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Cleavage of Members of the Synaptobrevin/VAMP Family by Types D and F Botulinal Neurotoxins and Tetanus Toxin*

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Tetanus toxin (TeTx) and the various forms of botulinal neurotoxins (BoNT/A to BoNT/G) potently inhibit neurotransmission by means of their L chains which selectively proteolyze synaptic proteins such as synaptobrevin (TeTx, BoNT/B, BoNT/F), SNAP-25 (BoNT/A), and syntaxin (BoNT/C1). Here we show that BoNT/D cleaves rat synaptobrevin 1 and 2 in toxified synaptosomes and in isolated vesicles. In contrast, synaptobrevin 1, as generated by *in vitro* translation, is only a poor substrate for BoNT/D, whereas this species is cleaved by BoNT/F with similar potency. Cleavage by BoNT/D occurs at the peptide bond Lys^{59}-Leu^{60} which is adjacent to the BoNT/F cleavage site (Gln^{58}-Lys^{59}) and again differs from the site hydrolyzed by TeTx and BoNT/B (Gln^{76}-Phe^{77}). Cellubrevin, a recently discovered isoform expressed outside the nervous system, is efficiently cleaved by all three toxins examined. For further characterization of the substrate requirements of BoNT/D, we tested amino- and carboxyl-terminal deletion mutants of synaptobrevin 2 as well as synthetic peptides. Shorter peptides containing up to 15 amino acids on either side of the cleavage site were not cleaved, and a peptide extending from Arg^{47} to Thr^{116} was a poor substrate for all three toxins tested. However, cleavability was restored when the peptide is further extended at the NH_2 terminus (Thr^{27}-Thr^{116}) demonstrating that NH_2 terminally located sequences of synaptobrevin which are distal from the respective cleavage sites are required for proteolysis. To further examine the isoform specificity, several mutants of rat synaptobrevin 2 were generated in which individual amino acids were replaced with those found in rat synaptobrevin 1. We show that a Met^{46} to Ile^{46} substitution drastically diminishes cleavability by BoNT/D and that the presence of Val^{76} instead of Gln^{76} dictates the reduced cleavability of synaptobrevin isoforms by TeTx.

The anaerobic bacteria *Clostridium tetani* and *Clostridium botulinum* produce several structurally related neurotoxins which are known to be potent inhibitors of the exocytotic release of neurotransmitters from synaptic vesicles at nerve terminals. The neurotoxin molecules are synthesized as single chain polypeptides which are proteolytically activated to generate di-chain toxins in which a heavy (H) chain (M_r 100,000) remains attached to a light (L) chain (M_r 50,000) by a single disulfide bond. It is generally accepted that the H chains control neuroselective binding, internalization of the entire toxin moiety, intraneuronal sorting, and, finally, translocation of the L chains into the cytosol (Niemann, 1991).

Seven serologically distinct botulinal neurotoxins (BoNT/A, B, C1, D, E, F, G) are known which all act (due to the specific properties of their H chains) on peripheral motoneurons in which they cause a blockade of acetylcholine release and thus produce the clinical manifestation of botulism. In contrast, tetanus toxin (TeTx)[1] undergoes retrograde axonal transport to the central nervous system where it blocks the release of inhibitory neurotransmitters resulting in the clinical manifestation of tetanus.

Despite these differences, it is known that the L chains of all clostridial neurotoxins are the active components that block exocytosis as soon as they are released into the cytoplasm. Here, the L chains exert their detrimental function as zinc-dependent proteases: TeTx, BoNT/B, and BoNT/F cause selective degradation of synaptobrevin 2 (Schiavo *et al.*, 1992; Link *et al.*, 1992; Schiavo *et al.*, 1993), BoNT/A and BoNT/E selectively proteolyze the synaptosome-associated protein SNAP-25 (Blasi *et al.*, 1993b; Binz *et al.*, 1994), and BoNT/C1 causes a selective breakdown of syntaxin/HPC-1 (Blasi *et al.*, 1993a). Synaptobrevin (also termed VAMP for vesicle-associated membrane protein, Trimble *et al.*, 1988) exists in two isoforms in the nervous tissue both of which are cytoplasmatically oriented integral membrane proteins that are anchored in the vesicle membrane by a single carboxyl-terminal transmembrane domain. Synaptobrevins are highly conserved in evolution from invertebrates to mammals suggesting a common function (Archer *et al.*, 1990; Südhof *et al.*, 1989; Südhof and Jahn, 1991; Jahn and Südhof, 1993). Furthermore, cellubrevin, a synaptobrevin isoform detected in vesicles of all non-neuronal cells examined, is also cleaved by TeTx suggesting that it could play a role in docking and fusion of these vesicles to target membranes (McMahon *et al.*, 1993). This view is further supported

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[1] The abbreviations used are: TeTx, tetanus toxin; BoNT/A to G, botulinal neurotoxin A to G; VAMP-1, VAMP-2, vesicle-associated membrane proteins 1 and 2 (acronyms for synaptobrevin 1 and 2); SNAP-25, synaptosome-associated protein of 25 kDa; NSF, *N*-ethylmaleimide-sensitive factors; SNAPs, soluble NSF attachment proteins; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-[*N*-morpholino]propane sulfonic acid.

by the detection of several syntaxin isoforms with broad tissue distribution and distinct intracellular transport properties (Bennett *et al.*, 1993). Recent evidence suggests that the three substrates of clostridial neurotoxins indeed form the core of a multiprotein complex which, composed of different subsets of isoforms of synaptobrevin, syntaxin, and SNAP-25, mediates fusion of carrier vesicles to target membranes in all eukaryotic cells. This protein complex contains, in addition, cytoplasmic proteins including NSF and α-, β-, γ-SNAPs (Söllner *et al.*, 1993a, 1993b) and may be further controlled by additional proteins such as Munc-18 (Hata *et al.*, 1993), synaptotagmin, and neurexins. According to a current concept (Südhof *et al.*, 1993), structurally distinct isoforms of synaptobrevin, syntaxin, and SNAP-25 dictate the intracellular targeting, whereas both NSF and SNAPs, factors that were originally identified by their ability to restore activity to a mammalian intra-Golgi transport assay system (for review see Rothman and Orci, 1992), appear to act in all intracellular fusion events.

In this study, we show that BoNT/D selectively proteolyzes synaptobrevin 2 but at a site different from that cleaved by either TeTx and BoNT/B or BoNT/F, respectively. In contrast, the synaptobrevin 1 isoform is cleaved *in vitro* only at high BoNT/D concentrations. In addition, we have prepared modified synthetic substrates (synthetic peptides and recombinant proteins) in order to characterize the substrate requirements for BoNT/D in comparison to those of TeTx and BoNT/F. Our results show that despite the high specificity of the respective toxins for synaptobrevin, subtle differences exist. Furthermore, sequences that are located farther away at the NH_2-terminal side of the respective cleavage sites may determine isoform cleavability indicating complex structural requirements for toxin action.

