Thermotropic Properties of Phosphatidylethanols

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ABSTRACT Phosphatidylethanol is formed when ethanol substitutes in the transphosphatidylation reaction catalyzed by phospholipase D. The structural and thermotropic properties of dipalmitoylphosphatidylethanol and dimyristoylphosphatidylethanol have been studied using differential scanning calorimetry, fluorescence spectroscopy, and ^{31}P nuclear magnetic resonance. These lipids exist in a bilayer phase with no indication of nonbilayer phase formation, as shown by ^{31}P nuclear magnetic resonance. It was found that the phase behavior of these phospholipids before and during the main chain melting transition is different in 50 mM Tris buffer compared to salt solutions. The phase transition behavior and the 6-propionyl-2-(dimethylamino)naphthalene (Prodan) fluorescence spectra for both lipids are consistent with the formation of the interdigitated gel phase under certain conditions. Both lipids become interdigitated in Tris-HCl, and ethanol enhances the formation of this phase. Comparative studies of the 6-propionyl-2-(dimethylamino)naphthalene spectra in dipalmitoylphosphatidylglycerol, dielaidoylphosphatidylethanolamine, and dipalmitoylphosphatidylcholine further elucidate the value and limitations of this probe as a diagnostic tool for lipid structure.

INTRODUCTION

Ethanol substitutes for water in the transphosphatidylation reaction catalyzed by phospholipase D, forming Peth instead of phosphatidic acid (Gustavsson and Alling, 1987). This phenomenon has been studied from the perspective of monitoring and understanding the activity of phospholipase D (Kobayashi and Kanfer, 1987; Moehren et al., 1994). It has more recently been shown that phosphatidylethanol accumulates in the membranes of animals exposed to ethanol in animal models of alcoholism (Gatalica et al., 1983; Alling et al., 1984; Gustavsson, 1995). In these animals it has been shown that Peth can reach its maximum concentration in the presence of 300–400 mM ethanol, and the amounts of Peth can vary between 0.2 and 2 nmol/μmol of total phospholipid (Gustavsson, 1995). This unique phospholipid with its small anionic headgroup has unusual physical properties that may have important functional effects on the cell membranes. It is important to understand these properties and their influence on the properties of the membranes in which they reside.

Very few studies have been done on the properties of individual pure phosphatidylethanols. In one study it was shown that the gel to liquid crystal phase transition of DPPeth and DMPeth were similar to that of the PC (Omodeo-Sale' et al., 1989). Several recent studies have been done on the effects of adding Peth to membranes, with mixed results. Omodeo-Sale' showed that Peth increased the fluidity of membranes from natural and artificial sources, and suggested that it protected the membranes from fluidization by ethanol for PC but not PE (Omodeo-Sale' et al., 1991). Another interesting report by Victorov et al. (1994) based on NMR studies, indicates that the rate of transbilayer movement of Peth is greater than any of the other naturally occurring phospholipids near physiological pH. These studies suggest that it is important to achieve an understanding of the properties of this unusual class of phospholipid.

In the present investigation, we studied the properties of pure DPPeth and DMPeth by DSC, by fluorescence using the probe Prodan, and by ^{31}P NMR, in comparison with phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol. These studies suggest that this class of lipids has some unique properties, including a tendency to form an interdigitated gel phase. To further characterize its tendency for interdigitation, we have also included the effects of ethanol and of Tris-HCl in these studies.

MATERIALS AND METHODS

Chemicals

DPPeth, DMPeth, DPPC, DPPG, and DEPE were obtained from Avanti, Birmingham, AL, the ethanol was purchased from PhaRmco, Linfield, PA, the fluorescence probe Prodan was obtained from Molecular Probes (Eugene, OR), and deuterium oxide (99.8%) was obtained from Aldrich (Milwaukee, WI).

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Abbreviations used in this article: DPPeth, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol (sodium salt); DMPeth, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanol (sodium salt); DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3 [phospho-rac-(1-glycerol)] (sodium salt); DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; Peth, phosphatidylethanol; QELS, quasi-elastic light scattering; MLV, multilamellar vesicles; LUVET, extruded vesicles; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PG, phosphatidylglycerol; T_m, phase transition midpoint; PC, phosphatidylcholine.

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Sample preparation

MLV or LUVET were used. Bangham's method was used to prepare the MLV (Bangham et al., 1967). Chloroform stock solutions of lipid were dried under a steady stream of nitrogen gas, and then kept overnight on a vacuum pump to remove all residual chloroform. The stock suspensions were hydrated with water or the required NaCl or Tris-HCl solution, at a temperature above the phase transition temperature of the particular lipid for at least 2 h with occasional vortexing. LUVET were prepared by extrusion of multilamellar vesicles through two stacked polycarbonate filters of 400-nm pore size at a temperature above the phase transition of the lipids, using the thermostatted extruder from Lipex (Vancouver, BC). The extrusion was repeated 10 times. Lipid concentrations were determined by Bartlett's method (Bartlett, 1959).

QELS

The size of LUVET was determined by NICOMP model 370 submicron particle sizer (Pacific Scientific Co., Silver Spring, MD). The accumulation time was 30 min.

DSC

DSC was performed using the MC-2 scanning calorimeter from Microcal, Inc., Amherst, MA. The calorimeter is interfaced with an IBM compatible computer, and the software used is Origin, provided by Microcal. A Haake refrigerated bath controlled by the computer is connected to the calorimeter for temperature control during cooling scans. The scan rates were 20°C/h for both heating and cooling scans. The final concentrations of lipids for DSC studies were from 2.0 to 4.5 mM. The data were analyzed using Origin software from Microcal.

Fluorescence measurements

Fluorescence studies using the probe Prodan were performed using the SLM 8300 spectrofluorometer which is interfaced with an IBM computer. The lipid/probe ratio used was 500:1 unless specified otherwise. The concentration of lipids used in fluorescence experiments was 1.1–1.2 mM. In the Prodan experiments the excitation wavelength was 359 nm. The fluorescence spectra were measured from 400 to 610 nm. A control sample prepared without a fluoroprobe had less than a 1% scattering signal.

^{31}P NMR

The phosphorus NMR experiments were carried out on a Bruker AM-500 (Billerica, MA) instrument operating at 202.46 Mhz for ^{31}P.

RESULTS

DSC

At neutral pH, Peth has a negative charge that affects its behavior in the membrane. Using DSC we investigated the thermotropic behavior of both DPPeth and DMPeth LUVET in water and in several concentrations of Tris-HCl buffer and NaCl.

