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Calcium-induced condensation-reorganization phenomena in multilamellar vesicles of phosphatidic acid. pH potentiometric and ^{31}P-NMR, Raman and ESR spectroscopic studies

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In biological membranes, the anionic characteristics of the polar headgroup of phosphatidic acids are responsible for structural changes induced by Ca^{2+} in many cellular processes. The very simple headgroup structure of dipalmitoylphosphatidic acid (DPPA) offers particular advantages as a model to study the interactions between Ca^{2+} and natural phosphatidic acids such as cardiolipin and phosphatidylserine. The effects of calcium ions on DPPA membranes have been studied as a function of temperature by potentiometry and by Raman, ESR and ^{31}P-NMR spectroscopies. The protons in monosodic DPPA liposomes have been considered as a probe to detect pH variations resulting from introduction of Ca^{2+} inside the membrane. This method has also allowed us to determine the stoichiometry of this reaction: 2 DPPA(H) + Ca^{2+} → $Ca(DPPA)_2$ + 2H$^+$. ^{31}P-NMR spectroscopy has been used to detect reorganization-condensation phenomena in multilamellar vesicles of DPPA under the influence of calcium and temperature. Furthermore, the temperature profiles obtained from Raman spectra for $Ca(DPPA)_2$ membranes provide conclusive evidence that Ca^{2+} induces major reorganization of the phosphatidic acid component into a highly ordered phase. Quantitative estimates of the degree of motional restriction of spin-labeled soaps embedded inside membranes composed of DPPA with or without Ca^{2+} have been made using ESR technique. These results are discussed and compared to those found previously for a natural phosphatidic acids such as phosphatidylserine.

Introduction

The combination Ca^{2+} and negatively charged phospholipids in biological membranes may play an important role in various membrane processes: vesicle fusion [1–3]; phase separations [4–8]; liquid-crystalline to solid lamellar phase transitions [9]. Out of the many acidic phospholipids, phosphatidylserine (PS) alone or in combination with other lipids has often been used for such membrane process studies. It was thus demonstrated that the interaction of Ca^{2+} with PS vesicles leads to lipid phase changes and lipid vesicle

Abbreviations: DPPA, dipalmitoylphosphatidic acid; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; 5-PG, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-*N*-oxyl)-stearoyl)]-*sn*-glycero-3-phosphoglycerol; (7,6)PG, (7,6)PA and (7,6)PS, 1-acyl-2-[(7,6)-palmitoyl]-*sn*-glycerol-3-phosphoglycerol, -phosphoric acid and -phosphoserine, respectively; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DMPS, 1,2-dimyristoyl-*sn*-glycerol-3-phosphoserine; ESR, electron spin resonance; T_c, gel to fluid liquid-crystal phase-transition temperature; $A_{\parallel}$ and $A_{\perp}$, outer and inner hyperfine splittings, respectively; S, order parameter.

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aggregations and fusion [10–17], Also, it has been shown to produce a phase composed of $Ca(PS)_2$ [18], of low water content and highly ordered acyl chains [19]. However, the use of phosphatidic acid membranes is more appropriate for the determination of the mode of ion binding to phospholipid membranes than the use of natural phosphatidic acid membranes. Indeed, although phosphatidic acid is one of the least common lipids found in natural membranes, the very simple headgroup of phosphatidic acid offers particular advantages in defining and modelling in detail the aspects of the interactions between calcium and a negatively charged lipid: phosphatidic acid has only one dissociable polar group below pH 7.0, and has no other bulky polar groups as do phosphatidylserine, phosphatidylinositol or phosphatidylglycerol. For these reasons, and on account of various properties attributed to phosphatidic acid in the membrane fusion processes, we have used a model system of multilamellar vesicles composed of dipalmitoylphosphatidic acid (DPPA) and have examined the effects of Ca^{2+} binding between lamellae by means of potentiometry ^{31}P-NMR, Raman and ESR spectroscopies.

pH potentiometry has allowed us to survey the transport of Ca^{2+} across DPPA liposomes and to indicate the implications of this insertion on the properties of the membrane. The uptake of Ca^{2+} inside liposomes has been analyzed in terms of H^+ fluxes from the membrane to the outside aqueous medium. ^{31}P-NMR spectroscopy has been used to detect changes in the structures of the liposomes and especially in the phosphate headgroups under the effect of calcium and temperature.

Raman study of Ca^{2+}-DPPA liposomes has been undertaken in the Raman CH-stretch region to learn about the dynamic fluctuations of the ordered and disordered phases or states in hydrocarbon chains and chain packing.

ESR spectroscopy has been used to evaluate the extent of the motional restriction of spin-labeled probes incorporated in the multilamellar vesicles composed of DPPA alone or with Ca^{2+}.

