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A New Method for Membrane Reconstitution: Fusion of Protein-Containing Vesicles with Planar Bilayer Membranes Below Lipid Phase Transition Temperature

W. Hanke[1], H. Eibl[2], and G. Boheim[1]

[1] Ruhr-Universität Bochum, Lehrstuhl für Zellphysiologie, D-4630 Bochum, Federal Republic of Germany
[2] Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen, Federal Republic of Germany

Abstract. A new method of membrane reconstitution was developed by fusion of channel protein containing vesicles with planar bilayer membranes. The fusion process only occurred below and near the phase transition temperature of the lipid used. We obtained the following results: 1. Our system is solvent-free and vesicles do not come into contact with the air/water interface. This obviates a possible denaturation of hydrophobic proteins. 2. Channel forming proteins and protein complexes, respectively, are active in a frozen lipid matrix. 3. We detected an unknown channel in cilia fragments. 4. Purified acetylcholine receptors form fluctuating channels in a membrane consisting of a pure synthetic lecithin (two component system).

Key words: Planar bilayer membrane – Lipid phase transition – Fusion – Membrane reconstitution – Channel proteins

Introduction

Fusion of solvent-free phospholipid vesicles has been reported to occur under two different experimental conditions: 1. in the presence of negatively charged lipids and millimolar concentrations of Ca^{2+} (Papahadjopoulos et al. 1976a, 1977) and 2. near the phase transition temperature of pure phospholipids (Lawaczeck et al. 1975; Papahadjopoulos et al. 1976b). In both cases it has been argued that structural defects due to lipid phase changes might initiate the fusion, in particular because divalent cations increase the transition temperature of negatively charged lipids by charge neutralization (Träuble and Eibl 1974). The incorporation of intrinsic membrane proteins into planar bilayer membranes by fusion of protein-containing vesicles with planar bilayers was successfully demonstrated using negatively charged lipids and Ca^{2+} (Miller 1978; Cohen et al. 1980). However, an additional osmotic gradient was needed to

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achieve a high fusion rate. Planar bilayers were formed with n-decane as stabilizing solvent (Mueller et al. 1963) and with lipids in the fluid state.

Recently we reported the formation of virtually solvent-free planar bilayer membranes in the solid state using mixed-chain lipids. Pore forming antibiotics caused similar effects above and below the lipid phase transition temperature (t_c) (Boheim et al. 1980). These results suggested that fusion of vesicles with planar bilayers might be possible using a pure lipid near its t_c. In this paper we report the successful application of this new method of reconstitution utilizing channel forming proteins as fusion indicators.

Materials and Methods

We used three different vesicle preparations containing the following hydrophobic channel proteins: 1. a cationic channel from fragmented cilia of *Paramecium tetraurelia* (cilia-channel), 2. membrane fragments rich in acetylcholine receptor from *Torpedo marmorata* (Ach-channel), and 3. the corresponding acetylcholine receptor protein purified by affinity chromatography and incorporated into vesicles of the same pure lipid as used for the planar bilayers (pur. Ach-channel). Fragment vesicles of cilia from *Paramecium tetraurelia* were isolated by Dr. J. Schultz (Universität Tübingen) as described elsewhere (Boheim et al. in prep.) and frozen in liquid nitrogen in small aliquots. Each sample was thawed only once and used the same day. Acetylcholine receptor-rich membrane fragments from *Torpedo marmorata* were prepared by Dr. G. Fels (MPI Dortmund) (Fels and Maelicke in prep.). Channel fluctuations were observed with both fresh and frozen electric organ prior to fragmentation if fragments were used within 2 days. Purified receptor preparations were prepared in Dr. A. Maelicke's laboratory (MPI Dortmund) by affinity chromatography as described elsewhere (Rüchel et al. 1981). These samples were still active at least 1 week after preparation. Carbamylcholine chloride, hexamethonium chloride and all buffer reagents (p.A. grade) were obtained from Merck.

Virtually solvent-free planar bilayer membranes were formed according to the method of Montal and Mueller (1972) using a $\geqslant 99\%$ pure mixed-chain lecithin. This lipid, 1-stearoyl-3-myristoyl-glycero-2-phosphocholine, was synthesized following a method described elsewhere (Eibl 1980). The temperature ranges for its phase transition (t_c) from fluid to solid and solid to fluid state are 30–27° C and 30–33° C, respectively, as determined by differential scanning calorimetry (Boheim et al. 1980). Bilayers were made at 25–26° C if not otherwise indicated. The mechanical and electronic equipment used has been described elsewhere (Boheim 1974). Polarity is defined as in electrophysiology: a negative applied voltage refers to the potential of the rear compartment (inside) being more negative than that of the front compartment (outside). Current is designated negative if cation transfer occurs from front to rear (inward current).

