Studies of the Role of Group VI Phospholipase A₂ in Fatty Acid Incorporation, Phospholipid Remodeling, Lysophosphatidylcholine Generation, and Secretagogue-induced Arachidonic Acid Release in Pancreatic Islets and Insulinoma Cells

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Phospholipases A₂ (PLA₂) catalyze hydrolysis of the sn-2 fatty acid substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (1–7). PLA₂ are a diverse group of enzymes, and the first members to be well characterized have low molecular masses (~14 kDa), require millimolar [Ca²⁺] for catalytic activity, and function as extra-cellular secreted enzymes designated sPLA₂ (3, 6). The first PLA₂ to be cloned is that active at [Ca²⁺] that can be achieved in the cytosol of living cells is an 85-kDa protein classified as a group IV PLA₂ and designated cPLA₂ (3, 5). This enzyme is induced to associate with its substrates in membranes by rises in cytosolic [Ca²⁺] within the range achieved in cells stimulated by extracellular signals that induce Ca²⁺ release from intracellular sequestration sites or Ca²⁺ entry from the extracellular space, is also regulated by phosphorylation, and prefers substrates with sn-2 arachidonoyl residues (5).

Recently, a second PLA₂ that is active at [Ca²⁺] that can be achieved in cytosol has been cloned (8–10). This enzyme does not require Ca²⁺ for catalysis, is classified as a group VI PLA₂, and is designated iPLA₂ (3, 4). The iPLA₂ enzymes cloned from hamster (8), mouse (9), and rat (10) cells represent species homologs, and all are 84-kDa proteins containing 751–752 amino acid residues with highly homologous (~95% identity) sequences. Each contains a GXSGX lipase consensus motif and eight stretches of a repeating sequence motif homologous to a repetitive motif in the integral membrane protein binding domain of ankyrin (8–10). The substrate preference of these iPLA₂ enzymes varies widely with the mode of presentation (8), but each is susceptible to inhibition (8–10) by a bromoenol lactone (BEL) suicide substrate (11, 12) that is not an effective inhibitor of sPLA₂ or cPLA₂ enzymes at comparable concentrations (4, 11–14).

Proposed functions for iPLA₂ include a housekeeping role in phospholipid remodeling that involves generation of lysophospholipid acceptors for incorporation of arachidonic acid into phospholipids of murine P388D1 macrophage-like cells (4, 15, 16), although signaling functions of iPLA₂ have been suggested in other cells (10, 17–21). The proposed housekeeping function for iPLA₂ (4) has been deduced from experiments involving inhibition of iPLA₂ activity in group IV phospholipase A₂; ECL, enhanced chemiluminescence; ESI, electrospray ionization; GC, gas chromatography; GPC, glycerophosphocholine; HBSS, Hank’s balanced salt solution; iPLA₂, group VI phospholipase A₂; LPC, lysophosphatidylcholine; MS, mass spectrometry; NEM, N-ethylmaleimide; XMMMA, N⁰-monomethyl-L-arginine acetate; NP-HPLC, normal phase high performance liquid chromatography; PA, phosphatidic acid; PAP, phosphatidate phosphohydrolase; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; RP-HPLC, reverse phase high performance liquid chromatography; RT, reverse transcriptase; PCR, polymerase chain reaction; IL, interleukin; bp, base pair; BAPTA-AM, 1,2-bis(O-amino-phenoxylethane-N,N',N⁰,N⁰'-tetracetic acid tetra(acetoxymethyl) ester.

An 84-kDa group VI phospholipase A₂ (iPLA₂) that does not require Ca²⁺ for catalysis has been cloned from Chinese hamster ovary cells, murine P388D1 cells, and pancreatic islet β-cells. A housekeeping role for iPLA₂ in generating lysophosphatidylcholine (LPC) acceptors for arachidonic acid incorporation into phosphatidylcholine (PC) has been proposed because iPLA₂ inhibition reduces LPC levels and suppresses arachidonate incorporation and phospholipid remodeling in P388D1 cells. Because islet β-cell phospholipids are enriched in arachidonate, we have examined the role of iPLA₂ in arachidonate incorporation into islets and INS-1 insulinoma cells. Inhibition of iPLA₂ with a bromoenol lactone (BEL) suicide substrate did not suppress and generally enhanced [³H]arachidonate incorporation into islet or INS-1 cell phospholipids. Inhibition of islet iPLA₂ did not alter the phospholipid head-group classes into which [³H]arachidonate was initially incorporated or its subsequent transfer from PC to other lipids. Electrospray ionization mass spectrometry measurements indicated that inhibition of INS-1 cell iPLA₂ accelerated arachidonate incorporation into PC and that inhibition of islet iPLA₂ reduced LPC levels by 25%, suggesting that LPC mass does not limit arachidonate incorporation into islet PC. Gas chromatography/mass spectrometry measurements indicated that iPLA₂ but not propranolol suppressed insulin secretagogue-induced hydrolysis of arachidonate from islet phospholipids. In islets and INS-1 cells, iPLA₂ is thus not required for arachidonate incorporation or phospholipid remodeling and may play other roles in these cells.
P388D1 cells with BEL (15) or with an antisense oligonucleotide (16). Inhibition of iPLA2 activity in P388D1 cells suppresses incorporation of \(^{3}H\)arachidonic acid into phospholipids by about 60%, but \(^{3}H\)palmitic acid incorporation is reduced only slightly (15, 16). Incorporation of \(^{3}H\)arachidonic acid into P388D1 cells is Ca\(^{2+}\)-independent and unaffected by chelation of extracellular or intracellular Ca\(^{2+}\) (15). Inhibition of iPLA2 also reduces \(^{3}H\)lysophosphatidylcholine (LPC) levels by about 60% in \(^{3}H\)choline-labeled P388D1 cells, and this is thought to represent the mechanism whereby iPLA2 inhibition reduces incorporation of \(^{3}H\)arachidonic acid into P388D1 cell phospholipids (15, 16). Such incorporation (15, 16) reflects a deacylation/reacylation cycle (22, 23) of phospholipids into P388D1 cell phosphatidylcholine (PC) (15, 16). Activity of iPLA2 appears to be required to maintain sufficient levels of LPC in P388D1 cells to support \(^{3}H\)arachidonic acid incorporation into PC (15, 16).

