Evidence for the Extended Phospholipid Conformation in Membrane Fusion and Hemifusion

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ABSTRACT Molecular-level mechanisms of fusion and hemifusion of large unilamellar dioleoyl phosphatidic acid/phosphocholine (DOPA/DOPC, 1:1 molar ratio) vesicles induced by millimolar Ca^{2+} and Mg^{2+}, respectively, were investigated using fluorescence spectroscopy. In keeping with reduction of membrane free volume V_f, both divalent cations increased the emission polarization for 1,6-diphenyl-1,3,5-hexatriene (DPH). An important finding was a decrease in excimer/monomer emission intensity ratio (I_e/I_m) for the intramolecular excimer-forming probe 1,2-bis[(pyren-1-)yl]decanoyl-sn-glycero-3-phosphocholine (bis-PDPC) in the course of fusion and hemifusion. Comparison with another intramolecular excimer-forming probe, namely, 1-[(pyren-1)-yl]decanoyl-2-[(pyren-1)-yl]tetradecanoyl-sn-glycero-3-phosphocholine (PDPTPC), allowed us to exclude changes in acyl chain alignment to be causing the decrement in I_e/I_m. As a decrease in V_f should increase I_e/I_m for bis-PDPC and because contact site between adhering liposomes was required we conclude the most feasible explanation to be the adoption of the extended conformation (P.K.J. Kinnunen, 1992, Chem. Phys. Lipids 63:251–258) by bis-PDPC. In this conformation the two acyl chains are splaying so as to become embedded in the opposing leaflets of the two adhered bilayers, with the headgroup remaining between the adjacent surfaces. Our data provide evidence for a novel mechanism of fusion of the lipid bilayers.

INTRODUCTION

Membrane fusion is in a key role in a very large number of cellular processes, such as fertilization, membrane recycling, protein trafficking within the cell, exocytosis, and enveloped virus infection. So far, the role of proteins in fusion has received major attention (e.g., Hoekstra, 1990; Zaks and Creutz, 1990; White, 1992; Creutz, 1992). However, at some stage in the course of the fusion process merging of the lipid bilayers must take place. Accordingly, also the properties of the lipids can be anticipated to be of importance. This was verified in a recent study by Chernomordik et al. (1997), demonstrating that depending on their three-dimensional molecular shape, different lipids either inhibited or promoted influenza virus fusion. The role of the hemagglutinin protein was to initialize membrane fusion by promoting the formation of a lipid intermediate. Wilschut et al. (1995) demonstrated that the fusion of Semliki Forest virus with cholesterol-containing liposomes also requires 1–2 mol % of sphingolipid to be present in the membrane. A recent review on the role of non-bilayer-forming lipids in biological fusion (Chernomordik, 1996) concludes that the lipid composition affects the fusion downstream to the activation of the fusion proteins and upstream to fusion pore formation and that the ability of fusion-promoting lipids is strongly correlated with their geometrical shape. As has already been pointed out (Kinnunen, 1992), this area bears major biomedical relevance as elucidation of the detailed mechanisms may also open novel possibilities for therapeutic intervention.

Membrane fusion is a highly localized process progressing via distinct and specific steps. Before fusion, two vesicles must first come into close contact, thus requiring short-range repulsive undulation and electrostatic and/or hydration forces to be overcome (Rand and Parsegian, 1986; Chernomordik et al., 1987). This contact is followed by membrane adherence, which is essential yet not sufficient for fusion (Zimmerberg et al., 1993). Partial dehydration of the adhering surfaces precedes the formation of an intermediate state and hemifusion, which allows for lipid mixing between vesicles in the absence of coalescence of the aqueous contents (Zimmerberg et al., 1993; Arnold, 1995). A fusion pore then opens into the septum separating the internal cavities of the two original vesicles. This pore subsequently widens, thus resulting in the merging of their aqueous contents (Zimmerberg et al., 1993).

Most ambiguity in fusion mechanisms concerns the exact nature of the intermediate state and the involved molecular arrangements. Three models have been forwarded. The first one was based on the formation of inverted micellar intermediates (IMIs) (Verkleij et al., 1979; Siegel, 1984). However, after more revealing techniques were exploited it has been rather generally accepted that IMIs are not required for fusion (Papahadjopoulos et al., 1990; Siegel, 1993). The second mechanism is based on the formation of semitoroidal structures, stalks (Markin et al., 1984), estimated to have diameters of >4 nm and lifetimes <1 ms (Siegel, 1993). These structures have so far escaped direct, affirmative observation. Stalks have been suggested to be involved in hemifusion (Siegel, 1993). The lipid organization in the

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base of the stalk would be promoted by lipids with negative spontaneous curvature (Chernomordik et al., 1995). However, within its medial part a stalk requires positive membrane curvature for the lipids in its outer monolayer. Comparing the above model, stalks have been estimated to require less free energy than IMIs (Siegel, 1993). Notably, both IMI and stalk arrangements involve a transient increase in free energy, and a stochastic nature for the fusion intermediate has been suggested (Arnold, 1995; Papahadjopoulos et al., 1990). A third mechanism is based on the so-called extended lipid conformation (Kinnunen, 1992). In brief, in this conformation the two acyl chains should be embedded in the opposing contacting leaflets of the two adjacent bilayers, with the headgroup remaining in the interface. Based on considerations arising from lipid-packing constraints the extended conformation is anticipated to be promoted by a decrease in the effective size of the lipid headgroup by dehydration, for instance, as well as by lipids favoring inverted nonlamellar phases, i.e., lipids with negative spontaneous curvature (Gruner, 1985). It is important to note that there is no a priori physical reason prohibiting the extended conformation. On the contrary, it is directly supported by nuclear magnetic resonance (NMR) data on the glycerol backbone configuration (Hauser et al., 1980, 1988) and has been shown in lipid crystals (Hybl and Dorset, 1971; Jensen and Mabis, 1966; Larsson, 1963, 1986).

