Interfacial Catalysis by Human 85 kDa Cytosolic Phospholipase A2 on Anionic Vesicles in the Scooting Mode†

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ABSTRACT: Analysis of phospholipases A2 on model phospholipid bilayers in which enzyme is essentially irreversibly bound at the lipid—water interface, termed “scooting mode”, is a useful tool for studying the kinetic properties of interfacial enzymes. It is shown that human cytosolic 85 kDa phospholipase A2 (cPLA2) hydrolyzes sn-2-arachidonoyl-containing phospholipids or the γ-linolenoyl ester of 7-hydroxy-coumarin (GLU) dispersed in vesicles of 1,2-dioleoyl-sn-glycero-3-phosphocholine (L-DOPM) in the scooting mode. Trapping of cPLA2 on L-DOPM vesicles is rapid and independent of product formation. Slowing of cPLA2-catalyzed hydrolysis of substrates present in phosphatidymethanol and phosphatidylcholine vesicles is primarily due to apparent inactivation rather than to substrate depletion. cPLA2 phosphorylated on serine 505 by mitogen-activated protein kinase displays a 30% increase in the rate of sn-2-arachidonoylphosphatidylcholine hydrolysis in the scooting mode compared to that of the nonphosphorylated enzyme. Kinetic parameters of cPLA2 acting on a variety of different phosphatidymethanol vesicles were evaluated, and the results are discussed in terms of active site affinities for substrates and of lateral organization of substrates in the bilayer. A key result is that the sigmoidal kinetics reported previously using 1,2-dimyrystoyl-sn-glycero-3-phosphomethanol (DMPM) vesicles are most prominent near the phase transition temperature of DMPM. No sigmoidal kinetics was observed using L-DOPM vesicles. The results of kinetic experiments and the behavior of a fluorescent substrate analog are consistent with nonideal mixing of substrate in DMPM vesicles, but not in L-DOPM vesicles, suggesting that apparent saturation and sigmoidal kinetics are more a result of nonideal mixing of substrate in DMPM vesicles than of active site binding of substrate. The fluorescence assay described using L-DOPM/GLU vesicles is useful for evaluating the interfacial behavior of cPLA2, including its substrate preferences and the effect of active site-directed inhibitors.

The 85 kDa cytosolic phospholipase A2 (cPLA2) is part of a signal transduction cascade in many mammalian cell types leading to the liberation of arachidonic acid from membrane phospholipids for the biosynthesis of eicosanoids (Lin et al., 1992; Kramer et al., 1993; Mayer & Marshall, 1997; Qiu et al., 1993; Dennis, 1994; Clark et al., 1995). The binding of cPLA2 to membranes requires micromolar amounts of Ca2+ (Channon & Leslie, 1990; Ghomashchi et al., 1992; Wijkander & Sundler, 1992), and this occurs via the enzyme’s Ca2+-dependent phospholipid binding domain (Clark et al., 1991; Sharp et al., 1991), which is distinct from its catalytic domain (Nalefski et al., 1994). Recent microscopic studies indicate that cPLA2 translocates from the cytosol to the nuclear envelope and endoplasmic reticulum in stimulated cells (Glover et al., 1995; Schievella et al., 1995). In addition to Ca2+, phosphorylation of cPLA2 is necessary for maximal activation of this enzyme, and MAP kinase has been shown to phosphorylate cPLA2 on serine 505 to activate this enzyme in cells (Lin et al., 1993). Sites of phosphorylation other than the MAP kinase site have recently been determined (de Carvalho et al., 1996); the functional significance of all of these sites remains to be established. Recent studies with cPLA2 inhibitors have shown that it is possible to use a pharmacological approach to block arachidonic acid release in platelets that have been stimulated with thrombin to produce eicosanoids (Bartoli et al., 1994; Riendeau et al., 1994). The hydrolytic mechanism of cPLA2 is not fully understood. Current evidence favors the formation of an acyl enzyme (Reynolds et al., 1993; Trimble et al., 1993; Hanel & Gelb, 1995) with serine 228 as the residue of acyl attachment (Sharp et al., 1994; Huang et al., 1996).
Characterization of the interfacial enzymology of cPLA₂ acting on phospholipid vesicles in vitro is at an early state. cPLA₂ binds more tightly to vesicles that contain anionic lipids than to those that are charge neutral (Diez et al., 1992; Ghomashchi et al., 1992; Hanel et al., 1993; Witmer et al., 1995). The binding of cPLA₂ to anionic vesicles can be sufficiently tight such that the enzyme hydrolyzes many phospholipids without leaving the vesicle surface. This type of processive interfacial catalysis is termed scooting (Jain et al., 1986). Through study of the action of interfacial enzymes in the scooting mode, it is possible to describe the reaction progress in terms of the well-known Michaelis–Menten formalism and to extract most, if not all, of the rate and equilibrium constants for the steps that make up the reaction cycle (Jain et al., 1986; Gelb et al., 1995). This has been extensively carried out with secreted 14 kDa PLA₂ acting on vesicles of the anionic phospholipid DMPM (Berg et al., 1991; Bayburt et al., 1995). Since DMPM is not a substrate for cPLA₂, owing to the preference of this enzyme for sn-2-arachidonyl-containing phospholipids (Hanel et al., 1993; Mayer & Marshall, 1993; Clark et al., 1995), it is necessary to study covesicles of DMPM with sn-2-arachidonyl-containing cPLA₂ substrates. In this study, the kinetic features of cPLA₂ acting on anionic phosphatidylmethanol vesicles are reported, and the results are compared to related and recent studies (Diez et al., 1992; Burke et al., 1995; Witmer et al., 1995).

### MATERIALS AND METHODS

**Materials.** Recombinant human cPLA₂ was overexpressed in an Sf9-baculovirus system and purified by anion-exchange and hydrophobic interaction chromatographies (Street et al., 1990) and was further purified by CM-cellulose chromatography (Kates, 1986). DTPM was a gift from Dr. Ian Street (Merck-Frosst Center for Therapeutic Research). Purified active recombinant MAP kinase was a generous gift from Prof. E. Krebs (University of Washington).

**Synthesis of Phosphatidylmethanols.** L-DOPM, HOPM, and SAPM were prepared by phospholipase D-catalyzed transphosphatidylation (Comfurius & Zwaal, 1977). Cabbage phospholipase D acetone precipitate was prepared as described (Davidson & Long, 1958) and stored as a lyophilized powder at −20 °C. The corresponding phosphatidylcholine was suspended in ethanol at 10 mg/mL. Following the addition of 0.2 volume of methanol, 1 volume of a 10 mg/mL suspension of cabbage phospholipase D acetone powder in buffer [0.2 M sodium acetate and 80 mM CaCl₂ (pH 5.6)] was added. The mixture was stirred vigorously overnight in a vessel sealed under argon. After the ether phase was evaporated under a stream of N₂, 0.2 volume of 0.5 M EDTA (pH 8.5) was added to the aqueous phase, which was then extracted twice with chloroform/methanol. The organic extract was dried down under a stream of N₂, and the product was purified by silica gel chromatography using chloroform/methanol/glacial acetic acid (65:15:2 by volume) as the solvent. Column fractions were monitored by TLC using the same solvent. [³H]SAPM (80 Ci/mol) and [³H]DOPM (60 Ci/mol) were made from the corresponding radiolabeled phosphatidylcholines using a scaled-down reaction and were purified by TLC using chloroform/methanol/glacial acetic acid (65:15:2 by volume) as the solvent.

**Synthesis of D-DOPM.** This phospholipid was synthesized from rac-1,2-dioleoylglycerol as follows. The following distillations were performed under N₂: tetrahydrofuran (from ketyl) and methanol and triethylamine (from CaH₂); POCl₃ was redistilled immediately before use. rac-1,2-Dioleoylglycerol in chloroform was dried with a stream of N₂ and placed in vacuo for 1 h just before use. The reaction was performed under argon in an oven-dried glass vial containing a small Teflon stir bar and fitted with a Teflon-lined septum cap. All additions were made by syringe. Tetrahydrofuran (160 µL), POCl₃ (18 µL), and triethylamine (40 µL) were added to the vial on ice. rac-1,2-Dioleoylglycerol (100 mg) in tetrahydrofuran was added dropwise with stirring. Triethylamine (40 µL) was added after 20 min, followed by excess methanol added dropwise at room temperature. After the reaction vial was placed in a 35 °C water bath and the mixture stirred for 40 min at 35 °C, a few milliliters of isopropyl ether were added. The solution was filtered, and the filtrate was washed with distilled water. The organic phase was dried by rotary evaporation, and the residue was placed in vacuo overnight. The residue was taken up in n-hexane, the suspension filtered, and the filtrate dried by rotary evaporation. Acetone (3 mL) and LiBr (35 mg) were added, and the solution was refluxed under argon for 6 h. The solution was dried down with a stream of N₂, and the residue was placed in vacuo for several hours. The enantiomer of DOPM was removed by PLₐ₃ digestion as follows. Ten milliliters of buffer [0.1 M Tris (pH 8.0) and 4 mM CaCl₂] containing 100 µg of bee venom PLₐ₃ (Sigma) was added, and the reaction mixture was stirred overnight. The reaction was complete as judged by TLC. Lipids were extracted with chloroform/methanol, and the organic phase was dried under a stream of N₂. D-DOPM was purified from the dried residue by silica gel chromatography using
chloroform/methanol/glacial acetic acid (65:15:2) as the eluant. The product migrated as a single spot on TLC ($R_f$ 0.4) using chloroform/methanol/glacial acetic acid (65:15:2) as the solvent. The proton NMR spectrum corresponded to the spectrum of commercially available L-DOPM. D-DOPM was stored in chloroform at −80 °C.

**Synthesis of 1-Oleoyl-2-[9,10-^3H]oleoyl-sn-glycero-3-phosphocholine.** 1-Oleoyl-sn-glycero-3-phosphocholine was prepared from DOPC (30 mg) by overnight digestion with 100 μg of Naja naja venom PL A 2 (Sigma) in 2 mL of buffer [50 mM Tris (pH 8.0) and 5 mM CaCl$_2$]. The reaction mixture was extracted three times with chloroform/methanol; the organic extracts were combined, and the solvent was removed under a stream of N$_2$. The product was purified by CM-cellulose column chromatography (Kates, 1986). After the column was washed with 10% (v/v) methanol in chloroform to remove oleic acid and DOPC, the lysophosphatidylcholine was eluted with 30% (v/v) methanol in chloroform. 1-Oleoyl-2-[9,10-^3H]oleoyl-sn-glycero-3-phosphocholine was synthesized from 1-oleoyl-sn-glycero-3-phosphocholine and [9,10-^3H]oleic acid as described for [^3H]SAPC (Hanel et al., 1993).

