Probing the Ethanol-Induced Chain Interdigitations in Gel-State Bilayers of Mixed-Chain Phosphatidylcholines

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ABSTRACT Using high-resolution differential scanning calorimetry (DSC), we have studied the effects of ethanol concentrations, [EtOH], on the main phase transition temperatures (T_m) of the following mixed-chain phosphatidylcholines (PCs): C(15):C(17)PC, C(17):C(15)PC, and C(12):C(20)PC. These lipids have a common molecular weight; however, their apparent acyl chain-length differences between the *sn*-1 and *sn*-2 acyl chains, ΔC, are distinctively different. The ΔC values for these three mixed-chain PCs are, respectively, 0.5, 3.5, and 6.5 C-C bond lengths. DSC results show that the T_m profiles for C(15):C(17)PC and C(17):C(15)PC bilayers in the plot of T_m versus [EtOH] are V-shaped biphasic curves, with the minimum T_m occurring at 50 and 73 mg/ml of ethanol, respectively. In contrast, the C(12):C(20)PC bilayer exhibits a nearly linear decrease in T_m with increasing [EtOH]. In addition, x-ray diffraction experiments were also performed to assess the structural changes of these three mixed-chain PCs in the gel-state bilayers, at 20°C, in response to high concentrations of ethanol. X-ray diffraction data indicate that, in the absence of ethanol, these three lamellar lipids are all packed in the normal ($L_{\beta'}$) gel phase in aqueous media. In the presence of 120 mg/ml of ethanol, however, the C(15):C(17)PC and C(17):C(15)PC lamellae are packed in the fully interdigitated ($L_{\beta I}$) gel phase. The V-shaped T_m curves detected calorimetrically for these two lipids in response to [EtOH] can thus be explained by the ethanol-induced $L_{\beta'} \rightarrow L_{\beta I}$ isothermal phase transition. Interestingly, the results of x-ray diffraction study reveal, for the first time, that an ethanol-induced $L_{\beta'} \rightarrow L_{MI}$ (mixed interdigitated phase) isothermal phase transition occurs in the gel-state bilayer of highly asymmetrical C(12):C(20)PC. Therefore, the chain asymmetry is recognized to play an important role in the ethanol-induced chain interdigitation at $T < T_m$.

INTRODUCTION

By using one-component multilamellar lipid vesicles (MLVs) composed of saturated identical-chain phosphatidylcholines (PCs) as the bilayer system and by applying various physical techniques, it has been shown quite exhaustively that high concentrations of ethanol can induce the isothermal transformation of the normal lipid bilayer in the $L_{\beta'}$ phase to the fully interdigitated bilayer in the $L_{\beta I}$ phase at $T < T_m$ (Simon and McIntosh, 1984; Nambi et al., 1988; Ohki et al., 1990; Roth and Chen, 1991; Zeng and Chong, 1991; Kinoshita and Yamazaki, 1996). The well-known biphasic effect of ethanol on the main phase transition temperature (T_m) of MLVs composed of dipalmitoylphosphatidylcholines or C(16):C(16)PC (Rowe, 1983, 1992) is a manifestation of such a $L_{\beta'} \rightarrow L_{\beta I}$ isothermal phase transition (Simon and McIntosh, 1984). Based on results obtained with identical-chain PC/ethanol complexes in excess H_2O using x-ray diffraction, Adachi et al. (1995) proposed a structural model for the equilibrium state of the fully interdigitated bilayer in the $L_{\beta I}$ phase. In this model, the packing unit is a binary mixture of PC/ethanol at a molar ratio of 1:2. Specifically, the two linear all-*trans* acyl chains of each PC molecule (e.g., C(16):C(16)PC) penetrate through the hydrophobic core of the gel-state bilayer with their terminal methyl groups located on the opposing side of the bilayer. Moreover, each of the chain terminal methyl groups is in line with the methyl group of an ethanol, and the hydroxyl end of the same ethanol is coplanar with the *sn*-1 carbonyl groups of neighboring C(16):C(16)PC molecules, facing the ethanol-containing aqueous medium.

Until now, the investigation of the ethanol effect on the T_m of MLVs composed of phosphatidylcholines has been confined to lipids with saturated identical fatty acids esterified at the *sn*-1 and *sn*-2 positions of the glycerol backbone. To approach a broader understanding of the ethanol effect on the bilayer property, we have recently examined the possibility that ethanol induces the $L_{\beta'} \rightarrow L_{\beta I}$ isothermal transition for MLVs composed of mixed-chain phosphatidylcholine or C(X):C(Y)PC using molecular mechanics simulations (Li et al., 1996). Here C(X):C(Y)PC refers to saturated phosphatidylcholines with *sn*-1 and *sn*-2 acyl chains of unequal length. The overall symmetry/asymmetry of each molecular species of C(X):C(Y)PC packed in the gel-state bilayer can be described quantitatively by a structural parameter, ΔC, defined as the effective chain-length difference between the *sn*-1 and *sn*-2 acyl chains. The absolute value of ΔC, in C-C bond lengths, can be related to the X and Y values in C(X):C(Y)PC as follows (Huang and Mason, 1986): $\Delta C = |X - Y + 1.5|$, where X and Y are the numbers of carbons in the *sn*-1 and *sn*-2 acyl chains, respectively, and 1.5 is the effective chain-length difference between the *sn*-1 and *sn*-2 acyl chains, in C-C bond lengths, for identical-chain PCs (e.g., C(16):C(16)PC). For a series of C(X):

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C(*Y*)PCs with a common molecular weight identical to that of C(16):C(16)PC, such as C(15):C(17)PC, C(17):C(15)PC, and C(12):C(20)PC, the larger the value of the asymmetry parameter (ΔC), the more asymmetrical is the molecular geometry of the lipid structure. The molecular mechanics computations employed by Li et al. (1996) were based on Allinger's MM2(85) program to calculate the overall stabilization energy difference between bilayers of C(*X*):C(*Y*)PC with the $L_{\beta'}$ packing motif and those with the $L_{\beta I}$ motif. The results of this computational study predict that MLVs composed of C(*X*):C(*Y*)PC with modest asymmetry ($\Delta C < 4.2$) can be induced by ethanol to form the $L_{\beta I}$-state bilayer at $T < T_m$, whereas highly asymmetrical C(*X*):C(*Y*)PCs ($\Delta C > 4.2$) cannot be converted by ethanol into the $L_{\beta I}$ bilayer.

