Cleavage of SNAP-25 by Botulinum Toxin Type A Requires Receptor-Mediated Endocytosis, pH-Dependent Translocation, and Zinc

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Received July 31, 2000; accepted November 28, 2000

ABSTRACT
Previously we reported that SNAP-25, synaptobrevin II, and syntaxin I, the intracellular substrates of botulinum toxin originally identified in nontarget tissues, were present in a recognized mammalian target tissue, the mouse hemidiaphragm. Furthermore, we reported that SNAP-25, syntaxin I, and synaptobrevin II were cleaved by incubation of the intact hemidiaphragm in botulinum serotypes A, C, and D, respectively. The objective of the current study was to use the mouse phrenic nerve-hemidiaphragm preparation and botulinum serotype A to investigate 1) the relationship of substrate cleavage to toxin-induced paralysis, and 2) the relevance of substrate cleavage to the mechanism of toxin action. Immunoblot examination of tissues paralyzed by botulinum toxin type A (10^{-8} M) revealed ≤10% loss of SNAP-25 immunoreactivity at 1 h postparalysis, and ≥75% loss at 5 h postparalysis. Triticum vulgaris lectin, an agent that competitively antagonizes toxin binding, antagonized toxin-induced paralysis as well as SNAP-25 cleavage. Methylamine hydrochloride, an agent that prevents pH-dependent translocation, also antagonized toxin-induced paralysis and SNAP-25 cleavage. Furthermore, zinc chelation antagonized toxin-induced paralysis and SNAP-25 cleavage. These results demonstrate that cleavage of SNAP-25 by botulinum serotype A fulfills the requirements of the multistep model of botulinum toxin action that includes receptor-mediated endocytosis, pH-dependent translocation, and zinc-dependent proteolysis. Furthermore, the minimal amount of SNAP-25 cleavage at 1 h postparalysis suggests that inactivation of only a small but functionally important pool of SNAP-25 is necessary for paralysis.

Botulinum toxin, a neurotoxin that selectively targets peripheral cholinergic nerve endings, is widely recognized as the most potent biological poison. Seven distinct serotypes of toxin have been identified and designated A to G (Hathaway, 1990; Oguma et al., 1995). Considerable information on the cellular and molecular aspects of toxin action has accumulated. It is well established that the principal target of botulinum toxin is the cholinergic nerve ending of the neuromuscular junction, where inhibition of acetylcholine release results in neuromuscular blockade and paralysis (Ambache, 1949; Burgen et al., 1949; Rao et al., 1976; van Ermenegem, 1979; Simpson, 1981). Using a series of pharmacological manipulations combined with electrophysiology, a multistep scheme was developed to describe the mode of toxin action at the neuromuscular junction (Simpson, 1980, 1981). Accordingly, after first binding to serotype-specific receptors on the surface of cholinergic nerve endings, the toxin is internalized by endocytosis. Following internalization, the toxin translocates from the endosome to the cytosol by a pH-dependent process, where it is then free to act on its intracellular targets. Proceeding through this sequence of events is required for toxin-induced paralysis in vertebrate animals (Simpson, 1980; Bakry et al., 1991). The intracellular targets of botulinum toxin, a zinc-dependent endoprotease, have been shown to be proteins of the presynaptic SNARE fusion complex (Schiavo et al., 1992a; Simpson et al., 1993; Sollner et al., 1993; Schiavo et al., 1994a). These include the plasma membrane proteins syntaxin I and synaptosomal-associated protein of 25 kDa (SNAP-25), and the vesicular protein synaptobrevin II. With the exception of serotype C, each of the botulinum toxin serotypes targets only one of the three proteins (Schiavo et al., 1992b, 1993a,b, 1994b; Blasi et al., 1993a,b). Serotypes A and E cleave SNAP-25. Serotypes B, D, F, and G cleave synaptobrevin II. Serotype C cleaves primarily syntaxin I, and also cleaves SNAP-25 in some cell types (Foran et al., 1996; Williamson et al., 1996). These findings have greatly advanced our understanding of the cellular biology of exocytosis; however, the use of nontarget preparations in the majority of these studies prevented accurate

ABBREVIATIONS: SNAP-25, synaptosomal-associated protein of mol. wt. 25 kDa; MEPP, miniature endplate potential; TVL, Triticum vulgaris lectin; MAH, methylamine hydrochloride; TPEN, tetrakis(2-pyridylmethyl)ethylenediamine.