**Synthesis of GLU.** GLU was synthesized essentially as described (Huang et al., 1994). γ-Linolenic acid (250 mg) and 7-HC (178 mg) were placed in a round bottom flask along with 15 mL of methylene chloride and a stir bar. N,N-Dimethylformamide was added dropwise to dissolve the solids. (Dimethylamino)pyridine (99.4 mg) and dicyclohexylcarbodiimide (483 mg) were added, and the vessel was capped with a rubber septum and flushed with argon and the mixture stirred at room temperature for 24 h. The reaction mixture was washed three times with water, and the organic phase was dried by rotary evaporation. The residue was taken up in a small volume of low-boiling petroleum ether/ether/acetic acid (70:30:1 by volume) and applied to a flash column (1 × 8 in.) of silica gel 60 (EM Science) pressurized by N$_2$. Products were eluted with low-boiling petroleum ether/ether/acetic acid (70:30:1 by volume), and fractions were screened by TLC using the elution solvent. GLU-containing fractions ($R_f$ ≈ 0.2) were combined and dried by rotary evaporation. Chloroform was added, and the mixture was washed with several volumes of water. The chloroform layer was filtered and then dried down under a stream of N$_2$. A small amount of nonfluorescent solid was removed from the clear, oily product by dissolving the product in toluene and filtering through a 0.42 µm PTFE syringe filter. TLC of the product showed a trace amount of fluorescent substance with the same $R_f$ as 7-HC [petroleum ether/ether/acetic acid (70:30:1), $R_f$ for GLU = 0.36 and $R_f$ for 7-HC = 0.13; 20% ethyl acetate in petroleum ether, $R_f$ for GLU = 0.5 and $R_f$ for 7-HC = 0.125]. The 1H-NMR spectrum was identical to the previously published spectrum (Huang et al., 1994). Working stock solutions in chloroform were made and stored under argon at −80 °C.

**Formation of Vesicles.** Chloroform solutions of phospholipids were made by weight, and the concentrations were verified by phosphate analysis (Bartlett, 1959). PyAPC concentrations were determined by absorbance at 345 nm in ethanol using an extinction coefficient of 40 000 M$^{-1}$ cm$^{-1}$ (Bayburt et al., 1995). Phospholipid solutions were dried down in glass tubes under a stream of N$_2$, and the residues were placed in vacuo for at least 1 h. Typically, buffer [50 mM Tris (pH 8.0) and 150 mM NaCl] was added to the lipid film to give millimolar phospholipid concentrations, and the lipid was suspended by vortexing. SUVs were made from phospholipid suspensions using bath sonication (Laboratory Supplies Inc. model GI15SPIT instrument) until the solutions were almost clear (several minutes) (Jain & Gelb, 1991). LUVs were made by extrusion (10 passes) of the phospholipid suspension through two stacked 0.1 µm pore size polycarbonate filters using a Liposofast extruder (Avestin, Inc.). Extrusion of DMPC vesicles was performed at ~45 °C by immersing the extruder in a water bath. DMPC vesicle stock solutions were kept at the assay temperature. All vesicle stock solutions were kept under argon and were used the same day.

**Fluorescence cPLA$_2$ Assays.** Fluorescence cPLA$_2$ assays were performed using GLU (Huang et al., 1994; Bayburt et al., 1995). The assay buffer was 50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mM EGTA, 0.3 mM CaCl$_2$, and 30% glycerol (v/v) unless indicated otherwise. In most cases, GLU was added from a DMSO stock solution [final amount of DMSO in assay was 0.2% (v/v)] to vesicles in the assay buffer, which gives identical results compared to vesicles formed from a dried lipid film of phospholipid and GLU. Assays were performed at 30 °C (unless stated otherwise) using a Jasco 821-FP spectrofluorimeter equipped with a thermostated cell holder and a magnetic stirrer. Excitation was at 375 nm, and emission was at 460 nm with monochromator band widths of 18 nm. No time-dependent change in fluorescence signal was detectable at these wavelengths when 0.1 μg of cPLA$_2$ was added to 100 μM L-DOPM LUVs without GLU at the most sensitive settings used (a limit of detection of approximately 80 pM 7-HC). Background GLU hydrolysis rates were measured before enzyme addition and were subtracted from the cPLA$_2$-catalyzed rate. The background rate of GLU hydrolysis in 100 μM DOPM LUVs is 0.7 nM per minute per 0.01 mole fraction and increases linearly from 0.001 to 0.25 mole fraction GLU. The cPLA$_2$-catalyzed rate is ~26 nM/min in a typical 1 mL reaction mixture containing 100 μM DOPM/0.01 mole fraction GLU and 0.1 μg of cPLA$_2$ (40-fold higher than the background rate). Fluorescence was calibrated against standard curves made by adding known amounts of 7-HC to assay mixtures containing all components except enzyme. The slope of the calibration curve for 0–500 nM 7-HC in the absence of L-DOPM was found to be 0.6% lower than the slope in the presence of 100 μM L-DOPM, which is probably within the error of the measurement. Excess cPLA$_2$ was found to quantitatively hydrolyze GLU presented in the form of DOPM/GLU LUVs, on the basis of fluorescence. The cPLA$_2$-dependent release of γ-linolenate was confirmed by gas chromatography/mass spectrometry. Monoacylglycerol is known to be formed from ester substrates in the presence of glycerol (Hanel & Gelb, 1995), but this was not examined with GLU as the substrate.

**Test for Scooting.** LUVs composed of phospholipid mixtures to be tested were prepared and diluted to 600 μM or 1 mM total phospholipid. The chase LUVs had the same phospholipid composition, except for the presence of a small amount of radiolabeled substrate, and were also diluted to 600 μM or 1 mM phospholipid. Five microliters of the chase vesicles contained approximately 50 000 cpm of [^3H]SAPC (400 Ci/mol) or [^3H]HAPC (1300 Ci/mol). Five microliters of the LUVs to be tested as a scooting matrix was added to 45 μL of buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mM EGTA, 0.3 mM CaCl$_2$, and 30% (v/v) glycerol] in either
cPLA₂ in the Scooting Mode

silanized glass tubes or polypropylene microfuge tubes. After the mixture was vortexed briefly, cPLA₂ was added (typically 2 µL of a 0.05 mg/mL stock solution), and the solutions were gently mixed. After a specified time, 5 µL of the chase LUVs was added to each tube, and the contents of the tubes were mixed gently. For the zero time points, cPLA₂ was added last to a mixture of 5 µL of LUVs without radiolabel, 5 µL of chase LUVs, and 45 µL of buffer. The reactions were quenched after either 10 or 20 min and worked up as described previously (Ghomashchi et al., 1992).

cPLA₂ Inactivation Measurements. The activity of cPLA₂ after incubation with phospholipid vesicles was measured after disruption of vesicles with Triton X-100 and substrate or by polymyxin B-induced fusion of vesicles with substrate-containing vesicles. For activity measurements using Triton X-100 disruption, cPLA₂ (0.1 µg) was added to polypropylene microfuge tubes containing 1.1 mL of a solution of vesicles (100 µM phospholipid) in 50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mM EGTA, 0.3 mM CaCl₂, and 30% glycerol (v/v). After a specified time at 30 °C, 1 mL was placed in a glass fluorescence cuvette followed by addition of 0.1 mL of a mixture of 0.26% (w/v) Triton X-100, 200 µM DTPM, and 30 µM GLU in deionized water. Initial reaction velocities were measured as described above for the fluorescence assays. Zero-time point measurements were obtained by placing 1.1 mL of buffer containing 100 µM phospholipid in the cuvette followed by 0.11 mL of the Triton X-100/DTPM/GLU mixture and starting the reaction by addition of 0.1 µg of cPLA₂. Blanks containing no enzyme were measured for background GLU hydrolysis rates. For measurement of activity using polymyxin B-induced vesicle fusion, reactions were performed as for trapping assays, with the addition of 2 µL of 0.625 mg/mL polymyxin B sulfate (in water, Sigma) immediately after chase vesicles were added. Polymyxin B (25 µg/mL) was found to induce complete mixing of L-DOPM/N-(7-nitro-2-benzoxa-1,3-diazol-4-yl)dipalmitoylphosphatidylethanolamine and L-DOPM/N-(lissamine rhodamine B sulfonfonyl)-dipalmitoylphosphatidylethanolamine LUVs as measured by resonance energy transfer (Düzgünes et al., 1987).

Partitioning of GLU into L-DOPM LUVs. Partitioning of GLU was measured by centrifugation of sucrose-loaded LUVs (Buser et al., 1994; Ghomashchi et al., 1995). Two hundred eighty micrograms of L-DOPM and 2.2 µCi of 1-palmitoyl-2-[9,10-³H]palmitoyl-sn-glycero-3-phosphocholine (55 Ci/mmol) were dried down under a stream of N₂, placed in vacuo for 1 h, and resuspended in 75 µL of 50 mM Tris/HCl (pH 8.0), 180 mM sucrose, and 60 mM NaCl, and LUVs were formed as described above. Polyallylamine microfuge tubes (1.5 mL, Beckman) were treated with Sigmaticote (Sigma, according to the manufacturer’s directions). Varying amounts of LUVs were added to tubes containing 1 mL of centrifugation buffer [50 mM Tris/HCl (pH 8.0) and 150 mM NaCl]. The volume was adjusted to 1.01 mL with the LUV sucrose buffer and 1 µL of 50 µM GLU added to each tube from a stock solution in DMSO, and the contents of the tubes were mixed by inverting several times. Samples were centrifuged for 1.5 h at 30 °C at 30 000 rpm (75 000g average) in a Kompasin KA-30 rotor. The supernatant (0.8 mL) was transferred to glass tubes, and the remaining buffer and pellet were vortexed for 30 s. One hundred microliters of the supernatant and 20 µL of the resuspended pellet were transferred to scintillation vials and counted. Ninety percent or more of the counts added to each tube were pelleted. The supernatant (0.7 mL) and the resuspended pellet (170 µL) were assayed for GLU as follows. Centrifugation buffer was added to the pellet fraction to give a total volume of 0.7 mL, and DOPM LUVs (without 1-palmitoyl-2-[9,10-³H]palmitoyl-sn-glycero-3-phosphocholine) were added to each tube for a total of 17 nmol per tube (including the DOPM pellet, pelleting was assumed to be quantitative). Buffer (0.4 mL) [50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mM EGTA, 0.3 mM CaCl₂, and 30% glycerol (v/v)], 10 µL of 10% (w/v) Triton X-100, and 0.1 mL of 1 N NaOH were added to each tube, and the tubes were vortexed. After 5 min, fluorescence was measured against a blank. Fluorescence was calibrated with known amounts of 7-HC as described above. GLU hydrolysis was found to be quantitative in 2–3 min using this procedure. The amounts of GLU in the supernatant and pellet were corrected for the phospholipid adsorbed to the walls of the tubes and unpelleted phospholipid as described previously (Ghomashchi et al., 1995).

