Calcium-independent phospholipase A$_2$ (iPLA$_2$) participates in numerous diverse cellular processes, such as arachidonic acid release, insulin secretion, calcium signaling, and apoptosis. Herein, we demonstrate the highly selective iPLA$_2$-catalyzed hydrolysis of saturated long-chain fatty acyl-CoAs (palmitoyl-CoA ≈ myristoyl-CoA ≫ stearoyl-CoA ≫ oleoyl-CoA ≈ arachidonoyl-CoA) present either as monomers in solution or guests in host membrane bilayers. Site-directed mutagenesis of the iPLA$_2$ catalytic serine (S465A) completely abolished acyl-CoA thioesterase activity, demonstrating that Ser-465 catalyzes both phospholipid and acyl-CoA hydrolysis. Remarkably, incubation of iPLA$_2$ with oleoyl-CoA, but not other long-chain acyl-CoAs, resulted in robust stoichiometric covalent acylation of the enzyme. Moreover, S465A mutagenesis or pretreatment of wild-type iPLA$_2$ with (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one unexpectedly increased acylation of the enzyme, indicating the presence of a second reactive nucleophilic residue that participates in the formation of the fatty acyl-iPLA$_2$ adduct. Radiolabeling of intact S9 cells expressing iPLA$_2$ with [H]$^+$oleic acid demonstrated oleoylation of the membrane-associated enzyme. Partial trypsinolysis of oleoylated iPLA$_2$ and matrix-assisted laser desorption ionization mass spectrometry analysis localized the acylation site to a hydrophobic 25-kDa fragment (residues ~400–600) spanning the active site to the calmodulin binding domain. Intriguingly, calmodulin-Ca$^{2+}$ blocked acylation of iPLA$_2$ by oleoyl-CoA. Remarkably, the addition of low micromolar concentrations (5 μM) of oleoyl-CoA resulted in reversal of calmodulin-mediated inhibition of iPLA$_2$ phospholipase A$_2$ activity. These results collectively identify the molecular species-specific acyl-CoA thioesterase activity of iPLA$_2$, demonstrate the presence of a second active site that mediates iPLA$_2$ autoacylation, and identify long-chain acyl-CoAs as potential candidates mediating calcium influx factor activity.

Phospholipases A$_2$ (PLA$_2$s) catalyze the hydrolysis of ester-linked fatty acids from glycerophospholipids, thereby regulating cellular signaling pathways through the generation of lysophospholipids, free fatty acids (e.g. arachidonic acid), and their downstream metabolites. In eukaryotes, PLA$_2$s are broadly categorized into three families: secretory, cytosolic, and calcium-dependent phospholipases A$_2$ (iPLA$_2$). Secretory PLA$_2$s are low molecular mass (~12–15 kDa) extracellular enzymes that require high micromolar to millimolar concentrations of Ca$^{2+}$ for catalysis (2, 3). Six cytosolic phospholipases A$_2$ (α, β, γ, δ, ε, and ζ) have been characterized at present, five of which (α, β, δ, ε, and ζ) contain C2 domains that require submicromolar Ca$^{2+}$ for membrane association (4–7). Calcium-independent PLA$_2$s are intracellular, do not require calcium ion for membrane association or catalysis, and currently comprise seven family members (α, β, γ, δ, ε,ζ, η) (8–11), all of which contain conserved nucleotide-binding (GXXGXXG) and lipase (GXGXXG) sequence motifs. Studies of calcium-independent phospholipase A$_2$ have revealed its importance in agonist-induced arachidonic acid release (12, 13), apoptosis (14, 15), lymphocyte proliferation (16), fat cell differentiation (17), insulin secretion (18), and lysolipid production mediating capacitative calcium influx (19, 20). In addition, recent experiments utilizing transgenic mice selectively overexpressing iPLA$_2$ in myocardium have provided evidence that cardiac ischemia activates iPLA$_2$, precipitating ventricular tachyarrhythmias, which can be suppressed by pretreatment with the mechanism-based iPLA$_2$ inhibitor, (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL) (21). Previously, we have demonstrated that iPLA$_2$,β activity is regulated through calmodulin-mediated inhibition of phospholipase A$_2$ activity in the presence of physiologic concentrations of calcium (~200 nM) (22). Subsequent experiments identified the calmodulin binding domain of iPLA$_2$, containing “1-9-14” and IQ sequence motifs, located ~160 and 240 amino acid residues, respectively, from the active site (23). During cellular stimulation and the depletion of intracellular Ca$^{2+}$ stores, activation of iPLA$_2$,β has been proposed to occur through dissociation of the iPLA$_2$,β-CaM complex (13, 20), potentially through the actions of a low molecular weight cellular component known as calcium influx factor (CIF) (20, 24). Although CIF was first described and partially characterized more than 10 years ago as a diffusible messenger released upon intracellular Ca$^{2+}$ store depletion, which stimulated Ca$^{2+}$ influx through the plasma membrane (25, 26), the precise molecular identity of CIF is unknown.

Multiple fatty acyl-CoA thioesterases have been purified from mammalian cytosol, peroxisomes, and mitochondria and have been cloned.
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and characterized with respect to substrate selectivity, enzyme kinetics, and sensitivity to various inhibitors (27). In general, fatty acyl-CoA thioesterases have been classified as those that are induced by peroxisome proliferators (Type-I or Type-II thioesterases) and those that do not share significant sequence homology with these isoforms (27). Several other mammalian enzymes, such as lysophospholipases (28), secretory phospholipase A₂ (29), and palmitoyl-protein thioesterases (30–32), have also been shown to exhibit acyl-CoA hydrolase activity. Interestingly, hepatocyte nuclear factor-4α has been recently demonstrated to hydrolyze fatty acyl-CoAs, followed by binding of the fatty acid product to hepatocyte nuclear factor-4α, thereby allowing cross-talk between the acyl-CoA and free fatty acid binding domains (33).

In addition to its regulation by calmodulin, iPLA₂β binds ATP through a conserved nucleotide binding motif (GXGXXG), resulting in both enzyme stabilization and activation (34, 35). From this perspective, we considered the possibility that iPLA₂β might also bind and hydrolyze long-chain acyl-CoAs, given the structural similarity of the 3’-phosphoadenosine moiety of CoA to ATP. In this paper, we demonstrate that iPLA₂β catalyzes the hydrolysis of saturated long-chain acyl-CoAs present as either monomers or as guests in host membrane vesicles at physiologic concentrations (1–5 mol %). Moreover, highly selective autocytolysis of iPLA₂β by oleoyl-CoA occurred at a second nucleophilic site(s) within the catalytic domain, which is protected from oleoylation by Ca²⁺-activated calmodulin. Finally, oleoyl-CoA was found to attenuate calmodulin-mediated inhibition of iPLA₂β phospholipase A₂ activity. In summary, these results describe the previously unrecognized fatty acyl-CoA thioesterase activity and fatty acyl-CoA dependent covalent acylation of iPLA₂β, thereby revealing additional levels of complexity in this multifunctional signaling enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1-14C]palmitoyl-CoA, [1-14C]oleoyl-CoA, and 1-palmitoyl-2-[1-14C]oleoyl-sn-glycero-3-phosphocholine were obtained from PerkinElmer Life Sciences. [1-14C]Arachidonoyl-CoA, [1-14C]myristoyl-CoA, [1-14C]stearoyl-CoA, and [methyl-14C]human albumin were obtained from American Radiolabeled Chemicals. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phospho-i-serine (DOPS) were purchased from Avanti Polar Lipids. Sf9 cell culture reagents and 2-decanoyl-1-(O-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)undecyl-sn-glycero-3-phosphocholine (BODIPY-PC) were purchased from Invitrogen. High purity bovine calmodulin was obtained from Calbiochem. Most other materials were obtained from either Sigma or Fisher. BEL was purchased from Cayman Chemical and separated into individual enantiomers as described previously (36).

