Surface Charge Markedly Attenuates the Nonlamellar Phase-Forming Propensities of Lipid Bilayer Membranes: Calorimetric and ^{31}P-Nuclear Magnetic Resonance Studies of Mixtures of Cationic, Anionic, and Zwitterionic Lipids

Ruthven N. A. H. Lewis and Ronald N. McElhaney
Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

ABSTRACT The lamellar/nonlamellar phase preferences of lipid model membranes composed of mixtures of several cationic lipids with various zwitterionic and anionic phospholipids were examined by a combination of differential scanning calorimetry and ^{31}P NMR spectroscopy. All of the cationic lipids utilized in this study form only lamellar phases in isolation. Mixtures of these cationic lipids with zwitterionic strongly lamellar phase-preferring lipids such as phosphatidylcholine form only the lamellar liquid-crystalline phase even at high temperatures, as expected. Moreover, mixtures of these cationic lipids with strongly nonlamellar phase-preferring zwitterionic lipids such as phosphatidylethanolamine exhibit a markedly reduced propensity to form inverted nonlamellar phases, again as expected. However, when mixed with anionic lipids such as phosphatidylserine, phosphatidylglycerol, cardiolipin, or phosphatidic acid, a marked enhancement of nonlamellar phase-forming propensity occurs, despite the fact both components of the mixture are nominally lamellar phase-preferring. An examination of the lamellar/nonlamellar phase transition temperatures and the nature of the nonlamellar phases formed, as a function of temperature and of the composition of the mixture, indicates that the propensity to form inverted nonlamellar phases is maximal in mixtures where the mean surface charge of the membrane surface approaches neutrality and decreases markedly with increases in the density of positive or negative charge at the membrane surface. Moreover, the onset temperatures of the reversed hexagonal phase rise more steeply than do those of the inverted cubic phase as the ratio of cationic and anionic lipids is varied, suggesting that the formation of inverted hexagonal phases is more sensitive to this surface charge effect. These results indicate that surface charge per se is a significant and effective modulator of the lamellar/nonlamellar phase preferences of membrane lipids and that charged group interactions at membrane surfaces may have a major role in regulating this particular membrane property.

INTRODUCTION

The complex mixture of lipids present in eukaryotic cell membranes typically forms only a liquid-crystalline lamellar (L_α) phase under physiologically relevant conditions (see Gennis, 1989; Lewis et al., 1997). However, the individual lipid classes present in such membranes can form either lamellar or nonlamellar lipid phases when dispersed in excess water (see Rilfors et al., 1984; Lewis et al., 1997; Thurmond and Lindblom, 1997). Specifically, although the zwitterionic phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) prefer the lamellar phase, as do the anionic phospholipids phosphatidylserine (PS), phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidic acid (PA), the zwitterionic phospholipid phosphatidylethanolamine (PE) prefers the inverted hexagonal (H_{II}) phase, while the complex glycosphingolipids (gangliosides) prefer the normal micellar phase at neutral pH and physiological ionic strength. A considerable body of evidence has now accumulated that indicates that these nonlamellar phase-preferring lipid components, as well as the various lamellar phase-preferring lipids, perform important structural and functional roles in eukaryotic membranes (for a review, see Hui, 1997).

The phase that a fully hydrated membrane lipid prefers under a given set of conditions can be rationalized by considering the geometric packing of lipid molecules in various aggregates, which can in turn be described by a packing parameter or shape factor characteristic of the lipid molecule under these conditions (see Cevc and Marsh, 1987; Israelachvili, 1992). For a series of different phospholipids having the same number and type of hydrocarbon chains, this parameter is in turn determined primarily by the optimal area occupied by the polar headgroup at the lipid-water interface. If the conformations of the various phospholipid polar headgroups are generally similar, the optimal area occupied by the polar headgroups should be approximately proportional to headgroup size (Lee et al., 1993; Foht et al., 1995). However, second-order but nevertheless potentially important interactions can also effect the optimal headgroup area and thus the effective shape of the lipid molecule, including electrostatic interactions between adjacent polar headgroups. These electrostatic interactions can potentially be attractive in the case of zwitterionic phospholipids but should always be repulsive in the case of anionic lipids. These repulsive electrostatic interactions presumably account for the fact that anionic phospholipids with relatively small polar headgroups, such as PA, CL, and PG,

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Address reprint requests to Dr. Ronald N. McElhaney, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-2413; Fax: 780-492-0095; E-mail: rmcelhan@gpu.srv.ualberta.ca.

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which might otherwise assume inverted conical shapes at higher temperature and form inverted cubic or hexagonal phases, nevertheless prefer the lamellar liquid-crystalline phase at neutral pH and low salt concentration in the absence of divalent metal cations (see Rilfors et al., 1984; Lewis et al., 1997; Thurmond and Lindblom, 1997).

