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Activation, Inhibition, and Regiospecificity of the Lysophospholipase Activity of the 85-kDa Group IV Cytosolic Phospholipase A_2*

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The 85-kDa Group IV calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$) catalyzes the hydrolysis of palmitoylglycero-3-phosphocholine to palmitic acid and glycero-3-phosphocholine. Palmitoylglycero-3-phosphocholine exists as a 9:1 equilibrium mixture of the *sn*-1 and *sn*-2 isomers, with the fatty acid predominately at the *sn*-1 position. We have monitored this reaction by ^{31}P NMR to determine which palmitoylglycero-3-phosphocholine isomer is processed by $cPLA_2$. When both lysophospholipid isomers are present in a 1:1 mixture under conditions in which acyl migration is minimized, $cPLA_2$ rapidly consumes both isomers. However, 1-palmitoylglycero-3-phosphocholine is consumed seven times faster than the 2-palmitoylglycero-3-phosphocholine isomer. We have previously reported that this lysophospholipase reaction is accelerated in the presence of glycerol. We now find that this apparent increase in activity is accounted for, in part, by glycerol acting as an alternative acceptor for the cleaved fatty acid, as is the case for this enzyme's phospholipase A_2 (PLA_2) activity. In contrast, dioleoylglycerol, which accelerates the PLA_2 activity, does not act as an acceptor in either the lysophospholipase or the PLA_2 reaction, but can affect enzyme activities by altering substrate presentation. We also show that a known inhibitor of the PLA_2 activity of $cPLA_2$ is able to inhibit its lysophospholipase activity with a similar IC_{50} to its PLA_2 activity. However, the effect of inhibitors is dependent on the manner in which they are presented to the enzyme.

Phospholipase A_2 (PLA_2)[1] comprises a family of enzymes that catalyze the hydrolysis of fatty acids from the *sn*-2 position of phospholipids (1). The Group IV calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$) is an 85-kDa member of this family that displays a preference for phospholipids that contain arachidonic acid (2–7). Since arachidonic acid is a second messenger for a number of cellular functions and is also a precursor for a variety of proinflammatory eicosanoids (8), $cPLA_2$ has been the subject of a large number of studies directed toward understanding both its mechanism and regulation (see Ref. 9 for review).

Mechanistically, $cPLA_2$ exhibits a number of other activities in addition to its namesake phospholipase A_2 activity, including transacylase (10, 11), esterase (11, 12), phospholipase A_1 (2, 7, 11), and lysophospholipase (7, 10, 13, 14) activities. However, both the transacylase and esterase activities are a small fraction of the PLA_2 activity, while the PLA_1 activity is only observed when there is no hydrolyzable acyl chain present at the *sn*-2 position. In fact, only the lysophospholipase activity is quantitatively significant and comparable with the PLA_2 activity. We (10) and others (7, 13, 14) have previously shown that the lysophospholipase activity is actually greater than the PLA_2 activity under certain conditions. Interestingly, the substrate often used for assaying the lysophospholipase activity is 1-palmitoylglycero-3-phosphocholine (1-PGPC). Thus, in contrast to the PLA_2 activity, the lysophospholipase activity of $cPLA_2$ presumably acts at the *sn*-1 position, suggesting that $cPLA_2$ is a positionally sloppy enzyme.

There are many conflicting views as to the relationship between the two main activities exhibited by $cPLA_2$. It was initially suggested that the two activities may be catalyzed by distinct sites on the enzyme when antibodies were shown to inhibit the PLA_2 activity, but not the lysophospholipase activity (14). A model of separate enzymatic sites would be consistent with the apparently different positions of reactivity on the glycerol backbone of the two phospholipid substrates. However, more recent data by Sharp *et al.* (15) suggest that the two activities share at least a common catalytic residue. They have shown that a single serine residue is essential for both the PLA_2 and lysophospholipase activities. How then can a single catalytic site accommodate a PLA_2 activity acting at the *sn*-2 position of a phospholipid substrate and a lysophospholipase activity acting at the *sn*-1 position of a lysophospholipid substrate? Actually, lysophospholipids (16) can exist as two positional isomers in which the fatty acid is acylated to either the *sn*-1 or *sn*-2 position of a glycerophosphate backbone (Fig. 1). Therefore, the lysophospholipase activity that has been reported to date may merely be a result of the normal PLA_2 activity acting exclusively on the 2-palmitoylglycero-3-phosphocholine isomer. If that were the case, then $cPLA_2$ would not be the positionally sloppy enzyme it had been believed to be.

In this report, we try to shed light on the link between the lysophospholipase and PLA_2 activities of $cPLA_2$. Most important, we first examine the regiospecificity of the lysophospholipase activity of $cPLA_2$. We have monitored this reaction by ^{31}P NMR to determine which palmitoylglycero-3-phosphocholine isomer is processed by $cPLA_2$ (17). We then compare the various modes of activation and inhibition of the two activities.

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; 1-PGPC, 1-palmitoylglycero-3-phosphocholine; 2-PGPC, 2-palmitoylglycero-3-phosphocholine; AA-TFMK, arachidonyl trifluoromethyl ketone; PA-TC, palmityltricarbonyl; PAPC, 1-palmitoyl-2-arachidonoylglycero-3-phosphocholine; PC, phosphatidylcholine; DAG, 1,2-dioleoylglycerol; GPC, glycero-3-phosphocholine; fid, free induction decay.

