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Function, Activity, and Membrane Targeting of Cytosolic Phospholipase $A_2\zeta$ in Mouse Lung Fibroblasts*

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Group IVA cytosolic phospholipase A_2 ($cPLA_2\alpha$) initiates eicosanoid production; however, this pathway is not completely ablated in $cPLA_2\alpha^{-/-}$ lung fibroblasts stimulated with A23187 or serum. $cPLA_2\alpha^{+/+}$ fibroblasts preferentially released arachidonic acid, but A23187-stimulated $cPLA_2\alpha^{-/-}$ fibroblasts non-specifically released multiple fatty acids. Arachidonic acid release from $cPLA_2\alpha^{-/-}$ fibroblasts was inhibited by the $cPLA_2\alpha$ inhibitors pyrrolidine-2 (IC_{50}, 0.03 μM) and Wyeth-1 (IC_{50}, 0.1 μM), implicating another C2 domain-containing group IV PLA_2. $cPLA_2\alpha^{-/-}$ fibroblasts contain $cPLA_2\beta$ and $cPLA_2\zeta$ but not $cPLA_2\epsilon$ or $cPLA_2\delta$. Purified $cPLA_2\zeta$ exhibited much higher lysophospholipase and PLA_2 activity than $cPLA_2\beta$ and was potently inhibited by pyrrolidine-2 and Wyeth-1, which did not inhibit $cPLA_2\beta$. In contrast to $cPLA_2\beta$, $cPLA_2\zeta$ expressed in Sf9 cells mediated A23187-induced arachidonic acid release, which was inhibited by pyrrolidine-2 and Wyeth-1. $cPLA_2\zeta$ exhibits specific activity, inhibitor sensitivity, and low micromolar calcium dependence similar to $cPLA_2\alpha$ and has been identified as the PLA_2 responsible for calcium-induced fatty acid release and prostaglandin E_2 production from $cPLA_2\alpha^{-/-}$ lung fibroblasts. In response to ionomycin, EGFP-$cPLA_2\zeta$ translocated to ruffles and dynamic vesicular structures, whereas EGFP-$cPLA_2\alpha$ translocated to the Golgi and endoplasmic reticulum, suggesting distinct mechanisms of regulation for the two enzymes.

Mammals contain a number of phospholipases A_2 (PLA_2)[2] that include secreted forms, intracellular group IV cytosolic PLA_2s (GIV $cPLA_2$), and group VI calcium-independent PLA_2s (GVI $iPLA_2$) (1, 2). The presence of diverse PLA_2s provides cells with differentially regulated pathways for the hydrolysis of fatty acids from phospholipid. Intracellular PLA_2s exhibit multiple enzymatic activities (PLA_2, PLA_1, lysophospholipase, transacylase) to varying degrees, which can potentially result in the formation of a diverse number of phospholipid breakdown products (3, 4). The direct products of PLA_2 action, lysophospholipids and fatty acids, can themselves act as cellular mediators or serve as precursors for the formation of mediators such as platelet-activating factor and eicosanoids.

There are six enzymes classified as GIV PLA_2s: $cPLA_2\alpha$ (GIVA), $cPLA_2\beta$ (GIVB), $cPLA_2\gamma$ (GIVC), $cPLA_2\delta$ (GIVD), $cPLA_2\epsilon$ (GIVE), and $cPLA_2\zeta$ (GIVF) (4). These enzymes contain a conserved Ser/Asp active site dyad and an Arg residue, which are critical for catalytic activity. $cPLA_2\alpha$ has been studied extensively because it is the only PLA_2 that exhibits specificity for hydrolysis of *sn*-2 arachidonic acid from phospholipids (4–6). Arachidonic acid is the precursor of a large number of biologically active oxygenated metabolites including prostaglandins and leukotrienes. $cPLA_2\alpha$ is a highly regulated enzyme, which is important in controlling the availability of free arachidonic acid in cells for the production of eicosanoids (7). $cPLA_2\alpha$ is regulated by phosphorylation and an increase in intracellular calcium. Calcium binds to the calcium- and phospholipid-binding C2 domain on $cPLA_2\alpha$, which promotes its translocation from the cytosol to the Golgi, endoplasmic reticulum, and nuclear envelope, where it can access substrate (4, 8–11). $cPLA_2\alpha$ is phosphorylated on serine residues in the catalytic domain. Phosphorylation of Ser-505 by mitogen-activated protein kinases occurs in response to diverse agonists and is required for $cPLA_2\alpha$-mediated release of arachidonic acid in stimulated cells (12, 13).

Much less is known about the regulation and physiological function of the other GIV PLA_2s ($cPLA_2\beta$, -γ, -δ, -ϵ, -ζ) (4). $cPLA_2\gamma$ is the only GIV enzyme that does not contain a C2 domain (14, 15). It contains fatty acyl and C-terminal farnesyl groups, and is constitutively bound to membrane (16, 17). Human $cPLA_2\gamma$ is expressed most abundantly in heart and skeletal muscle; however, its role in these tissues is unknown. In

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[2] The abbreviations used are: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent PLA_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGE_2, prostaglandin E_2; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; Sf9, *Spodoptera frugiperda*; MLF, mouse lung fibroblasts; IMLF, immortalized MLF; EGFP, enhanced green fluorescent protein; GIV, group IV; RACE, rapid amplification of cDNA ends; MOPS, 4-morpholinepropanesulfonic acid.

contrast, the mouse cPLA$_2\gamma$ homologue is only 50% homologous to human cPLA$_2\gamma$ and is expressed exclusively in oocytes (18). cPLA$_2\delta$, cPLA$_2\epsilon$, and cPLA$_2\zeta$ form a gene cluster near cPLA$_2\beta$ in humans and mice and have more homology to cPLA$_2\beta$ than to cPLA$_2\alpha$ (4, 19, 20). Human cPLA$_2\delta$ is associated with psoriatic lesions and is expressed in stratified squamous epithelium (19). Human cPLA$_2\beta$ is widely expressed and occurs as multiple splice variants (14, 21, 22). It contains a novel, N-terminal-truncated JmjC domain immediately upstream of the C2 domain. We have recently found that the principle form of cPLA$_2\beta$ translated in human cells is a novel splice variant (cPLA$_2\beta$3) that contains an internal deletion in the catalytic domain (22). cPLA$_2\beta$3 exhibits calcium-dependent PLA$_2$ activity but is constitutively bound to mitochondria and early endosomes in cells, suggesting a mechanism of regulation and function distinct from cPLA$_2\alpha$. cPLA$_2\delta$, cPLA$_2\epsilon$, and cPLA$_2\zeta$ have been cloned from mouse tissues; however, only preliminary information is available about their biochemical properties, and nothing is known of their functional roles (20).

It is well documented that cPLA$_2\alpha$ functions to release arachidonic acid for the production of eicosanoids. However, eicosanoid production is not completely ablated in the cPLA$_2\alpha$ knock-out mouse indicating a role for other PLA$_2$s in mediating arachidonic acid release (23, 24). We previously isolated mouse lung fibroblasts (MLF) from cPLA$_2\alpha$ wild type and knock-out mice and demonstrated a primary role for cPLA$_2\alpha$ in mediating arachidonic acid release and prostaglandin E$_2$ (PGE$_2$) production (25). However, we found that cPLA$_2\alpha^{-/-}$ MLFs (MLF$^{-/-}$) release lower, but significant, levels of arachidonic acid and produce PGE$_2$ in response to calcium ionophore and serum (25). We have identified cPLA$_2\beta$ and cPLA$_2\zeta$ in MLF$^{-/-}$ and provide evidence here that cPLA$_2\zeta$ is the enzyme that mediates calcium-dependent arachidonic acid release.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (100 Ci/mmol), 1-palmitoyl-2-[^{14}C]arachidonyl-phosphatidylcholine (PC) (48 mCi/mmol), 1-[^{14}C]palmitoyl-2-lyso-PC (55 mCi/mmol), 1-palmitoyl-2-[^{14}C]arachidonyl-phosphatidylethanolamine (PE) (48 mCi/mmol), and 1-palmitoyl-2-[^{14}C]oleoyl-PC (55 mCi/mmol) were from PerkinElmer Life Sciences. 1-Palmitoyl-[^{14}C]linoleoyl-PE (55 mCi/mmol) was from Amersham Biosciences. 1-Palmitoyl-2-arachidonyl-PE, 1-palmitoyl-2-linoleoyl-PE, and 1-hexadecyl-2-arachidonyl-PC were from Avanti Polar Lipids. 1-Palmitoyl-2-arachidonyl-PC, dioleoylglycerol, bovine serum albumin (BSA), fatty acid-free BSA, pluronic acid, and anti-His$_6$ monoclonal antibody were from Sigma. 1-Arachidonyl-2-hexadecyl-PC was prepared as described (26). PLA$_2$ inhibitors indoxam, pyrrolidine-2, and Wyeth-1 were synthesized as described previously (27–30). Dulbecco's modified Eagle's medium (DMEM) was from BioWhittaker. Penicillin-streptomycin-L-glutamine solution was from Invitrogen. Fetal bovine serum was from Irvine Scientific. Bromoenol lactone was purchased from Biomol. Silica gel LC-Si SPE columns were from Sigma-Supelco. Protease inhibitor mixture tablets were from Roche Applied Science. The Total RNA Isolation kit was purchased from Promega, and the Advantage reverse transcription-PCR kit, BD SMART RACE cDNA amplification kit, and EGFP vector were from Clontech. The TA cloning vector was from Invitrogen. The plasmid isolation kit, RNeasy mini kit, and nickel-nitrilotriacetic acid-agarose beads were from Qiagen. iScript cDNA synthesis kit was obtained from Bio-Rad.

