Mass Spectrometric Characterization of Arachidonate-Containing Plasmalogens in Human Pancreatic Islets and in Rat Islet β-Cells and Subcellular Membranes†

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ABSTRACT: Pancreatic islets, when stimulated with D-glucose, secrete insulin by processes requiring glycolytic metabolism and generation of ATP. Hydrolysis of membrane phospholipids also occurs in glucose-stimulated islets, resulting in accumulation of nonesterified arachidonate, which facilitates Ca^{2+} entry and the rise in β-cell [Ca^{2+}] that triggers insulin secretion. Glucose-induced hydrolysis of arachidonate from islet phospholipids is mediated in part by an ATP-stimulated, Ca^{2+}-independent (ASCI) phospholipase A_2 (PLA_2) which prefers plasmenyl over diacyl phospholipid substrates. Here we characterize the endogenous plasmalogen content of islet cells and subcellular membranes. Fast atom bombardment mass spectrometric analyses demonstrated that three of the most abundant molecular species of ethanolamine phospholipids in rat pancreatic islets were plasmalogens with *sn*-2 arachidonate residues and palmitic, oleic, or stearic aldehyde residues, respectively, in the *sn*-1 position. Purified populations of β-cells prepared by fluorescence-activated cell sorting were also found to contain these plasmenylethanolamine molecular species in abundance similar to that in intact islets and greater than that in islet α-cells. Both islet plasma membranes (PM) and endoplasmic reticulum (ER) also contained these plasmenylethanolamine species, which accounted for 42% (PM) to 64% (ER) of the ethanolamine phospholipid arachidonate content of these membranes, as measured by stable isotope dilution mass spectrometry. Plasmenylethanolamine species were also abundant constituents of human pancreatic islets (accounting for 58% of their ethanolamine phospholipid arachidonate content) and were hydrolyzed more rapidly than diacyl ethanolamine phospholipids by human islet cytosolic ASCI-PLA_2. Both secretagogue-induced eicosanoid release and insulin secretion from human islets were attentuated by an ASCI-PLA_2 suicide substrate which sterically resembles plasmalogens. These observations are consistent with the hypotheses that islet β-cell ASCI-PLA_2-catalyzed hydrolysis of arachidonate from endogenous plasmenylethanolamine substrates may occur in membrane compartments which participate in regulation of the β-cell cytosolic [Ca^{2+}] and that this may be an intermediary biochemical event in the induction of insulin secretion.

Pancreatic islets, when stimulated with high concentrations of D-glucose, secrete insulin by processes involving entry of D-glucose into islet β-cells, glycolytic metabolism, generation of ATP, and a rise in β-cell cytosolic [Ca^{2+}] (Malaisse et al., 1979; Ashcroft, 1980; Hedeskov, 1980; Wollheim & Sharp, 1981; Ashcroft et al., 1984; Cook & Hales, 1984; Rorsman & Trube, 1985; Sturgess et al., 1985; Meglasson & Matschinksy, 1986; Arkhammar et al., 1987; Cook et al., 1988; Gylfe, 1988a,b). Signal transduction processes involving phospholipid hydrolysis and the accumulation of nonesterified arachidonate in β-cell membranes appear to amplify the D-glucose-induced rise in β-cell [Ca^{2+}]. Upon stimulation with D-glucose, arachidonate is rapidly hydrolyzed from islet membrane phospholipids, and nonesterified arachidonate accumulates (Wolf et al., 1986, 1991). Glucose-induced hydrolysis of arachidonate from islet phospholipids is dependent upon the metabolism of D-glucose but is in part independent of Ca^{2+} influx (Metz, 1985; Wolf et al., 1991; Turk et al., 1992). Nonesterified arachidonate appears to facilitate 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 augmentation of the rise in β-cell [Ca^{2+}] by arachidonate (Metz et al., 1987; Ramanadham et al., 1992) amplifies depolarization-induced insulin secretion (Wolf et al., 1991) and appears to participate in the insulin secretory response to D-glucose (Ramanadham et al., 1993).

D-Glucose-induced hydrolysis of arachidonate from islet membrane phospholipids is mediated in part by a phospholipase A_2 (PLA_2)[1] (Gross et al., 1993; Ramanadham et al., 1993) which is stimulated by ATP and active in the absence of Ca^{2+}. The islet ATP-stimulated, Ca^{2+}-independent (ASCI) PLA_2 prefers *sn*-2 arachidonate residues over other *sn*-2 fatty acyl substituents and prefers plasmalogen over 1,2-diacyl-*sn*-

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[1] Abbreviations: ASCI, ATP-stimulated Ca^{2+}-independent; PLA_2, phospholipase A_2; HELSS, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; PC, choline phospholipids; PE, ethanolamine phospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; SM, sphingomyelin; PA, phosphatidic acid; NL, neutral lipids; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2(*N*-morpholino)ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; KRB, Krebs Ringer bicarbonate buffer; 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; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; NP, normal phase; RP, reverse phase; PM, plasma membrane; ER, endoplasmic reticulum; SG, secretory granules; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

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phospholipid substrates (Gross et al., 1993). The plasmalogen substrate preference of ASCI-PLA_2 has facilitated the utilization of a haloenol lactone suicide substrate (HELSS) which sterically resembles plasmalogens and irreversibly inactivates ASCI-PLA_2 at concentrations which do not influence the activities of Ca^{2+}-dependent phospholipases A_2 from several sources (Hazen et al., 1991b). HELSS-induced inhibition of β-cell ASCI-PLA_2 results in suppression of D-glucose-induced hydrolysis of arachidonate from islet membrane phospholipids, of the rise in β-cell [Ca^{2+}], and of insulin secretion but does not influence islet glucose oxidation or muscarinic agonist-induced activation of phosphoinostide–phospholipase C (Ramanadham et al., 1993).

Because ASCI-PLA_2 prefers plasmalogen substrates containing *sn*-2 arachidonate residues and because the enzyme may be a component of the β-cell glucose-sensor apparatus, the islet content of plasmalogen phospholipids with *sn*-2 arachidonate residues which might serve as endogenous substrates for ASCI-PLA_2 has recently been examined (Ramanadham et al., 1993b). Intact rat pancreatic islets were found to contain substantial amounts of arachidonate-containing plasmenylethanolamine molecular species which underwent hydrolysis in secretagogue-stimulated islets and which were hydrolyzed more rapidly than phosphatidylethanolamine substrates by islet ASCI-PLA_2 in vitro. At present, the cellular distribution among islet cells, the subcellular location within membrane compartments, and the human islet content of these plasmenylethanolamine molecular species are not known. These issues are addressed in the studies described here.

EXPERIMENTAL PROCEDURES

Materials. The compounds [$^3H_8$]arachidonic acid (100 Ci/mmol), L-α-1-palmitoyl-2-[1-^{14}C]linoleoyl)phosphatidylcholine (60 mCi per mmol), and L-α-1-palmitoyl-2-[1-^{14}C]-arachidonoylphosphatidylethanolamine (60 mCi/mmol) were purchased from Du Pont New England Nuclear; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), and sphingomyelin (SM) were from Avanti Polar Lipids (Birmingham, AL); phosphatidic acid (PA) was from Serdary Lipids; cholesterol, EGTA, and common buffer reagents were from Sigma Chemical (St. Louis, MO); 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).

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, D.C.).

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 from male Sprague-Dawley rats and cultured overnight at 37 °C, as described elsewhere (Lacy et al., 1972; Gross et al., 1993). Human pancreatic islets were prepared in the laboratory of Dr. David Scharp and cultured for 48 h at 37 °C as described elsewhere (Ricordi et al., 1988, Gross et al., 1993). 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; Ramanadham et al., 1993b).

Perifusion of Human Islets and Measurement of Insulin and of $PGF_{2\alpha}$. Isolated 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 chambers of a perifusion apparatus and 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 15 min. Perifusion effluent was collected and analyzed for content of insulin by double antibody radioimmunoassay with ^{125}I-labeled insulin (Wright et al., 1971) and for content of $PGF_{2\alpha}$ by enzyme immunoassay with anti-$PGF_{2\alpha}$ antibody and acetylcholinesterase-labeled $PGF_{2\alpha}$ obtained from Cayman Chemical (Ann Arbor, MI), as previously described (Pradelles et al., 1985; Turk et al., 1992a).

Preparation of β-Cell and Non-β-Cell Populations from Isolated Islets by Fluorescence-Activated Cell Sorting. Isolated pancreatic islets were dispersed into individual cells with dispase as described elsewhere (Ono et al., 1977; Ramanadham et al., 1992; Gross et al., 1993). Dispersed cells were then filtered and subjected to autofluorescence-activated cell sorting (FACS) to yield separate β-cell enriched and α-cell enriched populations of cells as described elsewhere (Pipeleers, 1984; Wang & McDaniel, 1990; Ramanadham et al., 1992).