Experimental

Dipalmitoylphosphatidic acid (DPPA), 5-doxylstearic acid and 16-doxylstearic acid were purchased from Sigma. Aqueous suspensions of DPPA liposomes were prepared by vigorous stirring, for at least 3 h, with a vortex mixer at the desired temperature. These lipid dispersions were formed by mixing about 31–32 mg of phospholipid with 60 cm^3 of triply distilled water.

pH Measurements

pH measurements were performed with a 4500 Beckman pH-meter. The experimental temperature and the agitation of the liposomal media were maintained constant during the course of the experiments. The pH in our samples was measured with a glass pH electrode (Orion No. 916100) and a calomel reference electrode (Orion No. 900600). The greatest source of error in pH measurement is temperature, resulting from: (i) mechanical effects (changes of the Nernstian electrode slope and also because of the thermal response of the electrode); (ii) chemical effects (changes in chemical equilibrium with variation in T). Buffer and sample pH values vary with T on account of their T-dependent chemical equilibria. Therefore, pH values are reported, in this paper, along with the temperature at which the measurement was made.

^{31}P-NMR measurements

^{31}P-NMR spectra of the liposome samples (the NMR tube contained 80–90 mg of phospholipid dispersed as described above) were obtained with a General Electric GN 300 NMR spectrometer equipped with a variable temperature unit and operating with proton decoupling during acquisition time. Accumulated free induction decays were obtained from 200 transients on 3 cm^3 samples in a 10 mm NMR tube, using 100 s interpulse time and 1.02 s acquisition time. A sweep width of 4000 Hz and a 10 μs 30° pulse were used. Before registering each ^{31}P-NMR spectrum, the liposome sample was maintained at the desired temperature for 30 min.

Raman measurements

Aliquots of liposome were transferred to capillaries for Raman measurements. The Raman sample was centrifuged before use. The Raman measurements were recorded with a computer-controlled SPEX 14018 double monochromator equipped with a spatial filter to reduce stray light.

The 514.5 nm line of a CR8 argon ion laser was used for sample excitation. Spectral bandpass was set at 5 cm^{-1}. The temperature was monitored by a thermistor controlled by a DCR4 interface to the computer. Sample integrity was also monitored by measuring acyl vibrational Raman bands (C=O stretch) at each temperature. These bands showed no significant changes which might indicate sample deterioration.

ESR measurements

ESR spectra were recorded on a Varian E-109 spectrometer (with a nitrogen gas flow temperature regulation) at a microwave power of 2 mW, a microwave frequency of 9.385 GHz, with a field sweep of 100–200 G centered at 3350 G, scan time = 4 min, and a 100 kHz field modulation amplitude of 0.63–1.25, depending on the spectral line-widths. Aqueous suspensions of DPPA plus 5 (or 16-) doxylstearic acid at a probe : lipid ratio of about 0.005–2 were prepared by vigorous stirring at $T = 60–63\,°C$ (which is superior to the transition temperature, T_c(DPPA)), for at least 1 h, with a vortex mixer. Excess calcium chloride (or nitrate) was subsequently added into the liposomal suspensions at T=45 °C in order to form $Ca(DPPA)_2$. Under these experimental conditions, a small amount of the spin-labeled soaps used as a probe is embedded inside the membrane composed of DPPA alone or in combination with Ca^{2+}.

The extent of motional restriction of the spin labeled soaps (16- and 5-doxylstearic acid) was estimated by calculating the order parameter, S. This parameter is a measure of the amplitude of the motion of the molecular long axis concerning the average orientation of the fatty acid chains in the lipid bilayer. It was obtained from the anisotropic hyperfine splittings, $A_{\|}$ and $A_{\perp}$, measured on the ESR spectra, as illustrated in Fig. 5c. To a first approximation, $A_{\|}$ and $A_{\perp}$ are equal to the half separation of the outer and inner hyperfine extrema, respectively, of the experimental ESR spectra.

The mathematical expression of S is [20]:

$$S = (\alpha/\beta)(A_{\|} - A_{\perp} - C) \Big/ (A_{zz} - A_{xx}/2 - A_{yy}/2) \quad (1)$$

where A_{xx}, A_{yy} and A_{zz} are the principal elements of the hyperfine tensor, C is a correction term allowing for the difference between the measured and true inner hyperfine splitting, $\alpha = (A_{xx} + A_{yy} + A_{zz})/3$ and $\beta = (A_{\|} + 2A_{\perp} + 2C)/3$. If $A_{\perp}$ cannot be determined from the experimental spectra, it is possible to assume $\alpha = \beta$. Hence S can be expressed as follows [17,20]:

$$S = \left(3A_{\|}/2 - 3A_{xx}/4 - 3A_{yy}/4\right) \Big/ \left(A_{zz} - A_{xx}/2 - A_{yy}/2\right) - 0.5 \quad (2)$$

