Cooperativity and Binding in the Mechanism of Cytosolic Phospholipase A_2

James R. Burke,*,‡ Mark R. Witmer,*,§ Jeffrey Tredup,§ Radmila Micanovic,§ Kurt R. Gregor,‡ Joydeep Lahiri,|| Kenneth M. Tramposch,‡ and Joseph J. Villafranca§

Departments of Dermatology Discovery Research, Buffalo, New York, 14213, and Enzymology, Macromolecular Structure Department, Princeton, New Jersey 08543, Bristol-Myers Squibb Pharmaceutical Research Institute, and Department of Chemistry, Princeton University, Princeton, New Jersey 08540

Received May 23, 1995; Revised Manuscript Received August 24, 1995⊗

ABSTRACT: Cytosolic phospholipase A_2 ($cPLA_2$) hydrolyzes the *sn*-2 ester of phospholipids and is believed to be responsible for the receptor-regulated release of arachidonic acid from phospholipid pools. The enzyme was assayed using vesicles containing arachidonate-containing phospholipid substrate, such as 1-palmitoyl-2-arachidonoylphosphatidylcholine (PAPC) or 1-stearoyl-2-arachidonoylphosphatidylinositol (SAPI), dispersed within vesicles of 1,2-dimyristoylphosphatidylmethanol (DMPM). We report here that the enzyme shows an apparent cooperative effect with respect to the mole fraction of arachidonate-containing phospholipids within these covesicles. The data can be fit to a modified Hill equation yielding Hill coefficients, *n*, of 2−3. This effect is unusual in that it is dependent on the nature of the *sn*-2 ester as opposed to the phosphoglycerol head group. This cooperativity is independent of both the concentration of glycerol, which greatly increases enzyme activity and stability, and the concentration of calcium, which facilitates the fusion of the covesicles. Surprisingly, 1-palmitoyl-2-arachidonoylphosphatidylethanolamine (PAPE) does not show the same cooperative effect, although the rate at which it is hydrolyzed is much greater when PAPC is present. Moreover, PAPE has a dissociation constant from the active site (K_D* = 0.7 mol %) which is comparable to that of PAPC and SAPI (K_D* values of 0.3 and 0.3 mol %, respectively). These results are consistent with the presence of an allosteric site that, when occupied, induces a change in the enzyme which facilitates enzymatic hydrolysis. If so, PAPC and SAPI, but not PAPE, must be able to bind to this allosteric site. Alternatively, this effect may result from changes in the physical nature of the bilayer which result upon increasing the bilayer concentration of arachidonate-containing phospholipids. This previously unobserved effect may represent another mechanism by which cells can regulate the activity of $cPLA_2$.

The lipases and phospholipases represent two classes of enzymes which process hydrophobic or amphiphilic molecules that form aggregates, such as vesicles and membranes. Phospholipase A_2 catalyzes the hydrolysis of the *sn*-2 ester of phospholipids. Several different types of phospholipases A_2 have been identified and characterized, the best known of which are the 14-kDa secreted enzyme ($sPLA_2$),[1] the 85-kDa cytosolic enzyme ($cPLA_2$), and a calcium-independent enzyme (CaI-PLA_2) found in myocardial tissue (Kramer, 1994).

Because of its putative role in the generation of arachidonic acid, which is the biosynthetic precursor to leukotrienes and prostaglandins, PLA_2s have received considerable medicinal interest. While the relative roles of these enzymes in eicosanoid production remain unclear, $sPLA_2$ has been implicated in the production of prostaglandins in some cell types such as macrophage-like $P388D_1$ cells (Barbour & Dennis, 1993), TNF-stimulated endothelial cells (Murakami *et al.*, 1993), and rat mesangial cells (Schalkwijk *et al.*, 1992). However, the $sPLA_2$ is not thought to be directly involved in the receptor-mediated release of arachidonate (Kramer *et al.*, 1990). The CaI-PLA_2 has been implicated in the release of arachidonate from pancreatic islet cells as well as aortic smooth muscle cells (Lehmann *et al.*, 1993; Turk *et al.*, 1993), although the general role of this enzyme in arachidonate mobilization remains unclear.

$cPLA_2$ is a calcium-dependent enzyme that is present in a number of different tissues and cells including monocytes, neutrophils, and platelets (Nakamura *et al.*, 1992; Kramer *et al.*, 1993b; Ramesha & Ives, 1993). The enzyme is

* Authors to whom correspondence should be addressed; the contributions of these authors should be considered equal.
‡ Dermatology Discovery Research, Buffalo.
§ Macromolecular Structure, Princeton.
|| Department of Chemistry, Princeton University.
⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; CaI-PLA_2, calcium-independent phospholipase A_2; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; SAPI, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PAPE, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine; 1-alkyl-2-AA-PC, 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SAPC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PSPC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ^{14}C-PAPC, 1-palmitoyl-2-arachidonoyl-[*arachidonoyl*-1-^{14}C]-*sn*-glycero-3-phosphocholine; ^{14}C-PAPE, 1-palmitoyl-2-arachidonoyl-[*arachidonoyl*-1-^{14}C]-*sn*-glycero-3-phosphoethanolamine; ^{14}C-POPC, 1-palmitoyl-2-oleoyl-[*oleoyl*-1-^{14}C]-*sn*-glycero-3-phosphocholine; ^{14}C-SAPC, 1-stearoyl-2-arachidonoyl-[*arachidonoyl*-1-^{14}C]-*sn*-glycero-3-phosphocholine; ^{14}C-SAPI, 1-stearoyl-2-arachidonoyl-[*arachidonoyl*-1-^{14}C]-*sn*-glycero-3-phosphoinositol; ^{3}H-1-alkyl-2-AA-PC, 1-*O*-hexadecyl-2-arachidonoyl-[*arachidonoyl*-5,6,8,9,11,12,14,15-^{3}H(N)]-*sn*-glycero-3-phosphocholine; DTT, 1,4-dithiothreitol; EDTA, ethylenedinitrilotetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; BSA, bovine serum albumin; HSA, human serum albumin; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; THF, tetrahydrofuran; TLC, thin layer chromatography.

0006-2960/95/0434-15165$09.00/0

normally located in the cytosol, but translocates to the membrane in response to submicromolar concentrations of calcium (Channon & Leslie, 1990; Clark *et al.*, 1991). Unlike the $sPLA_2$s, this enzyme has been shown to be selective for arachidonate-containing phospholipids (Diez *et al.*, 1992, 1994). Mounting evidence indicates that phosphorylation of $cPLA_2$ and activation by an increase in the cytosolic calcium concentration is regulated by receptor-mediated stimuli (Lin *et al.*, 1992; Kramer *et al.*, 1993a,b; Qiu *et al.*, 1993). Both PKC-dependent and -independent pathways have been implicated in the receptor-mediated regulation of $cPLA_2$ (Bonventre *et al.*, 1990; Godson *et al.*, 1993; Qiu & Leslie, 1994). Additionally, evidence supports the idea that regulation of $cPLA_2$ can occur through direct control by G proteins (Xing & Mattera, 1992). Because of the involvement of receptors in its regulation, $cPLA_2$ is believed to play a role in receptor-mediated eicosanoid production. Indeed, arachidonyl trifluoromethyl ketone, a tight-binding inhibitor of $cPLA_2$, but not of $sPLA_2$, dose-dependently inhibits arachidonate release from thrombin-stimulated human platelets (Bartoli *et al.*, 1994).

The role of $cPLA_2$ in the liberation of arachidonate from cellular pools and its overall role in inflammation is currently the subject of intense investigation. The biochemical properties of human $cPLA_2$, including kinetic behavior and regulatory mechanisms, are also being revealed since several groups have cloned and expressed the human cDNA for $cPLA_2$ and purified the enzyme from recombinant sources, including insect cells (de Carvehlo *et al.*, 1993; Sharp *et al.*, 1994).

Since the phospholipid substrate on which the enzyme acts is in the form of an aggregate rather than water-soluble monomers, the enzyme must first bind to the surface of the lipid/water interface before a phospholipid molecule can bind to the active site (Verger, 1980). To accomplish this interfacial binding step, the enzyme contains a calcium-dependent interfacial binding site, separate from the active site, which is responsible for the translocation of the enzyme to the interface. The location of this interfacial binding site appears to be at the N-terminal end of the enzyme. This portion of the enzyme has significant sequence homology to the lipid binding domains of both protein kinase C and phospholipase $C_{\gamma 1}$ (Clark *et al.*, 1991).

