Effect of Physical Constraints on the Mechanisms of Membrane Fusion: Bolaform Lipid Vesicles as Model Systems

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ABSTRACT Bolaform lipid vesicles were used to study the effect of physical constraints on membrane fusion. In these vesicles the membrane is organized in a single monolayer, because of the presence of covalent bonds in its middle plane. Therefore, the formation of fusion intermediates is subject to higher energy barriers and greater geometrical constraints than is usual in bilayer membranes. Bolaform lipids were extracted from the thermophilic archaeon *Sulfolobus solfataricus*. These lipids can be divided into two classes, the monosubstituted molecules, in which one of the polar heads is glycerol, and the bisubstituted molecules, endowed with two complex polar heads. The fusion process in vesicles composed of different mixtures of monosubstituted/bisubstituted molecules was studied by means of fluorescence techniques. Ca^{2+} or poly(ethylene glycol) was employed as a fusogenic agent. We found that fusion of such constrained membranes is still possible, provided that molecules able to mediate a structural rearrangement of the membrane are present. This condition is fulfilled by monosubstituted molecules, which are able to partition the glycerol headgroup in the apolar moiety. In addition, the presence of traces (~5%) of the monopolar compound diphytanylglycerol is an important factor for fusion to occur. On the contrary, vesicles formed by bisubstituted molecules are unable to fuse, irrespective of the fusogen employed.

INTRODUCTION

It is generally accepted that membrane fusion proceeds through two fundamental steps. The first step consists of the establishment of membrane contact and is triggered by agents like divalent ions (Düzgüneş et al., 1987; Bentz and Düzgüneş, 1985), poly(ethylene glycol) (PEG) (Boni et al., 1984; Hui and Boni, 1991), proteins and (poly)peptides (Blumenthal, 1987; Bentz and Ellens, 1988; Tournois et al., 1990), and viruses (White, 1990; Wilschut and Bron, 1993). The second step involves membrane merging, and it is most likely trigger independent (Vogel et al., 1993). At this step the membrane leaves the bilayer structure transiently, locally assuming nonlamellar configurations that mediate the molecular rearrangement in the fusion pore. These intermediate structures have been the object of intensive studies. Two different mechanisms have been discussed in the literature (Markin and Hudspeth, 1993). In the first one, fusion proceeds via a semitoroidal structure, named stalk (Chernomordik et al., 1987; Markin et al., 1984; Siegel, 1993; Zimmerberg et al., 1993). The second mechanism is based on the formation of inverted micellar intermediates (IMI) (Verkleij et al., 1979a,b). It has been shown that the stalk mechanism requires a lower free energy than an inverted micellar structure (Siegel, 1993). Both mechanisms, however, assume that the two monolayers that constitute the membrane can act independently. Therefore it is interesting to investigate the strictness of the independence requirement by considering membranes in which the two monolayers are covalently bound.

To this purpose, we have performed studies with an unusual class of lipids, extracted from the thermophilic archaeon *Sulfolobus solfataricus* (De Rosa et al., 1986; Gambacorta et al., 1995). These lipids are termed bipolar or bolaform to recall the presence of a polar head at both ends of the same molecule. The length of the apolar chains is about twice the average length of the aliphatic components of a classic lipid (Fig. 1). Bipolarity allows the plasma membrane of this archaeon to be organized in a simple monolayer (De Rosa et al., 1986; Gliozzi et al., 1983). The same basic molecular organization is found when vesicles are formed from these lipids; in fact, the lack of a preferential fracture plane of the membrane is observed (De Rosa et al., 1986; Gliozzi and Relini, 1996). The polar lipid extract (PLE) and several lipid fractions have been isolated and their phases characterized; it was shown that at high temperature in the disordered chain conformation, the unsubstituted glycerol headgroups segregate into the hydrocarbon matrix (Gulik et al., 1985, 1988). This led to the conclusion that the lipids (Fig. 1) can be divided into two classes: the monosubstituted molecules (monosubstituted bolaform phospholipid (P1), monosubstituted bolaform glycolipid (GL), monosubstituted bolaform sulfolipid (SL)) and the bisubstituted molecules (bisubstituted bolaform phosphoglycolipid, P2), with, respectively, one substituted OH at one extremity or substituted OH groups at both extremities. A third type of molecule, diphytanyl glycerol

