Effects of Temperature and Glycerides on the Enhancement of *Agkistrodon piscivorus piscivorus* Phospholipase A_2 Activity by Lysolecithin and Palmitic Acid†

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ABSTRACT: The effect of temperature and various glycerides to modulate the ability of lysolecithin and fatty acid to promote high phospholipase A_2 activity was studied using dipalmitoylphosphatidylcholine large unilamellar vesicles as substrate. The length of the lag phase prior to the accumulation of sufficient hydrolysis products (lysolecithin and fatty acid) to support high phospholipase activity was shortest at temperatures near the thermotropic phase transition of the phospholipid substrate. A reduction in the lag phase correlated with a reduction in the requirement for hydrolysis products at the phase transition temperature, where the bilayer exists in a state of fluctuating domains of gel and liquid crystal. Dipalmitoylglycerol and tripalmitoylglycerol also reduced the length of the lag phase. This reduction was both concentration-dependent and temperature-dependent relative to the phase transition in the presence of the glycerides. As with the effect of temperature, the ability of di- and triglycerides to decrease the lag time correlated with a decrease in the amount of reaction products necessary to promote high phospholipase activity. This effect coincided with the tendency of the glycerides to form domains in the bilayer. Glycerides that did not form domains either had no effect (monopalmitoylglycerol) or increased the length of the lag phase (dicaprylglycerol). These data suggest that the effect of the reaction products to increase phospholipase A_2 activity is aided by the presence of fluctuations in lipid domains within the bilayer.

An important principle that has received increased attention recently in the study of membrane biochemistry is the effect of the lateral distribution of bilayer lipids on the function of proteins bound to the bilayer surface. For example, the binding of certain extrinsic proteins to bilayers of mixed composition appears to involve the formation of domains of specific lipids (Birrell & Griffith, 1976; Nelsestuen & Broderius, 1977; Lentz et al., 1983; Wiener et al., 1985; Wang et al., 1993). Also, the hydrolytic activity of a variety of lipases depends on the lateral distribution of lipid components within the bilayer (Bhat & Brockman, 1982; Tsujita et al., 1987; Cunningham et al., 1989; Zidovetzki et al., 1992; Smaby et al., 1994). Interest in the problem has not been limited to experimental research. Numerous theoretical studies have focused on such topics as percolation theory and the dynamics of fluctuations of bilayer domains (Freire & Biltonen, 1978; Saxton, 1982; Biltonen, 1990; Mouritsen, 1991; Sperotto & Mouritsen, 1991; Smaby et al., 1994).

Soluble phospholipase A_2 from snake venom or mammalian pancreas (PLA_2)[1] appears to be an excellent model system for studies of the effects of bilayer composition and lipid distribution on enzyme activity (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). When mixed with large vesicles composed of saturated phosphatidylcholine, this enzyme displays limited activity unless certain additives are present in sufficient quantity in the bilayer. For example, the products of phospholipid hydrolysis, lysolecithin and fatty acid, greatly enhance the activity of the enzyme (Apitz-Castro et al., 1982; Bell & Biltonen, 1989b; Jain et al., 1989; Burack et al., 1993). Consequently, the rate of hydrolysis of pure phosphatidylcholine vesicles proceeds slowly until sufficient reaction products have accumulated in the bilayer. At that point, the enzyme activity suddenly increases by 1 or more orders of magnitude (Apitz-Castro et al., 1982; Bell & Biltonen, 1989b).

It has been proposed that molecules that tend to segregate into domains in the membrane increase the ability of lysolecithin to enhance PLA_2 activity (Bent & Bell, 1995). Consistent with this idea, previous studies have shown that the fatty acid produced during the hydrolysis of phosphatidylcholine bilayers separates into domains and also reduces the amount of lysolecithin required to increase PLA_2 activity (Jain et al., 1989; Burack et al., 1993; Bent & Bell, 1995). However, the negative charge of the fatty acid complicates the interpretation. It is well-documented that negative charge enhances the activity of PLA_2, probably by increasing the ability of the enzyme to bind to the bilayer (Jain et al., 1986;

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[1] Abbreviations: PLA_2, low molecular weight soluble phospholipase A_2; DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicles; SUV, sonicated unilamellar vesicles; C-16 MAG, 1-monopalmitoylglycerol; C-10 DAG, 1,2-dicaprylglycerol; C-16 DAG, 1,2-dipalmitoylglycerol; C-16 TAG, tripalmitoylglycerol; τ, time of onset of increased PLA_2 activity; dansyl-DHPE, *N*-[5-(dimethylamino)-naphthyl-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; X_p, minimum mole percent of reaction products at which τ equals zero; K_p, lysolecithin lipid/water partition coefficient.

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Scott et al., 1994). Therefore, it is not clear whether the fatty acid acts by conferring negative charge on the membrane or by segregating lysolecithin as proposed earlier. The two possibilities are not mutually exclusive.

To study the effect of domain formation on the ability of lysolecithin to increase the activity of PLA_2 in the absence of the complication of charge, we have used neutral mono-, di-, and triacylglycerols as additives to dipalmitoylphosphatidylcholine (DPPC) large unilamellar vesicles (LUV) and the alkaline aspartate 49 phospholipase A_2 purified from the venom of *Agkistrodon piscivorus piscivorus* as our experimental system. Several studies have shown that diacylglycerols can increase PLA_2 activity in both cellular systems and model membranes (Dawson et al., 1983, 1984; Billah & Siegel, 1987; Kramer et al., 1987; Cunningham et al., 1989; Roldan & Mollinedo, 1991; Zidovetzki et al., 1992; Rosenthal et al., 1993). Furthermore, some of these diacylglycerols have been demonstrated to phase-separate in phosphatidylcholine bilayers (De Boeck & Zidovetzki, 1989, 1992; Zidovetzki et al., 1992; Heimburg et al., 1992; López-García et al., 1994). However, the details of the effects of diacylglycerols and other glycerides on the kinetics of vesicle hydrolysis by PLA_2 and the ability of the reaction products to promote the enzyme's activity have not been studied. Also, since the tendency of the glycerides to phase-separate in the bilayer is temperature-dependent, the temperature dependence of the effects of the glycerides on PLA_2 activity was also determined.

MATERIALS AND METHODS

DPPC and monopalmitoyllysolecithin (lyso-PC) were purchased from Avanti Polar Lipids (Birmingham, AL). To form vesicles, DPPC in chloroform was dried and then hydrated in 35 mM KCl, 3 mM NaN_3, and 10 mM $CaCl_2$ for 1 h at 45−55 °C with periodic vortex mixing. To form LUV, the mixture was 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.) To form SUV (sonicated unilamellar vesicles), the hydrated phospholipid mixture was sonicated twice at 70 °C for 3 min with a titanium probe. Titanium granules were removed by centrifugation for 30 s at 13 000 rpm in a microfuge. The concentration of vesicles was expressed in terms of the bulk phospholipid concentration, which was determined by assay of the phosphate content (Bartlett, 1959). 1-Monopalmitoylglycerol (C-16 MAG), 1,2-dicaprylglycerol (C-10 DAG), 1,2-dipalmitoylglycerol (C-16 DAG), and tripalmitoylglycerol (C-16 TAG) were codispersed with the DPPC in chloroform solution prior to the preparation of vesicles. For diacylglycerols, this procedure appears to lead to the incorporation of more than 90% of the glyceride into the bilayer (Ortiz et al., 1988). In the case of C-16 TAG, however, only about 3 mol % is reported to partition into phosphatidylcholine bilayers. Above that concentration, separate emulsion particles composed of triglyceride appear to form (Hamilton, 1989).

