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Clostridial Neurotoxins and Substrate Proteolysis in Intact Neurons

BOTULINUM NEUROTOXIN C ACTS ON SYNAPTOSOMAL-ASSOCIATED PROTEIN OF 25 kDa*

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Clostridial neurotoxins are zinc endopeptidases that block neurotransmission and have been shown to cleave, *in vitro*, specific proteins involved in synaptic vesicle docking and/or fusion. We have used immunohistochemistry and immunoblotting to demonstrate alterations in toxin substrates in intact neurons under conditions of toxin-induced blockade of neurotransmitter release. Vesicle-associated membrane protein, which colocalizes with synaptophysin, is not detectable in tetanus toxin-blocked cultures. Syntaxin, also concentrated in synaptic sites, is cleaved by botulinum neurotoxin C. Similarly, the carboxyl terminus of the synaptosomal-associated protein of 25 kDa (SNAP-25) is not detectable in botulinum neurotoxin A-treated cultures. Unexpectedly, tetanus toxin exposure causes an increase in SNAP-25 immunofluorescence, reflecting increased accessibility of antibodies to antigenic sites rather than increased expression of the protein. Furthermore, botulinum neurotoxin C causes a marked loss of the carboxyl terminus of SNAP-25 when the toxin is added to living cultures, whereas it has no action on SNAP-25 in *in vitro* preparations. This study is the first to demonstrate in functioning neurons that the physiologic response to these toxins is correlated with the proteolysis of their respective substrates. Furthermore, the data demonstrate that botulinum neurotoxin C, in addition to cleaving syntaxin, exerts a secondary effect on SNAP-25.

Clostridial neurotoxins (CNTs)[1] are synthesized as single polypeptides of 150 kDa and subsequently are cleaved to active disulfide-linked dichain toxins. The heavy chain (100 kDa) carries the receptor binding and transmembrane domains of the toxin, and the light chain (50 kDa) contains the catalytic domain that blocks neurotransmitter release (1, 2). Clostridial neurotoxins are zinc endopeptidases (3–5), which cleave specific proteins thought to be involved in the synaptic vesicle docking-fusion complex. Vesicle-associated membrane protein (VAMP), also known as synaptobrevin, is cleaved by the majority of the CNTs including tetanus toxin (TeNT) and botulinum neurotoxins (BoNTs) B, D, F, and G (3, 5–9). Botulinum neurotoxins A and E cleave SNAP-25 (synaptosomal-associated protein of 25 kDa) (6, 10–12), and BoNT C cleaves syntaxin (13, 14).

VAMP, SNAP-25, and syntaxin interact with *N*-ethylmaleimide-sensitive fusion protein and soluble *N*-ethylmaleimide-sensitive fusion protein attachment proteins (SNAPs), cytosolic elements essential for intracellular membrane fusion (15). Since SNAPs must bind to membrane receptors prior to *N*-ethylmaleimide-sensitive fusion protein attachment, the SNAP receptors have been designated as SNAREs, with the vesicular protein (VAMP/synaptobrevin) as the v-SNARE and the target membrane proteins (SNAP-25 and syntaxin) as the t-SNAREs (15). Furthermore, it has been demonstrated that VAMP, SNAP-25, and syntaxin themselves form a stable complex (16–18).

Two isoforms of VAMP in neuronal tissue and a nonneuronal homologue cellubrevin have been identified in a number of animal species (19–22). VAMP is comprised of three major domains: the NH_2 terminus is a variable domain, the middle or B domain is highly conserved, and the COOH terminus contains the transmembrane spanning region with a short projection into the synaptic vesicle lumen. Tetanus toxin cleaves the peptide bond Gln^{76}–Phe^{77} within the conserved domain of VAMP (3). Recent *in vitro* studies have shown that VAMP can bind to syntaxin (17), to SNAP-25 (17), and to synaptophysin (23–25).

Syntaxin has two isoforms that are anchored to membranes by a single COOH-terminal transmembrane domain (26, 27). Syntaxin binds to presynaptic calcium channels and to synaptotagmin located in the synaptic vesicle membrane (26, 28, 29). Botulinum neurotoxin C cleaves syntaxin at a site near the transmembrane domain (13, 14, 30).

SNAP-25 is a membrane-associated cytoplasmic protein implicated in the fusion of synaptic vesicles with the presynaptic membrane (15) and in membrane addition leading to constitutive axonal growth (31). SNAP-25 is a hydrophilic protein that is palmitylated at one to four of its closely spaced cysteine residues and acts as an integral membrane protein (32, 33). Botulinum neurotoxin A cleaves SNAP 25 at a site nine amino acids from the COOH terminus between residues Gln^{197} and Arg^{198} (6, 10–12).

