Uptake of Botulinum Neurotoxin into Cultured Neurons

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ABSTRACT: Botulinum neurotoxins (BoNTs) act within the synaptic terminal to block neurotransmitter release. The toxin enters the neuron by binding to neuronal membrane receptor(s), being taken up into an endosome-like compartment, and penetrating the endosome membrane via a pH-dependent translocation process. Once within the synaptic cytoplasm, BoNT serotypes A and E cleave separate sites on the C-terminus of the neuronal protein SNAP-25, one of the SNARE proteins required for synaptic vesicle fusion. In this study, we measured the effect of brief toxin exposure on SNAP-25 proteolysis in neuronal cell cultures as an indicator of toxin translocation. The results indicate that (1) uptake of both BoNT-A and -E is enhanced with synaptic activity induced by K^+ depolarization in the presence of Ca^{2+} and (2) translocation of BoNT-A from the acidic endosomal compartment is slow relative to that of BoNT-E. Polyclonal antisera against each toxin protect cells when applied with the toxin during stimulation but has no effect when added immediately after toxin exposure, indicating that toxin endocytosis occurs with synaptic activity. Both serotypes cleave SNAP-25 at concentrations between 50 pM and 4 nM. IC_{50} values for SNAP-25 cleavage are approximately 0.5 nM for both serotypes. Inhibition of the pH-dependent translocation process by pretreating cultures with concanamycin A (Con A) prevents cleavage of SNAP-25 with IC_{50} values of ∼25 nM. Addition of Con A at times up to 15 min after toxin exposure abrogated BoNT-A action; however, addition of Con A after 40 min was no longer protective. In contrast, Con A inhibited, but did not prevent, translocation of BoNT-E even when added immediately after toxin exposure, indicating that pH-dependent translocation of BoNT-E is rapid relative to that of BoNT-A. This study demonstrates that uptake of both BoNT-A and -E is enhanced with synaptic activity and that translocation of the toxin catalytic moiety into the cytosol occurs at different rates for these two serotypes.

Botulinum neurotoxins (BoNTs)[1] represent a class of immunologically distinct proteins that act at the presynapse of motoneurons causing muscle paralysis (*1*−*3*). This effect is believed to be due to toxin-induced cleavage of neuronal SNARE complex proteins, which disrupts synaptic vesicle function and prevents neurotransmitter release (*4*). BoNTs (∼150 kDa) are comprised of three independent domains that mediate neuron intoxication via five steps: (1) interaction of the H_C domain with the neuronal surface, (2) uptake and sequestration within endosomal compartments, (3) pH-dependent channel formation by the H_N domain allowing (4) translocation of the light chain (LC) domain across the endosomal membrane, and (5) proteolysis of SNARE proteins by the LC (*5*−*7*). Toxin binding appears to involve high- and low-affinity sites with dissociation values at the low nanomolar and sub-nanomolar level (*8*−*10*). Following toxin binding and sequestration, the endosome is acidified, triggering the H_N domain to interact with the lumenal face of the membrane eventually forming a pore which behaves like a voltage-gated Na^+/K^+ channel (*11*−*13*). It has been demonstrated recently that the 50 kDa LC moves through this pore and assumes a functional conformation upon reaching a neutral-pH environment (*14*). After translocation, BoNT-A and -E LCs cleave SNAP-25 at specific peptide bonds, causing neuromuscular blockade (*15*−*18*).

BoNT-A forms channels at low pH in a variety of lipid membranes (*11*, *12*, *19*, *20*). Disruption of endosomal pH gradients in neuromuscular preparations slows toxin translocation (*21*−*23*). Those studies, in part, sought to demonstrate the requirement for endosomal acidification as part of the toxin entry mechanism, and in achieving a uniform population of toxin-loaded vesicles for studying BoNT translocation. "Toxin synchrony" was a term proposed by Simpson (*22*) for achieving a uniform toxin population at each step of the intoxication process (*22*). Binding synchrony, for example, was achieved with a reduced temperature or elevated Mg^{2+} and reduced Ca^{2+} levels, allowing toxin to accumulate on external receptor(s). In contrast, endosomal synchrony was achieved partially but proved to be difficult to examine because disruption of endosomal acidification also caused muscle paralysis (*22*−*24*).

Toxin serotypes A and E are particularly interesting because BoNT-A has become a valuable medical therapeutic

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[1] Abbreviations: BoNT, botulinum neurotoxin; bafA1, bafilomycin; Con A, concanamycin A; MEM, minimum essential medium; SDS, sodium dodecyl sulfate; SNAP-25, 25 kDa synaptosomal-associated protein; VAMP, vesicle-associated membrane protein; LC and HC, light chain and heavy chain of botulinum neurotoxin, respectively.