Culture of MLF and Assays for Fatty Acid Release and PGE$_2$ Production—Lung fibroblasts were isolated from wild type (MLF$^{+/+}$) and cPLA$_2\alpha$ knock-out (MLF$^{-/-}$) mice, and SV40 immortalized MLF (IMLF) were generated as described previously (31). Fibroblasts were plated in 24-well tissue culture plates at a density of 2.5 × 10^4 cells/well in supplemented DMEM (10% fetal bovine serum, 0.1% nonessential amino acids, 1 mM sodium pyruvate, and 1% penicillin-streptomycin-L-glutamine solution) and incubated for 6 h at 37 °C with 5% CO$_2$. Cells were washed twice with serum-free DMEM and incubated in serum-free DMEM containing 0.1% BSA and radiolabeled fatty acids (0.2 μCi of [^{3}H]arachidonic acid, 0.5 μCi of [^{3}H]palmitic acid, 0.25 μCi of [^{3}H]oleic acid, and 0.5 μCi of [^{14}C]linoleic acid/well). After incubation overnight, the cells were washed three times with DMEM containing 0.1% fatty acid-free BSA and stimulated with agonists in albumin-containing medium for the times indicated. For inhibitor experiments, cells were preincubated with inhibitors for 15 min prior to stimulation. The culture medium was removed and centrifuged at 15,000 rpm for 15 min. Cells were solubilized with 0.1% Triton-X-100. The level of radioactivity in the culture medium and in the cells was measured, and the amount released was calculated as a percentage of the total (released plus cellular) radioactivity.

To measure the effect of inhibitors on PGE$_2$ production, MLF$^{-/-}$ were incubated overnight in serum-free medium containing transforming growth factor β to up-regulate cyclooxygenase-2 as reported previously (25). The cells were incubated with and without pyrrolidine-2 and Wyeth-1 (both at 10 μM) for 15 min and then stimulated with A23187 (2 μg/ml) for 45 min. PGE$_2$ in the culture medium was quantified by enzyme-linked immunosorbent assay (Elisa Tech, Aurora, CO).

Quantitative Real-time PCR—The primers and probes used for real-time PCR of mouse cPLA$_2\beta$, cPLA$_2\zeta$, cPLA$_2\delta$, and cPLA$_2\epsilon$ were obtained from Applied Biosystems (premade Taqman gene expression assays). Assay IDs for mouse cPLA$_2\beta$, cPLA$_2\zeta$, cPLA$_2\delta$, and cPLA$_2\epsilon$ are Mm 01271073_g1, Mm 01338177_g1, Mm 01279782_m1, and Mm 00625711_m1, respectively. Total RNA was isolated from MLF and IMLF using a Qiagen RNeasy mini kit, and 1 μg of total RNA was used to make cDNA using iScript cDNA synthesis kit from Bio-Rad following the manufacturer's instructions. Each PCR reaction (25 μl) contained 500 ng of cDNA, PCR master mix, and premade Taqman gene expression assay components containing a 6-carboxyfluorescein reporter dye at the 5′-end of the Taqman probe and a nonfluorescent quencher at the 3′-end of the probe. Rodent glyceraldehyde-phosphate dehydrogenase was used as a control to verify the quality of cDNA template. Real-time PCR was performed and analyzed by the dual-labeled fluorogenic probe method using an ABI Prism 7000 sequence detector from Applied Biosystems.

Cloning of Mouse cPLA$_2\beta$ and cPLA$_2\zeta$—To clone cPLA$_2\beta$ cDNA from IMLF$^{-/-}$, cells were cultured in supplemented

DMEM; total RNA was isolated, 1 μg of which was used to generate cDNA. PCR analysis was performed using 10 μl of cDNA for cPLA$_2\beta$ and 5 μl of cDNA for glyceraldehyde-phosphate dehydrogenase following the manufacturer's instructions (Clontech Advantage reverse transcription-PCR kit). Specific primers used for mouse cPLA$_2\beta$ were as follows: 5′-gtctacaagcttatgcaggcaaaggtg-3′, 5′-gccaactttggcggtaccggcaagagc-3′, 5′-gctcttgccggtaccgccaaagttggc-3′, and 5′-cagctgggatcctcactccggcctaaac-3′. The primers were designed based on the mouse cPLA$_2\beta$ genomic sequence available from NCBI (gi:211429) to amplify the full-length cDNA in two fragments. The PCR products were cloned into the TA cloning vector, and the fragments were sequenced and then assembled into the full-length clone using the internal KpnI site present in the PCR products.

Mouse cPLA$_2\zeta$ was cloned from IMLF$^{-/-}$ cells and from mouse thyroid using the following primer sets: 5′-ctgggacctgagctgctactgctgg-3′, 5′-gaatactactcccgggaaaagagag-3′, 5′-ctctcttttcccgggagtagtattc-3′, and 5′-gtttaaagtcttccctctccctcag-3′. These were designed based on the mouse cPLA$_2\zeta$ sequence (NCBI NM_001024145) to amplify the full-length cDNA in two fragments. PCR products were cloned into the TA cloning vector, and the fragments were sequenced and then assembled into the full-length cPLA$_2\zeta$ clone using the internal SmaI site present in the PCR products. For immunofluorescence microscopy, cPLA$_2\zeta$ and cPLA$_2\beta$ cDNAs were cloned into the EGFP vector in the XhoI/HindIII and XhoI/BamHI sites, respectively.

Production of Recombinant Baculoviruses and Expression in Sf9 Cells—Mouse cPLA$_2\beta$ cDNA was cloned into the baculovirus vector pAcHLT in the StyI/NotI sites and cPLA$_2\zeta$ in the XhoI/SacI sites. Recombinant baculovirus was generated by cotransfection of Sf9 cells with cPLA$_2$-containing constructs and linearized baculovirus DNA (Baculogold) following the manufacturer's instructions (BD Biosciences-Pharmingen), and amplified by standard protocols. To determine the expression of cPLA$_2$s, Sf9 cells were plated in a 12-well tissue culture plate at a density of 0.5×10^6 cells/well and infected with recombinant viruses at different multiplicities of infection for 1 h. The virus-containing medium was replaced with fresh medium, and cells were incubated for 48 h. Expression of His$_6$-cPLA$_2\beta$ and His$_6$-cPLA$_2\zeta$ was determined by Western blot analysis using anti-His$_6$ monoclonal antibodies. His$_6$-cPLA$_2\beta$ and His$_6$-cPLA$_2\zeta$ expressed in Sf9 cells were affinity-purified using nickel-agarose beads following the manufacturer's instructions (Qiagen). The concentration of the enzymes in eluted fractions was determined by comparing the intensity of Coomassie-stained bands on SDS-polyacrylamide gels with a standard curve made with BSA and also by the bicinchoninic acid method.

Western Blotting—Cells were washed with phosphate-buffered saline and then scraped into ice-cold lysis buffer (50 mM Hepes, pH 7.4, 150 mM sodium chloride, 1.5 mM magnesium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 200 μM sodium vanadate, 10 mM terasodium pyrophosphate, 100 mM sodium fluoride, 300 nM *p*-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubation on ice for 30 min, lysates were centrifuged at 15,000 rpm for 15 min, and protein concentration in the supernatant was determined. Lysates were boiled for 5 min in Laemmli electrophoresis sample buffer, and the proteins (15–25 μg total protein) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. After blocking with 5% milk for 1 h, membranes were incubated overnight at 4 °C with monoclonal anti-His$_6$ antibody in 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween containing 5% milk and then incubated with anti-mouse IgG horseradish peroxidase antibody (1:5000 dilution) for 30 min at room temperature. The immunoreactive proteins were detected using the Amersham Biosciences ECL system.

Assay for Arachidonic Acid Release from Sf9 Cells—Sf9 cells were plated in 24-well plates (2.5×10^5 cells/well) in 500 μl of TNM-FH medium containing 10% fetal bovine serum (complete medium) and incubated at 27 °C for 15 min. The medium was removed and baculoviruses added in 150 μl of complete medium. After incubation for 1 h, complete medium (350 μl/well) was added and the cells incubated 25–30 h followed by incubation overnight in complete medium (500 μl/well) containing 0.2 μCi [^{3}H]arachidonic acid. The cells were washed three times with serum-free TNM-FH medium containing 0.1% human serum albumin and stimulated for 45 min with A23187 (2 μg/ml). The level of released [^{3}H]arachidonic acid was determined as described above for fibroblasts. A portion of the Triton lysate was used for Western blotting to determine the relative level of expression of the cPLA$_2$s.

Enzyme Assays—PLA$_2$ activity was assayed using 1-palmitoyl-2-[^{14}C]arachidonyl-PC, 1-palmitoyl-2-[^{14}C]oleoyl-PC, 1-palmitoyl-2-[^{14}C]arachidonyl-PE, and 1-palmitoyl-2-[^{14}C]linoleoyl-PE as substrates. To prepare substrate, solvents were evaporated from the lipid mixture under a stream of nitrogen, 50 mM Hepes buffer, pH 7.4, was added and the lipid mixture sonicated at 4 °C for 10 s on ice using a microprobe (Braun Instruments) to form small unilamellar vesicles. The reaction mixture (50 μl final volume) contained 30 μM phospholipid substrate (100,000 dpm), 9 μM dioleoylglycerol (which was co-sonicated with the substrate), 150 mM sodium chloride, 1 mg/ml fatty acid free BSA, 1 mM EGTA, and 5 mM $CaCl_2$. For assays with palmitoyl-arachidonyl-PE, dioleoylglycerol was not added. Reactions were started by the addition of affinity-purified enzyme (50 ng-1 μg) and incubated at 37 °C for the times indicated. Free fatty acids were extracted using Dole reagent (propan-2-ol:heptane:1 N H_2SO_4, 20:5:1) and separated by silicic acid chromatography as described previously using unlabeled oleic acid (25 μg) as carrier lipid (32).