Tetanus toxin, BoNT A, and BoNT C act *in vivo* to block neurotransmitter release. Both BoNT A and TeNT have been shown previously to block synaptic transmission in spinal cord cell cultures (34, 35). TeNT-induced disappearance of inhibitory and excitatory postsynaptic potentials coincides in the same system with the blockade of inhibitory and excitatory neurotransmitter release (36). In the present study, we have characterized the effect of TeNT, BoNT A, and BoNT C on the

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[1] The abbreviations used are: CNT, clostridial neurotoxins; VAMP, vesicle-associated membrane protein; TeNT, tetanus toxin; SNAP, synaptosomal-associated protein; BoNT, botulinum neurotoxin; NGS, normal goat serum.

vSNARE, VAMP/synaptobrevin, and the tSNARES, SNAP-25 and syntaxin, in cells in which we have demonstrated the arrest of neurotransmitter release. This is the first study to examine VAMP, SNAP-25, and syntaxin in intact functioning neurons, where it has been possible to observe the action of BoNT C on two of the three SNARE proteins.

EXPERIMENTAL PROCEDURES

Materials—Purified tetanus toxin (2×10^7 mouse lethal doses/mg of protein) was provided by Dr. William Habig (Food and Drug Administration, Bethesda, MD). Purified BoNT A was from List Biological Laboratories, Inc., Campbell, CA, and BoNT C was from the Centre for Applied Microbiology and Research, Porton Down, UK (5.2×10^7 and 1.0×10^7 mouse lethal doses/mg of protein, respectively). [^{3}H]Glutamine (specific activity, 52 Ci/mmol) and [^{3}H]glycine (specific activity, 12.2 Ci/mmol) were from Amersham Corp., Arlington Heights, IL. 5-Fluoro-2′ -deoxyuridine was a gift from Hoffman-LaRoche Inc., Nutley, NJ. Horse antibodies against BoNT A and against BoNT C were from the United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD. Mouse monoclonal antibody against synaptophysin was from Boehringer Mannheim. Anti-VAMP antibodies were produced in rabbits to synthetic peptides corresponding to amino acids 1–32 of the VAMP variable domain from each of the two isoforms found in rat brain and affinity purified using the synthetic peptides.[2] Monoclonal antibody against the NH_2 terminus of SNAP-25 was from Chemicon, Temecula, CA. Polyclonal antibodies were raised in rabbits against a peptide consisting of the COOH-terminal 12 amino acids of SNAP-25 conjugated to keyhole limpet hemocyanin and affinity purified from columns containing the peptide coupled to Sepharose. Antibodies specific for syntaxin were obtained by immunizing rabbits with recombinant syntaxin 1A and purifying the IgG fraction.

Spinal Cord Cell Cultures—Spinal cords from 13-day fetal mice were dissociated and plated in 35-mm Vitrogen- (Collagen Corp., Palo Alto, CA) coated culture dishes as described previously (38, 39). Cultures were grown for 3 weeks in a humidified 10% CO_2 atmosphere at 35 °C. On the 5th day after plating, 5-fluoro-2′ deoxyuridine was added to cultures for 96 h to inhibit nonneuronal cell growth.

Evoked Neurotransmitter Release—Spinal cord cultures were rinsed once in minimum essential medium and incubated for 20 h in 0.06 nM TeNT, BoNT A, or BoNT C in serum-free culture medium at 35 °C. Cell cultures were rinsed and radiolabeled with 2 μCi/ml [^{3}H]glycine for 30 min or 5 μCi/ml [^{3}H]glutamine for 60 min in isosmotic HEPES-buffered salts solution (HBSS) containing 136 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 0.1% (w/v) bovine serum albumin. The pH was adjusted to 7.25, and the osmolality was adjusted with sucrose to 325 ± 5 mmol/kg. To evoke neurotransmitter release, cultures were exposed sequentially to 1.25 ml of the following solutions for 4.5-min intervals at 35 °C as described previously (36): HBSS without calcium and with 0.5 mM EGTA; HBSS containing 56 mM KCl, 83 mM NaCl and no $CaCl_2$; HBSS containing 56 mM KCl, 83 mM NaCl, and 2 mM $CaCl_2$; and finally HBSS containing 0.5 mM EGTA and no calcium. In a previous study, we demonstrated by thin-layer chromatography that virtually all of the radioactivity, [^{3}H]glycine or [^{3}H]glutamate, released under these conditions co-migrates with free glycine or free glutamate, respectively (36). Cell-associated radioactivity was assayed after dissolving the cells in 0.2 N NaOH and neutralizing with HCl. Calcium-dependent release was calculated as the amount of radiolabel released in the presence of calcium minus the amount released in the absence of calcium and normalized to the total radioactivity in the culture at the start of the release assay.

