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Neuroscience Letters 196 (1995) 37–40

NEUROSCIENCE LETTERS

Botulinum toxin type A inhibits Ca^{2+}-dependent transport of acetylcholine in reconstituted giant liposomes made from presynaptic membranes from cholinergic nerve terminals

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Received 31 March 1995; revised version received 4 July 1995; accepted 4 July 1995

Abstract

Giant liposomes were made from a mixture of asolectin phospholipid vesicles and presynaptic plasma membranes isolated from *Torpedo* cholinergic nerve endings. Acetylcholine filled giant liposomes were able to release neurotransmitter upon stimulation by the Ca^{2+} ionophore A23187 and Ca^{2+}. Botulinum neurotoxin type A inhibited this Ca^{2+}-dependent acetylcholine transport. Additionally, Botulinum toxin type A decreased membrane fluidity of liposomes. These results suggest that Botulinum toxin can interact directly with components of the presynaptic plasma membrane and inhibit acetylcholine translocation. Furthermore, since the reconstituted liposomes do not have synaptic vesicle components, the observed effects may account for the action of Botulinum toxin on the non-quantal release of acetylcholine from motor nerve terminals.

Keywords: *Torpedo*; Clostridial toxins; non-quantal transmitter release

Botulinum neurotoxin type A (BoNT/A) is a two polypeptide chain protein that blocks acetylcholine (ACh) release from the neuromuscular junction [4], the electric organ of *Torpedo* [8] and nerve endings in the central nervous system [24]. It has been suggested that BoNT/A may act similarly to other bacterial toxins (for review, see Refs. [14,22]) in three different steps: (i) binding of the protein to the presynaptic membrane; (ii) internalization inside the nerve terminal by endocytosis; and (iii) lytic step in which the toxin impairs the regulated neuroexocytosis. As an alternative to this pathway, Simpson [21] has proposed that entrance of the toxin would not need the endocytic pathway to exert its effects. In this model, the toxin light chain would be able to cross the plasma membrane and gain the cytoplasm of the nerve terminal, blocking the vesicle exocytotic process.

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Quantal release of ACh results from exocytosis of synaptic vesicles. However, it has been demonstrated that there is a molecular release or leakage of ACh from nerve terminals, which may account for approximately 90% of the ACh released in resting conditions. Botulinum toxin interferes with both quantal and non-quantal transmitter secretion [6]. Non-quantal transmitter release should be a mechanism linked with presynaptic plasma membranes. An experimental model that can account for such a transport system is one developed by Israël et al. [11] who successfully incorporated a Ca^{2+}-dependent transport system of ACh into proteoliposomes by dissolving presynaptic plasma membrane from cholinergic nerve terminals in lipids and organic solvents. This or similar experimental models may be useful tools to identify the subcellular components involved in the non-quantal release of ACh. In this paper, we have tested the inhibiting effect of BoNT/A on an ACh transport system present in presynaptic plasma membranes (PSPM) reconstituted into giant liposomes, in order to explore whether BoNT/A acts

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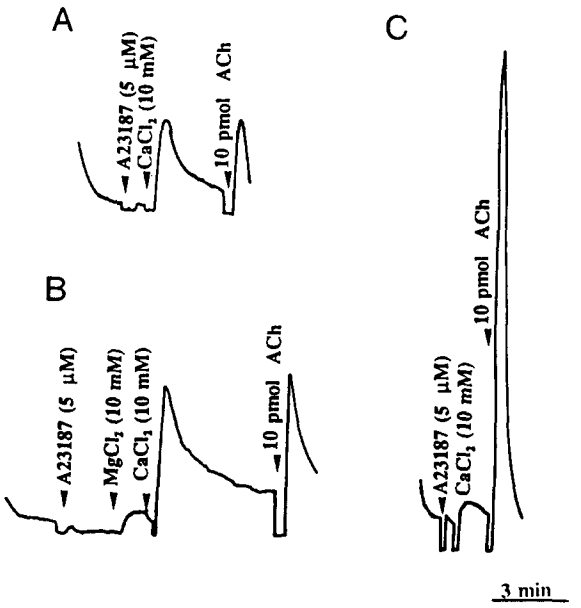

