Effects of Stable Suppression of Group VIA Phospholipase A2 Expression on Phospholipid Content and Composition, Insulin Secretion, and Proliferation of INS-1 Insulinoma Cells*

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Studies involving pharmacologic inhibition or transient reduction of Group VIA phospholipase A2 (iPLA2) expression have suggested that it is a housekeeping enzyme that regulates cell 2-lyso-phosphatidylcholine (LPC) levels, rates of arachidonate incorporation into phospholipids, and degradation of excess phosphatidylcholine (PC). In insulin-secreting islet β-cells and some other cells, in contrast, iPLA2β signaling functions have been proposed. Using retroviral vectors, we prepared clonal INS-1 cell lines in which iPLA2β expression is stably suppressed by small interfering RNA. Two such iPLA2β knockdown (iPLA2β-KD) cell lines express less than 20% of the iPLA2β of control INS-1 cell lines. The iPLA2β-KD INS-1 cells exhibit impaired insulin secretory responses and reduced proliferation rates. Electrospray ionization mass spectrometric analyses of PC and LPC species that accumulate in INS-1 cells cultured with arachidonic acid suggest that 18:0/20:4-glycerophosphocholine (GPC) synthesis involves sn-1 remodeling via a 1-lyso/20:4-GPC intermediate. The iPLA2β-KD INS-1 cells exhibit impaired insulin secretory responses and reduced proliferation rates. Electrospray ionization mass spectrometric analyses also indicate that the PC and LPC content and composition of iPLA2β-KD and control INS-1 cells are nearly identical, as are the rates of arachidonate incorporation into PC and the composition and remodeling of other phospholipid classes. These findings indicate that iPLA2β plays signaling or effector roles in β-cell secretion and proliferation but that stable suppression of its expression does not affect β-cell GPC lipid content or composition even under conditions in which LPC is being actively consumed by conversion to PC. This calls into question the generality of proposed housekeeping functions for iPLA2β in PC homeostasis and remodeling.

Phospholipases A2 (PLA2) catalyze hydrolysis of the sn-2 fatty acid constituent from glycerophospholipid substrates to yield a free fatty acid (e.g. arachidonic acid) and a 2-lysophospholipid (1, 2) that have intrinsic mediator functions (3, 4) and can initiate synthesis of other mediators (5). Arachidonic acid, for example, is converted to prostaglandins, leukotrienes, and epoxyprostagens, and acetylation of 2-lysophosphatidylcholine yields platelet-activating factor (PAF) (5).

Secretory PLA2 (sPLA2) are low molecular weight enzymes that require millimolar [Ca2+] for catalysis and affect inflammation and other processes, and the PAF-acetylhydrolase PLA2 family exhibits substrate specificity for PAF and oxidized phospholipids (1). Of Group IV cytosolic PLA2 (cPLA2) family members (1), cPLA2α was the first identified and prefers substrates with sn-2 arachidonoyl residues, catalyzes arachidonate release for subsequent metabolism, associates with its substrates in membranes upon rises in cytosolic [Ca2+], and is also regulated by phosphorylation (6). There are additional members of the cPLA2 family that arise from separate genes (7–10).

The Group VI PLA2 (iPLA2) enzymes (11–13) do not require Ca2+ for catalysis and are inhibited by a bromoelanol lactone (BEL) suicide substrate (14) that does not inhibit sPLA2 or cPLA2 at similar concentrations (14–17). The Group VIA PLA2 (iPLA2β) resides in the cytoplasm of resting cells, but Group VIB PLA2 contains a peroxisomal targeting signal and is membrane-associated (18, 19). These enzymes belong to a larger class of serine lipases that are encoded by multiple genes (20, 21). The iPLA2β enzymes cloned from various species are 84–88-kDa proteins that contain a GXXG lipase consensus sequence and eight stretches of a repetitive motif homologous to that in the protein-binding domain of ankyrin (11–13).

It has been proposed that iPLA2β plays housekeeping roles in phospholipid metabolism (22, 23), such as generating lysophospholipid acceptors for incorporating arachidonic acid into phosphatidylcholine (PC) of murine P388D1 macrophage-like cells, based on studies involving reducing iPLA2β activity with BEL or an antisense oligonucleotide, which suppresses [3H]arachidonate incorporation into PC and reduces [3H]lysophosphatidylcholine (LPC) levels (24, 25). Arachidonate incorporation involves a deacylation/reacylation cycle of phospholipid remodeling (26, 27), and the level of LPC is thought to limit the [3H]arachidonate incorporation rate into P388D1 cell PC (24, 25).

Another housekeeping function for iPLA2β in PC homeostasis has been proposed from studies of overexpression of CTP:phosphocholine cytidylyltransferase (28, 29), which catalyzes the rate-limiting step in PC synthesis. Cells that overexpress CTP:phosphocholine cytidylyltransferase exhibit increased rates of PC biosynthesis and degradation and little net change in PC levels, suggesting that PC degradation is up-regulated to prevent excess PC accumulation. Increased PC degradation in CTP:phosphocholine cytidylyltransferase-overexpressing cells is prevented by BEL, and iPLA2β protein and activity increase, suggesting that iPLA2β is up-regulated (28, 29).

If general, this could be important, because PC synthesis is involved in regulating the cell cycle and apoptosis (30, 31), but studies involving iPLA2β overexpression give a different perspective on this issue (32, 33).
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The activity of iPLA$_2$B has been reported to vary with the cell cycle, to be required for proliferative responses in lymphocyte cell lines (34, 35), and to play a role in membrane biogenesis (36).

