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Hydrocarbon chain packing and the effect of ethanol on the thermotropic phase behavior of mixed-chain phosphatidylglycerols

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Abstract

Previous studies in this laboratory have delineated the relationship between the acyl chain asymmetry of mixed-chain phosphatidylcholines and the effect of ethanol concentration ([EtOH]) on their melting behavior (Li et al., Biophys J., 70 (1996) 2784–2794). This present investigation extends these findings to another phospholipid family by using high-resolution differential scanning calorimetry (DSC) to characterize the effect of ethanol concentration on the main phase transition temperature (T_m) of five molecular species of mixed-chain phosphatidylglycerol (PG). For C(14):C(18)PG, C(15):C(17)PG, C(16):C(16)PG, and C(17):C(15)PG, a biphasic profile in the T_m versus [EtOH] plot was observed, and the minimum in the plot for each PG occurred at 33, 15, 19, and 36 mg/ml, respectively. This biphasic behavior is typical of phospholipids whose acyl chain asymmetry is fairly small. For C(18):C(14)PG, only a linear decrease in the T_m was observed as a function of ethanol concentration; this effect is characteristic of highly asymmetric phospholipids. Our DSC results obtained with mixed-chain PG in the presence of ethanol demonstrate that the acyl chain asymmetry of the five lipids studied can be ranked as follows: C(15):C(17)PG < C(16):C(16)PG < C(14):C(18)PG < C(17):C(15)PG < C(18):C(14)PG, confirming independent results from this laboratory that the overall acyl chain structure of PG in the hydrated gel phase is opposite to that detected in the single crystals. In fully hydrated bilayers, the acyl chain conformation of PG is most likely to be similar to that of phosphatidylcholine.

Keywords: Differential scanning calorimetry; Interdigitation; Acyl chain asymmetry

1. Introduction

The classic biphasic effect of ethanol on the main phase transition temperature (T_m) of identical-chain phosphatidylcholine has been thoroughly investigated over the past two decades. Rowe first observed the biphasic effect of ethanol on the T_m of C(16):C(16)PC [1]. It was shown that as the ethanol concentration ([EtOH]) is increased up to about 50 mg/ml, the T_m of C(16):C(16)PC decreases almost linearly. However, above an ethanol concentration of 50 mg/ml, the T_m increases as the [EtOH] is increased up to 120 mg/ml. The value of the ethanol concentration at which the minimum in the T_m versus [EtOH] occurs is called the 'threshold concentration' and is referred to herein as $[EtOH]_{TC}$. This biphasic behavior is related to the bilayer packing motif of the acyl chains in the gel phase. When $[EtOH] \leq [EtOH]_{TC}$, the acyl chains of an identical-chain PC are in the partially interdigitated packing motif at $T < T_m$ [2]. In a partially interdigitated bilayer, the *sn*-1 chain of one PC molecule is juxta-

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posed to the *sn*-2 chain of another PC molecule from the opposing leaflet. When the [EtOH] > $[EtOH]_{TC}$, the acyl chains of the bilayer are in the fully interdigitated bilayer at $T < T_m$. In this packing motif, both acyl chains of a lipid extend across the length of the whole hydrocarbon core. This biphasic effect in the T_m versus [EtOH] plot was observed by Rowe for a large number of identical-chain PCs with different acyl chain lengths [1]. The threshold concentration generally decreased as the length of the acyl chains of PC increased from 14 carbons to 22 carbons.

Recently, Huang and McIntosh investigated the effect of ethanol on the thermotropic phase behavior and acyl chain interdigitation of several saturated mixed-chain C(X):C(Y)PCs, where C(X) refers to the number of carbons in the *sn*-1 acyl chain, and C(Y) refers to the number of carbons in the *sn*-2 chain [3]. The effect of ethanol on mixed-chains PCs was clearly shown to be related to the acyl chain asymmetry of each PC. The acyl chain asymmetry, designated by ΔC, is a measure of the difference in effective length between the two acyl chains of a phospholipid in the gel-state bilayer. For any saturated C(X):C(Y)PC in the hydrated gel phase, the ΔC value in carbon–carbon bond lengths can be estimated directly from the chemical formula by the following equation: $\Delta C = |X - Y + 1.5|$ [13]. Huang and McIntosh demonstrated that for C(X):C(Y)PC with $\Delta C < 4.2$ C–C bond lengths, the classic biphasic effect of ethanol on the main phase transition temperature is observed [3]. The value of $[EtOH]_{TC}$ is not only different for each C(X):C(Y)PC, but also dependent on the ΔC value of each phospholipid. Specifically, when $\Delta C < 4.2$, the $[EtOH]_{TC}$ increases as the ΔC increases. For example, C(15):C(17)PC has a ΔC of 0.5, and the $[EtOH]_{TC}$ of C(15):C(17)PC is about 50 mg/ml. For C(17):C(15)PC, whose ΔC is 2.5, the $[EtOH]_{TC}$ is 73 mg/ml. However, for PCs with $\Delta C > 4.2$, the biphasic effect in the T_m versus [EtOH] plot is not observed. Rather, the T_m of highly asymmetric phospholipids merely decreases continuously as the [EtOH] is increased up to a concentration of 120 mg/ml.

