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Fusion of synaptic vesicles and plasma membrane in the presence of synaptosomal soluble proteins

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Abstract

Fusion between synaptic vesicles and plasma membranes isolated from rat brain synaptosomes is regarded as a model of neurosecretion. The main aim of current study is to investigate whether the synaptosomal soluble proteins are essential members of Ca^{2+}-triggered fusion examined in this system.

Fusion experiments were performed using fluorescent dye octadecylrhodamine B, which was incorporated into synaptic vesicle membranes at self-quenching concentration. The fusion of synaptic vesicles, containing marker octadecylrhodamine B, with plasma membranes was detected by dequenching of the probe fluorescence. Membrane fusion was not found in Ca^{2+}-supplemented buffer solution, but was initiated by the addition of the synaptosomal soluble proteins. When soluble proteins were treated with trypsin, they lost completely the fusion activity. These experiments confirmed that soluble proteins of synaptosomes are sensitive to Ca^{2+} signal and essential for membrane fusion. The experiments, in which members of fusion process were treated with monoclonal antibodies raised against synaptotagmin and synaptobrevin, have shown that antibodies only partially inhibited fusion of synaptic vesicles and plasma membranes in vitro. These results indicate that other additional component(s), which may or may not be related to synaptobrevin or synaptotagmin, mediate this process. It can be assumed that fusion of synaptic vesicles with plasma membranes in vitro depends upon the complex interaction of a large number of protein factors.

Keywords: Membrane fusion; Exocytosis; SNARE proteins; Cell-free model system

1. Introduction

Stimulation of the nerve terminal leads to calcium influx (Kostyuk, 1986) that triggers membrane fusion resulting in neurotransmitter release via exocytosis. Ca^{2+}-triggered fusion of synaptic vesicles (SVs) with the presynaptic plasma membrane (PM) is regarded as a final step of exocytosis. Recent genetic and biochemical studies have revealed that this highly regulated fusion process involves a cascade of protein–protein and protein–lipid interactions (Sudhof, 1995; Lin and Scheller, 2000). According to the SNARE hypothesis, protein complex known as the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) is of fundamental role for synaptic vesicle fusion process (Sollner et al., 1993). Although the functional importance of the SNARE complex in membrane fusion is well established, its precise role in the fusion process remains unclear. It has been proposed that the formation of the SNARE complex in a trans configuration pulls the apposing membranes into close contact and provides a driving force for membrane fusion (Hanson et al., 1997). This process is triggered by Ca^{2+}(Chen et al., 1999). A large number of membrane and soluble proteins have been identified at nerve terminals as participants of the exocytosis (Bajjalieh, 1999; Mochida, 2000; Zimmerberg et al., 2000). In the work with artificial system the assembly of SNARE complexes reconstituted in the liposomes has been shown to serve as the minimal machinery for membrane fusion (Weber et al., 1998). Membrane fusion events seem to include the interactions of vesicle SNARE proteins with SNAREs present on the plasma membrane (Rothman, 1994). To achieve the extraordinary speed, precision, and plasticity of neurotransmission, additional proteins have to be involved to regulate the function of these SNAREs and to control temporal and spatial formation of SNARE complexes. The soluble proteins of synaptosomes appear to operate at different transport steps within the nerve terminal, exerting an essential regulatory functions in membrane fusion events and in the mediation of vesicle tethering to target membranes (Rothman, 1994;

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Bajjalieh, 1999). However, the roles of these proteins in molecular pathway of membrane fusion are poorly understood because most assays fail to differentiate between steps of the exocytosis. To study the final fusion step of exocytosis in isolation from concomitant processes, such as vesicle mobilization from reserve pools, priming, and recycling, an isolated membrane system is required. That model retains the capacity for Ca^{2+}-triggered fusion and is amenable to a variety of biochemical manipulations (Crabb and Jackson, 1985).

Martin and Kowalchyk (Martin and Kowalchyk, 1997) studied Ca-activated exocytosis in cell-free system using radioactive ligands. It have been developed an in vitro assay for the fusion of pancreatic zymogen granules with the PM (Lee et al., 1994). The lipid-soluble fluorescent probe octadecylrhodamine was loaded into the granule membrane, and the granules were then incubated with unlabeled plasma membranes (PMs) (Edwardson, 1998). Fusion between Saccharomyces cerevisiae secretory vesicles and cytoplasmic-side-out plasma membrane vesicles was studied with fluorescent probe R18 (Arrastua et al., 2003).

Fusion between SVs and PMs isolated from rat brain synaptosomes is regarded as a cell-free model of neurosecretion (Trikash, 2002). The fusion of SVs containing fluorescent marker octadecylrhodamine B (R18) with PMs is detected. The method is based on the principle of dequenching of the fluorescence signal of a marker that has been incorporated into the membrane phospholipids of SVs. Earlier, in the model experiments we have found that calcium at micromolar concentrations triggers fusion between SVs and target membranes (Terletska and Trikash, 1998).

