

Polymorphism of phosphatidylethanolamine-phosphatidylserine model systems: influence of cholesterol and Mg^{2+} on Ca^{2+} -triggered bilayer to hexagonal (H_{II}) transitions

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Previous work has shown that Ca^{2+} can trigger bilayer to hexagonal (H_{II}) polymorphic phase transitions in (unsaturated) phosphatidylserine (PS)-phosphatidylethanolamine (PE) model systems. In this work we examine the influence of cholesterol and Mg^{2+} on the phase preferences of PS-PE systems. Subsequently, the influence of cholesterol and Mg^{2+} on the levels of Ca^{2+} required to trigger bilayer- H_{II} transitions in these mixed systems is studied. It is shown that at 30°C the presence of equimolar (with respect to phospholipid) levels of cholesterol engenders formation of the H_{II} phase for PE-PS systems containing 15 and 30 mol% PS, whereas bilayer structure is maintained for PE-PS-cholesterol (1:1:2) dispersions. However, the polymorphic phase preferences of the latter system are much more sensitive to the presence of monovalent and divalent cations. In the absence of cholesterol, Mg^{2+} and high salt concentrations do not affect the polymorphic phase preferences of PE-PS (1:1) systems. In contrast, 8 mM or higher Mg^{2+} levels or salt concentrations greater than 1.0 M induce H_{II} -phase formation in PE-PS-cholesterol (1:1:2) systems. Further, lower Mg^{2+} concentrations (2 mM) act as a powerful adjunct to Ca^{2+} triggering of H_{II} -phase structure in such systems, reducing the Ca^{2+} concentration required from 4 to 0.25 mM. These results are discussed in terms of Ca^{2+} concentrations required for fusion events and the influence of cholesterol on the structural preferences of the inner monolayer lipids of the erythrocyte membrane.

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Des travaux antérieurs ont montré que le Ca^{2+} peut provoquer des passages de la phase bilamellaire à une phase hexagonale (H_{II}) polymorphe dans les systèmes modèles (insaturés) phosphatidylsérine (PS) - phosphatidyléthanolamine (PE). Nous examinons ici l'influence du cholestérol et du Mg^{2+} sur les choix de phase par les systèmes PS-PE. Par la suite, nous étudions l'influence du cholestérol et du Mg^{2+} sur les taux de Ca^{2+} requis pour déclencher les passages de bicouche à H_{II} dans ces systèmes mixtes. Nous démontrons qu'à 30°C, la présence de quantités équimolaires (quant aux phospholipides) de cholestérol engendre la formation de la phase H_{II} dans les systèmes PE-PS contenant 15 et 30 mol% de PS alors que la structure bilamellaire est maintenue dans les dispersions PE-PS-cholestérol (1:1:2). Cependant, les choix de la phase polymorphe dans ce dernier système sont beaucoup plus sensibles à la présence de cations monovalents et divalents. En absence de cholestérol, le Mg^{2+} et les concentrations salines élevées n'affectent pas le choix de phase polymorphe des systèmes PE-PS (1:1). Par contre, des concentrations de Mg^{2+} 8 mM ou plus élevées ou des concentrations salines plus grandes que 1,0 M induisent la formation de la phase H_{II} dans les systèmes PE-PS-cholestérol (1:1:2). De plus, des concentrations plus faibles (2 mM) de Mg^{2+} agissent comme de puissants auxiliaires du Ca^{2+} pour provoquer la phase H_{II} dans de tels systèmes, réduisant la quantité de Ca^{2+} requise de 4 à 0,25 mM. Nous discutons de ces résultats en fonction des concentrations de Ca^{2+} requises pour les phénomènes de fusion et de l'influence du cholestérol sur les préférences structurales des lipides de la monocouche interne de la membrane thyrocytaire.