Many other iPLA$_2$B functions have been proposed (37–50), and the facts that multiple splice variants are differentially expressed among cells and form hetero-oligomers with distinct properties suggest that iPLA$_2$B gene products might have multiple functions (49–51). Proposed functions include signaling in secretion (47, 48, 52–57), and BEL attenuates glucose-induced insulin secretion, arachidonate release, and rises in cytosolic $[Ca^{2+}]$ in pancreatic islet $\beta$-cells and insulinoma cells (48, 52–57).

Many cells, including $\beta$-cells, express multiple distinct PLA$_2$ enzymes (13, 16, 17, 58–60), which might reflect redundancy or specific functions of individual PLA$_2$s. The mechanism-based iPLA$_2$ inhibitor BEL and its enantiomers inhibit iPLA$_2$, at concentrations lower than those required to inhibit sPLA$_2$, or cPLA$_2$ (14–17, 39), and this has been widely exploited to discern potential biological roles for iPLA$_2$ (37–57, 61–63). BEL also inhibits enzymes other than iPLA$_2$, however, including serine proteases (64) and phosphatidate phosphohydrolase-1 (65), which accounts for some of its biological effects (66). In addition, BEL inhibits Group VIB PLA$_2$ (18) and at least four other serine lipases (20, 21).

The ambiguity of pharmacologic studies with BEL makes manipulating iPLA$_2$B expression by molecular biologic means an attractive alternative to study iPLA$_2$B functions. Transient suppression of iPLA$_2$B activity with antisense oligonucleotides has been useful in monocyte-macrophages and vascular myocytes (25, 37, 41, 42, 47), but substantial suppression of expression is not readily achieved in all cells in this way. Insulinoma cell lines experience toxicity from antisense oligonucleotides at concentrations that fail to reduce iPLA$_2$B activity (66).

Physiological roles for PLA$_2$s can also be studied with genetic gain- or loss-of-function manipulations. Transgenic overexpression of iPLA$_2$B in cardiac myocytes indicates a role in ventricular arrhythmias (67), and stable iPLA$_2$B overexpression in insulinoma cells has provided evidence for participation in exocytosis, cell proliferation, and apoptosis (32, 68, 69). Transient suppression of Group VI PLA$_2$ expression has been achieved with small interfering RNA (siRNA) in some cells (48, 59), and we have now prepared stably transfected insulinoma cells in which siRNA directed against iPLA$_2$B mRNA suppresses iPLA$_2$B expression. These cell lines have been used to examine iPLA$_2$B participation in insulin secretion, cell proliferation, and regulation of phospholipid content and composition.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-hydroxy-sn-glycerophosphocholine (16:0-LPC), 18:0-LPC, 1,2-dimyristoyl-sn-glycerophosphocholine (14:0/14:0-GPC), 20:4/20:4-GPC, 18:0/22:6-GPC, and other phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). (1–20:4, 2-lyso)-GPC was prepared from 20:4/20:4-GPC with phospholipase A$_2$ from Naja naja (Sigma). Arachidonic acid was obtained from Nu-Chek Prep (Elysian, MN); [5,6,8,9,11,12,14,15-3H]arachidonic acid (100 Ci/mmol) was from Amersham Biosciences; BEL (E=6-(bromomethyl)tetrahydro-3-(1-naphthalenyl)2H-pyran-2-one was from Cayman Chemical (Ann Arbor, MI); culture media (PRMI-1066 and Dulbecco’s modified Eagle’s medium), penicillin, streptomycin, and$L$-glutamine were from Invitrogen (Carlsbad, CA); fetal bovine serum was from Hyclone (Logan, UT); Pentex bovine serum albumin (fatty acid-free, fraction V) was from ICN Biomedical (Aurora, OH); ATP, ampicillin, and kanamycin were from Sigma; and forskolin was from Calbiochem. Krebs-Ringer bicarbonate buffer (KRB) contained 25 mM HEPES (pH 7.4), 115 mM NaCl, 24 mM NaHCO$_3$, 5 mM KCl, and 1 mM MgCl$_2$.

Cell Culture—INS-1 insulinoma cells were cultured as described (66, 68) in RPMI 1640 medium. RetroPack PT 67 cells (Clontech) were maintained in Dulbecco’s modified Eagle’s medium (4.5 mg/ml glucose) containing 10% fetal bovine serum, 4 mL/l-Gln, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin.

Establishing iPLA$_2$B Knockdown INS-1 Insulinoma Cell Lines Using siRNA (70) —Two hairpin-forming oligonucleotides directed against iPLA$_2$B mRNA were cloned into RNAi-Ready pSIREN Retro-Q as per the manufacturer’s protocol (BD Biosciences Clontech). Targeting sequences within the synthetic oligonucleotides are italicized and underlined below. The sequence of the first was gatceGACAGCAAGA ATGAGAGTTCAGAGACTCTCATTCTGTGCTGTTTT-

TTTG. The second oligonucleotide was gatceGGTGAAGAGGT-

gAACAAATTCAAGAGATTGGTACCTGATTCAGGCTTTTTG. Constructs that express the siRNAs are pSIREN-iPLA$_2$-1 and pSIREN-
iPLA$_2$-2. Retroviruses were packaged in PT67 cells and used to infect INS-1 cells. Cells were selected with 0.4 $\mu$g/ml puromycin. A construct that encoded scrambled RNA was used to prepare control INS-1 cell lines.