EXPERIMENTAL PROCEDURES

Plasmids—Clones encoding rat VAMP-1 and VAMP-2 (Elferink *et al.*, 1989) were kindly provided by Dr. Richard H. Scheller (Stanford, CA). The generation of a cDNA clone encoding rat cellubrevin was described previously (McMahon *et al.*, 1993). For generation of 5′ deletion mutants of synaptobrevin 2 by polymerase chain reaction the following primers were used: T27T116, 5′-CACACTCGAGATGACTAGTAACAGGAGACTGCAG-3′; A37T116, 5′-CACACTCGAGATGGCCCAGGTGGATGAGGTGGTG-3′; R47T116, 5′-CACACTCGAGATGAGGGTGAACGTAGACAAGGTC-3′; L54T116, 5′-CACACTCGAGATGTTGGAGCGGGACCAGAAGCTG-3′. As downstream primer we used 5′-ATCGATACGCGTGGTACCAGATCCG-3′. Amplified fragments were cleaved with *Xho*I and *Kpn*I and used to replace the corresponding fragment in the synaptobrevin 2 clone. Point mutations of synaptobrevin 2 were generated by polymerase chain reaction using suitable primers. All mutants were confirmed by resequencing of the entire coding region.

Antibodies—Monoclonal antibodies against syntaxin/HPC-1 and SV2 were the kind gifts of Drs. C. Barnstable and K. Buckley, respectively (Barnstable *et al.*, 1985; Buckley and Kelly, 1985). Monoclonal antibody directed against synapsin I was kindly provided by Dr. P. Greengard. Monoclonal antibodies against other proteins were described previously: synaptophysin (Jahn *et al.*, 1985), synaptobrevin (Baumert *et al.*, 1989), synaptotagmin (Brose *et al.*, 1992), and rab3A (Matteoli *et al.*, 1991).

Clostridial Neurotoxins—BoNT/D holotoxin was isolated from strain 1873 according to Miyazaki *et al.* (1977). BoNT/F was isolated from *C. botulinum* type F following the procedures of Ohishi and Sakaguchi (1974). The Hall strain of *C. botulinum* type A was used for production of BoNT/A holotoxin following the protocol of Sugii and Sakaguchi (1975). L chains were produced from the individual holotoxins following the protocols by Sathyamoorthy and DasGupta (1985). To express a recombinant TeTx L chain, we made use of a resynthesized gene (Eisel *et al.* (1993)) encoding the entire L chain and a COOH-terminal His_6 tag. The gene was expressed in *Escherichia coli* M15 and purified by binding to N^5-nitrilotriacetic acid agarose according to the protocols of the manufacturer (DIAGEN, Düsseldorf, Federal Republic of Germany). L chains were concentrated by ultrafiltration using Amicon Filtron omega NMWL 30K ultrafilters retaining material with molecular masses larger than 30 kDa. Protein concentrations were determined according to Bradford (1976). Approximately 6 mg of L chain were obtained from 1 liter of culture fluid.

Preparation and Incubation of Subcellular Fractions—Synaptosomes were prepared as described previously by means of differential and Ficoll-gradient centrifugation (McMahon *et al.*, 1992). In all experiments, synaptosomes were preincubated for 90 min at 37 °C in the absence or presence of holotoxin (150 nM) 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 wave-length 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 of incubation buffer were lysed by addition of 2.7 ml of H_2O, followed by rapid homogenization. A first membrane fraction, LPI, was recovered from lysed synaptosomes by centrifugation for 10 min at 12,000 × *g*. The resulting supernatant was centrifuged for 20 min at 200,000 × *g* in a Beckman TLA 100.3 rotor. The pellet, LPII, was resuspended and immediately analyzed by SDS-PAGE and immunoblotting (Jahn *et al.*, 1985).

Synaptic vesicles were prepared according to Hell *et al.* (1988). Vesicles (90 μg) were suspended in 55 μl of 10 mM MOPS buffer (MOPS/KOH, pH 7.3, containing 320 mM sucrose) and incubated for 60 min at 37 °C with BoNT/D (15 nM) or BoNT/F L chain (30 nM). Samples were processed by electrophoresis and immunoblotting using the ECL Western blotting detection system (Amersham Buchler, Braunschweig, F.R.G.).

Proteolysis of in Vitro Translated Synaptobrevin Homologues—Plasmids encoding synaptobrevin homologues were linearized downstream from the coding regions, and mRNA was synthesized according to the manufacturer's protocols. Translations were performed in reticulocyte lysate (Promega). To demonstrate membrane integration, previous protocols were applied (Mayer *et al.*, 1988). Membrane fractions were recovered from diluted translation mixtures (addition of 40 μl of 50 mM Tris-HCl, 100 mM NaCl, pH 7.5, to 25 μl of translation mix) by sedimentation through sucrose cushions at pH 7.5, as detailed previously (Mayer *et al.*, 1988).

In vitro translated free or membrane-associated synaptobrevin was incubated for 1–16 h with various neurotoxin preparations. Material was separated on 15% SDS-PAGE according to Laemmli (1970), and bands were visualized by autoradiograhy. Where appropriate, gels were scanned with an LKB model 2202 ultroscan laser densitometer (Pharmacia, Freiburg, F.R.G.). In experiments with inhibitors, the individual L chain preparation was incubated for 30 min at 37 °C with the individual inhibitor prior to addition of membrane-associated substrates also containing the inhibitor. Dipicolinic acid, EDTA, *o*-phenanthroline, and captopril ([2S]-1-[3-mercapto-2 methyl-propionyl]-L-proline) were all from Sigma (München, F.R.G.).

Other Methods—Synthetic peptides were synthesized on a Milligen (Bedford, MA) model 9050 peptide synthesizer using Fmoc chemistry. The 93- and the 43-mer peptides were synthesized on a Applied Biosystems model 431A peptide synthesizer using Fmoc chemistry and HMP resin. The peptide was purified on a C4 Vydac 5-μm column (220 × 10 mm) applying a linear gradient of 10–90% of solvent B in 30 min (solvent A: H_2O, 0.1% trifluoroacetic acid; solvent B: acetonitrile, H_2O, trifluoroacetic acid, 70, 30, and 0.9%).

Cleavage products were isolated by reverse phase chromatography using a Nucleosil 5 μm C8 column (250 × 4 mm) from Macherey and Nagel (Düren, F.R.G.). Amino acid sequences were determined on a model 473A protein sequencer from Applied Biosystems (Foster City, CA).

