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The effect of protons or calcium ions on the phase behavior of phosphatidylserine-cholesterol mixtures

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The influence of protons or calcium ions on the miscibility of cholesterol in phosphatidylserine has been examined using differential scanning calorimetry and X-ray diffraction. At pH 2.6, where the carboxyl group of the serine moiety is protonated, two endothermic transitions are observed in cholesterol-phosphatidylserine mixtures. The midpoint of the first is at 35°C in the absence of cholesterol and decreases to approx. 15°C for molar fraction of cholesterol 0.5. The second transition is centered at approx. 44°C, almost independent of cholesterol content. The two lower temperature phases are lamellar and the high temperature phase has hexagonal symmetry. Cholesterol is more miscible in protonated phosphatidylserine than in the sodium form: cholesterol crystals are detected at a molar ratio of phosphatidylserine to cholesterol of about 1.7:1 as compared to about 2.3:1 at neutral pH. In the presence of calcium ions (1.3 Ca^{2+} per phosphatidylserine), a lamellar phase is observed with layer spacing 53 Å which is independent of temperature (25°C–65°C) and of cholesterol content. Calcium ions cause reduced cholesterol solubility: crystallites are detected already at a molar ratio of 4:1.

Introduction

Phosphatidylserine (PS) is one of the most prevalent negatively charged membrane phospholipids. Its anionic character under physiological conditions renders it a particularly important participant in ion–membrane interactions. The net negative charge of −1 may also be expected to influence the way in which the PS molecule interacts with other components of the mammalian membrane, e.g., proteins and sterols. Of the latter, cholesterol is the most important representative.

Model systems of PS bilayers, as studied by DSC and X-ray diffraction, show limited solubility for cholesterol [1–3]. Phase separation into a PS-cholesterol phase and an almost pure cholesterol phase takes place at molar ratios of approximately 2:1 phospholipid to cholesterol and below. Similar effects are observed with phosphatidic acid [3]. In bilayers of zwitterionic phospholipids, cholesterol solubility is higher: phase separation takes place at a molar ratio of about 1:1 phosphatidylcholine (PC) to cholesterol [4,5] and 1.5:1 phosphatidylethanolamine (PE) to cholesterol [6]. The presence or absence of the net negative charge therefore seems to be one of the important factors in determining the miscibility of cholesterol in the phospholipid bilayers. Nevertheless, comparison among different phospholipids cannot rule out other influences, e.g. of hydrogen bonding. In this report, we focus on the question of charge alone: we use DSC and X-ray diffraction to monitor the effects of protonation of the carboxyl group of the serine moiety, or the effects of Ca^{2+}. The latter study is a continuation of earlier work [3]. We show how these ions affect the miscibility and thermotropic behavior of PS-cholesterol mixtures and compare these properties with those characteristic of the charged state, i.e. PS-Na at pH 7.

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Materials and Methods

Lipids

Phosphatidylserine from bovine spinal cord (Grade I) monosodium salt was purchased from Lipid Products

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(South Nutfield, U.K.). The phospholipid was converted into the protonated form by shaking: twice with HCl/methanol/H_2O (H_2O/methanol, 1:1, v/v) and twice with H_2O/methanol (1:1, v/v). This procedure (recommended by the Lipid Products Company) was followed by lyophilization after evaporation of the solvents with a nitrogen stream. Cholesterol (extra pure grade) was purchased from Merck (Darmstadt, F.R.G) and was recrystallised twice from ethanol. The interaction products were prepared by mixing solutions of PS-either protonated or as the sodium salt-with the appropriate volumes of cholesterol solution. Both cholesterol and PS were dissolved in chloroform/methanol (2:1, v/v). The solvents were driven off by a stream of nitrogen and the samples were kept under high vacuum for 3 h.