Analyses of INS-1 Cell iPLA$_2$B mRNA—Northern blots of iPLA$_2$B mRNA were performed as described (71). For quantitative real time reverse transcription-PCR, total RNA was isolated with an RNAeasy kit (Qiagen Inc.). The SuperScript First Strand Synthesis System (Invitrogen) was used to synthesize cDNA in 20-$\mu$L reactions that contained DNase I-treated total RNA (2 $\mu$g). The cDNA product was treated (20 min, 37 °C) with RNase H (2 units; Invitrogen) and heat-inactivated (70 °C for 15 min). A reaction without reverse transcriptase was performed to verify the absence of genomic DNA. PCR amplifications were performed using SYBR Green dye in an ABI 7000 detection system (Applied Biosystems). Product sizes were determined on 3% (w/v) agarose-TAE gels.

Assay of INS-1 Cell iPLA$_2$B Activity—$Ca^{2+}$-independent PLA$_2$-specific activity was determined in cytosol by monitoring hydrolysis of 16:0/18:2-GPC [14C]linoleate as measured by TLC and liquid scintillation spectrometry as described (55).

Determination of Insulin Secretion by INS-1 Cells—At confluence, INS-1 cells were detached from T75 flasks, and aliquots (10$^5$ cells) were added to 24-well plates. Culture medium was removed the next day, and cells were washed twice in KRB containing 1 mM glucose and 0.1% bovine serum albumin. Cells were incubated (1 h, 37 °C, under 95% air, 5% CO$_2$), and medium was then removed to measure insulin by radioimmunoassay. Secretion was normalized to cell protein measured with Coomassie reagent (Pierce) (72).

Determination of INS-1 Cell Proliferation Rate—One assay used to measure INS-1 cell proliferation rates is based on fluorescence enhancement when CyQuant GR binds to nucleic acids, which reflects the amount of cell DNA (73). Cells were seeded onto 96-well plates (3 $\times$ 10$^3$ cells/well). Medium was removed after 1 or 3 days, and cells were frozen (−20 °C). DNA was measured with a CyQuant assay kit (Molecular Probes, Inc., Eugene, OR) with reference to a standard curve. CyQuant GR solution (200 $\mu$L) was added to each well and incubated (5 min, room temperature). Fluorescence was measured on a microplate fluorimeter (excitation, 480 nm; emission, 538 nm). A second assay is based on incorporation of thymidine analog 5-bromo-2'-deoxyuridine (BrdUrd) into DNA in proliferating cells (74). Cells were seeded (10$^4$ cells/well) and cultured (3 days) before assay with BrdUrd labeling and
an enzyme-linked immunoassay detection kit III (Roche Applied Science).

Extraction INS-1 Cell Phospholipids and Quantitation of Phosphorus—Lipids were extracted (75), and their lipid phosphorus content was measured (76), as described.

Incubation of INS-1 Cells with [3H]Arachidonic Acid—Incubation studies involved [3H]arachidonic acid addition (final concentration 0.5 μCi/ml, 5 nCi) to medium and incubation (10–60 min, 37 °C). Cells were washed three times in KRB containing 5.5 mM glucose and 0.1% bovine serum albumin to remove unincorporated [3H]arachidonate. Cell viability exceeded 98% by trypan blue exclusion. [3H]Arachidonate incorporation into phospholipid extracts was then determined by TLC and liquid scintillation spectrometry (66).

Electrospray Ionization Mass Spectrometric Analyses of Glycerophosphocholine Lipids—PC and LPC were analyzed as Li+ adducts by positive ion ESI/MS on a Finnigan (San Jose, CA) TSQ-7000 triple stage quadrupole mass spectrometer with an ESI source controlled by Finnigan ICIS software. Phosphoglycerides were dissolved in methanol/chloroform (2:1, v/v) containing LiOH (10 pmol/μl), infused (1 μl/min) with a Harvard syringe pump, and analyzed as described (77–79). For tandem MS, precursor ions selected in the first quadrupole were accelerated (32–36-eV collision energy) into a chamber containing argon (2.3–2.5 millitorrs) to induce collisionally activated dissociation (CAD), and product ions were analyzed in the final quadrupole. The identities of GPC species were determined from their tandem spectra (77–79), and their quantities were determined relative to the internal standards 14:0/14:0-GPC and 18:0/22:6-GPC by interpolation from a standard curve (66, 80). To quantitate LPC species, constant neutral loss of 59 scanning was performed. The intensity of the ion for the 14:0/14:0-GPC internal standard (m/z 684) was compared with intensities of ions for endogenous 16:0-LPC (m/z 502), 18:1-LPC (m/z 528), 18:0-LPC (m/z 530), and 20:4-LPC (m/z 550). Standard curve studies in which a constant amount of 14:0/14:0-GPC and varied amounts of 16:0-, 18:1-, and 18:0-LPC were added to a series of tubes and analyzed as Li+ adducts by ESI/MS/MS were linear over a wide range that included levels in INS-1 cells.

Incubating INS-1 Cells with Arachidonic Acid to Induce Phospholipid Remodeling—INS-1 cells (1.2 × 10⁶/condition) were cultured in RPMI containing penicillin, streptomycin, fungizone, and gentamicin (0.1%, w/v) each. Cells were then cultured (37 °C) in fresh medium containing no other supplements or containing various concentrations of arachidonic acid (0, 1, 5, 10, 30, or 70 μM). After 0, 6, or 24 h, cells were washed twice with PBS, suspended in homogenization buffer, and disrupted by sonication. Lipids were extracted (75) and analyzed by ESI/MS/MS.