This work was supported in part by National Institutes of Health Grant 1 R01 ES10182-01 awarded to J.A.C.

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assessments of the relationship between intracellular substrate proteolysis, and paralysis of the target tissue. This is not a trivial matter, especially in light of reported differences in the central and peripheral intracellular targets of botulinum toxin, a functionally related clostridial neurotoxin (Fachiano et al., 1993; Ashton et al., 1995; Coffield et al., 1993). This type of assessment would be of value to both clinical medicine and cell biology, and is best done using a mammalian neuromuscular preparation that is suitable for both electrophysiology and protein chemistry. In the past, such study has been hampered by the perception that the amounts of protein in such a preparation are below detection limits. However, recent studies conducted in our laboratory revealed that SNAP-25, as well as syntaxin I and synaptobrevin II are detectable in measurable quantities in the mouse phrenic nerve-hemidiaphragm preparation using enhanced chemiluminescence (Kalandakanond and Coffield, 2001). Furthermore, we confirmed that these proteins served as substrates for botulinum toxin types A, C, and D action at the mammalian neuromuscular junction. These data indicate that this neuromuscular preparation is suitable for correlative electrophysiology and protein immunochemistry. In the current study, we have used the mouse phrenic nerve-hemidiaphragm preparation and botulinum toxin type A to investigate 1) the temporal relationship of substrate cleavage to toxin-induced paralysis, and 2) the dependence of substrate cleavage on the multistep mode of toxin action. The findings reported herein confirm our previous findings that SNAP-25 is an intracellular substrate of botulinum toxin type A in the phrenic nerve-hemidiaphragm, by demonstrating that SNAP-25 cleavage, like neuromuscular paralysis, requires receptor-mediated endocytosis, pH-dependent translocation, and the presence of zinc. Furthermore, our findings indicate that the temporal correlation between substrate proteolysis and paralysis is nonlinear, since the onset of paralysis is associated with minimal substrate cleavage. This suggests that inactivation of only a small but functionally important pool of SNAP-25 is necessary for nearly complete inhibition of transmitter release at the neuromuscular junction.

Materials and Methods

Tissue Preparation. Hemidiaphragm and brain tissues were isolated from NIH Swiss adult male mice (30 g) following decapitation. All procedures were approved by the University’s Institutional Animal Care and Use Committee. The hemi-preparation was removed from the mouse with muscle fibers remaining intact via connective tissue attachments on one end and bony attachments on the opposite end.

Electrophysiology. Phrenic nerve-hemidiaphragm preparations were used to monitor spontaneous miniature endplate potentials (MEPPs) and stimulus-evoked muscle twitch as previously reported (Simpson et al., 1993). Briefly, isolated hemi-diaphragms were pinned in a Sylgard-coated recording chamber and perfused with oxygenated physiological solution (1–2 ml/min; 32–34°C) of the following composition: 137.0 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgSO₄, 24.0 mM NaHCO₃, 1.0 mM NaH₂PO₄, and 11.0 mM d-glucose. The solution was augmented with gelatin (0.015%) as an auxiliary protein to diminish nonspecific adsorption or inactivation of toxin. The trunk of the phrenic nerve was drawn up into a suction electrode connected to a single channel stimulus isolator and stimulated at 0.3 Hz. Glass microelectrodes (20–40 MΩ) filled with 3 M KCl were used for intracellular recording of endplate activity. MEPPs were recorded with a high-input impedance amplifier (A-M Systems, Everett, WA). Activity was sampled every 15 min and a minimum of three endplate regions was sampled per time point. Toxin-induced paralysis was defined as a 90% reduction in MEPP frequency and twitch response.

Toxin Incubation. Botulinum toxin type A was purchased from WAKO Chemicals (Richmond, VA). Triticum vulgaris lectin (TVL), tetraakis(2-pyridylmethyl) ethylenediamine (TPEN), methylamine hydrochloride (MAH), and Ca-EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Botulinum toxin type A was added to the tissues at a final concentration of 10⁻⁸ M. Unbound toxin was removed from the bath within 10 to 20 min after paralysis was observed. Control and toxin-treated tissues were incubated for an additional 1 or 5 h in the oxygenated perfusion bath. In experiments to correlate the cleavage of substrate with the multistep model of toxin action, tissues were treated with TVL (100 µM), MAH (25 mM), or EDTA (200 µM) + TPEN (15–20 µM) before and during toxin incubation. Tissues continued to be incubated in MAH or EDTA + TPEN after toxin was removed. TVL was removed when toxin was removed. All incubations were terminated at 6 h after the addition of toxin.

