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Interfacial Kinetic and Binding Properties of Mammalian Group IVB Phospholipase A_2 (cPLA$_2\beta$) and Comparison with the Other cPLA$_2$ Isoforms*[S]

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The cytosolic (group IV) phospholipase A_2 (cPLA$_2$s) family contains six members. We have prepared recombinant proteins for human α, mouse β, human γ, human δ, human ϵ, and mouse ζ cPLA$_2$s and have studied their interfacial kinetic and binding properties *in vitro*. Mouse cPLA$_2\beta$ action on phosphatidylcholine vesicles is activated by anionic phosphoinositides and cardiolipin but displays a requirement for Ca^{2+} only in the presence of cardiolipin. This activation pattern is explained by the effects of anionic phospholipids and Ca^{2+} on the interfacial binding of mouse cPLA$_2\beta$ and its C2 domain to vesicles. Ca^{2+}-dependent binding of mouse cPLA$_2\beta$ to cardiolipin-containing vesicles requires a patch of basic residues near the Ca^{2+}-binding surface loops of the C2 domain, but binding to phosphoinositide-containing vesicles does not depend on any specific cluster of basic residues. Human cPLA$_2\delta$ also displays Ca^{2+}- and cardiolipin-enhanced interfacial binding and activity. The lysophospholipase, phospholipase A_1, and phospholipase A_2 activities of the full set of mammalian cPLA$_2$s were quantified. The relative level of these activities is very different among the isoforms, and human cPLA$_2\delta$ stands out as having relatively high phospholipase A_1 activity. We also tested the susceptibility of all cPLA$_2$ family members to a panel of previously reported inhibitors of human cPLA$_2\alpha$ and analogs of these compounds. This led to the discovery of a potent and selective inhibitor of mouse cPLA$_2\beta$. These *in vitro* studies help determine the regulation and function of the cPLA$_2$ family members.

It is well established that cytosolic phospholipase A_2-α (cPLA$_2\alpha$)[2] (also known as group IVA cPLA$_2$) liberates arachidonic acid from the *sn*-2 position of membrane phospholipids in agonist-stimulated mammalian cells for the biosynthesis of eicosanoids. With the completion of the mouse and human genomes, it became clear that these mammals also contain other proteins homologous to cPLA$_2\alpha$, namely the β, γ, δ, ϵ, and ζ isoforms (groups IVB-F cPLA$_2$s) (1, 2). This study is focused on the β isoform of mouse cPLA$_2$ (m-cPLA$_2\beta$) and its comparison with the other isoforms. m-cPLA$_2\beta$ and cPLA$_2\alpha$ have a similar modular structure with an N-terminal C2 domain linked to a catalytic domain containing the active site serine involved in the lipolytic catalysis. Human cPLA$_2\beta$ differs in that it contains an N-terminal truncated JmJc domain of unknown function followed by a C2 domain and a catalytic domain. Furthermore, human cPLA$_2\beta$ exists as two splice forms, human cPLA$_2\beta$1 and human cPLA$_2\beta$3, with the latter being the major protein form expressed in a large variety of human tissues (3). The differential splicing leads to a change in a portion of the catalytic domain that is probably not part of the active site cleft. As far as we know, m-cPLA$_2\beta$ exists as a single splice form (4).

An intriguing property of m-cPLA$_2\beta$, human cPLA$_2\beta$1, and human cPLA$_2\beta$3 is that they display specific activities for the hydrolysis of lysophosphatidylcholine and lysophosphatidylethanolamine that are ~100-fold higher than for the hydrolysis of phosphatidylcholine and phosphatidylethanolamine (3, 4). One possibility is that the active sites of the β isoforms differ from that of cPLA$_2\alpha$ in such a way that only the lysophospholipid is hydrolyzed by the former enzymes. An alternative possibility is that cPLA$_2\alpha$ can bind to the membrane interface of phosphatidylcholine or phosphatidylethanolamine vesicles but the β isoforms cannot. In this scenario, only the former enzyme can hydrolyze these vesicles, and the relatively high activity of the β isoforms for lysophospholipids reflects the hydrolysis of monomeric substrate in the aqueous phase (due to the relatively high solubility of lysophospholipids compared with phos-

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[2] The abbreviations used are: cPLA$_2\alpha$, cytosolic phospholipase A_2-α (group IVA); h, human; h-cPLA$_2\alpha$-C2, C2 domain of h-cPLA$_2$ α; [^{14}C]P-LPC, 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; h-cPLA$_2\gamma$, human cPLA$_2$ γ isoform (group IVC); h-cPLA$_2\delta$, human cPLA$_2$ δ isoform (group IVD); h-cPLA$_2\epsilon$, human cPLA$_2$ ϵ isoform (group IVE); h-cPLA$_2\zeta$, human cPLA$_2$ ζ isoform (group IVF); m-cPLA$_2\beta$, mouse cPLA$_2$ β isoform (group IVB); m-cPLA$_2\beta$-C2, C2 domain of m-cPLA$_2\beta$, m-cPLA$_2\zeta$, mouse cPLA$_2$ ζ isoform (group IVF); PAPC, 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine; [^{14}C]PAPC, PAPC labeled at C-1 of the *sn*-2 arachidonyl with ^{14}C; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-serine; RACE, rapid amplification of cDNA ends; Ni-NTA, nickel-nitrilotriacetic acid; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; POPI, 1-palmitoyl-2-oleoyl-phosphatidylinositol; CL, cardiolipin.

pholipids). This is consistent with the finding that cPLA$_2\alpha$ also displays high lysophospholipase activity (5) showing that the active site of the α and β isoforms can accommodate lysophospholipids. The problem with this hypothesis is that both the α and β isoforms contain a C2 domain that, in the case of cPLA$_2\alpha$, has been shown to be the basis of calcium-dependent binding to the surface of phospholipid vesicles and to cellular membranes (6, 7).

Here, we show that m-cPLA$_2\beta$ is as much a phospholipase A$_2$ as it is a lysophospholipase in that the enzyme displays high phospholipase A$_2$ on phosphatidylcholine vesicles that contain specific anionic phospholipids. This enzyme also has significant phospholipase A$_1$ (PLA$_1$) activity. We also compare the interfacial enzymatic properties of m-cPLA$_2\beta$ to the other cPLA$_2$ isoforms.

EXPERIMENTAL PROCEDURES

Materials—The following phospholipids are from Avanti Polar Lipids, Inc. (Alabaster, AL): cardiolipin (bovine heart, mostly tetralinoleoyl); PAPC; PI (dioleoyl); PI(4)P (brain, mostly *sn*-1-stearoyl, *sn*-2-arachidonyl); PI(3,4)P$_2$ (dioleoyl); PI(3,5)P$_2$ (dioleoyl); PI(4,5)P$_2$ (dioleoyl); PI(3,4,5)P$_3$ (dioleoyl); POPA; and POPS. PI(3)P (dipalmitoyl) is from Echelon Biosciences, Inc. (Salt Lake City, UT). [^{14}C]PAPC (50 mCi/mmol) and [^{14}C]P-LPC (50 mCi/mmol) are from PerkinElmer Life Sciences. Stock solutions of phosphoinositides were prepared in chloroform/methanol/water (4:4:1.2) at 0.1 mg/ml and stored at −80 °C in Teflon septum capped vials under argon. Stock solutions of other phospholipids were prepared in chloroform except for P-LPC, which was in chloroform/methanol (2:1); these stock solutions were stored as for phosphoinositides. Phospholipid concentrations in stock solutions were determined by inorganic phosphate assay using the ammonium molybdate procedure on acid-degraded samples (8). Phosphoinositides were analyzed for loss of phosphate due to hydrolysis by negative ion electrospray mass spectrometry (Bruker Esquire Ion Trap); no hydrolysis was detected. Purity of phosphoinositides is stated to be >99% by Avanti Polar Lipids, Inc. h-cPLA$_2\alpha$, h-cPLA$_2\gamma$, and m-cPLA$_2\zeta$ (>90% pure) were prepared as described previously (4, 9, 10). Bovine serum albumin is fatty acid-free (Sigma catalog no. A6003). Wyeth-1 (also known as ecopladib), Wyeth-2 (also known as giripladib), Wyeth-3, pyrrolidine-1, and pyrrolidine-2 (also known as pyrrophenone) were prepared as described previously (11, 12). The other inhibitors listed in supplemental Table 1 were prepared as described previously (13–15).

Production of m-cPLA$_2\beta$—The recombinant baculovirus expressing m-cPLA$_2\beta$ bearing an N-terminal His$_6$-tagged has been described previously (4). This protein contains the sequence MSPIDPMGHHHHHHGRRRASVAAGILVPRGSPGLDGICSRNSGLLAFKL fused to the N-terminal Met of m-cPLA$_2\beta$. The baculovirus was plaque-purified and amplified. Protein was produced in *Sf*9 cells in 1 liter of complete supplement Grace's medium (Invitrogen) containing 10% heat-inactivated FBS and 1% lipid concentrate (Invitrogen) and penicillin/streptomycin/fungizone (100-fold dilution of Invitrogen catalog no. 15240-062) in a 3-liter spinner flask. For each virus stock, a small scale expression test was carried out using 0.5 × 10^6 *Sf*9 cells in each well of a 12-well plate. Cells were incubated for 3 days post-infection, and cell lysates were analyzed by SDS-PAGE to find the optimal level of expression (the cPLA$_2$ band was located by Coomassie Blue staining and confirmed by Western blot using anti-His$_6$ antiserum (Sigma)). For large scale production, virus infection was carried out when cells reached a density of ~1.5 × 10^6/ml and then infected with the appropriate amount of virus stock. Cells were incubated at 27 °C with stirring at ~90 rpm for typically 50–60 h (until ~15–25% of the cells were dead as judged by trypan blue analysis). Cells were collected by centrifugation at 600 × *g* at room temperature. The cell pellet was stored at −80 °C.

