Enhancement of *Agkistrodon piscivorus piscivorus* Venom Phospholipase A_2 Activity toward Phosphatidylcholine Vesicles by Lysolecithin and Palmitic Acid: Studies with Fluorescent Probes of Membrane Structure†

Mark J. Sheffield,‡ Brian L. Baker,§ Du Li,§ Noel L. Owen,§ Marilyn L. Baker,‡ and John D. Bell*,‡

Departments of Zoology and Chemistry, Brigham Young University, Provo, Utah 84602

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ABSTRACT: The activity of phospholipase A_2 from snake venom to hydrolyze bilayers of phosphatidylcholines is greatly enhanced by the presence of the hydrolysis products, lysolecithin and fatty acid, in the bilayer. The fluorescence of several probes of membrane structure was used to monitor changes in bilayer physical properties during vesicle hydrolysis. These changes were compared to emission spectra and fluorescence polarization results occurring upon direct addition of lysolecithin and/or fatty acid to the bilayer. The excimer to monomer ratio of 1,3-bis(1-pyrene)propane was insensitive to vesicle hydrolysis, suggesting that changes in the order of the phospholipid chains were not relevant to the effect of the hydrolysis products on phospholipase activity. The fluorescence of 6-propionyl-2-(dimethylamino)-naphthalene (Prodan) suggested that the polarity of the bilayer in the region of the phospholipid head groups increases as the hydrolysis products accumulate in the bilayer. The fluorescence of 6-dodecanoyl-2-(dimethylamino)naphthalene (Laurdan) confirmed that such effects were restricted to the bilayer surface. Furthermore, the lysolecithin appeared to be the product most responsible for these changes. These results suggested that lysolecithin increases the activity of phospholipase A_2 during vesicle hydrolysis by disrupting the bilayer surface, making the phospholipid molecules more accessible to the enzyme active site.

Phospholipase A_2 research is important because of increasing evidence of the enzyme's role in transduction of hormone signals, membrane homeostasis, and the pathology of numerous inflammatory diseases (Jain & Berg, 1989; Kudo et al., 1993). Furthermore, studies using small soluble phospholipases A_2 (PLA_2)[1] from pancreatic juices and snake venoms (which are related structurally to the enzyme secreted by platelets and present in synovial fluid; Kudo et al., 1993; Dennis, 1994) with various types of phospholipid substrates have provided useful information pertinent to general understanding of lipid/protein interactions.

An interesting feature shared by PLA_2 and other lipases has been termed "interfacial activation" [reviewed in Verheij et al. 1981)]. Interfacial activation refers to the fact that PLA_2 activity is much higher toward aggregated compared to monomeric substrate. A related phenomenon is the observation that PLA_2 activity toward aggregated substrate also depends on the physical properties of the aggregate. For example, in the case of zwitterionic substrate, PLA_2 activity is highest when the phospholipid is in micellar form (Verheij et al., 1981). If the phospholipid is organized into a bilayer, the PLA_2 activity is influenced by the curvature of the bilayer, the physical state of the lipids, and the presence of other molecules in the bilayer (Verheij et al., 1981; Jain & Berg, 1989; Vernon & Bell, 1992). A general hypothesis to account for these phenomena is that PLA_2 is sensitive to the "quality of the interface", which limits the ability of the enzyme to interact productively with substrate molecules within the membrane (Verger et al., 1973). However, a molecular understanding of the meaning of "quality of the interface" has remained elusive, although much effort has been applied toward achieving that understanding [e.g., see Verheij et al. (1981), Jain and Berg (1989), and Vernon and Bell (1992) for reviews].

Bilayers of saturated phosphatidylcholine have been used as a model for studying how changes in the physical properties of aggregated substrate affect the activity of PLA_2. The rate of hydrolysis of these bilayers is initially very low after the addition of PLA_2. However, the enzyme activity abruptly increases by 1 or more orders of magnitude after a latency period of variable length ranging from seconds to hours (Tinker & Wei, 1979; Menashe et al., 1981; Apitz-Castro et al., 1982; Bell & Biltonen, 1989b). A variety of evidence indicates that the sudden increase in hydrolytic rate results from the accumulation of the hydrolysis products, lysolecithin and fatty acid, in the bilayer (Apitz-Castro et al., 1982; Jain et al., 1989; Bell & Biltonen, 1992; Burack et al., 1993). The length of the lag phase preceding this event can be manipulated by several experimental perturbations such as the exogenous addition of hydrolysis products (Apitz-Castro et al., 1982; Jain & De Haas, 1983; Bell & Biltonen, 1992; Brown et al., 1993; Bent & Bell, 1995), temperature relative to thermotropic phase transitions (Menashe et al., 1981; Lichtenberg et al., 1986; Bell & Biltonen, 1989b), the transmembrane osmotic pressure (Lichtenberg et al., 1986), membrane curvature (Verheij et al., 1981;

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* To whom correspondence should be addressed.

‡ Department of Zoology.

§ Department of Chemistry.

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[1] Abbreviations: PLA_2, low molecular weight soluble phospholipase A_2; AppD49, basic monomeric aspartate-49 phospholipase A_2 from *Agkistrodon piscivorus piscivorus* venom; DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicles; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; Laurdan, 6-dodecanoyl-2-(dimethylamino)naphthalene; bis-pyrene, 1,3-bis(1-pyrene)propane; lyso-PC, monopalmitoyllysophosphatidylcholine; PA, palmitic acid.

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Lichtenberg et al., 1986; Menashe et al., 1986; Gheriani-Gruszka et al., 1988), and the membrane composition (Gheriani-Gruszka et al., 1988; Zidovetzki et al., 1992). In spite of considerable progress and many models (Verger et al., 1973; Roberts et al., 1977; Tinker & Wei, 1979; Scott et al., 1990; Bell & Biltonen, 1992; Burack et al., 1993; Jain et al., 1993), a comprehensive hypothesis capable of rationalizing the broad range of observations at the molecular level has not yet been achieved.

This paper describes experiments designed to examine physical conditions present in the bilayer at the end of the latency period when sufficient lysolecithin and fatty acid are present to induce the abrupt increase in PLA_2 activity. Our experimental system consists of the alkaline aspartate-49 PLA_2 from *Agkistrodon piscivorus piscivorus* (AppD49), dipalmitoylphosphatidylcholine large unilamellar vesicles (DPPC LUV), and three environment-sensitive fluorescent probes, Prodan, Laurdan, and bis-pyrene. With these probes, we are able to monitor bilayer properties in real time during vesicle hydrolysis. Thus, we can test the relevance of ideas concerning the vesicle "interface quality" to actual hydrolysis kinetics. Our results are then applied in an effort to reconcile various hypotheses regarding the effects of physical state, reaction products, and other additives on PLA_2 activity.

MATERIALS AND METHODS

Fluorescent probes (Prodan, Laurdan, bis-pyrene) were obtained from Molecular Probes (Eugene, OR). Stock solutions of the probes were prepared in dimethylformamide and stored in the dark at −20 °C. DPPC and monopalmitoyllysolecithin (lyso-PC) were purchased from Avanti Polar Lipids (Birmingham, AL). To form LUV, 20 mg of DPPC in chloroform was dried and then hydrated in 50 mM KCl, 3 mM NaN_3, and 10 mM $Na_2B_4O_7$, pH 8, for 1 h at 45 °C with periodic vortexing. (NaN_3 was included in all solutions to prevent bacterial growth.) The multilamellar vesicles formed were then extruded through 0.1-μm polycarbonate filters 10 times at 55 °C to produce LUV (Hope et al., 1985). The concentration of DPPC vesicles was expressed in terms of the bulk phospholipid concentration, which was determined by assay of the phosphate content (Bartlett, 1959). Prodan or Laurdan was added directly to the DPPC/chloroform solution at a phospholipid to probe mole ratio of 0.002 and thus codispersed during vesicle preparation. Bis-pyrene (dissolved in dimethylformamide) was added to preformed vesicles at a mole ratio of 0.001. The vesicles were then heated and cooled repeatedly through the phase transition temperature (about 42 °C) and incubated for several days to equilibrate the probe with the bilayer.

