The Highly Selective Production of 2-Arachidonoyl Lysophosphatidylcholine Catalyzed by Purified Calcium-independent Phospholipase A_2γ

IDENTIFICATION OF A NOVEL ENZYMATIC MEDIATOR FOR THE GENERATION OF A KEY BRANCH POINT INTERMEDIATE IN EICOSANOID SIGNALING*§

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Herein, we report the heterologous expression of the human peroxisomal 63-kDa calcium-independent phospholipase A_2γ (iPLA_2γ) isoform in Sf9 cells, purification of the N-terminal His-tagged enzyme by affinity chromatography, and the identification of its remarkable substrate selectivity that results in the highly selective generation of 2-arachidonoyl lysophosphatidylcholine. Mass spectrometric analyses demonstrated that purified iPLA_2γ hydrolyzed saturated or monounsaturated aliphatic groups readily from either the sn-1 or sn-2 positions of phospholipids. In addition, purified iPLA_2γ effectively liberated arachidonic acid from the sn-2 position of plasmalogen-containing substrates. In contrast, incubation of iPLA_2γ with 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine resulted in the rapid release of palmitic acid and the selective accumulation of 2-arachidonoyl lysophosphatidylcholine (LPC), which was not metabolized further by iPLA_2γ. The putative regiospecificity of the 2-arachidonoyl LPC product was authenticated by its diagnostic fragmentation pattern during tandem mass spectrometric analysis. To identify the physiological relevance of iPLA_2γ-mediated 2-arachidonoyl LPC production utilizing naturally occurring membranes, we incubated purified rat hepatic peroxisomes with iPLA_2γ and similarly identified the selective accumulation of 2-arachidonoyl LPC. Furthermore, tandem mass spectrometric analysis demonstrated that 2-arachidonoyl LPC is a natural product in human myocardium, a tissue in which iPLA_2γ expression is robust. Because 2-arachidonoyl LPC represents a key branch point intermediate that can potentially lead to a variety of bioactive molecules in eicosanoid signaling (e.g. arachidonic acid, 2-arachidonoylglycerol), these results have uncovered a novel eicosanoid selective pathway through iPLA_2γ-mediated 2-arachidonoyl LPC production to amplify and diversify the repertoire of biologic lipid second messengers in response to cellular stimulation.

Phospholipases A_2 (PLA_2) constitute a large family of enzymes that catalyze the hydrolisis of the sn-2 ester bond of phospholipids to produce free fatty acid and lysophospholipids (1, 2). Previously, a novel human calcium-independent phospholipase A_2 (GenBank accession no. AF263613), termed iPLA_2γ (also known as group VIB phospholipase A_2), has been cloned from the human genome and expressed in Sf9 cells (3, 4). In addition to its dual signature lipase and nucleotide binding motifs that define the iPLA_2 subfamily of enzymes, the iPLA_2γ polypeptide also contains a C-terminal peroxisomal tripeptide localization sequence (-SKL). The first identification of the iPLA_2γ protein from non-recombinant tissues demonstrated that it was present in the peroxisomal fraction of rat liver predominantly as a 63-kDa polypeptide (5). Direct mass spectrometric analysis of the lipidome of hepatic peroxisomes demonstrated the high content of arachidonate-containing choline glycerophospholipid species (5). Thus, these findings suggested that iPLA_2γ might participate in the generation of lipid second messengers through the mobilization of arachidonic acid in response to cellular stimuli. However, because iPLA_2γ did not selectively cleave arachidonate directly from the sn-2 position (3), the molecular mechanism(s) potentially responsible for iPLA_2γ-mediated release of arachidonic acid were not clear.

Historically, the ability of PLA_2s to selectively mobilize arachidonic acid from endogenous phospholipid storage depots has served as an important and defining characteristic in identifying enzymes contributing to eicosanoid-mediated signaling processes. It is well established that cPLA_2α possesses high hydrolytic selectivity toward lipids containing arachidonic acid in the sn-2 position (6–9), making it an important and intensely investigated enzymatic candidate for intracellular eicosanoid signaling studies (10, 11). In addition, calcium-independent PLA_2β (iPLA_2β) has also been shown to participate in the release of arachidonic acid upon cellular stimulation (12–

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1 The abbreviations used are: PLA_2, phospholipase A_2; PLA_1, phospholipase A_1; PLC, phospholipase C; iPLA_2γ, calcium-independent phospholipase A_2γ; cPLA_2α, cytosolic phospholipase A_2; LPC, lysophosphatidylcholine; 2-AG, 2-arachidonoylglycerol; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; PPDPC, 1-palmitoyl-2-docosahexaenoyl-sn-glycerol-3-phosphocholine; BEL, (E)-6-(bromomethylene)-4-(1-naphthalenyl)-2H-tetrahydropyran-2-one; COX, cyclooxygenase; ESI/MS, electrospray ionization mass spectrometry; MALDI/TOF-TOF MS, matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry; DAG, 1,2-diacylglycerol; pFB, pFASTBac.
peroxisomal membranes with purified recombinant iPLA\textsubscript{2}\textgamma. Collectively, these results implicate iPLA\textsubscript{2}\textgamma as a likely proximal mediator of the production of 2-arachidonoyl LPC in vivo, which has the potential to integrate multiple eicosanoid signaling cascades in response to cellular stimulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tonal cobalt-charged affinity resin was purchased from BD Biosciences. 1-Palmitoyl-2-[1\textsuperscript{14}C]oleoyl-sn-glyero-3-phosphocholine ([\textsuperscript{14}C]POPC) was purchased from PerkinElmer Life Sciences. Diacyl-phospholipids, plasmalogents, and phospholipid internal standards for ESI/MS were obtained from Avanti Polar Lipids (Alabaster, AL). Deuterated fatty acid (i.e., 7,7,8,8-d\textsubscript{4}-16:0 fatty acid), used as an internal standard for the quantitation of non-esterified fatty acids, was purchased from Cambridge Isotope Laboratory Inc. (Andover, MA). The ProteoFilter trypsin in-gel digestion kits were purchased from Sigma-Aldrich. High-performance liquid chromatography grade organic solvents and channeled LKGD Silica Gel 60–\textalpha thin layer chromatography plates (Whatman) were obtained from Fisher Scientific. Chloroform and methanol used for lipid extraction were purchased from Burdick & Jackson (Muskegon, MI). SF9 cell culture media and reagents were obtained from Invitrogen. All other chemicals and reagents were typical batches obtained from either Fisher Scientific or Sigma-Aldrich.

