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Evidence That Synaptobrevin Is Involved in Fusion between Synaptic Vesicles and Synaptic Plasma Membrane Vesicles

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We have developed a model system, consisting of rat brain synaptic vesicles and rat brain synaptic plasma membrane vesicles, to study the fusion process associated with the exocytotic release of neurotransmitters. Our results show a significant increase in the extent of fusion when the reaction takes place in cytosol compared to that obtained when fusion is carried out in buffer. This effect is mediated by cytosolic proteins, although *N*-ethylmaleimide-sensitive factor does not play a role in fusion. We also registered an almost complete inhibition of fusion when synaptic vesicles were pre-incubated with botulinum toxin B, indicating that synaptobrevin plays an important role in the coalescence of membrane lipids of the interacting membranes.

The exocytotic release of neurotransmitters involves the regulated fusion between vesicles that store neurotransmitters, the synaptic vesicles, and the presynaptic plasma membrane. Most of the proposed theories to explain the mechanism of binding and fusion that occurs between synaptic vesicles and the plasma membrane of neuronal cells during neurotransmitter exocytosis, essentially involve an initial specific recognition and binding between synaptic vesicle SNARE (synaptobrevin) and complementary SNARE proteins of the plasma membrane (SNAP-25 and syntaxin). The subsequent steps of the process are still a matter of debate. Recently, several issues have been addressed, mainly the role of each of these proteins in the lipid intermixing that precedes the fused state, the effect of cytosolic factors on the prefusion complex, as well as the Ca^{2+} sensor and timing of Ca^{2+} action in the exocytotic process (1). To study the fusion that occurs during the exocytotic release of neurotransmitters we used a cell-free model system consisting of synaptic vesicles and synaptosomal plasma membrane (SPM) vesicles, both isolated from rat brain cortex. Many studies on the mechanism of exocytosis have relied on the fact that neurotoxins produced by *Clostridium botulimum* bacteria are zinc-dependent endoproteases extremely specific for vesicular and target membrane SNAREs (2). Botulinum neurotoxin type B specifically cleaves the synaptic vesicle protein synaptobrevin, which has been proposed to be part of the fusion machinery involved in fusion between synaptic vesicles and the presynaptic plasma membrane (2). Clostridial neurotoxins are produced as inactive polypeptide chains of 150 kdal and are subsequently cleaved by bacterial and tissue proteinases which generate an active di-chain toxin, composed of a 100 kdal chain (H - heavy chain) and a 50 kdal chain (L - light chain), bridged by a single interchain disulfide bond. While the heavy chain is responsible for cell binding and penetration, the light chain possesses the intracellular catalytic activity. Inside nerve cells reduction of the disulfide bond takes place, releasing the active light chain in the cytosol, that can thus act as a Zn^{2+}-dependent endoprotease (2). Selective removal of synaptobrevin from synaptic vesicles by the zinc-dependent endoproteases botulinum toxins B and F, and tetanus toxin blocks neurotransmitter release, suggesting an important role for this protein in synaptic vesicles docking and fusion (3). Therefore, we studied the effect of botulinum toxin B on the fusion process that occurs between synaptic vesicles and SPM vesicles.

Membrane fusion was measured continuously by using a fluorescence assay that monitors the mixing of

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Abbreviations: DTT, dithiothreitol; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive factor; R_{18}, octadecylrhodamine B chloride; α-SNAP, soluble NSF attachment protein; SNAP-25, synaptosomal-associated protein of 25 KDa; SNARE, SNAP receptor; SPM, synaptic plasma membrane.

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membrane lipids of the interacting membranes. This assay is based on the fluorescence quenching of octadecylrhodamine B chloride (R_{18}), when this molecule is incorporated in a lipid bilayer at high concentrations. Fusion between a R_{18}-labelled membrane and an unlabelled membrane causes dilution of the probe and a relief of fluorescence quenching, with a consequent increase in fluorescence (4). This assay has been used successfully to monitor membrane fusion kinetics of enveloped viruses (5,6) and it has been proven to be reliable in other fusion model systems (7). In this work we followed the increase in fluorescence resulting from fusion between R_{18}-labelled synaptic vesicles and SPM vesicles devoid of fluorescent probe.

