Biochimica et Biophysica Acta, 642 (1981) 182—195
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BBA 79158

STUDIES ON THE MECHANISM OF MEMBRANE FUSION

ROLE OF HEAD-GROUP COMPOSITION IN CALCIUM- AND MAGNESIUM-INDUCED FUSION OF MIXED PHOSPHOLIPID VESICLES

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(Received August 22nd, 1980)

Key words: Ca^{2+}; Membrane fusion; Head-group composition; Mg^{2+}; Phospholipid vesicle;

Summary

We have investigated the contribution of various phospholipids to membrane fusion induced by divalent cations. Fusion was followed by means of a new fluorescence assay monitoring the mixing of internal aqueous contents of large (0.1 μm diameter) unilamellar liposomes. The rate and extent of fusion induced by Ca^{2+} in mixed phosphatidylserine/phosphatidylcholine vesicles were lower compared to those in pure phosphatidylserine vesicles. The presence of 50% phosphatidylcholine completely inhibited fusion, although the vesicles aggregated upon Ca^{2+} addition. When phosphatidylserine was mixed with phosphatidylethanolamine, however, rapid fusion could be induced by Ca^{2+} even in mixtures that contained only 25% phosphatidylserine. Phosphatidylethanolamine also facilitated fusion by Mg^{2+} which could not fuse pure phosphatidylserine vesicles. In phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine mixtures, in which the phosphatidylcholine content was kept at 25%, phosphatidylethanolamine could not substitute for phosphatidylserine, and the fusogenic capacity of Mg^{2+} was abolished by the presence of merely 10% phosphatidylcholine. The initial rate of release of vesicle contents was slower than the rate of fusion in all the mixtures used. The presence of phosphate effected a considerable decrease in the threshold concentration of Ca^{2+} and also enhanced

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Tes, tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

the rate and extent of fusion. Mg^{2+} had a synergistic effect on Ca^{2+}-induced fusion of phosphatidylserine/phosphatidylethanolamine vesicles. We suggest that the role of phospholipids in membrane fusion is related to their ability to form dehydrated intermembrane complexes with divalent cations.

The complexity of biological membranes has hampered the identification of specific molecules and mechanism(s) involved in membrane fusion. Secretion and exocytosis, for example, occur in many cell types by Ca^{2+}-mediated fusion of intracellular vesicles with the plasma membrane [1,2]. To understand the biophysics and biochemistry of membrane fusion, simple model systems have been utilized where the membrane composition and ionic environment can be controlled. In particular, studies with liposomes have shown that acidic phospholipids and divalent cations are important elements in membrane fusion (for a review, see Ref. 3). Ca^{2+} induces extensive fusion of phosphatidylserine vesicles which results in the formation of large cochlear lipid cylinders [4] and causes a phase transition of the lipid bilayer into a highly ordered crystalline structure [5—7]. This transition is dependent on the formation of a Ca^{2+} complex between apposed bilayers [7] and may be involved in the fusion of the vesicles [6—9]. After two vesicles aggregate, their membranes fuse to form a continuous structure which allows their internal aqueous contents to intermix [10].

Phosphatidylserine vesicles provide a useful model system for studying the biophysics of membrane fusion. However, phosphatidylserine comprises only a small percentage of the lipids of biological membranes [11]; therefore, it is necessary to determine how the presence of other lipids in the liposome membrane affects fusion characteristics. For example, glycolipids with bulky head groups [7] and phosphatidylcholine [12] have inhibitory effects on Ca^{2+}-induced fusion.

Many techniques have been used to study membrane fusion in phospholipid vesicles, such as monitoring the mixing of phospholipids and membrane proteins, or the increase in vesicle size (for reviews, see Refs. 3 and 13). Most of these techniques have not been entirely satisfactory in distinguishing fusion, aggregation and molecular exchange and in providing information on the kinetics of fusion. Membrane fusion is expected to involve the communication between the internal aqueous compartments of two fusing vesicles; therefore, a reliable assay for fusion should meet this criterion. We have recently developed such an assay which monitors the interaction of Tb^{3+} entrapped in one population of vesicles and dipicolinic acid entrapped in another [10,13]. Tb^{3+} and dipicolinic acid produce a complex which results in an enhancement of four orders of magnitude of the Tb fluorescence [14]. The formation of this complex is inhibited outside the vesicles by the presence of EDTA and Ca^{2+}. Therefore, only the reaction protected from the outside environment by a non-leaky membrane is recorded as fusion, providing unequivocal evidence for membrane fusion. With this method fusion events occurring within a few seconds may be detected.

In this communication we describe our studies on the fusion of large (approx. 1000 Å in diameter) unilamellar phospholipid vesicles induced by Ca^{2+}

and Mg^{2+}. We have used various mixtures of phosphatidylserine with phosphatidylethanolamine and/or phosphatidylcholine in the liposome membrane to elucidate the role of each of these phospholipids in membrane fusion, and to determine the specificity of Ca^{2+} and Mg^{2+} for inducing the fusion of these liposomes.