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for treating a growing number of muscle disorders (*25*, *26*). Both serotypes share extracellular receptor components (*27*, *28*), similar pH profiles for membrane association (*29*), and channel and gating properties (*13*), SNAP-25 as a substrate, and both are stabilized by tyrosine phosphorylation (*30*, *31*). However, in contrast to these common traits, BoNT-A survives in nerve terminals remarkably longer than BoNT-E (*32*−*35*) which prevents the useful application of the E serotype in a medical setting. The molecular basis underlying the long-term activity of BoNT-A has remained unclear.

We analyzed toxin uptake and translocation using cultures of dissociated spinal cord neurons (*36*, *37*). Brief chemical depolarization of neurons caused uniform uptake of each BoNT, helping achieve a significant degree of endosomal synchrony. The data demonstrate that, although both serotypes are bound and endocytosed during brief exposure, BoNT-A translocation requires an acidic pH for a longer interval after uptake than BoNT-E as estimated using concanamycin A (Con A) to block acidification. This time-dependent pH effect mechanistically distinguishes these two serotypes and may indicate that each BoNT translocates from distinct endosomal pools.

MATERIALS AND METHODS

Materials. The monoclonal antibody to SNAP-25 (SMI-81) was obtained from Sternberger Monoclonals, Inc. (Lutherville, MD). Concanamycin A, the monoclonal antibody to syntaxin, and the horseradish peroxidase-labeled anti-mouse antibody were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All electrophoresis and Western blot reagents were from Bio-Rad (Hercules, CA). Equine antitoxin was a gift from J. E. Brown (USAMRIID, Frederick, MD). Preparations of BoNT-A and -E toxin complex were from Wako Chemicals, Inc. (Richmond, VA) with reported activities of 2.0×10^7 LD_{50} and 1.0×10^7 LD_{50} per milligram of protein, respectively. BoNT-E (1 mg/mL) was activated according to the manufacturer's instructions, i.e., incubation of the toxin with 0.3 mg/mL trypsin (type XI, bovine pancreas) in 30 mM HEPES (pH 6.75) for 0.5 h at 37 °C (*38*). Trypsin was inhibited by adding 0.5 mg/mL soybean trypsin inhibitor (type I-S). Toxins were aliquoted and stored at −20 °C; each experiment utilized a new aliquot of toxin to ensure uniform activity. Molar concentrations stated in the text were based upon toxin masses of 500 and 300 kDa for BoNT-A and -E, respectively, as supplied by Wako Chemicals.

Spinal Cord Cultures and Toxin Application. Timed pregnant C57BL/6NCR mice were obtained from the Frederick Cancer Research and Development Center (Frederick, MD). Spinal cord cell cultures were prepared as described previously (*39*, *40*). Briefly, spinal cords were removed from fetal mice at gestation day 13, dissociated with trypsin, and plated on Vitrogen 100-coated dishes (Collagen Corp., Palo Alto, CA) at a density of 10^6 cells/dish. Cultures were maintained for 3 weeks at 37 °C in a humidified atmosphere of 90% air and 10% CO_2 before addition of toxins. Growth medium consisted of Minimum Essential Medium (MEM, formula 82-0234AJ; Invitrogen, Inc., San Diego, CA) supplemented with 5% heat-inactivated horse serum and a mixture of complex factors (*41*). Generally, cultures were incubated with BoNT diluted in stimulation buffer, i.e., 10 mM HEPES (pH 7.4) containing 56 mM KCl, 82 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ adjusted to 325 mOsm with sucrose for 4 min at 37 °C. Resting buffer [10 mM HEPES (pH 7.4) containing 3 mM KCl, 138 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$] or Ca^{2+}-depleted buffer [10 mM HEPES (pH 7.4) with 3 mM KCl, 138 mM NaCl, 1 mM $MgCl_2$, and 0.1 mM EGTA adjusted to 325 mOsm] was used as indicated in the figure legends. BoNT-containing buffer was removed by aspiration, and cells were rinsed with toxin-free MEM. Cultures were incubated at 37 °C in MEM for an additional 2.5 h and then prepared for Western blot analysis or neurotransmitter release measurement.

Protein and Western Blot Analysis. Protein was prepared by dissolving cells in 1% SDS with 1 mM EDTA and 1 mM EGTA. The slurry was transferred to 1.5 mL microcentrifuge tubes, incubated in a 95 °C water bath for 4 min to inactivate proteases, and then stored at −20 °C. Immediately prior to electrophoresis, samples were thawed, mixed with equal volumes of Tris-Tricine sample buffer (Bio-Rad), and heated at 95 °C for 4 min. Protein was separated using 16.5% SDS−polyacrylamide gels by the method of Laemmli using 0.1 M Tris-Tricine running buffer (pH 8.3) (*42*, *43*). Western blot transfer utilized a polyvinylidene difluoride membrane with a buffer containing 192 mM glycine, 25 mM Tris (pH 8.3), and 12% methanol. Membrane development was performed using the chemiluminescent developing reagent ECL Plus (Amersham Bioscience, Piscataway, NJ). To measure the rate of potassium-depolarized Ca^{2+}-dependent neurotransmitter release, cultures were labeled with [^{3}H]glycine for 0.5 h as described previously (*36*). Baseline levels of glycine secretion were determined by collecting resting buffer at 5 min intervals. Vesicular glycine release was elicited by addition of stimulation buffer for 5 min at 37 °C. The level of depolarization-dependent release was determined by subtracting the baseline level of glycine secretion from the total radioactivity released during stimulation.

