

Calcium binding by phosphatidylserine headgroups

Deuterium NMR study

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ABSTRACT The binding of calcium to headgroup deuterated 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) was investigated by using deuterium magnetic resonance in pure POPS membranes and in mixed 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/POPS 5:1 (m:m) bilayers. Addition of CaCl₂ to pure POPS bilayers led to two component spectra attributed, respectively, to liquid-crystalline POPS (< 15 kHz) and POPS molecules in the calcium-induced dehydrated phase (cochleate) (≈ 120 kHz). The liquid-crystalline component has nearly disappeared at a Ca²⁺ to POPS ratio of 0.5, indicating that, under such conditions, most of the POPS molecules are in the precipitated cochleate phase. After dilution of the POPS molecules in zwitterionic POPC membranes (POPC/POPS 5:1 m:m), single component spectra characteristic of POPS in the liquid-crystalline state were observed in the presence of Molar concentrations of calcium ions (Ca²⁺ to POPS ratio > 50), showing that the amount of dehydrated cochleate PS-Ca²⁺ phase, if any, is low (< 5%) under such conditions. Deuterium NMR data obtained in the 15–50°C temperature range with the mixed PC/PS membranes, either in the absence or the presence of Ca²⁺ ions, indicate that the serine headgroup undergoes a temperature-induced conformational change, independent of the presence of Ca²⁺. This is discussed in relation to other headgroup perturbations such as that observed upon change of the membrane surface charge density.

INTRODUCTION

Calcium interaction with phosphatidylserine (PS)¹ headgroup is an essential step in several membrane-related events. For instance interbilayer PS-Ca²⁺ complexes observed upon Ca²⁺ binding to PS-containing bilayers may trigger the membrane fusion process (Papahadjopoulos, 1978; Portis et al., 1979). More recently, it has been proposed that the specific interaction of PS-Ca²⁺ complexes with blood clotting factors may explain the excellent procoagulant properties of membranes containing phosphatidylserine (Rosing et al., 1988). There is also a growing interest in a group of proteins, known under the various names of their subclasses (*calpactin*, *annexin*, *lipocortin*) which have been found to interact with phospholipid bilayers in the presence of calcium, and conversely show a high affinity for Ca²⁺ ions in the presence of negatively charged membranes. A preferential interaction of phosphatidylserine in such ternary lipid/calcium/peptide interaction has been reported in

several cases (for reviews see Klee, 1988; Burgoyne and Geisow, 1989).

Numerous studies of the interaction of Ca²⁺ ions with the serine headgroup have been carried out either with macroscopic (differential scanning calorimetry, freeze-fracture, binding studies, etc . . .) and microscopic techniques (³¹P NMR and infra-red spectroscopy) (Portis et al., 1979; Hauser and Shipley, 1984; McLaughlin et al., 1981; Feigenson, 1986, 1989; Mattai et al., 1989). Most of the work reported at the molecular level was done on pure phosphatidylserine membranes showing mainly a dehydration and a strong immobilization of the serine headgroup due to the formation of high-melting PS-Ca²⁺ complexes (Hope and Cullis, 1980; Tilcock and Cullis, 1981; Casal et al., 1987*a, b*). The PS fraction in real biomembranes does not exceed 20% of the total phospholipid composition. For this reason it is important to get information at the molecular level on the interaction of Ca²⁺ with headgroups of PS molecules diluted in phospholipid membranes, in addition to that collected in pure PS bilayers. This problem has not been addressed yet, partly due to the lack of a specific probe which would distinguish the PS headgroups in mixtures of phospholipids. A solution is provided by ²H NMR of specifically deuterated phosphatidylserine headgroups, which allow one to monitor unambiguously the serine headgroup in mixtures of PS with another unlabeled phospholipid, such as phosphatidylcholine. This method

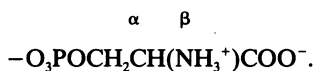
¹Abbreviations used in this paper: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; EDTA, ethylenediamine-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPA, 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphate; POPC, 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine.

has proven to be very useful in the study of the interaction of peptides and proteins with PS-containing membranes (Devaux et al., 1986; Roux et al., 1988, 1989; Bitbol et al., 1989; Dempsey et al., 1989). In this paper, we discuss ^2H NMR data describing the interaction of Ca^{2+} ions with PS headgroups in both pure POPS and mixed POPC/POPS 5:1 membranes.

