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Structural Determinants of the Specificity for Synaptic Vesicle-associated Membrane Protein/Synaptobrevin of Tetanus and Botulinum Type B and G Neurotoxins*

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Rossella Pellizzari‡, Ornella Rossetto‡, Luisa Lozzi§, Silvia Giovedi'‡, Eric Johnson¶, Clifford C. Shone‖, and Cesare Montecucco‡**

From the ‡Centro Consiglio Nazionale delle Ricerche di Biomembrane and Dipartimento di Scienze Biomediche, Università di Padova, Via Trieste 75, Padova, Italy, the §Dipartimento di Biologia Molecolare, Le Scotte, Università di Siena, Italy, the ¶Food Research Institute, University of Wisconsin, Madison, Wisconsin 52706, and ‖Center for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom

Tetanus and botulinum neurotoxins type B and G are zinc-endopeptidases of remarkable specificity. They recognize and cleave a synaptic vesicle-associated membrane protein (VAMP)/synaptobrevin, an essential protein component of the vesicle docking and fusion apparatus. VAMP contains two copies of a nine-residue motif, also present in SNAP-25 (synaptosomal-associated protein of 25 kDa) and syntaxin, the two other substrates of clostridial neurotoxins. This motif was suggested to be a determinant of the target specificity of neurotoxins. Antibodies raised against this motif cross-react among VAMP, SNAP-25, and syntaxin and inhibit the proteolytic activity of the neurotoxins. Moreover, the various neurotoxins cross-inhibit each other's proteolytic action. The role of the three negatively charged residues of the motif in neurotoxin recognition was probed by site-directed mutagenesis. Substitution of acidic residues in both copies of the VAMP motif indicate that the first one is involved in tetanus neurotoxin recognition, whereas the second one is implicated in binding botulinum B and G neurotoxins. These results suggest that the two copies of the motif have a tandem association in the VAMP molecule.

Tetanus neurotoxin (TeNT)[1] and botulinum neurotoxins (BoNTs, seven types from A to G) are three-domain protein toxins that bind selectively to the neuronal presynaptic membrane. They are internalized inside intracellular compartments from which the amino-terminal 50-kDa domain (termed L chain) enters into the cytosol (1–4). The L chains of TeNT and BoNTs are zinc-endopeptidases that cleave specifically three proteins of the neuroexocytosis apparatus, thereby blocking neurotransmitter release (4–7). TeNT and BoNT/B, BoNT/D, BoNT/F, and BoNT/G recognize and cleave specifically a synaptic vesicle-associated membrane protein (VAMP, also referred to as synaptobrevin) at different single peptide bonds (4, 8–12). BoNT/A and BoNT/E specifically recognize and cut SNAP-25 (synaptosomal-associated protein of 25 kDa) at two different peptide bonds near the COOH terminus (10, 13, 14), whereas BoNT/C cleaves syntaxin (15, 16) and SNAP-25 (17–19). VAMP, SNAP-25, and syntaxin are collectively termed SNARE proteins, because they act as receptors of soluble *N*-ethylmaleimide-sensitive factor accessory proteins, involved in vesicle-membrane fusion (5–7).

Sequence comparison of the L chains of the eight clostridial neurotoxins show strong similarities (20), which are even more extensive at the level of predicted secondary structure (21). These similarities suggest that they derive from a common ancestral metalloproteinase. On this basis, to account for their different substrate specificity, we considered the possibility that the three SNAREs contain a common neurotoxin recognition site in addition to the cleavage sites specific for each neurotoxin type. We identified a nine-residue-long motif (SNARE motif) present in eukaryotes only in the three proteins known to be proteolytic substrates of the neurotoxins (22). The SNARE motif is included within regions predicted to adopt an α-helical conformation in the three SNAREs (23). This motif is characterized by the presence of three negatively charged residues and three hydrophobic residues spaced in such a way that the Edmundson wheel plot shows a negatively charged surface contiguous to a hydrophobic face. Preliminary experiments showed that peptides corresponding to the motif sequence of the three SNARES inhibit neurotoxin activity both *in vitro* and *in vivo* in injected *Aplysia* neurons (22). We also suggested that the specificity of the clostridial neurotoxin's action is based on a double recognition of their substrates via the SNARE motif and via the segment containing the cleavage site (4, 22, 24).

Here we report on studies focusing on the interaction among TeNT, BoNT/B and BoNT/G, and VAMP. Results obtained with different experimental approaches provide strong evidence for the involvement of the SNARE motif in this specific interaction. Moreover, they show that negatively charged residues of the motif play a major role in this interaction.

