Pancreatic Islets Express a Ca\(^{2+}\)-independent Phospholipase A\(_2\) Enzyme That Contains a Repeated Structural Motif Homologous to the Integral Membrane Protein Binding Domain of Ankyrin*  

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Pancreatic islets express a Ca\(^{2+}\)-independent phospholipase A\(_2\) (CaI-PLA\(_2\)) activity that is sensitive to inhibition by a haloenol lactone suicide substrate that also attenuates glucose-induced hydrolysis of arachidonic acid from islet phospholipids and insulin secretion. A cDNA has been cloned from a rat islet cDNA library that encodes a protein with a deduced amino acid sequence of 751 residues that is homologous to a CaI-PLA\(_2\) enzyme recently cloned from Chinese hamster ovary cells. Transient transfection of both COS-7 cells and Chinese hamster ovary cells with the cloned islet CaI-PLA\(_2\) cDNA resulted in an increase in cellular CaI-PLA\(_2\) activity, and this activity was susceptible to inhibition by haloenol lactone suicide substrate. The domain of the islet CaI-PLA\(_2\) from amino acid residues 150–414 is composed of eight stretches of a repeating sequence motif of approximately 33-amino acid residues in length that is highly homologous to domains of ankyrin that bind both tubulin and integral membrane proteins, including several proteins that regulate ionic fluxes across membranes. These findings complement previous pharmacologic observations that suggest that CaI-PLA\(_2\) may participate in regulating transmembrane ion flux in glucose-stimulated \(\beta\)-cells.

Glucose-induced insulin secretion from pancreatic \(\beta\)-cells requires that glucose be transported into the \(\beta\)-cell and metabolized (1). Signals derived from glucose metabolism result in inactivation of plasma membrane ATP-sensitive K\(^+\) channels (2), membrane depolarization, activation of voltage-operated Ca\(^{2+}\) channels, influx of Ca\(^{2+}\), and a rise in cytosolic [Ca\(^{2+}\)], which triggers insulin exocytosis (2). Stimulation of islets with glucose also induces hydrolysis of arachidonic acid from islet membrane phospholipids (3), and the resultant accumulation of nonesterified arachidonic acid (4) may facilitate Ca\(^{2+}\) entry into \(\beta\)-cells (5) and amplify depolarization-induced insulin secretion (6).

Hydrolysis of arachidonic acid from membrane phospholipids in glucose-stimulated islets appears to be mediated in part by a phospholipase A\(_2\) (PLA\(_2\)) enzyme that is catalytically active in the absence of Ca\(^{2+}\) and that is inactivated by a haloenol lactone suicide substrate (HELSS) (7–10). Treatment of islets with HELSS results in attenuation of the glucose-induced rise in \(\beta\)-cell cytosolic [Ca\(^{2+}\)] and in inhibition of insulin secretion (8–10). The structure of islet Ca\(^{2+}\)-independent phospholipase A\(_2\) (CaI-PLA\(_2\)) is not known, but a CaI-PLA\(_2\) enzyme has recently been cloned from CHO cells (11) and its sequence determined (GenBank accession number 115470). We report here the cloning, expression, and sequence analysis of a homologous enzyme from a rat pancreatic islet cDNA library (12).

