Functional Identification of the Active-Site Nucleophile of the Human 85-kDa Cytosolic Phospholipase A_2

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ABSTRACT: Ser-228 has been shown to be essential for the catalytic activity of the human cytosolic phospholipase A_2 (cPLA$_2$). However, its involvement in catalysis has not yet been demonstrated. Using site-directed mutagenesis, active-site directed irreversible inhibitors, and the novel fluorogenic substrate 7-hydroxycoumarinyl γ-linolenate, evidence is presented to show that the hydroxyl group of Ser-228 is the catalytic nucleophile of cPLA$_2$. Replacement of Ser-228 by Ala, Cys, or Thr resulted in the inability of these mutants to mediate calcium ionophore induced PGE_2 production in COS-7 cells cotransfected with the cPLA$_2$ mutants and cyclooxygenase-1. Cell lysates from these transfected cells also had undetectable levels of cPLA$_2$ phospholipid hydrolyase activity as did the affinity column purified S228A and S228C cPLA$_2$ mutants overexpressed in insect cells. The loss in activity was not due to the inability of the mutant enzymes to translocate to the substrate lipid interface since the purified S228C cPLA$_2$ mutant, like the wild type, translocated to the phospholipid membrane in the presence of calcium as judged by fluorescence energy transfer. However, when an activated substrate, 7-hydroxycoumarinyl γ-linolenate (p$K_a \approx 7.8$ for its leaving group) was used as substrate, there was a significant level of 7-hydroxycoumarin esterase (7-HCEase) activity (about 1% of wild type) associated with the purified S228C cPLA$_2$ mutant. The S228A cPLA$_2$ mutant was catalytically inactive. Contrary to wild type cPLA$_2$, the 7-HCEase activity of the thio-cPLA$_2$ was not titrated by the irreversible active-site-directed inhibitor methyl arachidonyl fluorophosphonate, but rather titrated by one equivalent of arachidonyl bromomethyl ketone, an arachidonyl binding site directed sulfhydryl reagent. These results are compatible with the hydroxyl of Ser-228 being the catalytic nucleophile of cPLA$_2$ and that cysteine can replace serine as the nucleophile, resulting in a thiol-cPLA$_2$ with significantly reduced catalytic power.

Phospholipases A_2 (PLA_2) (EC 3.1.1.4) are hydrolytic enzymes which catalyze the cleavage of the ester bond at the *sn*-2 position of phospholipids to generate lysophospholipid and free fatty acid. In mammals, PLA_2s are present as a structurally diverse group of enzymes that include both calcium-dependent and -independent members (Ackermann et al., 1994; Hazen et al., 1990). The best-studied PLA_2s are the secretory PLA_2s (sPLA$_2$)[1] with an average molecular weight of 14 kDa (Glaser & Vadas, 1995; Kramer, 1994). Conversely, cytosolic PLA_2 (cPLA$_2$) is an 85-kDa cytosolic enzyme (Clark et al., 1991; Sharp et al., 1991). A comparison of the biochemical properties of these enzymes reveals that there is very little structural or mechanistic similarity between them. The sPLA$_2$s show no selectivity toward the fatty acid moiety at the *sn*-2 position and require millimolar concentrations of calcium for activity. Calcium is not only involved in sPLA$_2$ membrane association but is also a part of the catalytic machinery. Crystal structure analysis of the phosphono transition state analog/sPLA$_2$ complex has demonstrated the presence of a calcium ion in the active site which is involved both in catalysis and substrate binding (Scott et al., 1990; Wery et al., 1991). In contrast, cPLA$_2$ selectively catalyses the hydrolysis of cis polyunsaturated fatty acids such as arachidonic acid from the *sn*-2 position of phospholipids (see Scheme 1) and is activated by submicromolar concentrations of calcium (Clark et al., 1990; Kramer et al., 1991). Calcium does not participate in the catalytic mechanism directly but is required for its membrane association (Wijkander & Sundler, 1992; Reynolds et al., 1993; Huang et al., 1994). In addition to its calcium-dependent PLA_2 activity, cPLA$_2$ also possesses a calcium-independent lysophospholipase activity and a calcium stimulated 7-hydroxycoumarin esterase (7-HCEase) activity (see Scheme 1) (Leslie, 1991; Huang et al., 1994). Furthermore, a number of transition state inhibitors of sPLA$_2$ are inactive against cPLA$_2$ (Trimble et al., 1993). Conversely, the transition state analog arachidonyl trifluoromethyl ketone ($AACOCF_3$) of cPLA$_2$ is inactive against sPLA$_2$ (Street et al., 1993). This data would suggest that even though these two enzymes catalyze the same reaction, there must be some fundamental differences in their mechanism of catalysis.

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[1] Abbreviations: γ-LLCU, 7-hydroxycoumarinyl γ-linolenate; 7-HC, 7-hydroxycoumarin; 7-HCEase, 7-hydroxycoumarin esterase activity; AABrMK, arachidonyl bromomethyl ketone; $AACOCF_3$, arachidonyl trifluoromethyl ketone; BAEE, benzyl-L-arginine ethyl ester; Cox-1, cyclooxygenase-1; cPLA$_2$, cytosolic phospholipase A_2; dansyl-PE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; DTEPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; MAFP, methyl arachidonyl fluorophosphonate; PGE_2, prostaglandin E_2; PMSF, phenylmethylsulfonyl fluoride; PyAPC, 1-(10-pyrenedecanyl)-2-arachidonyl-*sn*-glycerolphosphatidyl choline; OSPC, 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; sPLA$_2$, secretory phospholipase A_2.

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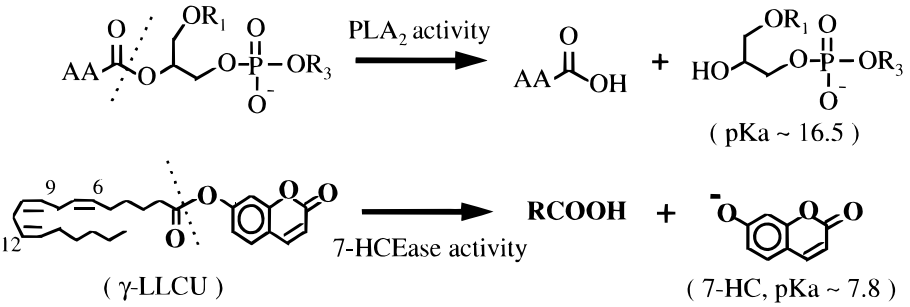
Scheme 1. Enzyme Activities of $cPLA_2$



Recently, Trimble et al. (1993) reported on the NMR spectrum of the $AA^{13}COCF_3/cPLA_2$ complex and found it to be similar to that previously observed for the binding of a peptidyl trifluoromethyl ketone inhibitor to α-chymotrypsin. The ^{13}C chemical shift of the carbonyl carbon of this complex was close to that of the anion of the methyl hemiketal of $AACOCF_3$, suggesting that the $cPLA_2$-AACOCF3 complex existed as an ionized hemiketal-like species. This led the authors to propose that there may be similarity in the catalytic mechanisms of the two enzymes and that a serine nucleophile maybe involved in catalysis. $cPLA_2$ has also been shown to catalyze transacylation reactions which was also suggestive of an acyl-enzyme intermediate in the catalytic pathway of $cPLA_2$ (Hanel & Gelb, 1995; Reynolds et al., 1993). Assuming that a serine nucleophile is involved, it is very likely that this residue would be contained within a sequence similar to the conserved pentapetide sequence G-X-S-X-G found in the catalytic site of the serine proteinases, esterases and lipases (Derewenda & Derewenda, 1992; Derewenda & Sharp, 1993). However, this sequence is not present in $cPLA_2$, although there are four sites with the sequence G-X-S-G. Two of these sites have the sequence G-(L,V)-S-G-S which is identical to a second serine concensus active site sequence, G-X-S-X-S, found in the Aeromonas hydrophila lipase/acyltransferase (G-D-S-L-S) and *Escherichia coli* thioesterase I (Hilton & Buckley, 1991; Robertson et al., 1994; Cho & Cronan, 1993). While this work was in progress Sharp et al. (1994) recently showed, that mutagenesis of Ser-228 of $cPLA_2$ which is present in the sequence G-L-S(228)-G-S, results in an inactive enzyme. This result suggested that this residue was critical for the activity, but there was no direct proof for this being the active-site nucleophile. However, by using the recently described $cPLA_2$ substrate, 7-hydroxycoumarinyl γ-linolenate (Huang et al., 1994) and novel active-site-directed inhibitors we prove conclusively that Ser-228 is the active-site nucleophile of $cPLA_2$.