Synaptic Protein Preparation. Following toxin incubation, excess muscle tissue from control and toxin-treated preparations was carefully removed so that only tissue immediately surrounding the visible innervation zone of the phrenic nerve remained. Enriched synaptic protein fractions were prepared from the hemidiaphragm preparation according to the following procedures. All steps were performed on ice. The isolated tissues were minced in homogenization buffer containing 255 mM sucrose, 1 mM EDTA, protease inhibitor cocktail (Sigma Chemical Co.) and 20 mM Hepes (pH 7.4). The resulting suspension was homogenized with a hand-held electronic homogenizer (Omni 1000) at 15,000 rpm for 1 min. The homogenate was fractionated by centrifugation. Homogenates were initially spun at 1000 g for 5 min using a tabletop centrifuge. The resulting supernatant (S₁) was then centrifuged at 10,000 g for 10 min. The second supernatant (S₂) was centrifuged at 250,000g for 1 h in an Optima ultracentrifuge (Beckman Coulter, Fullerton, CA), and the resulting pellet (P₃) was resuspended in homogenization buffer. Sample protein concentration was determined by the modified Lowry method (Bio-Rad, Hercules, CA). Samples of the S₁, P₂, and S₃ fractions (20–75 µg of protein/lane) were resolved by SDS-polyacrylamide gel electrophoresis.

Immunodetection. Subsequent to separation, proteins were electrophoretically transferred to polyvinylidene difluoride membranes in Tris-glycine transfer buffer. Blotted membranes were then blocked overnight (4°C) with 5% nonfat powdered milk in Tris-buffered saline. For identification of proteins, membranes were washed (two times) and incubated with the primary antibodies diluted in 0.5 to 1.0% milk. Following the primary antibody incubation, the membranes were washed and then incubated in a horseradish peroxidase-conjugated secondary antibody at room temperature. This incubation step was terminated with several washes and the immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL Plus; Amersham-Pharcia, Arlington Heights, IL) according to manufacturer’s instructions. Membranes were exposed to film (Hyperfilm-ECL) for times adequate to visualize chemiluminescent bands. Comparisons were made with known molecular weight standards. In addition, as a test of substrate specificity, blots were reprobed with a cocktail of antibodies to synaptobrevin II and syntaxin I. Differences in protein immunoreactivity between control and toxin-treated tissues were determined by scanning densitometry (Scion Image; Scion Corporation, Frederick, MD).

Primary Antibodies. Antibodies to SNAP-25 were purchased from Sternberger Monoclonals, Inc. (Baltimore, MD) and Sigma Immunochemicals (St. Louis, MO) and used at dilutions ranging between 1:5,000 and 1:20,000. A rabbit polyclonal antibody to synaptobrevin II was purchased from WAKO Chemicals and used at dilutions ranging between 1:5,000 and 1:10,000. A monoclonal antibody to syntaxin I was purchased from Sigma Immunochemicals and
used at a working dilution of 1:10,000. Horseradish peroxidase-labeled secondary antibodies were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and Biosource (Camarillo, CA) and used at working dilutions of 1:5,000.

**Controls.** Immunoblot samples of mouse brain tissue were processed simultaneously with samples of neuromuscular tissue and served as the positive control. Furthermore, as a control for nonspecific background and the immunoblotting technique, companion blots were processed without the primary and/or secondary antibodies.

**Statistical Analyses.** A minimum of three treated and three untreated tissues were used per experiment. The substrate cleavage data were expressed as the percentage difference in densitometry between treated (TVL, MAH, or TPEN/EDTA) and untreated tissues exposed to toxin. Statistical differences were determined using one-tailed Student's *t* test. *P* values <0.05 were considered significant.

