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Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin

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The anaerobic bacterium *Clostridium botulinum* produces several related neurotoxins that block exocytosis of synaptic vesicles in nerve terminals and that are responsible for the clinical manifestations of botulism. Recently, it was reported that botulinum neurotoxin type B as well as tetanus toxin act as zinc-dependent proteases that specifically cleave synaptobrevin, a membrane protein of synaptic vesicles (Link *et al.*, *Biochem. Biophys. Res. Commun.*, 189, 1017–1023; Schiavo *et al.*, *Nature*, 359, 832–835). Here we report that inhibition of neurotransmitter release by botulinum neurotoxin type C1 was associated with the proteolysis of HPC-1 (= syntaxin), a membrane protein present in axonal and synaptic membranes. Breakdown of HPC-1/syntaxin was selective since no other protein degradation was detectable. *In vitro* studies showed that the breakdown was due to a direct interaction between HPC-1/syntaxin and the toxin light chain which acts as a metallo-endoprotease. Toxin-induced cleavage resulted in the generation of a soluble fragment of HPC-1/syntaxin that is 2–4 kDa smaller than the native protein. When HPC-1/syntaxin was translated *in vitro*, cleavage occurred only when translation was performed in the presence of microsomes, although a full-length product was obtained in the absence of membranes. However, susceptibility to toxin cleavage was restored when the product of membrane-free translation was subsequently incorporated into artificial proteoliposomes. In addition, a translated form of HPC-1/syntaxin, which lacked the putative transmembrane domain at the C-terminus, was soluble and resistant to toxin action. We conclude that HPC-1/syntaxin is involved in exocytotic membrane fusion. Furthermore, these results indicate that the botulinum neurotoxins have evolved as related proteins recognizing the exocytotic fusion machine whereby individual toxin components target different components of the membrane fusion complex.

Key words: clostridial neurotoxins/exocytosis/glutamate release/HPC-1/syntaxin/synaptic proteins

Introduction

Neurons release neurotransmitter by exocytosis of synaptic vesicles from nerve endings. When an action potential arrives, the presynaptic plasma membrane depolarizes and voltage-gated Ca^{2+} channels become activated. The rise in intracellular Ca^{2+} triggers membrane fusion with a delay time of only 200–300 μs, indicating that the proteins involved in membrane fusion must exist in a preassembled and activated state [for review, see Ceccarelli and Hurlbut (1980), Augustine *et al.* (1987)].

In recent years, a great deal has been learned about the protein composition of synaptic vesicles, which currently can be regarded as the most completely characterized trafficking organelle [reviewed by Trimble *et al.* (1991) and Südhof and Jahn (1991)]. The membrane of synaptic vesicles contains a set of small protein families with unique properties, some of which are likely to be involved in exocytotic membrane fusion. Docking and fusion of intracellular membranes presumably requires the regulated interaction between components of the fusing membranes and of cytosolic factors that are recruited for the final step. Thus, major efforts are directed towards an understanding of the protein–protein interactions in which synaptic vesicle proteins participate. The Ca^{2+}-binding protein synaptotagmin (Brose *et al.*, 1992) has recently been shown to bind at least two different plasma membrane proteins: neurexin I and HPC-1/syntaxin (Petrenko *et al.*, 1991; Bennett *et al.*, 1992; Yoshida *et al.*, 1992). Neurexin I is probably identical to the receptor for the active component of black widow spider venom, α-latrotoxin, which causes massive, Ca^{2+}-independent exocytosis (Ushkaryov *et al.*, 1992). HPC-1 or syntaxin, originally discovered as a marker for amacrine cell development in the retina (Barnstable *et al.*, 1985), has recently been identified as a membrane protein primarily localized in axonal plasma membranes. HPC-1/syntaxin appears to be associated with binding sites for ω-conotoxin, indicating an interaction with presynaptic Ca^{2+} channels (Bennett *et al.*, 1992; Inoue and Akagawa, 1992; Yoshida *et al.*, 1992). Furthermore, Rothman and co-workers have recently demonstrated that HPC-1/syntaxin is part of a protein complex that interacts in an ATP-dependent manner with soluble protein factors, NSF and SNAPs (Söllner *et al.*, 1993). NSF and SNAPs are required for several intracellular membrane fusion events, and are highly conserved throughout evolution, being the mammalian equivalents of the yeast proteins Sec18 and Sec17, respectively (reviewed by Rothman and Orci, 1992). The other components of the complex include the synaptic vesicle protein synaptobrevin (VAMP) (Trimble *et al.*, 1988; Baumert *et al.*, 1989) and the membrane-associated protein SNAP-25 (Oyler *et al.*, 1989).

Strong support for a direct role of synaptobrevin in exocytosis was recently provided by the finding that the light