RESULTS

BoNT/D-induced Inhibition of Neurotransmitter Release Correlates with Breakdown of Synaptobrevin—Nerve terminals (synaptosomes) from rat cerebral cortex were used as a test system to study the effects of BoNT/D. The preparation, as purified by means of differential and Ficoll density gradient centrifugation, responds to potassium-induced depolarization with a Ca^{2+}-dependent release of the excitatory neurotransmitter, glutamate, monitored on-line with a coupled enzymatic detection system. Fig. 1*A* shows that depolarization induces a rapid release of glutamate, reaching a plateau value after approximately 5 min. Release was clearly dependent on the presence of Ca^{2+} and, in agreement with previously published results (Nicholls and Sihra, 1986), no release was observed when

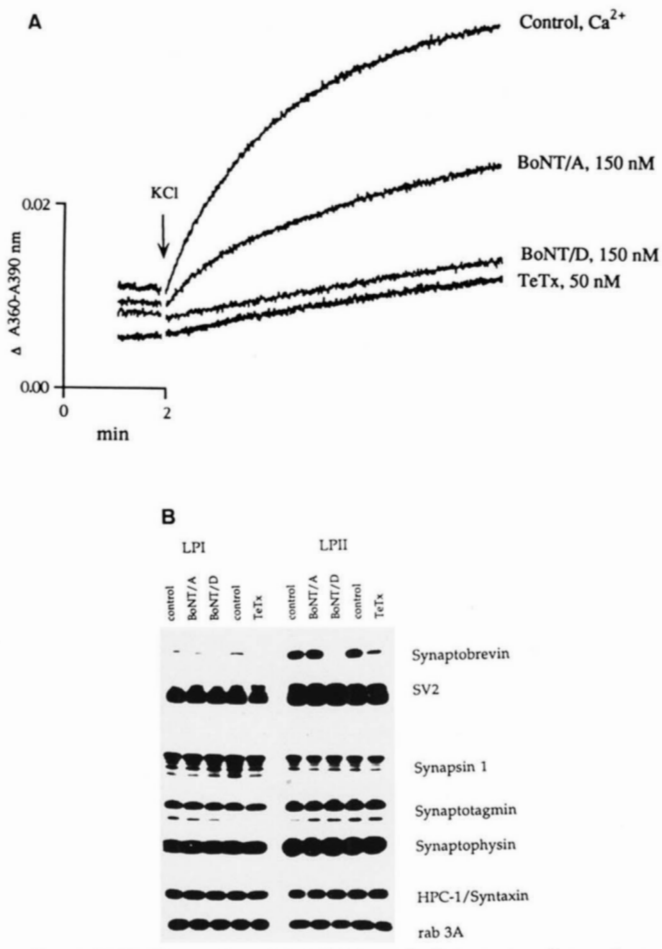


FIG. 1. **BoNT/D-mediated inhibition of glutamate release from isolated nerve terminals is associated with cleavage of synaptobrevin.** *A*, BoNT/D inhibits glutamate release from isolated nerve terminals. Rat brain synaptosomes (1.5 mg of protein/assay) were preincubated for 90 min in the absence or presence of BoNT/D, BoNT/A, or TeTx and then stimulated by the addition of 50 mM KCl (final concentration, *arrow*). Release was measured spectrophotometrically by following the conversion of glutamate by glutamate dehydrogenase. Traces are corrected for the base-line shifts associated with the addition of KCl. BoNT/D inhibits release as efficiently as TeTx whereas inhibition by BoNT/A is not complete. *B*, immunoblots of membrane fractions isolated from toxified synaptosomes. BoNT/D-toxified synaptosomes of *A* were lysed and subfractioned into a heavy membrane fraction, LPI, and a light membrane fraction, LPII, containing synaptic vesicles. Aliquots (5 μg of protein) were analyzed by SDS-PAGE and immunoblotting with monoclonal antibodies as indicated. As a control, untreated synaptosomes were analyzed in the same manner. Note the complete disappearance of synaptobrevin in BoNT/D-toxified membranes. In contrast, TeTx causes only a reduction in the synaptobrevin content, whereas BoNT/A does not affect synaptobrevin.

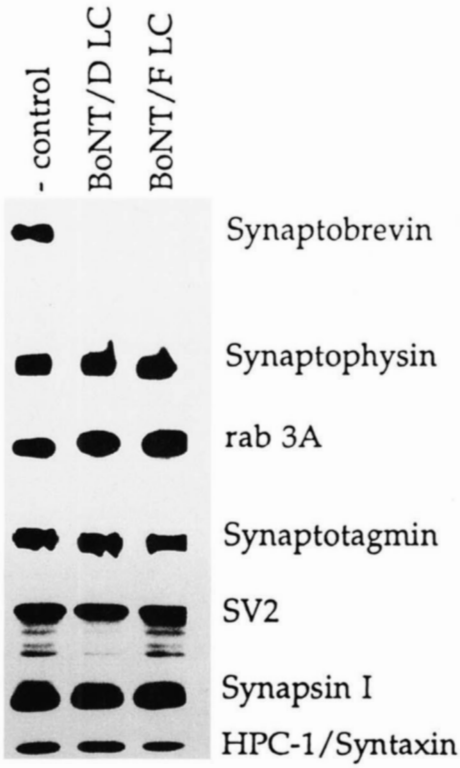


FIG. 2. **BoNT/D degrades synaptobrevin in isolated synaptic vesicles.** Rat brain synaptic vesicles (90 μg of protein) were treated for 60 min at 37 °C with 15 nM BoNT/D or 30 nM BoNT/F L chains (final concentration). The material was separated by SDS-PAGE on 15% gels, and Western blots were analyzed with monoclonal antibodies as indicated. Immunoreactive bands were visualized by the ECL method.

Ca^{2+} was omitted or EGTA was present (not shown). Preincubation of the preparation with 150 nM BoNT/D for 90 min at 37 °C resulted in a blockade of Ca^{2+}-dependent transmitter release. As controls we applied BoNT/A (150 nM) and TeTx (50 nM). Whereas BoNT/D and TeTx caused a complete depression of glutamate release (Fig. 1*A*), inhibition by BoNT/A was only partial. This observation is in agreement with our previous finding that this neurotoxin attacks a different intracellular target (Blasi *et al.*, 1993b).

Toxin-treated synaptosomes were then lysed and subfractioned into a heavy membrane fraction, LPI, that contained presynaptic membrane fragments and into a synaptic vesicle fraction, LPII. We then analyzed by immunoblotting the two membrane fractions for the synaptic vesicle proteins synaptobrevin, SV2, synapsin I, synaptotagmin, synaptophysin, rab3A, and for the synaptic membrane protein syntaxin/HPC-1. Synaptobrevin was selectively degraded in BoNT/D-treated material whereas all other proteins examined remained unaltered (Fig. 1*B*).