MATERIALS AND METHODS

Materials. Chemicals were from Sigma, Aldrich, and Avanti Polar Lipids. The synthesis and purification of methyl arachidonyl fluorophosphonate (MAFP) and [^{14}C]-methyl arachidonyl fluorophosphonate will be described elsewhere (Huang et al., manuscript in preparation).

Synthesis of Arachiodonyl Bromomethyl Ketone (AABrMK). In a 50 mL round bottom flask, 150 mg of arachidonyl chloride, 15 mL of ethyl ether, and 2 mL of freshly generated ethereal solution of diazomethane at room temperature were added. The formation of the arachidonyl diazomethyl ketone was monitored by TLC and completed in 30 min. Excess diazomethane was quenched with 0.5 mL of acetic acid. The resulting reaction mixture was partitioned between 150 mL of diethyl ether and cold brine. The ether layer was concentrated on a rotary evaporator with a cold water bath (10 °C) to minimize decomposition. The concentrated product was immediately loaded onto a 15 cm × 2 cm silica column and eluted with 20% ethyl acetate in hexanes; 100 mg of pure arachidonyl diazomethyl ketone was recovered. ^1H NMR analysis indicated it was >98% pure. ^1H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, $J = 7$ Hz), 1.30 (6H, m), 1.70 (2H, m), 2.06 (4H, m), 2.35 (2H, m), 2.80 (6H, m), 5.20 (1H, bs), 5.37 (8H, m).

A 100 mg amount of the purified arachidonyl diazomethyl ketone in 10 mL of dry THF was mixed with 2 mL of THF presaturated with hydrogen bromide (gas) at 0 °C in a round bottom flask. The conversion of the diazo compound to arachidonyl bromomethyl ketone was monitored immediately on TLC to prevent the hydrobrominations on the double bonds (20% EtOAc in hexanes, arachidonyl diazomethyl ketone $R_f \approx 0.3$, arachidonyl bromomethyl ketone $R_f \approx 0.7$). Upon the completion of the reaction (approximately 10 min), the excess hydrogen bromide was quenched by the addition of 25 mL of ice-cold 200 mM potassium phosphate buffer (pH 7.0). The product was partitioned into 150 mL of diethyl ether and washed twice with 150 mL of phosphate buffer. The ether layer was dried over anhydrous sodium sulfate and purified on a 2 × 15 cm silica gel column. Upon elution with 5% ethyl acetate in hexanes, 90 mg of arachidonyl bromomethyl ketone was recovered. ^1H NMR analysis indicated it was pure with no noticeable hydrobromination on the double bonds. ^1H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, $J = 7$ Hz), 1.35 (6H, m), 1.75 (2H, m), 2.05 (4H, m), 2.65 (2H, $J = 7.3$ Hz), 2.80 (6H, m), 3.87 (2H, s), 5.37 (8H, m).

Site-Directed Mutagenesis. The human $cPLA_2$ cDNA was cloned by PCR and inserted into the eukaryotic expression vector pSG5 (Abdullah et al., 1995a). This vector has an f1 orgin of replication for the production of single-stranded DNA. The human Cox-1 cDNA cloned into pcDNA1 (Invitrogen) was a generous gift of Dr. Colin Funk, Vanderbilt University. Table 1 lists the amino acid changes that were made in $cPLA_2$. All oligonucleotides used in the mutagenesis were synthesized on an Applied Biosystems 380B DNA synthesizer. Site-directed mutagenesis was performed according to the method of Kunkel et al. (1987), and positive clones were identified by sequencing.

COS-7 Cell Transfection and $cPLA_2$-Cox-1 Coupling Assay. COS-7 cells (5×10^5 cells/plate) were transfected using a modified calcium phosphate procedure described by Chen and Okayama (1988). The $cPLA_2$-Cox-1 coupling assay was performed by transfecting 5 μg of each cDNA into COS-7 cells. Four plates were used for each transfec-

Table 1: Oligonucleotides Used for the Mutagenesis of $cPLA_2$ and the Amino Acid Replacements

SER 195 -> **ALA**	5'-ATA TTG GGT **GCT** GGT GGG GGT-3'
SER 206 -> **ALA**	5'-GTG GGA TTC **GCT** GGT GTG ATG-3'
SER 228 -> **ALA**	5'-GCT GGT CTT **GCT** GGC TCC ACC-3'
ALA 228 -> **SER**	5'-GCT GGT CTT **TCT** GGC TCC ACC-3'
SER 228 -> **CYS**	5'-GCT GGT CTT **TGT** GGC TCC ACC-3'
SER 228 -> **THR**	5'-GCT GGT CTT **ACA** GGC TCC ACC-3'
ALA 228 -> **SER**	5'-GCT GGT CTT **TCT** GGC TCC ACC-3'
SER 230 -> **ALA**	5'-CTT TCT GGC **GCT** ACC TGG TAT-3'
SER 408 -> **ALA**	5'-TTG GGC GTT **GCT** GGT TCA CAA-3'
SER 505 -> **GLU**	5'-TCT TAT CCA CTG **GAG** CCT TTG AGT GAC-3'
SER 729 -> **ALA**	5'-TGC TCT GTT **GCT** CTT AGT AAT-3'

tion. Two days after transfection the media was removed, the cells were washed 3 times with Hank's media, and 2 mL of Hank's media was then added to each plate. The calcium ionophore A23187 in dimethyl sulfoxide was added (final concentration 1 μM) to two of the plates and the remaining two plates received vehicle. The plates were incubated at 37 °C for 10 min, and the amount of PGE_2 released into the media was measured by RIA (Amersham). The cells were then scraped from the plate and frozen in liquid N_2 for later immunoblot and $cPLA_2$ activity analysis. Transfection controls for the coupling assay were pSG5/$cPLA_2$ and pcDNA1, 5 μg each, pcDNA1/Cox-1 and pSG5, 5 μg each, and pcDNA1 and pSG5, 5 μg each.

Baculovirus Expression. Overexpression of $cPLA_2$ mutants in Sf9 cells using a recombinant baculovirus was performed as described previously (Abdullah et al., 1995a). Briefly, the baculovirus vector pJVETLZ-$hcPLA_2$ was digested with *Nco*I and gel purified. This digestion removes the 1.6 kb *Nco*I fragment from the coding region of $cPLA_2$. The 1.6 kb *Nco*I fragment from the appropriate pSG5/$cPLA_2$ mutants was then cloned into the *Nco*I digested baculovirus vector. The mutant was confirmed by sequencing and used for the generation of a recombinant baculovirus.

Preparation of COS-7 Cell Lysates and Purification of $cPLA_2$ from Sf9 Cells. The frozen samples from the COS-7 cell transfection experiments were thawed and resuspended in 0.1 mL of lysis buffer (phosphate-buffered saline, 5 mM EDTA, 1 mM PMSF, 1 mM BAEE, 1 mM benzamidine, and 1 μg/mL each of leupeptin and aprotinin). The cells were disrupted by freeze thawing, followed by centrifugation for 1 h at 100 000*g* at 4 °C. $cPLA_2$ activity and immunoblot analysis were performed on an aliquot of the cytosol as described below. Wild type and mutant $cPLA_2$ were purfied from infected Sf9 cells by affinity chromotography using the conditions as previously described (Abdullah et al., 1995b). Briefly 100 mL of Sf9 cells (2×10^6 cells/mL) were infected with the recombinant baculovirus at an moi (multiplicity of infection) of 5−10. The cells were processed 2 days after infection, and approximately 0.5−1 mg of enzyme was purified per 100 mL of infected cells. However, since the same affinity column was used for both wild type and mutant $cPLA_2$ enzyme preparations, there was the possibility that the mutant enzyme preparations were being contaminated with a very small amount of wild type enzyme that remained on the column even after washing with 1% CHAPS (Abdullah et al., 1995b). Therefore a second mutant $cPLA_2$ enzyme preparation was made, and in order to reduce wild type $cPLA_2$ contamination of the mutant enzymes, the columnwas treated with protease and washed extensively with 1% SDS after each purification (see Table 2).

