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STRUCTURAL PREFERENCES OF PHOSPHATIDYLINOSITOL AND PHOSPHATIDYLINOSITOL-PHOSPHATIDYLETHANOLAMINE MODEL MEMBRANES

INFLUENCE OF Ca^{2+} AND Mg^{2+}

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The structural preferences of soya phosphatidylinositol in isolation and in mixtures with soya phosphatidylethanolamine, and the influence of Ca^{2+} and Mg^{2+} on these preferences, have been examined employing ^{31}P-NMR and freeze-fracture techniques. It is shown that phosphatidylinositol assumes the bilayer organization on hydration both in the presence and absence of Ca^{2+} and Mg^{2+}. In mixed systems with (H_{II} phase) phosphatidylethanolamine, phosphatidylinositol induces lipidic particle structure at low (<10 mol%) concentrations and bilayer structure at higher levels. In systems containing 15 or 20 mol% phosphatidylinositol, Ca^{2+} (but not Mg^{2+}) can induce H_{II} phase structure. The results indicate that phosphatidylinositol is a more effective agent than other acidic phospholipids for stabilizing bilayer structure, particularly when high levels of divalent cations are present. These findings are discussed in terms of functional roles of phosphatidylinositol and mechanisms whereby Ca^{2+} induces structural reorganizations in mixed systems containing acidic phospholipids and phosphatidylethanolamine.

Introduction

The phosphatidylinositol effect accompanying receptor mediated membrane phenomena clearly indicates a direct lipid involvement in cellular responses to external stimuli [1,2]. Briefly, stimulation resulting from the presence of an extracellular ligand (such as acetylcholine or adrenalin) which produce increases in the cytosol levels of Ca^{2+} are accompanied by breakdown of phosphatidylinositol to diacylglycerol. This is subsequently phosphorylated to form phosphatidic acid from which phosphatidylinositol is resynthesized. However, the functional significance of this cyclic process to the stimulatory event is not understood. Two possibilities are apparent, namely (1) that phosphatidylinositol and/or one or more derivatives are directly involved in Ca^{2+} permeation, or (2) that the breakdown and resynthesis of phosphatidylinositol provides feedback regulation of a receptor associated Ca^{2+} translocating protein. Results supporting the former hypothesis have been presented which suggest that phosphatidic acid acts as a Ca^{2+} ionophore [3,4], although other workers [5] indicate that for some systems formation of phosphatidic acid occurs after Ca^{2+} entry.

It is clear that an understanding at the molecular level of the functional roles of phosphatidylinositol and its derivatives requires a detailed understanding of relevant physical properties of the various components. In this regard it has been

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shown [6,7,8] that phosphatidic acid can adopt the hexagonal (H_{II}) phase in the presence of Ca^{2+} and other divalent cations, behaviour consistent with a role as a Ca^{2+} ionophore [9]. In this work we extend such studies to include phosphatidylinositol. Our aim is to determine the structural preferences of hydrated phosphatidylinositol in pure and mixed systems, and to ascertain the sensitivity of these structural preferences to divalent cations. We show that soya phosphatidylinositol prefers a lamellar organization in isolation, maintaining such structure in the presence of Ca^{2+} and Mg^{2+}. In mixed systems with unsaturated (H_{II} phase) phosphatidylethanolamine, phosphatidylinositol is able to stabilize a lamellar organization at lower concentrations than other acidic phospholipids, indicating a pronounced proclivity for the bilayer phase. The addition of Ca^{2+} to systems containing 20 mol% or less phosphatidylinositol can trigger structural reorganizations in these systems, but higher concentrations are required than for phosphatidylethanolamine systems stabilized by other acidic phospholipids.

