

GAD65-mediated Glutamate Decarboxylation Reduces Glucose-stimulated Insulin Secretion in Pancreatic Beta Cells*

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Mitochondrial metabolism plays a pivotal role in the pancreatic beta cell by generating signals that couple glucose sensing to insulin secretion. We have demonstrated previously that mitochondrially derived glutamate participates directly in the stimulation of insulin exocytosis. The aim of the present study was to impose altered cellular glutamate levels by overexpression of glutamate decarboxylase (GAD) to repress elevation of cytosolic glutamate. INS-1E cells infected with a recombinant adenovirus vector encoding GAD65 showed efficient overexpression of the GAD protein with a parallel increase in enzyme activity. In control cells glutamate levels were slightly increased by 7.5 mM glucose (1.4-fold) compared with the effect at 15 mM (2.3-fold) versus basal 2.5 mM glucose. Upon GAD overexpression, glutamate concentrations were no longer elevated by 15 mM glucose as compared with controls (–40%). Insulin secretion was stimulated in control cells by glucose at 7.5 mM (2.5-fold) and more efficiently at 15 mM (5.2-fold). INS-1E cells overexpressing GAD exhibited impaired insulin secretion on stimulation with 15 mM glucose (–37%). The secretory response to 30 mM KCl, used to raise cytosolic Ca²⁺ levels, was unaffected. Similar results were obtained in perfused rat pancreatic islets following adenovirus transduction. This GAD65-mediated glutamate decarboxylation correlating with impaired glucose-induced insulin secretion is compatible with a role for glutamate as a glucose-derived factor participating in insulin exocytosis.

Mitochondrial metabolism plays a pivotal role in the pancreatic beta cell by generating signals that couple glucose sensing to insulin secretion (1–3). Initially, mitochondrial metabolism generates ATP, which promotes the closure of ATP-sensitive K⁺ channels and, as a consequence, the depolarization of the plasma membrane (4). This leads to Ca²⁺ influx through voltage-gated Ca²⁺ channels and a rise in cytosolic Ca²⁺ ([Ca²⁺]_c)¹

triggering insulin exocytosis (4–6). However, the Ca²⁺ signal alone is not sufficient to reproduce the complete and sustained secretion elicited by glucose (7–9). Therefore, glucose metabolism must generate other factors participating in the stimulation of insulin secretion. Such metabolites may be generated in different compartments, the mitochondrion being the most likely source (10–12).

In permeabilized INS-1 cells, mitochondrial activation is associated with a marked stimulation of insulin release, which depends both on activation of the mitochondrial respiratory chain and on provision of carbons for the tricarboxylic acid cycle (13, 14). This secretory response requires a factor generated by mitochondrial metabolism distinct from ATP (13, 15) proposed to be glutamate (14). This was deduced from the finding that in permeabilized cells under conditions of permissive, clamped [Ca²⁺]_c, glutamate directly stimulated insulin exocytosis (14). It was concluded that glutamate acts downstream of mitochondrial function, participating in the coupling of glucose metabolism to insulin secretion (11). Glutamate can be formed in the mitochondria from the tricarboxylic acid cycle intermediate α -ketoglutarate by glutamate dehydrogenase (17). In the beta cell, we have shown that glutamate is generated by the mitochondria during glucose stimulation (14, 16), although such changes have not been observed by others (18). The inherent problem in studies on cellular glutamate contents is the absence of methods allowing determination of glutamate levels in the relevant cellular compartment, *i.e.* the cytosol. Hence, because the net cellular changes are still controversial (11, 14, 18), further substantiation of the role of glutamate requires modulation of its cytosolic levels, which is the aim of the present study.

Results obtained with a transgenic mouse model have indirectly highlighted the putative role of intracellular glutamate in insulin secretion. In these mice, overexpression of the glutamate-decarboxylating enzyme GAD65 in the beta cells resulted in glucose intolerance without any sign of insulinitis or loss of beta cells. Their islets showed impaired glucose-stimulated insulin secretion, whereas the response to the Ca²⁺-raising agent KCl was preserved (19). Although the authors have not measured the glutamate levels in the pancreatic islets of these transgenic mice, they discussed the possibility of reduced cellular glutamate levels as one possible explanation for the diminished response to glucose. Hence, the decrease in cellular glutamate levels can theoretically be achieved by overexpression of glutamate decarboxylase. Upon appropriate expression, this cytosolic enzyme decarboxylates glutamate produced by the mitochondria after its release into the cytosol.

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¹ The abbreviations used are: [Ca²⁺]_c, cytosolic [Ca²⁺]; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GABA, γ -aminobu-

tyric acid; GAD, glutamate decarboxylase; KRBH, Krebs-Ringer bicarbonate HEPES buffer; PFU, plaque-forming unit.

Consequently, cytosolic glutamate could be specifically reduced even during glucose stimulation without affecting major metabolic pathways. The γ -aminobutyric acid (GABA) thus formed is not believed to affect insulin secretion (20). In the present study the smaller isoform of glutamate decarboxylase, GAD65, predominantly expressed in rat pancreatic islets (21), has been overexpressed in rat insulinoma INS-1E cells and rat pancreatic islets. To control the expression of GAD65 a recombinant adenovirus vector encoding GAD65 was prepared. Overexpression of GAD65 in beta cells resulted in reduced glutamate levels and impaired glucose-stimulated insulin secretion.