Results and Discussion

The occurence of single and multiple fusion events of solvent-free vesicles with solvent-free planar bilayer membranes a few degrees below t_c is shown in Fig. 1a and b, respectively. While the presence of Ca^{2+} ions is not a prerequisite of fusion, application of an osmotic gradient appears to be necessary for high fusion rates. If there is no osmotic gradient between vesicle and aqueous solution fusion events occur quite seldom. By using high vesicle concentrations (~ 100 µg protein per ml of compartment solution) and an osmotic gradient steady state membrane conductances are obtained which are equivalent to simultaneous fluctuations of 100, 1,000 or even more channels. However, fusion of many vesicles was often accompanied by an additional, artifactual increase in membrane conductance which became larger with time after vesicle preparation. In order to achieve a correct correlation between multi-channel and single-channel fluctuations noise analysis has to be done. We are planning to quantify the fusion rates by applying this method. The addition of cholesterol to the membrane lipid in a molar ratio of 5 : 1 (lecithin : cholesterol) stops fusion completely. Maximum fusion rates are observed at the calorimetric phase transition temperature but membranes tend to break. Lowered temperature leads to reduced fusion rates and stable membranes. Above t_c ($> 30°$ C) the fusion rate decreases sharply with increasing temperature. Bimolecular lipid membranes containing decane could not be formed below t_c.

By diluting the vesicle preparations (1–10 µg protein per ml depending on the vesicle preparation) the fusion rate can be reduced to the point where very few, sometimes only one, events occur before the osmotic gradient between vesicle and aqueous solution is dissipated and fusion stops. Figure 2 shows single channel current fluctuations at constant applied voltage of three different vesicle preparations and with two different channel proteins. Note that channel properties in Fig. 2a and b differ, whereas channel properties in Fig. 2b and c are not influenced by endogenous lipid incorporated into the planar bilayer by fragment fusion. We take this as evidence for the observed fluctuations being no artifacts due to hydrophobic protein or lipid. It was a surprise to us to observe the cationic channel (Fig. 2a), because only a voltage-dependent Ca^{2+}-channel was claimed to be present in the cilia (Machemer and Ogura 1979). Further experiments are required to clearly establish the various channels that may exist in this preparation.

A detailed account of the electrochemical properties of acetylcholine receptor-rich and purified receptor preparations is published elsewhere (Boheim et al. in prep.). As shown in Fig. 1b many Ach-channels are activated and desensitized within a few seconds by the application of high concentrations of carbamylcholine. However, carbamylcholine concentrations below the desensitizing level lead to continuous sequences of single Ach-channel events (Fig. 2b). These results agree with those published by Schindler and Quast (1980). Furthermore, we can demonstrate (Fig. 2c), that the receptor protein purified by affinity chromatography and incorporated into vesicles of mixed-chain lipid causes equivalent channel fluctuations as those obtained with receptor-rich membrane fragments. The properties of the reconstituted acetylcholine receptor

