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CALCIUM-INDUCED AGGREGATION AND FUSION OF MIXED PHOSPHATIDYLCHOLINE-PHOSPHATIDIC ACID VESICLES AS STUDIED BY ^{31}P NMR

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Summary

1. The transbilayer distribution of the phospholipids in sonicated egg phosphatidylcholine-phosphatidic acid vesicles and the interaction of Ca^{2+} with these vesicles was studied by ^{31}P NMR.

2. Over a wide composition range the bilayer of these vesicles has a symmetrical phospholipid composition.

3. With ratios of Ca^{2+} to phosphatidic acid in the outer monolayer of the vesicles up to 0.3, Ca^{2+} induces vesicle aggregation. The extent of aggregation is increased by the Ca^{2+} concentration in the medium and the outer monolayer concentration of phosphatidic acid. The vesicle aggregation can be fully reversed by chelating Ca^{2+}.

4. When the ratio exceeds 0.5 Ca^{2+} induces vesicle fusion. The fusion is maximal for vesicles containing both phosphatidylcholine and phosphatidic acid. The data suggest that Ca^{2+}-induced lateral phase separations make the bilayer more susceptible to fusion.

Introduction

In many biological processes membrane fusion plays an important role. The mechanism by which two membranes undergo fusion is still obscure. The presence of calcium ions in the medium is thought to be essential for the fusion process (see ref. 1 and references therein). Divalent cations, in particular Ca^{2+}, have a strong interaction with negatively charged phospholipids leading to an overall rigidifying of the phospholipid molecule [2—6]. The idea that the interaction of Ca^{2+} and acidic phospholipids in the membrane is required for membrane fusion is especially born out by the extensive studies of Papahadjopoulos

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et al. [5—7] on the effect of divalent cations on the structure and physical chemical properties of model membrane systems prepared from neutral and acidic phospholipids. These authors proposed that the interaction of Ca^{2+} and the negatively charged phospholipid causes a transient destabilization of the bilayer which then becomes susceptible to fusion at boundaries between domains of more fluid and more rigid lipids in the bilayer.

Several questions concerning the Ca^{2+}-induced fusion of lipid vesicles prepared from mixed neutral and acidic phospholipids are unanswered: (1) Is the Ca^{2+} concentration required to cause vesicle fusion related to the concentration of the negatively charged phospholipid in the outer monolayer of the vesicle? (2) Is the fusion process preceded by vesicle aggregation and is this vesicle aggregation reversible?

In this study we report ^{31}P NMR measurements on sonicated unilamellar mixed egg phosphatidylcholine-phosphatidic acid vesicles which show (a) that the bilayer is symmetrical in phospholipid composition, (b) that the Ca^{2+}-induced vesicle fusion is preceeded by a reversible vesicle aggregation and (c) that the Ca^{2+} concentration required to cause vesicle fusion is related to the surface concentration of phosphatidic acid in the outer monolayer of the vesicle.

Experimental

Materials

Egg phosphatidylcholine was isolated from hen eggs according to standard procedures. Phosphatidic acid was prepared from egg phosphatidylcholine by the action of partially purified phospholipase D from cabbage [8,9]. The purified phosphatidic acid was subsequently converted to the disodium salt [10]. All other chemicals were of analytical grade.

Methods

Preparation of lipid vesicles. Single bilayer vesicles were prepared by ultrasonication at 0°C of 1.5 ml of a phospholipid dispersion (40 mM phospholipid) in ^2H_2O containing 0.2 mM EDTA to complex possible traces of paramagnetic ions and 25 mM Tris/acetic acid, p^2H 7.0, unless otherwise state [11]. The p^2H of the solution was sometimes slightly decreased by the sonication. In those cases the p^2H was restored by addition of NaOH and resonication of the vesicles. In some experiments the p^2H of the vesicle solution was varied by the addition of small quantities of HCl or NaOH followed by resonication.

The vesicles were centrifuged at 0°C for 30 min at 20 000 $\times g$ in order to remove residual unbroken liposomes and metal particles from the probe.

Gel filtration. Gel filtration of the vesicles on Sepharose 4B (Pharmacia) was performed as described before [12].

