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CONTROL OF MEMBRANE FUSION BY PHOSPHOLIPID HEAD GROUPS

II. THE ROLE OF PHOSPHATIDYLETHANOLAMINE IN MIXTURES WITH PHOSPHATIDATE AND PHOSPHATIDYLINOSITOL

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Membrane fusion induced by Ca^{2+} and Mg^{2+} in large unilamellar vesicles composed of mixtures of phosphatidylethanolamine with phosphatidate and phosphatidylinositol was studied by means of a fluorescence assay for the intermixing of internal aqueous contents of the vesicles. The threshold concentrations of Ca^{2+} or Mg^{2+} required for fusion increased only moderately when up to 80 mol% phosphatidylethanolamine was included with phosphatidate at pH 7.4, but no fusion could be detected in vesicles containing 70 mol% phosphatidylcholine even at high concentrations of Ca^{2+} or Mg^{2+} . Phosphatidate-phosphatidylethanolamine (1 : 4) vesicles could be induced to fuse by 0.1 mM Ca^{2+} in the presence of a Mg^{2+} concentration which alone was insufficient for fusion. When equimolar amounts of phosphatidylethanolamine was included with phosphatidylinositol, the vesicles were susceptible to fusion by Ca^{2+} , although pure phosphatidylinositol vesicles themselves merely aggregate and do not fuse (Sundler, R. and Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta* 649, 743–750, accompanying paper). The role of phosphatidylethanolamine acyl chains, and hence the possible involvement of the bilayer-hexagonal (H_{II}) transition in membrane fusion, was examined by the temperature dependence of Ca^{2+} -induced fusion in phosphatidylinositol-dimyristoylphosphatidylethanolamine (1 : 1) vesicles. Fusion was strictly dependent on the gel-liquid crystalline transition of the mixture and not on the phase behavior of the phosphatidylethanolamines. Comparable fusion rates were obtained for both egg yolk phosphatidylethanolamine and dimyristoylphosphatidylethanolamine at 50°C. As the dimyristoylphosphatidylethanolamine does not convert to a non-bilayer phase in this temperature range, we conclude that the bilayer-hexagonal transition is not necessary for membrane fusion. We propose that the dehydration characteristics of the phospholipids and their metal ion complexes are the critical factors determining fusion susceptibility of phospholipid membranes.

Introduction

In most studies utilizing phospholipid vesicles as a model for Ca^{2+} -induced membrane fusion, emphasis has been given to the acidic phospholipids because of their relatively high binding capacity for divalent

metal ions [1,2]. These studies have shown that vesicles composed of phosphatidate, phosphatidylserine, or phosphatidylglycerol (suspended in 0.1 M NaCl, pH 7.4) can fuse rapidly following addition of Ca^{2+} at concentrations of 0.2, 2.0 and 10.0 mM, respectively [3,4]. In the accompanying paper we have shown that vesicles composed of phosphatidylinositol do not fuse, even at high Ca^{2+} (or Mg^{2+}) concentrations (up to 50 mM) although they aggregate immediately under these conditions [5].

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The inability of phosphatidylinositol vesicles to fuse has provided us with the opportunity to investigate further the role of phosphatidylethanolamine in membrane fusion. It was recently shown that vesicles composed of phosphatidylserine mixed with phosphatidylethanolamine fuse at similar concentrations of Ca^{2+} compared to pure phosphatidylserine vesicles, while mixed phosphatidylserine-phosphatidylcholine vesicles require much higher concentrations of Ca^{2+} [6]. This indicates a possible role of phosphatidylethanolamine in membrane fusion, but does not answer the question as to whether phosphatidylethanolamine participates actively during the fusion event, since phosphatidylserine alone tends to fuse in the presence of Ca^{2+} . In the study presented here, we have investigated the role of phosphatidylethanolamine in membrane fusion by comparing the fusion characteristics of vesicles composed of phosphatidylethanolamine mixed with phosphatidylinositol, and phosphatidylethanolamine (or phosphatidylcholine) mixed with phosphatidate. We have found that phosphatidylethanolamine, in contrast to phosphatidylcholine, has only a small inhibitory effect on phosphatidate fusion, but has a very large enhancing effect on phosphatidylinositol fusion, indicating a direct role for phosphatidylethanolamine in fusion phenomena.