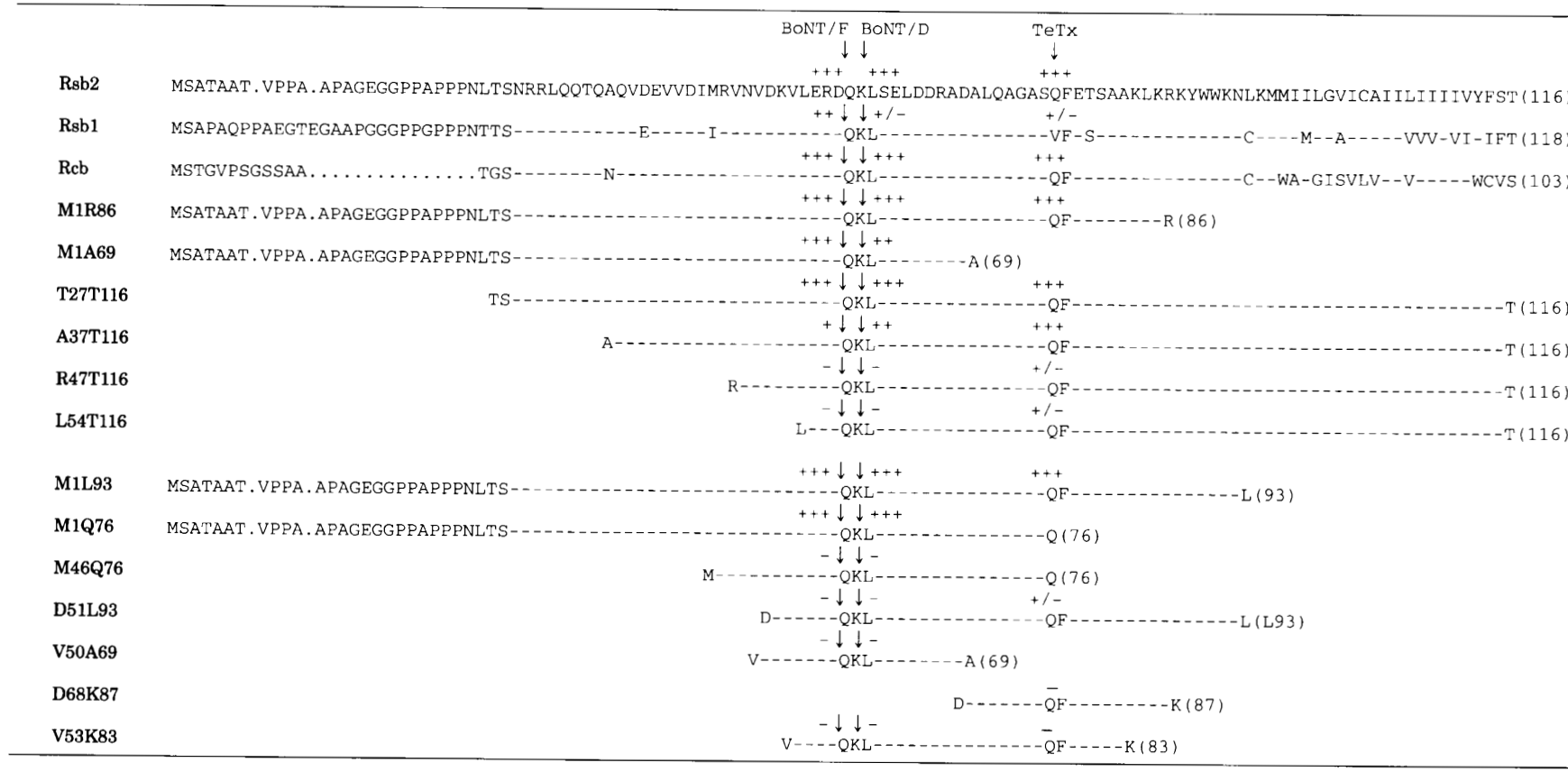
To examine whether BoNT/D-induced breakdown of synaptobrevin is due to a direct interaction between the toxin L chain and synaptic vesicles, we incubated synaptic vesicles with highly purified L chain of BoNT/D. As control, the L chain of BoNT/F was used which was recently shown to degrade both neuronal isoforms of synaptobrevin (Schiavo *et al.*, 1993). With both L chains synaptobrevin was degraded indicating that both synaptobrevin isoforms were degraded in synaptic vesicles (Fig. 2). In contrast, none of the other vesicle proteins examined were proteolyzed.

Determination of the BoNT/D-specific Cleavage Site in Rat Synaptobrevin 2—To identify the peptide bond of synaptobrevin 2 attacked by BoNT/D, the synthetic peptide M1L93 rather than the native protein was used as substrate. M1L93 represents the entire NH_2-terminal domain that up to Leu^{93} but lacks the COOH terminally located transmembrane domain (Table I). This peptide migrated as a single peak in reverse phase HPLC (Fig. 3, *panel A*). Incubation with BoNT/D L chain (5 ng of purified L chain/10 μg of peptide in 30 μl) resulted in the rapid formation of two smaller-sized peptides (*panel B*) which accumulated over a period of 2 h when cleavage was nearly complete. Microsequencing revealed that the material underlying peak fraction II corresponded to the NH_2-terminal fragment, whereas peak fraction I contained a new NH_2 terminus and sequencing over 12 steps yielded the sequence LSELDDRADALQ. For control, degradation by L chains of TeTx and BoNT/F was also analyzed followed by determination of the NH_2 termini of the cleavage products after separation by reverse-phase HPLC. In accordance with previously published data (Schiavo *et al.*, 1992, 1993), TeTx cut the Gln^{76}-Phe^{77} bond,

TABLE I

Minimal essential domains of rat synaptobrevin 2 required for cleavage by TeTx, BoNT/D, and BoNT/F

Rat synaptobrevin 2 (Rsb2), 1 (Rsb1), or cellubrevin (Rcb) were generated by *in vitro* transcription/translation and tested as membrane-associated substrates for cleavage by TeTx, BoNT/D, or BoNT/F, respectively. Substrates were at ~1 nM final concentration. With the exception of synaptobrevin 1 cleaved by BoNT/D, where the L chain was applied at 500 nM final concentration, L chain concentrations 10-fold higher than required for 50% cleavage (Table I) were used. After incubation for 1 h at 37 °C, cleavage was assessed by SDS-PAGE and autoradiography. In addition, deletion mutants M1R86 (prepared by *Bsg*I digestion), M1A69 (prepared by digestion with *Bgl*I), and mutants T27T116, A37T116, R47T116, and L54T116 were tested as *in vitro* translation products. M1L93, M1Q76, K52A67, D68R86, V53K83, and D51L93 were tested as synthetic peptide substrates at 20 μM final concentration applying 500 nM TeTx L chain or 50 nM of the type D and F L chains. Cleavage products were analyzed by HPLC or SDS-PAGE. +++, >75% cleavage; ++, about 50% cleavage; +, >25% cleavage; +/–, about 30% cleavage after 18 h; –, no breakdown products detectable after 18 h incubation at 37 °C.



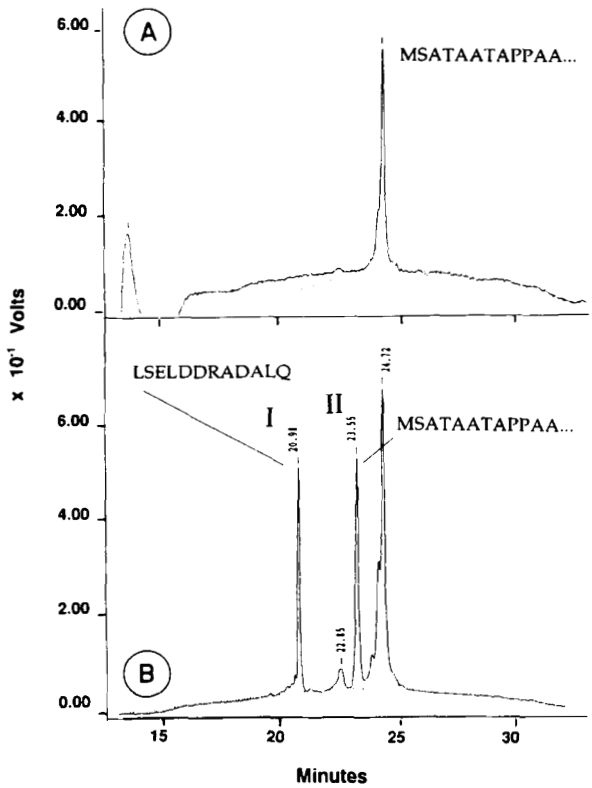


FIG. 3. **Determination of the cleavage site by microsequencing.** A synthetic 93-mer peptide encompassing residues Met^1 to Leu^{93} of rat synaptobrevin 2 was used as a substrate. Cleavage was analyzed by reverse-phase HPLC using a Nucleosil C8 column (250 × 4 mm) applying a linear gradient from 10 to 100% solvent B in 30 min (solvent A, 90% H_2O, 10% acetonitrile, 0.1% trifluoroacetic acid; solvent B: 90% acetonitrile, 10% H_2O, 0.1% trifluoroacetic acid). *Panel A*, 5 μg of untreated peptide control. *Panel B*, 10 μg of peptide were treated for 1 h at 37 °C in 20 mM HEPES, 100 mM NaCl, pH 7.0, containing 5 nM purified BoNT/D L chain. Peak fractions were collected and characterized by microsequencing.