Differential scanning calorimetry

The dry phospholipids or phospholipid-cholesterol mixtures were weighed directly (1–2 mg) into the aluminum pans of the calorimeter and an excess (about 4-fold) of solution of $1.5 \cdot 10^{-1}$ M NaCl in $2.5 \cdot 10^{-3}$ M HCl was added. The samples were shaken on a Vortex mixer and incubated for 0.5 h at approx. 70°C. DSC experiments were performed on the Du Pont 990 Thermal Analyser equipped with cell base 2 and a home made cooling device. A scanning rate of 5 C°/min was generally used; however to determine if there was any scan rate dependence, some experiments were also performed at 2 C°/min. To look for hysteresis effects, several experiments were scanned in both the heating and cooling modes.

X-ray diffraction experiments

For the X-ray diffraction experiments, samples were prepared in the following ways. To a dry sample of PS-Na was added an excess of solution of $5 \cdot 10^{-1}$ M NaCl in 10^{-2} M Tris-HCl (pH 7.4); to the dry sample of PS-H was added either $5 \cdot 10^{-1}$ M NaCl in $2.5 \cdot 10^{-3}$ M HCl, or $1.5 \cdot 10^{-1}$ M NaCl in $2.5 \cdot 10^{-3}$ M HCl to give a final concentration of lipids of 5–10 mg/ml. The samples were incubated for 0.5 h at 75°C followed by centrifugation in an Eppendorf centrifuge at 14000 rpm for 15 min. An aliquot of the resulting pellet plus excess supernatant were then gently centrifuged into either a quartz or Li glass X-ray capillary. In some cases the dry lipid mixtures were introduced into the X-ray capillary and an excess of NaCl + HCl solution was added, followed by incubation. The two methods of sample preparation produced cholesterol-PS mixtures which we found to be indistinguishable by X-ray diffraction. For the experiments in the presence of Ca^{2+} ions, dry lipids were dispersed in 10^{-2} M $CaCl_2$ + $1.5 \cdot 10^{-1}$ M NaCl in 10^{-2} M Tris buffer pH 7.4 to yield a Ca:PS molar ratio of 1.3:1. The dispersions were incubated for 0.5 h at approx. 75°C with frequent vortexing followed by three cycles of freezing and thawing and centrifugation for 15 min in an Eppendorf centrifuge.

Low angle X-ray diffraction experiments were performed on a Philips sealed-tube fine focus generator operating at 40 kV and 34 mA producing copper radiation. Monochromatization was provided by a nickel filter and one Franks mirror and the beam was collimated to 4 mm height, 350 μm width in the plane of the specimen. The specimen to detector distance was 460 mm. The diffraction pattern was recorded by a linear position sensitive detector of the delay line type [7]. Exposure times were generally 1–2 h, but occasionally 16 h when very weak diffraction lines were sought. During acquisition, the data were histogrammed in 256 channels and stored in a Z-80 based microprocessor unit. Following completion of the experiment, the data were transferred to an IBM 3090 computer for analysis. Wide-angle diffraction patterns were obtained on a Searle camera equipped with Franks optics affixed to an Elliott GX6 rotating anode generator operating at 40 kV, 30 mA and producing Cu radiation with a 200 μm focus. The camera could be used either with X-ray film (Kodak DEF-5) or with the electronic detector. For both the wide and low angle experiments, the temperature of the specimen was controlled by coolant flowing through the brass block capillary support.

Results

DSC experiments

Fig. 1 presents the thermograms of PS-H alone and in the presence of increasing concentrations of choles-

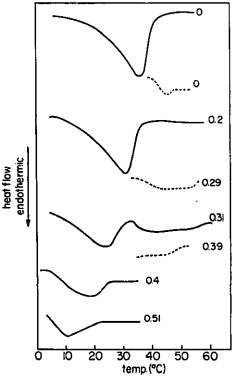

Fig. 1. Thermograms of PS-H/cholesterol mixtures at pH 2.6 obtained at a heating rate of 5 C°/min as described in Materials and Methods. The numbers indicate molar fraction of cholesterol.

