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The Influence of Ca^{2+} on the Subgel Phase and Transitions of Dipalmitoylphosphatidylcholine

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The influence of low molarity (10–50 mM) $CaCl_2$ solutions on the phases and transitions of dipalmitoylphosphatidylcholine was determined using calorimetry and x-ray diffraction. In all cases, a subgel phase was induced before characterization was initiated. The presence of 20–50 mM $CaCl_2$ was shown to have little influence on the pre- and main-phase transitions in DPPC bilayers. However, as the $CaCl_2$ solution concentration is increased, the enthalpy of the subgel to gel bilayer phase transition decreases until no calorimetric endotherm was observed for DPPC in 50 mM $CaCl_2$. An observed broadening of the pre-transition for DPPC in 10 mM $CaCl_2$ can be correlated with a second order phase transition mechanism involving a continuous change in acyl chain packing with no change in mesophase spacing.

INTRODUCTION

The role of ions, particularly divalent ions, on membrane structure and function has been the subject of much research and conjecture. Model membrane studies have been particularly fruitful in defining the role of Ca^{2+} on the packing within

bilayers of a variety of phospholipids. Current dogma allows for the binding of Ca^{2+} to both zwitterionic and charged lipids. Specifically, Ca^{2+} binds to acidic lipids such that the aqueous spacing between bilayers can be eliminated and the lipid acyl chains pack in a denser subcell.[1-11] On the other hand, Ca^{2+} binds to phosphatidylcholines in such a way that a net charge resides on the bilayers, bilayers imbibe large quantities of solvent between themselves, and the packing of the lipid acyl chains within a bilayer is changed.[12-14] Recently, the subgel phase has been characterized as the stable low temperature phase in phospholipids.[15-20] Thus far, no studies have examined the effect of Ca^{2+} or any ion on the structure of the subgel phase and the transitions involving it.

For this report, scanning calorimetry and x-ray diffraction techniques were used to examine the effect of low molarity (10–50 mM) $CaCl_2$ solutions on "fully hydrated" dipalmitoylphosphatidylcholine. These low molarity $CaCl_2$ solutions have previously been shown[12-14] to greatly affect the stability of the DPPC multi-lamellar array with little perturbation of the acyl chain structures in the gel and liquid crystalline bilayer phases. Although a previous calorimetry study[21] had indicated that similar dispersions can produce two main thermal transition characteristics of the Ca^{2+} bound and free fractions of DPPC, recent reports[22] produce contradictory evidence. We show that the presence of low molarity (10 mM) $CaCl_2$ solutions causes a restructuring of the DPPC subgel phase via an increase in the bilayer repeat spacing, a change in the transition properties as measured by calorimetry and a change in the transition mechanisms as shown by real time x-ray diffraction techniques.

MATERIALS AND METHODS

All phospholipids used in this study were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Lipid dispersions containing 20 wt/vol % lipid were prepared by suspending the lipid in distilled water or $CaCl_2$ solutions. All salts were reagent grade. The samples were heated above their transition temperatures for one hour and then cooled to room temperature. The resulting sample appeared to be a homogeneous lipid-water mixture with no evidence of residual lipid powder or a reservoir of solvent. All dispersions were then equilibrated at $\sim 0°C$ for at least three days. A similar method[13] has been previously shown to produce equilibrated samples for this system. Samples for calorimetric measurements were hermetically sealed in aluminum pans.

Transition temperatures and enthalpies were measured by a Perkin-Elmer DSC-2C. Analysis of the measured thermograms were made with a Perkin Elmer Thermal Analysis Data Station (TADS) using the supplied standard program to determine enthalpy and onset temperature of any transition. Enthalpy per unit area was calibrated using Indium (99.999% pure). The heating rate was 2.5°C/min for samples and calibration. All thermograms were reproducible for two or more samples. Transition temperatures were reproducible to $\pm 1°C$ and enthalpies to less than 10% of their measured values.