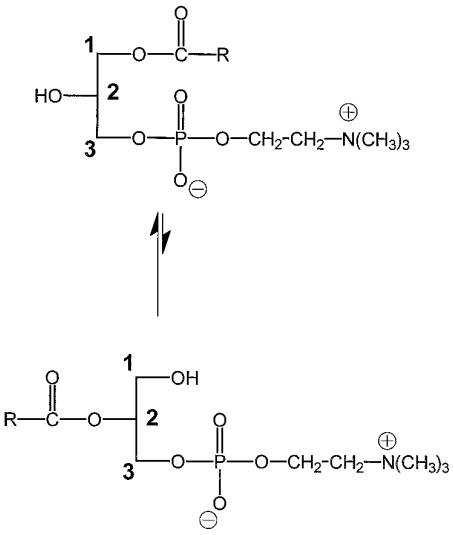

FIG. 1. **Regioisomers of palmitoylglycero-3-phosphocholine.** Shown are two natural regioisomers of palmitoylglycero-3-phosphocholine (depicted as Fischer projections; R = -$(CH_2)_{14}CH_3$) that are possible substrates for the lysophospholipase activity of cPLA$_2$.

EXPERIMENTAL PROCEDURES

Materials—L-α-1-[^{14}C]Palmitoylglycero-3-phosphocholine and L-α-1-palmitoyl-2-[^{14}C]arachidonoylglycero-3-phosphocholine were purchased from NEN Life Science Products. L-α-1-Palmitoyl-2-[^{14}C]palmitoylglycero-3-phosphocholine was purchased from Amersham Corp. Nonradioactive lipids were purchased from Avanti Polar Lipids. All-*cis*-5,8,11,14-nonadecatetraenyl trifluoromethyl ketone (arachidonyl trifluoromethyl ketone (AA-TFMK)) (18) and 2,3-dioxooctadecanoic acid *tert*-butyl ester monohydrate (palmityltricarbonyl (PA-TC)) (19) were prepared as described elsewhere. *Rhizopus arrhizus* lipase was purchased from Boehringer Mannheim. Deuterated solvents were from Cambridge Isotopes. Recombinant cPLA$_2$ was generously provided by Dr. Ruth Kramer (Lilly Research Laboratories).

2-Palmitoylglycero-3-phosphocholine (2-PGPC) was prepared by treating 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine with *Rhizopus* lipase following published procedures (20). Conversion of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine to 2-PGPC did not proceed to completion. However, palmitoylglycero-3-phosphocholine could be separated from a mixture with the starting material by chromatography on Sephadex LH-20. 2-PGPC prepared in this manner gave a single spot by analytical TLC, but showed two peaks by ^{31}P NMR corresponding to a 4:1 mixture of palmitoylglycero-3-phosphocholine isomers (the minor isomer being 1-PGPC). Similarly, commercial 1-PGPC contains ~10% of the corresponding 2-PGPC isomer. These cross-contaminations have been documented and are attributed to migration of the fatty acyl chain during the preparation and purification of lysophospholipids (20).

^{31}P NMR Assay—Samples were made up in a 35:35:30 (v/v/v) mixture of 200 mM Hepes (pH 7, with 10 mM $CaCl_2$ and 150 mM NaCl), D_2O, and glycerol. Appropriate amounts of 1-PGPC and 2-PGPC were combined to give a 1:1 mixture of isomers. Lysophospholipids were stored as chloroform solutions and were thus prepared by first removing the chloroform *in vacuo* at 25 °C. The resulting film was taken up in 500 μl of the reaction buffer and completely dissolved by vortexing and bath sonication to give a clear and colorless solution (10 mM). Similarly, cPLA$_2$ solutions (4 mg/ml) were diluted in the reaction buffer (typically, 5 μl in 100 μl of buffer). The final concentrations of reagents were 8.3 mM substrate and 33 μg/ml enzyme in 600 μl of buffer.

Substrate was preincubated at 40 °C in a 5-mm NMR tube, and at zero time, spectra were obtained. Enzyme was then added to this NMR tube to initiate the reaction. In addition, an insert containing 10 mM pyrophosphate in D_2O was used as an external standard.

^{31}P NMR spectra were obtained on a General Electric spectrometer operating at 121.5 MHz. A 66° pulse with a 2-s delay and a spectral width of 4000 Hz and 16,000 data points was used. Broad-band proton decoupling was utilized. Spectra were obtained at varying time intervals at 40 °C. Typically, 128 transients were obtained over 8 min. The resulting fid was apodized with a gaussian multiplication with line broadening of 5 Hz. The chemical shifts of the phosphorus-containing compounds are listed in Table I. Chemical shifts are reported relative to 85% phosphoric acid. Peak integrals were taken to represent the relative concentrations of the phosphorus-containing species in solution.

TABLE I
^{31}P chemical shifts of phosphorus-containing compounds

Compound	Chemical shift (δ)
	ppm
1-PGPC	0.10
2-PGPC	0.26
GPC	0.55
Pyrophosphate	−5.66

[a] R-relative to 85% phosphoric acid.