Fig. 1 shows examples of DSC heating scans of DPPeth in several solvents including 50 mM Tris-HCl (pH 7.4), 1 M Tris-HCl (pH 7.4), 0.5 M NaCl, 0.05 M NaCl, and pure water. These data show clearly that the solvent composition has an effect on the thermotropic properties of this lipid. Fig. 2 shows the corresponding data for DMPeth. In both cases the solvent has a large effect. For both lipids, the scans in water showed a transition temperature several degrees above those at the higher ionic strengths. For DPPeth, for all of the solvent compositions, the transitions showed a high temperature shoulder whose temperature depended on the solvent. For DMPeth, the transitions were narrower, without such a high temperature

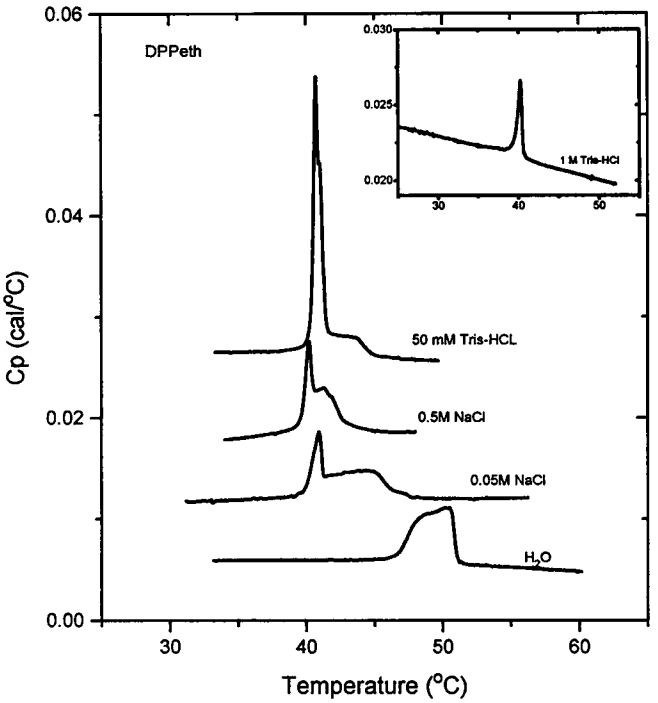

FIGURE 1 DSC scans of DPPeth extruded vesicles in several solvents, which are indicated on the curves.

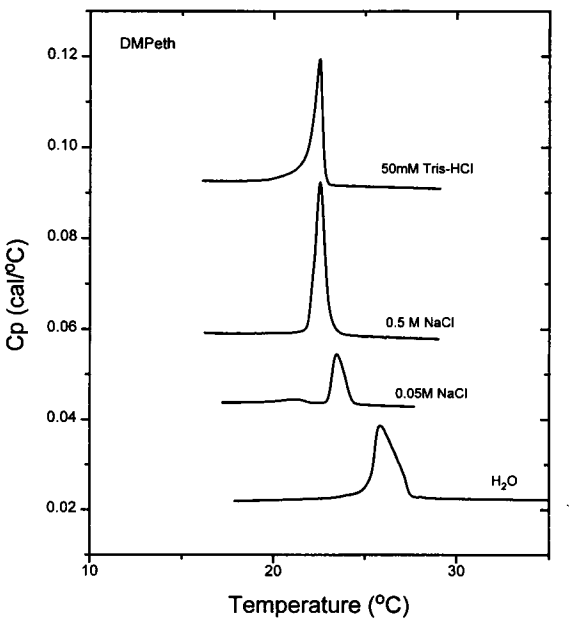

FIGURE 2 DSC scans of DMPeth extruded vesicles in several solvents, which are indicated on the curves.

shoulder. The scans in water proved to be of low reproducibility. These effects of ionic strength are typical of charged lipids (Epand and Hui, 1986).

The effect of ionic strength on the size of the extruded vesicles was studied by QELS for both DMPeth and DPPeth. Fig. 3 shows the size distributions of DPPeth liposomes in water and in Tris-HCl (pH 7.4) before DSC scanning (*A*, *C*) and after five heating and cooling scans (*B*, *D*). These data show that the vesicles prepared in Tris-HCl were stable during thermal cycling, whereas the vesicles prepared in water became smaller and more broadly disperse after thermal cycling. Similar results were obtained with DMPeth (data not shown). Because of the instability and irreproducibility of the behavior of these lipids in pure water, we have restricted the remainder of the investigation to 0.5 M NaCl and Tris-HCl solvents.

To explore the structural preferences of DMPeth and DPPeth, the effect of ethanol on the thermotropic properties was studied by DSC. Figs. 4 *A* and 5 *A* show the effects of ethanol on the T_m of DPPeth in NaCl and in Tris-HCl buffer. In NaCl there is a slight increase in T_m as a function of ethanol concentration, with no significant hysteresis. In comparison, in the presence of 50 mM Tris-HCl, at ~50 mg/ml ethanol, there is the beginning of a significant hysteresis between the transition temperatures for heating and cooling. This behavior in Tris-HCl is similar to that observed for the PCs when interdigitation is induced by ethanol (Rowe, 1985; Rowe and Cutrera, 1990).

For DMPEth, the results are qualitatively different than for DPPeth. Fig. 4 *B* shows that in the presence of 0.5 M NaCl an inflection as well as hysteresis in the T_m behavior appears at ~40 mg/ml ethanol. In the presence of 50 mM Tris-HCl, shown in Fig. 5 *B*, hysteresis is constant across the entire range of ethanol concentrations. The hysteresis induced in the presence of NaCl by ethanol, and the hysteresis present at all concentrations of ethanol in the presence of Tris-HCl, are characteristic of the PCs when they are in the ethanol-induced interdigitated phase (Rowe, 1985).

The enthalpies of the main transitions for DMPeth and DPPeth are summarized in Table 1. The enthalpy did not significantly change for DMPeth and DPPeth in Tris-HCl and for DPPeth in NaCl. However, ethanol caused a significant increase in the enthalpy for DMPeth in the presence of 0.5 M NaCl.

Prodan fluorescence measurements

Prodan fluorescence has recently been shown to be useful in detecting the various phases of PC, particularly in distinguishing between interdigitated and noninterdigitated phases (Zeng and Chong, 1991, 1995; Chong, 1988, Chong et al., 1989; Rottenberg, 1992). To further pursue the investigation of the structure of DPPeth and DMPeth, the spectra of Prodan in these lipids was compared with several other lipids whose structures are known.

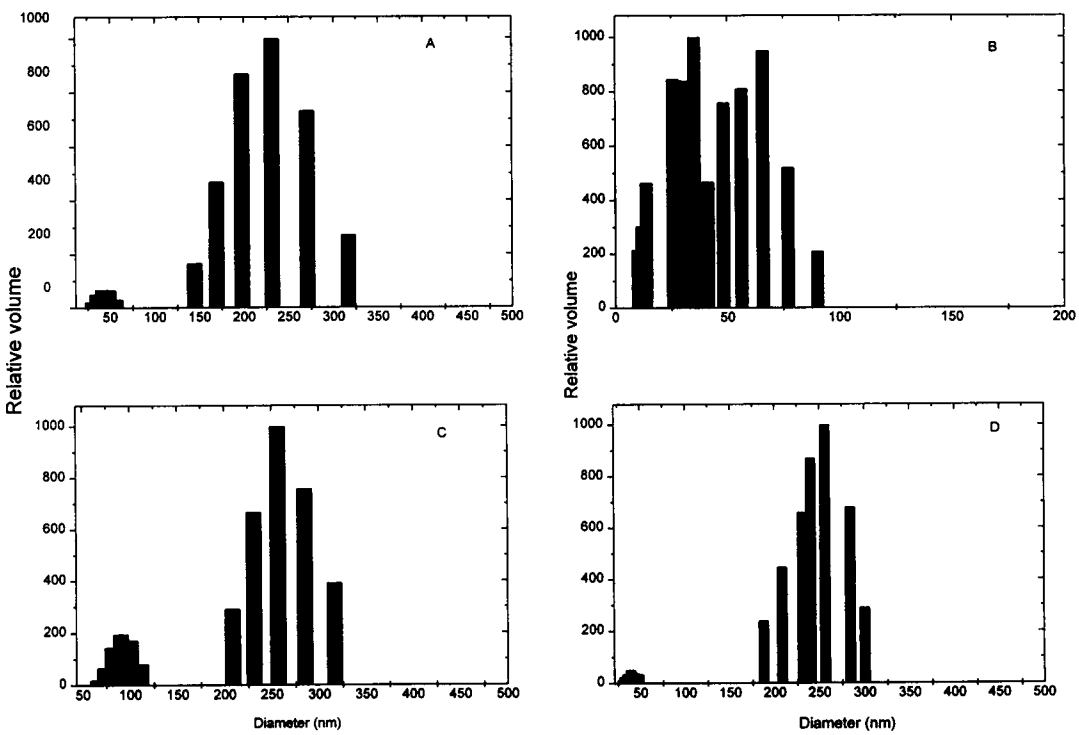

FIGURE 3 QELS results for DPPeth extruded vesicles at 25°C. The accumulation time was 30 min. (*A*) Distribution analysis for DPPeth in water before DSC scans; (*B*) distribution analysis for DPPeth in water after five DSC scans; (*C*) distribution analysis for DPPeth in 50 mM Tris-HCl buffer (pH 7.4) before DSC scans; (*D*) distribution analysis for DPPeth in 50 mM Tris-HCl buffer (pH 7.4) after five DSC scans.