To ensure that we studied the entire range of TAG concentrations possible in the vesicles, and to control for the possibility that the emulsion particles would interfere with our results, we repeated the various experiments with TAG concentrations up to about 7 mol %. At concentrations above 4 mol %, the expected emulsion particles did appear. However, calorimetric data and the hydrolysis experiments indicated that the behavior of the vesicles was independent of the emulsion particles and that this did not influence the experimental results. The concentrations of C-16 TAG reported in this paper refer to the total mole percent of triglyceride added to the system. In the case of all bilayer additives (lysolecithin, fatty acid, glycerides, and fluorescent probes), the mole percent was calculated on the basis of only the concentrations of DPPC and that additive.

Since the vesicles are used repeatedly for experiments over many days and are incubated at room temperature to avoid complications with the subtransition of DPPC, we could not prevent the migration of 1,2-diacylglycerols to form 1,3-diacylglycerols. We verified with thin-layer chromatography that such migration did occur in our system to the expected equilibrium near 1:1 of each isomer (Kodali et al., 1990). Control experiments indicated that the data were not dependent on the length of time between the preparation of vesicles and the experiment. Therefore, either the results were not affected by the isomerization or equilibration between the isomers was rapid enough to be complete for all experiments.

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 mg^{-1} cm^{-1}).

Measurements of the length of the lag phase preceding rapid hydrolysis (τ) were done by pH-stat titration and fluorescence spectroscopy, as described previously (Bell & Biltonen, 1989b; Bent & Bell, 1995). Reactions were performed at pH 8 in a volume of 2 mL. Sample temperatures were measured using an NIST-traceable thermometer. The reaction mixture contained 35 mM KCl, 10 mM $CaCl_2$, and 3 mM NaN_3. $Na_2B_4O_7$ (10 mM) was added as a buffer for reactions in the spectrofluorometer (Bell & Biltonen, 1989b; Bent & Bell, 1995). Samples containing reaction cocktail and lipids were incubated for about 10 min for temperature equilibration prior to the addition of PLA_2 (360 nM in fluorometer and 36 nM in pH-stat). For pH-stat experiments, τ was defined as the time from injection of the enzyme to the time at which the rate of hydrolysis suddenly increased. The concentrations of monopalmitoyllysolecithin and palmitic acid required to reduce τ to zero were determined by using fluorescence spectroscopy because of the better time resolution (about 2 s at the time of addition of enzyme and 1 s thereafter) than the pH-stat method. In such experiments, equimolar amounts of lysolecithin and palmitic acid were added during temperature equilibration prior to adding PLA_2. Methanol contributed by the stock solutions of lysolecithin and palmitic acid was generally less than 0.5% and always less than 1% (v/v). Control experiments with and without methanol indicated that no bias was introduced by the alcohol. τ was measured as the time from the addition of the enzyme to the commencement of the increase in emission intensity corresponding to elevated PLA_2 activity (Bell & Biltonen, 1989b; Bent & Bell, 1995). The excitation wavelength was 280 nm and the emission wavelength was 340 nm.

The apparent lipid/water partition coefficient of monopalmitoyl lysolecithin was determined by a competitive binding assay using bovine serum albumin, as described previously (Bent & Bell, 1995). The partition coefficient was obtained at several concentrations of vesicles. No correlation was observed between vesicle concentration and the value of the partition coefficient.

The apparent binding of PLA_2 to the surface of vesicles was monitored by fluorescence spectroscopy. For the binding to SUV, the intrinsic tryptophan fluorescence of the PLA_2 was measured at 25 °C, as described earlier, before and after the addition of various concentrations of SUV. EDTA (10 mM) was included in the reaction mixture instead of $CaCl_2$ to prevent hydrolysis of the vesicles during the experiment. The measurements were repeated without PLA_2 to assess the amount of light contributed by light scattering. Binding was assumed to be proportional to the increase in PLA_2 fluorescence intensity upon the addition of vesicles after subtracting the light scattering (Bell & Biltonen, 1989a).

Binding of PLA_2 to LUV was investigated by fluorescence energy transfer from the tryptophan of the enzyme to dansyl-DHPE (Molecular Probes, Eugene, OR) present in the phospholipid bilayer at a concentration of 2 mol %. The intensity of dansyl-DHPE fluorescence emission was then measured at 510 nm (excitation = 280 nm) before and after the addition of PLA_2 under the conditions described earlier for fluorometric measurements during vesicle hydrolysis (42 °C). The vesicle concentration was 100 μM DPPC, and the PLA_2 concentration was 360 nM. Binding was assumed to be proportional to the increase in dansyl-DHPE emission upon the addition of PLA_2 (but prior to time τ). LUV composed of a 1:1 mixture of DPPC and dipalmitoylphosphatidylglycerol were used as a positive control for the experiment. In this case, the concentration of calcium in the reaction mixture was reduced from 10 mM to 1 μM to avoid aggregation of the vesicles. That the observation of increased dansyl-DHPE emission obtained upon the addition of PLA_2 to this positive control represented energy transfer was verified by the simultaneous observation of quenching of the PLA_2 fluorescence (emission = 340 nm, excitation = 280 nm).

Calorimetry experiments were accomplished with a heat conduction scanning calorimeter (Model 7707, Hart Scientific, Pleasant Grove, UT). The scan rate was approximately 5 °C/h. In all cases, both up scans and down scans were obtained to test for hysteresis. Furthermore, scans were repeated on subsequent days to verify the stability of the samples. Temperatures at the beginning, midpoint, and end of the phase transitions shown in the figures are averages of the values from the up scans and down scans weighted by the exact scan rates. Temperature was monitored by the NIST-traceable thermometer in the calorimeter.

