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Botulinum Neurotoxin Serotype F Is a Zinc Endopeptidase Specific for VAMP/Synaptobrevin*

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Botulinum neurotoxin serotype F contains the zinc binding motif of zinc endopeptidases. Atomic adsorption analysis of highly purified toxin preparation revealed the presence of one atom of zinc per molecule of toxin, which could be removed with EDTA or *o*-phenanthroline.

The light chain of the neurotoxin was shown to have a zinc-dependent protease activity specific for VAMP/synaptobrevin, an integral membrane protein of synaptic vesicles. Both isoforms of rat VAMP were cleaved at the same site corresponding to the single Gln-Lys peptide bond present in their sequences. This proteolytic activity was inhibited by EDTA, *o*-phenanthroline, and captopril as well as by VAMP peptides spanning the cleavage site.

Botulinum neurotoxins (BoNT)[1] cause the flaccid paralysis of botulism by blocking acetylcholine release at the neuromuscular junction. BoNT are the most potent toxins known (mouse LD_{50} <0.1 ng/kg) and are produced by bacteria of the genus *Clostridium* in seven different serotypes: A, B, C, D, E, F, and G. BoNT are released in an inactive form as a single chain toxin that is activated by proteolytic cleavage at an exposed loop to generate two disulfide-linked chains, as shown in Fig. 1. The heavy chain (*H*, 100 kDa) is involved in neurospecific binding and membrane translocation, while the light chain (*L*, 50 kDa) enters the cytosol and blocks neuroexocytosis (1–4).

Recently we found that BoNT/A, BoNT/B, and BoNT/E, as well as tetanus neurotoxin (TeTx), contain one atom of zinc bound to the L chain and coordinated via two histidine residues of a highly conserved zinc binding motif of zinc endopeptidases (5, 6). For TeTx and BoNT/B it was also possible to demonstrate that the blockade of neuroexocytosis occurred via their zinc-dependent proteolytic cleavage of rat VAMP/synaptobrevin isoform 2 (VAMP-2) (7).[2]

The cDNA-derived sequence of BoNT/F (8) shows that the zinc binding motif is also present in this toxin serotype (Fig. 1). Here we report that BoNT/F is a zinc endopeptidase that cleaves both isoforms of rat VAMP at a single peptide bond, different from the one hydrolyzed by TeTx and BoNT/B.

MATERIALS AND METHODS

Preparation of Toxins, Synaptic Vesicles, and Other Materials—BoNT/F and BoNT/C were purified as described before (9). BoNT/A, BoNT/B, and BoNT/E were a kind gift of Dr. B. R. DasGupta (University of Wisconsin). TeTx was prepared as described (10). All toxins were further purified by immobilized metal ion affinity chromatography to remove traces of contaminant protease activity as described before (11). Captopril ((2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline) was from Squibb (Italy). Peptides DQKL and VLERDQKLSELD were synthesized using an automated, solid-phase peptide synthesizer (model 431A, Applied Biosystems Inc.) with Applied Biosystems FastMoc™ chemistry. Peptides were purified by reverse phase chromatography on a C8 Ultra-Sphere preparative column (Beckman).

Small synaptic vesicles were isolated from rat cerebral cortex omitting the glass bead chromatography step (12) and used immediately.

Determination of Metal Content—The content of zinc, nickel, iron, copper, cobalt, and manganese was measured, after dialysis of the toxin in metal-free buffers as previously described (6), by atomic adsorption with a Perkin-Elmer 4000 atomic adsorption flame spectrophotometer with impact bed loading. Zinc was removed by 3 × 8 h of dialysis at 4 °C against 20 mM HEPES-Na, 150 mM NaCl, 2 mM *o*-phenanthroline (OP), pH 7.4, and subsequent extensive dialysis at the same temperature with the same buffer without OP. Zinc reuptake by apo-BoNT/F was performed in 100 μM zinc sulfate, 20 mM HEPES-Na, 150 mM NaCl, pH 7.4; after 24 h at 4 °C, samples were extensively dialyzed against the same buffer without zinc, and metal content was determined as above.