Electrophoresis and Immunoblotting. SDS−PAGE analysis of samples was done according to Laemmli's procedure (Laemmli, 1970). The $cPLA_2$ phosphorylation bandshift analysis was performed on 10% polyacrylamide SDS gels (11×15 cm) which were electrophoresed an additional 1.5 h after the bromophenol blue dye front came off the gel. Immunoblot analysis was done using a $cPLA_2$ peptide antisera, described previously (Abdullah et al., 1995a), and developed by ECL (Amersham) using the conditions suggested by the manufacturer. Quantitation of immunoblots were performed on a Molecular Dynamics laser densitometer.

Fluorescence Energy Transfer. Fluorescence energy transfer was carried out as described (Stryer, 1968; Nalefski et al., 1994). Small unilamellar vesicles were prepared as follows. The chloroform solution of dansyl-PE (dansyl DHPE, D-57, Molecular Probe) and OSPC was mixed in 1:9 molar ratio, dried under nitrogen, resuspended in HEPES buffer (50 mM HEPES, 0.3 mM EDTA, pH 7.2) at a final concentration of 2 mM, and sonicated. The calcium-induced translocation of the S228C $cPLA_2$ mutant to the sonicated vesicles was monitored by the excitation spectra scan (slit 5 nm) with emission at 515 nm (slit at 10 nm) in a stirred cuvette containing 50 mM HEPES, 0.3 mM EDTA at pH 7.2. The spectra intensity of the $cPLA_2$ mutant with and without calcium were normalized against the excitation spectrum intensity at 335 nm of the mixed vesicle to account for the dilution induced intensity variations. The calcium induced translocation of the wild type $cPLA_2$ and its calcium concentration titration to dansyl-PE/OSPC vesicles were similar to those reported by Nalefski et al. (1994). The time course of calcium-induced translocation of the S228C mutant to the dansyl-PE/OSPC mixed vesicles was monitored using excitation at 285 nm (slit: 5 nm) and emission at 515 nm (slit: 10 nm). Calcium chloride (final concentration 1 mM) was added in the middle of the time course via a concentrated stock, the sample dilution induced a negligible intensity decrease as judged from a corresponding control.

Enzyme Activity Assays. PLA_2 activity was determined by monitoring the hydrolysis of the fluorogenic substrate 1-(10-pyrenedecanyl)-2-arachidonyl-*sn*-glycerolphosphatidyl choline (PyAPC) continuously on a Perkin-Elmer LS-50B fluorospectrometer as previously described (Abdullah et al., 1995a).

The $cPLA_2$ 7-hydroxycoumarin esterase (7-HCEase) activity assay has been described in detail by Huang et al. (1994). Briefly, 7-HCEase activity was measured in a sonicated 1 mL of DTEPM (1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol)/Triton X-100 high-affinity mixed micelle solution containing 960 μM Triton X-100, 40 μM DTEPM, 20 μM 7-hydroxycoumarinyl γ-linolenate (γ-LLCU), 50 mM HEPES, pH 7.2, 0.3 mM EDTA, and 1 mM Ca^{2+}. The formation of the product, 7-hydroxycoumarin (7-HC), was monitored continuously by a Perkin-Elmer fluorospectrometer with excitation at 335 nm and emission at 450 nm. The change in fluorescent intensity was converted to the changes in 7-HC based on a standard curve.

Active-Site Titration of $cPLA_2$ with Methyl Arachidonyl Fluorophosphonate (MAFP). A detailed account of the synthesis and inhibition kinetics of MAFP will be published elsewhere (Huang et al., manuscript in preparation), but here we outline its use as an active-site titrator of $cPLA_2$. Purified wild type $cPLA_2$ (0.75 μM, determined by A_{280} absorbance and a $cPLA_2$ extinction coefficient of 0.827 cm^{-1} mg^{-1} (Huang et al., 1994) in DTEPM/Triton X-100 mixed micelle

solution described above without substrate was incubated at room temperature for 3 h with increasing concentrations of MAFP (up to 5 equiv). The residual 7-HCEase activity of the preincubation mixture was detected by diluting 10 μL of preincubation mixture into 1 mL of 7-HCEase activity-assay buffer (20 μM of γ-LLCU, 40 μM DTEPM, 940 μM Triton, 1 mM Ca, 0.3 mM EDTA, and 50 mM HEPES at pH 7.2).

Active-site titration of the residual 7-HCEase activity of S228A and S228C cPLA$_2$ with MAFP was carried out as follows. Affinity-purified mutant enzyme ($\sim$3.5 μM) was incubated at room temperature with increasing concentrations of MAFP (up to 5.3 equiv) in the above mixed micelle preincubation buffer for 10 h. The residual activity was determined by diluting 60 μL of the preincubation mixture into 1 mL of the 7-HCEase activity assay buffer.

Active-Site Titration of cPLA$_2$ with Arachidonyl Bromomethyl Ketone (AABrMK). Purified wild type ($\sim$1.0 μM) or S228C mutant ($\sim$3.0 μM) cPLA$_2$ was incubated at room temperature for 4 h in the mixed micelle buffer described above containing increasing concentrations of AABrMK (up to 12 μM). The residual 7-HCEase activity in the S228C reaction mixture was determined by diluting 70 μL of the reaction mixture into 1 mL of assay buffer in a cuvette and assayed as described above. Because of the much higher activity of the wild type enzyme a plate reader assay was used to determine the residual 7-HCEase activity in the wild type reaction. Briefly, 10 μL of the cPLA$_2$/AABrMK reaction mixtures were added to 200 μL of assay buffer in a 96-well plate. Fluorescence was determined on the same fluorospectrometer equipped with a plate reader attachment. The plate reader assay could only be done with the wild type enzyme since it was not sensitive enough to detect the mutant enzyme activity.

RESULTS

Mutation of Ser-228 Uncouples A23187-Induced PGE Production in COS-7 Cells Transfected with cPLA$_2$ and Cox-1. Recently, Sharp et al. (1994) reported that Ser-228 was essential for the catalytic activity of cPLA$_2$. It was found that replacement of Ser-228 with alanine resulted in an inactive enzyme, suggesting that this residue could be the active-site nucleophile of cPLA$_2$. However, this was only circumstantial evidence since other possibilities such as conformational change which renders the enzyme inactive could not be ruled out. We had also been using site-directed mutagenesis to identify the active site serine residue of cPLA$_2$. Our approach was very similar and was based on the concensus active-site motif G-X-S-X-G found in many hydrolytic enzymes (Derewenda & Derewenda, 1992; Derewenda & Sharp, 1993). Four putative serines were identified: three were found within the sequence G-X-S-G (S206, S228, S408), and one was found within the sequence G-S-X-G (S195). Although none of these sites was completely identical to the G-X-S-X-G motif, two of these sites, S228 and S408, had sequences identical to a second concensus serine active site, G-X-S-X-S, found in a lipase/acetyltransferase from *Aeromonas hydrophila* and thioesterase I from *E. coli* (Hilton & Buckley, 1991; Robertson et al., 1994; Cho & Cronan, 1993). A fifth serine in the middle of the sequence S-X-S-X-S (S729) was also mutated as a control mutation to determine the effect of a random serine mutation. One other serine that was also replaced was Ser-505. This serine is present in a concensus sequence for MAP kinase phosphorylation. It has been shown that this serine is phosphorylated by MAP kinase in response to agonist stimulation of the cell, resulting in an apparent increase in enzyme activity (Lin et al., 1992, 1993). This cPLA$_2$ mutant was also used as a control. Alanine was used to replace each of these serine residues.