Subcellular Fractionation of Islets, Preparation of Endoplasmic Reticulum, and Preparation of Plasma Membranes by Sucrose Density Gradient Analysis. Islets (ca. 9000) which had been labeled with [$^3H_8$]arachidonic acid were transferred to a teflon-glass Potter-Elvehjem tissue grinder (size 0019), rinsed twice with fractionation buffer, and resuspended in this buffer (0.8 mL) at 4 °C. All subsequent operations were performed at 4 °C. The composition of fractionation buffer was 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] and 250 mM sucrose, pH 7.2. The islets were then homogenized (4 °C) with 14 strokes of the pestle at 1170 rpm (Polyscience RX R10 at setting 3). The homogenate (H) was then transferred to a 10 × 75 mm pyrex tube and centrifuged (600*g*, 5 min). The supernatant (S1) was transferred to a separate 10 × 75 mm pyrex tube. The pellet (P1) was resuspended in fractionation buffer (1 mL) and again centrifuged (600*g*, 5 min). The supernatant was combined with S1 and centrifuged (20000*g*, 20 min). The resulting supernatant (S2) was transferred to a Beckman cellulose nitrate centrifuge tube (Ultraclear 13 × 51 mm) and diluted to 4.0 mL with fractionation buffer. The pellet (P2) was resuspended in 0.2 mL of 10 mM MES with 1 mM EGTA, pH 6.0. S2 was then centrifuged (150000*g*, 90 min) to yield a supernatant (S4) and pellet (P4). P4 is enriched in endoplasmic reticulum, and S4 corresponds to cytosol (McDaniel et al., 1983). P4 was resuspended in fractionation buffer (0.4 mL) and rehomog-

enized (14 strokes, 1170 rpm). P2 was then rehomogenized as above and layered (in a final volume of 0.5 mL of 10 mM MES with 1 mM EGTA, pH 6.0) atop a discontinuous sucrose gradient, the layers of which contained 1 mL each of sucrose at densitites of 1.14, 1.16, 1.18, and 1.20 in a Beckman cellulose nitrate centrifuge tube (Ultraclear 13 × 51 mm). This tube was then centrifuged (150000*g*, 90 min), and the bands collecting at the interfaces were designated from top to bottom as B1, B2, B3, and B4. Bands B1 and B2 are enriched in plasma membranes, and bands B3 and B4 contain mitochondria and secretory granules (McDaniel et al., 1983). These bands were then harvested separately, resuspended in 4.0 mL each of 10 mM MES (with 1.0 mM EGTA, pH 6.0), centrifuged (150000*g*, 60 min) to remove sucrose, and resuspended in 0.5 mL of 10 mM MES without EGTA (pH 6.0). An aliquot (10%) of fractions H, S1, S2, S4, P4, B1, B2, B3, and B4 were removed for protein determination, and the remainder was processed for measurement of acid-extractable insulin, 5′-nucleotidase activity, cholesterol, and phospholipid content as described below.

Preparation of Insulin Secretory Granules by Percoll Density Gradient Analysis. All of the following operations were performed at 4 °C. Islets (ca. 2700) were homogenized as above in SG buffer [0.8 mL of 260 mM sucrose, pH 6.5, with 10 mM MOPS (4-morpholinepropanesulfonic acid)] (Jones et al., 1987). The homogenate (H′) was transferred to a 10-mL glass vial and diluted with 7.2 mL of SG buffer. One milliliter of this suspension was then placed in each of eight Eppendorf 1.5-mL polypropylene tubes and centrifuged (900*g*, 5 min) to yield a pellet (P1′) and a supernatant (S1′). Each 1 mL of S1′ was then centrifuged (13000*g*, 10 min) in an Eppendorf 1.5-mL polypropylene tube to yield a pellet (P2′) and a supernatant (S2′). P2′ was then resuspended in 0.6 mL of SG buffer, and 0.1 mL of the suspension was layered atop each of six Percoll density gradients [which consisted of a layer (50 μL) of 100% Percoll and a layer (250 μL) of 35% Percoll in a 400-μL Eppendorf polypropylene tube] and centrifuged (13000*g*, 10 min). The Percoll had been dialyzed for 24 h against SG buffer before preparation of the gradients. The lower 150 μL of the gradient, which contained the secretory granule (SG) fraction (Jones et al., 1987), was then removed with a 500-μL Hamilton syringe. The remaining 250 μL of the gradient contained a mixed membrane (M) fraction (Jones et al., 1987). The Percoll content of the SG and M fractions was then reduced by dilution with 1 mL of SG buffer and centrifugation (13000*g*, 10 min). An aliquot of fractions H′, S1′, P1′, S2′, P2′, SG, and M was removed for measurement of protein, and the remainder was processed for measurement of acid-extractable insulin and phospholipid content as described below.

Extraction of Phospholipids. Phospholipids from human islets, from FACS-purified rat islet β-cells or non-β islet cells, or from the membrane fractions from subcellular fractionation procedures were extracted with chloroform/methanol under neutral conditions as described elsewhere (Bligh & Dyer, 1959; Ramanadham et al., 1993b). Control experiments with islet-free medium and with sham subcellular fractions prepared from islet-free medium revealed no detectable phospholipid contamination in the HPLC and mass spectrometric analyses described below.

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 as desribed elsehwere (Blank & Snyder, 1983; Ramanadham et al., 1993b). 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.

Fast Atom Bombardment Mass Spectrometric Characterization of Islet Choline and Ethanolamine Phospholipids. Choline and ethanolamine phospholipids derived from NP-HPLC analyses of phospholipids of islets (ca. 9000) from 30 rats were dissolved in 10 μL of 1/1 chloroform/methanol, and 2 μL was mixed with 3 μL of glycerol on a metal probe. Desorption of phospholipid ions from the glycerol matrix was accomplished with a xenon fast atom bombardment (FAB) gun mounted on a VG ZAB-SE mass spectrometer. The FAB gun was operated at 8 kV and 1 mA. A dwell time of 90 ms was employed at each *m/z* value in the region of interest.

Reverse-Phase HPLC Analysis of Islet Phospholipid Head-Group Classes into Molecular Species and Notation for the Designation of Phospholipid Molecular Species. Resolution of molecular species of arachidonate-containing phospholipids from individual head-group classes was achieved by analysis on an octadecylsilicic acid HPLC column as previously described (Patton et al., 1982; Chilton & Murphy, 1986; Ramanadham et al., 1993b). The ^{3}H (and, where appropriate, ^{14}C) content of aliquots of each fraction was determined by liquid scintillation spectrometry. Approximate retention times for phospholipid standards observed in this system were as follows: (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, where 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. As used below, the letter "p" denotes a plasmalogenic linkage in the *sn*-1 position, and the letter "e" denotes an alkyl ether linkage in the *sn*-1 position.

Identification of Resolved Phospholipid Molecular Species by Gas Chromatography–Positive Ion Methane Chemical Ionization–Mass Spectrometry. Individual phospholipid peaks from RP-HPLC analyses were subjected to acid methanolysis, which causes release of fatty acid residues as fatty acid methyl esters (FAME) and of *sn*-1 fatty aldehyde residues in plasmalogens as dimethylacetals (DMA) (Gross, 1984). FAME and DMA were then extracted and analyzed by capillary column gas chromatography (GC)–mass spectrometry (MS) in the positive ion methane chemical ionization (PCI) mode, as described elsehwere (Ramanadham et al., 1993b). 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, the corresponding values for ($M - 31$) ions and GC retention times are 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. Quantitation of the fatty acyl mass in the small amounts of phospholipids obtained from islets and subcellular membrane fractions was achieved by alkaline hydrolysis in the presence of [$^{2}H_{8}$]arachidonate as internal standard, conversion of the liberated fatty acids to pentafluorobenzyl ester (PFBE) derivatives, and stable isotope dilution negative ion chemical ionization (NICI) mass spectrometry (MS), as

described elsehwere (Ramanadham et al., 1993b). 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).

Cholesterol Measurement by Stable Isotope Dilution Mass Spectrometry. Internal standard (100 ng of [$^{13}C_5$]cholesterol) was added in $CHCl_3$, and samples were extracted by the method of Bligh and Dyer (1959). The lipid extract was then concentrated to dryness, reconstituted in heptane (0.5 mL), and centrifuged. The supernatant was aspirated into a 1-mL ReactiVial (Pierce) and concentrated to dryness. Cholesterol was then converted to the pentafluorobenzoate derivative with pentafluorobenzoyl chloride (10 μL) and 4% pyridine in toluene (50 μL) (90 min, 80 °C). The reaction mixture was concentrated to dryness, water (0.2 mL) was added, and the cholesterol–pentafluorobenzoate was extracted with hexane (0.4 mL). Aliquots of the reaction products were then 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 300 °C) mass spectrometry (Hewlett-Packard 5988) in the methane (source pressure 1 torr) negative ion-chemical ionization mode. Cholesterol–pentafluorobenzoate was detected by monitoring its molecular ion (m/z 580) at the characteristic retention time (ca. 10.1 min) and was quantitated relative to the [$^{13}C_5$]cholesterol internal standard (molecular ion m/z 585). Cholesterol standard curves were linear from below 50 ng to above 1000 ng and exhibited a linear regression relationship of $y = 6.2 + 119(x)$, $r = 0.999$, where y is cholesterol mass in ng and x is the ratio of the peak area observed for native cholesterol divided by that observed for the [$^{13}C_5$]cholesterol internal standard.

