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Engineering of Glycerol-stimulated Insulin Secretion in Islet Beta Cells

DIFFERENTIAL METABOLIC FATES OF GLUCOSE AND GLYCEROL PROVIDE INSIGHT INTO MECHANISMS OF STIMULUS-SECRETION COUPLING*

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Insulin secretion from beta cells in the islets of Langerhans can be stimulated by a number of metabolic fuels, including glucose and glyceraldehyde, and is thought to be mediated by metabolism of the secretagogues and an attendant increase in the ATP:ADP ratio. Curiously, glycerol fails to stimulate insulin secretion, even though it has been reported that islets contain abundant glycerol kinase activity and oxidize glycerol efficiently. We have reinvestigated this point and find that rat islets and the well differentiated insulinoma cell line INS-1 contain negligible glycerol kinase activity. A recombinant adenovirus containing the bacterial glycerol kinase gene (AdCMV-GlpK) was constructed and used to express the enzyme in islets and INS-1 cells, resulting in insulin secretion in response to glycerol. In AdCMV-GlpK-treated INS-1 cells a greater proportion of glycerol is converted to lactate and a lesser proportion is oxidized compared with glucose. The two fuels are equally potent as insulin secretagogues, despite the fact that oxidation of glycerol at its maximally effective dose (2–5 mM) occurs at a rate that is similar to the rate of glucose oxidation at its basal, nonstimulatory concentration (3 mM). We also investigated the possibility that glycerol may signal via expansion of the glycerol phosphate pool to allow enhanced fatty acid esterification and formation of complex lipids. Addition of Triacsin-C, an inhibitor of long-chain acyl-CoA synthetase, to AdCMV-GlpK-treated INS-1 cells did not inhibit glycerol-stimulated insulin secretion despite the fact that it blocked glycerol incorporation into cellular lipids. We conclude from these studies that glycerol kinase expression is sufficient to activate glycerol signaling in beta cells, showing that the failure of normal islets to respond to this substrate is due to a lack of this enzyme activity. Further, our studies show that glycerol signaling is not linked to esterification or oxidation of the substrate, but is likely mediated by its metabolism in the glycerol phosphate shuttle and/or the distal portion of the glycolytic pathway, either of which can lead to production of ATP and an increased ATP:ADP ratio.

According to the fuel hypothesis of insulin secretion, release of the hormone is stimulated only by metabolic fuels that are actively metabolized in islet beta cells. In support of this notion, glucose and glyceraldehyde stimulate insulin secretion in proportion to their rates of metabolism (1). A working model of stimulus-secretion coupling in the beta cell is that fuel metabolism leads to an increase in the ATP:ADP ratio, which causes closure of ATP-sensitive K^+ channels (K_{ATP} channels) and activation of voltage-gated Ca^{2+} channels, influx of extracellular Ca^{2+}, and activation of the exocytotic machinery (reviewed in Refs. 1 and 2). Early studies in which mitochondrial uncouplers were found to block glucose-stimulated insulin secretion led to the assumption that fuel oxidation and mitochondrial ATP production were key events in insulin release (reviewed in Refs. 1–3). In the context of this model, the poor insulinotropic effect of pyruvate, despite its efficient oxidation in islets, has never been adequately explained. Subsequently, several lines of investigation have suggested that ATP produced from glycolysis constitutes the critical signal for K_{ATP} channel closure and initiation of insulin secretion, while ATP produced via mitochondrial metabolism plays a secondary role of maintaining sufficient energy to drive secretory granule exocytosis (4, 5). That "glycolytic" ATP more effectively regulates K_{ATP} channels than "mitochondrial" ATP is a concept originally introduced from studies in isolated myocytes (6). However, since other recent studies have provided data that seem to affirm the importance of mitochondrially derived ATP in beta cell stimulus-secretion coupling (7), the matter remains unresolved.

In addition to fuel signaling via ATP produced by glycolysis or fuel oxidation, two other pathways for glucose-stimulated insulin secretion have been considered. First, a link between glucose and fatty acid metabolism has been implicated as a critical component of the glucose sensing response (2, 8–10). Glucose has been shown to increase the level of malonyl-CoA, which causes inhibition of carnitine palmitoyltransferase I and diversion of long-chain acyl-CoAs away from oxidation and into esterification (8–10). Phospholipids derived in this way could participate in stimulation of insulin secretion by allowing membrane lipid turnover during granule exocytosis, or by generation of bioactive byproducts such as diacylglycerol or inositol 1,4,5-trisphosphate. Second, because of the very high activities of mitochondrial glycerol phosphate dehydrogenase in islets cells, it has been suggested that transfer of reducing equivalents from the cytosol to the mitochondria may occur with high efficiency in such cells, and that FADH produced as a byproduct of the mitochondrial glycerol phosphate dehydrogenase reaction can serve a signaling function by entering site II of the electron transport chain, resulting in generation of ATP (11–13).

In the current study, we have sought to create a new model

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of insulin secretion that can be used to determine which of the four signaling pathways named above are actually operative in fuel-stimulated beta cells. Despite the potent effects of glyceraldehyde on insulin secretion, islets are unable to respond to another simple triose, glycerol. This is puzzling in light of studies by Malaisse and co-workers suggesting that islets and insulinoma cell lines contain substantial levels of glycerol kinase and oxidize glycerol efficiently (14, 15). Our laboratory has been developing the recombinant adenovirus system as a tool for metabolic engineering of mammalian cells, including islet beta cells (16–18). To determine the effect of glycerol kinase expression on beta cell responses to glycerol, we have used the adenovirus system to introduce the gene encoding the bacterial form of the enzyme into isolated rat islets or well differentiated insulinoma cells. While control rat islets or INS-1 cells metabolize glycerol at a very low rate and exhibit no response to it as a secretagogue, adenovirus-mediated expression of glycerol kinase in such cells markedly enhances glycerol flux and confers glycerol-stimulated insulin secretion. Clear differences between the metabolic fates of glucose and glycerol in such engineered cells provide new insight into the important metabolic signaling events for fuel-mediated insulin secretion.

