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CALCIUM-INDUCED INTERACTION OF PHOSPHOLIPID VESICLES AND BILAYER LIPID MEMBRANES

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Summary

The interaction of unilamellar phospholipid vesicles with bilayer lipid membranes has been studied by observing the electrical conductance of the planar membrane. The presence of phosphatidylcholine vesicles as well as phosphatidylcholine/phosphatidylserine (1 : 1) vesicles on one side compartment of the bilayer membrane, but not phosphatidylserine vesicles, causes discrete fluctuations in the phosphatidylserine membrane conductance, which is also increased by at least an order of magnitude. These events are dependent on vesicle concentration as well as the presence of Ca^{2+}. The results are interpreted in terms of the incorporation of domains of phosphatidylcholine into the membrane, which confer a higher conductance state.

Introduction

Membrane fusion is a vital aspect of several cellular processes such as synaptic transmission, secretion, endocytosis and plasma membrane assembly [1]. It is also important in the fusion of cultured mammalian cells [2] and the administration to these cells of drugs entrapped in phospholipid vesicles [3]. The molecular mechanisms of the membrane fusion reaction are still not clearly understood. Several theories have been put forth [4—7] and model membrane systems have been used to elucidate the role which lipids play in this reaction [8—12].

In this study we have investigated the interaction of unilamellar phospholipid vesicles with planar phospholipid bilayers, especially with respect to Ca^{2+} concentration, as a model for the fusion of vesicles (secretory or extracellular) with plasma membranes. This system, originally described by Pohl and coworkers [13], and recently by others [14—16], also promises to be a method by which membranes reconstituted in the form of proteoliposomes can be in-

corporated into bilayer lipid membranes for studying their electrical properties [17,18].

Methods

Bovine brain phosphatidylserine was obtained from Supelco (Pa.) and egg yolk phosphatidylcholine was obtained from Applied Science Laboratory (Pa.) and also prepared by Dr. W. Naccarato. The samples showed a single spot on thin-layer chromatographic plates. Unilamellar phospholipid vesicles were prepared by the method of Papahadjopoulos and co-workers [2]. Phospholipids were suspended at a concentration of 10 μmol/ml in 100 mM NaCl (Fisher Chemical) containing 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, Calbiochem) buffer, pH 7.4, vortexed for 10 min and sonicated for 1 h in a bath type sonicator (Heat Systems, Ultrasonics). The final suspension exhibited very little turbidity ($A_{400\,nm}$ = 0.1 for 1 μmol/ml of phosphatidylcholine). These operations were carried out at 24°C and under a N_2 atmosphere. The vesicles were stored at 5° C under N_2 until use.

Bilayer lipid membranes were made as previously described [19]. Membrane conductance was measured with a Hewlett-Packard 425A Micro Volt-Ammeter by applying a constant 20 mV potential across the membrane through Ag/AgCl electrodes and was monitored continuously on a Bausch and Lomb VOM-7 recorder. Normally, the applied potential was more positive in the inside compartment than the outside. However, the results obtained in this way were almost identical as in the case where the direction of the applied voltage across the membrane was reversed. The electrodes, the cell and the glass cup were washed thoroughly after each experiment to avoid any contamination. All solutions were made with triple distilled water and passed through HA (0.45 μm) Millipore filters to eliminate debris from reagents.

Vesicles or divalent cations were introduced with microsyringes (Hamilton) connected via polyethylene tubing to the aqueous chamber bathing one side of a phosphatidylserine membrane after black film formation. Membrane bulging was corrected for by adding the bathing buffer solution to the other side. Only those membranes which initially had a very high resistance ($>10^{10}$ Ω) were used for the subsequent experiment. This resistance was constant for at least 1 h if no vesicles were introduced, even with an asymmetric distribution of Ca^{2+} across the membrane. Both the inner and outer chambers were stirred for 1 min at 5-min intervals by means of magnetic stirrers. All experiments were done at 25 ± 1°C.