MATERIALS AND METHODS

Proteins, Peptides, and Chemicals—TeNT and BoNT/A, BoNT/B, BoNT/C, BoNT/E, and BoNT/G were prepared as detailed before (25–27). Immobilized metal ion affinity chromatography was used to remove traces of contaminant proteases (28). Synaptosomes were isolated from rat brain cortex as detailed before (10). Peptides V2 (ELDDRADALQ), S3 (MLDEQGEQLER), and X2 (LEDMLESGN) were prepared with an SMP 350 automatic synthesizer (Zynsser Analytic, Frankfurt), employing an Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry, and were purified by reverse phase chromatography on a C8 Ultra-Sphere preparative column (Beckman). The composition of soybean mixed lipids (asolectin) was as described previously (29). Liposomes were obtained by mixing chloroform/methanol (2:1) stock solutions of lipids. After

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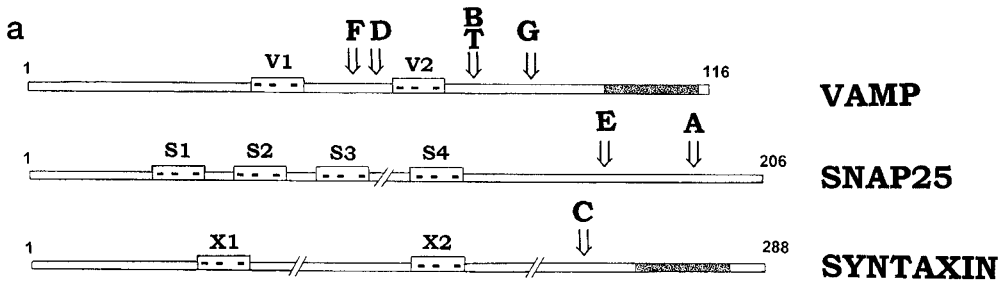
‡ To whom correspondence should be addressed: Dipartimento di Scienze Biomediche, Via Trieste 75, 35100 Padova, Italy. Tel.: 39-49-8286058; Fax: 39-49-8276049.

[1] The abbreviations used are: TeNT, tetanus neurotoxin; GST, glutathione *S*-methyl transferase; BoNT, botulinum neurotoxin; VAMP, vesicle-associated membrane protein; VAMP-2, VAMP isoform 2; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive factor accessory protein receptor.



b

V1	^{38}Q	V	D	E	V	V	D	I	M	R ^{47}
V2	^{62}E	L	D	D	R	A	D	A	L	Q ^{71}
S1	^{21}L	A	D	E	S	L	E	S	T	R ^{30}
S2	^{35}L	V	E	E	S	K	D	A	G	I ^{44}
S3	^{49}M	L	D	E	Q	G	E	Q	L	E ^{58}
S4	^{145}E	M	D	E	N	L	E	Q	V	S ^{154}
X1	^{29}F	M	D	E	F	F	E	Q	V	E ^{38}
X2	^{164}E	L	E	D	M	M	L	E	S	G ^{173}

x h - - x h - x h p

FIG. 1. **Schematic structure of VAMP, SNAP-25, and syntaxin with cleavage sites of neurotoxins and positions of SNARE motif (*a*).** The SNARE motif is composed of nine residues and contains three conserved carboxylate residues (−) at positions 2, 3, and 6; in an Edmundson wheel plot of the motif Glu or Asp residues clusters on one-third of the helix. This motif is present in two copies in VAMP-2 (V1 and V2), two copies in syntaxin (X1 and X2), and four in SNAP-25 (S1, S2, S3, and S4). *Capital letters* correspond to the proteolytic cleavage site of tetanus toxin (*T*) and of the seven botulinum neurotoxins from *A* to *G*. *Dark dots* identify the transmembrane region of VAMP and syntaxin. The *lower part* of the *figure* reports the sequences of the various copies of the SNARE motif (*b*).

drying under N_2 flux, lipids were resuspended in diethyl ether, dried, and sonicated until optical clarity was achieved.

Antibodies and Immunoblotting—Rabbit polyclonal antisera against peptides V2, S3, and X2 were prepared as described by Kreis (30). The antibodies were purified by affinity chromatography using activated CH-Sepharose 4B (Pharmacia Biotech Inc.) conjugated with each peptide. Antibody concentration was determined by the Bradford method (31). Synaptosomes were transferred onto nitrocellulose as described elsewhere (10) and treated with anti-V2, anti-S3, and anti-X2 antisera (1:200). Primary antibodies were detected by immunostaining with an anti-rabbit antibody (1:10,000 dilution, Boehringer Mannheim) conjugated with alkaline phosphatase (1:1,000; Sigma).