Results

Potentiometric study of the interaction between Ca^{2+} and monosodic DPPA

The addition of calcium chloride (or nitrate) into liposomes aqueous suspensions leads to pH variations indicating changes in the membrane. To understand more about the ionization characteristics of the DPPA liposomes containing Ca^{2+}, we have investigated proton release from the membrane in response to calcium inserted. The proton of the monosodic DPPA has therefore been considered as a probe to detect modifications and/or perturbations in the liposomal structures as a result of Ca^{2+} uptake inside the membrane. At the thermodynamic equilibrium of the liposomal medium, the proton concentration (or pH) has been measured as a function of added Ca^{2+} using pH potentiometry. Fig. 1 represents the plot of pH against the Ca^{2+}/DPPA molar ratio at the experimental temperature $T = 45\,°C$. We have chosen this temperature because insertion of Ca^{2+} inside liposomes is easier at $T > 43–44\,°C$. Under this condition, a sharp decrease in pH occurs just before the value of 0.5 of the Ca^{2+}/DPPA ratio, and this indicates an abrupt structural changes in the membrane. At a Ca^{2+}/DPPA molar ratio of at least 0.5, the pH reaches a plateau, and little change is noted over a wide range of added calcium. These pH phenomena can be explained as follows. The increase in the medium acidity can be attributed to a displacement of protons on the membrane surface, induced by the Ca^{2+} attack. This implies structural changes of the bilayers which affect probably the size of the liposomes and hence the surface area of the vesicles. Indeed, the more phosphate groups that are exposed, the more hydrogen ions calcium can displace. Under

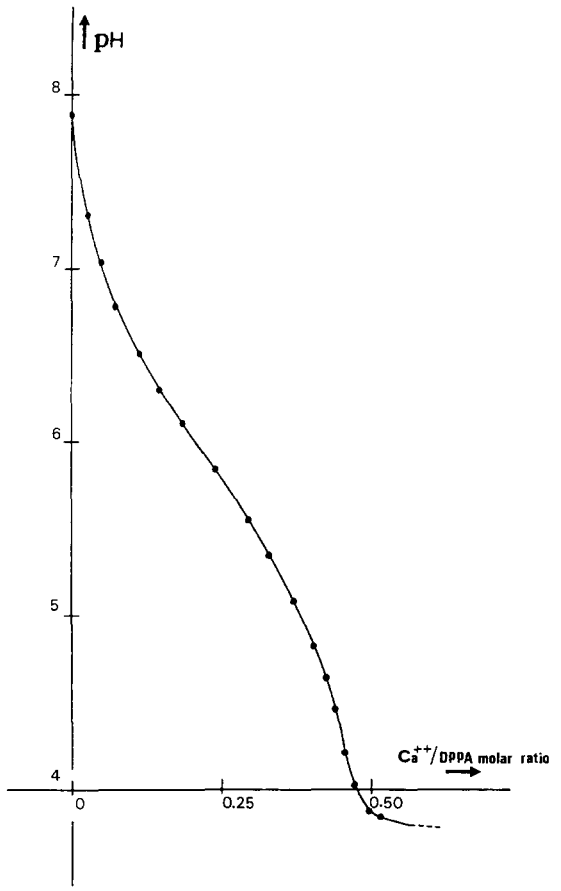

Fig. 1. pH variations vs. Ca^{2+}/DPPA molar ratio obtained from aqueous dispersions (liposomes) or monosodic DPPA in response to calcium at $T = 45\,°C$. 90 mg (or about $15 \cdot 10^{-5}$ mol) were dispersed in 80 cm^3 of pure water, for at least 3 h, at room temperature. pH was measured at equilibrium after each injection of a known volume of a titrated $Ca(NO_3)_2$ (or $CaCl_2$) solution $2 \cdot 10^{-2}$ mol·dm^{-3}.

such conditions, the reaction between monosodic DPPA and calcium can proceed to completion according to:

$$2\,DPPA(H) + Ca^{2+} \rightarrow Ca(DPPA)_2 + 2H^+ \qquad (3)$$

Accordingly, at the end of the potentiometric titration, the composition of the gel obtained is $Ca(DPPA)_2$, as already mentioned by Feigenson et al. [17,18] dealing with the interactions Ca^{2+}–phosphatidylserine: $Ca(PS)_2$.