Metal-cation-induced fusion of phosphatidic acid (PA)-containing vesicles provides a well established and thoroughly investigated model system (e.g., Düzgünes et al., 1987; Hong et al., 1982; Liao and Prestegard, 1979; Ohki and Zschörnig, 1993; Papahadjopoulos et al., 1990; Park et al., 1992; Simmonds and Halsey, 1985; Smaal et al., 1987). Interestingly, upon decreasing the mole fraction of PA (X_{PA}) below 0.50 in binary 1,2-dioleoyl-sn-glycero-3-phosphatidic acid (DOPA)/1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes, only hemifusion, i.e., lipid mixing between vesicles, is induced by Mg^{2+} whereas neither leakage nor mixing of their aqueous contents is manifested. In contrast, Ca^{2+} induces complete fusion (Leventis et al., 1986). When X_{PA} in PC vesicles exceeds 0.50, both Ca^{2+} and Mg^{2+} induce membrane fusion (Leventis et al., 1986).

We report here on lipid dynamics during fusion and hemifusion of DOPA/DOPC (1:1 molar ratio) vesicles induced by Ca^{2+} and Mg^{2+}, respectively, as monitored by pyrene-containing fluorescent lipid analogs. The rationale for their use is based on the photophysics of pyrene excimer formation (for recent reviews on the uses of pyrene-labeled lipids see Duportail and Lianos, 1996; Kinnunen et al., 1993). In brief, monomeric excited-state pyrene may relax back to the ground state by emitting with a maximum at ~380 nm (I_m), the exact peak energy and spectral fine structure depending on solvent polarity. During its lifetime the excited-state pyrene may also form a characteristic short-lived complex, excimer (excited dimer) with a ground state pyrene. This complex relaxes back to two ground-state pyrenes by emitting quanta as a broad and featureless band centered at ~480 nm (I_e). At sufficiently low probe concentrations excimer fluorescence of the above dipyrene phospholipids, such as 1,2-bis[(pyren-1)-yl]decanoyl-sn-glycero-3-phosphocholine (bis-PDPC) and 1-[(pyren-1)-yl]decanoyl-2-[(pyren-1)-yl]tetradecanoyl-sn-glycero-3-phosphocholine (PDPTPC), containing pyrene moieties at both ends of their acyl chains, is intramolecular and concentration independent (Sunamoto et al., 1980). More specifically, for these probes the rate of excimer formation depends on the alignment of the acyl chains (Thurén et al., 1984; Eklund et al., 1992), intramolecular thermal motion (Sunamoto et al., 1980), and changes in membrane free volume (Lehtonen and Kinnunen, 1994). In addition to the above also the extended phospholipid conformation would strongly reduce the excimer emission.

Materials and Methods

Materials

Hepes, EDTA, and dioleoyl phosphatidic acid were from Sigma Chemical Co. (St. Louis, MO) and dioleoyl phosphatidylcholine from Princeton Lipids (Princeton, NJ). NaCl, $CaCl_2$ dihydrate, and $MgCl_2$ hexahydrate were obtained from Merck (Darmstadt, Germany). Polyethylene glycol (PEG) with an average molecular weight of 6000 was from Fluka (Buchendorf, Switzerland). 1-Palmitoyl-2[(pyren)-1-yl]decanoyl-sn-glycero-3-phosphocholine (PPDPC), PDPTPC, and bis-PDPC were from K&V Bioware (Espoo, Finland), and 1,6-diphenyl-1,3,5-hexatriene (DPH) was from EGA Chemie (Steinheim, Germany). The purity of the above lipids was checked by thin-layer chromatography on silicic acid-coated plates (Merck) using chloroform/methanol/water (65:25:4, v/v) as a solvent system. Examination of the plates after iodine staining or, when appropriate, by fluorescence illumination revealed no impurities. The concentrations of PPDPC, bis-PDPC, and PDPTPC were determined spectrophotometrically using 42,000 cm^{-1} (for PPDPC) and 84,000 cm^{-1} (for bis-PDPC and PDPTPC) at 342 nm and for DPH 91,000 cm^{-1} at 354 nm as the respective molar extinction coefficients. Phospholipid concentrations were determined gravimetrically using a high-precision electrobalance (Cahn, Cerritos, CA).

Liposome preparation

Appropriate amounts of the lipid stock solutions were mixed in chloroform to obtain the desired compositions with either bis-PDPC (mole fraction $X = 0.001$), PDPTPC ($X = 0.001$), PPDPC ($X = 0.02$), or DPH ($X = 0.002$) included as a fluorescent probe. The resulting mixtures were then evaporated to dryness under a stream of nitrogen, and traces of solvent subsequently were removed by evacuating under reduced pressure for 24 h. The lipid residues were hydrated at 50°C in 5 mM Hepes, 0.1 mM EDTA, pH 7.4, to yield a lipid concentration of 3 mM and maintained at this temperature for 30 min. The suspensions were thereafter irradiated for 2 min in a bath-type ultrasonicator (NEY Ultrasonik 104H, Yucaipa, CA). The resulting dispersions were subsequently processed to large unilamellar vesicles (LUVs) by extrusion through a stack of two Millipore (Bedford, MA) 0.1-μm pore-size polycarbonate filters using a Liposofast-low pressure homogenizer (Avestin, Ottawa, Canada) essentially as described (Olson et al., 1979; MacDonald et al., 1991).

Measurement of I_e/I_m

Fluorescence emission spectra for LUVs labeled with the different pyrene-containing probes were recorded with a Perkin-Elmer LS50B spectrofluorometer equipped with a magnetically stirred, thermostatted cuvette com-

partment. Excitation wavelength was 344 nm, and the excitation and emission bandwidths were 2.5 nm for PPDPC and 7.5 nm for bis-PDPC and PDPTPC, respectively. Two milliliters of liposome solution (90 nmoles of lipid) in a four-window quartz cuvette was used in each measurement with temperature maintained at 25°C. After the addition of appropriate amounts of 1 M Ca^{2+}- or Mg^{2+}-containing buffer to induce fusion or hemifusion, respectively, samples were equilibrated for 3 min before recording of spectrum. For the steady-state measurements, five scans were averaged and the emission intensities at ~380 and 470 nm were taken for I_m and I_e, respectively. As only relative values were of interest the measured spectra were not corrected for instrument response.