**Cloning, Expression, and Affinity Purification of iPLA\textsubscript{2}\textgamma/His\textsubscript{6} from SF9 Cells**—The cDNA encoding the N-terminal His-tagged 63-kDa iPLA\textsubscript{2}\textgamma was prepared as follows: sense primer (5\textsuperscript{\prime}-AAAAATGCGACATCGACATTGCACTACATCTCTCACAAGAAAGAAATGAGAC-3\textsuperscript{\prime}) and antisense primer (5\textsuperscript{\prime}-GATGCCGATGCTCACAATTTTTGAAAAAGATGGAGAAGG-3\textsuperscript{\prime}) were paired to amplify a 1.7-kb product from a full-length iPLA\textsubscript{2}\textgamma pFASTBac1 construct (3) for cloning via Sall/Sphi sites into vector pFASTBac1 (Invitrogen). The Bac-to-Bac Baculovirus Expression System (Invitrogen) was subsequently utilized for bacmid preparation and CellFECTIN/mediated transfection of Spodoptera frugiperda (SF9) cells to produce baculovirus, which was amplified and filtered through the Neutral Red agar overlay method. Expression of the N-terminal His-tagged iPLA\textsubscript{2}\textgamma was initiated by infection of 300 ml of S. frugiperda cells (1 \times 10\textsuperscript{6} cells/ml) with recombinant virus at a multiplicity of infection of \textasciitilde 1. After incubation for 48 h at 27 °C, cells were pelleted by centrifugation, resuspended in ice-cold Grace’s insect medium without serum, and repelleted. The cell pellet was then resuspended in 30 ml of lysis buffer (25 mM sodium phosphate, pH 8.0, containing 0.25 M sucrose, 0.2 mM dithiothreitol, 5 \mu M aprotinin, and 5 \mu M leupeptin), sonicated (20 \times 1 s bursts utilizing a Vibra-cell sonicator at 30% output), and centrifuged at 150,000 \times g for 1 h. The pellet was resuspended in an equal volume of lysis buffer and saved as the membrane fraction. Recombinant iPLA\textsubscript{2}\textgamma was purified from SF9 cell cytosol by using Tonal Co\textsuperscript{58} affinity column chromatography (BD Biosciences) employing an imidazole gradient generated using an Amersham Biosciences fast protein liquid chromatography system. Fractions were monitored for activity utilizing [\textsuperscript{14}C]POPC as substrate as described below. Active samples were flash frozen in liquid nitrogen and stored at \textasciitilde 80 °C.

**Cloning, Expression, and Affinity Purification of iPLA\textsubscript{2}\textbeta/His\textsubscript{6} from SF9 Cells**—Sequence encoding iPLA\textsubscript{2}\textbeta with a C-terminal His tag was generated by PCR amplification of the native iPLA\textsubscript{2}\textbeta cDNA utilizing sense (5\textsuperscript{\prime}-AAAAATGCGACATCGACATTGCACTACATCTCTCACAAGAAAGAAATGAGAC-3\textsuperscript{\prime}) and antisense (5\textsuperscript{\prime}-AAACTCCGTACAGTCACTACATCTCTCACAAGAAAGAAATGAGAC-3\textsuperscript{\prime}) primers containing SalI and XhoI sites, respectively. The PCR product was subcloned into pFASTBac1 for preparation of recombinant baculovirus as described above for iPLA\textsubscript{2}\textgamma/His\textsubscript{6}. Following infection of 300 ml of SF9 cells (1.5 \times 10\textsuperscript{6} cells/ml) with baculovirus encoding iPLA\textsubscript{2}\textbeta/His\textsubscript{6}, for 48 h, cells were harvested by centrifugation (900 \times g for 10 min), washed once with Grace’s insect medium without serum, and resuspended in 30 ml of 25 mM sodium phosphate, pH 7.8, 20% glycerol, 2 mM \beta-mercaptoethanol, 5 \mu M aprotinin, and 5 \mu M leupeptin. After lysing the cells by sonication (30 \times 1 s bursts), the homogenate was centrifuged at 100,000 \times g for 1 h to obtain the cytosol to which NaCl was added to a final concentration of 250 mM. Recombinant iPLA\textsubscript{2}\textbeta in SF9 cell cytosol was purified by using an imidazole column (BD Biosciences) employing an imidazole gradient generated using an Amersham Biosciences fast protein liquid chromatography system. Column fractions were assayed for iPLA\textsubscript{2}\textbeta activity utilizing [\textsuperscript{14}C]POPC as substrate as described below, pooled, and dialyzed overnight against Buffer A (25 mM imidazole, pH 7.8 containing 20% glycerol, 1 mM dithiothreitol, and 1 mM EGTA). The dialyzed sample was applied to a 2.5-ml column of ATPagarose equilibrated with Buffer A and washed with Buffer A contain-
ing 1 mM AMP and 50 mM NaCl. Bound iPLA2(His)_6 was eluted with Buffer A containing 2 mM ATP and 100 mM NaCl, and diazylated against Buffer A (EGTA concentration was reduced to 0.1 mM) containing 50 mM NaCl. Active samples were flash frozen in liquid nitrogen and stored at −80 °C.

Calcium-independent Phospholipase A_2 and Lysophospholipase Activity Assays—Recombinant iPLA2 and iPLA2β were routinely assayed by incubating protein samples with 5 μM [14C]POPC, injected in 2 μl of ethanol, in 200 μl of assay buffer (100 mM Tris, pH 8.0, for iPLA2γ, or 100 mM Tris-HCl, pH 7.2, 1 mM EGTA for iPLA2β) for 0.5−2 min at 37 °C. Reactions were terminated by addition of 100 μl of butanol and vortexing. Products were separated by TLC using petroleum ether/ethyl ether/acetic acid (70:30:1) and fatty acid was quantified by liquid scintillation spectrometry.

