Relationships between Bilayer Structure and Phospholipase A_2 Activity: Interactions among Temperature, Diacylglycerol, Lysolecithin, Palmitic Acid, and Dipalmitoylphosphatidylcholine†

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ABSTRACT: Bilayers composed of phosphatidylcholine initially resist catalysis by phospholipase A_2. However, after a latency period, they become susceptible when sufficient reaction products (lysolecithin and fatty acid) accumulate in the membrane. Temperatures near the main bilayer phase transition and saturated long-chain diacylglycerol in the bilayer modulate the effectiveness of the reaction products. The purpose of this study was to identify possible mechanisms for these effects of temperature and diacylglycerol. Various fluorescent probes were used to assess changes in the ability of the reaction products to perturb the bilayer and promote enzyme binding to the membrane surface. Temperature appeared to cause three effects. First, the degree of binding of enzyme at the end of the latency period was greatest near the phase transition temperature where the latency period was shortest. Second, the bilayer was more sensitive to perturbation by reaction products near the transition. Third, the disturbance provoked by the products was confined to the membrane surface below the transition but affected deeper regions at higher temperature where the latency period was greater. The latter two effects of temperature required the presence of calcium. Diacylglycerol promoted lateral segregation of reaction products in the bilayer. This effect corresponded with the tendency of diacylglycerol to reduce the length of the latency period at temperatures below the phase transition. Therefore, it appeared that temperature affects the latency period by altering the binding of the enzyme and the depth and magnitude of the bilayer perturbation caused by reaction products. Alternatively, diacylglycerol may enhance the effectiveness of reaction products by inducing them to segregate in the bilayer and thus create local regions of increased impact on the bilayer surface.

A common focus in membrane biochemistry is the relationship between the composition and organization of bilayer lipids and the activity of proteins interacting with the bilayer surface. One popular model for studying this relationship is the soluble phospholipases A_2 from mammalian pancreas or snake venom (PLA_2)[1] (Gheriani-Gruszka et al., 1988; Cunningham et al., 1989; Bell & Biltonen, 1989b; Grainger et al., 1989; Jain et al., 1989; Biltonen, 1990; Zidovetzki et al., 1992; Burack et al., 1993; Bell et al., 1995). Membranes composed exclusively of saturated phosphatidylcholine are resistant to hydrolysis by PLA_2. However, when certain additives are present in sufficient quantity in the bilayer, the phosphatidylcholine is readily hydrolyzed by the enzyme. For example, the products of phospholipid hydrolysis, lysolecithin and fatty acid, can provoke high PLA_2 activity (Apitz-Castro et al., 1982; Bell & Biltonen, 1992; Jain et al., 1989; Burack et al., 1993). Consequently, when PLA_2 is mixed with pure phosphatidylcholine vesicles, the rate of catalysis is very low initially. After a period of time, identified in this report as "τ", sufficient reaction products (lysophospholipid and fatty acid) accumulate from the early slow hydrolysis to induce a change in the bilayer that renders it highly susceptible to the enzyme. At that point, the hydrolytic activity abruptly increases by a factor of 10 or greater (Apitz-Castro et al., 1982; Bell & Biltonen, 1989b; see Figure 1 for an illustration of this phenomenon). With some of the venom PLA_2, the elevated activity is accompanied by an increase in the intensity of the intrinsic tryptophan fluorescence of the enzyme (Bell & Biltonen, 1989b).

The length of τ can be modified by various conditions that affect the structure, composition, and/or dynamics of the bilayer. Hence, these perturbations influence the susceptibility of the membrane to PLA_2, and an understanding of the mechanisms involved is germane to understanding the enzyme. Two such conditions are the subject of this study: temperature and dipalmitoylglycerol (DAG). Temperature affects the bilayer by inducing a transition from the gel to the liquid crystalline phase of the phospholipid. In the region of the transition temperature, domains of lipid in one or the other of the two phases coexist (Freire & Biltonen, 1978; Mouritsen, 1991). Interestingly, the length of τ is reduced at temperatures adjacent to the transition temperature (Me-

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[1] Abbreviations: PLA_2, low molecular weight soluble phospholipase A_2; τ, time of onset of increased PLA_2 activity; DAG, 1,2-dipalmitoylglycerol; DPPC, dipalmitoylphosphatidylcholine; lyso-PC, monopalmitoyllysophosphatidylcholine; LUV, large unilamellar vesicles; PRODAN, 6-propionyl-2-(dimethylamino)naphthalene; LAURDAN, 6-dodecanoyl-2-(dimethylamino)naphthalene; dansyl-DHPE, *N*-(5-(dimethylamino)naphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; PA, palmitic acid; pyrene-lyso, 1-(1-pyrenedecanoyl)-2-hydroxy-*sn*-glycero-3-phosphocholine; pyrene-FA, 1-pyrenedecanoic acid; DPPG, dipalmitoylphosphatidylglycerol; GP, generalized polarization.

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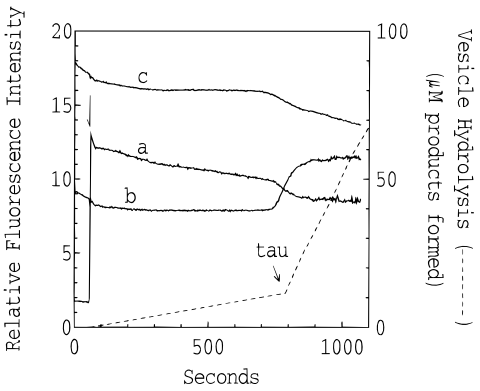

FIGURE 1: Energy transfer from PLA_2 to dansyl-DHPE in DPPC LUV during vesicle hydrolysis at 38 °C. Hydrolysis (dashed curve) was monitored by pH−stat titration (10 mM $CaCl_2$) simultaneously with the fluorescence of PLA_2 (added at the first arrow) and dansyl-DHPE as described in Materials and Methods. Curve a: PLA_2 fluorescence; excitation = 280 nm, emission = 340 nm. Curve b: (energy transfer) dansyl-DHPE fluorescence; excitation = 280 nm, emission = 510 nm. Curve c: intrinsic dansyl-DHPE fluorescence; excitation = 340 nm, emission = 510 nm. (Curves a and b cross at time τ.)

nashe et al., 1981; Lichtenberg et al., 1986; Bell & Biltonen, 1989b; Bell et al., 1995). DAG influences the bilayer by altering the temperature and breadth of the phase transition (López-García et al., 1994; Bell et al., 1995) and by segregating into discrete domains (De Boeck & Zidovetzki, 1992). The presence of modest (2−10 mol %) concentrations of DAG in the bilayer reduces τ considerably at temperatures at or below the phase transition (Bell et al., 1995).

Recently, an attempt has been made to identify mechanisms for the effect of temperature and DAG on the kinetics of vesicle hydrolysis by PLA_2 (Bell et al., 1995). The length of τ appears to depend on three interrelated factors: the binding of enzyme to the vesicle surface, the hydrolysis rate, and the concentration of reaction products necessary to provoke susceptibility of the membrane to PLA_2. While temperature and DAG both influenced the rate of hydrolysis, their effect on τ correlated best with alterations in the concentration of reaction products at time τ. Specifically, under conditions at which τ was reduced by temperature or DAG, the amount of products necessary to provoke rapid hydrolysis was also reduced. Concerning the binding, DAG was shown not to influence the initial binding of the enzyme to the bilayer. However, the effects of temperature or DAG on the binding at time τ were not considered.

