

# Glucose and Pharmacological Modulators of ATP-Sensitive $K^+$ Channels Control $[Ca^{2+}]_c$ by Different Mechanisms in Isolated Mouse $\alpha$ -Cells

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**OBJECTIVE**—We studied how glucose and ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channel modulators affect  $\alpha$ -cell  $[Ca^{2+}]_c$ .

**RESEARCH DESIGN AND METHODS**—GYM mice (expressing enhanced yellow fluorescent protein in  $\alpha$ -cells) and NMRI mice were used.  $[Ca^{2+}]_c$ , the  $K_{ATP}$  current ( $I_{KATP}$ , perforated mode) and cell metabolism [NAD(P)H fluorescence] were monitored in single  $\alpha$ -cells and, for comparison, in single  $\beta$ -cells.

**RESULTS**—In 0.5 mmol/l glucose,  $[Ca^{2+}]_c$  oscillated in some  $\alpha$ -cells and was basal in the others. Increasing glucose to 15 mmol/l decreased  $[Ca^{2+}]_c$  by  $\sim 30\%$  in oscillating cells and was ineffective in the others.  $\alpha$ -Cell  $I_{KATP}$  was inhibited by tolbutamide and activated by diazoxide or the mitochondrial poison azide, as in  $\beta$ -cells. Tolbutamide increased  $\alpha$ -cell  $[Ca^{2+}]_c$ , whereas diazoxide and azide abolished  $[Ca^{2+}]_c$  oscillations. Increasing glucose from 0.5 to 15 mmol/l did not change  $I_{KATP}$  and NAD(P)H fluorescence in  $\alpha$ -cells in contrast to  $\beta$ -cells. The use of nimodipine showed that L-type  $Ca^{2+}$  channels are the main conduits for  $Ca^{2+}$  influx in  $\alpha$ -cells.  $\gamma$ -Aminobutyric acid and zinc did not decrease  $\alpha$ -cell  $[Ca^{2+}]_c$ , and insulin, although lowering  $[Ca^{2+}]_c$  very modestly, did not affect glucagon secretion.

**CONCLUSIONS**— $\alpha$ -Cells display similarities with  $\beta$ -cells:  $K_{ATP}$  channels control  $Ca^{2+}$  influx mainly through L-type  $Ca^{2+}$  channels. However,  $\alpha$ -cells have distinct features from  $\beta$ -cells: Most  $K_{ATP}$  channels are already closed at low glucose, glucose does not affect cell metabolism and  $I_{KATP}$ , and it slightly decreases  $[Ca^{2+}]_c$ . Hence, glucose and  $K_{ATP}$  channel modulators exert distinct effects on  $\alpha$ -cell  $[Ca^{2+}]_c$ . The direct small glucose-induced drop in  $\alpha$ -cell  $[Ca^{2+}]_c$  contributes likely only partly to the strong glucose-induced inhibition of glucagon secretion in islets. *Diabetes* 58:412–421, 2009

**G**lucagon secretion is normally inhibited by hyperglycemia and stimulated by hypoglycemia, but alterations of its physiological regulation contribute to abnormal glucose homeostasis in diabetes (1,2). The cellular mechanisms controlling glucagon secretion are still unclear. In particular, whether

glucose directly or indirectly influences  $\alpha$ -cells remains disputed. An indirect inhibition of glucagon secretion by glucose has variably been ascribed to glucose-induced release of an inhibitory paracrine messenger from  $\beta$ - or  $\delta$ -cells, such as insulin (3–5),  $\gamma$ -aminobutyric acid (GABA) (4,6–9),  $Zn^{2+}$  (10,11), or somatostatin (12,13).

In contrast, the models attributing glucose inhibition of glucagon secretion to a direct action in  $\alpha$ -cells implicate a decrease of  $\alpha$ -cell  $[Ca^{2+}]_c$  by the sugar (14). A first mechanism attributes a key role to ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels. In  $\beta$ -cells, the metabolism of glucose increases the cytosolic ATP-to-ADP ratio, which closes  $K_{ATP}$  channels in the plasma membrane. This leads to plasma membrane depolarization, opening of high-threshold voltage-dependent  $Ca^{2+}$  channels (VDCC, mainly of the L-type),  $Ca^{2+}$  influx, and increase in  $[Ca^{2+}]_c$ , which triggers insulin secretion. According to the model, the  $K_{ATP}$  current ( $I_{KATP}$ ) in  $\alpha$ -cells is already small at low glucose, so that the plasma membrane is slightly depolarized to the threshold for activation of low-threshold voltage-dependent  $Na^+$  channels and VDCCs participating in action potential generation. At high glucose, further closure of  $K_{ATP}$  channels depolarizes the  $\alpha$ -cell plasma membrane to a potential where low-threshold voltage-dependent channels inactivate, preventing action potential generation, arresting  $Ca^{2+}$  influx, lowering  $[Ca^{2+}]_c$  and eventually inhibiting glucagon secretion (15,16). An alternative mechanism of direct inhibition of  $\alpha$ -cells by glucose suggests that the arrest of  $Ca^{2+}$  influx occurs independently of a modulation of  $K_{ATP}$  channels and is mediated by a hyperpolarization of the plasma membrane resulting from glucose-induced reduction of a depolarizing store-operated current ( $I_{SOC}$ ) (17,18).

One major reason for this lack of consensus is that identification of living  $\alpha$ -cells among other islet cells is not straightforward. We recently developed a new model, the GYM mouse, allowing rapid identification of living  $\alpha$ -cells thanks to their specific expression of the enhanced yellow fluorescent protein (EYFP) (19). In the present study, we used this model to evaluate the impact of glucose on cell metabolism [NAD(P)H fluorescence],  $I_{KATP}$ , and  $[Ca^{2+}]_c$  in isolated  $\alpha$ -cells. The responses of  $\alpha$ -cells were compared with those of  $\beta$ -cells. We also evaluated the effects of  $K_{ATP}$  channel modulators and candidate paracrine factors released by  $\beta$ -cells on  $\alpha$ -cell  $[Ca^{2+}]_c$ .

## RESEARCH DESIGN AND METHODS

Most experiments were performed with our mouse models expressing EYFP specifically in  $\alpha$ - or  $\beta$ -cells and referred to as GYM and RIPYY mice, respectively (19). NMRI mice were used as controls. The study was approved by our Commission d'Éthique d'Expérimentation Animale.

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**Preparations and solutions.** Islets were obtained by collagenase digestion of the pancreas, and single cells were prepared by dispersion in a  $\text{Ca}^{2+}$ -free medium. Islet cells were cultured for 1–4 days on coverslips in RPMI 1640 containing 7 mmol/l glucose.

The extracellular solution contained 120 mmol/l NaCl, 4.8 mmol/l KCl, 1.5 mmol/l  $\text{CaCl}_2$ , 1.2 mmol/l  $\text{MgCl}_2$ , 24 mmol/l  $\text{NaHCO}_3$ , and 1 mg/ml BSA (pH 7.4). It was gassed with  $\text{O}_2/\text{CO}_2$  (94:6%). The 2.5-mmol/l amino acid mixture used in some experiments contained 0.5 mmol/l alanine, 0.5 mmol/l leucine, 0.75 mmol/l glutamine, and 0.75 mmol/l lysine. For  $I_{\text{KATP}}$  and membrane potential recordings, the extracellular medium was devoid of BSA and supplemented with 5 mmol/l HEPES. Pipette solution contained 70 mmol/l  $\text{K}_2\text{SO}_4$ , 10 mmol/l NaCl, 10 mmol/l KCl, 3.7 mmol/l  $\text{MgCl}_2$ , and 5 mmol/l HEPES (pH 7.1).

**Identification of  $\beta$ -cells of GYY mice with DsRed.** To identify  $\beta$ -cells from GYY mice, islet cells were infected with the AdRIPBgliDsRed adenovirus ensuring a  $\beta$ -cell specific expression of DsRed (a description is available in an online appendix at <http://dx.doi.org/10.2337/db07-1298>).

