Characterization of a Vertebrate Neuromuscular Junction That Demonstrates Selective Resistance to Botulinum Toxin

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ABSTRACT

Botulinum toxin blocks transmitter release by proceeding through a series of four steps: binding to cell surface receptors, penetration of the cell membrane by receptor-mediated endocytosis, penetration of the endosome membrane by pH-induced translocation, and intracellular proteolysis of substrates that govern exocytosis. Each of these steps is essential for toxin action on intact cells. Therefore, alterations in cell structure or cell function that impede any of these steps should confer resistance to toxin. In the present study, screening for susceptibility to four serotypes of botulinum toxin revealed that the cutaneous-pectoris nerve-muscle preparation of Rana pipiens is resistant to type B botulinum toxin. Resistance was demonstrated both by electrophysiologic techniques and by dye-staining techniques. In addition, resistance to serotype B was demonstrated at toxin concentrations that were 2 orders of magnitude higher than those associated with blockade produced by other serotypes. In experiments on broken cell preparations, type B toxin cleaved synaptobrevin from frog brain synaptosomes. However, the toxin did not bind to frog nerve membranes. These findings suggest that resistance is due to an absence of cell surface receptors for botulinum toxin type B. The fact that cutaneous-pectoris preparations were sensitive to other botulinum toxin serotypes (A, C, and D), as well as other neuromuscular blocking agents (a-latrotoxin, b-bungarotoxin), indicates that botulinum toxin type B receptors are distinct.

Botulinum toxin acts in the cytosol of vulnerable cells to block spontaneous and evoked transmitter release. Although the toxin exerts this effect on most nerve cells, it acts preferentially on cholinergic nerve endings. Dose-response experiments indicate that the vertebrate neuromuscular junction is the site that is most sensitive to toxin action (for reviews, see Simpson 1989; Montecucco, 1994).

To produce blockade of neuromuscular transmission, botulinum toxin proceeds through a series of steps (Simpson, 1980, 1981). This sequence involves binding to the plasma membrane, internalization by receptor-mediated endocytosis, escape to the cytosol by pH-induced translocation, and eventual expression of zinc-dependent metalloendoprotease activity. The substrates for the protease activity of botulinum toxin are synaptobrevin, synaptosomal-associated protein of 25 kDa (SNAP-25), and syntaxin, each of which is essential for transmitter release (Schiavo et al., 1994). Of the seven toxin serotypes, four act on synaptobrevin (types B, D, F, and G), two cleave SNAP-25 (types A and E), and one cleaves both syntaxin and SNAP-25 (type C).

There is a substantial literature showing that almost all cholinergic neuromuscular junctions are sensitive to botulinum toxin. In fact, every junction that has been tested is poisoned by at least one of the seven toxin serotypes. Because of the near universality of toxin action, relatively little attention has been devoted to identification of cholinergic neuromuscular junctions that are resistant. This is unfortunate, because a comparison of sensitive and resistant cell lines could shed light on the complex sequence of events that underlies toxin action. In addition, a comparison of sensitive and resistant lines could provide novel insights into fundamental aspects of cell biology.

At least in theory, there are four alterations in cell structure or function that could produce resistance to botulinum toxin: 1) absence of cell surface receptors; 2) loss of receptor-mediated endocytosis; 3) loss of the endosomal proton pump; and 4) absence of substrate. Each of these corresponds to the four steps that underlie toxin action. To date, no cholinergic neuromuscular junctions have been identified that are resistant to toxin action because of alterations in receptor-mediated endocytosis or pH-induced translocation. However, the discovery of such cells is conceptually possible because drugs that neutralize endosomal pH (Simp-
son, 1982, 1983) or block the endosomal proton pump (Simpson et al., 1994) do inhibit toxin action. A small number of cholinergic neuromuscular junctions have been identified that are resistant to toxin action because of an absence of substrate. For example, Burgen et al. (1949) reported that the rat phrenic nerve-hemidiaphragm preparation is relatively resistant to serotype B. Interestingly, rat synaptobrevin has a mutation (Gln → Val) at the site of toxin-induced proteolysis, suggesting that the rat neuromuscular junction is relatively resistant to botulinum toxin type B due to an absence of vulnerable substrate (Patarnello et al., 1993).