Regression Analysis and Curve Fitting. Scanned images of Western blots were produced with the STORM 860 fluorescent detector (Molecular Dynamics, Eugene, OR) and quantified using ImageQuant software (Molecular Dynamics). Nonlinear regression analysis of data was performed with GraphPad Prism.

RESULTS

Stimulation and Calcium-Dependent Uptake of BoNT-A and -E. Figure 1 demonstrates proteolysis of SNAP-25 by BoNT-E and -A in cultured mouse spinal cord cells by Western blot analysis. Cultures were incubated at 37 °C for 4 min in buffer containing either BoNT-A or -E (500 pM) and various concentrations of K^+ and Ca^{2+}. Following toxin exposure, cultures were washed with MEM and incubated for an additional 2.5 h in MEM at 37 °C. Medium was then removed from the cultures by vacuum aspiration, and cells were prepared for Western blot analysis. SNAP-25 and toxin-cleaved SNAP-25 were visualized using a single monoclonal antibody that recognizes an epitope common to both proteins. In the first set of experiments, each buffer/toxin mixture contained either 80, 56, or 3 mM KCl and 2 mM $CaCl_2$. For both BoNTs, depolarization of neurons with 80 or 56 mM KCl in the presence of Ca^{2+} led to toxin-specific cleavage

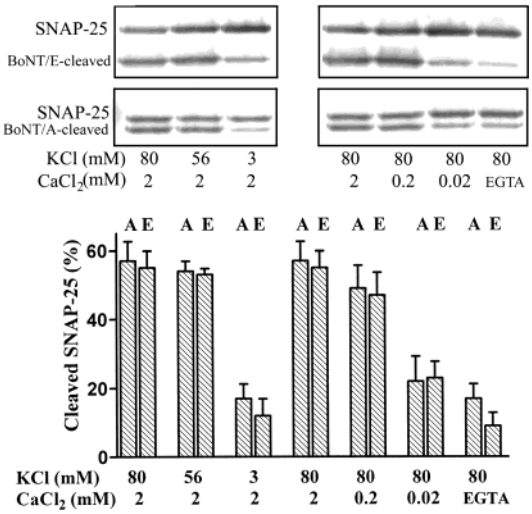

FIGURE 1: Action of BoNT on SNAP-25 following a brief exposure to toxin. Mouse spinal cord cultures were depolarized in the presence of BoNT-A or -E (500 pM) for 4 min as described in Materials and Methods. This concentration of toxin cleaves approximately half of the total SNAP-25 2.5 h after toxin exposure. Either lowering KCl concentrations while maintaining the Ca^{2+} concentration at 2 mM or reducing the Ca^{2+} concentration while maintaining the KCl concentration at 80 mM protected SNAP-25. These titrations indicate that increased availability of the BoNT-A and -E receptor(s) is dependent on both membrane depolarization and Ca^{2+}. Western blots were screened using a monoclonal antibody that recognizes both normal and toxin-cleaved SNAP-25.