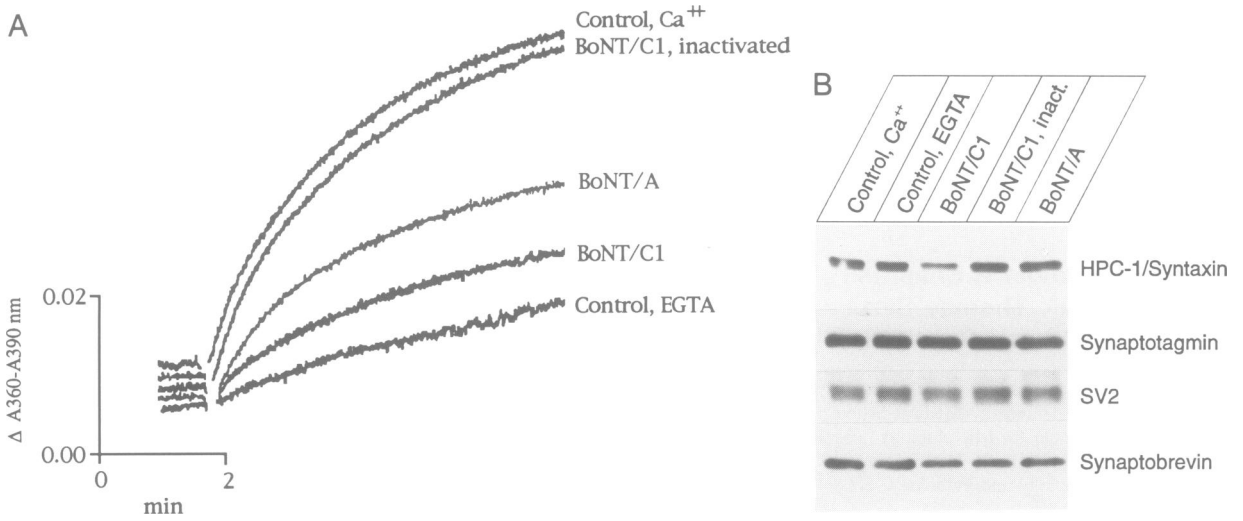

Fig. 1. Inhibition of neurotransmitter release from isolated nerve terminals is associated with the selective breakdown of HPC-1/syntaxin. Synaptosomes (1.5 mg of protein/assay), isolated from rat cerebral cortex, were preincubated for 90 min in the absence or presence of BoNT/C1 (80 nM) or BoNT/A (150 nM) and then stimulated by the addition of KCl. (**A**) Release of glutamate from synaptosomes, monitored by an on-line photometric assay. In the EGTA control, Ca^{2+} was omitted and replaced by 1 mM EGTA. (**B**) Immunoblots of a membrane fraction obtained from the synaptosomes shown in (A) after the end of the release experiment. A total of 5 μg of protein was separated on a 12% SDS–polyacrylamide gel, transferred to nitrocellulose and probed for HPC-1/syntaxin and for the synaptic vesicle proteins synaptotagmin, SV2 and synaptobrevin. All toxin-containing fractions were depolarized in the assay buffer containing Ca^{2+}, no difference was observed in the protein pattern when depolarization occurred in the presence of EGTA.

(L) chains of tetanus toxin (TeTx) and botulinum neurotoxin type B (BoNT/B) act as zinc-dependent proteases that cleave synaptobrevin with an extraordinary degree of selectivity (Link *et al.*, 1992; Schiavo *et al.*, 1992a,b; Wright *et al.*, 1992; H.Niemann *et al.*, in preparation). These toxins cause a long-lasting inhibition of synaptic vesicle exocytosis, without any impairment of other cellular functions such as energy levels, membrane potential or ion currents [reviewed by Niemann (1991)]. Thus, loss of synaptobrevin renders synaptic vesicles incapable of fusing with the presynaptic plasma membrane.

Whereas *Clostridium tetani* produces a single toxin species, *Clostridium botulinum* strains synthesize at least seven serologically distinct neurotoxins, designated BoNT/A, B, C1, D, E, F and G, that are complexed with a defined subset of non-toxigenic components, including proteins with hemagglutinating properties. Biochemical studies and molecular cloning revealed that tetanus toxin and the botulinal neurotoxins share a common basic structure. All toxins are synthesized as polypeptides of 150 kDa which are subsequently activated by proteolysis involving endogenous host proteases to yield a light (L) chain of 50 kDa and a heavy (H) chain of 100 kDa, held together by a single disulfide bond. The H chains are thought to be responsible for neuroselective binding, internalization, intraneuronal sorting and, finally, translocation of the L chains from an acidic compartment into the cytoplasm. The L chains, which are released in the reductive environment of the cytoplasm, are responsible for the block of transmitter release. The L chains of clostridial neurotoxins share between 32% (TeTx and BoNT/A) and 52% (TeTx and BoNT/B) identity, including a motif (HExxHxxH) that is presumably involved in Zn^{2+} binding [Jongeneel *et al.*, 1989; Vallee and Auld, 1990; Kurazono *et al.*, 1992; for review see Niemann (1991)].

The similarity between TeTx and the botulinum neurotoxins that block transmitter release suggests that they all evolved as Zn^{2+}-dependent proteases from a common ancestral gene. Interestingly, neither BoNT/A nor BoNT/E caused breakdown of synaptobrevin (Schiavo *et al.*, 1992a), suggesting that the targets of these toxins are different proteins. Here we report that inhibition of neurotransmitter release by BoNT/C1 is associated with a highly selective breakdown of HPC-1/syntaxin, strongly supporting the view that syntaxin as well as synaptobrevin participates in exocytotic membrane fusion. These data suggest that the botulinum neurotoxins have evolved as proteases that recognize the protein complex mediating fusion whereby individual toxins interact with different components of the complex.

Results

BoNT/C1-induced breakdown of HPC-1/syntaxin correlates with inhibition of neurotransmitter release

In the first series of experiments, we utilized nerve terminals isolated from rat cerebral cortex (synaptosomes) as a model system to study the effects of BoNT/C1. Synaptosomes are re-sealed nerve endings which retain normal metabolic and endo-exocytotic activity for hours *in vitro*. Therefore, this preparation provides an accessible means to study Ca^{2+}-dependent exocytotic release of neurotransmitters (Nicholls, 1989). Synaptosomes were purified by means of differential and Ficoll density gradient centrifugation, and preincubated for 90 min at 37°C in the absence or presence of purified BoNT/C1 (holotoxin, consisting of H and L chains linked by a disulfide bond). Transmitter release was then initiated by depolarization with KCl and the release of glutamate, the major excitatory transmitter of the mammalian CNS, was monitored with a coupled enzymatic detection system. Figure 1A shows that depolarization leads to a rapid release of glutamate that tapers off after 5 min. Release is dependent on Ca^{2+}, with no release being observed when Ca^{2+} is omitted and the chelator EGTA is present, which