The calcium dependence of the PLA$_2$ activity of cPLA$_2\alpha$ and cPLA$_2\zeta$ was measured using 1-palmitoyl-2-[^{14}C]arachidonyl-PC. Vesicles were made by extrusion through two 0.2-μm Nucleopore membranes as described previously (33) in buffer (10 mM MOPS, 100 mM KCl, 0.5 mM EGTA, pH 7.2) to give 4–4.5 mM total phospholipid at a final specific radioactivity of 2.7 Ci/mol. The phospholipid concentration in the stock solution after extrusion was calculated from the initial concentration and the yield of radioactivity. The same buffer containing various concentrations of free calcium from 0 to 20 μM was prepared by fluorimetric titration using fluo-3 and Calcium Green 5N as described previously (34). A small aliquot of extruded vesicle stock was added to give 200 μM total phospholipid in each assay. Reactions (80 μl) were carried out in various calcium buffers supplemented with 0.5 mg/ml fatty acid-free BSA for 2 min at 37 °C. Reactions were quenched and analyzed for radiolabeled free arachidonic acid as described earlier (35).

Reactions to study the PLA_2 and PLA_1 activities were carried out as follows. 1-Hexadecyl-2-arachidonyl-PC or 1-arachidonyl-2-hexadecyl-PC was sonicated at 60 μM in 50 mM Hepes, pH 7.4, to form a stock solution of small unilamellar vesicles as described (36). Reaction mixtures contained 30 μM phospholipid in 250 μl of 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mg/ml fatty acid-free BSA, 1 mM EGTA, 5 mM $CaCl_2$, and either $cPLA_2\alpha$ (10 ng), $cPLA_2\beta$ (7.5 μg), or $cPLA_2\zeta$ (20 ng). After 20 min at 37 °C, reactions were processed for arachidonic acid analysis using gas chromatography/mass spectrometry as described with d_8-arachidonic acid (Cayman Chemicals, Inc.) as an internal standard (37).

Lysophospholipase activity was measured using 1-[^{14}C]palmitoyl-2-lyso-PC sonicated in 50 mM Hepes, pH 7.4, to make micelles as described previously (38). Assays contained 50 μM substrate (120,000 dpm), 1 mM EGTA, and 5 mM $CaCl_2$ in a final volume of 50 μl. Reactions were started by adding affinity-purified enzyme and incubated at 37 °C for the times indicated. Free fatty acid product was extracted using Dole reagent. After vortexing, the upper heptane phase was removed and dried under a stream of nitrogen, and 0.5 ml of heptane added. Radiolabeled free fatty acids were measured by liquid scintillation spectrometry. For inhibitor experiments, enzymes were preincubated for 2 min at 37 °C with inhibitors, and reactions were started by addition of substrate.

Immunofluorescence Microscopy—$IMLF^{-/-}$ were transfected with 10 μg of EGFP-$cPLA_2\zeta$ and EGFP-$cPLA_2\alpha$ cDNA using nucleofection technology (Amaxa Biosystems), with solution T, following the manufacturer's instructions. Transfected fibroblasts were plated in 35-mm glass-bottom MatTek plates at a density of $0.5 \times 10^6/cm^2$ and incubated for 48 h. Cells were then washed twice with serum-free DMEM and incubated in serum-free DMEM containing 0.1% BSA overnight. For live cell imaging, fibroblasts were washed with and incubated in Hanks' balanced salt solution buffered with 25 mM Hepes. Cells were stimulated with 0.5 μM ionomycin and imaged at 37 °C with an inverted Zeiss 200M microscope with a 175-watt xenon lamp using a ×63 oil immersion objective and GFP filters. Images were acquired every 5 s for a total of 10 min with a charge-coupled device camera from Sensicam, and data were analyzed using Intelligent Imaging Innovations Inc. (3I) software.

RESULTS

Fatty Acid Release from $IMLF^{+/+}$ and $IMLF^{-/-}$ and Effect of PLA_2 Inhibitors—We previously reported that primary $MLF^{-/-}$ and immortalized $MLF^{-/-}$ release arachidonic acid and produce PGE_2 in response to A23187 and serum indicating the presence of a calcium-regulated PLA_2 that can initiate lipid mediator production (25). To characterize the PLA_2, we compared the types of fatty acids released from stimulated $IMLF^{+/+}$ and $IMLF^{-/-}$. The cells were incubated with radiolabeled fatty acids to determine the acyl chain specificity of the PLA_2 (Fig. 1, *A* and *B*). $IMLF^{+/+}$ stimulated with A23187 or mouse serum release relatively greater amounts of arachidonic acid than

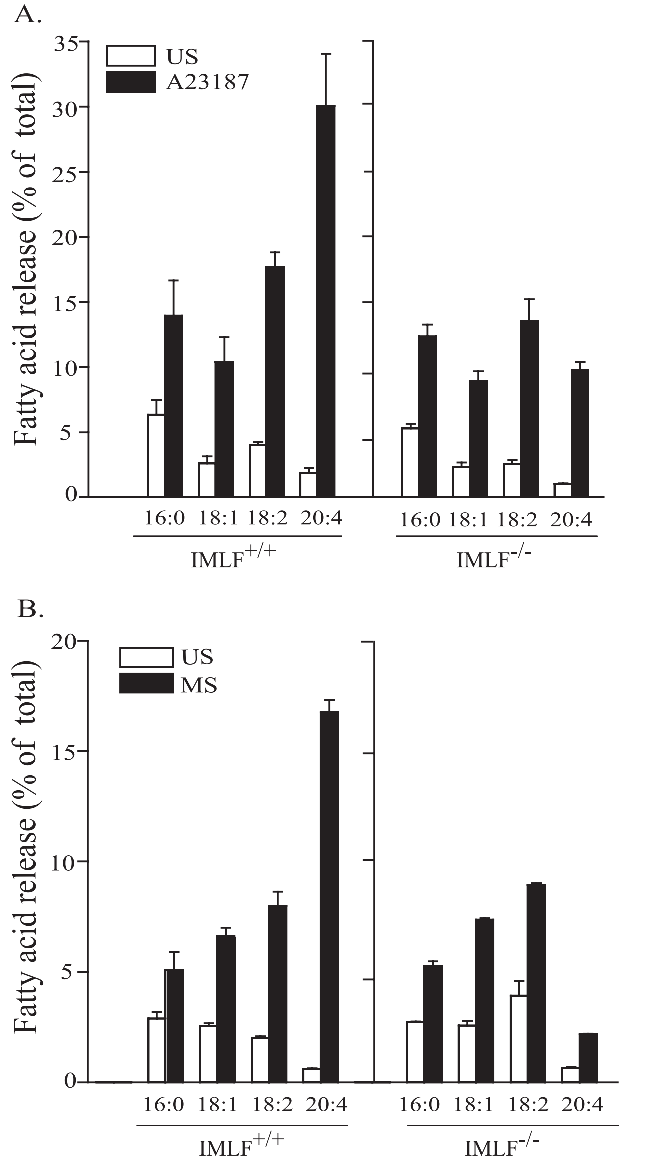

FIGURE 1. **Comparison of fatty acids released from $IMLF^{+/+}$ and $IMLF^{-/-}$.** $IMLF^{+/+}$ and $IMLF^{-/-}$ were incubated overnight in medium containing various radiolabeled fatty acids and then either left unstimulated (*US*, *white bar*) or stimulated with A23187 (2 μg/ml) (*black bar*) for 45 min (*A*) or with 10% mouse serum (*MS*, *black bar*) for 30 min (*B*). The amount of fatty acids released into the medium is expressed as a percentage of the total cellular incorporated radioactivity. Results are the average ± S.E. of four independent experiments.

other fatty acids. This is consistent with a primary role for $cPLA_2\alpha$ in mediating arachidonic acid release (25). In contrast $IMLF^{-/-}$ stimulated with A23187 do not preferentially release arachidonic acid but release similar levels of saturated, monounsaturated, and polyunsaturated fatty acids. As previously shown, the release of arachidonic acid from $IMLF^{-/-}$ stimulated with mouse serum is lower than with A23187 (25); however, the results also show nonspecific release of multiple fatty acids (Fig. 1*B*). Thus the PLA_2 in $IMLF^{-/-}$ does not exhibit acyl chain specificity. It is interesting to note that the release of arachidonic acid from $IMLF^{-/-}$ is about 65% lower than from $IMLF^{+/+}$; however, the % release of the other fatty acids (16:0, 18:1, 18:2) is similar in $IMLF^{-/-}$ and $IMLF^{+/+}$. This suggests that the release of these fatty acids from $IMLF^{+/+}$ is not due to