The PLA_2 used in these experiments was isolated and purified according to the procedure of Maraganore et al. (1984) from the venom of *Agkistrodon piscivorus piscivorus* (water moccasin) obtained from the Miami Serpentarium (Punta Gorda, FL) and stored as a lyophilized powder at −20 °C. The enzyme was suspended in stock solutions of 50 mM KCl and 3 mM NaN_3 prior to use. Enzyme concentration was determined by the absorbance at 280 nm (extinction coefficient = 2.2 $mL \cdot mg^{-1} \cdot cm^{-1}$). The final PLA_2 concentration in experiments was 5 μg/mL.

Samples for fluorescence spectroscopy were prepared in 2-mL volumes with 50 mM KCl, 3 mM NaN_3, and 10 mM $Na_2B_4O_7$, pH 8, and the indicated temperatures and phospholipid and $CaCl_2$ concentrations. The lowest calcium concentration employed (1 μM) represents no added $CaCl_2$ and is based on measurement of the concentration of Ca^{2+} as a contaminant in the samples assessed by the fluorescence of a calcium indicator dye as described (Grynkiewicz et al., 1985; Bent & Bell, 1995). Actual Ca^{2+} concentrations determined in this way were typically near 1 μM (Bell & Bent, 1995). Furthermore, only one buffer stock was generally used in each experiment so that the calcium concentration was the same within that experiment. Addition of LUV and/or PLA_2 did not change the concentration of Ca^{2+} in the solution. Temperatures were controlled by a circulating water bath. Unless specified otherwise, experimental temperatures were 40−40.5 °C. Samples were stirred by magnetic stirring to maintain homogeneity. Emission and excitation wavelengths are listed in the figure legends. Bandwidths were 4.25 nm for emission spectra and time course data and 16 nm for anisotropy measurements. All emission spectra are uncorrected. Light scattering due to sample turbidity was small enough at the vesicle concentrations used so as not to interfere with the interpretation of the results. Experiments involving time courses with data acquired from multiple excitation and emission wavelengths were accomplished by rapid sluing of the monochromator mirrors (FluoroMax, Spex Industries, Edison, NJ). The time resolution in such experiments was 3−5 s.

Samples were incubated in the fluorometer sample chamber for about 10 min for temperature equilibration prior to initiation of the experiment. Lyso-PC and/or palmitic acid (PA) (10 mM stock solutions in methanol) were added directly to the sample. In such experiments, the methanol concentration did not exceed 0.8% (v/v). Control experiments showed that such did not alter the behavior of the PLA_2 and caused only minor effects on the spectroscopy that were independent of the presence of the reaction products and did not influence the interpretation of the results (see Figure 7). Previous measurements of the lyso-PC and PA partition coefficients (Brown et al., 1993; Bent & Bell, 1995) indicate that at least 75% of the lyso-PC and nearly 100% of the PA partition into the bilayer under the conditions used in this study. Lyso-PC and PA concentrations reported in the presentation of the results refer to total concentrations uncorrected for partitioning. When PLA_2 was added, it was done after the temperature equilibration of the other components.

Simultaneous measurements of fluorescence and vesicle hydrolysis were obtained by pH-stat titration at pH 8.0 in the fluorometer cell as described (Bell & Biltonen, 1991). In such experiments, the reaction volume was increased to 3 mL and $Na_2B_4O_7$ was deleted from the reaction mixture.

Anisotropy measurements were obtained in the L-format using a PC1 fluorometer from ISS (Urbana, Illinois) equipped with Glan-Thompson polarizers. *G*-factors and the anisotropy were obtained and calculated according to standard procedures (Lakowicz, 1983).

All ^{31}P NMR experiments were conducted with a Varian VXR-500 spectrometer equipped with a Sun 4/360 workstation. Vesicles were used at 13.3 mM DPPC in the same buffer solution described above for fluorescence spectroscopy (at both 1 μM and 1 mM calcium) with 25% (v/v) D_2O and in a total volume of 0.7 mL. Spectra were obtained at 40 °C. Lyso-PC and/or PA (prepared by direct hydration of dried lipids rather than being dissolved in methanol) were

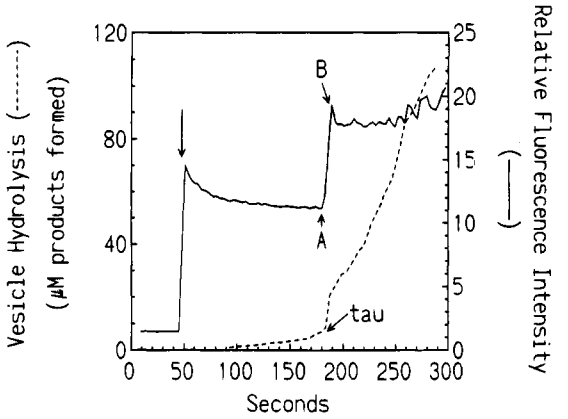

FIGURE 1: Time course of fluorescence of PLA_2 during vesicle hydrolysis. PLA_2 was added to vesicles (100 μM DPPC) at the time indicated by the arrow in the presence of 1 mM calcium, and vesicle hydrolysis (dashed curve) was measured concurrent with enzyme fluorescence (solid curve) as described in Materials and Methods. Fluorescence wavelengths were excitation = 280 nm, emission = 340 nm.

added incrementally to the sample after removal from the NMR tube and mixed before reintroduction to the tube between spectra.

RESULTS

The addition of AppD49 PLA_2 to DPPC LUV at 40 °C results in a time course of vesicle hydrolysis exhibiting an initial phase of slow hydrolysis followed by an abrupt increase in activity of 1 or more orders of magnitude (Bell & Biltonen, 1989b; Figure 1, dashed curve). Concurrent with the onset of the increased activity, the intrinsic tryptophan fluorescence of the protein also increases sharply by about 50% (Bell & Biltonen, 1989b; Figure 1, solid curve). The time of onset of increased activity and PLA_2 fluorescence is designated throughout as τ according to previous convention (Bell & Biltonen, 1992).

The purpose of this study was to use fluorescent probes to identify what physical properties of the bilayer might induce the high activity of PLA_2 at τ. In accordance, the basic strategy was to determine which probes detect changes in bilayer properties that correlate with the approach to time τ. Furthermore, since the state of high PLA_2 activity also occurs at low calcium concentration (albeit with a longer lag phase preceding time τ; Bent & Bell, 1995) or in the presence of sufficient exogenously added reaction products (either lyso-PC alone or with PA) (Apitz-Castro et al., 1982; Jain & De Haas, 1983; Bell & Biltonen, 1992; Brown et al., 1993; Bent & Bell, 1995), we examined the fluorescence of the probes under those conditions as well. The rationale was that if a given bilayer property was relevant to the ability of the reaction products to promote high PLA_2 activity, it should be evident under each condition at which the high activity is achieved.

Fluorescence measurements of the state of the PLA_2 (solid curve, Figure 1) provide better resolution than pH-stat titration of the hydrolysis reaction (dashed curve, Figure 1) for making the temporal correlations germane to this study. Consequently, we used the PLA_2 intrinsic fluorescence as a marker of τ in experiments with the probes. In order to obtain quantitative information regarding the degree of hydrolysis at τ, we repeated the experiment of Figure 1 several times and measured the percentage of vesicle hydrolysis at the beginning and end of the PLA_2 fluorescence change (points A and B in Figure 1). In six such experiments at 1 mM calcium, 8.3% ± 3.5% (mean ± SE) of vesicle phospholipid was hydrolyzed at the time of onset of the PLA_2 fluorescence change (point A, Figure 1). This value agreed well with previous estimates of the amount of vesicle hydrolysis at τ (Apitz-Castro et al., 1982; Bell & Biltonen, 1989b; Burack & Biltonen, 1994). An average of 18.1% ± 2.3% was hydrolyzed when the sudden rise in PLA_2 fluorescence was complete (point B, Figure 1). Light scattering data indicates that major changes in vesicle structure may occur beyond that point (Bell & Biltonen, 1989b). At the low calcium concentration used in this study (1 μM), the pH-stat method does not provide reliable quantitative information about the reaction time course beyond the early phase of hydrolysis (Lathrop & Biltonen, 1992; Bent & Bell, 1995). Nevertheless, previous investigations into the effect of calcium concentration suggest that the percentage of vesicle hydrolysis at the onset of τ (point A, Figure 1) is probably a few percent higher than at 1 mM calcium (Bent & Bell, 1995). This observation is substantiated by experiments presented later in this paper (see Table 1 below). Since the rate of vesicle hydrolysis at 1 μM calcium is only about one-tenth the rate at 1 mM calcium (Lathrop & Biltonen, 1992; Bent & Bell, 1995) but the rate of rise of PLA_2 fluorescence appears independent of calcium concentration (see Figure 5 and Table 1 below), less than 18% of the vesicle must be hydrolyzed at point B at 1 μM calcium. Since we were interested in the events leading up to the sudden increase in PLA_2 activity at τ, which corresponds to the interim between points A and B in the fluorescence time course, we confined our analysis to a study of the vesicles up to point B in the time course.