EXPERIMENTAL PROCEDURES

Materials—Enhanced chemiluminescence detection reagents, [\(^{32}\)P]dCTP (3000 Ci/mmol), [\(^{35}\)S]dATP (>1000 Ci/mmol), and t-\(\alpha\)-1-palmitoyl-2-\([\(^{14}\)C]linoleoyl-phosphatidylcholine (50 mCi/mmol) were purchased from Amersham Corp. Male Harlan Sprague Dawley rats (180–220 g) were obtained from Sasco (O’Fallon, MO); collagenase was from Boehringer Mannheim; tissue culture media (CMRL-1066 and MEM); penicillin, streptomycin, Hank’s balanced salt solution, heat-inactivated fetal bovine serum, and L-glutamine were from Life Technologies, Inc.; Pentex bovine serum albumin (BSA, fatty acid-free, fraction V) was from Miles Laboratories (Elkhart, IN); rodent Chow 5001 was fromRalston Purina (St. Louis, MO); ampicillin and kanamycin were from Sigma; and \(\beta\)-glucose was from the National Bureau of Standards. Media included KRB (Kreb’s-Ringer bicarbonate buffer; 25 mM HEPES, pH 7.4, 115 mM NaCl, 24 mM NaHCO\(_3\), 5 mM KCl, 2.5 mM Ca\(_{\text{Cl}}\), 1 mM MgCl\(_2\)); nKRB (KRB supplemented with 3 mM L-glutamine); cCMRL-1066 (CMRL-1066 from Life Technologies, Inc. supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine and 1% (w/v) each of penicillin and streptomycin); and Hank’s balanced salt solution supplemented with 0.5% penicillin/streptomycin. The HELSS is \((\text{E})-6-(\text{bromomethylene})\text{tetrahydro-3-(1-naphthalenyl)-2H-pyran-2}\)-one and was purchased from Cayman Chemical (Ann Arbor, MI).  

Isolation of Pancreatic Islets, Preparation of \(\beta\)-Cells and Non-\(\beta\)-cells, and Culture of COS-7, CHO, and HIT-T15 Cells—Islets were isolated aseptically from male Harlan Sprague Dawley rats, as described (13), by collagenase digestion of excised, minced pancreas, density gradient isolation, and manual selection under microscopic visualization (10). Isolated islets were transferred to Falcon Petri dishes containing 2.5 ml of cCMRL-1066, placed under an atmosphere of 95% air, 5% CO\(_2\), and cultured overnight at 37 °C. Purified populations of islet \(\beta\)-cells and non-\(\beta\)-cells were prepared by fluorescence-activated cell sorting of dispersed cells obtained by treating islets with the enzyme dispase, as described previously (5, 7, 10, 14, 15). COS-7 and CHO cells were cultured in Dulbecco’s modified Eagle’s medium (MEM) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin sulfate. HIT-T15 cells were obtained from ATCC (Bethesda, MD) and were cultured in Ham’s F12K medium (Life Technologies, Inc.) containing 7 \(\mu\)g/ml L-glucose, 10% dialyzed horse serum, and 2.5% fetal bovine serum.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) US19898.  

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1 The abbreviations used are: K\(_{\text{ATP}}\), ATP-sensitive K+ channel; BSA, bovine serum albumin; Ca\(^{2+}\), calcium; CHO, Chinese hamster ovary; HELSS, haloenol lactone suicide substrate; KRB, Krebs-Ringer bicarbonate buffer; MEM, modified Eagle’s medium; PCR, polymerase chain reaction; PLA\(_{\text{A}}\), phospholipase A\(_{\text{2}}\); RT, reverse transcriptase; TBS-T, Tris-buffered saline-Tween; bp, base pair(s); AMP-PCP, adenosine 5’-(\(\beta\),\(\gamma\)-methylene)triphosphate; TCR, target to competitor ratio.
RNA isolation, Reverse Transcription, and Polymerase Chain Reaction—Total RNA was isolated from cells and tissues after solubilization in guanidinium thiocyanate by extraction (phenol/chloroform/isooamyl alcohol) and precipitation (isooamyl alcohol) (16). First strand cDNA was transcribed with avian myeloblastosis virus reverse transcriptase (RT) obtained from Boehringer Mannheim. The restriction analysis of the cloned cDNA was performed as described elsewhere (12) in the Lambda ZAP Express system (Stratagene). Clones that hybridized with the probe were isolated and further plaque-purified. The pBK-CMV plasmid containing the Cal-PLA2 cDNA sequence was then excised in vitro using restriction enzymes EcoRI and XhoI. The cloned cDNA in pBK-CMV was sequenced from the double strand by the method of Sanger et al. (17) using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.) with T3 and T7 primers and specific oligonucleotide primers.