For studies employing ESI/MS, phospholipid substrates were co-sedimented to homogeneity with 1,2-dioleoylglycerol (DAG) (37) in the assay buffer. In the assay for cPLA2α, 100 mM Tris acetate containing 5 mM of CaCl2, pH 8.0, was used to replace the EGTA-containing buffer. Lipid substrates were then mixed with phospholipase (0.5−1 μg of purified iPLA2γ or iPLA2β, or 70 μg of cytosolic protein of Sf9 cells expressing cPLA2α) in a total volume of 200 μl, and the final concentrations for phospholipase substrate and DAG were 30 and 10 μM, respectively. The mixture was incubated at 37 °C for the indicated times in the figures. Reactions were terminated by addition of 4 ml of chloroform/methanol (1:1, v/v) containing internal standards, followed by addition of 2 ml of 50 mM aequous LiCl (38) and extraction of lipid species into the chloroform layer by the Bligh and Dyer method (39). The solvent in the lipid extracts was then evaporated using a nitrogen stream, and the samples were redissolved in chloroform and filtered with Millex-FG 0.20-μm filters (Millipore, Bedford, MA). Extracted lipid samples were routinely stored in 200 μl of chloroform/methanol (1:1, v/v) under nitrogen at −20 °C. Hydrolysis of peroxisomal phospholipids catalyzed by iPLA2γ was examined by incubation of 500 ng of purified iPLA2γ with isolated rat liver peroxisomes (5) at 37 °C for the indicated times using identical assay conditions.

Lipase activity was assayed by incubating 1 μg of enzyme (iPLA2γ or iPLA2β), with 50 μM of LPC substrate, injected in 2 μl of ethanol, in 100 μl of assay buffer at 37 °C for 20 min. The reaction was terminated by addition of chloroform/methanol (1:1, v/v), and the products were analyzed by ESI mass spectrometry.

ESI-MS of Phospholipids—ESI-MS analysis was performed using a Thermo Electron TSQ Quantum Ultra spectrometer (San Jose, CA) equipped with an electrospray ion source as previously described in detail (24). Samples were diluted with chloroform/methanol (1:1, v/v, −2 pmol/μl) prior to direct infusion into the ESI source at a flow rate of 2 μl/min with a 2-min period of signal averaging. Phosphatidylcholine and lysophosphatidylcholine molecular species were quantitated by multidimensional mass spectrometry (14:1−14:1 PC and 17:0 LPC as internal standards) after correction for 31P isotopomer differences in the positive ion mode as previously described (38, 42, 43). After addition of the appropriate amount of LiOH in methanol (38, 44), fatty acid species were quantitated by comparison to the deuterated internal standard (7,7,8,8-d4−16:0 fatty acid) in the negative ion mode. Tandem mass scanning of neutral loss of 59 atomic mass units (loss of trimethylamine) was performed at a collision energy of 24 eV and a collision gas pressure of 1.0 mTorr. Product ion spectra of sodiated LPC species were acquired at a collision energy of 30 eV and a collision gas pressure of 1.0 mTorr. The determination of the regiospecificity of LPC species by mass scanning of neutral loss of 59 atomic mass units (loss of trimethylamine) was performed at a collision energy of 24 eV and a collision gas pressure of 1.0 mTorr. The determination of the regiospecificity of LPC species by mass scanning of neutral loss of 59 atomic mass units (loss of trimethylamine) was performed at a collision energy of 24 eV and a collision gas pressure of 1.0 mTorr.

RESULTS

Expression of the 63-kDa iPLA2γ in Sf9 Cells—Previous work demonstrated that expression of the full-length iPLA2γ utilizing a pFASTBac (pFB) promoter proximal to the ATG translation initiation site coding the 88-kDa isoform resulted in a relatively modest level of expression of only the membrane associated 77- and 63-kDa proteins (3). However, truncation of the nested transcriptional inhibitory domain in exon 5 resulted in high levels of expression of the 63-kDa peroxisomal iPLA2γ isoform (Fig. 1) (47). Accordingly, we constructed a baculovirus encoding the 63-kDa iPLA2γ with an N-terminal His tag utilizing a pFB vector and examined the level of expression in Sf9 cells. Western analysis demonstrated a strong immunoreactive band at 63-kDa in both cytosolic and membrane fractions of cells expressing recombinant 63-kDa iPLA2γ, but not in control cells infected with empty pFB vector (Fig. 2A). Other immunoreactive bands were also present, with most predominantly in the membrane fraction, indicating proteolytic degradation and/or other post-translational modifications. The cells expressing the 63-kDa isoform of iPLA2γ showed robust calcium-independent phospholipase A_2 activity in both cytosolic and membrane fractions in comparison to control cell fractions (Fig. 2B). Collectively, these results indicated that the 63-kDa iPLA2γ(His)_6 was successfully expressed in Sf9 cells as a highly active calcium-independent phospholipase A_2.

Affinity Purification of the Cytosolic 63-kDa iPLA2γ from Sf9 Cells—The soluble recombinant 63-kDa iPLA2γ in Sf9 cell cytosol was purified by Talon Co^2+ affinity chromatography utilizing an imidazole gradient (Fig. 3A), which resulted in a purification of iPLA2γ of over 400-fold to a final specific activity of 1.2 μmol/mg protein·min^-1 as assayed by [14C]oleic acid release from [14C]POPC. Multiple attempts at further chromatographic purification were largely unsuccessful in removing the small residual amounts of contaminating proteins. Approaches utilized included a secondary metal affinity step as well as hydrophobic interaction, ion exchange, hydroxylapatite, and other non-metal affinity columns (e.g. heparin). Although iPLA2γ contains a nucleotide binding motif homologous to the sequence that greatly facilitates the purification of iPLA2β (48), iPLA2γ expressed in Sf9 cells did not bind to ATP- or ADP-agarose resins in our hands. SDS-PAGE silver staining revealed the presence of a predominant 63-kDa protein and a minor band at 70-kDa (Fig. 3B). The identities of these proteins were verified by MALDI-TOF/TOF mass spectrometry to be iPLA2γ and the heat shock protein, HSP70, respectively (see Table I and supplemental material).
Inhibition of purified iPLA$_2$$\gamma$ by BEL—The mechanism-based inhibitor, (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL), has been previously shown to inhibit phospholipase $A_2$ activity in crude membrane preparations obtained from Sf9 cells transfected with the full-length iPLA$_2$$\gamma$ coding sequence (3). Subsequent experiments with the $R$- and $S$-enantiomers of BEL utilizing crude membrane fractions revealed that ($R$)-BEL was $\sim$5-fold more potent than ($S$)-BEL (49). We performed similar inhibition experiments with ($R$)- and ($S$)-BEL for the purified soluble 63-kDa iPLA$_2$$\gamma$. Over 80% inhibition of PLA$_2$ activity was manifest with 5 $\mu$m ($R$)-BEL, whereas only 20% inhibition was manifest with the same concentration of ($S$)-BEL (Fig. 4). The $IC_{50}$ for ($R$)-BEL was $\sim$1 $\mu$m. Thus, the enantiomerically selective inhibition of iPLA$_2$$\gamma$ by ($R$)-BEL resulted from the chiral interactions of the BEL molecule with the purified 63-kDa iPLA$_2$$\gamma$ protein in solution and did not result from diastereotopic interactions of the chiral inhibitor with the chiral membrane microenvironment (49).