The model of the effect of ethanol on the packing motif and thermotropic phase behavior of phosphatidylcholine has been well characterized. It is important to extend this model to other phospholipids with different headgroups. Phosphatidylglycerol (PG) is an anionic phospholipid that has been shown to be a requirement for various membrane functions as well as cell viability [4]. The headgroup of PG contains a chiral center, and only one enantiomer is found in nature [5]. The crystal structure of dimyristoylphosphatidylglycerol (DMPG) contains both enantiomers of PG, and each enantiomer has a distinct acyl chain conformation [6]. The natural form of DMPG (1′-DMPG) contains an acyl chain conformation that is opposite to that found in phosphatidylcholine [6,7]. If this difference in chain conformation obtained with single crystals persists in the fully hydrated gel phase, then the response of *rac*-C(X):C(Y)PG to the presence of ethanol is expected to be very different to the response of C(X):C(Y)PC.

Fig. 1 shows the diacyl glyceride moieties of the energy-minimized crystal structures of C(14):C(14)PC and C(14):C(14)PG. It can be seen that the acyl chain conformations of these two lipids are different. Specifically, the *sn*-1 chain of C(14):C(14)PC is in the all-*trans* conformation while the *sn*-2 chain contains a bend at the C(2) position. In

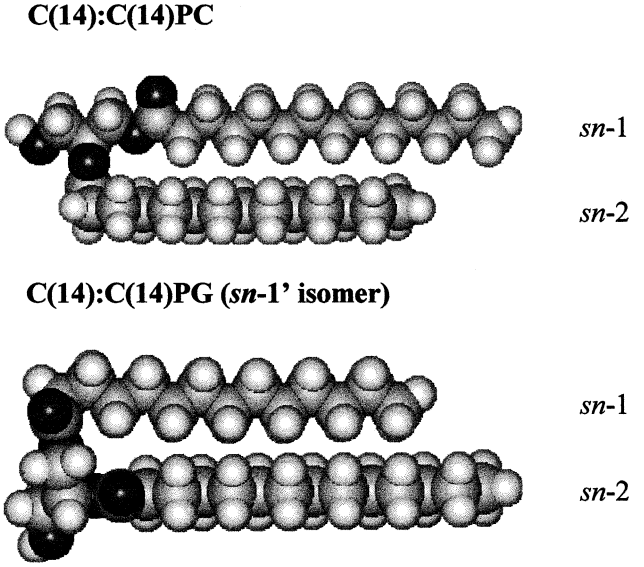

Fig. 1. Molecular graphics representations of the diacyl glyceride moieties of the energy minimized crystal structures of C(14):C(14)PC and 1′-C(14):C(14)PG. Initial atomic coordinates were taken from previously published reports [6,7]. Structures were minimized using Allinger's MM2 program [25]. Note that the *sn*-1 chain of C(14):C(14)PC is straight while the *sn*-2 chain is bent; in 1′-C(14):C(14)PG, the *sn*-1 chain contains the bend while the *sn*-2 chain is straight. Figures were generated using HyperChem 5.0 (Hypercube, Gainesville, FL).

C(14):C(14)PG, the *sn*-1 chain contains a bend while the *sn*-2 chain is completely straight. This apparently subtle difference in the acyl chain conformation has important implications with regards to the effect of acyl chain asymmetry on the behavior of these lipids. For example, if one methylene group was added to the *sn*-1 chain and one methylene group was removed concomitantly from the *sn*-2 chain, it would be expected, based on the crystal structures, that the unnormalized chain asymmetry of C(15):C(13)PC would be higher than that of C(14):C(14)PC, whereas the asymmetry of C(15):C(13)PG would be lower than that of C(14):C(14)PG.

Under the model developed in this laboratory of how chain asymmetry affects the chain melting behavior of lipids [14] it would be expected that the T_m of C(15):C(13)PC would be lower than that of C(14):C(14)PC and the T_m of C(15):C(13)PG would be higher than that of C(14):C(14)PG. It would be further expected, based on the work of Huang and McIntosh [3], that the $[EtOH]_{TC}$ of C(15):C(13)PG would be lower than C(14):C(14)PG. Similarly, one can predict that the T_m of C(17):C(15)PG would be higher (and the $[EtOH]_{TC}$ lower) than that of C(16):C(16)PG. Guided by these predictions, DSC studies were carried out to determine the effect of ethanol on the thermotropic phase behavior of five molecular species of C(*X*):C(*Y*)PG, each with a molecular weight homologous to C(16):C(16)PG. Nearly all previous work with PG has been limited only to identical-chain PG [8]. This, together with the accompanying paper, represents the first study that has ever been carried out on mixed-chain PGs. Each of the five PGs studied in this investigation has a different acyl chain composition, but they all have the same total number of carbon atoms in the two acyl chains. The results indicate that the acyl chain conformation of *rac*-C(*X*):C(*Y*)PG in the hydrated gel phase is very similar to the acyl chain structure of C(*X*):C(*Y*)PC.

2. Materials and methods

2.1. Semi-synthesis of saturated mixed-chain phosphatidylglycerols

All chemicals used in the semi-synthesis of C(*X*):C(*Y*)PG were of reagent grade, and all solvents were of spectroscopic grade. Saturated C(16):C(16)PG was obtained from Avanti Polar Lipids (Alabaster, AL). The following four saturated mixed-chain phosphatidylglycerols were semi-synthesized from their corresponding PC: C(14):C(18)PG, C(15):C(17)PG, C(17):C(15)PG, and C(18):C(14)PG. Initially, C(*X*):C(*Y*)PC was prepared by the modified procedure of Mena and Djerassi as previously described [9,10]. The fatty acids used in the reaction were purchased from Sigma (St. Louis, MO) and lysophosphatidylcholines were purchased from Avanti. Once the C(*X*):C(*Y*)PC was purified, the corresponding C(*X*):C(*Y*)PG was prepared via transphosphatidylation using phospholipase D from cabbage (Sigma) as previously described by Dawson [11]. The C(*X*):C(*Y*)PG was isolated via consecutive washings with 200 mM EDTA, 50 mM HCl and distilled water, followed by column chromatography using Silica Gel 60 Å (mesh size, 230–400) (Sigma). All lipids synthesized were judged to be ~99% pure by analytical thin-layer chromatography [12].