Methods

Lipids

Phosphatidylserine was prepared from bovine brain and phosphatidylcholine from egg yolk as before [8,15]. Phosphatidylethanolamine, obtained by transesterification of egg phosphatidylcholine, was purchased from Avanti Polar Lipids (Birmingham, AL). All lipids showed single spots on thin-layer plates with two different solvent systems, chloroform/methanol/water (65 : 25 : 4, v/v) and hexane/propanol/water (6 : 8 : 1, v/v). The purity of phosphatidylserine was also checked by two-dimensional thin-layer chromatography [16]. Lipids were stored under Ar or N_2 in sealed ampules and kept at −50°C.

Chemicals

$TbCl_3$ was obtained from Alfa (Danvers, MA), dipicolinic acid from Sigma and carboxyfluorescein from Eastman Kodak (Rochester, NY). L-Histidine and Tes were purchased from Sigma; NaCl, sodium citrate, chloroform and diethyl ether were from Mallinckrodt and $CaCl_2$, $MgCl_2$, EDTA and EGTA were from Fisher. Carboxyfluorescein was recrystallized according to the method of Blumenthal et al. [17]. Water was twice distilled, the second time in an all-glass apparatus.

Preparation of vesicles

Large unilamellar vesicles were prepared by a reverse-phase evaporation technique [18], as modified by Wilschut et al. [13].

The 'Tb vesicles' were prepared in 2.5 mM $TbCl_3$, 50 mM sodium citrate, 2 mM L-histidine, 2 mM Tes (pH 7.4). Citrate was necessary as a chelator of Tb^{3+} to prevent its interaction with the negatively charged vesicle surface [10]; the absence of any interaction of Tb^{3+} with phosphatidylserine was also checked by examining the phase transition characteristics of phosphatidylserine by differential scanning calorimetry [13]. After the vesicles were formed they were sized to 0.1 μm by extrusion through polycarbonate filters (Uni-Pore, Bio-Rad) [19,20] and were separated from non-encapsulated material on a Sephadex G-75 column (elution buffer: 100 mM NaCl, 2 mM L-histidine, 2 mM Tes ('NaCl buffer') containing 1 mM EDTA to prevent Tb^{3+} from binding to the outer surface of the vesicles). A fraction of the vesicles was rechromatographed on a separate column by eluting with the buffer without EDTA for calibration of the fluorimeter to 100% fluorescence and to determine the amount of encapsulated material. The latter was in the range of 1.2—4.9 μl/μmol phospholipid for the various preparations; this is consistent with the size range of these vesicles [20].

The 'dipicolinic acid vesicles' were prepared in 50 mM sodium dipicolinate, 20 mM NaCl, 2 mM L-histidine and 2 mM Tes, pH 7.4. Since the large unila-

mellar vesicles used in this study were osmotically active, the encapsulated material needed to be maintained at the same osmolarity as the outside aqueous medium. The dipicolinic acid vesicles were subsequently separated from non-encapsulated material by the same procedure utilized for the Tb vesicles.

The 'carboxyfluorescein vesicles' contained 50 mM carboxyfluorescein, 2 mM L-histidine, 2 mM Tes, 0.1 mM EDTA and were separated from non-encapsulated material in the same way except that the elution buffer contained 0.1 mM EDTA in addition to the NaCl buffer.

These vesicles were shown to have a single bilayer and to be of uniform size by freeze-fracture electron microscopy and ^{1}H-NMR (Ref. 20 and unpublished data). The uniformity of the bilayer composition in a population of vesicles was checked by electrophoresis on cellulose acetate plates (unpublished data). The concentration of lipid in the various vesicle preparations was determined by assaying for P_i [21].

Assays for fusion and release of contents

Mixing of internal aqueous contents of vesicles upon introduction of divalent cations was measured by the fluorescence assay developed previously [10,13]. Equimolar amounts of Tb and dipicolinic acid vesicles were mixed in a quartz cuvette at a total lipid concentration of 50 μM in 1 ml of 100 mM NaCl, 2 mM L-histidine, 2 mM Tes, 0.1 mM EDTA, pH 7.4; in experiments where Mg^{2+} was the only divalent ion, 0.1 mM EGTA was added to this mixture. The cell was stirred constantly by a Teflon-coated magnetic stir bar and the temperature maintained at 25.0 ± 0.1°C. Fluorescence was measured in an SLM-4000 fluorimeter (SLM Instruments, Champaign-Urbana, IL). The excitation wavelength for the fusion assay was 276 nm; the fluorescence above 520 nm was measured by using a Corning 3-68 cut-off filter. Calibration for 100% Tb fluorescence was made in the presence of 20 μM free dipicolinic acid and 25 μM Tb vesicles, separated from external EDTA by gel filtration, and the contents of which were released by 1% (w/v) sodium cholate (recrystallized, Sigma). The sensitivity of the assay allowed the detection of fusion between as little as 1% of the vesicles.