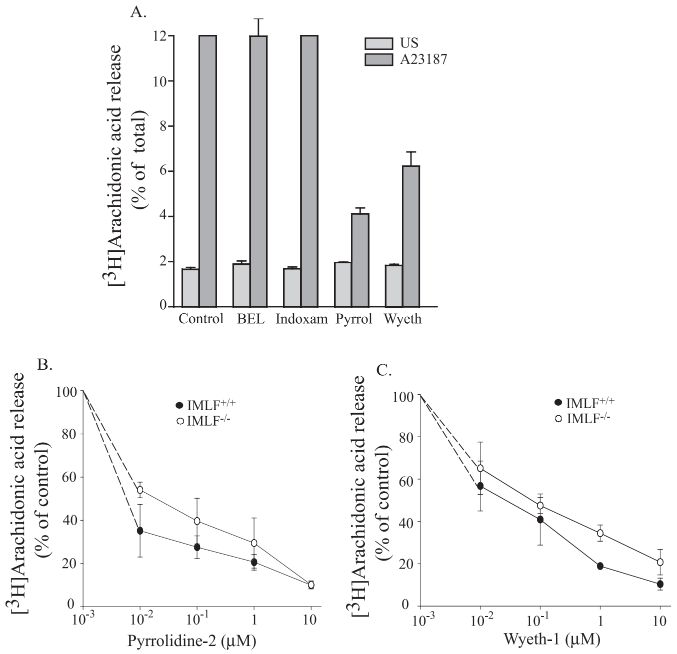

FIGURE 2. **Effect of PLA$_2$ inhibitors on arachidonic acid release from IMLF$^{+/+}$ and IMLF$^{-/-}$.** *A*, [^{3}H]arachidonic acid-labeled IMLF$^{-/-}$ were treated with PLA$_2$ inhibitors (10 μM) or Me$_2$SO (vehicle control) for 15 min and then left unstimulated (*US*, *light gray bars*) or incubated with A23187 (2 μg/ml) (*dark gray bars*) for 45 min. The amount of [^{3}H]arachidonic acid released into the medium is expressed as a percentage of the total cellular incorporated radioactivity. IMLF$^{+/+}$ and IMLF$^{-/-}$ were preincubated with the indicated amounts of pyrrolidine-2 (*B*) or Wyeth-1 (*C*) for 15 min and then stimulated with 2 μg/ml A23187. The amount of [^{3}H]arachidonic acid released is expressed as a percentage of the total incorporated radioactivity after subtracting the % release from unstimulated cells treated with inhibitors. Data shown are the average $\pm$ S.E. of three independent experiments.

cPLA$_2\alpha$ but is mediated by another PLA$_2$ that is present in both IMLF$^{-/-}$ and IMLF$^{+/+}$.

To determine the type of PLA$_2$ involved in release of arachidonic acid from IMLF$^{-/-}$, the cells were treated with PLA$_2$ inhibitors and stimulated with A23187. The iPLA$_2$ inhibitor bromoenol lactone, the sPLA$_2$ inhibitor indoxam, and the cPLA$_2\alpha$ inhibitors pyrrolidine-2 and Wyeth-1 were tested (29, 30). Although IMLF$^{-/-}$ lack cPLA$_2\alpha$, pyrrolidine-2 and Wyeth-1 were used because they may inhibit another GIV cPLA$_2$ present in IMLF. Pyrrolidine-2 and Wyeth-1 are the only inhibitors that block A23187-stimulated arachidonic acid release from IMLF$^{-/-}$ (Fig. 2*A*). Pyrrolidine-2 and Wyeth-1 inhibit arachidonic acid release from IMLF$^{+/+}$ with an IC$_{50}$ of ~0.01 and 0.05 μM, respectively, and from IMLF$^{-/-}$ with an IC$_{50}$ of ~0.03 and 0.1 μM, respectively (Fig. 2, *B* and *C*).

We previously reported that MLF$^{-/-}$ produce PGE$_2$ in response to A23187 and serum (25), suggesting that arachidonic acid released by the PLA$_2$ couples to cyclooxygenases for PGE$_2$ production. This is supported by data showing that pyrrolidine-2 and Wyeth-1 block PGE$_2$ production from A23187-stimulated MLF$^{-/-}$ by 91 and 50%, respectively (Table 1). MLF$^{-/-}$ were incubated overnight with transforming growth factor β prior to stimulation to up-regulate cyclooxygenase-2. These experiments were carried out with MLF$^{-/-}$ rather than IMLF$^{-/-}$ because IMLF$^{-/-}$ produce very little PGE$_2$ due to a defect in the expression of microsomal PGE synthase from inactivation of p53 by SV40 (25).

TABLE 1
PGE$_2$ production from A23187-stimulated MLF$^{-/-}$

MLF$^{-/-}$ were cultured overnight with transforming growth factor β (TGFβ) (2 ng/ml) to up-regulate cyclooxygenase 2 and then left unstimulated (US) or treated with A23187 (2 μg/ml) for 45 min. Fibroblasts were incubated with 10 μM pyrrolidine-2 (Pyr-2) or Wyeth-1 for 15 min prior to stimulation with A23187. PGE$_2$ levels in the culture medium were measured by enzyme-linked immunosorbent assay. A representative experiment of triplicate samples is shown $\pm$ S.D.

Sample	PGE$_2$
	pg/ml
US	30.4 ± 2.5
TGFβ	28.0 ± 1.4
TGFβ + A23187	187.7 ± 7.3
TGFβ + Pyr-2	25.0 ± 0.3
TGFβ + A23187 + Pyr-2	43.7 ± 7.1
TGFβ + Wyeth-1	32.7 ± 0.6
TGFβ + A23187 + Wyeth-1	94.0 ± 3.5

Identification of cPLA$_2\beta$ and cPLA$_2\zeta$ in IMLF$^{-/-}$—Because pyrrolidine-2 does not inhibit iPLA$_2\beta$ or sPLA$_2$s (39) and the PLA$_2$ in IMLF$^{-/-}$ is calcium-regulated, the results suggested another C2 domain-containing GIV PLA$_2$ may be responsible for arachidonic acid release in these cells. Real-time PCR analysis revealed that IMLF$^{-/-}$ contain transcripts for cPLA$_2\beta$ (GIVB) and cPLA$_2\zeta$ (GIVF) but not cPLA$_2\epsilon$ (GIVE) or cPLA$_2\delta$ (GIVD) (Fig. 3*A*). The level of expression of cPLA$_2\beta$ and cPLA$_2\zeta$ is similar in IMLF$^{+/+}$ and IMLF$^{-/-}$, indicating that the ablation of cPLA$_2\alpha$ does not result in their up-regulation. Similar results were obtained for primary MLF$^{+/+}$ and MLF$^{-/-}$ (data not shown).

Cloning of cPLA$_2\beta$ cDNA from IMLF$^{-/-}$ demonstrated that it has a C2 domain as well as a catalytic domain that contains the active site residues previously identified in human cPLA$_2\beta$1 (14, 21). An alignment of the amino acid sequence of mouse cPLA$_2\beta$ and human cPLA$_2\beta$1 is shown in Fig. 3*B*. The C2 and catalytic domains of mouse cPLA$_2\beta$ have 82% amino acid identity with human cPLA$_2\beta$1. We have recently identified three transcripts of human cPLA$_2\beta$ in BEAS-2B lung epithelial cells (22). cPLA$_2\beta$1 is identical to the form originally cloned (14, 21), and cPLA$_2\beta$2 and cPLA$_2\beta$3 contain internal deletions in the catalytic domain (22). We found that only cPLA$_2\beta$3 is translated in BEAS-2B cells. Only one transcript of mouse cPLA$_2\beta$ is found in IMLF$^{-/-}$, and its catalytic domain is most similar to human cPLA$_2\beta$1 because it does not contain the internal deletion. This was determined by 3′-RACE PCR using three gene-specific primers to different regions in the catalytic domain of mouse cPLA$_2\beta$ and the universal primer. One striking difference between mouse and human cPLA$_2\beta$ is that mouse cPLA$_2\beta$ does not contain the N-terminal extension with the truncated JmjC domain found in human cPLA$_2\beta$ (Fig. 3*B*) (14, 21). We confirmed the lack of a truncated JmjC domain in mouse cPLA$_2\beta$ by 5′-RACE analysis. This is consistent with the prediction in the NCBI Database that separate adjacent genes encode a complete JmjC domain (accession number BC016255) and cPLA$_2\beta$ (accession number BC098210) on mouse chromosome 2.

Mouse cPLA$_2\zeta$ cloned from IMLF$^{-/-}$ is homologous to the sequence previously reported for mouse cPLA$_2\zeta$ cloned from thyroid with the exception of a number of base differences leading to amino acid changes. We also cloned cPLA$_2\zeta$ from mouse thyroid and confirmed that it is identical to cPLA$_2\zeta$ in