**Construction of Recombinant Baculoviruses Encoding His-tagged Wild-type and S465A Mutant iPLA₂β Proteins**—cDNA encoding wild-type *C. griseus* (Chinese hamster) iPLA₂β (9) was used as template for PCR amplification of the sequence to introduce six in-frame His codons followed by a stop codon and XhoI restriction site (3′ primer) for subcloning into pFASTBac1. The 5′ primer contained a Sall site and Kozak sequence (GCCACC) 5′ of the ATG start codon. The His-tagged S465A iPLA₂β construct was created by PCR amplification of the mutant cDNA (9) utilizing a 5′ primer containing an EcoRI restriction site and a 3′ primer encoding a His₉ tag followed by a Sall site for subcloning into pFASTBac1. The 2.4-kb products were each inserted into a bacmid shuttle vector and sequenced in both directions to confirm the integrity of the constructs. *S. frugiperda* (Sf9) cells in 35-mm plates containing supplemented Grace’s medium were transfected and incubated at 27 °C for 72 h to produce high titer baculoviral stocks for infection of Sf9 cells.

**Expression and Affinity Purification of iPLA₂βHis₉ from Sf9 Cells**—Sf9 cells were grown as 100 ml of suspended cultures (in 1-liter plastic bottles) in supplemented Grace’s insect medium containing 10% heat-inactivated fetal bovine serum and 0.1% Pluronic F-68. Following infection of 3 X 10⁶ cells/ml with baculovirus encoding iPLA₂βHis₉ for 48 h, cells were harvested by centrifugation (250 x 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 β-ME, 5 μg/ml aprotinin, 5 μg/ml leupeptin. After lysing the cells by sonication (30 1-s bursts), the homogenate was centrifuged at 100,000 × g for 1 h to obtain the cytosol, to which NaCl was added to a final concentration of 250 mM. The cytosol was then mixed by inversion with 3 ml of HIS-Select-Co₂⁺ affinity resin (Sigma) for 1 h, and the cytosol-resin suspension was poured into an Amersham Biosciences 1 × 10-cm column. Following washing of the settled resin with 30 ml of Buffer A (25 mM sodium phosphate, pH 7.8, containing 500 mM NaCl, 20% glycerol, and 2 mM β-ME), bound protein was eluted from the column at a flow rate of 0.25 ml/min utilizing a 250 mM imidazole gradient in Buffer A (50-ml total volume) generated using an Amersham Biosciences fast protein liquid chromatography system. Column fractions were assayed for iPLA₂ activity as described below, pooled, and dialyzed overnight against Buffer B (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 ATP-agarose equilibrated with Buffer B and washed with Buffer B containing 1 mM AMP and 50 mM NaCl. Bound iPLA₂βHis₉ was eluted with Buffer B containing 2 mM ATP and 100 mM NaCl, dialyzed against Buffer B (EGTA concentration was reduced to 0.1 mM) containing 100 mM NaCl, flash frozen in liquid nitrogen, and stored at —80 °C. Approximately 1 mg (65% yield) of iPLA₂βHis₉, with a specific activity of 0.5 μmol of oleic acid/min/mg, utilizing 5 mM [14C]POPC as substrate, was typically recovered from 300 ml of cultured Sf9 cells by this procedure.

**Phospholipase A₂ Activity and Acyl-CoA Hydrolysis Enzymatic Assays**—Purified recombinant iPLA₂βHis₉ (0.1–1 μg) was incubated with radiolabeled phospholipid or acyl-CoA in 25 mM Tris-HCl, pH 7.2, containing 1 mM EGTA (200 μl final volume) for 1–2 min at 37 °C. In experiments using acyl-CoAs as guests in host phospholipid bilayers, radiolabeled acyl-CoAs were incorporated into POPC/DOPS (90:10 mol %) vesicles (100 μM) before addition to the reaction mix. Long-chain acyl-CoAs have been previously demonstrated to integrate into lipid bilayers within seconds (37). Reactions were terminated by extraction of the released radiolabeled fatty acids into 100 μl of butanol, separation of fatty acids from unreacted substrate by thin layer chromatography, and quantitation by scintillation spectroscopy as previously described (38). For experiments examining the effects of acyl-CoAs on calmodulin-mediated inhibition of iPLA₂β, phospholipase A₂ activity was continuously measured utilizing a SPECTRAMax GEMINI XS Dual-Scanning Microplate Spectrofluorometer (Molecular Devices). BODIPY-PC substrate (1.17 mM in Me₂SO, 5 μM final concentration) was co-incubated (10 min at 40% power, 50% duty cycle) with POPC (95 μM final concentration) in 25 mM HEPES, pH 7.2. Oleoyl-CoA and CaCl₂ were added at the indicated concentration to the lipid vesicles before addition to iPLA₂β with or without CaM (preincubated on ice for 10 min) present in individual wells of a black 96-well microtiter plate. Fluorescence readings were acquired at 15-s intervals for 2 min at 37 °C utilizing excitation/emission wavelengths of 495/515 nm, respectively.

**Electrospray Ionization (ESI)/Mass Spectral (MS) Analyses**—Purified iPLA₂β (0.25 μg) was incubated with POPC (95 μM) vesicles containing BODIPY-PC (5 μM) for 5 min at 37 °C as described above. The reactions were stopped by the addition of 4 ml of chloroform/methanol (1:1, v/v)
containing internal standards (i.e. 14:1–14:1 PC and 17:0 LPC), and the lipid species were extracted into the chloroform layer as described previously (39). Extracted lipid samples were filtered with Millipore 0.20-μm filters (Millipore, Bedford, MA) and were routinely stored in 200 μl of chloroform/methanol (1/1, v/v) under nitrogen at −20 °C. ESI/MS analysis was performed using a Thermo Finnigan TSQ Quantum Plus spectrometer (San Jose, CA) equipped with an electrospray ion source as previously described in detail (40, 41). Samples were diluted with chloroform/methanol (1:1, v/v) (−5 pmol/μl) prior to direct infusion into the ESI ion source at a flow rate of 4 μl/min with a 2-min period of signal averaging. 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 millitor.

