

Increased glucose sensitivity of both triggering and amplifying pathways of insulin secretion in rat islets cultured for one week in high glucose

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Running head: β -cell function after one week culture in high glucose

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ABSTRACT

Chronic hyperglycemia has been shown to induce either a lack of response or an increased sensitivity to glucose in pancreatic β -cells. We reinvestigated this controversial issue in a single experimental model by culturing rat islets for one week in 10 or 30 mmol.L⁻¹ glucose (G10 (Controls) or G30 (High-glucose islets)) before testing the effect of stepwise glucose stimulation from G0.5 to G20 on key β -cell stimulus-secretion coupling events. Compared with Controls, the glucose sensitivity of High-glucose islets was markedly increased, leading to maximal stimulation of oxidative metabolism and both triggering and amplifying pathways of insulin secretion in G6 rather than G20, hence to loss of glucose effect above G6. This enhanced glucose sensitivity occurred despite a ~2 fold increase in islet UCP2 mRNA expression. Besides this increased glucose sensitivity, the maximal glucose stimulation of insulin secretion in High-glucose islets was reduced by ~50%, proportionally to the reduction of insulin content. In High-glucose islets, changes in ⁴⁵Ca²⁺ influx induced by glucose and diazoxide were qualitatively similar but quantitatively smaller than in Control islets, and paradoxically did not lead to detectable changes in the intracellular Ca²⁺ concentration measured by microspectrofluorimetry (furaPE3). In conclusion, after one week culture in G30, the loss of glucose stimulation of insulin secretion in the physiological range of glucose concentrations (G5 to G10) results from the combination of an increased sensitivity to glucose of both triggering and amplifying pathways of insulin secretion, and an ~50% reduction in the maximal glucose stimulation of insulin secretion.

INTRODUCTION

Type 2 diabetes is characterized by the association of insulin resistance and impaired glucose-stimulation of insulin secretion (GSIS)¹ (9; 11; 15). The defect of GSIS seems to result from the combination of a moderate decrease in β -cell mass (10; 58; 66) and functional alterations of the remaining β -cells. The latter could be precipitated by chronic overstimulation and/or exposure to hyperglycemia/hyperlipidemia (23; 33; 37; 50), and involve global changes in β -cell gene expression (β -cell loss of differentiation) such as those observed in islets from hyperglycemic animals (31; 62; 64).

Previous studies have shown that prolonged *in vivo* or *in vitro* exposure to high glucose concentrations causes parallel decreases in maximal GSIS and β -cell insulin content, and abolishes GSIS within the physiological range of glucose concentrations (5 to 10 mmol.L⁻¹) (32; 37; 52). However, the cause of this loss of GSIS remains controversial. In one group of studies, β -cells exposed to high glucose concentrations became **insensitive** to subsequent acute glucose stimulation, i.e. for changes in ATP production, intracellular Ca²⁺ concentration ([Ca²⁺]_i) and insulin secretion (5; 7; 8; 13; 23; 39; 51), defects that were tentatively attributed to an increased expression of the mitochondrial uncoupling protein UCP2 (34; 47). In sharp contrast, in another group of studies, β -cells exposed to high glucose concentrations became **hypersensitive** to subsequent acute glucose stimulation, i.e. for the acceleration of mitochondrial metabolism, proinsulin biosynthesis and insulin secretion (1; 38; 40-42; 48; 60). In association with β -cell degranulation, this increased sensitivity to glucose resulted in an already stimulated secretion at low, fasting glucose concentrations (≤ 5 mmol.L⁻¹), and a lack of further stimulation of insulin secretion by physiological, postprandial glucose concentrations (5-10 mmol.L⁻¹).

These two apparently irreconcilable explanations for the absence of GSIS after prolonged exposure to high glucose concentrations have distinct and even opposite implications for the development of new therapeutic strategies to improve GSIS in type 2 diabetes. Therefore, to clarify this issue, we reinvestigated the effects of prolonged exposure to high glucose concentrations on key β -cell stimulus-secretion coupling events in a single experimental

model, the *in vitro* cultured rat islet, with a particular focus on changes in islet energetic metabolism and both triggering and amplifying pathways of GSIS (27; 59).

MATERIALS AND METHODS

Solutions. Most experiments were performed at 37°C with a bicarbonate-buffered Krebs solution containing 120 mmol.L⁻¹ NaCl, 4.8 mmol.L⁻¹ KCl, 2.5 mmol.L⁻¹ CaCl₂, 1.2 mmol.L⁻¹ MgCl₂ and 24 mmol.L⁻¹ NaHCO₃, and various glucose concentrations (Gx). This solution was supplemented with 1g.L⁻¹ bovine serum albumin, and was continuously gassed with O₂:CO₂ (94:6) to maintain pH at 7.4. When the concentration of KCl was raised to 30 mmol.L⁻¹, that of NaCl was reduced to 94.8 mmol.L⁻¹ to keep the medium osmolarity unchanged. Ca²⁺-free solutions were prepared by substituting MgCl₂ for CaCl₂ and chelating remaining free Ca²⁺ with 0.1-2 mmol.L⁻¹ EGTA. Na⁺-free solutions were prepared by substituting choline chloride for NaCl and choline bicarbonate for NaHCO₃, and were supplemented with 10 µmol.L⁻¹ atropine to prevent activation of muscarinic receptors by choline. Diazoxide, kindly provided by Schering-Plough Avondale (Rathdrum, Ireland), was dissolved to 50 mmol.L⁻¹ in NaOH 0.1N and used at the final concentration of 100-250 µmol.L⁻¹.