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Introduction

Previous work has shown that unsaturated PE's of natural and synthetic origin preferentially adopt the H_{II} configuration at physiological temperatures (1). Alternatively, an acidic (negatively charged) phospholipid

ABBREVIATIONS: H_{II} , hexagonal; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NMR, nuclear magnetic resonance; HEPES, 2-(N-2-hydroxyethyl)piperazin-N'-yl)ethanesulfonic acid; TLC, thin-layer chromatography.

such as PS can stabilize a bilayer organization for mixed PE - acidic phospholipid systems (2-5). Such systems are of interest not only because they can mimic the lipid composition of certain membranes (such as the inner monolayer of the human erythrocyte membrane (6)), but also because their structural preferences (bilayer ↔ nonbilayer) can be modulated or regulated by physiologically relevant factors such as pH and divalent cations (2-7). Given evidence that nonbilayer lipid structures play intermediate roles in membrane fusion processes (7, 8) and their possible involvement in lipid "flip-flop"

and other forms of transbilayer transport (9), an understanding of such regulation could provide potentially important information regarding the functional roles of lipids and the methods by which those functions are regulated.

In this regard it has been shown that bilayer to H_{II} transitions in multilamellar (soya) PE-PS (4: 1) systems can be triggered by 2 mM or higher concentrations of Ca^{2+} (5). Fusion between sonicated unilamellar versions of these systems requires similar levels of Ca^{2+} (10). Such Ca^{2+} concentrations are rather high and would not be expected to occur in most biological systems, particularly in the cytoplasm where the free Ca^{2+} concentrations are less than 100 μM (11). In this communication we therefore examine the influence of two factors which may be expected to act as adjuncts to the Ca^{2+} effect, namely Mg^{2+} and cholesterol. Mg^{2+} does not induce bilayer to H_{II} transitions in PE-PS systems, but does induce aggregation, a step which is vital to obtaining the close apposition between bilayers apparently required before H_{II} -phase formation can proceed (9). In (unsaturated) bilayer PE-PC systems, on the other hand, cholesterol can induce formation of the hexagonal phase (12, 13) and thus may also be considered to facilitate H_{II} -phase formation. We show here that cholesterol can induce H_{II} -phase structure in previous bilayer PE-PS systems and that, in combination, the presence of (2mM) Mg^{2+} and equimolar cholesterol can reduce the Ca^{2+} concentrations required to induce such transitions by more than an order of magnitude.

Materials and methods

Soya PE and soya PS were obtained from soya bean PC employing the head-group-exchange capacity of phospholipase D (14). The PS was purified using carboxymethyl-cellulose column chromatography and subsequently converted to the sodium salt as described previously (15). PE was purified employing silicic acid preparative liquid chromatography. All lipids were shown to be greater than 99% pure as indicated by two-dimensional thin-layer chromatography. Cholesterol was obtained from Sigma (St. Louis, MO). Phospholipase D was partially purified from the inner leaves of savoy cabbage employing established procedures (16).

Samples for ^{31}P -NMR studies were prepared from an appropriate mixture of the lipids in chloroform in a 10-mm NMR tube. Chloroform was evaporated under nitrogen and the sample was then kept under high vacuum for approximately 2 h. The dry lipid mixtures were hydrated with 0.7 mL of buffer (100 mM NaCl, 10% (v/v) 2H_2O , and 10 mM HEPES (pH 7.4)) by vortex mixing at 4°C in the presence of a glass agitator to facilitate lipid dispersal. Samples were titrated with calcium by adding aliquots of 100 mM stock solution of the chloride salt. To ensure equilibration of the divalent cation with the lipid, the samples were subjected to three freeze-thaw cycles employing liquid nitrogen. Samples requiring dialysis were sealed in 0.6-cm-diameter dialysis tubing following

hydration and equilibrated against the required Ca^{2+} and (or) Mg^{2+} concentration at 4°C for 6 h. Control experiments employing ^{45}Ca revealed maximal binding of Ca^{2+} to soya PS occurred within 4 h. A 10-fold molar excess of the divalent cation in the dialysate over PS was utilized in all dialysis experiments. Lipid degradation following the 6 h of dialysis was found to be less than 1%, as determined by TLC.

