A CORE FACILITY FOR THE STUDY OF NEUROTOXINS OF BIOLOGICAL ORIGIN

Annual Report

February 15, 1992

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Supported by:

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

Contract No. DAMD17-86-C-6161

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**Title:** A Core Facility for the Study of Neurotoxins of Biological Origin

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**Type of Report:** Annual

**Date of Report:** February 15, 1992

**Page Count:** 85

**Subject Terms:**
- Neurophysiology
- Toxin
- RAI
- Central Nervous System
- Mechanism

**Abstract:**
Studies have been done on the variety of toxins that affect the nervous system. The toxins of major interest have been dendrotoxin, tetrotoxin, saxitoxin, botulinum neurotoxin, tetanus toxin, and ceratin. Experiments have been conducted mainly on three types of tissue preparations: 1) brain synaptosomes, 2) phrenic nerve-hemidiaphragm, and 3) cells grown in tissue culture, including neuroblastoma cells and adrenal medullary tumor cells. The major accomplishments have been: 1) isolation of homogeneous preparations of dendrotoxin, 2) partial characterization of dendrotoxin binding sites, 3) partial characterization of tetotoxin and saxitoxin binding properties using channels inserted into lipid membranes, 4) further characterization of the intracellular actions of clostridial toxins, 5) development of binding assays for clostridial toxins, and 6) study of structure-function relationships in clostridial neurotoxins and snake neurotoxins.
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In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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The Core Facility for the Study of Neurotoxins is composed of several scientific projects, and these are supported by a central administrative staff. The scientific projects conduct research that falls into three broad categories: i.) the development of \textit{in vitro} systems to assay toxin activity, ii.) exploratory and definitive studies to determine mechanism of toxin action, and iii.) screening efforts to identify agents that will protect against or reverse the effects of poisoning.

During the past year, a number of new projects have been initiated, as follows:

- Dr. Nabil Bakry has developed techniques for labeling clostridial toxins and for studying their binding to brain membrane preparations. He has found that several previously published and widely used methods for measuring toxin binding are flawed, and he has proposed revised methods that measure binding to authentic receptors.

- Dr. Roger Sorensen has isolated and characterized several dendrotoxins, as well as structurally and functionally similar toxins from other sources. He has used iodinated preparations of these toxins to extract and solubilize receptors, which appear to be potassium channels.

- Dr. Lee Chabala has used the lipid bilayer technique to study the interaction between small molecular weight toxins and ion channels. He has found that several of the physical properties of channels that account for their gating behavior also account for their affinity for neurotoxins.

- Dr. Joseph Sherwin has continued to study the intracellular effects of clostridial toxins, and in the process has developed an improved method for measuring protein kinase activity. His work indicates that toxins alter intracellular disposition of the kinase, although the underlying mechanism remains obscure.

- Dr. Lance Simpson has studied the structure of clostridial toxins, including the native and nicked molecules. He has discovered that there is a structural distinction between activation and aging of the neurotoxins. He has also employed a new method for measuring hydrophobicity of proteins and used this technique to characterize pH-induced changes in toxin structure.
Scientific Progress During the Year

A. Specific Aims

Botulinum neurotoxin and tetanus toxin act on nerve endings to block release of transmitters (Simpson, 1989). The molecular basis for blockade of exocytosis has not been established, but several events involved in the process have been determined (see reviews by Habermann and Dreyer, 1986; Simpson, 1986). Botulinum neurotoxin and tetanus toxin bind with high affinity to receptors on the plasma membrane. During the natural process of poisoning, the receptors of interest are those on peripheral cholinergic nerve endings. However, synaptic membranes of central nervous system origin also possess receptors for clostridial neurotoxins. After binding, the toxins enter nerve endings by the process of receptor-mediated endocytosis. A fall in endosomal pH causes the toxins to expose hydrophobic domains, and these domains facilitate transfer of toxin molecules from the endosomal compartment to the cytosol. During the final step in the sequence, botulinum neurotoxin and tetanus toxin act inside the cell to modify a substrate that governs transmitter release (Bittner et al., 1989 a,b).

Numerous attempts have been made to develop broad spectrum antagonists of clostridial neurotoxins, especially those that would prevent toxin association with nerve endings, but these efforts have met with little success. For example, several laboratories have tried to generate antibodies that recognize more than one toxin and that produce universal neutralization, but no epitopes have been found that are common to all toxins and no antibodies have been identified that have a universal effect. In a similar vein, efforts have been made to find drugs that prevent receptor binding by all serotypes of botulinum neurotoxin as well as tetanus toxin, but again there have been no reports of success.

In spite of the real and assumed difficulties in finding drugs that block toxin association with nerve endings, the authors have been systematically testing a host of agents. One of the approaches
has been to examine drugs that interact with carbohydrates in general and with sialic acid in particular. This search has been based on the premise (see Discussion) that toxin receptors may not be identical but they may possess certain elements of commonalty (viz., receptors are sialoglycoproteins).

In the report that follows, data are presented that show that two lectins, one of plant origin and the other of animal origin, are capable of blocking toxin action. These lectins, which appear to exert their effects at the level of receptors on the plasma membrane, are antagonists of all clostridial neurotoxins.

B. Methods

Lectins, toxins and other reagents. Lectins from Triticum vulgaris, Ricinus communis, Glycine max, Canavalia ensiformis and Anguilla anguilla were purchased from Sigma Chemical Co.; lectins from Limax flavus, Datura stramonium and Artocarpus integrifolia were purchased from E. Y. Laboratories. Botulinum neurotoxin types A and B were isolated and purified by previously described methods (Sakaguchi, 1983). Botulinum neurotoxin types C, D, E and F were purchased from WAKO Pure Chemical Industries. Tetanus toxin was purchased from Calbiochem; β-bungarotoxin, N-acetylglucosamine and N-acetylneuraminic acid were purchased from Sigma Chemical Co. All other reagents were from Fisher Scientific Co. or Sigma Chemical Co.

Binding studies. Proteins were iodinated with Bolton-Hunter reagent according to standard methods. Triticum vulgaris lectin (500 μg), botulinum neurotoxin type B (150 μg) or tetanus toxin (150 μg) in sodium borate buffer (100 mM, pH 7.9) was mixed with 125I-Bolton-Hunter reagent (1 mCi) for 30 minutes at room temperature (final volume, 1 ml). The reaction was terminated by adding 200 mM glycine. The radioiodinated protein was separated from reactants on a Sephadex G-50 column. Protein concentration was determined by the methods of Lowry et al. (1951) or Bradford (1976), and toxicity was bioassayed by the methods of Hardegree (1965) and Kondo et al. (1984). Residual toxicity of iodinated preparations was 65 to 85 percent.
Brain membrane preparations were obtained from adult Sprague-Dawley rats. Dissected brains were washed and homogenized in iced Tris-HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged for 10 min at 1000 x g, and the resulting supernatant was recentrifuged for 45 min at 40,000 x g. The pellets were re-suspended in Tris-HCl buffer (as above).

Binding of toxins and lectins to brain membrane preparations was measured by using a centrifugation assay. Labelled ligand was mixed with the specified amount of membrane protein (see Results) in 1.0 ml of pH 7.4 buffer containing 50 mM Tris-HCl, 100 mM sodium chloride, and 1 mg/ml BSA. The binding reaction was done at 22° C for the amount of time necessary to reach equilibrium, unless otherwise indicated. The reaction was terminated by centrifugation (15,000 x g, two min), after which the pellet was washed and recentrifuged in fresh buffer. The bottom of the microtube was cut, and the amount of 125I-ligand was quantified. Data were corrected for non-specific binding.

The results were evaluated by using the equilibrium binding data analysis program (McPherson, 1982). When more that one binding site was apparent, the SCAFIt program was used for non-linear regression analysis (Munson and Rodbard, 1980)

In Vitro studies. Drug antagonism experiments were conducted on the mouse phrenic nerve-hemidiaphragm preparation (N = 3 or more per experiment). Tissues were excised and suspended in a physiological buffer that was bubbled with 95% O2, 5% CO2 and maintained at 35° C, unless otherwise indicated. The physiological solution had the following composition (millimoles): NaCl, 137; KCl, 5; CaCl2, 1.8; MgSO4, 1.0; NaHCO3, 24; NaH2PO4, 1.0; glucose, 11; and gelatin, 0.01%. Phrenic nerves were stimulated continuously (0.2 Hz square waves of 0.1 to 0.3 msec duration) and muscle twitch was recorded. Toxin-induced paralysis was measured as a 50% reduction in muscle twitch response to nerve stimulation.
C. Results

**Antagonism of toxin binding.** Eight lectins with affinities for various carbohydrates (Table 1) were tested for their abilities to antagonize the binding of 

\[^{125}\text{I}-\text{botulinum neurotoxin type B}\] (0.5 nM) or \[^{125}\text{I}-\text{tetanus toxin}\] (0.3 nM) to brain membrane preparations. Representative findings are presented in Table 2. At a concentration of \(5 \times 10^{-6} \text{ M}\), only two of the lectins produced 50% or greater inhibition of toxin binding. *Limax flavus* lectin (LFL) almost completely abolished binding by the two toxins, and *Triticum vulgaris* lectin (TVL) produced substantial blockade of botulinum toxin binding and approximately 50% blockade of tetanus toxin binding. In control experiments, a molar excess of unlabeled toxin (1 x \(10^{-7} \text{ M}\)) antagonized binding of homologous but not heterologous labelled toxin.

Increasing concentrations of lectins (10^{-8} to 10^{-4} M) were tested for their abilities to antagonize the binding of iodinated toxins (botulinum neurotoxin type B, 0.5 nM; tetanus toxin, 0.3 nM). When tested against botulinum neurotoxin type B, LFL had an IC\(_{50}\) of \(-1 \times 10^{-6} \text{ M}\) and TVL had an IC\(_{50}\) of \(-3 \times 10^{-7} \text{ M}\) (Fig 1A). When tested against tetanus toxin, LFL and TVL had IC\(_{50}\) values of, respectfully: \(-3 \times 10^{-7} \text{ M}\) and \(-3 \times 10^{-6} \text{ M}\) (Fig. 1B). The other six lectins had IC\(_{50}\) values of \(1 \times 10^{-5} \text{ M}\) or greater (results not illustrated).

Experiments were done to characterize more fully the interaction between lectins and clostridial toxins. Various concentrations of \[^{125}\text{I}-\text{botulinum neurotoxin}\] were incubated with brain membrane preparations (200 \(\mu\text{g/ml}\) for 90 min at 22\(^\circ\) C). Scatchard analysis of the data revealed a single binding site with a \(K_d\) of 2.22 nM and a \(B_{\text{max}}\) of 1.3 pmol/mg protein (Fig 2). When the experiment was repeated in the presence of LFL (10^{-6} M), the apparent \(K_d\) was increased almost four-fold (9.45 nM) but the \(B_{\text{max}}\) was unchanged (1.37 pmol/mg protein). Analysis of the results revealed a \(K_i\) of 3.1 x \(10^{-7} \text{ M}\), which is in reasonable agreement with the IC\(_{50}\) value. When the experiment was done in the presence of TVL (3 x \(10^{-7} \text{ M}\), the apparent \(K_d\) was increased almost two-fold (3.91 nM), but again the \(B_{\text{max}}\) was unchanged (1.25 pmol/mg protein). The calculated \(K_i\) for TVL was 3.75 x \(10^{-7} \text{ M}\).
Similar experiments were done with $^{125}$I-tetanus toxin. In this case, brain membranes (50 μg/ml) were incubated with various concentrations of toxin for 30 min at 22° C. Scatchard analysis of the data revealed two binding sites, as follows: high affinity site, $K_d = 0.032$ to 0.040 nM and $B_{max} = 0.35$ to 0.50 pmol/mg protein; low affinity site, $K_d = 0.57$ to 0.83 nM and $B_{max} = 2.15$ to 3.76 pmol/mg protein. Experiments were done in the presence of LFL (2 x $10^{-7}$ M) or TVL (1 x $10^{-6}$ M), and the results were similar to those obtained with botulinum toxin. The lectins increased the apparent $K_d$ for tetanus toxin binding but they had little effect on $B_{max}$ (Table 3). The calculated $K_i$ for LFL-induced inhibition of tetanus toxin binding to the high and low affinity sites was $1.5 x 10^{-7}$ M. The $K_i$ values for TVL were: high affinity site, $1 x 10^{-6}$ M; low affinity site, $1.8 x 10^{-6}$ M. These values approximate those obtained graphically from the concentration-effect curves above (Fig. 1A and 1B).