Frozen cell pellet from 0.5 liter of cell culture was thawed on ice and resuspended in 20 ml of ice-cold buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol and freshly added 2 mM 2-mercaptoethanol and protease inhibitors (1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A)). Cells were disrupted in a Dounce homogenizer on ice, and the suspension was ultracentrifuged at 100,000 × *g* for 1 h at 4 °C. Ni-NTA (Qiagen) affinity resin was washed with water and then resuspended in ice-cold buffer A. The ultracentrifuge supernatant was added to 1 ml of packed and equilibrated Ni-NTA resin, and the suspension was gently mixed for 2 h at 4 °C and then transferred to a glass column with the aid of some ice-cold buffer A. The column was washed five times each with 1 ml of ice-cold buffer A (gravity flow). The column was then washed five times each with 1 ml of ice-cold buffer A but with 25 mM imidazole (gravity flow). m-cPLA$_2\beta$ was eluted with 5 ml of ice-cold cell homogenization buffer A containing 250 mM imidazole. Fractions (8–10) were collected and examined for protein purity by SDS-PAGE. Appropriate fractions were pooled, and the sample was concentrated in an Amicon Ultra-15 ultrafilter at 4 °C, and buffer was exchanged to 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM fresh DTT by three rounds of addition and concentration. Glycerol was added to give 30% (v/v), and the sample was stored at −20 °C. The protein concentration was estimated with the Bradford dye binding assay (Bio-Rad) using bovine serum albumin as a standard. The enzyme was judged to be ~80% pure by SDS-PAGE (7.5% gel) with Coomassie Blue staining. The specific activity of the purified m-cPLA$_2\beta$ using the radiometric [^{14}C]P-LPC assay (see below) was ~350 (nmol/min)/mg (Table 1). The yield of purified protein is ~5 mg/liter of *Sf*9 cell culture.

RACE Analysis of the h-cPLA$_2\epsilon$ Gene—A commercial human cDNA multiple tissue panel (Clontech catalog no. PT3158-1) was used in PCRs with the primers 5′-ccatctacctcaccatcaatgtc and 5′-aactcctccgaggtgtgtgacag to determine which tissues express a relatively high level of h-cPLA$_2\epsilon$. Based on this, we selected lung tissue and used commercial RACE-ready cDNA made from human lung (Clontech catalog no. 639308). RACE PCR was carried out according to the manufacturer's instructions using the following primer pair in the first round of PCR: gene-specific primer 5′-gtcctttaccacatgtctccgagcc and the adapter primer 5′-ccatcctaatacgactcactatagggc. In the second round of PCR using the first round PCR product as template, the primers used were gene-specific primer 5′-gcagccccagcaggtggccatac and adapter primer 5′-actcactatagggctcgagcggc.

The PCR product was gel-purified, cloned into the pGEM-T Easy vector after A tailing according to the manufacturer's directions (Promega catalog no. A-1360), and submitted to DNA sequencing.

Production of h-cPLA$_2\delta$ and h-cPLA$_2\epsilon$—Synthetic genes coding for these enzymes were prepared by Genscript (Piscataway, NJ) (the DNA sequences are given in the supplemental material). The genes was subcloned into the baculovirus transfer plasmid pAc-HLT (BD Biosciences) using the flanking restriction sites EcoRI and NotI. Baculoviruses were prepared using the BaculoGold kit (BD Biosciences). Proteins were expressed in *Sf*9 cells as for m-cPLA$_2\beta$ (see above). h-cPLA$_2\delta$ was purified as for m-cPLA$_2\beta$. The yield is 5–7 mg/liter of cell culture. h-cPLA$_2\epsilon$ was purified as for m-cPLA$_2\beta$ with the following exception. After washing the Ni-NTA gel with buffer A, a 10–250 mM imidazole gradient in buffer A (total volume 15 ml) was run at 0.25 ml/min, collecting 4-min fractions. The yield of h-cPLA$_2\epsilon$ is 0.25 mg/liter of cell culture. Estimated purities (supplemental Fig. 1) are >90% for h-cPLA$_2\delta$ and ~80% for h-cPLA$_2\epsilon$.

Production of m-cPLA$_2\beta$-C2—The C2 domain was identified based on the x-ray structure of the C2 domain from h-cPLA$_2\alpha$ (residues 1–130 for cPLA$_2\beta$-C2) (16). The gene coding for m-cPLA$_2\beta$-C2 was amplified using PCR and the full-length gene in pAcHLT (4) as template. The primers used are as follows: 5′-CCG CAT ATG CAG GCA AAG GTG CCA GAG ACA-3′ and 5′-CCT CTC GAG TCA TGT CAG AGT CTG TAG CCG-3′, which introduce an NdeI site immediately upstream of the ATG start codon and a XhoI site immediately downstream of the stop codon. The PCR product was digested with NdeI/XhoI, and the product was ligated into the pET15b expression vector (Novagen), which encodes a 20-residue N-terminal extension MGSSHHHHHHSSGLVPRGSH.

The C2 domain was expressed as a soluble protein in *Escherichia coli* BL21(DE3) (Novagen). Cells were grown at 37 °C in 1 liter of Luria broth containing 100 μg/ml ampicillin, and protein was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 25 °C for 12 h when the A_{600} reached ~0.5. Cells were harvested by centrifugation at 4000 × *g* at 4 °C, and the pellets were stored at −80 °C. Frozen pellets were thawed and resuspended in ice-cold 50 mM NaH_2PO_4 buffer, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol, and freshly added 5 μM 2-mercaptoethanol and protease inhibitors (1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A). The suspension was sonicated on ice to disrupt cells, and the mixture was centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant was gently shaken with 2 ml of Ni-NTA resin (50% slurry) (Qiagen) at 4 °C for 1 h, and the resin was then packed into a column. The resin was washed with 10 ml of extraction buffer containing 20 mM imidazole at 4 °C. The protein was eluted with 10 ml of extraction buffer containing 120 mM imidazole at 4 °C. The protein solution was concentrated to ~10 mg/ml in a centrifugal ultrafilter (Millipore, 5-kDa cutoff), and buffer was exchanged into 20 mM Tris-HCl, pH 7.5, 150 mM NaCl for storage at −20 °C. Protein concentration was estimated using the Bradford dye binding assay (Bio-Rad) using bovine serum albumin as a standard. The purified protein was estimated to be 90% pure for m-cPLA$_2\beta$-C2 by SDS-PAGE with Coomassie Blue staining. The yield is about 5 mg of purified protein/liter of bacterial culture. h-cPLA$_2\alpha$-C2 was produced as above using the plasmid described previously (17). Mutants of m-cPLA$_2\beta$-C2 were prepared using the QuikChange kit (Stratagene), and the full-length coding regions were sequenced to confirm the products.

Radiometric Assay of m-cPLA$_2\beta$ with [^{14}C]P-LPC—This assay was routinely used to quantify m-cPLA$_2\beta$. The 100-μl assay mixture in a polypropylene microcentrifuge tube contained 25 μM P-LPC, 120,000 dpm [^{14}C]P-LPC in 10 mM MOPS, pH 7.2, 0.5 mM EGTA, 100 mM KCl, 4 mM $CaCl_2$, and 0.5 mg/ml bovine serum albumin. After adding enzyme, the mixture was incubated at 37 °C for 20 min. The mixture was analyzed for released fatty acid as described previously (18).

Because the addition of diglycerides was found to increase the activity of h-cPLA$_2\epsilon$ acting on P-LPC, inhibition studies with this enzyme were carried out with 30 μM [^{14}C]P-LPC (45,000 dpm per assay), 10 μM 1,2-dioleoyl-*sn*-glycerol (Avanti Polar Lipids) in 80 μl of 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 5 mM $CaCl_2$ without albumin for 30 min at 37 °C. Reactions were analyzed for free fatty acid analysis as described above.

Vesicle Binding Studies—Interfacial binding of m-cPLA$_2\beta$ to 0.2-μm sucrose-loaded vesicles was measured as described previously (18). Vesicles were extruded through 0.2-μm membranes in 10 mM MOPS, pH 7.2, 176 mM sucrose, prespun, quantified, and diluted in 10 mM MOPS, pH 7.2, 100 mM KCl, 0.5 mM EGTA (18). Extruded vesicles were prepared fresh daily. m-cPLA$_2\beta$ binding reactions were prepared in 1.5-ml polyallomar microcentrifuge tubes and contained 10 mM MOPS, pH 7.2, 100 mM KCl, 0.5 mM EGTA with 0.5 mg/ml bovine serum albumin and various amounts of free calcium in the range 0–20 μM (measured with a Ca^{2+}-sensing dye (18)), phospholipid (200 μM total phospholipid), and 62 ng of m-cPLA$_2\beta$ in a total volume of 0.5 ml. Samples were centrifuged at 100,000 × $g_{average}$ at 21 °C for 1 h, and two 90-μl aliquots of supernatant were submitted to duplicate radiometric m-cPLA$_2\beta$ assays by adding 10 μl of P-LPC stock solution (250 μM P-LPC containing 1,200 dpm [^{14}C]P-LPC per μl, 10 mM MOPS, pH 7.2, 0.5 mM EGTA, 100 mM KCl, 40 mM $CaCl_2$). The results were compared with a standard curve of [^{14}C]P-LPC hydrolysis *versus* the amount of m-cPLA$_2\beta$ (0–64 ng, linear response obtained, data not shown) to obtain the amount of enzyme in the supernatant. See above for other details of the radiometric [^{14}C]P-LPC assay. The amount of m-cPLA$_2\beta$ was compared with the amount measured in an ultracentrifuged mixture containing all of the above components except vesicles to obtain the percentage of total enzyme remaining in the supernatant above pelleted vesicles. Recovery of m-cPLA$_2\beta$ from this ultracentrifuged sample lacking vesicles was ~100%, showing minimal binding of enzyme to the tube wall. Ultracentrifugation of vesicles doped with a small amount of [^{14}C]PAPC and scintillation counting of the supernatant showed that virtually all of the phospholipid was pelleted.

Binding of m-cPLA$_2\beta$-C2 to vesicles was measured as described previously (19). Reaction mixtures (500 μl) contained 5 μg of m-cPLA$_2\beta$-C2 and 200 μM phospholipid as vesicles in 10 mM MOPS, pH 7.2, 100 mM KCl, 0.5 mM EGTA with 0.5

mg/ml bovine serum albumin and various amounts of free calcium in the range 0–20 μM (18). After incubation for 10 min at 37 °C, the mixture was centrifuged at 100,000 × *g* at 21 °C for 1 h, and the pellet was submitted to SDS-PAGE on a 15% gel to estimate the amount of vesicle-bound protein. The same procedure was used to measure binding of h-cPLA$_2\delta$ to vesicles.

Kinetic Studies—Extruded vesicles were prepared as described above and diluted in 80 μl of 10 mM MOPS, pH 7.2, 100 mM KCl, 0.5 mM EGTA containing various concentrations of free calcium (18). Reactions were carried out in polypropylene microcentrifuge tubes at 37 °C for 10 min. Vesicle composition and concentration are given in the figure and table legends. Reaction workup to determine the amount of released radiolabeled arachidonic acid was carried out as described previously (18).