To observe I_e/I_m versus time emission, intensities at 380 and 470 nm were first recorded separately under essentially identical conditions after the addition of the indicated divalent cation concentrations. As only relative changes in I_e/I_m were of interest the data were normalized and I_e/I_m calculated using these values. Effects of PEG were measured essentially as described previously (Lehtonen and Kinnunen, 1994). All measurements were repeated at least three times. The data were analyzed using Microsoft Excel (Microsoft Co., Redmond, WA).

Fluorescence polarization

DPH was included into liposomes to yield a lipid:DPH molar ratio of ~500:1 (Lakowicz et al., 1979a,b; Prendergast et al., 1981). Polarized emission was measured in L-format using Polaroid-film-type filters. Excitation at 360 nm and emission at 450 nm were selected with monochromators and using 5-nm bandwidths. The samples were maintained in the cuvette for 3 min before measuring polarization averaged over a 10-s interval. After the addition of Ca^{2+} or Mg^{2+} the cuvette contents were allowed to equilibrate for 2 min after which fluorescence intensity was measured. All measurements were repeated at least three times. Values of steady-state fluorescence polarization P were calculated using routines of the software provided by Perkin-Elmer.

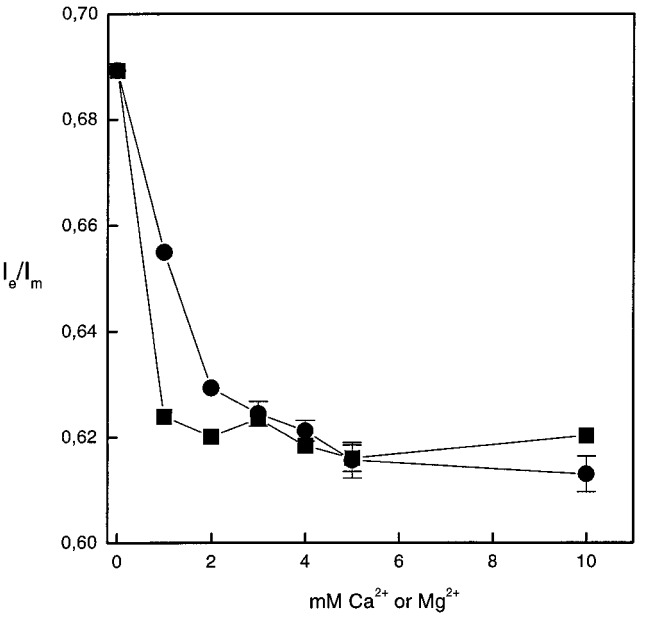

FIGURE 1 Pyrene excimer to monomer emission intensity ratio I_e/I_m for bis-PDPC ($X = 0.001$) residing in binary DOPA/DOPC (1:1 molar ratio) LUVs measured at increasing Ca^{2+} (■) and Mg^{2+} (●) concentrations. Final total phospholipid concentration was 45 μM in 5 mM Hepes, 0.1 mM EDTA, pH 7.4. Temperature was maintained at 25°C. Values shown and the illustrated average errors represent the mean for at least three separate measurements. For the sake of clarity error bars contained within the symbols are not shown.

RESULTS

To investigate the feasibility of the involvement of the extended lipid conformation in membrane fusion and hemifusion the intramolecular pyrene excimer-forming probe bis-PDPC was included at $X = 0.001$ in DOPA/DOPC (1:1 molar ratio) liposomes. The addition of mmolar Ca^{2+} decreased I_e/I_m for bis-PDPC by ~10% (Fig. 1). The same effect and of nearly equal magnitude was observed for Mg^{2+} (Fig. 1). Compared with Ca^{2+}, slightly higher concentrations of Mg^{2+} were needed, in keeping with somewhat less efficient dehydration of the anionic phospholipid by the latter divalent cation (Düzgünes et al., 1987). To elucidate that lipid concentration is not determining the behavior of I_e/I_m for bis-PDPC we used liposomes containing bis-PDPC (labeled, or hot, liposomes) and liposomes lacking the probe (nonlabeled, or cold, liposomes). When varying the stoichiometry of the hot and cold liposomes, from 1:0 to 1:2, the decrease in I_e/I_m for bis-PDPC was within 1% the same, ~11% (data not shown). This result shows that the fraction of bis-PDPC molecules that can adopt the extended conformation is independent of the area of aggregated membrane surface at constant X_{PA} and supports the view that only part of the bis-PDPC molecules are able to adopt the extended conformation. This fraction of bis-PDPC increases with X_{PA}, as shown in Fig. 3 (see below). The changes in I_e/I_m were rapid, being completed within ~10 s (Fig. 2 *A*). This time scale is, within resolution of our data, identical to that for the kinetics of fusion and hemifusion of DOPA/DOPC vesicles reported by Leventis et al. (1986).

The magnitude of the decrease in I_e/I_m for bis-PDPC induced by Ca^{2+} and Mg^{2+} did depend on the content of PA in the binary PA/PC vesicles (Fig. 3). More specifically, for neat DOPC LUVs no change in I_e/I_m after the addition of 5 mM Ca^{2+} or Mg^{2+} was observed in concordance with previous reports on lack of either fusion or hemifusion under these conditions (Leventis et al., 1986). Upon increasing X_{DOPA} in LUVs a progressively augmenting decrease in I_e/I_m was evident due to the divalent metal cations (5 mM) and at $X_{DOPA} = 1.0$, an ~30% decrease in I_e/I_m was seen.