Results and Discussion

When phosphatidylcholine or phosphatidylserine/phosphatidylcholine (1 : 1 or 1 : 2) vesicles were incubated with the phosphatidylserine-decane planar membrane in the presence of certain amounts of Ca^{2+}, step increases in and discrete fluctuations of the membrane conductance were observed [16]. The behavior of the membrane conductance in a typical experiment is shown in Fig. 1. In preliminary experiments membranes were made in 100 mM NaCl, 5 mM HEPES, pH 7.4 ("NaCl buffer") which contains residual amounts of Ca^{2+}

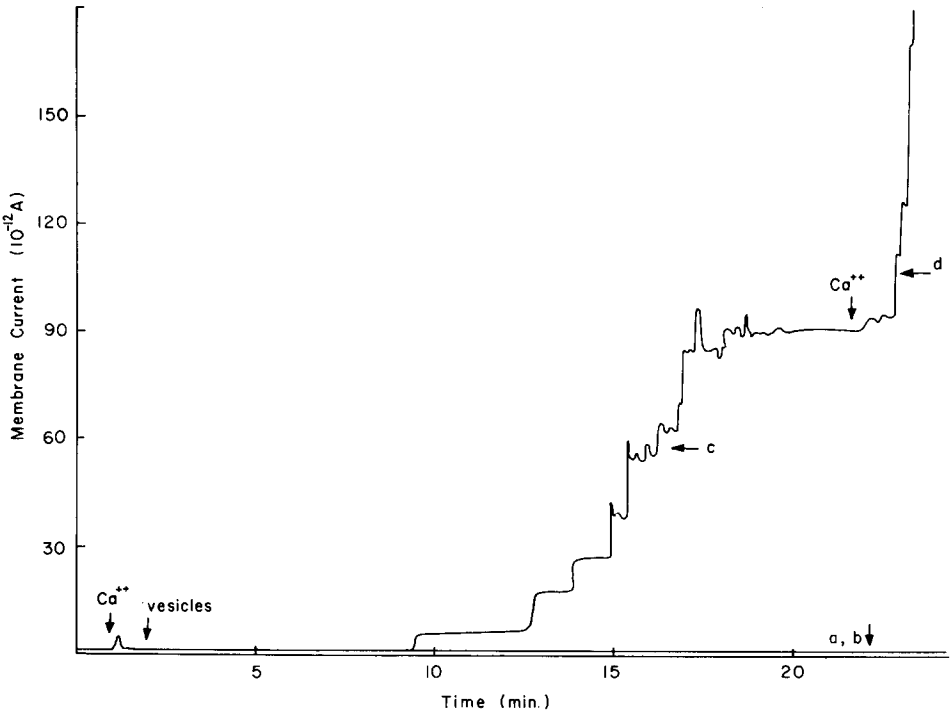

Fig. 1. The membrane current at 20 mV applied direct current potential of a phosphatidylserine-decane bilayer as a function of time as Ca^{2+} and vesicles are introduced into the inner chamber. The membrane was made in NaCl buffer containing 10 μM Ca^{2+}. Membrane incubated with a, 0.5 μmol phosphatidylcholine in unilamellar vesicles; final concentration 136 μM; b, 0.5 mM Ca^{2+} inside, with no vesicles, and 0.1 mM Ca^{2+} outside; c, the same Ca^{2+} concentration as in b, but with 136 μM phosphatidylcholine vesicles in the inside compartment; d, an additional 0.5 mM Ca^{2+} was introduced to the inside after the membrane attained a high conductance level.

($\approx$0.7 μM) from the NaCl; this concentration of Ca^{2+} conferred a high resistance to the phosphatidylserine membrane (cf. ref. 19), but was not sufficient for the phosphatidylcholine vesicle-induced conductance increase. When the Ca^{2+} concentration was further increased in the inner chamber it was necessary to increase the concentration in the outer chamber proportionately (e.g. 1/10 of inside) in order to maintain stability [20]. In later experiments phosphatidylserine membranes were routinely made in NaCl buffer containing 0.1 mM $CaCl_2$.

Phosphatidylserine vesicles, however, did not lead to conductance increases of the phosphatidylserine membrane at $[Ca^{2+}]$ = 0.1—5 mM. The vesicle types which did cause conductance increase and fluctuations seemed to be those which did not flocculate in the presence of Ca^{2+} (followed by measurement of turbidity at 400 nm in a Beckman DU-Gilford spectrophotometer). Fig. 2 shows that above 1 mM Ca^{2+}, phosphatidylserine vesicles start flocculating (possible aggregation or fusion), whereas phosphatidylcholine and phosphatidylserine/phosphatidylcholine (1 : 1) vesicles do not. Although phosphatidylcholine vesicles do aggregate at $[Ca^{2+}] > 10$ mM when no other salt is present [21], this is not the case when the medium contains 100 mM NaCl. If the con-