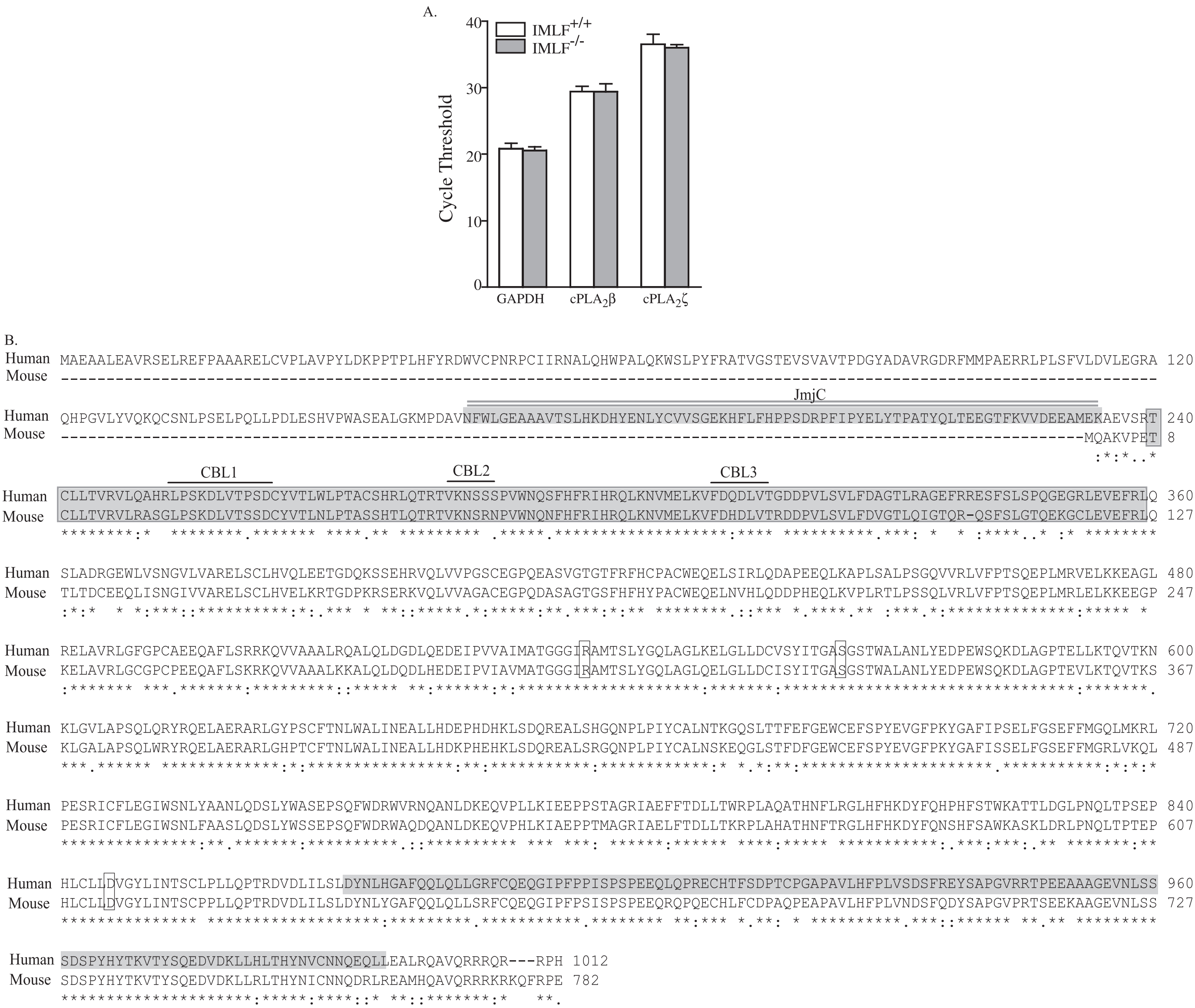

FIGURE 3. **Real-time PCR of GIV cPLA$_2$s in IMLF$^{+/+}$ and IMLF$^{-/-}$.** *A*, real-time PCR was carried out using Taqman gene expression assays for mouse GIV cPLA$_2$s using iScript cDNA from IMLF$^{+/+}$ and IMLF$^{-/-}$. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a cDNA template control. The data are represented as cycle threshold values, which is the cycle number that a signal was detected for a specific cDNA and is inversely proportional to the abundance of the transcript. Results are the average ± S.E. of six independent experiments. *B*, amino acid alignment of human cPLA$_2\beta$1 and mouse cPLA$_2\beta$. The N-terminal extension containing the truncated JmjC domain (*highlighted*, with *double overline*) in human cPLA$_2\beta$1 is not present in mouse cPLA$_2\beta$. The calcium-binding loops (*CBL*) in the C2 domains (*highlighted* and *boxed*) of human and mouse cPLA$_2\beta$ are shown. Residues required for catalytic activity, including the active site Ser (human Ser-566; mouse Ser-333) and Asp (human Asp-846; mouse Asp-613) catalytic dyad and Arg (human Arg-538; mouse Arg-305) are shown in *open boxes*. The region in the catalytic domain of human cPLA$_2\beta$1 that is deleted in the human cPLA$_2\beta$3 splice variant (22) is highlighted in *gray*.

IMLF$^{-/-}$. The sequences of cPLA$_2\beta$ (accession number DQ888308) and cPLA$_2\zeta$ (accession number DQ904008) from IMLF$^{-/-}$ can be found in the NCBI Database.

Enzymatic Properties and Inhibitor Sensitivity of Purified cPLA$_2\beta$ and cPLA$_2\zeta$—To determine whether cPLA$_2\beta$ and cPLA$_2\zeta$ have characteristics of the PLA$_2$ responsible for mediating arachidonic acid release from IMLF$^{-/-}$, the enzymes were expressed as N-terminal His$_6$-tagged proteins in Sf9 cells using baculoviruses, affinity-purified, and their enzymatic properties and inhibitor sensitivity characterized. cPLA$_2\beta$ has lysophospholipase activity against palmitoyl-lyso-PC and lower PLA$_2$ activity against both palmitoyl-arachidonyl-PC and palmitoyl-arachidonyl-PE (Fig. 4, *A* and B). We tested the ability of pyrrolidine-2 and Wyeth-1 to inhibit the activity of cPLA$_2\beta$ using palmitoyl-lyso-PC as substrate (Fig. 4*C*). The results demonstrate that concentrations of pyrrolidine-2 and Wyeth-1 that are more than sufficient to inhibit arachidonic acid release from IMLF$^{-/-}$ have no effect on the activity of mouse cPLA$_2\beta$. In contrast, the activity of cPLA$_2\zeta$ under the same assay conditions is inhibited by over 90% by pyrrolidine-2 and Wyeth-1 (Fig. 4*C*).

Affinity-purified His$_6$-tagged cPLA$_2\zeta$ exhibits greater lysophospholipase and PLA$_2$ activity than cPLA$_2\beta$ (Fig. 5, *A* and *B*). cPLA$_2\zeta$ does not exhibit *sn*-2 acyl chain specificity, because it hydrolyzes both *sn*-2 arachidonic acid and oleic acid from PC with similar activity (Fig. 5*B*). cPLA$_2\zeta$ hydrolyzes arachidonic acid from PE with slightly lower activity than from PC (Fig. 5*C*). The PLA$_2$ activity of cPLA$_2\zeta$ is very sensitive to inhibition by

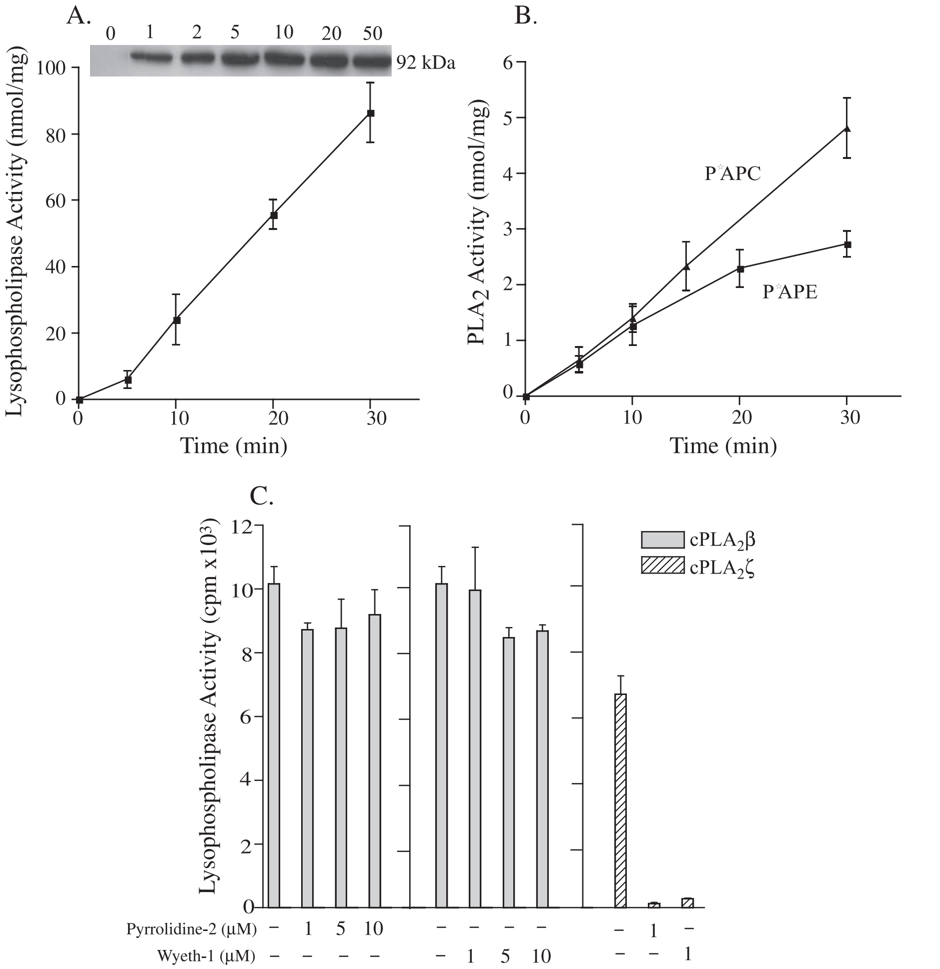

FIGURE 4. **cPLA$_2\beta$ has low enzymatic activity and is insensitive to pyrrolidine-2 and Wyeth-1.** *A*, lysophospholipase activity of affinity purified His$_6$-cPLA$_2\beta$ (1 μg) was assayed using 1-[^{14}C]palmitoyl-2-lyso-PC for the indicated times. Expression of His$_6$-cPLA$_2\beta$ in Sf9 cells infected with increasing volumes (μl) of baculovirus is shown in the *inset*. *B*, PLA$_2$ activity of His$_6$-cPLA$_2\beta$ (1 μg) was measured at the indicated time points using 1-palmitoyl-[^{14}C]arachidonyl-PE or -PC as described under "Experimental Procedures." *C*, the effect of pyrrolidine-2 and Wyeth-1 on lysophospholipase activity of cPLA$_2\beta$ (1 μg) or cPLA$_2\zeta$ (50 ng) was studied by incubating purified enzyme at 37 °C for 2 min with different doses of inhibitors followed by the addition of the substrate. Results shown are the average ± S.D. of two independent experiments.

pyrrolidine-2 and Wyeth-1 with IC$_{50}$ values of 0.002 and 0.02 μM, respectively (Fig. 5*D*). The analog of pyrrolidine-2 in which the ketone carbonyl has been reduced to the tertiary alcohol did not inhibit cPLA$_2\alpha$ or cPLA$_2\zeta$ when tested *in vitro* at a concentration up to 1 μM (data not shown). This is consistent with a mechanism of inhibition of both enzymes in which the active site serine residue (Ser-228 in cPLA$_2\alpha$) adds to the ketone carbonyl of pyrrolidine-2 to form a hemi-ketal. This also explains why removal of the 2-fluorine atoms from pyrrolidine-2 renders the compound inactive as an inhibitor (30).

