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Effect of Cholesterol on the Formation of an Interdigitated Gel Phase in Lysophosphatidylcholine and Phosphatidylcholine Binary Mixtures[1]

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We previously reported that 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) forms an interdigitated gel phase in the presence of 1-palmitoyl-*sn*-glycero-3-phosphocholine (16:0LPC) at concentrations below 30 mol%. In the present investigation, fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), X-ray diffraction, and differential scanning calorimetry (DSC) were used to investigate the effect of cholesterol on the phase behavior of 16:0LPC/DPPC binary mixtures. At 25°C, 30 mol% 16:0LPC significantly decreases the DPH fluorescence intensity during the transition of DPPC from the $L_{\beta'}$ phase to the $L_{\beta I}$ phase. However, the addition of cholesterol to 16:0LPC/DPPC mixtures results in a substantial increase in fluorescence intensity. The changes in DPH fluorescence intensity reflect the probe's redistribution from an orientation parallel to the acyl chain to the center of the bilayer, suggesting a bilayer structure transition from interdigitation to noninterdigitation. The normal repeat period of small angle X-ray diffraction patterns can be restored and a reflection appears at 0.42 nm with a broad shoulder around 0.41 nm in wide angle X-ray diffraction patterns when 10 mol% cholesterol is incorporated into 30 mol% 16:0LPC/DPPC vesicles, indicating that the mixtures are in the gel phase ($L_{\beta'}$). Moreover, DSC results demonstrate that 10 mol% cholesterol is sufficient to significantly decrease the main enthalpy, cooperativity and lipid chain melting of 30 mol% 16:0LPC/DPPC binary mixtures, which are $L_{\beta I}$, indicating that the transition of the interdigitated phase is more sensitive to cholesterol than that of the noninterdigitated phase. Our data imply that the interdigitated gel phase induced by 16:0LPC is prevented in the presence of 10 mol% cholesterol, but unlike ethanol, an increasing concentration of 16:0LPC is not able to restore the interdigitation structure of the lipid mixtures.

Key words: cholesterol, DSC, fluorescence, interdigitation, 16:0LPC/DPPC, X-ray diffraction.

Cholesterol is the major neutral lipid component of biological membranes of most eukaryotic cells. The primary role of cholesterol is as a modulator of the physical properties of the plasma membrane phospholipid bilayer; in particular, it induces large changes in the structure of the phospholipid bilayer (*1*). Cholesterol acts as a "disordering agent" for the lipid bilayer, which results in the main phase transition broadening and disappearance with increasing concentration. On the other hand, it acts as an "ordering agent," and causes reductions in lipid chain mobility and the average area per lipid molecule (*2–4*). Lipid bilayers containing cholesterol may also exhibit phase separation into cholesterol-rich (liquid ordered) and cholesterol-poor (liquid disordered) domains (*5–7*). When interacting with non-lamellar lipids, cholesterol destabilizes the lamellar phase, and consequently stabilizes the hexagonal II phase (*8*). Cholesterol has been shown to be involved in the modulation of a variety of plasma membrane functions (*4, 8–12*). In recent years, a new aspect of cell membrane structure has been presented, *i.e.* that cholesterol and sphingolipid can form "rafts" that move within the fluid bilayer. It is proposed that these rafts function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction (*13, 14*).

Some studies have shown that cholesterol has a great effect on the interdigitated bilayer structures, which are induced in several different mechanisms. It has been demonstrated that dispersion of DHPC occurs in the interdigitated lamellar gel phase. At low concentrations, cholesterol perturbs DHPC interdigitation, leading to the presence of coexisting interdigitated and noninterdigitated lamellar gel

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Abbreviations: DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 16:0LPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; C(18):C(10)PC, 1-stearoyl-2-capryl-*sn*-glycero-3-phosphocholine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine, T_m, lipid chain melting temperature; ΔH, lipid phase transition enthalpy; CHOL, cholesterol; DSC, differential scanning calorimetry; DPH, 1,6-diphenyl-1,3,5-hexatriene; nAS, *n*-(9-anthroyloxyl)-stearic acid; 16AP, 16-(9-anthroyloxyl)-palmitic acid; $L_{\beta'}$, tilted-chain bilayer gel phase; $P_{\beta'}$, rippled gel phase; $L_{\beta I}$, interdigitated gel phase; L_α, liquid-crystalline bilayer phase; PKC, protein kinase C.

phases; at higher concentrations, *i.e.* 20–50 mol%, cholesterol eliminates the hydrocarbon chain interdigitation in the gel phase of DHPC (*15–17*). Saturated symmetric phospholipids such as DPPC, which are normally noninterdigitated lipids, can also be converted to form an interdigitated bilayer in the presence of inducing agents (*18–22*). Cholesterol prevents the induction of interdigitation in DPPC by ethanol (*15, 23*). An asymmetric mixed-chain phosphatidylcholine, C(18):C(10)PC, is known to form highly ordered mixed interdigitated bilayers below the transition temperature (T_m), and partially interdigitated bilayers above T_m. Sterols have been shown to cause a disordering effect on the packing of the chains in the mixed interdigitated bilayer (*24*).

