Rat and Human Pancreatic Islet Cells Contain a Calcium Ion Independent Phospholipase A_2 Activity Selective for Hydrolysis of Arachidonate Which Is Stimulated by Adenosine Triphosphate and Is Specifically Localized to Islet β-Cells†

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ABSTRACT: The recent demonstration that myocardial Ca^{2+}-independent phospholipase A_2 exists as a complex of catalytic and regulatory polypeptides that is modulated by ATP has suggested a novel mechanism through which alterations in glycolytic flux can be coupled to the generation of eicosanoids which facilitate insulin secretion. To determine the potential relevance of this mechanism, we examined the kinetic characteristics, substrate specificities, and cellular locus of phospholipase A_2 activity in pancreatic islets. Rat pancreatic islets contain a Ca^{2+}-independent phospholipase A_2 activity which is optimal at physiologic pH, preferentially hydrolyzes phospholipid substrates containing a vinyl ether linkage at the *sn*-1 position, and prefers arachidonic acid compared to oleic acid in the *sn*-2 position. Rat islet Ca^{2+}-independent phospholipase A_2 activity is inhibited by the mechanism-based inhibitor (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one and is stimulated by ATP. Purification of β-cells from dispersed pancreatic islet cells by fluorescence-activated cell sorting demonstrated that β-cells (but not non-β-cells) contain Ca^{2+}-independent, ATP-stimulated phospholipase A_2 activity. Remarkably, clonal RIN-m5f insulinoma cells, which possess a defect in glucose-induced insulin secretion, contain a Ca^{2+}-independent phospholipase A_2 which is not modulated by alterations in ATP concentration. Collectively, these results and those of an accompanying paper [Ramanadham et al. (1993) *Biochemistry* (following paper in this issue)] implicate Ca^{2+}-independent phospholipase A_2 as a putative glucose sensor which can couple alterations in glycolytic metabolism to the generation of biologically active eicosanoids and thereby facilitate glucose-induced insulin secretion.

Activation of intracellular phospholipases A_2 results in the hydrolysis of fatty acid residues from the *sn*-2 position of phospholipids, thereby initiating a cascade of enzymatic reactions which culminates in the generation of biologically active eicosanoids (e.g., Needleman et al., 1986; Davidson & Dennis, 1990; Schalwijk et al., 1990; Clark et al., 1991; Leslie, 1991; Takayama et al., 1991). Recent studies have demonstrated the presence of several novel intracellular phospholipases A_2 which are either responsive to physiologic alterations in calcium ion concentration (Loeb & Gross, 1986; Kramer, 1988; Clark et al., 1990, 1991; Diez & Mong, 1990; Leslie, 1991; Takayama et al., 1991) or alternatively can catalyze hydrolysis of their phospholipid substrates in an entirely Ca^{2+}-independent fashion (Wolf & Gross, 1985a; Hazen et al., 1990, 1991a,b; Hazen & Gross, 1991).

Although arachidonic acid and its 12-lipoxygenase metabolites appear to facilitate glucose-induced insulin secretion by pancreatic islet β-cells (Metz, 1983, 1985, 1988, 1991; Wolf et al., 1986; Metz et al., 1987; Turk et al., 1987; Laychock, 1990), the biochemical mechanisms responsible for arachidonic acid release after glucose stimulation of β-cells are unknown. Traditional dogma has considered alterations in calcium ion content as the predominant mechanism responsible for the mobilization of arachidonic acid after exposure of β-cells to D-glucose. While alterations in calcium ion content clearly contribute to some phospholipid hydrolytic events in islets (Laychock, 1982; Dunlop & Larkins, 1984; Biden et al., 1987), recent studies demonstrate that β-cells can be induced to release arachidonic acid upon stimulation with fuel secretagogues even under conditions where there is no rise in β-cell cytosolic $[Ca^{2+}]$, such as in the presence of Ca^{2+}-channel blockers or in Ca^{2+}-free medium (Wolf et al., 1991, Turk et al, 1992). Furthermore, since D-glucose must be metabolized via glycolysis to induce the hydrolysis of arachidonic acid from endogenous islet cell phospholipids (Metz, 1985; Turk et al., 1992), it seems clear that glycolytic and lipolytic metabolism must be coupled in some fashion in this cell type.

The recent identification of a neutral-active Ca^{2+}-independent phospholipase A_2 in myocardium which is selective for the hydrolysis of arachidonic acid and which is activated by ATP (Wolf & Gross, 1985a; Hazen et al., 1990, 1991a,b; Hazen & Gross, 1991) suggests a novel biochemical mechanism through which biologically active eicosanoids can be generated in response to physiologically relevant alterations in glucose concentration. In this study, we demonstrate that rat pancreatic islets contain a Ca^{2+}-independent phospholipase A_2 which is neutral-active, preferentially hydrolyzes substrates with *sn*-2-arachidonoyl over *sn*-2-palmitoyl residues, is activated by ATP, and is present in substantial quantities in β-cells but not in non-β-cells derived from pancreatic islets. In an accompanying paper (Ramanadham et al., 1993), we demonstrate through specific mechanism-based inhibition of

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Ca^{2+}-independent phospholipase A_2 that attenuation of Ca^{2+}-independent phospholipase A_2 activity results in the concordant dose- and time-dependent inhibition both of hydrolysis of arachidonate from islet cell phospholipids and of insulin secretion in response to D-glucose.

EXPERIMENTAL PROCEDURES

Materials. The radiolabeled compounds [^{3}H]oleic acid and [^{3}H]arachidonic acid were purchased from Dupont–New England Nuclear, and [^{14}C]arachidonic acid, [^{14}C]oleic acid, and [^{14}C]linoleic acid were purchased from Amersham (Arlington Heights, IL). Bovine heart lecithin and 1-palmitoyl-2-lysophosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL). Phosphatidic acid was purchased from Serdary Lipids. Glycerol, EGTA,[1] and other common buffer reagents were obtained from Sigma Chemical Co. (St. Louis, MO). All HPLC columns were purchased from P. J. Cobert. Dicyclohexylcarbodiimide and *N,N*-dimethyl-4-aminopyridine were obtained from Aldrich (Milwaukee, WI). Male Sprague-Dawley rats (180–220 g body weight) were purchased from Sasco (O'Fallon, MO). Collagenase was obtained 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 obtained from Miles Laboratories (Elkhart, IN). Rodent chow 5001 was purchased from Ralston Purina (St. Louis, MO). D-Glucose was purchased from the National Bureau of Standards (Washington, DC).

Isolation of Islets by Collagenase Digestion and Density Gradient Centrifugation. Islets were isolated aseptically from male Sprague-Dawley rats fed ad libitum, as described elsewhere (McDaniel et al., 1983). In brief, the pancreas was inflated with HBSS medium, excised, freed from adherent lymphatic, vascular, and adipose tissue, and minced. The supernatant was removed, and collagenase (8 mg/mL) was added. Digestion was allowed to proceed with shaking at 37 °C for 17 min. The digested tissue was diluted with HBSS, and the tubes were centrifuged three times at 2000 rpm. After each centrifugation, the supernatant was removed and fresh HBSS was added. The tissue pellet was suspended in 8 mL of 27% Ficoll, transferred to a gradient tube, and layered atop the gradient, which consisted of layers of 23%, 20.5%, and 11% Ficoll. The gradient tubes were centrifuged in a swinging bucket rotor at 800*g* for 10 min. Islets which collected at the 11% interface (major fraction) and the 20% interface (minor fraction) were harvested, rinsed with HBSS, and centrifuged for 1 min at 400*g*. The supernatant was discarded and the islet-containing pellet was resuspended in cCMRL-1066 medium, transferred into Falcon petri dishes each containing 2.5 mL of cCMRL-1066, placed under an atmosphere of 95% air/5% CO_2 for 10 min, and cultured overnight either at room temperature (for experiments with intact islets) or at 37 °C (for experiments with isolated β-cells). This procedure typically yielded 300 islets per rat.

