus toxin is rapidly internalized, in a temperature dependent manner , into a subcellular vesicular compartment that does not appear to be lysosomal. It is clear that more quantitative methods need to be developed so that this uptake process and the intracellular sites of toxin sequestration may be characterized. To that end, the principal investigator has developed methods that differentiate surface bound toxin from internalized toxin. These assays have provided a useful tactic to study the toxin uptake process in cultured cells (see next section).

There is growing evidence that tetanus toxin is localized in vesicles after it has been translocated across the plasma membrane (Montesano et al., 1982; Critchley et al., 1985). Studies in model membrane systems suggest that a low pH environment may cause the insertion of tetanus toxin into the lipid membrane, facilitating the passage of toxin into the cytosol (Hoch et al., 1985; Roa and Bouquet, 1985). Analogous behavior has been reported for diphtheria toxin (Draper and Simon, 1980). While it is tempting to speculate that tetanus toxin gains access to other intracellular compartments via a low pH-mediated translocation from endocytotic vesicles, there is no clear evidence at this time that these events occur in neuronal cells.

One of the striking features of the mechanism of action of tetanus toxin is that there is a characteristic latency period before the onset of the toxic effects. It is clear that part of this delay is the result of the

translocation of the toxin in the central nervous system. Price et al. (1977) reported that tetanus toxin travels centripedally from the periphery to the CNS via retrograde intraaxonal transport after it has been internalized by motor neurons. Other elegant studies have clearly demonstrated that tetanus toxin undergoes further translocation in the CNS via a retrograde transsynaptic transfer through a chain of at least two neurons (Schwab et al., 1979; Dumas et al., 1979). Taken together these results indicate that tetanus toxin is uniquely translocated within the CNS. The translocation mechanisms and intracellular sites of toxin localization are not known. Further, it is not known if other neurotoxic agents are delivered to their sites of action by analogous behavior.

(3) 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 terminals (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 is to characterize and utilize such a system.

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

Research efforts during the past year have focused on the characterization of the binding-internalization reaction of tetanus toxcin with a neuroblastoma hybrid cell line, N18-RE-105. These studies are important because it is essential to develop an intact cell system in order to directly assess the physiological relevance of the tetanus toxin receptor in the intoxication process and to study the events that occur after initial binding. 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 principal investigator's laboratory has recently identified a neuroblastoma hybrid cell line, N18-RE-105, that has a ganglioside composition similar to that found in brain.

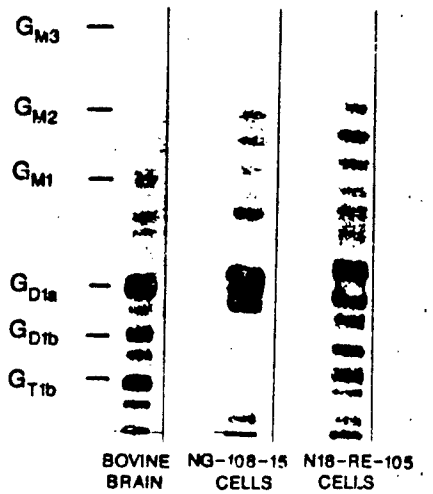

FIGURE 1. TLC chromatogram showing the ganglioside pattern found for two neuroblastoma cell lines compared to mammalian brain.

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., 1985 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., 1985). 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).

COMPOUND ADDED	% TOTAL ^{125}I-TETANUS TOXIN BOUND (B/B_o)
UNLABELED TETANUS TOXIN (1 μM)	0
UNLABELED TETANUS TOXIN (10 nM)	53
TETANUS TOXOID (1 μM)	90
TETANUS ANTITOXIN (2 UNITS)	5
MIXED GANGLIOSIDES (20 μM)	25
LECTINS	
CONCONAVALIN A (150 μg)	95
HELIX POMATIA (50 μg)	80
WHEAT GERM LECTIN (25 μg)	10

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.

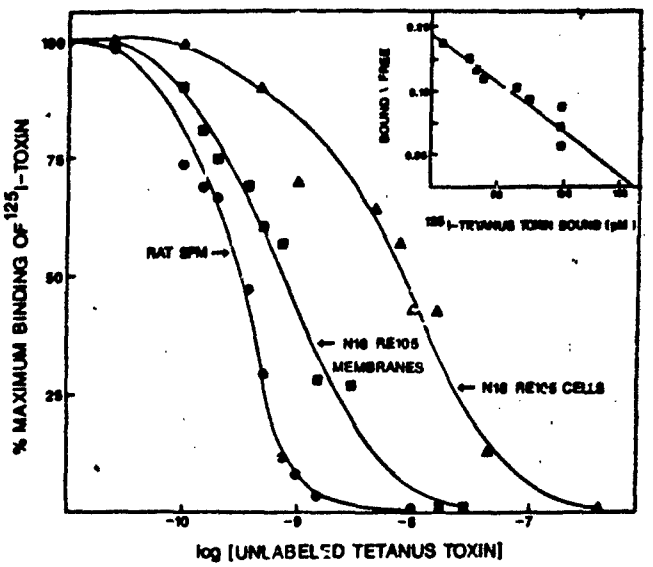

FIGURE 2. Competition binding of ^{125}I-tetanus toxin with unlabeled tetanus toxin to membranes from rat brain(●), N18-RE-105 cells(■), and intact N18-RE-105 cells (▲) at 0°C.

^{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).

PREPARATION	CONCENTRATION OF PRONASE (μg/ml)	INCUBATION TEMPERATURE	% ^{125}I-TETANUS TOXIN RELEASED
MICROSOMES	5	0° 37°	69% 73%
MICROSOMES	20	0° 37°	98% 98%
N18 RE105 CELLS	5	0° 37°	49% 34%
N18 RE105 CELLS	20	0° 37°	94% 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.

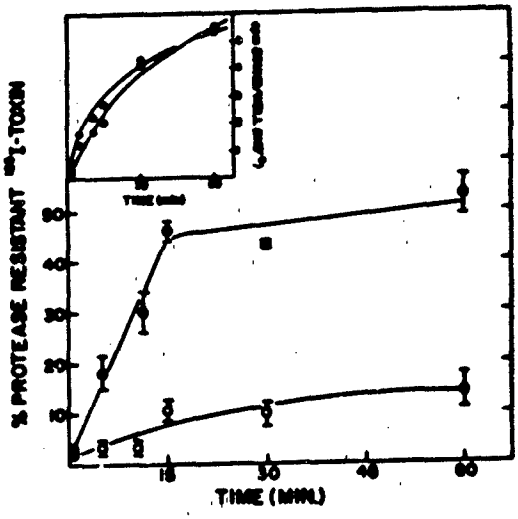

FIGURE 3. Characterization of ^{125}I-tetanus toxin internalization with N18-RE-105 cells incubated at either 0°C (○) or 37°C (●).

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.

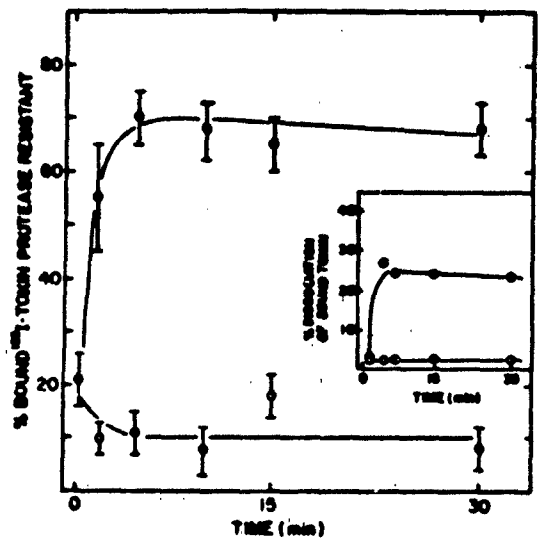

FIGURE 4. Kinetics of ^{125}I-tetanus toxin internalization. After preincubating the N18-RE-105 cells with toxin at 0°C, the labeled cells were either warmed to 37°C (●) or maintained at 0°C (○).