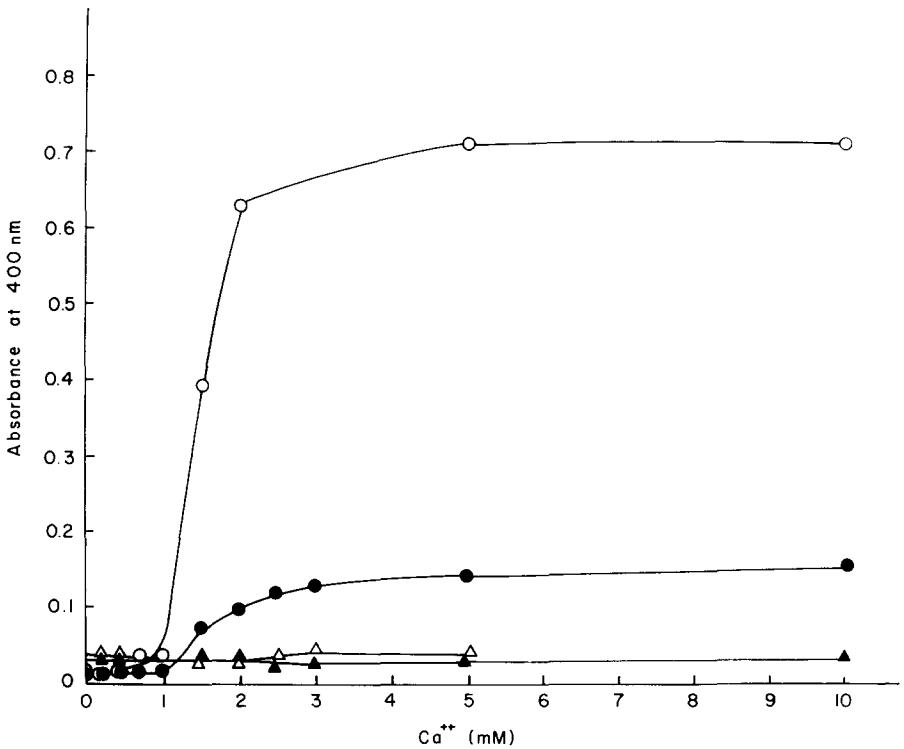

Fig. 2. The absorbance at 400 nm of a suspension of phospholipid vesicles in NaCl buffer as a function of Ca^{2+} concentration. The absorbance was measured at equilibrium after the introduction of Ca^{2+}. ○———○, phosphatidylserine, 250 μM; ●———●, phosphatidylserine, 50 μM; △———△, phosphatidylserine/phosphatidylcholine (1 : 1), 500 μM; ▲———▲, phosphatidylcholine, 138 μM.

ductance increases and fluctuations were due to the fusion process of individual vesicles one would expect to observe them with phosphatidylserine vesicles, which are known to fuse [9]. Since only the vesicles which contained phosphatidylcholine lead to the increase in conductance of the phosphatidylserine membrane, we investigated the effect of membrane composition on membrane conductance. A planar bilayer membrane made of phosphatidylcholine only had a conductance which was an order of magnitude higher than a membrane made only of phosphatidylserine (confirming earlier results [19]). The absolute value of the conductance is somewhat higher than that reported by some laboratories for pure phosphatidylcholine, but in agreement with others. In this study only the difference between two types of membrane is of importance. Fig. 3 shows that as the phosphatidylcholine content of the membrane forming solution is increased the planar bilayer membrane conductance increases and attains levels comparable to those obtained in Fig. 1 (c and d) (approx. $5 \cdot 10^{-9}$ Ω^{-1}).

The conductance increase and fluctuations were dependent on both vesicle and Ca^{2+} concentrations. Lower concentrations of vesicles required more time to initiate the altered conductance behavior of the planar membrane; below a critical concentration, which varied with different vesicle preparations, no conductance increase was observed for more than 40 min (see Fig. 4). In some