In addition to the extended conformation (Kinnunen, 1992), a decrease in the intramolecular I_e/I_m for bis-PDPC may result from increased free volume V_f of the membrane, leading to diminished collision frequency of the two pyrene moieties (Lehtonen and Kinnunen, 1994) or from an altered alignment of the acyl chains (Thurén et al., 1984; Eklund et al., 1992). To distinguish between the above possibilities, changes in lipid dynamics caused by Ca^{2+} and Mg^{2+} were investigated using other probes. As expected from dehydration and screening of the charges of DOPA by Ca^{2+} and Mg^{2+} causing enhanced lipid packing (for a review see, e.g., Arnold, 1995) polarization of DPH was significantly increased by both divalent cations (Fig. 4). Similarly to their effect on the emission on bis-PDPC, slightly higher concen-

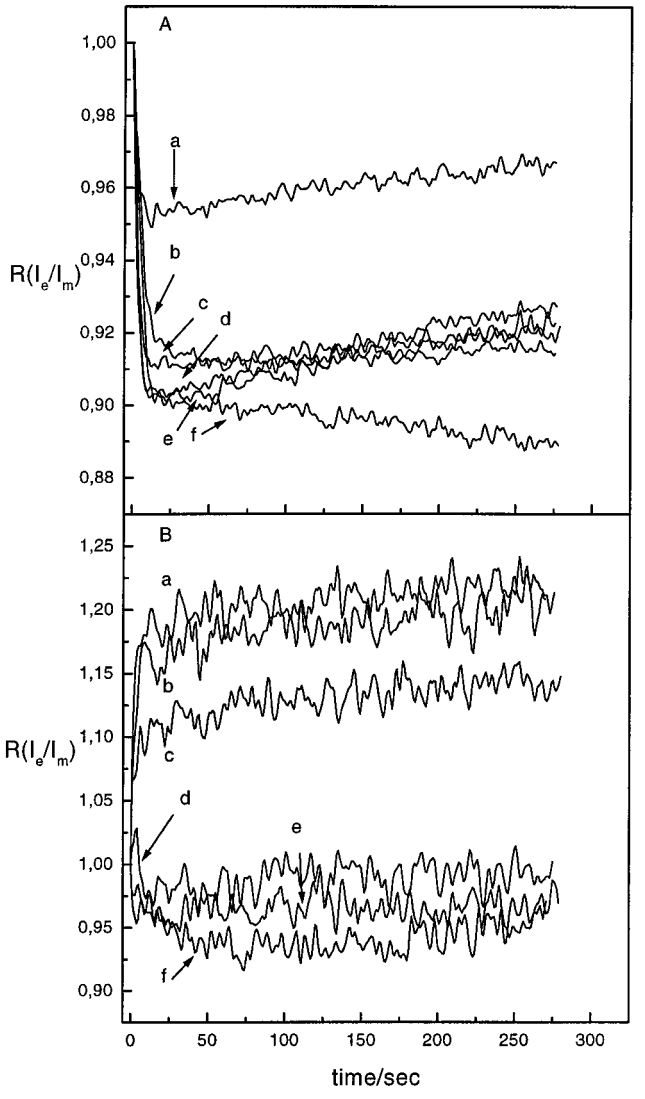

FIGURE 2 (*A*) Relative changes in I_e/I_m as a function of time for bis-PDPC ($X = 0.001$) residing in DOPA/DOPC (1:1 molar ratio) binary liposomes. From top to bottom the traces are for (*a*) 1 mM Mg^{2+}; (*b*) 2 mM Mg^{2+}; (*c*) 1mM Ca^{2+}; (*d*) 2 mM Ca^{2+}; (*e*) 5 mM Mg^{2+}; and (*f*) 5 mM Ca^{2+}. (*B*) Same experiment as in *A* but using PPDPC ($X = 0.02$) as the fluorescent probe. From top to bottom the traces are for (*a*) 5 mM Ca^{2+}; (*b*) 2 mM Ca^{2+}; (*c*) 1 mM Ca^{2+}; (*d*) 1 mM Mg^{2+}; (*e*) 2 mM Mg^{2+}; and (*f*) 5 mM Mg^{2+}. Otherwise the conditions were essentially identical to those described in the legend for Fig. 1.

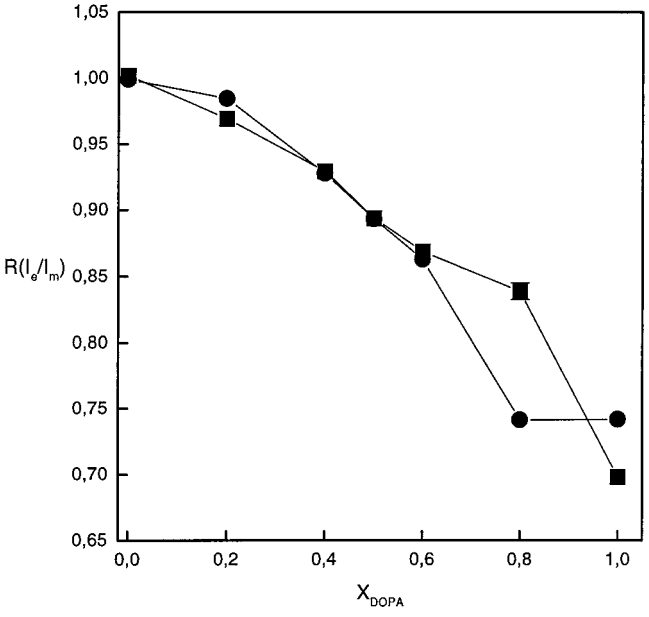

FIGURE 3 Values of I_e/I_m for bis-PDPC ($X = 0.001$) as a function of X_{DOPA} and measured at 5 mM Ca^{2+}(■) and Mg^{2+} (●). Otherwise the conditions were essentially identical to those described in the legend for Fig. 1.

trations of Mg^{2+} were required, and also the final increase in P induced by Mg^{2+} was somewhat less than for Ca^{2+}.

Under isothermal conditions, an increase in DPH polarization is a consequence of decrease in membrane free volume V_f, which is the difference between the effective and the van der Waals volumes per molecule (Bondi, 1954; Turnbull and Cohen, 1970). For a phospholipid bilayer, V_f arises from short-lived, mobile structural defects due to *trans-gauche* isomerization of the lipid acyl chains created because of packing constraints as well as by thermal motion (briefly reviewed in Xiang, 1993). Reducing V_f attenuates the amplitude of the thermal motion of the pyrenedecanoyl chains, i.e., decreases the extent of their splaying. According to Maxwell, velocity distribution for molecules of identical mass at the same temperature is constant, regardless of the state of the matter (gas, fluid, or solid). In other words, the equipartition theorem requires the contribution by each