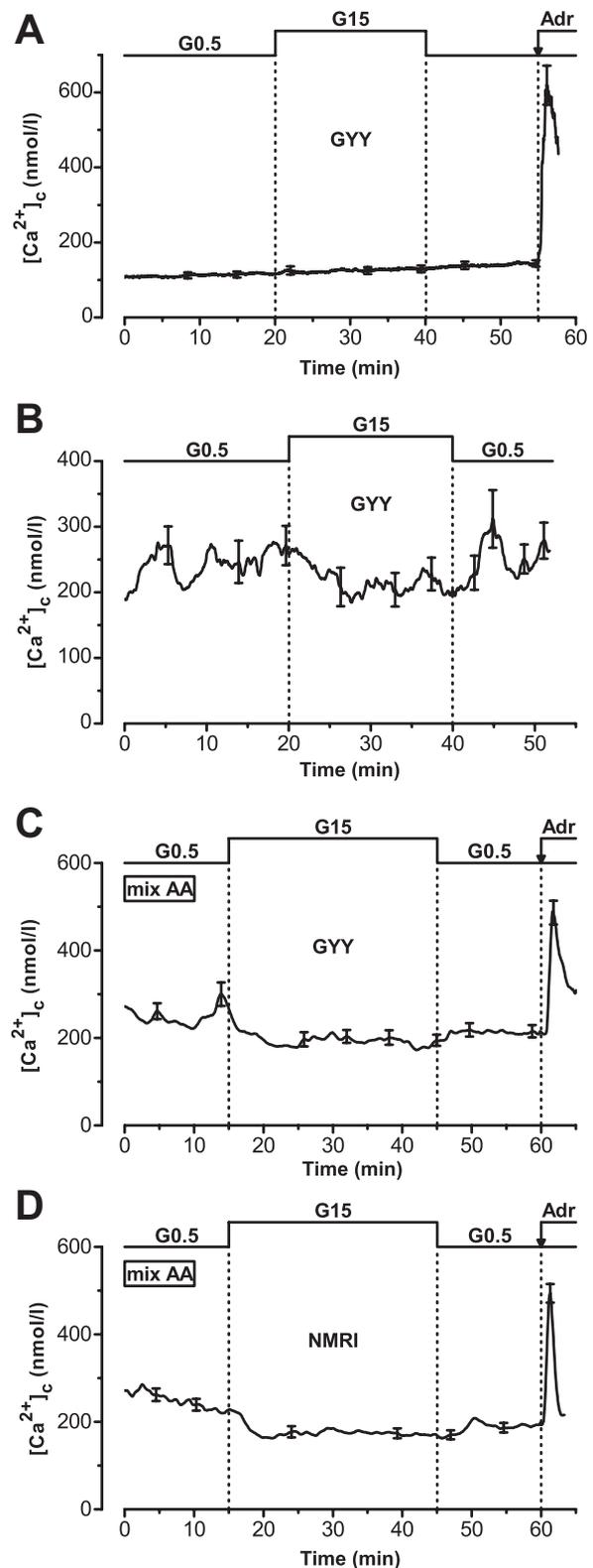
**$[\text{Ca}^{2+}]_c$ , NAD(P)H,  $I_{\text{KATP}}$ , and glucagon secretion measurements.** Cells expressing EYFP (excitation, 490 nm; emission, 535 nm) or DsRed (excitation, 540 nm; emission, 610 nm) were first selected.  $[\text{Ca}^{2+}]_c$  (fura-PE3 or fura-2) and NAD(P)H fluorescences were monitored at 37°C as described previously (19). It was verified that EYFP fluorescence did not contaminate  $[\text{Ca}^{2+}]_c$  and NAD(P)H signals.  $I_{\text{KATP}}$  was recorded at 31–32°C in the perforated mode by applying 100-ms-duration pulses of  $\pm 20$  mV from a holding potential of  $-80$  mV as reported previously (20). Membrane potential measurements were performed at 33°C in the perforated mode in current-clamp. Glucagon secretion from batches of 200 islets of GYY mice was monitored in perfusion experiments as described previously (19).

**Statistical analysis.** Data are shown as representative traces or means  $\pm$  SE of results obtained with the indicated number of cells or batches of 200 islets (Fig. 7D only) from at least three different cultures. The statistical significance of differences between means was assessed by paired Student's *t* test.

## RESULTS

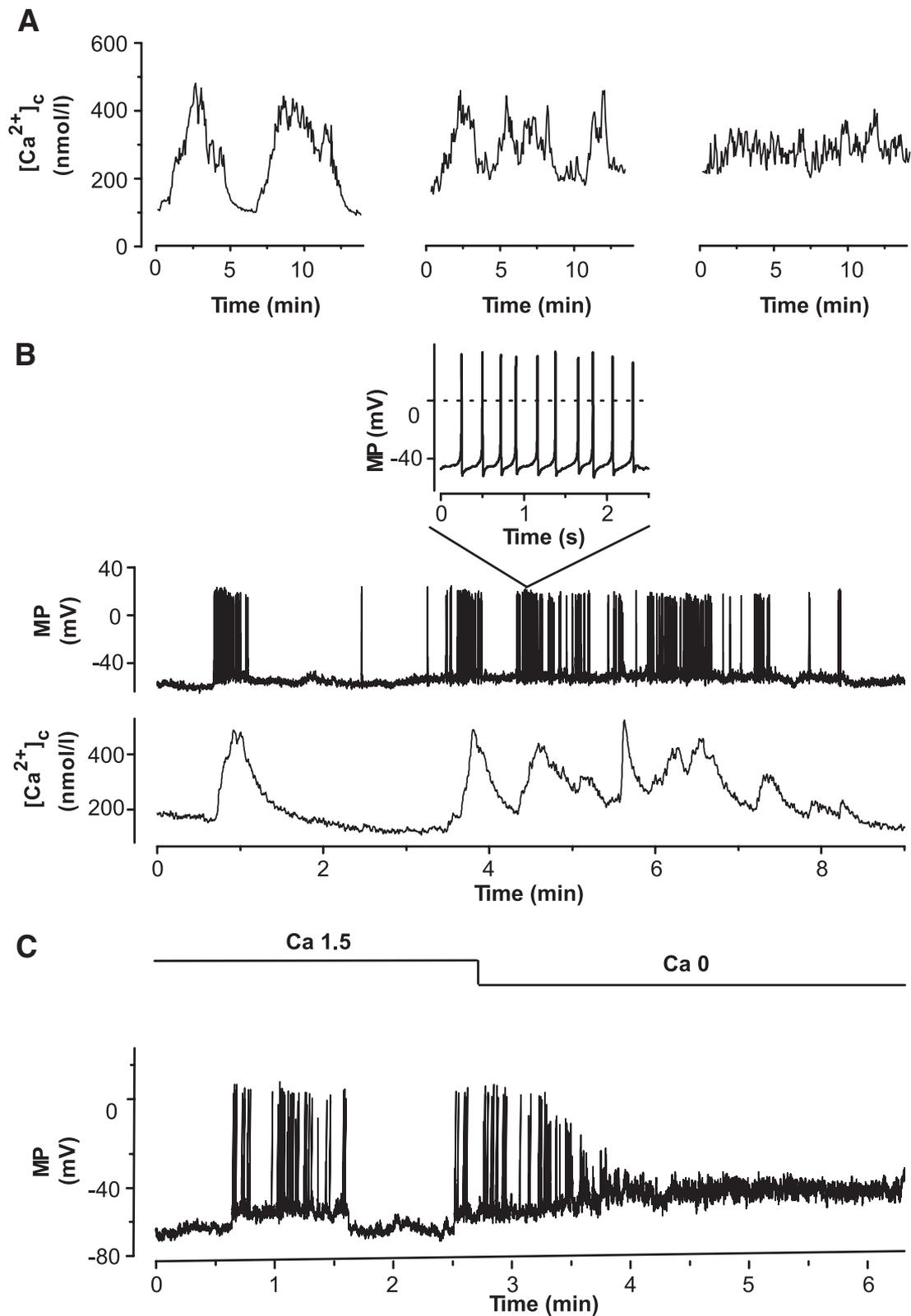
**Effects of glucose on  $[\text{Ca}^{2+}]_c$  in  $\alpha$ -cells.** Because EYFP and fura-PE3 excitation spectra do not overlap,  $[\text{Ca}^{2+}]_c$  could be easily monitored in EYFP-expressing  $\alpha$ -cells. In the presence of 0.5 mmol/l glucose,  $[\text{Ca}^{2+}]_c$  oscillated in 31% (175 of 555) of  $\alpha$ -cells (Fig. 1B) and was stable at basal levels in the others (Fig. 1A). Non-oscillating and oscillating  $\alpha$ -cells were equally responsive to arginine and adrenaline, indicating that they are both physiologically normal (not shown). Increasing the glucose concentration from 0.5 (G0.5) to 15 mmol/l (G15) did not affect  $[\text{Ca}^{2+}]_c$  in non-oscillating  $\alpha$ -cells (Fig. 1A;  $n = 21$ ) and slightly decreased it in oscillating  $\alpha$ -cells (Fig. 1B). In the latter group, average  $[\text{Ca}^{2+}]_c$  integrated over the last 17 min of perfusion with G15 was 28% lower than average  $[\text{Ca}^{2+}]_c$  in G0.5 ( $n = 35$ ,  $P < 0.05$ ).

