The interaction of synaptic vesicle-associated membrane protein/synaptobrevin with botulinum neurotoxins D and F

Rossella Pellizzari[a], Silvia Mason[a], Clifford C. Shone[b], Cesare Montecucco[a,*]

[a]*Centro CNR Biomembrane and Dipartimento di Scienze Biomediche, Università di Padova, Via G. Colombo 3, 35100 Padova, Italy*
[b]*Center for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, UK*

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Abstract Botulinum neurotoxins type D and F are zinc-endopeptidases with a unique specificity for VAMP/synaptobrevin, an essential component of the exocytosis apparatus. VAMP contains two copies of a nine residue motif, termed V1 and V2, which are determinants of the interaction with tetanus and botulinum B and G neurotoxins. Here, we show that V1 plays a major role in VAMP recognition by botulinum neurotoxins D and F and that V2 is also involved in F binding. Site-directed mutagenesis of V1 and V2 indicates that different residues are the determinants of the VAMP interaction with the two endopeptidases. The study of the VAMP-neurotoxins interaction suggest a pairing of the V1 and V2 segments.

Key words: Tetanus; Botulism; Neurotoxin; Zinc-endopeptidase; VAMP; SNARE motif

1. Introduction

Clostridial neurotoxins form a novel group of zinc-endopeptidases that act in the cytosol of neurons and block neurotransmitter release [1]. They consist of two disulfide-linked chains: the larger one (H chain, 100 kDa) binds specifically to synaptic terminals and promotes the entry of the shorter chain (L chain, 50 kDa) to the cytosol. The metalloproteolytic activity of these neurotoxins is associated to the L chain [2–5]. The only known proteolytic substrate of tetanus neurotoxin (TeNT) and of botulinum neurotoxins serotype B, D, F and G (BoNT/B, BoNT/D, BoNT/F, BoNT/G) is VAMP/synaptobrevin (VAMP) (Fig. 1), which is cleaved at single different peptide bonds [6–14]. VAMP is a tail-anchored membrane protein of exocytotic vesicles and the cellular consequences of its cleavage demonstrate that it is an essential component of the exocytotic machinery [4,15,16]. VAMP is a family of different isoforms [17–22] and neurotoxin-sensitive isoforms are present in many non-neuronal tissues [23,24]. Consequently, the lack of activity of clostridial neurotoxins on non-neuronal cells is due to the fact that they do not bind to these cells because of their lack of cell surface neurotoxin receptors, rather than to the fact that they do not possess the neurotoxin intracellular substrates.

TeNT, BoNT/B, BoNT/D, BoNT/F and BoNT/G cleave VAMP within a conserved region and it has been suggested that their substrate specificity is due to the recognition of the segment encompassing the cleavage site and of a nine residue motif, termed SNARE motif from the collective name of SNAREs given to the three clostridial neurotoxin substrates [16,17,25]. The motif is present in two copies (V1 and V2) in the VAMP molecule (see Fig. 1). Recently, we have obtained experimental evidence for the involvement of V1 in VAMP-TeNT interaction, whereas V2 is a determinant of the VAMP proteolysis by BoNT/B and BoNT/G [26,27]. Here, we extend these studies to BoNT/D and BoNT/F and show that their interaction with VAMP differs in terms of ionic strength sensitivity and of SNARE motif residues involved. These results not only uncover the molecular basis of the recognition and cleavage of VAMP by the clostridial neurotoxins, but also provide novel information on the possible spatial arrangements of the SNARE motifs of VAMP.

2. Materials and methods

2.1. Proteins, peptides and chemicals

BoNT/D and BoNT/F were prepared as detailed before [28,29] and immobilized-metal-ion affinity chromatography was used to remove traces of contaminant proteases [30].