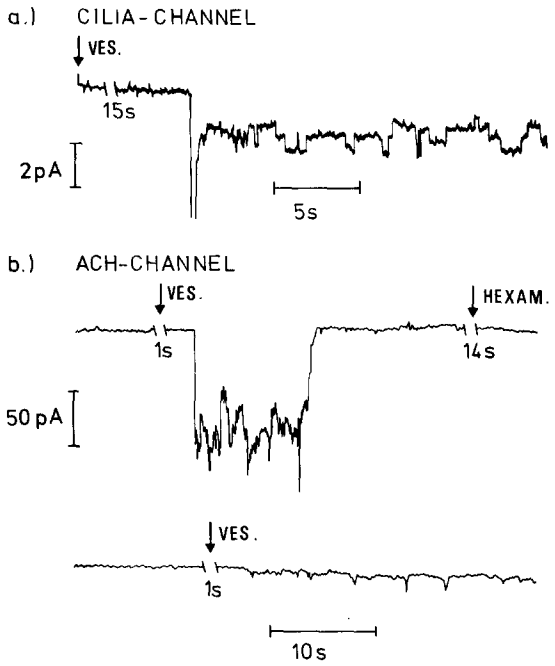

Fig. 1a and b. Fusion of vesicles with planar bilayer membranes. Bilayers were formed from 1-stearoyl-3-myristoyl-glycero-2-phosphocholine using the Montal-Mueller technique at 25° C. **a** 10 μg protein of cilia fragments from *Paramecium tetraurelia* are added to the front (outside) compartment (arrow). A few seconds later and indicated by a spontaneous conductance increase, a single fusion event is observed. At the end of the fusion spike, stepwise conductance changes are discernible which result from pore state fluctuations. These were followed by consecutive fusion processes (not shown). Fragment vesicles were equilibrated in 200 mM sucrose, 10 mM MOPS-buffer, pH 7.2. Aqueous solutions: front compartment 0.2 M KCl, 1 mM $CaCl_2$, 2 mM Na, K-phosphate buffer, pH 7.0; rear compartment 0.1 M KCl, 1 mM $CaCl_2$, 2 mM Na, K-phosphate buffer, pH 7.0. Membrane potential −17.8 mV, inversion potential +7.2 mV. Downward deflections refer to ion transfer from front to rear (inward current). **b** Approximately 10 μg protein (~ 10 pmol toxin binding sites) of acetylcholin receptor-rich membrane fragments from *Torpedo marmorata* were added to the front compartment at the position of the first arrow mark. To the same compartment, 2 min earlier, 4 μM carbamylcholine (final concentration) were added. Fusion is indicated by the large increase in membrane conductance occurring shortly after addition of fragment vesicles. Within 10–15 s the conductance decreased again to its initial value, apparently due to desensitization of the carbamylcholine activated channel by the high concentration of agonist. At the second arrow mark 10 μM hexamethonium were added to the front compartment. Supplementary addition of 10 μg protein to the same compartment, indicated by the third arrow mark, caused only a slight conductance increase because the receptor was blocked by the antagonist. Fragment vesicles were equilibrated for at least 1 h in 1 M KCl, 5 mM Na, K-phosphate buffer, pH 7.0, 1 M sucrose. Aqueous solutions in both compartments were identical, but without 1 M sucrose. We emphasize that the presence of Ca^{2+} is not needed for the fusion of vesicles with a pure lipid planar bilayer membrane ~ 4° C below its phase transition temperature. Applied voltage: −50 mV