X-ray diffraction patterns were also obtained using the 0.150 nm x-radiation at

station 7.2/3 of the synchrotron radiation source at the SERC Daresbury Laboratory.[23] A cylindrically bent single crystal of Ge[24] and a long float mirror were used for monochromatization and horizontal focusing, providing about 2×10^9 photons $\cdot s^{-1}$ down a 0.2 mm collimator at 2.0 GeV and 100 to 200 mA of electron beam current. A Keele flat plate camera was used with a sample path of 1 mm. Scattered x-rays were recorded on a linear detector constructed at the Daresbury Laboratory. The dead time between data acquisition frames was 50 μs with a temporal resolution of 1.2s for each frame. X-ray scattering has been plotted as a function of reciprocal space ($s = 2\sin\theta/\lambda$) using Teflon (0.48 nm) as a calibration standard.[25] All mesophase and subcell spacings were calculated using Bragg's Law.[26]

Temperature scans were produced by water baths connected internally to the sample mount of x-ray camera. The temperature of the sample was monitored internally using a thermocouple placed adjacent to the sample in the x-ray sample holder.

RESULTS AND DISCUSSION

Gravimetric samples were prepared for DPPC dispersed in various $CaCl_2$ solutions. It has been previously shown[12-14] that the presence of low molarity $CaCl_2$ solution allowed DPPC headgroups to bind Ca^{2+} and swell to large interbilayer separations. Unlimited swelling was observed in DPPC bilayers when ~ lmM $CaCl_2$ was present.[12] As more $CaCl_2$ was added to the bathing media, the DPPC bilayer *d*-spacing was observed to slowly decrease until, in the presence of 500 mM $CaCl_2$, it reached the value for fully hydrated DPPC in water. The observed decrease in bilayer swelling with increasing $CaCl_2$ content was a reflection of the greater screening ability of the solvent with increasing $CaCl_2$ content in relation to the increasing bilayer surface charge due to an increase in Ca^{2+} binding also with increasing $CaCl_2$ concentration.[13,14] In addition, a change in the gel state DPPC bilayer packing was not observed until greater than 800 mM $CaCl_2$ was used. There have been conflicting reports as to the effect of low molarity $CaCl_2$ solutions on the DPPC thermodynamic parameters. We have attempted to standardize these results by inducing the DPPC subgel phase which has been shown to be the low temperature equilibrium phase, when DPPC is dispersed in water.[15-20]

The thermodynamic parameters were obtained for these samples with the initial phase being the L_C and the $L_{\beta'}$ phase. Table I and Figure 1 indicate that all three DPPC transitions (i.e., sub-, pre- and main) were affected by the presence of 10 mM $CaCl_2$ when used in place of water. The transition temperatures were lowered while the enthalpies increased for DPPC when 10 mM $CaCl_2$ was present. It was not surprising that changes in the DPPC bilayer thermal properties were observed when 10 mM $CaCl_2$ was present but it was surprising that they did so in the manner shown in Table I. It was previously shown that DPPC bilayers in this $CaCl_2$ solution swelled[12] the most for the concentrations used in this study. It would be expected that DPPC bilayers in 10 mM $CaCl_2$ would be influenced the most by thermal undulations, akin to a disordered rippling, in the bilayer, and have the weakest interbilayer forces. This would lead to a destabilization of the order between bi-

TABLE I

Thermodynamic parameters for multi-bilayer arrays of dipalmitoylphosphatidylcholine in $CaCl_2$ solutions. All samples contained 80 vol/wt% [$CaCl_2$] solution and were equilibrated to obtain an initial subgel phase. All scan rates were 2.5°/min.

[$CaCl_2$] mM	T_1 (°K)	ΔH_1 (Kcal/mole)	T_2 (°K)	ΔH_2 (Kcal/mole)	T_3 (°K)	ΔH_3 (Kcal/mole)
0	294.0	2.53	308.3	0.98	314.3	7.26
10	291.7	3.59	303.1	1.36	312.8	9.72
20	293.6	3.32	308.9	0.86	314.3	7.76
30	289.8	2.40	307.0	1.02	313.1	7.91
40	293.6	1.12	308.2	0.92	314.8	7.55
50	--	--	309.8	1.10	314.8	8.32