Nuclear magnetic resonance (NMR). ^{31}P NMR spectra were recorded on a Bruker WH 90 spectrometer operating at 36.4 MHz on ^{31}P as described before [11]. Accumulated free induction decays were obtained at 25°C, under conditions of 3 W broad band proton decoupling from 500 transients with a 1.7 interpulse time using 90° pulses. A sweep width of 1200 Hz was used. For some samples spectra were recorded, with identical results, using gated decou-

pling with 10-s waiting times between subsequent pulses. A chloroform solution of triphenylphosphine in a central capillary was used as an external reference. The error in the determination of the linewidth and peak intensities is estimated to be 10% maximally.

Analytical methods. Lipid phosphorus was measured after perchloric acid destruction of the lipids via the Fiske Subba Row procedure [13].

Results

Transbilayer distribution of phosphatidylcholine and phosphatidic acid in mixed vesicles. To understand the mechanism of the Ca^{2+}-induced vesicle aggregation and fusion it is essential to know (1) the charge of the phosphatidic acid in the vesicles and (2) the transbilayer distribution of the phospholipids.

The ionisation state of phosphatidic acid was investigated by measuring the pH dependence of the chemical shift of the phosphatidic acid resonance in mixed phosphatidic acid/phosphatidylcholine (1 : 1) vesicles. In Fig. 1A the ^{31}P NMR spectrum of these vesicles at p^2H 7.0 shows that the phosphatidic acid resonance (−5.2 ppm) and the phosphatidylcholine resonance (−4.7 ppm) are well separated and that each resonance is split as was observed previously [14], for other lipid vesicles. This splitting is caused by a slight difference in packing in the polar headgroup region of the lipid molecules in the outer and inner layer of the vesicles. Consequently there is a small difference in chemical shift for the resonance of the molecules in the outher layer (low field peak) and the resonance of the molecules in the inner layer (high field peak). The pH dependence of the low field phosphatidic acid resonance is shown in Fig. 2. In the pH range 7—9 there is a large downfield shift of this resonance demonstrating the protonation of the phosphate group to form a single charged phosphatidic acid

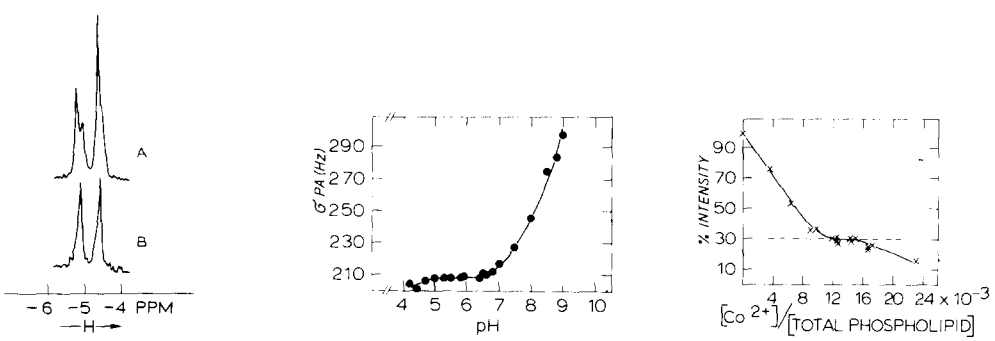

Fig. 1. 36.4 MHz ^{31}P NMR spectra of phosphatidylcholine-phosphatidic acid (1 : 1) vesices in the absence (A) and in the presence (B) of 0.68 mM $CoCl_2$ (Co^{2+}/total phospholipid = 0.012). Chemical shifts in ppm downfield from external triphenylphosphine.

Fig. 2. pH dependence of the chemical shift of the ^{31}P NMR resonance originating from the phosphatidic acid (PA) molecules in the outer monolayer of phosphatidylcholine-phosphaticid acid (1 : 1) vesicles. Chemical shifts in Hz downfield from external triphenylphosphine.

Fig. 3. Intensity of the sum of both the ^{31}P NMR signal of phosphatidylcholine and phosphatidic acid in phosphatidylcholine-phosphatidic acid (1 : 1) vesicles as a function of the Co^{2+} concentration.

molecule. This is in good agreement with the titration studies of Träuble and Eibl [15] on pure phosphatidic acid vesicles and recent calorimetric investigations on liposomes prepared from synthetic phosphatidic acid species [16]. Below pH 5 phosphatidic acid becomes neutral [15,16]. This is indicated by a further downfield shift of the phosphatidic acid resonance below pH 5 (Fig. 2). In order to avoid lipid hydrolysis, measurements were not performed above p^2H 9 or below p^2H 4. From Fig. 2 it can be concluded that phosphatidic acid in the outer monolayer of the mixed phosphatidic acid/phosphatidylcholine (1 : 1) vesicles bears one negative charge at pH 7.

