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THE POLYMORPHIC PHASE BEHAVIOUR OF MIXED PHOSPHATIDYLSERINE-PHOSPHATIDYLETHANOLAMINE MODEL SYSTEMS AS DETECTED BY ^{31}P-NMR

EFFECTS OF DIVALENT CATIONS AND pH

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Summary

1. The influence of divalent cations and pH on the polymorphic phase behaviour of aqueous dispersions of phosphatidylethanolamine-phosphatidylserine systems have been investigated employing ^{31}P-NMR techniques.

2. Phosphatidylserines, derived from both egg and soya phosphatidylcholines, stabilize a bilayer organization at 30°C in mixtures with soya phosphatidylethanolamine (which assumes the hexagonal (H_{II}) phase on hydration) when the phosphatidylserine constitutes 15 mol% or more of the phospholipid.

3. The addition of Ca^{2+} to equimolar soya phosphatidylserine/soya phosphatidylethanolamine mixtures triggers complete H_{II} phase formation as detected by ^{31}P-NMR at Ca^{2+} : phosphatidylserine ratios, R, of 1.0 or larger. In contrast, Mg^{2+} is ineffective even at Mg^{2+} : phosphatidylserine ratios of 10.0. In mixtures containing 15 mol% phosphatidylserine, Ca^{2+} triggers H_{II} phase formation at $R = 0.25$. The Ca^{2+}-induced polymorphic phase transitions appear to occur as a result of a structural segregation of phosphatidylserine by Ca^{2+} into crystalline domains, leaving the phosphatidylethanolamine free to adopt the H_{II} phase it prefers in isolation.

4. The polymorphism of soya phosphatidylserine/soya phosphatidylethanolamine systems is markedly sensitive to the pH of the aqueous medium. At 30°C equimolar mixtures exhibit a bilayer-H_{II} transition as the pH is decreased below

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Abbreviation: DMSO, dimethylsulfoxide.

4.0, whereas mixtures containing 15 mol% phosphatidylserine exhibit detectable H_{II} phase structure at pH values below 5.5.

5. ^{31}P-NMR studies suggest that the binding of Ca^{2+} to phosphatidylserine to produce crystalline structures is sensitive to the unsaturation of the acyl chains, with more unsaturated species requiring higher Ca^{2+} : phosphatidylserine ratios for formation of crystalline Ca^{2+}-phospholipid complexes. Studies of the binding of Ca^{2+} with soya phosphatidylserine indicate half maximal binding at 0.3 mM in the absence of salt, which is increased to approx. 0.8 mM in the presence of 100 mM NaCl.

6. These results suggest that the effectiveness of phosphatidylserine as a bilayer-stabilizing agent can be modulated by local changes in such biologically relevant parameters as pH, ionic strength and/or divalent cation concentrations, and are discussed in relation to membrane fusion processes.

Introduction

Many species of naturally occurring membrane lipids preferentially adopt the hexagonal (H_{II}) phase at physiological temperatures when fully hydrated. Examples include major neutral lipid species such as unsaturated phosphatidylethanolamine [1] and monoglucosyl diglyceride [2] as well as acidic phospholipids (such as cardiolipin [3,4], phosphatidic acid [5] and phosphatidylserine [6]) under appropriate conditions of pH and/or divalent cation concentration. Such behaviour is of interest because, as indicated elsewhere [7], the ability of lipids to assume non-bilayer configurations has been employed to suggest dynamic roles of lipid in many functional abilities of membranes, including fusion [8,9] and transport [10] processes.

It is clear that in order for non-bilayer lipid structures to be functionally important, mechanisms must exist for their isothermal regulation and control. Plausible agents for such regulation include membrane protein, the availability of divalent cations and pH, all of which can affect the polymorphic preferences of certain lipid dispersions [7]. In the case of phosphatidylserine (the major acidic phospholipid of mammalian cell membranes) for example, the addition of Ca^{2+} to bilayer model systems results in precipitation and formation of crystalline 'cochleate' [11] structures. Alternatively, decreasing the pH below 4.0 results in formation of the H_{II} phase [6].

It is of obvious interest to extend these observations to include mixed lipid systems containing phosphatidylserine where the macroscopic organisation may be expected to be sensitive to the structural preferences and distribution of the phosphatidylserine component. Mixtures of phosphatidylserine with (unsaturated) phosphatidylethanolamines provide an important example. Preliminary investigations [12] on such systems show that phosphatidylserine can stabilize a bilayer organisation for phosphatidylethanolamine which would otherwise adopt the H_{II} phase and that the addition of Ca^{2+} can trigger bilayer to H_{II} phase transitions in such systems. It is of interest to note that Ca^{2+} can induce similar effects in model erythrocyte 'inner monolayer' systems, where phosphatidylethanolamine and phosphatidylserine are major components [33].