Light Scattering. Light scattering measurements were taken on a Perkin-Elmer 650 fluorimeter using excitation and emission wavelengths of 650 nm (Bazzi et al., 1992) with monochromator band widths of 5 nm. Twenty microliters of 10 mM L-DOPM LUVs was added to buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mM EGTA, 0.3 mM CaCl₂, and 30% glycerol (v/v)], followed by the addition of 4 µL of 0.1 mg/mL cPLA₂. Scattering due to buffer alone was subtracted from scattering in the presence of vesicles and enzyme. No time-dependent change in light scattering was observed for buffer, vesicles, or enzyme alone.

Pyrene Fluorescence. Fluorescence emission spectra of L-DOPM/PyAPC (SUVs) and DMPM/PyAPC (LUVs) in 50 mM Tris (pH 8.0), 150 mM NaCl, 0.3 mM CaCl₂, 0.2 mM EGTA, and 30% (v/v) glycerol were taken on a Perkin-Elmer 650 fluorimeter using excitation at 348 nm with 2 nm excitation and 5 nm emission bandwidths. The temperature was held constant using a thermostated cuvette holder. After subtraction of background fluorescence of blanks containing buffer and vesicles (0 mole fraction PyAPC), excimer to monomer fluorescence ratios were measured from spectra by measuring the peak height at 398 and 486 nm for monomer and excimer, respectively. The concentrations of PyAPC were in the linear range of fluorescence intensity at both wavelengths.

Preparation of Dephosphorylated and MAP Kinase-Phosphorylated cPLA₂. To avoid degradation of cPLA₂, phosphatase was treated with protease inhibitors and protease inhibitors were present in reaction mixtures containing phosphatase. Potato acid phosphatase ammonium sulfate suspension (type III, Sigma) was pelleted, dissolved in 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 100 mM NaCl, 4 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM Na₅p-tosyl-L-lysine chloromethyl ketone, and 50 µg/mL N-tosyl-L-phenylalanine chloromethyl ketone, and left on ice for 30 min. The phosphatase was then treated with α₂-macroglobulin gel (Boehringer), which had been washed with 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 100 mM NaCl, and 4 mM MgCl₂, by transferring the phosphatase to a polypropylene microfuge tube containing a pellet of α₂-macroglobulin gel (100 µL per 100 units of phosphatase) and stirring at room temperature for 30 min. The α₂-macroglobulin-treated phosphatase was applied to a spin column of P6-DG desalting gel (Bio Rad) equilibrated with 200 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5),
150 mM NaCl, 4 mM MgCl₂, and 2 mM DTT. Dilutions of the phosphatase were added to 1 mL of 0.1 M sodium acetate (pH 4.8) and 5 mM p-nitrophenyl phosphate for determination of phosphatase activity. After 30 min at 37°C, 0.1 mL of 0.5 M NaOH was added to each assay tube, and the absorbance was measured at 405 nm. Phosphatase units of activity were calculated using an extinction coefficient for p-nitrophenol of 1.8 × 10⁵ M⁻¹ cm⁻¹, where 1 unit hydrolyzes 1 µmol of p-nitrophenyl phosphate per minute. The following conditions were used for dephosphorylation of cPLA₂: To provide cPLA₂ containing less than 0.1 µmol phosphate per enzyme on the basis of phosphoserine analysis (Niedbalski & Ringer, 1986) using a recombinant cPLA₂ preparation containing approximately 50% phosphoserine 505 cPLA₂. To 25 µL of cPLA₂ [0.25 mg/mL in 50 mM Tris (pH 8.0), 0.3 M NaCl, 1 mM EDTA, 1 mM EGTA, 30% ethylene glycol, 40 µg/mL aprotinin, and 40 µg/mL leupeptin] was added 25 µL of 0.01 unit/µL potato acid phosphatase in 200 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 2 mM DTT, and 4 mM MgCl₂. After 30 min at 30°C, 20 µL aliquots of the reaction mixture were transferred to two fresh tubes, and 20 µL of MAP kinase [7.5 µg/mL in 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30% ethylene glycol, 40 µg/mL aprotinin, and 1.2 mM sodium orthovanadate] was added to each tube. The MAP kinase preparation had an activity of 0.544 µmol min⁻¹ mg⁻¹ toward myelin basic protein in the assay described previously (Ahn et al., 1990). Two microliters of 4 mM ATP was added to one tube to generate phosphoserine 505 cPLA₂ from the dephosphorylated cPLA₂, and water was added to the other tube. Both tubes were incubated at room temperature for 1.5 h. Approximately 90% of the phosphatase-treated cPLA₂ was dephosphorylated at serine 505, and the kinase reaction was approximately 90% complete on the basis of the band shift observed on SDS-PAGE gels across the appropriate bands of Coomassie-stained SDS-PAGE gels. Fractions were collected every 0.2 min. Activity was determined in the Triton X-100 mixed micelle assay described below. Active fractions were pooled, concentrated to 1 mL using a Centriprep 30 apparatus (Amicon), diluted to 14 mL of buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA], and concentrated to 1 mL. This process was repeated twice more, resulting in >3000-fold reduction in phosphate with quantitative recovery of protein.

**RESULTS**

**Trapping of cPLA₂ on Phosphatidylmethanol-Containing Vesicles.** Previously, cPLA₂ has been shown to bind to vesicles composed of the anionic phospholipid DMPM in preference to *Escherichia coli* membranes (Diez et al., 1992) and to be trapped on DMPM vesicles (Witmer et al., 1995). The kinetic test for trapping (very slow dissociation of enzyme from vesicles) utilized in the present study involves chasing the vesicle-bound enzyme with radiolabeled substrate vesicles. Reversible binding, with a preference for binding to the vesicles being tested, rather than trapping can be ruled out by using chase vesicles of the same phospholipid on anion-exchange HPLC. A 0.48 × 10 cm HPLC column of Poros HQ 20 µm media (Perseptive Biosystems) was run on a Rainin HPLC apparatus (titanium with peek tubing) at room temperature at 10 mL/min. The Poros column was equilibrated with buffer [50 mM Tris buffer (pH 8.0)]. Hydroxylapatite-purified cPLA₂, dephosphorylated as described above, was loaded. The following gradient program was run after loading: 125 mM NaCl in buffer (0 to 3 min), a linear gradient from 125 to 250 mM NaCl in buffer (3 to 3.2 min), 250 mM NaCl in buffer (3.2 to 5 min), and a linear gradient from 250 to 1000 mM NaCl in buffer (5 to 10 min). Fractions were collected every 0.2 min. Activity was determined in the Triton X-100 mixed micelle assay described below. Active fractions were pooled, concentrated using a Centriprep 30 (Amicon) apparatus, and placed at −20°C for storage. Purity was estimated to be ~98% on the basis of Coomassie-stained SDS-PAGE gels.

**Triton X-100 Mixed Micelle Assay.** To make 100× micelle stock, GLU and DTMP were dried down together from organic stock solutions under a stream of N₂ and placed in vacuo for 1 h. Triton X-100 [as a 10% (w/v) stock in water] and enough water were added to give 40 mM Triton X-100 (assuming a MW of 646), 2 mM DTMP, and 300 µM GLU. This micelle stock solution was made the day of use and used within several hours. The micelle stock and assay buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.3 mM CaCl₂, 0.2 mM EGTA, and 30% glycerol] were mixed together just before use. The complete reaction mixture (1 mL) was placed in disposable glass tubes and an aliquot of the cPLA₂ column fraction added. A blank reaction was also made up using the column buffer. After 30 min at room temperature, the fluorescence was read within 2–3 min against the blank (excitation at 375 nm and emission at 460 nm).

**RESULTS**

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composition as the test vesicles (Hanel et al., 1993). An experiment was performed to test for trapping on L-DOPM vesicles in which the enzyme was allowed to bind to L-DOPM vesicles for an initial time period followed by addition of chase L-DOPM vesicles containing a trace amount of radiolabeled substrate. Release of radiolabeled arachidonate was measured in a second time period. With L-DOPM LUVs, the presence of 150 mM NaCl along with 30% glycerol provides an approximately 20-fold increase in the initial rate and rates at any time during or after the fast initial phase of hydrolysis (defined below) (Bayburt et al., 1995). Therefore, trapping experiments were performed in the presence of glycercol and NaCl.

Figure 1 shows that a 5 s incubation of cPLA2 with L-DOPM LUVs (circles) is sufficient to inhibit the release of arachidonic acid from chase vesicles by 90%. The presence of 0.04 mole fraction [3H]SAPC in the chase vesicles does not affect this result, since the presence of SAPC in the trapping vesicles (up to 0.5 mole fraction) gave identical results in experiments performed as described in Materials and Methods (not shown). Incomplete inhibition of arachidonic acid release after incubation of cPLA2 with L-DOPM vesicles was interpreted as slow hopping of enzyme from L-DOPM to L-DOPM/[3H]SAPC vesicles over the time of the chase. As expected, a longer chase period (20 min) results in less inhibition (squares). Thus, there is a rapid and slowly reversible binding of cPLA2 to L-DOPM LUVs. Similar results were obtained in experiments performed as described in Materials and Methods with L-DOPM SUVs (not shown).

Tight binding of cPLA2 to phosphatidylmethanol vesicles may be partly due to the negative charge of this phospholipid (Leslie & Channon, 1990; Diez et al., 1992; Ghomashchi et al., 1992; Witmer et al., 1995). The fact that little inhibition of arachidonate release from chase vesicles occurs after incubation of cPLA2 with SUVs of the zwitterionic phospholipid DOPC (Figure 1, triangles) is consistent with this hypothesis. One of the products of L-DOPM hydrolysis, oleic acid, is negatively charged and could play a role in enzyme trapping on L-DOPM vesicles. But the reaction rate per enzyme for cPLA2-catalyzed L-DOPM hydrolysis using pure [3H]DOPC vesicles is 0.004 s⁻¹, indicating that only 0.02 product per enzyme is formed during the binding period of 5 s shown in Figure 1.