MATERIALS AND METHODS

Lipids and peptides

POPA, POPC, and POPS were purchased from Avanti Polar Lipids (Birmingham, AL). Headgroup deuterated POPS was prepared from POPA according to Browning and Seelig (1980) and Roux and Neumann (1986), and purified on preparative Thin Layer Chromatography (5717, 20 cm \times 20 cm \times 0.5 cm; Merck, Rahway, NJ). The following nomenclature is employed for the deuteration sites of the serine headgroup:



Sample preparation

Liposomes were prepared from chloroform solutions of POPC and POPS mixed in an appropriate ratio, giving samples of 45 μmol of total lipid. The solvent was removed by evaporation under N_2 , the solid residues dried under high vacuum (10^{-2} mm Hg) for 12 h and dispersed with continuous vortexing at 40°C in 500 μl of Hepes buffer (50 mM in deuterium depleted water, pH 7.5, 40 mM NaCl), containing either zero or 1 M CaCl_2 for the POPC/POPS 5:1 samples, giving 90 mM lipid dispersions. The resulting samples were submitted to five freezing (liquid nitrogen) and thawing (25°C) cycles, centrifuged at 25°C (300,000 g, 2 h), and the resulting pellet transferred directly into the NMR tubes. For the experiments carried out with pure POPS, the calcium concentrations were raised by supplementing the pellet with appropriate amounts of concentrated CaCl_2 solutions, followed by freezing and thawing of the membranes.

NMR experiments

^2H NMR experiments were done at 46 MHz on a home-made NMR spectrometer (Davis, 1979; Sternin, 1985). Spectra were acquired with a dwell time of 2 μs (POPS) or 5 μs (POPC) with 4,096 data points and a repetition time of 150 ms. A quadrupolar echo pulse sequence (Davis et al., 1976) was employed with pulse length of 4 μs and pulse separation τ of 60 μs . The free induction decay (FID) was shifted by some fraction of the dwell time to ensure that its effective starting time for the Fourier transforms (FT) corresponded to the top of the quadrupolar echo (Davis, 1983). The Fourier transforms were performed without any exponential multiplication of the FID, unless otherwise stated in the text. All the experimental values of the deuterium quadrupolar splittings were measured from dePaked spectra, which were obtained by the numerical dePakeing procedure described by Sternin et al. (1983).

RESULTS

Effect of Ca^{2+} on POPS headgroups in pure POPS membranes

Fig. 1 displays ^2H NMR spectra (46 MHz) at 25°C of pure POPS membranes in the presence of various amounts of CaCl_2 . In absence of CaCl_2 the spectrum shows a typical powder lineshape characteristic of liquid-crystalline bilayers with three distinct quadrupolar splittings. In light of previous data obtained with headgroup deuterated PS (Browning and Seelig, 1980; Roux and Bloom, 1990), two of these quadrupolar splittings, $\Delta\nu_1$ and $\Delta\nu_2$, can be associated with the two deuterons of the α - CD_2 methylene, denoted by α_1 and α_2 , respectively, and a third one $\Delta\nu_3$ with the β - CD of the serine moiety. The smaller quadrupolar splitting, which we have denoted $\Delta\nu_2$ (3.4 kHz) is attributed to one of the α deuterons (α_2), whereas the larger quadrupolar splittings (14.5 and 16.2 kHz) are attributed to the β and the other α deuterons (α_1). Generally, the β quadrupolar splitting ($\Delta\nu_3$) is larger than the α_1 splitting ($\Delta\nu_1$), although the opposite situation was observed at low temperature (-5°C), with unsaturated headgroup deuterated DOPS (Browning and Seelig, 1980). On this basis, and on the ground of experimental results discussed below, we assume that the smaller splitting (14.5 kHz) is associated with the α_1 deuteron and attribute the splitting of 16.2 kHz to the β deuteron.