Mass Spectrometric Determination of the Substrate Selectivity of Purified iPLA$_2$$\gamma$—Initial rate analyses of 1-palmitoyl-2-[1-$^{14}$C]arachidonyl-sn-glycero-3-phosphocholine ([1$^{14}$C]PAPC) hydrolysis catalyzed by the purified 63-kDa iPLA$_2$$\gamma$ resulted in a dramatically lower amount of release of [1$^{14}$C]arachidonic acid, in comparison to release of [1$^{14}$C]oleic acid from [1$^{14}$C]POPC substrate (data not shown). To determine the biochemical mechanisms underlying this observation (e.g. regiospecificity and aliphatic chain selectivity), we utilized the analytical power of ESI/MS and tandem mass spectrometry to concurrently monitor the production of individual metabolites, and loss of substrate during the hydrolysis of PAPC liposomes catalyzed by iPLA$_2$$\gamma$. First, in the positive ion mode, ESI/MS analyses demonstrated that the decreasing peak intensity of PAPC substrate (observed as the sodiated ion, [M+Na$^+$], $m/z$ 804) was highly correlated with the emerging peak intensity of 2-arachidonoyl LPC product ([M+Na$^+$], $m/z$ 566), whereas only diminutive amounts of 1-palmitoyl LPC ([M+Na$^+$], $m/z$ 518) were produced (Fig. 5, A–C). Similarly, spectra acquired in the negative ion mode demonstrated the rapid and near stoichiometric production of palmitic acid (observed as the deprotonated ion, [M-H]$^-$, $m/z$ 255), with only diminutive increases in arachidonic acid ([M-H]$^-$, $m/z$ 303) (Fig. 5, D–F). All metabolites, as well as PAPC, were readily quantitated by comparisons of their ion peak intensities to those of the corresponding internal standards. Thus, the predominant products from the iPLA$_2$$\gamma$-catalyzed PAPC hydrolysis were 20:4 LPC and palmitic acid (Fig. 6), with their molar amounts correlating with the amounts of hydrolyzed PAPC. The initial rate of iPLA$_2$$\gamma$-catalyzed PAPC hydrolysis was 2.4 $\mu$mol$\cdot$min$^{-1}$$\cdot$mg$^{-1}$ based upon the decrease in PAPC peak intensity and the increase in 2-arachidonoyl LPC peak intensity.

The presence of diolein (DAG) in the artificial liposomes was found not to affect the substrate selectivity of iPLA$_2$$\gamma$. Multiple experiments utilizing PAPC liposomes in the absence of DAG showed the same selectivity toward 2-arachidonoyl LPC production, but with $\sim$3-fold less total phospholipase activity (data not shown).

We also examined the hydrolysis of 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (DPDC) catalyzed by iPLA$_2$$\gamma$ in a similar manner. Incubation of DPDC liposomes with iPLA$_2$$\gamma$ resulted in rapid production of 2-docosahexaenoyl LPC ($\sim$1.0 $\mu$mol$\cdot$min$^{-1}$$\cdot$mg$^{-1}$) but only diminutive amounts of palmitoyl LPC were produced. As anticipated, palmitic acid, but not docosahexaenoic acid, was released as the predominant fatty acid, with the amount of palmitic acid released closely corresponding to the amount of 2-docosahexaenoyl LPC produced.

To determine the regiospecificity of the lysolipid products, we employed tandem mass spectrometry, which provides diagnostic fragmentation patterns to discriminate between the sn-1 and sn-2 isomers of LPC as previously described (45). First, 1- and 2-arachidonoyl LPC standards were each prepared by sPLA$_2$ and sn-1-specific lipase treatment of diarachidonoyl-PC, respectively, and the resultant products were subsequently purified by high-performance liquid chromatography as previously described (46). The tandem mass spectra of the sodiated sn-1 and sn-2 arachidonoyl LPC standards ([M+Na$^+$], $m/z$ 566) demonstrated that the relative intensities of the peaks at $m/z$
Fractions from the TALON Co²⁺ affinity column with the highest iPLA₂ activity were concentrated and resolved by 8% SDS-PAGE. Protein bands corresponding to iPLA₂ (≈63 kDa) and HSP70 (≈70 kDa) were subjected to trypsinolysis and subsequent MALDI-TOF/TOF analyses employing an Applied Biosystems 4700 Proteomics Analyzer (for details and sample MS/MS spectra see supplemental materials).

**Table 1**

MALDI TOF/TOF identification of iPLA₂ and copurifying protein, HSP70

| Protein | No. | Calc. mass | Obs. mass | Deviation | Sequence | Ion score | Mod |
|---------|-----|------------|-----------|-----------|----------|-----------|-----|
| iPLA₂   |     |            |           |           |          |           |     |
| 1       | 770.485 | 770.524 | 46 | ALVQLAR | 99.3 |
| 2       | 804.419 | 804.455 | 45 | MQYAIR  | 85.8 |
| 3       | 887.571 | 887.616 | 50 | LIPYLLR | 99.7 |
| 4       | 926.589 | 926.637 | 51 | ALVQLARR | 84.2 |
| 5       | 1662.906 | 1662.889 | 4 | LGSDDVFSQNV10VTK | 100.0 |
| 6       | 1675.840 | 1675.884 | 26 | LKTMYBGLPFFSFK | 70.2 |
| 7       | 1691.835 | 1691.903 | 40 | LKTMYBGLPFFSFK | 69.6 |
| 8       | 1787.921 | 1787.993 | 40 | VEELTHLIFEPFKRK | 100.0 |
| 9       | 1868.867 | 1868.948 | 43 | FNPVCMNPLDESR | 100.0 |
| 10      | 1884.862 | 1884.940 | 41 | FNPVCMNPLDESR | 100.0 |
| 11      | 2168.103 | 2168.175 | 33 | CLMVDPVEPCLVISLGTGR | 100.0 |
| 12      | 2243.023 | 2243.102 | 41 | MSNHSHFYDSQTVENILK | 98.0 |
| 13      | 2514.151 | 2514.234 | 33 | MSNHSHFYDSQTVENILKRD | 91.7 |
| 14      | 2530.146 | 2530.232 | 34 | MSNHSHFYDSQTVENILKRD | 91.9 |
| HSP 70  |     |            |           |           |          |           |     |
| 5       | 1855.969 | 1856.055 | 46 | MDKSQQIDLIVGQSTR | 100.0 |
| 6       | 1952.060 | 1952.141 | 42 | DNNLLGKFGPQIPAPR | 100.0 |
| 7       | 2325.169 | 2325.202 | 36 | APAVGILDLLTSCVQPFQIK | 85.5 |
| 8       | 2758.332 | 2758.399 | 24 | QYQFPYGLVSQDPGVIQFQER | 100.0 |
| 9       | 2981.463 | 2981.523 | 20 | TLSSSTQASIDEISLFLBDIFYTSITR | 100.0 |

*Calculated peptide mass.
*bObserved peptide mass.
*cCovalent modification of peptide.
*dMethionine oxidation.

