Succinate Is a Preferential Metabolic Stimulus-Coupling Signal for Glucose-Induced Proinsulin Biosynthesis Translation

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The secondary signals emanating from increased glucose metabolism, which lead to specific increases in proinsulin biosynthesis translation, remain elusive. It is known that signals for glucose-stimulated insulin secretion and proinsulin biosynthesis diverge downstream of glycolysis. Consequently, the mitochondrial products ATP, Krebs cycle intermediates, glutamate, and acetoacetate were investigated as candidate stimulus-coupling signals specific for glucose-induced proinsulin biosynthesis in rat islets. Decreasing ATP levels by oxidative phosphorylation inhibitors showed comparable effects on proinsulin biosynthesis and total protein synthesis. Although it is a cofactor, ATP is unlikely to be a metabolic stimulus-coupling signal specific for glucose-induced proinsulin biosynthesis. Neither glutamic acid methyl ester nor acetoacetic acid methyl ester showed a specific effect on glucose-stimulated proinsulin biosynthesis. Interestingly, among Krebs cycle intermediates, only succinic acid monomethyl ester specifically stimulated proinsulin biosynthesis. Malonic acid methyl ester, an inhibitor of succinate dehydrogenase, also specifically increased glucose-induced proinsulin biosynthesis without affecting islet ATP levels or insulin secretion. Glucose caused a 40% increase in islet intracellular succinate levels, but malonic acid methyl ester showed no further effect, probably due to efficient conversion of succinate to succinyl-CoA. In this regard, a GTP-dependent succinyl-CoA synthetase activity was found in cytosolic fractions of pancreatic islets. Thus, succinate and/or succinyl-CoA appear to be preferential metabolic stimulus-coupling factors for glucose-induced proinsulin biosynthesis translation. *Diabetes* 51: 2496–2504, 2002

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Received for publication 3 August 2001 and accepted in revised form 24 April 2002.

DNP, dinitrophenol; GDH, glutamate dehydrogenase; HPLC, high-performance liquid chromatography; IC_{50}, half-maximal inhibitory concentration; K_{ATP}-channel, ATP-sensitive K^+ channel; KRBH, Krebs-Ringer bicarbonate buffer with HEPES; LDH, lactate dehydrogenase; SCS, succinyl-CoA synthetase; TCA, trichloroacetic acid.

Normal glucose homeostasis depends on the regulated production and secretion of insulin by the pancreatic β-cell. Generally, when insulin release is increased by a nutrient secretagogue, there is an accompanying upregulation of proinsulin biosynthesis at the translational level to rapidly replenish the β-cell insulin stores. However, several differences in the stimulus-coupling signal pathways for these two β-cell processes have been reported (1). For example, sulfonylureas (2) and fatty acids (3,4) stimulate insulin secretion but have no effect on upregulating proinsulin biosynthesis. Nevertheless, one characteristic that insulin secretion and proinsulin biosynthesis have in common is a requirement for glucose metabolism. Glucose is metabolized in the pancreatic β-cell via glycolysis in the cytosol, resulting in pyruvate production. Pyruvate is then transported into the mitochondria for Krebs cycle metabolism, supporting subsequent oxidative phosphorylation and ATP production (5–7). Generation of ATP by increased glucose metabolism promotes a rise in the cytosolic ATP/ADP ratio, closure of the ATP-dependent K^+ channel, opening of the voltage-sensitive Ca^{2+} channel, and a subsequent increase in the cytosolic free calcium concentration, resulting in a triggering of insulin release (8). In contrast, glucose-stimulated proinsulin biosynthesis is independent of extracellular calcium (1). Anaplerosis is an important requirement for glucose-induced insulin secretion (9–12) and is necessary for glucose-induced proinsulin biosynthesis (3). Anaplerosis may accelerate Krebs cycle activity in the β-cell (9) and thus the production of ATP; however, other products leave the cycle in response to anaplerosis and may act as metabolic stimulus-coupling factors for β-cell functions (8,9). For example, citrate has been proposed as a secondary metabolic coupling factor to stimulate insulin release (13,14). The efflux of citrate from the mitochondria causes an increase of cytosolic malonyl-CoA, which inhibits fatty acid oxidation and results in a rise of cytosolic long-chain fatty acyl-CoA, that has been proposed to be an insulin secretion coupling factor (13,15). However, free fatty acids, which stimulate insulin secretion, have no effect on basal proinsulin biosynthesis and moderately inhibit glucose-stimulated proinsulin biosynthesis (3,4). Hence, it is unlikely that malonyl-CoA and/or long-chain fatty acyl-CoA are signals of the stimulus-coupling mechanism specific for glucose-induced pro-

insulin biosynthesis. It has also been proposed that glutamate, which is formed in the mitochondria from α-ketoglutarate and then exported to the cytosol, is a metabolic coupling signal for the regulation of glucose-stimulated insulin exocytosis (16). However, glutamate only slightly increased glucose-stimulated insulin secretion at intermediate glucose concentrations (16,17), and its possible role as a secondary stimulus-coupling signal for glucose-induced insulin release remains controversial (18). Regardless, whether glutamate or citrate acts as a metabolic coupling factor for glucose-stimulated proinsulin biosynthesis has not been examined.

Glucose regulation of proinsulin biosynthesis (1) and insulin secretion (14) both require β-cell metabolism and anaplerosis. However, it should be realized that the respective distal targets of either the protein synthesis translational machinery or the exocytosis apparatus in the β-cell are quite distinct. Thus, the metabolic stimulus-coupling pathways for glucose-induced proinsulin biosynthesis and insulin secretion possibly branch away from each other at a point beyond anaplerosis. Whereas the signaling pathway for glucose-induced insulin secretion has been, in part, relatively well described (8,9), the signaling pathway for translational control of proinsulin biosynthesis is poorly understood. In this study, we have investigated whether stimulus-coupling signal(s) originating in the mitochondria can be implicated in the regulation of proinsulin biosynthesis induced by glucose. Among those investigated have been Krebs cycle intermediates, glutamate, acetoacetate, and ATP. We have compared their effect on proinsulin biosynthesis with total protein synthesis and insulin secretion in the same isolated pancreatic islets in order to better identify potential regulatory differences between metabolic stimulus-coupling pathways for these β-cell functions.

RESEARCH DESIGN AND METHODS

Materials. EasyTag Expre^{35}S^{35}S Protein Labeling Mix (NEN, Boston, MA) containing 73% of L-[^{35}S]methionine was used for islet metabolic radiolabeling. (We will refer to it here as [^{35}S]methionine.) Guinea pig anti-bovine insulin serum was from Sigma. Mitochondrial Krebs cycle intermediates, oxidative phosphorylation inhibitors, and other reagents were from Sigma or Aldrich.

Islet isolation and subcellular fractionation. Islets of Langerhans were isolated from pancreata of ~250-g male Sprague-Dawley rats as previously described (19,20).

