Interaction of botulinum and tetanus toxins with the lipid bilayer surface

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The interaction of botulinum neurotoxins serotypes A, B and E (from *Clostridium botulinum*) and of tetanus neurotoxin (from *Clostridium tetani*) with the surface of liposomes made of different lipid compositions was studied by photolabelling with a radioiodinated photoactive phosphatidylethanolamine analogue [¹²⁵I-dipalmitoyl (3,4-azidosalicylamido)phosphatidylethanolamine]. When the vesicles were made of negatively charged lipids (asolectin), each of these neurotoxic proteins was radioiodinated, thus providing evidence for their attachment to the membrane surface. The presence of gangliosides on liposome membranes enhanced fixation of the neurotoxic proteins to the lipid vesicle surface. Both the heavy and light chains of the clostridial neurotoxins were involved in the attachment to the lipid bilayer surface. Each of the toxins tested here attached poorly to liposomes made of zwitterionic lipids (egg phosphatidylcholine), even when polysialogangliosides were present. The data suggest that the binding of botulinum and tetanus neurotoxins to their target neuronal cells involves negatively charged lipids and polysialogangliosides on the cell membrane.

INTRODUCTION

Tetanus (TeNT) and botulinum neurotoxins (BoNT, seven different serotypes, i.e. types A, B, C1, D, E, F and G) are the most potent microbial protein toxins (Wellhoner, 1982; Sakaguchi, 1983; Simpson, 1986; Habermann & Dreyer, 1986). They are extremely specific for nerve cells, and part of this specificity is probably due to their ability to bind specific polysialogangliosides richly distributed in the nervous tissue (Wiegandt, 1985).

The ganglioside-binding ability of these neurotoxins was demonstrated by various methods, including binding of the radioactively labelled toxin to gangliosides adsorbed to thin-layer silica plates or to plastic microtitre plates (Van Heyningen & Miller, 1961; Mellanby *et al.*, 1968; Simpson & Rapport, 1971; Helting *et al.*, 1977; Holmgren *et al.*, 1980; Kitamura *et al.*, 1980; Habermann & Tayot, 1985; Critchley *et al.*, 1985; Kamata *et al.*, 1986; Ochanda *et al.*, 1986; Lazarovici *et al.*, 1987).

These neurotoxins, produced by *Clostridium tetani*

Fig. 1. (a) Schematic drawing of the structure of clostridial neurotoxins showing their single- (top) and di-chain (middle) forms and the proteolytic fragments B and C of tetanus (bottom) and (b) structural formula of ¹²⁵I-ASA-PE, the lipid bilayer surface photoactive phospholipid probe used in the present study

Abbreviations used: TeNT, tetanus neurotoxin; BoNT, botulinum neurotoxins; ¹²⁵I-ASA-PE, ¹²⁵I-dipalmitoyl (3,4-azidosalicylamido)-phosphatidylethanolamine; BPB, Bromophenol Blue.

and *Clostridium botulinum*, have a similar structure (Fig. 1a). They are composed of two polypeptide chains, H (100 kDa) and L (50 kDa), held together by a disulphide bridge(s) and non-covalent interactions. Tetanus toxin can be cleaved by papain in the middle of the H-chain, thus generating a 100 kDa fragment, termed 'B' (the L-chain and the N-terminal half of the H-chain) and a 50 kDa fragment, termed 'C' (the C-terminal half of the heavy chain). The ganglioside-binding site of TeNT is confined to the C fragment (Helting & Zwisler, 1977; Helting *et al.*, 1977; Bizzini *et al.*, 1980). The H-chain of BoNT appears to have the receptor binding site (Bandyopadhyay *et al.*, 1987; Simpson, 1986). These neurotoxins are thought to have a similar mode of action: after binding, the toxin or part of it enters the cell and then blocks neurotransmitter release (Simpson, 1986; Habermann & Dreyer, 1986).

The importance of the membrane binding in the mode of action of these neurotoxins led us to investigate further the binding step on liposomes with or without gangliosides, a model system more closely related to the synaptosomal membranes than micellar or plastic- or silica-adsorbed gangliosides. The interaction of the neurotoxins with the liposome surface was monitored by photolabelling with a photoactivatable phospholipid analogue (Bisson & Montecucco, 1985; Montecucco, 1987), ^{125}I -dipalmitoyl (3,4-azidosuccinylamido)phosphatidylethanolamine (^{125}I -ASA-PE). This approach is based on the interdispersion of trace amounts of the phospholipid probe among the other lipids of the membrane. The reagent used here, ^{125}I -ASA-PE, shown in Fig. 1(b), carries a ^{125}I -arylazido group on its polar head group. Illumination generates a very reactive intermediate able to form covalent bonds with neighbouring molecules, thereby labelling them radioactively. Owing to the restricted location and hydrophilic nature of the reactive group of ^{125}I -ASA-PE, the interaction of proteins at the surface of a lipid bilayer can be monitored, thus providing information on the protein regions involved in membrane binding.

