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Docking and fusion of synaptic vesicles in cell-free model system of exocytosis

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ABSTRACT

The present study involves the testing and characterization of synaptic vesicle (SV) docking and fusion as the steps of exocytosis using two different approaches in vitro.

The interaction of SVs was determined by the changing of particles size in suspensions by the method of dynamic light scattering (DLS). Fluorescence assay is represented for studying the mechanism of SV membrane fusion. The sizes of membrane particles were shown to increase in the medium containing cytoplasmic proteins of synaptosomes. Therefore, the cytosolic proteins are suggested to promote the SVs into close proximity where they may become stably bound or docked. The specific effect of synaptosomal cytosolic proteins on the interaction of SVs in the cell-free system was demonstrated. The incubation of SVs with liver cytosol proteins or in the bovine serum albumin solution did not lead to the enlargement of the particles size. The fusion reaction of the SVs membranes occurred within the micromolar range of Ca^{2+} concentrations. Our studies have shown that in vitro process of exocytosis can be divided into Ca^{2+}-independent step, termed docking and followed by fusion step that is triggered by Ca^{2+}. The role of cytosolic proteins of synaptosomes in docking and fusion of SVs in cell-free system was further confirmed.

1. Introduction

Regulated exocytosis is a result of many molecular events.

Synaptic vesicles (SVs) loaded with neurotransmitters target the specific regions on the plasma membrane called active zones (Zhai and Bellen, 2004; Mundige and De Camilli, 1994). Fusion of docked secretory vesicles with the plasma membrane is triggered by the rapid elevation of the intracellular calcium concentration. The targeting, docking and fusion of vesicles with the acceptor membranes require specific recognition between integral membrane proteins known as t- and v-SNAREs (syntaxin 1A, SNAP25 and synaptobrevin2/VAMP2, respectively) (Söllner et al., 1993) and recruitment of cytosolic docking complexes (Waters and Pfeffer, 1999). This mechanism transfers proteins and lipids to plasma membrane and supports secretion of signal molecules from the cell (Llona, 1995; Zimmermann, 1997; Chapman, 2002).

A large number of molecules, including n-Sec1/munc18-a, Rabs, complexin, munc-13, tomosyn, and SNAP-29 have been suggested to regulate the availability of SNARE proteins used to form functional neurotransmitter release machinery (Carr and Munson, 2007; Tsuboi and Fukuda, 2006). Some of them were found to be soluble, cytoplasmic proteins. NSF/Sec18p acts prior to membrane fusion to prime specific SNARE complexes for docking of vesicles with their targets, while the SNAREs are likely to be the minimal membrane-fusion machinery (Barr, 2000). Interactions between these proteins are reversible, and are often associated with conformational changes that probably represent elementary reactions in the pathway leading from vesicle docking to membrane fusion (Avery et al., 2000). Whereas some basic understanding of fusion has been achieved (Jahn and Südhof, 1999), the role of the proteins mediating the preceding steps is still obscure.

Interestingly that experiments with nerve terminals poisoned by tetanus or botulinum toxins, which are highly selective and specifically proteolyze SNAREs, synaptobrevin or syntaxin 1A or SNAP-25 does not result in depletion of docked SVs. These observations indicate, that SNARE proteins are not required for SV docking (Südhof et al., 1993; Hunt et al., 1994). These results imply that other factors are responsible for, or at least contribute to, docking specificity. However, despite the isolation of large numbers of regulatory proteins essential for neurotransmitter release, the relations between SVs docking and Ca^{2+}-triggering fusion events of regulated exocytosis remain rather obscure. A major obstacle to further progress in the fields of the sequential molecular steps leading to membrane fusion is the limited availability of assays.

Biochemical studies of separate steps of exocytosis require model systems that retain the capacity for Ca^{2+}-triggering fusion and amenable to biochemical manipulation. The most convenient systems are permeabilized cell preparations that are all else quite complicated systems by themselves (Avery et al., 2000), and cell-free preparations described only for a few specialized systems