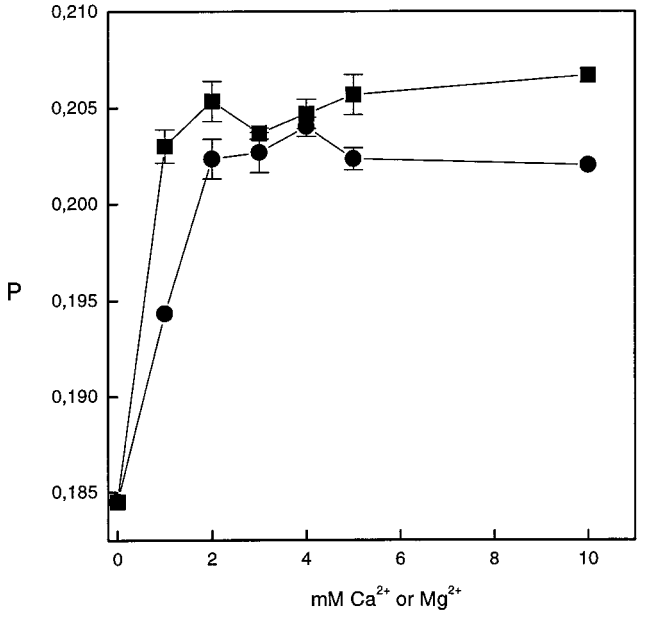

FIGURE 4 Changes in fluorescence polarization (*P*) for DPH ($X = 0.002$) incorporated in DOPA/DOPC (1:1 stoichiometry) alloys as induced by the indicated Ca^{2+} (■) and Mg^{2+} (●) concentrations. Measurement temperature was 25°C and values for *P* represent averages of a 10-s exposure.

of the quadratic terms to be identical, with variation only in potential energy. This means that with reduction in splaying amplitude the Debye frequency for the molecule and thus also the frequency of collisions between pyrenes (and I_e/I_m) should actually increase. When the thermal excitation remains constant (i.e., the frequency of chain motion is not reduced) the probability of collisional intramolecular excimer formation during the lifetime of the excited state of pyrene increases upon reduction in V_f (Lehtonen and Kinnunen, 1994). The enhanced lipid packing and acyl chain rigidification (Papahadjopoulos et al., 1977) induced by Mg^{2+} and Ca^{2+} and signaled by augmented DPH polarization (Fig. 4) excludes the possibility that an increase in membrane free volume (Lehtonen and Kinnunen, 1994) was causing the decrease in intramolecular excimer formation by bis-PDPC.

Possible changes in the alignment of the acyl chains were investigated using another intramolecular excimer-forming lipid analog, PDPTPC. In this probe the length of the sn-2 chain exceeds that of the sn-1 chain by four methylene segments. Compared with bis-PDPC, ~19% lower I_e/I_m is measured in the absence of either Ca^{2+} or Mg^{2+} for PDPTPC residing in DOPA/DOPC vesicles. This difference has been interpreted to be due to the pyrene at the end of tetradecanoyl chain to reside deeper in the bilayer than the pyrene of the decanoyl chain (Eklund et al., 1992). Notably, for PDPTPC, an ~30% decrease in I_e/I_m was induced by mmolar Ca^{2+} whereas for Mg^{2+}, an ~10% decrease was evident (Fig. 5). Dehydrating DOPC bilayers by 10 w/w % PEG decreases membrane free volume and causes an ~2-fold increase in I_e/I_m for bis-PDPC (Lehtonen and Kinnunen, 1994). Instead, under these conditions a minor (<5%) decrease in I_e/I_m is evident for PDPTPC (data not shown). This is in keeping with increasing acyl chain order in the membrane upon dehydration (Arnold, 1995; Rand and Parsegian, 1989), which in turn increases the effective length of the acyl chain. As a consequence, the pyrene moiety at the end of the tetradecanoyl chain in the sn-2 position becomes embedded deeper into the membrane so as to diminish the excimer formation with the pyrene at the end of the decanoyl spacer in the sn-1 chain. Accordingly, although decrease in free volume augments I_e/I_m, a concomitant decrement in the number of gauche conformers decreases I_e/I_m for PDPTPC, resulting in a minor net effect on I_e/I_m. Finally, it could also be argued that augmented lateral packing could favor the orientation of the glycerol backbone parallel to the sn-1 chain and perpendicular to the layer plane, similarly to the conformation observed for phospholipid crystals (Thurén et al., 1984; Eklund et al., 1992). In this case, both cations should increase I_e/I_m for PDPTPC, contrary to what is observed.

To obtain further insight into the changes in lipid dynamics induced by Mg^{2+} and Ca^{2+} we investigated the response of the intermolecular excimer-forming probe PPDPC. It should be emphasized that we used only a single population of vesicles so that the effects observed were not due to lipid mixing between labeled and unlabeled vesicles. Addition of Ca^{2+} enhanced I_e/I_m for PPDPC maximally by ~1.2-fold whereas Mg^{2+} had no effect (Fig. 6). The changes in I_e/I_m for this probe were complete within the same time range, ~10 s, as observed for the intramolecular excimer-forming probe, bis-PDPC (Fig. 2 *B*). In the absence of possible quantum mechanical effects (Kinnunen et al., 1987) and the

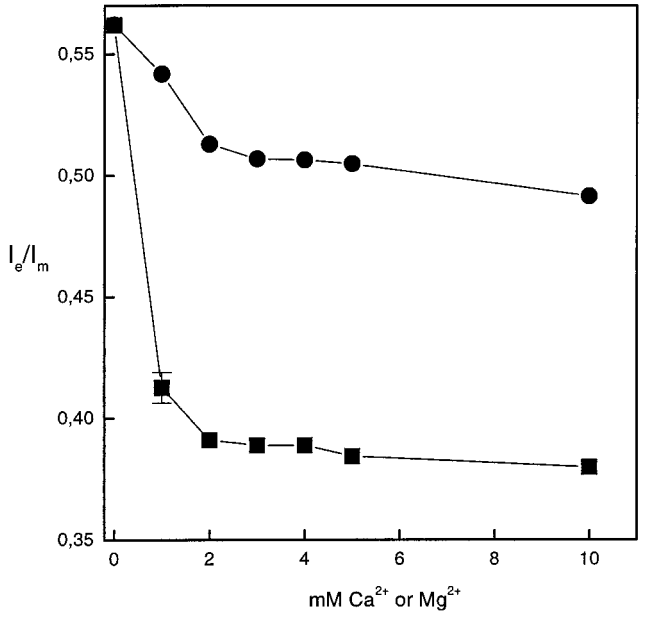

FIGURE 5 Similar experiment as illustrated in Fig. 1 but using PDPTPC ($X = 0.001$) as the fluorescent lipid analog. Concentrations of Ca^{2+} (■) and Mg^{2+} (●) were as indicated.