In addition, we have found that phosphatidylinositol-phosphatidylethanolamine mixtures can fuse irrespective of the acyl chain composition of phosphatidylethanolamine indicating no direct relationship to the formation of a hexagonal (H_{11}) phase. Finally, we have studied the fusion of phosphatidate with phosphatidylserine vesicles (hybrid fusion) and compared it with the fusion of mixed phosphatidate-phosphatidylserine vesicles. We have interpreted the results obtained from these studies in terms of the importance of head-group hydration for promoting or inhibiting fusion phenomena.

Materials and Methods

Phosphatidylinositol (from soy bean), phosphatidate (prepared from egg yolk phosphatidylcholine) and egg yolk phosphatidylethanolamine were purchased from Avanti Polar Lipids, Birmingham, AL. Phosphatidylserine was isolated from bovine brain [7]. Dimyristoylphosphatidylethanolamine was ob-

tained from Fluka AG, Switzerland. Purity of the lipids was assessed by thin-layer chromatography.

Unilamellar vesicles (approx. $0.1\ \mu\text{m}$ diameter) were prepared by reverse-phase evaporation, using diethyl ether as solvent, followed by passage through a $0.1\ \mu\text{m}$ (pore diameter) Uni-Pore membrane (Bio-Rad) [8]. Encapsulation of Tb^{3+} ions (2.5 mM TbCl_3 -100 mM nitrilotriacetic acid), of 100 mM dipicolinic acid and of carboxyfluorescein (50 mM carboxyfluorescein-100 mM NaCl) were carried out as described [5]. All encapsulated solutions as well as the external medium (0.2 M NaCl) contained 2 mM L-histidine and 2 mM tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid and had a final pH of 7.4.

Assays for vesicle fusion were carried out at 25°C , except where indicated, and contained vesicles with encapsulated Tb and dipicolinic acid, respectively (25 nmol vesicle lipid each) in 1 ml of external medium supplemented with 0.1 mM EDTA. Changes in 90° light scattering (at 276 nm) and the formation of Tb-dipicolinic acid complex upon vesicle fusion was followed in an SLM-4000 fluorimeter (SLM Instruments, Champaign-Urbana, IL) as described [9]. Release of vesicle contents was followed by the relief of selfquenching upon dilution of encapsulated carboxyfluorescein into the external medium [9–11]. Fusion is presented as percent of maximal fluorescence; in the fusion assay the latter represents the fluorescence obtained upon lysis of the same amount of Tb-containing vesicles in an excess of dipicolinic acid (20 μM).

Vesicles composed of phosphatidylinositol-dimyristoylphosphatidylethanolamine (50 : 50), prepared as described above but using isopropylether as solvent, were studied by differential scanning calorimetry using a Perkin-Elmer DSC-2. Vesicles were pelleted by centrifugation and transferred in a 15 μl volume to calorimeter pans. The heating (cooling) rate was 5 K/min and the sensitivity 1 mcal/s. Hydrated samples of pure phosphatidylinositol showed no significant heat uptake in the range of 0 – 50°C while pure dimyristoylphosphatidylethanolamine gave one endothermic peak (midpoint 49°C) in heating scans, as expected.

