In Vitro Characterization of Botulinum Toxin Types A, C and D Action on Human Tissues: Combined Electrophysiologic, Pharmacologic and Molecular Biologic Approaches

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ABSTRACT

Human exposure to botulinum toxin typically occurs in two settings: 1) as an etiologic agent in the disease botulism and 2) as a therapeutic agent for the treatment of dystonia. Epidemiologic studies on botulism suggest that the human nervous system is susceptible to five toxin serotypes (A, B, E, F and G) and resistant to two (C and D). In the past, these epidemiologic findings have been used as the basis for selecting serotypes that should be tested as therapeutic agents for dystonia. Epidemiologic data have been utilized because there are no studies of botulinum neurotoxin action on isolated human nerves. In the present study, electrophysiologic techniques were used to monitor toxin effects on neuromuscular transmission in surgically excised human pyramidalis muscles, ligand binding studies were done to detect and characterize toxin receptors in human nerve membrane preparations, and molecular biologic techniques were used to isolate and sequence a human gene that encodes a substrate for botulinum neurotoxin. The results demonstrated that stable resting membrane potentials (−61.5 mV; S.E.M. ± 0.7) were maintained in individual fibers of pyramidalis muscle for up to 6 hr at 33°C. The rate of spontaneous miniature endplate potentials was low in physiologic solution (0.14 sec⁻¹) but increased in response to elevations in extracellular potassium concentration. In keeping with epidemiologic findings, botulinum toxin type A (10⁻⁸ M) paralyzed transmission in human preparations (ca. 90 min). In contrast to epidemiologic findings, serotype C (10⁻⁸ M) also paralyzed human tissues (ca. 65 min). Iodinated botulinum toxin displayed high-affinity binding to receptors in human nerve membrane preparations (serotype A high-affinity site: Kd = 0.3 nM, Bmax = 0.78 pmol/mg protein; serotype C high-affinity site: Kd = 1.96 nM, Bmax = 8.9 pmol/mg protein). In addition, the human nervous system was found to encode polypeptides that are substrates for botulinum neurotoxin types A (syntaptosomal-associated protein of M, 25,000) and C (syntaxin 1A). These data have important implications bearing on: 1) the development and administration of vaccines against botulism and 2) the testing of toxin serotypes for the treatment of dystonia.

Botulinum neurotoxin exists in seven different serotypes, designated A, B, C, D, E, F and G. All seven serotypes are large proteins that act on cholinergic neuromuscular junctions to block transmitter release. Research on laboratory animal preparations has shown that the toxins produce this effect by proceeding through a sequence of four steps: 1) binding to receptors on the plasma membrane, 2) penetration of the plasma membrane by receptor-mediated endocytosis, 3) penetration of the endosome membrane by pH-induced translocation and 4) intracellular expression of an enzymatic action that culminates in blockade of exocytosis (Simpson, 1980; Simpson, 1981; Habermann and Dreyer, 1986; Simpson, 1989; Schiaivo et al., 1994b).

A great deal of attention has recently been focused on botulinum neurotoxin. This is due in part to the discovery that the various serotypes are zinc-dependent endoproteases that cleave synaptic proteins implicated in docking and fusion of vesicles (Schiavo et al., 1994b). Serotypes A and E cleave SNAP-25 (Blasi et al., 1993a; Schiaivo et al., 1993a; Binz et al., 1994); serotype C acts on syntaxin (Blasi et al., 1993b); serotypes B, D, F and G act on synaptobrevin (Schiavo et al., 1993a; Schiaivo et al., 1992; Yamasaki et al., 1994a; Schiaivo et al., 1993b; Yamasaki et al., 1994b; Schiaivo et al., 1994a). SNAP-25, syntaxin and synaptobrevin, along with several other polypeptides, are thought to be essential for exocytosis (Söllner et al., 1993b; Südhof et al., 1993; Söllner et al., 1993a; Pevsner et al., 1994).

Another reason for the recent focus on botulinum neurotoxin is its introduction as a therapeutic agent for the treatment of dystonia (Jankovic and Brin, 1991; Jankovic and

Abbreviations: MEPP, miniature endplate potential; SNAP-25, syntaptosomal-associated protein of M, 25,000.