INS-1 Cell Lysophosphatidylcholine Analyses—INS-1 cells were washed twice, resuspended, and incubated (30 min, 37 °C) in KRB containing 5.5 mM glucose and 0.1% bovine serum albumin. Cells were then placed in fresh medium, incubated (30 min, 37 °C), washed twice with phosphate-buffered saline, and extracted (75). The aqueous phase contained 150 mM LiCl, and the chloroform phase contained a 14/0/14-GPC internal standard. Concentrated extracts were analyzed by silica gel G TLC on heat-activated (30 min, 80 °C) plates with chloroform/methanol/ammonium hydroxide (65/30/0.8) to separate LPC (RF = 0.38), LPE (RF = 0.46), and PC (RF = 0.60). LPC was analyzed by ESI/MS/MS as described (77–79).

ESI/MS/MS Analyses of Anionic Phospholipids—Glycerophosphoethanolamine (GPE), glycerophosphoglycerol (GPG), and glycerophosphoinositol (GPI) were analyzed as [M – H]+ ions by negative ion ESI/MS/MS (69, 72) relative to a 14/0/14:0-GPC internal standard (80), and their tandem spectra were obtained as described.

Knockdown of iPLA₂β Reduces Secretion and Proliferation

FIGURE 1. Suppression of iPLA₂β expression in iPLA₂β knockdown INS-1 cells. INS-1 cell lines were prepared with retroviral vectors containing an insert encoding scrambled RNA (control) or siRNA against iPLA₂β mRNA to generate iPLA₂β knockdown cell lines, and iPLA₂β mRNA was analyzed by Northern blots (A, lane 1, vector control; lane 2, iPLA₂β-KD1; lane 3, iPLA₂β-KD2; lane 4, parental cells) and real time PCR (B). Activity of iPLA₂β (C) was measured without Ca²⁺ in the presence of EGTA and without (open bars) or with 1 mM ATP alone (cross-hatched bars) or with ATP and 10 μM BEL (solid bars). The leftmost bar (B) or set of bars (C) reflects control cells, and the center and rightmost bar or set of bars reflects iPLA₂β-KD1 and iPLA₂β-KD2 cells, respectively.

Statistical Analyses—Two groups were compared by Student’s t test, and multiple groups were compared by one-way analysis of variance with post hoc Newman-Keul’s analyses.

RESULTS

Establishing iPLA₂β Knockdown Cell Lines—INS-1 insulinoma cells were infected with retroviral constructs containing inserts that produced either scrambled RNA (control) or siRNA directed against sequences in iPLA₂β mRNA. Selection of puromycin-resistant cells resulted in isolation of two clones that had stably incorporated knockdown constructs and expressed less than 20% of the control cell iPLA₂β mRNA content when analyzed by Northern blots (Fig. 1A) or by real time PCR (Fig. 1B). The iPLA₂β activity in control INS-1 cells was stim-
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Knockdown of iPLA2β activity in islets and other insulinoma cells (13). The iPLA2β knockdown (iPLA2β-KD) cell lines exhibited reduced iPLA2β activity, and the reduction of activity was comparable with that of iPLA2β mRNA. The iPLA2β expression level was a stable property of control and iPLA2β-KD INS-1 cells that persisted on serial passage in culture.

Insulin Secretion Is Attenuated in iPLA2β Knockdown Cells—Glucose is the dominant insulin secretagogue, and pancreatic islets and insulinoma cells exhibit greater insulin secretory responses in the presence of forskolin (32, 68). When treated with forskolin (2.5 μM), control INS-1 cells secreted more insulin than either iPLA2β-KD INS-1 cell line (Fig. 2). This effect increased with the medium glucose concentration over the range of 3–20 mM. Reduced insulin secretory responses were observed with both iPLA2β-KD cell lines under all conditions, supporting the proposal that iPLA2β participates in insulin secretion (2, 32, 68).

Cell Proliferation Rate in iPLA2 Knockdown Cells—Pharmacologic inhibition of iPLA2 reduces cell proliferation rates, and iPLA2β overexpression results in increased proliferation (32, 34, 35). It might thus be predicted that iPLA2β-KD cells would proliferate less rapidly than control INS-1 cells. To test this possibility, INS-1 cell proliferation was measured using an indicator that exhibits strong fluorescence enhancement upon association with nucleic acids (73). Identical numbers of INS-1 cells were seeded at time 0, and their growth rates were monitored for 1–3 days. INS-1 iPLA2β-KD lines proliferated at rates that were significantly lower than those for control INS-1 cells (Fig. 3A). Proliferation was also measured by BrdUrd incorporation into DNA (74). INS-1 cells were again seeded in identical numbers at time 0, and the increase in BrdUrd signal was monitored for 3 days. INS-1 iPLA2β-KD lines were again found to proliferate more slowly than control INS-1 cells, and similar results were obtained when seeding was performed at either of two different initial cell densities at time 0 (Fig. 3B).

Incorporation of [3H]Arachidonic Acid into INS-1 Cell Phospholipids—[3H]arachidonate incorporation in murine macrophage-like P388D1 cells is reduced by pharmacologic inhibition of iPLA2β or by antisense suppression of its expression, suggesting that iPLA2β plays a housekeeping role in phospholipid remodeling (22–25). To determine whether iPLA2β plays this role in β-cells, we compared [3H]arachidonic acid incorporation into INS-1 cell phospholipids. Control (closed symbols) or iPLA2β-KD (open symbols) INS-1 cells were preincubated (30 min, 37 °C) and then incubated with [3H]arachidonate acid for 10–60 min. The [3H] content of extracted phospholipids was then determined and expressed as dpm/10^5 cells (n = 6).
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Acid incorporation into control and iPLA₂β-KD INS-1 cells and observed indistinguishable time courses (Fig. 4).

