Biochimica et Biophysica Acta, 1062 (1991) 70–76

ADONIS 000527369100098L

BBAMEM 75125

Cholesterol does not remove the gel-liquid crystalline phase transition of phosphatidylcholines containing two polyenoic acyl chains

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(Received 7 August 1990)

Key words: Lipid–cholesterol interaction; Model membrane; Transformed cell; Liposome; DSC

Homoacid (single-acid) phosphatidylcholines containing two linoleate (18:2), arachidonate (20:4), or docosahexaenoate (22:6) chains were dispersed in water to form multilamellar vesicles. The influence of cholesterol on the gel to liquid phase transitions was studied by differential scanning calorimetry (DSC). The pure dipolyenoic phosphatidylcholines exhibited very broad endothermic transitions (widths of 28 to 38 C° at scanning rates of 5 C°/min) in the temperature range of about −80°C to −30°C. The mixing of cholesterol into the phospholipids in proportions up to 50 mol% had little effect on the temperatures, widths or enthalpy changes associated with the phase transitions. The data suggest that cholesterol does not interfere with the packing of these lipids in the gel state, possibly because the packing is already irregular or because the solubility of cholesterol in these lipids is low, or both.

Introduction

Cholesterol is a major constituent of biological membranes, its proportion being especially high in the surface membranes of mammalian cells [1]. Studies of the influence of cholesterol on the physicochemical properties of membranes have indicated that cholesterol modifies the packing of phospholipids in membranes. In the gel state cholesterol disrupts the regular packing of chains of phospholipids. In the liquid crystal the sterol restricts the motion of the normally mobile acyl chains resulting in an increase in membrane viscosity. Since most lipids in most natural membranes are in the liquid crystalline state, cholesterol is generally considered to restrict motion in biological membranes.

Previous studies have indicated that the quantitative effect of cholesterol on physicochemical parameters in model membranes depends upon the structure of the phospholipid that is in association with the sterol (e.g., Refs. 2, 3). Not all membranes are likely to be affected to the same extent by a given proportion of cholesterol. It is necessary to study examples of cholesterol interacting with phospholipids of different acyl chain distribution in order to obtain a complete quantitative understanding of lipid–cholesterol interactions in membranes.

Certain transformed and cancer cells have been found to display altered microviscosities, or cholesterol contents, or both [4–6]. In some of these cells, especially in solid tumors, a partial loss of the usual positional specificity in the phospholipids has been observed. There are higher than normal proportions of phospholipids containing two unsaturated acyl chains, including some lipids with two polyenoic chains. Dipolyenoic lipids have also been found in some normal tissues (e.g., Refs. 7–10), and they are in particularly high amounts in retinal rod membranes [9,10].

Previous studies on lipid–cholesterol interactions in model membranes containing diunsaturated phospholipids have, to our knowledge, included only phospholipids with two oleate chains. Because dipolyunsaturated phospholipids have unusual physicochemical characteristics of themselves [11], we have investigated the influence of cholesterol on the gel to liquid-crystalline phase transition of a series of dipolyunsaturated PC, 18:2-18:2 PC, 20:4-20:4 PC and 22:6-22:6 PC.

Abbreviations: DSC, differential scanning calorimetry (ic); PC, phosphatidylcholine; 18:2-18:2 PC, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine; 20:4-20:4 PC, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine; 22:6-22:6 PC, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine.

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Materials

Lipids were obtained from Avanti Polar Lipids (Birmingham, AL) and silica gel G (Kieselgel 60G, Merck) was obtained from British Drug Houses (Toronto, Canada). Other chemicals and solvents were reagent grade or better and were purchased from Sigma (St. Louis, MO), Fisher Chemical (Dartmouth, Canada) and GSW Consolidated Alcohols (Toronto, Canada). Solvents were distilled in glass before use. Water was deionized and distilled twice in glass, the second time from dilute potassium permanganate solution. Cholesterol (Sigma) was recrystallized from 95% ethanol, vacuum-dried over P_2O_5 for 16 h, and stored at −20°C.

