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Lecithin:cholesterol acyltransferase regulation. Effect of fluidity of dimyristoylphosphatidylcholine vesicles

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The regulation of lecithin:cholesterol acyltransferase by changes in phospholipid bilayer fluidity was investigated using pyrene excimer fluorescence to measure fluidity. Fluidity of dimyristoylphosphatidylcholine (DMPC) unilamellar vesicles was decreased by the addition of up to 20% (mol/mol) cholesterol and increased by the addition of up to 10% (mol/mol) lysoDMPC. When both cholesterol and lysoDMPC are present in the bilayer, their individual effects on fluidity are altered. These changes can be explained by complex formation between cholesterol and phospholipid as in the model of Presti et al. (Presti, F.C., Pace, R.J. and Chan, S.I. (1982) Biochemistry 21, 3831–3335). Lecithin:cholesterol acyltransferase activity with these vesicles as substrates was measured to determine whether activity can be modulated by the fluidity changes of the bilayer on which the enzyme acts. When 10% lysoDMPC, a known lecithin:cholesterol acyltransferase inhibitor, is added to the vesicles, inhibition of activity is observed. When 7.5% lysoDMPC is added to vesicles which contain either 5 or 10% cholesterol, lecithin:cholesterol acyltransferase activity increases. This increase in lecithin:cholesterol acyltransferase activity due to vesicle-fluidity increase is sufficient to overcome the decrease in activity due to lecithin:cholesterol acyltransferase inhibition. This is the first report of the ability of lysoDMPC to increase lecithin:cholesterol acyltransferase activity.

The presence of other molecules in a phospholipid bilayer is known to modify the bilayer fluidity. The addition of cholesterol rigidifies the bilayer above the transition temperature and fluidizes it below the transition temperature [1]; the addition of lysoPC fluidizes the bilayer [2]. However, there are no reports on the effect of lysoPC and cholesterol on bilayer fluidity when both are present in the bilayer. The enzyme lecithin:cholesterol acyltransferase (EC 2.3.1.43) uses as substrates PC and cholesterol and produces lysoPC and cholesterol esters. Lecithin:cholesterol acyltransferase activity has been reported to be regulated by the fluidity of the bilayer [3]. In this report, we explore what fluidity changes occur when cholesterol, a lecithin:cholesterol acyltransferase substrate, and lysoPC, a lecithin:cholesterol acyltransferase product, are present in the bilayer; and examine how these fluidity changes affect lecithin:cholesterol acyltransferase activity.

There are two problems in studies of fluidity with the use of native lipoproteins, which serve as lecithin:cholesterol acyltransferase substrates in vivo. (1) Each subclass of lipoproteins consists of a range of particle sizes, with only an average composition known. (2) Because of the probability of protein interaction with the probe, it is more

Abbreviations: PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; lysoDMPC, 1-myristoylphosphatidylcholine; T_c, critical temperature; I_m, pyrene monomer fluorescence intensity (390 nm); I_e, pyrene excimer fluorescence intensity (470 nm).

straightforward to interpret fluidity measurements in a protein-free system.

The model system we have chosen is the unilamellar vesicle, prepared by ethanol injection [4] and purified by ultracentrifugation [5]. These vesicles can act as lecithin:cholesterol acyltransferase substrates. The system has been demonstrated to be free of multilamellar liposomes and is highly reproducible. The maximum amounts of lysoPC and cholesterol that were added to the vesicles were limited to 10 and 20 mol%, respectively, in order not to greatly alter the vesicle shape or bilayer thickness [2,6,7]. We chose DMPC as our phospholipid because its critical temperature is convenient.

We chose to examine fluidity by pyrene excimer fluorescence [8], which measures lateral diffusion of a pyrene probe. There are other methods for measurement of lateral diffusion, such as pulsed NMR [9] and fluorescence recovery after photobleaching [1]. Pyrene excimer fluorescence presents the considerable advantages of simplicity over the alternative approaches.

