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FUSION OF SMALL UNILAMELLAR LIPOSOMES WITH PHOSPHOLIPID PLANAR BILAYER MEMBRANES AND LARGE SINGLE-BILAYER VESICLES

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Summary

Small unilamellar phosphatidylserine/phosphatidylcholine liposomes incubated on one side of planar phosphatidylserine bilayer membranes induced fluctuations and a sharp increase in the membrane conductance when the Ca^{2+} concentration was increased to a threshold of 3—5 mM in 100 mM NaCl, pH 7.4. Under the same ionic conditions, these liposomes fused with large (0.2 μm diameter) single-bilayer phosphatidylserine vesicles, as shown by a fluorescence assay for the mixing of internal aqueous contents of the two vesicle populations. The conductance behavior of the planar membranes was interpreted to be a consequence of the structural rearrangement of phospholipids during individual fusion events and the incorporation of domains of phosphatidylcholine into the Ca^{2+}-complexed phosphatidylserine membrane. The small vesicles did not aggregate or fuse with one another at these Ca^{2+} concentrations, but fused preferentially with the phosphatidylserine membrane, analogous to simple exocytosis in biological membranes. Phosphatidylserine vesicles containing gramicidin A as a probe interacted with the planar membranes upon raising the Ca^{2+} concentration from 0.9 to 1.2 mM, as detected by an abrupt increase in the membrane conductance. In parallel experiments, these vesicles were shown to fuse with the large phosphatidylserine liposomes at the same Ca^{2+} concentration.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

Many exocytotic processes, such as histamine secretion in mast cells and neurotransmitter release in synapses, are activated by calcium and involve the fusion of secretory vesicles with the cell membrane [1—4]. The mechanism of these calcium-induced membrane fusion reactions is not understood clearly. Freeze-fracture electron microscopy studies have shown that during fusion, membrane proteins are excluded from the zone of contact of the two membranes, thus enabling lipid bilayer regions to interact and to fuse with each other [5]. The implications for the role of the lipid bilayer in membrane fusion have been supported further by the observation that liposomes made of acidic phospholipids fuse in the presence of physiological concentrations of Ca^{2+} [6]. Several model systems have been used to study the effect of Ca^{2+} on the interaction and fusion of phospholipid membranes [6]. The interaction of unilamellar liposomes with planar phospholipid bilayers is of special interest, because it is not only has a geometrical resemblance to the exocytotic vesicle-plasma membrane system, but also allows for the study of the interaction between membranes which have different molecular compositons [7—11].

Small unilamellar phospholipid vesicles cause a sharp increase in the conductance of planar phosphatidylserine bilayers at a threshold concentration of Ca^{2+} [8]. Since the presence of phosphatidylcholine in the vesicle membrane is necessary for this conductance behavior to be exhibited, and since bilayers made of a phosphatidylcholine/phosphatidylserine mixture in which the former is in excess have a higher membrane conductance than pure phosphatidylserine membranes, the conductance increase induced by vesicles was interpreted as being the result of the incorporation of phosphatidylcholine into the planar membrane. It was not possible, however, to demonstrate unequivocally that membrane fusion had occurred [11]. We subsequently showed that phospholipid vesicles decrease the surface tension of phosphatidylserine monolayers at a threshold Ca^{2+} concentration which depends on the vesicle and monolayer compositions and on the area per molecule in the monolayer membrane [12]; this change in surface tension was interpreted to be a manifestation of the fusion of the vesicles with the monolayer. In the present study, we have demonstrated that vesicles made of a 1 : 1 mixture of phosphatidylcholine and phosphatidylserine fuse with large, single-bilayer phosphatidylserine liposomes in a Ca^{2+} concentration range identical to that which induces conductance fluctuations in planar phosphatidylserine bilayers. We have used a fluorescence assay which detects the intermixing of the internal aqueous compartments of the two populations of vesicles. The assay utilizes the enhancement of the fluorescence of Tb^{3+} when it complexes with dipicolinic acid, these substances being entrapped in different vesicle populations [13,14]. We have also shown a correlation between the fusion of small unilamellar phosphatidylserine vesicles with large vesicles and with planar bilayers made of phosphatidylserine, monitoring the interaction with the planar membrane by means of the conductance increase caused by the transfer of gramicidin A from the vesicle membrane [10].

Experimental Procedure

Materials

Bovine brain phosphatidylserine was purchased from Avanti Polar Lipids (Birmingham, AL) and Serdary Biochemicals (Ontario) and egg phosphatidylcholine from Avanti; these lipids were also prepared in our laboratory according to the method of Paphadjopoulos and Miller [15]. Samples were tested for impurities with thin-layer chromatography on Silica gel G (Brinkman). Chemicals and organic solvents were of the highest purity commerically available. Water was distilled twice for the fusion experiments and three times for the bilayer experiments including distillation from alkaline permanganate.