PLA$_2$ Mixed Micelle Assay—Assays were performed in a standard buffer composed of 80 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM $CaCl_2$, 1 mg/ml bovine serum albumin, and 1 mM dithiothreitol. The mixed micelle assay also contained 1 mM 1-palmitoyl-2-arachidonoylglycero-3-phosphocholine (PAPC) (with 100,000 cpm [^{14}C]PAPC), 2 mM Triton X-100, 30% glycerol, and 3.75 μg/ml cPLA$_2$ in a volume of 200 μl. The substrate solution was prepared as described previously (19). Assays were run for 40 min at 40 °C. The reaction was quenched and worked up also as described previously (19).

Lysophospholipase Mixed Micelle Assay—The lysophospholipase activity was measured in a mixed micelle assay that contained 1 mM commercial 1-palmitoylglycero-3-phosphocholine (lyso-PC)[2] (with 100,000 cpm lyso-[^{14}C]PC), 2 mM Triton X-100, 30% glycerol, and 0.5 μg/ml cPLA$_2$ in 200 μl of the standard buffer described above. The substrate was vortexed to clarity instead of probe sonicating. Assays were also run for 40 min at 40 °C and quenched using the modified Dole procedure (21).

Dual-substrate Assay—Assays containing both PAPC and lyso-PC in mixed micelles were composed of 1 mM PAPC, 1 mM lyso-PC (with 100,000 cpm either [^{14}C]PAPC or lyso-[^{14}C]PC), 4 mM Triton X-100, 30% glycerol, and 3.75 μg/ml cPLA$_2$ in 200 μl of the standard buffer. Assays were prepared and run analogous to the PC mixed micelle assay described above. Those assays containing [^{14}C]PAPC were worked up as described for the PLA$_2$ mixed micelle assay, whereas those with lyso-[^{14}C]PC were worked up as described for the lysophospholipase mixed micelle assay.

PC/DAG Assay—The PC/DAG assay was similar to that described by Kramer *et al.* (4), but utilized a higher concentration of substrate. This assay contained 20 μM PAPC (with 100,000 cpm [^{14}C]PAPC), 10 μM 1,2-dioleoylglycerol, and 15–25 ng/ml cPLA$_2$ in 200 μl of the standard buffer. The substrate was prepared by adding 3 × assay buffer to the dried PAPC/DAG and probe sonicating. Assays were run for 15 min at 40 °C and extracted using the Dole procedure as described above for the PLA$_2$ mixed micelle assay.

TLC Assay—Assays that required separation of fatty acid and monoglyceride or triglyceride products utilized the same substrate, buffer components, and assay conditions as described above. The reactions were quenched with 500 μl of chloroform/methanol/acetic acid (2:4:1, v/v/v) instead of Dole reagent and then vortexed. To this, 1.0 ml of water and 500 μl of chloroform were added, and the mixture was vortexed again. This solution was then centrifuged (1000 × *g* for 2 min) to separate the organic and aqueous layers. 500 μl of the organic layer was transferred to another test tube, and the solvent was evaporated in a vacuum oven overnight at 40 °C. The resulting lipid film was resuspended in 50 μl of chloroform/methanol (2:1, v/v) and loaded onto TLC prep plates. Unlabeled fatty acid, monoglyceride, and/or triglyceride standards were added to the prep plate. The TLC plate was then developed in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The resulting TLC plate was visualized with iodine, and zones corresponding to fatty acid, starting phospholipid, monoglyceride, and/or triglyceride were scraped and counted. Blanks were conducted for every data point and substracted from the corresponding data point.

RESULTS

Regiospecificity of the Lysophospholipase Activity of cPLA$_2$—Under the experimental conditions described, 1-PGPC, 2-PGPC, and GPC displayed distinct ^{31}P NMR signals (Table I). This permitted simultaneous *in situ* monitoring of the three species by ^{31}P NMR. Fig. 2 shows the effect of cPLA$_2$ on an equimolar mixture of the two lysophospholipid isomers. In the presence of

[2] Palmitoylglycero-3-phosphocholine used in the radiochemical assays was a mixture of *sn*-1 and *sn*-2 regioisomers and is generally referred to as lyso-PC in the text.

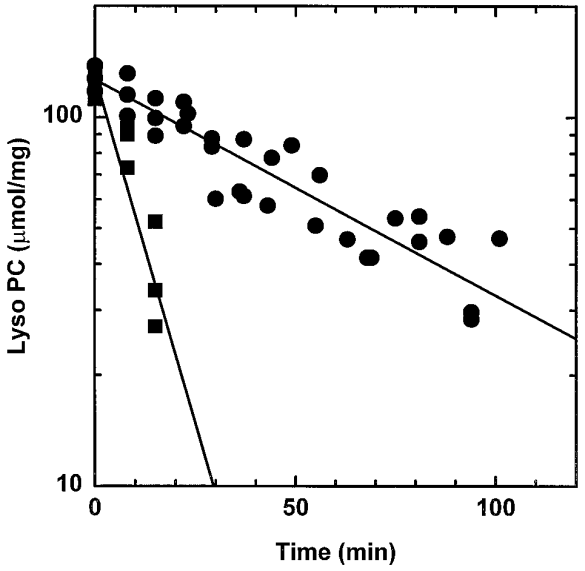

FIG. 2. **Time course for the cPLA$_2$-catalyzed hydrolysis of the two regioisomers of palmitoylglycero-3-phosphocholine.** Shown is a semilog plot of the time course for the hydrolysis of a 1:1 mixture of 1-PGPC and 2-PGPC. The data points from three separate experiments are shown representing the disappearance of 1-PGPC (■) and 2-PGPC (●) as monitored by ^{31}P NMR. The *lines* represent first-order fits to 1-PGPC and 2-PGPC hydrolysis, respectively.