Bacterial Strains, Plasmid Construction, and VAMP Mutagenesis—The VAMP-2 gene was generated by inserting a PCR-derived DNA fragment from the rat VAMP-2 cDNA clone characterized previously (32). This DNA fragment covers the complete sequence of the VAMP-2 gene and has BamHI and *Eco*RI restriction sites at the 5′ and 3′ ends, respectively. It was inserted into the pEMBL8- plasmid (33). Uracil containing single-stranded DNA was produced within BW313, an *Escherichia coli* dut- ung- strain, for generation of VAMP-2 mutants by site-directed mutagenesis. The *in vitro* reactions were performed as described by Kunkel *et al.* (34). The following oligonucleotides were used to generate the corresponding mutants: 5′-TGCGCGATTATTCAGTTCC-3′ for VAMP-2 D64N,D65N; 5′-GGAGGGCATTTGCGCGAT-3′ for VAMP-2 D68N; the combination of the two previous oligonucleotides for VAMP-2 D64N,D65N,D68N; 5′-CTGCGCGACTACTCAGTTCC-3′ for VAMP-2 D64S,D65S, and 5′-TCCACCACCTGATTCACCTGG-3′ for VAMP-2 D40N,E41Q. The sequence of the mutated genes was checked by dideoxy sequencing using the fmole DNA sequencing System (Promega). Wild type VAMP-2 and mutants were subcloned into *Bam*HI and *Eco*RI sites of pGEX-KG vector (35) and transformed into the AB1899 strain of *E. coli*.

VAMP Protein Expression and Purification—Rat VAMP-2 and rat VAMP-2 mutants were expressed as GST fusion proteins and were purified by affinity chromatography on GSH-agarose matrix (Pharmacia) as before (12).

Assay of Proteolytic Activity—GST-fusion VAMPs (50 μg/ml final concentration) were incubated with a sonicated clear liposome suspension (final concentration, 1 mg of soya bean mixed phospholipids/ml) at 37 °C for 1 h. After treatment with dithiothreitol (10 mM for 30 min at 37 °C), TeNT (200 nM), BoNT/B, or BoNT/G (40 nM) was added to the reaction mixture, containing one of the different GST fusion proteins. Proteolysis was carried out in 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, at 37 °C for variable periods of time. In some experiments 1 μg of GST-VAMP-2 was preincubated for 30 min with affinity-purified antibodies (1 μM), specific for the different peptides, before neurotoxin addition and proteolysis, as described above. Samples were analyzed in a 15% polyacrylamide SDS gels and, after silver staining of proteins, scanned with a dual wavelength Shimadzu CS-630 densitometer.

For neurotoxin competition experiments, GST-VAMP-2 was preincubated for 1 h at 37 °C with each one of the three neurotoxins, BoNT/A, BoNT/E, and BoNT/C, which do not cleave VAMP-2 (1 μM) in 150 mM NaCl, 10 mM Na_2HPO_4, 0.3 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4. Proteolytic cleavage was carried out for 2 h at 37 °C with TeNT 200 nM or BoNT/B 40 nM. All neurotoxins were preincubated with dithiothreitol 10 mM in the same cleavage buffer for 30 min at 37 °C.

RESULTS

Anti-SNARE Motif Antibodies Specifically Recognize VAMP, SNAP-25, and Syntaxin—Available models for neuroexocytosis assign a central role to VAMP, SNAP-25, and syntaxin, the three targets of clostridial neurotoxins (5–7). These proteins form an SDS-resistant heterotrimeric complex able to bind SNAPs and *N*-ethylmaleimide-sensitive factor (23, 36, 37). Fig. 1 depicts the three SNAREs and includes membrane-spanning segments and sites of cleavage of clostridial neurotoxins. There are two copies of the motif in VAMP (V1 and V2), four copies in SNAP-25 (S1, S2, S3, and S4), and two copies in syntaxin (X1 and X2). Little structural information is available for isolated SNARE proteins. If the motif is to serve as neurotoxin recognition site, it has to be exposed at the protein surface, where it may act as an epitope and bind antipeptide specific antibodies. To test this possibility, rabbits were immunized with keyhole limpet hemocyanin coupled with the V2, S3, or X2 peptide, and IgGs were purified after booster immunization with the same antigens. Fig. 2 shows that the rabbit IgGs specific for V2 do recognize VAMP, but they also stain syntaxin and SNAP-25,

although with lower intensity. In parallel, anti-S3 rabbit IgGs show binding to syntaxin and to VAMP in addition to the expected recognition of SNAP-25, and a similar pattern of staining is observed with X2-specific antibodies. The immunogenicity of the SNARE motif, at the basis of the SNARE cross-reactivity, is also supported by the fact that rabbit polyclonal antisera prepared against recombinant VAMP or SNAP-25 or syntaxin stain all three SNARE proteins (Fig. 2). This result puts a word of caution on the interpretation of immunofluorescence studies performed with antibodies raised against an entire SNARE protein.