Proteolytic Activity of BoNT/F—30 μg of SSV in 30 μl of 5 mM HEPES-Na, 0.3 M glycine, 0.3 M NaCl, 0.02% NaN_3 were treated for 60 min at 37 °C with 80 ng of native BoNT/F or BoNT/F previously reduced with 10 mM DTT for 30 min at 37 °C. In some samples reduced toxin was preincubated for 30 min at 37 °C with different effectors (captopril, 1 mM; *o*-phenanthroline, 1 mM; EDTA, 1 mM; peptides DQKL and VLERDQKLSELD, 0.7 mM). Some samples (30 μl) were diluted with 5 mM HEPES-Na, 10 mM EDTA to a final volume of 100 μl and centrifuged on 10 mM HEPES-Na pH 7.4, 120 mM NaCl for 60 min at 350.000 × *g* in a TL-100 centrifuge (Beckman). Supernatants were dialyzed extensively against 10 mM HEPES-Na pH 7.4 with Spectra/Por dialysis tubes (Spectrum, Houston; 500 daltons cut-off), lyophilized, and then dissolved in 8% SDS, 10 mM Tris-Ac, pH 8.2, 0.1 mM EDTA. Pellets were solubilized in the same buffer and boiled for 2 min. Samples were applied on 13–18% polyacrylamide gradient gel (7) and stained with Coomassie Blue or with silver staining.

Time course of VAMP proteolysis and production of 8-kDa fragment were determined on Coomassie Blue stained gel by scanning with a dual-wavelength Shimadzu CS-630 densitometer.

For sequence analysis 8-kDa fragments were electroeluted from polyacrylamide gels, applied to ProSpin tubes, and sequenced in a pulsed liquid Applied Biosystems model 477A protein sequenator. Amino acid analysis was performed by using the Pico-Tag amino acid analysis system (Waters).

RESULTS AND DISCUSSION

Fig. 1 shows that BoNT/F, like the other botulinum neurotoxins, contains the zinc binding motif of zinc endopeptidases (4–6, 8, 13). Measurement of the heavy metals content of highly purified preparations of BoNT/F by atomic adsorption gave an average content of 1.0 ± 0.1 atoms of zinc per toxin molecule, while nickel, cobalt, iron, copper, and manganese were below detection.

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[1] The abbreviations used are: BoNT, botulinum neurotoxins; TeTx, tetanus neurotoxin; SSV, small synaptic vesicles; VAMP-1, VAMP/synaptobrevin isoform 1; VAMP-2, VAMP/synaptobrevin isoform 2; OP, *o*-phenanthroline; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

[2] B. Poulain, O. Rossetto, F. Deloye, G. Schiavo, L. Tauc, and C. Montecucco, submitted for publication.

