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Determination of the phase behaviour of phosphatidylethanolamine admixed with other lipids and the effects of calcium chloride: implications for protein kinase C regulation

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The phase behaviour of 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) was studied by differential scanning calorimetry and ^{31}P-NMR spectroscopy. Modulation of the phase behaviour of POPE by 1-palmitoyl-2-oleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1,2-diolein (DOG), $CaCl_2$, $MgCl_2$, and combinations of these substances was studied. The bilayer-forming lipids, POPS and POPC, raise the bilayer-to-hexagonal phase-transition temperature of POPE. The POPC has a greater effect than POPS, probably because the former lipid is more miscible with POPE. Addition of 10 mM $CaCl_2$ has little effect on the phase-transitions of POPE/POPC mixtures, but it greatly decreases the effectiveness of POPS in raising the bilayer-to-hexagonal phase-transition temperature of POPE. The effectiveness of DOG in lowering the phase-transition temperature of POPE is also greatly reduced in the presence of 10 mM $CaCl_2$. This phenomenon may play a role in the negative feedback regulation of protein kinase C.

Introduction

There has been considerable interest in determining the phase behaviour of lipid mixtures in order to obtain information about the mixing of the components in these systems. It is possible that biological membranes contain domains which are enriched in particular lipids. This lateral phase separation of lipids probably affects membrane function. One of the properties which is thought to affect membrane function is the variation of lipid composition, which regulates the ability of the membrane to undergo morphological rearrangements, such as formation of the hexagonal phase [1]. Although the hexagonal phase is probably never a prominent feature of biological membranes, changes in the propensity to form the hexagonal phase can often be correlated with changes in membrane properties. For example, conditions which favour hexagonal phase formation would make the membrane more prone to fusion [2,3], and would also result in the activation

Abbreviations: DOG, 1,2-diolein; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulphonic acid; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; FID, free induction decay; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

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of protein kinase C [4,5]. Protein kinase C is an enzyme involved in signal transduction [6]. Both Ca^{2+} and phosphatidylserine are required for the activity of this enzyme and the activity is enhanced by diacylglycerols [7]. Substances such as dolichols [8] and diacylglycerols [9], are potent promoters of the hexagonal phase in pure phosphatidylethanolamines. Uncharged substances which induce hexagonal phase formation are activators of protein kinase C [4,5]. It was therefore of interest to extend earlier studies on the propensity for hexagonal phase formation in lipid mixtures [10–12] by using well-defined synthetic lipid components with both saturated and unsaturated acyl chains, and including those components required for protein kinase C activation. Phosphatidylserine is the most effective stimulator of protein kinase C activity [13]. The presence of phosphatidylethanolamine enhances the activity of this enzyme further in the presence of phosphatidylserine [14]. Calcium ions are also required for protein kinase C activity. Lower concentrations of Ca^{2+} are required when the enzyme is activated by diacylglycerols in the presence of phosphatidylserine and phosphatidylethanolamine [15]. Lipid mixtures containing high mole fractions of unsaturated phosphatidylethanolamine can be used to study the bilayer to hexagonal phase transition. Earlier results on phosphatidylserine/phosphatidylethanolamine mixtures were obtained with dielaidoyl [16], or with dioleoyl, or dilinoleoyl [17] lipid species. It was shown that calcium ion-induced lipid phase separation was strongly dependent on the nature of the acyl side-chains [17]. In the present work, 1-palmitoyl, 2-oleoyl lipids were studied, which are closer to structures found in mammalian membranes. We have not only looked at the phase behaviour of these lipids, but also at the effects of DOG, Ca^{2+}, Mg^{2+} and phosphatidylcholine on lipid phase properties.

Experimental procedures

Materials

All phospholipids used were obtained from Avanti Polar Lipids, Birmingham, AL, in the 1-palmitoyl-2-oleoyl form. Their purity was confirmed by their phase behaviour and by TLC using chloroform/methanol/water (16:7:1) as the developing solvent. DOG was purchased from Sigma Chemical Co., St. Louis, MO, and from Nu Check Prep. Inc., Elysian, MN.

Sample preparation

The phospholipids, with or without DOG, were dissolved together in a solution of chloroform and methanol (2:1, v/v). The solvent was evaporated with a stream of dry nitrogen in order to deposit the lipid as a film on the walls of a glass test tube. Last traces of solvent were removed into a liquid nitrogen trap by placing the samples in a vacuum oven at 40°C. The apparatus was maintained under high vacuum for at least 90 min. The lipid film was then suspended in a buffer of 20 mM Pipes/150 mM NaCl/0.02 mg/ml NaN_3 (pH 7.4). The buffer also contained either 1 mM EDTA, 10 mM $MgCl_2$, or up to 10 mM $CaCl_2$. The tube was warmed to about 45°C and vortexed vigorously for about 30 s. The final concentration of POPE was 5 mg/ml with varying amounts of other lipids admixed. All lipid compositions given in this paper are in mol% and not weight%. The buffer and lipid suspensions were degassed under vacuum before being loaded into the calorimeter.