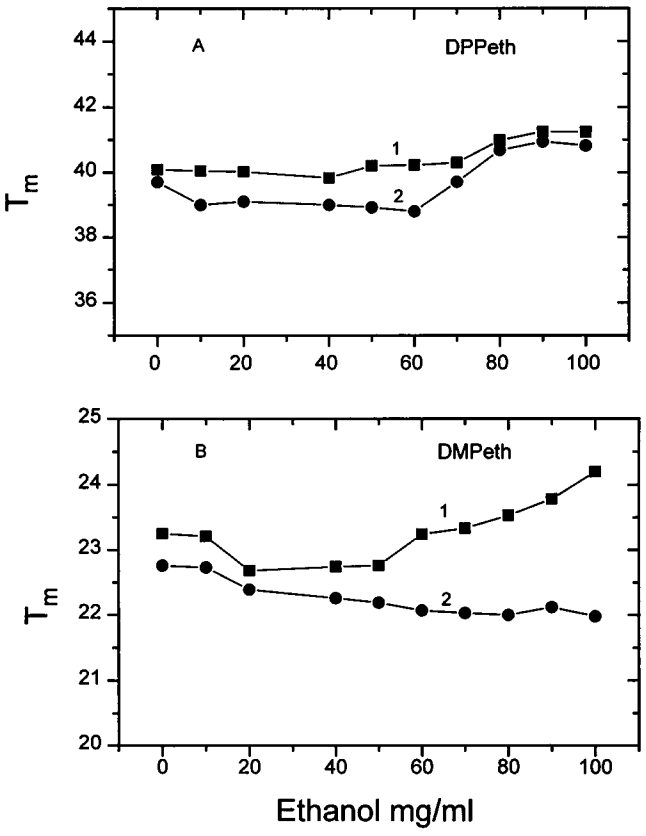

FIGURE 4 Effect of ethanol on the temperature of the main phase transition of Peth in 0.5 M NaCl. (*A*) Effect of increasing concentration of ethanol on the temperature of the phase transition of DPPeth in 0.5 M NaCl (*1*, heating scans; *2*, cooling scans); (*B*) effect of increasing concentration of ethanol on the temperature of the phase transition of DMPeth in 0.5 M NaCl (*1*, heating scans; *2*, cooling scans).

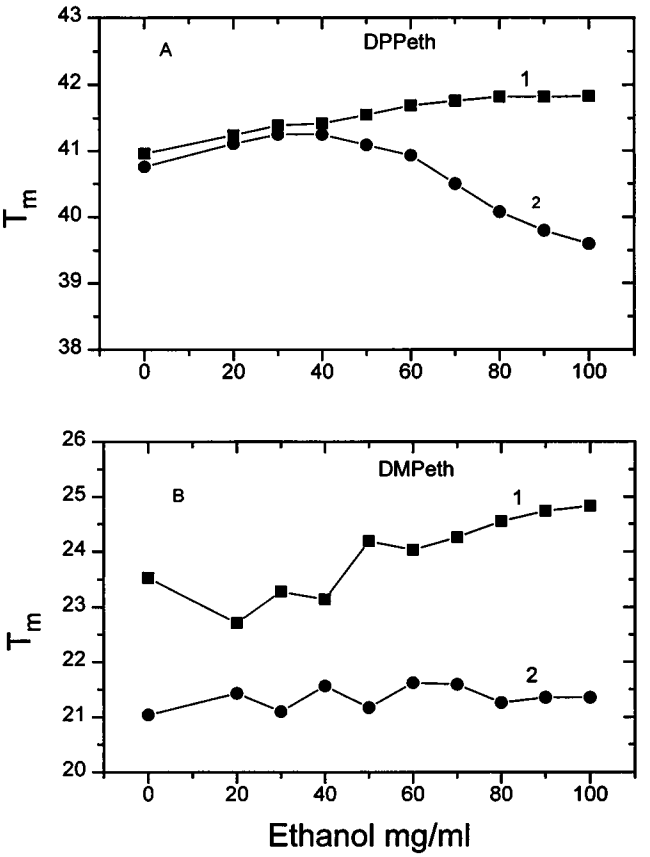

FIGURE 5 Effect of ethanol on the temperature of the main phase transition of Peth in 50 mM Tris-HCl (pH 7.4). (*A*) Effect of increasing concentration of ethanol on the temperature of the phase transition of DPPeth in 50 mM Tris-HCl (*1*, heating scans; *2*, cooling scans); (*B*) effect of increasing concentration of ethanol on the temperature of the phase transition of DMPeth in 50 mM Tris-HCl (*1*, heating scans; *2*, cooling scans).

Fig. 6 *A* shows the Prodan spectrum in DPPC MLV in the presence and absence of ethanol. The DPPC spectra show clearly the large difference in the Prodan spectrum between the normal L'_β gel phase and the interdigitated $L_{\beta I}$ phase induced by ethanol. In the interdigitated phase, the maximum is shifted from 435 to 507 nm. This shift is due to the change in the partitioning of the Prodan between the solvent and the lipid in these two phases (Zeng and Chong, 1991); the spectrum in the presence of ethanol is primarily that of Prodan in water, whereas the spectrum with the maximum at 435 represents Prodan in the lipid environment (Weber and Farris, 1979; Massey et al., 1985).

Fig. 6 *B* shows the Prodan spectrum in DEPE MLV under several conditions. The spectrum in 0.5 M NaCl is similar to that in DPPC, reflecting the same partitioning of Prodan in the gel phase for DEPE. The addition of ethanol causes a small decrease in the maximum at 435 nm and a small increase at 507 nm. As a control, a similar amount of water was added to a parallel sample, which also resulted in a decrease, although smaller, of the 435 nm intensity. This change can be attributed to the change in partitioning of Prodan due to the decrease in lipid concentration on the addition of water. The greater change that occurs with ethanol is probably not a structural change, but it may reflect a difference in the solubility of the probe in the ethanol-containing solvent compared to water. At 70°C, where DEPE is in the nonbilayer inverted hexagonal phase, the Prodan spectrum resembles that of DPPC in the interdigitated phase.

