Mass Spectrometric Identification and Quantitation of Arachidonate-Containing Phospholipids in Pancreatic Islets: Prominence of Plasmenylethanolamine Molecular Species†

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ABSTRACT: D-Glucose induces insulin secretion from β-cells of pancreatic islets by processes involving glycolytic metabolism and generation of ATP. Glucose also induces hydrolysis of β-cell membrane phospholipids and accumulation of nonesterified arachidonate, which facilitates Ca^{2+} entry and the rise in β-cell Ca^{2+} concentration that is a critical signal in the induction of insulin secretion. Glucose-induced hydrolysis of arachidonate from β-cell phospholipids is mediated in part by an ATP-stimulated, Ca^{2+}-independent (ASCI)-phospholipase A_2 (PLA_2), which, in vitro, prefers plasmalogen over diacylphospholipid substrates, but it is not known whether islets contain plasmalogens. We have identified and quantitated the major species of arachidonate-containing phospholipids in pancreatic islets by high-performance liquid chromatographic and mass spectrometric analyses. Arachidonate has been found to constitute 30% of the total islet glycerolipid fatty acyl mass. Ethanolamine phospholipids contain 30% of total islet arachidonate, and 44% of that amount resides in three plasmenylethanolamine molecular species with residues of palmitic, oleic, or stearic aldehydes in the *sn*-1 position. These endogenous islet plasmenylethanolamine species are hydrolyzed more rapidly than phosphatidylethanolamine species by islet ASCI-PLA_2 in vitro and are also hydrolyzed in intact islets stimulated with secretagogues. ASCI-PLA_2-catalyzed hydrolysis of islet plasmenylethanolamine species in vitro is inhibited by a selective haloenol lactone suicide substrate (HELSS) which is sterically similar to plasmalogens, and HELSS also inhibits all temporal phases of both eicosanoid release and insulin secretion from secretagogue-stimulated pancreatic islets. Islet β-cell ASCI-PLA_2-catalyzed hydrolysis of arachdionate from endogenous plasmenylethanolamine substrates may be an intermediary biochemical event in the induction of insulin secretion.

When stimulated with high concentrations of D-glucose, pancreatic islets secrete insulin by a process that requires entry of D-glucose into islet β-cells and glycolytic metabolism (Malaisse et al., 1979; Ashcroft, 1980; Hedeskov, 1980; Meglasson & Matschinksy, 1986). Among the signals derived from the metabolism of D-glucose is the generation of ATP in compartments accessible to a β-cell plasma membrane K^+-channel (K_{ATP}) which is inactivated by ATP (Ashcroft et al., 1984; Cook & Hales, 1984; Rorsman & Trube, 1985; Sturgress et al., 1985). The resultant decline in membrane K^+ permeability causes a rise in membrane potential and the activation of voltage-operated Ca^{2+} channels (Arkhammar et al., 1987; Gylfe, 1988a,b; Keahey et al., 1989). This results in Ca^{2+} influx and a rise in β-cell cytosolic [Ca^{2+}], which is thought to be a critical signal in the induction of insulin secretion (Wollheim & Sharp, 1981; Arkhammar et al., 1987; Gylfe, 1988a,b).

The D-glucose-induced rise in β-cell Ca^{2+} concentration appears to be amplified by a signal transduction process involving phospholipid hydrolysis and the accumulation of nonesterified arachidonate in β-cell membranes. Upon stimulation with D-glucose, arachidonate is rapidly hydrolyzed from β-cell membrane phospholipids, and nonesterified arachidonate accumulates to concentrations of at least 35–70 μM (Wolf et al., 1986, 1991). This process requires the metabolism of D-glucose and is in part independent of Ca^{2+} influx (Metz, 1985; Wolf et al., 1991; Turk et al., 1992). Nonesterified arachidonate appears to faciliate Ca^{2+} influx into β-cells (Wolf et al., 1991; Ramanadham et al., 1992), perhaps by altering the voltage sensitivity of voltage-operated Ca^{2+} channels (Vacher et al., 1989). The resultant amplification of the rise in β-cell Ca^{2+} concentration by arachidonate (Metz et al., 1987; Ramanadham et al., 1992) amplifies depolarization-induced insulin secretion (Wolf et al., 1991) and appears to play a role in the insulin secretory response to D-glucose (Ramanadham et al., 1993).

A component of the D-glucose-induced hydrolysis of arachidonate from islet membrane phospholipids has recently been demonstrated to be catalyzed by a phospholipase A_2 (PLA_2)[1] enzyme (Gross et al., 1993; Ramanadham et al., 1993) similar or identical to an enzyme first described in cardiac myocytes (Wolf & Gross, 1985; Hazen et al., 1990, 1991a,b; Hazen & Gross, 1991), the activity of which is independent of Ca^{2+} and stimulated by ATP. Like the myocardial enzyme, the islet ATP-stimulated, Ca^{2+}-independent (ASCI)-PLA_2 prefers *sn*-2 arachidonyl residues over other *sn*-2 fatty acyl substituents and prefers plasmalogen over 1,2-diacyl-*sn*-phospholipid substrates (Gross et al., 1993). The preference of ASCI-PLA_2 for plasmalogen substrates has facilitated the utilization of a haloenol lactone suicide substrate (HELSS) which is sterically similar

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to plasmalogens and which irreversibly inactivates ASCI-PLA_2 at concentrations which have no influence on the activities of Ca^{2+}-dependent phospholipases A_2 from a variety of sources (Hazen et al., 1991b). Selective inhibition of β-cell ASCI-PLA_2 with HELSS suppresses D-glucose-induced hydrolysis of arachidonate from islet membrane phospholipids, the rise in β-cell Ca^{2+} concentration, and insulin secretion at concentrations which do not influence islet glucose oxidation or muscarinic agonist-induced phosphoinositide–phospholipase C activation (Ramanadham et al., 1993).

The preference of ASCI-PLA_2 for plasmalogen substrates containing *sn*-2 arachidonyl residues and the possibility that this enzyme is a component of the β-cell glucose-sensor apparatus raise the question of whether islets contain plasmalogen phospholipids with *sn*-2 arachidonoyl residues which might serve as endogenous substrates of ASCI-PLA_2. At present nothing is known about the plasmalogen content of islets. Here we report the results of studies in which the islet content of plasmalogens and of other phospholipids with *sn*-2 arachidonoyl residues has been determined by liquid chromatographic and mass spectrometric analyses.

EXPERIMENTAL PROCEDURES

Materials. The compounds [$^3H_8$]arachidonic acid (100 Ci/mmol), L-α-1-palmitoyl-2-([1-^{14}C]linoleoyl)phosphatidylcholine (60 mCi/mmol), and L-α-1-palmitoyl-2-([1-^{14}C]-arachidonoyl)phosphatidylethanolamine (60 mCi/mmol) were purchased from Dupont New England Nuclear; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), and sphingomyelin (SM) were from Avanti Polar Lipids (Birmingham, AL); standard ethanolamine–phospholipid from bovine brain containing 65% plasmalogen was from Sigma (St. Louis, MO); phosphatidic acid (PA) was from Serdary Lipids; EGTA and other common buffer reagents were from Sigma; and HPLC columns were from Alltech (Deerfield, IL). The haloenol lactone suicide substrate (HELSS) [(*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one] was prepared as described (Hazen et al., 1991), transferred to its diluent vial as an ethanol solution, concentrated to dryness, and reconstituted in albumin-free buffer immediately before use.

Male Sprague-Dawley rats (180–220 g body weight) were purchased from Sasco (O'Fallon, MO); collagenase was from Boehringer Mannheim (Indianapolis, IN); tissue culture medium (CMRL-1066), penicillin, streptomycin, Hanks' balanced salt solution (HBSS), heat-inactivated fetal bovine serum, and L-glutamine were from Gibco (Grand Island, NY); pentex bovine serum albumin (fatty acid free, fraction V) was from Miles Laboratories (Elkhart, IN); rodent Chow 5001 was from Ralston Purina (St. Louis, MO); and D-glucose was from the National Bureau of Standards (Washington, DC).

Media. Media included KRB (Krebs-Ringer Bicarbonate buffer; 25 mM HEPES, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$); nKRB ("normal" KRB, which contained 3 mM D-glucose); cCMRL-1066 ("complete" CMRL-1066, which was supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% (w/v) each of penicillin and streptomycin); and HBSS (Hanks' balanced salt solution supplemented with 0.5% penicillin–streptomycin).

Isolation of Islets and Labeling with [$^3H_8$]Arachidonic Acid. Islets were isolated aseptically from male Sprague-Dawley rats fed ad libitum (McDaniel et al., 1983). Surgically exposed pancreata were inflated with HBSS medium instilled via the common bile duct, excised, freed from adherent tissue, minced, and digested with collagenase (8 mg/mL, 37 °C, 15 min). Islets were then isolated from the digestate by centrifugation through a discontinuous ficoll gradient as described elsewhere (Ramanadham et al., 1993). The isolated islets were then resuspended in cCMRL-1066 medium, transferred into Falcon Petri dishes which contained 2.5 mL of cCMRL-1066, placed under an atmosphere of 95% air/5% CO_2, and cultured overnight (37 °C). Human pancreatic islets were prepared in the laboratory of Dr. David Scharp as described (Ricordi et al., 1988) and then cultured (48 h, 37 °C) under an atmosphere of 5% CO_2/95% O_2 in CMRL-1066 containing 10% fetal bovine serum, penicillin (100 U/mL), glutamine (2 mM), and HEPES (25 mM) at pH 7.4. For radiolabeling studies, isolated islets (ca. 9000) were incubated overnight at 37 °C in cCMRL-1066 medium with [$^3H_8$]arachidonic acid [50–200 μCi, 100 Ci/mmol) and then washed free of unincorporated radiolabel as previously described (Turk et al., 1986). Disappearance of radiolabel from the culture medium ranged between 30 and 62% after this period of culture.

Perifusion of Islets, Measurement of Insulin and PGE_2, and Extraction of Phospholipids. Isolated, cultured islets were washed three times in nKRB medium supplemented with 0.1% fatty acid-free BSA and saturated under an atmosphere of 95% air/5% CO_2. Islets (500 per condition) were then transferred onto millipore filters of each of four chambers of a perifusion apparatus and were perifused with nKRB medium (1 mL/min) at 37 °C, as described elsewhere (Wolf et al., 1991). The composition of the medium was then either maintained or changed to contain a higher D-glucose concentration (17 mM) and 500 μM carbachol, and perifusion was continued for an additional 30–240 min. Perifusion effluent was collected continuously (1–10-mL aliquots) and was subsequently analyzed for content of insulin by double antibody radioimmunoassay with ^{125}I-labeled insulin (Wright et al., 1971) and of PGE_2 by enzyme immunoassay with anti-PGE_2 antibody and acetylcholinesterase-labeled PGE_2 obtained from Cayman Chemical (Ann Arbor, MI), as previously described (Pradelles et al., 1985; Turk et al., 1992b). At the end of the perifusion period, filters containing the islets were removed from the perifusion chambers, and phospholipids were extracted with chloroform/methanol under neutral conditions (Bligh & Dyer, 1959). In studies with the haloenol lactone suicide substrate (HELSS) [(*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one], islets were pretreated with HELSS (25 μM) for 30 min at 37 °C in nKRB and then resuspended in fresh medium before application to the filters of the perifusion apparatus.