Characteristics of lectin binding. Limited availability of LFL precluded further studies, but TVL was studied in some detail. When $^{125}$I-TVL (3 nM) was incubated with increasing concentrations of rat membrane protein, there were corresponding increases in the amount of bound lectin (Fig. 3). An apparent plateau was attained at 200 μg membrane protein/ml. A concentration of 20 μg membrane protein/ml was used in subsequent experiments.

When $^{125}$I-TVL (3 nM) was incubated with a fixed amount of membrane protein for varying lengths of time, an equilibrium was attained at approximately 60 min (Fig. 4A). Transformation of the data (Fig 4B) revealed a nonlinear relationship between length of incubation and amount of bound ligand. The data indicate a fast initial rate of association ($t_{1/2} = 0.16 x \text{min}^{-1}$) and a slower subsequent rate of association ($t_{1/2} = 0.045 x \text{min}^{-1}$).

When increasing amounts of $^{125}$I-TVL were incubated (60 min) with a fixed amount of membrane protein (20 μg/ml), the saturation isotherm revealed two binding sites (Fig. 5). The high affinity site had a $K_d$ of 1.4 nM and $B_{max}$ of 75 pmol/mg protein; the low affinity site had a $K_d$ of 57 nM and a $B_{max}$ of 386 pmol/mg protein. A second strategy was used to confirm these constants. Varying concentrations of unlabeled lectin ($10^{-10}$ to $10^{-6}$ M) were mixed with a fixed concentration of labelled lectin (3 x $10^{-9}$ M), and the amount of labelled material bound to
membranes was determined. The results, which were corrected for dilutions in specific activity, are shown in Figure 6. The displacement technique gave the following results: high affinity site, $K_d = 0.44 \text{ nM}$ and $B_{\text{max}} = 55 \text{ pmol/mg protein}$; low affinity site, $K_d = 77 \text{ nM}$ and $B_{\text{max}} = 437 \text{ pmol/mg protein}$.

The specific sugars for which TVL has affinity are N-acetylglucosamine and N-acetylsialic acid. Simultaneous incubation of these sugars and $^{125}\text{I-} \text{TVL}$ with brain membranes led to inhibition of binding. The IC50 values were: N-acetylglucosamine, $3 \times 10^{-3} \text{ M}$; N-acetylsialic acid, $5 \times 10^{-3} \text{ M}$. By contrast, clostridial toxins had only a negligible effect. Even at concentrations as high as $3 \times 10^{-7} \text{ M}$, botulinum neurotoxin and tetanus toxin produced only fractional inhibition of $^{125}\text{I-} \text{TVL}$ binding ($3 \times 10^{-9} \text{ M}$; Table 4).

**Studies on neuromuscular transmission.** Unlabeled lectin (TVL) was added to phrenic nerve-hemidiaphragm preparations at concentrations ranging from $10^{-6}$ to $10^{-3} \text{ M}$, and tissue responses were monitored for at least 200 min. Even at the highest concentration tested, the lectin did not block or enhance neuromuscular transmission (results not illustrated).

When TVL was added to phrenic nerve-hemidiaphragm preparations simultaneously with botulinum neurotoxin type B ($7 \times 10^{-12} \text{ M}$), the lectin produced concentration-dependent antagonism of the onset of toxin-induced paralysis (Fig. 7). At a concentration of $3 \times 10^{-5} \text{ M}$, TVL produced a highly statistically significant effect ($p < 0.001$). When this same concentration of lectin was tested against neurotoxin types A, C, D, E, and F, as well as tetanus toxin, it produced significant antagonism ($p < 0.01$) in all cases (Table 5). By contrast, the lectin did not protect tissues against the neuromuscular blocking effects of a snake neurotoxin ($\beta$-bungarotoxin).

A series of experiments were performed to confirm that TVL was antagonizing clostridial toxins by virtue of blocking the receptor. For this purpose, tissues were incubated at $4^\circ \text{ C}$ during exposure to toxin and/or lectin. Incubation at low temperature allows binding to proceed but it prevents the subsequent step of internalization. After incubation at $4^\circ \text{ C}$ (and see below), tissues
were washed and transferred to baths at 35°C. Phrenic nerves were stimulated and the rate of toxin-induced paralysis was monitored.

In the first experiment, tissues were incubated with or without TVL \((3 \times 10^{-5} \text{ M})\) for 30 minutes, after which botulinum neurotoxin type B \((7 \times 10^{-12} \text{ M})\) was added and tissues were incubated for an additional 60 minutes. Tissues were then washed and suspended in baths, and paralysis times were monitored. The paralysis times of tissues exposed only to toxin was \(90 \pm 5\) min; the paralysis times of tissues exposed first to lectin and then to toxin was \(> 200\) min.

In the second experiment the order of addition was reversed. Tissues were incubated with or without botulinum neurotoxin for 30 minutes, after which TVL was added and tissues were incubated for another 60 minutes. In this case, the paralysis times of tissues exposed only to toxin was \(100 \pm 7\) min, and this was closely similar to the paralysis times of tissues exposed first to toxin and then to lectin \((98 \pm 9\) min).

In the final experiment, a somewhat different approach was used. TVL was incubated with or without N-acetyl-\(\beta\)-glucosamine \((1 \times 10^{-1} \text{ M})\) for 30 min. This mixture was then incubated with tissues for 30 min at 4°C, after which botulinum neurotoxin was added and incubation was continued for another 60 min. After being washed, tissues were suspended in baths and paralysis times were monitored. A control group of tissues that was incubated only with toxin had a paralysis time of \(96 \pm 10\) min. The tissues incubated with lectin in the absence of carbohydrate had a paralysis time of \(> 200\) min; tissues incubated with lectin plus carbohydrate had a paralysis time of \(110 \pm 8\) min.

D. Discussion

Botulinum neurotoxin and tetanus toxin act peripherally and centrally to block the release of transmitter substances (Habermann and Dreyer, 1986; Simpson, 1986). The sequence of events involved in blockade of exocytosis is complex, but it begins with binding of the toxins to presynaptic membranes. It is not known whether receptors in the central nervous system and
peripheral nervous system are the same, nor is there any evidence that the various toxins share the same receptor. Therefore, a ligand binding assay has been used to characterize receptor interactions with nerve membranes of central origin, and a functional assay has been used to study receptor interactions with peripheral tissues. In addition, numerous serotypes of the toxins have been tested.

Although it is unknown whether the central and peripheral receptors for the various toxins are the same, these receptors may share certain properties. For example, there is the possibility that the receptors possess at least one sialic acid residue. This hypothesis stems from the observation that gangliosides, which are sialic acid-containing glycosphingolipids, bind to toxins and cause loss of activity (Simpson and Rapport, 1971; van Heyningen, 1974). It is unlikely that gangliosides are receptors for the toxins, but the ganglioside data could be an indication that other classes of sialic acid-containing molecules, such as glycoproteins, are the true receptors (Middlebrook, 1989).

One test of the hypothesis that sialic acid-containing molecules are receptors for clostridial toxins is to examine lectins as potential antagonists. This is an experimental approach that has drawn only limited attention in the past (Marconi et al., 1982; Evans et al., 1986). In the present study a variety of lectins with differing carbohydrate affinities were examined for their abilities to antagonize iodinated toxin binding to brain membranes, and separately to antagonize toxin action on isolated neuromuscular preparations. The results showed that two lectins with affinity for sialic acid could block the binding of toxins to brain membranes and biologic activity of toxins at the neuromuscular junction. These lectins appear to be the first known universal antagonists for preventing toxin association with nerve membranes.

Lectin-induced antagonism of toxin binding to brain membranes. Among the lectins tested were those that have affinity for α-fucose, α-galactose, β-galactose, α-glucose, α-mannose, N-acetyl-α-galactosamine, N-acetyl-β-galactosamine, N-acetyl-α-glucosamine and N-acetyl-β-glucosamine. None of these lectins was a potent antagonist of botulinum neurotoxin or tetanus toxin, indicating that the carbohydrates for which they have affinity are not accessible components of the binding sites of clostridial neurotoxin receptors. Furthermore, these carbohydrates are not
sufficiently close to receptors such that binding by a lectin could produce steric hindrance of binding by a toxin.

Two lectins, LFL and TVL, were effective antagonists of toxin binding. LFL possesses affinity for N-acetyl-α-sialic acid, whereas TVL has affinity for both N-acetyl-β-glucosamine and N-acetyl-α-sialic acid. However, the fact that the lectin from Datora stramonium was only a poor antagonist appears to eliminate N-acetyl-β-glucosamine from consideration.

Both LFL and TVL antagonized the binding of iodinated toxin to brain membrane preparations. Analysis of the data for high and low affinity sites indicated that the lectins were competitive antagonists. When these experiments were done in reverse the toxins were only modestly effective in antagonizing iodinated lectin binding (TVL), but this was the expected outcome. The lectins can bind to many classes of molecules that have sialic acid, but the toxins can bind only to those molecules that are their receptors. Toxin receptors could represent no more than a fraction of the total sialic acid-containing molecules in the nervous system.

**Lectin Binding.** A detailed discussion of iodinated toxin binding to brain membranes will be presented elsewhere (Bakry at al., submitted for publication), but a brief discussion of iodinated lectin binding is warranted here. Due to a limited availability of the lectin from Limax flavus, most of the work dealt with the lectin from Triticum vulgaris. $^{125}$I-TVL demonstrated all the characteristics of binding to a receptor, including specificity and saturability. When incubated with a fixed amount of membrane protein, the lectin attained equilibrium within 60 min. Interestingly, the data indicated two rate constants for association that differed by 3- to 4-fold. The data also revealed a high affinity and a low affinity binding site with $K_d$ values of 1.4 nM and 57 nM and $B_{max}$ values of 75 pmol/mg protein and 386 pmol/mg protein. No effort was made to determine whether there was a relationship between the two rate constants for association and the two binding sites. Nevertheless, there are a number of reports that confirm that TVL has multiple binding sites (Gurd, 1977; Bharanandan and Katlic, 1979; Wright, 1984; Gallagher et al., 1985).
The carbohydrate specificities for TVL are N-acetyl-glucosamine and N-acetylsialic acid. Both blocked the binding of $^{125}$I-TVL to brain membranes. Inhibition of binding was complete, indicating that both high affinity and low affinity sites were blocked. On the other hand, neither botulinum neurotoxin nor tetanus toxin, when tested at reasonable concentrations, completely blocked TVL binding, as discussed above. At most, the extent of inhibition was only ~20%. This is in keeping with the presumption that toxins bind to only a limited number of sialic acid-containing molecules.

Lectin-induced antagonism of neuromuscular blockade. TVL did not itself alter neuromuscular transmission, but it was an antagonist of the serotypes of botulinum neurotoxin and tetanus toxin. The lectin was effective when incubated with tissues and free toxin, but it was ineffective when incubated with tissue that had already bound toxin. This indicates that the lectin was acting at the receptor rather than some later step in the poisoning process (e.g., endocytosis).

In keeping with its known characteristics, the lectin ceased to be effective as an antagonist when incubated in the presence of a molar excess of N-acetyl-β-glucosamine. Similar experiments could not be performed in the presence of N-acetyl-α-sialic acid, due to the complicating fact that the toxins have affinity for sialic acid in these compounds.

Strategies for implicating sialic acid. There are at least three approaches that could be used to implicate a sialic acid moiety in the toxin receptor. These approaches involve the use of neuraminidase, antibodies against sialic acid-containing molecules, and lectins with affinity for sialic acid. Numerous authors have already tried to use neuraminidase, but the results have been contradictory (for review of literature, see Habermann and Dreyer, 1986; Middlebrook, 1989). The reasons for these problems are not clear, although one issue has drawn considerable attention. Only rarely have investigators shown that neuraminidase treatment produced exhaustive cleavage of sialic acid. In the absence of such data, experimental findings can be difficult to interpret.
The authors are not aware of any systematic efforts to evaluate antibodies as toxin antagonists. However, several groups have reported isolation and characterization of antibodies against sialic acid-containing molecules, and these antibodies should be tested.

Finally, lectins with affinity for sialic acid are obvious candidates for study. In the present report two such lectins have been evaluated, and they have emerged as universal antagonists of clostridial neurotoxins. These are the first compounds to be identified as broad spectrum antagonists of neurotoxin association with nerve membranes.

Additional lectins have been reported to have affinity for sialic acid, and others are likely to be discovered. Ideally, lectins will be found that are specific not only for sialic acid but also for the nature of the linkage between sialic acid and the adjoining carbohydrate residues. Data comparing the ability of these lectins to antagonize toxin binding would help to provide distinguishing characteristics of receptors when they are ultimately isolated.