Phospholipids used for studying the PLA$_1$ activity using mass spectrometry were purified as follows to remove trace amounts of lysophospholipids. POPC, POPA, POPS, POPE, and POPG (5 mg, Avanti Polar Lipids) were individually injected onto a normal phase HPLC column (Varian MonoChrom 5 μm, Si, 1 × 25 cm, catalog no. A0501-250-X100) in 1:1 solvent A (30:40, v/v, *n*-hexane/isopropyl alcohol)/solvent B (43:47:10, v/v, *n*-hexane/isopropyl alcohol, 100 mM ammonium formate in water). The solvent program is as follows: 0–5 min, 25% B; 5–35 min, 25–70% B; 35–36 min, 70–99% B, 36–45 min, 99% B; 45–46 min, 99 to 25% B at 3 ml/min. A splitter was used to divert a portion of the column eluant (0.05 ml/min) to the mass spectrometer; the main stream was connected to a fraction collector. Phospholipid and lysophospholipid molecular species were monitored by mass spectrometry as described previously (20) except that full scan (400–900 atomic mass units) mass spectrometry was carried out instead of tandem mass spectrometry. POPI (Avanti) was not purified because no lysophospholipid contaminant was detected by mass spectrometry. Reaction mixtures contained extruded phospholipid vesicles (50 μM total phospholipid) in 10 mM MOPS, pH 7.2, 100 mM KCl, 0.5 mM EGTA, and sufficient $CaCl_2$ to give ~100 μM free Ca^{2+} (monitored using Calcium Green^{TM}-2, Invitrogen). Reactions were started by adding 2 μg of each cPLA$_2$ and incubated for 10 min at 37 °C. Reactions were extracted for lysophospholipid analysis as described previously (20) (because the sample did not contain plasmalogens, the two-stage extraction procedure was not used; rather the sample was acidified and extracted with organic solvent). The 1-palmitoyl-lysophospholipids and the 2-oleoyl-lysophospholipids were quantified by liquid chromatography/tandem mass spectrometry as described previously (20).

Inhibition Studies—Reaction mixtures contained [^{14}C]PAPC vesicles (4.9 μM, 58 Ci/mol) in 80 μl of 10 mM MOPS, pH 7.2, 100 mM KCl, 0.5 mM EGTA, 1.5 mM $CaCl_2$, and 0.5 mg/ml fatty acid-free bovine serum albumin. Vesicles were prepared by adding [^{14}C]PAPC in DMSO (2 μl per 80-ml reaction) and mixing with a vortexer immediately after dilution into buffer (to form unilamellar vesicles of nonuniform size). Reactions also contained 1–2 μM inhibitor (added in 1 μl of DMSO). Reactions were started by addition of cPLA$_2$ (10 ng of h-cPLA$_2\alpha$, 100 ng of m-cPLA$_2\beta$, 400 ng of h-cPLA$_2\gamma$, 1 μg of h-cPLA$_2\delta$, or 10 ng of m-cPLA$_2\zeta$) and were incubated at 37 °C for 30 min. Released fatty acid was quantified as for the kinetic studies described above. Inhibition studies with h-cPLA$_2\epsilon$ (1 μg per assay) were carried out with the [^{14}C]P-LPC/1,2-dioleoyl-*sn*-glycerol assay (as described above) because the activity of this isoform on [^{14}C]PAPC was very low.

RESULTS

Preparation of cPLA$_2$ Proteins—All cPLA$_2$ proteins were prepared as recombinant proteins using the baculovirus/*Sf*9 cell expression system. All proteins bear an N-terminal His_6 tag except for h-cPLA$_2\alpha$, which bears a C-terminal His_6 tag. A complete cDNA sequence for h-cPLA$_2\epsilon$ was not available in the public databases, so we obtained the full-length sequence by RACE PCR analysis of mRNA obtained from human lung. We used either native genes or synthetic genes (with optimized codons for expression in *Sf*9 cells, see under "Experimental Procedures" and supplemental material). The purity of all proteins was >90% as determined by SDS-PAGE analysis (supplemental Fig. 1) except for m-cPLA$_2\beta$ and h-cPLA$_2\epsilon$ (~80% pure).

Kinetic Properties of m-cPLA$_2\beta$—When human cPLA$_2\beta$ was first cloned, it was shown using crude lysates from baculovirus-infected insect cells that it had a very low specific activity on PAPC vesicles compared with cPLA$_2\alpha$ (21). We previously reported using purified human cPLA$_2\beta$ that it displays a specific activity on vesicles composed of 75% PAPC and 25% 1,2-dioleoyl-*sn*-glycerol that is about 30-fold lower than that of h-cPLA$_2\alpha$ and that human cPLA$_2\beta$ is about 15-fold more active on [^{14}C]P-LPC (presumably as micelles) than on [^{14}C]PAPC (sonicated liposomes) (3). We also reported that purified m-cPLA$_2\beta$ is about 25-fold more active on [^{14}C]P-LPC micelles than on vesicles of 75% PAPC and 25% 1,2-dioleoyl-*sn*-glycerol (4). Furthermore, the specific activity of human cPLA$_2\beta$ on [^{14}C]PAPC vesicles was about 30-fold lower than that of h-cPLA$_2\alpha$ (3). These initial studies suggested that the human and mouse β isoforms behave more as a lysophospholipase than as a phospholipase.

Table 1 gives the specific activities of m-cPLA$_2\beta$ and h-cPLA$_2\alpha$ acting on various substrates. Specific activities are based on initial reaction velocities because reactions were quenched after 10 min, which is in the linear part of the product *versus* time curve for both enzymes (supplemental Fig. 2). In the absence of Ca^{2+}, m-cPLA$_2\beta$ is 1500-fold more active on [^{14}C]P-LPC than on [^{14}C]PAPC (Table 1, entries 1 and 2). These values do not change significantly if there is 20 μM free Ca^{2+} in the buffer (Table 1, entries 1 and 2). By comparison, h-cPLA$_2\alpha$ is slightly less active on [^{14}C]P-LPC *versus* [^{14}C]PAPC and shows a clear increase in activity on [^{14}C]PAPC in the presence of Ca^{2+} (Table 1, entries 13 and 14).

The lack of phospholipase A$_2$ activation by Ca^{2+} is surprising because in an earlier study it was reported that 1 mM Ca^{2+} activates human cPLA$_2\beta$ by 5–8-fold when acting on sonicated liposomes of [^{14}C]PAPC vesicles containing 30 mol % 1,2-dioleoyl-*sn*-glycerol (21), and in a later study an ~6-fold activation of human cPLA$_2\beta$ by 4 mM $CaCl_2$ was reported when acting on [^{14}C]1-palmitoyl-2-arachidonyl-phosphatidylethanolamine vesicles containing 30 mol % 1,2-dioleoyl-*sn*-glycerol (3). There are no earlier data on the Ca^{2+} dependence of m-cPLA$_2\beta$. We also tested our preparation of m-cPLA$_2\beta$ using the exact sub-

TABLE 1
Interfacial kinetic properties of m-cPLA$_2\beta$ and h-cPLA$_2\alpha$

Entry	Enzyme	Substrate	Specific activity (0 μM Ca^{2+})	Specific activity (20 μM Ca^{2+})
			nmol/min/mg protein	*nmol/min/mg protein*
1	m-cPLA$_2\beta$	[^{14}C]P-LPC	381, 365[a]	394, 336
2	m-cPLA$_2\beta$	[^{14}C]PAPC	0.24, 0.25	0.28, 0.31
3	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% POPS	0.25, 0.25	1.0, 0.7
4	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% POPA	0.6, 0.6	0.9, 1.1
5	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% PI	0.44, 0.38	0.36, 0.37
6	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% PI(3)P	1.4, 1.6	1.8, 1.4
7	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% PI(4)P	4.3, 4.5	4.0, 4.4
8	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% PI(3,4)P_2	2.9, 2.9	5.5, 6.4
9	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% PI(3,5)P_2	5.9, 6.4	6.0, 6.0
10	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% PI(4,5)P_2	3.5, 3.6	3.7, 4.0
11	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% PI(3,4,5)P_3	3.6, 3.5	3.7, 3.7
12	m-cPLA$_2\beta$	[^{14}C]PAPC, 10% CL	0.93, 0.9	11.0, 11.4
13	h-cPLA$_2\alpha$	[^{14}C]P-LPC	135, 154	129, 122
14	h-cPLA$_2\alpha$	[^{14}C]PAPC	1.2, 1.0	230[b]
15	h-cPLA$_2\alpha$	[^{14}C]PAPC, 10% POPS	1.5, 1.6	220, 200
16	h-cPLA$_2\alpha$	[^{14}C]PAPC, 10% POPA	1.6, 2.0	400, 390
17	h-cPLA$_2\alpha$	[^{14}C]PAPC, 10% PI(4,5)P_2	11.2, 10.6	1,030[2]
18	h-cPLA$_2\alpha$	[^{14}C]PAPC, 10% CL	2.1, 1.9	200, 205

[a] Duplicate assay values are given. Reactions with [^{14}C]P-LPC contained 25 μM P-LPC (100,000 dpm [^{14}C]P-LPC per assay) in 100 μl of 10 mM MOPS, pH 7.2, 0.5 mM EGTA, 100 mM KCl, 0.5 mg/ml bovine serum albumin, a free Ca^{2+} concentration of 0 or 20 μM, and 100 ng of enzyme at 37 °C. Reactions with vesicles contained 200 μM total phospholipid (as extruded vesicles) in 80 μl of the above buffer at 37 °C and 0.8–1 μg of m-cPLA$_2\beta$ or 250 ng of h-cPLA$_2\alpha$.
[b] Data were taken from our previous studies using identical reaction conditions as for m-cPLA$_2\beta$ (18).

strate composition ([^{14}C]1-palmitoyl-2-arachidonyl-phosphatidylethanolamine/1,2-dioleoyl-*sn*-glycerol-sonicated vesicles) and buffer conditions reported previously (3) and found a 3-fold activation by 4 mM Ca^{2+} (data not shown). When this assay was repeated in the buffer used throughout this study in the presence of 0 *versus* 20 μM free Ca^{2+}, the specific activities in duplicate measurements were 2.0 and 2.2 (nmol/min)/mg of protein in the absence of Ca^{2+} and 2.4 and 2.5 (nmol/min)/mg in the presence of 20 μM free Ca^{2+}. When sonicated vesicles of [^{14}C]PAPC/1,2-dioleoyl-*sn*-glycerol were used, the specific activities were 0.7 and 0.5 (nmol/min)/mg in the absence of 20 μM free Ca^{2+} and 0.7 and 0.5 (nmol/min)/mg in the presence of 20 μM free Ca^{2+}. Thus, we conclude that m-cPLA$_2\beta$ is not activated by physiologically relevant concentrations of Ca^{2+} when these substrate preparations are used. It may be noted that cPLA$_2\alpha$ shows a phase of Ca^{2+} activation in the 0–20 μM range and a second phase of activation if the concentration of Ca^{2+} is increased into the millimolar range (for example see Ref. 22). The first phase is due to Ca^{2+}-dependent binding of the C2 domain of h-cPLA$_2\alpha$ to vesicles *in vitro* and to cellular membranes *in vivo* (18, 23). The origin of the effect of the supraphysiological Ca^{2+} level on cPLA$_2\alpha$ activity is not known but perhaps is due to an effect of this cation on the physical state of the substrate aggregate. It is interesting to note that addition of the Ca^{2+} ionophore A23187 to *Sf*9 cells expressing the ζ isoform of cPLA$_2$, a highly calcium-dependent enzyme, causes an ~10-fold increase in arachidonic acid release, whereas calcium ionophore treatment increases arachidonate release in *Sf*9 cells expressing m-cPLA$_2\beta$ by only ~2-fold (4). All together, the data show that m-cPLA$_2\beta$ is not activated by low micromolar concentrations of Ca^{2+} in the presence of phosphatidylcholine and phosphatidylethanolamine vesicles, and previous data on modest Ca^{2+} activation of this enzyme is probably the result of an effect of millimolar Ca^{2+} concentration on vesicle structure.