The structure of the 16:0LPC molecule can be regarded structurally as an extreme case of an asymmetric phosphatidylcholine system in which the entire *sn*-2 acyl chain is substituted by a hydrogen atom, and the wedge-shaped 16:0LPC molecules can form an interdigitated lamellar structure in the gel state or a micelle arrangement at a temperature above T_m (*25–27*). Our initial study on 16:0LPC/DPPC binary mixtures demonstrated that DPPC can form an interdigitated bilayer in the presence of 16:0LPC at concentrations below 30 mol% at the gel phase, and finally the 16:0LPC/DPPC binary mixtures are packed in micelles rather than bilayers with LPC concentrations up to 60 mol% (*28*). Since biological membranes are heterogeneous entities, consisting of different lipid species together with a variety of proteins, it is necessary to use a more complex model membrane system to elucidate the biological implications of the interdigitated structure of membranes. Considering cholesterol's high content and important functions in biological membranes, we examined whether or not cholesterol prevents 16:0LPC/DPPC binary mixtures from forming highly ordered interdigitated structures, just like it prevents other types of interdigitation mentioned above. By using DPH fluorescence, X-ray diffraction and DSC, we show the physical effects of cholesterol on a ternary lipid model membrane system of 16:0LPC/DPPC/CHOL.

MATERIALS AND METHODS

Materials—DPPC, 16:0LPC, DPH, and cholesterol were obtained from Sigma Chemical (St. Louis, MO). All materials were used without further purification. All other reagents used were of analytical grade. Doubly distilled water was used to make all samples.

Samples—Stock solutions of the lipids were prepared in chloroform. The concentrations of the stock solutions of DPPC, 16:0LPC and cholesterol were 3.4, 4.0, and 1 mM, respectively. The cholesterol molar percentage (X) is calculated with

$$X = \frac{[CHOL]}{[LPC] + [DPPC]}$$

Appropriate volumes of chloroform solutions of lipids and cholesterol were mixed to give mixtures with various ratios. The mixtures were dried under a stream of nitrogen gas to give thin films on the bottoms of the tubes, and then were evaporated in a lyophilizer overnight at 4°C to remove all residual chloroform. Tris-HCl buffer (20 mM Tris, 150 mM NaCl, pH 7.4) was added to each thin film and the resulting suspension was hydrated at 50°C (above the chain melting temperature of the lipid) for least 1 h. During this incubation, the samples were vortexed periodically for 10 min. The suspensions were vortexed at 50°C for another 10 min after freezing. The samples were then kept overnight at 4°C.

Fluorescence—Fluorescence experiments were performed with a Hitachi F-4010 fluorescence spectrophotometer. The methodology was described previously (*29*). DPH powder was dissolved in chloroform. Lipids and the probe were mixed to give a lipid to probe ratio of 500 to 1. The total lipid concentrations were 1.0 mM, at which samples without the probe showed that the signal from stray scattered light was less than 1% of the signal from the samples with the probe at the same lipid concentration. The excitation wavelength was 360 nm, and the emission wavelength was 425 nm for all samples. The experimental temperatures were kept at 25 and 50°C, which are below and above the chain melting temperatures of DPPC, respectively.

X-Ray Diffraction—Mixtures with various ratios of LIPIDS/CHOL were centrifuged at 100,000 $\times g$ for 30 min. The supernatants were removed. The final concentrations of the samples were about 750 mM. The samples were placed in the holding slot between two Mylar windows of an aluminum sample holder. X-ray diffraction experiments were carried out with a RIGAKU D/ max-RB X-ray diffractometer (X-ray Laboratory, Materials Science Institute, Tsinghua University, Beijing), using CuKα radiation, $\lambda = 0.154$ nm, 40 kV, 120 mA. Fine-focus line source X-rays were monochromatized with a graphite crystal. Both small-angle (0.8°–6°) and wide-angle (20°–23°) diffraction patterns were recorded with a scintillation counter at 25°C. The data were plotted with ORIGIN 5.0 software from Microcal. The repeat period d can be calculated with

$$2d\sin\frac{\theta_s}{2} = h\lambda \quad h = 1,\ \lambda = 1.54,\ \theta_s = 2\theta$$

Calorimetry—Calorimetric analysis was performed with a Microcal MC-2 high-sensitivity differential scanning calorimeter interfaced to a personal computer for automatic data collection and analysis. A scan rate of 60°C/h was used for all experiments. The final concentrations of phospholipid/cholesterol mixtures for DSC were 1.0 mM. It was important that the base line obtained with the pure buffer was subtracted from the curves obtained with the lipid dispersions prior to data evaluation. The transition temperatures and calorimetric enthalpies were evaluated with the software package supplied by Microcal.