The interfacial binding step has presented a great challenge when kinetic analysis of the enzyme is performed. However, the analysis of lipase catalysis at the interface has received considerable investigation and has led to kinetic schemes describing the action of phospholipases (Ramirez & Jain, 1991; Yu & Dennis, 1992). The kinetics can be described using classical Michaelis–Menten kinetic theory applied to interfacial catalysis (Berg *et al.*, 1991), which facilitates determination of numerous kinetic parameters and analysis of inhibitors (Gelb *et al.*, 1992).

We have recently reported expression and purification to homogeneity of a deca-histidine tagged construct of human $cPLA_2$ from *Escherichia coli*, called His-$cPLA_2$ (Witmer *et al.*, 1995), and while comparing the kinetic behaviors of this protein to native $cPLA_2$ observed an interesting behavior, previously unreported, with both proteins. To explore further the kinetic properties of $cPLA_2$, we have used purified native recombinant $cPLA_2$ and an anionic vesicle system to determine binding and apparent active site-dissociation constants, and investigated conditions that support maximal activity on these vesicles. In this paper, we provide a detailed kinetic characterization of the action of $cPLA_2$ on covesicles containing DMPM and a radiolabeled, arachidonate-containing phospholipid such as ^{14}C-PAPC. This analysis implicated a cooperative mechanism in the action of $cPLA_2$ which may indicate the existence of a mechanism of regulation for the enzyme that had previously been unobserved.

MATERIALS AND METHODS

Materials. All nonradiolabeled phospholipids were obtained from Avanti Polar Lipids except for DMPM, which was from Calbiochem and 1-alkyl-2-AA-PC which was from Biomol. The radiolabeled compounds were from Amersham (^{14}C-SAPC) or Dupont/NEN (^{14}C-PAPC, ^{3}H-1-alkyl-2-AA-PC, ^{14}C-PAPE, ^{14}C-SAPI, ^{14}C-POPC), with typical specific radioactivities of ~50 mCi/mmol. All reagents and buffers were obtained from Sigma, except as noted. 1-Monoarachidonylglyceride was obtained from NuChekPrep. Gels for SDS–PAGE were obtained from Novex. Aminopropyl BondElut columns (500 mg, 3 mL) were obtained from Varian Sample Prep. Protein purity was assessed by SDS–PAGE and silver (Biorad kit) or Coomassie staining.

Purification of $cPLA_2$. The cDNA encoding native $cPLA_2$ was obtained as described elsewhere (Witmer *et al.*, 1995). The cDNA for the full-length 749 amino acid protein was subcloned into the baculovirus expression vector (InVitroGen) pVL1392, and recombinant protein was expressed and purified from insect Sf9 cells. Purification was achieved following procedures similar to reported conditions (de Carvalho *et al.*, 1993; Street *et al.*, 1993). Protein used for these studies was ≥90% pure based on silver stained SDS–PAGE gels and showed specific activity of ≥8 milliunits/mg. Enzyme was stored in 25 mM Tris, pH 8, containing 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% v/v glycerol, at ≥0.10 mg/mL. The protein was stored at −80 °C and was stable for months under these conditions. Native $cPLA_2$ was used for all experiments described below.

Assays. Small, sonicated phospholipid covesicles of uniform size, comprised of DMPM and containing ^{14}C-PAPC or other phospholipids, were prepared using general methods (Jain & Berg, 1989; Jain & Gelb, 1991; Diez *et al.*, 1992). Vesicle samples were prepared by bath sonication to yield covesicles with an average diameter of 25–30 nm as measured by laser-light scattering. Alternatively, larger vesicles of relatively uniform size were formed by extrusion through two stacked polycarbonate membranes (Poretics Corporation) of 200-nm pore size using an Amicon ultrafiltration cell (Berg *et al.*, 1991). Samples were extruded 8 times, and preincubated at 50 °C for 1–2 min before the initial pass and between passes. The addition of ≤1 mM $CaCl_2$ and/or 30% v/v glycerol did not affect the size distribution of the vesicles at 37 °C for at least 8 h.

As measured by laser-light scattering, higher calcium concentrations (7–10 mM) promote rapid fusion of these SUVs. This agrees with Jain *et al.* (1986), who demonstrated both this effect and the continuous exchange of phospholipids at this higher calcium concentration. In the present study, both nonfusing (i.e., 0.5 mM Ca^{2+}) and fusing (7–10 mM Ca^{2+}) conditions were used in the enzymatic assays.

Unless otherwise noted, enzymatic assays were performed by adding $cPLA_2$, at 37 °C, to 25 mM BisTris Propane, pH 8.0, containing 0.5–0.7 mM $CaCl_2$ (i.e., nonfusing conditions) or 7–10 mM $CaCl_2$ (i.e., fusing conditions), and 0.4–

0.5 mg/mL albumin (BSA or HSA, essentially fatty acid free), 30% glycerol (by volume), and radiolabeled covesicles (typically 300−800 μM total phospholipid, with 0.1−10.0 mol % radiolabeled substrate and the remainder DMPM). All components except enzyme were incubated at 37 °C for 5 min before the addition of enzyme. At various times, 100-μL aliquots were removed and quenched by addition into 1.9 mL of THF. The hydrolyzed, radiolabeled product was then isolated using aminopropyl solid-phase extraction columns as described previously (Tramposch *et al.*, 1992).

TLC Analysis of the Products of Enzymatic Hydrolysis. Samples from the assay of cPLA$_2$ using covesicles of ^{14}C-PAPC/DMPM which had been quenched with THF were evaporated to dryness and redissolved in 30 μL of $CHCl_3$. The radiolabeled components were separated by TLC in hexane/diethylether/formic acid (90:60:6 v/v/v), then visualized using an imaging scanner (Model System 200, Bioscan, Washington, D.C.) and I_2 vapor. The areas corresponding to phospholipid, monoarachidonoyl glyceride, and arachidonic acid were scraped off the plate and quantitated by liquid scintillation counting. With this TLC system, the R_F values for synthetic standards of these components were 0.10, 0.18, and 0.56, respectively.

Identification of Monoarachidonylglyceride by Mass Spectrometry. The enzymatic assay was scaled up to a volume of 500 μL with a phospholipid concentration of 500 μM DMPM:PAPC (90%:10%). Enzyme (1 μg) was added and the reaction incubated at 37 °C for 15 min before extracting with 2 × 500 μL volumes of $CHCl_3$:MeOH (2:1 v/v). The aqueous layer was extracted with 500 μL $CHCl_3$, and the combined organic layers evaporated to dryness under N_2 and redissolved in $CHCl_3$ (500 μL). The sample was loaded onto an Aminopropyl BondElut column, and the column was eluted with 4 mL $CHCl_3$:2-propanol (2:1) then 4 mL diethyl ether:acetic acid (98:2). These eluates were collected separately and dried under N_2. The residues were dissolved in 200 μL of methanol for electrospray mass spectrometry and TLC analysis with comparison to authentic standards. The monoarachidonylglyceride eluted with the $CHCl_3$:2-propanol step and arachidonic acid eluted with the ether: acetic acid step. Electrospray mass spectrometry samples were introduced by infusion in a solution of 10 mM NH_4-OAc in methanol, with positive mode ion detection, which showed peaks for the monoglyceride at m/z = 379 $(M + H)^+$, 396 $(M + NH_4)^+$ and 401 $(M + Na)^+$, and for arachidonic acid at m/z = 322 $(M + NH_4)^+$ and 327 $(M + Na)^+$.

Laser-Light Scattering Studies. A Wyatt Dawn F laser photometer (Wyatt Instruments, Santa Barbara, Cal.) was used to determine mean radius of vesicles using 15 preset angles, 3 vesicle concentrations per sample, and software obtained from the manufacturer. Scintillation vials that had been washed with 0.02 μm-filtered water and 0.02 μm-filtered methanol and inverted to dry were used for light scattering samples. Samples were prepared by microfuging the phospholipid sample at 15000g for 3 min and pipetting aliquots into scintillation vials containing prefiltered (0.02 μm) 25 mM BTP buffer, pH 8, containing 30% glycerol.

Monolayer Studies. A computer-controlled KSV Langmuir microbalance with teflon trough (15 × 50 cm) and teflon-coated barrier was used for measuring isotherms. Samples (25 μg, in 5 μL of $CHCl_3$) were applied by syringe from stock solutions onto the buffer subphase (25 mM BisTris Propane, pH 8, 10 mM $CaCl_2$, 30% v/v glycerol) and were allowed to evaporate at room temperature for 15 min. Reproducible traces were obtained by running three compression and decompression isotherms at a sweep rate of 30 mm/min. DPPC was used to calibrate the instrument. Mean molecular areas were calculated using the KSV software, and final isotherms were plotted using IgorPro.