As expected, in the control experiments when the ^{125}I-tetanus toxin surface-labeled cells were maintained at 0°C, most of the toxin was degraded. In contrast, when the cells were warmed, ^{125}I-tetanus toxin rapidly disappeared from the cell surface and within 10 min about 70% of the bound ^{125}I-tetanus toxin was pronase-resistant (Staub et al., 1985).

Recent experiments have focused on characterizing the internalization process using the N18-RE-105 cells and the internalization assay. Apparently ^{125}I-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 ^{125}I-tetanus toxin migrates with authentic toxin on SDS gels (Staub et al., 1985). 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.

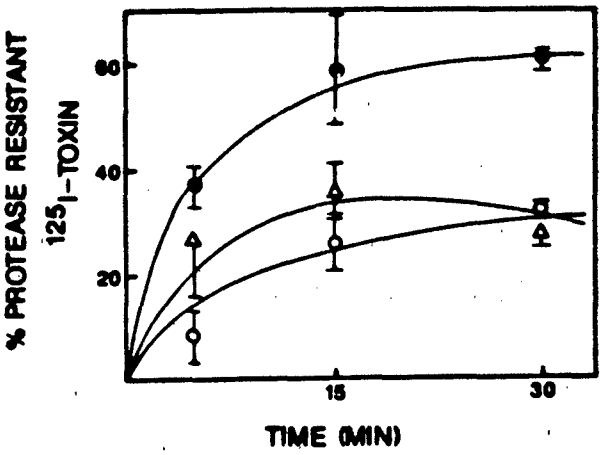

FIGURE 5. Effect of metabolic inhibitors on ^{125}I-tetanus toxin internalization. Protease resistant toxin was monitored in cells at 0°C (○), cells at 37°C (●), or cells pretreated with oligomycin-rotenone at 37°C (△).

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.

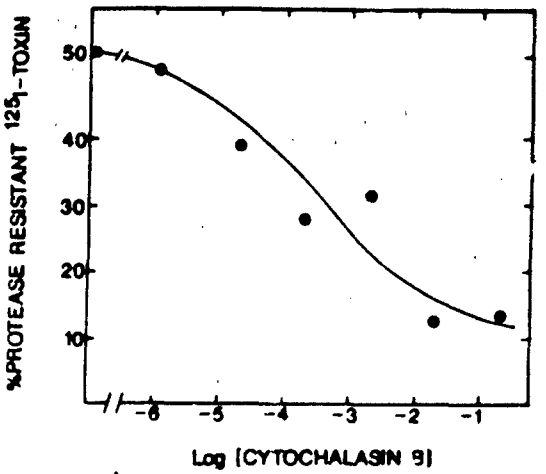

FIGURE 6. Effects of cytochalasin B pretreatment on tetanus toxin uptake into N18-RE-105 cells.

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 (Halouf and Schnaar, 1984). Therfore it would be very useful to have a cell line that binds tetanus toxin and has a well defined neurotransmitter release system. 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. As shown in Table 3, ^{125}I-tetanus toxin binding is increased nearly 280% by culturing the cells with NGF.

EFFECT OF DIFFERENTIATION ON ^{125}I-TETANUS TOXIN BINDING TO PC12 CELLS

Neurochemical Change	Differentiation Condition	^{125}I-Tetanus Toxin Bound (fmol/mg protein)
Control	Sparse	189 ± 23
Cholinergic and Noradrenergic (TH +, CAT +)	Dense	312 ± 35
Cholinergic (CAT +)	NGF (sparse)	523 ± 45
Noradrenergic (TH +)	Dexamethasone (sparse)	194 ± 16

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". Further experiments need to be performed to determine if these changes in binding are the result of changes in the affinity of the receptor or in B_{max}.

However, 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. They should prove useful to study tetanus toxin effects. It is noteworthy that a recent brief report indicates that tetanus toxin does inhibit the release of catecholamines from NGF treated PC12 cells (Figliomeni and Grasso, 1985). A major goal in the present proposal is to exploit this promising system to characterize the toxic mechanism of tetanus.

Conclusions and Recommendations

The major accomplishment during the past year has been to characterize a neuroblastoma cell line to use in modeling the interactions of the *Clostridial* neurotoxins with their physiological target tissues. The N18-RE-105 cell line has receptors that are analogous to those found in synaptic membranes both in their affinity and specificity. Further, a significant result in the past year was the identification of a specific tetanus toxin uptake mechanism that is coupled to high affinity binding interactions. This internalization process has been charaterized in some detail. (1) The internalization is rapid, with a half life of 5 min. (2) The internalization is temperture dependent and finally, (3) since metabolic inhibitors such as oligomycin or rotenone inhibit the uptake, intracellular ATP is required for this process.

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 precedes 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). 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.

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

(1). The internalization process will 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 will also be studied by immunocytochemical techniques. It should be possible to identify the subcellular location of tetanus toxin at the light microscopic level.

(3). The binding and internalization of tetanus toxin by nerve growth factor-treated cultured PC12 cells will be examined. The effects of tetanus toxin on neurotransmitter release will be examined and correlated to the biochemical findings.

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APPENDIX

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Characterization of the Binding and Internalization of Tetanus Toxin in a Neuroblastoma Hybrid Cell Line

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Tetanus toxin is known to bind neuronal tissue selectively. To study the interactions of this potent neurotoxin in an intact cell system, the binding of ^{125}I-tetanus toxin was characterized in a neuroblastoma retina hybrid cell line, N18-RE-105. The binding of ^{125}I-tetanus toxin to membranes prepared from N18-RE-105 cells showed many similarities to the interactions of ^{125}I-toxin with rat synaptic membranes. The binding was decreased with increasing temperature, ionic strength, and pH. ^{125}I-Toxin bound to membranes with high affinity: $K_D = 0.62 \pm 0.05$ nM; $B_{max} = 196 \pm 45$ pmol/mg protein. Quantitative thin-layer chromatography and acid-degradation analysis revealed that N18-RE-105 cells contained polysialogangliosides GD_{1a} and GT_{1b} in high concentrations. An assay was developed to quantitate surface-bound and internalized ^{125}I-tetanus toxin by exploiting the observation that surface-bound ^{125}I-toxin is susceptible to pronase digestion. When cells were incubated with ^{125}I-tetanus toxin at 0°C, all of the bound ^{125}I-toxin could be degraded with pronase. In contrast, when the incubations were performed at 37°C, within 10 min about 50% of the total cell-associated ^{125}I-toxin was pronase-resistant. Temperature pulse experiments demonstrated that ^{125}I-tetanus toxin that was bound to cells at 0°C rapidly disappeared from the surface when the cells were warmed to 37°C, as revealed by the appearance of pronase-resistant radioactivity. This internalization was sensitive to metabolic inhibitors. Thus, N18-RE-105 cells are one of the few neuroblastoma cell lines that possess a ganglioside pattern similar to that found in normal brain, and they have tetanus binding sites that exhibit properties resembling those described in synaptic membranes. These cells provide an excellent model to study the events that occur in the intoxication process subsequent to initial surface binding interactions.

Tetanus toxin is an extremely potent protein neurotoxin, with lethal doses in the range of 1 ng/kg in rodents (Habermann, 1973; Mellanby and Green, 1981; Wellhoner, 1982). The toxin's major effect on the CNS is presynaptic and is thought to involve an inhibition of the evoked and spontaneous release of neurotransmitter (Bergey et al., 1983; Collingridge et al., 1980; Curtis and DeGroat, 1968; Davies and Tongroach, 1979). Although very little is known about the molecular mechanism of toxic action, the intoxication process probably involves a number of steps: (1) specific high-affinity binding between the toxin and neuronal cell surface receptors; (2) internalization of the toxin; (3) translocation of the toxin via retrograde intraaxonal as well as transsynaptic transport to its toxic site of action; and finally, (4) specific perturbation of the neurotransmitter release process (Price et al., 975; Schwab et al., 1979).

It has been known for some time that tetanus toxin is selectively bound by neural tissue (Dimpfel et al., 1977; Habermann et al., 1973). More recently, high-affinity receptors for radiolabeled tetanus toxin, with affinities in the nanomolar range, have been identified and characterized on brain membranes (Goldberg et al., 1981; Lee et al., 1979; Rogers and Snyder, 1981). These reports suggest that the receptor is comprised of complex gangliosides, such as GD_{1b} and GT_{1b} (Holmgren et al., 1980; Stoeckel et al., 1977).