In the present work a detailed study of the polymorphic properties of mix-

tures of unsaturated phosphatidylethanolamine and phosphatidylserine and the influence of divalent cations and pH is presented. Questions which are addressed concern the amounts of phosphatidylserine required to stabilize a bilayer arrangement, the mechanism whereby Ca^{2+} triggers H_{II} phase formation and the amounts of Ca^{2+} required to induce these effects. It is shown that as little as 15 mol% of (soya) phosphatidylserine stabilizes a bilayer arrangement for soya phosphatidylethanolamine at 30°C and that the polymorphic preferences of such systems become progressively more sensitive to variations in pH and available Ca^{2+} as the phosphatidylserine content is lowered from 50 to 15 mol%. Further, Ca^{2+} appears to induce its effects via a structural separation of Ca^{2+}-phosphatidylserine complexes into separate crystalline domains. These results are discussed with regard to fusion processes requiring Ca^{2+} (such as exocytotic release of secretory vesicles) as well as those dependent on pH (such as those occurring in secondary lysosomes).

Materials and Methods

Soya phosphatidylethanolamine, soya phosphatidylserine and egg phosphatidylserine were derived from their respective phosphatidylcholines employing the headgroup exchange capacity of phospholipase D [13]. The phosphatidylserines were purified by carboxymethylcellulose chromatography and converted to their respective sodium salts as described previously [6]. Soya phosphatidylethanolamine was purified by preparative liquid chromatography [14]. All lipids were more than 99% pure as determined by thin-layer chromatography. Methyl esters for fatty acid analysis were prepared by heating lipid samples at 70°C in 5% (v/v) H_2SO_4 in methanol for 2 h [15]. Fatty acid analyses were performed using a Hewlett Packard 7610A high efficiency gas chromatograph (fitted with a column of ethylene glycol succinate) operated at 170°C.

Samples for ^{31}P-NMR studies were prepared from an appropriate mixture of the lipids in chloroform in a 10 mm NMR tube, the chloroform being evaporated under nitrogen, and subsequently under high vacuum for 30 min. The dry lipid was hydrated with 0.7 ml of buffer (10 mM Tris-acetic acid/100 mM NaCl/2 mM EDTA/10% (v/v) ^2H_2O, pH 7) by vortex mixing. Titrations with calcium or magnesium were performed by adding aliquots of 100 mM stock solutions of their chloride salts, following by three cycles of freeze-thawing of the samples to ensure equilibration of the titrant. In certain instances the calcium ionophore A23187 (E.I. Lilly Corp.; 10 μl, 2 mg/ml in DMSO) was added to the samples prior to the addition of calcium, giving identical results. Where signal intensities were measured, triphenyl phosphite (10% v/v in $CHCl_3$) was used as a standard, and was located in the NMR sample tube in a 3 mm central insert. Samples for dialysis were sealed in 0.6 cm diameter dialysis tubing and equilibrated against the required calcium concentration at 4°C for 24 h; in all instances there was a 10-fold molar excess of calcium over phosphatidylserine. Variation of the pH of the samples was accomplished by the addition of 100 mM HCl or 100 mM NaOH to a dispersion of the lipid in buffer, the pH being measured at the temperature of signal accumulation. Degradation of lipids to their lyso derivatives during the time course of a typical pH variation experiment was found to be less than 2%.

^{31}P-NMR spectra were recorded employing a Bruker WP-200 Fourier transform spectrometer. Accumulated free induction decays were obtained for up to 2000 transients using an interpulse time of 0.8 s, an 11 μs 90° pulse and a sweepwidth of up to 50 kHz. All spectra were recorded at 30°C in the presence of broad-band proton decoupling.

For ^{45}Ca binding studies, samples of soya phosphatidylserine (as a dry lipid film) were dispersed in solutions of calcium chloride of concentrations between 0.1 mM and 10 mM, each containing 0.05 μCi of ^{45}Ca (spec. act. 10 Ci/g calcium), either in the presence or absence of 100 mM NaCl, such that the molar ratio of phosphatidylserine to calcium was 2 : 1 in each sample. Following dispersal by extensive vortexing and incubation at room temperature for 30 min, samples were centrifuged (20 000 × g, 120 min) and aliquots (0.1—0.5 ml) of the supernatant removed for counting and phosphate analysis. Less than 1% of total lipid phosphorus was detectable in the supernatant following centrifugation.

Results

The bilayer or non-bilayer preferences of the fully hydrated lipid mixtures employed were monitored at 30°C employing ^{31}P-NMR techniques [7]. The aqueous dispersions of pure (soya) phosphatidylserine showed the characteristic bilayer ^{31}P-NMR lineshape with a low field shoulder and high field peak separated by approx. 50 ppm (Fig. 1a) consistent with results obtained for (egg) phosphatidylserine [6]. Alternatively, (soya) phosphatidylethanolamine adopts the hexagonal (H_{II}) phase at 30°C, as indicated by the characteristic lineshape which has reversed asymmetry compared to the 'bilayer' lineshape, and is narrower by a factor of 2, again in agreement with previous results [7].