Scheme 1: Kinetic Scheme for Binding of cPLA$_2$ to Phospholipid[a]

$$E + A \underset{}{\overset{K_S}{\rightleftharpoons}} EA^* + S \overset{K_M^*}{\rightleftharpoons} EAS^* \xrightarrow{k_{cat}} \text{products}$$

[a] E is defined as the free enzyme, A is the phospholipid vesicle, EA* is the enzyme bound to the lipid/water interface, S is the phospholipid substrate within the vesicle, and EAS* is the interface-bound enzyme containing an active site−bound phospholipid substrate.

Table 1: Apparent Dissociation Constants (K_M^{app}) for cPLA$_2$ from the Interface of Covesicles of DMPM and ^{14}C-PAPC[a]

X_{PAPC} (mol %)	K_M^{app} (μM)
2	102 ± 19
4	72 ± 12
6	67 ± 11
8	16 ± 6
10	16 ± 4

[a] Enzyme (260 ng/mL) was assayed in a solution containing radiolabeled covesicles of DMPM and ^{14}C-PAPC (160−800 μM phospholipid). The K_M^{app} values were obtained by nonlinear regression analysis of the rate versus bulk phospholipid concentration data (Cleland, 1979). See Materials and Methods for details.

RESULTS

Binding of cPLA$_2$ to the Interface. The generally accepted concept of equilibrium binding of lipases, such as cPLA$_2$, to the lipid/water interface, is dependent on two processes as defined in Scheme 1 (Verger, 1980). These include the intrinsic equilibrium binding to the interface (defined by a dissociation constant, K_S) and the equilibrium binding of phospholipid substrate to the active site at the interface (defined by a dissociation constant, K_M^*).

This scheme can also be represented by a standard Michaelis−Menten kinetic scheme with substrate being the phospholipid vesicle. In that case, however, the presence of the two binding steps results in an apparent Michaelis−Menten dissociation constant (K_M^{app}) equal to $K_S K_M^*/X_S$ where X_S, in units of mole fraction of the interface, equals the concentration of substrate.

Using covesicles of DMPM and ^{14}C-PAPC as substrate for cPLA$_2$, initial rate studies were conducted in which the mole fraction of ^{14}C-PAPC substrate in the interface (X_{PAPC}) is held constant while the total bulk concentration of phospholipid (DMPM *and* ^{14}C-PAPC) is varied. Hyperbolic plots of rate versus bulk phospholipid concentration were obtained (plots not shown) which yield Michaelis constants equal to K_M^{app} (see Table 1). As expected, increasing the mole fraction of the PAPC results in the enzyme binding more tightly to the interface. That is, more of the enzyme is in the EAS* form, which shifts the enzyme away from the E form and increases the total (EA* and EAS*) enzyme bound to the interface.

Dependence of Rate on the Mole Fraction of Arachidonate-Containing Phospholipid. The converse experiment, where the bulk phospholipid concentration is held constant while the mole fraction of ^{14}C-PAPC is varied, was per-

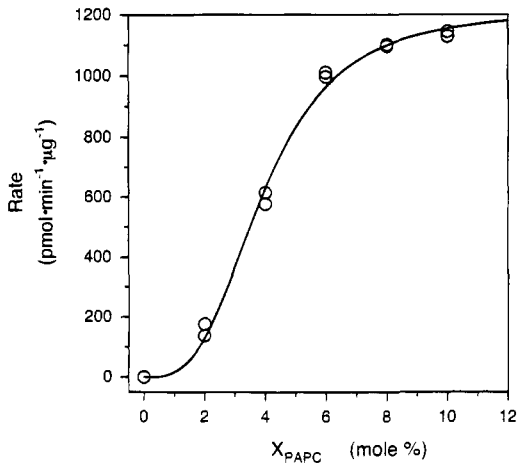

FIGURE 1: Effect of concentration of ^{14}C-PAPC (X_{PAPC}) on the rate of hydrolysis of DMPM/^{14}C-PAPC covesicles by cPLA$_2$. Enzyme (260 ng/mL) was assayed in a solution containing radiolabeled covesicles under fusing conditions ([phospholipid] = 800 μM). Note that X_{PAPC} has concentration units of mol % of the phospholipid bilayer. The curve was obtained by fitting the data to eq 1 yielding a Hill coefficient, n, of 3.2 ± 0.2. See Materials and Methods for details.

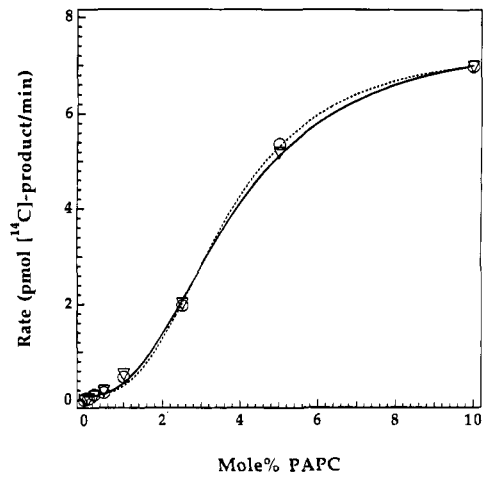

FIGURE 2: Effect of DMPC on the rate of hydrolysis of DMPM/^{14}C-PAPC covesicles by cPLA$_2$. Enzyme (250 ng/mL) was assayed in a solution containing radiolabeled covesicles under fusing conditions ([phospholipid] = 300 μM). The concentration of phosphatidylcholine was kept constant at 10 mol % by using DMPC. The data sets, shown as triangles (PAPC experiment) or circles (PAPC + DMPC experiment) were fitted to eq 1, shown as a solid line (PAPC experiment) or a dotted line (PAPC + DMPC experiment). The values of n were determined from equation 1 to be 2.7 ± 0.2 and 2.8 ± 0.2 for PAPC and PAPC + DMPC experiments, respectively.

formed as well. As long as the bulk phospholipid concentration is large enough to ensure that all of the enzyme is at the interface, plots of rate versus X_{PAPC} should be hyperbolic as well. However, Figure 1 indicates that this is not the case. Instead, sigmoidal plots indicative of a cooperative mechanism were obtained (Segel, 1975). Indeed, the data could be fit to the Hill equation:

$$v_0 = \frac{V_{max}(X_S^{\circ})^n}{(X_S^{\circ})^n + K'} \quad (1)$$

where v_0 is the initial rate; V_{max} is the maximum rate at infinite PAPC mol % (not necessarily attainable under experimental conditions); X_S° is the mole fraction of PAPC substrate; n is the Hill coefficient; and K' is a constant comprising interaction factors and the intrinsic dissociation constant (Segel, 1975).

It should be noted that this sigmoidal plot does not result from poor binding of the enzyme to the interface at, for instances, the lower mole percentages of ^{14}C-PAPC. Even at ^{14}C-PAPC bilayer concentrations as low as 2 mol %, the K_M^{app} value shown in Table 1 corresponds to 90% of the enzyme bound to the interface at a bulk phospholipid concentration of 800 μM (see Table 1).

Other arachidonate-containing phospholipids were also analyzed in this manner. Covesicles of DMPM containing ^{14}C-SAPI or ^{3}H-1-alkyl-2-AA-PC showed similar sigmoidal plots of rate versus X_S (results not shown). In order to test whether the presence of the choline or inositol head group within covesicles of phosphatidylmethanol is the cause of the apparent cooperativity, covesicles of DMPM with ^{14}C-PAPC containing DMPC were used as substrate. As shown in Figure 2, if the total phosphatidylcholine concentration was kept constant at 10 mol %, then the sigmoidal character associated with the plot of rate versus X_{PAPC} was unchanged. This indicates that the presence of the phosphatidylcholine head group is not responsible for the apparent cooperative behavior. Instead, the cooperativity appears to result from the presence of the arachidonate ester in the *sn*-2 position of the phospholipid.

Similarly, titration of ^{14}C-PAPC from 0 to 10 mol % in preformed 200 nm vesicles under nonfusing conditions (i.e., 0.5 mM $CaCl_2$) also fitted to a sigmoidal curve (data not shown). This result indicates the sigmoidal phenomenon is not due to vesicle fusion at high calcium ion concentrations.

Activation by Glycerol. The cPLA$_2$ assays used in this study typically include 30% (v/v) glycerol which greatly enhances the rate of hydrolysis (Ulevitch *et al.*, 1988). Glycerol was also shown to enhance greatly the stability of dilute solutions of the enzyme (results not shown). Light scattering studies on SUV at 0.5 mM $CaCl_2$ indicated that neither glycerol concentrations up to 30% nor PAPC content up to 10 mol % altered the size distribution of the DMPM vesicles (data not shown).