**ESI/MS Analyses of INS-1 Cell GPC Lipids**—iPLA₂β has been proposed to regulate GPC lipid fatty acid composition (22–25) and to cooperate with CTP:phosphocholine cytidylyltransferase to maintain cell PC content at appropriate levels (28, 29). This suggests that suppressing iPLA₂β expression might perturb the amount or composition of PC, and we evaluated this possibility with quantitative ESI/MS analyses of GPC lipids from control and iPLA₂β-KD INS-1 cells as Li⁺ adducts (77–79), using both 14:0/14:0-GPC and 18:0–22:6 as internal standards to correct for the effects of fatty acid chain length and unsaturation on ESI/MS ion intensity [81].

The most abundant ion in mass spectra of control INS-1 cell GPC lipids is m/z 766 (Fig. 5A) and represents 16:0/18:1-GPC [M + Li]⁺, as shown by ions in its tandem spectrum (77–79). Identities of other GPC lipids represented by ions in Fig. 5A were similarly determined and include 18:0/18:1-GPC (m/z 794), 16:1/18:1-GPC (m/z 792), 16:0/18:1-GPC (m/z 764), and 16:0/16:1-GPC (m/z 738). The ions of m/z 684 and m/z 840 represent the internal standards 14:0/14:0-GPC and 18:0/22:6-GPC, respectively. Fig. 5B shows the ESI/MS spectrum of GPC lipid Li⁺ adducts from iPLA₂β-KD cells and is virtually identical to that for control INS-1 cells (Fig. 5A). Table 1 shows that amounts of GPC lipid species are nearly identical in control and iPLA₂β-KD INS-1 cells.

**ESI/MS/MS Analyses of Arachidonic Acid Incorporation into INS-1 Cell GPC Lipids**—Arachidonic acid-containing GPC lipids are not abundant in INS-1 cells cultured in standard medium, as reflected by the low abundance of ions at m/z 788 and 816 (Figs. 5, A and B) that correspond to 16:0/20:4-GPC and 18:0/20:4-GPC [M + Li]⁺, respectively. Fig. 4 shows that [³H]arachidonic acid is readily incorporated into INS-cell phospholipids at nanomolar concentrations within minutes, and the possibility that suppressing iPLA₂β expression in iPLA₂β-KD cells might impair GPC lipid remodeling and synthesis of arachidonate-containing species, as suggested for some cells (22–25), was examined rigorously by ESI/MS (Figs. 5–8).

Li⁺ adducts of GPC lipids from control and iPLA₂β-KD INS-1 cells incubated with various concentrations of arachidonic acid for 24 h were analyzed by ESI/MS/MS scanning for parent ions that undergo loss of phosphorus (P) was measured. Internal standard 14:0/14:0-GPC was added, and amounts of glycerophosphocholine lipid species in INS-1 cells with different levels of iPLA₂β expression were similarly determined and are tabulated.

**TABLE 1**

| Value of m/z  | Predominant GPC species | Control amount | iPLA₂β-KD amount |
|--------------|-------------------------|----------------|-----------------|
| 738          | 16:0/16:1-GPC           | 366 ± 37       | 322 ± 32        |
| 764          | 16:1/18:1-GPC           | 115 ± 11       | 107 ± 11        |
| 766          | 16:0/18:1-GPC           | 234 ± 24       | 214 ± 21        |
| 788          | 16:0/20:4-GPC           | 19 ± 2         | 19 ± 2          |
| 792          | 18:1/18:1-GPC           | 152 ± 15       | 121 ± 12        |
| 794          | 18:0/18:1-GPC           | 70 ± 7         | 72 ± 7          |
| 814          | 18:1/20:4-GPC           | 19 ± 2         | 20 ± 2          |
| 816          | 18:0/20:4-GPC           | 14 ± 1         | 14 ± 1          |

FIGURE 5. Electrospray ionization mass spectrometric analyses of INS-1 cell glycerophosphocholine lipids. Phospholipids from control (A) or iPLA₂β-KD1 INS-1 cells (B) were analyzed as Li⁺ adducts by positive ion ESI/MS. In C and D, iPLA₂β-KD1 INS-1 cells were incubated for 24 h with 10 or 30 μM supplemental arachidonic acid, respectively, in the culture medium before lipids were extracted and analyzed.
FIGURE 6. Tandem mass spectra of arachidonate-containing glycerophosphocholine lipids in INS-1 cells cultured with arachidonic acid. GPC lipid-Li²⁺ adducts from control or iPLA₂β-KD1 INS-1 cells incubated for 24 h with arachidonic acid were analyzed by positive ion ESI/MS/MS. In A and B, the ion m/z 788 was subjected to CAD, and product ions were analyzed. A, ions from m/z 50 to 800; B, displays ions from m/z 300 to 620. In C and D, the ion at m/z 816 was subjected to CAD, and product ions were analyzed and displayed as above.

FIGURE 7. Electrospray ionization mass spectrometric analyses of glycerophosphocholine lipids in iPLA₂β knockdown and control INS-1 cells incubated with arachidonic acid for various intervals. Control (A–C) or iPLA₂β-KD (D–F) INS-1 cells were incubated with arachidonic acid for 0 h (A and D), 6 h (B and E), or 24 h (C and F). Extracted GPC lipid-Li⁺ adducts were analyzed by positive ion ESI/MS/MS scanning for constant neutral loss of 183.
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FIGURE 8. Time course and concentration dependence of appearance of arachidonate-containing glycerophosphocholine lipids in INS-1 cells incubated with arachidonic acid. Control (closed symbols) or iPLA$_2$-KD INS-1 cells (open symbols) were incubated with 70 mM arachidonic acid for 0, 6, or 24 h (A) or with varied concentrations (0, 1, 5, 10, 30, or 70 mM) arachidonic acid for 24 h (B). Extracted GPC lipids were analyzed as Li$^+$ adducts by positive ion ESI/MS. The fraction of total ion current represented by 16:0/20:4-GPC (m/z 788), 18:1/20:4-GPC (m/z 814), and 18:0/20:4-GPC (m/z 816) was then determined for each condition. Displayed values are mean ± S.E. (n = 6). No values were significantly different between control and iPLA$_2$-KD INS-1 cells except for the arachidonate incorporation value at 5 mM arachidonic acid in B (p < 0.05).

