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Role of cholesterol in the modulation of interdigitation in phosphatidylethanols

Olga P. Bondar [a,b], Elizabeth S. Rowe [a,b,*]

[a] *Veterans Affairs Medical Center, Kansas City, MO 64128, USA*
[b] *Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66103, USA*

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Abstract

Phosphatidylethanol (Peth) is formed in biological membranes when ethanol replaces water in the transphosphatidylation reaction catalyzed by phospholipase D. This charged lipid accumulates in the presence of ethanol, and it has unusual properties that can influence membrane structure and function. We have previously shown that dimyristoylphosphatidylethanol (DMPeth) and dipalmitoylphosphatidylethanol (DPPeth) form the interdigitated gel phase in the presence of Tris–HCl [O.P. Bondar, E.S. Rowe, Biophys. J., 71 (1996) 1440–1449]. In the present investigation, differential scanning calorimetry (DSC) and fluorescence have been used to investigate the effect of cholesterol on the phase behavior of DPPeth and DMPeth. Our results show that cholesterol prevents the formation of the interdigitated phase in the presence of Tris–HCl, and that ethanol counters this influence and restores the ability of these lipids to interdigitate. Pyrene-PC fluorescence probe was used in this investigation and gave results that were in agreement with the conclusions based on the DSC study. © 1998 Elsevier Science B.V.

Keywords: Cholesterol; Interdigitation; Lipid phase behavior; Differential scanning calorimetry; Pyr-PC fluorescence

1. Introduction

Phosphatidylethanol (Peth) is an anionic phospholipid that has been found in a wide variety of tissues in the presence of ethanol [1–5]. Ethanol replaces water as a substrate for transphosphatidylation catalyzed by phospholipase D, and diverts phospholipase D activity from phosphatidic acid formation to the formation of Peth. Peth is a charged lipid that accumulates in membranes and can alter the physical properties of the membranes that control such functions as aggregation, fusion and transport processes [6–8]. Understanding the interactions of Peth with other membrane components such as proteins and cholesterol is necessary to elucidate its effects on

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPeth, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol (sodium salt); DMPeth, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanol (sodium salt); DPPG, 1,2-dipalmitoyl-*sn*-glycero-3 [phospho-*rac*-(1-glycerol)] (sodium salt); DHPC, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; Pyr-PC, 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine; Peth, phosphatidylethanol; PC, phosphatidylcholine; PG, phosphatidylglycerol; MLV, multilamellar vesicles; T_m, phase transition midpoint

* Corresponding author. VA Medical Center, 4801 Linwood Blvd., Kansas City, MO 64128, USA. Fax: +1-816-861-1110; E-mail: erowe@kuhub.cc.ukans.edu

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membrane functions. Many lipid compositional changes, including changes in cholesterol content and distribution, have been shown to occur in animal models of alcoholism [9–13]. Investigation of the interactions of Peth with cholesterol will help to elucidate these compositional adaptations to the chronic presence of alcohol.

The role of cholesterol in the function and structure of membranes has been the subject of numerous investigations using a wide variety of methods [14–19]. These studies have shown that cholesterol has profound effects on the physical properties of bilayers, suggesting that it plays a major role in the regulation of the properties of biological membranes. Although most of the studies have focused on PCs, there have been some studies of other lipid classes, including charged lipids. Some previous studies have shown that the miscibility of cholesterol in bilayers of negatively charged phospholipids is limited and strongly dependent on the acyl chain length and charge of the phospholipid [20–24]. For DPPG, it was shown that the miscibility of cholesterol in this phospholipid is significantly lower than for the corresponding PC bilayers and decreases with increasing acyl chain length of the phospholipid [23]. Recent studies on the effect of acyl chain length of PCs on the miscibility of cholesterol also show chain length dependence [18,19]. These studies all indicate that cholesterol interactions vary with individual lipid species, suggesting that cholesterol may play a role in the lateral distribution of lipid species or domains. These studies demonstrate the importance of understanding the interactions of cholesterol with particular lipid species such as the Peths.

Individual phospholipids exist in a variety of phase structures when examined in model membranes of simple compositions; their phase behavior provides information about the contributions of each lipid to the physical properties of biological membranes. Among the interesting phase structures that synthetic lipids can adopt is the fully interdigitated gel phase. This phase has now been observed in a variety of lipid classes. It occurs in like chain PCs in the presence of a variety of solvents including ethanol [25–33], and in dihexadecylphosphatidylcholine (DHPC) and 1,3-DPPC in the absence of any additives [26,34–37]. It also occurs in dipalmitoylphosphatidylglycerol (DPPG) in the presence of Tris–HCl, as observed by X-ray diffraction and fluorescence [38]. The role of cholesterol in lipid phase behavior has been studied for a variety of lipids, and it has been shown to abolish the interdigitated gel phase in DPPC and in DHPC [37,39].

We have been studying the physical properties of DMPeth and DPPeth, and have demonstrated that both of these lipids are interdigitated in the presence of Tris–HCl [40]. In the present report, we describe the effects of cholesterol on the behavior of DMPeth and DPPeth as a function of solvent conditions including ethanol, using DSC and Pyrene-PC fluorescence.

2. Materials and methods

2.1. Chemicals

The dipalmitoylphosphatidylethanol (DPPeth), dimyristoylphosphatidylethanol (DMPeth) dipalmitoylphosphatidylglycerol (DPPG), and dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti, Birmingham, AL. Ethanol was purchased from PhaRmco, Linfield, PA. The fluorescence probe 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine (Pyr-PC) was obtained from Molecular Probes (Eugene, OR). Cholesterol was purchased from Sigma Chemical (St. Louis, MO).