EXPERIMENTAL PROCEDURES

Materials. R_{18} was obtained from Molecular Probes Inc. (Eugene, OR). Botulinum toxin type B, trypsin, and soybean trypsin inhibitor were obtained from Sigma Chemical (St. Louis, MO).

Isolation of rat brain synaptic vesicles and cytosol. Rat brain synaptic vesicles were prepared according to Huttner et al. (8), with omission of the sucrose density gradient centrifugation and the glass chromatography. The synaptic vesicle pellet was resuspended in 145 mM NaCl, 10 mM HEPES, 1 mM EGTA, pH 7.4. A rat brain cytosolic fraction was also prepared as described by these authors (8) and it consisted of the supernatant from an ultracentrifugation, with cytosolic components dissolved in buffered sucrose. As in other membrane fusion studies (7), sucrose containing media produces a strong inhibition of the fusion reaction in this model system, which makes it necessary to eliminate this component from the rat brain cytosolic fraction. This was accomplished by submitting the cytosol to a centrifugation for 120 min at 3000 rpm in Centriprep-3 concentrators (Amicon Inc., Beverly, MA), that additionally concentrates the cytosolic protein components. Protein concentration was determined by the method of Sedmak et al. (9). We found no loss of protein components from the cytosol into the sucrose fraction which was discarded.

Isolation of rat brain synaptic plasma membrane (SPM) vesicles. Rat brain SPM vesicles were isolated as previously described (10) with the modifications of Coutinho et al. (11). The synaptic plasma membrane (SPM) vesicle fraction was resuspended in 145 mM NaCl, 10 mM HEPES, 1 mM EGTA, pH 7.4 and a portion of the pellet was resuspended in buffered sucrose for enzymatic analysis.

Labelling of synaptic vesicles with R_{18}. Rat brain synaptic vesicles were labelled with octadecylrhodamine B chloride (R_{18}) by injecting an ethanolic stock solution containing this fluorescent probe into a synaptic vesicle suspension while vortexing (4). The final concentration of probe was about 1 mol% of total vesicle lipid and that of ethanol was 1% (v/v). The mixture was incubated for 60 min on ice, in the dark. R_{18} that was not incorporated into synaptic vesicles was removed by centrifugation for 15 min at $165000 \times g_{av}$. The labelled-synaptic vesicle pellet was resuspended in 145 mM NaCl, 10 mM HEPES, 1 mM EGTA, pH 7.4.

Pre-treatment of cytosol and membranes with N-ethylmaleimide (NEM). Previously to fusion assays, cytosolic fractions, synaptic vesicles, and SPM vesicles were incubated for 15 min at 37°C in the presence of 1 mM NEM, after which 2 mM dithiothreitol was added to inhibit NEM (12).

Pre-treatment of synaptic vesicles with botulinum toxin B. The incubation of synaptic vesicles in the presence of botulinum toxin B and the previous activation of the latter were performed according to Braun et al. (13). Botulinum neurotoxin B was activated by incubating an aliquot of 10 μg of toxin in the presence of 5 μg of trypsin, 0.05 M sodium acetate, 0.2 M NaCl, pH 6.0 at 37°C. After 30 min incubation, 10 μg of trypsin inhibitor were added and the toxin was further incubated for 30 min at 37°C with dithiothreitol (10 mM). Treatment of synaptic vesicles with botulinum toxin B was performed by incubating 35 μg of synaptic vesicle protein with active toxin, at a toxin/vesicular protein ratio (w/w) of 1/10, in the presence of 2 μM zinc acetate. These conditions were shown previously to specifically cleave synaptobrevin (13). In parallel, synaptic vesicles were treated with active toxin that had been pre-incubated with 10 mM EGTA. This procedure does not cause VAMP cleavage since EGTA binding of Zn^{2+} renders this Zn^{2+}-dependent endoprotease inactive.