[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HELSS, haloenol lactone suicide substrate [(*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one]; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FACS, fluorescence-activated cell sorting; FAD, flavin adenine dinucleotide; KRB, Krebs–Ringer bicarbonate buffer.

Labeling of Isolated Rat Pancreatic Islets with [$^3H_8$]-Arachidonic Acid, Extraction of Phospholipids, Purification of Rat Islet Phosphatidylethanolamine and Phosphatidylcholine by Normal-Phase HPLC, and Determination of Specific Radioactivity. Islets isolated from 30 rats (ca. 9000) were incubated overnight at 37 °C in complete CMRL-1066 tissue culture medium (supplemented as described above) with [$^3H_8$]arachidonic acid (100 μCi, Dupont–NEN, 100 Ci/mmol) and washed free of unincorporated radiolabel as previously described (Turk et al., 1986). Phospholipids were extracted with chloroform/methanol (Bligh, & Dyer, 1959) and analyzed by normal-phase HPLC (Patton et al., 1982) on a silicic acid HPLC column (LiChrospher Si-100, 10 μm, 250 × 4.6 mm, Alltech) in hexane/2-propanol/(25 mM potassium phosphate buffer, 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. Aliquots (50 μL) of each fraction (1 mL) were subjected to liquid scintillation counting for ^{3}H. Phosphatidylethanolamine (PE) eluted at 13 min and phosphatidylcholine (PC) at 100 min. Aliquots (1%) of the recovered [^{3}H]-PE and [^{3}H]-PC were spiked with [$^2H_8$]arachidonic acid (10 ng) and subjected to alkaline hydrolysis in 3 N LiOH (Turk et al., 1986). The liberated fatty acids were extracted, converted to the pentafluorobenzyl esters, and quantitated by gas chromatography–mass spectrometry in the negative ion chemical ionization mode as previously described (Wolf et al., 1986, 1990). The purified islet PE and PC contained 99 and 135 nmol of arachidonate, respectively, and the specific radioactivity of the esterified [^{3}H]arachidonate in these phospholipids was 1.21×10^4 dpm/nmol.

Preparation of β-Cell and Non-β-Cell Populations from Isolated Islets by Fluorescence-Activated Cell Sorting (FACS). Isolated pancreatic islets were dispersed into individual cells by incubation with dispase (0.33 mg/mL) in Ca^{2+}- and Mg^{2+}-free HBSS medium for 15 min (Ono et al., 1977). Dispersed cells were removed at 5-min intervals, and fresh dispase solution was added to the pellet. The islet cells were then filtered through a 60-μm nylon screen to remove aggregated cells and subjected to autofluorescence-activated cell sorting (FACS) using a FACS-IV instrument (Pipeleers, 1984). An argon laser illuminated the cells at 488 nm, and emission at 510–550 nm reflected endogenous FAD content. Suspensions consisting predominantly (>90%) of single β-cells were prepared in this way, as verified by immunocytochemical staining for insulin, glucagon, somatostatin, and pancreatic polypeptide, as previously described (Pipeleers, 1984; Wang & McDaniel, 1990).

Preparation of Human Pancreatic Islets. Human pancreatic islets were prepared from a pancreas obtained from a life-supported cadaver at the time other organs were obtained for transplantation by permission of the donor's family. Islets were then isolated from the pancreas in the laboratory of Dr. David Scharp by methods described elsewhere (Ricordi et al., 1988) and were cultured for 48 h at 37 °C before use under an atmosphere of 5% CO_2/95% O_2 in tissue culture medium 1066 (Gibco, Grand Island, NY) containing 10% fetal bovine serum, penicillin (100 units/mL), glutamine (2 mM), and HEPES (25 mM) at pH 7.4.

Culture of Insulinoma Cells. RIN-m5f cells (Cell Culture Support Center, Washington University) were cultured at 37 °C in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal calf serum, with 1% (w/v) each of glutamine, nonessential amino acids, sodium pyruvate, glucose, and 0.10% (w/v) penicillin–streptomycin. The cells were grown in T175 flasks (Falcon) to about 95% confluency. On the day of the

experiment, the culture medium was decanted, and the cells were detached from the flask with 15 mL of trypsin–EDTA (0.05%–0.02% w/v) with shaking at 37 °C. The cells were then transferred to a 50-mL Falcon tube, diluted with an equal volume of CMRL-1066, and centrifuged for 90 s at 1700 rpm. The resulting cell pellet (ca. 10^8 cells) was resuspended in CMRL-1066 and centrifuged as above three times.

Measurement of Insulin Secretion. Isolated human pancreatic islets were washed 3 times in KRB medium containing 3 mM D-glucose, and 30 islets were transferred into each of three 10- × 75-mm siliconized test tubes and preincubated for 30 min at 37 °C in 0.2 mL of KRB medium with 3 mM D-glucose. At the end of the preincubation period, the medium was removed and the islets were incubated for 30 min at 37 °C in KRB medium containing either 3 or 20 mM D-glucose. The medium was then removed for measurement of insulin content. Aliquots (100 μL) of the medium were placed in 12- × 17-mm borosilicate tubes and stored at −20 °C for radioimmunoassay. Appropriate dilutions were then prepared, and insulin was measured by double-antibody radioimmunoassay employing ^{125}I-labeled insulin as described elsewhere (Wright et al., 1971).

Subcellular Fractionation. Subcellular fractionation of isolated rat and human islets, cultured insulinoma cells, and FACS-purified β-cells and non-β-cells was performed after homogenization in buffer (0.25 M sucrose and 10 mM histidine, pH 7.2, adjusted with HCl) by three 15-s bursts from a Polytron apparatus at a setting of 6. Nuclei and cellular debris were removed by centrifugation (1000*g*, 10 min). The mitochondrial pellet obtained after further centrifugation (10000*g*, 10 min) was discarded. The resultant 10000*g* supernatant was then centrifuged (170000*g*, 60 min) to separate the microsomal (membranous) fraction (pellet) from the cytosolic fraction (supernatant). The membranous fraction (microsomes) was then resuspended in homogenization buffer.

