brief communication

Cholesterol interacts with all of the lipid in bilayer membranes

Implications for models

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ABSTRACT The interaction of cholesterol with lipid membranes has been studied by differential scanning calorimetry on liposomes, a technique which involves only the natural lipids, with no exogeneous probes. The influence of cholesterol at different molar percent concentrations c on the enthalpy ΔH of the main gel to liquid crystal phase transition of saturated phosphatidylcholines of acyl chain length n = 12–20 was well represented by $\Delta H = -9.43 + 1.01n - 0.268c$ kcal/mol. The linear dependence of ΔH simultaneously upon chain length n and upon cholesterol concentration c shows clearly that cholesterol interacts with the deeper part of the lipids, as well as the superficial parts. This observation is not accommodated in any of the current models of cholesterol–lipid interactions.

INTRODUCTION

Most of the cholesterol in animal cells resides in the plasma membrane, where cholesterol is believed to be a functionally essential component. Because of its ubiquity in mammalian membranes, and its correlation with atherosclerosis in humans, its properties have been extensively studied and a number of phospholipid–cholesterol packing models have been proposed (1). However, there are relatively few experimental techniques which are free of introduced probes (2). Space-filling models, based on x-ray and neutron diffraction data, show that the position of the cholesterol molecule is such that its hydroxyl group is close to the ester carbonyl group of the phospholipid, and its opposite end is near the methyl phospholipid end of an extended lipid of chain length n of 14 carbons (2). The liposome (a multibilayer structure of lipids in water) provides a widely used model system for membranes, and a wide variety of naturally occurring membrane lipids and proteins have been successfully incorporated (3). The main phase transition of phospholipids, from a rigid gel to fluid liquid crystal phase as temperature is increased, in the mimetic liposome is due to a cooperative order–disorder change in the acyl chains. Cholesterol reduces the cooperativity and change in enthalpy ΔH of this transition. Differential scanning calorimetry (d.s.c.), determining ΔH directly, has the advantage of measuring cholesterol–lipid interactions thermodynamically, unperturbed by any probes.

MATERIALS AND METHODS

The purity of the saturated synthetic phospholipids (Avanti Polar Lipids Inc., Birmingham AL) and cholesterol (99+% purity, Sigma Chemical Co., St. Louis, MO) was checked by thin layer chromatography; each showed a single spot. Liposomes were prepared by a standard technique (4) of drying down a chloroform solution of phosphatidylcholine (of chain length n carbons "CnPC") with the appropriate cholesterol concentration c (as mol cholesterol/(mol cholesterol + mol lipid)) in a rotary evaporator of some 5 cm^2 surface area, and then left under a vacuum of <200 mTorr overnight. The lipid–cholesterol mixture was hydrated by Vortexing in double-distilled water at a temperature above that of the main transition. Liposomes so prepared have been examined by quasielastic light scattering and electron microscopy (5). 20-μl samples of 10 wt% (lipid/water) were loaded into 40-μl pans in a TA 2000B d.s.c. (Mettler Instrument Corp., Hightstown, NJ) (6). The instrument was calibrated with diphenyl ether (7). Second and subsequent scans (always 1.2 K/min) of a pan agreed with the first scan. Masses were gravimetrically determined after d.s.c. measurements. Enthalpy changes were determined as detailed in reference 4.

RESULTS AND DISCUSSION

The results, given in Figs. 1 and 2 and Table 1, can be summarized by the equation $\Delta H = -9.43 + 1.01n - 0.268c$ kcal/mol, where ΔH is the enthalpy of the main transition, n is the acyl chain length, and c is the cholesterol concentration in molar percent. The data show clearly that (a) ΔH is linearly dependent upon cholesterol concentration c for all chain lengths; (b) the initial slopes of ΔH vs. c (Table 1, column b) are parallel for n = 13 through n = 20; (c) the initial slope for C12PC is quite different from that for the other CnPCs; and (d) there is no sign of a break in the slope, i.e., in this work there is no evidence of phase separation as has been indicated for a restricted range n (1, 2). The finding of equal slopes for C13PC through C20PC indicates that cholesterol solubility is independent of n and that, for a given value of ΔH,

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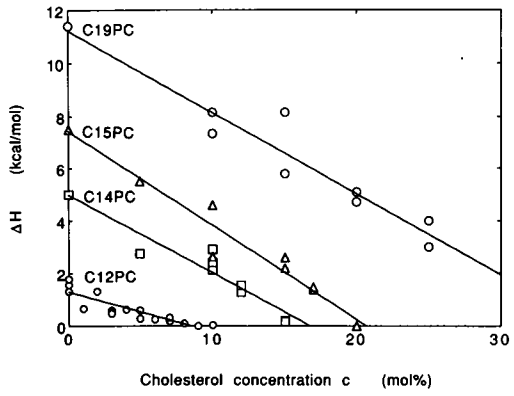

FIGURE 1 Cholesterol causes a linear change in the enthalpy change ΔH of the main transition of bilayers of phosphatidylcholines C*n*PC. Four acyl chain lengths are illustrated here; the parameters for all acyl chain lengths n = 12–20 are given in Table 1.