Designed to experimentally test the computational predictions, the main phase transition temperatures of a series of C(*X*):C(*Y*)PCs with different ΔC values but with a common molecular weight identical to that of C(16):C(16)PCs were determined in the presence of various concentrations of ethanol by high-resolution differential scanning calorimetry. When the calorimetrically determined T_m values were plotted against the ethanol concentration, a V-shaped biphasic curve was observed for C(*X*):C(*Y*)PCs with $\Delta C < 4.2$. In contrast, a linear curve was detected for highly asymmetrical C(*X*):C(*Y*)PCs with $\Delta C > 4.2$ (Li et al., 1996). Hence the experimental results and the computational predictions were in excellent agreement. However, despite important implications of the agreement, the exact structure of C(*X*):C(*Y*)PC at high concentrations of ethanol remains unknown. Therefore we extended our earlier DSC study and, furthermore, carried out x-ray diffraction experiments to assess the structural features of MLVs composed of various C(*X*):C(*Y*)PCs in the presence and absence of high concentrations of ethanol. In particular, we present in this report the calorimetric and x-ray diffraction data obtained with three C(*X*):C(*Y*)PC/H_2O-ethanol systems. Our data demonstrate unequivocally, for the first time, that the ethanol-induced $L_{\beta'} \rightarrow L_{\beta I}$ isothermal phase transition can indeed occur for a pair of positional isomers, C(15):C(17)PC and C(17):C(15)PC, with $\Delta C < 4.2$ at $T < T_m$. Moreover, such an isothermal phase transition does not take place in MLVs composed of highly asymmetrical C(12):C(20)PC with $\Delta C > 4.2$ in the presence of high concentrations of ethanol. Instead, C(12):C(20)PC, in response to high concentrations of ethanol, forms a mixed interdigitated phase (L_{MI}), where the longer hydrocarbon chain spans the entire width of the bilayer and the shorter chain aligns with or abuts the shorter chain from the opposing monolayer (McIntosh et al., 1984; Hui et al., 1984).

MATERIALS AND METHODS

Phospholipids

Three mixed-chain phosphatidylcholines, i.e., C(15):C(17)PC, C(17):C(15)PC, and C(12):C(20)PC, were semisynthesized at room temperature using a modified procedure of Mena and Djerassi (1985) as described previously (Lin et al., 1990). The fatty acids and lysophosphatidylcholine required for the semisynthesis were purchased from Sigma (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL), respectively. The purity of the position isomers, C(15):C(17)PC and C(17):C(15)PC, was found to be extremely high (>99%), as judged from the DSC scans (see Results) and analytical thin-layer chromatography as reported previously (Li et al., 1996).

High-resolution DSC measurements

The lipid samples containing various concentrations of ethanol were prepared in buffer solution containing 50 mM NaCl, 1 mM EDTA, and 5 mM phosphate buffer, pH 7.4, as described in detail elsewhere (Li et al., 1996). After a minimum of 24 h incubation at 4°C, each lipid sample was subjected to DSC experiments using a high-resolution MC-2 differential scanning microcalorimeter (Microcal, Northampton, MA). Each lipid sample was scanned at least three times at a scan rate of 15°C/h. To ascertain that the same thermal history pertained to all lipid samples, only the T_m value from the second DSC heating scan was reported in this study. Detailed procedures for the DSC experiments were described in our earlier publications (Li et al., 1996; Lin et al., 1990).

X-ray diffraction

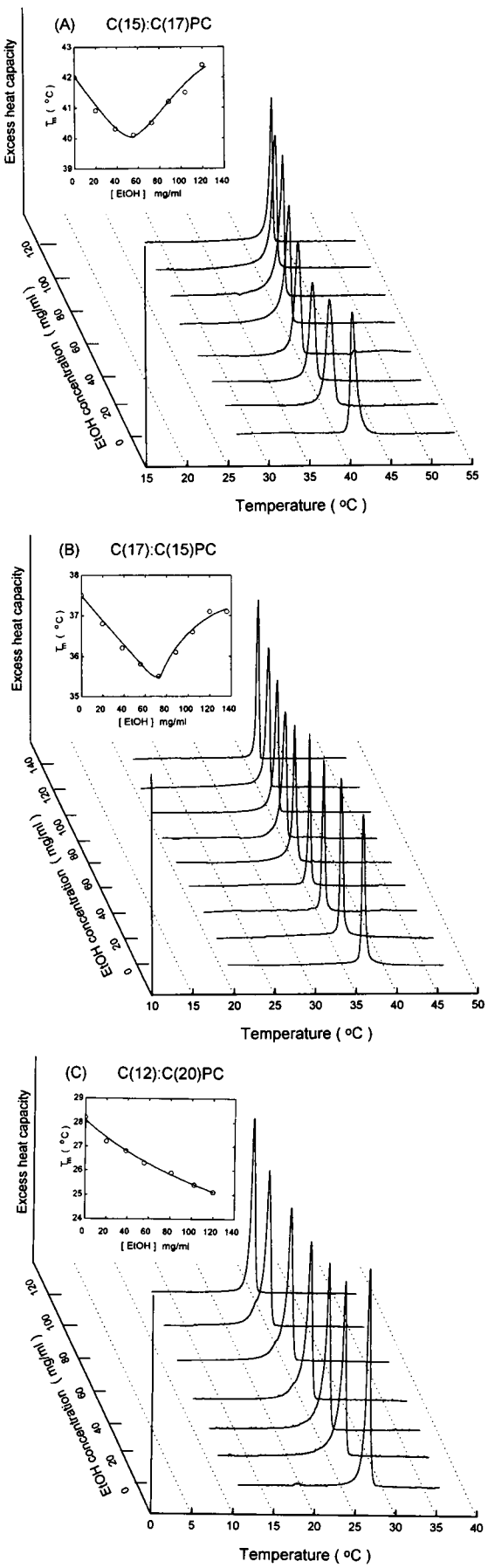
For x-ray diffraction analysis, multiwalled vesicles of C(15):C(17)PC, C(17):(15)PC, or C(12):C(20)PC were prepared by adding dry lipid to excess buffer (100 mM NaCl, 20 mM HEPES, pH 7.0) or excess buffer containing 120 mg/ml ethanol. The suspensions were heated to 50°C three times for 30 min and extensively vortexed. The suspensions were then pelleted with a bench centrifuge, sealed in quartz glass x-ray capillary tubes, and mounted in a point collimation x-ray camera. X-ray diffraction patterns were recorded on stacks of Kodak DEF x-ray film, which were densitometered with a Joyce-Loebl microdensitometer. After background subtraction, integrated intensities, $I(h)$, were obtained for each order h by measuring the area under each diffraction peak as described previously (McIntosh, 1980; McIntosh and Simon, 1986). For these patterns from unoriented suspensions, the structure amplitudes $F(h)$ were set equal to $\{h^2 I(h)\}^{1/2}$. Electron density profiles, $p(X)$, on a relative electron density scale were calculated from

