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Biochimica et Biophysica Acta 1414 (1998) 95–107

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Reconstitution of membrane fusion between pancreatic islet secretory granules and plasma membranes: catalysis by a protein constituent recognized by monoclonal antibodies directed against glyceraldehyde-3-phosphate dehydrogenase

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Received 21 July 1998; accepted 13 August 1998

Abstract

An isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isolated and purified from rabbit brain cytosol has previously been demonstrated to catalyze membrane fusion (Glaser and Gross, Biochemistry 33 (1994) 5805–5812; Glaser and Gross, Biochemistry 34 (1995) 12193–12203). Herein, we provide evidence suggesting that this GAPDH isoform can reconstitute in vitro protein-catalyzed fusion between naturally occurring subcellular membrane fractions involved in insulin exocytosis. Utilizing purified rat pancreatic β-cell plasma membranes and secretory granules, we show that a brain cytosolic factor catalyzed the rapid and efficient fusion of these two purified membrane fractions which could be inhibited by a monoclonal antibody directed against the brain isoform of GAPDH. Moreover, the brain cytosolic factor also catalyzed the fusion of reconstituted vesicles prepared from lipid extracts of islet plasma membranes and secretory granules. Although the brain cytosolic factor rapidly catalyzed membrane fusion between islet plasma membranes and secretory granules, it did not catalyze fusion between one secretory granule population with another. To identify the potential importance of brain cytosolic factor catalyzed membrane fusion in islet cells, we examined extracts of hamster insulinoma tumor cells (HIT cells) for fusion-catalyzing activity. A protein constituent was present in HIT cell cytosol which was immunologically similar to the rabbit brain GAPDH isoform. Although native HIT cell cytosol did not catalyze membrane fusion, removal of an endogenous protein inhibitor unmasked the presence of the protein which catalyzed membrane fusion activity and such fusion was ablated by a monoclonal antibody directed against the brain isoform of GAPDH. Collectively, these results suggest the possibility that an isoform of brain GAPDH, also evident in HIT cells, can catalyze fusion between the two naturally occurring subcellular membrane compartments involved in insulin secretion and suggest a novel paradigm potentially coupling glycolytic flux with insulin release.

Abbreviations: FB, fractionation buffer (50 mM MES, 250 mM sucrose, pH 7.2); FCGI, fusion-catalyzing GAPDH isoform; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIT, hamster insulinoma tumor; PB, preparation buffer (10 mM MES, 1 mM EGTA, pH 6.0); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserines; R18, octadecyl rhodamine B-chloride; SG, secretory granules; SUV, small unilamellar vesicles

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00154-0

Keywords: Membrane fusion; Pancreatic islet; Fluorescence dequenching; GAPDH

1. Introduction

Insulin is stored in secretory vesicles closely juxtaposed to the pancreatic β-cell plasma membrane. Fuel secretagogues, including D-glucose must be metabolized by pancreatic β-cells to facilitate the release of insulin which is mediated by the fusion of secretory granules with pancreatic β-cell plasma membranes [1–3]. However, the chemical mechanisms coupling secretagogue metabolism with β-cell secretory granule-plasma membrane fusion are not known. Moreover, the fusion of pancreatic β-cell secretory granules with plasma membranes has never been reconstituted in vitro. Recently, we have identified the ability of one isoform of an enzyme playing a prominent regulatory role in glucose metabolism, glyceraldehyde-3-phosphate dehydrogenase from brain, to catalyze the fusion of phospholipid vesicles comprised of plasmalogen molecular species in vitro [4]. Membrane fusion catalyzed by this isoform possessed an obligatory dependence on plasmalogens for catalytic activity and required the presence of physiologic amounts of cholesterol and phosphatidylserine [4]. Plasmalogen molecular species contain a vinyl ether linkage at the *sn*-1 position of the glycerol backbone and typically contain polyunsaturated fatty acids at the *sn*-2 position [5,6] resulting in a decrease of the cross sectional area at the hydrophobic–hydrophilic interface and an increased steric bulk in the hydrophobic portion of the molecule [7,8]. In neurons, both synaptic vesicles and presynaptic membranes are enriched in plasmenylethanolamine containing polyunsaturated *sn*-2 content species [9–12] and this is also the case with β-cell plasma membrane and secretory granule membrane [13,14].

The fusion of membrane bilayers is facilitated by the transient formation of an intermediate structure similar to an inverted micelle where the phospholipid head groups are oriented towards the interior of the structure, and the aliphatic side chains are directed outward (i.e. an inverted hexagonal phase II (H_{II})) [15–20]. The importance of this structure in membrane fusion is supported by the demonstration of accelerated rates of membrane fusion in bilayers containing constituents which promote formation of the H_{II} phase [17,20–22]. Phospholipids predisposed to adopt the H_{II} configuration are those in which the steric bulk of the hydrophobic region greatly exceeds that of the polar head group [18,23].

Although prior observations demonstrated that a brain cytosolic GAPDH isoform catalyzes fusion of model plasmenylethanolamine-containing vesicles, it is not yet known whether this protein can catalyze fusion between naturally occurring biologic membranes which actually participate in exocytosis in vivo. It is also not known whether the fusion-catalyzing GAPDH isoform (FCGI) is expressed only in brain or whether it is also present in other secretory cells. Identification of proteins which participate in fusion between pancreatic β-cell plasma membranes and secretory granules could have implications for the regulation of insulin secretion and perhaps for the understanding of insulin secretory defects in type II diabetes mellitus. Both islets and neurons express a family of proteins involved in the interactions between secretory granules and plasma membranes, including syntaxin, synaptobrevin, and SNAP-25 [24]. This suggests that there are common biochemical features between neurotransmitter release and insulin secretion. Despite the importance of the identification of proteins which facilitate insulin exocytosis, no candidate protein has yet been demonstrated to catalyze the fusion of secretory granules and plasma membranes isolated from pancreatic islets despite early suggestions that such a factor might exist. In view of the biochemical similarities between neurotransmitter release and insulin secretion, and of the similar phospholipid composition of β-cell and neuronal membranes, we have examined the possibility that the fusion-catalyzing GAPDH isoform isolated from brain might also catalyze fusion between insulin secretory granules and islet plasma membranes. Herein we demonstrate the ability of a protein present in brain to effectively catalyze the fusion of pancreatic islet plasma membranes with secretory granules in vitro and that this activity is ablated by monoclonal antibody directed

against GAPDH. Moreover, we demonstrate the presence of a similar protein constituent in HIT cells which is chromatographically and immunologically similar to the GAPDH isoform and thus represents a candidate protein for catalyzing the exocytosis of insulin from β-cells in vivo.