It is not yet certain that the observations with P388D1 cells reflect a general mechanism for incorporation of arachidonic acid into phospholipids of all cells, and P388D1 cells exhibit some atypical features of arachidonate incorporation. Arachidonate represents about 25% of the total esterified fatty acyl mass in native murine macrophage phospholipids (25, 26) but only 3% of that in murine P388D1 macrophage-like cells (27–29). A significant portion of exogenous arachidonate provided to P388D1 cells is also found in the intracellular free fatty acid fraction (29), and this is not the case in other cell types (30–32), in which nonesterified arachidonate is maintained at a very low level by an efficient esterification system (26, 33–34).

In many cells, nonesterified arachidonate imported from the extracellular space or released intracellularly by PLA\(_2\) enzymes is quickly converted to arachidonoyl-CoA in an ATP-dependent step and then rapidly incorporated into phospholipids by acyltransferases (35, 36). The low levels of esterified arachidonate and the relatively high levels of nonesterified arachidonate observed in P388D1 cells (27–29) suggest that they may be deficient in arachidonate incorporation mechanisms expressed by other cells.

One biomedically important cell that may require especially effective arachidonate incorporation mechanisms is the insulin-secreting pancreatic islet beta cell, the function of which is impaired in diabetes mellitus. Arachidonate represents 30–36% of the total esterified fatty acyl mass in phospholipids of normal rat and human islets and isolated beta cells (37, 38), and arachidonate-containing species are the most abundant components of all major islet phospholipid head-group classes (39). The abundance of arachidonate-containing phospholipid species is higher in islets than in many other tissues (39), and islet plasma membranes and secretory granule membranes are especially enriched in such species (38, 39). Fusion of secretory granule and plasma membranes is the final event in insulin exocytosis, and the high content of certain arachidonate-containing phospholipids in those membranes may facilitate their fusion (39, 40). Because both rat and human beta cells also express iPLA\(_2\) (10, 41), we have examined the participation of iPLA\(_2\) in arachidonic acid incorporation into islet and insulinoma cell phospholipids, and our findings differ from those in P388D1 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The compounds [5,6,8,9,12,14,15-\(^{3}H\)arachidonic acid (100 Ci/mmol), [9,10-\(^{3}H\)]palmitic acid (54 Ci/mmol), [methyl-\(^{14}C\)]nicotine, [\(\beta\)-dipalmitoyl-glycerol]-U-\(^{14}C\)phosphatidic acid (200 mCi/mmol) were obtained from NEN Life Science Products. ECL detection reagents and 1-palmitoyl-2-[\(\beta\)-dichloroethyl]-sn-glycerol-3-phosphocholine (55 mCi/mmol) were purchased from Amersham Pharmacia Biotech. The 1-palmitoyl-2-hydroxy-sn-glycero-phosphocholine (16:0-LPC), 1-heptadecanoyl-2-hydroxy-sn-glycero-phosphocholine (17:0-LPC), and 1-steinoyl-2-hydroxy-sn-glycero-phosphocholine (18:0-LPC) and standard phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine, and phosphatidic acid (PA) were obtained from Avanti Polar Lipids (Birmingham, AL). [5,6,8,9,11,12,14,15-\(^{3}H\)]Arachidonic acid was prepared by catalytic reduction of eicos-5,8,11,14-tetraynoic acid with \(^{2}H\)gase (42).

Arachidonic acid, palmitic acid, triolein, diolein, and monoolein were obtained from Nu-Chek Prep (Elysian, MN). The bromoenol lactone (BEL) iPLA\(_2\) suicide substrate (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one was purchased from Cayman Chemical (Ann Arbor, MI). Male Sprague-Dawley rats (180–220 g) were obtained from Harlan Breeders (Indianapolis, IN) and collagenase from Roche Molecular Biochemicals. Tissue culture media (CMRL-1066, RPMI, and minimal essential medium), penicillin, streptomycin, Hank's balanced salt solution (HBSS), and l-glutamine were purchased from Life Technologies, Inc. Fetal bovine serum was obtained from HyClone (Logan, UT), and Pentex bovine serum albumin (BSA, fatty acid free, fraction V) from ICN Biomedicals (Aurora, OH). Rodent Chow 5001 was obtained from Ralston Purina (St. Louis, MO) and \(\beta\)-glucose from the National Bureau of Standards (Washington, D. C.). ATP, ampicillin, propranolol, and kanamycin were obtained from Sigma. BAPTA-AM and N-nonoethyl-\(\beta\)-arginine acetate (NMMA) were obtained from Calbiochem and interleukin-1\(\alpha\) (IL-1) from Cistron Biotechnology (Pine Brook, NJ). Ringer's bicarbonate buffer (pH 7.4, 115 mM NaCl, 24 mM NaHCO\(_3\), 5 mM KCl, 1 mM MgCl\(_2\)) and cCMRL (CMRL-1066 supplemented with 10% heat-inactivated fetal bovine serum, 1% l-glutamine, 1% penicillin, 1% streptomycin) and HBSS supplemented with 0.5% penicillin/streptomycin.