104 (choline ion, [C₂H₅NO]⁺) and m/z 147 (sodiated cyclic ethylene phosphate, [C₂H₅PO₄Na]⁻) were dramatically different for the two regioisomers (i.e. m/z 104 to m/z 147 ratio ratio is 6:1 for 1-arachidonoyl LPC and 1:7 for 2-arachidonoyl LPC) (Fig. 7, A and B), in accordance with the fragmentation mechanisms identified in previous studies for oleoyllysophosphatidylcholine (45). In comparison, the tandem mass spectrum (MS/MS) of the sodiated 20:4 LPC produced by iPLA₂-catalyzed PACP hydrolysis showed several salient features. First, these tandem mass spectra contained typical peaks characteristic of the phosphocholine head group (i.e. m/z 507, [M+Na⁻]₉; m/z 383, [M+Na⁻]₁₉; m/z 381, [M+Na⁻]₂₀⁻) and m/z 361, [M+Na⁻]₂₀⁻ (50). Second, the relative intensity of the peak at m/z 147 was 5-fold higher than that of the peak m/z 104 (Fig. 7C), indicating that the dominant species of arachidonoyl LPC produced by iPLA₂-catalyzed PACP hydrolysis was the 2-acyl isomer during the entire reaction interval studied (45).

To determine whether the observed sn-1 selectivity of iPLA₂ toward PACP was unique to iPLA₂, we performed ESI/MS analyses of the products of two other prominent intracellular PL₂ enzymes, iPLA₂β and cPLA₂α, under similar conditions. Hydrolysis of PACP liposomes (30 μM) catalyzed by purified iPLA₂β resulted in the production of both 1-palmityl LPC (0.8 μmol·min⁻¹·mg⁻¹) and 2-arachidonoyl LPC (0.3 μmol·min⁻¹·mg⁻¹) with the subsequent release of palmitic and arachidonic acid. This result is in accordance with the previous results from kinetic studies of iPLA₂β (48). To examine the substrate selectivity of cPLA₂α, the cytosolic fraction of Sf9 cells expressing cPLA₂α was assayed employing mass spectrometry in the presence of CaCl₂ utilizing PACP liposomes. Only 1-palmityl LPC, without detectable amounts of 2-arachidonoyl LPC, was produced during incubations of the cytosol of Sf9 cells expressing cPLA₂α, accompanied by stoichiometric amounts of arachidonic acid release (6.7 μmol·min⁻¹·mg⁻¹), which was not observed at comparable initial rates with pFB control cytosol.

To further substantiate the unique molecular selectivity of iPLA₂, hydrolysis of liposomes containing equimolar amounts of PACP and 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) was examined by mass spectrometry. ESI/MS spectra of PC and LPC were acquired in the positive ion mode as a function of time after addition of purified iPLA₂ (Fig. 8, A–C). Spectra were also acquired in the negative ion mode to measure the initial rate of fatty acid release (Fig. 8, D–F). SOPC was hydrolyzed at a similar rate as PACP, as measured by similar overall decreases in the amounts of each substrate as a function of time (Fig. 9A). For SOPC, the intrinsic PLA₁ and PLA₂ activity of iPLA₂ resulted in the production of both 18:1 and 18:0 LPC. Palmitic, stearic, and oleic acids were all rapidly released with
only diminutive amounts of arachidonic acid produced. The degree of accumulation of LPC species followed the rank order 20:4 > 18:1 ~ 18:0 > 16:0 (Fig. 9A), which was closely correlated with the observed release of fatty acid species (Fig. 9B). Collectively, these results indicated that iPLA₂γ is capable of hydrolyzing saturated or mono-unsaturated acyl chains from either the sn-1 or sn-2 position of the phosphatidylcholine species (e.g. SOPC hydrolysis). More importantly, the results from incubations of pure (PAPC or PDPC) and mixed (PAPC/SOPC) phosphatidylcholine liposomal substrates with iPLA₂γ also demonstrated that iPLA₂γ possesses a unique capacity to selectively catalyze the generation of 2-polyunsaturated LPC species, including 2-arachidonoyl LPC.

**Intrinsic PLA₂ and Lyso phospholipase Activity of Purified iPLA₂γ**—To further investigate the substrate selectivity of iPLA₂γ for polyunsaturated acyl chains at the sn-2 position of the substrate, hydrolysis of mixed liposomes containing diacyl and plasmalogen PC molecular species were examined. Liposomes containing equimolar amounts of PAPC and 1-O-1'-[Z]-hexadecenyl-2-arachidonoyl-sn-glycero-3-phosphocholine (16:0–20:4, plasmenylcholine) were incubated with iPLA₂γ, and the accumulation of reaction products was analyzed by ESI/MS. The robust release of arachidonic acid from 16:0–20:4 plasmenylcholine was demonstrated in concert with an increase in the lysoplasmamylcholine peak ([M+Na]+, m/z 502), indicating that iPLA₂γ is capable of effectively hydrolyzing the sn-2 arachidonate ester in plasmalogen substrate (Fig. 10). In contrast, hydrolysis of the diacyl substrate, PAPC, resulted in the accumulation of 2-arachidonoyl LPC and only modest amounts of palmitoyl LPC (Fig. 10). The initial rates of the iPLA₂γ-catalyzed hydrolysis of mixed liposomes containing equimolar amounts of diacyl PAPC and 16:0–20:4 plasmenylcholine were similar for both substrates. Thus, these results demonstrated that purified iPLA₂γ can catalyze the hydrolysis of PC plasmalogen species (based on its intrinsic PLA₂ activity) as well as the hydrolysis of diacyl PC substrates (predominantly from its PLA₂ activity). Moreover, when incubated with arachidonate-containing plasmalogen substrate, unlike with diacyl PAPC, iPLA₂γ is capable of effectively releasing arachidonic acid directly from sn-2 position in a bilayer comprised of mixed phospholipid substrates.