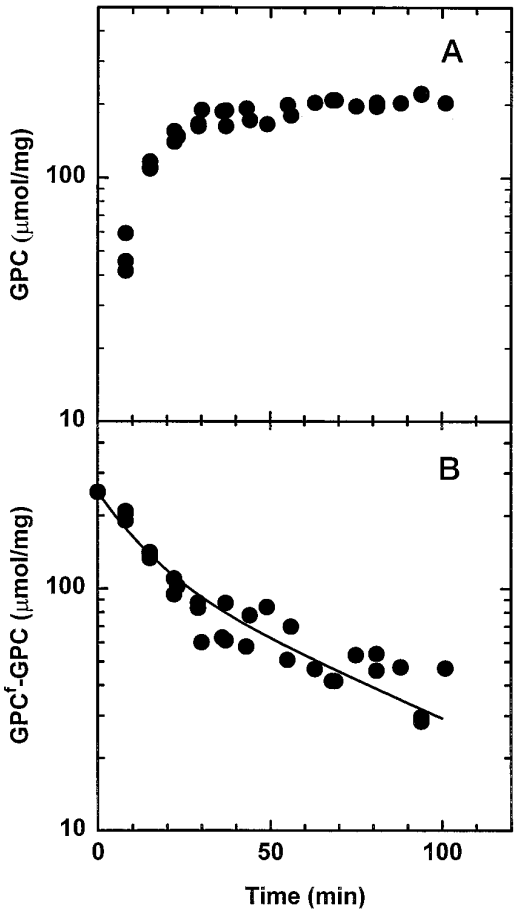

FIG. 3. **Time course for the concomitant formation of glycero-3-phosphocholine during cPLA$_2$-catalyzed hydrolysis of the two regioisomers of palmitoylglycero-3-phosphocholine.** *A*, semilog plot of the time course for GPC formation from a 1:1 mixture of 1-PGPC and 2-PGPC; *B*, replot of *A* plotting $[GPC]^f - [GPC]$ as a function of time (where $[GPC]^f = [GPC]$ at completion of the reaction). The *line* represents a best fit to the data using the equation described under "Results": $v = k_1[1\text{-PGPC}] + k_2[2\text{-PGPC}]$.

cPLA$_2$, both palmitoylglycero-3-phosphocholine isomers were rapidly consumed (<20% of the total starting palmitoylglycero-3-phosphocholine remained after 2 h). In addition, the consumption of palmitoylglycero-3-phosphocholine coincided with the expected appearance of GPC (Fig. 3*A*). In the absence of cPLA$_2$, no GPC formation was observed even after 24 h.

The time courses for the disappearance of both palmitoylglycero-3-phosphocholine isomers can be fit to first-order rates of decay. The hydrolysis of 1-PGPC displays an apparent first-order rate constant of 0.087 ± 0.008 min^{-1}, whereas the hydrolysis of 2-PGPC has an apparent first-order rate constant of 0.013 ± 0.001 min^{-1}. Thus, in the presence of cPLA$_2$, 1-PGPC is consumed seven times faster than its 2-PGPC isomer.

By comparison, the formation of GPC is not a simple first-order exponential process. Instead, it appears to be composed of a combination of two first-order processes (Fig. 3*B*). There is an initial fast formation of GPC, followed by a slower rate of GPC production. This result is consistent with a picture of GPC being produced via two non-equivalent simultaneous first-order reactions (22). In other words, cPLA$_2$ is catalyzing the hydrolysis of both 1-PGPC and 2-PGPC directly to GPC. Thus, the rate of GPC production can be described by $v = k_1[1\text{-PGPC}] + k_2[2\text{-PGPC}]$. A best fit of the data for GPC formation to this equation yields a k_1 of 0.079 ± 0.009 min^{-1} and a k_2 of 0.015 ± 0.001 min^{-1} (Fig. 3*B*), both of which compare favorably with the experimentally determined apparent rate constants for 1-PGPC and 2-PGPC disappearance. The initial phase of GPC production is the result of both 1-PGPC and 2-PGPC being converted to product. A slower rate of GPC formation results when all the 1-PGPC is consumed, and the formation of GPC can occur only from hydrolysis of 2-PGPC.

Effect of Glycerol on the Lysophospholipase Activity of cPLA$_2$—cPLA$_2$ catalyzes the transfer of the acyl group from palmitoylglycero-3-phosphocholine to glycerol in addition to water. Fig. 4*A* shows that when 30% glycerol by volume is present in an assay, both palmitoylglycerol and palmitic acid are generated from cPLA$_2$ activity. Thus, in the presence of glycerol, the total observed lysophospholipase activity in an assay can be accounted for by the combined rates of formation of both palmitoylglycerol and palmitic acid when monoglyceride and fatty acid products are not separated by TLC.