**Isolation and Culture of Pancreatic Islets and Insulinoma Cells**—Islets were isolated aseptically from male Sprague-Dawley rats by collagenase digestion of minced pancreas, density gradient isolation, and manual selection under microscopic visualization (43). Isolated islets were placed in cCMRL-1066 under a humidified atmosphere of 95% air, 5% CO\(_2\) and cultured at 37 °C. Islet total insulin and protein content were determined as described previously (44). Islets were isolated from human pancreas in the Core Laboratory of the Washington University Diabetes Research and Training Center (45) and were cultured as described (39). INS-1 insulinoma cells provided by Dr. Christopher Newgard (University of Texas-Southwestern, Dallas, TX) were cultured under previously described conditions (46–48).

**Incubation of Islets and Insulinoma Cells with Radiolabeled Fatty Acids, BEL, BAPTA, IL-1, and Other Additives**—Islets or INS-1 cells were washed three times in KRB medium containing 5.5 mM glucose and 0.1% BSA, resuspended in that medium, and preincubated for 30 min at 37 °C. The islets (100 per condition) or INS-1 cells (2 \(\times\) 10\(^5\) per condition) were then placed in fresh KRB medium that contained glucose (5.5, 11, or 22 mM), 0.1% BSA, and either 2.5 mM CaCl\(_2\) or 1 mM EGTA and no added Ca\(^{2+}\) and were incubated (30 min, 37 °C) after addition of vehicle only (control) or BEL (1–25 \(\mu\)g/mL). In experiments examining iPLA\(_2\) activity, control and BEL-treated islets or INS-1 cells were washed in another homogeneous or preincubated in KRB medium and incubated at 37 °C for 8–68 h before homogenization, and iPLA\(_2\) activity was determined in homogenates. In some experiments examining radiolabeled fatty acid incorporation, effects of loading islets or INS-1 cells with BAPTA-AM (100 \(\mu\)M, 30 min) or propranolol (250 \(\mu\)M, 30 min) under described conditions (49) before adding radiolabeled fatty acid were determined. After the preincubation and loading steps described above, fatty acid incorporation experiments were initiated by adding either \(^{3}H\)arachidonic acid (final concentration 0.5 \(\mu\)Ci/ml, 5 nmol) or \(^{3}H\)palmitic acid (final concentration 0.5 \(\mu\)Ci/ml, 10 nmol) to the medium, and incubation was performed for 10–60 min at 37 °C. Islets or INS-1 cells were then washed three times in KRB medium containing 5.5 mM glucose and 0.1% BSA to remove unincorporated \(^{3}H\)-fatty acid. In some experiments, cellular lipids were then immediately extracted by the method of Bligh and Dyer (50) and analyzed by TLC or HPLC. In other experiments, after initial incubation with \(^{3}H\)arachidonic acid, islets were washed to remove unincorporated radiolabel, placed in cCMRL medium, and incubated (24 h, 37 °C) in the absence of \(^{3}H\)-fatty acid. The islets were then washed and their lipids extracted and analyzed. In experiments examining effects of IL-1 on insulin secretion, islets were incubated (24 h, 37 °C) with no additions, with IL-1 (5 units/ml) alone, or with IL-1 plus NMMA (0.5 mM) under described conditions (51). The islets were then washed, preincubated, and incubated with \(^{3}H\)-fatty acids as above. Under all loading and incubation conditions, islet and INS-1 cell viability exceeded 98% by trypan blue exclusion performed as described (49).

**Assay of iPLA\(_2\) Activity in Islets and INS-1 Cells**—Control and BEL-
treated islets and INS-1 cells were homogenized by sonication as described (52). Protein content was measured with Coomassie reagent (Pierce) against bovine serum albumin as standard. Ca\(^{2+}\)-independent PL\(_A\) activity was assayed by incubating (30 min, 37 °C) aliquots of homogenized insulinoma in buffer (100 mM Heps, pH 7.5, 5 mM EDTA, 400 μM Triton X-100, with or without 1 mM ATP). The substrate was prepared in mixed micelles of Triton X-100/phospholipid at a molar ratio of 4/1, obtained by a combination of heating, vortex mixing, and batch sonication. Products were analyzed by TLC as described below, and PL\(_A\) specific activity was calculated from disintegration of released \[\text{[^{32}P]}\text{inositol and protein content as described (52).}

Chromatographic Analyses of Radiolabeled Phospholipids—Incorporation of \(^{3}H\) fatty acids into islet and INS-1 cell phospholipids was determined by TLC (15, 16) or by HPLC (37, 49, 53). TLC was performed on silica gel G plates with hexane/diethyl ether/acetic acid (70/30/1). Phospholipids remain at the origin and are resolved from diglycerides (R\(_f\) 0.21–0.24), free fatty acids (R\(_f\) 0.58), and triglycerides. Phospholipid head-group classes were separated by NP-HPLC (37, 49, 53) analyses on a silicic acid column (LiChrosphere Si-100, 10 μm, 250 × 4.5 mm, Alltech, Deerfield, IL) with the solvent system hexane/2-propanol/25 mM potassium phosphate, pH 7.0 (ethanol/acetic acid (367/490/82/100/0.6) at a flow of 0.5 ml/min for 60 min and then 1 ml/min of the same solvent mixture) or by extraction of standards with toluene/ethanol (100/0.5) (2.5 min, five times) and incubated (1 h, room temperature) with a polyclonal antibody (1:4000 dilution in TBS-T) to PL\(_A\), provided by Dr. Simon Jones (Genetics Institute, Boston). The nylon membrane was then washed in TBS-T (5 min, five times) and incubated (1 h, room temperature) with a secondary antibody coupled to horseradish peroxidase (Roche Molecular Biochemicals) at 1:20,000 dilution in TBS-T containing 5% BSA. The antibody was visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Isolation of RNA from INS-1 Cells, Reverse Transcription, and Polymerase Chain Reaction Amplification of an iPLA\(_2\) cDNA Fragment—Total RNA was isolated from INS-1 cells and first strand cDNA prepared by reverse transcription (RT) using standard methods (8, 55). Polymerase chain reactions (PCR) were performed and products analyzed on agarose gels with ethidium bromide (8). PCR primers were designed to amplify specific DNA fragments of unknown sequence. The expected fragment length was 582 bp, and digestion with restriction endonuclease SmaI is expected to yield 164- and 384-bp fragments. Attempt at Suppressing INS-1 Cell iPL\(_A\) Activity with Antisense Oligonucleotides—Many attempts to suppress INS-1 cell iPL\(_A\) expression with antisense oligonucleotides were performed using different oligonucleotide sequences. One (5'-AGCGCTTCCAG-
**Results**