Immunohistochemistry—Control and toxin-treated (0.06 nM for 20 h) cultures were fixed in 2% paraformaldehyde in HBSS without calcium, magnesium, or bovine serum albumin for 30 min at room temperature. After rinsing, 0.1 M glycine in phosphate-buffered saline (PBS) was added for 30 min to block free aldehyde groups, after which cells were permeabilized with 0.05% saponin in PBS for 30 min at room temperature. The cultures were incubated overnight at 4 °C in a primary antibody solution containing a mixture of mouse anti-synaptophysin (1:70) with either affinity-purified rabbit anti-VAMP-1 (1:400), anti-VAMP-2 (1:400), anti-SNAP-25 (1:400), or anti-syntaxin (1:500) in PBS containing 5% normal goat serum (NGS). After rinsing 3 times (10 min each) in PBS/NGS, cultures were incubated in a mixture of goat anti-rabbit IgG-rhodamine and goat anti-mouse IgG-fluorescein (each 1:50) in PBS/NGS for 60 min at 35 °C. Cultures were rinsed twice (10 min each) in PBS/NGS, twice in PBS, and stored at 4 °C in *n*-propyl gallate in glycerol to prevent fluorescence photobleaching (40).

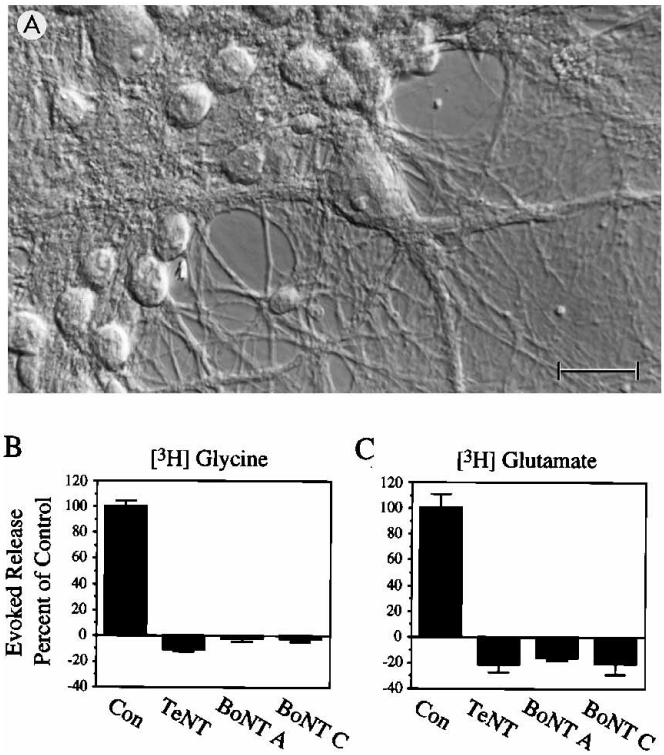

FIG. 1. **Neurotransmitter release in spinal cord cell cultures.** *A*, interference contrast photomicrograph shows that spinal cord cell cultures are a heterogeneous mixture of neurons. *Magnification bar*, 25 μm. *B* and *C*, control and toxin-exposed cultures are radiolabeled and assayed for potassium-stimulated calcium-dependent release of glycine and glutamate. Tetanus toxin and botulinum neurotoxins A and C (0.06 nM for 20 h) completely block release of the inhibitory neurotransmitter glycine (*B*) and the excitatory neurotransmitter glutamate (*C*).

Electrophoresis and Immunoblot Analysis—After incubation with toxins (0.06 nM or 0.3 nM; see Figs.), spinal cord neurons were detached from tissue culture dishes by trypsinization and washed once with PBS. Cells were dissolved by boiling for 5 min in electrophoresis sample buffer containing 2% SDS and dithiothreitol (DTT). Protein samples were run on 10–20% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h in Tris buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 5% nonfat dry milk and 0.05% Tween 20 (TTBS), incubated sequentially with primary antibodies (dilutions were the same used for immunohistochemistry) in TTBS and the appropriate alkaline phosphatase conjugated secondary antibody (Bio-Rad), and developed using alkaline phosphatase color development reagents.