No one has yet identified a cholinergic neuromuscular junction that is resistant to botulinum toxin because of an absence of cell surface receptors. Therefore, a concerted effort has been made in the present study to find a tissue that is relatively or absolutely resistant to at least one, but not all, of the seven toxin serotypes. This search has resulted in the discovery that the cutaneous-pectoris nerve-muscle preparation of *Rana pipiens* is resistant to botulinum toxin type B, and resistance is related to an absence of binding sites on the cell surface. This discovery will make it possible to address three related aspects of botulinum toxin action, as follows: 1) the role of synaptotagmin as a receptor for serotype B; 2) the competence of high-affinity and low-affinity toxin-binding sites in evoking neuromuscular blockade; and 3) the relationship between the receptor for serotype B and receptors for other botulinum toxin serotypes (i.e., A, C, and D) and other neuromuscular blocking agents (i.e., α-latrotoxin and β-bungarotoxin).

### Materials and Methods

**Toxins.** Botulinum toxin types A and B were isolated and tested for potency as described previously (Simpson et al., 1988); serotypes C and D were purchased from WAKO Fine Chemicals (Dallas, TX). Serotypes A, C, and D were in the nicked and activated form. Serotype B was activated by adding it to N-tosyl-phenylalanine chloromethylketone-treated trypsin that was coupled to agarose beads [trypsin-toxin, 1:40 (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 centrifugation and aspiration of activated toxin. The homogeneity and molecular structure of the toxins were confirmed by polyacrylamide gel electrophoresis in the presence of SDS, and biological activity of the toxins was measured on mouse phrenic nerve-hemidiaphragm preparations as described previously (Simpson and DasGupta, 1983).

**Neuromuscular Preparations.** Frog cutaneous-pectoris preparations were exposed to botulinum neurotoxin under two conditions. In the initial experiments, various serotypes of botulinum toxin were added to tissues at 30°C, nerve stumps were stimulated, and twitch responses were recorded. The purpose of the experiments was to determine which serotypes block frog neuromuscular transmission. In the second set of experiments, toxin was added to tissues, and spontaneous miniature endplate potentials (MEPPs) were monitored. Standard intracellular recordings were obtained using glass microelectrodes filled with 3 M KCl (tip resistance, 20–40 MΩ). The purpose of these experiments was to determine the rate at which various serotypes could paralyze tissues in which vesicles had previously been stained with FM1–43 (see below). Nerve stimulation was not applied during development of paralysis so that vesicles would not destain.

**Visualization of Exocytosis and Toxin Action.** Procedures for staining vesicles with the styryl dye FM1–43 and for monitoring stimulation-evoked exocytosis have been described in detail (Betz et al., 1992; Betz and Bewick, 1993). Briefly, cutaneous-pectoris nerve-muscle preparations were dissected from *R. pipiens* and pinned in Sylgard-lined dishes. Unless otherwise noted, tissues were maintained at room temperature (about 23°C) in Ringer solution consisting of 120 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, and 2.4 mM NaHCO3. Vesicles were stained by adding 2 μM FM1–43 to the incubation medium and stimulating nerves continuously (10 Hz) for 4.5 to 5 min. Tissues were then washed for 30 to 90 min at 4°C to remove dye associated with surface membranes.

Images of stained or destained nerve terminals were obtained as previously described (see above). All images were top views of surface motor nerve terminals; that is, the terminals lay on the uppermost surface of superficial muscle fibers. Preparations were viewed with a Leitz Laborlux epifluorescence microscope fitted with a Zeiss 40× water immersion objective. Excitation was from a 100-W Hg lamp through a 2 to 10% transmittance neutral density filter and 420- to 440-nm bandpass filter; emission was through a 500- to 600-nm bandpass filter. Images were captured with a Photometrics Star I camera (gain 4); exposures usually were for 3 s. Image processing was performed using Silicon Graphics (Mountain View, CA). Personal Iris computer software was obtained from G.W. Hannaway (Boulder, CO). Prints were made with a Kodak X/7700 printer (Rochester, NY).

**Iodination of Botulinum Toxin and Ligand-Binding Experiments.** Botulinum toxin type B was radioiodinated with Bolton–Hunter reagent. Purified toxin (100 μg) was mixed with 1 mCi of [125I]-Bolton–Hunter reagent in 100 mM borate buffer, pH 8.0, for 30 min at room temperature. Iodinated toxin was separated from free iodine by fractionation on Sephadex G-50 columns. Preparations of labeled material typically had a specific activity of 600 to 900 Ci/mmol and a residual toxicity of 70 to 90%.