RESULTS

Consistent with previous reports, the time course of hydrolysis of DPPC LUV by PLA_2 at 40 °C was composed of an initial phase of slow reaction followed by a sudden onset of rapid vesicle hydrolysis until substrate was depleted (Figure 1, solid curves; Bell & Biltonen, 1989b). The time to onset of rapid hydrolysis is designated as τ. The presence of C-10 DAG in the vesicle inhibited the action of PLA_2, as shown by the increase in τ (Figure 1A, dashed curves). An

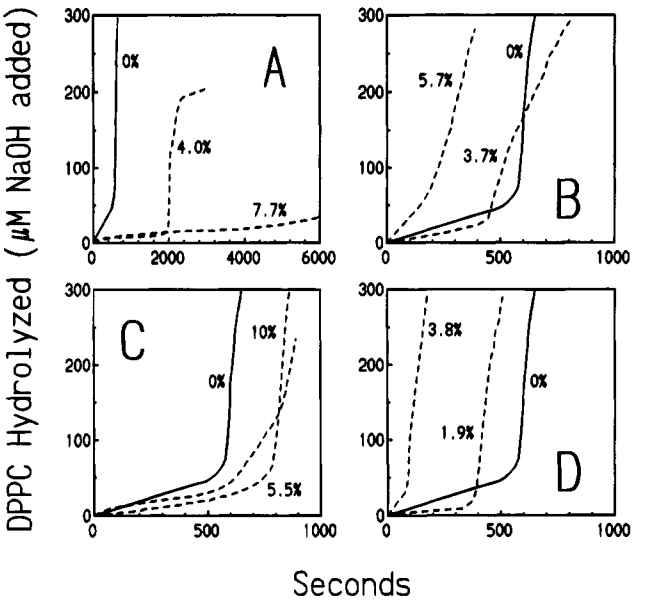

FIGURE 1: Effect of glycerides on the time course of hydrolysis of DPPC LUV by PLA_2 at 40 °C. Hydrolysis of vesicles (0.5 mM DPPC) by PLA_2 (0.5 μg/mL) was monitored by pH-stat titration of acid produced in the reaction. Vesicles contained the indicated mole percentages of C-10 DAG (A), C-16 DAG (B), C-16 MAG (C), or C-16 TAG (D). A representative time course in the absence of any additives (solid curve) is included in each panel. The instrument baseline (0.015−0.03 μM/s) was subtracted from the data.

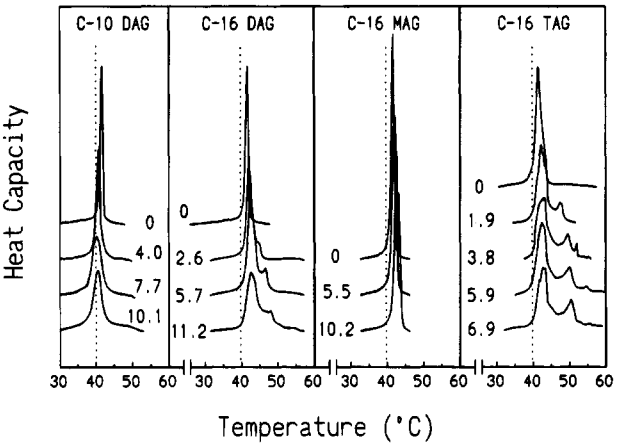

FIGURE 2: Effect of glycerides on the thermotropic phase transition of DPPC LUV. The excess heat capacity of DPPC LUV as a function of temperature was determined by differential scanning calorimetry at the indicated mole percentages of C-10 DAG, C-16 DAG, C-16 MAG, or C-16 TAG. The data shown were obtained as the temperature was lowered in the calorimeter. The linear instrument baseline was subtracted. The vertical dotted line at 40 °C is for visual reference.

increase in the length of the diglyceride chains from 10 to 16 carbons produced the opposite effect and reduced τ compared to control (Figure 1B). The number of acyl chains on the glycerol also affected τ. C-16 MAG had no consistent effect on τ (Figure 1C), whereas C-16 TAG was more effective than C-16 DAG at reducing τ (Figure 1D). (The small increase in τ apparent in Figure 1C was not reproducible; see Figure 3D.)

Figure 2 shows scanning calorimetry data for each of the four glycerides. In the case of C-10 and C-16 DAG, the data were qualitatively comparable to those recorded previously for multilamellar vesicles (Ortiz et al., 1988; Heimburg

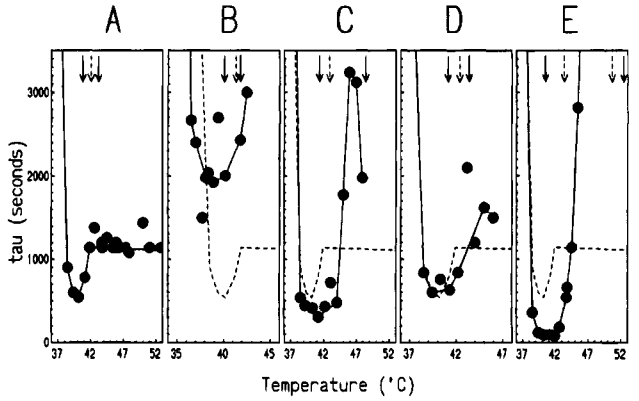

FIGURE 3: Temperature dependence of τ for DPPC LUV hydrolysis in the presence of various glyceride additives. τ was determined from hydrolysis time courses such as those shown in Figure 1 at the indicated temperatures. The arrows represent the extremes (solid arrows) and the peak(s) (dashed arrows) of the corresponding phase transitions shown in Figure 2. The control values of τ from panel A are reproduced (dashed lines) in panels B–E for comparison: (A) no additives; (B) 4.0 mol % C-10 DAG; (C) 5.7 mol % C-16 DAG; (D) 5.5 mol % C-16 MAG; (E) 3.8 mol % C-16 TAG. Lines reaching the top of the ordinate extend to values of τ greater than 3500 s. The patterns shown in the figure were reproduced in many additional experiments at various concentrations of the additives.

et al., 1992; López-García et al., 1994). C-10 DAG broadened the phase transition and shifted it toward lower temperatures. C-16 DAG also broadened the phase transition, but shifted it toward higher temperatures. Evidence for two peaks in the curve was apparent at the higher concentrations. As reported, the low-temperature boundary of the phase transition remained essentially constant throughout the range of C-16 DAG concentrations tested. Such results generally are interpreted to indicate immiscibility or phase separation of the phospholipid and diglyceride in the low-temperature (gel) phase (Heimburg et al., 1992; López-García et al., 1994).

C-16 MAG elevated the temperature of and broadened the phase transition. No evidence for phase separation was apparent from the data. C-16 TAG broadened the phase transition to a greater extent than did C-16 DAG. Again, the low-temperature boundary of the transition was constant at all concentrations of C-16 TAG tested, suggesting gel phase immiscibility of DPPC and C-16 TAG. Phase separation was also evident in the two peaks clearly resolved in the data. Only minor changes occurred in the data at concentrations of TAG above 3.8 mol %. This behavior reflects the observation that only about 3 mol % TAG partitions into bilayers (Hamilton, 1989). At higher concentrations of TAG, the excess triglyceride forms emulsion particles. These particles were visibly apparent in the samples and were found to melt at temperatures around 60 °C. These emulsion particles apparently did not interfere with the behavior of the vesicles in the phase transition region, as seen by the calorimetric data in Figure 2.

