FEBS Letters 542 (2003) 132–136 FEBS 27195

VAMP/synaptobrevin cleavage by tetanus and botulinum neurotoxins is strongly enhanced by acidic liposomes

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Received 6 February 2003; revised 2 April 2003; accepted 3 April 2003

First published online 15 April 2003

Edited by Felix Wieland

Abstract Tetanus and botulinum neurotoxins (TeNT and BoNTs) block neuroexocytosis via specific cleavage and inactivation of SNARE proteins. Such activity is exerted by the N-terminal 50 kDa light chain (L) domain, which is a zinc-dependent endopeptidase. TeNT, BoNT/B, /D, /F and /G cleave vesicle associated membrane protein (VAMP), a protein of the neurotransmitter-containing small synaptic vesicles, at different single peptide bonds. Since the proteolytic activity of these metalloproteases is higher on native VAMP inserted in synaptic vesicles than on recombinant VAMP, we have investigated the influence of liposomes of different lipid composition on this activity. We found that the rate of VAMP cleavage with all neurotoxins tested here is strongly enhanced by negatively charged lipid mixtures. This effect is at least partially due to the binding of the metalloprotease to the lipid membranes, with electrostatic interactions playing an important role.

Key words: Tetanus neurotoxin; Botulinum neurotoxin; Vesicle associated membrane protein/synaptobrevin; Liposome

1. Introduction

Clostridial neurotoxins form a highly homologous family of zinc metalloendopeptidase proteins endowed with unique characteristics. They comprise tetanus neurotoxin (TeNT) and seven botulinum neurotoxins (BoNT from A to G). TeNT acts within the spinal cord and causes the spastic paralysis of tetanus, whereas the BoNTs act peripherally by blocking acetylcholine release at the neuromuscular junction, causing the characteristic flaccid paralysis of botulism. These neurotoxins are released by bacteria of the genus Clostridium as inactive 150 kDa polypeptide chains. Specific proteolytic cleavage generates disulfide-linked di-chain toxins which are the most toxic substances known, with mouse LD_{50} values in the 0.1–1 ng/kg range.

The structural architecture of these neurotoxins is very similar and it is related to their mode of neuronal intoxication [1]. The heavy chain (H, 100 kDa) consists of a C-terminal 50 kDa domain responsible for the specific binding to the presynaptic membrane and of an N-terminal domain of 50 kDa responsible for the subsequent translocation of the light chain (L, 50 kDa) across the vesicular membrane into the cytosol. Here, the L chain displays a metalloprotease activity specific for three proteins, collectively termed SNARE proteins, which are core components of the machinery that mediates small synaptic vesicles (SSV) fusion and release of neurotransmitters from nerve terminals. In particular, BoNT/A and /E cleave SNAP-25, whereas BoNT/C cleaves both syntaxin and SNAP-25, which are located on the cytosolic face of the plasma membrane. TeNT, BoNT/B, /D, /F and /G cleave vesicle associated membrane protein (VAMP)/synaptobrevin, an integral membrane protein of SSV [2,3].

VAMP belongs to a large and growing family of proteins implicated in the fusion of vesicles with target membranes within the cell [4]. The VAMP isoforms involved in neuroexocytosis consist of four parts: (a) a non-conserved 33 residues long N-terminal portion, (b) a highly conserved 33–96 domain, which contains the *core* of the fusion machinery [5], (c) a 20 residues long transmembrane domain and (d) an intraluminal tail of variable size [6,7]. The VAMP-specific clostridial neurotoxins cleave at single different sites within the conserved region forming two fragments. The toxin-cleaved VAMP is unable to form the heterotrimeric syntaxin–VAMP–SNAP-25 complex which mediates the fusion of the neurotransmitter-containing SSV with the presynaptic membrane.

The proteolytic activity of the L chain of the VAMP-specific clostridial neurotoxins can be conveniently assayed with recombinant VAMP or VAMP fragments, with synaptic vesicles, or by injecting the L chain inside neurons [8–11]. Comparison of the rate of VAMP cleavage or recombinant isolated VAMP or VAMP fragments in vitro with the rate of blockade of neurotransmitter release in vivo and with that of VAMP bound to synaptic vesicles indicates that the toxin L chain is more active under the latter two conditions [2,3]. It has been reported that BoNT/A and /E are phosphorylated

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Abbreviations: BoNT, botulinum neurotoxin; LSB, Laemmli sample buffer; PBS, phosphate-buffered saline; SSV, small synaptic vesicles; TeNT, tetanus neurotoxin; VAMP, vesicle associated membrane protein

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00365-X

inside neurons and that the phosphorylated toxin is more active [12]. However, comparable information is not available for the VAMP-cleaving neurotoxins. Considering that they act on an integral membrane protein which resides in a lipid environment, we have tested the possibility that lipids enhance the activity of these metalloproteases as a consequence of their binding to membranes. Here, we show that negatively charged lipids strongly promote VAMP cleavage by TeNT, BoNT/B, /D and /F. The L chain of TeNT and the cytosolic 1–96 segment of VAMP are capable of binding to negatively charged liposomes via electrostatic as well as other interactions, creating conditions that largely favor their encounter. These findings unveil an additional feature of the activity of this unique class of metalloproteases which is relevant to their in vivo activity.