We next tested the effect of glucose on  $\alpha$ -cell  $[\text{Ca}^{2+}]_c$  in the presence of a 2.5-mmol/l mixture of amino acids that potentiate glucagon secretion (21). This mild stimulatory condition increased the proportion of  $\alpha$ -cells displaying  $[\text{Ca}^{2+}]_c$  oscillations in G0.5 to 70% (180 of 255), which made it easier to study the inhibitory effect of high glucose. Increasing the glucose concentration from 0.5 to 15 mmol/l decreased  $[\text{Ca}^{2+}]_c$  (integrated over the last 27 min in G15) to a similar extent (by 24%;  $n = 54$ ,  $P < 0.05$ ) as without amino acids (Fig. 1C). We also monitored  $[\text{Ca}^{2+}]_c$  in NMRI  $\alpha$ -cells identified by their response to adrenaline applied at the end of the experiment (17,22). In the presence of G0.5,  $[\text{Ca}^{2+}]_c$  oscillated in 20% (16 of 82) of  $\alpha$ -cells in the absence of amino acids and in 67% (67 of 100) of  $\alpha$ -cells in the presence of 2.5-mmol/l amino acid mixture. The effect of G15 on  $[\text{Ca}^{2+}]_c$  in NMRI  $\alpha$ -cells was similar to that observed in GYY  $\alpha$ -cells (Fig. 1D), with a 32% drop in average  $[\text{Ca}^{2+}]_c$  (integrated over the last 27 min in G15) compared with initial  $[\text{Ca}^{2+}]_c$  in low glucose ( $n = 46$ ,  $P < 0.01$ ).



**FIG. 1.** Glucose slightly decreases oscillating  $[\text{Ca}^{2+}]_c$  in  $\alpha$ -cells isolated from GYY (A–C) and NMRI mice (D, adrenaline-responsive cells). A–D: The glucose (G) concentration was changed between 0.5 and 15 mmol/l, and adrenaline (Adr, 10  $\mu\text{mol/l}$ ) was added as indicated. C and D: The perfusion medium was supplemented with a 2.5-mmol/l mixture of amino acids (mix AA). Data are means  $\pm$  SE of results obtained in 21 (A), 35 (B), and 54 (C) isolated GYY  $\alpha$ -cells and in 46 isolated NMRI  $\alpha$ -cells (D).

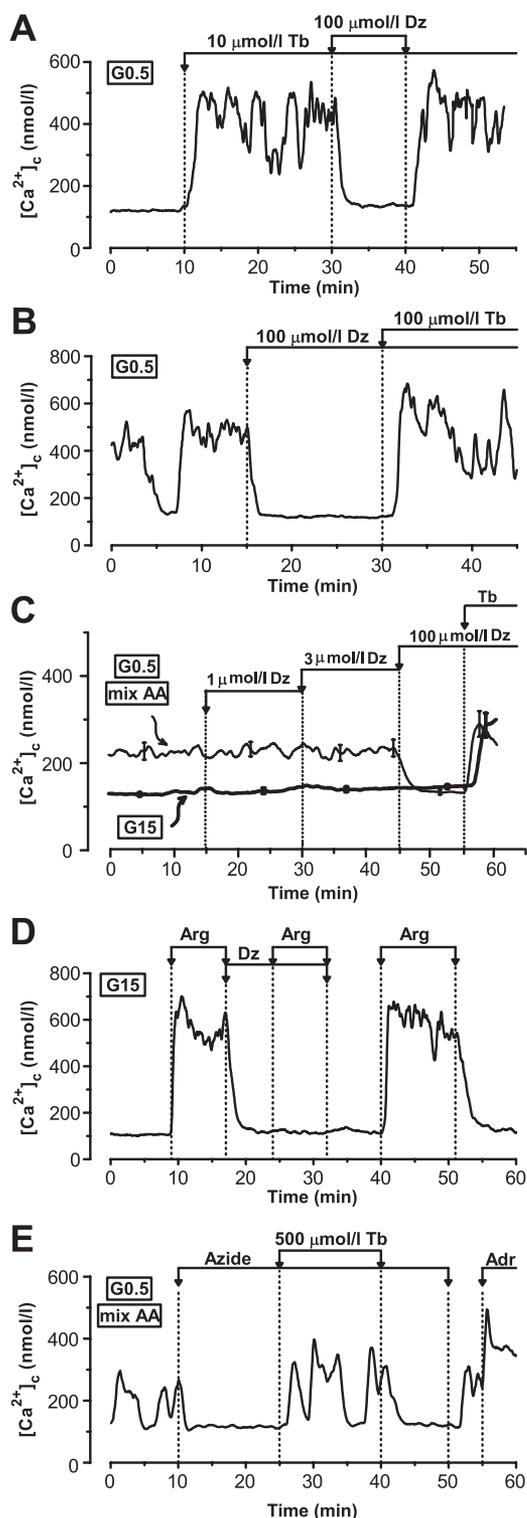
$[\text{Ca}^{2+}]_c$  oscillations had very heterogeneous patterns, being irregular or mixed, composed of oscillations of various amplitude and frequency (Fig. 2A and beginning of



**FIG. 2.** Spontaneous  $[Ca^{2+}]_c$  oscillations in isolated mouse  $\alpha$ -cells are highly variable and result from synchronous and intermittent  $Ca^{2+}$ -dependent spiking electrical activity.  $\alpha$ -Cells were perfused with a medium containing 0.5 mmol/l glucose (G0.5) and a 2.5-mmol/l mixture of amino acids (mix AA). **A:** Examples of  $[Ca^{2+}]_c$  oscillations in three isolated  $\alpha$ -cells. **B and C:** The membrane potential (MP) was recorded in single  $\alpha$ -cells in the perforated mode of the patch-clamp technique, and in **B**, it was simultaneously monitored with  $[Ca^{2+}]_c$ . Note the overshoots in the spikes, a typical feature of  $\alpha$ -cells (inset in **B**). In **C**,  $\alpha$ -cells were first perfused with a medium containing 1.5 mmol/l  $Ca^{2+}$  (Ca1.5) and then, when indicated, with a  $Ca^{2+}$ -free medium supplemented with 200  $\mu$ mol/l EGTA (Ca0). Traces are representative of results obtained in 910 (**A**), 8 (**B**), and 3 (**C**)  $\alpha$ -cells from GYY mice.