Preparation of Synthetic Phospholipid Substrates. Homogeneous 1-*O*-(*Z*)-hexadec-1′-enyl-glycerophosphocholine was obtained by alkaline methanolysis of bovine heart choline glycerophospholipids, purified by silicic acid column chromatography, and resolved into individual molecular species by isocratic reverse-phase HPLC as previously described (Creer & Gross, 1985). Synthesis of *sn*-2 radiolabeled plasmenylcholine was performed by dicyclohexylcarbodiimide-mediated synthesis of radiolabeled fatty acid anhydride followed by its condensation to the *sn*-2 hydroxyl of 1-*O*-(*Z*)-hexadec-1′-enyl-glycerophosphocholine utilizing *N*,*N*-dimethyl-4-aminopyridine as catalyst (Wolf & Gross, 1985a). Each radiolabeled choline glycerophospholipid molecular species was first purified by preparative TLC (Wolf & Gross, 1985b) and then by Partisil SCX-HPLC (Gross & Sobel, 1980).

Preparation of (E)-6-(Bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one. The haloenol lactone (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one was prepared essentially as described (Daniels et al., 1983). The compound was purified by preparative reverse-phase HPLC in a solvent system of acetonitrile/water (50/50 v/v) at a flow rate of 8 mL/min on an octadecylsilicic acid column (250 × 10 μm, Alltech) and exhibited a retention time of 20 min. The structure of the purified product was verified by ^{1}H NMR spectroscopy by comparisons with published spectra (Daniels et al., 1983).

Phospholipase A_2 Activity Measurements. The phospholipase A_2 activity contained in subcellular fractions (150 μL of cytosol or 100 μL of membranous fraction with average protein contents of 70 μg) from rat and human pancreatic islets, FACS-purified islet β-cells and non-β-cells, and RIN-m5f cells was assayed by ethanolic injection (5 μL) of 2.5 μM (final concentration) synthetic radiolabeled phospholipid substrate in assay buffer (final conditions 400 μL total volume, 200 mM Tris, pH 7.5, and either 10 mM EGTA or 10 mM $CaCl_2$). The assay mixture was incubated for 2 min at 37 °C, and the reaction was terminated by addition of butanol (100 μL) and vortexing (twice for 10 s each). The organic (upper) phase was separated by centrifugation (2000*g* for 2 min) and a 25-μL aliquot was applied to channeled TLC plates. Samples were not acidified before butanol extraction, and the butanol extract was not concentrated before application to the TLC plate. Efficiency of fatty acid extraction under these conditions exceeds 95% (Gross & Sobel, 1982). TLC was performed on silica gel G plates with petroleum ether/ethyl ether/acetic acid (70/30/1), which resolved fatty acid (R_f 0.58) from 1-alkenyl-2-acyl-*sn*-glycerol (R_f 0.24) and diacylglycerol (R_f 0.21). The region of the TLC plates corresponding to fatty acid, 1-alkenyl-2-acyl-*sn*-glycerol, or diacylglycerol was scraped into a scintillation vial, and hydrolysis products were quantified by liquid scintillation spectrometry after addition of Universol (3 mL). Under these conditions, phospholipase A_2 reaction velocities were linear with respect to time and enzyme concentration for each substrate examined.

pH Dependence. Assays to identify the pH optima of cytosolic and membranous phospholipase A_2 activities were performed as described above except that reactions in the pH 4–6 range were buffered by 200 mM BisTris, and reactions at pH 7–9 were buffered by 200 mM Tris-HCl.

Inhibition by a Haloenol Lactone Suicide Substrate. The haloenol lactone, (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one, was injected (5 μL) into buffer (100 mM Tris-Cl, pH 7.5, and 10 mM EGTA) containing phospholipase A_2 after dissolution of an appropriate concentration of the inhibitor in ethanol. After preincubation with the haloenol lactone for 3 min at 20 °C, the phospholipase A_2 solution was diluted with assay buffer, and phospholipase A_2 activity was quantified by injection of phospholipid substrate (2.5 μM final concentration) in ethanol (5 μL). Phospholipase A_2 assays were performed by incubation of substrate and enzyme for 2 min at 37 °C and were terminated by addition of butanol (100 μL). Products were analyzed by TLC and quantified by liquid scintillation spectrometry as described above.

Influence of ATP. The influence of ATP on phospholipase A_2 activity was determined in assays performed as above except that ATP was included in assay buffer (final [ATP] 0, 0.01, 0.1, 0.5, 1, or 10 mM).

Protein Measurements. Protein content was measured with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

RESULTS

Rat pancreatic islet cytosolic or membranous subcellular fractions, when incubated with the radiolabeled phospholipid substrate 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadecanoyl-*sn*-glycero-3-phosphocholine (plasmenylcholine), catalyzed the release of [^{3}H]oleic acid with a specific enzymatic activity of 136 (cytosol) or 1003 (membranes) pmol mg^{-1} min^{-1}, respectively (Table I). Phospholipase A_2 activities in both subcellular fractions were maximal in the presence of EGTA and were modestly inhibited by supraphysiologic concentrations (10 mM) of calcium ion. Inhibition was less pronounced

Table I: Isolated Pancreatic Islets from Rats Express a Ca^{2+}-Independent Phospholipase A_2 Activity[a]

condition	phospholipase A_2 specific activity (pmol mg^{-1} min^{-1}) cytosol	membranes	n
10 mM $CaCl_2$	48 ± 30	699 ± 388	7
10 mM EGTA	136 ± 17	1003 ± 276	14

[a] Phospholipase A_2 assays were performed with cytosolic (150 μL) and membranous (100 μL) fractions prepared from isolated rat pancreatic islets in assay buffer (200 mM Tris-HCl, pH 7.5) containing either 10 mM $CaCl_2$ or 10 mM EGTA (final volume 400 μL). Reactions were initiated by injection in ethanol (5 μL) of radiolabeled substrate 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine. Final substrate concentration was 2.5 μM (specific activity = 1.1×10^3 dpm/pmol). Assay mixtures were then incubated at 37 °C for 2 min and terminated by addition of butanol (100 μL). Reaction products in the butanol extract were analyzed by TLC, and released [^{3}H]oleic acid was quantified by liquid scintillation spectrometry as described in Experimental Procedures. Radiolabeled product release was converted to a specific enzymatic activity value after quantification of the protein content from cytosol or membranes in each assay tube as described in more detail in Table II.

at 10 μM Ca^{2+}, but the highest level of phospholipase A_2 activity with any of three distinct phospholipid substrates was always observed with 10 mM EGTA (Table II). The only radiolabeled product formed under these conditions during incubation of phospholipid substrates with islet membranous or cytosolic subcellular fractions was radiolabeled fatty acid, with no observable radiolabeled diglyceride, lysophospholipid, or phosphatidic acid. These observations demonstrate that rat pancreatic islets contain a Ca^{2+}-independent phospholipase A_2 activity in both cytosolic and membranous subcellular fractions which does not possess an obligatory requirement for calcium ion.

The substrate selectivity of rat islet Ca^{2+}-independent phospholipase A_2 activity was examined by comparing fatty acid release from plasmenylcholine, phosphatidylethanolamine, and phosphatidylcholine substrates containing different *sn*-2 acyl substituents (Table III). The release of fatty acid from phospholipid substrate was most rapid for plasmenylcholine and phosphatidylethanolamine substrates, with smaller amounts of activity manifest utilizing phosphatidylcholine substrate. The release of fatty acid from substrate containing arachidonic acid at the *sn*-2 position was greater than that observed from phosphatidylcholine substrate containing oleic acid or palmitic acid at the *sn*-2 position (Table III). Rat islet cytosolic Ca^{2+}-independent phospholipase A_2 also catalyzed hydrolysis of [^{3}H]arachidonate from endogenous islet phosphatidylethanolamine and phosphatidylcholine that had been extracted and purified from prelabeled islets (Table IV). The optimum pH of rat islet Ca^{2+}-independent phospholipase A_2 was pH 7–8, with little activity manifest at pH 5 and only about 23% at pH 9 (Table V).