2. Materials and methods

2.1. Materials

TeNT was isolated from culture filtrates of *Clostridium tetani* as detailed before [13]. BoNT/B, /D and /F were produced and purified as routinely performed in the laboratory of the University of Wisconsin, Madison, WI, USA [14]. BoNT/B was nicked with 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Serva) as described [15] and the cleavage was blocked by adding a four-fold excess of soybean trypsin inhibitor. Toxicity tests performed on mice hemidiaphragm preparations showed that all neurotoxin preparations used here were highly active.

Rat VAMP-2 full-length (1–116) and cytosolic (1–96) domains were expressed in *Escherichia coli* as a glutathione S-methyltransferase fusion protein and were purified by affinity chromatography on GSH-agarose matrix (Sigma). The TeNT L chain was expressed and purified as described previously [16]. SSV were isolated from rat cerebral cortex [17]. Phospholipids purified from bovine brain were prepared as described [18]. Asolectin, cholesterol, phosphatidic acid and dioleoyl L-α-phosphatidylcholine were purchased from Sigma. Goat anti-rabbit antibodies conjugated with peroxidase were from Calbiochem.

2.2. Preparation of liposomes

Soybean asolectin, dioleoyl L-α-phoshatidylcholine, bovine brain phospholipids or PA lipid mixture (dioleoyl L-α-phosphatidylcholine 70%, phosphatidic acid 20% and cholesterol 10%) were dissolved in chloroform, dried to a thin film under a gentle N_2 flow and vacuum pumped for at least 2 h to remove residual traces of organic solvent. The dried lipid film was then resuspended at the concentration of 10 mg/ml in 10 mM sodium phosphate buffer, pH 7.3, containing 150 mM NaCl. Glass beads were then added and vortexed for 15 min until optical clarity was achieved. Alternatively, the lipid suspension was sonicated inside a glass vial under N_2 until optical clarity was attained.

2.3. Proteolytic activity

The proteolytic activity of TeNT, BoNT/B, /D and /F was tested on recombinant VAMP-2/1–116 in buffer or incorporated in liposomes. Briefly, 20 μg of recombinant glutathione-S-transferase (GST)–VAMP-2/1–116 in 20 μl of 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.3 (cleavage buffer) were incubated at 37°C with toxin (40 nM BoNT/B, 20 nM BoNT/D, 40 nM BoNT/F or 100 nM TeNT) previously reduced with 10 mM dithiothreitol (DTT) for 30 min at 37°C. In some experiments recombinant GST–VAMP-2/1–116 was preincubated with gentle stirring with 20 μg of lipid vesicles for 1 h at room temperature. After different time periods, the reaction was stopped by adding sodium dodecyl sulfate (SDS) Laemmli sample buffer (LSB) and samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (13% polyacrylamide), and stained with Coomassie blue.

SSV (250 μg) in 110 μl of 5 mM HEPES–Na, 0.3 mM glycine, pH 7.4 were incubated with toxin and, after different time periods, 60 μg of sample were mixed with LSB and analyzed in a 13–18% polyacrylamide gradient gel. Gels were stained in Coomassie blue. To determine the time course of VAMP proteolysis, gels were scanned with a Gel Doc 2000 Bio-Rad system.

2.4. High-speed sedimentation assay

The binding of proteins to liposomes was assayed in 10 mM NaH_2PO_4 with 150 mM NaCl as described previously [19]. Briefly, 2–3 μg of GST–VAMP-2/1–116, of VAMP-2/1–96 and of TeNT L chain were incubated with 20 μg of liposomes for 1 h at room temperature, and then samples were layered onto a sucrose cushion (5% sucrose in the same buffer) and subjected to ultracentrifugation at $200\,000 \times g$ for 15 min in a Beckman TLA-100 ultracentrifuge to separate the pelleted bound protein from the free protein in the supernatant. Pellets were resuspended in 15 μl of LSB, whilst the protein present in the supernatant fraction was recovered by precipitation with trichloroacetic acid, followed by resuspension in LSB. Samples were subjected to SDS–PAGE stained with Coomassie blue and the bound and free proteins were quantified by scanning with a Gel Doc 2000 Bio-Rad system.