TABLE 1 Influence of cholesterol on the enthalpy change ΔH of the main phase transition of saturated phospholipids C*n*PC, of acyl chain length *n*

n (No. carbons)	*b*	Intercept a/b at $\Delta H = 0$	*R*
	kcal/mol of lipid per mol% of cholesterol	*mol%*	
12	−0.151	8.50	0.88
13	−0.317	13.8	0.89
14	−0.298	16.8	0.95
15	−0.358	20.7	0.98
16	−0.227	26.5	0.89
17	−0.237	25.1	0.82
18	−0.307	32.3	0.90
19	−0.309	36.3	0.96
20	−0.212	43.3	0.72

The parameters of a least-squares fit to the data $\Delta H = a + bc$ are given, where c = cholesterol concentration as (mol cholesterol)/(mol cholesterol + mol lipid) and R is the correlation coefficient.

cholesterol concentration must be a linear function of n. Essentially, the variation of ΔH with cholesterol concentration probes the cholesterol–lipid interactions in the plane of the bilayer, and the variation of ΔH with n (at a given cholesterol concentration) probes the cholesterol–lipid interactions perpendicular to (across) the bilayer. The linearity of ΔH with concentration shows that cholesterol–lipid interactions are not shielded by cholesterol in the concentration range studied, i.e., cholesterol molecules appear to mix uniformly with the lipid molecules and with each other. The linearity of ΔH (at a given c) with n shows that, overall, terminal CH_2 groups of the lipid molecule interact with cholesterol as much as groups that are closer to the membrane surface. This point will be discussed later and is quite consistent with space-filling models and measurements of bilayer thickness (2).

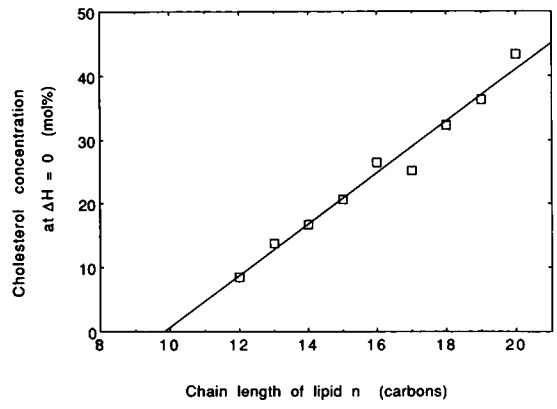

FIGURE 2 The special cholesterol concentration, at which $\Delta H = 0$, is linearly dependent on the chain length n of the phospholipids. Details are given in Table 1, where "Intercept" is the abscissa of this figure. The fit is to $c(\Delta H = 0) = -39.8 + 4.04n$; ($R = 0.99$).

The main peak of dilaurylphosphatidylcholine (C12PC) exhibits quite a different behavior to that of the other lipids (Fig. 1 and Table 1). Its slope b of 0.151 is less than the average of 0.283 of the other lipids. (There is no trend of b with n [$R = 0.03$].) The main transition of pure C12PC has been reported to show (8) in addition to the sharp d.s.c. peak typical of the PCs of higher n, a broad shoulder (which is however not always observed [9]). We have confirmed that the shoulder exists in C12PC of high purity, and that the transition in C12PC is otherwise different from the other C*n*PCs (10).

It has long been known that the enthalpy of the main transition in pure phosphatidylcholines increases linearly with chain length n (11). We find (Fig. 2) that this dependence is still satisfyingly linear even in the presence of cholesterol concentrations which are so high that they almost eliminate the main transition, eliminating it at a special (at which $\Delta H = 0$) concentration. Also, the line of Fig. 2 extrapolates to $n = 9.8$, showing that ~10 carbons of each acyl chain are inactive in the transition; this number is within a carbon of previous data (11) and is better established than data provided by nuclear magnetic resonance (2). Hence there is considerable confidence in the utility of this enthalpic approach. If the "sphere of influence" (actually a circle in a two-dimensional membrane) of cholesterol were short range, then one would expect ΔH vs. cholesterol concentration c to vary from a straight line at higher cholesterol concentrations, when cholesterol–cholesterol interactions become more frequent and important. Because the experimental results are so linear, the circle of influence must be large. Again, that ΔH depends linearly on the fraction of total area covered by cholesterol is consistent with a long-range influence of cholesterol.