MATERIALS AND METHODS

Cell Culture—Clonal INS-1E cells (22), derived and selected from the parental rat insulinoma INS-1 cell line (23), were cultured in RPMI 1640 medium with 5% fetal calf serum. Adenovirus amplification was performed in HEK-293 cells cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Adherent cultured INS-1E cells were infected with recombinant adenovirus for 90 min at 37 °C, washed once, and further cultured in complete RPMI 1640 medium for 18–20 h before experiments were performed. Pancreatic islets were isolated by collagenase digestion from male Wistar rats weighing 200–250 g (24) and cultured free floating in RPMI 1640 medium for 24 h before adenovirus transduction.

Adenovirus Construction—The recombinant human 65-kDa isoform of glutamic acid decarboxylase (GAD65) was used for the adenovirus construct (25). Recombinant adenovirus encoding GAD65 under the chicken actin promoter (26) was generated as previously described (27). The plasmid hGAD65-pcDNA3 containing full-length human GAD65 cDNA was kindly provided by Drs. F. C. Schuit and F. Gorus (Brussels, Belgium), originally obtained from Drs. A. Lernmark and A. Falorni when at the Karolinska Institute (Stockholm, Sweden) (28). Following *Bam*HI-*Xba*I digestion, the insert with blunt ends was subcloned in a cosmid pAdCAG (27), previously opened by *Swa*I. The presence and right orientation of the insert was checked by restriction enzyme digestions using *Cla*I and *Bgl*II. The cosmid containing hGAD65 (pAd-CAG-hGAD65) and the adenovirus type 5 DNA terminal protein complex were co-transfected using the calcium phosphate method (CellPfect, Amersham Pharmacia Biotech) in HEK-293 cells, which were seeded in 96-well plates. Ten days after transfection the cell lysate was used to infect 24-well plates, and the adenoviral DNA was extracted from the cells and analyzed by DNA digestion with *Cla*I and *Xho*I to check for the presence of hGAD65. The cell lysate containing the virus with full-length hGAD65 (AdCA-GAD65) was used to infect two 138-mm dishes of HEK-293 cells. The virus was purified by CsCl ultracentrifugation. AdCA-lacZ, which expresses bacterial β -galactosidase, was used as a control adenovirus.

Immunoblotting and Immunofluorescence—Cultured INS-1E cells were infected with different clones of recombinant adenovirus (from HEK-293 cell homogenates) for 90 min in a tissue culture incubator the day prior to analysis. Immunoblotting was performed following SDS-polyacrylamide gel electrophoresis using 10- μ g proteins of INS-1E cell extract per lane on an 11% gel. Proteins were transferred onto nitrocellulose membrane and incubated overnight at 4 °C in the presence of rabbit anti-GAD polyclonal antibody (1:2000) raised against human GAD65 (Chemicon, Temecula, CA). The membrane was then incubated for 1 h at room temperature with donkey anti-rabbit IgG antibody (1:5000) conjugated to horseradish peroxidase (ECL, Amersham Pharmacia Biotech), and the GAD protein was revealed by chemiluminescence (Pierce). The adenovirus clone 11 was selected, purified, and used for the following experiments.

For immunofluorescence, cells were grown on polyornithine-treated glass coverslips for 3 days prior to infection with AdCA-LacZ or AdCA-GAD (~100 PFU/cell) for 90 min. The next day, cells were fixed as previously described (29) before incubation with anti-GAD (1:1000) and then goat anti-rabbit IgG rhodamine (1:200) antibodies (Chemicon). Cells were viewed using a Zeiss laserscan confocal 410 microscope.

GAD Activity and Glutamate Level Determination—INS-1E cells were cultured 2–3 days in 6-well plates, infected with AdCA-GAD65 or AdCA-lacZ in 1 ml of RPMI 1640 medium for 90 min at ~40 PFU/ml, and thereafter supplemented with fresh medium. After 18 h the cells were washed with cold phosphate-buffered saline, collected with a rubber policeman in 50 μ l of a lysis buffer (0.5 M HEPES-NaOH, pH 7.0, 10 mM NaF, 2 mM aminoethylisothiuronium bromide hydrobromide, 2 mM phenylmethylsulfonyl fluoride, 0.05% Triton X-100), and kept on ice for 1 h in 1.5-ml tubes. Cells were then centrifuged at 13,000 rpm for 30

min at 4 °C to eliminate cellular membranes. After centrifugation, the supernatant was collected and diluted 1:10 in lysis buffer without Triton before protein determination (Bradford's assay). Aliquots (100 μ l) of this protein sample were injected into cups containing 100 μ l of a glutamate mixture (0.5 M HEPES-NaOH, pH 7.0, 0.2 mM pyridoxal phosphate, 2 mM glutamate, 0.1 μ Ci of L-[1-¹⁴C]glutamate (ARC, St. Louis, MO)), and placed in sealed vials. After a 1-h incubation at 37 °C the reaction was stopped by adding 100 μ l of 7% perchloric acid, and CO₂ was captured by the addition of 300 μ l of benzethonium hydroxide. Diluted lysis buffer was used as a blank. After 5 h the cups were removed and 10 ml of scintillation liquid was added to the vials. After 24 h the radioactivity was measured in a β counter (LS6500, Beckman Coulter, Fullerton, CA).