Identification of 1-O-Hexadecylglycerol Derived from Alkyl Ether Phospholipids by Negative Ion Chemical Ionization Gas Chromatography–Mass Spectrometry. Standard 1-*O*-hexadecyl-*sn*-glycerol was obtained from Sigma Chemical (St. Louis), converted to the bispentafluorobenzoate derivative with pentafluorobenzoyl chloride, and analyzed by GC-NICI-MS as described above for cholesterol–pentafluorobenzoate. The 1-*O*-hexadecyl-2,3-pentafluorobenzoylglycerol was detected by monitoring its molecular ion (m/z 704) and eluted at a characteristic retention time of 9.25 min under these conditions. The complete NICI mass spectrum of this material consisted essentially exclusively of the molecular ion. For determination of the 1-*O*-hexadecylglycerol content of islet phospholipid peak a (Figure 6B), an aliquot of the peak was digested with *Bacillus cereus* phospholipase C to yield the diglyceride (Turk et al., 1992). 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 (800g, 5 min), the ether phase containing the diglyceride product was aspirated into a separate vial and concentrated to dryness. The diglyceride was then saponified with LiOH in dimethoxyethane as described elsewhere (Turk et al., 1992b). The product was then converted to the bispentafluorobenzoate derivative and analyzed by GC-NICI-MS as described above.

Determination of Acid Lability of Phospholipids. Two aliquots of [^{3}H]arachidonate-containing islet phospholipid samples were prepared, spiked with 10 μg of (C16:0a/C18:2)-PC as internal standard, concentrated, treated with acid or with vehicle, and analyzed by RP-HPLC as described elsehwere (DaTorre & Creer, 1991; Ramanadham et al., 1992b). Plasmalogens are destroyed by acid treatment and appear only in the chromatogram of the vehicle-treated sample, but diacyl- and alkylacylphospholipids are stable under these conditions and appear in the chromatograms of both acid-treated and vehicle-treated samples.

Measurement of 5′-Nucleotidase Activity. The method of Avruch et al. (1971) as modified by Naber et al. (1980) was used to measure the activity of the plasma membrane enzyme 5′-nucleotidase. This procedure measures the 5′-nucleotidase-catalyzed liberation of [^{3}H]adenosine from [^{3}H]AMP after precipitation of [^{3}H]AMP with $Zn(SO_4)$ and $Ba(OH)_2$. An aliquot (10 μL) of sample containing 2–20 μg of protein was added to 100 μL of assay bufffer (50 mM Tris-HCl, pH 8.4, 0.12 mg/mL BSA, 1.5 mM $MgCl_2$) containing 0.1 μCi of [^{3}H]AMP (0.2 mM) in a 1.5-mL Beckman microfuge-B tube. Blank samples contained no enzyme. This mixture was then incubated for 60 min at 37 °C, and the reaction was terminated by addition of 20 μL of 0.3 M $ZnSO_4$ and 20 μL of 0.3 M $Ba(OH)_2$ and centrifugation (1 min, Beckman-B microfuge). Addition of $ZnSO_4$ and $Ba(OH)_2$ was then repeated, and the samples were centrifuged again. The ^{3}H content of an aliquot (0.1 mL) of the supernatnant was then determined by liquid scintillation spectrometry, and 5′-nucleotidase activity was calculated as nmol of released [^{3}H]adenosine/(μg of protein·h).

Acid-Extractable Insulin. Membrane-enclosed insulin was extracted with acid–ethanol as previously described (McDaniel et al., 1983). Samples (20 μL) were extracted with 40 μL of 1.5% sulfuric acid in 75% ethanol overnight at 4 °C. Immunoreactive insulin was then measured by double-antibody radioimmunoassay (Wright et al., 1971) in 100-μL aliquots of 200- and 2000-fold dilutions of the extract.

Protein. Protein content was measured either with Coomassie protein assay reagent (Pierce, Rockford, IL) against bovine serum albumin as standard according to instructions from the manufacturer or with a Bio-Rad protein assay kit.

Preparation of Human Islet Cytosol and Measurement of Phospholipase A_2 Activity against Endogenous Ethanolamine Phospholipid Substrates. Isolated human pancreatic islets were homogenized, and the homogenate was subjected to ultracentrifugation to yield a cytosolic supernatant and a membraneous pellet as described elsewhere (Gross et al., 1993; Ramanadham et al., 1993b). The ATP-stimulated, Ca^{2+}-independent phospholipase A_2 activity contained in human islet cytosol (125 μL, 29.3 μg of protein) was assayed (final conditions: 400-μL total volume, 200 mM Tris, pH 7.5, 10 mM EGTA, and 1 mM ATP) by ethanolic injection (5 μL, final ethanol concentration 1.25%) of [^{3}H]arachidonate-labeled ethanolamine phospholipid substrates (specific activity 72.2 dpm/pmol, final concentration 0.33 μM) isolated from human islets that had been prelabeled overnight with [^{3}H]-arachidonate (200 μCi) at 37 °C. The assay mixture was incubated, the reaction was terminated by addition of butanol, and released radiolabeled fatty acid was quantified by TLC and liquid scintillation spectrometry as described elsewhere (Gross et al., 1993; Ramanadham et al., 1993b). Phospholipase A_2 (PLA_2) specific acitivity was calculated from the measured cpm of radiolabeled fatty acid released from the phospholipid substrate and the measured protein content of the assay tube as previously described (Gross et al., 1993; Ramanadham et al., 1993b).

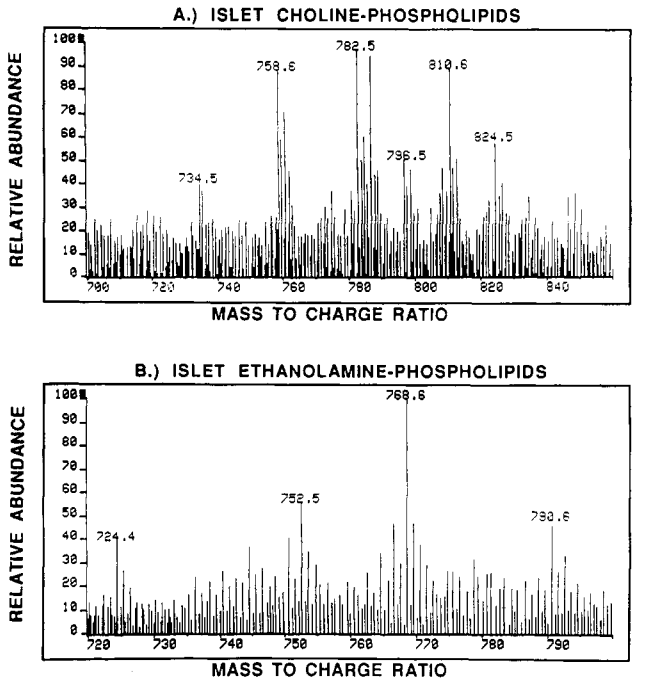

FIGURE 1: Fast atom bombardment mass spectra of ethanolamine and choline phospholipids from isolated rat pancreatic islets. Freshly isolated islets (ca. 9000) from 30 rats were pooled and subjected to neutral phospholipid extraction, and the phospholipids were separated into head-group classes by NP-HPLC, as described under Experimental Procedures. The choline (upper panel) and ethanolamine phospholipids (lower panel) were collected separately and subjected to xenon fast atom bombardment (FAB) from a FAB gun mounted on a VG ZAB-SE mass spectrometer as described under Experimental Procedures.

RESULTS

Previous analyses of islet phospholipids have revealed that 84% of the rat islet content of arachidonate resides in choline phospholipids (PC) and ethanolamine phospholipids (PE) (Ramanadham et al., 1993b). Partial characterization of rat islet PC and PE by reverse-phase (RP) HPLC suggested that the majority of PC arachidonate resides in the molecular species (C16:0a/C20:4)-PC and (C18:0a/C20:4)-PC, using the notation for phospholipid designation described under Experimental Procedures. These analyses also suggested that the three most abundant arachidonate-containing molecular species of rat islet PE are (C18:0a/C20:4)-PE, (C18:0p/C20:4)-PE, and (C16:0p/C20:4)-PE. The tentative identification of these molecular species was achieved by inference from analyses of the acid methanolysis products of individual peaks from RP-HPLC, but these analyses did not directly demonstrate residence of the indicated *sn*-1 and *sn*-2 substituents on individual phospholipid molecular species.