2.2. Bacterial strains, plasmid construction and VAMP mutagenesis

The PCR product of rat *VAMP-2* gene was cloned into a pEMBL vector as detailed before [26] and used to produce mutated or deleted proteins. The D41N,E41Q mutant of rat VAMP-2 was obtained as previously described [26]. Uracil containing ssDNA was produced within BW313, an *E. 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. [31]. The following oligonucleotides were used to generate the corresponding mutants: 5′-ATTCACCCTCGCGATGTCCACCA-3′ for VAMP-2M46A, 5′-ATGATGTCCGCCACCTCATC-3′ for VAMP-2V43A, 5′-AGGGCATCTACGCGATCATCC-3′ for VAMP-2A67V, 5′-CCTCATGATGTTAACCACCTCATC-3′ for VAMP-2 D44N, and 5′-CTTGTCCACCCTCACCTGGGCCTGGGTCTGCT-3′ to make the deletion D40-46 by looping out. The sequence of the mutated genes was checked by dideoxy sequencing using fmole DNA sequencing system (Promega). Wild-type VAMP-2 and mutants were subcloned into *Bam*HI and *Eco*RI sites of pGEX-KG vector [32] and transformed into the AB1899 strain of *E. coli*.

Rat VAMP-2 and its mutants were expressed as GST-fusion proteins and purified by affinity chromatography on GSH-agarose matrix (Pharmacia).

2.3. Assay of proteolytic activity

After preincubation with 10 mM DTT in 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, for 30 min at 37°C, BoNT/F (100 nM) or BoNT/D (25 nM) was added to the reaction mixture containing one of the different GST-fusion proteins (50 μg/ml). Proteolysis was carried out for 2 h in 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, at 37°C [12]. Under these conditions of toxin concentration and incubation time, the extent of proteolysis is nearly complete with wild-type GST-VAMP, thus allowing a valuable comparison of data obtained with the different VAMP mutants. To test the ionic strength sensitivity of

*Corresponding author. Fax: (39) 49-8276049.
E-mail: cesare@civ.bio.unipd.it

Abbreviations: DTT, dithiothreitol; GST, glutathione *S*-methyl transferase; BoNT, botulinum neurotoxin; TeNT, tetanus neurotoxin; VAMP, vesicle-associated membrane protein; VAMP-2, VAMP isoform 2; SNARE, soluble *N*-ethyl-maleimide-sensitive factor accessory proteins receptor

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the proteolytic activity of BoNT/D and BoNT/F, buffers containing 10 mM Na_2HPO_4, pH 7.4, and NaCl in the range of concentration of 0.1, 0.2, 0.5 or 1 M were used. The time course of GST-VAMP proteolysis was estimated by separation of the VAMP fragments by SDS-PAGE and quantitation of the Coomassie Blue stained bands with a dual wavelength Shimadzu CS-630 densitometer, as described before [12].

3. Results and discussion

BoNT/F and BoNT/D cleave adjacent peptide bonds of VAMP: they hydrolyse the Gln^{58}/Lys^{59} and Lys^{59}/Leu^{60} peptide bonds (rat VAMP-2 numbering), respectively [7,9,14]. Together with the fact that their L chains have closely similar sequences [33], this suggested the possibility that their interaction with VAMP is based on similar residues and forces. However, a preliminary analysis of the effects of ionic strength on VAMP proteolysis indicates that this is not the case. Fig. 2 shows that 0.5 M NaCl abolishes the proteolytic activity of BoNT/F, whereas it has no effect on BoNT/D activity. Such inhibition of BoNT/F proteolytic activity is not due to its denaturation induced by high molarity solutions of NaCl because VAMP is fully proteolysed by a sample of BoNT/F, which was treated with 0.5 M NaCl and then diluted to a final NaCl concentration of 0.1 M (not shown). The BoNT/B cleavage of VAMP was previously found to be influenced by ionic strength and this effect was dependent on the negatively charged residues of the V2 motif [13,27].

To identify VAMP residues which are determinants of the interaction with BoNT/D and BoNT/F and which could account for such a diverse effect of ionic strength, a site-directed mutagenesis approach was undertaken. Previous results had shown that the specific recognition of VAMP by clostridial neurotoxins is mediate by: (i) binding of the VAMP residues at and around the cleavage site [13,34] and (ii) two segments of VAMP, which are located amino terminal with respect to the cleavage site [25–27]; these two segments are the V1 and V2 copies of the SNARE motif present in the VAMP molecule (see Fig. 1). The SNARE motif is characterized by an alternation of aliphatic residues and of three negatively charged amino acids. The peptide bonds of VAMP hydrolysed by BoNT/F and BoNT/D are comprised between V1 and V2. The importance of V1 in BoNT/D and BoNT/F proteolysis of VAMP was indicated in preliminary experiments with a V1 deleted VAMP-2 mutant, which was not cleaved at all by these two proteases (left panels of Figs. 3 and 4). Hence, mutants of the six carboxylate residues, present in the two rat VAMP-2 SNARE motifs, were designed together with a mutant of Met46, a residue previously shown to affect BoNT/D activity [14]. These VAMP-2 mutants were expressed as fusion proteins with GST in *E. coli* and purified from bacterial extract by affinity chromatography on GSH-Sepharose. GST-VAMP is a good substrate for the neurotoxins [12,26,35].