When assayed with 1-palmitoyl LPC substrate, iPLA₂γ showed a lysophospholipase activity of ~120 nmol min⁻¹ mg⁻¹ (Table II). Remarkably, iPLA₂γ hydrolyzed the same concent-

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**FIG. 5.** Novel PLA₂ activity of iPLA₂ γ toward PAPC and accumulation of arachidonoyl LPC. Liposomes containing 1-palmitoyl-2-arachidonoyl-

sn-glycero-3-phosphocholine (PAPC, 30 μM) and 1,2-dioleoylglycerol (DAG, 10 μM) were prepared by sonication, and the lipid substrate was incubated with purified iPLA₂γ (0.5 μg) at 37 °C for the indicated times as described under “Experimental Procedures.” The reaction was terminated by addition of chloroform/methanol (1:1) containing di-14:1 PC, 17:0 LPC, and d4–16:0 fatty acid as internal standards. All lipids were extracted into chloroform by the method of Bligh and Dyer. Lipid extracts were diluted by chloroform/methanol (1:1) to a total lipid concentration of ~2 pmol/μl prior to direct infusion into an ESI source using a syringe pump at a flow rate of 2 μl/min. A 2-min period of signal averaging was employed for spectrum acquisition. A–C, spectra acquired under positive ion mode for samples at the start of hydrolysis (0 min, A) and after 1 min and 5 min of hydrolysis (B and C, respectively). PC and LPC species were detected as their sodiated adduct ions ([M-H]⁻, Na⁺), respectively). Results are representative of three independent experiments.

**FIG. 6.** Time course of PAPC hydrolysis catalyzed by purified iPLA₂γ. Concentrations of PAPC and LPC at indicated time points after addition of iPLA₂γ were calculated from positive ion ESI mass spectra (Fig. 5, A–C) by comparing their sodiated ion peak intensities to those of the corresponding internal standards (di-14:1 PC and 17:0 LPC) after correction for 13C isotopomer differences. Similarly, the concentrations of released fatty acids were calculated from negative ion ESI spectra (Fig. 5, D–F) by comparing the [M-H]⁻ peak intensities of fatty acids to that of the internal standard (d₄–16:0 fatty acid) after correction for 13C isotopomer differences. Results are representative of three independent experiments.
Identification of PLA2γ-catalyzed Accumulation of Arachidonoyl LPC in Naturally Occurring Membrane Systems—Because the localization of the 63-kDa iPLA2γ has previously been documented in hepatic peroxisomes (5), additional experiments were performed to determine if a similar substrate selectivity was present utilizing native peroxisomal membranes. Rat liver peroxisomes were purified by buoyant density gradient centrifugation (5) and were incubated with purified iPLA2γ. Tandem MS spectrometric analyses demonstrated the presence of a small amount of arachidonoyl LPC (m/z 566) in peroxisomes at the beginning of the reaction (Fig. 12A). After the incubation of peroxisomes with iPLA2γ, the arachidonoyl LPC became the predominant LPC species produced during peroxisomal membrane phospholipid hydrolysis (Fig. 12, B and C). Moreover, product ion mass spectrometry of the arachidonoyl LPC product, based on its diagnostic fragmentation patterns, demonstrated that the arachidonoyl moiety was initially present at the sn-2 linkage of the glycerol backbone (data not shown). These results also indicated substantial production of docosahexaenoyl LPC (m/z 590) from the peroxisomes after incubation with iPLA2γ, which was in agreement with the previously determined substrate selectivity of iPLA2γ to generate polyunsaturated lysolipids from bilayer membranes. Thus, these results confirmed that iPLA2γ is able to mediate the selective production of 2-polyunsaturated LPC in its natural membrane context.

**DISCUSSION**

The present study describes the purification of the 63-kDa peroxisomal iPLA2γ and identifies its novel substrate selectivity that results in the selective synthesis of a key branch point metabolite in eicosanoid signaling, 2-arachidonoyl LPC. These studies employed ESI/MS and tandem mass spectrometry to unambiguously identify the regiospecific lipid products, which are difficult to observe utilizing conventional assay methods. Detailed kinetic analyses of the substrate selectivity of highly purified iPLA2γ utilizing both synthetic phospholipids as well as naturally occurring bilayer membrane substrates, which differed in their acyl substituents, phospholipid subclasses, and membrane physical properties, could be achieved by this method. The co-purification of HSP70 and 63-kDa iPLA2γ (Table I) likely reflects the tight association of these two proteins. HSP70 is known to participate in peroxisomal import by regulating the interaction between the peroxisomal targeting signal type 1 of cargo proteins and the peroxisomal targeting signal type 1 receptor (e.g. Pex5p) (51). Because iPLA2γ contains a C-terminal peroxisomal targeting signal type 1 sequence (SKL) and was previously shown to be enriched in peroxisomes (5), it is likely that the import of iPLA2γ into peroxisomes is regulated in part by the molecular chaperone HSP70 in vivo. Purified iPLA2γ readily released saturated and mono-unsaturated fatty acids at nearly equal rates from either the sn-1 or sn-2 position in diacyl phosphatidylethanolamine substrates (e.g. SOPC, Figs. 8 and 9). In contrast, iPLA2γ was largely unable to cleave polyunsaturated fatty acids from the sn-2 position of diacyl phosphatidylethanolamine, and the rapid hydrolysis of the sn-1-saturated fatty acyl ester resulted in the production of 2-polyunsaturated LPC (Figs. 5 and 6). We point out that this observation is unique to iPLA2γ, because purified iPLAβ and the cytosol from S9 cells expressing cPLAα did not demonstrate the same selectivity. Clearly, iPLA2γ showed mixed PLA1/PLA2 activities toward PAPC, and the resultant 1-palmitoyl LPC that is produced appears at an initial rate /H18528 greater than that of the 2-arachidonoyl LPC. The predominant

**Identification of Arachidonoyl LPC as a Natural Product in Human Heart Tissue**—Previously, the mRNA of iPLA2γ was shown to have the highest abundance in human heart tissue in comparison to multiple other tissues (3). Accordingly, to gain insight into the biological relevance of the unique substrate selectivity of iPLA2γ to produce 2-polyunsaturated LPC species, we analyzed human myocardial lipids by tandem mass spectrometry. The mass spectrometric analysis of human heart explant tissue clearly indicated arachidonoyl LPC (m/z 566) as a major constituent in the LPC pool (Fig. 11). Thus, arachidonoyl LPC is a natural product in human myocardium, a prominent site of iPLA2γ gene expression.