Expression and Purification of Cal-PLA2—After removal of the 5'- untranslated region, the full-length rat isoform Cal-PLA2 cDNA was sub-cloned in-frame into the EcoRI and XhoI sites of PET-28c (Novagen) using standard techniques. The fidelity of the construct was verified by restriction analysis and sequencing. The pet28P-CaI-PLA2 construct then was transformed into the bacterial expression host Escherichia coli strain BL21 (DE3) (Novagen). This system yields a fusion protein with polyclastidine and T7 epitope tags sequences that hybridize to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expressed protein was affinity-purified by nickel-nitrilotriacetic acid agarose (Qiagen) and analyzed by 10% agarose gel electrophoresis and visualized with ethidium bromide (16).

cDNA Cloning and Sequencing—A pair of PCR primers (sense, 5'- TATGCGTGGTGTTGTACQMG-230) located at the C-terminus of CaI-PLA2 activity—was designed based on the cDNA sequence of the CHO cell CaI-PLA2 (11) and used in RT-PCR reactions with RNA from HIT T15 insulinoma cells. These reactions yielded a 820-bp product. A 32P-labeled form of this product was prepared by randomly primed labeling and used to screen an islet cDNA library prepared as insert served as negative controls. Expression was induced by incubation of E.coli Escherichia coli then was transformed into the bacterial expression host plate, and the cells were then cultured for an additional 2 days. At 72 h after transformation, cells were harvested after washing with ice-cold phosphate-buffered saline. For Cal-PLA2 enzyme activity assays, the cells were collected by centrifugation (Beckman table-top centrifuge, 3000 rpm, 2 min, room temperature). These cells were then resuspended and washed three times with ice-cold phosphate-buffered saline and once with homogenization buffer (250 mM sucrose, 40 mM Tris-HCl, pH 7.5). The cells were pelleted again, resuspended in homogenization buffer, and sonicated. The homogenates were centrifuged (170,000 × g, 60 min, 4 °C) to obtain a cytosolic supernatant. The protein content of the cytosolic fraction was measured by Bio-rad assay. Cal-PLA2 activity was measured in aliquots of cytosol (100 μl, approximately 25 μg of protein) added to assay buffer (200 mM Tris-HCl, pH 6.0, 10 mM EGTA, total assay volume 200 μl). As described in the appropriate figure legends, effects of preincubation (2 min, room temperature) with various concentrations of cyclooxygenase inhibitors on Cal-PLA2 activity were also determined, as were effects of varying the assay solution pH and effects of including nucleotide phosphates such as ATP in the assay solution. Reactions were initiated by injection of an ethanolic solution (5 μl) of 1α-1-palmitoyl-2-[14C]linoleoyl-phosphatidylethanolamine substrate (specific activity 50 mCi/mmol, final concentration 5 μM). Assay mixtures were then incubated (3 min, 37 °C, with shaking), and reactions were terminated by addition of butanol (100 μl) and vortexing (8 s). After centrifugation (2000 × g, 2 min), reaction products in 25 μl of the upper (butanol) layer were analyzed by silica Gel G thin layer chromatography in the solvent system petroleum ether/ethyl ether/acetic acid (80:20:1). The region of the TLC plate containing free fatty acid (Rf 0.58) was identified with iodine vapor and scraped into scintillation vials, and the 14C-labeled content was then determined by liquid scintillation spectrometry. The amount of 14C]Linoleate released was then converted to a PLα2, specific activity value (pmol/mg protein × min) as described elsewhere (7).