Interaction of vesicles with planar bilayers

Phosphatidylserine, dried from chloroform and from ether, was dissolved in *n*-decane (Fluka) for bilayer formation (5—10 mg/ml) and the solution kept in the cold under N_2 (maximum 2—3 days). The phosphatidylserine sample from Serdary was previously washed with EDTA to facilitate black film formation.

Planar bilayer membranes were formed in a solution of 100 mM NaCl and 5 mM Hepes (Calbiochem and Sigma, respectively) at pH 7.4. Bilayer formation and electrical measurements were as described in detail elsewhere [11,16]. The conductance of the membranes was monitored by recording the current generated by applying a 20 mV potential across the membrane with the outer chamber as ground. For the experiments with valinomycin (Calbiochem), bilayers were made in 100 mM KCl, 5 mM Hepes, pH 7.4, containing 10^{-9} M ionophore added in a concentrated ethanol solution, and the membrane current was measured at a 10 mV applied potential. Additions of equivalent amounts of ethanol did not alter the conductance. After each experiment, the electrodes were washed successively with chloroform/methanol, ethanol, and water to eliminate any adhering vesicles. The cell and glass cup were washed with detergent and rinsed thoroughly with water.

Chloroform solutions of the phospholipids were dried under a stream of N_2 or in a vacuum, suspended in 100 mM NaCl, 5 mM Hepes (pH 7.4) by vortex mixing for 10 min, and used as the multilamellar vesicle preparation. Unilamellar vesicles were made by sonicating a vortex mixed preparation (10 μmol/ml) for 1 h under a positive pressure of N_2 or Ar at 20—24°C in a bath-type sonicator [11,15] and sedimenting any remaining large vesicles by centrifugation at 100 000 $\times g$ for 1 h. Phosphatidylserine/gramicidin A vesicles were prepared in 100 mM NaCl, 0.01 mM EDTA, 0.2 mM Tris-HCl at pH 7.2, by adding an ethanolic solution of the ionophore (Nutritional Biochemicals, OH) to multilamellar vesicles (at a final concentration corresponding to about 1 gramicidin A molecule per unilamellar vesicle). This suspension was then sonicated and centrifuged as described above. These vesicles were incubated overnight at 5°C to facilitate the partitioning of gramicidin A into the membrane, and in some experiments passed through a Sephadex LH-20 (Pharmacia, NJ) column to eliminate free ionophore [17]. In the experiments with phosphatidylserine/gramicidin A vesicles, the planar phosphatidylserine membranes were formed in the buffer used for vesicle preparation.

After a black film was formed, materials were added to the inner and outer

aqueous chambers through polyethylene tubes connected to microsyringes. The hydrostatic pressure head that developed across the membrane upon addition of vesicles or $CaCl_2$ was countered by adding an appropriate amount of the NaCl buffer solution to the opposite side of the membrane.

Experiments were performed at room temperature (24—28°C).

The assay for fusion

The fusion of small and large unilamellar vesicles was followed by using the assay described by Wilschut and co-workers [13,14], with some modifications. The small vesicles (either pure phosphatidylserine or its 1 : 1 mixture with phosphatidylcholine) were prepared by sonication, as described above, in the presence of 15 mM $TbCl_3$ (Alfa Products, Dover, MA), 150 mM sodium citrate, 5 mM Hepes, pH 7.4. To check for fusion among small vesicles, another preparation was made in 150 mM dipicolinic acid, 5 mM Hepes, pH 7.4. Large unilamellar vesicles were made by reverse-phase evaporation according to the method of Szoka and Papahadjopoulos [18] with some alterations. Phosphatidylserine (10 μmol) was dissolved in 1 ml redistilled diethyl ether; 0.3 ml of 50 mM dipicolinic acid (Sigma, sodium salt), 20 mM NaCl, 5 mM Hepes, pH 7.4, was added and the mixture sonicated for 5 min under Ar. The ether was removed slowly from the emulsion in a rotary evaporator kept at 30°C under controlled vacuum (350 mmHg); the resulting gel was broken up by vortex mixing, and the ether was further evaporated until an aqueous suspension resulted. More aqueous solution was added and any residual ether was evaporated at 10 mmHg for 20 min. The vesicles were extruded through polycarbonate filters (Unipore, BioRad; Richmond, CA) of 0.2 μm pore diameter to achieve a uniform size distribution [19]. Non-encapsulated material was separated from the vesicles by gel filtration on Sephadex G-75 (Pharmacia) columns freshly packed for each vesicle preparation. The elution buffer was 100 mM NaCl, 5 mM Hepes, pH 7.4, containing 1 mM EDTA to prevent the interaction of Tb^{3+} with phosphatidylserine.

