A fluorescence energy transfer study of lecithin-cholesterol vesicles in the presence of phospholipase C

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Abstract **We demonstrate Förster resonance energy transfer from dehydroergosterol to dansylated lecithin in lecithin-cholesterol vesicles and characterize the vesicles in the presence of the pro-nucleating enzyme, phospholipase C (PLC). Exposure to phospholipase C causes a temporary decrease in the dehydroergosterol to dansyl fluorescence ratio followed by an increase to and above the initial value. The temporary decrease in the fluorescence ratio results from an increase in the dansylated lecithin intensity that coincides with a dansyl blue shift. The extent of the blue shift correlates with the level of diacylglycerol generated in situ by PLC, suggesting an increased association between dansylated lecithin and cholesterol as membrane fluidity increases and membrane polarity decreases. The subsequent increase in the fluorescence ratio results from both an increase in the dehydroergsterol intensity and a concomitant decrease in the dansylated lecithin intensity of equal magnitude. This signifies a reduction in energy transfer from dehydroergosterol to dansylated lecithin and indicates an increased separation between the two fluorophores. The increase in the fluorescence ratio persists beyond the time scales for vesicle aggregation and fusion, as measured by turbidity, and precedes the onset of macroscopic cholesterol crystals observed with an optical microscope. Thus, the increased separation between dehydroergosterol and dansylated lecithin is consistent with a mechanism of cholesterol nucleation from the vesicles. Moreover, the onset and rate of increase in the fluorescence ratio correlate with the cholesterol:lecithin mole ratio of the vesicles. Fluorescence energy transfer from dehydroergosterol to dansylated lecithin therefore shows potential as a methodology for measuring cholesterol nucleation in model bile.**—Wrenn, S. P., E. W. Kaler, and S. P. Lee. **A fluorescence energy transfer study of lecithin-cholesterol vesicles in the presence of phospholipase C. *J. Lipid Res.* 1999.** 40: **1483–1494.**

Supplementary key words gallstones • dehydroergosterol • dansyl • nucleation

Lecithin-cholesterol vesicles are the predominant carriers of cholesterol in bile and allow the temporary existence of bile that is supersaturated with cholesterol yet void of cholesterol crystals (1). The vesicles are thermodynamically metastable and eventually revert to a lamellar phase which accommodates a maximum cholesterol:lecithin ratio of unity (2). The cholesterol:lecithin ratio of the parent vesicles can be as high as 2:1 (3), however, and any excess cholesterol nucleates from the vesicles during or soon after the transition to a lamellar phase (4). Once formed, the cholesterol nuclei grow into macroscopic cholesterol monohydrate crystals that are the precursors to gallstones.

Although this sequence is expected to be the same in both healthy and diseased individuals, the rate of cholesterol nucleation from lecithin-cholesterol vesicles varies and appears to be the determining factor in gallstone formation (5). The reason for the variance in the rate of cholesterol nucleation remains unknown and is not simply related to cholesterol supersaturation. For biles with a given cholesterol supersaturation or cholesterol saturation index (CSI), nucleation in patients with stones is more rapid than in healthy individuals (6). Recent studies attribute this variance in the nucleation rate to a variety of "pro-nucleating" factors in diseased individuals and "anti-nucleating" factors in healthy individuals (7–10).

Phospholipase C (PLC) is a well known pro-nucleating factor in model bile that has recently been detected in the gallbladder bile of humans (11). PLC is a Ca^{2+}-requiring enzyme that hydrolyzes the phosphoester bond of lecithin to yield a water-insoluble diacylglycerol (DAG) and the zwitterionic head group, phosphorylcholine. The pro-nucleating action of PLC is likely due to the DAG, which destabilizes lipid bilayers and accelerates vesicle aggregation and fusion (12, 13). Recently, a detailed analysis of PLC-induced vesicle aggregation and fusion kinetics was performed on a model system of sonicated lecithin-cholesterol vesicles using a combination of turbidity, QLS, and fluorescence quenching measurements (14).

Abbreviations: L, egg yolk lecithin; CH, cholesterol; DHE, dehydroergosterol; DL, 1-(O-(11-(5-dimethylaminonaphthalene-1-sulfonyl)amino) undecyl)-2-decanoyl-*sn*-glycero-3-phosphocholine; PLC, phospholipase C; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid; DAG, diacylglycerol.

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Although the "pro-aggregating" and "pro-fusogenic" properties are well established, the "pro-nucleating" aspects of PLC, as with any pro-nucleating factor, are not understood. Crystal formation is usually reported in terms of a nucleation time, which is the time required for crystals to become apparent with an optical microscope (6). The method is only semi-quantitative and intrinsically can provide no information about nucleation kinetics (15). The purpose of this work is to characterize the pro-nucleating effects of PLC in model systems of sonicated lecithin-cholesterol vesicles with cholesterol:lecithin mole ratios ranging from 0.5 to 1.6. To do this, a new approach is taken in which the lecithin-cholesterol vesicles are labeled with the fluorescent analogs, dehydroergosterol (DHE) and dansylated lecithin (DL). The dansyl excitation spectrum overlaps the DHE emission spectrum, allowing the possibility of energy transfer between the two fluorophores within the vesicles. As DHE co-crystallizes with cholesterol (16), the nucleation of cholesterol and DHE out of the vesicles should separate the DHE from DL and dissipate energy transfer.

The use of DHE as a fluorescent analog of cholesterol is well established, and numerous studies confirm that DHE is a non-invasive membrane probe (17–21) that can replace cholesterol (22–24). The use of DL as a lecithin probe is also well known, and fluorescence emission from the dansyl chromophore depends heavily on solvent properties (25–28). In this work dansylated lecithin quenches the fluorescence from DHE because the spectra of the two fluorophores overlap. The DL is therefore not intended as a lecithin probe, and in principle any quencher should suffice. However, the use of dansylated lecithin avoids the usual concerns that arise when probe molecules do not resemble native lipids, and neither DHE nor DL is expected to perturb the model bile system to any appreciable extent. At least one study specifically addresses the incorporation of both DHE and DL into lecithin-cholesterol vesicles and finds these molecules to be suitable membrane probes (29). We now report the use of a new fluorescence technique involving energy transfer from DHE to DL to study lecithin-cholesterol vesicles in the presence of PLC.

MATERIALS AND METHODS

Fluorescence studies

Egg yolk lecithin (L), cholesterol (CH), dehydroergosterol (DHE), and ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid (EGTA), all of analytical grade, were purchased from Sigma (St. Louis, MO) and used without further purification. Phospholipase C (EC 3.1.4.3) (PLC) of highest purity from *Clostridium perfringens* was also purchased from Sigma and used as received. 1-(O-(11-(5-dimethylaminonaphthalene-1-sulfonyl) amino) undecyl)-2-decanoyl-*sn*-glycero-3-phosphocholine (DL) was purchased from Molecular Probes (Eugene, OR). The glycerol assay kit (Cat. No. 148-270) was purchased from Boehringer Mannheim (Indianapolis, IN).

Mixtures of CH, L, DHE, and/or DL were coprecipitated from chloroform to give lipid films of desired composition. In all cases, DHE accounted for 28 mole% of the total sterol, and DL comprised 4.5 mole% of the total lecithin. Films were hydrated with an aqueous buffer solution (0.15 m NaCl, 5 mm $CaCl_2$, 5 mm HEPES, and 0.02 wt % NaN_3, pH = 7.4) and directly sonicated (Heat Systems Ultrasonics Model W-225) for 90 min. Sonication was done in an ice/water bath under a nitrogen blanket to prevent lipid degradation. Sonicated dispersions were centrifuged at 32,000 *g* and 25°C for 2 h (DuPont Sorval RB), and the supernatant was diluted with buffer solution to yield unilamellar vesicles at an overall lipid concentration of 0.15 mm. All experiments were performed at 25°C, and samples were maintained under a nitrogen atmosphere to prevent lipid oxidation.