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Hallett, 1994). Medically supervised administration of toxin is used to produce local blockade of transmission at sites of excessive efferent activity. Botulinum neurotoxin is now regarded as the treatment of choice for disorders such as blepharospasm, strabismus and hemifacial spasm, and it is likely to be accepted as the treatment of choice for many other neurological disorders (American Academy of Neurology, 1990; NIH Consensus Statement, 1991).

In spite of the fact that botulinum neurotoxin is both an agent that causes disease and a drug that has been approved for medicinal use, its actions have never been systematically studied on viable human tissues, such as the neuromuscular junction. As a result, there is a profound lack of knowledge about many of the most fundamental properties of the toxin. For example, there exist no data on the comparative dose-response characteristics of the seven toxin serotypes. Indeed, there are no convincing data to demonstrate whether the human neuromuscular junction is actually sensitive to all seven serotypes. As a consequence, epidemiologic findings on the occurrence of botulism have been used as the basis for deciding which serotypes should be tested as therapeutic agents. To date, epidemiologic data suggest that serotypes A, B, E, F and G cause adult botulism, whereas serotypes C and D do not (Gangarosa et al., 1971; Dowell, Jr. 1984).

The goal of the present study was to undertake the first systematic analysis of botulinum toxin action on isolated and viable human neuromuscular junctions. Serotype A, which has often been implicated in naturally occurring botulism, and serotypes C and D, which have rarely if ever been implicated, were selected for evaluation. Because this is the first study to undertake a detailed examination of toxin action on living human tissues, the work was divided into two phases: 1) identification and characterization of a human preparation that is suitable for analyzing neuromuscular transmission and 2) examination of the action of botulinum toxin types A, C, and D on this preparation.

Materials and Methods

Human tissues. Institutional Review Board approval was obtained for protocols in which striated muscle was removed during surgical procedures (e.g., removal of pyramidalis muscle from patients undergoing laparotomies). Informed consent was obtained whenever removal of tissue was not an essential part of the surgical procedure.

Excised tissues were immersed in chilled physiologic solution of the following composition (mM): NaCl, 138.8; KCl, 4.0; KH₂PO₄, 1.0; NaHCO₃, 12.0; CaCl₂, 2.0; MgCl₂, 1.0; d-glucose, 11.0. Depending on size, each preparation was divided into smaller but intact fascicles approximately 5 mm wide and 1 mm thick. Individual fascicles were pinned in a 35-mm Sylgard-coated Petri dish and continuously perfused (3 ml/min) with fresh physiologic solution equilibrated with 95% O₂ and 5% CO₂. The bath temperature was maintained at 33°C unless otherwise stated.

Electrophysiology. Standard electrophysiologic techniques were used to record endplate activity. Glass microelectrodes filled with 3 M KCl (tip resistance 20–40 MΩ) were connected to a high-input impedance amplifier. The output from the amplifier was further amplified, filtered through a low-pass filter and digitized through an A/D converter interfaced with a computer. Data were acquired, stored and analyzed with AXOTAPE and PCLAMP software (Axon Instruments, Inc., Foster City, CA).

Endplate regions were localized by following the course of tiny intramuscular nerve branches along the surface of muscle fibers. Microelectrodes were inserted as close as possible to endplate regions, and the rate of spontaneous MEPPs was monitored at various levels of extracellular potassium (see “Results”). During a typical experiment, MEPPs were measured for a base-line period of 60 to 90 min before addition of toxin.

Two protocols were used for exposing tissues to toxin. In the first, toxin was merely added to normal physiologic solution (33°C) and maintained for the duration of the experiment. The rate of spontaneous MEPPs was monitored continuously throughout the experiment. In the second, the temperature of the bath was lowered to 7°C, after which toxin was added for 60 min. At the end of incubation, tissues were washed to remove unbound toxin, temperature was raised to 33°C, and recording of MEPPs was resumed.

Neuromuscular blockade was defined as a 90% reduction in the rate of spontaneous MEPPs. If blockade did not occur within 240 min, then tissues were considered resistant to toxin at the concentration tested. Control experiments were done to demonstrate the viability of preparations in the absence of toxin.

Receptor binding studies. Institutional Review Board approval was obtained for a protocol to acquire human brain tissue at autopsy. Binding of toxin to human brain membrane preparations was measured as previously described (Bakry et al., 1991). Membrane preparations were obtained by homogenizing tissue in iced Tris·HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged for 10 min at 1000 × g, and the resulting homogenate was resuspended in fresh buffer and recentrifuged for 45 min at 40,000 × g. The final pellet was resuspended in Tris·HCl buffer, as described above.