**Covalent Modification of iPLA2β with 14C-labeled Long-chain Acyl-CoAs—**Purified recombinant iPLA2βHis6 was incubated with POPC vesicles (90 μM) containing 10 mol % [1-14C]acyl-CoA for 1 h at 37 °C. In some experiments, iPLA2βHis6 was preincubated with BES (3 min at 23 °C), N-ethylmaleimide (5 min at 30 °C) or iodoacetamide (5 min at 30 °C) prior to the addition of radiolabeled acyl-CoA. Chloroform/methanol precipitation of some samples was performed as described (42), utilizing 15 μg of bovine serum albumin as carrier. In experiments to examine the nature of the covalent linkage between oleic acid and iPLA2β, acid (HCl), base (NaOH), and hydroxylamine were added to the indicated concentrations, and the samples were incubated at 30 °C for 1 h. Bovine serum albumin (15 μg) and SDS-PAGE loading buffer were then added to each sample prior to dialysis against 50 mM Tris-HCl, pH 6.8, containing 10% glycerol and 1% SDS for 4 h. Samples were electrophoresed by SDS-PAGE, fixed (40% methanol containing 10% acetic acid), stained with Coomassie Blue R-250, incubated in fixation solution. Gels containing the radiolabeled peptide fragments were soaked in Amplify fluorogenic reagent (Amersham Biosciences), dried, and exposed to Eastman Kodak Co. Biomax MR film for 2–5 days at −80 °C.

**Partial Trypsinolysis of Oleoylated iPLA2β—**Purified iPLA2βHis6 (10 μM) was incubated with 50 μM [1-14C]oleoyl-CoA or unlabeled oleoyl-CoA in 25 mM imidazole, pH 7.8, containing 50 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, and 20% glycerol for 1 h at 37 °C. Excess [1-14C]oleoyl-CoA was removed by using a Micro Bio-Spin (Bio-Rad) column equilibrated with the above buffer. Recovered iPLA2β was partially digested with trypsin (1:25, w/w) for 1–30 min at 37 °C. Tryptic peptides were separated by SDS-PAGE, fixed in 40% methanol, 10% glacial acetic acid, stained with Coomassie Blue, and destained in the fixation solution. Gels containing the radiolabeled peptide fragments were soaked in Amplify fluorogenic reagent (Amersham Biosciences), dried, and exposed to film. In parallel experiments utilizing unlabeled oleoyl-CoA, the band corresponding to the ~25-kDa radiolabeled fragment was excised, cut into ~1 × 1-mm pieces, and destained further by washing with 50% acetonitrile at 37 °C. The gel pieces were then dried in a SpeedVac, resuspended in 50 mM ammonium bicarbonate (100 μl) containing 0.5 μg of sequencing grade modified trypsin (Promega), and incubated for 12 h at 37 °C. After aliquoting the supernatant solution to a separate tube, residual peptides in the gel pieces were extracted into 50% acetonitrile, 20% isopropyl alcohol, 0.1% trifluoroacetic acid, combined with the supernatant solution, and concentrated utilizing a SpeedVac.

**MALDI-TOF Analysis of iPLA2β Tryptic Fragments—**Concentrated peptide samples were diluted with 0.5% trifluoroacetic acid, absorbed to a C18 Zip-Tip (Millipore), and desorbed with a solution composed of 50% acetonitrile, 20% isopropyl alcohol, 0.1% trifluoroacetic acid, and containing in addition 5 mg/ml α-cyano-4-hydroxycinnamic acid. Samples were applied to 192-spot sample plates (ABI) and allowed to air-dry. MS analyses were performed utilizing an Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA), which possesses a 200-Hz Nd:YAG laser operating at 355 nm. The mass accuracy of the instrument was externally calibrated to the 4700 Proteomics analyzer calibration mixture of peptides. For MALDI-MS analysis, spectra were obtained by the accumulation of 2500 consecutive laser shots at a collision energy of 1 kV with air serving as the collision gas. Calculations of predicted peptide and peptide fragment masses were performed using programs developed at the University of California, San Francisco, Mass Spectrometry Facility (available on the World Wide Web at prospector.ucsf.edu).

**Metabolic Labeling of iPLA2β in Intact Sf9 Cells—**Cultures (100 ml) of Sf9 cells (1.5 × 10⁶ cells/ml) were infected with control (pPB empty vector) or iPLA2βHis6-encoding baculovirus and incubated at 27 °C for 45 h. The cells were then pelleted by centrifugation (250 × g for 10 min), resuspended in 10 ml of Grace’s insect medium (Sigma) containing 1% heat inactivated fetal bovine serum, 0.1% Pluronic F-68, and 1 μCi of [1-14C]oleic acid. Following incubation at 27 °C for 3 h, cells were harvested by centrifugation (250 × g for 10 min) and resuspended in 5 ml of 25 mM sodium phosphate, pH 7.8, containing 20% glycerol, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. After sonication, cytosol and membrane fractions were separated by ultracentrifugation as described above.

**Other Procedures—**SDS-PAGE was performed according to Laemmli (43). ECL Western analyses for iPLA2βHis6 were performed utilizing a monoclonal anti-His antibody (BD Biosciences) in conjunction with an anti-mouse IgG-horseradish peroxidase conjugate. Silver staining of SDS-polyacrylamide gels was performed as described (44). Protein concentration was determined by a version of the Bradford protein assay (Bio-Rad) with bovine serum albumin as a standard.

**RESULTS**

**iPLA2β Hydrolyzes Palmitoyl-CoA and Is Inhibited at Submicellar Concentrations of Substrate—**Due to the structural similarity between ATP and the 3’-phosphoadenosine moiety of CoA, we hypothesized that iPLA2β could bind to, and potentially hydrolyze, the thioester linkage of long-chain fatty acyl-CoAs. Accordingly, we overexpressed iPLA2βHis6 in Sf9 cells and purified the enzyme to apparent homogeneity (as determined by SDS-PAGE/silver staining) by sequential cobalt and ATP affinity chromatographies as described under “Experimental Procedures.” Initial assays with iPLA2β utilizing supramicellar concentrations of palmitoyl-CoA (100 μl) typically used for acyl-CoA thioesterases revealed very low rates of hydrolysis (Fig. 1A). Remarkably, robust palmitoyl-CoA thioesterase activity was demonstrated at low micromolar concentrations of substrate with a maximal rate of ~250 nmol of palmitic acid/min/mg at 25.5 μM palmitoyl-CoA (Fig. 1A). Similar acyl-CoA-mediated substrate inhibition was observed in previous studies of a purified rabbit heart mitochondrial thioesterase (45) and peroxisomal acyl-CoA thioesterase 2 (46). It should be noted that significant inhibition of iPLA2β by palmitoyl-CoA occurs below the critical micelle concentration (10–20 μM) (47) and implies the presence of a second acyl-CoA binding site on the enzyme (i.e. SES intermediate) (48).

