Glucose or Insulin, but not Zinc Ions, Inhibit Glucagon Secretion From Mouse Pancreatic α-Cells

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The mechanisms by which hypoglycemia stimulates glucagon release are still poorly understood. In particular, the relative importance of direct metabolic coupling versus paracrine regulation by β-cell secretory products is unresolved. Here, we compare the responses to glucose of *1*) α-cells within the intact mouse islet, *2*) dissociated α-cells, and *3*) clonal αTC1-9 cells. Free cytosolic concentrations of ATP ($[ATP]_c$) or Ca^{2+} ($[Ca^{2+}]_c$) were imaged using α-cell–targeted firefly luciferase or a green fluorescent protein–based Ca^{2+} probe ("pericam"), respectively. Consistent with a direct effect of glucose on α-cell oxidative metabolism, an increase in glucose concentration (from 0 or 3 mmol/l to 20 mmol/l) increased $[ATP]_c$ by 7–9% in α-cells within the intact islet and by ~4% in αTC1-9 cells. Moreover, glucose also dose-dependently decreased the frequency of $[Ca^{2+}]_c$ oscillations in both dissociated α-cells and αTC1-9 cells. Although the effects of glucose were mimicked by exogenous insulin, they were preserved when insulin signaling was blocked with wortmannin. Addition of $ZnCl_2$ slightly increased the frequency of $[Ca^{2+}]_c$ oscillations but failed to affect glucagon release from either islets or αTC1-9 cells under most conditions. We conclude that glucose and insulin, but not Zn^{2+} ions, independently suppress glucagon secretion in the mouse. *Diabetes* 54:1789–1797, 2005

Glucagon is the key counterregulatory hormone responsible for opposing the glucose-lowering effects of insulin (1). Thus, glucagon stimulates both glycogen breakdown and gluconeogenesis by the liver (2) while decreasing hepatic triglyceride synthesis (3,4). Appropriate stimulation of glucagon release from pancreatic islet α-cells is particularly important to minimize the impact of acute insulin-induced hypoglycemia, a major complication and the cause of 2–4% of all deaths in type 1 diabetes (5,6).

Glucagon release is normally stimulated as blood glucose concentrations fall, a response that is progressively diminished in type 1 diabetes (7,8). This loss of responsiveness, which leads to increased incidence and severity of hypoglycemia with time, does not involve an apparent change in total α-cell mass (9,10) but rather the development of "hypoglycemia blindness" in existing α-cells.

Although the molecular mechanisms involved in the regulation of insulin secretion are increasingly well understood (11–13), knowledge of those that mediate the inhibition of glucagon release remains fragmentary. In particular, the respective roles of *1*) glucose, acting directly on individual α-cells; *2*) insulin (5,14,15); or *3*) other factors, including γ-aminobutyric acid (16) secreted from neighboring β-cells, are uncertain, as is *4*) the contribution of autonomic inputs (5). Against an important role for a paracrine mechanism, the stimulation of glucagon release from the perfused rat pancreas after a decrease (5.5–4.4 mmol/l) in glucose concentration was unaffected by perfusion with anti-insulin antiserum (17). Similarly, a larger decrease in glucose concentration (5.5–1.4 mmol/l) prompted the same increment in glucagon release in pancreata from control or streptozotocin-induced diabetic rats (18). On the other hand, retrograde perfusion at slightly elevated glucose concentrations increased both insulin and glucagon release, suggesting an inhibitory role for β-cell factors at elevated glucose concentrations (19). Moreover, cessation of β-cell secretion is required for the activation of glucagon release during hypoglycemia in rats (20). Finally, a recent study (21) implicated the release of Zn^{2+} ions from β-cells, based on the ability of micromolar concentrations of these ions to reverse the stimulatory effects of pyruvate on glucagon release from the perfused rat pancreas and the reversal of the effects of monomethyl succinate with calcium EDTA, a broad-range divalent metal ion chelator.

Glucose-induced increases in total cellular ATP content and in the ATP-to-ADP(AMP) ratio have been reported in isolated pancreatic β-cells (22) as well as rodent islets (23–25). These increases seem likely to inhibit ATP-sensitive K^+ channels (K_{ATP} channels) (12) and to inhibit AMP-activated protein kinase (26). Whereas the former leads to plasma membrane depolarization, Ca^{2+} influx, and the activation of vesicle fusion at the cell surface (27), the latter may allow vesicle mobilization and sustained insulin secretion (28). Correspondingly, total islet ATP content (24,29) and free β-cell ATP (30–32) increase in response to glucose. By contrast, no changes in total ATP were reported in purified rat α-cells at elevated glucose concentrations (22), although Ishihara et al. (21) recently demonstrated detectable increases in the free cytosolic concentration of ATP ($[ATP]_c$) under these conditions.

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$[ATP]_c$, cytosolic concentration of ATP; $[Ca^{2+}]_c$, cytosolic concentration of Ca^{2+}; K_{ATP} channel, ATP-sensitive K^+ channel; KRBB, Krebs-Ringer bicarbonate buffer; PPG, preproglucagon.