Iodinated ligand (1.0 nM), prepared as described below, was mixed with 50 µg of membrane protein in 0.1 ml of pH 7.4 buffer containing 50 mM Tris·HCl, 100 mM NaCl and 1 mg/ml bovine serum albumin. The binding reaction was done at 23°C for 60 min, which was the amount of time necessary to reach equilibrium. The reaction was terminated by centrifugation (15,000 × g; 2 min), after which the pellet was washed and recentrifuged. The amount of iodinated ligand associated with membranes was quantified, and the results were corrected for nonspecific binding.

Molecular biology studies. A human brain library (Human Brain 5′-stretch plus cDNA, Clonetech) was screened twice with an 800-base pair fragment of open reading frame obtained from an initial screening with rat syntaxin 1A cDNA (kindly provided by Dr. R. Scheller, Stanford University). A cDNA clone, designated pCD-HS.2, was selected and analyzed by primer walking of both strands and was found to have 2088 base pairs. Dideoxynucleotide sequencing was used to automate Tth cycle sequencing, and the results were confirmed by manual dideoxynucleotide sequencing (Sanger et al., 1977). Additionally, the 5′ end sequencing was confirmed by polymerase chain reaction applied to library cDNAs with sets of primers designed to overlap the cloning sites. A putative start codon was located at nucleotide 2, and the open reading frame (nucleotide positions 2–868) suggested a coding region containing 288 translated amino acids (GenBank accession No. U12918).

The cloned human gene for syntaxin 1A was expressed in vitro by using a rabbit reticulocyte lysate preparation (Promega TnT Transcription and Translation System). The circular DNA was inserted into a pCR II vector with a T7 promoter, and expression was carried out in the presence of [35S]Methionine according to the manufacturer’s instructions. The resulting preparation was fractionated on a sodium dodecylsulfate polyacrylamide gel (15 percent) and submitted to autoradiography (48 hr; −20°C). The molecular weight of the expression product was deduced by comparison with a set of molecular weight standards.

Neurotoxins and antibodies. Botulinum neurotoxin types A, C and D were isolated as previously described (Simpson et al., 1988). Samples of botulinum neurotoxin type C were also provided by Dr. Y. Kamata (University of Osaka Prefecture). Human pentavalent (AB–CDE) immune globulin against botulinum neurotoxin was obtained from Connaught Laboratories (Swiftwater, PA).
Samples of botulinum neurotoxin were radioiodinated with Bolton-Hunter reagent. Purified toxin (100 μg) was mixed with 1 mCi (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 percent.

Expression of human substrates and proteolysis by botulinum toxin. Human syntaxin 1A was cloned and sequenced (see above and “Results”), and 1 μg of DNA for the gene was added to 25 μl of TNT Coupled Reticulocyte Lysate (Promega, Madison, WI), 1 μl of T7 RNA polymerase (per kit instructions), and 20 μCi 35S-labeled methionine. The reaction mixture was incubated for 90 min at 30°C, after which it was centrifuged (12,000 × g) for 15 min. The pellet was washed twice and then resuspended in proteolysis buffer (25 mM HEPES, pH 7.4; 50 mM NaCl; 10 μM ZnCl₂).

Human SNAP-25 was obtained by polymerase chain reaction, using template DNA from a human brain cDNA library (Human brain 5'-stretch plus cDNA; Clonetech). The primers were 5'-ATGGCCGAAAGACGCAGAC-3' and 5'-GCACACTTAACCACTTCC-3’, which cover the entire open reading frame (nucleotides 89–805; Bark and Wilson, 1994). The authenticity of the product was confirmed by sequencing. The polymerase chain reaction product was cloned into the TApCR vector (Invitrogen, San Diego, CA) and subcloned into the EcoRI site of Bluescript SK- (Stratagene, La Jolla, CA). Human SNAP-25 was transcribed and translated in vitro with the TNT System and T3 RNA polymerase. The product was centrifuged, washed and suspended in proteolysis buffer, as described above.

In proteolysis experiments, botulinum neurotoxin types A and C were prerduced with 10 mM DTT (45 min at 37°C). Toxin was then incubated with substrate for 60 min at 37°C. The reaction was terminated by boiling in sodium dodecylsulfate sample buffer for 3 min and then run on a polyacrylamide gel (12%). Dried gels were exposed to X-ray film for 16 hr. The molecular weights of substrates and reaction products were determined by comparison with standards.