^{31}P -NMR experiments were performed on a Bruker WP-200 Fourier transform spectrometer. Accumulated free induction decays were obtained from up to 1000 transients employing an interpulse time of 0.8 s, an 11 μs 90° pulse, and a sweep width of 20 kHz. All spectra were recorded employing broad-band proton decoupling at temperature of 30°C.

Results

The phase preferences of the lipid mixtures were monitored employing ^{31}P -NMR techniques as indicated elsewhere (7). Briefly, phospholipids in large (diameter > 2000 Å) bilayer systems exhibit broad asymmetric ^{31}P -NMR spectra with a low field shoulder and high field peak separated by approximately 40 ppm. (See the 30 mol% PS spectrum of Fig. 1 in the absence of cholesterol.) H_{II} -phase phospholipids, on the other hand, exhibit spectra with reversed asymmetry which are a factor of two narrower (see the pure PE spectrum of Fig. 1). Finally, phospholipids in small lamellar systems or nonbilayer structures allowing isotropic motional averaging (such as inverted micelles (17) or cubic phases (18) give rise to narrow, symmetric spectra (see spectrum of Fig. 1 for 30 mol% PS where the cholesterol to phospholipid ratio is 0.2).

The influence of cholesterol on the polymorphic phase preferences of various soya PE – soya PS mixtures is illustrated in Fig. 1. The results presented there clearly illustrate an ability of cholesterol to destabilize a bilayer organization of such systems, particularly at PS contents below 50 mol%. Thus for the 30 mol% PS – 70 mol% PE sample a bilayer line shape is obtained in the absence of cholesterol. Increasing cholesterol contents lead first to a structure allowing isotropic motional averaging and subsequently to the ^{31}P -NMR line-shape characteristic of the H_{II} -phase. This ability of cholesterol to engender H_{II} -phase formation in PE-PS systems is also illustrated by the freeze-fracture micrographs of Fig. 2. Large fracture planes characteristic of bilayer structures are observed for the 30 mol% PS – 70 mol% PE system in the absence of cholesterol (Fig. 2A), whereas the striated pattern characteristic of H_{II} -phase structures is observed in the presence of equimolar cholesterol (Fig. 2B). As indicated earlier, the narrow ^{31}P -NMR resonance may arise from small lamellar structures or nonlamellar structures such as inverted micelles (17) which can play intermediary roles in bilayer- H_{II} transitions (19). Freeze-fracture studies revealed the presence of small (diameter < 1000 Å) vesicles in these systems which appear to form spontaneously on hydration. This