Normal-Phase HPLC Separation of Islet Phospholipids into Head-Group Classes. Phospholipid solutions were concentrated to dryness under nitrogen, reconstituted in 200 μL of hexane/isopropranol (1:1), and analyzed by normal-phase HPLC (Patton et al., 1982) on a silicic acid HPLC

[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; SM, sphingomyelin; PA, phosphatidic acid; NL, neutral lipids; HELSS, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; KRB, Krebs Ringer bicarbonate buffer; PGE_2, prostaglandin E_2; ASCI, ATP-stimulated Ca^{2+}-independent; PLA_2, phospholipase A_2; FAME, fatty acid methyl ester; DMA, dimethylacetal; GC, gas chromatography; FID, flame ionization detection; MS, mass spectrometry; EI, electron impact; PCI, positive ion chemical ionization; NICI, negative ion chemical ionization; HPLC, high-performance liquid chromatography; NP, normal phase; RP, reverse phase.

column (LiChrospher Si-100, 10 μm, 250 × 4.6 mm, Alltech) in hexane/isopropanol/(25 mM potassium phosphate, pH 7.0)/ethanol/acetic acid (367:490:62:100:0.6) at a flow of 0.5 mL/min for 60 min and then 1.0 mL/min with flow-through UV monitoring at 205 nm. Aliquots (50 μL) of each fraction (1 mL) were subjected to liquid scintillation spectrometry to determine ^3H content (and, where appropriate, ^{14}C content). Standard phospholipids exhibited the following approximate retention times under these conditions: PE, 13 min; PA, 23 min; PI, 28 min; PS, 39 min; CL, 62 min; PC, 101 and 105 min (bifid peak); and SM, 125 min. An alternate normal-phase solvent system employed in some analyses consisted of a gradient between solvent system A (hexane/isopropanol/water 100:100:3) and B (hexane/isopropanol/water 100:100:7) starting with 100% A for 6 min followed by a linear gradient to 50% B over 24 min at a flow rate of 2.0 mL/min (Blank & Snyder, 1983). Under these conditions, the following approximate retention times were observed for phospholipid standards: PE, 8 min; PI, 25 min; PS, 30 min; and PC, 55 min. Recovery of internal standard ^{14}C-labeled phospholipids from the NP-HPLC analyses averaged 72 ± 2%.

Analysis of the Diglyceride Moiety of Islet Phospholipids. An aliquot of each phospholipid head-group class was digested with *Bacillus cereus* phospholipase C to yield the diglycerides (Turk et al., 1992). This enzyme is active against diacyl, alkenylacyl, and akylacyl species of PC and PE (Sugiura et al., 1983; Wey et al., 1986; Ramesha & Pickett, 1987; Purdon et al., 1987). To the dry sample was added diethyl ether (1 mL) and buffer (pH 8.0, 0.25 mL), which was composed of water (300 mL), sodium tetraborate (9.15 g), sodium chloride (0.97 g), and calcium chloride monohydrate (35 mg). *B. cereus* phospholipase C (25 units) (Sigma P-4014) was then added, followed by incubation (90 min, 37 °C). After centrifugation (800*g*, 5 min), the ether phase containing the diglyceride product was aspirated into a separate vial and concentrated to dryness. The diglycerides were then converted to the acetate derivatives with acetic anhydride (0.1 mL) and pyridine (0.02 mL) (room temperature, 90 min) (Wey et al., 1986). After concentration to dryness and reconstitution in hexane (50 μL), the acetyldiglyceride products were analyzed by TLC (Analtech silica gel G plates) by developing first with hexane/diethyl ether (1:1) and then, after drying, with toluene. Visualization was achieved with phosphomolybdic acid (Sigma). Under these conditions, alklenylacyl- (R_f 0.47), alkylacyl- (R_f 0.42), and diacyl- (R_f 0.37) acetylglycerols were clearly separated (Wey et al., 1986).

Reverse-Phase HPLC Analysis of Islet Phospholipid Head-Group Classes into Molecular Species. An aliquot of each phospholipid head-group class was analyzed on an octadecylsilicic acid HPLC column (Ultrasphere ODS 4.6 × 250 mm, Alltech) with 20 mM choline chloride in methanol/water/acetonitrile (90.5:7:2.5) at a flow rate of 2.0 mL/min (Patton et al., 1982) at 40 °C (Chilton & Murphy, 1986) with flow-through UV monitoring (205 nm). The ^3H (and, where appropriate, ^{14}C) content of aliquots (50 μL) of each fraction (1 mL) was determined by liquid scintillation spectrometry. Phospholipids with differing *sn*-1 and *sn*-2 substituents are separated into molecular species in this system (Patton et al., 1982; Chilton & Murphy, 1986). For phospholipids with a given fatty acid residue (e.g., arachidonate) in the *sn*-2 position and a saturated 16-carbon chain in the *sn*-1 position, the order of elution is diacyl < alkenylacyl < alkylacyl, with baseline resolution among these species (Chilton & Murphy, 1986). The following approximate retention times for commercially available 1,2-diacylphospholipid standards were observed in this system: (C16:0a/C20:4)-PC, 33 min; (C16:0a/C18:2)-PC, 34 min; (C16:0a/C18:1)-PC, 46 min; (C18:0a/C20:4)-PC, 53 min; (C18:0a/C18:1)-PC, 74 min; (C16:0a/C18:2)-PE, 28 min; (C16:0a/C18:1)-PE, 36 min. The numbers in parentheses denote the carbon length and number of carbon–carbon double bonds in the fatty acyl residues, and the letter "a" denotes an acyl linkage in the *sn*-1 position. Recovery of internal standard ^{14}C-labeled phospholipids from the RP-HPLC analyses averaged 70 ± 5%.

Determination of Acid Lability of Phospholipids. Phospholipid samples were divided into two aliquots and concentrated to dryness. One aliquot was then treated with acid (1 N HCl in methanol/chloroform (1:1), total volume 1 mL, 45 min, room temperature), and the other was sham-treated (chloroform/methanol (1:1), 1 mL). Samples were then neutralized (0.5 mL of 1 M Na_2CO_3), extracted (1 mL of $CHCl_3$), concentrated, reconstituted in methanol (180 μL), and analyzed by RP-HPLC. Diacyl- and alkylacylphospholipids are stable under these conditions and appear in the chromatograms of both acid-treated and sham-treated samples. Plasmalogens are destroyed by acid treatment and appear only in the chromatogram of the sham-treated sample (DaTorre & Creer, 1991).

Identification of Resolved Phospholipid Molecular Species by Gas Chromatography–Positive Ion Methane Chemical Ionization–Mass Spectrometry. Individual peaks from reverse-phase HPLC analyses were subjected to acid methanolysis (1 N HCl in methanol, 1 mL, under nitrogen, 90 °C, 90 min) (Gross, 1984). Under these conditions, fatty acyl residues are released from glycerolipids as fatty acid methyl esters (FAME). Plasmalogens contain *sn*-1 fatty aldehyde residues in a vinyl ether linkage, which are released as dimethylacetals (DMA) by acid methanolysis. FAME and DMA were then extracted with heptane (1 mL) from the neutralized (excess Na_2CO_3) methanolysate after addition of water (1 mL). The extracts were concentrated to dryness, reconstituted in heptane (10–100 μL), and analyzed by capillary column (Hewlett-Packard Ultraperformance, 8 m, 0.17 μm dimethylsilicone film, i.d. 0.31 mm) gas chromatography (initial temperature 85 °C for 0.5 min, followed by a 20 °C/min increasing ramp to 240 °C) mass spectrometry (Hewlett-Packard 5988) in the methane (source pressure, 1 torr) positive ion chemical ionization mode. Under these conditions, FAME exhibit the following (M+1) ions and approximate retention times: palmitate (m/z 271, 3.47 min); stearate (m/z 299, 4.80 min); oleate (m/z 297, 4.63 min); linoleate (m/z 295, 4.58 min); arachidonate (m/z 319, 5.25 min). For DMA derived from the fatty aldehyde residues of plasmalogens, the corresponding values for (M-31) ions and GC retention times are as follows: palmitic aldehyde (m/z 255, 4.23 min); oleic aldehyde (m/z 281, 4.90 min); and stearic aldehyde (m/z 283, 5.08 min).

Quantitation of the Phospholipid Content of Arachidonate and Other Fatty Acids by Stable Isotope Dilution Negative Ion Chemical Ionization Gas Chromatography–Mass Spectrometry. To quantitate the fatty acyl mass in the small amounts of phospholipids obtained from islet incubations and perifusions, alkaline hydrolysis was performed in the presence of [$^2H_8$]arachidonate as an internal standard, and the liberated fatty acids were extracted, converted to their pentafluorobenzyl ester (PFBE) derivatives, and quantitated by stable isotope dilution negative ion chemical ionization (NICI)–mass spectrometry (MS). Phospholipid peaks from HPLC analyses

were spiked with [$^2H_8$]arachidonic acid (250 ng) and concentrated to dryness. Dimethoxyethane (0.5 mL) and 3 N LiOH (0.1 mL) were added, and samples were heated (60 °C, 90 min) with stirring and then allowed to cool to room temperature, whereupon water (3.5 mL) was added. After vortex-mixing, methylene chloride (1 mL) was added, vortex-mixing was repeated, and the methylene chloride phase was aspirated into a separate vial. To the dry sample, 20 μL of a solution consisting of *N*,*N*-dimethylacetamide/tetramethylammonium hydroxide/methanol (1:0.5:1.5) and 20 μL of a solution consisting of pentafluorobenzyl bromide/*N*,*N*-dimethylacetamide (1:3) were then added, and the tubes were votex-mixed and incubated (room temperature, 15 min). After concentration to dryness, water (50 μL) was added, vortex-mixing was performed, methylene chloride (200 μL) was added, vortex-mixing was repeated, and the aqueous phase was discarded. The methylene chloride phase was then concentrated to dryness. Samples were then reconstituted in heptane (0.5 mL) and analyzed by capillary column (Hewlett-Packard Ultraperformance, 8 m, 0.17 μm dimethylsilicone film, i.d. 0.31 mm) gas chromatography (initial temperature 85 °C for 0.5 min, followed by a 20 °C/min increasing ramp to 240 °C) mass spectrometry (Hewlett Packard 5988) in the methane (source pressure, 1 torr) negative ion–chemical ionization mode. Fatty acid PFBE were detected by their M-PFB (M-181) ion at characteristic retention times and were quantitated relative to the [$^2H_8$]arachidonate internal standard: palmitate (*m*/*z* 255, 5.3 min); oleate (*m*/*z* 281, 6.85 min); stearate (*m*/*z* 283, 7.0 min); linoleate (*m*/*z* 279, 6.6 min); arachidonate (*m*/*z* 303, 7.9 min); [$^2H_8$]arachidonate (*m*/*z* 311, 7.85 min). Quantitation was performed by integrating the area of each of these peaks, dividing by the peak area for the [$^2H_8$]arachidonate internal standard, subtracting the blank value of the peak ratio for individual fatty acids, and interpolating from standard curves for each fatty acid. Values for blank values of peak ratios for individual fatty acids were determined from duplicate samples to which internal standard had been added and which were processed in parallel with the phospholipid samples but which contained no phospholipid. Six point standard curves for each fatty acid species were generated by adding constant amounts of [$^2H_8$]arachidonate internal standard to each of six vials and varied concentrations (0, 0.15, 0.75, 1.5, 3, or 6 μg) of palmitate, stearate, oleate, linoleate, and arachidonate.