Although fully hydrated anionic phospholipid dispersions form only lamellar phases at neutral pH and physiological ionic strength, various workers have shown that PS (Eins, 1972; Hope and Cullis, 1980; de Kroon et al., 1990), PG (Cullis and de Kruijff, 1976; Harlos and Eibl, 1980), CL (Rand and Sengupta, 1972; Cullis et al., 1978; Rainier et al., 1979; van Venetie and Verkleij, 1981; Seddon et al., 1983b; Powell and Marsh, 1985; Sankaram et al., 1989), and PA (Harlos and Eibl, 1981; Verkleij et al., 1982; Farren et al., 1983; Miner and Prestegard, 1984) can form inverted cubic or hexagonal phases at low pH or in the presence of high salt concentrations or divalent metal cations such as Ca^{2+}. These observations have often been rationalized by invoking electrostatic effects, particularly the neutralization or shielding of the repulsive negative charges present on the anionic phospholipid headgroup at neutral pH or in the absence of Ca^{2+} or at high salt concentrations, respectively, which would favor the formation of the H_{II} over the L_α phase, because of the greater electrostatic repulsion in the more tightly packed headgroup in the inverted phase (Seddon et al., 1984). However, Seddon et al. (1983a,b) and others (Rand and Sengupta, 1972; Cevc and Marsh, 1987) have pointed out that electrostatic effects may not be the most important interactions in this regard. For example, in a dialkyl PE, the L_α/H_{II} phase transition temperature decreases at low pH, despite the fact that the PE polar headgroup, which is zwitterionic at neutral pH, becomes positively charged at low pH (Seddon et al., 1983a). It has therefore been suggested that variations in pH, salt concentration, and Ca^{2+} concentration may also affect the lamellar/hexagonal phase equilibria by altering polar headgroup hydration, headgroup-water interactions, and the strength of hydrogen bonding between adjacent polar headgroups on the surface of the lipid aggregates (Eibl and Wooley, 1979; Boggs et al., 1981), and that these effects may dominate the electrostatic effects in some circumstances. In fact, theoretical considerations suggest that changes in the nature of the electrostatic interactions between polar headgroups will have only a relatively weak effect on the relative stabilities of the L_α and H_{II} phases (Cevc and Marsh, 1987). However, as the determination of the L_α/H_{II} phase transition temperature of the dialkyl PE as a function of pH was performed in 2.5 M NaCl (Seddon et al., 1983a), electrostatic effects would be markedly diminished at any rate. Therefore, the relative importance of electrostatic interactions in determining the lamellar/inverted nonlamellar phase equilibria of individual phospholipids is not completely clear at present.

We adopt a different approach to investigating the effect of the surface charge on the lamellar/nonlamellar phase equilibrium of the various phospholipids in the present study. Instead of varying the effective surface charge by changing the charge on the phospholipid polar headgroup by variations in pH, ionic strength, or the addition of divalent cations, we do so by the addition of a cationic phospholipid or lipid analog to the major zwitterionic and anionic phospholipid components of eukaryotic membranes. In this way we hope to minimize the changes in the hydration and hydrogen-bonding ability of the polar headgroup that accompany alterations in pH and ionic strength, and to avoid the precipitation of essentially dehydrated lamellar crystalline phases that can accompany the addition of Ca^{2+} to anionic phospholipids. Using this approach, we show that the addition of double-chained cationic lipids inhibits inverted phase formation when added to zwitterionic phospholipids capable of forming such phases but markedly potentiates inverted cubic and hexagonal phase formation when added to anionic phospholipids. In fact, we demonstrate that mixtures of nominally lamellar phase-preferring cationic and anionic lipids can readily form inverted nonlamellar phases. These results suggest that lipid aggregate surface charge per se can be a major determinant of lamellar/nonlamellar phase equilibrium and thus that electrostatic interactions can have important effects on phospholipid phase preference.

The cationic phospholipid that we use here, *P-O*-ethyldioleoylphosphatidylcholine (Et-DOPC), retains the relatively large polar headgroup and presumably a generally similar extended polar headgroup conformation at the lipid aggregate surface of the natural zwitterionic eukaryotic membrane lipid PC. However, the negative charge on the phosphate group normally present in PC at neutral pH has been removed, and the addition of the alkyl group to the phosphate oxygen should also reduce or abolish intermolecular hydrogen bonding (MacDonald et al., 1999). The other three synthetic cationic lipids used, 1,2-dimyristoyloxy-3-*N,N,N*-trimethylaminopropane (DM-TAP), 1,2-dioleoyloxy-3-*N,N,N*-trimethylaminopropane (DO-TAP), and 1,2-dioleoyloxy-3-*N,N*-dimethylaminopropane (DO-DAP), lack the phosphate moiety of natural phospholipids and have much smaller cationic polar headgroups that are analogs of the choline moiety of PC. Moreover, these small cationic headgroups are tethered closely to the surface of the lipid aggregate and should be rather conformationally restricted. Furthermore, the cationic polar headgroup of DO-DAP is somewhat smaller than that of DM-TAP or DO-TAP and, unlike the latter, has the capability of acting as a hydrogen bond donor. These cationic lipid analogs and related compounds have been used extensively to deliver DNA, RNA, and various proteins into eukaryotic cells (see references in MacDonald et al., 1999).

MATERIALS AND METHODS

The phospholipids and cationic amphiphiles used in this study were obtained from Avanti Polar Lipids (Alabaster, AL) and were used without

further purification. Sample preparation for the differential scanning calorimetry (DSC) experiments was as follows. Stock solutions of the phospholipids and cationic lipids in chloroform/methanol (2:1) were mixed in the required ratios to produce samples containing 4–5 mg of total lipid. The solvent was removed with a stream of nitrogen while the temperature of the sample was kept near 50°C to prevent any fractional crystallization that might occur upon cooling. The last traces of solvent were removed in vacuo overnight. Subsequently, the lipid samples were warmed to temperatures above the hydrocarbon chain-melting phase transition temperature (T_m) and hydrated by the addition of 0.6 ml of a preheated sample of a buffer containing 50 mM Tris, 100 mM NaCl, and 1 mM EDTA (pH 7.4). Next, the sample was dispersed by vigorous vortexing (or by gentle bath sonication in some cases) at temperatures above the expected T_m, and a 0.5-ml aliquot was introduced into the Hastelloy capsules for DSC analysis. DSC thermograms were recorded with a high-sensitivity multicell scanning calorimeter (Calorimetry Sciences Corporation, Provo, UT) operating at heating and cooling rates of 10°C/h. The data obtained were analyzed and plotted with the Origin software package (Microcal Software, Northampton, MA).