In order to directly assess the physiological role of tetanus receptors in the intoxication process, an intact cell system is essential. In the present study we have identified a hybrid cell line (N18-RE-105) of neuronal origin that not only has membranes that contain a pattern of polysialogangliosides similar to those found in mammalian brain, but which also displays a high capacity for tetanus toxin binding. Most reported cell lines do not contain significant amounts of complex gangliosides, nor do they express significant levels of high-affinity tetanus receptors (Dimpfel et al., 1977; Mirsky et al., 1978; Rebel et al., 1980; Yavin and Habig, 1984). The tetanus receptor in the N18-RE-105 cells was found to be similar to the tetanus receptor characterized in mammalian brain membranes (Lee et al., 1979; Rogers and Snyder, 1981). This cell line has also been useful in the study of the events that occur subsequent to initial binding. Using these cells, we have developed methods that permit distinction between surface binding and toxin uptake, and we report here the characteristics of a receptor-mediated toxin internalization process. Preliminary reports of this work have been published (Rogers, 1983; Staub et al., 1984).

Materials and Methods

Materials

Mixed brain gangliosides, BSA (recrystallized), oligomycin, 2-deoxyglucose, phenylmethyl sulfonyl fluoride (PMSF), benzamidine, γ-aminocaproic acid, and pronase (*Streptomyces griseus*, Type XIV) were obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from Hazlton Dutchland (Denver, PA). Tetanus toxin was the kind gift of Dr. R. O. Thomson, Wellcome Research Laboratories (Beckenham, England). Horse tetanus antitoxin was purchased from Sclavo Labs (Wayne, NJ). ^{125}I-Bolton-Hunter reagent was purchased from New England Nuclear. All other chemicals

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were purchased as reagent grade. The following tissue culture plasticware was used: 75 cm² tissue culture flasks (Corning); 17 mm multiwell plates (Falcon); and 35 mm multiwell plates and 35 mm petri dishes (Nunc).

Cell culture

N18-RE-105 cells (mouse neuroblastoma clone N18TG-2-Fischer rat 18 d embryonic neural retina) were cultured as recently described (Malouf et al., 1984a, b). The day before experiments, cells were transferred to one of the following: 35 or 17 mm well multicluster dishes or 35 mm petri dishes. Cells were subcultured by removing the growth medium and replacing it with 10 ml of Ca^{2+},Mg^{2+}-free PBS: 137 mM NaCl, 5.22 mM KCl, 0.168 mM Na_2HPO_4, 0.22 mM KH_2PO_4, pH 7.4. After 10 min, the cells were removed from the flasks by agitation, collected by centrifugation at 250 × *g* for 5 min, and reseeded in growth medium at densities specified below for each experiment.

Membrane preparation

Membranes were prepared from N18-RE-105 cells in the following manner. Cells were removed from the culture flasks and collected as described above. The cells were resuspended in 0.25 M sucrose, 20 mM Tris, 30 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.0, and homogenized for 30 sec with a Brinkman Polytron, setting 7. This homogenate was centrifuged at 50,000 × *g* for 10 min at 4°C. The supernatant was removed by aspiration. The pellet was resuspended in fresh buffer by homogenization and centrifuged as above for 10 min. The washed pellet was resuspended with a Teflon–glass homogenizer to an approximate final concentration of 2 mg protein/ml. Aliquots were frozen on dry ice and stored at −70°C until use. Rat (Sprague-Dawley) synaptic plasma membranes (SPM) were prepared by the method of Rogers and Snyder (1981).

Ganglioside extraction and purification

Gangliosides from N18-RE-105 cells (20 confluent 75 cm² tissue culture flasks) were extracted and partially purified by solvent partitioning and dialysis as described previously (Dahms and Schnaar, 1983). Ganglioside analysis was performed by thin-layer chromatography (TLC) on silica gel–60 coated glass plates (E. Merck #5763) using chloroform : methanol : 0.25% aqueous KCl (60:35:8) as solvent. Gangliosides were detected with an acid/resorcinol reagent specific for sialic acid and quantitated with a scanning densitometer as described previously (Dahms and Schnaar, 1983). Purified bovine brain ganglioside standards were prepared by the methods of Fredman (1980).

N18-RE-105 gangliosides were purified further by DEAE–Sepharose chromatography and Iatrobead (Iatron Chemical Co., Tokyo) silicic acid chromatography. Briefly, the gangliosides were evaporated to dryness, resuspended in 0.5 ml of chloroform : methanol : water (120:60:9), and placed on a 1 ml DEAE–Sepharose column pre-equilibrated with the same solvent. The column was washed first with 5 ml of the same solvent, then 5 ml of methanol, and gangliosides were eluted with a step-gradient consisting of 10, 20, and then 30 mM potassium acetate in methanol (10 ml/step). Each fraction was evaporated, resuspended in a small volume of water, and dialyzed overnight at 4°C to remove the salt.

The N18-RE-105 gangliosides cochromatographing with the bovine brain standard GD_{1a} and GT_{1b} were purified further using Iatrobead silicic acid chromatography. The DEAE–Sepharose fractions containing GD_{1a} or GT_{1b} were dissolved in 10 μl of chloroform : methanol : water (65:25:4) and chloroform : methanol : 2.5 M NH_4OH (60:32:7), respectively, applied to a 2 × 75 mm Iatrobead column, and eluted in 30 drop fractions of the same solvent. The eluant was examined by TLC and appropriate fractions pooled.

Partial acid hydrolysis of gangliosides

N18-RE-105 GD_{1a} and GT_{1b} were evaporated in 12 × 75 mm glass tubes, resuspended in 0.2 ml of 0.1 *N* aqueous formic acid, and heated at 100°C for 20 min. The acid was neutralized by addition of 20 μl of 1.0 *N* NaOH; then methanol (1.47 ml) and chloroform (2.93 ml) were added. The solution was desalted on a 0.5 ml column of Sephadex G-25 pre-equilibrated with chloroform : methanol : water (120:60:9) as described previously (Dahms and Schnaar, 1983). The effluent was evaporated, resuspended in a small volume of chloroform : methanol : water (4:8:3) and examined by TLC.

Gel electrophoresis

SDS gel electrophoresis was performed using the method of Laemmli (1970). All samples were incubated in a denaturation buffer of 50 mM Tris, 0.3 mM β-mercaptoethanol, 4% SDS, pH 6.8, for 10 min at 100°C. Generally, 10,000 cpm of ^{125}I-tetanus toxin were layered onto the tracks of the SDS slab gel, which was prepared with a 7–15% linear gradient of acrylamide. Gels were dried and autoradiograms prepared by incubating the dried gel with unexposed X-ray film at −70°C for 4 hr in cassettes equipped with intensifying screens (Quanta 3, Dupont, Wilmington, DE).

Bioactivity of toxin

Bioassays of ^{125}I-tetanus toxin were performed as previously described (Rogers and Snyder, 1981). Groups of mice were injected with serial dilutions of ^{125}I-tetanus toxin stock solutions or radiolabeled toxin that was dissociated from N18-RE-105 cells after binding at 0°C. A minimal lethal dose was defined as the highest dilution of ^{125}I-toxin that caused death in all three mice after 96 hr.