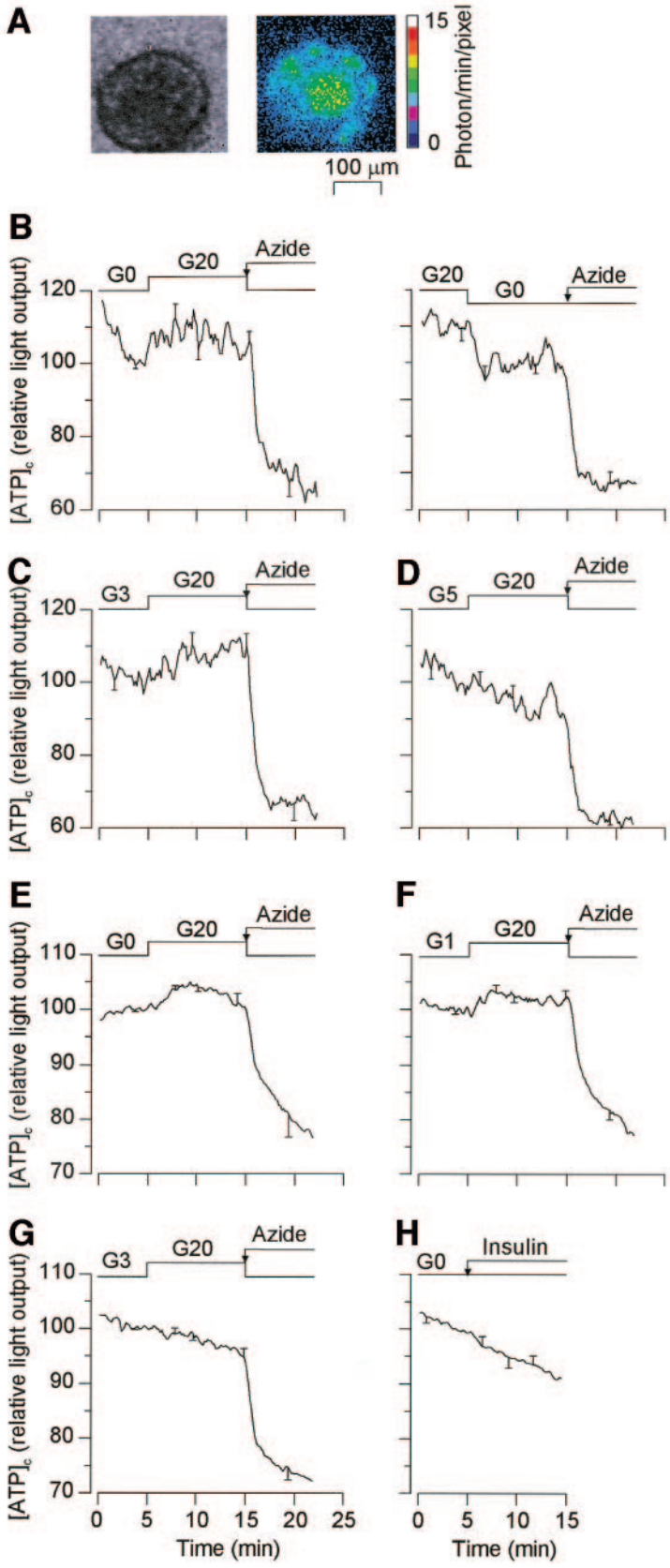

FIG. 1. Effects of glucose on $[ATP]_c$ changes in α-cells within intact mouse islets (*B–D*) and in αTC1-9 cells (*E–H*). *A*: An intact mouse islet was infected with the PPG-Luc adenovirus, and bioluminescence was imaged in the presence of luciferin, as described in RESEARCH DESIGN AND METHODS. Photon production is represented in pseudocolor (dark blue-yellow), representing the range 0–15 photon · min^{-1} · $pixel^{-1}$ (in 20 mmol/l glucose). *B–G*: The concentration of glucose (G) was either increased or decreased from 0, 1, 3, or 5 to 20 mmol/l as indicated, whereas 2 mmol/l azide was applied after 15 min to inhibit complex IV of the respiratory chain, and thus mitochondrial ATP synthesis. *H*: αTC1-9 cells were perifused throughout in the absence of glucose. After 5 min, 17 nmol/l insulin was applied for 10 min. Values are means ± SE for 10–20 islets from four separate cultures or 5–16 independent experiments for αTC1-9 cells.

Here, we developed dynamic imaging approaches to monitor free ATP and Ca^{2+} with targeted luciferase and the green fluorescent protein–based Ca^{2+} probe pericam (33), respectively. Expressed under the preproglucagon (PPG) promoter, using recombinant adenoviruses, these probes have allowed the effects of glucose and other stimuli to be examined in dissociated primary and clonal mouse α-cells under conditions where the potentially

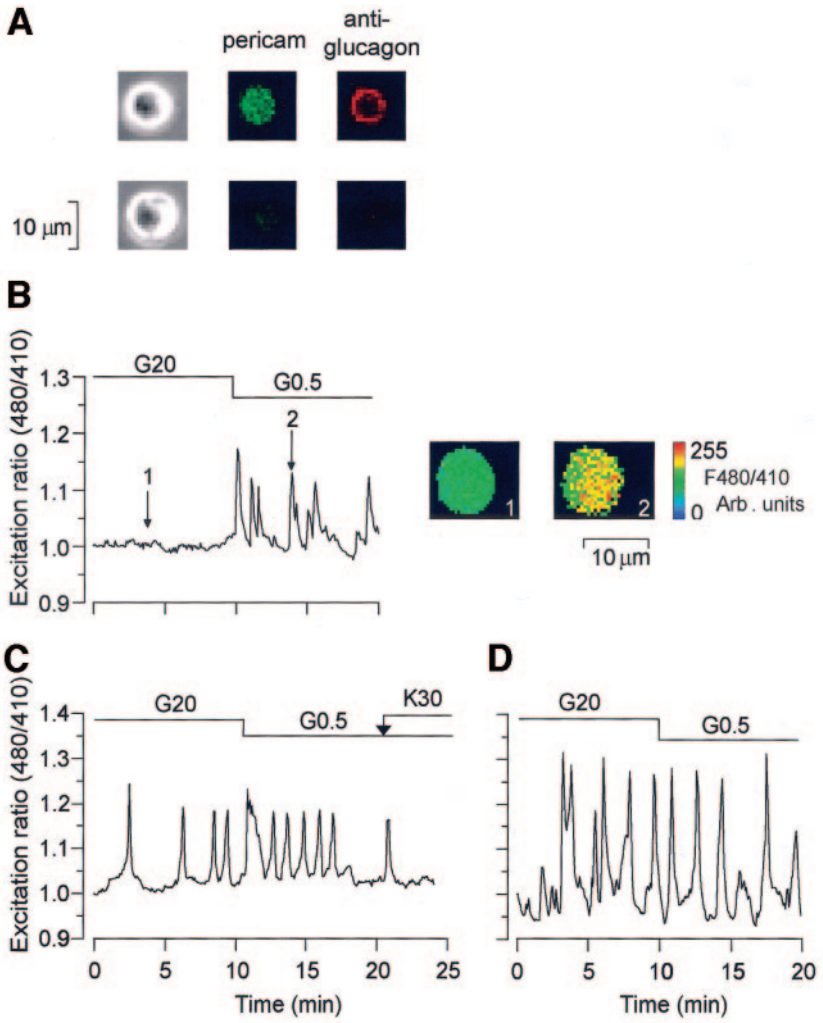

FIG. 2. Effects of glucose on $[Ca^{2+}]_c$ oscillations in primary mouse α-cells expressing the ratiometric pericam under the control of the PPG promoter. *A*: Immunocytochemistry performed in dispersed mouse islet cells. The *left panels* illustrate bright field, the *middle panels* illustrate the GFP (green fluorescent protein) fluorescence of the ratiometric pericam (excited at 480 nm), and the *right panels* illustrate glucagon immunoreactivity. *B–D*: $[Ca^{2+}]_c$ oscillations in three distinct single α-cells. Glucose concentration (G) was reduced from 20 to 0.5 mmol/l, and 30 mmol/l K^+ (K30) was applied as indicated. Arrows 1 and 2 in *panel B* indicate time points of *image 1* and *2*. Arb., arbitrary.

confounding effects of β-cell products can be reduced or eliminated.