2. Materials and methods

2.1. Materials

Octadecyl rhodamine B-chloride (R18) was purchased from Molecular Probes (Eugene, OR) and purified on an Altex Ultrasphere-CN column as described previously [4]. Bovine brain phosphatidylserine (PS) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Birmingham, AL) and purified on an Altex Ultrasphere-Si column as described previously [4]. Plasmenylethanolamine from bovine heart ethanolamine glycerophospholipids was purified by reverse phase HPLC [23]. All phospholipids were quantified by capillary gas chromatography after acid methanolysis by comparison with methyl arachidate internal standard as described previously [25]. Most other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Preparation of rat islet secretory granules and plasma membranes

Rat islet plasma membranes, secretory granules and mitochondria were prepared by sucrose density gradient centrifugation [14]. Briefly, rat islets (ca. 9000) were transferred to a tissue grinder, rinsed twice with fractionation buffer (FB, 50 mM MES, 250 mM sucrose, pH 7.2), resuspended in 0.80 ml of FB, and homogenized with 14 strokes of the pestle at 1170 rpm (Polyscience RX R10 at setting 3). The homogenate was centrifuged at $600\times g_{max}$ for 5 min and the resultant supernatant was centrifuged at $20\,000\times g_{max}$ for 20 min. The resulting pellet was resuspended in 0.20 ml of preparation buffer (PB, 10 mM MES with 1 mM EGTA, pH 6.0) and was rehomogenized (14 strokes, 1170 rpm), layered atop a discontinuous sucrose gradient comprised of 1 ml each of sucrose at relative densities of 1.14, 1.16, 1.18, and 1.20 in a Beckman cellulose nitrate centrifuge tube (Ultraclear 13×51 mm), and centrifuged at $150\,000\times g_{max}$ for 90 min. The bands enriched in plasma membranes (top two interfaces) and secretory granules (present at the bottom two interfaces) were harvested separately [26]. Next, each fraction was resuspended in 4.0 ml of PB and centrifuged at $150\,000\times g_{max}$ for 60 min (to remove sucrose) and the resulting pellets were resuspended in 0.50 ml of 10 mM MES buffer (pH 6.0). Both acid-extractable insulin (marker for secretory granules) and 5′-nucleotidase activity (marker for plasma membranes) were measured as previously described [14].

2.3. Preparation of insulin secretory granules by Percoll density gradient fractionation

To obtain a homogeneous population of secretory granules free of mitochondria, the following procedure was employed. All operations were performed at 4°C. Islets (ca. 9000) were homogenized as described above in secretory granule (SG) buffer (0.8 ml of 260 mM sucrose, pH 6.5, with 10 mM MOPS (4-morpholinepropanesulfonic acid)) [27]. The homogenate (H) was transferred to a 10 ml glass vial and diluted with 7.2 ml of SG buffer. One ml of this suspension was then placed in each of eight Eppendorf 1.5 ml polypropylene tubes and centrifuged at $900\times g_{max}$ for 5 min to yield a pellet (P1) and a supernatant (S1). Each 1 ml of S1 was then centrifuged ($13\,000\times g_{max}$, 10 min) separately in a Eppendorf 1.5 ml polypropylene tube to yield a pellet (P2) and a supernatant (S2). P2 was then resuspended in 0.6 ml of SG buffer and 0.1 ml of the suspension was layered atop each of 6 Percoll density gradients. Each consisted of a layer (50 μl) of 100% Percoll and a layer (250 μl) of 35% Percoll in a 400 μl Eppendorf polypropylene tube. The Percoll had been dialyzed for 24 h against SG buffer before preparation of the gradients. After centrifugation at $13\,000\times g_{max}$ for 10 min, the lower 150 μl of the gradient (which contained the secretory granule fraction [27]) was then removed with a 500 μl Hamilton syringe. The Percoll content of the SG fraction was

then reduced by dilution with 1 ml of SG buffer and was subsequently centrifuged at $13\,000 \times g_{max}$ for 10 min. An aliquot of fractions H, S1, P1, S2, P2, SG, were removed for measurement of protein, and the remainder was processed for measurement of acid-extractable insulin [14].

2.4. Preparation of the crude GAPDH isoform membrane fusion activity from rabbit brain cytosol and HIT insulinoma cell cytosol

Crude fusion-catalyzing GAPDH isoform (FCGI) from rabbit brain cytosol was prepared as described previously [4]. Briefly, rabbit brains harvested from New Zealand white rabbits were homogenized with a Brinkman PT 10/35 Polytron apparatus. Crude cytosol was prepared by sequential centrifugation of the homogenate at $10\,000 \times g_{max}$ for 20 min and the resultant supernatant was further centrifuged at $100\,000 \times g_{max}$ for 60 min. The supernatant (cytosol) was twice dialyzed against 500 vols. of buffer A (50 mM Tris-HCl, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT, pH 7.0 at 4°C) for 6 h. The dialyzed cytosol was applied to a DE-52 (2.6 cm × 20 cm) column previously equilibrated with buffer A. After loading the cytosol, the column was washed with buffer A and the flow-through fractions were assessed for, and contained, membrane fusion activity as previously described [4]. To determine whether β-cells express an analogous fusion-catalyzing protein, dialyzed cytosol from HIT insulinoma cells (ca. 3×10^9) was prepared in a manner similar to that described above. Briefly, HIT insulinoma cells were resuspended in 5 ml of homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, and 1 mM DTT, pH 7.4 at 25°C) and cells were homogenized by brief sonication (10 × 1 s pulse) at 0°C. Crude cytosol was obtained by sequential centrifugations of the homogenate first at $10\,000 \times g_{max}$ for 20 min and subsequent centrifugation of the supernatant at $100\,000 \times g_{max}$ for 60 min. The resultant cytosolic supernatant cytosol was twice dialyzed against 500 vols. of buffer A for 6 h. The dialyzed HIT cell cytosol was applied to a DE-52 (1 cm × 10 cm) column previously equilibrated with buffer A. The void fractions were collected and assayed for membrane fusion-catalyzing activity as described below.

