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GIANT PROTEOLIPOSOMES PREPARED BY FREEZING-THAWING WITHOUT USE OF DETERGENT: RECONSTITUTION OF BIOMEMBRANES USUALLY INACCESSIBLE TO PATCH-CLAMP PIPETTE MICROELECTRODE

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SUMMARY: When sarcoplasmic reticulum membrane vesicles or synaptosomes were mixed with sonicated phospholipid vesicles and subjected to freezing-thawing, giant vesicles of up to 50 μm in diameter were formed. When the biomembrane vesicles were labeled with a covalently binding fluorescent dye, the resultant giant vesicles were fluorescent, thereby suggesting that the freezing-thawing process induces fusion of phospholipid and biomembrane vesicles. When membranes of the giant proteoliposomes thus prepared were studied using the patch-clamp technique, potassium channels of the biomembranes were detectable. The present method of the giant proteoliposome preparation is simple and rapid, and provides a system suitable for the study of ion channels of various biomembranes usually inaccessible to a patch-pipette microelectrode.

Giant proteoliposomes prepared by the freezing-thawing technique have been used for electrophysiological studies on ion channels of biomembranes (1-4). We reported the optimum condition for the formation of giant proteoliposomes, determined using this method, with reference to the phospholipid composition, ionic strength and protein-to-lipid ratio (5). In all these studies, small proteoliposomes were first prepared by the detergent/dialysis method: vesicles were solubilized with detergent together with exogenous phospholipid, and then reconstituted into small proteoliposomes (about 100 nm in diameter) by removing the detergent with dialysis or with adsorption on Bio-Beads. The resultant proteoliposomes were frozen in liquid nitrogen or dry-ice and thawed on ice or in air. Giant proteoliposomes had formed.

The method of reconstitution used in the studies cited above is based on the freeze-thaw/sonication technique originally described by Kasahara and Hinkle (6) in a reconstitution procedure of a glucose-transporter from eryth-

Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; EGTA, ethylene glycol bis(-aminoethylether)-N,N,N',N'-tetraacetic acid; FITC, fluorescein-5-isothiocyanate; SR, sarcoplasmic reticulum; FT, freezing-thawing.

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rocytes. Recently, Kasahara and coworkers reported the reconstitution of glucose transport activity from subcellular fractions of adipocytes by a newly devised freeze-thaw/sonication method which utilizes no detergent (7). This finding implies that the fusion of biomembrane vesicles and sonicated phospholipid vesicles can occur in the process of freezing and thawing without the need of detergent. This prompted us to examine the feasibility of the freezing-thawing (FT) technique, without using detergent/dialysis process, in a reconstitution of ion channels of isolated biomembrane vesicles into giant proteoliposomes suitable for the application of a patch pipette. When sarcoplasmic reticulum (SR) vesicles or synaptosomes in which intrinsic membrane proteins had been fluorescence-labeled were mixed with sonicated phospholipid vesicles and subjected to the FT treatment, the resultant giant vesicles were fluorescence-labeled. This suggests fusion of these biomembrane vesicles and exogenous phospholipid vesicles. Patch-clamp experiments revealed the incorporation of ion channels in the membrane of the giant proteoliposomes.

MATERIALS AND METHODS

Chemicals - Asolectin (soy bean phospholipid, type II-S) and fluorescein-5-isothiocyanate (FITC) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Asolectin was partially purified according to Kagawa and Racker (8).

Biomembrane preparations - Sarcoplasmic reticulum (SR) vesicles were prepared from rabbit skeletal muscle according to Miller and Rosenberg (9). Synaptosome was prepared from cortex of rat brain according to Dunkley et al. (10). Briefly, the cortex from six male Sprague-Dawley rats, 7 - 8 weeks old, was homogenized in a solution of 0.32 M sucrose and 10 mM Tris-HCl (pH 7.0) with a Teflon homogenizer at the speed of 800 rpm. The membranous P2' fraction was obtained from the homogenate by centrifugation and placed on Percoll density gradient: 3 %, 10 %, 15 %, and 23 % Percoll in 0.32 M sucrose. After ultracentrifugation at 32,500 x g for 5 min, the turbid band at the interface of 15 % and 23 % layers was taken as the synaptosome fraction. The purity of the synaptosome was checked by electron microscopy. Usually synaptosomes of about 70 % purity were obtained and myelin was the dominant contaminant.