A summary of the specific activities of cPLA$_2\beta$ and cPLA$_2\zeta$ is shown in Table 2. Overall cPLA$_2\zeta$ has 300-fold greater lysophospholipase and 400–800-fold greater PLA$_2$ activity than cPLA$_2\beta$. The release of *sn*-2 fatty acids could occur through a PLA$_2$ mechanism and/or by sequential deacylation of the *sn*-1 fatty acid (PLA$_1$ reaction) followed by release of *sn*-2 fatty acid by lysophospholipase activity. We measured the specific activity of the various cPLA$_2$s for hydrolysis of 1-hexadecyl-2-arachidonyl-PC (PLA$_2$ activity) or 1-arachidonyl-2-hexadecyl-PC (PLA$_1$ activity) using sonicated vesicles of the pure phospholipid. The use of ester/ether phospholipids ensures that only one round of lipolysis can occur, *i.e.* no lysophospholipase activity is possible because phospholipases do not cleave ether-linked aliphatic groups. Estimates of the specific activities came from a single time point assay (20 min). The results show that all three cPLA$_2$ isoforms (α, β, ζ) have PLA$_2$ and PLA$_1$ activity, with the former being about 2-fold higher than the latter (Table 3). To be sure that the 1-arachidonyl-2-hexadecyl-PC was not contaminated by the positional isomer in which the arachidonyl group is in the *sn-2* position, we submitted the 2-hexadecyl-PC synthetic precursor to combined reverse-phase high pressure liquid chromatography/electrospray ionization mass spectrometry using the previously reported method for lysophospholipid analysis (40). Based on the signal-to-noise ratio for the 2-hexadecyl-PC peak, we estimated that there was less than 2% of the positional isomer present. Based on this estimate and the data in Table 3, we conclude that cPLA$_2\alpha$ and cPLA$_2\zeta$ display significant PLA$_1$ activity. The activity for cPLA$_2\beta$ was very low; however, the amount of arachidonic acid generated was 30- and 4-fold higher than that measured with no enzyme control in the reactions with 1-arachidonyl-2-hexadecyl-PC and 1-hexadecyl-2-arachidonyl-PC, respectively.

cPLA$_2\zeta$ Expressed in Sf9 Cells Mediates A23187-induced Arachidonic Acid Release—Our results suggest that cPLA$_2\zeta$ is the PLA$_2$ in IMLF$^{-/-}$ responsible for fatty acid release in response to A23187. To compare the ability of cPLA$_2\zeta$ and cPLA$_2\beta$ to release arachidonic acid in cells in response to A23187, the enzymes were expressed in Sf9 cells. We have used this cell model to study the properties and regulation of cPLA$_2\alpha$, which releases arachidonic acid in response to A23187 when expressed in Sf9 cells (41, 42). As shown in Fig. 6*A*, His$_6$-cPLA$_2\zeta$ has a much greater capacity than cPLA$_2\beta$ to release arachidonic acid in response to A23187 when expressed in Sf9 cells. Sf9 cells were infected with two concentrations of baculovirus, resulting in similar levels of expression of cPLA$_2\zeta$ and cPLA$_2\beta$ as shown by Western blot analysis using anti-His$_6$ antibody. In Sf9 cells expressing His$_6$-cPLA$_2\zeta$, A23187-stimulated arachidonic acid release is 10-fold greater than vector control cells but is only slightly above control levels in cells expressing His$_6$-cPLA$_2\beta$. As we observed for arachidonic acid release from

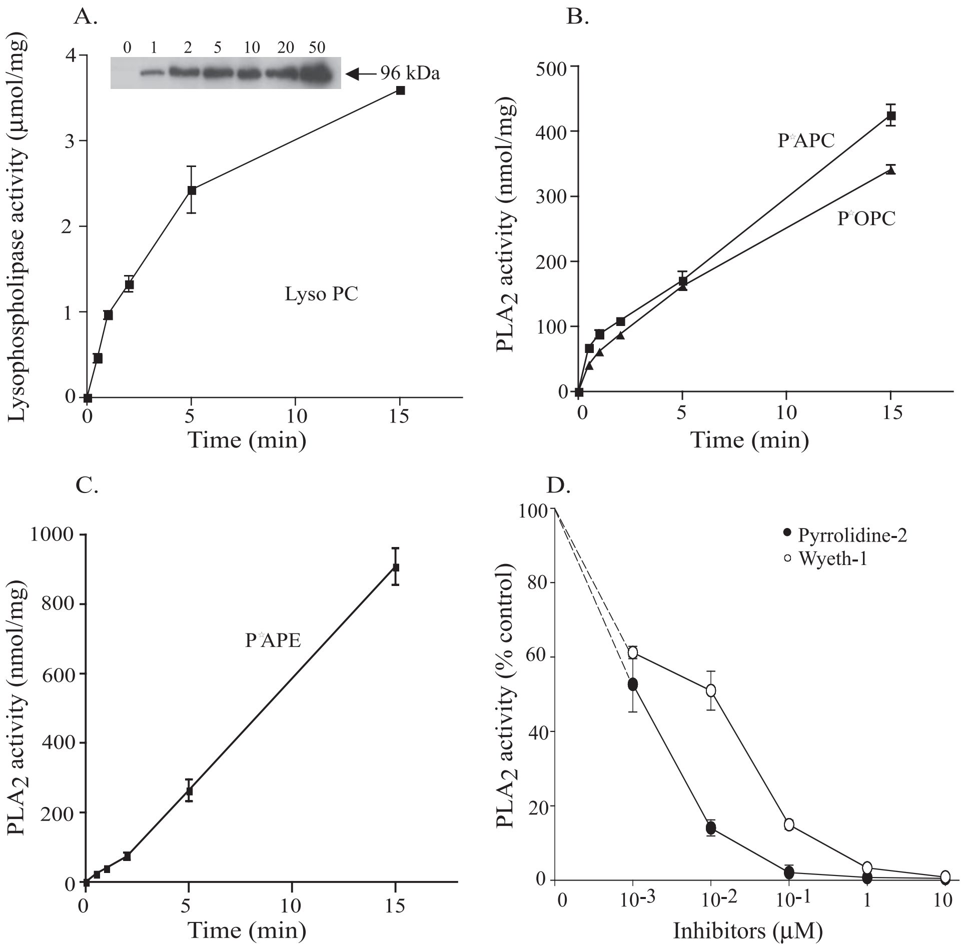

FIGURE 5. **Enzymatic properties of cPLA$_2\zeta$ and inhibition by pyrrolidine-2 and Wyeth-1.** *A*, lysophospholipase activity of affinity-purified His$_6$-cPLA$_2\zeta$ (50 ng) was assayed using 1-[^{14}C]palmitoyl-2-lyso-PC. Expression of His$_6$-cPLA$_2\zeta$ in Sf9 cells infected with increasing volumes (μl) of baculovirus is shown in the *inset*. *B* and *C*, PLA$_2$ activity (150 ng) was assayed using 1-palmitoyl-2-[^{14}C]arachidonyl-PC (P*APC) and 1-palmitoyl-2-[^{14}C]oleoyl-PC (P*OPC) (*B*) and 1-palmitoyl-[^{14}C]arachidonyl-PE (P*APE) (*C*) for the indicated times. *D*, the effect of pyrrolidine-2 and Wyeth-1 on PLA$_2$ activity of His$_6$-cPLA$_2\zeta$ (150 ng) was studied by incubating the purified enzyme at 37 °C for 2 min with different amount of inhibitors followed by the addition of the substrate (P*APE). Results shown are the average ± S.D. of two independent experiments.

TABLE 2
Specific activity of cPLA$_2\beta$ and cPLA$_2\zeta$

Lysophospholipase and PLA$_2$ activity of affinity-purified His$_6$-cPLA$_2\beta$ and His$_6$-cPLA$_2\zeta$ were assayed with different substrates as described under "Experimental Procedures." Values represent the average of two independent experiments in triplicate ± S.D.

Substrate	Specific activity	
	cPLA$_2\beta$	cPLA$_2\zeta$
	nmol min^{-1} mg^{-1}	
1-[^{14}C]16:0–2-lyso-PC	2.93 ± 0.5	960.0 ± 7.9
1–16:0–2-[^{14}C]20:4-PC	0.16 ± 0.03	142.5 ± 7.4
1–16:0–2-[^{14}C]18:1-PC		110.0 ± 2.5
1–16:0–2-[^{14}C]20:4-PE	0.13 ± 0.03	58.2 ± 6.2
1–16:0–2-[^{14}C]18:2-PE		22.2 ± 2.5

IMLF$^{-/-}$, the release of arachidonic acid from A23187-stimulated Sf9 cells expressing His$_6$-cPLA$_2\zeta$ is inhibited by pyrrolidine-2 (IC$_{50}$ 0.1 μM) and Wyeth-1 (IC$_{50}$ 1.0 μM) (Fig. 6*B*).