2.2. Sample preparation

The multilamellar liposomes were prepared using Bangham's method [41]. Chloroform stock solutions of lipids or lipid:probe were dried under a gentle stream of nitrogen gas and then kept overnight on a vacuum pump to remove all residual chloroform. Distilled water or 50 mM Tris–HCl buffer was added to the dry film, and the stock suspensions were hydrated at a temperature above the temperature of the phase transition for at least 2 h with occasional vortexing. Lipid concentrations were determined by the Bartlett's method [42].

2.3. High-sensitivity differential scanning calorimetry

DSC was performed using the MC-2 scanning calorimeter from Microcal, Amherst, MA. The

calorimeter is interfaced with an IBM computer, and the software used is that provided by Microcal. A Haake refrigerated bath controlled by the computer is connected to the calorimeter for temperature control during cooling scans. The scan rates were 20°C/h for both heating and cooling scans. The final concentrations of lipids for DSC studies were from 2.0 to 4.0 mM. The data were analyzed using ORIGIN software from Microcal, Amherst, MA.

2.4. Fluorescence measurements

The fluorescence experiments were performed using the SLM 8300 spectrofluorometer. The temperature of samples was monitored with an Instrulab Model 700 digital thermometer, with the thermistor placed in a parallel reference cuvette. The sample was magnetically stirred during the measurements. For Pyr-PC fluorescence studies, MLVs were used, prepared as described above. The total lipid and probe concentrations in all Pyr-PC experiments were 0.12–0.13 mM and 1.2 μM, respectively, giving a lipid:probe ratio of 1:100. The sample solution, 3.0 ml, was placed in a fluorescence cuvette, and the cuvette was then sealed with the silicon cap. The sample was deaerated by flushing nitrogen gas through the sample solution for about 10 min. All low temperature measurements were protected from condensation by dry nitrogen. The sample was cooled to about 4°C prior to the addition of ethanol. The excitation wavelength was 325 nm, and the emission spectrum was scanned in increments of 0.1 nm. The excitation and emission slits were 16 nm and 2 nm, respectively. A control sample prepared without a fluoroprobe has less than 1% scattering signal for all fluorescence experiments.

3. Results

As we have shown previously, both DMPeth and DPPeth in 50 mM Tris–HCl exist in the interdigitated phase, and the effect of ethanol is to enhance the tendency to interdigitate [40]. In the present investigation, we have extended our characterization of the Peths to determine the effect of cholesterol on the phase behavior and phase structure of DPPeth and DMPeth in the presence and absence of ethanol. In order to conduct a thorough investigation, we used two methods, DSC and Pyr-PC fluorescence, and compared the results with those of other lipids where the structures have been determined using independent methods such as X-ray diffraction.

3.1. Differential scanning calorimetry

Fig. 1 shows the series of DSC heating scans of DMPeth in 50 mM Tris–HCl as a function of cholesterol content, showing a broadening and a slight decrease in transition temperature, accompanied by a decrease in area with increasing cholesterol content. At 50 mol% cholesterol, there is still a small peak at the lipid phase transition, and a new peak representing separation of pure cholesterol appears at 32°C. The cooling transitions were similar, and are not shown.

The DSC heating scans for DPPeth are shown in Fig. 2. As reported previously, DPPeth exhibits a high temperature shoulder in 50 mM Tris–HCl in the absence of cholesterol [40]. This shoulder disappears at 10 mol% cholesterol. Increasing cholesterol con-

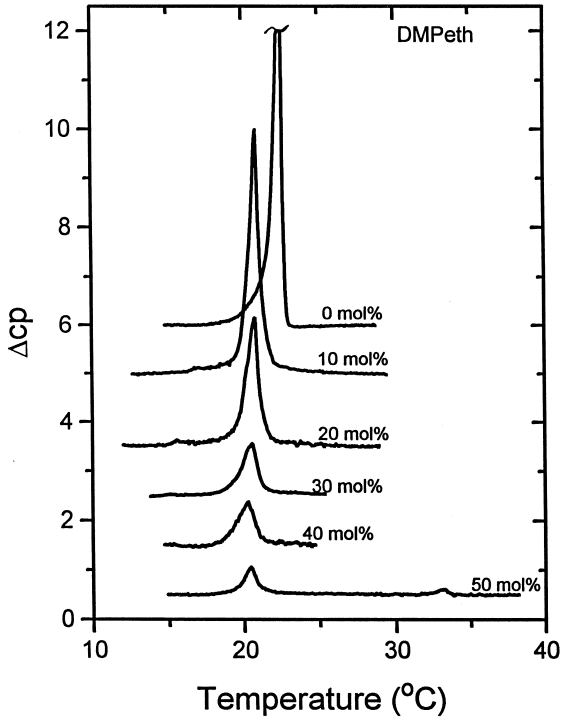

Fig. 1. DSC scans of DMPeth MLVs in 50 mM Tris–HCl, pH 7.4 in the presence of increasing concentrations of cholesterol. The concentrations of cholesterol are indicated on the curves.