Subcellular Fractionation. Isolated pancreatic islets were homogenized in buffer (0.25 M sucrose, 40 mM Tris-HCl, pH 7.1, adjusted with HCl) by sonication in a Polytron apparatus (probe 419, 15-s burst, 1.5 output, 50% duty cycle, 2-s pulse). Nuclei and cellular debris were removed by centrifugation (1000*g*, 10 min). The mitochondrial pellet obtained after additional centrifugation (10000*g*, 10 min) was discarded. The resultant 10000*g* supernatant was then centrifuged (170000*g*, 60 min) to separate the membranous fraction (pellet) from the cytosolic fraction (supernatant). The membranous fraction was then resuspended in homogenization buffer.

Phospholipase A_2 Activity Measurements. The ATP-stimulated, Ca^{2+}-independent phospholipase A_2 activity contained in subcellular fractions (150 μL of cytosol or 100 μL of membranous fraction with average protein contents of 35 μg) from pancreatic islets was assayed by ethanolic injection (5 μL, final ethanol concentration 1.25%) of [^{3}H]arachidonate-labeled phospholipid substrates (final substrate concentration, 0.28 μM) isolated from islets in assay buffer (final conditions: 400-μL total volume, 200 mM Tris, pH 7.5, 10 mM EGTA, and 1 mM ATP). The assay mixture was incubated (5 min, 37 °C), and the reaction was terminated by addition of butanol (100 μL) and vortex-mixing. The organic phase was separated by centrifugation (2000*g* for 2 min), and an aliquot (25 μL) was applied to channeled TLC plates with a preadsorbent band for sample application. Samples were not acidified before butanol extraction, and the butanol extract was not concentrated before application to the TLC plate. Fatty acid extraction efficiency under these conditions exceeds 95% (Gross & Sobel, 1982). TLC was performed on Silica Gel G plates with petroleum ether/diethyl ether/acetic acid (70:30:1). Free fatty acid (R_f 0.58) was clearly resolved from 1-alkenyl-2-acyl-*sn*-glycerols (R_f 0.24) and 1,2-diacyl-*sn*-glycerols (R_f 0.21) under these conditions. The region of the TLC plates corresponding to free fatty acid was scraped into a scintillation vial and quantified by liquid scintillation spectrometric measurement of ^{3}H content after addition of Universol (3 mL). In experiments examining the influence of HELSS on the islet ATP-stimulated, Ca^{2+}-independent phospholipase A_2 activity, HELSS was dissolved in ethanol (5 μL), concentrated to dryness, reconstituted in incubation buffer, and injected into the assay mixture (final concentration, 10 μM). Protein content was measured with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

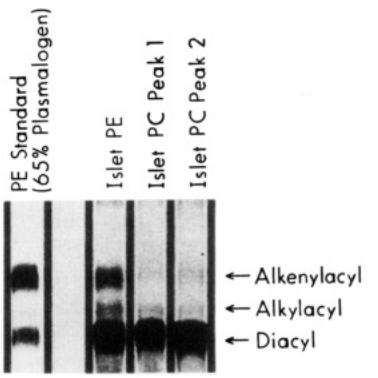

FIGURE 1: Thin layer chromatographic analyses of the diglyceride moieties of ethanolamine and choline phospholipids from isolated pancreatic islets. The PE peak and the two PC peaks from NP-HPLC analyses of isolated rat islet phospholipids were harvested separately and digested to the diglycerides with *B. cereus* phospholipase C along with standard bovine brain ethanolamine phospholipid which contains 65% plasmalogen. The diglycerides were then converted to the acetate derivatives with acetic anhydride and analyzed by TLC under conditions where alkenylacyl-, alkylacyl-, and diacylacetylglycerols are clearly separated as described under Experimental Procedures.

RESULTS

When pancreatic islets isolated from 15 rats were pooled and subjected to neutral Bligh-Dyer phospholipid extraction, normal-phase (NP) HPLC analysis under conditions where phospholipids are separated into head-group classes (Patton et al., 1982) revealed that the major rat islet phospholipids were phosphatidylethanolamine (PE, ca. 22.4 ± 2.3 pmol/islet) and phosphatidylcholine (PC, ca. 58.1 ± 9.1 pmol/islet). Smaller amounts of phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL) were observed. Identification of islet head-group classes were based on co-elution with standard phospholipids, and estimates of their relative abundance were based on UV absorbance. (This method provides only a rough estimate of phospholipid mass because it is affected by the amount of unsaturation in the *sn*-1 and *sn*-2 substituents.)

To determine whether any of these islet phospholipid classes contained plasmalogen species, the phospholipid peaks were

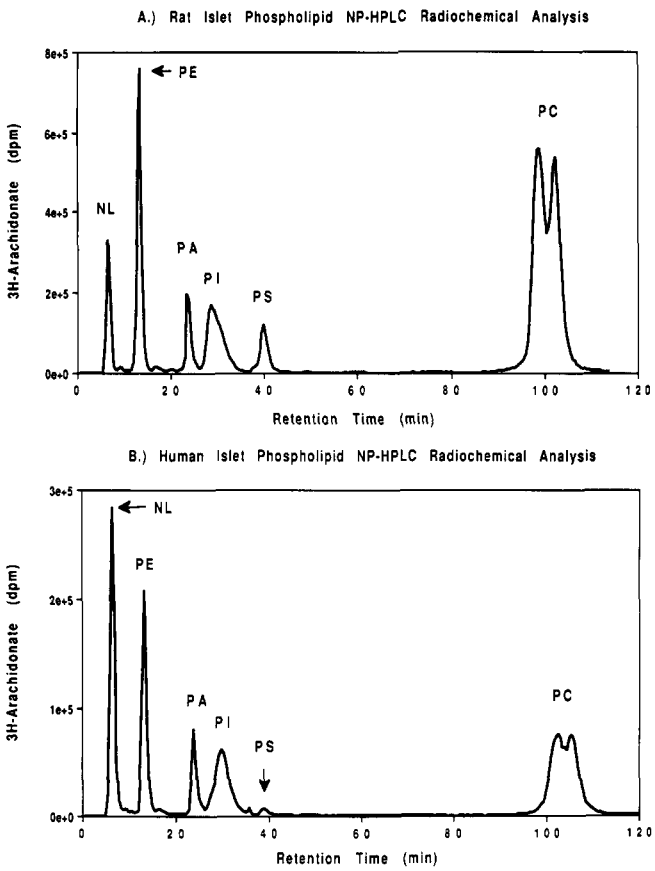

FIGURE 2: Normal-phase HPLC radiochemical analyses of arachidonate-containing phospholipids from isolated pancreatic islets incubated with [^{3}H]arachidonic acid. Isolated pancreatic islets (ca. 9000 per condition) from 30 rats (panel A) or a single human donor (panel B) were incubated with [^{3}H]arachidonic acid overnight at 37 °C and washed free of unincorporated radiolabel as described under Experimental Procedures. Phospholipids were then extracted under neutral conditions and analyzed by NP-HPLC. The ^{3}H content of aliquots (50 μL) of each eluant fraction (1 mL) was determined by liquid scintillation spectrometry. Abbreviations: NL, neutral lipids; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PC, phosphatidylcholine. The bifid peak for PC reflects partial separation of distinct molecular species of PC on the NP-HPLC column.

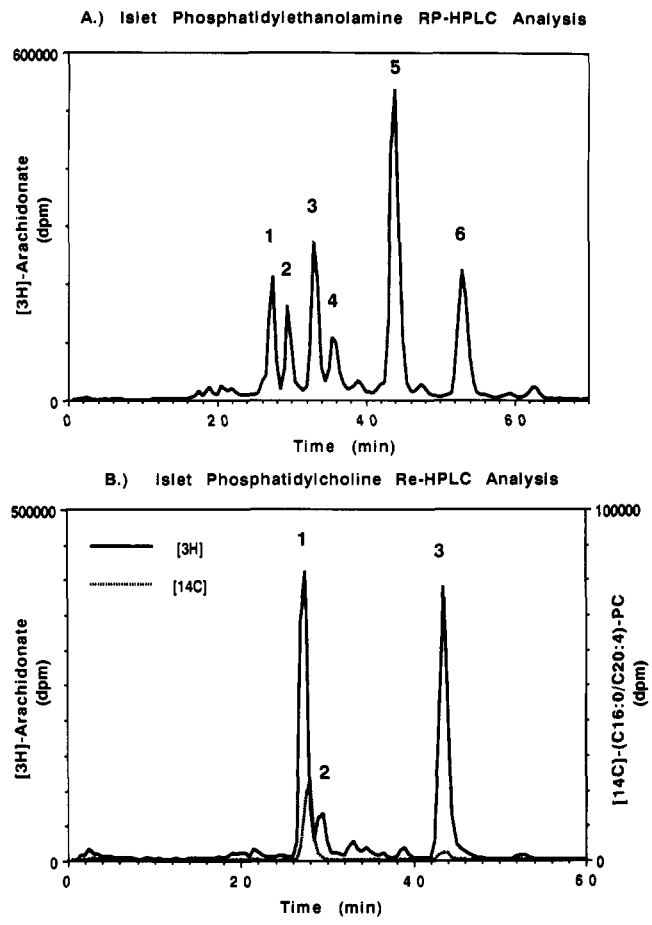

FIGURE 3: Reverse-phase HPLC analyses of ethanolamine and choline phospholipids from isolated pancreatic islets. The PE peak and the PC peak from Figure 2A were harvested separately and analyzed by RP-HPLC under conditions where molecular species of phospholipids are separated as described under Experimental Procedures.

harvested separately from NP-HPLC analyses, digested to the diglycerides with *B. cereus* phospholipase C, converted to the acetate derivatives with acetic anhydride, and analyzed by TLC under conditions where alkenylacyl-, alkylacyl-, and diacylacetylglycerols are separated (Wey et al., 1986). As illustrated in Figure 1, such treatment generated both alkenylacyl- and diacylacetylglycerols from standard bovine brain PE which contains 65% plasmalogen. In contrast, islet PC yielded only diacylacetylglycerol under these conditions. Islet PE, however, yielded substantial amounts of alkenylacylacetylglycerol upon such treatment, suggesting that islet PE contains plasmalogen molecular species. Islet PI, PA, and PS yielded only diacylacetylglycerols (not shown).

To facilitate identification of arachidonate-containing molecular species of islet phospholipids, isolated islets were incubated overnight at 37 °C with [$^3H_8$]arachidonic acid before Bligh-Dyer extraction. NP-HPLC analyses of the resultant extracts revealed substantial incorporation of [$^3H_8$]-arachidonic acid into neutral lipids (NL), PE, PA, PI, PS, and PC from both rat and human islets (Figure 2). Individual phospholipid classes were then harvested separately and subjected to reverse-phase (RP) HPLC analyses under conditions where individual molecular species of phospholipid species are resolved (Patton, 1982). As illustrated in Figure 3, such analyses revealed six major arachidonate-containing species of islet PE and three major arachidonate-containing species of islet PC.