For subcellular fractionation, batches of ~1,000 islets were homogenized in 0.25 mol/l sucrose, 10 mmol/l $MgCl_2$, 1 mmol/l EDTA, and 50 mmol/l HEPES buffer, pH 7.4, containing protease inhibitors [0.01 mmol/l each of phenylmethylsulfonyl fluoride, pepstatin-A, *N-p*-tosyl-L-lysine chloromethyl ketone, and *trans*-epoxysuccinyl-L-leucyl-amino-(4-guanidino) butane as well as 0.1 mmol/l leupeptin] by 10 strokes in a Potter-Elvehjen tissue grinder. Homogenates were spun at 1,000*g* for 5 min to remove nuclei and whole-cell debris. The postnuclei supernatant was centrifuged at 10,000*g* for 30 min, and this supernatant was further centrifuged at 100,000*g* for 60 min. The resulting supernatant (S100; cytosol-enriched fraction) was concentrated to 0.1 ml in a microcon-10 concentrator (Amicon-Millipore, Bedford, MA). The 10,000*g* pellet (P10), which contains, among other organelles, mitochondria, was reconstituted by sonication in 0.1 ml of 50 mmol/l HEPES, pH 8.0, containing 1% Triton X-100 and protease inhibitors. All procedures were carried out at 4°C. Enrichment of the cytosolic fraction was determined by a marker-enzyme assay of lactate dehydrogenase (LDH). The conversion of NAD (1 mg/ml) to NADH in the presence of 1 mmol/l lactate in 0.1 mol/l phosphate buffer, pH 7.4, by 10 μl (5–10 μg protein) of cytosol or P10 fraction was monitored at 340 nm. Mitochondria contamination in the cytosolic fraction was determined by analyzing the presence of glutamate dehydrogenase (GDH) marker enzyme activity. Conversion of NAD (0.5 mg/ml) to NADH by 10 μl (5–10 μg protein) of cytosol or P10 fraction in the presence of glutamate in 0.1 mol/l Tris, 0.4 mol/l hydrazine hydrate, 10 mmol/l $MgCl_2$, 5 mmol/l EDTA, pH 8.5, buffer was monitored at 340 nm. LDH activity was 8.0 ± 1.6 nmol of NADH · min^{-1} · mg^{-1} protein in the S100 cytosolic fraction compared with 0.11 ± 0.11 in the P10 mitochondrial-enriched fraction ($n \geq 2$). This represented a >80-fold enrichment of cytosol in the S100 fraction over the P10 mitochondrial-enriched fraction. In contrast, GDH activity was 0.32 ± 0.07 nmol of NADH · min^{-1} · mg^{-1} protein in the cytosol compared with 6.5 ± 3.7 in the P10 fraction ($n \geq 2$). This represented a >20-fold enrichment of mitochondrial marker enzyme activity in the P10 fraction over the S100 cytosolic fraction. Moreover, GDH activity in the cytosol was only ≤5% of that in the P10 fraction, indicating a very low mitochondria contamination in the S100 cytosolic fraction. The amount of insulin measured by radioimmunoassay in the S100 cytosolic fraction was 1.4 ± 0.1 μg/mg protein vs. 128.4 ± 1.4 in the P10 fraction ($n \geq 2$). This represented a ≤2% insulin secretory granule contamination in the S100 cytosol fraction that is comparable with the ≤5% low degree of mitochondrial contamination.

Proinsulin biosynthesis and insulin secretion in isolated rat islets. Proinsulin biosynthesis was examined in extracts of [^{35}S]methionine metabolically radiolabeled isolated islets, and insulin secretion was analyzed in the incubation medium of the same islets. Islet radiolabeling was essentially as previously described (20). Briefly, batches of 40 islets were preincubated at basal conditions (0 or 2.8 mmol/l glucose) for 1 h at 37°C in 0.2 ml modified Krebs-Ringer bicarbonate buffer, 20 mmol/l HEPES, and 0.1% BSA (KRBH). The incubation medium was then replaced with 0.2 ml fresh KRBH buffer containing the indicated reagent. After a 1-h incubation period at 37°C, 0.1 ml medium was collected and stored at −20°C until radioimmunoassay for insulin secretion. Immediately, 0.1 mCi [^{35}S]methionine was added and incubation continued for 30 min. Radiolabeled islets were lysed, (pro)insulin was immunoprecipitated from the lysate, and the immunoprecipitate was subjected to alkaline-urea PAGE and fluorography as previously described (19,20). Total protein synthesis was determined by trichloroacetic acid (TCA) precipitation in a 5-μl lysate aliquot (20). Another 5-μl aliquot was separated and stored at −20°C until radioimmunoassay for intracellular insulin content. Insulin radioimmunoassay was performed using rat insulin standards and antibodies from Linco (Linco Research, St. Charles, MO) and human ^{125}I-insulin from Eli Lilly (Indianapolis, IN). Insulin secretion was calculated as the percent of islet insulin content.

ATP content measurement in isolated rat islets. Islet cell total ATP content was determined as luciferase activity in islets expressing firefly (*Photinus pyralis*) luciferase cDNA (21). Batches of ~250 islets were infected with firefly luciferase adenovirus as previously described (22). Islets were then apportioned into 1.5-ml tubes (20 islet per tube), washed with 0.5 ml KRBH, preincubated in 0.1 ml KRBH at basal conditions, and incubated in 0.1 ml fresh medium containing the indicated agent. Islets were then lysed, and ATP-dependent luciferase activity was determined in the cell extracts using the Promega luciferase assay system (Promega, Madison, WI). Light emittance was detected in a luminometer (TD-20/20; Promega). In the linear range (0.5–5 mmol/l ATP), a 1-mmol/l increase in ATP concentration was equivalent to 10,000 relative luminescence units in an in vitro assay. This is in agreement with previous studies using luciferase activity as a relative analysis of ATP concentration (23). Luminescence was undetectable in lysates of uninfected or control β-galactosidase–infected islets.

Succinate, malate, and fumarate analysis. Islet total content of succinate, malate, and fumarate was measured in isolated islets incubated in the presence of 2.8 mmol/l glucose, 16.7 mmol/l glucose, or 16.7 mmol/l glucose plus 1 mmol/l malonic acid methyl ester. Batches of 200–500 islets were preincubated at 37°C for 1 h in KRBH (0.1 ml/100 islets) in the presence of basal 2.8 mmol/l glucose and then incubated for an additional hour in the corresponding volume of KRBH (0.1 ml/100 islets) containing the indicated agent. After incubations, islets were sonicated for 30 s in 10% TCA and incubated in ice for 15–30 min. The TCA soluble material was washed five times with ether to eliminate the TCA and raise the pH, and then lyophilized and stored at −80°C until analysis. Pools of two to four experiments (total of ~1,000 islets) were used as a single experiment for each assay. Succinate, malate, and fumarate islet content was analyzed in the TCA soluble fraction following the fluorometric enzyme methods described by Williamson and Corkey (24). Essentially, succinate was measured using an enzyme cycling/amplification method with succinate thiokinase (ITP-dependent; Roche Molecular Biochemicals, Mannheim, Germany), pyruvate kinase, and LDH, using a decrease in NADH fluorescence as a readout. The sensitivity of this assay enabled <1 nmol succinate to be measured (24). Malate levels were determined using malate dehydrogenase activity, and fumarate levels were determined using fumarase and malate dehydrogenase activities, with both assays using an increase in NADH fluorescence as a readout. The sensitivity of the malate and fumarate assays enabled ~1 nmol malate/fumarate to be measured (24). Values were normalized to DNA concentration. For DNA assay, TCA-precipitated material was dissolved in 0.1N NaOH, and DNA content was

determined using Hoechst dye 33258 (1 μg/ml PBS) as previously described by Labarca and Paigen (25).