^{31}P-NMR study vs. T of membranes composed of DPPA alone or with Ca^{2+}

^{31}P-NMR spectroscopy is recognized as a technique which yields information concerning both the dynamics and organization of phospholipid molecules in biological membranes and phospholipid dispersions. As pointed out previously [21–25], the line-width and line-shape of the ^{31}P-NMR spectra of phospholipids reflect the motional state of the molecules, and they are sensitive to changes in fluidity and the presence of perturbants in the membrane. Because of the similarity of the molecular motions in the region of the phosphate group found with liposomes and biological membranes, this technique was used to gain detailed conformational information concerning the phosphate region of the polar headgroup and perturbations induced by polyvalent ions and charged or non-charged organic compounds.

Unsonicated aqueous suspensions of phospholipids considered as model membrane systems are in a state of anisotropic motion intermediate between the isotropic motion characteristic of molecules in a homogeneous liquid medium and the static behavior of molecules in the solid state [21,23,26]. However, as mentioned previously in the case of sonicated phospholipid vesicles [27–30], the dimensions of the liposomes must be sufficiently large in order to prevent isotropic phenomena resulting from liposome tumbling and lateral diffusion of phospholipid [21,26]. Fig. 2 represents the ^{31}P-NMR spectra vs. T of $Ca(DPPA)_2$ ($Ca(DPPA)_2$ was prepared at 45 °C, as described above). The ^{31}P-NMR spectra were recorded with proton-decoupling. The ^{31}P-NMR spectra of $Ca(DPPA)_2$ have been compared with those obtained for monosodic DPPA and DPPA diacid. It is worth noting that we have used DPPA diacid (containing only protons) as a reference sample, because of the eventual implications of Na^+ ions in the liposomal structures of mono or disodic DPPA under the influence of temperature. Thus DPPA diacid has been prepared by addition of HCl solution into an aqueous suspension of monosodic DPPA liposomes.

As the temperature increases, the ^{31}P-NMR spectra of the aqueous suspensions of DPPA diacid and/or monosodic DPPA show a collapse to the pattern characteristic of axially symmetric shielding tensors, as described previously [21]. The residual anisotropy, $\Delta\sigma$, decreases from 25 to 17 ppm when the temperature increases from 45 to 80 °C. And when $T \geqslant 80\,°C$, only one peak, centered at 0 ppm (with reference to a standard sample containing 85% H_3PO_4), is observed in the

^{31}P-NMR spectra, indicating the formation of micelles and thereby of free phosphates, or more probably a degradation of the phosphatidic acid molecules.

Whereas the disturbing influence of calcium in the phosphate headgroups results in a large broadening of the ^{31}P-NMR line-width. This indicates that the characteristic 'solid state' line shape is enhanced by the chemical shift anisotropy of the phosphate phosphorus. Furthermore, at $T \geqslant 75\,^{\circ}$C the line-shape shift to lower field and the residual anisotropy, $\Delta\sigma$, decrease from 45 to 25 ppm, showing the characteristic line-width of the hexagonal phase structure (Fig. 2c). These observations suggest that Ca^{2+} immobilizes the phosphate headgroups of phospholipids, perhaps by forming bridges between neighboring phosphates of the same layer or two parallel layers. In addition, the temperature effects lead to reorganizations of the liposomal structures with condensation phenomena induced by the strong binding of calcium to phosphate groups. Indeed, we have observed that the volume of liposomes of the ^{31}P-NMR sample heated up to 85 °C has been reduced dramatically, about 5-fold, leading to a pellet suspended in water and constituted of aggregated lipids, suggesting a nearly anhydrous state, as mentioned previously [17] concerning $Ca(PS)_2$.

Raman study vs. T of the liposomal structures DPPA-Ca^{2+}

Raman spectroscopy is a very useful technique to follow phospholipid thermotropic phase transition and to monitor simultaneously and without using any perturbing probe the conformation and the chain-packing characteristics of each lipid in lipid mixtures [31–35]. We have thus undertaken a spectroscopic study in the Raman CH stretch region of DPPA-Ca^{2+} liposomes (containing a Ca^{2+} : DPPA molar ratio equal to 0.5), in order to examine the temperature dependence of bilayers under the perturbating influence of calcium inserted within membrane (Fig. 3). As pointed out previously [35], the ratio of peak heights for the Fermi-enhanced Raman-active antisymmetric CH_2 stretch at about 2880 cm^{-1} and the symmetric CH_2 stretch at 2850 cm^{-1} can be used to monitor the packing 'fluidity' of bilayers and the eventual