To test whether the effect of reaction products to promote high PLA_2 activity was a consequence of changes in the viscosity of the bilayer, we examined the effect of vesicle hydrolysis on the fluorescent probe bis-pyrene. Bis-pyrene fluorescence is sensitive to the freedom of motion in the region of the hydrophobic core of the bilayer (Melnick et al., 1981). The efficiency with which the adjacent pyrene moieties within the molecule can interact during the photoexcited state depends on the rate at which they can diffuse through the surrounding medium, which in this case is the phospholipid acyl chains. The frequency of pyrene/pyrene interactions is detected experimentally by the intensity of excimer fluorescence at 480 nm. Any changes in excimer fluorescence intensity should be paralleled by inverse changes in monomer fluorescence intensity at 376 nm. For example, the ratio of excimer to monomer intensities can be used to monitor the thermotropic phase transition of DPPC vesicles (Figure 2A). The elevated ratio occurring at higher temperatures indicated the decreased order of phospholipid acyl chains in the liquid crystalline phase.

The time course of bis-pyrene fluorescence emission at 376 nm (dashed line) and 480 nm (dotted line) was observed simultaneously with the PLA_2 tryptophan fluorescence (solid line) during the hydrolysis of DPPC-LUV at 40 °C and 1 mM calcium (Figure 2B). As shown in the figure, a small increase in the intensity of the monomer (376 nm) occurred initially after adding the PLA_2. However, no additional changes occurred in either excimer or monomer fluorescence during the time course at or after time τ. A similar result was obtained at 1 μM calcium (not shown). Therefore, it

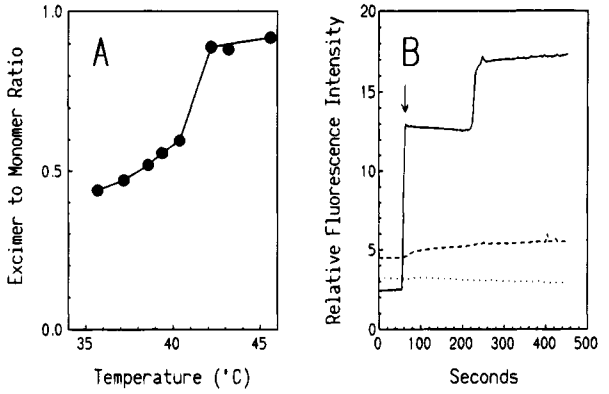

FIGURE 2: Effect of temperature and vesicle hydrolysis on bis-pyrene fluorescence. (Panel A) Bis-pyrene excimer (480 nm) to monomer (376 nm) ratio in DPPC LUV (100 μM DPPC, 100 nM bis-pyrene) as a function of temperature at 1 μM calcium. (Panel B) PLA_2 and bis-pyrene fluorescence were monitored during vesicle hydrolysis at 1 mM calcium using vesicles containing bis-pyrene. PLA_2 was added at the time indicated by the arrow. Wavelengths were as follows: PLA_2 (solid line), excitation = 280 nm, emission = 340 nm; bis-pyrene (dashed and dotted lines), excitation = 344 nm, emission = 376 nm (dashed line) or 480 nm (dotted line).

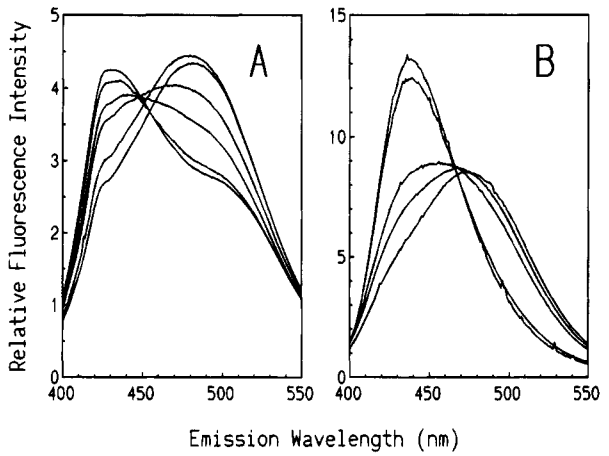

FIGURE 3: Temperature dependence of Prodan and Laurdan fluorescence emission spectra. (Panel A) LUV (200 μM DPPC) contained Prodan (400 nM), excitation wavelength = 370 nm. Temperatures were (top to bottom at 425 nm) 36.2, 38.3, 40.6, 41.4, 42.7, and 43.9 °C. (Panel B) LUV (100 μM DPPC) contained Laurdan (200 nM), excitation wavelength = 340 nm. Temperatures were (top to bottom at 425 nm) 36.9, 39.5, 41.5, 43.3, and 50.2 °C.

appeared that changes in the order of the phospholipid acyl chains were not required for the increase of PLA_2 activity at time τ.

Prodan and Laurdan are sensitive to the polarity of their immediate environments (Weber & Farris, 1979). They differ in the position they occupy within the bilayer and in how tightly the fluorescent aromatic rings anchor to the bilayer (Chong et al., 1989; Chong & Wong, 1993; Parasassi et al., 1994). As the state of the bilayer changes with increasing temperature from gel to liquid crystalline phases, the intensity of the emission spectrum of each probe decreases and the wavelength of maximum emission shifts from about 435 nm (less polar) to 480 nm (more polar) (Figure 3). The spectrum width was greatest in the phase transition region of the LUV (39–43 °C) due to the heterogeneous and fluctuating environment. Detailed descriptions of the molecular events thought to produce these effects have been published (Massey et al., 1985; Chong,

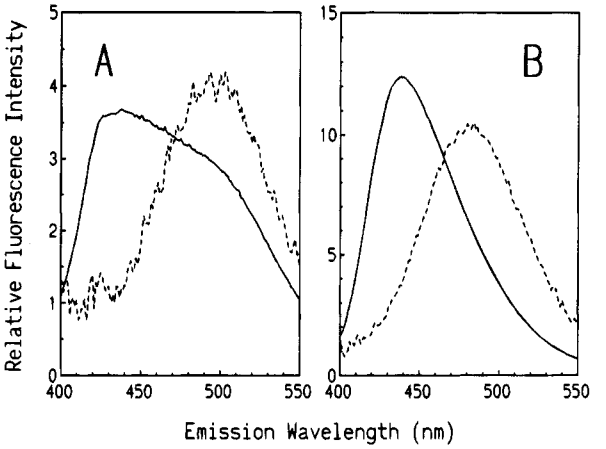

FIGURE 4: Effect of vesicle hydrolysis on the emission spectra of Prodan and Laurdan. Reaction conditions were identical to those in Figure 2B for vesicles (100 μM DPPC) containing 200 nM Prodan (panel A, excitation = 370 nm) or 200 nM Laurdan (panel B, excitation = 340 nm). Emission spectra were obtained prior to (solid curves) or following (dashed curves) vesicle hydrolysis by PLA_2.

1988; Chong et al., 1989; Chong & Wong, 1993; Parasassi et al., 1994).