Fig. 7, *A* and *B* show the spectra of Prodan in DPPG MLV. We have examined it in both 0.5 M NaCl and 50 mM Tris-HCl, in the presence and absence of ethanol. In 0.5 M

TABLE 1 DSC summary

	DPPeth	DPPeth + 2 M Ethanol	DMPeth	DMPeth + 2 M Ethanol
50 mM Tris-HCl				
ΔH kcal/mol Heating scan	9.38	9.049	5.76	6.45
ΔH kcal/mol Cooling scan	9.05	10.45	6.15	6.78
0.5 M NaCl				
ΔH kcal/mol Heating scan	8.98	7.63	5.54	8.6
ΔH kcal/mol Cooling scan	8.08	7.3	5.06	8.36

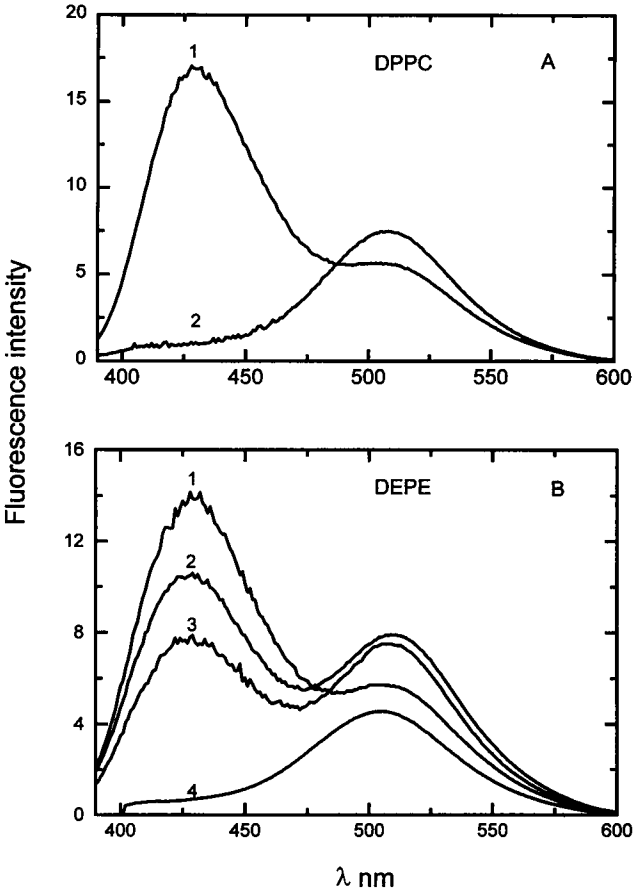

FIGURE 6 Prodan fluorescence spectra in DPPC and DEPE MLV. (*A*) Effect of ethanol on the emission spectra of Prodan fluorescence in DPPC in water at 22°C. *1*, 0 mg/ml ethanol; *2*, 100 mg/ml ethanol. (*B*) Effect of ethanol on the emission spectra of Prodan fluorescence in DEPE in water at 22°C. *1*, 0 mg/ml ethanol; *2*, addition of 100 mg/ml ethanol (0.380 ml); *3*, addition of 0.380 ml water; *4*, emission spectra of Prodan fluorescence in DEPE at 70°C.

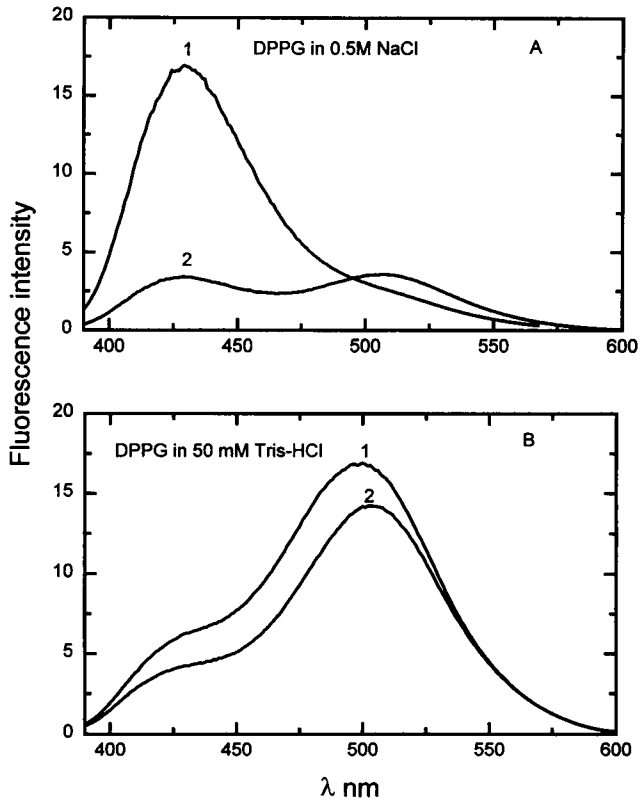

FIGURE 7 Emission spectra of Prodan fluorescence in DPPG MLV. (*A*) Effect of ethanol on the emission spectra of Prodan fluorescence in DPPG in 0.5 M NaCl at 22°C (*1*, 0 mg/ml ethanol; *2*, addition of 100 mg/ml ethanol). (*B*) Effect of ethanol on the emission spectra of Prodan fluorescence in DPPG in 50 mM Tris-HCl (pH 7.4) at 22°C (*1*, 0 mg/ml ethanol; *2*, 100 mg/ml ethanol).

NaCl the spectrum in the absence of ethanol is similar to that in DPPC. However, in the presence of ethanol, the spectrum in 0.5 M NaCl appears to be intermediate between the interdigitated and noninterdigitated spectra of DPPC, although the intensity is considerably reduced. In Fig. 7 *B*, the spectrum of DPPG in Tris-HCl buffer is similar to that of DPPC in the interdigitated phase, except that it has a significant shoulder at 435 nm. The structure of DPPG has been shown to be interdigitated in Tris buffer by x-ray diffraction (Wilkinson et al., 1987). The addition of ethanol reduces the intensity slightly but does not change the shape; the shoulder at 435 nm persists.

Fig. 8, *A* and *B* show the spectra of Prodan in DPPeth MLV in 0.5 M NaCl and 50 mM Tris-HCl, in the presence and absence of ethanol. In 0.5 M NaCl, DPPeth shows a Prodan spectrum similar to that of DPPC, although the 507 nm shoulder is somewhat larger than that of DPPC, suggesting a slightly lower partitioning of Prodan into DPPeth. In 0.5 M NaCl, the effect of ethanol on the Prodan spectrum is nearly identical to that in DPPC, giving a spectrum characteristic of interdigitation, without a residual shoulder at 435 nm. In the presence of 50 mM Tris-HCl, shown in Fig. 8 *B*, in the absence of ethanol, the Prodan spectrum in DPPeth is similar to that in 0.5 M NaCl plus ethanol for DPPG: it appears to be intermediate between the two spectral shapes. It shifts more toward the interdigitated spectrum with the addition of ethanol. In the presence of 1 M Tris, the spectrum becomes one similar to that in interdigitated DPPC, both in the presence and absence of ethanol.

The corresponding data for DMPeth MLV are shown in Fig. 9, *A* and *B*; they are qualitatively different from those for DPPeth. In 0.5 M NaCl the addition of ethanol produces a change in the spectrum similar to that in DPPG in which there is a partial shift to the interdigitated spectrum with a decrease in intensity. In the presence of 50 mM Tris-HCl, however, DMPeth has a spectrum similar to that of DPPeth in Tris-HCl plus ethanol in which the major peak is at 507 nm, with a shoulder at 435 nm, consistent with interdigitation. The addition of ethanol makes no additional changes in the spectrum in DMPeth in Tris-HCl, consistent with the DSC results suggesting that the DMPeth is fully interdigitated in Tris before the addition of ethanol.