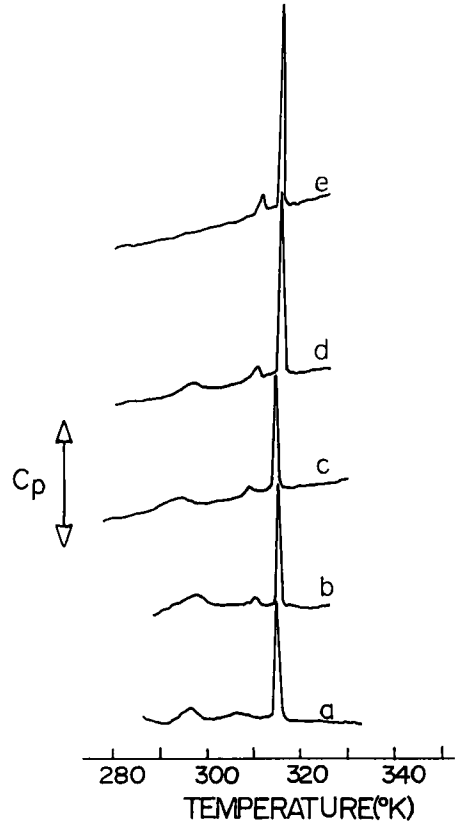

FIGURE 1 DSC scans of DPPC in a) 10, b) 20, c) 30, d) 40, and e) 50 mM $CaCl_2$ solutions. All samples had contained 80 vol/wt% solvent with sample equilibration at ~0°C for over 5 days. The samples were run at a scan rate of 2.5°C/min.

layers in the multi-lamellar array, and a decrease in the packing order of the acyl chains. These phenomena would be expected to cause a decrease in the transition enthalpies rather than the increase that was observed. This result cannot be simply explained by the increase in ordering of the acyl chain packing due to the binding of Ca^{2+} to the DPPC bilayers since it would predict a further increase in enthalpy as more $CaCl_2$ was added to the system. As the $CaCl_2$ concentration was increased from 10 to 20 mM, the pre- and main transition T_m's and enthalpies were observed to decrease and approach that of DPPC in water. The DPPC acyl chain packing has been previously shown[12] to be unaffected by the presence of increasing $CaCl_2$ until a concentration of 0.5M was used. It cannot be ruled out that the changes in transition temperature for the three phase transitions and transition enthalpy for the pre- and main phase transitions with $CaCl_2$ concentration are a reflection of some random variation. However, we also observed a continuous decrease in the enthalpy of the DPPC subgel to gel transition as the $CaCl_2$ concentration increased from 10 to 40 mM until it becomes negligible for DPPC in 50 mM $CaCl_2$. In addition, the pretransition for DPPC in 10 mM $CaCl_2$ was broader and more diffuse than for the other samples studied. A similar examination of DPPC in 1 mM $CaCl_2$ also resulted in the appearance of a broad pretransition. Again, it would be expected that DPPC bilayers in 1 or 10 mM $CaCl_2$ would be affected the most, of the samples used, by the destabilizing effect of the imbibing of large quantities of solvent between the bilayers on the long range order in the multi-lamellar array. In addition, there is no evidence for two transitions involving phases consisting of DPPC molecules within a bilayer having Ca^{2+} bound to them and those molecules without bound Ca^{2+} for the thermograms of the subgel, gel, rippled, and liquid crystal transitions. This is contrary to a previous report by Ganesan, *et al.*[21]

It is known that the presence of a 10 mM $CaCl_2$ solution allowed for the greatest DPPC bilayer swelling in this data set because its charge screening capabilities of the positive charge on the bilayer surface due to the binding of Ca^{2+} to the DPPC headgroup were the least. We are then left to hypothesize that extensive bilayer swelling may be a limiting factor in the development of the rippled bilayer phase. It has been previously shown that the pretransition for DPPC in H_2O is characteristic of the $L_{\beta'}$ (or gel) to rippled bilayer phase transformation even for samples undergoing temperature scans.[33,34] Transitions involving the rippled bilayer phase are, however, dependent on a number of external conditions of which time is the most significant.[27,28] It has been[27] shown, for example, that the $P_{\beta'} \rightarrow L_{\beta'}$ transition required $\sim$ 7 min. to be 90% complete.