Materials and Methods

Materials. Pyrene and cholesterol (reagent grade) were obtained from Sigma Chemical Co. (St Louis, MO) and were stored in ethanol solution in the dark at −15°C. DMPC and lysoDMPC were obtained from Avanti Polar Lipids (Birmingham, AL) and were stored in ethanol at 4°C. All lipids were checked for purity on silica-gel-coated plates (chloroform/methanol/water, 65 : 50 : 4, v/v) and used without further purification. The molecular weights used in calculations were 695 for DMPC and 467 for lysoDMPC. Tris, ultrapure grade, was obtained from Schwartz-Mann (Orangeburg, NY). NaCl and EDTA, reagent grade, were obtained from Baker Chemical Co. (Phillipsburg, NJ). Scintiverse and [^{3}H]cholesterol (spec. act. 34.6 Ci/mmol) were obtained from New England Nuclear (Boston, MA) DEAE-cellulose (Cellex D) was obtained from Bio-Rad (Richmond, CA); Sephadex was from Pharmacia (Piscataway, NJ).

Preparation of vesicles. To prepare DMPC vesicles for fluorimetric studies [4], typically 3 mg (4.2 μmol) DMPC, 40 μg pyrene and the proper amounts of other lipids, where appropriate, were mixed and dried under nitrogen. Then, 0.35 ml ethanol was added and the samples were mixed. When lysoDMPC was to be present in the bilayer, the moles of DMPC present were decreased by the number of moles of lysoDMPC added. Each ethanol solution was injected rapidly by pipette into 17.5 ml of rapidly stirring buffer (50 mM Tris/0.15 M NaCl/2 mM EDTA (pH 7.4), which was at 30°C. The vesicle solution was concentrated to 5 ml in an Amicon Diaflo cell with an XM-100 membrane, washed with three 15-ml portions of buffer and concentrated to a final volume of about 4 ml. There was no phosphate detectable in the wash solution. The vesicle solutions were purified by a modification of the method of Barenholz et al. [5]. Each vesicle solution was pipetted into a cellulose nitrate tube and spun at 105 000 × g for 1 h at 25°C in a Beckman L3-50 ultracentrifuge. The top portion of the supernatant was drawn off with a Pasteur pipette and used immediately. Final phospholipid concentration was 0.3 mM. Vesicle solutions were prepared in duplicate.

Vesicles for enzyme activity studies were prepared in the same manner except that there was no pyrene added, and that there was added about $8 \cdot 10^3$ dpm of [^{3}H]cholesterol. Assays were performed on the day of preparation.

Fluorescence studies. A Perkin-Elmer 44-A spectrofluorometer was used with a slitwidth of 2 nm. The excitation wavelength was 330 nm; emission of the monomer was read at 390 nm and of the excimer was read at 470 nm. Samples were maintained at constant temperature with 5–15 min allowed for equilibration to each new temperature. In a typical experiment, the temperature was varied from 10 to 40°C. I_e/I_m ratios were calculated and the results were plotted vs. temperature.

A theoretical treatment of pyrene excimer fluorescence can be found in Ref. 8. The ratio of excimer and monomer intensities is related to viscosity:

$$\frac{I_e}{I_m} = \frac{P \cdot K \cdot T}{\eta}$$

P is the pyrene concentration in the bilayer; K incorporates both theoretical constants and instrumental parameters; T is the absolute temperature and η is the viscosity. We define the term 'fluidity' in the context of these studies as the reciprocal of

the viscosity. Therefore, if the pyrene concentration in the bilayer and the temperature are constant, an increase in I_e/I_m indicates an increase in fluidity [1].

To test whether two lines were significantly different, we used a two-tailed t-test on comparisons of intercepts and slopes. If either the intercepts or slopes were significantly different, the two lines were as well.