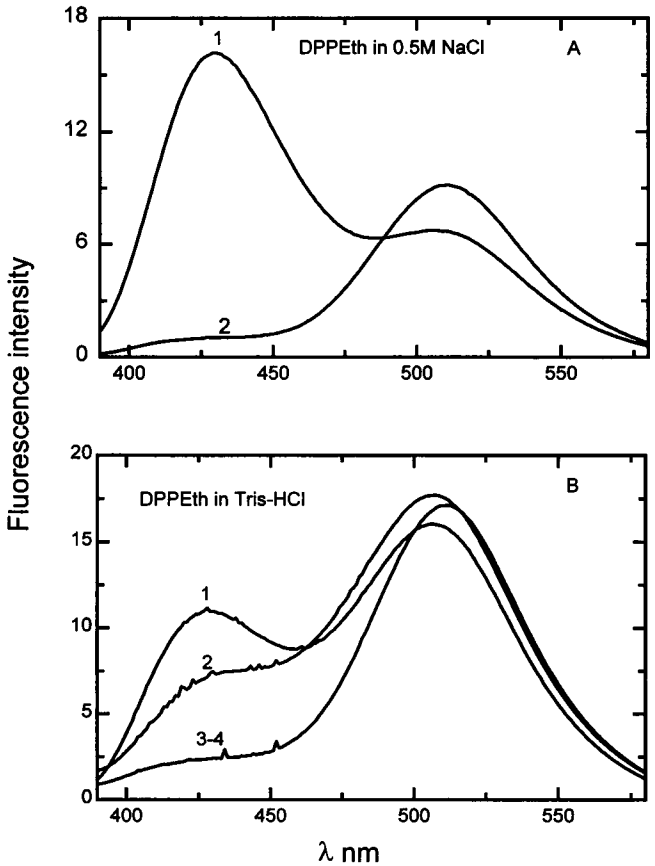

FIGURE 8 Emission spectra of Prodan fluorescence in DPPeth MLV. (*A*) Effect of ethanol on the emission spectra of Prodan fluorescence in DPPeth in 0.5 M NaCl at 22°C (*1*, 0 mg/ml ethanol; *2*, 100 mg/ml ethanol). (*B*) Effect of ethanol on the emission spectra of Prodan fluorescence in DPPeth in 50 mM Tris-HCl (pH 7.4) at 22°C (*1*, 0 mg/ml ethanol; *2*, 100 mg/ml ethanol) and in 1 M Tris-HCl (pH 7.4) at 22°C (*3*, 0 mg/ml ethanol; *4*, 100 mg/ml ethanol).

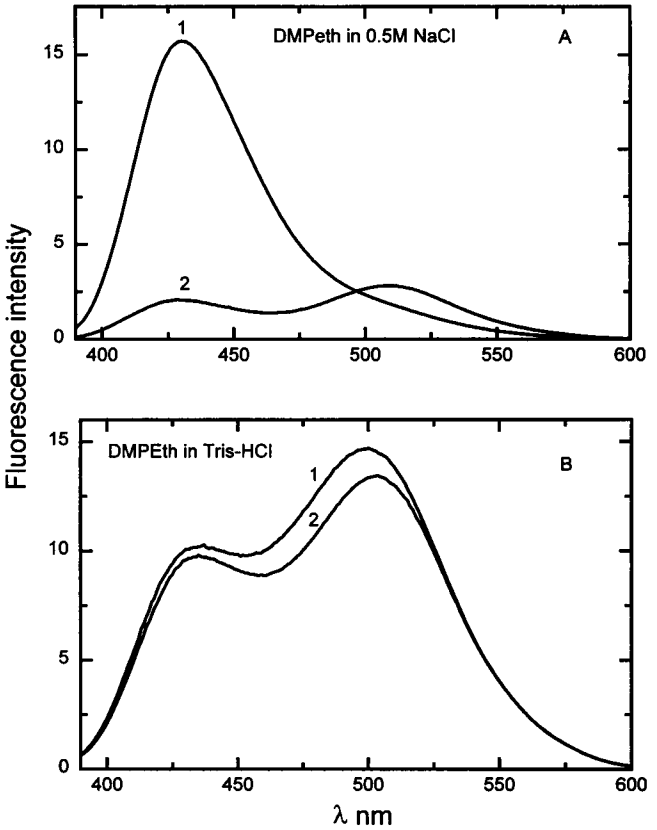

FIGURE 9 Emission spectra of Prodan fluorescence in DMPeth MLV. (*A*) Effect of ethanol on the emission spectra of Prodan fluorescence in DMPeth in 0.5 M NaCl at 15°C (*1*, 0 mg/ml ethanol; *2*, 100 mg/ml ethanol). (*B*) Effect of ethanol on the emission spectra of Prodan fluorescence in DMPeth in 50 mM Tris-HCl (pH 7.4) at 15°C (*1*, 0 mg/ml ethanol; *2*, 100 mg/ml ethanol).

^{31}P NMR

^{31}P NMR can differentiate among certain physical phases of phospholipids. Notably it can distinguish between the nonbilayer inverted hexagonal phase and the bilayer phases (Yeagle, 1990). Because the Prodan fluorescence spectrum of DEPE in the inverted hexagonal phase was similar to the spectrum in DPPC in the interdigitated phase, it was necessary to determine whether the DPPeth and DMPeth were in the inverted hexagonal phase or a bilayer phase. ^{31}P NMR can readily distinguish between these possibilities.

Fig. 10 shows the NMR spectra of DPPeth and DMPeth in Tris-HCl, and DEPE in water. Fig. 11 shows the NMR spectra of DPPC in water without ethanol and with 100 mg/ml ethanol at several temperatures. The spectra of all of the lipids except DEPE at 70°C are consistent with a bilayer structure. The spectrum of DEPE at 65°C (Fig. 10 *C*) is consistent with the well-established conversion of this lipid to the inverted hexagonal phase at high temperature (Cullis and De Kruijff, 1979; Veiro et al., 1989; Seddon, 1990; Yeagle, 1990). As shown in Fig. 11, the spectra of DPPC in the presence and absence of ethanol show that the ^{31}P NMR spectrum is relatively insensitive to the various bilayer phases including the interdigitated gel phase. These data

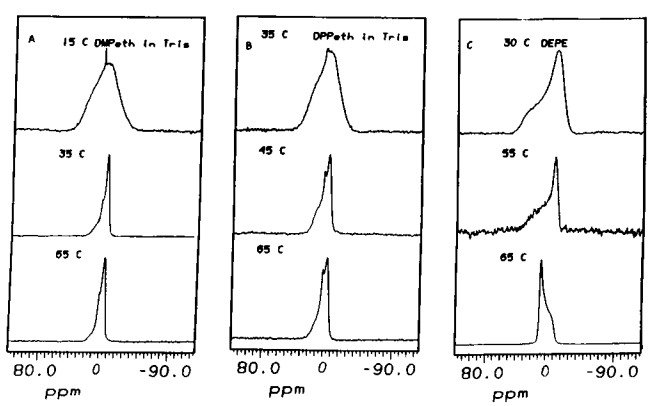

FIGURE 10 ^{31}P-NMR spectra of multilamellar vesicles of (*A*) DMPeth in 50 mM Tris-HCl (pH 7.4); (*B*) DPPeth in 50 mM Tris-HCl (pH7.4); (*C*) DEPE in water; at different temperatures, as indicated.