Results and Discussion

Mixed vesicles containing phosphatidate

We have observed previously that the inclusion

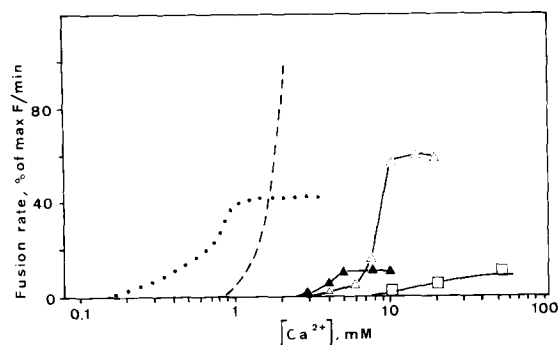


Fig. 1. Rate of fusion of phosphatidate-phosphatidylcholine mixed lipid vesicles as a function of Ca^{2+} concentration at pH 6.0 (open symbols) or pH 7.4 (filled symbols). Molar lipid composition was 50 : 50 (Δ , \blacktriangle) or 30 : 70 (\square). Corresponding data (from Ref. 5) for pure phosphatidate vesicles at pH 6.0 (broken line) and pH 7.4 (dotted line) are included for comparison. The rate of fusion is expressed as percent of maximal Tb-dipicolinic acid fluorescence (F) per min.

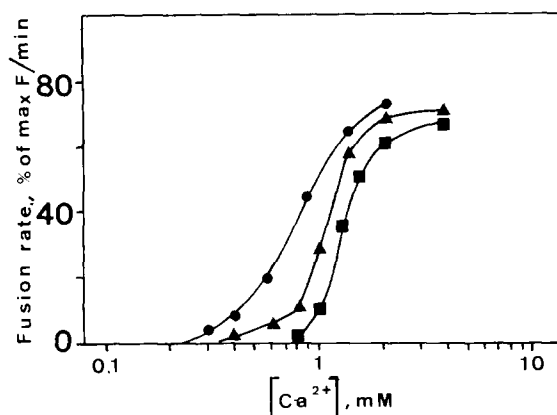


Fig. 2. Rate of fusion of phosphatidate-egg yolk phosphatidylethanolamine mixed lipid vesicles as a function of Ca^{2+} concentration at pH 7.4. Molar lipid composition was: 75 : 25 (\bullet), 50 : 50 (\blacktriangle), or 20 : 80 (\blacksquare).

of phosphatidylcholine in phosphatidylserine membranes is extremely inhibitory to Ca^{2+} -induced fusion and completely abolishes it at 50 mol% [6]. When 50 mol% phosphatidylcholine was included in phosphatidate vesicles the threshold Ca^{2+} concentration required for fusion observed at pH 6 as well as at pH 7.4 increased several-fold (Fig. 1) and the maximal Tb-dipicolinic acid fluorescence was reduced. At pH 8.5 no significant Tb-dipicolinic acid fluorescence could be detected. As discussed in the accompanying paper [5] the explanation for the low level of Tb-dipicolinic acid fluorescence appears to be the rapid release of vesicle contents once the 'threshold' concentration for fusion is reached (results not shown). Interestingly, in the presence of 5 mM (pH 7.4) or 10–12 mM (pH 6.0) Mg^{2+} the mixed phosphatidate-phosphatidylcholine vesicles aggregated but no fusion and no release of vesicle contents could be detected. When the proportion of phosphatidylcholine in the vesicles was increased further to 70 mol% the threshold Ca^{2+} concentration for aggregation and fusion was even further increased (Fig. 1). Significant levels of Tb-dipicolinic acid fluorescence could be detected in these vesicles at pH 6, but not at pH 7.4 or above. Release of vesicle contents was induced by Ca^{2+} at all three pH values, but the maximum rate of release that could be obtained by increasing the Ca^{2+} concentration at each pH-value was clearly lower than for

vesicles containing equimolar amounts of phosphatidate and phosphatidylcholine. The rate of Ca^{2+} induced leakage from these later vesicles was, in turn, significantly lower than for pure phosphatidate vesicles. In summary, the inclusion of phosphatidylcholine in phosphatidate vesicles: (a) raises the threshold concentration for Ca^{2+} -induced fusion, (b) abolishes Mg^{2+} -induced fusion, and (c) lowers the extent of fusion as well as the rate and extent of the release of vesicle contents.