Next, we examined the effect of anionic phospholipids on the specific activity of m-cPLA$_2\beta$ acting on extruded [^{14}C]PAPC vesicles. The addition of 10 mol % POPS or POPA to [^{14}C]PAPC vesicles had a modest effect on the specific activity (Table 1, entries 3 and 4). In the presence of 10% POPS, m-cPLA$_2\beta$ showed a 4-fold increase in specific activity when [Ca^{2+}] goes from 0 to 20 μM; the effect of calcium when POPA is present is only 1.6-fold. Addition of POPS had no effect on the specific activity of h-cPLA$_2\alpha$, and the addition of POPA increased the activity by 2-fold when calcium was present (Table 1, entries 15 and 16). PI(4,5)P_2 activates h-cPLA$_2\alpha$ about 8-fold (Table 1, entry 17) as reported previously (18). A number of phosphoinositides activate m- cPLA$_2\beta$ when present in PAPC vesicles at 10 mol % (Table 1, entries 5–11). The most effective is PI(3,5)P_2, which increases the specific activity by ~25-fold, but other phosphoinositides are almost as effective. PI, which contains no phosphate monoesters, activates m-cPLA$_2\beta$ by only ~1.5-fold. PI(3)P and especially PI(4)P are better activators. PI(3,4,5)P_3 is a slightly worse activator than PI(3,5)P_2. In all cases with phosphatidylinositols, there was no effect of adding 20 μM calcium on the specific activity. h-cPLA$_2\alpha$ shows activation by PI(4,5)P_2 anionic phospholipids (Table 1, entry 17). In all cases, and in marked contrast to m-cPLA$_2\beta$, h-cPLA$_2\alpha$ shows a high dependence on the presence of Ca^{2+}.

Next we tested the dependence of the specific activity of m-cPLA$_2\beta$ on the mol % of an activating phosphatidylinositol, PI(3,4,5)P_3, in [^{14}C]PAPC, and results are shown in Fig. 1. Again, essentially no effect of calcium was seen (Fig. 1). The results show that most of the activation occurs in the 4–6 mol % range, and the activation plateaus as the amount of PI(3,4,5)P_3 approaches 10 mol %. Based on these data, the phosphoinositide species were retested at 6 rather than 10 mol % to see if the phosphoinositide species activation selectivity increases when they are present at subsaturating concentrations in vesicles. A similar phosphoinositide activation specificity is seen with 6 mol % anionic phospholipid in [^{14}C]PAPC vesicles as that seen with 10 mol % (data not shown).

The effect of calcium and PI(3,4,5)P_3 on the activity of m-cPLA$_2\beta$ was also tested on mixed phospholipid vesicles composed of 23 mol % POPC, 40 mol % POPE, and 30 mol % POPS (the approximate composition of the inner leaflet or

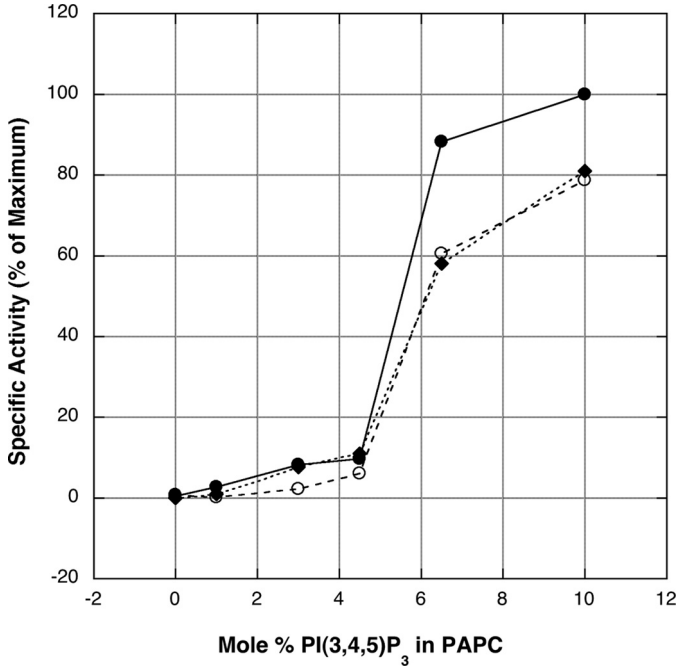

FIGURE 1. **Specific activity of m-cPLA$_2\beta$ on [^{14}C]PAPC vesicles as a function of the mol % of PI(3,4,5)P$_3$ (200 μM total phospholipids).** *Filled circles*, 0 μM Ca^{2+}; *open circles*, 2 μM Ca^{2+}; *filled diamonds*, 20 μM Ca^{2+}.

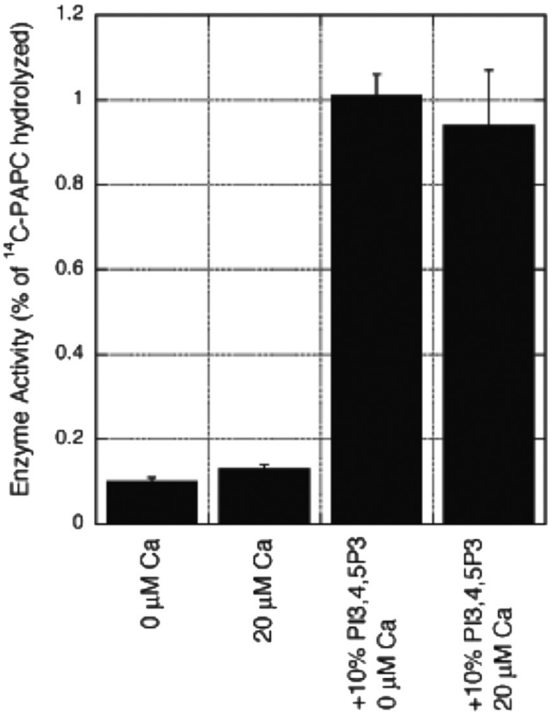

FIGURE 2. **Activity of m-cPLA$_2\beta$ on vesicles containing 23 mol % POPC, 40 mol % POPE, and 30 mol % POPS (200 μM total phospholipids) without PI(3,4,5)P$_3$ or vesicles containing 20 mol % POPC, 37 mol % POPE, 27 mol % POPS, and 10 mol % PI(3,4,5)P$_3$ in the absence or presence of 20 μM Ca^{2+}.** Vesicles also contained a trace of [^{14}C]PAPC (100,000 dpm). Activity is expressed as percent of [^{14}C]PAPC hydrolyzed.

erythrocyte plasma membranes (24)). As with [^{14}C]PAPC vesicles, the activity of m-cPLA$_2\beta$ was calcium-independent (Fig. 2). In these studies, the mixed phospholipid vesicles were doped with a small amount of [^{14}C]PAPC, and the activity of m-cPLA$_2\beta$ was reported as the % of total [^{14}C]PAPC hydrolyzed. A specific activity was not calculated because it is probable that POPC, POPE, and POPS compete with [^{14}C]PAPC for binding to the active site of the enzyme; thus, the specific activity was not comparable with that measured with vesicles composed only of [^{14}C]PAPC vesicles.

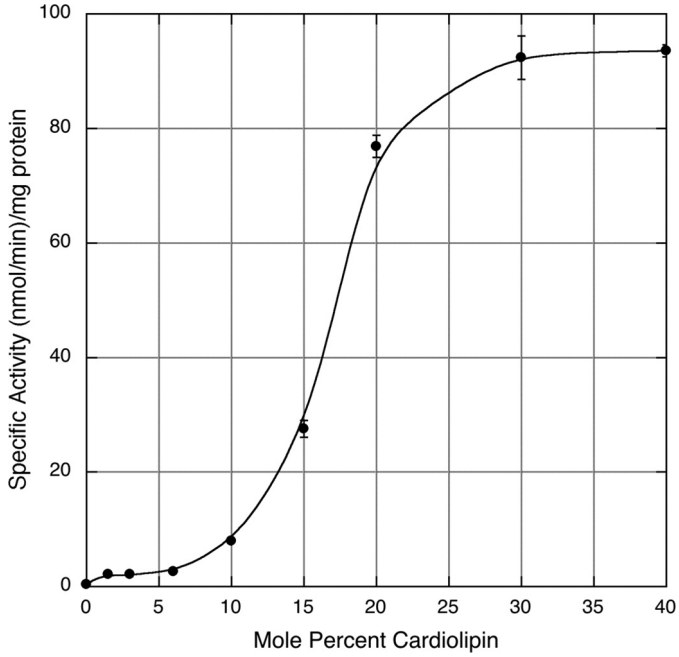

FIGURE 3. **Specific activity of m-cPLA$_2\beta$ on [^{14}C]PAPC vesicles as a function of the mol % of CL in vesicles.** Total phospholipid concentration was 200 μM. All reactions contained 20 μM Ca^{2+} and 2 μg of m-cPLA$_2\beta$ at 37 °C for 20 min.

Next, we examined the effect of addition of 10 mol % CL to [^{14}C]PAPC vesicles. Among the anionic phospholipids tested, CL was the best activator of m-cPLA$_2\beta$ (Table 1, entry 12). Remarkably, the enzyme now becomes highly Ca^{2+}-dependent when CL is present in [^{14}C]PAPC vesicles (Table 1, entry 12). The specific activity with [^{14}C]PAPC, 10 mol % CL vesicles in the presence of 20 μM Ca^{2+} is 35-fold higher than with [^{14}C]PAPC vesicles. In contrast, CL only activates h-cPLA$_2\alpha$ by 2-fold and only in the absence of Ca^{2+}.