Calcium Sensitivity of cPLA$_2\zeta$—Our results demonstrate that cPLA$_2\zeta$ is activated in cells in response to increases in intracellular calcium. The calcium dependence of cPLA$_2\zeta$ was investigated in more detail by measuring the effect of calcium on the activity of purified cPLA$_2\zeta$ *in vitro*. The PLA$_2$ activity of cPLA$_2\zeta$ assayed using palmitoyl-arachidonyl-PE is stimulated by calcium ~5-fold above the low level of activity observed in the absence of calcium (Fig. 7*A*). The effect of calcium concentration on PLA$_2$ activity of cPLA$_2\zeta$ and cPLA$_2\alpha$ was compared directly using palmitoyl-arachidonyl-PC as substrate (Fig. 7*B*). The results of three experiments show that the calcium dependence of cPLA$_2\alpha$ (K_{Ca} 0.82 ± 0.45, 1.5 ± 0.3, 0.87 ± 0.3 μM) and cPLA$_2\zeta$ (K_{Ca} 1.5 ± 0.3, 0.95 ± 0.15, 1.7 ± 0.4 μM) is comparable. At saturating calcium levels the specific activity of cPLA$_2\alpha$ is 2-fold higher than cPLA$_2\zeta$ (Fig. 7*B*). The activity in the absence of calcium is 16 and 7% of maximal activity at saturating calcium for cPLA$_2\alpha$ and cPLA$_2\zeta$, respectively.

Calcium-induced Translocation of EGFP-cPLA$_2\zeta$ and EGFP-cPLA$_2\alpha$ in IMLF$^{-/-}$—The ability of cPLA$_2\zeta$ to release arachidonic acid in response to calcium ionophore and the stimulation of its activity by physiological levels of calcium suggests that it may translocate to membrane in response to increases in intracellular calcium as reported for cPLA$_2\alpha$. This was investigated by comparing translocation of EGFP-cPLA$_2\zeta$ and EGFP-cPLA$_2\alpha$ in ionomycin-stimulated IMLF$^{-/-}$ by live cell imaging. EGFP-cPLA$_2\zeta$ exhibits primarily diffuse cytosolic localization in unstimulated cells (Fig. 8*A*, *a*). In response to ionomycin, EGFP-cPLA$_2\zeta$ rapidly translocates to membrane ruffles and to dynamic vesicular structures (Fig. 8*A*, *b–f*). An *enlarged image* of a portion of this cell (3.3 min after ionomycin) shows the presence of numerous fluorescent vesicles (Fig. 8*A*, *g*). A video of this cell shows the dynamic localization of cPLA$_2\zeta$ to these structures (Movie 1 in supplemental data). Images of another cell depicting the translocation of cPLA$_2\zeta$ to membrane ruffles in response to ionomycin are shown in Fig. 8*A*, *h–j*. In comparison, EGFP-cPLA$_2\alpha$ expressed in IMLF$^{-/-}$ rapidly translocates to Golgi and endoplasmic reticulum in response to ionomycin as observed in other cell types (Fig. 8*B*, *a–c*) (11, 43).

DISCUSSION

cPLA$_2\alpha$ plays an important role in production of eicosanoids through its ability to specifically release arachidonic acid from stimulated cells. However, there is ample evidence that other PLA$_2$s participate in the release of arachidonic acid for produc-

TABLE 3

Specific activity of three cPLA$_2$ isoforms

The PLA$_1$ and PLA$_2$ activity of affinity-purified cPLA$_2\alpha$, cPLA$_2\beta$, and cPLA$_2\zeta$ was investigated with different substrates as described under "Experimental Procedures."

Enzyme	Specific activity	
	1-Hexadecyl-2-arachidonyl-PC	1-Arachidonyl-2-hexadecyl-PC
	nmol min^{-1} mg^{-1}	
cPLA$_2\alpha$	590	368
cPLA$_2\beta$	1	0.9
cPLA$_2\zeta$	142	69

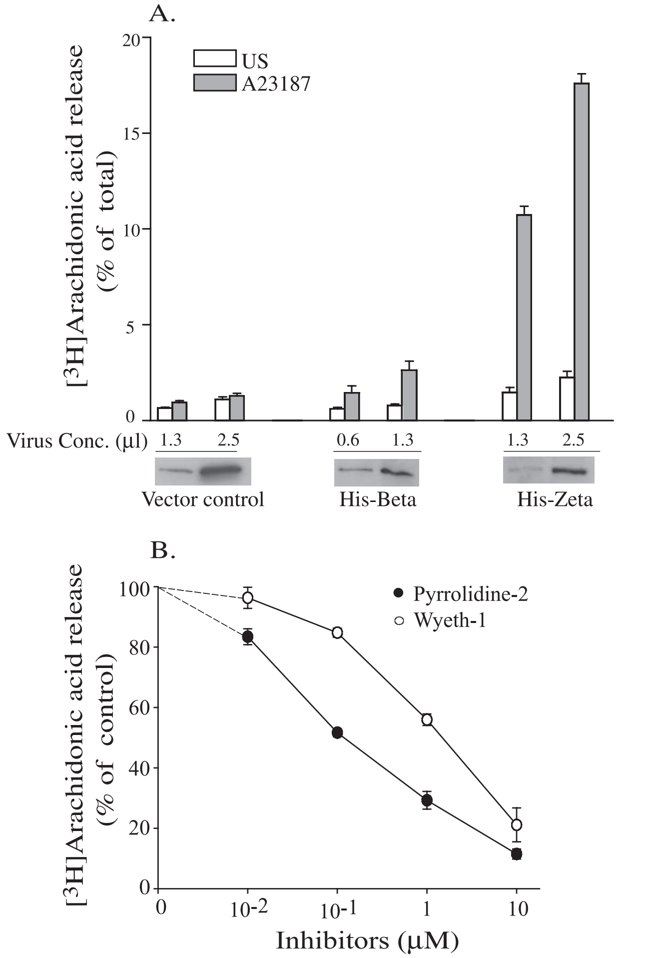

FIGURE 6. **A23187 stimulates arachidonic acid release from Sf9 cells expressing His-cPLA$_2\zeta$.** *A*, Sf9 cells were infected with different amounts of recombinant baculoviruses for expression of glutathione *S*-transferase-His (pAcGHLT, vector control), mouse His$_6$-cPLA$_2\beta$, or mouse His$_6$-cPLA$_2\zeta$. [^{3}H]Arachidonic acid-labeled Sf9 cells were stimulated with A23187 (2 μg/ml) for 45 min. The amount of [^{3}H]arachidonic acid released into the medium from unstimulated (*US*) or A23187-stimulated cells is expressed as a percentage of the total incorporated radioactivity. The *bottom panels* are representative immunoblots of Sf9 lysates showing levels of expression of glutathione *S*-transferase-His$_6$ (*vector control*), His$_6$-cPLA$_2\beta$ and His$_6$-cPLA$_2\zeta$ using antibody to His$_6$. *B*, [^{3}H]arachidonic acid-labeled Sf9 cells expressing His$_6$-cPLA$_2\zeta$ were treated with the indicated amounts of pyrrolidine-2 or Wyeth-1 for 15 min prior to stimulation with A23187. Arachidonic acid release was measured similarly as described in *A*. Arachidonic acid release from Sf9 cells infected with control vector treated similarly was subtracted as background. Results shown are the average ± S.D. of two independent experiments.

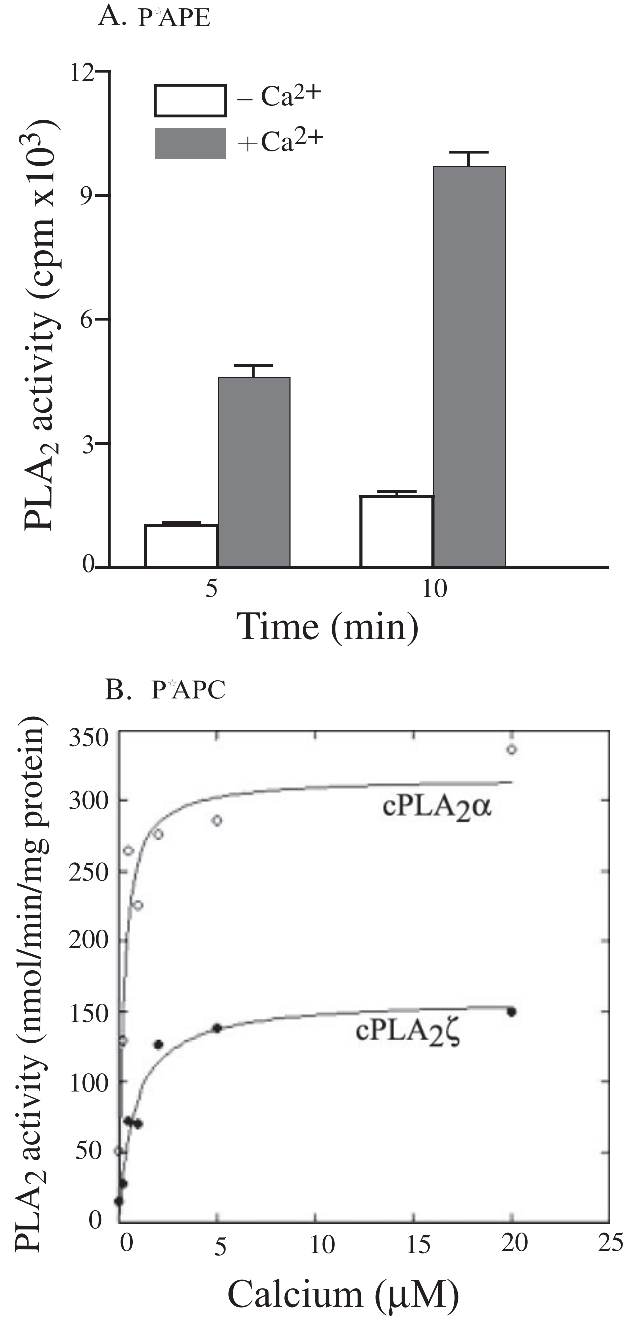

FIGURE 7. **Calcium dependence of cPLA$_2\zeta$ activity.** *A*, PLA$_2$ activity of His$_6$-cPLA$_2\zeta$ was measured with 1-palmitoyl-[^{14}C]arachidonyl-PE in the absence (1 mM EGTA) (*open bars*) or presence (1 mM EGTA with 5 mM CaCl$_2$) of calcium (*gray bars*). Results are the average ± S.D. of two independent experiments. *B*, cPLA$_2\alpha$ and His$_6$-cPLA$_2\zeta$ were assayed using 1-palmitoyl-2-[^{14}C]arachidonyl-PC vesicles as a function of the concentration of free calcium. A representative experiment of triplicate analyses is shown.