In the report that motivated our study, 25 μM BEL maximally inhibited iPLA₂ activity and suppressed [³H]arachidonic acid incorporation into P388D1 cell phospholipids during a 10-min incubation but only slightly suppressed [³H]palmitic acid incorporation, and [³H]arachidonic acid incorporation was unaffected by chelation of extracellular or intracellular [Ca²⁺] (15). To examine the generality of these phenomena, we performed similar experiments with islets. Control islets exhibited PLA₂ activity in the absence of Ca²⁺ that was stimulated by ATP, and activity was 98% inhibited in 25 μM BEL-treated islets (Fig. 1, left panel), consistent with properties of recombinant iPLA₂ expressed from cDNA cloned from an islet library (10). Despite effective iPLA₂ inhibition, no suppression of [³H]arachidonic acid incorporation into phospholipids was observed in BEL-treated islets during 10-min incubations in Ca²⁺-replete medium, in Ca²⁺-free EGTA-containing medium, or in islets that had been loaded with the intracellular Ca²⁺-chelator BAPTA-AM and incubated in Ca²⁺-free EGTA-containing medium (Fig. 1, center panel). [³H]Arachidonic acid incorporation was greater in Ca²⁺-free EGTA-containing me-
Corporation into islet phospholipids is not completely Ca\textsuperscript{2+}-dependent. Incorporation of [\textsuperscript{3}H]palmitic acid into islet phospholipids in Ca\textsuperscript{2+}-free medium is also true in Ca\textsuperscript{2+}-replete medium and that this also occurs with palmitate (67).

In Ca\textsuperscript{2+}-free EGTA-containing medium, non-BAPTA-loaded islets incorporated more [\textsuperscript{3}H]arachidonic acid into phospholipids than did BAPTA-loaded islets (Fig. 1, center panel), under conditions where BAPTA reduces islet beta cell cytosolic [Ca\textsuperscript{2+}] by 50% and prevents ionophore A23187-induced rises in cytosolic [Ca\textsuperscript{2+}] (49). This indicates that [\textsuperscript{3}H]arachidonic acid incorporation into islet phospholipids is not completely Ca\textsuperscript{2+}-independent. Incorporation of [\textsuperscript{3}H]palmitic acid into islet phospholipids (Fig. 1, right panel) exhibited features similar to those for [\textsuperscript{3}H]arachidonic acid, and incorporation of both fatty acids was generally higher in BEL-treated than control islets. This effect was observed at all incubation times between 10 and 60 min (Fig. 2), and no suppression of [\textsuperscript{3}H]arachidonic acid incorporation into islet phospholipids was observed at any BEL concentration that reduced islet iPLA\textsubscript{2} activity (Fig. 3). Incorporation of both fatty acids into human islet phospholipids was affected by BEL and by manipulating Ca\textsuperscript{2+} in ways similar to those with rat islets (not shown).

To determine whether it is possible to suppress fatty acid incorporation into islet phospholipids under our conditions, we examined effects of incubating islets with interleukin-1 (IL-1) (Fig. 4), which stimulates islet production of nitric oxide and causes accumulation of nonesterified arachidonic acid by an NO-dependent mechanism that is thought to reflect impaired arachidonoyl-CoA generation (51). Incubating islets with IL-1 impairs incorporation of [\textsuperscript{3}H]arachidonic acid into islet phospholipids in Ca\textsuperscript{2+}-replete medium (51), and we found that this is also true in Ca\textsuperscript{2+}-free medium and that incorporation of [\textsuperscript{3}H]palmitic acid is similarly suppressed (Fig. 4). Coincubation of islets with IL-1 and the nitric oxide synthase inhibitor NMMA prevented IL-1-induced suppression of fatty acid incorporation into islet phospholipids (Fig. 4), reflecting the NO dependence of this effect. Fatty acid incorporation into islet phospholipids thus can be suppressed, even though inhibiting islet iPLA\textsubscript{2} activity does not do so.

To attempt to inhibit beta cell iPLA\textsubscript{2} activity with antisense oligonucleotides, we examined INS-1 insulinoma cells because islets are a multicellular aggregate in which it is difficult to achieve effective intracellular levels of oligonucleotide. INS-1 cells are derived from rat islet beta cells (46–48) and were found to express a PLA\textsubscript{2} activity in the absence of Ca\textsuperscript{2+} that is stimulated by ATP and inhibited by BEL (Fig. 5A), an 84-kDa protein recognized by an iPLA\textsubscript{2} antibody (Fig. 5B), and an mRNA species that is amplified in RT-PCR reactions with rat iPLA\textsubscript{2}-specific primers and that contains the expected Smal restriction endonuclease cleavage site (Fig. 5C). We were unable to suppress INS-1 cell iPLA\textsubscript{2} activity or protein with an antisense oligonucleotide corresponding in sequence, except for minor differences between species, to one that suppresses P388D1 cell iPLA\textsubscript{2} expression (16). Two other antisense oligonucleotide sequences presented under a variety of conditions were also ineffective.