We studied the dependence of the specific activity of m-cPLA$_2\beta$ on the mol % of CL in [^{14}C]PAPC vesicles and the effect of calcium in more detail. As can be seen in Fig. 3, the activity shows a sigmoidal dependence on mol % CL with maximal activation seen at around 25 mol % CL (all in the presence of 20 μM Ca^{2+}). The specific activity of m-cPLA$_2\beta$ approaches that of the highly active h-cPLA$_2\alpha$ acting on pure [^{14}C]PAPC vesicles. With [^{14}C]PAPC, 10 mol % CL vesicles, the specific activity increases ~8-fold as the concentration of calcium increases from 0 to 100 μM with no evidence of saturation (Fig. 4, *top panel*). However, when the mol % CL is increased to 30%, the increase in specific activity saturates when [Ca^{2+}] reaches about 10 μM (Fig. 4, *bottom panel*). This response to calcium is near the physiologically relevant range of intracellular calcium variation.

We studied whether m-cPLA$_2\beta$ displays any PLA$_1$ activity. Vesicles of POPC were used, and liquid chromatography combined with electrospray tandem mass spectrometry was used to measure the amount of P-LPC (PLA$_2$) and 2-oleoyl-*sn*-glycero-

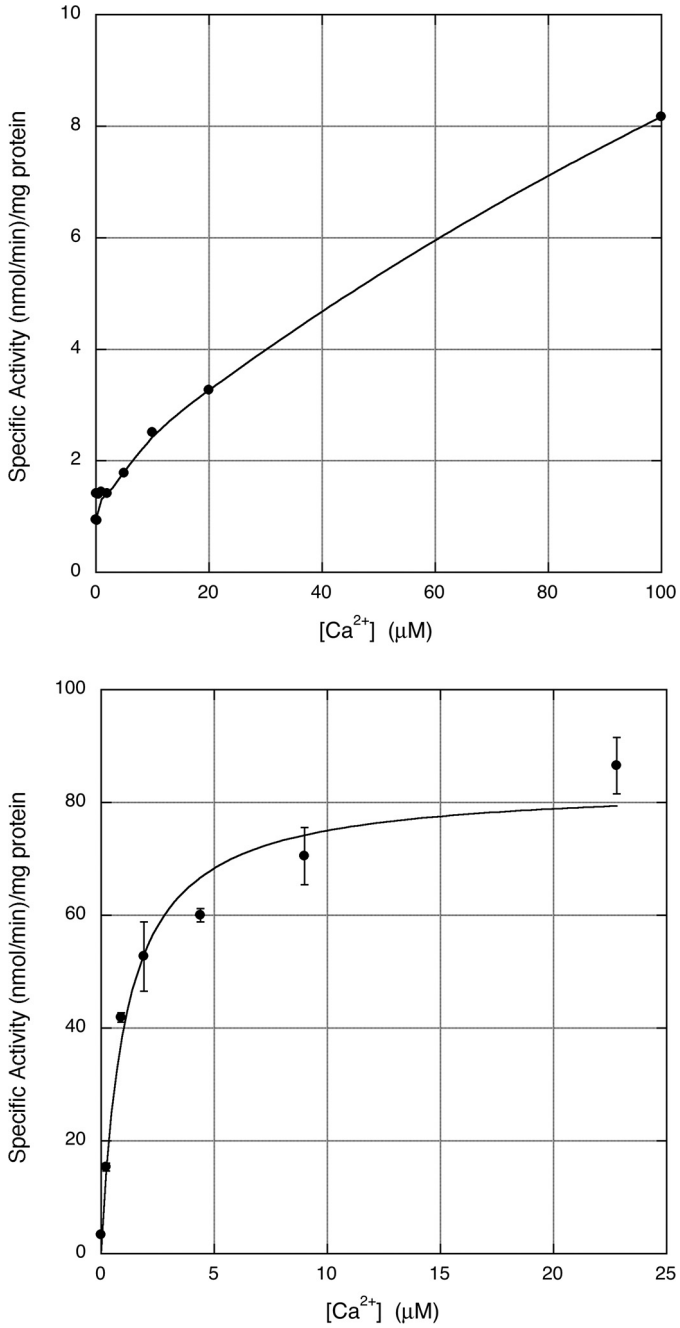

FIGURE 4. **Specific activity of m-cPLA$_2\beta$ on [^{14}C]PAPC/CL vesicles as a function of [Ca^{2+}].** *Top panel,* vesicles contain 10 mol % CL; *bottom panel,* vesicles contain 30 mol % CL. In both cases reactions contained 200 μM total phospholipid and 1 μg of m-cPLA$_2\beta$ at 37 °C for 10 min.

TABLE 2
PLA$_1$ versus PLA$_2$ activities of cPLA$_2$ (POPC vesicles)

Entry	Enzyme	PLA$_1$, specific activity (100 μM Ca^{2+})	PLA$_2$, specific activity (100 μM Ca^{2+})	PLA$_1$/PLA$_2$ activity ratio
		nmol/min/mg protein	*nmol/min/mg protein*	
1	m-cPLA$_2\beta$	0.08	0.06	1.3
2	h-cPLA$_2\alpha$	0.36	4.4	0.1
3	h-cPLA$_2\gamma$	0.02	0.10	0.2
4	h-cPLA$_2\delta$	6.0	1.13	5.3
5	h-cPLA$_2\epsilon$	0.02	0.00[a]	
6	m-cPLA$_2\zeta$	0.65	1.07	0.6

[a] Although no activity was detected by mass spectrometry, h-cPLA$_2\epsilon$ has detectable PLA$_2$ activity when measured using the more sensitive radiometric assay (Table 1).

TABLE 3
PLA$_1$ versus PLA$_2$ activities of cPLA$_2$ (mixed phospholipid vesicles)

Entry	Enzyme	Phospholipid species	PLA$_1$, specific activity (100 μM Ca^{2+})	PLA$_2$, specific activity (100 μM Ca^{2+})
			nmol/min/mg protein	*nmol/min/mg protein*
1	m-cPLA$_2\beta$	POPC	0.13, 0.05, 0.06	0.00, 0.00, 0.00
2		POPE	0.00, 0.00	0.03, 0.04
3		POPS	0.00, 0.00	0.13, 0.12
4		POPI	0.00, 0.00	0.03, 0.08
5		POPG	0.00, 0.00	0.24, 0.14
6		POPA	0.04, 0.01	0.25, 0.23
7	h-cPLA$_2\alpha$	POPC	0.03, 0.04	0.33, 0.27
8		POPE	0.00, 0.02	0.06, 0.04
9		POPS	0.00, 0.01	0.08, 0.04
10		POPI	0.00, 0.00	0.07, 0.07
11		POPG	0.00, 0.00	0.06, 0.02
12		POPA	0.01, 0.01	0.15, 0.05
13	h-cPLA$_2\gamma$	POPC	0.00, 0.01	0.00, 0.03
14		POPE	0.00, 0.03	0.00, 0.00
15		POPS	0.00, 0.00	0.00, 0.00
16		POPI	0.00, 0.00	0.00, 0.06
17		POPG	0.00, 0.00	0.00, 0.00
18		POPA	0.00, 0.00	0.00, 0.00
19	h-cPLA$_2\delta$	POPC	7.59, 5.10	1.13, 0.85
20		POPE	1.4, 1.45	0.24, 0.28
21		POPS	1.05, 1.25	0.16, 0.21
22		POPI	0.55, 0.63	0.00, 0.10
23		POPG	0.62, 0.36	0.07, 0.05
24		POPA	0.54, 0.60	0.14, 0.04
25	h-cPLA$_2\epsilon$	POPC	0.25, 0.26	0.00, 0.00
26		POPE	0.03, 0.05	0.00, 0.01
27		POPS	0.00, 0.00	0.00, 0.01
28		POPI	0.00, 0.05	0.00, 0.00
29		POPG	0.00, 0.00	0.00, 0.00
30		POPA	0.04, 0.04	0.00, 0.00
31	m-cPLA$_2\zeta$	POPC	0.48, 0.31	0.66, 0.67
32		POPE	0.22, 0.24	0.23, 0.21
33		POPS	0.08, 0.09	0.28, 0.22
34		POPI	0.00, 0.00	0.15, 0.12
35		POPG	0.07, 0.00	0.21, 0.09
36		POPA	0.24, 0.14	0.28, 0.27

3-phosphocholine (PLA$_1$) formation. All studies were carried out with 100 μM Ca^{2+}. Results summarized in Table 2 show that m-cPLA$_2\beta$ displays a PLA$_1$/PLA$_2$-specific activity ratio of 1.3 showing that it is a dual PLA$_1$/PLA$_2$ enzyme (Table 1, entry 1). In contrast, h-cPLA$_2\alpha$ is mainly a PLA$_2$, displaying a PLA$_1$/PLA$_2$ ratio of 0.1 (Table 1, entry 2). We also prepared mixed phospholipid vesicles composed of 50 mol % POPC and 10 mol % each of POPG, POPA, POPE, POPS, and POPI. We monitored the formation of palmitoyl-containing *sn*-1 lysophospholipid species (PLA$_2$) and oleoyl-containing *sn*-2 lysophospholipid species (PLA$_1$), and the results are summarized in Table 3. Interestingly, m-cPLA$_2\beta$ preferentially utilized anionic phospholipids (POPA, POPS, and POPG) over zwitterionic phospholipids (POPC and POPE) in mixed phospholipid vesicles, and the activity on anionic phospholipids is mainly as a PLA$_2$ and not as a PLA$_1$ (Table 3, entries 1–6). The activity of m-PLA$_2\beta$ on the POPC in these vesicles is mainly as a PLA$_1$, whereas in pure PAPC vesicles it displays both PLA$_1$ and PLA$_2$ activities (Table 2, entry 1). h-cPLA$_2\alpha$ displays a high PLA$_2$/PLA$_1$ ratio on all phospholipids in the mixed vesicles and prefers POPC (Table 3, entries 7–12).

Interfacial Binding Properties of m-cPLA$_2\beta$—We studied the binding of m-cPLA$_2\beta$ to vesicles loaded with sucrose, which can be pelleted by ultracentrifugation. With 200 μM PAPC vesicles in the presence or absence of 20 μM Ca^{2+}, no binding of m-cPLA$_2\beta$ was observed (Fig. 5). When PAPC vesicles contained 10 mol % PI(3,4,5)P$_3$, about 50% of the enzyme was bound in the presence or absence of 20 μM Ca^{2+} (Fig. 5).