Transbilayer distributions of phospholipids across vesicle bilayers can be determined by ^{31}P NMR using paramagnetic shift reagents (for review, see ref. 17). Low concentrations of the commonly used trivalent lanthanides Nd^{3+} and Eu^{3+} were found to cause vesicle fusion when more than 15 mol phosphatidic acid was present in the vesicles. Therefore, we used the divalent paramagnetic cation Co^{2+} which has previously been employed for the study of phospholipid asymmetry in neutral phospholipid vesicles [14]. Co^{2+}, when added in a sufficient amount to the vesicles, broadens the ^{31}P resonance of the molecules in the outer monolayer beyond detection, thus revealing only the resonances of the molecules in the inner monolayer [14]. This is shown in Fig. 1B for phosphatidic acid/phosphatidylcholine (1 : 1) vesicles at a Co^{2+}/phospholipid ratio of 0.012. To test the optimal Co^{2+} concentration required to broaden all resonances from the molecules in the outer monolayer, the intensity of the phospholipid resonances was tested as a function of the Co^{2+} concentration (Fig. 3). At Co^{2+}/phospholipid ratios of 0.010—0.016 the observed intensity is 30% of the original intensity and is independent of the Co^{2+} concentration. Under these conditions apparently only the resonances of the inner monolayer phospholipids are observed. Below ratios of 0.01 the Co^{2+} concentration is insufficient to eliminate the resonances of the outer monolayer phospholipids. Above a ratio of 0.016 a further decrease in intensity is observed. This is most likely caused by an inward leak of Co^{2+} and not due to vesicle fusion since, by the addition of a slight excess of EDTA the total intensity of the phospholipid resonances was observed again in a spectrum identical to the one shown in Fig. 2A. The addition of the low amounts of Co^{2+} did not cause vesicle aggregation or fusion sine (1) no visible increase in turbidity was observed and (2) chromatography of the vesicles on Sepharose 4B after the Co^{2+} addition revealed the same size distribution and phospholipid recovery as the control vesicles.

From experiments described in Figs. 2 and 3 the inner monolayer composition and the distribution of the total phospholipids over the outer and inner monolayer can be determined as a function of the phosphatidic acid/phosphatidylcholine ratio in the vesicles (Table I). For all vesicle compositions tested the inner monolayer composition was (nearly) identical to the total phospholipid composition demonstrating that the phosphatidic acid-phosphatidycholine vesicles have a symmetrical composition and that the outer monolayer phospholipid composition is almost identical to the total phospholipid composition of the vesicle. Also the number of phospholipid molecules in the outer monolayer with respect to the inner monolayer was virtually independent of the vesicle composition. This demonstrates that the size of the vesicles is hardly affected by the phosphatidic acid/phosphatidylcholine ratio.

TABLE I

TRANSBILAYER DISTRIBUTION OF PHOSPHOLIPIDS IN MIXED PHOSPHATIDIC ACID-PHOSPHATIDYLCHOLINE VESICLES

Total vesicle composition (mol % phosphatidic acid)	Inner monolayer composition (mol % phosphatidic acid)	Phospholipid outer monolayer / Phospholipid inner monolayer
100	100	2.4
84	75	2.3
80	80	2.5
67	63	2.6
50	54	2.5
33	36	2.3
25	24	2.3
20	19	2.4
0	0	2.2

Effect of Ca^{2+} on the vesicles. The linewidth ($\Delta\nu$) of the ^{31}P NMR resonance of phospholipids in vesicles is dependent on the vesicle size according to $\Delta\nu = ca^3 + d$ [11], where a is the vesicle radius and c, d are constants characteristic of chemical shift anisotropy and dipolar interactions experienced by the phosphate phosphorus. This relation is only valid when lateral diffusion is slow when compared to vesicle tumbling, a condition which holds in the case of egg phosphatidylcholine vesicles [18]. Linewidth increases caused by the addition of Ca^{2+} to mixed phosphatidic acid-phosphatidylcholine vesicles can be attributed to an increase in the size of the vesicle (aggregate) if the constants c and d are not affected by Ca^{2+}. For both multilayered liposomes of egg phosphatidylcholine and phosphatidic acid it was found that up to Ca^{2+}/phospholipid ratios of 0.5 the characteristic asymmetrical lineshape of the spectrum [20,21] was almost unchanged (de Kruijff, B., unpublished observations) demonstrating that neither the dipolar interaction between the phosphate and the neighbouring methylene group nor the magnitude of the effective chemical shift anisotropy [19—21] of the lipid phosphate was affected by the presence of Ca^{2+}. This demonstrates that the constants c and d are virtually independent of the presence of Ca^{2+} and that an increase in linewidth is most probably caused by a decrease in the vesicle tumbling as a result of an increase in size of the vesicle (aggregate).