Differential scanning calorimetry (DSC)

Lipid suspension or buffer were loaded into the sample or reference cell, respectively, of an MC-2 high-sensitivity scanning calorimeter (Microcal Co., Amherst, MA). A scan rate of 39 K/h was generally employed. The data were collected through an IBM-PC fitted with a Data Translation DT2801 board. Data were analyzed using software provided by the Microcal Co. The calorimeter was calibrated electrically. The amount of lipid transferred to the calorimeter cell was estimated, based on the cell size and the initial lipid concentration. Because of the non-homogeneous nature of POPE or POPS/Ca^{2+} suspensions, these enthalpy values are only precise to ± 20%. Second heating scans on the same sample were very similar to the first scan. This suggests that calcium equilibrates with all of the lipid, despite its impermeability. This occurs because the calcium is added to the lipid as a dry film, and neither POPE nor POPS/Ca^{2+} form well-sealed vesicles. Because of the high lipid concentration required to allow observation of the bilayer to

hexagonal phase transition, the calorimeter overshoots after the gel-to-liquid crystalline transition giving rise to an apparent exotherm. Partial phase diagrams were constructed using the onset- and completion-temperature for each transition. With transitions followed by an apparent exotherm, the completion temperature was taken as the point of intersection between the transition and a baseline extrapolated from the linear region following the exotherm.

^{31}P-NMR

NMR-spectra were recorded on a Bruker AM-500 spectrometer operating at 202.45 MHz. The probe temperature was maintained to within $\pm 1°C$ by a Bruker B-VT 1000 variable temperature unit. Temperatures were checked by thermocouple measurements. A 10 mm broad band probe was used. A 30 kHz sweep width was employed with an acquisition time of 0.28 s, and a relaxation delay of 0.3 s (16K data points). The 90° pulse width was 16.6 μs with composite pulse proton decoupling. FID's were processed using exponential multiplication (line broadening 15.0 Hz). Chemical shifts were expressed in ppm from an external reference of 85% phosphoric acid in ^2H_2O.

Results

A few representative DSC scans of the gel-to-liquid crystalline transition of POPE, in the presence and absence of various additives, are shown in Fig. 1. The presence of 10 mM $CaCl_2$ causes a small lowering of the transition temperature, with no change in the transition enthalpy (Fig. 1, curve B vs. A). Pure POPS has a transition temperature of 11.2°C and a transition enthalpy of 6.0 kcal/mol (Fig. 1, curve C). Calcium ions are known to induce the formation of crystalline cochleate structures in phosphatidylserine [18]. In the presence of 10 mM $CaCl_2$, no transition below 100°C is observable for POPS (not shown). A transition, corresponding to a phase highly enriched in POPS, is not observed in mixtures of this lipid with 91% POPE in the presence or absence of Ca^{2+} (Fig. 1, curves D and E). However, at high mole fractions of POPS (38%) in the presence of $CaCl_2$, a second, lower melting component is observed in the DSC curve (Fig. 1, curve F, see also the expanded inset, curve f). The magnitude of this lower melting component increases with increasing mole fraction of POPS, and is probably a result of the formation of domains in the membrane. This behaviour is not observed in the absence of $CaCl_2$, even at high mole fractions of POPS (not shown). Pure POPC has a phase-transition temperature at about −2°C [19]. The presence of POPC in preparations of POPE causes a lowering of the phase-transition temperature (Fig. 1, curves G, H and I) more

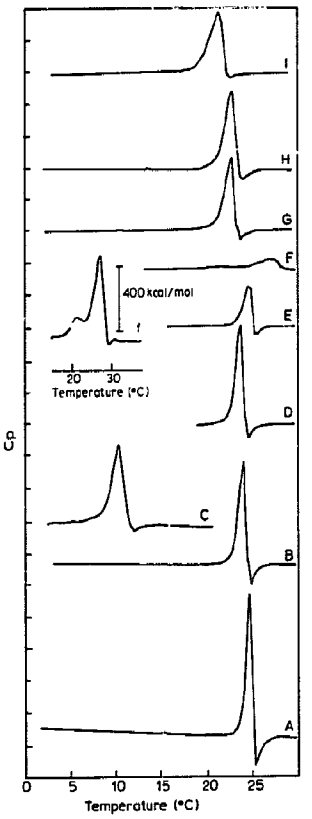

Fig. 1. Representative DSC scans of the gel-to-liquid crystalline phase transition. Heating scan rate 0.65 K/min. Buffer, 20 mM Pipes/0.15 M NaCl/0.02 mg/ml NaN_3 (pH 7.40) with either 1 mM EDTA, or 10 mM $CaCl_2$, when indicated. POPE concentration 5 mg/ml. (A) POPE; (B) POPE-Ca^{2+}; (C) POPS; (D) 91% POPE, 9% POPS; (E) 91% POPE, 9% POPS and Ca^{2+}; (F) 62% POPE, 38% POPS and Ca^{2+}, with an expanded version given as inset (curve f); (G) 91% POPE, 9% POPC; (H) 91% POPE, 9% POPC and Ca^{2+}; (I) 84% POPE, 16% POPC and Ca^{2+}. Each division on the vertical axis represents an increment in apparent heat capacity of 1.5 kcal/mol per K.

marked than does POPS. In the presence of $CaCl_2$, a very small, but reproducible, feature in the DSC curves appears at about 14°C (Fig. 1, curve H). As this component does not change in a systematic manner with increasing concentrations of POPC, we are uncertain about its significance.