2.5. Preparation and purification of monoclonal antibodies

Hybridomas were prepared from mouse spleen after injection of purified membrane fusion protein [4] and were used to generate ascites fluid by established methods [28]. The resultant IgG was purified from ascites fluid by a protein A-agarose affinity chromatography kit (Pierce, Rockford, IL). Briefly, diluted ascites fluid (diluted 1:1 (v/v) with supplied binding buffer) was centrifuged at $2000 \times g_{max}$ for 5 min and the supernatant was loaded onto a protein A-agarose column previously equilibrated with binding buffer. After being extensively washed with binding buffer, IgG was eluted with the supplied high salt eluent buffer. The salt eluate was subsequently loaded onto an Excellulose GF-5 desalting column previously equilibrated with PBS buffer, and the monoclonal antibody was eluted with PBS buffer.

2.6. Labeling of pancreatic islet secretory granules with R18

Labeling of pancreatic islet secretory granules with R18 was performed as described previously [29] with minor modification. Briefly, aliquots of a solution of R18 (0.5 mg/ml) in $CHCl_3$ were placed in glass test tubes, concentrated to dryness under nitrogen and then exhaustively evacuated at high vacuum for at least 30 min. A suspension of islet secretory granules was then added to the test tube and was incubated for 5 min at 37°C with gentle shaking. The resultant suspension was then transferred to a new test tube and centrifuged at $900 \times g_{max}$ for 10 min. The resultant pellet (which contained R18-labeled secretory granules) was resuspended in the fusion assay buffer (280 mM sucrose, 5 mM MES, pH 6.0) at a concentration of 0.2 mg protein/ml.

2.7. Membrane fusion assays

Fusion of rat islet secretory granules with islet plasma membranes (or secretory granules) was measured by R18 fluorescence-dequenching as described in detail previously [30]. This R18 fluorescence-dequenching technique has been commonly used for fusion assays in biological membrane systems [29,31–36]. Briefly, R18-labeled secretory granules

(typically, 10 μg protein) and unlabeled plasma membranes (typically, 4 μg protein) were added to a fluorescence cuvette containing 1 ml of fusion assay buffer and stirred at 37°C. Fluorescence was measured continuously utilizing an SLM-Aminco 4800C spectrofluorometer operating at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. After a stable baseline fluorescence was recorded (ca. 100 s), crude rabbit brain cytosolic FCGI (typically 100 μl) was added in the absence or presence of other reagents as indicated. The mixture was continuously stirred and fluorescence was monitored for an additional 500 s. Control assays utilizing combinations of labeled and unlabeled secretory granules or of labeled and unlabeled plasma membranes were also performed as indicated. Fluorescence-dequenching was expressed as a percentage of maximal fluorescence intensity, which was defined as that observed after addition of Triton X-100 (0.2%, v/v) to the assay cuvette.

To determine whether the void fractions from DE-52 chromatographic analysis of HIT cell cytosol contained fusion catalyzing activity, fluorescence-dequenching measurements were performed with model phospholipid vesicles [4]. Small unilamellar vesicles (SUV) were prepared which contained POPC (27 mol%), plasmenylethanolamine (27 mol%), PS (6 mol%), and cholesterol (40 mol%) at a final lipid concentration of 200 μM in fusion assay buffer (100 mM NaCl, 5 mM MES, 0.1 mM EGTA, pH 6.0). Preparation of the vesicles involved sonication utilizing a 40% duty cycle at a power level of 1.5 with a Vibra Cell sonicator equipped with a small tip. Labeled SUV containing R18 (2 mol%) were also prepared. Equimolar amounts of labeled and unlabeled SUV were loaded into one chamber of an SLM Aminco 4800C spectrofluorometer equipped with SLM stopped-flow apparatus. The other chamber was loaded with either dilution buffer alone (100 mM NaCl, 50 mM MES, and 0.1 mM EGTA, pH 6.0) or with protein-containing fractions diluted with dilution buffer in the absence or presence of other reagents as indicated. The contents of the two chambers were rapidly mixed in a 1:1 (v/v) ratio. Fusion was monitored by the temporal dependence of R18 fluorescence-dequenching observed at an emission wavelength of 590 nm with an excitation wavelength of 560 nm. The 0% fusion level was determined by monitoring fluorescence when the vesicles were mixed with sample dilution buffer only. The 100% fusion level was determined by preparing vesicles composed of the mixture which would result if all vesicles fused (e.g. 27% PC, 27% PE, 6% PS, 40% cholesterol, and 1% R18) and quantifying the resultant R18 fluorescence. The fluorescence tracings were normalized to the 0% and 100% fusion levels. Fusion of rat islet secretory granules with islet plasma membranes induced by the void fractions from DE-52 chromatography of HIT cell cytosol was determined by fluorescence dequenching as described above.

Fusion of reconstituted lipid extracts of rat islet secretory granules and plasma membranes was also examined by fast stopped-flow kinetic analysis. Briefly, total membrane lipids of rat islet secretory granules and plasma membranes were extracted by the Bligh and Dyer method [37]. Small unilamellar vesicles comprised of membrane lipid extracts were prepared by sonication in fusion assay buffer (100 mM NaCl, 5 mM MES, and 0.1 mM EGTA, pH 6.0) to achieve a final total lipid concentration of ~200 μM. R18-labeled (2 mol%) small unilamellar vesicles of extracted lipids were prepared similarly. In some experiments, Bligh and Dyer extracts were treated with HCl fumes for 30 min to destroy plasmalogens as described previously [19]. Fast stopped-flow kinetic analysis of FCGI-induced fusion of these SUV was performed as described above.

2.8. Miscellaneous procedures

Protein content was determined with a Bio-Rad protein assay using bovine serum albumin as standard. Trypsin-digestion of proteins or membranes was performed by incubation with 0.5% (w/w) of TPCK trypsin (Pierce) at 37°C for ≈1 h and terminated by addition of 0.1% (w/w) trypsin inhibitor.

3. Results

3.1. A rabbit brain cytosolic factor induces fusion of islet secretory granules with islet plasma membranes

When R18-labeled islet secretory granules were

mixed with unlabeled islet plasma membranes in the absence of any DE-52 eluent, no significant fluorescence-dequenching (other than a normal background drift) was observed (tracing A, Fig. 1), indicating that these two membrane populations do not spontaneously fuse at measurable rates. Addition of DE-52 chromatographic eluents of rabbit brain cytosol induced the rapid fusion of the islet secretory granules with plasma membranes in a concentration-dependent manner (Fig. 1, upper three tracings).