Quartz cuvettes were filled with 2.5-mL aliquots of the vesicle solution, and 10 μL of aqueous phospholipase C was added to induce vesicle aggregation and fusion. In one experiment, 40 μL of a 140 mm EGTA solution was added at various times to quench the action of PLC. Turbidity and fluorescence were monitored over time using a Perkin-Elmer Lambda 2 spectrophotometer and an SLM Aminco 8100 spectrofluorometer, respectively. The turbidity measurements were used to correct fluorescence data for the inner filter effect according to

$$I_{cor} = \frac{I_{obs}\, 10^{OD_{ex}\, x/l}\, dx\; 10^{OD_{em}\,(l-y)/l}\, dy}{\int_a^b dx \int_0^a dy} \qquad \textit{Eq. 1)}$$

where I_{obs} is the observed fluorescence intensity, I_{cor} is the corrected fluorescence intensity, and OD_{ex} and OD_{em} are the optical densities measured at the excitation and emission wavelengths, respectively. Excitation light travels in the x-direction and penetrates the entire path length, *l*. Emission light travels in the y-direction, but only the area from y = a to y = b is illuminated.

Phospholipase C kinetics

In separate experiments, lecithin-cholesterol vesicles were prepared that did not contain fluorescent labels. These vesicles were sonicated for 2 h and were not diluted, yielding vesicles with a L:CH molar ratio of 2:1 at an overall lipid concentration of 9 mM.[2] Lecithin concentration was measured by a modification of the Bartlett method for lipid phosphorus (30), and cholesterol content was determined using a cholesterol oxidase assay (31). The unlabeled vesicle solution was divided into 10-mL aliquots and added to vials equipped with stir bars. Phospholipase C of varying concentration was added to each vial, and 200-μL samples of the reacting mixtures were quenched periodically with EGTA. The amount of diacylglycerol generated in each sample was determined by first converting diacylglycerol to free glycerol by alkaline hydrolysis (32), and the free glycerol was measured with an enzymatic kit from Boehringer Mannheim (Cat. No. 148-270).

RESULTS

Fluorescence properties

Figure 1 shows the molecular structures and **Fig. 2** shows the fluorescence spectra for (a) dehydroergosterol

[2] The levels of diacylglycerol generated during the fluorescence studies were too small to be detected with the Boehringer-Mannheim kit. Thus, a separate study of PLC kinetics was performed in a more concentrated vesicle sample to generate higher DAG levels. The time scales for PLC activity in the kinetic studies do not apply directly to the fluorescence studies. Rather, PLC is active over longer times in the fluorescence studies due to the smaller concentrations used therein.

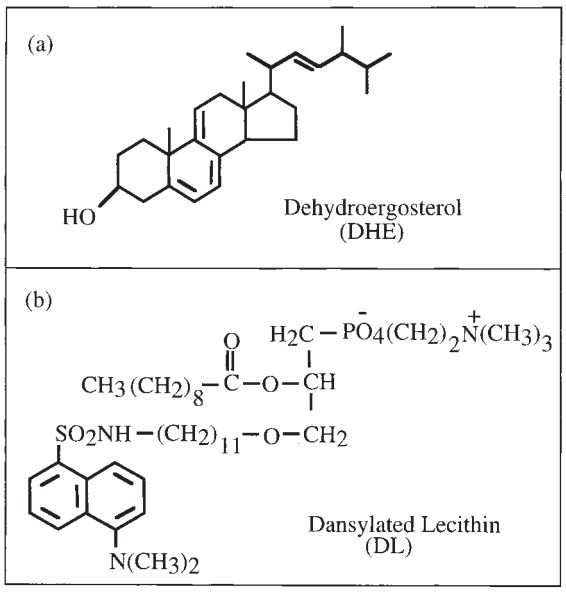

Fig. 1. Molecular structures of (a) dehydroergosterol (DHE) and (b) dansylated lecithin (DL).

(DHE) and (b) dansylated lecithin (DL). The spectra in Fig. 2a are from DHE-labeled lecithin-cholesterol vesicles at a concentration of 98 μm lecithin, 37 μm cholesterol, and 15 μm DHE, measured at an excitation wavelength of 300 nm (emission spectrum) and an emission wavelength of 430 nm (excitation spectrum). DHE exhibits one excitation maximum (329 nm), one excitation shoulder (341 nm), one emission shoulder (355 nm), and two emission maxima (373 and 393 nm). The small emission peak at 340 nm is Raman scattering from water. Note that DHE does not fluoresce above 500 nm (Fig. 2a). Unlike DHE, the dansyl chromophore exhibits single excitation and emission maxima, as shown for DL-labeled lecithin-cholesterol vesicles with lipid concentrations of 94 μm lecithin, 4 μm DL, and 52 μm cholesterol (Fig. 2b). The peak excitation and emission wavelengths of DL are 343 nm and 522 nm, respectively, as measured at an excitation wavelength of 343 nm (emission spectrum) and an emission wavelength of 522 nm (excitation spectrum).

Energy transfer

Figure 3a shows the fluorescence emission spectrum for a sample of lecithin-cholesterol vesicles labeled with both DHE and DL (bold line), along with the emission spectra for samples labeled with DHE alone (solid line) and DL alone (dashed line) from Fig. 2. Note that the excitation wavelength is 300 nm in Fig. 3, whereas an excitation wavelength of 343 nm was used in Fig. 2b. Lipid concentrations in the sample with both fluorophores were 94 μm lecithin, 4 μm DL, 37 μm cholesterol, and 15 μm DHE. The emission spectrum of this sample is not a simple algebraic sum of the samples labeled with single fluorophores. Rather, the DHE fluorescence maximum at 373 nm drops in the presence of DL to 68% of the value obtained with DHE alone. Moreover, the DL maximum at 522 nm grows in the presence of DHE by a factor of 2.5 when compared to the value obtained with DL alone. This is evidence of energy transfer from DHE to DL in labeled lecithin-cholesterol vesicles.

Figure 3b shows the fluorescence excitation spectra for the three samples described in Fig. 3a. Although spectra appear broadened due to a change in abscissa scale, the features of vesicle samples labeled with single fluorophores (from Fig. 2) remain unchanged. Hence, vesicles labeled only with DL (dashed line) exhibit a single excitation maximum at 343 nm when measured at an emission