Prior to calorimetric analyses the lipids were analyzed for purity by thin-layer chromatography, gas chromatography and ultraviolet spectroscopy. Each phospholipid contained exclusively the expected fatty acid. 18 : 2-18 : 2 PC showed essentially no oxidative breakdown on thin-layer and ultraviolet analyses. 20 : 4-20 : 4 PC and 22 : 6-22 : 6 PC had molar extinction coefficients of 1550 and 1850, respectively This was consistent with a slight amount of oxidation having taken place in these samples [12]. Thin-layer chromatography of these lipids also showed some traces of material which could be associated with oxidation products [12,13]. Since, in our hands, attempts to purify lipids with such a small amount of oxidation have been uniformly unsuccessful on scales required for this work, these lipids were used without further purification.

Methods

Ultraviolet spectra. Samples of 100 μl of the lipid extract of the DSC sample were dried under argon, and the residues were redissolved in 1.0 ml of absolute ethanol. Absorbance was read from 350 to 200 nm with absolute ethanol as a reference using a Beckman DU-50 spectrophotometer.

Fatty acid analysis. Samples containing 100–250 μg lipid were transmethylated for 3 h at 60–65°C in 6% H_2SO_4 in 99.9 mol% methanol which contained a small amount of recrystallized (from ethanol) hydroquinone. After extraction of the methyl esters and their dissolution in CS_2 [14], the samples were analyzed on 10% SP2330 (Supelco, Bellefonte, PA) in a 30 m glass capillary column at 180°C in a Perkin-Elmer 8310 gas chromatograph with a flame ionization detector.

Phosphorus was determined and thin-layer chromatography was performed as described previously [11]. Cholesterol was measured by the method of Rudel and Morris [15].

Differential scanning calorimetry. Lipids were mixed in chloroform and the solvent was removed under a stream of Ar followed by evacuation overnight in the dark over P_2O_5. Just prior to DSC analysis, the lipids were dispersed at 33% by weight in argon-saturated, doubly-distilled water at room temperature. DSC was performed with a Perkin-Elmer DSC-2. All samples were heated and cooled over the range −125 to 27°C to ensure that excess water was present and that transitions were reproducible. Water was used in the reference pans. High sensitivities (0.5 or 1 mcal/s) were employed. Scanning rates will be given where appropriate in Results. Experimental thermograms were normalized per mol of lipid phosphorus as previously described [2].

After DSC analysis, the DSC pans were opened and the contents extracted with $CHCl_3/CH_3OH$ (1 : 1, v/v) and made up to a final volume of 10 mL. The extract was analyzed for phosphorus, and studied by thin-layer chromatography and ultraviolet spectroscopy.

Sepharose 2B and 4B (Pharmacia) were washed with N_2-purged water to remove all traces of the phosphate buffer in which the materials were suspended by the supplier. Liposomes in water were eluted from columns of Sepharose of dimensions 15 cm length × 0.9 cm diameter. Samples containing 18 : 2-18 : 2 PC and cholesterol in 1 : 1 molar ratio were prepared by the method described above. They were either applied directly to the column after vortexing or they were sonicated. Sonication was done using a Branson sonifier with a microtip using setting 6. The suspensions were held in an ice bath. There were 15 cycles of 30 s of sonication followed by 15 s of cooling. Sonicated samples were centrifuged for 2 min in an Eppendorf Microfuge to remove large particles. The supernatants containing smaller particles were applied to the columns. The columns were eluted with water and fractions of 0.5 ml (0.05 bed volumes) were collected. Dispersions made by vortexing did not pass through the columns.

Results

Analyses of the lipids (Fig. 1 and Table I) indicated that little oxidative or lytic degradation occurred during calorimetric analysis. In some samples there was a trace of lyso PC. In our previous work, we have observed that such small amounts of breakdown occur randomly in samples undergoing calorimetric study, even when the phospholipids have contained only saturated chains. In some samples there was a slight increase in oxidation, and these samples also occurred randomly.