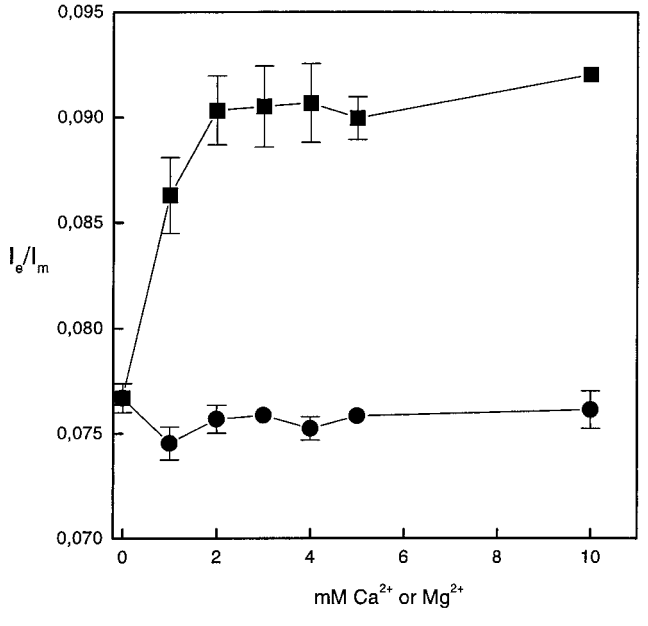

FIGURE 6 I_e/I_m for PPDPC (X = 0.02) for binary liposomes composed of DOPA/DOPC (1:1 molar ratio) measured in the presence of the indicated Ca^{2+} (■) or Mg^{2+} (●) concentrations. Except for the probe the conditions were identical to those described in the legend for Fig. 1.

formation of superlattices, I_e/I_m for a single pyrene moiety containing a phospholipid analog such as PPDPC depends on the intermolecular collision frequency of pyrenes (Förster, 1969). Consequently, the value for I_e/I_m reflects the lateral mobility (Galla and Sackmann, 1974; Galla et al., 1979) as well as the local concentration of the fluorophore in the membrane (Eklund et al., 1988; Galla and Hartmann, 1980; Hresko et al., 1986; Somerharju et al., 1985). Accordingly, either enhanced rate of lipid lateral diffusion or segregation of the probe into microdomains must be induced by Ca^{2+}. As DPH polarization reveals a decrease in membrane fluidity, enhanced lateral diffusion can be abandoned. As a consequence, Ca^{2+} enrichment of PPDPC must occur, in keeping with previous studies reporting Ca^{2+}-induced phase separation of PA in binary alloys with PC (Leventis et al., 1986; Eklund et al., 1988; Kouaouci et al., 1985; Silvius, 1990). The lack of effect by Mg^{2+} must only be apparent (Fig. 6). More specifically, as DPH reveals membrane rigidification to be caused by Mg^{2+}, it follows that also for Mg^{2+} the rate of lateral diffusion of PPDPC must be attenuated. Therefore, as no changes in I_e/I_m are evident accompanying slower lateral diffusion, microscopic enrichment of PPDPC due to Mg^{2+} is required, the opposite signals balancing each other out. Yet, compared with Ca^{2+}, the extent of segregation of lipids by Mg^{2+} must be less pronounced, in keeping with previous reports (Leventis et al., 1986). This is readily explained by the different coordination numbers for Ca^{2+} and Mg^{2+} as well as by the more efficient lipid surface dehydrating effect exerted by Ca^{2+} (Arnold, 1995).

DISCUSSION

The experiments presented here addressed the molecular level events of fusion and hemifusion of PA/PC vesicles induced by Ca^{2+} and Mg^{2+}, respectively, as revealed by the intramolecular excimer-forming pyrene-labeled phospholipids bis-PDPC and PDPTPC. In addition, DPH was used to elucidate changes in membrane fluidity and the intermolecular excimer-forming probe PPDPC to assess alterations in lateral segregation and diffusion. Our data allow the following, well established events to be distinguished. In brief, both cations increase lateral lipid packing in the vesicle bilayers, evident as an increase in polarization for DPH. In concordance with earlier data, this effect is somewhat smaller for Mg^{2+}. Both cations cause the formation of microscopic domains enriched in PPDPC and, conversely, domains enriched in PA. In the latter type of domains, the charges of PA are neutralized by divalent metal cation (Me^{2+}), and the surface is dehydrated, thus overcoming the repulsion between the vesicles and allowing for the formation of a contact site between the vesicles (Arnold, 1995). An intriguing novel finding reported here is the decrease in the intramolecular I_e/I_m for bis-PDPC (and PDPTPC) upon increasing [Me^{2+}]. This result could be accounted for by three distinct lipid conformations, as follows.

First, a phospholipid conformation caused by augmented lateral lipid packing in which the glycerol backbone parallels the sn-1 acyl chain, thus causing this chain to lie deeper in the membrane, whereas the sn-2 chain starts perpendicular to the glycerol backbone, after which the carbon segments bend and align with the sn-1 chain (Thurén et al., 1984). However, combination of probes (bis-PDPC and PDPTPC) allows us to exclude changes in chain alignment as a cause for the decrease in I_e/I_m for bis-PDPC. More specifically, to investigate the possibility that this conformation was causing the observed decrease in I_e/I_m for bis-PDPC we used another pyrene probe, PDPTPC. This probe contains a pyrene decanoyl chain at sn-1 and pyrene tetradecanoyl chain at the sn-2 position. If the above conformation was causing the decrease in I_e/I_m for bis-PDPC using PDPTPC, one should instead observe an increase in I_e/I_m because the pyrene moieties in the latter probe would reside approximately at the same level. This was not the case, but a decrement in I_e/I_m was evident for PDPTPC.