Nonetheless, because INS-1 cell iPLA\textsubscript{2} activity is inhibited by BEL, we compared effects of BEL on fatty acid incorporation into phospholipids of islets and INS-1 cells. We also examined effects of propranolol because both BEL and propranolol inhibit phosphatidate phosphohydrolase (PAPH) in some cells (57, 70). At 250 \muM, propranolol maximally inhibits PAPH in islets (71) and amniotic WISH cells (70) and suppressed islet de novo synthesis of triacylglycerol, as do PAPH inhibitors in P388D1 cells (29, 57). BEL did not suppress incorporation of [\textsuperscript{3}H]arachidonic acid or [\textsuperscript{3}H]palmitic acid into islet (Fig. 6, A and B) or INS-1 cell phospholipids (Fig. 6, C and D) in Ca\textsuperscript{2+}-replete or Ca\textsuperscript{2+}-free EGTA-containing medium, and incorporation of neither fatty acid was significantly affected by propranolol in islets or INS-1 cells (Fig. 6).

One possible explanation for the failure of BEL to suppress
fatty acid incorporation into islet phospholipids is that, by inhibiting PAPH, BEL causes accumulation of phosphatidic acid (PA) and enhances de novo phosphatidylinositol (PI) synthesis (24, 72). This might mask suppression of [3H]arachidonic acid incorporation into GPC lipids by deacylation/reacylation. We first determined whether BEL inhibits islet PAPH activity. PAPH isoforms include PAPH-1, which is Mg2+ -dependent and inhibited by NEM (58, 73) and BEL, and PAPH-2, which is Mg2+ -independent, NEM-insensitive, and resistant to BEL (57). Islets were found to express Mg2+ -dependent, NEM-sensitive PAPH activity both in cytosol and membranes (Table I), consistent with a previous report (74). BEL (25 μM) reduced islet cytosolic Mg2+ -dependent, NEM-sensitive PAPH activity by 48% but had no effect on membranous Mg2+ -dependent, NEM-insensitive PAPH activity (Table I). Neither BEL nor NEM affected islet PAPH activity in the absence of Mg2+, and the combination of BEL and NEM achieved no greater inhibition of cytosolic PAPH activity than NEM alone (not shown).

The inhibition of islet cytosolic PAPH-1 activity by BEL caused us to examine the possibilities that BEL stimulates incorporation of fatty acids into PA and PI via de novo synthesis while it reduces incorporation into phosphatidylcholine (PC) by deacylation/reacylation. Islet phospholipids into which [3H]arachidonic acid is incorporated were analyzed by NP-HPLC (Fig. 7). After 10 min of incubation with [3H]arachidonic acid, the majority of [3H] was incorporated into PC in both control, as previously observed (63), and BEL-treated islets. BEL-treated islets exhibited greater [3H]arachidonic acid incorporation into PC than control islets, and the profile of [3H]arachidonic acid-labeled phospholipid head-group classes was similar in the two groups (Fig. 7A). Upon removal of exogenous [3H]arachidonic acid and continued culture, a time-dependent decline in the [3H]arachidonate content of PC and a rise in that of phosphatidylethanolamine (PE) occurred, as previously observed in islets (63) and other cells (34), and these effects occurred in both BEL-treated and control islets (Fig. 7B). RP-HPLC analyses of PC molecular species labeled with [3H]arachidonic acid were also similar in control and BEL-treated islets (Fig. 7C). PI contained only a minor fraction of [3H]arachidonic acid incorporated into phospholipids in BEL-treated or control islets. These findings indicate that accumu-
Pancreatic Islet iPLA\textsubscript{2} and Arachidonate Incorporation

![Graph A](image1.png)  
**Fig. 5.** Expression of iPLA\textsubscript{2} by INS-1 insulinoma cells. A, homogenates of INS-1 cells were assayed for iPLA\textsubscript{2} activity in medium containing 1 mM EGTA and no added Ca\textsuperscript{2+} without BEL or ATP (EGTA) or in medium supplemented either with 10 \textmu M BEL (EGTA + BEL) or with 1 mM ATP (EGTA + ATP). B, aliquots of INS-1 cell cytosol containing 1 \mu g (lane 1), 10 \mu g (lane 2), or 20 \mu g (lane 3) of protein were analyzed by SDS-PAGE, and immunoblotting was performed with an antibody against iPLA\textsubscript{2} provided by Dr. Simon Jones. C, RNA was prepared from INS-1 cells and used as template in RT-PCR reactions with rat iPLA\textsubscript{2}-specific primers that were expected to yield a product of 528 bp in length, based on the rat iPLA\textsubscript{2} cDNA sequence (10). RT-PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide. Lane 1 represents a control reaction performed without an RT step to exclude contamination of the RNA with genomic DNA. Lanes 2 and 3 represent reaction products obtained from RT-PCR reactions performed with two separate preparations of INS-1 cell RNA. Lanes 4 and 5 represent products obtained from digestion of the DNA species visualized in lanes 2 and 3 with the restriction endonuclease Smal, which was expected to yield products of 164 and 364 bp lengths, based on the rat iPLA\textsubscript{2} cDNA sequence (10).

lation of PA and PI does not mask a suppression by BEL of \textsuperscript{3}H\textsubscript{2} arachidonate incorporation into islet PC and PE and that BEL has little effect on the phospholipid head-group classes into which \textsuperscript{3}H\textsubscript{2} arachidonate is initially incorporated or on its subsequent transfer from PC to other lipids.

To examine further any contribution of \textit{de novo} phospholipid synthesis (24) to \textsuperscript{3}H\textsubscript{2} arachidonate incorporation into islet phospholipids, effects of medium glucose concentration were determined (Table II, top). \textit{De novo} phospholipid synthesis from glucose requires glycolytic metabolism to triose phosphates and acylation of glycerol 3-phosphate and then lysophosphatidic acid to yield PA (24). Kinetic properties of the predominant islet glucose transporter and glucokinase cause islet glycolytic flux to rise severalfold as the medium glucose concentration increases from 5 to 20 mM (75), and this promotes islet \textit{de novo} glycerolipid synthesis (60). Incorporation of \textsuperscript{3}H\textsubscript{2} arachidonic acid into phospholipids was found not to be significantly affected by medium glucose concentration in control or BEL-treated islets, although the latter exhibited greater incorporation at each glucose concentration (Table II, top). This indicates that \textit{de novo} synthesis is not a major contributor to incorporation of \textsuperscript{3}H\textsubscript{2} arachidonic acid into islet phospholipids under our conditions and cannot account for effects of BEL on incorporation. This is supported by the finding that conversion of \textsuperscript{13}C\textsubscript{12}glucose to \textsuperscript{13}C\textsubscript{12}glycerolipids is reduced rather than increased in BEL-treated islets (Table II, bottom).