2.2. Preparation of lipid/alcohol samples

Lyophilized lipid powder (4.0 mg) was dispersed in 2 ml of aqueous solution containing 150 mM NaCl, 50 mM HEPES, 1 mM EDTA, pH 7.4. The aqueous sample was first heated to 15°C above the T_m of the phospholipid, and was then subjected to ultrasonic irradiation in an ultrasonic water bath for up to 30 min. Aliquots of absolute ethanol (10–360 μl) were added to the lipid solution, and the sample was vortexed for several minutes and stored at 4°C until just prior to injection into the sample cell of the differential scanning calorimeter. All samples were stored for at least 24 h prior to DSC measurements.

2.3. High-resolution differential scanning calorimetry

The thermotropic phase behavior of the lipid/alcohol samples was studied using a high-resolution Microcal MC-2 differential scanning calorimeter (Microcal, Northampton, MA). A constant heating rate of 15°C/h was used for all samples, and for each sample, the reference cell of the calorimeter was filled with buffer solution containing the same

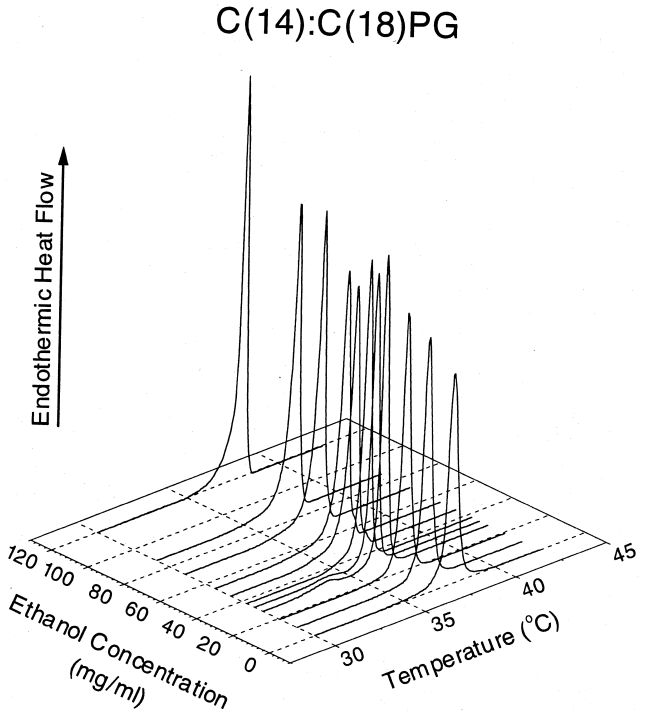

Fig. 2. A series of DSC heating scans of aqueous dispersions of C(14):C(18)PG in the presence of ethanol. The molar heat capacity is plotted as a function of temperature and ethanol concentration. Freshly dispersed lipid samples were heated to 15°C above T_m and sonicated for up to 30 min. Samples were then stored at 4°C for several days, and appropriate amounts of absolute ethanol were added at least 24 h prior to injection into the calorimeter. The ethanol concentration was varied from 0 to 120 mg/ml. The nominal scan rate was 15°C/h. Only the third heating scan is presented here. No thermal hysteresis was observed subsequent to the second heating scan.

amount of ethanol as the lipid sample. The first heating scan was followed by a cooling scan and then two consecutive heating scans. Only the third heating scan is presented in this report, and no thermal history-dependent transition behavior was observed after the cooling scan. Raw data from the DSC experiments were analyzed using software provided by Microcal. The T_m of each sample was defined as the temperature at which the heat capacity reached a maximum. The $\Delta T_{1/2}$ of the sample was defined as the peak width that is measured at one-half the maximal height of the transition peak in the heat capacity plot. The T_m of each lipid/alcohol sample was reproducible to within ± 0.1°C.

3. Results

Fig. 2 shows the DSC scans of C(14):C(18)PG in the presence of various concentrations of ethanol. Throughout the full range of ethanol concentrations, the peaks in the DSC scans of C(14):C(18)PG are all fairly sharp, and no thermal history-dependent transition behavior is seen in the DSC plots on successive scans. In the absence of ethanol, the DSC heating curves for the aqueous dispersion C(14):C(18)PG is characterized by a T_m of 37.6°C with a $\Delta T_{1/2}$ of 0.5°C. As the ethanol concentration is increased up to about 33 mg/ml, the T_m decreases curvilinearly. However, above 33 mg/ml of ethanol, the T_m of C(14):C(18)PG increases with further increases in the ethanol concentration. Thus, a minimum in the T_m versus [EtOH] plot is seen for C(14):C(18)PG at $[EtOH]_{TC} \cong 33$ mg/ml, where the T_m of C(14):C(18)PG is 36.6°C. It is interesting to note that the $[EtOH]_{TC}$ of C(14):C(18)PC is about 63 mg/ml [12]. Reasons for why the $[EtOH]_{TC}$ of C(X):C(Y)PG is less than the corresponding C(X):C(Y)PC are discussed in a later section.