For ^{31}P NMR spectroscopy, samples containing 10–15 mg of phospholipid were prepared and dried by methods similar to those applied to the DSC sample. The dried sample obtained was warmed to temperatures above the lipid T_m and hydrated with 0.6 ml of preheated buffer, as described for the DSC sample, and dispersed by gentle bath sonication at elevated temperatures. Subsequently the sample was introduced into a 10-mm NMR tube and equilibrated by three cycles of freezing to −80°C and rewarming. Finally, the sample was recooled to −80°C and rewarmed to temperatures near 0–4°C before the start of the NMR experiment. The above procedure was applied to ensure that all samples were cooled to gel-phase temperatures before the start of the NMR experiment. This step was required to ensure that cubic phases formed during the sample hydration and sample dispersal process were completely converted to the lamellar phase before the start of the experiment. NMR spectra were recorded with a Varian Unity 300 wide-bore spectrometer operating at a frequency of 121.41 MHz for ^{31}P. Data were acquired by single pulse acquisition techniques, with other data acquisition parameters similar to those previously used in this laboratory (Lewis et al., 1988). The data obtained were processed as described previously (Lewis et al., 1988) and plotted with the Origin software package.

At the completion of the DSC and NMR experiments, the samples were analyzed by thin-layer chromatography to check for possible chemical degradation due to exposure to high temperatures. However, no such degradation was detectable.

RESULTS

Illustrated in Fig. 1 is a series of ^{31}P NMR spectra showing the temperature-dependent changes in the shape of the ^{31}P NMR powder pattern exhibited by an equimolar mixture of 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) and the cationic lipid DO-TAP. At temperatures up to 10°C, the mixture exhibits ^{31}P NMR powder patterns characteristic of liquid-crystalline lipid lamellae in which motion of the phosphate headgroup is fast and symmetrical about the axis of reorientation, the bilayer normal. Upon heating to temperatures near 25°C, the overall shape of the spectra changes significantly, now appearing as a superposition of components with peaks centered near 8 ppm downfield, 2 ppm downfield, and 13 ppm upfield (see Fig. 1). These peaks coincide with the resonance maxima normally exhibited by phospholipid H_{II}, inverted cubic, and lamellar phases, respectively (Tilcock, 1986; Tilcock et al., 1986),

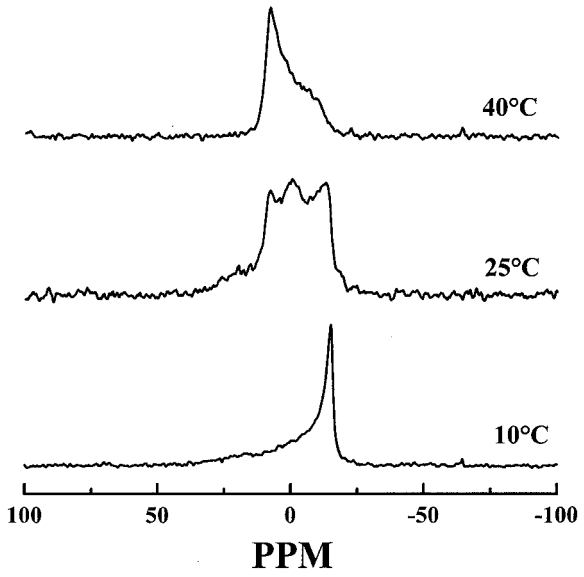

FIGURE 1 Proton-decoupled ^{31}P NMR spectra of aqueous dispersions of an equimolar mixture of POPG and DO-TAP. Spectra were acquired in the heating mode at the temperatures indicated.

suggesting that all three phospholipid phases coexist at temperatures near 25°C. Upon heating to temperatures above 40°C, the resonance maxima attributable to the lamellar and cubic phases disappear completely and only powder patterns characteristic of the H_{II} phase are observed (see Fig. 1). This result provides strong evidence that equimolar mixtures of POPG and DO-TAP form inverted nonlamellar phases under conditions of physiologically relevant pH, hydration, and ionic strength, a surprising observation given that hydrated dispersions composed of pure POPG or pure DO-TAP form only lamellar liquid-crystalline phases under comparable experimental conditions (Tilcock, 1986; Koltover et al., 1998, 1999). The demonstration that inverted nonlamellar phases can be formed by interactions between two nominally lamellar phase-preferring double-chained lipids of opposite charge is novel, and the phenomenon was further investigated by an examination of temperature-dependent changes in the shapes of the ^{31}P NMR spectra exhibited by mixtures of DO-TAP with a range of other phospholipid classes, and by mixtures of other cationic amphiphiles with various phospholipids.