**iPLA2β Catalyzes the Highly Selective Hydrolysis of Myristoyl-CoA and Palmitoyl-CoA Present as Guests in Host Phospholipid Bilayers—**Since iPLA2β would probably be expected to encounter acyl-CoAs in a membrane bilayer in vivo, we examined whether the enzyme could hydrolyze palmitoyl-CoA present as a guest (at a low mol %) in phospholipid host vesicles. Purified iPLA2β effectively hydrolyzed palmitoyl-CoA at physiologically relevant concentrations of acyl-CoA (i.e. 1–5 mol %) present in POPC/DOPS vesicles (Fig. 1B). This was surprising, since POPC is an excellent substrate for iPLA2β and would probably be expected to encounter acyl-CoAs in a membrane bilayer in vivo, we examined whether the enzyme could hydrolyze palmitoyl-CoA present as a guest (at a low mol %) in phospholipid host vesicles. Purified iPLA2β effectively hydrolyzed palmitoyl-CoA at physiologically relevant concentrations of acyl-CoA (i.e. 1–5 mol %) present in POPC/DOPS vesicles (Fig. 1B). This was surprising, since POPC is an excellent substrate for iPLA2β and would be expected to efficiently compete with the palmitoyl-CoA as substrate. To deter-
Selective \( \text{iPLA}_2 \beta \) Acyl-CoA Hydrolysis and Autoacylation

![FIGURE 1. Palmitoyl-CoA thioesterase activity of \( \text{iPLA}_2 \beta \). A, substrate inhibition of \( \text{iPLA}_2 \beta \) palmitoyl-CoA hydrolytic activity. B, marked preference for hydrolysis of saturated acyl-CoA substrates (14–16 carbon length) in comparison with unsaturated acyl-CoA molecular species (C18:1 and C20:4) in the presence of membrane bilayers.](image)

![FIGURE 2. Substrate selectivity of \( \text{iPLA}_2 \beta \) long-chain acyl-CoA hydrolysis activity in the presence of phospholipid vesicles.](image)

Effect of \( \text{pH} \) and Determination of the Active Site Nucleophile Mediating Palmitoyl-CoA Hydrolysis—Hydrolysis of either POPC or palmitoyl-CoA was examined over a wide \( \text{pH} \) range (5.5–8.5) to determine potential mechanistic differences between the two substrates relative to the surrounding hydrogen ion concentration. A plateau of maximal activity was observed with both substrates between \( \text{pH} \) 6.5 and 7.5, indicating a similar \( \text{iPLA}_2 \beta \)-mediated hydrolytic mechanism for POPC and palmitoyl-CoA.

Site-directed mutagenesis of the lipase catalytic serine (GTSGKV in \( \text{iPLA}_2 \beta \) to alanine has been previously demonstrated to ablate PLA2 activity (10). To determine if Ser-465 was equally crucial for \( \text{iPLA}_2 \beta \) acyl-CoA hydrolytic activity, we generated and purified the identical mutant (S465A) and compared the PLA2 and palmitoyl-CoA hydrolytic activities with those of its wild-type counterpart. Importantly, the S465A \( \text{iPLA}_2 \beta \) His<sub>6</sub> bound to ATP-agarose (as determined by Western analysis), indicating that the mutant protein was properly folded around the nucleotide binding site (G31GGKVGGK<sup>436</sup>), which is ~30 amino acid residues away from the lipase site. As expected, calcium-independent PLA2 activity was abolished in the S465A mutant utilizing POPC as substrate (Fig. 3A). Hydrolysis of palmitoyl-CoA incorporated into POPC/DOPS vesicles was also virtually eliminated in the S465A mutant (Fig. 3A), indicating that the \( \text{iPLA}_2 \beta \) active site serine hydroxyl probably serves as the primary nucleophile for both phospholipase A<sub>2</sub> and acyl-CoA thioesterase reactions catalyzed by \( \text{iPLA}_2 \beta \).

Chiral Mechanism-based Inhibition of Acyl-CoA Hydrolysis by (R)- and (S)-BEL—In previous work, we have demonstrated that racemic BEL potently inhibits both \( \text{iPLA}_2 \beta \) (IC<sub>50</sub> ~ 0.2 \( \mu \)M) and \( \text{iPLA}_2 \gamma \) (IC<sub>50</sub> ~ 3 \( \mu \)M) phospholipase A<sub>2</sub> activities (11, 49, 50). Through resolving the enantiomers of BEL by chiral high pressure liquid chromatography, we have further shown that (S)- and (R)-BEL are selective for \( \text{iPLA}_2 \beta \) and \( \text{iPLA}_2 \gamma \), respectively (36). To determine if (R)- and (S)-BEL had similar effects on \( \text{iPLA}_2 \beta \)-palmitoyl-CoA hydrolytic activity, \( \text{iPLA}_2 \beta \) His<sub>6</sub> was preincubated with each BEL enantiomer prior to the addition of radio-labeled palmitoyl-CoA incorporated into POPC/DOPS bilayers. As seen in Fig. 3B, (S)-BEL inhibited \( \text{iPLA}_2 \beta \) palmitoyl-CoA hydrolytic activity with an IC<sub>50</sub> of ~0.1 \( \mu \)M, whereas (R)-BEL was ~8-fold less effective (IC<sub>50</sub> = 0.8 \( \mu \)M). Thus, the selectivity of the BEL enantiomers for inhibiting \( \text{iPLA}_2 \beta \) palmitoyl-CoA thioesterase activity is virtually identical to that previously observed for inhibition of \( \text{PLA}_2 \) activity (36). Collectively, these results suggest that both long-chain acyl-CoA and phospholipid substrates utilize the same mechanism and hydrolytic site (binding domain and catalytic residue(s)) in \( \text{iPLA}_2 \beta \) for catalysis.