Dot Matrix Analysis of the Islet Cal-PLA2, Amino Acid Sequence and Comparison to Other Sequences—Dot matrix plots (21) were constructed with the UWGG programs COMPARE and DOTPLOT using a window size of 30 and stringency of 15. Segments from the horizontal axis were compared with segments from the vertical axis, and a dot was placed in the appropriate position whenever the total score of aligned sequences exceeded a value of 15. These plots were used to examine internal repeating motifs in the islet Cal-PLA2 sequence and to compare the sequences of ankyrin and of related proteins to the Cal-PLA2 sequence.
Cloning the Rat Islet Ca\textsuperscript{2+}-independent PLA\textsubscript{2} cDNA—An oligonucleotide probe was generated by RT-PCR using HIT-T15 insulinoma cell RNA as template and primers designed from the CHO cell Ca\textsuperscript{2+}-PLA\textsubscript{2} sequence. Both CHO cells and HIT-T15 cells are hamster-derived lines, and HIT-T15 cells express a Ca\textsuperscript{2+}-PLA\textsubscript{2}.

**Fig. 1.** Nucleotide sequence and deduced amino acid sequence of cloned rat pancreatic islet Ca\textsuperscript{2+}-PLA\textsubscript{2} cDNA. The putative methionine start codon (ATG) and the presumptive polyadenylation signal (AATAAA) present 14-bp upstream of the poly(A) tail are displayed in **bold type**. Regions of amino acid sequence homologous to ankyrin are **underlined**. Arrowheads (R1–R8) mark the beginning of each of the eight consecutive strings of repeating sequence motif.
activity similar to that in islets (22). A $^{32}$P-labeled form of the resultant 820-bp RT-PCR product was generated by randomly primed labelling and used to screen an islet cDNA library. Screening about 900,000 plaque-forming units resulted in the isolation of five positive clones that were isolated, plaque-purified, and excised in vivo. The size of the cDNA inserts from each of the five clones was then determined after release of the inserts by digestion with the restriction enzymes EcoRI and XhoI. Each clone contained an insert of about 3.3 kilobases.

Sequencing the inserts from both 5'- and 3'-ends revealed that each contained an identical 5'-sequence and an identical 3'-sequence ending in a poly(A) tail. A putative initiation codon (ATG) was observed 475 bp downstream of the 5'-end. Nucleotide sequencing of the entire 3288-bp insert from one clone revealed a single long open reading frame (2256 bp), which was predicted to encode a protein with 751 amino acid residues. The alignment of the repeats in the islet CaI-PLA2 enzyme has been cloned from CHO cells (11). One absolutely conserved region is 95% identity of amino acid sequence to the CaI-PLA2 recently cloned from CHO cells (11). A BLAST search of the islet CaI-PLA2 cDNA—

**Deduced Amino Acid Sequence of the Rat Islet CaI-PLA2**

The encoded protein has a calculated molecular mass of 83,591 daltons and a predicted isoelectric point of 6.6. The amino acid sequences of known Ca$^{2+}$-dependent PLA2 enzymes, including the 85-kDa cytosolic PLA2 or secretory PLA2 (23). The islet CaI-PLA2 exhibits 90% identity of nucleotide sequence and 95% identity of amino acid sequence to the CaI-PLA2 recently cloned from CHO cells (11). One absolutely conserved region is that between His421 and Glu551, which flanks and includes a Ca$^{2+}$-lipase consensus sequence (11, 24, 25). This sequence occurs in the islet CaI-PLA2 as G$^{462}$STG$^{466}$. The encoded protein has a calculated molecular mass of 83,591 daltons and a predicted isoelectric point of 6.6. The amino acid sequences of known Ca$^{2+}$-dependent PLA2 enzymes, including the 85-kDa cytosolic PLA2 or secretory PLA2 (23). The islet CaI-PLA2 exhibits 90% identity of nucleotide sequence and 95% identity of amino acid sequence to the CaI-PLA2 recently cloned from CHO cells (11). One absolutely conserved region is that between His421 and Glu551, which flanks and includes a Ca$^{2+}$-lipase consensus sequence (11, 24, 25). This sequence occurs in the islet CaI-PLA2 as G$^{462}$STG$^{466}$. The encoded protein has a calculated molecular mass of 83,591 daltons and a predicted isoelectric point of 6.6. The amino acid sequences of known Ca$^{2+}$-dependent PLA2 enzymes, including the 85-kDa cytosolic PLA2 or secretory PLA2 (23). The islet CaI-PLA2 exhibits 90% identity of nucleotide sequence and 95% identity of amino acid sequence to the CaI-PLA2 recently cloned from CHO cells (11).