We show that *1*) glucose increases $[ATP]_c$ in α-cells both within the islet and in clonal αTC1-9 cells, implying a direct effect of the sugar on α-cell oxidative metabolism; *2*) glucose and insulin, but not IGF-1, inhibit Ca^{2+} oscillations in isolated or clonal α-cells; and *3*) release of Zn^{2+} ions is unlikely to mediate the effects of β-cell activation on glucagon release from mouse α-cells.

RESEARCH DESIGN AND METHODS

Adenoviruses were constructed and amplified, using the pAdEasy system (34) as previously described (31). The 1.6-kb rat PPG promoter was digested using *Sac*I and subcloned into pEGFP-C2 (Clontech). The resulting plasmid was then digested with *Xho*I/*Hin*dIII and transferred into pShuttle (pShuttle-PPG). cDNA encoding cytosolic luciferase (pGL3-Basic; Promega) was digested with *Hin*dIII/*Sal*I and transferred into pShuttle-PPG (pShuttle-PPG-Luc). cDNA encoding cytosolic ratiometric pericam (33) was digested with *Hin*dIII/*Xba*I, and the restricted fragment was subcloned into pGL3-Basic. The resulting plasmid was then partially digested using *Hin*dIII/*Sal*I and transferred into pShuttle-PPG (pShuttle-PPG-ratiometric pericam). Recombination with pAdEasy-1, transfection into HEK 293 cells, and viral amplification were performed as described (31).

Preparation, solutions, and adenoviral infection. αTC1-9 cells (passage 35–45; American *Type Culture* Collection) were grown in Dulbecco's modified Eagle's medium containing (in mmol/l): 18 $NaHCO_3$, 16 glucose, 0.1 nonessential amino acids, 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Islets were aseptically isolated by collagenase digestion of the pancreas of female CDI mice and selected by hand-picking. Islets were then cultured for 1 day in RPMI 1640 medium containing 10 mmol/l glucose, 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (35). To obtain clusters or single cells, islets were dissociated after incubation for 5 min in Ca^{2+}-free medium. After brief centrifugation, this solution was replaced by culture medium, and the islets were disrupted by gentle pipetting through a siliconized glass pipette. Clusters and single cells were then cultured for 1 day on circular glass coverslips. The control medium (Krebs-Ringer bicarbonate buffer [KRBB]) used for islet isolation contained (in mmol/l) 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 10 glucose, and 1 mg/ml BSA and was gassed with O_2/CO_2 (95/5) to maintain a pH of 7.4. The same medium was used for all imaging experiments. Where the concentration of KCl was increased to 30 mmol/l, the concentration of NaCl was decreased accordingly to maintain iso-osmolarity. αTC1-9 cells and islets or dispersed islet cells (2nd day of culture) were infected with adenovirus at a multiplicity of infection of 50–100 infectious particles per cell for 4 h, and then the medium was changed. Imaging was performed 2–3 days after infection.

Measurement of $[ATP]_c$. Changes in $[ATP]_c$ were measured in mouse α-cells within intact islets or in αTC1-9 cells after infection with PPG-luciferase adenovirus. Cells were perifused in KRBB containing 100 μmol/l beetle luciferin and imaged at 37°C using a single-cell photon-counting imaging device (ICCD218 intensified camera; Photek, Lewes, U.K.) (31) mounted on an Olympus IX-70 microscope fitted with a 10× objective lens. Photon release was monitored continuously at a video rate (60 frames/s) and data pooled over 10 s followed by three points moving average were performed off-line to optimize the signal-to-noise ratio (30).

Measurement of cytosolic concentration of Ca^{2+} with the ratiometric pericam. Changes in cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_c$) were measured in dispersed mouse α-cells and in αTC1-9 cells after infection with PPG-ratiometric pericam-encoding adenovirus. Cells were perifused in KRBB and imaged at 37°C using an Olympus IX-70 with an Imago charge-coupled device camera (Till Photonics, Grafelfing, Germany) controlled by TILLvisION software (Till Photonics). Cells were illuminated alternatively for 5 ms (mouse α-cells) or 15 ms (αTC1-9 cells) at 410 and 480 nm, and the emitted light was

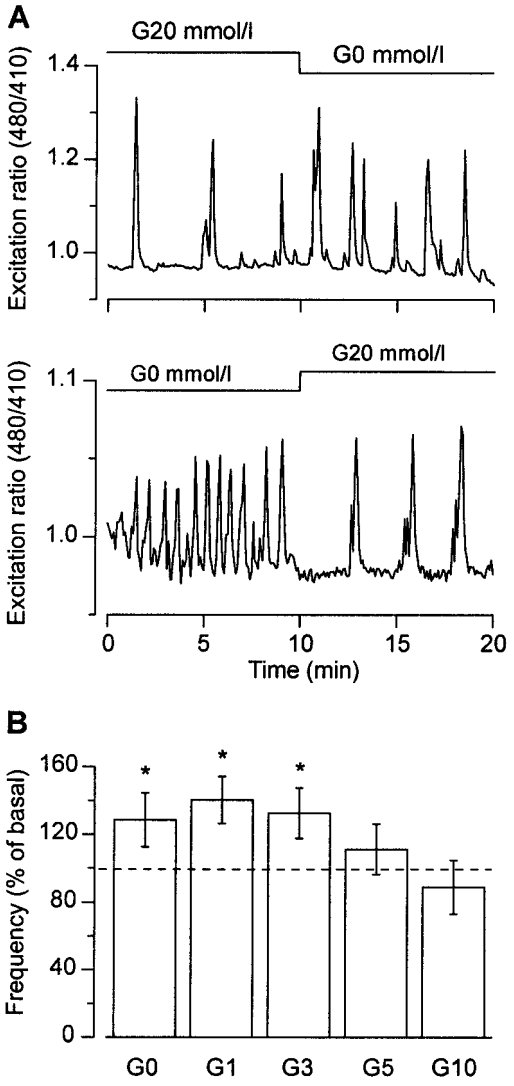

FIG. 3. Effects of glucose on $[Ca^{2+}]_c$ changes in αTC1-9 cells. *A*: The glucose concentration (G) was either decreased or increased from 20 to 0 mmol/l as indicated. *B*: Quantification of changes in $[Ca^{2+}]_c$ oscillation frequency of the experiments above. The dashed line illustrates the frequency, expressed as 100%, measured at 20 mmol/l glucose from 10 to 20 min in each experiment, whereas the empty bars (□) illustrate the frequency at 0, 1, 3, 5, or 10 mmol/l glucose during the first 10 min of perifusion. Values are the means ± SE for 18–52 cells from 9 to 15 independent experiments. *$P < 0.05$ for the effects of glucose.

filtered at 535 nm. The ratio images were used to calculate $[Ca^{2+}]_c$ off-line, as previously described (33).