MATERIALS AND METHODS

Construction of AdCMV-GlpK—A plasmid pWT165 containing the gene encoding *Escherichia coli* glycerol kinase (*glpK*) was obtained from Dr. Donald Pettigrew, Texas A & M University (19). The intact glycerol kinase insert was amplified by polymerase chain reaction using oligonucleotides 5′-GAAGGTACCTTCATGACTGAAAAAAAATATATCGTT-3′ and 5′-TGCAAGCTTTTATTCGTCGTGTTCTTCCCACGCCAT-3′. The oligonucleotides used for amplification contained 5′ *Kpn*I and 3′ *Hin*dIII endonuclease sites, which allowed ligation into similarly treated pACCMV.pLpA (20). The new recombinant virus (AdCMV-GlpK) was produced by homologous recombination of the pACCMV.pLpA plasmid containing the glycerol kinase insert with pJM17 (21) in cotransfected 293 cells and purified by CsCl density equilibrium ultracentrifugation as described previously (16).

Culture and Viral Treatment of INS-1 Cells—INS-1 rat insulinoma cells were obtained from Drs. Claes Wollheim and Philippe Halban, University of Geneva (22). Cells were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (22). Experiments were conducted by plating the cells into 12-well dishes and treating them at approximately 75% confluence with AdCMV-GlpK virus or, as a control, the AdCMV-βGAL virus containing the bacterial β-galactosidase gene (23). Cells were exposed to one or the other of the recombinant viruses for 1 h in the supplemented RPMI medium described above. The virus-containing medium was then aspirated, and the cells were washed once with fresh medium and then cultured in fresh medium for 48 h to allow the transgenes to be expressed.

RNA Blot Hybridization Analysis—Total RNA was extracted from AdCMV-GlpK or AdCMV-βGAL-treated INS-1 cells with the TRIzol kit (Life Technologies, Inc.). Samples of 20 μg of RNA were resolved on agarose/formaldehyde gels, transferred to nylon supporting membranes by capillary action, and cross-linked by UV irradiation. Randomly primed ^{32}P-labeled probes for rat GLUT-2 (a 1063-base pair *Hin*dIII fragment within the protein coding region of the cDNA) (24), and bacterial glycerol kinase (the full-length 1514-base pair gene) (19) were hybridized to RNA-containing membranes using previously described procedures (25), and hybridization signals were detected by autoradiography.

Glycerol Kinase Activity Assay—Cells were suspended in extraction buffer (50 mM HEPES, 40 mM KCl, 11 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, pH 7.8) and snap frozen in liquid nitrogen. The cell suspensions were thawed on ice, and cellular debris pelleted by centrifugation (15 min, 20,000 $\times$ *g*, 4 °C). Protein levels in the resultant supernatant were quantified by the Bradford assay (Bio-Rad) (26). Glycerol kinase activity was measured by modification of a radioisotopic assay that is routinely used for measurement of glucose phosphorylation (27). Briefly, samples of the extract supernatant containing 10 μg of total protein in 7–10 μl of extraction buffer were added to 50-μl assay buffer (100 mM Tris, 5 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 2.5 mM dithiothreitol, 4 mM glycerol, pH 7.2) for 2 h at room temperature, after which the enzymatic reaction was terminated by the addition of 100 μl of 97% ethanol, 3% methanol. 30 μl of the alcohol-treated reaction mix were spotted onto circular DEAE filters (Schlieicher & Schuell) and allowed to air dry. Filters were washed overnight in distilled water and allowed to air dry the next morning. Dried filters were placed in scintillation vials and counted in 10 ml of BioSafe II scintillation mixture (Research Products International, Mount Prospect, IL).

Insulin Secretion Assay—Cells were preincubated in 1 ml of Hepes/bicarbonate balanced salt solution (HBBSS)[1] consisting of 20 mM HEPES, 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, 0.2% bovine serum albumin, pH 7.4, with 2 mM glucose for 1 h prior to a 3-h incubation with 500 μl of HBBSS containing various secretagogues. In some experiments, 10 μM Triacsin-C (BioMol, Plymouth Meeting, PA) was included in both the preincubation and secretagogue buffers. Following the 3-h incubation, the HBBSS medium was transferred to Eppendorf-type tubes and centrifuged for 3 min at 3,000 $\times$ *g*. An aliquot of the supernatant was transferred to a new tube, and 50 μl were diluted 1:4 for radioimmunoassay utilizing tubes precoated with anti-human insulin IgG (Diagnostic Products Corporation, Los Angeles, CA). A standard curve was generated with rat insulin and was linear over the range of 5–400 microunits/ml insulin. Insulin secretion was normalized to total cellular protein (26).

Metabolic Assays—Lactate accumulation was measured by sampling of 50–75 μl of HBBSS at the conclusion of the 3-h static incubation studies described above. Oxidation assays were performed as described previously (28). Briefly, approximately 5×10^5 INS-1 cells ($\approx$100 μg of protein) were transferred to a center well plastic cup connected to a rubber sleeve stopper. Approximately 10^5 cpm of ^{14}C-labeled substrate ([U-^{14}C]glucose or [U-^{14}C]glycerol) was added to the suspension, and the cup was sealed within a 20-ml borosilicate glass scintillation vial by means of the sleeve stopper. Vials were incubated at 37 °C for 2 h, after which 100 μl of $HClO_4$ were added to the cells in the cup, followed by addition of 300 μl of methanolic hyamine hydroxide (1 M) to the bottom of the vials (both compounds were injected into the sealed vials through the sleeve stopper). Following a further 2 h of incubation at 37 °C, vials were unsealed, cups were removed and discarded, and 10 of ml BioSafe II scintillation mixture were added. Vials were allowed to equilibrate overnight, prior to liquid scintillation counting. Glycerol usage was assayed by inclusion of tracer [2-^{3}H]glycerol ($\sim$2500 cpm/nmol) in the aforementioned insulin secretion assay and measurement of ^3H_2O production by previously described methods (17, 18).