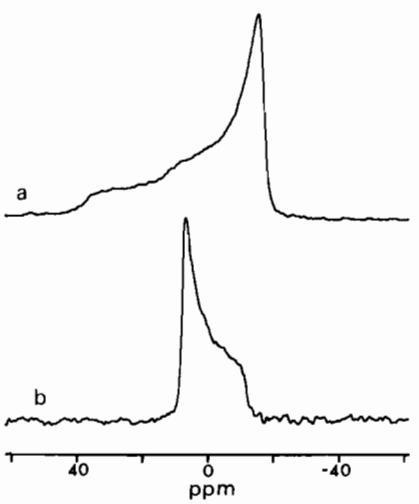

Fig. 1. 81.0 MHz ^{31}P-NMR spectra at 30°C obtained from aqueous dispersions of (a) soya phosphatidylserine and (b) soya phosphatidylethanolamine. Both lipid species were obtained from soya phosphatidylcholine (see Materials and Methods). Spectra were recorded employing broad-band proton decoupling and a 50 Hz line-broadening function was applied. For other details see Materials and Methods.

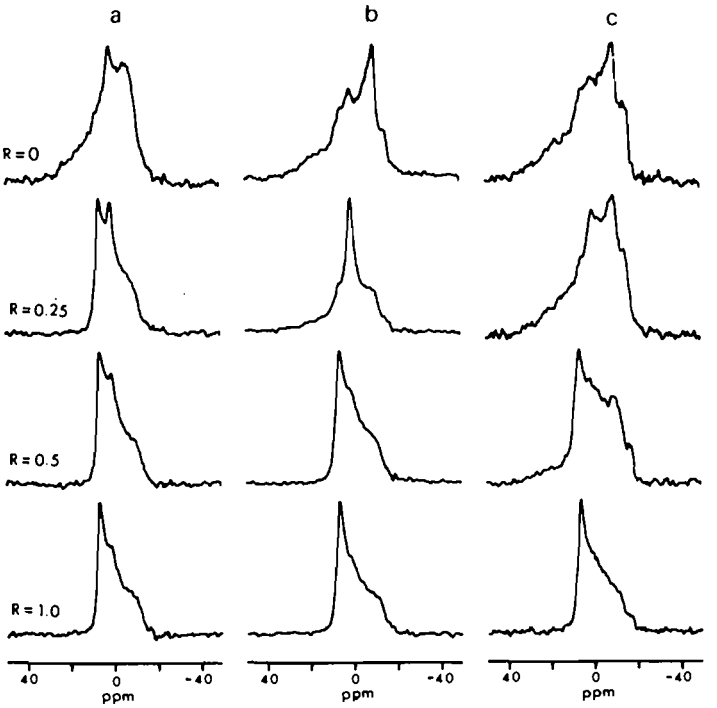

Fig. 2. 81.0 MHz ^{31}P-NMR spectra obtained at 30°C from aqueous dispersions of mixtures of soya phosphatidylserine and soya phosphatidylethanolamine: (a) sample containing 15 mol% phosphatidylserine; (b) sample containing 30 mol% phosphatidylserine; and (c) sample containing 50 mol% phosphatidylserine. The ratios R refer to the molar ratios of Ca^{2+} to phosphatidylserine, where the Ca^{2+} was added to the preformed lipid system as aliquots of a 100 mM stock solution. Other conditions as for Fig. 1.

The ability of 15 mol% or more (soya) phosphatidylserine to stabilize a bilayer arrangement of mixed systems containing soya phosphatidylethanolamine is demonstrated in Fig. 2, which also indicates the influence of Ca^{2+}. It may be noted that the larger effective chemical shift anisotropy $\Delta\sigma_{CSA}^{EFF}$ (measured as the spectral separation between the high field peak and low field shoulder of the bilayer ^{31}P-NMR lineshape) of phosphatidylserine (approx. 54 ppm, see Fig. 1a) as compared to the value of 42 ppm for phosphatidylethanolamine in the bilayer organization (see Fig. 1 of Ref. 1) can result in partial resolution of the high field peaks of these two phospholipids in the mixed lipid systems, particularly in the absence of Ca^{2+}. As the molar ratio of Ca^{2+} to phosphatidylserine is increased, the ^{31}P-NMR spectra reflect a transition from a bilayer to a hexagonal (H_{II}) organisation. In the system containing 15 mol% phosphatidylserine a predominantly H_{II} ^{31}P-NMR spectrum is observed at Ca^{2+} : phosphatidylserine ratios of 0.25, whereas in the mixtures containing 30 and 50 mol% phosphatidylserine Ca^{2+} : phosphatidylserine ratios of 0.5 and 1.0, respectively, were required to produce equivalent effects.