whereas BoNT/F cleaved the Gln^{58}-Lys^{59} bond. Together, our results indicate that BoNT/D cleaves the peptide bond between Lys^{59} and Leu^{60} in rat synaptobrevin 2 which is different from the sites cleaved by TeTx and BoNT/F, respectively.

Comparison of the Substrate Specificity of BoNT/D with That of TeTx and BoNT/F—To employ an *in vitro* system that facilitates cleavage studies with the individual toxins and allows analyses of mutant substrates, synaptobrevin 2, synaptobrevin 1, and the non-neuronal isoform cellubrevin were generated as radiolabeled substrates by *in vitro* transcription/translation of the corresponding cDNAs. The substrates were incorporated into canine microsomal membranes and purified from the soluble material by sedimentation through a sucrose cushion. The resuspended membrane fractions were then incubated with the isolated toxin L chains. The toxin concentrations were adjusted to yield >50% cleavage (Fig. 7). After proteolysis the material was resedimented through a sucrose cushion allowing separate analysis of the released fragment in the resulting supernatant (S) and of the membrane-anchored fragment present in the pellet (P) fractions. Since synaptobrevin 1 lacks methionine residues in its NH_2-terminal domain, fragment analysis was restricted to the COOH-terminal, membrane-anchored fragments.

The results in Fig. 4 allow the following conclusions. First, the assay allows a quick comparison of the cleavage sites attacked by the individual toxins. Even hydrolysis at adjacent peptide bonds by BoNT/D and BoNT/F can be detected by the different electrophoretic migration of the released and membrane-anchored fragments (*panel A*). Second, despite differences in the susceptibilities, cleavage of the three substrates by a particular toxin affects peptide bonds in identical positions. Third, cellubrevin displays similar overall sensitivity to BoNT/D, BoNT/F, and TeTx, thus resembling in its substrate properties synaptobrevin 2 (compare *panels A* and *C*). Fourth, in agreement with the observation that synaptobrevin 1 is not cleaved by TeTx under physiological conditions (Fig. 1*B*; Schiavo *et al.*, 1992), a high concentration (5 μM) was required to obtain >90% cleavage of this isoform *in vitro* (*panel B*). Fifth, synaptobrevin 1 was remarkably resistant to treatment with BoNT/D, yielding about 50% breakdown at a toxin concentration of 500 nM. This contrasts with the situation *in vivo* (Fig. 1*B*) or with isolated synaptic vesicles (Fig. 2) where complete degradation of this isoform was achieved with 150 or 15 nM of BoNT/D, respectively. While it is possible that cleavability in synaptic vesicles is facilitated by an association of synaptobrevin 1 with other proteins, we cannot exclude post-translational modification that could induce a conformational change of synaptobrevin 1 that is more favorable for cleavage.

Using the above experimental approach and BoNT/D L chain concentrations that yielded 50% (0.15 nM) or 90% cleavage (1.5 nM; Fig. 7) of membrane-anchored rat synaptobrevin 2, respectively, we then studied the effects of various inhibitors of metalloproteases. Whereas 0.15 nM BoNT/D L chain was inactivated by dipicolinic acid (0.2 mM), captopril (2 mM), *o*-phenanthroline (20 mM), and EDTA (1 mM), only *o*-phenanthroline could inhibit the activity of 1.5 nM BoNT/D (data not shown). This finding indicates that the BoNT/D L chain indeed acts as a metalloprotease.

Delineation of the Minimal Essential Domains of Synaptobrevin 2 Required for Cleavage by BoNT/D, TeTx, and BoNT/F—Before obtaining additional insights into substrate and isoform specificity, we first wanted to define the minimal essential regions of the synaptobrevin 2 molecule that are recognized by BoNT/D, BoNT/F, and TeTx. The distal NH_2-terminal regions of rat cellubrevin (residues 1–14), synaptobrevin 1 (residues 1–29), and synaptobrevin 2 (residues 1–27) show considerable structural divergence, suggesting that this portion of the molecule should not influence cleavability. In addition, the 93-mer peptide M1L93 which lacked the membrane anchor region turned out to be an excellent substrate for each of the toxins tested here. Together, these data suggested to us that the high selectivity of BoNT/D and the other clostridial neurotoxins cleaving members of the synaptobrevin family should be dictated by the strongly conserved core portion of synaptobrevins (residues 28–93 in rat synaptobrevin 2). To dissect this region more specifically, we generated NH_2-terminal deletion mutants, designated T27T116, A37T116, R47T116, and L54T116. The individual mutants were applied as radiolabeled membrane-associated substrates and incubated with the individual L chains. The results are summarized in Fig. 5 and Table I.

No significant reduction in cleavability by BoNT/D and the two control L chains was observed with the T27T116-mutant. The A37T116-deletion mutant yielded about 50% breakdown with BoNT/D, whereas it was completely cleaved by TeTx and degraded to ~20% by BoNT/F. Proteolysis of the R47T116 mutant by BoNT/D was difficult to evaluate because the cleavage product and the uncleaved substrate migrated to the same position during electrophoresis. However, a synthetic peptide extending from Met^{46} to Gln^{76} showed no signs of degradation in HPLC even after 18 h of incubation with the BoNT/D L chain (Table I).

Similarly, after the 1-h incubation period no breakdown of the R47T116 mutant could be demonstrated with TeTx or BoNT/F, as evidenced by the persistence of radiolabel in the substrate before and after treatment and the absence of cleav-