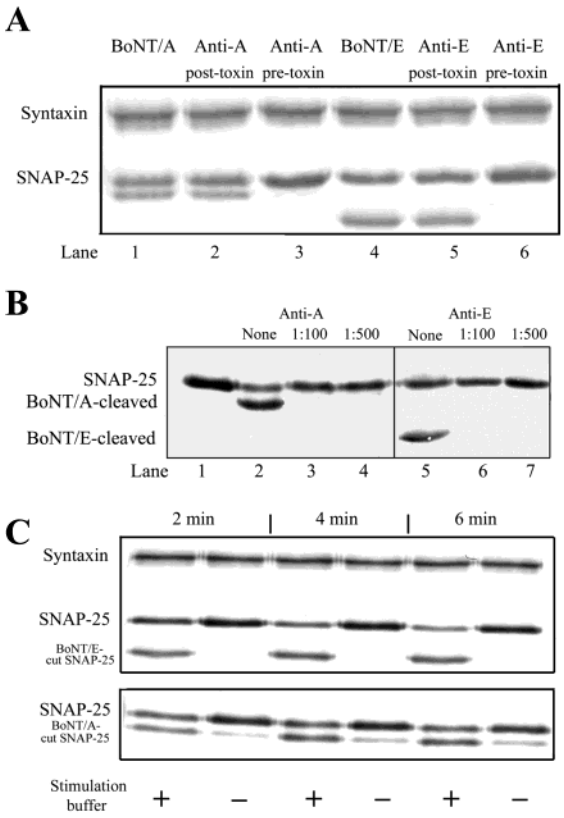

FIGURE 2: Effect of antitoxin and time on toxin uptake. (A) Spinal cord cultures were depolarized in the presence of 500 pM BoNT for 4 min. Representative Western blots show the effects of BoNT-A (lanes 1−3) and BoNT-E (lanes 4−6) on SNAP-25 after exposure: lanes 1 and 4, cultures treated with toxin only; lanes 2 and 5, cultures treated with equine antitoxin (1:500 dilution) immediately after removal of BoNT; and lanes 3 and 6, cultures treated with BoNT preincubated with antitoxin for 5 min. Neutralization of BoNT with antitoxin protected SNAP-25, whereas antitoxin applied immediately after toxin exposure did not substantially alter the extent of SNAP-25 proteolysis. (B) Cultures were incubated in ice-cold medium with either BoNT-A (lanes 2−4) or BoNT-E (lanes 5−7) for 0.5 h. After cultures had been washed to remove unbound toxin, ice-cold medium containing antitoxin was applied at the indicated dilutions for 10 min. The cultures were then kept at 37 °C for 3 h. Addition of antitoxin in this manner neutralizes bound toxin. (C) Cultures were exposed for the indicated times to BoNT-E or -A in either stimulation buffer (+) or Ca^{2+}-depleted buffer (−). The extent of SNAP-25 proteolysis increased only slightly as exposure times in stimulation buffer increased. In contrast, toxin exposure using Ca^{2+}-depleted buffer causes only a trace of SNAP-25 proteolysis, similar to Figure 1, indicating that BoNT binding and endocytosis are enhanced by depolarization in the presence of extracellular Ca^{2+} and not by increasing time of depolarization in the absence of Ca^{2+}. Syntaxin was monitored as a control and remained unaffected by the various treatments.

of 50−60% of cellular SNAP-25. When cultures were not depolarized (i.e., 3 mM KCl), much less toxin entered the neurons, presumably because of ongoing synaptic activity, and the level of SNAP-25 proteolysis was reduced to 10−20% of the total SNAP-25.

The second set of experiments kept the KCl concentration at 80 mM while reducing the extracellular $CaCl_2$ concentration. When the Ca^{2+} concentration was reduced 10-fold to 0.2 mM, little change in SNAP-25 cleavage was noted. When the Ca^{2+} concentration was reduced further to either 20 or 0 μM by addition of 0.1 mM EGTA, the amount of toxin-cleaved SNAP-25 was significantly reduced. Upon removal of the toxin/buffer solution, all cultures were incubated in MEM containing 3 mM KCl and 2 mM $CaCl_2$. Therefore, depolarization- and Ca^{2+}-related changes occurred during only the 4 min toxin exposure. The resulting proteolysis of SNAP-25 demonstrates that toxin uptake into cultured neurons correlates with synaptic activity.

Antitoxin and Time Dependence of BoNT Uptake. The enhanced proteolysis of SNAP-25 resulting from toxin exposure during depolarization may reflect either enhanced BoNT receptor(s) availability and thus greater toxin binding to neurons followed by slow internalization, or it may reflect enhanced receptor availability accompanied by rapid internalization. To discriminate between rapid and slow endocytosis, we examined the accessibility of toxin to neutralizing antibody during and after toxin exposure. Incubation of toxin with antitoxin prior to application to cultures protected SNAP-25 (Figure 2A, lanes 3 and 6), as expected. Other cultures were stimulated in the presence of BoNT, and after toxin washout, antitoxin was added to some cultures for the duration of the experiment. When antitoxin was added immediately following the 4 min toxin exposure, no significant change in SNAP-25 cleavage was observed 2.5 h after toxin removal (Figure 2A, lanes 2 and 5) or exhaustive washing (data not shown). This indicates that SNAP-25 proteolysis was due to rapid toxin binding that proceeded immediately to a state protected from antitoxin. To further address this question, new cultures were incubated on ice for 10 min, after which ice-cold MEM with BoNT was applied and remained on cultures for 0.5 h to allow for toxin binding but not internalization. BoNT-containing medium was removed. MEM with or without antitoxin was applied and remained on cultures for 10 min on ice. Cultures were returned to 37 °C and incubated for 3 h. The results show that each BoNT was bound in the cold and internalized upon