Initial heating endotherms were carried out at 5 C° per min. Our ability to discern heating endotherms was improved by scanning at 20 C° per min and increasing the speed of the chart recorder. In this work (Fig. 2 and Table I) and in a previous study [11], it was observed that the salient features of the very broad endotherms associated with the transitions of dipolyenoic lipids alone were not influenced significantly by scanning rates. Similarly, no substantial effect of scanning rate on the endotherm shapes, characteristic temperatures or

TABLE I

Calorimetric and analytical data on lipid mixtures

Lipid		Differential scanning calorimetry						Analytical data				
		5 C° per min [a]			20 C° per min [a]			ultraviolet		thin-layer chromatography		
		T_{max} [b] (°C)	range [c] (C°)	ΔH [d] ($\frac{kcal}{mol}$)	T_{max} [b] (°C)	range [c] (C°)	ΔH [d] ($\frac{kcal}{mol}$)	E_{230}^{mol} [e]	E_{270}^{mol} [e]	% PC [f]	% lyso PC [f]	% other [f]
18:2-18:2 PC												
prior to DSC								2050	450	100	0	0
mol% cholesterol	0	−57.0	27.8	1.4				1950	600	100	0	0
	5	−63.2	28.0	0.8	−62.0	32.9	1.3	2200	400	~100	0	trace
	9	−64.2	30.1	1.0	−63.0	46.6	1.8	950	300	96.6	0.6	2.9
	13	−65.0	29.8	0.8	−63.2	43.4	1.9	1200	400	99.2	0.4	0.4
	17	−66.4	33.6	0.6	−64.0	41.8	1.1	600	200	98.8	0.8	0.5
	23	−67.7	32.8	0.6	−65.0	40.6	1.0	750	150	96.6	1.1	2.4
	30	−67.7	33.2	0.8	−64.2	39.9	1.0	400	200	98.2	0.3	1.4
	50	−64.0	33.7	0.7	−63.2	41.1	1.0	1600	450	98.0	0.8	1.1
20:4-20:4 PC												
prior to DSC								1550	700	94.4	1.3	4.4
mol% cholesterol	0	−69.2	38.0	0.6	−69.0	46.0	0.8	1300	900	93.1	0.5	6.4
	5	−72.2	39.6	0.8	−71.0	44.9	0.9	2250	1150	91.7	1.0	7.3
	9	−69.7	38.5	0.7	−71.7	46.6	0.9	1550	1100	97.8	trace	2.2
	13	−70.7	38.5	0.6	−68.7	47.6	1.1	1450	1100	91.8	1.4	6.8
	30	−74.2		0.8	−70.7	52.3	1.0	1850	1050	89.2	3.3	7.5
	40	−73.0	38.4	0.9	−70.0	38.1	0.7	1750	1150	91.4	1.8	6.7
	50	−69.7	38.2	0.9	−69.0	40.0	0.7	2600	1300	92.7	1.6	5.7
22:6-22:6 PC												
prior to DSC								1850	1100	92.6	1.9	5.5
mol% cholesterol	0	−68.4	34.9	0.5	−66.4	37.5	0.8	2200	1450	86.3	3.4	10.3
	5	−68.2	34.2	0.6	−66.2	40.3	0.9	2300	1800	91.6	0.2	8.3
	9	−69.4	35.1	0.6	−67.4	47.8	1.0	3100	2050	93.8	0.8	5.6
	13	−66.2	34.8	0.5	−64.2	44.0	0.8	3550	2000	90.2	1.2	9.0
	30	−67.7	33.6	0.5	−65.4	40.6	0.7	2650	1550	87.4	1.2	11.5
	50	−66.2	32.3	0.6	−63.2	35.0	1.1	3250	1850	91.3	1.5	7.2

[a] Scanning rate.
[b] Temperature of maximum heat flow into sample, as determined from normalized curves.
[c] Width of exotherm in degrees.
[d] Change in enthalpy associated with the gel to liquid-crystal transition.
[e] Molar extinction coefficients taken at maxima at or near 230 and 270 nm.
[f] Phosphorus content measured in thin-layer chromatography spots.

enthalpies was observed for these lipids in the presence of cholesterol (Figs. 2 and 3).

