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Phosphatidylcholine–cholesterol interactions: bilayers of heteroacid lipids containing linoleate lose calorimetric transitions at low cholesterol concentration

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Model membranes composed of cholesterol plus one of two phosphatidylcholines (PC), each containing a saturated and a dienoic acyl chain, have been studied by differential scanning calorimetry. The gel to liquid-crystalline phase transition temperature of 1-palmitoyl-2-linoleoyl PC was −19.5°C and that of 1-stearoyl-2-linoleoyl PC was −13.7°C. The addition of cholesterol to the phosphatidylcholines in aqueous dispersion resulted in the progressive removal of the phase transition as observed by differential scanning calorimetry. Per mole of sterol in the membrane, cholesterol was more effective at reducing the enthalpy change of the phase transitions of these bilayers containing dienoic phosphatidylcholines than it is in eliminating the transition of membranes made with other phospholipids that contain more saturated chains. No transitions in membranes made with palmitoyl-linoleoyl PC or stearoyl-linoleoyl PC could be detected calorimetrically when 17 mol% cholesterol was present.

Biological membranes contain cholesterol in a wide range of concentrations (for a review, see for example, Ref. 1), and the study of phospholipid–cholesterol interactions in model membranes has received considerable attention. Various physicochemical properties of model membranes of all types of phospholipids are influenced by cholesterol. While the qualitative aspects of interactions with cholesterol appear to be similar for most phospholipids, various studies suggest that there are quantitative differences which depend upon the phospholipid headgroup, the acyl chain-length, the degree of saturation and the position of the unsaturated chain in heteroacid (mixed-acid) lipids (see, for example, Refs. 2–5). Investigations of bilayer systems containing pure lipids have been done primarily with phospholipids of the homoacid type, that is, lipids with two identical chains. Many biological membranes, however, contain phospholipids that have a saturated chain plus an unsaturated chain in which there are two or more double bonds. In this report we present findings of the DSC behaviour of two such systems. The bilayers contained cholesterol plus either SLPC or PLPC. The results suggest that the gel to liquid-crystalline phase transitions of these lipids are more profoundly influenced by cholesterol than are those of monoenoic heteroacid or homoacid phosphatidylcholines.

Materials and Methods

Two separate lots of SLPC and one of PLPC were obtained from Avanti Polar Lipids, Pelham, AL. They were analyzed by thin-layer and gas-liquid chromatography, and for their ultraviolet spectra in absolute ethanol. These lipids were pure PC, containing the designated acids, but they had undergone very slight amounts of oxidation. Cholesterol was obtained from Sigma Chemical, St. Louis, MO, and it was recrystallized twice from 95% ethanol at 4°C. The PC were found to have about 20% of their respective 'reversed' positional isomers [6], that is, the corresponding PC in which the linoleate chain was in the *sn*-1 position and the saturated chain was in the *sn*-2 position of glycerol.

Phospholipids and cholesterol were mixed in $CHCl_3$, dried under N_2, and evacuated over P_2O_5 overnight. The

Abbreviations: DSC, differential scanning calorimetry; PC, phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; SLPC, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; T_c, gel to liquid-crystalline phase transition temperature; T_{max}, temperature of maximum excess specific heat or maximum heat flow into a sample in a calorimetric transition.

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dried lipids were dispersed at approx. 30 weight percent in deionized, doubly distilled water (second distillation from dilute potassium permanganate solution) by vortexing at room temperature.

DSC was carried out using a Perkin-Elmer DSC-2 as described previously [3] at scanning rates of 5 C°/min. Normalization of the observed endotherms for the amount of phospholipid was done as before [3]. After calorimetry, the dispersed lipids were extracted into 10 ml of $CHCl_3/CH_3OH$ (1:1, v/v). The phosphorus content was determined and the extracts were analyzed by thin-layer chromatography and for ultraviolet absorption [7]. Some samples were also analyzed by gas-liquid chromatography [7,8].