Glycerol Incorporation into Cellular Lipids—Following a 1 h preincubation in culture medium containing 2 mM glucose with or without 10 μM Triacsin-C, cells were incubated in culture medium with added [2-^{3}H]- or [U-^{14}C]glycerol for 3 h. The reaction was terminated by aspiration of the culture medium and addition of 20% methanol in phosphate-buffered saline. Cells were collected, washed with phosphate-buffered saline, resuspended in 250 μl of 0.2 M NaCl and snap-frozen in liquid nitrogen. 750 μl of chloroform:methanol (2:1) were added to the thawed cell suspension, and the mixture was vortexed and centrifuged for 5 min at 6,000 $\times$ *g*. The top (aqueous) fraction was transferred to new tubes, and the bottom (lipid) layer was washed once with methanol:chloroform:water (48:3:47). [2-^{3}H]Glycerol incorporated into these fractions was quantified by liquid scintillation counting in 10 ml of BioSafe II scintillation mixture.

Islet Perifusion—Pancreatic islets were isolated from 150–200-g Wistar rats, placed in culture, and treated with the AdCMV-GlpK and AdCMV-βGAL recombinant adenoviruses as described previously (17, 18). After exposure to virus, islets were cultured for an additional 48 h, after which 300–600 islets were transferred into Swinnex chambers and submerged in a 37 °C water bath. Islets were perifused at a flow rate of 0.8 ml/min, beginning with a 15-min equilibration with HBBSS supplemented with 2 mM glucose, and followed by perifusion with HBBSS supplemented with various secretagogues as indicated in the legend to Fig. 2. Fractions were collected at 1-min intervals, and 200 μl of each sample were used for insulin radioimmunoassay as described above.

Statistical Analysis—Two-tailed, two-sample Student's *t* test with pooled estimator of common variance was used for statistical comparison of experimental groups.

[1] The abbreviations used are: HBBSS, Hepes/bicarbonate balanced salt solution; m.o.i., multiplicity of infection.

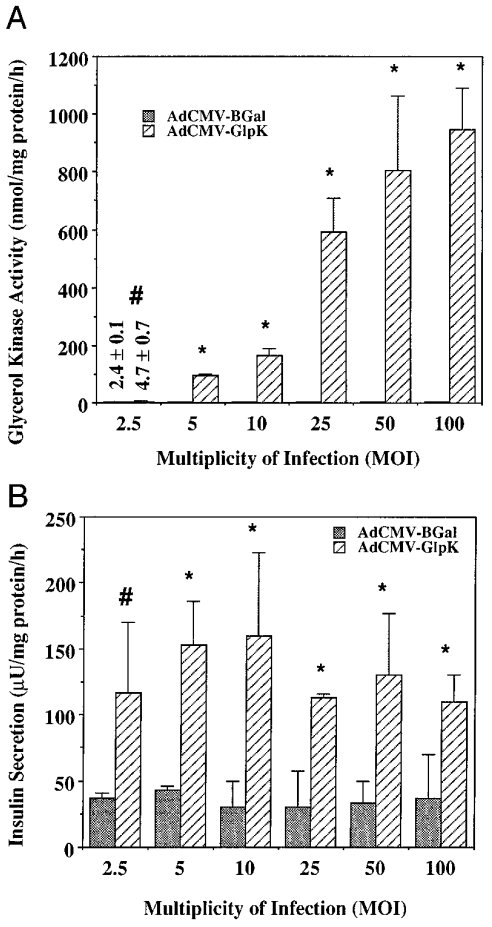

FIG. 1. **Glycerol kinase activity and glycerol-stimulated insulin secretion from AdCMV-GlpK-treated INS-1 cells.** *A*, INS-1 cells were treated with AdCMV-βGAL or AdCMV-GlpK at the indicated multiplicity of infection (*MOI*) for 1 h and studied 48 h later. Glycerol kinase enzyme activity was measured as described under "Materials and Methods." *B*, the same cells studied in *A* were subjected to static incubation assays for insulin secretion prior to homogenization for measurement of glycerol kinase enzyme activity. AdCMV-βGAL- or AdCMV-GlpK-treated cells were incubated in 3 mM glucose + 5 mM glycerol. Note the enhanced response to this secretagogue mixture in all cells treated with the AdCMV-GlpK virus. For both panels, values represent the mean ± S.E. for three independent experiments, and differences between the AdCMV-GlpK and AdCMV-βGAL groups at levels of significance of $p < 0.1$ (#) and $p < 0.05$ (*) are indicated.

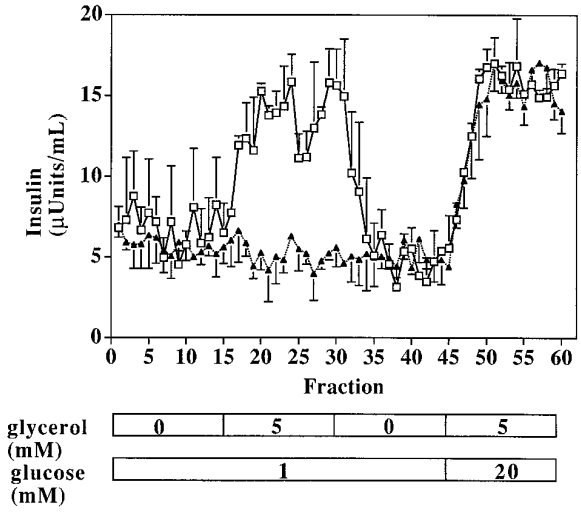

FIG. 2. **Glycerol-stimulated insulin secretion from perifused rat islets.** Freshly isolated rat islets were treated with AdCMV-βGAL (▲) or AdCMV-GlpK (□) as described under "Materials and Methods," and perifusion studies were conducted 48 h after viral treatment. Islets were perifused at a flow rate of 0.8 ml/min, and fractions were collected at 1-min intervals for measurement of insulin. The perifusate additions are noted at the *bottom* of the figure. Note that only AdCMV-GlpK-treated islets increased insulin secretion in response to the addition of 5 mM glycerol to the basal perifusate (containing 1 mM glucose), while both groups of islets responded to the combination of 5 mM glycerol and 20 mM glucose. Data represent the mean ± S.E. for three independent groups of islets per viral treatment.