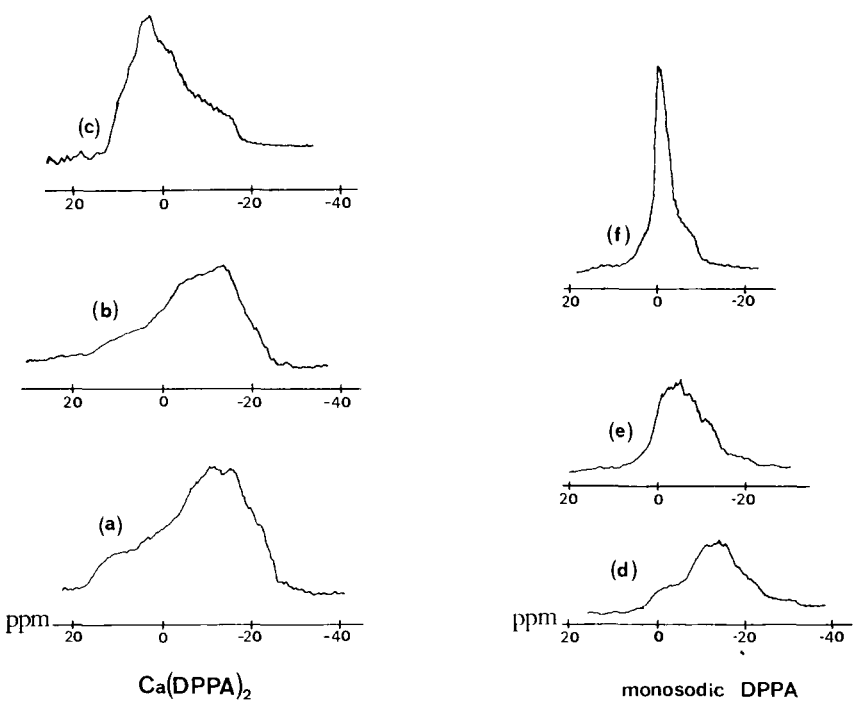

Fig. 2. ^{31}P-NMR spectra vs. T obtained from aqueous suspensions of monosodic DPPA and Ca^{2+}-DPPA complex (in a 2:1 molar ratio). Ca^{2+}-DPPA complex (2:1): (a) 35 °C; (b) 55 °C; (c) 75 °C. Monosodic DPPA: (d) 45 °C; (e) 75 °C; (f) 85 °C.

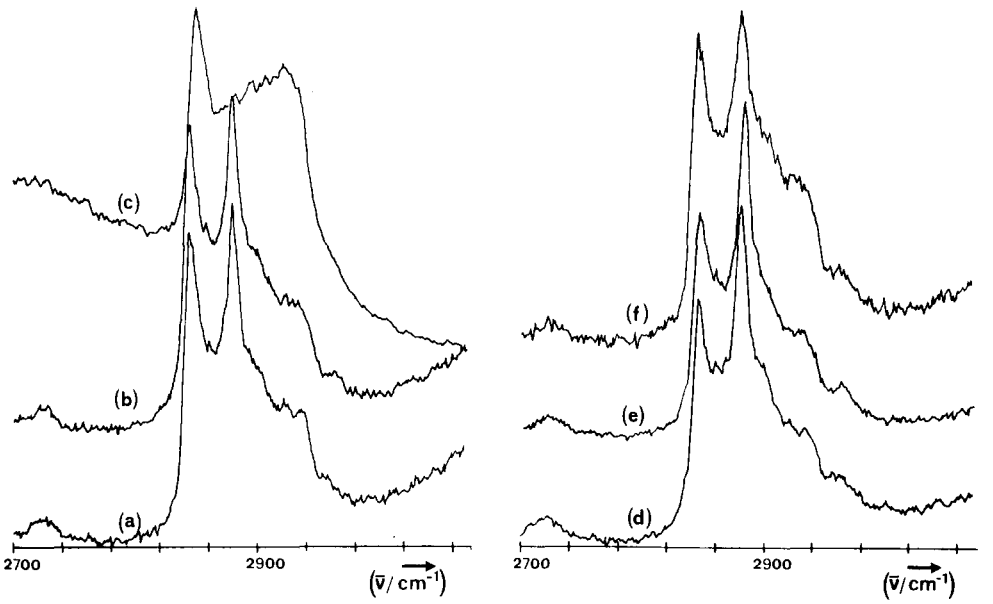

Fig. 3. Raman spectra vs. T obtained from aqueous suspensions of DPPA diacid and Ca^{2+}-DPPA complex (in a 2:1 molar ratio). DPPA diacid: (a) 30.5; (b) 40.8; (c) 62.0 °C. $Ca(DPPA)_2$: (d) 26.2; (e) 45.2; (f) 61.3 °C.