Fig. 3*B* and *E*). The pattern was not obviously affected by glucose or amino acids. The oscillations resulted from concomitant membrane potential oscillations with bursts

of spikes (Fig. 2*B*). The electrical spiking involved  $Ca^{2+}$  channels because it was abolished in a  $Ca^{2+}$ -free medium (Fig. 2*C*).



**FIG. 3.** Effects of drugs on  $[Ca^{2+}]_c$  in  $\alpha$ -cells. Closure of  $K_{ATP}$  channels by tolbutamide induced  $[Ca^{2+}]_c$  oscillations, whereas opening of  $K_{ATP}$  channels with diazoxide and blockade of cell metabolism with sodium azide lowered  $[Ca^{2+}]_c$  to basal levels in isolated  $\alpha$ -cells from GYY mice. The perfusion medium contained 0.5 (A–C and E) or 15 mmol/l (C and D) glucose (G) and a 2.5-mmol/l mixture of amino acids (mix AA) (C, top trace, and E). A: Sequential addition of 10  $\mu$ mol/l tolbutamide (Tb) and 100  $\mu$ mol/l diazoxide (Dz). B: Sequential addition of 100  $\mu$ mol/l diazoxide (Dz) and 100  $\mu$ mol/l tolbutamide (Tb). C: The diazoxide concentration (Dz) was increased stepwise, and 100  $\mu$ mol/l tolbutamide (Tb) was applied as indicated. D: Arginine (Arg; 10 mmol/l) and 100  $\mu$ mol/l diazoxide (Dz) were applied when indicated. E: Sodium azide (2 mmol/l), 500  $\mu$ mol/l tolbutamide (Tb), and 10  $\mu$ mol/l adrenaline (Adr) were applied as indicated. Traces are representative of results obtained in 9 (A), 8 (B), 8 (G15; C), 46 (G0.5 + mix AA; C), 12 (D), and 19 (E)  $\alpha$ -cells from GYY mice.

### Closure of $K_{ATP}$ channels increases $[Ca^{2+}]_c$ in $\alpha$ -cells.

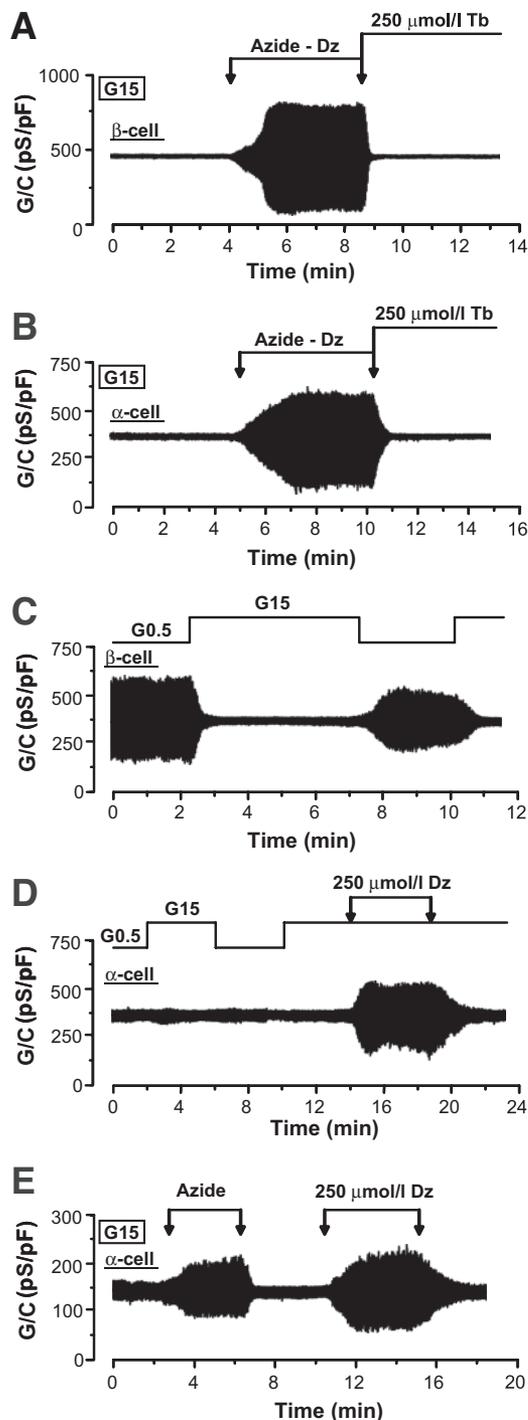
We next tested modulators of  $K_{ATP}$  channels on  $\alpha$ -cell  $[Ca^{2+}]_c$ . When  $[Ca^{2+}]_c$  was low and stable in G0.5, addition of 10  $\mu$ mol/l tolbutamide increased  $[Ca^{2+}]_c$  (Fig. 3A). Subsequent addition of 100  $\mu$ mol/l diazoxide reversed the effects of tolbutamide (Fig. 3A). When  $[Ca^{2+}]_c$  was oscillating in low glucose, 100  $\mu$ mol/l diazoxide decreased  $[Ca^{2+}]_c$  to basal levels, and this effect was reversed by 100  $\mu$ mol/l tolbutamide (Fig. 3B). Similar results were observed in the presence of 2.5-mmol/l amino acid mixture (19). Low diazoxide concentrations (1–3  $\mu$ mol/l) failed to affect oscillating or basal  $[Ca^{2+}]_c$  (Fig. 3C). Diazoxide was also ineffective at 10  $\mu$ mol/l in non-oscillating cells perfused with G15 ( $n = 8$ , not shown). Figure 3D shows that 100  $\mu$ mol/l diazoxide prevented 10 mmol/l arginine from increasing  $[Ca^{2+}]_c$  in  $\alpha$ -cells. Overall, these data indicate that  $[Ca^{2+}]_c$  oscillations in the presence of glucose alone and  $Ca^{2+}$  influx induced by arginine occur only when most  $K_{ATP}$  channels are closed.

We next tested whether  $\alpha$ -cell  $[Ca^{2+}]_c$  was affected by perturbation of cell metabolism. In the presence of 2.5-mmol/l amino acid mixture, the mitochondrial poison azide reversibly abolished spontaneous  $[Ca^{2+}]_c$  oscillations occurring in G0.5 and lowered  $[Ca^{2+}]_c$  to basal levels. Subsequent closure of  $K_{ATP}$  channels with 500  $\mu$ mol/l tolbutamide reversed this inhibition (Fig. 3E). A similar effect of azide was observed in the absence of amino acids and occurred in glucose-stimulated  $\beta$ -cells (not shown). The comparable response of  $\alpha$ - and  $\beta$ -cells to azide suggests that the membrane potential is influenced by metabolism in both cell types. We therefore compared their  $I_{KATP}$ .

**$\alpha$ -Cells possess  $K_{ATP}$  channels that are insensitive to glucose.** EYFP-expressing cells from GYY mice had a capacitance of  $4.5 \pm 0.19$  pF/cell ( $n = 42$ ) versus  $6.77 \pm 0.37$  pF/cell ( $n = 18$ ) for those from RIPYY mice. These results agree with previous reports showing that  $\alpha$ -cells are smaller than  $\beta$ -cells (14,23,24).

$I_{KATP}$  was measured in the perforated mode of the patch-clamp technique in  $\alpha$ - and  $\beta$ -cells from GYY mice and in  $\beta$ -cells from RIPYY mice. For some experiments, islet cells from GYY mice were infected with the recombinant adenovirus AdRIPBgliDsRed 2 days before the experiments to permit easy identification of  $\beta$ -cells (red) and  $\alpha$ -cells (yellow) before recordings. Insulin immunodepletion showed that DsRed was exclusively targeted to  $\beta$ -cells (supplementary Fig. 1A–C, available in the online appendix) but that only ~45%  $\beta$ -cells were fluorescent for DsRed (43 of 94). Importantly, DsRed and EYFP were consistently expressed in distinct cell types (supplementary Fig. 1D).