For cellular glutamate determination INS-1E cells were cultured 2–3 days in 10-cm Petri dishes, infected with AdCA-GAD65 or AdCA-lacZ for 90 min, and supplemented with fresh medium. After 18 h the cells were preincubated for 2 h in glucose- and glutamine-free RPMI 1640 medium at 37 °C. Cells were then incubated for 30 min at 37 °C in Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing (in mM): 135 NaCl, 3.6 KCl, 10 HEPES (pH 7.4), 5 NaHCO₃, 0.5 NaH₂PO₄, 0.5 MgCl₂, 1.5 CaCl₂, and 2.5/7.5 or 15 glucose. The stimulation period was stopped by putting the cells on ice. After discarding the incubation buffer 1 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM CDTA, 0.2% Tween 20) was added to the cells. Glutamate levels were measured in cell homogenates by monitoring NADH fluorescence as previously described (14).

Mitochondrial Membrane Potential and ATP Generation—INS-1E cells were cultured 2–3 days, infected with AdCA-GAD65 or AdCA-lacZ for 90 min at ~40 PFU/ml, and used for experiments the next day. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured as described (13) using rhodamine-123. Cells were used as a suspension in KRBH buffer, gently stirred in a fluorimeter cuvette at 37 °C, and fluorescence, excited at 490 nm, was measured at 530 nm. Cytosolic ATP levels were monitored in cells expressing the ATP-sensitive bioluminescent probe luciferase following infection with the specific viral construct (30, 31). ATP generation was also assessed in 6-well plates following a 2-h preincubation period in glucose-free RPMI 1640. Cells were incubated for 10 min in KRBH with 2.5 mM or 15 mM glucose before the stimulation was stopped on ice by washing with ice-cold KRBH and the addition of 0.4 N HClO₄. ATP levels were determined using a bioluminescence assay kit (HS II, Roche Diagnostic, Rotkreuz, Switzerland).

Insulin Secretion Assay—INS-1E cells cultured in 24-well plates were infected over a 90-min period with either AdCA-GAD65 or AdCA-LacZ adenovirus at ~40 PFU/cell and assayed the next day. Prior to the experiments, cells were maintained for 2 h in glucose- and glutamine-free culture medium. The cells were then washed and preincubated further in glucose-free KRBH, supplemented with 0.1% bovine serum albumin (Sigma) added as carrier, before the incubation period (30 min at 37 °C) with different glucose concentrations or KCl. For GABA experiments, cells were cultured and incubated in the same way except for adenovirus infection.

Isolated rat islets were infected with either AdCA-GAD65 or AdCA-LacZ adenovirus over a 90-min period the day after isolation and further cultured for 24 h before the secretion assay. Islet perfusions were carried out using 20 islets per chamber of 250 μ l volume thermostated at 37 °C (Brandel, Gaithersburg, MD). The flux was set at 0.5 ml/min, and fractions were collected every minute following a 20-min washing period at basal glucose (2.8 mM). Insulin secretion was determined by radioimmunoassay using rat insulin as standard (14).

Statistical Analyses—Where applicable, the results were expressed as means \pm S.E., and differences between groups were analyzed by Student's *t* test.

RESULTS

Assessment of Adenovirus-mediated GAD65 Overexpression—Several clones of adenoviruses were amplified in HEK-293 cells before testing their ability to overexpress GAD65 in target cells. Insulinoma INS-1E cells cultured in 6-well plates for 4 days were infected over a 90-min period with homogenates of HEK-293 cells used to amplify adenovirus. Immunoblotting performed the next day using anti-human GAD65 antibody revealed a band at the expected size of 65 kDa. As shown on one representative immunoblot (Fig. 1A), clone 11 gave a clean signal at 65 kDa detected as a likely doublet (32). The recombinant adenovirus was further validated by DNA digestion and selected for virus amplification, purification, and use through-