Since the effects of temperature and DAG appear related to the ability of reaction products to cause the bilayer to become susceptible to PLA_2, our objective was to determine how temperature and DAG might alter the perturbations induced by the products. We focused on three factors previously identified as being associated with the mechanism of the reaction products. These are (1) the binding of PLA_2 to the vesicle surface at τ, (2) the structure of the bilayer surface, and (3) the lateral distribution of the reaction products in the bilayer (Jain et al., 1982, 1989; Bell & Biltonen, 1989b; Burack et al., 1993, 1995; Burack & Biltonen, 1994; Sheffield et al., 1995). Our strategy was to use fluorescent probes to monitor changes in these factors during vesicle hydrolysis and as a function of temperature and DAG concentration.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC) and monopalmitoyllysolecithin (lyso-PC) were obtained from Avanti Polar Lipids (Birmingham, AL). DPPC in chloroform was dried under N_2 and then lyophilized overnight. To form large unilamellar vesicles (LUV), the lipid was first hydrated in the relevant diluent (35 or 50 mM KCl, 3 mM NaN_3, and 10 or 0 mM $CaCl_2$) for 1 h at 45−55 °C with periodic vortexing. 10 mM $Na_2B_4O_7$ (pH 8) was included in the diluent during preparation of vesicles to be used exclusively in fluorescence experiments. The suspension was then extruded through 0.1 μm polycarbonate filters 10 times at 55−65 °C (Hope et al., 1985). (NaN_3 was included in all solutions to prevent bacterial growth.) Phospholipid concentration was determined by phosphate assay (Bartlett, 1959). When used, 1,2-dipalmitoylglycerol (DAG) and/or the fluorescent probes PRODAN, LAURDAN, or dansyl-DHPE (Molecular Probes, Eugene, OR) was/were mixed with the DPPC in chloroform solution prior to the preparation of vesicles. For some experiments with PRODAN, the fluorophore was added externally to preformed vesicles. This difference in protocol did not appear to influence the results. As explained peviously, no attempt was made to avoid the migration of 1,2-diacylglycerol to the 1,3-isomer. However, the effect of DAG on PLA_2 kinetics appears not to be affected by the length of time between preparation of vesicles and the experiment (Bell et al., 1995). This suggests either that the results are not affected by the migration or that equilibration between the isomers is rapid enough to be complete for all experiments. The concentrations of all additives were expressed as a mole percentage of the DPPC concentration. For all experiments, the final concentration of phospholipid was 100 μM. PRODAN and LAURDAN concentrations were 1/500 the phospholipid concentration or 0.2 μM. Dansyl-DHPE was incorporated at a concentration of 2 mol % or 2 μM. The concentrations of DAG are listed in the individual figures.

PLA_2 was purified from water moccasin (*Agkistrodon piscivorus piscivorus*) venom obtained from the Miami Serpentarium (Punta Gorda, FL) as described (Maraganore et al., 1984) and stored as a lyophilized powder at −20 °C. The enzyme was dissolved in stock solutions of 50 mM KCl and 3 mM NaN_3 and stored at 4 °C prior to use. PLA_2 concentration was assessed by the absorbance at 280 nm (absorbance = 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 the diluents used to prepare vesicles (including the $Na_2B_4O_7$). The $CaCl_2$ concentrations are indicated in the individual figures. When lyso-PC and palmitic acid (PA) (1 mM each in stock solution containing 20% methanol) were included, they were added directly to the sample after the vesicles. This procedure has been used extensively in the past in attempt to mimic at steady state the condition that exists at time τ during vesicle hydrolysis (Jain & De Haas, 1983; Bell & Biltonen, 1992; Brown et al., 1993; Bent & Bell, 1995). Control experiments have demonstrated that lyso-PC and PA incorporate rapidly into the bilayer and produce a steady state that remains stable over the course of the experiment (Elamrani & Blume, 1982; Jain et al., 1989; Bell et al., 1992, 1995). Furthermore, as assessed by PRODAN and LAURDAN fluorescence, the

state of the bilayer produced by this method resembles the state occurring at time τ during vesicle hydrolysis both quantitatively and qualitatively (Sheffield et al., 1995). In such experiments, the final methanol concentration did not exceed 0.5% (v/v), an amount that does not interfere with the results (Sheffield et al., 1995). On the basis of the published partition coefficients for lyso-PC and PA (Brown et al., 1993; Bent & Bell, 1995), 72% of the lyso-PC and nearly 100% of the PA partition into the bilayer under the conditions used in this study. Also the partition coefficient for lyso-PC is not sufficiently sensitive to temperature to influence the interpretation of the data in this study (Bell et al., 1995). However, the concentrations of these molecules shown in the figures refer to total concentrations uncorrected for partitioning. Samples were incubated for approximately 10 min in the fluorimeter sample chamber to equilibrate the temperature prior to initiating the experiment. Temperature was regulated by a circulating water bath, and sample homogeneity was maintained by magnetic stirring in the cuvette.

Emission and excitation wavelengths are listed in the figure legends. Bandwidths were generally 4.25 nm for fluorescence spectra and time course data and 16 nm for anisotropy measurements. Excitation spectra were corrected automatically by the fluorimeters using a lamp reference. Emission spectra are uncorrected. Light scattering due to sample turbidity was subtracted from the raw intensities in the calculation of generalized polarization and excimer/monomer ratios (see Figure legends).

In time course experiments, PLA_2 was added last to the sample and after temperature equilibration. In these experiments, fluorescence data were acquired at multiple excitation and emission wavelengths by rapid sluing of the monochromator mirrors (FluoroMax, Spex Industries, Edison, NJ). The time resolution in such experiments was 3−5 s. In some time courses, vesicle hydrolysis was also monitored simultaneously with the fluorescence by pH−stat titration (pH 8.0) in the fluorimeter cell as described previously (Bell & Biltonen, 1991). In such experiments, $Na_2B_4O_7$ was excluded from the reaction mixture, and the sample volume was increased to 3 mL.

Anisotropy measurements were obtained using Glan−Thompson polarizers in a PC1 fluorometer in the L-format (ISS, Urbana, IL). G-factors were also measured for each datum. Anisotropy values were calculated according to standard procedures (Lakowicz, 1983).

The binding of PLA_2 to the surface of vesicles was assessed during hydrolysis time courses by resonance energy transfer between the tryptophan of the enzyme to dansyl-DHPE in the phospholipid bilayer (2 mol %) as described (Burack et al., 1995). The intensity of dansyl-DHPE fluorescence emission was measured at 510 nm with excitation at both 280 nm (for energy transfer) and at 340 nm (for measurement of direct dansyl-DHPE fluorescence) before and after addition of PLA_2 under the conditions described above for time course experiments. Quantitatively, the magnitude of energy transfer at time τ was defined as the maximum emission intensity (excitation at 280 nm) after time τ ($F_{280,max}$) minus the intensity due to intrinsic probe fluorescence at 280 nm excitation ($F_{280,i}$). This calculation required that $F_{280,i}$ be corrected to account for the abrupt decrease in the intrinsic dansyl-DHPE fluorescence that occurred at time τ during the experiment (probably caused by changes in the polarity of the probe environment due to the accumulation of hydrolysis products, see Figure 1 and associated text). This correction was accomplished by using a calibration curve to infer $F_{280,i}$ from the fluorescence intensity of the probe excited at 340 nm and measured at the same time point as $F_{280,max}$ ($F_{340,max}$). The calibration curve was obtained from numerous independent observations of dansyl-DHPE fluorescence emission excited at the two wavelengths. This correction is valid only if the shape or wavelength of the dansyl-DHPE excitation spectrum does not change during the experiment. This assumption was verified by control experiments evaluating the excitation spectrum before and after the addition of lyso-PC and PA. Furthermore, the data were also normalized to the intensity of both the dansyl-DHPE fluorescence (excitation at 280 nm) prior to energy transfer ($F_{280,0}$) and the PLA_2 tryptophan fluorescence immediately prior to τ ($F_{PLA2(\tau-\delta)}$) to account for experimental variation in the concentrations of these molecules and for adsorption of the enzyme to the cuvette walls. Since we could not determine whether the emission due to energy transfer was also affected by the polarity of the environment, we calculated the energy transfer twice using the following two equations:

$$ET = \frac{F_{280,max} - F_{280,i}}{F_{280,0}F_{PLA2(\tau-\delta)}} \quad (1)$$

$$ET_{adj} = \frac{ET}{F_{340,max}/F_{340,0}} \quad (2)$$

ET is the magnitude of energy transfer at time τ, ET_{adj} is the energy transfer adjusted for changes in probe fluorescence due to environment polarity, and $F_{340,0}$ is the probe emission intensity (excitation at 340 nm) prior to time τ. Comparison of the data calculated as ET or ET_{adj} revealed identical qualitative results. Therefore, the interpretations did not depend on whether the fluorescence due to energy transfer was affected by the probe environment as was the intrinsic probe fluorescence. The data shown in Figures 4 and 9 represent ET_{adj}.