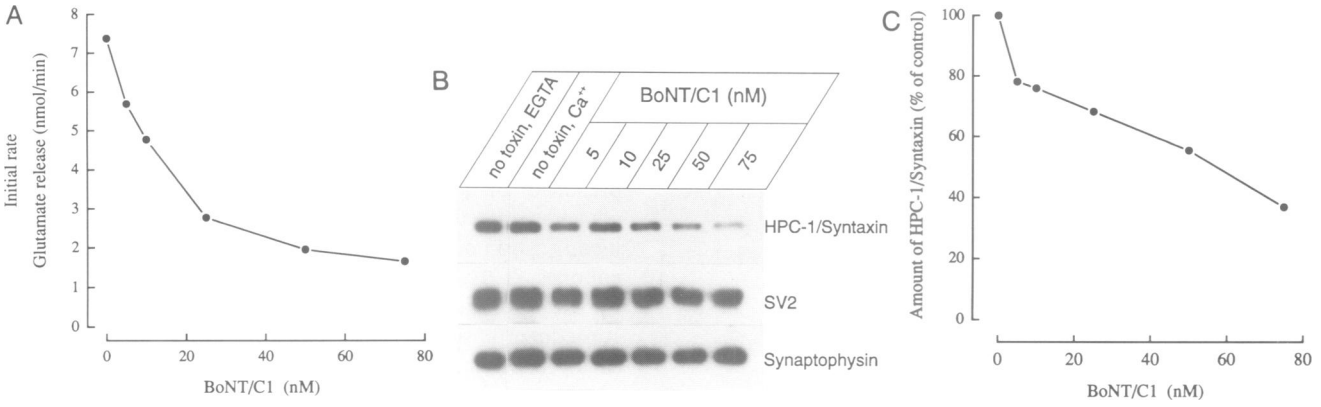

Fig. 2. The concentration range of BoNT/C1 required for inhibition of transmitter release correlates with that required for HPC-1/syntaxin breakdown. Synaptosomes isolated from rat cerebral cortex were utilized and treated in the same way as described in Figure 1. (**A**) Dependency of BoNT/C1 concentration on the initial rate of glutamate release. The initial rate was graphically determined from recordings such as those shown in Figure 1A by determining ΔA in the first minute after KCl addition. To each incubation, 10 nmol of glutamate were added 6 min after KCl addition as an internal standard for quantitation (1.5 mg synaptosomal protein/assay). (**B**) Immunoblot of the same experiments after isolation of a membrane fraction, showing that the amount of HPC-1/syntaxin decreases with increasing BoNT/C1 concentrations (5 μg of protein/lane). (**C**) Quantitation of HPC-1/syntaxin by densitometry. The values are normalized to synaptophysin to correct for inequalities in the protein recovery. Normalization to SV2 or synaptotagmin resulted in virtually identical curves (not shown).

is in agreement with previously published results (Nicholls and Sihra, 1986). Preincubation of synaptosomes with 80 nM BoNT/C1 leads to an almost complete inhibition of Ca^{2+}-dependent transmitter release (Figure 1A), demonstrating that the toxin is effective in this preparation. BoNT/A that was used for comparison also inhibits release, but was less effective under these conditions (see e.g. McMahon *et al.*, 1992).

To analyze the effects of the toxin on synaptic proteins, synaptosomes were subfractionated after the release experiments to yield a light membrane fraction that contained presynaptic plasma membrane fragments as well as synaptic vesicles. This fraction was analyzed by immunoblotting for HPC-1/syntaxin and several synaptic vesicle proteins, including synaptobrevin. As shown in Figure 1B, BoNT/C1 caused a selective reduction of the amount of HPC-1/syntaxin without affecting the levels of the synaptic vesicle proteins synaptobrevin, synaptotagmin, SV2, synaptophysin, rab3, synapsin 1, p29, or the endosomal protein rab5 (Figures 1B and 2, and not shown). In addition, no change in the overall protein pattern was detectable (not shown), demonstrating that BoNT/C1 action was not associated with non-specific proteolysis. Heat inactivation of the toxin prior to the incubation prevented inhibition of transmitter release as well as breakdown of HPC-1/syntaxin. In contrast, BoNT/A had no significant effect on the levels of any of the proteins examined.

A dose–response analysis of BoNT/C1 was performed to investigate the degree of correlation between inhibition of glutamate release and HPC-1/syntaxin breakdown. Relatively high concentrations of BoNT/C1 were required to inhibit glutamate release as compared with the high potency of the toxin observed in *in vivo* studies. This is likely due to low-efficiency uptake of the toxin by synaptosomes. For various BoNT/C1 concentrations, the initial rate of glutamate release was determined and compared with the amount of HPC-1/syntaxin that remained at the end of the incubation period (Figure 2). Starting with 5 nM, BoNT/C1 caused a progressive inhibition of neurotransmitter release that was virtually complete at 75 nM (Figure 2A). Breakdown of HPC-1/syntaxin showed a corresponding dose

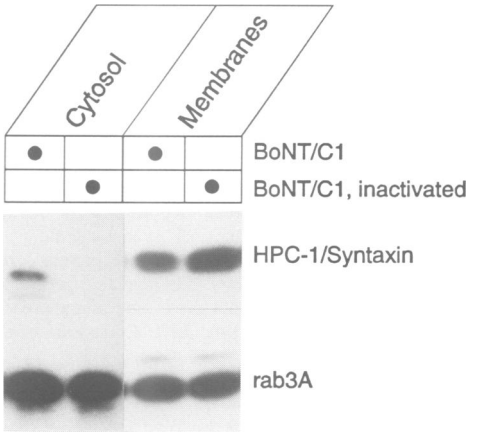

Fig. 3. BoNT/C1-induced cleavage of HPC-1/syntaxin generates a soluble fragment. Synaptosomes were incubated as described in Figure 1 and subfractionated to yield a membrane fraction and a soluble fraction (synaptosomal cytosol). Both fractions were analyzed by SDS–PAGE and immunoblotting (5 μg of protein/lane, 12% gel). For comparison, the small GTP-binding protein rab3A, which occurs both in a membrane-bound and a soluble pool, is also shown. Note that the cytosol lanes were exposed 8 times longer than the membrane lanes in order to visualize the fragment due to its weaker immunoreactivity.

dependency (Figure 2B and C), reaching a maximum of 60% degradation. The extent of degradation could not be further increased by increasing the toxin concentration or extending the incubation time.