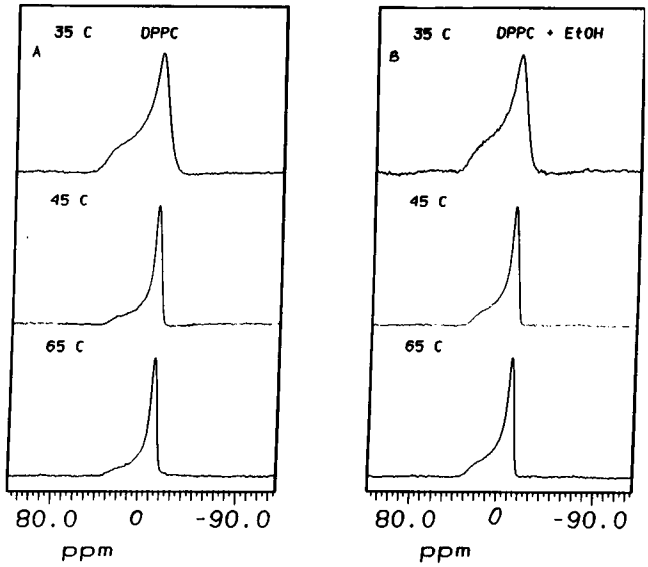

FIGURE 11 ^{31}P-NMR spectra of multilamellar vesicles of (*A*) DPPC in water; (*B*) DPPC in water with 100 mg/ml ethanol at different temperatures, as indicated.

rule out the possibility that DMPeth and DPPeth exhibit nonbilayer behavior.

DISCUSSION

We have investigated the physical properties of two representative phosphatidylethanols, DPPeth and DMPeth, using DSC, fluorescence, and ^{31}P NMR. Our results demonstrate the unusual properties of these lipids, particularly a tendency to become interdigitated under certain conditions. The conditions under which each lipid is interdigitated are slightly different. Both lipids become interdigitated in the presence of Tris-HCl, behavior similar to that of DPPG (Wilkinson et al., 1987). For DMPeth, full interdigitation occurs at 50 mM Tris-HCl; for DPPeth, the data suggest that only partial interdigitation occurs at this concentration; however, at 1 M Tris-HCl, interdigitation appears to be complete. The addition of ethanol also increases the interdigitation of DPPeth in 50 mM Tris-HCl. With 0.5 M NaCl as solvent, both lipids become interdigitated in the presence of 100 mg/ml ethanol. The interdigitation of DMPeth occurs more readily than for DPPEth. The phase behavior of these lipids was studied by two methods, and there are some differences in the details of results from the two methods that are discussed in detail below.

DSC

In our studies DSC was used for the investigation of the thermodynamic properties of the negatively charged phospholipids DPPeth and DMPeth. The results demonstrate that the thermotropic behavior of both phosphatidylethanols strongly depend on the ionic strength and the presence of large cations in the solution. The effect of cations on the structure of anionic phospholipids has been the subject of several earlier investigations (Epand and Hui, 1986; Hauser, 1984; Maggio et al., 1987; Wilkinson et al., 1987). For both DPPeth and DMPeth the main melting transition occurs at a higher temperature in water than in higher ionic strength solutions. QELS measurements on the vesicles show that in water, the vesicular structure is unstable, and the vesicles become smaller as a function of cycling through the phase transition. Similar instability at low ionic strengths has been reported for phosphatidylglycerol (Epand and Hui, 1986; Hauser et al., 1986). It is suggested that at low ionic strengths the charge repulsions create the potential for high enough curvature to seal the edges of disks or shells. This results in instability in extruded vesicles, and a decrease in the cooperativity of phase transitions. In the presence of NaCl or Tris-HCl, the transitions of both lipids become more cooperative, and the temperature of the transition for both lipids approach that of the corresponding PC; for LUVET, the vesicle size is stable also. The melting temperature behavior for both DMPeth and DPPeth suggests that both of these lipids occur in the interdigitated gel phase under certain conditions. Based on previously reported criteria for interdigitation in DPPC, the large hysteresis observed in Figs. 4 *B* and 5 *B* for DMPeth and Fig. 5 *A* for DPPeth are characteristic of interdigitation (Rowe, 1985; Rowe and Cutrera, 1990; Boggs et al., 1989). For DMPeth it appears that in NaCl, interdigitation occurs in the presence of ethanol, beginning at ~50 mg/ml ethanol. In the presence of Tris-HCl, DMPeth exhibits large hysteresis at all concentrations of ethanol, including no ethanol, suggesting that Tris-HCl alone is sufficient to cause interdigitation in this lipid. For DPPeth, in NaCl there is a slight inflection in the melting temperature as a function of ethanol concentration, and no hysteresis; the lack of hysteresis does not rule out interdigitation, but it indicates that if there is interdigitation, the kinetics of reformation of this phase on cooling in these solvent conditions is not slow as it is in the other lipids studied. In Tris-HCl, the transition temperature of DPPeth exhibits an inflection and hysteresis beginning at ~50 mg/ml ethanol, consistent with the induction of interdigitation.

^{31}P NMR

To determine whether DMPeth or DPPeth could exist in a nonbilayer phase, ^{31}P NMR experiments were performed. The ruling out of nonbilayer phases was necessitated by the finding that inverted hexagonal phase DEPE exhibits Pro-dan spectra similar to interdigitated DPPC. These ^{31}P NMR spectra show that these lipids are in a bilayer phase, and there is no evidence for the nonbilayer inverted hexagonal phase. The small spike in some of the higher temperature spectra suggests the presence of a small amount of small vesicles. The line shape is not the same as that of DPPC,

suggesting a more restricted headgroup motion (Yeagle, 1990). Previous NMR studies of phospholipids with short alkyl headgroups, including DPPeth, showed that the line width was a function of the length of the alkyl chain (Browning, 1981).

Prodan fluorescence

Prodan fluorescence was used to further investigate the structure of DPPeth and DMPeth. Prodan is a fluorescence probe sensitive to the polarity of its environment, and it has been widely studied (Zeng and Chong, 1995, 1991; Chong et al., 1989; Rottenberg, 1992; Chong, 1988). In various membranes in the liquid crystalline phase it exhibits various appearances that have been interpreted as showing variations in the polarity and/or the probe locations in these membranes (Rottenberg, 1992). In the gel phase, it has been shown that there is a major change in the spectrum of Prodan in DPPC when it undergoes the ethanol-induced transition to the interdigitated phase (Zeng and Chong, 1991). This change has been shown to be attributable to a change in the partitioning of Prodan into the lipid, although these authors suggest that some redistribution of the location of Prodan in the interfacial region may also contribute to the large spectral change (Zeng and Chong, 1995).

Previous studies of Prodan fluorescence in gel phase lipids have not included phosphatidylethanolamines or phosphatidyl glycerol. Our results show that the spectra in DPPG have similar characteristics to those in DPPC, in both interdigitated and noninterdigitated gel phases, although there are some minor differences. However, although in DEPE (Fig. 6 *B*) the spectrum in the gel phase is also similar to DPPC in the noninterdigitated gel phase, at 70°C, where this lipid is known to be in the inverted hexagonal phase (Cullis and De Kruijff, 1979; Veiro et al., 1989), the spectrum is very similar to that in DPPC in the interdigitated phase (Fig. 6 *A*). This suggests that Prodan interacts differently with the interfacial region of this inverted hexagonal phase, or it does not partition well into this phase. The similarity of the Prodan spectrum in these two phases shows that the use of Prodan to identify the interdigitated phase must be combined with other methods, because the interdigitated spectrum is not unique. In the case of the Peths, we have ruled out the nonbilayer phase by ^{31}P NMR, so it is valid to interpret the Prodan spectra in terms of interdigitation.