EXPERIMENTAL PROCEDURES

Materials

BoNT serotypes A, B and E were prepared as reported previously (DasGupta & Rasmussen, 1983; DasGupta & Sathyamoorthy, 1984; DasGupta & Woody, 1984). BoNT and TeNT are synthesized by the bacteria as single-chain proteins that are cleaved by proteinases endogenous to the bacteria, thus yielding the two-chain neurotoxin composed of H- and L-chains; in the absence of endogenous cleavage (e.g. BoNT type B and type E), the isolated toxin is a single-chain protein. When needed, the type B and E BoNT were cleaved with tosylphenylalanylchloromethane ('TPCK')-treated trypsin (Serva) as described by Sathyamoorthy & DasGupta (1985). TeNT was purified by chromatography on DEAE-cellulose (Whatman) and on Ultrogel ACA-44 (LKB). It was stored at -80°C at a protein concentration of 2 mg/ml in 10 mM-sodium phosphate buffer, pH 7.4. Egg lecithin (phosphatidylcholine) and soya-bean asolectin were from Sigma. Asolectin, after purification by the method of Kagawa & Racker (1971), had the following phospholipid composition (w/w): phosphatidylcholine, 24%; phosphatidylethanolamine, 39%; phosphatidylserine, 19%; phosphatidic acid, 6%; phosphatidylinositol, 1%; lyso derivatives, 9%; other phospholipids, 2%. Mixed brain gangliosides and purified bovine brain lipids were kindly given by Dr. L. Poletti (Fidia Research Laboratories, Abano, Italy). Bovine brain lipids had the following composition (w/w): gangliosides, 6%; phospholipids, 34%; cerebroside and sulphatides, 48%; cholesterol, 4%; and proteins, 0.2%. ^{125}I -ASA-PE (sp. radioactivity 200 Ci/mmol) was prepared by modifying dipalmitoylphosphatidylethanolamine with 4-azidosalicylic acid and subsequent radioiodination, as reported elsewhere (Gao & Bauerlein, 1987).

Liposome preparation

All operations were under a red safety light. Liposomes were prepared by mixing egg lecithin (0.5 mg/sample),

Fig. 2. Labelling of clostridial neurotoxins in the presence of photoactive liposomes

(a) Shows the Coomassie Blue-stained SDS/polyacrylamide gels of BoNT type A (17 μg ; left) and TeNT (10 μg ; right) after photolabelling with ^{125}I -ASA-PE interdispersed in liposomes of egg lecithin (E), egg lecithin plus gangliosides (EG), asolectin (A) and asolectin plus gangliosides (AG). (b) Shows the corresponding autoradiographic patterns obtained after drying the gel and exposure to a photographic film. The amount of labelling is correlated with the intensity of the dark spots corresponding to the protein bands as stained in (a). The slight impurity present in the TeNT preparation, which runs between the H- and L-chains (100 and 50 kDa respectively), is not labelled. The TeNT preparation also shows a small amount of uncleaved material (150 kDa), which is visible above the H-chain.

asolectin (0.525 mg/sample) or bovine brain lipids (0.48 mg/sample), with or without mixed gangliosides (35 µg/sample), with ^{125}I -ASA-PE (0.033 µg/sample) in chloroform/methanol (1:1, v/v). After drying under an N_2 flux, resuspension in diethyl ether and vacuum pumping, the lipids (0.5 ml/sample) were sonicated in 125 mM-NaCl/10 mM phosphate/10 mM-sodium citrate/1.5 mM-EDTA, pH 7.4, in a stoppered vial under N_2 until optical clarity was achieved.

