Multiple Enzymatic Activities of the Human Cytosolic 85-kDa Phospholipase A_2: Hydrolytic Reactions and Acyl Transfer to Glycerol†

Arthur M. Hanel‡ and Michael H. Gelb*

Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195

Received January 6, 1995; Revised Manuscript Received March 21, 1995⊗

ABSTRACT: The recombinant human 85-kDa cytosolic phospholipase A_2 ($cPLA_2$), when assayed in the presence of glycerol, catalyzes the transfer of acyl chains of radiolabeled phosphatidylcholine and *para*-substituted phenyl esters of fatty acids to glycerol, in addition to hydrolyzing these substrates. The product of the transacylation reaction is monoacylglycerol (MAG), and the acyl chain is predominantly esterified (≥95%) to a primary hydroxyl group of glycerol (*sn*-1/3); the stereochemistry is not known. Increasing concentrations of glycerol accelerate enzyme turnover both by providing an additional mechanistic pathway for the enzyme−substrate complex to form products and by increasing the intrinsic hydrolytic and transacylation activities of the enzyme. Significant enzymatic hydrolysis of *sn*-1/3-arachidonylmonoacylglycerol was measured, while *sn*-1/3-α-linolenoyl- and *sn*-2-arachidonylmonoacylglycerols were not detectably hydrolyzed. 1,3-Propanediol also serves as an acyl acceptor for the enzyme. $cPLA_2$ hydrolyzes an analog of lysophosphatidylcholine that lacks the *sn*-2 hydroxyl group. The enzyme will hydrolyze *sn*-1-acyl chains of *rac*-1-(arachidonyl, α-linolenoyl, palmitoyl)-2-*O*-hexadecyl-glycero-3-phosphocholine lipids and transfer the acyl chain to glycerol. Thus, $cPLA_2$ has phospholipase A_1 activity but only if an ether linkage rather than an ester linkage is present at the *sn*-2 position, and it is shown that the *sn*-1 acyl chains of both enantiomers of phosphatidylcholine are hydrolyzed. Phenyl [^{14}C]-α-linolenate and five *para*-substituted phenyl esters of [^{3}H]-α-linolenic acid with pK_a values ranging from 7.2 to 10.2 for the phenol leaving groups were incorporated into 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol/Triton X-100 mixed micelles as substrates for the transacylation/hydrolysis reactions of the enzyme. Average product ratios, which are defined as the amount of monoacylglycerol formed to phenyl ester hydrolyzed, were 2.1 ± 0.1 (n = 5) for the *para*-substituted phenyl esters and 2.0 ± 0.3 (n = 7) for phenyl α-linolenate. The similarity of the ratios, despite the range of pK_a values for the leaving groups, is consistent with the formation of a common enzyme intermediate that partitions to give either fatty acid or MAG. That intermediate may be a covalent acyl enzyme. Finally, the acyl chain specificity of $cPLA_2$ was investigated to better understand the preference of the enzyme for phospholipids with *sn*-2-arachidonyl chains.

The hydrolysis of arachidonic acid from the phospholipids of mammalian cell membranes is the first in a series of reactions that leads to the formation of the eicosanoids. Enzymes that hydrolyze fatty acids esterified at the *sn*-2 position of phospholipids are the phospholipases A_2. The secreted small molecular mass 14-kDa phospholipases A_2 ($sPLA_2$)[1] are well studied, and the binding of these enzymes to phospholipid interfaces and hydrolysis of the bound substrate are well understood (Dennis, 1983; Gelb et al., 1992; Jain & Berg, 1989; Verheij et al., 1981; Waite, 1987). Recently, a cytosolic phospholipase A_2 that preferentially hydrolyzes arachidonyl chains of phospholipids ($cPLA_2$) (Clark et al., 1990; Diez et al., 1994; Diez & Mong, 1990; Hanel et al., 1993; Kramer et al., 1991) was identified in and isolated from a number of types of mammalian cells (Clark et al., 1990; Diez & Mong, 1990; Gronich et al., 1990; Kim et al., 1991a,b; Kramer et al., 1987, 1991; Leslie et al., 1988; Wijkander & Sundler, 1991). The $cPLA_2$ has a molecular mass of ~85 kDa, shows no sequence homology with $sPLA_2$'s, and requires micromolar concentrations of calcium to bind to membranes (Channon & Leslie, 1990; Clark et al., 1991; Nalefski et al., 1994; Sharp et al., 1991); calcium is not required for the hydrolytic reaction (Ghomashchi et al., 1992; Nalefski et al., 1994; Wijkander &

† Supported by a grant and a Research Career Development Award to M.H.G. from the National Institutes of Health (HL50040 and GM562) and a postdoctoral fellowship from the American Cancer Society (PF 3670) to A.M.H.

* Author to whom correspondence should be addressed.

‡ Present address: RedCell, Inc., 270-B Littlefield Ave., South San Francisco, CA 94080.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1995.

[1] Abbreviations: AA, arachidonic acid; $AACOCF_3$ and $AACOCH_3$, ketone analogues of arachidonic acid in which COOH is replaced by either $COCF_3$ or $COCH_3$, respectively; [^{3}H]AAPC, 1-arachidonyl-2-[5,6,8,9,11,12,14,15-^{3}H(N)]arachidonyl-*sn*-glycero-3-phosphocholine; *rac*-[^{3}H]AHPC, *rac*-1-[5,6,8,9,11,12,14,15-^{3}H(N)]arachidonyl-2-*O*-hexadecyl-glycero-3-phosphocholine; [^{3}H]AHPC, 1-[5,6,8,9,11,12,14,15-^{3}H(N)]arachidonyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; [^{14}C]-APPC, 1-arachidonyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; $cPLA_2$, cytosolic human 85-kDa phospholipase A_2; [^{14}C]DPPC, 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; GC, gas chromatography; [^{3}H]-HAPC, 1-*O*-hexadecyl-2-[5,6,8,9,11,12,14,15-^{3}H(N)]arachidonyl-*sn*-glycero-3-phosphocholine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; K_M*, interfacial Michaelis constant; *rac*-[^{14}C]-LHPC, *rac*-1-[1-^{14}C]-α-linolenoyl-2-*O*-hexadecyl-glycero-3-phosphocholine; MAG, monoacylglycerol; OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; [^{14}C]PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine; *rac*-[^{14}C]PHPC, *rac*-1-[^{14}C]palmitoyl-2-*O*-hexadecyl-glycero-3-phosphocholine; S-lyso-PC, 1-stearoyl-lyso-*sn*-glycero-3-phosphocholine; [^{3}H]SAPC, 1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H(N)]arachidonyl-*sn*-glycero-3-phosphocholine; [^{14}C]SLPC, 1-stearoyl-2-[1-^{14}C]-α-linolenoyl-*sn*-glycero-3-phosphocholine; SAG, 1-stearoyl-2-arachidonyl-*sn*-glycerol; $sPLA_2$, 14-kDa phospholipase A_2; TLC, thin-layer chromatography; 16:0, palmitic acid; 18:1, oleic acid, 18:3, α-linolenic acid; 20:4, arachidonic acid; kDa, kilodaltons.

0006-2960/95/0434-7807$09.00/0

Sundler, 1992). In contrast, millimolar concentrations of calcium are required as a cofactor for $sPLA_2$ catalysis (Scott et al., 1991). The $cPLA_2$ is regulated by signal transduction pathways whose apparent purpose is to control the ability of the cell to synthesize eicosanoids by regulating the release of esterified arachidonic acid from membrane phospholipids [for example, see Kramer et al. (1993), Lin et al. (1992), Nemenoff et al. (1993), and Xing and Mattera (1992)]. The differences between the regulation, amino acid sequences, and calcium requirements of the $cPLA_2$ and $sPLA_2$ enzymes suggest that their mechanisms of hydrolysis also differ.

The hydrolytic mechanism of $cPLA_2$ is not well understood. The kinetics of hydrolysis of unilamellar vesicles, which contain *sn*-2-arachidonyl phospholipids, is biphasic; initial rapid hydrolysis is followed by the slowing and ultimate cessation of the hydrolytic reaction before all of the available substrate is consumed (Diez et al., 1992; Ghomashchi et al., 1992; Leslie, 1991). The presence of lysophospholipid and fatty acid in the vesicles promotes tight binding of the enzyme to the interface and hydrolysis of phospholipid substrates (Ghomashchi et al., 1992). The enzyme also hydrolyzes lysophospholipids (Fujimori et al., 1993; Leslie, 1991; Nalefski et al., 1994; Reynolds et al., 1993).

Three recent reports provide evidence that an acyl enzyme intermediate forms during the $cPLA_2$-catalyzed hydrolysis of phospholipid substrates. Dennis and co-workers observed the synthesis of a small amount of DPPC resulting from a transacylation reaction when reaction mixtures contained only 1-palmitoyl-*sn*-lysophosphocholine as substrate; palmitic acid, which is the product of the lysophospholipase reaction, is the major product (Reynolds et al., 1993). The authors propose that a covalent acyl enzyme intermediate, which contains a palmitoyl acyl chain, is formed. Subsequently, either water or a second molecule of 1-palmitoyl-*sn*-lysophosphocholine reacts with the intermediate to give either palmitic acid or DPPC, respectively.

The second report (Trimble et al., 1993) describes NMR studies that measure the binding of the trifluoromethyl ketone derivative of arachidonic acid ($AACOCF_3$) to $cPLA_2$. $AACOCF_3$ is a slow-binding inhibitor of the enzyme (Street et al., 1993), and the kinetics of inhibition of phospholipid hydrolysis by this analogue is similar to the kinetics of inhibition of serine proteases by trifluoromethyl ketone peptide inhibitors that bind slowly (Imperiali & Abeles, 1986; Stein et al., 1987). X-ray crystallographic studies show that the peptide inhibitor forms a stable hemiketal enzyme–inhibitor complex with chymotrypsin (Brady et al., 1990). NMR measurements of the $cPLA_2$•$AA^{13}COCF_3$ complex and of model compounds of the inhibitor alone suggest that $AACOCF_3$ forms a hemiketal adduct with the enzyme. The authors propose that a residue at the active site of the enzyme, which is responsible for displacing the lysophospholipid product from the phospholipid substrate to give the acyl enzyme intermediate, may be responsible for the formation of the hemiketal adduct of the inhibitor with the enzyme. The third report describes site-directed mutagenesis studies of $cPLA_2$ that identify serine-228 as an amino acid that is essential for catalytic activity and possibly an active site nucleophile (Sharp et al., 1994).

In this paper, we describe multiple enzymatic activities of the $cPLA_2$ with phospholipid, lysophospholipid, and *para*-substituted phenyl esters of α-linolenic acid as substrates. The reactions were conducted in the presence of glycerol, which activates the enzyme, and we report that glycerol serves as an acyl acceptor for these substrates. The mechanistic implications of this fact are discussed.