Binding experiments

Radiolabeled tetanus toxin was prepared to a specific radioactivity of 400–600 Ci/mmol by incubating 100 μg of toxin with 1 mCi of ^{125}I-*p*-hydroxyphenylpropionic *N*-succinimidyl-ester (Bolton-Hunter reagent) by methods adapted from Bolton and Hunter (1973) as previously described (Rogers and Snyder, 1981). Binding studies with N18-RE-105 membranes and rat SPM preparations were performed using a microcentrifugation assay as previously described (Rogers and Snyder, 1981). The binding buffer, unless otherwise indicated, consisted of 0.25 M sucrose, 20 mM Tris, 30 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25% BSA, pH 7.0. The rinse buffer was the binding buffer without BSA. The binding of ^{125}I-tetanus toxin was linear up to 20 ng of protein for rat SPM and up to 500 ng of protein for the N18-RE-105 membranes. The specific binding was determined as the difference between the total binding and the nonspecific binding. The latter was estimated by incubating membranes in an identical manner except that 50 nM unlabeled toxin was added. Using these incubation conditions, at least 90% of the nonspecific binding was due to ligand binding to the tubes alone, as determined in control experiments. Incubations in which three units of antitoxin were added gave identical values to those obtained in the presence of excess cold toxin. Therefore, antitoxin was used in most experiments as a measure of nonspecific binding. This method has been used previously to determine ^{125}I-toxin nonspecific binding (Lee et al., 1979; Yavin et al., 1981). Typically, when 20 ng of rat SPM protein or 200 ng of N18-RE-105 membrane protein was included in the incubation with 0.2 nM ^{125}I-tetanus toxin (50,000 cpm) at 0°C, the total binding reached a plateau after 2 hr and was about 2500 cpm; the nonspecific binding was about 500 cpm. Scatchard plots were performed by incubating fixed concentrations of ^{125}I-tetanus toxin with increasing concentrations of cold toxin and analyzing the data as previously reported (Rogers and Snyder, 1981). Protein was determined by the method of Bradford (1976) using BSA as a standard.

In binding experiments with cells attached to culture dishes, growth medium was removed and replaced with binding buffer (described above) containing ^{125}I-tetanus toxin in a concentration range of 0.1–0.4 nM. Incubations were terminated by removing the incubation medium and replacing it with 1–2 ml of a rinse buffer (binding buffer without BSA). After 5 min, the rinse buffer was gently aspirated and the cells were solubilized in 1% SDS, 0.5 *N* NaOH. The solutions were then transferred to test tubes and counted in a gamma radiation counter at 59% efficiency. Protein was determined in an aliquot of a 0.005% SDS extract from individual wells using the Coomassie Blue assay according to Bradford (1976). The protocols were designed so that greater than 90% of cell protein remained bound to the dishes during the various incubations and rinses required. Specific binding was determined as described above. All data points were performed in duplicate or triplicate, with a variation of 10% or less, and each experiment was repeated three times. In a typical experiment, when 0.2 nM ^{125}I-tetanus toxin (50,000 cpm) was incubated with N18-RE-105 cells (30,000 cells/1.7 cm well, 20 μg of cell protein) in 1 ml of binding buffer, total binding was approximately 2000 cpm and nonspecific binding was about 500 cpm.

Cell viability

Changes in cell viability were assessed by two independent methods. In the first procedure, the ability of cells to exclude 0.04% Trypan Blue

was used as a measure of viability. A more quantitative method was also employed, which used the amount of lactate dehydrogenase released into the medium as a measure of nonviable cells (Schnaar and Schaffner, 1981).

ATP assay

ATP content of intact N18-RE-105 cells attached to culture dishes was determined by first extracting ATP from the cells and then quantitating the ATP content of the extract using a luciferin–luciferase fluorimetric assay according to Stanley and Williams (1969). To quantitate ATP in cells attached to culture dishes, the cells (generally 30,000 1.7 cm well) were fast-frozen in a dry ice–alcohol bath and then placed on ice. An extraction solution (6% perchloric acid, 2.5 mM EDTA) was added, and the resulting slurry scraped from the plate with a rubber policeman. The suspension was transferred to a 1.5 ml microfuge tube, and the samples were centrifuged for 2 min at 12,000 × *g*. The supernatant was then neutralized with 5 M K_2CO_3. The entire sample was recentrifuged at 12,000 × *g* for 2 min, and the supernatant cell extract was removed and frozen on dry ice. Extracts were stored at −70°C and used within 48 hr. Control experiments indicated that no ATP was lost during this storage procedure. Bioluminescence measurements were made on a model A3330 Packard Tricard liquid-scintillation spectrophotometer. ATP levels were found to be 10–11 nmol ATP/mg of cell protein for cells attached to culture dishes.

Protease digestion of ^{125}I-tetanus toxin

In preliminary experiments, optimal conditions for the degradation of unbound ^{125}I-tetanus toxin were determined by assessing toxin degradation on SDS slab gels after the ligand had been exposed to a variety of enzymes as described below. The conditions that produced complete degradation of free ^{125}I-labeled toxin were 5.0 μg/ml pronase incubated for 10 min at 37°C in sucrose binding buffer without BSA. Control experiments showed that the proteolytic activity of this protease preparation could be completely stopped by adding an inhibitor cocktail that contained 1 mM PMSF, 1 mM benzamidine, and 5 mM γ-aminocaproic acid.

The experiments with membranes were done in a similar manner. N18-RE-105 membranes were incubated with ^{125}I-labeled toxin as described above. The binding reactions were terminated by centrifugation at 12,000 × *g* for 2 min (Beckman Microfuge No. 12). The incubation medium was removed by aspiration, and the pellet was resuspended in rinse buffer containing 20 μg/ml pronase. The suspension was incubated for 10 min at 37°C. After the inhibitor cocktail was added to terminate the reaction, the membranes were collected by centrifugation. The pellets were rinsed with 1 ml of rinse buffer and then recentrifuged. The amount of radioactivity still bound to the membranes was determined by counting the pellets in a gamma counter.

For degradation of ^{125}I-tetanus toxin bound to N18-RE-105 cells, cells attached to 35 mm multiwell dishes were incubated with ^{125}I-tetanus toxin in 2 ml of incubation buffer for various time periods. The incubation medium was removed by aspiration, and the cells were rinsed with 2 ml of rinse buffer. The cells were incubated at 37°C in 2 ml of rinse buffer containing either 20 or 40 μg/ml of pronase for 10 or 5 min, respectively. The inhibitor cocktail was added to terminate the reaction, and the cells, 90% of which remained attached (measured as cell protein), were gently rinsed with 2 ml of rinse buffer at 37°C. The cells were then removed from the dishes and counted in a gamma counter, as described above. All of the data were corrected for the small losses in protein (less than 15%) that were observed.

Results

Preliminary studies indicated that N18-RE-105 cells incubated with 0.1 nM ^{125}I-tetanus toxin display a high capacity for ^{125}I-toxin binding (Staub et al., 1984). In fact, these cells have a much higher capacity for tetanus toxin binding compared to the NCB-20 cell line, which has been cited to contain the highest levels of toxin receptor of any reported cell line (Yavin and Habig, 1984). When both cell lines were incubated with ^{125}I-tetanus toxin under identical conditions (0.1 nM ^{125}I-toxin, 37°C, 2 hr), the N18-RE-105 cells bound sixfold more toxin (3.2 ± 0.1 pmol/mg protein) than the NCB-20 cells (0.52 ± 0.02 pmol/mg protein).

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

Compound added	Total ^{125}I-tetanus toxin bound (B/B_o) (%)
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

N18-RE-105 cells (20,000 cells/tube) were incubated with 0.2 nM ^{125}I-tetanus toxin in 1.0 ml binding buffer for 2 hr at 0°C in the presence of the compounds as indicated. The specific binding was quantitated as described under Materials and Methods. ^{125}I-tetanus toxin binding is expressed as the percentage bound relative to control values (0.52 ± 0.05 pmol/mg protein). These ^{125}I-toxin experiments were repeated three times with a variation of 10%.

A number of studies were performed to determine if the tetanus receptor on the N18-RE-105 cells is similar to the receptor previously characterized on mammalian brain membranes (Lee et al., 1979; Rogers and Snyder, 1981). First, the binding properties of the tetanus toxin receptor on microsomal preparations from N18-RE-105 cells were examined. The binding of ^{125}I-tetanus toxin to either N18-RE-105 membranes or rat SPM was stimulated threefold when the pH was decreased from pH 8 to 5.5. The binding of ^{125}I-toxin to either membrane preparation was decreased by increasing ionic strength. For example, relative to salt-free controls, the binding of 0.1 nM ^{125}I-tetanus toxin to both membranes was decreased 10-fold when 125 mM NaCl was added to the incubation buffer. The regulation of ^{125}I-tetanus toxin binding by NaCl appears to be an ionic strength effect since similar results were obtained with KCl, choline chloride, and $CaCl_2$ (data not shown). As a further comparison, the effect of incubation temperature on ^{125}I-tetanus toxin binding to membranes was also determined. The binding of ^{125}I-toxin to both membrane systems was decreased by 60% when the incubation temperature was increased from 4°C to 37°C. These results demonstrate that the tetanus receptor on the N18-RE-105 cells is similar to the receptor characterized on mammalian brain membranes.