^{31}P NMR powder patterns exhibited by equimolar mixtures of DO-TAP and dioleoylphosphatidylethanolamine (DOPE), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), or 1-palmitoyl-2-oleoylphosphatidic acid (POPA) are illustrated in Fig. 2. The spectra shown reveal a number of interesting points. First, equimolar mixtures of DO-TAP with POPC (a zwitterionic, strongly bilayer phase-preferring phospholipid) exhibit ^{31}P NMR spectra consistent with the retention of lamellar phases at all temperatures examined. Second, equimolar mixtures of DO-TAP with DOPE

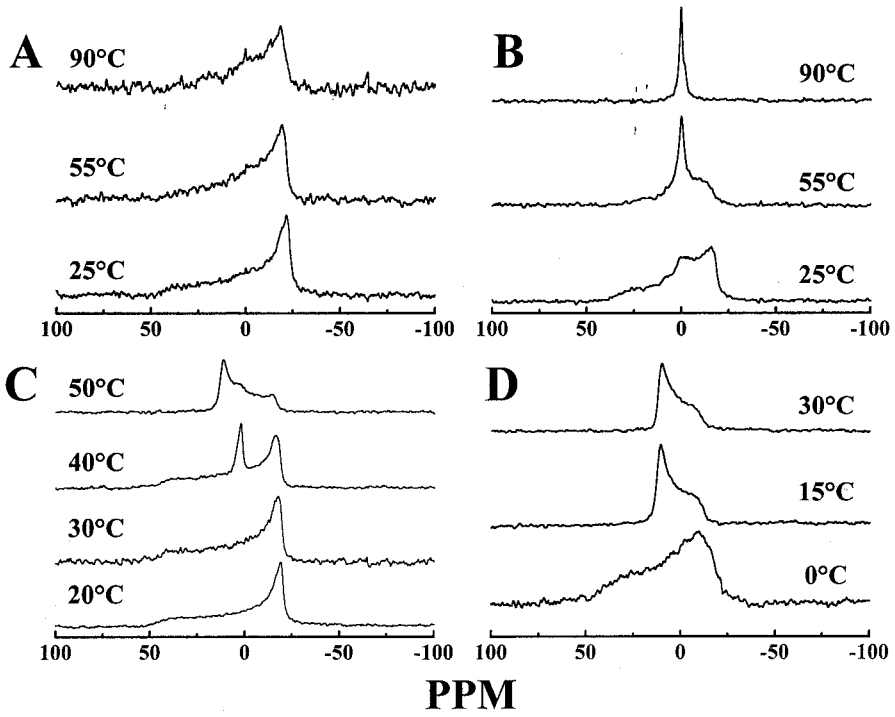

FIGURE 2 Proton-decoupled ^{31}P NMR spectra of aqueous dispersions of mixtures of DO-TAP with various phospholipids. Data are presented for POPC:DO-TAP (1:1) (*A*); DOPE:DO-TAP (1:1) (*B*); POPS:DO-TAP (1:1) (*C*); POPA:DO-TAP (1:1) (*D*). Spectra were acquired in the heating mode at the temperatures indicated.

(a zwitterionic, strongly H_{II} phase-preferring phospholipid) exhibit ^{31}P NMR powder patterns consistent with their being predominantly lamellar at temperatures up to 25°C. However, upon heating to temperatures near 55°C, the relative intensity of the lamellar phase signal decreases and is replaced by a spectral component whose resonance maximum is close to the so-called isotropic frequency. Upon further heating, this latter component progressively increases in intensity at the expense of the lamellar phase component and is essentially the only component existing at temperatures near 90°C. There was no evidence that the DOPE:DO-TAP mixture formed H_{II} phases under these experimental conditions. Given that pure DOPE exhibits its L_α/H_{II} phase transition at temperatures near 10°C (see Lewis et al., 1997), it is evident that the interactions of DO-TAP with DOPE markedly decrease the nonlamellar phase-forming propensity of DOPE, in marked contrast to what is observed when DO-TAP interacts with POPG. Third, at high temperatures, equimolar mixtures of DO-TAP with POPA or with POPS (nominally lamellar phase-preferring anionic phospholipids) both exhibit ^{31}P NMR spectra consistent with the formation of H_{II} phases. Specifically, at temperatures near 0°C, POPA:DO-TAP mixtures exhibit ^{31}P NMR powder patterns with basal linewidths near 90 ppm, characteristic of lamellar gel (L_β) phases. However, upon warming to temperatures above 10°C, these powder patterns narrow significantly and assume the shape associated with the H_{II} phase. Moreover, the L_α phase of the POPA:DO-TAP mixture apparently converts directly to the H_{II} phase upon heating, suggesting that its L_α phase is unstable with respect to the H_{II} phase at all temperatures above the T_m. Finally, equimolar mixtures of DO-TAP with POPS exhibit axially symmetrical ^{31}P NMR powder patterns consistent with the existence of the L_α phase at temperatures between 0° and 20°C. However, upon further heating, a small isotropic component appears, but the overall shape of the powder pattern remains consistent with the system being predominantly lamellar under those conditions. Moreover, at temperatures above 50°C, the ^{31}P NMR spectroscopic signatures of lamellar and cubic phase components disappear completely and are replaced by powder patterns consistent with the formation of the H_{II} phase. These results indicate that nonlamellar phases can be formed when cationic lipids such as DO-TAP interact with a wide range of anionic lipids, and that the tendency of such cationic lipids to induce nonlamellar phase formation upon interaction with anionic phospholipids varies with the structure of the anionic phospholipid polar headgroup. From the variation in the temperature ranges over which equimolar DO-TAP mixtures with POPG, POPA, and POPS form inverted nonlamellar phases (Figs. 1 and 2, respectively), and from the results of similar experiments with mixtures of DO-TAP with dioleoylphosphatidic acid (DOPA), tetraoleoylcardiolipin, and dioleoylphosphatidylglycerol (DOPG) (data not presented), the propensities of these anionic lipids for forming such phases as surface charge density is reduced decrease in the order PA > CL > PG > PS.