Progress curves under fusing conditions obtained by adding 10 mM Ca^{2+} to sonicated SUVs showed a linear rate of product formation for extended times (>15 min), and the linearity was unaffected by glycerol. However, the rate of the enzymatic reaction was increased dramatically, by up to 20-fold.

The presence of glycerol (30%) additionally leads to the formation of both free arachidonic acid and monoarachidonoyl glyceride as enzymatic reaction products. Electrospray mass spectrometric analysis of the reaction products of the enzymatic reaction indicated the presence of the ester, and TLC analysis of the radiolabeled products indicated that there were 0.3 molecules of monoarachidonoyl glyceride for every molecule of arachidonate released by cPLA$_2$ from covesicles of DMPM and ^{14}C-PAPC. The regiochemistry of monoarachidonoyl glyceride was not identified in this work, although recently has been demonstrated to be ≥95% of the *sn*-1/3 regioisomer and ≤5% of the *sn*-2 regioisomer (Hanel & Gelb, 1995).

The formation of monoarachidonoyl esters by cPLA$_2$ suggests that the polyol, in competition with water, can act as a nucleophile to give the alternate product instead of the free acid. This nucleophilic attack can come about by direct

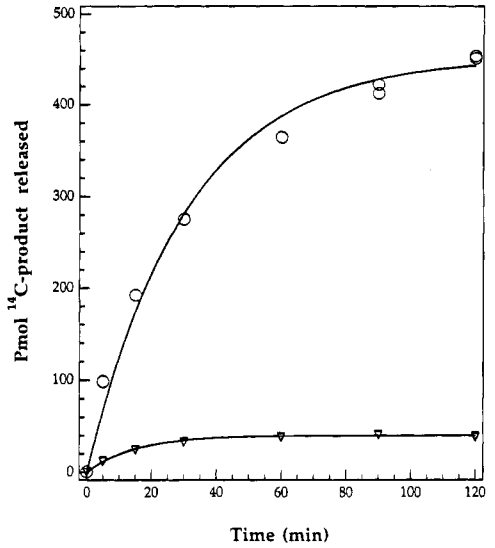

FIGURE 3: Dependence of enzymatic activity on the presence of glycerol using 200-nm nonfusing vesicles of DMPM/^{14}C-PAPC. Samples without glycerol are shown as triangles; samples with glycerol are shown as circles; and the curves were obtained by a first-order fit to the data. The assay, under nonfusing conditions with DMPM/^{14}C-PAPC ([phospholipid] = 500 μM, [^{14}C-PAPC] = 5 mol % or 25 μM) and 0.5 mM $CaCl_2$, is as described in Materials and Methods.

attack at the ester carbonyl of either the phospholipid substrate or the acyl-enzyme intermediate through which the mechanism may proceed (Reynolds *et al.*, 1993; Trimble *et al.*, 1993; Sharp *et al.*, 1994; Hanel & Gelb, 1995).

Under nonfusing conditions, progress curves using preformed DMPM/^{14}C-PAPC covesicles of narrow size distribution (i.e., mean size of 200 nm) demonstrated significant differences between assay conditions that contained glycerol and those that did not. As shown in Figure 3, the addition of 30% glycerol to these LUVs increased the enzymatic rate and extended the active phase of cPLA$_2$ on these vesicles.

Interestingly, the enzyme appeared to become inactivated on SUVs. For example, after less 20 min in the presence of 25-nm covesicles, the enzyme becomes incapable of further hydrolysis of substrate when the vesicles are subsequently fused upon addition of calcium (results not shown). This may explain the small amount of hydrolysis observed with SUVs in the absence of glycerol.

Effect of Glycerol on the Apparent Cooperativity. In order to determine whether glycerol is having an effect on the apparent cooperativity, the dependence of glycerol concentration on the shape of sigmoidal curves was measured under fusing conditions (i.e., 10 mM $CaCl_2$). While the rates of hydrolysis were diminished as the glycerol concentration was decreased, Figure 4 shows that the sigmoidal character of plots of rate versus X_{PAPC} was unchanged even when glycerol was absent.

Determination of Equilibrium Dissociation Constants from the Active Site. Regardless of the mechanism by which the apparent cooperative behavior is brought about, this covesicle system allows for the determination of the equilibrium dissociation constants of phospholipids from the active site at the interface. For example, ^{14}C-PAPC was used as the substrate and nonradiolabeled SAPC as the inhibitor codispersed within DMPM vesicles. Using these covesicles, the concentration of each was varied while measuring the effect on the hydrolysis of ^{14}C-PAPC by cPLA$_2$. Additionally, the

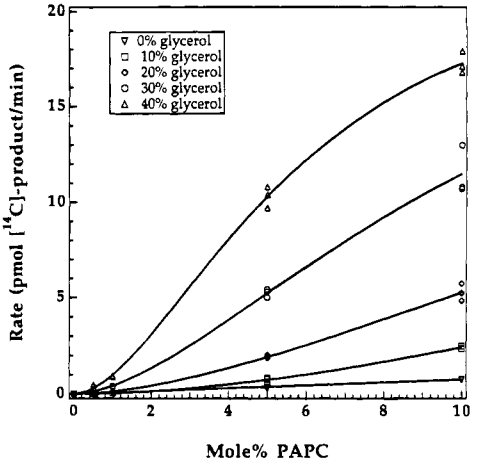

FIGURE 4: Effect of glycerol on the rate of hydrolysis of DMPM/^{14}C-PAPC covesicles by cPLA$_2$. Enzyme (20 ng/mL) was assayed in a solution containing radiolabeled covesicles under fusing conditions ([phospholipid] = 500 μM). The concentration of glycerol present was (inverted triangles) no glycerol; (squares) 10% glycerol; (diamonds) 20% glycerol; (circles) 30% glycerol; (triangles) 40% glycerol. The curves were obtained by fitting to eq 1, and n values were the same within experimental error, ranging from n = 1.6 to 1.9.

concentration of ^{14}C-PAPC was kept above 6 mol % to ensure that all of the cooperativity was manifested (i.e., in the plateau region of the sigmoidal curve as shown in Figure 1). As long as the bulk phospholipid concentration was kept far above the K_M^{app} to ensure that essentially all of the enzyme is at the interface (e.g., at 270 μM phospholipid over 94% of the enzyme is interface-bound), the following equation describing competitive inhibition at the interface is valid (Burke *et al.*, 1995),

$$\frac{(v_0)^o}{(v_o)^I} = 1 + \left(\frac{1 + 1/K_I^*}{1 + \frac{X_S^o}{K_M^*} + \frac{X_L^o}{K_L^*}}\right) X_I/(1 - X_I)) \qquad (2)$$

where $(v_0)^0/(v_0)^I$ is the ratio of initial rates in the absence to that in the presence of a competitive inhibitor; K_I^* is the dissociation constant for the nonradiolabeled inhibitor for the active site at the interface; K_L^* is the dissociation constant for DMPM at the interface; and X_S^o, X_I, and X_L^o are the mole fraction of radiolabeled substrate, nonradiolabeled inhibitor, and DMPM, respectively. The unit of concentration in the interface is mole fraction which is related to the surface concentration of substrate, rather than bulk concentration which has units of molarity.

If DMPM has little or no affinity for the active site (as shown to be true later), then eq 2 can be simplifed to

$$\frac{(v_0)^0}{(v_0)^I} = 1 + \left(\frac{1 + 1/K_I^*}{1 + X_S^o/K_M^*}\right) X_I/(1 - X_I)) \qquad (3)$$

With ^{14}C-SAPC as substrate and PAPC as inhibitor, the dose-dependent inhibition was plotted in Figure 5 as $(v_0)^o/(v_0)^I$ versus $X_I/(1 - X_I)$ at each X_S^o value. As predicted by eqs 2 and 3, linear correlations were obtained. Nonlinear regression analysis of the data as fit to eq 3 yielded K_M^* (SAPC) and K_I^* (PAPC) values of 0.2 ± 0.1 and 0.3 ± 0.1

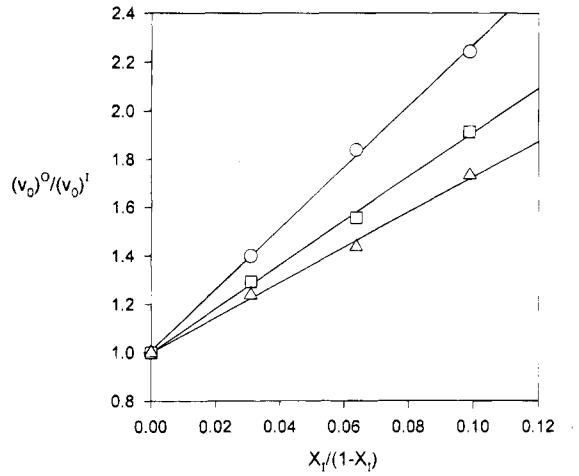

FIGURE 5: Correlation of the inhibition of hydrolysis of ^{14}C-SAPC/DMPM covesicles with the concentration of PAPC (X_I). Enzyme (390 ng/mL) was assayed in a solution containing radiolabeled covesicles under fusing conditions ([phospholipid] = 270 μM). These DMPM/^{14}C-SAPC covesicles contained 0−0.09 mol % PAPC and had X_S^o values (in terms of ^{14}C-SAPC concentrations) of (open circles) 6 mol %; (open squares) 8 mol %; (open triangles) 10 mol %. The data were fit to eq 3, which describes competitive inhibition within the bilayer.