We repeated the experiments shown in Figure 1 at several temperatures in the regions of the phase transitions. Figure 3 shows τ plotted as a function of temperature for vesicles of pure DPPC (Figure 3A) or vesicles containing C-10 DAG (Figure 3B), C-16 DAG (Figure 3C), C-16 MAG (Figure 3D), or C-16 TAG (Figure 3E). The arrows in the figure represent the temperatures at the extremes or the midpoint of the peak(s) of the heat capacity curves corresponding to the vesicles used to obtain the data for each panel. The data from Figure 3A are also reproduced in Figure 3B–E for comparison. As reported previously for PLA_2, τ for the hydrolysis of pure DPPC LUV varied with temperature (Lichtenberg et al., 1986; Bell & Biltonen, 1989b). τ was very long for vesicles in the pure gel phase and was reduced at temperatures approaching the onset of the main thermotropic phase transition of the bilayer. The temperature at which τ was at a minimum consistently occurred near the low-temperature boundary and below the midpoint of the phase transition (i.e., about 40–41 °C). As the temperature was raised further, τ increased until about midway through the phase transition. Thereafter, the value of τ was insensitive to temperature. The same basic motif was observed in the presence of each of the glyceride additives, with certain differences noted in the following.

In the case of C-10 DAG, the temperature range over which τ was near the minimum was broader than that with pure DPPC (Figure 3B). Also, the temperature of the minimum τ was shifted systematically toward lower temperature as the concentration of C-10 DAG was increased. These observations reflect the fact that the phase transition for these vesicles was also broader and shifted toward lower temperatures. Thus, although τ was increased at temperatures greater than 38 °C by the addition of C-10 DAG, it was reduced at temperatures below 38 °C.

The temperature for minimum τ in the presence of C-16 DAG was at a slightly higher temperature relative to the phase transition than for pure DPPC (Figure 3C). Also, the temperature range at which τ was minimal was widened by the C-16 DAG again in correlation with the wider phase transition. Consistent with the observations in Figure 1, τ was shorter in the optimal temperature range with C-16 DAG in the bilayer. At temperatures above the main peak of the heat capacity curve, however, τ increased greatly in the presence of C-16 DAG. Thus, for vesicles in the liquid crystalline phase, τ was increased by C-16 DAG. Hence, each diacylglycerol tested appeared inhibitory (caused τ to increase relative to control vesicles) at some temperatures and stimulatory (shortened τ compared to control vesicles) at other temperatures.

C-16 MAG caused the least change in the temperature profile of the glycerides tested (Figure 3D). Like its effect on the thermotropic phase transition, C-16 MAG appeared to broaden slightly the temperature range for minimal τ. Also, as with C-16 DAG, C-16 MAG appeared to be inhibitory when the bilayer was in the liquid crystalline phase since the value of τ was increased compared to that of pure DPPC.

C-16 TAG caused essentially the same response as C-16 DAG, except that it appeared to be more effective at lowering the value of τ at the optimal temperature (Figure 3E). Again, as observed with each of the other glyceride additives, C-16 TAG broadened the range of temperatures for minimal τ.

Several of the models that have been proposed to explain the interactions of PLA_2 with phospholipid vesicles assume that the rate of vesicle hydrolysis is constant prior to the changes in the bilayer induced by the reaction products at time τ (Bell & Biltonen, 1992; Jain et al., 1993). Much evidence indicates that the sudden onset of high PLA_2 activity occurring at time τ results from the accumulation of a particular mole percentage (X_p) of the hydrolysis products, lysolecithin and fatty acid, in the bilayer (Apitz-Castro et al., 1982; Bell & Biltonen, 1989b; Jain et al., 1989; Burack

et al., 1993; Bent & Bell, 1995). In fact, τ may be reduced to zero by the addition of either lysolecithin or lysolecithin plus fatty acid to the bilayer (Jain & De Haas, 1983; Jain et al., 1989; Bent & Bell, 1995). At constant substrate concentration, the hydrolysis reaction would be described by the following relationship:

$$\text{product} = k_{obs}t \quad (1)$$

where k_{obs} is the observed catalytic efficiency of the enzyme at a given substrate concentration. At time τ, the product concentration is described by X_p. Hence,

$$\tau = X_p/k_{obs} \quad (2)$$

If this simple model describes the mechanism by which PLA_2 acts on phospholipid bilayers, and if that mechanism is not changed by temperature or the presence of glyceride additives, then the glycerides could reduce τ by changing either X_p, k_{obs}, or both (at constant substrate concentration). However, eqs 1 and 2 do not consider the initial interaction between the enzyme and the surface of the vesicles, since they apply to the condition of constant phospholipid concentration. Several estimates of the binding affinity of PLA_2 to the vesicle surface have been made. For the venom enzyme used in this study with phosphatidylcholine bilayers, the estimates of the dissociation constant range from about 10^{-4} to 10^{-3} (Bell & Biltonen, 1989a, 1992; Burack & Biltonen, 1994). Although the binding affinity probably increases at τ when sufficient reaction products are present (Jain et al., 1989), the initial low-affinity binding is relevant to the events leading up to τ and, therefore, the length of τ. At the concentration of vesicles used in Figures 1 and 3 (0.5 mM DPPC), not all of the enzyme would be bound. Thus, another possibility for the effect of the glycerides to reduce τ could be an increase in the binding of PLA_2 to the vesicle surface.

We examined the possibility that C-16 DAG reduces τ by increasing the binding of PLA_2 to the vesicle surface by using three separate methods: fluorescence energy transfer (Figure 4A), measurements of PLA_2 fluorescence upon binding to SUV (Figure 4B), and kinetic analysis of hydrolysis time courses (Figure 5). The energy transfer experiments were based on the idea that effective transfer of fluorescence energy from the tryptophan of the PLA_2 to dansyl-DHPE introduced into the LUV would occur only if the enzyme were adsorbed to the vesicle surface. Thus, binding of PLA_2 to the vesicles would be observed in these experiments as an increase in dansyl-DHPE fluorescence emission (excited at the tryptophan excitation maximum) upon the addition of PLA_2. Curve a in Figure 4A shows a positive control verifying that the experiment was successful. The addition of PLA_2 to vesicles composed of a 1:1 mixture of DPPC and the anionic dipalmitoylphosphatidylglycerol caused a substantial increase in the dansyl-DHPE emission. This result was consistent with the previous observation of high-affinity binding of PLA_2 to vesicles containing anionic phospholipids (Jain et al., 1989; Burack & Biltonen, 1994; Scott et al., 1994). However, when the experiment was done with DPPC LUV, there was no measurable energy transfer upon the addition of enzyme to the vesicles (Figure 4A, curve b). The same result also occurred regardless of the presence of C-16 DAG (curves c and d). In replicate experiments,