**Expression of the Protein Encoded by the Cloned Islet CaI-PLA2 cDNA**—The open reading frame of the putative islet CaI-PLA2 cDNA was subcloned in-frame into the bacterial expression vector pET-28c (Novagen) to generate a fusion protein containing the islet CaI-PLA2 sequence and both polyhistidine and T7 epitope tag sequences. Upon isopropyl-1-thio-β-D-galactopyranoside induction of bacterial cultures transfected with vector containing the islet CaI-PLA2 insert, strong expression of the expected 87-kDa fusion protein occurred, and this material was not observed in control cultures (Fig. 4). The expressed protein was recognized by a monoclonal antibody raised against a synthetic peptide contained within the islet CaI-PLA2 sequence.

**Expression of CaI-PLA2 Activity in COS-7 and CHO Cells After Transient Transfection with the Cloned Islet CaI-PLA2 cDNA**—For eukaryotic expression, the islet CaI-PLA2 cDNA was inserted into the vector pcDNA3.1 (Invitrogen), and this construct (pcDNA3.1-CaI-PLA2) was used to transfect both COS-7 and CHO cells. At 72 h after transfection with this construct or with vector containing no insert, CaI-PLA2 activity was measured in cytosolic fractions prepared from these cells. No measurable CaI-PLA2 activity was observed in the cytosol of either untransfected COS-7 cells (not shown) or COS-7 cells transfected with vector containing no insert (Fig. 5A). In contrast, COS-7 cells transfected with the pcDNA3.1-CaI-PLA2 construct exhibited substantial amounts of cytosolic CaI-PLA2 activity (Fig. 5A). This activity was pretreated by pretreatment of cytosol with a suicide substrate (HELS) (Fig. 5A) that has previously been demonstrated to inhibit CaI-PLA2 activity in islet cytosol (7). Similar results were obtained with CHO cells. Both untransfected CHO cells (not shown) and CHO cells transfected with vector containing no insert exhibited measurable CaI-PLA2 activity (Fig. 5B). This was expected because a CaI-PLA2 enzyme has been cloned from CHO cells (11). Transient transfection of CHO cells with the pcDNA3.1-CaI-PLA2 construct, however, resulted in a 5.5-fold increase in cytosolic...
CaI-PLA2 activity, and this activity was inhibited by treatment of cytosol with HELSS (Fig. 5B).

Characterization of CaI-PLA2 Activity in COS-7 Cells Transiently Transfected with the Islet CaI-PLA2 cDNA Construct—The concentration dependence of inhibition by HELSS of CaI-PLA2 activity in COS-7 cells transfected with the islet CaI-PLA2 cDNA construct was found to be very similar to that of the CaI-PLA2 activity in native HIT insulinoma cell cytosol (Fig. 6). Like the CaI-PLA2 activity in islet and HIT cell cytosol (7, 22), the CaI-PLA2 activity in cytosol from transiently transfected COS-7 cells was optimal near neutrality and was stimulated by including 1 mM ATP in the assay solution (Fig. 7). The effects of ATP to stimulate cytosolic CaI-PLA2 activity in transiently transfected COS-7 cells was optimal near neutrality and was stimulated by including 1 mM ATP in the assay solution (Fig. 7). The effects of ATP to stimulate cytosolic CaI-PLA2 activity in transiently transfected COS-7 cells was optimal near neutrality and was stimulated by including 1 mM ATP in the assay solution (Fig. 7). The effects of ATP to stimulate cytosolic CaI-PLA2 activity in transiently transfected COS-7 cells was optimal near neutrality and was stimulated by including 1 mM ATP in the assay solution (Fig. 7).