RESULTS

1) DPH Fluorescence Measurements—DPH has been used as a good probe for studying the structures and dynamics of biological membranes due to its sensitivity to the environment and the fact that it interferes very little with the lipid bilayer (*30*). The fluorescence quenching of DPH in model membranes, represented by increasing F_0/F, is used to study the phase transition from the noninterdigitated to the interdigitated phase (*29*), where F_0 and F are the fluorescence intensities in the absence and presence of different concentrations of 16:0LPC and cholesterol, respectively. The change of F_0/F in 16:0LPC/DPPC/CHOL ternary vesicles is plotted as F_0/F against the cholesterol concentration

in Fig. 1. Figure 1A reveals that the F_0/F value of 16:0LPC/DPPC is about 1 in the presence of 0–10 mol% 16:0LPC and in the absence of cholesterol at 25°C, indicating that the system is in non-interdigitated bilayer phases. The gradual increase of F_0/F with increasing 16:0LPC concentration to 30 mol% in the absence of cholesterol suggests the formation of interdigitated bilayer phases. Above 30 mol% 16:0LPC concentration, the abrupt decrease of F_0/F indicates the system likely existing in micelles. It has been demonstrated that at 16:0LPC concentrations higher than 30 mol% in 16:0LPC/DPPC binary, the lyso component gradually disrupts the bilayer, leading to micellization of the bilayer (*28, 31*). Figure 1A also shows that cholesterol has no significant effect on non-interdigitated bilayer phases and micelles at concentrations between 0–10 mol%. However, when the transitions involve the interdigitated phases, *i.e.* for 30 mol% 16:0LPC/DPPC, an abrupt decrease in the F_0/F ratio occurs, the F_0/F value changing to about 1 in the presence of 10 mol% cholesterol. It is possible that under this condition 16:0LPC/DPPC/CHOL ternary undergoes a phase transition from interdigitation to non-interdigitation. These data demonstrate that cholesterol facilitates the conversion from an interdigitated to a non-interdigitated bilayer phase. Figure 1B shows that 16:0LPC/DPPC binary exists in a non-interdigitated bilayer phase and micelles in the presence of 16:0LPC at concentrations below and above 30 mol% at 50°C, respectively. The addition of cholesterol to the lipid bilayer phases and micelles did not change the F_0/F ratio values, in other words, it did not significantly change the environment of the DPH probe, because these lipids did not undergo the transition changes. This is not an unexpected result because LPC/DPPC binary mixtures exist in interdigitated bilayer structures only at temperatures below T_m.

2) X-Ray Diffraction Measurements—In order to verify that what is shown in Fig. 1 is the transition between the non-interdigitated and interdigitated bilayer phases, X-ray diffraction measurements of 16:0LPC/DPPC/CHOL systems were carried out at 25°C. The small angle X-ray diffraction patterns are shown in Fig. 2. The lamella repeat period of pure DPPC vesicles is 7.35nm (Fig. 2a). The lamella repeat period of 30 mol% 16:0LPC/DPPC binary is 5.52 nm (Fig. 2b). This is a result of 16:0LPC inducing the transition of DPPC vesicles from the non-interdigitated to

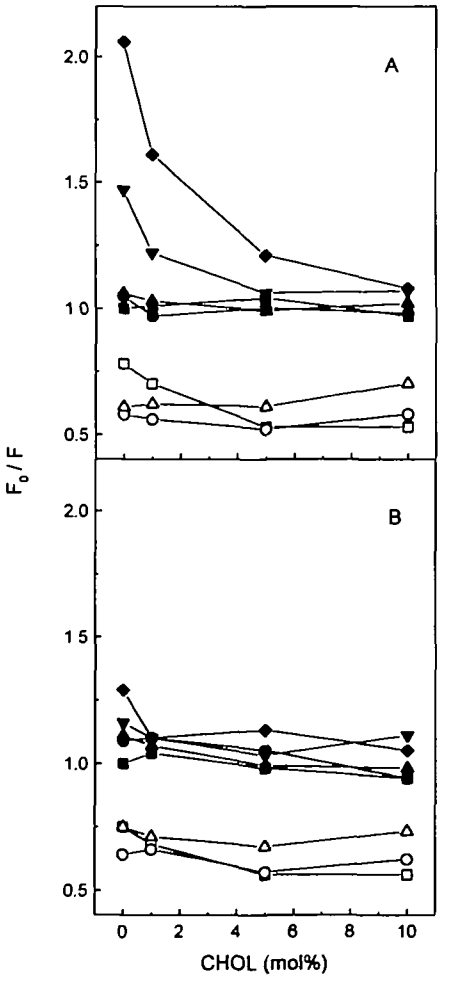

Fig. 1. **Effect of cholesterol on the apparent quenching of DPH in DPPC multilamellar vesicles containing different amounts of 16:0LPC at 25°C (A) and 50°C (B).** 16:0LPC concentrations: ■, 0 mol%; ●, 5 mol%; ▲, 10 mol%; ▼, 20 mol%; ◆, 30 mol%; □, 40 mol%; ○, 50 mol%; △, 60 mol%.