The enzyme was also found to be trapped on DMPM SUVs at room temperature using the trapping test described in Materials and Methods, but only in the absence of 150 mM NaCl (data not shown). Increasing the salt concentration of the buffer used to test for trapping (see Materials and Methods) results in faster dissociation times from DMPE vesicles at room temperature, indicating that electrostatic enzyme-vesicle interaction is involved in trapping. Equilibration among DMPE test and chase vesicles occurs rapidly at 150 mM NaCl (less than 10 min). The dissociation half-time with L-DOPM LUVs in the presence of 150 mM NaCl, on the other hand, is on the order of 30 min on the basis of the data of Figure 1. Reactions using DMPE vesicles without salt were not pursued further because under these conditions apparent enzyme inactivation occurs rapidly [essentially complete inactivation within 10 min after the addition of cPLA2 to DMPE SUVs in buffer containing 30% (v/v) glycerol at pH 8.0 and 30 °C, not shown], thus making kinetic studies difficult.

**Apparent Inactivation by Phosphatidylmethanol.** Enzyme inactivation or inhibition during the reaction time course was a concern because the extent of [3H]SAPC hydrolysis in L-DOPM/[3H]SAPC vesicles was lower than expected. The scooting model predicts complete hydrolysis of substrate present in the outer monolayer of vesicles containing a trapped enzyme. In a 50 μL reaction mixture in buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mM EGTA, 0.3 mM CaCl₂, and 30% glycerol (v/v)] at room temperature containing 100 mM L-DOPM/0.025 mole fraction [3H]SAPC (400 Ci/mol) LUVs and 0.15 μg of cPLA2 with an average of 36 enzymes per LUV, on the basis of the size of LUVs of 100 000 phospholipids, [3H]SAPC hydrolysis approached only 3.5% of the predicted 50% hydrolysis (not shown). This observation suggests enzyme inactivation or inhibition. Experiments were performed to determine whether loss of enzyme activity occurs over the time course of hydrolysis. In these experiments, cPLA2 was incubated with vesicles followed by the addition of Triton X-100, DTPM, and GLU. DTPM was included because negative charge appears to be required for high rates of GLU hydrolysis in Triton X-100 mixed micelles. It was assumed that detergent induces mixing of all lipid components, allowing the enzyme access to the GLU substrate. Reaction rates in this Triton X-100 mixed micelle assay system are linear over many minutes, and the initial GLU hydrolysis rate was used as a measure of remaining cPLA2 activity. The loss of activity over time on L-DOPM LUVs (Figure 2A, squares) is biphasic with a fast phase having a rate constant of 0.056 s⁻¹. The rate constant for the slow phase of loss of activity measured at longer times is 3.1 × 10⁻⁴ s⁻¹. Similar inactivation time courses were obtained by measuring residual rates ([3H]-arachidonate formation) after addition of polymyxin B and L-DOPM/[3H]HAPC LUVs (Figure 2B). Polymyxin B is known to promote phospholipid exchange at low concentrations or fusion when used at >0.05 mole fraction (Jain et al., 1991a,b; Cajal et al., 1995, 1996). The amount of polymyxin B used in the assays was >0.05 mole fraction, which was found to cause complete mixing of vesicles containing fluorescent probes (see Materials and Methods). The rate constants of the fast and slow phases measured using polymyxin B-induced fusion are 0.066 and 2.6 × 10⁻⁴ s⁻¹, respectively. Similar kinetics for the loss of activity were also seen in the initial rate of GLU hydrolysis when 2 μM
GLU (from a DMSO stock solution) was added at various times after addition of 0.11 µg of cPLA$_2$ to 100 µM L-DOPM LUVs in 1.1 mL of the assay buffer described in Materials and Methods (not shown). At the indicated times, Triton X-100 was added to disrupt the vesicles to give mixed micelles. GLU and DTPM were added along with Triton X-100 so that the enzymatic activity could be measured. Enzymatic activity is expressed as the percentage of activity of the enzyme added directly to the mixed micelle assay. (B) The percent remaining active enzyme as a function of time in the presence of L-DOPM was measured by inducing fusion between L-DOPM vesicles and L-DOPM/[³H]HAPC vesicles with polymyxin B (see Materials and Methods).

GLU (from a DMSO stock solution) was added at various times after addition of 0.11 µg of cPLA$_2$ to 100 µM L-DOPM LUVs in 1.1 mL of the assay buffer described in Materials and Methods (not shown). A slow loss of activity is observed over 5 min in the presence of DOPC LUVs (Figure 2A, triangles); an estimated rate constant of $1.3 \times 10^{-4}$ s$^{-1}$ was obtained from measurements at longer times. Vesicles composed of 0.5 mole fraction L-DOPM in DOPC are able to trap cPLA$_2$ in the trapping test described in Materials and Methods. However, the apparent inactivation shows biphasic kinetics identical to those using pure L-DOPM vesicles when activity was measured by disruption of vesicles with Triton X-100. Dissociation is more rapid with 0.25 mole fraction L-DOPM in DOPC vesicles in the trapping test described in Materials and Methods (not shown).

Reaction time courses using GLU as substrate could be dominated by loss of enzyme activity and not by substrate depletion, in some cases, because GLU can exchange rapidly among vesicles (see below). Shown in Figure 3A is the reaction time course for cPLA$_2$ acting on L-DOPM/GLU LUVs (solid line) and its first derivative (circles). Figure 3B shows the plots at early time points. The first-derivative plot (rate as a function of time) emphasizes the biphasic nature of the reaction time course. The fast initial phase has a rate constant of 0.077 s$^{-1}$, consistent with the fast loss of activity measured in Figure 2A,B. The slow phase of decreasing rate has a rate constant of $6.5 \times 10^{-4}$ s$^{-1}$, slightly larger than the values measured from Figure 2A,B. GLU hydrolysis rates using DOPC/GLU LUVs also decrease substantially, well before a large proportion of GLU in the assay has been hydrolyzed. The fast initial phase of product formation and decreasing activity observed with DOPM vesicles is not observed with DOPC vesicles (Figure 3B, dashed line and open squares; Figure 2A, triangles). Instead, a slow decrease in rate is seen. The estimated rate constant for the decrease in the rate of GLU hydrolysis over time on DOPC LUVs is $5.4 \times 10^{-4}$ s$^{-1}$, which is similar to the slow phase of decreasing rate on L-DOPM LUVs but larger than the rate constant of $1.3 \times 10^{-4}$ s$^{-1}$ measured by the method of Figure 2A.

The possibility of inactivation due to a protease or an interfering protein contaminant in the cPLA$_2$ preparation was considered. No loss of band intensity or appearance of proteolysis products was detected on Coomassie-stained gels of cPLA$_2$ after incubation of cPLA$_2$ for 1 h with L-DOPM.
LUVs in assay buffer. Furthermore, no difference in the kinetics of decreasing rate was observed in the L-DOPM/GLU hydrolysis time course in the presence or absence of protease inhibitors (5 µg/mL leupeptin, 5 µg/mL aprotinin, 1 µg/mL pepstatin, and 0.125 mM phenylmethanesulfonyl fluoride). A similar time-dependent decrease in rate was also observed using cPLA₂ preparations further purified by hydroxylapatite and anion-exchange HPLC (see Materials and Methods). Inactivation due to the presence of a protease or interfering contaminant in the cPLA₂ preparation is unlikely. Biphasic loss of activity due to the presence of different forms of enzyme, such as phosphorylated versus unphosphorylated cPLA₂, is also unlikely because the kinetics of the apparent inactivation are the same for dephosphorylated and MAP kinase-phosphorylated cPLA₂. Also, the extent of the loss of activity during the fast initial phase on HOPM differs from the extent of the loss on L-DOPM during the fast initial phase, which argues against two forms of enzyme in the preparation that inactivate at different rates.

An abrupt increase in light scatter was seen when cPLA₂ was added to L-DOPM LUVs (8.9% of the scattering intensity of vesicles alone). A small amount of time-dependent change in light scattering was also seen (2% of the scattering intensity of vesicles alone) that stabilized after ~100 s. From the time dependence of this process, it is tempting to speculate that the fast phase of apparent inactivation is due to formation of an enzyme–vesicle aggregate. However, this phenomenon was not studied further.

The loss of activity on L-DOPM vesicles occurs on a time scale much greater than that of trapping of cPLA₂ on L-DOPM vesicles. Trapping occurs in less than 5 s (Figure 1, circles), while more than 80% of the enzyme activity remains at 20 s in the inactivation experiment (Figure 2A, squares). The inhibition of hydrolysis of L-DOPM/[³H]-SAPC chase vesicles is mainly the result of trapping of active enzyme on L-DOPM vesicles rather than enzyme inactivation over the binding period. The fact that enzyme catalyzes the hydrolysis of many substrates without leaving the vesicle indicates that it is acting in the processive scooting mode (Jain et al., 1986).

**Vesicle Partitioning and Exchange of GLU Substrate.**

cPLA₂-catalyzed hydrolysis rates were measured in the presence of increasing concentrations of LUVs composed of L-DOPM/0.02 mol fraction GLU. A 30–60 s lag in the rate of GLU hydrolysis occurred at low phospholipid concentrations (less than 20 µM phospholipid), presumably due to time-dependent binding of cPLA₂ to vesicles at low vesicle concentrations. The lags at the concentrations of vesicles used in the assays are consistent with a second-order rate constant of ~3 × 10⁹ M⁻¹ s⁻¹ which is near the values reported for the nearly collisional-limited binding of annexin to LUVs (Lu et al., 1995). The rates of cPLA₂-catalyzed GLU hydrolysis were measured after the lag. A plot of the rate versus L-DOPM/GLU concentration gave hyperbolic curves with half-maximal rates at 4 µM L-DOPM (Figure 4, circles). Unless binding sites are limiting, these results are not due to an increasing concentration of vesicle-bound enzyme since it was already shown that cPLA₂ binds tightly to L-DOPM vesicles. On the basis of the estimates for binding and dissociation rates using the data of Figure 1, the upper limit for the dissociation constant calculated using the molar L-DOPM concentration is 50 nM. If binding sites are limiting, the curves would reflect titration of enzyme with binding sites on the vesicles. In the experiments shown in Figure 4, however, concentrations of L-DOPM required for half-saturation of the rate with 0.02 µg (circles) and 0.1 µg of cPLA₂ (diamonds) are similar (4 and 7 µM, respectively). Furthermore, saturation of the rate occurs at a large lipid to enzyme ratio (85 000 in the case of 0.02 µg of cPLA₂), suggesting that the accessibility of the membrane-binding surface is not rate-determining.