Islet isolation and culture. Islet isolation was performed using sterile-filtered Krebs solution containing 10 mmol.L⁻¹ glucose, 5 mmol.L⁻¹ HEPES, 100 UI.mL⁻¹ penicillin, 100 µg.mL⁻¹ streptomycin and 0.3 µg.mL⁻¹ fungizone. Briefly, male Wistar rats (180-200 g) were anesthetized by intra-peritoneal injection of 25 mg.kg⁻¹ pentobarbital (Nembutal, Abbott Laboratories) and laparotomized. After the animals were killed by incision of the heart, the pancreas was inflated by injecting 5 mL of medium containing 1.5 mg.mL⁻¹ collagenase (Serva, Heidelberg, Germany) through the common pancreatic duct. After collagenase digestion, the islets were separated from the digest by density gradient centrifugation using Histopaque 1077 (Sigma). The islets were washed thrice and hand-picked under a stereomicroscope to ensure high purity of the preparation. They were then separated in two batches and cultured at 37°C in RPMI 1640 medium containing 10 (Control) or 30 mmol.L⁻¹ glucose (High-glucose) and 5 g.l⁻¹ bovine serum albumin instead of 10 % fetal calf serum, to prevent proliferation of fibroblasts and attachment and flattening of islets on the bottom of the dish. Unless otherwise specified, the islets were cultured for 7-8 days, and the medium was renewed after 1 day, then every other day. Islets with

a diameter > 150-200 μm , which usually develop central necrosis during culture, were discarded. High-glucose and Control islets of similar size were then chosen for functional comparison. Although mycoplasma contamination was not specifically tested, culture contamination was routinely excluded by phase contrast microscopy (20-40X objective).

All animal experimentations performed for this study were conducted in accord with accepted standards of humane animal care, and were approved by the Institutional Committee on Animal Experimentation from the Faculty of Medicine of the University.

Optical and electron microscopy analysis of islets. After 1 week of culture in G10 or G30, the islets were washed in RPMI without BSA, fixed for 4h in Bouin's fluid or in 2.5% glutaraldehyde solution for light and electron microscopy respectively. For light microscopy analysis, four μm -thick sections of paraffin embedded tissue were mounted on glass slides and processed routinely for hematoxylin and eosin staining. Structures containing fragments of basophilic chromatin material were considered characteristic of "apoptotic bodies". For electron microscopy analysis, ultrathin sections (80 nm) of epoxy embedded islets were counterstained with aqueous uranyl acetate and lead citrate. They were then examined on a Zeiss 109 transmission electron microscope (Carl Zeiss, NY, USA).

Insulin secretion and insulin content measurements. After culture, batches of 20-30 islets were perfused ($\sim 1 \text{ mL} \cdot \text{min}^{-1}$) with a medium containing various glucose concentrations. Insulin was measured in 2 min effluent collections by radio-immunoassay using rat insulin as a standard. After perfusion, the islets were disrupted by sonication in TNE buffer (Tris 10 $\text{mmol} \cdot \text{L}^{-1}$, NaCl 0.2 $\text{mol} \cdot \text{L}^{-1}$, EDTA 10 $\text{mmol} \cdot \text{L}^{-1}$) for measurement of their insulin (RIA) and DNA contents (fluorescent assay using bisbenzimidazole)(35).

$[\text{Ca}^{2+}]_i$ measurements. $[\text{Ca}^{2+}]_i$ was measured by microspectrofluorimetry using Quanticell 700m (Visitech, Sunderland, U.K). Islets were loaded for 2-3 h at 37°C with 2 $\mu\text{mol} \cdot \text{L}^{-1}$ furaPE3

acetoxymethylester (Teflabs, Austin, Tex. USA) in RPMI medium containing the same glucose concentration as that used during culture. They were then transferred into a thermostated (37°C) chamber mounted on the stage of an inverted microscope used in the epifluorescence mode with a 20× objective, and perfused at a flow rate of ~1 mL.min⁻¹. The islets were alternately excited at 340 nm and 380 nm by a monochromator. A dichroic mirror centered at 430 nm reflected the light of excitation to the perfusion chamber and transmitted the emitted fluorescence to a CCD video camera (Photonic Science Ltd., Tunbridge Wells, U.K.) through a 510 nm filter. The data were automatically corrected for background fluorescence. $[Ca^{2+}]_i$ was calculated by comparing the ratio of fluorescence at each pixel to a calibration curve based on the equation of Grynkiewicz (24). The mean $[Ca^{2+}]_i$ in the islet was then calculated by averaging the $[Ca^{2+}]_i$ of all pixels of the islet.

⁴⁵Ca²⁺ efflux measurements. After 1 week culture in RPMI medium containing 10 or 30 mmol.L⁻¹ glucose, the islets were loaded overnight with ⁴⁵CaCl₂ (80 µCi.mL⁻¹, Amersham Pharmacia Biotech, U.K.) in the same medium. After several washes to remove the extracellular tracer, batches of 30 islets were perfused with a medium containing various glucose concentrations. The radioactivity lost by the islets was measured in 2 min effluent collections by liquid scintillation using Pico-fluor 15 (Packard Bioscience, Meriden, CT, USA). At the end of the experiment, the islets were collected to measure their total ⁴⁵Ca²⁺ radioactivity. The fractional rate of ⁴⁵Ca²⁺ efflux was calculated by dividing the radioactivity lost in each sample by the total sum of radioactivity present in the islets just before collection of that sample (14).