Release of vesicle contents was measured by following the fluorescence of carboxyfluorescein as it was released into the medium and was no longer self-quenched [7,22]. The excitation wavelength was 430 nm and the emission above 520 nm was detected using the 3-68 Corning filter. Maximal carboxyfluorescein fluorescence was determined by lysing the vesicles (50 μM lipid) with 0.1% Triton X-100 (Sigma).

Light scattering at 90° was simultaneously measured in each of the fusion or release measurements, at 276 and 430 nm, respectively, by means of a second photomultiplier located opposite the fluorescence channel. The outputs for fluorescence and light scattering from the fluorimeter were continuously recorded on an Omniscribe chart recorder (Houston Instruments).

The fusion reaction induced by Ca^{2+} or Mg^{2+} could be stopped by the addition of EDTA, and the fluorescence level reached by that time was maintained inside the fused vesicles, effectively sequestered from the external medium (data not shown).

Results

Phosphatidylserine/phosphatidylcholine vesicles

It is now well established that phosphatidylcholine vesicles above the phase transition temperature do not fuse in a physiological aqueous environment even in the presence of Ca^{2+} [12,23—25]. When phosphatidylcholine is mixed with acidic phospholipids in the liposome membrane, the threshold concentration of divalent cations required for aggregation and fusion is higher compared to that of vesicles made only of the acidic phospholipid [12,26]. We therefore examined in detail the effect of including phosphatidylcholine in phosphatidylserine membranes on Ca^{2+}-induced fusion. Pure phosphatidylserine vesicles fused extensively in the presence of 10 mM Ca^{2+} with very fast kinetics. The inclusion of even 25% phosphatidylcholine, however, drastically inhibited the initial rate of Tb fluorescence increase as well as the maximal fluorescence attained (Fig. 1A). When the phosphatidylcholine content was increased to 50%, no fusion was detected up to 50 mM Ca^{2+}, even though the vesicles formed large aggregates. Freeze-fracture electron microscopy also confirmed that the vesicles retained their original size and shape (not shown).

Mg^{2+} (up to 20 mM) was ineffective in causing any fusion in either the 3 : 1 or 1 : 1 phosphatidylserine/phosphatidylcholine mixtures as well as in pure phosphatidylserine vesicles, although it produced extensive aggregation. Mg^{2+} also inhibited the fusogenic capacity of Ca^{2+}; in 3 : 1 phosphatidylserine/phosphatidylcholine mixtures, 5 mM Mg^{2+} reduced by about 3-fold the initial rate of fusion induced by 10 mM Ca^{2+}.

The kinetics of the release of vesicle contents into the medium during fusion are shown in Fig. 1B. The initial rate of release was much lower than the rate of fusion, and there was no detectable release from phosphatidylserine/phosphatidylcholine (1 : 1) vesicles indicating that aggregation does not necessarily lead to leakage of vesicle contents.

Light scattering at 276 nm recorded simultaneously during the fusion assay exhibited a sharp but limited increase followed by an immediate decrease with

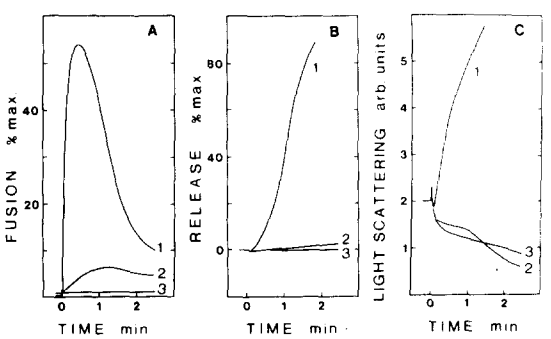


Fig. 1. Ca^{2+}-induced fusion of pure phosphatidylserine vesicles (curve 1) and vesicles made of 3 : 1 (curve 2) and 1 : 1 (curve 3) mixtures of phosphatidylserine/phosphatidylcholine. 25 μM Tb vesicles and 25 μM dipicolinic acid vesicles were suspended in 1.0 ml NaCl buffer containing 0.1 mM EDTA. 10 mM Ca^{2+} was added at t = 0. A, fusion; B, release of aqueous contents (carboxyfluorescein) and C, 90° light scattering at 276 nm. The maximal fluorescence (>520 nm) was determined as described in Methods.

vesicles composed of phosphatidylserine/phosphatidylcholine mixtures (Fig. 1C). The extensive aggregation of the vesicles reduces the number of scattering particles and also produces interference effects on the scattered light. As large visible aggregates are formed the light path is cleared, which further contributes to the decrease in scattering. In the case of phosphatidylserine vesicles the time course of light scattering is more complex. The initial, sharp increase may be due to vesicle dimerization or a structural change of the membrane. The subsequent decrease is an indication of the aggregation and resulting interference effects, as argued above. The third stage of the light scattering is the dramatic increase which is most likely due to the complete collapse of the internal space of the vesicles leading to the formation of a crystalline anhydrous phosphatidylserine-Ca^{2+} complex [5—7] which would have a different refractive index than the initial bilayer membrane [13]. Phosphatidylserine/phosphatidylcholine vesicles, however, do not form this tight complex to any appreciable extent.

