THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 18, pp. 14880–14895, April 27, 2012

Activation of Mitochondrial Calcium-independent Phospholipase $A_2\gamma$ ($iPLA_2\gamma$) by Divalent Cations Mediating Arachidonate Release and Production of Downstream Eicosanoids*[S]◆

Received for publication, December 21, 2011, and in revised form, February 22, 2012 Published, JBC Papers in Press, March 2, 2012, DOI 10.1074/jbc.M111.336776

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Background: Calcium-independent $PLA_2\gamma$ is a major phospholipase in cardiac mitochondria that modulates multiple mitochondrial functions, but its mechanism of activation is unknown.
Results: Divalent cations activate $iPLA_2\gamma$ leading to release of eicosanoids and lysolipids from mitochondrial phospholipids.
Conclusion: Divalent cation-activated mitochondrial $iPLA_2\gamma$ initiates the production of biologically active signaling metabolites.
Significance: $iPLA_2\gamma$ contributes to regulation of myocardial bioenergetic and electrophysiologic functions by production of eicosanoids.

Calcium-independent phospholipase $A_2\gamma$ ($iPLA_2\gamma$) (PNPLA8) is the predominant phospholipase activity in mammalian mitochondria. However, the chemical mechanisms that regulate its activity are unknown. Here, we utilize $iPLA_2\gamma$ gain of function and loss of function genetic models to demonstrate the robust activation of $iPLA_2\gamma$ in murine myocardial mitochondria by Ca^{2+} or Mg^{2+} ions. Calcium ion stimulated the production of 2-arachidonoyl-lysophosphatidylcholine (2-AA-LPC) from 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine during incubations with wild-type heart mitochondrial homogenates. Furthermore, incubation of mitochondrial homogenates from transgenic myocardium expressing $iPLA_2\gamma$ resulted in 13- and 25-fold increases in the initial rate of radiolabeled 2-AA-LPC and arachidonic acid (AA) production, respectively, in the presence of calcium ion. Mass spectrometric analysis of the products of calcium-activated hydrolysis of endogenous mitochondrial phospholipids in transgenic $iPLA_2\gamma$ mitochondria revealed the robust production of AA, 2-AA-LPC, and 2-docosahexaenoyl-LPC that was over 10-fold greater than wild-type mitochondria. The mechanism-based inhibitor (*R*)-(*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one (BEL) ($iPLA_2\gamma$ selective), but not its enantiomer, (*S*)-BEL ($iPLA_2\beta$ selective) or pyrrolidine (cytosolic $PLA_2\alpha$ selective), markedly attenuated Ca^{2+}-dependent fatty acid release and polyunsaturated LPC production. Moreover, Ca^{2+}-induced $iPLA_2\gamma$ activation was accompanied by the production of downstream eicosanoid metabolites that were nearly completely ablated by (*R*)-BEL or by genetic ablation of $iPLA_2\gamma$. Intriguingly, Ca^{2+}-induced $iPLA_2\gamma$ activation was completely inhibited by long-chain acyl-CoA (IC_{50} ~20 μM) as well as by a nonhydrolyzable acyl-CoA thioether analog. Collectively, these results demonstrate that mitochondrial $iPLA_2\gamma$ is activated by divalent cations and inhibited by acyl-CoA modulating the generation of biologically active metabolites that regulate mitochondrial bioenergetic and signaling functions.

Phospholipases A_2 (PLA_2s)[2] catalyze the hydrolysis of fatty acyl moieties at the *sn*-2 position of glycerophospholipids. Mammalian phospholipases serve critical roles in transducing cellular signals into biologically active lipid second messengers such as arachidonic acid and lysophospholipids (1). In mitochondria, the products of PLA_2s and their downstream metabolites modulate numerous functions, including mitochondrial bioenergetics, signaling, and apoptosis (2–6). Despite their key roles in multiple mitochondrial functions, the molecular identity of the phospholipase(s) responsible for the hydrolysis of mitochondrial phospholipids and their mechanism of activation are unknown. In this study, we utilize $iPLA_2\gamma$ gain of function and loss of function models in conjunction with high mass accuracy mass spectrometry to identify the prom-

* This work was supported, in whole or in part, by National Institutes of Health Grants 5PO1HL57278 and RO1HL41250. R. W. G. has financial relationships with LipoSpectrum and Platomics.
◆ This article was selected as a Paper of the Week.
[S] This article contains supplemental Fig. 1.
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[2] The abbreviations used are: PLA_2, phospholipases A_2; AA, arachidonic acid; 2-AA-LPC, 1-lyso-2-arachidonoyl-*sn*-glycero-3-phosphocholine; AMPP, *N*-(4-aminomethylphenyl)pyridinium; BEL, (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one; $cPLA_2$, cytosolic phospholipase A_2; EET, epoxyeicosatrienoic acid; FFA, free fatty acid; HETE, hydroxyeicosatetraenoic acid; $iPLA_2$, calcium-independent phospholipase A_2; LPC, lysophosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TG, $iPLA_2\gamma$ transgenic; MPT, mitochondrial permeability transition; OA, oleic acid.

inent role of divalent cation-mediated activation of iPLA$_2\gamma$ resulting in the generation of multiple biologically active eicosanoid metabolites.

Since the original identification over 2 decades ago of an intracellular calcium-independent phospholipase A$_2$ activity and its subsequent purification and cloning (7–10), the family of mammalian calcium-independent phospholipases A$_2$ (iPLA$_2$s) has been defined by the presence of a patatin homology domain that contains a nucleotide-binding motif (G*X*G*XX*G) and a lipase consensus site (G*X*S*X*G) separated by a 10–40-amino acid residue spacer linkage (10). This family of proteins, now known as the patatin-like phospholipases, serves multiple signaling and bioenergetic functional roles. Unlike the cPLA$_2$ and secretory PLA$_2$ subfamilies, members of the iPLA$_2$ family have not previously been shown to be regulated by calcium ion either through facilitating membrane binding or augmenting catalytic activity.

Calcium is a central regulator of numerous integrated biochemical, contractile, and signaling functions in myocardium, including cardiac myocyte bioenergetics, excitation-contraction coupling, action potential generation, and hemodynamic function (11–13). Excitation of cardiac myocytes results in calcium influx through L-type Ca^{2+} channels leading to calcium-induced calcium release through the sarcoplasmic reticulum ryanodine receptor (14). Recent studies have focused on spatially localized increases in calcium ion in the intradyadic space and resultant increases in intramitochondrial calcium modulating mitochondrial bioenergetics and signaling functions (15, 16). Adaptive physiologic increases in mitochondrial Ca^{2+} concentration lead to the activation of TCA cycle flux (17, 18), increases in oxidative phosphorylation (19–21), and stimulation of ATP production during hemodynamic stress (22, 23). In contrast, pathologic elevation of calcium ion concentration promotes the opening of the mitochondrial permeability transition (MPT) pore resulting in the swelling of the mitochondrial matrix leading to rupture of the outer mitochondrial membrane, the release of cytochrome *c*, and the induction of the intrinsic pathway of apoptosis (24). Previously, Pfeiffer and coworkers (6) implicated a calcium-independent phospholipase activity in liver mitochondria as an important participant in the calcium-mediated opening of the MPT pore. However, the molecular identity of this and possibly other mitochondrial phospholipases activated by calcium ion have yet to be defined. Because of the importance of phospholipase-derived lipid second messengers in heart disease, recent attention has focused on the types, roles, and mechanisms of activation of mitochondrial phospholipases whose downstream products modulate cardiac bioenergetic and signaling functions that promote apoptosis with resultant cardiac myocyte dropout precipitating the progression of heart failure.

Previously, we identified and characterized a novel membrane-associated calcium-independent iPLA$_2$ present in myocardium (now termed iPLA$_2\gamma$ (also known as PNPLA8 (HUGO nomenclature)). Detailed molecular biologic analysis of this gene identified multiple translation initiation codons, splice variants, and post-translational modifications suggesting its importance in cellular regulatory processes (25). Remarkably, mass spectrometric analyses of iPLA$_2\gamma$ activity demonstrated that iPLA$_2\gamma$ possessed highly selective PLA$_1$ activity with phosphatidylcholine molecular species containing polyunsaturated (*e.g.* 20:4 and 22:6), but not monounsaturated, fatty acids esterified to the *sn*-2 position (26). This intriguing property of iPLA$_2\gamma$ identified a previously unanticipated metabolic pathway leading to the generation of 2-arachidonoyl-lysophosphatidylcholine (2-AA-LPC), which serves as a central branch point metabolite in several signaling cascades (26). Specifically, 2-AA-LPC can be transformed into endocannabinoids (*e.g.* 2-arachidonoyl-glycerol by the action of lysophospholipase C present in the endoplasmic reticulum), arachidonic acid by lysophospholipase activity (present in cytosol, mitochondria, and many other membrane-delimited compartments), or 2-AA lysophosphatidic acid by the action of lysophospholipase D (*e.g.* autotaxin present in the sarcolemmal membrane) (27–29). Notably, 2-AA-LPC is the most abundant lysolipid molecular species in failing human hearts thus implicating a central role of iPLA$_2\gamma$ in the metabolism of AA-containing phospholipids in myocardium (26).

Using cardiac myocyte-specific transgenic expression of iPLA$_2\gamma$ in conjunction with iPLA$_2\gamma^{-/-}$ mice and analyses of lipid metabolites by high mass accuracy mass spectrometry, we now demonstrate that iPLA$_2\gamma$ activity in mitochondria from murine myocardium mice is robustly activated by either Ca^{2+} or Mg^{2+} ions leading to the release of AA, the production of 2-AA-LPC, and the generation of multiple biologically active eicosanoid metabolites. Moreover, the current results demonstrate marked decreases in eicosanoid production in mitochondria from iPLA$_2\gamma^{-/-}$ mice. Collectively, these gain of function and loss of function studies demonstrate that iPLA$_2\gamma$ is regulated by Ca^{2+} and Mg^{2+} ions and catalyzes the coordinated release of arachidonic acid and the production of downstream signaling metabolites from mitochondria that collectively orchestrate cellular bioenergetic and signaling responses to external stimuli.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-3-glycerophosphocholine (55 mCi/mmol) was purchased from Perkin-Elmer Life Sciences. 1-Palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (58 mCi/mmol) was obtained from Amersham Biosciences. Racemic (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one (BEL) and internal standards for eicosanoid analyses, including thromboxane B$_2$-d_4, prostaglandin E$_2$-d_4, leukotriene B$_4$-d_4, and 12(*S*)-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic-5,6,8,9,11,12,14,15-d_8 acid (12(*S*)-HETE-d_8), were purchased from Cayman Chemicals. The (*R*)- and (*S*)-enantiomers of BEL were prepared as described previously (30). *N*-(4-Aminomethylphenyl)pyridinium (AMPP) was obtained from Alchem Laboratories Corp. *N*-[(2*S*,4*R*)-4-(Biphenyl-2-ylmethylisobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl]-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide was purchased from EMD Biosciences. Anti-cPLA$_2\alpha$ and anti-rabbit HRP-IgG antibodies were from Cell Signaling Technology, Inc., and Santa Cruz Biotechnology, Inc., respectively (Santa Cruz, CA). Antibodies against iPLA$_2\beta$ and iPLA$_2\gamma$ were prepared in our laboratory as described previously (31, 32).