Another explanation for the results shown in Figure 4 is that GLU does not partition fully into vesicles of L-DOPM at the lower concentrations of vesicles. To test this, GLU solvsolysis rates were measured in buffer at pH 9.0 in the presence and absence of L-DOPM vesicles. The hydrolysis rate in the presence of increasing amounts of L-DOPM approaches a small proportion of the rate in the absence of L-DOPM (Figure 5, triangles). This behavior may reflect partitioning of GLU between the aqueous and lipid phases, in which hydrolysis rates differ. Half-partitioning occurs at 1 µM L-DOPM, which is similar to the L-DOPM concentration required for half-maximal cPLA₂-catalyzed rates. A more direct measure of partitioning of GLU into L-DOPM LUVs was obtained after separation of aqueous and phospholipid components by centrifugation (Figure 5, squares).
In the absence of L-DOPM, most of the GLU was found on the walls of the centrifuge tubes; the recovery of GLU from the aqueous phase was only 3%. The amount of GLU found associated with phospholipid approaches the total amount of GLU present as the concentration of L-DOPM is increased. The curve in Figure 5 can be fit to a hyperbola, giving a concentration for half-partitioning of GLU at 9.7 µM L-DOPM. These results strongly suggest that the hyperbolic curve of Figure 4 for the cPLA₂-catalyzed rate is caused by a hyperbolic increase in the vesicle-bound concentration of GLU that trapped cPLA₂ "sees".

Several observations indicate that GLU exchanges rapidly between vesicles. GLU hydrolysis attains a maximal rate at the earliest measurable time (a few seconds) after addition of 100 µM DOPM/0.01 mole fraction GLU to 0.2 µg of enzyme trapped on 100 µM L-DOPM LUVs in assay buffer at 30 °C (not shown). Hydrolysis of GLU also begins rapidly and without a lag when 2 µM GLU is injected from a stock solution in DMSO into a solution of 2 µg of cPLA₂ bound to 60 µM L-DOPM LUVs in 1 mL of assay buffer at 30 °C (not shown). These results indicate that dissociation and association of GLU with phospholipid bilayers occur rapidly on the time scale of enzyme-catalyzed hydrolysis.

**Application of Michaelis-Menten Kinetics.** Reaction kinetics for interfacial enzymes such as PLA₂ have been shown to obey the Michaelis-Menten formalism (Berg et al., 1991). The Michaelis-Menten parameters for enzyme associated with the bilayer interface are denoted by an asterisk. The Michaelis constant, $K_M^*$, and substrate concentration are expressed in units of mole fraction. Only enzyme bound to the interface is catalytically competent, and the use of scooting mode assays avoids complications due to the enzyme-vesicle binding equilibrium. Therefore, it is possible that values of $K_M^*$ and the interfacial maximal velocity, $V_m^*$, may be obtained by measuring the dependence of the reaction rate on the mole fraction of a given substrate in L-DOPM vesicles. $K_M^*$ values for enzymes that utilize phospholipids as substrates are usually apparent values ($K_M^{app}$) because the active site of the enzyme may have some affinity for the diluent phospholipid used to vary the mole fraction of the substrate.

An attempt was made to measure $K_M^{app}$ and $V_m^*$ values for GLU and [³H]HAPC using L-DOPM as the diluent phospholipid in a scooting mode assay. Figure 6A shows the dependence of the initial rate of GLU hydrolysis on the mole fraction of GLU in L-DOPM LUVs (closed circles). Inactivation is of no concern in these experiments as long as it is not dependent on the mole fraction of GLU. Rates were measured at early time points when the active enzyme concentration is close to the initial value (from approximately 0 to 10 s), which would attenuate the possible effect of GLU concentration on inactivation. Similar curves were obtained by measuring the rate after the fast initial phase of inactivation, indicating that altering the mole fraction of GLU does not change the inactivation kinetics. The curve can be fit to a hyperbola with $K_M^{app} = 0.09 \pm 0.02$ mole fraction and $V_m^*$ of 2600 ± 300 nmol min⁻¹ mg⁻¹. Values for $K_M^{app}$ of 0.06 mole fraction GLU and $V_m^*$ of 470 nmol min⁻¹ mg⁻¹ were obtained using highly purified dephosphorylated cPLA₂, suggesting that potential interfering protein contaminants in the cPLA₂ preparation do not significantly affect these results. The values of $K_M^{app}$ of 0.11 ± 0.04 mole fraction and $V_m^*$ of 830 ± 160 nmol min⁻¹ mg⁻¹ were obtained for the ester of arachidonic acid with 7-HC. Results of the analogous experiment with [³H]HAPC as substrate in L-DOPM LUVs are shown in Figure 6B. In contrast to the results with GLU, the reaction rate is linear up to the maximum mole fraction tested, establishing that in L-DOPM vesicles the $K_M^{app}$ for HAPC is greater than 0.15 mole fraction. Similar results were obtained with 100 µM [³H]SAPC/L-DOPM LUVs in the same assay at room temperature (not shown).

**Effect of MAP Kinase Phosphorylation.** Phosphorylation of cPLA₂ on serine 505 by MAP kinase has previously been shown to cause a small increase in activity (Lin et al., 1993; Nemenoff et al., 1993). In those studies, pure phosphatidylcholine vesicles or Triton X-100/phosphatidylcholine mixed micelles were used as substrate. Recombinant cPLA₂ expressed in insect cells is extensively phosphorylated at multiple sites (Becker et al., 1994; Abdullah et al., 1995; de Carvalho et al., 1996). Therefore, cPLA₂ was nearly quantitatively dephosphorylated in vitro by treatment with potato acid phosphatase and nearly quantitatively phosphorylated on serine 505 by treatment with MAP kinase. The activity of cPLA₂ phosphorylated at serine 505 was compared to the activity of dephosphorylated cPLA₂ in the scooting mode using L-DOPM/[³H]HAPC LUVs. Serine 505-phosphorylated enzyme gives a 30% larger rate of [³H]HAPC hydrolysis compared to dephosphorylated enzyme when measured using L-DOPM/[³H]HAPC vesicles in the scooting mode assay. Therefore, this modest increase in activity is...
not due to a change in the equilibrium binding of enzyme to vesicles or micelles, to an artifact due to differences in trapping and substrate depletion in assays using pure phosphatidylcholine vesicles, or to a change in the rate of exchange of enzyme between mixed micelles (Jain et al., 1993). Inspection of reaction progress curves using L-DOPM/GLU LUVs as the substrate revealed no differences in inactivation rates for either form of the enzyme.

**Kinetics Using Other Phosphatidymethanol Vesicles.** For rigorous kinetic studies of lipolytic enzymes, a diluent phospholipid that has little, if any, affinity for the catalytic site of the vesicle-bound enzyme is desirable. $K_{M,*}$ for substrates, rather than $K_{M,app,*}$, can be obtained with such a diluent. Such a lipid has been termed a “neutral diluent” by Jain and co-workers (Jain et al., 1991a,b). In order to gauge whether L-DOPM is a neutral diluent, hydrolysis of substrates dispersed in vesicles of other phosphatidymethanols was measured. Differences in the $K_{M,*}$ (or $K_{d,*}$) values between the different phosphatidymethanols could result in differences in the rates of hydrolysis of [H]SAPC or GLU present in vesicles of the phosphatidymethanols. All phosphatidymethanols, with the exception of DMPM (at room temperature), were found to trap cPLA2 on the basis of the trapping experiment described above in buffer containing 150 mM NaCl and 30% (v/v) glycerol.

D-DOPM was selected as a candidate neutral diluent since it was found that d-phospholipids are not hydrolyzed by cPLA2 and thus may have poor affinity for the enzyme’s active site. The hydrolysis of d-[H]HAPC present at 0.08 mole fraction in L-DOPM SUVs was found to be 0.2 ± 0.8% of the activity toward L-DOPM/0.08 mole fraction [H]-SAPC. Likewise, the rate of hydrolysis of pure d-[H]HAPC vesicles was found to be 0.2% of that of pure [H]SAPC vesicles. Surprisingly, progress curves obtained with [H]-SAPC as substrate in D-DOPM (Figure 7A, diamonds) or in L-DOPM (closed circles) SUVs were found to be similar. Identical results were obtained with D- and L-DOPM/GLU LUVs (Figure 7B).

[ [H]SAPC SUVs are hydrolyzed 300 times faster than [H]DOPM SUVs (0.9 versus 0.003 μmol min$^{-1}$ mg$^{-1}$) using pure substrate vesicles. The specificity for SAPM may be due to a smaller $K_{M,*}$, a larger $k_{cat,*}$, or a combination of both. In contrast to the hyperbolic dependence of the velocity of GLU hydrolysis on the mole fraction of GLU in L-DOPM SUVs (Figure 6A, closed circles), a linear dependence is seen for GLU/SAPM SUVs (Figure 6A, open circles). This suggests that SAPM has a higher affinity than L-DOPM for the active site of vesicle-bound cPLA2, resulting in a larger $K_{M,app,*}$ for GLU. However, the values of $k_{cat,*}/K_{M,app,*}$ for GLU, estimated from the initial portions of the curves, only differ by 2-fold.

DMPM is not detectably hydrolyzed at either the sn-1 or sn-2 position (Diez et al., 1992). Use of DMPM as the diluent phospholipid gave a $K_{M,app,*}$ for GLU of approximately 0.28 mole fraction and a $V_{max,*}$ of approximately 1300 nmol min$^{-1}$ mg$^{-1}$ at 30 °C. At 40 °C, there was no evidence of saturation in the rate with increasing GLU up to 0.1 mole fraction using 100 μM DMPM LUVs and 0.2 μg of cPLA2 in 1 mL of the assay buffer described in Materials and Methods (rate of GLU hydrolysis = 100 nmol min$^{-1}$ mg$^{-1}$ at 0.1 mole fraction GLU) (not shown). In these experiments, 150 mM NaCl was included to prevent rapid enzyme inactivation (see above). The apparent variation in $K_{M,app,*}$ with temperature using DMPM vesicles will be developed in more detail below.