MATERIALS AND METHODS

Human recombinant $cPLA_2$ (Trimble et al., 1993) was obtained from baculovirus-infected Sf9 cells and purified to near homogeneity (>80%, as determined by SDS–PAGE with a specific activity of 9 μunits/μg). The fraction of total enzyme that is phosphorylated is typically 0.6, and the specific activity of the phosphorylated enzyme is ≤2-fold greater than that of the nonphosphorylated enzyme (unpublished data from I. Street, Merck-Frosst, Inc). One microunit of activity is defined as the amount of enzyme that produces 1 pmol of [^{14}C]20:4 per minute from [^{14}C]PAPC at 37 °C, using the assay described by Gronich and co-workers (Gronich et al., 1990). This assay is far less sensitive than other assays reported in the literature, but it is still useful as the amount of product in the assay is linearly proportional to the amount of enzyme added. In addition, the activity of the purified $cPLA_2$ can be titrated to 0 when 1 ± 0.1 equiv of $AACOCF_3$ is added (Street et al., 1993), which indicates that the enzyme is mostly in a native form.

Enzyme Assays. All enzyme assays were conducted at 30 ± 0.5 °C in silanized glass tubes unless otherwise noted. The standard assay buffer consisted of 50 mM HEPES (pH 8.0), 1 mM $CaCl_2$, and 0.1 mM EGTA. Glycerol (30%, by volume) was present in all assays except where noted. Substrate stocks were prepared as 10-fold concentrated solutions of either mixed micelles or unilamellar vesicles by first taking to dryness *in vacuo* the appropriate volumes of the desired fatty acid and phospholipid components, which were dissolved in toluene and $CHCl_3$, respectively. The dry lipid film was mixed with the appropriate volume of either 4 mM Triton X-100 or H_2O to give 10-fold concentrated stock solutions of micelles and vesicles, respectively, and dispersed using a vortex mixer. Suspensions of micelles were sonicated with a bath sonicator (Lab Supplies, Model G11 2SPIT) for 1 min and used within 15 min. Unilamellar vesicles were formed by freeze–thaw sonication as previously described (Hanel et al., 1993). Reactions were initiated by the addition of substrate, and 50-μL aliquots were withdrawn at the times given in the text and figures and mixed with 0.37 mL of $CHCl_3$/CH_3OH/concentrated HCl (200:100:1, by volume) to quench the reaction. Reaction blanks did not contain enzyme and were measured at the times given in the figure legends. Mass standards for 18:3 and 18:3-MAG (5 μg each) were then added. The labeled substrate and the MAG and fatty acid products were extracted as described previously (Ghomashchi et al., 1992) and separated on silica gel TLC plates (20 × 20 cm, silica H, Analtech) by using a solvent system of 60:40:1 hexane/diethyl ether/acetic acid. The regions of the plate containing the labeled products were identified by staining with I_2 vapor and scraped into individual scintillation vials, and the silica gel was shaken with 0.2 mL of methanol for ≥30 min to elute the products. Recovery of the products using this method was 85–90% of the products applied. Six milliliters of BioSafe II scintillation cocktail (Research Products International) was added, and the amount of radioactivity was measured with a Beckman 1801 scintillation counter. Hydrolysis reactions that did not contain glycerol were quenched and extracted as described earlier. The extracts were taken to dryness with nitrogen, and the residue was

dissolved in 0.2 mL of hexane/diethyl ether/acetic acid (70:30:0.1) and applied to a 0.5 × 2 cm column of silica gel 60 (EM Science) equilibrated with the same solvent. The fatty acid product(s) was eluted with 1.2 mL of the same solvent and collected in an 8-mL scintillation vial, the solvent was evaporated, and the residue was redissolved in 6 mL of BioSafe II scintillation cocktail and counted as described above. Positional isomers of MAG were separated on boric acid-impregnated silica H TLC plates that were developed with $CHCl_3$/acetone (94:6, by volume; Christie, 1982) and eluted with methanol as described earlier. R_f values of the fatty acid, *sn*-2-MAG, and *sn*-1/3-MAG reaction products were typically 0.5, 0.3, and 0.2, respectively.

The hydrolysis of synthetic *sn*-1/3-[^{14}C]18:3-MAG, *sn*-1/3-[^{3}H]20:4-MAG, and unlabeled *sn*-2-20:4-MAG was determined by using the standard reaction conditions described earlier. The radiolabeled hydrolysis products, [^{14}C]18:3 and [^{3}H]20:4, were isolated and quantified by TLC (see earlier). The unlabeled hydrolysis product 20:4 was measured by combined gas chromatography/electron capture chemical ionization mass spectrometry, as described previously (Bartoli et al., 1994; Riendeau et al., 1994). One microgram of [$^2H_8$]20:4 was added to the quenched reactions as an internal standard, and the fatty acids were converted to their pentafluorobenzyl ester derivatives. Increases in the amount of nondeuterated 20:4 above the levels of nondeuterated 20:4 that were measured for the samples containing internal standard only (~0.4% or 3.2 pmol) represented the amount of *sn*-2-20:4-MAG hydrolyzed by the enzyme.

Isolation of $cPLA_2$ with Bound Label. Centrifuge columns of BioGel P6 (Bio-Rad) were prepared by first making a 1:1 slurry (by volume) of settled gel with distilled H_2O. Approximately 0.7 g of the slurry was transferred to a 0.5-mL polypropylene microfuge tube, which had a hole in its bottom that was covered by glass wool. The tubes were placed on top of a 1 × 7.5 mL glass receiving tube, and the water was forced out of the resin by centrifugation for 1 min at 3800*g* in a clinical tabletop centrifuge. One 0.3-mL volume of 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 0.1 mM EDTA, 30% glycerol (by volume), and 1 mg/mL γ-globulin (Sigma, catalog no. G 3486) was equilibrated with the gel and removed by centrifugation. The columns were then washed three times with 0.3 mL of the same buffer that did not contain γ-globulin. After the final application, the columns were spun until no more liquid eluted.

Enzyme reaction mixtures for Figure 5 contained 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 0.1 mM EDTA, 30% glycerol (by volume), and 16 μg of $cPLA_2$ in a final volume of 0.8 mL. The reactions were initiated by the addition of [^{14}C]18:3 phenyl ester in DMSO to give final concentrations of 1.1 μM labeled substrate and 4% DMSO in the assay. Aliquots of 0.05 mL of the reaction mixture were withdrawn at increasing reaction times and either quenched immediately by mixing with 0.37 mL of a solution of $CHCl_3$/CH_3OH/concentrated HCl (200:100:1.6, by volume) or applied within 10 s to a centrifuge column resting on a silanized glass receiving tube, which contained the same volume of quench solution. The protein with bound radiolabel was eluted from the column by spinning at 3800*g* for 40 s. The presence of 150 mM NaCl in the buffers and pretreatment of the gel with γ-globulin increased the recovery of enzyme from the column. The radiolabeled substrate and the products of column eluates and directly quenched reactions were extracted, isolated, and quantified as described earlier.

Recovery of enzyme from the column was measured by applying 1 μg of enzyme in 48 μL of assay buffer that did not contain labeled substrate to the column and collecting the enzyme during centrifugation in a glass tube that contained 2 μL of a DMSO solution of [^{14}C]PAPC (55 mCi/mmol), which gave final concentrations of 22 μM [^{14}C]PAPC and 4% DMSO (by volume). The reactions were quenched after reaction time t = 10 min, and the products of the reactions were quantified as described earlier. In separate reactions, a standard curve that describes the increase in formation of total products with increasing amounts of enzyme was determined by adding known amounts of enzyme directly to the assay buffer, which contained 22 μM [^{14}C]PAPC. Recoveries of $cPLA_2$ activity from the columns were 45−50% of the total activity applied.

RESULTS

Acyltransferase Activity of $cPLA_2$. Panels A and B of Figure 1 show that $cPLA_2$ catalyzes the transfer of the *sn*-2 acyl chains of [^{3}H]SAPC and [^{14}C]SLPC, respectively, to glycerol in addition to catalyzing the hydrolysis of the *sn*-2 esters. The phospholipid substrates were present together in mixed micelles that were formed from 0.4 mM Triton X-100 and 20 μM DTPM, and the reaction mixture contained 30% glycerol by volume. DTPM is a negatively charged phospholipid that apparently increases the fraction of total enzyme bound to micelles (Street et al., 1993), and negatively charged phospholipids have been shown to increase the activity of the enzyme (Leslie & Channon, 1990). Figure 1A shows the time course for the formation of the hydrolysis product [^{3}H]20:4 and the acyl transfer products *sn*-1/3-[^{3}H]-20:4-MAG from [^{3}H]SAPC. Chromatography of the monoacylglycerol products on boric acid-impregnated silica resolves the structural isomer 2-MAG from 1-MAG and 3-MAG; the two enantiomers are not resolved by this technique. It is not known which enantiomer is formed, and the product(s) is designated *sn*-1/3-MAG to reflect this uncertainty. $cPLA_2$ preferentially transfers [^{3}H]20:4 from the *sn*-2 position of [^{3}H]SAPC to the *sn*-1/3 position of glycerol; approximately 18-fold more *sn*-1/3-[^{3}H]20:4-MAG is formed than *sn*-2-[^{3}H]-20:4-MAG at reaction times t > 20 min. The ratio of the sum of the rates of formation of MAG acyl transfer products to the rate of formation of the fatty acid hydrolysis product during the initial period of the reaction is 0.46. Control experiments with *sn*-2-[^{3}H]18:1-MAG and no enzyme showed that significant acyl migration from the *sn*-2 position to the *sn*-1/3 position of glycerol, which is thermodynamically favored (K_{eq} ~ 4) (Serdarevich, 1967), does not occur either during the reaction or during the extraction and isolation of the products (data not shown).