2.5. Lipid binding plate assay

Microtiter plates were coated with different lipids mixture prepared as described above and resuspended in water at a final concentration of 10 ng/μl and 1 ng/μl; 100 μl of these suspensions were added to each well. Samples were allowed to dry overnight at 37°C, prior to saturation with rat serum for 4 h at 4°C. 0.5–2.5 μg of TeNT L chain in phosphate-buffered saline (PBS) containing 0.5% bovine albumin (binding buffer) was added and incubated for 3 h at room temperature. In some experiments, NaCl from a 4 M stock solution was added to reach the final desired salt concentration. After six consecutive washings with binding buffer, the amount of bound protein was estimated by incubation with a polyclonal anti-TeNT antibody for 3 h at room temperature under continuous mixing. After four washings with PBS, the samples were incubated with a goat anti-rabbit IgG peroxidase conjugate for 1 h at room temperature and washed again. 100 μl of peroxidase substrate buffer (NaH_2PO_4 50 mM pH 5.0, Triton X-100 0.1%, *o*-dianisidine 0.5 mM, H_2O_2 0.003%) were added to each well and the absorbance was measured at 405 nm after 1 h. The absorbance due to the primary antibody bound to the lipid film in the absence of protein was subtracted. Each assay was performed in triplicate.

3. Results

Preliminary experiments performed with TeNT showed a very large difference between the amount of VAMP cleaved by TeNT after 1 h incubation when using VAMP alone or VAMP inserted in synaptic vesicles. Therefore, we investi-

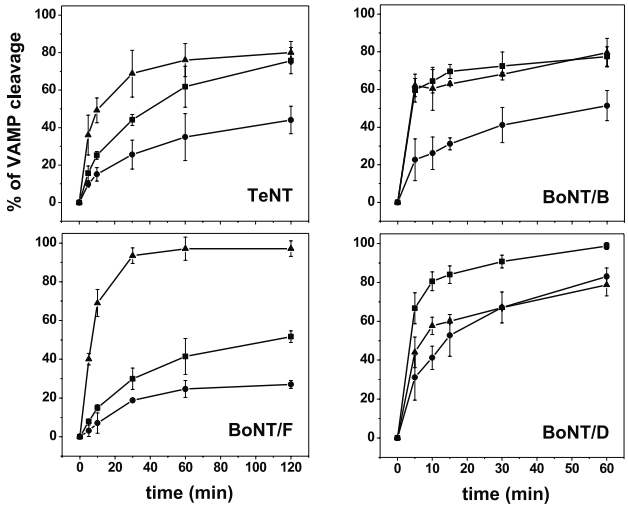

Fig. 1. The proteolytic activity of the VAMP-specific neurotoxins is enhanced by the presence of lipid membrane. The proteolytic activity of TeNT, BoNT/B, /D and /F was tested on VAMP-2/1–116 alone (●) or incorporated in asolectin liposomes (■) or on the VAMP present on highly purified SSV (▲). Bars are the ±S.D. of three different experiments.

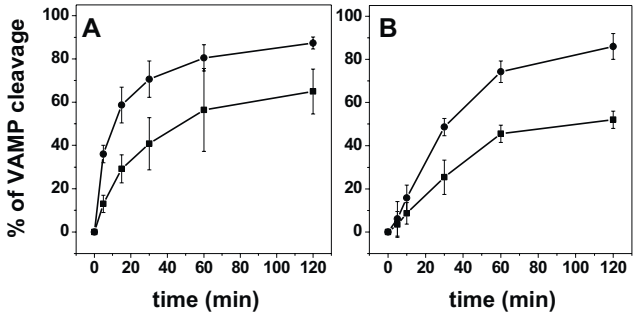

Fig. 2. The lipid activation of VAMP cleavage by clostridial neurotoxins does not depend on VAMP transmembrane domain. The proteolytic activity of TeNT L chain was measured at 37°C on full-length VAMP-2 (A) or on its cytosolic domain (fragment 1–96) (B) in the presence (●) or absence (■) of asolectin liposomes. Bars are the ± S.D. of three different experiments.