The emission spectrum of each probe was obtained at 40 °C and 1 mM calcium before and after vesicle hydrolysis by PLA_2 (Figure 4). In each case, the emission spectrum was shifted toward higher wavelength, indicating that the environment of the probes had become more polar after hydrolysis (Weber & Farris, 1979). To determine whether this change in fluorescent emission reflected molecular circumstances inducing the high activity of PLA_2 at time τ or the subsequent breakdown of the vesicles, we monitored the probe fluorescence as a function of time during vesicle hydrolysis (Figure 5). Emission wavelengths were selected that allowed observation of the shifts in the probe emission spectra (shown in Figure 4) in order to indicate at what point during vesicle hydrolysis these fluorescence changes occurred. With Prodan, the intensity of fluorescence at both 435 and 480 nm began to decrease immediately upon addition of PLA_2 (Figure 5A,B). The rate of decline increased rapidly in the vicinity of time τ. This decrease in intensity was more pronounced at 435 nm than at 480 nm. Thus, the ratio of intensity at 480 nm to that at 435 nm increased. These results were qualitatively similar with either 1 mM or 1 μM calcium except for a later increase in the intensity of fluorescence at 480 nm in the presence of 1 mM calcium (Figure 5A).

Laurdan gave very different results compared to Prodan. At 1 mM calcium, the Laurdan fluorescence was stable during the latency phase of the time course up to time τ (Figure 5C). About 50 s after the increase in PLA_2 fluorescence at time τ, the fluorescence changed dramatically with a large drop in the intensity at 425 nm accompanied by an increase in the intensity at 510 nm. Experiments such as that shown in Figure 1 with vesicles containing Laurdan revealed that over 70% of the vesicle was hydrolyzed at the point where the intensities at the two wavelengths are equal (71.3% $\pm$ 2.2%, $n = 5$). Interestingly, the profile was very different when 1 μM calcium was used in the reaction mixture (Figure 5D). In that case, the fluorescence at 425 nm increased upon addition of enzyme up to time τ. At the onset of PLA_2 activation, the fluorescence at 425 nm

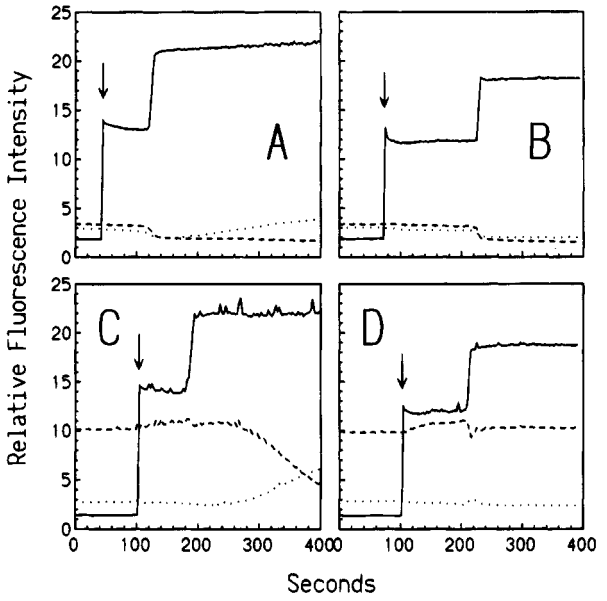

FIGURE 5: Time course of fluorescence of PLA_2 and Prodan or Laurdan during hydrolysis of DPPC LUV. Reaction conditions were identical to those in Figure 4 with vesicles containing Prodan (panels A and B) or Laurdan (panels C and D) at 1 mM (panels A and C) or 1 μM (panels B and D) calcium. PLA_2 was added at the time indicated by the arrow. Wavelengths were as follows: PLA_2 (solid lines), excitation = 280 nm, emission = 340 nm; Prodan (dashed and dotted lines, panels A and B), excitation = 370 nm, emission = 435 nm (dashed lines) or 480 nm (dotted lines); Laurdan (dashed and dotted lines, panels C and D), excitation = 340 nm, emission = 425 nm (dashed lines) or 510 nm (dotted lines).

suddenly decreased and thereafter remained stable. The intensity at 510 nm was essentially stable throughout the time course with the exception of a small transient increase in intensity at time τ. On the basis of these results, it appeared that the large shift in the emission spectrum shown in Figure 4 for Laurdan was largely a consequence of events occurring late in the hydrolysis reaction and probably a reflection of vesicle dissolution.

The results with Prodan were particularly encouraging since they demonstrated a change in the bilayer that appeared to anticipate time τ at both high and low calcium concentrations. Therefore, they could represent conditions in the membrane facilitating the increased PLA_2 activity at τ. To improve the resolution of the experiment to verify this important result, we used high concentrations of KCl to slow the hydrolysis time course (Fernández et al., 1991). Figure 6 displays time courses of PLA_2 tryptophan and concurrent Prodan fluorescence at 150 mM and 350 mM KCl with 1 μM Ca. As shown in the figure, the increasing salt concentration slowed the reaction. The improved resolution of these slower reactions demonstrated clearly that the abrupt decreases in Prodan fluorescence did precede τ.

Figure 7 shows the effect of incremental addition of equimolar lyso-PC and PA to DPPC LUV on the emission spectrum of Prodan at 40 °C and 1 mM calcium (Figure 7A), 6 μM calcium (Figure 7B), or 1 μM calcium (Figure 7C). Initially, the addition of these reaction products caused an overall decrease in spectrum intensity. As more lyso-PC and PA were added, the decrease was greater at the lower wavelengths such that the ratio of intensity at 480 nm to that at 435 nm was increased. This trend was most obvious at high calcium concentration where the intensity at 480 nm increased prominently at reaction product concentrations

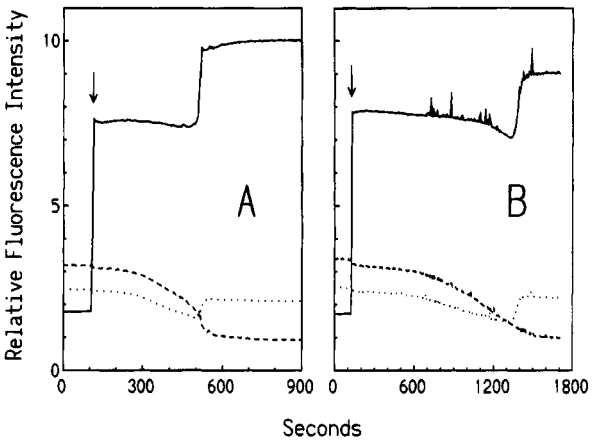

FIGURE 6: Increased resolution of the time course of PLA_2 and Prodan fluorescence during hydrolysis of DPPC LUV in the presence of increased KCl concentration. Reaction conditions, fluorometer wavelengths, and line types were identical to those in Figure 5B with the addition of 50 μL of saturated KCl (panel A, 100 mM total final) or 150 μL of saturated KCl (panel B, 350 mM total final). PLA_2 was added at the time indicated by the arrow.

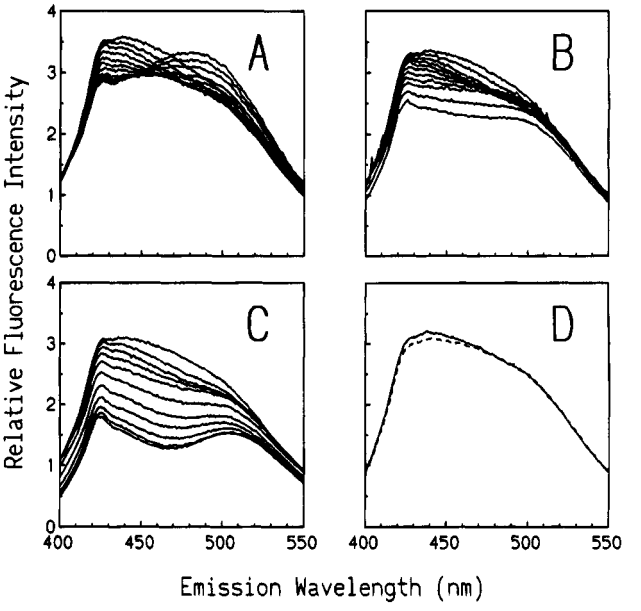

FIGURE 7: Prodan emission spectra as a function of added reaction products. (Panels A–C) Lyso-PC and PA at 0, 1.9, 3.7, 5.4, 6.9, 8.3, 9.7, 10.9, 12.1, 13.2, or 14.3 mol % each (top to bottom at 450 nm) were added sequentially to vesicles containing Prodan (100 μM DPPC and 200 nM Prodan) and the emission spectra were recorded (excitation = 370 nm). Calcium concentrations were 1 mM (panel A), 6 μM (panel B), and 1 μM (panel C). Panel D represents the effect of addition of the volume of methanol contributed by the highest concentration of reaction products tested (0.4% v/v).

greater than about 6 mol %. The addition of an equivalent amount of the reaction product diluent (methanol) caused only a minor alteration in the emission spectrum (Figure 7D).