Acyl-CoAs (acetyl-, 16:0-, 18:1-, and 20:4-CoA) and CoASH were purchased from Sigma, and *S*-hexadecyl-CoA was prepared in our laboratory as described previously (33, 34).

General Animal Studies—Animal protocols were conducted in strict accordance with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the Animal Studies Committee of Washington University.

Isolation of Heart Mitochondria—The iPLA$_2\gamma^{-/-}$ mice and cardiac myocyte-specific iPLA$_2\gamma$ transgenic mice expressing human iPLA$_2\gamma$ were generated in our laboratory as described previously (3, 35). Following euthanasia of wild-type and iPLA$_2\gamma$ transgenic mice by cervical dislocation, hearts were removed and washed extensively in ice-cold isolation buffer (10 mM HEPES, 0.25 M sucrose, 1 mM EGTA, 0.4% fatty acid-free BSA (pH 7.4)) and finely minced with a razor blade. The minced heart tissue was homogenized with a 7-ml glass homogenizer (60 strokes) on ice. The homogenates were first centrifuged at 700 × *g* for 10 min to pellet nuclei and cellular debris. The supernatant was centrifuged at 12,000 × *g* for 10 min to pellet mitochondria. The resultant mitochondria were then washed with fresh isolation buffer without EGTA and BSA and repelleted by centrifugation at 10,000 × *g*. The final mitochondrial pellet was reconstituted in buffer as indicated for the following assays.

Mass Spectrometric Analyses of Phospholipase Activity in Sonicated Mitochondria—For measuring PLA activity in isolated mitochondria, the mitochondrial pellet was resuspended in ice-cold HEPES buffer (10 mM HEPES (pH 7.4), 1 mM DTT, 10% glycerol) and sonicated (20 times with 1-s pulses) at 30% power. The concentration of mitochondrial protein was determined by a Bradford protein assay (Bio-Rad), and the homogenates were assayed for phospholipase activity within 1 h. Mitochondrial phospholipase activity was initiated by addition of $CaCl_2$, $MgCl_2$, or EGTA (control) at the indicated concentrations and times at 35 °C. Because a number of calcium-chelating agents (*e.g.* proteins, nucleotides, etc.) are present in mitochondria, the final free calcium ion concentration in mitochondrial sonicates after addition of $CaCl_2$ was determined using a calcium calibration buffer kit and FURA-2 calcium indicator obtained from Invitrogen. In experiments with PLA_2 inhibitors, mitochondria were preincubated with the indicated inhibitors or DMSO vehicle alone for 15 min at 23 °C. Reactions were terminated by addition of 2 ml of chloroform/methanol (1:1, v/v) followed by addition of internal standards (16:0-FFA-d_4 and 17:0-LPC, di-14:1-PC) and 700 μl of 50 mM LiCl. Lipids in the chloroform layer were dried down under a N_2 stream and re-extracted in 3 ml of chloroform/methanol/water (1:1:1, v/v/v). Following isolation and evaporation of the chloroform layer under a N_2 stream, extracted mitochondrial lipids were reconstituted in chloroform/methanol/isopropyl alcohol (1:2:4, v/v/v). Samples were loaded into an automated nanospray apparatus (TriVersa Nanomate, Advion Biosciences, Ithaca, NY) in line with a TSQ Quantum ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA) for mass spectrometric analysis. Free fatty acids (FFAs) and lysophosphatidylcholines (LPCs) were quantified in either the negative or positive ions modes, respectively, as described previously (26, 36).

For the measurement of mitochondrial phospholipase activity with radiolabeled substrates, mitochondrial homogenates (0.1 mg of protein) in 300 μl of HEPES buffer were incubated with radiolabeled phosphatidylcholine substrates (~1 × 10^5 dpm of 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine or 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) in the presence of 4 mM EGTA or 150 μM free Ca^{2+} (final) for 10 min. Phospholipase reactions were terminated by addition of 2 ml of chloroform/methanol/acetic acid (50:48:2, v/v/v) followed by addition of 700 μl of water. Lipids were extracted into the chloroform and loaded onto Partisil LK6D silica gel 60-Å plates and resolved using petroleum ether/ethyl ether/glacial acetic acid (70:30:1, v/v/v) with oleic acid as a fatty acid standard as described previously (37). For assays measuring both [^{14}C]lysophosphatidylcholine (PLA_1 activity) and ^{14}C-fatty acid (PLA_2 activity) production, lipid extracts were resolved by TLC using channeled silica plates developed in chloroform/acetone/methanol/glacial acetic acid/water (6:8:2:2:1, v/v) after addition of oleic acid and 1-oleoyl-LPC standards. Regions containing fatty acid and lysophosphatidylcholine were identified with iodine vapor and scraped into vials, and the amount of radioactivity was determined by liquid scintillation spectrometry.

For the determination of membrane-bound Ca^{2+}-dependent PLA_2 activities, mitochondrial homogenates were incubated with either 1 mM EGTA or 5 μM free Ca^{2+} and centrifuged at 100,000 × *g* for 1 h. The resultant pellet was resuspended in HEPES buffer and briefly sonicated, and PLA_2 activity was determined using [^{14}C]PAPC as described above.

Determination of Phospholipase Activity in Intact Mitochondria—Isolated mitochondria were resuspended in buffer containing 3 mM HEPES (pH 7.4), 0.07 M sucrose, 0.23 M mannitol, 5 mM succinate, 2.5 μM rotenone, and 1 mM KH_2PO_4. Intact mitochondria were exposed to 70 μM exogenous Ca^{2+} (a concentration of calcium known to be present in the intradyadic space) following preincubation with different PLA_2 inhibitors or DMSO vehicle alone for 10 min at 23 °C. In control reactions, EGTA (50 μM) was added instead of 70 μM Ca^{2+}. The reactions were stopped by addition of 2 ml of chloroform/methanol (1:1, v/v), and lipidomic analyses were performed as described previously (26, 36).

Isolation and Quantitation of Eicosanoids—Mitochondrial homogenates (1 mg/ml) in HEPES buffer were preincubated with (*R*)-BEL (20 nmol/mg protein) or DMSO vehicle alone for 10 min at 23 °C and then exposed to either 2 mM EGTA or 6 μM free measured Ca^{2+} for 20 min. For the experiments with intact mitochondria, 1 mg/ml energized mitochondria were prepared as described above and incubated in the presence of either 50 μM EGTA or 70 μM exogenous Ca^{2+} at 23 °C for 10 min. Reactions were stopped by addition of methanol (20% final concentration) on ice and diluted to 0.5 mg of protein/ml with water after addition of internal standards (500 pg each of thromboxane B_2-d_4, prostaglandin E_2-d_4, leukotriene B_4-d_4, and 12(*S*)-HETE-d_8). Sample solutions were immediately applied to a Strata-X solid phase extraction cartridge (Phenomenex, CA) preconditioned with 1 ml of methanol followed by 1 ml of 10%

methanol. The cartridge was then washed with 1 ml of 5% methanol twice, and the remaining solvent was removed by flushing with N_2 at a pressure of 5 p.s.i. Eicosanoids were eluted with 1 ml of methanol containing 0.1% acetic acid. All steps utilizing solid phase extraction cartridges were carried out using a vacuum manifold attached to a house vacuum line. After evaporating the methanol solution using a SpeedVac, recovered eicosanoids were derivatized with AMPP as described previously by Gelb and co-workers (38). Briefly, eicosanoids were dissolved in 12.5 μl of ice-cold acetonitrile/*N*,*N*-dimethylformamide (4:1, v/v) followed by addition of 12.5 μl of ice-cold 640 mM (3-(dimethylamino)propyl)ethyl carbodiimide hydrochloride in HPLC grade water. Eicosanoid derivatization was initiated by addition of 25 μl of 5 mM *N*-hydroxybenzotriazole, 15 mM AMPP and incubated at 60 °C for 30 min. Quantitative analysis of the derivatized eicosanoids was performed by LC/MS/MS by comparisons with stable isotope internal standards using selected reaction monitoring with high mass accuracy product ion determination using an LTQ-Orbitrap (Thermo Scientific). Briefly, the derivatized eicosanoids were separated on a C18 reversed phase column (Ascentis Express, 2.7 μm particles, 150 × 2 mm) at 23 °C using a linear gradient of solvent A (0.1% glacial acetic acid in water) and solvent B (0.1% glacial acetic acid in acetonitrile) at a flow rate of 0.2 ml/min. The solvent gradient program was as follows: 0.0–1.0 min, 5–20% B; 1.0–7.0 min, 20–25% B; 7.0–7.1 min, 25–40% B; 7.1–20 min, 40–60% B; 20–21 min, 60–100% B; 21–24 min, 100% B; 24–25 min, 100% B to 5% B. The derivatized eicosanoids were analyzed using a hybrid tandem mass spectrometer (LTQ-Orbitrap) with selected reaction monitoring in the positive ion mode with high mass accuracy determination of product ions. Sheath, auxiliary, and sweep gas flows of 30, 5, and 1, respectively, were utilized. The capillary temperature was set to 275 °C, and the electrospray voltage was 4.1 kV. Capillary voltage and the tube lens voltage were set to 2 and 100 V, respectively. Setting of instrument parameters and data acquisition were performed using Thermo Fisher Xcalibur Version 2.1 software.

Statistical Analyses—Values are expressed as mean ± S.E. The significance of experimental observations was determined by a Student's *t* test, and results were considered significant at $p < 0.05$.

RESULTS

Calcium-mediated Activation of Mitochondrial iPLA$_2\gamma$—Considering the importance of calcium in the precise spatiotemporal integration of mitochondrial bioenergetics and signaling in myocardium, we sought to determine whether iPLA$_2\gamma$ activity in myocardial mitochondria was modulated by calcium ion. Accordingly, we measured the calcium dependence of the initial rate of mitochondrial iPLA$_2\gamma$ activity from mitochondrial homogenates isolated from myocardium of wild-type, cardiac myocyte-specific TG iPLA$_2\gamma$ and iPLA$_2\gamma^{-/-}$ mice.