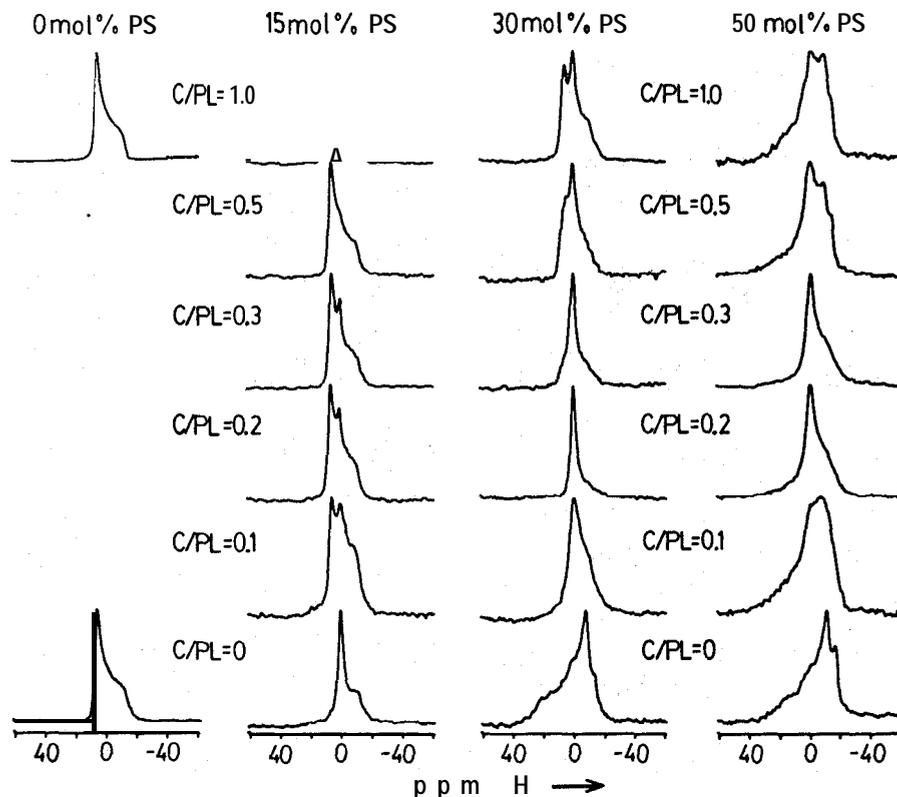


FIG. 1. ^{31}P -NMR spectra (81.0 MHz) obtained at 30°C from aqueous dispersions of soya PE in the presence of varying amounts of soya PS and cholesterol. C/PL refers to the molar ratio of cholesterol to phospholipid. The 0-ppm position corresponds to the chemical shift of sonicated PC vesicles. For other details see Materials and methods.

would be consistent with the behaviour of the systems containing 50 mol% PS, where intermediate ($0.5 < \text{cholesterol/phospholipid} < 0.1$) cholesterol contents appear to generate formation of somewhat smaller lamellar structures, but do not induce H_{II} -phase organization even for cholesterol to phospholipid ratios of 1.0.

As indicated in the Introduction, bilayer to H_{II} transitions can be triggered in PE-PS systems by the addition of Ca^{2+} (2, 5). Given the ability of cholesterol to encourage H_{II} -phase formation, it may be expected that lower Ca^{2+} levels are required to trigger such transitions if cholesterol is present. Results showing this to be the case are illustrated in Fig. 3 for equimolar mixtures of soya PS – soya PE in the presence of varying amounts of cholesterol. Whereas Ca^{2+}/PS ratios R of 0.5 and higher are required to induce appreciable H_{II} -phase formation in the absence of cholesterol, similar effects are observable in samples containing 25 and 50 mol% cholesterol at Ca^{2+} levels as low as $R = 0.125$.

It has been shown previously (5) that in contrast to Ca^{2+} , Mg^{2+} is unable to trigger bilayer- H_{II} transitions in equimolar soya PS – soya PE dispersions even at Mg^{2+}/PS ratios as high as 10.0. However, the presence

of equimolar cholesterol enables Mg^{2+} -induced bilayer- H_{II} transitions to occur as illustrated in Fig. 4 for 8 mM Mg^{2+} concentrations. This leads to the possibility that lower levels of Mg^{2+} can act synergistically with Ca^{2+} to produce Ca^{2+} -induced phase transitions at lower net Ca^{2+} concentrations. Results supporting this possibility are illustrated in Fig. 5 for equimolar soya PS – soya PE systems, which were dispersed in the presence of 2 mM Mg^{2+} and subsequently dialyzed against a buffer containing 2 mM Mg^{2+} and various concentrations of Ca^{2+} (see Materials and methods). The addition of Ca^{2+} had little effect on the structural preference of systems containing no cholesterol. Even at 8 mM Ca^{2+} levels such systems evidenced largely lamellar ^{31}P -NMR lineshapes with a small (10%) H_{II} -phase component superimposed (see Fig. 5). However, some H_{II} -phase formation is visible for Ca^{2+} concentrations as low as 0.25 mM when equimolar (with respect to phospholipid) cholesterol is present. Thus Mg^{2+} and cholesterol in combination can act as important adjuncts for Ca^{2+} -induced phase changes in PS-PE systems.