Measurement of glucagon and insulin secretion. Islets (2nd day of culture) or αTC1-9 cells were preincubated for 1 h at 37°C in control KRBB medium containing 10 mmol/l glucose. Islets were then distributed in batches of six and incubated for 1 h in 400 μl of medium containing test substances, whereas αTC1-9 cells were incubated for 30 min in 700 μl in a 12-well plate. At the end of experiments, an aliquot (350 μl) of the medium was taken and saved until glucagon and insulin were measured by radioimmunoassay (Linco Research, St. Charles, MO).

Immunocytochemistry. Cells were fixed and incubated with primary and secondary antibodies as previously described (36). Glucagon was probed with polyclonal rabbit anti-glucagon antibody (1:250; DakoCytomation, Cambridgeshire, U.K.) and revealed with Alexa Fluor 568 goat anti-rabbit IgG (1:3,000; Molecular Probes, Eugene, OR). Images were captured on a Leica SP2 laser-scanning confocal microscope using a 63× oil immersion objective.

Statistical analysis. Data are the means ± SE for the number of observations indicated. Statistical significance and differences between means were assessed by Student's *t* test (paired or unpaired, as appropriate) or by ANOVA followed by a Newman-Keuls test when more than two groups were compared.

RESULTS

Imaging α-cell ATP concentration in the intact islet. Expression of luciferase under the control of the rat PPG promoter (PPG-Luc) led to efficient expression of the photoprotein in islets (Fig. 1*A*). Because we could not perform immunocytochemistry for luciferase on islets due to antibody cross-reactivity, we compared the ability of the PPG-Luc virus to infect clonal α- versus β-cells. In six independent experiments, 56% of αTC1-9 cells were infected with PPG-Luc and 56% with CMV (cytomegalovirus)-Luc (i.e., 100% efficiency after normalization). By contrast, 7% of MIN6 β-cells were infected with PPG-Luc versus 55% with CMV-Luc (13% efficiency after normalization), demonstrating α-cell selectivity, and this was consistent with α-cell–restricted expression of the pericam probe (see below).

We used photon-counting imaging to monitor the effect of glucose on luciferase luminescence, and hence $[ATP]_c$ (30–32), within α-cells in individual islets. Although this technique did not allow resolution at the level of individual cells over short integration times (10–20 s), recordings could nevertheless be made from the surface of the whole islet with a resolution of ~30 μm, corresponding to 1–6 single α-cells (Fig. 1*A*). Increasing the glucose concentration from 0 to 20 mmol/l increased the luminescence from α-cell–localized luciferase by 9% (100 ± 0.5 vs. 109 ± 3.0 units, $P < 0.01$) (Fig. 1*B*). Consistent with previous measurements in groups of islets (21), we also observed an increase of 7% in $[ATP]_c$ when the glucose concentration was increased from 3 to 20 mmol/l (100 ± 0.4 vs. 107 ± 2 units, $P < 0.01$) (Fig. 1*C*). By contrast, an increase from 5 to 20 mmol/l glucose did not produce any further increase in $[ATP]_c$ (100 ± 0.3 vs. 96 ± 3.0 units, NS) (Fig. 1*D*), suggesting that $[ATP]_c$ reached a maximum between 3 and 5 mmol/l glucose.

Imaging free ATP concentrations in αTC1-9 cells. To explore the potential role of β-cell secretory products in mediating the effects of glucose, we sought to measure changes in $[ATP]_c$ in α-cells in the absence of other islet cell types. Because the luminescence signals were too weak to be recorded from individual α-cells after islet disruption (data not shown), we instead monitored the effects of the sugar on $[ATP]_c$ in small populations of 30–100 αTC1-9 cells. In these clonal cells, an increase in glucose concentration from 0 or 1 mmol/l to 20 mmol/l induced an increase in $[ATP]_c$ of 4 or 3%, respectively ($P < 0.05$) (Fig. 1*E* and *F*), whereas an increase from 3 to 20 mmol/l glucose did not produce any further increase in $[ATP]_c$ (Fig. 1*G*). The addition of 17 nmol/l insulin either in the absence (Fig. 1*H*) or in the presence (not shown) of glucose (3 mmol/l) failed to elicit any change in $[ATP]_c$.

Regulation of Ca^{2+} dynamics by glucose in isolated primary α-cells. To determine whether glucose may be able to affect intracellular $[Ca^{2+}]_c$ in the absence of β-cell secretory products, we used a recombinant ratiometric pericam (33,37) expressed under the control of the PPG promoter (PPG-pericam). As expected, the ratiometric pericam was targeted efficiently and almost exclusively to glucagon-positive α-cells (26 of 28 cells examined, 93%) (Fig. 2*A*), allowing selective measurements of $[Ca^{2+}]_c$ in this cell type.

