Evidence for Association of an ATP-Stimulatable Ca^{2+}-Independent Phospholipase A_2 from Pancreatic Islets and HIT Insulinoma Cells with a Phosphofructokinase-like Protein†

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ABSTRACT: Glucose-induced insulin secretion from pancreatic islets requires metabolism of glucose within islet β-cells, and ATP has attracted interest as a messenger of glucose metabolism within β-cells. Glucose-induced insulin secretion from islets and HIT insulinoma cells is accompanied by activation of an ATP-stimulatable Ca^{2+}-independent phospholipase A_2 (ASCI-PLA_2) enzyme, the catalytic activity of which resides in a 40 kDa protein. An analogous PLA_2 enzyme in myocardium was recently found to consist of a complex of a 40 kDa catalytic protein with a tetramer of an isoform of the glycolytic enzyme phosphofructokinase (PFK). Association of the PFK isoform with the myocardial PLA_2 catalytic protein was found to confer ATP sensitivity onto the enzyme complex. Here we demonstrate that the majority of HIT cell and islet ASCI-PLA_2 catalytic activity elutes from a gel filtration column in a region corresponding to 400 kDa, suggesting that the 40 kDa β-cell ASCI-PLA_2 catalytic protein exists as part of a larger molecular mass complex. Islet and HIT cell ASCI-PLA_2 activities were immunoprecipitated by antibodies directed against PFK, and the immunoprecipitates contained 40 and 85 kDa proteins which correspond to the molecular masses of the PLA_2 catalytic protein and of a PFK monomer, respectively. Islet and HIT cell ASCI-PLA_2 activities were selectively and reversibly adsorbed to affinity matrices containing immobilized PFK but not to similar matrices containing immobilized transferrin or bovine serum albumin. Addition of free PFK prevented binding of HIT cell ASCI-PLA_2 activity to immobilized PFK matrices and promoted desorption of activity previously bound to such matrices. These results suggest that β-cell ASCI-PLA_2, like the myocardial enzyme, exists as a complex comprised of a catalytic protein and a PFK-like protein and raise the possibility that the ASCI-PLA_2 complex may represent a component of the β-cell glucose sensor, which links glycolysis, phospholipid hydrolysis, and membrane electrochemical events involved in glucose-induced insulin secretion.

To induce insulin secretion, glucose must be transported into and metabolized within β-cells (Meglasson & Matschinksy, 1986). Subsequent glucose metabolism induces closure of β-cell plasma membrane K^+ channels (K_{ATP}) which govern the β-cell resting membrane potential and which are inactivated by ATP (Cook & Hales, 1984; Cook et al., 1988; Ashcroft et al., 1984; Rorsman & Trube, 1985; Misler et al., 1986). K_{ATP} channel closure induces a rise in membrane potential, opening of voltage-operated Ca^{2+} channels, influx of Ca^{2+} from the extracellular space (Arkhammar et al., 1987; Gylfe, 1988), and a rise in β-cell cytosolic Ca^{2+} concentration which is required for induction of insulin secretion (Prentki & Matschinsky, 1987; Wollheim & Scharp, 1981). Glucose metabolism also induces hydrolysis of arachidonate from β-cell membrane phospholipids (Turk et al., 1992; Konrad et al., 1993) and accumulation of nonesterified arachidonate (Wolf et al., 1986, 1991). Arachidonate potentiates both depolarization-induced Ca^{2+} entry into β-cells (Ramanadham et al., 1992) and depolarization-induced insulin secretion (Wolf et al., 1991), and compounds which prevent glucose-induced hydrolysis of arachidonate from β-cell phospholipids suppress both insulin secretion (Laychock, 1982; Dunlop & Larkins, 1984; Metz, 1987, 1988, 1991; Metz & Dunlop, 1990; Konrad et al., 1992a,b; Ramanadham et al., 1993a) and the rise in β-cell cytosolic Ca^{2+} concentration (Ramanadham et al., 1993a).

Islet β-cells and HIT insulinoma cells express a phospholipase A_2 (PLA_2)[1] enzyme with distinctive properties that appears to mediate glucose-induced hydrolysis of arachidonate from β-cell phospholipids (Gross et al., 1993; Ramanadham et al., 1993a−c, 1994). Activity of the enzyme is stimulated by ATP and does not require Ca^{2+} ions, and it

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[1] Abbreviations: ASCI, ATP-stimulated Ca^{2+}-independent; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting; HBSS, Hank's balanced salt solution; HELSS, (*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-PFK, liver phosphofructokinase; MEM, minimum essential medium; M-PFK, muscle phosphofructokinase; PLA_2, phospholipase A_2; TBS, Tris-buffered saline.

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is therefore designated as an ATP-stimulated Ca^{2+}-independent (ASCI)-PLA_2. A selective suicide substrate which inactivates ASCI-PLA_2 suppresses glucose-induced eicosanoid release and insulin secretion from islets and HIT cells and attenuates the glucose-induced rise in β-cell cytosolic Ca^{2+} concentration (Ramanadham et al., 1993a−c, 1994). These findings suggest that ASCI-PLA_2 may be a component of the β-cell glucose sensor apparatus.