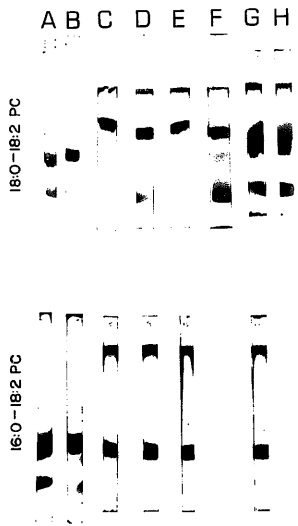

Fig. 1. Thin-layer chromatograms of the lipids in dispersions after DSC. The lanes represent the following: A, F, H, DPPC and lyso PC standards; B, the pure PC; C, PC/chol (95:5); D, PC/chol (91:9); E, PC/chol (87:13); G, PC/chol (83:17). Samples were obtained after DSC. Samples were run on different plates at different times after calorimetry and the figures are composites. Staining in the region of lyso PC in 18:0-18:2 PC lanes D and G and 16:0-18:2 PC lane B most likely came from spillover from lyso PC standards applied in adjacent lanes. This is concluded on the basis of the appearance of the original plates and the non-symmetric distribution of these spots in the experimental lanes.

Some PLPC was purified by column chromatography on CM-52 cellulose (Whatman, Clifton, NJ) [6,9] using solvents which had been purged with argon prior to mixing. Purified PLPC was analyzed by thin-layer and gas chromatography, and by ultraviolet spectroscopy. Fatty acid compositions were determined, after transmethylation, [7,8] on a 0.25 mm × 30 m column coated with SP330 (Supelco, Bellefonte, PA) in a Perkin-Elmer 8310 chromatograph. The oven temperature was 180 °C, and the temperature of the injection port and the flame ionization detector oven were maintained at 230 °C. Fatty acid compositions were calculated using the data handling components of the chromatograph.

Results

DSC studies of the first series of membranes made with SLPC plus cholesterol yielded the unexpected finding that an endotherm associated with a gel to liquid-crystalline transition could not be discerned in mixtures containing 17 mol%, or more, cholesterol. Because this finding could have some impact on the current concepts of the influence of cholesterol on lipid

TABLE I

Calorimetric and ultraviolet data for lipid mixtures

Each entry line represents a separate lipid dispersion. Samples of SLPC III were made with a different synthetic sample than samples I and II. n.d., Not detected. E^{233}_{mol} and E^{270}_{mol}, extinctions per mole lipid phosphorus for 1 cm path at 233 and 270 nm, respectively.

Lipid	PC/cholesterol (mol:mol)		ΔH (kcal·mol^{-1})	T_{max} (°C)	E^{233}_{mol}	E^{270}_{mol}
PLPC	100:0	I	2.6	−19.2	273	67
		II	3.2	−19.7	589	126
	95:5	I	1.6	−20.2	322	161
		II	1.9	−16.7	3120	523
	91:9	I	0.9	−18.9	313	77
		II	0.7	−16.6	2680	392
	87:13	I	0.4	−16.9	239	69
		II	0.4	−16.6	2930	510
	83:17	I	n.d.	n.d.	355	115
		II	n.d.	n.d.	2680	385
SLPC	100:0	I	4.8	−14.5	566	127
		II	4.5	−14.5	514	171
		III	4.5	−12.2	303	83
	95:5	I	2.5	−13.2	349	125
		II	1.9	−14.0	1125	500
		III	2.4	−14.2	327	172
	91:9	I	1.3	−13.4	676	132
		II	2.2	−13.9	722	164
		III	1.8	−14.0	800	200
	87:13	I	1.0	−13.1	805	1229
		II	0.9	−13.1	58	39
		III	0.4	−11.7	987	395
	83:17	I	n.d.	n.d.	5150	1170
		II	n.d.	n.d.	529	74
		III	n.d.	n.d.	675	183