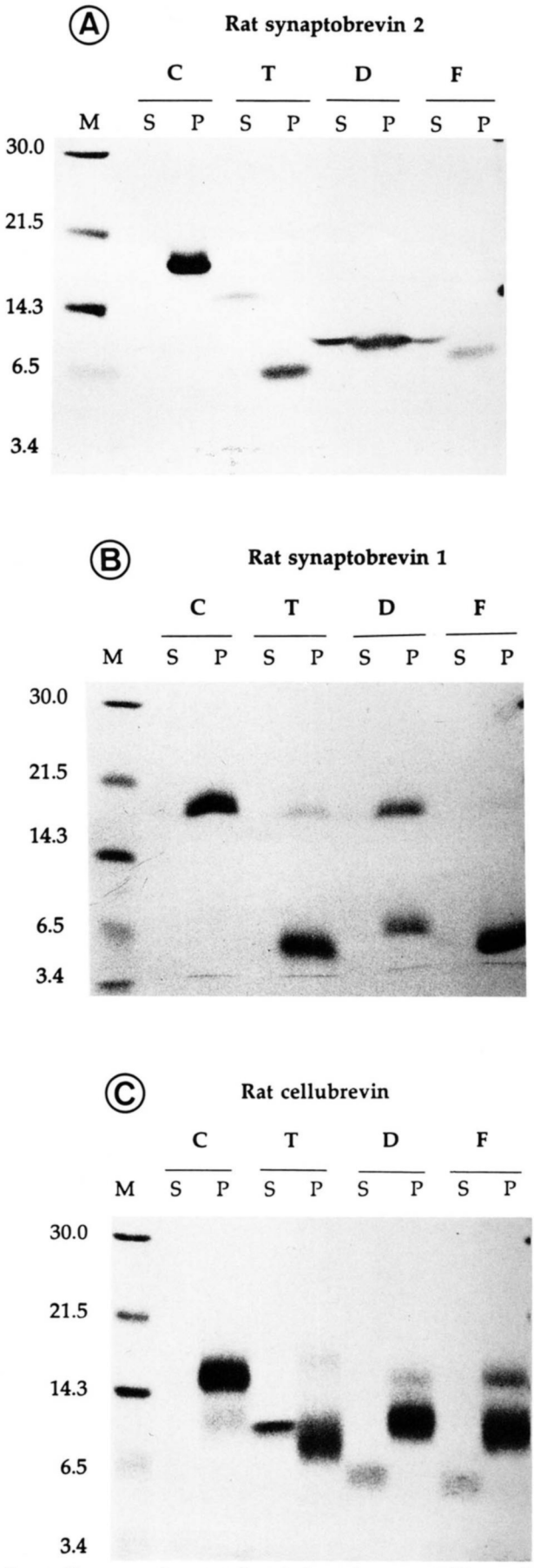


FIG. 4. **Cleavage of synaptobrevin homologues.** mRNA encoding rat synaptobrevin 2 (*panel A*), synaptobrevin 1 (*panel B*), or rat cellubrevin (*panel C*) was translated in the presence of microsomal membranes, and the membrane fractions were recovered by centrifugation through a sucrose cushion. After resuspension in 20 mM HEPES, 100 mM NaCl, pH 7.0, samples containing synaptobrevin 2 or cellubrevin were incubated for 60 min at 37 °C with the isolated L chains of TeTx (*T*, 50 nM), BoNT/D (*D*, 5 nM), or BoNT/F (*F*, 5 nM). For synaptobrevin 1, the L chain concentrations were 5 μM (TeTx), 500 nM (BoNT/D), 50 nM (BoNT/F). Samples were placed onto 100-μl sucrose cushions and centrifuged for 5 min at 50,000 revolutions/min in a Beckman TLA100 rotor. Released fragments in the supernatants were precipitated with 10% trichloroacetic acid using bovine serum albumin as a carrier. Supernatant (*S*) and pellet (*P*) fractions were analyzed by SDS-PAGE using 15% gels. *C*, untreated control.

age products. Similar observations were made with the 43-mer peptide D51L93. Again, this peptide was resistant against BoNT/D and BoNT/F and was only partially cleaved (28%) by TeTx after 18 h of incubation (Table I). Thus, this peptide was hydrolyzed by TeTx about 40-fold slower than the 93-mer peptide encompassing the entire NH_2-terminal region.

To obtain insights into the sequences required at the COOH-terminal side of the cleaved bonds, we linearized synaptobrevin 2-specific DNA at particular restriction sites in order to generate 3′-deleted mRNAs. In addition, we tested whether M1Q76, the NH_2-terminal cleavage product generated from M1L93 by TeTx, could be cleaved by BoNT/D or BoNT/F.

The M1R86 derivative ending in Arg^{86} was still efficiently cleaved by BoNT/D, BoNT/F, and TeTx, and mutants ending in Gln^{76} or Ala^{69} were cleaved by both BoNT/D and BoNT/F indicating that cleavability is maintained with only 10 or 11 residues at the COOH-terminal side provided that an intact NH_2-terminal core region is present. This clearly contrasts our observations made with shorter soluble peptides lacking sequences beyond Ala^{37}. Both BoNT/D and BoNT/F failed to proteolyze the 20-mer peptide V50A69. Similarly, TeTx could not hydrolyze the 20-mer peptide D68K87, whereas it did cleave M1R86 quite efficiently.

Together, our results suggest that cleavage by BoNT/D, TeTx, and BoNT/F requires the presence of at least 10 residues on the COOH-terminal side of the corresponding scissile bonds. Apparently, however, cleavage is not only controlled by the residues present at the cleavage sites or in their ultimate proximity, but, in addition, by more distal residues located NH_2 terminally within the highly conserved core region.

Single Amino Acid Substitutions Control Cleavability of Synaptobrevin Isoforms—Our observation that proteolysis of synaptobrevin 1 by BoNT/D required about 3,700-fold higher toxin concentration than needed for cleavage of the synaptobrevin 2 isoform (Fig. 7) was surprising because 13 amino acid residues toward the NH_2 and 16 residues toward the COOH terminus are identical in both isoforms. Within the conserved core region of synaptobrevins only three amino acid substitutions, Asp^{40} to Glu^{40}, Met^{46} to Ile^{46}, and/or Gln^{76} to Val^{76} could account for this difference (Table I). To determine which of the three amino acid residues influenced cleavability by BoNT/D and TeTx, we replaced them individually by those found in rat synaptobrevin 1. All mutations were verified by DNA sequencing of the entire coding region. To test cleavability of the resulting mutants, we applied each L chain at 5 nM final concentration using membrane-associated substrates. The results are summarized in Fig. 6 and are further supported by dose-response curves shown in Fig. 7.

First, a replacement of aspartic acid in position 40 by glutamic acid had no influence on cleavability by BoNT/D, BoNT/F, and TeTx (data not shown).

Second, as a consequence of the replacement of Met^{46} by Ile^{46} (in RSB-2 I^{46}), NH_2-terminal fragments produced by the three toxins were no longer detectable. This mutation, however, virtually abolished cleavability by BoNT/D and even 500 nM BoNT/D caused only about 40% breakdown (Fig. 7, *panel A*).