Phosphatidylserine/phosphatidylethanolamine vesicles

The effect of various concentrations of Ca^{2+} on the time course of the fusion reaction for phosphatidylserine/phosphatidylethanolamine (1 : 1) vesicles is shown in Fig. 2A. The 'threshold' Ca^{2+} concentration for inducing fusion appeared to be 3—4 mM, which is only slightly higher than that for pure phosphatidylserine vesicles (2.5 mM [13]). Again, the rate of release of contents was lower than the initial fusion rate (Fig. 2B). The initial rate of fusion increased with the Ca^{2+} concentration. At 10 mM Ca^{2+}, however, lower maximal Tb fluorescence was observed compared to that at 5 mM, since the rate of release had also increased. Vesicles containing only 25% phosphatidylserine were also able to fuse extensively in the presence of 3 mM or more Ca^{2+} (Fig. 3), the release of vesicle contents being slower than the fusion reaction. Compared to pure phosphatidylserine vesicles these initial rates of fusion were significantly lower. In the presence of 10 mM Ca^{2+}, they were 240, 32 and 14% maximal fluorescence/min for phosphatidylserine and its 1 : 1 and 1 : 3 mixtures with phosphatidylethanolamine, respectively; for 5 mM Ca^{2+} these rates were 96, 12 and 11, respectively (cf. Ref. 13).

In contrast to pure phosphatidylserine or mixed phosphatidylserine/phosphatidylcholine vesicles, Mg^{2+} was effective in inducing fusion of phosphatidylserine/phosphatidylethanolamine membranes. About 28% of maximal fluorescence was attained with 6 mM Mg^{2+}, albeit at a slow rate (Fig. 4). The rates of fusion and leakage obtained with Mg^{2+} were slower than those observed with Ca^{2+} at the same concentration (cf. Fig. 2), and the kinetics exhibited a biphasic behavior. The reasons for the latter are not entirely clear. When vesicles contained 25% phosphatidylserine, fusion could again be induced by Mg^{2+}, the threshold concentration being 4 mM (Fig. 5). More than 40% of maximal fluorescence was attained in 5 min with 5 mM Mg^{2+}. The extent and initial rates of fusion induced by Mg^{2+} in the 1 : 3 mixture of phosphatidylserine and phosphatidylethanolamine appeared to be higher than in the 1 : 1 mixture, which may be explained by the lower rate and extent of release, and the lower repulsive electrostatic forces between these vesicles compared to the 1 : 1 vesicles.

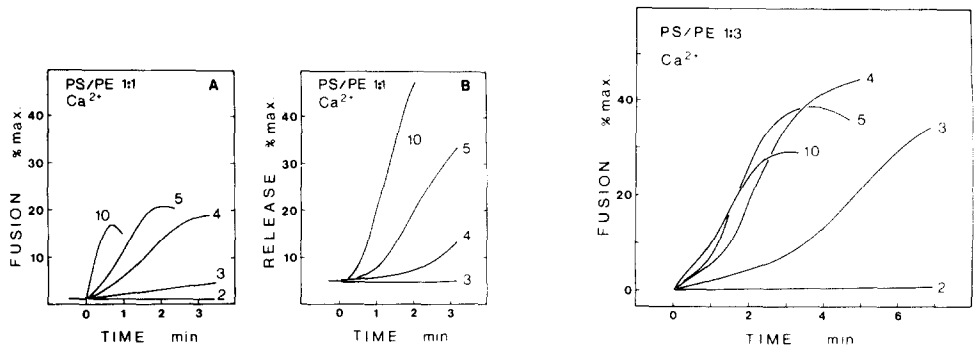


Fig. 2. The effect of various concentrations of Ca^{2+} (mM) on the time course of fusion (A) and release of aqueous contents (B) in phosphatidylserine (PS)/phosphatidylethanolamine (PE) (1 : 1) vesicles. Ca^{2+} was added at $t = 0$.

Fig. 3. The time course of fusion in phosphatidylserine/phosphatidylethanolamine (1 : 3) vesicles induced by various concentrations of Ca^{2+} (mM) added at $t = 0$.

The effect of Ca^{2+} in the presence of Mg^{2+}

Under aggregating conditions, Ca^{2+} binds more effectively to small unilamellar phosphatidylserine vesicles in the presence of Mg^{2+} than in its absence [7]. Mg^{2+} also reduces the threshold Ca^{2+} concentration required to induce aggregation of phosphatidylserine vesicles and release of their contents [7,27,28]. As reported above for phosphatidylserine/phosphatidylcholine vesicles, if Mg^{2+} itself does not cause fusion it will inhibit the effect of Ca^{2+}. In the case of phosphatidylserine/phosphatidylethanolamine vesicles, however, Mg^{2+} does induce fusion, and it also enhances the fusogenic effect of Ca^{2+}. Fig. 5 shows a series of experiments in which fusion was induced by various combinations of Ca^{2+} and Mg^{2+}. Either 2 mM Ca^{2+} or 3 mM Mg^{2+} alone did not produce any observable fusion; added together they caused considerable fusion, comparable to

