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 zine 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 vivo 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.

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† 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.
**Clostridial Neurotoxin Action in Intact Neurons**

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 x 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 x 10^7 and 1.0 x 10^7 mouse lethal doses/mg of protein, respectively). [3H]Glycine (specific activity, 52 Ci/mmol) and [3H]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 NH₂-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% CO2 atmosphere at 35°C. The cultures exposed to these toxins are radiolabeled with [3H]glycine (B and C) and the excitatory neurotransmitter glutamate (C), in press.

Figu. 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).

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).

Electrophoresis and Immunoblot Analysis—After incubation with toxins (0.06 nM or 0.3 nM; see Figs.), spinal cord neurons were detached from the 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. 1A). 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 [3H]glycine or [3H]glutamate. Neurotransmitter release is evoked by potassium-induced depolarization in the presence of calcium. With sodium stimulation, control cultures release 25–30% of the total [3H]glycine (Fig. 1B) and 4–5% of the total [3H]glutamate (Fig. 1C) 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...
Syntaxin 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. 2B) co-localizes with synaptophysin immunoreactivity (Fig. 2A). In TeNT-exposed cultures, although synaptic terminals are stained with anti-synaptophysin (Fig. 2C), there is no synaptic labeling with anti-VAMP-1 (Fig. 2D). 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. 2F) 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. 5C) do not stain with antibodies against the COOH terminus of SNAP-25 (Fig. 5D). 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. 5F). In contrast, the immunoreactivity of the SNAP-25 COOH terminus is markedly reduced in BoNT C-exposed neurons (Fig. 5H).

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. 6A). Botulinum neurotoxin C exposure causes a similar loss of the COOH terminus of SNAP-25, consonant
with the immunohistochemistry of intact neurons. In contrast, TeNt has no effect on SNAP-25 when analyzed by immunoblot (Fig. 6A). 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. 6B); 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

![Figure 5](http://www.jbc.org/Download/fig5.jpg)

**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. 4B); 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.

![Figure 6](http://www.jbc.org/Download/fig6.jpg)

**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).

![Figure 7](http://www.jbc.org/Download/fig7.jpg)

**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.
conditions, immunoblots using antibodies against either the NH₂ 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 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, 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 spasms disorders.

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