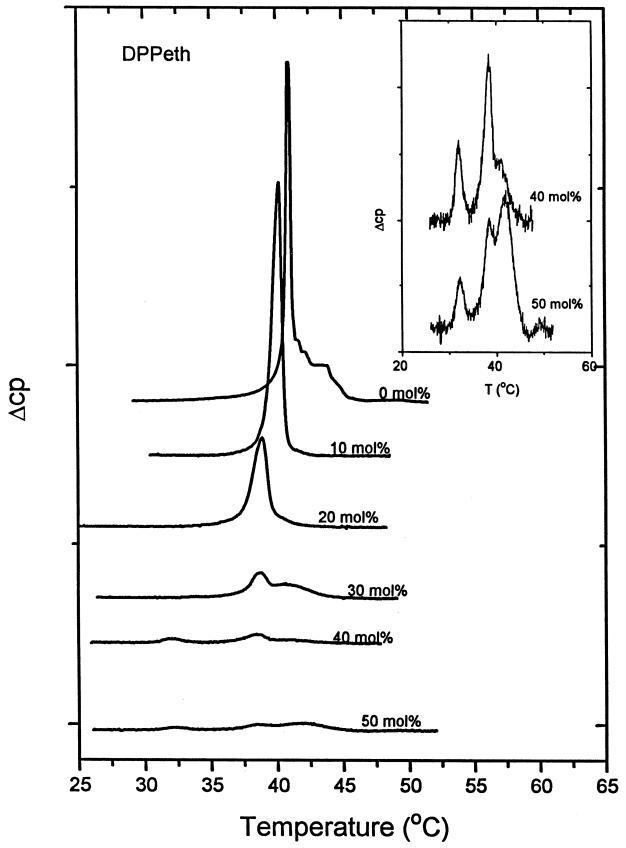

Fig. 2. DSC scans of DPPeth MLVs in 50 mM Tris–HCl, pH 7.4 in the presence of increasing concentrations of cholesterol. The concentrations of cholesterol are indicated on the curves.

tent progressively reduces the area, and broadens the transition. At 30 mol% cholesterol, the transition has a high temperature shoulder. At 40 mol% cholesterol the transition is very small, but on the expanded scale, shows the resolution of a pure cholesterol transition at 37°C [21] as well as the two-component main transition. At 50 mol% cholesterol, there is still a discernable series of transitions.

The temperatures for both heating and cooling scans for DMPeth are plotted in Fig. 3A as a function of cholesterol. Similar experiments were done in 100 mg/ml ethanol, and are also shown. Hysteresis of the main phase transition has been well established and indicates the presence of interdigitated gel phase [43,44]. As in our previous study, there is some hysteresis in the absence of cholesterol, suggesting the presence of the interdigitated phase. As shown in Fig. 3A, the hysteresis is greatly reduced at 5 mol% cholesterol and has completely disappeared at 10 mol% cholesterol, suggesting that interdigitation has been abolished by the presence of cholesterol. Also shown in Fig. 3A are the results in the presence of 100 mg/ml ethanol, showing that there is a large hysteresis present at all concentrations of cholesterol, suggesting that all of the lipid that participates in the transition is in the interdigitated phase.

The effect of cholesterol on the phase transition enthalpy of DMPeth is shown in Fig. 3B. The enthalpy decreases with cholesterol concentrations up to 30 mol%. In the presence of 100 mg/ml ethanol, the enthalpy decreased gradually until 20 mol% of cholesterol, and then decreased more rapidly. This suggests that the fraction of lipid participating in the phase transition decreases with increasing cholesterol concentration. The greater enthalpy in the presence of

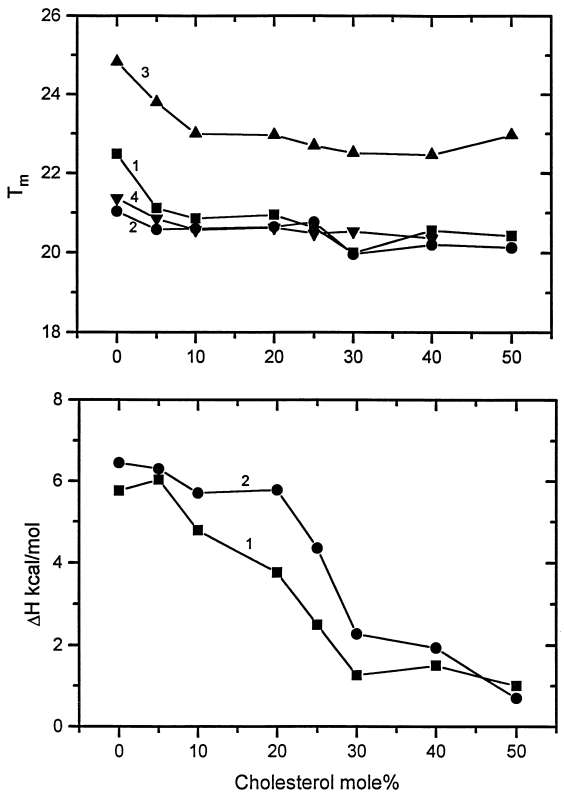

Fig. 3. Effect of the cholesterol on the temperature (A) and enthalpy (B) of the main phase transition of DMPeth (50 mM Tris–HCl pH 7.4) in the presence and absence of ethanol: (A) Effect of cholesterol on the temperature of the main phase transition of DMPeth (1: heating scan; 2: cooling scan; 3: heating scan in the presence of 100 mg/ml ethanol; 4: cooling scan in the presence of 100 mg/ml ethanol); (B) Effect of cholesterol on the enthalpy of the main phase transition of DMPeth (1: heating scan; 2: heating scan in the presence of 100 mg/ml ethanol).