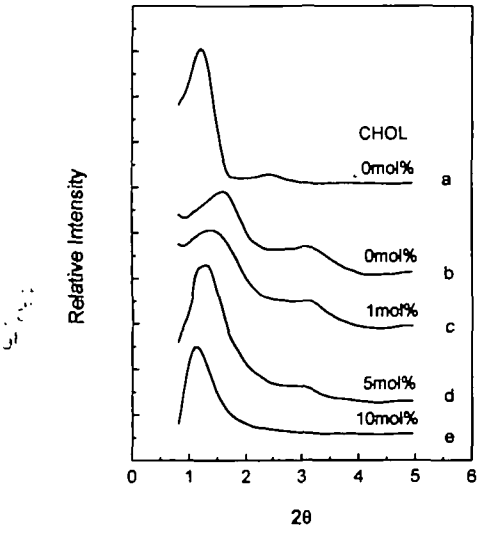

Fig. 2. **Small-angle X-ray diffraction patterns of DPPC (a) and 30 mol% 16:0LPC/70 mol% DPPC (b–e) multilamellar vesicles containing various cholesterol molar concentrations at 25°C with a total lipid concentration of 750 mM.**

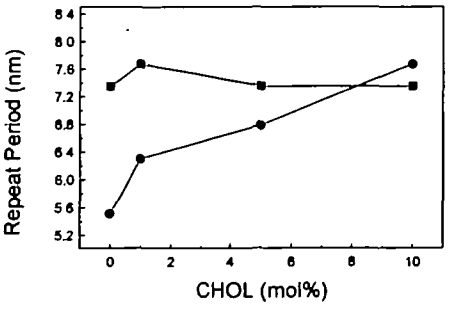

Fig. 3. **The dependence of the repeat period in DPPC (■) and 30 mol% 16:0LPC/70 mol% DPPC (●) multilamellar vesicles on the cholesterol concentration.**

the interdigitated bilayer phases. The addition of cholesterol to the binary results in an increase of the repeat period with increasing cholesterol concentrations (Fig. 2, c–e). The changes in the lamellar repeat period as a function of cholesterol are plotted in Fig. 3. It is clear that the normal repeat period can be restored when 10 mol% cholesterol is incorporated into a 30 mol% 16:0LPC/DPPC binary system (Fig. 3). These results obtained with small angle X-ray diffraction are consistent with those of DPH fluorescence intensity measurement (Fig. 1).

The wide-angle X-ray diffraction patterns of DPPC and 30 mol% 16:0LPC/DPPC/CHOL systems are shown in Fig. 4. A reflection appears at 0.42 nm with a broad shoulder around 0.41 nm in pure DPPC, indicating that this sample is in the ($L_{\beta'}$) (Fig. 4a). In the 30 mol% 16:0LPC/DPPC system, a single reflection appears at 0.41 nm (Fig. 4b), which is indicative of an interdigitated gel phase. The sharp and symmetrical single peak suggests that the hydrocarbon chains are packed in a hexagonal lattice, and the direction of chains is normal as to the membrane surface (*20*). In the 30 mol% 16:0LPC/DPPC/CHOL system containing 5 mol% cholesterol, a broad shoulder around 0.42 nm of reflection can be observed (Fig. 4d). When the cholesterol concentration is 10 mol%, the features of the reflection return to the original pattern seen in the absence of 16:0LPC, *i.e.* a reflection peak at 0.42 nm with a broad shoulder around 0.41 nm (Fig. 4e). This indicates that a 30 mol% of 16:0LPC/DPPC binary system containing 10 mol% cholesterol exists in $L_{\beta'}$ rather than $L_{\beta I}$. This result is consistent with that of small angle X-ray diffraction described above.

3) DSC Measurements—Figure 5 shows the endothermic phase transition for binary mixtures of 16:0LPC/DPPC. The multilamellar vesicles of DPPC alone exhibit a pretransition ($L_{\beta'}$ to $P_{\beta'}$) at 34.8°C and a main phase transition ($P_{\beta'}$ to L_{α}) at 41.8°C (Fig. 5a). The pretransition shifts to 29.9°C at a 16:0LPC concentration of 5 mol% (Fig. 5b), and appears to be abolished at 10 mol% (Fig. 5c), while the main transition temperature just shifts slightly to a lower temperature. The main transition temperature increases slightly with increasing concentration of 16:0LPC up to 40 mol%. It is interesting that Fig. 5 shows the main melting