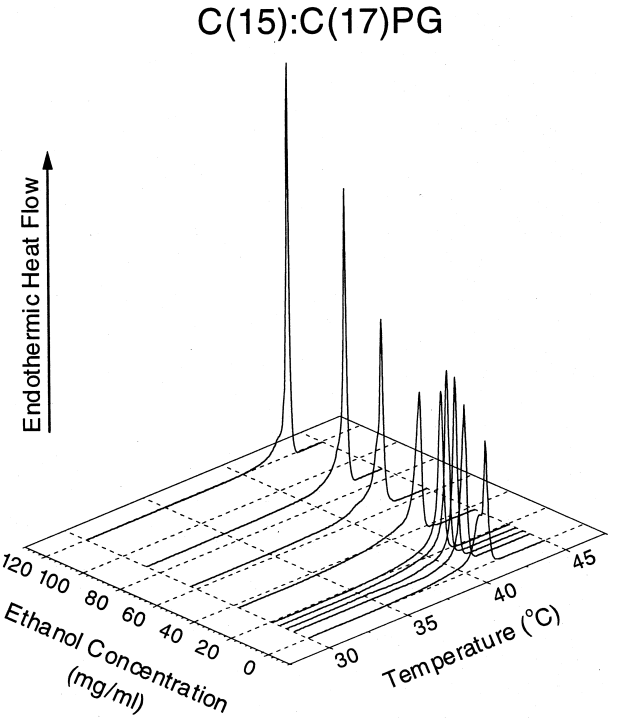

Fig. 3. DSC scans of aqueous dispersions of C(15):C(17)PG in the presence of various concentrations of ethanol. Experimental conditions were exactly as described for Fig. 2.

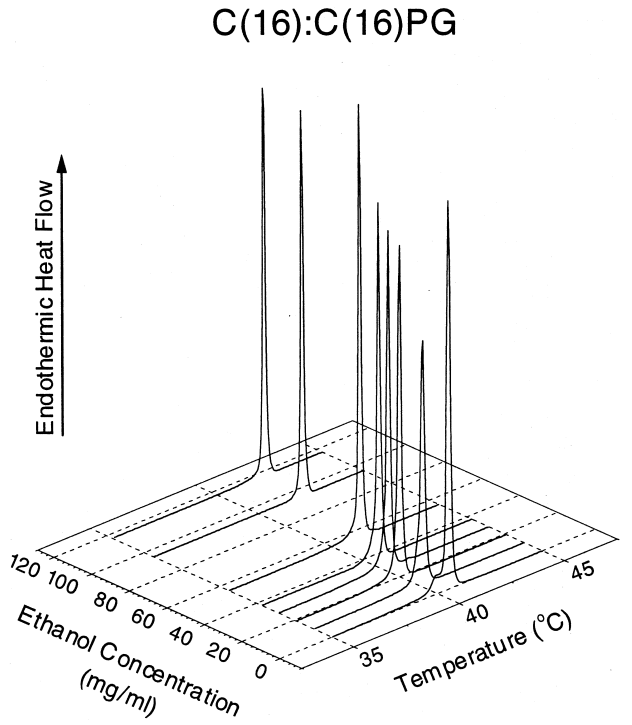

Fig. 4. DSC scans of aqueous dispersions of C(16):C(16)PG in the presence of various concentrations of ethanol. Experimental conditions were exactly as described for Fig. 2.

The DSC scans of C(15):C(17)PG in the absence and presence of ethanol are shown in Fig. 3. In the absence of ethanol, the aqueous dispersion of C(15):C(17)PG exhibits a sharp peak at 41.3°C with a small shoulder on the low-temperature side. In the presence of ethanol, this low-temperature shoulder disappears. The T_m of C(15):C(17)PG decreases with increasing amounts of ethanol up to a concentration of $[EtOH]_{TC} \cong 15$ mg/ml. Above this $[EtOH]_{TC}$, the T_m of C(15):C(17)PG increases with further increases in the ethanol concentration. Fig. 4 shows the DSC scans of C(16):C(16)PG in the presence and absence of ethanol. Similar to the first two lipids discussed, the T_m of C(16):C(16)PG decreases with increasing ethanol concentration until a minimum is reached at $[EtOH]_{TC} \cong 19$ mg/ml. Above $[EtOH]_{TC}$, the T_m of C(16):C(16)PG increases with increasing [EtOH]. For C(17):C(15)PG, shown in Fig. 4, a similar scenario is seen as in the previous three lipids, and the value $[EtOH]_{TC}$ for C(17):C(15)PG is about 36 mg/ml. The phase transition behavior shown by all four lipids discussed thus far (Figs. 2–5) is characteristic of lipids going through an isothermal phase transition from the normal gel phase ($L_{\beta'}$) to the fully interdigitated ($L_{\beta I}$) packing motif under the conditions of $T < T_m$ and $[EtOH] > [EtOH]_{TC}$. Most interestingly, the DSC scans of C(18):C(14)PG (Fig. 6) show only a linear response to ethanol concentrations up to 120 mg/ml. This behavior is associated with highly asymmetric lipids that cannot be induced into the fully interdigitated bilayer by ethanol.