Recovery of iPLA\textsubscript{2} activity in BEL-treated islets occurs slowly and substantial inhibition persists for many hours (51), raising the possibility that long-term arachidonate incorporation studies can be performed without repeated manipulation of the cells. When islets were exposed to 25 \mu M BEL and then cultured for various periods, 99, 92, and 80% inhibition of iPLA\textsubscript{2} activity was observed at 0, 8, and 24 h after treatment, respectively (Fig. 8). Mean iPLA\textsubscript{2} activity for the entire 24-h period following BEL treatment was about 10% that in control islets. Similar experiments with INS-1 cells indicated that iPLA\textsubscript{2} activity was inhibited by 99, 92, and 60% at 0, 8, and 24 h, respectively, after treatment and that the mean iPLA\textsubscript{2} activity in the 24-h period following BEL treatment was about 20% of control values (Fig. 8).

Similar experiments were performed to examine effects of iPLA\textsubscript{2} inhibition on incorporation of arachidonate mass into INS-1 cell phospholipids to complement the radiochemical data. Electrospray ionization (ESI) mass spectrometric (MS) analyses of control INS-1 cell glycerophosphocholine (GPC) lipids as Li\textsuperscript{+} adducts indicated that arachidonate-containing species are not abundant in unsupplemented cells but that their abundance increases substantially within hours after adding arachidonic acid to the medium (Fig. 9). Fig. 9A is the ESI/MS total positive ion current of GPC-Li\textsuperscript{+} lipid species from control INS-1 cells. Tandem MS analyses (62) established that the major ions in this profile represent 16/0:16:1-GPC (m/z 738), 16:1/18:1-GPC (m/z 764), 16:0/18:1-GPC (m/z 766), 18:1/18:1-GPC (m/z 792), and 18:0/18:1-GPC (m/z 794). Arachidonate-containing GPC species at 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) are not abundant. Fig. 9B illustrates that, when INS-1 cells are incubated with arachidonic acid, by 8 h there is substantial accumulation of 16:0/20:4-GPC (m/z 788). Fig. 9C illustrates that at 24 h the abundance of this species increases further and that 18:0/20:4-GPC (m/z 816) also becomes abundant, a sequence consistent with reports that 16:0/20:4-GPC is a primary remodeling product and that 18:0/20:4-GPC is a secondary product from remodeling at both sn-1 and sn-2 positions (76–80). Remodeling of INS-1 cell phospholipids with arachidonate was not suppressed by BEL treatment and was accelerated at 8 h after treatment (Fig. 10), despite the fact INS-1 cell iPLA\textsubscript{2} activity was inhibited by an average of about 96% during the first 8 h after treatment (Fig. 8).

Suppression of arachidonate incorporation by iPLA\textsubscript{2} inhibition in P388D1 cells is proposed to reflect reduction in lysophosphatidylcholine (LPC) acceptor molecules, the abundance of which is thought to limit the rate of arachidonate incorporation and to be governed by iPLA\textsubscript{2} activity (15, 16). To determine effects of iPLA\textsubscript{2} inhibition on islet LPC mass, ESI tandem MS experiments were performed (Fig. 11). After incubation of islets with BEL or vehicle, internal standard 17:0-LPC, which does not occur naturally in islets, was added to islet lipid extracts, and LPC species were isolated by TLC and analyzed as Li\textsuperscript{+} adducts by ESI/MS/MS scanning for neutral loss of trimethylamine, an approach that yields a better signal to noise ratio than does monitoring the total ion current. Comparing relative intensities of ions for 17:0-LPC-Li\textsuperscript{+} (m/z 516) and 16:0-LPC-Li\textsuperscript{+} (m/z 502) or 18:0-LPC-Li\textsuperscript{+} (m/z 530) yields a linear standard curve over a wide concentration range that includes LPC levels observed in islets.
Fig. 11 illustrates that the two most abundant LPC species in islets are 16:0-LPC and 18:0-LPC, consistent with a previous report (63), that treatment of islets with BEL induces a modest decline in the abundance of these species relative to the internal standard, and that treatment with bee venom sPLA2 induces the expected increase in abundance of these species. A series of similar experiments indicated that the resting islet LPC mass measured by this method (5.11 pmol/islet) corresponds closely to a measurement of 5.55 pmol/islet based on GC/MS quantitation islet LPC fatty acid content (63) and that the mean fall in LPC mass in BEL-treated islets is about 25% (Table III, top). This corresponds closely to a 21% decrement induced by BEL in [3H]LPC levels in [3H]choline-labeled islets (Table III, bottom). Both methods indicate that BEL induces a smaller decline in islet LPC levels than the 60% decrement for P388D1 cells (15, 16). Although BEL induces a modest decline in islet LPC content (Table III), this is not associated with reduced [3H]arachidonic acid incorporation into islet PC (Fig. 7), suggesting LPC level does not limit arachidonate incorporation into islet PC.