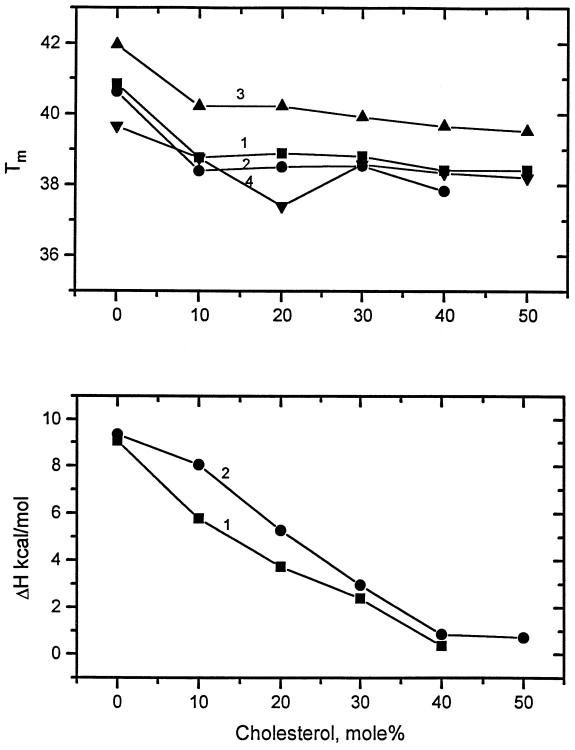

Fig. 4. Effect of the cholesterol on the temperature (A) and enthalpy (B) of the main phase transition of DPPeth (50 mM Tris–HCl pH 7.4) in the presence and absence of ethanol: (A) Effect of cholesterol on the temperature of the main phase transition of DPPeth (1: heating scan; 2: cooling scan; 3: heating scan in the presence of 100 mg/ml ethanol; 4: cooling scan at the presence of 100 mg/ml ethanol); (B) effect of cholesterol on the enthalpy of the main phase transition of DMPeth (1: heating scan; 2: heating scan in the presence of 100 mg/ml ethanol).

ethanol for 10 mol% to 30 mol% of cholesterol may be due to a larger enthalpy for transition in the presence of ethanol, a greater proportion of the lipid participating in the transition, or both.

Fig. 4A,B show the corresponding data for DPPeth in 50 mM Tris–HCl. As seen in Fig. 4A, there is a slight decrease in T_m for both heating and cooling scans as a function of cholesterol concentration and no hysteresis. Beginning at 40 mol% of cholesterol, we observed a separation of the phases and an additional peak appeared at the melting temperature of cholesterol [21]. As in the case of DMPeth, adding 100 mg/ml of ethanol led to hysteresis in the main phase transition, consistent with interdigitation of the lipid bilayer [43]. The enthalpy of the main transition decreased for all concentrations of cholesterol in the absence and presence of ethanol. As with DMPeth in the presence of ethanol, the enthalpy of the transition was greater at intermediate cholesterol contents.

To determine the ethanol dependence of the induction of interdigitation in the Peth–cholesterol system, we studied DMPeth and DPPeth MLVs with 20 mol% cholesterol in Tris–HCl buffer. As shown in Fig. 5A for DMPeth, significant hysteresis in the main transition appears by 30 mg/ml ethanol. Increasing concentrations of ethanol led to an increase in the amplitude of hysteresis. Fig. 5B shows the effect of ethanol on the enthalpy of the transition of DMPeth–cholesterol liposomes in Tris–HCl buffer. The enthalpy increased with increasing concentration of ethanol both for heating and cooling scans.

Similar results were obtained for DPPeth with 20 mol% cholesterol. As seen in Fig. 6A, increasing the concentration of ethanol increases the difference of the T_m for heating and cooling scans. This suggests

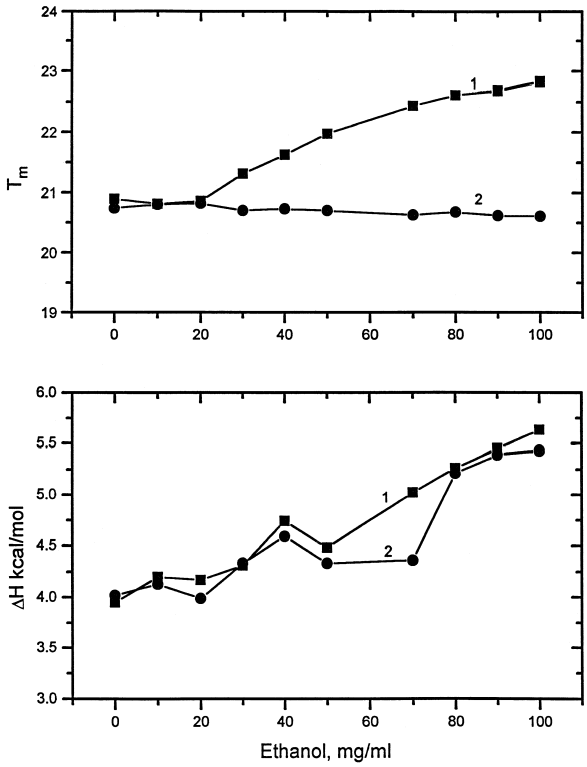

Fig. 5. Effect of ethanol on the temperature (A) and enthalpy (B) of the main phase transition of DMPeth–20 mol% cholesterol in 50 mM Tris–HCl, pH 7.4: (A) Effect of ethanol on the temperature of the main phase transition of DMPeth–20 mol% cholesterol (1: heating scan; 2: cooling scan); (B) effect of ethanol on the enthalpy of the phase transition of DMPeth–20 mol% cholesterol (1: heating scan; 2: cooling scan).