Enzyme preparation and assay. The enzyme-preparation procedure was adapted from Soutar et al. [10]. Protein in the final preparation was determined by the method of Lowry et al.; activity was determined as described below. The preparation was then adjusted to a final activity of 65 pmol cholesterol ester formed/0.1 ml per h, using purified egg PC vesicles containing 5% cholesterol as substrate. The protein concentration was about 200 μg/ml, representing about an 800-fold purification. About 5–10% of the protein was present as apoprotein A1 as determined by immunodiffusion [10]. There was less than 2 μg/ml cholesterol in any preparation [11]. The enzyme was then frozen and stored at 0°C. Thus stored, the enzyme is stable for at least 1 year. We used a partially purified lecithin:cholesterol acyltransferase preparation because we are pricipally interested in the regulation of the biological activity, which includes cofactors.

We used the enzyme assay of Soutar et al. [8], except that each tube contained 37.4 nmol DMPC. Lecithin:cholesterol acyltransferase activity is expressed as pmol cholesterol esterified/0.1 ml per h. All assays were performed in triplicate; all assays contained blank tubes from which enzyme was omitted. The data shown in the figures are averages of at least three separate experiments. Standard statistical methods were used in calculating averages and their standard errors [12]. All tests were two-tailed. All percentages are expressed as mol%, unless otherwise stated.

Results

The pyrene concentration in DMPC vesicles prepared with and witout 20% cholesterol was varied from 0 to 6.1% (mol/mol), and the I_e/I_m ratio was determined at 19 and 30°C. These temperatures are below and above the DMPC transition temperature of about 24°C. The curves in all vesicle preparations are linear ($P < 0.01$) up to at least 5.4% (mol/mol) (data not shown). This lin-

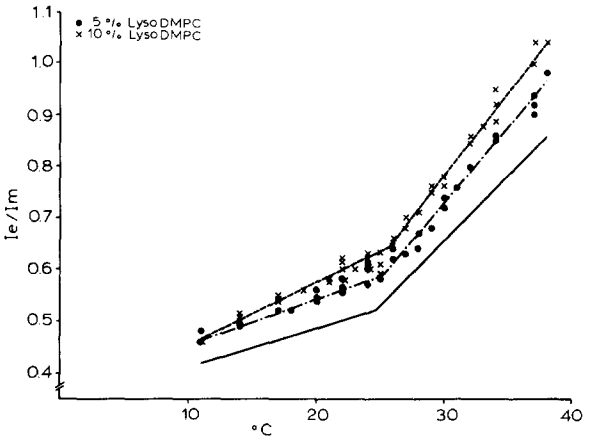

Fig. 1. The I_e/I_m ratio vs. temperature curves for DMPC vesicles containing various levels of lysoDMPC. The lines are the linear regression best fit to the data points. The solid line is the linear regression line for unmodified DMPC vesicles. The correlation coefficient and number of points are stated first for the temperature range 10–23°C and then for the temperature range 25–40°C. ●–·–●, 5% lysoDMPC ($r = 0.998$, $n = 18$; $r = 0.996$, $n = 17$). ×------×, 10% lysoDMPC ($r = 0.992$, $n = 21$; $r = 0.998$, $n = 21$).

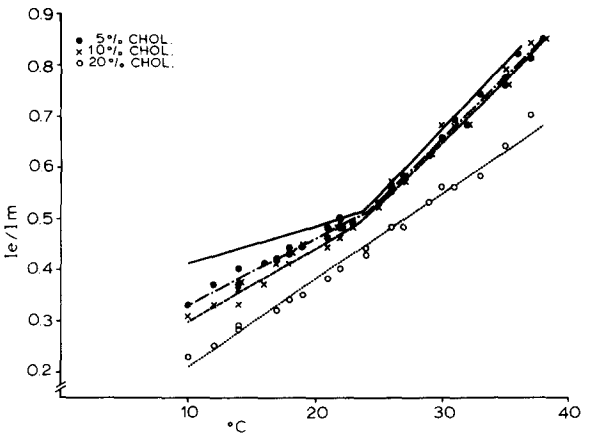

Fig. 2. The I_e/I_m ratio vs. temperature curves for DMPC vesicles containing various levels of cholesterol. The lines are the linear regression best fit to the data points. The solid line is the linear regression line for unmodified DMPC vesicles. The correlation coefficient and the number of points are stated first for the temperature range 10–23°C and then for the temperature range 25–40°C. ●–·–●, 5% cholesterol ($r = 0.992$, $n = 12$; $r = 0.004$, $n = 16$). ×------×, 10% cholesterol ($r = 0.990$, $n = 9$; $r = 0.992$, $n = 15$). O·····O, 20% cholesterol (one line, see text, $r = 0.994$, $n = 22$).