To investigate the effect of variations in the structure of the cationic lipid component on its ability to induce inverted nonlamellar phases in anionic phospholipids, we studied the

effect of three different cationic lipids on the lamellar/nonlamellar phase preference of a single anionic phospholipid. Illustrated in Fig. 3 are the temperature-dependent changes in the ^{31}P NMR spectra exhibited by equimolar mixtures of POPA with the cationic lipids DO-TAP, Et-DOPC, and DO-DAP. It is clear that there are significant differences between the capacities of the various cationic amphiphiles for inducing nonlamellar phase formation upon interaction with this anionic phospholipid. With the DO-TAP:POPA mixture, the H_{II} phase is observed at all temperatures above the T_m. With the Et-DOPC:POPA mixture, predominantly L_α phases are observed at temperatures between 0° and 20°C and H_{II} phases at temperatures near and above 30°C. Finally, the ^{31}P NMR spectra exhibited by the DO-DAP:POPA mixture suggest that cubic phases predominate over a fairly wide temperature range, and there was no evidence that either lamellar or H_{II} phases were formed within the temperature range accessible to the NMR spectrometer (0–90°C). It is clear from these results that the formation of nonlamellar phases upon interaction with anionic phospholipids is not unique to molecules like DO-TAP, because nonlamellar phases are also formed when structurally different cationic amphiphiles interact with anionic phospholipids, although to varying extents. The possibility that these experimental observations are a manifestation of a more fundamental property of lipid membranes was examined in the experiments described below.

The data presented above indicate that equimolar mixtures of DO-TAP with zwitterionic POPC bilayers exhibit no discernible enhancement in their propensity to form an inverted nonlamellar phase, whereas equimolar mixtures of the same cationic lipid with the zwitterionic, strongly nonlamellar phase-preferring lipid DOPE exhibit a substantial attenuation of the capacity to form an inverted nonlamellar phase. However, equimolar mixtures of DO-TAP and the nominally lamellar phase-preferring anionic lipids exhibit a marked increase in the propensity to form inverted nonlamellar phases, despite the fact that both components of the mixture are nominally lamellar phase-preferring under the conditions examined. Although the above results are those expected because of electrostatic considerations, the possibility that we were actually observing a membrane surface charge-related effect was investigated further by an examination of how the nonlamellar phase-forming propensities of mixtures of anionic and cationic lipids are affected by the ratio of cationic and anionic lipids in the binary mixture.

The left side of Fig. 4 shows a series of DSC heating thermograms exhibited by binary mixtures of dimyristoylphosphatidic acid (DMPA) and DM-TAP. Each of these mixtures exhibits a major endothermic transition arising from its gel/liquid-crystalline phase transition and one or more weakly energetic endothermic events at temperatures above its gel/liquid-crystalline phase transition. These latter endothermic events are attributable to the formation of one or more inverted nonlamellar phases (see ^{31}P NMR data presented below). The DSC thermograms also show that the midpoint temperature of these higher temperature endothermic transitions approaches a minimum with mixtures containing equimolar proportions of the anionic and cationic lipids and rises sharply as the content of either the anionic or the cationic component increases. Given that any significant imbalance between the molar amounts of cationic and anionic lipids present will result in a build-up of surface charge, this demonstration that the lamellar/nonlamellar phase transition temperature approaches a minimum as the net surface charge of the membrane approaches neutrality

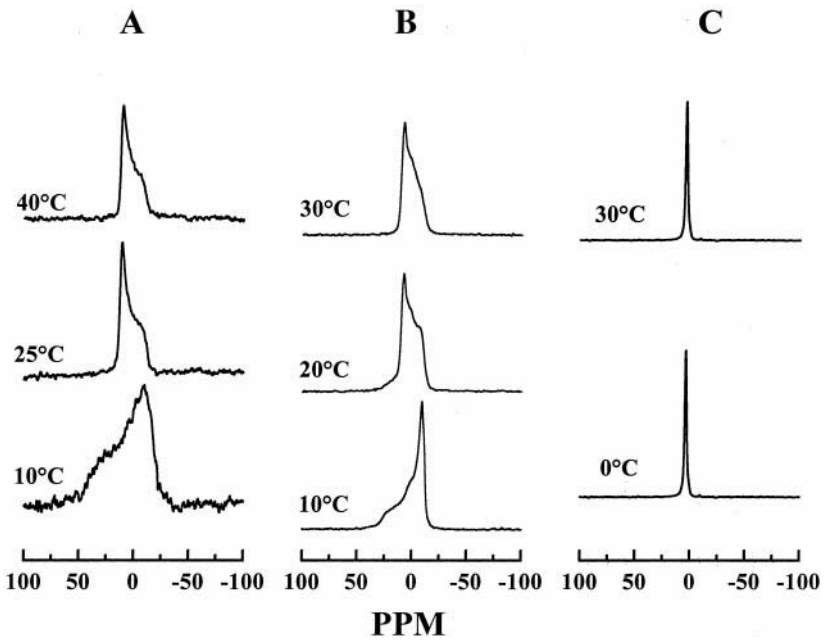

FIGURE 3 Proton-decoupled ^{31}P NMR spectra of aqueous dispersions of POPA with various cationic lipids. Data are presented for POPA: DO-TAP (1:1) (*A*); POPA: Et-DOPC (1:1) (*B*); POPA: DO-DAP (1:1) (*C*). Spectra were acquired in the heating mode at the temperatures indicated.