The hydrolysis of [^{14}C]SLPC by $cPLA_2$ to give [^{14}C]18:3 and the transfer of [^{14}C]18:3 from the *sn*-2 position of [^{14}C]-SLPC to the *sn*-1/3 and *sn*-2 positions of glycerol are shown in Figure 1B. The predominant MAG product that is formed from [^{14}C]SLPC is *sn*-1/3-[^{14}C]18:3-MAG; the rate of formation is 35-fold greater than the rate of formation of *sn*-2-[^{14}C]18:3-MAG. The reaction of $cPLA_2$ with [^{14}C]-SLPC is 4-fold slower than the reaction of enzyme with [^{3}H]-SAPC, and Figure 1B shows that the ratio of the amount of

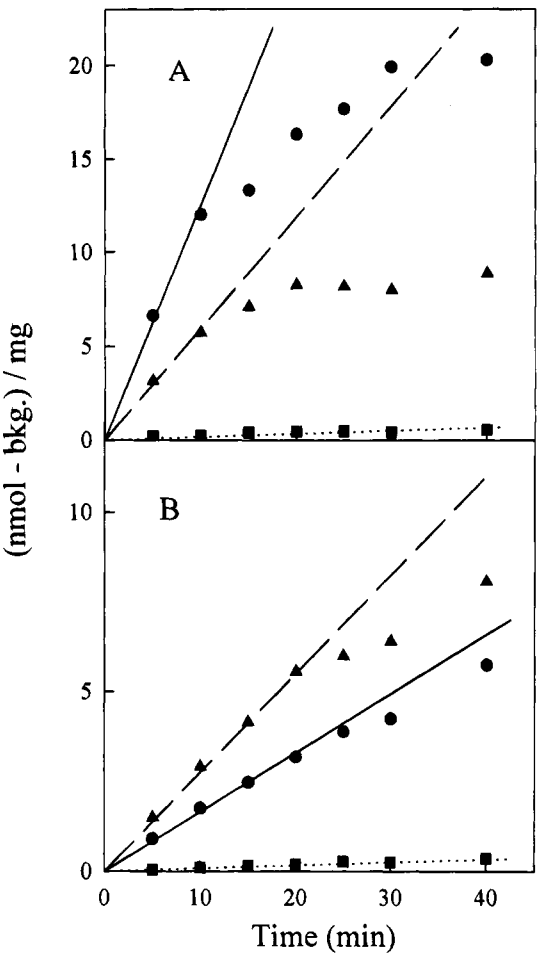


FIGURE 1: $cPLA_2$-catalyzed hydrolysis of [^{3}H]SAPC and [^{14}C]-SLPC and acyl transfer reactions to glycerol. (A) Kinetics of formation of [^{3}H]20:4 (●), *sn*-1/3-[^{3}H]20:4-MAG (▲), and *sn*-2-[^{3}H]20:4-MAG (■) from [^{3}H]SAPC. Background values for the products were 0.15 ± 0.03, 0.09 ± 0.01, and 0.11 ± 0.02 pmol, respectively. The solid, dashed, and dotted lines are drawn for rates of formation of 1.3, 0.6, and 0.02 nmol/min/mg, respectively. (B) Kinetics of formation of [^{14}C]18:3 (●), *sn*-1/3-[^{14}C]18:3-MAG (▲), and *sn*-2-[^{14}C]18:3-MAG (■) from [^{14}C]SLPC. Background values for the products were 0.50 ± 0.15, 0.12 ± 0.03, and 0.13 ± 0.01 pmol, respectively. The dashed, solid, and dotted lines are drawn for rates of formation of 0.28, 0.17, and 0.01 nmol/min/mg, respectively. Reaction mixtures contained 50 mM Tris (pH 8.0), 1 mM $CaCl_2$, 0.1 mM EGTA, 30% glycerol (by volume) and 8 μg of $cPLA_2$ in a total volume of 0.40 mL at 30 °C. The reactions were initiated by the addition of substrate-containing micelles to give final concentrations of 0.4 mM Triton X-100, 20 μM DTPM, 1.1 μM [^{3}H]SAPC (400 mCi/mmol) and 1.1 μM [^{14}C]SLPC (55 mCi/mmol) in the assay. Aliquots of 0.05 mL were removed and quenched at the times given in the figures. Reaction blanks did not contain enzyme and were quenched at 5, 20, and 40 min.

acyl transfer products formed to the amount of hydrolysis product, which are all derived from [^{14}C]SLPC, is 1.7; this ratio is approximately 4-fold greater than the ratio that describes similar reactions with [^{3}H]SAPC as the substrate.

Similar experiments were conducted with 1,3-propanediol as a potential acyl acceptor and [^{3}H]SAPC in DTPM-Triton micelles as the substrate. 1,3-Propanediol, at a concentration of 30%, stimulates the hydrolysis of [^{3}H]SAPC by the enzyme by approximately 3-fold, and a radiolabeled acyl-propanediol adduct was formed during the reaction (data not shown). Both the stimulation of hydrolysis and the amount of formation of the acyl-propanediol adduct increase with increasing concentrations of 1,3-propanediol in the assay. These results show that 1,3-propanediol is an acyl acceptor for the enzyme, and they are consistent with the observation that *sn*-1/3-MAG is the major acylation product formed from glycerol since 1,3-propanediol lacks a secondary hydroxyl group.

The ratio that describes the amount of MAG products formed in relation to fatty acid product formed during the linear part of the reactions is a measure of the relative rates of the two reactions only when the MAG product is not hydrolyzed by the enzyme. Otherwise, the ratio is an underestimation of the rate of acyl transfer and an overestimation of the rate of hydrolysis of a given substrate. To determine whether and to what extent the enzyme hydrolyzes the MAG products that are formed, 10 pmol of *sn*-1/3-[^{14}C]-18:3-MAG (0.2 μM) in DTPM-containing micelles was incubated with $cPLA_2$ in the presence of 30% glycerol; this amount of MAG is approximately equal to that formed in 30 min for the experimental conditions described for Figure 1B. The rate of enzyme-catalyzed hydrolysis of *sn*-1/3-[^{14}C]-18:3-MAG was ~0.005 nmol/min/mg (data not shown), which is at least 34-fold slower than the formation of [^{14}C]-18:3 shown in the figure. The measured slow rate of hydrolysis of *sn*-1/3-[^{14}C]18:3-MAG by $cPLA_2$ indicates that significant hydrolysis of the *sn*-1/3-[^{14}C]18:3-MAG product formed from [^{14}C]SLPC did not occur, and the ratio of the rates of the acyl transfer reactions to the hydrolysis reaction is an accurate measure of the amounts of the products that are formed from [^{14}C]SLPC by the enzyme.

Similar experiments with 45 pmol of *sn*-1/3-[^{3}H]20:4-MAG and 55 pmol of [^{14}C]PAPC (0.9 and 1.1 μM, respectively), which were present together in DTPM-containing micelles, showed that *sn*-1/3-20:4-MAG was hydrolyzed at a rate of 0.23 nmol/min/mg (data not shown). Separate experiments with unlabeled *sn*-2-20:4-MAG, in which the hydrolysis products were quantified by gas chromatography/mass spectrometry, showed that there was no detectable hydrolysis of *sn*-2-20:4-MAG by the enzyme (data not shown). The rate of hydrolysis of *sn*-1/3-20:4-MAG can be significant, and an interpretation of experiments that measure the formation of 20:4-MAG by the enzyme must address the likelihood that the amount of 20:4-MAG that is measured is an underestimation of what is actually formed.

Activation of $cPLA_2$ by Glycerol. $cPLA_2$-catalyzed hydrolysis of phospholipids is activated by glycerol (Clark et al., 1990; Reynolds et al., 1993; Sharp et al., 1994; Street et al., 1993), and Figure 2 shows that an increase in the concentration of glycerol in the assay from 0% to 30% (by volume) increases both the rate of hydrolysis of [^{14}C]SLPC and the rate of acyl transfer of *sn*-2-[^{14}C]18:3 to glycerol. The rates of the reactions were obtained from the initial linear portions of plots of product formation versus time, which are similar to those shown in Figure 1. The observed increase in the rate of formation of MAG is expected for a concentration dependent reaction of glycerol with enzyme-bound phospholipid substrate to give the MAG products. Glycerol also increases the rate of hydrolysis of [^{14}C]SLPC. Ratios of the rate of transacylation to the rate of hydrolysis are given for the concentrations of glycerol tested, and the solid line is drawn for a proportional increase in the ratio with an increasing concentration of glycerol in the assay and for a ratio of 0 when glycerol is not present. The proportional increase is consistent with a concentration dependent second-order reaction of glycerol with bound substrate.

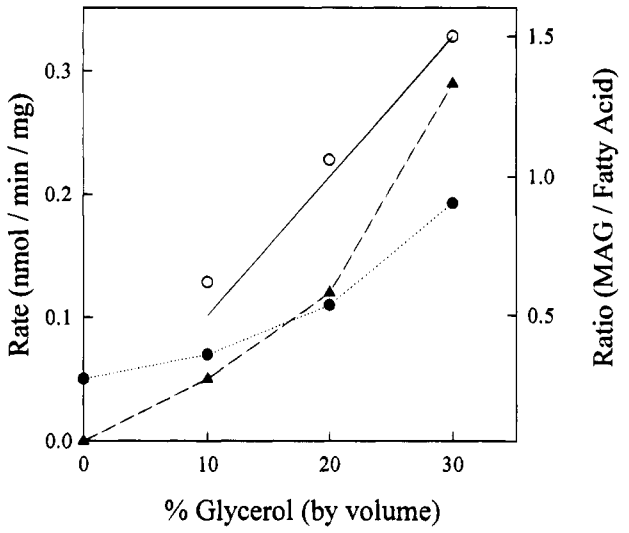


FIGURE 2: Increased rates of hydrolysis of [^{14}C]SLPC and acyl transfer to glycerol with increasing concentrations of glycerol. The rates of hydrolysis and acyl transfer are given by ● and ▲, respectively. The ratios of the rates of acyl transfer to the rate of hydrolysis are given by ○. The solid line describes a ratio of MAG/fatty acid of 0 at 0% glycerol and a proportional increase in the ratio with increasing concentrations of glycerol. The reaction conditions are as described for Figure 1B, with the exceptions that 1.1 μM [^{14}C]SLPC (55 mCi/mmol) was the only radiolabeled substrate present, and the concentrations of glycerol in the assays are given in the figure.

Table 1: Substrate Specificity of cPLA$_2$: Relative Values of k_{cat}/K_M* for Labeled Substrates in Product-Containing Vesicles[a]

labeled substrate[b]	relative k_{cat}/K_M*
[^{14}C[PAPC	1.0
[^{3}H]HAPC	0.84 ± 0.07
[^{3}H]AAPC	0.78 ± 0.07
[^{3}H]SAPC	0.61 ± 0.07
[^{14}C]APPC	0.29 ± 0.10
[^{3}H]DPPC	0.12 ± 0.08
L-[^{3}H]AHPC	0.61 ± 0.07
rac-[^{3}H]AHPC	0.71 ± 0.06

[a] ^{3}H- and ^{14}C-labeled substrates (approximately 10 mol % each, 6 μM total phospholipid) were both present in sonicated vesicles of 70 mol % OPPC that contained 10 mol % S-lyso-PC and unlabeled 20:4. [b] Pairs of ^{14}C and ^{3}H substrates tested were, respectively, PAPC/HAPC, PAPC/AAPC, PAPC/SAPC, APPC/SAPC, PAPC/DPPC, PAPC/L-AHPC, and PAPC/*rac*-AHPC.