Fig. 7 shows the plot of T_m versus [EtOH] for each of the five PGs studied in this investigation. C(14):C(18)PG, C(15):C(17)PG, C(16):C(16)PG, and C(17):C(15)PG all show biphasic behavior in response to increasing amounts of ethanol; however, C(18):C(14)PG has only a linear response to ethanol concentration. There is an important correlation to note in this plot. Namely, there is a direct relationship between the T_m of each lipid at [EtOH] = 0 mg/ml and the $[EtOH]_{TC}$ of each lipid. With the exception of C(18):C(14)PG, if any two PGs are compared, then the C(*X*):C(*Y*)PG with the *higher* T_m at [EtOH] = 0 mg/ml has a *lower* value for its corresponding $[EtOH]_{TC}$. For example, in the absence of

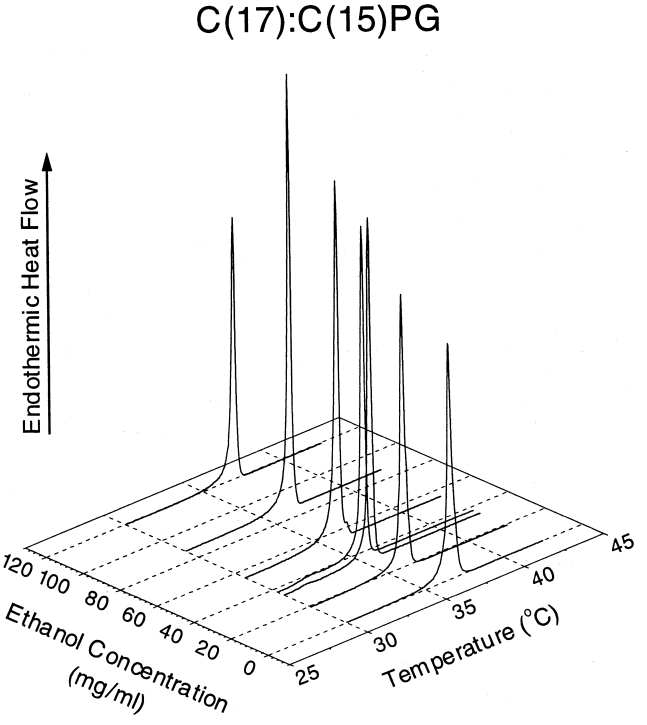

Fig. 5. DSC scans of aqueous dispersions of C(17):C(15)PG in the presence of various concentrations of ethanol. Experimental conditions were exactly as described for Fig. 2.

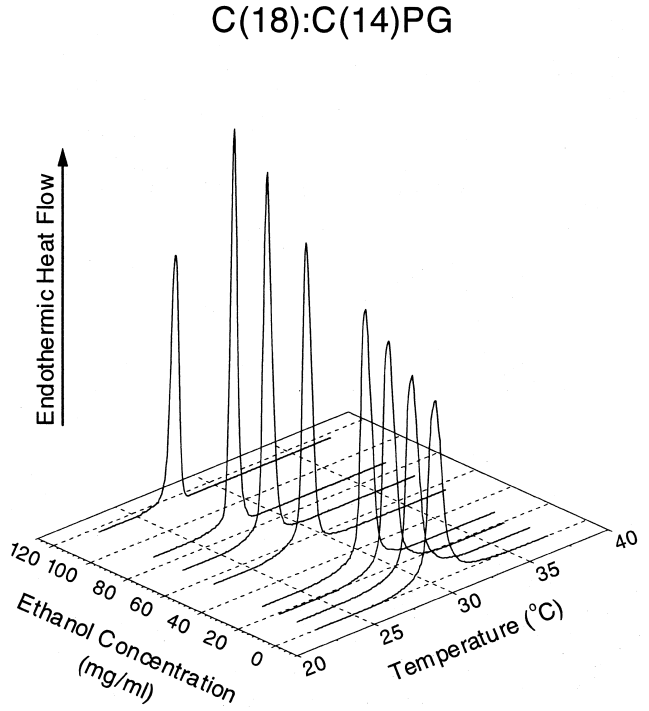

Fig. 6. DSC scans of aqueous dispersions of C(18):C(14)PG in the presence of various concentrations of ethanol. Experimental conditions were exactly as described for Fig. 2.

ethanol, the T_m of C(15):C(17)PG is 41.3°C, and the T_m of C(16):C(16)PG is 40.6°C. The $[EtOH]_{TC}$ of C(15):C(17)PG is 15 mg/ml, and the $[EtOH]_{TC}$ of C(16):C(16)PG is 19 mg/ml. With respect to C(18):C(14)PG, its T_m in the absence of ethanol (30.3°C) is lower than any of the other four lipids studied in this investigation, and it has no $[EtOH]_{TC}$. Both the T_m and the $[EtOH]_{TC}$ of diacyl phospholipids have been shown to be dependent on the acyl chain asymmetry of the phospholipid [3,13], and the ramification of these dependencies with regards to the acyl chain structure of C(X):C(Y)PG will be discussed in the next section.

4. Discussion

In this investigation, we have studied the effect of ethanol on the thermotropic phase behavior of five saturated mixed-chain phosphatidylglycerols in excess buffered aqueous solution. Before we can begin analyzing the implications of the surprising results presented in this investigation, it is important to summarize the basic features of the theoretical framework outlined by Huang and McIntosh regarding the behavior of mixed-chain phosphatidylcholines in the presence and absence of ethanol [3]. The most salient feature of Huang and McIntosh's framework is the impact of ethanol on the free energy of a PC bilayer both in the partially interdigitated $L_{\beta'}$ and in the fully interdigitated $L_{\beta I}$ packing motifs. In the absence of ethanol, phospholipids whose two acyl chains are of similar length assume the $L_{\beta'}$ packing motif because the free energy of the partially interdigitated structure is much less than the free energy of the $L_{\beta I}$ structure. As ethanol concentration is increased, the free energy of the partially interdigitated system increases, whereas the free energy of the fully interdigitated system decreases. At one point, both the $L_{\beta I}$ and the $L_{\beta'}$ packing motifs will have the same free energy, and above this ethanol concentration, the $L_{\beta I}$ motif will be the more stable structure.