For the assay of vesicle fusion, equimolar (based on lipid phosphorus [20]) amounts of Tb vesicles and dipicolinic acid vesicles were suspended in 1 ml of 100 mM NaCl, 5 mM Hepes, 0.1 mM EDTA, pH 7.4, in a quartz cell stirred magnetically and maintained at 25°C. Fluorescence measurements were made in an SLM-4000 fluorimeter. The excitation wavelength was set at 276 nm; fluorescence was detected at 545 nm after transmission through a Corning 3-68 cut-off filter which eliminated possible contributions from light scattering. The presence of EDTA (and Ca^{2+}, during the fusion reaction) outside the vesicles quenched the fluorescence such that only the Tb/dipicolinic acid reaction occurring inside fusing vesicles registered as fusion. The aggregation of vesicles was followed by 90° light scattering simultaneously with the fusion measurements by the use of a second photomultiplier in a T-configuration and a Corning 7-54 band pass filter. The fluorescence resulting from the interaction with dipicolinic acid of all the Tb^{3+} in a certain amount of vesicles was designated as 100% and was determined as follows. A portion of the Tb-containing vesicles were freed of EDTA by a second gel filtration on Sephadex G-75 by elution with NaCl buffer containing no EDTA. The appropriate amount of this vesicle preparation was suspended in 1 ml of 100 mM NaCl, 5 mM Hepes, pH 7.4,

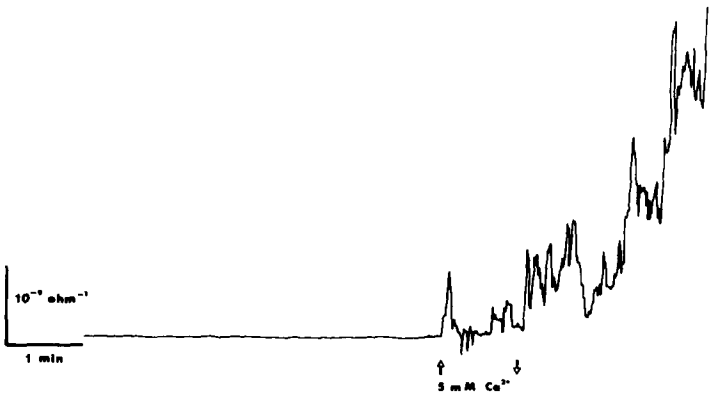

Fig. 1. The time course of the conductance (Ω^{-1}) of a planar phosphatidylserine bilayer in the presence of sonicated unilamellar phosphatidylserine/phosphatidycholine (1 : 1) vesicles. The aqueous solution was 100 mM NaCl, 5 mM Hepes, pH 7.4. First, the conductance of the bilayer was checked and ascertained to be steady; then the vesicles were added to the inner chamber at a final concentration of 0.1 μmol lipid/ml. Ca^{2+} was introduced in the following scheme: 0.1 mM inside and outside, wait 6 min; 1 mM inside and outside, wait 6 min; 3 mM inside, wait 6 min; 5 mM inside. The trace shows only the latter part of the experiment; the conductance did not change until the introduction of 5 mM Ca^{2+}, except for the transitory effect of Ca^{2+} on the planar membrane itself, as established in control experiments. Both chambers were stirred for 1 min after vesicle and Ca^{2+} additions. The arrows indicate the stirring period. The membrane eventually ruptured.

excess dipicolinic acid was added and the contents of the vesicles were released by lysing them with 1% (w/v) cholate (Sigma, recrystallized). Other details of the fusion assay are given elsewhere [14].

Results

Phosphatidylserine/phosphatidylcholine vesicles

We had observed earlier that sonicated vesicles made of various mixtures of phosphatidylserine and phosphatidylcholine incubated in the aqueous chamber on one side of a planar phosphatidylserine bilayer caused fluctuations in the conductance of the membrane when the Ca^{2+} concentration was increased in the same chamber [8,11]. The results of a representative experiment employing small (approx. 25 nm diameter) unilamellar phosphatidylserine/phosphatidylcoline (1 : 1) vesicles are shown in Fig. 1. When these vesicles were added to