The enthalpy of the main transition of pure POPE is 4.5 kcal/mol. This transition enthalpy is not altered by the addition of POPS, or DOG and/or 10 mM $CaCl_2$ to POPE. In the case of POPC, the enthalpy is constant for different POPE/POPC mixtures in the absence of $CaCl_2$, but shows a trend toward increasing values as the mole fraction of POPC increases. At 16% POPC and 84% POPE in the presence of 10 mM $CaCl_2$, the enthalpy is 5.5 kcal/mol. This change is within the experimental error of ± 0.5 kcal/mol. This is also within the error of the enthalpy of POPE/POPS mixtures in the absence of $CaCl_2$. The error in enthalpy determinations for the other systems is ± 1 kcal/mol. This large uncertainty arises because of the aggregated nature of the lipid samples with either high POPE or POPS/Ca^{2+} content.

Representative scans of the bilayer-to-hexagonal phase transition are shown in Fig. 2. As previously noted [9], this transition is more sensitive to the presence of additives than is the gel-to-liquid crystalline transition. For example, the addition of 10 mM $CaCl_2$ reduces the temperature of the transition to the hexagonal phase (Fig. 2, curve B vs. A) to a greater extent than it reduces the temperature of the gel-to-liquid crystalline transition (Fig. 1, curve B vs. A). DOG markedly lowers and broadens the bilayer-to-hexagonal phase transition of POPE, both in the presence (Fig. 2, curve D) and in the absence (Fig. 2, curve C) of 10 mM $CaCl_2$. POPS has a more marked effect in increasing the temperature of this transition in the absence (Fig. 2, curve E) than in the presence (Fig. 2, curve F) of 10 mM $CaCl_2$. DOG also lowers the transition temperature of POPE/POPS mixtures. The effect of DOG is greater in the absence (Fig. 2, curve G) than in the presence (Fig. 2, curve H) of 10 mM $CaCl_2$.

The enthalpy of the bilayer-to-hexagonal phase transition of POPE is 400 cal/mol. In addition to the aggregation of the lipid and the difficulty of introducing a uniform sample to the calorimeter,

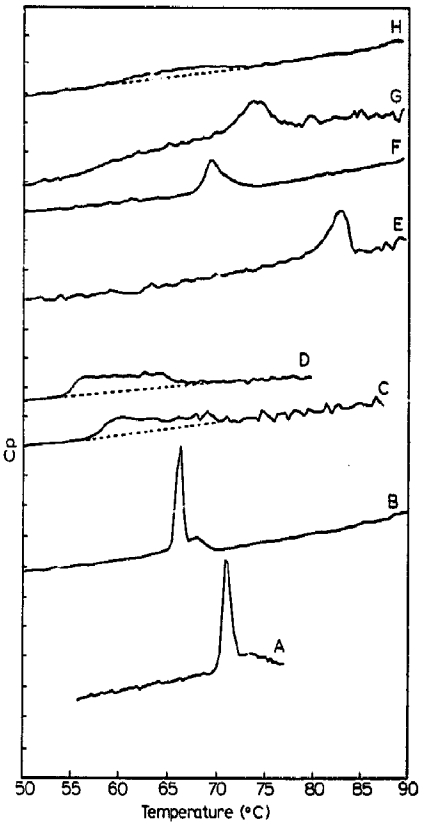

Fig. 2. Representative DSC-scans of the bilayer-to-hexagonal phase transition. Conditions as for Fig. 1. (A) POPE; (B) POPE-Ca^{2+}; (C) 99% POPE, 1.25% DOG; (D) 99% POPE, 1.25% DOG and Ca^{2+}; (E) 91% POPE, 9% POPS; (F) 91% POPE, 9% POPS and Ca^{2+}; (G) 90% POPE, 9% POPS, 1.15% DOG; (H) 90% POPE, 9% POPS, 1.15% DOG and Ca^{2+}. Each division on the vertical axis represents an increment in apparent heat capacity of 0.1 kcal/mol per K.

the enthalpy of the bilayer-to-hexagonal phase transition is susceptible to considerable error, because of its low magnitude and because of the broadness of the transition in the presence of additives. Addition of DOG to POPE, with or without $CaCl_2$, or the addition of POPS to POPE in the absence of $CaCl_2$, had no consistent effect on the transition enthalpy. However, in the presence of 10 mM $CaCl_2$, the POPS/POPE samples exhibited a marked reduction in the enthalpy of the hexagonal phase transition, particularly over the range of 15–20% POPS. Above 20% POPS, the enthalpy gradually decreased from a value of 100