Although iPLA2 activity is not required for arachidonate incorporation or phospholipid remodeling in islets, we have suggested that iPLA2 might participate in insulin secretagogue-induced hydrolysis of arachidonic acid from islet phos-
phospholipids, based on the finding that BEL suppresses insulin secretagogue-induced release of arachidonate metabolites from islets (81). To determine whether this might reflect inhibition of PAPH-1 (4, 57) rather than iPLA2, we compared effects of BEL and the PAPH inhibitor propranolol (29, 70) on accumulation of nonesterified arachidonate in islets stimulated with the insulin secretagogue 17 mM glucose alone or in combination with the muscarinic agonist carbachol (Fig. 12). As measured by isotope dilution GC/MS with [2H8]arachidonate as internal standard, 17 mM glucose induced a doubling in accumulation of nonesterified arachidonic acid in islet incubations (Fig. 12), consistent with previous reports (82, 83), and this response was greatly amplified by carbachol (Fig. 12). The effect of 17 mM glucose alone was completely prevented by BEL, and the response to the combination of 17 mM glucose plus
carbachol was reduced by about 70%. Propranolol did not reduce accumulation of nonesterified arachidonate induced either by 17 mM glucose alone or by the combination of 17 mM glucose plus carbachol (Fig. 12).

**DISCUSSION**

A housekeeping role for iPLA₂ in phospholipid remodeling has been suggested from iPLA₂ inhibition studies in P388D1 cells (4, 15, 16), but we find no evidence that iPLA₂ activity is required for incorporation of arachidonate or phospholipid remodeling in pancreatic islets or insulinoma cells. A difficulty in studying iPLA₂ functions is that the most effective pharmacologic inhibitor of the enzyme, BEL (8–12), also inhibits PAPH-1 (57), although BEL does not inhibit sPLA₂ or cPLA₂ enzymes at concentrations that effectively inhibit iPLA₂ (11–14, 57). One approach to this problem is to compare effects of BEL and of the
PAPH inhibitor propranolol to distinguish functional consequences of inhibition of iPLA$_2$ or of PAPH (29, 70), and a second employs antisense oligonucleotide suppression of iPLA$_2$ expression (16). We used the first approach here but were unable to suppress iPLA$_2$ activity in INS-1 insulinoma cells with antisense oligonucleotides using conditions applied to P388D1 cells (16) and several variations on them. It has been suggested that the efficacy of antisense suppression may be affected by iPLA$_2$ expression level (29). Anoxic WISH cells, for example, apparently express much higher iPLA$_2$ levels than P388D1 cells (29), and our inability to suppress INS-1 cell iPLA$_2$ with antisense oligonucleotides might reflect high iPLA$_2$ expression. Others have also encountered difficulties with lack of efficacy and lack of specificity of antisense approaches (84), which may offer no greater selectivity than conventional pharmacologic agents even when suppression of the target is achieved (85).

Despite difficulties in achieving completely selective iPLA$_2$ inhibition, the fact that incorporation of arachidonic acid into islet or INS-1 cell phospholipids is not suppressed when iPLA$_2$ is inhibited by 98% indicates that iPLA$_2$ activity is not required for this process in these cells. Pathways for arachidonate incorporation into islet cells appear to differ in several respects from those in P388D1 cells. Such differences might be expected from the marked difference in the esterified arachidonate content in islet phospholipids, which is among the highest of any known tissue (37–39), compared with that of P388D1 macrophage-like cells, which contain far lower levels of esterified arachidonate (27–29) than do native macrophages (25, 26) and higher levels of nonesterified arachidonate (29) than other cells (30–32). P388D1 cells may be deficient in arachidonate incorporation mechanisms employed by other cells and forced to use mechanisms not employed by cells that can maintain a high content of arachidonate-containing phospholipids.

Although arachidonate incorporation is unaffected by chelation of extracellular or intracellular Ca$^{2+}$ in P388D1 cells (15), chelation of intracellular Ca$^{2+}$ reduces but does not eliminate incorporation of arachidonate and palmitate into islets, when incorporation is compared in non-BAPTA-loaded islets and in BAPTA-loaded islets incubated with $[^{3}$H]-fatty acid in Ca$^{2+}$-free EGTA-containing medium. This suggests that both Ca$^{2+}$-dependent and Ca$^{2+}$-independent processes participate in fatty acid incorporation into islet phospholipids. Although Ca$^{2+}$-independent hydrolysis of phospholipids by iPLA$_2$ to generate lysophospholipid acceptors is proposed to be the Ca$^{2+}$-independent event that limits arachidonate incorporation into P388D1 cells (15, 16), only about 60% suppression of arachidonate incorporation into P388D1 cells is achieved under conditions where iPLA$_2$ activity is completely inhibited (15). This suggests that P388D1 cells also employ Ca$^{2+}$-independent mechanisms other than iPLA$_2$ to generate a substantial fraction of LPC acceptors that limit arachidonate incorporation into these cells (15, 16). Ca$^{2+}$-independent mechanisms for generating lysophospholipids that do not involve iPLA$_2$ exist in other cells (86). The Ca$^{2+}$-dependent processes that participate in fatty acid incorporation into islet cells have not yet been identified, but islet beta cells express at least two classes of Ca$^{2+}$-dependent iPLA$_2$ enzymes that might contribute to islet lysophospholipid content (14, 87).

The role of LPC content in regulating fatty acid incorporation into GPC lipids and the contribution of iPLA$_2$ to LPC levels also differ between islets and P388D1 cells. BEL induces a more modest reduction in LPC levels in islets than in P388D1 cells, and this reduction does not impair arachidonate incorporation into islet PC but does so in P388D1 cells (15, 16). This suggests that iPLA$_2$ makes only a modest contribution to islet LPC content and that islet LPC is maintained at sufficiently high levels by non-iPLA$_2$-dependent mechanisms that arachidonate incorporation into islet PC is not limited by LPC level. ESI/MS/MS measurements here and previous measurements by other methods (63) yield similar estimates of islet LPC content and indicate that LPC levels in rat islets are substantially higher than that in rat liver (53), and this may be one means employed by islets to maintain a higher content of arachidonate-containing phospholipids than liver (39) or P388D1 cells (27–29). Although LPC levels do not limit arachidonate incorporation into islet phospholipids, our experiments with IL-1 here and elsewhere (51) indicate that generation of arachidonoyl-CoA may limit such incorporation under circumstances where production of ATP is impaired or CoASH degradation is accelerated. IL-1 stimulates islet production of nitric oxide, which impairs ATP generation by islet mitochondria (68) and reacts with free thiols groups (69), such as those of CoASH. Both ATP and CoASH are required to form arachidonoyl-CoA from arachidonic acid (28, 33, 34), an obligatory step for its incorporation into phospholipids (35, 36).