Pyrene-labeled lyso-PC (pyrene-lyso) and decanoate (pyrene-FA) were synthesized by hydrolysis of 1,2-bis(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (Molecular Probes) with PLA_2. Pyrene-labeled phospholipid was suspended (1 mg/mL) in 35 mM KCl, 10 mM $CaCl_2$, 3 mM NaN_3, 55 μM phenol red, and 0.6% lubrol PX. PLA_2 was added (43 μg/mL), and the pH was adjusted to approximately 8 based on the phenol red color. The sample was then incubated at 43 °C until the color changed to yellow, indicating hydrolysis of the sample (about 20 min). The suspension was lyophilized, and the lipids were extracted from the powder with methanol. Pyrene-lyso and pyrene-FA were separated by thin-layer chromatography on K6 silica gel (Whatman) using chloroform/methanol/water (65:35:4) as the mobile phase. The resolved probes were visualized under ultraviolet light, scraped from the chromatography plates, extracted with methanol, and stored at −20 °C. In experiments, the probes were added directly to the sample cuvette after the vesicles as described above for lyso-PC and PA (estimated final concentration was ≤2 μM or ≤2 mol %). Experiments were initiated after the probe intensity became relatively stable, indicating that steady state had been reached between the probe and the vesicles.

RESULTS

Effects of Reaction Products at Time τ. As stated in the introduction, the goal of this study was to assess the influence of temperature and DAG on three potentially relevant events provoked by the reaction products (lyso-PC and PA) at time τ. Before presenting the effects of temperature and DAG, we show in this section the behavior of these events under control conditions: temperature below the phospholipid phase transition temperature and in the absence of DAG.

Figure 1 depicts a typical time course of hydrolysis of DPPC LUV by PLA_2 (dashed curve). Time τ is obvious in such experiments as the boundary between the initial slow phase of hydrolysis while the bilayer is resistant to attack by PLA_2 and the subsequent rapid phase when the bilayer is susceptible. As reported previously, this enhancement of activity is a consequence of the accumulation of a specific concentration of reaction products in the bilayer (Apitz-Castro et al., 1982; Jain et al., 1989; Bell & Biltonen, 1992; Burack et al., 1993).

The binding of PLA_2 to the bilayer surface was studied by monitoring the fluorescence of a membrane-bound probe (dansyl-DHPE) during the time course of vesicle hydrolysis. In this way, we were able to observe PLA_2 binding both initially and at time τ. This energy transfer experiment reflects binding as the tryptophan residue(s) of the PLA_2 donate(s) excited state energy to the dansyl moiety upon interaction between PLA_2 and the bilayer (Burack et al., 1995; Bell et al., 1995). Curve b in Figure 1 displays the dansyl-DHPE fluorescence excited at 280 nm (the tryptophan maximum), the condition required for detection of energy transfer. The PLA_2 intrinsic tryptophan fluorescence is also shown (curve a, enzyme added at the first arrow). As reported previously (Jain et al., 1989; Burack & Biltonen, 1994; Burack et al., 1995; Bell et al., 1995), very little of the enzyme appeared to bind initially to the bilayer since no increase in dansyl fluorescence representing potential energy transfer (curve b) was observed at the time of enzyme addition. That conditions were appropriate to detect binding was verified using reference vesicles composed of 50% DPPC and 50% dipalmitoylphosphatidylglycerol (DPPG). Such vesicles bind essentially 100% of the enzyme at the concentration used (100 μM phospholipid; Burack & Biltonen, 1994; Burack et al., 1995) and consequently display a large increase in dansyl-DHPE fluorescence (excited at 280 nm) immediately upon addition of PLA_2 [not shown, but compare DPPC and DPPC:DPPG vesicles in Bell et al. (1995)].

The situation changed at time τ. As shown in Figure 1, the PLA_2 fluorescence (curve a) decreased during the hydrolysis time course immediately prior to time τ (dashed curve). [The slow decrease prior to τ is an artifact present in all studies of PLA_2 tryptophan fluorescence and represents adsorption of some of the enzyme to the walls of the sample cell; e.g., Bell and Biltonen (1989b)]. This decrease at τ differed from the sudden *increase* in PLA_2 fluorescence that normally occurs at τ (Bell & Biltonen, 1989b, 1992; Sheffield et al., 1995) and presumably reflected the transfer of excited state energy from tryptophan to dansyl-DHPE. This interpretation was verified by the rise in the dansyl-DHPE fluorescence excited at 280 nm (Figure 1, curve b) concurrent with the decrease in PLA_2 fluorescence. We concluded, then, that this sudden increase in energy transfer reflects the rapid enhancement of binding of the enzyme to the lipid bilayer at τ.

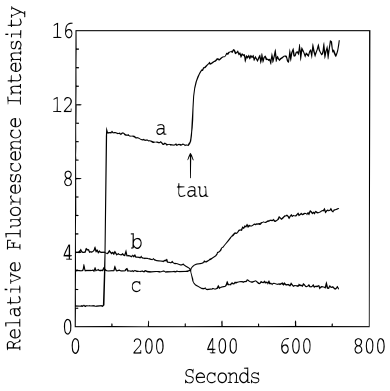

FIGURE 2: Time courses of PLA_2 and PRODAN fluorescence during hydrolysis of DPPC LUV at 39 °C. PLA_2 was added to DPPC vesicles containing PRODAN at time equals 80 s. The reaction mixture contained 10 mM $CaCl_2$. Curve a: PLA_2 fluorescence; excitation = 280 nm, emission = 340 nm. Curves b and c: PRODAN fluorescence; excitation = 350 nm, emission = 435 nm (curve a) or 500 nm (curve c). (Curves b and c cross at time τ.)

We also monitored simultaneously the dansyl-DHPE intrinsic fluorescence excited at 340 nm where no energy transfer occurs and observed a decrease in the intensity at τ (Figure 1, curve c). Apparently the intensity of dansyl fluorescence is attenuated by increases in the polarity of the probe's environment (Johnson et al., 1980; Ghiggino et al., 1981). The reduction in intrinsic dansyl fluorescence shown in Figure 1, then, corroborated the results obtained previously with PRODAN, suggesting that the polarity of the bilayer surface increases at time τ (Sheffield et al., 1995).

It has long been assumed that the increase in PLA_2 activity at τ was due to a change in bilayer structure and/or dynamics provoked by the reaction products. Insight into the nature of that change has recently been obtained using the fluorescent probes PRODAN and LAURDAN. Specifically, the accumulation of lyso-PC and PA causes a shift in the PRODAN emission spectrum toward longer wavelengths as τ is approached (Sheffield et al., 1995). Since PRODAN residues in superficial regions of the bilayer (Chong et al., 1989; Chong & Wong, 1993), we concluded that the result represents a disturbance of the bilayer surface in response to the presence of the reaction products (Sheffield et al., 1995). In support of that interpretation, a similar probe, LAURDAN, that occupies a deeper region of the bilayer (Chong and Wong, 1993; Parasassi et al., 1994b) was less sensitive to the effect of reaction products.

Figure 2 shows the temporal relationship between PRODAN fluorescence and τ during vesicle hydrolysis. Time τ was identified by the increase in PLA_2 fluorescence that typically accompanies the elevated hydrolysis rate (curve a, Figure 2; Bell & Biltonen, 1989b, 1992; Sheffield et al., 1995). The PRODAN emission intensity was monitored at both 435 nm (curve b) and 500 nm (curve c) to allow observation of any spectral shifts. As reported, an increase in the ratio of intensity at 500 nm compared to 435 nm occurred as τ was approached (Sheffield, et al., 1995). Such data with PRODAN have been interpreted as an increase in the polarity of the probe's environment (Weber & Farris, 1979).