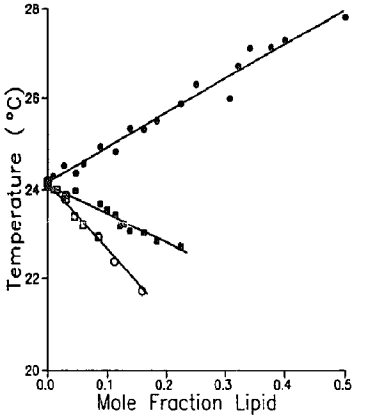

Fig. 3. The gel-to-liquid crystalline phase-transition temperature of POPE as a function of the percentage of POPS (■), POPS plus 10 mM $CaCl_2$ (●), POPC (□), or POPC plus 10 mM $CaCl_2$ (○) added to the membrane.

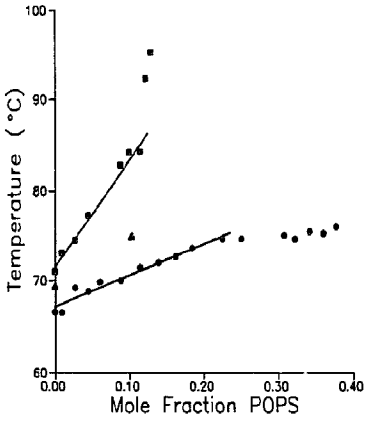

Fig. 4. The effect of POPS on the bilayer-to-hexagonal phase-transition temperature of POPE (■), and with the addition of 10 mM $CaCl_2$ (●), or 10 mM $MgCl_2$ (▲).

± 50 cal/mol and finally approached zero enthalpy at about 40% POPS with 10 mM $CaCl_2$. This is probably a result of the transition becoming less cooperative and therefore broader. As a result, the full transition is not discernible above the baseline. In the case of POPC/POPE mixtures, the enthalpy was independent of the presence of $CaCl_2$, but increased in proportion to the fraction of POPC added, reaching a value of 800 cal/mol at 10% POPC, having had a value of 400 cal/mol for pure POPE.

The DSC transitions were fitted to a single van 't Hoff component. In the case of the gel-to-liquid crystalline transition, POPS raised the transition temperature of POPE in the presence of $CaCl_2$, but lowered it in the absence of this salt (Fig. 3). The direction of the change is in accord with the addition of a higher melting component (Ca^{2+}/POPS, $T_c > 100°C$), or a lower melting component (POPS, $T_c = 11°C$). The gel-to-liquid crystalline transition of POPE was more greatly perturbed by POPC (Fig. 3) than by POPS. In the latter case, however, the presence or absence of $CaCl_2$ made no difference.

The bilayer-to-hexagonal phase-transition temperature of POPE was raised by the presence of the bilayer-forming POPS, both in the presence and in the absence of $CaCl_2$ (Fig. 4). The effect of POPS in the absence of $CaCl_2$ was greater than in its presence. The addition of 10 mM $MgCl_2$ also lowered the effectiveness of POPS in raising the hexagonal phase-transition temperature of POPE but not to such a great extent as did $CaCl_2$ (Fig. 4). In the case of the bilayer-forming POPC, the hexagonal phase-transition temperature of POPE was also raised, but in this case, $CaCl_2$ and $MgCl_2$ had little effect (Fig. 5). The effect of POPC on the hexagonal phase transition of POPE was similar to that previously observed for the effect of dielaidoylphosphatidylcholine on the hexagonal phase transition of dielaidoylphosphatidylethanolamine [13]. In contrast to these bilayer-stabilizing phospholipids, DOG promoted forma-

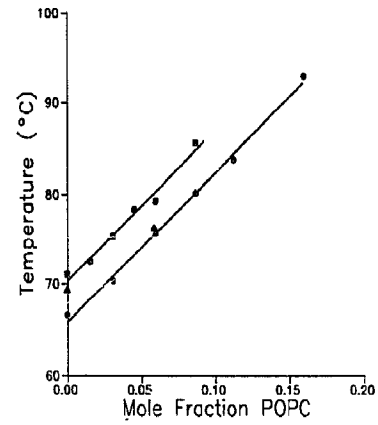

Fig. 5. The effect of POPC on the bilayer-to-hexagonal phase-transition temperature of POPE (■), and with the addition of 10 mM $CaCl_2$ (●), or 10 mM $MgCl_2$ (▲).

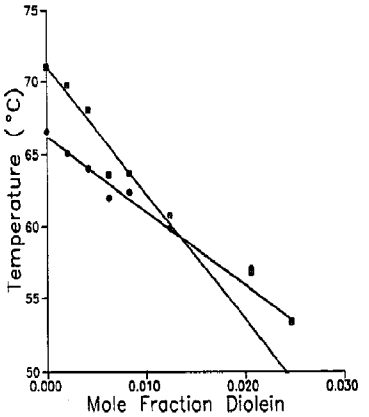

Fig. 6. The effect of DOG on the bilayer-to-hexagonal phase-transition temperature of POPE (■), and with the addition of 10 mM $CaCl_2$ (●).