Results

Selection of tissues. A variety of innervated muscle preparations were evaluated, including tissues from the head and neck, trunk and upper and lower limbs. Of the many preparations tested, the only one that proved suitable was the pyramidalis muscle (see “Discussion”). This tissue, which is located along the lower abdominal wall at the base of the rectus abdominis, is relatively small, surgically accessible and reasonably available (see “Discussion”).

Characteristics of the human pyramidalis muscle preparation. Each muscle was probed with microelectrodes in an effort to localize endplate regions. Intracellular recordings in a large series of endplates (n = 107) revealed an

Fig. 1. A) Intracellular analysis of endplate activity in human pyramidalis muscle. Intracellular electrodes were used to record spontaneous MEPPs from muscle bathed in normal physiologic solution (panel a; 4 mM KCl) or in modified physiologic solution (panel b; 12.5 mM KCl). Evoked activity was recorded from muscle bathed in moderately elevated potassium solution (panel c; 25 mM KCl). In both panels b and c, NaCl concentration was adjusted appropriately to maintain isotonicity. B) Graphical analysis of endplate activity in human pyramidalis muscle. MEPP frequencies (ordinate) were compared at increasing concentrations of KCl (abscissa). The mean frequency in normal physiologic solution (4 mM KCl) was 0.14 ± 0.03 MEPPs sec⁻¹. Mean frequency increased to 1.5 ± 0.12 MEPPs sec⁻¹ in 12.5 mM KCl solution and to 14.5 ± 2.7 MEPPs sec⁻¹ in 25 mM KCl. Inset: Amplitude histogram from a representative endplate region of the pyramidalis muscle, illustrating the Gaussian nature of spontaneous MEPPs. The mean amplitude of MEPPs at this particular endplate was 1.96 mV ± 0.021.
average resting membrane potential of $-61.5 \pm 0.7$ mV. Resting potentials were well maintained for periods of 4 to 6 hr when tissues were kept at 33°C. Membrane potential and tissue responsiveness tended to diminish when experiments were done for comparable lengths of time at 37°C.

The rate of spontaneous MEPPs in physiologic solution was $0.14 \pm 0.03$ sec$^{-1}$ ($n = 11$; temperature, 33°C). This rate increased in a concentration-dependent manner with elevations in extracellular potassium (fig. 1). The mean amplitude of spontaneous MEPPs was $2.4 \pm 0.08$ mV ($n = 27$), and the amplitude distribution was Gaussian in nature (fig. 1). The mean duration of spontaneous MEPPs was $3.4 \pm 0.19$ msec ($n = 24$).

**Characteristics of evoked responses.** Surgically excised preparations of human pyramidalis muscle frequently did not have a sufficient nerve stump to permit direct, microelectrode-induced stimulation. Therefore, mild potassium-induced depolarization was used as an alternative. In a typical experiment, tissues were maintained in 12.5 mM potassium for a base-line period of 30 to 60 min, and the frequency of MEPPs was monitored. Under these conditions, the base-line rate of MEPPs was $1.5 \pm 0.12$ sec$^{-1}$ ($n = 50$). At the end of the base-line period, tissues were transiently exposed to 25 mM potassium (1–3 min). This caused the rate of MEPPs to increase by approximately one order of magnitude ($14.5 \pm 2.7$ sec$^{-1}$). When tissues were returned to 12.5 mM potassium, the rate of MEPPs fell to base-line levels.

Human pyramidalis muscle responses to 12.5 mM and 25 mM potassium were well maintained for extended periods of time (fig. 2A). When continuously perfused in 12.5 mM potassium solution, tissues displayed a stable MEPP rate for periods of 240 to 300 min. When tissues were briefly exposed to 25 mM potassium at intervals of approximately 60 min, the magnitudes of responses were comparable (see below). These stable responses permitted an analysis of botulinum neurotoxin action.