X-ray diffraction patterns were collected in real time for DPPC dispersed in 10 mM $CaCl_2$ while the sample underwent a temperature scan of $\sim$ 7.5°C/min. so that the broadened pre-transition observed calorimetrically (Figures 2 & 3) could be characterized. An initial L_c phase was obtained which had a mesophase d-spacing of 10.14 nm with acyl chain scattering reflections at 0.383 and 0.416 nm. The same phase observed for DPPC in water[20] had acyl chain reflections at 0.375 and 0.430 nm. The unit cell bases vectors and areas per molecule are thus similar (within 4%) for DPPC in water and in 10 mM $CaCl_2$. Thus, the presence of 10 mM $CaCl_2$ allows the DPPC subgel (or L_C) state bilayer to become charged and to imbibe a large amount of salt solution in the interbilayer space without changing the acyl

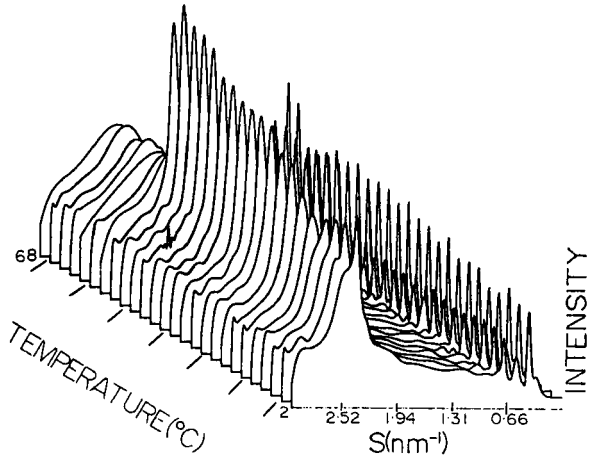

FIGURE 2 X-ray diffraction patterns obtained for DPPC in 10 mM $CaCl_2$ undergoing a temperature scan of ~ 7.5°C/min from 2 to 68°C. Every tenth frame of 2s duration from a data set of 255 frames is shown. The temperature scale indicates every 10 degrees between 5 and 65°C.

chain packing. It has been previously shown that the presence of such a low concentration of $CaCl_2$ did not cause a substantial charge in the bilayer thickness or acyl chain packing when the DPPC molecules were in a gel state. The observation of an increase in DPPC subgel (or L_C) state bilayer swelling with the presence of 10 mM $CaCl_2$ indicates that the initial suggestion of Ruocco and Shipley[18,19] that the L_C phase was crystalline in three dimensions with residual water mediating

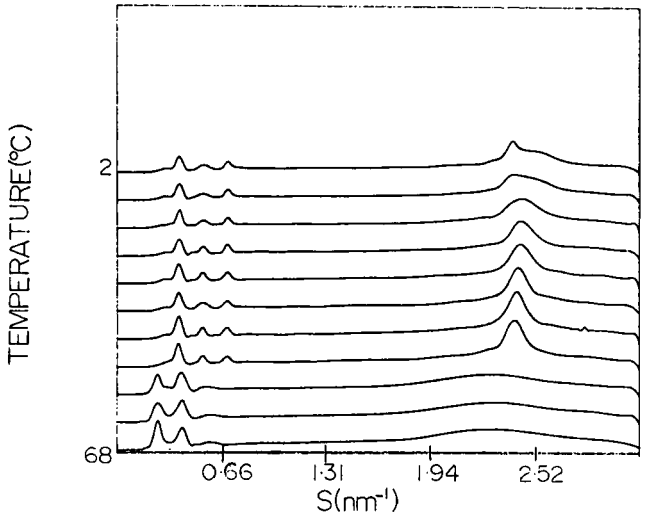

FIGURE 3 Selected diffraction patterns for the data set in Figure 3 plotted as a function of inverse *d*-spacing. Every twenty-fifth frame is shown.