The ability of Mg^{2+} to induce polymorphic bilayer- H_{II} transitions when cholesterol is present and its in-

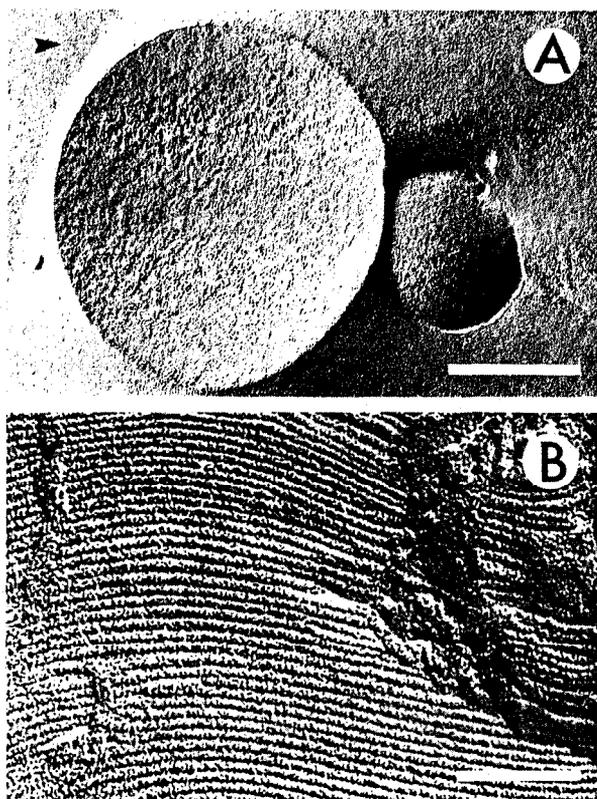


FIG. 2. Freeze-fracture micrographs of soya PE - soya PS mixtures (30 mol% PS) in the presence (B) and absence (A) of equimolar cholesterol. The white bars represent 200 (A) and 100 nm (B). The direction of shadowing is indicated by the arrowhead in each micrograph.

ability to segregate PS in PE-PS systems in the absence of cholesterol suggests either of two possibilities. First, it may be that cholesterol facilitates PS segregation. Alternatively, Mg^{2+} may act to decrease the local charge density at the membrane surface, thereby facilitating H_{II} -phase formation (see Discussion). If the latter possibility is correct, high salt concentrations should also be able to exert similar effects. This is indeed the case as indicated in Fig. 6, where it is shown that 1 M NaCl concentrations cause a large proportion of the PE-PS-cholesterol (1: 1:2) dispersions to adopt the H_{II} configuration, behaviour which does not occur for the systems not containing cholesterol.

Discussion

The data presented here shows clearly that the presence of cholesterol in (unsaturated) PE-PS systems promotes formation of H_{II} -phase structure. This is expressed either as a direct result of the presence of cholesterol, or as an increased sensitivity of the structural preferences of PE-PS systems to the presence of divalent cations or increased ionic strength. We first

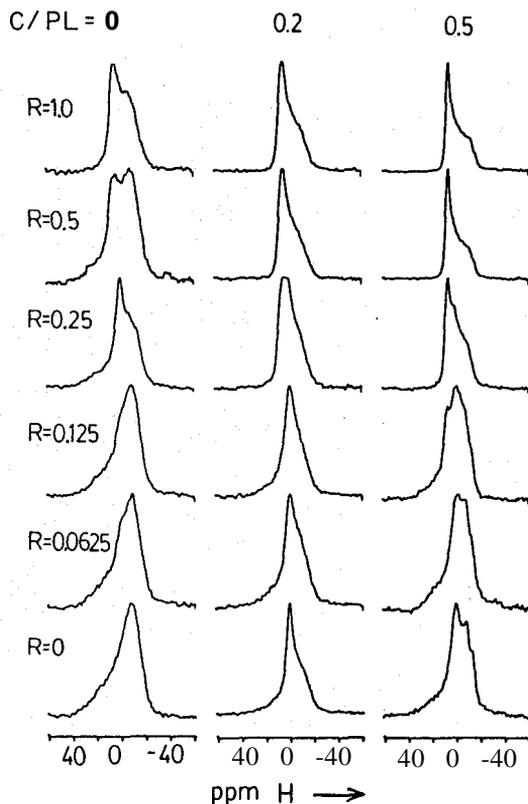


FIG. 3. ^{31}P -NMR spectra (81.0 MHz) obtained at 30°C from aqueous dispersions of equimolar mixtures of soya PS and soya PE in the presence of varying amounts of cholesterol and Ca^{2+} . The ratio C/PL refers to the molar ratios of cholesterol to phospholipid, whereas the ratio R refers to the molar ratio of Ca^{2+} to PS. For details of sample preparation see Materials and methods. The Ca^{2+} was added to the (0.7 mL) aqueous lipid dispersions as aliquots from a 100 mM stock solution of $CaCl_2$.