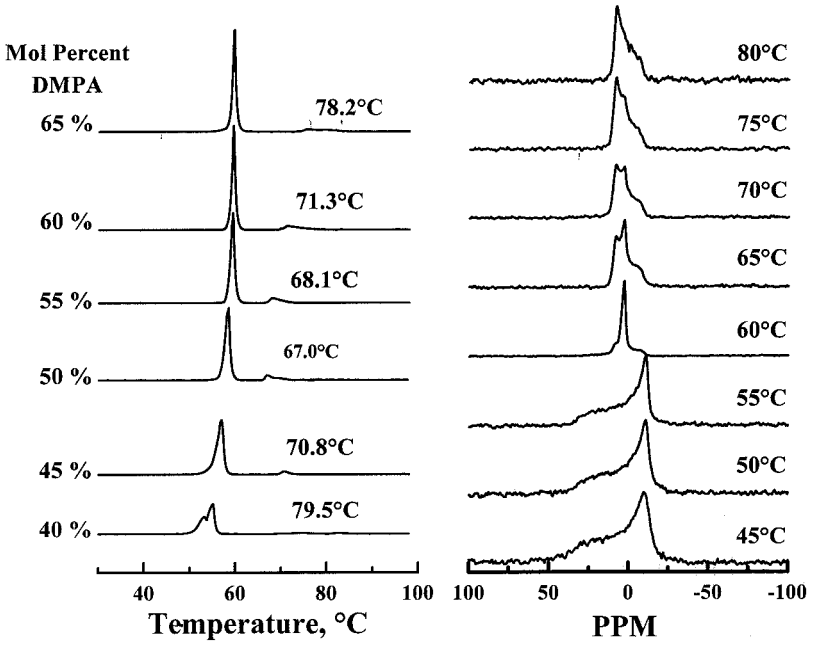

FIGURE 4 Effect of lipid composition on the phase behavior and phase preferences of mixtures of DMPA with DM-TAP. (*Left*) Series of DSC thermograms exhibited by binary mixtures of DMPA and DM-TAP. The mole fraction of DMPA ranges from 40 mol% (*bottom*) to 65 mol% (*top*) in 5-mol% increments. The temperatures indicate the estimated midpoint temperatures of the observed lamellar/nonlamellar transition endotherms. (*Right*) Representative set of ^{31}P NMR spectra of one of these mixtures (55 mol% DMPA). Spectra were acquired in the heating mode at the temperatures indicated.

provides strong evidence that membrane surface charge is an important determinant of the nonlamellar phase-forming propensity of these lipid model membranes.

An examination of the ^{31}P NMR spectra exhibited by these DM-TAP/DMPA mixtures (for an example, see Fig. 4, *right*) provides further insight into the relationship between membrane lamellar/nonlamellar phase preference and membrane surface charge. With all of the lipid mixtures examined, ^{31}P NMR spectra consistent with the existence of lamellar lipid phases are observed at the onset and completion temperatures of the higher-enthalpy, lower-temperature phase transitions observed by calorimetry. Upon heating to temperatures near the onset of the weakly energetic higher-temperature transitions, the coexistence of an isotropic component and the axially symmetrical lamellar phase powder pattern is observed, and the relative intensity of the isotropic component grows progressively at the expense of the axially symmetrical component as the temperature increases. Interestingly, with mixtures containing 45–55 mol% DMPA, further heating eventually results in the complete disappearance of the axially symmetrical and isotropic NMR signals and their replacement with powder patterns characteristic of the H_{II} phase. With the other lipid compositions shown, further heating also results in the complete disappearance of the axially symmetrical component. However, with these mixtures, the disappearance of the lamellar phase is concomitant with the appearance of spectral components attributable to both isotropic and H_{II} phases, the relative proportions of which appear to be determined by the temperature and composition of the mixture. These data indicate that the isotropic component tends to predominate at lower temperatures and at relatively higher surface charge densities, whereas the H_{II} phase tends to predominate at higher temperatures and at relatively lower surface charge densities. This effect is vividly illustrated by the partial temperature-composition phase diagram constructed from our ^{31}P NMR spectroscopic observations (see Fig. 5). The data clearly show that the onset temperatures of both nonlamellar phases rise sharply as the surface charge of the membrane deviates from near neutrality, but that the lower boundary temperatures of the H_{II} phases curve more sharply upward as the membrane surface charge increases. This suggests that the formation of the H_{II} phase is more sensitive to the putative effects of membrane surface charge than is the formation of the inverted cubic phase. Interestingly, mixtures of DM-TAP with tetramyristoyl cardiolipin (TMCL) exhibit similar behavior (see below), although the minimum temperatures for the onset of the inverted nonlamellar phase are slightly higher than for mixtures with DMPA, while mixtures of DM-TAP with dimyristoylphosphatidic acid and dimyristoylphosphatidylserine do not form inverted nonlamellar phases below 90°C (data not presented).

To further evaluate the correlation between membrane surface charge density and the various phenomena described above, the lamellar/nonlamellar phase-forming properties of mixtures of DM-TAP and TMCL were also investigated. The rationale for this experiment is that because TMCL is a doubly negatively charged molecule, near-neutral membranes would be formed by mixtures composed of two parts DM-TAP and one part TMCL. Thus, if the neutralization of surface charge is driving the phenomena described above, the observed nonlamellar phase-forming propensity should be at maximum with binary DM-TAP:TMCL mixtures containing 33 mol% TMCL and should decline sharply as the lipid composition deviates from this value. Fig. 6 shows the DSC heating thermograms