Succinyl-CoA synthetase activity assay. Succinyl-CoA synthetase (SCS) activity was measured in an islet cytosol fraction in the direction of succinyl-CoA to succinate reaction. The release of CoA-SH from succinyl-CoA was monitored spectrophotometrically at 415 nm on its reaction with DTNB (Ellman's reagent) over 15 min using a microwell plate reader (Packard, Meriden, CT). Reactions were started by adding 0.2 ml assay buffer (10 mmol/l Tris, pH 7.1, 10 mmol/l $MgCl_2$, 0.2 mmol/l DTNB, and 1 mmol/l succinyl-CoA) ± 0.1 mmol/l GTP or ATP to 10 μl (5–10 μg protein) of the islet cytosolic fraction. In parallel, as a control for succinyl-CoA–specific hydrolysis, the same reaction was performed in the presence of acetyl-CoA or acetoacetyl-CoA. Control values in the absence of islet fraction were subtracted to correct the unspecific hydrolysis of the CoA derivatives, and the final values were normalized to total protein concentrations.

High-performance liquid chromatography analysis of succinyl-CoA. SCS activity in islet cytosol was also investigated by high-performance liquid chromatography (HPLC) analysis of in vitro formation of succinyl-CoA from succinate and CoA. Reactions were carried out for 5 min at 30°C in 0.1 ml of 50 mmol/l succinate, 110 mmol/l Tris, pH 7.4, 10 mmol/l $MgCl_2$, and 0.1 mmol/l CoA ± 0.1 mmol/l GTP or ATP, containing ~8 μg protein of the islet cytosolic fraction. In parallel assays, the formation of acetoacetyl-CoA and acetyl-CoA from acetoacetate and acetate was investigated. Immediately after the reaction period was completed, samples were subjected to HPLC, as previously described (26), but with a slightly modified elution gradient, as described in the legend of Fig. 6.

Statistical analysis. Statistically significant differences between groups were analyzed using the Student's *t* test for unpaired samples.

RESULTS

Effect of Krebs cycle intermediates on islet proinsulin biosynthesis, insulin secretion, and ATP content. It was investigated whether Krebs cycle intermediates may act as specific stimulus-coupling signals for glucose-stimulated proinsulin biosynthesis. Esters of the carboxylic acids citrate, succinate, fumarate, malate, and oxaloacetate were used to facilitate their cellular uptake. The esters of carboxylic acids were then processed to their acid form intracellularly (27). Stimulation of proinsulin biosynthesis by 16.7 mmol/l glucose was 5.7 ± 0.9-fold more than that by 2.8 mmol/l glucose ($P < 0.01$, $n = 13$). Succinic acid monomethyl ester (20 mmol/l) significantly stimulated proinsulin biosynthesis by 2.6 ± 0.4-fold ($P < 0.01$, $n = 12$) (Fig. 1*A*), independently of glucose. However, none of the other Krebs cycle intermediates (also at a 20-mmol/l concentration) had any measurable effect on stimulating proinsulin biosynthesis. Only 16.7 mmol/l glucose significantly stimulated islet total protein synthesis (1.6 ± 0.1-fold; $P < 0.01$, $n = 13$) (Fig. 1*B*). Insulin secretion was significantly stimulated (16.8 ± 2.5-fold; $P < 0.01$, $n = 17$) by 16.7 mmol/l glucose as well as by citric acid triethyl ester (3.0 ± 0.6-fold; $P < 0.01$, $n = 9$) and by succinic acid monomethyl ester (5.2 ± 1.0-fold; $P < 0.01$, $n = 11$) (Fig. 1*C*). The stimulation of proinsulin biosynthesis and insulin secretion evoked by succinate was 46 and 31%, respectively, of the glucose-induced response. Intracellular ATP levels were examined in islets expressing firefly luciferase cDNA by measuring ATP-dependent luciferase activity. Only stimulatory (16.7 mmol/l) glucose augmented islet ATP levels significantly (Fig. 1*D*). This increase was 40% above the ATP level observed at 2.8 mmol/l glucose ($P < 0.01$, $n = 6$). In contrast, ATP content in islets incubated in the presence of citric acid triethyl ester or oxaloacetic acid diethyl ester was 36 and 26%, respectively, lower than that observed at 2.8 mmol/l glucose.

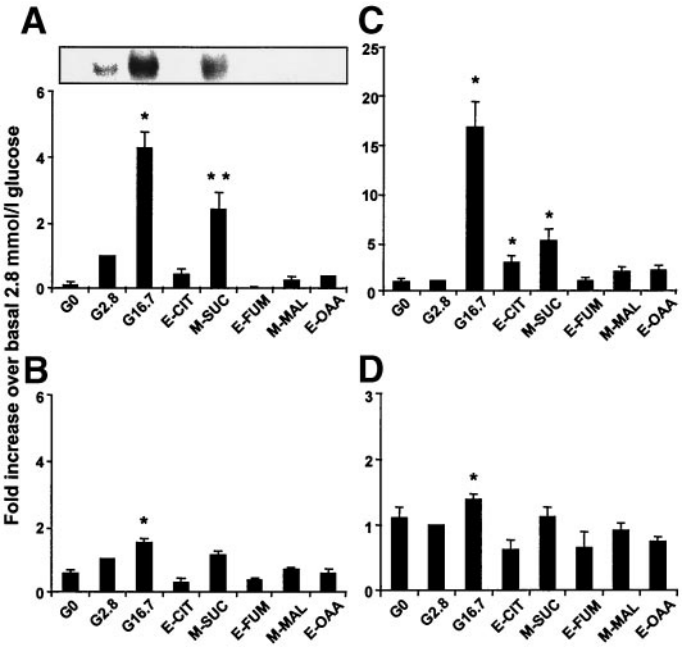

FIG. 1. Effect of various esters of carboxylic acids on isolated rat islet proinsulin biosynthesis, total protein synthesis, insulin secretion, and ATP content. Islets were preincubated for 1 h in the absence of glucose or any other agent and then incubated for 1 h at basal (2.8 mmol/l) glucose (G2.8), stimulatory (16.7 mmol/l) glucose (G16.7), or 20 mmol/l of the indicated carboxylic acid ester. Proinsulin biosynthesis (*A*) was determined in immunoprecipitates of [^{35}S]methionine radiolabeled islet extract by densitometric scanning of alkaline-urea PAGE fluorographs. A representative fluorograph is shown at the top. Bars represent the means ± SE of the densitometric analysis of more than three independent experiments. Total protein synthesis (*B*) was calculated as TCA-precipitable radioactivity in a 5-μl aliquot of the [^{35}S]methionine radiolabeled islet extract. Insulin secretion (*C*) was determined in the islet incubation medium by radioimmunoassay. Results are the means ± SE of 6–19 separate experiments. The islet ATP content (*D*) was measured as ATP-dependent luciferase activity in lysates of transient luciferase-expressing islets. Results are the means ± SE of four to six experiments. Values are shown as fold stimulation over basal 2.8 mmol/l glucose. E-CIT, citric acid triethyl ester; E-FUM, fumaric acid monoethyl ester; E-OAA, oxaloacetic acid diethyl ester; G0, absence of glucose or other agent; M-MAL, malic acid dimethyl ester; M-SUC, succinic acid monomethyl ester. *$P < 0.01$ and **$P < 0.05$ vs. 2.8 mmol/l glucose.