The second possibility is chain reversal (Ben-Shaul et al., 1984) as also in this case a decrease in I_e/I_m would be observed for bis-PDPC. The driving force for chain reversal is most easily understood in the context of the lateral pressure profile (Cantor, 1997). As pointed out by Cantor (1997), on a molecular level the magnitude of forces acting in membranes are enormous. As shown for 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-acyl chain containing lipid probes, the hydrophilicity of the NBD moiety favors chain reversal so as to bring this aromatic group into the interface (Chattopadya and London, 1987; Abrams and London, 1993). Similar behavior was recently suggested for a dansyl-moiety-containing phospholipid probe (Bartlett et al., 1997) as well as for other fluorescent lipid analogs (Epand et al., 1996). Likewise, due to its polarizability, pyrene could also under proper conditions (see below) accommodate into the interface. Dehydration of the phospholipid surface results in an increased packing within the hydrocarbon region of the bilayer (Lehtonen and Kinnunen, 1994). However, this does not decrease I_e/I_m for bis-PDPC as would be expected if chain reversal were taking place. Instead, an increase in I_e/I_m for this probe is measured, in keeping with diminished membrane free volume and location of the pyrene moieties in the membrane interior, i.e., absence of chain reversal (Lehtonen and Kinnunen, 1994).

The third possibility is the extended conformation, in which the acyl chains of the lipid are spread apart from each other so that the time-averaged angle between them may maximally approach 180° (Kinnunen, 1992). During membrane fusion the acyl chains have been suggested to become embedded into the opposing leaflets of the bilayers of contacting vesicles whereas the lipid headgroup should remain in the interface. More specifically, due to the fact that the hydration layer of the membrane is absent at the contact site of the adhering liposomes there should be a very low energy barrier for chain extension, resulting in the intercalation of one of the phospholipid alkyl chains into the opposing bilayer. In keeping with the requirement of the extended lipid conformation for fusion, an inability to fuse has been recently shown for pure bolaform lipids, which

have polar headgroups at both ends of the same molecule (Relini et al., 1994). However, the presence of small amounts of monopolar lipids was sufficient to allow fusion by Ca^{2+} or PEG (Relini et al., 1994, 1996). Notably, Ca^{2+} and PEG did induce lipid mixing between liposomes composed of the bolaform lipids (Relini et al., 1994) when measured with lipid probes with the normal two-chain configuration for which the extended conformation is possible.

A contact site is necessary for the decrease in I_e/I_m for bis-PDPC upon fusion as well as hemifusion. More specifically, dehydration of the surface of DOPC vesicles by PEG at concentrations not causing their fusion increases I_e/I_m for bis-PDPC (Lehtonen and Kinnunen, 1994), in contrast to the conditions where fusion or hemifusion are observed, as reported here. At $X_{DOPA} = 0.50$ the reduction in I_e/I_m for bis-PDPC induced by millimolar Ca^{2+} and Mg^{2+} is ~10%. Although part of the probe could be partitioned into PA-enriched domains and adopt the extended conformation at the contact sites it is likely that some of the bis-PDPCs also reside in the membrane domains enriched in PC. Due to generally augmented lipid packing (revealed by DPH) I_e/I_m for bis-PDPC should actually be increased by the divalent cations. Not excluding the partitioning of the probe into the Ca^{2+}-DOPA domains it is also possible that bis-PDPC favors localization in the interface between regions enriched in PC and PA. Upon increasing X_{DOPA} from 0 to 1.0 the relative values for intramolecular I_e/I_m for bis-PDPC decrease from 1.0 to 0.70 and 0.74 for 5 mM Ca^{2+} and Mg^{2+}, respectively. These data indicate that the number of bis-PDPC molecules in the extended conformation would increase upon increasing the extent of vesicle aggregation, and thus the fraction of the surface constituted by Me^{2+}-DOPA-enriched domains (Leventis et al., 1986). Interestingly, compared with bis-PDPC the decrease in I_e/I_m for PDPTPC induced by Ca^{2+} is ~3-fold whereas for Mg^{2+} a similar decrement is observed for both probes. In keeping with PDPTPC being more perturbing one may also expect larger free energy gain upon adoption of the extended conformation by this probe. Accordingly, taking into account the more efficient dehydration of PA by Ca^{2+} this difference is readily comprehensible. For reasons remaining unclear at present, subsequent addition of an excess of EDTA (final concentration, 0.1–1 mM) could reverse only ~50% of the decrease in I_e/I_m caused by the divalent cations (data not shown). This effect did depend on the content of PA, however, and for neat egg PA liposomes EDTA could fully reverse the changes in I_e/I_m.

What is the driving force promoting the extended lipid conformation? As repulsion between the headgroups is reduced due to charge neutralization and/or dehydration, the packing pressure within the hydrocarbon interior will, respectively, increase (Cantor, 1997). The lateral pressure within the membrane interior can be first relieved by chain reversal provided that the membrane free volume distribution is such that there is allocating space in the interfacial region. At this point it is relevant also to consider the relationship between membrane fusion and membrane negative spontaneous curvature. The latter concept was introduced by Gruner and co-workers (Gruner, 1985, 1989) and provides a quantitative description of the consequences of the molecular geometries of lipids, forwarded by Israelachvili et al. (1980) who emphasized the (headgroup area)/(projected area of the hydrocarbon chains) in the interface in providing a qualitative approach to the correlation between lipid packing and the formation of distinct three-dimensional structures, i.e., micelles, lamellar, and inverted phases by different lipids. As has been pointed out by us previously, the largest relief in packing within the hydrocarbon region is achieved when the chains extend into the two opposing leaflets forming the contact site between two adhering bilayers (Kinnunen, 1992, 1996b), as in this case the projected interfacial area for the acyl chains is halved. Accordingly, there is a gain in free energy upon lipids adopting the extended conformation at the contact site of adhering vesicles. The concluded changes in the orientation of the lipid acyl chains are schematically illustrated in Fig. 7.