First, we investigated the Ca^{2+} dependence and regioselectivity (*i.e.* PLA_1 *versus* PLA_2) of mitochondrial iPLA$_2\gamma$ activity using conventional radiolabeled assay systems. Incubations of sonicates of wild-type heart mitochondria with 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine ([^{14}C]PAPC) revealed a substantial increase in calcium-activated production of 2-[1-^{14}C]arachidonoyl-lysophosphatidylcholine (2-[^{14}C]AA-LPC), indicative of PLA_1 activity in comparison with samples containing EGTA (Fig. 1*A*). In contrast, parallel incubations with 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]POPC) resulted in a greater release of [1-^{14}C]oleic acid ([^{14}C]OA) in comparison with 2-[1-^{14}C]oleoyl-lysophosphatidylcholine (2-[^{14}C]OA-LPC), indicative of the substrate selective regiospecificity of hydrolysis catalyzed by iPLA$_2\gamma$ (Fig. 1*B*). Transgenic expression of iPLA$_2\gamma$ in myocardium resulted in its localization to the mitochondrial compartment as shown previously (25). Mitochondrial soni-

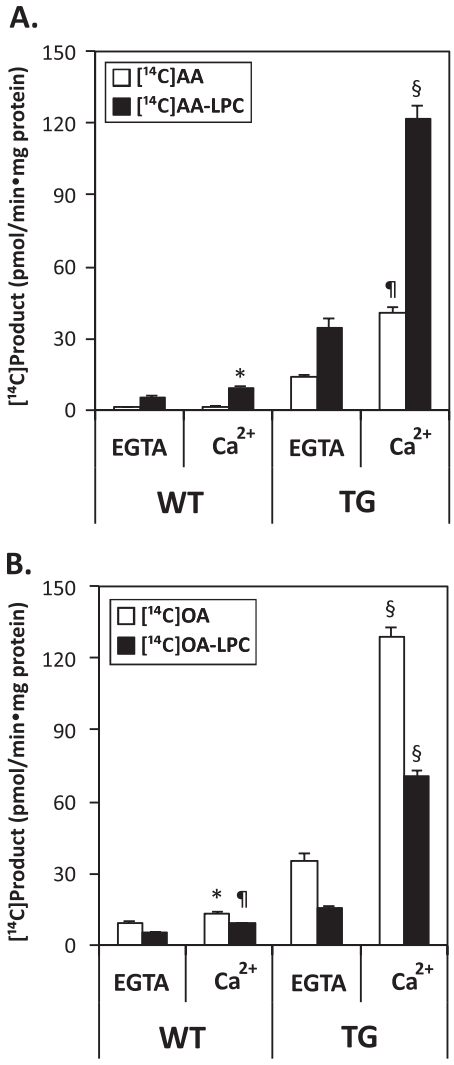


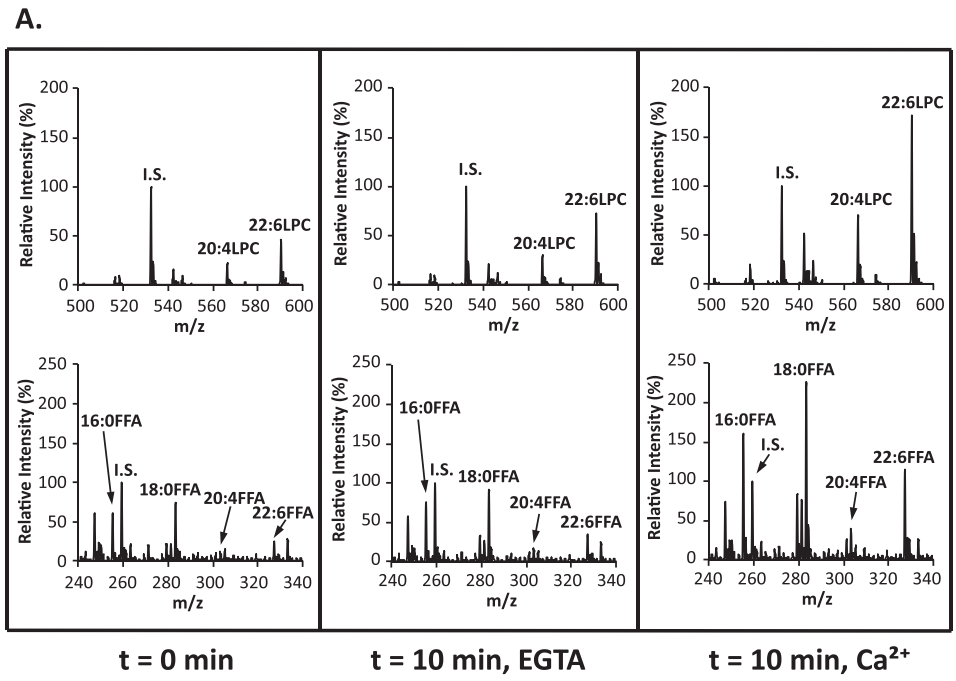
FIGURE 1. **Calcium activates robust phospholipase activity in myocardial mitochondria from transgenic mice selectively expressing iPLA$_2\gamma$ in cardiac myocytes.** Myocardial mitochondria from wild-type and iPLA$_2\gamma$ transgenic mice were isolated by differential centrifugation as described under "Experimental Procedures" and homogenized by sonication. Exogenous 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (*A*) or 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (*B*) (final 1% ethanol vehicle) was then added in the presence of either 4 mM EGTA or 150 μM free Ca^{2+} and incubated at 35 °C for 10 min. Reactions were terminated by adding chloroform/methanol (1:1, v/v), and lipids extracted into the chloroform layer were resolved by TLC by developing with chloroform/acetone/methanol/glacial acetic acid/water (6:8:2:2:1). Values are the average of four independent preparations ± S.E. *, $p < 0.01$; ¶, $p < 0.0001$; §, $p < 10^{-5}$ when compared with EGTA treatment.

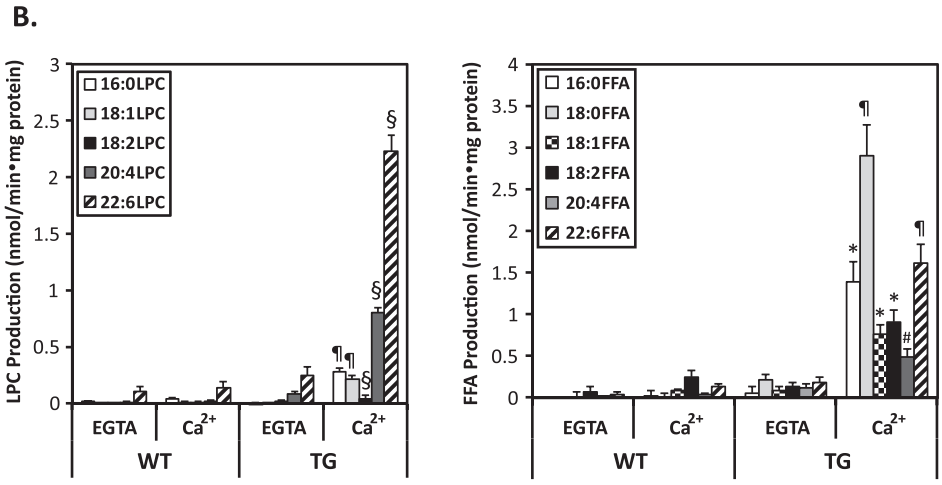
cates containing transgenic iPLA$_2\gamma$ exhibited 6.3- and 9.3-fold higher rates of production of 2-[^{14}C]AA-LPC and [^{14}C]AA, respectively, from [^{14}C]PAPC in the presence of EGTA in comparison with WT controls (Fig. 1*A*). Moreover, TG iPLA$_2\gamma$ mitochondria possessed 3.0- and 3.9-fold greater rates of 2-[1-^{14}C]oleoyl-lysophosphatidylcholine (2-[^{14}C]OA-LPC) production and [1-^{14}C]oleic acid ([^{14}C]OA) release, respectively, in comparison with WT samples in the presence of EGTA (Fig. 1*B*). Remarkably, addition of calcium to TG iPLA$_2\gamma$ mitochondria using either [^{14}C]PAPC or [^{14}C]POPC as substrate resulted in robust 12.8- and 7.7-fold increases in the release of 2-[^{14}C]AA-LPC and 2-[^{14}C]OA-LPC, respectively, in comparison with mitochondria isolated from wild-type littermates (Fig. 1). Furthermore, the calcium-mediated increases in the release of 2-[^{14}C]AA-LPC from [^{14}C]PAPC substrate and 2-[^{14}C]OA-LPC from [^{14}C]POPC substrate catalyzed by TG iPLA$_2\gamma$ mitochondrial sonicates were 3.5- and 4.5-fold greater, respectively, in comparison with reactions containing EGTA (Fig. 1). Mitochondrial homogenates prepared from genetically engineered mice expressing iPLA$_2\gamma$ in a cardiac myocyte-specific fashion displayed 3-fold greater calcium-dependent PLA$_1$ activity with [^{14}C]PAPC substrate by production of higher amounts of 2-[^{14}C]AA-LPC in comparison with the release of [^{14}C]AA (indicative of PLA$_2$ activity) (Fig. 1*A*). In contrast, cleavage of the oleoyl group at the *sn*-2 position of [^{14}C]POPC was 2-fold greater than hydrolysis of the palmitoyl group at the *sn*-1 position to produce 2-[^{14}C]OA-LPC (Fig. 1*B*). Collectively, these results demonstrate substrate-dependent differences in the regiospecificity of hydrolysis (*i.e.* the ratio of PLA$_1$/PLA$_2$ activities) of human recombinant mitochondrial iPLA$_2\gamma$ in the presence of Ca^{2+}, which are qualitatively similar to results with the soluble 63-kDa iPLA$_2\gamma$ previously studied (26).

Next, we examined whether Ca^{2+} altered the rate and regiospecificity of iPLA$_2\gamma$-mediated hydrolysis of endogenous mitochondrial phospholipid substrates. Mitochondria isolated from wild-type and transgenic iPLA$_2\gamma$-expressing myocardium by differential centrifugation were briefly sonicated and incubated in the presence of either 4 mM EGTA or 150 μM measured free Ca^{2+}. Changes in the levels of endogenous FFA and LPC molecular species were quantified by ratiometric comparisons with internal standards by mass spectrometry as described previously (26, 36). Remarkably, calcium ion dramatically increased mitochondrial phospholipase A$_1$ activity in mitochondria prepared from TG iPLA$_2\gamma$ myocardium resulting in 10- and 9-fold increases in 2-AA-LPC (20:4-LPC) and 2-docosahexaenoyl-LPC (22:6-LPC) production from endogenous mitochondrial phospholipids, respectively, in comparison with EGTA-treated controls (Fig. 2, *A* and *B*). Mass spectrometric determination of the regiospecificity of the product lysolipids (*i.e.* 1-acyl *versus* 2-acyl lysolipids) was determined by tandem mass spectrometry utilizing diagnostic product ion analysis (39) of the calcium-induced iPLA$_2\gamma$-mediated production of polyunsaturated LPC molecular species. The results demonstrated that the polyunsaturated fatty acyl chains were predominantly esterified to the *sn*-2 position as ascertained by the ratio of the diagnostic product ion peaks at *m/z* 147 (five-membered sodiated cyclophosphane, $[C_2H_5PO_4Na]^+$) and at *m/z* 104 (choline ion, $[C_5H_{14}NO]^+$), which are the predominant product ions of 1-hydroxy-2-acyl-*sn*-glycero-3-phosphocholine and 1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholine, respectively (Fig. 2*C*) (39). Markedly elevated amounts of saturated fatty acids such as stearic acid (18:0-FFA) and palmitic acid (16:0-FFA) were also present resulting in 11- and 23-fold increases, respectively, in the calcium-mediated activation of iPLA$_2\gamma$ in mitochondria prepared from TG iPLA$_2\gamma$ myocardium (Fig. 2). Docosahexaenoic acid (22:6-FFA) and arachidonic acid (20:4-FFA) were the predominant polyunsaturated fatty acids released by Ca^{2+} stimulation from endogenous mitochondrial phospholipids either through direct PLA$_2$-catalyzed hydrolysis or alternatively by sequential PLA$_1$ and lysophospholipase activities (Fig. 2).