In Fig. 4 the linewidth of the phosphatidic acid and phosphatidylcholine resonance of mixed phosphatidic acid-phosphatidylcholine vesicles is shown as a function of the Ca^{2+} concentration in the buffer. For pure phosphatidylcholine vesicles the linewidth is not influenced by Ca^{2+} (Fig. 4B), demonstrating that Ca^{2+} does not aggregate or fuse these vesicles. Incorporation of increasing amounts of phosphatidic acid into the phosphatidylcholine vesicles results in a broadening of the linewidth of both the phosphatidic acid resonance (Fig. 4A) and phosphatidylcholine resonance (Fig. 4B) upon addition of Ca^{2+} to the vesicles. Furthermore, the vesicle solutions become visibly more turbid. This is interpreted as a Ca^{2+}-induced vesicle aggregation or fusion, caused by the interaction of Ca^{2+} with phosphatidic acid in the outer monolayer of the vesicle. For pure phosphatidic acid vesicles a sharp increase in line-

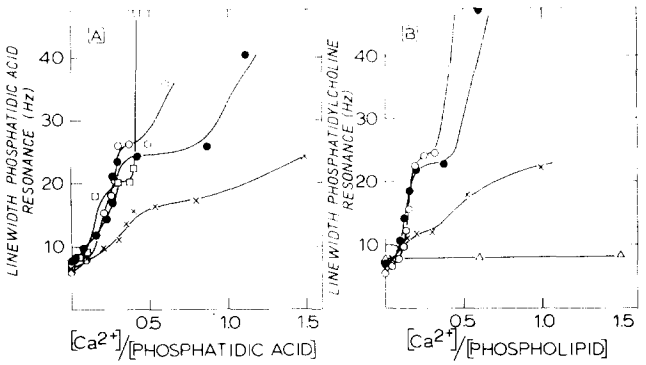

Fig. 4. Effect of Ca^{2+} addition on the linewidth of the phosphatidic acid (A) and phosphatidylcholine (B) resonance of various phosphatidylcholine-phosphatidic acid vesicles. The spectrum was recorded immediately after the addition of an aliquot of a 100 mM $CaCl_2$ solution. Further incubation had no effect on the linewidths. The amounts of Ca^{2+} indicated are the amounts of free Ca^{2+} which are calculated from the amount of Ca^{2+} added and the 0.2 mM EDTA present in the buffer. △———△, pure phosphatidylcholine vesicles X———X, 33 mol% phosphatidic acid; ●———●, 50 mol% phosphatidic acid; ○———○, 67 mol% phosphatidic acid; □———□, pure phosphatidic acid vesicles.

width occurs at a Ca^{2+}/phosphatidic acid ratio of 0.4. From the outside-inside distribution of phosphatidic acid in these vesicles (Table I) it can be concluded that this corresponds to a Ca^{2+}/phosphatidic acid ratio in the outer monolayer of 0.57, thus close to a value where full charge neutralisation of phosphatidic acid in these vesicles occurs. The dependence of the resonance linewidth on the Ca^{2+} concentration is complex which might be due to Ca^{2+}-induced lateral phase separations occurring in these vesicles [22]. This could affect the charge distribution in the vesicles and correspondingly the tendency of the vesicles to aggregate or to fuse. Furthermore, the observed linewidth increase could be due either to vesicle aggregation or fusion. To discriminate between these possibilities the reversibility of the linewidth increase was tested by the addition of excess EDTA to the vesicles (Fig. 5). Independent of the vesicle composition, for Ca^{2+}/phosphatidic acid ratios up to 0.2, the linewidth increase can