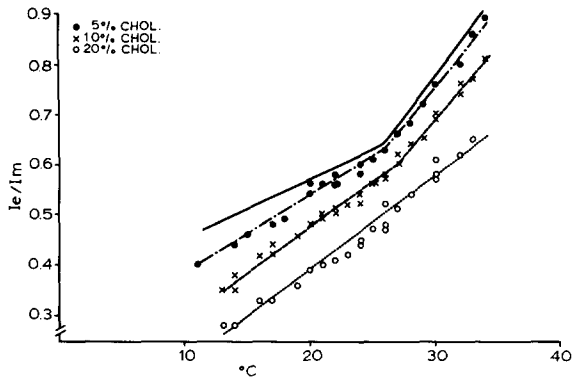

Fig. 3. The I_e/I_m ratio vs. temperature curves for DMPC vesicles with 10% lysoDMPC and varying amounts of cholesterol. The lines are the linear regression best fit to the data points. The solid line is the linear regression line for unmodified DMPC vesicles. The correlation coefficient and the number of points are stated first for the temperature range 10–23°C and then for the temperature range 25–40°C. ●–·–●, 5% cholesterol ($r = 0.993$, $n = 11$; $r = 0.994$, $n = 15$). ×------×, 10% cholesterol ($r = 0.006$, $n = 14$; $r = 0.998$, $n = 18$). ○·····○, 20% cholesterol (one line, see text, $r = 0.993$, $n = 23$).

earity demonstrates that in our system, which uses 4.4% pyrene, pyrene excimer formation is diffusion-controlled.

The I_e/I_m vs. temperature curve for DMPC vesicles consists of two discrete straight lines ($P < 0.01$) intersecting at a temperature of about 24°C, which is the transition temperature (data not shown). The linearity of all the DMPC vesicle plots reveals that there is no pretransition, which has previously been noted for unilamellar vesicles [13]. There was no hysteresis observed in the I_e/I_m vs. temperature curves, which is in agreement with previous reports [3].

The effects of the I_e/I_m ratio of adding lysoDMPC to vesicles are presented in Fig. 1. The data points in all figures are those from several experiments on the given composition, conducted on different days. The addition of lysoDMPC fluidizes the bilayer and the more lysoDMPC added the larger the increase in fluidity. We compare the lines above and below the transition temperature separately. Each line is significantly different ($P < 0.01$) from any other line by t-test on intercepts. There is no significant change in the transition temperature.

Fig. 2 presents the results of adding cholesterol to DMPC vesicles. Above T_c, the presence of up to 10% cholesterol in the vesicle has no effect on the fluidity of the vesicle; there is no significant difference in either slopes or intercepts. Below T_c, the presence of 5% cholesterol produces a significant decrease in fluidity; the presence of 10% cholesterol produces a further decrease in fluidity. At the level of 20% cholesterol, both above and below T_c, there is a large decrease in fluidity, and the phase transition disappears.

The results of adding cholesterol to DMPC vesicles already containing 10% lysoDMPC are shown in Fig. 3. Above T_c, the presence of 5% cholesterol in these vesicles does not significantly decrease the fluidity; the presence of 10% cholesterol does decrease the fluidity ($P < 0.01$ on intercept). The presence of 20% cholesterol de-