V1 plays a major role in the TeNT recognition of VAMP, whereas V2 is involved in BoNT/B and BoNT/G binding [26,27]. For the remaining two neurotoxins having VAMP-2 as a target, we found that single substitutions of any of the V1 Asp or Glu residues with the corresponding Asn or Gln abolish the proteolytic activity of BoNT/F (Fig. 3), whereas such substitutions do not affect BoNT/D proteolytic activity (Fig. 4). The replacement of the carboxylate residues of the V2 segment with the corresponding amide residues also influences the cleavage of VAMP-2 by BoNT/F, but the effect of such

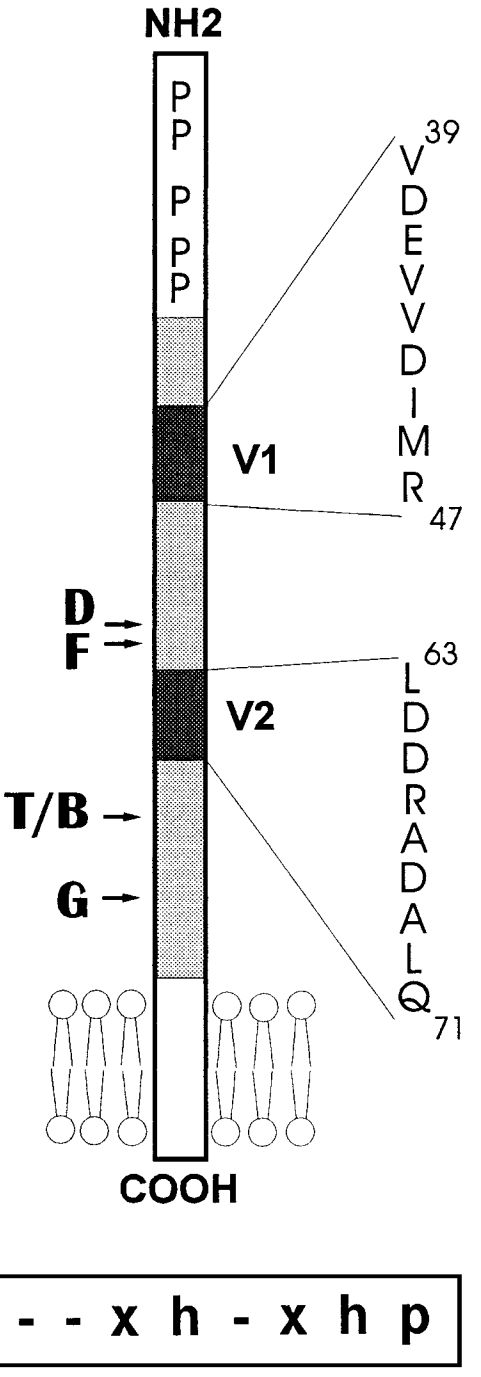

Fig. 1. Schematic structure of rat VAMP-2. The N-terminal part is proline rich and is not conserved among VAMP isoforms. The central grey region is highly conserved and includes the two copies of the SNARE motif, indicated by V1 and V2. The positions of the capital letters correspond to the cleavage sites of tetanus neurotoxin (T) and of botulinum neurotoxins type B, D, F and G. The lower part of the figure represents the SNARE motif (with -, carboxylate residues; h, aliphatic residues; p, polar residues; x, any residue).

mutations is less pronounced than that of mutating the corresponding residues of the V1 segment (Fig. 3). All the VAMP-2 mutants in the V2 segment are cleaved by BoNT/D at the same rate as the control. Taken together, these results indicate that carboxylate residues of the V1 segment play a major role in the interaction of BoNT/F and that also the corresponding residues of the V2 segment are implicated. Electrostatic interactions of positively charged residues of