Materials and Methods

Phosphatidylinositol was purified from crude soya phosphatidylinositol (Sigma, St. Louis, MO) employing first preparative high pressure liquid chromatography (Waters Prep 500) using chloroform/methanol/water/25% ammonium hydroxide (60:40:2:1, v/v) as the mobile phase. This material was further purified employing carboxymethyl-cellulose column chromatography [10] by washing with 10 column volumes of chloroform/methanol (4:1, v/v) and the phosphatidylinositol was subsequently eluted with chloroform/methanol (2:1, v/v). The final product gave one spot on two-dimensional thin-layer chromatography (application of 1 mg material) and was more than 97% pure with respect to phosphorus. The sodium salt was obtained as indicated elsewhere for phosphatidylserine [11]. Soya phosphatidylethanolamine was obtained from soya phosphatidylcholine employing the headgroup exchange capacity of phospholipase D [11] and was purified as indicated elsewhere [12]. Aqueous dispersions of this lipid revealed a bilayer to hexagonal (H_{II}) transition as indicated by ^{31}P-NMR as the temperature was increased through 10°C.

Fatty acid analyses were performed by obtaining the methyl esters, which involved incubating lipid samples at 80°C in 20% (by vol.) borane trifluoride in methanol for 1 h followed by pentane extraction [13]. Fatty acid analyses were then performed using a Hewlett Packard 7610A gas chromatograph (fitted with an ethylene glycol column) operated in the program mode employing a temperature gradient from 150°C to 180°C.

Samples for ^{31}P-NMR studies were prepared from appropriate mixtures of .phospholipid (50 μmol total phospholipid) in chloroform. The chloroform was evaporated under a stream of nitrogen and the sample was then stored under vacuum for 2 h. The lipid was hydrated in 0.9 ml of a buffer (10% ^2H_2O) containing 100 mM NaCl, 10 mM Hepes (pH = 7.4) by vortex mixing. Additions of Ca^{2+} and Mg^{2+} (chloride salts) were performed by introducing appropriate aliquots from 100 mM stock solutions. ^{31}P-NMR spectra were obtained employing a Bruker WP-200 Fourier transform NMR spectrometer operating at 81.0 MHz. Free induction decays were accumulated from up to 1000 transients employing a radiofrequency (rf) pulsewidth of 11 μs, a sweepwidth of 20 or 50 kHz, a 0.8 s interpulse time and gated high power (setting 2 Hz) broad band proton decoupling. An exponential filter corresponding to 50 Hz line-broadening was applied prior to Fourier transformation.

The divalent cation binding determinations were performed at 20°C employing equilibrium dialysis according to established protocols [14]. The extent of Ca^{2+} binding was determined employing ^{45}Ca whereas the extent of Mg^{2+} binding was measured employing a Varian Techtron AA-5 atomic absorption spectrophotometer. In order to ensure an equilibrium distribution of divalent cations the lipid samples used for these binding experiments contained the ionophore A-23187 (E. Lilly Co. Ltd.) at an ionophore to phospholipid molar ratio of 1:100. Control experiments employing ^{31}P-NMR revealed that such ionophore levels did not affect the polymorphic properties of the phospholipids. Samples were also freeze-thawed three times during the 6-h dialysis, again to ensure an equilibrium cation distribution.

Freeze-fracture studies were performed employ-

ing a Balzers BAF 301 apparatus. Samples were quenched from 20°C in the presence of 25% (by vol.) glycerol, and replicas were viewed employing a Phillips 400 electron microscope.

Results

The polymorphic phase preferences of phosphatidylinositol were investigated employing ^{31}P-NMR and freeze-fracture techniques (for review, see Ref. 15). Identification of bilayer or hexagonal (H_{II}) phase structure via ^{31}P-NMR relies on equivalent values of the 'rigid lattice' (no motion) chemical shift anisotropy (CSA) tensor of phospholipid phosphodiesters of various phospholipids. The ^{31}P-NMR spectrum of the anhydrous sodium salt of phosphatidylinositol is shown in Fig. 1a and is effectively identical to the rigid lattice ^{31}P-NMR spectra of phosphatidylcholine [16], phosphatidylserine [11], phosphatidylglycerol [17] and sphingomyelin [12] indicating a similar conformation in the phosphate region for these different phospholipids. On hydration (Fig. 1c) a broad asymmetric ^{31}P-NMR spectrum with a low-field shoulder and high-field peak is observed, which is characteristic of liquid crystalline phospholipids in a bilayer organization [15]. It may be noted that the effective chemical shift anisotropy $\Delta\sigma_{eff}$ (measured as the separation between the low-field shoulder and high-field peak) of approx. 60 ppm is approx. 15–20 ppm broader than is observed for other phospholipid species. This may reflect a slightly different conformation and/or reduced motion in the phosphate region of the phosphatidylinositol polar headgroup.