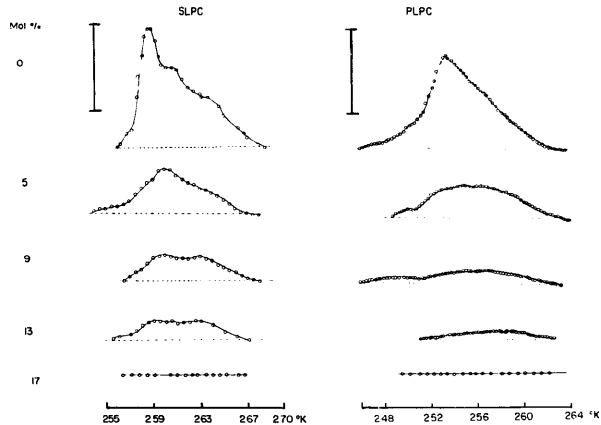

Fig. 2. Normalized DSC endotherms for dispersions containing PLPC or SLPC in the presence of cholesterol. The bars represent 600 and 400 mcal·deg^{-1}·mol^{-1} PC for SLPC and PLPC, respectively. At the high sensitivity employed, the original DSC-2 traces had considerable curvature over the range 200–270 K in which the transitions occurred. While it may have been possible to construct baselines which would have showed a very small transition in the presence of 17 mol% cholesterol, our judgement was that there were essentially no observable transitions in these samples.

transitions, we wished to ensure ourselves that the observation, though repeatable with that particular sample of SLPC, was not due to some unknown contaminant in that SLPC. Therefore, measurements were repeated using liposomes made of cholesterol plus three separate lots of heteroacid phosphatidylcholines, two different lots of SLPC and one of PLPC.

Investigation of all the DSC samples after calorimetry by thin-layer chromatography (Fig. 1), ultraviolet analysis (Table I), and fatty acid analysis in some cases, showed that little oxidation or hydrolytic breakdown had occurred in the samples during DSC analysis. In Fig. 1, two lanes of samples of SLPC (lanes D and G) and one of PLPC (lane B) show traces of material in the lyso PC region. We feel that these mostly come from spillover from large amounts of standards applied in adjacent lanes.

Fig. 2 shows normalized endotherms from scans of membranes made with one batch each of SLPC and PLPC after column chromatography. DSC analysis of dispersions made of the second sample of SLPC and the PLPC before chromatography gave traces similar to those presented. The SLPC showed a T_{max} near −14 °C, and a shoulder on the high-temperature portion of the endotherm. These findings are consistent with observations on dispersions of pure SLPC studied previously [10]. The normalized endotherms of pure PLPC (containing no cholesterol) also were asymmetric. They were

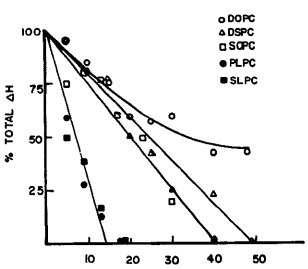

Fig. 3. Effect of cholesterol on the enthalpy change of the gel to liquid-crystalline phase transition of various PC. ■——■, SLPC; ●——●, PLPC; □——□, 1-stearoyl-2-oleoyl PC; ○——○, 1,2-dioleoyl PC; Δ——Δ, 1,2-distearoyl PC. The enthalpy change is given as a percentage of the change for the transition of the dispersion of the pure lipid. Data for 1-stearoyl-2-oleoyl PC, distearoyl PC and dioleoyl PC were taken from Ref. 4.

skewed to temperatures higher than T_{max}, but the shoulders were not as pronounced as those seen with SLPC. SLPC showed a greater excess enthalpy at T_{max} than did PLPC. Dispersions of PLPC alone had T_{max} at a mean value of $-19.5°C$, whereas those of SLPC were at an average of $-13.7°C$ (Table I). The average values for the enthalpy changes associated with the gel to liquid-crystalline transitions of the individual lipids were 2.9 and 4.6 kcal · mol^{-1} for PLPC and SLPC, respectively.