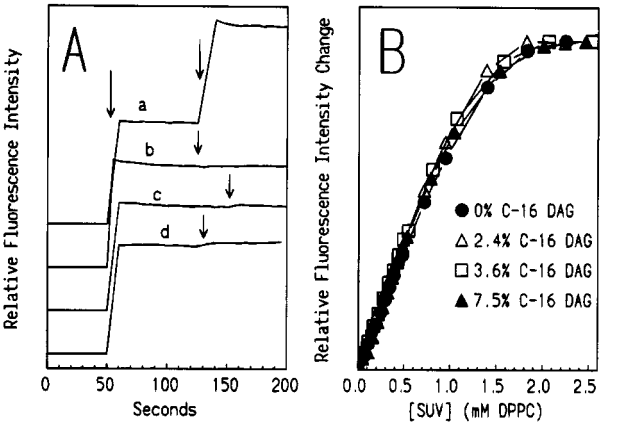

FIGURE 4: Lack of an effect of C-16 DAG on the association of PLA_2 to the surface of LUV (A) or SUV (B). (A) Fluorescence energy transfer from the protein tryptophan (excitation = 280 nm) to dansyl-DHPE (emission = 510 nm) in the vesicle bilayer (2 mol %) was measured as described in Materials and Methods. Vesicles (100 μM phospholipid) containing 1:1 DPPC and dipalmitoylphosphatidylglycerol (curve a), DPPC alone (curve b), 3.1 mol % C-16 DAG (curve c), or 4.9 mol % C-16 DAG (curve d) were added to the reaction mixture at the first arrow. PLA_2 was added at the second arrow in each curve. The curves are drawn on the same scale in each case, but are offset from each other along the ordinate for clarity of presentation. These data are representative of 2–6 experiments for each condition. (B) The increase in tryptophan fluorescence of PLA_2 was determined upon the addition of the indicated concentrations of SUV. The reaction conditions are described in Materials and Methods.

the hint of an increase in the intensity of curves c and d upon the addition of PLA_2 (arrows at 150 and 130 s) either was not reproducible or was not enhanced by higher vesicle concentrations, as would be expected for a true binding phenomenon.

One possible reason for the inability of the energy transfer experiment to unequivocally demonstrate the effects of C-16 DAG on PLA_2 binding could be the lack of sensitivity of the experiment at vesicle concentrations appropriate for fluorescence spectroscopy due to low-affinity binding. In an effort to obtain a more sensitive measurement, we took advantage of the previous report that PLA_2 binding could be observed by an increase in the PLA_2 tryptophan fluorescence upon the addition of vesicles by using SUV instead of LUV (Bell & Biltonen, 1989a). Figure 4B demonstrates the effect of SUV concentration on the tryptophan fluorescence of PLA_2, with vesicles containing various concentrations of C-16 DAG. (These experiments were done in the absence of calcium to avoid vesicle hydrolysis; see Materials and Methods for details.) Regardless of the concentration of C-16 DAG, the binding curves were identical. Thus, no evidence for an effect of the diglyceride on the binding affinity of the PLA_2 to the vesicle surface prior to reaction product accumulation could be found. These experiments do not exclude the possibility that the glycerides could increase the binding affinity in the presence of reaction products.

For the kinetic analysis, we repeated the experiments of Figure 1B at a higher (2 mM DPPC) and a lower (0.2 mM DPPC) concentration of vesicles. If the effect of the glyceride on τ were due to an increase in the binding affinity of the enzyme, then that effect would be reduced by increasing the vesicle concentration since more PLA_2 would be bound. Such should be true even if this hypothetical

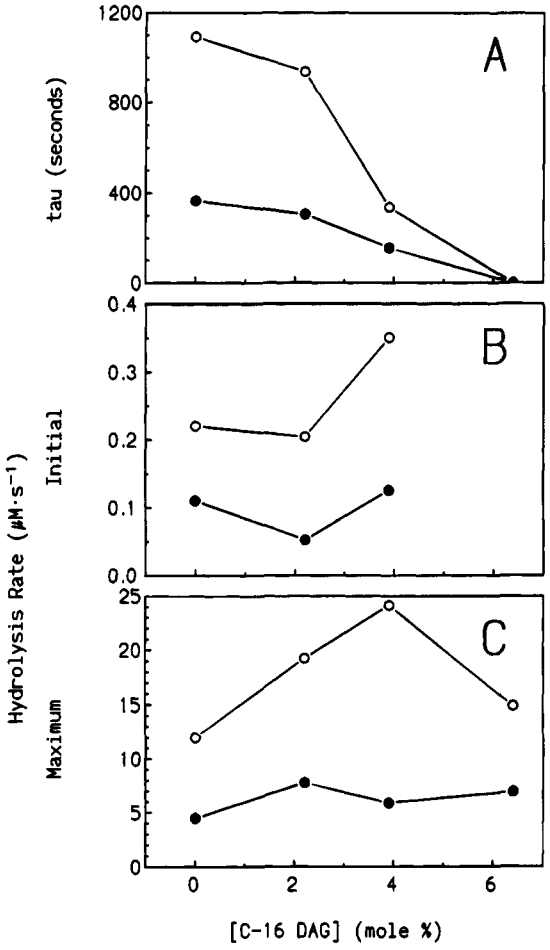

FIGURE 5: Effect of C-16 DAG on τ (A), the initial rate of vesicle hydrolysis (B), and the maximum rate of vesicle hydrolysis (C). The experiments of Figure 1B were repeated using 0.2 (●) or 2 mM DPPC (○) at 42 °C. The initial rate of hydrolysis (B) was determined by linear regression of the early linear portion of the time course. The maximum hydrolysis rate (C) was determined from derivatives of the time course data immediately after time τ. The pH-stat instrument baseline was measured in the absence of PLA_2 and subtracted from the raw hydrolysis rates (0.03 μM/s).

enhancement of binding affinity requires the presence of the reaction products. Figure 5A displays the relationship between τ and C-16 DAG concentration at the low and high vesicle concentrations at 42 °C. τ was longer at 2 mM DPPC than at 0.2 mM DPPC, as reported previously and as predicted theoretically for any model in which the increased PLA_2 activity depends on the mole fraction of reaction products in the bilayer (Bell & Biltonen, 1992). That the increased vesicle concentration enhanced the binding of the enzyme is shown in Figure 5B,C, where the higher vesicle concentration produced a substantial increase (average of about 3-fold) in both the initial and maximum rates of vesicle hydrolysis (k_{obs}). This result was consistent with an enzyme/vesicle surface dissociation constant of 1 mM, which agrees well with previous estimates. Nevertheless, C-16 DAG reduced τ proportionally at each vesicle concentration. Hence, the vesicle concentration did not appear to alter the effectiveness of the glyceride. Therefore, we concluded that, taken together, the various binding and kinetic experiments revealed that the effect of the glycerides probably was not simply on the binding affinity of the PLA_2 for the vesicle surface.

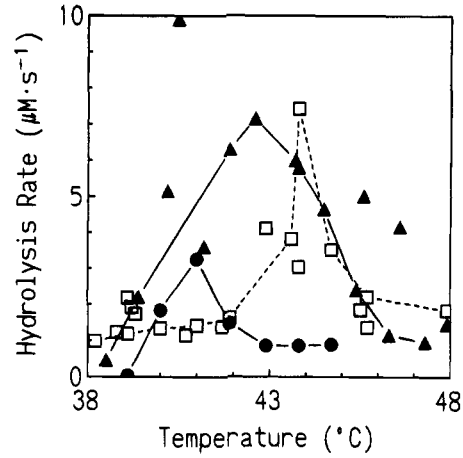

FIGURE 6: Temperature dependence of the maximum rate of DPPC LUV hydrolysis in the presence of no additives (●), C-16 DAG (□) and C-16 TAG (▲). The maximum rate of vesicle hydrolysis immediately after time τ was determined from derivatives of reaction time courses such as those shown in Figure 1 at the indicated temperatures.