E. References


<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specific Sugar(s)</th>
</tr>
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<tbody>
<tr>
<td>Anguilla anguilla</td>
<td>α-Fucose</td>
</tr>
<tr>
<td>Artocarpus integrifolia</td>
<td>α-Galactose, N-acetyl-α-galactosamine</td>
</tr>
<tr>
<td>Canavalia ensiformis</td>
<td>α-Glucose, α-mannose, N-acetyl-α-glucosamine</td>
</tr>
<tr>
<td>Datora stramonium</td>
<td>N-Acetyl-β-glucosamine</td>
</tr>
<tr>
<td>Glycine max</td>
<td>N-Acetyl-α-galactosamine, N-acetyl-β-galactosamine</td>
</tr>
<tr>
<td>Limax flavus</td>
<td>N-Acetyl-α-sialic acid</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>β-Galactose, N-acetyl-β-galactosamine</td>
</tr>
<tr>
<td>Triticum vulgaris</td>
<td>N-Acetyl-β-glucosamine, N-acetyl-α-sialic acid</td>
</tr>
</tbody>
</table>
# TABLE 2

The inhibitory effects of various lectins on the binding of iodinated botulinum neurotoxin or tetanus toxin to rat brain membranes

<table>
<thead>
<tr>
<th>Lectin&lt;sup&gt;a&lt;/sup&gt; or Toxin&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percent Inhibition&lt;sup&gt;c&lt;/sup&gt; of Botulinum Toxin Binding&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Percent Inhibition&lt;sup&gt;c&lt;/sup&gt; of Tetanus Toxin Binding&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anguilla anguilla</td>
<td>16 ± 4</td>
<td>6 ± 5</td>
</tr>
<tr>
<td>Artocarpus integrifolia</td>
<td>23 ± 4</td>
<td>3 ± 5</td>
</tr>
<tr>
<td>Canavalia ensiformis</td>
<td>18 ± 5</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Datora stramonium</td>
<td>20 ± 5</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Glycine max</td>
<td>29 ± 2</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>Limax flavus</td>
<td>98 ± 4</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>12 ± 3</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Triticum vulgari</td>
<td>85 ± 3</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>Botulinum Neurotoxin</td>
<td>100 ± 1</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Tetanus Toxin</td>
<td>15 ± 3</td>
<td>100 ± 1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lectins were tested at a concentration of 5 x 10<sup>-6</sup> M
<sup>b</sup> Toxins were tested at a concentration of 1 x 10<sup>-7</sup> M
<sup>c</sup> Values represent mean ± SEM of 3 or more observations
<sup>d</sup> Protein concentration in binding assay was 200 µg/ml
<sup>e</sup> Protein concentration in binding assay was 50 µg/ml
TABLE 3

Binding constants describing the interaction between tetanus toxin and rat brain membranes in the absence and presence of lectins

<table>
<thead>
<tr>
<th></th>
<th>High Affinity Site</th>
<th>Low Affinity Site</th>
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<tbody>
<tr>
<td></td>
<td>( K_d )</td>
<td>( B_{\text{max}} )</td>
</tr>
<tr>
<td></td>
<td>(nM)</td>
<td>(pmole/mg protein)</td>
</tr>
<tr>
<td>Tetanus Toxin(^a)</td>
<td>0.032</td>
<td>0.35</td>
</tr>
<tr>
<td>Toxin + LFL(^b)</td>
<td>0.070</td>
<td>0.35</td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td>0.040</td>
<td>0.50</td>
</tr>
<tr>
<td>Toxin + TVL(^b)</td>
<td>0.080</td>
<td>0.50</td>
</tr>
</tbody>
</table>

\(^a\) Iodinated tetanus toxin was used at various concentrations ranging from 1 \times 10^{-11} M to 1 \times 10^{-9} M. Brain membranes were used at a single concentration (50 \( \mu \)g protein/ml).

\(^b\) Experiments were done in the presence of 2 \times 10^{-7} M LFL or 1 \times 10^{-6} M TVL.
TABLE 4

**Effects of sugars and clostridial toxins on the binding of $^{125}$I-TV to rat brain synaptosomes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylglucosamine</td>
<td>$3 \times 10^{-3}$ M</td>
<td>50 ± 1</td>
</tr>
<tr>
<td></td>
<td>$10^{-1}$ M</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>N-Acetylsialic acid</td>
<td>$5 \times 10^{-3}$ M</td>
<td>50 ± 2</td>
</tr>
<tr>
<td></td>
<td>$1.2 \times 10^{-2}$ M</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td>$3 \times 10^{-8}$ M</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-7}$ M</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>Botulinum neurotoxin B</td>
<td>$3 \times 10^{-8}$ M</td>
<td>24 ± 3</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-7}$ M</td>
<td>25 ± 2</td>
</tr>
</tbody>
</table>
TABLE 5

The amount of time necessary for various toxins to cause neuromuscular blockade in the absence of TVL

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Concentration (amt/ml)</th>
<th>Paralysis Time</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- lectin</td>
<td>+ lectin</td>
</tr>
<tr>
<td>Botulinum toxin type A</td>
<td>0.7 ng/ml</td>
<td>78 ± 4</td>
<td>128 ± 4</td>
</tr>
<tr>
<td>Botulinum toxin type B</td>
<td>1.0 ng/ml</td>
<td>56 ± 10</td>
<td>88 ± 11</td>
</tr>
<tr>
<td>Botulinum toxin type C</td>
<td>5.0 ng/ml</td>
<td>75 ± 14</td>
<td>95 ± 15</td>
</tr>
<tr>
<td>Botulinum toxin type D</td>
<td>0.7 ng/ml</td>
<td>73 ± 5</td>
<td>113 ± 9</td>
</tr>
<tr>
<td>Botulinum toxin type E</td>
<td>2.0 ng/ml</td>
<td>40 ± 6</td>
<td>72 ± 20</td>
</tr>
<tr>
<td>Botulinum toxin type F</td>
<td>8.0 ng/ml</td>
<td>60 ± 5</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td>0.2 μg/ml</td>
<td>80 ± 15</td>
<td>125 ± 15</td>
</tr>
<tr>
<td>β-Bungarotoxin</td>
<td>0.5 μg/ml</td>
<td>52 ± 20</td>
<td>55 ± 17</td>
</tr>
</tbody>
</table>

a Lectin (3 x 10^-5 M) and toxin (concentration above) were added to mouse phrenic nerve-hemidiaphragm preparations as described in the text.

b The amount of time (min; mean ± SEM) necessary for toxin to produce 50% blockade of transmission. Group n = 3 or more per experiment.
FIGURE CAPTIONS

Figure 1. Effect of different concentrations (10^{-8} M to 10^{-4} M) of LFL (∅) and TVL (∅) on specific binding of 125I-botulinum toxin (A) and 125I-tetanus toxin (B). 125I-Botulinum toxin (0.5 nM) was incubated with 200 μg of rat brain membrane protein/assay, 1 ml, for 90 min while 125I-tetanus toxin (0.3 nM) was incubated with 50 μg of rat brain membrane protein/assay, 1 ml, for 30 min., both at 22° C. Nonspecific binding was measured in the presence of 100-fold excess of unlabeled toxin. Each data point is the mean of two experiments done in triplicate.

Figure 2. Scatchard plot of 125I-botulinum toxin specific binding to rat brain membranes in absence or presence of LFL (10^{-6} M; ∅) or TVL (3 x 10^{-7} M; ∅). The experimental conditions were similar to those described for Figure 1. The respective constants are given in the text.

Figure 3. Rat brain membrane protein concentration-dependent specific binding of 125I-TVL (3 nM). The radiolabeled ligand was incubated with increasing amounts of brain protein for 60 min at 22° C. Specific binding was determined by subtracting nonspecific binding measured in the presence of 3 x 10^{-6} M TVL.

Figure 4. Rate of association of 125I-TVL with rat brain membranes; (A) percent of control 125I-TVL (3 nM) specific binding to rat brain membranes (20 μg/assay, ml) versus incubation time at 22° C; (B) pseudo-first-order association rate curve for the same data. Symbols are means of two experiments, each done in triplicate.

Figure 5. Scatchard plot of 125I-TVL specific binding to brain membranes. The experiments were done under the same conditions as in Figure 4, except that incubation
time was fixed at 60 min. The insert illustrates concentration-dependent specific binding of the lectin to membranes. Scatchard analysis revealed the presence of two classes of binding sites. The respective constants for the binding sites are given in the text.

**Figure 6.** Displacement of specific binding of $^{125}$I-TVL (3 nM) by increasing concentrations of unlabeled lectin (10$^{-10}$ M to 10$^{-6}$ M). Experimental conditions were similar to those described for Figure 5. Each data point is the mean of two experiments done in triplicate.

**Figure 7.** The effect of increasing concentrations of TVL (10$^{-6}$ M to 3 x 10$^{-4}$ M) on the paralysis times induced by botulinum neurotoxin type B. Experimental details are presented in the text. Each data point is the mean ± SEM of three or more experiments.
A. Specific Aims

The aims of this project are to describe the molecular interactions between several snake venom polypeptides, dendrotoxins, isolated from green mamba, *Dendroaspis angusticeps*, venom and potassium (K) channels. Specifically, these aims include:

1. **Structural identification of the toxin binding site of dendrotoxin-sensitive K channels;** and

2. **Structural identification of the K channel recognition sites of the dendrotoxins.**

For the first aim, cloned K channels that are sensitive to block by the dendrotoxins are identified. The toxin binding sites are determined by preparation of chimeric channel proteins, and by site-directed mutagenic methods.

For the second aim, synthetic toxin genes are prepared. The recombinant toxin polypeptides are overexpressed and isolated. Finally, the toxin structure is manipulated at the DNA level by site-directed mutagenesis.

B. Background

Many K channels have been cloned that can be distinguished by their amino acid sequences, their gating kinetics and their pharmacologies. The differences in toxin sensitivity between these cloned K channels can be used to identify the toxin binding sites of the channel molecules. For example, the Shaker B (ShB) K channel is sensitive towards block by charybdotoxin (ChTX). Using site-directed mutagenesis methods, MacKinnon and colleagues (4,5) made several amino acid substitutions that resulted in changing the affinity
of ChTX for block of the K channel, thereby supporting the involvement of these amino acids for ChTX binding.

Random site-directed mutagenesis methods have also been used to determined the external and internal binding sites for tetraethylammonium (TEA) on Shaker K channels (2,3). However, having several K channel clones with greatly differing sensitivities towards block by a toxin can simplify the choices of those amino acids to alter. Kavanaugh et.al. (1) used sequence data between a TEA-sensitive and a TEA-insensitive K channel to identify amino acids that could be responsible for TEA binding. Initially, recombinant chimeric channels between the two K channels of differing toxin sensitivities were prepared. From the chimeric channels, it was determined that the external TEA binding site was located on the C-terminal half of the TEA-sensitive channel. The C-terminal half contains the putative ion pore sequence of the channel. Comparison of the ion pore sequences between the two K channels revealed 7 amino acid differences. Of these 7 residues, substitution of one, val-379 (found in the TEA-insensitive clone), for tyr (at the equivalent position of the TEA-sensitive channel) reduced TEA affinity for the TEA-sensitive channel by 300-fold. The reverse modification to the TEA-insensitive clone likewise re-established TEA sensitivity to this otherwise TEA-insensitive channel.

The above studies show one method, altering K channel structure, by which toxin/K channel interactions can be studied. However, it should be possible to do the converse, that is, make structural modifications to the toxins themselves. In this case, DNA encoding the sequence of the toxin could be obtained by normal methods of probe hybridization screening of cDNA or genomic libraries prepared from the venomous animal. Yet, because these toxins are relatively small polypeptides, it may be possible to directly synthesize the DNA for the toxins. The latter method was employed by Park et.al. (7) to prepare a synthetic gene for ChTX.
Both K channel and dendrotoxin (DaTX) modifications, to describe dendrotoxin/K channel interactions, were addressed in this project.

C. Methods

Methods for the preparation of cDNA, and for the expression and recording of cloned K channels from Xenopus oocytes have been described in previous quarterly reports.

**Chimeric channels:** Bst BI chimeric K channels were prepared by replacing both N-terminal and C-terminal DNA sequences between the dendrotoxin-insensitive K channel, Kv1, and the dendrotoxin-sensitive K channel, Kv3. N-terminal sequences of the cloned K channels were collected between a Sfi I restriction site located in the polycloning region of the vector [modified pGEM-9Zf(-), ref. 6], upstream from the 5' end of the channels, and a Bst BI site common to both channels. The N-terminal sequence from one channel was isolated by agarose gel electrophoresis and subsequently subcloned into the same site of the other channel. Similarly, C-terminal sequences of the channels were collected between the Bst BI site and a Spe I site located in the polycloning region of the vectors, downstream from the 3' end of the channel inserts. The ligated plasmid vectors containing the chimeric inserts were used to transform competent JM109 cells. cDNAs were prepared and expressed in Xenopus oocytes as described for the unmodified cloned K channels.