The magnitude of the polarity change is determined quantitatively from PRODAN data such as shown in Figure

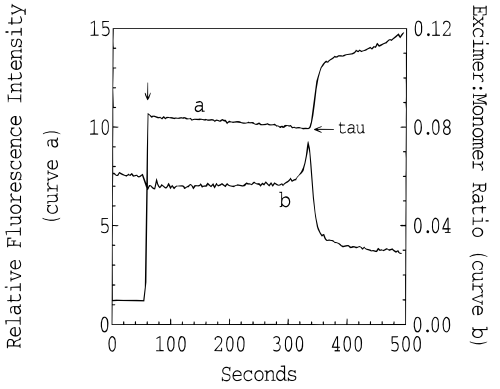

FIGURE 3: Time course of pyrene-lyso excimer to monomer fluorescence ratio during vesicle hydrolysis at 38 °C. DPPC LUV containing pyrene-lyso were hydrolyzed by PLA_2, (added at the first arrow) in the presence of 10 mM $CaCl_2$. Curve a: PLA_2 fluorescence (excitation = 280 nm, emission = 340 nm). Curve b: Ratio of pyrene lyso excimer to monomer fluorescence measured simultaneously (monomer, excitation = 344 nm, emission = 397 nm; excimer, excitation = 344 nm, emission = 480 nm).

2 using a mathematical description known as generalized polarization (GP):

$$\mathrm{GP} = \frac{I_{435} - I_{500}}{I_{435} + I_{500}} \tag{3}$$

GP is useful for quantitative analyses since the values of GP (in contrast to ratios) are additive and proportional to changes in the spectrum (Parasassi et al., 1991). GP, then, is a measure of the perturbation of the bilayer. A decrease in the value of GP signifies increased polarity in the bilayer. In the data of Figure 2, the value of GP decreased at time τ from 0.15 at the time of enzyme addition to 0.02 at τ.

Recent work has employed pyrene-FA as a probe of the lateral organization of fatty acid in the bilayer (Burack et al., 1993). The excimer emission (480 nm) reflects collisions among probe molecules while the monomer fluorescence (396−397 nm) describes non-interacting pyrene molecules. Thus, an increase in the excimer to monomer ratio would reveal an increase in segregation of the probe and presumably the analogous lipid. As reported by Burack et al. (1993) for pyrene-FA, the excimer to monomer ratio increases during the hydrolysis time course during the approach to τ. Those data were interpreted as suggesting that lateral segregation of fatty acid during the lag phase of the time course was involved with the mechanism by which reaction products enhanced the activity of PLA_2 at τ.

Figure 3 shows that a similar phenomenon was observed with pyrene-lyso at 38 °C (curve b). The decay of excimer to monomer ratio after τ probably reflects dilution of the probe by the rapid accumulation of the reaction products (Burack et al., 1993). In replicate experiments comparing pyrene-lyso and pyrene-FA, the apparent segregation of the pyrene-lyso probe at τ generally appeared less prominent than that of pyrene-FA.

Effects of Temperature. We examined the dansyl-DHPE fluorescence upon PLA_2 addition at a variety of temperatures (38−47 °C) to assess whether the effects of temperature on τ could reflect changes in either the initial binding of PLA_2 or the binding at time τ. At all temperatures tested, the behavior was qualitatively similar to Figure 1. No evidence of energy transfer was detectable initially upon addition of

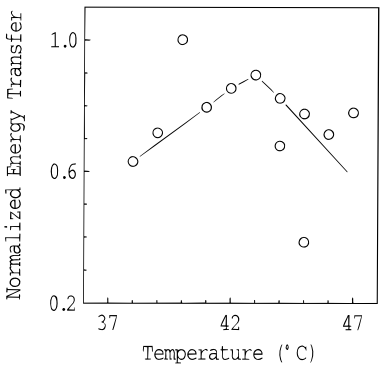

FIGURE 4: Effect of temperature on PLA_2 binding (energy transfer) to DPPC LUV at time τ during vesicle hydrolysis. Experiments and spectroscopy were identical to Figure 1 at the indicated temperatures. The amount of energy transfer was quantified and normalized at time τ (see Figure 1) as described in eq 2.

enzyme, but an increase (more abrupt at higher temperature) occurred at time τ. As shown in Figure 4, the magnitude of the energy transfer at τ depended on temperature. Up to 42 °C, the energy transfer at τ increased as a function of temperature. However, at higher temperatures, the amount of energy transfer declined. Nevertheless, at all temperatures, the magnitude of energy transfer was less than the reference (DPPC:DPPG) vesicles. At 42 °C, the apparent amount of PLA_2 bound was approximately two-thirds of the amount assessed with DPPC:DPPG (not shown). Comparison of the dansyl emission spectra obtained with DPPC at τ to that obtained with DPPC:DPPG vesicles showed that the difference in intensity was a difference in the amount of energy transfer (i.e., binding) and not a difference in spectrum shape or wavelength.

To test whether temperature influenced the nature of the perturbation to the bilayer surface provoked by the reaction products, the experiment shown in Figure 2 with the probe PRODAN was repeated at various temperatures. Although the initial intensities at 500 and 435 nm varied with temperature (due to the phase transition; Massey et al., 1985; Sheffield et al., 1995), the trend as a function of time was similar to that shown in Figure 2 at each temperature. Likewise, similar trends in the PRODAN emission spectrum occurred upon exogenous addition of reaction products to DPPC LUV. A summary of these data is illustrated in Figure 5A using GP values. The decrease in GP above the transition temperature (41.5 °C) presumably represents increased access of the probe to water in the liquid crystalline phase of the phospholipid. Consistent with the result described for Figure 2, the addition of 20 mol % each of lyso-PC and PA lowered the values of GP at all temperatures (solid circles). Therefore, temperature appeared not to change the general nature of the perturbation to the bilayer surface provoked by the reaction products.

To test whether temperature altered the sensitivity of the bilayer to the products, the relationship between GP values and reaction product concentration was assessed throughout the same temperature range. As shown in Figure 5B, the relationship between GP and product concentration was approximately linear at each temperature. However, the slope of the relationship varied with temperature and reached an extreme value at about 40−41 °C (Figure 5C). This maximum in the amplitude of the slope near the phase transition temperature suggested that fewer reaction products

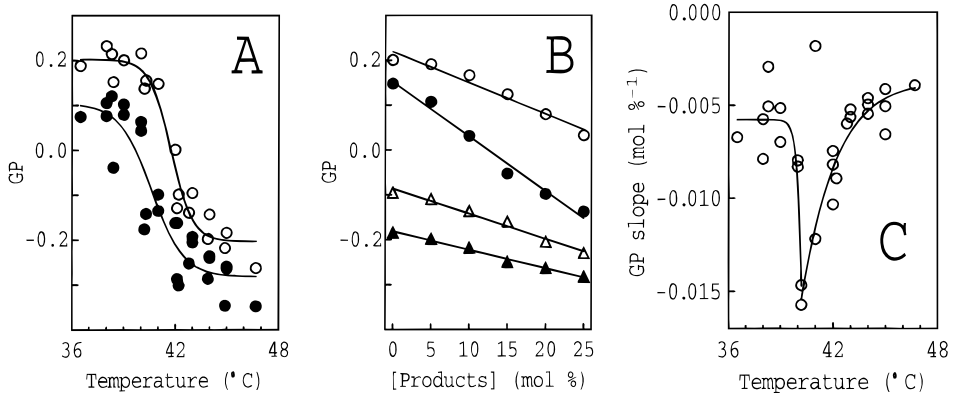

FIGURE 5: Relationship between PRODAN GP and temperature in DPPC LUV. GP was calculated from PRODAN emission intensities at 435 and 500 nm (excitation = 350 nm). All samples contained 10 mM $CaCl_2$. Panel A: open circles, no reaction products added; solid circles, 20 mol % each of lyso-PC and PA. Panel B displays representative relationships between GP and added reaction products at 39 (open circles), 41 (solid circles), 43 (open triangles), and 45 °C (solid triangles). Panel C: Slopes of data such as shown in panel B at several temperatures.