terol. Two endothermic transitions are observed; one strong (peak I) and the second weak and broad (peak II). The midpoint temperature of peak I is 35°C. If we compare T_m(I) with T_m of the sodium salt of bovine spinal cord phosphatidylserine (14°C), then we observe a positive shift of approx. 20°C due to protonation. This is similar to the shift observed in other protonated phosphatidylserines [8–10], and stems from a decrease in the electrostatic repulsion. T_m(I) is independent of scan rate: similar values were obtained at scan rates of 5 C°/min and 2 C°/min. Upon cooling, a slight hysteresis is seen, i.e. there is a difference in the position of the peak upon heating and cooling of 3°C. In addition to the main peak at 35°C a second peak at around 44°C is seen (indicated with broken lines on the figure). The second peak is due to the transition to the inverted hexagonal H_{II} phase, identified from X-ray data (see below). Such a transition has heretofore been inferred using NMR [11,12] in protonated phosphatidylserines from egg and from erythrocytes. Both the position and the large width of peak I (the latter being characteristic of natural phospholipids) preclude the observation in DSC of the polymorphic transition of anhydrous cholesterol at 37°C [13]. Upon addition of cholesterol to PS-H, there is a gradual decrease of T_m(I) and broadening of the peak; and the second peak becomes so broad that in some cases it is very difficult to distinguish from the base line. In the presence of cholesterol no dependence on scan rate was seen for T_m(I); for molar fraction of cholesterol, X(chol), equal to 0.2 there is a weak hysteresis upon cooling.

Fig. 2A shows the transition temperatures T_m as a function of the molar fraction of cholesterol. The data presented are from several different batches of PS-H. As seen from the figure, the maximal decrease of T_m(I) (at X(chol) = 0.5) is about 20°C. The effect of cholesterol on PS-H differs from its effect on PS-Na as presented in Fig. 2 of Ref 1. In the case of PS-

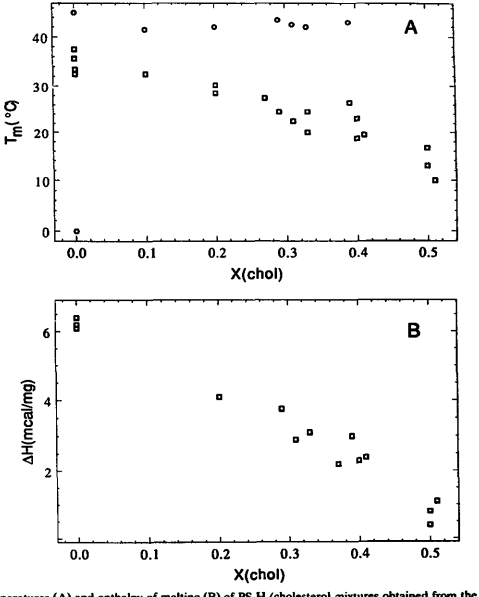

Fig. 2. The transition temperatures (A) and enthalpy of melting (B) of PS-H/cholesterol mixtures obtained from the thermograms of Fig. 1 and plotted as a function of molar fraction of cholesterol: □, First transition; ○, second transition.

Na/cholesterol mixtures, a small decrease of T_m of about 4 C° is seen up to $X(\text{chol}) = 0.2$ and at higher cholesterol ratio no further decrease of temperature is seen. Such an invariance is indicative of the appearance of an additional phase and indeed cholesterol crystallites were detected in X-ray measurements at about $X(\text{chol}) = 0.31$ (see below).

In Fig. 2A are also presented the T_m values for the transition to the H_{II} phase. These T_m(II) values were estimated from the thermograms: due to the breadth and weakness of the peaks the uncertainty in T_m(II) is quite large. However, it seems that T_m(II) does not change with the addition of cholesterol. This finding is in agreement with the results of Hope and Cullis [11] who found by NMR that the polymorphic transition to the H_{II} phase of egg PS-H is unaffected by cholesterol at a molar ratio of 1:1.

Fig. 2B presents the enthalpy of melting for PS-H/cholesterol mixtures as a function of cholesterol. The values shown for high cholesterol content may be somewhat too low as part of the peak is unaccounted for due to the shift of T_m(I) to low temperatures where it is superposed by the melting peak of water. However, it seems that the effect of cholesterol on ΔH of PS-H is stronger than in the case of PS-Na and that the enthalpy of melting becomes zero at a cholesterol content which is lower in the case of PS-H than in the case of PS-Na (Fig. 2b in Ref.1).