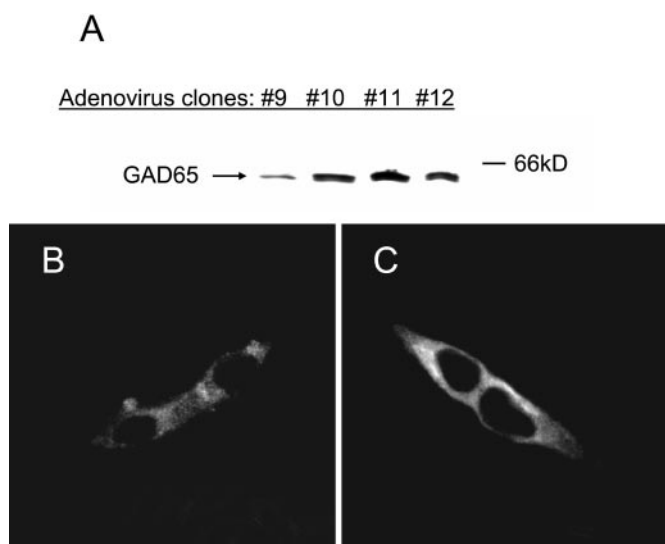


FIG. 1. Adenovirus-mediated GAD65 overexpression. *A*, immunoblotting using anti-GAD65 antibody in insulinoma INS-1E cells. These were cultured in 6-well plates for 4 days, infected over a 90-min period with homogenates of different clones of adenovirus-containing HEK-293 cells, and collected the following day for immunoblotting. Clone 11 was selected for use throughout the study and referred to AdCA-GAD65. *B*, cellular localization of GAD65 in INS-1E cells investigated by immunofluorescence using anti-GAD65 antibody performed the day after infection with purified virus preparations. Control INS-1E cells were infected with AdCA-LacZ virus. *C*, cells overexpressing GAD were infected with the AdCA-GAD65 virus. Observations are representative of three to five independent experiments.

out the study. This virus preparation was then referred to as AdCA-GAD65. The control adenovirus, which expresses bacterial β -galactosidase, was called AdCA-LacZ.

Cellular localization of GAD65 in control and INS-1E cells infected with AdCA-GAD65 was investigated by immunofluorescence performed the day after a 90-min infection using purified virus preparations. Reetz *et al.* (33) have shown that in pancreatic beta cells GAD is localized around synaptic-like microvesicles, probably without direct connection. Immunofluorescence on control INS-1E cells using anti-GAD65 antibody revealed a punctate pattern for these cells expressing only basal levels of GAD65, suggesting co-localization with vesicles (Fig. 1*B*). In contrast, cells overexpressing GAD65 following AdCA-GAD65 infection exhibited strong, diffuse fluorescence mainly in the cytosolic compartment (Fig. 1*C*).

GAD Activity and Glutamate Levels in INS-1E Cells—INS-1E cells were infected with either AdCA-GAD65 or AdCA-LacZ adenovirus and analyzed the next day. GAD activity in cell extracts was measured as the GAD-mediated decarboxylation of L-[1- 14 C]glutamate to 14 CO $_2$. It was dramatically increased by overexpression of GAD65 (Fig. 2*A*). Infection of INS-1E cells with the control AdCA-LacZ virus did not modify GAD activity, neither at 40 PFU/cell nor at 400 PFU/cell, compared with non-infected cells (0.09 ± 0.03 pmol of CO $_2$ /min/ μ g of protein). On the other hand, infection with AdCA-GAD65 virus resulted in a marked and dose-dependent increase in GAD activity: 26-fold at 40 PFU/cell and up to 102-fold at 400 PFU/cell compared with corresponding AdCA-LacZ controls ($p < 0.0001$ for both). These are *in vitro*, optimal enzyme activities from cell lysates, probably higher than the effective cellular activity. For the following experiments we selected an infection titer of 40 PFU/cell, which was sufficient for high GAD activity without noticeable virus-mediated cell toxicity.

Next, cellular glutamate contents were determined, because the aim of overexpressing GAD was to affect the cytosolic