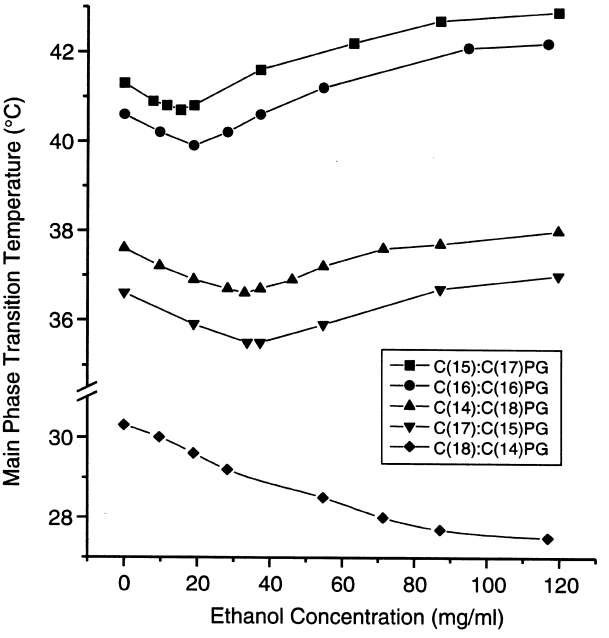

Fig. 7. Plot of T_m as a function of ethanol concentration for five molecular species of C(X):C(Y)PG: C(14):C(18)PG, C(15):C(17)PG, C(16):C(16)PG, C(17):C(15)PG, and C(18):C(14)PG. Four of the lipids shown demonstrate biphasic behavior in the presence of ethanol, whereas one lipid has only a linear response. For the four PGs that show biphasic behavior, the ethanol concentration at which the T_m reaches a minimum is designated as the $[EtOH]_{TC}$. Interestingly, the $[EtOH]_{TC}$ for each PG is dependent on the acyl chain asymmetry. The implications of this observation are outlined in Section 4.

The point at which the two packing motifs have the same free energy is called the threshold concentration of ethanol, or $[EtOH]_{TC}$. If the acyl chain asymmetry of the lipid is increased, then the $L_{\beta I}$ motif will become more unstable – its free energy will increase – because in the fully interdigitated structure, there will be greater exposure of acyl chain hydrocarbon to the aqueous solvent. Since the free energy difference (ΔG) in the absence of ethanol between the $L_{\beta I}$ and the $L_{\beta'}$ motifs will be higher for lipids with higher acyl chain asymmetry, the ethanol concentration at which $G^{L_{\beta'}} - G^{L_{\beta I}}$ for the lipid (i.e. the $[EtOH]_{TC}$) will be higher than that of a lipid with a smaller ΔC. This framework provides a useful means of studying lipids with acyl chains of comparable length, but with unknown ΔC values because the $[EtOH]_{TC}$ for each lipid will be extremely sensitive to the ΔC. If the difference in the number of carbons in the acyl chains of a lipid was very large, then only a linear decrease in the T_m of the lipid will be seen with increasing ethanol concentration. However, if there is only a slight difference in the number of carbon atoms – for example, less than 4 carbon atoms – then a biphasic profile would be expected in the T_m versus [EtOH] plot, and the $[EtOH]_{TC}$ of each lipid would be proportional to the ΔC of that lipid. We can utilize this framework as a virtual assay to determine the relative acyl chain asymmetry of the five C(*X*):C(*Y*)PGs studied in this investigation.

The results of the DSC experiments in this investigation show that C(18):C(14)PG was the only lipid studied that did not have a biphasic profile in the T_m versus [EtOH] plot. Furthermore, C(18):C(14)PG had the lowest T_m in the absence of ethanol among the five lipids. All of the other four lipids showed biphasic behavior in the presence of ethanol. As such, we can conclude that C(18):C(14)PG must have the largest acyl chain asymmetry of the five PGs studied. Among the remaining four lipids, C(15):C(17)PG had the smallest $[EtOH]_{TC}$, followed by C(16):C(16)PG, C(14):C(18)PG, and C(17):C(15)PG (Table 1). Furthermore, it has long been established from previous work in this laboratory that, given two lipids with different acyl chain asymmetries, but with the same total number of carbons, the lipid with the *lower* chain asymmetry will have the *higher* T_m [14]. Under this framework, assuming that the $[EtOH]_{TC}$ of each lipid is directly proportional to the ΔC value, it can be deduced that the order of acyl chain asymmetry from lowest to highest is:

C(15):C(17)PG < C(16):C(16)PG < C(14):C(18)PG < C(17):C(15)PG < C(18):C(14)PG.

Table 1
Values of $[EtOH]_{TC}$ for molecular species of C(*X*):C(*Y*)PG

Lipid	$[EtOH]_{TC}$ (mg/ml)
C(14):C(18)PG	33
C(15):C(17)PG	15
C(16):C(16)PG	19
C(17):C(15)PG	36
C(18):C(14)PG	N/A[a]

Values are estimated from the least-squares polynomial fit of the T_m versus [EtOH] plot for each phospholipid.
[a]N/A: not applicable. For C(18):C(14)PG, only a linear response to increasing ethanol concentration is observed, so no value of $[EtOH]_{TC}$ is reported.