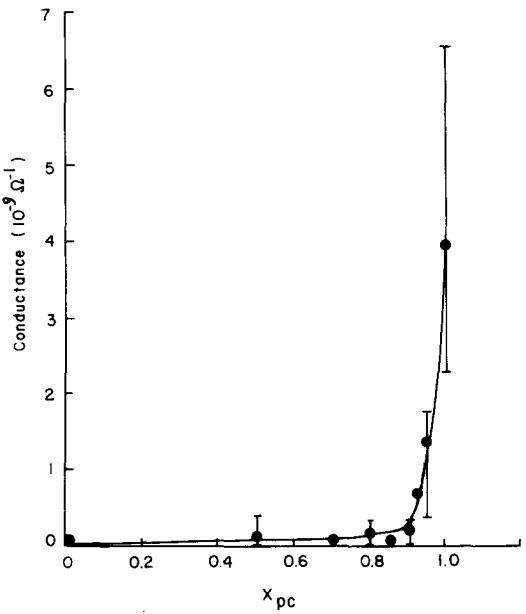

Fig. 3. Average membrane conductance (Ω^{-1}) as a function of membrane composition, indicated as the mol fraction of phosphatidylcholine (PC) in phosphatidylserine. Decane solutions (5—7.5 mg/ml) of the phospholipids were mixed in the designated proportions and used as the membrane-forming solution. The aqeous chamber contained 0.1 mM Ca^{2+} in NaCl buffer. Points represent the average of at least four experiments in which the conductance was taken as the mean value of the fluctuations. The bars represent the spread of these mean values.

experiments the time of onset was about 1 min; this was the lower limit of these measurements probably because of the initial stirring period. When a membrane broke and another one was made in the presence of vesicles, the time course was similar to those observed when the vesicles were introduced after black film formation. The formation of a stable membrane with a high resistance in the presence of vesicles also indicates that the conductance increase is not due to an instability caused by the presence of vesicles. Calcium concentrations between 0.1 and 10 mM resulted in conductance increases about the same time after the addition of phosphatidylcholine vesicles. The minimum Ca^{2+} concentration to cause this conductance increase seemed to be between 10 and 100 μM. This calcium-dependent conductance increase was observed not only for the case of asymmetrical distribution of Ca^{2+} with respect to the phosphatidyserine membrane, but also in the same manner for the case of the symmetrical distribution. At 10 mM Ca^{2+}, shiny microscopic patches appeared on the membrane, perhaps due to the adhesion of aggregated vesicles. In the case of phosphatidylserine/phosphatidylcholine (1 : 1) vesicles the minimum concentration of Ca^{2+} was about 4 mM for a similar conductance increase of the membrane. Unsonicated multilamellar phosphatidylcholine liposomes affected the conductance in a manner identical to unilamellar vesicles, but a five times greater amount of phospholipid was required. This may be due to the slower diffusion through the unstirred layer and smaller surface area per mol of lipid of multilamellar liposomes compared to unilamellar vesicles