A crude membrane preparation was obtained by homogenizing frog brain in iced Tris-HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged for 10 min at 1000g, and the resulting homogenate was resuspended in fresh buffer and recentrifuged for 45 min at 40,000g. The final pellet was resuspended in Tris-HCl buffer (as above).

The binding of iodinated botulinum toxin type B to membrane preparations was measured by a centrifugation assay as described previously (Bakry et al., 1991b; Coffield et al., 1997). The ligand was mixed with a specified concentration of membrane (see Results) in 100 μl of buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mg/ml BSA. The binding reaction was allowed to proceed at room temperature (about 23°C) for the amount of time necessary to reach equilibrium (see Results). The reaction was terminated by centrifugation (15,000g, 2 min), after which the pellet was washed with fresh buffer and recentrifuged. Membrane-associated ligand was collected and quantified, and the results were corrected for nonspecific binding. The data were evaluated by using the equilibrium binding analysis program of McPherson (1982).

**Vesicle Isolation and Incubation with Toxin.** Frog or mouse brain vesicles were isolated according to standard procedures. All isolation steps were performed at 3°C (on ice). Briefly, frog or mouse brains were excised and minced in homogenization buffer (255 mM sucrose, 1 mM EDTA, 20 mM HEPES, pH 7.4). The resulting suspensions were homogenized in a glass-Teflon homogenizer (20 strokes). The homogenate was fractionated using an SS-34 rotor in a Sorvall RC5B centrifuge (Sorvall, Inc., Newtown, CT). Homogenates were spun at 1000g for 5 min. The resulting supernatant (S-1) was recentrifuged at 10,000g for 10 min. The second supernatant (S-2) was recentrifuged at 250,000g for 1 h, and the resulting pellet (P-3; brain vesicle fraction) was resuspended in homogenization buffer and used for digestion experiments.

Nicked, reduced (10 mM dithiothreitol; 2 h) toxin was preincubated with 200 μM tetraakis(2-pyridylmethyl)ethylenediamine (TPEN) for 2 h at room temperature. A sample of toxin without TPEN was processed in parallel. Subsequently, these toxin preparations were added to aliquots of frog or mouse brain vesicles. Vesicles were incubated for 4 h with 1 × 10^−9 M nicked toxin in the presence
or absence of a final concentration of 30 μM TPEN. Aliquots processed identically but without toxin served as controls.

After incubation, reducing sample buffer was added to each tube, and the digests were separated using polyacrylamide gel electrophoresis.

**Peptide Antibodies and Western Blot Analysis.** Monoclonal antibodies against VAMP-1 and VAMP-2 were generously provided by Dr. Reinhard Jahn (Boyer Center for Molecular Medicine and Howard Hughes Medical Institute, Yale University). Antibodies against syntaxin and SNAP-25 were purchased from Sigma (St. Louis, MO) and Sternberger Monoclonals Inc. (Baltimore, MD), respectively.

Samples for Western blot analysis were separated in 12% Tris-tricine gels (Schägger and von Jagow, 1987). Subsequent to separation, proteins were transferred to NitroPure membranes (Micron Separations Inc., Westboro, MA) in Tris-glycine transfer buffer at 50 V for 30 to 45 min. Blotted membranes were rinsed in distilled water and stained for 1 min with 0.2% Ponceau S in 1% acetic acid. Following a brief rinse with distilled water, molecular weight markers and transferred proteins were identified. Membranes were destained in PBS-Tween (pH 7.5; 0.1% Tween 20), blocked with 5% nonfat powdered milk in PBS-Tween overnight at 3°C. For identification of membrane or vesicle proteins, membranes were washed again (3×) and incubated with primary antibodies at the appropriate dilution in 0.05 to 1.0% milk for 2 to 5 h at room temperature.

For visualization of synaptobrevin in toxin digestion experiments, membranes were incubated in 5% milk with a 1:2000 dilution of Cl 10.1 anti-synaptobrevin monoclonal antibody for 6 h at room temperature.

Membranes were washed again (3×) and visualized using enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Membranes were exposed to film (Hyperfilm-ECL) for times adequate to visualize enhanced chemiluminescence bands. Peptides were identified by comparison with known standards.