Comparison of these results to Figures 5 and 6 revealed that similar changes in Prodan fluorescence occurred regardless of whether the lyso-PC and PA accumulate in the bilayer during vesicle hydrolysis or whether they are added from the outside. As shown in Table 1, the similarity was also quantitative. At 1 mM calcium, the addition of about 7 mol % reaction products was sufficient to reduce the length of τ to zero. A higher concentration (about 11 mol %) was required at 1 μM calcium, consistent with a prior report (Bent

Table 1: Ratio of Prodan Fluorescence Intensity at 480 nm to That at 435 nm upon Addition of Sufficient Lyso-PC and PA To Reduce τ to Zero or at Time τ during Vesicle Hydrolysis[a]

	Lyso-PC/PA Addition ratio (480 nm/435 nm)		
	before[b]	after[c]	mol %
1 mM calcium (4)[d]	0.88 ± 0.02	0.96 ± 0.06	7.0 ± 2.2
1 μM calcium (9)	0.86 ± 0.05	0.98 ± 0.13	10.9 ± 1.9

	Vesicle Hydrolysis ratio (480 nm/435 nm)			
	before	τ_1[e]	τ_2[f]	Δt[g] (s)
1 mM calcium (9)	0.82 ± 0.07	0.80 ± 0.05	0.94 ± 0.06	7.9 ± 2.8
1 μM calcium (38)	0.88 ± 0.04	0.89 ± 0.09	1.22 ± 0.11	6.1 ± 2.5

[a] Prodan fluorescence upon addition of reaction products (lyso-PC and PA) was obtained from data such as that shown in Figure 7 using the minimum mol % of products that, when added to vesicles in parallel experiments, caused immediate high fluorescence of PLA_2 without a lag phase. Fluorescence during vesicle hydrolysis was obtained from data such as that shown in Figure 5. Data are expressed as the mean ± standard deviation. [b] Ratio prior to addition of reaction products or PLA_2. [c] Ratio at the indicated mol % lyso-PC and PA. Statistically different from the value in the "before" column by paired Student's two-tailed t test. [d] Number of samples. [e] Ratio at the last time point before the increase in the tryptophan fluorescence of PLA_2 during vesicle hydrolysis (point A in Figure 1). [f] Ratio at the first time point after the increase in the tryptophan fluorescence of PLA_2 during vesicle hydrolysis (point B in figure 1). Statistically different from the value in the "before" column by paired Student's two-tailed t test. [g] Time differential between the data points represented in columns τ_1 and τ_2.

& Bell, 1995). The presence of reaction products at these concentrations increased the ratio of Prodan intensity at 480 nm to that at 435 nm from about 0.87 to about 0.96 (Table 1). Likewise, the 480-nm to 435-nm ratio increased by the same increment coincident with the increase in PLA_2 fluorescence and activity at time τ. Interestingly, at 1 mM calcium, this increase in the ratio occurred exactly at the time at which the PLA_2 fluorescence first reached its maximum (corresponding to point B in Figure 1 at 18% hydrolysis), whereas it occurred prior to that point at 1 μM calcium. The reason for the slight time difference at high compared to low calcium is not known. However, the average time interval during which the PLA_2 fluorescence increased at time τ (6−7 s) was of the same magnitude as the time resolution of the experiment. Also, on the basis of the data of Figure 1, the increased ratio at 1 mM calcium appeared to correspond to a somewhat higher percent hydrolysis of the vesicles (i.e., 18 %) compared to the mole fraction of products that produced the same change when added exogenously (7 mol % Table 1). This difference could indicate a small time dependence in the changes measured during vesicle hydrolysis or a difference in the ability of exogenously added products to perturb the bilayer compared to products created *in situ* during hydrolysis. In any case, these results indicate that the exogenous addition of reaction products to vesicles probably qualifies as a useful model for studying the condition of the bilayer at τ when changes occur too rapidly during vesicle hydrolysis for adequate analysis.

Since the addition of lyso-PC alone is sufficient to reduce τ to zero (Jain & De Haas, 1983; Bell & Biltonen, 1992; Bell & Bent, 1995), we tested whether the results shown in Figure 7 required the presence of PA (Figure 8). Additions of lyso-PC alone at either 1 mM or 1 μM calcium produced a series of emission spectra that resembled the effect of

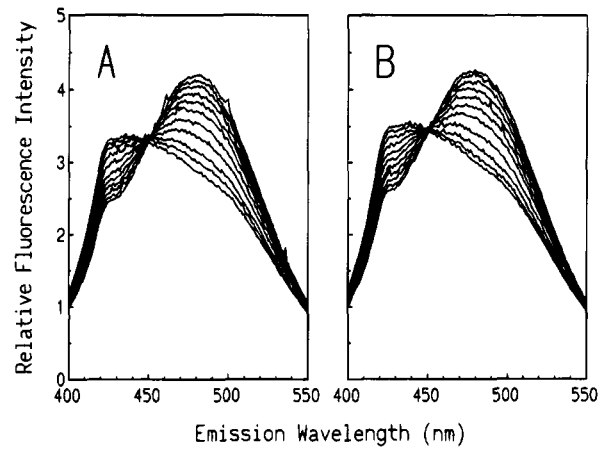

FIGURE 8: Prodan emission spectra as a function of added lyso-PC. Lyso-PC at 0, 2.0, 3.8, 5.7, 7.4, 9.1, 10.7, 12.3, 13.8, 15.3, or 16.7 mol % (top to bottom at 425 nm) was added to vesicles containing Prodan as described for Figure 7 in the presence of 1 mM (panel A) or 1 μM (panel B) calcium.

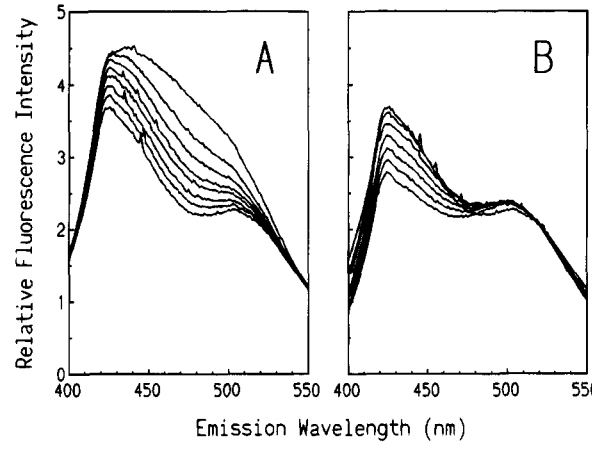

FIGURE 9: Prodan emission spectra as a function of added PA followed by lyso-PC. (Panel A) PA at 0, 4.8, 7.0, 9.1, 11.1, 13.0, 14.9, or 16.7 mol % (top to bottom) was added to vesicles containing Prodan as described for Figure 7 in the presence of 1 μM calcium. (Panel B) Following the final addition of PA, lyso-PC at 5.9, 7.7, 9.4, 11.1, 12.7, or 14.3 mol % (top to bottom at 425 nm) was added sequentially to the same vesicles. The final concentration of PA in the vesicles was 14.3 mol % following the addition of lyso-PC.

temperature on the Prodan fluorescence shown in Figure 3A. Addition of PA alone to the vesicles caused a progressive loss of fluorescence intensity and increased resolution of the emission spectrum into the two components (Figure 9A). In contrast to the effect of adding both reaction products together, PA caused a decrease in the ratio of intensities (480 nm:435 nm) from 0.83 to 0.64. Subsequent addition of lyso-PC produced a sequential loss of intensity at 435 nm while the intensity at the higher wavelengths rose slightly (Figure 9B). Consequently, the ratio (480 nm:435 nm) increased up to 0.86. The final spectrum obtained when the concentration of each product was equivalent (bottom spectrum, Figure 9B) was essentially identical to that obtained when both reaction products were added together at 1 μM calcium (bottom spectrum, Figure 7C). Therefore, both the fatty acid and the lyso-PC appeared to contribute to the shapes of the emission spectra, and the same end point was reached regardless of whether the products were added together or whether the fatty acid was added first.