Fusion between R_{18}-labelled synaptic vesicles and unlabelled SPM vesicles. Fusion monitored continuously as the increase in R_{18} fluorescence at 590 nm (4), was initiated by rapid injection of R_{18}-labelled synaptic vesicles, at a final concentration of 12.5 μg/ml, into a cuvette containing the reaction medium (saline buffer or cytosol), 75 μg/ml of SPM vesicles and additional agents as described under Results, in a final volume of 2 ml. The fluorescence scale was calibrated such that the initial fluorescence of R_{18}-labelled synaptic vesicles and SPM vesicles was set at 0% fluorescence. The value obtained by lysing vesicle membranes at the end of each experiment with 1% (v/v) of Triton X-100 was set at 100% fluorescence.

RESULTS

Characterisation of the Model System

Since this model system for the exocytotic release of neurotransmitters has not been used before for fusion studies, we decided to start by characterizing both the synaptic vesicle and the SPM vesicle preparations, in terms of their morphology and purity, by electron microscopy. It is reasonable to assume that the SPM vesicle preparation is composed of a mixture of right-side-out and inside-out vesicles. Kanner (14) has isolated and characterised a SPM vesicle fraction mainly composed of right-side-out vesicles, while Gill (15) was able to prepare SPM vesicle fractions with almost only inside-out vesicles, and these differences cannot be ascribed to substantial variations in the isolation procedures. Given this unpredictability, a parameter worth evaluating was the membrane leaflet orientation in SPM vesicles since fusion of synaptic vesicles with SPM vesicles is physiologically more relevant for SPM vesicles that have resealed inside out.

The method described to isolate rat brain synaptic vesicles has been designed to prepare synaptic vesicles of high purity (8). To achieve a balance between a good yield and a reasonable purity, we omitted the final two steps of purification. However, according to the authors the primary subfractionation of the brain homogenate removes most of small vesicles except those contained in nerve endings and, so, the vast majority of synaptic vesicles in the washed synaptosomal fraction are synaptic vesicles, which, upon osmotic lysis, are released in the medium. As revealed by electron microscopy, the synaptic vesicle preparation used in this work is composed of sealed vesicles, homogeneous in size, with a mean diameter ranging from 50 to 75 nm (not shown), which agrees with the dimensions described for neu-

ronal synaptic vesicles (16). Owing to the small size of the vesicles seen in the electron micrographs, it seems unlikely that they result from the fragmentation and resealing of larger membranes.

SPM vesicles have been isolated according to a method that has been optimised in order to yield a SPM vesicle fraction mainly free from contaminants, and therefore we found appropriate to analyse the purity of the preparation only by morphological criteria. An electron micrograph of a SPM vesicle preparation shows a population of vesicles heterogeneous in size, even though the majority of the vesicles have dimensions similar to those of synaptosomes, and free from major contaminants. These morphological characteristics of the SPM vesicle preparation (not shown) present great similarities to those of preparations of high purity and characterised in detail (17).

To analyse the leaflet orientation in SPM vesicle preparations we assayed the activity of the Na^+/K^+-ATPase, by monitoring the production of H^+ (15,18,19). Since the active site for ATP is inside the vesicles in right-side-out orientation, the activity of the Na^+/K^+-ATPase in SPM vesicle preparations will result from the functioning of this enzyme in inside-out vesicles upon addition of ATP to the medium. By expressing this activity as a percentage of the total activity, obtained upon lysis of all vesicles with a detergent, we can have an indication of the fraction of the vesicle preparation that has resealed inside-out. The Na^+/K^+-ATPase activity of SPM vesicle fractions was determined for each preparation, and the sealed vesicles exhibit about 70% of the activity obtained upon lysis of SPM vesicles with saponin (not shown), which indicates that the SPM vesicle preparation is predominantly oriented inside-out. This fact led us to consider dispensable the separation of right-side-out from inside-out vesicles, a procedure that would reduce significantly the yield of the preparation.