To infect individual α-cells, mouse islets were dispersed

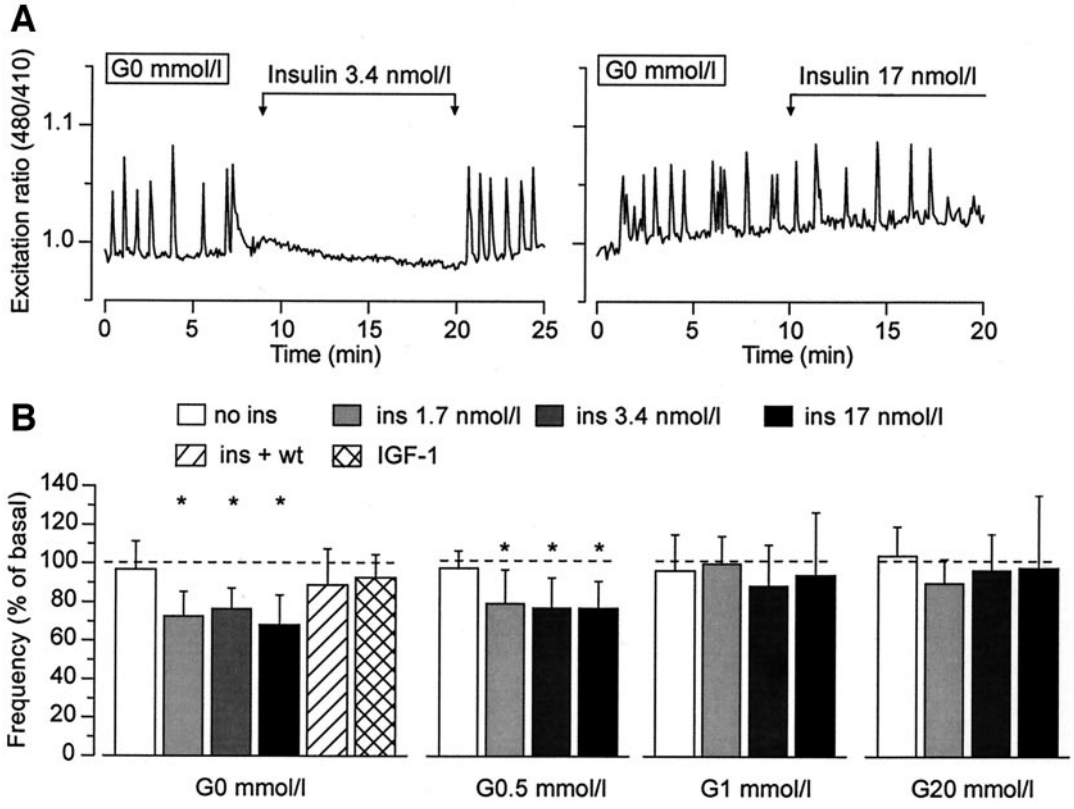

FIG. 4. Effects of insulin on $[Ca^{2+}]_c$ changes in αTC1-9 cells. These panels illustrate the impact of insulin (1.7, 3.4, or 17 nmol/l) on the frequency of $[Ca^{2+}]_c$ oscillations at a given concentration of glucose (G; 0, 0.5, 1, or 20 mmol/l). *A*: Panels show the effects of 3.4 or 17 nmol/l of insulin on $[Ca^{2+}]_c$ changes in the absence of glucose. *B*: Quantification of the experiments above. The dashed line illustrates the frequency of $[Ca^{2+}]_c$ oscillations in each experiment, expressed as 100%, before addition of insulin during the first 10 min of perifusion at a given concentration of glucose, as indicated. The empty bars illustrate control experiments where insulin (ins) was not added, whereas the light gray, dark gray, and black bars show the effects of 1.7, 3.4, or 17 nmol/l insulin added at 10 min for 10 min. The hatched and crossed bars show the effects of insulin in the presence of 100 nmol/l wortmannin (wt) and IGF-1, respectively. Values are means ± SE for 11–31 cells from 4 to 9 independent experiments. *$P < 0.05$ for the effects of insulin.

and then treated with the PPG-pericam–bearing adenovirus (Fig. 2). We then monitored $[Ca^{2+}]_c$ changes in single, well-dispersed primary α-cells. At the low cell densities used (<1 cell/mm^2), we anticipated that, under constant perifusion, local paracrine effects were unlikely to mediate any effects of glucose on individual cells. Of the 26 single primary α-cells examined in seven separate preparations, 31% (8 of 26 cells) displayed $[Ca^{2+}]_c$ oscillations at the lower glucose concentration tested (0.5 mmol/l), with a frequency of 0.2–1 oscillations/min. At a high glucose concentration (20 mmol/l), single α-cells displayed either a steady, low $[Ca^{2+}]_c$ value (2 of 8 cells) (Fig. 2*B*) or detectable $[Ca^{2+}]_c$ oscillations (6 of 8 cells) (Fig. 2*C*). In nonoscillating α-cells, a decrease in glucose concentration from 20 to 0.5 mmol/l induced oscillations of $[Ca^{2+}]_c$ (2 of 8 cells) (Fig. 2*B*), a response similar to that observed in mixed clusters of α- and non–α-cells (see below) (Fig. 5*D*). By contrast, in cells already displaying oscillations, the frequency was either increased by ≥15% (4 of 6 cells) (Fig. 2*C*) or remained unchanged (2 of 6 cells) (Fig. 2*D*), whereas the amplitude of oscillations was not affected (Fig. 2*C* and *D*). The addition of 30 mmol/l K^+ induced either a sustained (56%) or a transient (31%) increase of $[Ca^{2+}]_c$ in almost all (14 of 16, 88%) α-cells examined, even in non–glucose-responsive cells.

Regulation of Ca^{2+} dynamics by glucose in clonal αTC1-9 cells. To investigate further the ability of glucose to influence Ca^{2+} oscillations in the α-cell in the absence of β-cells, we used clonal mouse αTC1-9 cells. As shown in Fig. 3*A*, ~50% of αTC1-9 cells displayed clear oscillations in $[Ca^{2+}]_c$ in the complete absence of exogenous glucose, with a frequency of 0.1–2.4 oscillations/min. These $[Ca^{2+}]_c$ oscillations were either completely suppressed or markedly decreased in frequency when glucose concentrations were raised (Fig. 3 and 5*A*), demonstrating a direct effect of the sugar that did not require the release of β-cell products. Analysis of the dose response for these effects (Fig. 3*B* and 5*A*) revealed saturation at glucose concentrations >3–5 mmol/l.