RESULTS

Spinal cord cell cultures contain a heterogeneous population of neurons growing on a monolayer of nonneuronal cells (Fig. 1*A*). To confirm that TeNT, BoNT A, and BoNT C have blocked synaptic neurotransmission, spinal cord cultures are assayed for inhibitory and excitatory neurotransmitter release. Cultures exposed to these toxins are radiolabeled with [^{3}H]glycine or [^{3}H]glutamine. Neurotransmitter release is evoked by potassium-induced depolarization in the presence of calcium. With potassium stimulation, control cultures release 25–30% of the total [^{3}H]glycine (Fig. 1*B*) and 4–5% of the total [^{3}H]glutamate (Fig. 1*C*) taken up by cultures. Tetanus toxin, BoNT A, and BoNT C completely block potassium-evoked release of both neurotransmitters (Figs. 1, *B* and *C*).

Tetanus toxin abolishes synaptic immunostaining in mouse spinal cord neurons of both VAMP-1 (Fig. 2) and VAMP-2 (not shown). Loss of VAMP from neuronal terminals after TeNT

[2] Rossetto, O., Gorza, L., Schiavo, G., Schiavo, N., Scheller, R.H., and Montecucco, C. (1996) *J. Cell Biol.*, in press.

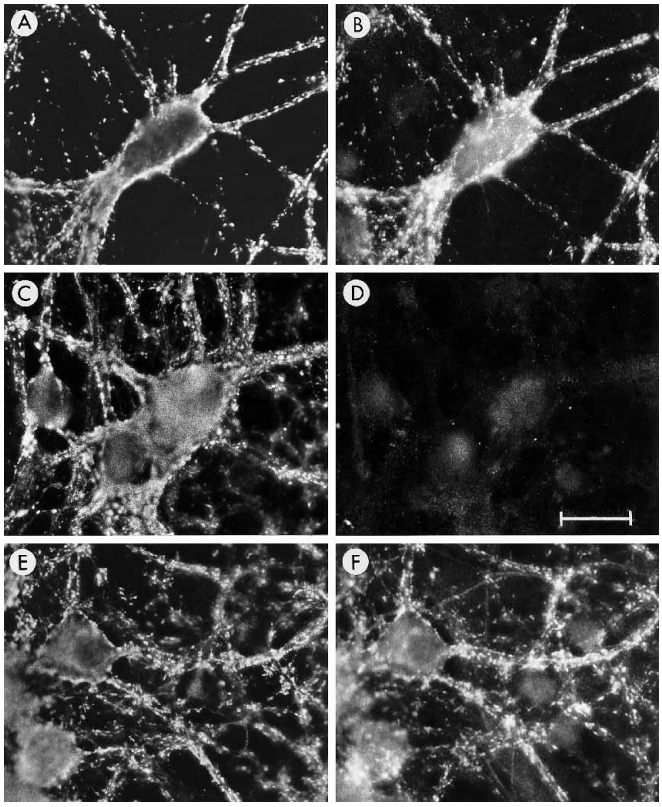

FIG. 2. **Cell cultures double-labeled with antibodies against VAMP and against synaptophysin.** Control cultures show synaptophysin immunostaining (*A*) in synaptic terminals; VAMP immunoreactivity (*B*) has an almost identical distribution. In cultures synaptically blocked by TeNT (0.06 nM for 20 h), synapses are identified clearly with anti-synaptophysin (*C*), although VAMP immunostaining (*D*) is totally abolished. In contrast, in BoNT A-blocked cultures (0.06 nM for 20 h), synapses are stained with both anti-synaptophysin (*E*) and with anti-VAMP (*F*) antibodies. *Magnification bar*, 25 μm.

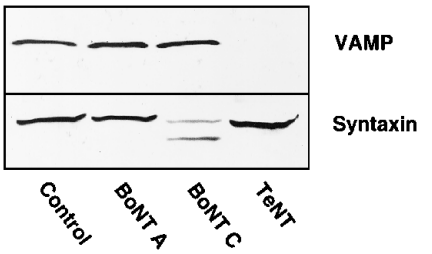

FIG. 3. **Immunoblot analysis for VAMP and syntaxin in toxin-treated cultures.** Spinal cord cell cultures were incubated with BoNT A, BoNT C, or TeNT (0.06 nM) for 16 h. Homogenates were prepared and analyzed for VAMP and syntaxin as described under "Experimental Procedures." VAMP is lost completely from cultures exposed to TeNT. Syntaxin is cleaved to a lower molecular weight by BoNT C.