We first compared  $I_{KATP}$  in  $\alpha$ - and  $\beta$ -cells from GYY mice. During perfusion with G15,  $I_{KATP}$  was small in  $\alpha$ -cells ( $39.5 \pm 9.5$  pS/pF,  $n = 5$ ) and  $\beta$ -cells ( $21.5 \pm 3.5$  pS/pF,  $n = 5$ ) (Fig. 4A and B). The maximal density of  $I_{KATP}$  was estimated by perfusing the cells with 250  $\mu$ mol/l diazoxide and 1 mmol/l sodium azide.  $I_{KATP}$  was 30% smaller (but not statistically different) in  $\alpha$ - ( $490 \pm 72$  pS/pF) than  $\beta$ -cells ( $695 \pm 130$  pS/pF). Closure of  $K_{ATP}$  channels by 250  $\mu$ mol/l tolbutamide in the presence of G15 decreased  $I_{KATP}$  to slightly lower, but not significantly different, values ( $29.7 \pm 7$  pS/pF in  $\alpha$ -cells and  $15.2 \pm 1.5$  pS/pF in  $\beta$ -cells) than those measured in G15 alone (Fig. 4A and B). This indicates that in the sole presence of G15, most, although not all,  $K_{ATP}$  channels are closed in  $\alpha$ -cells and in  $\beta$ -cells (25). To determine whether infection had



**FIG. 4.**  $\alpha$ -Cells possess a  $I_{K_{ATP}}$  with similar characteristics to those of  $\beta$ -cells, except for its insensitivity to glucose.  $I_{K_{ATP}}$  was monitored by pulses of  $\pm 20$  mV from a holding potential of  $-80$  mV using the perforated mode of the patch-clamp technique. The current density was obtained by dividing the amplitude of the current by the membrane capacitance of the cell.  $I_{K_{ATP}}$  was recorded in  $\beta$ -cells (A) and  $\alpha$ -cells (B) from GYY mice infected with AdRIPBgliDsRed, in noninfected  $\beta$ -cells from RIPYY mice (C), and in noninfected  $\alpha$ -cells from GYY mice (D and E). The glucose (G) concentration was 15 mmol/l throughout (A–B and E), or changed between 0.5 and 15 mmol/l as indicated (C and D). Sodium azide (1 mmol/l in A and B or 2 mmol/l in E), 250  $\mu$ mol/l diazoxide (Dz; A, B, D, and E), and 250  $\mu$ mol/l tolbutamide (Tb; A and B) were applied when indicated. Each trace is representative of five experiments.

affected the current density, we performed similar experiments in noninfected  $\alpha$ -cells from GYY mice. No effect of infection was observed:  $I_{K_{ATP}}$  was  $34.2 \pm 4.5$  pS/pF ( $n = 6$ ) in G15,  $432 \pm 92$  pS/pF in the presence of diazoxide and

azide, and  $23 \pm 3$  pS/pF after addition of 250  $\mu$ mol/l tolbutamide (not shown). Overall, these results highlight the similarities of  $I_{K_{ATP}}$  between  $\alpha$ - and  $\beta$ -cells.

We next investigated whether glucose influences  $\alpha$ -cell  $I_{K_{ATP}}$ . To validate our measurements,  $I_{K_{ATP}}$  was first recorded in  $\beta$ -cells from RIPYY mice. As expected, the large  $I_{K_{ATP}}$  in G0.5 was reversibly inhibited from  $242 \pm 72$  to  $27.5 \pm 8$  pS/pF by G15 ( $n = 5$ ; Fig. 4C) and from  $211 \pm 106$  to  $30 \pm 15$  pS/pF by 250  $\mu$ mol/l tolbutamide ( $n = 4$ , not shown). By contrast, glucose did not affect  $I_{K_{ATP}}$  in  $\alpha$ -cells ( $38.5 \pm 8.7$  and  $39.2 \pm 9.5$  pS/pF ( $n = 5$ ) in G0.5 and G15, respectively; Fig. 4D). However,  $\alpha$ -cell  $I_{K_{ATP}}$  was reduced by 30% by 250  $\mu$ mol/l tolbutamide (from  $33.2 \pm 8.7$  to  $21.0 \pm 1.7$  pS/pF,  $n = 12$ , not statistically different, not shown) and increased by diazoxide (from  $38.5 \pm 8.7$  to  $141 \pm 51$  pS/pF,  $n = 5$ ; Fig. 4D). In the presence of G15, 2 mmol/l azide reversibly increased  $I_{K_{ATP}}$  from  $33 \pm 8.2$  to  $138 \pm 39$  pS/pF (Fig. 4E;  $n = 5$ ,  $P < 0.05$ ) indicating that  $K_{ATP}$  channels in  $\alpha$ -cells can be controlled by changes in cell metabolism.

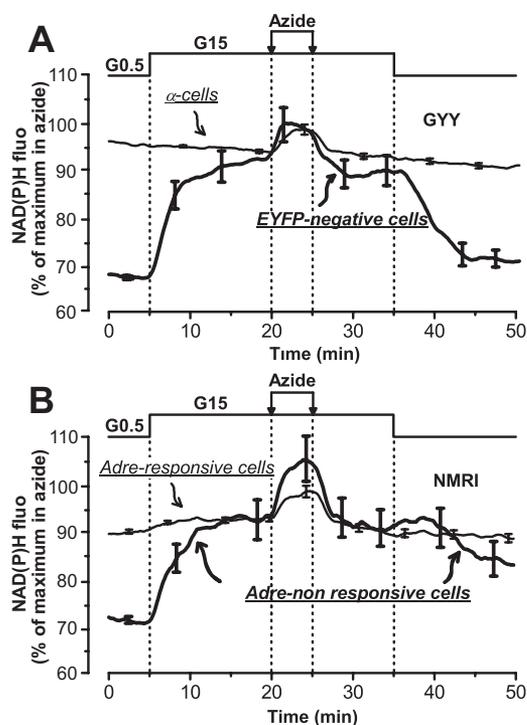
**Glucose does not affect NAD(P)H fluorescence in  $\alpha$ -cells.** NAD(P)H fluorescence can be used to monitor nutrient-induced changes in  $\beta$ -cell metabolism. We therefore compared the influence of glucose on NAD(P)H fluorescence in isolated  $\alpha$ -cells and EYFP-negative islet cells (most of them presumably being  $\beta$ -cells). Figure 5A shows that increasing the glucose concentration from 0.5 to 15 mmol/l induced a reversible increase of NAD(P)H fluorescence in EYFP-negative cells ( $\beta$ -cells) ( $n = 14$ ) but had no effect on  $\alpha$ -cell NAD(P)H fluorescence ( $n = 30$ ). Azide, which blocks the electron transport chain and inhibits NAD(P)H oxidation, evoked a small increase of NAD(P)H fluorescence in  $\alpha$ - and  $\beta$ -cells. Similar results were observed in the presence of 2.5-mmol/l amino acid mixture (not shown).

The same experiment was performed in NMRI islet cells. After each NAD(P)H measurement, cells were loaded with fura-2 on the stage of the microscope, and the  $[Ca^{2+}]_c$  response to adrenaline was monitored to distinguish  $\alpha$ -cells from non- $\alpha$ -cells. Figure 5B shows that G15 reversibly raised NAD(P)H fluorescence in adrenaline-nonresponsive cells (most of them presumably being  $\beta$ -cells,  $n = 18$ ), whereas it barely affected NAD(P)H fluorescence in adrenaline-responsive cells (presumably  $\alpha$ -cells,  $n = 33$ ). These results suggest that oxidative metabolism, hence ATP synthesis, is not significantly accelerated by high glucose in  $\alpha$ -cells and may explain the lack of effect of glucose on  $I_{K_{ATP}}$  in  $\alpha$ -cells.