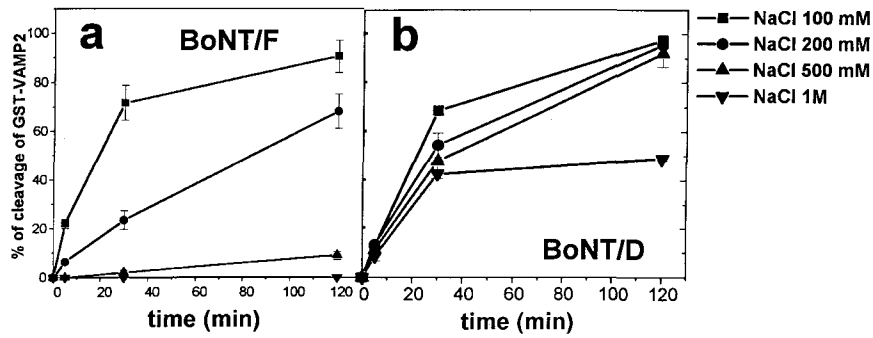

Fig. 2. Effect of ionic strength on the rate of VAMP proteolysis of BoNT/F and BoNT/D. Proteolysis of GST-VAMP2 was carried out in 10 mM Na_2HPO_4, pH 7.4, with different concentrations of NaCl, as indicated in the picture. The rate of cleavage of GST-VAMP2 protein was determined as reported in Section 2. Samples were removed at 5, 30 and 120 min, electrophoresed, and the amount of protein was determined by densitometric scanning of Coomassie stained gels. Data are the average of three different experiments, and bars represent SD values.

BoNT/F with negatively charged residues of VAMP provide an explanation for the strong effect of ionic strength on BoNT/F proteolysis. Conversely, the lack of effect of such mutations on BoNT/D activity is in agreement with its lack of sensitivity to ionic strength.

Recombinant rat VAMP-1 is proteolysed very slowly by BoNT/D, and a Val for Met substitution at position 46 is held responsible for its resistance to BoNT/D [14]. Fig. 4 shows that a recombinant rat VAMP-2 presenting the single substitution of Met^{46} with an Ala residue is completely resistant to BoNT/D. These results identify Met^{46} as the key residue of the V1 segment in the interaction of BoNT/D with VAMP.

The present study completes the analysis of the effect of mutations of V1 and V2 residues on the proteolytic activity of the clostridial neurotoxins specific for VAMP. As in the case of TeNT, BoNT/B and BoNT/G, the SNARE motifs of VAMP are major determinants of the remarkable specificity of these metalloproteases. However, in addition to this general conclusion, results described here clearly show that different residues play different roles in the recognition of VAMP by BoNT/F and BoNT/D. The different mode of interaction mechanism of these two toxins with their substrate is due to structural differences among the two proteases that are difficult to identify. However, it is difficult to attribute the effects described here to particular residues of the BoNT/D and BoNT/F molecules. There are several positions which are occupied by a positively charged residue in BoNT/F sequence and by an uncharged residue in the sequence of BoNT/D. In particular, around residue 30 of BoNT/F there are three lysines, not conserved in BoNT/D, spaced as the three carboxylates of the SNARE motif, suggesting possible sites for a mutagenesis analysis of BoNT/F VAMP recognition.

The present findings that mutation of negatively charged amino acids of both the V1 and V2 segments of VAMP influence the rate of VAMP proteolysis by BoNT/F are in keeping with the suggestion that V1 and V2 may be arranged in tandem within the VAMP molecule [26]. This particular structural arrangement may be induced by the interaction with clostridial neurotoxins during the cleavage, or be one of the conformations adopted by VAMP-2, whose analysis by circular dichroism and NMR has not revealed elements of secondary structure [10]. The possibility of a pairing of the V1 and V2 motifs of VAMP-2 is in keeping with a recent study on the effect of deletions on the capability of VAMP of supporting exocytosis of insulin [34]. The most deleterious deletion was found to be that of segment 51–60 which is included in the 48–62 region between V1 and V2 (Fig. 1). The simplest ex-