The addition of a five fold molar excess of

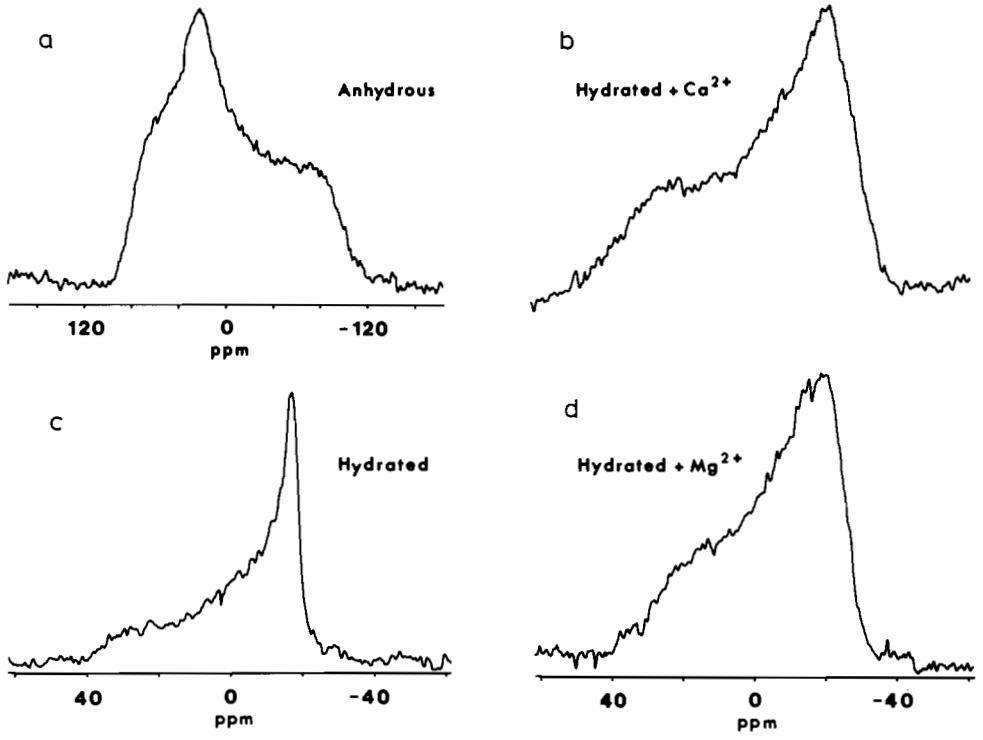

Fig. 1. 81.0 MHz ^{31}P-NMR spectra of soya phosphatidylinositol at 30°C (a) in the anhydrous (sodium salt) form and (c) fully hydrated in the presence of excess aqueous buffer, (b) in the presence of excess buffer and Ca^{2+} to obtain a Ca^{2+}/phosphatidylinositol ratio of 5 mol/mol, and (d) in the presence of a 5-fold molar excess of Mg^{2+}. The spectrum of the anhydrous sodium salt was obtained from 200 μmol phospholipid employing a 50 kHz sweep width, a 20 s interpulse time and high power gated proton decoupling. The remaining spectra were obtained employing 50 μmol phospholipid, a 20 kHz sweep width, a 0.8 s interpulse time and gated proton decoupling.