We then investigated whether a fragment of HPC-1/syntaxin could be detected after incubation of synaptosomes with BoNT/C1. Since HPC-1/syntaxin possesses only one putative transmembrane region at the C-terminus, fragments were likely to be released from the membrane. Therefore, we examined both the membrane and the soluble fraction for the presence of HPC-1/syntaxin fragments. As shown in Figure 3, BoNT/C1 resulted in the appearance of a fragment with higher electrophoretic mobility which was present in the cytosol (apparent mol. wt shift 2–4 kDa, depending on the gel system used). This suggested that the primary cleavage site of BoNT/C1 was located close to the

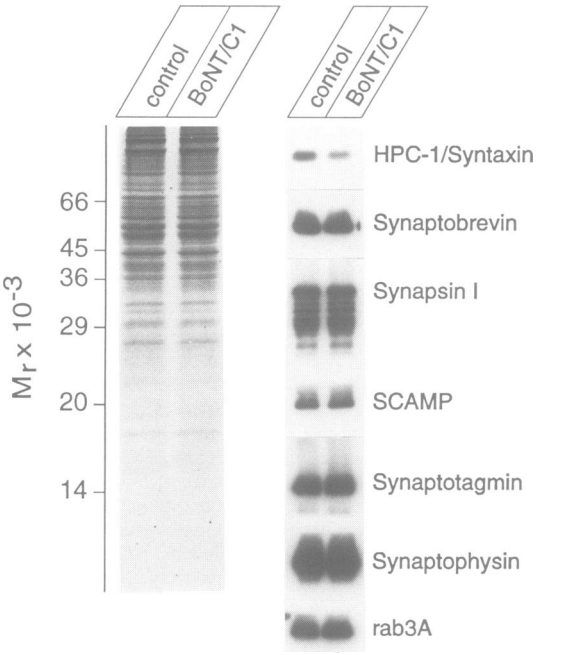

Fig. 4. BoNT/C1 selectively cleaves HPC-1/syntaxin when incubated with isolated membranes. Fifty micrograms of a membrane fraction isolated from synaptosomes were incubated for 60 min at 37°C with 50 nM BoNT/C1 in 20 mM HEPES (pH 7.0), 140 mM NaCl, 5 mM dithiothreitol. At the end of the incubation, the reaction was stopped by the addition of electrophoresis sample buffer and the samples were analyzed by SDS–PAGE and immunoblotting (5 μg of protein/lane). The figure shows segments of various blots probed for the synaptic vesicle proteins synaptobrevin, synapsin I, synaptotagmin, synaptophysin, rab3A and SCAMP, a membrane protein common to trafficking organelles of regulated and non-regulated pathways (Brand *et al.*, 1991). In addition, we did not detect any immunoreactive fragments derived from these proteins. **Left:** Coomassie blue staining; **right:** immunoblots.

C-terminus of HPC-1/syntaxin. However, the immunoreactivity of the antibody with the fragment was weaker than with the intact protein. Since the epitope of the monoclonal antibody is not yet known, it cannot be excluded that during BoNT/C1 incubation additional fragments were generated that escaped detection.

BoNT/C1 selectively cleaves HPC-1/syntaxin in a cell-free assay

The experiments described so far demonstrate that BoNT/C1 causes breakdown of HPC-1/syntaxin in parallel to inhibition of neurotransmitter release. However, it is not clear whether this breakdown is due to a direct proteolytic attack of the toxin on HPC-1/syntaxin or whether an intermediate step is involved, e.g. activation of an endogenous protease. To address this issue, we investigated whether BoNT/C1 was able to cleave HPC-1/syntaxin when it was directly incubated with isolated synaptic membranes. To liberate the L chain, BoNT/C1 was incubated with dithiothreitol. Figure 4 shows that under these conditions a selective breakdown of HPC-1/syntaxin was observed, whereas all other proteins examined remained unaffected, again demonstrating the high selectivity of BoNT/C1 for HPC-1/syntaxin. As in intact nerve terminals, breakdown of HPC-1/syntaxin was only partial (reduction between 40 and 90% in this fraction, see also Figure 7) and could not be increased either by extending the incubation time or by augmenting the amount of toxin. Since virtually all HPC-1/syntaxin is accessible to other proteases in this preparation (C.Walch-Solimena and R.Jahn, unpublished observations), this indicates the existence of a toxin-resistant pool (see Discussion).

To characterize the BoNT/C1-induced cleavage of HPC-1/syntaxin in more detail, we investigated the action of BoNT/C1 using HPC-1/syntaxin translated *in vitro* in the presence of [^{35}S]methionine. This technique has the advantage that the substrate is uniformly labeled, permitting identification of cleavage products independent of antibody epitopes. HPC-1/syntaxin a and b were separately translated in the presence of microsomes. The assay mix was subsequently subjected to ultracentrifugation to separate membrane-bound from soluble components. In addition, constructs of both isoforms were expressed that lacked the C-terminally located putative transmembrane region (TMR). As a control, the synaptic vesicle protein synaptotagmin I was co-translated in the same assay mixture.