The partitioning of the palmitoyl group from palmitoylglycero-3-phosphocholine to glycerol increases with increasing concentration of glycerol in the presence of cPLA$_2$. Fig. 4*B* shows that monoglyceride formation increases linearly when the amount of glycerol in the assay is increased from 0 to 30% (v/v). The amount of monoglyceride produced corresponds to an increase of ~1 μmol/mg for every 1% of glycerol (v/v).

Effect of DAG on cPLA$_2$ Activities—DAG does not accept the acyl group from a diacyl or lysophospholipid in the presence of cPLA$_2$. In the presence of cPLA$_2$ under a variety of conditions, no transfer of a palmityl group from palmitoylglycero-3-phosphocholine to DAG, by separating possible labeled triglyceride from palmitic acid on TLC, was detected. Similarly, no transfer of an arachidonyl group from PAPC to DAG was detected in the presence of cPLA$_2$. Hence, unlike lysophospholipid (10) or glycerol (11), DAG is not able accept the fatty acid from a diacyl or lysophospholipid substrate in the presence of cPLA$_2$.

This result is not surprising considering that in a mixed micelle assay, DAG does not even activate the activities of cPLA$_2$ as glycerol does. In fact, Fig. 5 shows that DAG actually inhibits the PLA$_2$ activity of cPLA$_2$ at high concentration. Interestingly, DAG has no effect on its lysophospholipase activity even at 10 mol % of total lipid. These experiments with DAG were carried out with mixed micelles of Triton X-100 contain-

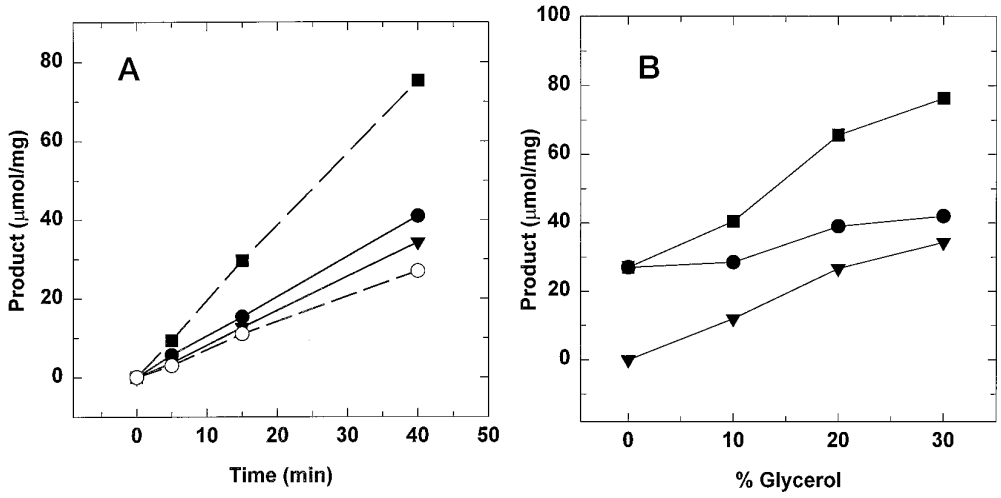

FIG. 4. **Effect of glycerol on the lysophospholipase activity of cPLA$_2$.** *A*, shown is a time course of the lysophospholipase activity of cPLA$_2$ as determined by the TLC assay in the absence (○) and presence (▼, ●, ■) of 30% (v/v) glycerol. The corresponding palmitic acid (○, ●) and palmitoylglycerol (▼) produced are plotted. The total Dole product (■) represents the combination of palmitic acid and palmitoylglycerol products from the reaction in glycerol. Each point represents the average of duplicates. *B*, the palmitic acid (●), palmitoylglycerol (▼), and total Dole product (■) produced by cPLA$_2$ action on lyso-PC with increasing glycerol are plotted. Each point represents the average of duplicates.

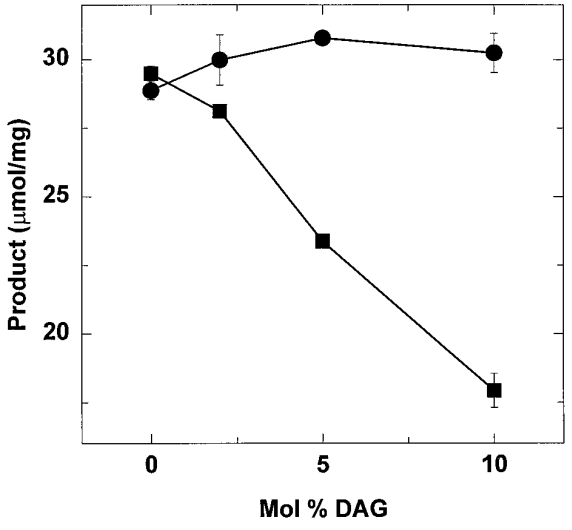

FIG. 5. **Effect of DAG on the PLA$_2$ and lysophospholipase activities of cPLA$_2$.** The total Dole product formed by cPLA$_2$ action on PAPC (■) and lyso-PC (●) substrates in a 1:1 PAPC/lyso-PC mixed micelle assay is plotted as a function of DAG. Each point represents the average of duplicates.

ing both lyso-PC and PAPC substrates. In contrast, DAG does activate both the PLA$_2$ and lysophospholipase activities when Triton X-100 is not present (data not shown).