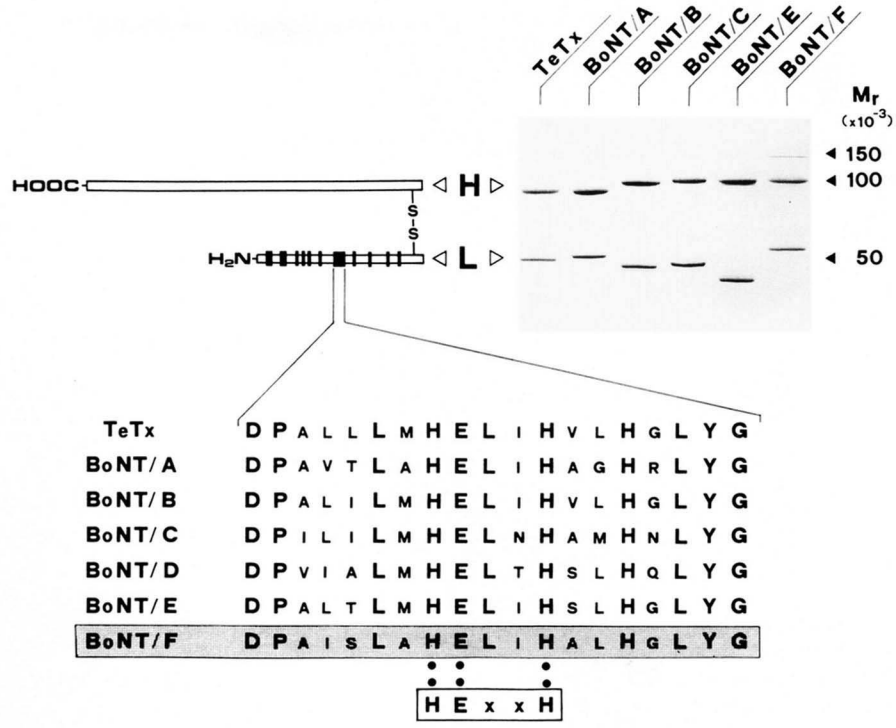
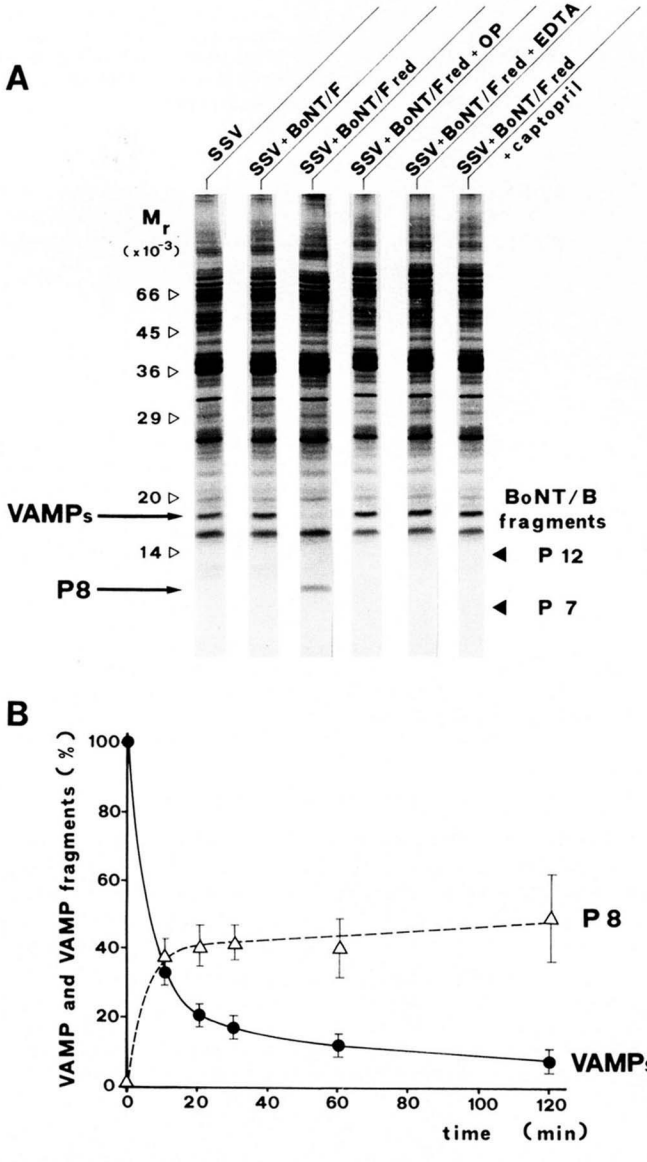


FIG. 1. **Schematic structure, electrophoretic profile, and sequence similarity of clostridial neurotoxins.** The *upper panel* shows the Coomassie Blue-stained profile of the indicated clostridial neurotoxins, highly purified as described in Ref. 11, after SDS-PAGE. *H* refers to the heavy chain, *L* to the light chain, and the *thick boxes* in the scheme of the L chain indicate position and size of the segments of similar sequence among the seven clostridial neurotoxins, whose sequence is available (4, 8). The *lower panel* reports the sequence of the highly similar segment, located in the central part of the L chain, which contains the HE*XX*H zinc binding motif of zinc endopeptidases (13).



As previously found for BoNT/A, BoNT/B, and BoNT/E (6), the atom of zinc of BoNT serotype F could be removed upon incubation with EDTA or OP, thus generating an apo-BoNT/F, and could be regained by incubation in zinc-containing buffers (not shown).

Recently it was found that TeTx and BoNT/B are zinc endopeptidases specific for VAMP (7). VAMP is a small integral protein of synaptic vesicles (14) first identified in the *Torpedo* electric organ (15) and later cloned from rat, bovine, human, *Drosophila*, and yeast (16–20). Rat brain VAMP consists of two isoforms, termed VAMP-1 and VAMP-2, of 118 and 116 amino acids, respectively (16). VAMP is present in neuronal and neuroendocrine tissues as well as in adipocytes (21, 22). VAMP is anchored to vesicles by a COOH-terminal hydrophobic tail, while the bulk of the molecule, including the NH_2 terminus, is exposed to the cytosol. Both TeTx and BoNT/B hydrolyze specifically the peptide bond between Gln-76 and Phe-77 of rat VAMP-2. Rat VAMP-1 is toxin-resistant, most likely because of a Val for Gln replacement at the cleavage site. No other substrate was found in other membrane or soluble fractions of the nervous tissue (7).