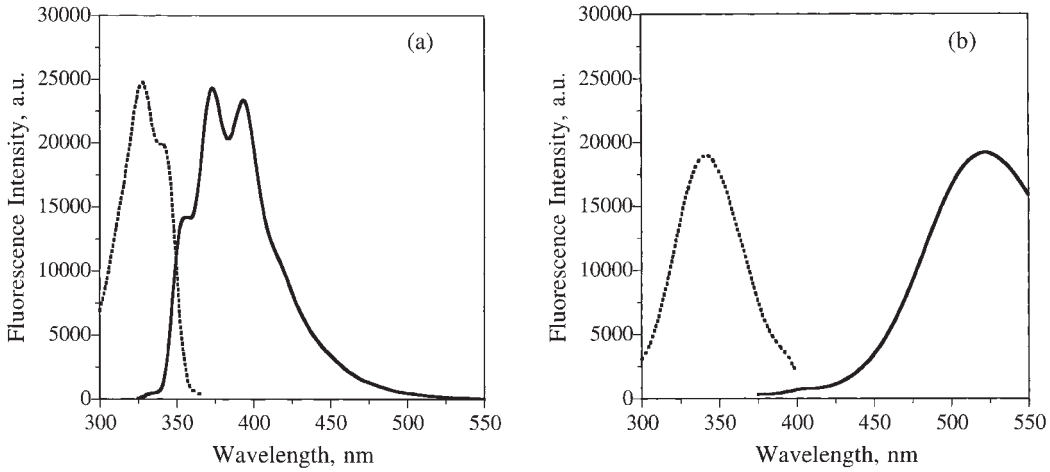

Fig. 2. Fluorescence spectra of lecithin-cholesterol vesicles labeled with (a) DHE and (b) DL. (a) Lipid concentrations in the DHE sample were 98 μm lecithin, 37 μm cholesterol, and 15 μm DHE. The DHE excitation spectrum (dashed) was measured at an emission wavelength of 430 nm, and the DHE emission spectrum (solid) was generated with 300 nm excitation light. DHE exhibits an excitation maximum at 329 nm, an excitation shoulder at 341 nm, an emission shoulder at 355 nm, and emission maxima at 373 nm and 393 nm. The small emission peak at 340 nm is Raman scattering of water. (b) Lipid concentrations in the DL sample were 94 μm lecithin, 4 μm DL, and 52 μm cholesterol. The DL excitation spectrum (dashed) was measured at an emission wavelength of 522 nm, and the DL emission spectrum (solid) was generated with 343 nm excitation light. DL exhibits a single excitation maximum at 343 nm and a single emission maximum at 522 nm.

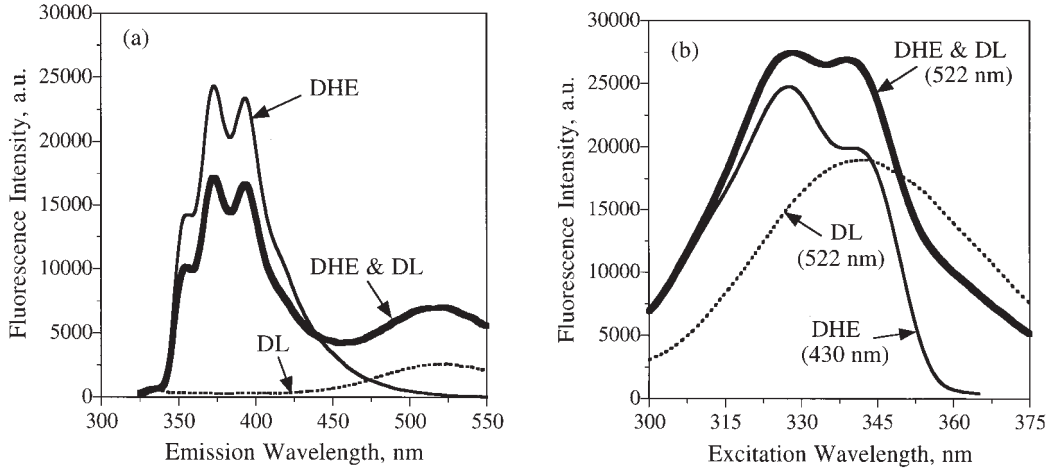

Fig. 3. (a) Emission spectra obtained at an excitation wavelength of 300 nm are given for three samples of labeled lecithin-cholesterol vesicles. The solid spectrum is that of vesicles labeled only with DHE at lipid concentrations matching those in Fig. 2a. The dashed spectrum is that of vesicles labeled only with DL at lipid concentrations matching those in Fig. 2b. The bold spectrum is that of vesicles labeled with both fluorophores at lipid concentrations of 94 μm lecithin, 4 μm DL, 37 μm cholesterol, and 15 μm DHE. The spectrum from vesicles containing both fluorophores is not the algebraic sum of spectra for vesicles labeled with individual fluorophores. Rather, the combination of DHE and DL causes the DHE intensity at 373 nm to decrease by 32% and enhances the DL intensity by a factor of 2.5 This is evidence of energy transfer from DHE to DL. (b) Excitation spectra are given for the three vesicle samples of Fig. 3a. The spectrum of vesicles labeled only with DL (dashed) was measured at an emission wavelength of 522 nm and exhibits a single maximum. The spectrum of the sample labeled only with DHE (solid) was measured at 430 nm and exhibits two maxima. The spectrum of the sample containing both fluorophores (bold) was measured at an emission wavelength of 522 nm, a wavelength at which DHE does not fluoresce, yet appears similar to the spectrum of the sample labeled with DHE alone. This is further evidence of energy transfer from DHE to DL.

wavelength of 522 nm, and vesicles labeled only with DHE (solid line) exhibit two excitation maxima, at 329 nm and 341 nm. Recall, however, that DHE does not fluoresce above 500 nm, and the sample containing DHE alone was measured at an emission wavelength of 430 nm. The excitation spectrum from the vesicle sample containing both fluorophores (bold line) also exhibits two maxima (at 330 nm and 340 nm) but was measured at an emission wavelength of 522 nm. As DHE does not fluoresce at this emission wavelength, the latter spectrum must be the excitation signal from DL (in the presence of DHE), which now exhibits the spectral characteristics of DHE. This is the clear signature of non-radiative (Förster) energy transfer from DHE to DL in lecithin-cholesterol vesicles.

Effects of phospholipase C

Figure 4 shows the fluorescence changes in vesicles containing 94 μm lecithin, 4 μm DL, 37 μm cholesterol, and 15 μm DHE (i.e., 35 mole% sterol) when exposed to PLC at 0.3 nm. This enzyme loading corresponds to approximately one PLC molecule for every 15 vesicles, where the lecithin aggregation number per vesicle is approximately 23,000 (unpublished quasi-elastic light scattering data). Figure 4a shows temporal changes in the fluorescence emission spectrum (excitation at 300 nm) during the first 2 h after enzyme addition. During this time scale the DHE maximum at 373 nm decreases in value by 7%, whereas the DL maximum nearly doubles in value and undergoes a 32 nm blue shift. These trends are temporary, and beyond 2 h the DHE maximum grows by approximately 5000 intensity units (Fig. 4b). Similarly, the DL intensity decreases by approximately 5000 intensity units and undergoes an additional 3 nm blue shift (Fig. 4b). The excitation spectra exhibit similar trends. Figure 4c shows temporal changes in the excitation spectrum during the first 2 h after enzyme addition, and Fig. 4d shows the changes that occur beyond 2 h. These excitation spectra were measured at an emission wavelength of 500 nm and correspond to DL fluorescence. As is the case for the emission spectrum, the DL fluorescence intensity increases during the first 2 h after PLC addition and subsequently decreases for the duration of the experiment. In addition, the shape of the DL excitation spectrum changes throughout the experiment, with the excitation maximum at 330 nm (peak I) steadily decreasing in value relative to the maximum at 340 nm (peak II). Thus, the excitation spectrum steadily evolves toward a single maximum commensurate with that of vesicles labeled with DL alone. **Figure 5** quantifies these changes via the I/II peak ratio, which is initially greater than 1 but begins a sustained decrease just 40 min after enzyme addition. The slope of the trace of the peak I/II ratio versus time is a measure of the extent to which energy transfer from DHE to DL diminishes.