gated the possibility that this difference in proteolytic activity is due to the presence of a lipid membrane environment around VAMP, and we extended this analysis to three VAMP-specific BoNTs. Fig. 1 shows that indeed VAMP is cleaved much faster by TeNT, BoNT/B and BoNT/F when it is inserted in SSV; in contrast this lipid-activating effect is smaller in the case of BoNT/D. In the presence of liposomes made of soybean mixed lipids (asolectin), the rate of VAMP cleavage was enhanced in all cases, though to a variable extent for the four clostridial neurotoxins. Neurons are enriched in specific classes of negatively charged lipids such as gangliosides and cerebrosides that are absent from soybeans. Therefore, purified bovine brain lipid mixtures were also tested and found to activate TeNT very similarly to asolectin (not shown). Thus, it appears that neurotoxin cleavage does not require gangliosides or other lipids enriched in the nervous tissue. This lipid-promoting effect of VAMP-specific neurotoxin activity is consistent with the previous finding that BoNT/C-induced syntaxin proteolysis occurs efficiently only when syntaxin is inserted in lipid bilayers [20,21].

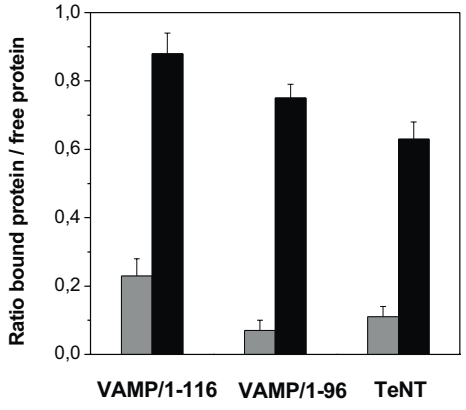

Fig. 3. Liposome binding of VAMP-2/1–116, of VAMP-2/1–96 and of the L chain of TeNT. The binding of the proteins to asolectin liposomes was quantified by determining the amount of protein present in the supernatant and in the pellet of samples of protein incubated with liposomes (black bars) or with buffer alone (gray bars) layered onto a sucrose cushion and ultracentrifuged as detailed in Section 2. Pellet (bound protein) and supernatant (free protein) were recovered and analyzed in SDS–PAGE. Data are given as ratios of the amount of protein present in the supernatant and in the pellet. Bars are the ± S.D. of three different experiments.

In order to investigate whether this lipid-promoting effect is dependent on the presence of the H chain of the toxin, full-length VAMP-2 was incubated with the L chain of TeNT in the presence or absence of asolectin liposomes. Fig. 2 shows that: (a) VAMP-2 is cleaved faster when it is inserted in asolectin liposomes also in the absence of the H chain of TeNT and (b) this lipid-promoting effect does not depend on the transmembrane segment of VAMP because it is still present when the cytosolic domain 1–96 of VAMP-2 is used as proteolytic substrate (Fig. 2B).

However, these results do not discriminate between an enhancing effect due to a lipid interaction with the metalloprotease and a lipid interaction with the substrate. Therefore, the ability of the recombinant TeNT L chain and of recombinant substrate VAMP to bind liposomes was tested. Liposome binding assay of TeNT L chain, of VAMP-2/1–116 and of VAMP-2/1–96 was performed by incubating the proteins with asolectin liposomes followed by ultracentrifugation to separate lipid-bound from unbound protein. Fig. 3 shows that all three proteins are able to bind to asolectin liposomes. This result is in agreement with the reports that VAMP/1–96 does interact with lipids [5,22,23].

Toxin binding to lipid mixtures of different compositions was further investigated with a well-established and simple assay which allows one to test a large array of conditions at the same time [24]. Lipids are coated onto the flat bottom of plastic wells and after saturation of unspecific binding sites with serum, the protein under test is added. The amount of lipid-bound protein was determined with a specific antibody. Fig. 4 shows that the L chain of TeNT does bind to asolectin-coated plastic wells and that high salt significantly reduces this binding. This binding is not significantly influenced by the composition of the acidic lipid mixture used since very similar results were obtained with bovine brain phospholipids or with a phosphatidylcholine–phosphatidic acid mixture (data not shown).

The effect of the net charge of liposomes on the rate of VAMP cleavage by the TeNT was investigated by extending the cleavage experiments to liposomes made only of the zwitterionic phospholipid dioleyl-phosphatidylcholine. Fig. 5 shows that such liposomes are poor activators of the metalloproteolytic activity of TeNT indicating that electrostatic in-

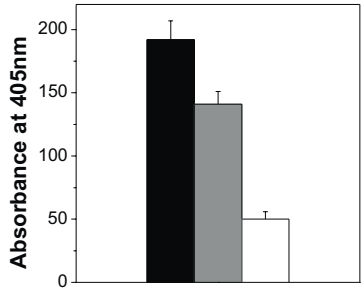

Fig. 4. The TeNT L chain binds to asolectin-coated plate. TeNT L chain was allowed to bind to microtiter plates coated with asolectin in buffer alone (black bar) or in buffer with 400 mM NaCl (gray bar). The amount of the lipid-bound TeNT L chain was determined with a polyclonal anti-TeNT antibody and a secondary anti-rabbit peroxidase-conjugated antibody; the absorbance due to the primary antibody bound to the lipid film in the absence of L chain was subtracted (white bar).