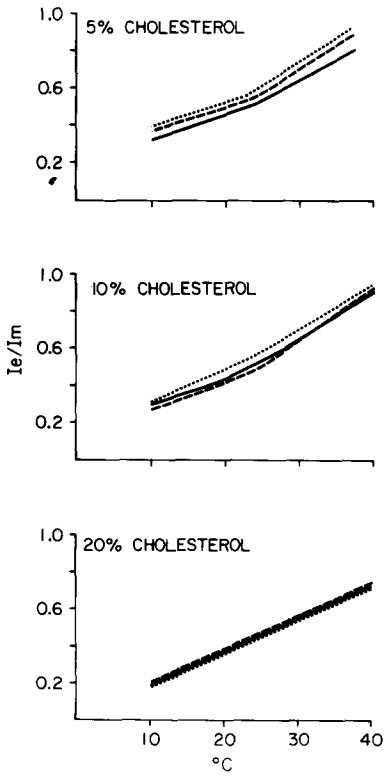

Fig. 4. The I_e/I_m ratio vs. temperature curves for DMPC vesicles with lysoDMPC and cholesterol present. Each panel presents the results on DMPC vesicles containing a constant level of cholesterol, with 0% (———), 5% (------), or 10% (·····) lysoDMPC.

creases the fluidity further ($P < 0.01$ slope and intercept), and removes the phase transition. Below T_c, each successive addition of cholesterol produces a significant decrease in fluidity. The results of adding cholesterol to vesicles containing 5% lysoDMPC produce the same sort of pattern (data not shown).

We can use the same data to compare vesicles containing the same cholesterol concentration (Fig. 4). Adding lysoDMPC to vesicles containing 5% cholesterol produces a proportional increase in fluidity. Adding lysoDMPC to vesicles containing 10% cholesterol produces a significant change only at the 10% lysoDMPC level. With vesicles containing 20% cholesterol, there is no change in fluidity at up to 10% lysoDMPC.

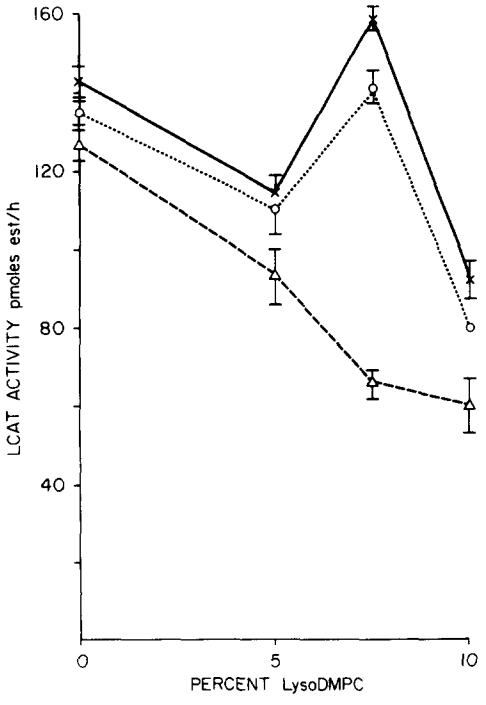

Fig. 5. Lecithin:cholesterol acyltransferase (LCAT) activity, expressed in pmol cholesterol esterified per h, vs. percent lysoDMPC present in DMPC vesicles. Each point is an average of at least three different experiments. Each vesicle composition was assayed in triplicate in each experiment, as described in Materials and Methods. × ——— ×, Vesicles containing 5% cholesterol and varying amounts of lysoDMPC, O····· O, vesicles containing 10% cholesterol and varying amounts of lysoDMPC, Δ- - - - -Δ, vesicles containing 20% cholesterol and varying amounts of lysoDMPC.

In Fig. 5 are presented the lecithin:cholesterol acyltransferase activities on vesicles containing various mixtures of cholesterol and lysoDMPC, plotted vs. percent lysoDMPC. For vesicles containing 5% cholesterol, the enzyme activities at each level of lysoDMPC are significantly different from the enzyme activities at 10% cholesterol, which follow the same pattern. For vesicles containing 20% cholesterol, the fall in enzyme activity on the addition of increasing amounts of lysoDMPC is linear ($P < 0.05$).