It was clear from these studies that the "optimum" binding conditions of low pH and ionic strength were not physiological and thus hazardous to cell viability. Therefore, the incubation buffer (see Materials and Methods) used in most of the experiments is a compromise between conditions that "optimize" toxin-cell interactions and conditions that maintain viable cells for a reasonable period of time.

Several experiments were performed to verify that the N18-RE-105 cells and microsomal preparations were binding authentic ^{125}I-tetanus toxin. The bound ligand was separated from the free ligand and analyzed by SDS gel electrophoresis autoradiograms. The radioactivity bound to intact cells or broken cell membrane preparations migrated identically with ^{125}I-tetanus toxin. The unbound radioactivity remaining in the supernatant was analyzed in the same manner. Autoradiograms of SDS gels showed that this unbound radioactive material migrated as intact ^{125}I-labeled toxin (data not shown). This indicates that no significant proteolysis of the ligand occurred during the course of normal incubations.

To further analyze the biological relevance of the toxin–cell interactions, the biological activity of the bound radioactivity was determined. This is an important control since it is well known that preparations of ^{125}I-labeled tetanus toxin also contain radiolabeled biologically inactive toxoid (Lee et al., 1979; Rogers and Snyder, 1981). ^{125}I-tetanus toxin that was bound to

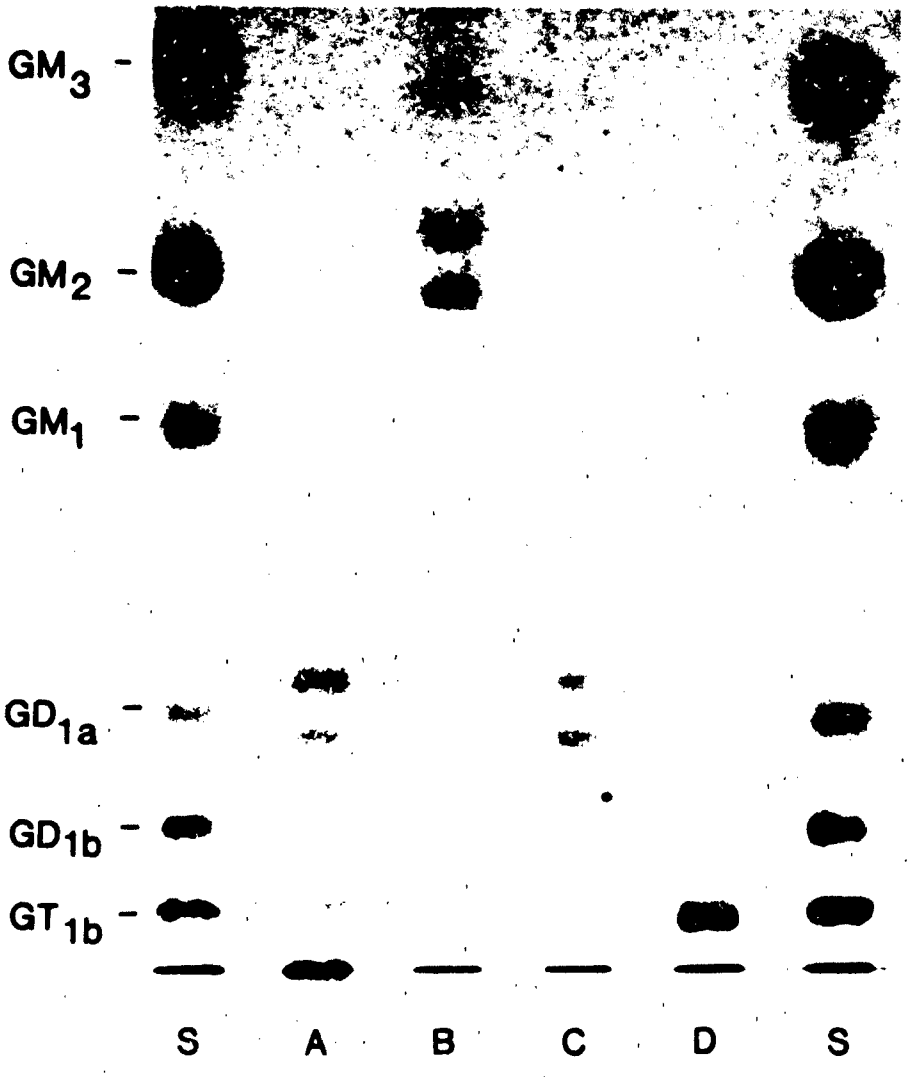

Figure 1. Purification and separation of N18-RE-105 gangliosides. Gangliosides from N18-RE-105 cells were extracted and purified by DEAE–Sepharose and Iatrobead silicic acid chromatography as described in the text. The gangliosides were subjected to TLC in chloroform : methanol : 0.25% (wt/vol) potassium chloride in water (60:35:8) and visualized with a resorcinol spray reagent: *S*, bovine brain standards as indicated at *left*; *A*, total N18-RE-105 gangliosides (0.7 nmol); *B*, *C*, and *D*, purified mono-, di-, and trisialoganglioside fractions, respectively (0.3 nmol/lane).

intact cells at 0°C was recovered, and the biological potency was determined as described under Materials and Methods. The bound toxin had a potency of 590 cpm/lethal dose. The native ^{125}I-labeled toxin stock solution had a potency of 650 cpm/lethal dose. Therefore, the N18-RE-105 cells bind biologically active toxin under the incubation conditions used in these experiments.

The specificity of the N18-RE-105 tetanus toxin receptor was determined in incubations using intact cells. As shown in Table 1, unlabeled toxin and tetanus antitoxin inhibited binding, whereas biologically inactive tetanus toxoid did not compete for the receptor. Further, mixed brain gangliosides were very effective at inhibiting binding to N18-RE-105 cells. These results are consistent with previous studies on ^{125}I-toxin interactions with rat brain membranes and primary cultured neurons (Rogers and Snyder, 1981; Yavin et al., 1981).

Since complex gangliosides have been implicated as receptors for tetanus toxin (Holmgren et al., 1980; Rogers and Snyder, 1981), we extracted the gangliosides from N18-RE-105 cells and examined them by TLC (Fig. 1). In contrast to previously characterized cell lines, which contain principally simple mono- and disialogangliosides, N18-RE-105 cells contain material that cochromatographs with standard GT_{1b}, GD_{1a}, and GD_{1b} (slight), as well as GM_1 and GM_2. The gangliosides from N18-RE-105 cells were further characterized by separation into three pools (tentatively called mono-, di-, and trisialogangliosides) via DEAE–Sepharose and Iatrobead silicic acid chromatography (Fig. 1). The species that cochromatographed with bovine brain standards GD_{1a} and GT_{1b} were subjected to TLC analysis after partial formic acid hydrolysis under conditions that remove a portion of the sialic acid residues (Fig. 2). It should be noted that the appearance of "doublet" ganglioside species is common with cultured cells and has previously been shown to reflect variation in the ceramide portion of gangliosides having identical carbohydrate chains (Dahms and Schnaar, 1983). The ganglioside doublet that cochromatographed with bovine brain GD_{1a} produced one new resorcinol-positive hydrolysis product (doublet) with a mobility similar to that of bovine brain GM_1 (panel A). In contrast, the ganglioside doublet which cochromatographed with bovine brain GT_{1b} produced three doublet products with mobilities similar to GD_{1b}, GD_{1a}, and GM_1 standards (panel B). These hydrolysis patterns are consistent with the designation of the two purified N18-RE-105 gangliosides as GD_{1a} and GT_{1b}.