Fig. 7, *A* and *B* showing the Prodan spectra in DPPG in a variety of solvent conditions, confirms and extends the validity of this method of diagnosing interdigitated gel phases. It has been shown by x-ray diffraction that DPPG is interdigitated in the presence of Tris-HCl (Wilkinson et al., 1987). The spectra of Prodan in Fig. 7 *B* are consistent with this, except that there is a shoulder at 435 in the Tris-HCl spectrum that is not present in the interdigitated DPPC spectrum. The effects of ethanol on DPPG have not previously been reported. The data of Fig. 7, *A* and *B* suggest that ethanol enhances the ability of DPPG to become interdigitated, but may not induce full interdigitation at 100 mg/ml ethanol. However, it is possible that the DPPG is completely interdigitated, but that Prodan does not behave exactly the same in DPPG as it does in DPPC (see discussion below). The shoulder at 435 nm in Fig. 7 *B*, under conditions where x-ray diffraction indicates full interdigitation, supports this possibility.

The results of the Prodan study on the Peths support the conclusion that both DPPeth and DMPeth are interdigitated under certain conditions, in light of the NMR data that rule out the nonbilayer phase. For DPPeth in 0.5 M NaCl, the conversion to interdigitation is complete in 100 mg/ml ethanol, with spectral changes very similar to those in DPPC. This is consistent with the DSC results if the kinetics of the transition are rapid in this solvent. In the presence of Tris DPPeth appears to be partially interdigitated in the absence of ethanol, becoming nearly completely interdigitated at 100 mg/ml ethanol. If the Tris-HCl concentration is increased to 1 M, then the spectrum appears fully interdigitated, and there is no further change when ethanol is added.

For DMPeth, there are some qualitative differences compared to DPPeth. In 0.5 M NaCl, the addition of ethanol leads to a spectral change similar to that of DPPG, in which the intensity is decreased and the resulting spectrum has peaks at both 435 and 507 nm, suggesting only partial interdigitation. In Tris-HCl, the spectrum still has two peaks, although with a shift toward the higher wavelength peak, and there is no further effect of ethanol on the spectrum. The DSC results indicated that DMPeth is fully interdigitated in Tris alone, with no further effects of ethanol. The Prodan spectra would be consistent with this interpretation if Prodan does not behave in the same way with DMPeth as it does with DPPeth and DPPC. For example, the spectral shape in Fig. 9 *B* could occur if Prodan retains significant partitioning into DMPeth in the interdigitated phase. The behavior of Prodan in DMPeth is similar to that in DPPG in Tris-HCl (Fig. 7 *B*) which is known from x-ray diffraction to be interdigitated (Wilkinson et al., 1987).

Our results on the spectra of Prodan in four different lipids in several phases under a variety of solvent and temperature conditions demonstrate the variability of these spectra as a function of small differences in conditions. These spectra are also very sensitive to Prodan/lipid ratio and total lipid concentration. These variations are due in part to changes in the partitioning of Prodan between the aqueous solvent and the lipid, which may be affected not only by lipid phase structure and chemical structure, but also by the solvent composition. The spectra are probably also affected by small changes in the polarity of their location when they are bound to the lipid. These considerations demonstrate the potential usefulness of Prodan for detecting small variations in lipid properties, but they also demonstrate the caution that must be exercised in interpreting changes in Prodan spectra, particularly in more complex biological systems. The finding that DEPE in the nonbilayer inverted hexagonal phase gives the same Prodan spectrum

as DPPC in the interdigitated phase also demonstrates the value of using more than one method to diagnose lipid structures.

Lipid interdigitation mechanisms

Our results provide strong evidence that DPPeth and DMPeth form the interdigitated gel phase in the presence of Tris-HCl buffer, and that ethanol enhances the formation of this phase. The Peth headgroup structure is similar to that of DPPG, in that it is negatively charged, and relatively small; DPPG also becomes interdigitated in the presence of Tris-HCl (Wilkinson et al., 1987). In the study of DPPG it was suggested that the large cation Tris causes interdigitation by binding between the headgroups of the lipid, creating an increased area per headgroup, which then allows interdigitation to take place (Wilkinson et al., 1987). It was found that the presence of sodium ions reduced the capability of Tris to induce interdigitation, presumably by replacing the large Tris cation with small cations. We have shown for the first time that ethanol enhances interdigitation in DPPG. It appears to induce partial interdigitation in the presence of 0.5 M NaCl; however, in view of the uncertainty about Prodan partitioning in this particular lipid, complete interdigitation under these conditions is not ruled out. For DPPeth and DMPeth, the mechanism of the induction of interdigitation by Tris is probably similar to that for DPPG. The role of ethanol in facilitating interdigitation in DPPG and the Peths is probably similar to that in DPPC of interacting in the interfacial region, and reducing the undesirability of exposing the terminal methyl groups to the aqueous interfacial region.

Our results on DMPeth and DPPeth indicate that DMPeth has a greater tendency to become interdigitated than does DPPeth. This is opposite to the behavior of the PCs in ethanol in which the longer the chain length, the greater the tendency to become interdigitated (Rowe, 1983; Nambi et al., 1987). In the case of the PCs, the increased stabilization of the interdigitated phase by increased chain length is attributed to the tighter packing of the acyl chains in the interdigitated phase compared to the noninterdigitated rippled phase or the L'_β phase (Simon et al., 1986). This consideration suggests that the packing contribution may be less significant in the Peths, or that the packing effects favor the noninterdigitated phase. There are no data available on the chain length effects on the interdigitation of PG.

The induction of interdigitation in DPPC by ethanol has been attributed to the combined effects of a large headgroup that forces a tilted gel phase, and the binding of alcohol to reduce the undesirability of exposing the terminal methyls to the aqueous phase in the interdigitated phase (Nambi et al., 1988). A similar mechanism may be occurring in DPPG, in which charge repulsions lead to tilted acyl chains in the regular gel phase (Wilkinson et al., 1987). The interdigitated structure relieves the headgroup repulsions by its larger area per headgroup. The presence of ethanol also increases the tendency of DPPG to become interdigitated by reducing the unfavorable effects of exposing the terminal methyls to the aqueous phase. The similar behavior of the Peths suggests that they may also have tilted acyl chains in the gel phase. Further elucidation of the structure of the Peths can be addressed by x-ray diffraction.

Biological considerations

Phosphatidylethanol is a unique, anionic phospholipid formed in cell membranes only in the presence of ethanol. The discovery of Peth in the brains of ethanol-treated rats (Gustavsson and Alling, 1987; Kobayashi and Kanfer, 1987) and the finding that phospholipase D is a component of signal transduction cascades (Bocckino et al., 1987; Pai et al., 1987) raises the possibility that this may be one of the ways through which ethanol affects the function of the brain and other organs. It has been shown that in the presence of ethanol the signal transduction via phospholipase D is changed and Peth is formed. Thus ethanol may lead to functional disturbances in receptor systems where phospholipase D is the dominating pathway for cell signaling. However, receptor-induced Peth formation is mainly located in plasma membranes (Gelas et al., 1989). High activity of phospholipase D has also been found in the synaptosomal membrane (Kobayashi and Kanfer, 1987), and in an axolemma preparation from rat brain (De Vries et al., 1983). The formation and accumulation in cell membranes of this unusual negatively charged phospholipid may induce changes in membrane-associated processes, especially protein-lipid interactions, and also affect membrane properties such as surface charge.