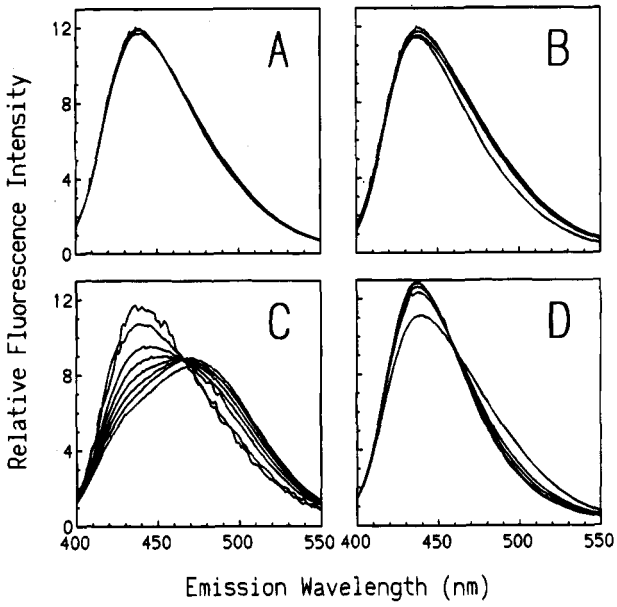

FIGURE 10: Laurdan emission spectra as a function of added reaction products. Lyso-PC and PA at 0, 3.7, or 5.4 mol % each (bottom to top at 440 nm, panel A); lyso-PC and PA at 5.4, 8.3, 10.9, or 14.3 mol % each (top to bottom at 440 nm, panel B); lyso-PC at 0, 2.0, 3.8, 5.7, 7.4, 9.1, 10.7, or 16.7 mol % (top to bottom, panel C); or PA alone at 0, 4.8, 9.1, 13.0, or 16.7 mol % (bottom to top at 440 nm, panel D) were added sequentially to vesicles (100 μM DPPC) containing Laurdan (200 nM) as described for Figure 7. The calcium concentration was 1 μM and the excitation wavelength was 340 nm.

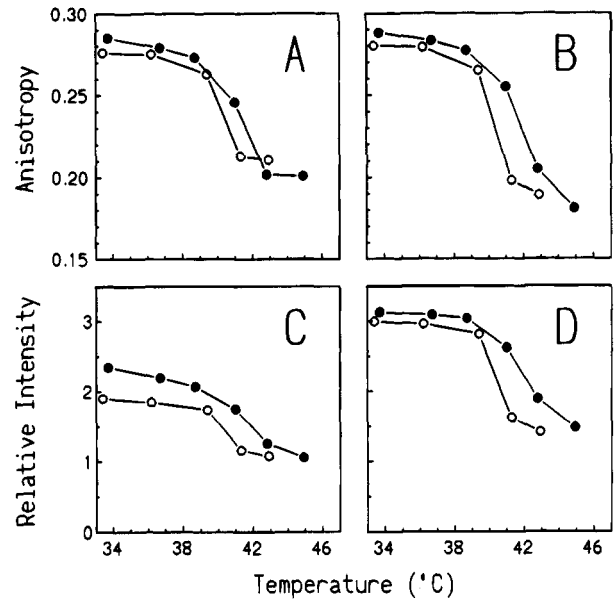

FIGURE 11: Anisotropy and intensity of Prodan and Laurdan fluorescence as a function of temperature in the absence and presence of 13.8 mol % lyso-PC. The fluorescence anisotropy was measured for vesicles (500 μM DPPC) containing Prodan (1 μM, panel A; excitation 370 nm, emission 435 nm) or Laurdan (1 μM, panel B; excitation 340 nm, emission 425 nm) as described under Materials and Methods. The calcium concentration was 1 μM. Panels C and D show the corresponding total emission intensity for Prodan and Laurdan, respectively. Solid circles: vesicles before the addition of lyso-PC. Open circles: vesicles after the addition of lyso-PC.

These experiments were repeated with vesicles containing Laurdan in the presence of 1 μM calcium (Figure 10). The addition of up to about 6 mol % of both reaction products caused a slight increase in spectral intensity (Figure 10A). Further addition caused a decrease in the intensity (Figure 10B). These changes were reminiscent of those obtained in the corresponding hydrolysis time course in Figure 5D. Addition of lyso-PC alone caused a progressive loss of peak intensity at about 435 nm and a shift of the spectrum peak toward 475 nm (Figure 10C). PA alone resulted in a systematic increase in intensity and narrowing of the emission peak at about 435 nm (Figure 10D). The addition of equivalent amounts of diluent (methanol) caused a minor decrease in fluorescence intensity comparable to that shown in Figure 7D for Prodan.

For both Prodan and Laurdan, the effect of lyso-PC on the emission spectra (Figures 8 and 10C) appeared similar to the effect of increasing temperature shown in Figure 3. Since previous data from calorimetric and NMR studies indicated that lyso-PC alone lowers the temperature of the main thermotropic phase transition of the DPPC bilayer (Klopfenstein et al., 1974; Jansson et al., 1990), we reasoned that the data of Figures 8 and 10C could be explained by such a phenomenon. To test that result, we measured the anisotropy of both Prodan and Laurdan as a function of temperature in the presence and absence of lyso-PC (Figure 11). As shown in the figure, the anisotropy of both probes decreased abruptly at the melting temperature of the DPPC. [The midpoint of the main phase transition for DPPC LUV is about 41.5 °C, (Wong et al., 1982).] Apparently, lyso-PC did cause a decrease in the melting temperature sufficient to account for the results of Figures 8 and 10C. Nevertheless, the anisotropy of both Prodan and Laurdan was essentially unaffected by lyso-PC outside the region of the phase

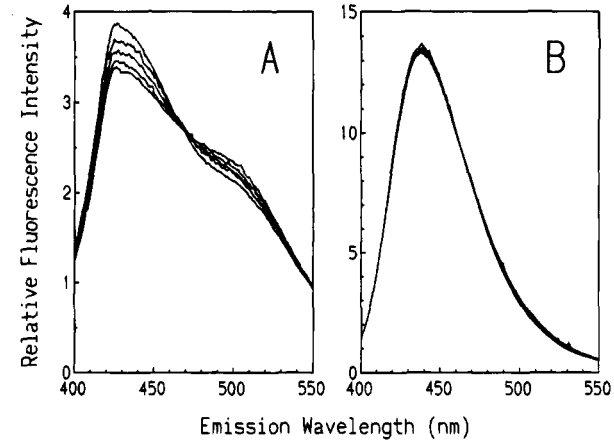

FIGURE 12: Prodan and Laurdan emission spectra as a function of added lyso-PC at 35 °C. The experiments of Figure 8B (panel A, Prodan) and Figure 10B (panel B, Laurdan) were repeated at 35 °C. Lyso-PC concentration was (top to bottom at 440 nm) 0, 4.8, 9.1, 13.0, or 16.7 mol %.

transition. Also, addition of both lyso-PC and PA did not affect the Prodan or Laurdan anisotropy below the transition (not shown).

Intensity measurements from the anisotropy experiments suggested that lyso-PC caused an additional perturbation to the fluorescence of Prodan beyond the effects on the phase transition (Figure 11A). In accordance, we reobtained the emission spectra of Prodan and Laurdan as a function of added lyso-PC at a temperature low enough to avoid the problem of shifts in the phospholipid melting temperature (35 °C, Figure 12). Under such conditions, lyso-PC caused changes to the Prodan that were analogous to those caused by both reaction products at 40 °C, i.e., an increase in the

ratio of fluorescence intensity (480 nm:435 nm) from 0.64 to 0.76 (also compare Figures 7 and 12A). In the case of Laurdan, the addition of lyso-PC resulted in negligible changes in the emission spectrum (Figure 12B).