In order to quantitate the binding interactions of ^{125}I-tetanus toxin with N18-RE-105 membranes, competition binding stud-

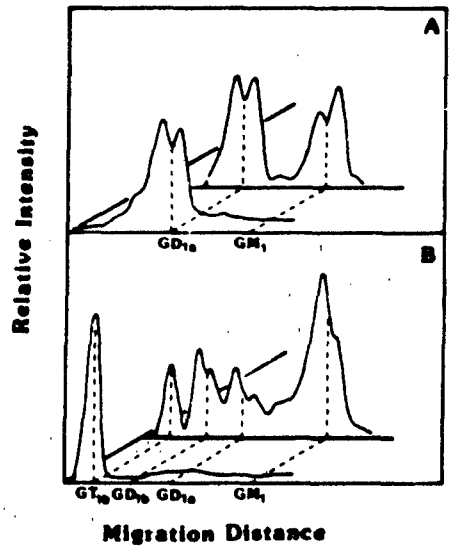

Figure 2. Partial acid hydrolysis of purified N18-RE-105 gangliosides. Purified disialogangliosides (*A*) and trisialogangliosides (*B*) were partially hydrolyzed with formic acid, neutralized, desalted, and subjected to TLC [in chloroform : methanol : 0.25% (wt/vol) potassium chloride in water (60:35:8)] as described in the text. Unhydrolyzed ganglioside fractions (*foreground traces*) and matched partial hydrolysates (*background traces*) were visualized using a resorcinol spray reagent and quantitated using a Kontes Fiber Optic Scanner. Mobilities of bovine brain ganglioside standards chromatographed on the same plate are indicated at the bottom of the figure.

ies were performed. As shown in Figure 3, unlabeled tetanus toxin was a potent inhibitor of ^{125}I-toxin binding to N18-RE-105 membranes and to rat SPM with K_i's in the subnanomolar range. Analogous to the results with rat SPM, the dose-inhibition curves for N18-RE-105 membranes were monophasic and were analyzed as a single class of high-affinity binding sites by the use of Scatchard plots (Fig. 3, inset). The high-affinity binding parameters from three separate experiments for N18-RE-105 membranes and rat SPM were $K_D = 0.62 \pm 0.05$ nM, $B_{max} = 196 \pm 45$ pmol/mg protein and $K_D = 0.39 \pm 0.05$ nM, $B_{max} = 520 \pm 61$ pmol/mg of protein, respectively.

Competition binding curves with intact N18-RE-105 cells were markedly different from the results with broken cell membranes. As shown in Figure 3, the dose-inhibition curves for the intact cells at 0°C were broader than those generated using membranes. Scatchard plots of these binding results were not monophasic and were difficult to interpret. Furthermore, at 37°C, virtually none of the ^{125}I-toxin was displaced by unlabeled toxin even when 500 nM tetanus toxin (a 2000-fold excess) was added (data not shown). The lack of binding inhibition at 37°C was not the result of increased ^{125}I-toxin metabolism, as revealed by two experiments: (1) supernatant radioactivity comigrated with authentic ^{125}I-toxin on SDS gels; (2) supernatant ^{125}I-toxin could still bind when exposed to fresh cells at 0°C. Antitoxin was effective in preventing binding of ^{125}I-toxin to intact cells at 0 and 37°C.

The lack of saturability of ^{125}I-tetanus toxin binding at 37°C suggested that tetanus toxin was being internalized in these intact cells. We reasoned that if ^{125}I-tetanus toxin was being transferred from the surface of the cell, then it should become resistant to proteolytic digestion. In the next series of experiments, we optimized conditions that would degrade ^{125}I-tetanus toxin bound to membranes. Pronase at 5 μg/ml could completely degrade free ^{125}I-toxin in 5 min at 37°C. Fourfold higher concentrations of pronase (20 μg/ml) were required to completely degrade toxin that had been bound to N18-RE-105 microsomes at 37 or 0°C (Table 2). To test for toxin internalization, ^{125}I-tetanus toxin was incubated at either 0 or 37°C for 2 hr with cells attached to culture dishes. After removal of unbound ligand, the cells were treated with pronase and the amount of ^{125}I-toxin remaining with the cells was determined. As shown in Table 2, when the incubations are done at 0°C with 20 μg/ml pronase, nearly all of the ^{125}I-toxin was degraded. These data

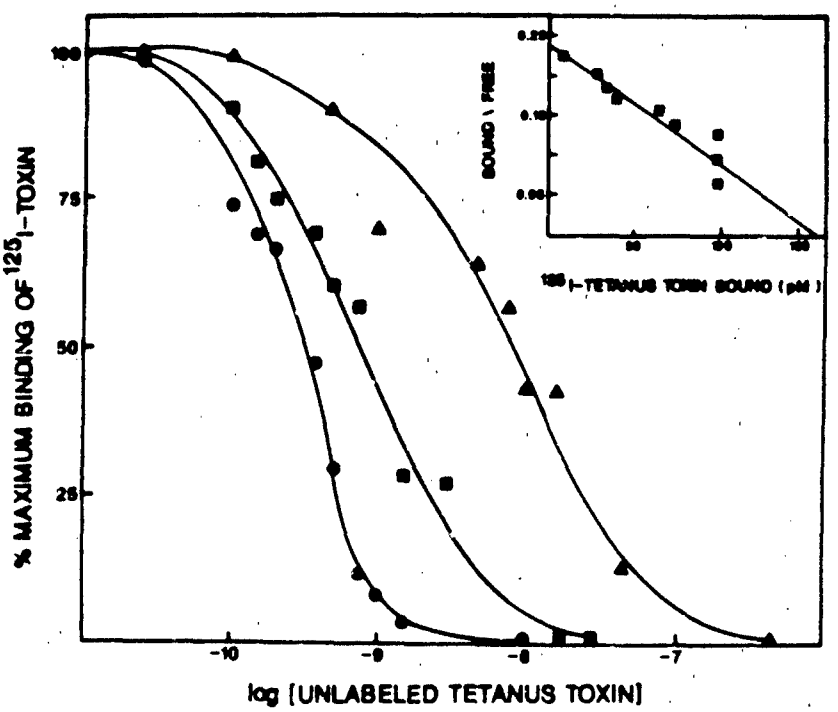

Figure 3. Competition of ^{125}I-tetanus toxin binding with unlabeled toxin to rat SPM, N18-RE-105 membranes, and intact N18-RE-105 cells at 0°C. Binding conditions are described under Materials and Methods. ^{125}I-tetanus toxin concentration was 0.2 nM. Protein concentrations were 20 ng/0.2 ml, 500 ng/0.2 ml, and 20 μg/1.7 cm well for rat SPM (●), N18-RE-105 membranes (■), and intact N18-RE-105 cells (▲), respectively. *Inset*, N18-RE-105 membrane data recalculated to fit a Scatchard plot. The experiments were repeated three times.

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

N18-RE-105 cells, plated to a density of 10^6 cells/35 mm dish, or N18-RE-105 membranes (200 μg) were incubated with 0.1 nM ^{125}I-tetanus toxin for 2 hr at 0 or 37°C as described under Materials and Methods. At the end of the incubations, the bound ligand was exposed to pronase, at the concentrations indicated, for 10 min at 37°C as described in detail under Materials and Methods. The percentage of released ^{125}I-toxin was determined relative to controls in which the tissue was not exposed to pronase. Control levels of tetanus toxin binding in these experiments were 0.095 pmol/mg protein and 0.034 pmol/mg protein for microsomes and intact cells, respectively. The results are the means of three experiments with a variation of 10%.

are in agreement with the broken cell membrane experiments. In contrast, when ^{125}I-tetanus toxin was bound to cells at 37°C, only 55% of the bound ^{125}I-toxin was accessible to pronase; while under identical conditions, all of the membrane bound ^{125}I-tetanus toxin was pronase-accessible.