Succinic acid methyl ester–stimulated proinsulin biosynthesis is independent of increased insulin secretion. To demonstrate that stimulation of proinsulin biosynthesis by succinic acid methyl ester as well as by glucose was not a consequence of increased insulin release, isolated islets were incubated under conditions in which insulin secretion was inhibited, such as in the absence of extracellular calcium or presence of somatostatin. Neither removal of extracellular calcium from the islet incubation medium nor addition of 1 μmol/l somatostatin affected preproinsulin mRNA levels (Fig. 2*A*) or glucose-stimulated and succinic acid methyl ester–stimulated proinsulin biosynthesis (Fig. 2*B*). In contrast, as expected, in the same islets, both the absence of extracellular calcium and the presence of somatostatin significantly inhibited glucose-stimulated insulin secretion, 5.8 ± 1.0-fold and 3.0 ± 0.2-fold, respectively (Fig. 2*C*). Moreover, succinic acid methyl ester–stimulated insulin secretion was also significantly decreased, 2.4 ± 0.6-fold by calcium depletion and 2.3 ± 0.3-fold by 1 μmol/l somatostatin (Fig. 2*C*).

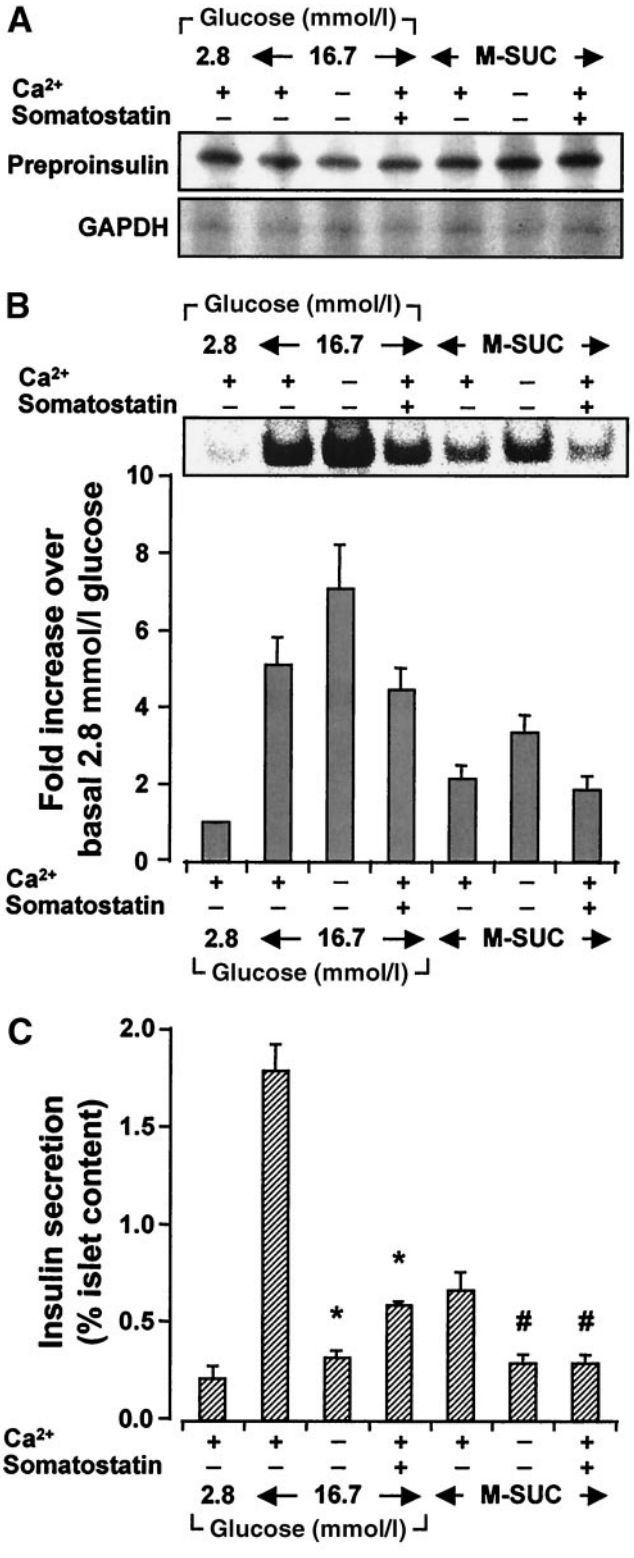

FIG. 2. Effect of Ca^{2+} depletion and somatostatin (1 μmol/l) on glucose-stimulated and succinic acid methyl ester–stimulated islet preproinsulin mRNA level, proinsulin biosynthesis, and insulin secretion. Isolated islets were preincubated for 1 h at basal (2.8 mmol/l) glucose and then incubated for 1 h at the indicated condition. Islet preproinsulin mRNA levels (*A*) were determined by RNase protection assay as previously described (22). Control GAPDH mRNA levels are shown. Proinsulin biosynthesis (*B*) and insulin secretion (*C*) were measured as stated in the Fig. 1 legend. Results are means ± SE of three independent experiments. *$P < 0.02$ vs. 16.7 mmol/l glucose in the presence of Ca^{2+}; #$P < 0.05$ vs. 20 mmol/l succinic acid methyl ester (M-SUC) in the presence of Ca^{2+}.

Effect of glutamic acid methyl ester on glucose-stimulated proinsulin biosynthesis, insulin secretion, and islet ATP content. Glucose-stimulated proinsulin biosynthesis was not changed in the presence of 5 mmol/l glutamic acid methyl ester at any glucose concentration and, if anything, was slightly decreased by 20 mmol/l glutamic acid methyl ester at glucose concentrations >2.8 mmol/l (Fig. 3*A*), although this was not statistically significant. Total protein synthesis was unaffected by either 5 or 20 mmol/l glutamic acid methyl ester. Glutamic acid methyl ester (5 mmol/l) slightly augmented glucose-stimulated insulin secretion at intermediate glucose concentrations, whereas no effect in the absence of glucose or on 16.7 mmol/l glucose–stimulated insulin secretion was observed (Fig. 3*B*), in agreement with previous reports (17). However, although 20 mmol/l glutamic acid methyl ester did not affect insulin secretion at low glucose concentrations, it decreased glucose-stimulated insulin secretion (Fig. 3*B*). This effect was statistically significant ($P < 0.02$, $n = 8$) at 16.7 mmol/l glucose. Islet total ATP content was significantly ($P < 0.05$) augmented by glucose at concentrations >5.5 mmol/l versus basal glucose (2.8 mmol/l). Glutamic acid methyl ester inhibited the glucose-induced islet ATP content increase at glucose concentrations >8 mmol/l, with this effect being more potent at 20 mmol/l than at 5 mmol/l glutamic acid methyl ester ($P < 0.05$ for 20 mmol/l) (Fig. 3*C*).