To determine whether rat pancreatic islet-derived Ca^{2+}-independent phospholipase A_2 activity from the cytosolic and membranous subcellular fractions was susceptible to mechanism-based inhibition by the haloenol lactone suicide substrate (HELSS) (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one, additional experiments were performed (Figure 1). Preincubation of cytosolic or membranous subcellular fractions with the indicated concentrations of HELSS resulted in the concentration-dependent inhibition of fatty acid release from the radiolabeled substrates with similar concentration–response profiles. Ca^{2+}-independent phospholipase A_2 from rat pancreatic islets was exquisitely sensitive to mechanism-based inhibition, with 70–80% inhibition achieved at a HELSS concentration of 100 nM under the conditions employed utilizing either plasmenylcholine, phosphatidylethanolamine, or phosphatidylcholine substrate (Figure 1).

Since prior experiments have demonstrated that cytosolic Ca^{2+}-independent phospholipase A_2 from myocardium exists as a catalytic complex comprised of regulatory and catalytic polypeptides whose activity is modulated by alterations in ATP concentration (Hazen & Gross, 1991), the ability of ATP to induce alterations in the release of radiolabeled fatty acid from plasmenylcholine, phosphatidylethanolamine, or phosphatidylcholine substrates catalyzed by islet subcellular fractions was examined. ATP induced a concentration-dependent increase in Ca^{2+}-independent phospholipase A_2 activity derived from cytosol but not from membranes (Figure 2). Importantly, this increase in Ca^{2+}-independent phospholipase A_2 activity was manifest utilizing substrates which were present in either the liquid crystal (plasmenylcholine and phosphatidylcholine) or inverted hexagonal phase (phosphatidylethanolamine). The observations summarized in Tables I–V and Figures 1 and 2 collectively demonstrate that isolated pancreatic islets from rats contain a Ca^{2+}-independent phospholipase activity which catalyzes hydrolysis of fatty acids from the *sn*-2 position of phospholipid substrates and which shares several functional characteristics with the myocardial enzyme (Wolf & Gross, 1985a; Hazen et al., 1990, 1991a,b; Hazen & Gross, 1991).

Isolated islets are aggregates of about 2000 cells surrounded by a limiting capsule. The quantitatively predominant cell type in islets is the insulin-secreting β-cell, which accounts for about 75% of islet cell mass (Turk et al., 1987). Islets also, however, contain glucagon-secreting α-cells, somatostatin-secreting ∂-cells, and vascular endothelial cells. To determine the cellular source of the Ca^{2+}-independent phospholipase A_2 activity within the islets, dispersed islet cells were separated into a virtually homogeneous population of β-cells and into a population enriched in α-cells by fluorescence-activated cell sorting (FACS) (Ramanadham et al, 1992). Islet β-cells are distinguishable from non-β-cells on FACS because of the higher endogenous content of flavin adenine dinucleotide and the larger size of β-cells (Van De Winkel et al., 1982). As illustrated in Table VI, the Ca^{2+}-independent, ATP-stimulated phospholipase activity which catalyzed release of [^{3}H]oleate from the *sn*-2 position of plasmenylcholine was observed in the FACS-purified β-cell population but not in the non-β-cell population. This indicates that β-cells are the source of the Ca^{2+}-independent phospholipase activity observed with intact isolated rat islets.

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 organ (Ricordi et al, 1988; Turk et al., 1988). As illustrated in Figure 3A, the human islets secreted insulin in response to an increase in glucose concentration from 3 to 20 mM. Figure 3 also illustrates that both cytosolic (panel B, upper bar graph) and membranous (panel B, lower bar graph) subcellular fractions from the isolated human islets exhibited Ca^{2+}-independent phospholipase activity which catalyzed release of radiolabeled fatty acid from the *sn*-2 position of both plasmenylcholine and phosphatidylcholine substrates. As with rat islets, the specific activity of the Ca^{2+}-independent phospholipase A_2 in human islets was higher in the membranous than in the cytosolic subcellular fraction, and ATP stimulated the Ca^{2+}-independent phospholipase A_2 activity of the cytosolic fraction but not of the membranous fraction.

Table II: Pancreatic Islet Phospholipase A_2 Specific Activity Measurements[a]

	cytosol			membranes		
	10 mM EGTA	10 μM $CaCl_2$	10 mM $CaCl_2$	10 mM EGTA	10 μM $CaCl_2$	10 mM $CaCl_2$
	Substrate (16:0p/18:1)-PC[b]					
raw cpm	1597 ± 280	906 ± 157	181 ± 32	1425 ± 184	1411 ± 254	764 ± 164
net dpm	4219 ± 751	2371 ± 377	113 ± 22	3207 ± 455	3172 ± 650	1556 ± 392
PLA_2 specific activity (pmol mg^{-1} min^{-1})	132 ± 33	74 ± 17	4 ± 2	232 ± 41	244 ± 53	125 ± 34
	Substrate (16:0a/20:4)-PE[c]					
raw cpm	916 ± 105	425 ± 92	168 ± 35	1170 ± 154	1042 ± 187	427 ± 16
net dpm	1045 ± 136	412 ± 134	80 ± 30	1182 ± 176	1041 ± 215	357 ± 18
PLA_2 specific activity (pmol mg^{-1} min^{-1})	264 ± 65	109 ± 34	28 ± 12	801 ± 205	707 ± 170	280 ± 100
	Substrate (16:0a/20:4)-PC[d]					
raw cpm	254 ± 81	145 ± 49	122 ± 38	429 ± 108	406 ± 86	178 ± 47
net dpm	247 ± 103	106 ± 61	79 ± 41	408 ± 118	382 ± 96	128 ± 50
PLA_2 specific activity (pmol mg^{-1} min^{-1})	62 ± 33	26 ± 11	23 ± 14	270 ± 74	252 ± 126	75 ± 21

[a] Phospholipase A_2 assays were performed with cytosolic or membranous fractions from isolated rat pancreatic islets as described in Table I except that buffer contained either 10 mM $CaCl_2$, 10 μM $CaCl_2$, or 10 mM EGTA. Reactions were initiated by injection of radiolabeled substrate: 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC], 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphoethanolamine [(16:0a/20:4)-PE], or 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine [(16:0a/20:4)-PC]. (The letter p in the substrate abbreviation denotes plasmalogen, i.e., an *sn*-1 vinyl ether linkage, and a denotes an *sn*-1 acyl linkage.) Final substrate concentration was 2.5 μM. Incubations were performed and terminated and products analyzed as in Table I. Phospholipase A_2 (PLA_2) specific activity was calculated from the measured counts per minute 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 picomoles), P is the assay tube protein content (in milligrams), and T is assay duration (in minutes). Assay duration was 2 min, and mean measured assay tube protein contents ranged from 24 to 82 μg. The parameter R was calculated as $4DS$, where the factor 4 accounts for the fraction (25 μL of 100 μL) of the butanol extract containing released fatty acid analyzed, S is the specific radioactivity of the phospholipid substrate in disintegrations per minute per picomole, and D is the net disintegrations per minute 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 and 0.9 for ^{14}C). The parameter S is designated "raw cpm", the parameter B "blank cpm", and the parameter D "net dpm". [b] n = 4; blank cpm = 142 ± 21. [c] n = 3; blank cpm = 106 ± 12. [d] n = 3; blank cpm = 63 ± 5.