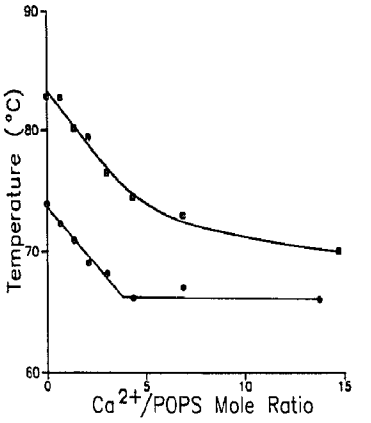

Fig. 8. The effect of Ca^{2+}/POPS molar ratio on the bilayer-to-hexagonal phase-transition temperature of a 91% POPE/9% POPS lipid mixture (■), and in the presence of 1.1 mol% DOG (●). The lipid concentration was 8 mM and the concentration of $CaCl_2$ was adjusted to give the indicated molar ratio.

tion of the hexagonal phase. This effect was greater in the absence than in the presence of $CaCl_2$ (Fig. 6). Calcium ions have a particularly marked effect in promoting hexagonal phase formation in pure POPE/POPS mixtures, or those containing low molar fractions of DOG (Fig. 7). The sensitivity of this system to the molar ratio of $CaCl_2$ to POPS was measured both in the presence and absence of DOG (Fig. 8). The slopes of the plots of the bilayer-to-hexagonal phase-transition temperature vs. mole fraction of additive are summarized in Table I.

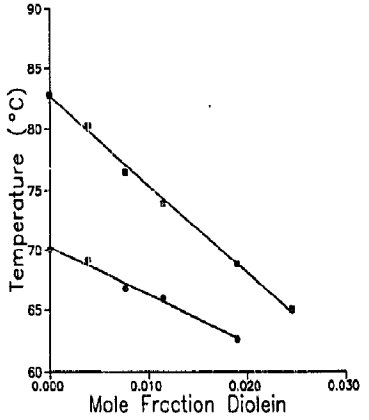

Fig. 7. The effect of DOG on the bilayer-to-hexagonal phase-transition temperature of a mixture of 91% POPE, 9% POPS (■), and with the addition of 10 mM $CaCl_2$ (●).

The DSC results give accurate information about the transition temperature and the transition enthalpy, and hence can be used to describe the phase changes. However, calorimetry cannot give information about the morphology or molecular arrangements within each phase. We therefore investigated the phase behaviour of these lipid systems by ^{31}P-NMR spectroscopy. Pure POPE exhibits a ^{31}P-NMR spectrum typical of a bilayer phase at 66 °C, and typical of the hexagonal phase arrangement at 74 °C (Fig. 9A). The temperature at which the shape of the spectra change is slightly

TABLE I

SENSITIVITY OF THE BILAYER TO HEXAGONAL PHASE TRANSITION TEMPERATURE OF POPE TO THE PRESENCE OF VARIOUS ADDITIVES

Lipid maintained constant	Lipid additive	$[Ca^{2+}]$ (mM)	Slope (K/mole fraction)
POPE	POPS	0	118 ± 7
POPE	POPS	10	33 ± 3
POPE	POPC	0	168 ± 10
POPE	POPC	10	168 ± 5
POPE	DOG	0	−870 ± 100
POPE	DOG	10	−515 ± 50
91% POPE/9% POPS	DOG	0	−730 ± 20
91% POPE/9% POPS	DOG	10	−396 ± 25