Effects of insulin and Zn^{2+} ions on Ca^{2+} dynamics in αTC1-9 cells and primary mouse α-cells. We next sought to determine whether there may be a separate role for insulin, or cosecreted Zn^{2+} ions, to potentiate the above direct effects of glucose. Added to αTC1-9 cells in the absence of glucose, where $[Ca^{2+}]_c$ oscillations were maximally stimulated, low concentrations (1.7–17 nmol/l) of insulin led to complete or partial suppression of $[Ca^{2+}]_c$ transients (Fig. 4*A* and *B*). The effects of insulin were also apparent at 0.5 mmol/l glucose, but they were no longer evident as the glucose concentration was raised to 1 or 20 mmol/l (Fig. 4*B*). Consistent with the actions of insulin via classical insulin receptors, and signal transduction via phosphatidylinositol 3′ kinase, the effects of the hormone were not mimicked by IGF-1 but were suppressed by

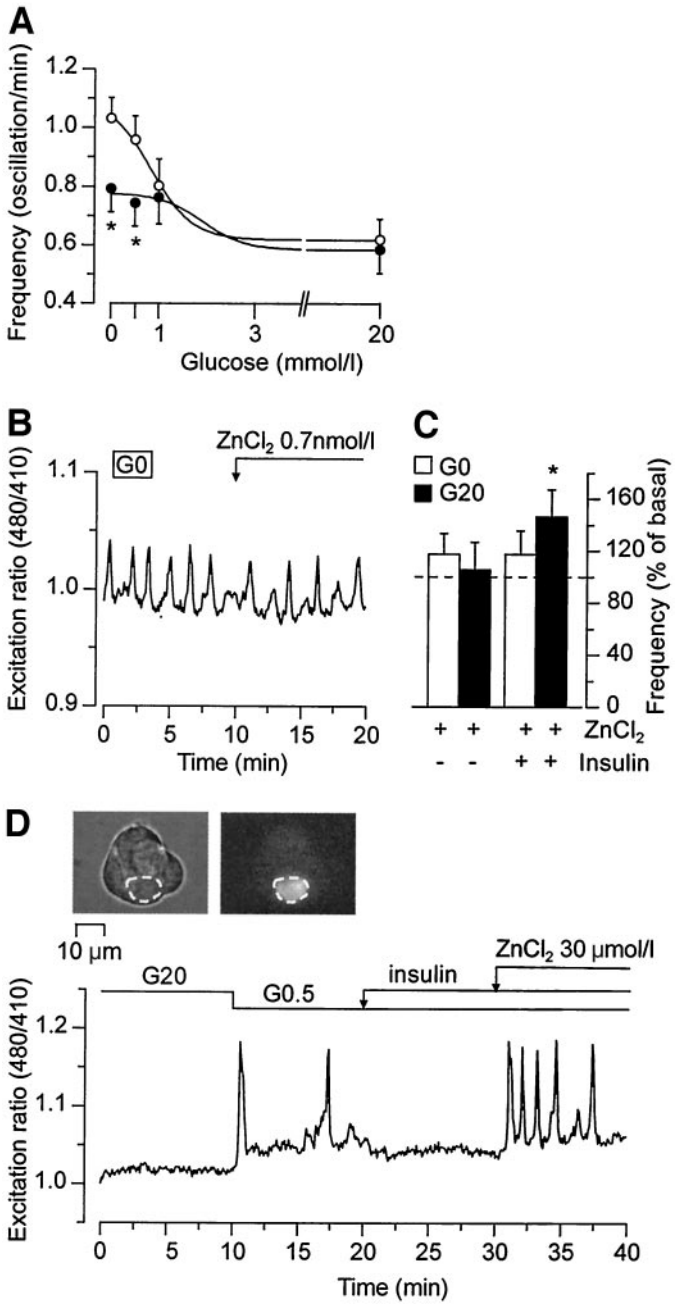

FIG. 5. Effect of $ZnCl_2$ and insulin on $[Ca^{2+}]_c$ oscillations in αTC1-9 cells (*A–C*) and mouse α-cells (*D*). *A*: Frequency of $[Ca^{2+}]_c$ oscillations in the absence (○) or in the presence (●) of insulin at a given concentration of glucose in αTC1-9 cells. *B*: After 10 min of perifusion, 0.7 nmol/l $ZnCl_2$ was applied. *C*: The quantification of experiments similar to those performed in *B*. Cells were perifused for 10 min in 0 or 20 mmol/l glucose, in the presence or in the absence of insulin, as indicated. After 10 min, 30 µmol/l of $ZnCl_2$ was added. The dashed line illustrates $[Ca^{2+}]_c$ oscillation frequency, expressed as 100%, under basal conditions during the first 10 min of perifusion, and bars illustrate the frequency after the addition of $ZnCl_2$. *D*: $[Ca^{2+}]_c$ changes in primary mouse α-cells within a cluster of four cells, with 17 nmol/l and 30 µmol/l $ZnCl_2$ applied as indicated. Values are means ± SE for 11–29 cells from 4 to 9 independent experiments. *$P < 0.05$ for the effects of insulin or $ZnCl_2$. G, glucose.

100 nmol/l wortmannin (Fig. 4*B*) or LY294002 (data not shown). Figure 5*A* illustrates the decrease in frequency of $[Ca^{2+}]_c$ oscillations at a given glucose concentration in the presence or absence of insulin. Insulin decreased the frequency of $[Ca^{2+}]_c$ oscillations at 0 or 0.5 mmol/l to the same extent as was observed at 1 mmol/l glucose in the absence of insulin, indicating that the effects of insulin and glucose were not additive.

To determine whether the effects of insulin might largely be explained by the presence of cocrystallized Zn^{2+} ions (insulin contained ~0.5% zinc), we added 0.7 nmol/l $ZnCl_2$, the concentration present after the dissolution of 1.7 nmol/l insulin. This maneuver exerted no effect on the frequency of Ca^{2+} oscillations in clonal αTC1-9 cells (100 ± 13 vs. 92 ± 15, NS) (Fig. 5*B*). Similarly, at either 0 or 20 mmol/l glucose, 30 µmol/l of $ZnCl_2$ (as used previously in the perfused pancreas) (21), exerted either no effect or had a minor stimulatory action on the frequency of Ca^{2+} oscillations in the presence or absence of 17 nmol/l insulin in αTC1-9 cells (Fig. 5*C*). Similar results were obtained in isolated primary mouse α-cells: insulin either completely suppressed (Fig. 5*D*) or decreased (not shown) the frequency of $[Ca^{2+}]_c$ oscillations. The stimulatory effect of Zn^{2+} ions was more evident in primary α-cells from dissociated mouse islets, where 30 µmol/l $ZnCl_2$ reversed the inhibitory effect of insulin (Fig. 5*D*).