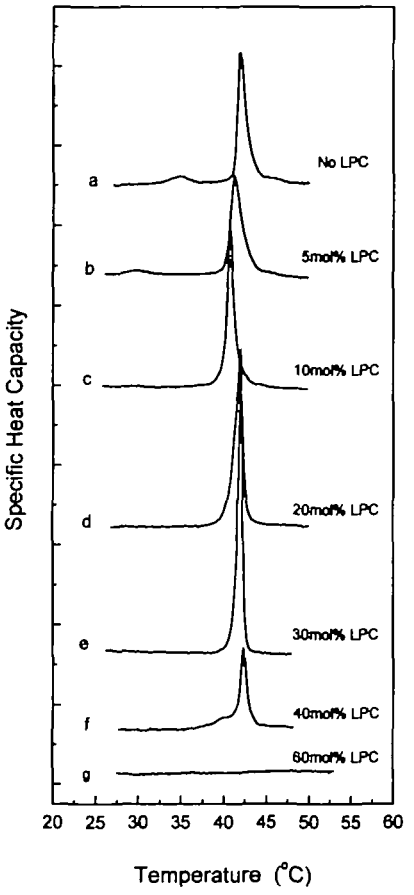

Fig. 5. **Representative DSC scans of DPPC multilamellar vesicles with various 16:0LPC molar concentrations.** DSC scans of DPPC vesicles containing 15, 25, 35, and 45 mol% 16:0LPC were also performed, but are not illustrated here.

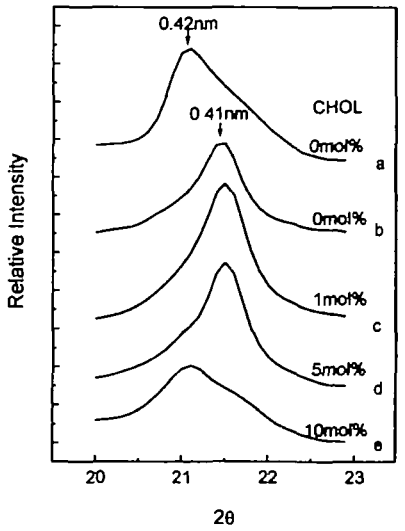

Fig. 4. **Wide-angle X-ray diffraction patterns of DPPC (a) and 30 mol% 16:0LPC/70 mol% DPPC (b–e) multilamellar vesicles containing various cholesterol molar concentrations at 25°C with a total lipid concentration of 750 mM.**

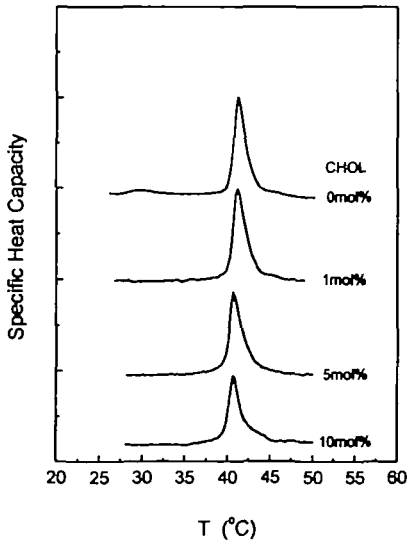

Fig. 6. **Representative DSC scans of 5 mol% LPC/95 mol% DPPC multilamellar vesicles with various cholesterol molar concentrations.**

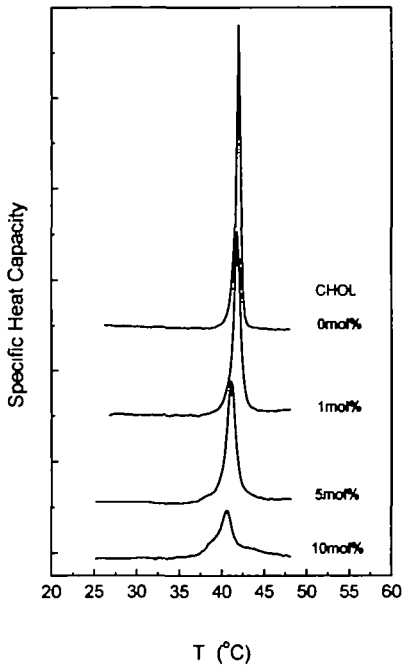

Fig. 7. **Representative DSC scans of 30 mol% 16:0LPC/70 mol% DPPC multilamellar vesicles with various cholesterol molar concentrations.**

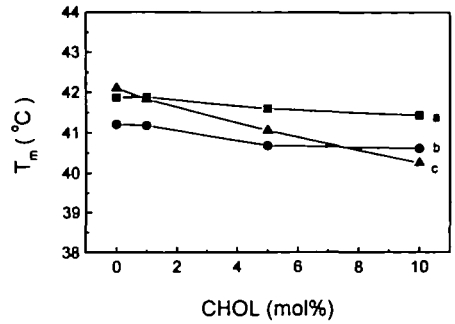

Fig. 8. **Effects of increasing amounts of cholesterol on the main transition temperatures of 16:0LPC/DPPC multilamellar vesicles.** 16:0LPC concentrations: ■, 0 mol%; ●, 5 mol%; ▲, 30 mol%.