Translation of HPC-1/syntaxin led to the appearance of a major band with an apparent M_r of 35 000 (Figure 5, left lanes). This band exhibited an electrophoretic mobility very similar to that of native HPC-1/syntaxin (not shown). Most of this band was recovered in the pellet after centrifugation together with synaptotagmin (compare P and S lanes on the left). Incubation of the assay mixture with BoNT/C1 led to the appearance of a fragment with an apparent M_r that is ~2–4 kDa smaller than that of the intact product (Figure 5, arrows). No additional fragments were detected despite the use of a Tricine-based variant of SDS–PAGE that permits the resolution of fragments >4 kDa (Schägger and von Jagow, 1987). Identical fragments were observed when synaptotagmin I DNA was omitted from the reaction mix, demonstrating that they are derived from HPC-1/syntaxin. Both isoforms of HPC-1/syntaxin generated fragments of similar size (compare upper and lower parts of Figure 5). In contrast to the intact molecule, the fragments remained in the supernatant upon ultracentrifugation. The efficiency of cleavage was low and could not be enhanced by extending the incubation time or the amount of toxin. This may be due to an inefficient incorporation of syntaxin into microsomal membranes (see below).

The right side of Figure 5 shows the results obtained with the HPC-1/syntaxin constructs that lacked the putative transmembrane domain. The major translation products were proteins with slightly increased mobilities when compared with the full-length constructs, but clearly separated from the fragment generated by BoNT/C1, in particular when a different gel system was used (not shown). These proteins were almost completely recovered in the supernatant, consistent with the prediction that the hydrophobic C-terminal region serves as the membrane anchor. Here, BoNT/C1 caused no shift in the position of the translated mutant proteins, indicating that the presence of the transmembrane domain is required for BoNT/C1 action.

The requirement for an intact transmembrane domain prompted us to investigate whether HPC-1/syntaxin needs to be incorporated into membranes in order to be recognized by the toxin. Preliminary results suggested that detergent solubilization of HPC-1/syntaxin-containing membranes completely inhibited toxin action (not shown). To address this issue in more detail, full-length HPC-1/syntaxin was translated *in vitro* in the presence or absence of microsomes and subjected to BoNT/C1 treatment. As shown in Figure 6A, primary translation products of the same size

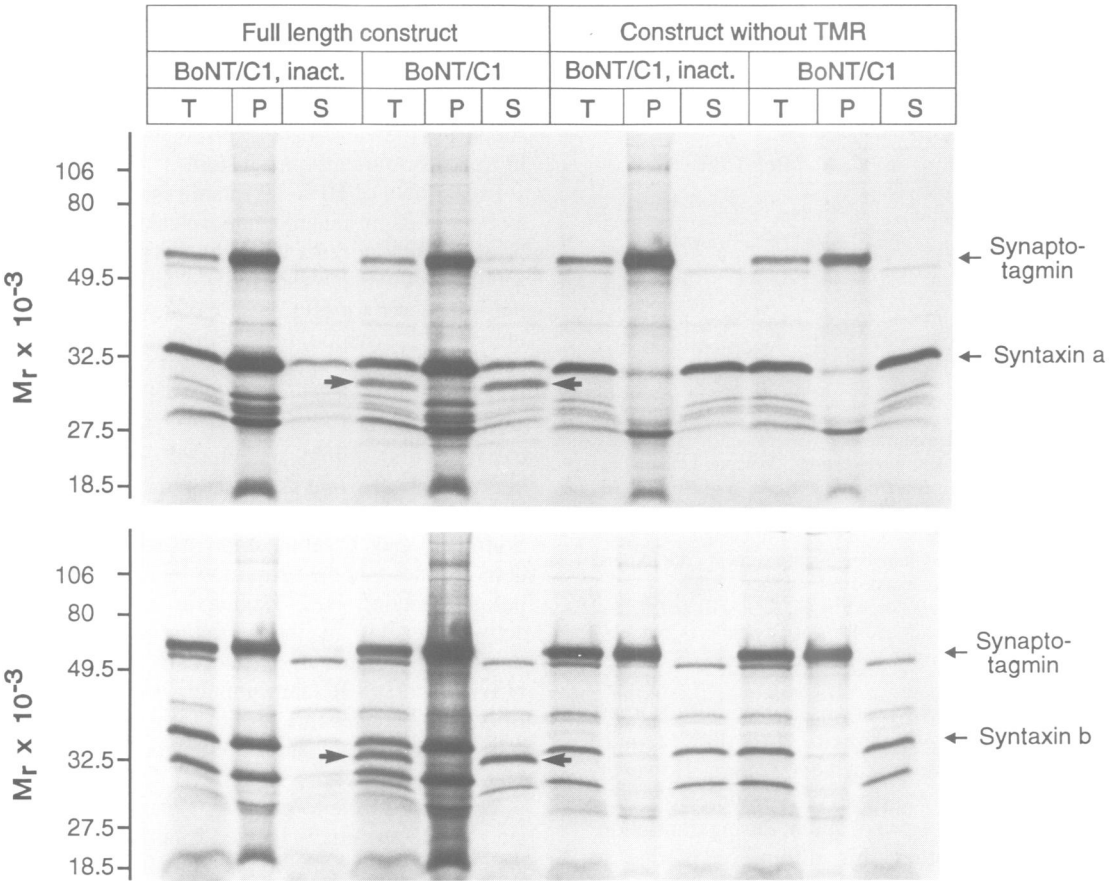

Fig. 5. BoNT/C1 cleaves HPC-1/syntaxin translated *in vitro*: cleavage requires the transmembrane domain. HPC-1/syntaxin was translated *in vitro* in the presence of microsomes. As a control, the synaptic vesicle protein synaptotagmin I was co-translated in the same assay. At the end of the translation reaction, the samples (T) were centrifuged in order to separate membrane-bound (pellet fraction P) from soluble (supernatant fraction S) translation products. All samples were analyzed by Tricine SDS−PAGE and [^{35}S]methionine-labeled proteins were visualized by fluorography. The upper panel shows the results obtained with syntaxin isoform a, the lower panel those obtained with syntaxin isoform b. The breakdown products of HPC-1/syntaxin are indicated by arrows. Note that the protein obtained from the full-length construct is associated with microsomes, whereas the protein obtained from the construct lacking the putative transmembrane region (TMR) is soluble.