implications of Ca^{2+} insertion on the liposomal structure. Fig. 4 represents the peak height ratios, I_{2880}/I_{2850}, plotted against temperature. We can observe changes of the line shape of the I_{2880}/I_{2850} vs. T curve for DPPA-Ca^{2+} liposomes (Fig. 4b) by comparison with the curve obtained for DPPA diacid liposomes (Fig. 4a). In agreement with ^{31}P-NMR investigations, perturbations of the liposomal structures of DPPA-Ca^{2+} become high in the temperature range of about 55–66 °C. As mentioned previously [35], the temperature profiles obtained from Raman spectra for the phosphatidic acid-Ca^{2+} complex provide conclusive evidence that Ca^{2+} induces a major reorganization of the phospholipids into a highly ordered phase. As shown in Figs. 3 and 4, the DPPA-Ca^{2+} complex (2:1) displays Raman spectra below 50 °C with a very high I_{2880}/I_{2850} spectral index, with reference to those corresponding to DPPA diacid and/or monosodic DPPA, thus indicating that the lipid acyl chains are packed very tightly and give rise to high intermolecular vibrational coupling. These Raman results are in good agreement with previous works [35] which shows that calcium causes a reduction of the mobility of the acyl chains of dimyristoylphosphatidic acid and natural PA (proceeding from egg PC) and induces a closer packing of the phospholipid molecules. However, the domains of the C–H region are highly modified in the temperature range of about 55–66 °C, since they give Raman spectra with a

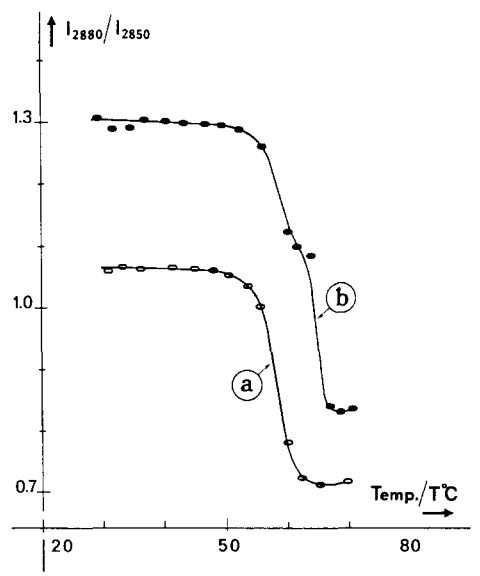

Fig. 4. The peak height ratio of the 2880 and 2850 cm^{-1} Raman bands plotted against temperature of the aqueous suspensions of: (a) DPPA diacid; (b) DPPA-Ca^{2+} complex (in a 2:1 molar ratio).

very high decrease in I_{2880}/I_{2850} ratio, resulting in a thermotropic transition. Accordingly, the thermotropic behavior of DPPA liposomes in the presence of calcium reinforces the conclusions from the preceding sections. Indeed, we believe that calcium incorporated in the DPPA bilayers induces reorganizations of the liposomal structures into more ordered states.

ESR spectral characteristics of spin-labeled soaps inside DPPA membranes in response to Ca^{2+}

The spin-label probes 5- and 16-doxylstearic acid were used to investigate the Ca(DPPA) gel phase. It should be noted that increasing probe concentration in the gel environment of $Ca(DPPA)_2$ can lead to spin–spin interactions (dipolar and exchange) in the ESR spectrum of 5- (or 16)-doxylstearic acid which cause the spectral lines to broaden and eventually to merge, as mentioned recently [17]. Thus, spectral broadening increases with increasing spin concentration until a single broad line is observed at a mole fraction of 5- (or 16-)doxylstearic acid superior or equal to 1 : 100. However, at a probe : lipid ratio inferior or equal to 1 : 200, no broadening can be observed and the ESR spectrum reflects the degree of ordering of the acyl chains. In these conditions, the inner and outer hyperfine splittings $A_\perp$ and $A_\parallel$, respectively, can be measured on the ESR spectra to gain insight into the structural aspects of the Ca^{2+}-DPPA combination by studying the motional constraint of 5- (or 16)-doxystearic acid inside membranes. Assuming rapid anisotropic motion of the spin-label about the long axis of the probe, increased ordering of the lipid acyl chains and, therefore, increased angular constraint of the spin-labeled soaps should be reflected in the ESR spectra by an increase in $A_\parallel$ accompanied by a decrease in $2A_\perp$ of equal magnitude, required by the rotational invariance of the trace of the hyperfine tensor [36]. The degree of motional restriction of the spin-labeled soaps in $Ca(DPPA)_2$ compared to thermotropic gel-phase lipids was estimated by measuring the $A_\perp$ and $A_\parallel$ parameters and by calculating the order parameter, S, as defined above (see Eqns. 1 and 2). The effective order parameter, which assumes fast motion, can be considered as only an apparent value, but it is nonetheless useful for making intercomparison between membranes and lipid dispersions or between different membranes. The results are reported in Table I and compared with those found

TABLE I

HYPERFINE SPLITTINGS AND ORDER PARAMETERS

Outer hyperfine splittings ($A_\parallel$) and order parameters (S) for 0.002 mole fraction of spin-labeled soaps (or phospholipids) in multilamellar vesicles composed of acidic phospholipids alone or in combination with calcium. All spectra were measured at about room temperature except for egg phosphatidic acid (PA) [44], which was at 37 °C. DSA, doxylstearic acid.