In several respects, vesicles containing phosphatidate mixed with phosphatidylethanolamine instead of phosphatidylcholine behaved quite differently. The threshold concentration for Ca^{2+} -induced fusion at pH 7.4 was increased only moderately even when 80 mol% of the vesicle lipids was phosphatidylethanolamine (Fig. 2). Furthermore, the release of vesicle contents was delayed relative to fusion in these vesicles and the fluorescent Tb-dipicolinic acid complex was therefore much better retained than in pure phosphatidate vesicles at neutral pH (Fig. 3). A low degree of selectivity for Ca^{2+} over Mg^{2+} in inducing fusion, as observed with pure phosphatidate vesicles, was also maintained when phosphatidylethanolamine was included and similar high rates of fusion could be obtained with vesicles containing either 25, 50 or 80 mol% phosphatidylethanolamine in the presence of 2–4 mM Ca^{2+} or Mg^{2+} at pH 7.4. Vesicles containing

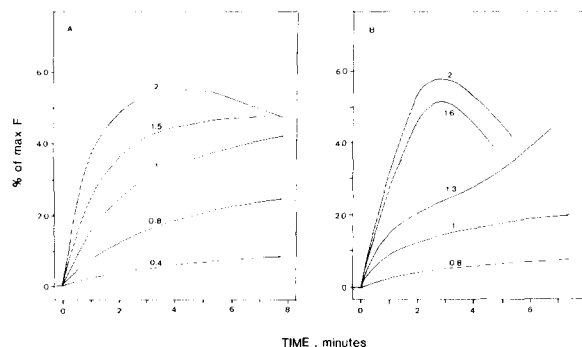


Fig. 3. Kinetics of Ca^{2+} -induced fusion of phosphatidate-egg yolk phosphatidylethanolamine mixed vesicles at pH 7.4. Molar lipid composition was 50 : 50 (A) or 20 : 80 (B). The concentration of Ca^{2+} (mM) in each experiment is indicated in the figure.

20 mol% phosphatidate in phosphatidylethanolamine did not fuse significantly when exposed to 0.9 mM Mg^{2+} but subsequent addition of only 0.1 mM Ca^{2+} was sufficient to induce rapid vesicle fusion (results not shown). The concentration of Mg^{2+} used in these experiments is similar to the intracellular concentration of free Mg^{2+} in frog muscle cells (0.6 mM [12]).

Mixed vesicles containing phosphatidylinositol

In the accompanying paper [5], we have shown that pure phosphatidylinositol vesicles do not undergo Ca^{2+} -induced fusion. When egg yolk phosphatidylethanolamine was included in vesicles at equimolar amounts with phosphatidylinositol, the vesicles became susceptible to Ca^{2+} fusion at high (≥ 10 mM) concentrations of Ca^{2+} (Fig. 4). In this case, the threshold Ca^{2+} concentration for fusion was significantly higher than that for vesicle aggregation. However, if we compare the results obtained with phosphatidylinositol-phosphatidylethanolamine (50 : 50) vesicles with those of Fig. 6 in the accompanying paper, we can conclude that phosphatidylethanolamine promoted Ca^{2+} -induced fusion at least as well as did phosphatidylserine in these mixed vesicles. The effect of phosphatidylethanolamine in promoting fusion is also seen in Fig. 5, which shows the effects of both Ca^{2+} and Mg^{2+} on the fusion of two different mixtures of phosphatidylinositol with phosphatidylethanolamine. As phosphatidylinositol was diluted in these mixed vesicles from 50% to 20%, there was a