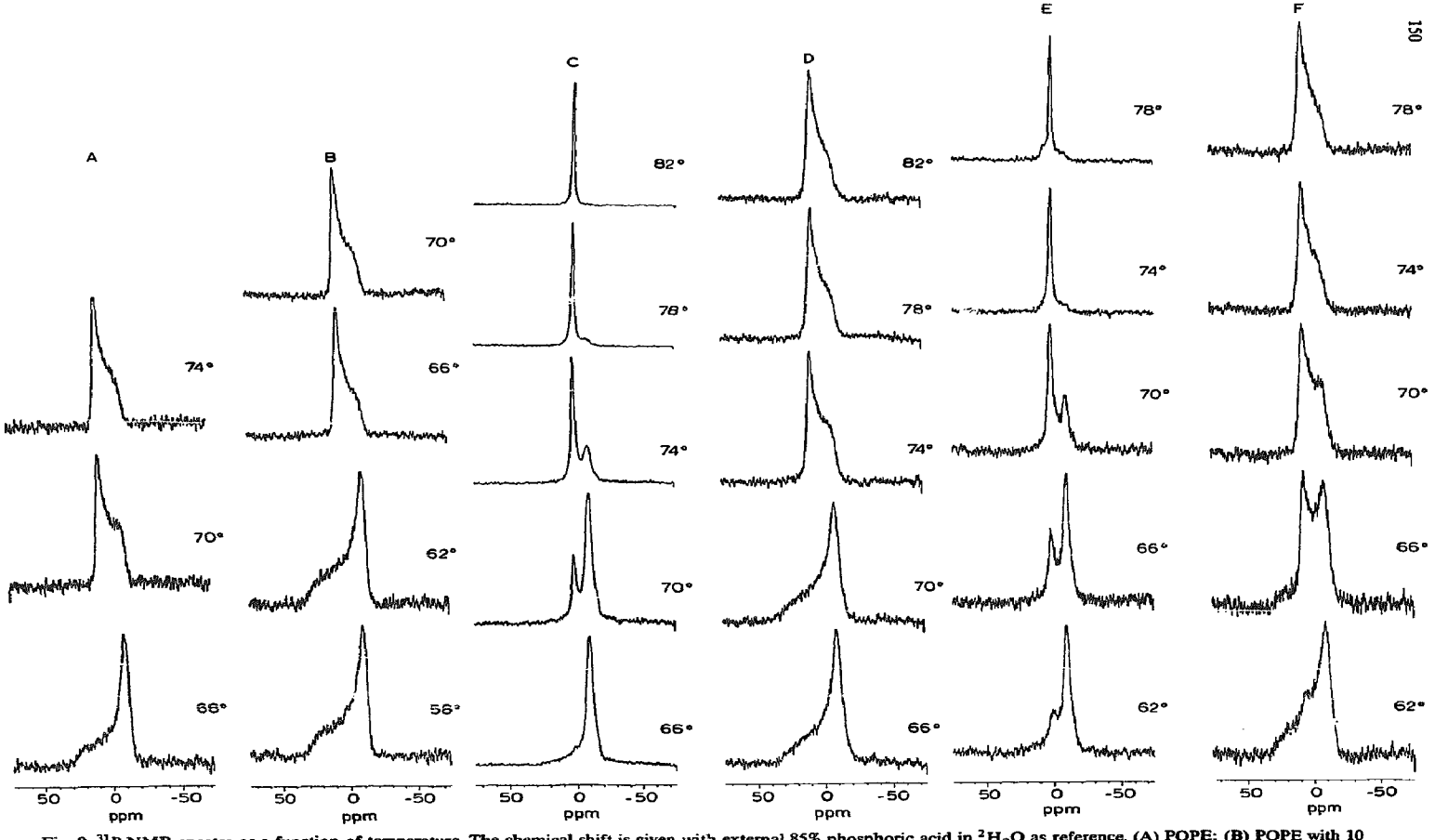

Fig. 9. ^{31}P-NMR spectra as a function of temperature. The chemical shift is given with external 85% phosphoric acid in $^{2}H_2O$ as reference. (A) POPE; (B) POPE with 10 mM $CaCl_2$; (C) 85% POPE, 15% POPS; (D) 85% POPE, 15% POPS with 10 mM $CaCl_2$; (E) 84% POPE, 15% POPS, 1.5% DOG; (F) 84% POPE, 15% POPS, 1.5% DOG with 10 mM $CaCl_2$.

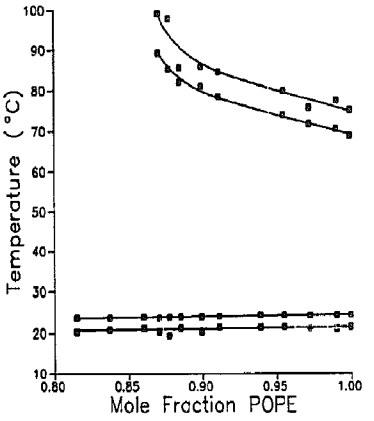

Fig. 10. Phase diagram for POPE/POPS.

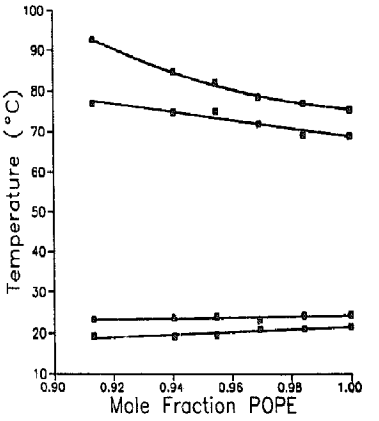

Fig. 12. Phase diagram for POPE/POPC.