**Synthetic dendrotoxin gene:** The synthetic dendrotoxin gene was assembled by the sequential ligation of 12 overlapping oligonucleotides (figure 1). The DNA was cloned into the Eco RI and Pst I sites of the filamentous bacteriophages M13mp18 and M13mp19, which were used to prepare single stranded DNA for sequencing to confirm the proper construction of the dendrotoxin gene.

The dendrotoxin gene was removed from the M13 vectors by restriction endonuclease digestion, isolated by agarose gel electrophoresis, and directly subcloned into the bacterial
expression vectors pKK233-2 and pPROK-1 (Clontech Laboratories, Palo Alto, CA) via Nco I/Pst I and Eco RI/Pst I sites, respectively.

The dendrotoxin gene was subcloned into pMAL-c2 and pMAL-p2 vectors (New England Biolabs, Beverly, MA) by the following procedure. A blunt 5' end of the dendrotoxin gene, necessary for blunt end ligation into a Xmn I site, was prepared by the polymerase chain reaction. A 18-base oligonucleotide coding from the 5' glu amino acid of the dendrotoxin sequence, and the universal reverse (40) primer, which anneals downstream of the 3' end of the toxin sequence, were used to amplify the dendrotoxin gene sequence. The amplification product was collected by agarose gel electrophoresis and digested with Pst I to prepare a 3' sticky end. The cut product was ligated into Xmn I/Pst I cut pMAL vectors. The ligated pMAL vectors containing the dendrotoxin gene inserts were used to transform several E. coli host strains (TB1, TG1, DH5a).

IPTG (isopropyl-b-D-thiogalactopyranoside)-induced expression of the dendrotoxin gene from the bacterial expression vectors was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10-15% acrylamide gels.

D. Results

Dendrotoxin-sensitive K channels: Four mammalian cloned K channels, the "delayed-rectifier-type" channels, Kv1, Kv2, Kv3, and the "A-type" channel, mShal, have been expressed in Xenopus oocytes and K channel currents measured by two electrode voltage-clamp. These currents are shown in figure 2.

Figure 3 shows that Kv2 and Kv3 K channels are sensitive to block by DaTX (alpha-DaTX). Kv1 and mShal were insensitive to DaTX block.
Chimeric K channels: We have begun preparing chimeric channels as a first step towards identifying the DaTX binding site on the toxin-sensitive (Kv2, Kv3) K channels. A chimeric channel was prepared by ligating DNA sequences of the DaTX-insensitive K channel, Kv1, with DNA sequences of the DaTX-sensitive K channel, Kv3, through a common Bst BI restriction endonuclease site located just prior to the first putative membrane spanning domain, S1, of the channels. Block by DaTX was observed for those chimeric channels retaining the C-terminal portion of Kv3. This result is in agreement with other toxin binding results that have shown TEA (1-3) and ChTX (4,5) binding to be influenced by mutations of amino acids located in the S5-S6 linker region of the K channels. This region would reside on the C-terminal parts of the Bst BI chimeric channels.

Synthetic dendrotoxin gene: A synthetic gene for DaTX has been assembled from 6 pairs of overlapping oligonucleotides that encode the entire coding sequence (fig. 1). The DNA sequence also includes restriction endonuclease sites at both 5' and 3' ends for cloning into plasmid vectors, and both start and stop codons. The dendrotoxin gene was initially cloned into the Eco RI and Pst I sites of the vectors M13mp18 and M13mp19, and the assembled sequence confirmed by DNA sequencing.

The dendrotoxin gene has been subcloned into several expression vectors as both non-fusion and fusion proteins for the purpose of overexpressing the recombinant protein. The gene was cloned into the Nco I and Pst I sites of pKK233-2, and the Eco RI and Pst I sites of pPROK-1. These bacterial expression vectors contain IPTG-inducible promoters that regulate expression of foreign genes as non-fusion proteins. Unfortunately, the dendrotoxin gene appears to be toxic to the host (E. coli strains TB1, DH5a) cells. SDS-PAGE failed to show a IPTG-induced increase in expression of a 7000 dalton polypeptide as expected for dendrotoxin, and cell growth (plasmid recovery) was greatly restricted in the transformed cells containing the inserted gene.
The dendrotoxin gene was subcloned into both pMAL-c2 and pMAL-p2 vectors as described in METHODS. These bacterial expression vectors produce fusion of foreign proteins linked to maltose binding protein (MBP). Expression is regulated under the IPTG-inducible tac promoter. Additionally, the pMAL vectors contain the sequence Ile-Glu-Gly-Arg located downstream from the MBP protein. This sequence is recognized by the blood coagulation Factor Xa protease, which enables the foreign protein to be cleaved and isolated from the MBP fusion. The MBP-dendrotoxin fusion protein has been expressed from both pMAL vectors. We are presently optimizing conditions for the isolation of the recombinant toxin from the fusion.

F. References


Figure 1: Synthetic alpha-DaTX

**EcoRI  BamHI Met Glu Pro Arg Arg Lys Leu**

A1 5’ - AATTCCGGATCCATGGGCGCGCCGGAAGCT
A2 3’ - GCTAGCTACCTCTGCCGCCTGCTGATACG

Cys Ile Leu His Arg Asn Pro Gly Arg Cys Tyr Asp

B1  A TGC ATC CTG CAT AGG AAC CCT GCC CGG TGC TAT G
B2  TAG GAC GTA TCC TTG GGA CCG GCC ACG ATA CTG TT

Lys Ile Pro Ala Phe Tyr Tyr Asn Glu Lys Lys

C1  AC AAG ATT CCT GCC TTC TAC TAC AAG CAG AAG AA
C2  C TAA GGA CGG AAG ATG ATG TTG GTC TTC TTT TTC

Gln Cys Glu Arg Phe Asp Trp Ser Gly Cys Gly

D1  A AAG CAG TGT GAG AGG TTT GAC TGG AGT GCC TGG TG G
D2  GTC ACA CTC TCC TAC ATG ACG GCC CAG ACA CCG CCG

Gly Asn Ser Asn Arg Phe Lys Thr Ile Glu Glu Cys

E1  C GCC AAC AGT AAC AGG TTT AAG ACC ATT GAG GAG
E2  TG TCA TGG TTC AAA TCC TGG TAA CTC CTC AC GC

Arg Arg Thr Cys Ile Gly * KpnI PstI

F1  TGC CGG CGG ACC TGC ATT GCC TAA GGTACC CTGCA - 3’
F2  CC GCC TGG ACG TAA CCG ATT CCATGG G - 5’

Shown are the amino acid and deduced DNA sequences of the synthetic DaTX gene. Six pairs of complimentary oligonucleotides (A thru F) were ligated via four base 5’-overhangs. Eco RI/Bam HI (5’-terminal) and Kpn I/Pst I (3’-terminal) recognition sequences have been added for cloning into expression vectors. The sequence also includes start and stop codons.
Figure 2: Expression of Cloned K Channels in Xenopus Oocytes

A. Families of macroscopic outward currents for Kv1, Kv2 and Kv3 in response to 15 depolarizing steps (300 ms in duration, 10 s between pulses) are shown in the top three panels from a holding potential of -90 mV. The test potential was stepped from -90 mV to +50 mV in 10 mV increments. Current traces were filtered at 2 kHz. Capacity compensation was not employed, but series resistance compensation (averaging about 660 ohms) was used. B. A fit of the normalized peak conductance vs. membrane potential to a simple two-state Boltzmann function. The gating parameters (V, the midpoint potential; K, the slope factor) are as follows: for Kv1 (open squares), V = -40.9 mV and K = 11.0 mV/e-fold change; for Kv2 (open circles), V = -2.9 mV and K = 9.3 mV/e-fold change; and for Kv3 (closed circles), V = -6.7 mV and K = 6.3 mV/e-fold change. C. A family of mial currents induced by 15 steps (one second duration) from -90 mV to +50 mV in 10 mV increments is shown in the inset. Normalized peak G-V relationship is shown in the main figure with a single Boltzmann fit, V = -17.4 mV and K = 11.9 mV/e-fold change.

Figure 3: Block of Kv1 and Kv3 by alpha-DaTX

Dansh current traces for Kv1 in the absence (A) and presence of 100 nM alpha-DaTX (B), and current traces for Kv3 in the absence (C) and presence of 100 nM alpha-DaTX (D). After addition of the toxins, the currents begin to decline. Alpha-DaTX blocks both Kv1 and Kv3 currents at membrane potentials suggesting a voltage-dependent effect.
Lee D. Chabala, Ph.D.
Assistant Professor of Medicine
Annual Report for Core Facility

A. **Specific Aims.** As previously indicated.

B. **Methods.** As previously outlined.

C. **Results.** The two sections below describe some of our observations on cloned and expressed K⁺ channels that are being prepared for publication (cf. Chabala & Soren-sen, 1991; Sorensen & Chabala, 1991).

1) **Gating Properties of the Kv Clones.** We characterized the macroscopic kinetic properties of three cloned rat brain K⁺ channels referred to as Kv1, Kv2, and Kv3). Those three clones have only a few unmatched residues in the entire S4-S6 region, although the gating properties are quite distinct. Our objective is to characterize functional regions and amino acid residues that give rise to the different gating properties. Toward that end we have studied the wild-type channels and chimeric channels in which different regions of one clone are transferred to another clone. We are also in the process of preparing point mutations in each of the clones.

a) Kv1 shows the most rapid activation followed by slowly developing inactivation (Figs. 1 & 2). Recovery from inactivation is (comparatively) rapid and is usually complete within 8 sec (not shown). Steady-state inactivation is incomplete at depolarized membrane potentials (Fig. 2).

b) Kv2 has much slower rates of activation but shows inactivation rates and recovery rates that are similar to Kv1 (Figs. 1 & 2). Steady-state inactivation is incomplete at positive membrane potentials, and the channel apparently reopens at large positive potentials (Fig. 2). The reopening may reflect a redistribution of occupancy such that the steady-state probability of being open is increased.

c) Kv3 shows intermediate rates of activation and pronounced inactivation (Figs. 1 & 2). Onset of inactivation is relatively rapid, but recovery is so slow that 20-25
sec are required between pulses in standard I-V protocols. Development of inactivation leads to a loss of the inactivating component of the current (Fig. 2C).

d) Gating parameters (slope factors and midpoint potentials) are unstable when estimated from conductance-voltage (g-V) relations (Fig. 1). More stable estimates of the gating parameters are obtained when a prepulse inactivation (PPI) protocol is used (Fig. 2). When a PPI protocol is used, the slope factors converge to a common value of 4-5 mV/\text{e-fold change} for each of the Kv clones, and the midpoint potentials are shifted about 30 mV in the hyperpolarizing direction relative to midpoint potentials of the g-V curves.

e) Chimeric channels formed by swapping a restriction fragment just prior to the S1 region between Kv1 and Kv3 transfer essentially all of the observed gating properties to the chimeric channel with the C-terminal region of Kv1 or Kv3. Thus, the regions modulating the differences in gating properties appear to be determined downstream from the restriction site.