Islet ⁴⁵Ca²⁺ uptake measurements. After 1 week culture, Control and High-glucose islets were divided in 3 batches of 30. For each type of islets, one batch was used for duplicate DNA content measurement, and the other two groups were loaded for 18h with ⁴⁵CaCl₂ in the same medium, with eventual addition of 1 µmol.L⁻¹ thapsigargin (Tg) for the last hour of culture. The islets were then rinsed thrice in modified Krebs solution without bicarbonate containing 5 mmol.L⁻¹ Tris pH 7.4, and supplemented with 2 mmol.L⁻¹ LaCl₃ to block Ca²⁺ fluxes (25). Each group of islets was

then divided in batches of 8 for triplicate determination of their $^{45}\text{Ca}^{2+}$ content. After normalization for variations in the total radioactivity concentration of the loading medium and for differences in islet DNA content, the results were expressed in percent of the $^{45}\text{Ca}^{2+}$ content of Control islets within the same experiment.

Reduced pyridine nucleotide fluorescence measurements. After culture, the islets were transferred to the same setup as for $[\text{Ca}^{2+}]_i$ measurements. The reduced forms of NAD and NADP, collectively referred to as NAD(P)H, were excited at 360 nm, and the fluorescence emitted through a dichroic mirror centered at 400 nm was filtered at 470 nm (22). After background subtraction, the results were normalized to the maximal fluorescence level measured 2-5 min after addition of 5 mmol.L⁻¹ azide in the presence of 20 mmol.L⁻¹ glucose.

Mitochondrial membrane potential measurements. The islets were loaded for 20 min with 10 µg.mL⁻¹ rhodamine 123 (Molecular Probes, Eugene, OR) in the presence of the same glucose concentration as that during culture, and changes in the fluorescence of rhodamine 123 were measured by spectrofluorimetry (excitation/emission 490/530 nm, 505 nm long pass dichroic mirror)(55). After background subtraction, the rhodamine 123 fluorescence in each islet was normalized to the maximal fluorescence level measured 2-5 min after addition of 5 mmol.L⁻¹ azide in the presence of 20 mmol.L⁻¹ glucose. A decrease in rhodamine fluorescence reflects mitochondrial membrane hyperpolarization.

Measurements of islet adenine nucleotides. After culture, batches of 5-8 islets were preincubated for 30 min in 500 µl of medium containing 0.5 mmol.L⁻¹ glucose. They were then incubated for 30 min in 1 mL of medium containing 0.5 to 20 mmol.L⁻¹ glucose. At the end of the experiment, the islets were disrupted with trichloroacetic acid (5% vol/vol) and their ATP and ADP contents (pmol/islet) were measured by a luminometric method (17). The fraction of the sum (ATP + ADP) that was in the form of ATP in the islets was calculated (ATP/(ATP + ADP)) and used as an indicator of islet cell energetic level (48).

Measurements of islet mRNA expression of mitochondrial uncoupling protein 2 (UCP2).

The UCP2/cyclophilin mRNA ratio was determined by reverse transcription and duplex radioactive semiquantitative PCR in Control and High-glucose islets as described (31). Primers for cyclophilin (31) and UCP2 (sense 5'-GGCCTCTACGACTCTGTAAAGC-3'; antisense 5'-TGGCATTTCGGGCAACATTG-3') were used at a concentration of 200 nmol.L⁻¹. The thermal cycle profile was a 10 min denaturing step at 94°C followed by cycles of amplification (1 min denaturation at 94°C, 1 min annealing at 60°C, 1 min extension at 72°C) and a final extension step of 10 min at 72°C. PCR were stopped in the middle of the exponential phase of amplification of both genes (21 cycles)(31). The amplimers were separated on a 6% PAGE and their [α^{32} P]dCTP content was quantitated by a Cyclone Storage Phosphor System (Packard Meriden, CT). The ratio UCP2/cyclophilin mRNA ratio was calculated for each sample, and normalized to the ratio measured in islets cultured in G10.

Data Analysis. High-glucose and Control islets from the same preparation were always tested simultaneously within a single experiment (equal number of islets (at least 2) of each type in each experiment). The results are means $\pm$ SEM for the indicated number of islets from at least 3 different preparations. The statistical significance of differences between means was assessed by one-way ANOVA followed by a test of Newman-Keuls, or by unpaired t test, unless specified otherwise. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Survival and maintenance of the phenotype of rodent islet β -cells *in vitro* is optimal in the presence of G10, but markedly decreases after a few days of culture in G3-G5 (18; 30; 56). We therefore chose to compare key β -cell stimulus-secretion coupling events in rat islets cultured for 1 week in RPMI medium containing G10 (Control islets) or G30 (High-glucose islets).

Morphological and functional characteristics of Control islets. Optical and electron microscopy of Control islets showed well preserved islet architecture, no morphological signs of β -cell apoptosis, and normal ultrastructure of β and non β -cells with uncondensed and unfragmented nuclei, well-defined endoplasmic reticulum (ER), Golgi complex, and mature secretory granules (Fig. 1A,C,E). In these islets, a stepwise increase in glucose concentration from G0.5 to G20 stimulated key GSIS coupling events as expected (27; 46; 65) : a glucose concentration-dependent increase in islet NAD(P)H autofluorescence, mitochondrial membrane hyperpolarization, and ATP/(ATP+ADP) ratio ; a transient decrease in $[Ca^{2+}]_i$ reflecting increased Ca^{2+} pumping by the ER (61) without changes in insulin secretion at sub-threshold glucose concentrations (G3-G6), and a parallel increase in $[Ca^{2+}]_i$ and insulin secretion above that threshold (Fig. 2). Although $[Ca^{2+}]_i$ did not reach steady state within the short duration of each glucose step, we can estimate that the glucose sensitivity of $[Ca^{2+}]_i$ and insulin secretion changes in Control islets was similar to that previously reported in freshly isolated rat islets (2), with half-maximal and maximal effective glucose concentrations close to G10 and G20 respectively. These glucose-induced $[Ca^{2+}]_i$ changes were similar after 1 to 21 days of culture in G10 (data not shown).