Fig. 1. Ca^{2+}-dependent translocation of ACh from giant liposomes derived from presynaptic plasma membrane fractions and filled with ACh. (A) Giant proteoliposomes translocate ACh from the inside to the outside media when Ca^{2+} reaches the intraliposomal compartment by means of the Ca^{2+} ionophore A23187. (B) Mg^{2+} does not produce such ACh translocation. (C) Giant proteoliposomes filled with ACh, but made in the absence of PSPM, are not able to translocate ACh. In this and all the other figures, the sequential addition of reagents with time proceeded from left to right. Presynaptic plasma membranes (PSPM) from cholinergic nerve endings of the electric organ of *Torpedo* marmorata were obtained according to Morel et al. [13]. Lipid vesicles and giant liposomes were obtained as described previously [16]. Acetylcholine was determined using the chemiluminescent method [10].

on quantal and non-quantal secretion following the same or different molecular mechanisms.

Giant liposomes, obtained by fusion of asolectin phospholipid vesicles and presynaptic plasma membrane fractions from cholinergic nerve terminals from *Torpedo* electric organ, have a large diameter (10–25 μm) similar to those obtained using a fraction of postsynaptic membranes from *Torpedo* [16]. Such giant liposomes are able to translocate ACh upon addition of the Ca^{2+} ionophore A23187 followed by Ca^{2+} (Fig. 1A). Conversely, addition of Ca^{2+} alone is not able to promote the translocation of ACh (data not shown), suggesting that it is necessary that the divalent cation reaches the internal space of the proteoliposomes by the action of the A23187 ionophore. In addition, when Ca^{2+} was substituted by Mg^{2+} (Fig. 1B) there was no triggering of ACh translocation in spite of the presence of the A23187 ionophore. Depending upon the different batches of giant liposomes prepared in these studies, the ACh released in response to stimulation by the A23187 ionophore followed by Ca^{2+} accounted for 10–30% of the total ACh entrapped in the liposomes, as determined by measurements of the total ACh content released upon solubilization of the liposomes by 3% Triton X-100. Such apparent restriction in the amount of ACh that can be translocated was expected to be based on the multilamellar nature of the giant liposomes, which is likely to result in trapping of ACh inside layers of the liposome to which the ionophore plus Ca^{2+} stimulus can not gain access. Also, the ACh translocation activity can be unequivocally ascribed to the protein components of the PSPM since giant liposomes prepared in the absence of such membranes and containing entrapped ACh, were not able to translocate ACh in response to the Ca^{2+} plus ionophore stimulus. Other experiments made in the absence of acetylcholinesterase in the enzyme cocktail used in the luminescence assay demonstrated that (i) the entrapped ACh does not undergo spontaneous breakdown inside the liposomes and (ii) the translocated ACh need to be hydrolyzed by the externally added cholinesterase to produce a luminescence signal. In summary, the reconstituted giant liposomes behave similarly to the small proteoliposomes reported previously [11] with respect to ACh transport, which has been shown to depend on the presence of the so-called 'mediatophore', a protein belonging to the family of proton-translocating vacuolar ATPases [2].

The ACh transport from the internal to the extraliposomal media is dependent on the external concentration of Ca^{2+} applied after A23187 Ca^{2+} ionophore treatment (Fig. 2A,B). The transport saturates and reaches its maximal value at approximately 12 mM $CaCl_2$ added to the media. The saturation of this process probably reflects that it is also dependent on the internal Ca^{2+} concen-