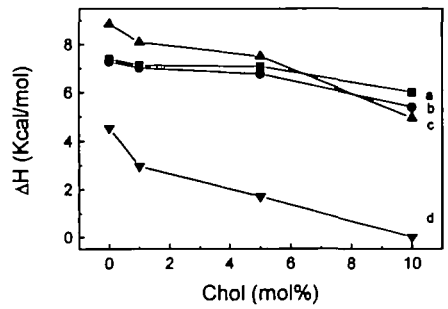

Fig. 9. **Effect of increasing amounts of cholesterol on the main transition enthalpies of 16:0LPC/DPPC multilamellar vesicles.** 16:0LPC concentrations: ■, 0 mol%; ●, 5 mol%; ▲, 30 mol%; ▼, 40 mol%.

transition of 16:0LPC/DPPC binary in the presence of 30 mol% 16:0LPC, a concentration at which the lipid is in the interdigitated phase prior to melting, so that the observed transition is the $L_{\beta I}$ to L_α one. This transition is very high and narrow for the heating scan (0.61°C width at half-height, Fig. 5e), *i.e.* good cooperation can be observed. The enthalpy of the main transition increases as a function of the 16:0LPC concentration up to 30 mol%. With a further increase in the 16:0LPC content, the transition enthalpy and scan height abruptly decrease, and the peak is finally abolished at 60 mol% 16:0LPC (Fig. 5, f and g).The thermotropic behavior of DPPC/CHOL vesicles has been previously investigated (*6, 23, 32, 33*). According to the data obtained, in the presence of an increasing cholesterol content, the phase transition broadens, and the transition enthalpy decreases before it is finally totally abolished. In order to study the thermotropic behavior of 16:0LPC/DPPC/CHOL ternary mixtures, DPPC vesicles containing a fixed 16:0LPC concentration and increasing cholesterol contents were compared. Figure 6 shows that a low concentration of cholesterol (<10 mol%) has no significant effect on the transition patterns of 5 mol% 16:0LPC/DPPC vesicles, which are in the non-interdigitated gel phase prior to melting. However, the thermotropic behavior of 30 mol% 16:0LPC/DPPC vesicles, which are in the interdigitated phase prior to melting, is strongly affected by low amounts of cholesterol (Fig. 7). The height and enthalpy of the main transition peak abruptly decrease with increasing cholesterol content. Nevertheless, the main transition temperature is shifted to a lower temperature. It is well suggested that 30 mol% 16:0LPC/DPPC vesicles undergo a phase transition from the interdigitated to the non-interdigitated gel phase in the presence of low concentrations of cholesterol.

Figure 8 shows the effects of the addition of cholesterol on the phase transition temperature (T_m) of 16:0LPC/DPPC vesicles. The results show that low amounts of cholesterol cause a significant change in T_m of only 30 mol% LPC/DPPC vesicles, which are in the interdigitated phase prior to melting (Fig. 8c), but not in that of DPPC and 5 mol% LPC/DPPC vesicles (Fig. 8, a and b).

The effects of cholesterol on the phase transition enthalpy (ΔH) of 16:0LPC/DPPC binary are shown in Fig. 9. The results show that cholesterol is more effective for the enthalpy of 30 mol% 16:0LPC/DPPC bilayers (Fig. 9c) than for that of DPPC and 5 mol% 16:0LPC/DPPC bilayers (Fig. 9, a and b). 10 mol% cholesterol decreases the former enthalpy from 8.8 to 5.1, suggesting that interdigitation is abolished in the presence of cholesterol. With 40 mol% 16:0LPC, the vesicle bilayer structure is disrupted, leading to the formation of micelles (*28, 31*).

DISCUSSION

The relatively large polar group of the lysoPC molecule relative to its hydrophobic chains gives it a wedge shape. This shape allows the molecules to pack into a micellar phase with a high water content and an interdigitated lamellar phase at low temperature (*27*). With 40 mol% 16:0LPC, the lyso compound disrupts the bilayer, leading to the formation of small aggregates. It has been indicated that the 16:0LPC/DPPC binary mixture is a homogeneous mixture of the two components (*31*). In a previous report from our

group, it was described that DPPC forms an interdigitated gel phase in the presence of 16:0LPC at concentrations below 30 mol%, and that above this concentration micellization of the bilayers occurs (*28*). In order to gain further insight into the phase behavior of 16:0LPC/DPPC/CHOL ternary vesicles, we employed, in the present study, three complementary approaches to characterize the physical properties of the ternary mixtures.

(i) It has been demonstrated that DPH fluorescence intensity can be used to monitor the transition between noninterdigitation and interdigitation (*29*). A significant decrease in DPH fluorescence intensity occurs during the transition of DPPC from the $L_{\beta'}$ phase to the $L_{\beta I}$ phase due to the addition of 30 mol% 16:0LPC. The phase transition may thus cause the change in the fluorescence intensity of DPH, and this change could be due to greater exposure of the DPH chromophore to the aqueous solvent due to the proximity to the interfacial region of the lipid (*30*). However, the addition of cholesterol to 16:0LPC/DPPC mixtures results in a significant increase in fluorescence intensity. This reflects the redistribution of DPH from an orientation parallel to the acyl chain to the center of the bilayer due to the transition from interdigitation to noninterdigitation.