The appearance rate of the protease-resistant toxin was characterized by incubating the cells with ^{125}I-tetanus toxin for various times and then exposing the labeled cells to pronase. Within 5 min, a significant fraction of nonreleasable ^{125}I-tetanus toxin appeared in cells incubated at 37°C compared to controls incubated at 0°C. After 15 min, the fraction of toxin that was pronase-resistant reached a maximum of about 45%. However, the total cell-associated toxin levels continued to increase (Fig. 4, inset), so that uptake and binding of toxin were still occurring. By the end of the experiment, approximately 11,000 molecules of ^{125}I-tetanus toxin per cell had been transferred to a compartment inaccessible to pronase. It is interesting that the total amount of cell-associated ^{125}I-toxin at 0°C, at which little internalization occurred, was identical to the amount bound at 37°C, at which considerable internalization was measured (Fig. 4, inset). These data indicate that a rate-limiting binding step is followed by a more rapid uptake of toxin. These data suggest that receptor recycling did not occur during the course of the experiments. Further studies are needed to confirm this possibility.

Temperature-pulse experiments were performed so that the internalization of surface bound ^{125}I-toxin could be studied separately from the initial receptor binding process. In these experiments, N18-RE-105 cells were incubated for 10 min at 0°C with ^{125}I-tetanus toxin. The unbound ^{125}I-toxin was removed by washing, and the labeled cells were either warmed to 37°C or maintained at 0°C. The amount of releasable ^{125}I-label was quantitated over time. The results are shown in Figure 5. As expected, when the cells were maintained at 0°C, about 90% of the radiolabel remaining on the cell was releasable by pronase treatment. In contrast, at 37°C, ^{125}I-toxin rapidly disappeared from the surface, and within 10 min, approximately 70% of the total cell-associated ^{125}I-toxin was pronase-resistant. These results reveal that the uptake of ^{125}I-toxin, once it is bound to the cell surface, is rapid at 37°C.

To further characterize the apparent internalization, the effect of metabolic inhibitors on the uptake of ^{125}I-tetanus toxin

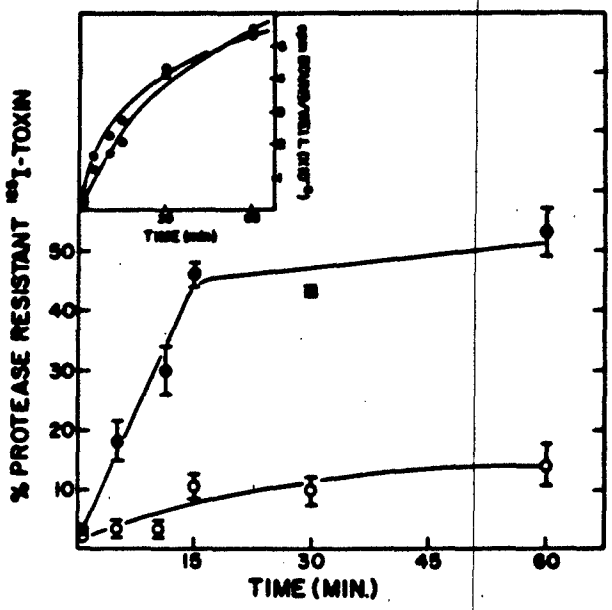

Figure 4. Characterization of ^{125}I-tetanus toxin internalization. N18-RE-105 cells (5×10^5 cells plated onto 35 mm dishes, 500 μg cell protein) were incubated with 0.4 nM ^{125}I-tetanus toxin in 2 ml of incubation buffer at either 0°C (○) or 37°C (●). At the indicated times, the cells were rinsed and incubated with pronase as described in detail in Materials and Methods. Each point is expressed as a percentage of control values, which represent the specific binding of ^{125}I-toxin bound to cells not treated with pronase. The data points are the means of three experiments (±SE), each performed in duplicate. *Inset*, Specific binding of ^{125}I-tetanus toxin to untreated cells at 0°C (○) or 37°C (●). Nonspecific binding, which was identical at either temperature, was based on inhibition by antitoxin and has been subtracted from the total binding values.

by intact cells was examined. In these experiments, cells were pretreated with oligomycin–rotenone under conditions that consistently reduced ATP levels by 95% in control experiments. The cells were then incubated with ^{125}I-toxin, and the amount of pronase-resistant label was quantitated. As shown in Figure 6, treatment of the cells with metabolic inhibitors resulted in an inhibition of ^{125}I-toxin internalization. After 30 min at 37°C, the amount of toxin internalized was identical to that observed in control experiments with untreated cells at 0°C. No difference was detected in the total amount of cell-associated ^{125}I-toxin between cells treated with oligomycin–rotenone and untreated controls. Binding studies on N18-RE-105 membranes clearly demonstrated that oligomycin–rotenone had no effect on ^{125}I-toxin binding at either 0°C (control, 2.50 pmol toxin bound/mg protein; oligomycin–rotenone, 2.40 pmol/mg protein) or 37°C (control, 2.75 pmol toxin/mg protein; oligomycin–rotenone, 2.35 pmol/mg protein). Taken together, these data demonstrate that the metabolic inhibitors alter a process that occurs after initial binding interactions.

Discussion

The major goal of this study was to characterize events in the tetanus toxin intoxication process that occur after initial receptor binding. We have identified a neuronal cell line that has high-affinity tetanus toxin receptors analogous to those identified in brain tissue. This cell line has been exploited to provide insights into the binding-internalization process. The major result is that a process has been identified in a homogeneous population of cells that involves a relatively slow binding of tetanus toxin to high-affinity receptor sites followed by a rapid

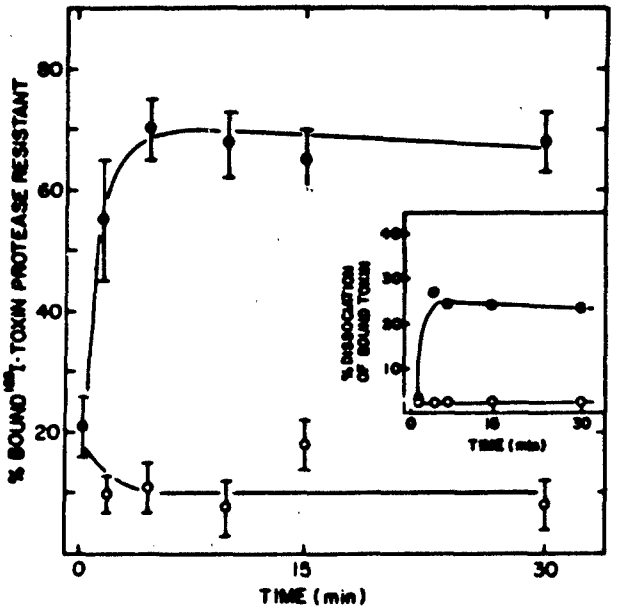

Figure 5. Kinetics of ^{125}I-tetanus toxin internalization. N18-RE-105 cells (5 × 10^5 cells attached to 35 mm dishes, 500 μg cell protein) were incubated with 0.8 nM ^{125}I-tetanus toxin in 2 ml of incubation buffer for 10 min at 0°C. At the end of this time, the dishes were rinsed with 2 ml of ice-cold rinse buffer and then 2 ml of rinse buffer at 0°C was added to each dish. The dishes were either rapidly warmed up 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 periods and then exposed to pronase as described under Materials and Methods. Each data point is expressed as the percentage of bound toxin that is resistant to pronase digestion relative to controls 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 (±SE) from three separate experiments. *Inset.* Amount of bound toxin that dissociated during the incubations at 0°C (○) and 37°C (●) relative to the values of bound ^{125}I-toxin at time zero.

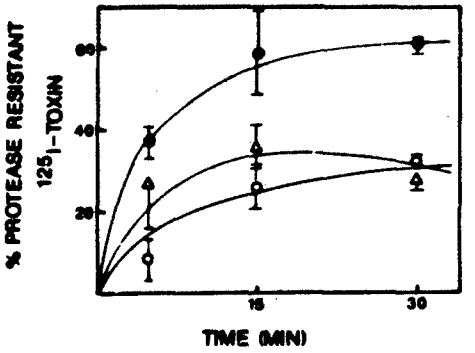

Figure 6. Effect of metabolic inhibitors on ^{125}I-tetanus toxin internalization. N18-RE-105 cells (5 × 10^5 cells plated onto 35 mm dishes, 500 μg cell protein) were preincubated for 1 hr in incubation buffer (2 ml) at either 0°C (○) or 37°C (●). A third set of dishes was incubated for 1 hr at 37°C in 0.4 nM rotenone and 0.4 ng/ml oligomycin (△). After a 1 hr preincubation, ^{125}I-tetanus toxin was added (0.4 nM), and the cells were incubated for the times indicated. The cells were then rinsed and incubated with pronase as described in detail in Materials and Methods. Each point is expressed as a percentage of control values, which represent ^{125}I-toxin bound to cells not treated with pronase. The data points are the means of three experiments (±SE), each performed in duplicate.

receptor-mediated internalization. Further results reported here demonstrate that this specific internalization of toxin is dependent on temperature and intracellular ATP.