Islet and HIT cell ASCI-PLA_2 activities exhibit chromatographic properties that are similar to each other (Ramanadham et al., 1994) and to those of a myocardial ASCI-PLA_2 enzyme, which was the first characterized PLA_2 of this class (Wolf & Gross, 1985; Hazen et al., 1990, 1991a,b; Hazen & Gross, 1991). All three activities are associated with a protein with an apparent molecular mass of 40 kDa (Hazen et al., 1990; Ramanadham et al., 1994). These findings suggest that ASCI-PLA_2 activities from HIT cells, islets, and myocardium are closely related enzymes. Purified myocardial ASCI-PLA_2 catalytic protein is insensitive to ATP (Hazen & Gross, 1991), and stimulation of the native enzyme complex by ATP requires association of the catalytic subunit with a tetrameric regulatory protein, the monomeric components of which exhibit molecular masses of 85 kDa (Hazen & Gross, 1993). The myocardial ASCI-PLA_2 regulatory protein has recently been identified as an isoform of the glycolytic enzyme phosphofructokinase (PFK), on the basis of amino acid sequence analysis, co-immunoprecipitation of ASCI-PLA_2 activity with anti-PFK antibodies, and other evidence (Hazen & Gross, 1993).

Signals derived from glucose metabolism and the effector systems with which they interact to induce insulin secretion are incompletely understood. In this regard, the possibility that β-cell ASCI-PLA_2 is also associated with a PFK isoform is of great interest because of the known involvement of glucose metabolism in signaling events which lead to insulin secretion and because PFK is a rate-limiting enzyme for flux through glycolysis in many cells. We have therefore explored the possible interaction of PFK with β-cell ASCI-PLA_2 by performing gel filtration analyses of ASCI-PLA_2 activity from islets and from HIT insulinoma cells, by examining the ability of anti-PFK antibodies to remove β-cell ASCI-PLA_2 activity from solution, and by examining interaction of β-cell ASCI-PLA_2 activity with PFK affinity matrices.

EXPERIMENTAL PROCEDURES

Materials. Male Sprague−Dawley rats (180−220 g body weight) were purchased from Sasco (O'Fallon, MO) and White Leghorn laying hens from Tyson Research Center (St. Louis, MO). Rodent Chow 5001 was purchased from Ralston Purina (St. Louis, MO). Clonal HIT-T15 cells were obtained from ATCC (NIH, Bethesda, MD) and maintained by the Tissue Culture Support Center at Washington University School of Medicine (St. Louis, MO). Other materials were obtained from the following sources: rainbow protein molecular mass standards and [^{125}I]protein A (Amersham, Arlington Heights, IL); microcon microconcentrators (Amicon, Beverly, MA); sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) supplies (BioRad, Richmond, CA); collagenase (Type P, Boehringer Mannheim, Indianapolis, IN); transferrin (Calbiochem, San Diego, CA); enlightening solution (Dupont-NEN, Boston, MA); tissue culture media (CMRL-1066, HBSS, and MEM), penicillin, streptomycin, heat-inactivated fetal bovine serum, and L-glutamine (Gibco, Grand Island, NY); pentex bovine serum albumin (fatty acid free, fraction V, Miles Laboratories, Eckert, IN); cyanogen bromide-activated Sepharose (4% agarose) 4B beads and Superose 6 column (Pharmacia LKB Biotechnology, Piscataway, NJ); dialysis cartridges (10 kDa cutoff, Pierce, Rockford, IL); and anti-chicken IgG, ATP−agarose (4%) matrix, rabbit skeletal muscle and liver phosphofructokinase, and molecular mass markers for gel filtration chromatography (Sigma, St. Louis, MO). Plasmenylcholine substrate containing ^{3}H-labeled oleate as the *sn*-2 substituent (16:0p/18:1-PC; specific activity of 1.1×10^3 dpm/pmol) was synthesized as previously described (Hazen et al., 1991b).

Media. Media were constituted as follows: KRB buffer [25 mM HEPES (pH 7.4), 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM D-glucose (pH 7.3)], assay buffer [200 mM Tris-HCl (pH 7.5)], homogenization buffer [250 mM sucrose, 40 mM Tris-HCl (pH 7.1)], TBS [1 mM Tris-base, 0.14 M NaCl (pH 7.4)], buffer A [400 mM KPO_4 buffer containing 25% glycerol, 1 mM dithiothreitol (DTT) (pH 7.0)], buffer B [1 M Tris-HCl (pH 6.8), 69 mM SDS, 10% glycerol, 0.01% bromophenyl blue, 1% 2-mercaptoethanol], and buffer C [10 mM imidazole, 25% glycerol, 1 mM DTT (pH 8.3)].

Preparation of Pancreatic Islets and HIT Insulinoma Cells and Subcellular Fractionation. Pancreatic islets were isolated from male Sprague−Dawley rats as described (Ramanadham et al., 1993b). Briefly, the pancreas was inflated with HBSS (supplemented with 0.5% penicillin−streptomycin), excised, freed from adherent tissues, and minced. The minced pancreatic tissue was digested with collagenase (7 mg/pancreas at 39 °C for 12.5 min), rinsed with HBSS, and centrifuged on a discontinuous Ficoll gradient (27, 23, 20.5, and 11%). Islets were collected from the 11 and 20.5% and the 20.5 and 23% interfaces, washed in complete CMRL-1066 (supplemented with 1% penicillin−streptomycin, 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 5.55 mM D-glucose), and cultured overnight in petri dishes containing 2.5 mL of complete CMRL-1066 medium at 24 °C under an atmosphere of 5% CO_2/95% air. HIT insulinoma cells ($7-8 \times 10^8$) were detached from T-175 flasks with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA), washed with MEM, gently pelleted by centrifugation, and sonicated in homogenization buffer, as described (Gross et al., 1993). Islet and HIT cell cytosolic fractions were prepared after homogenization by ultracentrifugation, as described (Gross et al., 1993; Ramanadham et al., 1994).