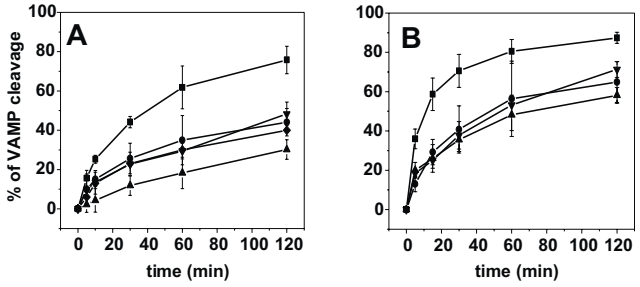

Fig. 5. The increase of metalloprotease activity of the L chain of TeNT caused by liposomes depends on the lipid composition and is abolished by high salt. The proteolytic activity of TeNT (A) and of its L chain (B) was measured using the full-length VAMP-2/1–116 in cleavage buffer alone (●), or containing asolectin liposomes (■), or asolectin liposomes plus 400 mM NaCl (▼), or in the presence of dioleyl-phosphatidylcholine liposomes (▲). In A the proteolytic activity of TeNT was also tested in cleavage buffer containing 400 mM NaCl (♦). Bars are the ±S.D. of three different experiments.

teractions are involved in the lipid-activating effect shown above. This conclusion is strongly supported by the finding that increasing the ionic strength of the medium by including 400 mM NaCl abolishes the activation brought about by asolectin liposomes. These effects are evident both with the entire TeNT (Fig. 5A) and with the L chain (Fig. 5B). Fig. 5A also shows that 400 mM NaCl does not affect the proteolytic activity of TeNT.

4. Discussion

The results reported here document that the metalloproteolytic activity of the clostridial neurotoxins specific for VAMP is strongly enhanced by the presence of lipid membranes. This effect provides an explanation for the fact that these neurotoxins are more active on VAMP inserted into SSV and in neurons than on the isolated VAMP molecule. This lipid-enhancing effect is brought about by negatively charged lipids with no apparent specificity for brain lipids. The lack of effect of zwitterionic phospholipids and the blockade of the lipid-activating effect with high salt indicate that electrostatic interactions play an important role in the lipid activation. Among VAMP-specific clostridial metalloproteases, only the structure of BoNT/B is available [25]. An analysis of the potential surface of BoNT/B and of BoNT/B L chain reveals the presence of a cluster of positive residues (Lys, Arg) which are exposed upon removal of the H chain belt, which wraps around the L chain. Unfortunately, the atomic structure of full-length TeNT, as well as BoNT/D and BoNT/F, is not available and further investigations are required to identify the toxin residues involved in lipid interaction.

The cytosolic domain of VAMP, which includes the peptide bonds cleaved by clostridial neurotoxins does bind to negatively charged liposomes and we cannot exclude that part of the lipid-activating effect is due to a substrate conformational change which makes it more prone to cleavage. Indeed, segment VAMP/77–90 was recently shown to bind phospholipids via a cluster of positive charges (K_{83}, K_{87}) and a triplet of aromatic residues ($_{88}YWW_{90}$) and to change conformation thereafter [22,23]. Interestingly, TeNT and BoNT/B cleave VAMP at the Q_{76}–F_{77} peptide bond connecting this phospholipid binding segment to the N-terminal helical region. However, at least part of the lipid activation is due to the binding of the toxin metalloprotease domain to lipids as documented here with different approaches. Such binding reduces the dimensionality of the enzyme–substrate system from the three dimensions of the buffer solvent to the two dimensions of the membrane surface. Clearly, under these conditions the probability of encounter between the protease toxin and its membrane-bound substrate is greatly augmented and this would probably suffice to account for the large increase in the rate of VAMP cleavage documented here, even in the absence of conformational effects on the substrate induced by its membrane insertion [26–28]. Such 'dimensionality' effect would also contribute to explain the yet uninterpreted fact that BoNT/C cleaves its substrate syntaxin efficiently only when it is inserted in an acidic lipid environment [20,21].

Acknowledgements: work performed in the authors' laboratory is supported by Telethon-Italia grant no. GP0272Y01, by progetto strategico MURST 01.00459.ST97, and by the Armenise-Harvard Medical School Foundation.

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