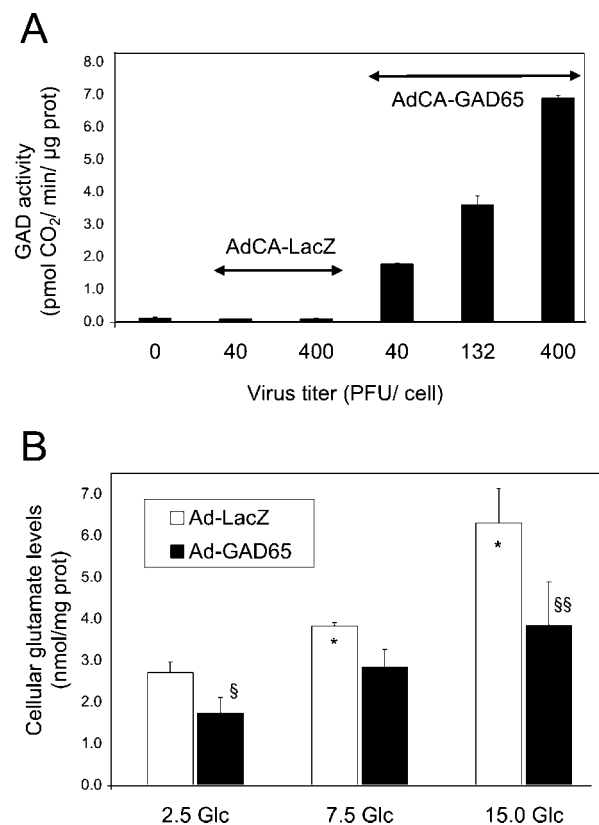


FIG. 2. GAD activity and glutamate levels in INS-1E cells. INS-1E cells were infected over a 90-min period with either AdCA-GAD65 or AdCA-LacZ adenovirus and analyzed the next day. *A*, GAD activity in cell extracts was measured as the GAD-mediated decarboxylation of L-[1- 14 C]glutamate to 14 CO $_2$. Values are means \pm S.D. representing one of a total of four experiments. An infection titer of 40 PFU/cell was selected for the following experiments. *B*, cellular glutamate levels were determined following a 30-min incubation in the presence of basal (2.5 mM) or stimulatory (7.5 and 15 mM) glucose concentrations. Values are the means \pm S.E. for three independent experiments, *, $p < 0.05$ versus 2.5 mM glucose; §, $p < 0.05$; §§, $p < 0.02$ versus AdCA-LacZ at corresponding glucose concentrations.

glutamate levels, especially during glucose stimulation. In control cells (AdCA-LacZ-infected) cellular glutamate content was elevated, as expected, after a 30-min glucose stimulation (Fig. 2*B*). Compared with 2.5 mM glucose (2.69 ± 0.25 nmol of glutamate/mg of protein), incubation at 7.5 and 15 mM glucose resulted in 1.4-fold ($p < 0.05$) and 2.3-fold ($p < 0.05$) glutamate increases, respectively. In cells overexpressing GAD following infection with AdCA-GAD65 virus, the basal levels were reduced by 36% ($p < 0.05$). Glucose stimulation failed to increase efficiently cellular glutamate contents at both 7.5 and 15 mM glucose. This resulted in a 40% reduction of glutamate levels at 15 mM glucose compared with stimulated control cells ($p < 0.02$). Using a higher titer of AdCA-GAD65 virus (120 PFU/cell), there was no further decrease of glutamate levels (data not shown).

Mitochondrial Membrane Potential and ATP Generation—In control cells mitochondrial membrane potential was hyperpolarized by raising glucose from 2.5 to 15 mM and by the addition of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) resulted in a rapid depolarization. Similar effects were observed in cells overexpressing GAD (Fig. 3*A*). Activation of the electron transport chain, measured here as the potential of the mitochondrial membrane, leads to the formation of ATP. In accordance with mitochondrial membrane potential, ATP generation was not affected by GAD overexpression. Indeed, cytosolic ATP levels monitored in cells expressing

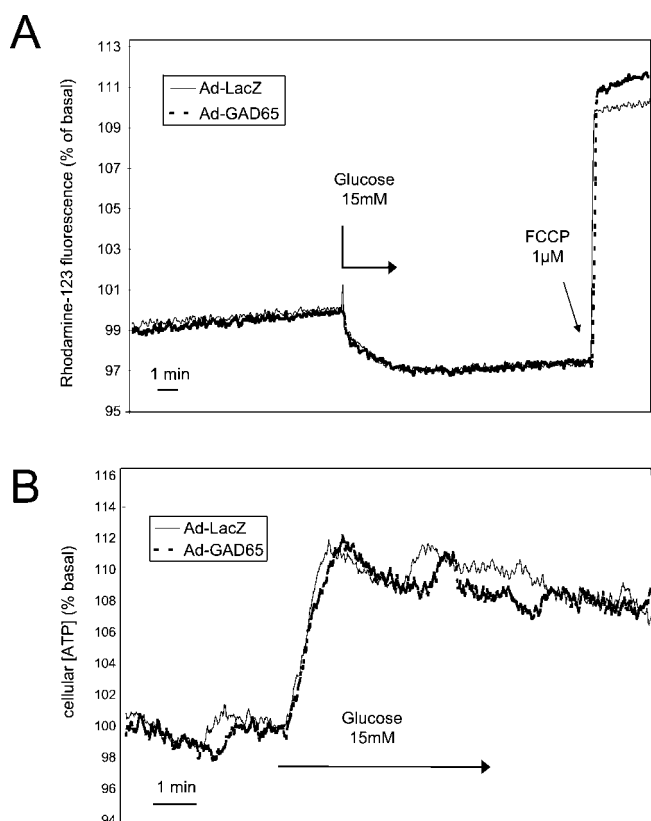


FIG. 3. Mitochondrial membrane potential and ATP generation. INS-1E cells were cultured 2–3 days, infected with AdCA-GAD65 or AdCA-lacZ for 90 min at ~ 40 PFU/ml, and used for experiments the next day. *A*, mitochondrial membrane potential was monitored as rhodamine-123 fluorescence. Each trace started at 2.5 mM glucose, which was then raised to 15 mM before complete depolarization induced by 1 μ M FCCP. *B*, cytosolic ATP levels were monitored in cells expressing the ATP-sensitive bioluminescent probe luciferase. Traces are representative of three independent experiments. See “Results” for data and statistics on static incubations.

luciferase were elevated to similar extents by raising glucose from 2.5 to 15 mM (Fig. 3*B*). ATP generation was also measured in cell lysates following a 10-min incubation period at low and high glucose. ATP levels at 15 mM glucose compared with 2.5 mM were elevated by 23.1% (367 ± 5 versus 298 ± 1 nmol/mg of protein, respectively, $p < 0.005$) in AdCA-LacZ control cells and by 27.9% (413 ± 22 versus 322 ± 14 nmol/mg of protein, respectively, $p < 0.05$) in AdCA-GAD65 cells (means of three independent experiments). Therefore, GAD overexpression did not alter the activity of the electron transport chain.