Table 2: Dissociation Constants for Various Phospholipids from the Active Site of cPLA$_2$ at the Interface[a]

phospholipid	K_D* (mol %)
PAPC	0.3 ± 0.1
SAPC	0.2 ± 0.1
SAPI	0.3 ± 0.1[b]
1-alkyl-2-AA-PC	0.3 ± 0.1[c]
PAPE	0.7 ± 0.1[c]
POPC	0.6 ± 0.1[c]
DMPC	>100[c]
PSPC	>100[c]

[a] Conditions were identical to those in Figure 5. [b] K_M* value was determined using PAPC as the inhibitor. [c] K_I* value was determined using PAPC as the substrate.

mol %, respectively. The converse experiment in which SAPC was used as the *inhibitor* while ^{14}C-PAPC was used as the *substrate* yielded K_M* (SAPC) and K_I* (PAPC) values of 0.3 ± 0.1 and 0.3 ± 0.1 mol %, respectively. Obtaining identical dissociation constants regardless of whether a phospholipid was used as a substrate or as an inhibitor demonstrated the internal consistency of this method. It also demonstrates that DMPM has no appreciable affinity for the active site. If it had, then it would not have been possible to simplify eq 2 into eq 3. In that case, the dissociation constants obtained from eq 3 when using one phospholipid as substrate would have differed from the value obtained when it was then used as an inhibitor.

With this technique, the dissociation constants for a number of phospholipids at the interface were obtained. As shown in Table 2, the K_D* values are nearly identical for 2-arachidonoyl-containing phospholipids regardless of the phospholipid head group. This is consistent with both the results of Diez *et al.* (1992) which indicated that arachidonate-containing phospholipids had similar inhibitory potency, as well as the results of Hanel *et al.* (1993) which showed identical k_{cat}/K_M values for SAPI and SAPC.

The dissociation constant at the interface for PAPE is somewhat higher than PAPC and PAPI. However, this may result from increased hydrogen bonding interactions which

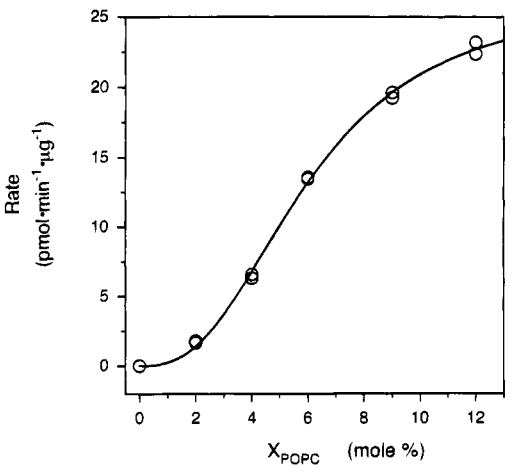

FIGURE 6: Effect of concentration of ^{14}C-POPC (X_{POPC}) on the rate of hydrolysis of DMPM/^{14}C-POPC covesicles by cPLA$_2$. Enzyme (2.2 μg/mL) was assayed in a solution containing radiolabeled covesicles under fusing conditions ([phospholipid] = 500 μM). The curve was obtained by fitting the data to eq 1 yielding a Hill coefficient, n, of 2.6 ± 0.1.

the phosphatidylethanolamine head group makes with neighboring phospholipids. That is, the value of a dissociation constant at the interface is a measure of the relative affinity of the molecule for the active site as compared to the affinity of the molecule for the bilayer (Jain *et al.*, 1993; Lin & Gelb, 1993; Burke *et al.*, 1995). Therefore, the increased interactions that PAPE makes with the bilayer as compared to PAPC or SAPI may explain its apparent larger dissociation constant from the active site.

As evidenced by the K_D* values for DMPC and PSPC, the results from Table 2 are also consistent with the conclusion that phospholipids containing saturated fatty acid esters have little or no affinity for the active site. It is interesting to note that POPC, which is hydrolyzed by cPLA$_2$ at less than 5% of the rate at which PAPC is hydrolyzed, binds very tightly to the active site (K_D* = 0.6 mol %). This indicates that the ineffectiveness of POPC as a substrate results from an effect on k_{cat} rather than on K_M*.

Using this technique for determining active site dissociation constants, the presence of products (i.e., 1:1 mixture of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine and arachidonic acid) at bilayer concentrations as high as 8 mol % did not significantly inhibit the enzyme-catalyzed hydrolysis of these DMPM/^{14}C-PAPC covesicles (X_S^o = 8 mol %).

PAPE and POPC as Substrates for cPLA$_2$. When ^{14}C-POPC codispersed in DMPM vesicles was used as a substrate for cPLA$_2$, a sigmoidal plot of rate versus concentration of POPC was obtained (see Figure 6). It differed from that obtained for PAPC in that apparent saturation did not occur until X_{POPC} reached a value of about 12 mol % as compared to 6 mol % for PAPC. POPC, while being a poor substrate of cPLA$_2$, nonetheless also shows an apparent cooperativity in this system.

However, when ^{14}C-PAPE was used as a substrate in the same way, a very unusual result was obtained. This substrate was hydrolyzed at only about one-fifth the rate of PAPC at X_S = 0.6 mol %. Figure 7 shows that a plot of rate versus concentration of PAPE is nearly linear (open circles) rather than sigmoidal. In order to determine whether this effect results from a small fraction of the enzyme being bound to

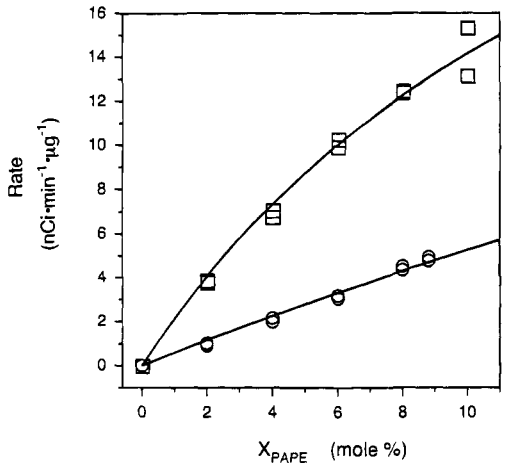

FIGURE 7: Effect of concentration of ^{14}C-PAPE (X_{PAPE}) on the rate of hydrolysis of DMPM/^{14}C-PAPE covesicles by cPLA$_2$. Enzyme (325 ng/mL) was assayed in a solution of radiolabeled covesicles ([phospholipid] = 500 μM) of ^{14}C-PAPE (28.5 mCi/mmol) and DMPM containing (open circles) no PAPC; (open squares) 6 mol % (unlabeled) PAPC. Rates of hydrolysis are presented as amount of ^{14}C-labeled arachidonate released, in nCi/min mg.

the interface, the dependence of the rate on the bulk phospholipid concentration was measured. With these covesicles of ^{14}C-PAPE and DMPM, the K_M^{app} values at X_{PAPE} = 4, 6, and 8 mol % were measured to be 100, 94, and 104 μM, respectively. This indicates that over 80% of the enzyme is bound to the interface of the DMPM: ^{14}C-PAPE covesicles at a bulk phospholipid concentration of 500 μM.