Ca^{2+} or Mg^{2+} to the phosphatidylinositol dispersion resulted in precipitation of the lipid, but the ^{31}P-NMR spectra maintained the basic lineshape consistent with lamellar structure. It may be noted, however, that the spectra are somewhat broader and exhibit a more pronounced low-field shoulder (with Ca^{2+} producing the largest effects), suggesting reduced local motion in the phosphate region. Such spectra are similar to those observed for phospholipids in the gel phase [12]. The effects of Ca^{2+} and Mg^{2+} on phosphatidylinositol dispersions are comparable to the effects of Ca^{2+} on (unsaturated) phosphatidylglycerol systems [17], and contrast with the influence of Ca^{2+} on cardiolipin [18] and (unsaturated) phosphatidic acid [7,8] for which hexagonal (H_{II}) phase structures can be induced. For phosphatidylserine, on the other hand, Ca^{2+} induces crystalline 'cochleate' structure [19]. An inability of Ca^{2+} to induce such a crystalline structure for phosphatidylinositol may arise in part from the relatively unsaturated nature of the acyl chains (16:0, 28% by wt.; 18:0, 11%, 18:1, 13%; 18:2, 43%; 20:0, 5%). Alternatively, the inability of divalent cations to trigger H_{II} phase formation may be attributed to the large size of the inositol headgroup.

Previous studies have shown that phospholipids preferring the bilayer phase in isolation can stabilize a net bilayer organization in mixtures with unsaturated (H_{II} phase) phosphatidylethanolamines [12,20]. Soya phosphatidylinositol exhibits a similar ability in the presence of (soya) phosphatidylethanolamine (which adopts the H_{II} phase above 10°C) at 30°C as indicated in Fig. 2. The presence of 10 mol% or 15 mol% phosphatidylinositol results in elimination of the hexagonal phase component as indicated by the absence of a peak at 7 ppm which would correspond to the low-field peak arising from H_{II} phase phospholipids. Such phosphatidylinositol concentrations result in a bilayer lineshape on which a narrow symmetric component indicative of phospholipid in structures allowing isotropic motional averaging is superimposed. Finally, the presence of 20 or 50 mol% phosphatidylinositol results in bilayer ^{31}P-NMR lineshapes where two distinct high-field peaks are observed due to the different values of $\Delta\sigma_{eff}$ for phosphatidylinositol ($\Delta\sigma_{eff} \simeq 60$ ppm) and phosphatidylethanolamine ($\Delta\sigma_{eff} \simeq 42$ ppm). The

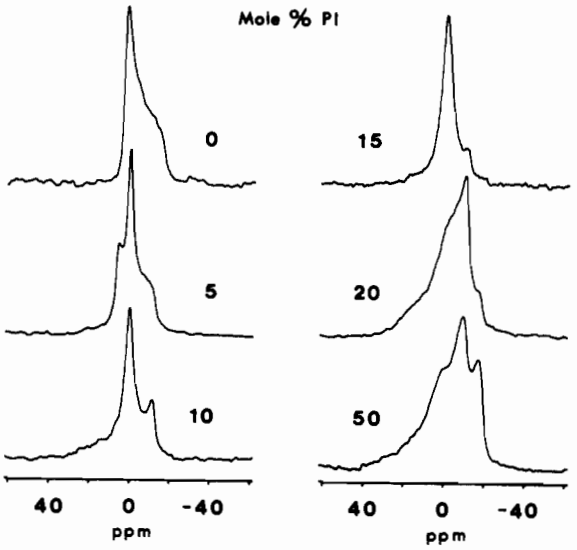

Fig. 2. 81.0 MHz ^{31}P-NMR spectra obtained at 30°C from aqueous dispersions of mixtures of soya phosphatidylinositol and soya phosphatidylethanolamine, where phosphatidylinositol represents 0, 5, 10, 15, 20 and 50 mol% of the lipid mixtures. Spectra were collected as described in the legend to Fig. 1 and Methods.

peak at the highest field may be assigned to phosphatidylinositol.