interbilayer coupling is not valid for this system. It would be expected that intracrystalline forces would be strong enough to limit the opportunity for Ca^{2+} to bind to the DPPC headgroups and/or overcome the weak electrostatic force between bilayers due to a charged bilayer surface. The acyl chains are in a crystalline packing, however, the bilayers show a definite mesophase character in their ability to imbibe greater amounts of solvent when charged. The temperature scan clearly produces a transition from the L_c to L_β phases (mesophase d-spacing of 10.14 nm and acyl chain spacing of 0.409 nm) beginning at ~10°C which is lower than our previous calorimetric results. The L_β acyl chain spacing then increases from 0.409 to 0.416 nm over a temperature range of ~36°C while there is no apparent change in the mesophase structure (See Figure 3). The absence of change in the DPPC mesophase d-spacing upon the transition between the subgel and gel bilayers is consistent with the lack of a reservoir of solvent in contact with the sample. It is well documented that DPPC bilayers imbibe greater amounts of solvent (when available) as the disorder in the acyl chains increases. It can be further inferred that the bilayer dimensions remain constant during the $L_C \rightarrow L_\beta$ phase transition. The bilayer thickness for DPPC gel state bilayers in the presence of any concentration of $CaCl_2$ has been shown previously [13] to be 4.4 nm. The formation of the $L_{\beta'}$ phase (gel state bilayers with tilted chains) from the L_β phase (gel state bilayers with untilted chains) has been shown to manifest itself by the presence of a shoulder on the L_β acyl chain wide angle x-ray diffraction peak. This transition required ~12 minutes when fully hydrated DPPC bilayers in water were allowed to equlibrate at 22°C. None of the diffraction patterns collected in real time for DPPC gel state bilayers in the presence of 10 mM $CaCl_2$ were found to contain this feature. Thus, the non-tilted acyl chain DPPC gel state bilayer was the phase present after the transformation from the L_C phase, and until the transformation into the L_α phase occurred. There is also no evidence for the formation of a rippled bilayer phase in this system as shown by the lack of suitable changes in the small angle scattering pattern.[33,34] A second order thermodynamic process involving a continuous change in the acyl chain packing was thus responsible for the broadened pre-transition observed in this system. The main thermal transition at ~45°C involved a change from the L_β phase to an L_α phase with a mesophase spacing of 9.72 nm and an acyl chain spacing of 0.445 nm.

CONCLUSIONS

The interaction of ions, particularly $CaCl_2$, with DPPC bilayers has been examined via a variety of physical techniques. Previous reports have indicated that the presence of Ca^{2+} caused DPPC bilayers to become positively charged[29,30] and to swell to large interbilayer separations at low $CaCl_2$ concentrations.[12-14] In addition, it has previously been indicated that $CaCl_2$ solutions can cause changes in the pre- and main transition parameters at high concentrations,[31,32] and may cause the presence of multiple transition peaks at low concentrations.[21] It has been shown that the presence of 10 mM $CaCl_2$ causes the DPPC subgel bilayer phase to become charged, and imbibe a significantly greater amount of solution than observed when

only water was present. It can be inferred that the subgel phase in these systems is not crystalline in three dimensions. We have unambiguously shown that the presence of low (i.e., 20–50 mM) $CaCl_2$ concentration solutions does not, in essence, affect the endotherms indicative of the DPPC pre- and main phase transitions. It is also clear that endothermic peaks from bound and unbound DPPC are not observed at these $CaCl_2$ concentrations which is contrary to a previous report.[21] The increase in the amount of $CaCl_2$ clearly causes a decrease in the transition enthalpy for the DPPC subgel to gel phase transition until at 50 nM $CaCl_2$, when the endotherm characteristic of this transition is no longer indicated. At low $CaCl_2$ molarity, it can be inferred that the gel to liquid crystalline phase transition does not proceed via first order processes in the usual $L_{\beta'} \rightarrow P_{\beta'} \rightarrow L_\alpha$ transition sequence but via a second order thermodynamic transition mechanism involving the continuous expansion of the acyl chain subcell from the L_β to L_α phases. It is clear that real time x-ray diffraction measurements are more sensitive to the presence of broad or low enthalpy phase transitions than non-adiabatic scanning calorimetry.

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