Interestingly, when nonradiolabeled PAPC was present at a concentration of 6 mol %, the rate of hydrolysis of ^{14}C-PAPE is activated rather than competitively inhibited (see Figure 7, open squares). In this case, the plot becomes hyperbolic and can be fit to an apparent K_M* value of 16 mol % in the presence of PAPC (V_{max} = 1270 pmol min^{-1} mg^{-1}). This apparent K_M* value is consistent with the dissociation constants shown in Table 2. That is, if PAPC is acting to increase the apparent K_M* value for ^{14}C-PAPE by a factor of (1 + X_I/K_I*), which would be predicted from simple Michaelis–Menten kinetics, then an apparent K_M* value of 15 mol % can be calculated from the K_D* values shown in Table 2. This, of course, is in close agreement with the value of 16 mol % actually measured in the presence of nonradiolabeled PAPC.

Physical Characterization of Monolayers. To investigate whether addition of PAPC to DMPM significantly altered the physical properties of the membrane, monolayer studies were done. Comparisons were made between monolayers of DMPM (100%), DMPM:PAPC (90%:10%) and PAPC (100%) using a Langmuir microbalance system. Well-behaved isotherms were recorded with a subphase buffer containing 25 mM BisTris Propane, 30% glycerol, and 10 mM $CaCl_2$. The presence of 10% PAPC significantly altered the compression behavior of the monolayer relative to pure DMPM, as seen by the transition pressure between the liquid-expanded and liquid-compressed states in Figure 8. From this figure, the mean molecular areas occupied by the molecules in the liquid condensed states of the monolayers were determined by extrapolation to be approximately 31, 46, and 40 Å^2, for DMPM, PAPC, and DMPM:PAPC, respectively.

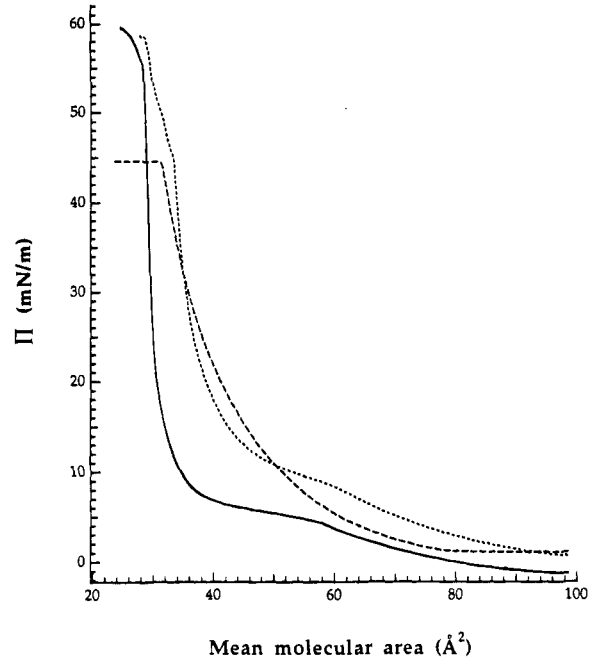

FIGURE 8: Pressure–surface area isotherms of DMPM, DMPM/PAPC, and PAPC monolayers. Monolayers were prepared on a subphase of 25 mM BisTris Propane, pH 8, containing 30% glycerol and 10 mM $CaCl_2$. The isotherm for DMPM is shown as a solid line, DMPM:PAPC (90%:10%) is shown as a dotted line, and PAPC is shown as a dashed line. The surface pressure is represented by Π. Extrapolation to the x-axis of the portions of isotherms showing the sudden pressure increases gives the mean molecular areas occupied by the liquid crystalline states. The extrapolated values are 31 ± 3 Å^2 for DMPM, 40 ± 3 Å^2 for DMPM:PAPC, and 46 ± 3 Å^2 for PAPC.

DISCUSSION

The principal objective of the present research was to investigate the mechanism by which cPLA$_2$ acts on covesicles composed of DMPM and arachidonate-containing phospholipids. The high selectivity for unsaturated *sn*-2 fatty acid side chains of the target phospholipid precludes DMPM from being a substrate for cPLA$_2$. The enzyme, however, will bind to DMPM vesicles and cleave suitable unsaturated phospholipids incorporated into the anionic vesicles (Diez *et al.*, 1992; Witmer *et al.*, 1995). Because DMPM does not bind to the active site of cPLA$_2$, it can be thought of as a neutral diluent (Jain *et al.*, 1991). That is, DMPM forms a phospholipid matrix that does not interfere with the binding of substrate phospholipids to the active site.

Assay Conditions. Glycerol had a dramatic effect on the enzymatic activity and stability of the enzyme. The rate of product release was stimulated up to 20-fold at 30% glycerol, relative to no glycerol, under fusing conditions (i.e., 10 mM $CaCl_2$), as seen in Figure 4. Moreover, the presence of 30% glycerol resulted in release of about 30% of the product as monoarachidonylglyceride, which indicates that glycerol binds to the enzyme. The enzymatic generation of monoarachidonyl glyceride by cPLA$_2$ has recently been described (Hanel & Gelb, 1995).

cPLA$_2$ is rapidly inactivated on these anionic SUVs under nonfusing conditions in the absence of glycerol. A high degree of surface curvature, most pronounced at small vesicle radii, may be denaturing to cPLA$_2$. However, the enzyme is not inactivated under Ca^{2+}-induced fusing conditions, which facilitates the anionic vesicles to increase from a mean

radius of ~30 nm to >200 nm and consequently reduces the vesicle surface curvature. This inactivation, regardless of its origin, is also prevented by adding glycerol to the assay buffer. As seen in Figure 3, glycerol also markedly increased the activity and extended the active phase of $cPLA_2$ on preformed 200-nm-diameter LUVs under nonfusing conditions. In summary, the highest enzymatic activity was supported in the presence of 30% glycerol and on large vesicles, either preformed or generated by Ca^{2+}-induced fusion of SUVs. We recently have shown similar kinetic properties for His-$cPLA_2$, a recombinant form of the protein containing a deca-histidine sequence (Witmer *et al.*, 1995).

Apparent Cooperativity. The present research provides previously unobserved results concerning an apparent cooperative effect in the hydrolysis of arachidonate-containing phospholipid substrates. At 7−10 mM $CaCl_2$, plots of rate versus mole fraction of substrate phospholipid were sigmoidal when covesicles of DMPM and arachidonate-containing phospholipids such as PAPC were used.

This result was unexpected since it had been reported that plots of rate versus X_S were hyperbolic (Diez *et al.*, 1992). However, instead of an impure preparation of $cPLA_2$ from U937 cells as was used in that work, the present study utilized highly purified recombinant enzyme. Using recombinant native $cPLA_2$ under the exact conditions previously reported (Diez *et al.*, 1992), the enzyme failed to show normal hyberbolic plots (results not shown). Moreover, this sigmoidal behavior was reproducible under numerous experimental conditions. These include different enzyme samples and forms [i.e., partially purified protein from U937 cells, and highly purified His-$cPLA_2$ expressed in *E. coli* (Witmer *et al.*, 1995), data not shown], buffers (i.e., BisTris Propane, Hepes), components (i.e., glycerol), phospholipid concentrations (i.e., 100−500 μM), and several different substrate molecules (i.e., PAPC, SAPC, 1-alkyl-2-AA-PC, SAPI, POPC; data not shown for some). In addition, an initial rate versus X_{PAPC} experiment using preformed 200-nm covesicles of DMPM/^{14}C-PAPC under nonfusing conditions also showed sigmoidal behavior, indicating that vesicle fusion alone is not responsible for this result (data not shown).

It is interesting, however, that the active site dissociation constants for arachidonate-containing phosphatidylcholine determined here (0.3 mol %) are in good agreement with the value of 0.34 mol % estimated by Diez *et al.* (1992). It may be that the impure protein used by Diez and co-workers contained a component that may have masked the cooperative behavior. This is important since it may may mean that there is some heretofore unrecognized regulatory component within cells.

Lateral Diffusion Rate of PAPC. The lateral diffusion of PAPC is not expected to be rate-limiting at low mole fraction of PAPC. The maximal observable catalytic turnover rate when lateral diffusion is rate-limiting is expected to be at least 200 s^{-1} at X_{PAPC} = 2 mol % [calculated according to Jain and Berg (1989)]. Since the enzyme has a turnover number of 0.2 s^{-1} at this concentration of PAPC, this would argue that lateral diffusion is not a rate-limiting step in the enzymatic reaction and therefore would not account for the kinetic data.