Three explanations for near-identical rates of GLU or SAPC hydrolysis using D-DOPM, SAPM, DMPM, and L-DOPM vesicles were considered. The first is that the interfacial $K_{M,*}$’s or $K_{d,*}$’s (for nonsubstrates) for all four phosphatidymethanols are approximately the same. The second possibility is that the interfacial $K_{M,*}$’s (or $K_{d,*}$’s) for phosphatidymethanols are large compared to unity, and thus, most of the vesicle-bound cPLA2 does not contain phosphatidymethanol in its active site. In such a case, the effect on GLU or [H]SAPC hydrolysis rates due to differences in the $K_{M,*}$ (or $K_{d,*}$) values for the different phosphatidymethanols would not be observed. The third possibility is that lateral separation of substrate is occurring and that enzyme is binding to a segregated domain of substrate and does not “see” the diluent phospholipid. The curves shown in Figure 7 were obtained using high-specific activity [H]SAPC at a L-DOPM/[H]SAPC ratio of 40 000. Therefore, most vesicles have no [H]SAPC, and some have at most one [H]SAPC molecule, establishing that segregation of the radioactive substrate cannot explain the similarity of the curves in the case of D- and L-DOPM. Nonideal mixing has not been ruled out in the other cases, but segregation of substrate at low-mole fraction substrate and above the transition temperature of the diluent phospholipid seems unlikely (however, see the studies below with DMPM).
Hydrolysis of Substrates Present in DMPM LUVs. In light of the observed linear dependency of the velocity for the hydrolysis of \(^{3}H\)HAPC in L-DOPM vesicles (Figure 6B), we were intrigued by reports that \(K_{M,app}^{*}\) values of less than 0.01 mole fraction for \(sn\)-2-arachidonyl-containing phospholipids such as HAPC could be measured using vesicles of DMPM (Diez et al., 1992; Burke et al., 1995). Therefore, we carried out a careful kinetic analysis using DMPM/HAPC LUVs. If saturation of the active site of cPLA\(_2\) bound to DMPM LUVs by concentrations of HAPC near 0.01 mole fraction occurs, increasing amounts of nonradiolabeled HAPC from 0 to 0.12 mole fraction in an assay consisting of DMPM LUVs containing a fixed amount of \(^{3}H\)HAPC should inhibit the release of \(^{3}H\)arachidonic acid. This experiment was performed above (Figure 8A, open circles, 37 °C) and near the main transition temperature of DMPM (Figure 8A, closed circles, 30 °C) [29 °C (Jain et al., 1986)]. At 30 °C, increasing the mole fraction of HAPC causes inhibition of \(^{3}H\)arachidonic acid release, consistent with a \(K_{M,app}^{*}\) for HAPC of approximately 0.02 mole fraction. At 37 °C, the inhibition is much less dramatic. Figure 8B shows the calculated rate of total arachidonic acid release (labeled and unlabeled) as a function of the mole fraction of total HAPC.

**Figure 8:** Substrate competition in DMPM LUVs. (A) Inhibition of the release of \(^{3}H\)arachidonic acid from \(^{3}H\)HAPC (290 Ci/mol) present in 200 \(\mu\)M DMPM/\(^{3}H\)HAPC/HAPC LUVs containing 0.01 mole fraction \(^{3}H\)HAPC and varying amounts of unlabeled HAPC. The reaction was performed in 75 \(\mu\)L of buffer with 0.075 \(\mu\)g of cPLA\(_2\) at 37 °C (open circles) or 30 °C (closed circles) for 10 min. Inhibition of the initial GLU hydrolysis rate by SAPC in 100 \(\mu\)M DMPM/GLU/SAPC LUVs at 30 °C (open triangles) and at 40 °C (open triangles). The mole fraction of GLU was 0.005, and 0.2 \(\mu\)g of cPLA was used in 1 mL of reaction mixture. (B) Rate of total arachidonate release from experiment in panel A after correcting for dilution of the specific activity of \(^{3}H\)HAPC by unlabeled HAPC.

HAPC present in DMPM LUVs. At 30 °C (filled circles), the rate appears to level off above 0.02 mole fraction, and at 37 °C (open circles), the rate levels off at 0.08 mole fraction. This apparent saturation is reproducible and occurs at a rate of approximately 20 nmol min\(^{-1}\) mg\(^{-1}\) (37 °C) and 50 nmol min\(^{-1}\) mg\(^{-1}\) (30 °C). Similar results were obtained using DMPM/SAPC/GLU vesicles where the rate of hydrolysis of a fixed amount of GLU present in vesicles at 0.005 mole fraction (i.e. well below its \(K_{M,app}^{*}\) of approximately 0.28 mole fraction) was measured in the presence of increasing concentrations of SAPC. At 30 °C, half-inhibition of GLU hydrolysis occurs when 0.04 mole fraction SAPC is present (Figure 8A, closed triangles), but at 40 °C (Figure 8A, open triangles), no inhibition of GLU hydrolysis by up to 0.1 mole fraction SAPC is seen. No inhibition of GLU hydrolysis under the conditions of Figure 8A is seen with an increasing mole fraction of SAPC in L-DOPM LUVs at any of the temperatures tested (not shown).

If the apparent saturation of the rate of HAPC hydrolysis seen with HAPC concentrations in DMPM LUVs above 0.08 mole fraction at 37 °C (Figure 8B, open circles) is due to saturation of the active site of cPLA\(_2\), the rate of HAPC hydrolysis should remain constant as the HAPC concentration is increased from 0.08 to 1 mole fraction. As shown in Figure 9A, this is clearly not the case. An upward curvature in Figure 9A is seen, consistent with a 2-fold activation of cPLA\(_2\) activity. This is more apparent when the counts per minute of \(^{3}H\)arachidonate from a fixed amount of \(^{3}H\)-HAPC is plotted against increasing amounts of unlabeled...
HAPC hydrolysis (Figure 9A) is due simply to a linear HAPC in DMPM LUVs (Figure 9B). If the increase in cPLA2 in the Scooting Mode
Anomalous nonlinear behavior occurs in DMPM LUVs at observed as the mole fraction of PyAPC is increased.
of the excimer to monomer fluorescence ratio would be pyrene-containing SAPC analog occurs, anomalous behavior DMPM. The expectation was that, if clustering of the vesicles might occur near the transition temperature of clustering of phosphatidylcholine substrates in DMPM DMPM/PyAPC vesicles to address the possibility that excimer to monomer fluorescence ratios were measured using f2-fold increase is observed; this phenomenon was not studied further.

**DISCUSSION**
cPLA2 has been shown to bind with high affinity to L-DOPM vesicles in the presence of glycerol and physiological amounts of salt, allowing high rates of hydrolysis. The estimated rate constant for dissociation of cPLA2 from L-DOPM LUVs is $3.8 \times 10^{-4}$ s$^{-1}$. The hydrolysis rate for GLU (260 nmol min$^{-1}$ mg$^{-1}$ at 0.01 mole fraction) and [3H]HAPC (0.6 nmol min$^{-1}$ mg$^{-1}$ at 0.01 mole fraction) shows that the enzyme is behaving processively (acting in the scooting mode), since it is capable of hydrolyzing 970 GLU molecules or 2.2 [3H]HAPC molecules at 0.01 mole fraction before dissociating.

Many peripheral membrane proteins require negatively charged phospholipids for high-affinity interaction with membranes (Kinnunen et al., 1994). Negatively charged phospholipids are known to activate cPLA2, probably by promoting association of the enzyme with the lipid–water interface (Leslie & Channon, 1990). Trapping of cPLA2 on phosphatidylmethanol vesicles but not on phosphatidylcholine vesicles is consistent with high-affinity binding to negatively charged membrane bilayers. Product trapping on phosphatidylcholine vesicles may also be at least partly due to the accumulation of anionic fatty acid product (Ghomashchi et al., 1992). Product formation is not required for scooting on phosphatidylmethanol vesicles, since scooting occurs on vesicles of DMPM, which are not detectably hydrolyzed (Diez et al., 1992), and trapping occurs on L-DOPM vesicles well before a stoichiometric amount of product has formed. The observation that increasing NaCl concentrations result in faster dissociation rates from DMPM vesicles is also consistent with a role for negative charge in binding of enzyme to phosphatidylmethanol vesicles.

Kinetic analysis of the reaction progress curve for cPLA2 acting on L-DOPM vesicles in the scooting mode is more difficult than for secreted PLA2s acting on DMPM vesicles in the scooting mode (Jain & Berg, 1989; Berg et al., 1991; Jain & Gelb, 1991). Cessation of reaction and the linear dependence of the extent of the reaction on the amount of enzyme present in the reaction are, in part, consistent with scooting behavior (Ghomashchi et al., 1992; Bayburt et al., 1995). However, the extent of reaction does not reach the value expected for depletion of sn-2-arachidonyl substrates present in the outer monolayer of enzyme-associated vesicles or for complete hydrolysis of GLU, which can exchange rapidly among vesicles. The kinetics of decreasing activity on L-DOPM LUVs (Figure 2A,B) are similar to the rate decelerations during the progress curve for GLU hydrolysis (Figure 3). The decays in the rate of GLU hydrolysis over time in L-DOPM vesicles are identical to the decays in the rate of [3H]SAPC hydrolysis in L-DOPM vesicles over time. It is apparent from the progress curves for cPLA2 acting on DMPM vesicles reported previously (Diez et al., 1992; Witmer et al., 1995) that cessation of the reaction progress also occurs before exhausting the substrate in the outer monolayer of vesicles. In contrast to L-DOPM vesicles, cPLA2 does not act processively on DMPM vesicles in the presence of 150 mM NaCl. In the absence of salt, the decrease in enzyme activity on DMPM vesicles is so rapid that it is difficult to probe for processivity with trapping

**FIGURE 10:** Excimer to monomer fluorescence ratios of PyAPC in phosphatidylmethanol vesicles as a function of probe concentration. (A) Excimer to monomer ratios were measured as described in Materials and Methods. Curves are shown for DMPM/PyAPC LUVs at 30 °C (closed circles) and 37 °C (open circles) and L-DOPM/PyAPC SUVs (diamonds) at ambient temperature. (B) Excimer to monomer ratios as a function of PyAPC in DMPM LUVs below 0.01 mole fraction. The symbols are the same as those in panel A.

HAPC in DMPM LUVs (Figure 9B). If the increase in HAPC hydrolysis (Figure 9A) is due simply to a linear increase in the amount of cPLA2-HAPC complex formed at the DMPM–HAPC interface, the plot in Figure 9B should have been flat over the entire range of HAPC. Rather, a 2-fold increase is observed; this phenomenon was not studied further.