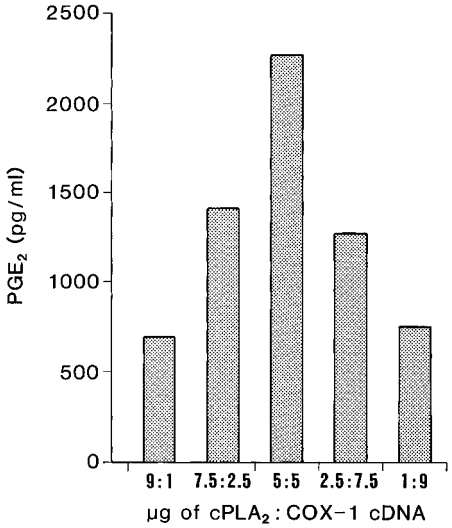


FIGURE 1: In order to quickly assess the cPLA$_2$ activity of the various mutants, an assay was developed that coupled the selective release of arachidonic acid by cPLA$_2$ to the cyclooxygenase activity of Cox-1. COS-7 cells were transfected with the indicated amounts of cPLA$_2$ and Cox-1 cDNAs. Two days after transfection, the amount of PGE$_2$ released into the media was measured following ionophore challenge (A23187, 1 μM). To assay the activity of the cPLA$_2$ mutants, 5 μg of both cDNAs were transfected.

In order to quickly assess the activity of the cPLA$_2$ mutants we developed a semiquantitative assay by coupling the arachidonic acid release activity of cPLA$_2$ to the cyclooxygenase activity of human Cox-1 and measuring PGE$_2$ released from transfected COS-7 cells. Shown in Figure 1 is the amount of PGE$_2$ released from COS-7 cells after transfecting varying amounts of cPLA$_2$ and Cox-1 cDNA plasmids and challenging with A23187. There was no PGE$_2$ production when only the pSG5/cPLA$_2$ vector was transfected and only about a 2-fold over background level (5−10 pg/mL) when only pcDNA1/Cox-1 was transfected. The highest level of PGE$_2$ release was when 5 μg of each plasmid was transfected which was greater than 100$\times$ background levels. The PGE$_2$ production of cells not treated with calcium ionophore was about 5-fold less than ionophore-treated cells. To check the cPLA$_2$ activity of the various mutants, cells were transfected with 5 μg each of the cPLA$_2$ and Cox-1 cDNA plasmids and then calcium ionophore challenged. The amount of PGE$_2$ released from the transfected cells for each of the cPLA$_2$ mutants based on 100% for wild type cPLA$_2$ is shown in Figure 2. The S228A cPLA$_2$ mutant was the only Ser to Ala mutant of the five serines identified above that did not couple with Cox-1 to produce PGE$_2$. In addition, conservative substitution of Ser-228 by cysteine or theronine also resulted in inactive enzymes as determined by this assay. The lack of PGE$_2$ production in mutant Ser-228 cPLA$_2$-transfected cells suggests that these cPLA$_2$s are incapable of mediating the A23187-induced arachidonic acid release and that Ser-228 is essential for cPLA$_2$ activity within the cell.

In order to prove that the inactivity of the S228A cPLA$_2$ mutant was due to the replacement of Ser-228 by Ala and

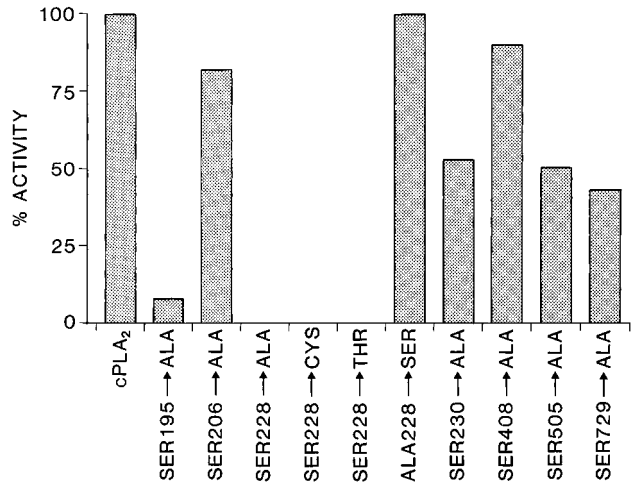


FIGURE 2: Relative levels of PGE_2 produced in COS-7 cells transfected with Cox-1 and the various $cPLA_2$ mutants. Results are presented as percent of wild type $cPLA_2$+Cox1 PGE_2 levels following ionophore challenge (A23187, 1 μM). Experiments were done in duplicate, and the results are the average of two to five experiments.

not due to some other random mutation that occurred during its preparation, the S228A $cPLA_2$ was reverted back to wild type. The reversion of the S228A $cPLA_2$ back to wild type results in wild type $cPLA_2$ levels of PGE_2 production (Figure 2). Another explanation for the S228A $cPLA_2$ mutant's loss of activity would be that this mutation causes some sort of conformational change that inactivates the enzyme. If this was the case, then mutation of a serine residue close to Ser-228 may have the similar affect on activity. However, replacement of Ser-230 by Ala had a minimal effect on activity, reducing PGE_2 levels to 50% of wild type.

$cPLA_2$ Activity in Transfected COS-7 Cytosols. In order to quantify the effect these mutations had on the hydrolytic activity of $cPLA_2$, the total phospholipid-hydrolyzing activity in the cytosol of the transfected COS-7 cells was determined by a fluorescence-based vesicle assay using PyAPC as its substrate. The level of $cPLA_2$ expressed in the cell was determined by densitometry quantitation of immunoblots, and this value was used to normalize the $cPLA_2$ activity data (Figure 3A). Most of the $cPLA_2$ mutants had specific activities comparable to wild type. The obvious exceptions were mutations of Ser-228 which all had undetectable PyAPC-hydrolyzing activity. Under these assay conditions, an activity of $>0.1\%$ of that of wild type would be detected.

An immunoblot of the $cPLA_2$ mutants expressed in COS-7 cells is shown in Figure 3B. All mutants are expressed, although there is some variablity in the level of expression. The wild type $cPLA_2$ transiently expressed in COS-7 cells is present as a doublet as are most of the expressed mutants. The slower migrating band has been shown to be the result of phosphorylation on Ser-505 by MAP kinase (Lin et al., 1992, 1993). This phosphorylation event normally occurs in cells exposed to various agonists, but $cPLA_2$ overexpressed in COS-7 and Sf9 cells has been found to be extensively phosphorylated on Ser-505 (Abdullah et al., 1995a). Phosphorylation of $cPLA_2$ on Ser-505 can increase enzyme activity, and it is thought that this is one of the steps that leads to activation of the enzyme in the cell. On the basis of the relative intensity of the gel-shifted species, mutation of Ser-228 seems to have an affect on the level of phosphorylation of Ser-505 by MAP kinase (Figure 3B,C). Replacement of Ser-228 by Ala was the least affected of these mutant