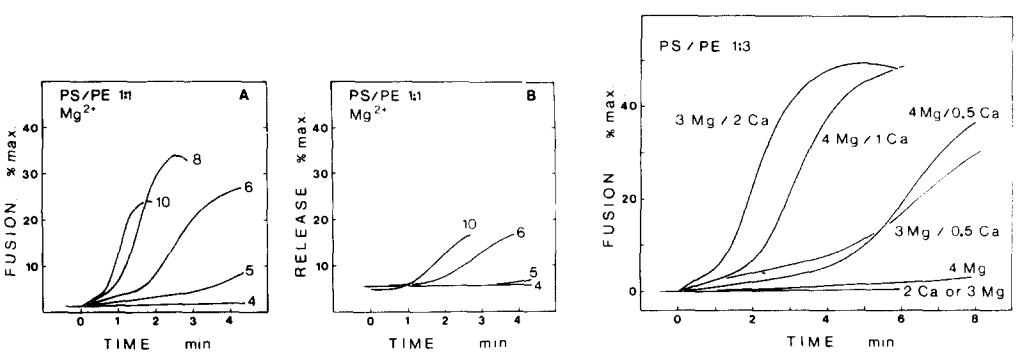


Fig. 4. The effect of various concentrations of Mg^{2+} (mM) on the time course of fusion (A) and release of aqueous contents (B) in phosphatidylserine/phosphatidylethanolamine (1 : 1) vesicles. Mg^{2+} was added at $t = 0$.

Fig. 5. Fusion of phosphatidylserine/phosphatidylethanolamine (1 : 3) vesicles induced by the combined action of Ca^{2+} and Mg^{2+}. The divalent cations were added simultaneously at $t = 0$ up to the indicated final concentrations (mM), using pre-mixed stock solutions.

the rate attained by 4 mM Ca^{2+} alone (cf. Fig. 3). In the presence of 3 mM Mg^{2+}, only 0.5 mM Ca^{2+} was sufficient to initiate fusion. The initial rate of fusion for 1 mM Ca^{2+}/4 mM Mg^{2+} was less than for 5 mM Ca^{2+} alone, but higher than for 5 mM Mg^{2+} alone, indicating that the relative effectiveness of these ions is still maintained when they are added together.

The time of pre-incubation with Mg^{2+} before the addition of Ca^{2+} considerably altered the kinetics of fusion (Fig. 6). When 1 mM Ca^{2+} was added to phosphatidylserine/phosphatidylethanolamine (1 : 3) vesicles 1 min after the introduction of Mg^{2+} (4 mM), fusion was rather slow and exhibited the characteristic biphasic behavior. When the pre-incubation period was extended to 8 min, however, the rate of fusion increased by more than 30-fold. A similar effect was observed for 0.5 mM Ca^{2+} which caused an 8-fold enhancement of fusion compared with the case where it was added simultaneously with Mg^{2+}. It should be noted that the free Ca^{2+} concentration in these experiments was about 0.3 mM because of the presence of 0.1 mM EDTA and 0.1 mM EGTA in the reaction medium.

Phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine vesicles

While the above data demonstrate that phosphatidylethanolamine sustains the ability of phosphatidylserine to fuse, it is important to establish whether phosphatidylethanolamine can still be effective in the presence of phosphatidylcholine. Vesicles were made from phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine mixtures with a constant phosphatidylcholine content (25% of total lipids) and a variable ratio of phosphatidylserine to phosphatidylethanolamine. The initial rates of fusion induced by the addition of various concentrations of Ca^{2+} to vesicles composed of 3 : 0 : 1, 2 : 1 : 1 or 1 : 2 : 1 mixtures of phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine are shown in Fig. 7 as a function of the mole fraction of phosphatidylserine. If part of the phosphatidylserine was substituted for by phosphati-

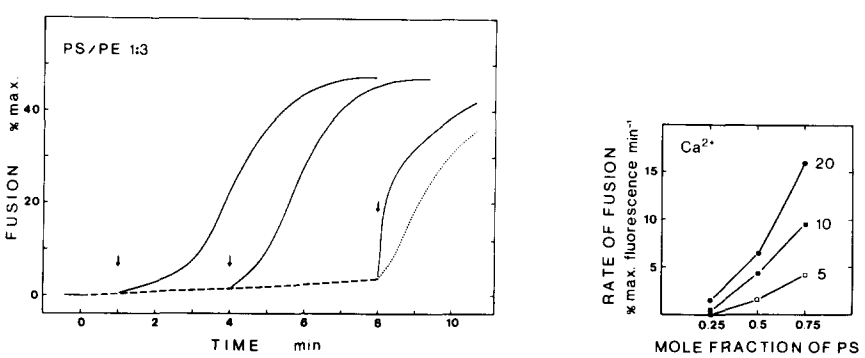


Fig. 6. The effect of pre-incubation with 4 mM Mg^{2+} on fusion induced by 1 mM Ca^{2+}. 4 mM Mg^{2+} was added at t = 0 and resulted in limited fusion (- - - - -). 1 mM Ca^{2+} was added at various times indicated by the arrows (———). Fusion could also be induced by 0.5 mM Ca^{2+} (. . . . .).