Morphological alterations in High-glucose islets. The islet architecture and non β -cell morphology were not altered in High-glucose islets (Fig. 1B,F). In contrast, β -cells were hypertrophied (Fig. 1B), had only few mature insulin granules, and contained numerous electron-dense particles characteristic of glycogen in their cytosol (Fig. 1D,G). The presence

of a few typical apoptotic bodies scattered throughout the islets likely reflected an increase in β -cell apoptosis under these culture conditions (Fig. 1B), as reported by others (19; 43; 49).

Insulin and DNA content of Control and High-glucose islets. On average, the insulin content was ~50% lower in High glucose than in Control islets (23.5 ± 2.2 and 48.4 ± 4.7 ng/islet respectively, $n = 23$, $p < 0.0001$), whereas the DNA content was similar in both types of islets (87.8 ± 9.5 and 87.5 ± 9.5 ng/islet, $n = 23$).

High-glucose islets have a high energetic state at low glucose despite increased UCP2 mRNA levels. As in Control islets, a stepwise increase in glucose concentration from G0.5 to G20 induced concentration-dependent increases in NAD(P)H autofluorescence, mitochondrial membrane hyperpolarization, and ATP/(ATP+ADP) ratio in High-glucose islets (Fig. 2A-C). However, these effects of glucose were of much smaller amplitude than in Control islets. To permit direct comparison of the semi-quantitative changes in NAD(P)H and rhodamine 123 fluorescence between the two types of islets, the results were normalized to the maximal fluorescence level observed 2-5 min after addition of azide to G20. At the concentration of 5 mmol.L^{-1} , this inhibitor of complex IV of the electron transport chain depolarized the mitochondrial membrane (maximal level of rhodamine 123 fluorescence), transiently increased NAD(P)H autofluorescence, and reduced the ATP/(ATP+ADP) ratio to the level measured in Control islets incubated in G0.5 (Fig. 2C). The NAD(P)H autofluorescence, mitochondrial membrane hyperpolarization, and ATP/(ATP+ADP) ratio measured in the presence of G0.5 were all significantly higher in High-glucose than in Control islets ($p < 0.05$ or less), whereas the maximal and minimal energetic levels, measured in the presence of G20 and G20+azide respectively, were similar in both types of islets. These results are not compatible with the hypothesis that prolonged exposure to high glucose concentrations abolishes glucose-induced rise in ATP production by increasing the expression of the mitochondrial uncoupling protein UCP2 (47). We therefore compared the expression of UCP2 in High-glucose and Control islets by RT-PCR, using cyclophilin as

internal control gene. After 1-2 weeks of culture, the UCP2/cyclophilin mRNA ratio was significantly higher in High-glucose than Control islets (174 ± 12 and 100 ± 9 % respectively, $p < 0.001$).

These results therefore indicate that the lower glucose-responsiveness of mitochondrial metabolism in High-glucose islets results from an increase in mitochondrial metabolism and ATP production in G0.5, rather than from its reduction in G20, despite a ~ 1.7 -fold increase in islet UCP2 mRNA expression. The functional significance of the latter increase is not clear (36). Although we agree that a large overexpression of UCP2 can be detrimental to GSIS in any type of islets (12), our results suggest that, as in other cell types, a moderate (less than 2-fold) increase in UCP2 expression in rat β -cells does not impair ATP production (53). It is even possible that a limitation in the production of reactive oxygen species at complex I and III by mild mitochondrial uncoupling explains the surprising observation that mild UCP2 overexpression rather improves β -cell dysfunction from Zucker Diabetic Fatty rats (63).

Increased glucose sensitivity of insulin secretion in the presence of a stable elevation of $[Ca^{2+}]_i$ in High-glucose islets. Compared with Control islets, $[Ca^{2+}]_i$ was ~ 50 nmol.L⁻¹ higher in High-glucose islets perfused with G0.5, and did not further increase upon glucose stimulation, except for a slow regular increase that varied between experiments (compare Figs. 2D and 4C) and likely resulted from the progressive leakage of furaPE3 from the islets (3; 22). These $[Ca^{2+}]_i$ alterations developed progressively during culture in G30 (normal response after 1 day, partial alteration after 3 days, and complete lack of response after 7-21 days). They were fully reversible upon further culture in G10 (no correction after 1 day, partial and complete normalization after 3 and 7 days respectively) (data not shown, $n=4-6$ islets from 2-3 independent cultures). Despite the sustained and glucose-insensitive elevation of $[Ca^{2+}]_i$, High-glucose islets displayed a clear GSIS that was rapidly inhibited by addition of 5 mmol.L⁻¹ azide. Compared with Control islets, insulin secretion from High-glucose islets was similar in G0.5, higher in G6 ($p < 0.05$), not different in G10, and lower in G20 ($p < 0.001$), with nearly maximal stimulation in G10 (Fig. 2E). After correction for the decrease in islet insulin

content, insulin secretion was much larger in High-glucose than Control islets during perfusion with G6-G10, but was not different in G20 (Fig. 2F).