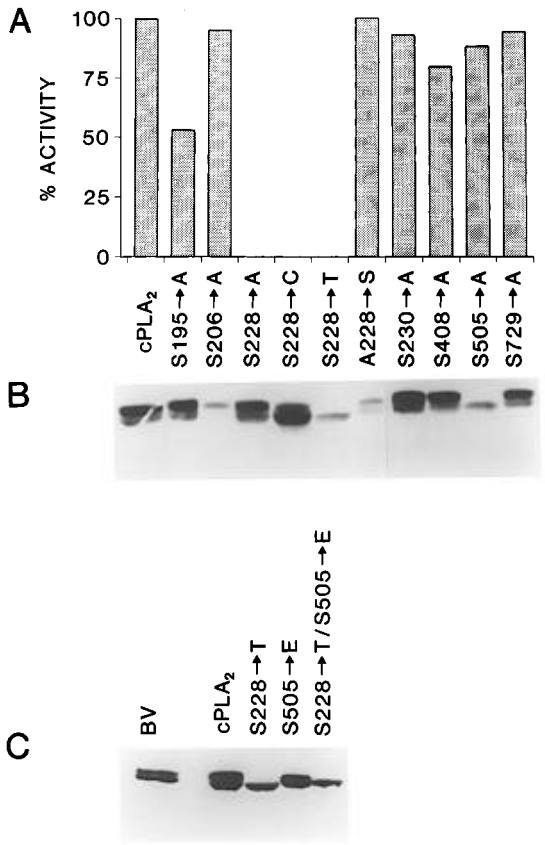


FIGURE 3: Enzyme activity and expression levels of $cPLA_2$ and $cPLA_2$ mutants in transfected COS-7 cells. (A) Normalized $cPLA_2$ activities found in the cytosol of transfected COS-7 cells. $cPLA_2$ activity was determined on an aliquot of cytosol using the PyAPC continuous fluorescence assay. $cPLA_2$ activity was then normalized to the level of $cPLA_2$ enzyme expressed in the cell by quantitation of the immunoblot. (B) Immunoblot analysis of the various $cPLA_2$s expressed in transfected COS-7 cells. A 10 μL amount of cytosol from COS-7 cells transfected with the indicated $cPLA_2$ mutant was electrophoresed on a 10% polyacrylamide SDS gel. Immunoblots were done using a $cPLA_2$ peptide antisera and developed by ECL (Amersham). Quantitation of the various $cPLA_2$ mutants was performed on the immunoblot autoradiograph by laser densitometry. (C) Immunoblot demonstrating by gel mobility shift that the S228T $cPLA_2$ mutant has an undetectable level of phosphorylation on Ser-505. BV, baculovirus-expressed wild type $cPLA_2$.

$cPLA_2$s and was expressed as the doublet similar to the levels of wild type. This would suggest that it is also phosphorylated on Ser-505 to the same extent as wild type. The S228C mutant had a reduced level of the gel-shifted species, suggesting a reduced level of phosphorylation on Ser-505. In contrast the S228T mutant appeared only to be expressed as the faster migrating unphosphorylated $cPLA_2$ (the lower band or the S505A $cPLA_2$), suggesting that this mutation results in a $cPLA_2$ that is no longer a substrate for MAP kinase. There is the possibility that the S228T $cPLA_2$ was phosphorylated, but the change in amino acid at position 228 had now altered the mobility of the Ser-505 phosphorylated species to migrate at the position of the unphosphorylated form. We had found that replacement of Ser-505 by Glu produced a $cPLA_2$ with an SDS polyacrylamide gel mobility similar to the Ser-505 phosphorylated $cPLA_2$ (Figure 3C), suggesting that the reduced mobility was due to the presense of a negative charge at this position. If the S228T $cPLA_2$ was phosphorylated but because of this mutation migrated as the lower band, then the double mutant S228T,S505E $cPLA_2$ would also migrate as the lower band. The result in Figure 3C shows that this was not the case, since the double

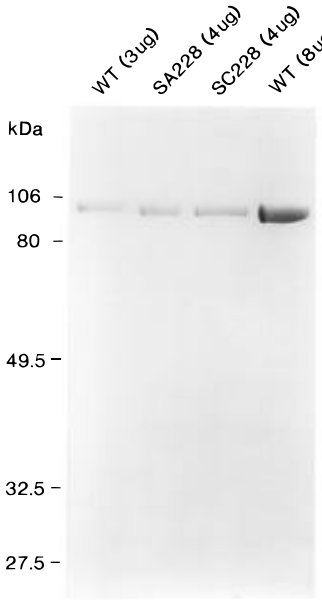


FIGURE 4: Analysis of the affinity column purified cPLA$_2$ enzymes. The wild type (WT) and the S228A and S228C cPLA$_2$ enzymes overexpressed in insect cells were purified on an Affi-665 affinity column. The indicated amounts were analyzed on a 10% polyacrylamide SDS gel and then stained with Coomassie Blue.

mutant had the mobility of the phosphorylated form. Therefore, one possible explanation of this result is that the Ser-228 to Thr mutation has some sort of long range effect on the efficiency of phosphorylation of the Ser-505 MAP kinase site.

Hydrolytic Activity of S228A and S228C cPLA$_2$s. Even though the Ser-228 cPLA$_2$ mutants have no detectable activity in the two assays described above, the data are still insufficient to establish Ser-228 as a residue involved in the catalytic machinery. In order to prove that Ser-228 was the catalytic nucleophile, the effect on the catalytic efficiency of the enzyme due to the replacement of the hydroxyl group of serine by the sulfhydryl group of cysteine was evaluated. In order to obtain large amounts of pure enzyme for such an analysis, both the S228A and S228C mutant cPLA$_2$s were over expressed in insect cells using a recombinant baculovirus. We and others have shown that active cPLA$_2$ is abundantly expressed and extensively phosphorylated on Ser-505 when overexpressed in Sf9 cells (Abdullah et al., 1995a; Becker et al., 1994; de Carvalho et al., 1993). The overexpressed cPLA$_2$ and Ser-228 mutants were purified from insect cells using Affi-665 affinity chromatography as described by Abdullah et al. (1995b,c) (Figure 4). The purified enzyme preparations were tested for phospholipase A$_2$ activity using the PyAPC substrate in both vesicle and mixed micelle assay conditions. No enzyme activity was observed, under both assay conditions in which >0.1% of wild type activity would be detectable, for both of the Ser-228 mutants (Table 2). Sharp et al. (1994) have also reported that insect cell extracts expressing these cPLA$_2$ mutants were inactive in PLA$_2$ and lysophospholipase assays.

Another explanation for the Ser-228 mutants' loss of catalytic activity is that these mutations do not affect the catalytic mechanism but prevent the translocation or association of the enzyme with the substrate lipid interface. In order to determine if these mutations have any affect on membrane association, the calcium induced membrane association of S228C cPLA$_2$ was determined by fluorescence energy transfer using a 10 mole % dansylPE/OSPC lipid interface (Stryer, 1968; Nalefski et al., 1994). Upon the addition of calcium, an instantaneous increase in the energy transfer efficiency from the intrinsic fluorescence of the S228C cPLA$_2$ to the dansyl probe was observed (Figure 5). This would be consistent with calcium inducing the membrane association of cPLA$_2$ (Nalefski et al., 1994). Thus the lack of PLA$_2$ activity for the S228C cPLA$_2$ was not due to its inability to translocate to the substrate-containing phospholipid interface.

It has been shown for the serine proteases, such as trypsin and subtilisin, that mutation of the serine nucleophile to cysteine reduces the catalytic activity by a factor of 10^5 with minimal changes in K_m (Hagaki et al., 1989). The residual activity of the cysteine-mutant enzymes (i.e., thiol-trypsin) can be detected by using activated ester substrates such as N^{α}-carbobenzoxy-L-lysine ρ-nitrophenyl ester (Hagaki et al., 1989). Recently, we reported that in addition to phospholipase A$_2$ and lysophospholipase activities, cPLA$_2$ catalyzes the hydrolysis of 7-hydroxycoumarin esters of polyunsaturated fatty acids (7-HCEase activity) to produce free fatty acids and the highly fluorescent 7-hydroxycoumarin (Huang et al., 1994). Comparison of the PLA$_2$ and the 7-HCEase activities of cPLA$_2$ indicates they are from the same active site since (1) similar substrate preference were observed, (2) identical calcium sensitivity were observed, (3) identical reaction progress curves were observed when the radiolabeled phospholipid substrate 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine and the 7-HCEase substrate 7-hydroxycoumarinyl γ-linolenate (γ-LLCU) were codispersed in the same lipid interface, and (4) parallel inhibitions of both 7-HCEase activity and PLA$_2$ activity were observed by the active site directed reversible inhibitor AACOCF$_3$. These and other kinetic evidence indicated that the three hydrolytic activities of cPLA$_2$ (7-HCEase, PLA$_2$, and the lysophospholipase activities) occur in the same active site through the use of the same catalytic nucleophile. These 7-hydroxycoumarin ester substrates are analogous to the activated ester substrates of the serine proteases since the 7-hydroxycoumarin is a better leaving group (p$K_a \approx 7.8$) than the secondary alcohol of glycerol phosphate (p$K_a \approx$ 16.5). Therefore, the Ser-228 mutants were tested for their ability to catalyze the hydrolysis of 7-hydroxycoumarinyl γ-linolenate (Table 2). Both Ser-228 cPLA$_2$ mutants had a detectable esterase activity, athough considerably less than wild type. The fact that the S228A cPLA$_2$ mutant had activity would suggest that Ser-228 is not the active-site nucleophile of cPLA$_2$. However, this assay is very sensitive to contaminating esterases, and there was the possibilty that some nonspecific esterase may have been carried through in the purification. Furthermore, it is very likely that this esterase activity maybe due to wild type cPLA$_2$ contaminating the mutant cPLA$_2$ enzyme preparations since the same affinity column was used for both wild type and mutant purifications. In an attempt to remove the possible esterase contaminating activity from the mutant cPLA$_2$ preparations, a second batch of enzyme was purified under more rigid purification conditions. In this case the 7-HCEase activity of the S228A cPLA$_2$ was reduced to 0.1% of wild type whereas, the S228C cPLA$_2$ mutant still retained approximately 1% of wild type activity (Table 2).