Figure 5B,C also addresses the possibility that the effect of the glyceride is on k_{obs}, the hydrolysis rate. While it appeared that C-16 DAG might alter the hydrolysis rate, a simple consistent trend with C-16 DAG concentration was not observed. This agreed with the data shown in Figure 1, where a simple relationship between the concentration of glycerides and the initial or maximum hydrolysis rate also was not apparent. With the initial rate, the situation may be compounded by a poor signal to noise ratio, since the instrument baseline is of the same magnitude as the initial hydrolysis rate. To test whether the relationship might be clarified by considering the effect of temperature, we repeated these experiments at several temperatures using C-16 DAG and C-16 TAG (Figure 6). Only the maximum hydrolysis rate is shown. Again, a simple relationship between the hydrolysis rate and the presence of the glycerides was not found. Nevertheless, the highest rates of hydrolysis did appear to correlate in each case with the temperatures surrounding the thermotropic phase transition of the DPPC. Also, these peak rates were greater in the presence of the glycerides.

The third possibility for the effect of the glycerides on τ is that X_p is reduced. This possibility was offered in the introduction as a general hypothesis that bilayer additives that segregate into domains reduce τ by lowering the amount of lysolecithin needed to induce high activity of PLA_2. Thus, we repeated the experiments of Figure 3 with the systematic addition of the hydrolysis products. X_p was determined from the minimum mole percent of added hydrolysis products sufficient to reduce τ to zero. Figure 7 displays X_p as a function of temperature and in the presence of the various glycerides.

In the absence of glycerides, X_p was lowest at the temperature of the main phase transition (39−42 °C, Figure 7). At higher temperatures, X_p increased. X_p also appeared to increase at lower temperatures, but such studies were more difficult to interpret unambiguously because discerning the point at which no lag time occurs becomes increasingly difficult as the temperature is lowered, due to coinciding decreases in the rate of vesicle hydrolysis (not shown).

In the presence of either C-16 DAG or C-16 TAG, X_p was decreased at temperatures in the region of the phospho-

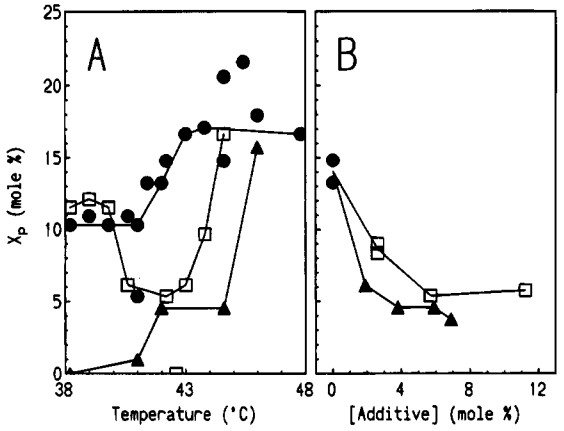

FIGURE 7: X_p as a function of temperature (A) and of concentrations of C-16 DAG and C-16 TAG at 42 °C (B). τ was measured by fluorescence spectroscopy of PLA_2 (5 μg/mL) during vesicle (0.1 mM DPPC) hydrolysis. X_p was determined as the minimum mole percentage each of lysolecithin and palmitic acid that when added to the vesicles caused τ to equal zero. Symbols: ●, no additives; □, C-16 DAG (5.7 mol %, panel A); ▲, C-16 TAG (3.8 mol %, panel A).

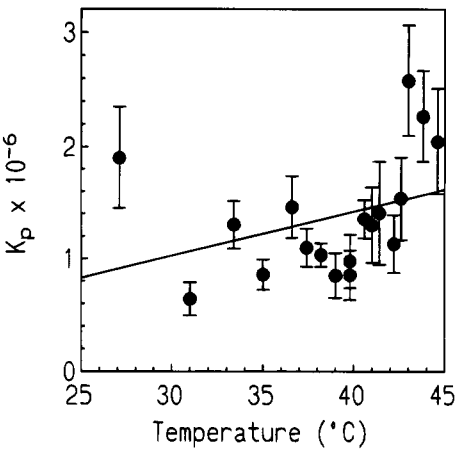

FIGURE 8: Lipid/water partition coefficient for lysolecithin in DPPC LUV as a function of temperature. Each data point represents the mean ± SE of five determinations of K_p. The correlation coefficient for the linear regression shown is 0.34, which corresponds to a p value of 0.16 and therefore is not statistically significant. The overall average values of K_p was (1.4 ± 0.1) × 10^6 (mean ± SE, n = 18).

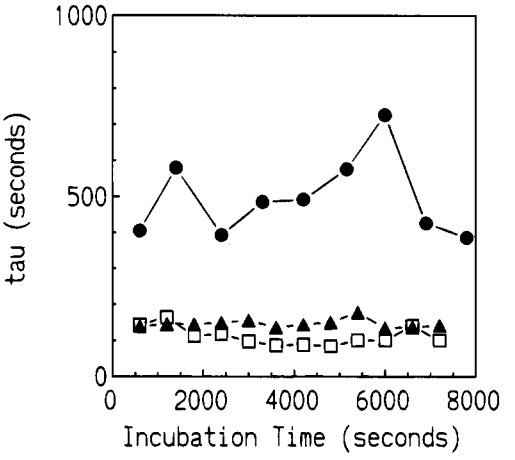

FIGURE 9: Time dependence of the ability of reaction products to reduce τ. τ was determined from PLA_2 fluorescence during vesicle (0.25 mM DPPC) hydrolysis in the presence of 3.7 mol % lysolecithin and 3.7 mol % palmitic acid. PLA_2 (5 μg/mL) was added at the indicated times after the addition of the two reaction products to the vesicles. Temperatures were 42.2 (●), 40.6 (▲), and 39.0 °C (□).

lipid phase transition (Figure 7A). Above the transition, the required mole percent was similar to that with DPPC alone. Figure 7B demonstrates that the effect of C-16 DAG and TAG to reduce X_p was reproducible and concentration-dependent. The TAG was more effective than C-16 DAG at reducing the value of X_p. Furthermore, the effect was maximal to about 3.8 mol %, suggesting that the effect of TAG was not influenced by the presence of emulsion particles produced by excess triglyceride not partitioned into the bilayer. Results similar to those shown in Figure 7 were also obtained by using lysolecithin alone to reduce the lag time to zero. If a glyceride such as C-10 DAG that did not cause phase separation in the bilayer was used, X_p was not reduced (not shown).