Identification of Specific Autoacylation of \( \text{iPLA}_2 \beta \) by Oleoyl-CoA—Since prior work has demonstrated that various proteins, such as rhodopsin (51), G-protein \( \alpha \) subunits (52, 53), and protein kinase C (54) are autoacylated in the presence of palmitoyl-CoA, we sought to determine if \( \text{iPLA}_2 \beta \) could become similarly acylated in the presence of saturated and unsaturated long-chain acyl-CoA substrates. Remarkably, although incubations with [1-<sup>14</sup>C]myristoyl-CoA, [1-<sup>14</sup>C]palmitoyl-CoA, and [1-<sup>14</sup>C]stearoyl-CoA displayed either no observable or only diminutive acylation of \( \text{iPLA}_2 \beta \) following SDS-PAGE, those containing [1-<sup>14</sup>C]oleoyl-CoA resulted in 10–100-fold greater radiolabeling of \( \text{iPLA}_2 \beta \) in comparison (Fig. 4A). Furthermore, incubations with [1-<sup>14</sup>C]arachidono-
Acyl-CoA Hydrolysis and Autoacylation

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FIGURE 3. Elimination of iPLA2β palmitoyl-CoA hydrolase activity by site-directed mutagenesis of Ser-465 and inhibition of iPLA2β palmitoyl-CoA hydrolase activity by (R)- and (S)-BEL. A, equivalent amounts of purified iPLA2βH11001, iPLA2βH11001 (546SA) were incubated with either 1-palmitoyl-2-[1-14C]oleoyl-sn-glycero-3-phosphocholine (5 μM) or 1-14Cpalmitoyl-CoA (5 μM) for 2 min at 37 °C. Radiolabeled fatty acids from the reaction were extracted into butanol, resolved by TLC, and quantified by liquid scintillation spectrometry as described under “Experimental Procedures.” Results represent the average ± S.E. of four separate determinations. B, purified iPLA2βH11001 was preincubated with the indicated concentrations of either enantiomer of BEL or ethanol vehicle for 5 min at 37 °C in 100 mM Tris-HCl, pH 7.2, containing 1 mM EDTA. [1-14C]palmitoyl-CoA guest (5 μM) in POPC (1 μM) host vesicles was added to the samples, which were incubated for 2 min at 37 °C and processed as described above. Results are representative of three separate experiments performed in duplicate.

Effects of chemical treatments on oleoylated iPLA2β

Selective iPLA2β acylation with oleoyl-CoA resulted in ~10-fold-less signal intensity than with [1-14C]oleoyl-CoA, but iPLA2β was still arachidonoylated under these conditions (Fig. 4A, top). To our surprise, similar experiments with S465A iPLA2β displayed a shift in the selectivity of acylation (i.e. autoacylation was greatest with stearoyl-CoA, labeling with palmitoyl-CoA became clearly detectable, and the enzyme exhibited reduced reactivity with oleoyl-CoA in comparison with wild-type iPLA2β (Fig. 4A, middle). Similar acylation experiments with BEL-pretreated iPLA2β revealed marked increases in acylation with palmitoyl-CoA and stearoyl-CoA that were not observed with the wild-type protein (Fig. 4A, bottom).

Thus, inactivation of the catalytic site through either site-directed mutagenesis or pretreatment with BEL does not abolish iPLA2β acylation, supporting the existence of a second active site, which catalyzes autoacylation.

In order to determine the stoichiometry of iPLA2β acylation with oleoyl-CoA, we compared the radiolabeling intensity of iPLA2β incubated with increasing amounts of [1-14C]oleoyl-CoA with that of a standard curve of [1-14C]methyl-human serum albumin of known specific activity (Fig. 4B). Approximately 1 mol of [1-14C]oleic acid was incorporated per mol of iPLA2β in the presence of POPC vesicles containing up to a 5-fold molar excess of [1-14C]oleoyl-CoA relative to iPLA2β. One potential consequence of iPLA2β oleoylation is alteration of catalytic activity, either toward phospholipid or acyl-CoA substrates. To address this possibility, iPLA2β was incubated with or without oleoyl-CoA and then purified by Co2⁺ affinity chromatography to remove residual oleoyl-CoA. Results from these experiments indicated that oleoylation did not significantly affect either iPLA2β-catalyzed POPC or palmitoyl-CoA hydrolysis (data not shown). Thus, iPLA2β autoacylation with oleoyl-CoA occurs at a site that does not block accessibility of substrate or inhibit release of products from the active site.

To further establish that the modification of iPLA2β with oleoyl-CoA was covalent, [1-14C]oleoyl-iPLA2β was precipitated with CHCl3/CH3OH and extensively washed with 70% acetone before SDS-PAGE (Fig. 4C). This treatment did not result in an appreciable decrease in signal intensity, indicating that the [1-14C]oleate is covalently bound to iPLA2β. Since esterification of fatty acids to proteins has been shown to occur through either amide, oxyster, or thioester bonds that can be distinguished through chemical treatment with strong acid (HCl), strong base (NaOH), or neutral hydroxylamine, additional experiments...
Selective iPLA$_2{\beta}$ Acyl-CoA Hydrolysis and Autoacylation

were performed to determine the nature of the covalent linkage. In the case of [1-14C]oleoyl-iPLA$_2{\beta}$, the addition of either 1 N HCl or 1–2 N neutral hydroxylamine did not result in a significant decrease in radiolabeling, whereas the addition of 1 N NaOH completely eliminated the majority of covalently bound [1-14C]oleate (Fig. 4C). The insensitivity of [1-14C]oleoyl-iPLA$_2{\beta}$ to hydroxylamine and HCl would indicate the absence of thioester and oxyester linkages, respectively, whereas the disappearance of radiolabeling in the presence of NaOH is consistent with an amide linkage. Pretreatment of iPLA$_2{\beta}$ with N-ethylmaleimide or iodoacetamide decreased [1-14C]oleoylation (Fig. 4C), indicating that free thiol (cysteine) groups are important for either the formation of oleoyl-iPLA$_2{\beta}$ intermediate(s) or for subsequent transfer to the terminal acceptor residue(s). Interestingly, acylation of iPLA$_2{\beta}$ was not detectable utilizing 1-palmitoyl-2-[1-14C]oleoyl-sn-glycero-3-phosphocholine (with or without CoASH) (Fig. 4C). This suggests that the acyl-enzyme intermediate formed with POPC, which is readily hydrolyzed by water, is fundamentally distinct from that which leads to the relatively stable iPLA$_2{\beta}$ acyl adduct(s) formed with oleoyl-CoA.

To determine whether oleoylated iPLA$_2{\beta}$ could undergo acyl group exchange in the presence of additional amounts of oleoyl-CoA, we first preincubated iPLA$_2{\beta}$ with a molar excess of unlabeled oleoyl-CoA guest in host POPC vesicles and then monitored incorporation of [1-14C]oleoyl acid utilizing [1-14C]oleoyl-CoA. Remarkably, robust radiolabeling of iPLA$_2{\beta}$ still occurred in a time-dependent manner after preacylation with unlabeled oleoyl-CoA (Fig. 4D), indicating the ability of iPLA$_2{\beta}$ to catalyze acyl cycling in the presence of additional exogenous oleoyl-CoA.

**Oleoylation of iPLA$_2{\beta}$ in Sf9 Cells**—Although iPLA$_2{\beta}$ was robustly acylated by oleoyl-CoA in vitro, it remained to be determined if the enzyme could be oleoylated in an intact cell. Accordingly, we incubated control and iPLA$_2{\beta}$His$_6$-containing Sf9 cells with $^3$H]oleic acid for 3 h and analyzed the cytosolic and membrane fractions by SDS-PAGE followed by autoradiography. A unique radiolabeled protein band was observed in the membrane fraction of cells expressing iPLA$_2{\beta}$ but not in the membranes of control cells (Fig. 5, left). A corresponding radiolabeled protein was not observed in the cytosol of cells containing iPLA$_2{\beta}$ (data not shown), indicating that the majority of the acylated protein is localized to cellular membranes. To confirm the identity of the radiolabeled band, Western blotting for iPLA$_2{\beta}$ was performed. A robust immunoreactive band representing iPLA$_2{\beta}$ was detected in Sf9 cell membranes at the same molecular weight as that of the unique radiolabeled band (Fig. 5, right).