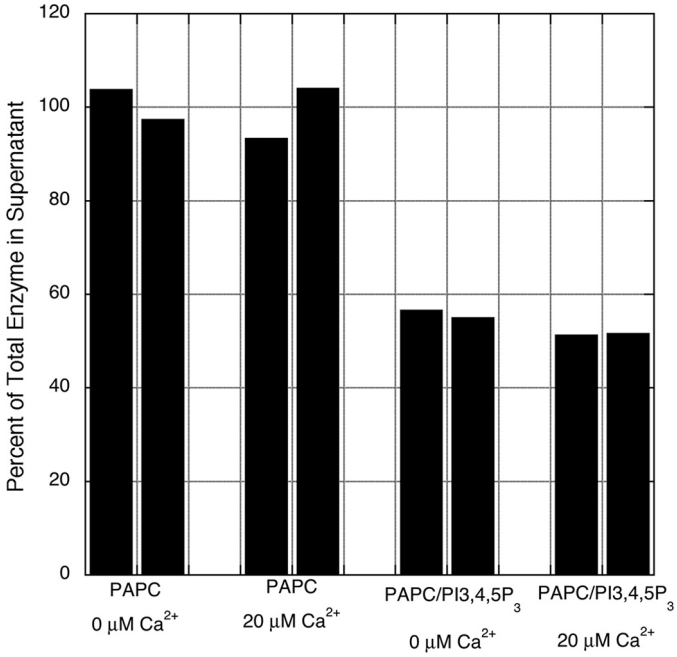

FIGURE 5. **Binding of m-cPLA$_2\beta$ to vesicles.** Binding reactions contained 200 μM total phospholipid as vesicles and 62 ng of enzyme in buffer with or without 20 μM free Ca^{2+}. See "Experimental Procedures" for more information. Plotted is the amount of enzyme in the supernatant above pelleted vesicles expressed as a percentage of the amount of enzyme measured in a binding reaction that lacked vesicles. See "Experimental Procedures" for other details.

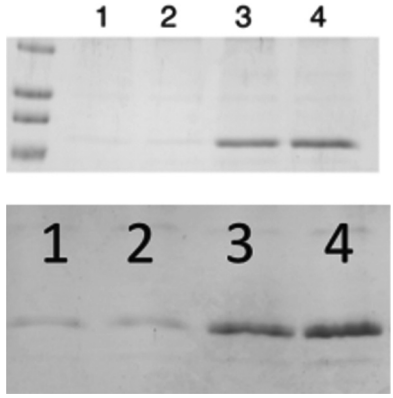

FIGURE 6. **Interfacial binding to vesicles.** *Top panel,* SDS-PAGE analysis of m-cPLA$_2\beta$-C2 in the pelleted vesicle fraction: *lane 1,* PAPC vesicles + 0 μM Ca^{2+}; *lane 2,* PAPC vesicles + 20 μM Ca^{2+}; *lane 3,* PAPC, /10 mol % PI(3,4,5)P_3 vesicles + 0 μM Ca^{2+}; *lane 4,* PAPC, 10 mol % PI(3,4,5)P_3 vesicles + 20 μM Ca^{2+}. *Bottom panel,* SDS-PAGE analysis of h-cPLA$_2\alpha$-C2 in the pelleted vesicle fraction: *lane 1,* PAPC vesicles + 0 μM Ca^{2+}; *lane 2,* PAPC/10 mol % PI(3,4,5)P_3 vesicles + 0 μM Ca^{2+}; *lane 3,* PAPC vesicles + 20 μM Ca^{2+}; *lane 4,* PAPC/10 mol % PI(3,4,5)P_3 vesicles + 20 μM Ca^{2+}. Data shown are representative of two independent SDS-PAGE analyses.

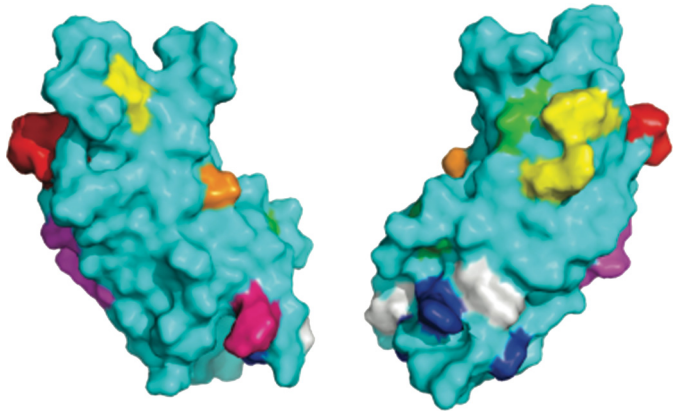

FIGURE 7. **Basic residues of m-cPLA$_2\beta$-C2.** Basic residues mutated to asparagine are as follows: Arg-17/Arg-55 (*red*); Lys-24/Arg-49/Lys-52 (*yellow*); Arg-14/Arg-107/Lys-117 (*magenta*); Lys-78 (*orange*); His-44/His-82 (*green*); Arg-66/Arg-69/Arg-125 (*blue*); His-64/His-68 (*gray*); Lys-72 (*pink*). The Ca^{2+}-binding surface loops are shown at the *top panel*. The *left image* is rotated ~180° along the vertical axis to give the *right image*.

Because there is no binding to PAPC vesicles in the absence of PI(3,4,5)P_3, it cannot be determined whether this phosphoinositide activates the enzyme bound to the interface. However, it is certainly the case that PI(3,4,5)P_3 enhances the binding of enzyme to vesicles, and this explains at least part of the activation by this phosphoinositide. The lack of an effect of Ca^{2+} on the extent of interfacial binding of enzyme is consistent with the lack of Ca^{2+} dependence on the enzymatic activity.

We also studied interfacial binding of m-cPLA$_2\beta$-C2 to vesicles. In the presence of 200 μM PAPC vesicles with 0 or 20 μM Ca^{2+}, no binding of m-cPLA$_2\beta$-C2 was observed when the pelleted vesicles were subjected to SDS-PAGE analysis to detect the protein (Fig. 6, *top panel*). When 10 mol % PI(3,4,5)P_3 was added to PAPC vesicles, interfacial binding of m-cPLA$_2\beta$-C2 was observed, and the extent of binding was not modulated by addition of 20 μM Ca^{2+} (Fig. 6, *top panel*). Based on the gel band intensity relative to that from a known amount of m-cPLA$_2\beta$-C2 loaded on to the gel, we estimate that about 50% of the m-cPLA$_2\beta$-C2 is bound to PAPC/PI(3,4,5)P_3 vesicles. When 10 mol % POPS or POPA was added to PAPC vesicles, no increase in binding of m-cPLA$_2\beta$-C2 was seen in the absence or presence of 20 μM Ca^{2+} (data not shown). As shown in Fig. 6 (*bottom panel*), h-cPLA$_2\alpha$-C2 binds to PAPC only in the presence of Ca^{2+} (20 μM), and binding in the presence and absence of Ca^{2+} is not modulated by the presence of 10 mol % PI(3,4,5)P_3 in the PAPC vesicles. All of these data are consistent with the kinetic studies of m-cPLA$_2\beta$, which shows that PI(3,4,5)P_3 but not POPA or POPS activates the enzyme in a Ca^{2+}-independent manner. Binding studies with h-cPLA$_2\alpha$-C2 are consistent with our early studies showing that Ca^{2+} but not phosphoinositides induce the binding of full-length cPLA$_2\alpha$ to PAPC vesicles (18) (Fig. 6, *bottom*).

Next, we prepared a number of m-cPLA$_2\beta$-C2 mutants in which surface cationic residues (arginine, lysine, and histidine) were mutated to the neutral residue asparagine to gauge the importance of cationic residues in supporting PI(3,4,5)P_3 dependence of the binding of the protein to vesicles. The circular dichroism spectra of all mutants were similar to that from wild type m-cPLA$_2\beta$ (data not shown) suggesting that all proteins are properly folded. Also, all mutants were expressed in *E. coli* as soluble proteins. The location of the mutants on the structure of m-cPLA$_2\beta$-C2 (homology model based on the x-ray structure of other C2 domains) is shown in Fig. 7. Remarkably, all mutants bound to PAPC, 10 mol % PI(3,4,5)P_3 vesicles to the same extent as the wild type m-cPLA$_2\beta$-C2 as judged by the protein band intensities after the same type of SDS-PAGE analysis as shown in Fig. 6 (data not shown). Thus, enhanced binding of m-cPLA$_2\beta$-C2 to PAPC vesicles caused by addition of PI(3,4,5)P_3 cannot be attributed to any single cluster