$$p(x) = (2/d) \sum \exp\{i\phi(h)\} \cdot F(h) \cdot \cos(2\pi x h/d)$$

where x is the distance from the center of the bilayer, d is the lamellar repeat period, $\phi(h)$ is the phase angle for order h, and the sum is over h. As described below, phase angles were determined by comparison with previous diffraction analyses (McIntosh et al., 1983, 1984). The electron density profiles were calculated at resolutions ($d/2h_{max}$) of 7–9 Å.

RESULTS

The second DSC heating curves of C(15):C(17)PC, C(17):C(15)PC, and C(12):C(20)PC dispersions obtained in the presence of various concentrations of ethanol are shown in Fig. 1 *A–C*, respectively; these calorimetric scans include some of those that have been cited, but not shown, in an earlier report (Li et al., 1996). The main phase transition temperatures of C(15):C(17)PC and C(17):C(15)PC are observed initially to decrease progressively with increasing [EtOH] or concentration of ethanol, and the T_m values are at a minimum when [EtOH] is at about 50 mg/ml and 73 mg/ml, respectively. The [EtOH] at the minimum T_m in the plot of T_m versus [EtOH] is referred to as the threshold concentration or $[EtOH]_{TC}$. Beyond this, the T_m values are observed to increase with increasing [EtOH], resulting in



the V-shaped biphasic T_m profiles shown in the insets in Fig. 1, *A* and *B*. It should be pointed out that the $[EtOH]_{TC}$ for C(17):C(15)PC is 73 mg/ml, as shown in Fig. 1 *B*, which is higher than that of 65 mg/ml reported earlier (Li et al., 1996). This is due to the fact that in this study new and reproducible T_m values are determined calorimetrically over a wider range of [EtOH]. The ΔC values for C(15):C(17)PC and its positional isomer, C(17):C(15)PC, are 0.5 and 3.5 C-C bond lengths, respectively. It should be emphasized that the biphasic curves observed in Fig. 1, *A* and *B*, are strikingly similar in shape to the one detected calorimetrically for identical-chain C(16):C(16)PC with ΔC of 1.5 C-C bond lengths (Ohki et al., 1990; Roth and Chen, 1991). In the case of C(16):C(16)PC bilayers, the V-shaped biphasic curve is well known to reflect two different types of lipid phase transitions: the $L_{\beta'} \rightarrow L_\alpha$ phase transition at $[EtOH] < [EtOH]_{TC}$ and the $L_{\beta I} \rightarrow L_\alpha$ phase transition at $[EtOH] > [EtOH]_{TC}$ (Simon and McIntosh, 1984). These two different phase transitions are a result of the $L_{\beta'} \rightarrow L_{\beta I}$ isothermal phase transition underlying the C(16):C(16)PC bilayer as [EtOH] increases beyond $[EtOH]_{TC}$ at $T < T_m$. Consequently, it is reasonable to infer that mixed-chain C(15):C(17)PC and C(17):C(15)PC at $T < T_m$ are most likely to undergo the $L_{\beta'} \rightarrow L_{\beta I}$ isothermal phase transition at $[EtOH] > [EtOH]_{TC}$.

The phase transition behavior in the presence of various concentrations of ethanol of MLVs composed of highly asymmetrical C(12):C(20)PC (ΔC = 6.5 C-C bond lengths) is shown in Fig. 1 *C*. Again, the T_m values are determined over a range of [EtOH], shown in Fig. 1 *C*, that is wider than that reported earlier (Li et al., 1996). The dependence of the main phase transition on the [EtOH], shown in the inset, is characterized by a progressive decrease in T_m as the [EtOH] increases over a wide range of concentrations. Clearly, no biphasic effect of ethanol on the T_m of MLVs composed of highly asymmetrical mixed-chain C(12):C(20)PC is observed, suggesting that the $L_{\beta'} \rightarrow L_{\beta I}$ isothermal phase transition in the presence of high concentrations of ethanol is not an energetically favorable process for highly asymmetrical C(*X*):C(*Y*)PC.

To explicitly verify these structural assignments, we have performed x-ray diffraction analyses of the mixed-chain PC as a function of ethanol concentration. In buffer in the absence of ethanol, both C(17):C(15)PC and C(15):C(17)PC gave diffraction patterns that contained a broad wide-angle reflection centered at 4.20 Å and several sharp low-angle reflections that indexed as orders of a lamellar phase. Such patterns are characteristic of multibilayers in the gel phase (McIntosh, 1980; Tardieu et al., 1973). The

FIGURE 1 The effects of the addition of increasing concentrations of ethanol on the phase transition behavior of multilamellar lipid vesicles composed of (*A*) C(15):C(17)PC, (*B*) C(17):C(15)PC, and (*C*) C(12):C(20)PC. The phase transition curves are the second DSC heating curves, and the T_m value of each DSC curve for the same lipid species is plotted against [EtOH] as shown in the inset. [EtOH] denotes the concentration of ethanol.