Determination of whether any of the islet arachidonate-containing PE molecular species were plasmalogens was accomplished by determining their acid lability and by mass spectrometric analyses. Plasmalogens are distinguished from diacyl- and alkylacylphospholipids by the lability of the alkenyl moiety upon exposure to acid (DaTorre & Creer, 1991). A second feature of plasmalogens is that, under conditions of acid methanolysis, the *sn*-1 fatty aldehyde residue is released as a dimethylacetal (DMA) while acylated fatty acid residues are released as fatty acid methyl esters (FAME) (Gross, 1984). Under conditions of positive ion methane chemical ionization (PCI)–mass spectrometry (MS), DMA species yield mass spectra which are dominated by ions generated by the loss of an OCH_3 moiety (M–31) and which also exhibit both (M–1) and (M–[2 × {CH_3OH}]) ions. FAME yield PCI–mass spectra whose base peak corresponds to the protonated molecular ion (M+1) and which also exhibit (M–1), ($M+C_2H_5$), and ($M+C_3H_5$) ions. Individual molecular species of DMA and FAME are clearly separated from each other on gas chromatography (GC) and can be visualized by selected monitoring of the appropriate (M–31) and (M+1) ions with methane PCI–MS.

Application of these procedures to individual islet PE peaks from the RP-HPLC analyses illustrated in Figure 3A revealed

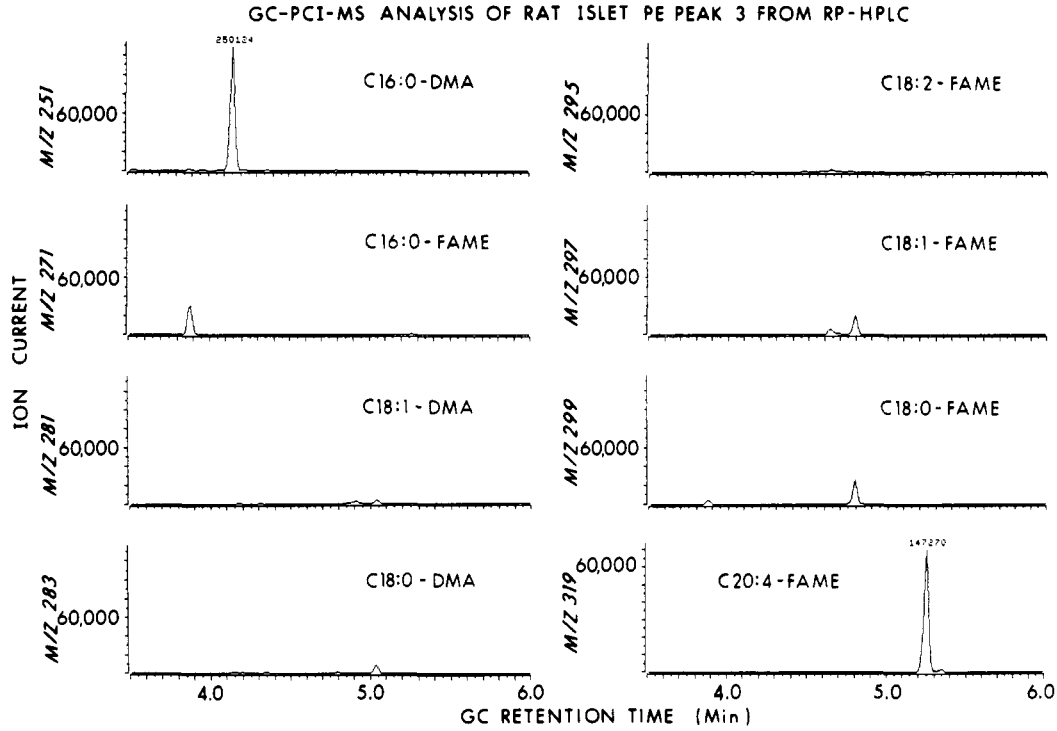

FIGURE 4: Gas chromatographic positive ion methane chemical ionization mass spectrometric analysis of the acid methanolysate of pancreatic islet ethanolamine-phospholipid RP-HPLC peak 3. Peak 3 from Figure 3A was subjected to acid methanolysis, and the methanolysate was analyzed by GC-PCI-MS as described under Experimental Procedures.

that peaks 3, 4, and 6 were acid labile and that peaks 1, 2, and 5 were acid stable. GC–PCI–MS analysis of the acid methanolysis products from the individual islet PE peaks revealed that the acid-labile peaks 3 (Figure 4), 4, and 6 contained (C16:0p/C20:4)-PE (Figure 4), (C18:1p/C20:4)-PE, and (C18:0p/C20:4)-PE, respectively, where the numbers in parentheses designate the carbon chain length and number of carbon–carbon double bonds in the fatty aldehyde or fatty acid residues in the *sn*-1 and *sn*-2 positions and the letter "p" denotes a plasmenyl linkage in the *sn*-1 position. GC–PCI–MS analyses of the acid methanolysis products of the acid-stable islet PE peaks 1, 2, and 5 revealed that these peaks contained (C16:0a/C20:4)-PE, (C18:1a/C20:4)-PE, and (C18:0a/C20:4)-PE, respectively, where the letter "a" denotes an acyl linkage in the *sn*-1 position. This is illustrated for peak 5 in Figure 5. Application of these procedures to individual islet PC peaks from the RP-HPLC analyses in Figure 3B revealed that islet PC peaks 1, 2, and 3 were all acid stable. GC–PCI–MS analyses of the acid methanolysis products of these peaks revealed that islet PC peaks 1, 2, and 3 contained (C16:0a/C20:4)-PC, (C18:1a/C20:4)-PC, and (C18:0a/C20:4)-PC, respectively. The bifid nature of the islet PC peak observed on NP-HPLC analysis (Figure 2) was found to reflect partial separation of (C16:0a/C20:4)-PC (later eluting peak) and (C18:0a/C20:4)-PC (earlier eluting peak) on the NP-HPLC column. Freshly isolated pancreatic islets that had not been subjected to radiolabeling or ex vivo incubation procedures were also found to contain substantial amounts of plasmenylethanolamine molecular species, as illustrated in Figure 6.

Attempts to determine whether stimulation of islets with insulin secretagogues induced detectable loss of arachidonate from individual arachidonate-containing phospholipid molecular species were complicated by the fact that static incubations of the relatively large numbers of islets required for phospholipid analyses resulted in a poor insulin secretory response, which may be attributable to accumulation of inhibitory factors such as somatostatin in the incubation medium (Wolf et al., 1991). It was found that up to 500 islets could, however, be loaded onto each filter of a perifusion apparatus and that perifusion of the islets with secretagogues induced a robust insulin secretory response under these conditions (Figure 7A). A rapidly evolving first phase of insulin secretion was first detectable 4 min after addition of secretagogues to the perifusion medium, peaked at 6 min, and declined to a trough value which remained above the basal secretory rate between 8 and 10 min. Thereafter, a progressive rise in the secretory rate occurred between 10 and 20 min, which was followed by a sharp increase to a plateau value between 20 and 40 min. This was followed by another sharp increase to a new pleateau value between 50 and 90 min.

The multiphasic time course of the insulin secretory response occurred in parallel with a similar multiphasic pattern of secretagogue-induced eicosanoid release from the islets (Figure 7B), which was significantly higher than the basal rate by 3 min after addition of secretagogues to the perifusion medium, increased to a peak value between 6 and 8 min, and declined to a trough value which remained well above basal rates of release at 10 min. This was followed by two phasic increases in eicosanoid release of increasing peak amplitudes between 10 and 20 min and between 20 and 40 min, respectively, and then by a sharp increase to a plateau value between 50 and 90 min. All temporal phases of both secretagogue-induced insulin secretion (Figure 7A) and eicosanoid release (Figure 7B) were suppressed to a similar degree by a haloenol lactone suicide substrate (HELSS) of the islet ATP-stimulated, Ca^{2+}-independent (ASCI) phospholipase A_2 (PLA_2).

Sequential NP-HPLC and then RP-HPLC analyses were performed on phospholipids obtained from islets which had been prelabeled with [$^3H_8$]arachidonate and then perifused. Internal standard [^{14}C]-(C16:0/C18:2)-PC and [^{14}C]-(C16:0/C20:4)-PE were included in these analyses to monitor recovery of the [^{3}H]arachidonate-labeled phospholipids. As indicated in Table I, perifusion of islets with secretagogues

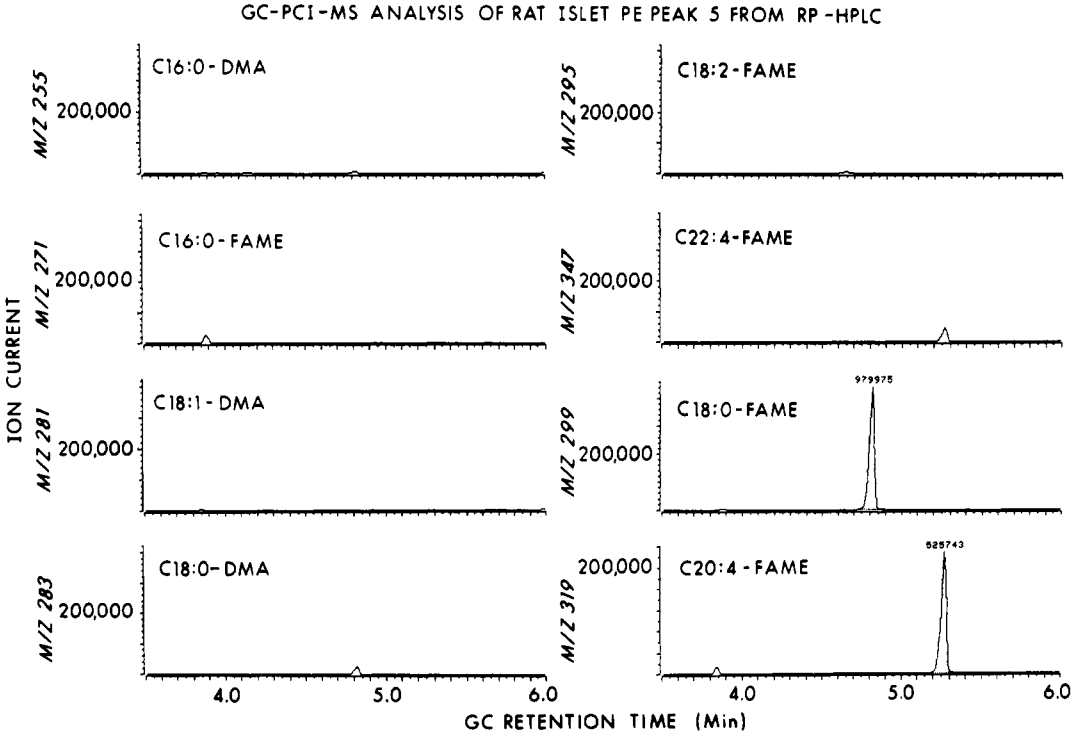

FIGURE 5: Gas chromatograhic positive ion methane chemical ionization mass spectrometric analysis of the acid methanolysate of pancreatic islet ethanolamine-phospholipid RP-HPLC peak 5. Peak 5 from Figure 3A was subjected to acid methanolysis, and the methanolysate was analyzed by GC-PCI-MS as described under Experimental Procedures.