Expression of CaI-PLA2 mRNA in Rat Islet Cells—To determine whether islet CaI-PLA2 mRNA is specifically expressed in β-cells within islets, a competitive RT-PCR (18, 19) method was developed. A competitor DNA was prepared that shares with CaI-PLA2 cDNA sequences recognized by the primers but that yields a smaller product than that derived from the CaI-PLA2 cDNA (Fig. 8A). The ratio of signals from the target and competitor was found to correspond to the input DNA over a wide range of concentrations (Fig. 8B). Single cell suspensions were then prepared from isolated islets and analyzed by fluorescence-activated cell sorting (5, 7, 10, 14, 15) to yield two populations of cells. One population contains over 90% β-cells, and the second contains 10–15% β-cells and about 70% α-cells (5, 7, 10, 14, 15). RNA was then prepared from the two populations of cells and used as template in RT-PCR reactions with CaI-PLA2-specific primers and the competitor DNA. At equivalent amounts of input RNA, a substantially higher target to competitor ratio was observed with RNA from the β-cell-enriched population than with that from the α-cell-enriched population.

Fig. 2. Repeating sequences in islet CaI-PLA2. A, dot matrix analysis. Dot matrix analyses of the islet CaI-PLA2 sequence was performed with the UWCG programs COMPARE and DOTPLOT with a window of 30 and stringency of 15 to search for regions with internal homology. Such regions are represented by the more densely populated regions of the plot, corresponding to residues 150–414. B, comparison of the eight aligned strings of repeating sequence motif in islet CaI-PLA2. Identical residues (black boxes) and conservative changes (open boxes) are indicated for the corresponding amino acid residues. The consensus sequence is identified in the lower portion of the figure.
This suggests that the RNA species amplified in RT-PCR reactions with the b-cell-enriched population did not arise from contaminating non-b-cells and that b-cells contain CaI-PLA2 mRNA. Expression of CaI-PLA2 mRNA was also examined in other tissues by competitive RT-PCR. At equivalent amounts of input RNA, the highest target to competitor ratio (TCR) among tissues examined was observed in brain (TCR 1.3). Target signal was also observed in lung (TCR 0.6), spleen (TCR 0.4), kidney (TCR 0.4), liver (TCR 0.2), heart (TCR 0.1), and skeletal muscle (TCR 0.1).

DISCUSSION

The studies described here indicate that pancreatic islet b-cells express an 84-kDa CaI-PLA2 enzyme that is highly homologous to an enzyme recently cloned from CHO cells (11). The occurrence of ankyrin-like repeats in the islet CaI-PLA2 sequence raises interesting possibilities about the function of this protein in b-cells. The domain of ankyrin that contains similar repeats is responsible for the binding of ankyrin to a number of integral membrane proteins that govern ionic fluxes across cellular membranes (29), including the Na\(^+/K\)\(^+\)-ATPase of renal basolateral membranes (31–33), a renal amiloride-sensitive Na\(^+\) channel (34), the red cell anion exchanger (35, 36), a cerebellar inositol-trisphosphate receptor (37), and a voltage-dependent Na\(^+\) channel in myelinated neurons (29).

Regulation of ionic fluxes at the b-cell plasma membrane is critical in the control of insulin secretion, and both inactivation of KATP channels and activation of voltage-operated Ca\(^{2+}\) channels are required for the induction of insulin secretion by glucose (2). One product of the action of PLA2 on islet phospholipids is nonesterified arachidonic acid. Arachidonic acid is the vastly predominant sn-2 fatty acid substituent in islet phospholipids and comprises 36% of the total fatty acyl mass in islet phospholipids (9, 10). Nonesterified arachidonic acid facilitates...
Ca²⁺ entry into cells through both voltage-operated (38) and receptor-operated (39) Ca²⁺ channels and induces a rise in β-cell cytosolic [Ca²⁺] that is dependent on Ca²⁺ entry from the extracellular space (5). Both exogenous PLA₂ and the products of its action suppress K ATP channel activity in excised and cell-attached patches of plasma membranes from HIT insulinoma cells (40).