spacing and width of the wide-angle reflection indicates that the lipid hydrocarbon chains are tilted with respect to the plane of the bilayer (McIntosh, 1980; Tardieu et al., 1973), meaning that the bilayers are in the $L_{\beta'}$ phase. For C(17):C(15)PC the lamellar repeat period was 72.0 Å ± 0.9 Å (three experiments, ± standard deviation), and for C(15):C(17)PC the repeat period was 71 Å (single experiment). The intensity distribution for the lamellar reflections from these lipids (data not shown) was similar to that previously observed (McIntosh and Simon, 1986) for C(16):C(16)PC in excess buffer. In the case of C(12):C(20)PC in excess buffer the diffraction pattern contained a broad wide-angle reflection centered at 4.20 Å, consistent with tilted hydrocarbon chains, and three very broad low-angle bands centered at 41 Å, 22 Å, and 15 Å. These low-angle bands are at spacings corresponding to the positions of the first three peaks in the continuous Fourier transform of C(16):C(16)PC in the $L_{\beta'}$ phase (McIntosh and Simon, 1986).

The addition of 120 mg/ml ethanol to the buffer changed both the wide-angle and low-angle patterns from each of these mixed-chain PCs. For C(17):C(15)PC, C(15):C(17)PC, and C(12):C(20)PC in 120 mg/ml ethanol, the wide-angle pattern consisted of a single very sharp reflection (d_s) at 4.12 ± 0.02 Å (see Table 1), characteristic of lipids packed in a gel phase with no hydrocarbon chain tilt (McIntosh, 1980; Tardieu et al., 1973). For each of these specimens, three sharp low-angle reflections were observed that indexed as orders of a lamellar repeat period. In 120 mg/ml ethanol, the lamellar repeat periods (d) for C(17):C(15)PC, C(15):C(17)PC, and C(12):C(20)PC were 49.9 ± 0.6 Å (two experiments), 50.2 Å (one experiment), and 57.6 Å ± 0.2 Å (two experiments), respectively (see Table 1). The wide-angle reflections (d_s), repeat periods, and intensity distributions of the lamellar reflections for both C(15):C(17)PC and C(17):C(15)PC (see Table 1) were similar to those previously observed for the interdigitated phase ($L_{\beta I}$) of C(16):C(16)PC in ethanol (Simon and McIntosh, 1984), as well as to those of the $L_{\beta I}$ phase of C(16):C(16)PC induced by other surface-active molecules such as tetracaine (McIntosh et al., 1983). The wide-angle diffraction, repeat period, and intensity distribution for C(12):C(20)PC in 120 mg/ml of ethanol (see Table 1) were similar to those previously observed for C(18):C(10)PC in excess water, which is in a mixed interdigitated phase, where the long hydrocarbon chain spans the entire width of the bilayer and short chains from opposing monolayers align (McIntosh et al., 1984).

Because of the similarities in repeat periods and intensity distributions noted above, for C(17):C(15)PC in buffer, C(17):C(15)PC and C(15):C(17)PC in 120 mg/ml ethanol, and C(12):C(20)PC in 120 mg/ml ethanol, phase angles for the x-ray reflections for these lipids were chosen to be the same as those previously obtained for C(16):C(16)PC in the $L_{\beta'}$ phase (McIntosh and Simon, 1986), C(16):C(16)PC in the $L_{\beta I}$ phase (McIntosh et al., 1983), and C(18):C(10)PC in the mixed interdigitated phase (McIntosh et al., 1984), respectively. Electron density profiles for C(17):C(15)PC in buffer and 120 mg/ml ethanol are shown in Fig. 2. For each profile the center of the bilayer is at the origin. The high electron density peaks, located at ± 21 Å (*dotted vertical lines*) for C(17):C(15)PC in buffer and at ±15 Å for C(17):C(15)PC in 120 mg/ml ethanol, correspond to the lipid headgroups. The medium density regions on the outer edges of each profile correspond to the fluid spaces between adjacent bilayers. In the case of C(17):C(15)PC in buffer there is a sharp dip in electron density in the center of the bilayer, which corresponds to the localization of the low-electron-density terminal methyl groups (Lesslauer et al., 1972; McIntosh and Simon, 1986). For C(17):C(15)PC in buffer the distance between headgroup peaks across the bilayers ($d_{pp} \approx 43$ Å) is similar to that observed for C(16):C(16)PC in the $L_{\beta'}$ gel phase ($d_{pp} \approx 42$ Å) (McIntosh, 1980; McIntosh and Simon, 1986). In the case of C(17):C(15)PC in 120 mg/ml EtOH, there is no terminal methyl dip in the center of the bilayer. The shape of this profile is similar to that previously observed for interdigitated phase bilayers (Kim et al., 1987). Moreover, for this profile the value of $d_{pp} \approx 31$ Å is in close agreement with the value of $d_{pp} \approx 30$ Å previously observed for C(16):C(16)PC in the fully interdigitated ($L_{\beta I}$) gel phase (McIntosh et al., 1983). Thus the addition of ethanol causes a marked decrease in bilayer thickness (≈ 12 Å) because of the full interpenetration or interdigitation of hydrocarbon chains from opposing monolayers in the bilayer.

TABLE 1 X-ray diffraction data for lipids in 120 mg/ml EtOH

Lipid	d_s (Å)	A_c (Å^2)	A (Å^2)	d (Å)	Structure factors F(1)	F(2)	F(3)
C(15):C(17)PC	4.12	19.6	78.4	50.2	−3.36	−5.04	+3.69
C(17):C(15)PC	4.12	19.6	78.4	49.9	−3.21	−5.11	+3.68
C(12):C(20)PC	4.12	19.6	58.8	57.4	−3.91	−4.88	+4.22

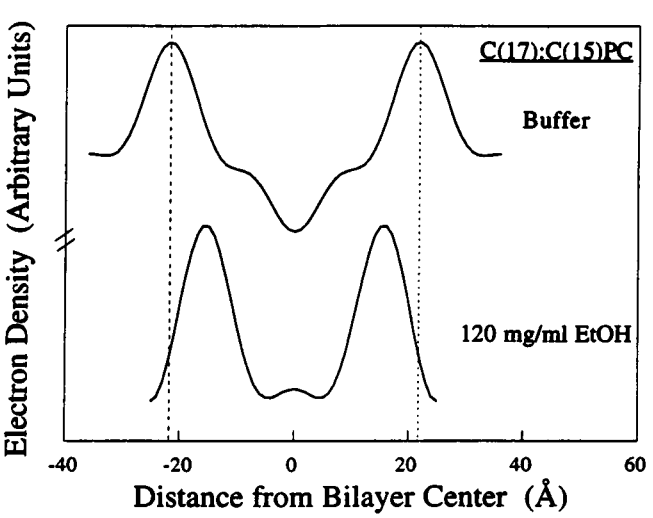


FIGURE 2 Electron density profiles of C(17):C(15)PC in excess buffer (*top*) and in 120 mg/ml ethanol in buffer (*bottom*). The vertical dotted lines are drawn through the middle of the headgroup peaks in the profile of C(17):C(15)PC in buffer.