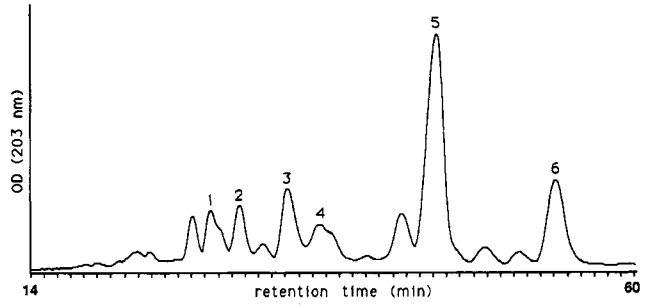

FIGURE 6: Reverse-phase HPLC analysis of ethanolamine phospholipids from freshly isolated rat pancreatic islets. Islets (ca. 4500) which had been freshly isolated from 15 rats were immediately subjected to Bligh-Dyer extraction, and phospholipids were analyzed by NP-HPLC. The PE peak was harvested separately and analyzed by RP-HPLC as in Figure 3 with flow-through UV monitoring at 203 nm. Individual peaks were harvested separately, subjected to acid methanolysis, and analyzed by GC-PCI-MS as in Figures 4 and 5. The numbered peaks contained (C16:0a/C20:4)-PE (peak 1), (C18:1a/C20:4)-PE (peak 2), (C16:0p/C20:4)-PE (peak 3), (C18:1p/C20:4)-PE (peak 4), (C18:0a/C20:4)-PE (peak 5), and (C18:0p/C20:4)-PE (peak 6). Peaks in the chromatogram that are not numbered were found not to contain arachidonic acid.

did not induce a detectable decline in the [^{3}H]arachidonate content of islet PA, PI, or PS or in any of the three identified molecular species of islet PC which contained arachidonate. A secretagogue-induced decrement in the [^{3}H]arachidonate content of two molecular species of islet PE was observed, and these two species were islet RP-HPLC peak 3 [C16:0p/C20:4-PE] and peak 6 [C18:0p/C20:4-PE] (Table I), as illustrated in the RP-HPLC radiochromatograms in Figure 8.

To quantitate the content of arachidonate in phospholipids from perifused islets, islet phospholipids were subjected to saponification in the presence of [$^2H_8$]arachidonate as an internal standard. Liberated arachidonate and other fatty acids were then converted to their pentafluorobenzyl ester derivatives and quantitated by stable isotope dilution gas chromatography–mass spectrometry in the negative ion–chemical ionization (NICI) mode, as illustrated in Figure 9. This method of analysis results in about a 200-fold enhancement of signal for arachidonate relative to electron impact or PCI analyses and yields a linear standard curve for arachidonate liberated from phospholipids at levels from below 300 pmol to above 20 nmol ($y = 5.691x - 0.34$, $r = 1.00$).

The combined arachidonate content of the major islet phospholipid species PE, PC, PA, PI, and PS was found to be 57.9 pmol/islet, and the total fatty acyl mass in these phospholipids was found to be 168.8 pmol/islet (Table II). Of the total arachidonate content in these phospholipids, 51% was found to reside in PC, 30% in PE, 9% in PI, 4% in PA, and 3% in PS. The overall specific radioactivity of [^{3}H]-arachidonate in islets (9000) incubated for 18 h with that radiolabeled fatty acid (200 μCi) was 156 ± 16 dpm/pmol. The specific radioactivity of neither islet PE nor PC differed significantly from that value, although the value for PC was higher than that for PE (Table II).

GC–NICI–MS analysis of the saponification products of individual phospholipid species from RP-HPLC analysis of islet PE revealed that, in islets perifused with a nonstimulatory concentration (3 mM) of glucose, 44% (8.0 pmol/islet) of the total arachidonoyl content of islet PE resided in the three plasmalogen-PE species [(C160:p/C20:4)-PE, (C18:1p/C20:4)-PE, and (C18:0p/C20:4)-PE] and that 56% resided in the three diacyl-PE species [(C16:0a/C20:4)-PE, (C18:1a/C20:4)-PE, and (C18:0a/C20:4)-PE] (Table III). Prolonged perifusion of the islets with secretagogues resulted in a decline in the arachidonoyl content of islet plasmalogen-PE species of 5.9 pmol/islet and a decline in the arachidonoyl content of islet diacyl-PE species of 2.1 pmol/islet, with the largest fractional decrements occurring in the (C16:0p/C20:4)-PE and (C18:0p/C20:4)-PE plasmalogen species (Table III). In experiments in which endogenous [^{3}H]arachidonate-labeled PE molecular species (prepared from isolated islets by sequential NP-HPLC and RP-HPLC) were used as substrates in assays of islet phospholipase A_2 activity, the plasmalogen

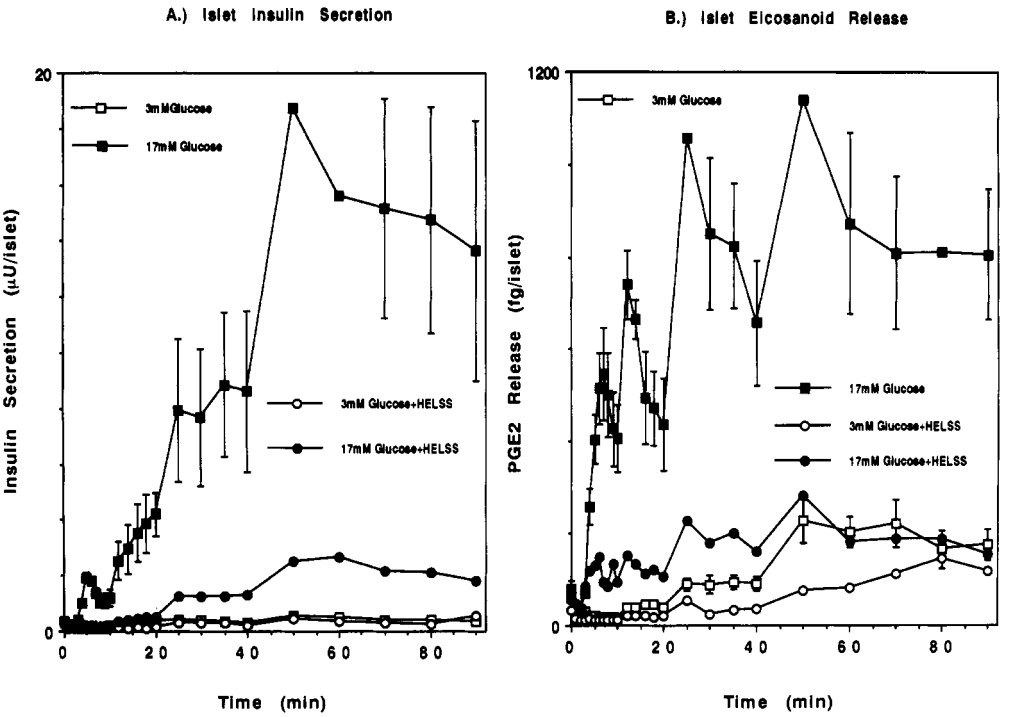

FIGURE 7: Secretagogue-induced insulin secretion and eicosanoid release from perifused isolated pancreatic islets. Isolated pancreatic islets (500 per condition) were loaded onto the filters of chambers of a perifusion apparatus and were perifused with 3 mM D-glucose in nKRB for 30 min after pretreatment with vehicle (squares) or with the haloenol lactone suicide substrate (HELSS) of ASCI-PLA_2 (circles) (25 μM, 30 min, 37 °C) as described under Experimental Procedures. At the time designated as zero in the figure, the medium composition was maintained (open symbols) or was changed to contain 17 mM D-glucose and 500 μM carbachol (closed symbols). The insulin content (panel A) of perifusion effluent was measured by radioimmunoassay, and the PGE_2 content was measured by enzyme immunoassay as described under Experimental Procedures. Values represent means ■ SEM from four experiments.

species (C16:0p/C20:4)-PE and (C18:0p/C20:4)-PE were hydrolyzed more rapidly than the diacyl species (C18:0a/C20:4)-PE by a Ca^{2+}-independent, HELSS-inhibited PLA_2 activity in both islet cytosolic and membranous subcellular fractions (Table IV).

DISCUSSION

The absolute and relative abundances of the islet phospholipids PC, PI, and PS measured in these studies correspond well to the limited amount of previously published information on the phospholipid content of pancreatic islets. Based on phosphate content, values for PC between 45.6 and 62.2 pmol/islet, for PI between 3.6 and 4.9 pmol/islet, and for PS between 3.5 and 4.7 pmol/islet have been observed for mouse pancreatic islets, with relative abundances of PC/PI/PS of 0.57/0.05/0.04 (Hallberg, 1984). The corresponding values from the studies described here for rat pancreatic islets based on fatty acyl content are 52.9 pmol/islet for PC, 5.9 pmol/islet for PI, and 3.4 pmol/islet for PS, with relative abundances of PC/PI/PS of 0.57/0.06/0.04. Pancreatic islets from mice and rats also exhibit similar values for protein content, DNA content, and volume (Erecinska et al., 1992) in addition to their apparent similarity in phospholipid content.

The fatty acid composition of islet phospholipids observed in the studies described here is also in general similar to those observed in the only two previous reports on this issue (Montague & Parkin, 1980; Turk et al., 1986), with the exception that the islet arachidonate content observed here (57.9 pmol/islet) is higher than previously reported (31 pmol/islet). In part, this reflects the fact that, in the studies described here, deuterium-labeled arachidonate was employed as an internal standard in the measurement of arachidonate by stable isotope dilution mass spectrometric methods. This corrects the measured mass of arachidonate for losses in absolute arachidonate content during sample processing which are not detected with GC–FID measurements employed in earlier studies (Montague & Parkin, 1980; Turk et al., 1986). Arachidonate was the most abundant fatty acid measured in islet phospholipids in the studies described here and constituted 34% of the combined fatty acyl content of PC, PE, PA, PI, and PS. The fatty acyl contents of islet neutral lipids (NL), sphingomyelin (SM), and cardiolipin (CL) were not measured in this study, but previous measurements indicate that the fatty acyl contents of these lipids in islets are 12.2 pmol/islet for NL (5% arachidonate), 3.4 pmol/islet for CL (4% arachidonate), and 6.6 pmol/islet for SM (0% arachidonate) (Hallberg, 1984; Turk et al., 1986). From these values and the measurements reported here, it can be estimated that arachidonate constitutes 30% of the total fatty acyl mass of islet glycerolipids, which is among the highest arachidonate contents for any tissue. For comparison, arachidonate comprises 25% of the fatty acyl content of macrophages (Scott et al., 1980), 23% of liver (Patton et al., 1982), 19% of platelets (Mahadevappa & Holub 1982), 15% of pancreatic exocrine tissue (Akpan et al., 1981), and 13% of testes (Coniglio, 1977).