To determine whether both membrane populations are required in the fusion event, two additional experiments were performed. First, R18-labeled islet secretory granules were mixed with unlabeled islet secretory granules and fluorescence-dequenching was monitored after addition of brain cytosolic fusion-catalyzing factor. After an initial decrease in fluorescence (tracing B in Fig. 2) minimal fluorescence-dequenching, similar to the control tracing A in Fig. 1 and probably reflecting a background drift or dilution effect, was observed. The absence of significant dequenching above background therefore indicates that secretory granules do not spontaneously fuse with each other. Fusion did occur when brain cytosolic factor was added to a mixture of unlabeled

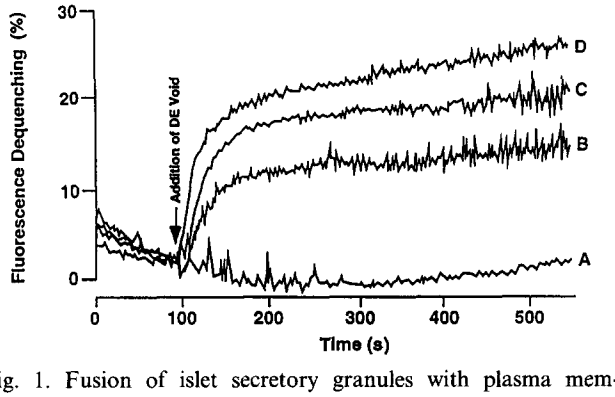

Fig. 1. Fusion of islet secretory granules with plasma membranes is induced by a rabbit brain cytosolic factor. R18-labeled islet secretory granules (10 μg protein) were mixed with islet plasma membranes (4 μg protein), and fluorescence-dequenching was monitored after addition of varied amounts of fusion-catalyzing protein (A to D, 0, 7.5, 15, 30 μg of protein, respectively) obtained from DE-52 chromatography of dialyzed rabbit brain cytosol. Fluorescence was measured continuously with an SLM-Aminco 4800C spectrofluorometer operated at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Fluorescence-dequenching is expressed as a percentage of the fluorescence intensity after addition of Triton X-100 (0.2%, v/v) to the assay cuvette at the end of the experiment.

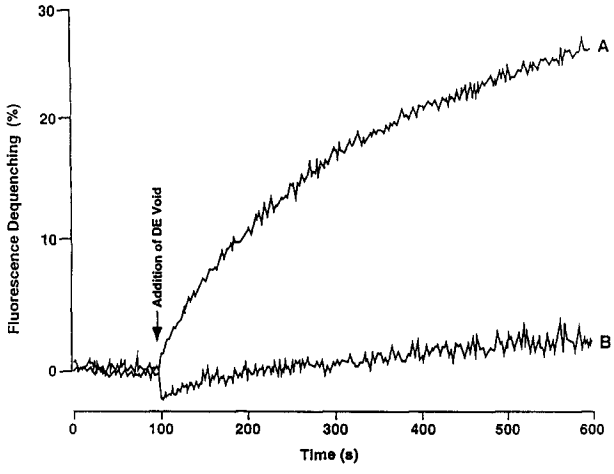

Fig. 2. The rabbit brain cytosolic factor catalyzes fusion of R18-labeled islet secretory granules with unlabeled islet plasma membranes but not with unlabeled islet secretory granules. R18-labeled islet secretory granules (10 μg protein) were mixed with unlabeled islet plasma membranes (4 μg protein, A) or with unlabeled secretory granules (10 μg protein, B), and fluorescence was recorded before and after addition (at arrow) of an aliquot (30 μg protein) of the void volume fractions from DE-52 chromatography of dialyzed rabbit brain cytosol. Fluorescence was quantitated as described in the legend of Fig. 1.

plasma membranes and R18-labeled secretory granules (tracing A in Fig. 2). This indicates that plasma membranes are required for the fusion event. In the second experiment, the effects of varying the concentrations of secretory granules on membrane fusion were examined. As illustrated in Fig. 3, fusion between islet secretory granules and plasma membranes was dependent on the concentration of plasma membrane. Similarly, in experiments where the concentration of plasma membrane was fixed, the amplitude of fusion was observed to increase as the concentration of secretory granules was increased (data not shown). This demonstrates that both islet membrane compartments are required for fusion induced by the brain cytosolic factor.

3.2. *The fusion-catalyzing factor from brain cytosol is a protein*

To determine whether the activity from brain cytosol which catalyzes the fusion between islet plasma membranes and secretory granules was mediated by a protein, the DE-52 void fractions (which contained the activity) were pretreated with trypsin as described

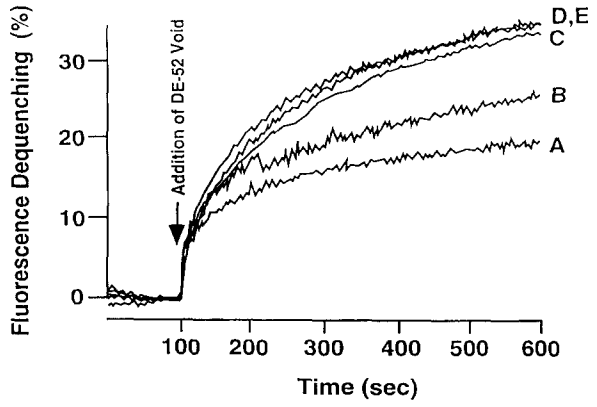

Fig. 3. The rate of membrane fusion induced by brain cytosolic fusion-catalyzing factor depends on the concentration of both islet plasma membranes and secretory granules. A fixed amount of R18-labeled islet secretory granules (10 μg protein) was mixed with varied amounts of unlabeled islet plasma membranes. The amounts of plasma membranes employed contained 2, 4, 8, 12 or 16 μg protein in tracings A, B, C, D, or E, respectively. Fluorescence was monitored before and after addition (at arrow) of an aliquot of fusion-catalyzing protein (30 μg) contained in the void volume fractions from DE-52 chromatography of dialyzed rabbit brain cytosol. Membrane fusion was quantified as described in the legend of Fig. 1.

in Section 2. Fluorescence-dequenching was then monitored after adding the trypsin-treated DE-52 void fractions to a mixture of R18-labeled islet secretory granules and unlabeled islet plasma membranes (Fig. 4, top panel). Fusion-catalyzing activity of the brain cytosolic factor was completely ablated by trypsin-treatment (tracing B) although non-trypsin-treated cytosolic factor did catalyze fusion (tracing A). This indicates that a protein constituent in the DE-52 void fraction was responsible for catalyzing fusion between islet plasma membranes and secretory granules.