**Localization of the iPLA$_2{\beta}$ Oleoylated Peptide by Partial Trypsinization and Mass Spectrometric Identification of the Oleoylated Domain of iPLA$_2{\beta}$**—To initially localize the region of iPLA$_2{\beta}$ modified by oleoyl-CoA, we partially trypsinized [1-14C]oleoyl-iPLA$_2{\beta}$ in solution and separated the radiolabeled peptide fragments by SDS-PAGE. Results from these experiments revealed that the majority of the radioactivity was contained within a 25-kDa proteolytic fragment (Fig. 6). In-gel tryptic digestion and subsequent MALDI-MS analysis of the 25-kDa polypeptide determined that it encompassed residues 408–578, which contains both the nucleotide binding domain and the active site (Table 1). Next, we utilized MALDI-MS to examine tryptic digests of the protein, specifically searching for unique peptide peaks that were 264.2 mass units (i.e. C18:1-H$_2$O) greater than their respective parent peak. Despite multiple attempts utilizing a wide range of conditions (e.g. in-gel digests, solution digests, multiple proteases, combinations of proteases, organic solvent and detergent extraction/solubilization techniques, etc.), we were unable to identify potential candidate peaks for MALDI-MS/MS analysis despite achieving 70% sequence coverage of iPLA$_2{\beta}$.

The addition of oleate to would be expected to increase the calculated water-octanol partition coefficient (log($P$) value) of the modified peptide by 2.23, representing a significant increase in nonpolarity (for reference, log($P$) for phenylalanine = 1.000). The difficulty in ionization of hydrophobic peptides is well documented (55–57) and has recently been discussed in detail (58). Potential nucleophilic amino acid residues within the 25-kDa peptide acylated by oleoyl-CoA include Cys-428, Lys-435, Lys-448, and Lys-455 near the nucleotide binding and lipase consensus sequence motifs.

**Effect of Calcium-activated Calmodulin on iPLA$_2{\beta}$-mediated Acyl-CoA Hydrolysis**—Calcium-bound calmodulin has been previously demonstrated to bind to iPLA$_2{\beta}$ and potently inhibit the phospholipase A$_2$ activity of the enzyme (22, 59). We were therefore interested to determine if Ca$^{2+}$-CaM would have a similar effect on the acyl-CoA thioesterase activity of iPLA$_2{\beta}$. Although Ca$^{2+}$-CaM inhibited the PLA$_2$ activity of recombinant iPLA$_2{\beta}$ by $\sim$70–80%, the palmitoyl-CoA thioesterase activity was unaffected under similar conditions (Fig. 7A). Thus, whereas the phospholipase A$_2$ activity of iPLA$_2{\beta}$ is responsive to changes in intracellular calcium (via calmodulin), iPLA$_2{\beta}$
would be expected to hydrolyze acyl-CoA thioesters independent of calcium concentration or the presence of calmodulin.

**Calmodulin-mediated Protection of iPLA₂β against Oleoylation by Oleoyl-CoA**—The proximity of the oleoylated iPLA₂β 25-kDa trypsic fragment to the calmodulin binding domain next led us to investigate whether Ca²⁺-CaM could protect the enzyme against covalent acylation by oleoyl-CoA. Although the addition of either Ca²⁺ or CaM in the presence of EGTA alone did not alter the extent of oleoylation of iPLA₂β (Fig. 7B), the combination of Ca²⁺ and CaM significantly decreased autoacylation of the enzyme. From these results, acyl-CoA-mediated acylation would be predicted to primarily occur after dissociation of the iPLA₂β-CaM complex.

**Oleoyl-CoA-mediated Reversal of the Inhibition of iPLA₂β by Calmodulin**—Depletion of intracellular calcium stores has been previously shown to initiate the influx of extracellular calcium through a poorly understood process, potentially involving an unknown cellular metabolite referred to as calcium influx factor (CIF) (25, 26, 60). Recent studies have demonstrated that CIF activates iPLA₂β by reversal of Ca²⁺-calmodulin inhibition of the enzyme (19, 20, 24). To determine if acyl-CoA could mitigate the inhibition of iPLA₂β by CaM, we utilized a real-time fluorescence assay employing the PLA₂ substrate, BODIPY-PC, to monitor the kinetic effects of oleoyl-CoA (guest in POPC 95 mol %)/BODIPY-PC (5 mol %) host vesicles on CaM inhibition of iPLA₂β phospholipase A₂ activity. In the absence of calmodulin, iPLA₂β efficiently hydrolyzes BODIPY-PC present at 5 mol % in a POPC background as demonstrated by a robust time-dependent increase in fluorescence intensity (Fig. 8A). The presence of calcium ion did not appreciably affect the phospholipase A₂ activity of iPLA₂β under these conditions (data not shown). In contrast, the presence of Ca²⁺-bound CaM inhibited iPLA₂β-catalyzed hydrolysis of BODIPY-PC by ~70–80% (Fig. 8A). Remarkably, the addition of 1 mol % oleoyl-CoA could activate CaM-inhibited iPLA₂β (~40% of initial activity) (Fig. 8B), and the presence of 2.5–5 mol % oleoyl-CoA completely eliminated CaM-mediated inhibition of iPLA₂β (Fig. 8, C and D) under these conditions. To confirm that iPLA₂β was in fact hydrolyzing BODIPY-PC and that the increase in fluorescence observed was not due to either protein-fluorophore or acyl-CoA-fluorophore interactions, the reaction substrates and products were extracted into chloroform/methanol in the presence of internal standards and subsequently quantified by shotgun lipidomics (61). As anticipated, the production of lyso-BODIPY-PC, 16:0-LPC, and 18:1-LPC was dependent upon the presence of iPLA₂β, and the amount of each product was diminished (~80%) by the presence of Ca²⁺-bound CaM (Fig. 8, E–G). Importantly, the addition of 5 mol % of oleoyl-CoA to the POPC/BODIPY-PC vesicles in the presence of Ca²⁺/CaM/iPLA₂β completely reversed the inhibition of iPLA₂β by Ca²⁺/CaM as evidenced by the recovery of similar amounts of 16:0-LPC, 18:1-LPC, and lyso-BODIPY-PC to that observed with iPLA₂β alone (Fig. 8F). Finally, to establish that the effects of oleoyl-CoA on Ca²⁺/CaM-mediated inhibition of iPLA₂β were not dependent on the presence of BODIPY-PC, similar experiments were performed with 1-palmitoyl-2-[1-¹⁴C]oleoyl-syr-glycero-3-phosphocholine as substrate. Similar to the real-time fluorescence assays, CaM inhibited iPLA₂β activity by ~70%, and the presence of oleoyl-CoA alone caused moderate inhibition (20%) of iPLA₂β activity (Fig. 8I), presumably due to interactions with iPLA₂β at or near the substrate binding site. As anticipated, the presence of oleoyl-CoA significantly attenuated the CaM-mediated inhibition of iPLA₂β, resulting in 75% of the activity observed with oleoyl-CoA alone (Fig. 8I). Collectively, these results demonstrate the rescue of the calmodulin-inhibited iPLA₂β activity by oleoyl-CoA by three independent methods and identify fatty acyl-CoAs as potential candidates for calcium influx factor.