### Results

**Paralysis by Botulinum Serotype A Precedes Significant Cleavage of SNAP-25.** We have previously reported that a synaptic protein-enriched fraction (P3) of the hemidiaphragm can be used to measure the proteolysis of botulinum toxin substrates in neuromuscular tissue (Kalandakanond and Coffield, 2001). In the current study, the relationship between substrate proteolysis, neuromuscular paralysis, and the multistep mode of toxin action was examined. In keeping with previous work, paralysis was defined as a 90% reduction in evoked and spontaneous endplate activity (Simpson et al., 1993; Coffield et al., 1999). Following the addition of botulinum toxin type A (10⁻⁸ M) to the bathing medium, MEPP frequency decreased to 50% of control activity within 35 min, and to less than 10% of control activity within 45 min (Fig. 1A). At this point muscle twitch evoked by stimulation of the phrenic nerve was completely inhibited. Examination of SNAP-25 immunoreactivity in the P3 fraction at 1 h after paralysis indicated that less than 10% of the protein had been cleaved by the toxin (Fig. 1B). When tissues were allowed to incubate in the oxygenated recording media for an additional 5 h after paralysis, cleavage of SNAP-25 increased (Fig. 1B). Examination of the P3 fraction at 5 h postparalysis indicated that approximately 76% of the SNAP-25 protein had been cleaved by the toxin. At both 1 and 5 h postparalysis, syntaxin I and synaptobrevin II immunoreactivities were unchanged, confirming that the cleavage was specific to SNAP-25 (data not shown; Kalandakanond and Coffield, 2001).

**Cleavage of SNAP-25 by Botulinum Toxin Serotype A Is Antagonized by Triticum vulgaris Lectin.** *Triticum vulgaris* lectin has been shown to antagonize the binding of botulinum toxin to tissue receptors (Bakry et al., 1991). In the current study, incubation of phrenic nerve hemidiaphragm preparations in TVL (10⁻⁴ M) for 45 min before the addition of serotype A (10⁻⁸ M) antagonized the action of the toxin as demonstrated by an increased latent period preceding paralysis (Fig. 2A). The time to paralysis in TVL-treated tissues was 240 min, approximately 5-fold longer than in toxin-treated tissues without TVL (~45 min). Furthermore, the antagonism of toxin binding by TVL was correlated with significant antagonism of substrate cleavage in these same tissues. Examination of the P3 fraction from lectin-treated and untreated tissues exposed to toxin type A revealed a significantly greater loss of SNAP-25 immunoreactivity in tissues that were treated with toxin alone, than in tissues that were treated with toxin plus TVL (Fig. 2B). In tissues treated with toxin without TVL, SNAP-25 immunoreactivity was reduced by 63.5% compared with control tissues treated with lectin alone. In the toxin plus TVL-treated tissues, SNAP-25 immunoreactivity was reduced by only 5.6% compared with TVL-treated controls, and this reduction was not significantly different from control values (Fig. 2C).

**Cleavage of SNAP-25 by Botulinum Serotype A Is Inhibited by Methylamine Hydrochloride.** Methylamine hydrochloride, which prevents endosomal acidification and toxin translocation, antagonizes the paralytic action of botulinum toxin at the neuromuscular junction (Simpson, 1983; Coffield et al., 1999). In the current study, incubation of phrenic nerve-hemidiaphragm preparations in MAH (25 mM) for 60 min before the addition of serotype A (10⁻⁸ M) significantly prolonged the onset of toxin action as demonstrated by an increased latent period preceding paralysis. The time to paralysis in toxin-treated tissues plus MAH was 73.3 min, approximately twice as long as in toxin-treated tissues without MAH (Table 1). Furthermore, this effect of
endosomal neutralization on paralysis time was correlated with a significant reduction in substrate cleavage in these same tissues. Examination of the P3 fraction from MAH-treated and untreated tissues exposed to toxin revealed a significantly greater loss of SNAP-25 immunoreactivity in tissues treated with toxin alone, than in tissues treated with toxin plus MAH (Fig. 3A). In tissues treated with toxin alone, SNAP-25 immunoreactivity was reduced by 56.7% compared with control tissues treated with MAH alone (Fig. 3B). In the toxin plus MAH-treated tissues, SNAP-25 immunoreactivity was reduced by only 13.3% compared with MAH-treated controls, and this reduction was not statistically significant from control values.