As may be seen from Fig. 1, increasing the concentration of CaCl_2 leads to a gradual decrease of the liquid-crystalline spectral intensity, without any large changes in the size of the quadrupolar splittings. A closer look at the NMR spectra (Fig. 2, traces d, e, f) indicates that this decrease of the area of the liquid-crystalline spec-

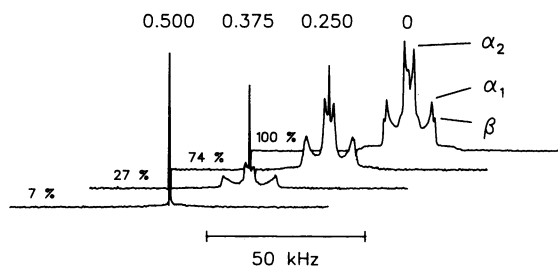


FIGURE 1 ^2H NMR spectra at 46 MHz of pure POPS α , β (d_3) membranes recorded at various concentrations of CaCl_2 (mole per mole of POPS) at pH 7.5. The spectra are normalized according to their number of scans, and their integrated areas expressed as a percentage of the original intensity measured in the absence of calcium. The narrow central peak, which is very likely due to deuterated water molecules was omitted in the integration. Measuring temperature was 25°C.

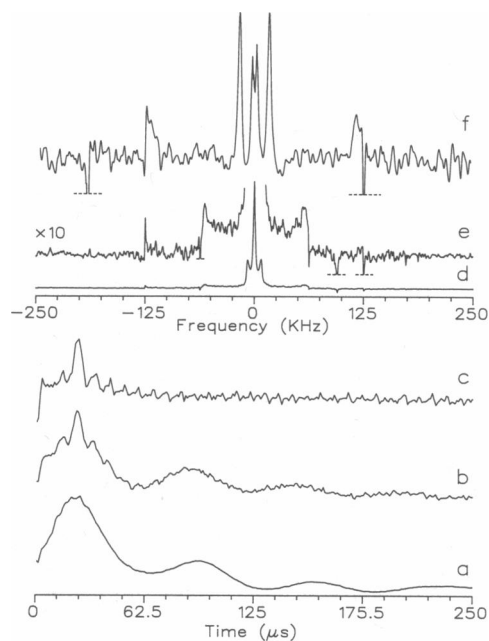


FIGURE 2 ^2H NMR signals at 46 MHz of pure POPS α , β (d_3) membranes at 25°C, pH 7.5: Free Induction Decay in the absence (a) and in the presence of 0.375 (b) and 0.5 (c) CaCl_2 per POPS. The Fourier transform of b, performed after exponential multiplication with a time constant of 300 μs , is plotted in d and e ($\times 10$ vertical enhancement). dePaking of spectrum d lead to trace f.

trum is related to the appearance of a second component, with a large quadrupolar splitting of ~ 120 kHz. The latter component is also clearly observable, before taking the Fourier transform, in the time domain as a narrow component on the top of the echo (Fig. 2, trace b, c). This narrow signal is not observed in the absence of CaCl_2 (trace a). Spectral distortions have precluded accurate quantitative meaningful estimates of the intensity of this calcium-induced component. However, the integrated intensity of the spectrum, recorded at 0.5 CaCl_2 per POPS is close to zero (see Fig. 1), indicating that very few PS molecules remain in the liquid-crystalline state at this CaCl_2 concentration. These results match those observed previously (Tilcock et al., 1984) in related ^{31}P NMR experiments, describing the interaction of unsaturated DOPS with Ca^{2+} ions.