Several reports document that tetanus toxin specifically binds to receptors on purified neural membranes, synaptosomes, nervous tissue slices, and primary neurons in culture (Habermann, 1973; Lee et al., 1979; Rogers and Snyder, 1981; Yavin et al., 1981). The identification of neuronal cell lines that interact with tetanus toxin would be extremely valuable in the characterization of this toxin's molecular mechanism of action. Unfortunately, most transformed cell lines do not have the capacity to bind the toxin (Dimpfel et al., 1977; Mirsky et al., 1978; Yavin, 1984). Yavin and Habig (1984) have reported that a somatic-neural hybrid line, NCB-20, binds more ^{125}I-labeled tetanus toxin than any other cell line so far examined. However, the receptor level is about sevenfold lower than that found on primary neurons in culture, and these cells have some toxin-binding properties at variance with those found on brain tissue. Two striking features regarding the N18-RE-105 cell line are that (1) these cells express a receptor density that is sixfold higher than the NCB-20 cells (crude microsomal membranes prepared from the N18-RE-105 cells have only a 2.6-fold less binding capacity than synaptic membrane preparations); and (2) unlike NCB-20 cells, the high-affinity receptors are very similar to those reported on brain membranes.

In order to understand the internalization process in intact cells, it is essential to have detailed quantitative information on the receptor sites in these cells. Accordingly, studies were performed that showed that tetanus toxin receptors on this transformed cell line are closely related to those found on normal brain tissue. Thus, in agreement with previous studies of mammalian brain membranes and primary cultured neurons (Lee et al., 1979; Rogers and Snyder, 1981; Yavin et al., 1981), ^{125}I-tetanus toxin binding was regulated by increasing ionic strength, pH, and temperature of the incubation medium. Moreover, ^{125}I-tetanus toxin binding to N18-RE-105 cells was inhibited by unlabeled toxin, antitoxin, and gangliosides but not by tetanus toxoid (Table 1). The radioactive material that was bound to cells at 0°C appeared to be authentic toxin. Competition binding experiments revealed that tetanus toxin bound to a single class of high-affinity receptor sites on broken cell membrane preparations. The binding affinity of ^{125}I-toxin for N18-RE-105 membranes was nearly as high as that observed for rat SPM, which was measured simultaneously in these studies (Fig. 3). Bioassays showed that the radiolabel that was bound by N18-RE-105 cells and then recovered was at least as toxic to mice as native solutions of ^{125}I-labeled tetanus toxin. This is an important point since it is well known that preparations of ^{125}I-labeled tetanus toxin contain 20–35% radiolabeled, biologically inactive toxoid materials (Lee et al., 1979; Rogers and Snyder, 1981). These results support the conclusion that the N18-RE-105 cells express a physiologically relevant tetanus toxin binding determinant.

Previously published data indicate that complex gangliosides, notably GD_{1b} and GT_{1b}, may act as receptors for tetanus toxin (Holmgren et al., 1980; Rogers and Snyder, 1981). Since previously published studies on neuroblastoma gangliosides have only reported the presence of mono- and disialogangliosides (Rebel et al., 1980), gangliosides that are much less potent in binding tetanus toxin (Holmgren et al., 1980), and since most neuronal cell lines do not bind significant amounts of tetanus toxin, we analyzed the ganglioside composition of the N18-RE-105 cell line. Initial examination showed that the cells contain gangliosides that cochromatograph with bovine mono-, di-, and trisialoganglioside species (Fig. 1). Some of these species appear as doublets, such as the gangliosides that cochromatograph with GM_1 and GD_{1a}. This is a common occurrence in neuronal cell lines (Dahms and Schnaar, 1983) and has been attributed to differences in the ceramide portion of the molecules (Walton and Schnaar, unpublished observations). Ganglioside doublets

with mobilities similar to those of GD_{1a} and GT_{1b} were purified and subjected to partial acid hydrolysis since each ganglioside species has a distinct partial hydrolysis pattern that can be used for its identification. The patterns yielded by these two gangliosides are consistent with their designation as GD_{1a} and GT_{1b} (Fig. 2). Thus, the N18-RE-105 cell line not only contains the ganglioside (GT_{1b}) thought to be a potent tetanus toxin receptor (Holmgren et al., 1980), but also is one of the few neuronal cell lines whose ganglioside species are similar to those found in mammalian brain.

Although the N18-RE-105 tetanus toxin receptor has been extensively characterized in this report, the major goal of these studies was to provide insight into the events that occur after initial receptor occupancy. The lack of saturability of ^{125}I-toxin binding to intact cells at 37°C, in the absence of any detectable ligand degradation, strongly suggested that internalization of the toxin occurred. In order to investigate this possibility, a technique was developed that effectively differentiates between surface-bound toxin and toxin that has been translocated from the surface. This method exploits the susceptibility of surface-bound toxin to pronase digestion and is analogous to methods from previous reports on the release of surface-bound epidermal growth factor and diphtheria toxin by proteolytic treatment (Aharonov et al., 1978; Dorland et al., 1978). The pronase-resistant radiolabel has been defined operationally as internalized toxin, although it is clear that these experiments cannot distinguish between toxin that is actually internalized and toxin that is sequestered in some other manner. Further studies are needed to explore these possibilities.

This pronase-digestion assay has been valuable in defining characteristics of a tetanus toxin internalization process. First, intact cells are required; ^{125}I-labeled toxin remains on the surface of broken cell membrane preparations (Table 2). Second, toxin internalization is dependent on temperature; little internalization of toxin was observed at 0°C, while as much as 80% of the surface-bound toxin was internalized at 37°C (Table 2). Moreover, temperature pulse studies indicated that the internalization of surface-bound toxin was quite rapid with a half-life of 5 min at 37°C (Figs. 4, 5). Finally, the rapid uptake mechanism is dependent on intracellular ATP. When ATP levels were decreased by 95% by metabolic inhibitors, tetanus toxin uptake levels were reduced to 0°C values (Fig. 6).

It is important to know if the internalization process described here is related to tetanus toxin's mechanism of action *in vivo*. The best demonstration of this correlation would be to show a direct relationship between toxin internalization and an appropriate functional response; that is, in this case, the inhibition of neurotransmitter release. Direct functional studies with the N18-RE-105 cells have not been performed since it has not yet been possible to identify their neurotransmitter characteristics (Malouf et al., 1984a).

Despite this limitation, the high-affinity binding-internalization process that has been quantitated in this report is complementary to and expands on the results of previous studies with analogous systems. Several reports have provided qualitative evidence that primary cultured neurons appear to internalize tetanus toxin in a temperature-dependent manner (Yavin et al., 1981, 1983). Schmitt et al. (1981) have postulated that a temperature-mediated internalization step precedes tetanus toxin-induced blockade of neurotransmission in isolated neuromuscular junctions. Second, there is some evidence that the uptake of Clostridial neurotoxins can be rapid. A rapid internalization (on the order of minutes) of tetanus into primary cultures of spinal cord neurons has been recently observed (Critchley et al., 1985). However, because of the relatively high concentrations of tetanus toxin used in these experiments (66 nM), it is not clear if high-affinity receptors mediate this process. Botulinum toxin becomes inaccessible to antitoxin in neuromuscular junctions 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 toxin into intact neuromuscular junctions. All of these observations can be explained by the mechanisms characterized in this report. Taken together, these results document the relevance of the toxin-entry process identified on N18-RE-105 cells.

This study establishes that N18-RE-105 cells are a valuable system for studying tetanus toxin's mechanism of action. Experiments are now in progress to study in more detail the uptake process and the subcellular localization of internalized toxin.

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