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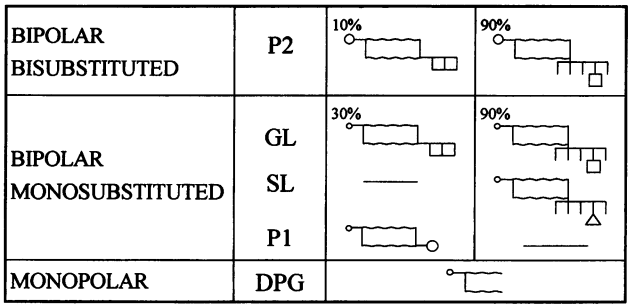

FIGURE 1 Schematic structure of the molecules constituting the polar lipid extract (PLE). Wriggles, hydrocarbon chains; small circles, unsubstituted glycerol; comb, nonitol; large circles, phosphomyoinositol; square, β-D-glucopyranose; double square, β-D-glucopyranosyl-β-D-galactopyranose; triangle, β-D-glucopyranosyl-sulfate. The apolar core of bolaform lipids consists of two C_{40} diphytanylic chains with variable degrees of cyclization.

(DPG) (Fig. 1), is present in small amounts (~5%). In a previous work (Relini et al., 1994) it was shown that Ca^{2+}-induced fusion occurs for vesicles prepared with PLE, a mixture displaying a complex polymorphic behavior (Gulik et al., 1985). On the contrary, no fusion was observed for vesicles of the bisubstituted fraction P2 (Fig. 1), a fraction displaying only the lamellar phase (Gulik et al., 1988). The use of these compounds as model lipids subject to geometrical constraints can shed some light on the molecular mechanisms of fusion. For instance, the free energy of the intermediate structure can be strongly affected by the presence of hydrophobic interstices. In the presence of apolar molecules like long-chain alkanes, these interstices are reduced or filled and fusion is facilitated (Siegel, 1993; Walter et al., 1994). The effect of apolar lipids on the energy cost of fusion intermediates brings up the question of the role of different lipids. Therefore it seems important to explore the fusion process with lipids that are endowed with a variety of particular features and construct model systems to test the theory. To this purpose, we have formed vesicles with several artificial mixtures similar to PLE. We found that the presence of particular components is determinant of the fusion process.

Finally, attention is devoted to the triggering process. Results obtained in the presence of Ca^{2+} are compared with those obtained in the presence of PEG. It is found that although the mechanisms of action of these fusogenic agents are different, giving rise to a different degree of membrane destabilization, the completion of the fusion event is governed by the headgroup polarity.

MATERIALS AND METHODS

Materials

The PLE was obtained from the membrane of *Sulfolobus solfataricus* by an overnight cold extraction in chloroform/methanol (1:1). PLE is a mixture of several fractions (Fig. 1), which derive from two backbone molecules, glycerol dialkyl glycerol tetraether and glycerol dialkyl nonitol tetraether. The mean weight composition of PLE is 48% P2, 30% GL, 10% P1, 7% SL, and 5% DPG. The method of extraction may lower the amount of P2 by 10–15% with respect to the native membrane.

Egg phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabaster, AL). Terbium chloride hexahydrate (99% pure) was obtained from Aldrich Chimica (Milan, Italy); PEG (average molecular weight 3350), dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), and calcein were purchased from Sigma Chimica (Milan, Italy). Sephadex G-50 was obtained from Pharmacia (Milan, Italy). All other chemicals were analytical grade. Water was purified by means of a Millipore Milli-Q system including a terminal 0.22-μm filter.

Vesicle preparation

Unilamellar vesicles were prepared by sonication. Hydration of the dry lipid film was made easier by employing a bath-type sonicator, Transonic 130 C (ACAD, Genoa, Italy). The liposomal dispersion was then sonicated with a probe-type sonicator (Ultrasonics, England) at 60°C for 10 min. To remove large lipid aggregates and titanium impurities, the preparation was centrifuged for 10 min at 11,500 × *g* in a Sorvall RC-SB Superspeed Centrifuge (Du Pont Instruments, Newtown, CT).