The intensity changes from Figs. 4a and 4b are summarized in **Fig. 6a**, which gives the DHE intensity (at 373 nm) and the DL intensity (at the maximal wavelength) as a function of time after enzyme addition. It is clear that DHE and DL exhibit opposite behavior in general, but the magnitudes of the changes are not always equivalent. For example, the initial decrease in the DHE intensity is just 40% in magnitude relative to the initial increase in the DL intensity. Moreover, the decrease in DHE intensity

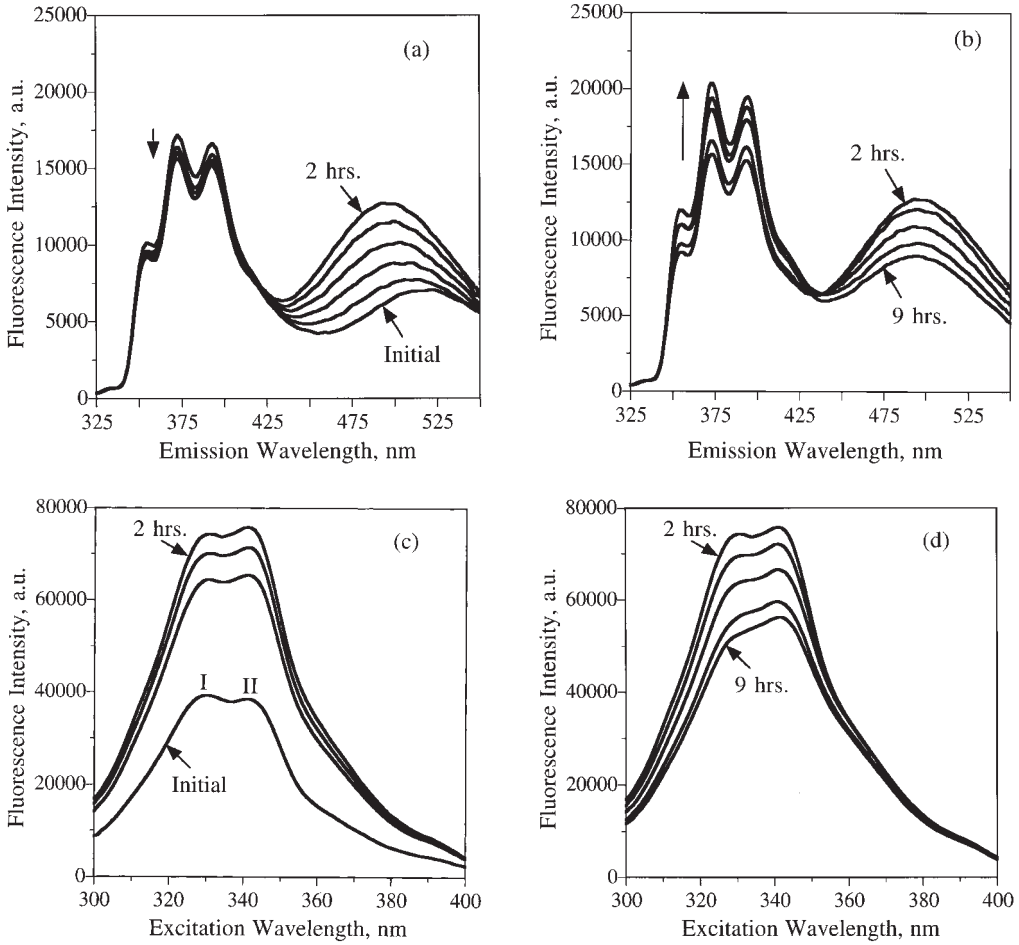

Fig. 4. Temporal changes in fluorescence spectra are shown for lecithin-cholesterol vesicles labeled with DHE and DL at lipid concentrations matching those in Fig. 3 when exposed to PLC at a concentration of 0.3 nm. (a) Emission spectra obtained 0, 10, 20, 40, 60, and 120 min after PLC addition. (b) Emission spectra at 2, 3, 5, 7, and 9 h after PLC addition. (c) Excitation spectra obtained 0, 10, 20, 40, 60, and 120 min after PLC addition. Maxima at 330 nm and 340 nm are defined as peaks I and II, respectively. (d) Excitation spectra at 2, 3, 5, 7, and 9 h after PLC addition. Emission spectra were obtained with excitation at 300 nm, and excitation spectra were measured at an emission wavelength of 500 nm.

persists for just 1 h, whereas the DL increase lasts for 2 h. Beyond 2 h, however, DHE and DL intensities change by nearly the same magnitude and appear as mirror images of one another. These latter changes are consistent with the diminishing I/II excitation peak ratio and signify a reduction in energy transfer from DHE to DL.

As sample turbidity is known to decrease fluorescence intensity via the well known inner filter effect (equation 1), optical density was recorded for each fluorescent sample. Figure 6b shows optical density as a function of time at the excitation wavelength (300 nm) and both emission wavelengths (DHE at 373 nm, DL at 515 nm). The onset of maximal turbidity occurs 3 h, 4 h, and 7 h after PLC addition at 300 nm, 373 nm, and 515 nm, respectively, and turbidity remains essentially constant thereafter. The turbidity profiles in Fig. 6b were used to correct the fluorescence intensities of Fig. 6a for the inner filter effect, as shown in Fig. 6c.

Figure 6c quantifies intensity changes in both fluorophores through the use of a single parameter, the fluorescence ratio (R_F), which is simply the DHE fluorescence intensity at 373 nm divided by the maximal DL fluorescence intensity. Using the intensities of Fig. 6a, R_F (solid line) decreases from an initial value of 2.33 to a minimum of 1.18 in the first 2 h after PLC addition. Beyond 2 h, R_F increases for the duration of the experiment to a final value of 2.03. Using equation 1 to correct the fluorescence intensities in Fig. 6a for inner filter effects, Fig. 6c also gives a corrected R_F (dashed line). Similar to the uncorrected fluorescence ratio, the corrected R_F decreases to a minimum at 2 h and then increases for the duration of the experiment. Thus, the fluorescence changes in Fig. 6a are clearly not an artifact of turbidity, and inner filter effects cannot account for the observed increase in the fluorescence intensities.

Figure 7 shows the changes in R_F for multiple PLC loadings measured in vesicle samples with lipid concentrations of 72 μm lecithin, 3 μm DL, 54 μm cholesterol, and 21 μm DHE. Four enzyme (PLC) loadings were used, and R_F was recorded as a function of time for each. As shown, the general trace of R_F in all samples is similar to that in Fig. 6c. However, the minimum shifts to shorter times and the

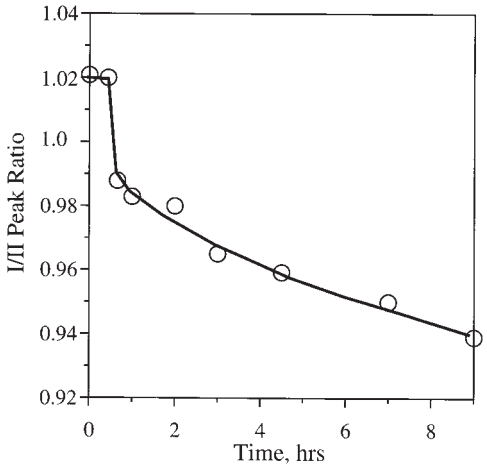

Fig. 5. The excitation peak ratio (I/II) defined in Fig. 4c is plotted as a function of time after PLC addition. A decrease in this ratio begins after 40 min and continues for the duration of the experiment. This signifies a loss of energy transfer from DHE to DL and an increased separation between the two fluorophores.

slope of the R_F trace beyond the minimum becomes steeper with increased enzyme loading.