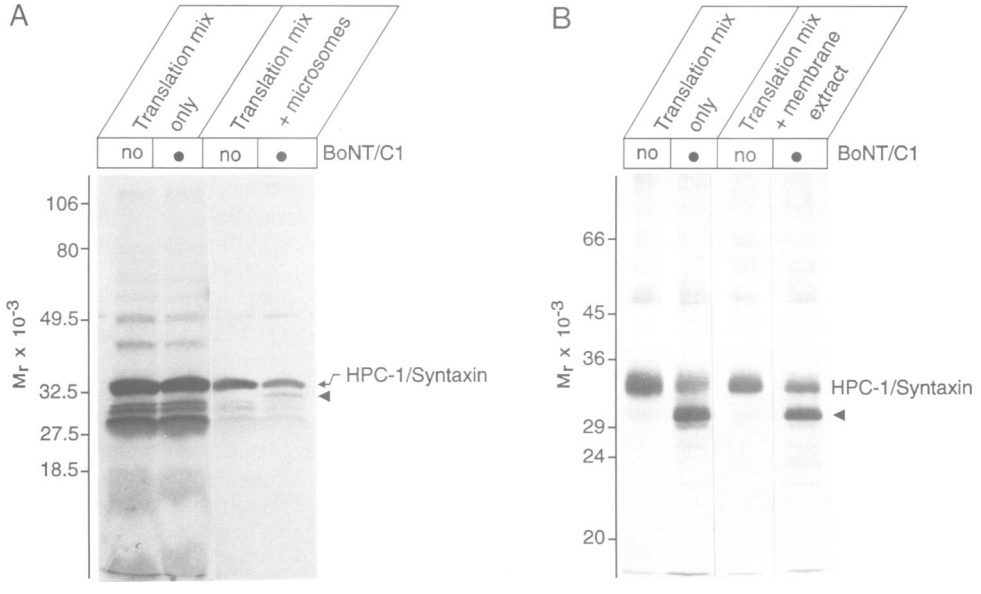

Fig. 6. Membrane binding of HPC-1/syntaxin is required for susceptibility to BoNT/C1. (**A**) BoNT/C1 action on HPC-1/syntaxin translated *in vitro* in the absence or presence of microsomes. The experimental design was identical to that described in Figure 5 except that synaptotagmin I DNA was omitted. Note that translation in the presence of microsomes reduced translation efficiency. The arrowhead indicates the position of the fragment. (**B**) BoNT/C1 action on HPC-1/syntaxin translated *in vitro* in the absence of microsomes and incorporated subsequently into artificially generated liposomes, either without or with synaptic membrane protein extracts. The arrowhead indicates the position of the fragment.

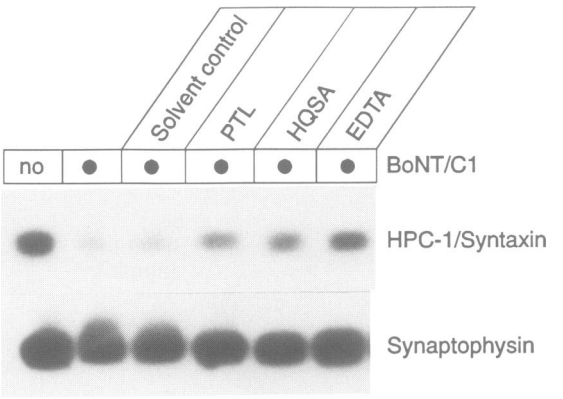

Fig. 7. BoNT/C1-dependent cleavage of HPC-1/syntaxin is partially prevented by chelators for divalent metal cations. Synaptic membranes (2.5 μg of protein each) were incubated in the presence of BoNT/C1 (100 nM final concentration) and the following inhibitors where indicated: 20 mM phenanthroline (PTL, dissolved in ethanol, 2% final concentration), 20 mM 8-hydroxyquinoline-5-sulfonic acid (HSQA) and 20 mM EDTA. Solvent control contained 2% ethanol (final concentration). All samples were analyzed by SDS–PAGE and immunoblotting for HPC-1/syntaxin and for synaptophysin as control for recovery (2.5 μg of protein/lane).

were generated in the presence or absence of microsomes, demonstrating that HPC-1/syntaxin does not require microsomes for biosynthesis to reach completion. However, no cleavage product was observed upon BoNT/C1 incubations when microsomes were absent during translation. We then examined whether this difference is due to membrane binding itself or to an unknown post-translational modification caused by the microsomes. For this purpose, HPC-1/syntaxin was incorporated into artificially generated liposomes after translation in the absence of microsomes and then subjected to toxin treatment. As shown in Figure 6B (left lanes), BoNT/C1 treatment led to a reduction of the HPC-1/syntaxin band that was accompanied by a concomitant increase in the amount of the fragment. A virtually identical result was obtained when detergent extracts of synaptic membranes, containing endogenous HPC-1/syntaxin, were added before formation of the proteoliposomes (Figure 6B, right lanes).