The origin of the 'isotropic' components observed at low phosphatidylinositol concentrations cannot be ascertained by ^{31}P-NMR. Possibilities include lipidic particles [21,22] (inverted micelles [21,22], intermembrane attachment sites [23,24]) which appear to occur as intermediary structures between the bilayer and H_{II} phases [25], as well as small (diameter < 200 nm) lamellar systems. Freeze-fracture visualizations can give less ambiguous indications of the structures present, however, as indicated in Fig. 3 for systems containing 7.5 mol% phosphatidylinositol (Figs. 3A,B) and 20 mol% phosphatidylinositol (Fig. 3C). The 7.5 mol% mixture reveals large lipid structures with many lipidic particles distributed across the fracture face. Alternatively, at 20 mol% phosphatidylinositol some small bilayer vesicles are apparent and lipidic particles are not observed. Thus the isotropic spectral component may be attributed to lipidic particles and associated macroscopic structures such as the 'honeycomb' arrangement [9] at low (< 10%) phosphatidylinositol contents, and to small vesicu-

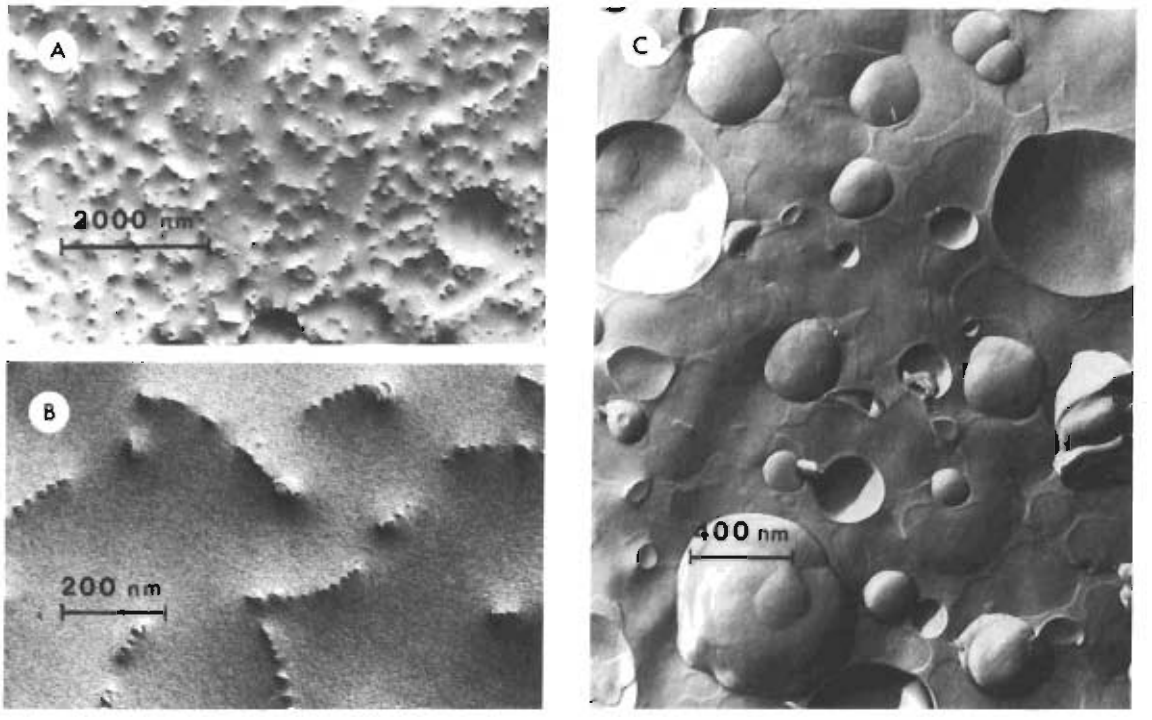

Fig. 3. Freeze-fracture micrographs of aqueous dispersions of (A) soya phosphatidylethanolamine containing 7.5 mol% phosphatidylinositol, (B) the same fracture face at higher magnification, and (C) soya phosphatidylethanolamine containing 20 mol% phosphatidylinositol. Samples were quenched from room temperature in the presence of 25% glycerol.

lar structures at higher concentrations of the bilayer preferring species. It may be noted that the amplitude of the 'isotropic' ^{31}P-NMR component in the systems containing 15 and 20 mol% phosphatidylinositol was somewhat variable (compare spectra of Figs. 2 and 4). This arises due to a variable generation of smaller vesicular structures which appear particularly sensitive to the exact phosphatidylinositol concentration in this range.