**Clustering of Phosphatidylcholine in DMPM LUVs.** Excimer to monomer fluorescence ratios were measured using DMPM/PyAPC vesicles to address the possibility that clustering of phosphatidylcholine substrates in DMPM vesicles might occur near the transition temperature of DMPM. The expectation was that, if clustering of the pyrene-containing SAPC analog occurs, anomalous behavior of the excimer to monomer fluorescence ratio would be observed as the mole fraction of PyAPC is increased. Anomalous nonlinear behavior occurs in DMPM LUVs at 30 °C (closed circles) and at 37 °C (open circles), in which the curves approach an excimer to monomer ratio of 0 only at very low mole fraction of PyAPC (Figure 10A,B). The dependence of the excimer to monomer ratio on the mole fraction of PyAPC in L-DOPM SUVs is linear, with the line passing through the origin (Figure 10A, diamonds). Such linear behavior is expected for random mixing of the probe in the bilayer (Galla & Sackmann, 1974; Somerharju et al., 1985). The dependence of the excimer to monomer ratio of PyAPC in DMPM LUVs suggests nonideal mixing of the PyAPC at the phase transition temperature and, to a lesser extent, above the phase transition temperature of DMPM.
experiments. Membrane–associated enzymes such as phospholipase C are also known to be inactivated on highly negatively charged vesicles (Xu & Nelsestuen, 1992). Assays of cPLA₂ using pure phosphatidylycholine vesicles also cease prematurely (Leslie, 1991; Ghomashchi et al., 1992). The results of Figures 2A and 3 indicate that apparent inactivation of cPLA₂ also occurs in the presence of DOPC vesicles.

The apparent inactivation on L-DOPM is not detrimental to its use for kinetic analysis of cPLA₂. The advantage of using L-DOPM is that all of the enzyme is bound tightly and undergoes very slow intervesicle exchange, which allows all cPLA₂ molecules to experience the same environment over time (Jain & Berg, 1989; Berg et al., 1991; Jain & Gelb, 1991). Since the amount of product formed over time is proportional to the enzyme concentration, the L-DOPM-based assay is suitable for measuring $K_{\text{app}}^* \text{ or } K_{\text{app}}^* \text{ for inhibitors}$ (Bayburt et al., 1995). It is of no consequence that the rate of hydrolysis changes over time. The rate at any point along the reaction time course is proportional to the amount of active enzyme. The rate at the same time point in the reaction time course in the presence of competitive inhibitors of cPLA₂ will decrease according to the standard rate equation for competitive inhibition. This is true for any instantaneous rate along the reaction time course as long as the presence of an inhibitor does not change the kinetics of the apparent enzyme inactivation. It may be possible for an inhibitor to change the time course of the apparent inactivation, but the effect of this would be small at early time points where rate measurements are commonly made and most of the enzyme is still active. The same is true for measurement of substrate preferences. The substrate specificity of phospholipase A₂ is best measured in the scooting mode using two competing substrates present in the same vesicle (Ghomashchi et al., 1991). A decrease in the amount of active enzyme over time cannot affect the ratio of products formed from the competing substrates (the substrate preference).

The molecular basis for this apparent enzyme inactivation remains to be established. It is unlikely that inactivation is due to a protein contaminant or protease present in the cPLA₂ preparation. The fact that loss of activity occurs on vesicles of the nonhydrolyzable phospholipid HOPM indicates that this is not the result of covalent acylation of enzyme by diester phospholipids, nor is it due to isolation of enzyme on an island of products that lack substrate. Apparent inactivation may be due to the formation of an inactive complex between enzyme and phosphatidylmethanol, which appears to be at least partially stable because it is not disrupted by treatment with Triton X-100. The slow loss of activity on L-DOPM might be partially reversible with Triton X-100 treatment, since the decrease in Triton-recoverable activity is slightly less than the decrease in rate during the time course of GLU hydrolysis. In contrast, on DOPC LUVs, the loss of activity measured after addition of Triton X-100 is 4-fold slower than the decay of rate seen during the time course of GLU hydrolysis. The slower loss of activity on DOPC vesicles measured after disruption with Triton X-100 is consistent with the recovery of much of the activity on phosphatidylycholine vesicles after addition of salt, bovine serum albumin, and diacylglycerol-containing vesicles at long times (Ghomashchi et al., 1992).

It is clear from previous studies (Bayburt et al., 1995; Burke et al., 1995) that inclusion of glycerol in the assay buffer significantly increases the enzymatic activity of cPLA₂. Although the mechanism of this activation is not known, it may be noted that addition of 30% (v/v) glycerol to the assay buffer described in Materials and Methods increases the specific activity of cPLA₂ acting on L-DOPM/GLU LUVs without changing the rate of enzyme inactivation (not shown). The reaction progress curves recently reported by Burke et al. (1995) seem to show less inactivation than the assay reported in the present study. However, inactivation also appears to be occurring under certain conditions in this system, especially in the absence of glycerol (Witmer et al., 1995). These workers used an assay buffer containing serum albumin (0.5 mg/mL) and glycerol (30%) but no salt. With this buffer, we confirmed the findings of Burke et al. that the reaction progress is linear over 30 min with DMPM LUVs under vesicle-fusing conditions (10 mM CaCl₂). However, under nonfusing conditions (0.5 mM CaCl₂), we find that the reaction slows to near-zero velocity on DMPM LUVs after only 4% of the total substrate is hydrolyzed. At a ratio of 0.5 enzyme per vesicle and assuming a Poisson distribution of enzyme, one would expect 20% of the substrate to be hydrolyzed if all of the substrate in the outer monolayer of enzyme-containing vesicles was hydrolyzed. We found that serum albumin, glycerol, and 10 mM CaCl₂ are all required to obtain a linear initial velocity.

The use of GLU provides a continuous and sensitive fluorescence-based assay using model phospholipid bilayers of L-DOPM (Huang et al., 1994; Bayburt et al., 1995). Partitioning of GLU (Figures 4 and 5) must be accounted for and can be guarded against by always using >50 μM L-DOPM. The possibility that the enzyme-catalyzed rate may reflect the exchange rate of GLU among vesicles was considered. The association of hydrophobic molecules with vesicles is typically diffusion-controlled (Jain, 1988). A rate constant for dissociation of GLU from L-DOPM LUVs of about 0.1–1 s⁻¹ can then be calculated from the amount of L-DOPM required for half-partitioning of GLU in Figure 5, using a diffusion-limited second-order association rate constant with vesicles of $10^{10}$ s⁻¹ M⁻¹. The measured turnover number for GLU of 3.7 s⁻¹ is near the estimated rate constant for exchange of GLU among vesicles. However, the decrease in rate over time (i.e. fast initial phase of product formation) does not reflect depletion of GLU in vesicles containing bound enzyme. This is based on the observation that the kinetics of the fast initial phase of product formation are very similar to the kinetics of inactivation (see Figures 2A and 3B). The fact that no burst of GLU hydrolysis is seen with DOPC vesicles is also consistent with the interpretation of the fast initial phase of product formation resulting from loss of activity rather than substrate depletion. Lastly, the GLU concentration in enzyme-bound vesicles in Figure 3 is not depleted significantly over the fast initial phase of product formation, as determined in the following discussion.

An estimate of the effect of GLU depletion on the rate during the fast initial phase of product formation in Figure 3 for cPLA₂ acting on L-DOPM/GLU must take into consideration the distribution of enzyme among vesicles (Jain & Berg, 1989). For example, in the case of Figure 3 for cPLA₂ acting on L-DOPM/0.005 mole fraction GLU LUVs, the moles of product formed after the fast initial phase at 1 min is 15 pmol. The reaction mixture contained ~1 pmol of enzyme, so the average number of products formed per enzyme at 1 min is 15, or 3% of the 500 GLU substrates
present initially in each vesicle of approximately 100,000 lipids. Assuming that there is no exchange of GLU between vesicles (worst case scenario), the percent remaining substrate in vesicles containing one enzyme can be estimated as 97%, two enzymes as 94%, three enzymes as 91%, etc. The enzyme is assumed to be distributed on vesicles according to the Poisson distribution, $P_j = \frac{je^{-J/P_j}}{j!}$, where $P_j$ is the probability of a vesicle having $j$ bound enzymes and $J$ is the enzyme to vesicle ratio (Jain & Berg, 1989). The proportion of enzyme bound to a vesicle containing $j$ enzymes is $jP_j/\Sigma jP_j$, and the rate for each enzyme molecule is proportional to the percent remaining substrate. The sum of the rates for the population of enzymes is then $\Sigma jP_jV_j/\Sigma jP_j$, where $V_j$ is the rate for enzymes present on vesicles with $j$ enzymes, is estimated as the percent remaining substrate. According to this treatment, in the experiment of Figure 3 for cPLA2 acting on L-DOPM/GLU, an estimated 6% of the decrease in rate over the first minute is due to substrate depletion if one ignores the fact that GLU can exchange among vesicles. At lower enzyme to vesicle ratios, $V_{obs}$ would be closer to 100%. Identical fast initial phases of product formation on L-DOPM/GLU are found at enzyme to vesicle ratios of 0.3–1.5, supporting the assertion that substrate depletion does not contribute to the initial fast phase of GLU hydrolysis.

It is unlikely that the cPLA2-catalyzed rate of reasonable enzyme to vesicle ratios reflects the exchange rate of GLU. This conclusion is based on an estimate of the mole fraction of GLU in enzyme-containing vesicles at steady state. The analysis assumes that GLU exchanges among vesicles through the aqueous phase, that the bulk GLU concentration is not significantly depleted, and that the products of GLU hydrolysis are not involved in the equilibria. Using the value of $k_{cat}/K_{M,app}$ of 40 s$^{-1}$, a first-order rate constant for GLU dissociation from vesicles of 0.1 s$^{-1}$, and a diffusion-limited second-order association rate constant with vesicles of $10^{10}$ s$^{-1}$ M$^{-1}$ (see above), it is found that the steady-state mole fraction of GLU in vesicles containing one enzyme is 98.5% of the mole fraction at time 0 and that the steady state is attained with a half-time of 7 s. With more than one enzyme on some vesicles, as is the case in Figure 3 with L-DOPM/GLU, an analysis similar to the one described above using a Poisson distribution of enzyme among vesicles suggests that the overall steady-state rate is 98% of the initial rate at an enzyme to vesicle ratio of 1. Thus, as long as the bulk GLU concentration is not depleted significantly, the rate of replenishment is not a major concern when measuring the GLU hydrolysis rate.