We assayed BoNT/F for a protease activity with several synthetic peptides and subcellular preparations and found that it cleaved specifically VAMP. Fig. 2*A* shows that BoNT/F, incubated with SSV isolated from the rat cerebral cortex, caused the disappearance of VAMP and the appearance of an 8-kDa protein band. No smaller peptides could be detected even in

FIG. 2. **Zinc-dependent proteolytic activity of botulinum neurotoxin serotype F.** *A*, Coomassie Blue-stained SDS-PAGE profile of SSV alone or after incubation with unreduced BoNT/F (*+BoNT/F*), DTT-reduced BoNT/F (*+BoNT/F red*), DTT-reduced BoNT/F in the presence of 1 mM OP (*+OP*), DTT-reduced BoNT/F in the presence of 1 mM EDTA (*+EDTA*), or DTT-reduced BoNT/F in the presence of 1 mM captopril (*+captopril*). The position of VAMP and of the BoNT/F induced fragments (*P8*) are indicated by *arrows*. *Triangles* on the *right-hand side* show the positions of fragments generated by BoNT/B or TeTx. *B*, time course of the proteolysis of VAMPs by BoNT/F and of the parallel appearance of 8-kDa fragments. For unknown reasons, the amount of Coomassie Blue staining of the 8-kDa fragment does not correspond to the actual amount of protein in the same way as for the VAMP band. This is not the case with silver staining, not shown here because of the difficulties in obtaining quantitative densitometric scanning.

highly cross-linked polyacrylamide gels. BoNT/F displayed its proteolytic activity only after reduction, as in the case of all bacterial protein toxins with intracellular targets (23). Its activity was blocked by EDTA and OP, which are both well characterized general inhibitors of zinc proteases.

A very relevant finding is the inhibition of BoNT/F proteolytic activity by captopril, a widely used anti-hypertensive agent, which blocks specifically the angiotensin-converting enzyme, a well characterized zinc endopeptidase (24, 25). This result opens the possibility of finding specific inhibitors of BoNT/F, which may be evaluated for the treatment of botulism.

Fig. 2*B* shows that rat VAMP was completely degraded, with the parallel appearance of an 8-kDa protein band, suggesting that both isoforms of rat VAMP are proteolyzed. These results indicate that BoNT/F is a zinc endopeptidase, which cleaves VAMP at a single site different from the peptide bond hydrolyzed by TeTx and BoNT/B, which convert VAMP into two fragments of 12 and 7 kDa (7). The BoNT/F cleavage site is common to VAMP-1 and VAMP-2, and it appears to be such that two fragments of overlapping electrophoretic mobility are generated.

To separate and isolate the two fragments, after incubation with the BoNT/F, SSV were diluted, loaded on a saline solution, and ultracentrifuged. Supernatant and pellet fractions were electrophoresed. Fig. 3 shows that both the supernatant and the pellet contain a 8-kDa band as expected if BoNT/F proteolysis generates both an 8-kDa NH_2-terminal VAMP fragment (released in the medium) and a COOH-terminal VAMP fragment (attached to the vesicles via its hydrophobic trans-membrane segment). The two 8-kDa protein bands were electroeluted and analyzed for amino acid content and sequence. Amino acid analysis showed that prolines are present only in the supernatant fragment, while isoleucine residues are highly enriched in the pellet fragment. This amino acid distribution among the two 8-kDa fragments indicates that the supernatant portion is the NH_2-terminal one, while the pellet one contains the COOH terminus.

Amino acid sequencing gave no sequence for the supernatant VAMP fragment, as expected from the fact that its NH_2 terminus is blocked (7). At variance, sequencing of the pellet fragment gave the sequence KLSELDDRADALQAGASQ(V)FES-(T)SAA (reported in larger letter size in Fig. 3), which corresponds to both rat VAMP-1 and -2 starting from Lys-61 of VAMP-1 and Lys-59 of VAMP-2. The cleavage of both rat VAMP isoforms, already suggested by the data of Fig. 2*B*, is demonstrated by the finding that at the 18th step of sequencing both Gln and Val are found and that at the 21st step both Ser and Thr are released. Neuronal rat VAMP-1 and -2 differ for these residues at these points of their sequences.

Here we have provided evidence that BoNT/F is a zinc endopeptidase specific for neuronal VAMP. VAMP is cleaved at a unique Gln-Lys peptide bond present in both rat VAMP isoforms. This is at variance from TeTx and BoNT/B, which hydrolyze a Gln-Phe peptide bond present only in rat VAMP-2. Hence the zinc-dependent protease activity of these clostridial neurotoxins appears to be very specific in terms of peptide bond. In this sense it is worth noting that the VAMP sequence shows the presence of the segment DKVLERD highly similar to the one recognized by BoNT/F, QKLSELD. Nonetheless, only the Q–K bond was cleaved, while the D–K bond was not hydrolyzed. Moreover only peptides spanning the cleavage site, such as DQKL and VLERDQKLSELD, were found to inhibit the VAMP-specific proteolysis of BoNT/F (not shown). A common feature of the peptide bond proteolyzed by TeTx, BoNT/B, and BoNT/F appears to be the presence of a Gln residue at the P_1 position. When the target specificity of the remaining BoNT serotypes is established, it will be seen if this is a general feature of clostridial neurotoxins protease activity.