183, reflecting net elimination of phosphocholine. This facilitates visualizing accumulation of arachidonate-containing GPC lipids because they eliminate phosphocholine in preference to its Li$^+$ salt (Fig. 6), but the converse is true for GPC lipids with shorter side chains or fewer double bonds (77–79). Such MS/MS scans indicate that 16:0/20:4-GPC (m/z 788) and 18:0/20:4-GPC (m/z 816) are not abundant in un-supplemented INS-1 cells (Fig. 5, A and B), but the abundances of [MLi]$^+$ ions of these compounds increased after 24 h of culture with 10 mM arachidonic acid (Fig. 5C) and increased further upon culture with 30 mM arachidonic acid (Fig. 5D).

That ions m/z 788 and 816 represent 16:0/20:4- and 18:0/20:4-GPC rather than regioisomers or isobaric compounds is shown by their tandem spectra (Fig. 6). CAD of [MLi]$^+$ (m/z 788) of 16:0/20:4-GPC yields spectra (Fig. 6, A and B) that contain ions reflecting neutral losses of trimethylamine plus either the sn-1 or sn-2 substituent (MLi$^+$ – 315) or the sn-2 substituent (MLi$^+$ – 363) as free fatty acids at m/z 473 and 425, respectively. The former is more abundant than the latter, indicating that palmitate and arachidonate are the sn-1 and sn-2 substituents, respectively. Fig. 6, A and B, also shows neutral losses of the sn-1 substituent as a free fatty acid (MLi$^+$ – 284) or as a Li$^+$ salt (MLi$^+$ – 290) at m/z 532 and 526, respectively. Neutral losses of the sn-2 substituent as a free fatty acid (MLi$^+$ – 304) or as a Li$^+$ salt (MLi$^+$ – 310) are seen at m/z 484 and 478, respectively.

The ion m/z 313 (MLi$^+$ – 475) in Fig. 6, A and B, reflects net elimination of LiPO$_4$(CH$_2$)$_2$N(CH$_3$)$_3$ and loss of the sn-2 substituent as a ketone. An analogous m/z 341 ion (MLi$^+$ – 475) is seen in the tandem spectrum 18:0/20:4-GPC-Li$^+$ (Fig. 6, C and D). Other diagnostic ions include those for loss of trimethylamine (m/z 729) or net loss of phosphocholine or its Li$^+$ salt from [M + Li]$^+$ at m/z 605 and 599, respectively; loss of the sn-1 substituent as a free fatty acid or a Li$^+$ salt (m/z 532 and 526, respectively); loss of trimethylamine plus the sn-1 substituent as a free fatty acid (m/z 473); and loss of trimethylamine plus the sn-2 substituent as a free fatty acid (m/z 506).

Upon culture with arachidonic acid, the abundances of [MLi]$^+$ ions of 16:0/20:4-GPC (m/z 788) and 18:0/20:4-GPC (m/z 816) increase after 6 h (Fig. 7B) and are the most abundant in the spectra after 24 h (Fig. 7C). The time course of accumulation is virtually identical in iPLA$_2$-KD (Fig. 7, D–F) and control INS-1 cells (Figs. 7, A–C), which rigorously confirms radiochemical evidence (Fig. 4) that stable suppression of iPLA$_2^\beta$ expression does not impair arachidonate incorporation into iPLA$_2^\beta$-KD INS-1 cells.

Expressing accumulation of 20:4-containing GPC species as the sum of ion currents for m/z 788 (16:0/20:4-GPC), m/z 814 (18:1/20:4-GPC), and m/z 816 (18:0/20:4-GPC) divided by the total ion current shows that such species accumulate at least as rapidly in iPLA$_2$-KD as in control INS-1 cells (Fig. 8A). Similarly, the concentration dependence of arachidonate incorporation into GPC lipids indicates that iPLA$_2^\beta$-KD cells incorporate arachidonate at least as readily as do control INS-1 cells (Fig. 8B). The findings illustrated in Fig. 8 conform over a wide range of concentrations and incubation periods the radiochemical evidence (Fig. 4) that stable suppression of iPLA$_2^\beta$ does not impair arachidonate incorporation into iPLA$_2^\beta$-KD INS-1 cells.

ESI/MS Analyses of LPC Species in INS-1 Cell Lines Incubated with Arachidonic Acid—It has been proposed that iPLA$_2^\beta$ both regulates levels of 2-LPC, which serves as precursor to 20:4-GPC lipids (22–25) and degrades excess PC to maintain homeostasis (29, 30). It might thus be predicted that suppressing iPLA$_2^\beta$ expression would reduce LPC levels, as reported in P388D1 cells after decreasing iPLA$_2^\beta$ activity (24, 25).