A second source of underestimation of islet arachidonate content in previous studies is that the acid chromatographic conditions used for analysis of islet phospholipids (Montague & Parkin, 1980; Turk et al., 1986) would have resulted in the loss of acid-labile plasmenylethanolamine species, which are shown here to contain a total of 8 pmol of arachidonate per islet or 14% of the total islet arachidonate content and 44% of the islet PE content of arachidonate. This also accounts for the higher estimate of total islet PE content derived from the studies described here (23.7 pmol/islet) compared to the previous estimate of 14.0 pmol/islet (Turk et al., 1986). The identification of islet plasmenylethanolamine species rests on the demonstration that islet PE contains species with alke-

Table I: Influence of Perfusion with Insulin Secretagogues on the ^3H Content of Phospholipids from Isolated Pancreatic Islets Prelabeled with [^3H]Arachidonate[a]

lipid	[glucose] (mM)	[^3H] (dpm × 10^4)	[^{14}C] (dpm × 10^3)	[^3H]/[^{14}C]	ratio to 3 mM glucose
		(A) Normal-Phase HPLC Analyses			
PE	3	9.90 ± 1.24	8.66 ± 0.55	11.73 ± 1.98	1.0
PE	17	9.62 ± 0.96	8.25 ± 0.68	11.95 ± 1.60	1.09 ± 0.20
PA	3	2.31 ± 0.07	na	2.46 ± 0.21	1.0
PA	17	2.28 ± 0.22	na	2.42 ± 0.11	1.00 ● 0.13
PI	3	5.78 ± 0.37	na	6.78 ± 0.68	1.0
PI	17	4.92 ± 0.22	na	5.87 ± 0.67	0.92 ± 0.06
PS	3	1.62 ± 0.16	na	1.88 ± 0.20	1.0
PS	17	1.82 ± 0.43	na	2.13 ± 0.45	1.14 ± 0.07
PC	3	15.41 ± 1.57	5.05 ± 0.33	18.29 ± 2.60	1.0
PC	17	14.58 ± 1.40	4.81 ± 0.18	17.82 ± 1.63	1.04 ± 0.19
		(B) Reverse-Phase HPLC Analyses of PC			
(16:0a/18:2)-PC	3	na	6.23 ± 1.65	na	na
(16:0a/18:2)-PC	17	na	6.30 ± 1.61	na	na
(16:0a/20:4)-PC	3	7.55 ± 2.15	na	11.56 ± 2.19	1.0
(16:0a/20:4)-PC	17	7.01 ± 2.01	na	10.81 ± 0.60	1.05 ± 0.23
(18:1a/20:4)-PC	3	1.63 ± 0.49	na	2.41 ± 0.46	1.0
(18:1a/20:4)-PC	17	1.52 ± 0.46	na	2.29 ± 0.20	1.12 ± 0.32
(18:0a/20:4)-PC	3	8.34 ± 2.35	na	12.81 ± 2.34	1.0
(18:0a/20:4)-PC	17	7.87 ± 2.26	na	12.15 ± 0.66	1.06 ± 0.22
		(C) Reverse-Phase HPLC Analyses of PE			
(16:0a/20:4)-PE	3	0.50 ± 0.64	4.77 ± 0.56	1.04 ± 0.14	1.0
(16:0a/20:4)-PE	17	0.40 ± 0.52	3.85 ± 0.46	1.00 ± 0.13	0.96 ± 0.13
(18:1a/20:4)-PE	3	0.47 ± 0.99	na	0.99 ± 0.21	1.0
(18:1a/20:4)-PE	17	0.42 ± 0.89	na	1.10 ± 0.14	1.11 ± 0.21
(16:0p/20:4)-PE	3	0.52 ± 0.52	na	1.08 ± 0.11	1.0
(16:0p/20:4)-PE	17	0.25 ± 0.25	na	0.80 ± 0.08	0.74 ± 0.10
(18:1p/20:4)-PE	3	0.31 ± 0.53	na	0.65 ± 0.11	1.0
(18:1p/20:4)-PE	17	0.19 ± 0.33	na	0.50 ± 0.09	0.77 ± 0.17
(18:0a/20:4)-PE	3	2.83 ± 5.09	na	5.93 ± 1.07	1.0
(18:0a/20:4)-PE	17	1.99 ± 3.60	na	5.16 ± 0.93	0.87 ± 0.18
(18:0p/20:4)-PE	3	0.80 ± 0.32	na	1.67 ± 0.07	1.0
(18:0p/20:4)-PE	17	0.31 ± 0.12	na	0.80 ± 0.03	0.48 ± 0.04

[a] Isolated rat pancreatic islets (ca. 9000) from 30 rats per experiment were labeled with [^3H]arachidonate (50 μCi) overnight at 37 °C, washed free of unincorporated radiolabel, loaded onto filters (500 islets/condition) in chambers of a perfusion apparatus, and perifused for 120 min with 3 mM D-glucose or with 17 mM D-glucose plus 500 μM carbachol as in Figure 7. At the end of the perifusion, phospholipids were extracted from the islets on the filters after addition of L-α-1-palmitoyl-2-([1-^{14}C]linoleoyl)-PC, and L-α-1-palmitoyl-2-([1-^{14}C]arachidonoyl)-PE as internal standards and then analyzed by sequential NP-HPLC as in Figure 2A and RP-HPLC as in Figure 3. The ^3H and ^{14}C contents of aliquots of each HPLC eluant fraction were determined by liquid scintillation spectrometry. Values represent the means of four experiments ± SEM. The meaning of the designation "na" is "not applicable". In the NP-HPLC analyses in part A, the [^3H]/[^{14}C] ratios were calculated by dividing the [^3H] dpm for a given phospholipid head-group class by the [^{14}C] dpm for the [^{14}C]-(16:0a/20:4)-PE internal standard. Although [^{14}C]-(16:0a/18:2)-PC was also added as an internal standard, all of the tabulated [^3H]/[^{14}C] ratios in section A (including those for PC) were calculated on the basis of the [^{14}C] dpm in the [^{14}C]-(16:0a/20:4)-PE internal standard to facilitate comparison of the relative abundance of [^3H]arachidonate in the head-group classes. The same procedure was used on RP-HPLC analyses of PE molecular species in part C, where the [^3H] dpm for each molecular species of PE was divided by the [^{14}C] dpm for the [^{14}C]-(16:0a/20:4)-PE internal standard to yield the tabulated [^3H]/[^{14}C] ratio. In part B, [^3H] dpm for each molecular species of PC on RP-HPLC analysis was divided by the [^{14}C] dpm observed for the [^{14}C]-(16:0a/18:2)-PC internal standard to yield the tabulated [^3H]/[^{14}C] ratio. Slight discrepancies encountered in using the tabulated [^3H] dpm and [^{14}C] dpm to arrive at the tabulated [^3H]/[^{14}C] ratio reflect the fact that this ratio was calculated as the mean of individual ratios in each experiment and not as the ratio of mean [^3H] dpm divided by mean [^{14}C] dpm.

nylacylglycerol moieties, that three arachidonate-containing islet PE species exhibit RP-HPLC chromatographic behavior compatible with their designation as plasmenylethanolamine species, that these three peaks exhibit the acid lability characteristic of plasmalogens, and that acid methanolysis of these peaks yields dimethylacetals derived from *sn*-1 fatty aldehyde residues of plasmalogens.

That plasmenylethanolamine molecular species contain 14% of total rat islet arachidonate and 44% of rat islet PE arachidonate is remarkably similar to the observation that plasmenylethanolamine comprises 14% of the total phospholipid and 45% of the PE phospholipid of rat cerebral cortical neurons (Freysz et al., 1968). Both islet β-cells and cerebral cortical neurons are electrically excitable cells and may share a common embryologic origin from the neuroectoderm (Escurat et al., 1991), although an endodermal origin has also been suggested for β-cells (Le Douarin, 1988). Neither rat islets nor rat cerebral cortical neurons contain substantial amounts of plasmenylcholine species, although myocardium, which is also an electrically active tissue, contains large amounts of both plasmenylcholine and plasmenylethanolamine (Dawson et al., 1962; Scott et al., 1967; Gross, 1984, 1985; DaTorre, & Creer, 1991). In contrast, liver, an electrically inactive tissue, contains only 2–3.6% plasmalogen (Dawson et al., 1962; Scott et al., 1967).

The electrical and secretory properties of pancreatic islet β-cells and phospholipid hydrolytic events are intimately related. Stimulation of islets with fuel secretagogues induces depolarization of β-cell plasma membranes (Ashcroft et al., 1984; Arkhammar et al., 1987) and hydrolysis of arachidonate from membrane phospholipids (Wolf et al., 1986, 1990; Metz, 1991), and both processes require glycolytic metabolism of glucose within the β-cell (Metz, 1985; Turk et al., 1992). Metabolism of glucose in β-cells generates ATP in compartments accessible to the plasma membrane, which interacts with at least two target effector systems: (1) A K^+ channel (K_{ATP}) which accounts for most of the resting membrane permeability to K^+ is inactivated by ATP (Cook & Hales 1984; Cook et al., 1988); and (2) an ATP-stimulated Ca^{2+}-independent phospholipase A_2 (ASCI-PLA_2) is activated by ATP and catalyzes hydrolysis of arachidonate from β-cell membrane phospholipids (Gross et al., 1993; Ramanadham

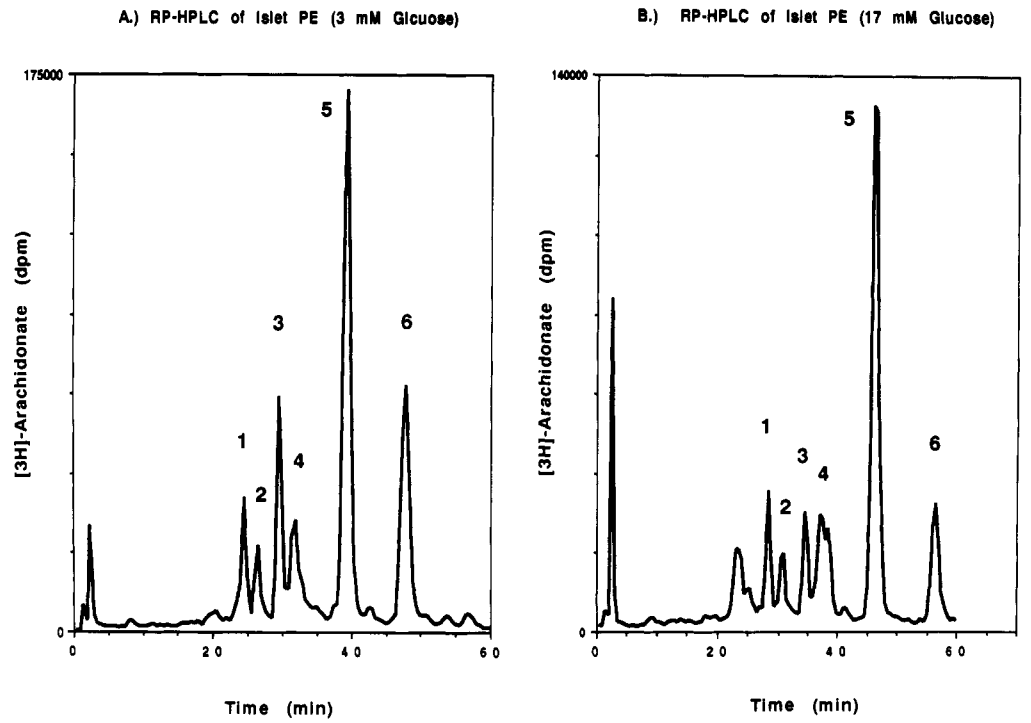

FIGURE 8: Reverse-phase HPLC analysis of ethanolamine phospholipids from pancreatic islets prelabeled with [^{3}H]arachidonic acid and perifused with secretagogues. Isolated pancreatic islets were labeled with [^{3}H]arachidonic acid as in Table I and perifused with 3 mM D-glucose (panel A) or with 17 mM D-glucose plus 500 μM carbachol (panel B) as in Figure 7. Phospholipids were then extracted and analyzed by NP-HPLC as in Figure 2A. The PE peaks were harvested and analyzed by RP-HPLC as in Figure 3A.