To obtain direct verification of the identity of these molecular species, phospholipids were extracted under neutral conditions from freshly isolated islets from 30 rats and then separated into head-group classes by normal-phase (NP) HPLC. The PC and PE peaks were harvested separately and anlayzed by fast atom bombardment (FAB)–mass spectrometry (Figure 1). The FAB mass spectrum of islet PC (Figure 1A) exhibited prominent protonated molecular ions for (C16:0a/C20:4)-PC (*m/z* 782) and (C18:0a/C20:4)-PC (*m/z* 810) and for a number of non-arachidonate-containing molecular species (Table I).

The four most abundant ions in the FAB mass spectrum of rat islet PE corresponded to the protonated (*m/z* 768) and sodiated (*m/z* 790) molecular ions of (C18:0a/C20:4)-PE and to the protonated molecular ions of (C16:0p/C20:4)-PE (*m/z* 724) and of (C18:0p/C20:4)-PE (*m/z* 752) (Figure 1B). This is in agreement with previous assignments of the identity of the most abundant species of rat islet PE based on RP-HPLC followed by acid methanolysis and GC–MS analyses in the positive ion methane chemical ionization (PCI) mode (Ramanadham, 1993b), although FAB-MS molecular ion analyses are known to underestimate the abundance of plasmalogen species relative to diacylphospholipids because of the greater tendency of the former to undergo fragmentation (Gross, 1984).

Table I: Fast Atom Bombardment Mass Spectrometric Analyses of Pancreatic Islet Choline and Ethanolamine Phospholipids[a]

ion (*m/z*)	relative abundance (%)	assignment
	(A) Choline Phospholipids	
734 (MH^+)	40	(C16:0a/C16:0)-PC
758 (MH^+)	87	(C16:0a/C18:2)-PC
760 (MH^+)	70	(C16:0a/C18:1)-PC
782 (MH^+)	96	(C16:0a/C20:4)-PC
784 (MH^+)	60	(C18:0a/C18:1)-PC
786 (MH^+)	94	(C18:0a/C18:2)-PC
788 (MH^+)	45	(C18:0a/C18:1)-PC
796 (MH^+)	50	(C18:0e/C20:4)-PC
808 (MH^+)	46	(C18:1a/C20:4)-PC
810 (MH^+)	88	(C18:0a/C20:4)-PC
824 (MH^+)	57	(C18:0e/C22:4)-PC
	(B) Ethanolamine Phospholipids	
724 (MH^+)	41	(C16:0p/C20:4)-PE
740 (MH^+)	26	(C16:0a/C20:4)-PE
744 (MH^+)	35	(C18:0a/C18:2)-PE
750 (MH^+)	40	(C18:1p/C20:4)-PE
752 (MH^+)	55	(C18:0p/C20:4)-PE
766 (MH^+)	47	(C18:1a/C20:4)-PE
768 (MH^+)	99	(C18:0a/C20:4)-PE
790 (MNa^+)	50	(C18:0a/C20:4)-PE

[a] Choline and ethanolamine phospholipids from isolated pancreatic islets were prepared and analyzed by fast atom bombardment mass spectrometry as in Figure 1. Conventions for designation of molecular species are explained under Experimental Procedures.

Intact pancreatic islets contain several types of cells, of which insulin-secreting β-cells are the most abundant (70–80%) (Erecinska et al., 1992). Other islet cell types include glucagon-secreting α-cells (ca. 20–30%) and smaller numbers of somatostatin-secreting δ-cells, vascular endothelial cells, fibroblasts, and resident phagocytic cells. Because β-cells are both larger and contain higher amounts of FAD than do other types of islet cells, virtually homogeneous populations of β-cells can be prepared from dispersed islet cells by autofluorescence-activated cell sorting (FACS) (Pipeleers et al., 1984; Wang & McDaniel, 1990). To determine the specific phospholipid composition of rat islet β-cells, FACS-purified populations of β-cells were prepared and labeled by incubation with [$^3H_8$]arachidonate. The β-cell phospholipids were then extracted under neutral conditions and separated into head-group classes by NP-HPLC. The β-cell PC and PE were collected separately and then analyzed by reverse-phase (RP) HPLC under conditions permitting resolution of individual molecular species. The rat islet β-cell PC was found to contain three predominant arachidonate-containing molecular species (Figure 2), which were identified as (C16:0a/C20:4)-PC (peak 1), (C18:1a/C20:4)-PC (peak 2), and (C18:0a/C20:4)-PC (peak 3) upon acid methanolysis and GC–PCI–MS analyses.

The rat islet β-cell PE was found to contain six predominant arachidonate-containing molecular species (Figure 3), which were identified as (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

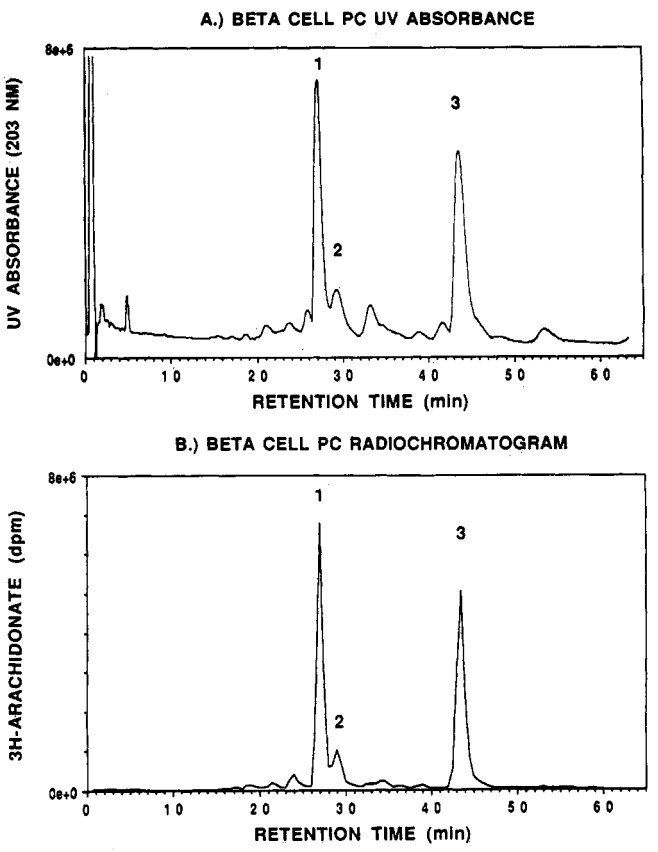

FIGURE 2: Reverse-phase HPLC analysis of choline phospholipids from FACS-purified rat pancreatic islet β-cells. Islets (ca. 9000) were isolated from 30 rats and dispersed into single cell suspensions with dispase, as described under Experimental Procedures. Dispersed cells were then subjected to fluorescence-activated cell sorting, and the β-cell population was collected separately and incubated overnight with 100 μCi of [$^3H_8$]arachidonic acid. Phospholipids were then extracted under neutral conditions and separated into head-group classes by NP-HPLC. The choline phospholipids were collected separately and analyzed by RP-HPLC under conditions permitting resolution of individual molecular species as described under Experimental Procedures. The upper panel represents UV absorbance (203 nm) recorded from a flow-through cell, and the lower panel represents [^{3}H]arachidonate content measured in aliquots of eluant fractions by liquid scintillation spectrometry. Aliquots of the indicated peaks were subjected to acid methanolysis and GC–PCI–MS analysis as described under Experimental Procedures to establish the identities of peak 1 as (C16:0a/C20:4)-PC, of peak 2 as (C18:1a/C20:4)-PC, and of peak 3 as (C18:0a/C20:4)-PC.