Ca^{2+}-Independent Phospholipase A_2 Activity Measurements. Aliquots of cytosol obtained before and after immunoprecipitation or affinity column analyses were incubated in assay buffer containing 10 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) (total assay volume of 400 μL). Reactions were initiated by injection of plasmenylcholine (16:0p/18:1-PC) substrate containing [^{3}H]oleate as the *sn*-2 substituent dissolved in ethanol (2.5 μM in 5 μL). Assay mixtures were incubated at 37 °C for 3 min, and the assay was terminated by addition of *n*-butanol (100 μL). Assay mixtures were centrifuged (200*g*, 4 min), and the reaction products in 25 μL of the butanol layer were separated by silicic acid thin layer chromatography (mobile phase, acetic acid/ethyl ether/petroleum ether, 1/20/80, v/v).

Hydrolyzed [^{3}H]oleate was identified with iodine vapor, scraped from the TLC plate, and quantified by liquid scintillation spectrometry, as described (Ramanadham et al., 1994). Protein concentrations were determined by Bio-Rad assay.

Gel Filtration Analysis of the ASCI-PLA$_2$ Enzyme Complex. Gel filtration analysis was performed on a SMART (Pharmacia) system using a Superose 6 column (0.32 × 30 cm), with an optimal separation range of 5×10^3 to 5×10^6 Da. Aliquots (50−100 μL) of ASCI-PLA$_2$ preparations were loaded onto the column, and ASCI-PLA$_2$ activity was eluted with buffer A at a flow rate of 30 μL/min. Two minute fractions were collected, and enzymatic activity in each fraction was assayed. A standard curve of elution volume/void volume (V_e/V_o) versus molecular mass (kilodaltons) was constructed using thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa). Blue dextran (2×10^3 kDa) was used to determine the V_o of the column. The molecular mass corresponding to the V_e of fractions containing peak enzymatic activity was then interpolated from the standard curve.

Preparation of Anti-PFK IgG Antibodies. Three isomeric forms of PFK are recognized and are designated on the basis of their abundance in tissues (L, liver; M, muscle; and C, central nervous system) (Dunaway & Kasten, 1985; Dunaway et al., 1988). Both L-PFK and M-PFK are commercially available, but C-PFK is not. Antibodies directed against L-PFK and M-PFK were generated, as described (Hazen & Gross, 1993). Rabbit skeletal muscle and liver PFK preparations (from Sigma) were dialyzed (overnight at 4 °C) against 10 mM ammonium bicarbonate solution, lyophilized, and purified by SDS−PAGE (10%). The region of the gel containing the PFK isoform was excised, and PFK was recovered from the gel using an elution apparatus (Hoefer Scientific), concentrated, and injected (im) into hens with Freund's complete adjuvant, as described (Hazen & Gross, 1993). The hens were boosted by injection with the PFK antigens prepared in Freund's incomplete adjuvant every 3 weeks, and preimmune and postimmune blood samples were collected. The serum was used to assess antibody response by Western analysis, as described below. At various times following administration of PFK antigen, eggs were collected and crude preparations of IgG were isolated from egg yolk, as described (Hazen & Gross, 1993). Chicken antibodies against L-PFK or against M-PFK were then obtained by affinity purification using immobilized L-PFK or M-PFK coupled to agarose matrices, as described (Hazen & Gross, 1993). Preparations of IgG isolated from eggs collected before administration of antigens served as the preimmune antibody.

Characterization of Affinity-Purified Anti-PFK Antibodies by SDS−PAGE and Western Analysis. Commercially available L-PFK and M-PFK preparations and cytosol prepared from rabbit liver or myocardium were diluted in buffer B and analyzed by SDS−PAGE (10%). Resolved proteins were then transferred from gels onto immobilon-P poly(vinylene difluoride) (PVDF) membranes using a Hoefer blotting apparatus. The electroblots were blocked with TBS buffer containing 5% powdered milk protein and sequentially incubated with affinity-purified chicken antibodies directed against L-PFK or against M-PFK, then with rabbit anti-chicken IgG antibody, and finally with [^{125}I]protein A, as described (Hazen & Gross, 1993). The ^{125}I-labeled proteins were then visualized by autoradiography.

Coupling of Anti-PFK IgG Antibodies to Agarose Matrices and Immunoprecipitation of Islet and HIT Insulinoma Cell ASCI-PLA$_2$ Activity. Preimmune antibody and affinity-purified anti-PFK antibodies were separately coupled to cyanogen bromide (CNBr)-activated agarose beads (5−10 mg of protein/mL of agarose matrix), according to instructions from the manufacturer. Preimmune antibody−agarose matrix and anti-PFK−agarose matrices were incubated with aliquots of islet or HIT cell cytosols for 30 min at 4 °C, with gentle shaking at 5 min intervals. Antibody−agarose matrices were then pelleted by centrifugation, and residual ASCI-PLA$_2$ activity in supernatants was determined.

Visualization of HIT Cell Proteins Immunoprecipitated by Anti-PFK−Agarose Matrices. To facilitate visualization of proteins immunoprecipitated from cytosol by anti-PFK−agarose matrices, HIT cells in T-175 flasks were metabolically labeled with [^{35}S]methionine. Cells were first incubated in culture medium containing only 10 μM methionine (4 h, 37 °C, under 5% CO_2/95% air). Cells were then incubated overnight (37 °C under 5% CO_2/95% air) in medium supplemented with 500 μCi [^{35}S]methionine. Cells were then washed five times with PBS to remove free [^{35}S]methionine, detached with trypsin/EDTA solution, and washed five times before preparation of cytosol, as described above. Preimmune antibody−agarose matrix and anti-PFK−agarose matrices were then incubated with aliquots of cytosol (twice the volume of agarose matrix), as described above. At the end of the incubation period, agarose matrices were pelleted by centrifugation and residual ASCI-PLA$_2$ activity in supernatants was measured. Agarose matrices were then washed extensively with buffer C, diluted in buffer B, and boiled. Beads were then pelleted by centrifugation, and an aliquot of supernatants was analyzed by SDS−PAGE (10%). Gels were then acid-fixed, treated with rapid autoradiography-enhancing solution, rehydrated, and dried. ^{35}S-labeled proteins were then visualized by autoradiography.