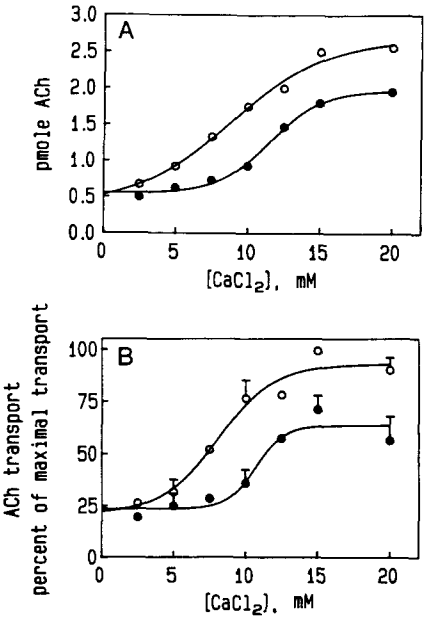

Fig. 2. Effects of BoNT/A on Ca^{2+}-dependent translocation of ACh from giant liposomes. (A) Plot of a titration experiment done in a single batch of giant proteoliposomes. (B) Plot of the results obtained using three different batches of giant proteoliposomes. Empty circles, non-treated, 'control' proteoliposomes. Filled circles, same proteoliposomes as in (A) but preincubated with BoNT/A, under the conditions described in Fig. 3.

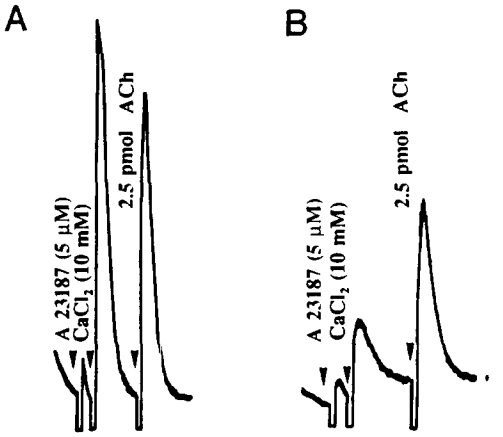

Fig. 3. Effect of BoNT/A on ACh translocation in giant proteoliposomes. (A) Non-treated, control proteoliposomes incubated with BoNT/A (2 nM) during 5 min at pH 7.4, before the addition of the luminescent solution. BoNT/A was purified from cultures of *Clostridium botulinum* (NTC 2916) according to Refs. [5,25] using a FLPC chromatographic system. The purified neurotoxin was utilized in this work.

tration because we have used the same A23187 concentration throughout the experiments.

This reconstituted system of ACh transport is sensitive to the action of BoNT/A. Figs. 2 and 3 show that BoNT/A (2 nM) clearly inhibits the Ca^{2+} dependent transport of ACh since an incubation of 5 min in the presence of the toxin is sufficient to shift the curve of transport to the right and decrease its maximal level. The specificity of this effect is highlighted by the fact that BonT/A do not affect the amount of ACh entrapped into the liposomal suspension.

Taking into account that we are utilizing a Ca^{2+} ionophore, the effect of BoNT/A must not be related to an action on the ionic channels presumably involved in the process under physiological conditions. On the contrary, according to the Ca^{2+}-receptor model [19], it must reflect a decrease in the affinity of Ca^{2+} for the reconstituted ACh transport system. Our results lend support to the hypothesis proposed by Simpson [21] in which the BoNT/A may act through direct interaction with the presynaptic plasma membrane, thus making the putative endocytic pathway unnecessary. Our view is that such the non-endocytic pathway should be related to the action of the toxin on non-quantal release. Nevertheless, it has been reported that BoNT/A, blocks non-quantal release at neuromuscular junctions only when it is present at high concentrations [6].