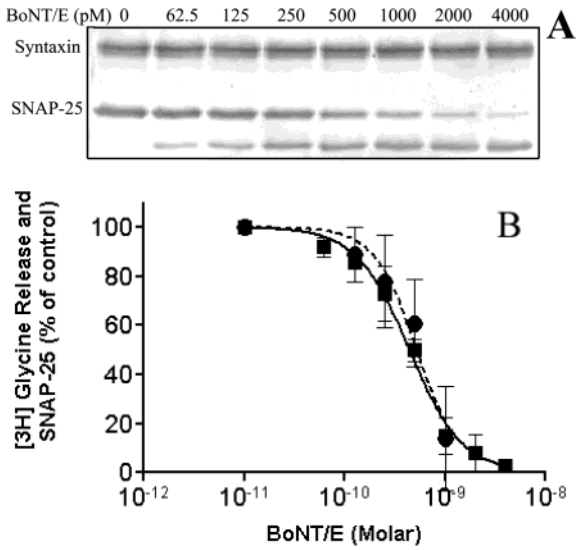

FIGURE 3: Dose response of BoNT-E on SNAP-25 proteolysis and neurotransmitter release. (A) Western blots from cultures treated with the indicated concentrations of BoNT-E in stimulation buffer for 4 min followed by incubation for 2.5 h in toxin-free resting medium. Antibodies to SNAP-25 and syntaxin were used. (B) Densitometric values show that BoNT-E cleaves SNAP-25 in a concentration-dependent manner (■) with an IC_{50} value of 455 ± 22 pM. Following toxin exposure, sister cultures were incubated with [^{3}H]glycine; K^+- and Ca^{2+}-dependent neurotransmitter release was assessed 2.5 h after toxin washout for 0, 125, 250, 500, and 2000 pM BoNT-E (●). The level of neurotransmitter release closely correlates with the amount of SNAP-25 cleavage by the toxin.

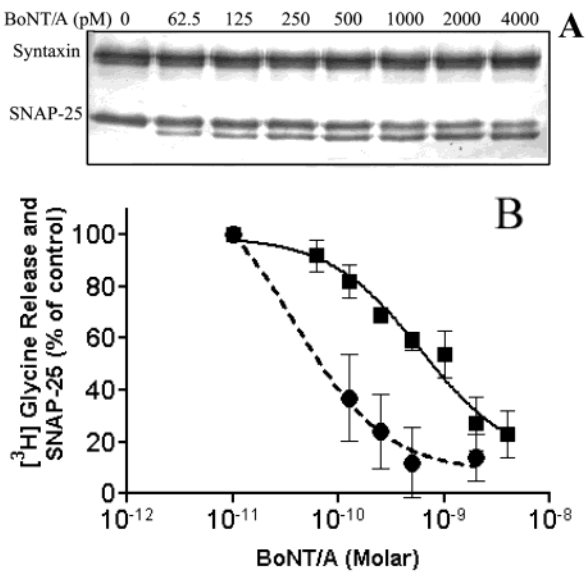

FIGURE 4: Dose response of BoNT-A on SNAP-25 proteolysis and neurotransmitter release. (A) Western blots from cultures treated with the indicated concentrations of BoNT-A as described in the legend of Figure 3. (B) The extent of [^{3}H]glycine release and densitometric values for SNAP-25 proteolysis were plotted (■). BoNT-A concentrations of 0, 125, 250, 500, and 2000 pM were tested for [^{3}H]glycine release (●). Unlike with BoNT-E, the evoked level of neurotransmitter release was substantially reduced by BoNT-A relative to SNAP-25 proteolysis. Regression analysis indicates that BoNT-A inhibits neurotransmitter release with an IC_{50} value of 40 ± 16 pM and cleaves SNAP-25 with an IC_{50} of 589 ± 62 pM. Error bars are one standard deviation unit determined from three experimental values.

warming in the absence of antitoxin (Figure 2B, lanes 2 and 5). Application of antitoxin totally protected SNAP-25 (Figure 2B, lanes 3, 4, 6, and 7). Taken together, these findings suggest that synaptic activity favors rapid and tight toxin binding. If synaptic depolarization simply leads to enhanced receptor numbers at the nerve terminal plasma membrane, reversible toxin binding does not follow since antitoxin added after toxin exposure at 37 °C did not protect SNAP-25 (Figure 2A,B).

Timed exposure of cultures to toxin in stimulation buffer at 37 °C showed that BoNT uptake is essentially complete within 4 min of exposure (Figure 2C). Incubating for an additional amount of time, namely, 6 min, did not lead to substantially greater proteolysis of SNAP-25. Given that both serotypes were present at sub-nanomolar concentrations applied for only several minutes, BoNT appears to be rapidly internalized by a high-affinity receptor-mediated endocytic process. Toxin exposure in Ca^{2+}-depleted buffer for as long as 6 min did not facilitate BoNT-E uptake and allowed only trace amounts of BoNT-A uptake as shown also in Figure 1. On the basis of this result, stimulation facilitates toxin binding and uptake with a half-time of just <2 min, whereas exposure without stimulation led to little BoNT uptake.