Glucose-stimulated proinsulin biosynthesis and insulin secretion are differentially inhibited by mitochondrial oxidative phosphorylation inhibitors. To investigate whether ATP was a stimulus-coupling signal specific for glucose-stimulated proinsulin biosynthesis, mitochondrial oxidative phosphorylation was inhibited at several stages in the electron transport chain. The inhibitors used were rotenone for complex I (NADH dehydrogenase), antimycin A1 for complex III, dinitrophenol (DNP), a mitochondria membrane uncoupler that diffuses the proton gradient across the mitochondrial inner membrane, and oligomycin, an ATP synthase (complex V) inhibitor. Rotenone, antimycin A1, DNP, and oligomycin all caused a dose-dependent reduction in total cell ATP content and inhibited glucose-stimulated proinsulin biosynthesis, total protein synthesis, and insulin secretion. Figure 4 depicts the dose-response inhibition curves for oligomycin. Rotenone, antimycin A1, and DNP showed inhibition curves qualitatively similar to these (not shown), although the range of inhibitory concentrations varied for each inhibitor. From such titration inhibition curves, the half-maximal inhibitory concentration (IC_{50}) was calculated (Table 1). Rotenone, antimycin A1, and DNP induced a parallel decrease in glucose-stimulated proinsulin biosynthesis, total protein synthesis, and ATP content, with comparable IC_{50} values, which were not significantly different from each other (Table 1). However, in the same islets, the IC_{50} values for glucose-stimulated insulin secretion inhibition were quite different from those of proinsulin biosynthesis inhibition. The IC_{50} for insulin secretion in the presence of rotenone was 3-fold lower than that for proinsulin biosynthesis ($P < 0.01$), 10-fold lower with DNP ($P < 0.01$), 7-fold higher with antimycin A1 ($P < 0.05$), and 4-fold higher with oligomycin ($P < 0.05$). For oligomycin, the IC_{50} value for proinsulin biosyn-

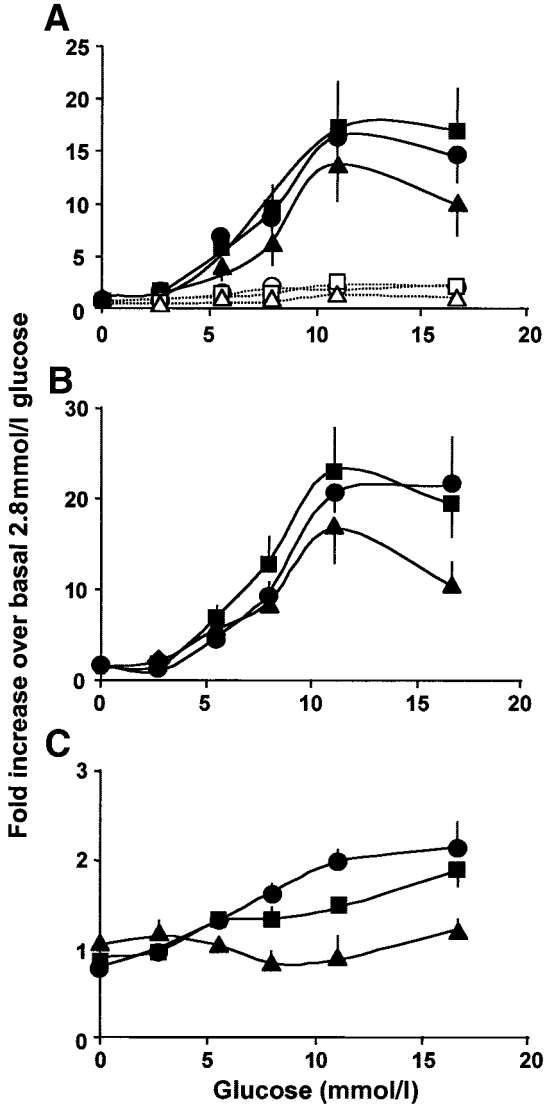

FIG. 3. Effect of glutamic acid methyl ester on proinsulin biosynthesis, insulin secretion, and ATP content in isolated islets. Islets were preincubated for 1 h in the absence of glucose and then incubated for 1 h at increasing glucose concentrations in the absence (●) or presence of 5 mmol/l (■) or 20 mmol/l (▲) glutamic acid methyl ester. Proinsulin biosynthesis (*A*, solid lines), total protein synthesis (*A*, dashed lines), insulin secretion (*B*), and ATP content (*C*) were determined in islet lysates or islet incubation media as stated in the Fig. 1 legend. Results are means ± SE of three to nine independent experiments.

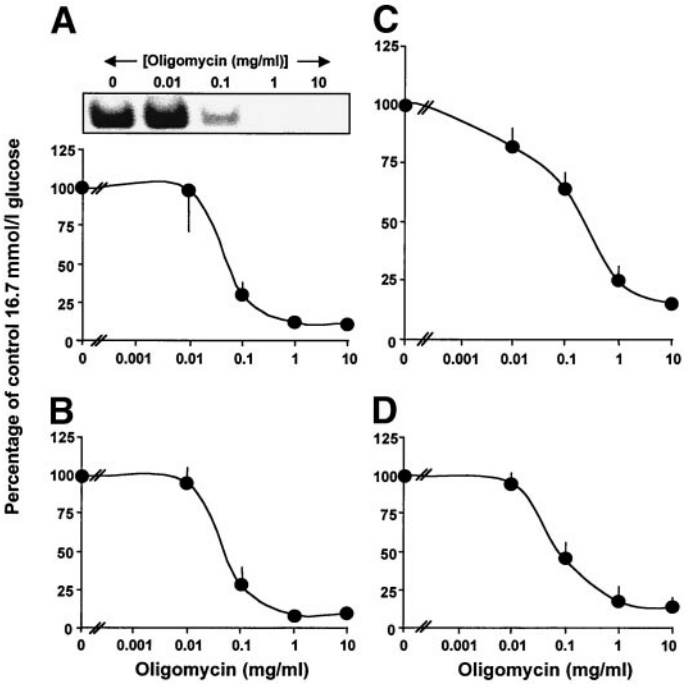

FIG. 4. Oligomycin dose-response effect on glucose-stimulated proinsulin biosynthesis, total protein synthesis, insulin secretion, and ATP content in isolated rat islets. Islets were preincubated for 1 h at basal (2.8 mmol/l) glucose and then incubated for 1 h in the presence of 16.7 mmol/l glucose and the indicated concentration of oligomycin. Proinsulin biosynthesis (*A*), total protein synthesis (*B*), insulin secretion (*C*), and ATP content (*D*) were determined in islet lysates or islet incubation media as stated in the Fig. 1 legend and RESEARCH DESIGN AND METHODS. Results are means ± SE of three to seven independent experiments.

thesis and total protein synthesis was similar, whereas that for islet ATP content was approximately fourfold higher. This might be due to an inhibition of the cell protein synthesis machinery by oligomycin that is independent of the electron transport chain. In fact, oligomycin had been previously reported to be an inhibitor of protein synthesis translation (28).