BoNT/C1-LC shares the motif His-Glu-x-x-His-x-x-His, which appears to be involved in Zn^{2+} binding (see Introduction), with the other clostridial toxins. We therefore investigated whether a divalent metal cation is required as a cofactor for HPC-1/syntaxin breakdown. Synaptic membranes were incubated with BoNT/C1 in the presence of various chelators. As shown in Figure 7, all chelators partially protected HPC-1/syntaxin from BoNT/C1-induced proteolysis. These effects are similar to those observed previously with TeTx-LC and BoNT/B-LC (Link *et al.*, 1992; Schiavo *et al.*, 1992a), although a somewhat lower degree of protection, perhaps due to a higher affinity of the toxin for zinc, was observed for BoNT/C1. These data indicate that BoNT/C1 requires Zn^{2+} as a cofactor for proteolytic activity.

Discussion

The data presented here clearly demonstrate that BoNT/C1 selectively cleaves the synaptic membrane protein HPC-1/syntaxin. Although it cannot be excluded that additional, hitherto unknown proteins are affected, we believe that the block in neurotransmission observed after BoNT/C1 poisoning can be directly attributed to the degradation of this protein. Therefore, our results lend strong support to the view that HPC-1/syntaxin is directly involved in exocytotic membrane fusion.

Breakdown of HPC-1/syntaxin is not due to the action of a contaminating metalloendoprotease (a risk that is always present when working with proteins purified from bacteria), for the following reasons. (i) The action of BoNT/C1 is highly specific for HPC-1/syntaxin, whereas none of a large number of synaptic membrane proteins examined were affected, including synapsin I and synaptotagmin that are notoriously sensitive to proteolysis (Perin *et al.*, 1991; Valtorta *et al.*, 1992). (ii) HPC-1/syntaxin is cleaved in experiments using unreduced holotoxin on intact synaptosomes, while under the same conditions the protein is inaccessible to externally added proteases (C.Walch-Solimena and R.Jahn, unpublished observations). This demonstrates that the toxin must be internalized and reduced before cleaving HPC-1/syntaxin. (iii) *In vitro*, cleavage activity resides in the L-chain of the toxin, whereas unreduced holotoxin or H chain are inactive (J.Blasi, H.Niemann and R.Jahn, unpublished observations). Thus, BoNT/C1 behaves in a fashion similar to TeTx where only the reduced free L chain is proteolytically active (Link *et al.*, 1992; Schiavo *et al.*, 1992a).

We were unable to precisely determine the cleavage site since the C-terminally located fragment of HPC-1/syntaxin containing the transmembrane domain could not be detected, preventing isolation and sequencing. Our data suggest that the toxin cleaves at a single site, although it is possible that additional cuts occur C-terminally of the observed cleavage site. This site is localized close, but not directly adjacent, to the putative transmembrane domain since the translation products of the constructs lacking this domain still exhibited a higher apparent M_r than the fragments generated by the toxin.

In synaptosomes, BoNT/C1 caused a maximum of 60% reduction in HPC-1/syntaxin content. This value is similar to that of synaptobrevin breakdown after TeTx poisoning of synaptosomes. The lack of complete degradation is probably due to the fact that our preparation contains a certain amount of non-functional nerve terminals that are incapable of sequestering the toxins by endocytosis. However, several lines of evidence show that HPC-1/syntaxin is susceptible to cleavage by BoNT/C1 only under certain conditions. The degree of cleavage differed in different subcellular fractions obtained upon synaptosomal subfractionation (J.Blasi and R.Jahn, unpublished observations). This suggests that BoNT/C1 selectively interacts with a specific subcellular pool of HPC-1/syntaxin, an issue currently under investigation. It is possible that HPC-1/syntaxin undergoes homo- or hetero-oligomeric protein–protein interactions, resulting in complexes that block toxin-mediated proteolysis. In addition, cleavage appears to require that HPC-1/syntaxin is inserted into membranes since (i) detergent solubilization led to loss of cleavage (unpublished observations), (ii) no cleavage is observed when the transmembrane domain is lacking and (iii) cleavability can be restored by incorporating the protein into proteoliposomes following its generation by *in vitro* translation in the absence of microsomes. Furthermore, cleavage is generally less

efficient with recombinant protein, probably reflecting misfolding and/or aggregation during expression. Together, these observations indicate that substrate recognition and/or proteolysis by BoNT/C1 is dependent on complex structural features of the substrate. We propose that toxin action consists of two steps: first recognition and binding to the substrate complex, and second the proteolytic action that may require substrate binding for activation. If this is the case, the protease itself may be relatively non-specific, cleaving the closest available site in the complex once it is bound. Preliminary observations in our laboratories suggest that this may actually be the case, at least for some of the clostridial neurotoxins.

The precise function of HPC-1/syntaxin in exocytosis remains to be established. HPC-1/syntaxin is related to a protein discovered in mesenchymal cells (epimorphin; Hirai *et al.*, 1992) whose subcellular localization and membrane orientation are controversial (Hirai *et al.*, 1993; Pelham, 1993). In addition, it bears some similarity to yeast proteins with a similar structure which have been implicated to function in membrane traffic. The genes encoding these yeast proteins include: (i) SED5, a multicopy suppressor of defects in ERD2, the yeast HDEL receptor that is involved in retention of proteins in the endoplasmic reticulum, (ii) PEP12, a gene apparently involved in targeting proteins to vacuoles and, more significantly, (iii) SSO1 and SSO2 which act as multicopy suppressors of the secretion-deficient mutant sec1 [reviewed by Bennett and Scheller (1993)]. The biochemical interactions of these yeast gene products are not understood. As outlined in the Introduction, HPC-1/syntaxin has been invoked in several protein−protein interactions, including binding to Ca^{2+} channels, to synaptotagmin (Bennett *et al.*, 1992; Yoshida *et al.*, 1992) and to a complex consisting of synaptobrevin, SNAP-25 and the soluble factors NSF and SNAPs that are required for intracellular membrane fusion events (Söllner *et al.*, 1993). In our hands, HPC-1/syntaxin is tightly complexed with SNAP-25 in the presence or absence of ATP and cytosol (C.Walch-Solimena and R.Jahn, manuscript in preparation). The fact that both synaptobrevin and HPC-1/syntaxin are targeted by clostridial neurotoxins provides strong evidence for their direct involvement in exocytosis as part of a membrane fusion machine. These findings may provide a framework that explains how the individual clostridial neurotoxins, all probably derived from one ancestral gene, have evolved to interact with different proteins. During evolution, the substrate specificity of individual toxins may have diverged, while the proteolytic activity was retained, allowing them to attack other components of the fusion machine and thus blocking exocytosis.

