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THE LIGHT CHAIN OF BOTULINUM NEUROTOXIN FORMS CHANNELS IN A LIPID MEMBRANE

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Summary: The ability of botulinum neurotoxin and its isolated subunits, the heavy and light chains, to bind to a lipid membrane and to form channels in the membrane was examined. At pH 4.0, the neurotoxin caused aggregation of calcein-containing liposomes, providing evidence of binding of the neurotoxin to the surface of the outer lipid membrane. Aggregation was followed by the release of calcein, as a result of the formation of channels. The heavy chain evoked the same responses as those of the neurotoxin. The light chain did not cause aggregation of the liposomes but did evoke the release of calcein. The channel-forming ability of the light chain appeared to be higher than that of the neurotoxin or the heavy chain. This novel property of the light chain may help us to understand the mechanism of action of botulinum neurotoxin.

Botulinum neurotoxin, synthesized by *Clostridium botulinum*, is recognized not only as an extremely toxic substance but also as a greatly useful tool for research of neuro-biology (1). The neurotoxin is a protein molecule with a molecular mass of 150 kDa. It has been classified into seven serotypes (A to G), and it targets presynaptic sites of neurons. The neurotoxin binds to a receptor on synaptic membranes, enters the cytosol, and inhibits the exocytosis of neurotransmitter from synaptic vesicles (1,2). Recently, ganglioside-associating synaptotagmin, a membrane-protein in synaptic vesicles, was identified as the receptor for

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type B neurotoxin (3). The neurotoxin is composed of two subunits, a heavy (100 kDa) and a light (50 kDa) chain, that are linked by a disulfide bond. The carboxyl-terminus of the heavy chain has been recognized as a receptor-binding site (2). Channel-forming activity in artificial planar lipid membranes and liposomes has also been observed with isolated heavy chain, in particular, the amino-terminal half of the heavy chain (4). The heavy chain requires acidic conditions (pH 4 - 5) for its channel-forming activity. The light chain was recently demonstrated to exhibit proteolytic activity against the NSF/SNAP receptors that are associated with the docking/fusion mechanism of synaptic vesicles (1,5). For example, the light chain of botulinum type F neurotoxin cleaves VAMP/synaptobrevin (6), type C1 HPC-1/syntaxin (7), and type B VAMP/synaptobrevin (8). Evidence from studies of the activity of the light chain supports predictions made by the A(active like enzyme)-B(binding) toxin theory proposed for other bacterial toxins, such as cholera toxin, diphtheria toxin, and pseudomonas exotoxin A (2,9). Recently, we found that the light chain could bind to an acidic phospholipid (10). In this communication, we describe the binding and channel-forming ability of the neurotoxin and its isolated subunits, in particular the light chain, as determined in a study with lipid membranes of liposomes.

Materials and Methods

The neurotoxin and its subunits. Single-chain botulinum type B neurotoxin was isolated from a culture of *Clostridium botulinum* type B strain Okra by the method reported elsewhere (11). The neurotoxin was treated with trypsin to convert it to the two-chain form. The heavy and light chains were prepared by the method of Sathyamoorthy and DasGupta (12). Concentrations of protein in the preparations of the neurotoxin and the separate chains were determined by the method of Bradford (13).

Preparation of calcein-containing liposomes. Calcein-containing liposomes were prepared by the method of Forti and Memesteina (14). Calcein and a liposome kit (negatively charged

product) were purchased from Sigma (St Louis, Mo, USA). Calcein (10 mM) was dissolved in a solution of 10 mM sodium citrate, 10 mM sodium phosphate, 125 mM sodium chloride, and 1.5 mM EDTA, pH 4.0. The solution of calcein was added to a lipid film in a round-bottomed flask, which has been prepared with a rotary evaporator, and the mixture was vigorously mixed on a vortex mixer for 60 sec for preparation of multilamellar liposomes. Free calcein was separated from calcein-containing liposomes by gel filtration on a column of Sephadex G-75 (Pharmacia, Tokyo, Japan) that had been equilibrated with the above mentioned buffer. The concentration of the liposomes was determined from the turbidity (absorbance) at 334 nm.