Other alternatives could also be considered in an attempt to explain the experimental results. For instance, it has been recently shown that fusion of cell membranes is the result of interaction of different soluble proteins and receptors located at the surface of secretory granules and plasma membranes [23]. It has also been demonstrated that clostridial toxins possess a zinc-dependent peptidase activity [19]. The specific target of the peptidase activity associated with the light chain of the tetanus toxin is synaptobrevin II [18], a synaptic vesicle-associated membrane protein. Blasi et al. [3] have demonstrated that the target protein for BoNT/A is the plasma membrane-associated protein SNAP-25 [15]. SNAP-25 is present in *Torpedo* electromotoneurons [17], and perhaps the effect that we have found on ACh transport in the giant proteoliposomes may be due to the interaction of the BoNT/A with SNAP-25. A possibility, therefore, is that the PSPM fractions contain part of the machinery needed for membrane fusion and since giant liposomes are multilamellar, the observed transport of ACh could be due to the fusion of membranes leaflets induced by Ca^{2+}. Assuming this working hypothesis as certain, the blocking effects of BoNT/A could be interpreted based on an interference with such fusion mechanism.

Another possibility is that, physiologically, synaptic vesicles would fuse transiently with presynaptic plasma membrane. In these transient fusion events, the neurotransmitter does not diffuse freely [1]. Then, some molecule of the synaptic vesicle membrane should bring the force to expel the neurotransmitter out of the vesicle. The mediatophore may be a good candidate for such a role. We can figure out how BoNT/A can interfere with the mediatophore by different means: (i) interacting directly over the mediatophore; (ii) decreasing lipid fluidity that permits molecular conformational changes needed for the acetylcholine transport; and (iii) it is possible that the mediatophore needs to interact with SNAP-25 to be activated physiologically; the cleavage of SNAP-25 by

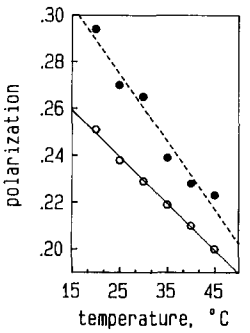

Fig. 4. Effect of BoNT/A on liposome fluidity. BoNT/A (filled circles) decreases the fluidity of proteoliposomes. Empty circles represent non-treated, control proteoliposomes. The apparent fluidity of the liposomes was measured in terms of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization as previously reported [4]. Proteoliposomes, diluted in Tris 10 mM (pH 7.4), 50 mM NaCl, at a final concentration of 10 mM of phospholipids were sonicated and degassed under vacuum for 30 min. The sonicated suspension was incubated in the dark at room temperature with 10 nM DPH (dissolved in acetonitrile) for 30 min, and BoNT/A (2 nM) or saline solution were added and the samples incubated for an additional period during 30 min. A SLM 8000 spectrofluorimeter equipped with calcite prism polarizers in the excitation an emission beams and a thermostated cell holder was used in these studies. The liposomal suspension was placed in a quartz cuvette, excited at 360 nm and the 'perpendicular' and 'parallel' polarized fluorescence intensities were monitored by using Corning 3-74 cut-off filters to eliminate scattered light.

BoNT/A would result in an inactivation of the acetylcholine transport.

A separate observation is that the fluidity of the reconstituted liposomes is decreased by BoNT/A, in the same range of concentrations used to depress ACh transport (Fig. 4). At the present time, we do not understand the basis of this effect, although it seems clear that it constitutes additional evidence for a direct interaction of the toxin with the reconstituted membrane. It has been reported that BoNT/A has the capacity to form channels in phospholipid vesicles [9] through the cooperation among four toxin molecules. However, it is difficult to envisage how the formation of such channels could decrease the fluidity of phospholipid bilayers. Nevertheless, the fluidity of the membrane should be an important element in the process of membrane fusion, since 'dimpling' and hemifusion of membranes [12] play a central role in the initiation of exocytosis.

In conclusion, even when the experimental model that we have utilized in this work is an artificial reconstituted system, we feel that it has been useful to demonstrate that the action of clostridial toxins may be more complicated than we thought and that some of its effects may be mediated by direct interaction with the external surface of the presynaptic plasma membrane.

This work is supported by grants from DGICYT (Ministerio de Educación, Spain) to C.S., J.M. and J.M.G.R. Elena López-Alonso is a fellow of Generalitat de València.

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