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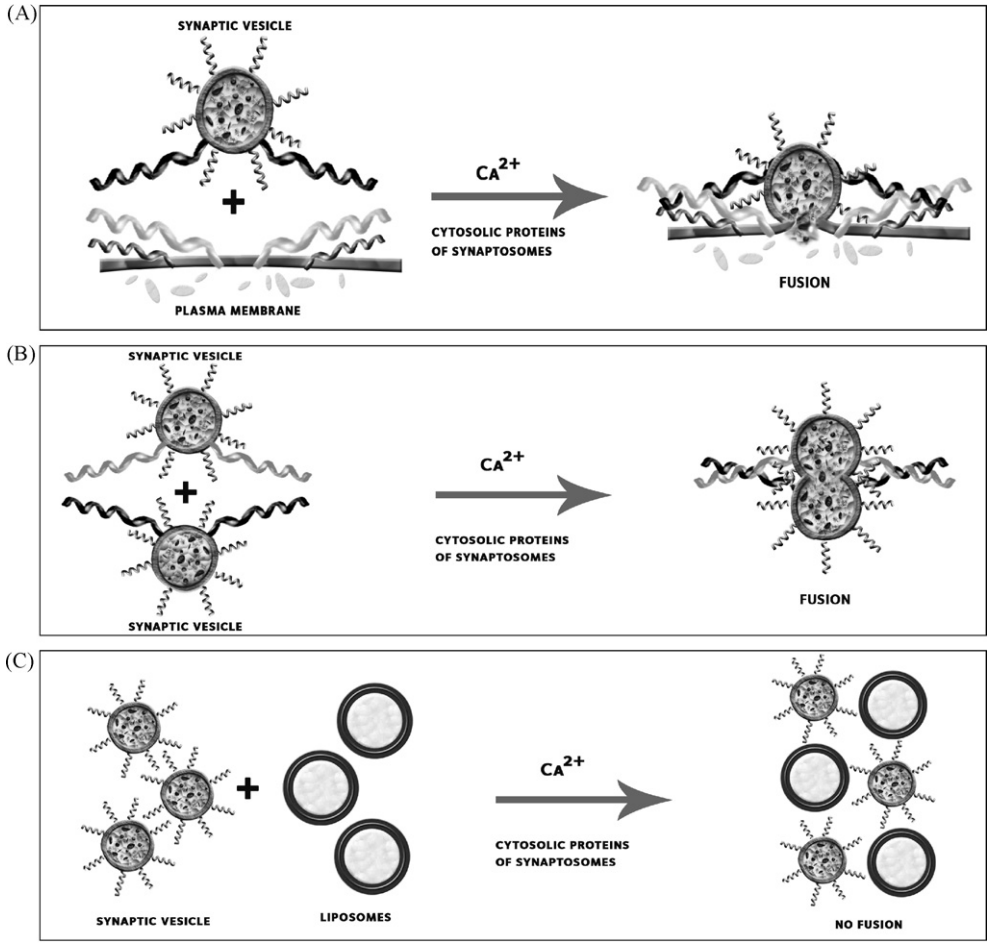

Fig. 1. Model systems of synaptic vesicles fusion with various target membranes: (A) plasma membranes of synaptosomes; (B) synaptic vesicles; (C) liposomes.

like the sea urchin eggs (Zimmerberg et al., 2000). Fig. 1 demonstrates a scheme of SVs interactions with different target membranes based on our previous experiments. We have found that in the presence of Ca^{2+} and cytosolic proteins of synaptosomes SVs fuse in vitro with plasma membrane (heterotypic fusion) (Fig. 1A), as with each other (homotypic fusion) (Fig. 1B) (Trikash et al., 1997, 2004; Terletska and Trikash, 1998). The cytosolic proteins of synaptosomes accelerate the fusion in dose-dependent manner. Conversely, when these proteins are not added SVs do not fuse (Trikash and Kolchinskaya, 2006). We studied the fusogenic properties of SVs using artificial lipid membranes as acceptors (Volynets and Trikash, 2007). It was shown that liposomes could not serve as target membranes for Ca^{2+}-induced fusion of SVs in the medium containing cytosolic proteins (Fig. 1C). This suggests that the fusion observed in Fig. 1A and B is protein-specific process that requires both membrane bound and cytosolic proteins. Taking into account that the kinetics of Ca^{2+}-dependent SVs fusion are similar in heterotypic and homotypic fusion systems, this work was carried out on homotypic fusion system that may reveal some exocytosis aspects of general importance (Guatimosim and von Gersdorff, 2002; Pickett and Edwardson, 2006).

Ability of SVs for homotypic fusion has been confirmed in *in vitro* experiments with purified SVs of Drosophila photoreceptor cells (Shimizu et al., 2003). The authors have also noted that after incubation of SVs in cytosol, particle size in SVs suspension dramatically increased. Together, these data indicate a possibility for multivesicular, compound exocytosis not only for sensory neurons, but also for brain neurons.

In this research our main efforts were aimed at resolving the steps leading towards fusion in nerve cells and clarifying the role of cytosolic proteins in these processes. We offer the system consisted of SVs and cytosolic proteins isolated from rat brain synaptosomes that allow the reconstitution of vesicle docking and fusion under cell-free conditions. The combination of different approaches, fluorescent spectroscopy with octadecyl rhodamine B chloride (R18) and dynamic light scattering (DLS), allowed to show the critical role of cytosolic proteins for aggregation/docking step and separate this step from Ca^{2+}-dependent membrane fusion.

2. Experimental procedures

The study was performed according to the guidelines of National Academy of Sciences of Ukraine and the Institutional Animal Care and Use Committee (IACUC).

Wistar rats were fasted overnight with free access to water and food before studies. They were anesthetized using chloroform.

2.1. Synaptosome preparation

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 SVs and cytosolic proteins of synaptosomes

To select SVs pool of monodispersed size we have isolated the SVs by two different methods. Crude synaptosomes were lysed by rapid resuspension in 1 mM