Substrate Specificity of cPLA$_2$. Table 1 gives relative values of k_{cat}/K_M* for radiolabeled phosphatidylcholine substrates when low concentrations of substrate are present in vesicles of OPPC that contain 10 mol % each 20:4 and S-lyso-PC. The enzyme binds tightly to these product-containing vesicles, and the measured substrate preferences reflect the substrate specificity of cPLA$_2$ at the interface rather than differential binding of enzyme to vesicles that differ in their composition (Ghomashchi et al., 1991a; Hanel et al., 1993). The substrates are ranked in decreasing order relative to the value of k_{cat}/K_M* for [^{14}C]PAPC, which was hydrolyzed most rapidly and gave the greatest extent of hydrolysis. The values were determined according to eq 1 by measuring the rates of hydrolysis of pairs of labeled substrates (S^x), one of which is ^{14}C labeled and the other is ^{3}H labeled, that were present in product-containing vesicles. The reaction velocities, v^x, are those obtained for the ^{14}C- and ^{3}H-radiolabeled substrates.

$$\frac{(k_{cat}/K_M^*)^{^3H}}{(k_{cat}/K_M^*)^{^{14}C}} = \frac{v^{^3H}}{v^{^{14}C}} \frac{[S^{^{14}C}]_{t=0}}{[S^{^3H}]_{t=0}} \quad (1)$$

The results of these competitive substrate experiments suggest that for phosphocholine substrates, which have *sn*-2-arachidonyl acyl chains, an increase in the length of a saturated acyl chain at position 1 decreases the value of k_{cat}/K_M* (PAPC, HAPC > SAPC). However, this decrease in the value of k_{cat}/K_M* apparently is offset when the *sn*-1 acyl chain is unsaturated (AAPC > SAPC, APPC > DPPC). DPPC is hydrolyzed by the enzyme when present in product-containing vesicles, albeit 10-fold more slowly than PAPC, while vesicles comprised solely of DPPC are not hydrolyzed (Ghomashchi et al., 1992). Finally, the enzyme shows little if any preference for substrates with an *sn*-1 acyl chain over the corresponding substrate with an *sn*-1 alkyl chain (PAPC ≈ HAPC) [Table 1 and Diez et al. (1992)]. Separate experiments in which the relative and absolute concentrations of pairs of substrates were changed by up to 3-fold showed that eq 1 is valid for the analysis of relative values of k_{cat}/K_M* of labeled substrates present as a small mole fraction of product-containing vesicles. Competitive double-labeled experiments have also defined the substrate specificities of both cPLA$_2$ and sPLA$_2$ with regard to *sn*-2 acyl chain (Ghomashchi et al., 1991a; Hanel et al., 1993) and the specificity of cPLA$_2$ with regard to the polar head group of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phospholipid substrates (Hanel et al., 1993).

The influence of the structure of the *sn*-1 acyl chain of phosphatidylcholine substrates, which have radiolabeled palmitic acid at the *sn*-2 position, on the transacylation and hydrolysis reactions was investigated with the substrates [^{3}H]-DPPC and [^{14}C]APPC present in micelles. The initial rate of acyl transfer from [^{3}H]DPPC was 1.8−2.4-fold greater than the initial rate of hydrolysis (data not shown). The initial rates of hydrolysis and acyl transfer for [^{14}C]APPC were approximately 2-fold greater than those observed for [^{3}H]DPPC; this is consistent with the relative values of k_{cat}/K_M* of 0.29 and 0.12, respectively, given in Table 1 when the two substrates were contained in vesicles. These results show that structural differences in the *sn*-1 acyl chains of phosphatidylcholine substrates affect both the rate and the extent of the hydrolysis and transacylation reactions.

Phospholipase A$_1$ Activity of the Enzyme. Table 1 shows that the enzyme efficiently catalyzes the hydrolysis of the *sn*-1-arachidonyl acyl chain of *rac*-[^{3}H]AHPC. The enzyme has phospholipase A$_1$ activity. Expression of this activity in baculovirus-infected Sf9 insect cells was dependent on the presence of the gene encoding cPLA$_2$ (data not shown). The relative k_{cat}/K_M* value for the racemic substrate is 0.71. The L-enantiomer, L-[^{3}H]AHPC, was chemically synthesized, and Table 1 shows that the relative value of k_{cat}/K_M* of 0.61 for the L enantiomer is similar to that measured for the racemic mixture. This fact and the observation that both substrates are hydrolyzed at similar rates and to similar extents when present at concentrations of 10 mol % (data not shown) demonstrate that both enantiomers of AHPC are substrates for the enzyme. If the D enantiomer were either an inhibitor of the enzyme or simply not a substrate, then, in both cases, the relative value of k_{cat}/K_M* measured for the racemic mixture would be one-half of the value measured

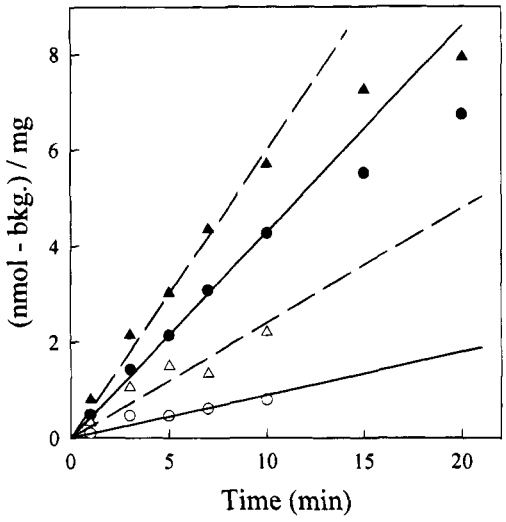


FIGURE 3: Phospholipase A_1 activity of $cPLA_2$ with *rac*-[^{3}H]AHPC; kinetics of formation of [^{3}H]20:4 (○, ●) and total [^{3}H]20:4-MAG (△, ▲) in either the absence (●, ▲) or presence of 5 μM $AACOCF_3$ (○, △). The dashed lines describe rates of acyl transfer of 0.60 and 0.24 nmol/min/mg in the absence and presence of $AACOCF_3$, respectively. The solid lines describe rates of hydrolysis of 0.43 and 0.09 nmol/min/mg in the absence and presence of $AACOCF_3$, respectively. Background values for the products were 0.27 ± 0.05 and 0.30 ± 0.10 pmol, respectively. The reaction conditions are as described in the legend of Figure 1. The reactions were initiated by the addition of the substrates in mixed micelles to give final concentrations in the assay of 0.4 mM Triton X-100, 20 μM DTPM, 1.1 μM [^{3}H]AHPC (400 mCi/mmol), 1.1 μM [^{14}C]LHPC (55 mCi/mmol) (●, ▲), and 5 μM $AACOCF_3$ (○, △). Reaction blanks did not contain enzyme and were measured at 1, 10, and 20 min.

for the L enantiomer alone. This is because the velocities of the hydrolysis reactions are proportional to both the concentrations of respective substrates for the reactions and the values of k_{cat}/K_M* for the substrates (eq 1). The concentration of L enantiomer of 10 mol % *rac*-[^{3}H]AHPC is one-half that of 10 mol % L enantiomer, and a relative value of k_{cat}/K_M* for the L enantiomer of the racemic mixture of 0.30 would reflect this difference in concentration. Inhibition of the reaction by the D enantiomer would result in a decrease in the rates of formation of both [^{3}H]20:4 and [^{14}C]20:4; neither observation was made.

The phospholipase A_1 activity of the enzyme was also measured for diacylphospholipid substrates. The reaction mixtures did not contain glycerol, and the reaction products of $cPLA_2$-catalyzed hydrolysis of either [^{3}H]AAPC or [^{14}C]-PAPC (label present in the *sn*-2 acyl chain) present in product-containing vesicles were analyzed by TLC, which resolved the phospholipid substrate from the fatty acid and lysophospholipid products. No detectable radioactivity above background levels comigrated with the lysophospholipid product, even though 13% of the initial label from [^{14}C]-PAPC and 4% of initial label from [^{3}H]AAPC comigrated with the fatty acid product after the addition of enzyme (data not shown).

Figure 3 shows that the phospholipase A_1 activity of the enzyme catalyzes the transacylation reaction that results in the formation of [^{3}H]20:4-MAG from *rac*-[^{3}H]AHPC when 30% glycerol is present in the assay and the substrate is contained in DTPM mixed micelles. Hydrolysis of the substrate also occurs. A lower limit for the ratio of MAG products to fatty acid product is 1.4 for the phospholipase A_1 reaction with *rac*-[^{3}H]AHPC; it is a lower limit because 20:4-MAG is hydrolyzed by the enzyme. The presence of 5 μM $AACOCF_3$ in substrate-containing micelles inhibits the phospholipase A_1 hydrolytic activity and the transacylase activity. The mixed micelles also contained *rac*-[^{14}C]LHPC, and this phospholipid both is hydrolyzed by the enzyme and is an acyl donor for the acylation of glycerol (data not shown). The rates of hydrolysis and transacylation obtained with *rac*-[^{14}C]LHPC were 6- and 3.5-fold slower, respectively, than the rates obtained with *rac*-[^{3}H]AHPC. A value of 2.4 is estimated for the ratio of MAG products to 18:3 hydrolysis product. The presence of 5 μM $AACOCF_3$ completely inhibited both the hydrolysis and acyl transfer reactions of the enzyme with *rac*-[^{14}C]LHPC as a substrate. Similar experiments with *rac*-[^{14}C]PHPC that were done in the absence of $AACOCF_3$ gave no measurable hydrolysis, and the rate of acyl transfer to glycerol was 0.03 nmol/min/mg (data not shown).

Esterase Activity of $cPLA_2$. The mechanism of hydrolysis by $cPLA_2$ is not known; however, three reports in the literature suggest that a covalent acyl enzyme intermediate is formed during the hydrolytic reaction (Reynolds et al., 1993; Sharp et al., 1994; Trimble et al., 1993). A kinetic approach that tests for the existence of an acyl enzyme intermediate measures how the intermediate, which is formed from different substrates, reacts with competing nucleophiles that are present in the assay (Jencks, 1969). Fatty acid ester substrates of the $cPLA_2$ that are similar in structure but differ in the basicity of the leaving group, and hence differ in their reactivity with water, will give the same ratio of products formed with the nucleophiles, if a common enzyme intermediate is generated during the reaction. Figures 1−3 show that glycerol competes with water as a nucleophile.