**Effects of glucose on  $[Ca^{2+}]_c$  in depolarized  $\alpha$ -cells.**

The above-described experiments showed that glucose slightly decreased  $[Ca^{2+}]_c$  without affecting  $I_{K_{ATP}}$ . Our conclusion is supported by experiments performed after maximal closure of  $K_{ATP}$  channels with 500  $\mu$ mol/l tolbutamide. Increasing the glucose concentration from 0.5 to 15 mmol/l under these conditions again induced a 42% drop of average  $[Ca^{2+}]_c$  (Fig. 6A;  $n = 26$ ,  $P < 0.01$ ). To determine whether this  $[Ca^{2+}]_c$  decrease results from a direct inhibition of VDCCs, glucose was tested in  $\alpha$ -cells depolarized with 30 mmol/l  $K^+$ . Under these conditions,  $[Ca^{2+}]_c$  was steadily elevated because of the forced opening of VDCCs, and glucose was ineffective (Fig. 6B;  $n = 19$ ).

**GABA and zinc did not decrease  $\alpha$ -cell  $[Ca^{2+}]_c$ , and insulin, although lowering  $[Ca^{2+}]_c$ , very modestly, did not affect glucagon secretion.** We tested the effect of three candidate paracrine factors released by  $\beta$ -cells, GABA, zinc, and insulin, on  $\alpha$ -cell  $[Ca^{2+}]_c$  in a medium



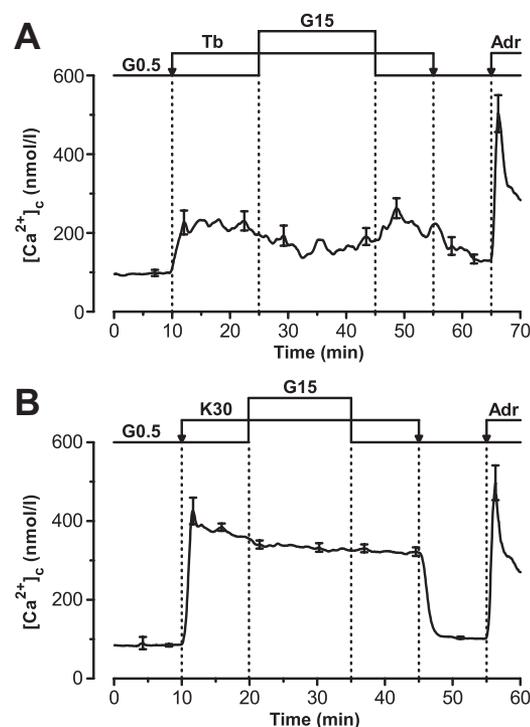
**FIG. 5.** Glucose increases NAD(P)H fluorescence in isolated  $\beta$ -cells but not in isolated  $\alpha$ -cells. NAD(P)H fluorescence is expressed as a percentage of the maximum signal recorded in the presence of azide. The glucose (G) concentration was changed between 0.5 and 15 mmol/l, and 5 mmol/l sodium azide was added when indicated. For all experiments, NAD(P)H fluorescence was first monitored. Thereafter, islet cells were loaded with fura-2/AM for 20 min on the stage of the microscope and then challenged with 10  $\mu$ mol/l adrenaline. Cells that responded to adrenaline were considered as  $\alpha$ -cells. **A:**  $\alpha$ -Cells from GYY mice were identified by their EYFP fluorescence before NAD(P)H fluorescence measurement, and their  $[Ca^{2+}]_c$  responsiveness to adrenaline was verified thereafter. The EYFP-negative cells were not responsive to adrenaline, and most of them were presumably  $\beta$ -cells. **B:** Isolated cells from NMRI mouse islets were monitored for NAD(P)H fluorescence, and their  $[Ca^{2+}]_c$  responsiveness to adrenaline was verified thereafter. Adrenaline-responsive and -nonresponsive cells were considered as  $\alpha$ - and  $\beta$ -cells, respectively. Data are means  $\pm$  SE of results obtained in 30  $\alpha$ -cells and 14 EYFP-negative cells from GYY mice (**A**) and in 18 adrenaline-nonresponsive cells and 33 adrenaline-responsive cells from NMRI mice (**B**).

containing G0.5 and 2.5-mmol/l amino acid mixture. GABA (100  $\mu$ mol/l) and zinc (3 and 30  $\mu$ mol/l) did not affect  $[Ca^{2+}]_c$  except for an initial, small, transient increase by zinc (Fig. 7A and B). Addition of 100 nmol/l insulin decreased  $[Ca^{2+}]_c$  in only 5 of 21 cells, leaving  $[Ca^{2+}]_c$  unchanged in the others. On average, insulin slightly decreased  $[Ca^{2+}]_c$  from  $234 \pm 10$  to  $212 \pm 12$  nmol/l (Fig. 7C;  $n = 21$ ). However, it failed to affect glucagon secretion and did not prevent the strong (70%) inhibitory effect of glucose on glucagon secretion (Fig. 7D).

**Ca<sup>2+</sup> influx through L-type VDCCs.** We finally evaluated the importance of Ca<sup>2+</sup> influx through L-type VDCCs in  $\alpha$ -cells. As illustrated by Fig. 8, 1  $\mu$ mol/l nimodipine inhibited the  $[Ca^{2+}]_c$  increase occurring during the spontaneous  $[Ca^{2+}]_c$  oscillations in G0.5 (by 74%) or induced by tolbutamide or arginine (by 85% for both agents). By contrast, as expected (22), the drug did not prevent the  $[Ca^{2+}]_c$  elevation elicited by 10  $\mu$ mol/l adrenaline.

## DISCUSSION

In this study, we used our GYY mouse model expressing EYFP in  $\alpha$ -cells (19) to study the mechanisms by which glucose controls  $\alpha$ -cell  $[Ca^{2+}]_c$ . The validation of our GYY

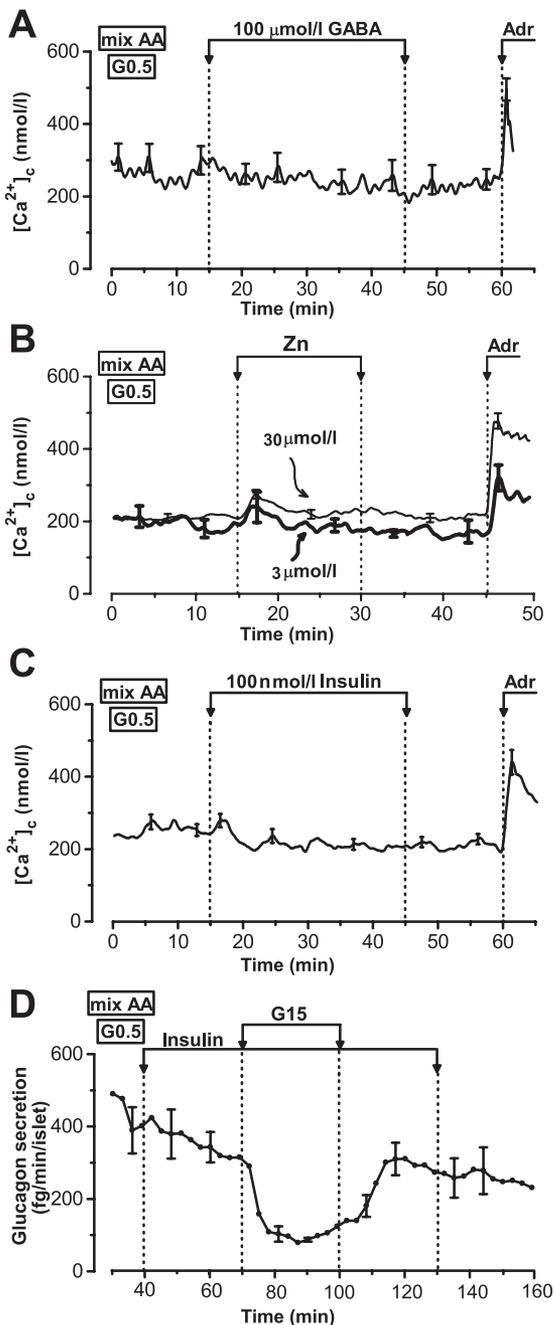


**FIG. 6.** Glucose decreases  $[Ca^{2+}]_c$  in  $\alpha$ -cells independently from an action on  $K_{ATP}$  and VDCCs. The glucose (G) concentration was changed between 0.5 and 15 mmol/l, and 10  $\mu$ mol/l adrenaline (Adr) was added as indicated. **A:** Tolbutamide (Tb; 500  $\mu$ mol/l) was applied as indicated. **B:** The KCl concentration (K) in the perfusion medium was changed from 4.8 to 30 mmol/l as indicated. Data are means  $\pm$  SE of results obtained in 26 (**A**) and 19 (**B**)  $\alpha$ -cells from GYY mice.

mouse is extended here by the similarity of key results in GYY  $\alpha$ -cells and NMRI  $\alpha$ -cells identified by their response to adrenaline (22).