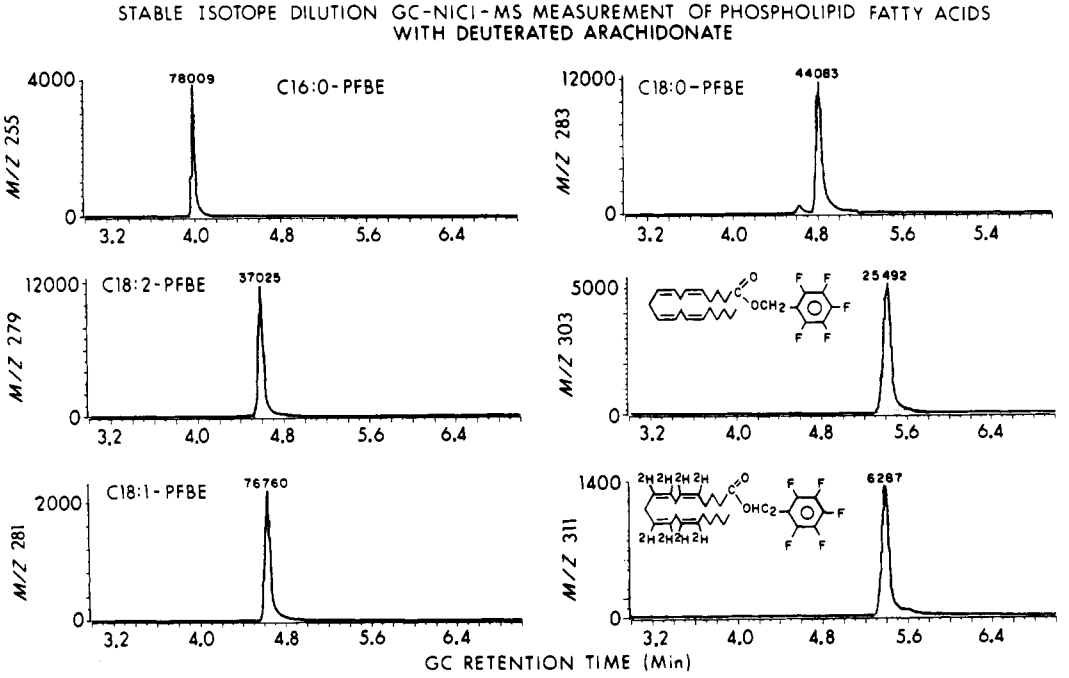

FIGURE 9: Stable isotope dilution gas chromatographic negative ion chemical ionization mass spectrometric analysis of pentafluorobenzyl ester derivatives of fatty acids derived from saponification of pancreatic islet phospholipids. Pancreatic islet PC from NP-HPLC analyses such as that in Figure 2A was saponified in the presence of [$^2H_8$]arachidonic acid as an internal standard, and the liberated fatty acids (FA) were converted to their pentafluorobenzyl ester (PFBE) derivatives and analyzed by GC-NICI-MS with selected monitoring of ions corresponding to the (M–181) moieties of FA-PFBE as described under Experimental Procedures.

et al., 1993), resulting in the accumulation of nonesterified arachidonate at concentrations of at least 35–70 μM (Wolf et al., 1986, 1991). Closure of the K_{ATP} channel induces depolarization of the β-cell plasma membrane (Cook & Hales, 1984; Aschcroft et al., 1984), and ASCI-PLA$_2$-mediated accumulation of nonesterified arachidonate facilitates Ca^{2+} entry into β-cells (Ramanadham et al., 1992), possibly by lowering the degree of depolarization required to activate voltage-operated Ca^{2+} channels (Vacher et al., 1989). The resultant rise in cytosolic Ca^{2+} concentration in the glucose-stimulated β-cell is a critical signal in the induction of insulin secretion (Wollheim & Scharp, 1981; Arkhammar et al., 1987; Prentki & Matschinsky, 1987; Gylfe, 1988a,b).

The only cell other than the pancreatic islet β-cell in which ASCI-PLA$_2$ has been demonstrated to date is the cardiac myocyte, where the enzyme was first discovered (Wolf &

Table II: Fatty Acid Composition and [^{3}H]Arachidonate Specific Activity of Phospholipids from Isolated Pancreatic Islets Prelabeled with [^{3}H]Arachidonic Acid[a]

lipid	fatty acyl mass (pmol/islet)						specific activity [^{3}H]-C20:4 (dpm/pmol)
	C16:0	C18:2	C18:1	C18:0	C20:4	Total	
PE	1.32 ± 0.10	3.00 ± 0.39	3.82 ± 0.27	13.37 ± 1.88	17.96 ± 1.58	39.47 ± 3.52	121 ± 24
PA	0.80 ± 0.20	0.29 ± 0.02	0.53 ± 0.10	1.03 ± 0.21	2.33 ± 0.15	4.97 ± 0.46	134 ± 12
PI	0.82 ± 0.12	0.26 ± 0.05	0.46 ± 0.05	4.54 ± 0.40	5.59 ± 0.57	11.87 ± 0.97	159 ± 14
PS	0.55 ± 0.14	0.61 ± 0.05	0.63 ± 0.10	3.46 ± 0.39	1.53 ± 0.15	6.78 ± 0.98	205 ± 18
PC	19.32 ± 4.28	17.97 ± 1.90	15.74 ± 2.48	22.13 ± 0.56	30.49 ± 2.85	105.7 ± 9.10	178 ± 12

[a] Isolated pancreatic islets were prelabeled with [^{3}H]arachidonic acid as in Table I. Phospholipids were extracted, analyzed by NP-HPLC, and assayed for ^{3}H content as in Figure 2. Phospholipid peaks were saponified in the presence of [$^2H_8$]arachidonate as internal standard. Liberated fatty acids were converted to pentafluorobenzyl esters and quantitated by stable isotope dilution gas chromatography–mass spectrometry in the negative ion chemical ionization mode as in Figure 9. Values are means ± SEM from six experiments.

Table III: Arachidonate Content of Pancreatic Islet Ethanolamine Phospholipid Molecular Species[a]

lipid	[glucose] (mM)	[^{3}H]-(C20:4)		C20:4 mass	
		(dpm × 10^{-3})	(dpm/pmol)	pmol	pmol/islet
(16:0a/20:4)-PE	3	6.26	31.7	197 ± 47	1.03 ± 0.24
(16:0a/20:4)-PE	17	5.12	35.7	143 ± 35	0.73 ± 0.18
(18:1a/20:4)-PE	3	5.04	35.2	143 ± 39	0.74 ± 0.20
(18:1a/20:4)-PE	17	3.35	31.8	105 ± 31	0.54 ± 0.16
(16:0p/20:4)-PE	3	13.93	38.7	360 ± 69	1.88 ± 0.36
(16:0p/20:4)-PE	17	4.05	60.0	67 ± 25	0.35 ± 0.12
(18:1p/20:4)-PE	3	10.40	22.4	467 ± 68	2.43 ± 0.36
(18:1p/20:4)-PE	17	11.12	31.1	357 ± 46	1.86 ± 0.24
(18:0a/20:4)-PE	3	39.91	25.3	1575 ± 165	8.20 ± 0.86
(18:0a/20:4)-PE	17	35.44	28.1	1260 ± 138	6.56 ± 0.72
(18:0p/20:4)-PE	3	21.10	29.8	707 ± 96	3.68 ± 0.50
(18:0p/20:4)-PE	17	7.69	40.2	191 ± 48	0.99 ± 0.25

[a] Isolated pancreatic islets from 30 rats were prelabeled with [^{3}H]arachidonate as in Figure 2 and perifused as in Figure 7. Phospholipids were then extracted in the presence of 10^5 dpm L-α-1-palmitoyl-2-([1-^{14}C]linoleoyl)-PC as an internal standard and analyzed by normal-phase HPLC as in Figure 2A. The PE peak was harvested separately, spiked with 10^5 dpm L-α-1-palmitoyl-2-([1-^{14}C]linoleoyl)-PC, and analyzed by RP-HPLC as in Figure 3A. Individual PE molecular species were collected separately, spiked with [$^2H_8$]arachidonate, and saponified as in Figure 9. Liberated arachidonate was then converted to the pentafluorobenzyl ester derivative and quantitated by stable isotope dilution gas chromatography–mass spectrometry in the negative ion chemical ionization mode as in Figure 9. Values are means of triplicates ± SEM.

Gross, 1985) and demonstrated to consist of a 40-kDa catalytic subunit and a separate 360-kDa regulatory subunit, which is the ATP-sensing component of the complex (Hazen & Gross, 1991). The expression of this novel PLA_2 in two such different cell types raises the question of what common role the enzyme might play in the physiologic function of these cells. Both islet β-cells and cardiac myocytes have substantial amounts of metabolic machinery dedicated to the regulation and sensing of alterations in glycolytic flux, and both cells also express K_{ATP} channels (Noma, 1983; Cook & Hales, 1984; Weiss & Lamp, 1987), which are found only in a restricted number of cell types. Both β-cells and cardiac myocytes also exhibit a periodic physiological response (insulin secretion or contraction) that is driven by electrical activity and changes in the cytosolic Ca^{2+} concentration.

In addition, as demonstrated here, β-cells, like cardiac myocytes (Gross, 1984, 1985), contain substantial amounts of plasmalogen molecular species with *sn*-2 arachidonoyl residues. Such compounds are the preferred substrates for ASCI-PLA_2 from β-cells (Gross et al., 1993) and cardiac myocytes (Hazen et al., 1990) in vitro, and the data presented here suggest that endogenous plasmalogen species also undergo hydrolysis in secretagogue-stimulated islets. The preference of ASCI-PLA_2 for plasmalogen substrates facilitated the utilization of a haloenol lactone suicide substrate (HELSS) [(*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one], which is sterically similar to plasmalogens and selectively inactivates ASCI-PLA_2 from β-cells or myocardium at concentrations that have no influence on the activity of Ca^{2+}-dependent phospholipases A_2 from a variety of sources (Hazen et al., 1991b; Gross et al., 1993). Treatment of isolated islets with HELSS suppresses glucose-induced arachidonate hydrolysis from islet membrane phospholipids, the rise in β-cell cytosolic Ca^{2+} concentration, and insulin secretion under conditions where islet oxidation of [^{14}C]glucose to CO_2, carbachol-induced activation of islet phosphinositide–phospholipase C, and depolarization-induced rises in β-cell Ca^{2+} concentration are unaffected (Ramanadham et al., 1993).