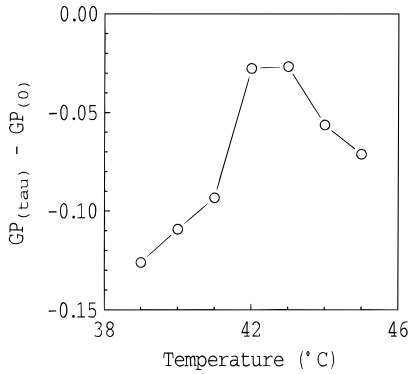

FIGURE 6: Temperature dependence of the change in PRODAN GP from time zero to time τ during hydrolysis of DPPC LUV. The value of GP calculated from PRODAN emission prior to addition of enzyme was subtracted from that calculated at τ using time courses such those shown in Figure 2 at the indicated temperatures. The GP value at τ was obtained using data corresponding to the last time point before the PLA_2 intrinsic fluorescence deviated from the base line.

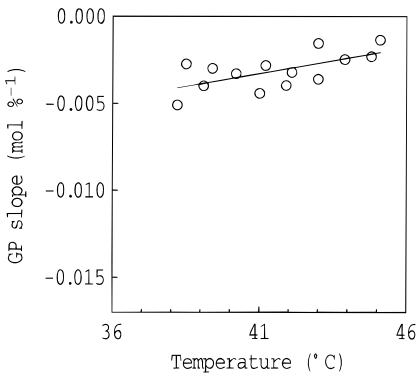

FIGURE 7: Temperature dependence of the effect of reaction products on PRODAN GP in the absence of added calcium. Reaction conditions and spectroscopic parameters were identical to Figure 5 with the exception that no $CaCl_2$ was added. The slope of GP as a function of concentration of added reaction products was calculated exactly as described in Figure 5 and plotted as a function of temperature as in Figure 5C.

were required at that temperature to elicit the same perturbation attained at temperatures away from the transition.

To test the relationship between the result shown in Figure 5C and the actual events during hydrolysis time courses, we compared the value of PRODAN GP at τ with the value prior to the addition of enzyme (Figure 6). In contrast to the result shown in Figure 5C, the difference in GP at τ did not become more negative at temperatures near the phase transition. In fact, the trend was in the opposite direction. Although reaction products appeared to perturb the bilayer more per molecule at the temperature range where τ is shortest (Figure 5C), the data of Figure 6 suggested that the same or even a smaller perturbation may actually be required for increased PLA_2 activity at such temperatures.

One possible interpretation of the result in Figure 5C is that reaction products shift the phase transition to lower temperature. We investigated this possibility by examining the rotational mobility (anisotropy) of PRODAN under the conditions of Figure 5. As with other probes, increasing temperature causes an abrupt decrease in the PRODAN anisotropy which corresponds to the lipid phase transition (Bell et al., 1995). The presence of reaction products appeared to shift the phase transition in such experiments toward lower temperatures (approximately 1 °C at 20 mol % products). Such a shift was consistent quantitatively with the result shown in Figure 5C.

This shift in phase transition temperature was surprising in light of the published calorimetry data indicating that the low-temperature boundary of the transition remains constant as a function of lyso-PC and PA (Burack et al., 1993). However, those data were obtained at low (10 μM) calcium concentration, and the data of Figure 5 were acquired at 10 mM $CaCl_2$. We therefore repeated these experiments in the absence of added calcium. In that case, the slope of GP as a function of reaction products did not exhibit a local extreme value (Figure 7) and the phase transition temperature assessed by PRODAN anisotropy was likewise insensitive to reaction product concentration (not shown).

We tested whether additional information regarding the effects of temperature could be obtained using LAURDAN. Figure 8A shows the slope of GP calculated from LAURDAN emission spectra (solid circles) as a function of reaction product concentration in the bilayer (the data from Figure 5C for PRODAN are included in the figure for comparison, open circles). Consistent with prior observations, little effect of reaction products on LAURDAN GP was observed at temperatures below 41 °C (i.e., GP slope $\approx$ 0). However, a substantial effect of reaction products on LAURDAN GP occurred at temperatures greater than 41 °C. In fact, the

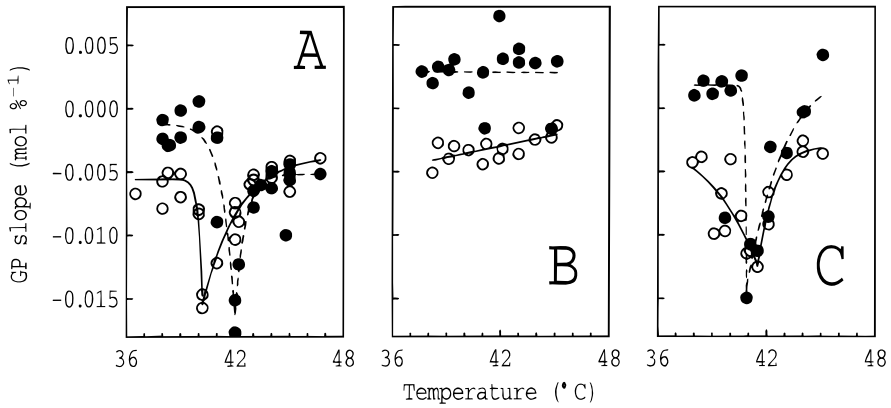

FIGURE 8: Comparison of the effects of reaction products and temperature on PRODAN and LAURDAN GP. The experiments of Figure 5 were repeated using LAURDAN at various calcium concentrations. Panel A: The slopes of LAURDAN GP as a function of reaction product concentration were determined as in Figure 5 and plotted as a function of temperature at 10 mM $CaCl_2$ (solid circles). The open circles represent the corresponding data collected with PRODAN (from Figure 5C). Panels B and C: The experiments of panel A were repeated in the absence of added $CaCl_2$ (B) or with ~55 μM $CaCl_2$ (C).

magnitude of the change in GP was identical to that observed for PRODAN in the liquid crystalline state of the bilayer. Therefore, although the effect of reaction products to disturb the bilayer surface (PRODAN GP) was qualitatively similar at all temperatures tested (i.e., GP slope < 0), the influence of that perturbation on the rest of the bilayer (LAURDAN GP) was temperature-dependent.

Interestingly, as shown in Figure 8B, this temperature effect on LAURDAN fluorescence was confined to high calcium concentration. When calcium was not added to the sample, the PRODAN and LAURDAN GP changes as a function of reaction products were displaced at all temperatures. Experiments at ~55 μM $CaCl_2$ revealed that this difference was not simply related to the effect of calcium on the phase transition shifts elicited by the reaction products. Similar to the result obtained at 10 mM $CaCl_2$ (Figure 5C), the magnitude of the GP slope reached an extreme value at about 41 °C (Figure 8C). Nevertheless, at the same condition, the PRODAN and LAURDAN GP slopes were still displaced at both low and high temperatures. Thus, the behavior of the phase transition at ~55 μM calcium followed that of high calcium, but the relative effects of reaction products on PRODAN and LAURDAN fluorescence at the temperature extremes resembled low calcium.

Possible effects of temperature on the lateral organization of lyso-PC and PA in the bilayer could only be studied at temperatures below the phase transition. This is because pyrene-labeled probes selectively partition into liquid crystalline phase lipid irrespective of the partitioning of the analogous lipid represented by the probe (Galla & Sackman, 1974). Thus, the presence of liquid crystalline domains at temperatures above about 40 °C invalidate interpretation of lateral bilayer organization using pyrene probes.