Taken together, the observations summarized in the preceding two paragraphs raise the possibility that the ankyrin-like repeat domains of the islet Ca²⁺-independent PLA₂ might serve to attach the (last three lanes), which contains the islet Ca²⁺-independent PLA₂ cDNA as insert. All assays were performed in buffer containing 10 mM EGTA. In the 1st (VO) and 4th (VI) lanes, cytosol was not pretreated before Cal-PLA₂ activity was measured. In the 3rd (VO+E) and 6th (VI+H) lanes, cytosol was incubated with the Cal-PLA₂ inhibitor HELSS before Cal-PLA₂ activity was measured. In the 2nd (VO+H) and 5th (VI+E) lanes, cytosol was incubated with ethanolic vehicle that did not contain HELSS before Cal-PLA₂ activity was measured. The meanings of the designations on the x axis of the panel are as follows: VO, vector only; VO+E, vector only plus ethanol; VO+H, vector only plus HELSS; VI, vector with insert; VI+E, vector with insert plus ethanol; VI+H, vector with insert plus HELSS; B, Cal-PLA₂ activity expressed in transfected CHO cells. In B, the left bar (VO) represents Cal-PLA₂ enzymatic activity in cytosol prepared from CHO cells that had been transfected with pcDNA3.1-Ca²⁺-independent PLA₂ cDNA as insert. All assays were performed in buffer containing 10 mM EGTA. In the 1st (VO) and 4th (VI) lanes, cytosol was not pretreated before Cal-PLA₂ activity was measured. In the 3rd (VO+E) and 6th (VI+H) lanes, cytosol was incubated with the Cal-PLA₂ inhibitor HELSS before Cal-PLA₂ activity was measured.
enzyme to β-cell K_{ATP} channels or to voltage-operated Ca^{2+} channels or to anchor the enzyme in the membrane in close proximity to such channels. The α-subunits of voltage-operated Ca^{2+} channels exhibit striking similarities in sequence to voltage-operated Na^{+} channels (41, 42) that interact with ankyrin through its repeating domain (29). Ankyrin is distributed in close proximity to voltage-operated Ca^{2+} channels in the triad junctional structures of skeletal myocytes, although direct interaction of ankyrin with these channels has not been demonstrated (43).

The repeat sequences in ankyrin are also responsible for its binding to the cytoskeletal component tubulin (29). Although the precise role of the cytoskeletal apparatus in insulin secretion is not clearly established, a number of agents that interfere with microtubule assembly or disassembly inhibit insulin secretion (44–47), and morphological evidence suggests a direct association of microtubules with insulin secretory vesicles (48). Electron microscopic evidence demonstrates secretory granules associated with microtubules and directed toward the plasma membrane (48). An association of the islet PLα2 with microtubular structures mediated by its ankyrin-like repeat domain might facilitate interaction of the enzyme with secretory granule membranes and/or plasma membranes. It is of interest in this regard that treatment of parotid acinar secretory granules with PLα2 greatly increases their tendency to fuse with plasma membranes (49), which is the final event in exocytosis.

The sensitivity of the islet Ca^{2+-independent} PLα2 to inhibition by the HELSS suggests that this enzyme could be the target within β-cells that is responsible for the ability of HELSS to inhibit the glucose-induced rise in β-cell cytosolic [Ca^{2+}] and insulin secretion (7–10), although HELSS has also recently been demonstrated to inhibit an isozyme of phosphatidate phosphohydrolase (50). HELSS does not inhibit any known Ca^{2+}-dependent PLα2 group I through IV (51–54), but Ca^{2+}-independent PLα2 activities from several sources are sensitive to inhibition by HELSS. These include the 84-kDa CHO cell Ca^{2+-independent} PLα2 (11) and a PLα2 from the macrophage-like P388D1 cell line (51, 52, 55, 56), which has recently been demonstrated to represent the mouse homolog of the CHO cell Ca^{2+-independent} PLα2 (61). In addition, Ca^{2+-independent}, HELSS-sensitive PLα2 activities thought to reside in 40-kDa proteins have been described in myocardium (53), HIT cells (22), and renal proximal tubular cells (57).