lower than the transition temperatures defined more accurately by DSC. Addition of 10 mM Ca^{2+} lowers the temperature at which the bilayer is converted to the hexagonal phase (Fig. 9B). Somewhat higher concentrations of POPS (15%) were used for the ^{31}P-NMR studies, since we were not limited by the broadness of the transition to examine the apparent morphology. In the presence of POPS, the shape of the bilayer spectrum is altered, with the upfield shoulder being less prominent (Fig. 9C, 66°C). This may result from the formation of smaller vesicles. However, the most marked alteration is in the higher-temperature spectra, which are characteristic of isotropic motion of the phosphate group (Fig. 9C, 82°C). This property is markedly altered in the presence of Ca^{2+}, which causes the high-temperature spectra of POPE/POPS mixtures to revert to those typical of a hexagonal phase arrangement (Fig. 9D). These spectra are quite similar to those of POPE alone, suggesting that Ca^{2+}/POPS may be forming a separate phase. A similar situation arose upon addition of 1.5% DOG to the POPE/POPS mixtures, either with or without 10 mM $CaCl_2$ (Fig. 9E and F). The temperature at which the phase change occurs is about 4 K lower in the presence of DOG, compared with the same lipid mixture in the absence of DOG (Fig. 9C and D).

The results of the calorimetric studies are summarized as partial-phase diagrams (Figs. 10–13).

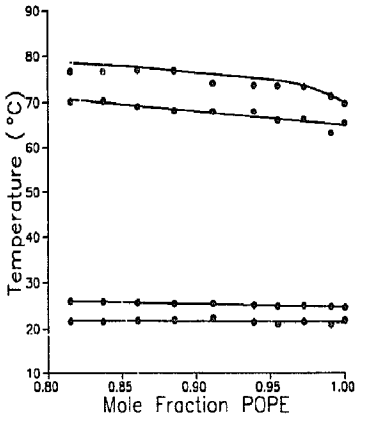

Fig. 11. Phase diagram for POPE/POPS, in the presence of 10 mM $CaCl_2$.

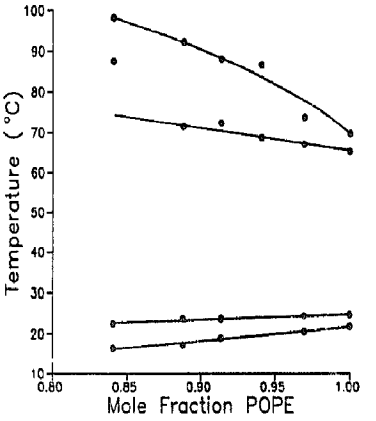

Fig. 13. Phase diagram for POPE/POPC, in the presence of 10 mM $CaCl_2$.

For the purpose of these figures, we do not differentiate between non-bilayer phases which give rise to ^{31}P powder patterns characteristic of the hexagonal phase and those patterns arising from isotropic motion.

Discussion

In this work, we are primarily concerned with the temperature range over which lipid mixtures which are in the bilayer phase are destabilized and undergo a morphological change. These temperatures can be accurately measured with DSC. In the case of pure POPE, it was established that the lipid is in a lamellar phase at 40°C, and converts to a hexagonal phase arrangement by 80°C [11,20]. In some lipid mixtures such as POPE with POPS (Fig. 9C), or with POPC [21], a narrow linewidth ^{31}P-NMR spectrum is obtained at high temperatures. Cubic-phase lipid, inverted micelles, lipidic particles or small hexagonal phase aggregates can give rise to such spectra through motional averaging of the chemical shift anisotropy. Addition of bilayer-forming lipids to phosphatidylethanolamine will decrease the intrinsic curvature and lead to the formation of a hexagonal phase containing cylinders of a larger diameter [22]. As these hexagonal phase cylinders become larger, a point will be reached where neither the bilayer nor the hexagonal phase is stable, and hence intermediate structures such as inverted micelles and cubic phases are formed, giving rise to an isotropic ^{31}P-NMR signal. However, the temperature and enthalpy of the phase transition appear to be independent of the morphology of the high-temperature phase, i.e., whether the morphology type gives rise to an isotropic or hexagonal phase ^{31}P-NMR powder pattern. This is shown by the fact that there is no abrupt change in the slope of a plot of the transition temperature or the transition enthalpy vs. mole fraction of bilayer lipid. Therefore, the equilibrium between the bilayer and the non-bilayer phase is relatively independent of the morphology of the non-bilayer phase.

The bilayer-to-hexagonal phase equilibrium is determined by the intrinsic curvature of a lipid monolayer, hydration, and the constraints of hydrocarbon packing [23]. Bilayer-forming lipids, such as POPS and POPC, with low intrinsic curvature and high hydration tend to raise the bilayer-to-hexagonal phase-transition temperature, while more hydrophobic substances, such as DOG, which can relieve hydrocarbon packing constraints, lower this transition temperature. In contrast, the gel-to-liquid crystalline transition is shifted according to whether the additive is a lower- or higher-melting component. For example, POPS lowers the gel-to-liquid crystalline-transition temperature of POPE, but raises that of the bilayer-to-hexagonal phase transition of this lipid. In comparison, Ca^{2+}/POPS raises the temperature of both transitions. Thus, the factors affecting each of these transitions are quite different.