EGTA quenching

PLC requires calcium to function, and **Fig. 8** shows the effect of deactivating PLC at various times by chelating calcium with EGTA. Forty μL of a 140 mm EGTA solution was added to 2.5 mL of lecithin-cholesterol vesicles labeled with both fluorophores at lipid concentrations of 72 μm lecithin, 3 μm DL, 54 μm cholesterol, and 21 μm DHE. EGTA was injected into one of four samples at 20 min, 45 min, 1 h, and 3 h after enzyme addition, and no EGTA was added to a fifth (control) sample. Figure 8a shows the corresponding DL fluorescence intensity profiles. The profile in all samples follows the control curve up to the point of EGTA injection, after which the DL intensity decreases. Figure 8b shows the effect of PLC deactivation on DHE intensity profiles. Without PLC deactivation, the DHE intensity in the control sample decreases slightly to a minimum at 1 h and then increases by 2000 units. In general, the consequence of PLC deactivation is to prolong and magnify the initial decrease, and lessen the subsequent increase, in the DHE fluorescence intensity.

Phospholipase C kinetics

Due to the small concentrations used in the fluorescence studies, kinetics were measured in the more highly concentrated lipid systems of **Fig. 9** (see footnote 2). This figure shows the reaction profiles for PLC hydrolysis of lecithin-cholesterol vesicles containing 33 mol% cholesterol at an initial lecithin concentration of 6.2 mm, for PLC concentrations of 50 nm, 245 nm, 485 nm, 970 nm, and 1940 nm. Reaction conversion to diacylglycerol (DAG) reaches a limiting value below 100% for all but the highest PLC concentration.

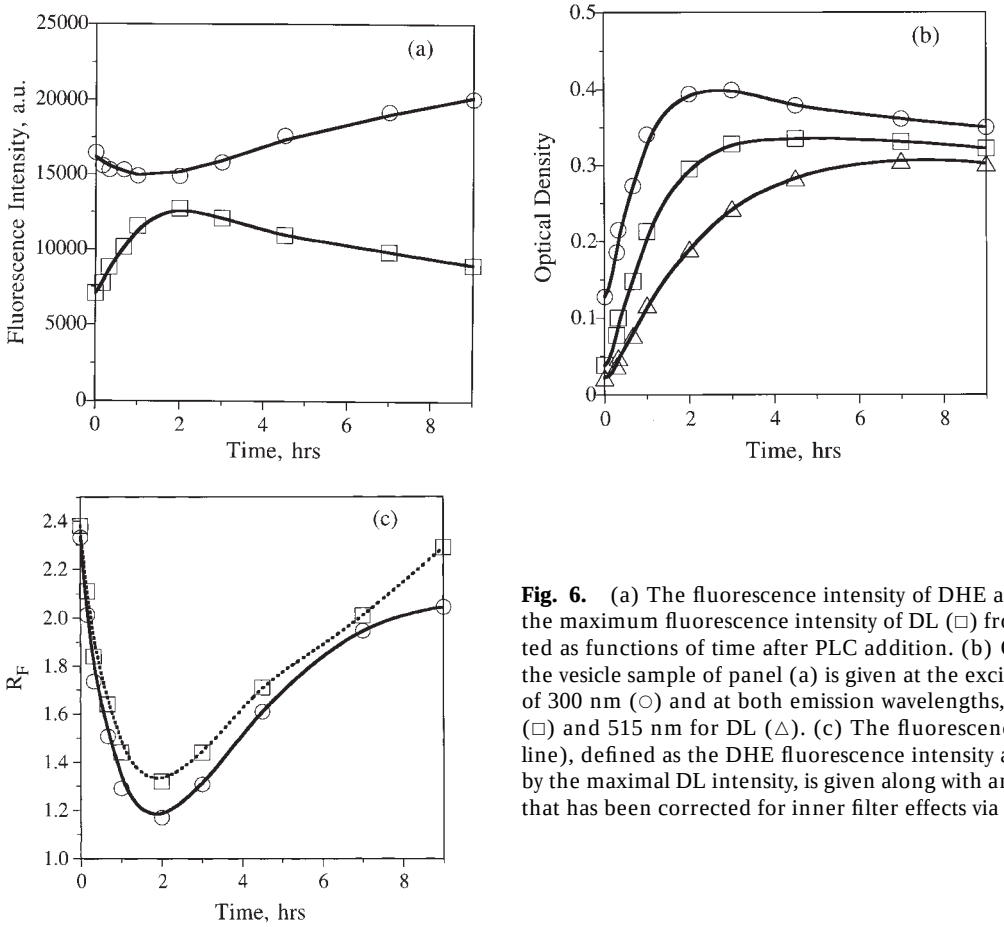

Fig. 6. (a) The fluorescence intensity of DHE at 373 nm (○) and the maximum fluorescence intensity of DL (□) from Fig. 4 are plotted as functions of time after PLC addition. (b) Optical density in the vesicle sample of panel (a) is given at the excitation wavelength of 300 nm (○) and at both emission wavelengths, 373 nm for DHE (□) and 515 nm for DL (△). (c) The fluorescence ratio, R_F (solid line), defined as the DHE fluorescence intensity at 373 nm divided by the maximal DL intensity, is given along with an R_F (dashed line) that has been corrected for inner filter effects via equation 1.

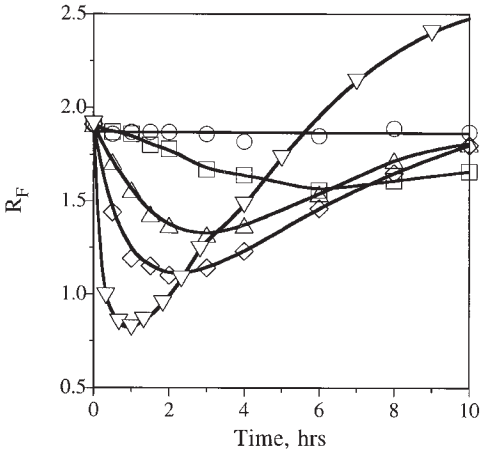

Fig. 7. R_F is plotted as a function of time after PLC addition for PLC concentrations of 0.01 nm (□), 0.1 nm (△), 0.2 nm (◇), and 0.6 nm (▽) and for a control sample with no PLC (○). Lipid concentrations were 72 μm lecithin, 3 μm DL, 54 μm cholesterol, and 21 μm DHE. Higher PLC loadings shift the R_F minimum to shorter times and increase the slope of the R_F trace beyond the minimum.

The limiting conversion is 20% at 120 min for the lowest PLC loading but moves to higher values and appears sooner in time with increasing levels of PLC. Complete hydrolysis occurs after 20 min when the PLC loading is 1940 nm.