Preparation of giant proteoliposomes - Phospholipid vesicles were prepared by a sonication method. Namely, a phospholipid suspension (10 mg lipid/ml) in a solution (FT buffer) containing 0.1 M KCl and 10 mM MOPS-KOH (pH 7.0) was sonicated with a bath-type sonicator, model NS50-05U of Nihon Seiki (Tokyo, Japan), under a nitrogen atmosphere. It became almost clear in 5 min. A standard membrane fusion experiment was carried out as follows: biomembrane vesicles were mixed with sonicated phospholipid vesicles with the ratio of protein/exogenous lipid = 1/10 (w/w) in the FT buffer. The mixture was frozen rapidly with liquid nitrogen and thawed on ice. In a control experiment, small proteoliposomes were prepared by the deoxycholate/dialysis method as described in (5) and subjected to the FT treatment to form giant proteoliposomes.

Labeling of biomembrane with FITC - Membranes were fluorescence-labeled by suspending them (2 mg protein/ml) in a solution containing 0.25 M sucrose, 0.1 mM EGTA, 25 mM Tris-HCl (pH 9.0) and FITC (0.25 mg/ml). After 30 min incubation at room temperature, the labeling reaction was stopped by neutralization and unreacted FITC was removed by washing the membranes twice with a repetition of sedimentation by ultracentrifugation and resuspension in the FT buffer. Fluorescence-labeled membrane vesicles were fused with sonicated phospholipid vesicles, by the standard procedure.

RESULTS

Fusion or binding of biomembrane vesicles and sonicated phospholipid vesicles was demonstrated using fluorescence-labeled biomembranes. Sarcoplasmic reticulum (SR) membranes were labeled with the fluorescent dye, FITC, which reacts with free amino groups to form a covalent bond (11). A suspension of SR vesicles (2 mg protein/ml) labeled with FITC and sonicated asolectin vesicles (10 mg lipid/ml) were subjected to the FT treatment. The resultant giant proteoliposomes were observed with a phase-contrast or fluorescence light microscope. Figure 1A reproduces a phase-contrast micrograph of a typical example of a giant vesicle prepared from SR vesicles and asolectin vesi-

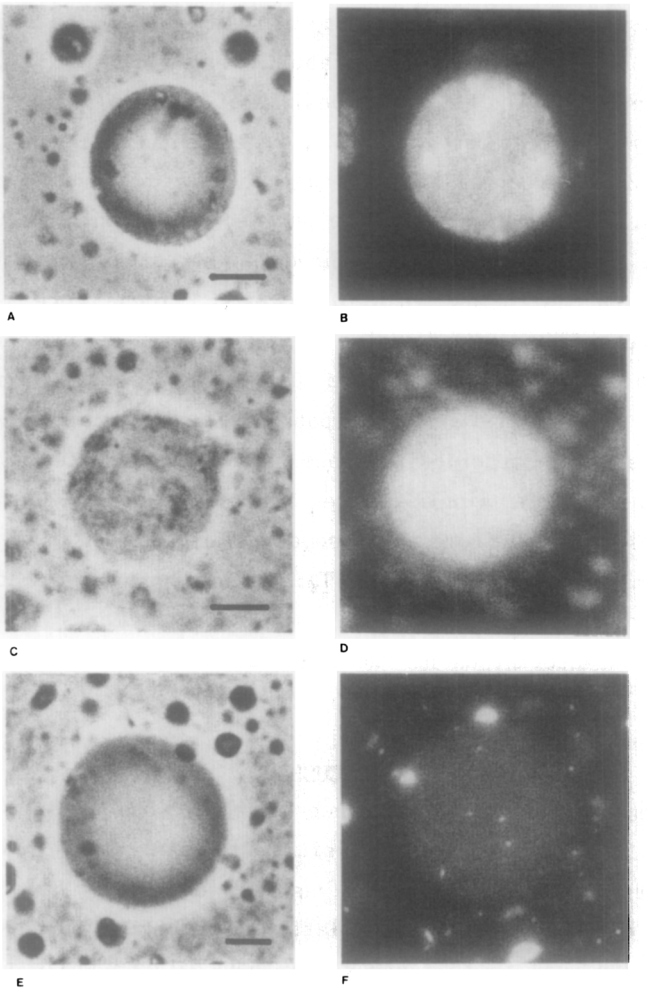

Fig. 1. Micrographs of giant vesicles observed with a phase-contrast (A, C, E) and a fluorescence (B, D, F) microscope. (A, B) SR vesicles labeled with FITC were mixed with sonicated asolectin vesicles and subjected to FT treatment to form giant proteoliposomes. (C, D) FITC-labeled SR vesicles were solubilized and reconstituted with asolectin by the deoxycholate/dialysis method followed by the FT treatment. (E, F) FITC-labeled SR vesicles were mixed with giant asolectin liposomes. bar: 10 μm.

cles. In Fig. 1B is shown a fluorescence micrograph of the same vesicle of Fig. 1A. The fluorescence distributed almost homogeneously over the whole vesicle membrane indicates the incorporation of SR membrane into the giant vesicle membrane.