Although the actual value of ΔC for each lipid cannot be determined from these experiments, a fair amount of structural information can be deduced from the relative acyl chain asymmetries of these five PGs. For example, it is very interesting to note that C(15):C(17)PG appears to have a smaller ΔC value than C(16):C(16)PG, based on their respective values of T_m and $[EtOH]_{TC}$. This implies that although C(15):C(17)PG has two more carbons in its *sn*-2 acyl chain than in its *sn*-1 acyl chain, while C(16):C(16)PG has an equal number of carbons in each chain, the distance between the terminal methyl groups of C(15):C(17)PG in the gel phase is less than the distance between the terminal methyls of C(16):C(16)PG. This situation is only possible if some structural feature exists in the hydrated gel phase of C(*X*):C(*Y*)PG which decreases the effective length of the *sn*-2 chain, such as a kink or a bend. Comparing the behavior of C(15):C(17)PG with its positional isomer, C(17):C(15)PG, further illustrates the existence of a kink or bend in the *sn*-2 chain of C(*X*):C(*Y*)PG.

If the acyl chain conformation seen in the single crystal structure of DMPG were similar to the chain structure in the hydrated gel phase, then it would be expected that the acyl chains of C(17):C(15)PG would be more symmetrical than C(15):C(17)PG due to an expected bend in the *sn*-1 chain. This bend would shorten the effective length of the *sn*-1

chain, causing the terminal methyls of C(17):C(15)PG to be closer together than the terminal methyls of C(16):C(16)PG. Also, the bend in the *sn*-1 chain of the single crystal would push the terminal methyls of C(15):C(17)PG farther apart along the long axis of the molecule. If it was assumed that the acyl chain conformation of the hydrated gel phase of C(*X*):C(*Y*)PG is similar to the single crystal structure, it would be expected that C(17):C(15)PG would have a smaller ΔC value than C(16):C(16)PG or C(15):C(17)PG. This would further imply that the $[EtOH]_{TC}$ of C(17):C(15)PG should be lower than the $[EtOH]_{TC}$ of either C(16):C(16)PG or C(15):C(17)PG. However, the data presented in this investigation clearly show that this predicted behavior, which is based on the crystal structure of PG, is nearly opposite to the observed behavior.

In reality, the $[EtOH]_{TC}$ of C(15):C(17)PG is lower – and in parallel, the ΔC of C(15):C(17)PG is deduced to be lower – than that of C(17):C(15)PG. Furthermore, if the gel phase acyl chain structure was similar to that found in the single crystal, then it would be expected that the ΔC (and the $[EtOH]_{TC}$) of C(18):C(14)PG would be lower than that of C(14):C(18)PG. However, results presented in this work clearly show that C(18):C(14)PG does not have an $[EtOH]_{TC}$, since the lipid only shows a linear response to increasing ethanol concentration. The implication of this profile in the T_m versus [EtOH] plot, based on the model outlined above, is that C(18):C(14)PG must have a relatively large ΔC, thus preventing ethanol-induced interdigitation in C(18):C(14)PG bilayers. On the other hand, C(14):C(18)PG has an $[EtOH]_{TC}$ value of 33 mg/ml, indicating that this lipid has a ΔC that must be lower than that of any lipid that cannot be induced by ethanol to interdigitate. Thus, C(14):C(18)PG must have a lower ΔC than C(18):C(14)PG, which is opposite to the prediction made based on the acyl chain conformation of the DMPG crystal structure.

The most important conclusion to be drawn from this investigation is that all predictions of behavior that are based on the crystal structure of DMPG have been proven to be incorrect. This suggests that perhaps the gel phase acyl chain conformation of C(*X*):C(*Y*)PG is different from that found in the single crystal. The crystal structure of DMPG by Pascher and co-workers has an acyl chain conformation that is nearly opposite to the chain conformation found in the single crystal structure of DMPC [6,7]. These different acyl chain structures imply that the behavior of PG and PC in the gel phase should be different as well. However, it is very interesting to note that the trend in the behavior of the five C(*X*):C(*Y*)PGs studied in this investigation is very similar to the trend seen in the corresponding C(*X*):C(*Y*)PCs. For example, C(15):C(17)PC has an $[EtOH]_{TC}$ of about 50 mg/ml, and C(17):C(15)PC has an $[EtOH]_{TC}$ of about 65 mg/ml [15]. The ΔC values for C(15):C(17)PC and C(17):C(15)PC are 0.5 and 3.5 C–C units, respectively.

If it is assumed that the ΔC of C(15):C(17)PG is lower than the ΔC of C(17):C(15)PG, then there must be some structural artifact that draws the terminal methyls of C(15):C(17)PG closer together than the terminal methyls of C(17):C(15)PG. It is important to remember that C(*X*):C(*Y*)PC has a bend in the *sn*-2 chain that effectively shortens the length of this acyl chain, and it is this structural element that causes C(15):C(17)PC to have a smaller ΔC than C(16):C(16)PC or C(17):C(15)PC. Thus, it is reasonable to speculate that perhaps the *sn*-2 chain of *rac*-C(*X*):C(*Y*)PG contains a bend that is similar to the one found in C(*X*):C(*Y*)PC, and the *sn*-1 chain of C(*X*):C(*Y*)PG must be in the all-*trans* conformation, just as in C(*X*):C(*Y*)PC. It is theoretically possible that both chains in C(*X*):C(*Y*)PG might contain some kinks or bends, but if this were the case, then the area per lipid in the bilayer plane would be much larger due to the larger volume occupied by multiple kinks in the acyl chains. This larger volume would then lead to an increased randomness of the lipid's acyl chains in the gel state bilayer, since there would be less interchain van der Waals contacts and greater rotational freedom for the dihedral angles of the chains at $T < T_m$. The increased entropy or randomness at $T < T_m$ would be detected by calorimetric measurements as a broadened peak during the gel-to-liquid crystalline phase transition.