RESULTS AND DISCUSSION

Adenovirus-mediated Expression of Glycerol Kinase in INS-1 Cells—The capacity of AdCMV-GlpK to direct expression of glycerol kinase mRNA in INS-1 cells was evaluated by RNA blot hybridization analysis and measurement of enzyme activity. A radiolabeled probe prepared from the GLUT-2 glucose transporter cDNA hybridized to a single 2.6-kilobase pair transcript in both AdCMV-βGAL- and AdCMV-GlpK-treated INS-1 cells. In contrast, a probe prepared from the bacterial glycerol kinase gene hybridized to a 2.2-kilobase pair transcript only in the AdCMV-GlpK-treated cells (data not shown). The level of glycerol kinase enzymatic activity was proportional to the multiplicity of infection (m.o.i.) of AdCMV-GlpK (Fig. 1*A*). Thus the lowest m.o.i. tested (2.5 plaque forming units/cell) caused an approximate doubling of glycerol kinase activity above the background level measured in AdCMV-βGAL-treated cells. Treatment of cells with higher viral titers caused a progressive increase in glycerol kinase activity, such that at an m.o.i. of 100, the activity was more than two orders of magnitude higher than that measured in cells treated with AdCMV-βGAL. Use of the AdCMV-βGAL virus at an m.o.i. of 25 and staining of the cells with the β-galactosidase chromogenic substrate revealed essentially 100% efficiency of gene transfer in INS-1 cells (data not shown), as we have previously shown for other cell lines (29).

Glycerol-stimulated Insulin Secretion from AdCMV-GlpK-treated INS-1 Cells—Insulin secretion in response to the combination of 3 mM glucose and 5 mM glycerol was assayed in cells treated with AdCMV-GlpK or AdCMV-βGAL over the same range of m.o.i. values as studied for glycerol kinase activity. We chose to include 3 mM glucose because this concentration is itself not stimulatory for insulin secretion, but does provide a basal level of the hexose (22) (see also data of Fig. 3). All INS-1 cells treated with the control virus AdCMV-βGAL secreted the same amount of insulin (approximately 35 microunits/mg of protein/h) regardless of the m.o.i. used (Fig. 1*B*). Treatment of cells with AdCMV-GlpK increased insulin secretion by approximately 4-fold relative to AdCMV-βGAL-treated cells, to around 150 microunits/mg of protein/h. Interestingly, for the range of m.o.i. chosen, the lowest viral titer used was equally effective as the highest in conferring enhanced responsiveness to the combination of fuels. We conclude that expression of glycerol kinase in INS-1 cells, even at relatively low levels, confers a glycerol-stimulated insulin secretion response that is absent in control cells. An m.o.i. of 25, falling in the middle of the range studied, was chosen for all subsequent experiments.

Glycerol-stimulated Insulin Secretion from AdCMV-GlpK-treated Rat Islets—To determine whether glycerol kinase expression confers glycerol-stimulated insulin secretion in normal islets as it does in INS-1 cells, pancreatic islets were isolated from normal Wistar rats and treated with AdCMV-GlpK or AdCMV-βGAL. Four days later, insulin secretion was studied by perifusion (Fig. 2). Basal insulin secretion during perifusion with 1 mM glucose was the same in AdCMV-GlpK- and AdCMV-βGAL-treated islets. Addition of 5 mM glycerol to this basal perifusate resulted in an approximate 2-fold increase in insulin secretion over the base line from AdCMV-GlpK-treated cells (from 6.9 ± 1.2 to 13.7 ± 1.7 microunits/ml, measured as the average insulin secreted over the respective

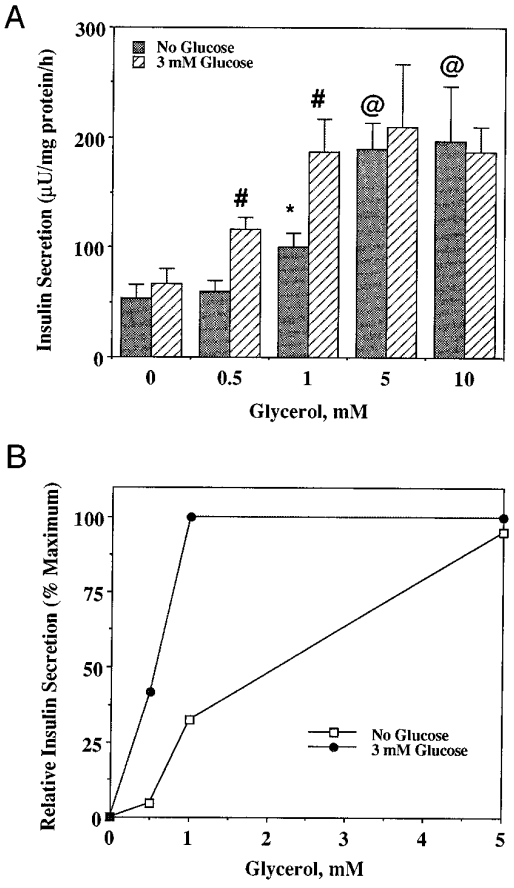

FIG. 3. **Dose response curve for glycerol-stimulated insulin secretion in the presence and absence of 3 mM glucose.** INS-1 cells were treated with AdCMV-GlpK, and insulin secretion was studied in response to a range of glycerol concentrations in the presence or absence of 3 mM glucose. *A*, insulin secretion expressed as microunits/mg of protein/h. Note the legend at the *top left side* of the panel. Data represent the mean ± S.E. for six independent experiments. Symbols indicate significant differences between cells incubated with and without glucose (#$p < 0.001$) or differences between groups incubated with glycerol alone relative to incubation in the absence of glycerol (*$p < 0.01$; @$p < 0.001$). *B*, the data of *A* are replotted as relative insulin secretion, defined as the percent of maximal insulin secretion achieved at each substrate concentration in the no glucose and 3 mM glucose groups, respectively. Note the left shift in glycerol response induced by incubation with 3 mM glucose, despite the lack of secretory effect of 3 mM glucose alone.