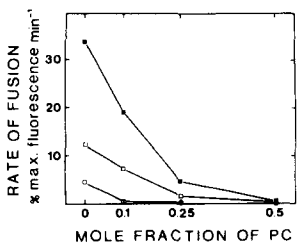
Fig. 7. The effect of membrane composition on the initial rate of fusion induced by various concentrations of Ca^{2+} (mM). The vesicles contained 25% phosphatidylcholine; the remaining 75% consisted of various molar ratios of phosphatidylserine (PS) and phosphatidylethanolamine. The membrane composition is given as the mole fraction of phosphatidylserine among total phospholipids.

dylethanolamine the initial rate of fusion was diminished. The extent of fusion, i.e., the maximal Tb fluorescence intensity attained, was also lower (data not shown). Thus, the presence of phosphatidylethanolamine in the membrane could not overcome the inhibitory effect of phosphatidylcholine, nor could it substitute for phosphatidylserine.

No fusion could be induced in these mixtures by Mg^{2+} although the vesicles aggregated following addition of appropriate amounts of Mg^{2+}. We have shown above that phosphatidylserine/phosphatidylethanolamine vesicles fuse extensively in the presence of 5—6 mM Mg^{2+}. Phosphatidylcholine is therefore extremely inhibitory for the effect of this ion even at a mole fraction of the total lipids of 0.1. Fig. 8 summarizes the dependence of the initial rate of fusion on membrane composition and divalent cation concentrations. Here, the phosphatidylserine content of the membranes was kept at 50% and the mole fractions of phosphatidylcholine and phosphatidylethanolamine were varied from 0 to 0.5. No fluorescence increase was detected in a 5 : 4 : 1 mixture of phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine with 10 mM Mg^{2+}; about 3—4% maximal fluorescence was observed with 20 mM Mg^{2+}. The initial rates of fusion induced by various concentrations of Ca^{2+} became appreciable as the amount of phosphatidylethanolamine in the membrane was increased at the expense of phosphatidylcholine and was maximal with the 1 : 1 phosphatidylserine/phosphatidylethanolamine mixture.

Enhancement of fusion by phosphate

Ionic factors other than divalent cations may also modulate the fusion of phospholipid membranes. Phosphate ions, for example, have been shown to be effective in promoting the fusion of erythrocyte ghosts in the presence of Ca^{2+} [29]. The effect of phosphate was determined for each of the phospholipid mixtures used in this study. A representative experiment is shown in Fig. 9.



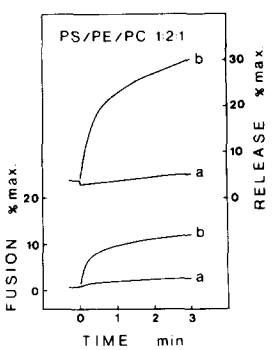


Fig. 8. The variation in the initial rate of fusion with membrane composition and divalent cation. The vesicles contained 50% phosphatidylserine and the remaining 50% consisted of various molar ratios of phosphatidylcholine (PC) and phosphatidylethanolamine. The membrane composition is given as the mole fraction of phosphatidylcholine among total phospholipids. ■———■, 10 mM Ca^{2+}; □———□, 5 mM Ca^{2+}; ○———○, 10 mM Mg^{2+}.

Fig. 9. Fusion induced by phosphate in vesicles made of a 1 : 2 : 1 mixture of phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine. a, 20 mM Ca^{2+} added at t = 0; b, 10 mM phosphate (pH 7.7) added at t = 0 after a 2 min pre-incubation with 2 mM Ca^{2+} which itself caused no fusion. Bottom curves, fusion; top curves, release of aqueous contents.

The addition of 2 mM Ca^{2+} caused no fusion or release of contents from vesicles made of a 1 : 2 : 1 mixture of phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine; however, if 10 mM phosphate was subsequently added to the medium, rapid fusion was induced and was accompanied by a fast release of contents. In comparison, even 20 mM Ca^{2+} could bring about very little fusion. The details of the effect of phosphate in promoting Ca^{2+}-induced fusion of phospholipid vesicles are given elsewhere [30].

Discussion

The fusion assay

In the assay used in our experiments, the increase in Tb fluorescence is observed only when the internal aqueous compartments of two vesicles mix while their membranes form one continuous membrane entrapping the aqueous contents of both vesicles. In this respect, we differ from Palade [31] in the use of the term 'fusion'. The close contact between polar head groups (less than 10 Å), the formation of a pentalaminar structure or of a single bilayer between two membranes are not included in our definition of fusion. We consider these as the initial necessary steps towards the ultimate fusion, which would involve both mixing of the membrane lipids and communication between the two previously distinct aqueous environments.