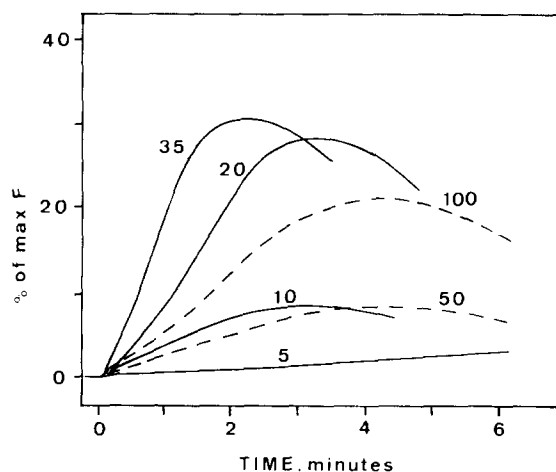


Fig. 4. Kinetics of fusion of phosphatidylinositol-egg yolk phosphatidylethanolamine (50 : 50) vesicles induced by Ca^{2+} (5–35 mM, solid lines) or Mg^{2+} (50 or 100 mM, broken lines). Temperature, 25°C; pH 7.4.

dramatic decrease in the threshold concentration of either cation required to induce fusion. Of equal importance was the difference in effectiveness between Ca^{2+} and Mg^{2+} which was also reduced. A similar effect on $\text{Ca}^{2+}/\text{Mg}^{2+}$ specificity was seen earlier [6] when it was observed that phosphatidylserine-phosphatidylethanolamine mixed vesicles could fuse with Mg^{2+} or Ca^{2+} , while pure phosphatidylserine

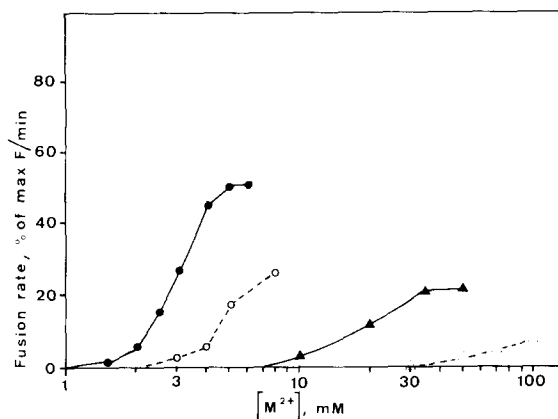


Fig. 5. Rate of fusion of phosphatidylinositol-egg yolk phosphatidylethanolamine vesicles as a function of concentration of Ca^{2+} (solid lines) or Mg^{2+} (broken lines). Molar composition of vesicles was 50 : 50 (▲, △) or 20 : 80 (●, ○).

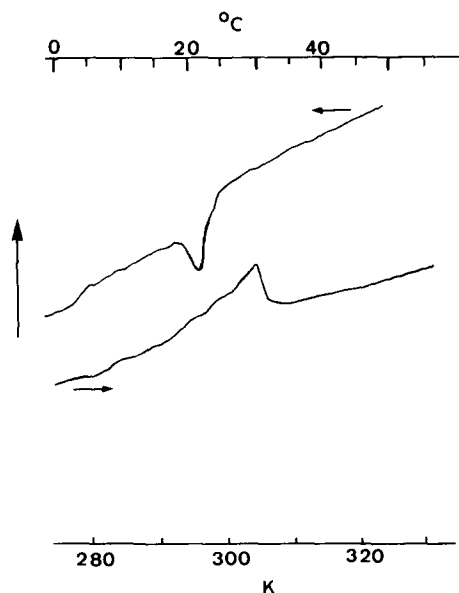


Fig. 6. Calorimetric heating and cooling scans of lipid vesicles composed of equimolar amounts of phosphatidylinositol and dimyristoyl-phosphatidylethanolamine. Vertical arrow indicates heat uptake.

vesicles could fuse only with Ca^{2+} [13]. In the latter case, however, the inclusion of phosphatidylethanolamine (with phosphatidylserine) did not enhance the Ca^{2+} -induced fusion of phosphatidylserine vesicles, while in the case presented in Fig. 5, phosphatidylethanolamine clearly enhanced fusion, since the other component (phosphatidylinositol) did not undergo fusion on its own.