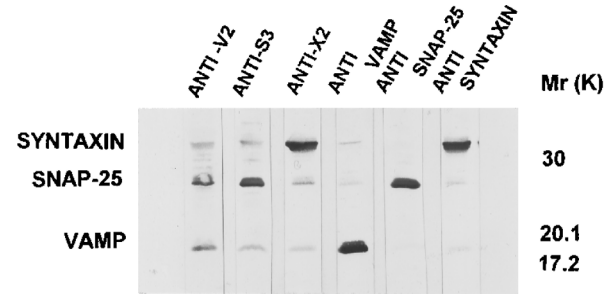


FIG. 2. **Antibody cross-reactions among VAMP, SNAP-25, and syntaxin.** Rat brain synaptosomes were electrophoresed and blotted onto nitrocellulose membranes. Samples were incubated with rabbit anti-SNARE motif-specific polyclonal antibodies anti-V2, anti-S3, and anti-X2 affinity-purified as detailed under "Materials and Methods." The last three samples were incubated with rabbit anti-VAMP-2, anti-SNAP-25, or antisyntaxin antisera and were stained with the appropriate alkaline phosphatase-conjugated anti-IgG antibodies.

Anti-SNARE Motif Antibodies Interfere with VAMP Proteolysis by Tetanus and Botulinum Neurotoxins—The involvement of the SNARE motif in neurotoxin binding leads to the prediction that antimotif-specific antibodies should inhibit the proteolytic activity of neurotoxins. Fig. 3*a* shows that antibodies prepared against V2, S3, or X2 have an inhibitory effect on TeNT and BoNT/B proteolysis of VAMP. The extent of protection is variable and incomplete. This may be accounted for by variable affinities of the different antibodies and by the fact that the antibody competes with the neurotoxin for only one of the two sites of interaction between VAMP and the neurotoxin.

SNAP-25 and Syntaxin-specific Neurotoxins Inhibit Tetanus and Botulinum Neurotoxin Proteolysis of VAMP—Another prediction of the model, outlined in the Introduction, is that each clostridial neurotoxin should be able to bind any SNARE protein, although only one of them (two in the case of BoNT/C) would be cleaved, depending on the fitting of a particular sequence into each neurotoxin active site (4). As a consequence, binding of any neurotoxin type to a SNARE should inhibit its proteolysis by the specific neurotoxin. Fig. 3*b* shows the results of neurotoxin cross-inhibition experiments performed with