To check whether the lipid composition of the sonicated vesicles is the same as the composition of the bulk lipid, we performed equilibrium density gradient experiments followed by thin-layer chromatography (TLC). Vesicles were prepared either in 30% (w/w) sucrose or in Milli-Q water at a concentration ranging from 0.5 to 2 mg/ml and then incubated for 2 h at a final concentration of 30% sucrose. Vesicles were gently layered in a centrifuge tube containing a linear gradient of 3–40% (w/w) sucrose. Vesicles were either layered from the top or, while the gradient was prepared in the tube, at a position corresponding approximately to 30% sucrose concentration. The samples were centrifuged at 10^4 × *g* in a Sorvall RC-SB Superspeed centrifuge (Du Pont Instruments) for 1 h. In all cases a single band was observed. Increasing the centrifugation time and/or the centrifuge speed did not cause a fractionation of the band. For very long centrifugation times (18 h) at 10^5 × *g* in a Beckman Ultracentrifuge LC65B, the single band was shifted toward the top of the tube because of the gradual loss of sucrose from the vesicles, which at the end were in equilibrium at the lowest concentration. In all cases the band was collected in three separate samples. TLC was performed according to a procedure described previously (De Rosa and Gambacorta, 1994). The samples collected from the band were compared with the original bulk lipid and the corresponding vesicle preparation. No significant difference in composition was detected. This indicates that sonication does not induce any heterogeneity in the vesicle population.

Electron microscopy

The sample containing 30% of glycerol as a cryoprotectant was maintained at the desired temperature, and then a small drop was deposited on a copper planchet held at the same temperature and rapidly frozen in liquid propane. The samples were fractured at −125°C and shadowed with platinum and carbon in a Balzers freeze-etch unit. The replicas were observed in a Philips 301 transmission electron microscope.

Fluorescence measurements

Fluorescence measurements were performed using an Aminco Bowman spectrofluorometer equipped with water bath-operated circulation around the jacketted cuvette to achieve temperature control. A temperature sensor (LM35DZ; National Semiconductors, RS Components, Milan, Italy) was used to continuously monitor the temperature on the outside walls of the cuvette. Control measurement of the temperature of the sample in the cuvette, performed with a thermocouple thermometer, showed that at equlibrium there is no difference between the temperature inside and on the walls of the cuvette. The solution in the cuvette was continously stirred. A long pass filter (GG4995; Oriel, Milan, Italy) was employed to eliminate contribution to the signal from light scattering.

Fusion assay

The mixing of vesicle contents subsequent to fusion was followed by means of the Tb/DPA method (Wilschut et al., 1980). Two vesicle populations were prepared, containing either 15 mM $TbCl_3$ and 150 mM sodium citrate or 150 mM DPA. In addition, both encapsulation media contained 2 mM histidine and 2 mM 2-tris(hydroxymethyl)methylamino-1-ethanesulfonic acid (Tes) adjusted to pH 7.4. Vesicles were separated from nonencapsulated material by using the minicolumn centrifugation technique (Lelkes, 1984). The columns (1.0 × 10 cm; Pierce, USA) were filled with Sephadex G-50 swollen in 2 mM histidine, 2 mM Tes, 100 mM NaCl, 1.0 mM EDTA (pH 7.4) and centrifuged twice for 1 min at 3000 × *g* in an ALC VISMARA 4225 rotor to remove excess buffer. The vesicle preparation was divided in aliquots of 100 μl per column and was filtered by centrifugation at 3500 × *g* for 1 min. Measurements were performed in a final volume of 1.5 ml of 100 mM NaCl, 2 mM histidine, 2 mM Tes, 0.1 mM EDTA (pH 7.4). A 1:1 mixture of Tb and DPA vesicles at a total lipid concentration of 0.05 mM was considered. The sample was excited at 276 nm, and the fluorescence emission was detected at 545 nm. The data are expressed as a percentage of the maximum fluorescence, which was determined by adding 0.5% (w/v) sodium cholate, in the presence of 20 μM external DPA, to Tb vesicles filtered without EDTA. The vesicle concentration employed in the calibration procedure was the same as in the fusion assay.