**Botulinum neurotoxin type A action.** The addition of botulinum neurotoxin type A to human pyramidalis muscle preparations produced irreversible blockade of transmission (i.e., 90% or greater reduction in MEPP frequency). This could be demonstrated by using two different paradigms and two different measures of outcome. When toxin ($1 \times 10^{-8}$ M) was added to tissues maintained at 33°C in 12.5 mM potassium solution, the base-line rate of MEPPs remained constant for about 30 to 40 min and then began to decay. Within 60 to 90 min, the base-line rate of MEPPs fell to levels that were too low to measure (fig. 2B). This evidence of toxin-induced blockade was confirmed by immersing tissues in 25 mM potassium solution. The spike in MEPP frequency ordinarily associated with potassium depolarization was almost completely abolished (fig. 2B).

A second paradigm generated very similar results. Tissues were initially immersed in medium at 33°C, and both base-line responses to 12.5 mM potassium and evoked responses to 25 mM potassium were monitored. Tissues were then transferred to physiologic solution at 7°C, after which toxin was added ($1 \times 10^{-8}$ M) for an incubation period of 60 min. After incubation, tissues were washed to remove unbound toxin, temperature was raised to 33°C, and MEPP frequency was monitored in 12.5 mM potassium solution. As before, the toxin produced an irreversible decay in the rate of MEPPs (not illustrated). Also as before, the normal response to elevated potassium (25 mM) was virtually abolished (not illustrated).

**Botulinum neurotoxin type C action.** Experiments identical to those done with botulinum toxin type A were done with botulinum toxin type C ($1 \times 10^{-8}$ M). Quite unexpectedly, this serotype also produced blockade of neuromuscular transmission. Regardless of whether toxin was present continuously at 33°C or was present for only 60 min at 7°C, the sequence of events was the same. There was an initial lag period, after which the base-line rate of MEPPs in 12.5 mM potassium evoked activity was returned to normal limits. All data points represent the mean activity of at least three endplates recorded per time-point. B) Base-line spontaneous and evoked endplate responses under control conditions (no toxin). Tissue was perfused with 12.5 mM KCl, and base-line MEPP frequency was monitored. At time-points a, b and c, the tissue was exposed briefly to 25 mM KCl, and evoked activity was recorded. Between these time points, MEPP frequency returned to within normal limits. All data points represent the mean activity of at least three endplates recorded per time-point. B) Base-line spontaneous and evoked endplate responses before and during toxin exposure (serotype A, $1 \times 10^{-8}$ M). Both spontaneous and K$^+$-evoked activity (time-point a) were monitored intracellularly over a period of 60 min. Toxin was then added to the bath at time-point b, and base-line activity was monitored. After a lag period, base-line activity began to decline, and it eventually disappeared within 90 min of the addition of toxin. At time-point c, exposure to 25 mM KCl did not evoke a measurable response.
potassium decayed and eventually became almost unmeasurable (fig. 3A).

To ensure that the observed response was authentic and could be attributed to serotype C, we did two control experiments. In the first, the neurotoxin was isolated for a second time from a different strain of Clostridium botulinum. Like the original serotype C, this batch of toxin produced neuromuscular blockade (not illustrated). In the second experiment, serotype C was preincubated with neutralizing antibody before being added to tissues. In this case, there was no neuromuscular blockade (fig. 3B).

**Botulinum neurotoxin type D action.** At concentrations equivalent to those tested with serotypes A and C, botulinum neurotoxin type D (1 × 10⁻⁸ M) did not block human neuromuscular transmission (n = 3). The rate of spontaneous and evoked MEPPs remained stable in the presence of toxin for periods up to 4 hr (not illustrated). Even when toxin concentration was increased an order of magnitude, there was still no evidence of paralysis. These results indicate that the human neuromuscular junction is resistant to serotype D.

**Binding of botulinum toxin to human nerve cell membranes.** The fact that botulinum toxin types A and C blocked transmission implies that the human nervous system has cell surface receptors. Therefore, work was done to verify the existence of these receptors and to characterize toxin-receptor interactions.

Preliminary experiments with serotypes A and C and membrane preparations from several areas of human brain (prefrontal cortex, anterior temporal cortex, superior parietal cortex, putamen, globus pallidus and cerebellum) revealed that the cerebellum typically had the highest density of toxin binding sites. Therefore, this region of the human nervous system was examined in some detail.

The binding of ¹²⁵I-botulinum neurotoxin type A to human cerebellar membranes increased as a function of protein; an apparent plateau was reached at 100 to 200 μg/assay (100 μl/assay). Binding also increased as a function of time, an apparent equilibrium being reached at 15 to 20 min (fig. 4).