These results indicate that one week of culture in G30 instead of G10 has two apparently opposite effects on rat β -cell function in the same experimental model. First, it increases the sensitivity to glucose for the acceleration of mitochondrial metabolism and for the stimulation of insulin secretion under physiological conditions, and it reduces the maximal glucose stimulation of insulin secretion in proportion with the reduction in islet insulin content. This combination leads to an apparent loss of GSIS within the physiological range of glucose concentrations (G5-G10), as previously reported (41; 42; 48). Second, it leads to a sustained increase in basal $[Ca^{2+}]_i$ that is not affected by subsequent glucose stimulation, as described previously in rat and human islets (5; 23).

Increased glucose sensitivity of the amplifying pathway of GSIS in High-glucose islets.

Besides its ability to increase Ca^{2+} influx (triggering pathway of GSIS), glucose also amplifies the efficacy of Ca^{2+} on insulin granule exocytosis by a mechanism that is independent from the closure of K^+ -ATP channels (amplifying pathway) (20; 21; 26; 27; 54). In normal rodent islets, both triggering and amplifying pathways correlate with changes in the islet ATP/ADP ratio (16; 20) and contribute simultaneously to GSIS under physiological conditions (27). We therefore tested whether the amplifying pathway of GSIS is more sensitive to glucose in High-glucose than in Control islets.

During perfusion with a medium containing 0, 6 or 20 mmol.L⁻¹ glucose (G0, G6 or G20) and 250 μ mol.L⁻¹ of the K^+ -ATP channel opener diazoxide (DZ), $[Ca^{2+}]_i$ was higher in High-glucose than in Control islets, but insulin secretion remained below RIA detection limit in both types of islets (Fig. 3). Upon membrane depolarization with 30 mmol.L⁻¹ K^+ (K30), $[Ca^{2+}]_i$ rapidly increased to a plateau that was similar in High-glucose and Control islets and was unaffected by the glucose concentration (Fig. 3A,B). In Control islets, the stimulation of insulin secretion by K30 was markedly glucose-dependent, with a monophasic increase in the absence of glucose, a slightly larger and more sustained increase in G6, and a much

larger increase in G20 (Fig. 3C). In High-glucose islets, however, K30-induced insulin secretion was already sustained in G0, resembling the response observed in G6 with Control islets, and the maximal amplifying effect of glucose was observed in G6 instead of G20 (Fig. 3D). Thus, in the presence of G6K30DZ, insulin secretion was ~4 fold larger in High-glucose than in Control islets, even though $[Ca^{2+}]_i$ was not different between both types of islets. In the presence of G20K30DZ, the absolute rate of secretion was similar in both types of islets, despite the ~50% lower insulin content of High glucose islets (329 ± 4 and 645 ± 8 pg insulin/ng DNA in High-glucose and Control islets respectively, $p < 0.0001$). These results therefore demonstrate that High-glucose islets are also more sensitive to glucose under conditions measuring the amplifying pathway of GSIS only, and that their maximal rate of secretion, measured under these conditions, is not reduced in parallel with their insulin content.

Dissociation between $[Ca^{2+}]_i$ and Ca^{2+} influx in High-glucose islets under non-stimulating conditions. The stimulation of insulin secretion that an increase of the glucose concentration from 0.5 to 6-20 mmol.L⁻¹ produces without changing the already elevated $[Ca^{2+}]_i$ (Fig. 2D-F) might suggest that GSIS in High-glucose islets mainly results from the amplifying action of glucose. This interpretation implies that the triggering pathway is already maximally stimulated in G0.5 and has become glucose-insensitive between G0.5 and G6-G20. However, it has recently been suggested that insulin secretion is better correlated with an increase in Ca^{2+} influx than with a global rise in $[Ca^{2+}]_i$, perhaps because of concentration gradient within the cell (57). It was important, therefore, to evaluate the effects of glucose and DZ on Ca^{2+} influx by a method other than furaPE3 microspectrofluorimetry, the fractional efflux of $^{45}Ca^{2+}$. This method measures exchange of intracellular radioactive $^{45}Ca^{2+}$ (loaded islets) for cold extracellular Ca^{2+} , so that a stimulus-induced acceleration of $^{45}Ca^{2+}$ efflux reflects a stimulation of Ca^{2+} influx when it is abrogated in the absence of extracellular Ca^{2+} (28).

In Control islets perfused with G0.5, $^{45}\text{Ca}^{2+}$ efflux was low and stable (Fig. 4B), and $[\text{Ca}^{2+}]_i$ was $\sim 120 \text{ nmol.L}^{-1}$ and slowly increased with time (Fig. 4C). Under these conditions, $^{45}\text{Ca}^{2+}$ efflux and $[\text{Ca}^{2+}]_i$ were significantly higher in High-glucose than Control islets ($p < 0.001$) (Fig. 4B,C). In both types of islets, they were unaffected by addition of DZ to the medium, but decreased in parallel upon removal of extracellular Ca^{2+} (Fig. 4D-F), so that $^{45}\text{Ca}^{2+}$ efflux and $[\text{Ca}^{2+}]_i$ remained higher in High-glucose vs Control islets even in the absence of extracellular Ca^{2+} . These results indicate that the elevations of $^{45}\text{Ca}^{2+}$ efflux and $[\text{Ca}^{2+}]_i$ in High-glucose islets perfused with G0.5 do not result from a stimulation of Ca^{2+} influx, and therefore reflect an elevation of resting $[\text{Ca}^{2+}]_i$. This may explain why the rate of insulin secretion was not higher in High-glucose than Control islets during perfusion with G0.5 (Fig. 4A).