Membrane fusion and lamellar→H_{II} phase transition have been suggested to be linked via common intermediating structures (Kinnunen, 1992; Ellens et al., 1986; Siegel

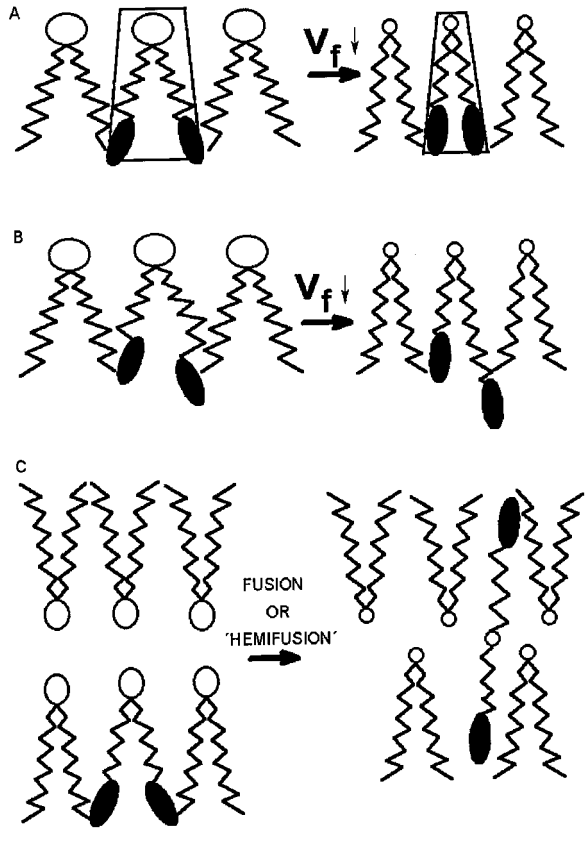

FIGURE 7 A schematic illustration of the changes in the acyl chain alignment for bis-PDPC (*A*) and PDPTPC (*B*) upon decreasing the membrane free volume by dehydration of the polar headgroup by PEG or Me^{2+}. Hemifusion of bilayers and the adoption of the extended conformation by bis-PDPC is depicted in *C*. The pyrene moiety is represented by the filled black ellipse.

and Epand, 1997). The effective shape of inverted hexagonal H_{II}-phase-forming lipids such as diacylglycerols and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) is conical with a smaller headgroup compared with the hydrocarbon part of the molecule, thus resulting in negative spontaneous curvature. This type of lipid also promotes fusion when introduced into bilayers composed of lipids forming lamellar phases (for a review, see Kinnunen, 1996b). In contrast, micelle-forming lipids with positive spontaneous curvature, such as lysophospholipids having a large headgroup relative to the hydrophobic part, inhibit fusion (Chernomordik et al., 1995). Intervesicle contacts are required for liposomes to undergo lamellar-to-hexagonal phase transition (Ellens et al., 1986). The rate of fusion of PEs is highest for bilayers in the poorly understood intermediate phase existing between the L_α and H_{II} phases (Ellens et al., 1989). In this intermediate phase thermal motion increases the effective size of the hydrocarbon region and causes negative spontaneous curvature (Tate et al., 1991). As lamellar arrangement is maintained despite the negative spontaneous curvature the membrane is defined as frustrated (Kinnunen, 1996a,b). To this end, PA complexed with divalent cations undergoes at elevated temperatures transition into the H_{II} phase (Miner and Prestegard, 1984). In keeping with previous studies on similar systems (Farren et al., 1983; Verkleij et al., 1982) these authors presented evidence from ^{31}P-NMR and low-angle x-ray scattering experiments for an unidentified, nonbilayer phase that was not a mixture of lamellar and hexagonal phases as had been suggested previously (Farren et al., 1983). Miner and Prestegard (1984) concluded that the Me^{2+}-PA complexes adopt a fundamentally different phase at intermediate temperatures that somehow resembles but is not identical to the H_{II} phase and proposed this intermediate to be equivalent to that formed during fusion.

Vesicle size has a large effect on membrane fusion, and sonicated PA/PC vesicles undergo fusion more easily than LUVs (Liao and Prestegard, 1979). Compared with LUVs the packing pressure within the hydrocarbon region of the outer monolayer and chain-chain interactions are increased in small unilamellar vesicles (SUVs). Intriguingly, Xu and Cafiso (1986) showed by ^{1}H-NMR dipolar interactions between the terminal methyl group and the polar headgroup in sonicated phosphatidylcholine SUVs (1986). This coupling was suggested to be caused by acyl chain interdigitation or chain reversal for a fraction of lipids in the vesicles. Interestingly, decreasing vesicle size increases lipid mixing between vesicles (Leventis et al., 1986). To this end, feasibility of the extended lipid conformation in the mechanism of lipid exchange between liposomes has been suggested (Kinnunen, 1996b).

To summarize, similar to the 1) introduction of lipids with negative spontaneous curvature into lamellar phases, 2) dehydration, 3) decrease in vesicle size (i.e., positive curvature of the outer monolayer), and 4) acyl chain unsaturation (Yang et al., 1997), all enhance chain-chain interactions and promote fusion, providing that the vesicles are adhering, i.e., there is no barrier for contact caused by hydration, electrostatic repulsion, or undulation. Importantly, all these factors are also anticipated to promote chain reversal (thus increasing the hydrophobicity of the liposome surface) as well as ultimately the adoption of the extended conformation. Increased surface hydrophobicity, i.e., exposure of the hydrophobic regions by stressing the membranes has been concluded to be essential for membrane adhesion and fusion (Arnold, 1995; Düzgünes et al., 1987; Helm et al., 1992; Leikin et al., 1987; Rand and Parsegian, 1986; Ohki and Arnold, 1990; Ohki and Düzgünes, 1979; Portis et al., 1979;) whereas even strong van der Waals or electrostatic forces alone are not sufficient (Helm et al., 1992; Marra, 1986a,b). This has been suggested to result from the latter forces acting on the headgroup region and not on the hydrophobic parts of the membrane. A fusogenic state of the plasma membrane having a lifetime of seconds to minutes has been demonstrated after exposure of cells to a high-voltage pulse used to trigger electrofusion. Reorganization in the headgroup region of the cell membrane concomitant with an increase in hydrophobicity of the surface was observed (reviewed in Dimitrov, 1995).

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