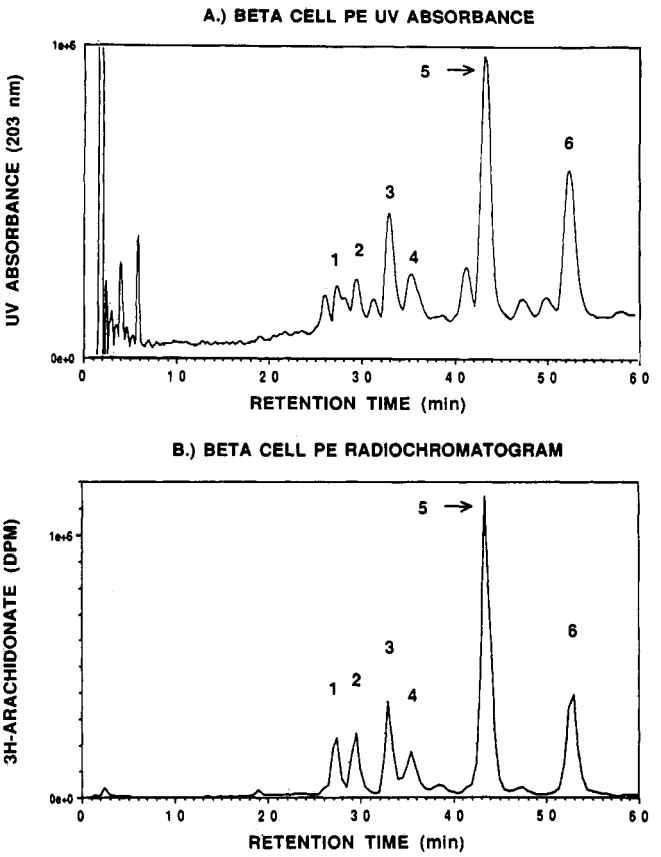

FIGURE 3: Reverse-phase HPLC analysis of ethanolamine phospholipids from FACS-purified rat pancreatic islet β-cells. FACS-purified β-cells were prepared and labeled with [$^3H_8$]arachidonic acid, and their ethanolamine phospholipids were isolated by NP-HPLC and further analyzed by RP-HPLC as in Figure 3. The upper panel represents UV absorbance (203 nm) recorded from a flow-through cell, and the lower panel represents [^{3}H]arachidonate content measured in aliquots of eluant fractions by liquid scintillation spectrometry. Aliquots of the indicated peaks were subjected to acid methanolysis and GC–PCI–MS analysis as described under Experimental Procedures to establish the identities of peak 1 as (C16:0a/C20:4)-PE, of peak 2 as (C18:1a/C20:4)-PE, of peak 3 as (C16:0p/C20:4)-PE, of peak 4 as (C18:1p/C20:4)-PE, of peak 5 as (C18:0a/C20:4)-PE, and of peak 6 as (C18:0p/C20:4)-PE.

(C18:0p/C20:4)-PE (peak 6) upon acid methanolysis and GC–PCI–MS analyses. The α-cell-enriched populations of islet cells were found to exhibit a phospholipid profile which was in general similar to that observed for β-cells, with the exception that the plasmenylethanolamine species were less abundant in the α-cell populations. The plasmalogen PE peak 6 from the α-cell population, for example, contained an arachidonate mass of 0.06 fmol/cell, while the plasmalogen PE peak 6 from the β-cell population contained an arachidonate mass of 1.11 fmol/cell. These observations indicate that the observed plasmalogen content of the FACS-purified β-cells cannot be attributed to the small residual content (ca. 5%) of contaminating α-cells.

Accumulation of nonesterified arachidonate in the plasma membranes of glucose-stimulated β-cells is thought to influence the activity of plasma membrane Ca^{2+} channels and to facilitate the entry of Ca^{2+} from the extracellular space, and hydrolysis of arachidonate from β-cell membrane phospholipids in glucose-stimulated islets is mediated in part by an ASCI-PLA_2 enzyme which prefers plasmalogen over diacylphospholipid substrates (Ramanadham et al., 1992, 1993a,b; Gross et al., 1993). To determine whether islet cell plasma membranes contain plasmalogens which might serve as endogenous substrates for this enzyme, islet cells were subjected to subcellular fractionation procedures which yield a plasma membrane-rich fraction, and the membranous fractions resulting from these procedures were examined for markers of plasma membranes. One such marker is cholesterol, which is distributed predominantly in the plasma membranes of mammalian cells (Lange et al., 1989). The cholesterol content of the membranous fractions was quantitated by stable isotope dilution mass spectrometry using [$^{13}C_5$]cholesterol as an internal standard in a procedure involving conversion to the pentafluorobenzoate derivative and GC–MS analyses in the negative ion chemical ionization (NICI) mode with selected ion monitoring of the molecular ion of native cholesterol and of the heavy isotope-labeled standard (Table II).

Islet cell membranous compartments, including plasma membrane, endoplasmic reticulum, secretory granules, mitochondria, and nuclei, can be partially separated by a procedure involving homogenization followed by differential centrifugation through sucrose density gradients (McDaniel et al., 1983). In part, differential behavior upon centrifugal

Table II: Subcellular Fractionation of Membranous Compartments from Isolated Pancreatic Islets by Centrifugation through Sucrose Density Gradients[a]

fraction	composition	insulin (milliunits/ μg of protein)	5′-nucleotidase activity [nmol/(μg of protein·h)]	cholesterol (ng/ μg of protein)	fatty acyl mass (pmol/μg of protein)					
					C16:0	C18:2	C18:1	C18:0	C20:4	total
H	homogenate	2.4	0.98	5	39.1	24.4	15.0	62.5	77.9	218
S1		5.8	nd	2	45.5	31.6	23.4	74.1	96.9	271
P1	nuclei	5.0	1.07	12	134	114	78.2	174	164	664
S2		10.5	nd	0.8	81.6	36.7	39.3	129	152	438
P2		14.0	nd	nd	223	162	125	465	567	1544
B1	plasma membrane	3.8	10.5	77	951	425	357	1932	1835	5503
B2	plasma membrane	5.8	16.6	34	383	249	190	730	803	2356
B3	mitochondria and secretory granules	8.5	3.78	13	248	432	234	517	687	2120
B4	mitochondria and secretory granules	14.0	4.23	26	167	175	116	382	433	1275
S4	cytosol	1.0	0.15	0.3	25.3	5.6	11.2	44.7	26.9	113
P4	endoplasmic reticulum	0.75	1.16	3.6	307	182	199	263	685	1636

[a] Pancreatic islets (ca. 9000) were isolated from 30 rats, homogenized, and subjected to the differential centrifugation and sucrose density gradient analysis procedure described under Experimental Procedures. The tabulated experiment is representative of five experiments.

Table III: Preparation of Insulin Secretory Granules from Isolated Pancreatic Islet Homogenates by Centrifugation through Percoll Density Gradients[a]

fraction	composition	insulin/protein (milliunits/μg)
H′	homogenate	2.38
S1′	900*g* supernatant	5.50
P1′	900*g* pellet	0.68
S2′	13000*g* supernatant	1.65
P2′	13000*g* pellet	3.00
SG	secretory granules	28.80
M	mixed membranes	0.25

[a] Pancreatic islets (ca. 2700) were isolated from nine rats, homogenized, and subjected to the differential centrifugation and Percoll density gradient analysis procedure described under Experimental Procedures.

analysis reflects the relative abundance of phospholipids and protein in various membranous compartments as reflected by the fatty acyl mass to protein ratio (Table II). Plasma membranes exhibited a relatively low density on sucrose gradient analysis and a fatty acyl mass to protein ratio of 2356–5503 pmol/μg, while the more dense secretory granules and mitochondria exhibited a fatty acyl to protein ratio of 1275–2120 pmol/μg. Arachidonate (C20:4) comprised 36% of the total fatty acyl mass of islet homogenates, in close correspondence to previous estimates (Ramanadham et al., 1993b), and comprised 34% of the fatty acyl mass of plasma membranes. Linoleate (C18:2) was enriched in fractions containing mitochondria, reflecting the facts that cardiolipin is distributed virtually exclusively in mitochondria and contains predominantly linoleate as its fatty acyl substituent (Hoestler et al., 1971; Turk et al., 1986). Both cholesterol and the plasma membrane enzyme 5′-nucleotidase were highly enriched in plasma membrane fractions compared both to homogenate and to intracellular membranes such as endoplasmic reticulum (Table II). An alternate procedure involving centrifugation through Percoll gradients was used to prepare an enriched population of secretory granules (Jones et al., 1987). This procedure resulted in a 12-fold enrichment in the insulin/protein ratio in the secretory granule fraction compared to homogenate (Table III), in close correspondence to reported values (Jones et al., 1987) for the procedure.

Phospholipids from plasma membranes obtained from sucrose density gradient analysis and from secretory granules obtained from Percoll gradient analysis were then extracted under neutral conditions and separated into head-group classes by NP-HPLC. The PE peak from these analyses was then harvested separately and subjected to RP-HPLC under conditions permitting resolution of molecular species (Figure 4). Plasma membrane PE was found to contain substantial amounts of the plasmalogen species represented in PE peak 3 (C16:0p/C20:4-PE) and in peak 6 (C18:0p/C20:4-PE) (Figure 5). Quantitation of the arachidonate mass in individual PE peaks was accomplished by a procedure involving addition of [$^2H_8$]arachidonate as an internal standard, followed by saponification, conversion of the liberated arachidonate to the pentafluorobenzyl ester derivative, and stable isotope dilution GC–MS measurements in the NICI mode. Of the total arachidonate mass esterified in plasma membrane PE,

Table IV: Arachidonate Content of Ethanolamine Phospholipids in Islet Subcellular Membrane Fractions[a]

peak no. from RP-HPLC	PE species	arachidonate mass (pmol/μg of protein)			arachidonate ratio to peak 5		
		plasma membrane	secretory granules	endoplasmic reticulum	plasma membrane	secretory granules	endoplasmic reticulum
1	(C16:0a/C20:4)-PE	1.51	1.21	1.31	0.17	0.11	0.17
2	(C18:1a/C20:4)-PE	1.62	1.40	0.84	0.19	0.13	0.17
3	(C16:0p/C20:4)-PE	2.91	0.96	3.37	0.33	0.09	0.67
4	(C18:1p/C20:4)-PE	2.39	nd	2.83	0.27	nd	0.56
5	(C18:0a/C20:4)-PE	8.71	10.65	5.04	1.00	1.00	1.00
6	(C18:0p/C20:4)-PE	3.36	1.61	6.61	0.39	0.15	1.31
	total PE arachidonate (pmol/ μg of protein)	20.0	15.8	20.5			
	fraction of PE arachidonate in plasmalogen peaks	0.42	0.16	0.64			

[a] Islet plasma membranes were prepared as in Table II and secretory granules as in Table III. Ethanolamine phospholipids (PE) from these membranes were isolated by NP-HPLC and analyzed by RP-HPLC as in Figure 5. Individual PE peaks were collected separately, mixed with [$^2H_8$]arachidonate, and saponified. Liberated arachidonate was converted to the pentafluorobenzyl ester derivative and quantitated by GC–NICI–MS as described under Experimental Procedures. "Not detected" is denoted as "nd".