A limiting conversion is common in enzymatic systems and occurs when a product of the enzymatic reaction competes with a substrate for the given enzyme (33). The fact that DAG resembles lecithin certainly makes DAG inhibition of PLC activity plausible, and fluorescence assays examining the fusogenic properties of PLC support this view (34). The conversion profiles of Fig. 9, therefore, suggest a simple two-step mechanism for the kinetics of PLC hydrolysis in lecithin-cholesterol vesicles. The first step is production of DAG according to the traditional MichaelisMenten framework, described by the maximal rate, V_{max}, the total enzyme concentration, $[E]_T$, the first order rate constant, k_2, and the Michaelis constant, K_M. The second step is an irreversible binding between DAG and PLC to account for the apparent inhibition of PLC activity. This binding is modelled as a second order process with rate constant, k_3. The solid lines in Fig. 9 represent the hydrolysis rates as predicted by the modified Michaelis-Menten model, using fitted parameters $\{V_{max}/[E]_T\} = k_2 = 1000\ min^{-1}$, $K_M = 2.0$ mm, and $k_3 = 0.035\ (mm \cdot min)^{-1}$. The Michaelis-Menten parameters reported in this work are consistent with results of other authors (35, 36) but are several orders of magnitude greater than the values reported in vivo (37).

Dansyl blue shift

Using the modified Michaelis-Menten model to calculate DAG levels generated during the fluorescence studies, **Fig. 10** shows the dependence of the DL blue shift on DAG conversion. This dependence is unique so that a single curve is obtained, independent of enzyme loading. This shows that the DL blue shift depends simply on the amount of DAG present in the vesicle bilayer, a fact that is easily understood given the solvent polarity dependence of the dansyl Stokes shift. DAG forms by removal of polar lecithin head groups, and the resulting drop in polarity within the vesicle bilayer diminishes solvent relaxation to produce the observed blue shift.

Additional insight regarding the dansyl blue shift comes from **Fig. 11**. Here the dependence of the dansyl emission wavelength is shown as a function of cholesterol loading in the vesicles, in the absence of PLC. The addition of cholesterol induces a dansyl blue shift, and the blue shift increases sharply when the cholesterol loading reaches 43 mole%. The change in slope at 43 mole% cholesterol is consistent with previous X-ray diffraction, nuclear magnetic resonance, and calorimetry studies that

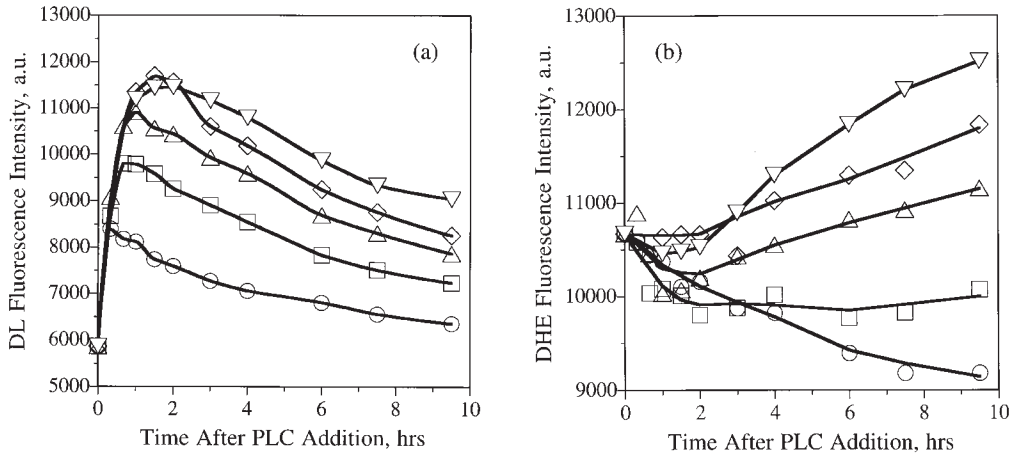

Fig. 8. The effect of deactivating PLC with EGTA at various times after enzyme addition is shown for lecithin-cholesterol vesicles labeled with DHE and DL at lipid concentrations identical to those in Fig. 7. The PLC loading was 0.3 nm, and the EGTA quenching concentration was 2.2 mm. The fluorescence intensity profiles of DL (a) and DHE (b) are shown for samples quenched with EGTA 20 min (○), 45 min (□), 1 h (△), and 3 h (◇) after PLC addition and for a control sample in which no EGTA was added (▽). PLC deactivation terminates the increase in DL intensity and suppresses the increase in DHE intensity.

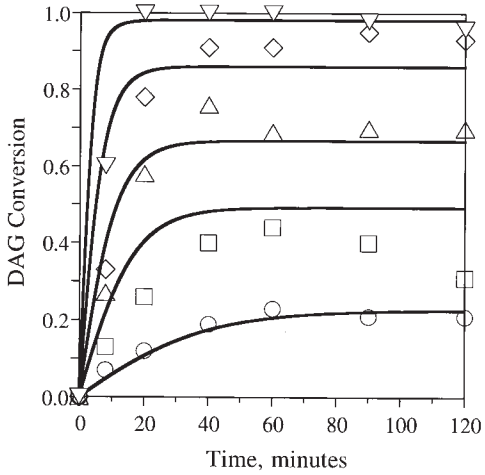

Fig. 9. The kinetics of lecithin hydrolysis by PLC in lecithin-cholesterol vesicles is shown in the form of conversion to 1,2-diacylglycerol (DAG) versus time for various PLC loadings (○ 50 nm, □ 245 nm, △ 485 nm, ◇ 970 nm, and ▽ 1940 nm). Initial lecithin concentration is 6.2 mm, and vesicle composition is 33 mole% cholesterol. Predicted rates of hydrolysis corresponding to these PLC loadings are shown as solid lines, using the following modified Michaelis Menten parameters: $k_2 = 1000\ min^{-1}$, $k_3 = 0.035\ (mm \cdot min)^{-1}$, and $K_M = 2.0$ mm (see text for details).

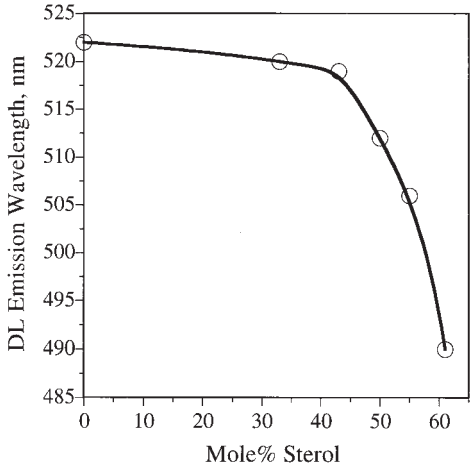

Fig. 11. The effect of cholesterol content on the maximal DL emission wavelength is shown for lecithin-cholesterol vesicles labeled with DHE and DL. Total lipid concentration was fixed at 0.15 mm, DL accounted for 4.5 mole% of lecithin species, and DHE accounted for 28 mole% of sterols. Excitation was at 300 nm. The addition of cholesterol to the vesicle bilayer induces a significant dansyl blue shift when the cholesterol loading exceeds 33%.

indicate abrupt changes in the physical properties of lecithin-cholesterol membranes (38–40).

Influence of CH on DL fluorescence

The cholesterol-driven blue shift indicates an interaction between DL and cholesterol, and this interaction might also account for the increase in DL intensity seen in Fig. 4a. **Figure 12a** compares the DL intensity profile from Fig. 6a (upper curve in Fig. 12a) with results for two additional vesicle samples; one containing no sterol (lower curve) and one containing cholesterol but no DHE (middle curve). There is no change in DL emission intensity (excitation at 300 nm) for vesicles containing no sterol, whereas the initial DL intensity is enhanced from 1400 units in the absence of sterol to 2800 units in the presence of cholesterol alone. Moreover, when the sample containing cholesterol but no DHE is subjected to the action of PLC, the DL intensity grows sharply during the first 2 h, reaches a maximum at 4 h, then gradually decreases for the duration of the experiment. This behavior shows that the initial rise in the DL intensity requires cholesterol but not DHE. If the initial DL intensities in the two samples containing cholesterol are normalized (Fig. 12b), then the DL profiles are identical for the first 2 h. Beyond 2 h the DL intensity decreases in the sample containing DHE but increases until 4 h in the sample without DHE. This shows that the DL intensity enhancement factor of 2.5, owing to energy transfer from DHE, steadily decreases after 2 h, and the point at which a loss in energy transfer commences coincides almost exactly with the minimum in R_F.