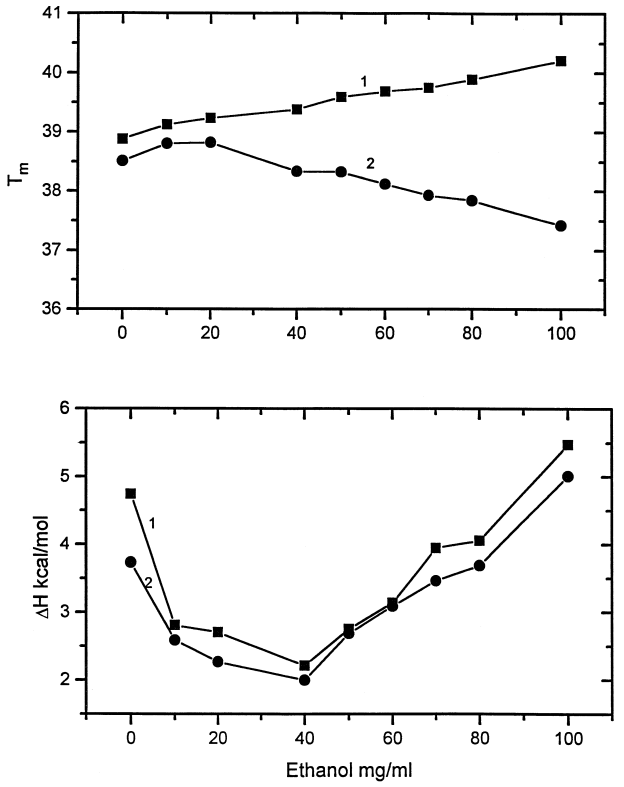

Fig. 6. Effect of ethanol on the temperature (A) and enthalpy (B) of the main phase transition of DPPeth–20 mol% cholesterol in 50 mM Tris–HCl, pH 7.4: (A) Effect of ethanol on the temperature of the main phase transition of DPPeth–20 mol% cholesterol (1: heating scan; 2: cooling scan); (B) effect of ethanol on the enthalpy of the phase transition of DPPeth–20 mol% cholesterol (1: heating scan; 2: cooling scan).

that DPPeth with 20 mol% of cholesterol is also interdigitated with more than 30 mg/ml ethanol. The behavior of the enthalpy of the transition for DPPeth was different than for the DMPeth-cholesterol vesicles. As shown in Fig. 6B, increasing the concentration of ethanol caused a decrease in enthalpy up to 20 mg/ml ethanol followed by an increase above 40 mg/ml.

3.2. Fluorescence studies

In order to confirm the DSC results on the influence of cholesterol on the interdigitated gel phase of both Peths in Tris–HCl, and the possibility of restoring interdigitation by ethanol, we used Pyr-PC fluorescence measurements that has been previously used to monitor the transition to the interdigitated phase in PCs [37]. The fluorescence spectrum of Pyr-PC has five main peaks at 376.5, 382.5, 387.5, 393.0 (shoulder), and 397.0 nm, which reflect the environment of this probe in the phospholipid bilayers. The intensity ratio $F_{387.5}$(III):$F_{376.5}$(I) is used to quantify the degree of polarity of the probe environment. According to previous studies a low value of the III:I ratio corresponds to a high level of polarity. This method was used previously in our laboratory to identify the interdigitated phase in PC and PC-cholesterol mixtures [37].

Fig. 7 demonstrates the behavior of Pyr-PC in lipids with phase behavior known from X-ray diffraction [29,37]. Fig. 7A shows the behavior of the III:I ratio during the heating of DPPC in water and DPPG in 50 mM Tris–HCl in the presence and absence of ethanol. As seen here, the III:I ratio shows no inflection at the pretransition (32°C) and main transition temperatures (41°C) for DPPC in the non-interdigitated bilayer phases. However, when the transitions involve the interdigitated phase, i.e., for DPPC the $L_{\beta'}$ to L_βI phase transition (12°C) and the main L_βI to L_α phase transition (41°C), as shown by X-ray diffraction [29], significant changes in the III:I ratio occur. These results were previously interpreted to indicate that the probe is forced into a more polar environment in the interdigitated phase, whereas in the bilayer phases, it remains buried in the bilayer and is not sensitive to the phase structure [37]. In the case of the negatively charged DPPG, which, in Tris–HCl is already in the interdigitated phase, as shown by X-ray diffraction, the behavior of the III:I ratio during the main transition is similar to that of this ratio for DPPC in the presence of ethanol. Adding ethanol to DPPG did not change the behavior of the III:I ratio, other than giving a slightly lower polarity above the main transition. Fig. 7B shows DPPG with 5 mol% cholesterol. Cholesterol broadened and decreased the magnitude of the change in the III:I ratio of the probe at the transition, but 100 mg/ml ethanol restored a steeper increase in the III:I ratio near the temperature of the main phase transition. Fig. 7C shows DPPG with 20 mol% cholesterol. These data indicate the elimination of the interdigitation of DPPG in Tris–HCl in the presence of 20 mol% cholesterol. Adding ethanol did not change the effect of cholesterol significantly. The data that were obtained for DPPG are similar to the results that were reported by