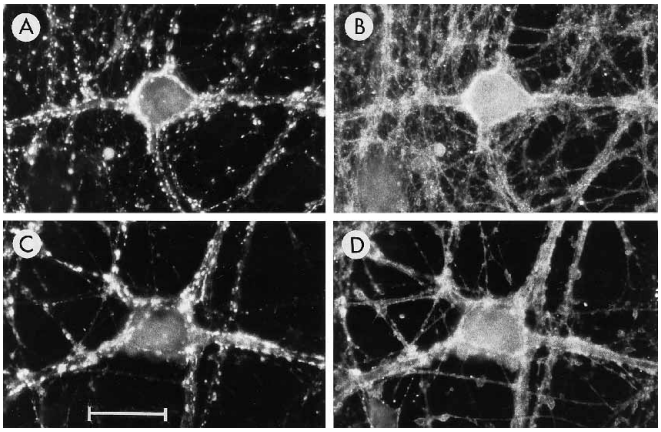

FIG. 4. **Cell cultures double-labeled with antibodies against syntaxin and against synaptophysin.** *A*, synaptophysin immunostaining defines synaptic terminals in control cultures. *B*, syntaxin is associated with the neuronal membrane including that of axonal branches, although intense immunofluorescence tends to coincide with synaptic sites. In BoNT C-blocked cultures, syntaxin immunoreactivity (*D*) persists, both at synaptophysin-positive sites (*C*) and along axonal membranes. *Magnification bar*, 25 μm.

proteolysis is demonstrated clearly by double-labeling experiments using mouse anti-synaptophysin, a marker for synaptic terminals, detected with fluorescein (Fig. 2, *A*, *C*, and *E*) and rabbit anti-VAMP-1 detected with rhodamine (Fig. 2, *B*, *D*, and *F*). In control cultures, VAMP-1 immunostaining of synaptic terminals (Fig. 2*B*) co-localizes with synaptophysin immunoreactivity (Fig. 2*A*). In TeNT-exposed cultures, although synaptic terminals are stained with anti-synaptophysin (Fig. 2*C*), there is no synaptic labeling with anti-VAMP-1 (Fig. 2*D*). An additional control for the specificity of TeNT action on VAMP was obtained using BoNTs A and C, which also are zinc endopeptidases, but which cleave other components of the vesicle docking-fusion complex (15). VAMP immunoreactivity (Fig. 2*F*) is unaffected by BoNT A exposure at a time when neurotransmitter release is completely blocked. Similar results were observed in BoNT C-exposed cultures (data not shown). Consistent with the immunohistochemistry, VAMP is absent from immunoblots of cultures exposed to TeNT (Fig. 3).

In control cultures, syntaxin is localized at the neuronal surface, particularly along axonal membranes, but also appears concentrated at synaptic membrane sites marked by synaptophysin immunoreactivity (Fig. 4, *A* and *B*). However, syntaxin staining persists in cultures known to be intoxicated by BoNT C (Fig. 4, *C* and *D*). This is consistent with the persistence of syntaxin on immunoblots (Fig. 3). Botulinum neurotoxin C cleaves syntaxin near the transmembrane domain producing a soluble fragment of syntaxin that is not degraded further (13). Immunoblots of homogenates prepared from BoNT C-exposed spinal cord cell cultures show syntaxin cleaved to a lower molecular weight band. None of the other toxins have any effect on syntaxin (Fig. 3).

The localization of SNAP-25 and the effects of BoNT A, TeNT, or BoNT C on its distribution were analyzed by double-label immunohistochemistry using antibodies against synaptophysin (Fig. 5, *A*, *C*, *E*, and *G*) and against the COOH terminus of SNAP-25 (Fig. 5, *B*, *D*, *F*, and *H*). The pattern of immunostaining for SNAP-25 in control cultures is similar to that of syntaxin (Fig. 5, *A* and *B*); *i.e.* presence along axonal and synaptic membranes. Botulinum neurotoxin A cleaves the last nine amino acids from the COOH terminus of SNAP-25 (6, 10–12). Synaptic terminals identified by synaptophysin immunostaining in BoNT A-exposed cultures (Fig. 5*C*) do not stain with antibodies against the COOH terminus of SNAP-25. (Fig. 5*D*). SNAP-25 is lost not only from the synaptic membranes but also from the other neuronal surface membranes including those of axons and cell bodies. Unexpectedly, alterations in SNAP-25 are seen also when cultures are exposed to TeNT or BoNT C. In TeNT-exposed cultures, SNAP-25 immunofluorescence clearly is more intense than in control cultures (Fig. 5*F*). In contrast, the immunoreactivity of the SNAP-25 COOH terminus is markedly reduced in BoNT C-exposed neurons (Fig. 5*H*).