With 18:2-18:2 PC, increasing concentrations of cholesterol caused the endotherms to become bimodal where a higher-temperature component became visible (maximum at 13 mol%) and then disappeared (not visible by 30 mol%). The presence of the secondary peak did not significantly change the T_{max} of the principle, lower-temperature endotherm (−64.9°C at 5 C°/min, −62.6°C at 20 C°/min) nor did it significantly alter the total ΔH or the endotherm (Table I).

The change in enthalpy associated with the observed endotherms of 18:2-18:2 PC essentially did not vary with cholesterol content, but all appeared to be a bit less than that observed for the pure lipid dispersed in water. Measurements of enthalpy change are influenced by the selection of the baseline. In the studies conducted here the baselines were generally curved, and so the baselines were subject to some potential observer bias. These curved baselines are unavoidable under the conditions necessary to see these transitions (see Ref. 11). Given our experience with differential scanning calorimetry of these lipids, we believe that our selection of baselines were reasonable ones which neither exaggerate nor underestimate the total ΔH of the transitions.

The addition of cholesterol to dispersions of 20:4-20:4 PC and 22:6-22:6 PC appeared to have no effect of either T_{max} or ΔH (Figs. 2 and 3 and Table I). Small, high-temperature components of endotherms were noted with 20:4-20:4 PC and somewhat larger ones were seen with 22:6-22:6 PC (Figs. 2 and 3). For these highly unsaturated lipids, the relative magnitude of the high and low temperature components, were not in-

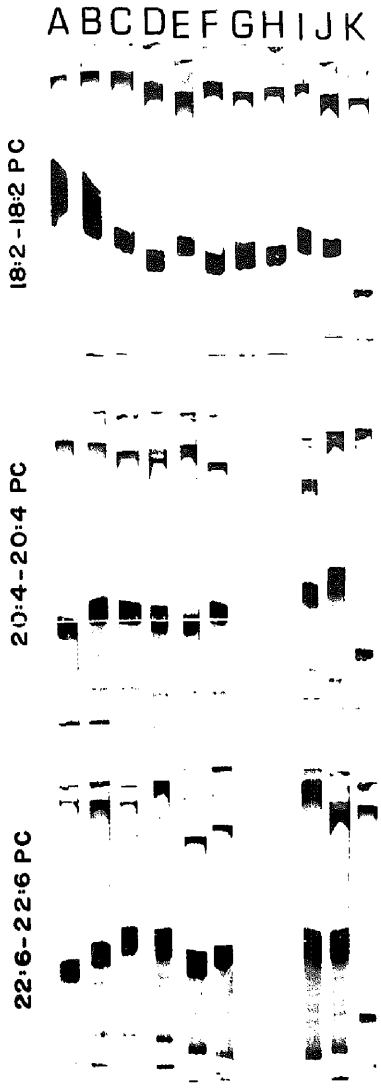

Fig. 1. Thin-layer chromatography of DSC samples. The figure is a composite of lanes from chromatograms run under slightly different conditions of humidity and plate activation. The major spot of each sample had a mobility of PC as judged from the mobility of a PC standard in an adjacent lane. A, dipalmitoyl PC standard; B, lipid prior to DSC analysis; C–J, lipids following DSC: C, 0 mol% cholesterol, D, 5 mol% cholesterol, E, 9 mol% cholesterol; F, 13 mol% cholesterol; G, 17 mol% cholesterol, H, 23 mol% cholesterol; I, 30 mol% cholesterol; J, 50 mol% cholesterol; K, lyso stearoyl PC standard.

fluenced by the cholesterol content as they were from 18:2-18:2 PC.