**Results**

**Botulinum Toxin Blocks Frog Neuromuscular Transmission.** A number of elegant studies have been published showing that botulinum toxin blocks frog neuromuscular transmission. However, most of these studies were published more than two decades ago. This means that most of the work was done 1) before the discovery that there is a multistep sequence of events that underlies toxin action and 2) before the discovery that all serotypes of botulinum neurotoxin are metalloendoproteases. A further limitation on these studies is that, with few exceptions, attention has been limited to serotype A.

In the present work, three toxin serotypes were selected for study based on substrate specificity as follows: serotype A, SNAP-25; serotype B, synaptobrevin; and serotype C, syntaxin. Each serotype was added to tissues (n = 5) at a concentration of 3 × 10⁻⁹ M, and nerve-stimulus-evoked muscle twitch was monitored for at least 180 min.

Botulinum toxin types A and C produced irreversible blockade of transmission. The respective paralysis times were serotype A, 165 ± 12 min; serotype C, 116 ± 9 min. In contrast, serotype B had no observable effect on evoked twitch, even when monitored for 180 min.

A similar set of experiments was done by monitoring spontaneous MEPP frequency, except that serotype D—which cleaves synaptobrevin—was added to the study. The results demonstrated that serotypes A, C, and D blocked transmission (Fig. 1), but serotype B had no effect.

**Fig. 1.** MEPPs were recorded from excised cutaneous-pectoris nerve-muscle preparations before and after botulinum toxin exposure using standard intracellular recording techniques (30°C; 0.3 Hz stimulation). Toxin types A, B, C, and D were tested at 10⁻⁹ M. Type B was further tested at 10⁻⁶ M and 10⁻⁷ M. Paralysis was defined as a 90% or greater reduction in MEPP frequency compared with control tissue. Types C and D produced paralysis within 60 min, and type A within 180 min. Type B did not show any change from control tissue at any concentration tested. Experiments were terminated at 240 min. Data points represent the mean of a minimum of three experiments per toxin type. Type A: [10⁻⁹ M], solid line; type B: [10⁻⁷ M], dashed-dotted line; type C: [10⁻⁹ M], dotted line; type D: [10⁻⁹ M], dashed, dotted line.

To determine the extent of resistance to type B, experiments were done with a 100-fold higher concentration than that previously tested (3 × 10⁻⁷ M). Interestingly, there was still no evidence that this serotype had an effect on neuromuscular transmission.

For those serotypes that did act on frog neuromuscular junctions, the characteristics of toxin-induced blockade were the same as those routinely observed at mammalian neuromuscular preparations. For example, lowering temperature had a profound effect on toxin-induced paralysis. When serotypes A, C, or D (3 × 10⁻⁹ M) were added to tissues at 4°C, there was no onset of paralysis within a 12-h observation period. The marked effect of temperature has been interpreted to mean that toxin-induced paralysis is an energy-dependent process (viz., receptor-mediated endocytosis; see Discussion).

Another similarity between toxin action on mammalian and frog neuromuscular junctions is the existence of a lag time. As shown in Fig. 1, there was an interval between addition of toxin to tissues and the initial signs of neuromuscular blockade. This interval has been interpreted to mean that there is a lag time necessary for the toxin to be productively internalized and reach its intracellular substrate (see Discussion).

**Toxin Action at the Frog Neuromuscular Junction Can Be Demonstrated by Visual as Well as Electrophysiologic Techniques.** FM1–43 is a fluorescent dye that can be used to stain the membranes of recycling synaptic vesicles. The dye has been previously used to study normal exocytotic activity at neuromuscular junctions (Betz and Bewick, 1992), as well as abnormal activity in the presence of neuromuscular blocking agents such as α-latrotoxin (Henkel and Betz, 1995) and botulinum toxin (Henkel et al., 1996). In the present study, nerve terminals were loaded with FM1–
43, and nerve terminals were photographed to obtain baseline values for intensity and distribution of vesicle staining. Botulinum toxin types A, B, C, or D were subsequently added to tissues (10^{-8} M), which were allowed to incubate for at least 2 h at 23°C. Single nerve pulses were applied at 120 min, and at approximately 20-min intervals thereafter, until transmission was blocked.

Nerve terminals were reimaged after onset of paralysis to ensure that there had been negligible loss of dye. Nerves were then stimulated at 10 Hz for 5 to 10 min, and nerve terminals were imaged a final time.