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(20–30 $\mu$M) has been shown to antagonize the paralytic action of botulinum toxin in the mouse hemidiaphragm (Simpson et al., 1993). In this earlier work, it was found that incubation of both the toxin and the tissue was necessary to demonstrate antagonism. In the current set of experiments, a combination of TPEN and Ca-EDTA was used because in preliminary experiments it was determined that prolonged incubation (~2.5 h) of the tissues in 20 to 30 $\mu$M TPEN was detrimental to the health of the tissues. Thus, to reduce the concentration of TPEN used, type A toxin ($10^{-8}$ M) was pretreated with 200 $\mu$M Ca-EDTA and 15 $\mu$M TPEN for 60 min, and the mixture of toxin and chelators was then added to tissues that had been pretreated with 20 $\mu$M TPEN for 120 min. Chelation significantly prolonged the onset of toxin action as demonstrated by an increased latent period preceding paralysis (Table 1). The time to paralysis in chelator-treated tissues was 81.3 min, approximately 2.5 times longer than in toxin-treated tissues without chelation. Furthermore, as with TVL and MAH treatment, the effect of chelation on paralysis time was correlated with a reduction in substrate cleavage. Examination of the P3 fraction from chelator-treated and untreated tissues exposed to toxin revealed a significantly greater loss of SNAP-25 immunoreactivity in tissues treated with toxin alone, than in tissues treated with toxin plus chelation (Fig. 4A). In tissues treated with toxin alone, SNAP-25 immunoreactivity was reduced by 74.6% compared with control tissues treated with chelators alone (Fig. 4B). In the toxin plus chelator-treated tissues, SNAP-25 immunoreactivity was reduced by only 40.3% compared with chelator-treated controls. Although the reduction in SNAP-25 immunoreactivity in this latter treatment group was still significantly different from control values, it was also significantly different from the toxin only treatment group.

**Discussion**

The primary target site of botulinum serotype A is the cholinergic nerve ending of the neuromuscular junction. It was determined several years ago that intoxication (i.e., paralysis) of the neuromuscular junction proceeds sequentially through a series of events that include receptor-binding, productive internalization, and translocation to the cytosol (Simpson, 1980, 1981). The final stage of intoxication is inactivation of an intracellular target essential for transmitter release, resulting in paralysis. Based on a number of biochemical studies, SNAP-25 was proposed as the intracellular target of botulinum serotype A in nontarget tissues (Blasi et al., 1993a; Schiavo et al., 1993a). To validate that SNAP-25 is the target substrate of botulinum type A at the principal site of toxin action, the neuromuscular junction, the following criteria should be met. First, SNAP-25 must be present and detectable in a neuromuscular junction-containing preparation. Second, SNAP-25 in this preparation must be susceptible to specific cleavage by toxin type A. Third, cleavage of SNAP-25 must be dependent upon toxin binding, internalization, and translocation. Fourth, SNAP-25 cleavage should lead to and correlate with neuromuscular paralysis. Recently, we reported that SNAP-25 is a specific intracellular target of botulinum serotype A in the mouse hemidiaphragm (Kalandakanond and Coffield, 2001). In the present study, examination of hemidiaphragm tissues paralyzed by botulinum toxin type A revealed that the amount of SNAP-25 cleavage observed at 1 h after paralysis was less than 10%, raising some concern about its functional relevance. To address this concern, we then investigated whether SNAP-25 cleavage by botulinum serotype A fulfilled the requirements of the multistep mechanism mediating intoxication of the neuromuscular junction, by selectively antagonizing the receptor binding, translocation, and proteolytic steps. If cleavage of SNAP-25 represents the final event leading to paralysis, then selective disruption of the stages of intoxication should result in antagonism of both paralysis and SNAP-25 cleavage.

The cascade of events leading to paralysis begins with binding of botulinum toxin to serotype-specific receptors on the cholinergic nerve ending. Although the existence of high-affinity receptors for botulinum serotype A is well documented (Black and Dolly, 1986a,b), their identification remains elusive. Thus, serotype-specific antagonism of toxin binding as a means of disrupting the first step of intoxication is not feasible. However, one may take advantage of the fact that certain lectins competitively antagonize the binding of all botulinum toxin serotypes. This is based on the observation that toxin binding is enhanced by the presence of sialogangliosides, substances that also bind animal and plant lectins (Marxen et al., 1989; Schengrund et al., 1992, 1993;
Kitamura et al., 1999). In particular, lectins from Triticum vulgaris and Limax flavus have been used to competitively antagonize the binding of botulinum toxin to brain and hemidiaphragm tissues (Bakry et al., 1991). In the current study, TVL significantly antagonized the onset of paralysis in the hemidiaphragm tissue. Furthermore, TVL significantly antagonized SNAP-25 cleavage in the same tissue. These results confirm that the cleavage of SNAP-25 by toxin type A in the mouse hemidiaphragm is mediated by a receptor-dependent process, the first step of neuromuscular intoxication.