For all three lipids cholesterol had very little influence on the overall transition widths.

With the exception of the bimodality of the endotherms induced in 18:2-18:2 PC, the lack of any substantial influence of the cholesterol on other aspects of the transitions of these lipids suggested that cholesterol may not have mixed with the lipids. We endeavoured to determine if cholesterol was being excluded in some way into separate liposomes or regions of liposomes. We have scanned a dispersion of 18:2-18:2 PC plus 50 mol% cholesterol upto 90°C and failed to find evidence of an endothermic transition near 40°C that could be attributed to cholesterol itself [16,17]. In another preliminary experiment, we saw some evidence for the presence of small amounts of crystal structures which were birefringent when a dispersion of 18:2-18:2 PC plus cholesterol was viewed with crossed polarizers in a light microscope. Were these crystals to be cholesterol, then some or all of the cholesterol might have been excluded from the bilayers. The appearance of this dispersion was somewhat like that of a dispersion of dipalmitoyl PC-cholesterol 1:2, but different from dispersions of dipalmitoyl PC-cholesterol 2:1 or dipalmitoyl PC alone above and below the phase transition.

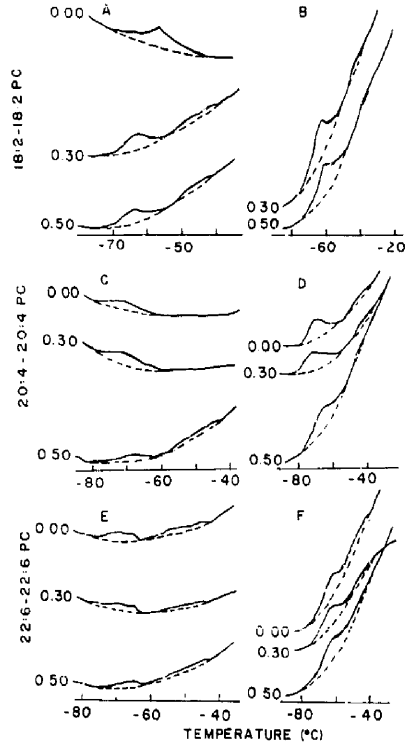

Fig. 2. Examples of endotherms from the DSC scans. Scans are presented as they were obtained from the DSC and are not normalized for phosphorus content. Scanning rates were 5 C° per min (A, C, E) and 20 C° per min (B, D, F). The number next to each endotherm indicates the molar proportion of cholesterol present.

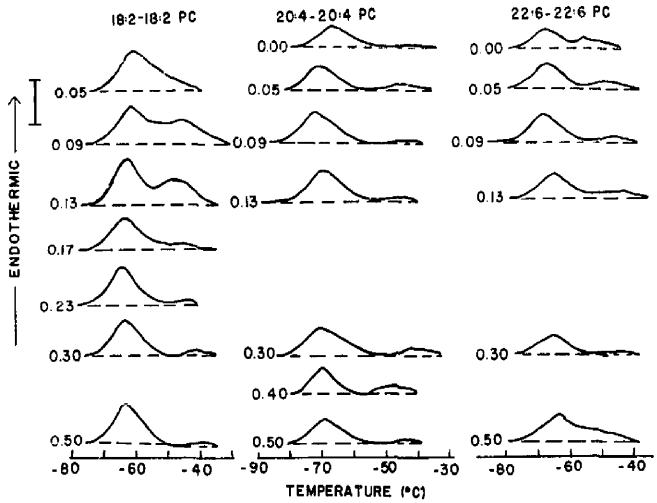

Fig. 3. Normalized endotherms for the gel to liquid-crystal transitions observed at a scanning rate of 20 C° per min. The endotherms are normalized per mol of lipid phosphorus. The number next to each endotherm indicates the molar proportion of cholesterol present. The bar indicates 0.1 kcal·mol^{-1}·(C°)$^{-1}$.