Consequently, the experiment shown in Figure 3 was repeated only in the range between 37 and 40 °C with either the pyrene-lyso or pyrene-FA probes. Data qualitatively similar to that shown in Figure 3 were obtained for both probes in the temperature range. Nonetheless, we found that the increased excimer to monomer ratio at τ was most obvious at 38 and 39 °C and was nearly absent at 40 °C. This was especially true for pyrene-lyso where the effect is smaller and more difficult to reproduce when a low signal to noise ratio exists. In any case, the initial ratio of excimer to monomer fluorescence was independent of temperature

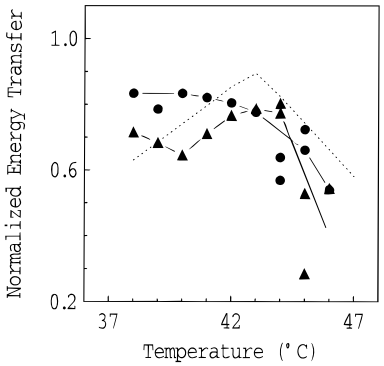

FIGURE 9: Effect of DAG concentration on the temperature dependence of PLA_2 binding (energy transfer) to DPPC LUV at time τ during vesicle hydrolysis. Reaction conditions and calculations of data were identical to Figure 4. DAG concentrations were 3.2 mol % (triangles) and 5.2 mol % (circles). The dotted line represents the consensus response from vesicles without DAG (directly from Figure 4) for comparison.

throughout the range for both probes as assessed by analysis of variance (pyrene-lyso, $p = 0.65$, $n = 15$; pyrene-FA, $p = 0.72$, $n = 15$).

Effects of DAG. The experiments for which the effect of temperature was investigated were repeated with vesicles containing DAG. The range of DAG concentration corresponded to that for which substantial effects on the hydrolysis kinetics are observed [see Bell et al. (1995)]. Previous work already established that DAG does not affect the initial amount of PLA_2 bound to the vesicle surface (Bell et al., 1995). Repetition of the experiment of Figure 3 at various DAG concentrations and temperatures confirmed that result (not shown). In addition, little or no consistent effect of DAG on the overall amount of energy transfer at τ was observed (Figure 9; the dotted line represents the data of Figure 4 for comparison). In particular, energy transfer was not enhanced near the phase transition temperature even though DAG has a substantial effect to reduce the length of τ at such temperatures (Bell et al., 1995). Nevertheless, the presence of DAG did appear to alter the temperature dependence of the energy transfer. For example, only the decline in energy transfer at high temperature was apparent at the highest DAG concentration (5.2 mol %).

We repeated the experiments with PRODAN and LAURDAN (Figures 5, 6, and 8 and the anisotropy experiments) at various DAG concentrations up to about 5 mol % with

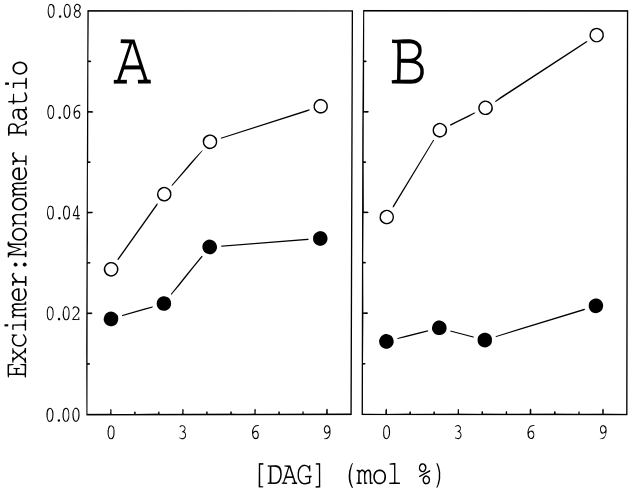

FIGURE 10: Ratio of initial excimer to monomer ratio for pyrene-lyso and pyrene-FA as a function of added DAG at 37 °C (panel A) and 40 °C (panel B). Refer to Figure 3 for reaction conditions. Excimer to monomer ratios of pyrene-lyso (open circles) or pyrene-FA (solid circles) immediately after PLA_2 addition were plotted as a function of DAG concentration.

10 mM $CaCl_2$. However, no additional effect of DAG beyond its influence on the DPPC phase transition was observed. Likewise, DAG did not alter the effects of the reaction products on PRODAN and LAURDAN fluorescence. In other words, the data were essentially identical to those shown in these figures with the exception that temperature ranges were broadened and shifted according to the effect of DAG on the phase transition (López-García et al., 1994; Bell et al., 1995).

DAG did increase the apparent segregation of both pyrene-lyso and pyrene-FA. Figure 10 details the effect of DAG concentration on the initial excimer to monomer ratio for each of the two probes at 37 and 40 °C. The trend as a function of DAG concentration was analyzed for each experiment by linear regression. The resulting slopes were independent of temperature in this range by analysis of variance (pyrene-lyso, $p = 0.37$; pyrene-FA, $p = 0.54$). Therefore, we pooled all the data in a single analysis for each probe and found that the effect of DAG was statistically significant (measured both from time courses as well as emission spectra, Table 1). We applied the same analysis to the maximum excimer/monomer ratio near time τ and obtained comparable results: temperature (37−40 °C) had no effect (pyrene-lyso, $p = 0.14$; pyrene-FA, $p = 0.57$), and the trends with DAG were significant (Table 1).

Table 1: Slope of the Pyrene-Lyso and Pyrene-FA Excimer to Monomer Ratio at Initial Time and at Time τ as a Function of DAG Concentration[a]

condition	slope (mol $\%^{-1} \times 10^3$)	slope = 0? [b] (p)	n[c]
initial ratio[d]			
pyrene-FA	1.51 ± 0.26	<0.0001	15
pyrene-lyso	2.91 ± 0.53	<0.0001	15
ratio at τ[e]			
pyrene-FA	1.48 ± 0.57	0.036	8
pyrene-lyso	3.87 ± 0.71	0.0009	8

[a] Table entries represent the slope of data such as that shown in Figure 10 for experiments obtained at 37, 38, 39, and 40 °C. The pooling of these data was justified by separate analysis indicating that there was no trend as a function of temperature in this range. Data are expressed as the mean ± SE. [b] Probability that the slope equals 0 based on one-sample Student's *t*-test (two-tailed). [c] Number of independent slopes. [d] The ratio was measured immediately upon addition of PLA_2 or in the absence of PLA_2. [e] The ratio was measured at the time adjacent to τ where its value was maximal (see Figure 3).

DISCUSSION

The purpose of this study was to clarify possible mechanisms by which temperature and DAG affect the kinetics of the activity of PLA_2 toward DPPC LUV. The basic assumption is that the effects of both temperature and DAG are mediated by the effects of the reaction products (Bell et al., 1995). Efforts by various investigators to understand the mechanism by which reaction products render the bilayer susceptible to PLA_2 have explored a variety of possible actions of the products. The results of such studies have narrowed the likely possibilities to four factors that seem capable of explaining the effect [e.g., Jain et al. (1982, 1989), Burack et al. (1993, 1995), Burack and Biltonen, (1994), and Sheffield et al. (1995)]: (1) Products increase the binding of PLA_2 to the vesicle surface. (2) The fatty acid makes the charge of the bilayer surface more negative, promoting enzyme binding electrostatically. (3) The integrity of the bilayer surface is perturbed by the products, perhaps due to local increases in membrane curvature. (4) The reaction products segregate laterally into domains in the bilayer. This segregation may enhance the ability of the products to induce the other three effects. We used fluorescent probes to investigate the effects of temperature and DAG on each of these factors except for charge (since neither should alter the surface charge of the bilayer). In summary, our data show that temperature affects the binding at time τ as well as the ability of the reaction products to perturb the bilayer surface. DAG appears to influence the lateral distribution of the reaction products in the bilayer. We now discuss these points individually.

Effect of Temperature on Binding at Time τ. At least some of the mechanism by which temperature alters τ appeared to involve changes in the binding of PLA_2 to the vesicle surface at time τ. The maximum binding of the enzyme at τ increased up to about 42 °C. Above 42 °C, it decreased systematically with temperature (Figure 4).