2) Neurotoxin and Pharmacological Sensitivity in the Kv Clones. The sensitivity to different neurotoxins in identical or nearly identical cloned K+ channels often varies by a factor 10-1000 in different laboratories. The reasons for these large differences are not clear, but it is clear that the differences must be resolved if the various neurotoxin receptor sites are to be mapped out. Rather large differences in pharmacological profiles have also been reported. During routine screening of the three Kv clones, Kv1, Kv2, and Kv3, we encountered several apparent contradictions with respect to neurotoxin and pharmacological sensitivity published by several other groups. The present work identifies some of the apparent contradictions and offers suggestions that may explain some of the differences. Many of the studied neurotoxins are postulated to bind in the H5 and adjacent linker regions, although it is difficult to believe that the very large differences in sensitivity to the toxins shown below for the Kv clones are completely explained by the few
amino acid differences in the adjacent L5 and L6 regions. Our objective is to map out regions that modulate pharmacological and neurotoxin sensitivity. We have studied wild-type channels and chimeric channels that transfer pharmacological and toxin sensitivity from one clone to another, and we are in the process of preparing point mutations to further identify the functional regions involved.
a) Kv2 and Kv3 are both sensitive to dendrotoxin (α-DTX) and to β-bungarotoxin (β-BuTX), while Kv1 is insensitive to those toxins. Other groups, however, report that Kv3 is not sensitive to α-DTX. We have tried samples of α-DTX prepared by two different groups and find similar sensitivities. The origin of the differences in α-DTX sensitivity are not clear. We have, however, shown that the currents from Kv2 and Kv3 show progressive growth in some cases, and that may account for some of the different observations. Stripping the oocyte of the vitelline envelope increases the apparent sensitivity to α-DTX by a factor of perhaps 2-3 fold.
b) We have observed complete toxin failures in several cases. Those observations may or may not be explained by observed progressive growth in the current.
c) Kv3 is sensitive to charybdotoxin (CTX), but we find that clone is considerably less sensitive than reported by other groups.
d) We also find a large fraction of toxin-insensitive current at high toxin concentrations. A consequence of this finding is that the binding isotherms are fit better with a single-site model plus a constant. A force-fit to a single site model without a baseline leads to a very large decrease in the apparent toxin sensitivity. Part of the residual current is probably due to progressive growth in the current, but that does not appear to be the entire explanation. A single-site model without a baseline, however, generally gives good fits for TEA and 4-AP.
e) α-DTX, β-BuTX, and CTX all appear to remove or slow down the inactivation normally seen in Kv3. This effect on gating can be reversed when the oocyte is washed with toxin-free solution.
f) 4-AP and TEA also remove or slow down inactivation in Kv3. TEA has a similar
effect on Kv1 when longer (10-sec) current pulses are recorded.

g) There is a surprisingly large (=200 fold) difference in TEA sensitivity between
Kv1 and Kv2 (Table 1). Kv2 is very sensitive to TEA at low concentrations.

h) All of the Kv clones appear to be insensitive to MCDP and to IBTX (see Table 1),
although other groups report that Kv2 is rather sensitive to MCDP.

i) Chimeric channels formed by swapping N- and C-terminal regions of Kv1 and Kv3
transfer pharmacological and toxin sensitivity to the construct with the wild-type
C-terminal region. This is in agreement with work reported by other groups.

Table 1

<table>
<thead>
<tr>
<th>cRNA</th>
<th>4-AP (mM)</th>
<th>TEA (mM)</th>
<th>DTX (nM)</th>
<th>β-BuTX (nM)</th>
<th>CTX (nM)</th>
<th>IBTX (nM)</th>
<th>MCDP (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1</td>
<td>0.43</td>
<td>180</td>
<td>&gt;200</td>
<td>&gt;400</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Kv2</td>
<td>0.22</td>
<td>1</td>
<td>25</td>
<td>90</td>
<td>&gt;200</td>
<td>&gt;200</td>
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<tr>
<td>Kv3</td>
<td>0.52</td>
<td>20</td>
<td>24</td>
<td>20</td>
<td>25</td>
<td>&gt;200</td>
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<tr>
<td>S/B31 (chimer)</td>
<td>22</td>
<td>30</td>
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<tr>
<td>S/B13 (chimer)</td>
<td>175</td>
<td>&gt;200</td>
<td></td>
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</table>

Complete I-V protocols were recorded for 1 sec test pulses from -90 to +50 mV fol-
lowing the addition of each pharmacological agent or toxin. The numbers in the
table refer to IC₅₀ values computed from binding isotherms at +50 mV. The data for
4-AP and TEA were fitted to a single binding isotherm. In the case of α-DTX, β-
BuTX, and CTX, however, there is substantial residual toxin-insensitive current at
high toxin concentrations. The data for toxin-induced current inhibition were thus
fitted to a single isotherm plus a constant. A 3 ml solution containing the pharma-
cological agent or toxin was perfused into the chamber. 4-AP was varied in steps of
0.2, 0.4, 0.6, 0.8, and 1.0 mM. TEA was substituted for an equivalent amount of
NaCl such that [TEA] + [NaCl] = 96 mM. The solutions contained 0.312, 0.625, 1.25,
2.5, 5, 10, 20, 40, or 80 mM TEA. α-DTX was purified from the venom of the green
Mamba. Two sources of the purified toxin were used. The first toxin sample was
purified by Dr. Sorensen at Jefferson, while the second sample was purified in Prof.
Blausteins' laboratory. Similar methods were used in each case to fractionate the
venom, but other groups have found different results using the Baltimore toxin.
Both purified toxins had identical effects on Kv2 and Kv3 currents.
FIGURE 1. Voltage dependence of conductance activation. A) The top panel shows the evoked currents (250 msec pulses, 20 sec recovery time), the reversal potential determination (250 msec pulses, 20 sec recovery time) from tail currents (-70 to -75 mV), and the normalized peak conductance-voltage curve for Kv1. B) The middle panel shows the data and plot for Kv2. C) The bottom panel shows the data and plot for Kv3. Notice that Kv3 shows full recovery during each prepulse during the reversal potential determination. As a first approximation, a Marquardt least-squares fit of the data to a single Boltzmann function was used to estimate the gating parameters, which include the midpoint potential (V_m) and the slope factor (k). In this experimental protocol, the slope factors (k) show considerable variation, which would not be expected from channels with identical S4 regions (i.e., the putative voltage sensing region). And estimates of the midpoints of voltage activation (V_m) are quite positive. The currents, however, are clearly not in a steady-state, so it is not surprising that the estimates of the gating parameters are unstable using this protocol. The fits can be improved by using multi-level Boltzmann functions, but that does not cause the slope factors to converge to a common value. Much better estimates of the gating parameters are obtained by using a prepulse inactivation protocol as in Fig. 2.
FIGURE 2. Prepulse-inactivation (PPI) protocol. A) Top panel shows a 10 sec prepulse followed by a 500 msec test pulse to 0 mV, a time expansion of the test pulse region, and the normalized PPI curve for Kv1. B) Middle panel shows data and plot for Kv2. C) Bottom panel shows data and plot for Kv3. Data were fitted to a single Boltzmann function plus a constant. Using this protocol, the currents are more nearly in a true steady-state. Transitions after channel opening are assumed to be voltage independent (cf. Zagotta & Aldrich, 1990), so the PPI protocol is a measure of channel activation. Slope factors are similar for the three Kv clones, which have identical S4 regions. The midpoint potentials are 20-30 mV hyperpolarized with respect to estimates from the g-V curves shown in Fig. 1. The midpoint potentials, however, show more variation than is suggested by the figures (Kv3 is usually more depolarized). In this case the last 30 points in each current trace were averaged as an estimate of the steady-state current, although the currents can be measured at any point during the test pulses with similar results. Estimates of the gating parameters were nearly identical, for instance, when Kv3 was measured at the peak level (Vₐ = -24.6 and k = -3.8), although the steady-state level of current was reduced to 0.16. Kv1 and Kv2 show a similar large residual steady-state level of current, but Kv2 appears to reopen at more positive potentials. Kv3 shows a 2-3 fold lower level of steady-state current but does not appear to reopen at positive potentials.
Joseph R. Sherwin, Ph.D.
Associate Professor of Physiology

Scientific Progress During The Year

A. Specific Aims

The overall objective of this project is to screen toxins for actions mediated through cyclic AMP, cyclic GMP or other second messenger systems. As part of this objective toxins are tested on either untreated cells, cell fractions or cells treated with various hormonal or pharmacological agents. In this manner we hope to obtain cells or cell systems that respond to the neurotoxins so as to be able to determine their mechanism of action. During the last year experiments were conducted to:

1. Further examine the effect of tetanus toxin pretreatment on the activity of membrane associated PKC induced by direct addition of 10 μM neurotensin.

2. Characterize K+-mediated PKC activation and the effect of tetanus toxin pretreatment on this depolarization-induced mobilization.

3. Assess the effect of botulinum C2 toxin on acetylcholine release.

4. Examine the effect of C2 toxin-mediated inhibition of 14C-acetylcholine release on the activity of membrane associated PKC.

5. Characterize the effect on membrane PKC activity of the direct addition of tetanus toxin to the cytosol of digitonin-permeabilized cells.

6. Screen the snake neurotoxin β-bungarotoxin for an effect on K+-induced 14C-acetylcholine release from NG-108 cells.

7. Examine the activity of membrane PKC following pretreatment with Botulinum neurotoxin A.

8. Characterize the effect of PKC inhibitors on membrane PKC using the synthetic peptide/permeabilized cell assay
B. Methods

Membrane PKC Activity  Membrane PKC activity was measured using the permeabilized cell/KRTLRR peptide assay described in previous reports. The cells were pretreated 2 h with $10^{-10}$M tetanus toxin, or 3 h with $10^{-9}$M C2 toxin in serum free culture medium. The cells were then stimulated with $0.1 \mu$M PMA, 10 $\mu$M neurotensin, or 44 mM K+ by direct addition of the agent to the toxin-containing medium. This medium was then removed and replaced with a digitonin-supplemented buffer containing calcium, ATP and the KRTLRR peptide. Phosphorylation of the peptide is a measure of membrane PKC activity in that the enzyme is only active in the presence of the phospholipids at the cell membrane.

Direct Addition of Tetanus Toxin/Membrane PKC  For these experiments, tetanus toxin and PMA were added directly to the digitonin-supplemented buffer described above. Therefore, the PMA-mediated increase in membrane PKC activity occurred during the 10 min permeabilization and peptide phosphorylation reaction period. The digitonin permeabilization, which occurs almost immediately, provided access for tetanus toxin to the cytosol and therefore, any tetanus toxin effect on membrane PKC activity also occurred during the 10 min reaction period.

14C-acetylcholine release  Acetylcholine release was measured by modification of the method of McGee et al. (1978). NG-108 cells were grown in 60 mm dishes and differentiated for 6-10 days with dibutyryl cAMP (dbcAMP) as previously described. The cells were labelled with $1.5 \times 10^{-6}$M [methyl 14C]-choline (50mCi/mmol, NEN) in differentiation medium containing dbcAMP for 36-48 h prior to release studies. Following the labelling period, the radioactive medium was removed and replaced with serum-free DMEM containing C2 toxin or 3-bungarotoxin. After toxin exposure, the cells were removed from the dish by gentle pipetting and washed 3 times by centrifugation and resuspension in DMEM containing 0.1 mM eserine sulfate. To induce depolarization, 44 mM NaHCO3 in the DMEM was replaced with 44 mM KHCO3. The cells were stimulated in suspension for 5 min, spun down, and an aliquot of the
release medium taken for determination of $^{14}$C-acetylcholine content. This aliquot was treated with choline kinase to phosphorylate any $^{14}$C-choline present, and the $^{14}$C-acetylcholine extracted from the reaction mixture with 30 mM tetraphenylboron in 3-heptanone. An aliquot of the organic phase was counted for $^{14}$C and the data normalized to cellular protein.

C. Results

Further examine the effect of tetanus toxin pretreatment on the activity of membrane associated PKC induced by direct addition of 10 µM neurotensin.

In a previous report we provided preliminary data that suggested that the basal activity of PKC in NG-108 cells fluctuated as a function of medium changes. We further demonstrated that direct addition of PMA to the pretreatment medium, rather than addition in fresh culture medium, resulted in a greater mobilization of PKC to the membrane. Since medium changes appear to influence membrane PKC activity, we reassessed, by direct addition, the effect of neurotensin on PKC mobilization. As shown in Table 1, using the direct addition protocol, the basal level of membrane PKC activity was 4276±1002 cpm/mg/10 min. A 5 min exposure to 10 µM neurotensin significantly increased the membrane PKC activity ($p<0.005$; Student's paired t-test comparison). A 2 h pretreatment with 10^{-10}M tetanus toxin had no effect on the basal level of membrane PKC. Although neurotensin significantly increased membrane PKC activity in control cells, the ability of the hormone to increase membrane PKC in toxin-pretreated cells was completely attenuated. This is in agreement with our previous observation, obtained by addition of neurotensin in fresh culture medium, as well as the data obtained by measurement of the cytosolic activity in the presence of toxin and neurotensin.

Characterize $K^+$-mediated PKC activation and the effect of tetanus toxin pretreatment on this depolarization-induced mobilization.

To depolarize the cells we added 10 µl of 4.4 M KCl directly to the pretreatment medium (serum-free DMEM) to obtain a final
concentration of 44 mM K+. Control cells received 10 μl of 4.4 M NaCl. The cells were depolarized for 2 min, the medium removed and membrane PKC activity measured with the peptide assay.