Various concentrations of iodinated botulinum neurotoxin were incubated with membrane preparations (50 μg/100 μl protein; 60 min), and the resulting data were used to generate a saturation isotherm and a Scatchard plot (fig. 5).

Graphical analysis of the data revealed two classes of binding sites. The characteristics of the high-affinity binding site were \( K_d = 0.3 \text{ nM} \) and \( B_{\text{max}} = 0.78 \text{ pmol/mg protein} \). The characteristics of the low-affinity site were \( K_d = 3.3 \text{ nM} \) and \( B_{\text{max}} = 3.18 \text{ pmol/mg protein} \). In related experiments, various concentrations of unlabeled serotype A were used as competitive antagonists of the binding of labeled serotype A (fig. 5). The apparent IC₅₀ value for the unlabeled toxin, as deduced by graphical analysis, was 3.5 nM, which is in agreement with the \( K_d \) value for the bulk of binding sites in these membranes.

By contrast, a substantial molar excess (0.3 μM) of heterologous toxin (i.e., serotype C) did not antagonize the binding of iodinated type A (and see below).

The binding of botulinum neurotoxin type C to nerve membranes was studied under conditions equivalent to those used with serotype A. The results indicated the presence of specific binding sites; toxin association with these binding sites increased as a function of time (equilibrium ca. 30–60 min) and protein (half-maximal binding ca. 50–100 μg/100 μl).

Various concentrations of iodinated botulinum toxin type C were incubated with nerve membranes (50 μg/100 μl; 60

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**Fig. 3.** Effects of botulinum toxin type C on spontaneous and evoked endplate activity in human muscle. A) Base-line spontaneous and evoked endplate responses before and during toxin exposure (serotype C, 1 × 10⁻⁸ M). Both spontaneous and K⁺-evoked activity (time-point a) were monitored over a period of 85 min. Toxin was then added to the bath at time-point b, and base-line activity was monitored. After approximately 45 min, MEPP frequency began to decrease, and within 60 min, transmission was completely blocked. At time-point c, exposure to 25 mM KCl failed to evoke a response. B) Base-line spontaneous and evoked endplate responses before and during exposure to toxin (type C, 1 × 10⁻⁸ M) that had been preincubated with an excess of toxin-specific neutralizing antibody. Both spontaneous and K⁺-evoked activity (time-point a) were monitored intracellularly over a period of 90 min. Toxin was then added to the bath at time-point b, and base-line activity was monitored. Note that in this case, base-line activity remained relatively constant during toxin exposure. At time-point c, exposure to 25 mM KCl evoked an increase in MEPP frequency comparable to that seen before toxin exposure (time-point a). Blockade of transmission had failed to occur in the presence of neutralized toxin, which indicates that the blockade illustrated in panel A was a specific effect of the toxin.
which the human gene has not been cloned and sequenced, and SNAP-25 (Williamson et al., 1996; Foran et al., 1996).

A gene for human syntaxin was isolated, cloned and sequenced, as described in "Materials and Methods" (fig. 7). The deduced amino acid sequence of the open reading frame revealed a polypeptide that was strikingly similar in length and primary sequence to rat syntaxin 1A (Zhang et al., 1995). The overall identity in amino acid sequences between the two syntaxins was approximately 98%. Therefore, the expression product was deduced to be human syntaxin 1A.

**Cleavage of human substrates.** SNAP-25 and syntaxin 1A were expressed in vitro, as described in "Materials and Methods." Serotypes A and C, each at $1 \times 10^{-7}$ M, were reduced with dithiothreitol (10 mM; 37°C; 45 min) and then incubated with substrates (37°C; 60 min). The reaction mixtures were submitted to polyacrylamide gel electrophoresis (12%), and the dried gels were subsequently exposed to X-ray film for 16 hr.

As shown in figure 8, serotypes A and C caused proteolytic cleavage of their respective human substrates. Furthermore, cleavage was in keeping with the zinc-dependent endoprotease action of the toxins. Thus incubation of toxin and substrate in the presence of a zinc chelator [50 $\mu$M tetrakis(2-pyridylmethyl)ethylenediamine] markedly diminished proteolysis of SNAP-25 and syntaxin 1A.