Fig. 3 shows electron density profiles for C(17):C(15)PC, C(15):C(17)PC, and C(12):C(20)PC in 120 mg/ml ethanol. The electron density profiles are almost identical for C(17):C(15)PC and C(15):C(17)PC in 120 mg/ml ethanol, indicating that both lipids are in the fully interdigitated $L_{\beta I}$ gel phase. The profile for C(12):C(20)PC also contains no terminal methyl trough in the center of the bilayer. However, the distance between headgroup peaks across the bilayer is larger ($d_{pp} \approx 36$ Å). This value is slightly larger than the value of $d_{pp} \approx 33$–34 Å previously observed for C(18):C(10)PC in the mixed interdigitated phase (McIntosh et al., 1984). This small difference in spacing is undoubtedly due to the larger number of methylene groups in C(12):C(20)PC.

Discrete lamellar reflections were not observed for C(12):C(20)PC in buffer. The absence of sharp reflections indicates that these bilayers did not stack in a regular array. The reasons for this disorderliness in the stacking are unknown, although it has been observed by Shah et al. (1990) that the similar mixed-chain C(10):C(18)PC forms a mixture of noninterdigitated and mixed interdigitated phases, with the relative amounts of the two phases depending upon the incubation and hydration conditions. Such a mixing of phases could cause disorderliness in the stacking of the multilayers. However, for C(12):C(20)PC the spacings of the three broad low-angle bands and the width and spacing of the wide-angle reflection indicate that this highly asymmetrical lipid is primarily in a tilted, noninterdigitated phase in excess buffer.

Thus both the wide-angle and low-angle patterns indicate that in buffer, at 20°C, C(17):C(15)PC, C(15):C(17)PC, and C(12):C(20)PC form the normal gel ($L_{\beta'}$)-phase lipid bilayers with tilted hydrocarbon chains. In the presence of 120 mg/ml ethanol, both C(17):C(15)PC and C(15):C(17)PC form the fully interdigitated ($L_{\beta I}$) gel phase, whereas highly asymmetrical C(12):C(20)PC forms a mixed interdigitated (L_{MI}) gel phase. Based on this assignment of phases, and the observed wide-angle reflections (d_s), we can calculate the area per hydrocarbon chain (A_c) and the area per lipid headgroup (A) for these three lipids in the presence of ethanol. In the case of untilted hydrocarbon chains with hexagonal packing, the area per hydrocarbon chain is given by $A_c = 2d_s^2/3^{1/2}$ (Table 1). In the case of the $L_{\beta I}$ phase, in which hydrocarbon chains from opposing bilayers completely interdigitate, the area per headgroup is $A = 4 \cdot A_c$ (McIntosh et al., 1983), whereas for the mixed interdigitated phase $A = 3 \cdot A_c$ (McIntosh et al., 1984). Therefore, in the presence of 120 mg/ml ethanol, the area per lipid headgroup at the interface is about 78 Å^2 for both C(15):C(17)PC and C(17):C(15)PC, and about 59 Å^2 for C(12):C(20)PC (Table 1). These areas can be compared to the values of $A = 48$ Å^2 and 63 Å^2 for DPPC in the gel ($L_{\beta'}$) and liquid crystalline (L_α) phases, respectively (Nagle et al., 1996).

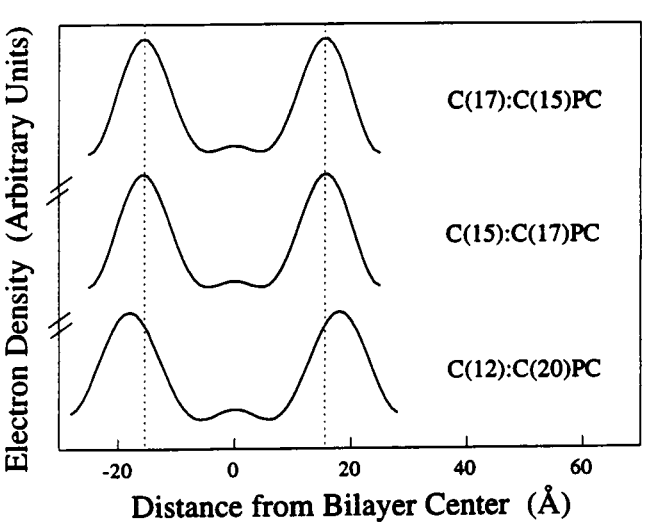


FIGURE 3 Electron density profiles of C(17):C(15)PC (*top*), C(15):C(17)PC (*middle*), and C(12):C(20)PC (*bottom*), all in excess 120 mg/ml ethanol. The vertical dotted lines are drawn through the middle of the headgroup peaks in the profiles of C(17):C(15)PC and C(15):C(17)PC.