Glucose-stimulated proinsulin biosynthesis is enhanced by malonic acid methyl ester in a dose-dependent manner. Isolated islets were incubated at 16.7 mmol/l glucose and increasing concentrations of malonate, which was used as the precursor form, and malonic acid methyl ester, to facilitate its cellular uptake. Malonate is an inhibitor of succinate dehydrogenase (29), the enzyme that catalyzes the succinate-to-fumarate reaction with formation of reduced flavin adenine dinucleotide ($FADH_2$) in the Krebs cycle. Inhibition of $FADH_2$ production may inhibit the mitochondria electron transport chain at complex II, where $FADH_2$ is an electron donor. It could be predicted that inhibition of succinate dehydrogenase and thus the reaction of succinate to fumarate might result in an increase of the intracellular content of succinate. Malonic acid methyl ester enhanced glucose-stimulated proinsulin biosynthesis in a dose-dependent manner (Fig. 5*A*). This increase was statistically significant at 0.5 and 1.0 mmol/l malonic acid methyl ester ($P < 0.05$, $n = 6$), the maximum effect being reached at 0.5 mmol/l with a value 55% more than that at 16.7 mmol/l glucose. At higher concentrations, malonic acid methyl ester showed no effect on glucose-stimulated proinsulin biosynthesis. Malonic acid methyl ester had no effect on proinsulin biosynthesis at 2.8 mmol/l glucose (data not shown). It also had no effect on basal (data not shown) or glucose-stimulated (Fig. 5*B–D*) total protein synthesis, insulin secretion, or ATP content at any of the concentrations studied.

Succinate levels are elevated in glucose-stimulated rat islets. We investigated whether glucose increased the content of the Krebs cycle intermediates succinate, fumarate, and malate in isolated islets. A concentration of 16.7 mmol/l glucose significantly increased the content of succinate in islet cells by 40 ± 10% compared with 2.8 mmol/l glucose ($P < 0.05$, $n = 5$), and 1 mmol/l malonic acid methyl ester had no further effect (Table 2). Malate levels were increased with 16.7 mmol/l glucose by 17 ± 4% ($n = 4$), but this increase was not statistically significant, and the additional presence of 1 mmol/l malonic acid methyl

TABLE 1
IC_{50} of oxidative phosphorylation inhibitors

	Proinsulin biosynthesis	Total protein synthesis	Insulin secretion	ATP content
Rotenone (nmol/l)	39.0 ± 2.5 (3)	39.0 ± 1.0 (3)	13.2 ± 4.3 (6)	43.3 ± 6.2 (3)
Antimycin A1 (nmol/l)	31.3 ± 9.0 (3)	43.3 ± 7.5 (3)	218 ± 54 (5)	54.7 ± 7.4 (3)
DNP (μmol/l)	29.3 ± 2.7 (3)	32.3 ± 5.5 (3)	2.0 ± 0.3 (5)	33.3 ± 11.7 (3)
Oligomycin (ng/ml)	40 ± 10 (3)	60 ± 10 (4)	180 ± 40 (7)	170 ± 70 (3)

Values are means ± SE (number of replicates). Islets were preincubated at basal (2.8 mmol/l) glucose for 1 h and then incubated at 16.7 mmol/l glucose in the presence of increasing concentrations of the indicated inhibitor for an additional hour. Proinsulin biosynthesis, total protein synthesis, insulin secretion, and islet ATP content were determined as stated in RESEARCH DESIGN AND METHODS.

ester showed no effect. Intracellular fumarate levels were undetectable, even in an extract derived from 1,000 islets.

Effect of acetoacetate methyl ester on glucose-stimulated proinsulin biosynthesis, insulin secretion, and islet ATP content. Succinyl-CoA reacts with acetoacetate to produce succinate plus acetoacetyl-CoA in a reversible reaction. To determine whether this reaction involving succinate and succinyl-CoA may be part of the stimulus-coupling signaling mechanism for glucose-induced proinsulin biosynthesis, isolated islets were incubated at increasing glucose concentrations, from basal 2.8 mmol/l to stimulatory 16.7 mmol/l, in the presence of 10 mmol/l acetoacetic acid methyl ester. Proinsulin biosynthesis was decreased in the presence of acetoacetate at all glucose concentrations (data not shown). However, this reduction occurred at the same extent for total protein synthesis. Thus, it is unlikely that acetoacetate or acetoacetyl-CoA are specific coupling signals for glucose-stimulated proinsulin biosynthesis. Furthermore, 10 mmol/l acetoacetic acid methyl ester had no effect on basal or glucose-stimulated insulin secretion and islet ATP content.

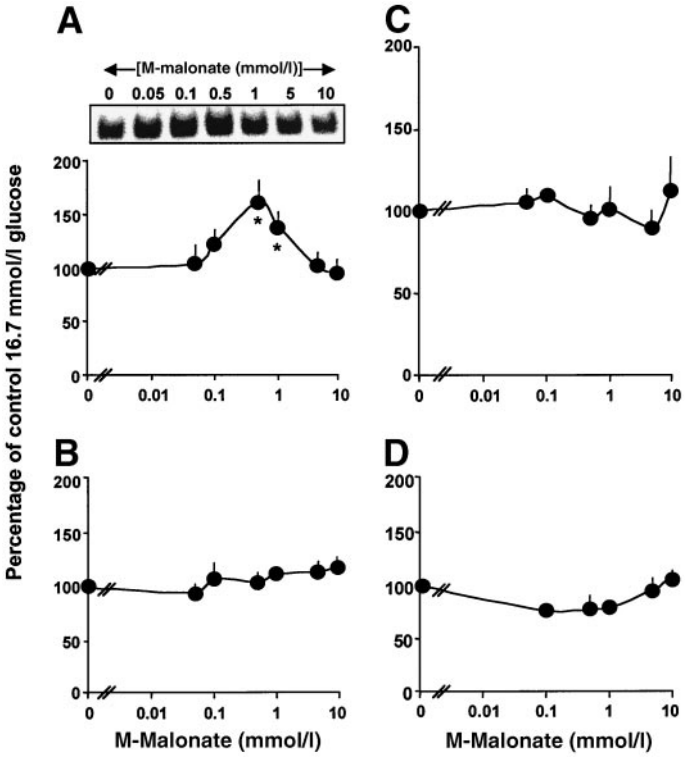

FIG. 5. Malonate dose-response effect on glucose-stimulated proinsulin biosynthesis, total protein synthesis, insulin secretion, and ATP content in isolated rat islets. Islets were preincubated for 1 h at basal (2.8 mmol/l) glucose and then incubated for 1 h in the presence of 16.7 mmol/l glucose plus the indicated concentration of malonic acid methyl ester (M-Malonate). Proinsulin biosynthesis (*A*), total protein synthesis (*B*), insulin secretion (*C*), and ATP content (*D*) were determined in islet lysates or islet incubation media as stated in the Fig. 1 legend and RESEARCH DESIGN AND METHODS. Results are means ± SE of three to eight independent experiments. *$P < 0.05$ vs. 16.7 mmol/l glucose.