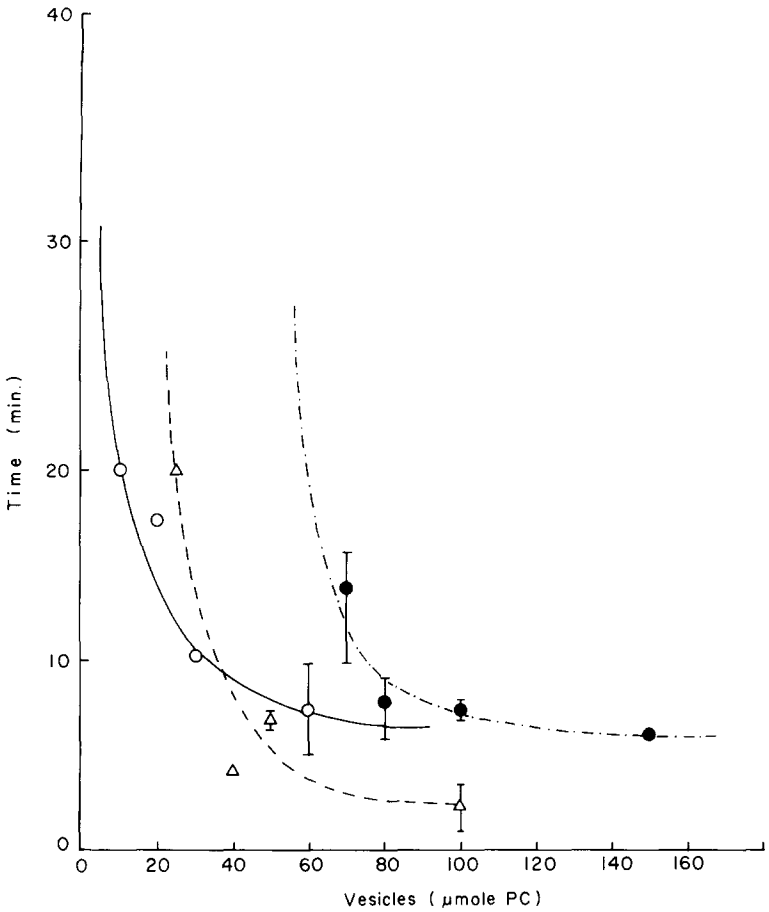

Fig. 4. The time of onset of the step conductance increase after the introduction of phosphatidylcholine (PC) vesicles as a function of the amount of vesicles in the inner chamber. The phosphatidylserine membrane was made in NaCl buffer containing 0.1 mM Ca^{2+}. Ca^{2+} (final concentration, 0.6 mM) was added to the inner chamber before vesicle addition. The bars represent the spread of 2—6 experiments; the different symbols refer to different vesicle preparations.

[22]. The exclusively unilamellar vesicles obtained from the supernatant by centrifugation of the vesicle suspension at 100 000 × *g* for 30 min, gave results identical to those described above.

Magnesium at concentrations of 0.1—5 mM and in the presence of phosphatidylcholine vesicles (1 μmol; 272 μM) caused drastic and initially almost linear increases in conductance (up to 10^{-7} Ω^{-1} before breakage) without the characteristic initial steps. The possibility that the conductance increase could be caused by lysolecithin usually found as an impurity in lecithin preparations was partially ruled out by the observation that when membranes were incubated in the presence of lysolecithin in the bathing solution at a concentration corresponding to a 1% impurity, only small fluctuations and no steps were observed.

We interpret the conductance increases caused by phosphatidylcholine vesicles to be due to the incorporation of increasingly larger amounts of phospha-

tidylcholine molecules into the phosphatidylserine bilayer, such that the planar membrane begins to exhibit a conductance level characteristic of membranes which contain a high level of phosphatidylcholine in the membrane-forming solution (about 90%, as shown in Fig. 3). However, the actual phosphatidylcholine content of the bilayer may not correspond to the mol fraction in the membrane-forming solution. The incorporation of phosphatidylcholine into the phosphatidylserine membrane could be through either membrane fusion or the transfer of individual molecules of phosphatidylcholine from the vesicles to the bilayer. Both of these mechanisms have been shown to be possible during the interaction of phospholipid vesicles with each other [9,10].

The Ca^{2+}-induced interaction of vesicles and planar membranes (Fig. 1) may be mediated by several mechanisms: (1) Ca^{2+} will neutralize the surface charge of the phosphatidylserine membrane. (2) The strong affinity of Ca^{2+} for the phosphatidylserine membrane polar surface will result in the removal of the water of hydration around the phospholipid head groups, rendering the membrane surface hydrophobic in nature [23]. This may be an enhancing factor in membrane fusion [7,24]. (3) The difference in surface energy between the planar membrane and vesicles (which have a low radius of curvature) may be an additional contributing factor. It should be pointed out that in some experiments conductance states much higher than that expected from a pure phosphatidylcholine-decane membrane were obtained (e.g. $2 \cdot 10^{-8}\ \Omega^{-1}$). This could be due to defective molecular packing in the membrane as the phosphatidylserine and phosphatidylcholine molecules diffuse laterally to form domains because of the presence of Ca^{2+} [25,26]. Defective molecular packing may also be one of the causes of the increase in conductance and eventual membrane breakage upon introduction of additional Ca^{2+} while the membrane is at a high conductance state (Fig. 1(d)). Mg^{2+} interacts with phosphatidylserine membranes in a different manner [9,25], and thus may affect the vesicle-planar bilayer interaction quite differently (vide supra).

We have thus demonstrated a Ca^{2+}-induced interaction of phospholipid vesicles with planar phospholipid membranes without the use of fluorescence, conductance or surface-charge probes [13—15]. The possible presence of organic solvent in the membrane in our system (planar bilayer) may affect the fusion process to a certain degree (cf. ref. 14). This factor must be considered in comparing our results with fusion between phospholipid vesicles. It is not yet certain at this moment, whether the experimental results here show actual membrane fusion between planar bilayer and vesicle membranes. However, our system bears a resemblance to those of many biological systems where Ca^{2+} introduction triggers exocytosis [27—29].

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