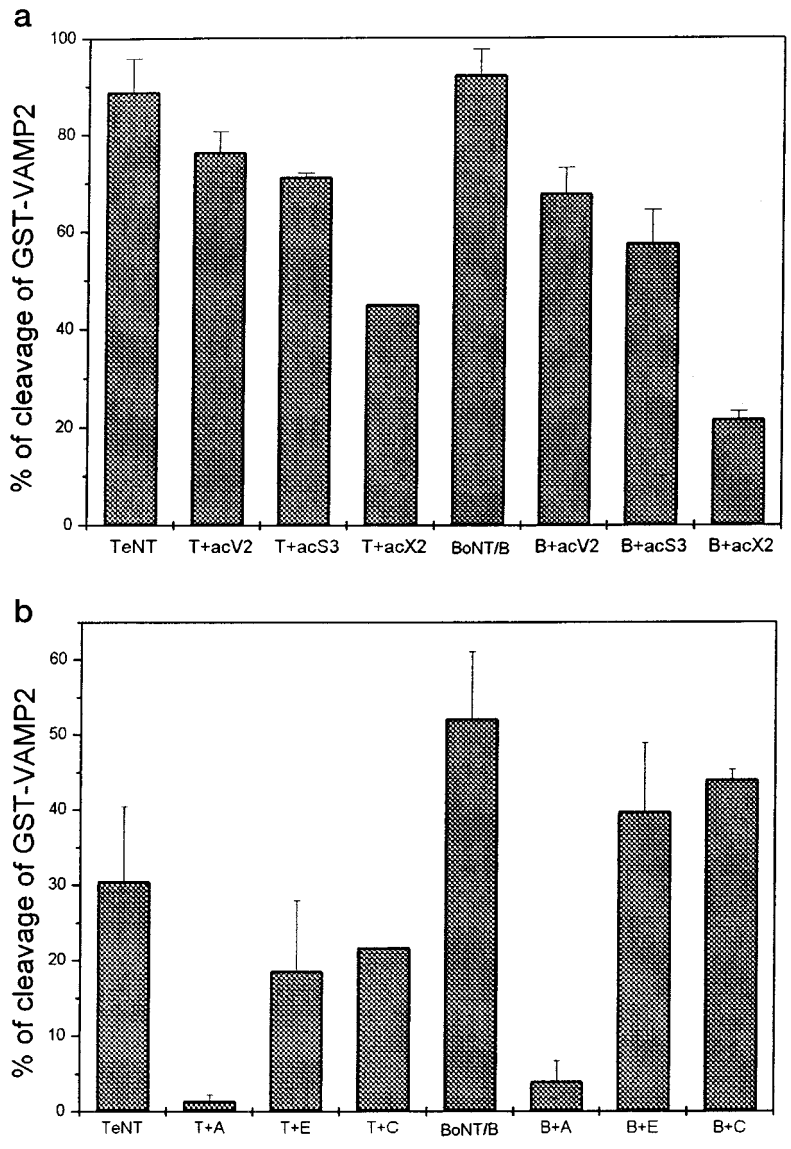


FIG. 3. **Inhibition of VAMP cleavage by tetanus and botulinum B neurotoxins with anti-SNARE motif-specific antibodies and non-VAMP-specific neurotoxins.** *a*, the amount of cleavage of GST-VAMP2 fusion protein incubated with tetanus neurotoxin (TeNT) or botulinum neurotoxin B (BoNT/B) alone or in the presence of affinity-purified antibodies against peptides V2, S3, and X2 after 2 h at 37 °C. *b*, GST-VAMP-2 was preincubated with BoNT/A, BoNT/E, or BoNT/C, previously reduced with dithiothreitol, and then reduced TeNT and BoNT/B were added and incubated for 2 h at 37 °C. Data are the average of three independent experiments, and *bars* represent S.D. values.

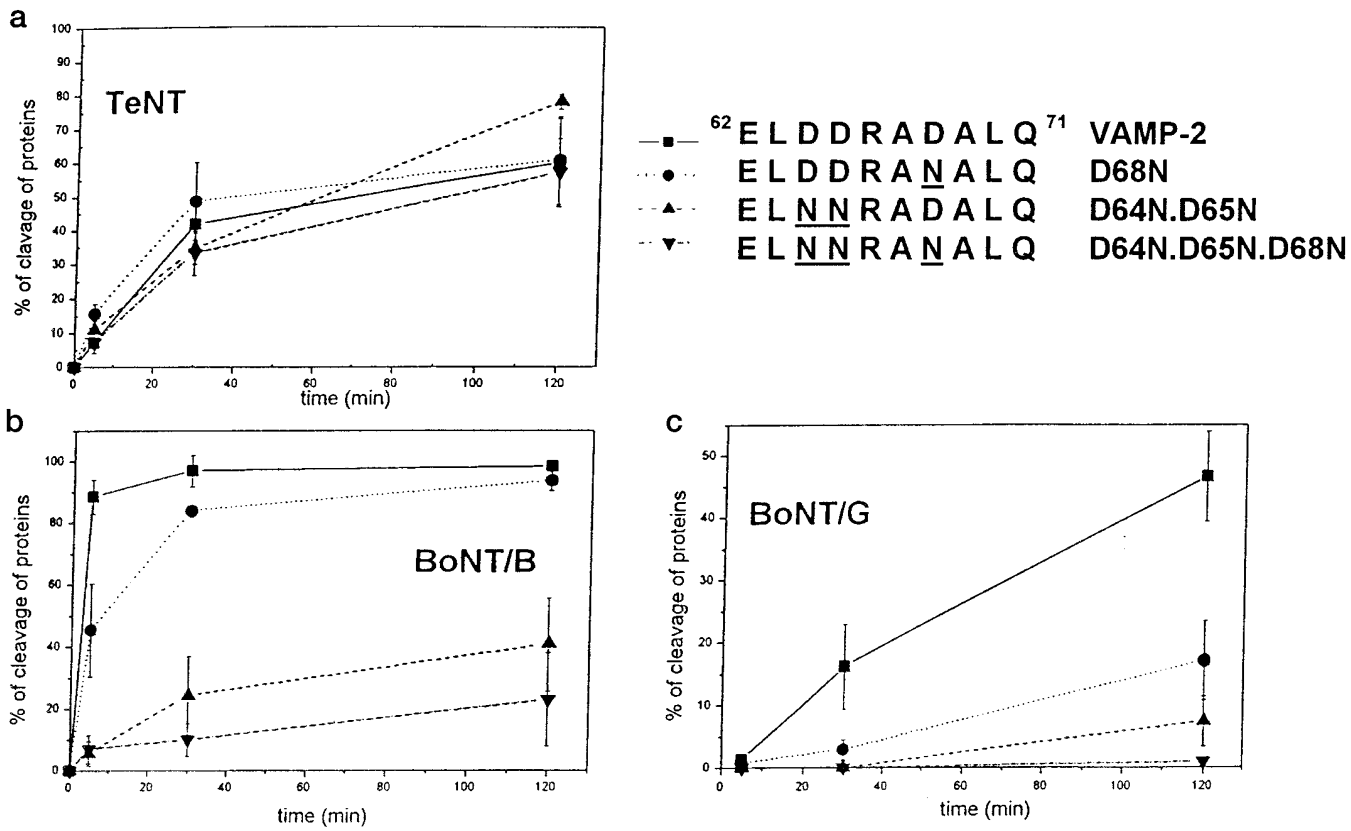


FIG. 4. **Neurotoxin proteolysis of VAMPs containing mutations that progressively remove the negative charges of the V2 SNARE motif.** The rate of proteolysis of GST-VAMP-2 and the GST-VAMP-2 mutants are indicated (*top right*) with TeNT (*panel a*), BoNT/B (*panel b*), and BoNT/G (*panel c*), determined as detailed under "Materials and Methods." Samples were removed at 5, 30, and 120 min, electrophoresed, and silver-stained, and the amount of protein was determined by densitometric scanning. Data are the average of four different experiments, and bars represent S.D. values.