DePaking of the FT-spectra of Fig. 1 allows a more precise analysis of the calcium dependence of the serine quadrupolar splittings of the POPS molecules in the liquid-crystalline state. Fig. 3 shows that one of the larger quadrupolar splittings of the serine headgroup (14.5 kHz) appears to separate into a second component (17.1 kHz) upon addition of CaCl_2 , whereas the other one (16.2 kHz) is barely modified. A similar situation is encountered for the small splitting (3.2 kHz) attributed

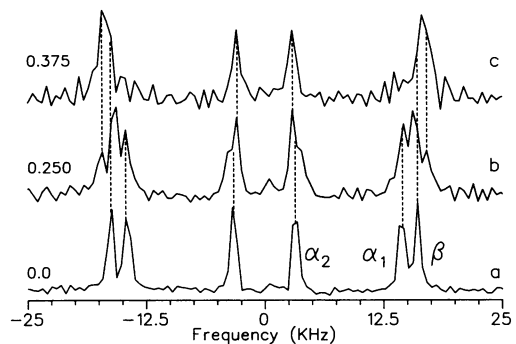


FIGURE 3 DePaked spectra obtained from the FT-spectra of Fig. 1.

to the α_2 deuteron, although the separation is comparatively less important. We note that the latter observation support the above hypothesis proposing that the quadrupolar splitting of 14.5 kHz should be associated with the α_1 deuteron. Increasing the CaCl_2 concentration up to 0.375 Ca^{2+} per POPS leads to a loss of the resolution of the large quadrupolar splittings, giving an average value of 16.8 kHz, with no intensity remaining below 15 kHz.

Effect of Ca^{2+} on POPS headgroups in POPC/POPS 5:1 membranes

Fig. 4 shows ^2H NMR spectra of POPC/POPS 5:1 membranes in the absence and in the presence of 1 M CaCl_2 , at 15°, 25°, and 50°C. The effect observed upon addition of CaCl_2 to POPC/POPS 5:1 membranes are in contrast with those recorded in pure POPS dispersions. Over the 15 to 50°C temperature range only one component was detected, showing that the rate of exchange of lipid between the perturbed and unperturbed states of

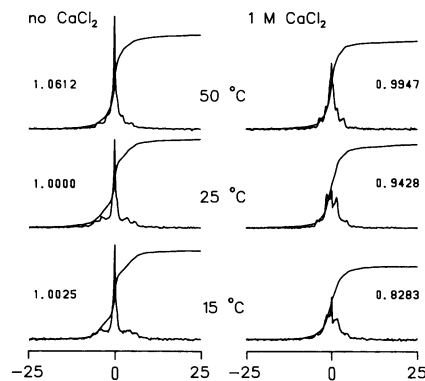


FIGURE 4 ^2H NMR spectra at 46 MHz of POPS α , β (d_3) recorded from POPC/POPS 5:1 (m:m) membranes at pH 7.5 in the absence or the presence of 1 M CaCl_2 . All spectra were recorded under the same conditions, with 100,000 scans.

the serine headgroup must be fast on the ^2H NMR time scale (10^{-5} – 10^{-6} s). The detection of the broad NMR component related to that observed with the pure POPS membrane is hampered by the low signal-to-noise ratio of the serine ^2H NMR spectra, and it is necessary to check whether all the signal intensity is in the liquid-crystalline component. This can be achieved by estimating the ratio of the liquid-crystalline spectrum areas measured in the presence and the absence of calcium. This ratio was calculated here in the 15–50°C temperature range, and found to be close to 1 giving strong evidence that a large majority of the POPS molecules were in the liquid-crystalline state in the presence of 1 M CaCl_2 , over this range of temperature. However, at 15°C this ratio appears to be appreciably smaller (0.83). At 2 M CaCl_2 (not shown), there is an important broadening of the NMR lines suggesting that the PS molecules undergo a liquid-crystalline-to-gel phase transition. This interpretation is confirmed by the observation that well-resolved quadrupolar splittings characteristic of axially symmetric liquid-crystalline spectra can be recovered by raising the temperature up to 40°C.