**Measurement of Interfacial Michaelis Constants.** The hyperbolic dependency of the reaction velocity for the hydrolysis of GLU in L-DOPM SUVs versus the mole fraction of GLU (Figure 6A) suggests that GLU binds to the active site of cPLA2 with a $K_{M,app}$ of 0.09 mole fraction. Surprisingly, the velocity for the hydrolysis of [3H]HAPC in L-DOPM SUVs increased linearly when substrate was increased up to 0.15 mole fraction, and thus, it was not possible to measure $K_{M,app}$ since $K_{M,app} > 0.15$ mole fraction. It is interesting that [3H]SAPC hydrolysis occurs with virtually identical rates when dispersed in L- or D-DOPM vesicles. This could arise if L- and D-DOPM do not significantly bind to the active site of cPLA2 or if the enantiomers do bind to the active site with similar affinity. The latter possibility may seem unlikely because it is well established that enzymes display high stereoselectivity in their binding of substrates. In fact, cPLA2 does not liberate arachidonic acid from [3H]-D-HAPC. However, it has recently been shown that cPLA2 has limited sequence similarity to Penicillium notatum phospholipase B (Saito et al., 1991; Sharp et al., 1994) and that both enzymes display phospholipase A$_1$ activity in a nonstereoselective fashion (Saito et al., 1991; Hanel & Gelb, 1995). For this reason, we cannot rule out the possibility that L- and D-DOPM bind with similar affinities to the active site of cPLA2.

Vesicles of pure [3H]SAPM are hydrolyzed by cPLA2 300-fold faster than vesicles of pure [3H]DOPM vesicles under conditions in which enzyme is in the scooting mode. DMPM vesicles are not detectably hydrolyzed by cPLA2 (Diez et al., 1992). Yet, GLU, HAPC, and SAPC hydrolysis rates are similar when these substrates are present in L-DOPM, SAPM, or DMPM LUVs. These results suggest either that these phosphatidylmethanols have poor affinity for the active site of cPLA2 ($K_{M,app} > 1$ mole fraction) or that they bind with similar affinity to the enzyme’s active site. If the rate of hydrolysis of [3H]HAPC dispersed at low mole fraction in L-DOPM vesicles (Figure 6B) is extrapolated to 1 mole fraction [3H]HAPC, the rate obtained of 63 nmol min$^{-1}$ mg$^{-1}$ is significantly lower than the rate of hydrolysis of pure HAPC vesicles (830 nmol min$^{-1}$ mg$^{-1}$, Figure 9A). This suggests that L-DOPM may be competing with [3H]HAPC for binding to the active site of cPLA2. It was also found that the rate of hydrolysis of [3H]SAPM present in L-DOPM LUVs extrapolates to the rate of hydrolysis of pure [3H]SAPM vesicles, suggesting that the $K_{M,app}$ values for [3H]SAPM and L-DOPM are nearly identical or both are much greater than 1 mole fraction. An alternative explanation for the inhibition of [3H]HAPC hydrolysis by L-DOPM is that $k_{cat}$ or $K_{M,app}$ for HAPC changes as the mole fraction of HAPC in L-DOPM vesicles approaches unity due to composition-dependent effects on the bilayer properties of mixed L-DOPM/HAPC vesicles. Other methods need to be developed to establish the $K_{M,app}$ values and $K_{M,app}$ values of phospholipids.

Most of the experiments described were performed using recombinant cPLA2 that is phosphorylated on multiple serines and has a mixture of phosphorylation states (de Carvalho et al., 1996). It is possible that the phosphorylation state can affect $k_{cat}$, $K_{M,app}$, or both. Only a small effect on $k_{cat}/K_{M,app}$ was seen for purely dephosphorylated versus purely MAP kinase-phosphorylated species or versus nontreated recombinant cPLA2 in the scooting mode assay using DOPM/[3H]-HAPC as the substrate. The effect of dephosphorylation and serine 505 phosphorylation on $K_{M,app}$ was not rigorously studied. However, the activity of serine 505-phosphorylated cPLA2, dephosphorylated cPLA2, and cPLA2 having a mixture of states on pure sn-2-arachidonylphosphatidylcholine substrate vesicles was found to be ~500-fold higher than the activity on L-DOPM/0.0065 mole fraction [3H]HAPC SUVs, suggesting that the $K_{M,app}$ for all three forms of enzyme is higher than 1 mole fraction. Thus, it seems likely that the bulk properties of the mixture of states used in these studies do not differ greatly from the properties of dephosphorylated or serine 505-phosphorylated pure forms of the enzyme. Further study of pure phosphorylated species is required.

$K_{M,app}$ measurements for cPLA2 using DMPM vesicles have appeared in the literature. In one report, DMPM vesicles in low-ionic strength buffer were used, and a $K_{M,app}$ of 0.003 mole fraction for [3H]HAPC was reported (Diez et al., 1992). We have not been able to reproduce these results using human recombinant cPLA2, either completely dephos-
phorylated, phosphorylated at serine 505, or having a mixture of phosphorylation states. It should be noted that at low ionic strength the reaction ceases abruptly after 10 min so that true rate measurements are not made, while altering the composition of vesicles may have an effect on the reaction time course and therefore the amount of product measured at the experimental time point of 10 min.

In the assay of Burke et al. where fast inactivation of cPLA2 is not occurring, a sigmoidal curve was obtained when the initial reaction velocity for the hydrolysis of PAPC was measured as a function of its mole fraction in DMPM vesicles (Burke et al., 1995). From such a curve, a value of $K_m^c$ of 0.003 mole fraction was obtained for PAPC. The authors suggested that PAPC binds in a cooperative fashion to the active site of cPLA2, but they also pointed out that the sigmoidal behavior could result from segregation of PAPC in DMPM vesicles with an effect of this segregation on the enzymatic activity of cPLA2. In this previous study, $K_m^c$ values for substrates other than PAPC were measured by monitoring the inhibition of $[^3H]$PAPC hydrolysis by increasing mole fraction of competing nonradiolabeled substrate in DMPM vesicles. From these results, it was concluded that phospholipids with fully saturated fatty acyl chains have a $K_m^c$ of >1 mole fraction and that $sn-2$-arachidonoyl- and oleoyl-containing phospholipids display similar affinities for the active site of cPLA2 ($K_m^c = 0.003–0.007$ mole fraction). Burke et al. also concluded that DMPM is a neutral diluent for cPLA2. However, we believe that this conclusion is invalid since it is based on a flawed mathematical approach (proof given as Supporting Information).

Several reasons exist to question whether saturation of the active site of cPLA2 by $sn-2$-arachidonoyl-containing phospholipids is occurring using a DMPM-based assay. When the mole fraction of $[^3H]$HAPC in DMPM LUVs is increased, an apparent saturation in rate occurs at about 0.08 mole fraction substrate (Figure 8B). However, the rate increases again when more HAPC is added, and there is no evidence for saturation even as the mole fraction of substrate approaches 1 mole fraction (Figure 9A). These results were obtained at 37 °C. Figure 8A shows that at 37 °C the presence of nonradiolabeled HAPC in DMPM LUVs containing 0.002 mole fraction $[^3H]$HAPC only slightly inhibits the hydrolysis of the tritiated substrate even when the amount of HAPC approaches 0.1 mole fraction. Similarly, at 40 °C, no inhibition of GLU hydrolysis is seen with up to 0.1 mole fraction SAPC in DMPM LUVs. These results unequivocally establish that the $K_m^c$ for HAPC is $>0.1$ mole fraction in DMPM vesicles. However, at 30 °C, a pronounced inhibition of $[^3H]$HAPC hydrolysis by nonradiolabeled HAPC is seen (Figure 8A). This dramatic temperature dependence of the inhibition by HAPC could be due to a temperature-dependent abrupt change in $K_m^c$ values for the competing substrates, but this seems highly unlikely for such a modest change in temperature as 7 °C. Rather, the results suggest that some form of segregation of phosphatidylcholines in DMPM LUVs is occurring, since such a process is highly cooperative and temperature sensitive, and that this nonideal mixing of components affects the kinetics of cPLA2-catalyzed lipolysis. Indeed, fluorescence studies with PyAPC in DMPM (Figure 10) show that clustering of $sn-2$-arachidonoylphosphatidylcholine is occurring in DMPM LUVs, and this clustering is more pronounced at 30 °C (near the transition temperature of DMPM) than at 37 °C. Furthermore, the PyAPC is ideally mixed in L-DOPM vesicles (Figure 10); L-DOPM is expected to have a phase transition temperature well below that of DMPM (Cevc & Marsh, 1987). Under these conditions, no apparent saturation of the active site of cPLA2 by $[^3H]$HAPC in L-DOPM LUVs is seen (Figure 6B), and no inhibition of GLU hydrolysis is seen when L-DOPM vesicles also contain increasing amounts of SAPC. The mechanism by which clustering of substrate affects the kinetics of cPLA2-catalyzed lipolysis remains to be established. One possibility explaining the results in Figures 8 and 9 is that cPLA2 is more active at the perimeter of a patch of $sn-2$-arachidonoylphosphatidylcholine substrate (the boundary region), and the velocity continues to increase with increasing patch size as more and more substrate is added to the DMPM vesicles. In this model, inhibition of GLU hydrolysis by SAPC in DMPM LUVs at 30 °C may occur if GLU is excluded from the boundary region, for example by preferential partitioning of GLU uniformly into the substrate patch.

All together, the results show that DMPM is not a good matrix for studying the kinetics of cPLA2-catalyzed hydrolysis of substrates present in DMPM vesicles. By using L-DOPM vesicles, the problems associated with nonideal mixing of cPLA2 substrates are avoided. However, an independent method is needed to determine if L-DOPM competes with substrates for the binding to the active site of cPLA2. The availability of a neutral diluent for cPLA2 will allow true equilibrium constants for enzyme–ligand interactions, rather than apparent ones, to be measured (Jain et al., 1991).

**Effect of Serine 505 Phosphorylation on cPLA2 Kinetics in the Scooting Mode.** Activities of dephosphorylated and MAP kinase-phosphorylated forms of cPLA2 were measured in the L-DOPM scooting mode assay. An increase in activity of 30% is near that reported using other assay systems, showing unequivocally that this small increase in rate occurs when the enzyme is fully bound to the interface. It is not known whether the effect of phosphorylation at serine 505 is due to increased substrate binding or a catalytic step or whether phosphorylation has an additional effect on the calcium-dependent membrane interaction. The possibility of other regulatory phosphorylation sites on cPLA2 is also open to further inquiry.

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**SUPPORTING INFORMATION AVAILABLE**

Evaluation of the mathematical approach used by Burke et al. (1995) for establishment of a neutral diluent (5 pages). Ordering information is given on any current masthead page.

**REFERENCES**


Bayburt and Gelb