Certain practical limitations of the studies reported here must be acknowledged. First, the decline of arachidonate content of islet plasmenylethanolamine species is observed only after prolonged perifusion with a combination of secretagogues. It is difficult to detect loss of arachidonate from particular phospholipid pools at short perifusion times because only a small fraction of the large reservoir of esterified arachidonate is released upon stimulation with secretagogues at early time points. Second, phospholipases A_2 in addition to ASCI-PLA_2 may participate in the release of arachidonate from phospholipids, including phosphatidylcholine, in secretagogue-stimulated islets (Laychock, 1982; Metz, 1991). Third, some of the plasmalogens observed in isolated islets may reside in islet cells other than β-cells. Although β-cells are the quantitatively predominant cell type in islets, islets also contain α-cells, δ-cells, and other cells. Fourth, these studies do not establish the subcellular location of islet plasmalogens, which may reside in plasma membrane, endoplasmic reticulum, secretory granule membrane, or other membranous compartments. These and related issues must be the subjects of future investigations.

Table IV: Hydrolysis of [^{3}H]Arachidonate from Endogenous Islet Ethanolamine Phospholipids by Islet Ca^{2+}-Independent Phospholipase A_2[a]

substrate	subcellular fraction	[HELSS] (μM)	Ca^{2+}-independent phospholipase A_2 activity [pmol/(mg·min)]
(16:0p/20:4)-PE	membranes	0	34.85 ± 2.52
(16:0p/20:4)-PE	membranes	10	8.01 ± 1.08
(16:0p/20:4)-PE	cytosol	0	10.30 ± 1.80
(16:0p/20:4)-PE	cytosol	10	2.21 ± 0.04
(18:0a/20:4)-PE	membranes	0	3.61 ± 0.30
(18:0a/20:4)-PE	membranes	10	0.04 ± 0.02
(18:0a/20:4)-PE	cytosol	0	0.04 ± 0.02
(18:0a/20:4)-PE	cytosol	10	0.02 ± 0.01
(18:0p/20:4)-PE	membranes	0	11.25 ± 2.52
(18:0p/20:4)-PE	membranes	10	1.73 ± 0.59
(18:0p/20:4)-PE	cytosol	0	1.86 ± 0.50
(18:0p/20:4)-PE	cytosol	10	0.95 ± 0.62

[a] Isolated pancreatic islets from 30 rats (ca. 9000) were prelabeled with [^{3}H]arachidonate (200 μCi) overnight at 37 °C, and phospholipids were extracted and analyzed by NP-HPLC as in Figure 2A. The PE peak was harvested and analyzed by RP-HPLC as in Figure 3A. Individual [^{3}H]arachidonate-labeled ethanolamine-phospholipid molecular species were used as substrates of phospholipase A_2 activity in cytosolic and membranous fractions prepared from a second population of islets (ca. 9000) isolated from 30 additional rats. Phospholipase A_2 assays were performed in the presence of 10 mM EGTA, 1 mM ATP, and no added Ca^{2+}, as described under Experimental Procedures. Reactions were initiated by injection of radiolabeled substrates as a final concentration of 0.28 μM. Mean measured substrate specific radioactivity was 451 ± 43 dpm/pmol ($n = 3$). Incubations were performed and terminated and products analyzed as described under Experimental Procedures. Phospholipase A_2 (PLA_2) specific activity was calculated from the measured cpm of radiolabeled fatty acid released from substrate and the measured protein content of the assay tube: PLA_2 specific activity is $[R/(PT)]$, where R is fatty acid released in pmol, P is the assay tube protein content in milligrams, and T is assay duration in minutes. Assay duration was 5 min, and mean measured assay tube protein contents ranged from 34.9 to 41.73 μg. The parameter R was calculated as $[4DS]$, where the factor 4 accounts for the fraction (25 of 100 μL) of the butanol extract containing released fatty acid analyzed, S is the specific radioactivity of the phospholipid substrate in dpm/pmol, and D is the net dpm of fatty acid released. The parameter D was calculated as $[(S - B)/E]$, where S is the measured cpm in the assay sample, B is the measured "blank" cpm value for fatty acid released from radiolabeled substrate when no source of enzyme was added, and E is the counting efficiency for the radioisotope (typically 0.4 for ^{3}H). Tabluated values are means of triplicates ± SEM.

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REFERENCES

Akpan, J. O., Hurley, M. C., & Lands, W. E. M. (1981) *Diabetes Lat. 18*, 147–156.

Arkhammar, P., Nilsson, T., Rorsman, P., & Berggren, P. O. (1987) *J. Biol. Chem. 262*, 5448–5454.

Ashcroft, F. M., Harrison, D. E., & Ashcroft, S. J. H. (1984) *Nature 312*, 446–448.

Ashcroft, S. J. H. (1980) *Diabetologia 18*, 5–15.

Blank, M. L., & Snyder, F. (1983) *J. Chromatogr. 273*, 415–420.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Chilton, F. H., & Murphy, R. C. (1986) *J. Biol. Chem. 261*, 7771–7777.

Coniglio, J. G. (1977) *Lipid Metabolism in Mammals 2* (Snyder, F., Ed.) pp 83–129, Plenum Press, New York.

Cook, D. L., & Hales, C. N. (1984) *Nature 311*, 271–273.

Cook, D. L., Satin, L. S., Ashford, M. L. J., & Hales, C. N. (1988) *Diabetes 37*, 495.

DaTorre, S. D., & Creer, M. H. (1991) *J. Lipid Res. 32*, 1159–1172.

Dawson, R. M. C., Hemington, N., & Davenport, J. B. (1962) *Biochem. J. 84*, 497–501.

Dunlop, M. E., & Larkins, R. G. (1984) *J. Biol. Chem. 259*, 8407–8411.

Erecinska, M., Bryla, J., Michalike, M., Meglasson, M. D., & Nelson, D. (1992) *Biochim. Biophys. Acta 1101*, 273–295.

Escurat, M., Djabali, K., Huc, C., Landon, F., Becourt, C., Boitaird, C., Gros, F., & Portier, M. (1991) *Dev. Neurosci. 13*, 424–432.

Freysz, L. F., Bieth, R., Judes, C., Sensenbrenner, M., Jacob, M., & Mandel, P. (1968) *J. Neurochem. 15*, 307–313.

Gross, R. W. (1984) *Biochemistry 23*, 158–165.

Gross, R. W. (1985) *Biochemistry 24*, 1662–1668.

Gross, R. W., & Sobel, B. E. (1982) *J. Biol. Chem. 258*, 15046–15053.

Gross, R. W., Ramanadham, S., Kruszka, K., Han, X., & Turk, J. (1993) *Biochemistry 32*, 327–336.

Gylfe, E. (1988a) *J. Biol. Chem. 262*, 5044–5048.

Gylfe, E. (1988b) *J. Biol. Chem. 263*, 13750–13754.

Hallberg, A. (1984) *Biochim. Biophys. Acta 796*, 328–335.

Hazen, S. L., & Gross, R. W. (1991) *J. Biol. Chem. 266*, 14526–14534.

Hazen, S. L., Stuppy, R. J., & Gross, R. W. (1990) *J. Biol. Chem. 265*, 10622–10630.

Hazen, S. L., Ford, D. A., & Gross, R. W. (1991a) *J. Biol. Chem. 266*, 5629–5633.

Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P., & Gross, R. W. (1991b) *J. Biol. Chem. 266*, 7227–7232.

Hedeskov, C. J. (1980) *Physiol. Rev. 60*, 442–509.

Keahey, H., Rajun, A., Boyd, A. E., & Kunze, D. L. (1989) *Diabetes 38*, 188–193.

Lacy, P. E., Walker, M. M., & Fink, C. J. (1972) *Diabetes 21*, 987–988.

Laychock, S. G. (1982) *Cell Calcium 3*, 43–54.

Le Douarain, N. (1988) *Cell 53*, 169–171.

Mahadevappa, V. G., & Holub, B. J. (1982) *Biochim. Biophys. Acta 713*, 73–79.

Malaisse, W. J., Sener, A., Herchuelz, A., & Hutton, J. (1979) *Metabolism 28*, 373–386.

McDaniel, M. L., Colca, J. R., Kotagal, N., & Lacy, P. E. (1983) *Methods Enzymol. 98*, 182–200.

Meglasson, M. D., & Matschinsky (1986) *Diabetes/Metab. Rev. 2*, 163–214.

Metz, S. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 198–202.

Metz, S. A. (1991) *Diabetes 40*, 1565–1573.

Metz, S. A., Draznin, B., Sussman, K. E., & Leitner, J. W. (1987) *Biochem. Biophys. Res. Commun. 142*, 251–258.

Montague, W., & Parkin, E. N. (1980) *Horm. Metab. Res., Suppl. Ser. 10*, 153–157.

Noma, A. (1983) *Nature 305*, 147–148.

Patton, G. M., Fasulo, J. M., & Robbins, S. J. (1982) *J. Lipid Res. 23*, 190–196.

Purdon, A. D., Patelunas, D., & Smith, J. D. (1987) *Lipids 22*, 116–120.

Pradelles, P., Grassi, I., & Maclouf, J. (1985) *Anal. Chem. 57*, 1170–1173.

Ramanadham, S., Gross, R. W., & Turk, J. (1992) *Biochem. Biophys. Res. Commun. 184*, 647–653.

Ramanadham, S., Gross, R. W., Han, X., & Turk, J. (1993) *Biochemistry 32*, 337–346.

Ramesha, C. S., & Pickett, W. C. (1987) *J. Lipid Res. 28*, 326–331.

Ricordi, C., Lacy, P. E., Finke, E. H., Olack, B. J., & Scharp, D. W. (1988) *Diabetes 37*, 413–420.

Rorsman, P., & Trube, G. (1985) *Pflugers Arch. 405*, 305–309.

Scott, T. W., Setchell, B. P., & Bassett, J. M. (1967) *Biochem. J. 104*, 1040–1047.
Scott, W. A., Zrike, J. M., Hamill, A. L., Kempe, J., & Cohn, Z. A. (1980) *J. Exp. Med. 152*, 324–335.
Sturgess, N. C., Cook, D. L., Ashford, M. L. J., & Hales, C. N. (1985) *Lancet ii*, 474–475.
Sugiura, T., Nakajima, M., Sekiguchi, N., Nakagawa, Y., & Walu, K. (1983) *Lipids 18*, 125–129.
Turk, J., Wolf, B., Lefkowith, J., Stump, W., & McDaniel, M. (1986) *Biochim. Biophys. Acta 879*, 399–409.
Turk, J., Bohrer, A., Stump, W. T., Ramanadham, S., & Mangino, M. J. (1992a) *J. Chromatogr. 575*, 183–196.
Turk, J., Mueller, M., Bohrer, A., & Ramanadham, S. (1992b) *Biochim. Biophys. Acta 1125*, 280–291.
Vacher, P., McKenzie, J., & Dufy, B. (1989) *Am. J. Physiol. 257*, E203–E211.
Weiss, J. N., & Lamp, S. T. (1987) *Science 238*, 67–69.
Wey, H. E., Jakubowski, J. A., & Deykin, D. (1986) *Biochim. Biophys. Acta 878*, 380–386.
Wolf, R. A., & Gross, R. W. (1985) *J. Biol. Chem. 260*, 7295–7303.
Wolf, B. A., Turk, J., Sherman, W. R., & McDaniel, M. L. (1986) *J. Biol. Chem. 261*, 3501–3511.
Wolf, B. A., Pasquale, S. M., & Turk, J. (1991) *Biochemistry 30*, 6371–6379.
Wollheim, C. B., & Sharp, G. W. G. (1981) *Physiol. Rev. 61*, 914–973.
Wright, P. H., Makuli, P. R., Vichick, P., Sussman, K. E. (1971) *Diabetes 20*, 33–45.