A series of *para*-substituted phenyl esters of radiolabeled 18:3, which had different substituents at the *para* position of the phenyl ring, was prepared as substrates for the $cPLA_2$. Figure 4A,B shows the kinetics of hydrolysis and transfer of labeled 18:3 to glycerol for the *p*-cyanophenyl [^{3}H]-α-linolenate and phenyl [^{14}C]-α-linolenate substrates, respectively. The reaction mixtures contained 30% glycerol, and the substrates were present together in mixed micelles containing DTPM. The acyl transfer product *sn*-1/3-18:3-MAG is the major product that is formed from both substrates, and the rate of acyl transfer to the primary alcohol of glycerol is 0.10 nmol/min/mg for each substrate. This rate is 28-fold greater than the enzyme-catalyzed rate of 0.004 nmol/min/mg for the formation of the *sn*-2-18:3-MAG product. The rates of formation of [^{3}H]18:3 and [^{14}C]18:3 are 0.056 and 0.049 nmol/min/mg, respectively (Figure 4A,B, respectively). Similar reactions with a concentration of glycerol of 10% (by volume) in the assay and phenyl [^{3}H]-α-linolenate as the substrate showed that the ratio of MAG products to fatty acid product decreases when the concentration of glycerol is lowered in the assay. The rates of hydrolysis and acyl transfer are 0.08 and 0.05 nmol/min/mg, respectively (data not shown). This corresponds to a partition ratio, which is defined as the ratio of the rate of formation of total MAG products (*sn*-1/3-MAG and *sn*-2-MAG) to the rate of formation of the hydrolysis product 18:3, of 0.67, and this ratio is ~3-fold less than the ratios given in Figure 4. This result is consistent with a second-order

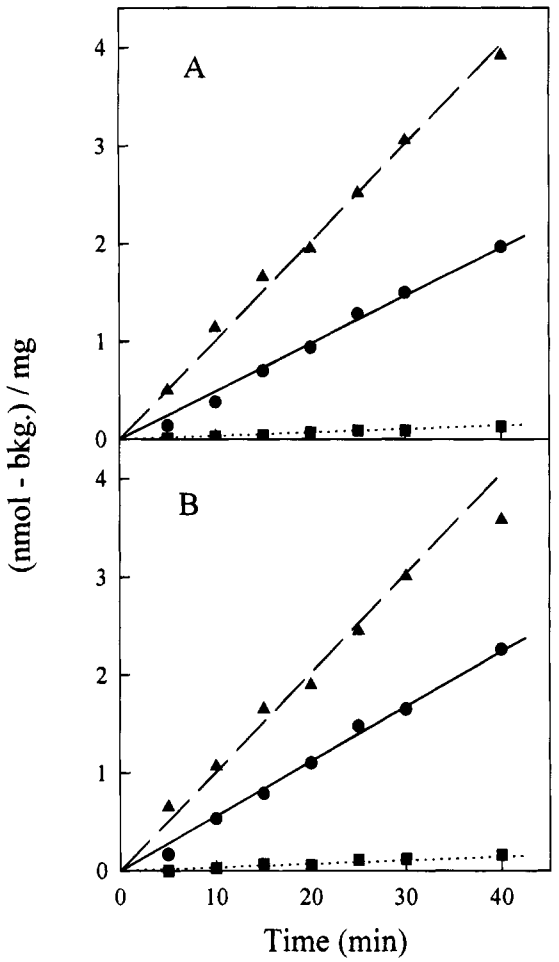


FIGURE 4: cPLA$_2$-catalyzed hydrolysis of *p*-cyanophenyl [^{3}H]-α-linolenate and phenyl [^{14}C]-α-linolenate and acyl transfer reactions to glycerol. (A) Kinetics of formation of [^{3}H]18:3 (●), *sn*-1/3-[^{3}H]18:3-MAG (▲), and *sn*-2-[^{3}H]18:3-MAG (■) from *p*-cyanophenyl [^{3}H]-α-linolenate. The dashed, solid, and dotted lines are drawn for rates of 0.10, 0.049, and 0.004 nmol/min/mg, respectively. Background values for the fatty acid, *sn*-1/3-MAG and *sn*-2-MAG products were 0.46 ± 0.02, 0.24 ± 0.03, and 0.12 ± 0.04 pmol, respectively. (B) Kinetics of formation of [^{14}C]18:3 (●), *sn*-1/3-[^{14}C]18:3-MAG (▲), and *sn*-2-[^{14}C]18:3-MAG (■) from phenyl [^{14}C]-α-linolenate. The dashed, solid, and dotted lines are drawn for rates of formation of 0.10, 0.056, and 0.004 nmol/min/mg, respectively. Background values for the fatty acid, *sn*-1/3-MAG, and *sn*-2-MAG products were 0.77 ± 0.05, 0.23 ± 0.03, and 0.15 ± 0.02 pmol, respectively. Reaction mixtures and conditions are as described in the legend of Figure 1. Reactions were initiated by the addition of substrate-containing micelles to give final concentrations in the assay of 0.4 mM Triton X-100, 20 μM DTPM, 1.1 μM phenyl [^{14}C]-α-linolenate (55 mCi/mmol), and 1.1 μM *p*-cyanophenyl [^{3}H]-α-linolenate (400 mCi/mmol). Reaction blanks did not contain enzyme and were quenched at 5, 20, and 40 min.

reaction of glycerol and bound substrate that is dependent on the concentration of glycerol, since a 3-fold decrease in the concentration of glycerol resulted in a 3-fold decrease in the partition ratio.

Figure 4 also shows that the rates of formation of the products derived from the ester substrates do not change significantly during the time of the assay. Table 2 gives the rates of formation of these products and the partition ratios for the phenyl ester substrate of 18:3 and five *para*-substituted phenyl ester substrates of 18:3 that were tested. The range of values for the pK_a of the *para*-substituted phenol leaving groups is between 7.1 and 10.2. Each reaction mixture contained the [^{14}C]18:3 phenyl ester and a single [^{3}H]18:3 *para*-substituted phenyl ester present together in mixed micelles containing DTPM. The presence of the [^{14}C]-18:3 unsubstituted phenyl ester in each assay provides a measure of the variation in the partition ratio that describes the hydrolysis of and acyl transfer from this substrate to glycerol.

Table 2 shows that the partition ratio for the ester substrates does not change significantly when the leaving group is changed. This result is consistent with the formation of an enzyme intermediate that is common to all of the *para*-substituted phenyl ester substrates tested, and this intermediate reacts with 30% glycerol approximately twice as fast as it does with water. The combined rates of the transacylation and hydrolysis reactions are affected by the substituent at the *para* position of the phenyl ester leaving group. The relative order of k_{cat}/K_M* for the substrates is as follows: Cl < NO_2 < OCH_3 ≈ F < H ≈ CN. The combined rates for the hydrolysis and transacylation reactions of the *para*-substituted substrates are compared to the corresponding combined rates for the unsubstituted phenyl [^{14}C]-α-linolenate substrate of the substrate pair, and small differences in concentration of the substrates are accounted for in accordance with eq 1. Attempts were made to measure the nonenzymatic acylation of glycerol by these substrates at pH 11 and 50% glycerol (by volume). Nonenzymatic formation of monoacylglycerol from any of the substrates was not detected, but the esters did hydrolyze: the rate of hydrolysis increased with decreasing basicity (lower values of pK_a) of the phenyl leaving group (data not shown).

AACOCF$_3$, which inhibits the cPLA$_2$-catalyzed hydrolysis of phospholipids [Figure 4 and Street et al. (1993)], also inhibits the hydrolysis of the phenyl ester of 18:3. Using the standard reaction conditions, the enzyme was incubated for 1 h with DTPM-containing micelles, which contained 20 μM AACOCF$_3$, and a DMSO suspension of [^{3}H]18:3 phenyl ester was then added to give final concentrations of radiolabeled substrate and DMSO of 1 μM and 4% (by volume), respectively. The reactions were quenched after 1 h. AACOCF$_3$ blocked the hydrolysis and transacylation reactions by 83%, while AACOCH$_3$ did not inhibit the reactions (data not shown).

Accumulation of the cPLA$_2$·[^{14}C]18:3 Phenyl Ester Complex. Figure 5A shows that ~95% of the labeled material that is bound to the enzyme is unreacted substrate during the reaction of cPLA$_2$ with [^{14}C]18:3 phenyl ester. The enzyme was mixed with approximately 5 equiv of labeled substrate for increasing reaction times, and equal volumes of the reaction mixture were either forced through a size-exclusion centrifugation column and quenched or quenched directly at the times given in Figure 5A,B, respectively. The column does not retain cPLA$_2$ with bound radiolabel, while radiolabeled substrate and products that are not bound to the enzyme remain on the column. A cPLA$_2$−substrate complex formed within 1 min, and the maximal extent of formation of 12 pmol after correction for the recovery of enzyme from the column corresponds to the total amount of enzyme applied. The complex persisted for ~5 min and then decreased with time to levels that were similar to those measured by passage of substrate alone through the column. Less than 1 pmol each of the hydrolysis reaction product [^{14}C]18:3 and the [^{14}C]MAG transacylation products were associated with the enzyme during the experiment. A similar experiment using substrate contained in Triton/DTPM mi-

Table 2: Reaction Rates and Partitioning Ratios for the cPLA$_2$-Catalyzed Hydrolysis and Transacylation Reactions[a]

leaving group	pK_a[b]	rate of formation of [^{3}H]18:3 (nmol/min/mg)	rate of formation of [^{3}H]MAG (nmol/min/mg)	rate of formation of [^{14}C]18:3 (nmol/min/mg)	rate of formation of [^{14}C]MAG (nmol/min/mg)	ratio[c] of [^{3}H]MAG/18:3	ratio[c] of [^{14}C]MAG/18:3
$-O-C_6H_4-p-NO_2$	7.15	0.024	0.040	0.046	0.096	1.7	2.1
$-O-C_6H_4-p-CN$[d]	7.96	0.056	0.11	0.053	0.10	2.0	1.9
$-O-C_6H_4-p-Cl$	9.42	0.012	0.026	0.060	0.13	2.2	2.2
$-O-C_6H_4-p-F$	9.88	0.038	0.069	0.058	0.12	1.8	2.1
$-O-C_6H_5$	9.90	0.050	0.10	0.048	0.095	2.0	2.0
$-O-C_6H_4-p-OCH_3$	10.19	0.026	0.054	0.042	0.093	2.1	2.2
S-lyso-PC[e]	~16[f]	no data	no data	0.17	0.29	no data	1.7

[a] The reaction conditions are as described in the legend of Figure 1. Each reaction contained ~1 μM [^{14}C]18:3 phenyl ester and ~1 μM [^{3}H]-*para*-substituted phenyl ester. The rates are taken from the linear portions of plots of product formation as a function of time. [b] Ritchie, 1990. [c] MAG is the rate of formation of total transacylation products (*sn*-1/3-MAG + *sn*-2-MAG). [d] The data are taken from Figure 5. [e] The data are taken from Figure 1B. [f] Reeve et al., 1979.

celles showed that all of the bound label was unreacted substrate (data not shown). Total product formation is also shown.