Methyl Arachidonyl Fluorophosphonate Titrations of the Active Site of cPLA$_2$ and Ser-228 Mutant cPLA$_2$s. On the basis of the arachidonyl selectivity of cPLA$_2$ and the possible involvement of a serine residue as the active-site nucleophile,

Table 2: Enzyme Activities and Active-Site Titrations of Wild Type, S228A, and S228C $cPLA_2$s[a]

sample	PLA_2 activity	7-HCEase activity	% of 7-HCEase sensitive to MAFP	equivalents of MAFP[a]	% of 7-HCEase activity sensitive to AABrMK	equivalents of AABrMK[b]
wild type	100%	100%	100%	1	0	
S228A						
P1	<DL	~0.8%	100%	≤0.05	ND	
P2	<DL	~0.1%	ND	ND	ND	
S228C						
P1	<DL	~1%	25%	≤0.05	75%	~1
P2	<DL	~1%	10%	≤0.05	90%	~1

[a] Activities are presented as a percentage of wild type $cPLA_2$ activity. The two purified preparations of the $cPLA_2$ mutants are identified as P1 and P2. The second sample of purified enzyme was prepared using more rigid purification conditions than the initial preparation (see Materials and Methods). The enzyme activity assays and the active-site titrations were perfomed as described in Materials and Methods. ND, not determined: <DL, below the detection limit of the PyAPC assay. [b] Number of equivalents of MAFP required for complete inhibition of MAFP-sensitive activity. [c] Number of equivalents of AABrMK required for complete inhibition of MAFP-less sensitive 7-HCEase activity of S228C $cPLA_2$.

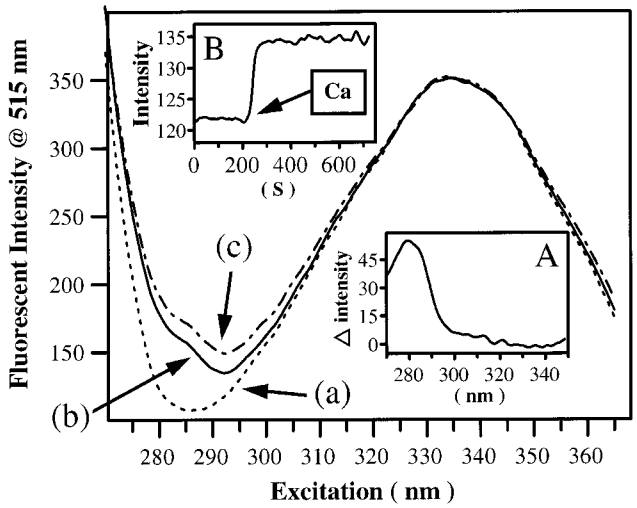


FIGURE 5: Calcium-induced translocation of S228C $cPLA_2$ to dansyl-PE/OSPC vesicles monitored by fluorescence energy transfer. Excitation spectra (emission at 515 nm) of a buffer solution containing (a) 50 μM dansyl-PE/OSPC (1:9) sonicated mixed vesicles; (b) normalized excitation spectrum after the addition of 15 μg of purified S228C mutant to solution a; (c) normalized excitation spectrum after the addition of 1 mM $CaCl_2$ to solution b. The intensity of spectra b and c was normalized against the intensity of spectrum a at 335 nm to cancel the dilution-induced intensity variations due to the addition of mutant enzyme and calcium chloride. Calcium chloride had negligible effect on spectrum a. Inset A: The difference spectra generated by subtracting b from c. With an excitation maximum centered around 290 nm, it is consistent with the involvement of tryptophan residues in the energy transfer. The magnitude of the difference spectra is linearly proportional to the S228C mutant concentration used. Inset B: The time course of calcium chloride induced S228C mutant translocation. An instantaneous translocation of S228C mutant to the mixed vesicles surface was observed after the addition of calcium. S228C (15 μg) was added to 20 μM sonicated dansyl-PE/OSPC (1:9) mixed vesicles in buffer (50 mM HEPES, 0.3 mM EDTA, pH 7.2) at ~50 s. Calcium chloride (final concentration, ~1 mM) was introduced at ~200 s. The energy transfer fluorescence signal was monitored with excitation at 285 nm (slit, 5 nm) and emission at 515 nm (slit, 10 nm). The short lag phase immediately following the calcium addition is due to the limited mixing rate of the stirred cuvette.

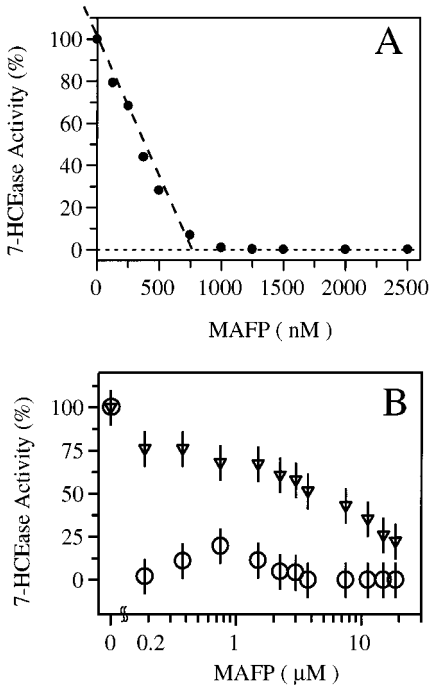


FIGURE 6: Active-site titration of wild type, S228A and S228C $cPLA_2$ by methyl arachidonyl fluorophosphonate (MAFP). (A) The 7-HCEase activity of purified wild type $cPLA_2$ (750 nM) was titrated with increasing concentrations of MAFP as described in Materials and Methods. Complete inhibition was obtained when $cPLA_2$ was incubated with 1 equiv (750 nM) of MAFP. (B) Titration of the 7-HCEase activity of the purified S228A (○) and S228C (▽) $cPLA_2$ enzymes (~3.5 μM, preparation 1) with MAFP. The two 7-HCEase activites that were present in this S228C $cPLA_2$ preparation were identified as MAFP-sensitive, inhibited with 0.05 equiv of MAFP, and MAFP-less sensitive, not inhibited with 0.05 equiv of MAFP.

we recently designed and synthesized methyl arachidonyl fluorophosphonate (MAFP) as a $cPLA_2$ inhibitor (Huang et al., manuscript in preparation). MAFP was found to be a potent time-dependent irreversible inhibitor of $cPLA_2$. It irreversibly inhibited both the PLA_2 and 7-HCEase activities of $cPLA_2$ stoichiometrically, making it an excellent active-site titrator for the $cPLA_2$ hydrolytic activities. Furthermore, the reversible $cPLA_2$ active-site inhibitor arachidonyl trifluoromethyl ketone could protect $cPLA_2$ from being inreversibly inactivated by MAFP. The mechanism of this inhibition is probably via the formation of an ester bond between the electrophilic phosphorous atom and the active-site nucleophile. Therefore, in order to further clarify the origin of the residual 7-HCEase activity of the Ser-228 mutants, the 7-HCEase activity of the purified preparations was titrated with MAFP.