We tested whether the effect of temperature on X_p was due to alterations in the distribution of the products between the bilayer and the aqueous phase of the system. We focused on lysolecithin since previous studies have shown that the fatty acid binds much tighter to the bilayer, making lysolecithin the limiting hydrolysis product (Bent & Bell, 1995). Also, similar temperature dependence occurred whether lysolecithin was added alone or with fatty acid to the bilayer (see above). Figure 8 displays the temperature dependence of the apparent partition coefficient (K_p) for the binding of lysolecithin to the bilayer. The trend was not statistically significant on the basis of linear regression of the data. Therefore, no evidence for the temperature dependence of K_p could be found. Furthermore, the range of K_p values was insufficient and in the wrong direction to account for the 1.5-fold increase in the amount of reaction product required to reduce τ to zero observed in Figure 7A between 41 and 44 °C. A reduction in the value of K_p from the average value of 1.4 × 10^6 (shown in Figure 8) to 5 × 10^5 would be required over the same temperature interval to account for the data of Figure 7. In addition, no effect of C-16 DAG or C-16 MAG on K_p was observed (not shown).

Another possible explanation for the effect of temperature on X_p is that the kinetics of partitioning of the products with the bilayer or the rate of development of putative physical changes in the bilayer relevant to high PLA_2 activity is sensitive to temperature. If such were true, τ would decrease exponentially for a series of hydrolysis reactions initiated at increasing times after the addition of reaction products to the bilayer at a concentration below X_p. Furthermore, this hypothesis predicts that the rate of decay of τ would vary with temperature in correlation with the apparent value of X_p shown in Figure 7. Figure 9 displays the results of experiments at three temperatures. No decay in the length of τ was observed regardless of temperature. Thus, the effect of temperature on X_p presumably depends on the average physical state of the bilayer in the presence of the reaction products rather than on the kinetics or thermodynamics of the partitioning of the products between the aqueous phase and the bilayer.

The data of Figures 6 and 7 suggested that the effect of C-16 DAG and C-16 TAG on τ could be explained, at least under some circumstances, by effects on either k_{obs}, X_p, or both. To determine which of these possibilities applied, we examined the correlation between τ and these observables at all of the temperatures and glyceride concentrations

studied. These relationships are shown in Figure 10. According to eq 2, k_{obs} will be inversely proportional to τ if it is the sole determinant of τ (i.e., if X_p is constant). Alternatively, the relationship between τ and X_p will be linear if X_p is solely responsible for the effects of temperature and glycerides. As presented in Figure 10A,B the correlation between the hydrolysis rate (i.e., k_{obs}) was generally poor, although some data appeared to agree with the expected inverse relationship (curve). The same was true for either C-16 DAG (Figure 10A) or C-16 TAG (Figure 10B). In contrast, a strong relationship was observed between the values of τ and X_p for both control vesicles and those containing either C-16 DAG or C-16 TAG (Figure 10C,D). However, the relationship approached linearity only for low values of τ. In the longer hydrolysis time courses, τ appeared independent of X_p. Therefore, while it seemed clear that τ was largely determined by the amount of reaction products required for high PLA_2 activity, the relationship was not as simple as predicted by eq 2. Presumably, k_{obs} also contributes to the sensitivity of τ to temperature or glycerides, especially with the longer time courses. Other slow kinetic processes not considered in eq 2 may also play ancillary roles in determining τ under some of the conditions tested (Bell & Biltonen, 1992; Bent & Bell, 1995).

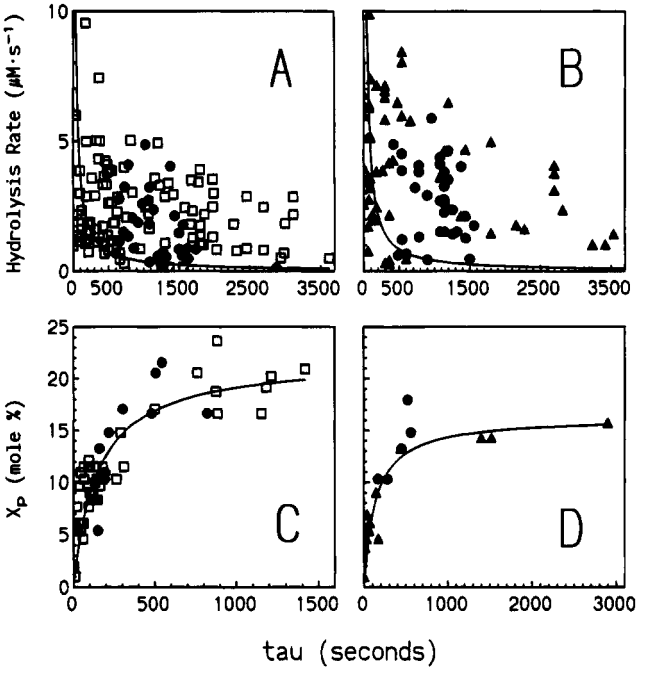

FIGURE 10: Relationships between τ and vesicle hydrolysis rate or X_p for DPPC LUV in the presence of C-16 DAG or C-16 TAG. The values of the maximum hydrolysis rate and τ (panels A and B) were determined from hydrolysis time courses exactly as described for Figure 6 for vesicles without additives (●) or with several concentrations of C-16 DAG (□, panel A) or C-16 TAG (▲, panel B) at multiple temperatures between 38 and 64 °C. The curves are least-squares nonlinear regression fits of the data to eq 2. The values of X_p and τ (panels C and D) were determined from fluorescence spectroscopy of PLA_2 during hydrolysis reactions exactly as described for Figure 7 for vesicles without additives (●) or with several concentrations of C-16 DAG (□, panel C) or C-16 TAG (▲, panel D) at multiple temperatures between 35 and 48 °C. The curves are least-squares nonlinear regression fits of the data to an arbitrary function.

DISCUSSION

Previous work has suggested that diacylglycerols can stimulate or inhibit phospholipase A_2 activity, depending on the chain length of the diglyceride (Zidovetzki et al., 1992). However, this generalization cannot be comprehensive since whether a diglyceride will be stimulatory or inhibitory depends on the physical state of the system in question (Zidovetzki et al., 1992). This study reinforces that point and adds further insight into the mechanism of the effect of diglycerides.

Consistent with prior studies, the C-10 DAG tended to be inhibitory and the C-16 DAG tended to be stimulatory at 40 °C, based on the effects of these molecules on τ. Effects on the maximum rate of hydrolysis were less clear. Thus, classification of a diglyceride as inhibitory or stimulatory requires careful definition of how those terms are applied to the reaction kinetics. Also, the data of Figure 3 demonstrate that the classification depends on the temperature. For example, at low temperature, C-10 DAG appeared stimulatory, and at high temperature, C-16 DAG appeared inhibitory, the opposite of the effects observed at 40 °C.

A variety of evidence supports the conclusion that certain diglycerides enhance PLA_2-catalyzed hydrolysis of phospholipid bilayers because of the tendency of the glyceride to form domains within the bilayer (Cunningham et al., 1989; Zidovetzki et al., 1992; De Boeck & Zidovetzki, 1992). The observations reported in this paper support that interpretation and extend it with data on C-16 MAG and C-16 TAG. The calorimetric experiments with monoglyceride did not show evidence for phase separation into domains, and likewise, this lipid did little to alter the kinetics of vesicle hydrolysis by PLA_2. In contrast, the calorimetric data with triglyceride strongly suggested phase separation, and triglyceride was very effective at reducing the length of the lag phase. Furthermore, the temperature range at which these glycerides were stimulatory was confined to temperatures below the midpoint of the main phase transition. Above the transition, random mixing of the glycerides and phospholipids apparently occurs, and the ability of the glyceride to reduce τ is lost.