SNAP-25 in control and toxin-treated cell cultures was analyzed further by immunoblotting (Fig. 6). Treatment of cultures with BoNT A for 24 h results in the loss of the COOH terminus of SNAP-25 (Fig. 6*A*). Botulinum neurotoxin C exposure causes a similar loss of the COOH terminus of SNAP-25, consonant

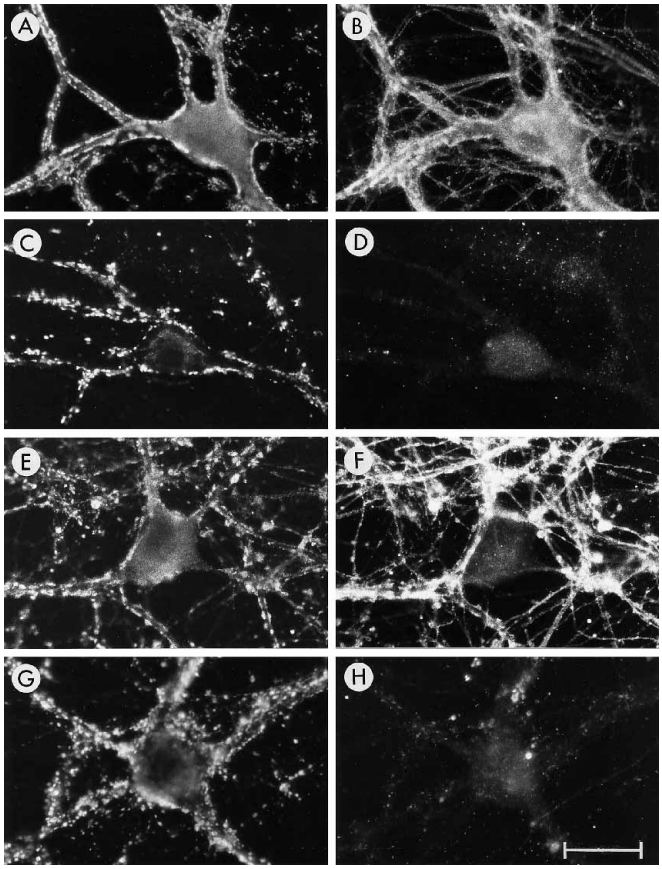

FIG. 5. **Cell cultures double-labeled with antibodies against the carboxyl terminus of SNAP-25 and against synaptophysin.** In control cultures, SNAP-25 immunostaining (*B*) shows a distribution pattern similar to that for syntaxin (compare with Fig. 4*B*); the most intense fluorescence co-localizes with synaptic sites marked by synaptophysin immunoreactivity (*A*). Synapses identified by synaptophysin immunoreactivity in BoNT A-blocked cultures (*C*) show no staining for the COOH terminus of SNAP-25 (*D*). Additionally, SNAP-25 immunostaining is lost from all neuronal surface membranes in BoNT A-treated cultures (*D*). In TeNT-blocked cultures, SNAP-25 immunoreactivity (*F*) over axonal membranes is more intense than in control cultures (compare with *panel B*; reacted, photographed, and printed under the same conditions), whereas synaptophysin staining (*E*) is similar to controls. In BoNT C-blocked cultures (0.06 nM for 20 h), staining for the COOH terminus of SNAP-25 (*H*) is almost totally eliminated from structures that stain with anti-synaptophysin antibodies (*G*). *Magnification bar*, 25 μm.

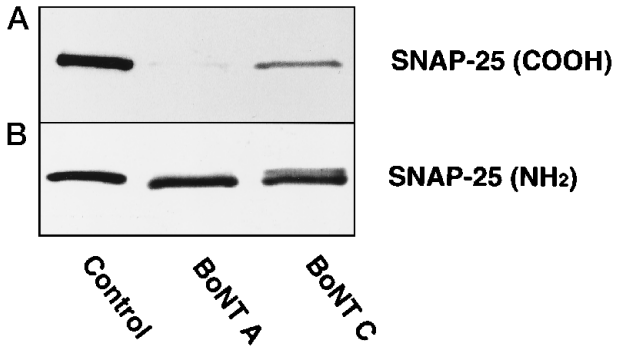

FIG. 6. **Immunoblot analysis of SNAP-25 in toxin-treated spinal cord cultures**. Cultures are incubated with BoNT A, BoNT C, or TeNT (0.06 nM) for 24 h (*A*) or 20 h (*B*). Homogenates are prepared and analyzed for SNAP-25 immunoreactivity using antibodies against the COOH or NH_2 termini. In BoNT A- or BoNT C-treated cultures, SNAP-25 (COOH terminus) immunoreactivity is lost completely (*A*). SNAP-25 immunoreactivity in TeNT-blocked cultures is similar to controls (*A*). In another set of BoNT A- or BoNT C-treated cultures, staining of the NH_2 terminus confirms SNAP-25 proteolysis (*B*).