15-min perifusion periods), but had no effect on insulin secretion from AdCMV-βGAL-treated cells. Return to the basal perifusate (1 mM glucose without glycerol) restored insulin secretion to the original base line in AdCMV-GlpK-treated cells. Finally, addition of 20 mM glucose and 5 mM glycerol to the perifusion buffer stimulated insulin secretion to a similar degree in AdCMV-GlpK- and AdCMV-βGAL-treated islets (2.7- and 2.9-fold, respectively). At the end of the study, islets were recovered from the perifusion chambers and assayed for glycerol kinase activity.

Homogenates from islets transduced with AdCMV-GlpK contained glycerol phosphorylating activity of 371 ± 36 nmol glycerol/mg of protein/h, while no glycerol kinase activity was detectable in homogenates from AdCMV-βGAL-transduced islets. These data demonstrate that INS-1 cells and isolated islets are similar in that they do not normally express glycerol kinase at detectable levels or respond to glycerol by secreting insulin, but both cell preparations gain a glycerol-stimulated insulin secretion response upon expression of the glycerol kinase gene. Based on these findings, all further studies were conducted with INS-1 cells.

Insulin Secretion in Response to Glucose and Glycerol—We next determined the glycerol concentration dependence of insulin secretion from AdCMV-GlpK-treated INS-1 cells. These studies were conducted in the presence and absence of 3 mM glucose and are summarized in Fig. 3. In the absence of glucose, 0.5 mM glycerol had no effect on insulin secretion, but 1 mM glycerol caused a 1.7-fold increase in insulin secretion relative to the base line level of 53 microunits/mg of protein/h, while 5 mM glycerol caused the maximal 3.6-fold enhancement (Fig. 3*A*). In other experiments that included glycerol concentrations between 1 and 5 mM, 2 mM glycerol was found to be sufficient to stimulate maximal insulin release (see Fig. 5). Addition of 3 mM glucose in the absence of glycerol had no effect on insulin secretion relative to cells incubated in the complete absence of substrates. However, inclusion of this nonstimulatory concentration of glucose caused a shift in the glycerol dose response, such that 0.5 mM glycerol now caused a 1.8-fold increase in secretion over the baseline of 67 microunits/mg of protein/h achieved by 3 mM glucose alone ($p < 0.001$). Furthermore, maximal insulin secretion, equivalent to that achieved with 5 or 10 mM glycerol alone, was achieved at 1 mM glycerol when 3 mM glucose was present (Fig. 3*A*). In parallel experiments, a maximal stimulatory effect of glucose on insulin secretion of approximately 4-fold above base line, similar to the maximal glycerol-stimulated response, was observed at concentrations of ≥ 10 mM glucose (data not shown). A clear illustration of the left shift in the glycerol response curve that is elicited by the inclusion of a glucose concentration (3 mM) that is itself not stimulatory is provided by plotting the data as relative insulin secretion *versus* glycerol concentration (Fig. 3*B*). Thus, glucose potentiates insulin secretion at low glycerol levels, but mixing of the two substrates does not increase maximal insulin output beyond that achieved with high levels of glucose or glycerol alone.

Comparison of Metabolic Fates of Glucose and Glycerol in AdCMV-GlpK-Treated Cells—Expression of glycerol kinase in islet beta cells allows glycerol to function as a secretagogue with a potency similar to that of glucose (see Figs. 1–3). To learn more about the signal transduction pathways involved, we carried out a series of experiments in which we compared the metabolic fate of glucose and glycerol in these cells. As shown in Fig. 4*A*, at the doses that are maximally effective for stimulation of insulin secretion, more [U-^{14}C]glucose is oxidized than [U-^{14}C]glycerol in AdCMV-GlpK-treated INS-1 cells, expressing the data as total nanomoles of CO_2 produced/mg of cellular protein/h. Thus, glucose was oxidized to produce 75 nmol of CO_2/mg of protein/h at basal glucose levels (3 mM) and 162 nmol of CO_2/mg of protein/h when glucose was raised to 10 mM (a further increase in glucose to 20 mM did not result in any further enhancement in glucose oxidation). In comparison, glycerol was oxidized at a rate of 44 nmol of CO_2/mg of protein/h at basal glycerol concentrations (0.5 mM), and at a rate of around 64 of nmol CO_2/mg of protein/h when glycerol was raised to a fully stimulatory concentration of 2–5 mM. Thus, glucose oxidation at 10 mM glucose was approximately 2.5 times that of glycerol oxidation at 5 mM glycerol, regardless of whether the data were expressed as nanomoles of CO_2 produced, as in Fig. 4*A*, or as triose equivalents oxidized (54 nmol of triose equivalents/mg of protein/h for 10 mM glucose *versus* 21 nmol of triose equivalents/mg of protein/h for 5 mM glycerol). Interestingly, the rate of glycerol oxidation at 2 or 5 mM glycerol (maximally stimulatory for insulin secretion) was approximately equal to the rate of glucose oxidation at 3 mM glucose (nonstimulatory for insulin secretion). Further, little change in the rate of glycerol oxidation was observed as glycerol was raised from a nonstimulatory concentration (0.5 mM) to a fully stimulatory level (5 mM). These data strongly suggest