Mitochondrial Ca^{2+}-activated PLA$_1$/PLA$_2$ Activities Demonstrate Enantiomeric Specific Inhibition by (R)-BEL but Not by (S)-BEL or Pyrrolidine—To substantiate the catalytic role of iPLA$_2\gamma$ in the observed Ca^{2+}-mediated activation of mitochondrial phospholipase activity present in iPLA$_2\gamma$ TG mitochondria (and not from altered expression or activation of other PLA$_2$ enzymes), the effects of different highly selective PLA$_2$ pharmacologic inhibitors on the Ca^{2+}-activated production of LPC and FFA from endogenous mitochondrial phospholipids were examined. Previously, we demonstrated a 10-fold selectivity of (*S*)-BEL for inhibition of recombinant iPLA$_2\beta$ and an approximate 10-fold selectivity of (*R*)-BEL for inhibition of recombinant iPLA$_2\gamma$ (30). Pyrrolidine, a relatively specific and widely utilized inhibitor of cPLA$_2\alpha$ activity, was used to assess the potential contributions of cPLA$_2\alpha$ to the release of fatty acids and lysolipids from mitochondria. Preincubation of TG iPLA$_2\gamma$ mitochondrial homogenates with 10 μM (*R*)-BEL resulted in the nearly complete inhibition of Ca^{2+}-induced production of polyunsaturated LPC molecular species and FFAs from endogenous phospholipids (Fig. 3*A*). In sharp contrast, (*S*)-BEL (10 μM) inhibited only ~10% of the measured mitochondrial Ca^{2+}-dependent PLA$_1$/PLA$_2$ activities. Pyrrolidine (2 μM) did not attenuate calcium-mediated hydrolysis of endogenous phospholipids present in cardiac mitochondrial membranes (Fig. 3*A*).

A previous study by Leslie and co-workers (40) showed that cPLA$_2\beta$3 was not inhibited by pyrrolidine and was bound to mitochondrial membranes in human BEAS-2B bronchial epithelial cells, and they demonstrated that cPLA$_2\beta$ could be readily extracted from cellular membranes with EGTA. To examine whether cPLA$_2\beta$ was responsible for the Ca^{2+}-dependent phospholipase activity present in transgenic iPLA$_2\gamma$ mitochondria, the sonicated mitochondrial homogenate was separated into membrane and soluble fractions by ultracentrifugation in the presence of either EGTA (Fig. 3*B*, *left panel*) or Ca^{2+} ion (Fig. 3*B*, *right panel*). Virtually all of the Ca^{2+}-dependent phospholipase activity remained in the membrane after either EGTA or Ca^{2+} treatment and did not require soluble (*i.e.* matrix) mitochondrial components to induce calcium-mediated activation. The overwhelming majority of the three major isoforms of iPLA$_2\gamma$ (50, 63, and 74 kDa) present in TG iPLA$_2\gamma$ mitochondria were localized in the mitochondrial membrane fraction after centrifugation as determined by Western blot analysis (supplemental Fig. 1). In addition, we measured the amounts of the intracellular PLA$_2$ enzymes, cPLA$_2\alpha$ and





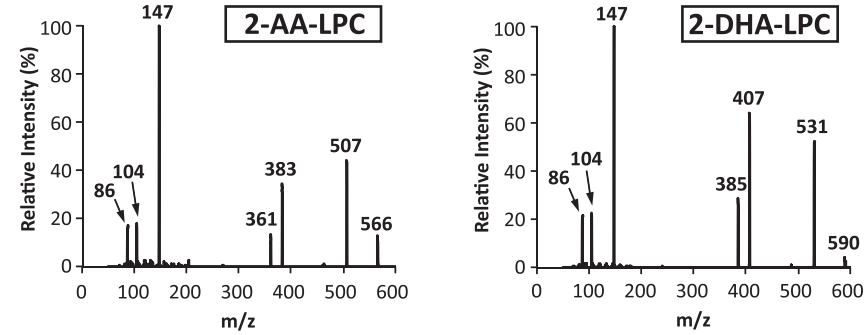


FIGURE 2. **Ca^{2+}-dependent generation of free fatty acid and lysophosphatidylcholine molecular species in iPLA$_2\gamma$ TG myocardial mitochondria.** Mitochondria were isolated by differential centrifugation from WT and iPLA$_2\gamma$ TG mouse myocardium and homogenized by sonication. Phospholipid hydrolysis was initiated by addition of Ca^{2+} (150 μM free Ca^{2+} in final) or 4 mM EGTA (control) followed by incubation at 35 °C for 10 min. Reactions were terminated by addition of chloroform/methanol (1:1, v/v), and resultant lipids were extracted in the presence of the following internal standards (*I.S.*: d_4-16:0-FFA, 17:0-LPC, and di-14:1-PC). Molecular species of LPC and FFA were quantitated by mass spectrometry as described under "Experimental Procedure." *A,* representative spectra for the relative intensities of molecular species of LPC and FFA after EGTA or Ca^{2+} treatment. *B,* LPC and FFA production rates were calculated by subtracting background levels of the appropriate lipids at t = 0 min from their concentrations at t = 10 min and normalized to the amount of mitochondrial protein. Changes in the predominant molecular species of endogenous LPCs and FFAs are shown in the *left* and *right panels*, respectively. #, $p < 0.01$; *, $p < 0.001$; ¶, $p < 0.0001$; §, $p < 10^{-7}$ when compared with EGTA treatment. Values presented are the mean ± S.E. of 7 preparations. *C,* product ion spectra of 2-AA-LPC and 1-lyso-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (*2-DHA-LPC*) at 30 eV collision energy were acquired from TG iPLA$_2\gamma$ mitochondrial homogenates after Ca^{2+} treatment for 10 min.

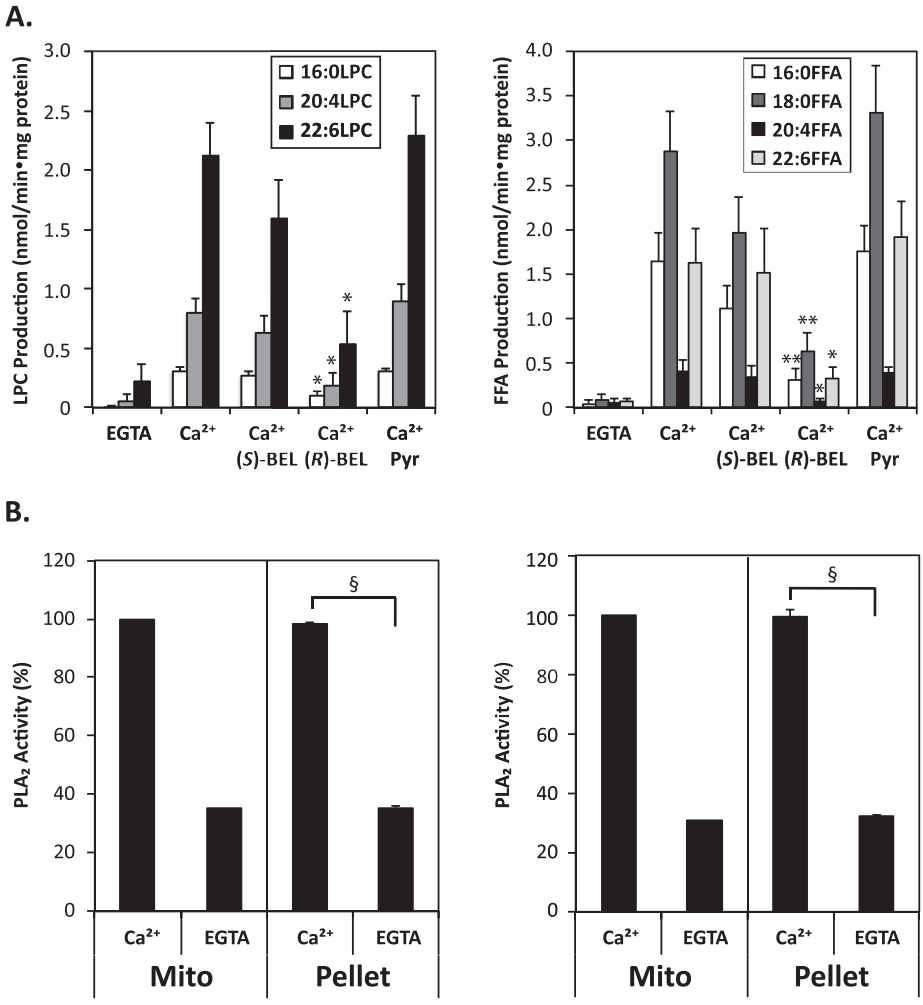


FIGURE 3. **Ca^{2+}-activated phospholipase A_1/A_2 activity in iPLA$_2\gamma$ transgenic mouse myocardial mitochondria is membrane-associated and inhibited by (*R*)-BEL but not by (*S*)-BEL or pyrrolidine.** Mitochondria were isolated from the myocardium of iPLA$_2\gamma$ transgenic mice by differential centrifugation and homogenized by sonication. Mitochondria homogenates were preincubated with either 10 μM (*S*)-BEL, 10 μM (*R*)-BEL, 2 μM of pyrrolidine (*Pyr*), or DMSO vehicle alone (1% v/v) for 15 min at 23 °C. Phospholipase activity was initiated by adding Ca^{2+} (150 μM free in final) or EGTA (4 mM) as control at 35 °C for 10 min and terminated by addition of chloroform/methanol (1:1, v/v). Lipids were extracted into chloroform with appropriate internal standards and lysophosphatidylcholine (*A, left panel*), and fatty acid (*A, right panel*) molecular species were quantified by mass spectrometry as described under "Experimental Procedures." Values are the mean ± S.E. of four preparations. *, $p < 0.05$; **, $p < 0.01$ when compared with Ca^{2+} treatment without inhibitor. To determine iPLA$_2\gamma$ distribution between soluble and membrane fractions, mitochondrial homogenates from iPLA$_2\gamma$ transgenic mouse heart were incubated with either 1 mM EGTA (*B, left panel*) or 5 μM free Ca^{2+} (*B, right panel*) prior to centrifugation at 100,000 × *g*. PLA$_2$ activities of the initial homogenate (*Mito*, designated as 100% activity in the presence of Ca^{2+}) and pellet fractions were determined using [^{14}C]PAPC (~1×10^5 dpm) as substrate in the presence of 4 mM EGTA or 150 μM free Ca^{2+} as described in Fig. 1. Values are the mean ± S.E. of three separate preparations with §, $p < 10^{-5}$.

iPLA$_2\beta$, to determine whether alterations in iPLA$_2\gamma$ expression resulted in compensatory changes in the expression levels of these enzymes. The protein levels of cPLA$_2\alpha$ and iPLA$_2\beta$ in iPLA$_2\gamma$ transgenic myocardial mitochondria were not significantly different from those present in their wild-type counterparts (data not shown). Collectively, these results strongly indicate that the Ca^{2+}-activated PLA$_1$/PLA$_2$ activities present in mitochondria prepared from either WT or TG iPLA$_2\gamma$ transgenic myocardium were catalyzed by iPLA$_2\gamma$.

Concentration Dependence of Ca^{2+}- and Mg^{2+}-mediated Activation of Mitochondrial iPLA$_2\gamma$ Activity—The sensitivity of Ca^{2+}-activated enzymes to alterations in calcium ion concentration is critical for their regulation within the physiologic range of calcium present in different subcellular compartments. Accordingly, we used cardiac mitochondria isolated from TG iPLA$_2\gamma$ mice to titrate the dependence of enzymatic activity on free calcium ion concentration. In the presence of 5.5 μM measured free Ca^{2+}, the activity of iPLA$_2\gamma$ was dramatically enhanced in comparison with EGTA (Fig. 4*A*). These results demonstrate that iPLA$_2\gamma$ activation likely occurs at physiologically relevant Ca^{2+} concentrations present in the matrix.