Inhibition of the Lysophospholipase Activity of cPLA$_2$—The ability of activated ketones to inhibit the lysophospholipase activity of cPLA$_2$ was evaluated. Fig. 6*A* shows the effect of AA-TFMK, PA-TC, and anandamide on the lysophospholipase activity. The activity of the cPLA$_2$ control in this assay was 1.5 μmol/min/mg, higher than the activity observed in a corresponding PLA$_2$ assay (0.2–0.7 μmol/min/mg) (19). This higher activity using a lyso-PC substrate has been noted previously (10).

In this assay, AA-TFMK inhibited the lysophospholipase activity of cPLA$_2$ with an IC$_{50}$ of 70 μM (0.023 mol fraction). PA-TC and anandamide did not inhibit the enzyme. This is in contrast to an assay of the enzyme's PLA$_2$ activity with a PAPC substrate, where AA-TFMK (19), PA-TC (19), and anandamide[3] all appeared to inhibit the enzyme. Instead, in this assay, both PA-TC and anandamide caused an increase in the lysophospholipase activity.

To clarify the observed differences in inhibition by these compounds of the PLA$_2$ and lysophospholipase activities of cPLA$_2$, activity was also examined under a different PLA$_2$ assay condition. In the PC/DAG assay, AA-TFMK inhibited enzyme activity with an IC$_{50}$ of 0.65 μM (0.021 mol fraction) (Fig. 6*B*), whereas the presence of PA-TC and anandamide had no effect in this assay.

In a final experiment, we assayed both activities under identical assay conditions. We ensured that the PAPC and lyso-PC substrates were presented in a similar form by preparing Triton X-100 mixed micelles containing a 1:1 mixture of both PAPC and lyso-PC substrates. In parallel experiments, we then observed both enzyme activities (Fig. 7). In this dual-substrate assay, AA-TFMK again inhibited cPLA$_2$, whereas PA-TC showed slightly weaker inhibition. More significantly, under these conditions, inhibition of the PLA$_2$ activity closely paralleled inhibition of the lysophospholipase activity. It is also interesting that, unlike the single-substrate experiments, under these conditions, the PLA$_2$ and lysophospholipase activities have similar specific activities. Most important, the surface IC$_{50}$ (in mole fractions) of AA-TFMK is similar under all of the different experimental conditions (Table II), as would be expected for a true active site-directed inhibitor (19).

DISCUSSION

There are a number of characteristic features of the PLA$_2$ activity of cPLA$_2$. Among them is an ability to use glycerol as an acceptor of the cleaved fatty acid to generate monoglyceride (11). The presence of glycerol also appears to yield an increase in the measured PLA$_2$ activity. DAG has been reported to also cause an increase in the PLA$_2$ activity (4), and activated ketones have been shown to inhibit that activity (19).

In contrast, there is much less known about the lysophospholipase activity of cPLA$_2$, and what little is known does not appear to be consistent with the mutagenesis data suggesting a common active site for the two activities. For instance, although the PLA$_2$ activity is known to prefer arachidonic acid-over palmitic acid-containing substrates (23), the rate of lysophospholipase activity has been shown to proceed at similar or greater rates on a palmitic acid-containing substrate than the rate of PLA$_2$ activity on arachidonic acid-containing substrates (10). The regiospecificity of the lysophospholipase reaction and

[3] L. J. Reynolds and E. A. Dennis, unpublished data.

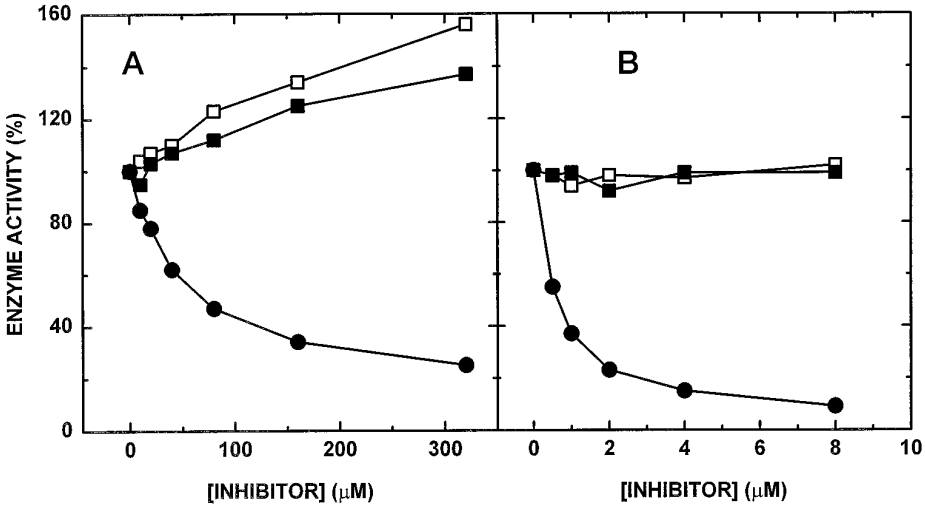

FIG. 6. **Differential inhibition of cPLA$_2$ by a variety of inhibitors.** cPLA$_2$ was assayed in the presence of increasing concentrations of PA-TC (□), anandamide (■), or AA-TFMK (●). *A*, in the standard lysophospholipase mixed micelle assay; *B*, in the PC/DAG assay measuring the PLA$_2$ activity. The enzyme activity is plotted as the percentage of the control activity, which was assayed in the absence of inhibitor; 100% activity represents 1.5 and 4.9 μmol/min/mg, respectively. Each point represents the average of duplicates or triplicates.