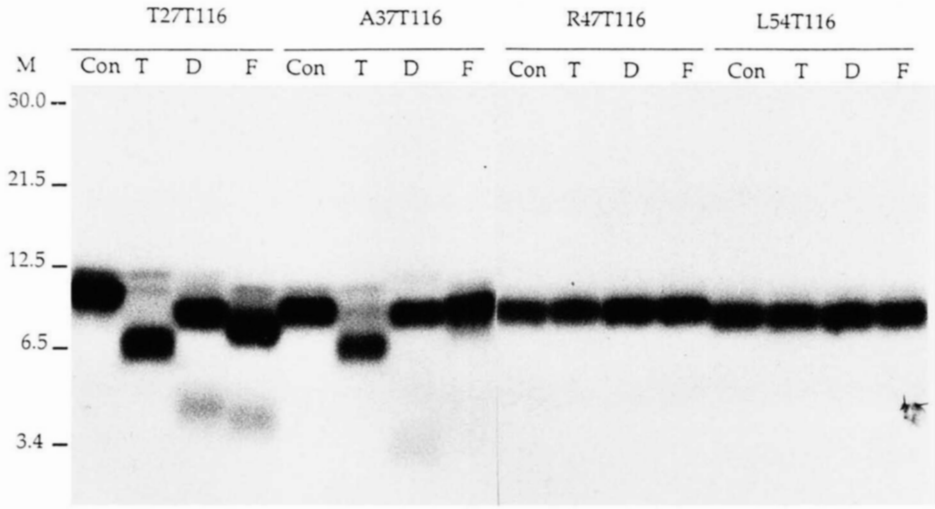


FIG. 5. **Cleavage of NH_2-terminal deletion mutants of rat synaptobrevin 2 by TeTx, BoNT/D, and BoNT/F.** NH_2 terminally deleted variants were obtained by polymerase chain reaction and *in vitro* transcription/translation in the presence of membranes. The membranes were recovered by centrifugation through a sucrose cushion and incubated either in the absence (*Con*) or presence of the L chains of TeTx (*T*, 5 nM final concentration), BoNT/D (*D*, 1.5 nM), or BoNT/F (*F*, 1.5 nM). Numbers specify both the NH_2- and the COOH-terminal amino acid.

Interestingly, cleavability of RSB-2 I^{46} by the BoNT/F L chain was reduced only by a factor of about 3 (Fig. 7, *panel B*). Furthermore, even cleavability by TeTx was affected, as evidenced by the persistence of about 2-fold more radiolabel in the uncleaved material (Fig. 6) and by dose-response curves (Fig. 7, *panel C*). Together our data indicate that the amino acid exchange in position 46 is indeed responsible for the poor *in vitro* substrate properties of rat synaptobrevin 1 for BoNT/D, and that this side group of residue 46 induces long range effects on the cleavability by BoNT/F and TeTx.

Third, the Gln^{76} to Val^{76} mutation in RSB-2 V^{76} did not significantly affect cleavability by BoNT/D and BoNT/F (Fig. 6). This is in agreement with the previous notion that COOH-terminal deletion mutants of synaptobrevin 2 lacking Gln^{76} were still efficiently cleaved. However, this mutant was cleaved by TeTx about 300-fold worse than RSB-2 (Fig. 6 and Fig. 7, *panel C*) thus providing the more trivial evidence that residues present in the scissile bond indeed affect cleavability.

DISCUSSION

Our data demonstrate that the L chain of BoNT/D, selectively cleaving the Lys^{59}-Leu^{60} peptide bond of rat synaptobrevin 2, acts as an *endo*protease and exhibits some properties of zinc metalloenzymes. Thus, BoNT/D is the fourth clostridial neurotoxin that exhibits specificity for this synaptic vesicle protein. TeTx and BoNT/B cleave the Gln^{76}-Phe^{77} peptide bond (Schiavo *et al.*, 1992), and BoNT/F attacks the Gln^{58}-Lys^{59} bond (Schiavo *et al.*, 1993).

Proteolysis was demonstrated both in intact synaptosomes under conditions where neurotransmitter release was blocked and in isolated vesicles. Furthermore, breakdown of a synthetic peptide representing the entire NH_2-terminal domain of rat synaptobrevin 2 including Leu^{93} indicated that no other cellular cofactors were required for the cleavage reaction.

To analyze proteolysis of other synaptobrevin isoforms, we employed an *in vitro* cleavage assay using *in vitro* translated radiolabeled substrates. We show that BoNT/D, BoNT/F, and TeTx also cleave cellubrevin, a synaptobrevin homologue present in all eukaryotic cells investigated (McMahon *et al.*, 1993). By contrast, rat synaptobrevin 1, presented either as a soluble or as a membrane-associated substrate, was only a poor substrate *in vitro*, requiring almost 3,700-fold higher concentrations of the BoNT/D L chain or about 300-fold larger amounts of the TeTx L chain for semiquantitative cleavage. As judged from the size of the membrane retained cleavage fragments, however, cleavage of rat synaptobrevin 1 by the three toxins involved the same peptide bonds as mapped in synaptobrevin 2 (Fig. 4, *panels A* and *B*).

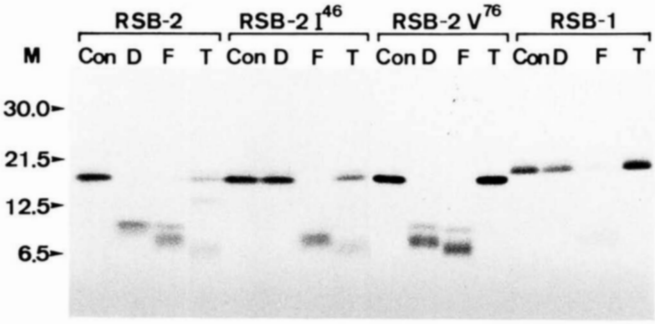


FIG. 6. **Single amino acid exchanges control cleavability of rat synaptobrevins.** Rat synaptobrevin 2 (*RSB-2*, *left panel*) and synaptobrevin 1 (*RSB-1*, *right panel*) were generated as radiolabeled substrates by *in vitro* translation in the presence of microsomal membranes. In addition, two synaptobrevin 2-point mutants carrying single synaptobrevin 1-specific amino acid substitutions (Met^{46} by Ile^{46} in RSB-2 I^{46}; Gln^{76} by Val^{76} in RSB-2 V^{76}) were translated in the same manner. To show differences in cleavability, the L chains of BoNT/D (*D*), TeTx (*T*), and BoNT/F (*F*) were added at 5 nM final concentration. Samples were incubated for 1 h at 37 °C, separated on a 15% SDS-polyacrylamide gel and processed by autoradiography. Due to substitution of Met^{46} by Ile^{46} in RSB-1 and in RSB-1 I^{46}, the released fragments can no longer be visualized in these variants. Note that the Met^{46} to Ile^{46} substitution nearly abolishes hydrolysis by BoNT/D whereas it has also some effect on cleavability by TeTx. In contrast, the Gln^{76} substitution by Val^{76} dramatically reduces cleavability by TeTx, whereas it does not influence cleavability by both BoNT/D and BoNT/F.

The structural requirements for cleavage of synaptobrevin homologues described here distinguish TeTx, BoNT/D, and BoNT/F from all other known metalloproteinases including the neutral endopeptidase 24.11 (EC 3.4.24.11), angiotensin-converting enzyme (EC 3.4.15.1), a peptidyl-dipeptidase (for review see Roques *et al.*, 1993). For these proteases short peptides serve as substrates and residues in the ultimate vicinity of the scissile bond, designated P3, P2, P1, P1′, P2′, and P3′ (Schechter and Berger, 1967), dictate cleavability. We show here that this is not the case with the clostridial neurotoxin L chains. Our studies on NH_2- and COOH-terminal deletion mutants show clearly that the L chains require at least 10 amino acid residues at the COOH-terminal side of the individual scissile bond. With respect to NH_2-terminal sequences required for cleavability, we noticed that the heterologous head structures (residues 1–27 in rat synaptobrevin 2) may be deleted without affecting cleavability. Deletions into the conserved core region, however, indicated that these play a critical role perhaps by establishing a cleavable substrate structure. Sequences between Ala^{37} and Arg^{47} were found to be of particular importance. We showed that a single amino acid substitution in po-