Involvement of Cytosolic Proteins in Fusion

Fusion between synaptic vesicles and SPM vesicles in a cytosolic fraction, isolated from rat brain cortex, is stimulated as compared to that in buffer, indicating the involvement of cytosolic factors in the fusion process (Fig. 1). In this model system, the extent of fusion shows a gradual increase as the cytosolic protein concentration is increased, reaching a plateau for cytosolic protein concentration above 0.9 mg/ml (not shown). These results indicate that the effect of cytosol may be mediated by soluble proteins. To further demonstrate that cytosolic proteins have an important function in fusion between synaptic vesicles and SPM vesicles, we performed fusion assays, in cytosol that had been pretreated with trypsin. As expected, under these conditions, fusion is drastically reduced, showing values which are similar to those obtained in buffer (Fig. 1).

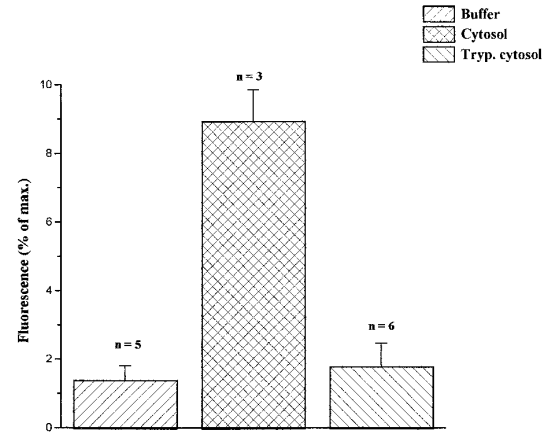

FIG. 1. Involvement of cytosolic proteins in fusion between synaptic vesicles and SPM vesicles. The fusion assay was carried out in 145 mM NaCl, 10 mM Hepes, 1 mM EGTA, pH 7.4, or in a rat brain cytosolic fraction, at a final protein concentration of 0.9 mg/ml. For some assays, previously to fusion, the cytosolic fraction was incubated, for 30 min at 37°C, in the presence of trypsin. The trypsin/protein ratio (w/w) used for the pre-treatment was 1/10. Fusion was monitored at 37°C, in a final volume of 2 ml. Values shown refer to fusion extent at 10 min and correspond to means ± SD of 3-6 assays.

Extents of fusion in untreated cytosol and in cytosol that had been incubated for 30 min at 37°C in the presence of the trypsin-trypsin inhibitor complex are not significantly different (not shown). We also assayed separately the influence of the presence of the trypsin-trypsin inhibitor complex and the effect of pre-incubating cytosol at 37°C for 30 min. The percentages of fusion observed under these conditions are not significantly affected as compared to control assays with untreated cytosol (not shown).

One likely candidate to mediate the effect of cytosol on fusion between synaptic vesicles and SPM vesicles could be NSF. This cytosolic ATPase has been shown to participate in several intracellular fusion events including regulated exocytosis (20), becoming inactive in the presence of *N*-ethylmaleimide (NEM). However we failed to observe an inhibition of fusion by NEM pretreatment of cytosolic fractions (Table I). Morgan and Burgoyne (21) state that NSF exists in a soluble form in cytosol and in a membrane bound form, and suggest that the fraction of NSF that remains associated to the membranes, that have been isolated from neuronal cells, may be sufficient to mediate fusion. For this reason, we also performed fusion experiments with synaptic vesicles and SPM vesicles that had been NEM-treated. However, even in assays with synaptic vesicles, SPM vesicles and cytosol that had been NEM pretreated, we could not observe significant changes in fusion extent (Table I).

The results shown in Fig. 1 also demonstrate that the assay used in this work indeed measures fusion of synaptic vesicles with SPM vesicles, since we would

not expect to observe changes in fusion extents for the different experimental conditions represented, if the fluorescence signal was due to R_{18} unspecific membrane transfer.

Effect of Botulinum Toxin B Pre-treatment of Synaptic Vesicles on Fusion

To evaluate the involvement of synaptobrevin in fusion between synaptic vesicles and SPM vesicles we performed fusion assays with synaptic vesicles that had been pre-incubated with botulinum toxin B. The toxin obtained in the inactive di-chain form was activated according to the method described by Braun et al. (13). These authors demonstrated, by SDS-PAGE, followed by Western Blots probed with an anti-synaptobrevin antiserum, that efficient cleavage of this protein occurs in a synaptic vesicle fraction isolated by the same method used in this work. Therefore, we used the same conditions as these authors to activate the toxin and pre-treat synaptic vesicles.