In Fig. 4 we show the elution profile of a suspension of 18:2-18:2 PC-cholesterol liposomes, prepared by bath sonication as described above, from columns of Sepharose 2B and 4B. The phospholipid and cholesterol coeluted. This suggests that the two components were in the same liposomes. Control suspensions of 18:2-18:2 PC liposomes eluted at the same volumes as did the mixed cholesterol-PC suspensions. We were unsuccessful in running proper cholesterol controls due to the crystalline nature of the material in the suspensions.

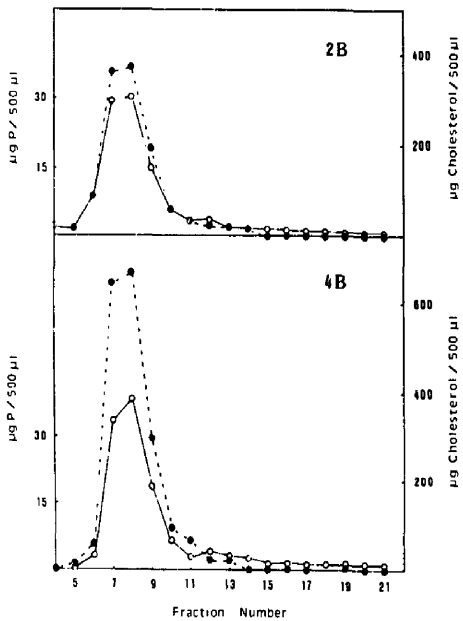

Fig. 4. Elution profile of dispersions containing equimolar mixtures of 18:2-18:2 PC and cholesterol from columns of Sepharose 2B and 4B. The sample preparation and elution conditions were described in Methods. ○——○, total phosphorus; ●——●, cholesterol.

Discussion

The results of the study indicate that there is a small influence of cholesterol on the gel to liquid-crystalline transition of 18:2-18:2 PC, and little or no influence on the transitions and endotherms of the other two more highly unsaturated lipids. The small effect on the enthalpy change of the transitions of the unsaturated lipids is in marked contrast to the influence of cholesterol on other lipid systems containing lecithins either with two saturated chains, two monoenoic chains, a saturated chain plus a monoenoic chain, or a saturated chain plus a dienoic chain [2,3,18]. The phase transitions of all of these latter types of lipids were progressively reduced in magnitude by the presence of cholesterol. Sufficient cholesterol results in the abolition of the detectable phase transition in all but one of these types of PC, dimonoenoic PC.

Cholesterol appears to be progressively less effective at removing the transitions with increasing chain unsaturation when it is combined with the series of lipids, 18:0-18:0 PC, 18:1-18:1 PC and 18:2-18:2 PC. Cholesterol at 50 mol% abolished the phase transition of 18:0-18:0 PC but in the presence of the same concentration of sterol about 40% of the original en-

thalpy of the transitions of 18:1-18:1 PC remained [2]. The current observations indicate that cholesterol is no more effective at removing the transition of 18:2-18:2 PC than it was from 18:1-18:1 PC, and probably less so. The sterol had little or no effect on the enthalpy change associated with the transitions of 20:4-20:4 PC or 22:6-22:6 PC. This could be interpreted to indicate that the packing of the more unsaturated lipids is sufficiently irregular in the gel state so that the presence of cholesterol does not further reduce the acyl chain packing order. This would be in contrast to the influence that cholesterol is generally thought to have on the gel phase of bilayers which contain more saturated lecithins. In this scenario, the multiple double bonds in each chain would be seen as reducing packing density to such an extent that cholesterol could intercolate between PC molecules by 'fitting' in among the kinked or bent chains such that there is little influence on the overall distribution of lipid contacts.