Because intrinsic membrane proteins are required for exocytosis [38,39], interaction of the fusion protein with islet plasma membranes or secretory granules may require proteins exposed on the surface of these membranes. Therefore, both labeled islet secretory granules and unlabeled islet plasma membranes were treated with trypsin prior to mixing, and fluorescence-dequenching induced by the partially purified fusion protein was monitored (Fig. 4, bottom panel). The rate of fusion with trypsin-treated islet secretory granules and trypsin-treated plasma membranes was nearly identical to that observed with islet secretory granules and plasma membranes that had not been treated with trypsin (tracings A and B).

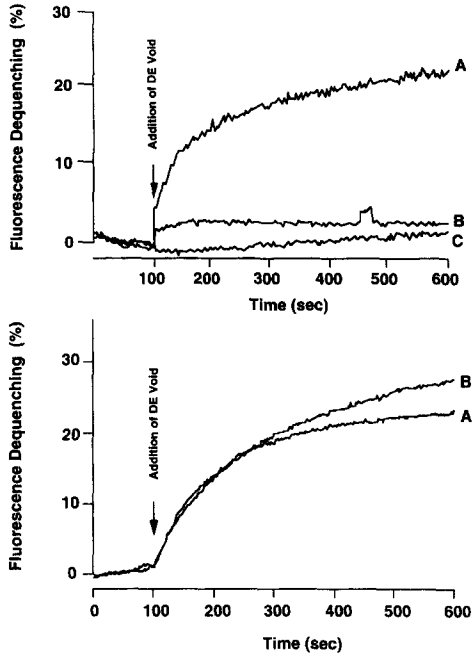

Fig. 4. Ablation of fusion of islet secretory granules and plasma membranes following trypsinization of brain cytosolic factor but not of plasma membrane. Top panel: R18-labeled islet secretory granules (10 μg protein) and unlabeled islet plasma membranes (4 μg protein) were mixed as in Fig. 1. Fluorescence was monitored before and after addition (at arrow) of an aliquot (30 μg protein) of void volume fractions from DE-52 chromatography of dialyzed rabbit brain cytosol (A), of trypsin-treated DE-52 eluate (B), or of buffer alone (C). Fusion was quantified as described in the legend of Fig. 1. Bottom panel: preparations of islet secretory granules and of plasma membranes were each divided into two aliquots. One aliquot of each membrane preparation was incubated with trypsin (0.5% (w/w), 1 h, 37°C) and the other aliquot was incubated (1 h, 37°C) without trypsin. The membranes were then collected by centrifugation and resuspended in trypsin-free buffer. This process was repeated to remove any residual trypsin. Next, secretory granules were then labeled with R18 as described in Section 2. Trypsin-treated labeled secretory granules were then mixed with unlabeled, trypsin-treated plasma membranes (tracing A). Control experiments involving mixing of non-trypsin-treated labeled secretory granules with non-trypsin-treated, unlabeled plasma membranes are illustrated in tracing B. Fluorescence was monitored before and after addition (at arrow) of an aliquot of fusion-catalyzing protein (30 μg) contained in the void volume fractions from DE-52 chromatography of dialyzed rabbit brain cytosol. Membrane fusion was quantified as described in the legend of Fig. 1.

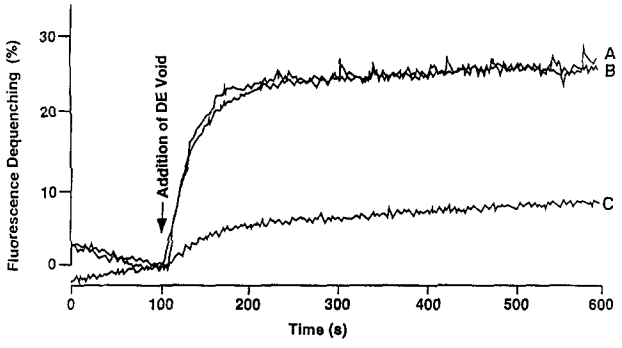

Fig. 5. Fusion of islet secretory granules and plasma membranes catalyzed by a brain cytosolic factor is ablated by antibody directed against glyceraldehyde-3-phosphate dehydrogenase. R18-labeled islet secretory granules (10 μg protein) were mixed with unlabeled islet plasma membranes (4 μg protein). Fluorescence was then monitored before and after addition (at arrow) of an aliquot of fusion-catalyzing protein (30 μg) contained in the void volume fractions from DE-52 chromatography of dialyzed rabbit brain cytosol that had been premixed with buffer control (tracing A), Ab 155.B5.4 (10 μg) (tracing B) or with anti-GAPDH antibody Ab 155.D2.2 (1 μg) (tracing C) as described in Section 2. Membrane fusion was quantified as described in the legend of Fig. 1.

3.3. *The protein catalyzing fusion between islet secretory granules and plasma membranes is neutralized by an antibody against glyceraldehyde-3-phosphate dehydrogenase*

A monoclonal antibody (Ab 155.D2.2) raised against GAPDH has been demonstrated to prevent fusion of plasmenylethanolamine-containing SUV that is otherwise induced by the rabbit brain cytosolic fusion-catalyzing factor [4]. In contrast, a second antibody also directed against GAPDH (Ab 155.B5.4) recognizes the protein in Western blots, but does not influence membrane fusion activity [4]. Fusion of islet plasma membranes with secretory granules induced by the rabbit brain cytosolic factor was prevented by Ab 155.D2.2, but was not attenuated by antibody Ab 155.B5.4 (Fig. 5). This indicates that the protein in rabbit brain cytosol which catalyzes fusion of islet plasma membranes and secretory granules shares epitopes with the isoform of GAPDH which facilitates membrane fusion. This is consistent with the possibility that the protein mediating the fusion of islet cell plasma membranes with secretory granules is a GAPDH isoform.