Discussion

The results presented in Fig. 1 show that lysoDMPC fluidizes the bilayer. LysoDMPC has the same headgroup as DMPC, but only one acyl chain. Thus, while a lysoDMPC molecule can replace a DMPC molecule in the bilayer with no headgroup change, the lack of a fatty acid chain breaks up the packing of the chains in the bilayer. The result could be a more fluid bilayer.

In order to interpret the effects on fluidity of the addition of cholesterol to the bilayer (Fig. 2), we use the model of Presti et al. [14]. With this model above T_c at the 5 and 10% levels of cholesterol, there is uncomplexed phospholipid available which is fluid, and there are no separate domains of 'rigid' cholesterol-phospholipid complex as occur below T_c. There are thus no barriers to pyrene diffusion and we observe no change in fluidity.

Below T_c when cholesterol is present, there are two phospholipid domains: gel-state phospholipid and relatively fluid complexed phospholipid. The 5 and 10% cholesterol curves in Fig. 2 in this temperature region can be interpreted as the sum of the probe reporting from the two domains. Each line is significantly different from each other line ($P < 0.01$ on intercepts).

At the level of 20% cholesterol, according to Presti et al., there is no uncomplexed phospholipid left, so there is no phase transition. The acyl chain motion of the complexed phospholipid is restricted by the cholesterol, so the fluidity of the bilayer should decrease. The 20% cholesterol curve supports this model. A previous study [1] reports no change in lateral diffusion coefficient in PC bilayers until the addition of 20% cholesterol, upon

which the diffusion coefficient significantly decreases.

Formation of a complex between lysophospholipid and cholesterol that lessens the effect of either molecule on properties of the phospholipid bilayer has been previously postulated. Rand et al. [15], suggested that a molecule of cholesterol can "fill the space of the missing fatty acyl chain" of the lysophospholipid. Ramsammy and Brockerhoff [16] found that when a 1 : 1 mixture of egg lysoPC and cholesterol is sonicated, it forms single bilayer vesicles whose physical parameters are between pure egg PC and 1 : 1 egg PC-cholesterol vesicles. These vesicles can incorporate no additional cholesterol.

A possible molecular basis for such a complex can be derived from the model of Presti et al. To form a complex with cholesterol according to this model, a phospholipid needs hydrocarbon chains for Van der Waals interaction with the planar alpha face of the cholesterol, and it needs a 2-glycerol oxygen available for a hydrogen bond to the cholesterol hydroxyl. The lysophospholipid retains the necessary characteristics although the complex might be less tight because of the availability of only one chain for Van der Waals interaction.

The existence of such a complex would not necessarily mean that the effects of the presence of a certain mol% of lysophospholipid in a bilayer would be cancelled completely by the addition of the same mol% of cholesterol. No assumption can be made that a complex between one lysophospholipid and one cholesterol will behave exactly as one phospholipid in the bilayer.

Complex formation can be used to explain the results presented in Fig. 3. Above T_c, adding 5% cholesterol to DMPC vesicles already containing 10% lysoDMPC does not significantly decrease the fluidity. This can mean that while cholesterol and lysoDMPC do complex, ther is not enough cholesterol complexing with the lysophospholipid to cause a measurable decrease in the fluidity. Adding 10% cholesterol reduces the fluidity; there now is sufficient cholesterol for complex formation to decrease the effect of lysoDMPC on the bilayer. Adding 20% cholesterol, according to the model, would cause both the lysophospholipid-cholesterol complex, which decreases the fluidizing effect of the lysoDMPC, and the phospholipid-cholesterol complex, which restricts phospholipid acyl chain motion, to form, with a resultant substantial decrease in fluidity. This decrease is observed experimentally. There is no phase transition at 20% cholesterol because there is no uncomplexed phospholipid domain available, as previously discussed.

Below T_c, the presence of 5, 10 and 20% cholesterol produce significant proportionate decreases in the fluidity. This is the same pattern as observed in Fig. 2, and can be explained in the same manner: the pyrene probe is reporting the sum of the two domains present. It cannot be deduced from the data whether the lysoDMPC is in either domain exclusively, or in both domains, since we cannot see each domain separately.