Photolabelling and radioactivity determination

Each neurotoxin (10–15 µg) was added to 0.5 ml of liposomal suspension and incubated in the dark for 5 min at room temperature. The samples were then illuminated for 10 min at room temperature as described previously (Montecucco, 1987), transferred to conical plastic tubes, and then the protein was recovered by precipitation with trichloroacetic acid (final concn. 7%) and centrifugation. The pellet was dissolved in 10% (w/v) SDS containing BPB, and the pH of the solution was adjusted with 2 M-Tris, the colour change of BPB being monitored. The samples were electrophoresed (Montecucco *et al.*, 1986) on 12-cm-long 8%-(w/v)-polyacrylamide gels overlayed with a 1-cm-long 4%-(w/v)-polyacrylamide step gel. Gels were stained with Coomassie Blue and, after destaining, their densitometric traces were recorded and the peaks integrated on a Shimadzu CS-930 dual-wavelength densitometer. Gels were dried at 80 °C and exposed to Kodak X-Omat films at –80 °C. The developed films were scanned and peaks integrated as described above. Labelling was expressed as the amount of film dark area per unit area of the corresponding Coomassie Blue-stained band of the gel, and hence it represented the specific radioactivity of each protein band. For comparison of different sets of experiments the specific labelling of the H-chain of any neurotoxin in the presence of ganglioside-containing liposomes was taken as 100%.

RESULTS

The Coomassie Blue-stained profiles of BoNT type A and of TeNT obtained by SDS/polyacrylamide-gel electrophoresis, after incubation with photoactive liposomes in the dark and illumination, are shown in Fig. 2(a); Fig. 2(b) shows the corresponding autoradiographic patterns. Both H- and L-chains of BoNT and TeNT were radiolabelled in the presence of asolectin liposomes, whereas a barely detectable radioactivity was found when these proteins were incubated with the egg-lecithin liposomes. This result indicates (i) that the two neurotoxins interact with the lipid bilayer surface when negatively charged lipids are present and (ii) that this interaction occurs via both the H- and the L-chain of the toxins.

From Fig. 2(b) it is also evident that (i) the neurotoxin is poorly labelled when gangliosides are included in lecithin liposomes, and (ii) the presence of gangliosides increase the labelling of the toxin on asolectin vesicles. A more quantitative estimation, reported in Table 1, indicates that a combination of negatively charged lipids and gangliosides enhances the interaction between BoNT and TeNT and the liposome surface. On the other hand, gangliosides do not appear to enhance the neurotoxin binding to zwitterionic lecithin vesicles; this latter finding may be related to the formation of

Table 1. Labelling of BoNTs and TeNT with liposomes of different compositions containing the surface photoactive reagent ^{125}I -ASA-PE expressed as percentage of the labelling of the H-chain found in the presence of the asolectin + ganglioside mixture taken as 100%

The ranges of values found in three separate sets of experiments (two for type E) are reported. E, EG, A, AG are as defined in Fig. 2; BBL, bovine brain lipids.

Toxin	Labelling (%)	
	H-chain	L-chain
BoNT		
Type A		
E	6–7	0.5–1.5
EG	6–16	2–3
A	78–85	5–7
AG	100	10–27
BBL	72–83	6–11
Type B		
E	6–8	1–2
EG	9–12	8–10
A	72–92	10–14
AG	100	13–19
BBL	43–52	8–13
Type E		
E	9–10	1
EG	10	2–4
A	80–87	4–5
AG	100	9–12
BBL	90–95	12–15
TeNT		
E	9–22	9–13
EG	6–15	7–13
A	36–93	84–112
AG	100	84–129
BBL	204	195
	B	C
Fragments		
E	2–3	2–3
EG	2–3	2–3
A	72–80	29–35
AG	100	100
BBL	107–119	35–47

ganglioside clusters (Thompson & Tillack, 1985), with a resulting lower availability for toxin binding.

Table 1 summarizes the results of parallel experiments performed with BoTx types A, B, E, TeNT (and its fragments B and C) analysed quantitatively by scanning the Coomassie Blue-stained gels together with the corresponding autoradiographic films. Patterns of radio-labelling similar to those obtained with BoNT type A in the presence of liposomes of different compositions were also found with BoNT type B and E. One general feature of BoNT was that labelling of the H-chain was significantly higher than that of the L-chain; this notable difference was not found among the chains of TeNT. We cannot discriminate between the possibility that these differences result from varying amounts of protein surface exposed to lipids or from the exposure to the photoactive group of different kinds of lateral amino acid residues with different reactivity.

The interaction of BoNT and TeNT with liposomes made of a purified bovine brain lipid extract resembled, in terms of patterns and amounts of labelling, those obtained with asolectin-ganglioside liposomes.

No significant differences were noticed in the liposome interactions of uncleaved and cleaved BoNT type B or E (results not shown).