3.4. *Fusion between vesicles prepared from lipid extracts of islet secretory granules and plasma membranes and the influence of acid treatment of the lipid extracts*

To determine whether lipid components of islet membrane preparations are important in the catalyzed fusion event, lipids were extracted from islet plasma membranes and secretory granules by the method of Bligh–Dyer and used to prepare SUV. The SUV prepared from the secretory granules extracts were labeled with R18 and mixed with unlabeled SUV prepared from islet plasma membranes. In some cases, the lipid extracts were treated with HCl vapor before preparation of SUV. Exposure to acid fumes under the conditions employed destroys the vinyl ether linkage present in plasmalogen molecular species [19]. After mixing the labeled SUV with unlabeled SUV, rabbit brain cytosolic fusion factor was added and fluorescence-dequenching was moni-

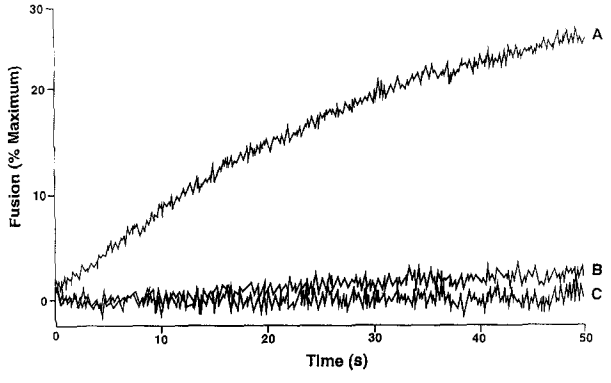

Fig. 6. Fusion of vesicles comprised of reconstituted lipid extracts of islet secretory granules and plasma membranes is catalyzed by the rabbit brain cytosolic factor. Islet secretory granules and plasma membrane lipids were extracted by the Bligh–Dyer method. These extracted lipids were used to prepare R18-labeled SUV and unlabeled SUV by sonication as described in Section 2. In some cases, the lipid extracts were treated with HCl fumes to destroy plasmalogen content before preparation of SUV. Fusion between various SUV preparations was then monitored by fluorescence-dequenching after addition of an aliquot (30 μg) of rabbit brain cytosolic fusion factor as in Fig. 1. In tracing A, R18-labeled SUV prepared from secretory granule lipid extracts were mixed with unlabeled SUV prepared from plasma membrane lipid extracts. In tracing B, labeled SUV prepared from secretory granule lipid extracts were mixed with unlabeled SUV prepared from HCl-treated plasma membrane lipid extracts. In tracing C, labeled SUV prepared from HCl-treated secretory granule lipid extracts were mixed with unlabeled SUV prepared from HCl-treated plasma membrane lipid extracts.

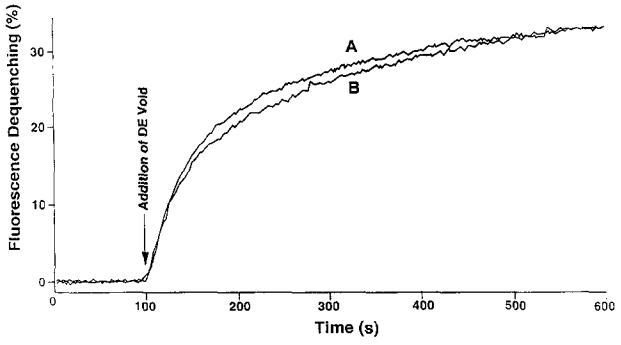

Fig. 7. Brain cytosolic factor-catalyzed fusion of islet plasma membranes with islet secretory granules prepared by Percoll density gradient analysis. A population of islet secretory granules free of mitochondria was prepared by Percoll density gradient analysis, as described in Section 2. An aliquot (10 μg protein) of R18-labeled secretory granules was mixed with plasma membranes (4 μg protein). Fluorescence was then monitored before and after addition (at arrow) of an aliquot of fusion-catalyzing factor (30 μg protein) contained in the void volume fractions from DE-52 chromatography of dialyzed rabbit brain cytosol (tracing A). Fusion was quantified as described in the legend of Fig. 1. In a separate experiment, both the secretory granule and plasma membrane fractions were treated with trypsin prior to use in the fusion assay (tracing B).

tored. Although fusion occurred readily between SUV prepared from extracts of islet secretory granules and SUV prepared from islet plasma membranes, pretreatment of the plasma membrane and secretory granule membrane extracts with HCl fumes ablated membrane fusion (Fig. 6). These observations suggest that an acid-labile phospholipid (i.e. plasmalogen) is a required component of the SUV in order for protein-catalyzed fusion to occur. However, the possibility that plasmalogen breakdown fragments (i.e. lysoglycerophospholipid and fatty aldehyde) inhibit the protein-catalyzed fusion cannot be excluded since prior studies demonstrated that lysophosphatidylcholine is a potent inhibitor of membrane fusion [40].

3.5. *Effects of Ca^{2+} on islet membrane fusion catalyzed by the brain cytosolic protein*

Fusion of plasmenylethanolamine-containing SUV catalyzed by the rabbit brain cytosolic GAPDH isoform does not require Ca^{2+} [4]. Similarly, rates of fusion of islet plasma membranes and secretory granules catalyzed by the rabbit brain cytosolic proteins were nearly identical in the absence and in the presence of 5 mM Ca^{2+} (data not shown).

3.6. *Demonstration of fusion between secretory granules prepared by Percoll density gradient analysis and plasma membranes*

Subcellular fractionation of islets by sucrose density gradient analysis yields a relatively pure population of plasma membranes. Secretory granule-containing fractions from this procedure also contain mitochondria [14,26]; islet secretory granules free of mitochondria were prepared by Percoll density gra-