In comparing the data on vesicles with the same cholesterol concentrations, (Fig. 4) it is evident that more lysoDMPC is needed to alter the fluidity of vesicles which contain more cholesterol, as would be expected if lysoDMPC and cholesterol form a complex. Both 5 and 10% levels of lysoDMPC increase the fluidity of 5% cholesterol vesicles. Up to 10% lysoDMPC does not alter the fluidity of 20% cholesterol vesicles.

In summary, cholesterol in a phospholipid bilayer decreases the fluidity; lysoDMPC increases the fluidity. When cholesterol and lysoDMPC are both present in the bilayer, their effects on fluidity cancel out, which can be explained by a complex of the type of Presti et al.

The data presented in Fig. 5 show that the lowest level of cholesterol has the highest lecithin:cholesterol acyltransferase activity. This reversal of the usual order is due to the nonlinearity of the lecithin:cholesterol acyltransferase activity vs. percent cholesterol plot above about 4% cholesterol.

Soutar et al. [3] have shown that the pattern of lecithin:cholesterol acyltransferase activity with respect to temperature at a constant level of cholesterol in various PCs can be explained in terms of the fluidity changes of those PCs with respect to temperature. Comparing two points on the temperature curve, the point at which a particular PC was more fluid was also the point of higher lecithin:cholesterol acyltransferase activity.

LysoDMPC increases the fluidity of a bilayer

(Fig. 2) so could it increase lecithin:cholesterol acyltransferase activity by that means. However, Fielding et al. [18] have reported that lysophospholipid inhibits lecithin:cholesterol acyltransferase, which obviously would decrease activity. Therefore, the observed increase or decrease of enzyme activity on the addition of lysoDMPC depends upon a balance between fluidization of the vesicle, which would increase enzyme activity; and inhibition, which would decrease activity.

The data presented in Fig. 5 can be interpreted at the 5 and 10% cholesterol levels in terms of fluidity changes (Fig. 4). There is a significant decrease in lecithin:cholesterol acyltransferase activity when comparing 5% cholesterol vesicles with 0% lysoDMPC to those with 5% lysoDMPC. If, at this vesicle composition, the decrease in enzyme activity due to inhibition by lysoDMPC is greater that the increase in activity due to bilayer fluidization by lysoDMPC, an inhibition would be observed on adding 5% lysoDMPC to these vesicles, which is the experimental result. For vesicles containing 5% cholesterol + 7.5% lysoDMPC, the experimental observation of an increase in activity can mean that the bilayer-fluidizing effect of lysoDMPC overcomes the inhibitory effect of lysoDMPC. For vesicles containing 5% cholesterol + 10% lysoDMPC, the enzyme activity decreases, which can mean that the inhibitory effect of lysoDMPC is dominant at this level of lysoDMPC.

The results for vesicles containing 10% cholesterol with varying levels of lysoDMPC follow the 5% cholesterol pattern and can be interpreted in the same manner. The enzyme activity for each lysoDMPC level is less than that for vesicles containing 5% cholesterol. This can be because the same level of lysoDMPC can increase the fluidity of vesicles containing 5% cholesterol more than those containing 10% cholesterol (Fig. 4); and increasing the fluidity of the vesicles means an increase in enzyme activity; as has been discussed previously.

The enzyme activities on the vesicles containing 20% cholesterol decrease with increase in lysoDMPC level. With the 5 and 10% cholesterol curves, there are fluidity increases (Fig. 4) to balance the inhibitory effect of lysoDMPC, but there are no such increases in fluidity at the 20% cholesterol level, so we observe a continual decrease in activity.

In summary, lecithin:cholesterol acyltransferase activity is affected by the fluidity of a bilayer. Vesicle bilayer fluidity changes can explain enzyme activity levels. At certain levels of cholesterol, adding lysoDMPC can result in an increase in observed lecithin:cholesterol acyltransferase activity. This is the first report of a rise in lecithin:cholesterol acyltransferase activity on the addition of an inhibitor.

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