Effects of GAD Overexpression on Insulin Secretion in INS-1E Cells—INS-1E cells cultured in 24-well plates were infected at ~ 40 PFU/cell and assayed the next day. Following a preincubation period and washes in glucose-free buffers, cells were challenged for 30 min with different glucose concentrations. Insulin secretion was also stimulated at basal 2.5 mM glucose with 30 mM KCl, as a Ca^{2+} raising agent, to assess for anaplerotic effects. In control cells (AdCA-LacZ-infected), raising glucose from basal 2.5 to 7.5 and 15 mM stimulated insulin secretion 2.5-fold ($p < 0.05$) and 5.2-fold ($p < 0.01$), respectively (Fig. 4*A*). Insulin release at basal (2.5 mM) or intermediate (7.5 mM) glucose concentration was not affected in cells overexpressing GAD (AdCA-GAD65). In contrast, insulin secretion stimulated by 15 mM glucose (2.6-fold versus basal glucose, $p < 0.05$) was inhibited by 37% ($p < 0.05$) compared with the corresponding control at high glucose. The effects of 30 mM KCl on insulin exocytosis were similar in control cells and cells overexpressing GAD. As shown in Fig. 4*B*, insulin secretion

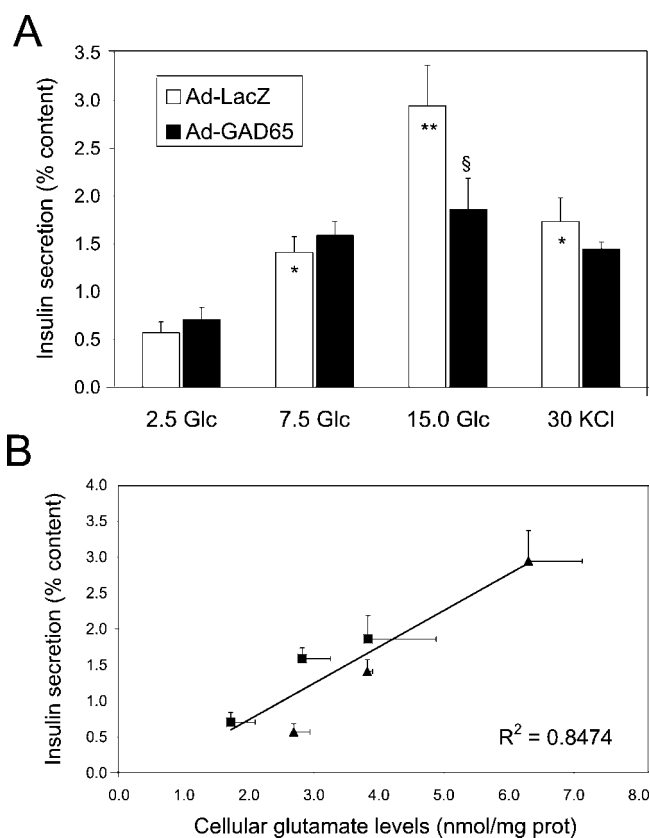


FIG. 4. Effect of GAD overexpression on insulin secretion in INS-1E cells. *A*, INS-1E cells cultured in 24-well plates were infected over a 90-min period with either AdCA-GAD65 or AdCA-LacZ adenovirus and assayed the next day. Following a preincubation period and rinsing in glucose-free buffer, cells were challenged for 30 min with 2.5, 7.5, and 15 mM glucose or 30 mM KCl (at 2.5 mM glucose). Values are the means \pm S.E. for four independent experiments, *, $p < 0.05$; and **, $p < 0.01$ versus 2.5 mM glucose; §, $p < 0.05$ versus AdCA-LacZ at 15 mM glucose. *B*, correlation between cellular glutamate levels and insulin secretion from data extracted from Figs. 2*B* and 4*A*, respectively. Triangles represent LacZ controls and squares the GAD overexpressing cells, each incubated at different glucose concentrations (2.5, 7.5, and 15 mM).

correlated positively with cellular glutamate levels ($R^2 = 0.8474$, $p < 0.05$), remarkably evident at high glucose concentration.

It has been shown that glutamate decarboxylation by GAD forms GABA in pancreatic islets (34), which may be released from synaptic-like microvesicles (35, 36). The effect of extracellular GABA on the beta cell is controversial; GABA has been postulated to inhibit insulin secretion (37), although this effect could not be confirmed (20). Cells overexpressing GAD following AdCA-GAD65 infection and stimulation with high glucose could therefore theoretically release significant amounts of GABA with a possible negative feedback on insulin secretion. In this context, it was felt necessary to check for any potential effect of GABA added in the medium during stimulation of insulin secretion. Exposure of INS-1E cells to GABA up to 100 μ M did not alter glucose-induced insulin secretion (Fig. 5), rendering unlikely a GABA-mediated inhibitory effect on exocytosis in cells overexpressing GAD.