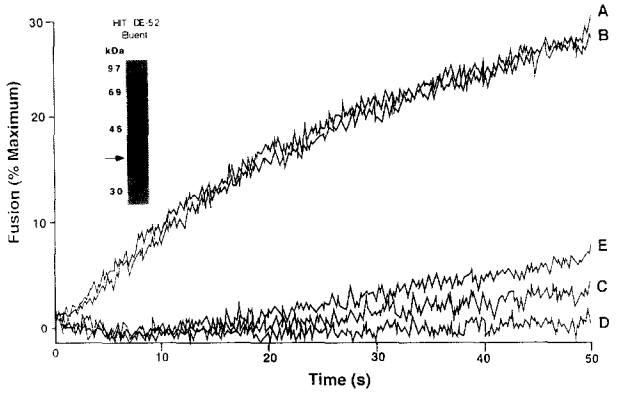

Fig. 8. Cytosol from HIT insulinoma cells contains a factor which catalyzes fusion of plasmenylethanolamine-containing small unilamellar vesicles. Small unilamellar vesicles (SUV) containing plasmenylethanolamine/POPC/PS/cholesterol (27:27:6:40 mol%) were prepared as described in Section 2. One population of this SUV was labeled with R18. Dialyzed HIT cell cytosol was prepared and then analyzed by DE-52 anion exchange chromatography as described in Section 2. Labeled and unlabeled SUV were then mixed and fluorescence dequenching was monitored as in Fig. 1 after addition of crude dialyzed HIT cell cytosol (tracing D) or of void-volume fractions obtained from DE-52 anion exchange analysis of dialyzed HIT cell cytosol (tracing A). In tracings B, C, and E the DE-52 void volume fractions were treated with Ab 155.B.5.4, Ab 155.D2.2 or with trypsin (0.5%, 1 h, 37°C), respectively, before addition to the SUV mixtures. Insert: DE-52 void fraction prepared from HIT cell cytosol containing fusion activity was analyzed by SDS-PAGE (10%) and the resolved proteins were transferred onto Immobilon-P PVDF membranes. The electroblot was blocked with TBS (1 mM Tris-HCl, 0.14 M NaCl, pH 7.4) buffer containing 5% powdered milk protein and 0.05% Tween 20, and sequentially incubated with Ab 155.D2.2, then with peroxidase-conjugated goat anti-mouse IgG antibody, followed by enhanced chemiluminescence reagent (for 1 min). The proteins were subsequently visualized by exposure of the blot to Hyperfilm ECL for 10 s.

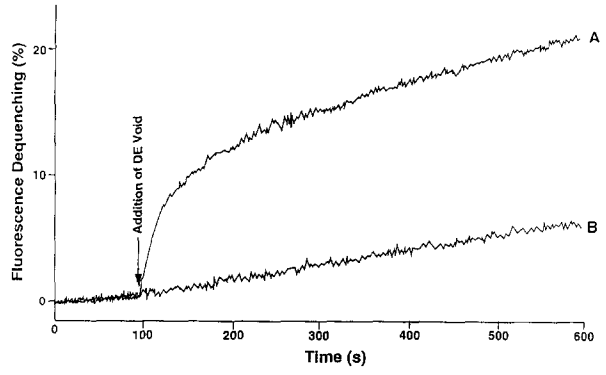

Fig. 9. Cytosol from HIT insulinoma cells contains a factor which catalyzes fusion of islet secretory granules and plasma membranes. R18-labeled islet secretory granules (10 μg protein) were mixed with unlabeled islet plasma membranes (4 μg protein). Fluorescence was monitored before and after addition (indicated by arrow) of crude dialyzed HIT cell cytosol (tracing B) or after addition of the void volume obtained from DE-52 anion exchange chromatography of dialyzed HIT cell cytosol (tracing A). Membrane fusion was quantified as described in the legend of Fig. 1.

dient analysis [14,27]. When R18-labeled secretory granules prepared by this method were mixed with plasma membranes, fluorescence-dequenching was observed upon addition of brain cytosolic fusion-catalyzing factor (Fig. 7), indicating the fusion of highly purified secretory granules with plasma membranes. The ability of the brain cytosolic fusion-catalyzing factor to promote fusion was not affected by prior treatment of secretory granules and plasma membranes with trypsin (Fig. 7), suggesting that the fusion event does not require membrane proteins that are digestible by trypsin under the conditions employed.

3.7. *Membrane fusion-catalyzing activity from HIT insulinoma cells*

These observations suggested the possibility that a GAPDH isoform in β-cells might catalyze fusion between secretory granule and β-cell plasma membranes. Crude, dialyzed HIT cell cytosol was prepared, but did not promote fusion of plasmenylethanolamine-containing SUV when utilized directly (Fig. 8). However, when the cytosol was subjected to DE-52 anion exchange chromatographic analysis, fusion-catalyzing activity was present in the void volume (Fig. 8). These results demonstrate that the fusion catalyzing activity in HIT cell cytosol, similar to that in brain cytosol, is latent and is observed only after removal of an endogenous inhibitor which binds to the anion exchange matrix [4]. Like the analogous activity from brain cytosol, HIT cell fusion catalyzing activity was trypsin-sensitive, and was neutralized by a monoclonal GAPDH antibody (Ab 155.D2.2) (Fig. 8). Western analyses (Fig. 8 insert) revealed the recognition by this antibody of a single protein band in the region corresponding to the molecular mass of GAPDH (38 kDa). Collectively, these results indicate that the HIT cell fusion-catalyzing protein, like that from brain cytosol, shares epitopes with GAPDH and may represent a GAPDH isoform. Further experiments were performed to examine whether this protein catalyzing membrane fusion from HIT insulinoma cells can also induce the fusion of rat islet secretory granules with islet plasma membranes. As before, crude dialyzed HIT cell cytosol did not induce fusion when used directly (tracing B in Fig. 9). However, when the void fractions from DE-52 chromatographic analysis of HIT cell cytosol were rapidly mixed with plasma membranes and secretory granules membrane fusion was manifest (tracing A in Fig. 9).

4. Discussion

The results of the present study demonstrate that pancreatic islet cell plasma membranes and secretory granules can be induced to fuse by a cytosolic protein constituent present in both rabbit brain and hamster insulinoma cells which is immunologically related to an isoform of GAPDH. This protein-mediated fusion of pancreatic islet cells plasma membrane and secretory granules is dependent upon the lipid components of each membrane since fusion can be reconstituted with vesicles prepared from lipid extracts of each membrane. The identification that an acid-labile constituent is necessary for GAPDH isoform catalyzed membrane fusion is consistent with the previous demonstration that the rabbit brain cytosolic factor only catalyzes fusion of membranes which contain plasmalogen molecules species [4]. The results described here are the first to demonstrate the in vitro reconstitution of membrane

fusion between the two naturally occurring subcellular compartments involved in insulin secretion in vivo.