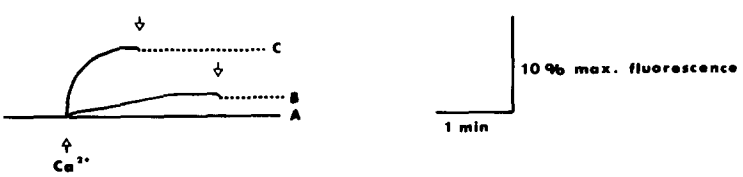

Fig. 2. Fusion of sonicated unilamellar phosphatidylserine/phosphatidylcholine (1 : 1) vesicles (0.1 μmol/ml) with large (approx. 0.2 μm) single bilayer phosphatidylserine vesicles (0.1 μmol/ml) in 1 ml of 100 mM NaCl, 5 mM Hepes (pH 7.4) and 0.1 mM EDTA. Ca^{2+} was added at the time indicated by the upward arrow. 2-fold excess EDTA was added at the maximal level of fluorescence (downward arrows). (A) 1 mM Ca^{2+}, (B) 3 mM Ca^{2+}, (C) 5 mM Ca^{2+}. The time-scale bar also represents the level of 0% fluorescence. 100% fluorescence was determined as described in Experimental Procedure.

TABLE I

INTERACTION OF PHOSPHATIDYLSERINE/PHOSPHATIDYLCHOLINE (1 : 1) LIPOSOMES WITH PHOSPHATIDYLSERINE MEMBRANES: EXPERIMENTAL CONDITIONS WHICH INDUCE FLUCTUATIONS AND INCREASE IN MEMBRANE CONDUCTANCE

SUV, small unilamellar vesicles; MLV, multilamellar vesicles.

Addition to the 'inner' chamber	Conductance	
	Fluctuations	Increase
0.1—5 mM Ca^{2+}	—	—
SUV	—	—
SUV + 1 mM Ca^{2+}	—	—
SUV + 5 mM Ca^{2+}	+	+
MLV	—	—
MLV + 10 mM Ca^{2+}	+	—

the inner chamber containing no Ca^{2+}, the conductance of the phosphatidylserine bilayer remained at a low level, identical to that of bilayers without added vesicles (about $10^{-10}\ \Omega^{-1}$, or $0.5 \cdot 10^{-8}\ \Omega^{-1} \cdot cm^{-2}$, shown by the initial part of the trace in Fig. 1). When the Ca^{2+} concentration was increased to 5 mM the conductance began to fluctuate and and to increase sharply. In control experiments, repetitive stirring with or without vesicles (no Ca^{2+} present), and the same sequence of Ca^{2+} additions (no vesicles present) did not alter the steady-state conductance of the membrane. These results are summarized in Table I. The Ca^{2+} gradient across the membrane was not sufficiently large at any stage of the experiment to cause an instability of the membrane [21]. In some experiments with the vesicles, 3 mM Ca^{2+} was sufficient to induce the conductance increase. The time of incubation of the vesicles with the planar bilayer before the addition of the threshold concentration of Ca^{2+} appeared to be a factor in eliciting this conductance behavior and is being investigated in detail. Phosphatidylserine/phosphatidylcholine multilamellar vesicles, at the same lipid concentration as unilamellar vesicles, elicited no appreciable increase in the conductance of planar bilayers up to 10 mM Ca^{2+}, but did cause small fluctuations (Table I). In these experiments, microscopic specks and thick patches formed on the planar membrane, indicating that the vesicles adhered to the surface of the membrane.

To demonstrate whether the vesicle membrane fused with the planar membrane and thus delivered its aqueous contents to the outside compartment is a difficult technical problem, especially in view of the small internal compartment of the vesicles and the dilution of any entrapped marker into the relatively large external compartment (see for example, Ref. 22). As an alternative, we investigated whether or not small phosphatidylserine/phosphatidylcholine vesicles fused with large, single-bilayer liposomes made of pure phosphatidylserine at the same Ca^{2+} concentration which initiated conductance changes in planar membranes (Fig. 2). At Ca^{2+} concentrations below 2 mM no fusion occurred between the two types of membrane. When the Ca^{2+} concentration was raised to 3—5 mM, however, the fluorescence intensity increased at a rate proportional to the concentration of the divalent ion. The fluorescence intensity could be arrested at the peak level by addition of excess EDTA, indicating

that the fluorescence reaction was occurring inside the aqueous compartment of the fusing vesicles [14]. The total surface area presented by the large unilamellar vesicles is significantly greater than the area of the planar membrane; therefore, the extent of fusion observed here cannot be related directly to the extent in the vesicle-planar bilayer system. Analogy in the two systems is limited to the similarity of the membrane compositions and the difference in the radius of curvature of the two types of membrane. Nevertheless, it is clear that small phosphatidylserine/phosphatidylcholine vesicles fuse with the relatively planar phosphatidylserine liposome membrane in the presence of 5 mM Ca^{2+}.

Small phosphatidylserine/phosphatidylcholine vesicles, at the same concentration used in the above experiments (0.1 μmol lipid/ml), did not fuse with each other in the presence of 5 mM Ca^{2+} (data not shown). This was demonstrated by utilizing the same fluorescence assay, but entrapping the reactant molecules in two populations of the small vesicles. The small vesicles, therefore, fuse preferentially with the phosphatidylserine bilayers or large liposomes when the Ca^{2+} concentration is between 3 and 5 mM.