The liposome aggregation test. Binding of the neurotoxin to the liposomes was examined by a liposome-aggregation test, as described by Papini *et al.* (15). The liposomes were initially diluted in the above mentioned buffer at pH 4.0 to give an absorbance of 0.25 at 334 nm. The suspension of diluted liposomes (1 ml) was placed in a plastic cuvette (Sigma) and 15 μg of neurotoxin or an equimolar amount of the heavy or the light chain was added. After mixing for 15 sec, the turbidity of the liposomes was monitored at 15-sec intervals.

The calcein-release test. The channel-forming activity of the neurotoxin was determined by a calcein-release test (14). The release of calcein from the liposomes was monitored in terns of the change in fluorescence intensity at an emission wavelength of 517.6 nm and with an excitation wavelength of 455 nm by a spectrofluorometer (model RF-5000; Shimadzu, Kyoto, Japan). The liposomes were diluted in the buffer at pH 4.0 to an absorbance of 0.01 at 334 nm. The liposomes were continuously mixed in a cuvette. After addition of the neurotoxin or of its heavy or light chain, fluorescence intensity was determined at 15-sec intervals.

Results and Discussion

The turbidity of the suspension of calcein-containing liposomes was determined 5 min after the addition of the neurotoxin at various pH values. There were no changes in turbidity at pH 7.0 or pH 6.0, but the turbidity started to increase at pH 5.0 (data not shown). At pH 4.0, the turbidity of the liposomes in the presence of the neurotoxin increased significantly (Fig. 1, upper panel). The heavy chain also increased the turbidity of the suspension at pH 4.0. The extent of the aggregation of liposomes by the heavy chain was almost the same as that caused by the neurotoxin. The addition of the light chain did not, however, affect the turbidity of the suspension of the liposomes. These findings indicate that the neurotoxin bound

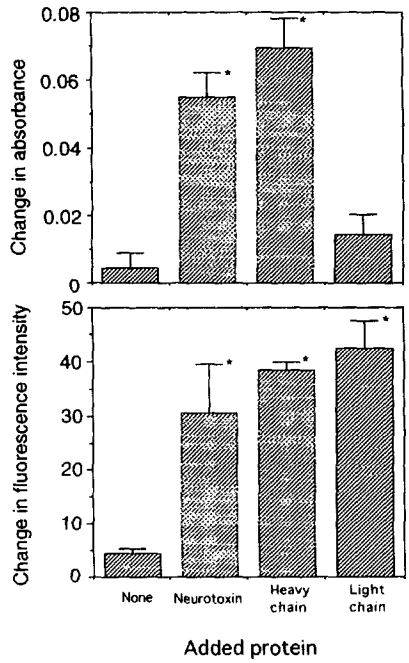

Fig. 1. Aggregation of calcein-containing liposomes (upper panel) and the release of calcein from liposomes (lower panel) by botulinum type B neurotoxin and its isolated subunits, the heavy and light chains. The test protein (0.1 μM) was added to a suspension of liposomes at pH 4.0. The change in absorbance (turbidity) at 334 nm was calculated, relative to the initial absorbance, 5 min after the addition. The change in fluorescence intensity at 517.6 nm with excitation at 445 nm was determined by subtraction of the initial fluorescence intensity from the intensity determined 5 min after addition of the protein. Statistic of analysis was carried out by Student's t-test. An asterisk indicates $P<0.001$ relative to the control.

to the outer membrane of the liposomes via the heavy chain. Using the same experimental system, we examined the channel-forming ability of the neurotoxin and its subunits. No spontaneous leakage of calcein was observed in the absence of the neurotoxin. As shown in Figure 1, the neurotoxin and the heavy chain evoked the release of calcein, indicating that these molecules formed channels in the liposome membrane. To our surprise, the light chain evoked the dramatic release of calcein. The amount of calcein released by the light chain was higher than that by the neurotoxin or the heavy chain.