We have shown elsewhere that mixtures of acidic phospholipids (such as phosphatidylserine [25], cardiolipin [27] and phosphatidylglycerol [17]) with phosphatidylethanolamine are sensitive to the presence of Ca^{2+} (and in some cases Mg^{2+}) which can trigger bilayer to hexagonal (H_{II}) phase transitions, in these systems. As indicated in Fig. 4, Ca^{2+} can also induce H_{II} phase structure in (soya) phosphatidylethanolamine systems where bilayer structure has been stabilized by the presence of 15 or 20 mol% phosphatidylinositol. There are, however, some rather unique features. First, the presence of low Ca^{2+} levels (Ca^{2+}/phosphatidylinositol molar ratios $R = 0.25$) results in the formation of structures allowing isotropic motional averaging. These structures are not intermediates between bilayer and H_{II} structures, however, as higher levels of Ca^{2+} ($R = 0.5$) results in precipitation of the phospholipid dispersion and a ^{31}P-NMR spectrum characteristic of bilayer structure. Freeze-fracture studies were therefore performed on the $R = 0.25$ samples, revealing the presence of small apparently bilayer vesicles with an average diameter of less than 200 nm. Thus the 'isotropic' ^{31}P-NMR components may be attributed to vesicular systems generated on addition of Ca^{2+}.

The second feature of interest concerns the high levels of Ca^{2+} required to induce H_{II} phase structure as revealed by ^{31}P-NMR. The addition of Ca^{2+} to attain $R = 5.0$ (equivalent to a Ca^{2+} concentration of 30 mM) results in predominantly H_{II} phase structure for the 15% phosphatidylinositol sample. However, the presence of such