To examine whether the role of Ca^{2+} in mediating the interaction of the small vesicles and the planar bilayer was to effect a structural change in the phosphatidylserine membrane itself, we used the carrier ionophore valinomycin as a probe of the fluidity of the hydrocarbon interior of the bilayer. If the membrane had undergone a phase transition from the liquid-crystalline to the gel state (as observed for phosphatidylserine liposomes at 1 mM Ca^{2+} [23]), the valinomycin-mediated conductance of the bilayer would have dropped several orders of magnitude, as demonstrated previously by Krasne and coworkers [24]. The valinomycin-mediated conductance of phosphatidylserine bilayers was reduced by about 60% when the Ca^{2+} concentration was increased from 0 to 1 mM (data not shown), a reduction for which the decrease in surface potential [25,26] and the resultant decrease in surface concentration of K^+ [9,27] can account. This observation does not necessarily imply that the Ca^{2+} did not cause any structural transformation in the bilayer. Haller and Freiser [28] found that solvent microlenses are formed in planar phosphatidylserine membranes by 0.5 mM Ca^{2+}; thus, it is possible that the valinomycin molecules are extruded into such microlenses as the phospholipids pack more tightly [25] and continue to contribute to the conductance. However, it is quite unlikely that single bilayers of phosphatidylserine would transform into the crystalline, anhydrous structure observed when phosphatidylserine vesicles are precipitated by Ca^{2+}, since the Ca^{2+}-induced shift to very high temperatures (greater than 100°C) in the gel-to-liquid crystalline transition temperature of phosphatidylserine is dependent on the formation of an intermembrane Ca^{2+} complex; if the membranes are kept apart by means of an extrinsic membrane protein, the endothermic transition occurs at about 32°C [29]. The absence of a drastic decrease in the ionophore-mediated conductance could thus be an indication that the bilayer is still fluid in the presence of Ca^{2+}.

Phosphatidylserine vesicles

The interaction of phosphatidylserine vesicles with phosphatidylserine planar bilayers was studied by incorporating a channel-forming ionophore, gramici-

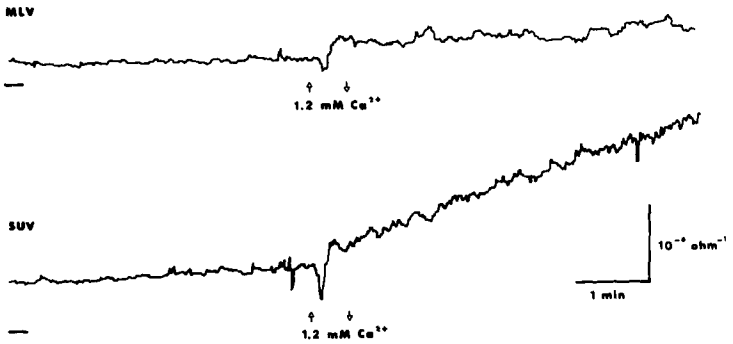

Fig. 3. The conductance (G) of a phosphatidylserine bilayer in the presence of phosphatidylserine/gramicidin A vesicles and the effect of increasing the Ca^{2+} concentration from 0.9 to 1.2 mM. The aqueous solution was 100 mM NaCl, 0.01 mM EDTA, 0.2 mM Tris-HCl, pH 7.2. 10 μl of sonicated vesicles (SUV) or multilamellar vesicles (MLV) were added to the inner chamber (final concentration 0.027 μmol lipid/ml), G was allowed to increase, 0.1 mM Ca^{2+} was added to both sides (which reduced G by about 90%) and G again gradually increased. The Ca^{2+} concentration was raised to 0.9 mM outside, and later inside; this lowered G to the value shown in the initial (left-hand side) portion of the trace. At the time indicated by the arrows, more Ca^{2+} was added (final concentration 1.2 mM) to the inner chamber. The downward arrow is the end of the stirring period. The short horizontal lines indicate $G = 0$.