To test whether these changes in energy transfer efficiency might explain the effect of temperature on τ, we examined the relationship between the two parameters. The values of normalized energy transfer shown in Figures 4 and 9 were compared to the values for τ from the same experiments. The comparison yielded a linear correlation with a negative slope that was statistically significant when the data of Figures 4 and 9 were pooled ($p = 0.0004$, $n = 34$). That is, decreased binding at time τ coincided with larger values of τ, as would be expected if temperature were altering τ by affecting the interaction between the enzyme and the bilayer. Nevertheless, the correlation was not as strong when individual experiments were considered (although the same trend was found in each case). Only the data for the two experiments shown in Figure 9 for vesicles with DAG displayed statistically significant correlations with τ when considered individually (0% DAG, $p > 0.05$, $n = 12$; 3.2% DAG, $p = 0.0002$, $n = 11$; 5.2% DAG, $p = 0.046$, $n = 11$). Likewise, when another condition at which τ is affected by temperature (pure DPPC LUV in the absence of added calcium) was considered, the energy transfer to dansyl varied with temperature as in Figure 4, but the correlation with τ

was not statistically significant ($n = 6$). Therefore, while it would appear that at least some of the effect of temperature on the length of τ involves the binding of PLA_2 at time τ, such probably does not explain the entire effect.

A subtlety in the bind data further supports the idea that all of the effects of temperature on the hydrolysis kinetics cannot be explained by differences in binding. As shown in formulations of models for PLA_2 activity, the concentration of enzyme bound to the bilayer surface should be proportional to τ^{-1} (Bell & Biltonen, 1992; Bent & Bell, 1995; Bell et al., 1995). In contrast, the experimental relationship was linear with a negative slope. Attempts to fit the data to a function of τ^{-1} were unsuccessful. Therefore, although an apparent causal relationship existed between τ and the binding of PLA_2, this relationship must represent only part of the mechanism by which temperature near the phase transition affects PLA_2 kinetics. (Of course, this interpretation assumes that the rate of energy transfer is directly proportional to the amount of enzyme bound.)

The high-temperature range of this result appears to substantiate older investigations proposing that the binding of PLA_2 to vesicle bilayers prefers the gel phase of the lipid (Menashe et al., 1986; Bell & Biltonen, 1989a). Furthermore, the result complements previous work on the effect of temperature on hydrolysis kinetics. In that study, we determined that temperature affected the observed hydrolysis rate. The highest rate was found near the phase transition temperature, although the correlation between τ and the apparent hydrolysis rate was very weak (Bell et al., 1995). The results described in this study regarding PLA_2 binding may help explain this temperature dependence of the hydrolysis rate. The reason why the apparent binding showed a somewhat better correlation with τ in this study than observed previously for the hydrolysis rate probably reflects the complexity of the measured hydrolysis rate. Surely, the rate reflects a variety of processes including product inhibition, substrate depletion, and surface diffusion limitations in addition to enzyme/vesicle binding (Lathrop et al., 1992; Burack & Biltonen, 1994).

It must be recognized that the energy transfer experiment does not provide fine details of the binding process. All that is measured is an increase in the proximity of enzyme tryptophan and the dansyl probe at the vesicle surface. However, the interpretation that the increase in energy transfer at time τ represents an increase in the total amount of PLA_2 adsorbed to the vesicle surface is substantiated by a variety of other observations. These include the fact that the intrinsic tryptophan fluorescence of the enzyme also increases at time τ (in the absence of dansyl-DHPE; Figures 2 and 3) as well as competition binding experiments (Burack et al., 1995), other measurements of PLA_2 fluorescence (Jain et al., 1982; Bell & Biltonen, 1989a; Burack et al., 1995; Bell et al., 1995) and column chromatography (Jain et al., 1982). Our interpretations also assume a homogeneous distribution of enzyme and reaction products among the vesicles. Ample evidence that both enzyme and products rapidly exchange between vesicles and between the lipid and aqueous phases (at least up to time τ) corroborates this assumption (Elamrani & Blume, 1982; Jain et al., 1989; Bell et al., 1992; Burack et al., 1995).

Effects of Temperature on the Perturbation Induced by Reaction Products. Qualitatively, the reaction products perturbed the bilayer surface (PRODAN fluorescence) similarly regardless of whether the vesicle was in the gel or the liquid crystalline state (Figure 5A). Therefore, the effect of temperature appeared not to reflect a fundamental change in the mechanism of the reaction product effect. The observation that the intensity of the intrinsic fluorescence of dansyl-DHPE decreased near τ (as in Figure 1) at all temperatures studied (38−47 °C) also supports this conclusion. In separate experiments not shown, we determined that this decrement in the dansyl fluorescence was accompanied by a red-shift in the emission spectrum indicative of an increase in the polarity of the dansyl environment (Johnson et al., 1980; Ghiggino et al., 1981).

It did appear that temperature affected the perturbation monitored by PRODAN quantitatively. Specifically, reaction products caused a larger perturbation of the bilayer near the phase transition temperature (Figure 5B,C). This result was rationalized by a reaction product-induced shift in the phospholipid phase transition toward lower temperature. While the quantitative differences could explain some of the temperature effect on τ, they appeared insufficient to rationalize all of the phenomenology. For example, the effect was absent at low calcium concentration (Figure 7) even though τ is affected similarly by temperature at both low and high calcium (not shown).

The data obtained with LAURDAN (Figure 8) provided further evidence that temperature influences aspects of the perturbation induced by reaction products. The differences between high and low temperature observed with LAURDAN could reflect the ability of phospholipids to accommodate a surface perturbation more readily in the liquid state than in the gel. Thus, in the gel state, the reaction products affected only the surface of the bilayer (detected by PRODAN). In the liquid crystalline state, the surface and the region occupied by LAURDAN molecules (probably near the glycerol backbone; Parasassi et al., 1994b) were equally perturbed. The fact that τ is larger in the liquid crystalline state (Bell et al., 1995) suggests that the reaction products are more effective at provoking membrane susceptibility to PLA_2 when the vesicle is less able to accommodate the perturbation. We are currently investigating the effects of reaction products on the dynamics of the bilayer in the regions occupied by PRODAN and LAURDAN using time-resolved fluorescence spectroscopy to try to address this issue more completely. Other issues such as fluctuations of bilayer internal organization and possible consequences of the phase transition on the tendency of products to form domains may also contribute to the mechanism of the effect of temperature (Freire & Biltonen, 1978; Burack et al., 1993; Smaby et al., 1994; Burack & Biltonen, 1994).

Effects of DAG on Lateral Segregation of Reaction Products in the Bilayer. The data obtained with pyrene-lyso (Figure 3) suggest that, like fatty acid, lyso-PC also segregates into domains in the bilayer near time τ in the hydrolysis time course. It has been proposed that such lateral segregation of the reaction products is beneficial or even necessary for the induction of high PLA_2 activity (Jain et al., 1989; Burack et al., 1993). The reason why the effect appeared more prominent with fatty acid than with pyrene-lyso is not known. However, we note that the excimer to monomer ratio was greater for pyrene-lyso than for pyrene-FA at the onset of the hydrolysis time course (Figure 10). Therefore, the larger effect at τ with fatty acid may reflect a difference in the initial conditions between the two probes.

It may also be that de-mixing of fatty acid in the bilayer during product accumulation is simply more complete than for lyso-PC which may remain more miscible with DPPC.

What was most clear from the data was that the presence of DAG in the bilayer enhanced the lateral segregation of pyrene-labeled reaction products (Figure 10 and Table 1). This result supports a recent hypothesis regarding the effect of DAG to reduce the length of τ at temperatures near or below the DPPC phase transition (Bell et al., 1995). The advantage to forming such domains could be to consolidate the perturbing effect of the reaction products to local areas on the bilayer. Thus, the size of individual perturbations would be greater per total number of phospholipids hydrolyzed than without lateral segregation (Bell et al., 1995). We believe that this effect of DAG is important for explaining its ability to reduce the length of τ. All other effects that could be identified in this study (binding and perturbations measured by PRODAN and LAURDAN) appeared to be solely a reflection of changes induced by DAG in the membrane phase transition. Since the ability of DAG to reduce τ is not simply a consequence of altering the phase transition temperature range (Bell et al., 1995), the effect on reaction product distribution is likely to be significant.