From the comparison of the results obtained with TeNT and with its two fragments, reported in Table 1, it clearly appears that the neurotoxin-ganglioside interaction is not solely responsible for the fixation of the toxin to the lipid bilayer surface, since fragment B, which lacks the ganglioside-binding fragment, is still able to interact with asolectin liposomes. Only a small increase in fragment B labelling was found when gangliosides were present, as expected on the basis of the increase in negative charge of the liposomes due to the presence of gangliosides. Fragment C shows a higher labelling when incubated with asolectin with respect to egg-lecithin liposomes, indicating that, in that case also, an electrostatic interaction with negative lipids is likely to be involved. With fragment C there is a much higher increase in labelling with ganglioside-containing liposomes as compared with liposomes without gangliosides, as expected from its ganglioside-binding property. This increase is much higher than that found with the intact TeNT molecule, most likely because of the greater relevance of ganglioside binding in its liposome interaction when compared with that of the intact toxin.

DISCUSSION

Clostridial neurotoxins have significant affinity for polysialogangliosides, and this interaction is thought to play a functional role in the binding of these neurotoxins to nerve cells (Wellhoner, 1982; Montecucco, 1986; Habermann & Dreyer, 1986). We considered the possibility that, in addition to the ganglioside-mediated binding, these neurotoxins interact with the head group of phospholipids, as has been shown to occur with diphtheria toxin (Alving *et al.*, 1980; Papini *et al.*, 1987). To probe the lipid interaction of the clostridial neurotoxins with membranes, we have used a simple and versatile method that provides information on protein-lipid binding and on the protein regions involved in the binding. Because of the intrinsic nature of the method, quantitative examinations of binding constants are very difficult to obtain. On the other hand, the very high sensitivity of the method allows one to monitor even very weak interactions.

We found that BoNT and TeNT interact with the surface of liposomes containing negatively charged lipids and negligibly with that of zwitterionic lipid vesicles in a buffer of ionic strength comparable with that of extracellular fluids. The amount of labelling indicates that both BoNT and TeNT are fixed more efficiently on the surface of polysialoganglioside-containing liposomes; this is consistent with the known ability of these neurotoxins to bind gangliosides.

The present study has shown that: (i) the attachment of clostridial neurotoxins to the nerve plasma membrane may involve negatively charged lipids in addition to G_{1b} gangliosides; (ii) this lipid interaction is not restricted to the ganglioside-binding fragment C, but also involves the L-chain and the N-terminal part of the H-chain. These observations are not negated by the finding that TeNT

binds strongly to pure ganglioside micelles (Lazarovici *et al.*, 1987) or gangliosides adsorbed on to plastic (Holmgren *et al.*, 1980), since the surface of these model systems is made highly negative by the presence of sialic acid residues on the oligosaccharide head of gangliosides (Rogers & Snyder, 1981; Agui *et al.*, 1983).

The ability of the clostridial neurotoxins to bind to the surface of negatively charged membranes provides an explanation for the their lack of ganglioside specificity of the kind shown by cholera toxin for G_{M1} ganglioside (Holmgren *et al.*, 1980; Kitamura *et al.*, 1980; Ochanda *et al.*, 1986; Kamata *et al.*, 1986) and for their poor ability to bind oligosaccharides (Habermann *et al.*, 1985).

The present study also helps to explain the finding that TeNT binds to gangliosides more strongly than its fragment C (Helting *et al.*, 1977), because fragment B, which lacks the ganglioside-binding site, is able to interact with negatively charged lipids. Binding of different negative lipids, including phosphatidylserine and gangliosides, to fragment B has also been recently shown to occur by monitoring their effect on the c.d. spectrum of the protein (Lazarovici *et al.*, 1987).

The possible relevance *in vivo* of a TeNT-lipid interaction in addition to that with polysialogangliosides is suggested by the finding that a ganglioside/cerebroside mixture was more potent than ganglioside alone in protecting mice from the toxicity of injected TeNT (Mellanby *et al.*, 1968).

Although the present work suggests that the bindings of BoNT and TeNT to the nerve membrane lipids are mediated by polysialogangliosides and by negatively charged lipids, the exact relative contribution from these two different interactions to the overall neurotoxin binding to the membrane remains to be determined. Another aspect that requires further investigation is that lipid interaction does not appear to be sufficient to ensure the binding of the clostridial neurotoxins to nerve-cell endings at the minute concentrations needed to give clinical symptoms. Growing evidence suggest that a protein component is also involved (Williams *et al.*, 1983; Yavin & Nathan, 1986; Evans *et al.*, 1986; Pierce *et al.*, 1986). A double-receptor model that takes into account both a lipid and a protein interaction with the neurotoxins has been recently proposed (Montecucco, 1986).

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