Increased glucose sensitivity, but reduced glucose responsiveness, of the triggering pathway of GSIS in High-glucose islets. When Control islets were stimulated with glucose, $^{45}\text{Ca}^{2+}$ efflux slightly decreased in G6, and markedly increased in G20 (Fig. 4B). Parallel changes in $[\text{Ca}^{2+}]_i$ were observed under these conditions (Fig. 4C). Accordingly, insulin secretion was not affected by G6 and stimulated ~ 30 fold by G20 (Fig. 4A). As expected (27; 28), the rises in $^{45}\text{Ca}^{2+}$ efflux, $[\text{Ca}^{2+}]_i$ and insulin secretion produced by G20 were completely inhibited by DZ in these Control islets (Fig. 4A-C). When the stimulation by G6 and G20 was applied in the presence of DZ or absence of extracellular Ca^{2+} , $^{45}\text{Ca}^{2+}$ efflux was inhibited in a concentration-dependent manner (Fig. 4D,E), likely as a result of the stimulation of Ca^{2+} pumping by the ER (61). These results therefore confirm that the rise in $^{45}\text{Ca}^{2+}$ efflux and $[\text{Ca}^{2+}]_i$ induced by G20 in Control islets results from the stimulation of Ca^{2+} influx following closure of K^+ -ATP channels and plasma membrane depolarization (27; 28).

In High-glucose islets, stimulation with G6-G20 induced parallel increases in $^{45}\text{Ca}^{2+}$ efflux and insulin secretion ($p < 0.001$) that were both completely inhibited by DZ (Fig. 4A,B). Surprisingly, these effects of glucose and DZ occurred in the absence of detectable $[\text{Ca}^{2+}]_i$ changes (Fig. 4C). Compared with Control islets, the stimulation of $^{45}\text{Ca}^{2+}$ efflux and insulin secretion in High-glucose islets occurred at a lower glucose concentration (G6 vs G20), and

was of smaller magnitude in G20. When the stimulation by G6 and G20 was applied in the presence of DZ or absence of extracellular Ca^{2+} , $^{45}\text{Ca}^{2+}$ efflux was inhibited in a concentration-dependent manner as in Control islets (Fig. 4D,E), but the maximal decrease of $^{45}\text{Ca}^{2+}$ efflux was observed at a lower glucose concentration than in Control islets (G6 vs G20).

These results demonstrate that, also in High-glucose islets, GSIS requires the closure of β -cell K^{+} -ATP channels and the stimulation of Ca^{2+} influx. They therefore suggest that, under normal ionic conditions, the strong stimulation of insulin secretion by G6 in High glucose islets reflects almost maximal glucose stimulation of both triggering and amplifying pathways.

Dissociation between $[\text{Ca}^{2+}]_i$ and Ca^{2+} influx/insulin secretion in High-glucose islets. In Control islets, glucose stimulation and DZ inhibition of Ca^{2+} influx were accompanied by the expected increase and decrease in $[\text{Ca}^{2+}]_i$ (27). This was not the case in High-glucose islets in which glucose stimulation and DZ inhibition of Ca^{2+} influx and insulin secretion occurred in the absence of detectable $[\text{Ca}^{2+}]_i$ changes (cfr previous paragraph).

What can be the cause of such a dissociation between $[\text{Ca}^{2+}]_i$ and Ca^{2+} influx/insulin secretion in High-glucose islets ? An experimental artifact of furaPE3 microspectrofluorimetry seems unlikely, because parallel changes in $[\text{Ca}^{2+}]_i$ were detected in High-glucose and Control islets upon stimulation with acetylcholine (ACh) in the absence of extracellular Ca^{2+} (Fig. 5A). Alternatively, the stimulation of Ca^{2+} influx by glucose could be smaller in High-glucose than in Control islets and escape detection by measuring $[\text{Ca}^{2+}]_i$ because of the already elevated mean $[\text{Ca}^{2+}]$ in the whole cytoplasm. It could, however, be sufficient to increase the Ca^{2+} concentration beneath the plasma membrane in the vicinity of VDCC and trigger exocytosis of insulin granules in their close proximity (6). This hypothesis is compatible with recent observations suggesting that insulin granule exocytosis may be better controlled by changes in Ca^{2+} influx through VDCC than by a rise in $[\text{Ca}^{2+}]_i$ (57).

Possible role of ER Ca^{2+} stores in the elevation of resting $[\text{Ca}^{2+}]_i$ in High-glucose islets.

The elevation of $[\text{Ca}^{2+}]_i$ in High-glucose islets perfused with a medium containing G0.5 did not result from a higher Ca^{2+} influx under these resting conditions (Fig. 4). Because the ER plays a crucial role in the control of resting $[\text{Ca}^{2+}]_i$ by normal β -cells (4; 61), we next tested whether the ER Ca^{2+} stores play a role in this elevation of $[\text{Ca}^{2+}]_i$, and whether a reduction in resting $[\text{Ca}^{2+}]_i$ would unmask a rise in $[\text{Ca}^{2+}]_i$ upon glucose stimulation of Ca^{2+} influx in High-glucose islets.