discuss the mechanism whereby cholesterol causes such effects and subsequently deal with potential biological ramifications.

The ability of cholesterol to destabilize bilayer structure in PS-PE systems is consistent with an ability to exert similar effects in (unsaturated) PC-PE systems (12, 13). In these latter systems, it was suggested (13) that cholesterol has a net cone shape which in combination with PC results in a complex more compatible with H_{II} -phase structure. Such a rationale may also apply to the effects of cholesterol on the PS-PE systems, where the cholesterol has been suggested to associate preferentially with the PS component (20). There are other factors which must be taken into account, however. First, cholesterol may be expected to exert a spacing effect in the mixed systems, thus reducing the electrostatic repulsion between the negatively charged serine head groups and thereby reducing the effective size of the

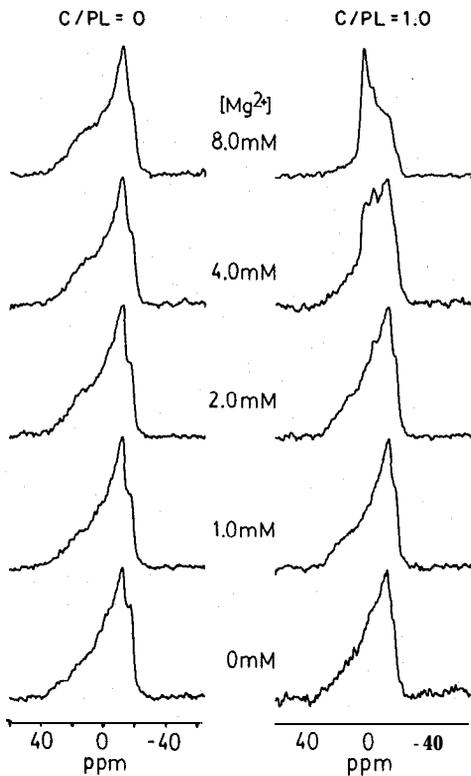


FIG. 4. ^{31}P -NMR spectra (81.0 MHz) obtained at 30°C from aqueous dispersions of equimolar mixtures of soya PS and soya PE after dialysis against the indicated concentrations of Mg^{2+} . The ratio C/PL refers to the molar ratio of cholesterol to phospholipid. For details of sample preparation see Materials and methods.

head group. Second, although the hydration state of cholesterol in a phospholipid matrix is not known, it may be expected to be relatively poorly hydrated in comparison to phospholipids. Cholesterol in water, for example, adopts a (crystalline) monohydrate form. Given that lipids which readily adopt the H_{II} phase hydrate poorly in comparison with "bilayer" phospholipids, the reduction in bound water per unit of membrane surface due to the presence of cholesterol could also play a role in promoting H_{II} -phase structure.

The ability of cholesterol to lower the amount of Ca^{2+} required to trigger bilayer- H_{II} transition in PS-PE dispersions poses interesting problems with regard to mechanism. In earlier work (5), it has been shown that in the absence of cholesterol Ca^{2+} appears to trigger H_{II} -phase formation in these systems by segregating PS into "cochleate" (21) domains, leaving the phosphatidylethanolamine free to adopt the H_{II} phase it prefers in isolation. Such Ca^{2+} -induced segregation may occur to some extent in the presence of cholesterol, but the results obtained when Mg^{2+} is present suggest an alternative possibility. In particular, high levels (50