From previous experience with phosphatidylserine-phosphatidylcholine or phosphatidate-phosphatidylcholine vesicles [14–16] it would be logical to assume that the additional requirement for fusion (in addition to vesicle contact), which is fulfilled by raising the Ca^{2+} concentration above the threshold for aggregation, constitutes a lateral phase separation. This could enhance fusion by creating phase boundaries [4] and in the case of phosphatidylinositol-containing vesicles also by creating fusion-competent regions enriched with another acidic phospholipid (phosphatidylserine, phosphatidate). In the case of phosphatidylinositol-phosphatidylethanolamine vesicles, formation of phosphatidylethanolamine-rich domains could promote fusion through their tendency to (i) establish close interbilayer contact [17],

or (ii) form inverted hexagonal (H_{II}) structures as proposed [18]. According to the latter explanation, mixed vesicles of phosphatidylinositol with a saturated phosphatidylethanolamine (which does not form H_{II} structures, according to Ref. 19) would not be fusion-competent.

Role of phosphatidylethanolamine acyl chains

In order to investigate the above possibility, we have prepared vesicles containing 50 mol% each of phosphatidylinositol and dimyristoylphosphatidylethanolamine. Such vesicles showed a relatively broad endothermic phase transition between 20 and 30°C in the absence of Ca^{2+} (Fig. 6) and no heat uptake at 48–50°C, the transition temperature for pure dimyristoylphosphatidylethanolamine. While these vesicles aggregated in the presence of Ca^{2+} in a manner indistinguishable from phosphatidylinositol-egg yolk phosphatidylethanolamine vesicles at all temperatures from 10 to 50°C, they differed with respect to vesicle fusion. The vesicles containing dimyristoylphosphatidylethanolamine were resistant to Ca^{2+} -induced fusion below 25°C (Fig. 7) but fusion rates increased rapidly above that temperature and were

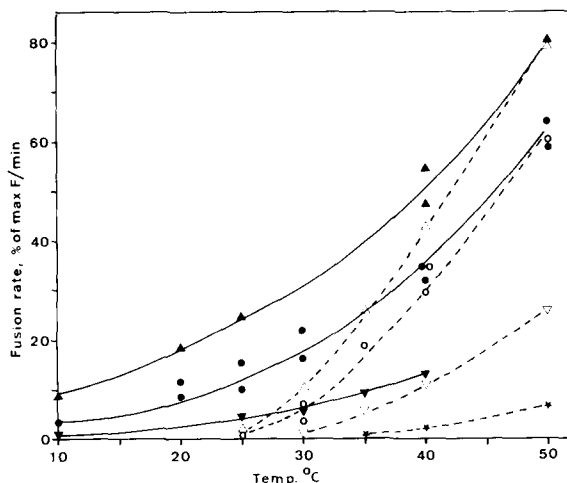


Fig. 7. Rate of Ca^{2+} -induced fusion of mixed phosphatidylinositol-phosphatidylethanolamine (50:50) vesicles as a function of temperature. The phosphatidylethanolamine component consisted of either egg yolk phosphatidylethanolamine (solid lines, filled symbols) or dimyristoylphosphatidylethanolamine (broken lines, open symbols). pH was 7.4. Concentrations of Ca^{2+} were: 10 mM (\blacktriangle , \triangle), 20 mM (\bullet , \circ), or 30 mM (\blacktriangledown , \triangledown).

similar to those for phosphatidylinositol-egg yolk phosphatidylethanolamine vesicles at 40 and 50°C. Thus, the fusion of vesicles containing equimolar amounts of phosphatidylinositol and phosphatidylethanolamine was clearly sensitive to the transition temperature for the phosphatidylinositol-phosphatidylethanolamine mixture rather than to that for the phosphatidylethanolamine component; since phosphatidylinositol vesicles do not fuse we can also conclude that fusion was probably not mediated via domains of pure phosphatidylinositol. Finally, since fusion was equally effective with either egg yolk phosphatidylethanolamine or dimyristoylphosphatidylethanolamine at higher temperatures, we may also conclude that Ca^{2+} -induced fusion of these vesicles does not require the formation of hexagonal phase (H_{II}) structures.