The result of a positive control experiment is shown in Figs. 1C and D, where giant vesicles were formed through the FT treatment of small proteoliposomes prepared from SR vesicles and asolectin by the deoxycholate/dialysis method. The microphotographs in Fig. 1C and 1D are similar to those in Fig. 1A and 1B, respectively. A negative control experiment was performed as follows: giant liposomes prepared from asolectin were mixed with FITC-labeled SR vesicles (Fig. 1E, F). The fluorescence intensity of these giant vesicles is negligibly weak. This indicates that incorporation of biomembranes in giant vesicles does not occur by simple mixing, but that FT treatment is required. The same results stated above were obtained with mixtures of synaptosomes and sonicated asolectin vesicles.

Electrophysiological experiments further supported the argument. As shown in Fig. 2, ion channel currents were observed from the membrane of the giant vesicles, when studied using the patch-clamp technique. Figure 2A shows single-channel current fluctuations through a sarcoplasmic reticulum K^+ channel reconstituted into giant proteoliposomes. These were recorded in the inside-out patch configuration. The single-channel conductances are 35, 90 and 123 pS in a symmetrical 100 mM KCl. This channel has higher open probability at more positive pipette potentials as seen from Fig. 2A. These characteristics are in accord with those previously reported (12, 13).

Figure 2B shows the single-channel K^+ current traces obtained from giant proteoliposomes prepared from synaptosomes. The single-channel conductance is 30 pS in a symmetrical 150 mM KCl. This K^+ channel is probably the same as one of those reported in the planar membrane experiments (14). A detailed study of the characteristics of the channels of synaptosomal membranes is in progress.

DISCUSSION

Fluorescence microscopic evidence revealed the fusion of biomembrane vesicles with sonicated asolectin vesicles to make giant proteoliposomes or binding (adsorption) of biomembrane vesicles on giant liposomes. Electrophysiological evidence suggests the latter possibility. The tip diameter of the patch pipette used in this study was about 0.5 μm while the diameters of SR vesicles and synaptosomes were 50 - 100 nm and about a few hundred nm, respectively. Since the diameter of the biomembrane vesicles is much smaller than that of the pipette tip, a giga-ohm seal and single-channel current records could not be obtained if the biomembrane vesicles had been adsorbed on the surface of giant liposomes.

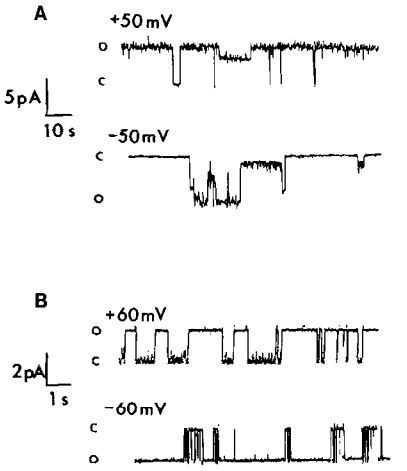

Fig. 2. Single-channel current fluctuations at two membrane potentials. Letters O and C indicate open and closed level, respectively. An inside-out patch of membrane was excised from a giant proteoliposome prepared from asolectin and biomembrane vesicles: (A) rabbit skeletal sarcoplasmic reticulum vesicles; (B) rat brain synaptosomes. Currents were measured under voltage-clamp conditions in a symmetrical solution: (A) 100 mM KCl, 1 mM $CaCl_2$ and 10 mM HEPES-KOH (pH 7.0); (B) 150 mM KCl and 10 mM HEPES-KOH (pH 7.0). Current records were filtered at (A) 100 Hz or (B) 320 Hz.

Reconstitution methods previously reported includes the process of membrane solubilization with detergent. The disadvantage of this method is the probable loss of orientation of intrinsic membrane proteins in the reconstituted membranes. When studying ion channels, knowledge of protein orientation is most important. Second, residual detergent molecules remaining in the reconstituted membrane may alter the lipid-protein interaction. Criado and Keller (12) reported a new method of biomembrane fusion into giant vesicles. It does not involve membrane solubilization with detergent, rather, use is made of a considerable amount of ethylene glycol as a fusogen, and the lipid-protein interaction may be altered. Giant proteoliposomes prepared without a detergent or any exogenous fusogen as described in the present paper circumvent these difficulties. Our method is simple and rapid. Reconstitution of biomembranes into giant proteoliposomes for patch-clamp studies is thus feasible.

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