DISCUSSION

The data presented in this paper reveal that specific changes in bilayer structure occur when relatively low concentrations of lyso-PC and fatty acid accumulate in the bilayer either during vesicle hydrolysis or upon exogenous addition of products. Both the positive and negative results are useful for interpreting the molecular nature of those perturbations and how they might induce high activity of PLA_2. For example, it appeared clear from the data that changes in bilayer fluidity were not associated with the increase in PLA_2 activity. No change occurred in the ratio of excimer to monomer fluorescence of bis-pyrene (Figure 2B). Furthermore, no change in Prodan nor Laurdan anisotropy was observed in the presence of reaction products other than the effect of lyso-PC on the thermotropic phase transition of the DPPC (Figure 11). These results coupled with calorimetry data published elsewhere (Burack et al., 1993) also indicated that the effect of reaction products at 40 °C was not a consequence of changes in the state of the bilayer from gel to liquid crystalline phases.

Insight into the bilayer changes responsible for increased PLA_2 activity can be obtained by interpretation of the Prodan and Laurdan emission spectra. Prior to hydrolysis or addition of reaction products, the emission spectrum of Prodan appeared to be composed of two components, the less polar component centered around 435 nm and the more polar at about 500 nm. This contributed to a spectral bandwidth much broader than those observed with pure solvents (Weber & Farris, 1979). Such a spectrum for Prodan has been suggested previously to result from subpopulations of Prodan molecules distributed at different elevations within the region of the polar head groups of the phospholipids in the bilayer (Chong, 1988). The addition of reaction products, and apparently the initial phase of vesicle hydrolysis, caused an overall loss of Prodan fluorescence intensity and an increase in the ratio of intensity at 480 nm to that at 435 nm. In general, these data are consistent with an elevation in the average polarity of Prodan's environment. More specifically, an increase in the similar ratios of high to low wavelength has been interpreted to represent a shift in the distribution of Prodan populations within the bilayer (Chong, 1988). Comparing the data of Figure 7 to those of Figures 9 and 12 suggests that lyso-PC was responsible for this presumed alteration in the distribution of Prodan populations.

In contrast to Prodan, Laurdan appears to remain tightly anchored to a single site deeper in the bilayer, possibly in the region of the glycerol backbones (Chong & Wong, 1993; Parasassi et al., 1994). The subtle changes in Laurdan fluorescence during the hydrolysis reaction at low calcium concentration appeared to reflect a decrease in the polarity of Laurdan's environment leading up to time τ, followed by a sudden increase in polarity at τ. Comparison of the emission spectra upon addition of either or both reaction products (Figure 10 and 12B) suggests that the fluorescence of Laurdan is affected more by the fatty acid than the lyso-PC during the hydrolysis time course. The abrupt reversal in the trend of the Laurdan fluorescence time course during vesicle hydrolysis at low calcium (Figure 5D) could thus reflect the sudden reorganization of fatty acid molecules into domains near time τ (Jain et al., 1989; Burack et al., 1993).

The main effect of fatty acid addition on the fluorescence of Prodan and Laurdan appeared to involve the intensity of the emission spectra (Figures 9A and 10C). Interestingly, the probes displayed opposite changes in spectrum intensity as fatty acid was added. The increase and narrowing of the Laurdan spectrum suggested a decrease in the accessibility or mobility of water molecules near the probe. Presumably, this reflected the ability of fatty acid (in the absence of lyso-PC) to stabilize the gel phase of DPPC vesicles (Lohner, 1991). This idea would also be consistent with the decrease in the 480 nm:435 nm ratio of the Prodan spectrum. However, the loss of Prodan intensity cannot be explained by the gel-like properties of the system. Instead, the decreased intensity may reflect the charge of the fatty acid. It also seemed likely, on the basis of the data of Figures 7, 9, and 10, that differences in the emission spectra of the probes at high *versus* low calcium concentration reflected differences in the magnitude of contribution of fatty acid to the spectra when both products were present. Specifically, the Prodan spectra with both products resembled those obtained with only fatty acid more at low than at high calcium, suggesting that the fatty acid made a larger contribution to the properties sensed by the probe at low calcium. This difference could be explained by interactions between calcium and fatty acid which alter the effect of fatty acid on the bilayer and thus on the environment of the probes.

On the basis of the results with Prodan and Laurdan, it seems reasonable to conclude that the effect of lyso-PC accumulation in the bilayer during the lag phase of vesicle hydrolysis is to disrupt the bilayer in the region of the phospholipid head groups. In particular, the data are consistent with an increase in the lateral spacing of the phospholipid head groups. This would allow an increase in the water penetration into that region which could account for the increase in the ratio of Prodan emission at 480 nm compared to that at 435 nm (Massey et al., 1985). The increased spacing of the polar heads could also allow Prodan molecules to migrate toward a more polar environment as has been postulated for other bilayer perturbations (Chong, 1988). Presumably, the disruption caused by the products has a much smaller effect on deeper regions of the bilayer where Laurdan partitions. Thus, these results may confirm the previous suggestions that PLA_2 activity is increased by changes in the lateral spacing of phospholipid head groups [reviewed in Verheij et al. (1981)]. Importantly, our experiments allow one to observe these changes in bilayer structure in real time while monitoring simultaneously the state of the enzyme.

One possible mechanism for the disruption of the head group region of the bilayer relates to changes in the average curvature of the bilayer. Burack and Biltonen recently suggested that an increase in positive membrane curvature could represent at least part of the mechanism of the product-induced increase in PLA_2 activity (Burack & Biltonen, 1994). In support of this idea, several lines of evidence suggest that lyso-PC can induce positive curvature into the bilayer. For example, in contrast to DPPC, which tends to form bilayers in aqueous solution, lyso-PC forms micelles (Stafford et al., 1989). This distinction is probably due to the conical shape of the lyso-PC molecule compared to the more cylindrical

DPPC molecule. In addition, ^2H and ^{31}P NMR spectroscopy experiments have also provided evidence that lyso-PC increases the curvature of the bilayer (Jansson et al., 1990). Data from other ^{31}P NMR studies have revealed similar results using both lyso-PC and fatty acid together in equimolar quantities (Burack & Biltonen, 1994). In the latter work, the amounts of the reaction products that induced the apparent increase in curvature corresponded to the amounts that induce high PLA_2 activity and were similar to those used in this study. We have repeated the ^{31}P NMR experiments using incremental exogenous additions of either lyso-PC alone or both reaction products together in equimolar quantities. At either 1 mM or 1 μM calcium, we obtained results comparable to those reported. Eight mole percent lyso-PC with or without equimolar fatty acid reduced the width of the ^{31}P line at half-height by about 50%, consistent with an increase in average bilayer curvature (not shown). Further additions of the reaction products caused smaller decrements in the line width. The observation in this study that similar changes in probe fluorescence occur during vesicle hydrolysis compared to exogenous addition of the reaction products suggest that these structural studies are relevant to conditions during the actual hydrolysis time course. Since many other studies also indicate that increased positive curvature of phospholipid aggregates promotes enhanced PLA_2 activity (Verheij et al., 1981; Menashe et al., 1986; Lichtenberg et al., 1986; Gheriani-Gruszka et al., 1988), we propose that lyso-PC acts by changing the average curvature of the bilayer during the initial phase of vesicle hydrolysis.

An interesting question is raised by these studies: why is the increase of PLA_2 activity during a time course of vesicle hydrolysis so abrupt? The change in Prodan fluorescence as a function of concentration of lyso-PC (Figure 12A) or both products (Figure 7) appears to be a continuous linear function of reaction product concentration in the range at which the PLA_2 activity is enhanced. To the extent that membrane curvature is the mechanism of action of lyso-PC and that it is directly related to the fluorescence data, one might imagine that PLA_2 activity would increase gradually during the latency phase of vesicle hydrolysis rather than increasing abruptly, as has been thoroughly documented. Consequently, one would anticipate that incremental addition of lyso-PC would cause a proportional increase in the initial rate of vesicle hydrolysis rather than the actual result, which is a systematic reduction in the length of the lag phase (Bell & Biltonen, 1992). However, the data make sense if one considers the possibility that the size of the PLA_2 molecule limits the accessibility of phospholipid molecules in the bilayer to the enzyme active site. Thus membrane curvature would have to reach a critical value before PLA_2 accessibility would be significantly altered. In support of this idea, data have been reported showing that increases in membrane curvature decrease τ as well as the amount of reaction product present at time τ (Gheriani-Gruszka et al., 1988). Presumably, sonicated vesicles in the gel phase possess sufficient curvature since no lag time is observed (Menashe et al., 1986).