In contrast to other stimulators, the PKC mobilization induced by high K+ was quite variable. Thus, as shown in Table 2, in 7 out of 15 experiments, K+ induced a significant increase in membrane PKC. In these 7 experiments, a 2 h tetanus toxin pretreatment, while not significantly altering basal PKC activity, did completely inhibit the K+-induced increase in membrane PKC. This is in agreement with our previous observations that tetanus toxin can block the increase in membrane PKC activity induced by PMA and neurotensin.

In an additional 8 experiments, K+ had no effect on membrane PKC. Thus, when all 15 experiments are included in the analysis, K+-induced membrane PKC activity was 7216±1539 pmol/mg/10 min compared to 5873±911 pmol/mg/10 min in the presence of 44 mM Na+. The activity in the presence of Na+ was slightly elevated over that from cells not exposed to additional ion (5251±869 pmol/mg/10 min).

Assess the effect of botulinum C2 toxin on acetylcholine release.

Botulinum C2 toxin is an ADP-ribosyltransferase toxin that modifies intracellular actin resulting in the depolymerization of the stress fibers and microfilaments. Since actin and the cytoskeleton have been implicated in the secretory process, we examined the effect of C2 toxin on the release of acetylcholine from NG-108 cells. As shown in Table 3, depolarization of the prelabeled cells with 44 mM K+ resulted in a significant release of 14C-acetylcholine (p<0.05; Student's paired t-test). A 3 h exposure to 10^-9 M C2 toxin did not appear to alter the basal release of acetylcholine. However, depolarization induced release was significantly attenuated following the toxin pretreatment period.

Examine the effect of C2 toxin-mediated inhibition of 14C-acetylcholine release on the activity of membrane associated PKC.

We examined the effect of C2 toxin on membrane PKC activity to determine if inhibition of secretion itself was sufficient to produce the alteration in PKC metabolism we have observed following tetanus toxin treatment. As shown in Table 4, a 15 min exposure to 0.1 μM PMA
resulted in a significant increase in the membrane associated PKC activity (p<0.05; Student's paired t-test comparison). Following a 3 h pretreatment with C2 toxin, the basal membrane PKC activity was elevated to almost the same level as that obtained with PMA alone. Although C2 toxin treatment elevated the basal membrane PKC activity, this increase was not significant in 5 experiments (Student's paired t-test comparison). Addition of 0.1 μM PMA to cells pretreated with the toxin did not elevate the membrane PKC to a level greater than that observed for the toxin treated controls. Because of the increase in the basal membrane PKC activity with C2 toxin treatment, it is not possible to assess whether the ability of PMA to translocate PKC has been compromised. It appears that C2 toxin stimulates the translocation of PKC, perhaps through its known effects on the cytoskeleton. Although, additional experiments are necessary to establish whether the C2 toxin-mediated increase in the basal PKC activity is significantly different from control, it appears that the effect of C2 toxin on PKC metabolism is different than that observed for tetanus toxin. This would suggest that inhibition of acetylcholine secretion, in and of itself, is not sufficient to produce the alteration in PKC metabolism that we attribute to tetanus toxin in this cell line.

Characterize the effect on membrane PKC activity of the direct addition of tetanus toxin to the cytosol of digitonin-permeabilized cells.

Membrane PKC activity is measured in the permeabilized cell assay in the presence of 50 μg/ml digitonin which creates large pores or holes in the plasma membrane. It was therefore of interest to test the hypothesis that direct addition of tetanus toxin to the cytosol would result in an attenuation of PMA-induced PKC mobilization during the 10 min reaction period. For this reason, tetanus toxin and PMA were added directly to the reaction mixture used to quantify membrane PKC activity. As shown in Table 5, 0.1 μM PMA significantly increased the activity of membrane PKC in the permeabilized cells during the 10 min reaction period. The increase in membrane PKC activity elicited by PMA during the reaction period was not different from that produced by a 10 min preincubation prior to permeabilization (preincubation: 1332±678
cpm/mg/10min PMA mobilized activity versus 1493±303 cpm/mg/10min PMA mobilized activity in the presence of digitonin). As previously observed, tetanus toxin (10^-10M) did not alter the basal membrane PKC activity during the 10 min reaction period; however, the ability of 0.1 μM PMA to increase membrane PKC was completely attenuated. This observation suggests that tetanus toxin alters PKC metabolism rapidly after gaining access to the cytosol and is in keeping with all of our previous work.

Screen the snake neurotoxin β-bungarotoxin for an effect on K^+-induced ^14C-acetylcholine release from differentiated NG-108 cells.

As shown in Table 1, exposure of the cells to 44 mM K^+ resulted in a significant increase in the release of ^14C-acetylcholine in each series of experiments (p<0.01; Student's paired t-test). A 2 h pretreatment with 10^-7M β-bungarotoxin significantly increased the basal acetylcholine release compared to that from non-toxin treated cells. Exposure to 44 mM K^+ did not increase the release of acetylcholine over the basal level. A 2 h exposure to 10^-8M bungarotoxin slightly elevated the basal release of acetylcholine; however, exposure of the toxin-treated cells to 44 mM K^+ resulted in secretion of acetylcholine that was not different from that observed for non-toxin treated cells. In the last series of experiments, a 2 h pretreatment with 10^-9M β-bungarotoxin did not alter the basal release nor the ability of 44 mM K^+ to increase secretion of acetylcholine. Although these observations suggest that at higher concentrations β-bungarotoxin has an effect on acetylcholine release, this effect is difficult to interpret. Therefore, it appears that the NG-108 cell is not a suitable model with which to investigate the effects of β-bungarotoxin on secretion.

Examine the activity of membrane PKC following pretreatment with Botulinum neurotoxin A.

As shown in Table 8, the basal membrane PKC activity in this series of experiments was 13.4±1.6 pmol/mg/10min. A 15 min exposure to PMA resulted in a significant elevation in the activity of membrane PKC (p<0.025; Student's paired t-test). Pretreatment of the cells for 4 h with 10^-8M botulinum A toxin had no effect on either the basal or PMA-
stimulated level of membrane PKC. These data suggest that either the NG-108 cell lacks a functional receptor for botulinum A or the toxin does not affect changes in PKC metabolism.

Characterize the effect of PKC inhibitors on membrane PKC using the synthetic peptide/permeabilized cell assay

In order to more fully characterize the synthetic peptide/permeabilized cell assay, we investigated the effect of staurosporine and calphostin C on the activity of membrane PKC. A 30 min preincubation with 2 μM staurosporine completely abolished both basal and PMA-stimulated PKC activity in these cells. A 30 min preincubation with 2 μM calphostin C had no effect on basal membrane PKC activity. As previously observed, a 15 min exposure to 0.1 μM PMA resulted in a doubling of the membrane PKC activity. Preincubation with calphostin C resulted in a 50% inhibition of the ability of PMA to increase the membrane PKC activity. These observations suggest that the synthetic peptide/permeabilized cell assay is a reliable measure of membrane PKC activity that responds to known inhibitors of PKC as would be expected.

E References


TABLE 1. KRTLRR Peptide Phosphorylation Induced by Direct Addition of 10 μM Neurotensin and the Effect of a 2 Hour Tetanus Toxin Pretreatment on this Phosphorylation

<table>
<thead>
<tr>
<th>Group</th>
<th>Membrane PKC Activity (cpm/mg/10 min)</th>
<th>Hormone-Activated membrane PKC (cpm/mg/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- hormone</td>
<td>+ hormone</td>
</tr>
<tr>
<td>Control 8</td>
<td>4276 ±1002</td>
<td>6070±1347</td>
</tr>
<tr>
<td>Tetanus toxin 10^-10M</td>
<td>4673 ±1157</td>
<td>4136 ±715</td>
</tr>
</tbody>
</table>

Cells were exposed to 10 μM neurotensin for 5 min by direct addition to the pretreatment medium. Values represent the mean ±SEM of the indicated number of experiments.

a p<0.005 by Student's paired t-test comparison to corresponding control cells not exposed to neurotensin.
b p<0.025 by Student's paired t-test comparison to cells not exposed to tetanus toxin.

TABLE 2. Depolarization-Induced Membrane PKC Activity and the Effect of Tetanus Toxin Pretreatment in Selected Experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Membrane PKC Activity (pmol/mg/10 min)</th>
<th>K+-Mobilized PKC Activity (pmol/mg/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Na+</td>
<td>+K+</td>
</tr>
<tr>
<td>Untreated 7</td>
<td>5401 ±1082</td>
<td>9906±2841</td>
</tr>
<tr>
<td>10^-10M toxin 2h</td>
<td>6394 ±1799</td>
<td>6542±1441</td>
</tr>
</tbody>
</table>

Values represent the mean ±SEM of the indicated number of experiments.
a p<0.05 by Student's paired t-test comparison to cells not exposed to 44 mM potassium.
b p<0.1 by Student's paired t-test comparison to cells not exposed to tetanus toxin.
### TABLE 3. Effect of a 3 h C₂ Toxin Pretreatment on K⁺-Stimulated Acetylcholine Release from Differentiated NG-108 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Acetylcholine Release (cpm/mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+Na⁺</td>
<td>+K⁺</td>
</tr>
<tr>
<td>Untreated</td>
<td>5</td>
<td>4390 ±546</td>
<td>5192a ±729</td>
</tr>
<tr>
<td>10⁻⁹M C₂ toxin 3 h</td>
<td></td>
<td>4102 ±350</td>
<td>3774 ±391</td>
</tr>
</tbody>
</table>

Values represent the mean ±SEM of the indicated number of experiments.  
*a p<0.05 by Students paired t-test comparison to cells not exposed to 44 mM potassium.

### TABLE 4. KRTLRR Peptide Phosphorylation Induced by Direct Addition of 0.1 μM PMA and the Effect of a 3 Hour Pretreatment with Botulinum C₂ Toxin

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Membrane PKC Activity (cpm/mg/10 min)</th>
<th>PMA-Activated membrane PKC (cpm/mg/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- PMA</td>
<td>+ PMA</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>2330 ±564</td>
<td>3647a ±682</td>
</tr>
<tr>
<td>C₂ Toxin 10⁻⁹M</td>
<td></td>
<td>3304 ±595</td>
<td>3263 ±338</td>
</tr>
</tbody>
</table>

Cells were exposed to 0.1 μM PMA for 15 min by direct addition to the pretreatment medium. Values represent the mean ±SEM of the indicated number of experiments.  
*a p<0.05; Student’s paired t-test comparison to corresponding control cells not exposed to PMA.  
*b p<0.1; Student’s paired t-test comparison to PMA-induced mobilization in cells not exposed to C₂ toxin.
TABLE 5. Addition of Tetanus Toxin to Digitonin Permeabilized NG-108 cells for 10 Min Completely Blocks the PMA-Mediated Increase in Membrane PKC Activity

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Membrane PKC Activity (cpm/mg/10min)</th>
<th>PMA-Activated membrane PKC (cpm/mg/10min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- PMA</td>
<td>+ PMA</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>2167</td>
<td>3661&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±470</td>
<td>±450</td>
</tr>
<tr>
<td>Tetanus Toxin</td>
<td>2455</td>
<td>2102</td>
<td>-353&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-10&lt;/sup&gt;M</td>
<td>±641</td>
<td>±666</td>
<td>±456</td>
</tr>
</tbody>
</table>

Tetanus toxin (10<sup>-10</sup>M) or PMA (0.1 µM) was added directly to the digitonin-containing reaction mixture. The differentiated NG-108 cells were exposed to the modified reaction mixture for 10 min and the activity of membrane PKC quantified by the phosphorylation of the KRTLRR peptide as previously described. Values represent the mean ±SEM of 10 experiments.

<sup>a</sup> p<0.0005; Student’s paired t-test comparison to corresponding control cells not exposed to PMA.