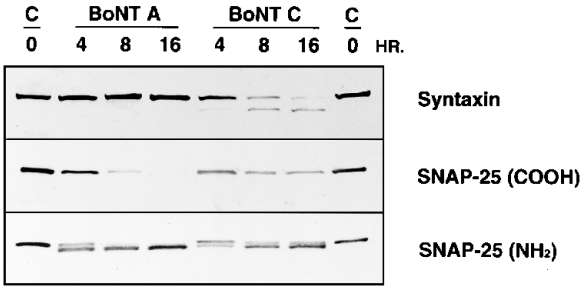

FIG. 7. **Time course analysis of SNAP-25 cleavage in BoNT A and BoNT C-treated cultures.** Spinal cord cultures were incubated in BoNT A or BoNT C (0.3 nM) for 4, 8 and 16 h. Homogenates were prepared and analyzed for SNAP-25 and syntaxin. BoNT A cleaves SNAP-25 more rapidly than BoNT C. Whereas there is total cleavage of SNAP-25 in BoNT A-treated cultures, some uncleaved SNAP-25 remains after 16 h with BoNT C. The proteolysis of SNAP-25 in BoNT C-treated cultures appears to follow the more complete cleavage of syntaxin.

with the immunohistochemistry of intact neurons. In contrast, TeNT has no effect on SNAP-25 when analyzed by immunoblot (Fig. 6*A*). Immunoblots prepared from another set of BoNT A or BoNT C-blocked cultures show two bands detected with a monoclonal antibody against the NH_2 terminus of SNAP-25 (Fig. 6*B*); the predominant band is the cleaved lower molecular weight form of SNAP-25 and the other band corresponds to the remaining uncleaved SNAP-25.

Cleavage of SNAP-25 was examined after 4, 8, and 16 h of toxin exposure to compare BoNT A and BoNT C effects (Fig. 7). Proteolysis of SNAP-25 by BoNT A is more rapid and more complete than by BoNT C as evidenced with both SNAP-25 antibodies. Some COOH terminus immunoreactivity persists after 16 h in BoNT C, whereas there is none left with BoNT A treatment. Similarly, antibodies against the NH_2 terminus indicate that some uncleaved SNAP-25 remains in BoNT C-treated cultures, although the BoNT A-exposed cultures show a clear progression with time to the total cleavage of SNAP-25. The time course of syntaxin cleavage demonstrates that virtually all of syntaxin is cleaved by BoNT C in 16 h, whereas more SNAP-25 remains intact. Thus, BoNT C action on SNAP-25 appears to follow its proteolysis of syntaxin.

To determine if cleavage of SNAP-25 in BoNT C-exposed cultures were due to contamination with BoNT A, toxins used in these studies were immunoblotted with antibodies against BoNT A. Preparations of BoNT C were not recognized by antibodies against BoNT A, providing evidence against the possibility of contamination by BoNT A (data not shown). Additionally, immunohistochemistry and immunoblots of BoNT A and BoNT C-treated cultures were repeated using a mixture of toxin with an excess of antibodies against BoNT A. When cultures are exposed to the BoNT A preparation premixed with antibodies against BoNT A, immunoreactivity for SNAP-25 persists, and no cleavage of SNAP-25 is detected by immunoblot, *i.e.* BoNT A is rendered ineffective. However, BoNT C premixed with anti-BoNT A is equally as effective as BoNT C alone in altering the staining patterns of both SNAP-25 and syntaxin (data not shown). These data demonstrate that the effect of BoNT C on SNAP-25 cannot be explained by the presence of contaminating amounts of BoNT A.

BoNT C action on SNAP-25 has not been described before, although the previous studies were carried out using subcellular preparations. We investigated the action of BoNT C *in vitro* on postnuclear supernatants prepared from spinal cord cell cultures. For *in vitro* studies, BoNT A and BoNT C (150 nM final concentration) are activated (3–5, 11) prior to addition to the postnuclear supernatants for 90 min at 37 °C. Under these

conditions, immunoblots using antibodies against either the NH_2 or COOH terminus demonstrate proteolysis of SNAP-25 by BoNT A but not by BoNT C (data not shown). Thus, the action of BoNT C on SNAP-25 is observed only when the toxin is added to intact neurons and gains access to synaptic proteins under physiologic conditions.