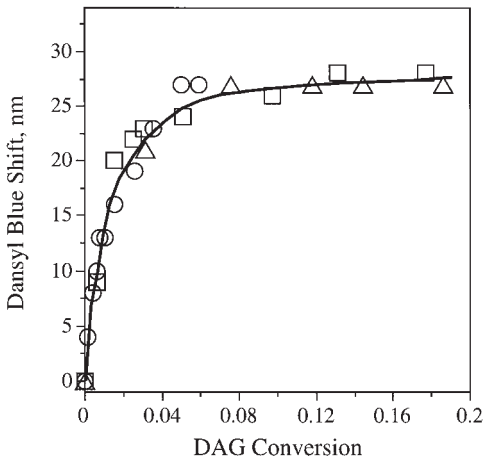

Fig. 10. A correlation of the dansyl blue shift with DAG content is shown. Lecithin-cholesterol vesicles labeled with DHE and DL at lipid concentrations matching those of Fig. 3 were injected with PLC, and the fluorescence emission spectrum (excitation at 300 nm) was recorded for 12 h after enzyme addition. The ensuing dansyl blue shifts for PLC loadings of 0.2 nm (○), 0.8 nm (□), and 3.8 nm (△) are plotted as a function of DAG conversion, where DAG levels were calculated with the modified Michaelis-Menten parameters of Fig. 9.

Effect of cholesterol loading

The effect of cholesterol loading on R_F was also measured, and **Fig. 13** shows the R_F profiles for lecithin-cholesterol vesicles containing 33, 45, and 61 mole% sterol at a fixed PLC loading of 0.3 nm. Cholesterol crsystals are eventually observed in each of these samples, indicating that they were initially supersaturated. The fact that samples with a cholesterol:lecithin mole ratio less than unity yield crystals is not bothersome for two reasons. One reason is that the effective cholesterol:lecithin mole ratio approaches unity in

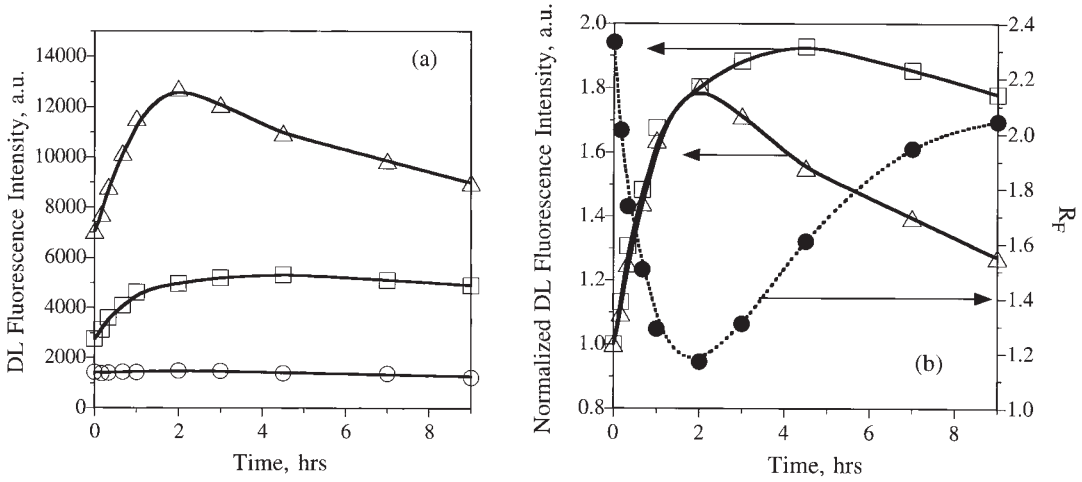

Fig. 12. (a) The DL fluorescence intensity is plotted as a function of time after PLC addition in three different vesicle samples: lecithin vesicles labeled only with DL at concentrations of 146 μm lecithin and 4 μm DL (○); lecithin-cholesterol vesicles labeled only with DL at concentrations of 94 μm lecithin, 4 μm DL, and 52 μm cholesterol (□); and the previous lecithin-cholesterol vesicle sample of Fig. 4 that contains both DHE and DL (△). The presence of cholesterol doubles the initial DL intensity in samples labeled only with DL, and the presence of DHE further enhances the initial DL intensity by a factor of 2.5. DL intensity rises to a maximum only in samples containing cholesterol, and the DL enhancement arising from the presence of DHE decreases after 2 h. (b) The DL intensity profiles of the lecithin-cholesterol vesicle samples from panel (a) are normalized and plotted with the fluorescence ratio (dashed line) from Fig. 6c. The point at which the DL enhancement factor of 2.5, owing to energy transfer from DHE, is lost coincides with the minimum in R_F.

these samples because DAG is produced at the expense of lecithin. Another reason is that the 50 mole% cholesterol saturation limit is valid only for a three component system comprising lecithin, cholesterol, and water. The generation of DAG and phosphocholine might shift the phase boundaries, and a rigorous phase behavior study of the five component system containing DAG and phosphocholine would be required if the saturation limit in a PLC system is desired. Figure 13 shows that the effect of increasing the cholesterol supersaturation is similar to the effect of increasing the loading of the pro-nucleating enzyme, PLC (Fig. 7). Higher cholesterol contents shift the minimum in R_F to earlier times and increase the slope of the R_F trace beyond the minimum. In contrast to PLC, the cholesterol-driven shift of the R_F minimum to earlier times reduces rather than intensifies the magnitude of the minimum.

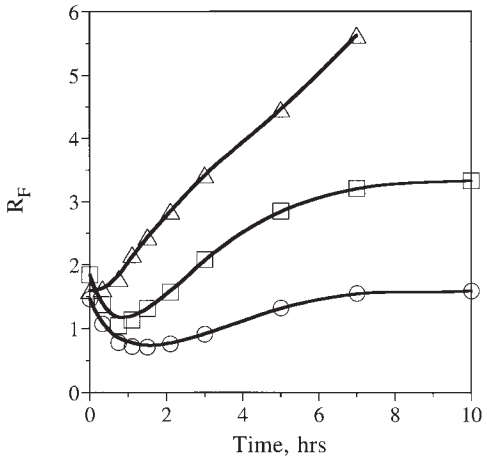

Fig. 13. The effect of varying cholesterol content on the fluorescence ratio is shown. Overall lipid concentration was fixed at 0.15 mm, DL accounted for 4.5 mole% of lecithin species, DHE accounted for 28 mole% of sterol, and PLC concentration was fixed at 0.3 nm. The fluorescence emission spectrum (excitation at 300 nm) is given as a function of time after enzyme addition for vesicles containing 33 mole% (○), 45 mole% (□), and 61 mole% (△) sterol. As with an increased PLC loading, increasing the cholesterol loading shifts the R_F minimum to shorter times and increases the slope of the R_F trace beyond the minimum.

