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 botulinum 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 Lys65-Leu66 which is adjacent to the BoNT/F cleavage site (Gln66-Lys67) and again differs from the site hydrolyzed by TeTx and BoNT/B (Gln76-Phe77). 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 Arg57 to Thr100 was a poor substrate for all three toxins tested. However, cleavability was restored when the peptide was further extended at the NH2 terminus (Thr57-Thr100) demonstrating that NH2 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 Met68 to Ile68 substitution drastically diminishes cleavability by BoNT/D and that the presence of Val88 instead of Gln88 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, 100,000) remains attached to a light (L) chain (M, 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 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; Bins et al., 1994), and BoNT/C1 causes a selective breakdown of syntaxin (Archer et al., 1993). 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 carboxy-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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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 (Sölter 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 (Schild et al., 1993), 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 proteolyses 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 NH₂-terminal side of the respective cleavage site may determine isoform cleavage indicating complex structural requirements for toxin action.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Clones encoding rat VAMP-1 and VAMP-2 (Eifert 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: 5'-TCTT116,5'-CACACTCGAGATGGCCCAGGTGGA-GAGACTCAGC3'-AATTT116,5'-CACACTCGAGATGGCCCAGGTGGA-TGAGGTGGGT3'-R41T116,5'-CACACTCGAGATGGGGTGAGTACAG-GACAAGGC5'-L54T116,5'-CACACTCGAGATGGGGTGAGTACAG-GACAAGGC5'-ACCAGAAGCTG-3'. As downstream primer we used 5'-ATCGAT-ACCCGCTGTCG3' and for the peptide we used 5'-ATCGATACCCGCTGTCG3'. Amplified fragments were cloned with KpnI and XbaI 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., 1988; Buckley and Kelly, 1985). Monoclonal antibody directed against synapsin I was kindly provided by Dr. P. Greenard. 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 8737 according to Miyazaki et al. (1977). BoNT/D was isolated from C. botulinum type F following the procedures of Oishi and Sakaguchi (1974). The Hall strain of Clostridium botulinum was purified by a resynthesized gene (Eisel et al., 1989) encoding the entire L chain and a COOH-terminal His₁₀ tag. The gene was expressed in Escherichia coli M15 and purified by binding to ω-nitrophenylacetic acid agarose according to the protocols of Nagel (Duren, 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 Ca²⁺-dependent release of the excitatory neurotransmitter, glutamate, monitored on-line with a coupled enzymatic detection system. Fig. 1A 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 Ca²⁺ 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 rat brain 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.

Ca2+ 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 Ca2+-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. 1A), 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, LP II. 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. 1B).

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 NH2-terminal domain that up to Leu96 but lacks the COOH-terminal NH2 terminus and sequencing over 12 steps yielded the sequence LSELD-DRADALQ. For control, degradation by L chains of TeTx and BoNT/F was also analyzed followed by determination of the NH2 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 Gln75-Phe77 bond,
**Table 1**

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

Rat synaptobrevin 2 (Rab2), 1 (Rab1), 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 BglII digestion), M1A69 (prepared by digestion with BglII), and mutants T27T116, A37T116, R47T116, and L54T116 were tested as in vitro translation products. M1L93, M1Q76, K52A67, D68R66, 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.

| Domain   | BoNT/F | BoNT/D | TeTx |
|----------|--------|--------|------|
| Rab2     | ++++++ | +      | -    |
| Rab1     | ++     | +++    | +    |
| Rcb      | ++++   | ++++   | +++  |
| M1R86    | ++++   | +      | R(86) |
| M1A69    | ++++   | QKL--- | A(69) |
| T27T116  | +      | QKL--- | T(116) |
| A37T116  | ++     | QKL--- | A(69) |
| R47T116  | +      | QKL--- | Q(76) |
| L54T116  | +      | QKL--- | L(93) |
| M1L93    | ++++   | +      | L(93) |
| M1Q76    | ++++   | QKL--- | Q(76) |
| M46Q76   | +      | QKL--- | M(76) |
| D51L93   | +      | QKL--- | D(76) |
| V50A69   | +      | QKL--- | V(83) |
| D68K87   | +      | QKL--- | K(87) |
| V53K83   | +      | QKL--- | K(87) |
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whereas BoNT/F cleaved the Gln58-Lys59 bond. Together, our results indicate that BoNT/D cleaves the peptide bond between Lys59 and Leu60 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 microsequencing.

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. 1B; 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. 1B) 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 μM) or 90% cleavage (1.5 μM) of membrane-anchored rat synaptobrevin 2, respectively, we then studied the effects of various inhibitors of metalloproteases. Whereas 0.15 μM 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 μM 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 NH2-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 NH2-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 Met66 to Gln76 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-
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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 NH₂-terminal and 16 residues toward the COOH terminus are identical in both isoforms. Within the conserved core region of synaptobrevins only three amino acid substitutions, Asp⁴⁰ to Gln⁴⁰, Met⁴⁶ to Ile⁴⁶, and/or Gln⁷⁶ to Val⁷⁶, 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 Met⁴⁶ by Ile⁴⁶ (in RSB-2 1₄₆), NH₂-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).

branes, 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 ºC 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 TL-A100 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.

[Figures and tables are not included in the natural text representation.]
Interestingly, cleavability of RSB-2 146 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 cleavage of RSB-2, whereas cleavability by TeTx, BoNT/D, and BoNT/F was not significantly affected (Fig. 6).

This in agreement with the previous notion that COOH-terminal deletion mutants of synaptobrevin 2 lacking Gln76 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). Together our data indicate that the amino acid exchange in position 46 is indeed responsible for the poor in vitro cleavage of RSB-2, whereas cleavability by TeTx, BoNT/D, and BoNT/F was not significantly affected (Fig. 6).

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).

**DISCUSSION**

Our data demonstrate that the L chain of BoNT/D, selectively cleaving the Lys56-Leu56 peptide bond of rat synaptobrevin 2, acts as an endoprotease and exhibits specificity for zinc metalloenzymes. Thus, BoNT/D is the fourth clostridial neurotoxin that exhibits specificity for this synaptic vesicle protein. TeTx and BoNT/B cleave the Gln56-Phe77 peptide bond (Schiavo et al., 1992), and BoNT/F attacks the Gln56-Lys99 bond (Schiavo et al., 1993).

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).
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Acknowledgments—We thank Naomi Yamasaki for excellent technical assistance and Drs. Eberhard Pfaff and Hubert Muller for synthesis of peptides. 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, F. (1993) Eur. J. Biochem. 218, 965–971).

Addendum—While this work was in the reviewing stage, Schiavo and co-workers (Schiavo, G., Rossetto, O., Catiasca, S., Polverino de Laureto, P., DauGupta, 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 Lys9–Leu10 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, F. (1993) Eur. J. Biochem. 218, 965–971).

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