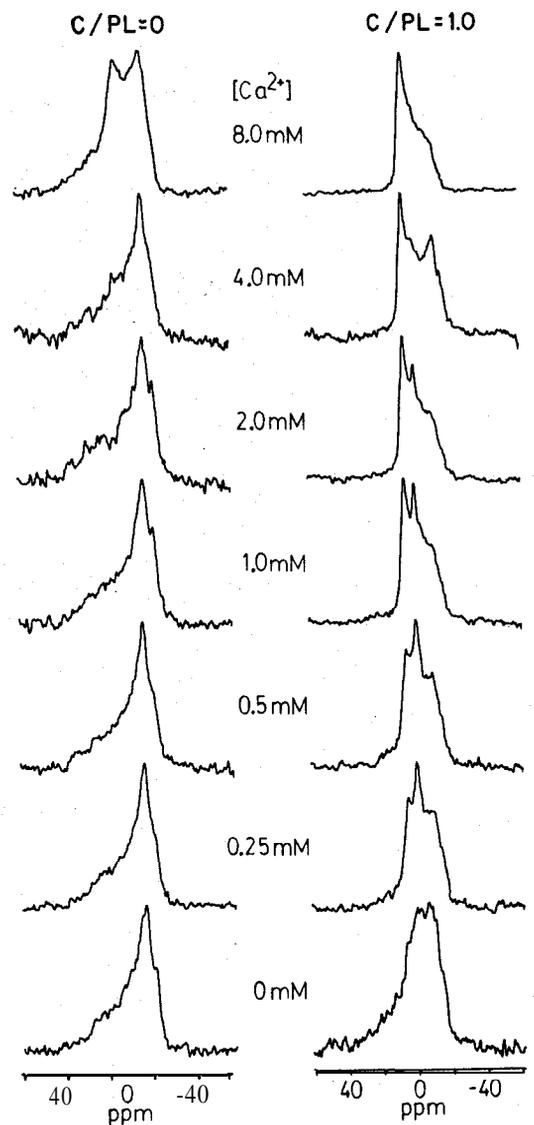


FIG. 5. ^{31}P -NMR spectra (81.0 MHz) obtained at 30°C from aqueous dispersions of equimolar mixtures of soya PS and soya PE after dispersion in the presence of 2 mM Mg^{2+} and subsequent dialysis against a buffer containing 2 mM Mg^{2+} and the indicated concentration of Ca^{2+} . The ratio C/PL refers to the molar ratio of cholesterol to phospholipid. For other details see Materials and methods.

mM) of Mg^{2+} are ineffective for inducing lateral segregation of PS in mixed PS-PC systems (22) and cannot trigger bilayer- H_{II} transition in mixed PS-PE systems (see Ref. 5 and Fig. 4). Thus the ability of 4.0 mM and higher Mg^{2+} concentrations to induce H_{II} -phase structure when cholesterol is present either suggests that cholesterol facilitates the lateral segregation of PS or that it promotes Mg^{2+} -dependent incorporation of the PS into the H_{II} phase. The latter

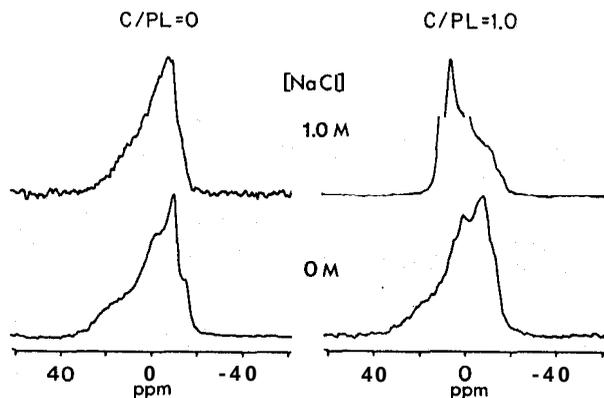


FIG. 6. ^{31}P -NMR spectra (81.0 MHz) obtained at 30°C from equimolar mixtures of soya PS and soya PE in the presence and absence of 1 M NaCl . The ratio C/PL refers to the molar ratio of cholesterol to phospholipid.