The special concentration, at which $\Delta H = 0$, seems to be of physical significance, and of greater usefulness for studies of cholesterol in membranes than the commonly used molar percent of cholesterol. For example, the maximum spectral splitting of an electron spin probe in fluid membranes of various fractions of cholesterol in C12PC, C14PC, C16PC, or C18PC membranes has been measured (12). A plot of maximum splitting at constant 30 mol% cholesterol vs. acyl chain length n is markedly curved. When we instead plot splitting at the special cholesterol concentration (for each n) vs. n, an excellent straight line results. This demonstrates that the special concentration is a natural experimental parameter. For any given n, the special concentration can be regarded thermally as the minimal concentration for which all the cholesterol interacts with all of the lipid. At higher concentrations, cholesterol–cholesterol interactions would occur.

The variation ΔH of C16PC (dipalmitoyl) with cholesterol has been measured by Mabrey et al. (13) who numerically resolved the ΔH-temperature d.s.c. peak into two components, each of which showed a linear variation like Fig. 1. However, for C14PC (dimyristoyl), two peaks were seen, which would require resolution into three components. As Mabrey et al. remark, because C14PC and C16PC are so similar in other respects, their C14PC findings weaken their interpretation of resolving peaks. Estep et al. (14) also examined cholesterol-C16PC mixtures by d.s.c., and found a ΔH-c result similar to our Fig. 1. They then resolved the peak into two components, one of which varied with c as Fig. 1, but the other showed a maximum, in contradiction with reference 13 and with us. The numerical resolution of d.s.c. peaks into components is fraught with difficulty when, as here, there is no well-established detailed theory of peak shapes. An empirical resolution is also difficult. Experimentally, the resolution of d.s.c. peaks also requires deconvolution of the instrument transfer function, which could depend on scan rate. Although we also see two peaks in some cholesterol-C14PC mixtures (unpublished results), C19PC-cholesterol samples (for example) show unambiguous single peaks, so one would have to posit a dependence of number of peaks upon chain length. Hence we have chosen to simply treat the total enthalpy of each transition.

Higher resolution d.s.c. may uncover a nonlinearity in ΔH vs. c at high cholesterol concentrations.

CONCLUSIONS

These results showing that cholesterol and lipid interact intimately with one another, present fundamental data that must be taken into account by theorists of cholesterol–lipid interactions (15). This data necessitates that cholesterol interacts "vertically" with a given lipid, either with its entire length or, at a minimum, with all of the CH_2 pairs that contribute to the enthalpy. Such vertical interactions have been ignored in previous models. Furthermore, the packing arrangement between cholesterol and a given CnPC could vary for different lengths n. However, whatever model is constructed must satisfy the observations that the change in ΔH with unit addition of cholesterol is independent of n (for $n > 12$), and that the special cholesterol concentration at which $\Delta H = 0$ is a linear function of n. We further suggest that this special cholesterol concentration is the one that should be used when comparing the effects of cholesterol on lipids of different lengths n.

It is pleasing that a Monte Carlo simulation of cholesterol with C14PC, C16PC, and C18PC suggests that "a plot of lipid phase transition enthalpy change versus cholesterol concentration should have an intercept of about one-third (i.e., $c = 25$ mol %) if extrapolated from low cholesterol concentration" (16). A recent theoretical paper by Ipsen et al. (17) predicts a cholesterol-C16PC phase diagram in close agreement with that determined experimentally. We hope that this work will be extended to other CnPCs.

We thank Ms. Marlene Young and Ms. Elise Singer for technical assistance, Dr. Esther Takeuchi (Wilson Greatbatch Ltd.) for the gift of equipment, Camu Zdat for manuscript and graph preparation, and the Medical Research Council (Canada) for financial support.

Received for publication 30 May 1989 and in final form 3 August 1989.

Note added in proof: We have extended the above analysis to the corresponding ethanolamines of chain length n "CnPE" and find parameters for Table 1 to be

n (No. carbons)	b	Intercept a/b at $\Delta H = 0$	R
	kcal/mol of lipid per mol% of cholesterol	*mol%*	
10	−0.392	4.54	0.87
12	−0.324	10.8	0.86
14	−0.224	25.1	0.91
16	−0.241	30.7	0.96

These results give a CnPE line nearly parallel to the CnPC line of Fig. 2 but intersecting at 9.2 carbons. This consistency with the CnPCs strengthens and supports our analysis, and gives information on the effect of lipid head groups.

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