Coupling of PFK and Other Proteins to Agarose Beads. Commercially available L-PFK, BSA, and transferrin proteins were dialysed overnight (4 °C) against 10 mM ammonium bicarbonate, lyophilized, and coupled to CNBr-activated agarose beads, according to the instructions from the manufacturer. M-PFK coupled to agarose beads (from Sigma) was preconditioned with sequential washes of TBS, 4.9 M $MgCl_2$, TBS, 100 mM glycine (pH 2.5), TBS, 1 M NaCl, 4.9 M $MgCl_2$, and TBS.

Adsorption of Islet and HIT Insulinoma Cell ASCI-PLA$_2$ Activity to Protein−Agarose Matrices. Islet and HIT cell cytosols were dialyzed overnight (4 °C) against buffer C. L-PFK−agarose or M-PFK−agarose matrices were then incubated overnight with aliquots of cytosol (4 °C with gentle 180° rotation). The specificity of association between ASCI-PLA$_2$ and PFK matrices was assessed using similarly prepared CNBr-activated agarose beads coupled to glycine or coupled either to transferrin or to BSA. At the end of the incubation period, residual activity in supernatants was measured and compared to activity in the original cytosol preparation. In two other sets of experiments, the specificity of interaction of HIT cell ASCI-PLA$_2$ activity with immobilized PFK matrices was assessed. In the first, cytosol was incubated (4 °C) with M-PFK−agarose matrix either in the absence or in the presence of free M-PFK (ca. 5 mg).

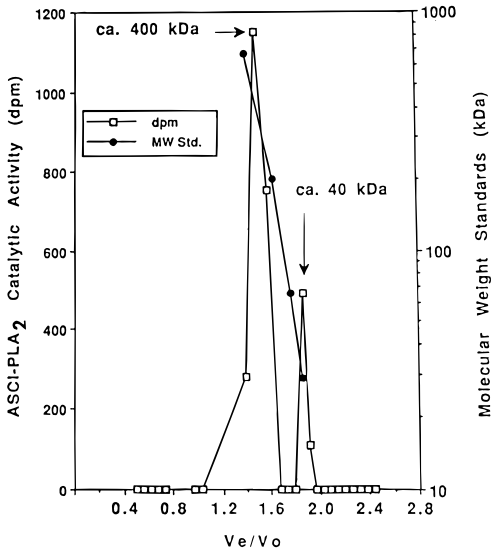

FIGURE 1: Gel filtration analysis of pancreatic islet ASCI-PLA_2 activity. Islet cytosol was prepared and applied to a Pharmacia Superose 6 PC 3.2/30 Smart gel filtration column as described in Experimental Procedures. The open squares represent ASCI-PLA_2 activity in eluant fractions and the closed circles the elution profile of protein standards. (V_e is the elution volume, and V_o is the void volume of Blue Dextran.) After derivation of a linear regression equation based on the V_e/V_o of the molecular mass standards, the V_e/V_o of fractions containing peak levels of ASCI-PLA_2 activity were substituted into the equation to obtain approximate molecular masses corresponding to the peaks of activity (indicated by arrows).

After overnight incubation, agarose matrices were pelleted by centrifugation, and effects of exogenous M-PFK on binding of HIT cell ASCI-PLA_2 activity to the M-PFK−agarose matrix were determined by comparison of residual activity in the two supernatants. In the second, HIT cell cytosol was first incubated (4 °C, overnight) with M-PFK−agarose matrix. The matrix was then pelleted by centrifugation, washed with buffer C, and incubated (4 °C, overnight) with buffer C alone or with buffer C to which free M-PFK, transferrin, or BSA had been added. Protein−agarose matrices were then pelleted by centrifugation, and the ability of the three free proteins to promote desorption of ASCI-PLA_2 activity was determined by measurement of the enzymatic activity in supernatants.

Statistical Analyses. The data were converted to mean and standard error of the mean (SEM) and analyzed by Student's t-test to determine significant differences (at $p < 0.05$).

RESULTS

Gel Filtration Analysis of Islet ASCI-PLA_2 Activity. Analysis of islet cytosolic ASCI-PLA_2 activity by gel filtration chromatography resulted in recovery of activity in two regions (Figure 1). The peak activities in the two regions corresponded to molecular masses of about 40 and 400 kDa. Similar analyses of HIT cell cytosolic ASCI-PLA_2 also resulted in two distinct peaks of activity corresponding to molecular masses of about 40 and 400 kDa (not shown). Purified HIT cell ASCI-PLA_2 catalytic protein exhibits a molecular mass of ca. 40 kDa (Ramanadham et al., 1994), and the two peaks of β-cell ASCI-PLA_2 activity observed upon gel filtration may reflect free catalytic subunit (ca. 40 kDa) and catalytic subunit in association with a larger complex (ca. 400 kDa), as previously observed with myocardial ASCI-PLA_2 (Hazen & Gross, 1994).