VAMP as a substrate: TeNT and BoNT/B proteolysis of VAMP is inhibited by BoNT/A, BoNT/C, and BoNT/E. The inhibitory effect is not complete and differs for BoNT/A, BoNT/C, and BoNT/E. This is to be attributed to the fact that BoNT/A, BoNT/C, and BoNT/E bind VAMP via a single interaction with the SNARE motif, whereas TeNT and BoNT/B interact with VAMP at two sites: the SNARE motif and the segment around the cleavage site. The result of Fig. 3*b* indicates that BoNT/A interacts with the SNARE motif more strongly than BoNT/C and BoNT/E. Similar inhibitory effects were found when TeNT or BoNT/B were present in assays of proteolysis of SNAP-25 by BoNT/A and BoNT/E (not shown).

Mutation of the Negatively Charged Residues of V2 Affects VAMP Cleavage by Botulinum B and G—As shown in Fig. 1, the SNARE motif is characterized by the presence of three negatively charged residues, which cluster on a face if the motif is arranged in an α-helix. As a first approach to the study of the role of the different residues of the motif in neurotoxin specificity, each of the three aspartic residues of the V2 segment of VAMP was replaced with asparagine. This mutation was chosen because it is the most conservative substitution compatible with the removal of the negative charge of the carboxylate residue. Such mutant VAMPs were purified as GST fusion proteins from *E. coli* extracts, and the proteolytic activity of VAMP-specific clostridial neurotoxins was tested. Previous studies with VAMP segments of different lengths (11, 23, 38–40) implicated V2 in BoNT/B and BoNT/G binding of VAMP (4, 24).

Fig. 4 shows that progressive replacement of the three Asp residues of the V2 segment of VAMP with Asn residues causes a progressive loss of proteolytic activity of BoNT/B and BoNT/G. The effect of the D68N replacement appears to be larger for BoNT/G than for BoNT/B, although the lower enzymic activity of BoNT/G may be partially responsible for this effect. On the contrary, TeNT cleaves the three VAMP mutants at similar rates. On the basis of the close structural similarity between clostridial neurotoxins, this result was unexpected. However, it is possible that TeNT binds V2 via hydrogen bonding interactions, which can be formed both with Asp and with Asn lateral chains. Therefore, the two Asp residues 64 and 65 of V2 were converted into Ser residues, which are hydrophilic but have a much smaller lateral chain. The VAMP-2 D64S, D65S mutant is, however, cleaved by TeNT at a rate very similar to that of wild type VAMP-2 (Fig. 5).

Mutation of the Negatively Charged Residues of V1 Affects VAMP Cleavage by Tetanus Neurotoxin, but Not by Botulinum B and G Neurotoxins—Another possibility that may account for the results of Fig. 5*a* is that TeNT recognizes VAMP via an interaction with V1, rather than V2. To test this possibility, Asp^{40} and Glu^{41} of VAMP-2 were replaced by the corresponding Asn and Gln residues. Fig. 6 shows that this VAMP-2 mutant is not cleaved by TeNT (*panel a*), while the same protein is proteolyzed by BoNT/B as efficiently as the wild type VAMP-2 (*panel b*). These results indicate that TeNT and BoNT/B, the only two clostridial neurotoxins that recognize and cleave the same target protein at the same peptide bond (8), actually differ from each other. They are identical with respect to the consequence of their proteolysis because the same two VAMP fragments are generated. But they differ in their mode of substrate recognition, because this recognition is mediated by two different segments of VAMP.

VAMPs mutated in V1 and V2 were also tested as proteolytic substrates of TeNT and BoNT/B in the presence of BoNT/A. The ability of this latter toxin to inhibit TeNT and BoNT/B