Although we never observe suppression of fatty acid incorporation into islet or insulinoma cell phospholipids when iPLA$_2$ activity is inhibited, stimulation of incorporation is often observed. The simplest explanation is that net incorporation of fatty acid reflects the difference between acylation and deacylation and that iPLA$_2$ inhibition reduces deacylation but does not affect acylation because iPLA$_2$-catalyzed generation of ly-
The fact that iPLA₂ is not required for arachidonate incorporation or phospholipid remodeling in beta cells suggests that it may play other roles in these cells. Various signaling functions for iPLA₂ have been suggested (10, 17–21), and as demonstrated here and elsewhere (81–83), insulin secretagogues induce hydrolysis of arachidonic acid from islet phospholipids. Our findings that insulin secretagogue-induced accumulation of nonesterified arachidonate in islet incubations is reduced by BEL corroborates the observation that insulin secretagogue-induced release of arachidonate metabolites from islets is reduced by BEL (81). The failure of the PAPH inhibitor propranolol (29, 70) to block insulin secretagogue-induced accumulation of nonesterified arachidonate in islet incubations suggests that PAPH is not the BEL-sensitive target that participates in insulin secretagogue-induced hydrolysis of arachidonate from islet phospholipids, although others (4, 57) have suggested that this process involves sequential actions of phospholipase D, PAPH, and diglyceride lipase. Our findings are consistent with observations by others that 250 μM propranolol maximally inhibits islet PAPH but does not block islet accumulation of nonesterified arachidonate and stimulates insulin secretion (71). PA produced by phospholipase D and hydrolyzed by PAPH may also be an unlikely source of arachidonic acid in signaling events because PA from this route contains primarily saturated or monounsaturated fatty acids and little arachidonate (89).

Signaling roles have been proposed for iPLA₂ in apoptosis induced by the Fas/Fas ligand system in U937 cells (20), in apoptosis induced by Ca²⁺ store depletion in beta cells (90), and in leukotriene production by granulocytes (17). The possibility that iPLA₂ plays different roles and is subject to different mechanisms of regulation in different cells is suggested by the fact that stimuli which induce iPLA₂-catalyzed arachidonate release and leukotriene production in human granulocytes fail to induce these events in human lymphocyte lines, even though both classes of cells express iPLA₂ and leukotriene biosynthetic enzymes (17, 21). The facts that the active form of iPLA₂ is an oligomer (8, 88), that multiple splice variants exist in some cells (21, 41), and that heterooligomeric complexes among different splice variants exhibit altered catalytic activity (21) suggest that iPLA₂ may be subject to complex regulatory mechanisms that differ among cell types. Our findings that iPLA₂ activity is not required for arachidonate incorporation into beta cell phospholipids, even though iPLA₂ appears to participate in such incorporation in P388D1 cells (15, 16), is consistent with the notion that the enzyme plays diverse roles in different cell types.

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FIG. 12. Effects of BEL and propranolol on insulin secretagogue-induced accumulation of nonesterified arachidonic acid in pancreatic islet incubations. Islets were treated with vehicle (cross-hatched bars), 25 μM BEL (stippled bars), or 250 μM propranolol (dark bars) for 30 min at 37°C. The islets were then incubated (30 min at 37°C) in medium containing 3 mM glucose, 17 mM glucose, or 17 mM glucose plus 0.5 mM carbachol. Propranolol (250 μM) was included in the incubation medium for islets that had been preincubated with propranolol. At the end of the incubation, lipids were extracted, mixed with 825 pmol of [3H]arachidonic acid internal standard, and analyzed by RP-HPLC to isolate arachidonic acid, which was converted to a pentafluorobenzyl (PFBE) ester derivative and analyzed by GC/MS in negative ion electron capture mode with selected monitoring of the carboxylate anions of endogenous arachidonic (m/z 303) and of the internal standard (m/z 311). Endogenous arachidonate content was determined from the ratio of the integrated areas of the two ion current peaks at the appropriate retention time by interpolation from a standard curve and normalized to islet protein content. Displayed values represent the arachidonate content in each sample divided by the mean content in islets that had been incubated with 3 mM glucose without BEL or propranolol (n = 6). Conditions that differed from the control with a p value of less than 0.05 are denoted with an asterisk.

sphospholipid acceptors is not required for fatty acid incorporation into islet phospholipids. The similar effects of BEL on incorporation of both arachidonate and palmitate are consistent with the fact iPLA₂ hydrolyzes phospholipids with palmitate substituents at least as readily as those with arachidonate substituents (8, 88). Although palmitate incorporation into P388D1 cells has been taken to reflect primarily de novo phospholipid synthesis (15), palmitate incorporation into islet phospholipids under our conditions proceeds predominantly by deacylation/reacylation because BEL impairs de novo phospholipid synthesis but does not suppress palmitate incorporation. Deacylation/reacylation reactions with palmitate occur as rapidly as those with arachidonate in some cells (76–80). We have not determined whether palmitate incorporated into islet phospholipids under our conditions resides primarily in the sn-1 or sn-2 position, but islets contain substantial amounts of GPC species with palmitate as the sn-2 substituent, including 16:0/16:0-GPC (39). Deacylation/reacylation of phospholipid sn-1 substituents also occurs (76–80), and iPLA₂ can catalyze hydrolysis of sn-1 substituents of phospholipids (8).