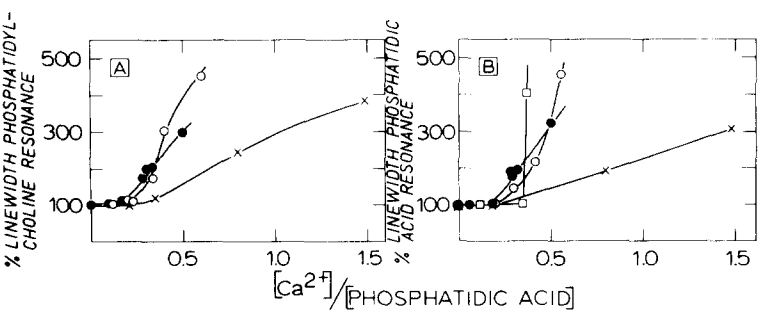

Fig. 5. Reversibility of the Ca^{2+}-induced changes in linewidth of the phosphatidylcholine (A) and phosphatidic acid (B) resonance of various phosphatidylcholine-phosphatidic acid vesicles. After the addition of Ca^{2+} the vesicles were incubated for 30 min at 25°C, then a 10% excess of EDTA was added as an aliquot of a 100 mM solution, whereafter the ^{31}P NMR spectrum was recorded immediately. No time-dependent changes in the linewidth were observed. X———X, 33 mol% phosphatidic acid; ●———●, 50 mol% phosphatidic acid; ○———○, 67 mol% phosphatidic acid; □———□, pure phosphatidic acid vesicles.

be fully reversed by the addition of excess EDTA, demonstrating that at the low concentrations of Ca^{2+} the vesicles only aggregate and do not fuse. A quantitative analysis of the sizes of these aggregates from the ^{31}P NMR linewidths is not possible because of the unknown tumbling behaviour of the entire aggregate and the individual vesicles within the aggregate. Above Ca^{2+}/phosphatidic acid ratios of 0.2 the linewidth increase can only be partly reversed by the addition of excess EDTA. This is interpreted as a Ca^{2+}-induced fusion of the vesicles. During this process the bilayers of different vesicles fuse. Considering the structure and morphology of the fused system it was reported that by the addition of excess Ca^{2+} to pure egg phosphatidic acid vesicles large aggregates were formed with X-ray spacings characteristic of a well-ordered lamellar phase [7]. Furthermore, freeze-fracture analysis of an equimolar mixture of dimyristoyl phosphatidic acid and dimyristoyl phosphatidylcholine in the presence of excess Ca^{2+} revealed the coexistence of liposomal bilayers and closely packed lamellae and cylinders [16]. In our experiments with phosphatidic acid-phosphatidylcholine (1 : 1) vesicles and a Ca^{2+}/phosphatidic acid ratio of 0.5 freeze-fracture analysis showed large, closely-packing lamellae as the only structure in the sample (Verkleij, A.J., unpublished observations). At Ca^{2+}/phosphatidic acid ratios of 0.2—0.4 the irreversibility of the Ca^{2+}-induced linewidth increase is largest for the phosphatidic acid/phosphatidylcholine 1 : 1 vesicles. For the pure phosphatidic acid vesicles at a Ca^{2+}/phosphatidic acid ratio of 0.35 the reversible vesicle aggregation changes to an irreversible vesicle fusion. This corresponds (see Table I) to a Ca^{2+}/phosphatidic acid ratio in the outer monolayer of 0.5 which is just enought Ca^{2+} to fully charge neutralize all phosphatidic acid molecules in the outer monolayer of the vesicles. The effect of Ca^{2+} on the linewidth of the ^{31}P NMR resonances of the phosphatidylcholine-phosphatidic acid vesicles was very similar when 100 mM NaCl was included in the buffer.

Discussion

The dynamic shape and charge of a lipid molecule determines its transbilayer distribution in curved bilayers [17]. In general, tapered molecules with a relatively large polar headgroup like lysophosphatidylcholine prefer the outer monolayer [23,24] whereas wedge-shaped molecules with a relatively small polar headgroup like phosphatidylethanolamine [14] and cholesterol [11,25] prefer the inner monolayer. The fatty acid composition of the lipid determines the molecular shape and therefore also determines the sidedness of phospholipids in vesicles [26]. From electrostatic considerations charged lipids should prefer the outer monolayer in which the average distance between the polar headgroups is maximal. This was found for mixed phosphatidylglycerol-phosphatidylcholine vesicles [27]. However, phosphatidylserine and phosphatidylinositol were found to prefer the inner monolayer in mixed vesicles with phosphatidylcholine [14]. This localisation was thought to be caused by the small polar headgroup size of these negatively charged phospholipids [14]. This conclusion has later been criticized [28].

Our ^{31}P NMR observations show that over a wide composition range sonicated mixed phosphatidylcholine-phosphatidic acid vesicles have a (nearly)

symmetrical phospholipid composition. Apparently, the effect of the small polar headgroup of phosphatidic acid which would favour an inner monolayer localisation is counterbalanced by the charge effect leading to a symmetrical bilayer.