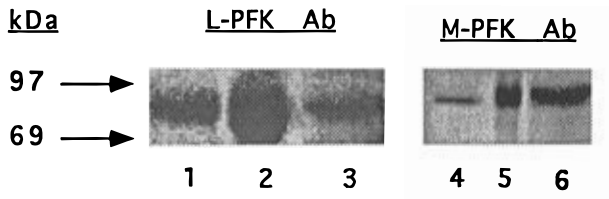

FIGURE 2: Examination of the ability of affinity-purified anti-PFK antibodies to recognize PFK isoforms by Western blotting analysis. Standard liver (lane 1) and skeletal muscle (lane 3) PFK preparations and cytosols from rabbit liver (lanes 2 and 4) and muscle (lanes 3 and 5) were analyzed by SDS−PAGE (10%) and then transferred to nitrocellulose by electroblotting. Electroblots of transferred proteins were probed with affinity-purified anti-PFK antibodies generated against purified rabbit liver (lanes 1−3) or muscle (lanes 4−6) PFK isoforms, as described in Experimental Procedures. The left panel shows affinity-purified anti-L-PFK antibody and the right panel affinity-purified anti-M-PFK antibody.

Immunoprecipitation of Islet and HIT Insulinoma Cell ASCI-PLA_2 Activity by Affinity-Purified Anti-PFK Antibodies Coupled to Agarose. To examine the possibility that the larger complex of β-cell ASCI-PLA_2 activity might reflect association of the catalytic subunit with phosphofructokinase (PFK)-like proteins, antibodies to PFK were generated in an attempt to effect immunoprecipitation of β-cell ASCI-PLA_2 activity. First, Western blotting analyses were performed to verify that the anti-PFK antibodies recognized PFK standards and PFK in cytosolic samples. Such analyses revealed that the affinity-purified anti-L-PFK antibody (Figure 2, left panel) and anti-M-PFK antibody (Figure 2, right panel) recognized both L-PFK and M-PFK standards and PFK in cytosols prepared from rabbit liver and heart muscle. The cross-reactivity between anti-L-PFK and anti-M-PFK antibodies is not unexpected since the amino acid sequences of the L-PFK and M-PFK isoforms exhibit 68% identity and 82% homology and therefore probably share many antigenic determinants (Lee et al., 1987). Subsequent incubations of islet (Figure 3A) and HIT cell (Figure 3B) cytosol with the anti-PFK−agarose matrices resulted in removal of nearly 80% of β-cell ASCI-PLA_2 activities from solution, relative to residual activity in supernatants from cytosol that had been incubated with preimmune antibody−agarose matrix.

Visualization of HIT Cell Cytosolic Proteins Immunoprecipitated with Anti-PFK−Agarose. Aliquots of cytosol prepared from HIT cells that had been metabolically labeled with [^{35}S]methionine were incubated either with preimmune antibody−agarose matrix or with anti-PFK−agarose matrix, under conditions described in the legend to Figure 3. ASCI-PLA_2 activity in supernatants from cytosol that had been incubated with anti-PFK−agarose was found to be reduced by nearly 80% relative to the activity remaining in supernatants from cytosol that had been incubated with preimmune antibody−agarose matrix. The ^{35}S-labeled proteins that had become associated with the antibody−agarose matrices during the incubations were then examined by SDS−PAGE and autoradiography. Two prominent protein bands were recovered from the anti-PFK−agarose matrix (Figure 4, lane 2) that were not observed with the preimmune antibody−agarose matrix (Figure 4, lane 1). These proteins exhibited apparent molecular masses of 85 and 40 kDa and may correspond to PFK monomer and ASCI-PLA_2 catalytic subunit, respectively.

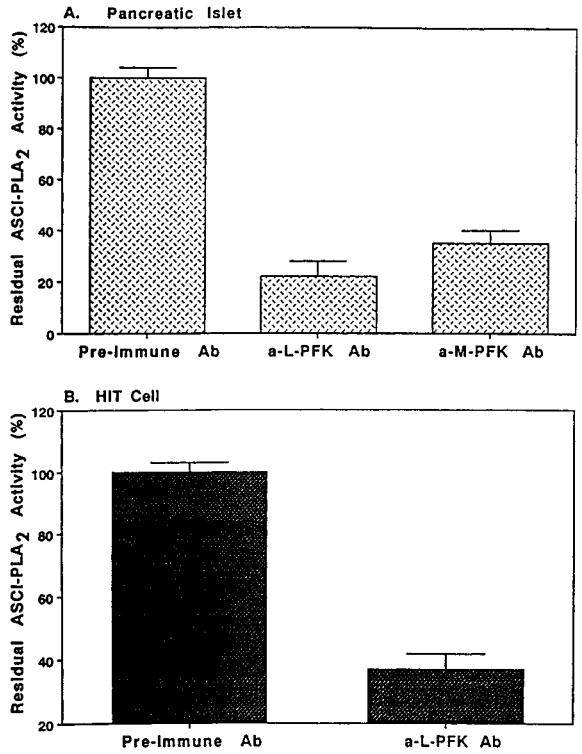

FIGURE 3: Immunoprecipitation of pancreatic islet and HIT cell ASCI-PLA_2 activity with affinity-purified anti-PFK−agarose matrices. Cytosols prepared from islets (A) or HIT cells (B) were incubated with agarose−antibody matrices that had been prepared by coupling to agarose either preimmune antibody or affinity-purified antibodies raised against L-PFK or M-PFK, as described in Experimental Procedures. After the incubations, the agarose matrices were removed by centrifugation, and residual ASCI-PLA_2 activity remaining in supernatants was measured. Activities are expressed as percent (mean ± SEM) of the mean value ($n = 3-7$) observed in supernatants from incubations performed with the matrix prepared from preimmune IgG.