Distinct roles of phosphatidylcholine and phosphatidylethanolamine

Our results show that the inclusion of neutral phospholipids in addition to phosphatidylserine in the vesicle membrane reduces the initial rate and extent of fusion compared to pure phosphatidylserine membranes (Figs. 1A and 2A). The two neutral lipids used in our studies behave very differently, however; phosphatidylcholine inhibits fusion, whereas phosphatidylethanolamine sustains it. This is clearly evidenced by a comparison of the 1 : 1 mixtures of phosphatidylcholine. The phosphatidylserine/phosphatidylethanolamine vesicles fuse extensively (Fig. 2A), whereas phosphatidylserine/phosphatidylcholine vesicles only aggregate and do not fuse (Fig. 1A). This difference between phosphatidylcholine and phosphatidylethanolamine is also seen in Fig. 8; the initial rate of fusion progressively increases as the phosphatidylethanolamine content of the membrane is increased while keeping the amount of phosphatidylserine constant. This observation is in agreement with the results of Miller and Racker [32] who utilized a reconstituted membrane protein system and an assay which detected the mixing of the membranes of two populations of vesicles. Phosphatidylethanolamine is a major component of many cellular membranes [11], and it is preferentially located in the inner monolayer of the erythrocyte membrane and, possibly, the plasma membranes of other cells [33—35]. Thus, it is likely that this phospholipid is involved in the interaction of intracellular membranes with the plasma membrane.

It is not possible at this time to identify unequivocally the molecular factor which dominates the difference in fusion capacity between phosphatidylserine/phosphatidylcholine and phosphatidylserine/phosphatidylethanolamine mixtures. Although phosphatidylcholine and phosphatidylethanolamine carry similar charges at neutral pH, they differ in their polar head-group structure and

water of hydration. Phosphatidylcholine has a large hydration shell around the head group [36] and a higher affinity for water [37]. The orientation of the polar groups in both phospholipids is parallel to the plane of the bilayer [38], but the hydrogen bonding between adjacent molecules must be different, as well as the bonding between these lipids and phosphatidylserine. Phosphatidylethanolamine vesicles made at high pH and relatively high ionic strength aggregate when the pH is lowered [39] while divalent cations aggregate phosphatidylethanolamine vesicles in a low ionic strength medium at neutral pH [40]. Vesicles made of egg phosphatidylcholine do not aggregate in the presence of millimolar Ca^{2+} [41], for which the dissociation constant with phosphatidylcholine is about 1 M [42]. Large repulsive forces between phosphatidylcholine bilayers would prevent their close approach [43], and the presence of Ca^{2+} increases the equilibrium distance between multilayers of dipalmitoyl phosphatidylcholine [44]. Fully hydrated phosphatidylcholine always maintains a lamellar structure, whereas phosphatidylethanolamine transforms into the hexagonal (H_{II}) phase at a characteristic temperature [45,46]. This bilayer-to-hexagonal transition is also affected by the extent of hydration of phosphatidylethanolamine [47]. The amount of Ca^{2+} bound per phosphatidylserine molecule appears to be an important determinant of fusion in sonicated unilamellar vesicles [48]; it is possible that the difference between phosphatidylserine/phosphatidylethanolamine and phosphatidylserine/phosphatidylcholine vesicle fusion reflects the differential binding capacity of Ca^{2+} to the two mixtures, since Ca^{2+} binds more strongly to phosphatidylethanolamine than to phosphatidylcholine monolayer membranes [49]. Moreover, the difference in the ability of Ca^{2+} to phase-separate the phospholipids in these two mixtures could be an important factor [3,12].

Specificity of Ca^{2+} and Mg^{2+}

Ca^{2+} has an absolute specificity over Mg^{2+} in its ability to induce fusion only for certain phospholipids. Vesicles made of pure phosphatidylserine (or vesicles which contain even small amounts of phosphatidylcholine) do not fuse in the presence of Mg^{2+}, although they aggregate at a threshold concentration. In contrast, sonicated vesicles made of the same phospholipid do undergo limited fusion with Mg^{2+} [50] and the threshold concentrations of Ca^{2+} required for aggregation and fusion of these vesicles are reduced in the presence of subthreshold concentrations of Mg^{2+} [7,27]. Large phosphatidylserine/phosphatidylethanolamine vesicles, however, can be fused with Mg^{2+}. The threshold concentration of Mg^{2+} necessary for aggregation and fusion is slightly higher than that of Ca^{2+}, in accordance with its lower binding constant to phosphatidylserine [28,48,51]. However, since Mg^{2+} is unable to fuse large phosphatidylserine vesicles, the role of phosphatidylethanolamine is particularly important. After the vesicles are aggregated by Mg^{2+}, contact between the phosphatidylethanolamine molecules in apposed bilayers may lead to fusion.