Effect of GAD Overexpression on Insulin Secretion in Rat Islets—Cultured rat islets infected with the control AdCA-LacZ virus responded to glucose stimulation by a sustained increased rate of insulin secretion at 8.3 mM glucose, which rose further at 16.7 mM glucose (Fig. 6*A*). Overexpression of GAD65, assessed by immunoblotting, had no significant effects at basal (2.8 mM) or intermediate (8.3 mM) glucose concentrations. In

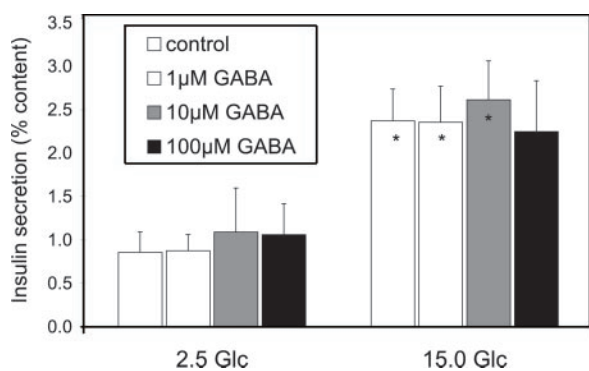


FIG. 5. Effect of GABA on insulin secretion in INS-1E cells. INS-1E cells cultured in 24-well plates were preincubated and washed in glucose-free buffer before incubation for 30 min at 2.5 or 15 mM glucose in the presence of increasing concentrations of GABA. Values are the means \pm S.E. for four independent experiments, *, $p < 0.05$ versus 2.5 mM glucose.

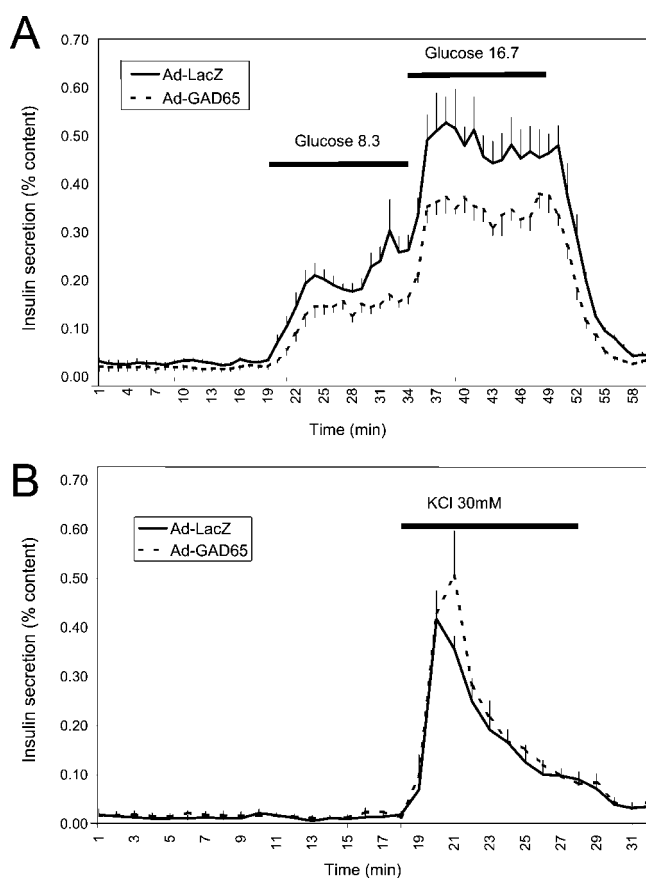


FIG. 6. Effect of GAD overexpression on insulin secretion in rat islets. Isolated rat islets were infected with either AdCA-GAD65 or AdCA-LacZ adenovirus over a 90-min period the day after isolation and further cultured for 24 h before secretion assay. Islet perfusions were carried out using 20 islets per chamber at 0.5 ml/min, and fractions were collected every minute. *A*, after a period at 2.8 mM glucose, insulin secretion was stimulated by raising glucose to 8.3 mM for 15 min directly followed by 15 min at 16.7 mM glucose, then returning to 2.8 mM. *B*, islets were exposed to 30 mM KCl for 10 min at the constant basal glucose concentration of 2.8 mM. Values are means \pm S.E. for three independent experiments, see "Results" for statistics.

contrast, at 16.7 mM glucose the secretory response was reduced by 31% in islets infected with AdCA-GAD65 compared with controls (area under the curve = 4.43 ± 0.63 versus 6.41 ± 1.23 (insulin release as % content), respectively, $p < 0.05$). Stimulation of insulin secretion by high potassium-induced membrane depolarization resulted in a transient elevation of the secretory response in control islets (Fig. 6*B*). Overexpres-

sion of GAD did not modify this non-metabolic response. These results are in accordance with those obtained in the clonal beta cell line INS-1E, *i.e.* inhibition of insulin secretion stimulated by glucose but not by potassium.