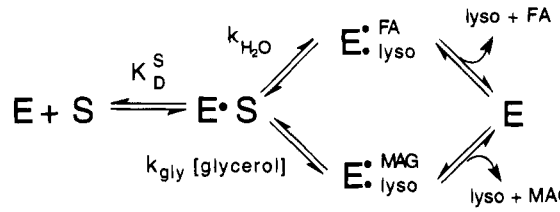
Total products of the reaction were measured by directly quenching aliquots of the reaction mixture at the times given in Figure 5B. The formation of the MAG transacylation products and the 18:3 fatty acid hydrolysis product is biphasic when the substrate is introduced as a suspension in DMSO; the kinetics of total product formation is described by a burst followed by a slower phase that ceases by 60 min. The cause of the burst is not known. It may be the result of the formation of a substrate aggregate that the enzyme rapidly hydrolyzes. The ratio of MAG products to fatty acid product decreases with increasing time of reaction; this suggests that 18:3-MAG is hydrolyzed significantly by cPLA$_2$ when Triton/DTPM micelles are absent. The kinetics and extent of formation of total products after 5 min (Figure 5A) are approximately the same as those that describe the time dependent decrease in the enzyme−substrate complex that elutes from the size-exclusion chromatography columns.

Lysophospholipase Activity of cPLA$_2$. cPLA$_2$ catalyzes the hydrolysis of lysophospholipids (Fujimori et al., 1993; Leslie, 1991; Reynolds et al., 1993, Nalefski et al., 1994). A ^{14}C-labeled analogue of 1-palmitoyl-*sn*-lysophosphocholine that does not have an *sn*-2 hydroxyl group, 1-[^{14}C]palmitoylpropanediol-3-phosphocholine, was synthesized and tested as a substrate for the lysophospholipase reaction. Figure 6A shows that this substrate is both hydrolyzed and serves as an acyl donor to glycerol. The rates of the reactions decrease with increasing time, but the ratio of transacylation products to hydrolysis product of ~2 does not change significantly during the reaction.

$AACOCF_3$ was tested as an inhibitor of the lysophospholipase reaction. The reaction of 1-[^{14}C]palmitoylpropanediol−3-phosphocholine with the enzyme is inhibited by 5 μM $AACOCF_3$; the compound inhibits both the hydrolysis and acyl transfer reactions (Figure 6A). The ratio of transacylation products to hydrolysis product formed remains ~2 for the inhibited reaction. $AACOCH_3$ did not inhibit the reactions (data not shown).

The reaction mixture also contained 0.7 μM [^{3}H]SAPC, and Figure 6B shows the formation of the hydrolysis product [^{3}H]20:4 and the [^{3}H]MAG products. The ratio of transacylation products to hydrolysis product is 0.40. This is in reasonable agreement with the ratio of 0.46 taken from Figure 1A, which is estimated from the initial rates of the reactions with [^{3}H]SAPC when [^{14}C]SLPC was present. $AACOCF_3$ blocks both the hydrolysis of [^{3}H]SAPC and the acyl transfer to glycerol, while $AACOCH_3$ did not inhibit the reactions (data not shown). These results are consistent with those of Street et al. (1993) for the inhibition of hydrolysis of SAPC.

Scheme 1



DISCUSSION

This report describes experiments that show that glycerol is an acyl acceptor for the transacylase activity of cPLA$_2$. The first report of transacylase activity of the enzyme described the formation of the diacylphospholipid, DPPC, by enzymatic transfer of the acyl group of 1-palmitoyl-*sn*-glycero-3-phosphocholine to a second molcule of lysophospholipid (Reynolds et al., 1993). Glycerol competes with water as a nucleophile at the active site of the enzyme (Figures 1−6), and the competition results in the formation of primarily *sn*-1/3-MAG. Acyl transfer to the *sn*-2 position of glycerol is ≥20-fold less favored. Glycerol is commonly used in assays of cPLA$_2$ activity because it activates the hydrolysis activity of the enzyme [Figure 2 and Clark et al. (1990), Reynolds et al. (1993), Sharp et al. (1994), and Street et al. (1993)]. Measurements of cPLA$_2$ activity in the presence of glycerol will underestimate the total activity of the enzyme if the MAG product of the reaction is not accounted for, and investigators should be aware of this.

Stimulation of cPLA$_2$ activity by glycerol was studied by measuring the effect of increasing concentrations of glycerol on the formation of fatty acid hydrolysis product and MAG transacylation products from [^{14}C]SLPC, which was present in mixed micelles (Figure 2). A simple model that explains, in part, the observed activation of cPLA$_2$ by glycerol is shown in Scheme 1. According to the scheme, the product ratio, which is defined as the ratio of the rate of formation of MAG and lysophospholipid (lyso) to the rate of hydrolysis of phospholipid substrate (S), will increase linearly with increasing concentrations of glycerol in the assay because $v_{MAG} = k_{gly}[E{\cdot}S][glycerol]$, while the rate of hydrolysis of bound substrate to give fatty acid (FA) is described by $v_{H_2O} = k_{H_2O}[E{\cdot}S]$. The solid line of Figure 2 shows that the data are in reasonable agreement with the proportional increase in the ratio expected for the model. Scheme 1 does not

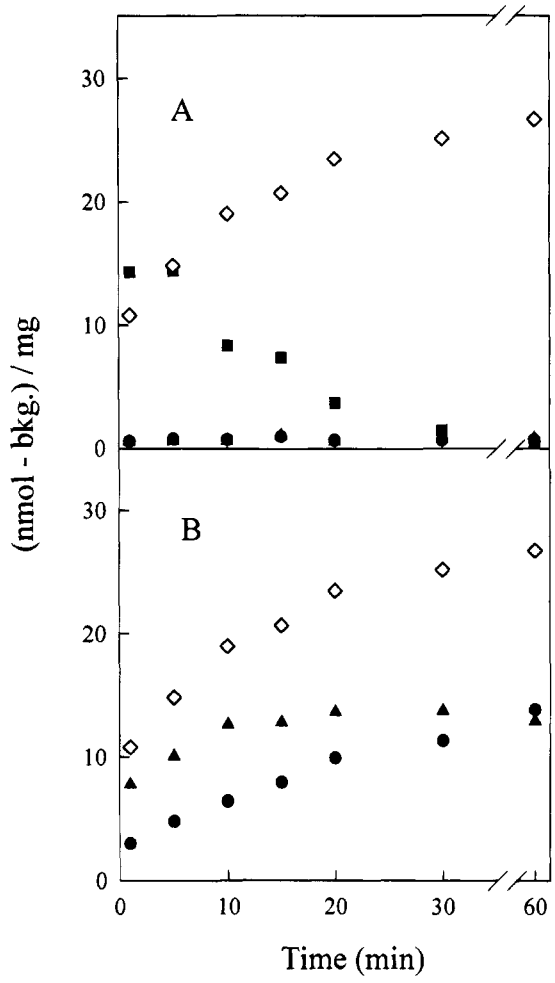


FIGURE 5: Isolation by rapid gel filtration of a cPLA$_2$–substrate complex during the reaction with phenyl [^{14}C]-α-linolenate. (A) Radiolabeled substrate and products eluting with the enzyme are phenyl [^{14}C]-α-linolenate (■), [^{14}C]18:3 (●), and [^{14}C]18:3-MAG (▲). Background values were 0.73 ± 0.2, 0.29 ± 0.03, and 0.28 ± 0.03 pmol, respectively. Total product formation (◇) is the sum of [^{14}C]18:3 (●) and total [^{14}C]18:3-MAG (▲) taken from Figure 6B. Approximately 12 pmol of enzyme was applied to the column at each time point. (B) Hydrolysis and acyl transfer products formed during the reaction, as measured by direct quenching: [^{14}C]-18:3 (●); total [^{14}C]18:3-MAG (▲). Total product formation (◇) is the sum of [^{14}C]18:3 and total [^{14}C]18:3-MAG. The background values for the reactions were 1.06 ± 0.15 and 1.16 ± 0.05 pmol, respectively. Reaction mixtures contained 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 0.1 mM EGTA, 30% glycerol (by volume), and 16 μg of cPLA$_2$ in a total volume of 0.80 mL at 30 °C. The reactions were initiated by the addition of phenyl [^{14}C]-α-linolenate (55 mCi/mmol) in DMSO to give final concentrations of 1.1 μM and 4% (by volume), respectively. Aliquots of 50 μL were removed at the times given in the figure and either applied to a gel filtration centrifuge spin column (A) or quenched directly (B). Reaction blanks did not contain enzyme and were measured at 1, 30, and 60 min.

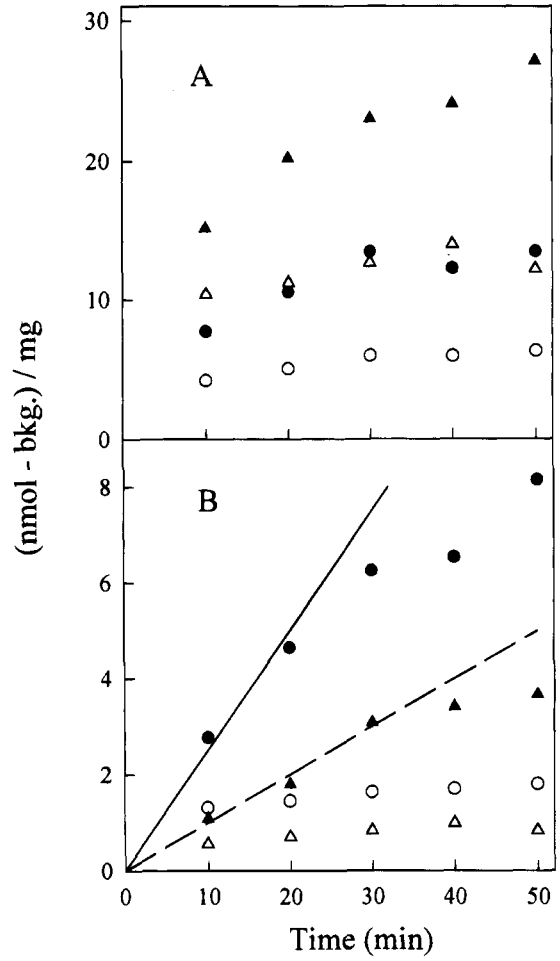


FIGURE 6: cPLA$_2$-catalyzed hydrolysis of 1-[^{14}C]palmitoylpropanediol-3-phosphocholine and [^{3}H]SAPC and acyl transfer reactions to glycerol. (A) Kinetics of formation of [^{14}C]16:0 (●, ○) and [^{14}C]16:0-MAG (▲, △) in either the absence (●, ▲) or presence of 5 μM $AACOCF_3$ (○, △). Background values for the fatty acid and MAG products were 0.28 ± 0.11 and 1.15 ± 0.06 pmol, respectively. (B) Kinetics of formation of [^{3}H]20:4 (●, ○) and [^{3}H]20:4-MAG (▲, △) from [^{3}H]SAPC in either the absence (●, ▲) or presence of 5 μM $AACOCF_3$ (○, △). The solid and dashed lines describe rates of 0.25 and 0.10 nmol/min/mg, respectively. Background values for the fatty acid and MAG products were 0.15 ± 0.05 and 0.66 ± 0.15 pmol, respectively. The reaction conditions are as described in the legend of Figure 1. The reactions were initiated by the addition of the substrates in mixed micelles to give final concentrations in the assay of 0.4 mM Triton X-100, 20 μM DTPM, 0.7 μM [^{3}H]SAPC, 1.1 μM 1-[^{14}C]palmitoylpropanediol-3-phosphocholine (●, ▲), and 5 μM $AACOCF_3$ (○, △). Reaction blank did not contain enzyme and were measured at 10 and 50 min.

explain why the rates of hydrolysis and transacylation reactions increase nonlinearly with increasing concentrations of glycerol. This activating effect of glycerol must affect the hydrolysis and transacylation reactions to similar extents; otherwise, the product ratio would not show the proportional increase with glycerol. Three possible explanations for this, which are given in the context of Scheme 1, are that glycerol either increases the concentration in the assay of the reactive species, E·S, proportionally increases the values of k_{H_2O} and k_{gly}, or both. Increases in k_{H_2O} and k_{gly} represent increases in the intrinsic reactivities of water and glycerol at the active site, which may result from a conformational change in either the enzyme, the bound substrate, or both. Increased formation of E·S could result from glycerol either increasing the fraction of total enzyme bound to micelles, decreasing the affinity of the enzyme for DTPM, or increasing the affinity of the enzyme for SLPC. These changes in affinity could result from a conformational change in the enzyme that is induced by glycerol.