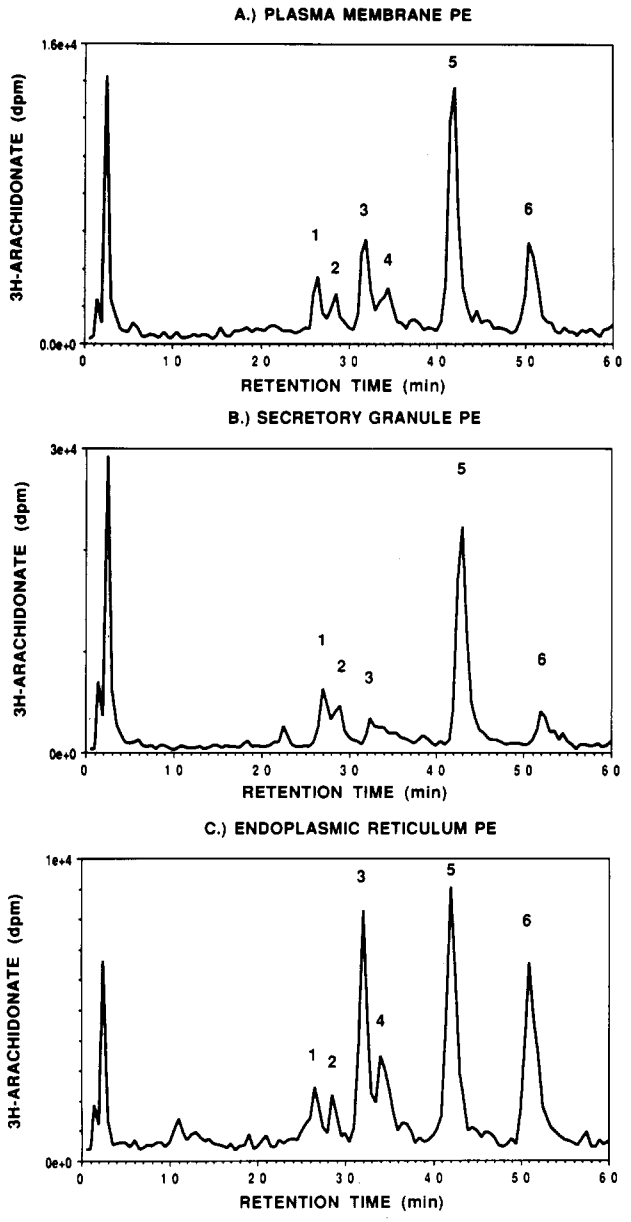

FIGURE 4: Reverse-phase HPLC analyses of ethanolamine phospholipids from rat pancreatic islet plasma membranes, secretory granules, and endoplasmic reticulum. Isolated pancreatic islets were labeled overnight with [$^3H_8$]arachdidonic acid as described under Experimental Procedures. Plasma membranes and endoplasmic reticulum were prepared from the labeled islets as in Table II, and secretory granules were prepared as in Table III. Each membrane fraction was then harvested separately and subjected to phospholipid extraction under neutral conditions. Phospholipids were then separated into head-group classes by NP-HPLC, and the ethanolamine phospholipid peaks were collected separately and subjected to RP-HPLC analyses as in Figure 3.

42% was found to reside in plasmalogen species (Table IV), which is virtually identical to the value observed for intact islets (Ramanadham et al., 1993b). In contrast, secretory granule PE was found to contain smaller amounts of plasmalogen species (Figure 4), and only 16% of the total arachidonate esterified in secretory granule PE resided in plasmalogens (Table IV). Endoplasmic reticulum PE was found to be highly enriched in plasmalogen species (Figure 4), which contained 64% of the total PE arachidonate mass (Table IV).

During the course of these studies, a human pancreas from a life-supported donor with irreversible loss of cerebral function became available to us, and islets were isolated from that

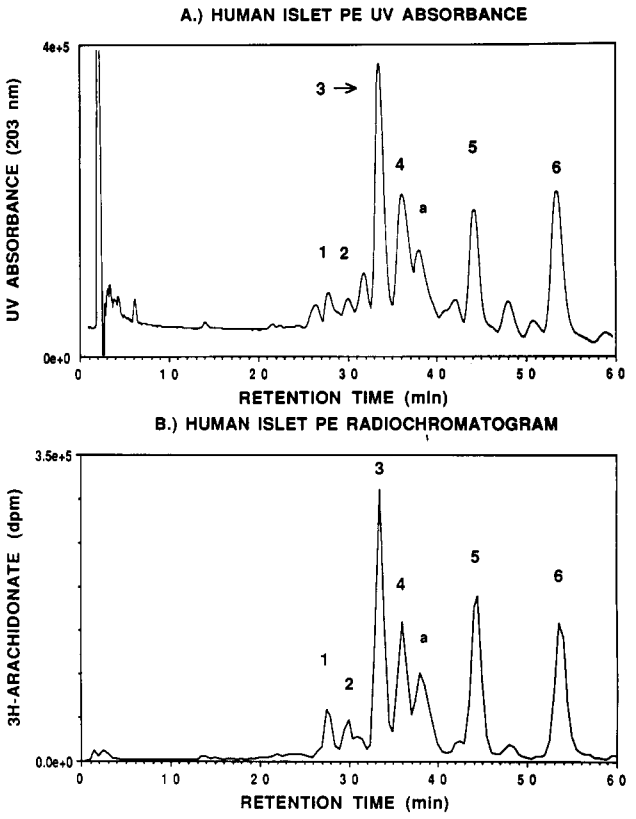

FIGURE 5: Human pancreatic islet ethanolamine phospholipids. Pancreatic islets (ca. 10 000) were isolated from a human donor and labeled overnight with [$^3H_8$]arachidonic acid. Two thousand of these islets were then removed for secretion studies. From the remainder, phospholipids were extracted under neutral conditions and separated into head-group classes by NP-HPLC. The ethanolamine phospholipid peak was harvested separately and analyzed by RP-HPLC as in Figure 3. Panel A represents UV absorbance (203 nm) recorded from a flow-through cell, and panel B represents [^3H]arachidonate content measured in aliquots of eluant fractions by liquid scintillation spectrometry.

organ. The human islets were then labeled by overnight incubation with [$^3H_8$]arachidonic acid. Phospholipids were then extracted under neutral conditions and separated into head-group classes by NP-HPLC. The PE and PC peaks were harvested and subjected to RP-HPLC, as illustrated in Figures 5 and 6. The plasmenylethanolamine molecular species represented by peaks 3, 4, and 6 were found to be prominent components of human islet PE (Figure 5) and contained 58% of the total PE arachidonate, as measured by stable isotope dilution GC–NICI–MS analyses with [$^2H_8$]-arachidonate after saponification and derivatization.

The diacylethanolamine phospholipid peak 5 (C18:0a/C20:4-PE) and the plasmenylethanolamine phospholipid peak 6 (C18:0p/C20:4-PE) from the human islets were collected separately and used as substrates in assays with cytosolic ASCI-PLA_2 activity prepared from the same human donor (Table V). As previously observed with rat islets (Ramanadham et al., 1993b), the human islet cytosolic ASCI-PLA_2 hydrolyzed the endogenous plasmenylethanolamine species more rapidly than the diacylethanolamine phospholipid species. When intact islets from this human donor were stimulated with secretagogues, a robust insulin secretory response occurred, and the eicosanoid $PGF_{2\alpha}$ was released into the perifusion medium, reflecting hydrolysis of arachidonate from membrane phospholipids (Table V). As previously observed with rat islets (Ramanadham et al., 1993a,b), both secretagogue-induced eicosanoid release and insulin secretion were attenuated in human islets that had been pretreated with the ASCI-

Table V: Human Pancreatic Islet ASCI-PLA_2-Catalyzed Hydrolysis of Endogenous Ethanolamine Phospholipid Substrates, Insulin Secretion, and Eicosanoid Release[a]

islet phospholipid substrate	ASCI-PLA_2 specific activity [pmol/(mg·min)]
(C18:0a/C20:4)-PE	43.6
(C18:0p/C20:4)-PE	173.7

glucose concentration (mM)	HELSS concentration (μM)	insulin secretion (μunits/islet)	$PGF_{2\alpha}$ release (pg)
3	0	3.3 ± 1.9	<6
17	0	88.1 ● 25.6	31.7 ± 2.8
3	25	<2	<6
17	25	30.9 ± 5.6	<6

[a] In the upper panel, human pancreatic islets from a single donor (ca. 4000) were prelabeled with [^{3}H]arachidonate (200 μCi) overnight at 37 °C, and phospholipids were extracted and analyzed by NP-HPLC as described under Experimental Procedures. The PE peak was harvested and analyzed by RP-HPLC as in Figure 3. The [^{3}H]arachidonate-labeled ethanolamine phospholipid peaks 5 and 6 were harvested separately, concentrated, and used as substrates of phospholipase A_2 (PLA_2) activity in the cytosolic fraction prepared from a second population of human islets (ca. 4000).