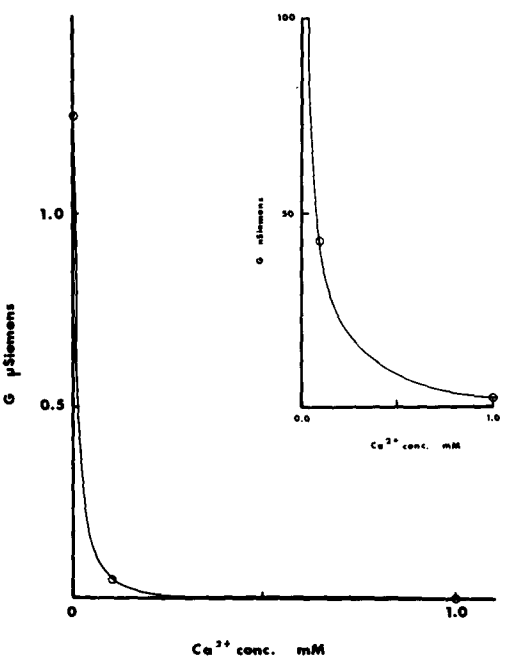

Fig. 4. Change in phosphatidylserine membrane conductance (G) with the Ca^{2+} concentration in the aqueous solution (100 mM NaCl, 0.01 mM EDTA, 0.2 mM Tris-HCl, pH 7.2), on both sides of the membrane. Gramicidin A was added to the inner chamber at a concentration of $1 \cdot 10^{-10}$ M. G was allowed to reach quasi-equilibrium before Ca^{2+} addition. The inset shows the conductance on an expanded scale. G is given in units of S (Ω^{-1}).

din A, into the membrane of the vesicles and following the transfer of the ionophore into the planar membrane [10]. Phosphatidylserine vesicles with no ionophore caused no change in the conductance up to 5 mM Ca^{2+} [11]. When ionophore-containing vesicles were incubated in the inner chamber, however, raising the Ca^{2+} concentration from 0.9 to 1.2 mM effected an increase in membrane conductance (Fig. 3). Above 1.2 mM Ca^{2+}, no further change was detected. Multilamellar phosphatidylserine liposomes containing the same ratio of gramidicin A per μmol lipid as sonicated vesicles caused a slight increase in the conductance.

If gramidicin A in an ethanol solution was added to the inner chamber in the absence of vesicles or Ca^{2+}, the conductance of the planar membrane increased to a quasi-equilibrium value shown in Fig. 4. When 1 mM Ca^{2+} was added, the conductance dropped to 0.03% of its initial value and no further change was observed above this Ca^{2+} concentration. A similar effect of Ca^{2+} on bilayers of total brain phospholipids has been reported by Liberman and Nenashev [30]. The lifetime of the transmembrane gramicidin A channel is sensitive to the thickness and deformability of the membrane [36]. The interaction of Ca^{2+} with the phosphatidylserine membrane could prevent the deformation of the membrane; alternatively, the closer packing of the lipid molecules could extrude the ionophore into solvent microlenses or the Plateau-Gibbs border. Ca^{2+} could also block the channel directly [32]. Furthermore, the Na^{+} concentration at the surface would be lowered due to the reduction of the surface potential, and so would the conductance of the channels. When the gramicidin A is incorporated first into the vesicle membrane and then added to one side of a planar phosphatidylserine bilayer, the Ca^{2+}-mediated transfer of the ionophore to the planar membrane must therefore be in sufficient quantities to overcome the conductance-reducing effect of the divalent ion. The transfer of gramicidin A could be brought about through adhesion of the vesicles to the planar membrane, fusion of apposed monolayers of the two membranes, or complete fusion of the two phospholipid bilayers. During fusion, any gramicidin A dimer in the vesicle membrane would contribute immediately to the conductance. Gramicidin A in liposomes has been proposed to exist preferentially in the dimer form [33]; a sub-population of the vesicles therefore could contain one or more dimers, even though there is, on the average, one ionophore per vesicle. At such a low ratio of ionophore to lipid, gramicidin A would

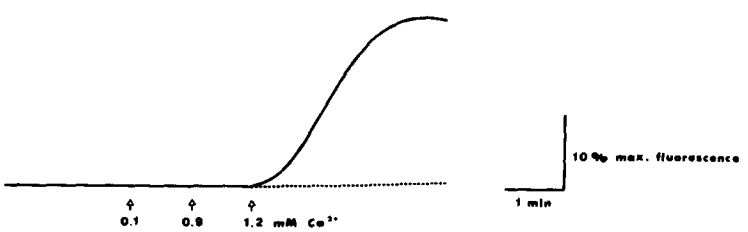

Fig. 5. Fusion of sonicated unilamellar phosphatidylserine vesicles (0.025 μmol/ml) with large single-bilayer phosphatidylserine vesicles (0.025 μmol/ml) in 1 ml of 100 mM NaCl, 5 mM Hepes (pH 7.4) and 0.1 mM EDTA. The Ca^{2+} concentration (above the 0.1 mM EDTA) was increased to the indicated values at the arrows. The dashed line shows the time course of fluorescence obtained with 0.9 mM Ca^{2+}. Other details were as described in Fig. 2 and in Experimental Procedure.