From the dePaked spectra of Fig. 5, it can be seen that, in contrast to pure POPS, the addition of 1 M CaCl_2 to POPC/POPS mixtures, leads to important variations of the serine headgroup quadrupolar splittings. These variations were studied in detail on a larger concentration range (0–2 M CaCl_2) and discussed in a previous paper (Roux and Bloom, 1990). We note that in the absence of CaCl_2 , the dilution of POPS in POPC membranes leads to a decrease of the three quadrupolar splittings of the serine headgroup (1.7, 8.0, and 12.7 kHz for, respectively, the α_2 , α_1 , and β deuterons at 25°C), as observed previously after dilution of saturated DMPS in

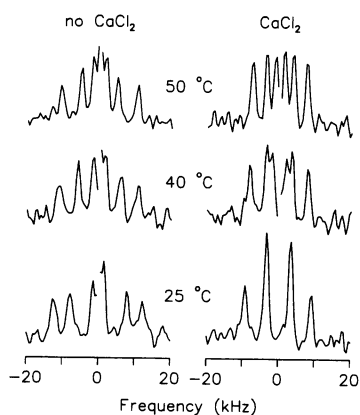


FIGURE 5 Depaked spectra obtained from the ^2H NMR FT-spectra recorded as a function of the temperature (see FT-spectra on Fig. 4, FT-spectrum recorded at 40°C not shown).

DMPC (Browning and Seelig, 1980; Roux and Neumann, 1986; Dempsey et al., 1989).

Finally, Fig. 5 shows that the serine headgroup is perturbed by temperature variations, as reflected in the temperature-induced changes of the α and β quadrupolar splittings, which are plotted on Fig. 6. In the absence of CaCl_2 , both α_1 and β quadrupolar splittings are reduced when the temperature is increased, whereas the α_2 splitting is slightly increased. This singular feature, i.e., the β and the α_1 splittings are decreased whereas the α_2 is almost constant, is also observed in the presence of 1 M CaCl_2 . Fig. 6 also shows that the slope of the temperature dependence of each splitting is barely modified by the addition of 1 M CaCl_2 .

DISCUSSION

POPS/ Ca^{2+} cochleate phase

It is well documented that PS molecules are able to form strong molecular complexes with Ca^{2+} ions, leading to the complete dehydration of the serine headgroup in lipid bilayers containing a high percentage of phosphatidylserine, and to the so-called *cochleate* structures (Papahadjopoulos et al., 1975; Portis et al., 1979; Feigenson, 1986; Casal et al., 1987a, b). This effect of calcium on phosphatidylserine is clearly observed on the POPS NMR spectra recorded with pure POPS membranes, on which two components can be detected in the presence of CaCl_2 .

One of the components is identical to the spectrum measured in the absence of CaCl_2 , whereas the other is very broad. Similar two-components ^{31}P NMR spectra

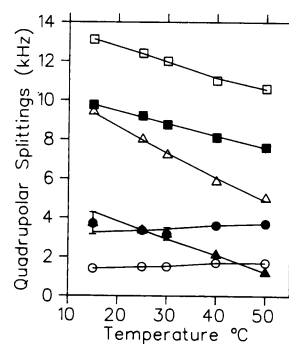


FIGURE 6 Temperature dependence of the α_1 (Δ , \blacktriangle), α_2 (\circ , \bullet), and β (\square , \blacksquare) POPS headgroup quadrupolar splittings obtained from POPC/POPS 5:1 (m:m) membranes at pH 7.5 in the absence (open symbols) or the presence (solid symbols) of 1 M CaCl_2 . The bars on symbols (\bullet , \blacktriangle) at 15 and 30°C, represent the error, due to the overlapping of the dePaked NMR lines, in the measurement of the α_1 and α_2 splittings at these temperatures.