An effect that was particularly noticeable for the systems containing 15 and 30 mol% phosphatidylserine is the narrow spectral feature at 0 ppm (indicative of isotropic motional averaging) at Ca^{2+} concentrations below those required to induce predominantly H_{II} phase structure. The observation of such

an intermediate is a common feature of lipid systems progressing from the bilayer to the hexagonal (H_{II}) arrangement, which may occur as a result of temperature variation in phosphatidylethanolamine/phosphatidylcholine/cholesterol systems [16] or as a result of Ca^{2+} addition to cardiolipin systems [4]. As a result, it is tempting to ascribe this narrow resonance to the occurrence of intrabilayer inverted micellar structures (lipidic particles [17]) which appear to be able to participate as intermediates in bilayer to H_{II} transitions [18]. However, this resonance could also arise due to the presence of smaller (diameter less than approx. 2000 Å) lipid structures which maintain a bilayer organisation [19].

The mechanism whereby Ca^{2+} triggers H_{II} formation in phosphatidylethanolamine/phosphatidylserine systems is naturally of interest. Given the ability of Ca^{2+} to induce structural segregation of phosphatidylserine in mixed phosphatidylcholine/phosphatidylserine model systems [20], it is logical to suggest that such segregation effects also occur in phosphatidylethanolamine/phosphatidylserine systems. This would remove the bilayer-stabilizing capacity of the phosphatidylserine and allow the phosphatidylethanolamine to revert to its preferred H_{II} organization. In this regard it has been noted elsewhere [6] that Ca^{2+} phosphatidylserine complexes display much longer T_1 values (approx. 16 s, as opposed to approx. 0.5 s in the absence of Ca^{2+}) and much broader 'rigid lattice' ^{31}P-NMR spectra than does phosphatidylserine in the liquid crystalline lamellar organisation. Under our experimental conditions it may be calculated that, in the presence of Ca^{2+}, the ^{31}P-NMR signal intensity obtained from phosphatidylserine (in the 'cochleate' form) in the spectral region normally occupied by the bilayer lineshape will be less than 2% of that in the absence of Ca^{2+}, and thus the signal effectively disappears. This is demonstrated in Fig. 3, which shows the spectral intensity observed for pure egg phosphatidylserine and soya phosphatidylserine dispersions as a function of added Ca^{2+}. In the case of egg phosphatidylserine the ^{31}P-NMR signal is virtually eliminated at Ca^{2+} : phosphatidylserine molar ratios of 0.5, whereas for soya phosphatidylserine greater than equimolar ratios of Ca^{2+} are required to produce equivalent effects. The results for egg phosphatidylserine are consistent with previous results [6] indicating a 1 : 2 Ca^{2+} to phosphatidylserine stoichiometry in the condensed 'cochleate' precipitate. The higher Ca^{2+} content required to eliminate the soya phosphatidylserine resonance may be tentatively attributed to the polyunsaturated nature of this lipid species. Fatty acid analysis of egg phosphatidylserine revealed the following composition (by wt.): 16 : 0 (19%); 18 : 0 (30%); 18 : 1 (17%); 18 : 2 (10%); 20 : 4 (17%). Soya phosphatidylserine is clearly more unsaturated: 16 : 0 (24%); 18 : 0 (4%); 18 : 1 (12%); 18 : 2 (52%); suggesting that more unsaturated species pack less easily into the condensed cochleate structure.

The extension of these results and techniques to mixed (soya) phosphatidylethanolamine/phosphatidylserine (1 : 1) systems is indicated in Fig. 4. Clearly, the influence of Ca^{2+} on the ^{31}P-NMR spectral intensity obtained from these mixed systems closely parallels the effects of Ca^{2+} on the corresponding pure phosphatidylserine system. Such effects are therefore consistent with a Ca^{2+}-induced structural segregation of phosphatidylserine into separate domains in the mixed lipid system, leaving (H_{II} phase) phosphatidylethanolamine as the

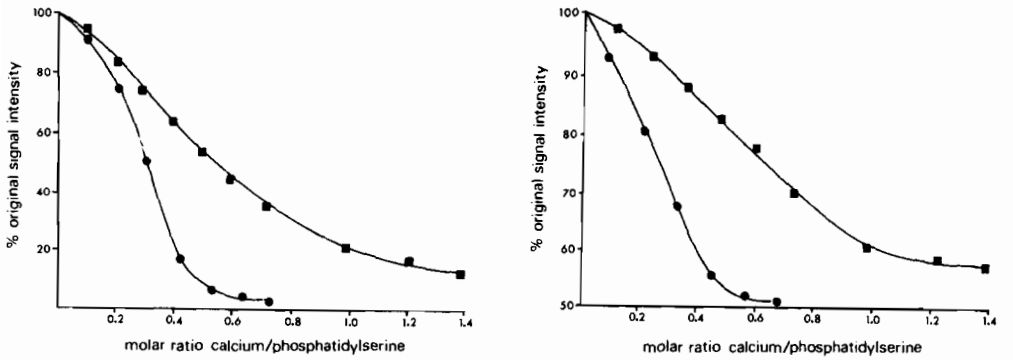

Fig. 3. Integrated ^{31}P signal intensity of egg phosphatidylserine (●) and soya phosphatidylserine (■) as a function of calcium content. For details of protocol see Materials and Methods.