<sup>b</sup> p<0.005; Student’s paired t-test comparison to PMA-induced mobilization in cells not exposed to tetanus toxin.
TABLE 6. EFFECT OF β-BUNGAROTOXIN ON BASAL AND K+-STIMULATED ACETYLCHOLINE RELEASE

<table>
<thead>
<tr>
<th>Group</th>
<th>$^{14}$C-Acetylcholine Release (cpm/mg protein)</th>
<th>K+-Stimulated $^{14}$C-Acetylcholine Release (cpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$+\text{Na}^+$</td>
<td>$+\text{K}^+$</td>
</tr>
<tr>
<td>Control</td>
<td>2610 ±290</td>
<td>3097 ±381</td>
</tr>
<tr>
<td>β-Bungarotoxin</td>
<td>3035 ±411</td>
<td>3107 ±451</td>
</tr>
<tr>
<td>10^{-7}M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9092 ±756</td>
<td>12275 ±1248</td>
</tr>
<tr>
<td>β-Bungarotoxin</td>
<td>9875 ±1145</td>
<td>12593 ±1646</td>
</tr>
<tr>
<td>10^{-8}M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6433 ±646</td>
<td>8263 ±871</td>
</tr>
<tr>
<td>β-Bungarotoxin</td>
<td>6786 ±861</td>
<td>8395 ±839</td>
</tr>
</tbody>
</table>

Cells were pretreated 2 h in the presence or absence of β-bungarotoxin followed by depolarization with 44 mM K+. Values represent the mean ±SEM of 9 (10^{-7}M toxin), 6 (10^{-8}M toxin), and 8 (10^{-9}M toxin) experiments. 

a $p<0.05$; Student's paired t-test comparison to cells not exposed to 44 mM K+. 
TABLE 7. BOTULINUM A TOXIN HAS NO EFFECT ON BASAL OR PMA-STIMULATED MEMBRANE PKC ACTIVITY

<table>
<thead>
<tr>
<th>Group</th>
<th>Membrane PKC Activity (pmol/mg/10min)</th>
<th>PMA-Stimulated PKC Activity (pmol/mg/10min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- PMA</td>
<td>+PMA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>13.4</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>±1.6</td>
<td>±1.4</td>
</tr>
<tr>
<td>Bot A</td>
<td>12.0</td>
<td>18.3</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt;M</td>
<td>±1.1</td>
<td>±0.6</td>
</tr>
</tbody>
</table>

Cells were pretreated 4 h in the presence or absence of 10<sup>-8</sup>M botulinum A toxin followed by incubation in the presence or absence of 0.1 μM PMA for 15 min. PMA mobilized PKC activity was calculated for each experiment as previously described in the Methods. Values represent the mean ±SEM of 3 experiments.

<sup>a</sup> p<0.05; Student's paired t-test comparison to cells not exposed to PMA.
A. Specific Aims

Clostridial neurotoxins are thought to enter cells by the process of receptor-mediated endocytosis (Simpson, 1986). Various lines of evidence support this belief, including immunologic work demonstrating that the toxins undergo antibody escape (Simpson, 1980), pharmacologic experiments showing that drugs like ammonium chloride, methylamine hydrochloride and chloroquine antagonize onset of toxin action (Simpson, 1982; 1983), and morphologic work indicating that toxin can be localized in endosomes (Black and Dolly, 1986 a, b).

Although there is a consensus that receptor-bound toxin crosses the plasma membrane by endocytosis, the mechanism by which the toxin crosses the endosomal membrane to reach the cytosol is unknown. There are, however, three experimental findings that may be relevant. First, the ability of the toxins to block exocytosis is antagonized by drugs that neutralize low pH in endosomes (see above). Second, the toxins appear to have hydrophobic domains, and exposure of these domains is enhanced by low pH (Boquet et al., 1984; Montecucco et al., 1986; 1989). Third, clostridial neurotoxins can be induced to insert into membranes and form channels, and this process is dependent on a pH gradient similar to that found across endosomal membranes (Boquet and Duflot, 1982; Borochov-Neori et al., 1984; Hoch et al., 1985; Donovan and Middlebrook, 1986; Blaustein et al., 1987; Shone et al., 1987). These data suggest that low pH induces the toxins to insert into endosomal membranes, after which a portion or perhaps the entire toxin molecule translocates into the cytosol.

In the present study, pH-induced conformational changes have been monitored in the botulinum neurotoxin molecule. This work was done with three goals in mind:
i.) to demonstrate that the magnitude, and even the existence, of pH-induced conformational changes depends on the technique used to monitor these changes, ii.) to further clarify the domains of the toxin molecule that participate in conformational changes, and iii.) to correlate structural changes in the molecule with expression of pharmacological activity.

B. Methods

Toxins. Types A and B botulinum neurotoxin were isolated from cultures of Clostridium botulinum strain 62A and strain OKRA. The crude neurotoxin was isolated by a series of procedures, including acid precipitation, ammonium sulfate precipitation, a second acid precipitation, protamine treatment, SP-Sephadex chromatography and Sephadex G-200 gel filtration, as previously described (Kozaki et al., 1974; Sugii and Sakaguchi, 1975; Sakaguchi, 1983). The pure neurotoxin was isolated by DEAE-Sephadex chromatography in 0.01 M sodium phosphate buffer, pH 7.5. Type A neurotoxin was obtained in the activated form, whereas type B was purified as the unactivated form. For activation of the latter, N-tosyl-phenylalanine choloromethylketone-treated trypsin was added to neurotoxin at a 1 to 40 ratio (trypsin:toxin, w:w). The mixture was incubated at 37°C for 15 min in 0.02 M sodium phosphate buffer, pH 7.0. The reaction was terminated by addition of leupeptin at a 1 to 1 ratio (w:w) with trypsin. Activated neurotoxins were separated into heavy and light chains by QAE-Sephadex chromatography in the presence of urea and dithiothreitol, as previously described (Sathyamorthy and DasGupta, 1985). The isolated chains were dialyzed against 0.02 M sodium phosphate buffer, pH 7.0, containing 0.5 M sodium chloride.

All botulinum neurotoxins and chains were used 1 to 2 weeks after purification. Protein determinations were done by the methods of Lowry (Lowry et al., 1951) or
Bradford (1976). The purity and molecular weights of proteins were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. In vivo bioassays were done as described below. The mouse LD50 values for the various toxins were: activated type A, $1.0 \times 10^8$ LD50/mg protein; unactivated type B, $5.5 \times 10^5$ LD50/mg protein; and activated type B, $3.5 \times 10^7$ LD50/mg protein.

Bioassays. The in vivo potency of clostridial toxins was determined by the methods of Hardegree (1965) and Kondo et al. (1984). In vitro toxicity was bioassayed on the mouse phrenic nerve-hemidiaphragm preparation as previously described (Simpson, 1980).

Intrinsic fluorescence. Solutions of toxin (1 x $10^{-7}$ M; 0.02 M phosphate buffer, pH 7.0) were added to quartz cells (10 mm path length), and intrinsic fluorescence was measured. Samples were excited at 290 nm, and the emission spectra were measured between 280 and 450 nm. Proteins were analyzed at least twice, and each experiment was done in duplicate or triplicate. Data were obtained at various pH values, and predetermined volumes of 10% citric acid were used to achieve the desired pH. The rate of onset and the magnitude of pH-induced changes are presented under Results.

Reporter group fluorescence. 2-p-Toluidinyl-naphthalene sulfonate (TNS) was used to monitor pH-induced changes in hydrophobicity. Solutions of toxin or other proteins (1 x $10^{-7}$ M) were prepared in 0.02 M phosphate buffer, pH 7.0. As before, reductions in pH were achieved by addition of predetermined volumes of 10% citric acid. Proteins were excited at 350 nm, and emission spectra were obtained from 380 to 580 nm. Protein solutions were analyzed at least twice, and each experiment was done in duplicate.

Partition experiments. Triton X-114 was prepared and used essentially as described previously (Bordier, 1981; Madshus and Collier, 1989). A 20% solution of
the detergent was created with 0.02 M phosphate buffer, pH 7.0, that contained 0.14 M NaCl. The suspension was left at 4°C overnight, after which temperature was raised to 37°C for 30 min. The aqueous phase was removed by aspiration and fresh phosphate buffer with NaCl was added. The procedure of incubating at 4°C, incubating at 37°C, and then aspirating was repeated three times, and the final solution of Triton X-114 was stored at 4°C.

During partitioning experiments, aqueous solutions of toxin (1 x 10⁻⁹ M; phosphate-buffered saline) were added to Triton X-114 at 4°C. Separation of phases was achieved by raising temperature to 37°C and centrifuging the mixture for 5 min at 6,000 x g. Both the aqueous and organic phases were recovered and analyzed for protein. When the intact toxins were used, the amount of protein in the individual phases was determined by bioassay (see above). When the isolated chains were used, the proteins were iodinated as previously described (Bakry et al., 1991). The amount of protein in the individual phases was then determined by quantifying the distribution of label.

C. Results

**TNS Fluorescence.** Conformational changes induced by low pH were monitored by using a dye (TNS) that associates with protein and is sensitive to surface changes in hydrophobicity. The dye was used to study two different clostridial toxins (botulinum neurotoxin and tetanus toxin), two different serotypes of botulinum neurotoxin (A and B), two different states of a single botulinum serotype (unactivated and activated), and two chains obtained from an activated serotype (heavy chain, Mr - 100,000; light chain, Mr - 50,000). Three proteins that do not undergo significant pH-induced conformational changes were included as controls (lactoglobulin, trypsin and chymotrypsin).
Table 1 summarizes the data for the major proteins of interest. As expected, the fluorescence due to TNS was similar at pH 7.0 and pH 4.0 when added to solutions of lactoglobulin, trypsin and chymotrypsin. This was in sharp contrast to results obtained with botulinum neurotoxin type A, type B and tetanus toxin. For each of the clostridial toxins there was an increase of two-fold or greater in fluorescence when pH was lowered from 7.0 to 4.0.

The full emission spectra for botulinum neurotoxin type A, type B and tetanus toxin are shown in Figure 1. There are three major points that emerge from the data. First, the peak emission for TNS shifted from -490 nm to -450 nm and the fluorescence intensity increased sharply when the dye was mixed with protein and pH was lowered to 4.0. Second, pH-induced effects were most marked within the range of 4.5 and 4.0. By comparison, pH-induced effects between 7.0 and 4.5 were modest. Third, the effect of pH was more marked on tetanus toxin than on botulinum neurotoxin.

The effect of pH on botulinum neurotoxin type B was studied in more detail. As shown in Figure 1, there was a notable difference between the unactivated and the activated form of the molecule. Prior to exposure to trypsin, the toxin did not undergo large conformational changes when exposed to low pH. After exposure to trypsin, conformational changes were substantial (see above).

The heavy and light chains of botulinum neurotoxin types A and B were isolated and then exposed to TNS. As shown in Figures 2 and 3, heavy and light chains were both affected by changes in pH. However, the behavior of the individual chains differed somewhat, and the difference was more obvious with serotype B. For the heavy chain, the apparent increases in hydrophobicity were relatively continuous with decreases in pH. For the light chain, the relationship between hydrophobicity and pH was more discontinuous.
The effects of pH on the conformation of types A and B botulinum neurotoxin were rapid in onset (Fig. 4). Effects could be detected within 30 sec, and a maximal or near maximal effect was obtained within 5 to 10 min.

Intrinsic fluorescence. Spectra were obtained for activated botulinum neurotoxin type A, unactivated type B and activated type B. The conditions that were used are those that detect the presence of, or change in state of, tryptophan residues. The results, which are shown in Fig. 5, were quite different from those obtained with TNS (see Discussion). Neither the activated nor the unactivated forms of the toxins were substantially affected by pH. Reducing the pH to 4.0 produced only a slight increase in fluorescence intensity for the active forms of serotypes A and B, and a slight decrease for the fluorescence intensity of unactivated type B.

Partitioning experiments. The rate and extent of botulinum neurotoxin partitioning from an aqueous phase to an organic phase (Triton X-114) were measured. Aliquots of toxin were added in an aqueous phase to a mixture with Triton X-114 at 40°C. The mixture was rapidly warmed (37°C) to produce separation of phases, and both the aqueous and organic phases were bioassayed (in vivo) for residual toxicity.

In the initial series of experiments, the effect of pH on partitioning of toxin into the organic phase was monitored. The results (Fig. 6) showed that lowering pH to 4.0 caused a substantial fraction of activated toxin (~35 to 45%) to appear in the organic phase. Under the same conditions only a small fraction of unactivated type B (~9%) moved to the organic phase. A similar but more limited series of experiments was done with the isolated heavy chain and light chain of type B neurotoxin. Because the isolated chains cannot easily be bioassayed, partitioning was monitored by radioiodinating the proteins and monitoring the amount of label in the aqueous and organic phases. At pH 7.0, virtually all (>98%) of the iodinated heavy chain and
iodinated light chain remained in the aqueous phase. At pH 4.0, 39% of the heavy chain and 31% of the light chain partitioned into the organic phase.

Partitioning of the intact toxins from the aqueous to organic phases was rapid and somewhat similar to that found for the TNS experiments illustrated in Figure 4. A near maximal effect was obtained within ~10 min, and a complete effect was obtained within 30 to 60 min (results not illustrated).