not be expected to alter the biophysical properties of the lipid bilayers [34]. Gramicidin A may be transferred from vesicle to vesicle, but whether this is through the aqueous phase, or by collision of the vesicles is not clear [17]. The following observations show that most of the ionophore was confined to the vesicle membrane and was not free in solution. The total amount of ionophore in the inner chamber in a typical experiment corresponded to a concentration of 10^{-8} M; this amount of ionophore added without the vesicles would cause a very sharp increase in conductance (approx. 152 $\mu\Omega^{-1} \cdot \min^{-1}$), unlike the gradual increase (approx. 0.35 $\mu\Omega^{-1} \cdot \min^{-1}$) observed when it was added as a component of the vesicle membrane. The latter rate was also about 3—6-fold slower than the increase caused by $2 \cdot 10^{-10}$ M gramicidin A, an upper limit for the free ionophore concentration in aqueous solution [31]. If multilamellar phosphatidylserine/gramicidin A vesicles were centrifuged and the supernatant added to the inner chamber only a very slow rise in conductance was seen.

Analogous experiments with the fusion assay were carried out with small phosphatidylserine vesicles containing Tb and large vesicles containing dipicolinic acid. Ca^{2+} was added in a sequence identical to that in the planar bilayer experiments. When the Ca^{2+} concentration was increased from 0.9 to 1.2 mM the aqueous contents of the small and large vesicles began to intermix due to the fusion of their membranes (Fig. 5). Addition of EDTA at the time of maximal fluorescence stopped the fusion reaction and the fluorescence intensity stayed at that level (data not shown). It appears, therefore, that there is a close correspondence of fusion as demonstrated by the fluorescence assay to the conductance behavior of planar membranes interpreted as arising from fusion.

Discussion

The fluctuations and increase in the electrical conductance of phosphatidylserine planar bilayer membranes incubated with mixed phosphatidylserine/phosphatidylcholine small, unilamellar vesicles and a threshold concentration of Ca^{2+} arise most likely from the fusion of the two types of membrane. It is evident from analogous experiments with the fusion assay that the small vesicles fuse with the large, single-bilayer phosphatidylserine liposomes at identical Ca^{2+} concentrations. Therefore, individual fusion events could be accompanied by the transient formation of ion leakage pathways across the planar membrane which would be observed as fluctuations in the membrane current (such as those occurring within the first 2 min after the addition of 5 mM Ca^{2+} in Fig. 1), and many such events occurring simultaneously would increase the overall conductance. These ion pathways could be formed during structural rearrangements in the regions of adhesion of the small unilamellar vesicles to the planar membrane. Molecular packing defects at the phase boundaries of phosphatidylserine-Ca^{2+} complexes and phosphatidylcholine domains in the vesicle membrane [23,35,36] could cause an increase in the permeability of the membrane [37]. Indeed, during Ca^{2+}-induced fusion of sonicated phosphatidylserine/phosphatidylcholine vesicles, there is a considerable, but transient, increase in the permeability of the membranes, measured as the leakage of carboxyfluorescein entrapped in the vesicles [38]. Antonov and co-workers [39] have presented evidence for fluctuations of membrane current, possibly

arising from lipid domain interactions, at the phase transition temperature of synthetic phospholipids. Sarcoplasmic reticulum fragments, when incubated on one side of a planar bilayer made of a mixture of phosphatidylserine and phosphatidylethanolamine, induce step conductance increases in the presence of 0.5—1.0 mM Ca^{2+} because of their high permeability to monovalent ions; this result has also been interpreted as the fusion of the vesicle membranes with the planar bilayer [40]. Phosphatidylcholine membranes exhibit a lower resistance than phosphatidylserine membranes in the presence of low concentrations of Ca^{2+} [11,16]. It is not unlikely, therefore, that the incorporation of domains of phosphatidylcholine into the phosphatidylserine membrane complexes with Ca^{2+} could give rise to an increase in conductance. That is, not only the fusing vesicles but also the now modified planar membrane would contribute to the conductance increase.

The use of the fluorescence assay for membrane fusion provides strong evidence that there is fusion between phosphatidylserine planar bilayers or large vesicles and the small, sonicated vesicles at Ca^{2+} concentrations which depend on the composition of the latter. The Tb/dipicolinic acid fluorescence does not, however, give an accurate estimate of the extent of fusion, because the contents of the large vesicles are slowly released into the medium, especially at higher Ca^{2+} concentrations, and are not available for complexation with the Tb in the small vesicles at later stages of fusion. Nevertheless, it is sufficient for our purposes to know that a relatively high percentage of the small vesicles have fused with the large ones at a threshold concentration of Ca^{2+}. The planar bilayer system appears to be very sensitive to small numbers of fusion events, since the surface area of the phosphatidylserine membrane available for interaction with the sonicated vesicles (0.1 μmol/ml in both systems) is about 3 orders of magnitude less than that in the vesicle-vesicle system.