Shown in Figure 6 is the active-site titration of the 7-HCEase activity of native $cPLA_2$ in response to increased concentrations of MAFP. The data shows that 1 equiv of MAFP (750 nM) was required and sufficient for the complete inactivation of the hydrolytic activities of $cPLA_2$. When the S228A $cPLA_2$ mutant (~3.5 μM, preparation 1) was treated similarly, the minimal 7-HCEase activity was inhibited with 0.2 μM (0.05 equiv) of MAFP (Figure 6B, Table 2). This

would indicate that the minimal esterase activity of this mutant which was sensitive to MAFP was due to some minor esterase contamination, possibly $cPLA_2$, and therefore the S228A $cPLA_2$ has no enzyme activity. In contrast, only 20−25% of the 7-HCEase activity in the S228C $cPLA_2$ purified sample (∼3.5 μM, preparation 1) was inhibited with 0.2 μM (0.05 equiv) of MAFP (Figure 6B and Table 2), indicating that this sample also contained a small amount of contaminating esterase activity. The remaining activity (75%) behaved in a different way toward increased concentrations of MAFP as compared with that of the wild type $cPLA_2$ activity. Prolonged incubation with more than 1 equiv of MAFP only gradually suppressed this activity, suggesting that a different mechanism of inhibition was occurring. MAFP did not completely inhibit this portion of the 7-HCEase activity of S228C even when more than 5 equiv of inhibitor was used. These two activities present in the S228C cPLA2 sample were defined as MAFP-sensitive, 7-HCEase activity inhibited by 0.05 equiv of MAFP, and MAFP-less sensitive, 7-HCEase activity that was not inhibited by 0.05 equiv of MAFP. Analysis of the second preparation of S228C $cPLA_2$ revealed that it had 10% MAFP-sensitive and 90% MAFP-less sensitive 7-HCEase activities (Table 2).

Arachidonyl Bromomethyl Ketone Titrations of the Active Site of $cPLA_2$ and S228C $cPLA_2$. In order to demonstrate that the MAFP-less sensitive 7-HCEase activity of S228C was due to the mutant and not to some esterase contamination in the sample, one would have to show that every mutant molecule possesses this activity. Again this could be achieved by the use of an active site-directed irreversible inhibitor of the mutant. It is known that α-haloketone analogs of various substrates are potent irreversible inhibitors of the cysteine proteases (Shaw, 1970). The mechanism of inactivation is through the covalent modification of the essential cysteine nucleophile (Bender & Brubacher, 1966) with the formation of a thioether linkage. By incorporating the $cPLA_2$'s recognition for the arachidonyl moiety, arachidonyl bromomethyl ketone (AABrMK) was prepared and evaluated as an inhibitor of the S228C $cPLA_2$. Prolonged preincubation of this compound even at micromolar concentrations was inactive toward the wild type $cPLA_2$ (Figure 7A). This result is analogous to that previously observed with the halomethyl ketone fatty acid $C_{19}H_{39}COCH_2Cl$ which was shown not to inhibit the wild type enzyme (Street et al., 1993). However, when AABrMK was tested against the MAFP-less sensitive 7-HCEase activity present in the purified S228C samples, an instantaneous dose-dependent inactivation of this activity was observed. The inactivation was irreversible over a 5 h period (data not shown) . Shown in Figure 7B is the active-site titration of the 7-HCEase activity of S228C $cPLA_2$ (∼3 μM, preparation 1) by AABrMK. The MAFP-less sensitive 7-HCEase activity of this preparation (75%) was completely inhibited by the preincubation of approximately 1 equiv (2.6 μM) of AABrMK. Increased concentrations of AABrMK had no further effect on the remaining ∼25% 7-HCEase activity, which was MAFP-sensitive and due to a minor esterase contamination of the purified sample. These results are consistent with the Cys-228 of the S228C $cPLA_2$ reacting with and being covalently modified by AABrMK. It is also very likely that the contaminating esterase activity in the purfied mutant $cPLA_2$ preparation was due to the presence of a small amount of wild type $cPLA_2$. This confirms that Ser-228 is the active-site nucleophile of $cPLA_2$ and that cysteine can replace serine as the nucleophile but that the thio-$cPLA_2$ is only catalytically active on the 7-hydroxycoumarin esters. The specific activity of this thio-$cPLA_2$ (0.04 nmol/mg/min, 2 mol % substrate) is about 1% of that of the wild type $cPLA_2$ activity under the same assay condition (20 μM γ-LLCU, 1 mM of high-affinity DTEPM/Triton X-100 mixed micelle, 1 mM Ca).

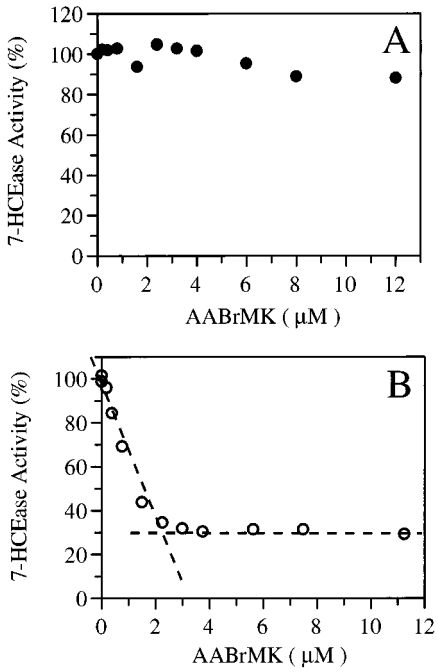


FIGURE 7: Active-site titration of wild type and S228C $cPLA_2$s by arachidonyl bromomethyl ketone (AABrMK). (A) The 7-HCEase activity of purified wild type $cPLA_2$ (∼1 μM) was titrated with increasing concentrations of AABrMK as described in Materials and Methods. (B) The same AABrMK titration of the 7-HCEase activity of affinity purified S228C $cPLA_2$ (∼3 μM). This preparation of S228C $cPLA_2$ contained a small amount of contaminating esterase activity that accounted for about 25% of the 7-HCEase activity of this sample. This activity was inhibited with ≤0.05 equiv of MAFP (MAFP-sensitive activity), and like wild type $cPLA_2$ it was not inhibited by AABrMK. The MAFP-less sensitive 7-HCEase activity of S228C $cPLA_2$ was inhibited with a near-equivalent amount of AABrMK (the AABrMK concentration at the point where the two linear lines intersect, ∼2.6 μM).

DISCUSSION

The results presented in this paper establish $cPLA_2$ as a serine hydrolase with Ser-228 as its catalytic nucleophile. A report recently by Sharp et al. (1994) showed that mutagenesis of Ser-228 resulted in an inactivated enzyme. Although they suggested that Ser-228 could be the active-site nucleophile of $cPLA_2$, other possibilities such as a conformational change of the enzyme due to the mutation can not be ruled out and could also explain this result. Using a novel series of $cPLA_2$ substrates and inhibitors, we provide evidence to show that Ser-228 is the active-site nucleophile of $cPLA_2$.

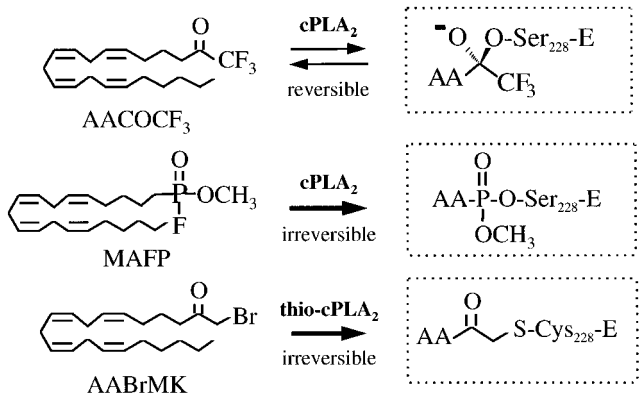
The initial identification of Ser-228 as a potentially important residue for $cPLA_2$ activity was based on its location within the sequence G-L-S^{228}-G-S (G-X-S-X-S) which was very similar to the so-called lipase consensus active-site motif, G-X-S-X-G, except that it contained a serine in place of the second glycine. This G-X-S-X-S active-site motif is also present in *A. hydrophila* lipase/acyltransferase (Hilton & Buckley, 1991; Robertson et al., 1994) and in *E. coli* thioesterase I (Cho & Cronan, 1993) and has been suggested

to represent an active-site consensus sequence for a second group of lipolytic serine esterases (Derewenda & Derewenda, 1992; Derewenda & Sharp, 1993). Interestingly, the *Aeromonas* lipase preferentially catalyzes acyl transfer from the *sn*-2 position of phospholipids and is activated by lipid/water interfaces. Like $cPLA_2$, it catalyzes the acyl transfer to various donors.