Materials and methods

Materials

cDNA clones for syntaxin a and b and for synaptotagmin I were gifts of Drs M.K.Bennett, R.H.Scheller (Stanford) and T.C.Südhof (Dallas), respectively. BoNT/C1 holotoxin was a gift from Dr S.Kozaki (Osaka, Japan). Monoclonal antibodies directed against HPC-1/syntaxin and SV2 were kind gifts of Drs C.Barnstable (New Haven) and K.Buckley (Boston), respectively (Barnstable *et al.*, 1985; Buckley and Kelly, 1985). Monoclonal antibodies directed against synapsin I and SCAMP were generously provided by Drs P.Greengard (New York) and D.Castle and S.Brand (Charlottesville), respectively. Monoclonal antibodies directed against the following proteins were described previously: synaptophysin (Jahn *et al.*, 1985), synaptobrevin (Baumert *et al.*, 1989), synaptotagmin (Brose *et al.*, 1992), rab3A (Matteoli *et al.*, 1991).

The Hall strain of *Clostridium botulinum* type A was used for the production of BoNT/A holotoxin following the protocol of Sugii and Sakaguchi (1975). BoNT/C1 was isolated from strain 003-9 according to Kurazono *et al.* (1985).

Preparation and incubation of subcellular fractions

Synaptosomes were prepared as described previously by means of differential and Ficoll-gradient centrifugation (Fischer von Mollard *et al.*, 1991; McMahon *et al.*, 1992). In all experiments, synaptosomes were preincubated for 90 min at 37°C in the absence or presence of toxins before transmitter release was initiated by the addition of KCl (50 mM final concentration). Glutamate release was monitored using an on-line photometric assay (Nicholls and Sihra, 1986) and a dual-wavelength recording protocol (Fischer von Mollard *et al.*, 1991). At the end of the incubation, synaptosomes were centrifuged for 10 min at 12 000 *g*. All subsequent steps were at 4°C. Pellets resuspended in 0.3 ml incubation buffer were lysed by the addition of 2.7 ml H_2O, followed by rapid homogenization. To prepare a membrane fraction, mitochondria and unbroken synaptosomes were removed by centrifugation for 10 min at 12 000 *g*. The resulting supernatant was centrifuged at 200 000 *g* for 20 min in a Beckman TLA 100.3 rotor. The pellet was collected by resuspension and immediately processed for electrophoretic analysis. The supernatant was kept as synaptosomal cytosol containing soluble proteins. For experiments with isolated membranes, membranes were prepared directly from freshly isolated synaptosomes using the same procedure.

In vitro translation assays and preparation of proteoliposomes

Since the clones for syntaxin a and b lacked the first three and four amino acids, respectively, the 5′ ends were filled with appropriately designed polymerase chain reaction (PCR) primers that contained an *Eco*RI restriction site. Transcription was placed under the control of a T7 promoter by subcloning into CDM8 (Seed, 1987) that was modified to contain *Eco*RI sites in the polylinker (Chapman *et al.*, 1992). For transcription−translation, the TNT-system was used (Promega, Madison, WI). DNA (0.5 μg) was incubated with T7 polymerase in a reaction mixture of 25 μl containing [^{35}S]methionine following the instructions of the manufacturer, with microsomes added where indicated. For the translation of HPC-1/syntaxin isoforms lacking the putative transmembrane region, 3′ PCR primers were designed to omit the C-terminal 23 and 24 amino acids for syntaxin a and syntaxin b, respectively.

For incorporation of *in vitro* translated HPC-1/syntaxin into proteoliposomes, 5 μl of the translation mix were brought to 50 μl in 1% sodium cholate, 20 mM HEPES (pH 7.0), 100 mM NaCl. After incubation for 30 min on ice, the mix was centrifuged at 50 000 r.p.m. for 10 min in a Beckman TLA 100.3 rotor to remove insoluble particles, 200 μl of the same buffer were added to the supernatant. When indicated, an identical volume of an extract of synaptic membranes (50 μg of protein, extracted in the same buffer) was added instead. The mix was then added to 1 mg dried phospholipids (65% phosphatidylcholine, 35% phosphatidylserine; obtained from Avanti, Alabaster, AL), followed by resuspension by means of vortexing and sonication until the solution appeared almost clear. Liposomes were then formed by dilution of the mix into 20 ml (35 ml in some experiments) of 20 mM HEPES (pH 7.0), 100 mM NaCl and incubated at room temperature for 20 min. Liposomes were then collected by centrifugation for 1.5 h at 60 000 r.p.m. in a Beckman Ti70 rotor.

Other methods

SDS−PAGE was performed according to standard procedures (Laemmli, 1970) using the Bio-Rad Protean II minigel system. Where indicated, a modification of SDS−PAGE was used that is based on replacing Tris−Cl with Tricine−Cl which allows the separation of small peptides (Schägger and von Jagow, 1987). Immunoblotting and visualization with [^{125}I]protein A was performed as described previously (Jahn *et al.*, 1985). For quantitation, the bands on the autoradiograms were integrated with a Bioimage Visage 2000 system linked to two Sun Sparc workstations using the linear range of standard curves. Protein concentrations were determined according to Bradford (1976).

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