The catalytic mechanism of cPLA$_2$ is not well understood. There is indirect evidence suggesting that the mechanism is similar to that of serine proteases. To achieve rate enhancements, serine proteases employ covalent catalysis by forming an acyl enzyme intermediate between bound substrate and a serine residue at the enzyme's active site. Mutagenesis of a single serine residue of cPLA$_2$, serine-228, results in the

complete loss of both phospholipase A_2 and lysophospholipase activities (Sharp et al., 1994). These results suggest that serine-228, which resides within a sequence of amino acids of the enzyme that shows homology to other hydrolytic enzymes and is referred to as the "nucleophile elbow", is an active site nucleophile. Formation of a hemiketal adduct between the enzyme and $AACOCF_3$, which is an inhibitor of $cPLA_2$ phospholipase A_2 activity [Figure 6B and Street et al. (1993)], phospholipase A_1 activity (Figure 3), and lysophospholipase activity (Figure 6A), has been detected by NMR spectroscopy (Trimble et al., 1993). The adduct is presumably formed with a nucleophilic residue at the active site, possibly serine-228, and it may mimic a high-energy tetrahedral intermediate of the hydrolysis reaction. Precedent for this mechanism of binding and resultant inhibition of enzymatic activity by trifluoromethyl ketones is described for the serine protease chymotrypsin (Liang & Abeles, 1987). Finally, the transacylase activity of the enzyme is suggestive of the formation of an acyl enzyme intermediate. The formation of DPPC from two molecules of 1-palmitoyl-*sn*-lysophosphocholine by an alternative mechanism—direct nucleophilic attack by the *sn*-2 hydroxyl group of one molecule of lysophospholipid on the *sn*-1 acyl chain of another—would require the simultaneous accommodation of both substrate molecules at the active site. In contrast, the formation of an acyl enzyme intermediate from 1-palmitoyl-*sn*-lysophosphocholine would permit the dissociation of the *sn*-glycero-3-phosphocholine product and the binding of a second molecule of lysophospholipid, which subsequently reacts with the acyl intermediate to form DPPC.

A common acyl enzyme intermediate will form from dissimilar substrates that have the same acyl chain (i.e., phenyl α-linolenate and *p*-cyanophenyl α-linolenate). The common intermediate will react with competing nucleophiles and partition to give products (Jencks, 1969). The ratio of the amounts of products that are formed, the partition ratio, will depend on the nucleophiles that are present, their respective concentrations, and the reactivity of the intermediate toward those nucleophiles. However, the partition ratio should be insensitive to the chemical differences between the substrates present at the interface, which are lost after the intermediate is formed at the active site of the enzyme. Different substrates forming a common enzyme intermediate in the presence of competing nucleophiles should give the same partition ratio.

$cPLA_2$ catalyzes both the hydrolysis of esters (Huang et al., 1994) and the transfer of the acyl group of esters to glycerol (Figure 4 and Table 2). The transacylase activity allowed the use of *para*-substituted phenyl esters of α-linolenic acid as substrates for $cPLA_2$ in partitioning experiments. These esters react with the enzyme approximately 3−12-fold more slowly than the phospholipid [^{3}H]SLPC, but the kinetics of the enzyme-catalyzed reactions are linear and significantly above background levels, which did not increase with time. They were chosen because of the range of values of pK_a for the leaving groups, and because the sizes of the phenyl leaving groups are approximately the same and are small relative to lysophospholipid, which is the leaving group of phospholipid substrates. The differences between the partition ratios of 1.4 for *rac*-[^{3}H]AHPC (Figure 3) and 0.4 for [^{3}H]SAPC (Figure 1A) and the partition ratios of 2.4 for *rac*-[^{14}C]LHPC (not shown) and 1.7 for [^{14}C]SLPC (Figure 1B) suggest that the size of the leaving group may influence the partition ratio. The partition ratios for five *para*-substituted substrates (average = 2.0 ± 0.3, n = 5) are the same, within experimental error, as the value obtained for phenyl α-linolenate (average = 2.1 ± 0.1, n = 7). The product of the transacylation reaction, 18:3-MAG, is only very slowly hydrolyzed by the enzyme, and thus the observed partition ratios accurately reflect the amounts of the products produced. Table 2 provides evidence for the formation of a common enzyme intermediate from *para*-substituted phenyl esters of α-linolenic acid.

The similarity of the partition ratios for the substrates given in Table 2 is supportive evidence for the existence of a common intermediate, but it must be emphasized that the similarity does not prove that an acyl enzyme is formed. Attempts to identify by denaturing gel electrophoresis a form of $cPLA_2$ containing a [^{3}H]SAPC-derived radiolabeled acyl chain were unsuccessful. It is possible, however, that radiolabeled forms of the enzyme either did not accumulate to detectable levels during turnover or were unstable during the analysis. Rapid gel filtration of radiolabeled enzyme, which was formed with phenyl [^{14}C]-α-linolenate, showed that most of the radiolabel associated with the enzyme was unreacted substrate (Figure 5). This result is consistent with the slow formation of a covalent intermediate relative to all of the following: (1) binding of substrate; (2) dissociation of bound substrate and products; (3) reaction of the intermediate with either glycerol or water to give products.

Alternative mechanisms that do not invoke the formation of a covalent intermediate and could explain the formation MAG from glycerol and the synthesis of phospholipid from two molecules of lysophospholipid (Reynolds et al., 1993) are as follows. The active site of the enzyme may be large enough to both accommodate and orient two molecules of lysophospholipid so that an amino acid residue at the active site acting as a general base catalyzes the nucleophilic attack of one molecule of lysophospholipid on the other. The results of Reynolds et al. (1993) show that the hydrolysis reaction is ~20-fold faster than the formation of phospholipid. Smaller nucleophiles, such as glycerol, would be more easily accommodated and thus appear more reactive. Indeed, twice as much 16:0-MAG is formed from 1-palmitoylpropanediol-3-phosphocholine than lysophospholipid hydrolyzed (Figure 6).

A second, albeit less likely, alternative mechanism would involve the generation of noncovalently bound fatty acid at the active site that reacts with added nucleophiles such as glycerol or lysophospholipid. This mechanism would permit the dissociation of bulky lysophospholipid and the binding of the smaller nucleophile glycerol, and the mechanism would not require that both substrate and an alternative acyl acceptor be present simultaneously at the active site in order for the transacylation reaction to occur. A partition ratio of 1 for the reaction of a noncovalently bound fatty acid intermediate with glycerol means that equivalent barriers exist for the dissociation of fatty acid and the combined formation and dissociation of monoacylglycerol. The difference in partition ratios for the bound arachidonyl acyl group (≥0.4, Figures 1 and 6) and the bound α-linolenoyl acyl group (~2.0, Figure 1 and Table 2) suggests that the dissociation of larger, more unsaturated fatty acids is favored over reaction with glycerol. Our results give no indication of whether changes in the ratio that result from increased interactions of larger more sterically constrained fatty acids with the enzyme are caused by a decrease in the kinetic

barrier for the dissociation of fatty acid or a thermodynamic destabilization of a bound fatty acid intermediate. The results of Figure 5A do show that the intermediate does not accumulate during turnover. Furthermore, attempts to form the intermediate, reverse the hydrolysis reaction, and synthesize [^{3}H]SAPC by incubating the enzyme with 1-stearoyllysophosphatidylcholine and [^{3}H]20:4, which are components of product-containing vesicles, were unsuccessful (data not shown).

cPLA$_2$ has phospholipase A$_1$ activity. This conclusion is based on the observation that the enzyme hydrolyzes the radiolabeled *sn*-1 acyl chain of phosphocholine substrates that have the nonhydrolyzable *O*-hexadecyl alkyl chain at the *sn*-2 position (Figure 3 and Table 1). The hydrolysis reaction is not stereospecific; values of k_{cat}/K_M* for racemic [^{3}H]AHPC and the L isomer of [^{3}H]AHPC relative to the value of k_{cat}/K_M* for [^{14}C]PAPC are approximately the same. The enzyme also catalyzes the acylation of glycerol with these substrates (Figure 3). A phospholipase B enzyme from *Penicillium notatum* that has phospholipase A$_1$ activity, which is not stereospecific, has been reported (Saito et al., 1991; Sugatani et al., 1980). Similar to cPLA$_2$, which does not hydrolyze the diglyceride 1-stearoyl-2-arachidonyl-*sn*-glycerol (Hanel et al., 1993), but does hydrolyze the monoglyceride *sn*-1/3-20:4-MAG, this phospholipase B hydrolyzes monoacylglycerol but not diacylglycerol. Recently, Sharp et al. (1994) describe similarities between the primary amino acid sequences of this phospholipase B (Masuda et al., 1991) and cPLA$_2$. They noted that serine-228 of cPLA$_2$ resides within a sequence that is conserved between four hydrolytic enzymes, including the phospholipase B. They speculate that these enzymes may have structurally similar active sites, and the similarities between the phospholipase B and cPLA$_2$ catalytic activities described in this report support their assertion.