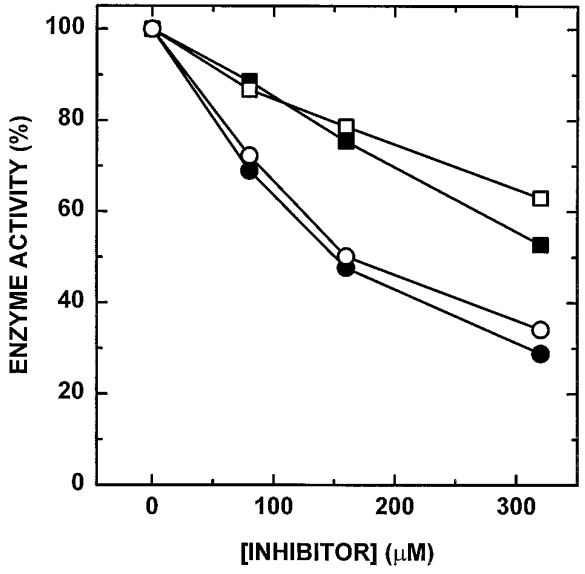

FIG. 7. **Inhibition of cPLA$_2$ in the dual-substrate assay.** The PLA$_2$ and lysophospholipase activities of cPLA$_2$ were assayed in a 1:1 PAPC/lyso-PC mixed micelle assay in the presence of increasing concentrations of AA-TFMK (●, ○) or PA-TC (■, □). The enzyme activity is plotted as the percentage of the control activity, which was assayed in the absence of inhibitor; 100% activity represents 0.97 μmol/min/mg in the PLA$_2$ assay (*closed symbols*) and 0.84 μmol/min/mg in the lysophospholipase assay (*open symbols*). Each point represents the average of triplicates.

TABLE II
Comparison of the inhibition of cPLA$_2$ by AA-TFMK under different assay conditions

IC$_{50}$ is expressed in bulk concentration units (μM) and surface mole fractions (χ_s)

Assay	IC$_{50}$	
	μM	χ_s
PC mixed micelles	64	0.021
Lysophospholipid micelles	70	0.023
PC/DAG	0.65	0.021
PC/lysophospholipid mixed micelles		
PLA$_2$ activity	150	0.024
Lysophospholipase activity	160	0.026

the effect of PLA$_2$ activators and inhibitors on the lysophospholipase activity are addressed below.

Regiospecificity—cPLA$_2$ catalyzes the hydrolysis of fatty acid from the *sn*-2 position of diacylphospholipids to yield a 1-acylglycero-3-phospholipid. In facilitating this reaction, cPLA$_2$ is highly specific for the *sn*-2 position of diacylphospholipid substrates. It has been reported that no fatty acid is released from the *sn*-1 position of diacylphospholipids (11). Only when the *sn*-2 ester was replaced with an unhydrolyzable ether linkage was cPLA$_2$-catalyzed hydrolysis of the remaining *sn*-1 ester bond detected. However, it was not known from which position cPLA$_2$ catalyzes the hydrolysis of fatty acids from lysophospholipids.

Lysophospholipids can exist as two positional isomers in which the fatty acid is acylated to either the *sn*-1 or *sn*-2 position (Fig. 1). In addition, the fatty acyl chain is readily able to migrate between the two hydroxyl groups of the glycerol backbone. At equilibrium, palmitoylglycero-3-phosphocholine exists as a 9:1 mixture of the two isomers, with the palmitic acid predominately at the *sn*-1 position (20). *In vitro* studies of the lysophospholipase activity of cPLA$_2$ typically utilize commercially available 9:1 equilibrium mixtures of palmitoylglycero-3-phosphocholine, and the reaction is generally only followed to a few percent conversion by measuring the formation of palmitic acid (7, 10, 13). Hence, these experiments have not addressed the regiospecificity of the lysophospholipase activity of cPLA$_2$. Indeed, the lysophospholipase activity of cPLA$_2$ could have been due exclusively to the processing of the minor 2-palmitoylglycero-3-phosphocholine isomer by cPLA$_2$, as might be expected for an analogue of its normal diacyl substrate.

We have found that cPLA$_2$ catalyzes the hydrolysis of 1-palmitoylglycero-3-phosphocholine seven times faster than its *sn*-2 isomer. Thus, the specificity of cPLA$_2$ for the *sn*-2 position of a diacylphospholipid substrate switches to a preference for the *sn*-1 position when the substrate is a lysophospholipid. The lysophospholipase *sn*-2 activity that is observed may in fact be a consequence of 2-PGPC acting as a poor analogue of a diacylphospholipid substrate for the phospholipase A$_2$ activity of cPLA$_2$.