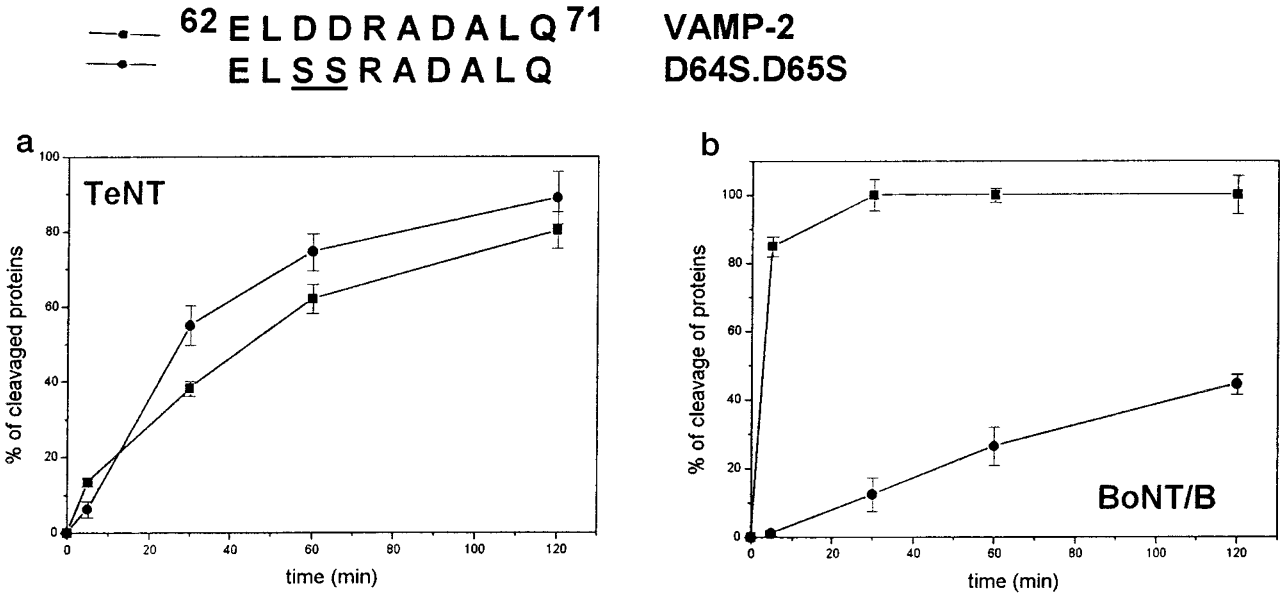


FIG. 5. **Tetanus and botulinum B neurotoxin proteolysis of VAMP mutants containing Asp → Ser substitutions in the V2 motif.** GST-VAMP-2 and mutants with Ser residues replacing Asp residues at positions 64 and 65 (*D64S.D65S*) were incubated with TeNT (*panel a*) or BoNT/B (*panel b*) as described in the legend to Fig. 4. Data are the average of three independent experiments, and *bars* are S.D. values.

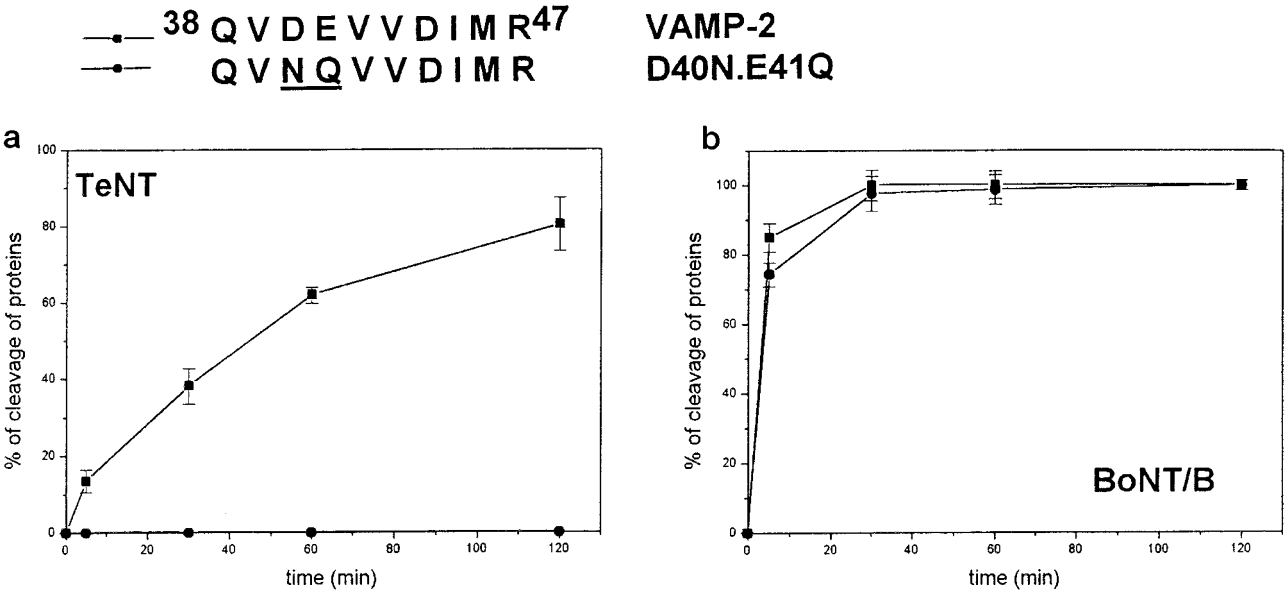


FIG. 6. **Substitution of the carboxylate residues in positions 40 and 41 of VAMP abolishes proteolysis with tetanus neurotoxin but not with botulinum B neurotoxin.** GST-VAMP-2 and GST-VAMP-2 mutants in which two carboxylated residues of the V1 motif were replaced with the corresponding amide groups (*D40N.E41Q*) were incubated with TeNT (*panel a*) or BoNT/B (*panel b*) under the same conditions of Fig. 4. Samples were removed at 5, 30, 60, and 120 min, electrophoresed, silver-stained, and quantitated by densitometric scanning. Data are averages of three independent experiments, and *bars* are S.D. values.

proteolysis of VAMP, documented in Fig. 3*b*, was reduced when VAMP-2 D64S,D65S mutant and the VAMP-2 Asp^{40} and Glu^{41} were assayed (not shown). However, results did not allow us to draw a clear conclusion of a possible preference of BoNT/A between V1 and V2.