Presence of a SCS activity in a rat islet cytosolic fraction. SCS has recently been identified in clonal β-cell mitochondria (30). SCS catalyzes the GDP(ADP)-dependent reversible reaction succinyl-CoA to succinate, which has been described to occur in the cell mitochondria (31). Here, we found the presence of SCS enzymatic activity in an enriched cytosolic fraction from isolated rat islets. The formation rate of CoA-SH from succinyl-CoA (0.31 ± 0.06 A^{415} units · min^{-1} · mg $protein^{-1}$) by an islet cytosol fraction was of the same extent as that from acetoacetyl-CoA (0.31 ± 0.06 A^{415} units · min^{-1} · mg $protein^{-1}$) but twofold faster than that from acetyl-CoA (0.14 ± 0.02 A^{415} units · min^{-1} · mg $protein^{-1}$). HPLC analysis of CoA-derivative formation from succinate, acetate, and acetoacetate by the S100 islet cytosol fraction showed that only succinyl-CoA is formed from succinate under these assay conditions (Fig. 6). The HPLC analysis showed no peak at the corresponding elution times for acetoacetyl-CoA and acetyl-CoA, regardless of whether succinate, acetate, or acetoacetate was used as a substrate (data not shown). However, a succinyl-CoA elution peak was observed after a 5-min incubation of succinate with the islet cytosol fraction in the presence of GTP (Fig. 6*B*). No succinyl-CoA peak was detected in the absence of GTP (Fig. 6*C*) or in the presence of ATP (not shown). These data indicate the presence of a GTP-dependent SCS activity in the islet cytosol fraction. From this HPLC analysis, the specific

TABLE 2
Succinate and malate content in glucose-stimulated rat pancreatic islets

	Succinate (pmol/μg of DNA)	Malate (pmol/μg of DNA)
n	5	4
2.8 mmol/l glucose	100.6 ± 4.8	52.1 ± 6.2
16.7 mmol/l glucose	140.1 ± 11.5*	61.3 ± 8.1
16.7 mmol/l glucose + 1 mmol/l malonate methyl ester	147.0 ± 16.9*	58.7 ± 10.0

Values are means ± SE. Isolated islets were preincubated for 1 h at 2.8 mmol/l glucose, followed by 1-h incubation in the presence of the indicated agent. Succinate and malate were extracted from islets with TCA and analyzed using a fluorometric enzyme assay as described previously (24) and in RESEARCH DESIGN AND METHODS. *$P < 0.05$ vs. 2.8 mmol/l glucose.

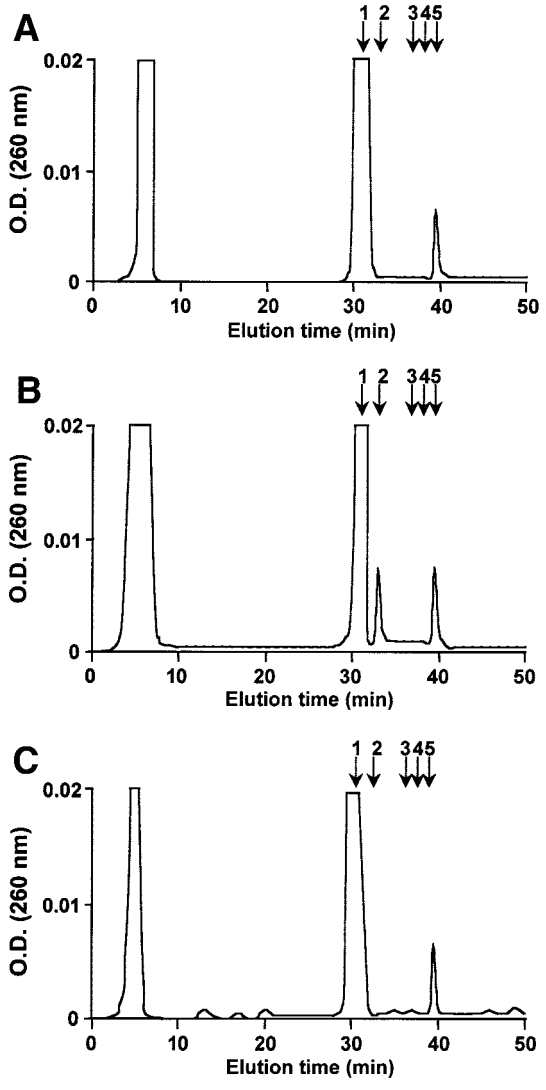

FIG. 6. HPLC analysis of succinyl-CoA formation from succinate by an islet cytosol fraction. Succinate to succinyl-CoA reaction was performed at 30°C in the presence of ~8 mg protein of islet cytosol fraction, 0.1 mmol/l CoA, 10 mmol/l Mg^{2+}, and 0.1 mmol/l GTP. *A*: Reaction time = 0 min. *B*: Reaction time = 5 min. *C*: Reaction time = 5 min, in the absence of GTP. The reaction products were analyzed in a Whatman EQC C-18 5-μm reverse-phase column over the following gradient: 0–5% solution B over 7 min, 5–12% solution B over 13 min, 12–20% solution B over 10 min, 20–25% solution B over 10 min, 25% solution B over 10 min. Solution B: 40% acetonitrile in 0.1 mol/l KH_2PO_4, pH 5. Arrows indicate the elution times of standards: CoA (1 and 5), succinyl-CoA (2), acetyl-CoA (3), and acetoacetyl-CoA (4). OD, optical density.

activity of SCS in the S100 islet cytosol fraction was 2.9 ± 0.3 nmol of succinyl-CoA · min^{-1} · mg $protein^{-1}$ vs. 10.2 ± 1.7 in the P10 mitochondrial-enriched fraction ($n \geq 3$). This represented ~25% of the total SCS specific activity recovered in the S100 cytosolic fraction, which did not correspond to the ≤5% mitochondrial contamination in this S100 fraction, as indicated by the mitochondria marker GDH activity (see RESEARCH DESIGN AND METHODS). As such, this suggested that a significant proportion of the SCS activity measured was derived from the islet cytosol and could not be accounted for by a nonspecific mitochondrial contamination in the S100 fraction.

DISCUSSION

The metabolic stimulus-coupling pathway for glucose-induced insulin release in the β-cell is reasonably well characterized and involves increased glucose metabolism, a rise in generation of mitochondrial ATP that consequently increases the cytosolic ATP/ADP ratio to close the ATP-sensitive K^+ channel (K_{ATP}-channel), which depolarizes the β-cell to open voltage-sensitive L-type Ca^{2+}-channels, causing an influx of Ca^{2+} to raise cytosolic $[Ca^{2+}]_i$, which acts as a major signal to trigger insulin exocytosis (8). However, the secondary metabolic coupling signals required to evoke proinsulin biosynthesis are essentially unknown.