Contents release assay

Vesicle leakage was monitored by means of calcein release (Allen and Cleland, 1980). Vesicles were prepared in 175 mM calcein, 2 mM histidine, 2 mM Tes, adjusted to pH 7.4. The minicolumn centrifugation technique was employed to separate vesicles from nonencapsulated material, as described in the fusion assay section. Measurements were performed at a lipid concentration of 0.05 mM, in a final volume of 1.5 ml of the same buffer used in the Tb/DPA assay. The sample was excited at 490 nm, and fluorescence emission was measured at 520 nm. The data are expressed as a percentage of the maximum fluorescence, determined by adding 0.5% (w/v) sodium cholate to the sample at the end of the experiment.

RESULTS AND DISCUSSION

A previous study (Relini et al., 1994) showed that Ca^{2+} induced the fusion of PLE but not of P2 vesicles. To gain further insight into the fusion process, freeze-fracture electron microscopy was performed on vesicles held at 60°C in the absence (Fig. 2, *A* and *B*) and in the presence (Fig. 2, *C* and *D*) of 15 mM Ca^{2+}. To our knowledge, it is the first time that fusion of bipolar lipid vesicles has been visualized. For PLE vesicles it was possible to observe the evolution with time after the addition of Ca^{2+}: after 5 min a very few small aggregates were formed (not shown); after 15 min it was possible to observe fusion (Fig. 2 *C*); after 40 min large, three-dimensional aggregates were formed (Fig. 2 *D*). Then the time scale for the process is on the order of tens of minutes, in agreement with fluorescence data of Fig. 3. The formation of extensive aggregates accounts for the observed 10-fold increase in light scattering (Relini et al., 1994). Such behavior is not exhibited by P2 vesicles, in agreement with previous experiments indicating that in these samples no aqueous contents mixing occurs. However, the infrequent presence of small aggregates of stacked vesicles retaining separate aqueous compartments confirms the prediction

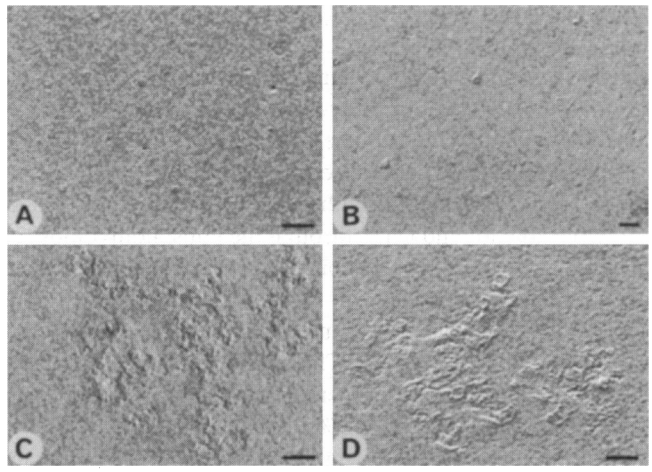

FIGURE 2 Electron micrographs of freeze fracture replicas of PLE and P2 vesicles. (*A*) PLE and (*B*) P2 vesicles in the absence of Ca^{2+}; (*C*) PLE 15 min and (*D*) PLE 40 min after the addition of Ca^{2+}. Bar = 100 nm.

made on the basis of fluorescence measurements, which showed the absence of leakage but a low amount of lipid mixing (Relini et al., 1994).

It might be surprising that PLE and P2 vesicles show a completely different behavior, in spite of the fact that P2 represents the main fraction (48%) of PLE. Fusion could be due to the presence of monosubstituted molecules and DPG, which confer a higher degree of plasticity on the membrane. To test this hypothesis, we performed experiments to characterize the fusion process on the basis of the molecular species present in the native mixture PLE. The Tb/DPA assay was used to monitor the Ca^{2+}-induced aqueous con-