DISCUSSION

The light chain of the eight clostridial neurotoxins have a unique specificity for VAMP, SNAP-25, and syntaxin, termed SNAREs (5–7). Their three-dimensional structure is not known. Available spectroscopic and biochemical data, as well as comparison of their primary and predicted secondary structure, indicate that they are closely similar. Nonetheless, they show unique proteolytic activity exerted on different peptide bonds of three different protein substrates. Short peptides encompassing the cleavage site of VAMP-2 cannot be cleaved by TeNT and BoNT/B. For proteolysis to occur, long VAMP peptides, including V2 and/or V1, are required (4, 38–41). Hence, the neurotoxin's specificity cannot be explained by the sole recognition of the cleavage site (4, 39). A negatively charged motif was identified in the three SNAREs, and it was suggested that it is implicated in their selective recognition by clostridial neurotoxins (22, 24). Here, we report strong evidence in favor of the involvement of the SNARE motif in the interaction between VAMP and TeNT and BoNT/B and BoNT/G. 1) Antibodies, raised against motif peptides or recombinant proteins, recognize and cross-react among the three SNAREs, despite subtle sequence differences in the specific SNARE motifs of VAMP, SNAP-25, and syntaxin. This result indicates that this is a

common structural element of SNAREs. 2) Occupation of the motif with an antibody or with a non-VAMP-specific neurotoxin inhibits VAMP proteolysis by TeNT and BoNT/B. The partial and variable inhibitory effects observed can be explained by the fact that antibodies and neurotoxins can compete for only one of the two sites involved in the specific binding of the appropriate neurotoxin type. 3) Mutation of the acidic residues of the motif leads to resistance of VAMP to the neurotoxin proteolysis. Based on the clostridial neurotoxin similarity mentioned above, we had assumed that TeNT and BoNT/B, which cleave VAMP at the same peptide bond, were also similar in their recognition. Hence, we were expecting that mutation in V2, the SNARE motif copy next to the cleavage site, would affect to a similar extent TeNT and BoNT/B proteolysis of VAMP. The results of the mutagenesis experiments reported here clearly show that TeNT, BoNT/B, and BoNT/G are all different from each other, not only from the serological point of view, but also with respect to their interaction with VAMP. The two botulinum neurotoxins interact with the same V2 segment but cleave VAMP at two different peptide bonds (8, 12). Conversely, TeNT and BoNT/B cleave VAMP at the same peptide bond (8) but recognize VAMP via two additional different segments: V1 and V2, respectively. This result explains the recent finding of Foran *et al.* (40) that BoNT/B, but not TeNT, cleaves a peptide corresponding to the VAMP-2-(55–94) segment and that TeNT requires an amino-terminal extension of 12 residues to cleave efficiently. The present results are potentially relevant also with respect to VAMP structure. All available data suggest that the L chains of TeNT and BoNT/B fold very similarly. If this is the case, a corresponding structural similarity must be present in the VAMP structure. In other words, the present results are best explained by assuming that V1 and V2 occupy spatially equivalent positions with respect to the Gln^{76}–Phe^{77} peptide bond cleaved by TeNT and BoNT/B. We suggest that V1 and V2 are arranged as a tandem association in the VAMP structure and that this particular three-dimensional organization plays a role in the biological function of VAMP. Recently, Kelly and colleagues (42, 43) have analyzed exoendocytosis in cells transfected with VAMP mutants deleted in various portions of the molecule. It was found that deletion of V1 leads to a deficient targeting of VAMP-2 to the synaptic vesicles. Hence, it appears that tetanus neurotoxin interacts with a crucial part of the VAMP molecule, a part that cannot be altered without negative consequences for a fundamental physiological process. Our findings are very relevant to the possible engineering of TeNT- or BoNT-cleavable domain in a protein whose function is to be abolished at any given time in a cell by toxin exposure. This is suggested by a recent study in which tetanus toxin light chain was expressed in *Drosophila* and it was found that only one of the two VAMP isoforms was cleaved by TeNT. The two *Drosophila* VAMPs have an identical sequence at the cleavage site, but in the TeNT-resistant VAMP isoform a Gly residues replaces the third Asp residue within the V1 motif (44).

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