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REFERENCES

Alling, C., Gustavsson, J. E. Mansson, G. Benthin, and E. Anggard. 1984. Phosphatidylethanol formation in rat organs after ethanol treatment. *Biochim. Biophys. Acta.* 793:119–122.

Bartlett, G. R. 1959. Phosphorus assay in collum chromatography. *J. Biol. Chem.* 234:466–470.

Bangham, A. D., J. DeGier, and G. D. Grevill. 1967. Osmotic properties and water permeability of phospholipid liquid crystals. *Chem. Phys. Lipid.* 1:225–246.

Bocckino, S. B., P. B. Wilson, and J. H. Exton. 1987. Ca^{2+} mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation. *FEBS Lett.* 225:201–204.

Boggs, J. M., J. R. Rangeraj, and A. Watts. 1989. Behavior of spin labels in a variety of interdigitated lipid bilayer. *Biochem. Biophys. Acta.* 981:243–253.

Browning, J. L. 1981. Motions and interactions of phospholipids head groups at the membrane surface. 1. Simple alkyl head groups. *Biochemistry.* 20:7123–7133.

Chong, P. L.-G. 1988. Effects of hydrostatic pressure on the location of Prodan in lipid bilayer an cellar membranes. *Biochemistry.* 27:399–404.

Chong, P. L-G, S. Capes, and P. T. T. Wong. 1989. Effects of hydrostatic pressure on the location of Prodan in lipid bilayer: a FT-IR study. *Biochemistry.* 28:8358–8363.

Cullis, P. R., and B. DeKruijff. 1979. Lipid polymorphysm and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta.* 559:399–420.

DeVries, G. H., R. J. Chalifour, and J. N. Kanfer. 1983. The presence of phospholipase D in rat central nervous system axolemma. *J. Neurochem.* 40:1189–1191.

Epand, R. M., and S.-W. Hui. 1986. Effect of electrostatic repulsion on the morphology and thermotropic transitions of anionic phospholipids. *FEBS Lett.* 209:257–260.

Gatalica, Z., G. Moehren, and J. B. Hoek. 1983. Unilateral nephrectomy selectively stimulates phospholipase D in the remaining kidney. *Biochim. Biophys. Acta.* 1177:87–92.

Gelas, P., G. Ribber, F. Terce, and H. Chap. 1989. Differential activation by fMet-Leu-Phe and phorbol ester of a plasma membrane phosphatidylcholine-specific phospholipase D in human neutrophil. *FEBS Lett.* 251:231–238.

Gustavsson, E. 1995. Phosphatidylethanol formation: specific effects of ethanol mediated via phospholipase D. *Alcohol Alcoholism.* 30: 391–406.

Gustavsson, L., and C. Alling. 1987. Formation of phosphatidylethanol in rat brain by phospholipase D. *Biochem. Biophys. Res. Commun.* 142: 958–963.

Hauser, H. 1984. Some aspects of the phase behavior of charge lipids. *Biochim. Biophys. Acta.* 772:37–50.

Hauser, H., N. Gains, H.-J. Eibl, M. Muller, and E. Wehrli. 1986. Spontaneous vesiculation of aqueous lipid dispersions. *Biochemistry.* 25: 2126–2134.

Kobayashi, M., and J. N. Kanfer. 1987. Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J. Neurochem.* 48:1597–1603.

Moehren, G., L. Gustavsson, and J. B. Hoek. 1994. Activation and Desensitization of phospholipase D in intact rat hepatocytes. *J. Biol. Chem.* 269:838–848.

Massey, L. B., H. S. She, and H. J. Pownall. 1985. Interfacial properties of model membranes and plasma lipoproteins containing ether lipids. *Biochemistry.* 24:6973–6978.

Maggio, B., J. M. Sturtevant, and R. K. Yu. 1987. Effect of calcium ions on the thermotropic behavior of neutral and anionic glucosphingolipids. *Biochim. Biophys. Acta.* 901:173–182.

Nambi, P., E. S. Rowe, and T. J. McIntosh. 1988. Studies of ethanol-induced interdigitated gel phase in phosphatidylcholines using the fluorophore 1,6-diphenyl-1,3,5-hexatriene. *Biochemistry.* 27:9175–9182.

Omodeo-Sale', M. F., C. Lindi, P. Palestini, and M. Masserini. 1991. Role of phosphatidylethanol in membranes. Effects on membrane fluidity, tolerance to ethanol, and activity of membrane-bound enzymes. *Biochemistry.* 30:2477–2482.

Omodeo-Sale', M. F., B. Cestaro, A. Mascherpa, D. Monti, and M. Masserini. 1989. Enzymatic synthesis and thermotropic behavior of phosphatidylethanol. *Chem. Phys. Lipids.* 50:135–142.

Pai, J., E. C. Liebl, C. S. Tettenborn, F. I. Ikegwuonu, and G. C. Mueller. 1987. 12-O-Tetradecanoylphorbol-13-acetate activates the synthesis of phosphatidylethanol in animal cells exposed to ethanol. *Carcinogenesis.* 8:173–178.

Rottenberg, H. 1992. Probing the interaction of alcohols with biological membranes with the fluorescent probe Prodan. *Biochemistry.* 31: 9473–9481.

Rowe, E. S. 1985. Phase transition hysteresis: specific alcohol-phosphatidylcholine interaction. *Biochem. Biophys. Acta.* 813:321–330.

Rowe, E. S., and T. Cutrera. 1990. Differential scanning calorimetric studies of ethanol interactions with distearoylphosphatidylcholine: transition to the interdigitated phase. *Biochemistry.* 29:10398–10404.

Seddon, J. M. 1990. Structure of inverted hexagonal H_{II} phase and nonlamellar phase transition of lipid. *Biochem. Biophys. Acta.* 1031:1–69.

Simon, S. A., T. J. McIntosh, and M. L. Hines. 1986. The influence of anesthetics on the structure and thermal properties of saturated lecithins. *In* Molecular and Cellular Mechanisms of Anesthetics. S. H. Roth and K. W. Miller, editors. Plenum, New York. 297–308.

Veiro, J. A., R. G. Khalifah, and E. Rowe. 1989. The polymorphic phase behavior of dielaidoylphosphatidylethanolamine. Effect of *n*-alkanols. *Biochem. Biophys. Acta.* 979:251–256.

Victorov, A. V., N. Janes, G. Moehren, E. Rubin, T. F. Taraschi, and J. B. Hoek. 1994. Rapid transbilayer movement of phosphatidylethanol in unilamellar phosphatidylcholine vesicles. *J. Am. Chem. Soc.* 116: 4050–4052.

Weber, G., and F. J. Farris. 1979. Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino) naphthalene. *Biochemistry.* 18:3075–3078.

Wilkinson, D. A., D. A. Tirrell, A. B. Turek, and T. J. McIntosh. 1987. Tris buffer acyl chain interdigitation in phosphatidylglycerol. *Biochim. Biophys. Acta.* 905:447–453.

Yeagle, P. L. 1990. Phosphorus NMR of membranes. *In* Biological Magnetic Resonance. L. J. Berliner and J. Reuben, editors. Plenum, New York. 1–59.

Zeng, J., and P. L.-G. Chong. 1991. Interactions between pressure and ethanol on the formation of interdigitated DPPC liposomes: a study with Prodan fluorescence. *Biochemistry.* 30:9485–9491.

Zeng, J., and P. L.-G. Chong. 1995. Effect of ethanol-induced lipid interdigitation on the membrane solubility of Prodan, Acdan, and Laurdan. *Biophys. J.* 68:567–573.