The phospholipase A$_1$ activity of cPLA$_2$ that this study describes may not be physiologically relevant. Experiments with diacylphosphatidylcholines, which had radiolabeled *sn*-2 acyl chains and were present in product-containing vesicles, showed that release of radiolabeled free fatty acid occurred as a result of the phospholipase A$_2$ reaction. However, no detectable radiolabeled lysophospholipid, which would have been the product of the phospholipase A$_1$ reaction, accumulated during the experiment. This result is consistent with the high preference of the enzyme for catalyzing the phospholipase A$_2$ reaction over the phospholipase A$_1$ reaction (Clark et al., 1990), and the kinetics of hydrolysis of 1-[^{14}C]-palmitoyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine reported by Leslie (1993) show that turnover of the phospholipase A$_2$ reaction is ≥10-fold faster than the combined rates of turnover of the phospholipase A$_1$ and lysophospholipase reactions, which may be occurring simultaneously in her assay. Furthermore, her results show that the dissociation of bound lysophospholipid, which is a product of the phospholipase A$_2$ reaction, is ≥10-fold faster than its reorientation at the active site and hydrolysis as a consequence of the enzyme's lysophospholipase activity. Significant phospholipase A$_1$ activity of cPLA$_2$ becomes apparent only when a nonhydrolyzable ether-linked *sn*-2 alkyl chain is present, and yet no naturally occurring *sn*-1 ester-, *sn*-2 ether-linked phospholipids are known.

The substrate specificity of the enzyme was investigated further by measuring relative values of k_{cat}/K_M* with competitive double-radiolabel experiments in which the substrates were present in product-containing vesicles. The results of Table 1 show that the enzyme does not discriminate dramatically between phosphocholine substrates with *sn*-2-arachidonyl acyl chains and different *sn*-1 acyl chains that vary in length and degree of unsaturation. The enzyme also does not discriminate greatly between [^{14}C]PAPC and [^{3}H]-HAPC, and this result confirms that of Diez et al. (1992): an ether bond at the *sn*-1 position to the glycerol moiety of a phospholipid does not significantly affect the substrate specificity of the enzyme. The results obtained with [^{14}C]-APPC and [^{3}H]DPPC show that, in the case of substrates with *sn*-2-palmitoyl acyl chains, the enzyme does discriminate between substrates that have *sn*-1-palmitoyl acyl chains and those that have *sn*-1-arachidonyl acyl chains. The preference of the enzyme for [^{14}C]APPC over [^{3}H]DPPC suggests that, for substrates with slowly hydrolyzed *sn*-2 acyl chains, longer and more unsaturated *sn*-1 acyl chains promote the hydrolysis of these substrates. The phospholipid [^{3}H]-DPPC is hydrolyzed when present in product-containing vesicles, although vesicles formed only from DPPC are not hydrolyzed (Ghomashchi et al., 1992). This result supports the proposal that the presence of the products in the vesicles promotes binding of the enzyme to the interface.

Substrate requirements of the lysophospholipase activity of cPLA$_2$ were investigated with the phospholipid 1-[^{14}C]-palmitoylpropanediol-3-phosphocholine, which lacks a hydroxyl group at the *sn*-2 position. This phospholipid is both hydrolyzed by the enzyme and is an acyl donor for the transacylase activity (Figure 6). These results show that the *sn*-2 hydroxyl group of lysophospholipid substrates is not required for the catalysis of the lysophospholipase reaction by cPLA$_2$. They also rule out mechanisms for the lysophospholipase reaction that require the migration of the acyl chain of either free or bound substrate from the *sn*-1 position to the *sn*-2 position before hydrolysis can take place; acyl migration cannot occur with this substrate. The lysophospholipase and phospholipase A$_1$ activities of the enzyme are similar in this respect: they become significant when the bound substrate does not have a hydrolyzable *sn*-2 ester bond.

The suggestion has been made that cPLA$_2$ has two active sites that exist on distinct and separate domains of the enzyme: one site catalyzes the phospholipase A$_2$ reaction and the other catalyzes the lysophospholipase reaction (Fujimori et al., 1993). This proposal, which is based on the observation that a monoclonal antibody inhibits the enzyme's phospholipase A$_2$ activity but not its lysophospholipase activity, has been questioned, because mutation of serine-228 of cPLA$_2$ inhibits both activities (Sharp et al., 1994). Several results of the present study suggest that a single active site is responsible for both activities. The acylation of glycerol occurs with substrates for the lysophospholipase, phospholipase A$_1$, and phospholipase A$_2$ reactions. Figure 6 shows that the lysophospholipase and phospholipase A$_2$ reactions are both inhibited by AACOCF$_3$, and inhibition is complete for both reactions before the first measurement is made at 10 min.

SUPPLEMENTARY MATERIAL AVAILABLE

Description of the synthesis and purification of radiolabeled fatty acid phenyl esters, 1-[^{14}C]palmitoyl-3-hydroxypropyl ester, 1-[^{14}C]palmitoylpropanediol-3-phosphocholine,

[^{3}H]20:4-MAG, [^{14}C]18:3-MAG, [^{3}H]AAPC, [^{14}C]APPC, and [^{3}H]AHPC and the commercial sources of materials (10 pages). Ordering information is given on any current masthead page.

REFERENCES

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

Brady, K., Wei, A., Ringe, D., & Abeles, R. H. (1990) *Biochemistry 29*, 7600−7607.

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

Christie, W. W. (1982) *Lipid Analysis*, Pergamon, New York.

Clark, J. D., Milona, N., & Knopf, J. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7708−7712.

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.

Dennis, E. A. (1983) *The Enzymes 16*, 307−353.

Diez, E., & Mong, S. (1990) *J. Biol. Chem. 265*, 14654−14661.

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.

Fujimori, Y., Kudo, I., Fujita, K., & Inoue, K. (1993) *Eur. J. Biochem. 218*, 629−635.

Gelb, M. H., Jain, M. K., & Berg, O. (1992) *Bioorg. Med. Chem. Lett. 2*, 1335−1342.

Ghomashchi, F., Yu, B.-Z., Berg, O., Jain, M. K., & Gelb, M. H. (1991a) *Biochemistry 30*, 7318−7329.

Ghomashchi, F., Yu, B. Z., Jain, M. K., & Gelb, M. H. (1991b) *Biochemistry 30*, 9559−9569.

Ghomashchi, F., Schüttel, S., Jain, M. K., & Gelb, M. H. (1992) *Biochemistry 31*, 3814−3824.

Gronich, J. H., Bonventre, J. V., & Nemenoff, R. A. (1990) *Biochem. J. 271*, 37−43.

Hanel, A. M., Schüttel, S., & Gelb, M. H. (1993) *Biochemistry 32*, 5949−5958.

Huang, Z., Laliberté, F., Tremblay, N. M., Weech, P. K., & Street, I. P. (1994) *Anal. Biochem. 222*, 110−115.

Imperiali, B., & Abeles, R. H. (1986) *Biochemistry 25*, 3760−3767.

Jain, M. K., & Berg, O. (1989) *Biochim. Biophys. Acta 1002*, 127−156.

Jain, M. K., Tao, W., Rogers, J., Arenson, C., Eibl, H., & Yu, B.-Z. (1991a) *Biochemistry 30*, 10256−10268.

Jain, M. K., Yu, B.-Z., Rogers, J., Ranadive, G. N., & Berg, O. (1991b) *Biochemistry 30*, 7306−7317.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.

Kim, D. K., Kudo, I., & Inoue, K. (1991a) *Biochim. Biophys. Acta 1083*, 80−88.

Kim, D. K., Suh, P. G., & Ryu, S. H. (1991b) *Biochem. Biophys. Res. Commun. 174*, 189−196.

Kramer, R. M., Checani, G. C., & Deykin, D. (1987) *Biochem. J. 248*, 779−783.

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., Sportsman, J. R., & Jakubowski, J. A. (1993) *J. Lipid Mediat. 6*, 209−216.

Leslie, C. C. (1991) *J. Biol. Chem. 266*, 11366−11371.

Leslie, C. C., & Channon, J. Y. (1990) *Biochim. Biophys. Acta 1045*, 261−270.

Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M., & Zelarney, P. T. (1988) *Biochim. Biophys. Acta 963*, 476−492.

Liang, T.-C., & Abeles, R. H. (1987) *Biochemistry 26*, 7603−7608.

Lin, L.-L., Lin, A. Y., & Knopf, J. L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6147−6151.

Masuda, N., Kitamura, N., & Saito, K. (1991) *Eur. J. Biochem. 202*, 783−787.

Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L., & Clark, J. D. (1994) *J. Biol. Chem. 269*, 18239−18249.

Nemenoff, R. A., Winitz, S., Qian, N., Van Putten, V., Johnson, G. L., & Heasley, L. E. (1993) *J. Biol. Chem. 268*, 1960−1964.

Reeve, W., Erikson, C. M., & Aluotto, P. F. (1979) *Can. J. Chem. 57*, 2747−2754.

Reynolds, L. J., Hughes, L. L., Louis, A. I., Kramer, R. M., & Dennis, E. A. (1993) *Biochim. Biophys. Acta 1167*, 272−280.

Riendeau, D., Guay, J., Weech, P. K., Laliberté, F., Yergey, J., Li, C., Desmarais, S., Perrier, H., Lui, S., Nicoll-Griffith, D., & Street, I. P. (1994) *J. Biol. Chem. 269*, 15625−15630.

Ritchie, C. D. (1990) *Physical Organic Chemistry*, Marcel Dekker, New York.

Saito, K., Sugatani, J., & Okumura, T. (1991) *Methods Enzymol. 197*, 446−456.

Scott, D. L., White, S. P., Browning, J. L., Rosa, J. J., & Gelb, M. H. (1991) *Science 254*, 1007−1010.

Serdarevich, B. (1967) *J. Am. Oil Chem. Soc. 44*, 381−393.

Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., Roberts, E. F., & Kramer, R. M. (1991) *J. Biol. Chem. 266*, 14850−14853.

Sharp, J. D., Pickard, R. T., Chiou, X. J., 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.

Stein, R. L., Strimpler, A. M., Edwards, P. D., Lewis, J. J., Mauger, R. C., Schwartz, J. A., Stein, M. M., Trainor, D. A., Wildonger, R. A., & Zottola, M. A. (1987) *Biochemistry 26*, 2682−2689.

Street, I. P., Lin, H.-K., Laliberté, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N. M., Huang, Z., Weech, P. K., & Gelb, M. H. (1993) *Biochemistry 32*, 5935−5940.

Sugatani, J., Okumura, T., & Saito, K. (1980) *Biochim. Biophys. Acta 620*, 372−386.

Trimble, L. A., Street, I. P., Perrier, H., Tremblay, N. M., Weech, P. K., & Bernstein, M. A. (1993) *Biochemistry 32*, 12560−12565.

Verheij, H. M., Slotboom, A. J., & De Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharmacol. 91*, 91−137.

Waite, M. (1987) *The Phospholipases*, Plenum, New York.

Wijkander, J., & Sundler, R. (1991) *Eur. J. Biochem. 202*, 873−880.

Wijkander, J., & Sundler, R. (1992) *Biochem. Biophys. Res. Commun. 184*, 118−124.

Xing, M., & Mattera, R. (1992) *J. Biol. Chem. 267*, 25966−25975.

BI950026G