The possibility that Ca^{2+} -interaction with the headgroup of phosphatidylethanolamine (reportedly a very weak interaction at neutral pH [20]) might be necessary for fusion, but not for aggregation, of the phosphatidylinositol-phosphatidylethanolamine vesicles should also be considered as an explanation for the higher ion concentration required for fusion (compared to aggregation). However, this possibility appears less likely for the following reasons. First, phosphatidylinositol-phosphatidylserine vesicles required at least as high a concentration of Ca^{2+} as did the phosphatidylinositol-phosphatidylethanolamine vesicles, although phosphatidylserine would be expected to interact more efficiently with Ca^{2+} than phosphatidylethanolamine. Secondly, vesicles in which the proportion of phosphatidylinositol was reduced to 20 mol% and the proportion of phosphatidylethanolamine correspondingly increased started to fuse at a significantly lower concentration of Ca^{2+} (Fig. 5). The electrostatic attraction of Ca^{2+} to the vesicle surface would be expected to be reduced as the proportion of phosphatidylinositol decreases.

Role of head-group hydration

It is evident from the results presented in this and the accompanying paper [5] that the polar head group structure of vesicle phospholipids dramatically affects the divalent cation-induced fusion of vesicles. The charge of the head group is not the only factor of importance in this context since lipids promoting

vesicle fusion (e.g. phosphatidate and phosphatidylethanolamine) as well as lipids which are clearly inhibitory (phosphatidylinositol and phosphatidylcholine) are found among both the neutral and the anionic phospholipids. A more conspicuous relationship that seems to emerge is one between hydrated size of the lipid headgroup and the effect on fusion. Among the lipids investigated, phosphatidylinositol and phosphatidylcholine carry bulky head groups that are likely to be highly hydrated. The polar head group of phosphatidylethanolamine is, from several lines of evidence, considered to be small (less hydrated) compared to that of phosphatidylcholine. It has been shown by X-ray diffraction [21] that the interbilayer distance in the fusing Ca^{2+} -phosphatidylserine system is smaller than in the non-fusing Mg^{2+} -phosphatidylserine system and this has been attributed to a more extensive dehydration of the phosphatidylserine polar group by Ca^{2+} than by Mg^{2+} . Furthermore, the repulsive hydration forces between bilayers appears to be considerably less for phosphatidylethanolamine than for phosphatidylcholine [17].

It is therefore possible that reduction of the effective size, or the polarity, of anionic lipid head groups through dehydration constitutes a critical step in divalent cation-induced fusion. We might further postulate that a sufficiently short inter-bilayer distance, or a sufficiently reduced polarity of the inter-bilayer space, must be allowed by the surface regions of contacting vesicles for fusion to occur.

Within this conceptual framework, the widely varying polar head group effects observed in this and the accompanying paper, as well as those previously reported [6], may be given a qualitative interpretation. Thus, for phosphatidate vesicles the minimum requirement would be fulfilled with either Ca^{2+} or (the probably more hydrated) Mg^{2+} [5], because either ion would sufficiently reduce the hydrated size or polarity of the simple head group. For phosphatidylinositol vesicles, not even Ca^{2+} could induce sufficiently close contact or a sufficient reduction of polarity. The inositol ring is chemically 'bulky' and its hydrated size and polarity may not be much affected by Ca^{2+} , which would be expected to interact with only the phosphate group. In the case of phosphatidylserine, both the phosphate and the ionized carboxyl group would be sites for ion binding and consequent reduction in hydrated size (or polar-

ity) of this head group would be expected to be more extensive than in the case of phosphatidylinositol. The polar headgroup of phosphatidylethanolamine which lacks the carboxyl group would be expected, according to the above postulate, to promote ion-induced fusion at least as well as phosphatidylserine. This is also found in the cases investigated. Mixed phosphatidylserine-phosphatidylethanolamine vesicles are equally susceptible to Ca^{2+} -induced fusion compared to pure phosphatidylserine vesicles [6]; mixed phosphatidylinositol-phosphatidylethanolamine vesicles (equimolar amounts) (Fig. 4) are at least as susceptible to Ca^{2+} -induced fusion as are phosphatidylinositol-phosphatidylserine vesicles [5].