Fig. 4. Integrated ^{31}P NMR signal intensity for soya phosphatidylethanolamine/egg phosphatidylserine (1 : 1) mixtures (●) and soya phosphatidylethanolamine/soya phosphatidylserine (1 : 1) mixtures (■) as a function of externally added calcium. For details of protocol see Materials and Methods.

dominant ^{31}P-NMR spectral feature at higher Ca^{2+} contents.

In previous work it has been shown that Mg^{2+} is unable to trigger bilayer-H_{II} transitions in egg phosphatidylethanolamine-bovine brain phosphatidylserine dispersions [12] and is also unable to induce crystalline 'cochleate' structures in pure phosphatidylserine dispersions [6,21]. In agreement with these results, soya phosphatidylethanolamine/soya phosphatidylserine (1 : 1) dispersions remained bilayer even at Mg^{2+} : phosphatidylserine ratios of 10 : 1 (Fig. 5).

An important aspect of the influence of Ca^{2+} on model systems containing phosphatidylserine concerns the concentrations of Ca^{2+} (rather than the molar ratios of Ca^{2+} to phosphatidylserine) which are required to induce the structural transitions observed. In the ^{31}P-NMR experiments it is most convenient to employ rather high lipid concentrations (50 μmol in 0.7 ml) and thus the addition of Ca^{2+} to obtain Ca^{2+} : phosphatidylserine ratios (R) in the range R = 0.25—1.0 corresponds to relatively high Ca^{2+} concentrations. For example, in order to obtain $R = 1$ for the equimolar phosphatidylethanolamine/phosphatidylserine systems, the Ca^{2+} concentration in the NMR tube was 25 mM. Even to obtain $R = 0.25$ for the 85% phosphatidylethanolamine/15% phosphatidylserine system required a Ca^{2+} concentration of 3 mM. A first approach to this problem was made employing a dialysis procedure as indicated in Materials and Methods. As shown in Fig. 6 for dispersions of 80 mol% soya phosphatidylethanolamine/20 mol% soya phosphatidylserine, more than 2 mM Ca^{2+} was required before significant H_{II} phase structure was induced. Similar results were obtained for a system containing 50 mol% soya phosphatidylserine, although the H_{II} phase formation for 5 mM Ca^{2+} was less complete. These results indicate that as an upper limit, 5 mM Ca^{2+} is required before Ca^{2+}-induced lateral segregation of the phosphatidylserine component occurs. This is not necessarily a lower limit, however, due to limitations of the dialysis technique as well as the multilamellar nature of the liposomal systems, which give rise to associated permeability problems. In order to ascertain the minimum Ca^{2+} concentrations