Next, in light of the known selectivity of the cPLA$_2$ family of enzymes to activation by Ca^{2+} (and not Mg^{2+}) through the presence of a conserved C2 domain (41), we sought to determine whether the Ca^{2+}-dependent activation of iPLA$_2\gamma$ was specific for calcium ion and not other physiologic divalent cations. Accordingly, we tested the effect of Mg^{2+} on the catalytic activity of the transgenic iPLA$_2\gamma$ present in mouse myocardial mitochondria. The activity of mitochondrial iPLA$_2\gamma$ increased in response to physiologic levels of magnesium ion (Fig. 4*B*). Collectively, these results suggest that the availability of free

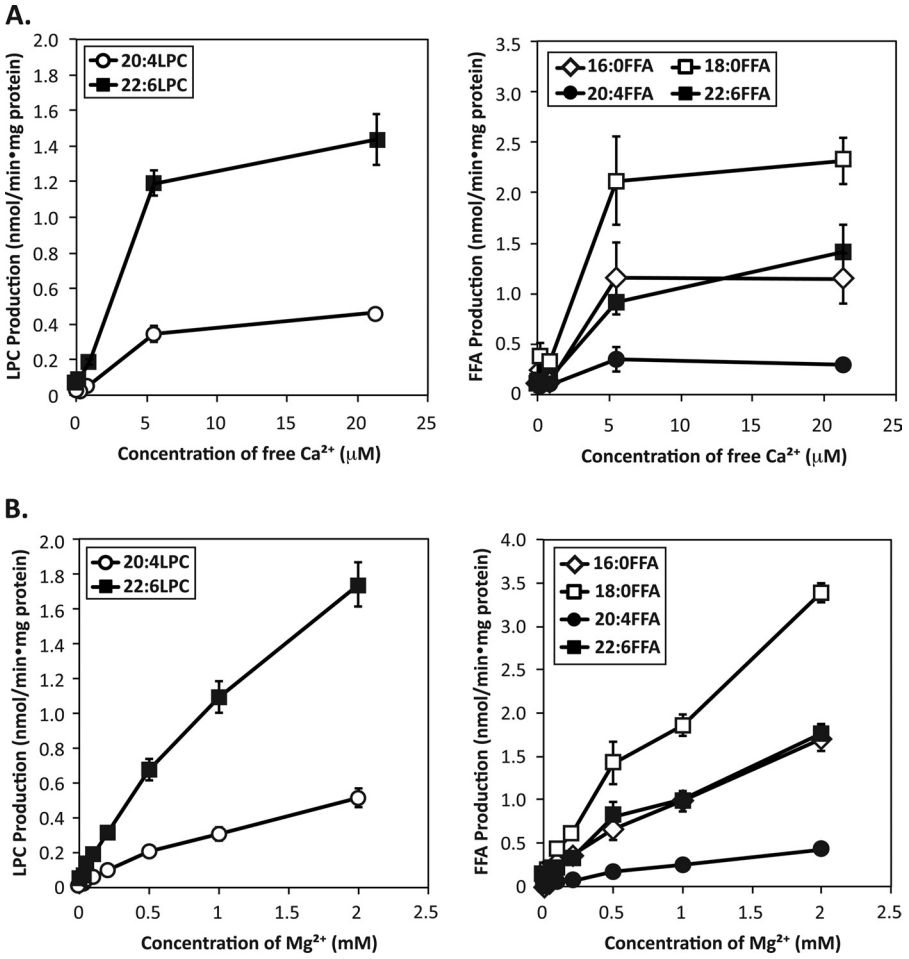


FIGURE 4. **Calcium and magnesium dependence of the phospholipase A_1/A_2 activities present in mitochondria isolated from iPLA$_2\gamma$ transgenic mouse myocardium.** Mitochondrial homogenates were incubated in the presence of either 0 (2 mM EGTA and 2 mM EDTA), 0.2, 0.9, 5.5, and 21 μM of free Ca^{2+} (*A*) or 0 (2 mM EGTA and 2 mM EDTA), 0.02, 0.05, 0.1, 0.2, 0.5, 1, or 2 mM of Mg^{2+} (*B*) at 35 °C for 10 min. Reactions were terminated by addition of chloroform/methanol (1:1, v/v) and lysophosphatidylcholine (*left panels*) and free fatty acid (*right panels*) molecular species extracted into chloroform in the presence of appropriate internal standards were analyzed and quantified by mass spectrometry as described under "Experimental Procedures." Values are the mean ± S.E. of three independent preparations.

Ca^{2+} and Mg^{2+} likely coordinately participate in the regulation of mitochondrial iPLA$_2\gamma$ activity.

Ca^{2+}-induced LPC and Fatty Acid Release in Intact Myocardial Mitochondria from Transgenic iPLA$_2\gamma$ Mice—Previously, pharmacologic approaches using racemic BEL were employed to suggest that an iPLA-type of activity was responsible for Ca^{2+}-activated fatty acid accumulation in intact rat liver and rabbit cortical mitochondria (6, 42, 43). To provide greater insight into the type of phospholipase involved and the mechanisms of activation, we examined the effects of (*R*)-BEL, (*S*)-BEL, and pyrrolidine on phospholipase activity in intact mitochondria from TG iPLA$_2\gamma$ myocardium at a physiologically relevant concentration of Ca^{2+}. Intact mitochondria isolated from TG iPLA$_2\gamma$ myocardium were placed in isotonic buffer containing succinate and exposed to either 50 μM EGTA or 70 μM exogenous Ca^{2+}. After incubation of intact mitochondria for 10 min at 23 °C, alterations in FFAs and LPC molecular species were quantified by mass spectrometry as described under "Experimental Procedures." Consistent with the results obtained with sonicated mitochondria, production of unsaturated LPC (20:4-LPC and 22:6-LPC) molecular species and saturated FFAs (16:0-FFA and 18:0-FFA) were markedly increased by Ca^{2+} at concentrations known to be present in the T-tubule intradyadic space (~200 μM) (15). Moreover, total phospholipase activity (released FFA + LPC) was completely inhibited by (*R*)-BEL, only modestly inhibited by (*S*)-BEL, and not affected by pyrrolidine (Fig. 5). After preincubation with (*R*)-BEL and subsequent calcium stimulation, LPC levels were even lower than nonstimulated levels suggesting the importance of iPLA$_2\gamma$ in modulating mitochondrial lipid second messenger production in intact mitochondria under basal as well as stimulated conditions.

Inhibition of Mitochondrial iPLA$_2\gamma$ Activity by Long-chain Fatty Acyl-CoAs—Because we had identified a novel mechanism of iPLA$_2\gamma$ activation having important implications for the regulation of mitochondrial bioenergetic and signaling functions, we next investigated whether fatty acyl-CoA, a central branch point intermediate in mitochondrial anabolic and catabolic lipid metabolism, could modulate mitochondrial iPLA$_2\gamma$ activity. To this end, sonicated myocardial mitochondria were prepared from TG iPLA$_2\gamma$ mice and were incubated with either nonesterified coenzyme A (CoASH) or acyl-CoAs of varying chain lengths prior to measurement of PLA_2 activity. Neither 50 μM of CoASH nor acetyl-CoA significantly affected

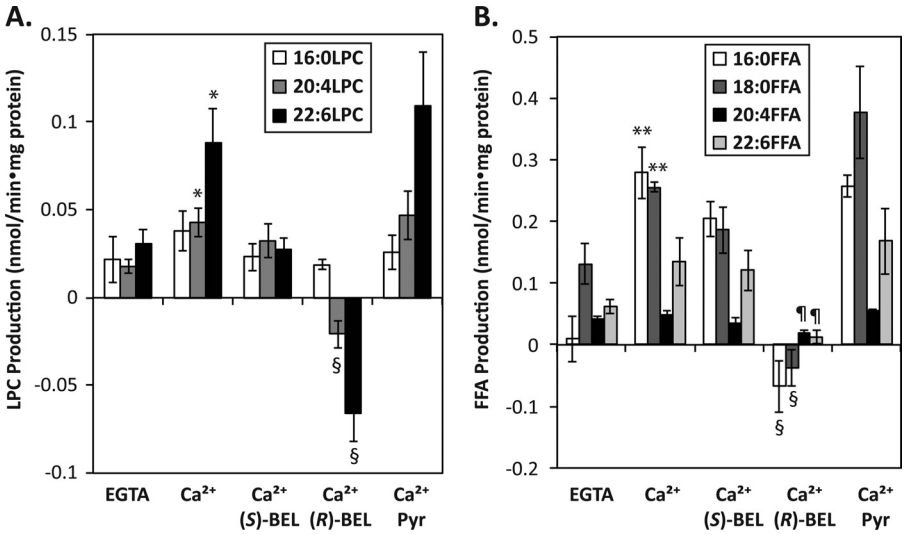


FIGURE 5. **Ca^{2+}-dependent activation of iPLA$_2\gamma$ in intact mitochondria results in liberation of lysophosphatidylcholine and free fatty acid molecular species that are ablated by (*R*)-BEL.** Myocardial mitochondria were isolated by differential centrifugation from iPLA$_2\gamma$ transgenic mice and resuspended in buffer containing 3 mM HEPES (pH 7.4), 0.23 M mannitol, 0.07 M sucrose, 1 mM KH_2PO_4, 5 mM succinate, and 2.5 μM rotenone. Intact mitochondria were then preincubated with either 10 μM (*S*)-BEL, 10 μM (*R*)-BEL, 2 μM pyrrolidine (*Pyr*), or DMSO vehicle alone (1% v/v) for 10 min prior to exposure to either 70 μM Ca^{2+} or 50 μM EGTA and incubation for 10 min at 23 °C. Phospholipase A_1/A_2 activity in intact mitochondria was terminated by addition of chloroform/methanol (1:1, v/v), and LPC (*A*) and FFA (*B*) molecular species extracted into chloroform with the appropriate internal standards (*I.S.*) were analyzed and quantified by mass spectrometry as described under "Experimental Procedures." Values are the mean ± S.E. of four preparations. *, $p < 0.05$; **, $p < 0.01$ when compared with EGTA treatment and ¶, $p < 0.05$; §, $p < 0.005$ when compared with Ca^{2+} treatment in the absence of inhibitors.

iPLA$_2\gamma$ catalytic activity (Fig. 6*A*). In sharp contrast, long chain fatty acyl-CoAs, including palmitoyl-CoA, oleoyl-CoA, and arachidonoyl-CoA, potently inhibited mitochondrial iPLA$_2\gamma$ at concentrations of acyl-CoA present within the mitochondrial matrix (Fig. 6*A*). To determine whether the observed acyl-CoA-mediated inhibition of iPLA$_2\gamma$ required covalent modification of the enzyme, we synthesized *S*-hexadecyl-CoA (a nonhydrolyzable analog of palmitoyl-CoA). *S*-Hexadecyl-CoA was as effective as palmitoyl-CoA in inhibiting iPLA$_2\gamma$ activity demonstrating that neither acyl-CoA hydrolysis nor enzyme thioesterification was necessary to mediate iPLA$_2\gamma$ inhibition. To further investigate the inhibition of iPLA$_2\gamma$ by fatty acyl-CoA, we incubated mitochondrial homogenates from TG iPLA$_2\gamma$ hearts with increasing concentrations of oleoyl-CoA (Fig. 6*B*). Concentrations of acyl-CoA present in the mitochondrial compartment potently inhibited iPLA$_2\gamma$-mediated hydrolysis of exogenous [^{14}C]PAPC (IC_{50} ~20 μM).