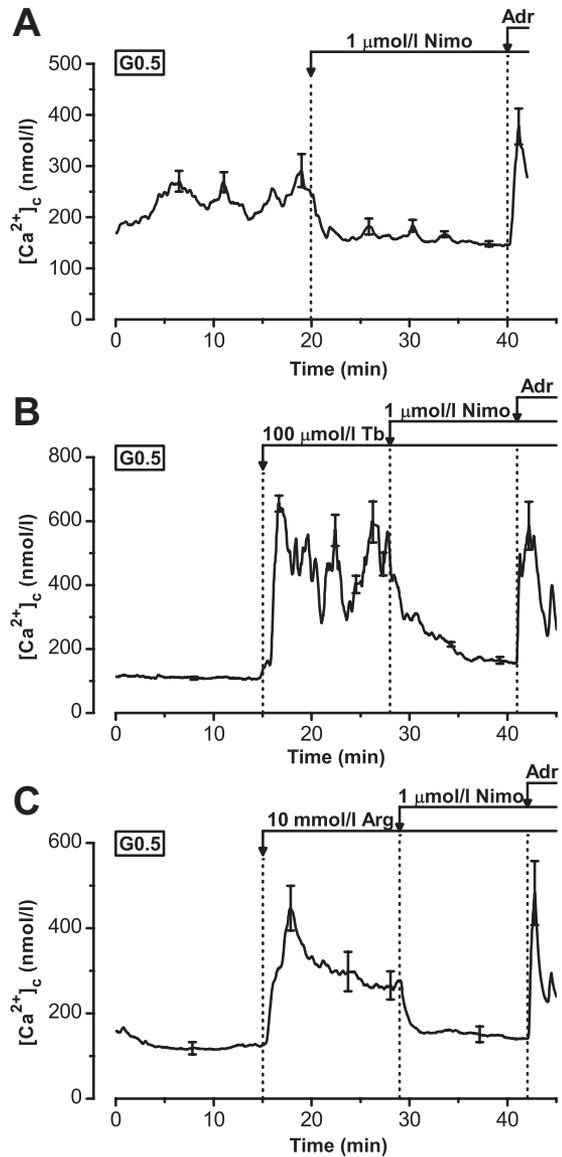
In the presence of a low glucose concentration,  $[Ca^{2+}]_c$  was found to oscillate in only  $\sim$ 30% of  $\alpha$ -cells; similar proportions were reported by others (3,17). As expected, the percentage of oscillating  $\alpha$ -cells increased to  $\sim$ 70% in the presence of a 2.5-mmol/l mixture of amino acids known to stimulate glucagon release (21). These oscillations resulted from rhythmic Ca<sup>2+</sup>-dependent spiking activity.

**Direct effects of glucose in  $\alpha$ -cells.** Whether the inhibition of glucagon secretion by glucose results from direct or indirect effects remains disputed (3,5,9,16,18,26–28). Our observation that high glucose induced a small  $[Ca^{2+}]_c$  decrease in isolated  $\alpha$ -cells supports a direct effect. Several mechanisms have been suggested to explain direct effects of glucose on  $\alpha$ -cells. One hypothesis implicates  $K_{ATP}$  channels. With only one exception (29), previous studies agree that  $\alpha$ -cells possess  $K_{ATP}$  channels (9,11,15,16,23,30,31). However, their possible role in stimulus-secretion coupling remains obscure. Thus, glucagon secretion has been shown to be stimulated (9,11), unaffected (32), or inhibited (15,33,34) on closure of  $K_{ATP}$  channels by sulfonylureas. Species differences can only partly account for these contradictions (9,14,30), because results are also controversial within the same species. For instance, tolbutamide was reported to decrease (33), not to affect (29), or to increase (17)  $[Ca^{2+}]_c$  in mouse  $\alpha$ -cells. In the present study, we demonstrated that  $\alpha$ -cells display several essential features of  $\beta$ -cells.  $I_{K_{ATP}}$  is inhibited by tolbutamide and increased by diazoxide. Its maximal amplitude is only 30% smaller than in  $\beta$ -cells. It is controlled



**FIG. 7.** GABA and zinc did not decrease  $\alpha$ -cell  $[Ca^{2+}]_c$ , and insulin, although lowering  $[Ca^{2+}]_c$  very modestly, did not affect glucagon secretion. The medium contained a 2.5-mmol/l amino acid mixture (mix AA) and 0.5 mmol/l glucose (G). GABA (100  $\mu$ mol/l), 3 or 30  $\mu$ mol/l  $ZnCl_2$ , 100 nmol/l insulin, and 10  $\mu$ mol/l adrenaline (Adr) were added when indicated. Data are means  $\pm$  SE of results obtained in 31 (A), 7 (3  $\mu$ mol/l  $ZnCl_2$ ; B), 43 (30  $\mu$ mol/l  $ZnCl_2$ ; B), and 31 (C)  $\alpha$ -cells from GYY mice and from four glucagon secretion experiments with 200 islets of GYY mice per chamber (D).

by cell metabolism because mitochondrial poisoning of the cells with azide reversibly increased it. As in  $\beta$ -cells,  $K_{ATP}$  channels control the membrane potential and  $[Ca^{2+}]_c$ . Thus, closing  $K_{ATP}$  channels with tolbutamide triggered a  $[Ca^{2+}]_c$  rise. In contrast, opening  $K_{ATP}$  channels with diazoxide or by decreasing cell metabolism with azide decreases  $[Ca^{2+}]_c$ , very likely as a result of plasma membrane hyperpolarization and arrest of  $Ca^{2+}$  influx through VDCCs. All of these results indicate that there is a coupling between  $K_{ATP}$  channel activity and  $Ca^{2+}$  influx through VDCCs in isolated mouse  $\alpha$ -cells as in  $\beta$ -cells.



**FIG. 8.** Spontaneous  $[Ca^{2+}]_c$  oscillations and the rise in  $[Ca^{2+}]_c$  elicited by tolbutamide or arginine result from  $Ca^{2+}$  influx through L-type channels in  $\alpha$ -cells. The glucose concentration was 0.5 mmol/l throughout. Nifedipine (Nimo; 1  $\mu$ mol/l), 10  $\mu$ mol/l adrenaline (Adr; A-C), 100  $\mu$ mol/l tolbutamide (Tb; B), and 10 mmol/l arginine (Arg; C) were added when indicated. Data are means  $\pm$  SE of results obtained in 10 (A), 4 (B), and 7 (C)  $\alpha$ -cells from GYY mice.