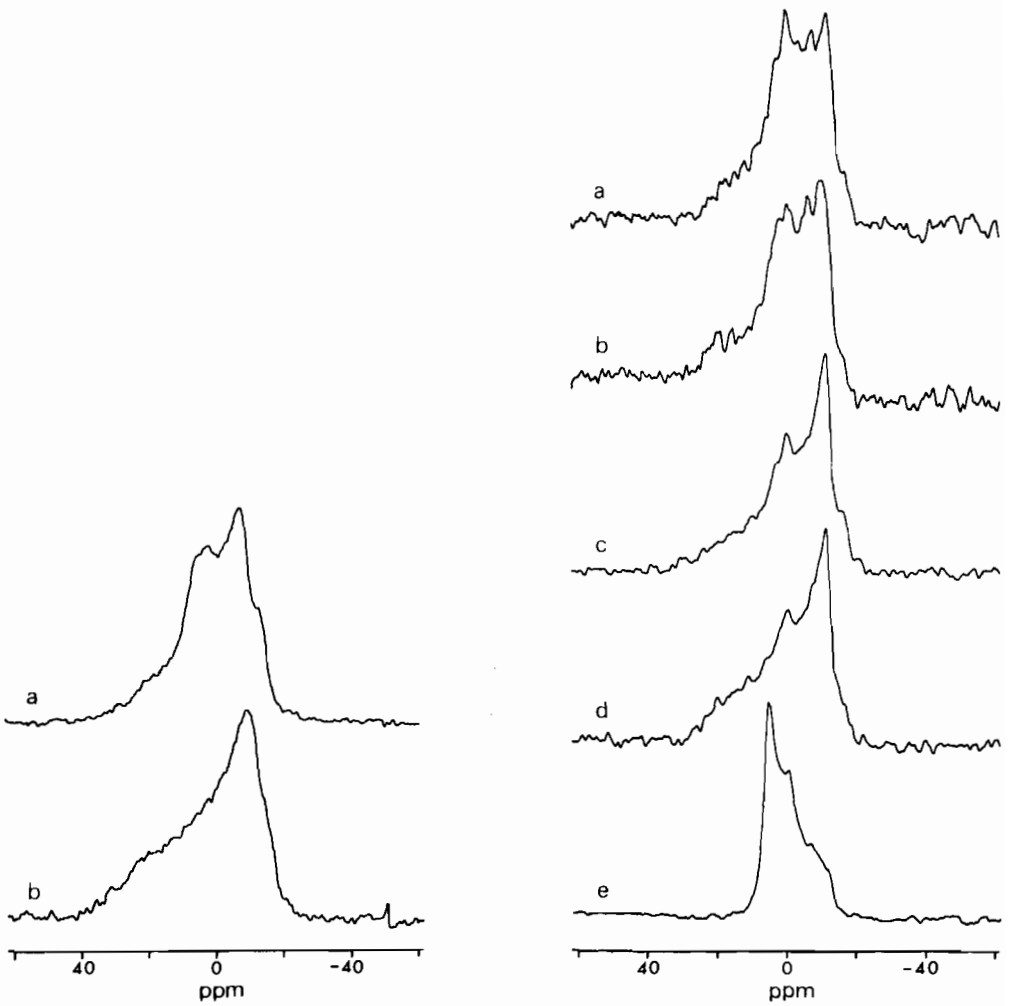

Fig. 5. 81.0 MHz ^{31}P-NMR spectra at 30°C arising from an aqueous dispersion of soya phosphatidylethanolamine/soya phosphatidylserine (1 : 1) mixtures (a) in the absence of Mg^{2+} and (b) in the presence of a 10-fold molar excess of Mg^{2+} with respect to phosphatidylserine. Spectra were collected as for Fig. 1.

Fig. 6. 81.0 MHz ^{31}P-NMR spectra at 30°C obtained from aqueous dispersions of 80 mol% soya phosphatidylethanolamine/20 mol% soya phosphatidylserine mixtures after dialysis against buffers containing $CaCl_2$ in the following concentrations: (a) 0.1 mM; (b) 0.5 mM; (c) 1.0 mM; (d) 2.0 mM; (e) 5.0 mM. For details of protocol see Materials and Methods.

required to form Ca^{2+}-phosphatidylserine (1 : 2) complexes, binding studies were therefore performed employing ^{45}Ca and soya phosphatidylserine (see Materials and Methods). The results obtained in the presence and absence of 100 mM NaCl are presented in Fig. 7, and show half maximal binding of Ca^{2+} to phosphatidylserine at 0.3 mM in the absence of salt, which is increased to 0.8 mM when 100 mM NaCl is present. These results indicate a minimum Ca^{2+} concentration of approximately 1—2 mM before the phosphatidylserine is fully complexed with Ca^{2+}, which correlates closely with the Ca^{2+} concentrations

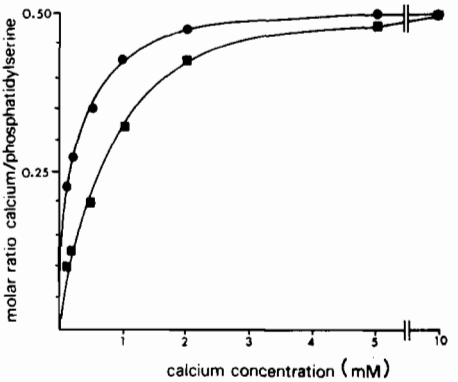

Fig. 7. Stoichiometry of Ca^{2+} binding to soya phosphatidylserine as a function of Ca^{2+} concentration (a) in the absence and (b) in the presence of 100 mM NaCl. Binding assays employing ^{45}Ca were performed as indicated in Materials and Methods.