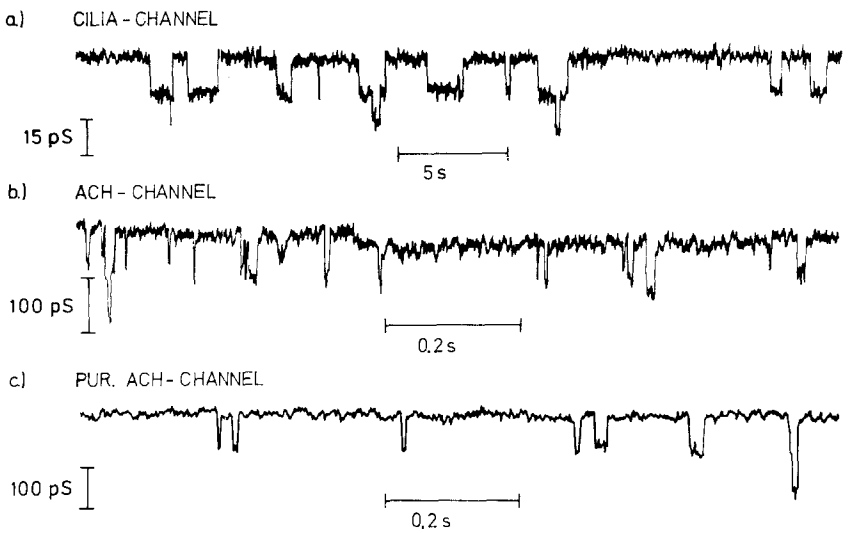

Fig. 2a–c. Single channel events obtained with different vesicle preparations. **a** Single channel fluctuations of a cationic channel from cilia fragments. Mean open channel conductance: 16 pS, mean lifetime: 0.5 s. Appl. voltage: −50 mV. Aqueous solutions (in both compartments): 0.1 M KCl, 1 mM $CaCl_2$, 2 mM Na, K-phosphate buffer, pH 7.0. Fragment vesicles were equilibrated in 200 mM sucrose, 10 mM MOPS-buffer, pH 7.2. **b** Single channel fluctuations of the cationic channel from acetylcholine receptor-rich membrane fragments. Fragment vesicles were given to the front compartment and no channel fluctuations occurred. The channels were then activated by the addition of 0.4 μM carbamylcholine (final concentration) to the same compartment. Mean open channel conductance: 79 pS, mean lifetime: 3.1 ms. Appl. voltage: −50 mV. Aqueous solutions (in both compartments): 1 M KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Na, K-phosphate buffer, pH 7.0. Vesicles were equilibrated at least for 1 h in the same solution supplemented with 1 M sucrose. **c** Single channel fluctuations of the acetylcholine receptor protein purified by affinity chromatography and incorporated into vesicles composed of the same pure mixed-chain lipid as the planar bilayers. After vesicle fusion channels were activated by the addition of 0.4 μM carbamylcholine to the front compartment. Mean open channel conductance: 90 pS, mean lifetime: 4.2 ms. Appl. voltage: −110 mV. Aqueous and vesicle equilibrating solutions same as in **b**. The difference of mean open channel conductance in **b** and **c** is mainly due to the different applied voltage. At −50 mV a value of 82 pS is found for the purified receptor preparation. Membrane lipid and membrane forming procedure were identical to those described in Fig. 1. Temperature 25° C. The experimental data were reproducible within ± 10%

also correspond to those observed in biological muscle cell membranes using the patch-clamp technique (Neher and Sakmann 1976; Boheim et al. in prep.).

The data presented here demonstrate an efficient new method of membrane reconstitution. The fusion of protein-loaded vesicles with planar bilayer membranes free of organic solvents is gentle and may be applicable to many integral membrane proteins and to many different kinds of vesicle preparations. Experiments with pore forming antibiotics showed that a few degrees below t_c the lipid molecules around a pore were in the fluid state, whereas domains of pure lipid were solid (Boheim et al. 1980; Chapman et al. 1977). Since lateral diffusion is two orders of magnitude slower in the frozen compared to the fluid membrane, endogenous lipids surrounding the channel protein may remain

associated with the protein for at least a few seconds, thus preserving its activity. It is demonstrated by the purified acetylcholine receptor protein incorporated into pure synthetic lipid vesicles that association with an extraneous lipid does not necessarily cause denaturation of an integral channel protein. Concerning the question of a specifically immobilized (Marsh and Barrantes 1978) of fluid (Bienvenüe et al. 1977) boundary layer surrounding the acetylcholine receptor protein it appears from our experiments with pore forming antibiotics (Boheim et al. 1980) that the protein associated lipid seems to be in a fluid state and that this protein-lipid patch is surrounded by a solid lipid phase.

There seem to be two requirements for the fusion of vesicles with planar bilayer membranes: 1. an osmotic gradient and 2. structural instabilities. A possible reason for the first condition was discussed by Cohen et al. (1980). Osmotic pressure arising from diffusion of water into the vesicle may facilitate rupture of the membranes in the region of vesicle-planar membrane contact and thus induce fusion. The second condition of structural defects might reflect a reduction in the activation barrier for such a local rupture. The existence of structural instabilities in a pure lipid system near or at t_c is well established (Lawaczeck et al. 1975; Boheim et al. 1980). The appearance of such defects following the addition of Ca^{2+} to a membrane system containing negatively charged lipids as a consequence of lateral phase separation is very likely (Papahadjopoulos et al. 1976a, 1977; Träuble and Eibl 1974).

An alternative method for membrane reconstitution without using any solvent has recently been published by Schindler and Quast (1980). The formation of a stable monolayer at the air/water interface of an aqueous vesicle suspension was utilized to obtain planar bilayers according to the method of Montal and Mueller (1972). Although successful reconstitution was demonstrated using acetylcholine receptor-rich membrane fragments, a disadvantage of this method is the partial air contact of the hydrophobic proteins when surrounded only by a lipid monolayer which might cause protein denaturation (Schindler and Quast 1980). The same arguments apply to the experiments of Nelson et al. (1980) with purified acetylcholine receptor preparations. With our method of vesicle fusion within the aqueous phase we do not see sidedness of the receptor activity. Furthermore, fusion experiments can be done with only a small amount of fragment vesicles.

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