'Hybrid' fusion

If the concept put forward is correct, one would predict that the susceptibility to fusion of multi-component vesicles (composed of mixed lipids with different head-group properties) among themselves would be the same as that between populations of one-component vesicles, each composed of the different lipids, as long as the same interbilayer distance or polarity of the interbilayer space is obtained in each case. We compared the fusion (among themselves) of mixed phosphatidate-phosphatidylserine (50 : 50) vesicles with the 'hybrid' fusion [22] between pure phosphatidate and phosphatidylserine vesicles. As shown in Fig. 8, a striking similarity was found between the two systems. Of special interest is that Mg^{2+} induced fusion between phosphatidylserine and phosphatidate vesicles while phosphatidylserine vesicles do not fuse among themselves when exposed to Mg^{2+} [13]. Also, it is to be noted that phosphatidylserine vesicles fused with phosphatidate vesicles at lower Ca^{2+} concentrations (0.6–1.0 mM) than those required for fusion of phosphatidylserine vesicles with each other. The similarity of the results obtained in Fig. 8 A and B indicate that in all likelihood, lateral phase separation is not necessary for fusion in the mixed phosphatidate-phosphatidylserine vesicles. Rather, fusion may be the result of the dehydration of the inter-membrane space following Ca^{2+} binding to the phosphatidate-phosphatidylserine mixed system. This suggestion is supported by the finding that Ca^{2+} induces fusion of mixed vesicles composed of phosphatidylinositol and dimyristoylphosphatidylethanolamine (Fig. 7) at temperatures where dimyris-

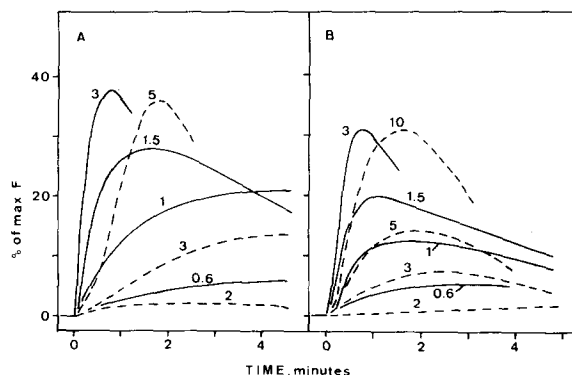


Fig. 8. Ca^{2+} - and Mg^{2+} -induced fusion among mixed phosphatidate-phosphatidylserine (50 : 50) vesicles (A) or between phosphatidate vesicles containing Tb and phosphatidylserine vesicles containing dipicolinic acid (B). Results obtained with phosphatidate vesicles containing dipicolinic acid and phosphatidylserine vesicles containing Tb were very similar to those shown in (B). Solid lines, Ca^{2+} (mM); broken lines, Mg^{2+} (mM).

toylphosphatidylethanolamine (if phase separated) would be in the gel state (Fig. 6) and phosphatidylinositol domains would not be expected to fuse. The same argument applies to the phosphatidylserine-phosphatidylethanolamine system which fuses in the presence of Mg^{2+} [6] without any evidence for phase separation either by calorimetry (Düzgüneş, N., unpublished data) or by phosphorus NMR [23].

Concluding remarks

The results presented here are fully compatible with a molecular mechanism of vesicle fusion involving formation of a dehydrated divalent ion 'trans' complex [21], since its formation would depend on a critical, sufficiently close, interbilayer contact. The role of phosphatidylethanolamine in promoting fusion also seems to be related to the relatively poor hydration of its head group. The close apposition of bilayer membranes allowed by the polar headgroups and created by the ion complex could then trigger fusion by reducing the polarity of the interbilayer space and thereby the energetic barrier towards intermixing of bilayer components.

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