The results of this study further our understanding of the mechanisms by which PLA_2 activity is modulated by bilayer structure and dynamics. It appears clear from several sources that the sudden increase in PLA_2 activity at time τ is a consequence of the accumulation of the hydrolysis products, lysolecithin and fatty acid, in the bilayer (Apitz-Castro et al., 1982; Bell & Biltonen, 1989b; Jain et al., 1989; Burack et al., 1993). Lysolecithin appears capable of inducing the high PLA_2 activity without the fatty acid (Jain & De Haas, 1983; Bell & Biltonen, 1992; Bent & Bell, 1995). However, the concentration of lysolecithin required to induce the high activity is lowered by the presence of fatty acid (Jain et al., 1989; Bent & Bell, 1995). Moreover, fluorescence studies have revealed that the fatty acid segregates into domains within the bilayer immediately prior to the sudden increase in activity at time τ (Jain et al., 1989; Burack et al., 1993). Thus, we propose that the ability of lysolecithin to enhance the activity of PLA_2 is assisted by the formation of domains in the bilayer. This hypothesis predicts that other perturbations to the bilayer that promote domain formation would also reduce τ by reducing the amount of lysolecithin required to increase the phospholipase activity. The results reported in this paper with the various glycerides were consistent with this prediction. It is also likely that the glyceride domains

would facilitate segregation of the fatty acid. The accumulation and segregation of fatty acid in the bilayer have also been postulated to contribute to the increased activity at τ by enhancing PLA_2 binding at that point due to favorable electrostatic interactions (Jain et al., 1989; Scott et al., 1994).

The alternative explanation that the glycerides reduced τ primarily by directly enhancing the binding of the enzyme to the vesicle surface was not supported by the data of Figures 4 and 5. This result was not surprising since the glycerides are neutral lipids, and additives that directly increase PLA_2 binding to vesicles are generally anionic (Jain et al., 1989; Burack & Biltonen, 1994; Scott et al., 1994). That the variation in lag times was determined exclusively by differences in the intrinsic hydrolytic activity of the enzyme under the various conditions also was not substantiated experimentally, although some effect of the glycerides on the hydrolytic rate was observed. Finally, the possibility that reductions in the required amount of reaction products resulted from changes in the kinetics or apparent thermodynamics of partitioning of the lysolecithin between the aqueous and lipid phases was also excluded.

The temperature dependence of PLA_2 kinetics also corroborated our hypothesis regarding the relationship among domains, reaction products, and τ. It has been argued that the main thermotropic phase transition of phospholipid bilayers involves finite cooperativity among the phospholipid molecules (Freire & Biltonen, 1978; Biltonen, 1990; Mouritsen, 1991). Consequently, the phase transition is broad. At temperatures near the transition, domains of solid and melted phospholipid would coexist. The preference of PLA_2 for phospholipid at the phase transition temperature has been known for several years (Op Den Kamp et al., 1974). That this preference relates to the presence of domains in the membrane has recently been observed directly in monolayers by fluorescence microscopy (Grainger et al., 1989). In accordance with our hypothesis, the lag time and the mole percent of reaction products required to enhance the activity of PLA_2 would be expected to be the least near the phase transition temperature. The former expectation has been verified previously for LUV (Lichtenberg et al., 1986). The latter is substantiated in this report on the basis of comparisons of temperatures near or above the phase transition (Figure 7).

It is important to note that although domain formation appears plausible as an explanation for the effects of both the glyceride and temperature on the ability of reaction products to increase the activity of PLA_2, the two effects are not identical. For example, even though segregation of C-16 DAG and TAG into domains should occur at all temperatures below the main phase transition (Heimburg et al., 1992; López-García et al., 1994), τ was not uniformly reduced at such temperatures. One explanation would be that gel phase lipid is a poor substrate for PLA_2, regardless of the presence of domains. The data of Figure 6 appear to support this idea. Alternatively, Biltonen and co-workers have suggested that the temperature dependence of the kinetics of vesicle hydrolysis by PLA_2 correlates with dynamic fluctuations of clusters of gel phase lipid that occur at temperatures near the phase transition (Freire & Biltonen, 1978; Lichtenberg et al., 1986; Biltonen, 1990). Perhaps such fluctuations are responsible for the effect of temperature to reduce the amount of reaction products required to increase PLA_2 activity on the surface of phosphatidylcholine LUV. In support of this thought, we note that C-10 DAG broadened the phase transition without inducing segregation of the lipids into domains. Such would represent a loss of cooperativity in the phase transition and, presumably, a decline in the magnitude of bilayer fluctuations (Mountcastle et al., 1978). If the fluctuations were important for the effect of reaction products, then C-10 DAG would be expected to prolong the lag phase of the hydrolysis time course, as was observed in Figures 1 and 3. In any case, the data at temperatures well below the phase transition argue that the formation of domains by C-16 DAG or C-16 MAG cannot substitute for the effect of temperature to reduce τ. Instead, there appears to be a synergism between the state of the bilayer near the phase transition and the presence of glyceride domains to enhance the ability of the reaction products to promote high PLA_2 activity.

We also acknowledge the observation of Smaby et al. (1994) that macroscopic phase separation in the strict sense may not be required for the phenomena discussed in this report. The average size of clusters or domains depends on the interaction energy between the various components of the system. This energy would be zero for systems that display ideal mixing and very large for systems that exhibit macroscopic phase separation. Even with ideal mixing, however, domains form as the concentration of additive approaches 50 mol %. If the ability of an additive to promote PLA_2 activity depends on domain size, then effective concentrations of that additive to promote the activity of PLA_2 through domain formation presumably would depend on the magnitude of the interaction energy among the additive and the other bilayer components (Gheriani-Gruszka et al., 1988). In other words, more additive would be required with systems that are more miscible.

Some authors have suggested that diacylglycerol may modulate PLA_2 activity in cells independent of protein kinase C (Billah & Siegel, 1987; Roldan & Mollinedo, 1991; Rosenthal et al., 1993) by altering the membrane structure (Dawson et al., 1984; Cunningham et al., 1989; Zidovetzki et al., 1992). Apparently, the concentrations of diacylglycerol used in this study are relevant to concentrations found in biological membranes (Kramer et al., 1987; Takuwa et al., 1987). However, attempts to interpret data from living cells directly with the results of investigations such as this are premature. Nevertheless, this study clearly demonstrates that the effects of the diacylglycerols in membranes greatly depend on the composition and physical properties of the bilayer in question. This emphasizes the need for continued physical and biochemical investigation of both model and biological systems. We anticipate that such studies will be of use for understanding both PLA_2 and other proteins modulated specifically by diacylglycerols or more generally by the lateral distribution of various lipids in the bilayer.

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