**DISCUSSION**

Since its initial purification and characterization (9, 10, 38), iPLA₂β has been demonstrated to be an important mediator of arachidonic acid release (12, 14, 62, 63), lymphocyte proliferation (16, 64), store-operated Ca²⁺ entry (13, 19, 20), insulin secretion (65, 66), myocardial phospholipid hydrolysis (67, 68), and ventricular arrhythmogenesis (21). Prior studies have identified ATP (9) and

**TABLE 1**

| m/z (observed) | m/z (calculated) | Modification | Sequence | Residues |
|---------------|------------------|--------------|----------|----------|
| 1076.61       | 1076.54          | Pyr-Glu      | (R)NYDAPEVIR(E) | 547–555 |
| 1093.65       | 1093.58          |              | (R)QPAELHLFR(N) | 538–546 |
| 1110.67       | 1110.61          |              | (R)QPAELHLFR(N) | 538–546 |
| 1149.72       | 1149.65          |              | (R)KPAFILSSMR(D) | 508–417 |
| 1458.82       | 1458.73          |              | (R)NYDAPEVIREP(F) | 547–558 |
| 1708.95       | 1708.85          |              | (R)GSRPYESGPLEEFLK(R) | 497–511 |
| 2184.28       | 2184.16          |              | (K)VMLTGTLSDRQPAELHLFR(N) | 528–546 |
| 2379.40       | 2379.26          |              | (R)QNHNLKPTQPADQLYVR(A) | 559–578 |

**FIGURE 7.** Ca²⁺-CaM blocks covalent acylation of iPLA₂β by oleoyl-CoA but does not inhibit palmitoyl-CoA thioesterase activity. A, iPLA₂β His₆ (0.5 μg) was preincubated in the presence of Ca²⁺ (1 mM) or Ca²⁺ -CaM (6 μg) on ice before addition to 95 μM POPC containing either 5 mol % [1-¹¹C]POPC or [1-¹¹C]palmitoyl-CoA in 100 μM Tris-HCl, pH 7.2, containing 0.25 mM CaCl₂. After incubation at 37 °C for 3 min, reactions were terminated by vortexing in the presence of butanol, and released radioactively fatty acids were resolved by TLC and quantitated by liquid scintillation spectrometry. Results are presented as the average ± S.E. of four separate determinations. B, purified iPLA₂β His₆ was incubated with [1-¹¹C]oleoyl-CoA (10 μM) and CaCl₂ (0.25 mM) for 1 h at 37 °C. Samples were resolved by SDS-PAGE and exposed to film as described under “Experimental Procedures.”
calmodulin binding domains (23), ankyrin repeats (10, 69), splice variants (70, 71), proteolytic products (38, 72, 73), phosphorylation (9, 74), and interaction with calmodulin kinase II/II (75), each of which serve as potential regulators of the pleiotropic functions of iPLA2. In this paper, we demonstrate that iPLA2 efficiently hydrolyzes saturated fatty acyl-CoAs at physiologically relevant concentrations, is selectively autoacylated by oleoyl-CoA, is protected from autoacylation by Ca\(^{2+}\)-CaM, and is rescued from calmodulin-mediated inhibition of phospholipase A2 activity by oleoyl-CoA.

Site-directed mutagenesis of Ser-465 or pretreatment of iPLA2 with BEL inhibit both thioesterase and phospholipase A2 activities to identical degrees, indicating that the same active site and nucleophile (Ser-465) are utilized for both hydrolytic reactions. Remarkably, iPLA2 autoacylation by saturated acyl-CoAs is dramatically increased by either mutagenesis of Ser-465 or pretreatment of the enzyme with BEL. One possible explanation for these effects is that a greater effective saturated acyl-CoA concentration for acylation is achieved near the iPLA2 active site, since hydrolysis is reduced or eliminated under these conditions. These results demonstrate the existence of a second nucleophilic site(s) (distinct from Ser-465 and the proximal nucleophilic residue(s) modified by BEL) in iPLA2. Furthermore, the presence of oleoylated iPLA2 in the membranes of an intact cell indicates that acylation may facilitate localization of iPLA2 to specific membrane compartments or microdomains.

A combination of partial trypsinolysis and MALDI-MS was utilized to localize the region of acylation to amino acid residues 400–600 (adjacent to the calmodulin binding domain), which includes the nucleotide and lipase consensus sequence motifs. Thus, acylation by oleoyl-CoA occurs near the catalytic domain of iPLA2, although it does not appear to inhibit or block substrate (i.e. palmitoyl-CoA or POPC) access to the active site serine (Ser-465) for catalysis. Interestingly, Ca\(^{2+}\)-CaM blocked covalent acylation of iPLA2 by oleoyl-CoA. In contrast, Ca\(^{2+}\)-CaM significantly inhibited only the phospholipase A2 activity of iPLA2, whereas the acyl-CoA thioesterase activity of the

FIGURE 8. Reversal of Ca\(^{2+}\)/CaM-mediated inhibition of iPLA2 activity by oleoyl-CoA. A–D, purified iPLA2His6 in the presence (filled circles) or absence (open squares) of Ca\(^{2+}\)/CaM was incubated with POPC/BODIPY-PC (95:5 mol%, 100 \(\mu\)M phospholipid) host vesicles for 2 min at 37 °C with the indicated concentrations of guest oleoyl-CoA. Relative fluorescence was recorded at 15-s intervals utilizing 495-nm excitation and 515-nm emission wavelengths as described under “Experimental Procedures.” Results are representative of four independent experiments performed in duplicate. E–H, ESI/MS analysis of phosphatidylcholine molecular species from the reactions described in A and D. Lipid species were extracted into chloroform in the presence of internal standards (I.S.; 14:1–14:1 PC and 17:0 LPC for phosphatidylcholine and lysophosphatidylcholine species, respectively) and were subjected to ESI tandem mass analysis as described under “Experimental Procedures.” Spectra were acquired by 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 million. The relative abundance of lipid species in each spectrum was normalized to that of the LPC internal standard (I.S., 14:1 PC). I, purified iPLA2His6 in the presence or absence of Ca\(^{2+}\)/CaM was incubated with 1-palmitoyl-2-[\(^{14}\)C]oleoyl-sn-glycero-3-phosphocholine (100 \(\mu\)M) host vesicles with or without guest oleoyl-CoA (10 \(\mu\)M) for 3 min at 37 °C. Radioabeled fatty acid was extracted into butanol, resolved by TLC, and quantified by scintillation spectrometry as described under “Experimental Procedures.” Results represent the average ± S.E. of four separate determinations.
enzyme was unaffected under similar conditions. This result suggests that the less sterically bulky hydrophobic portion of the acyl-CoA substrate may have greater access to the iPLA$_2$B active site (Ser-465) than the bulkier diacyl phospholipid substrate in the presence of Ca$^{2+}$/bound CaM.