Before studying the effect of botulinum toxin B on fusion in our model system, we have done fusion assays in order to test whether the agents used for toxin activation could, on their own, affect fusion. As illustrated in Fig. 2, the presence of these substances during the assay cause no changes in fusion as compared to control assays. Pre-treatment of synaptic vesicles with active botulinum toxin B in the presence of EGTA does not also alter significantly fusion extent, since EGTA complexes Zn^{2+} rendering the toxin unable to cleave synaptobrevin. However, when synaptic vesicles were pre-incubated with active botulinum toxin B, in the absence of EGTA, a strong reduction of fusion extent is observed.

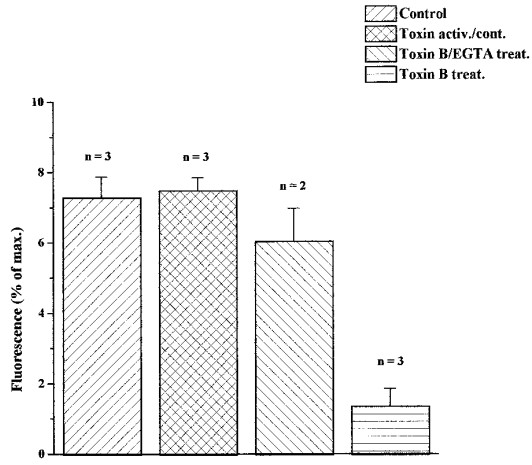

FIG. 2. Effect of botulinum neurotoxin B on fusion between synaptic vesicles and SPM vesicles. The fusion assay took place in cytosol, at a final protein concentration of 0.9 mg/ml, at 37°C, and in a final volume of 1.5 ml. Values represent the fusion extent, at 10 min, for control assays, and synaptic vesicles that have been pre-incubated with a mixture of substances used to activate the botulinum toxins (Toxin activ./cont.) and for synaptic vesicles that have been incubated with the active botulinum toxin B, in the presence of EGTA (Toxin B/EGTA treat.), or in the absence of EGTA (Toxin B treat.). Values are means ± SD of 2-3 experiments. For more details of botulinum neurotoxin B activation see Experimental Procedures.

DISCUSSION

In this work we have devised a model system consisting of synaptic vesicles and synaptic plasma membrane vesicles to study the membrane fusion event that mediates the exocytotic release of neurotransmitters.

Fusion between synaptic vesicles and SPM vesicles is mediated by proteins present in the cytosol. NSF is a cytosolic protein that has been implicated in neurotransmitter exocytosis (20). However, this protein does not play a role in the fusion step in this model system. These results are not so surprising in view of the models that have been recently proposed to explain the mechanism of binding and fusion between synaptic vesicles and the pre-synaptic plasma membrane. Several authors have recently reported NSF-independent fusion events and propose that ATP hydrolysis by NSF occurs upstream of Ca^{2+} action, and its role would be merely turning the SNAREs complex fusion-competent (22).

However, the major finding reported here concerns the fact that specific synaptobrevin cleavage by botulinum toxin B inhibits lipid mixing in our system. These results indicate that synaptobrevin may be the fusogenic component of the protein complex that allows exocytosis of neurotransmitters. Synaptobrevin protein structure possesses a putative peptide homologous to viral fusion peptides, suggesting that this synaptic protein may be the fusogenic agent (23) and that it could act similarly to hemagglutinin for influenza virus fusion (24).

TABLE I

Effect of NSF Inactivation of Fusion between Synaptic Vesicles and SPM Vesicles

	Fluorescence (% of max.)
Control	4.15 ± 0.92
NEM-treated	
Cytosol	4.45 ± 0.13
Synaptic vesicles, SPM vesicles	3.64 ± 0.60
Cytosol, synaptic vesicles, SPM vesicles	4.43 ± 1.34

Note. Fusion was followed as the increase of R_{18} fluorescence, at 590 nm, and the values shown refer to fusion extent at 10 min and correspond to means ± SD of 2-5 assays.

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