Once bound to its receptor, botulinum toxin is productively internalized by receptor-mediated endocytosis. However, before it can act on its intracellular substrate, the toxin must escape the endosomal compartment. During this process, acidification of the endosome results in rearrangement of the toxin and translocation into the cytosol. Inhibition of endosomal acidification prevents translocation and antagonizes toxin action at the neuromuscular junction (Simpson, 1983; Simpson et al., 1994; Coffield et al., 1999). In the present study, MAH significantly prolonged the onset of paralysis in the hemidiaphragm. Furthermore, inhibition of the translocation event significantly antagonized SNAP-25 cleavage. These data confirm that, in addition to being a receptor-mediated process, cleavage of SNAP-25 by botulinum toxin type A requires pH-dependent translocation.

The final event of botulinum intoxication is substrate cleavage. Botulinum toxin is a zinc metalloprotease (Schiavo et al., 1992a). Chelation of zinc antagonizes substrate cleavage in nontarget tissues, and inhibits toxin action in the mouse hemidiaphragm (Simpson et al., 1993; Schiavo et al., 1994a; Fu et al., 1998). In the final set of experiments of the present study, chelation of zinc significantly prolonged the onset of paralysis in the hemidiaphragm, and this was correlated with significant antagonism of SNAP-25 cleavage. Collectively, these data confirm that cleavage of SNAP-25 by botulinum toxin type A in the mouse hemidiaphragm requires 1) receptor-mediated endocytosis, 2) pH-dependent translocation, and 3) the presence of zinc.

In spite of the lack of temporal correlation between SNAP-25 cleavage and paralysis, the finding that cleavage of SNAP-25 by botulinum serotype A fulfills the requirements of the multistep mechanism mediating intoxication of the neuromuscular junction strongly supports a functional correlation between cleavage and paralysis. Furthermore, these findings suggest that cleavage of only a small fraction of the measurable pool of SNAP-25 is necessary for paralysis to occur at the mouse neuromuscular junction. This small but functionally significant pool of SNAP-25 would most likely be associated with the population of release-ready synaptic vesicles closest to the fusion event. Several lines of evidence support this (for review, see Martin, 1997; Humeau et al., 2000). Two populations of small synaptic vesicles were originally described by morphological studies. They included the “dock” pool, in which vesicles were found lined up along the active zone of the nerve terminal, and the “storage and recruitment” pool in which vesicles appeared in random clusters located away from the active zone. Recent biochemical and electrophysiological study indicates that within the “morphologically docked pool”, only a small subset of vesicles is immediately releasable upon stimulation of the nerve terminal. These latter data suggest that morphologically docked vesicles exist in various states of “fusion competence”, and must go through maturation and/or priming steps to achieve this competent state. Although the exact nature of the priming event is still unresolved, one theory is that a preformed SNARE complex dissociates temporarily, and then reassociates into a conformational state that is more permissible for lipid fusion. Interestingly, studies report that the SNARE proteins are only susceptible to botulinum toxin cleavage when in an “uncomplexed” state (Hayashi et al., 1994). Furthermore, other evidence indicates that botulinum toxin action does not antagonize vesicle docking, but acts downstream of docking, close to the final fusion event (Hunt et al., 1994; Banerjee et al., 1996). Given that a total of only 240 molecules of botulinum toxin is required to kill a mouse (Simpson, 1981), the number of molecules necessary to paralysis an individual nerve ending is exceedingly small. Thus, the toxin must act strategically in selecting not only its choice of substrate, but also its pool of substrate. The temporarily dissociated state of the fusion complex would provide one window of opportunity for such strategic toxin action. Since this step would be so close to the final fusion event, a small amount of cleavage by a few toxin molecules would result in a very large functional impact, i.e., blockade of the final fusion event and thus paralysis. With time, as more vesicles in the docked pool proceed through maturation/priming, more substrate would be cleaved. Intracellular electrophysiology is a very sensitive technique, capable of detecting a single fusion event. However, immunoblot detection methods, even with enhanced chemiluminescence, may not be sensitive enough to measure cleavage of a single, or even a few molecules of SNAP-25. Thus, the lack of a linear correlation between the onset paralysis and substrate proteolysis is likely due to a combination of factors, namely, 1) the small pool of SNAP-25 that is cleaved initially, and 2) the different sensitivities of the techniques used to measure paralysis and proteolysis.

Finally, it should be stated that the lack of temporal correlation between toxin-induced paralysis and proteolysis in the current study could also support the alternative hypothesis that paralysis of the neuromuscular junction is not mediated by proteolysis of SNAP-25, but rather, involves another yet to be identified mechanism. However, given the evidence already discussed in the preceding paragraphs, we believe this to be highly unlikely.

References


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