It is not yet known whether these 40-kDa proteins are related to the 84-kDa Ca^{2+-independent} PLα2 enzymes, although generation of the former from the latter by proteolytic processing is a formal possibility. It is also possible that the 40-kDa proteins are separate gene products that share sequence homology with the 84-kDa Ca^{2+-independent} PLα2 enzymes, perhaps including the serine lipase consensus sequence that is likely to confer sensitivity to HELSS. The 40-kDa proteins from myocardium (58) and HIT cells (22, 59) are thought to be associated with 85-kDa protein
subunits that are homologous to homoprostaglandin D_{2} synthase and that confer sensitivity to activation by ATP and to modulation of this effect by ADP. Experiments with the CaI-PLA_{2} enzymes from CHO cells (62) and P388D1 cells (51) have failed to demonstrate association with homoprostaglandin D_{2} synthase-like proteins, although both enzymes behave as 300–350-kDa multimers on gel filtration chromatography, as do CaI-PLA_{2} activities from myocardium (58) and HIT cells (59). Whether the 84-kDa CaI-PLA_{2} enzymes may exist as hetero-oligomers consisting of distinct catalytic and regulatory proteins in some cells but not others is a question that will require further examination.

Although the 84-kDa islet CaI-PLA_{2} expressed in transiently transfected COS-7 cells is sensitive to activation by ATP under the assay conditions employed in this study, the nature of this effect differs in several respects from that of CaI-PLA_{2} activities in islet and HIT cell cytosol and is in general quite similar to effects observed with the P388D1 cell CaI-PLA_{2} (51). The possibility that the differences in nucleotide phosphate sensitivity between the islet CaI-PLA_{2} expressed in transfected COS-7 cells and that in native islet cytosol might reflect the influence of an interacting protein that is expressed in islets but that is not abundantly expressed in COS-7 cells is under study. It is of interest that the CHO cell CaI-PLA_{2} after expression in an SF9 cell host, has recently been demonstrated to interact avidly and selectively with both ATP affinity matrices and with calmodulin affinity matrices (60), suggesting that the enzyme may bind ATP and interact with other cytosolic proteins.

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An Islet Ca\(^{2+}\)-independent PLA\(_{2}\)

54. Zupan, L. A., Weiss, R. H., Hazen, S. L., Parnas, B. L., Aston, K. W., Lennon, P. J., Getman, D. P. & Gross, R. W. (1993) *J. Med. Chem.* **36**, 95–100

55. Ackermann, E. J., Conde-Frieboes, K. & Dennis, E. A. (1995) *J. Biol. Chem.* **270**, 445–450

56. Ackermann, E. J. & Dennis, E. A. (1995) *Biochim. Biophys. Acta* **1259**, 125–136

57. Portilla, D., Shah, S. V., Lehman, P. A. & Creer, M. H. (1994) *J. Clin. Invest.* **93**, 1609–1615

58. Hazen, S. L. & Gross, R. W. (1993) *J. Biol. Chem.* **268**, 9892–9900

59. Ramanadham, S., Wolf, M. J., Ma, Z., Li, B., Wang, J., Gross, R. W. & Turk, J. (1996) *Biochemistry* **35**, 5464–5471

60. Wolf, M. J. & Gross, R. W. (1996) *J. Biol. Chem.* **271**, 30879–30885

61. Balboa, M. A., Balsinde, J., Jones, S. S. & Dennis, E. A. (1997) *J. Biol. Chem.* **272**, in press

62. Tang, J., Kritz, R. W., Hoffman, N., Shaffer, M., Seehra, J. & Jones, S. S. (1997) *J. Biol. Chem.* **272**, in press