The data in Fig. 3 and Table I indicate that there was a progressive reduction of the detectable enthalpy change of the transitions as the cholesterol content of the liposomes was increased. Some of the endotherms showed a shape which might be resolvable into two components. We have not carried out deconvolutions (for example, as in Ref. 4) to obtain enthalpy changes associated with the components because the PC alone also displayed asymmetric endotherms. There were essentially no scans where, under our experimental conditions, an enthalpy change above the baseline could be reliably detected for membranes that contained either PLPC or SLPC plus 17 mol% cholesterol.

To try to ensure that this observation was not due to the fact that the membrane transitions were taking place in the presence of ice, we examined a sample of PLPC/cholesterol (83 : 17) in ethylene glycol/water (1 : 1, v/v). There was no obvious endotherm, although there was a possibility that a low-enthalpy, broad transition may have occurred which was superimposed on a curving baseline that was itself a consequence of the very high operating sensitivity. It is noted that inclusion of ethylene glycol in aqueous dispersions of phospholipid can influence the calorimetric and ^{2}H-nuclear magnetic resonance properties, so interpretation of data in its presence must be approached with caution (see, for example, Refs. 11–14]. Consistent with this, the endotherm of pure PLPC was altered somewhat by the presence of ethylene glycol. It is unlikely that the presence of ice caused the disappearance of the transition at 17 mol% cholesterol since the transition of dioleoyl PC plus up to 50 mol% PC was observable in the presence of ice [4].

Discussion

Cholesterol is known for its ability to modulate the fluidity or order and motion of the acyl chains of biological and model membranes. It broadens the temperature range of the gel to liquid-crystalline phase transition and removes the discernable enthalpy change of that transition (for example, Ref. 15). Cholesterol also condenses the packing of some phospholipids in monolayers, especially if the lipids are above their respective bulk phase transition temperatures and in the liquid-expanded state in the monolayer (see, for example, Refs. 16 and 17). Cholesterol increases the microviscosity of membranes containing unsaturated lipids when they are above T_c (see, for example, Ref. 18). There is a reduction in the acyl chain motion and an increase in the order of the chains in the presence of cholesterol (see, for example, Ref. 19). Given that a high proportion of membranes which contain cholesterol also have lipids of varying degrees of unsaturation, it is important to know to what extent the different kinds of lipids are influenced by the same amount of cholesterol. This study was concerned with the effect of cholesterol on the calorimetrically observable gel to liquid-crystal transition of model membranes. The observed data are due to changes between gel and liquid crystal, and thus a interpretation of observed differences between systems could be ascribed to different molecular interactions in the gel, in the liquid-crystal, or in both states for any lipid/cholesterol system of interest.

The findings of this study were in general accord with those in other systems containing less saturated phospholipids (for example, Refs. 4, 5 and 19); that is, it was found that cholesterol removed the observable gel to liquid-crystalline phase transition. This observation implies that cholesterol interacts with PLPC and SLPC in a similar fashion to the way it does with other PCs. Presumably, it interferes with regular packing of the acyl chains of the lipids when they are in the gel state, and it restricts the motion of the chain in the liquid-crystalline state, as has been suggested for other lipid/cholesterol systems. It is remarkable how little cholesterol was necessary to remove the transition of PLPC and SLPC. This suggests that cholesterol has a large perturbing effect on the gel state of these lipids, or that it exerts a strong restriction on the motion of the chains in the liquid-crystalline state, or both.