Effects of BoNT on Neurotransmitter Release and SNAP-25 Cleavage. On the basis of the preceding results, the remaining experiments involved toxin exposure in stimulation buffer. BoNT-E treatment of cultures caused concentration-dependent cleavage of SNAP-25 (Figure 3A). A 4 min exposure caused a sigmoidal relationship between BoNT-E concentration and the level of SNAP-25 cleavage with an IC_{50} value of approximately 500 pM toxin. Cleavage of SNAP-25 occurred with a Hill coefficient of approximately 2, and at the highest BoNT-E concentration (4 nM), nearly all SNAP-25 was cleaved. Assessment of synaptic function (Figure 3B) indicates that K^+-evoked glycine release is inhibited to the same extent as SNAP-25 proteolysis. Neurotransmitter release was assessed 2.5 h after toxin exposure, the same incubation time as depicted in Figure 3A. However, we found that the first stimulation interval to facilitate toxin uptake inhibited the neuronal ability to accumulate or release neurotransmitter. Cultures that received no prior stimulation released 18−23% of the cellular [^{3}H]-glycine upon depolarization, whereas cultures depolarized 2.5 h prior to assessment of neurotransmission released only 12−17% of cellular [^{3}H]glycine upon depolarization. Data in Figure 3B are expressed as a percentage of release from these latter cultures.

Unlike BoNT-E, BoNT-A in these experiments could not cleave all SNAP-25 (Figure 4). The degree of inhibition of neurotransmitter release is greater than the amount of cleaved SNAP-25 (Figure 4B), and the IC_{50} values for blockade of glycine release and cleavage of SNAP-25 are 40 ± 16 and 589 ± 62 pM, respectively. In contrast, BoNT-E cleaved SNAP-25 and blocked glycine release with IC_{50} values of 493 ± 61 and 455 ± 22 pM, respectively. With the exception of a small SNAP-25 population, 4 nM BoNT-E seemed to encounter and act upon SNAP-25 with relative ease, whereas the highest concentrations of BoNT-A led to cleavage of only ~70−80% of the total SNAP-25 (Figure 4B).

Effect of Con A on BoNT Activity. The effect of endosome acidification on toxin translocation was examined by employing Con A, a specific high-affinity inhibitor of endosomal and vesicular acidification. SNAP-25 proteolysis was taken as a measure of successful translocation. The first

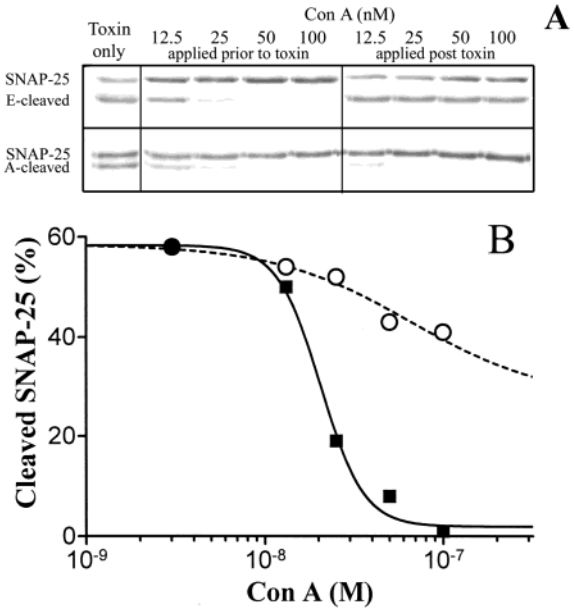

FIGURE 5: Blocking of entry of BoNT-A and -E into cell cytosol by Con A is concentration-dependent. (A) Western blot analysis demonstrates that preincubation of cultures for 5 min with Con A at the indicated concentrations blocks both BoNTs (500 pM) from acting on SNAP-25, presumably by disrupting the endosomal pH gradient and inhibiting translocation of toxin into the cytosol. Addition of Con A immediately after toxin washout protects SNAP-25 from BoNT-A but has a much weaker effect for BoNT-E, implying that pH-dependent channel formation for BoNT-E occurs rapidly relative to that for BoNT-A. (B) Densitometry of cleaved SNAP-25 plotted for BoNT-E demonstrates that BoNT-E becomes less sensitive to Con A with a shift in IC_{50} values from 16 nM when Con A was applied prior to toxin (■) and greater than 250 nM when Con A was applied after BoNT-E (○).