(ii) The addition of cholesterol to a 30 ml% 16:0LPC/DPPC binary mixture results in an increase in the repeat period with increasing cholesterol content. The normal repeat period (noninterdigitated bilayer) is restored when 10 mol% cholesterol is incorporated into 30 mol% 16:0LPC/DPPC vesicles. Wide-angle X-ray diffraction indicated a single shape reflection occurring at 0.41 nm for 30 mol% 16:0LPC/DPPC binary mixtures. This is indicative of an interdigitated gel phase. A reflection appears at 0.42 nm with a broad shoulder around 0.41 nm for 30 mol% 16:0LPC/DPPC/10mol%CHOL ternary mixtures, indicating that these mixtures are in the gel phase ($L_{\beta'}$). Interdigitation can be detected by X-ray diffraction, which is based on a concomitant decrease in the repeat period. It is an essential and direct method for characterizing the transition between noninterdigitation and interdigitation.

(iii) The incorporation of 30 mol% 16:0LPC in DPPC vesicles results in increases in the transition temperature and enthalpy. The increases in both the temperature and enthalpy are interpreted as increased stability of the DPPC lamellar structure (*34*). Generally, the main enthalpy, cooperativity and T_m of PC containing 12–20 carbon atoms decrease with increasing cholesterol content, as reported previously (*5*, *33*, *35*). But cholesterol at low concentrations up to 10 mol% in the present study had no significant effect on those of DPPC or 5 mol% 16:0LPC/DPPC vesicles (Figs. 6 and 9). However, 10 mol% cholesterol is sufficient to significantly decrease the enthalpy, cooperativity and T_m of 30 mol% 16:0LPC/DPPC binary mixtures, which are in $L_{\beta I}$ (Figs. 7 and 9). It is very clear that this is a phase transition contribution from interdigitated to noninterdigitated bilayer structures. From this point of view, the transition of interdigitated lipids is more sensitive to cholesterol than the phase transition of noninterdigitated lipids. All of the results described above reveal that the induction of the interdigitated gel phase by 16:0LPC is prevented in the presence of 10 mol% cholesterol. It should be emphasized that 16:0LPC is unlike ethanol, in that the latter can counter cholesterol's influence and restore the lipid interdigitation with increasing ethanol concentration (*35*).

The conversion of DPPC bilayers from a noninterdigitated phase to an interdigitated phase in the presence of 16:0LPC, and that of 16:0LPC/DPPC bilayers from an interdigitated to a noninterdigitated phase in the presence of cholesterol can be explained using a geometrical argument. The DPPC headgroup surface area in the noninterdigitated bilayers is 48 Å^2 at 20°C (*36*). The hydrocarbon chain cross-sectional area is approx. 40 Å^2 per molecule. Clearly, the packing surface area of hydrocarbon chains and the headgroup in DPPC are relatively well matched, leading to the spontaneous formation of noninterdigitated bilayer assemblies. The incorporation of 16:0LPC, in which the entire sn-2 acyl chain is substituted by a hydrogen atom, leads to a 16:0LPC/DPPC headgroup and hydrocarbon chain surface area mismatch. In the binary mixtures, 2 PC headgroups *vs.* 3 acyl chains (96 Å^2 *vs.* 60 Å^2) require an interdigitated assembly for a thermodynamically stable bilayer phase. It has been suggested that cholesterol, which is partitioned into the hydrophobic hydrocarbon portion of the bilayer and has a cross-sectional area of about 38 Å^2 (*16*), creates a more favorable match between the head group and hydrocarbon surface area in a 16:0LPC/ DPPC/ CHOL mixture. Thus this mixture favors the formation of a noninterdigitated phase instead of an interdigitated one. Also, cholesterol may reduce the headgroup crowding by intercalating between the lipid molecules and increasing the area per head group, thus reducing the steric destabilization of the noninterdigitated structures (*35*). As a structural model, it was proposed that cholesterol occupies the space left by the missing fatty acyl chain and makes lysophosphatidylcholine more cylindrical rather than wedge-shaped (*37*). These factors described above may contribute to the transition from the interdigitated phase (16:0LPC/DPPC) to the noninterdigitated phase (16:0LPC/DPPC/CHOL).