Comparing the effects of POPS and POPC on POPE in the absence of Ca^{2+}, we find that POPC has the greater effect on the main transition temperature (Fig. 3), as well as on the hexagonal phase-transition temperature. This occurs despite the fact that POPS has a larger head group than POPC, and because of its charge it is more hydrated and undergoes electrostatic repulsion, further expanding the head-group region. An explanation for the unexpectedly greater effect of POPC on the bilayer-to-hexagonal phase-transition temperature of POPE is that POPC mixes with POPE to a greater extent than does POPS. This is indicated by the more horizontal liquidus line for the conversion of POPS/POPE mixtures to the liquid crystalline phase (Fig. 10) compared with POPC/POPE (Fig. 12) and because of the greater effect of POPC (Fig. 12), compared with POPS (Fig. 10), on the cooperativity of the bilayer-to-hexagonal phase transition. When these phospholipids contain elaidoic acid as the acyl group, then phosphatidylserine appears to mix better with phosphatidylethanolamine than does phosphatidylcholine [12]. It is not clear what the basis of this difference is, but the lipids used in the present work are closer to those found in biological membranes.

The effects of Ca^{2+} in inducing lateral-phase separation of phosphatidylserine/phosphatidylethanolamine membranes also appears to be dependent on the acyl chain composition [17]. Calcium ions can induce the phase separation of the dioleoyl forms of phosphatidylserine and phosphatidylethanolamine [17], except at low mole fractions of one of the lipids [16]. However, mix-

tures of the dilinoleoyl forms of these lipids are not phase-separated by Ca^{2+} [17]. We find that POPS and POPE are more phase-separated than POPC and POPE, even in the absence of Ca^{2+} at low temperatures. This is indicated by the fact that both the temperature and cooperativity of the gel-to-liquid crystalline transition of POPE remain almost constant with the addition of POPS (Fig. 10). However, the phase separation is not complete because a POPS transition is not observed in the DSC scans of these mixtures, and there is some change in the main-phase transition temperature with increasing mole fraction of POPS (Fig. 3). Addition of 10 mM $CaCl_2$ leads to a marked reduction in the effect of POPS on the bilayer-to-hexagonal phase-transition temperature of POPE (Figs. 10 and 11, Table I). This can be explained by Ca^{2+} inducing increased lateral-phase separation, even at high temperatures, by forming stable Ca^{2+}/POPS domains. Thus, the small amount of mixing of POPS and POPE which occurs in the absence of Ca^{2+} is reduced even further in the presence of this ion.

Diacylglycerols are potent hexagonal-phase promoters [9,24]. In the present study, we have demonstrated that although Ca^{2+} itself lowers the bilayer-to-hexagonal phase-transition temperature of POPE (Fig. 6) or POPE/POPS (Fig. 7), it also markedly reduces the effectiveness of DOG in lowering the bilayer-to-hexagonal phase-transition temperature of POPE and POPE/POPS mixtures (Figs. 6 and 7, Table I). Calcium ions also lower the effectiveness of POPS in raising the hexagonal phase-transition temperature of POPE (Table I). This can be interpreted, as discussed above, as being a result of calcium ion-induced increased lateral-phase separation of POPS. Calcium ions do not affect the ability of POPC to raise the phase-transition temperature of POPE (Table I). Therefore, Ca^{2+} does not interact strongly with the zwitterionic POPC, and its interaction with POPE is not sufficient to alter the effects of POPC. The mechanism by which Ca^{2+} can stabilize POPE against the effect of DOG more than against the effect of POPC remains to be elucidated.

We would like to suggest some biological implications of our results. The lipids we have chosen are those required for protein kinase C activation. The activity of this enzyme is enhanced by agents which induce formation of the hexagonal phase [4,5]. However, extensive hexagonal phase formation is unlikely in biological membranes and similar effects of lipids can be studied in Triton-solubilized micelles. Therefore, it is not the formation of the hexagonal phase per se which is required for enzyme activation, but rather changes in membrane surface properties, which can be affected by agents that also promote formation of the hexagonal phase. In addition to this qualification, it should also be recognized that the model lipid system cannot mimic the specific interactions which may exist between lipids and the enzyme; nor is the lipid composition and ionic environment as complex as the one which would exist in a biological system. Nevertheless, there are properties of these lipid mixtures which probably are relevant for protein kinase C activation. Binding of protein kinase C to membranes is usually, but not necessarily, accompanied by enzyme activation [25]. The conformational dynamics of the protein are probably modulated by the lipid environment. The micromolar concentrations of Ca^{2+} required for activation of this enzyme are probably required for binding to specific sites on the protein. In addition to this, however, high concentrations of Ca^{2+} can down-regulate a diacylglycerol signal, since Ca^{2+} reduces the effectiveness of DOG in promoting hexagonal phase formation (Table I). This may explain why the calcium ionophore, ionomycin, inhibits the translocation of protein kinase C to cell membranes [26]. It would also suggest that the extracellular side of the plasma membrane, as well as the inner side of Ca^{2+}containing intracellular organelles, is less responsive to diacylglycerol signals.

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