Acyl migration can occur between the two positions of a lysophospholipid and could complicate or obscure our results. However, at pH 7 in the absence of the enzyme, the half-life for migration of palmitic acid between the two hydroxyl groups of GPC is 20 h (20). This is too slow to account for the observed rate of disappearance of 1-PGPC in our experiments; nor is cPLA$_2$

likely to be catalyzing the isomerization of 1-PGPC to 2-PGPC because the observed consumption of 1-PGPC would then have to proceed through a 2-PGPC intermediate, and then the observed rate of 1-PGPC disappearance could never exceed the rate of 2-PGPC disappearance. (At best, the observed rates of disappearance of 1-PGPC and 2-PGPC would be equivalent in the situation that the hydrolysis reaction is rate-determining.) We find the cPLA$_2$-catalyzed rate of 1-PGPC disappearance to be 7-fold greater than the rate of 2-PGPC disappearance. The above scenario would assume that the isomerization and hydrolysis reactions occur sequentially on the enzyme. In the case that the isomerization and hydrolysis are independent events on the enzyme and intermediate 2-PGPC is released, then the faster rate of 1-PGPC disappearance we observed should result in an initial build-up in the amount of 2-PGPC and an apparent slower initial rate of 2-PGPC disappearance. (If the isomerization step is slower, then the rate of 1-PGPC disappearance would remain constant, but the rate of 2-PGPC disappearance would initially appear fast as the existing 2-PGPC is depleted and would eventually slow to that of 1-PGPC disappearance because it is rate-determining.) In fact, the experimentally measured rates of disappearance of both isomers proceed at constant, but different rates. Thus, acyl migration does not appear to be occurring under our experimental conditions and cannot account for the results we observed.

The simplest explanation for the difference in positional specificity between the PLA$_2$ and lysophospholipase activities of cPLA$_2$ is that there are separate catalytic sites responsible for the two activities. However, there is considerable evidence that both activities share a common catalytic serine residue. The mechanism of cPLA$_2$ action has been studied by a number of groups, and there are now several pieces of evidence that implicate the involvement of an acyl-enzyme intermediate. Transfer of the fatty acid from both diacyl- and lysophospholipid substrates to acceptors other than water initially suggested the existence of such an acyl-enzyme intermediate (10, 11). Further corroboration came when NMR studies indicated the formation of a hemiketal between arachidonyl trifluoromethyl ketone (a potent inhibitor of both activities) and an enzyme hydroxyl group (24). Finally, a serine residue (Ser-228) was identified to be essential for the PLA$_2$ activity and was also shown to be the site of enzyme acylation (15, 25). This serine residue is required not only for the PLA$_2$ activity, but also for the lysophospholipase activity. The involvement of serine 228 in both activities of cPLA$_2$ argues against the likelihood of separate catalytic sites for its two activities.

An alternative explanation that accounts for the observed differences in positional specificity of the two activities of cPLA$_2$ and the common serine required for both activities is that there are different binding pockets on the enzyme for the two substrates that orient positionally different acyl chains toward the shared catalytic serine. This scenario might explain the anomalous report of an antibody to cPLA$_2$ that inhibits the PLA$_2$ activity and not the lysophospholipase activity (14). The antibody would have to be only blocking a region of the enzyme responsible for binding of diacylphospholipids and leaving free a lysophospholipid-binding site and the catalytic serine.

Effect of Glycerol, DAG, and Inhibitors—Based on our earlier studies (26) with a membrane-bound PLA$_2$, in which glycerol was shown to increase its activity, glycerol was also added to cPLA$_2$ assays at up to 70% by volume to improve the activity that was measured (2). More recently, it has been shown that glycerol is also able to act as a nucleophile and to accept the fatty acid from a diacylphospholipid substrate (11). In typical radiochemical assays during the isolation of the radiolabeled fatty acid product, the monoglyceride product from the acylation of glycerol also elutes with the free fatty acid formed through the normal hydrolysis pathway, and the two species are counted together. This results in an apparent hydrolysis activity that is greater than what would be measured if the monoglyceride and fatty acid products are separated. Thus, the apparent activity is actually a combination of the formation of the two products as well as an activation of the hydrolysis reaction with water itself. In contrast, with DAG, we have found no evidence for a second product and no activation in a mixed micelle assay.

We have previously shown that activated ketones can inhibit the PLA$_2$ activity of cPLA$_2$ (19). When we examined these same inhibitors in an assay of the lysophospholipase activity of cPLA$_2$, we found that they were behaving quite differently. AA-TFMK inhibits the lysophospholipase activity, but PA-TC and anandamide do not. This suggests that these inhibitors are differentiating between the two cPLA$_2$ activities. Furthermore, testing these same inhibitors against the PLA$_2$ activity in the DAG assay yielded the same unexpected results. The inhibitors only showed similar effects against both the PLA$_2$ and lysophospholipase activities when examined under identical conditions in which both PAPC and lyso-PC substrates were diluted into Triton X-100 mixed micelles. More important, under these conditions, the PLA$_2$ and lysophospholipase activities decreased in parallel in response to the inhibitors. Thus, some facet of the substrate presentation in the different assay conditions appears to affect the ability of the different compounds to inhibit cPLA$_2$ rather than inherent differences between the two activities. More important, AA-TFMK inhibits both the PLA$_2$ and lysophospholipase activities with the same surface IC$_{50}$ (in mole fractions) in all of the assays examined.

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Activation, Inhibition, and Regiospecificity of the Lysophospholipase Activity of the 85-kDa Group IV Cytosolic Phospholipase A_2

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