Studies on synthetic phosphatidylglycerols and natural phosphatidylserine have revealed that Ca^{2+} can have two effects on the properties of bilayers made of these lipids [2—7]. In increasing amounts of Ca^{2+} up to a Ca^{2+}/phospholipid ratio of 0.5 there is a gradual charge neutralisation causing an increase in the packing of the molecules which is, for instance, manifested in an increase in the transition temperature. At a ratio of 0.5, however, the bilayers undergo a structural change and are converted to cylindrical [2] or cochleated [3] structures composed of bilayers formed by the Ca^{2+}-phospholipid salt which have a very low water content and in which the acyl chains have a crystalline packing [6].

The effect of Ca^{2+} on the structural properties of mixed phosphatidylcholine-phosphatidic acid vesicles can be resolved into at least two processes: (1) a Ca^{2+}-induced vesicle aggregation occurring at Ca^{2+}/phosphatidic acid ratios of ≤ 0.2 which can be fully reversed by chelating Ca^{2+} and (2) vesicle fusion occurring at Ca^{2+}/phosphatidic acid ratios of ≥ 0.2. The extent of vesicle aggregation is increased by increasing the phosphatidic acid concentration in the vesicle and by increasing the Ca^{2+} concentration in the buffer. Charge neutralization of phosphatidic acid by Ca^{2+} would be expected to facilitate vesicle aggregation by decreasing the repulsive forces between the different vesicles. However, we consider it more likely that Ca^{2+}, via electrostatic interactions, is acting as a bridge between different vesicles. This is because (1) under the conditions of vesicle aggregation the amount of Ca^{2+} is low with respect to the phosphatidic acid concentration (Ca^{2+}/phosphatidic acid in the outer monolayer ≤ 0.3) so that the vesicles must still bear an overall negative charge and (2) neutral phosphatidylcholine vesicles do not aggregate upon the addition of Ca^{2+}.

At Ca^{2+}/phosphatidic acid ratios above 0.35 (e.g. Ca^{2+}/phosphatidic acid in outer monolayer ≥ 0.53) phosphatidylcholine vesicles containing more than 30 mol% phosphatidic acid fuse and form large bilayer structures. In analogy with studies on mixtures of dimyristoylphosphatidylcholine with dimyristoylphosphatidic acid [16] it can be suggested that under these conditions very rigid Ca^{2+}-(phosphatidic acid)$_2$ complexes are formed which destabilize the vesicle bilayer leading to vesicle fusion and structural reorganization of the lipids.

Interestingly, in the Ca^{2+}/phosphatidic acid range 0.2—0.35 (Ca^{2+}/phosphatidic acid in the outer monolayer 0.3—0.53) pure phosphatidic acid vesicles only undergo vesicles aggregation whereas mixed phosphatidylcholine-phosphatidic acid vesicles undergo fusion (Fig. 5B). The tact that in the presence of phosphatidylcholine less Ca^{2+} is required to cause vesicle fusion strongly suggests that Ca^{2+}-induced phase separations are required to make the bilayer susceptible for fusion. This is supported by experiments on the glucose-6-phosphate permeability of these vesicles. At Ca^{2+}/phosphatidic acid ratios of 0.05—0.2 Ca^{2+} does not affect the passive glucose-6-phosphate permeability of either pure phosphatidylcholine or pure phosphatidic acid vesicles, whereas a strong increase in glucose-6-phosphate permeability was observed for mixed phosphatidylcholine-phosphatidic acid (1 : 1) vesicles (Koter, M., unpublished).

This suggests that Ca^{2+} induces lateral phase separations thereby creating phase bounderies through which the glucose-6-phosphate can readily permeate. This is similar to previous observations on the enhancement of permeability through a bilayer in the vicinity of the transition temperature [29—32]. Furthermore, Galla and Sackman [22] have provided evidence that Ca^{2+} does increase the extent of phase separation in mixed phosphatidylcholine-phosphatidic acid vesicles. Our results are in good agreement with the studies of Papahadjopoulos and co-workers [5—7] on the effect of Ca^{2+} on phosphatidylserine-containing vesicles. These authors also observed that under conditions of phase transitions and phase separations phospholipid membranes become susceptible to fusion as a result of Ca^{2+}-induced changes in the molecular packing of negatively charged phospholipids.

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