**Discussion**

The literature describing electrophysiologic properties of excised human neuromuscular junctions is very limited. This is due in large measure to ethical constraints, which properly restrict the circumstances under which normal tissue can be removed from patients. One possible remedy that respects the ethical constraints is to harvest tissue that would ordinarily be removed or be damaged in situ during routine surgical procedures. However, this raises a host of experimental concerns. To be acceptable as an experimental preparation, a human tissue should possess the following characteristics: 1) reasonable availability, based on accessibility of muscle during various surgical procedures, 2) reasonable consistency in size, 3) ease of orientation after removal from patients, 4) ease of localization of endplate regions and 5) ability to survive and respond for substantial periods of time. These criteria were best satisfied by the pyramidalis muscle, which has one additional advantage (Chouke, 1935; Beaton and Anson, 1939; Monkhouse and Khalique, 1986). The muscle is generally regarded as nonessential, so partial or complete removal does not impair the donor.

Electrophysiologic studies of the pyramidalis muscle revealed that the endplate region was localized to discrete areas. Intracellular recordings at the endplate region demonstrated a resting membrane potential of $-61.5$ mV. This was well maintained over 4 to 6 hr at a constant temperature of 33°C. The rate of MEPPs in physiologic solution was 0.14 sec$^{-1}$, and this value is in keeping with that previously reported for other human neuromuscular junctions (Elmqvist and Quastel, 1965; Lambert and Elmqvist, 1971; Haynes, 1971; Maselli et al., 1991; Maselli et al., 1992; Slater et al., 1992). The amplitude distribution of spontaneous MEPPs was consistent with a Gaussian distribution. Elevations in extracellular potassium produced concentration-dependent increases in the rate of MEPPs. These evoked
increases in MEPP rate, like spontaneous MEPP rate, were well maintained over time. This gave us the opportunity to examine the action of botulinum neurotoxin.

**Mechanism of toxin action.** Botulinum toxin proceeds through a series of steps to produce its effects on cholinergic nerve endings. This series includes binding, productive internalization and eventual expression of an intracellular effect (see the Introduction for references). This general scheme for describing toxin action arose from studies on the murine phrenic nerve-hemidiaphragm preparation (Simpson, 1980), and with few exceptions (e.g., Aplysia; Poulain et al., 1989; Poulain et al., 1991), it has proved useful for describing toxin action on other cholinergic junctions. However, there is no way to know whether this model applies to the human neuromuscular junction, because there have been no studies on isolated human tissues. Therefore, one of the goals of this work was to analyze toxin action on excised human neuromuscular junctions. Serotypes A, C and D were selected for study.

Botulinum neurotoxin type A produced irreversible blockade of transmission in the pyramidalis preparation. This could be demonstrated by using a standard paradigm in which toxin was continuously present, and it could also be shown using a binding paradigm in which the toxin was present for only a limited time. The latter approach arose from studies on murine preparations (Simpson, 1980). When mouse phrenic nerve-hemidiaphragms are incubated with toxin at a low temperature (<10°C), the toxin binds to the tissue but does not cause paralysis. The absence of blockade is due to the fact that receptor-mediated endocytosis is arrested at low temperature, and thus toxin is not internalized. Transmission does not begin to fail until temperature is elevated.

The actions of botulinum neurotoxin type A on human tissues were entirely consistent with this model. When toxin was added to tissues at 7°C, there was no evidence of the onset of paralysis. When tissues were washed and then suspended in toxin-free medium at 33°C, there was a lag time that could account for internalization and then onset of blockade. Paralysis of transmission was demonstrated in two ways: 1) the rate of spontaneous MEPPs fell more than 90%, and 2) the sharp rise in MEPP rate due to elevated potassium was nearly abolished.

Botulinum neurotoxin types C and D were also studied on the human pyramidalis preparation. In contrast to serotype C, which appeared to block transmission similarly to serotype A, serotype D produced no observable effect. Even when used at concentrations 10-fold higher than those of the other two serotypes, type D still produced no measurable effect over a period of 4 to 5 hr.

The finding that serotype D does not poison human preparations is in keeping with epidemiologic data. There has never been a confirmed case of type D human botulism. On the other hand, the finding that serotype C blocked transmission was unexpected. There are no confirmed cases of type C poisoning in adults, and there is only one report of type C poisoning in a single infant (Oguma et al., 1990). In view of the apparent difference between epidemiologic data and isolated tissue data, a number of experiments were done to ensure that type C does indeed act on human tissues. These experiments included: 1) isolation and testing of type C toxin from two different strains of clostridia, 2) neutralization of toxicity with specific antibodies, 3) demonstration that the human nervous system has high-affinity binding sites for serotype C, 4) demonstration that the human nervous system has a gene encoding syntaxin 1A, the major substrate for serotype C, and 5) demonstration that serotype C cleaves the translation product of the human syntaxin 1A gene. Taken together, these results offer compelling evidence that isolated human tissues are susceptible to the toxin.