**Fig. 7. Identification of the nascent arachidonoyl LPC produced by iPLA2γ as the 2-acyl regioisomer.** Tandem MS spectra of the sodiated adduct ion of high-performance liquid chromatography purified 1-acyl and 2-acyl arachidonoyl LPC (A and B, respectively), and that of the arachidonoyl LPC produced after 2 min of iPLA2γ-catalyzed PAPC hydrolysis (C) were acquired under positive ion mode with a collision energy of 30 eV and a collision gas pressure of 1.0 mTorr. The values of all peaks were rounded to the nearest integer.
products identified from PAPC hydrolysis catalyzed by cytosolic cPLA2 in this report are 1-palmitoyl LPC and arachidonic acid, indicating cPLA2/H9251 is acting exclusively as a PLA2 enzyme toward PAPC in accordance with previous observations (52). By comparison, iPLA2/H9253 catalyzed the production of 2-arachidonyl LPC at least 10-fold faster than that of the 1-palmitoyl LPC (Fig. 6) from PAPC hydrolysis. Cytosolic PLA2/H9253 also has been shown not to function as a PLA1 enzyme (37), although its hydrolytic activity toward phospholipids is calcium-independent (37, 53–55). Multiple PLA1 activities have been previously reported, including the purified PLA1 from bovine brain (56), bonito muscle (57), as well as phosphatidylserine-selective (58) and phosphatidic acid-selective (59) PLA1 enzymes. In contrast to the strict sn-1 regiospecificity and lack of selectivity toward sn-2 acyl groups (56, 57) of the reported PLA1 enzymes, iPLA2/H9253 is distinct from these enzymes in that it possesses both PLA1 (Fig. 5) and PLA2 (Fig. 10) activities, and that it functions predominantly as a PLA1 enzyme in the presence of diacyl PC substrates containing an sn-2 polyunsaturated acyl group (Fig. 5).

The lysophospholipase activity of iPLA2/H9253 toward lysophospholipid substrates (e.g. palmitoyl LPC) with saturated acyl chains was robust (Table II). The substantial lysophospholipase activity of iPLA2/H9253 using palmitoyl LPC was similar to that manifested by iPLA2/H9252 (Table II) and cPLA2/H9251, which hydrolyzes lysolipids rapidly (27). However, although iPLA2/H9253 (Table II) and cPLA2/H9251 (25) readily hydrolyze lysolipids containing arachidonic acid at the sn-2 position, iPLA2/H9253 is inefficient toward these substrates by comparison (Table II). Therefore, cPLA2/H9252 possesses high selectivity for the hydrolysis of either phospholipids (6, 7) or lysophospholipids (25) possessing arachidonic acid at the sn-2 position, whereas iPLA2/H9253 readily hydrolyzes diacyl phospholipids at the sn-2 position except those that contain arachidonic acid or docosahexaenoic acid (Figs. 5 and 8). Thus, accumulation of 2-arachidonoyl LPC during iPLA2/H9253-catalyzed PAPC hydrolysis (Fig. 5) is likely the result of the combined effects of rapid and selective 2-arachidonoyl LPC generation (via PLA1 activity) and its much slower
degradation (via lysophospholipase activity) by iPLA$_2$$gamma$. Although these results identified unanticipated substrate selectivities in model liposome systems, it was equally important to know their relevance in naturally occurring membranes, especially in the context of the native subcellular locations. First, we employed tandem mass spectrometry to demonstrate the presence of 2-arachidonoyl LPC as one of the major naturally occurring LPC molecular species in human heart (Fig. 11), which is a predominant tissue of iPLA$_2$$gamma$. As far as we are aware, this is the first report of a phospholipase that has the capacity to selectively generate 2-arachidonoyl LPC. LPC plays an important regulatory role in cellular signaling cascades as a lipid second messenger, likely functioning through ion channel (60, 61) and G-protein-coupled receptors (62–65), in a broad range of biological processes. More specifi-

**Table II**

| Enzyme  | LPC          | Specific activity | LCP$^a$ | Free fatty acid$^b$ |
|---------|--------------|-------------------|---------|---------------------|
| iPLA$_2$$gamma$  | 1-Palmitoyl   | 125.8 ± 5.9       | 117.5 ± 8.1 |
|         | 1-Arachidonoyl | 23.4 ± 4.0       | 17.3 ± 3.1   |
|         | 2-Arachidonoyl | 6.5 ± 2.8        | 4.2 ± 1.2    |
| iPLA$_2$$beta$  | 1-Palmitoyl   | 174.1 ± 5.2       | 182.9 ± 4.5  |
|         | 1-Arachidonoyl | 153.3 ± 6.0      | 142.8 ± 7.2  |
|         | 2-Arachidonoyl | 98.3 ± 7.5       | 112.1 ± 5.7  |

$^a$ Specific activities were calculated based upon the decrease in the concentration of the different LPC species.

$^b$ Specific activities were calculated based upon the increase in released fatty acid. Values are shown as means ± S.D. (n = 4).

**Fig. 10.** iPLA$_2$$gamma$-catalyzed hydrolysis of mixed liposomes composed of diaely and plasmenyl PC species. Liposomes containing equimolar amounts of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) and 1-O-1'-([Z]-hexadecenyl-2-arachidonoyl-sn-glyceryl-3-phosphocholine (Plas-PAPC) (15 $mu$M each) and DAG (10 $mu$M) were incubated with purified iPLA$_2$$gamma$ (0.5 $mu$g) at 37 °C for the indicated times. All lipids were extracted into chloroform by the method of Bligh and Dyer and the ESI mass spectrometric analyses were performed under the same conditions for PAPC hydrolysis (Fig. 5). A–C, spectra acquired under positive ion mode for samples at the start of hydrolysis (0 min, A) and after 1 min and 5 min of hydrolysis (B and C, respectively). PC and LPC species, including 1-O-1'([Z]-hexadecenyl-2-lyso-sn-glycero-3-phosphocholine (16:0-L-PlasCho), were detected as their sodiated adduct ions ([M+Na]$^+$). D–F, spectra acquired under negative ion mode for samples at the start of hydrolysis (D) and after 1 min and 5 min of hydrolysis (E and F, respectively). Fatty acid species were detected as their deprotonated ion ([M-H]$^-$). Results are representative of two independent experiments.