Ca^{2+} Activation of Myocardial Mitochondrial iPLA$_2\gamma$ Activity from Wild-type and Transgenic Mice Leads to the Production of Multiple Eicosanoid Metabolites—The release of arachidonic acid by phospholipases is the rate-determining step in the production of numerous biologically active eicosanoid metabolites that play multiple discrete roles in cellular signaling and bioenergetic functions. Considering the potent effects of calcium ion on AA release from mitochondria catalyzed by iPLA$_2\gamma$, we measured alterations in downstream eicosanoid production by mass spectrometry after derivatization with *N*-(4-aminomethylphenyl) pyridinium using selected reaction monitoring with high mass accuracy product ion analysis. In the presence of 6 μM measured free Ca^{2+}, the rates of 12-hydroxyeicosatetraenoic acid (12-HETE) production were 1108 and 4054 pg/min·mg protein in WT and TG iPLA$_2\gamma$ mitochondrial sonicates, respectively, corresponding to 1.6- and 5.2-fold increases in comparison with incubations containing EGTA (Fig. 7*A*). This dramatic Ca^{2+}-activated production of 12-HETE was nearly completely blocked by (*R*)-BEL in both WT and TG iPLA$_2\gamma$ mitochondria suggesting iPLA$_2\gamma$ catalyzed the rate-limiting step in the production of this downstream oxygenated AA metabolite (Fig. 7*A*). Moreover, the production of multiple eicosanoid molecular species, including various HETEs, EETs, prostaglandins, and leukotrienes, was dramatically enhanced in TG iPLA$_2\gamma$ mitochondria when compared with WT controls in the presence of calcium ion (Fig. 7, *A* and *C*). Consistent with the results from pharmacologic inhibition of mitochondrial iPLA$_2\gamma$, genetic ablation of iPLA$_2\gamma$ resulted in the dramatic reduction of the majority of the eicosanoids produced after calcium stimulation of mitochondria prepared from wild-type and TG iPLA$_2\gamma$ myocardium (Fig. 7, *B* and *D*).

Finally, we measured Ca^{2+}-activated eicosanoid production in intact myocardial mitochondria isolated from WT and TG iPLA$_2\gamma$ mice. Energized intact mitochondria were incubated with either 50 μM EGTA or 70 μM exogenous Ca^{2+}. Calcium ion stimulation of both intact WT and TG mitochondria resulted in a predominant increase of 12-HETE with smaller increases in other eicosanoids. These results demonstrate that Ca^{2+}-induced activation of iPLA$_2\gamma$ mediates the production of 12-HETE, the most abundant eicosanoid produced in mouse myocardial mitochondria (Fig. 8). Consistent with these results, Ca^{2+}-mediated eicosanoid production from intact mitochondria prepared from iPLA$_2\gamma^{-/-}$ myocardium was markedly decreased in comparison with WT and TG mitochondria (Fig. 8). Collectively, these results demonstrate the metabolic coupling between calcium-activated iPLA$_2\gamma$ catalyzed arachidonic acid release and the production of downstream eicosanoids thereby establishing iPLA$_2\gamma$ as an important mediator of the production of signaling metabolites from mitochondria (Fig. 9).

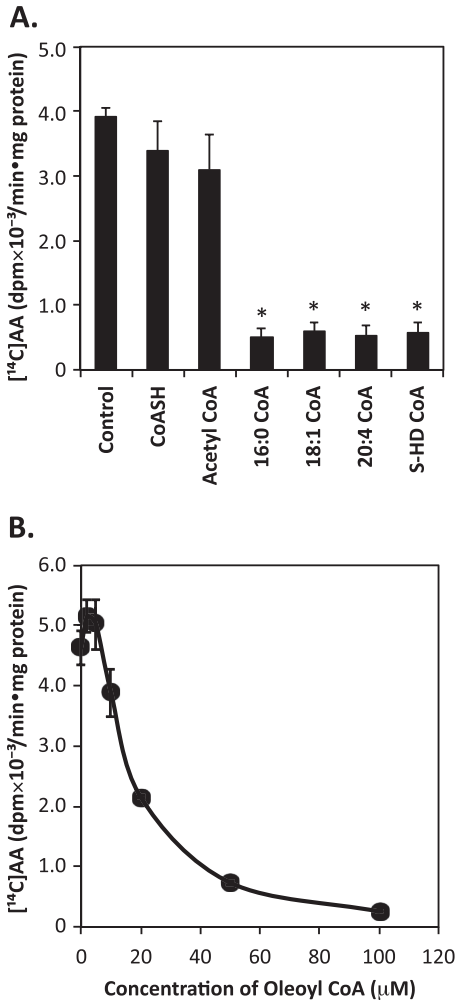


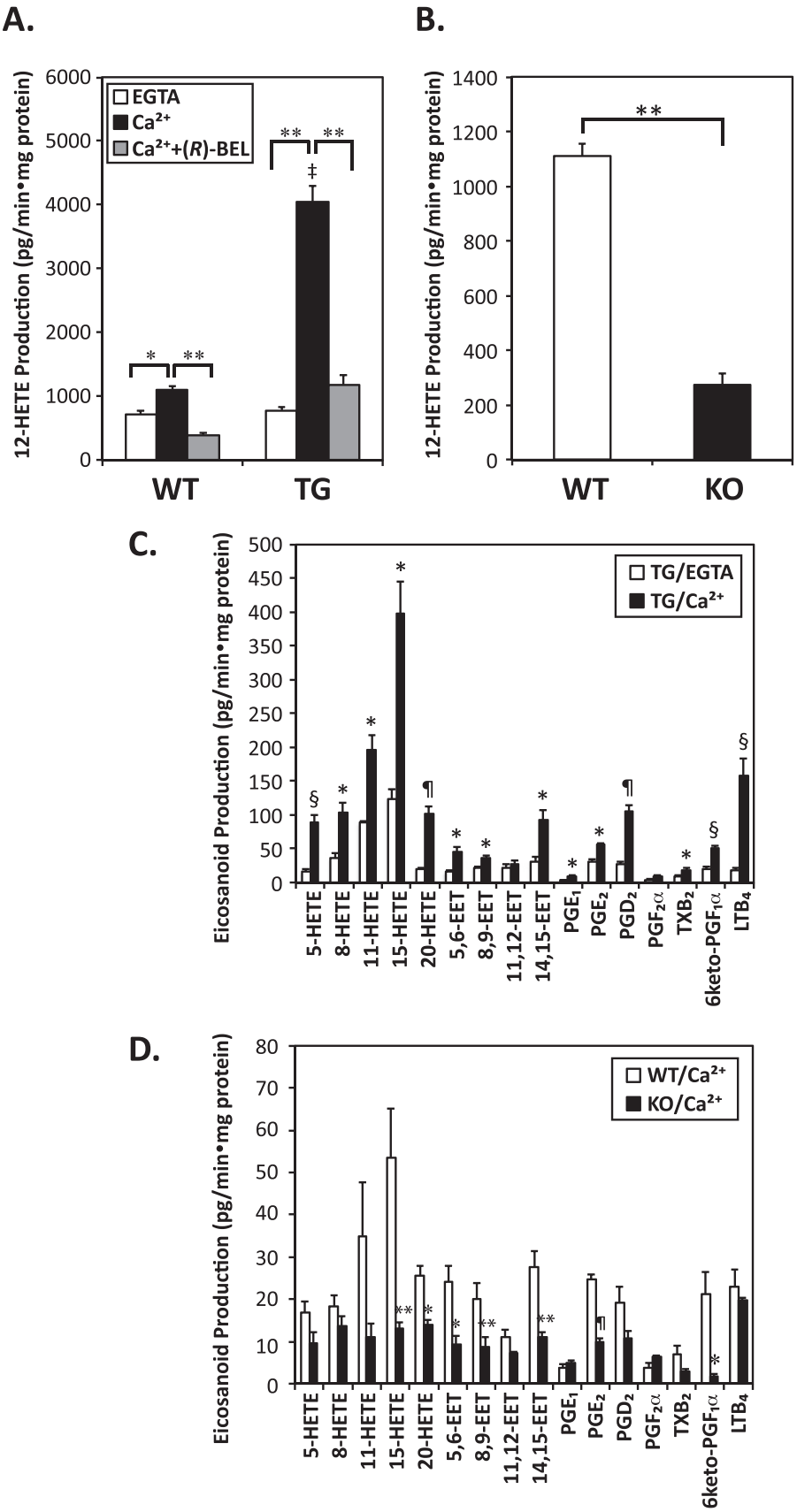
FIGURE 6. **Inhibition of mitochondrial iPLA$_2\gamma$ activity by long chain fatty acyl-CoAs.** *A,* mitochondrial homogenates from iPLA$_2\gamma$ transgenic myocardium were incubated with either 50 μM free CoASH, acetyl-CoA, palmitoyl(16:0)-CoA, oleoyl(18:1)-CoA, arachidonoyl(20:4)-CoA, or the nonhydrolyzable analog of palmitoyl-CoA, *S*-hexadecyl-CoA (*S-HD-CoA*), or buffer/H_2O alone (control) in the presence of 150 μM free Ca^{2+}. *, $p < 10^{-5}$ when compared with control. *B,* iPLA$_2\gamma$ transgenic mitochondrial homogenates were exposed to 0, 2, 5, 10, 20, 50, or 100 μM of oleoyl-CoA in the presence of 150 μM free Ca^{2+}, and PLA$_2$ reaction was immediately triggered by adding [^{14}C]PAPC ($\sim 1 \times 10^5$ dpm). [^{14}C]AA was collected by TLC and counted by liquid scintillation spectrometry for determination of PLA$_2$ activity. Values are the mean ± S.E. of four preparations.

DISCUSSION

Mitochondria play diverse functional roles in cellular bioenergetics and signaling, but the precise chemical mechanisms integrating mitochondrial responses to physiologic and pathophysiologic perturbations remain incompletely understood. In this study, we use integrated genetic and pharmacologic approaches to demonstrate the prominent role of mitochondrial iPLA$_2\gamma$ in the calcium-stimulated generation of biologically active eicosanoids and lysolipids from murine heart mitochondria. Multiple independent approaches using myocardial mitochondria isolated from wild-type mice, cardiac myocyte-specific transgenic iPLA$_2\gamma$-expressing mice, and mice in which iPLA$_2\gamma$ was genetically ablated were employed to determine that iPLA$_2\gamma$ can be activated by calcium ion leading to the generation of biologically active lipid second messengers from endogenous mitochondrial membrane lipids. First, incubation of exogenous [^{14}C]PAPC with mitochondrial membranes prepared from TG iPLA$_2\gamma$ mice with calcium resulted in over 10-fold increases in [^{14}C]arachidonic acid release and 2-[^{14}C]AA-LPC production in comparison with WT controls. Second, incubation of physiologically relevant concentrations of free calcium ion with mitochondrial sonicates from TG iPLA$_2\gamma$ mice resulted in a dramatic increase in the regiospecific hydrolysis of endogenous mitochondrial phospholipids, including the highly selective production of *sn*-2 polyunsaturated lysolipids (2-AA-LPC and 1-lyso-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine). Third, the calcium-stimulated production of 2-AA-LPC and 1-lyso-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine from endogenous mitochondrial lipids was completely inhibited by the mechanism-based inhibitor (*R*)-BEL (selective for iPLA$_2\gamma$ inhibition), whereas (*S*)-BEL (selective for iPLA$_2\beta$ inhibition) and pyrrolidine (selective for cPLA$_2\alpha$ inhibition) did not affect mitochondrial phospholipase A$_1$/A$_2$ activities. Fourth, the production of 12-HETE from myocardial mitochondrial homogenates was increased by calcium ion in WT and TG iPLA$_2\gamma$ mitochondria, and this increase was inhibited by (*R*)-BEL. Fifth, genetic ablation of iPLA$_2\gamma$ decreased calcium-induced production of 12-HETE from mitochondrial membrane sonicates by over 80%. Sixth, calcium activated the production of eicosanoids in intact mitochondria in WT hearts, and genetic ablation of iPLA$_2\gamma$ markedly reduced the calcium-activated production of eicosanoids in intact mitochondria. Collectively, these results provide compelling evidence that calcium activates iPLA$_2\gamma$ resulting in the generation of biologically active eicosanoids and lysolipids from mitochondrial phospholipids.