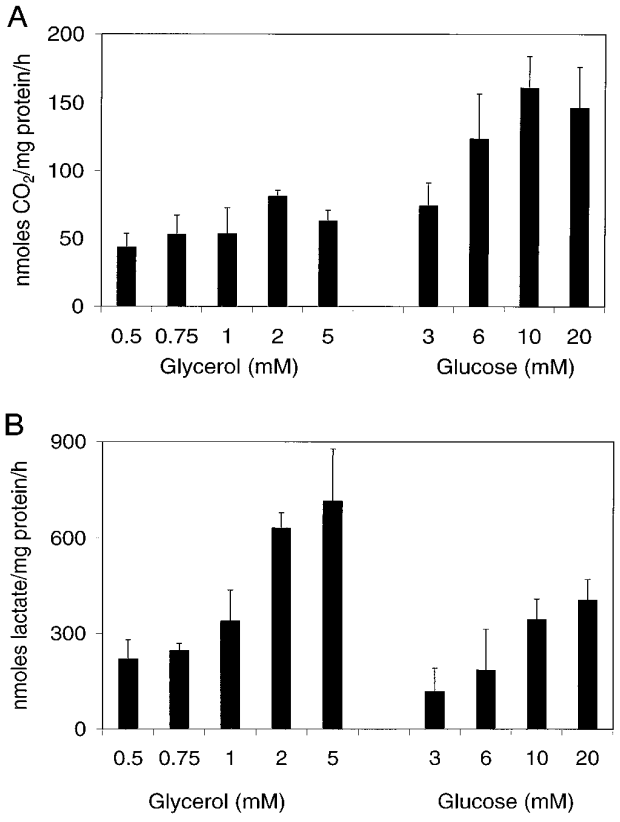

FIG. 4. **Glucose and glycerol metabolism in AdCMV-GlpK-treated INS-1 cells.** *A*, oxidation of varying concentrations of [U-^{14}C] glycerol (*left*) and [U-^{14}C]glucose (*right*) by AdCMV-GlpK-treated INS-1 cells. Data are expressed as total nanomoles of CO_2 produced/mg of protein/h for each substrate. Note that CO_2 production at concentrations of glycerol that are maximallly stimulatory for insulin secretion (2–5 mM) are similar to the CO_2 produced at a concentration of glucose that is nonstimulatory for insulin secretion (3 mM). *B*, lactate produced at varying concentrations of glycerol (*left*) and glucose (*right*) from the same AdCMV-GlpK-treated INS-1 cells studied in *A*. Note that more glycerol is converted to lactate than glucose, the inverse of the oxidation data. For both panels, data represent the mean ± S.E. of three independent groups of experiments, each performed in triplicate.

that fuel stimulation of insulin secretion is not strictly linked to a specific threshold of fuel oxidation.

The pattern for lactate production was opposite to that of oxidation (Fig. 4*B*). Thus, lactate output from AdCMV-GlpK-treated INS-1 cells incubated at basal glycerol (0.5 mM) was 223 nmol/mg of protein/h, while raising the glycerol concentration to 2–5 mM raised lactate output in these cells to 718 nmol/mg of protein/h. Note that glycerol metabolism to lactate was maximal in the same range of substrate concentrations required for maximal glycerol oxidation (2–5 mM), consistent with the relatively low K_m of bacterial glycerol kinase for glycerol (approximately 10–50 μM) (30). Lactate production from glucose was clearly lower, being 120 nmol/mg of protein/h at basal glucose (3 mM) and 409 nmol/mg of protein/h at a maximally stimulatory glucose concentration (20 mM).

We interpret our metabolic data as follows. The enhanced propensity for lactate production from glycerol may be related to the conversion of glycerol phosphate to dihydroxyacetone phosphate via the cytosolic glycerol phosphate dehydrogenase, with the attendant reduction of NAD to NADH. To the extent that glycerol phosphate is converted to dihydroxyacetone phosphate via this reaction rather than by mitochondrial glycerol phosphate dehydrogenase (which utilizes FAD as co-factor), a deficit in NAD as cofactor for running the glyceraldehyde phosphate dehydrogenase reaction may occur that must be compensated for by reoxidation of the surplus NADH in the lactate dehydrogenase reaction. Despite this diversion of pyruvate produced from glycerol away from mitochondrial oxidation and into lactate formation, glycerol retains full secretory potency. These results suggest that fuel-mediated insulin secretion is not dictated simply by the absolute rate of fuel oxidation, since rates of glycerol oxidation at maximally stimulatory concentrations of the fuel are equivalent to the rates of glucose oxidation observed at basal, nonstimulatory concentrations of the hexose. These data suggest that the common signal derived from glycerol and glucose metabolism is generated via enhanced flux through the glycerol shuttle or the distal portion of glycolysis and not from mitochondrial oxidation of pyruvate.

The FAD-linked mitochondrial glycerol phosphate dehydrogenase is one of several Ca^{2+}-dependent mitochondrial dehydrogenases (12, 31). In an effort to estimate the contribution of the glycerol phosphate shuttle to overall glucose and glycerol utilization in AdCMV-GlpK-treated INS-1 cells, we studied the effect of removal of Ca^{2+} on glycerol and glucose metabolism. Cells were incubated either in the standard secretion buffer containing 2.5 mM Ca^{2+} or in that buffer with Ca^{2+} ommitted and with the addition of 2 mM EGTA. Removal of Ca^{2+} resulted in no significant change in the rate of glucose or glycerol oxidation (oxidation of 5 mM glycerol was 64 ± 7 and 56 ± 12 nmol of CO_2/mg of protein/h and oxidation of 10 mM glucose was 161 ± 22 and 179 ± 39 nmol of CO_2/mg of protein/h in the presence and absence of Ca^{2+}, respectively). Removal of Ca^{2+} did, however, cause a 132% increase in lactate production in glycerol kinase expressing cells incubated with 5 mM glycerol (from 545 to 1263 nmol/mg of protein/h) and a 79% increase in lactate production from cells incubated with 10 mM glucose (from 314 nmol/mg of protein/h to 562 nmol/mg of protein/h). We suggest that this increase in lactate production reflects the carbon that would normally flow through the glycerol phosphate shuttle, but that is prevented from doing so in the absence of Ca^{2+} activation of the mitochondrial glycerol phosphate dehydrogenase. Unfortunately, most fuel-stimulated insulin secretion is dependent upon the presence of extracellular Ca^{2+}, preventing us from linking these metabolic changes to the secretory response. Nevertheless, these experiments suggest that flux through the glycerol phosphate shuttle is actively occurring in INS-1 cells, particularly when glycerol is the substrate in AdCMV-GlpK-treated cells. Since entry of carbon into the lower half of glycolysis is also very efficient, it is not yet possible to distinguish the relative importance of the glycerol phosphate shuttle and the lower half of glycolysis for fuel-stimulated insulin secretion. These conclusions are similar to those made by Dukes and colleagues in a recent study employing pharmacologic agents for evaluation of insulin secretion from mouse islets (5).