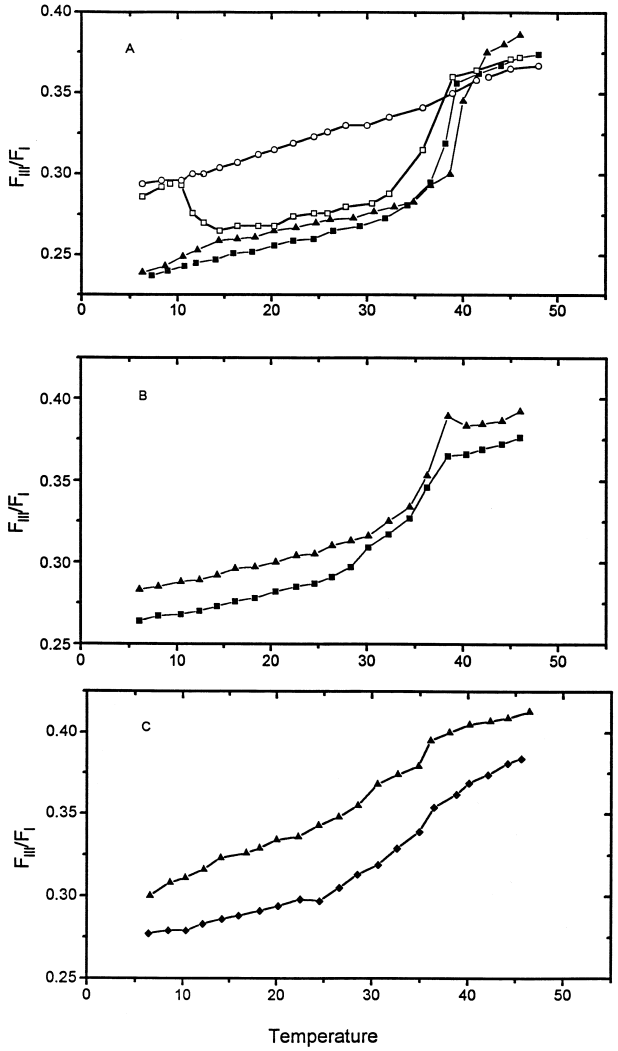

Fig. 7. III:I ratios for Pyr-PC as function of temperature in DPPG MLVs in 50 mM Tris–HCl, pH 7.4, and DPPC MLVs in water: (A) DPPC and DPPG MLVs (○: heating sequence without ethanol for DPPC; □: heating sequence with 100 mg/ml ethanol for DPPC; ■: heating sequence without ethanol for DPPG; ▲: heating sequence with 100 mg/ml ethanol for DPPG). (B) DPPG with 5 mol% cholesterol MLVs (■: heating sequence without ethanol; ▲: heating sequence with 100 mg/ml ethanol). (C) DPPG with 20 mol% cholesterol MLVs (■: heating sequence without ethanol; ▲: heating sequence with 100 mg/ml ethanol).

our laboratory earlier for DPPC cholesterol mixtures [37], in demonstrating the elimination of interdigitation by cholesterol.

The results presented in Fig. 8A show the changes in the III:I ratio with temperature for DMPeth in Tris–HCl buffer, in the presence and absence of 100 mg/ml ethanol. In the absence of ethanol, there is a change in the III:I ratio in the region of the main transition. In the presence of ethanol, the transition has a greater magnitude of change and becomes steeper. This is similar to the DPPG behavior and is consistent with interdigitation in Tris–HCl alone, which is enhanced in the presence of ethanol. Fig. 8B shows the effect of 5 mol% cholesterol on these transitions. The magnitude of the transition in Tris–HCl in the absence of ethanol is less than with no

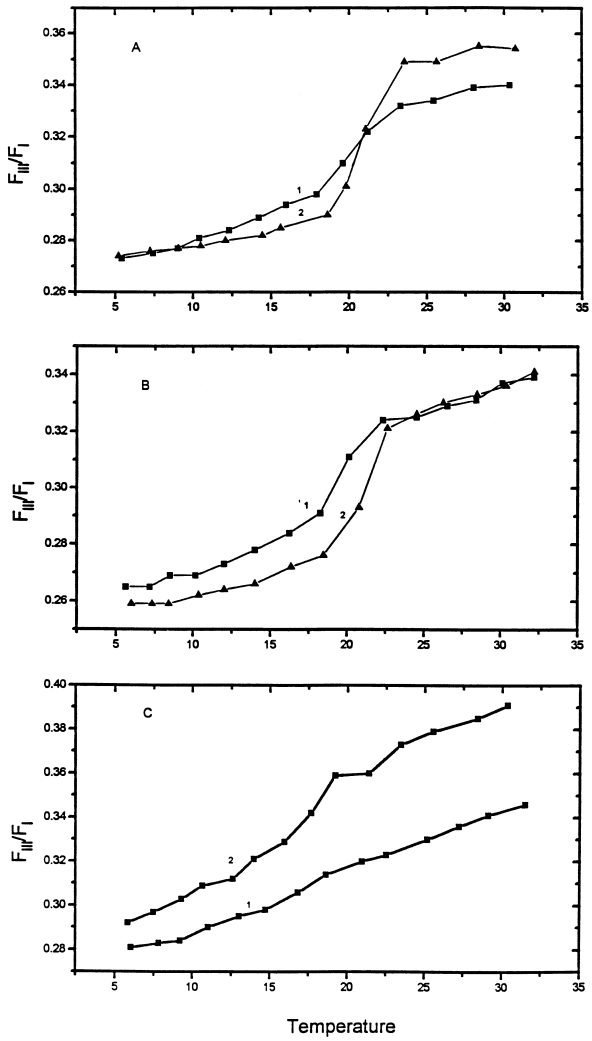

Fig. 8. III:I ratios for Pyr-PC as function of temperature in DMPeth MLVs in 50 mM Tris–HCl pH 7.4: (A) DMPeth MLVs (1: heating sequence without ethanol; 2: heating sequence with 100 mg/ml ethanol). (B) DMPeth–5 mol% cholesterol MLVs (1: heating sequence without ethanol; 2: heating sequence with 100 mg/ml ethanol). (C) DMPeth–20 mol% cholesterol MLVs (1: heating sequence without ethanol; 2: heating sequence with 100 mg/ml ethanol).

cholesterol, but it is still discernable. This suggests that some portion of the lipid is interdigitated. Again, the presence of ethanol produces a clear transition, indicating an increase in the proportion of the lipid that is interdigitated. Fig. 9C shows the results for 20 mol% cholesterol. Here, there is no transition observed in the absence of ethanol. This is consistent with the DSC results, showing that there is no interdigitation under these conditions. Again, the addition of ethanol provides a weak transition, consistent with the DSC results, which indicate that the small amount of the lipid that undergoes a transition is interdigitated. The values of the III:I ratios observed in the presence of cholesterol and ethanol are consistent with a non-interdigitated bulk phase.