Several models have been proposed to explain the increase in PLA_2 activity at time τ (Verger et al., 1973; Tinker & Wei, 1979; Jain et al., 1989; Bell & Biltonen, 1992; Burack at al., 1993). In recent years the diversity has decreased and a converging idea has emerged. Basically, the model proposes that PLA_2 first binds to the interface and then in a second step interacts with individual phospholipid molecules as shown below (Jain et al., 1993):

$$E + \text{vesicle} \rightleftharpoons E_B \rightleftharpoons E_B^*$$

where E, E_B, and E_B* are the free PLA_2, the enzyme bound to the surface of the interface, and the bound enzyme in a state on the vesicle in which substrate molecules are accessible to bind to the active site. The accessibility of substrate to interact efficiently with the enzyme could be limited by the ability of the enzyme to penetrate into the bilayer sufficiently (Verger et al., 1973), by the ability of phospholipid molecules to leave the plane of the bilayer and enter the hydrophobic groove in the enzyme (Scott et al., 1990), or by the conformation of the enzyme (Bell & Biltonen, 1992). If either the initial binding step or the substrate accessibility (second step) is weak, the rate of vesicle hydrolysis by PLA_2 will be slow. If both steps are limiting, a sudden shift in the equilibrium of either step 1 or step 2 to the right would cause an abrupt increase in PLA_2 activity. On the basis of previous kinetic analysis of time courses of vesicle hydrolysis by PLA_2 (Bell & Biltonen, 1992), we hypothesize that disruption of the bilayer surface by lyso-PC alters the equilibrium of step 2. By analogy, other perturbations that increase the curvature of the membrane would also enhance step 2. Placing negative charge in the bilayer or acylation of the enzyme with fatty acid is thought to enhance PLA_2 activity by promoting step 1 (Scott et al., 1994). For example, vesicles composed of pure anionic phospholipid are hydrolyzed immediately without a lag phase upon addition of enzyme (Jain et al., 1986). Likewise, similar results have been obtained for acylated enzyme (Shen et al., 1994).

Since the fluorescence emission of the snake venom PLA_2 increases at time τ, upon addition of sufficient lyso-PC, or with sonication of the vesicles (Figures 1, 2, and 5, this work; Bell & Biltonen, 1989a,b), one or more of the tryptophan residues in the protein is likely to be sensitive to step 2 in the scheme. Tryptophan 3 in the porcine pancreatic PLA_2 appears to be mostly sensitive to the initial binding of the enzyme to the bilayer interface (step 1; Kuipers et al., 1991). Recent work has shown that mutation of the pancreatic enzyme so that the tryptophan is found at position 31 instead of 3 changes the fluorescence properties so that the emission becomes more sensitive to the binding of the phospholipid molecules to the active site (step 2; Kuipers et al., 1991). One of the three tryptophans of the PLA_2 used in this study is located at position 31 (based on the numbering system applied to bovine pancreatic PLA_2; Maraganore & Heinrikson, 1986). Therefore, this explanation of the fluorescence of the enzyme seems plausible.

Our results complement data reported by Burack et al. (1993) and Jain et al. (1989). Those previous studies focused on the disposition of nascent fatty acid in the bilayer during the lag phase preceding time τ. Both studies revealed that the fatty acid segregates into domains immediately prior to the rapid phase of vesicle hydrolysis by PLA_2. This formation of fatty acid domains has been offered as at least part of the explanation for the reaction product-induced increase in PLA_2 activity at time τ. However, that interpretation cannot explain all of the mechanism of the effect of reaction products, since lyso-PC is clearly involved and

the effect can be mimicked by lyso-PC in the absence of fatty acid (Apitz-Castro et al., 1982; Jain & De Haas, 1983; Bell & Biltonen, 1992; Bent & Bell, 1995). Our data, then, provide missing information regarding the effect of nascent lyso-PC during the hydrolysis time course to more fully elucidate the process of reaction product-induced increases in PLA_2 activity.

A possible role that has been suggested for the fatty acid is an increase in binding of PLA_2 to the interface because of the negative charge on the fatty acid. This effect of fatty acid would be enhanced by the lateral segregation of fatty acid into domains (Jain et al., 1989; Scott et al., 1994). However, some researchers have expressed concern that such a mechanism would tend to sequester PLA_2 into regions of the bilayer poor in substrate (Burack at al., 1993). This phenomenon would set a limit as to how helpful the fatty acid could be before it became inhibitory. Also, it has been argued that an increase in binding of PLA_2 to the interface at time τ cannot solely account for several kinetic observations (Bell & Biltonen, 1992). Those kinetic observations were obtained with PLA_2 from *A. p. piscivorus* (the enzyme used in this study) and it is not yet clear to what extent they apply equally to PLA_2 from all sources. Consequently, it may be that reaction products exert a dual effect: increased binding of the enzyme to the vesicle surface promoted by the negative charge of the fatty acid and increased accessibility of substrate molecules to the bound enzyme promoted by lyso-PC as described in this paper. The relative contribution of each effect may vary quantitatively among the various soluble PLA_2. For example, much of the work on the effect of fatty acid to promote enzyme binding has focused on porcine pancreatic PLA_2 (Jain et al., 1989). The specific role of fatty acid in promoting the activity of porcine pancreatic PLA_2 was demonstrated in an experiment with sonicated vesicles (González-Martínez & Fernández, 1988). At temperatures corresponding to the gel phase of the phospholipid, such vesicles are normally hydrolyzed by PLA_2 without a lag phase in the time course. However, if the nascent reaction products are removed from the bilayer during the time course, a lag is introduced. In contrast to experiments with LUV and the venom PLA_2, this lag was abolished by replacement of the fatty acid but not the lyso-PC. Presumably, the membrane curvature was already sufficient, and lyso-PC was therefore unable to assist in promoting high PLA_2 activity. The fatty acid, then, produced during the first instances of the time course was probably important to promote PLA_2 binding to the interface.

Perhaps the formation of fatty acid domains during the hydrolysis time course fulfills another function in addition to or instead of increasing the binding of PLA_2 to the interface. One reason for considering another role for lateral segregation of molecules within the bilayer is that other molecules besides fatty acid that separate into domains also enhance the activity of PLA_2 (Zidovetzki et al., 1992). It is likely that phase separation of fatty acid also causes segregation of lyso-PC. It has been suggested that this could create discrete areas of high curvature of the membrane where the lyso-PC is located (Burack & Biltonen, 1994). Indeed, recent electron microscopic investigations have suggested that increased curvature induced by the addition of reaction products to vesicles may be confined to specific regions in the plane of the bilayer, causing a change in the shape of the vesicles from spheres to discs (Burack & Biltonen, 1994). However, these ideas concerning the role of domain formation and the events occurring at time τ would be better pursued using neutral lipids so that the additional factor of charge is not an issue.

In summary, we have established several important observations in this study. First, we have shown that changes in bilayer properties that correlate with increased PLA_2 activity at time τ can be monitored using the membrane probe Prodan. Second, it appears that some of these changes represent a disruption in the lateral spacing of phospholipid molecules, allowing increased access of water or movement of Prodan molecules to more polar regions of the bilayer. Third, this disruption appears to be confined to the region of the polar head groups of the bilayer. Fourth, such changes occur under several conditions at which PLA_2 activity is high: at time τ, upon addition of lyso-PC to the bilayer with or without PA, and at high or low calcium concentration. Therefore, we conclude that the changes detected by Prodan are relevant to the activity of PLA_2. Finally, our experiments have provided an opportunity to test in real time during vesicle hydrolysis the hypothesis that the effect of reaction products on PLA_2 activity relate to the "quality" of the lipid/aqueous interface of the vesicle and to the presence of "structural defects" in that interface (Verger et al., 1973; Op Den Kamp et al., 1974). The results of this study add further light to what those structural defects might be and how they might relate to reaction products (especially lysolecithin) in the bilayer.

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