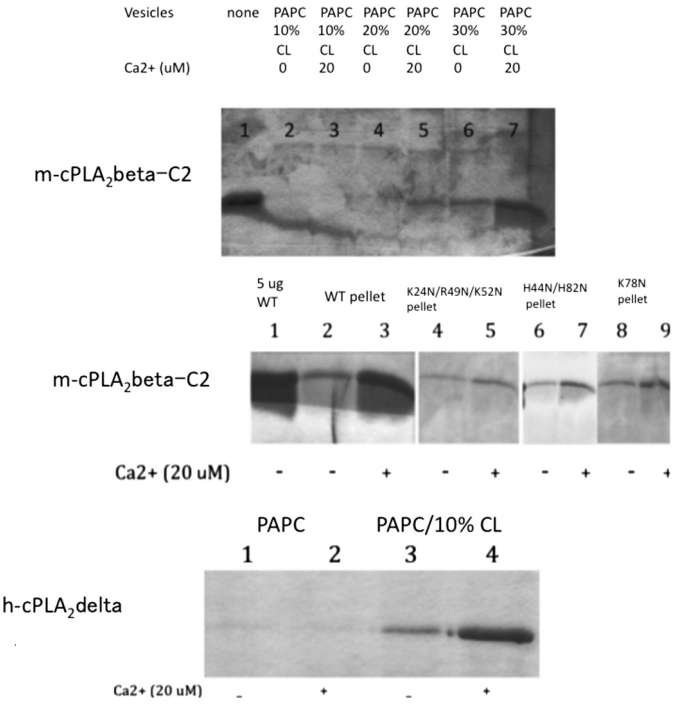

FIGURE 8. **Effect of CL and basic residue mutations on interfacial binding of m-cPLA$_2\beta$-C2 to vesicles.** *Top panel,* SDS-PAGE analysis of the pelleted vesicle fraction: *lane 1,* m-cPLA$_2\beta$-C2 (5 μg) in the absence of vesicles; *lane 2,* PAPC, 10 mol % CL vesicles + 0 μM Ca^{2+}; *lane 3,* PAPC, 10 mol % CL vesicles + 20 μM Ca^{2+}; *lane 4,* PAPC, 20 mol % CL vesicles + 0 μM Ca^{2+}; *lane 5,* PAPC, 20 mol % CL vesicles + 20 μM Ca^{2+}; *lane 6,* PAPC, 30 mol % CL vesicles + 0 μM Ca^{2+}; *lane 7,* PAPC, 30 mol % CL vesicles + 20 μM Ca^{2+}. *Middle panel, lane 1,* wild type m-cPLA$_2\beta$-C2 (5 μg) in the absence of vesicles; *lane 2,* vesicle pellet fraction using PAPC, 30 mol % CL vesicles with wild type m-cPLA$_2\beta$-C2 + 0 μM Ca^{2+}; *lane 3,* same as *lane 2* but + 20 μM Ca^{2+}; *lane 4,* same as *lane 2* but with K24N/R49N/K52N mutant of m-cPLA$_2\beta$-C2 + 0 μM Ca^{2+}; *lane 5,* same as *lane 4* but + 20 μM Ca^{2+}; *lane 6,* same as *lane 2* but with the H44N/H82N mutant of m-cPLA$_2\beta$-C2 + 0 μM Ca^{2+}; *lane 7,* same as *lane 6* but + 20 μM Ca^{2+}; *lane 8,* same as *lane 2* but with the K78N mutant of m-cPLA$_2\beta$-C2 + 0 μM Ca^{2+}; *lane 9,* same as *lane 8* but with 20 μM Ca^{2+}. *Bottom panel,* binding of h-cPLA$_2\delta$ to vesicles (SDS-PAGE analysis of the pelleted vesicle fraction): *lane 1,* PAPC vesicles + 0 μM Ca^{2+}; *lane 2,* PAPC vesicles + 20 μM Ca^{2+}; *lane 3,* PAPC, 10% CL vesicles + 0 μM Ca^{2+}; *lane 4,* PAPC, 10% CL vesicles + 20 μM Ca^{2+}.

of basic residues mutated in this study (all basic residues predicted to be on the protein surface of the homology model).

We also studied the effect of CL on binding of m-cPLA$_2\beta$-C2 domains to PAPC vesicles. As shown in Fig. 8 (*top panel*), increasing amounts of CL in PAPC vesicles increases the amount of m-cPLA$_2\beta$-C2 bound to vesicles, and the binding is enhanced in the presence of Ca^{2+}. In the presence of 30 mol % CL and 20 μM Ca^{2+} about 50% of the m-cPLA$_2\beta$-C2 is vesicle-bound (Fig. 8, *top panel*). We studied the binding of the m-cPLA$_2\beta$-C2 mutants to PAPC, 30 mol % CL vesicles in the presence and absence of 20 μM Ca^{2+}. As shown in Fig. 8 (*middle panel, lanes 4* and *5*), the triple mutant K24N/R49N/K52N does not show Ca^{2+}-enhanced binding to PAPC/30 mol % CL vesicles. The H44N/H82N mutant (Fig. 8, *middle panel, lanes 6* and *7*) and the K78N mutant (Fig. 8, *middle panel, lanes 8* and *9*) show some Ca^{2+}-enhanced binding, but it is less than that for the wild type C2 domain. All other basic residue mutants show the same Ca^{2+}-enhanced binding to PAPC, 30 mol % CL vesicles as does the wild type C2 domain (data not shown).

Kinetic Properties of Other cPLA$_2$ Isoforms—We measured the specific activities of the other cPLA$_2$ isoforms using the substrates and additives used to study m-cPLA$_2\beta$, and the results are summarized in Table 4. h-cPLA$_2\gamma$ displays lysophospholipase activity (Table 4, entry 1) on [^{14}C]P-LPC comparable with that of m-cPLA$_2\beta$ and h-cPLA$_2\alpha$, but it displays relatively low PLA$_2$ activity on [^{14}C]PAPC vesicles in the absence and presence of anionic phospholipids (Table 4, entries 2–12). Specific activities with 0 and 20 μM Ca^{2+} are essentially identical, consistent with the fact that this cPLA$_2$ lacks a C2 domain but rather contains a C-terminal farnesyl-cysteine methyl ester (9).

h-cPLA$_2\delta$ displays lysophospholipase A$_2$ activity comparable with the other isoforms (Table 4, entry 13) and relatively low PLA$_2$ activity (entries 14–24). Among the anionic additives, only the addition of 10% CL to [^{14}C]PAPC shows activation (8-fold) (Table 4), and the increase is Ca^{2+}-dependent (Table 4, entry 24). The latter result may be explained by the fact that h-cPLA$_2\delta$ has a C2 domain. To explore this further, we studied the binding of h-cPLA$_2\delta$ to vesicles. As shown in Fig. 8 (*bottom panel*), no binding of this enzyme to PAPC was seen in the absence or presence of 20 μM Ca^{2+}. Binding to PAPC, 10% CL vesicles was enhanced by the presence of 20 μM Ca^{2+}. These binding data explain at least part of the activation of h-cPLA$_2\delta$ seen by CL and Ca^{2+} (Table 4).

h-cPLA$_2\epsilon$ has very low specific activity as a lysophospholipase on [^{14}C]P-LPC and as a PLA$_2$ on [^{14}C]PAPC vesicles with or without anionic phospholipid additives (Table 4, entries 25–36), although some of the phosphoinositides (especially PI(3,4)P$_2$, PI(3,5)P$_2$, and PI(3,4,5)P$_3$) activate up to ~10-fold (Table 4, entries 32, 33, and 35). Specific activities in the absence and presence of 20 μM Ca^{2+} are similar.

m-cPLA$_2\zeta$ displays relatively high lysophospholipase activity on [^{14}C]P-LPC (Table 4, entry 37) and PLA$_2$ activity on [^{14}C]PAPC similar to that of h-cPLA$_2\alpha$ (entries 38–48). The PLA$_2$ activity but not the lysophospholipase A$_2$ is Ca^{2+}-dependent (as is the case for h-cPLA$_2\alpha$). m-cPLA$_2\zeta$ displays significant activation when most of the anionic phospholipids are added to [^{14}C]PAPC vesicles as follows: 3-fold by POPA; up to ~2-fold by most of the phosphoinositides, and 3-fold by CL. In all cases, the PLA$_2$ activity is Ca^{2+}-dependent consistent with the presence of a C2 domain.

We also studied the PLA$_1$ *versus* PLA$_2$ activity of these additional cPLA$_2$ isoforms, and the results with POPC vesicles are summarized in Table 2. h-cPLA$_2\gamma$ is a better PLA$_2$ than a PLA$_1$ (Table 2, entry 3). h-cPLA$_2\delta$ has much higher PLA$_1$ activity than PLA$_2$ activity (Table 2, entry 4). Again, h-cPLA$_2\epsilon$ has very low PLA$_2$ activity, and its PLA$_1$ activity is also relatively low (Table 2, entry 5). The PLA$_1$ and PLA$_2$ activities of m-cPLA$_2\zeta$ are comparable (Table 2, entry 6). With the mixed phospholipid vesicles, h-cPLA$_2\gamma$ displays low PLA$_1$ and PLA$_2$ activity on all phospholipid species (Table 4, entries 13–18). h-cPLA$_2\delta$ displays a high PLA$_1$/PLA$_2$ ratio and hydrolyzes all of the phospholipid species in the mixed vesicles with comparable efficiency (Table 4, entries 19–24) (note the higher values for POPC hydrolysis is probably due to the fact that POPC is present at a 5-fold higher concentration than the other phospholipid species in these mixed phospholipid vesicles). h-cPLA$_2\epsilon$ displays low PLA$_1$ and PLA activities on all phospholipid species except for POPC in which it acts mainly as a PLA$_1$ (Table 4, entries 25–30). m-cPLA$_2\zeta$ displays comparable PLA$_1$ and PLA$_2$

TABLE 4
Lipolysis by cPLA$_2$ isoforms

Entry	Enzyme	Substrate	Specific activity (0 μM Ca^{2+})	Specific activity (20 μM Ca^{2+})
			nmol/min/mg protein	*nmol/min/mg protein*
1	h-cPLA$_2\gamma$	[^{14}C]P-LPC	61, 55[a]	52, 48
2	h-cPLA$_2\gamma$	[^{14}C]PAPC	0.3, 0.3	0.3, 0.3
3	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% POPS	0.3, 0.3	0.3, 0.3
4	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% POPA	0.2, 0.3	0.2, 0.3
5	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% PI	0.4, 0.4	0.4, 0.4
6	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% PI(3)P	0.3, 0.3	0.3, 0.3
7	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% PI(4)P	0.4, 0.4	0.4, 0.4
8	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% PI(3,4)P$_2$	0.6, 0.6	0.6, 0.6
9	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% PI(3,5)P$_2$	0.6, 0.6	0.5, 0.5
10	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% PI(4,5)P$_2$	0.7, 0.7	0.7, 0.7
11	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% PI(3,4,5)P$_3$	0.6, 0.7	0.6, 0.6
12	h-cPLA$_2\gamma$	[^{14}C]PAPC, 10% CL	0.2, 0.2	0.2, 0.3
13	h-cPLA$_2\delta$	[^{14}C]P-LPC	23, 22	33, 35
14	h-cPLA$_2\delta$	[^{14}C]PAPC	0.06, 0.06	0.1, 0.1
15	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% POPS	0.06, 0.04	0.2, 0.1
16	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% POPA	0.1, 0.2	0.2, 0.3
17	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% PI	0.09, 0.08	0.1, 0.1
18	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% PI(3)P	0.2, 0.2	0.2, 0.2
19	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% PI(4)P	0.3, 0.3	0.3, 0.3
20	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% PI(3,4)P$_2$	0.2, 0.3	0.4, 0.4
21	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% PI(3,5)P$_2$	0.3, 0.3	0.4, 0.4
22	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% PI(4,5)P$_2$	0.3, 0.3	0.4, 0.4
23	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% PI(3,4,5)P$_3$	0.3, 0.3	0.3, 0.3
24	h-cPLA$_2\delta$	[^{14}C]PAPC, 10% CL	0.1, 0.1	0.8, 0.7
25	h-cPLA$_2\epsilon$	[^{14}C]P-LPC	0.22, 0.20	0.15, 0.14
26	h-cPLA$_2\epsilon$	[^{14}C]PAPC	0.02, 0.1	0.05, 0.03
27	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% POPS	0.05, 0.1	0.2, 0.05
28	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% POPA	0.06, 0.12	0.08, 0.12
29	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% PI	0.06, 0.05	0.02, 0.02
30	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% PI(3)P	0.14, 0.06	0.06, 0.5
31	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% PI(4)P	0.26, 0.31	0.3, 0.3
32	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% PI(3,4)P$_2$	0.4, 0.4	0.5, 0.5
33	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% PI(3,5)P$_2$	0.5, 0.4	0.5, 0.6
34	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% PI(4,5)P$_2$	0.5, 0.5	0.5, 0.5
35	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% PI(3,4,5)P$_3$	0.5, 0.5	0.5, 0.6
36	h-cPLA$_2\epsilon$	[^{14}C]PAPC, 10% CL	0.05, 0.09	0.05, 0.06
37	m-cPLA$_2\zeta$	[^{14}C]P-LPC	375, 359	391, 348
38	m-cPLA$_2\zeta$	[^{14}C]PAPC	2.3, 2.6	61, 59
39	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% POPS	2.7, 2.6	62, 62
40	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% POPA	3.3, 4.2	159, 159
41	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% PI	3.0, 2.8	85, 94
42	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% PI(3)P	4.9, 5.4	73, 69
43	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% PI(4)P	8.8, 8.1	94, 90
44	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% PI(3,4)P$_2$	8.3, 6.6	89, 103
45	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% PI(3,5)P$_2$	8.0, 8.2	104, 96
46	m-cPLA$_2\zeta$	[^{14}C]PAP, 10% PI(4,5)P$_2$	7.8, 8.1	93, 117
47	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% PI(3,4,5)P$_3$	8.2, 8.4	88, 99
48	m-cPLA$_2\zeta$	[^{14}C]PAPC, 10% CL	5.6, 5.8	192, 190