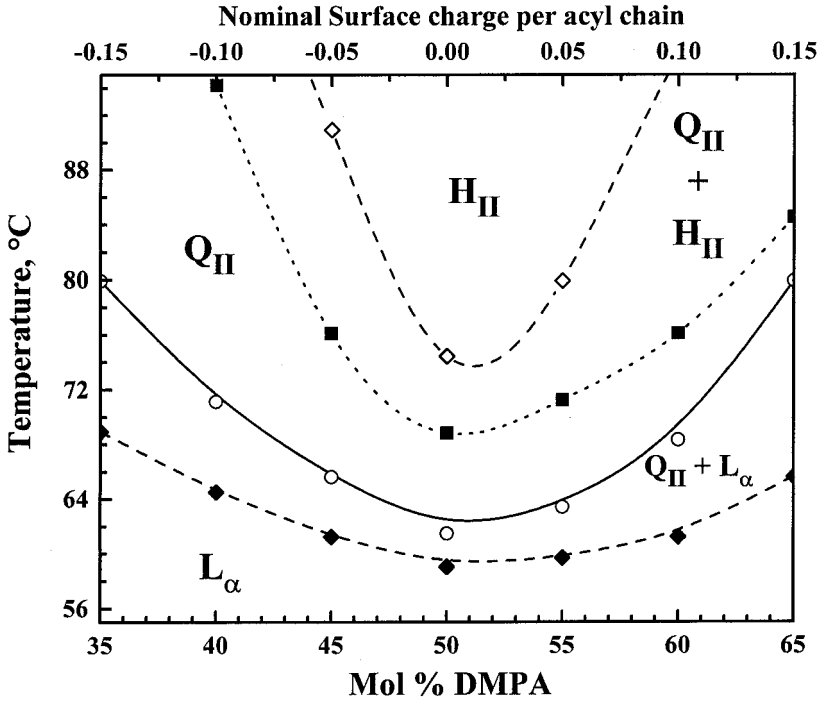

FIGURE 5 Pseudo phase diagram showing the effect of membrane composition on the lamellar/nonlamellar phase boundaries observed with of DMPA:DM-TAP mixtures. The diagram was constructed predominantly from ^{31}P NMR spectroscopic data. Phase boundaries illustrated: – –◆– –, onset temperature of the Q_{II} (cubic) phase; —○—, upper boundary of the lamellar liquid-crystalline phase; ···■···, onset temperature of the H_{II} phase; – –◇– –, upper boundary of the Q_{II} (cubic) phase.

exhibited by various mixtures of TMCL with DM-TAP. As observed with the DMPA:DM-TAP mixtures, these DM-TAP:TMCL mixtures each exhibit a highly energetic endotherm arising from a lamellar gel/liquid-crystalline phase transition and one or more weaker endothermic events at temperatures above the T_m. Moreover, as was the case with the DMPA:DM-TAP mixtures, the weakly energetic higher-temperature endothermic events shown in Fig. 6 have been correlated with ^{31}P NMR spectroscopic changes, indicating the formation of inverted nonlamellar phases (data not shown). However, in this case the estimated midpoint temperatures of the lamellar/nonlamellar transition endotherms are lowest with mixtures containing 33 mol % cardiolipin and rise sharply as the composition of the mixture deviates from this value. This observation is precisely what would be expected if the observed enhancement in the nonlamellar phase-preferring propensity were driven by the neutralization of the surface charge of the membrane.

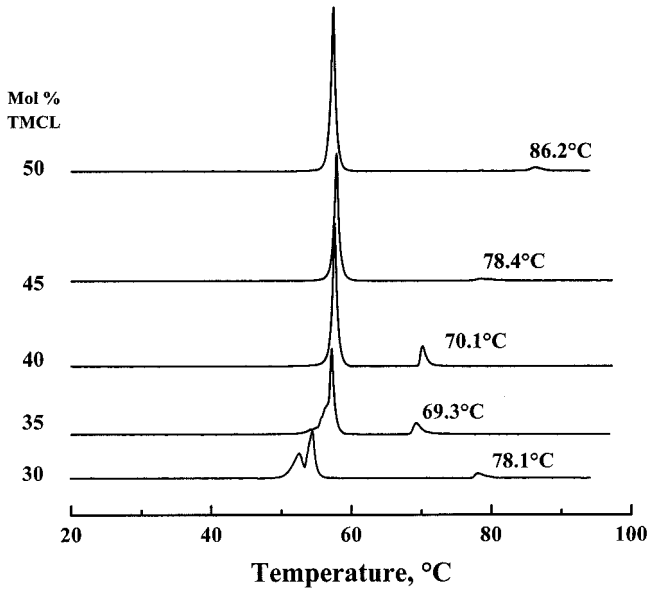

FIGURE 6 DSC thermograms exhibited by binary mixtures of DM-TAP and tetramyristoyl cardiolipin. The mole fraction of TMCL ranges from 30 mol% (*bottom*) to 50 mol% (*top*) in 5-mol% increments. The temperatures indicate the estimated midpoint temperatures of the observed lamellar/nonlamellar transition endotherms.

DISCUSSION

We have shown for the first time that when mixed in appropriate proportions, binary mixtures of double-chained cationic lipids and anionic lipids can form inverted nonlamellar phases, despite the fact that neither component of the mixture exhibits any discernible nonlamellar phase-forming propensity under the conditions examined. We also show that the propensity of such mixtures to form inverted nonlamellar phases is at maximum when the net surface charge of the membrane approaches neutrality and declines markedly as the mean surface concentration of either positive or negative charges increases. We also demonstrate that the incorporation of cationic lipids does not induce nonlamellar phase formation in zwitterionic bilayer phase-preferring phospholipids and markedly attenuates the formation of such phases when these lipids are mixed with zwitterionic nonlamellar-preferring phospholipids. Our experimental ob-

servations thus present a consistent picture in which the accumulation of positive or negative charges at a lipid bilayer surface markedly diminishes its propensity to form an inverted nonlamellar phase.