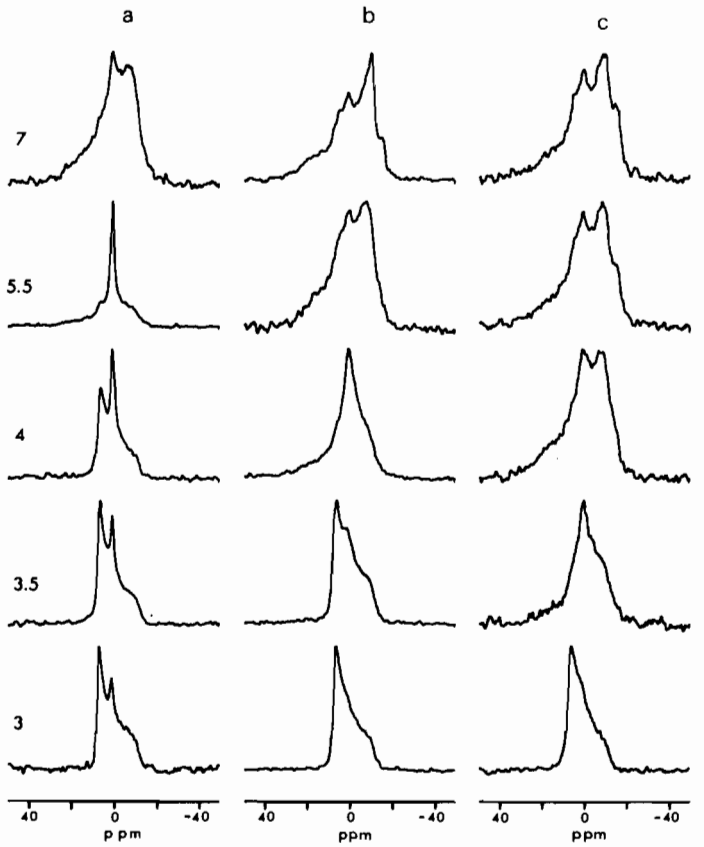

Fig. 8. 81.0 MHz ^{31}P-NMR spectra at 30°C obtained from aqueous dispersions of soya phosphatidylethanolamine containing (a) 15 mol% soya phosphatidylserine; (b) 30 mol% soya phosphatidylserine; (c) 50 mol% soya phosphatidylserine, at pH 7.0, 5.5, 4.0, 3.5 and 3.0. The pH was adjusted as indicated in Materials and Methods and the spectra were collected and analyzed as in Fig. 1.

required to induce fusion of phosphatidylserine vesicles [22] with associated permeability changes [22,23].

In previous work [6] it was shown that egg phosphatidylserine (above 40°C) adopts the hexagonal (H_{II}) phase below pH 4.0. In mixed systems where bilayer structure is stabilized by phosphatidylserine it would therefore be expected that reduction of the pH will also result in H_{II} phase formation. That this is the case is illustrated in Fig. 8 for soya phosphatidylserine/soya phosphatidylethanolamine mixtures containing 15, 30 and 50 mol% phosphatidylserine. For mixtures containing 50 mol% soya phosphatidylserine, between pH 4 and 7 the bulk of the lipid remains in the bilayer phase. At pH 3.5 an intermediate state is evidenced where isotropic motion occurs, whereas at pH 3 hexagonal (H_{II}) structure appears. For mixtures containing 15 and 30 mol% soya phosphatidylserine, a predominantly H_{II} phase occurs at pH 4 and 3.5, respectively, and there is an indication of some H_{II} phase structure at pH 5.5 for the system containing 15 mol% phosphatidylserine. The narrowness of the 0 ppm resonance for mixtures containing 15 mol% soya phosphatidylserine is suggestive of the formation of small vesicles since for these mixtures, at pH 5.5 and lower, the suspensions were translucent. Upon reversal of the pH to 7, it was not possible to reattain spectra identical to those obtained prior to variation of the pH for any of the mixtures, the spectra being dominated by a resonance at 0 ppm, although with subsequent freeze thawing it was possible to re-establish the original spectra.

Discussion

The results of this investigation illustrate clearly the sensitivity of the structural preferences of model systems composed of unsaturated phosphatidylserine and phosphatidylethanolamine to both the amount of phosphatidylserine incorporated as well as environmental factors such as Ca^{2+} concentration and pH. We discuss these results with regard to the structural and functional roles of phosphatidylserine in biomembranes with particular emphasis on fusion phenomena.

The observation that 15 mol% or more phosphatidylserine is able to induce a bilayer configuration for (H_{II} phase) phosphatidylethanolamine is clearly consistent with a structural 'bilayer-stabilizing' role of phosphatidylserine in vivo, serving to maintain membrane integrity. This behaviour is consistent with results obtained for other neutral (phosphatidylcholine [24], sphingomyelin [14] and charged phospholipids (including phosphatidylglycerol [25] and cardiolipin [26]) which adopt the bilayer phase in isolation and exhibit similar bilayer-stabilizing abilities in the presence of non-bilayer lipid.

An interesting feature of mixed lipid systems where the bilayer phase is stabilized by the presence of an acidic phospholipid species concerns the sensitivity of such systems to the presence of divalent cations such as Ca^{2+}. In particular in phosphatidylglycerol/phosphatidylethanolamine mixtures [23], cardiolipin/phosphatidylethanolamine systems [24] as well as the phosphatidylserine/phosphatidylethanolamine dispersions as described here and elsewhere [12], Ca^{2+} is able to trigger bilayer hexagonal (H_{II}) polymorphic phase transitions. However, in each of these systems the detailed mechanism involved

differs. In the cardiolipin-containing systems, for example, Ca^{2+} converts the cardiolipin from a bilayer to an H_{II}-preferring species. Alternatively, in systems stabilized by phosphatidylglycerol, the presence of Ca^{2+} does not appear to affect the lateral distribution or phase preferences of the phosphatidylglycerol component, but merely reduces its ability to stabilize a bilayer arrangement, allowing the H_{II} preference of the phosphatidylethanolamine to predominate. Both of these situations contrast with the phosphatidylserine-containing systems, where Ca^{2+}-induced lateral segregation of phosphatidylserine allows the phosphatidylethanolamine to revert to the H_{II} organization it prefers in isolation