Vesicle composition		$2A_\parallel$ (G): outer maximum hyperfine splitting	Order parameter	Ref.
spin-label	membrane			
5-PG	bovine PS dispersions	51–52	0.57–0.59	43
(7,6)PG	DOPS	50	0.53	17
(7,6)PA	egg PA/egg PC (2 : 1)	51	0.57	42
(7,6)PA	DOPS	50	0.53	17
(7,6)PS	DOPS	50	0.54	17
(7,6)PS	DMPS	53	0.64	17
5-DSA	DPPA	51 ± 1	0.58–0.57	this work
5-DSA	egg PA	~ 49	≈ 0.48	44
16-DSA	DPPA	34 ± 1	0.14 ± 0.01	this work
16-DSA	egg PA	35	≈ 0.12	44
(7,6)PG	$Ca(PS)_2$	56	0.69	17
(7,6)PA	$Ca(PS)_2$	58	0.75	17
(7,6)PS	$Ca(PS)_2$	59	0.79	17
5-DSA	$Ca(DPPA)_2$	68–67	0.88 ± 0.01	this work
16-DSA	$Ca(DPPA)_2$	43 ± 1	0.43 ± 0.02	this work

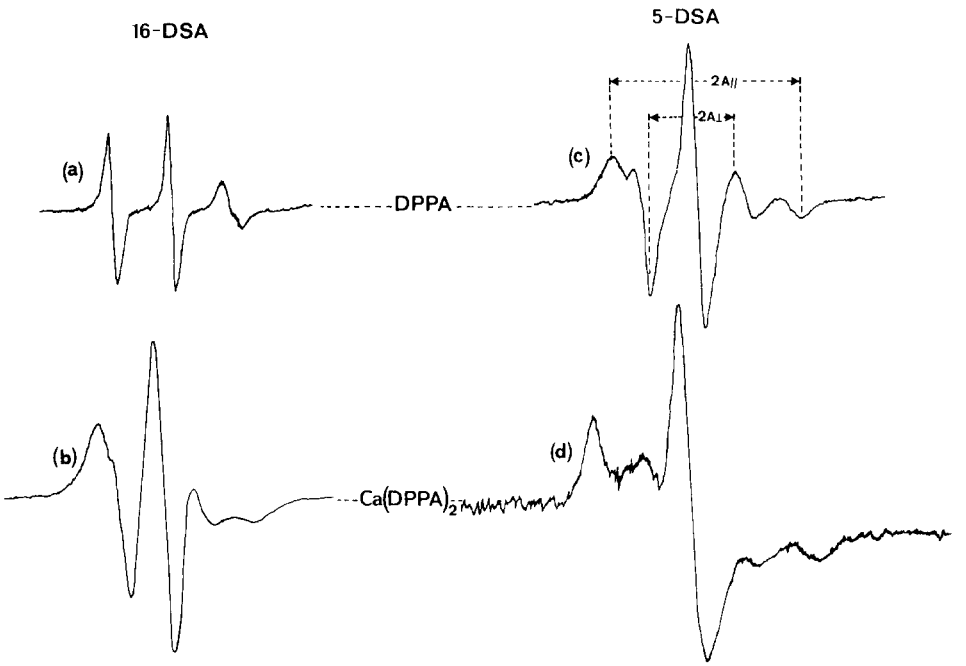

Fig. 5. ESR spectra of 0.002 mole fraction of 16- and 5-doxylstearic acid (DSA) in gel-phase DPPA, and in gel-phase $Ca(DPPA)_2$ at room temperature. Ca^{2+}-DPPA complex was prepared at 45 °C as described in the Experimental section. 16-Doxylstearic acid in vesicles of DPPA (a) and $Ca(DPPA)_2$ (b); 5-doxylsstearic acid in vesicles of DPPA (c) and $Ca(DPPA)_2$ (d). 2 $A_{||}$ and 2 $A_{\perp}$ are measured as indicated in Fig. 5c.