DISCUSSION

We begin our discussion by considering the T_m curves illustrated in the insets in Fig. 1, *A* and *B*, for C(15):C(17)PC and C(17):C(15)PC, respectively. The T_m in each of the curves decreases steadily with increasing [EtOH], reaching a nadir at $[EtOH]_{TC}$. Thereafter, it increases with increasing [EtOH], resulting in a V-shaped biphasic profile. This shape of the curve can be explained phenomenologically based on the concept of the freezing point depression/elevation of the solvent by solute as previously described for lamellar C(16):C(16)PC (Simon and McIntosh, 1984). Specifically, the linear decrease in T_m at low concentrations of ethanol can be explained as a freezing-point depression phenomenon caused by more ethanol partitioning into the liquid crystalline phase than into the gel phase. The sudden upward breaks in the curves, at [EtOH] of 50 mg/ml for C(15):C(17)PC and at 73 mg/ml for C(17):C(15)PC, are caused by the onset of the interdigitated phase. The induction of this phase causes these breaks in the T_m versus [EtOH] curves because in the $L_{\beta I}$ phase the hydrocarbon chains are at the hydrocarbon-water interface, increasing the area per lipid molecule (Table 1), providing more area for ethanol binding than in either the $L_{\beta'}$ phase or the L_α phase (Simon and McIntosh, 1984). Therefore, at ethanol concentrations above these critical values, the partitition coefficient becomes greater in the gel ($L_{\beta I}$) phase than the liquid crystalline phase, causing a freezing point elevation (Simon and McIntosh, 1984).

The structural basis of the formation of the $L_{\beta I}$ phase has been discussed previously for the C(16):C(16)PC bilayer (McIntosh et al., 1983; Simon and McIntosh, 1984). First, it is important to appreciate that ethanol is a small, amphipathic molecule. As such, it is located preferentially in the normal gel-state $L_{\beta'}$ bilayer at the hydrocarbon-water interface and does not extend too deeply into the bilayer interior. In this location, ethanol increases the lateral separation

between adjacent lipid headgroups. However, because the nonpolar moiety of ethanol is short compared to that of the lipid hydrocarbon chains, the addition of ethanol would potentially cause voids in the hydrocarbon region of the bilayer. To avoid the formation of these energetically unfavorable voids in the gel phase, the hydrocarbon chains from opposing monolayers interdigitate (Simon and McIntosh, 1984).

The spontaneous conversion of MLVs composed of C(15):C(17)PC or C(17):C(15)PC from the $L_{\beta'}$ gel phase to the $L_{\beta I}$ gel phase, at $T < T_m$, in the presence of high concentrations of ethanol must be driven thermodynamically by a negative free energy difference between the bilayer in the $L_{\beta I}$ phase in the presence of excess ethanol and the $L_{\beta'}$ bilayer in the absence of ethanol. To understand that the isothermally ethanol-promoted $L_{\beta'} \rightarrow L_{\beta I}$ phase transition is indeed energetically favorable, it is necessary to dissect and compare the free energy changes associated with the $L_{\beta'} \rightarrow L_{\beta I}$ phase transitions in the C(15):C(17)PC and C(17):C(15)PC bilayers. Specifically, we first describe the free energy levels of the $L_{\beta'}$ and $L_{\beta I}$ phases in the absence of ethanol. The changes in the free energies of the $L_{\beta'}$ and $L_{\beta I}$ phases as a function of [EtOH] will subsequently be considered. In particular, the concentration of ethanol at which the free energy difference (ΔG) of the $L_{\beta'} \rightarrow L_{\beta I}$ process is zero will be assessed for the bilayer of C(15):C(17)PC as well as C(17):C(15)PC.

Let us first examine the free energy changes associated with the C(15):C(17)PC bilayer, in which the lipid molecule at $T < T_m$ is quite symmetrical, with a ΔC value of 0.5 C-C bond lengths. In the absence of ethanol, the Gibbs free energy (G) of the $L_{\beta'}$ phase is assigned to be at a level that is considerably lower than that of the $L_{\beta I}$ phase (Fig. 4 *A*). One characteristic feature of the chain packing motif of the $L_{\beta I}$ phase is that each lipid acyl chain penetrates through the hydrophobic core of the gel-state bilayer with its terminal methyl group located on the opposing side of the bilayer. Part of the reason for the larger value of G for the $L_{\beta I}$ phase in the absence of ethanol lies in the energetically unfavorable exposure of the chain terminal methyl group to the polar aqueous medium. The assigned energy levels shown in Fig. 4 *A* are consistent with the data presented earlier by Kinoshita and Yamazaki (1996) for MLVs of C(16):C(16)PC. Specifically, based on the concept of the interaction energy χ parameter and the solubility data, Kinoshita and Yamazaki concluded that in the absence of ethanol, the chemical potential of the C(16):C(16)PC bilayer in the $L_{\beta'}$ phase is lower than that in the $L_{\beta I}$ phase.

As an increasing amount of ethanol is added successively to the aqueous solution containing MLVs of C(15):C(17)PC, the T_m value of the lipid sample is observed to decrease progressively with increasing [EtOH] up to the threshold concentration of 50 mg/ml (Fig. 1 *A*). This implies that the stability of the lipid assembly in the $L_{\beta'}$ phase is decreased by the presece of ethanol, which may be taken to reflect an ethanol-induced increase in the free energy content of the lipid assembly. Indeed, it has been shown experimentally that the C(16):C(16)PC/H_2O-ethanol system at [EtOH] < $[EtOH]_{TC}$ has a smaller free energy change (ΔG) for the $L_{\beta'} \rightarrow L_{\alpha}$ phase transition in comparison with the corresponding free energy change of the C(16):C(16)PC/H_2O system without ethanol, or that $\Delta G - \Delta G^{\circ} < 0$, where the superscript denotes the quantity in the absence of etha-