It is interesting to compare the relative tendencies of the four anionic phospholipids studied here to form inverted cubic or hexagonal phases when they are mixed with equimolar amounts of the cationic lipids DO-TAP or DM-TAP. Based on their relative lamellar/nonlamellar phase transition temperatures and the type of inverted nonlamellar phase formed (cubic or hexagonal), the tendency of these anionic phospholipids to form nonlamellar phases decreases in the order PA > CL > PG > PS. Because this order is also the order of increasing phospholipid headgroup size when expressed on a per mole of hydrocarbon chain basis (Lee et al., 1993; Foht et al., 1995), we can rationalize these results by recourse to the relative packing geometries or molecular shapes of these anionic phospholipids (see Cevc and Marsh, 1987; Israelachvili, 1992). However, it is likely that other factors, such as the conformation and hydration of the polar headgroups and the strength of hydrogen bonding between them, also play a role here.

The relative order of increasing tendency to form inverted phases observed here is compatible with the earlier results of Lindblom et al. (1991), who showed that aqueous dispersions of DOPA have a greater tendency to form the H_{II} phase at lower water contents and temperatures than do similar dispersions of bovine heart CL, and that DOPG and DOPS do not form reversed phases at water contents of 10–95 wt % and temperatures up to 55°C in the absence of NaCl. However, because the H_{II} phase can be induced at high temperatures in anhydrous PS bilayers by the addition of Li^+ (Cevc et al., 1985) or in fully dehydrated PS bilayers (Hauser et al., 1982), and because PG had been reported not to form nonlamellar phases, even in the presence of divalent cations (Farren and Cullis, 1980), these workers concluded that DOPS has a stronger tendency to form nonlamellar phases than does DOPG. However, even strongly lamellar-phase preferring lipids such as PC can form an H_{II} phase at low water content (see Sackmann, 1983), and the effect of the addition of Li^+ on the phase behavior of PG has not been studied. Moreover, in the study by Farren and Cullis (1980) of the ability of several PGs to form reversed phases, a temperature of only 30°C was employed. In fact, other studies have shown that PGs can form the H_{II} phase at higher temperatures in the presence of high salt concentration or at lower pH (Cullis and de Kruijff, 1976; Harlos and Eibl, 1980). Moreover, Lee et al. (1993) report that small quantities of dioleoyl PS are slightly more effective than similar quantities of DOPG in stabilizing the L_α phase relative to the H_{II} phase in a 1-palmitoyl-2-oleoyl-PE matrix. Furthermore, we have shown that although small quantities of dielaidoyl PG are more effective than dielaidoyl PS in increasing the onset temperature for the formation of the H_{II} phase in a dielaidoyl PE matrix, the addition of dielaidoyl PG induced the formation of an inverted cubic phase at temperatures just above the L_α/H_{II} phase transition temperature of dielaidoyl PE alone, whereas the addition of dielaidoyl PS did not (Foht et al., 1995). Considering the results of these previous studies and the findings presented here, we believe that PG has an intrinsically greater tendency to form inverted nonlamellar phases than does PS, at least at neutral pH and in the absence of high salt and divalent cations.

A comparison of the relative abilities of the various cationic lipids utilized here to induce nonlamellar phase formation in a single anionic phospholipid is also instructive. We find that DO-TAP and DO-DAP are both considerably more effective than is Et-DOPC in inducing the formation of nonlamellar phases in equimolar mixtures with POPA. Again, this would be expected, based on the much larger size of the polar headgroup of Et-DOPE relative to DO-TAP and especially to DO-DAP. However, the differences in the conformational freedom of the polar headgroups of these two types of cationic lipids and the possible localization of the positive charge at different distances from the polar/apolar interface of the bilayer may also be important in this regard. A comparison of the relative tendencies of DO-TAP and DO-DAP to induce nonlamellar phases is more difficult. DO-TAP induces the more highly curved H_{II} phase at all temperatures above that at which the L_β phase melts, so one could argue that it is more potent in this regard than is DO-DAP, which induces the less tightly curved inverted cubic phase in DOPA bilayers. However, the addition of equimolar DO-DAP abolishes L_α phase formation entirely in DOPA dispersions at all temperatures above 0°C. Certainly additional work will be necessary to resolve this question. In principle, the smaller polar headgroup of DO-DAP, and its ability to hydrogen bond with the polar headgroups of anionic phospholipids, would be expected to make it a more potent inducer of nonlamellar phase formation than DO-TAP.

Although zwitterionic lipids are the major lipid components of eukaryotic cell membranes, anionic phospholipids are also invariably present in considerable quantities and are known to be essential structural and functional components of such membranes (see Gennis, 1989; Lentz, 1999; Buckland and Wilton, 2000). In most eukaryotic membranes, for example, PS is the major anionic phospholipid present, and one of its primary functions seems to be to impart a negative charge to the surface of the membrane lipid bilayer. This negative surface charge is required for the binding and activation of various peripheral membrane proteins, including various phospholipases (see Buckland and Wilton, 2000), myristoylated proteins (see McLaughlin and Aderem, 1995), as well as components of the blood coagulation process (see Lentz, 1999). Moreover, PS is required for the activation of a variety of integral transmembrane proteins such as protein kinase C (see Newton, 1995) and various ion-transporting adenosine triphosphatases (see

Gennis, 1989). Our present results, which indicate that the accumulation of negative charge on the bilayer surface strongly inhibits inverted nonlamellar phase formation, suggest that anionic phospholipids may also function in eukaryotic cell membranes to stabilize the lipid bilayer phase in the presence of often considerable quantities of inverted phase-preferring phospholipids such as PE. Moreover, in eukaryotic plasma membranes, the anionic phospholipid lipid PS and the inverted nonlamellar phase-forming zwitterionic phospholipid PE are both localized primarily to the inner monolayer of the lipid bilayer, while the strongly lamellar phase-preferring PC and SM are localized primarily in the outer monolayer (see Op den Kamp, 1981), lending credence to this suggestion.

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