Calcium is a prominent regulator of multiple mitochondrial functions, including energy production (*e.g.* regulation of TCA cycle flux) (17, 18), cell fate decisions (*e.g.* apoptosis and necrosis by opening of the MPT pore) (44–47), and mitochondrial signaling to distant membrane compartments (*e.g.* regulation of sarcolemmal ion channels) by signaling molecules emanating from the mitochondrial compartment that function as mitokines (48). Previous work has emphasized the importance of reactive oxygen species (*e.g.* superoxide, hydrogen peroxide, and nitric oxide) and mitochondrial metabolites as mediators of mitochondrial inter-organelle communication (49–52). The results of this study identify iPLA$_2\gamma$ as the major enzymatic mediator for the calcium-activated hydrolysis of mitochondrial phospholipids leading either to the direct release of arachidonic acid or the subsequent generation of arachidonic acid by lysophospholipase-catalyzed hydrolysis of 2-AA-LPC (Fig. 9). The liberation of arachidonic acid is the rate-determining step in the production of a wide variety of eicosanoid metabolites that regulate multiple aspects of myocardial function. The current results highlight the previously unappreciated ability of mitochondria and their associated membranes to generate eicosanoid and lysolipid signaling metabolites through the calcium-mediated activation of iPLA$_2\gamma$.

Arachidonic acid and its downstream eicosanoid metabolites play critical roles in cardiac myocyte energy production and signaling functions. These include the regulation of ion channel kinetics (53–56), contractility (53, 57), metabolic flux, and bioenergetic efficiency (58–60). For example, lipoxygenase inhibi-


A.
12-HETE Production (pg/min•mg protein)
EGTA
Ca2+
Ca2+ +(R)-BEL
WT
TG
B.
12-HETE Production (pg/min•mg protein)
WT
KO
C.
Eicosanoid Production (pg/min•mg protein)
TG/EGTA
TG/Ca2+
5-HETE
8-HETE
11-HETE
15-HETE
20-HETE
5,6-EET
8,9-EET
11,12-EET
14,15-EET
PGE1
PGE2
PGD2
PGF2α
TXB2
6keto-PGF1α
LTB4
D.
Eicosanoid Production (pg/min•mg protein)
WT/Ca2+
KO/Ca2+

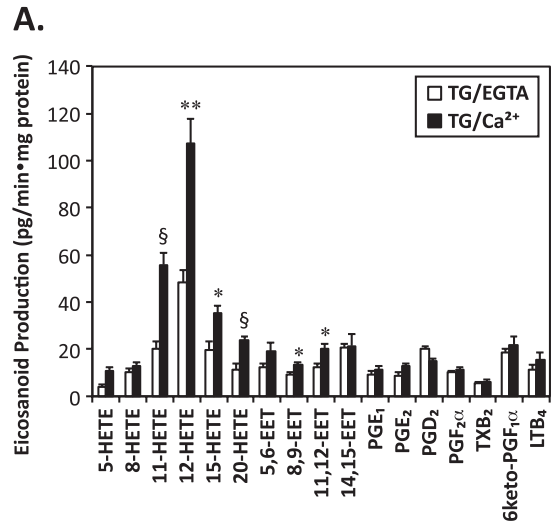
tion has been demonstrated to block insulin-mediated glucose uptake in cultured ventricular myocytes suggesting the importance of downstream lipoxygenase metabolites in insulin signaling (61). Furthermore, 12-HETE has been previously demonstrated to increase myocardial mitochondrial Ca^{2+} content as well as mitochondrial nitric-oxide synthase activity, which led to increased apoptosis in HL-1 cardiac myocytes (51, 62). Intriguingly, arachidonic acid has been implicated in MPT pore opening in several tissues, and inhibition of the release of AA from mitochondria prevented pore opening (42, 63). Although the increased generation of AA and HETEs in cardiac myocytes expressing iPLA$_2\gamma$ may be considered detrimental to myocardial function through activation of pro-inflammatory and pro-apoptotic pathways, HETEs and EETs serve multiple other cardioprotective functions. For example, 14,15-EET is cardioprotective during pressure overload, ventricular hypertrophy, and heart failure that has now been demonstrated through multiple approaches, including nonbiased genetic screens in the spontaneous hypertensive heart failure rat (64). Furthermore, 15(*S*)-HETE, 11(*S*)-HETE, and 5(*S*),15(*S*)-diHETE have been shown to increase the sensitivity of β-adrenergic receptor signaling in cardiomyocytes (65). Seubert *et al.* (66) have shown that increased EET production in mouse myocardium selectively expressing cytochrome P450 (CYP) 2J2 improved post-myocardial infarction recovery of left ventricular developed pressure that may be due to the higher mitochondrial K_{ATP} channel activity found in CYP2J2 TG cardiomyocytes in comparison with non-TG controls. Moreover, the mitochondrial initiated production of eicosanoids could serve to modulate vascular flow in stressed myocardium to increase substrate and oxygen delivery after hemodynamic stress. Collectively, these results demonstrate that calcium-induced activation of mitochondrial iPLA$_2\gamma$ can serve as an important enzymic mediator integrating mitochondrial metabolism with multiple aspects of myocardial function in discrete membrane-delimited compartments. Because most eicosanoid metabolites are readily diffusible across membrane bilayers, they are able to not only influence metabolic and signaling functions within mitochondria but can also transmit biologic information to other subcellular compartments within the cell of origin (autocrine effects) as well as distant targets in other cells (paracrine effects).

Previously, we identified the unusual substrate-dependent regiospecificity of iPLA$_2\gamma$ by mass spectrometry using a purified soluble 63-kDa isoform of recombinant iPLA$_2\gamma$ in combination with exogenous small unilamellar vesicles comprised of different molecular species of phosphatidylcholine as substrate (26). Unexpectedly, those studies demonstrated that soluble iPLA$_2\gamma$ catalyzes the selective hydrolysis of exogenous monounsaturated phospholipids at the *sn*-2 position, although it hydrolyzes the *sn*-1 aliphatic chain when polyunsaturated fatty acids are present at the *sn*-2 position. This study extends those results to the membrane-associated isoforms of iPLA$_2\gamma$ and demonstrates the calcium-activated regiospecific hydrolysis of endogenous mitochondrial membrane lipids leading to the generation of AA, 2-AA-LPC, and the resultant production of downstream signaling metabolites emanating from the mitochondrial compartment.

In this study, mass spectrometric analyses consistently demonstrated the robust accumulation of polyunsaturated lysophospholipids derived from the hydrolysis of the *sn*-1 acyl chain of endogenous mitochondrial phospholipids containing polyunsaturated aliphatic chains at the *sn*-2 position. This intriguing observation suggests a role for polyunsaturated lysolipids as a metabolic node in signaling processes promoting the release of arachidonic acid by lysophospholipase activity, the generation of endocannabinoids via 2-AA-monoacylglycerol through the action of lysophospholipase C (28), the production of lysophosphatidic acid (29), or the direct oxidation of the arachidonoyl moiety to provide oxidized lysolipids to effect downstream signaling processes (Fig. 9).

The flux of calcium into mitochondria and the precise regulation of both free and bound Ca^{2+} in the mitochondrial matrix are known modulators of numerous mitochondrial processes, including TCA cycle flux (17, 18), respiration (67–69), inner membrane potential (6), the phospholipase-mediated generation of free fatty acids (6, 42, 43), and the opening of the MPT pore (70). To the best of our knowledge, no known members of the patatin-like phospholipase family of enzymes have been previously demonstrated to be activated by calcium ion. In this study, we demonstrate that iPLA$_2\gamma$ can be activated by the divalent cations Ca^{2+} and Mg^{2+}. Many proteins are activated by divalent cations through a mechanism that typically exploits the bidentate nature of calcium and magnesium coordination to electron-rich residues that can increase the dipole of electrophilic centers such as the carbonyl groups in phospholipids to accelerate nucleophilic attack. In the case of the secretory PLA$_2$s, previous work has emphasized the importance of calcium-mediated polarization of the *sn*-2 carbonyl in phospholipids in facilitating hydrolysis (71, 72). By analogy, it seems likely that the effects of calcium and magnesium are due to formation of divalent complexes with the phospholipid carbonyl and the negatively charged phosphate moiety thereby polarizing the targeted carbonyl for nucleophilic attack by the active site serine residue and accelerating the rate-determining step in hydrolysis. It is tempting to speculate that differences in the hydrolytic activity of iPLA$_2\gamma$ result from differential coordination of the divalent cations with the carbonyl groups at the *sn*-1 or *sn*-2 position in monosaturated *versus* polyunsaturated

FIGURE 7. **Ca^{2+}-dependent eicosanoid production from wild-type, iPLA$_2\gamma$ transgenic, and iPLA$_2\gamma^{-/-}$ myocardial mitochondria.** Myocardial mitochondria were isolated from wild-type (*WT*), iPLA$_2\gamma$ transgenic (*TG*), and iPLA$_2\gamma^{-/-}$ (*KO*) mice and homogenized by sonication. Mitochondria homogenates (1 mg/ml) were preincubated with (*R*)-BEL (20 nmol/mg protein) or DMSO vehicle alone for 10 min at 23 °C and then exposed to either 2 mM EGTA or 6 μM free calcium ion for 20 min. Phospholipase activity and eicosanoid production were terminated by addition of methanol (20% final concentration). Eicosanoids were then isolated by solid phase extraction and derivatized with AMPP. Quantitative analysis was performed by LC/MS/MS via selected reaction monitoring (*SRM*) in the positive ion mode following separation of molecular species using a reverse phase column as described under "Experimental Procedures." Alterations in 12-HETE production are shown in *A* and *B* where *, $p < 0.05$ and **, $p < 0.0001$. ‡, $p < 0.00001$ in *A* when compared with Ca^{2+}-treated WT. Production of other identified eicosanoids is displayed in *C* and *D* where *, $p < 0.05$; **, $p < 0.01$; §, $p < 0.001$; and ¶, $p < 0.0001$ when comparing EGTA *versus* Ca^{2+} treatment (*C*) or WT *versus* KO (*D*). Values are the mean ± S.E. of 3–5 preparations. *LTB_4*, leukotriene B_4; *TXB*, thromboxane B; *PG*, prostaglandin.