X-ray diffraction

In Fig. 3A is shown the low angle X-ray diffraction pattern of PS-H in the lamellar phase at 28°C in the presence of cholesterol, $X(\text{chol}) = 0.41$. Three orders of the bilayer spacing are observed; in addition, a prominent peak at approx. 34 Å signals the presence of domains of crystalline cholesterol. Fig. 3B identifies the high temperature phase of pure PS-H as clearly being of hexagonal symmetry. The high temperature phase of mixtures also has hexagonal symmetry (Fig. 3C); however, as seen in the figure, the (11) reflection is not resolved from the 34 Å reflection of the cholesterol crystallites. Nevertheless, an approximately 70% increase in intensity at 34 Å upon passing from the lamellar phase to the hexagonal phase indicates the contribution of the (11) reflection at this position. The temperature dependence of the low-angle d spacings of a specimen with $X(\text{chol}) = 0.41$ is summarized in Fig. 4. A phase transition is clearly marked by a decrease in the layer spacing with midpoint at 17–18°C, which agrees well with the DSC result. The transition to the hexagonal phase may be noted first by the appearance of the (20) reflection at 35°C. The [10] reflection at this temperature is not resolved from the $l = 1$ reflection of the lamellar phase in our experiment, and is first clearly seen beginning at 45°C. There

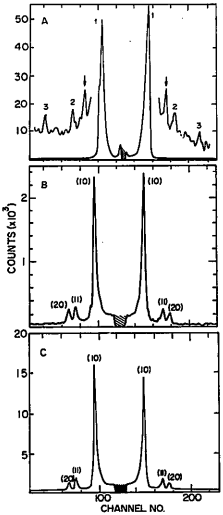

Fig. 3. Low-angle X-ray diffraction profiles from: (A) a PS-H/cholesterol mixture, $X(\text{chol}) = 0.41$ at 28°C in $5 \cdot 10^{-1}$ M NaCl in $2.5 \cdot 10^{-3}$ M HCl. The numbers indicate three orders of the lamellar spacing $d = 56$ Å, (i.e., 56 Å, 28 Å, 18.7 Å) and the arrows indicate the 34 Å reflection from crystalline cholesterol; (B) pure PS-H, at $T = 70$°C in the same buffer as (A). The indices of the first three reflections from a two-dimensional hexagonal lattice with unit cell dimension $a = 67$ Å are indicated: $d_{10} = 58$ Å, $d_{11} = 34$ Å, $d_{20} = 29$ Å; (C) a PS-H/cholesterol mixture, $X(\text{chol}) = 0.39$ at 56°C in the same buffer as (A). The indices of the first three reflections from a two dimensional, hexagonal lattice with unit cell dimension $a = 69$ Å are indicated. In all cases the symmetric profile is shown and the hatched region denotes the position of the beam stop.

is obviously a broad biphasic region and the pure hexagonal phase is observed only above 52°C.

High-angle diffraction experiments on PS-H (results not shown) verified the existence of three phases with three different chain packing arrangements. In pure PS-H, below 35°C a single sharp line is observed at 4.05 Å; at 42°C, two strong and sharp lines are observed at 4.2 Å and at 4.6 Å, as well as a number of other weak, sharp lines. There is also sometimes evidence of an admixture of the hexagonal phase at the latter temperature as would be expected from the low angle results. The acyl chains in the hexagonal phase at 62°C are clearly disordered, as is evidenced by the presence of a single diffuse ring at 4.4 Å. We tenta-

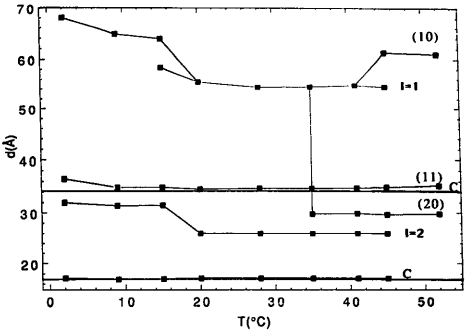

Fig. 4. The temperature dependence of the d spacings of the low angle X-ray reflections from a PS-H/cholesterol mixture in $5 \cdot 10^{-1}$ M NaCl in $2.5 \cdot 10^{-3}$ M HCl with $X(\text{chol}) = 0.41$. The instrumental resolution of 0.001 $Å^{-1}$ is due to the channel width.