**Fig. 11.** Arachidonoyl LPC is a natural product in human myocardium. Lipids from end-stage human heart tissue were extracted by the procedure of Bligh and Dyer with modifications as previously described (40) for ESI mass spectrometric analysis. Lipid extracts were diluted by chloroform/methanol (1:1) prior to direct infusion into an ESI source using a syringe pump at a flow rate of 2 $mu$l/min. A 2-min period of signal averaging was employed for spectrum acquisition. Scanning for the neutral loss of 59 atomic mass units in the positive ion mode at a collision energy of 24 eV and a collision gas pressure of 1.0 mTorr was employed to fingerprint LPC species ([M+Na]$^+$). Results are representative of three independent human heart samples.
FIG. 12. Selective accumulation of polyunsaturated LPC during iPLA\textsubscript{2}{γ}-mediated hydrolysis of phosphatidylethanolamine species in isolated rat liver peroxisomes. Rat liver peroxisomes were purified as previously described (5) and were incubated with purified iPLA\textsubscript{2}{γ} (0.5 μg) at 37 °C. Reactions were terminated by addition of chloroform/methanol (1:1, v/v) containing FC, LPC, and fatty acid internal standards. Extraction of lipids into chloroform by the method of Bligh and Dyer and the ESI mass spectrometric analyses were performed under similar conditions to those for PAPC hydrolysis (Fig. 5). Scanning for the neutral loss of 59 atomic mass units in the positive ion mode at a collision energy of 24 eV and a collision gas pressure of 1.0 mTorr was employed to fingerprint LPC species ([M + Na]\textsuperscript{+} at the start of hydrolysis A, and after 10 min of hydrolysis (B). C, increase in the concentrations of LPC species during iPLA\textsubscript{2}{γ}-catalyzed PC hydrolysis of peroxisomal membranes. Concentrations of LPC species at the indicated time points (0 and 10 min) after the addition of iPLA\textsubscript{2}{γ} were calculated by comparing their sodiated ion peak intensities to that of the internal standard (17:0 LPC) after correction for \textsuperscript{13}C isotopomer differences. Values shown are means ± S.D. of separate determinations from three liver samples.

Collectively, evidence from multiple lines of investigation has suggested that 2-arachidonoyl LPC is an important branch point metabolite in cellular eicosanoid metabolism due to the existence of several intracellular catabolic enzymes that can provide a diverse repertoire of biologically active eicosanoid products from this lysolipid (Scheme 1). First, the deacylation of 2-arachidonoyl LPC by highly active lysophospholipases (25) is an efficient way of generating non-esterified arachidonic acid (26). Thus, release of arachidonic acid from the sn-2 position of phospholipids (e.g. PAPC) could result from the sequential actions of the PLA\textsubscript{1} activity of iPLA\textsubscript{2}{γ} followed by the lysophospholipase activity of either cPLA\textsubscript{α}, iPLA\textsubscript{α} (25, 27), iPLA\textsubscript{β} (this study), or other lysophospholipases. Whatever the case, the sequential deacylation reactions catalyzed by iPLA\textsubscript{2}{γ} and lysophospholipase in certain tissues (e.g. heart) would lead to the highly selective release of arachidonic acid in a two-step process exploiting the unusual specificity of iPLA\textsubscript{2}{γ} uncovered in this study. Mass spectrometry unambiguously demonstrated that iPLA\textsubscript{2}{γ} can also hydrolyze phospholipids directly from the sn-2 position using plasmalogens containing arachidonic acid at the sn-2 position as substrate (Fig. 10). The results presented herein demonstrate the versatility of iPLA\textsubscript{2}{γ} in mediating direct and indirect arachidonic acid release in a substrate-dependent manner, i.e. iPLA\textsubscript{2}{γ} hydrolyzes arachidonate-containing plasmalogens or diacyl lipids through PLA\textsubscript{1} or PLA\textsubscript{2} activities, respectively, to release different forms of the signaling arachidonoyl species (i.e. free arachidonic acid or arachidonoyl LPC).

Previous work by Waku et al. (36) has demonstrated the presence of a mammalian lysophospholipase C activity, which can convert 2-arachidonoyl LPC into 2-arachidonoylglycerol (2-AG). The biological role of 2-AG as an endocannabinoid signaling molecule has been demonstrated by the binding of this endogenous lipid metabolite to cannabinoid receptors (28, 29) and by the subsequent initiation of downstream events (30). Moreover, 2-AG is an effective substrate for cyclooxygenase-2 (but not cyclooxygenase-1) (31) and the resultant glycerol-linked prostaglandin derivatives (32) have recently been shown by Marnett and colleagues (33) to have unique signaling functions in RAW 264.7 cells. 2-AG can also serve as substrate for 15-lipoxygenases, resulting in the production of 15-hydroperoxy metabolites that can be utilized for specific signaling functions (34). Thus, it seems likely that iPLA\textsubscript{2}{γ} can participate in the generation of endocannabinoid and glycerol-linked oxygenated eicosanoid metabolites through selective production of 2-arachidonoyl LPC and subsequent conversion of this lysolipid into 2-AG. Finally, 2-arachidonoyl LPC can be transported to other cellular destinations for further metabolism and/or signal transduction functions since the “off-rate” for lysolipids leaving their membrane bilayer of generation greatly exceeds that of fatty acids (66). It has been reported that the uptake of liver derived 2-arachidonoyl LPC from blood may be one of the most important arachidonate sources for extraplastic tissues in rat (67).

Collectively, these results identify multiple biologic products and signaling pathways that can be initiated by iPLA\textsubscript{2}{γ} in mammalian cells through the use of parallel pathways for ligand-stimulated phospholipid hydrolysis for the production of lipid second messengers. The diversity of products generated through this pathway underscore the complexity of possible hydrolytic events contributing to eicosanoid signaling in mammalian cells. Although the direct release of arachidonic acid from the sn-2 position is the most straightforward process, it seems clear that many other competing and complementary pathways contribute to the repertoire of lipid second messen-
The Highly Selective Production of 2-Arachidonoyl Lysophosphatidylcholine Catalyzed by Purified Calcium-independent Phospholipase Aγ: IDENTIFICATION OF A NOVEL ENZYMATIC MEDIATOR FOR THE GENERATION OF A KEY BRANCH POINT INTERMEDIATE IN EICOSANOID SIGNALING

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