It is necessary to determine why such a low concentration of cholesterol is sufficient to perturb or completely eliminate the hydrocarbon chain interdigitation. A possible mechanism has been described (*17*), *i.e.* that cholesterol molecules can locally induce the formation of noninterdigitated clusters at the site of their insertion into an interdigitated bilayer, which would result in the appearance of line boundaries between these clusters and the rest of the interdigitated bilayers. The line boundaries should be energetically very unfavorable due to the difference in the thicknesses of the noninterdigitated and interdigitated bilayers. This may result in unlimited growth of the noninterdigitated phase until the whole bilayers assume a noninterdigitated phase (*17*). Another mechanism suggested by Bonder *et al.* (*35*) is that cholesterol preferentially interacts with the noninterdigitated regions, because cholesterol itself must be shielded from the water solvent, and pulls the phase equilibrium toward the noninterdigitated structures. But they did not explain where the free energy comes from. The free energy is necessary to drive the "unlimited growth" or "pull" of the noninterdigitated domains. In the present study, on the incorporation of 16:0LPC into DPPC vesicles, the transition enthalpy and temperature increase with increasing concentrations of 16:0LPC. At 30mol% 16:0LPC, the enthalpy and temperature increase to 8.84 kcal/mol and 42.09°C from 7.38 and 41.20°C, respectively (Figs. 8 and 9). So, the process of the formation of an interdigitated bilayer can be considered to be a process of energy

storage. In contrast to this, with the addition of cholesterol to 30mol% 16:0LPC/DPPC vesicles, which are in the interdigitated phase, the transition enthalpy, and temperature decrease with increasing cholesterol content (Figs. 8 and 9). We suggest that this is a process of free energy release upon transition from the interdigitated to the noninterdigitated phase. Cholesterol substantially perturbs PC's interdigitation at concentrations below 5 mol%, where the coexistence of noninterdigitated and interdigitated lamellar gel phases is observed (*15–17, 35*). In this case, the noninterdigitated domains are not able to trigger the "pull" to convert the interdigitated to the noninterdigitated phase. They have to coexist in the range up to 5 mol%. But, for example, 10–20 mol% cholesterol, which is over the threshold value, in this study and other studies (*15, 16, 23*) was sufficient to trigger the transition from the interdigitated to the noninterdigitated phase due to the unfavorable hydrophobic mismatch between the interdigitated and noninterdigitated lipid domains, although noninterdigitated domains contain only 10–20% in total membrane structures. It is a spontaneous process of free energy release and a positive cooperative effect. Because the free energy is stored in interdigitated structures, the transition from the noninterdigitated phase in a low energy state to the interdigitated phase in a high energy state will not take place.

It has been reported that the repulsive interaction between the headgroups of lipids plays an important role in the formation of the $L_{\beta I}$ phase of DHPC vesicles. And, the conditions that reduce the repulsive interaction will stabilize the bilayer gel phase rather than the $L_{\beta I}$ phase (*38, 39*). But cholesterol mainly interacts with the hydrocarbon chains through hydrophobic interaction (*35, 40*). It is not necessary to explain the observed cholesterol-induced effects on the basis of the presence of hydrogen bonding between the 3β-OH of cholesterol and the headgroup region of a lipid (*40, 41*). As discussed above, in the case of the LPC/DPPC $L_{\beta I}$ phase, the stabilization factor is a geometrical mismatch. When cholesterol is present, it fills the space left by LPC's missing fatty acyl chain, which is filled by interdigitation in the $L_{\beta I}$ phase (*37*). Hence, the role of cholesterol is likely to be quite a bit more involved than simply as a spacer lipid, as in cholesterol/sphingolipid vesicles (*41, 42*).

The biological significance of interdigitation is not yet known in vivo, although interdigitation inhibits the activity of Ca^{2+}-ATPase and gramicidin *in vitro* (*43, 44*). However, if interdigitated bilayers really exist in biological membranes, it could have important functional consequences, which has been discussed by Slater and Huang (*45*). Modulation of a bilayer through the formation of interdigitated bilayer domains may serve to influence the behavior of integral membrane proteins. A thin bilayer results in more amino acid residues that were originally embedded in the hydrophobic region of noninterdigitated membranes to be exposed to the hydrophilic environment. This may bring about perturbations in the native structures of proteins, and lead to the inactivation of the enzymes (*43*). Also, lateral and vertical displacement or rotational mobility of the embedded proteins may by affected by interdigitation. Because of interdigitation, the midplane of a bilayer is lost (*45*). All of these factors may contribute to changes in the structure and function of biological membranes.

A second messenger system, the phospholipase A_2/PC system, in signal transduction has been reported (*46–48*). Lyso-PC and certain other lysophospholipids regulate PKC activity in a biphasic manner, *i.e.* they stimulate it at low concentration and conversely inhibit it at high concentration (*49, 50*). It is suggested that lyso-PC might represent a unique second messenger in that it can subserve as a positive or negative regulatory factor in signal transduction (*47*). A highly ordered interdigitated bilayer induced by 16:0LPC also inhibits the activities of membrane proteins (*43, 44*). In this present study, we confirmed that cholesterol prevents 16:0LPC/DPPC binary mixtures from forming interdigitated structures. Our data also suggest that LPC/CHOL might represent a regulatory system in signal transduction, in that the interdigitation and noninterdigitation of lipids regulated by Lyso-PC and CHOL can contribute to the biological implications as negative and positive factors.

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