The timecourses of the aggregation of liposomes and the release of calcein were examined in the presence of the neurotoxin and its subunits. When the neurotoxin was added to the suspension of liposomes at pH 4.0, aggregation was immediately observed, reaching a plateau within 4 min. The release of calcein in the presence of the neurotoxin was also initiated rapidly but it proceeded slowly, in contrast to the aggregation response (Fig. 2, left panel). The heavy chain evoked the aggregation of liposomes and the release of calcein in a manner resemble to the neurotoxin. Both responses were evoked by the heavy chain, reaching plateau values more rapidly than those evoked by the neurotoxin (Fig. 2, center panel). When the light chain was added, the release of calcein started immediately and a plateau was reached within 1 min (Fig. 2, right panel). The light chain released calcein from the liposomes more rapidly among the tested proteins.

Blaustein *et al.* reported that the heavy chain, in particular, the amino-terminal half of the heavy chain, generated channels in a planar lipid bilayer. However, they did not examine the capacity of the light chain for such formation of channels

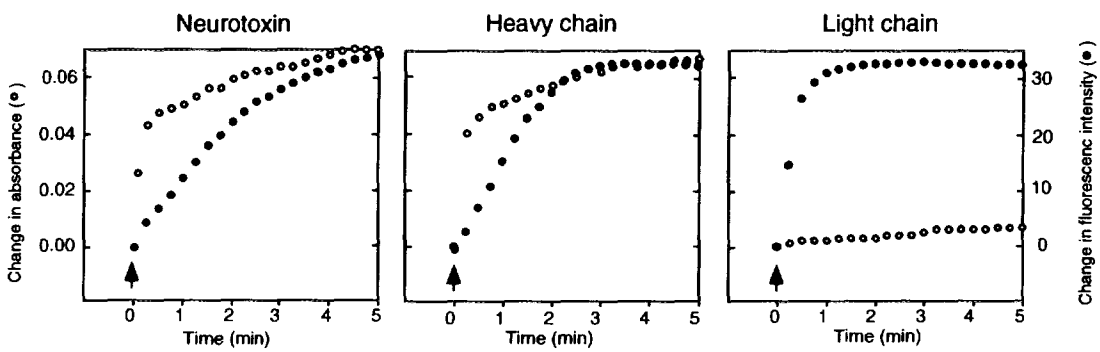

Fig. 2. Timecourses of the aggregation of liposomes and the release of calcein by botulinum type B neurotoxin and its subunits. Measurements were made every 15 sec. Arrows indicate the addition of the test protein to the suspension of liposomes. Details of the procedures are described in the legend to Fig. 1 and in the text.

(4). In a previous study, we determined the potential of the light chain to bind to an acid phospholipid (10). As described in the present communication, the light chain itself had greater channel-forming activity than the neurotoxin or the heavy chain. The heavy chain forms channels on the lipid membrane relatively slowly and it is retained on the membrane, with the resultant aggregation of liposomes. The light chain is probably embedded rapidly in the membranes and passes immediately through it. This scenario would explain why the light chain formed channels without the aggregation of liposomes. It is reported that the light chain of botulinum neurotoxin has proteolytic activity that is specific for proteins involved in the mechanism of neurotransmitter release (1). According to the A-B toxin theory, the light chain of botulinum neurotoxin passes through the channel formed by the heavy chain (2). From the accumulated evidence and the findings described in this communication, the following hypothesis is proposed for the molecular intoxication mechanism of botulinum neurotoxin: the heavy chain recognizes and binds to a receptor, such as synaptotagmin, and is retained on the membrane. The light chain is exposed to low pH by receptor-mediated endocytosis or recycling of synaptic vesicles and immediately passes through the lipid membrane. Then the light chain acts as a protease, cleaving a specific protein that is located at the site at which the light chain is now located. Experiments are in progress in our laboratory to validate this hypothesis.

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