In 1990 Karli et al. (Karli et al., 1990) firstly reported direct evidence of fusion of chromaffin granules with the PM by an elegant method of fluorescence dequenching of the lipid marker, R18, as developed by Hoekstra et al. (Hoekstra et al., 1984). The popularity of the assay reflects the fact that intact biological membranes can be easily labeled with R18 by simple injection of ethanolic solution of R18 into an aqueous buffer containing the membranes. This method was confirmed subsequently by several other studies (Morris et al., 1989; Nagao et al., 1995) to be valid. At the same time few authors stressed some problems associated with this assay (Stegmann et al., 1995; Ohki et al., 1998). It was shown that in fusion assays using labeled lipid molecules the increase of fluorescence intensity could be the result of non-specific probe transfer without fusion (Cobaleda et al., 1994). So, a special experiments are necessary to discriminate these two possibilities.

The studies presented here reveal some requirements for realization of fusion process at cell-free model system of exocytosis and show a role of soluble and membrane protein components of the exocytotic apparatus in fusion.

2. Experimental procedures

2.1. Synaptosome preparation

A Wistar rats (150–200 g body weight) were killed by decapitation, the brains were rapidly removed and kept on ice. Rat brains were weighed, cut into pieces, transferred to ice-cold 0.32 M sucrose, 10 mM Tris–HCl, pH 7.5 (9 ml/g of brain tissue) and gently homogenized using a glass homogenizer with a teflon plunger (0.25 mm clearance). All manipulations were performed at 0 °C. Synaptosomes were prepared by differential centrifugation of rat brain homogenate (De Lorenzo and Freedman, 1978). The homogenate was centrifuged at 1500 × *g* for 10 min and the supernatant was removed and centrifuged at 12,000 × *g* for 20 min. The resultant pellet is the fraction of crude synaptosomes.

2.2. Isolation of synaptic vesicles and soluble proteins of synaptosomes

SVs were obtained by a modification of previously described technique (De Lorenzo and Freedman, 1978). Crude synaptosomes were lysed by rapid resuspension in 1 mM EGTA, 10 mM Tris–HCl pH 8.1 (3 ml/g of brain tissue) and incubated at 4 °C for 60 min. The preparation was centrifuged at 20,000 × *g* for 30 min. The pellet (M_1) was used to separate the PMs of synaptosomes (De Lores Arnaiz et al., 1967). The supernatant was centrifuged again at 55,000 × *g* for 60 min. Soluble proteins plus synaptic vesicle supernatant were centrifuged at 130,000 × *g* for 60 min. The supernatant is the soluble proteins of synaptosomes. The synaptic vesicle pellet was suspended in 10 mM Tris–HCl pH 7.5. This preparation is refered to as crude SVs. This fraction was then further purified by filtering through glass microfibre filters Whatman GF/C and two-fold washing. After thorough washing SVs were used for fusion experiments. The composition of the vesicle fraction was controlled by PM marker (Na^+/K^+ ATPase). No PM contamination was present in the purified SV preparation since no Na^+/K^+ ATPase could be detected after purification. This fraction was characterized in detail by electron microscopy and lazer correlation spectroscopy previously (Trikash et al., 2004).

2.3. Isolation of plasma membranes

The M_1 fraction was resuspended in 0.32 M sucrose, 10 mM Tris–HCl pH 7.5 and layered on a discontinuous (0.8–1.1 M) sucrose gradient. Centrifugation was carried out in the bucket-rotor at 50,000 × *g* for 120 min. Membrane fraction was collected at the interface between 0.8 M and 1.1 M sucrose and 4 volume of 10 mM Tris–HCl pH 7.5 was added. This suspension was centrifuged at 130,000 × *g* for 10 min. Finally, the pellet was resuspended in 0.32 M sucrose, 10 mM Tris–HCl pH 7.5. Purified PMs were taken for the membrane fusion studies.The proteins were determined by modified method of Lowry (Markwell et al., 1978).

2.4. Incorporation of R18 into the synaptic vesicle membrane

Within a certain range of concentrations the self-quenching of R18 should be proportional to its concentration in the membrane, according to Hoekstra et al. (Hoekstra et al., 1984). The suspension of SVs was prepared as described above. An ethanolic solution of R18 was injected into 0.2 ml of vesicle suspension (2 mg/ml of 10 mM Tris–HCl pH 7.5). The final concentrations of the probe and ethanol were 20 μM and 0.5% (v/v), respectively. The suspension was incubated with R18 for 5 min at 37 °C in the dark. Unbound R18 was removed on a Sephadex G-75 column. R18-loaded SVs were kept on ice in dark until use.

2.5. R18 fusion assay

The fusion of the SVs with PMs was monitored by the R18 dequenching assay (Hoekstra et al., 1984). The assay is based on the relief of self-quenching of the probe which, when diluted into the target membrane as a result of fusion and probe transfer, gives rise to an increase of the fluorescence emission signal. R18 fluorescence of labeled vesicles was about 86% quenched. Measurement of the fluorescence of R18 can provide a continuous and a quantitative assessment of membrane fusion.

In the assay, 1 ml of soluble proteins of synaptosomes (1 mg/ml), 10 μl of PMs and 20 μl of the R18-labeled SVs were stirred with a microstirbar in a 1 ml cuvette and maintained at 37 °C with a circulating waterbath. Fluorescence was monitored in an spectrofluorimeter (Hitachi 650-10 S) at $\lambda_{excitation}$ = 560 nm, $\lambda_{emission}$ = 580 nm (slit widths of 5 and 6 nm, respectively), and a 530 nm cut-off filter in the emission beam. The increase in the fluorescence signal resulting from