have been reported during Ca^{2+} interaction with pure phosphatidylserine (Hope and Cullis, 1980; Casal et al., 1987b), in which the broad component has been attributed to the dehydrated $\text{POPS}_2(\text{Ca})$ complexes. In the latter studies, the broad spectrum is that of the axially asymmetric ^{31}P tensor with edge values close to those of crystalline phosphodiester, thus indicative of a completely rigid phosphate group. The value measured here (≈ 120 kHz), for the quadrupolar splitting between the 90° edges associated with the calcium-induced component, is very close to that of the maximum possible value for a C-D bond (120–135 kHz; Mantsch et al., 1977), indicating that there are essentially no averaging motions of the serine C-D bonds, and consequently that the $\text{C}_\alpha\text{-C}_\beta$ bond of the serine headgroup is also quasi rigid.

Earlier studies, carried out on PC/PS mixtures, seemed to indicate that the critical concentration of PS at which PS/ Ca^{2+} cochleate structures start to appear is between 20 and 30% (Hui et al., 1983; Silvius and Gagné, 1984). More recently, Feigenson (1989) has, however, reported x-ray diffraction patterns of filter-supported POPC/POPS 4:1 membranes, from which the author estimated that $\sim 60\%$ of the total PS was involved in a dehydrated $\text{POPS}_2(\text{Ca})$ complex. The ^2H NMR data obtained here at 25°C with POPC/POPS 5:1 membranes up to 1 M CaCl_2 (Ca^{2+} to PS ratio ≈ 65) show clearly that, in coarse multilamellar samples dispersed in calcium-containing buffer, the PS/calcium cochleate structures does not occur to a significant extent when the PS fraction in the membrane is below 17%. The small loss in the intensity of the “narrow” component of the PC/PS/calcium spectrum observed at 15°C (Fig. 4) could be due to POPS molecules undergoing a liquid-crystalline-to-gel phase transition, as detected at 25°C in the presence of 2 M CaCl_2 .

Liquid-crystalline POPS in the presence of the POPS-Ca^{2+} cochleate phase

In an earlier binding study of Ca^{2+} ions to pure POPS membranes, Feigenson (1986) has shown that during the formation of the cochleate phase, most of the added Ca^{2+} ions were involved in the dehydrated PS/ Ca^{2+} complex and that only a trace of “free” Ca^{2+} ions could bind to hydrated POPS. This observation is actually supported by the spectra of Fig. 1, which indicate that the amount of dehydrated PS/ Ca^{2+} complex is roughly proportional² to the amount of Ca^{2+} added, almost one equivalent of $\text{PS}_2(\text{Ca})$ complex being obtained with one

equivalent of POPS and 0.5 equivalent of CaCl_2 . However, POPS molecules remaining in the liquid-crystalline phase are significantly affected by the progressive addition of Ca^{2+} ions, as shown by the behavior of the serine α splittings which, unlike the β splitting, separates each into a second splitting (Fig. 3). In this regard the dePaked trace obtained after addition of 0.25 Ca^{2+} per POPS (Fig. 3 b) indicates that in such conditions, two forms of liquid-crystalline PS coexist in slow exchange at the NMR time scale. It is interesting to note that a very similar ^2H NMR spectrum was obtained from a racemic mixture of L-DL-phosphatidylserine (with racemic DL-serine headgroups) (Browning and Seelig, 1980). In this spectrum, each α splitting was also subdivided into two components while a single β component was detected. Each α subcomponent was shown to be attributed to one of the diastereoisomers. Indeed, the situation encountered here is very different because we have *only one* stereoisomer, and the ratio of the two components appears to depend on the experimental conditions, i.e., the amount of added CaCl_2 , one being observed in the absence of calcium (Fig. 3 a), whereas the other is predominant at high calcium level (Fig. 3 c). A simple interpretation would be that the effect detected on the ^2H NMR spectra of liquid-crystalline POPS is induced by the “free” Ca^{2+} ions, which are not involved in the cochleate dehydrated PS/ Ca^{2+} complex. This hypothesis is not completely satisfactory if we consider that the obtained effect is very different from that observed on the quadrupolar splittings of liquid-crystalline POPS in POPC/POPS 5:1 bilayers for which we have seen that (a) the PS molecules perturbed by calcium ions are in *fast* exchange with the other unperturbed PS, (b) the larger splittings (α_1 and β) of the serine headgroup are *decreased* by the addition of CaCl_2 (see Fig. 5 and Roux and Bloom, 1990). We propose that in pure POPS membranes, the appearance of a second liquid-crystalline form of POPS indicated by the ^2H NMR data, is related to the occurrence of the dehydrated-cochleate phase, considering that part of the PS molecules remaining in the liquid-crystalline state, associated with the second α_1 splitting (17.1 kHz), would “see” the cochleate phase. According to this interpretation, increasing the amount of cochleate phase should lead finally to the complete disappearance of the initial α_1 quadrupolar splitting (14.5 kHz), as observed experimentally (Fig. 3 c).