It seems contradictory that we were able to observe an effect of DAG on the bilayer perturbation caused by reaction products using the pyrene probes but not with PRODAN or LAURDAN. Part of the reason for this disparity may relate to the observation that the horizontal distribution of PRODAN and LAURDAN in the bilayer tends to be random even when lipid domains are present (Massey et al., 1985; Parasassi et al., 1993, 1994a). PRODAN and LAURDAN would therefore report global rather than local properties of the bilayer at their respective depths. In contrast, the pyrene probes apparently do segregate into domains (presumably with fatty acid or lyso-PC) and therefore report local properties along the plane of the bilayer. Thus, DAG may facilitate the effect of the reaction products on PLA_2 activity not by changing the average perturbation of the bilayer (sensed by PRODAN and LAURDAN). Instead, it may help consolidate individual perturbations caused by the products so that locally the bilayer disturbance is much larger at the same concentration of reaction products. Nevertheless, since DAG reduces the concentration of reaction products required to provoke rapid hydrolysis at time τ but did not appear to affect the reaction product dependence of GP, it seems logical that the magnitude of change in GP at time τ should have decreased as a function of DAG concentration similar to how it did at certain temperatures (Figure 5). However, this was not observed. The reason for this apparent discrepancy is not known, but it could reflect differences in the product dependence of GP in the presence versus the absence of enzyme.

Effect of Calcium. The effect of calcium on the ability of the reaction products to alter the phase transition of the bilayer was intriguing and, to our knowledge, unreported. It could relate to the tendency of calcium to bind to fatty acids in the bilayer at the alkaline pH used in these studies. The data probably do not reflect removal of fatty acid from the bilayer since most of the calcium effects were apparent at 55 μM, a concentration of calcium that does not alter the apparent fatty acid bilayer partition coefficient (Bell & Bent, 1995). Calcium also affected the ability of LAURDAN to detect the perturbation caused by reaction products independently of the effect on the phase transition. For example, at high calcium, the effect of reaction products altered the PRODAN and LAURDAN emission spectra similarly in the liquid crystalline phase (Figure 8A). Alternatively, at low calcium, the effect of reaction products as measured by the value of GP was opposite with LAURDAN compared to PRODAN (Figure 8B). That is, reaction products appeared to increase the polarity of PRODAN's environment but *decrease* the polarity of LAURDAN's environment. A complete understanding of these observations requires further study. Nevertheless, we note that other effects of calcium on the kinetics of vesicle hydrolysis by PLA_2 beyond its trivial role as an obligatory cofactor for the enzyme have been reported (Lathrop & Biltonen, 1992; Bent & Bell, 1995).

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REFERENCES

Apitz-Castro, R., Jain, M. K., & De Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 349−356.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.
Bell, J. D., & Biltonen, R. L. (1989a) *J. Biol. Chem. 264*, 225−230.
Bell, J. D., & Biltonen, R. L. (1989b) *J. Biol. Chem. 264*, 12194−12200.
Bell, J. D., & Biltonen, R. L. (1991) *Methods Enzymol. 197*, 249−258.
Bell, J. D., & Biltonen, R. L. (1992) *J. Biol. Chem. 267*, 11046−11056.
Bell, J. D., Brown, S. D., & Baker, B. L. (1992) *Biochim. Biophys. Acta 1127*, 208−220.
Bell, J. D., Baker, M. L., Bent, E. D., Ashton, R. W., Hemming, D. J. B., & Hansen, L. D. (1995) *Biochemistry 34*, 11551−11560.
Bent, E. D., & Bell, J. D. (1995) *Biochim. Biophys. Acta 1254*, 349−360.
Biltonen, R. L. (1990) *J. Chem. Thermodyn. 22*, 1−19.
Brown, S. D., Baker, B. L., & Bell, J. D. (1993) *Biochem. Biophys. Acta 1168*, 13−22.
Burack, W. R., & Biltonen, R. L. (1994) *Chem. Phys. Lipids 73*, 209−222.
Burack, W. R., Yuan, Q., & Biltonen, R. L. (1993) *Biochemistry 32*, 583−589.
Burack, W. R., Gadd, M. E., & Biltonen, R. L. (1995) *Biochemistry 34*, 14819−14828.
Chong, P. L.-G., & Wong, P. T. T. (1993) *Biochim. Biophys. Acta 1149*, 260−266.
Chong, P. L.-G., Capes, S., & Wong, P. T. T. (1989) *Biochemistry 28*, 8358−8363.
Cunningham, B. A., Tsujita, T., & Brockman, H. L. (1989) *Biochemistry 28*, 32−40.
De Boeck, H., & Zidovetzki, R. (1992) *Biochemistry 31*, 623−630.
Elamrani, K., & Blume, A. (1982) *Biochemistry 21*, 521−526.
Freire, E., & Biltonen, R. (1978) *Biochim. Biophys. Acta 514*, 54−68.
Galla, H.-J., & Sackmann, E. (1974) *Biochim. Biophys. Acta 339*, 103−115.
Gheriani-Gruszka, N., Almog, S., Biltonen, R. L., & Lichtenberg, D. (1988) *J. Biol. Chem. 263*, 11808−11813.
Ghiggino, K. P., Lee, A. G., Meech, S. R., O'Connor, D. V., & Phillips, D. (1981) *Biochemistry 20*, 5381−5389.

Grainger, D. W., Reichert, A., Ringsdorf, H., & Salesse, C. (1989) *FEBS Lett. 252*, 73−82.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
Jain, M. K., & De Haas, G. H. (1983) *Biochim. Biophys. Acta 736*, 157−162.
Jain, M. K., Egmond, M. R., Verheij, H. M. Apitz-Castro, R., Dijkman, R., & De Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 341−348.
Jain, M. K., Yu, B.-Z., & Kozubek, A. (1989) *Biochim. Biophys. Acta 980*, 23−32.
Johnson, J. D., Taskinen, M.-R., Matsuoka, N., & Jackson, R. L. (1980) *J. Biol. Chem. 255*, 3461−3465.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp 112−128, Plenum Press, New York.
Lathrop, B. K., & Biltonen, R. L. (1992) *J. Biol. Chem. 267*, 21425−21431.
Lichtenberg, D., Romero, G., Menashe, M., & Biltonen, R. L. (1986) *J. Biol. Chem. 261*, 5334−5340.
López-García, F., Villalaín, J., Gómez-Fernández, J. C., & Quinn, P. J. (1994) *Biophys. J. 66*, 1991−2004.
Maraganore, J. M., Merutka, G., Cho, W., Welches, W., Kézdy, F. J., & Heinrikson, R. L. (1984) *J. Biol. Chem. 259*, 13839−13843.
Massey, J. B., She, H. S., & Pownall, H. J. (1985) *Biochemistry 24*, 6973−6978.
Menashe, M., Lichtenberg, D., Gutierrez-Merino, C., & Biltonen, R. L. (1981) *J. Biol. Chem. 256*, 4541−4543.
Menashe, M., Romero, G., Biltonen, R. L., & Lichtenberg, D. (1986) *J. Biol. Chem. 261*, 5328−5333.
Mouritsen, O. G. (1991) *Chem. Phys. Lipids 57*, 179−194.
Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R. M., & Gratton, E. (1991) *Biophys. J. 60*, 179−189.
Parasassi, T., Ravagnan, G., Rusch, R. M., & Gratton, E. (1993) *Photochem. Photobiol. 57*, 403−410.
Parasassi, T., Di Stefano, M., Loiero, M., Ravagnan, G., & Gratton, E. (1994a) *Biophys. J. 66*, 120−132.
Parasassi, T., Di Stefano, M., Loiero, M., Ravagnan, G., & Gratton, E. (1994b) *Biophys. J. 66*, 763−768.
Sheffield, M. J., Baker, B. L., Li, D., Owen, N. L., Baker, M. L., & Bell, J. D. (1995) *Biochemistry 34*, 7796−7806.
Smaby, J. M., Muderhwa, J. M., & Brockman, H. L. (1994) *Biochemistry 33*, 1915−1922.
Weber, G., & Farris, F. J. (1979) *Biochemistry 18*, 3075−3078.
Zidovetzki, R., Laptalo, L., & Crawford, J. (1992) *Biochemistry 31*, 7683−7691.

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