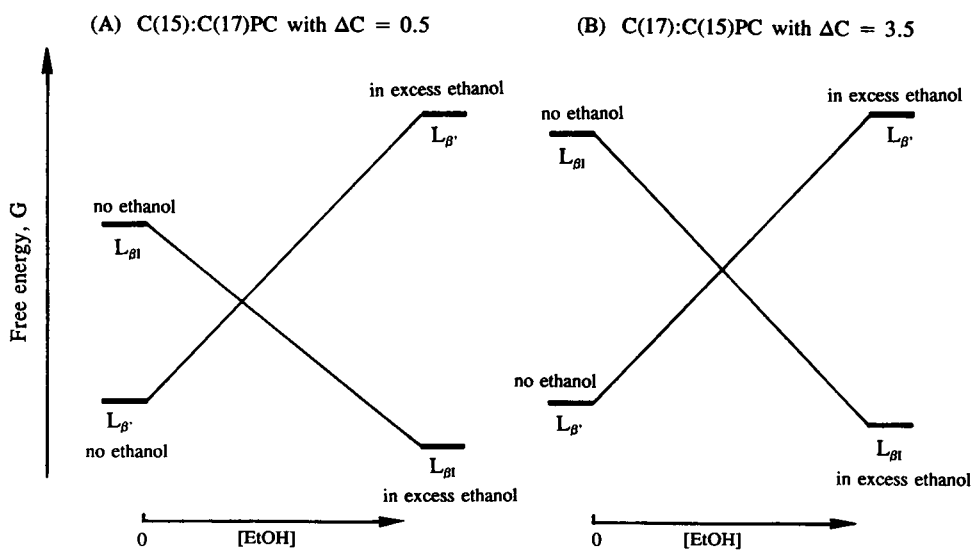


FIGURE 4 The proposed free energy changes of the gel-state bilayer in the $L_{\beta'}$ and $L_{\beta I}$ phases in response to the addition of increasing concentrations of ethanol. (*A*) The C(15):C(17)PC bilayer. The lipid molecule in this bilayer at $T < T_m$ is characterized by a ΔC value of 0.5 C-C bond lengths. (*B*) The C(17):C(15)PC bilayer. The lipid molecule is more asymmetrical, with a ΔC value of 3.5 C-C bond lengths. It should be noted that the cross-over point of the two lines in *A* and *B* occurs at the concentration of ethanol corresponding to $[EtOH]_{TC}$. It is apparent that $[EtOH]_{TC}$ shifts to a higher value of ethanol as the lipid molecule becomes more asymmetrical. This figure is intended to explain the isothermal $L_{\beta'} \rightarrow L_{\beta I}$ transition induced by different [EtOH] for C(15):C(17)PC and C(17):C(15)PC at $T < T_m$. The free energy levels of the L_{α} phase at various [EtOH] are not presented. It is assumed, however, that they are very similar and are located at a level far above the highest free energy level of $L_{\beta'}$ (in excess ethanol) shown in both *A* and *B*.

nol (Roth and Chen, 1991). Hence, if we make a reasonable assumption that the free energy difference of the L_α-C(16):C(16)PC bilayer in the presence and absence of ethanol ($G_\alpha - G^\circ_\alpha$) is significantly smaller than that of the $L_{\beta'}$ phase ($G_{\beta'} - G^\circ_{\beta'}$), we can then draw the conclusion that the free energy of the C(16):C(16)PC bilayer in the $L_{\beta'}$ phase will increase with increasing [EtOH]. Analogously, the free energy of C(15):C(17)PC in the $L_{\beta'}$ phase can also be inferred to increase with increasing [EtOH]. This increase in G for the $L_{\beta'}$ phase by ethanol is graphically illustrated in Fig. 4 *A*.

The free energy of the $L_{\beta I}$ phase, unlike the $L_{\beta'}$ phase just discussed above, will decrease with increasing [EtOH]. There are two important contributions to this free energy drop. For the $L_{\beta I}$ phase, the magnitude of the unfavorable interaction between the chain methyl end and water in the aqueous medium can be considered to be proportional to the interfacial surface tension times the exposed surface area (Nagle, 1980). As more ethanols are added to the $L_{\beta I}$ bilayer, an increasing amount of the small, amphipathic molecules will be strategically located between the terminal methyl groups of the lipid's acyl chains and water in solution. Consequently, the H_2O-accessible hydrocarbon surface in the $L_{\beta I}$ bilayer will decrease with increasing [EtOH]. In addition, the surface tension at the H_2O/hydrocarbon chain interface will also be decreased steadily by increasing [EtOH]. The combined effects of [EtOH] will thus diminish considerably the unfavorable interaction between the chain methyl groups and water in the $L_{\beta I}$ bilayer, leading to an increase in the stability and hence a decrease in free energy, G. This proposed decrease in G is in complete accord with the experimental evidence obtained with C(16):C(16)PC at [EtOH] > $[EtOH]_{TC}$ (Roth and Chen, 1991). The ethanol-induced decrease in G for the $L_{\beta I}$ phase is also depicted in Fig. 4 *A*.

Because of the overall opposing effect of ethanol on the stability of the gel-state bilayer in the $L_{\beta'}$ and $L_{\beta I}$ phases, the changes in the free energy as a function of [EtOH] are positive and negative, respectively, for $L_{\beta'}$ and $L_{\beta I}$, as illustrated in Fig. 4 *A*. At a characteristic concentration of ethanol, the two curves expressing the changes in free energy cross. At this point, the free energy difference (ΔG) for the $L_{\beta'} \rightarrow L_{\beta I}$ isothermal transition is zero. This means that the $L_{\beta'}$ and $L_{\beta I}$ phases are in equilibrium at this characteristic concentration of ethanol, which, in fact, corresponds to the experimentally determined $[EtOH]_{TC}$ of 50 mg/ml for C(15):C(17)PC. At [EtOH] > $[EtOH]_{TC}$, lamellar C(15):C(17)PC packed in the $L_{\beta I}$ phase is energetically more favorable because of the lower free energy (Fig. 4 *A*); hence the $L_{\beta'} \rightarrow L_{\beta I}$ isothermal transition can occur spontaneously at high concentrations of ethanol in MLVs composed of C(15):C(17)PC.