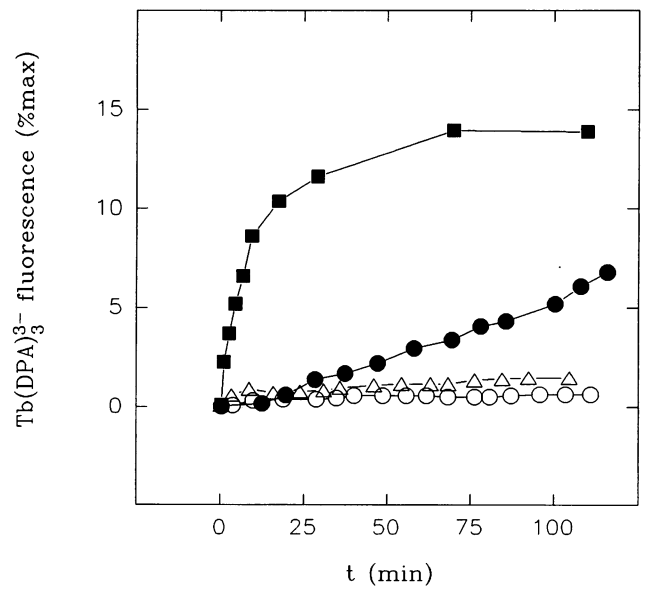

FIGURE 3 Fluorescence of the complex $Tb(DPA)_3^{3-}$ (% of max) as a function of time at 60°C after the addition of 15 mM Ca^{2+} to a 1:1 mixture of vesicles containing, respectively, Tb and DPA. Vesicle composition (w/w): ■, PLE; ●, P2/P1/GL/SL/DPG, 48:10:30:7:5; △, P2/P1/GL/SL/DPG, 57:8:20:10:5; ○, P2/P1/GL, 7:1:2.

tent mixing in sonicated vesicles. For geometrical reasons it is not possible to form vesicles composed only of monosubstituted molecules (Cavagnetto et al., 1992; Gliozzi and Relini, 1996). Therefore only mixtures of monosubstituted/bisubstituted molecules were considered. No fusion was observed in vesicles composed of P1/P2 at several weight ratios (1:9, 2:8, 3:7, 4:6), although both compounds are in principle able to bind Ca^{2+} through the phosphomyoinositol group (Fig. 1). When fusion occurs, subsequent to the addition of 15 mM Ca^{2+} at T = 60°C, the fluorescence peaks at 490 nm and 545 nm characteristic of the Tb-$(DPA)_3^{3-}$ complex appear. The presence of these peaks is an unambiguous proof of fusion, and therefore even when the extent is very small (~1%), it is significant.

We found that some molecular components are of crucial importance to fusion. The results are collected in Table 1 and can be summarized as follows.

a) Approximately 30% monosubstituted lipids is a necessary but not sufficient condition for fusion to proceed. Fusion occurs only when the monosubstituted fraction contains other lipids besides P1. This result might be explained by considering that the headgroups of these other lipids are sugars and as such are not involved in intermembrane bridges. This might allow an easier rearrangement of the interacting membrane patches.

b) Increasing the percentage of P2 decreases the extent of fusion.

c) The presence of DPG seems to be an important factor for increasing the extent of fusion. Indeed, diglycerides are known to destabilize the lamellar structure and may give rise to other structures, such as hexagonal and cubic (Luzzati et al., 1992; Basáñez et al., 1996).

The kinetic behavior of the fusion process for some lipid mixtures is compared with that of PLE in Fig. 3. These data indicate that fusion in these artificial systems is a very slow process, if compared with the usual lipids or even PLE. One of the lipid mixtures considered (Fig. 3, filled circles) was chosen to reproduce the composition of PLE. However, although on large time scales (~200 min) the fluorescence level reached is the same (data not shown), the kinetic behavior is different, indicating that the composition of the artificial mixture does not exactly match that of PLE. In fact, the relative ratios between the components of PLE can be determined with an accuracy not greater than 5–10%. Therefore, even small changes in the composition of this complex mixture induce relevant differences in the fusion process.