The present results provide a molecular understanding of the botulism caused by serotype F of botulinum neurotoxin, which has been recently used as a therapeutic agent in the treatment of torticollis (26). At the same time it provides a well defined tool for the study of the yet unknown cellular role(s) of VAMP.

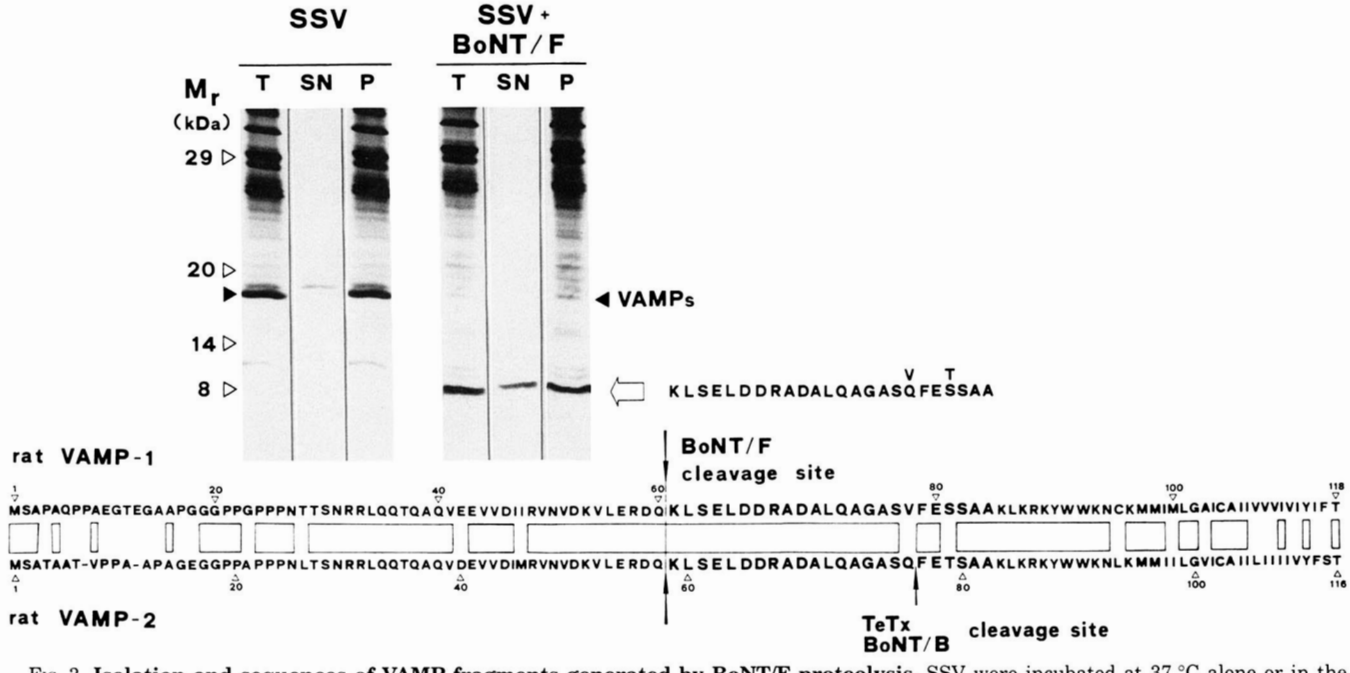


FIG. 3. **Isolation and sequences of VAMP fragments generated by BoNT/F proteolysis.** SSV were incubated at 37 °C alone or in the presence of BoNT/F. Half-sample was blocked with SDS-electrophoresis buffer (*T*), while the other half was ultracentrifuged. Pellet (*P*) and supernatant (*SN*) fractions were electrophoresed, and the BoNT/F-induced 8-kDa VAMP fragments were electroeluted and analyzed for amino acid compositions and sequences. The supernatant fragment gave no sequence, while the pellet fragment gave the sequence reported in *larger letters*. VAMP-1 and -2 sequences are reported in the *lower panel* for comparison, together with the deduced cleavage sites of BoNT/F (this work) and TeTx and BoNT/B (7).

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