Figure 2 shows representative images of a control nerve terminal and a poisoned nerve terminal. As expected, stimulation of control tissues produced destaining of nerve terminals (Fig. 2, panel 1, A and B). Stimulation of a tissue poisoned with botulinum toxin type A (10^{-8} M) produced little (<10%) destaining (Fig. 2, panel 2), and the same result was obtained in tissues poisoned with serotypes C and D. By contrast, tissues treated with botulinum toxin type B were indistinguishable from control tissues (Fig. 2, panel 1, C and D).

As indicated above, the characteristics of toxin action at the frog neuromuscular junction were the same as those at mammalian neuromuscular junctions. A particularly good example of this is shown in Fig. 3. TPEN is a zinc chelator that is an antagonist of all serotypes of botulinum toxin when tested on the mouse phrenic nerve-hemidiaphragm preparation. When frog tissues were pretreated with this drug and then exposed to botulinum toxin, the ability of the toxin to block destaining of frog nerve terminals was almost completely abolished.

**Serotype B Binds to Mouse But Not to Frog Membrane Preparations.** Studies that successfully quantify the rate constants for toxin association and dissociation at cholinergic neuromuscular junctions have not been reported. The absence of such work is due to the small amount of nerve ending membrane at neuromuscular junctions, the small number of receptors in these membranes, and the apparent high affinity of toxin for these receptors (i.e., low toxin concentrations needed to achieve binding, as deduced by toxicity assays). Therefore, as an alternative, investigators have studied toxin binding to central nervous system receptors. This approach is based on the observation that the toxin blocks transmitter release from central nerve endings by the same mechanism that accounts for blockade of transmitter release at peripheral nerve endings (Ahnert-Hilger and Bigalke, 1995; Schiavo et al., 1995).

**Fig. 2.** Cutaneous-pectoris nerve-muscle preparations were stained with the styryl dye FM1–43, as described in Materials and Methods. Tissues were subsequently stimulated to monitor exocytosis of dye. Panel 1 illustrates a control preparation that was loaded with dye (A), and the same preparation following intense nerve stimulation (B; 10 Hz stimulation; 10 min). Panel 2 illustrates a preparation that was loaded with dye (A), poisoned with botulinum toxin type A as described in the text (B), and then submitted to intense stimulation (C). Note that nerve stimulation produced substantial destaining, indicating that the chelator had antagonized toxin action. For comparison, panel 1 shows a stained preparation that was poisoned with botulinum toxin type B (C) and then submitted to intense stimulation (D). Note that the preparation treated with serotype B was substantially destained, indicating that exocytosis was not blocked.

**Fig. 3.** Cutaneous-pectoris nerve-muscle preparations were pretreated with TPEN (40 μM; 30 min) then stained with FM1–43. Tissues were subsequently exposed to botulinum toxin type A (10^{-8} M; 200 min). Tissues were then stimulated, and the extent of destaining was monitored. The figure illustrates a representative preparation after exposure to TPEN (A), after staining and subsequent exposure to toxin (B), and after stimulation (C). Note that nerve stimulation produced substantial destaining, indicating that the chelator had antagonized toxin action.
Botulinum neurotoxin type B was iodinated to high specific activity, and this ligand was used to study toxin binding to mouse and frog brain membrane preparations (experiments were done three times, with each experiment done in triplicate). The data obtained with mouse brain membranes were very similar to those previously reported for rat membrane preparations (Bakry et al., 1997) and human membrane preparations (Coffield et al., 1997). Binding was dependent on time, with a $T_{1/2}$ to equilibrium of approximately 30 min. Binding was also dependent on protein concentration, with half-maximal binding at approximately 40 to 50 $\mu$g/ml. The apparent $K_d$ value for association with high-affinity sites was 4.1 nm, which is similar to that observed in rat tissues (Bakry et al., 1997). The $B_{max}$ value was 8.4 pmol/mg protein, which is also similar to that previously reported.

When mouse membrane preparations were incubated in a 50-fold molar excess of unlabeled homologous toxin, the binding of labeled toxin was diminished an average of 87% (three experiments done in triplicate). When membranes were incubated in a 50-fold molar excess of heterologous toxin (serotype C), binding was never reduced by more than 12% (three experiments done in triplicate).

Identical experiments were done with frog membranes and iodinated serotype B, but the results were strikingly different. The amount of specific binding was so low as to be indistinguishable from background. Regardless of incubation time or membrane protein concentration, high-affinity binding sites for serotype B could not be detected.