Table III: Substrate Preference of Rat Pancreatic Islet Ca^{2+}-Independent Phospholipase A_2 Activity[a]

		PLA_2 specific activity (pmol mg^{-1} min^{-1})	
substrate	concn (μM)	cytosol	membranes
(16:0p/18:1)-PC	2.5	136 ± 17	969 ± 317
(16:0a/18:1)-PC	2.5	38	161
(16:0a/20:4)-PC	2.5	69 ± 15	293 ± 48
(16:0a/20:4)-PE	2.5	267 ± 33	726 ± 121
(16:0a/16:0)-PC	2.5	<0.5	60 ± 21
(16:0a/18:2)-PC	2.5	96	179
(16:0p/18:1)-PC	10	330	3453
(16:0a/18:1)-PC	10	36	541
(16:0a/20:4)-PC	10	282	800
(16:0a/16:0)-PC	10	6	143
(16:0a/18:2)-PC	10	280	500

[a] Phospholipase A_2 activity assays in cytosolic and microsomal fractions prepared from isolated rat pancreatic islets were performed in buffer containing 10 mM EGTA as described in Table I. Reactions were initiated by injection of one of the following substrates in ethanol (10 μL): 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC], 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine [(16:0a/20:4)-PC], 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphoethanolamine [(16:0a/20:4)-PE], 1-hexadecanoyl-2-[1-^{14}C]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0a/18:1)-PC], 1-hexadecanoyl-2-[1-^{14}C]octadeca-9′,12′-dienoyl-*sn*-glycero-3-phosphocholine [(16:0a/18:2)-PC], or 1-hexadecanoyl-2-[1-^{14}C]hexadecanoyl-*sn*-glycero-3-phosphocholine [(16:0a/16:0)-PC]. Released fatty acid was quantified and converted to PLA_2 specific activity as described in Table II. Tabulated values represent means. SEM (n = 9–14) is indicated where appropriate. Other values are the means of duplicate determinations.

Table IV: Rat Pancreatic Islet Cytosolic Ca^{2+}-Independent Phospholipase A_2 Activity Hydrolyzes Arachidonic Acid from Endogenous Islet Phospholipids[a]

		[^{3}H]arachidonate release		
substrate	assay condition	raw cpm	net dpm	PLA_2 specific activity (pmol mg^{-1} min^{-1})
islet [^{3}H]-(20:4)-PE	blank (no cytosol)	33	0	0
islet [^{3}H]-(20:4)-PE	cytosol + 10 mM EGTA	134 ± 8	253 ± 20	278 ± 16
islet [^{3}H]-(20:4)-PC	blank (no cytosol)	34	0	0
islet [^{3}H]-(20:4)-PC	cytosol + 10 mM EGTA	108 ± 33	188 ± 83	207 ± 91

[a] Phospholipase A_2 (PLA_2) assays were performed with rat pancreatic islet cytosol as in Table II except that (a) the substrates were phospholipids extracted and purified from isolated rat pancreatic islets (9000) that had been prelabeled with [$^3H_8$]arachidonic acid (100 μCi) by 18 h of incubation at 37 °C, (b) islet [^{3}H]-(20:4)-PE and [^{3}H]-(20:4)-PC were added at final concentrations of 33 and 45 μM, respectively, and (c) assay duration was 5 min. Cytosolic protein content of each assay tube was 60 μg. Measured [$^3H_8$]arachidonate-specific radioactivity in the phospholipids was 12.1 dpm/pmol. The purified radiolabeled rat islet phospholipids were prepared by extraction of the prelabeled rat islets and analysis by normal-phase HPLC as described in Experimental Procedures. In the table, phosphatidylethanolamine is designated PE and phosphatidylcholine PC. Tabulated values represent means ± SEM (n = 4).

These observations indicate that the expression of Ca^{2+}-independent, ATP-stimulated phospholipase A_2 activity by pancreatic islets is not confined to a single species and suggests the possibility that this may be a general property of insulin-secreting β-cells.

RIN-m5f is a clonal insulin-secreting cell line that proliferates in cell culture and is derived from a rat pancreatic radiation-induced neoplasm (Chick et al. 1977; Praz et al, 1983). As illustrated in Figure 4, RIN-m5f insulinoma cells also contained a Ca^{2+}-independent phospholipase activity in both membranes and cytosol that catalyzed the release of radiolabeled fatty acid from the *sn*-2 position of phospholipid substrates (panel A). Like the rat islet activity, the RIN-m5f cell Ca^{2+}-independent phospholipase A_2 activity exhibited a pH optimum near neutrality (panel B) and was inhibited by preincubation with HELSS for 5 min at 20 °C (panel C). Inhibition of the RIN-m5f cell Ca^{2+}-independent phospholipase A_2 activity was concentration-dependent, with over 50%

Table V: pH Dependence of Rat Pancreatic Islet Ca^{2+}-Independent Phospholipase A_2 Activity[a]

		phospholipase A_2 specific activity (pmol mg^{-1} min^{-1})	
expt	pH	cytosol	membranes
1	5.0	1	3
1	6.0	226	193
1	7.0	348	371
1	8.0	307	394
1	9.0	84	27
2	5.0	1	3
2	6.5	152	132
2	7.0	227	680
2	7.5	194	641
2	8.0	151	595
2	8.5	126	265
2	9.0	73	198

[a] Phospholipase A_2 activity measurements were performed with cytosolic or membranous fractions prepared from isolated rat pancreatic islets in buffer (pH 5–9) containing 10 mM EGTA as described in Table I. Reactions in the pH 4–6 range were buffered by 100 mM Tris–acetate, and reactions from pH 7 to 9 were buffered by 100 mM Tris-HCl. Reactions were initiated by injecting 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphoethanolamine [(16:0a/20:4)-PE] in ethanol (10 μL). Released fatty acid was quantified and converted to phospholipase A_2 specific activity as described in Table II. Values are the means of duplicate determinations.