Effects of glucose, insulin, and Zn^{2+} on glucagon secretion from αTC1-9 cells and islets. Glucagon secretion from αTC1-9 cells was dose-dependently decreased by glucose (Fig. 6*A*) and, in common with the observed $[Ca^{2+}]_c$ oscillations, significant inhibition was observed at glucose concentrations ≥1.0 mmol/l ($P < 0.05$). It is important to note that the release of insulin from αTC1-9 cells was essentially undetectable (≤50 pg · $ml^{-1} \cdot h^{-1} \cdot well^{-1}$) and was not affected by an increase in glucose concentration (not shown), consistent with a well-preserved α-cell phenotype. Again, the addition of insulin decreased glucagon secretion in the absence of glucose to approximately the same extent as 1 mmol/l glucose (Fig. 6*A*, Table 1), and the effects of insulin on glucagon secretion were suppressed by 100 nmol/l wortmannin (Table 1). Note, however, that the αTC1-9 cells used in this study displayed a left shift in the responses to glucose compared with primary α-cells in terms of $[ATP]_c$, $[Ca^{2+}]_c$, and glucagon secretion (see below).

The release of glucagon from primary mouse islets was dose-dependently decreased in the presence of glucose, although in this case the main inhibitory effect was observed between 3 and 5 mmol/l (Fig. 6*B*), a range of glucose concentrations that did not stimulate insulin secretion (Fig. 6*D*). These results are consistent with the range over which glucagon release is suppressed in vivo (18). As in αTC1-9 cells, insulin (17 nmol/l) suppressed glucagon secretion from intact islets, and these effects were completely reversed by the phosphatidylinositol 3′-kinase inhibitor wortmannin (Fig. 6*B* and *C*, Table 2). Wortmannin, in the absence of insulin, also tended to increase slightly the release of glucagon, possibly as a result of the suppression of an inhibitory effect of basal insulin release (66 pmol/l) at 0.5 mmol/l glucose (Fig. 6*C* and *D*). However, even in the presence of wortmannin, 10 mmol/l glucose still inhibited glucagon secretion, consistent with a direct, insulin-independent effect of the sugar on α-cells (Fig. 6*C*). In line with the absence of effect of $ZnCl_2$ on $[Ca^{2+}]_c$ changes in both αTC1-9 cells and mouse α-cells, 0.7 nmol/l (not shown) or 30 µmol/l $ZnCl_2$ (Tables 1 and 2) had no

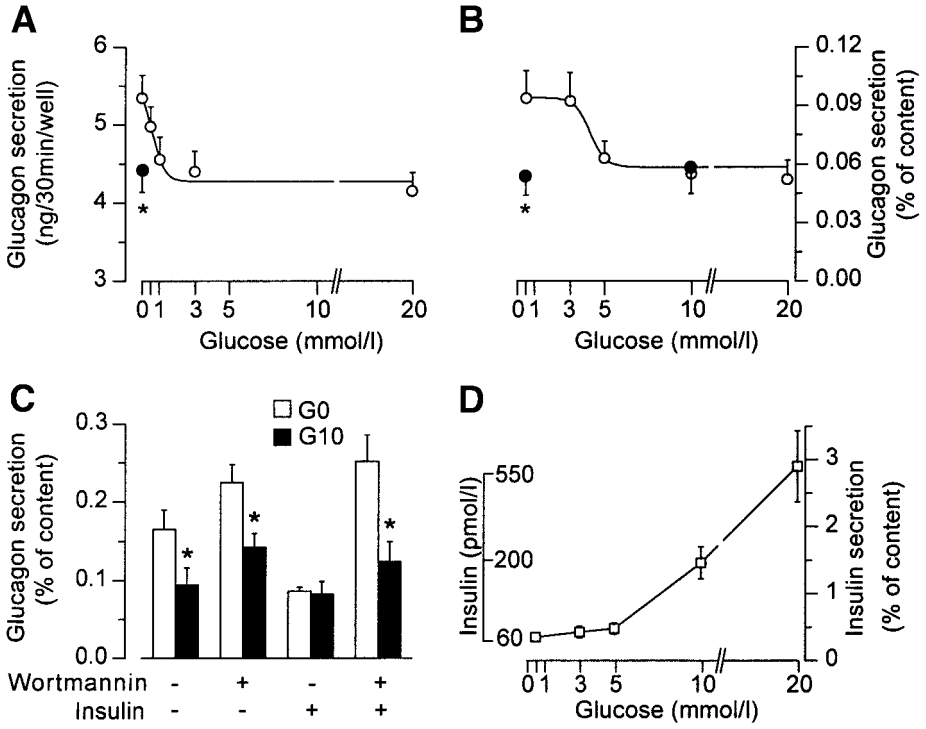

FIG. 6. Effect of glucose and insulin on glucagon secretion in αTC1-9 cells (*A*) and intact mouse islets (*B–D*). *A* and *B*: Glucagon secretion was measured at the given concentration of glucose, in the absence (○) or in the presence (●) of 17 nmol/l insulin in αTC1-9 cells (*A*) or in intact mouse islets (*B*). *C*: Impact of wortmannin on glucagon secretion in the absence or presence of insulin at 0.5 mmol/l (□) or 10 mmol/l (■) glucose (G). *D*: Insulin secretion from islets measured in samples from *B*. The scale bars illustrate the concentration of insulin released after 1 h of incubation. Values are means ± SE for four independent experiments. *$P < 0.05$ for the effects of 17 nmol/l insulin (*A* and *B*).

impact on glucagon secretion in either the absence or the presence of insulin and/or elevated glucose concentration.

DISCUSSION

Use of targeted luciferase to image free $[ATP]_c$ in α-cells. We demonstrate here the feasibility of imaging recombinant probes delivered selectively to either primary or clonal mouse α-cells under the control of the PPG gene promoter. We show firstly that fluctuations in luciferase luminescence may be used as a guide to intracellular free ATP concentrations in α-cells within the intact islet, as well as in clonal α-cells, as previously reported for β-cells (30,32). In the present studies, no attempt was made to provide a calibration of absolute free ATP concentrations in the α-cell. When such calibrations are performed after cell permeabilization (30), there are in any case considerable uncertainties as to the intracellular concentrations of luciferin and other luciferase regulators. However, total ATP concentrations have been reported to be ~2 mmol/l in αTC1-6 cells (38), compatible with free ATP concentrations (comprising ATP^{4-} and $Mg\text{-}ATP^{2-}$) in the range of 1–2 mmol/l (22).

Luciferase imaging revealed that glucose is able, in the absence of changes in β-cell secretion, to increase free cytosolic ATP concentrations in α-cells. This result is in apparent contrast to measurements of total cellular ATP content made in fluorescence-activated cell-sorted rat α-cells (22), but it is consistent with measurements of α-cell–restricted luciferase luminescence in rat islet populations (21). The magnitude of the observed increases (7–9% in primary α-cells in the intact islet, ~4% in αTC1-9 cells) was, however, significantly lower than those recorded in either β-cells within the intact islet or clonal MIN6 cells, possibly reflecting the poorer capacity for oxidative metabolism of α- versus β-cells (39,40).