Proof that Ser-228 is the active-site nucleophile of $cPLA_2$ is summarized as follows. Replacement of this residue by any of the amino acids tested resulted in the loss of its phospholipid-hydrolyzing capability as determined in both a cell-based $cPLA_2$/Cox-1 coupling assay and the PyAPC fluorescence assay. The lack of phospholipid-hydrolyzing activity was reconfirmed with the purified S228A and S228C $cPLA_2$ mutants. This loss of activity was not due to the inability of these $cPLA_2$ mutants to associate with the substrate interface, since the S228C $cPLA_2$ readily translocates to the lipid interface in the presence of calcium. Although the purified S228C $cPLA_2$ preparation lacked the phospholipid-hydrolyzing activity, it had a significant 7-HCEase activity. This activity was insensitive at stoichiometric concentrations to inhibition by the active-site $cPLA_2$ inhibitor MAFP. This suggested that the 7-HCEase activity of the S228C $cPLA_2$ was due to the introduced cysteine acting as the nucleophile in catalysis. That this was indeed the case was demonstrated by using arachidonyl bromomethyl ketone, an arachidonyl binding-site-directed sulfhydryl reagent. This compound inhibited stoichiometrically the MAFP-less sensitive 7-HCEase activity of S228C $cPLA_2$ but had no effect on the wild type enzyme. Thus, thio-$cPLA_2$ is catalytically active but with significantly reduced catalytic power.

The decreased catalytic activity of the thio-$cPLA_2$ is very analogous to that of thiol-trypsin and thiol-subtilisin. Replacement of the serine nucleophile by cysteine decreases their catalytic power by an average factor of 10^5 (Higaki et al., 1989). These modified proteases had no observable levels of activity using normal ester and amide substrates, whereas when using activated ester substrates with 4-nitrophenol or 7-aminocoumarin as leaving groups, a low level of activity could be observed (Higaki et al., 1989; Neet & Koshland, 1966; Polgar & Bender, Yokosawa et al., 1977). The thio-$cPLA_2$ was also incapable of hydrolyzing normal phospholipid substrates. It was however, capable of hydrolyzing the activated substrate γ-LLCU. The ability of thiol-trypsin to hydrolyze activated ester substrates supported the hypothesis that the presence of a good leaving group was required for the formation of the acyl-enzyme. The fact that thiol-$cPLA_2$ is inactive toward phospholipid substrates which have a poor leaving group ($pK_a \approx 16.5$) and active on 7-hydroxycoumarin esters which have a good leaving group ($pK_a \approx 7.8$) would suggest that its reduced catalytic efficiency, like that of thiol-trypsin, is due to a reduction in its ability to form the acyl−enzyme intermediate. This not only demonstrates that Ser-228 is the active-site nucleophile but also supports the NMR studies and transacylase activity of $cPLA_2$, that suggested the presence of an acyl−enzyme intermediate in the catalytic mechanism. Even though the sulfhydryl is a better nucleophile than the hydroxyl in solution, other mechanistic requirements appear to be necessary for efficient catalysis in the active site. One of the reasons suggested for the catalytic inefficiency of thio-trypsin was thought to be due to the disruption of the active-site electrostatics caused by the replacement of an uncharged

Scheme 2. Active-Site Inhibitors of $cPLA_2$ Mutants and Proposed Complex



polar residue for a negatively charged one (McGrath et al., 1989). In addition there also appears to be a blockage of the oxyanion hole by the sulfur anion (McGrath et al., 1989). An oxyanion hole has also been suggested to be present in the $cPLA_2$ catalytic mechanism based on the ^{13}C NMR chemical shift of the $AACOCF_3$/$cPLA_2$ complex (Trimble et al., 1993). Whether or not this blockage would also occur in the S228C $cPLA_2$ remains to be determined. However, the participation of a serine residue in the $cPLA_2$ catalytic mechanism and its location within an active-site sequence that is similar to the concensus hydrolase active-site motif of the serine proteases, lipases, and esterases, suggests the involvement of a catalytic triad in the hydrolysis mechanism. If catalysis of the ester bond by $cPLA_2$ does proceed through such a mechanism, then it could explain the catalytic inefficiency of the S228C $cPLA_2$ on phospholipid substrates.

Possible mechanisms of $cPLA_2$ inhibition by the various inhibitors are illustrated in Scheme 2. Inactivation of $cPLA_2$ by MAFP is most likely due to the phosphonylation of the active-site serine with the formation of a covalent phosphonoester bond. In contrast, MAFP does not titrate the thio-$cPLA_2$ 7-HCEase activity. Nevertheless, there does appear to be a dose dependent inhibition of the 7-HCEase activity of thio-$cPLA_2$ under high MAFP concentrations and prolonged incubation conditions. This inhibition could be due to a nonspecific protein modification of the enzyme because of the prolonged incubation conditions or alternatively, the phosphonylated thio-$cPLA_2$ linkage is being turned over slowly since the phosphono−thio-ester bond is hydrolytically much more labile. In contrast, reaction of AABrMK with thio-$cPLA_2$ most likely results in the formation of a non-hydrolyzable thioether linkage with the sulfhydryl of Cys-228. Studies are in progress to isolate the inhibitor labeled active-site peptide of $cPLA_2$ and thio-$cPLA_2$ so as to confirm that these compounds are reacting with amino acid 228.

The cell-based assay coupling the selective archidonic acid release activity of $cPLA_2$ to the prostaglandin synthesis activity of Cox-1 is a novel way of identifying mutations of $cPLA_2$. Mutations affecting $cPLA_2$ activity can be quickly detected, enzyme activity is assayed within a cellular environment, and the results are very reproducible. However, it is not possible to distinguish between mutations that interfere with the catalytic mechanism vs mutations that disrupt other enzyme functions within the cell such as translocation to the membrane. This can be accomplished by measuring the enzyme activity in the transfected cell lysate. This assay also indirectly provides support for the involvement of $cPLA_2$ in the inflammatory process. Al-

though it is an artificial system, the transient transfection of $cPLA_2$ and Cox-1 into COS-7 cells shows that the arachidonic acid released by $cPLA_2$ can be utilized by cyclooxygenase to produce prostaglandins. Both Cox-1 and Cox-2 can be utilized in this assay, but because of the instability of the Cox-2 mRNA in COS-7 cells, higher levels of PGE_2 production are observed with Cox-1 (B. Kennedy, unpublished results; Hla & Neilson, 1992).

It is of interest to note that replacement of Ser-228 by threonine appears to have an effect on the level of $cPLA_2$ phosphorylation as evident by the absence of the gel-shifted phosphorylated form in cells transfected with S228T $cPLA_2$ (Figure 2C). This would indicate that S228T $cPLA_2$ has no or an undectable level of phosphorylation on Ser-505 and that this mutation affects in some way the efficiency of MAP kinase to phosphorylate this site. It is quite possible that the addition of a methyl group within the active site of $cPLA_2$ significantly disrupts the conformation of the enzyme, resulting in a $cPLA_2$ structure that is no longer a substrate for MAP kinase. This is in contrast to cysteine replacement of Ser-228 in which the sulfhydryl side chain of cysteine is only slightly larger than the hydroxyl of serine. Therefore, it is possible that slight changes in the structure of the active site of $cPLA_2$ not only has significant affects on the catalytic activity but also on the overall conformation of the enzyme.

In summary, this report provides direct evidence that Ser-228 is the catalytic nucleophile for $cPLA_2$. Cysteine can replace serine as the active-site nucleophile, resulting in a thio-$cPLA_2$ with significantly reduced catalytic power. The use of these novel substrates and inhibitors, and the various $cPLA_2$ mutants should help in the further elucidation of the catalytic mechanism of $cPLA_2$.

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