PLA_2 suicide substrate HELSS (Table V), which is sterically similar to plasmalogens (Hazen et al., 1991b).

Human islet PE also contained one arachidonate-containing molecular species which was much more prominent than in rat islet PE and which is designated as peak a in the radiochromatogram in Figure 6B. This material was identified (C16:0e/C20:4)-PE, because digestion of peak a to the diglyceride with *B. cereus* phospholipase C, followed by saponification and treatment with penatfluorobenzoyl chloride resulted in production of the dipentafluorobenozoate derivative of 1-*O*-hexadecylglycerol, as visualized by GC–NICI–MS analyses. Including the contribution of peak a, 71% of the total arachidonate mass esterified in human islet PE resided in ether phospholipids and 29% in diacylphospholipids.

Human islet choline phospholipids also contained an arachidonate-containing molecular species which was much more prominent than in rat islet PC and which is designated as peak b in Figure 6. This material was identified as the plasmalogen (C16:0p/C20:4)-PC on the basis of several observations. First, acid treatment of an aliquot of peak b resulted in disappearance of the peak upon reanalysis by RP-HPLC under conditions where a vehicle-treated aliquot of peak b and internal standard (C16:0a/C18:2)-PC were stable. Acid lability is characteristic of plasmalogens, although diacyl- and alkylacylphospholipids are acid-stable (DaTorre & Creer, 1991). Second, acid methanolysis of peak b yielded C16:0-dimethylacetal, as visualized by GC–PCI–MS, which is the expected acid methanolysis product of a palmitic aldehyde residue (Gross, 1984, 1985). Third, the relative RP-HPLC retention time of Peak b [(C16:0p/C20:4)-PC] compared to peak 1 [(C16:0a/C20:4)-PC] in Figure 6B was quite similar to the relative RP-HPLC retention times of the analogous PE species in Figure 3B [compare peak 3 [(C16:0p/C20:4)-PE] to peak 1 [(C16:0a/C20:4)-PE]]. Other evidence supporting this assignment included the greater apparent abundance of peak b relative to peaks 1 or 2 on UV monitoring (Figure 6A) than on the radiochromatogram (Figure 6B) or on arachidonate mass measurements by GC–NICI–MS, reflecting the fact that the vinyl ether linkage imparts a higher UV extinction coefficient to plasmalogens than exhibited by analogous diacylphospholipids (Gross et al., 1984, 1985).

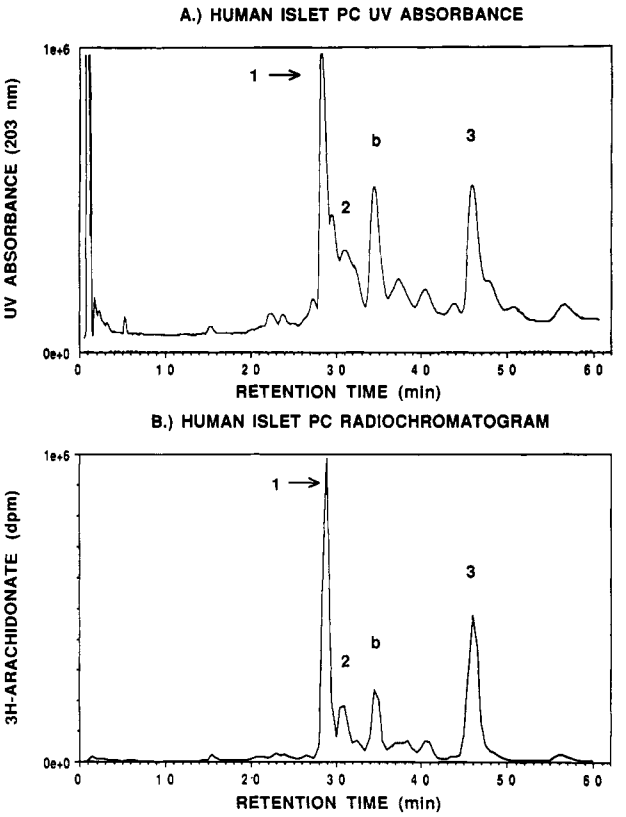

FIGURE 6: Human pancreatic islet choline phospholipids. In the experiment described in the legend of Figure 5, the PC peak from the NP-HPLC analysis of the human islet phospholipids was extracted and analyzed by RP-HPLC as in Figure 2. Panel A represents UV absorbance (203 nm) recorded from a flow-through cell, and panel B represents [^{3}H]arachidonate content measured in aliquots of eluant fractions by liquid scintillation spectrometry. Each labeled peak was collected separately and subjected to acid methanolysis and GC–PCI–MS analysis as described in the text under Results.

DISCUSSION

The studies described here establish that arachidonate-containing plasmalogens are prominent components of the ethanolamine phospholipids of human pancreatic islets and of rat islet β-cells, plasma membrane, and endoplasmic reticulum. The observation that these plasmenylethanolamine molecular species are more abundant in human than in rat islets is similar to previous observations that plasmenylethanolamine species are less abundant in rat central nervous system (CNS) tissue (Freysz et al., 1968) than in CNS tissue from other animal species (Dawson et al., 1962; Owens, 1966; Scott et al., 1967). The plasmenylethanolamine content of islets and of CNS tissue is quite similar (Freysz et al., 1968; Ramanadham et al., 1993b), and both islet β-cells and CNS neurons are electrically excitable cells which may share a common embryologic origin from the neuroectoderm (Escurat et al., 1991). Human islets were also found to contain an arachidonate-containing plasmenylcholine molecular species.

Myocardium, which is also an electrically active tissue, contains large amounts of both plasmenylethanolamine and plasmenylcholine (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). Myocardial plasmalogens are prominently distributed both in sarcolemma (Gross, 1984) and in sarcoplasmic reticulum (Gross, 1985), with a somewhat greater relative abundance in sarcoplasmic reticulum (53%) than in sarcolemma (40%). These observations are similar to those reported here that

islet plasmalogens are disbributed both in plasmalemma and endoplasmic reticulum, with a somewhat greater relative abundance in endoplasmic reticulum (64% of PE arachidonate) than in plasmalemma (42% of PE arachidonate).

Both the plasma membrane (Gilon & Henquin, 1992; Misler et al., 1992a,b) and endoplasmic reticulum (Wolf et al., 1988) participate in the regulation of the β-cell cytosolic [Ca^{2+}]. The rise in β-cell [Ca^{2+}] triggered by stimulation with secretagogues is thought to be a critical signal in the induction of insulin secretion, and such rises can be effected by opening of plasma membrane voltage-operated Ca^{2+} channels, which permits entry of Ca^{2+} from the extracellular space, or by release of Ca^{2+} sequestered in the endoplasmic reticulum. The former mechanism is thought to predominate in the rise in β-cell [Ca^{2+}] induced by fuel secretagogues and the latter in that induced by muscarinic agonists (Theler et al., 1993). Nonesterified arachidonate (2–20 μM) induces both entry of Ca^{2+} from the extracellular space across β-cell plasma membranes (Ramanadham et al., 1992) and release of Ca^{2+} from islet endoplasmic reticulum (Wolf et al., 1986). Nonesterified arachidonate accumulates in islets stimulated both with fuel secretagogues (Wolf et al., 1986, 1991; Konrad et al., 1992a, 1993) and with muscarinic agonists (Konrad et al., 1992b) and achieves concentrations exceeding 35–70 μM (Wolf et al., 1986, 1991).