tively interpret the combined low- and high- angle X-ray results , following Harlos and Eibl who observed similar behavior for phosphatidylglycerol in the presence of Ca^{2+} [14]: there is a transition from an ordered lamellar phase to a disordered lamellar phase and it is this change which gives rise to the major peak of the DSC experiments. However, on the time scale of the X-ray experiment, this latter phase is unstable, and a transition occurs to a second ordered lamellar phase, with a smaller interlayer spacing. The final transition is from the second lamellar phase to the hexagonal phase in which the chains are truly liquid crystalline.

In Fig. 5 we compare the dependence of the d-spacings in the lamellar phases and the onset of cholesterol phase separation in mixtures with PS-Na, PS-H and PS-Ca. Some of the data for PS-Na and PS-Ca have been discussed previously [1–3]. At pH 7, PS-Na forms bilayers in the gel phase which are spaced approx. 71 Å apart in $5 \cdot 10^{-1}$ M NaCl. This spacing decreases to 60 Å in the liquid crystalline phase. As cholesterol is added, the layer spacing in the gel phase decreases and that in the liquid crystalline phase increases. At $X(\text{chol}) = 0.31$ occurs the first observation of cholesterol crystallites. At pH 7, PS-Ca forms bilayers which are approx 53 Å apart. The layer spacing is independent of both temperature and cholesterol content between 25°C and 65°C. This is consistent with data in the literature [8] which show that Ca^{2+} causes crystallization of the acyl chains of PS and shifts T_m to temperatures above 80°C. The low temperature phase transition for PS-Ca observed in DSC [3,15] was not detected. Diffraction from cholesterol crystallites is first seen for $X(\text{chol}) = 0.2$. In the absence of cholesterol, the high-angle diffraction pattern (not shown) is not invariant. Two relatively sharp reflections are observed at 4.2 Å and 4.7 Å at room temperature and at 4.2 Å and 4.5 Å at 63°C. In the presence of cholesterol, the relevant numbers for $X(\text{chol}) = 0.1$ are 4.2 Å and 4.6 Å for both high and low temperatures. The high temperature pattern is always sharper than the low temperature pattern. Whether or not these changes are related to the low enthalpy transition mentioned above is not clear.

Protonation of the carboxyl group of PS results in an overall decrease of approx. 5 Å with respect to PS-Na in the layer spacings of the lamellar phases. The spacing in the higher temperature phase is 56–57 Å and is close to that found in the presence of Ca^{2+} [16]. The appearance of cholesterol diffraction is not seen until $X(\text{chol}) \approx 0.37$.

Discussion

The net negative charge which phosphatidylserine carries at physiological pH must influence the way in which the lipid participates in membrane related processes such as phase modulation and fusion events [16]. For example, cholesterol, a commonly occurring sterol in mammalian membranes, is known to modify the phase behavior of membrane lipids above the acyl chain melting transition by restricting their conformational freedom [5]. The solubility of cholesterol in the environment of any given phospholipid controls its effectiveness in this regard. At neutral pH, cholesterol is less soluble in PS than in zwitterionic phospholipids. It is known that the headgroup of PS is more rigid than that of PE or PC [12,17] . This may be a contributing factor to the lower solubility of cholesterol in PS.