It has been observed that cholesterol condenses monolayers of PLPC and SLPC [17,20,21]. In monolayers the condensations of PLPC and SLPC cholesterol were of similar magnitudes to those that cholesterol induced in palmitoyl-oleoyl PC or stearoyl-oleoyl PC when either phospholipid was a state where its chains were 'melted' corresponding to the liquid-crystalline state of a bilayer [17,20,21]. Using steady-state fluorescence polarization measurements of 1,6-diphenyl-1,3,5-hexatriene in liposomes of pure lipids in the liquid-crystal state at 25°C, it has been observed that palmitoyl-oleoyl PC is more susceptible to ordering by cholesterol than is PLPC [22]. These observations suggest that increased restriction of the motion of the chains of the dienoic lipid relative to those of the monoenoic lipid in the liquid crystal is not a facile explanation for the greater effect of cholesterol on the phase transition of the dienoic lipids.

In Fig. 3 the relative influence of cholesterol on ΔH of the transition of a number of different PCs is plotted. Since each of the pure lipids has a different value of ΔH

for its gel to liquid-crystalline transition, the values have been normalized to 100% of the ΔH of the transition of the pure lipid in each case. All lipids in the group distearoyl PC, dioleoyl PC, 1-stearoyl-2-oleoyl PC and 1-stearoyl-2-linoleoyl PC have the same length for both acyl chains, but the degree of unsaturation and unsaturated chain location varies in each.

The amount of cholesterol necessary to remove the calorimetrically observable transition from distearoyl-, stearoyl-oleoyl and stearoyl-linoleoyl PC decreases with increasing unsaturation in the *sn*-2 chain. The ΔH of the transition of the pure compounds also decreases with increasing unsaturation [4,13,23]. Assuming that there is no free energy change in these transitions, the energy, in this case ΔH, required for each transition is then reflected in ΔS. The transition temperatures are 258 K for SLPC, 279 K for stearoyl-oleoyl PC and 329 K for distearoyl PC [3,13,23], the highest being 28% above the lowest. The corresponding ΔH values (4.6, 5.4 and 9.2 kcal $\cdot$ mol^{-1}, respectively) increase by 100%. Thus, a progressively decreasing DS is associated with the phase transition as the degree of unsaturation in the *sn*-2-chain increases. If cholesterol causes additional disruption to the gel packing (above that caused by the presence of double bonds), and it restricts motion of the chains in the liquid crystal, then it is possible to comprehend that less cholesterol would be required to remove the gel to liquid-crystalline transition for the more unsaturated lipid. Some data [22] suggest that this attractive explanation may not be sufficient, and more information about lipid–cholesterol interactions in both the gel and liquid-crystal states of each of the phospholipid types will be necessary to fully understand the role of cholesterol in membranes containing phospholipids like those studied here.

The transition of dioleoyl PC, which also contains two double bonds but one in each chain, is not completely removed even by 50 mol% cholesterol (Fig. 3 and Ref. 4). The difference between the influence of cholesterol on dioleoyl PC and SLPC indicates that the distributions of double bonds among chains has as much effect, if not greater, on lipid–cholesterol interactions as does the total number of double bonds in one chain. The data suggest that cholesterol interacts differently with homoacid (one type of chain) unsaturated lipids than with homoacid saturated PC or heteroacid PC.

If some or all of the dissimilarities seen in the calorimetric scans of these various lipid-cholesterol systems were associated with distinctions between–cholesterol interactions when the lipids are in the liquid-crystal state, the state existing in biological membranes for the most part, the differences seen could have consequences for lipid–cholesterol interactions in membranes. In a membrane with a higher number of unsaturated chains, or with great extent of unsaturation in individual chains, a smaller amount of cholesterol may cause the same change in order and motion as would a larger amount of cholesterol in a more saturated membrane. This view is in keeping with the idea that the cholesterol content of a given membrane is influenced by, if not perhaps dictated by, the molecular species of phospholipids present in the membrane (see, for example, Ref. 22).

In summary, the data indicate that PC–cholesterol interactions, as measured by DSC scans of gel to liquid-crystal transitions, vary with the nature and location of acyl chains on the PC. Caution is advised in extrapolating data from one type of lipid/cholesterol system to another. Quantitative aspects of phospholipid–cholesterol interactions may have to be established with the individual phospholipid of interest.

Acknowledgement

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