DISCUSSION

The role of GAD in the beta cell is under intense study because it has been recognized as an autoantigen in insulin-dependent diabetes (38). GAD65 is the form predominantly expressed in rats and humans, localized mainly in brain and pancreatic islets (21). The purpose of the present study was to overexpress GAD65 in beta cells to lower the levels of glutamate through decarboxylation during glucose stimulation. This was achieved by the use of a recombinant adenovirus vector encoding human GAD65 in beta cells. Hence, we could substantiate the proposed role for glutamate as a metabolic coupling factor participating in glucose-induced insulin secretion (14).

In pancreatic beta cells, GAD was shown to be associated with synaptic-like microvesicles, although GAD was also found in the cytosol (33, 39). Michalik and co-workers (34) demonstrated that in fractions from homogenates of rat pancreatic islets, GAD activity was mainly recovered in the cytosol (51%) with lesser enrichment in the microsomes (17%). In our study, immunofluorescence of GAD65 revealed a punctuate pattern in control cells, whereas INS-1E cells overexpressing GAD65 exhibited strong cytosolic-like fluorescence. Therefore, it would seem that overexpression of GAD65 does not lead to a noticeable increase in vesicle attachment, essentially accumulating freely in the cytosol. This observation is in agreement with the previous proposal for an indirect or labile interaction of GAD with vesicles (33).

The control of insulin secretion by the pancreatic beta cell is achieved through a complex metabolic cascade converting glucose and other nutrients into signals leading to appropriate insulin release (3, 12). Mitochondrial metabolism is crucial in generating ATP leading to a rise in $[Ca^{2+}]_c$, which is the main and necessary signal triggering insulin exocytosis (4, 6). However, other factors generated by glucose participate in the stimulation of insulin secretion and have attracted much interest over the last years (9). In this context, the beta cells of a transgenic mouse model have recently been reported to exhibit impaired insulin secretion in response to glucose but not to KCl, a $[Ca^{2+}]_c$ -raising agent (19). These mice overexpressing GAD65 specifically in beta cells exhibited impaired glucose tolerance and inhibition of insulin secretion in response to glucose, without any insulinitis or loss of beta cells. Overexpression of the glutamate-decarboxylating enzyme in beta cells could theoretically lead to decreased cellular glutamate levels. The reduced insulin secretion observed in these mice is compatible with the proposal that mitochondrially derived glutamate participates in glucose-stimulated insulin secretion (11, 14).

Glutamate levels in control INS-1E cells were increased following glucose stimulation. Overexpression of GAD65 resulted in high GAD activity and reduced cellular glutamate levels, whereas the mitochondrial activation was preserved. The present study demonstrates the feasibility of specifically reducing cellular glutamate levels mediated by controlled GAD overexpression. This leads to an elevated glutamate decarboxylation rate, essentially in the cytosol (34). The decrease in cellular glutamate levels correlated with impaired insulin secretion stimulated by high glucose. It is noteworthy that the major effects were observed above basal and intermediate glucose concentrations, suggesting a threshold phenomenon for glutamate action. This is compatible with a role of a potentiator for glutamate in conditions of permissive $[Ca^{2+}]_c$. Indeed, the effect of the $[Ca^{2+}]_c$ -raising agent (30 mM KCl) was not affected

by GAD overexpression, also indicating that exocytosis *per se* was preserved. Very similar results were obtained using isolated rat pancreatic islets overexpressing GAD. This is in good agreement with pancreatic perfusions performed in mice overexpressing GAD65 in the beta cells, although this study did not examine the glutamate pathway (19). Taken together these data argue in favor of a role for glutamate as a factor derived from glucose participating in the stimulation of insulin secretion. One hypothetical mechanism is that glutamate would favor granule priming for exocytosis, a mechanism which might not be restricted to the beta cell. In this context it is of interest that clonal pancreatic alpha cells, secreting glucagon, have recently been shown to accumulate glutamate in their vesicles and to release it upon stimulation (40).

The high GAD activity resulting from GAD65 overexpression may substantially increase GABA generation and release. During high glucose culture, GABA was shown to be released from pancreatic beta cells (35, 36). Once in the extracellular space, GABA could act on neighboring islet alpha cells, inhibiting glucagon secretion through activation of GABA-A receptors (41). Although these receptors have not been identified on beta cells, it has been suggested that GABA inhibits insulin secretion in dogs (37), an effect that could not be reproduced in mice or rats (20). In this context, we checked for any potential effect of GABA on glucose-stimulated insulin secretion in INS-1E cells derived from a rat insulinoma. There was no effect of GABA added to the medium up to 100 μ M, a concentration far above the estimate of GABA release by beta cells (35, 36).

In the present study the overexpression of GAD65 was used to test the proposed role for glutamate as a coupling factor in glucose-stimulated insulin secretion. The data support the model in which glutamate would act as a potentiator of insulin release, sensitizing exocytosis to the effect of calcium. Indeed, the effect of elevated glucose concentration was inhibited by high GAD activity, whereas intermediate glucose or KCl stimulation was not affected by GAD overexpression. GAD overexpression could also clarify the role of GABA as a paracrine factor in the islet.

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