Triticum Vulgaris Lectin Antagonizes Toxin Binding and Activity. The receptor for botulinum toxin either possesses a sialic acid residue or is in close proximity to a sialic acid residue. One piece of evidence to support this concept is the finding that lectins with affinity for sialic acid compete with botulinum toxin for binding sites on rodent brain membranes and antagonize toxin action at rodent neuromuscular junctions (Bakry et al., 1991a).

In the present work, ligand binding studies with iodinated botulinum toxin types A and C were done as described above in the absence or presence of T. vulgaris lectin ($10^{-5}$ M). As shown in Fig. 4, the lectin produced a significant reduction in toxin binding. For both serotypes, the amount of binding in the presence of lectin was less than half of that in the absence of lectin. In companion experiments, the effect of botulinum toxin type C on spontaneous MEPPs was monitored in the absence and presence of lectin ($10^{-6}$ M). The lectin by itself did not affect the rate of spontaneous MEPPs. However, the presence of lectin significantly delayed the onset of poisoning by botulinum toxin (Table 1).

Methylamine Hydrochloride Antagonizes Toxin Activity. Work on other vertebrate neuromuscular junctions has shown that botulinum toxin must proceed through an acid-dependent step to block transmission. For example, drugs that block acidification of the endosomal lumen (e.g., bafilomycin) or neutralize acidification of the lumen (e.g., methylamine hydrochloride, chloroquine) substantially antagonize toxin action (see Introduction).

In the present work, methylamine hydrochloride was selected as a prototype agent to determine whether neutralization of frog endosomes would delay onset of toxin action. Tissues ($n = 5$) were pretreated with the drug (1 mM) for 30 min, after which they were exposed to botulinum toxin type C ($1 \times 10^{-8}$ M), and MEPPs were monitored. In the absence of the drug, the toxin blocked transmission in 53 ± 3 min; in the presence of the drug, the toxin blocked transmission in 114 ± 14 min. The difference in paralysis times is highly significant ($p < 0.01$).

Frog Nerve Endings Have the Three Principal Substrates for Botulinum Toxin. Frog and mouse brain synaptosomes were isolated as described in Materials and Methods and then lysed and submitted to polyacrylamide gel electrophoresis. The gels were subsequently used in immunoblots for SNAP-25, synaptobrevin 1, synaptobrevin 2, and syntaxin. As shown in Fig. 5, all three substrates were present in both frog and mouse. However, frog brain synaptobrevin 1 was present to the near exclusion of synaptobrevin 2.

A frog brain synaptic vesicle fraction was isolated and exposed to botulinum toxin type B that had been pre-exposed to dithiothreitol to reduce the interchain disulfide bond. Subsequent immunoblots with antibody against synaptobrevin 1 demonstrated substantial cleavage of substrate (Fig. 6). The proteolytic action of serotype B was significantly diminished when experiments were done in the presence of a zinc-chelating agent (TPEN).

**Discussion**

Mechanism of Toxin Action. There is substantial electrophysiological evidence that botulinum toxin type A acts on frog neuromuscular junctions to block transmission (e.g., Miledi and Spitzer, 1974). Thus, serotype A has been shown to reduce the magnitudes of nerve stimulus-induced endplate

**TABLE 1**

<p>| Effect of <em>T. vulgaris</em> lectin on botulinum toxin paralysis of neuromuscular junctions |
|---------------------------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th><strong>MEPP Frequency</strong></th>
<th><strong>Toxin</strong></th>
<th><strong>Toxin + MA</strong></th>
<th><strong>Toxin + TVL</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>50% reduction</td>
<td>33.3 ± 3.3</td>
<td>58.3 ± 3.5</td>
<td>47.7 ± 5.0</td>
</tr>
<tr>
<td>90% reduction</td>
<td>53.3 ± 3.3</td>
<td>114 ± 13.7</td>
<td>104 ± 10.7</td>
</tr>
</tbody>
</table>

$^a$ Spontaneous MEPP rate was measured for 30 min before addition of drug or toxin.

$^b$ Length of time (min ± S.E.M.) for tissues ($n = 3$) to be paralyzed by botulinum toxin type C ($10^{-9}$ M). Data are given for length of time for both 50% reduction in MEPP rate and 90% reduction in MEPP rate.