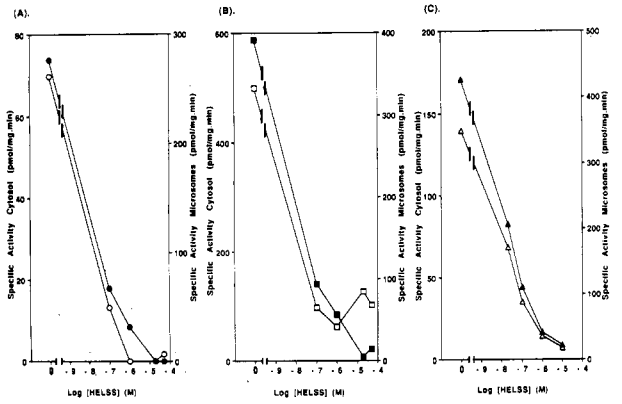

FIGURE 1: Inhibition of rat pancreatic islet Ca^{2+}-independent phospholipase A_2 activity by (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one. Phospholipase A_2 in cytosolic (open symbols) and membranous (closed symbols) fractions prepared from isolated rat pancreatic islets was preincubated with selected concentrations (0–100 μM) of the haloenol lactone phospholipase A_2 suicide substrate (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one. Residual phospholipase A_2 enzymatic activity was measured as described in Table I after addition of radiolabeled substrate: 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC] (panel A), 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphoethanolamine [(16:0a/20:4)-PE] (panel B), or 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine [(16:0a/20:4)-PC] (panel C). Released fatty acid was quantified and converted to phospholipase A_2 specific activity as described in Table II. Values are the means of duplicate determinations.

inhibition of the cytosolic and membranous activities achieved at HELSS concentrations of 100 nM or above (Figure 4, panel C). In contrast to the rat islet cytosolic Ca^{2+}-independent phospholipase A_2 activity, the RIN-m5f cytosolic activity was not stimulated by ATP with any of three phospholipid substrates tested (Figure 5). Also in contrast to the rat islet activity, which was nearly 8-fold greater in membranes than in cytosol, the RIN cell Ca^{2+}-independent phospholipase A_2 activity was distributed nearly equally between the cytosolic and membranous subcellular fractions (Figure 4, panel A, vs Table I).

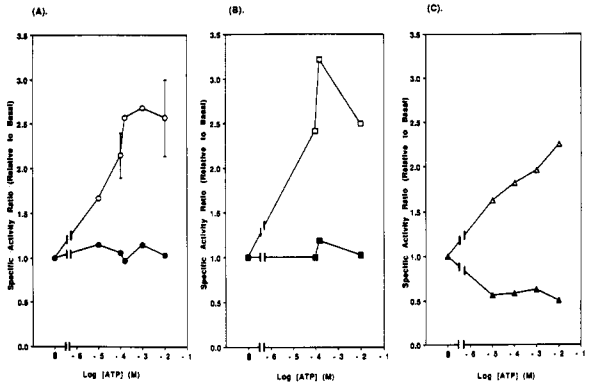

FIGURE 2: Activation of rat pancreatic islet Ca^{2+}-independent phospholipase A_2 actvity by ATP. Phospholipase A_2 activity was measured as described in Table I, with cytosolic (open symbols) and membranous (closed symbols) fractions prepared from isolated rat pancreatic islets in the presence of selected concentrations of ATP. Reactions were initiated by injection of radiolabeled substrates: 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC] (panel A), 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphoethanolamine [(16:0a/20:4)-PE] (panel B), or 1-hexadecanoyl-2-[1-^{14}C]-eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine [(16:0a/20:4)-PC] (panel C). Released fatty acid was quantified and converted to phospholipase A_2 specific activity as described in Table II. Phospholipase A_2 specific activity in the presence of ATP was divided by its specific activity for each substrate in the absence of ATP to yield the displayed "specific activity ratio (relative to basal)" value. Standard errors of the mean (n = 5) are indicated where appropriate. Other values are the means of duplicate determinations.

Table VI: Islet β-Cells Purified by Fluorescence-Activated Cell Sorting Express a Ca^{2+}-Independent Phospholipase A_2 Activity[a]

			PLA_2 specific activity (pmol mg^{-1} min^{-1})	
cell population	EGTA (mM)	ATP (mM)	cytosol	membranes
β-cells	10	0	203	1010
β-cells	10	10	491	945
non-β-cells	10	0	<30	<30
non-β-cells	10	10	<30	<30

[a] Phospholipase A_2 assays with cytosolic or membranous fractions from purified populations of β-cells and from populations of non-β-cells (predominantly α-cells) prepared from dispersed rat islet cells by fluorescence-activated cell sorting were performed as described in Table I. Assay buffer containing 10 mM EGTA ± 10 mM ATP was employed and reactions were initiated by injection of 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC]. Tabulated values represent means of duplicate determinations.

DISCUSSION

These observations indicate that isolated pancreatic islets from rats possess a phospholipase A_2 activity in both cytosol and membranes that is similar in several respects to a phospholipase A_2 enzyme recently described in myocardium (Wolf & Gross, 1985; Hazen et al., 1990, 1991a,b; Hazen & Gross, 1991). Both the islet and the myocardial phospholipases A_2 catalyze hydrolysis of arachidonate from the *sn*-2 position of choline and ethanolamine phospholipids, are fully active in the absence of Ca^{2+}, prefer plasmalogen over diacyl substrate, exhibit a pH optimum near neutrality, are activated by ATP, and are inhibited by the haloenol lactone (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one with a similar concentration–response profile. Pancreatic islets from both rats and humans express this Ca^{2+}-independent, ATP-stimulated phospholipase A_2 activity, and this activity is specifically present in pancreatic islet β-cells.

Cultured RIN-m5f insulinoma cells also express a Ca^{2+}-independent phospholipase A_2 activity that is similar to the

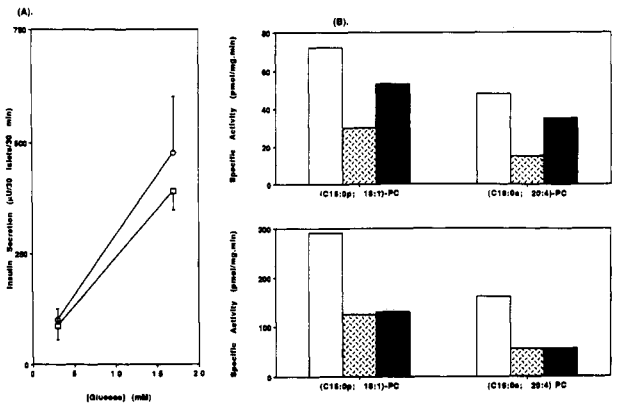

FIGURE 3: Human pancreatic islet cells which secrete insulin contain Ca^{2+}-independent phospholipase A_2 activity. Pancreatic islets were isolated from a human pancreas and cultured at 37 °C for either 24 h (open circles) or 48 h (open squares). Insulin secretion (panel A) was then measured by radioimmunoassay after static incubation of 30 islets per condition with either 3 or 17 mM glucose for 30 min at 37 °C, as described in the Experimental Procedures. Values represent means ± SEM of triplicate determinations. Phospholipase A_2 activity measurements were performed as described in Table I with cytosolic (panel B, upper bar graph) or membranous (panel B, lower bar graph) fractions from the isolated human pancreatic islets in assay buffer containing 10 mM $CaCl_2$ (open bars), 10 mM EGTA (stippled bars), or both 10 mM EGTA and 10 mM ATP (solid bars). Reactions were initiated by injection of radiolabeled substrate, 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-^{3}H]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC] or 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine [(16:0a/20:4)-PC] as indicated. Released fatty acid was quantified and converted to phospholipase A_2 specific activity as described in Table II. Values represent the means of duplicate determinations.