previously for multilamellar vesicles composed of acidic phospholipids in the presence or absence of Ca^{2+}. It should be noted that order parameters calculated from Eqn. 1 do not differ significantly from those evaluated from Eqn. 2. The ESR spectrum of 5-doxylstearic acid inside a DPPA membrane (Fig. 5) displays the largest increase in the separation of the outer hyperfine extrema, 2 $A_{||}$, from approx. 51 ± 1 G in DPPA without Ca^{2+} to 68–67 G in $Ca(DPPA)_2$ (Fig. 5 and Table I). Whereas, on account of the remote position of the doxyl group from the phosphate group in the 16-doxylstearic acid molecule, the motional restriction phenomena of this soap inside $Ca(DPPA)_2$ are less significant than those observed for 5-doxylstearic acid. Indeed, for 16-doxylstearic acid we found that the outer hyperfine separations increase only from 34 ± 1 G in DPPA without calcium, to 43 ± 1 G in $Ca(DPPA)_2$ (Fig. 5 and Table I). Nevertheless, for spin labels such as 16-doxylstearic acid, which have fast, nearly isotropic motion (2 $A_{||} = 34 \pm 1$ G, see Table I), calcium has a small effect on the order parameter of 16-doxylstearic acid incorporated inside $Ca(DPPA)_2$ membranes. We can notice that for 5-doxylstearic acid and 16-doxylstearic acid in $Ca(DPPA)_2$, the observed increase in $A_{||}$ (with reference to the results obtained with DPPA in the absence of Ca^{2+}, Table I) is not accompanied by any significant decrease in 2 $A_{\perp}$: approx. 20 G and 19 G for 5-doxylstearic acid and 16-doxylstearic acid, respectively. These aberrant values of 2 $A_{\perp}$ observed in the ESR spectra of 5-doxylstearic acid and 16-doxylstearic acid embedded inside $Ca(DPPA)_2$ membranes cannot be interpreted in terms of spin–spin interactions, as stated recently by Feigenson et al. [17] in their works concerning the interpretation of the ESR spectra of (7,6)PA, (7,6)PE and (7,6)PG in $Ca(PS)_2$.

Consequently, these results indicate that for a 'Ca^{2+}-DPPA' complex, the calcium ions have a much greater effect on fatty acid chain packing near the polar headgroup region than in the interior of the bilayer.

Conclusion

We have explored various aspects of the structural modifications of DPPA liposomes induced by the insertion of Ca^{2+} under the influence of the temperature. The results obtained from potentiometric titrations and from ^{31}P-NMR spectra vs. T provide conclusive evidence that Ca^{2+} reacts with DPPA to form a $Ca(DPPA)_2$ complex with major reorganization and condensation of the phosphatidic acid bilayers into a highly ordered phase. The temperature profiles obtained from the Raman spectra for the combination of Ca^{2+} and DPPA (Fig. 3) indicate that the lipid acyl chains are packed very tightly with reference to DPPA in the absence of Ca^{2+}, and give rise to high intermolecular vibrational coupling. Our results agree well with those obtained, either for natural phosphatidic acid derived from egg PC [37] using ESR technique, or by vibrational spectroscopies on the effect of Ca^{2+} on bovine brain PS [38] and on the properties of PS-CA^{2+} and phosphatidic acid-Ca^{2+} complexes [35,39].

We have subsequently studied the effect of calcium on the motional properties of spin-labeled soaps inside DPPA membranes by means of the electron spin resonance spectroscopy. With this technique, it has been possible to obtain detailed information on the degree of the motional restriction of spin labels in multilamellar vesicles of DPPA in response to Ca^{2+} by determining the anisotropic parameters, $A_{\parallel}$ and $A_{\perp}$, on the ESR spectra and thereby by calculating the order parameter, S. We may then question the structure or the molecular organisation of DPPA-Ca^{2+} bilayers under the influence of T. The effect of temperature on DPPA liposomes containing Ca^{2+} is to alter the conformational state of Ca^{2+}–phosphate binding in the hydrophilic region of the bilayers. This structural phenomenon is difficult to interpret; however, as suggested previously [40,41], the resulting structures are presumably composed of extended and flattened lipid cylinders of varying diameter. These suggestions can explain, in part, the more condensed structure of DPPA-Ca^{2+} combinations found at high temperatures, $T > 60\,^{\circ}C$.

The activity of calcium with synthetic membranes depends upon the class of the phospholipids used (phosphatidic acid $\gg$ PE and PC), the amount of phosphatidic acid inside a given membrane, the order in which these lipids are located on the bilayer, and so forth. This supports the view that the biological membrane possesses specific sites, essentially composed of phosphatidic acids, for reaction with cations. In this regard, the interaction of cations such as Ca^{2+}, Ba^{2+}, Mn^{2+}, Sr^{2+} and DPPA alone or together with neutral phospholipids (PC,PE) will be reported later.

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