The alternative explanation could be that cholesterol is nearly completely excluded from packing in these lipids. Our high temperature calorimeter scans and the column elutions suggest that cholesterol is not likely excluded to structures separate from those formed by the PC, at least from 18:1-18:2 PC. The limited microscopic data, however, gives some hint of regionalization of the cholesterol in the dispersions. Cholesterol has a rigid four-ring portion which extends eight to ten carbons along the acyl chains into the bilayer from the glycerol backbone region. Assuming all double bonds introduced similar perturbations, one might expect that lipids with double bonds located closer to the head group than C9, such as 20:4-20:4 PC or 22:6-22:6 PC, might be more affected by the rigid nucleus of cholesterol than lipids with double bonds more distant from the head group, such as 18:2-18:2 PC. On the contrary, 18:2-18:2 PC was the only lipid whose phase transition seemed to be influenced by the presence of cholesterol. This is consistent with the observation that 20 mol% cholesterol broadened the transition of the anologous of 18:2-18:2 PC with double bonds at positions 5 and 9 of the chains [19]. Our observations and those of others on the phase transitions of polyunsaturated PC [11,20–22] would suggest that double bonds in excess of two produce little additional disruption in the packing in the gel over that produced by two double bonds. The regions between the head group and the middle of the chains in 20:4-20:4 PC and 22:6-22:6 PC may have less 'flexibility' than the same region of 18:2-18:2 PC, and this could be the cause for the apparent small differences in the effect of cholesterol on 18:2-18:2 PC and on the other two lipids.

The small effect that cholesterol had on 18:2-18:2 PC, producing a high temperature component in the endotherm, might have occurred because of the presence of a small amount of oxidative products (e.g., Refs. 11-13). In addition to small amounts of oxidative products being present initially, oxidation could occur to different extents in individual samples during experimental study. We believe that oxidative products might cause, or at least contribute to, the higher temperature components of the transitions of these dipolyunsaturated PC (see Refs. 12, 13). Some oxidative products of 18:2-18:2 PC might have interacted with cholesterol in such a way as to produce the slight 'enhancement' of the high temperature component of the transition as seen in Fig. 3. We acknowledge, however, that there might be other explanations for this effect, including some special cholesterol–lipid interactions in the range of concentration of cholesterol where the bimodal endotherms of 18:2-18:2 PC-cholesterol are prominent.

In summary, cholesterol does not substantially influence the packing of these dipolyunsaturated lipids. This observation is consistent with the data, which indicates that cholesterol produces only a very small condensing effect in monolayers of 18:2-18:2 PC and 18:3-18:3 PC [23,24]. Cholesterol was also found not to influence the permeability of liposomes made from these lipids to glucose, erythritol or glycerol, nor did it modify their swelling rates, although cholesterol did modify both permeability and swelling of liposomes made from other PC [23]. It is very interesting that cholesterol has been found to have little or no influence on the enthalpy changes of transitions of some PC with long chains (24 and 26 carbons) containing one or two double bonds per chain [19,25,26]. It is also noteworthy that it has been recently suggested that there may be fluid-phase immiscibility in mixtures of 18:1:18:1 PC and cholesterol [27]. Before one considers the influence of cholesterol on the phospholipids of biological membranes, one must also be aware of the various molecular species with which cholesterol may or may not interact. Attempts to simply correlate cholesterol to phospholipid ratios with physical characteristics of a membrane should be approached with caution.

Selective interactions between cholesterol and lipids with varying kinds of acyl chains may be of biological significance. The apparent lack of effect of cholesterol on these polyunsaturated lipids may have significant consequences for those membranes in which positional specificity is lost (e.g., Ref. 6) or for membranes that contain large amounts of highly unsaturated lipids (e.g., Ref. 10). Would polyunsaturated lipid domains form in such membranes, and if so, would cholesterol preferentially interact with or be excluded from such domains?

Acknowledgement

This work was supported by the Medical Research Council of Canada.

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