$^c$ Tissues were pretreated with either methylamine hydrochloride (MA; $10^{-3}$ M) or *T. vulgaris* lectin (TVL; $10^{-6}$ M) for 45 min before addition of toxin. Both drugs produced a statistically significant increase ($p < 0.01$) in the amount of time necessary for the toxin to produce either a 50% or 90% reduction in spontaneous MEPP rate.
potentials and twitch, resulting in complete paralysis of transmission, and to reduce the frequency of spontaneous MEPPs to levels that are barely 1% of normal. These observations are suggestive evidence that botulinum toxin action at the frog neuromuscular junction is the same as its action at other vertebrate neuromuscular junctions. However, the marked differences between toxin action on certain mammalian nerve endings (e.g., mouse phrenic-nerve hemidiaphragm) and on certain nonmammalian preparations (e.g., Aplysia buccal ganglion) argue strongly that one must be cautious about the notion that a single scheme can account for all aspects of the cellular and subcellular effects of the toxin (see, for example, Poulain et al., 1989). Therefore, experiments were done to demonstrate that botulinum toxin action at the frog neuromuscular junction involves binding and internalization, pH-induced translocation to the cytosol, and zinc-dependent metalloprotease action. In addition, work was done to show that the pharmacological characteristics of these steps are similar to those at mammalian neuromuscular junctions.

Botulinum neurotoxin types A, C, and D blocked transmission in the frog cutaneous-pectoralis preparation. Electrophysiologic evidence showing blockade of evoked and spontaneous transmitter release was complemented by dye-staining experiments showing failure of vesicles to meld with the plasma membrane and discharge their contents.

Additional experiments with serotypes A and C confirmed that toxin-induced blockade in the frog preparation was essentially identical with that in mammalian preparations. The principal observations were 1) the toxin displays specific and saturable binding to frog nerve membranes; 2) the receptor for the toxin, or a molecule that is in close proximity to the receptor, has a sialic acid residue that can be occluded by T. vulgare lectin; 3) toxin action is markedly delayed when tissues are incubated at low temperature, implicating an energy-dependent receptor-mediated endocytosis step; 4) toxin action is delayed by drug treatment that neutralizes endosomal pH, implicating a pH-dependent translocation step; 5) there is a lag time in toxin action, reflecting the time necessary for the toxin to move from the extracellular site of binding to the intracellular site of substrate proteolysis; and 6) toxin action is antagonized by TPEN, implicating a zinc-dependent proteolysis step. In sum, the evidence suggests that botulinum toxin proceeds through the same sequence of events in blocking frog neuromuscular transmission as in blocking mammalian neuromuscular transmission.

Resistance to Serotype B. In contrast to serotypes A, C, and D, botulinum toxin type B did not block frog neuromuscular transmission. This result was obtained even when using toxin concentrations that were orders of magnitude higher than those used with other serotypes. By combining deduction and experimental findings, one can reasonably conclude that the absence of effect by type B was due to an absence of cell surface receptors.

Resistance to this one serotype could not have been due to an absence of receptor-mediated endocytosis. Endocytosis is a process upon which all active serotypes depend, and thus paralysis induced by any one serotype is good presumptive evidence for the existence of a mechanism for receptor-mediated endocytosis. In addition, resistance to serotype B could not have been due to an absence of endosomal acidification, as indicated by two observations. First, all active serotypes must proceed through pH-induced translocation to escape the endosome and reach the cytosol. The fact that several serotypes act on frog tissues is highly suggestive evidence that endosomes are acidified. Second, methylamine hydrochloride, which acts to neutralize endosomal lumens, was an antagonist of the toxin serotypes that act on frog tissues. Finally, resistance to serotype B could not have been due to an absence of susceptible substrate. Western blot analysis with appropriate antibodies showed that substrate was present and, furthermore, that this substrate was susceptible to zinc-dependent proteolysis by serotype B.

By process of elimination, the most plausible explanation for resistance to serotype B is an absence of—or alteration of—the cell surface receptor. The observation that iodinated serotype B did not bind to frog nerve membranes strongly reinforces the concept that resistance is linked to the receptor.

The fact that a specific type of nerve ending lacks receptors for a specific type of toxin creates the opportunity to address several issues that pertain to toxin action and nerve function. Some of the more important issues are described below.

1. Synaptotagmin may be a receptor for botulinum toxin type B. Several studies have been published suggesting that one or more of the synaptotagmins could be part of the receptor for serotype B (Nishiki et al., 1993, 1994, 1996). Synaptotagmin is enriched in membranes of synaptic vesicles, and ordinarily it would not be accessible to toxin on the cell surface. However, the luminal domain of synaptotagmin

Fig. 5. Synaptosomes from frog and mouse were isolated, submitted to electrophoresis, and then stained with antibodies for toxin substrates. Both sources of tissue contained syntaxin, SNAP-25, and one or both forms of synaptobrevin.