An effort was made to promote partitioning from the organic phase back into the aqueous phase. Experiments were done as before to promote movement of toxins into Triton X-114 (pH 4.0; 37°C; 60 min). The aqueous phase was then aspirated and replaced with solution that did not contain toxin. Temperature was lowered to 4°C, and at various times thereafter temperature was raised again to 37°C and the two phases were assayed for toxin. The results indicated that there was very little reverse partitioning. Even with intervals of 24 hr, less than 20% of the toxin in the organic phase moved back into the aqueous phase. Preliminary evidence suggests that failure to partition back into the aqueous phase is due to a tight association between detergent (Triton X-114) and toxin (Kamata and Simpson, unpublished data).

Reversible changes. Two experiments were done to show that pH-induced conformational changes were reversible and that pH did not cause significant losses in toxicity. In the first, TNS experiments were performed as before. Botulinum neurotoxin types A and B were examined at pH 7.0, pH 4.0, then again at 7.0. The results (Fig. 7) showed that changes in conformation induced by low pH were almost completely reversible.

In companion experiments, a solution of botulinum neurotoxin type A was divided into three aliquots such that one remained at pH 7.0, one was lowered from pH 7.0 to pH 4.0 for 30 min, and one was lowered from pH 7.0 to pH 4.0 for 30 min and then
raised back to pH 7.0. These samples were bioassayed for toxicity on the phrenic nerve-hemidiaphragm preparation. The final concentration of toxin applied to tissues was $1 \times 10^{-11}$ M. The paralysis times (mean ± SEM) of tissues exposed to toxin incubated at pH 7.0 was $89 \pm 6$ min (n=5); the paralysis times of tissues exposed to toxin that had been at pH 7.0 and then pH 4.0 was $93 \pm 9$ min (n=6); the paralysis times of tissues exposed to toxin that went from pH 7.0 to pH 4.0 and back to pH 7.0 was $91 \pm 10$ min (n=6).
D. Discussion

Clostridial toxins exert their pharmacologic effects in the cytosol of nerve endings. The mechanism by which these toxins reach the cell interior has not been fully explained, but a partial model has been advanced (Simpson, 1981; 1986). The initial step involves binding of toxins to receptors on the plasma membrane, and this is followed by the process of receptor-mediated endocytosis. The toxins then escape the endosome to reach the cytosol, but the molecular basis for escape remains unclear.

Perhaps the most revealing data that pertain to translocation of toxins from the endosome compartment to the cytosol compartment are those that relate to pH. This began with the observation that lysosomotropic drugs, which prevent the proton pump in endosome membranes from lowering pH, are effective antagonists of clostridial toxins (Simpson, 1982, 1983). This observation was complemented by the findings that acid pH causes the toxins to insert into artificial membranes, and acid pH induces holotoxins, as well as certain fragments of toxins, to create channels in artificial membranes (see Introduction for references). Taken collectively, the data suggest that a fall in pH induces toxins to insert into the endosome membrane, after which some or all of the toxin molecules penetrate to the cytosol. This would be in keeping with the mechanism by which other potent protein toxins, such as diphtheria toxin and pseudomonas toxin, reach the cytosol of vulnerable cells.

Data in the present study are supportive of the concept of pH-induced translocation of toxin molecules from the endosome to the cytosol. Furthermore, the data indicate which portions of the toxin molecules are likely to be associated with translocation, and they show that the nature and time-course of the conformational changes associated with translocation are in keeping with the known pharmacologic actions of the toxins.

Conformational changes in toxins. TNS is a widely used reporter group that helps to reveal the hydrophobic character of proteins. When TNS moves from the free state
in solution to the associated state with hydrophobic domains of proteins, the fluorescence maxima shifts (490 → 450 nm) and the intensity increases. The magnitude of the increase in intensity is proportional to the amount of bound TNS, which in turn is proportional to the amount of hydrophobicity displayed by a protein.

TNS has been used in the present study to detect and quantify pH-induced changes in the conformation of clostridial toxins. In addition, several comparisons have been made: i.) tetanus toxin versus botulinum neurotoxin, ii.) botulinum neurotoxin type A versus type B, iii.) unactivated type B versus activated type B, and iv.) isolated heavy chain versus isolated light chain. The data reveal that both tetanus toxin and botulinum neurotoxin undergo marked pH-induced conformational changes. The magnitude of effect for tetanus toxin was much greater than that for the two serotypes of botulinum neurotoxin.

The effect of pH on activated serotypes A and B was not significantly different, but this was not the case for unactivated versus activated type B. The unactivated form, which is one to two orders of magnitude less potent than the activated form, was only negligibly affected by pH. This observation may have important implications for understanding the pharmacologic actions of the toxin (see below).

The effect of pH was also studied on the isolated heavy and light chains of botulinum neurotoxin types A and B. These experiments showed that both chains experienced marked changes in conformation when exposed to low pH, and this may be an indication that both chains participate in the process of translocation. The effect of pH was somewhat dissimilar on the two chains. The heavy chain tended to display a discontinuous response whereas the light chain showed a more continuous response.

In contrast to findings with TNS, the measurement of intrinsic fluorescence did not reveal any marked conformational changes. This apparent anomaly can be
explained on the basis of the amino acid composition and the primary structure of clostridial neurotoxins. These toxins have relatively few tryptophan residues, and the residues are not concentrated in the domains that may be associated with translocation (i.e., light chain and aminoterminus of heavy chain; see below). The data on intrinsic fluorescence indicate that this measure may have serious limitations for providing insights into the structure-function relationships of the toxin molecule.

**Structural determinants of pharmacologic activity.** Clostridial neurotoxins are known to form pH-induced channels in artificial membranes. These channels could be interpreted as tunnel proteins through which a portion of the toxin reaches the cytosol, or they could be epiphenomena secondary to some other penetration mechanism. In either case, it is the light chain that must reach the cytosol, because this is the portion of the molecule that causes blockade of transmitter release (Bittner et al., 1989 a, b).

Electrophysiologic experiments have shown that the aminoterminus of the heavy chain is capable of forming pH-induced channels (Hoch et al., 1985; Blaustein et al., 1987; Shone et al., 1987). This indicates that the aminoterminus of the chain must have a pH sensor, must be able to insert into and span the membrane, and must be able to adopt a conformation that permits flux of solutes. The light chain by itself does not form channels, meaning it lacks one or more of the properties of the aminoterminus of the heavy chain. By extrapolation, this could also mean that the light chain - if it were hydrophilic - would have to rely on the heavy chain to reach the cytosol (viz., tunnel protein). However, recent work suggests that the light chain may not require the heavy chain for translocation. More correctly, the light chain may cooperate with the heavy chain to promote internalization.

Montecucco and his associates (Montecucco et al., 1988; 1989) have shown that clostridial neurotoxins will insert into artificial membranes. By using a labelling technique that detects insertion into the lipid phase, they were able to demonstrate that
both the heavy and the light chains of the intact toxin can reach the hydrophobic core of lipid bilayers. The present study supports and extends these findings. Experiments with the isolated chains have shown that the light chain has an occult hydrophobic region that, upon treatment with low pH, becomes exposed. This means that the heavy chain need not be a device for propelling the light chain through the membrane. To the contrary, the present results show that the light chain has a pH sensor, possesses a hydrophobic domain, and therefore is capable of inserting by itself. This opens the possibility that the two chains work cooperatively to achieve translocation.

The partitioning experiments provide graphic evidence of the efficacy of the hydrophobic domains. When exposed to pH values found in endosomes (pH 7.0 - 4.0), a large fraction of the toxin partitioned into the lipid phase. These experiments indicate that the fully activated toxin is well designed to penetrate a biological membrane. On the other hand, the unactivated toxin responded only poorly to reductions in pH. This is an interesting result, because unactivated toxin is between one and two orders of magnitude less potent than the activated toxin. The TNS and the partitioning experiments could mean that the relative absence of pharmacologic activity in the unactivated type B toxin is due to a relative absence of exposed hydrophobic domains. Prior to proteolysis-induced activation, the toxin may have molecular constraints that prevent a conformational change and consequent exposure of hydrophobic regions.

In those instances in which the toxin did respond to low pH, the time-course of the response was in keeping with the pharmacologic actions of the molecule. The fluorescence changes observed with TNS occurred within minutes, as did the partitioning behavior seen with Triton X-114. This is precisely the time-course one would expect of substances that exit the endosome to reach the cytosol. It is also noteworthy that the pH-induced changes did not produce detectable losses in toxicity.
When exposed to low pH for the amounts of time necessary to achieve membrane penetration, the toxin retained essentially full potency in producing neuromuscular blockade.

In summary, native clostridial toxins in the activated form and isolated chains obtained from native toxins undergo conformational changes when exposed to low pH. These molecular changes result in exposure of occult hydrophobic domains. The magnitude, time-course and reversibility of the changes suggest that the heavy and light chains of clostridial toxins act cooperatively to promote insertion and penetration from the endosome to the cytosol.

E. References


in planar lipid bilayers: Relevance to translocation of proteins across membranes. 


TABLE 1.

Fluorescence intensity of TNS in the presence of various proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fluorescence intensity</th>
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<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
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<tr>
<td>None</td>
<td>0.250</td>
</tr>
<tr>
<td>Lactoglobulin</td>
<td>0.790</td>
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<tr>
<td>Trypsin</td>
<td>0.260</td>
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<tr>
<td>Chymotrypsin</td>
<td>0.267</td>
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<tr>
<td>Botulinum Neurotoxin</td>
<td></td>
</tr>
<tr>
<td>Activated type A</td>
<td>0.290</td>
</tr>
<tr>
<td>Activated Type B</td>
<td>0.330</td>
</tr>
<tr>
<td>Tetanus Toxin</td>
<td>0.350</td>
</tr>
</tbody>
</table>

Proteins were dissolved in 0.02 M phosphate buffer at a concentration of 1 x 10^{-7}; TNS was used at a concentration of 1.5 x 10^{-4} M. The excitation wavelength was 350 nm. Emission was measured at 490 nm at pH 7.0 and 450 nm at pH 4.0. Fluorescence values are expressed in arbitrary units.
FIGURE CAPTIONS

**Figure 1.** The emission spectra for buffer solution (No Toxin), botulinum neurotoxin type A, tetanus toxin, unactivated botulinum neurotoxin type B and activated botulinum neurotoxin type B are shown. The emission spectra were obtained in the presence of TNS and at various pH levels. Fluorescence values are expressed in arbitrary units.

**Figure 2.** The emission spectra for botulinum neurotoxin type A, as well as the isolated heavy and light chains, are shown. Readings were obtained in the presence of TNS and at various pH levels. Fluorescence values are expressed in arbitrary units.

**Figure 3.** The emission spectra for botulinum neurotoxin type B, for the heavy chain and for the light chain are shown. See the captions to Figs. 1 and 2 and the Methods section for further details.

**Figure 4.** The rates of onset of pH-induced changes in buffer solutions (A.) and botulinum toxin solutions (B.) are shown. The fluorescence values of buffer solutions maintained at pH 7.0 for 30 min (○) or lowered from pH 7.0 to pH 4.0 for 30 min (○) were the same and did not change with time. Similarly, the fluorescence values for toxin solutions containing activated type A (□), activated type B (Δ) or unactivated type B (▽) at pH 7.0 did not change with time. However, when pH was lowered to 4.0, the fluorescence values increased rapidly and then remained stable. The magnitude of the induced changes was: activated type B (Δ) > activated type A (■) > unactivated type B (▽).

**Figure 5.** The emission spectra showing the intrinsic fluorescence of activated type A, unactivated type B and activated type B botulinum neurotoxins are presented. The spectra were obtained at pH 7.0 and 4.0. Fluorescence values are given in arbitrary units.

**Figure 6.** The partitioning of activated type B, unactivated type B and activated type A botulinum neurotoxin from an aqueous to an organic phase is shown. The aqueous phase was composed of phosphate buffered saline solution in which pH was lowered by addition of
citric acid. The organic phase was composed of the detergent Triton X-114. Toxicity was quantified by bioassay (see Methods).

**Figure 7.** The emission spectra of activated botulinum neurotoxin type A in the presence of TNS are shown. Spectra were obtained under four conditions: 1. buffer solution at pH 7.0; 2. toxin that was maintained in a solution at pH 7.0; 3. toxin that was maintained at pH 7.0 and then lowered to 4.0; 4. toxin that was maintained at pH 7.0, lowered to pH 4.0 and then returned to pH 7.0. Equivalent results were obtained for type B toxin (not illustrated).
No Toxin Tetanus Toxin

Botulinum Neurotoxin Type A

Unactivated Botulinum Neurotoxin Type B

Activated Botulinum Neurotoxin Type B
Toxicity in Detergent Phase (%) vs pH

- Activated Type B
- Unactivated Type B
- Activated Type A
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