INS-1 cell LPC levels were thus measured with an internal standard by ESI/MS/MS scanning for loss of trimethylamine, which greatly increases the signal/noise ratio (66, 78, 79) and permits LPC measure-
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FIGURE 9. Electrospray ionization mass spectrometric analyses of INS-1 cell lysophosphatidylcholine species. Extracted lipids from control (A) or iPLA$_2$αβ-KD1 INS-1 cells (B) at time 0 or after 24 h (D) of incubation with arachidonic acid were analyzed as Li$^+$ adducts by positive ion ESI/MS/MS for neutral loss of 59. In C, the ion m/z 528 from ESI/MS analyses in A or B was subjected to CAD, and product ions were analyzed. D, the ESI/MS/MS constant neutral loss of 59 scan for LPC from INS-1 cells incubated for 24 h with arachidonic acid.

The most abundant ions in such scans of LPC Li$^+$ adducts from resting control (Fig. 9A) and iPLA$_2$αβ-KD (Fig. 9B) INS-1 cells correspond to 16:0-LPC (m/z 502) and 18:1-LPC (m/z 528), respectively. The ion m/z 530 (18:0-LPC) is of low abundance, although in such scans from pancreatic islets, the most abundant ions are m/z 502 and 530 (66). This is consistent with the facts that 18:0-GPC species in INS-1 cells (Table 1) are less abundant than in islets (81). INS-1 cells cultured in standard medium contain little polyunsaturated fatty acid in GPC lipids (Table 1) are less abundant than in islets (81). INS-1 cells cultured in standard medium contain little polyunsaturated fatty acid in GPC lipids (Table 1) are less abundant than in islets (81).

Culturing INS-1 and expressing high levels of stearoyl-CoA desaturase-1, which converts 16:0 and 18:0 to 16:1 and 18:1, respectively (72, 82). INS-1 cells with arachidonic acid expression level is not the major regulator of their LPC content. Interestingly, incubating either control or iPLA$_2$αβ-KD INS-1 cells with arachidonic acid caused accumulation of material represented by an ion m/z 550 (Fig. 9D).

Little difference in the LPC content of resting control and iPLA$_2$αβ-KD INS-1 cells was observed (Fig. 9, A and B), indicating that iPLA$_2$αβ expression level is not the major regulator of their LPC content. Interestingly, incubating either control or iPLA$_2$αβ-KD INS-1 cells with arachidonic acid caused accumulation of material represented by an ion m/z 550 (Fig. 9D).

That m/z value is consistent with 20:4-LPC-Li$^+$, but, in attempts to obtain a CAD spectrum, the ion of m/z 550 was resistant to fragmentation at a collision energy of 32 eV, which produced ready fragmentation of other LPC species (Fig. 9C), or at higher values. There was modest attenuation of the parent ion and limited production of fragment ions (Fig. 10A). When this material was stored for several days or heated for 2 h, the CAD spectrum in Fig. 10B was obtained, which is similar to that reported for 1–20:4/2-lyso-GPC (79) and to that obtained from standard 1–20:4/2-lyso-GPC prepared from sPLA$_2$ from N. naja venom (Fig. 10C).

Fig. 10, B and C, identifies the parent ions as 1–20:4/2-lyso-GPC-Li$^+$, because they contain the Li$^+$-arachidonic acid ion (m/z 311) that identifies the fatty acid substituent; ions reflecting losses of trimethylamine (m/z 491) and of phosphocholine with H$^+$ or Li$^+$ (m/z 367 and 361, respectively) that identify the head group; and ions characteristic of
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Acyl-LPC species, including choline ion (m/z 104), loss of choline to yield a cyclized phosphate diester ([MLi-103]⁺, m/z 447), loss of arachidonate as a substituted ketene from m/z 447 to yield m/z 161, and a Li⁺-cyclic ethylene phosphate ion (m/z 131) (80). The relative abundance of ions m/z 104 and 367 reveal an sn-1 fatty acid substituent (79). It is believed that the material represented in Fig. 10A is 1-lyso-2-20:4-GPC and that it isomerizes upon storage or heating to yield 1-20:4/2-lyso-GPC. Acyl migration readily occurs in 2-monocacylphosphoglycerides to form the more stable primary ester (83).

Culturing INS-1 cells with arachidonic acid caused accumulation of 20:4-LPC by 6 h in both control and iPLA₂β-KD INS-1 cells that was not associated with a decline in other LPC species (Fig. 11A), although synthesis and accumulation of 20:4-GPC species occurred during this period (Figs. 5–8), presumably via consumption of 16:0-LPC, 18:1-LPC, and 18:0-LPC. INS-1 cells must thus have mechanisms to replenish LPC not affected by suppressing iPLA₂β expression, because 16:0-LPC, 18:1-LPC, and 18:0-LPC levels are maintained in iPLA₂β-KD INS-1 cells at values no less than those for control cells (Fig. 11A).

Continued culture of INS-1 cells with arachidonic acid for 24 h causes some further rise in 20:4-LPC levels, but other LPC species remain nearly constant (Fig. 11B), despite the fact that continued accumulation of 20:4-GPC species, and presumably consumption of LPC, occurs between 6 and 24 h (Figs. 5–8). The increasing abundance of 20:4-LPC (Fig. 11) must reflect its more rapid generation than consumption in synthetic or degradative reactions. No accumulation of 20:4/20:4-GPC (m/z 836 for MLi⁺) was observed.

ESI/MS Analyses of GPE, GPG, and GPI Lipids in INS-1 Cells Incubated with Arachidonic Acid—Arachidonic acid first incorporated into PC is then transferred to other phospholipids (66, 84). When cultured with arachidonic acid, both control and iPLA₂β-KD INS-1 cells replaced GPE, GPG, and GPI species that contained sn-2 18:1 with species that contained sn-2 20:4 without obvious differences between the cell lines (Fig. 12).