Oligomerization State of the Enzyme. A possible explanation for the apparent cooperativity is enzyme oligomerization, which leads to enhanced activity, induced by binding to arachidonate-containing phospholipid (Bell & Bell, 1988). Human $cPLA_2$ is monomeric in solution, based on migration in PAGE under native conditions and purification by gel permeation chromatography (Clark *et al.*, 1991; Kramer *et al.*, 1991; Rehfeldt *et al.*, 1993; Huang *et al.*, 1994). Moreover, various cross-linking experiments failed to detect significant amounts of oligomers at the phospholipid−water interface at the low protein concentrations (i.e., nanomolar range) used in kinetic studies (M. R. Witmer, J. Tredup, and R. Micanovic, unpublished experiments). Variation in enzyme concentration over a >100-fold range (i.e., 20 ng/mL−2.2 μg/mL, or 0.23−25.82 nM) used in our kinetic experiments all showed sigmoidal behavior as well. Cooperativity between enzyme subunits is therefore an unlikely explanation with these vesicles, although a clustering of the enzyme on zwitterionic PC vesicles has recently been proposed (Creaney *et al.*, 1995).

Apparent Cooperativity as a Result of Changes in Membrane Structure. The cooperative behavior is dependent on the unsaturated fatty acid ester component of the phospholipid. This contrasts with a more common dependence on the phosphoglycerol head group for other membrane-associated enzymes showing cooperative effects (*vide supra*). Although it may be surprising that an interface-bound enzyme could recognize structural elements buried within the bilayer, numerous physical studies of model systems illustrate that *cis*-unsaturated side chains alter the molecular phospholipid properties relative to fully saturated analogues, and consequently, PAPC is expected to have quite different properties from those of the fully saturated DMPM. The preferred side chain conformations, the range and rates of side chain motion, lateral diffusion coefficients, and occupied surface areas are all expected to differ between the two molecules (Stubbs & Smith, 1984).

Apparent Cooperativity as a Result of Changes in Membrane Fluidity. Another explanation for the sigmoidal behavior is that increasing the amount of arachidonate-containing phospholipids affects the physical nature of the bilayer which may facilitate enzymatic abstraction of the phospholipid molecules from the bilayer into the active site.

Phospholipids that contain unsaturated fatty acid esters are known to increase the fluidity of bilayers (Shinitzky, 1984), although this is an oversimplification of the physical properties (Stubbs & Smith, 1984). Changes in properties have been demonstrated by differential scanning calorimetry (Burack *et al.*, 1993) and microbalance surface tension measurements with fatty acids in carrier phospholipids (Tsujita *et al.*, 1987) and pure *cis*-unsaturated PCs, including POPC and PAPC (Stubbs *et al.*, 1981). Our monolayer experiments with 10% PAPC in the DMPM monolayer show a significantly different isotherm that pure DMPM, indicating that even this amount of PAPC affects packing of side chains. The addition of 10% PAPC to this anionic phospholipid is enough to change the pressure at which the molecules orient and become organized into a monolayer. Although this is a property of the monolayers, the bilayer properties are also expected to differ significantly, even over this narrow substrate range. This is supported by DSC experiments which showed that when either 10% PAPC or 10% PAPE was added to DMPM vesicles, significantly lower transition temperatures between gel and liquid crystalline states were observed, indicating differences in bilayer properties (M. R. Witmer, unpublished experiments).

Nonideal mixing of the substrate phospholipid within the DMPM vesicle may cause clustering and lateral phase separation of the arachidonate-containing phospholipid (Gennis, 1989). For instance, as X_{PAPC} is increased, clusters of PAPC may result which are recognized by the interfacial recognition site. Binding to these clusters may then lead to a deeper penetration of the enzyme into the bilayer, and abstraction of phospholipids out of the bilayer and into the active site would then be less energetically difficult. Indeed, lateral phase separation has been implicated to play a role in the activity of the 14-kDa snake venom sPLA$_2$ (Burack *et al.*, 1993). Similarly, the observed specificity of the PLA$_2$ from rabbit platelets toward arachidonyl-containing membranes of intact platelet membranes was attributed to arachidonyl-rich domains regulating enzymatic activity (Kannagi *et al.*, 1981).

This possibility is consistent with the fact that PAPE does not show the same cooperative behavior as do other phospholipids such as PAPC and SAPI. The amine headgroup of phosphatidylethanolamine has the unique ability to form hydrogen bonds with neighboring phospholipids, which is unavailable to phosphatidylcholine. Therefore, increasing the PAPE concentration within the DMPM covesicles should increase the fluidity to a lesser degree than with the same amount of PAPC (Shinitzky, 1984). This is supported by the demonstration that increasing ratios of PE versus PC causes a decrease in the fluidity of biological membranes (Esko, *et al.*, 1977; Gilmore *et al.*, 1979).

The increase in the hydrolysis rate of ^{14}C-PAPE seen when PAPC is present is consistent with this hypothesis. Addition of nonradiolabeled PAPC to DMPM vesicles containing ^{14}C-PAPE may lead to the increase in fluidity necessary to allow the enzyme to penetrate more deeply into the bilayer. Therefore, the binding affinity of PAPE to the active site would be greater in the presence of PAPC. If this is true, it may represent a previously unobserved way to regulate the action of cPLA$_2$. Indeed, increases in membrane fluidity resulting from receptor-stimulated pathways in many cell types are closely associated with arachidonate release and mobilization (Hirata & Axelrod, 1980).

The idea that membrane viscosity can be modulated by a cell in order to regulate enzymatic activities is not unprecedented. As reviewed by Stubbs and Smith (1984), lipid-associated enzymes such as adenylate cyclase and ATPases, as well as receptors such as the insulin receptor, have been shown to be sensitive to the fluidity of the membrane.

Apparent Cooperativity as a Result of Allosterism. The most intriguing explanation for the sigmoidal kinetic data is that the enzyme may possess a homotropic allosteric site separate from the active site (Hammes, 1982). In this scenario, binding of arachidonate-containing phospholipids to the allosteric site would induce a conformational change in the enzyme that facilitates hydrolysis catalyzed by the active site.

If an allosteric site exists, it must be that PAPE has, at best, only a very weak affinity for such a site since it does not show the same cooperative behavior. This explains why PAPE is hydrolyzed at a much slower rate. When nonradiolabeled PAPC is present in vesicles of DMPM containing ^{14}C-PAPE, however, binding of PAPC to the putative allosteric site could enhance the ability of the enzyme to hydrolyze ^{14}C-PAPE. Support for this explanation is the observation that pyruvate decarboxylase shows cooperativity with respect to its substrate, pyruvate. The presence of pyruvamide, which is able to bind to an allosteric site, greatly enhances the decarboxylase-catalyzed reaction (Schellenberger *et al.*, 1988).

The results with ^{14}C-PAPE indicate that the cooperative effect manifests itself through decreasing the value of K_M* rather than increasing the value of k_{cat}. This is evidenced by a decrease in the K_M* value for PAPE from approximately 60 mol % (open circles, Figure 7) to 0.6 mol % in the presence of PAPC (see Table 2 and Figure 7, open squares).

The putative allosteric site may in fact, be the interfacial recognition site. Marangoni recently has proposed that the cooperativity exhibited by some lipases may result from an association of the interfacial recognition site with a cluster of substrate molecules at the interface (Maragoni, 1994). This may then result in an interfacially-penetrated lipase bound to a cluster of substrate molecules.

CONCLUSIONS

Kinetic analysis of cPLA$_2$ using the DMPM vesicle system has revealed an intriguing result: a sigmoidal dependence of rate on substrate content at the interface. Several explanations for this behavior have been considered, including differential membrane properties and an allosteric effector site. Although the sigmoidal effect may be due, in part, to physical changes in the bilayer properties, the most interesting possibility is that cPLA$_2$ contains an allosteric site which is specific for unsaturated fatty acid side chains.

In conclusion, while the mechanism from which the apparent cooperativity results remains unclear, the present study indicates that this may be another way, along with phosphorylation and control of intracellular calcium levels, through which a cell is able to regulate the activity of cPLA$_2$.

ACKNOWLEDGMENT

The authors wish to thank Dr. Terry Stouch for insightful discussions about phospholipid properties and membrane dynamics, Dr. John Groves for facilitating use of the KSV Langmuir microbalance in his laboratory, and Bethanne Warrack for electrospray mass spectrometric analysis of monoarachidonylglyceride samples.

REFERENCES

Barbour, S. E., & Dennis, E. A. (1993) *J. Biol. Chem. 268*, 21875−21882.

Bartoli, F., Lin, H.-K., Ghomashchi, F., Gelb, M. H., Jain, M. K., & Apitz-Castro, R. (1994) *J. Biol. Chem. 269*, 15625−15630.

Bell, J. E., & Bell, E. T. (1988) *Proteins and Enzymes*, pp 370−377, Prentice Hall, Englewood Cliffs, NJ.