It is obvious from the DSC scans in this report that the peaks are indeed very sharp for C(*X*):C(*Y*)PG both in the absence and presence of ethanol, thus ruling out the possibility of there being multiple kinks in both chains. The most logical explanation for the observed behavior of C(*X*):C(*Y*)PG liquid dispersions is that the structural motif of the acyl

chains at $T < T_m$ must be similar in nature to that in C(X):C(Y)PC. This would corroborate the argument of Findlay and Barton that the similarity in the behavior of identical-chain PC and PG is due to common structural features [16]. It would also corroborate the observation of Seelig that the headgroup structure and motional properties of PC and PG are very similar [17,18]. It is important to point out that nearly all work previously done on PG has focused exclusively on identical-chain PGs. This present investigation constitutes the first study reported on saturated mixed-chain PGs, and we can now say that the similarity in PC and PG behavior extends to all acyl chain compositions of these lipids, not merely to identical-chain lipids. Clearly the most salient difference in the experimental conditions of the single crystal and the gel phase is the hydration of the system. The results of this work, as well as other results from this laboratory [19], seem to indicate that the influence of hydration on the structural arrangement of *rac*-C(X):C(Y)PG in a bilayer is very important. Indeed, it would seem that hydration plays a less influential role with regard to C(X):C(Y)PC than with C(X):C(Y)PG. The most likely explanation for this dichotomous nature of the influence of hydration on PC and PG involves the composition of the headgroups of PC and PG.

PC has a zwitterionic headgroup that has no hydrogen-bond donor, whereas PG contains an anionic headgroup with two free hydroxyl groups that have a strong tendency to form hydrogen bonds with neighboring molecules [20]. In the presence of excess water, PG will likely form hydrogen bonds with water molecules; however, in the single crystal, where there is little or no water present, PG will have a tendency to hydrogen bond with other PG molecules. Zhang et al. found that upon low-temperature incubation, identical-chain PGs form quasi-crystalline polymorphs in which the hydroxyl groups of PG seem to hydrogen bond with the polar phosphate moiety of adjacent PG molecules [21]. This large tendency to hydrogen bond could explain the difference in the acyl chain conformation of PG in the presence and absence of water.

Some mention should be made of the fact the values of $[EtOH]_{TC}$ for each C(X):C(Y)PG are far less than those of the corresponding C(X):C(Y)PC. The decreased value in $[EtOH]_{TC}$ indicates that the point at which $G^{L_{\beta'}} - G^{L_{\beta I}}$ has been reached at a lower [EtOH] for PG than for PC. This situation can arise under three possible conditions: (1) the free energy of the $L_{\beta'}$ phase is greater for PG than for PC; (2) the free energy of the $L_{\beta I}$ is less for PG than for PC; or (3) both (1) and (2) are true. It is our speculation that the most likely answer is that the third option is correct: that both $G_{PC}^{L_{\beta'}} < G_{PG}^{L_{\beta'}}$ and $G_{PC}^{L_{\beta I}} > G_{PG}^{L_{\beta I}}$. The source for both of these conditions is the same, namely, the charge repulsion in the PG bilayer. PG is an anionic phospholipid with a negative charge on the phosphate moiety. In a partially interdigitated bilayer, where headgroup–headgroup separation distance is the lowest among all packing motifs, there is discernible charge repulsion in a pure PG system [21–23]. As such, the charge repulsion would be relieved if the bilayer could be induced into an interdigitated gel state.

When the lipid goes through an isothermal phase transition to the $L_{\beta I}$ phase, there is a great increase in the headgroup–headgroup separation distance, and this would reduce the electrostatic repulsion between PG molecules. Furthermore, the fact that ethanol can form hydrogen bonds, and the fact that X-ray studies have found that ethanol 'binds' to interdigitated bilayers at the interfacial region [2], indicates that there is a high probability that ethanol forms a hydrogen bonding network with the headgroups of PG molecules in the fully interdigitated phase, further stabilizing the $L_{\beta I}$ phase and thus considerably lowering the free energy ($G^{L_{\beta I}}$) of PG. No such hydrogen bonding is found between the headgroup of PC and ethanol [24]. For this reason, ethanol can induce PG bilayers to undergo an isothermal phase transition from the $L_{\beta'}$ to the $L_{\beta I}$ phase at a much lower concentration than is needed to induce PC bilayers into interdigitation. The conclusions drawn from this investigation regarding the acyl chain conformation of C(X):C(Y)PG and the influence of charge repulsion on the behavior of PC and PG bilayers have significant implications with regard to the model of behavior of biological membranes as well as the interaction of membrane proteins with C(X):C(Y)PG. It will be very interesting to pursue greater insight into the interaction of binary mixtures of mixed-chain C(X):C(Y)PGs to further elucidate the interaction of multiple biological membrane components.

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