It has been proposed that although different fusion processes utilize different triggers, the highly localized fusion event involving membrane merging is trigger independent (Vogel et al., 1993). Therefore this study was extended to investigate the fusion process induced by the strongly water-binding polymer PEG (Boni et al., 1984; Hui and Boni, 1991; Yamazaki et al., 1989; Yamazaki and Ito, 1990) to compare the results obtained with both fusogenic agents. Data reported in Fig. 4 indicate that PEG (20% w/v) does not induce fusion with aqueous content mixing in vesicles formed by the fraction P2, a result that parallels that obtained with Ca^{2+}. A very limited extent of fusion at a very low rate is observed in PLE/P2 vesicles; in fact, this mixture contains a very low amount of monosubstituted molecules. An appreciable level of fusion at faster rates is induced by PEG in vesicles made of PLE/PC and PLE (Fig. 4). This behavior is only partially consistent with the results obtained with Ca^{2+}. In fact, PLE/PC vesicles do not fuse in the presence of Ca^{2+}, although lipid mixing is observed (Relini et al., 1994). This discrepancy can be explained by the perturbing effect of the strongly hygroscopic polymer. The PEG-induced dehydration of the phospholipids and consequently the diminished size of the polar headgroups may cause phase separation (Lehtonen and Kinnunen, 1995) and contribute to a rearrangement of lipids due to the change in packing conditions. A particular feature of the PLE/PC curve in Fig. 4 is the sudden fluorescence increase followed by a decrease. This increase can be attributed to the pres-

TABLE 1 Comparison of Ca^{2+}-induced aqueous content mixing for several lipid mixtures (by weight) at varying ratios of monosubstituted/bisubstituted molecules

Lipid mixture	M/B	$Tb(DPA)_3^{3-}$ fluorescence* (%) max
P2/P1#	#	0
P2/P1/GL 7:1:2	7:3	<1%
P2/P1/GL/SL 62:8:20:10	2:3	<2%
P2/P1/GL/SL/DPG 57:8:20:10:5	2:3	4%
P2/P1/GL/SL/DPG 48:10:30:7:5	1:1	9%
PLE	1:1	15%

*Asymptotic values.
#9:1, 8:2, 7:3, 4:6. M/B, Monosubstituted/bisubstituted.

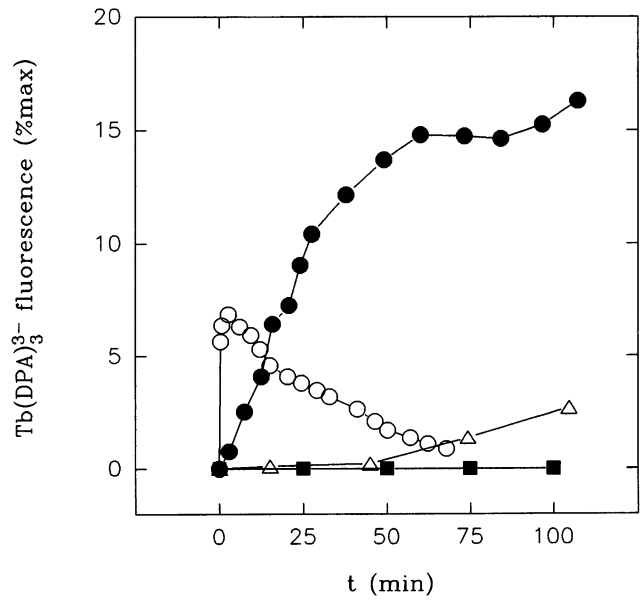

FIGURE 4 Fluorescence of the complex $Tb(DPA)_3^{3-}$ (% of max) as a function of time after the addition of 20% w/v PEG at T = 25°C. ●, PLE; ○, PLE/PC, 1:2 m.r.; △, PLE/P2, 1:2 m.r.; ■, P2.

ence of monopolar lipid, which gives rise to faster kinetics, whereas the decrease can be explained by the substantial PEG-induced leakage that occurs under these conditions (Fig. 5).

A more complex point is the one related to temperature effects. In fact, Fig. 4 shows that, in contrast to results obtained with Ca^{2+}, PEG is able to trigger fusion in PLE vesicles even at temperatures as low as 25°C, which correspond to the L_β phase (Gulik et al., 1985). Previous results indicated that at 25°C in the presence of Ca^{2+} no lipid mixing was observed in these vesicles (Relini et al., 1994). Therefore, in that case, fusion is not able to proceed, because even the first step of membrane merging does not occur. This finding may suggest that the forces due to polar head hydration are responsible for the repulsion between nonflexible vesicles at T = 25°C. In the presence of PEG the hydration layer is strongly reduced. Consequently, the probability that vesicles will aggregate and fuse is higher with PEG than with Ca^{2+}.