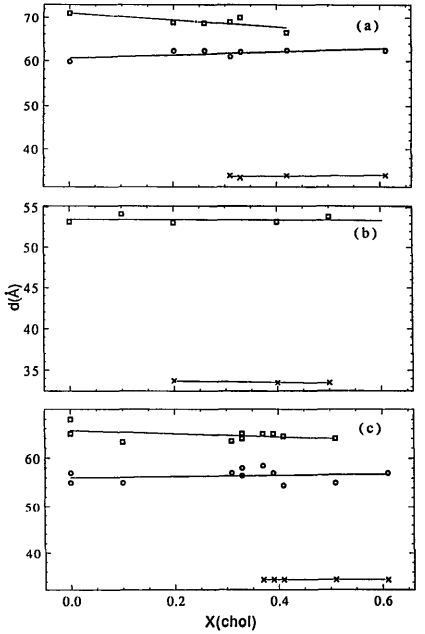

Fig. 5.Comparison of layer-spacings in the lamellar phases (□, low-temperature phase; ○, high-temperature phase) and the appearance of diffraction from separate cholesterol crystallites (×) as a function of cholesterol content for (a) PS-Na, (b) PS-Ca and (c) PS-H/cholesterol mixtures.

However, more specific contributions such as the effects of charge and hydrogen bonding have also been suggested [3].

To examine this latter proposal in more detail, we have used DSC and X-ray diffraction to characterize the phase modulation and phase separation in PS-cholesterol mixtures, at pH 2.6 where the carboxyl is protonated or in the presence of Ca^{2+} ions. We compared these results in parallel with those obtained at neutral pH in NaCl solution. At molar ratios of PS-Na/cholesterol of approx. 2:1 and below, a peak associated with a polymorphic transition of pure cholesterol is seen in DSC [1]. The limit of solubility of cholesterol in PS-Na as determined by X-ray diffraction is approx 2.3:1 (PS/cholesterol). In the case of PS-H/cholesterol mixtures, the cholesterol transition cannot be detected by DSC as it is superimposed either by the major transition or by the transition to the H_{II} phase. In X-ray diffraction experiments cholesterol crystallites were seen at about 1.7:1 molar ratio of PS-H/cholesterol. The continuous decrease of T_m of the major transition as detected in DSC experiments indicates that the effect of cholesterol on the PS chains in the gel state is much stronger than in the case of PS-Na. This is in agreement with X-ray data showing that the protonation of PS results in an increase of the lipid's solubilizing capacity for cholesterol as compared to that at neutral pH. As the pH is lowered, the intermolecular electrostatic repulsion is reduced and concomitantly, the strength of the H-bonding increases. At pH 2.6 it is the serine carboxyl group which is protonated as its pK is approx 4.5 ± 0.2 [18]. The pK

of the phosphate is 1 [18]. The result is a less hydrated headgroup more tightly packed. In this regard PS becomes similar to PE. Protonation raises the temperature of the chain melting transition and like PE, PS-H goes into an inverted hexagonal phase at temperatures above the lamellar phases. In addition, the solubility limit of cholesterol in PS-H is close to that in PE [6]. In X-ray diffraction experiments above T_m of the major transition, the acyl chains appear more ordered in PS-H than in PS-Na, although the interbilayer spacing is larger in the latter. The difference might be due to the lower hydration of the bilayer of protonated phosphatidylserine.

In our multilamellar preparation, PS-Ca has the lowest solubility for cholesterol of the mixtures studied. At $X(\text{chol}) = 0.2$, a separate cholesterol phase is just discernible. The crystallization of the acyl chains and their tilted orientation with respect to the plane of the bilayer are nearly unaffected by the presence of cholesterol; the interbilayer spacing is similarly unchanged. Ca^{2+} is known to bind to the phosphate group of PS [19] with a stoichiometry of 1 Ca^{2+} per 2 PS molecules [20]. There is evidence that the binding takes place between neighboring bilayers [16]. As a result of the binding, the serine headgroup rotates; the P-O bonds undergo a conformational change and the water of hydration is lost [19]. On the other hand, the carboxyl group remains hydrated and the formation of H-bonds to the ester carbonyl functional groups is induced causing a degree of immobilization [19]. It is possible that the free carboxyl group on the serine moiety contributes to low cholesterol solubility. Also the rigid chain conformation due to interaction with Ca^{2+} may be a further impediment to cholesterol-phospholipid interaction. Since Ca^{2+} and PS are participants in many dynamic membrane processes, the low solubility of cholesterol in PS-Ca may be significant in causing local environmental modification i.e. diffusion of cholesterol away from the PS rich regions, thereby enhancing the sterol's interaction with other phospholipids.

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