However, we found that  $I_{KATP}$  in  $\alpha$ -cells was not affected by glucose, remaining similarly low in the presence of 0.5 and 15 mmol/l glucose. This contrasts markedly with  $\beta$ -cells in which  $I_{KATP}$  is much larger in low than in high glucose. These results are in keeping with our NAD(P)H measurements, indicating that stimulation by high glucose did not significantly increase  $\alpha$ -cell metabolism while producing its expected acceleration of  $\beta$ -cell metabolism. The reasons for the lack of effect of glucose on  $\alpha$ -cell metabolism are unclear. Unlike  $\beta$ -cells,  $\alpha$ -cells do not express GLUT2 but the higher affinity GLUT1 transporter (35), and both cell types possess glucokinase (36). Our results are consistent with previous studies on  $\alpha$ -cell metabolism showing that glucose does not affect NAD(P)H (37) and flavin adenine dinucleotide fluorescence (38,39) or the ATP-to-ADP ratio (40). They are at variance with other data on  $\alpha$ -cells reporting a hyperpolarization of the mitochondrial membrane potential (41), a small increase

in ATP concentration (3,10), and an inhibition of  $I_{KATP}$  by glucose (9). The reasons for these discrepancies are unclear and could be due to differences in experimental procedures (including selection of cells) and/or species. The observation that  $\alpha$ -cell  $I_{KATP}$  was small in the presence of 0.5 mmol/l glucose suggests that the ATP-to-ADP ratio is high in low glucose. A previous report from our laboratory has shown that the ATP-to-ADP ratio is much higher in  $\alpha$ - than  $\beta$ -cells maintained in a low-glucose concentration (40). It is also possible that  $K_{ATP}$  channels are more sensitive to ATP inhibition in  $\alpha$ - than  $\beta$ -cells (23).

It is unclear why, at low glucose,  $[Ca^{2+}]_c$  oscillates in some  $\alpha$ -cells and remains at basal levels in others. The similar responsiveness of the two groups of  $\alpha$ -cells to adrenaline (this study) and arginine (19) rules out a trivial explanation of malfunctioning. Oscillating and silent cells could correspond to two populations of  $\alpha$ -cells either equipped with different sets of VDCCs (42) or maintaining small differences in input resistance (percentage of closed  $K_{ATP}$  channels). The high input resistance measured at 0.5 mmol/l glucose ( $>5$  G $\Omega$  in our whole-cell recordings) can explain why  $[Ca^{2+}]_c$  is elevated in one-third of the  $\alpha$ -cells in low glucose. This is consistent with the observation that tolbutamide, which only slightly increased input resistance, abruptly raised  $[Ca^{2+}]_c$  in all non-oscillating  $\alpha$ -cells. That  $[Ca^{2+}]_c$  oscillates in some  $\alpha$ -cells and remains basal in others is reminiscent of the situation found in isolated mouse  $\beta$ -cells perfused with 7–8 mmol/l glucose, a threshold concentration for  $\beta$ -cells. The high input resistance of  $\alpha$ -cells also explains why arginine increases  $[Ca^{2+}]_c$  in  $\alpha$ - but not in  $\beta$ -cells at a low glucose concentration. Thus, decreasing the  $\alpha$ -cell input resistance with diazoxide prevented the effect of arginine.

It has been suggested that closure of most  $\alpha$ -cell  $K_{ATP}$  channels might depolarize the plasma membrane to such an extent that voltage-dependent channels participating in action potential generation inactivate (15,16,33). Supporting this model, low diazoxide concentrations were reported to reverse glucose inhibition of glucagon secretion by slightly reactivating  $K_{ATP}$  channels and relieving the inactivation of voltage-dependent channels (16). Two of our observations argue against this proposal. Tolbutamide similarly increased  $[Ca^{2+}]_c$  at high and low concentrations, and low diazoxide concentrations did not increase  $[Ca^{2+}]_c$ , even in silent cells in G15.

Our findings that glucose did not significantly affect  $I_{KATP}$  and slightly decreased  $[Ca^{2+}]_c$  in the absence or presence of a high concentration of tolbutamide, which was expected to maximally close  $K_{ATP}$  channels, suggest that glucose decreases  $[Ca^{2+}]_c$  independently from an action on  $K_{ATP}$  channels. Its lack of effect during depolarization with KCl also indicates that glucose does not inhibit VDCCs. The small inhibitory effect of glucose on  $[Ca^{2+}]_c$  likely results from a change in membrane potential, the mechanisms of which remain to be identified.

**Indirect effects of glucose on  $\alpha$ -cells.** Other models propose that glucose-induced inhibition of glucagon secretion is indirect and mediated by  $\beta$ -cell-derived paracrine factors: GABA, insulin, or zinc (27). This hypothesis is also contested. GABA has been reported to inhibit glucagon secretion by activating GABA<sub>A</sub> receptor channels in  $\alpha$ -cells (6,8,43), but some studies (18,41,44), including the present one, failed to detect an effect of GABA in  $\alpha$ -cells. However, it is important to bear in mind that GABA<sub>A</sub> receptors quickly desensitize and that a prolonged application of the neurotransmitter might not reproduce the *in vivo* situa-

tion. Other experiments tested whether GABA<sub>A</sub> receptor antagonists prevent the inhibition of glucagon secretion by glucose, and again, conclusions in favor (6,43) or against (7,16) the hypothesis were reached. Species differences have been put forward to explain these conflicting results (6,16,27).

Because of the inverse regulation of glucagon and insulin secretion by glucose, insulin is an appealing candidate to mediate indirect inhibition of glucagon secretion. Several reports suggest that insulin can at least partly mediate the effect of glucose in  $\alpha$ -cells (3–5,9,45), but this hypothesis is refuted by others (18,21,46). In the present study, we found a very small inhibitory effect of insulin on  $[Ca^{2+}]_c$  that did not affect insulin secretion. Zinc, which helps insulin storage in secretory granules, is coreleased with insulin and has been suggested to mediate the indirect effect of glucose on  $\alpha$ -cells (10,46), possibly by opening  $\alpha$ -cell  $K_{ATP}$  channels (11). However, this hypothesis is at variance with the observations that chelation of zinc does not reverse glucose-induced inhibition of glucagon release in mouse islets (16) and that zinc does not decrease  $\alpha$ -cell  $[Ca^{2+}]_c$  (this study) or even accelerates  $[Ca^{2+}]_c$  oscillations (3,18). Again, species differences might explain these contradictory results.

Although we acknowledge that isolated and cultured  $\alpha$ -cells, as studied here, may behave differently from  $\alpha$ -cells within intact islets, the fact that inhibition of glucagon secretion by glucose largely occurs over a concentration range that is below the threshold for stimulation of insulin secretion (3,9,18,47,48) is difficult to reconcile with the proposed intervention of  $\beta$ -cell-derived paracrine factors.

The nature of the VDCCs present in  $\alpha$ -cells is another controversial issue. It has been reported that mouse  $\alpha$ -cells in freshly isolated islets possess at least three types of VDCCs, T-, N-, and L-type channels (14,16,49), and that in the absence of cAMP production, N-type channels are more important than L-type in the control of  $Ca^{2+}$  influx and exocytosis (49,50). However, others have been unable to identify N-type  $Ca^{2+}$  channels in mouse  $\alpha$ -cells (24) and have suggested that  $Ca^{2+}$  influx mainly occurs through L-type channels (17,24,34). Here, using nimodipine, a selective L-type  $Ca^{2+}$  channel blocker, we showed that  $Ca^{2+}$  influx stimulated by arginine and tolbutamide or occurring in the sole presence of glucose takes place mainly through L-type channels and, to a lesser extent, through non-L-type channels.

## CONCLUSIONS

Our data show that, as in  $\beta$ -cells,  $K_{ATP}$  channels can transduce changes in cell metabolism into changes in membrane potential and  $Ca^{2+}$  influx through VDCCs in mouse  $\alpha$ -cells. We also show that, in contrast to  $\beta$ -cells, isolated  $\alpha$ -cells are poorly responsive to glucose that slightly lowers  $[Ca^{2+}]_c$  without significantly affecting cell metabolism or  $K_{ATP}$  channel activity. Hence, glucose and  $K_{ATP}$  channel modulators exert distinct effects on  $\alpha$ -cell  $[Ca^{2+}]_c$ . The lowering of  $[Ca^{2+}]_c$  resulting from a direct action of glucose on  $\alpha$ -cells is modest and probably insufficient to account for the robust inhibition of glucagon secretion produced by glucose in whole islets (19). It is therefore likely that glucose-induced inhibition of glucagon secretion in the intact islets results from a combination of both effects of the sugar and indirect effects by islet factors.

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