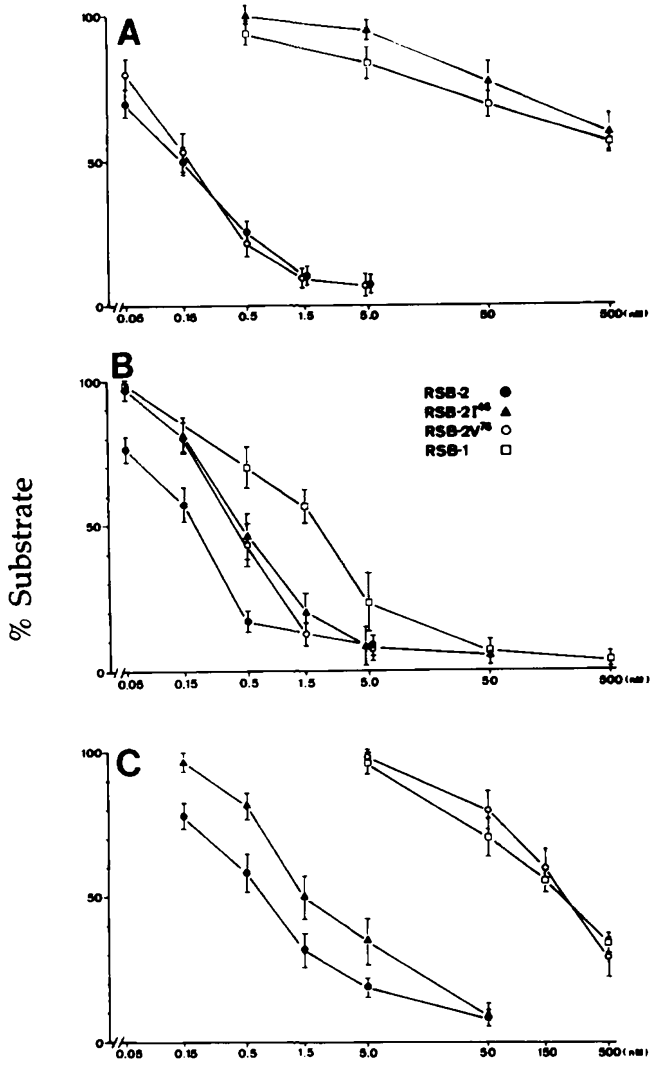


FIG. 7. **Dose dependence of cleavage of rat synaptobrevins and its mutants by BoNT/D, BoNT/F, and TeTx.** Equimolar amounts of radiolabeled rat synaptobrevin 2 (RSB-2, ●), RSB-2 I^{46} (▲), RSB-2 V^{76} (○), or RSB-1 (□) were incubated for 1 h at 37 °C with serial dilutions of L chains of BoNT/D (*panel A*), BoNT/F (*panel B*), or TeTx (*panel C*). The reaction products were analyzed by SDS-PAGE and radioactivity in individual lanes was determined by laser scan densitometry of autoradiographs. Each point represents the mean value ± S.D. of three independent experiments.

sition 46 accounts for the poor *in vitro* substrate properties of rat synaptobrevin 1 for BoNT/D. This finding is remarkable for three reasons. First, a replacement of a methionine residue (in synaptobrevin 2) by isoleucine (in synaptobrevin 1) must be considered a conservative exchange, whereby isoleucine is only slightly more hydrophobic than methionine. Second, BoNT/D cleaves only one peptide bond adjacent to that cut by BoNT/F which, however, is much less affected by the amino acid exchange in position 46. Third, the resistance of synaptobrevin 1 against proteolysis by BoNT/D is only observed with synaptobrevin 1 peptides generated *in vitro*, *i.e.* under conditions where other synaptic vesicle proteins are absent and some post-translational modifications are not carried out. At this moment we have no explanation for the different susceptibilities of brain derived and *in vitro* generated synaptobrevin 1.

With respect to the isoform specificity of TeTx, we provided evidence that cleavability of synaptobrevin 2 by TeTx is drastically reduced by a Gln^{76} to Val^{76} mutation (as found in synaptobrevin 1). Whereas this finding is not surprising because the mutation affects a residue in the scissile bond, it is noteworthy that the Met^{46} to Ile^{46} substitution also affects cleavability by TeTx. Does this indicate that the region between Ala^{37} and Arg^{47} of synaptobrevins folds back into a position close to the individual cleavage sites? NMR studies directed to determine the secondary structure of the M1L93 peptide did not reveal a highly structured conformation.[2] This suggests that the synaptobrevin isoforms gain the critical conformation required for cleavage only upon contact with the individual L chain. Alternatively, the above region including Met^{46} could play an important role in binding the individual L chains.

The discovery of proteolytic activity of clostridial neurotoxins, originally described for TeTx (Schiavo *et al.*, 1992; Link *et al.*, 1992) has recently provided a valuable set of tools that should contribute in deciphering the molecular mechanisms underlying vesicular exocytosis both in the two tightly regulated neuronal pathways and in the constitutive pathways of nonneuronal cells. With TeTx (BoNT/B), BoNT/D, and BoNT/F highly selective proteases are now available that cleave synaptobrevin homologues in neuronal and non-neuronal tissue. We have recently described that BoNT/A hydrolyzes SNAP-25 (Blasi *et al.*, 1993b) whereas syntaxin/HPC-1 is the target of BoNT/C1 (Blasi *et al.*, 1993a). Each of these substrates appears to be a member of a protein complex present in synaptic vesicles and, most likely in the form of specific isoforms, in transport vesicles involved in constitutive exocytosis and membrane trafficking in nonneuronal cells. This protein complex may contain, in addition, Munc-18, a protein whose precise function is unknown and which is highly conserved in evolution (Hata *et al.*, 1993). What are the additional requirements for the assembly of a fusion-competent machine? In the synapse the process of docking and fusion clearly involves proteins with control functions such as synaptotagmin which binds to neurexins (Petrenko *et al.*, 1991), acting as one of perhaps several Ca^{2+}-sensitive clamps that prevent fusion until the local Ca^{2+} concentration has reached a threshold level (Brose *et al.*, 1992). Binding of SNAPs and NSF are supposedly late events just preceding fusion of the vesicle with the target membrane (Söllner *et al.*, 1993a, 1993b). Several laboratories are trying to establish an *in vitro* fusion system based on the above components. Clearly, the neurotoxins could be ideal tools to study the proper assembly of the fusion machine.

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Addendum—While this work was in the reviewing stage, Schiavo and co-workers (Schiavo, G., Rossetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B. R., Benfenati, F., and Montecucco, C. (1993) *J. Biol. Chem.* **268,** 23784–23787) reported that in agreement with our finding, BoNT/D cleaved synaptobrevin at the Lys^{59}-Leu^{60} bond. Furthermore, Shone and co-workers (1993) studied the minimal essential domains of rat synaptobrevin 2 required for cleavage by BoNT/B. In agreement with our findings, BoNT/B also failed to cleave short peptides (Shone, C. C., Quinn, C. P., Wait, R., Hallis, B., Fooks, S. G., and Hambleton, P. (1993) *Eur. J. Biochem.* **218,** 965–971).

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