After stimulation with G10, removal of extracellular Ca^{2+} abrogated glucose-induced $[\text{Ca}^{2+}]_i$ rise in Control islets, and reduced $[\text{Ca}^{2+}]_i$ by $\sim 20 \text{ nmol.L}^{-1}$ in High-glucose islets (Fig. 5A), so that $[\text{Ca}^{2+}]_i$ remained elevated in High-glucose vs Control islets. Subsequent addition of ACh to the medium induced a similar transient rise in $[\text{Ca}^{2+}]_i$ in High-glucose and Control islets (58 ± 3 vs $54 \pm 1 \text{ nmol.L}^{-1}$ respectively)(Fig. 5A), followed by a prolonged lowering of $[\text{Ca}^{2+}]_i$ that was slightly larger in High-glucose than in Control islets (18 ± 2 vs $9 \pm 1 \text{ nmol.L}^{-1}$, $p < 0.05$).

Pretreatment of Control islets with the SERCA pump inhibitor thapsigargin (Tg) did not affect their resting $[\text{Ca}^{2+}]_i$ compared with non-pretreated islets from the same culture, but had three effects: the initial decrease in $[\text{Ca}^{2+}]_i$ induced by stimulation with G10 was abolished, the fall in $[\text{Ca}^{2+}]_i$ following omission of extracellular Ca^{2+} was faster and larger, and ACh was ineffective (both the initial transient peak and the subsequent lowering of $[\text{Ca}^{2+}]_i$ were abolished)(Fig. 5B). In High-glucose islets pretreated with Tg and perfused with G0.5, $[\text{Ca}^{2+}]_i$ was lower than in non-pretreated islets from the same culture, so that the difference in $[\text{Ca}^{2+}]_i$ between High-glucose and Control islets was reduced by $\sim 1/3$ from 50 ± 1 to $34 \pm 1 \text{ nmol.L}^{-1}$ ($p < 0.001$). Despite this reduction, the inability of G10 to increase $[\text{Ca}^{2+}]_i$ persisted. Both phases of the effect of ACh were also abolished in the absence of extracellular Ca^{2+} (Fig. 5B).

These results suggest that the elevation of $[\text{Ca}^{2+}]_i$ in High glucose islets could partly result from the overfilling of intracellular Ca^{2+} stores during culture in G30 (cytosolic Ca^{2+} binding proteins, ER and/or mitochondria). In support of this hypothesis, we observed that, after 18h loading with $^{45}\text{Ca}^{2+}$, conditions under which we assume that isotopic equilibrium was

reached, 1h treatment with Tg decreased the islet $^{45}\text{Ca}^{2+}$ content to a much larger extent in High-glucose than Control islets (Fig. 5D). However, the partial reduction of the elevation of $[\text{Ca}^{2+}]_i$ produced by Tg pretreatment was not sufficient to unmask a rise in $[\text{Ca}^{2+}]_i$ upon glucose stimulation of Ca^{2+} influx. It is, therefore, clear that factors other than ER Ca^{2+} overfilling contribute to the elevation of resting $[\text{Ca}^{2+}]_i$ and the absence of glucose-stimulated $[\text{Ca}^{2+}]_i$ rise in High-glucose islets.

General discussion. The present study demonstrates that High-glucose islets are more sensitive to glucose for the stimulation of both triggering (Ca^{2+} influx) and amplifying (augmentation of Ca^{2+} -induced exocytosis) pathways of GSIS, with a lower threshold glucose concentration for the stimulation of the triggering pathway than in Control islets, and nearly maximal stimulation of both pathways in G6-G10 rather than G20. A similar increase in glucose sensitivity was observed for the glucose-induced reduction of Ca^{2+} efflux in the presence of DZ or absence of extracellular Ca^{2+} . This increase in glucose sensitivity of High-glucose vs Control islets likely results from their higher energetic state in the presence of low glucose concentrations. Because energetic metabolism of High-glucose islets is almost maximally stimulated in the presence of G6, the changes in NAD(P)H production, mitochondrial hyperpolarization, ATP production, Ca^{2+} influx and insulin secretion that are expected to occur when the glucose concentration is raised from G6 to G10-G20 are strongly reduced, when not absent. This could have been interpreted as a lack of glucose responsiveness if the lower glucose concentration of G0.5 had not been tested. Our measurement of an increase in glucose sensitivity of High-glucose islets, which could partly result from the abnormal accumulation of glycogen in islet β -cells (41; 44; 67), are in good agreement with data obtained with rat and human islets cultured under similar conditions (40-42; 45; 60), with islets from 48h glucose infused rats (1), and with islets from *Psammomys obesus* made diabetic by 5 days on a high-energy diet (48). Other studies suggesting that β -cells exposed to high glucose concentrations become insensitive to glucose may be conflicting at first sight only. Thus, the basal glucose concentration used as a reference was

higher than here, and presumably already had induced nearly maximal stimulation of β -cells (7; 8; 13; 39; 47; 51).

Besides their increased glucose sensitivity, High-glucose islets displayed an ~50% decrease in maximal GSIS under physiological conditions (G20). This reduction, which could result from both β -cell degranulation and reduced glucose stimulation of Ca^{2+} influx, was no longer observed when $[\text{Ca}^{2+}]_i$ was further increased by plasma membrane depolarization with 30 mmol.L⁻¹ K⁺. Our results therefore suggest that correcting the triggering action of glucose (stimulation of Ca^{2+} influx through VDCC) could be more effective to improve GSIS in type 2 diabetes than further augmenting an already maximally stimulated amplifying action of the sugar. They also suggest that slightly reducing the basal rate of β -cell ATP production may restore GSIS within the physiological range of glucose concentrations while simultaneously decreasing fasting hyperinsulinemia and β -cell degranulation (23; 29).