Unlike the mixed phospholipid vesicles, phosphatidylserine vesicles (without added ionophore) do not induce conductance fluctuations in the presence of Ca^{2+} [11]. In view of the effect of Ca^{2+} on the apparent permeability of these vesicles to univalent ions [41], it might be expected that conductance pathways would be created during vesicle fusion with the planar bilayer. It has been shown recently, however, that the Ca^{2+}-induced increase in permeability (or release of entrapped molecules) is dependent on the aggregation and fusion of vesicles and is a result of the collapse of their internal aqueous space [29]. The actual permeability of the membrane of a single phosphatidylserine vesicle in the presence of Ca^{2+} may be rather low, as in the case of the planar membranes [16]. The Ca^{2+}-induced fusion of large unilamellar phosphatidylserine vesicles among themselves is essentially non-leaky [14]. The fusion of the small vesicles with the planar membrane could, therefore, be a similarly non-leaky event.

Sonicated unilamellar vesicles are strained structures with higher molecular mobility compared to multilamellar liposomes [42—44]. The addition of Ca^{2+} will not only neutralize the surface charge and reduce the electrostatic repulsion between the small vesicles and the planar membrane, but it may also increase the free energy of the vesicle membrane [45]. The difference in surface energy between the two membranes may be a driving force for the fusion reaction once molecular contact has been established, since the planar membrane is a thermodynamically stable structure with low surface tension [46],

and the coupled system would attain a state of lower free energy by undergoing fusion. The limited interaction of multilamellar vesicles with planar bilayers (Table I and Fig. 3) could be a reflection of their stability and large radius of curvature. It is possible that the large vesicles remain at the secondary minimum of the potential energy barrier, whereas the small vesicles establish molecular contact [47].

The range of Ca^{2+} concentrations in which phosphatidylserine vesicles interact with planar phosphatidylserine bilayers is the same as that in which vesicles aggregate and fuse with each other [11,41]. Phosphatidylserine vesicles also fuse with phosphatidylserine monolayers at about 1 mM Ca^{2+} at which the area per molecule in the monolayer (70 $Å^2$) corresponds to that estimated for bilayer membranes [12]. Hence, there seems to be no difference between vesicle-vesicle and vesicle-bilayer fusion with respect to the threshold Ca^{2+} concentration when the phospholipid composition of the vesicles and the planar membranes is identical. Large unilamellar phosphatidylserine vesicles require higher concentrations of Ca^{2+} to fuse with each other [14], but they fuse with small vesicles at the threshold concentration characteristic of the latter. The binding of Ca^{2+} to both membranes would lead to the removal of the water of hydration in the zone of contact [29] and the membranes would fuse essentially through a hydrophobic interaction of the membrane lipids. It has been proposed that hydrophobic interactions are essential for temperature-induced fusion of spherical bilayers [48] and that the entropy increase associated with divalent cation-induced dehydration may be the driving force for fusion [49]. We have suggested previously that the change in the hydrophobicity of the membrane surface, closely related to divalent metal binding to the surface, is an important determinant for membrane fusion [12]. It has also been suggested that Ca^{2+} causes a contact-mediated transformation of membrane structure which then initiates the membrane fusion reaction [29,50]. This suggestion is supported by the observation, in the experiments on the interaction of mixed vesicles with planar bilayers, that the planar membrane had been exposed to 1—3 mM Ca^{2+} prior to the initiation of fusion by 5 mM Ca^{2+} and thus had already undergone a contact-independent transformation. Therefore, what initiates the fusion reaction may be the further membrane alteration caused by the formation of the inter-bilayer Ca^{2+}-phosphatidylserine complex.

The preferential interaction of phosphatidylserine/phosphatidylcholine vesicles with planar phosphatidylserine membranes at a Ca^{2+} concentration insufficient to cause aggregation or fusion among each other may also provide an explanation for the fusion of exocytotic vesicles preferentially with the plasma membrane and not with each other in Ca^{2+}-induced simple exocytosis [4]. A difference in the lipid composition of the cytoplasmic monolayers of the secretory vesicle membrane and the plasma membrane could be one parameter controlling the selectivity of the membrane interaction. Our studies also indicate that attempts to reconstitute membrane protein function in planar membranes by fusing reconstituted proteoliposomes with the planar bilayer [51—53] must be approached with caution, because conductance changes may be induced by the lipid bilayer segments of these liposomes, especially if they contain phosphatidylcholine. Since phosphatidylserine vesicles (without ionophore) do not cause fluctuations in conductance, the effect of a channel-form-

ing membrane protein could be identified when this phospholipid is used for reconstitution.

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