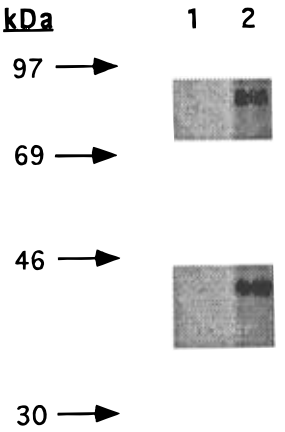

FIGURE 4: SDS−PAGE analysis and autoradiography of HIT cell cytosolic proteins immunoprecipitated by affinity-purified anti-L-PFK−agarose. Cytosol was prepared from HIT cells that had been labeled with [^{35}S]methionine. The cytosol was then incubated either with preimmune antibody−agarose matrix or with anti-L-PFK−agarose matrix, as in Figure 3. At the end of that incubation, agarose matrices were collected by centrifugation, diluted with buffer B, boiled, and again collected by centrifugation. Aliquots of the resultant supernatants were then analyzed by SDS−PAGE (10%), and [^{35}S]methionine-labeled proteins were visualized by autoradiography, as in Experimental Procedures. Lane 1 shows preimmune antibody−agarose matrix and lane 2 anti-L-PFK−agarose matrix.

Interaction of Islet and HIT Insulinoma Cell ASCI-PLA_2 Activity with Various Protein−Agarose Matrices. In light of the immunoprecipitation data suggesting that the β-cell ASCI-PLA_2 enzyme complex is recognized by antibodies generated against PFK, other attempts were made to confirm the association of the ASCI-PLA_2 catalytic protein with PFK. Affinity matrices were prepared by coupling M-PFK, L-PFK, glycine, or control proteins to agarose. Incubation of islet (Figure 5A) or HIT cell (Figure 5B) cytosols with agarose beads coupled to glycine did not result in removal of ASCI-PLA_2 activity from solution, but incubation with the PFK−agarose matrices resulted in significant loss of ASCI-PLA_2 activity from both islet and HIT cell cytosol. The specificity of this interaction was addressed using transferrin−agarose and BSA−agarose matrices. Transferrin (80 kDa) has a molecular mass similar to that of PFK, and BSA interacts with a number of small molecular mass substances. Neither transferrin−agarose nor BSA−agarose was effective in removing ASCI-PLA_2 activity from either islet or HIT cell cytosol.

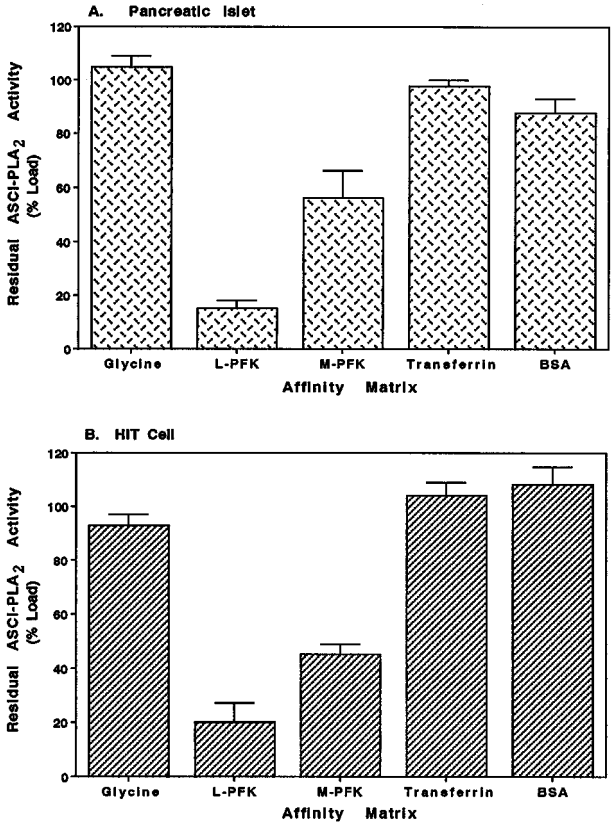

FIGURE 5: Adsorption of pancreatic islet and HIT cell cytosolic ASCI-PLA_2 activity to agarose matrices coupled to PFK or to other proteins. Cytosols prepared from islet (A) or HIT cells (B) were incubated with agarose matrices coupled either to glycine (first column) or to commercially available L-PFK, M-PFK, transferrin, or bovine serum albumin (BSA) standards, as described in Experimental Procedures. After overnight incubation, the agarose matrices were collected by centrifugation and residual ASCI-PLA_2 activity in supernatants was assayed. Data are expressed as a percentage (mean ± SEM) of initial activity ($n = 3-31$).

Addition of free M-PFK to cytosol almost completely prevented the binding of HIT cell ASCI-PLA_2 activity to the M-PFK−agarose matrix (Figure 6A). ASCI-PLA_2 activity that had been previously bound to M-PFK−agarose matrix was also selectively desorbed by the subsequent addition of free M-PFK (Figure 6B). Buffer alone, free transferrin, and free BSA were all ineffective in promoting desorption of ASCI-PLA_2 activity that had bound to the M-PFK affinity matrix. Bound ASCI-PLA_2 activity was also