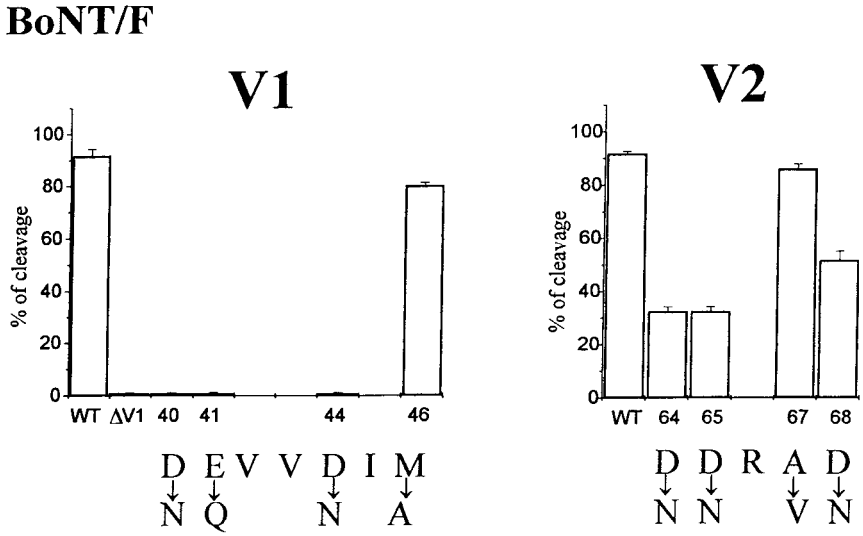

Fig. 3. Effect of substitution of charged residues of V1 and V2 motif of VAMP-2 on the proteolysis by BoNT/F. The percent of cleavage of GST-VAMP2 and GST-VAMP2 mutants of the V1 and V2 copies of the motif are compared. Numbers in horizontal axis indicate positions of VAMP residues that have been mutated and ΔV1 indicates the mutant deleted of segment 40–46. Substrates were incubated with BoNT/F 100 nM, as specified in Section 2. After 2 h at 37°C, samples were electrophoresed, Coomassie blue stained and quantitated by densitometric scanning. Data are averages of four independent experiments and error bars indicate SD values.

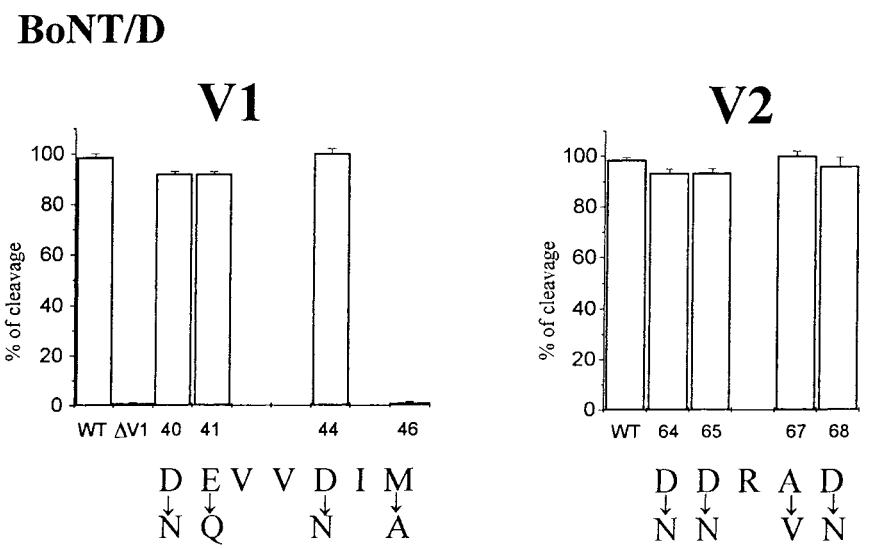

Fig. 4. Effect of substitution of the charged residues of V1 and V2 motif of VAMP-2 and of Met46 on the proteolysis by BoNT/D. Symbols and conditions are as in the legend of Fig. 3, except for the concentration of BoNT/D (25 nM). Data are averages of four independent experiments and error bars indicate S.D. values.

planation of this result is that any shortening of the segment connecting V1 to V2 prevents their parallel pairing. Deletions of V1 and V2 also gave non-functional VAMP mutants [34]. It was previously shown that deletion of V1 leads to a deficient targeting of VAMP-2 to the synaptic vesicles [35,36]. These studies indicate that the SNARE motifs play essential roles in VAMP localization and function, and hence they have been conserved during evolution, even in the presence of the selective pressure exerted on vertebrate animals by the widespread toxigenic Clostridia.

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