Implications for molecular mechanisms

While two bilayers approach each other due to initial charge neutralization by divalent ions, they will experience a repulsive force possibly due to the water of hydration around the polar groups [43,52]. Any molecular mecha-

nism we invoke must therefore take into consideration the necessity to overcome this hydration force. The water of hydration can be removed from phosphatidylserine membranes by Ca^{2+} [6,7,53] possibly forming an intermembrane '*trans*' complex [7]. The drastic reduction of the rate and extent of Ca^{2+}-induced fusion by inclusion of phosphatidylcholine in phosphatidylserine bilayers (and its complete inhibition at 50% phosphatidylcholine) may reflect the role of hydration forces and the inability of Ca^{2+} to remove the water around phosphatidylcholine.

The limited degree of hydration of phosphatidylethanolamine and the fact that it interacts with Ca^{2+} may be some of the reasons why phosphatidylserine/phosphatidylethanolamine membranes do fuse in the presence of Ca^{2+}. Cullis and Verkleij [54] have proposed that the induction of fusion by Ca^{2+} in 1 : 4 mixtures of phosphatidylserine/phosphatidylethanolamine at 37°C may be related to the formation of non-bilayer (H_{II} phase) intermediary structures, as detected by ^{31}P-NMR, and suggest that subsequent to the formation of the phosphatidylserine-Ca^{2+} complex, the phase-separated phosphatidylethanolamine molecules intermix via the formation of inverse micelles. However, this mechanism does not explain how Mg^{2+} can induce fusion of phosphatidylserine/phosphatidylethanolamine vesicles, since the H_{II} phase is not formed in the presence of Mg^{2+} even at higher concentrations than Ca^{2+} [54]. It is possible that the hexagonal phase induced by Ca^{2+} is the final equilibrium structure attained after a long incubation and that the fusion reaction itself is not strictly dependent on this phase change. This is certainly the case with phosphatidylserine vesicles which fuse in the presence of Ca^{2+} without undergoing a transition to a non-bilayer state. Moreover, the lamellar-to-hexagonal transition of the phosphatidylethanolamine used in our studies takes place at 40°C [55]; therefore, at 25°C, even if the lipids are phase-separated, phosphatidylethanolamine would not be transformed to the H_{II} phase. It is possible, however, that under dehydrating conditions the transformation of phosphatidylethanolamine to a hexagonal (or micellar) phase occurs at lower temperatures. We are currently investigating this problem in detail.

Implications for membrane fusion in exocytosis

Phospholipid vesicles are undoubtedly very simple structures compared to biological membranes; yet the physicochemical disposition of some types of vesicle to fuse in the presence of Ca^{2+} (and Mg^{2+}) raises the possibility that membrane fusion may be initiated between the lipid portions of exocytotic vesicles and plasma membranes. Since in most biological membranes the percentage of phosphatidylserine among total phospholipids is significant, but lower than 50% [11,56,57], the use of pure phosphatidylserine membranes for studying the mechanism of membrane fusion can be questioned. We have shown here and elsewhere [30], however, that vesicles containing only 10—25% phosphatidylserine are able to fuse extensively in the presence of Ca^{2+} (Fig. 3), or Ca^{2+} and phosphate (Fig. 9 and Ref. 30). In addition, the presence of Mg^{2+} lowers the concentration of Ca^{2+} required to induce fusion (Figs. 5 and 6). Millimolar concentrations of Mg^{2+} in the cell could very likely facilitate the fusogenic activity of Ca^{2+} by enhancing the rate of fusion and lowering the threshold for Ca^{2+} concentration at the site of fusion.

Based on their study of the interaction of isolated secretory vesicles, Gratzl and co-workers [58,59] have proposed that certain protein sites on intracellular membranes recognize each other and provide the specificity of fusion, for example, between the chromaffin granule membrane and the plasma membrane. While this is quite likely, specificity of fusion may also arise from the phospholipid composition of these membranes. Membrane proteins may aid in this process by creating domains of certain phospholipids [60] which would tend to fuse, even if the overall phospholipid composition of the membrane would classify it as a non-fusing membrane. Water-soluble proteins in the cytoplasm may also be involved in membrane fusion. For example, synexin, isolated from the adrenal medulla, induces the formation of a pentalaminar membrane between aggregated chromaffin granules in the presence of Ca^{2+} [61] and enhances the rate of fusion of phospholipid vesicles [62,63]. Elucidating the molecular mechanisms of membrane fusion at the cellular level therefore requires an understanding of the role of both phospholipids and proteins in the reaction.

Acknowledgements

This work was supported by Research Grant GM-26359 from the National Institutes of Health (D.P.), a Fellowship Grant from the Netherlands Organization for the Advancement of Pure Research (J.W.), N.I.H. Fellowship GM-07167 (R.F.), and N.I.H. Fellowship CA-06190 from the National Cancer Institute (N.D.). We wish to thank Drs. K. Hong (UCSF), S. Nir (Hebrew University of Jerusalem), C. Newton (Kalamazoo College), R. Sundler (Lund University), and K. Freeman (McMaster University) for discussions, H. Guillemin for her help in preparing the manuscript, and B. Abrams for technical assistance.

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