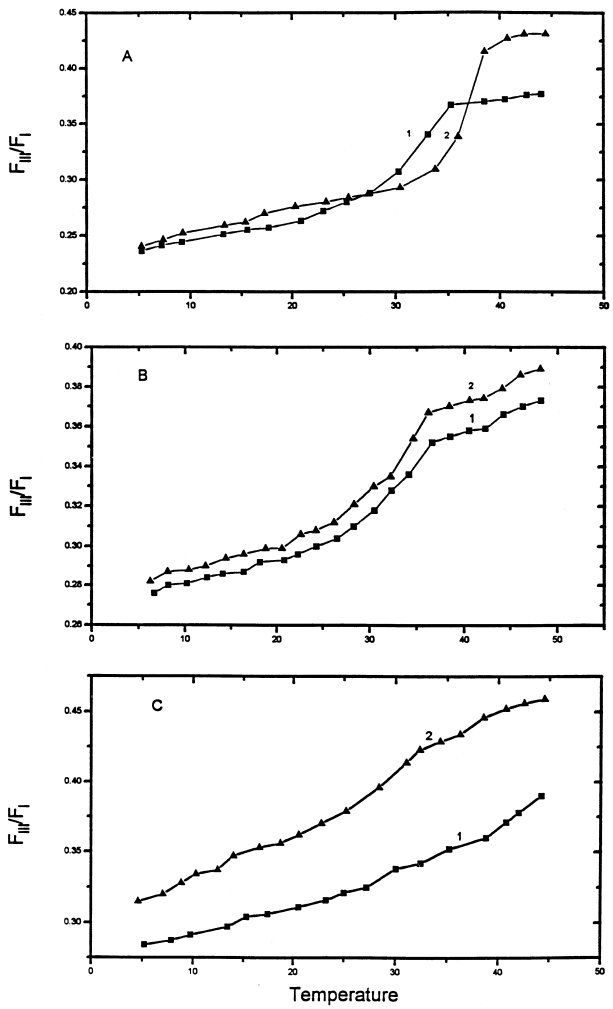

Fig. 9. III:I ratios for Pyr-PC as function of temperature in DPPeth MLVs in 50 mM Tris–HCl: (A) DPPeth MLVs (1: heating sequence without ethanol; 2: heating sequence with 100 mg/ml ethanol). (B) DPPeth–5 mol% cholesterol MLVs (1: heating sequence without ethanol; 2: heating sequence with 100 mg/ml ethanol). (C) DPPeth–20 mol% cholesterol MLVs (1: heating sequence without ethanol; 2: heating sequence with 100 mg/ml ethanol).

The data for DPPeth are shown in Fig. 9; they are similar to the DMPeth results. The Pyr-PC results for both DMPeth and DPPeth are consistent with the DSC results, indicating that interdigitation occurs in the presence of cholesterol in those lipids that undergo the phase transition. In addition, the values of the III:I ratio observed for the bulk lipid indicate that the lipids that do not undergo the transition, i.e., those in the cholesterol-rich regions, are not interdigitated also.

4. Discussion

In our recent investigation of the physical properties of the phosphatidylethanols DMPeth and DPPeth, we showed that these lipids form an interdigitated gel phase in the presence of 50 mM Tris–HCl, and that the tendency to form the interdigitated phase was enhanced by the presence of ethanol [40]. It was also shown that DPPG behaved similarly to the Peths in that its interdigitation in Tris–HCl, previously known from X-ray diffraction [38], was also enhanced by ethanol. In the present work, we have investigated the effects of cholesterol in this system, and shown that cholesterol prevents the formation of the interdigitated phase in DMPeth and DPPeth in the presence of 50 mM Tris–HCl. The addition of ethanol restores the interdigitated phase in the presence of cholesterol for the portion of the lipid that undergoes a transition. In the present investigation, DSC and Pyr-PC fluorescent probe gave similar results.

4.1. Differential scanning calorimetry (DSC)

It is well established that hysteresis, i.e., a difference in the main phase transition temperature between heating and cooling scans, is a reliable indicator of the presence of interdigitation in the gel phase, so that the main transition represents the transition from the interdigitated $L_{\beta}I$ phase to the uninterdigitated L_{α} phase [43,44]. The DSC results indicate that interdigitation in the presence of Tris–HCl is pre-

vented at cholesterol compositions as low as 10 mol%, in both DMPeth and DPPeth. The addition of 100 mg/ml ethanol restores interdigitation at all cholesterol compositions where the main phase transition is observed. Using the 20 mol% cholesterol composition, the ethanol concentration dependence of the induction of interdigitation showed that interdigitation appeared at approximately 20 mg/ml ethanol for both DMPeth and DPPeth. This behavior is similar to that previously observed in the DPPC–cholesterol system [37].

The effect of cholesterol on the enthalpy of the transitions indicated a broadening transition, with enthalpy decreasing with increasing cholesterol. This behavior is similar to the effect of cholesterol in other lipids, in which cholesterol removes lipid from the transition, eventually abolishing it. For DMPeth, a small transition was still observed at 50 mol% cholesterol; for DPPeth, there was also a very small transition remaining at 50 mol% cholesterol. This is in contrast to the PCs, for which the transition is abolished completely at 50 mol% cholesterol [18]. This may be explained by the different head group structure of the Peths, which may affect the miscibility of cholesterol in the lipid. It was previously shown that DPPG, also a charged lipid, also exhibited detectable phase transitions above 50 mol% cholesterol [23].