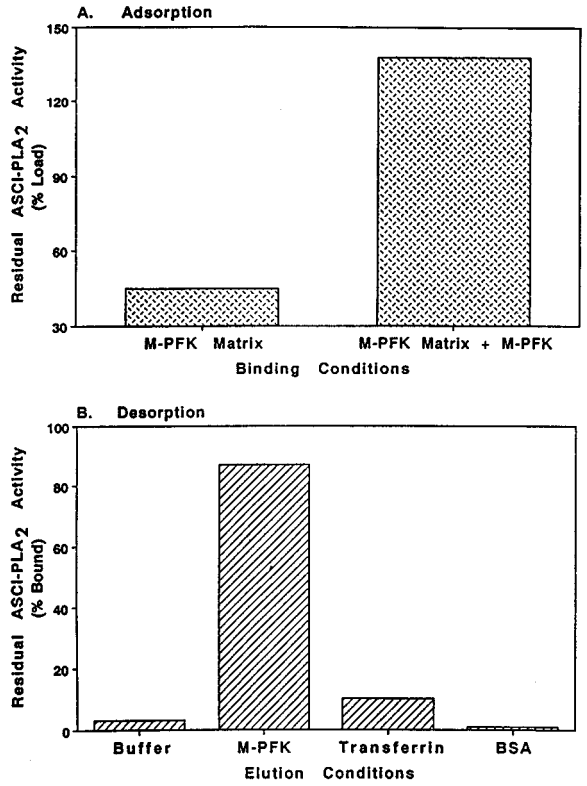

FIGURE 6: Effects of exogenous M-PFK in solution on adsorption of HIT cell cytosolic ASCI-PLA_2 activity to M-PFK−agarose matrix and on desorption of previously bound activity from the matrix. (A) Cytosol prepared from HIT cells was incubated with M-PFK−agarose matrix either in the absence or in the presence of exogenous free M-PFK in solution. Following an overnight incubation, the agarose matrix was collected by centrifugation and residual ASCI-PLA_2 activity remaining in the supernatant was measured. Data are expressed as a percentage of initial activity. (B) HIT cell cytosol was incubated with M-PFK−agarose matrix overnight in the absence of exogenous free PFK, and the agarose matrix was then collected by centrifugation. Following removal of the supernatant and washing of the M-PFK−agarose matrix with buffer C, the matrix was again incubated overnight with buffer alone or with buffer to which was added free M-PFK, transferrin, or BSA. After this second incubation, the agarose matrix was collected by centrifugation, and ASCI-PLA_2 activity in the supernatant was measured. ASCI-PLA_2 activity recovered in the supernatant is expressed as a percentage of bound activity.

desorbed by a high salt concentration (0.5 M NaCl) (not shown). The ability to desorb ASCI-PLA_2 activity from the PFK affinity matrix indicates that the loss of ASCI-PLA_2 activity from cytosol induced by incubation with the matrix reflects reversible association of the PLA_2 catalytic moiety with the matrix rather than matrix-induced inactivation of the enzyme.

DISCUSSION

Our observations suggest that the catalytic moiety of the β-cell ASCI-PLA_2 complex is associated with phosphofructokinase (PFK) or a related protein (Hazen & Gross, 1993). Evidence supporting this association includes the facts that islet and HIT insulinoma cell cytosolic ASCI-PLA_2 activities are immunoprecipitated by antibodies raised against PFK isoforms and are selectively and reversibly adsorbed to PFK affinity matrices. In addition, gel filtration analyses indicate that the majority of islet and HIT cell ASCI-PLA_2 activities migrate with an apparent molecular mass (400 kDa) exceeding that of the isolated β-cell ASCI-PLA_2 catalytic subunit (40 kDa) and roughly correspondent to that of a complex of a PFK tetramer with the catalytic subunit. The limited amounts of material obtainable from islets or HIT cells have precluded amino acid sequence analysis of components of the β-cell ASCI-PLA_2 complex, but amino acid sequences of two tryptic peptides from the regulatory subunit of myocardial ASCI-PLA_2 reveal high homology with skeletal muscle PFK (Hazen & Gross, 1993).

Properties reported here and previously for ASCI-PLA_2 activities from islets and HIT cells suggest that they reside in an enzyme closely related or identical to myocardial ASCI-PLA_2 but distinct from other characterized PLA_2 enzymes. Groups I, II, and III of PLA_2 have molecular masses of about 14 kDa, require a millimolar Ca^{2+} concentration for activity, exhibit little preference for specific *sn*-2 fatty acyl substituents in substrates, and function as extracellular, secreted enzymes (Dennis, 1994). Group IV PLA_2 has a molecular mass of 85 kDa, requires a micromolar Ca^{2+} concentration to associate with substrates in membranes, prefers substrates with *sn*-2 arachidonate residues, and functions as an intracellular enzyme involved in signaling (Dennis, 1994). The activity of a novel enzyme (iPLA_2) recently purified from P388D1 macrophage-like cells is independent of Ca^{2+} and stimulated by ATP (Ackerman et al., 1994, 1995). The activity of iPLA_2 is stimulated equally well by ATP, GTP, and ADP, however, while β-cell ASCI-PLA_2 is preferentially stimulated by ATP or a nonhydrolyzable ATP analog and is not stimulated by GTP or ADP (Ramanadham et al., 1994). Myocardial ASCI-PLA_2 exhibits a preference for activation by nucleotides similar to that of β-cell ASCI-PLA_2 (Hazen et al., 1991b). Macrophage iPLA_2 is also activated by Triton X-100 (Ackerman et al., 1994), but ASCI-PLA_2 activities from β-cells (S. Ramanadham, unpublished observations) and myocardium (Hazen et al., 1990) are inactivated by this detergent. The molecular mass of iPLA_2 is 80 kDa, and iPLA_2 does not adsorb to a PFK affinity matrix (Ackerman et al., 1994, 1995). These findings suggest that iPLA_2 is distinct from the ASCI-PLA_2 of β-cells and myocardium, but iPLA_2, like ASCI-PLA_2, is inactivated by a bromoenol lactone suicide substrate (Ackerman et al., 1995) that does not inhibit types I−IV of PLA_2 (Hazen et al., 1991b; Zupan et al., 1993). This and other features of similarity between iPLA_2 and ASCI-PLA_2 suggest that they may be members of a related family of Ca^{2+}-independent PLA_2 enzymes.