Hydrolysis of arachidonate from membrane phospholipids in secretagogue-stimulated islets appears to be mediated in part by a novel ATP-stimulated, Ca^{2+}-independent (ASCI) phospholipase A_2 (PLA_2) enzyme (Gross et al., 1993; Ramanadham et al., 1993a,b), which is similar or identicial to an enzyme first discovered in myocardium (Wolf & Gross, 1985; Hazen et al., 1990, 1991a,b; Hazen & Gross, 1991). In vitro, islet ASCI-PLA_2, like the myocardial enzyme (Wolf & Gross, 1985; Hazen et al., 1990), prefers plasmenyl- over diacylphospholipid substrates (Gross et al., 1993; Ramanadham et al., 1993b), and islet arachidonate-containing plasmalogens undergo hydrolysis upon stimulation of islets with secretagogues (Ramanadham et al., 1993b). The preference of ASCI-PLA_2 for plasmalogen substrates has 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 rat islets with HELSS suppresses glucose-induced arachidonate hydrolysis from islet membrane phospholipids, the rise in β-cell cytosolic [Ca^{2+}], and insulin secretion under conditions where islet oxidation of [^{14}C]glucose to [^{14}C]O_2, carbachol-induced activation of islet phosphoinositide–phospholipase C, and depolarization-induced rises in β-cell [Ca^{2+}] are unaffected (Ramanadham et al., 1993a). As reported here, HELSS also attentuates both secretagogue-induced eicosanoid release and insulin secretion from human pancreatic islets.

The distribution of the preferred substrate of ASCI-PLA_2 in islet cell membrane compartments which participate in the regulation of the β-cell cytosolic [Ca^{2+}] raises interest in the possibility that activation of this enzyme and the accumulation of nonesterified arachidonate in these membranes is involved in the rise in β-cell cytosolic [Ca^{2+}] induced by insulin secretagogues. Nonesterified arachidonic acid amplifies Ca^{2+} entry across the plasma membrane through voltage-operated Ca^{2+} channels in clonal GH_3 pituitary tumor cells (Vacher et al., 1989) and through *N*-methyl-D-aspartate-operated Ca^{2+} channels in cerebellar cells (Miller et al., 1992) and also induces release of Ca^{2+} from sarcoplasmic reticulum of striated myocytes (Cheah, 1981) and from endoplasmic reticulum of hepatocytes (Chan & Turk, 1987). These observations suggest the possibility that the level of nonesterified arachidonate in the membranes of organelles involved in Ca^{2+} homeostasis may play some general role in the regulation of Ca^{2+} gating.

The potential involvement of phospholipases A_2 in glucose-induced insulin secretion has long been suspected (Laychock, 1982; Dunlop & Larkins, 1984; Metz, 1987, 1988, 1991; Metz & Dunlop, 1990). The specific localization of ASCI-PLA_2 (Gross et al., 1993, Ramanadham et al., 1993a) and of arachidonate-containing plasmenylethanolamine molecular species within β-cells of pancreatic islets and in islets from both rats and humans suggest that the presence of both the enzyme and its preferred substrates may be a general property of insulin-secreting β-cells. This possibility is further supported by recent observations that clonal insulin-secreting β-cell lines both express ASCI-PLA_2 and contain abundant amounts of arachidonate-containing plasmalogen molecular species (Ramanadham et al., manuscript in preparation). The precise mechanisms coupling islet glycolytic metabolism to activation of ASCI-PLA_2, the molecular properties of β-cell ASCI-PLA_2, and the role of its activation in the secretory process are the subjects of ongoing studies.

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REFERENCES

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.

Avruch, J., & Wallach, D. F. H. (1971) *Biochim. Biophys. Acta 233*, 334–347.

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.

Chan, K. M., & Turk, J. (1987) *Biochim. Biophys. Acta 928*, 186–193.

Cheah, A. M. (1981) *Biochim. Biophys. Acta 648*, 113–119.

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

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) *Biochem. Biophys. Res. Commun. 120*, 820–827.

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.
Gilon, O., & Henquin, J. C. (1992) *J. Biol. Chem. 267,* 20713–20720.
Gross, R. W. (1984) *Biochemistry 23,* 158–165.
Gross, R. W. (1985) *Biochemistry 24,* 1662–1668.
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.
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.
Hoestler, K. Y., van den Bosch, H., & van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta 239,* 113–119.
Jones, P. M., Saermark, T., & Howell, S. I. (1987) *Anal. Biochem 166,* 142–149.
Keahey, H., Rajun, A., Boyd, A. E., & Kunze, D. L. (1989) *Diabetes 38,* 188–193.
Konrad, R. J., Jolly, Y. C., Major, C., & Wolf, B. A. (1992a) *Biochim. Biophys. Acta 1135,* 215–220.
Konrad, R. J., Jolly, Y. C., Major, C., & Wolf, B. A. (1992b) *Biochim. J. 287,* 283–290.
Konrad, R. J., Jolly, Y. C., Major, C., & Wolf, B. A. (1993) *Mol. Cell. Endocrinol. 92,* 135–140.
Lacy, P. E., Walker, M. M., & Fink, C. J. (1972) *Diabetes 21,* 987–988.
Lange, Y., Swaisgood, M. H., Ramos, B. V., & Steck, T. L. (1989) *J. Biol. Chem. 264,* 3786–3793.
Laychock, S. G. (1982) *Cell Calcium 3,* 43–54.
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, F. M. (1986) *Diabetes/Metab. Rev. 2,* 163–214.
Metz, S. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82,* 198–202.
Metz, S. A. (1987) *Biochem. J. 241,* 863–769.
Metz, S. A. (1988) *Diabetes 37,* 1453–1469.
Metz, S. A. (1991) *Diabetes 40,* 1565–1573.
Metz, S. A., & Dunlop, M. E. (1990) *Biochem. Biophys. Res. Commun. 167,* 61–66.
Metz, S. A., Draznin, B., Sussman, K. E., & Leitner, J. W. (1987) *Biochem. Biophys. Res. Commun. 142,* 251–258.
Miller, B., Sarantis, M., Traynelis, S. F., & Atwell, D. (1992) *Nature 335,* 722–725.
Misler, S., Barnett, D. W., Pressel, D. M., Gillis, K. D., Scharp, D. W., & Falke, L. C. (1992a) *Diabetes 41,* 662–670.
Misler, S., Barnett, D. W., Pressel, D. M., & Gillis, K. D. (1992b) *Diabetes 41,* 1221-1228.
Naber, S. P., McDonald, J. M, Jarrett, L., Ludvigsen, C. W., & Lacy, P. E. (1980) *Diabetologia 19,* 439.
Ono, J., Takaki, R., & Fukuma, M., (1977) *Endocrinol. Jpn. 24,* 265–270.
Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48,* 47–71.
Owens, K. (1966) *Biochem. J. 100,* 354–361.
Patton, G. M., Fasulo, J. M., & Robbins, S. J. (1982) *J. Lipid Res. 23,* 190–196.
Pipeleers, D. G. (1984) in *Methods in Diabetes Research* (Larner, J., Ed.) pp 185–191, John Wiley & Sons, New York.
Pradelles, P., Grassi, I., & Maclouf, J. (1985) *Anal. Chem. 57,* 1170–1173.
Purdon, A. D., Patelunas, D., & Smith, J. D. (1987) *Lipids 22,* 116–120.
Ramanadham, S., Gross, R. W., & Turk, J. (1992) *Biochem. Biophys. Res. Commun. 184,* 647–653.
Ramanadham, S., Gross, R. W., Han, X., & Turk, J. (1993a) *Biochemistry 32,* 337–346.
Ramanadham, S., Bohrer, A., Mueller, M., Jett, P., Gross, R. W., & Turk, J. (1993b) *Biochemistry 32,* 5339–5351.
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.
Sturgess, N. C., Cook, D. L., Ashford, M. L. J., & Hales, C. N. (1985) *Lancet ii,* 474–475.
Theler, J. M., Mollard, P., Guerineau, N., Vacher, P., Pralong, W. F., Schlegel, W., & Wollheim, C. B. (1992) *J. Biol. Chem. 267,* 18110–18117.
Turk, J., Wolf, B., Lefkowith, J., Stump, W., & McDaniel, M. (1986) *Biochim. Biophys. Acta 879,* 399–409.
Turk, J., Mueller, M., Bohrer, A., & Ramanadham, S. (1992a) *Biochim. Biophys. Acta 1125,* 280–291.
Turk, J., Bohrer, A., Stump, W. T., Ramanadham, S., & Mangino, M. J. (1992b) *J. Chromatogr. 575,* 183–196.
Vacher, P., McKenzie, J., & Dufy, B. (1989) *Am. J. Physiol. 257,* E203–E211.
Wang, J. L., & McDaniel, M. L. (1990) *Biochem. Biophys. Res. Commun. 66,* 812–828.
Wolf, B. A., Turk, J., Sherman, W. R., & McDaniel, M. L. (1986) *J. Biol. Chem. 261,* 3501–3511.
Wolf, B. A., Colca, J., Turk, J., Florholmen, J., & McDaniel, M. L. (1988) *Am. J. Physiol. 254,* E121–E136.
Wolf, B. A., Pasquale, S. M., & Turk, J. (1991) *Biochemistry 30,* 6371–6379.
Wolf, R. A., & Gross, R. W. (1985) *J. Biol. Chem. 260,* 7295–7303.
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.