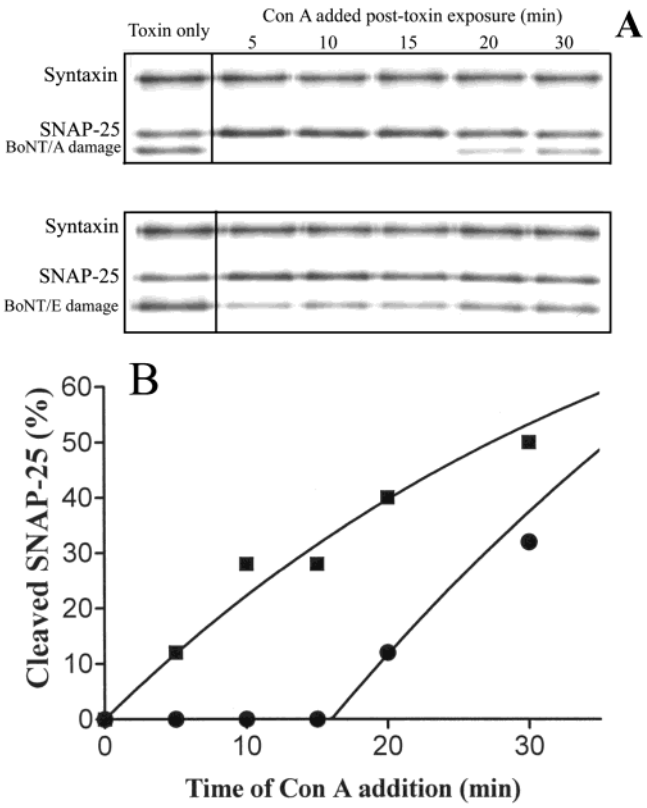

FIGURE 6: Time dependency of the blocking of toxin action by Con A. (A) Cultures were treated with 500 pM BoNT. At indicated times after toxin washout, Con A (250 nM) was applied to cultures. Cultures were incubated for 2.5 h and then analyzed by Western blotting. (B) This approach demonstrates that SNAP-25 proteolysis by BoNT-A is completely blocked by Con A for up to 15 min after toxin washout (●). Proteolysis by BoNT-E is blocked much less efficiently such that addition of Con A 20 or 30 min after toxin washout has a weaker effect (■). Both BoNTs show complete insensitivity to Con A 40 min after toxin washout.

approach involved titrated concentrations of Con A applied before or after BoNT (Figure 5). When cultures were preincubated with this drug for 5 min prior to toxin application, proteolysis of SNAP-25 by both serotypes was inhibited ($IC_{50} \sim 20$ nM). When BoNT-A was applied first and Con A applied immediately after toxin washout, SNAP-25 was completely protected. Under the same conditions, however, BoNT-E caused substantial SNAP-25 cleavage. The sensitivity of BoNT-E to Con A was shifted from an IC_{50} value of ~20 nM when it was added prior to toxin to >250 nM when added immediately after BoNT-E (Figure 5B). The time-dependent sensitivity of BoNT-E to endosomal acidification indicates that channel formation and light chain translocation occur more rapidly than BoNT-A translocation.

To ensure that Con A was not interfering with toxin binding, and to measure the BoNT-A residence time within the acidic compartment, cultures were exposed first to either BoNT-A or -E and then to a single high dose of Con A at different times after toxin washout (Figure 6). This second approach demonstrates that SNAP-25 proteolysis by BoNT-A is completely blocked by Con A at times up to 15 min, with only a trace of proteolysis of SNAP-25 occurring when Con A was added 20 min after toxin washout. This result indicates that BoNT-A has a latent period within the endosome of 15−20 min after toxin uptake. BoNT-E, on the other hand, was slowed much less than BoNT-A and demonstrated no measurable latency period. Addition of Con A 40 min after toxin washout failed to protect neurons from either BoNT-A or -E.

DISCUSSION

In this study, we examined the action of BoNT-A and -E in neuronal cultures to help understand how each serotype enters neurons and, perhaps, to indicate how BoNT-A derives a longer duration of action than does BoNT-E (*33*, *34*, *44*). Following endocytosis, both serotypes require acidification to enter the cytosol, as had been demonstrated previously in other model systems (*21*, *45*). Here we demonstrate that both BoNTs bind and enter neurons in a stimulation- and Ca^{2+}-dependent manner. BoNT-E shows a stricter requirement for Ca^{2+} than BoNT-A for this process. From that point, BoNT-A translocation into the nerve terminal cytosol is slower than BoNT-E translocation.

To study the effect of acidification on toxin translocation, toxin was applied for brief periods at 37 °C under conditions that stimulate neural activity. This approach created a toxin population that was inaccessible to neutralizing antibody. An alternative approach could have entailed incubating cells at a reduced temperature to allow synchronous toxin binding as was used in Figure 2B (*10*, *22*). We find that incubating BoNT with cultures at ice-cold temperatures creates a toxin population that is readily neutralized by antitoxin. Cold-bound BoNT does not dissociate following three 0.5 min ice-cold washes; however, this binding is reversible and following three 5 min washes, no SNAP-25 proteolysis

occurs during a subsequent incubation at 37 °C (data not shown). Because stimulation of synaptic activity at physiological temperatures protected BoNT from antitoxin, we presume these toxin molecules constitute a synchronous population within the uptake compartment. It is possible that cold binding for 0.5 h and depolarization at 37 °C for 4 min reveal two binding steps: a reversible binding step followed by a transition to an irreversible state. Extracellular Ca^{2+} at 37 °C enhanced the uptake of BoNT-A but was required for BoNT-E. It has been shown that BoNT binding in muscle tissue is independent of activity and extracellular Ca^{2+} concentration and uptake is enhanced (*6*, *46*). These earlier observations noted that diffusion of toxin into muscle tissue is hindered greatly because of the extracellular matrix. Non-neuronal tissue may block toxin delivery to synaptic terminals *in vivo*, especially when picomolar toxin concentrations are used. In our assay system, neuron terminals are exposed directly to the culture medium, allowing unobstructed toxin binding. Under these conditions, our data demonstrate that toxin uptake: 1 does not require substantial time for binding and 2 is enhanced by synaptic activity and extracellular Ca^{2+} concentration, similar to previous observations for tetanus neurotoxin (*45*). The activity dependence of toxin uptake observed in this study is consistent with a toxin receptor exposed at the cell surface during the process of synaptic vesicle recycling. Because some BoNT-A uptake occurs in the absence of depolarization and Ca^{2+}, BoNT-A and -E may have different receptors or some BoNT-A can enter neurons by a second Ca^{2+}-independent pathway.