Although ATP is necessary for protein synthesis, at least at the level of aminoacyl-tRNA and initiation complex formation (32,33), a role for ATP as a specific signal for glucose-stimulated proinsulin biosynthesis has not been proven. In this study, luciferase activity was used as relative indicator of intracellular ATP levels in islets, as previously described (21). This enabled the simultaneous measurement of insulin secretion, proinsulin biosynthesis, total protein synthesis, and fluctuations in ATP levels in the very same islets. However, although it is a convenient and sensitive method requiring fewer islet cells than more classical methods (21), it is recognized that this is a secondary measurement of ATP and does not reflect changes in the ATP/ADP ratio, which is the more relevant secondary signal for glucose-induced insulin release (8). Nonetheless, adenoviral-mediated expression of luciferase permitted comparative assessment of ATP levels in isolated islets, incubated under different conditions, relative to changes in proinsulin biosynthesis. Inhibitors of the electron transport chain at complexes I (rotenone) and III (antimycin A1) and the mitochondrial inner membrane uncoupler, DNP, provoked a parallel inhibition of islet ATP levels, total protein synthesis, and glucose-induced proinsulin biosynthesis. If ATP were to act as a stimulus-coupling factor for glucose-induced proinsulin biosynthesis, then one would predict that glucose-induced proinsulin biosynthesis would be more sensitive to inhibition by rotenone, antimycin A1, or DNP (i.e., have lower IC_{50} values) than total protein synthesis or ATP content. However, this was not the case because glucose-induced proinsulin biosynthesis, total protein synthesis, and islet ATP levels were similarly affected by rotenone, antimycin A1, or DNP (Table 1). This indicated that ATP was a necessary cofactor for general protein synthesis in islets but not a specific signaling intermediate for glucose-stimulated proinsulin biosynthesis translation. However, in contrast and somewhat surprisingly, glucose-stimulated insulin release was differentially affected by respiratory chain inhibitors and uncoupling effects by DNP when compared with glucose-induced proinsulin biosynthesis inhibition, total protein synthesis, and ATP levels. These data suggested that another signal originating from the electron transport chain, in addition to ATP, might also mediate metabolic stimulus coupling for glucose-stimulated insulin secretion. Such an additional mitochondrial-generated signal (e.g., reactive oxygen species [34]) would be in agreement with the existence of so-called K_{ATP}-channel and Ca^{2+}-independent pathways for the regulation of nutrient-stimulated insulin secretion (35,36).

Nevertheless, these data emphasize that metabolic stimulus-coupling pathways for glucose-induced proinsulin biosynthesis and insulin release are different downstream of glycolysis. Because a product of the β-cell mitochondrial electron transport chain/oxidative phosphorylation does not appear to act as a secondary coupling signal for glucose-stimulated proinsulin biosynthesis, such a signal is more likely to emanate from β-cell anaplerosis (3).

Certain esterified Krebs cycle metabolites are potent insulin secretagogues (37,38). Glutamate, formed in the mitochondria from α-ketoglutarate during glucose metabolism, has also been recently proposed to act as a stimulus-coupling factor for glucose-induced insulin release (16), although this is currently controversial (18). However, no effect of glutamate on glucose-induced proinsulin biosynthesis was found. As such, glutamate was unlikely to act as a coupling signal for glucose-induced proinsulin biosynthesis. Unlike insulin secretion, the only Krebs cycle metabolite that stimulated proinsulin biosynthesis independently of glucose was succinic acid monomethyl ester. Consistent with the majority of previous studies (1,2), neither glucose-induced nor succinic acid monomethyl ester–induced proinsulin biosynthesis could be attributed to a positive feedback of secreted insulin on the β-cell. Inhibition of glucose-induced and succinic acid monomethyl ester–induced insulin release by calcium depletion or somatostatin addition had no effect on stimulation of proinsulin biosynthesis in the very same islets. Thus, it was more likely that succinate-induced proinsulin biosynthesis was a direct consequence of increased succinate metabolism. Intriguingly, malonate, an inhibitor of the enzyme FAD-linked succinate dehydrogenase at complex II of the electron transport chain (29), potentiated glucose-stimulated proinsulin biosynthesis in a dose-dependent manner, whereas it had no effect on glucose-stimulated insulin secretion, total protein synthesis, or islet ATP content. Malonate-induced specific inhibition of succinate dehydrogenase could result in the accumulation of intracellular succinate in islet β-cells and consequently enhance glucose-stimulated proinsulin biosynthesis. The lack of effect of malonate at concentrations >1 mmol/l would likely be a result of additional inhibition of succinate export from the mitochondria, as both succinate and malonate are substrates of the same dicarboxylic acid transporter (39,40). Thus, the data are consistent with the idea that not only increased mitochondrial succinate generation but also its export from β-cell mitochondria to the cytosolic compartment are important for a metabolic coupling signal specific for proinsulin biosynthesis. This is an important consideration because a metabolic secondary signal for proinsulin biosynthesis would have to communicate with the protein synthesis translational control machinery present in the cytosol of β-cells (1).

As a candidate metabolic coupling factor for glucose-induced proinsulin synthesis, intracellular succinate should increase as glucose levels rise. Indeed, in this study, glucose caused a significant increase in the total islet content of succinate, whereas that of malate was unaffected. Besides, the intracellular concentration of succinate in islets was twofold higher than that of malate, and intracellular levels of fumarate were beyond the limit of detection and as such very low. This suggested that the succinate dehydrogenase catalytic step in the Krebs cycle of β-cell mitochondria was a rate-limiting point. However, malonate at a concentration that enhanced glucose-stimulated proinsulin biosynthesis did not further raise glucose-increased succinate levels. Nonetheless, this may be explained by the dynamic flux of succinate to succinyl-CoA. The presence of an enzymatic activity in islet cell cytosol that is able to synthesize succinyl-CoA specifically from succinate (but not from acetate, acetyl-CoA, acetoacetate, or acetoacetyl-CoA) opens the possibility of increased cytosolic succinyl-CoA acting as a metabolic coupling signal specific for glucose-induced increases in proinsulin biosynthesis. It has been shown that glucose increases succinyl-CoA levels in perifused rat islets (26) and the clonal pancreatic β-cell line HIT (12). Interestingly, the levels of malonyl-CoA, proposed to be a glucose stimulus-coupling signal for insulin secretion (12,13), were maximally increased after 10 min exposure to glucose, whereas the rise of succinyl-CoA levels was slower, with the maximum being reached after 30 min. It should be noted that the kinetic response of the β-cell to glucose is faster for the stimulation of insulin secretion (~2 min) than that of proinsulin biosynthesis, which has a 15- to 20-min lag period (20,41). As such, the kinetics for a glucose-induced increase in succinyl-CoA levels are compatible for upregulating proinsulin biosynthesis at the translational level rather than insulin secretion.

In summary, these data support the notion that succinate is accumulated in the β-cell mitochondria as a consequence of increased glucose metabolism and exported into the cytosol, where it is converted to succinyl-CoA and can then act as a stimulus-coupling secondary signal for glucose-induced proinsulin biosynthesis. Such a hypothesis is intriguing but requires further experimentation for substantiation. Nonetheless, we propose succinate and/or succinyl-CoA as the most promising candidates for a metabolic stimulus-coupling signal specific for glucose-induced proinsulin biosynthesis. Under this premise, it might be postulated that chronic hyperglycemia may result in a persistent accumulation of succinate/succinyl-CoA that in turn could cause dysregulation of the proinsulin biosynthesis observed in type 2 diabetes. Such a scenario could explain the marked increase of basal proinsulin biosynthesis observed in isolated islets from a hyperglycemic rat model of type 2 diabetes (42). In addition, this study presents a new point of divergence in the stimulus-coupling signaling for glucose-induced proinsulin biosynthesis versus insulin secretion at the level of anaplerosis, downstream of glycolysis and upstream of oxidative phosphorylation, where succinate would be the preferential anaplerotic signal specific for regulating glucose-induced proinsulin biosynthesis.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK47919 and DK50610, the Canadian Institute of Health Research, and a Juvenile Diabetes Foundation International Fellowship to B.W. M.P. is a Canadian Institute of Health Research scientist.

We thank Dr. Jude Deeney and Vera Schulz for their excellent assistance with succinate, fumarate, and malate islet content analysis.

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