Fig. 5 shows calcein release of PLE, PLE/P2, and PLE/PC vesicles in the presence and in the absence of PEG at T = 60°C and at T = 25°C. It appears that PEG causes an extensive degree of leakage and therefore a noticeable destabilization of the membrane structure in PLE and PLE/PC membranes. However, this extent of leakage is not directly related to fusion. This observation is even more clear from data of Fig. 6, which show the calcein release of P2 vesicles in the presence as well as in the absence of PEG. For comparison, leakage in the presence of Ca^{2+} is also reported. These data indicate that although the system is unable to fuse, the integrity of the membrane is affected by the dehydration action of PEG. In summary, although PEG causes destabilization and leakage in all vesicles irrespective of the lipid composition, the fusion process with aqueous content mixing occurs only in those vesicles composed of lipids that allow a local separation of the two half-planes of the membrane. This is the case of vesicles containing monosubstituted molecules, DPG or egg PC.

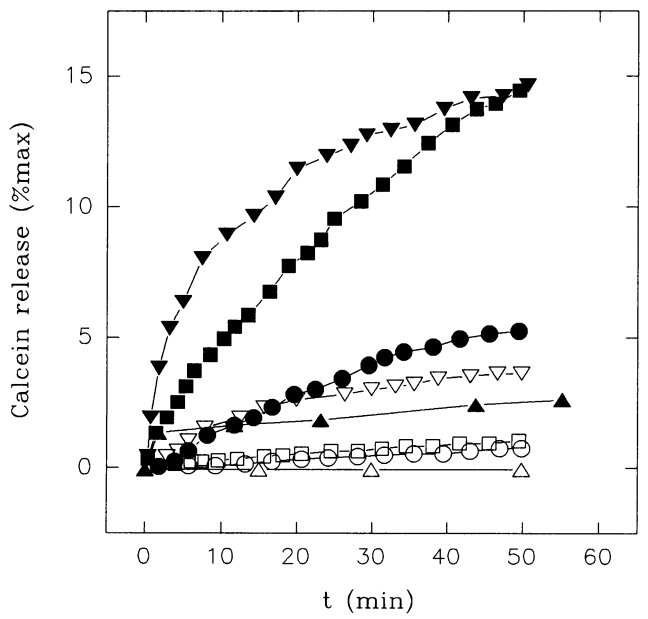

FIGURE 5 Calcein release (% of max) as a function of time in the absence (empty symbols) and in the presence (filled symbols) of PEG (20% w/v). ○,●, PLE at T = 25°C; △,▲, PLE/P2, 1:2 m.r. at T = 25°C; □,■, PLE/PC, 1:2 m.r. at T = 25°C; ▽,▼, PLE at T = 60°C.

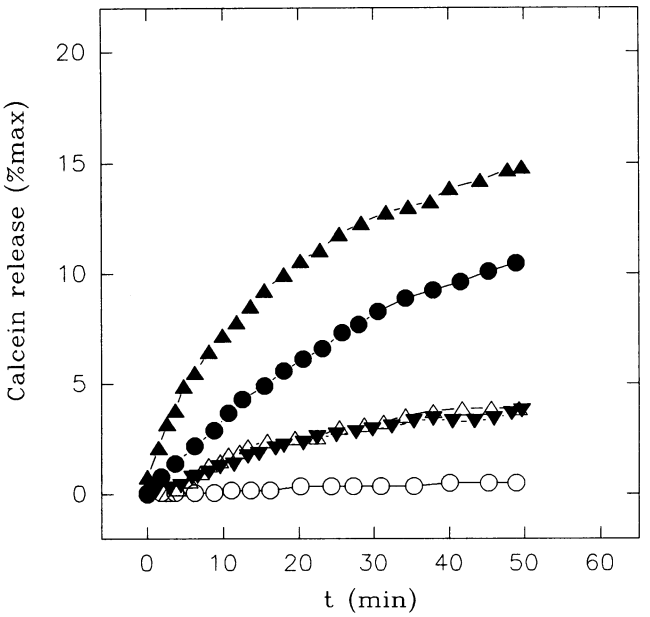

FIGURE 6 Calcein release (% of max) as a function of time for P2 vesicles in the absence (empty symbols) and in the presence (filled symbols) of destabilizing agents. △, ▲, ▼, T = 60°C; ○, ●, T = 25°C. ▲, in the presence of 20% w/v PEG; ▼, in the presence of 15 mM Ca^{2+}; ●, in the presence of 20% w/w PEG.