The fact that type C toxin does paralyze human neuromuscular transmission raises the question why the toxin does not
typically cause human botulism. Although there could be many possible explanations, two are particularly obvious. Apparent resistance may be due to ecologic factors (i.e., lack of human exposure) or to physiologic factors (i.e., poor human absorption). Experiments are currently in progress in an effort to answer this question.

**Therapeutic issues.** The data presented here bear on three interrelated issues of patient care: 1) the appropriateness of developing and administering a polyvalent vaccine against botulinum neurotoxin, 2) the inappropriateness of including serotype C in any vaccine formulation and 3) the need to evaluate serotype C as a therapeutic agent for dystonia.

There currently exist a number of experimental vaccines against botulinum toxin, including a pentavalent vaccine (A, B, C, D and E) distributed by the Centers for Disease Control. These vaccines were developed and were being administered long before it was realized that botulinum toxin has value as a therapeutic agent. As a result, there are vaccinated persons who, should they develop dystonia, would be unresponsive to botulinum toxin therapy. This is a serious matter given that 1) botulinum toxin is the only therapeutic intervention that gives satisfactory results for most patients with dystonia, and 2) vaccination can produce long-term resistance to toxin, i.e., a decade or longer. These facts argue strongly that one must be cautious and thoughtful about administering vaccine.

This argument takes on added weight when viewed in the context of recent immunology work. The complete primary structures of the botulinum serotypes have been determined, and all possess similarity to tetanus toxin. Recombinant molecular biology techniques have been used to generate experimental vaccines to tetanus (Fairweather et al., 1987; Chatfield et al., 1992; Boucher et al., 1994), and the same will probably occur for botulinum toxin. This creates the temptation to develop and administer a polyvalent vaccine against botulism and tetanus. However, such a course of action may not be in the best interest of patient care. The incidence of naturally occurring botulism is orders of magnitude less than the incidence of dystonia. If a polyvalent vaccine against botulinum toxin were to be widely administered, it would produce the negligible benefit of protecting against botulism and at the same time produce the substantial risk of causing all current and future dystonia patients to become unresponsive to the only medication that has authentic value. This risk-to-benefit analysis suggests that immunization should...
be provided only when there is an identifiable and meaningful clinical gain.

On a related issue, there is strong reason to argue that serotype C should not be included in any vaccine. Although the toxin does act on isolated human tissues, type C botulism rarely if ever occurs in adults. The explanation for the apparent absence of disease may be related to ecologic factors or physiologic factors, as discussed earlier. Whatever the explanation, the low incidence of type C botulism argues against the need for a vaccine.

On a more positive note, the data encourage the testing of serotype C as a therapeutic agent for dystonia and related neurologic disorders. In the past, the epidemiologic literature on naturally occurring botulism was used as a guide in selecting toxin serotypes to test as medicinal agents. Serotype A has long been implicated in human illness; it was the first serotype to be evaluated as a medicinal agent, and it is the only serotype that has been approved for clinical use (Jankovic and Brin, 1991). Other serotypes implicated in human illness (B, E and F) are now undergoing clinical trials (Jankovic and Hallett, 1994). However, the finding that serotype C blocks human neuromuscular transmission means that the practice of relying solely on epidemiology as a guide for choosing therapeutic agents is flawed. Clearly, serotype C warrants investigation for the treatment of neurologic disorders.

The possibility that serotype C is efficacious carries a hidden benefit. If a situation were to arise in which it was necessary to provide immunization against naturally occurring botulism, there would be no need to include serotype C in the vaccine. This would mean that it should be possible to protect patients against food-borne botulism (e.g., serotype A) while not depriving them of the ability to respond to antidysonxia medication (e.g., serotype C).

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References


BIANCHI, M. C., BLASI, J., CHAPMAN, E. R., ZEUS, L. S., KALASINSKA, M., YAMASAKI, S., DE CAMILLI, P., BARK, I. C. AND WILSON, M. C.: Human cDNA clones encoding two different ring botulism, there would be no need to include serotype C.


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