In resting iPLA₂β-KD (Fig. 12A) and control (Fig. 12C) INS-1 cells, negative ion ESI/MS analyses of lipid extracts revealed abundant ions for 16:0/18:1-GPE (m/z 716), 18:0/20:4-GPE (m/z 766), 16:1/18:1-GPG (m/z 745), 16:0/18:1-GPG (m/z 747), 18:1/18:1-GPG (m/z 773), 16:1/18:1-GPI (m/z 835), 18:1/18:1-GPI (m/z 863), and 18:0/20:4-GPI (m/z 885). Upon culture with arachidonate, ions representing 18:1-containing phospholipids declined markedly, and ions representing 20:4-containing species increased (e.g. m/z 795 (18:1/20:4-GPG), m/z 817 (20:4/20:4-GPG), and m/z 885 (18:0/20:4-GPI)) (Fig. 12, B and D). Lipid identities were assigned from tandem mass spectra (85, 86).

DISCUSSION

We have generated clonal INS-1 iPLA₂β-KD cell lines that stably express siRNA that reduces iPLA₂β expression to less than 20% of control INS-1 cell levels, and this property is stable on serial passage. This provides a tool to study β-cell iPLA₂β function, which is important because the best iPLA₂β pharmacologic inhibitor (BEL) also inhibits other enzymes (17, 18, 20, 21, 64, 65) and because antisense oligonucleotides are toxic to β-cells at concentrations that fail to reduce iPLA₂β expression (66).

Although iPLA₂β has been proposed to play the housekeeping role of maintaining membrane phospholipid homeostasis by degrading excess PC (28, 29), stable suppression of iPLA₂β expression in INS-1 cells does not result in a significant change in their PC content or composition. Another proposed iPLA₂β housekeeping role is to provide LPC acceptors for incorporating arachidonic acid into PC (22–25), but we also observe no change in LPC content or composition or in arachidonate incorporation rates into iPLA₂β-KD INS-1 cells.

Rather, we find that INS-1 iPLA₂β-KD cells incorporate arachidonate into GPC lipids as readily as control cells when cultured with arachidonic acid. In both control and iPLA₂β-KD INS-1 cells cultured in standard medium, GPC lipids contain primarily sn-2 oleate or palmitoleate, and such species are also abundant in anionic phospholipids. Upon culture with arachidonic acid, all of these lipids are remodeled, and species with sn-2 arachidonate become their most abundant components.

In INS-1 cells cultured with arachidonic acid, 16:0/20:4-GPC accumulation precedes that of 18:0-GPC (66, 68), and 16:0/20:4-GPC is thought to be a primary remodeling product, which is then remodeled at sn-1 to generate 18:0/20:4-GPC (87). Synthesis of 18:0/20:4-GPC in...
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FIGURE 11. Time course of appearance of lysophosphatidylcholine species in INS-1 cells incubated with arachidonic acid. The phosphorus contents of lipid extracts from control or iPLA₂-KD INS-1 cells after 0, 6, or 24 h of incubation with arachidonic acid were measured, and internal standard 14:0/14:0-GPC was added. LPC-Li⁺ adducts were analyzed by positive ion ESI/MS/MS for constant neutral loss of 39. LPC species were measured by determining the ion current for 16:0-LPC (m/z 502), 18:1-LPC (m/z 528), 18:0-LPC (m/z 530), and 20:4-LPC (m/z 550) relative to that for the internal standard (m/z 684) and interpolating from a standard curve. Values are normalized to lipid phosphorus. In A, the leftmost four bars at 0 and 6 h represent LPC analytes in control INS-1 cells, and the rightmost four bars at each time represent those from iPLA₂-KD cells. In B, the closed triangles represent 20:4-LPC, and the closed circles represent the sum of 16:0-, 18:1-, and 18:0-LPC.

FIGURE 12. Negative ion electrospray ionization mass spectrometric analyses of anionic glycerophospholipids in iPLA₂β knockdown and control INS-1 cells incubated with arachidonic acid. The lipid phosphorus content was measured in extracts from iPLA₂-KD (A and B) or control (C and D) INS-1 cells incubated with arachidonic acid for 0 h (A and C) or 24 h (B and D); internal standard 14:0/14:0-GPE was added; and the mixture was analyzed by negative ion ESI/MS.
β-cells is potentially important, because that is the most abundant GPC lipid in pancreatic islet β-cell secretory granule membranes (88).

The predicted intermediate in that pathway is 1-lyso-2–20:4-GPC, which is produced from 16:0/20:4-GPC by iPLA2γ (89), a distinct enzyme that arises from a gene different from that for iPLA2β (88). We do observe 1-lyso-2–20:4-GPC accumulation in iPLA2β-KD and control INS-1 cells incubated with arachidonate under conditions in which 16:0/20:4-GPC and 18:0/20:4-GPC are produced. This could reflect a role for iPLA2γ in phospholipid remodeling that is consistent with its phospholipase A1 action on 16:0/20:4-GPC (89), and this might explain effects of pharmacologic inhibition (57, 66) and molecular biologic suppression of iPLA2γ expression with antisense oligonucleotides (38). The reduced proliferation rates reported here for INS-1 cells that vary among tissues and cell types, perhaps dependent in part on splice variants (49–52) and proteolytic processing products (38, 57) of iPLA2β are expressed in a given cell and on what interacting proteins (102) are present in the cell compartment (68, 103) in which the iPLA2β isoform resides. Our findings do indicate that iPLA2β plays signaling or effector role(s) in stimulated insulin secretion from β-cells, and these results with molecular biologic suppression of iPLA2β expression provide an important independent test of inferences from studies with pharmacologic inhibition of iPLA2β activity (54–58, 72, 88).

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