It would be interesting to interpret the experimental results obtained on vesicle fusion by evaluating the energy cost associated with the formation of fusion intermediates. Unfortunately, there are no data in the literature concerning the bending modulus and spontaneous curvature of bolaform lipid membranes. Therefore it is not possible to calculate the curvature free energy of the intermediate according to the model proposed by Siegel (1993). From a qualitative point of view, it is expected that to form an intermediate structure like a stalk, bipolar molecules must transfer one polar head from one water interface to the other one, passing through the hydrocarbon phase. Therefore, for bisubstituted molecules, endowed with large headgroups, the energy barrier would be much higher than for monosubstituted molecules, which can just shift the OH headgroup. This would indicate that in the former case fusion is not energetically favored, in agreement with the experimental finding that P2 vesicles are not able to fuse.

The fusion process observed in PLE vesicles could be due to a local fluctuation in the composition of the membrane or to a fusogen-induced phase separation, causing an increase in the amount of monosubstituted molecules in the region of interest. Another possibility is that the lipid arrangement in

vesicles is similar to that proposed for the lamellar phase of PLE in bulk (Gulik et al., 1985). In this case the membrane would contain a heterogeneous mixture of two types of domains, monolayers of bisubstituted molecules and bilayers of monosubstituted molecules. Then the fusion event would be similar to that usually described for bilayer membranes, and the low extent of fusion might be related to the low probability that two similar patches would meet.

The effect of DPG in favoring the fusion process is in agreement with previous work on typical bilayer membranes. In fact, diacylglycerol has been observed to increase the lipid mixing rates between large unilamellar phosphatidylserine vesicles (Walter et al., 1994) and to promote membrane fusion in different model systems (Basáñez et al., 1996). All of these results are consistent with the proposal that traces of apolar lipids reduce the energy cost of the intermediate structures by filling hydrophobic voids (Siegel, 1993).

CONCLUSIONS

One of the purposes of this study was to investigate whether the independence of the two half-planes of the membrane is strictly required to achieve fusion. We found that when the two half-planes are not independent at all, fusion does not occur, irrespective of the fusogen employed. This is the case of P2 molecules, which have both polar heads strongly anchored to the aqueous interfaces. However, lipid mixing could be observed (Relini et al., 1994); this indicates that even if the trigger is able to produce membrane interaction, the free energy requirement for the formation of intermediate structures is too high. On the contrary, if the membrane contains molecules that are able to partition one of the polar heads in the apolar region, fusion may occur. However, even in this case, the constraints of bolaform molecules hinder the fusion process. Fusion is facilitated by the presence of very low amounts of DPG. As is usually the case with bilayer vesicles, fusion behavior is affected by the lipid mixture considered and results from the interplay of different factors, such as headgroup charge, hydration, polarity, and molecular geometry. A peculiar feature of the fusion process of bolaform vesicles is the very slow time scale (tens of minutes) as compared to monopolar lipid vesicles (tens of seconds) (Wilschut et al., 1980). Such long time scales can be related to ESR experiments which showed that the correlation times of the outermost hydrophobic portion of bolaform molecules are higher by a factor of up to 10^3 than those usually observed for monopolar lipids (Bruno et al., 1985).

The final point that deserves attention is the action of the trigger molecule. In fact, Ca^{2+} or PEG can change the system to a different extent. PEG dehydrates lipids and may change the molecular packing conditions. Our results indicate that this action appears to alter the fundamental mechanisms of membrane interaction and thus also induces fusion in cases in which Ca^{2+} does not. In spite of these differences, when lipid constraints are such that intermediate structures cannot be formed, the fusion process does not occur independently of the trigger conditions.

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