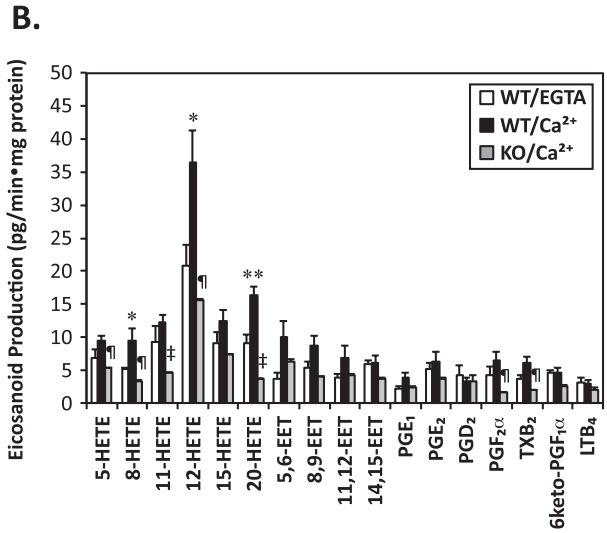


FIGURE 8. **Ca^{2+}-dependent eicosanoid production in intact wild-type, iPLA$_2\gamma$ transgenic, and iPLA$_2\gamma^{-/-}$ myocardial mitochondria.** Myocardial mitochondria were isolated by differential centrifugation from wild-type (*WT*), iPLA$_2\gamma$ transgenic (*TG*), and iPLA$_2\gamma^{-/-}$ (*KO*) mice and resuspended in buffer containing 3 mM HEPES (pH 7.4), 0.23 M mannitol, 0.07 M sucrose, 1 mM KH_2PO_4, 5 mM succinate, and 2.5 μM rotenone. Intact mitochondria were then exposed to either 70 μM Ca^{2+} or 50 μM EGTA and incubated for 10 min at 23 °C. Phospholipase activity and eicosanoid production were terminated by addition of methanol (20% final concentration). Intact mitochondria were homogenized by brief sonication, and eicosanoids were then isolated by solid phase extraction and derivatized with AMPP. Quantitative analysis was performed by LC/MS/MS via selected reaction monitoring (*SRM*) in the positive ion mode following separation of molecular species using a reverse phase column. Ca^{2+}-induced alterations in eicosanoids in wild-type (*WT*), iPLA$_2\gamma$ transgenic (*TG*), and iPLA$_2\gamma^{-/-}$ (*KO*) myocardial mitochondria are shown in *A* and *B* where *, $p < 0.05$; **, $p < 0.01$; and §, $p < 0.001$ when comparing EGTA *versus* Ca^{2+} treatment. ¶, $p < 0.05$, and ‡, $p < 0.005$ in *B* when comparing WT *versus* KO in the presence of Ca^{2+}. Values are the mean ± S.E. of 3–4 independent preparations. *LTB_4*, leukotriene B_4; *TXB*, thromboxane B; *PG*, prostaglandin.

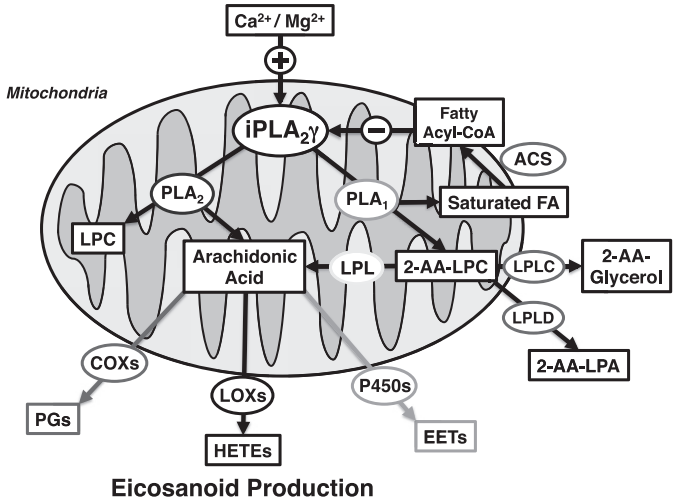


FIGURE 9. **Pleiotropic metabolic and signaling functions of iPLA$_2\gamma$ in mitochondria.** Calcium or magnesium ion activates iPLA$_2\gamma$ resulting in the hydrolysis of mitochondrial arachidonate-containing phospholipids to release arachidonic acid directly (by its PLA_2 activity) or to generate 2-AA-LPC (by its PLA_1 activity). Arachidonic acid liberated through mitochondrial iPLA$_2\gamma$ activation is subsequently oxidized to form multiple downstream eicosanoids such as HETEs by lipoxygenases (*LOXs*), prostaglandins (*PGs*) by cyclooxygenases (*COXs*), and EETs by cytochrome P450 epoxygenases (*P450s*). These eicosanoid metabolites serve as mitokines to regulate both intra- and extramitochondrial functions. Moreover, 2-AA-LPC has multiple metabolic fates, including conversion to 2-arachidonoyl-lysophosphatidic acid (*2-AA-LPA*) by lysophospholipase D (*LPLD*), transformation to the endocannabinoid 2-arachidonoyl-glycerol (*2-AA-glycerol*) by lysophospholipase C (*LPLC*), or hydrolysis by lysophospholipase (*LPL*) to liberate arachidonate. Importantly, fatty acyl-CoA inhibits mitochondrial iPLA$_2\gamma$ thereby establishing an interactive metabolic network that facilitates the coordinated regulation of cellular bioenergetics and signaling in response to external perturbations.

aliphatic chains that are dependent on the degree of unsaturation present in the *sn*-2 aliphatic chain. A corollary of these results is that it may not exclusively be intramitochondrial free calcium that is the predominant effector of iPLA$_2\gamma$ activation but rather the binding of Ca^{2+} to the highly anionic mitochondrial inner membrane bilayer where divalent cation coordination with phospholipid carbonyl moieties can accelerate the rate of hydrolysis. Thus, potential-driven flux of divalent cations into the mitochondrial matrix may serve as a prominent mechanism to initiate mitochondrial signaling through the generation of lipid second messengers as well as alterations in the surface charge, architecture, and dynamics of the mitochondrial inner membrane.

Many prior studies have examined alterations in mitochondrial calcium and their effects on mitochondrial bioenergetics and signaling. During an action potential transient, calcium concentrations in the cytosol typically reach 1–2 μM. However, recent work has emphasized that the intimate spatial relationship between the sarcoplasmic reticulum calcium release channels and the mitochondrial membrane facilitates localized 20–100-fold increases in calcium ion concentration in comparison with that present in the cytoplasm. When specific mitochondrial calcium probes have been targeted to the mitochondria, levels of free calcium ranging from 50 to 200 μM have been reported (73–75). It is important to note that the concentrations of Ca^{2+} and Mg^{2+} employed in this study are similar to previously determined values that occur in beating cells as measured by mitochondrial targeted fluorophores (73–75) and/or are calculated to be present in the intradyadic space where local calcium concentrations may be as high as 200 μM or more (15).

In early studies of the mitochondrial PLA$_2$(s) involved in mediating MPT pore opening, a low molecular weight (9.7 kDa) PLA$_2$ was purified from rat liver mitochondria, which was later identified as a type IIA PLA$_2$ (76, 77). The potential role of this enzyme in catalyzing mitochondrial phospholipid hydrolysis and mediating MPT pore opening, however, has been questioned considering its secretory signal sequence and the lack of a known mitochondrial localization sequence. Work by Pfeiffer and co-workers (6) has demonstrated the presence of a BEL-sensitive iPLA$_2$ activity in rat liver mitochondria. Exposure of hepatic mitochondria to calcium or disruption of the mitochondrial membrane potential resulted in the accumulation of free fatty acids, which promoted mitochondrial swelling by opening of the permeability transition pore (6). In addition, Schnellmann and co-workers (43) identified a phospholipase activity present in rabbit kidney cortex mitochondria that was modestly (~50%) activated by Ca^{2+} and was attenuated by racemic BEL suggesting the participation of a member of the iPLA$_2$ family of enzymes. Although the enzymatic activity generating the observed free fatty acids was categorized as an iPLA$_2$ due to its sensitivity to racemic BEL, this alone could not establish the molecular identity of the iPLA$_2$ family member involved or rule out potential off target effects of racemic BEL (78–81). In addition, the regiospecific hydrolysis of polyunsaturated phospholipids, the calcium sensitivity of iPLA$_2\gamma$ catalytic activity, and the production of downstream eicosanoids were not examined in that study. The results of the current study establish that the vast majority of the calcium-dependent release of polyunsaturated lysophosphatidylcholine molecular species and fatty acids from mitochondrial phospholipids is due to the calcium-mediated activation of mitochondrial iPLA$_2\gamma$.

Remarkably, the activity of iPLA$_2\gamma$ was potently inhibited by submicellar concentrations of fatty acyl-CoA, suggesting an integrated role of this enzyme in regulating substrate utilization, bioenergetic flux, and cellular signaling based upon the concentration of activated (thioesterified) fatty acid availability in the mitochondrial matrix. Inhibition of iPLA$_2\gamma$ by fatty acyl-CoAs may therefore represent a negative regulatory feedback mechanism to attenuate iPLA$_2\gamma$-mediated hydrolysis of phospholipids under conditions of high acyl-CoA content in mitochondria. It should be noted that concentrations of fatty acyl-CoAs in the mitochondrial matrix range from 0.02 to 1 mM depending upon mitochondrial substrate utilization, oxygen tension, bioenergetic demand, and redox state (82, 83). Moreover, acyl-CoA concentrations change dramatically in many disease states, including ischemia, diabetes, and obesity, which are characterized by mitochondrial dysfunction (83–86). Thus, iPLA$_2\gamma$-dependent release of 2-AA-LPC, arachidonic acid, and downstream eicosanoid production initiated in the mitochondrial compartment is anticipated to be coordinately regulated by intramitochondrial divalent cations and fatty acyl-CoA concentrations thereby integrating cellular signaling with mitochondrial bioenergetic function. Similarly, iPLA$_2\gamma$-mediated phospholipid remodeling would also be inhibited under conditions of elevated fatty acyl-CoA concentrations thereby promoting the dysfunctional regulation of membrane structure and dynamics at times of altered calcium ion homeostasis and/or during changes in membrane potential and mitochondrial redox state, which regulate fatty acid catabolic flux. Common disease states where such maladaptive regulation is known to occur resulting in mitochondrial dysfunction include diabetes, obesity, myocardial ischemia/reperfusion, and heart failure. Taken together, these results identify a mechanism by which a central branch point intermediate in lipid metabolism (fatty acyl-CoA) regulates a mitochondrial phospholipase responsible for the production of a multiplicity of lipid second messengers thereby integrating mitochondrial bioenergetics with lipid metabolic flux to coordinately regulate energy storage, utilization, and mitochondrial signaling functions.

Collectively, these results reveal a previously unknown divalent cation-dependent activation of iPLA$_2\gamma$ present in mitochondria that can serve to generate an extensive repertoire of eicosanoid and lysolipid signaling molecules that are known to regulate mitochondrial bioenergetic function, signaling, and ion channel kinetics. Moreover, the present results identify a mechanism through which complex mitochondrial functions can be integrated with multiple processes in other membrane-delimited compartments to collectively orchestrate cardiac myocyte responses to external perturbations in health and disease.

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