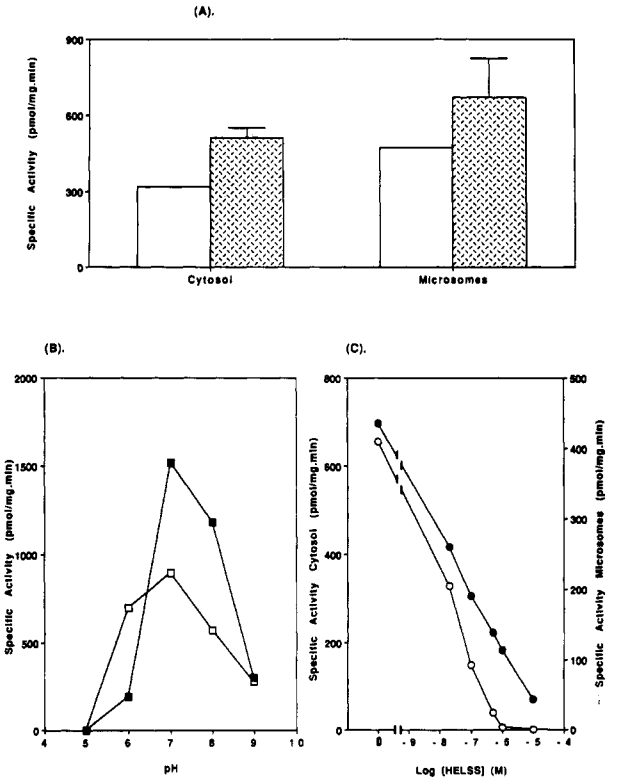

FIGURE 4: Clonal RIN-m5f insulinoma cells express a Ca^{2+}-independent phospholipase A_2 activity. Phospholipase A_2 activity was measured as described in Table I with cytosolic and membranous fractions prepared from cultured RIN-m5f insulinoma cells in assay buffer containing either 10 mM $CaCl_2$ (open bars) or 10 mM EGTA (stippled bars). In panel A, Ca^{2+}-independent phospholipase A_2 activity in cytosol or membranes (microsomes) from RIN-m5f cells as indicated was measured after injection of the radiolabeled substrate 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC]. In panel B, pH-dependence of the RIN cell Ca^{2+}-independent phospholipase A_2 activity was determined in assay buffer containing 10 mM EGTA after injection of the radiolabeled substrate 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,-11′,14′-tetraenoyl-*sn*-glycero-3-phosphoethanolamine [(16:0a/20:4)-PE]. In panel C, the concentration–response profile of phospholipase A_2 inhibition induced by the suicide substrate (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one was determined after injection of the radiolabeled substrate 1-*O*-(*Z*)-hexadec-1′-enoyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC]. In both panels B and C, the cytosolic fraction is represented by open symbols and the membranous fraction by closed symbols. Released fatty acid was quantified and converted to phospholipase A_2 specific activity as described in Table II. Standard errors of the mean are indicated where appropriate (n = 5). Other values are means of duplicates.

islet activity with respect to pH dependence and susceptibility to inhibition by the haloenol lactone suicide substrate. In contrast to the islet activity, however, RIN-m5f cell phospholipase A_2 activity is not stimulated by ATP. The ATP sensitivity of the myocardial Ca^{2+}-independent phospholipase A_2 enzyme is conferred by interaction with a 350-kDa ATP-responsive regulatory protein which is distinct from the 40-kDa phospholipase A_2 catalytic protein (Hazen & Gross, 1991). Such an ATP-sensing regulatory protein may be absent or defective in RIN-m5f cells, or the phospholipase A_2 catalytic protein may itself be altered and unable to interact with an ATP-sensing regulatory protein in these cells. RIN-m5f cells can be induced to secrete insulin by depolarization with KCl but do not secrete insulin in response to glucose (Praz et al., 1983). It is possible that the failure of glucose to induce insulin secretion by RIN-m5f cells may be attributable to the inability of the phospholipase A_2 catalytic complex to respond to alterations in local ATP concentrations derived from the glucose-induced increase in glycolytic flux. Conversely, the stimulation of the islet β-cell Ca^{2+}-independent phospholipase A_2 activity by ATP suggests that ATP derived from the glycolytic metabolism of glucose may potentially be responsible for the coupling of alterations in glucose concentration to the hydrolysis of arachidonate from endogenous membrane phospholipid pools (Wolf et al., 1986, 1991; Turk et al., 1992). These issues are addressed in the companion paper (Ramanadham et al., 1993).

The properties of the islet ATP-stimulated, Ca^{2+}-independent phospholipase A_2 and of the analogous myocardial enzyme distinguish them from other characterized phospholipases A_2. Low molecular weight (14–18 kDa) phospholipases A_2, such as those in pancreatic exocrine secretions (Type I) and those in inflammatory exudates (Type II), function as extracellular enzymes and require millimolar [Ca^{2+}] for activity (Dennis et al., 1991). One class of higher molecular weight (70–100 kDa) phospholipases A_2 comprises intracellular enzymes which are distributed in the cytosol of resting cells, become membrane-associated in activated cells in response to rising cytosolic [Ca^{2+}], require several hundred nanomolar to micromolar [Ca^{2+}] for optimal activity, and retain activity at millimolar [Ca^{2+}] (e.g. Leslie et al., 1991; Takayama et al., 1991; Clark et al., 1991). In contrast, islet ATP-stimulated phospholipase A_2 is fully active in the presence of 10 mM EGTA and is somewhat inhibited at millimolar [Ca^{2+}]. Other phospholipases A_2 which retain activity in the presence of EGTA have been described in intestinal brush border membrane (Gassama-Diagne et al., 1989), lung cytosol (Pierik et al., 1988; Nijssen et al, 1986), and other cells (Ross, et al., 1985; Ballou et al., 1986; Husebye & Flatmark, 1987; Yost et al., 1992). Although characterization of many of these phospholipases is incomplete, none has been reported to exhibit the ATP stimulation and plasmalogen preference characteristic

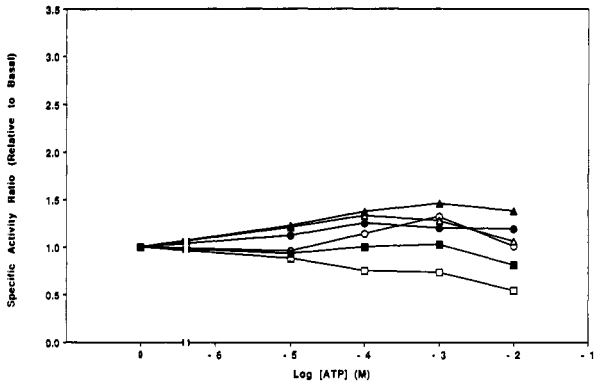

FIGURE 5: Lack of ATP activation of RIN-m5f insulinoma cell Ca^{2+}-independent phospholipase A_2 activity. Phospholipase A_2 activity measurements were performed as described in Table I with cytosolic (open symbols) or membranous (closed symbols) fractions prepared from cultured RIN-m5f insulinoma cells in assay buffer containing the indicated concentrations of ATP. Reactions were initiated by injection of radiolabeled substrate: 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,-10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC] (circles), 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenyl-*sn*-glycero-3-phosphoethanolamine [(16:0a/20:4)-PE] (squares), or 1-hexadecanoyl-2-[1-^{14}C]eicosa-5′,8′,11′,14′-tetraenoyl-*sn*-glycero-3-phosphocholine [(16:0a/20:4)-PC] (triangles). Released fatty acid was quantified and converted to phospholipase A_2 specific activity as described in Table I. Displayed values are the means of duplicate determinations.

of the islet and myocardial enzymes. Where such information is available, these other Ca^{2+}-independent phospholipases A_2 also appear to be distinguished from the myocardial enzyme on the basis of regiospecificity (Gassama-Diagne et al., 1989), pH optimum (Ross et al., 1985; Yost et al., 1992), and molecular mass (Gassama-Diagne et al., 1989).