Intracellular mechanisms involved in the regulation of α-cell Ca^{2+} homeostasis by glucose. Using an α-cell–targeted pericam, we analyzed here intracellular Ca^{2+} oscillations in primary and clonal mouse α-cells. Using this approach, we provide evidence that glucose is able to directly influence the activity of α-cells in the absence of β-cell secretory factors. Thus, although we were unable to

TABLE 1
Glucagon secretion from αTC1-9 cells

	0 mmol/l glucose (ng · 30 min^{-1} · $well^{-1}$)	20 mmol/l glucose (ng · 30 min^{-1} · $well^{-1}$)
No additions	5.34 ± 0.30	4.15 ± 0.24*
17 nmol/l insulin	4.42 ± 0.28*	—
17 nmol/l insulin + 100 nmol/l wortmannin	5.21 ± 0.40	—
17 nmol/l insulin + 30 μmol/l $ZnCl_2$	4.41 ± 0.21*	—

Data are the means ± SE for four separate experiments. The effects of insulin or $ZnCl_2$ were tested on glucagon secretion in either the absence or presence of 20 mmol/l glucose in αTC1-9 cells. *$P < 0.05$ vs. 0 mmol/l glucose.

TABLE 2
Glucagon secretion from mouse islets

	0.5 mmol/l glucose (% of content/h)	10 mmol/l glucose (% of content/h)
No additions	0.095 ± 0.011	0.055 ± 0.008*
17 nmol/l insulin	0.053 ± 0.008*	0.054 ± 0.009*
17 nmol/l insulin + 30 μmol/l $ZnCl_2$	0.069 ± 0.010	0.056 ± 0.006*
30 μmol/l $ZnCl_2$	0.097 ± 0.012	0.060 ± 0.010*

Data are the means ± SE for seven separate experiments. The effects of insulin, wortmannin, or $ZnCl_2$ were tested on glucagon secretion at either 0.5 or 10 mmol/l glucose in mouse islets. *$P \leq 0.05$ vs. 0.5 mmol/l glucose.

compare the efficiency of glucose to suppress Ca^{2+} oscillations in isolated α-cells versus α-cells within the intact islet (because of interference from β-cell autofluorescence in the latter case) (data not shown), the finding that a decrease in glucose concentration accelerated oscillations in most isolated α-cells is in line with previous observations that Ca^{2+} oscillations are inhibited in ~19% of all cells within the intact islet (41). Importantly, we demonstrate here for the first time that glucose and insulin modulate the frequency of Ca^{2+} oscillations in clonal mouse α-cells. This contrasts with earlier reports in lnR1-G9 glucagonoma cells (42), where nutrients were found principally to affect the amplitude of oscillations. Although the sample sizes in both the current and an earlier (41) study were not sufficiently large to quantify the changes in primary mouse α-cells, such frequency modulation nonetheless provides a potential mechanism through which glucose may regulate the pulsatile release of glucagon.

What intracellular signaling mechanisms may mediate the effects of glucose on α-cell Ca^{2+}? Several are possible. First, K_{ATP} channels may close as ATP concentrations (or the ATP-to-ADP ratio) increase. This has been proposed (43,44) to lead to the inactivation of Na^+ channels and thus the inhibition of action potentials. However, we observed little if any response to 500 μmol/l tolbutamide, the K_{ATP} channel blocker, in clonal α-cells (M.A.R and G.A.R., unpublished observations), which is consistent with previous reports that these channels are scarce (45) or insensitive to tolbutamide and diazoxide in mouse α-cells, at least at basal glucose concentrations (3 mmol/l) (15,46,47). On the other hand, electrical and secretory responses to glucose are lost in α-cells from $SUR1^{-/-}$ mice (44), arguing for an important role for these channels. Nevertheless, it is conceivable that additional sensors of cellular energy charge, such as AMP-activated protein kinase (26,48), are involved, as recently described for glucose-inhibited hypothalamic neurons (49). Alternatively, increases in ATP may also lower $[Ca^{2+}]_c$ by activating Ca^{2+} pumps, which in turn inhibit store-operated Ca^{2+} channels (46).

Roles of β-cell secretory factors in regulating glucagon release. The present studies considered the possibilities *1*) that the effects of glucose on glucagon release are indirect and mediated largely by insulin (or another β-cell–derived factor) and *2*) that they are direct, but that paracrine factors additionally affect glucagon release when glucose concentrations are low. With regards to the former, our findings indicate that glucose stimulates α-cell oxidative metabolism directly, elevating $[ATP]_c$ and in turn suppressing glucagon release. By contrast, Ishihara et al. (21) proposed that in the absence of a factor(s) secreted from β-cells, enhancement of α-cell metabolism by glucose would stimulate glucagon release. Although the reasons for these divergent conclusions are unclear, the current data argue that the release of a β-cell–derived factor is unlikely to be essential to inhibit glucagon release in response to glucose. Thus, the inhibitory effects of glucose were observed here both in αTC1-9 cells, where an effect of β-cell factors is excluded, and in mouse islets when insulin signaling was blocked with wortmannin (Fig. 6*C*). Correspondingly, glucagon secretion was fully inhibited at glucose concentrations (<5 mmol/l) that were clearly lower than those that began to stimulate the secretion of insulin (>5 mmol/l) (Fig. 6*B* vs. *D*) and presumably other β-cell factors. Concerning the possibility that the effects of glucose on glucagon are direct but that paracrine factors additionally affect glucagon release, insulin was found here to suppress glucagon release in the absence of glucose. Whereas the physiological relevance of this phenomenon is uncertain, it may ensure that insulin released in response to metabolizable amino acids such as leucine prevents the simultaneous activation of glucagon secretion (50).

In further contrast to recent observations in the perfused rat pancreas (21), we observed no or only small stimulatory effects of $ZnCl_2$ on $[Ca^{2+}]_c$ transients and glucagon release from either mouse αTC1-9 cells or mouse islets. One possible explanation for this apparent difference between rat and mouse is that the effects of Zn^{2+} ions may be mediated in the former case through a K_{ATP} channel–dependent mechanism, given that these channels are relatively abundant in rat, but not mouse, α-cells (see above). It is also conceivable that other targets for Zn^{2+} action, including γ-aminobutyric acid receptors (16,51), are important: although these have been characterized in part in rat α-cells (52), data are currently unavailable for mouse α-cells.

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