Is Accumulation of Acyl-CoA and Its Esterification to Glycerol Phosphate Necessary for Glucose-stimulated Insulin Secretion?—It has been proposed that stimulation of insulin secretion by glucose is mediated in part by accumulation of long-chain acyl-CoAs, which in turn act directly on the exocytotic machinery or are esterified to form lipid by-products with known signaling properties such as diacylglycerol or inositol-1,4,5-trisphosphate (8–10). A potential consequence of expansion of the glycerol phosphate pool in AdCMV-GlpK-treated beta cells could be to enhance such a lipid esterification-mediated signaling pathway. To test this idea, we evaluated the incorporation of [2-^{3}H]glycerol into bulk lipids and measured glycerol-stimulated insulin secretion in AdCMV-GlpK-treated cells in the presence and absence of Triacsin-C, a potent inhibitor of long-chain acyl-CoA synthetase (32). In AdCMV-GlpK-treated cells, the incorporation of [2-^{3}H]glycerol into the lipid

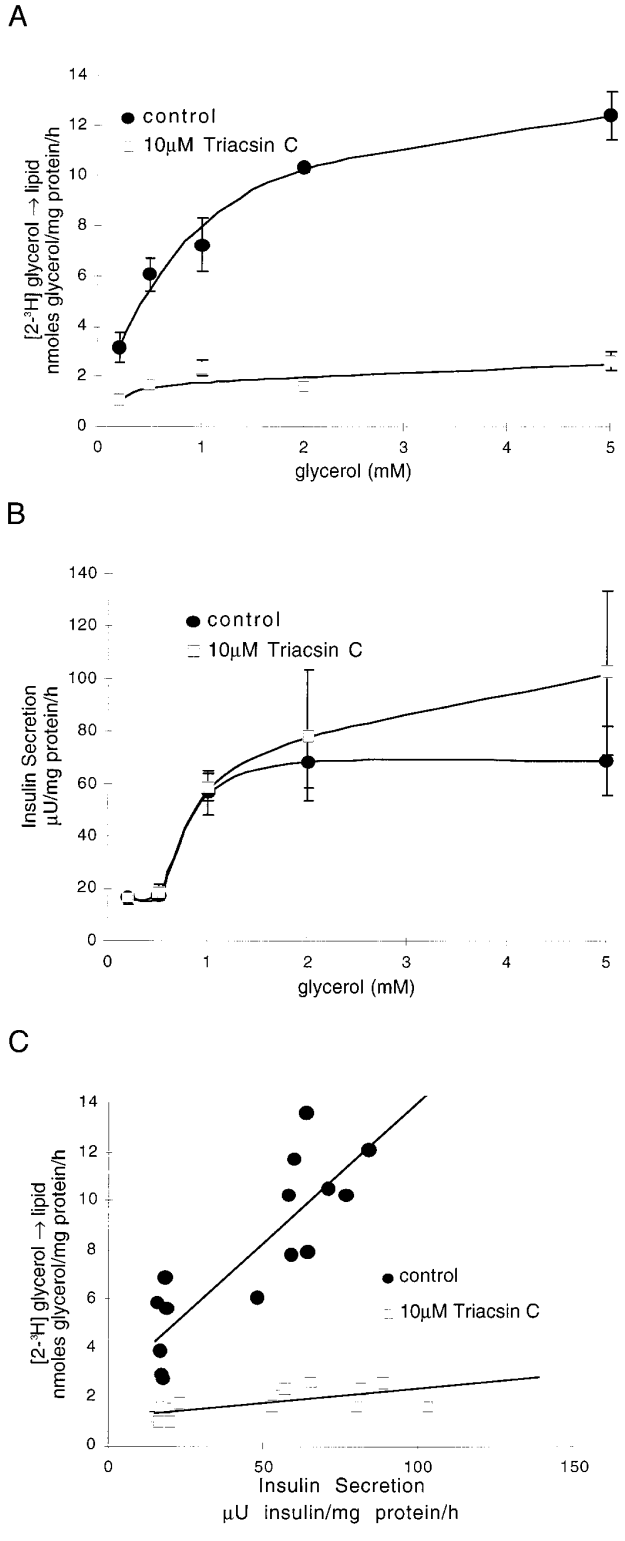

FIG. 5. **The effect of Triacsin-C on glycerol incorporation into cellular lipid and glycerol-induced insulin secretion in AdCMV-GlpK-treated INS-1 cells.** *A*, intact AdCMV-Glp-K treated INS-1 cells were incubated with [2-^{3}H]glycerol and incorporation of the tracer into cellular lipid was measured as described under "Materials and Methods," in the presence and absence of 10 μM Triacsin-C, an inhibitor of long-chain acyl-CoA synthetase. Note the potent inhibition of glycerol incorporation into lipid by the drug. *B*, glycerol-stimulated insulin secretion from the same cells studied in *A*. Note the absence of effect of Triacsin-C on insulin secretion. *C*, plot of insulin secretion *versus* glycerol incorporation into lipid (data taken from *A* and *B*), indicating the lack of correlation between glycerol incorporation into lipid and insulin release. For all panels, data represent the mean ± S.E. for four independent measurements per condition.