The observation that only the cytosolic and not the membrane-associated form of the islet Ca^{2+}-independent phospholipase A_2 activity is sensitive to ATP raises the possibility that the effect of ATP in the intact cell may be to induce translocation of cytosolic phospholipase A_2 to the membrane, where it can productively interact with its phospholipid substrate. The fact that the RIN-m5f cell cytosolic Ca^{2+}-independent phospholipase A_2 activity is insensitive to ATP and that the specific activity of the RIN-m5f cell Ca^{2+}-independent phospholipase A_2 is higher in cytosol and lower in membranes in comparison with the phospholipase A_2 activity in islets is consistent with a defective ATP-induced membrane translocation event in RIN-m5f cells. There are substantial precedents for induction of membrane association of enzymes by activating ligands, including Ca^{2+}-dependent phospholipases A_2 (Brooks et al., 1989; Channon & Leslie, 1990; Clark et al., 1991; Leslie, 1991; Takayama et al., 1991) and the arachidonate 5-lipoxygenase (Kargman et al., 1991; Wong et al., 1991), where association is induced by Ca^{2+} in both cases. Similarly, protein kinase C (Bazzi & Nelsestuen, 1989; Bell & Burns, 1991) undergoes membrane association when activated by 1,2-*sn*-diacylglycerol accumulation and elevations in cytosolic [Ca^{2+}].

Direct molecular comparisons of the cytosolic and membrane-associated forms of the Ca^{2+}-independent phospholipase A_2 enzyme from myocardium have so far been frustrated by the extreme sensitivity of the enzyme to a variety of detergents, which has prevented solubilization and purification of the membrane-associated enzyme (Hazen et al., 1990). Obstacles to purification of the islet enzyme include both the low abundance of the enzyme relative to other cellular proteins [154 000-fold purification is required to achieve homogeneity (Hazen et al., 1990) from myocardium] and the limited number of pancreatic islets it is feasible to isolate [30 rats yield 9000 islets with 1 μg of total protein each (Turk et al., 1987)]. Determination of the relationship between the cytosolic and membrane-associated forms of the enzyme in myocardium and between the cytosolic enzyme in myocardium and in islets will be facilitated by development of antibodies directed against the purified myocardial cytosolic enzyme.

The preference of the islet ATP-stimulated Ca^{2+}-independent phospholipase A_2 activity for *sn*-2-arachidonoyl residues is of interest in the context of changes in nonesterified fatty acid mass that occur in glucose-stimulated islets. Arachidonate is the most abundant unsaturated fatty acid in islets and accounts for 17% of the total fatty acyl mass and 34% of the *sn*-2 fatty acyl mass in islet glycerolipids (Turk et al., 1986). Upon stimulation with glucose, islets levels of nonesterified arachidonate rise significantly by about 2 pmol/islet (Wolf et al., 1986, 1991). The level of nonesterified palmitate does not change, and nonesterified oleate and linoleate rise by smaller amounts (Wolf et al., 1991). Our observation that islet cytosolic Ca^{2+}-independent phospholipase A_2 catalyzes hydrolysis of arachidonate from endogenous islet phosphatidylethanolamine and phosphatidylcholine is consistent with a role for the enzyme in mediating glucose-induced hydrolysis of arachidonate from islet phospholipids. More detailed kinetic characterization of the effects of membrane phospholipid composition on the activity of the islet ATP-stimulated Ca^{2+}-independent phospholipase A_2 is difficult to achieve because the limited available amounts of the islet enzyme have hindered its purification. Kinetic characterization of the purified, homogeneous myocardial Ca^{2+}-independent phospholipase A_2 has demonstrated that the enzyme catalyzes the selective hydrolysis of arachidonate from plasmenylcholine even in mixed bilayers consisting predominantly of phosphatidylcholine, indicating that the selectivity of the enzyme is independent of alterations in the physical properties and interfacial characteristics of aggregate substrate (Hazen et al., 1989).

The common expression of this novel ATP-stimulated phospholipase A_2 enzyme in islets and in myocardium raises the question of what functional role the enzyme might play in two such seemingly physiologically disparate tissues. We note that 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 another ATP response element, the ATP-sensitive K^+ channel (Noma, 1983; Cook & Hales, 1984; Weiss & Lamp, 1987), which is found only in a restricted number of cell types. Both β-cells and cardiac myocytes are also electrically active, and each cell has a periodic physiological response (insulin secretion or contraction) that is driven by changes in the cytosolic [Ca^{2+}]. In the companion paper (Ramanadham et al., 1993), we suggest a model for glucose-induced insulin secretion which invokes the activity of the β-cell ATP-stimulated, Ca^{2+}-independent phospholipase A_2 in the regulation of cytosolic [Ca^{2+}] and which is supported by the effects of the suicide substrate HELSS on these phenomena.

The results of the present studies demonstrate susceptibility of the islet Ca^{2+}-independent phospholipase A_2 enzyme to inhibition by the mechanism-based inhibitor (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one. This offers a tool to examine the possible participation of the enzyme in the signaling processes underlying glucose-induced insulin secretion (Ramanadham et al., 1993). This haloenol lactone suicide substrate (HELSS) inhibitor has been demonstrated

to be a bona fide mechanism-based inhibitor for Ca^{2+}-independent phospholipase A_2 because it induces a time-dependent irreversible inhibition and covalent modification of the enzyme as a result of in situ generation of the electrophilic α-bromomethyl ketone at the catalytic site of phospholipase A_2 (Hazen et al., 1991b). The high molar potency of HELSS in conjunction with its selectivity for Ca^{2+}-independent phospholipases A_2 is underscored by the fact that at HELSS concentrations up to 100 μM the activity of Ca^{2+}-dependent phospholipase A_2 enzymes from a variety of sources is not altered (Hazen et al., 1991b). The susceptibility of the islet Ca^{2+}-independent phospholipase A_2 enzyme to inhibition by HELSS is quite comparable to that of the myocardial enzyme, with about 70% inhibition of the islet enzyme achieved after a 5-min preincubation with 100 nM HELSS.

In contrast to other suicide inhibitors of phospholipases (Washburn et al., 1990a,b), HELSS does not contain a polar head group or other charged moieties (Hazen et al., 1991b). Thus, the nonpolar character of HELSS would be anticipated to facilitate its traversal of cellular membranes, resulting in a useful pharmacologic probe which can rapidly, effectively, and specifically target intracellular Ca^{2+}-independent phospholipases A_2 in intact cells (Hazen et al., 1991b). These properties have allowed the use of HELSS to demonstrate the importance of this Ca^{2+}-independent phospholipase A_2 in the mobilization of arachidonic acid in pancreatic islets after glucose stimulation and the subsequent role of released arachidonate in insulin secretion (Ramanadham et al., 1993).

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