DISCUSSION

This study is the first to demonstrate in physiologically relevant cells, *i.e.* in intact functioning neurons, a direct correlation between the clostridial neurotoxin-induced block in neurotransmitter release and the cleavage of toxin-specific protein substrates, VAMP, SNAP-25, or syntaxin. The cleavage of synaptic proteins may not be the only mechanism whereby these toxins induce their prolonged neuroparalysis (41–43). Nonetheless, our data clearly demonstrate by immunohistochemistry and immunoblot analysis that VAMP, SNAP-25, and syntaxin are cleaved by TeNT, BoNT A, or BoNT C, respectively, in the same neurons in which neurotransmitter release is shown to be blocked. These findings confirm that the principal mechanism of action of clostridial neurotoxins is proteolytic cleavage of specific synaptic proteins necessary for neurotransmitter release.

Additionally, this study provides insight into the interaction of these proteins preceding synaptic vesicle exocytosis in intact neurons. Treatment of cultures with TeNT results in the cleavage of VAMP and an increase in the intensity of SNAP-25 immunoreactivity as detected by immunohistochemistry with the COOH terminus antibody. This suggests that VAMP/synaptobrevin binds to the COOH terminus of SNAP-25. In contrast, immunohistochemistry of the NH_2 terminus of SNAP-25 in intact neurons remains unchanged (data not shown). Furthermore, immunoblot analysis of SNAP-25 (COOH terminus) did not show any difference between control and TeNT-exposed cultures. Thus, the increased immunoreactivity of the COOH terminus of SNAP-25 in TeNT-treated cultures is not due to increased levels of SNAP-25 but rather to increased accessibility to the antibody. Direct binding of SNAP-25 to VAMP and to syntaxin was demonstrated in an *in vitro* system using purified fusion proteins and deletion analysis (17, 18). Furthermore, Chapman *et al.* (17) reported that deletion of nine amino acids from the COOH terminus of SNAP-25 reduced VAMP binding by 73%. Our observations in spinal cord neurons are consistent with these findings.

When BoNT C is added to intact spinal cord neurons in culture, not only is syntaxin cleaved, as expected, but there is a concomitant loss of immunostaining for the COOH terminus of SNAP-25. The reduction in SNAP-25 immunoreactivity in intact neurons is seen both by immunohistochemistry and by immunoblotting. Furthermore, in support of this result, an antibody against the NH_2 terminus of SNAP-25 recognizes two bands due to SNAP-25 cleavage in BoNT C-treated cultures. Possible explanations for the effect include 1) that syntaxin proteolysis by BoNT C changes the conformation or accessibility of SNAP-25, increasing its susceptibility to endogenous proteases or 2) that BoNT C itself proteolytically cleaves SNAP-25 albeit at a slower rate than it cleaves syntaxin. The action of BoNT C on SNAP-25 apparently occurs only when living neurons are exposed to this toxin. SNAP-25 is not affected when BoNT C is added to spinal cord cell culture homogenates, although BoNT A cleaves SNAP-25 in the same homogenates. Furthermore, BoNT C does not cleave recombinant SNAP-25.[3] Thus, the action of BoNT C on SNAP-25 *in vivo* might occur only when SNAP-25 is complexed with another protein and/or plasma membranes. Alternatively, SNAP-25 may be protected from proteolysis by endogenous proteases when it is complexed to syntaxin, and the cleavage of syntaxin by BoNT C increases SNAP-25's susceptibility to proteolysis.

Recent findings demonstrate the presence of syntaxin (proposed as a t-SNARE) in purified synaptic vesicle fractions (37). Although the majority of syntaxin appeared in a plasma membrane fraction, BoNT C preferentially cleaved vesicular syntaxin leaving the plasma membrane syntaxin largely unaffected. In the spinal cord cultures, however, much lower concentrations of BoNT C cleaved almost all syntaxin. Differences between *in vivo* and *in vitro* preparations as well as differences in the conditions of BoNT C incubation might account for this discrepancy.

In summary, the findings reported here demonstrate the actions of the clostridial neurotoxins *in vivo*, seen together as the blockade of neurotransmitter release with the proteolytic cleavage of the respective toxin substrates. The data also provide evidence for the first time that, in addition to cleavage of syntaxin, BoNT C has a secondary action on the COOH terminus of SNAP-25. The finding that BoNT C is the only clostridial neurotoxin that acts on two of the three SNARE proteins might be significant in terms of its efficacy for the clinical treatment of muscle spasm disorders.

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