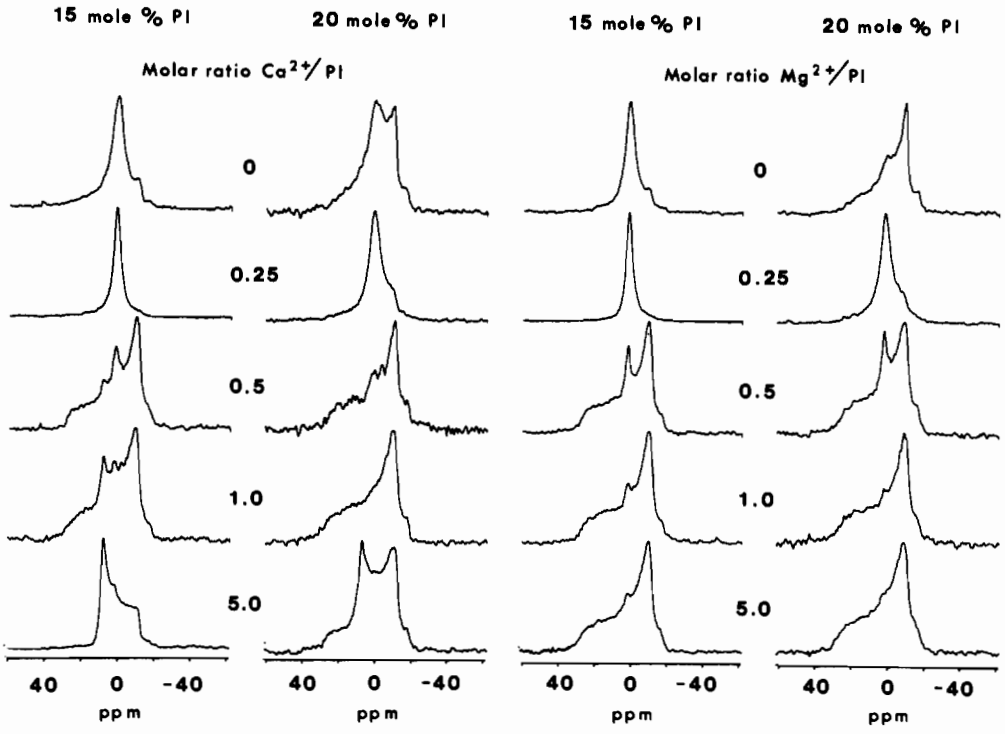

Fig. 4. 81.0 MHz ^{31}P-NMR spectra at 30°C obtained from aqueous dispersions of soya phosphatidylethanolamine containing 15 and 20 mol% phosphatidylinositol. The ratios R refer to the molar ratios of Ca^{2+} or Mg^{2+} to phosphatidylinositol where the divalent cation was added to the hydrated lipid systems as aliquots from a 100 mM stock solution. Other conditions as for Fig. 1.

Ca^{2+} levels in the 20 mol% phosphatidylinositol sample only results in a minority (approx. 20%) H_{II} phase component, the large majority (80%) of the phospholipid remaining in the bilayer organization. At higher phosphatidylinositol concentrations Ca^{2+} did not induce any H_{II} organization. These results contrast strongly with the behaviour of other acidic phospholipid-soya phosphatidylethanolamine mixtures, where such Ca^{2+} concentrations can induce apparently complete H_{II} organization in systems containing 30 mol% (soya) phosphatidylserine [26], 30 mol% (egg) phosphatidylglycerol [17] or 30 mol% (beef heart) cardiolipin [27]. This would suggest that phosphatidylinositol is a particularly effective bilayer stabilizing agent in the presence of Ca^{2+}.

A third point concerns the influence of Mg^{2+} on these systems which is also illustrated in Fig. 4. Again, at low Mg^{2+} concentrations ($R = 0.25$) a large symmetric resonance indicating structures allowing isotropic motional averaging is observed, which, by analogy with the Ca^{2+} induced behaviour may be attributed to a population of small lamellar vesicles. At higher Mg^{2+} levels, however, Mg^{2+} does not induce H_{II} phase components. Such behaviour is similar to that observed in (soya) phosphatidylethanolamine-phosphatidylserine systems [26] and indicates some specificity in the Ca^{2+}-phosphatidylinositol interaction. The different effects of Mg^{2+} and Ca^{2+} do not appear to be due to different affinities of Mg^{2+} and Ca^{2+} for phosphatidylinositol, or, indeed, phosphatidylserine as indicated by the results of equilibrium dialysis experiments presented in Fig. 5. The binding curves for Mg^{2+} and Ca^{2+} are clearly similar and do not explain the differing abilities of Mg^{2+} and Ca^{2+} to induce H_{II} phase structure at concentrations of $R = 5.0$, corresponding to cation concentrations of more than 30 mM. Such concentrations are well above the concentrations at which maximal binding is observed (2–3 mM). It would appear that the natures of the Ca^{2+}-

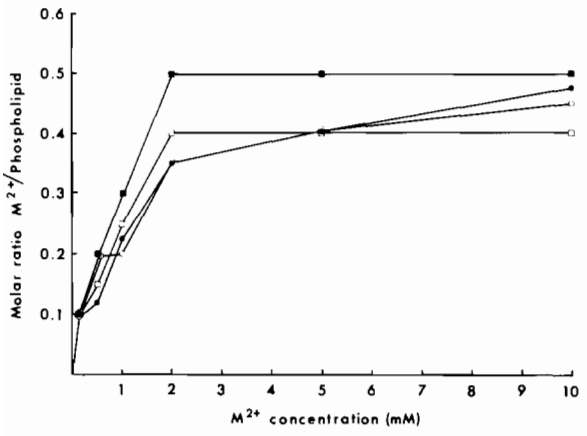

Fig. 5. The ratio of bound cation/phospholipid following equilibrium dialysis against various concentrations of Ca^{2+} or Mg^{2+}. ■, Ca^{2+} and phosphatidylserine; □, Ca^{2+} and phosphatidylinositol; ●, Mg^{2+} and phosphatidylserine; ○, Mg^{2+} and phosphatidylinositol. Experimental conditions are described in Materials and Methods

phosphatidylinositol and Mg^{2+}-phosphatidylinositol complexes formed are different. One possibility may be that the Ca^{2+} complexes tend to segregate more readily, thus effectively reducing the distributed concentration of the bilayer stabilizing phosphatidylinositol species.