The observed enthalpy of the transition is in part a measure of the proportion of lipid that is available to participate in the phase transition. The results presented here indicate that cholesterol reduces the tendency of DPPeth and DMPeth in the low-cholesterol range to be interdigitated in the presence of Tris–HCl. In the absence of ethanol, the lipid that undergoes the transition is not interdigitated above 10 mol% cholesterol, although the total enthalpy measurement suggests that as much as 75% of the lipid is in the low-cholesterol regions that undergo the transition. This indicates that the Tris-induced interdigitated state is highly cooperative. In the presence of 100 mg/ml ethanol, all of the lipid that undergoes the transition is interdigitated, and the enthalpy of the transition at the intermediate cholesterol concentrations is higher than in the absence of ethanol. This is also demonstrated in the effect of increasing ethanol on the enthalpy of the transition of the 20 mol% cholesterol mixtures. This greater enthalpy arises from two sources. The first is the greater enthalpy of the $L_{\beta}I$ to L_{α} transition compared to the transition to L_{α} from the non-interdigitated phase [40]. The second is a possible shift of the equilibrium between the low cholesterol and high cholesterol regions due to the reduced miscibility of cholesterol in the interdigitated gel compared to the non-interdigitated gel, so that more of the lipid is in the low-cholesterol regions in the presence of ethanol.

4.2. Fluorescence results

In Pyr-Pc, the chromophore is located at the end of the acyl chain, and it reports the polarity of its environment. It is buried in the interior of the bilayer for all of the bilayer phases, L_{α}, $L_{\beta'}$ and $P_{\beta'}$, and is not sensitive to the transitions among these phases [37]. However, in the interdigitated phase, it is located in the interfacial region, which is much more polar. As shown previously [37] and demonstrated in Fig. 7 for DPPC, the polarity, expressed in the III:I ratio, is sensitive to the transitions that involve the interdigitated phase. The results of the Pyr-PC study of DMPeth and DPPeth are consistent with the DSC results in identifying the presence of interdigitation under the same conditions in which the DSC results indicated that the lipid that undergoes the main melting transition is interdigitated in the gel phase. The Pyr-PC provides additional information regarding the lipid that does not participate in the transition and is in the cholesterol-rich regions. The III:I ratios that are observed in the higher cholesterol mixtures are more similar to those of the non-interdigitated bilayers, becoming even less polar with the addition of ethanol. This indicates that the lipid that does not undergo the main transition is also not interdigitated in the presence of cholesterol. This is consistent with the interpretation that cholesterol is not incorporated into interdigitated regions, but instead prevents the lipid from becoming interdigitated.

4.3. Mechanism of cholesterol effect on interdigitation

We have previously shown that the Peths are interdigitated in the presence of Tris–HCl, but not in salt or water [40]. Ethanol enhances interdigitation in these lipids, as well as in DPPG. Cholesterol has now been shown to shift the structure to the non-interdig-

itated gel in the Peths, as it has previously been shown to do for DPPC [37]. In addition to DPPC, DMPeth, and DPPeth, it was shown previously that the naturally interdigitated lipid, DHPC, also becomes non-interdigitated in the presence of 20 mol% cholesterol [31,37,39,45].

The thermodynamic influences that cause interdigitation in several like-chain phospholipids under various conditions has been discussed by several authors [29,44,46–48]. In the PCs, one of the factors is the mismatch between the headgroup and the cross-sectional area of the acyl chains, leading to a crowding of head groups that produces a tilted gel phase in the $L_{\beta'}$ phase, and the ripples of the $P_{\beta'}$ phase. The interdigitated phase is also favored by closer van der Waals interactions in the acyl chain region than the $L_{\beta'}$ or $P_{\beta'}$ structures. The increased area per headgroup of the interdigitated structure alleviates this crowding, and the function of ethanol or other inducer is to reduce the unfavorable effect of the exposure of the terminal methyl groups to the interfacial region by reducing its polarity. The 1,3-DPPC, and the ether-linked DHPC, also form interdigitated gel phases without any additives; again this may be due to a destabilizing headgroup crowding in the gel phase. For DPPG and the Peths, the head groups are not large by themselves, but are charged. These lipids can be interdigitated only in the presence of the large cation Tris, and it has been suggested that the large size of this counterion creates crowding in the head group region similar to that of the PCs [40]. The role of ethanol in enhancing this effect is again to reduce the unfavorable effect of the exposure of the terminal methyl groups to the polar interfacial region.

From this perspective, the role of cholesterol in this scheme is twofold. From the point of view of the model above, the cholesterol can reduce the headgroup crowding by intercalating between the lipid molecules and increasing the area per head group and thus reducing the steric destabilization of the non-interdigitated structure. Another probable effect is that the cholesterol itself must be shielded from the water solvent, and the smaller thickness of the interdigitated structure is not sufficient to completely shield the cholesterol molecule. In fact, it has been shown that cholesterol miscibility in varied chain-length PCs is sensitive to the bilayer thickness even over a relatively small range [18]. Thus, a probable mechanism by which cholesterol prevents the interdigitation of these lipids is by preferentially interacting with the non-interdigitated regions and pulling the phase equilibrium toward the non-interdigitated structure. The cooperative nature of the lipid phase equilibria between the interdigitated and non-interdigitated structure is indicated by the observation that the lipids remain completely non-interdigitated in the regions that undergo the melting transition (i.e., the cholesterol poor regions) at a bulk composition of only 10 mol% cholesterol. The effect of ethanol in the Tris–Peth system is to pull that equilibrium back toward interdigitation in the cholesterol-poor regions. As noted above, the increase in transition enthalpy as a function of ethanol concentration may be an indication that it is also removing lipids from the cholesterol-rich regions into the cholesterol-poor interdigitated regions.

The distribution and dynamics of cholesterol in membranes and lipid bilayers has been the subject of a great deal of study and speculation. There is also a growing body of evidence that cholesterol interacts differently with different lipids [17–19,23,49–51]. Our finding that cholesterol has a strong preference for non-interdigitated lipids provides yet another mechanism for a role of cholesterol in the control of membrane domains. It is clear from this consideration that the introduction of small regions of interdigitation due to the presence of Peths could lead to a change in cholesterol distribution in the membrane. Similarly, the presence of cholesterol in membranes can prevent the appearance of interdigitated phases.

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