DISCUSSION

Energy transfer

A key result of this work is the identification of (Förster) resonance energy transfer from dehydroergosterol (DHE) to dansylated lecithin (DL). Two features of Fig. 3 demonstrate the effect. First, Fig. 3a shows that the DL fluorescence intensity in the vesicle sample containing both DL and DHE is greater by a factor of 2.5 than in the sample containing DL alone. This enhancement of DL fluorescence intensity is accompanied by a decrease in the DHE fluorescence intensity of similar magnitude. This suggests that DL derives additional fluorescence from the presence of DHE and is evidence for energy transfer. Second, Fig. 3b shows that the sample containing both fluorophores yields an excitation spectrum matching that of DHE but at a wavelength at which DHE does not fluoresce. The (bold) excitation spectrum in Fig. 3b must be due solely to DL, which now exhibits the absorption characteristics of DHE. Thus, non-radiative energy transfer proceeds from DHE to DL in lecithin-cholesterol vesicles.

The existence of energy transfer from DHE to DL dem-

onstrated here provides a new way of characterizing the changes in lecithin-cholesterol vesicles upon exposure to PLC. This is because the efficiency of energy transfer drops sharply as the separation between molecules increases (41, 42), and by repeatedly measuring the extent of energy transfer one obtains the average separation between lecithin and cholesterol as the metastable vesicles attain equilibrium. This information is potentially very useful, as a significant increase in the separation between lecithin and cholesterol is expected when cholesterol nucleates from the vesicles.

Moreover, energy transfer from DHE to DL is special in that DL is itself fluorescent, and this provides additional insight into the modification of bilayer properties when vesicles are exposed to PLC. For example, the dansyl chromophore is extremely sensitive to solvent polarity (25), and the DL blue shift in Fig. 4a likely represents a decrease in bilayer polarity resulting from cleavage of polar phosphocholine head groups by PLC. The fact that the dansyl blue shift correlates with the level of DAG, regardless of PLC loading (Fig. 10), supports this claim. Thus, the use of a fluorescent energy acceptor provides additional information regarding the level of DAG and serves as an indirect indicator of membrane polarity and fluidity.

Also, it is unclear from Fig. 4 whether the initial increase in dansyl intensity, which is accompanied by a slight decrease in DHE intensity, is due to improved energy transfer or some other mechanism involving DL. Here the use of a fluorescent energy acceptor is essential, and the increasing DL intensity in the absence of DHE (Fig. 12) rules out improved energy transfer as the cause for the increase. Similarly, the lack of a DL increase in the absence of cholesterol clearly shows that the initial increase in DL intensity (Fig. 4) stems from an increased interaction between DL and cholesterol, which is not surprising (43). The fact that calcium chelation with EGTA inhibits this interaction suggests that the interaction of DL and DHE too stems from the generation of DAG, perhaps through fluidization of the vesicle bilayer. However, it remains unknown whether the interaction is a general effect involving unlabeled lecithin and cholesterol, is a specific interaction between DL and cholesterol, or is a more specific interaction between cholesterol and the diaclylglycerol version of DL. The latter scenario is plausible because dansyl is polar, and cleavage of the DL phosphocholine head group might induce a reorientation of the dansyl moiety within the vesicle bilayer.

Although the utility of intrinsic DL fluorescence is clear, it is energy transfer from DHE to DL that is potentially most beneficial for the investigation of lecithin-cholesterol vesicles. This is because energy transfer in lecithin-cholesterol vesicles dissipates shortly after exposure to PLC, and the rate and onset of energy transfer dissipation correlate with both enzyme loading and cholesterol supersaturation. This is a very interesting result, and any mechanism that separates DHE from DL might account for it.

The first possibility is diacylglycerol generation by PLC. If sufficient DAG is generated, then DL might partition into a new DAG-rich "oil" phase. This seems unlikely, however, for several reasons. First, the primary effect of DAG is to decrease rather than increase the fluorescence ratio, as shown by the increase in DL intensity and dansyl blue shift (Fig. 4a). Whereas both the initial drop in the fluorescence ratio and the dansyl blue shift correlate with DAG content, the subsequent increase in the fluorescence ratio does not. Second, the fluorescence ratio continues to increase even after the formation of DAG is terminated with EGTA. Unless partitioning into the putative DAG-rich phase is much slower than DAG generation itself, partitioning of probes cannot account for the continued reduction in energy transfer. Third, the fluorescence ratio does not increase upon direct addition of diacylglycerol (unpublished observations). If the increase in the fluorescence ratio is due to DAG, then the DAG formation must proceed in situ. Fourth, no macroscopic oil phase is observed, which agrees qualitatively with the observation that conversion to DAG never exceeds 20%. To answer unequivocally whether a DAG-rich phase forms would require rigorous phase behavior studies in a five component system, a time-consuming task that seems unnecessary in light of the above considerations. Fifth, and perhaps most compelling, we observe a similar dissipation of energy transfer in a PLC-free system comprising lecithin, cholesterol, and sodium taurocholate at near physiological levels (S. P. Wrenn, E. W. Kaler, and S. P. Lee, unpublished results). Thus, it appears that the reduction in energy transfer observed in this work is a general effect and is not related to DAG formation by PLC.

Another mechanism that would separate DHE and DL is cholesterol nucleation from vesicles, and several observations make cholesterol nucleation a likely candidate for the observed dissipation of energy transfer. First, the onset and increase in the fluorescence ratio correlate with PLC loading, as would be expected for any "pro-nucleator." Second, the onset and increase in the fluorescence ratio also correlate with the initial cholesterol content of the lecithin-cholesterol vesicles. As either the enzyme loading or the initial supersaturation increases, dissipation of energy transfer commences earlier and proceeds more rapidly, in agreement with nucleation theory. Third, the reduction of energy transfer continues beyond the time scales for vesicle aggregation and fusion, as measured by turbidity, and precedes the appearance of macroscopic crystals observed with an optical microscope. Thus, the time scale for the dissipation of energy transfer is consistent with a nucleation mechanism. Moreover, all samples in which the fluorescence ratio increased yielded crystals, and all samples in which the fluorescence ratio did not increase yielded no crystals (S. P. Wrenn, E. W. Kaler, and S. P. Lee, unpublished observations).

A third mechanism that is consistent with the observed dissipation of energy transfer is lateral phase separation of cholesterol and DHE within the vesicle bilayer. There is some evidence to suggest that lateral phase separation does proceed in metastable lecithin-cholesterol vesicles (44–46), but there is no clear way to discern whether the increasing fluorescence ratio results from lateral phase separation or sterol nucleation. It might be possible to dis-

tinguish the two mechansims based on the extent of energy transfer dissipation because lateral phase separation maintains DHE and DL at a distance less than or equal to the vesicle diameter. As energy transfer is active over distances of 80 nm, lateral phase separation should not eliminate energy transfer. On the other hand, the separation between DHE and DL will be much greater than 80 nm for direct nucleation from the vesicles and should dissipate energy transfer to a larger extent. However, neither mechanism will eliminate energy transfer completely, as some DHE and DL will reside in close proximity within the equilibrium lamellar phase. In any event, if the increase in the fluorescence ratio is due to lateral phase separation, then this must be considered a precursor step to nucleation because crystals are always observed after the increase. In fact, lateral phase separation could be viewed as the first step in nucleation.

Regardless of whether nucleation proceeds directly by exit of cholesterol from the vesicles or involves a preliminary lateral phase separation, dissipation of energy transfer from DHE to DL shows potential as a nucleation assay to assess putative pro- and anti-nucleating factors in model bile. Here we have reported the effect of the pro-nucleating enzyme, phospholipase C. The technique is currently being extended to model bile systems at physiological concentrations with the aim of measuring the influence of bile salts and other pro- and anti-nucleating agents.

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