Discussion

As indicated in the Introduction, the object of this study was to characterize the structural preferences of phosphatidylinositol in pure and mixed model systems. We have shown that phosphatidylinositol prefers a lamellar organization on hydration as indicated by ^{31}P-NMR. Also, whereas both Ca^{2+} and Mg^{2+} precipitate these lipid dispersions and induce effects consistent with reduced local motion in the phosphate region, the ^{31}P-NMR results indicate that bilayer structure is maintained. Further, in mixed systems with soya phosphatidylethanolamine, phosphatidylinositol is a particularly effective agent for stabilizing bilayer structure at 15 mol% and higher concentrations, inducing 'intermediary' structures such as lipidic particles at lower levels. Finally, in systems containing 20 mol% or less phosphatidylinositol Ca^{2+} (but not Mg^{2+}) can trigger H_{II} phase formation. We discuss these observations in terms of roles of phosphatidylinositol as related to the phosphatidylinositol effect as well as the mechanism whereby Ca^{2+} induces structural alterations in these systems.

The predilection of phosphatidylinositol for the bilayer organization both in the presence and absence of Ca^{2+} argues against a dynamic role of phosphatidylinositol per se in Ca^{2+} transport. This is in contrast to the phosphatidic acid generated during the phosphatidylinositol 'response', which can undergo structural reorganization in the presence of divalent cations [6–8] and has been implicated as a Ca^{2+} ionophore [28]. This would be consistent with a role of phosphatidylinositol in vivo which is primarily structural (serving to maintain an intact permeability barrier) but with the added feature that enzymatically generated derivatives can play dynamic roles in transbilayer transport.

The mechanism whereby Ca^{2+} induces a (partial) bilayer to hexagonal (H_{II}) transitions in (soya) phosphatidylethanolamine systems where bilayer organization is stabilized by phosphatidylinositol is of interest as it appears to proceed via a different mechanism than Ca^{2+} induced bilayer-H_{II} transitions in phosphatidylethanolamine systems stabilized by other acidic phospholipids. In systems stabilized by cardiolipin [27] and phosphatidic acid (Farren, S.B., Tilcock, C.P.S. and Cullis, P.R., in preparation) for example, Ca^{2+} triggers H_{II} phase formation by converting the bilayer stabilizing species to a species preferring the H_{II} organization. This contrasts with systems stabilized by phosphatidylserine, where Ca^{2+} segregates the phosphatidylserine into (anhydrous) crystalline domains, allowing the phosphatidylethanolamine to revert to the H_{II} phase it prefers in isolation [26]. Alternatively, in phosphatidylethanolamine systems stabilized by up to 30 mol% (unsaturated) phosphatidylglycerol, Ca^{2+} appears to reduce the bilayer stabilizing capacity of this acidic phospholipid resulting in a direct incorporation into the H_{II} phase matrix [17]. However, the behaviour of systems stabilized by phosphatidylinositol suggests that none of these mechanisms apply. For example, Ca^{2+} does not induce crystalline complexes in pure phosphatidylinositol systems, and therefore does not behave in a comparable manner to phosphatidylserine. Alternatively,

there is no real evidence that phosphatidylinositol actually enters the H_{II} phase matrix, as the system containing 15 mol% phosphatidylinositol in the presence of excess Ca^{2+} (Fig. 4) still appears to have a residual 'bilayer' ^{31}P-NMR component. We suggest that to a limited extent Ca^{2+} is able to segregate phosphatidylinositol in these mixed systems, where the phosphatidylinositol-Ca^{2+} aggregates remain in a hydrated lamellar structure.

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