Phosphofructokinase catalyzes the first committed step in glycolysis, the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, and it regulates glycolytic flux in many cells (Uyeda, 1979; Poorman et al., 1984). ATP is both a substrate and an allosteric modulator of the enzyme, and binding of ATP may induce conformational changes in the enzyme that could be transmitted to interacting proteins. Signals derived from the metabolism of glucose within β-cells are required for glucose-induced insulin secretion, and compounds, such as mannoheptulose, which prevent glycolytic metabolism of glucose also prevent glucose-induced insulin secretion (Meglasson & Matschinsky, 1986). ATP has attracted interest as a second messenger of glucose metabolism in β-cells and is among the modulators of β-cell ATP-sensitive K^+ channels (K_{ATP}), the closure of which is a critical event in induction of insulin secretion (Cook & Hales, 1984; Cook et al., 1988; Ashcroft et al., 1984; Rorsman & Trube, 1985; Misler et al., 1986; Arkhammar et al., 1987; Gylfe, 1988; Prentki & Matschinsky, 1987;

Wollheim & Scharp, 1981). It is possible that ASCI-PLA_2 is a second target for signals derived from glucose metabolism in β-cells and that such signals are communicated to the PLA_2 catalytic protein via interaction with an associated PFK-like subunit. Such an interaction could explain the findings that glucose induces hydrolysis of arachidonate from β-cell phospholipids and accumulation of nonesterified arachidonate (Wolf et al., 1986, 1991) by a process which requires glucose metabolism (Turk et al., 1992; Konrad et al., 1993) and that both glucose-induced phospholipid hydrolysis and insulin secretion are attenuated by a suicide substrate which inactivates β-cell ASCI-PLA_2 (Gross et al., 1993; Ramanadham et al., 1993a−c, 1994).

Several observations suggest that a rise in the β-cell [ATP]/[ADP] ratio rather than the absolute level of [ATP] may be the critical metabolic signal in glucose-stimulated islets that governs K_{ATP} channel activity (Ghosh et al., 1991). These observations include the facts that islet ATP concentration rises only modestly upon stimulation with glucose (Ghosh et al., 1991), that ADP concentration falls by a substantially greater amount (Meglasson et al., 1989; Ohta et al., 1990, 1991, 1992, 1993), and that ADP attenuates ATP-induced inactivation of β-cell K_{ATP} channels (Misler et al., 1986; Kakei et al., 1986). It is of interest in this regard that ADP and ATP also exert opposing effects on β-cell ASCI-PLA_2 activity (Ramanadham, 1994). ADP attenuates both ATP-induced activation of β-cell ASCI-PLA_2 and ATP-conferred protection of the enzyme against thermal inactivation (Ramanadham et al., 1994). Association of a regulatory PFK isoform with β-cell ASCI-PLA_2 could account for these opposing effects of ADP and ATP on the enzyme. The activity of PFK is allosterically modulated by ADP in a direction opposite to that of ATP, and ADP and ATP bind at different allosteric sites in the PFK tetramer (Poorman et al., 1984).

The products of ASCI-PLA_2-catalyzed phospholipid hydrolysis are nonesterified arachidonate and lysophospholipids. Nonesterified arachidonate amplifies depolarization-induced insulin secretion (Wolf et al., 1991) and depolarization-induced Ca^{2+} entry into β-cells (Ramanadham et al., 1992), and an ASCI-PLA_2 suicide substrate attenuates the glucose-induced rise in β-cell cytosolic Ca^{2+} concentration (Ramanadham et al., 1993a). These effects could reflect amplification of β-cell Ca^{2+} channel activity by arachidonate in a manner similar to that reported for voltage-operated Ca^{2+} channels in a pituitary cell line (Vacher et al., 1989) and for receptor-operated Ca^{2+} channels in cerebellar cells (Miller et al., 1992). Alternative targets for products generated by ASCI-PLA_2 are K^+ channels. Intracellular injection of nonesterified arachidonate or of purified myocardial ASCI-PLA_2 into Sf9 cells which express human recombinant delayed rectifier K^+ channel Kv1.1 accelerates channel activation and inactivation kinetics (Gubitosi-Klug et al., 1995). Exogenous PLA_2 also suppresses HIT insulinoma cell K_{ATP} channel activity in excised inside-out plasma membrane patches, and this effect is mimicked by arachidonate and lysophospholipids (Eddlestone, 1995). Lysophospholipids also reduce HIT cell K_{ATP} channel activity in cell-attached patches. These findings suggest that ASCI-PLA_2-catalyzed phospholipid hydrolysis products might participate in regulation of β-cell K_{ATP} channels.

Whether the material associated with ASCI-PLA_2 catalytic protein and recognized by anti-PFK antibodies represents glycolytically active PFK or a related but distinct protein of similar size which shares antigenic determinants with PFK is uncertain. Mammalian PFK is a tetramer composed of monomers with molecular masses of about 85 kDa (Uyeda, 1979; Poorman et al., 1984). Three distinct isoforms of the monomeric subunits exist and are differentially distributed among tissues (Lee et al., 1987; Gehnrich et al., 1988; Gekakis et al., 1994; Li et al., 1994). These isoforms exhibit 65−69% amino acid identity (Gekakis et al., 1994) and probably share antigenic determinants. Human islets express mRNA species encoding all three known PFK isoforms (Eto et al., 1994), and our preliminary data indicate that this is also the case in rat islets (Ma et al., submitted). The PFK isoform associated with myocardial ASCI-PLA_2 does not express PFK catalytic activity, at least when complexed to the PLA_2 catalytic subunit (Hazen & Gross, 1993). The possibility cannot be excluded that the ASCI-PLA_2-associated PFK isoform represents a PFK-related sequence that retains recognition sites for ATP, ADP, and perhaps other modulators but has diverged in evolution to serve a strictly regulatory function.

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