Temperature dependence of the POPS quadrupolar splittings in POPC/POPS 5:1 membranes

The serine headgroup quadrupolar splittings exhibit different sensitivities to the temperature variations. The

²This is not fully verified for the data recorded at 0.250 Ca^{2+} per POPS. We believe that this result should be related to the supersaturation effect described by Feigenson (1986), due mainly to the slow equilibration of Ca^{2+} ions through the multilamellar structures.

most striking feature is that one of the α quadrupolar splittings increases slightly whereas the other one, similarly to the β splitting, decreases when the temperature increases. Considering that the variation of the α_2 splitting is opposite to that of the α_1 and β quadrupolar splittings, the overall effect cannot be interpreted simply in terms of an increase of the angular fluctuations of the serine headgroup, but rather indicates that the headgroup undergoes a conformational change due to the temperature increase. The nature of the observed conformational change is the same in the absence and in the presence of 1 M CaCl_2 . In fact, this temperature-induced conformational change is somewhat related to that observed after adsorption of Ca^{2+} and positive charges of metallic cations or amphiphilic peptides at the surface of PC/PS 5:1 membranes, which also leads to a large decrease of the α_1 and β quadrupolar splittings (Roux et al., 1989; Roux and Bloom, 1990). Another feature shared by these two kinds of perturbations is that large variations of the α_1 quadrupolar splitting are coupled with small changes (or none) of the α_2 splitting. This particular effect detected with the two α deuterons was interpreted (Roux et al., 1989) in terms of a charge-induced rotation of the serine headgroup along a direction (*almost*) parallel to that of the C^2H bond direction associated with the unperturbed deuteron (α_2), yielding (*small*) no variations of the α_2 splitting.

In a more general way, it is interesting to note that the quadrupolar splittings of α deuterons of other phospholipid headgroup components such as the choline (Brown and Seelig, 1978), or the glycerol (Wohlgemuth et al., 1980), appear to be also rather insensitive to variations of temperature in the liquid-crystalline phase, as opposed to those of the neighboring β deuterons. Moreover, in situations where two distinct quadrupolar splittings were observed for the $\alpha - \text{CD}_2$ deuterons, for instance with PG (Wohlgemuth et al., 1980) or PC in the presence of cholesterol (Brown and Seelig, 1978), only one of the splittings was reduced when the temperature was raised, the other remaining unmodified. After x-ray and neutron diffraction experiments, it has been previously emphasized that headgroups of various phospholipids (PC, PE, PG) share a common orientation, essentially parallel to the bilayer surface (Büldt and Wohlgemuth, 1981; Seelig et al., 1987). On the basis of the experiments outlined above, it is possible to extend this finding, although the exact conformation of the PS headgroup is at the present time not known, and propose that the conformation of lipid headgroups could also share a common thermic response.

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