Berg, O. G., Yu, B. Z., Rogers, J., & Jain, M. K. (1991) *Biochemistry 30*, 7283−7297.

Bonventre, J. V., Gronich, J. H., & Nemenoff, R. A. (1990) *J. Biol. Chem. 265*, 4934−4939.

Burack, W. R., Yuan, Q., & Biltonen, R. L. (1993) *Biochemistry 32*, 583−589.

Burke, J. R., Gregor, K. R., & Tramposch, K. M. (1995), *J. Biol. Chem. 270*, 274−280.

Channon, J. Y., & Leslie, C. C. (1990) *J. Biol. Chem. 265*, 5409−5413.

Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., & Knopf, J. L. (1991) *Cell 65*, 1043−1051.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.

Creaney, A., Masters, D. J., Needham, M. B., Gordon, R. D., Mott, R., & Wilton, D. C. (1995) *Biochem. J. 306*, 857−864.

de Carvalho, M. S., McCormack, R. X., & Leslie, C. C. (1993) *Arch. Biochem. Biophys. 306*, 534−540.
Diez, E., Louis-Flamberg, P., Hall, R. H., & Mayer, R. J. (1992) *J. Biol. Chem. 267*, 18342−18348.
Diez, E., Chilton, F. H., Stroup, G., Mayer, R. J., Winkler, J. D., & Fonteh, A. N. (1994) *Biochem. J. 301*, 721−726.
Esko, J. D., Gilmore, J. R., & Glaser, M. (1977) *Biochemistry 16*, 1881−1890.
Gelb, M. H., Jain, M. K., & Berg, O. (1992) *Bioorg. Med. Chem. Lett. 2*, 1335−1342.
Gennis, R. B. (1989) *Biomembranes: Molecular Structure and Function*, pp 71−72, Springer-Verlag, New York.
Gilmore, R., Cohn, N., & Glaser, M. (1979) *Biochemistry 18*, 1042−1049.
Godson, C., Bell, K. S., & Insel, P. A. (1993) *J. Biol. Chem. 268*, 11946−11950.
Hammes, G. G. (1982) *Enzyme Catalysis and Regulation*, pp 156−161, Academic Press, Orlando, FL.
Hanel, A. M., Schuttel, S., & Gelb, M. H. (1993) *Biochemistry 32*, 5949−5958.
Hanel, A. M., & Gelb, M. H. (1995) *Biochemistry 34*, 7807−7818.
Hirata, F., & Axelrod, J. (1980) *Science 209*, 1082−1090.
Huang, Z., Laliberte, F., Tremblay, N. M., Weech, P. K., & Street, I. P. (1994) *Anal. Biochem. 222*, 110−115.
Jain, M. K., & Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127−156.
Jain, M. K., & Gelb, M. H. (1991) *Methods Enzymol. 197*, 112−125.
Jain, M. K., Rogers, J., Jahagirdar, D. V., Marecek, J. F., & Ramirez, F. (1986) *Biochim. Biophys. Acta 860*, 435−447.
Jain, M. K., Yu, B. Z., Rogers, J., Ranadive, G. N., & Berg, O. G. (1991) *Biochemistry 30*, 7306−7317.
Jain, M. K., Yu, B. Z., & Berg, O. G. (1993) *Biochemistry 32*, 11319−11329.
Kannagi, R., Koizumi, K., & Masuda, T. (1981) *J. Biol. Chem. 256*, 1177−1184.
Kramer, R. M., Johansen, B., Hession, C., & Pepinsky, R. B. (1990) *Adv. Exp. Med. Biol. 275*, 35−54.
Kramer, R. M., Roberts, E. F., Manetta, J., Putnam, J. E. (1991) *J. Biol. Chem. 266*, 5268−5272.
Kramer, R. M., Roberts, E. F., Manetta, J. V., Hyslop, P. A., & Jakubowski, J. A. (1993a) *J. Biol. Chem. 268*, 26796−26804.
Kramer, R. M., Roberts, E. F., Manetta, J. V., Sportsman, J. R., & Jakubowski, J. A. (1993b) *J. Lipid Mediators 6*, 209−216.
Kramer, R. M. (1994) in *Signal-Activated Phospholipases* (Liscovitch, M., Ed.) pp 13−30, R. G. Landes Co., Austin, TX.
Lehmann, J., Brown, K. A., Ramanadham, S., Turk, J., & Gross, R. W. (1993) *J. Biol. Chem. 268*, 20713−20716.
Lin, L.-L., Lin, A. Y., & Knopf, J. L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6147−6151.
Lin, H.-K., & Gelb, M. H. (1993) *J. Am. Chem. Soc. 115*, 3932−3942.
Marangoni, A. G. (1994) *Biochem. Biophys. Res. Commun. 200*, 1321−1328.
Murakami, M., Kudo, I., & Inoue, K. (1993) *J. Biol. Chem. 268*, 839−844.
Nakamura, T., Lin, L.-L., Kharbanda, S., Knopf, J. L., & Kufe, D. (1992) *EMBO J. 11*, 4917−4922.
Qiu, Z.-H., & Leslie, C. C. (1994) *J. Biol. Chem. 269*, 19480−19487.
Qiu, Z.-H., de Carvalho, M. S., & Leslie, C. C. (1993) *J. Biol. Chem. 268*, 24506−24513.
Ramesha, C. S., & Ives, D. L. (1993) *Biochim. Biophys. Acta 1168*, 37−44.
Ramirez, F., & Jain, M. K. (1991) *Proteins 9*, 229−239.
Rehfeldt, W., Resch, K., & Goppelt-Struebe, M. (1993) *Biochem. J. 293*, 255−261.
Reynolds, L. J., Hughes, L. L., Louis, A. I., Kramer, R. M., & Dennis, E. A. (1993) *Biochim. Biophys. Acta 1167*, 272−280.
Schalkwijk, C., Pfeilschifter, J., Marki, F. & van den Bosch, H. (1992) *J. Biol. Chem. 267*, 8846−8851.
Schellenberger, A., Hubner, G., & Sieber, M. (1988) in *Thiamin Pyrophosphate Biochemistry* (Schellenberger, A., & Schowen, R. L., Eds.) Vol. 1, pp 113−121, CRC Press, Boca Raton, FL.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 353−377, Wiley-Interscience, New York.
Sharp, J. D., Pickard, R. T., Chiou, X. G., Manetta, J. V., Kovacevic, S., Miller, J. R., Varshavsky, A. D., Roberts, E. F., Strifler, B. A., Brems, D. N., & Kramer, R. M. (1994) *J. Biol. Chem. 269*, 23250−23254.
Shinitzky, M. (1984) *Physiology of Membrane Fluidity*, Vol. 1, pp 1−51, CRC Press, Boca Raton, FL.
Street, I. P., Lin, H.-K., Laliberte, F., Ghomaschchi, F., Wang, Z., Perrier, H., Tremblay, N. M., Huang, Z., Weech, P. K., & Gelb, M. H. (1993) *Biochemistry 32*, 5935−5940.
Stubbs, C. D., Kouyama, T., Kinosita, K., Jr., & Ikegami, A. (1981) *Biochemistry 20*, 4257−4262.
Stubbs, C. D., & Smith, A. D. (1984) *Biochim. Biophys. Acta 779*, 89−137.
Tramposch, K. M., Steiner, S. A., Stanley, P. L., Nettleton, D. O., Franson, R. D., Lewin, A. H., & Carroll, F. I. (1992) *Biochim. Biophys. Res. Commun. 189*, 272−279.
Trimble, L. A., Street, I. P., Perrier, H., Tremblay, N. M., Weech, P. K., & Bernstein, M. A. (1993) *Biochemistry 32*, 12560−12565.
Tsujita, T., Smaby, J. M., & Brockman, H. L. (1987) *Biochemistry 26*, 8430−8434.
Turk, J., Gross, R. W., & Ramandham, S. (1993) *Diabetes 42*, 367−374.
Ulevitch, R. J., Watanabe, Y., Sano, M., Lister, M. D., Deems, R. A., & Dennis, E. A. (1988) *J. Biol. Chem. 263*, 3079−3085.
Verger, R. (1980) *Methods Enzymol. 64*, 340−392.
Witmer, M. R., Micanovic, R., Tredup, J., Lin, W., Hail, M., & Villafranca, J. J. (1995) *Arch. Biochem. Biophys. 318*, 430−438.
Xing, M., & Mattera, R. (1992) *J. Biol. Chem. 267*, 25966−25975.
Yu, L., & Dennis, E. A. (1992) *Bioorg. Med. Chem. Lett. 2*, 1343−1348.

BI9511604