Fig. 6. Frog or mouse brain vesicles were incubated for 4 h at room temperature with or without 1 × 10⁻⁸ M botulinum neurotoxin type B in the presence or absence of 30 μM TPEN. Synaptobrevin was visualized on Western blots using enhanced chemiluminescence. Lane 1, control vesicles; lane 2, vesicles incubated with toxin and TPEN; lane 3, vesicles incubated with toxin alone.
does become accessible during exocytosis. Indeed, de Camilli and colleagues (Kraszewski et al., 1995) have prepared a fluorescent derivative of an antibody against the luminal domain of synaptotagmin and used it to label synaptic vesicles.

The discovery that frog nerve terminals are resistant to serotype B by virtue of an absence of receptors presents a unique opportunity. It may be possible to prepare a family of antibodies directed individually against the family of synaptotagmin homologues and then use cytohistochemical techniques to determine whether any of the homologues is missing.

2. If a low-affinity receptor and a high-affinity receptor play a role in type B toxin binding, these receptors are 1) missing from frog membranes; 2) not capable of acting individually as fully competent receptors; or 3) both of the above. Montecucco (1986) has advanced the interesting idea that toxin binding may involve two separate receptors. A low-affinity receptor would play the role of collecting toxin molecules on the nerve surface, and a high-affinity receptor would play the role of initiating events that lead to toxin action (viz., receptor-mediated endocytosis). In the present study, frog nerve endings were resistant to high concentrations of, and lengthy exposure to, serotype B. If the dual receptor hypothesis is correct, it means that one or both of the following two premises must be correct. First, the frog is resistant because it has simultaneously lost both classes of receptor, or second, the frog is resistant because it has lost one receptor, and the remaining receptor—even when exposed to high toxin concentrations—is not competent to initiate subsequent steps in toxin action.

3. Resistance to type B cannot be due to the loss of a sialic acid residue that all toxin serotypes share to produce paralysis. Incubation of botulinum toxin with sialic acid-containing molecules such as gangliosides causes loss of toxicity (Simmons and Rapport, 1971; Kitamura et al., 1980; Kamata et al., 1993). Similarly, incubation of nerve membranes with lectins that have affinity for sialic acid blocks toxin binding and action (Bakry et al., 1991a). These observations suggest that there is a sialic acid residue in or close to receptors for each serotype. The fact that frog nerve endings are sensitive to types A, C, and D, but not to type B, means that resistance to any individual serotype cannot be explained on the basis that the membrane has lost a common sialic acid binding site. This conclusion is reinforced by the finding that Limax flavus lectin continued to act as an antagonist of type C toxin in the cutaneous-pectoris preparation. This means, for example, that the various serotypes cannot share a common low-affinity receptor (viz., a ganglioside) whose loss accounts for resistance to serotype B. There must be some additional deficit to explain resistance (see above).

4. The binding site whose loss accounts for resistance to type B is not essential for binding of other clostridial neurotoxins, β-bungarotoxin or α-latrotoxin. Ligand-binding studies reported by other investigators have demonstrated that, with the possible exception of serotypes C and D, the various toxin serotypes do not share a common receptor. The findings reported here strongly reinforce that point. Even in the absence of binding sites for serotype B, several other serotypes apparently bind quite well. This line of reasoning can be expanded to include other neuromuscular blocking agents. Both β-bungarotoxin (Abe et al., 1977; Caratsch et al., 1985) and α-latrotoxin (Pumpkin and Reese, 1977) act on frog nerve endings that are resistant to botulinum toxin type B, which likely reflects an absence of overlap in binding sites.

Nerve endings that lacked receptors for type B toxin were otherwise normal in their ability to store and release transmitter. Clearly, the toxin receptor is not essential for cell viability in general or to the process of exocytosis in particular. This is in stark contrast to the toxin substrate. In the absence of synaptobrevin, individual nerves would be unable to maintain excitation-secretion coupling, and intact organisms would be unable to survive. The challenge now is to determine what role type B toxin receptors play in the structure or function of nerve membranes. The answer to this question may be facilitated by the identification of other nerves that lack receptors for single serotypes of botulinum toxin. Work to identify such nerves is underway.

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