It is of interest to extrapolate these observations to consider possible functional roles of phosphatidylserine in Ca^{2+}-dependent membrane fusion processes. Other investigators [27] suggest that phosphatidylserines can play a critical role in fusion events by forming an interbilayer anhydrous Ca^{2+} complex, which would serve to bring adjacent membranes into close proximity. Fusion is then envisaged to occur employing the dislocation boundaries between crystalline phosphatidylserine · Ca^{2+} domains and surrounding liquid crystalline lipid as initiation sites. It is our contention, on the other hand, that membrane fusion proceeds via formation of non-bilayer structures such as inverted micelles (lipidic particles [16,17]) or elongated versions thereof, corresponding to short segments of inverted cylinders (H_{II} phase structure). An appealing feature of the latter mechanism is its generality, in that any influence which would be expected to favour formation of these non-bilayer alternatives for a portion of the endogeneous lipid would be expected to promote fusion, irrespective of the presence of a particular lipid species such as phosphatidylserine or even Ca^{2+}. Thus in cardiolipin/phosphatidylcholine model systems the presence of Ca^{2+} converts the cardiolipin to an H_{II}-preferring species and promotes fusion, with the associated appearance of lipidic particles (inverted micelles) at the fusion interface [9]. Alternatively, in the phosphatidylethanolamine/phosphatidylcholine vesicles systems an increase of temperature (to a temperature at which the phosphatidylethanolamine would adopt the H_{II} phase in isolation) again results in fusion and appearance of lipidic particles which are a ubiquitous feature of mixtures of bilayer and H_{II} phase lipid systems [7].

We therefore suggest that the rapid, Ca^{2+}-stimulated fusion observed for systems containing phosphatidylserine and phosphatidylethanolamine [28] may arise at least in part from the lateral segregation of the Ca^{2+} · phosphatidylserine, allowing the non-bilayer preferences of the phosphatidylethanolamine component to be expressed. It must be noted, however, that this may not be the whole story as Ca^{2+}-stimulated fusion can occur between model systems containing phosphatidylserine [29] where Ca^{2+} does not induce appreciable non-bilayer 'inverted' lipid structures. It is conceivable that the apparently anhydrous nature [27] of the phosphatidylserine-Ca^{2+} complexes encourages close apposition of adjacent bilayers, and that this condition alone is sufficient for fusion. In this regard lipids which prefer an H_{II} configuration hydrate poorly compared to bilayer phospholipids, and thus conditions which favour H_{II} phase formation would also be expected to favour aggregation and possibly fusion of membrane-bound systems by a similar rationale. We consider it most likely, however, that both conditions are important; firstly, regions of poorly

hydrated lipid which encourage close proximity of adjacent bilayers, and secondly, an ability to form non-bilayer structures as intermediates in the fusion event. It should be noted that only a very small fraction of lipid is required to participate in such intermediates and that almost all lipids (including phosphatidylcholine [30]) adopt H_{II} and other non-bilayer phase structures under appropriate conditions of hydration, temperature and pH [7]. Thus the absence of a typical H_{II}-preferring lipid species does not exclude the possibility that 'inverted' intermediate structures can occur in local environments.

The influence of pH on the polymorphism of phosphatidylserine/phosphatidylethanolamine systems is of interest with regard to a recent report [31] showing enhanced fusion between inner mitochondrial membrane preparations and model systems where phosphatidylserine is a major acidic phospholipid, as well as the pH-sensitive fusion processes occurring in secondary lysosomes. In the latter systems, fusion reactions between entrapped viruses and the lysosome are inhibited above pH 6 [32]. In both these cases, it may be that lower pH values permit non-bilayer structures to be assumed, thus promoting fusion processes. It may be suggested that the appearance of H_{II} phase structure as the pH is lowered arises due to the reduced affinity of phosphatidylserine for the bilayer organisation at lower pH values, as phosphatidylserine in isolation adopts the hexagonal H_{II} phase below pH 3.5 [16]. It would be expected that the effects of Ca^{2+} and lowering the pH would be synergistic, and that the polymorphism of relatively unstable systems (such as those containing only 15 mol% phosphatidylserine) would be particularly sensitive to variations in these parameters. In addition, the ability of salt to modulate the binding of Ca^{2+} to phosphatidylserine in these systems would also be expected to modulate the phase preferences of these systems. These possibilities are currently under active investigation.

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