[a] Duplicate assay values are given.

activities on all species except for POPI and POPG, where it shows mainly PLA$_2$ activity (Table 4, entries 31–36).

Inhibition Studies—A number of highly potent h-cPLA$_2\alpha$ inhibitors were tested on the full set of cPLA$_2$ isoforms. The inhibitors include pyrrolidine-1 and pyrrolidine-2 developed by Shionogi & Co., Ltd. (25, 26), which contain an electrophilic ketone group that may form a hemiketal adduct with the active site serine residue (working hypothesis) (structures of all inhibitors are given in supplemental Fig. 3). Wyeth-1, -2, and -3 are indole-based h-cPLA$_2\alpha$ inhibitors developed by Wyeth (12). Compounds in this series are undergoing clinical trials for various inflammatory disorders. All of the other compounds studied are analogs of the original h-cPLA$_2\alpha$ inhibitor, arachidonyltrifluoromethyl ketone (27), and contain an electrophilic ketone group (15).

The supplemental Table 1 gives the percent inhibition of each cPLA$_2$ isoform by each inhibitor present at 1 μM (pyrrolidine-1 and -2, and Wyeth-1, -2, and -3) or 2 μM (remaining compounds). All studies were done with [^{14}C]PAPC vesicles in the presence of Ca^{2+} except for studies with h-cPLA$_2\epsilon$, which made use of the assay with [^{14}C]P-LPC, 1,2-dioleoyl-*sn*-glycerol because this enzyme has almost no PLA$_2$ activity. Pyrrolidine-1 and -2 and Wyeth-1, -2, and -3 display high potency on h-cPLA$_2\alpha$ and m-cPLA$_2\zeta$ and are relatively inactive against all other cPLA$_2$ isoforms. A subset of the electrophilic ketone inhibitors was potent on h-cPLA$_2\alpha$; some show intermediate potency, and some are not active. The structure-activity data for these compounds against h-cPLA$_2\alpha$ and m-cPLA$_2\zeta$ are very similar. Among all the inhibitors tested, only a subset of the electrophilic ketone inhibitors was active on m-cPLA$_2\beta$, and Lf-80 emerges as a specific and reasonably potent inhibitor of this isoform. All of the inhibitors tested are relatively ineffective at blocking the activity of h-cPLA$_2\gamma$, h-cPLA$_2\delta$, and h-cPLA$_2\epsilon$. We also tested pyrrolidine-1 and -2 and Wyeth-1, -2, and -3 on m-cPLA$_2\beta$ using [^{14}C]PAPC vesicles containing 30 mol % CL (optimal activity conditions for this isoform), and again we found no inhibition by these compounds (data not shown).

DISCUSSION

The behavior of the C2 domain of m-cPLA$_2\beta$ is unusual among previously reported C2 domains. Interfacial binding of this C2 domain and the activity of the full-length protein are promoted by phosphoinositides, especially highly phosphorylated species, but Ca^{2+} is not needed. Presumably, cationic amino acids are required for forming favorable electrostatic interactions with the anionic phosphoinositides, but after mutating all cationic patches on the surface of the C2 domain, we were not able to locate a patch that is critical for interfacial binding. The only hypotheses we can put forward is that the C2 domain of m-cPLA$_2\beta$ binds to anionic phosphoinositides in lipid vesicles either in multiple orientations, making use of different clusters of cationic residues, or that many more than 2–3 basic residues form electrostatic interactions with the interface such that mutating only a few of them is insufficient to see a reduction in interfacial binding. These results are surprising because the amino acid sequence of this C2 domain, when aligned with other Ca^{2+}-responsive C2 domains, appears to have all the ligands that interact with Ca^{2+} ions. In fact the C2 domain of m-cPLA$_2\beta$ is responsive to Ca^{2+} when the anionic phospholipid CL is present in zwitterionic phosphatidylcholine vesicles. The concentration of Ca^{2+} required for maximal activation of m-cPLA$_2\beta$ depends on the mole fraction of CL in PAPC vesicles; higher mol % CL leads to a lower Ca^{2+} requirement. This suggests that CL and Ca^{2+} work synergistically together to promote interfacial binding of this cPLA$_2$ isoform to the membrane interface. It will be interesting to see where m-cPLA$_2\beta$ traffics in mammalian cells. One possibility suggested by the data of this study is that when intracellular Ca^{2+} is low, the enzyme associates with a membrane surface that is rich in phosphoinositides, but when intracellular Ca^{2+} rises, the enzyme may move to a membrane rich in CL such as mitochondrial membranes.

The PLA$_2$ activity of m-cPLA$_2\beta$ approaches its lysophospholipase activity only when a vesicle composition is used that supports a high level of interfacial enzyme binding (*i.e.* PAPC + CL + Ca^{2+}). PAPC plus Ca^{2+} is sufficient to support a high level of h-cPLA$_2\alpha$ interfacial binding. Like h-cPLA$_2\alpha$, m-cPLA$_2\zeta$ displays activity on PAPC vesicles in the presence of Ca^{2+} that approaches its lysophospholipase A$_2$ activity. However, m-cPLA$_2\zeta$ and h-cPLA$_2\alpha$ behave differently in that phosphoinositides activate only the latter (mainly by increasing the catalytic activity of the vesicle-bound enzyme rather than promoting interfacial binding of enzyme (18)). The relatively small amount of activation of m-cPLA$_2\zeta$ and h-cPLA$_2\alpha$ by CL distinguishes these enzymes from the behavior of m-cPLA$_2\beta$. h-cPLA$_2\gamma$ and h-cPLA$_2\delta$ display high lysophospholipase activity and low PLA$_2$/PLA$_1$ activities at least among the substrates tested in this study. h-cPLA$_2\epsilon$ displays much lower lysophospholipase activity relative to the other isoforms studied, and its PLA$_1$ and PLA$_2$ activities are also low. In the case of h-cPLA$_2\delta$, some increase in phospholipase activity was seen when CL was present in vesicles and Ca^{2+} was added, but the specific activity still remains relatively low. Interfacial binding studies show that CL and Ca^{2+} promote interfacial binding of h-cPLA$_2\delta$ to vesicles. Thus, the low activity suggests that the phospholipids present are not optimal substrates for this enzyme.

It has been previously reported that cPLA$_2\alpha$ displays PLA$_1$, PLA$_2$, and lysophospholipase activities (see for example Refs. 4, 5). This is in marked contrast to secreted phospholipases A$_2$, which are strict PLA$_2$s. The x-ray structure of phospholipid analogs bound to secreted PLA$_2$s shows how the *sn*-3 phosphate coordinates to the active site of Ca^{2+} to position the *sn*-2 ester close to the catalytic residues required for ester hydrolysis (28). There are no x-ray structures on phospholipid analogs bound to the active site of cPLA$_2$s, so it is not possible to understand why these enzymes have multiple phospholipase activities; this degree of multiple substrate tolerance is unusual for enzymes. Our studies of the full set of mammalian isoforms show that they all display PLA$_1$ and PLA$_2$ activities, but the relative amount of each is dramatically different among the isoforms. m-cPLA$_2\beta$ is both a PLA$_1$ and a PLA$_2$, and the ratio of these activities depends dramatically on the headgroup of the phospholipid structure. It is preferentially a PLA$_1$ on POPC but is preferentially a PLA$_2$ on anionic phospholipids (POPS, POPI, POPG, and POPA). m-cPLA$_2\zeta$ displays comparable PLA$_2$ and PLA$_1$ activities on phospholipids with different headgroups, an additional feature that distinguishes it from h-cPLA$_2\alpha$. Among the cPLA$_2$ isoforms, h-cPLA$_2\delta$ displays the strongest PLA$_1$ *versus* PLA$_2$ bias, and this extends to phospholipids with different headgroups.

With the realization that several cPLA$_2$ isoforms are present in mammals and in some cells several cPLA$_2$s are present (see for example Ref. 4) comes the concern that previously reported h-cPLA$_2\alpha$ inhibitors may not be specific to a single isoform. We have already shown that previously reported h-cPLA$_2\alpha$ inhibitors block arachidonate release in mouse lung fibroblasts that lack h-cPLA$_2\alpha$, a result attributed to inhibition of m-cPLA$_2\zeta$ (4). In this study, we have tested a large set of previously reported potent h-cPLA$_2\alpha$ inhibitors, and we failed to find a compound that can well distinguish h-cPLA$_2\alpha$ from m-cPLA$_2\zeta$. The overlap in structure-activity data for this large set of inhibitors is remarkable. Whether or not some of these compounds also inhibit human cPLA$_2\zeta$ remains to be determined. As of yet, we have not been able to express this human enzyme despite attempts using several expression systems. The inhibitors studied here do not significantly inhibit h-cPLA$_2\gamma$, h-cPLA$_2\delta$, and h-cPLA$_2\epsilon$, but a subset inhibits m-cPLA$_2\beta$. Among the latter, we discovered a few specific inhibitors of m-cPLA$_2\beta$, which should prove useful in cell studies to study the function of this enzyme.

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Enzymology:

Interfacial Kinetic and Binding Properties of Mammalian Group IVB Phospholipase A_2 ($cPLA_2\beta$) and Comparison with the Other cPLA $_2$ Isoforms

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