Now let us proceed to further consider the ethanol-induced $L_{\beta'} \rightarrow L_{\beta I}$ isothermal transition underlying MLVs composed of C(17):C(15)PC. In this particular case, the ΔC value of C(17):C(15)PC is 3.0 C-C bond lengths longer than that of C(15):C(17)PC. It should be recalled that the structural parameter ΔC reflects the openness region between the *sn*-1 and *sn*-2 acyl chains within each lipid molecule packed in the gel-state $L_{\beta I}$ bilayer. Moreover, the ability of a lipid molecule as a whole in the $L_{\beta I}$ bilayer to undergo the closest van der Waals interactions with its neighbors can be perturbed by this openness region. The magnitude of the perturbation is determined by the value of ΔC; namely, the longer the ΔC value, the greater is the perturbation imposed on the overall lateral lipid-lipid contact interaction. By comparing the ΔC value of C(15):C(17)PC with that of C(17):C(15)PC, it is clear that the overall lateral lipid-lipid van der Waals interaction in the $L_{\beta I}$ bilayer of C(17):C(15)PC must be weakened appreciably. Furthermore, the unfavorable energy in exposing the hydrocarbon surface to water, as stated earlier, is proportional to the interfacial surface tension times the surface area (Nagle, 1980). The surface tension decreases with increasing ethanol concentration, whereas the surface area of exposed hydrocarbon increases with increasing ΔC. Consequently, in the absence of ethanol, the free energy of lamellar C(17):C(15)PC in the $L_{\beta I}$ phase, due to its bigger ΔC value, is significantly larger than that of C(15):C(17)PC. This large difference in G between C(17):C(15)PC and C(15):C(17)PC can be recognized from the free energy levels illustrated in Fig. 4, *A* and *B*. In contrast, the ΔC region in the $L_{\beta'}$ phase is not an openness region, but is instead a filled region that a chain segment from a lipid molecule in the opposing leaflet occupies. Hence the free energy levels for C(15):C(17)PC and C(17):C(15)PC in the $L_{\beta'}$ phase differ only slightly, as indicated in Fig. 4, *A* and *B*.

If ethanol is now added to the solution containing C(17):C(15)PC bilayers, the free energies of the $L_{\beta'}$ and $L_{\beta I}$ phases begin to change simultaneously, but in opposite directions (Fig. 4 *B*). At the threshold concentration, $L_{\beta'}$ and $L_{\beta I}$ coexist. Beyond $[EtOH]_{TC}$, the $L_{\beta I}$ phase has a favorable free energy. As a result, the $L_{\beta'} \rightarrow L_{\beta I}$ isothermal transition occurs spontaneously at high concentrations of ethanol. It should be emphasized that, in the absence of ethanol, the $L_{\beta I}$ phase of lamellar C(17):C(15)PC has a considerably larger free energy; hence, in comparison with C(15):C(17)PC, the concentration of ethanol required to make the two free energy curves ($L_{\beta'}$ and $L_{\beta I}$) of lamellar C(17):C(15)PC cross can be expected to be greater than that required for the corresponding C(15):C(17)PC, as indicated in Fig. 4, *A* and *B*. Indeed, experimentally determined values of $[EtOH]_{TC}$ for C(15):C(17)PC and C(17):C(15)PC bilayers shown in Fig. 1, *A* and *B*, confirm our expectation. In fact, if two mixed-chain C(*X*):C(*Y*)PCs ($\Delta C < 4.2$) have a common molecular weight, the one with a higher ΔC value can be generalized, based on Fig. 4, to give rise to a larger value of $[EtOH]_{TC}$ in the T_m versus [EtOH] plot. Here the ΔC value is related to two additive features in the $L_{\beta I}$ bilayer: the lateral lipid-lipid perturbation and the H_2O-accessible hydrocarbon surface. It takes little imagination to extend the above generalization and to predict that a similar relationship is most likely to exist between the $\Delta C/CL$ value and the $[EtOH]_{TC}$ for MLVs composed of identical-chain C(*X*):C(*X*)PC with a common ΔC value. In this case, CL is the effective chain length of the longer acyl chain, in C-C

bond lengths, and $\Delta C/CL$ is the normalized chain-length difference between the *sn*-1 and *sn*-2 acyl chain (Huang, 1991). For identical-chain PC, the $\Delta C/CL$ term reflects the size of the openness region of the acyl chains (ΔC) within the hydrocarbon core of the $L_{\beta I}$ bilayer relative to the longer *sn*-1 acyl chain length; hence, $\Delta C/CL$ can be considered a normalized perturbation parameter in terms of lateral lipid-lipid interactions. This concept applies to phospholipids with the same headgroup. For instance, the $\Delta C/CL$ values for C(14):C(14)PC, C(16):C(16)PC, C(18):C(18)PC, and C(20):C(20)PC are 0.12, 0.10, 0.09, and 0.08, respectively; consequently, one can expect that their $[EtOH]_{TC}$ values would decrease in the same order as the normalized perturbation parameter as follows: C(14):C(14)PC > C(16):C(16)PC > C(18):C(18)PC > C(20):C(20)PC. This expectation is indeed borne out by experimental data (Rowe, 1983, 1992).

Finally, it should be emphasized that MLVs composed of C(12):C(20)PC do not exhibit calorimetrically the V-shaped curve in the plot of T_m versus [EtOH]. Consistent with the DSC data, x-ray diffraction results show that MLVs composed of highly asymmetrical C(12):C(20)PC do not undergo the ethanol-induced $L_{\beta'} \rightarrow L_{\beta I}$ transition. In addition, x-ray data demonstrate that a $L_{\beta'} \rightarrow L_{MI}$ (mixed interdigitated) isothermal transition takes place in MLVs of C(12):C(20)PC in response to high concentrations of ethanol. To the best of our knowledge, this is the first time that such an ethanol-induced $L_{\beta'} \rightarrow L_{MI}$ isothermal transition is detected experimentally for MLVs composed of saturated PC at $T < T_m$. Therefore, in the presence of 120 mg/ml EtOH, the area per headgroup in the mixed interdigitated gel phase of C(12):C(20)PC is ~59 Å^2 (Table 1), which is less than the area per molecule (63 Å^2) in the liquid-crystalline phase of DPPC (Nagle et al., 1996). In the case of C(12):C(20)PC we argue that there is more exposed hydrocarbon in the L_α phase than in either the $L_{\beta'}$ phase (at low [EtOH]) or in the mixed interdigitated phase (at high [EtOH]). Therefore, we expect a monotonic decrease in T_m with increasing [EtOH], as indeed is observed (Fig. 1 *C*). Thus it is important to realize that MLVs composed of one-component saturated PCs can undergo at least two types of isothermal transitions, at $T < T_m$, in response to high concentrations of ethanol. Moreover, the specific type of isothermal transition depends critically on the asymmetry parameter of the underlying lipid, ΔC, or the effective chain-length difference between the two acyl chains within the lipid molecule.

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