Increasing the time of exposure to toxin from 2 to 6 min did not increase the extent of SNAP-25 proteolysis, indicating that the rate of toxin uptake is too rapid to measure with this assay. Furthermore, toxin binding is irreversible because addition of antitoxin immediately following toxin exposure did not protect SNAP-25. Therefore, toxin exposure during elevated synaptic activity indicates that after 4 min, BoNT binding had proceeded beyond a simple ligand−receptor interaction. This activity-dependent interaction might explain how patients with spastic muscle disorders retain voluntary muscle function following BoNT-induced paralysis of overactive nerve terminals. If overactive nerve terminals preferentially bind and internalize BoNT while lower activity nerve terminals escape intoxication, then spastic symptoms will dissipate while normal, low-level neural activity will persist (*47*).

Cultured neurons are approximately equally sensitive to BoNT-A and -E in terms of SNAP-25 proteolysis. Both toxin serotypes cleave approximatrly the same amount of SNAP-25 in 2.5 h at approximately the same toxin concentrations, IC_{50} values of 590 and 450 pM for BoNT-A and -E, respectively. In a previous study (*48*), we showed greater sensitivity of these neuronal cultures to both serotypes (0.1 pM to 1 nM). The earlier study, however, relied on bath application of toxins, which remained in contact with the cultures for up to 24 h at 37 °C. Under those conditions, spontaneous network activity of the neurons most likely contributed to toxin uptake and the toxin was given a longer interval in which to act. The conditions of the current experiments provide for both brief toxin exposure and a shorter time to act. Nevertheless, the sensitivities observed in this study are similar to previously determined affinities for BoNT-A and -E (*6*, *8*, *10*, *46*).

Neurotransmitter release is blocked following uptake of both serotypes. The relationship between neurotransmission blockade and SNAP-25 cleavage seen in this study supports our previous observations. BoNT-E causes a level of synaptic inhibition equivalent to the amount of cleaved SNAP-25, whereas BoNT-A causes more inhibition than SNAP-25 proteolysis (*48*), consistent with additional effects of BoNT-A-cleaved SNAP-25 inhibiting the release process. In this study, however, we observe that maximal blockade by BoNT-A occurs when less than half of the total SNAP-25 is cleaved. This might be explained, in part, if activity-related uptake delivers BoNT-A to a select population of SNAP-25 residing within synaptic terminals to cause vesicle blockade, which is consistent with studies in muscle (*49*, *50*).

Simpson *et al*. (*21*) demonstrated that bafilomycin A1, an inhibitor of endosomal acidification, acts most prominently to protect muscle action from BoNT-A when the drug is applied prior to or during toxin exposure, and this protective effect is diminished if bafilomycin A1 is applied at times after toxin addition, consistent with our results with bafilomycin A1 (data not shown) and Con A (Figure 6). The study by Simpson could not be extended because concentrations of bafilomycin A1 that totally protected motor nerve terminals from BoNT caused muscle paralysis, imposing limitations for the characterization of pH and toxin entry. Results using Con A show that BoNT-A remains sensitive to endosomal pH for a longer interval than BoNT-E.

With neuronal cultures, we were able to test several conditions that demonstrate a different time course for BoNT-A and -E translocation. BoNT-A requires endosomal acidification for 10−15 min after toxin internalization for light chain translocation, whereas BoNT-E requires significantly less time. This delay for BoNT-A can reflect a mechanistically greater need for H^+ during channel formation. However, if the A and E channels form at similar pH values, then the current data suggest that the BoNT-A LC tranlocates more slowly than the BoNT-E LC. An attractive hypothesis is that BoNT-A remains associated with the uptake compartment, conferring protection from degradation while still allowing BoNT-A to contact SNAP-25. If BoNT-A is somewhat restricted while BoNT-E freely disperses into the cytosol, this would explain why BoNT-E can cleave nearly all SNAP-25 while BoNT-A can cleave only ∼70−80% of the SNAP-25 in 2.5 h. The distinct sensitivities to Con A might reflect the placement of BoNT-A in a confined location but in the proximity to part of the SNAP-25 trafficking pathway, while the catalytic domain of BoNT-E appears to have greater accessibility to SNAP-25 as had been shown previously (*48*).

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