phase was found to increase as a function of glycerol concentration, rising from a value of 3.2 ± 0.6 nmol/mg of protein/h at 0.2 mM glycerol to a maximum of 12.5 ± 1.0 nmol/mg of protein/h at 5 mM glycerol. Half-maximal glycerol incorporation occurred at approximately 0.5 mM glycerol (Fig. 5*A*). Incubation of these cells with 10 μM Triacsin-C resulted in reduction of glycerol incorporation at 0.2 mM glycerol to 1.1 ± 0.1 nmol/mg of protein/h, from which it rose to a maximum of 2.7 ± 0.4 nmol/mg of protein/h at 5 mM glycerol. This sharp reduction in glycerol incorporation is presumably due to the deficit in long-chain acyl-CoAs required for esterification to the glycerol phosphate backbone. Despite the large inhibition of fatty acid esterification induced by Triacsin-C, the drug had no effect on glycerol-stimulated insulin secretion, with a 4-fold stimulation induced by 2 or 5 mM glycerol in both Triacsin-C-treated and control groups (Fig. 5*B*). Still, it remained possible that the lack of secretory impact of Triacsin-C could be explained if only a small rate of fatty acid esterification was sufficient for signaling. This possibility appears unlikely, based on replotting of the data in Fig. 5, *A* and *B*, to generate Fig. 5*C*. Here all insulin secretion values for each of the individual wells studied in Fig. 5*B* are plotted against all of the individual values for glycerol incorporation into lipid summarized in Fig. 5*A*. The resulting plot clearly shows that insulin secretion from glycerol stimulated AdCMV-GlpK-treated INS-1 cells is independent of the incorporation of glycerol into lipid. Furthermore, when studied at nonstimulatory glycerol levels (0.2 mM), cells incubated in the absence of Triacsin-C exhibited higher rates of glycerol incorporation than cells treated with the drug, with no effect on insulin release. Finally, Triacsin-C had no effect on the rate of [2-^{3}H]glycerol usage in INS-1 cells (data not shown), consistent with the notion that entry of glycerol phosphate into the lower half of glycolysis and/or the glycerol shuttle are the major pathways for glycerol signaling in these cells.

Note that, in the absence of Triacsin-C, the amount of labeled glycerol incorporated into lipid was far less than the amount metabolized to lactate or oxidized to CO_2 (at 5 mM glycerol, 12.5 nmol glycerol/mg of protein/h are incorporated into lipid compared with production of 718 nmol of lactate/mg of protein/h and 64 nmol of CO_2/mg of protein/h). One concern with such a comparison is that use of [2-^{3}H]glycerol might have caused us to underestimate the amount of glycerol converted to lipid, to the extent that unlabeled glycerol phosphate is reformed from dihydroxyacetone phosphate. We chose to use [^{3}H]glycerol because it is available at much higher specific activity than ^{14}C-labeled material. However, in one series of experiments with [U-^{14}C]glycerol, we obtained a qualitatively similar estimate of glycerol incorporation into lipid (6.8 nmol of glycerol/mg of protein/h incorporated at a glycerol concentration of 2 mM and in the absence of Triacsin-C). Furthermore, Triacsin-C potently inhibited incorporation of the ^{14}C-labeled material as it did the ^{3}H-labeled glycerol. Thus, we conclude that incorporation of glycerol phosphate into lipid is a minor pathway for glycerol metabolism in AdCMV-GlpK-treated INS-1 cells and that insulin secretion is effectively stimulated even when this incorporation is markedly reduced. It is important to point out, however, that this result does not argue against the essentiality of lipids for stimulus/secretion coupling, as recently demonstrated by Stein *et al.* (33) in rodents rendered hypolipacidemic by treatment with nicotinic acid.

Comparison to Previous Findings—Our findings in islets and INS-1 cells agree only partially with two previous studies on glycerol metabolism in islet cells. Similar to us, Malaisse and co-workers reported that glycerol had no effect on insulin secretion from either isolated rat islets or the rodent insulinoma cell lines RINm5F (14, 15). These investigators also reported,

however, that islets and RIN cells contain high levels of glycerol kinase activity, and also presented data suggesting that [U-^{14}C]glycerol is oxidized in islets at the same rate as glucose. Using a radioisotopic assay similar to that employed by Malaisse and co-workers, we found glycerol kinase activity to be undetectable in islets or INS-1 cells, but easily detectable in fresh liver extracts, indicating that the assay method employed by our group was sufficiently sensitive to detect endogenous glycerol kinase activity in tissues known to express the enzyme. Yilmaz *et al.* (14) reported that islets contain 1.3 nmol/min/islet or approximately 1.8 nmol/min/μg of protein of glycerol kinase activity. This is very surprising, in light of our own data showing that even in islets treated with the highest m.o.i. of AdCMV-GlpK tested (100), glycerol kinase activity is only 0.016 nmol/min/μg of protein. Consistent with our measurements of enzyme activity, we could demonstrate only very low levels of glycerol metabolism in AdCMV-βGAL-treated INS-1 cells, such that their rate of [U-^{14}C]glycerol oxidation was only 1.2% of the rate observed in AdCMV-GlpK-treated cells (data not shown). That normal islets lack glycerol kinase is also supported by our findings that the lowest m.o.i. of AdCMV-GlpK tested in our studies, which caused a small rise in glycerol kinase activity, was sufficient to confer near-maximal insulin secretion in response to glycerol. Thus, our data are consistent with a model in which the explanation for the lack of glycerol-stimulated insulin secretion in normal islets or INS-1 cells is a lack of sufficient glycerol kinase activity to allow significant metabolism of the substrate.

Concluding Remarks—Our studies demonstrate that INS-1 or islet cells are not normally responsive to glycerol as an insulin secretagogue, but become so when the enzyme responsible for its phosphorylation is expressed. Glycerol is a highly effective secretagogue, despite the fact that it is less readily oxidized and more readily converted to lactate than glucose. Glycerol does not appear to signal insulin secretion via its conversion to glycerol phosphate and esterification with fatty acids to form triglycerides and other complex lipids. Rather, it appears that glycerol and glucose stimulate insulin secretion via a common signal that is produced in the distal reactions of glycolysis or via entry into the glycerol phosphate shuttle, or both.

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