tion of eicosanoids, including certain secreted and GVI PLA$_2$s (27, 29, 44, 45). Our study is the first to implicate another member of the GIV PLA$_2$ family as mediating agonist-induced arachidonic acid release and eicosanoid production. We previously reported that lung fibroblasts from the cPLA$_2\alpha$ knockout mouse release arachidonic acid and produce PGE$_2$ in response to A23187 and mouse serum (25). Our data indicates that this is due to the calcium-dependent activation of cPLA$_2\zeta$. The cPLA$_2\zeta$ transcript is highly expressed in mouse thyroid and at lower levels in stomach, large intestine, and prostate (20). The mRNA for cPLA$_2\zeta$ was not detected in whole mouse lung; however, the lung comprises over 20 different cell types, and

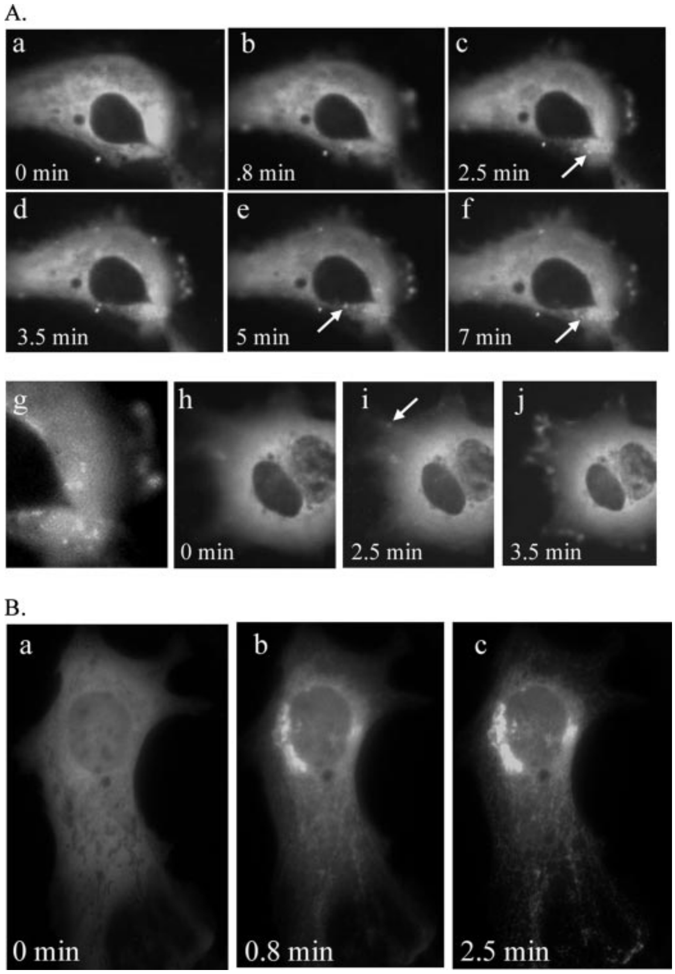

FIGURE 8. **Calcium-induced translocation of EGFP-cPLA$_2\zeta$ and EGFP-cPLA$_2\alpha$ in IMLF$^{-/-}$.** Ionomycin (0.5 μM) was added to IMLF$^{-/-}$ expressing EGFP-cPLA$_2\zeta$ (*A*) or EGFP-cPLA$_2\alpha$ (*B*), and images were taken every 5 s. The *arrows* in *A*, *a–f*, point to vesicles with EGFP-cPLA$_2\zeta$. A movie of this cell can be found in the supplemental data (http://www.jbc.org). An *enlarged portion* of the cell showing translocation of EGFP-cPLA$_2\zeta$ to vesicles 3.3 min after adding ionomycin is shown in *A*, *panel g*. Another cell depicting tranlocation of EGFP-cPLA$_2\zeta$ to ruffles is shown in *A*, *h–j*.

cell type-specific expression of cPLA$_2\zeta$ is possible. Mouse lung fibroblasts contain both cPLA$_2\beta$ and cPLA$_2\zeta$; however, the enzymatic properties of cPLA$_2\zeta$, its ability to mediate calcium-dependent release of arachidonic acid, and its sensitivity to inhibition by pyrrolidine-2 and Wyeth-1 support its role in mediating arachidonic acid release and PGE$_2$ production from cPLA$_2\alpha^{-/-}$ mouse lung fibroblasts. The ability of two structurally different inhibitors, pyrrolidine-2 and Wyeth-1, to block arachidonic acid release from cells and the activity of purified cPLA$_2\zeta$ with similar IC$_{50}$ values is strong evidence supporting a role for cPLA$_2\zeta$ in mediating arachidonic acid release from stimulated IMLF$^{-/-}$.

Our results demonstrate that cPLA$_2\zeta$ and cPLA$_2\alpha$ share similar properties. They both have lysophospholipase, PLA$_1$, and PLA$_2$ activity, they have similar specific activities, and they are activated by low micromolar levels of calcium. The data showing that cPLA$_2\zeta$ and cPLA$_2\alpha$ are both potently inhibited by pyrrolidine-2 and Wyeth-1 complicate the use of these inhibitors to specifically implicate cPLA$_2\alpha$ in mediating arachidonic acid release from cells. The similar inhibitor sensitivity of cPLA$_2\zeta$ and cPLA$_2\alpha$ is in contrast to the results with mouse cPLA$_2\beta$, which is not inhibited by pyrrolidine-2 and Wyeth-1. Human cPLA$_2\beta$ and human cPLA$_2\gamma$ also are not inhibited by pyrrolidine-2 and pyrrolidine-1, respectively (22, 39). The basis for the differences in sensitivity of the GIV PLA$_2$ family to pyrrolidine-2 and Wyeth-1 is not clear at this time.

One difference noted between cPLA$_2\zeta$ and cPLA$_2\alpha$ is that cPLA$_2\zeta$ does not have specificity for arachidonic acid but releases multiple fatty acids, including palmitic, oleic, linoleic, and arachidonic acids, in response to A23187. The release of these fatty acids may be through its PLA$_1$, PLA$_2$, and lysophospholipase activities acting sequentially to deacylate diacylphospholipids. We confirmed that pyrrolidine-2 inhibits the release of oleic and palmitic acids from A23187-stimulated IMLF$^{-/-}$ (data not shown), which is consistent with a role for cPLA$_2\zeta$ in mediating the nonspecific release of fatty acids. Despite the similarities in inhibitor sensitivities, cPLA$_2\zeta$ and cPLA$_2\alpha$ clearly have differences in the active site that influence acyl-chain specificity.

In contrast to cPLA$_2\zeta$, mouse cPLA$_2\beta$ has very low enzymatic activity and only weakly releases arachidonic acid in response to A23187 when expressed in Sf9 cells. We recently reported that human cPLA$_2\beta$ also has low enzymatic activity and that neither mouse nor human cPLA$_2\beta$ is inhibited by cPLA$_2\alpha$ inhibitors (22). Analysis of the mouse cPLA$_2\beta$ transcript present in lung fibroblasts demonstrates that it does not contain the truncated JmjC domain found in the N terminus of human cPLA$_2\beta$. The truncated JmjC domain of human cPLA$_2\beta$ is created by skipping exons 7 and 8 of the cPLA$_2\beta$ gene (4). However, a complete JmjC domain (image clone AAH25390) can be transcribed from the first 8 exons of the human cPLA$_2\beta$ gene (4). This transcript stops prematurely after the first 8 exons of the human cPLA$_2\beta$ gene and thus lacks the C2 and catalytic domains of cPLA$_2\beta$. In contrast separate adjacent genes on mouse chromosome 2 encode a complete JmjC domain and cPLA$_2\beta$. Both mouse and human cPLA$_2\beta$ are similarly situated near the gene cluster encoding cPLA$_2\epsilon$, -δ, and -ζ (4, 20). cPLA$_2\beta$ has very low enzymatic activity when assayed *in vitro*; however, it is possible that it has a unique substrate and/or cofactors *in vivo*. The physiological function and mechanisms of the regulation of cPLA$_2\beta$ remain to be elucidated.

The finding that lung fibroblasts contain three GIV PLA$_2$s suggests that they play different physiological functions and are regulated by distinct mechanisms. This is emphasized by the observation that cPLA$_2\alpha$ and cPLA$_2\zeta$ translocate to different subcellular sites in response to ionomycin. Although we were unable to express mouse EGFP-cPLA$_2\beta$ in IMLF$^{-/-}$ for microscopy, we had previously observed that human cPLA$_2\beta$ does not translocate in response to calcium ionophore but is constitutively localized on mitochondria and early endosomes (22). In addition to differences in localization of cPLA$_2\alpha$ and cPLA$_2\zeta$, the phosphorylation sites found in cPLA$_2\alpha$ are not conserved in cPLA$_2\zeta$. Thus cPLA$_2\zeta$ and cPLA$_2\alpha$ provide differentially regulated pathways for fatty acid release, eicosanoid production, and the regulated turnover of lysophospholipids.

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