To our knowledge, iPLA$_2$B is the only intracellular phospholipase A$_2$ to exhibit substantial amounts of long-chain acyl-CoA thioesterase activity and represents the first acyl-CoA thioesterase identified at the molecular level shown to efficiently hydrolyze membrane-associated acyl-CoAs. In contrast, in vitro assays with purified recombinant cytosolic PLA$_2$$\gamma$ (76) and iPLA$_2$$\gamma$ in our hands did not detect appreciable long-chain acyl-CoA hydrolysis activities. A 54-kDa acyl-CoA hydrolase from rat intestinal microsomes was found to cleave long-chain acyl-CoAs in the presence of phosphatidylcholine vesicles, although the sequence identity of this enzyme has not been described since its original purification (77).

Myocardial ischemia is known to be accompanied by activation of iPLA$_2$B (21), although the mechanism(s) contributing to such activation have been the subject of much debate. It is known that iPLA$_2$B largely exists as an inhibited complex in transgenic mice, since vast overexpression does not result in substantive increases in phospholipolysis (21). However, induction of myocardial ischemia results in the dramatic activation of the enzyme demonstrated by fatty acid release and lysolipid accumulation. The present results identify a likely mechanism contributing to the activation of iPLA$_2$B, since acyl-CoA is known to dramatically increase during myocardial ischemia due to the blockade of mitochondrial fatty acid oxidation (78). Accordingly, these results potentially link the activation of iPLA$_2$B in hearts during ischemia with that which occurs during the influx of extracellular calcium (see below) in other tissues.

Depletion of intracellular calcium stores in smooth muscle cells has been previously demonstrated to activate iPLA$_2$B through a mechanism hypothesized to involve the dissociation of CaM from the enzyme (13, 36). Store-operated calcium (cation) channels in the plasma membrane are then activated in response to agonist-stimulated intracellular calcium pool depletion for the purpose of replenishing the emptied calcium stores. Recent work by Bolotina and co-workers has provided additional details of this process by showing that iPLA$_2$B is required for activation of store-operated calcium (cation) channels through generation of lysophospholipids (20). Furthermore, the inhibitory complex between CaM and iPLA$_2$B could be disrupted by a partially purified preparation of CIF (20). Although attempts to elucidate the molecular identity of CIF over the past 10 years have not been successful, these studies have determined various chemical properties of calcium influx factor. In general, CIF is believed to be a nonprotein, diffusible, phosphorylated “sugar nucleotide,” which is resistant to heat, alkaline pH, and protease treatment and is retained on a C18 reverse phase matrix (79).

In this work, we demonstrate that oleoyl-CoA is able to mimic the properties of CIF by restoring the phospholipase A$_2$ activity of Ca$^{2+}$/CaM-inhibited iPLA$_2$B. The acute production of acyl-CoAs due to fatty acid influx or release (e.g. from phospholipids or triacylglycerol) and subsequent thioesterification in specific membrane microenvironments containing complexes of Ca$^{2+}$/CaM-inhibited iPLA$_2$B would probably be sufficient to mediate iPLA$_2$B activation through displacement of calmodulin in a temporally and spatially specific manner. Thus, this process would be predicted to initiate an amplification cascade by a subset of activated iPLA$_2$B, which initially releases fatty acids (from surrounding phospholipids) for thioesterification to CoA, thereby activating adjacent iPLA$_2$B/Ca$^{2+}$/CaM complexes. We specifically point out that many other CIF-like cellular constituents capable of reversing Ca$^{2+}$/CaM-iPLA$_2$B inhibition may exist and that other membrane components and conditions that occur in vivo (accessory proteins, membrane surface charge and curvature, membrane electrochemical potential, etc.) may facilitate this process.

Multiple acyl-CoA thioesterases have been cloned from mammalian sources and are classified on the basis of their subcellular localization (cytosolic, mitochondrial, or peroxisomal), sequence similarity, and ability to be induced by peroxisome proliferators. The majority of these thioesterases, as well as all known intracellular phospholipases A$_2$, contain the canonical GXGXXG lipase (esterase) consensus sequence motif. Amino acid sequence alignments of iPLA$_2$B with the known mammalian acyl-CoA thioesterases did not reveal any significant sequence homology outside of the GXGXXG consensus motif. This is not completely unexpected, given the diversity among the different classes of acyl-CoA thioesterases. Some of the established acyl-CoA thioesterase family members (e.g. mitochondrial acyl-CoA thioesterases and cytosolic acyl-CoA thioesterases) possess conserved putative nucleotide binding sequences (GXGXXG). Interestingly, calcium-independent phospholipase A$_2$B displays an acyl-CoA substrate selectivity (C14-C20) similar to the cytosolic Type-I thioesterase (27). In addition, iPLA$_2$B, like this cytosolic acyl-CoA thioesterase, is not inhibited by high concentrations of CoASH, indicating that these enzymes are probably not involved in “sensing” and regeneration of free CoASH through acyl-CoA hydrolysis, as has been ascribed to peroxisomal acyl-CoA thioesterase-2 (46). Calcium-independent phospholipase A$_2$B and other acyl-CoA thioesterases probably serve multiple pleotropic roles in metabolism and cellular signaling.

In this study, we demonstrate that purified recombinant iPLA$_2$B possesses robust palmitoyl-CoA hydrolyase activity in addition to its previously well characterized lysophospholipase and phospholipase A$_2$ activities. Moreover, iPLA$_2$B is covalently autoacylated in a highly substrate-specific fashion (by oleoyl- but not palmitoyl-CoA), which occurs at a second active site distinct from the hydrolytic lipase site (GXGXXG). Thus, iPLA$_2$B could potentially have multiple effects on the production of lipid metabolites (arachidonic acid and lysolipids) or alternatively through removal of saturated acyl-CoAs from specific cellular membrane compartments. Importantly, calcium-independent phospholipase A$_2$B is present in multiple subcellular compartments, most notably plasma and nuclear membranes, cytosol, and mitochondria (68, 74, 80). Collectively, these results identify novel enzymatic (acyl-CoA thioesterase), covalent (oleoylation), and regulatory (acyl-CoA reversal of CaM inhibition) properties of iPLA$_2$B that probably contribute to the multiple diverse signaling roles of this enzyme in cellular functions.

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