Pancreatic islet plasma membranes are highly enriched in arachidonic acid-containing plasmenylethanolamine molecular species [13,14] and this has also recently been demonstrated to be the case for insulin secretory granule membranes [76]. The importance of plasmenylethanolamine in facilitating both spontaneous and protein-catalyzed membrane fusion [4,19] likely reflects the propensity of plasmenylethanolamine molecular species to adopt a conformation similar to the inverted hexagonal phase. In the case of plasmenylethanolamine-containing membrane vesicles, spontaneous fusion occurs slowly, but can be accelerated over 1000-fold by a brain cytosolic protein constituent that is chemically, chromatographically, and immunologically similar to an isoform of GAPDH [4]. Previous studies have demonstrated that apposition of secretory granules with plasma membranes in neurons and in other cell types is facilitated by a complex set of interactions between docking proteins, proteins facilitating the ATP-dependent conformational reorganization of these proteins (e.g. NSF) and the proteins involved in catalyzing the fusion event itself [39,41–43]. In the current study, we supplanted the requirement for docking proteins by utilization of high concentrations of vesicles which interact through diffusion to study GAPDH isoform-catalyzed membrane fusion. Under these conditions, fusion from productive intervesicular encounters can be catalyzed by a cytosolic factor from brain and insulinoma cells which is distinct from known docking proteins. In the pancreatic β-cell, secretory granule movement along cytoskeletal elements towards the plasma membrane occurs [44], and it is likely that cytosolic proteins present in the β-cell are also involved in regulation of the exocytotic event, by analogy with other systems [38]. It is also likely that the cytosolic factor described here participates in catalyzing the actual membrane fusion event after docking of the membranes has occurred [24].

Insulin secretion from pancreatic islet β-cells requires the fusion of insulin-containing secretory granules with the β-cell plasma membrane. The molecular mechanisms underlying this fusion process are incompletely understood. Apposition of secretory granules with plasma membranes in neurons involves a complex set of interactions between proteins expressed in plasma membrane (e.g. syntaxin, SNAP-25, and synapotagmin) and in secretory granule membranes (e.g. synaptobrevin) [39]. These proteins are also expressed in pancreatic islets [24], suggesting that there are common features between synaptic vesicle exocytosis and insulin secretion. Our findings indicate that a brain cytosolic protein that is neutralized by an anti-GAPDH antibody can catalyze fusion of isolated secretory granule membranes with plasma membranes prepared from pancreatic islets. Our findings also demonstrate the presence of a similar fusion-catalyzing activity in the cytosol of HIT insulinoma cells.

These findings are of great interest with respect to the regulation of insulin secretion. The potential involvement of a GAPDH isoform in catalyzing fusion of islet secretory granules and plasma membranes is particularly intriguing because all known carbohydrate fuel insulin secretagogues yield glyceraldehyde-3-phosphate as an intermediate [2,45]. Carbohydrate fuels which enter glycolysis distal to glyceraldehyde-3-phosphate (e.g. lactate and pyruvate) are readily metabolized to CO_2, but fail to induce insulin secretion [2,45]. Glyceraldehyde is a more potent insulin secretagogue on a molar basis than is glucose [2,46] and its potency as an insulin secretagogue exceeds its suitability as a fuel [2]. The discrepancy between the fuel and secretagogue properties of glucose and glyceraldehyde in comparison with pyruvate and lactate, has long been interpreted to indicate that a signal triggering insulin secretion is generated in the glycolytic pathway between glyceraldehyde-3-phosphate and pyruvate [2,45]. The nature of this signal has not yet been established. The present results suggest the possibility that this signal might involve activation of a fusion-catalyzing GAPDH isoform in β-cells by glyceraldehyde-3-phosphate itself or by downstream metabolic products known to interact with other isoforms of GAPDH. Such activation might be imagined to involve dissociation of the fusion-catalyzing GAPDH isoform from an endogenous inhibitor which suppresses the latent fusion-catalyzing activity in cytosol from brain and HIT insulinoma cells.

Although the function of GAPDH in glycolysis is well-recognized, a variety of non-glycolytic activities

have been attributed to GAPDH, including nucleic acid binding and DNA repair and interactions with membranes and the cytoskeleton [47–57]. These observations suggest that isoforms of GAPDH may play multifunctional roles in cell biology, and the association of such isoforms with cell membranes [48,51,52] and with microtubular structures [49] is compatible with the possibility that one such function could be to facilitate fusion of microtubule-associated insulin secretory granules [44] with plasma membranes in glucose-stimulated islets. It is of interest, in this regard, that adaptation of native islet β-cells to chronic hyperglycemia is characterized by enhanced insulin secretory function [58,59]. Similarly, prolonged exposure of the clonal pancreatic β-cell line INS-1 to high concentrations of glucose results in augmentation of insulin secretion by a mechanism that requires gene transcription and mRNA translation [60]. In INS-1 cells, the enhanced insulin secretory function induced by prolonged exposure to glucose is associated with increases in the levels of GAPDH mRNA and protein that are similar in magnitude to the increase in secretory rate [60]. Although this could reflect a role for metabolic signals derived from the glycolytic activity of GAPDH in insulin secretion [61–63], it is also possible that up regulation of fusogenic isoforms GAPDH is required to support the greater rate of fusion between secretory granules and plasma membranes that occurs in the hypersecretory state.

Of equal interest is the possibility that modification of fusogenic GAPDH isoforms might be involved in the profound reduction in glucose-induced insulin secretion that occurs when islets are incubated with the cytokine interleukin-1 (IL-1). This IL-1-induced suppression of insulin secretion involves induction of the inducible isoform of nitric oxide synthase and overproduction of nitric oxide and is preventable by nitric oxide synthase inhibitors [64–73]. It is not yet known precisely how nitric oxide impairs glucose-induced insulin secretion, but NO is known to induce post-translational modifications of GAPDH which reduce its catalytic activity [74,75]. These modifications include *S*-nitrosylation at thiol groups and subsequent covalent attachment of NADH [74,75]. It is possible that such modifications of fusogenic isoforms of GAPDH by NO could reduce their ability to catalyze membrane fusion and that this phenomenon could contribute to the IL-1-induced suppression of insulin secretion.

Acknowledgements

The excellent technical assistance of Ms. Bingbing Li, Mr. Alan Bohrer, and Dr. Mary Mueller is gratefully acknowledged. This research was supported jointly by grants from the Juvenile Diabetes Foundation International File 996003 and the National Institutes of Health No. 1 PO1 HL57278, and a Career Development Award to S.R. from the American Diabetes Association and the National Institutes of Health (DK-34388)

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