possibility appears the most likely as there is no evidence for a Mg^{2+} -induced segregation of PS. In particular, it may be estimated that more than 80% of the phospholipid in the soya PS – soya PE (1:1) systems containing equimolar cholesterol adopt the H_{II} configuration in the presence of 8 mM Mg^{2+} (see Fig. 4). As soya PS in the presence of Mg^{2+} maintains the bilayer organization (as does equimolar PS-cholesterol in the presence of Mg^{2+} ; M. J. Hope, unpublished), Mg^{2+} -induced lateral segregation of PS in the sample of Fig. 4 should leave 50% of the lipid in a lamellar organization, contrary to experiment. We suggest that the presence of Mg^{2+} acts to decrease the charge density at the lipid-water interface. This may be considered to reduce the electrostatic repulsion between serine head groups, thus reducing the effective size of the serine head group and producing a net molecular “shape” more compatible with the H_{II} phase. This would allow the H_{II} -phase preferences of the PE to predominate and would correspond to the situation for Ca^{2+} -triggered H_{II} -phase formation in phosphatidylglycerol-PE systems (3). Experiments are now in progress employing ^2H -labelled PS to establish unambiguously the presence of the PS in the H_{II} organization in these mixed systems.

The Ca^{2+} -induced triggering of H_{II} -phase formation in the soya PS-PE-cholesterol (1:1:2 on a mole basis) systems may have a similar basis as the Mg^{2+} -induced transition. The situation where 2 mM Mg^{2+} acts as an adjunct to the Ca^{2+} effect, lowering the Ca^{2+} concentrations required to 0.25 mM , would be particularly likely to proceed via this charge neutralization mechanism. This is because such concentrations of Ca^{2+} are well below those required to induce lateral segregation of PS in mixtures with PE (5) and even below those required to trigger formation of “cochleate” crystalline PS- Ca^{2+} (2:1) complexes in pure PS systems (5). The ability of

high salt concentrations to induce H_{II} -phase structure in PS-PE-cholesterol (1:1:2) systems also corresponds to charge neutralization effects, and it therefore appears possible that the cation-dependent macroscopic structural alterations of these systems can proceed by a common mechanism which does not necessarily involve lateral segregation of individual components.

The results presented here have important implications for Ca^{2+} -induced fusion processes, as well as the compositions and properties of the inner monolayer of the erythrocyte membrane. We discuss these areas in turn. First, the observation that cholesterol and cytosol levels of Mg^{2+} can reduce the Ca^{2+} concentrations required to induce nonbilayer structures in appropriate systems to $250\text{ }\mu\text{M}$ or less supports our contention (10, 23, 24) that membrane fusion processes *in vivo* proceed by intermediate formation of inverted micelle and (or) inverted cylinder lipid configurations. Previously, the high (nonphysiological) Ca^{2+} levels of 2 mM or more required to induce nonbilayer structures in and induce fusion between mixed PE-PS vesicle systems posed major problems for extrapolating to *in vivo* situations. It should be noted that other factors may act to reduce the Ca^{2+} levels required even further. These include proteins such as synexin (25, 26), which appear to increase the effective Ca^{2+} concentration for fusion between model systems, and we are currently extending our studies to examine the Ca^{2+} levels required to trigger polymorphic transitions when synexin is present.

The second point concerns the composition and structural preferences of the inner monolayer of the erythrocyte membrane. First, it is clear that the sensitivity of the polymorphic preferences of this lipid composition to the presence of divalent cations will be markedly enhanced by the presence of cholesterol. This is in agreement with observations that Ca^{2+} cannot trigger H_{II} -phase formation in model systems mimicking the inner monolayer composition in the absence of cholesterol (M. J. Hope, unpublished observations). Although the transbilayer distribution of cholesterol in the erythrocyte membrane has not been established, it is likely that cholesterol is present at levels approaching equimolar (with respect to phospholipid) proportions, given the ability of cholesterol to redistribute rapidly across bilayer membranes (27). Thus the structural preferences of the inner monolayer may be dictated by relatively low cytosol levels of Ca^{2+} or even by the local cholesterol content.

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