In conclusion, after one week culture in G30, the loss of glucose stimulation of insulin secretion within the physiological range of glucose concentrations (from G5 in the fasting state to G10 in the postprandial period) results from the combination of two effects : first, an increased sensitivity to glucose of mitochondrial metabolism and both triggering and amplifying pathways of insulin secretion, which occur in spite of a ~2-fold increase in UCP2 mRNA expression; second, an ~50% reduction in the maximal glucose stimulation of the triggering pathway, hence of the maximal glucose-stimulated insulin secretion under physiological conditions. These alterations are associated with a paradoxical dissociation between glucose induced changes in Ca^{2+} influx and the absence of detectable $[\text{Ca}^{2+}]_i$ rise of unclear origin.

FOOTNOTES

¹Abbreviations used in this paper: ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; cDNA, complementary DNA; DZ, diazoxide; ER, endoplasmic reticulum; Gx, x mmol.L⁻¹ glucose; GSIS, Glucose-stimulated insulin secretion; K⁺-ATP channels, ATP-dependent K⁺ channels; mRNA, messenger ribonucleic acid.; Tg, thapsigargin; UCP2, uncoupling protein 2 (mitochondrial proton carrier); VDCC, voltage-dependent Ca^{2+} channels.

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LEGENDS TO FIGURES

Figure 1 : Islet morphology after prolonged culture in the presence of G10 or G30. Rat islets were cultured for 1 week in RPMI medium containing G10 (A,C,D) or G30 (B,D,F,G). After culture, the islets were fixed and processed for optical microscopy analysis of islet morphology (A, B) or for electron microscopy analysis of β -cells (C,D,G) and non β -cells (E,F). A, B: White stars indicate (at their lower right side) structures containing fragments of basophilic pyknotic chromatin material characteristic of apoptotic bodies. G: electron dense particles characteristic of glycogen were observed in β -cells from High-glucose islets only.

Figure 2 : Glucose-induced changes in energetic metabolism, $[Ca^{2+}]_i$ and insulin secretion in islets cultured for one week in RPMI medium containing G10 (Control) or G30 (High-glucose). A,B, after culture, the islets were perfused and stimulated by stepwise increases in glucose concentrations, followed by addition of 5 mmol.L⁻¹ azide to G20, as indicated on the top of each panel. Changes in NAD(P)H autofluorescence (A) and mitochondrial membrane potential (B) are shown as a percentage of the mean fluorescence level measured 2-5 min after addition azide. Please note that a decrease in rhodamine 123 fluorescence corresponds to mitochondrial membrane hyperpolarization. C, after 30 min preincubation in G0.5, batches of 5-8 islets were incubated for 30 min in the presence of G0.5-G20, or G20 + 5 mmol.L⁻¹ azide. The ATP/(ATP + ADP) ratio was calculated for each batch of islets from its absolute ATP and ADP contents expressed in pmol/islet. Data are means $\pm$ S.E. for 3-5 independent experiments.

D-F, After culture, the islets (eventually loaded with furaPE3 (D)) were perfused for 30 min with a medium containing 0.5 mmol.L⁻¹ glucose (G0.5). They were then stimulated by stepwise increases in the glucose concentration, followed by eventual addition of 5 mmol.L⁻¹ azide, as indicated on the top of each panel. Insulin secretion, measured in 2 min effluent

collections, is shown as absolute values (E) or as a percentage of the islet insulin content secreted per min (F). Data are means $\pm$ S.E. for 5 independent experiments.

Figure 3 : Amplifying action of glucose on high- K^+ induced insulin secretion in Control and High-glucose islets. After culture, the islets were perfused for 30 min with a medium containing 250 $\mu\text{mol.L}^{-1}$ diazoxide (DZ) and G0, G6 or G20. They were then stimulated with 30 mmol.L^{-1} K^+ (K30), as indicated on the top of each panel. A-D, $[\text{Ca}^{2+}]_i$ (A, B) and insulin secretion (C, D) from Control (A, C) and High-glucose islets (B, D). Data are means $\pm$ S.E. for 4-5 independent experiments.

Figure 4 : Effects of glucose, diazoxide, and removal of extracellular Ca^{2+} on insulin secretion (A), fractional $^{45}\text{Ca}^{2+}$ efflux (B, D, E) and $[\text{Ca}^{2+}]_i$ (C, F) in Control and High-glucose islets. After eventual loading with $^{45}\text{CaCl}_2$ for the last 18 h of culture (B, D, E) or with furaPE3 (C, F), the islets were perfused for 30 min with a medium containing G0.5 and stimulated by stepwise increases in glucose concentration followed by addition of 250 $\mu\text{mol.L}^{-1}$ diazoxide to G20 (A, B, C). The effect of glucose on $^{45}\text{Ca}^{2+}$ efflux was also tested in the presence of extracellular Ca^{2+} after addition of 250 $\mu\text{mol.L}^{-1}$ diazoxide (DZ) (D), or after removal of extracellular Ca^{2+} (Ca^{2+} omission + 100 $\mu\text{mol.L}^{-1}$ EGTA, $\text{Ca}^{2+}\emptyset$) (E). At the end of each experiment, the islets were stimulated with 30 mmol.L^{-1} K^+ (K30) in the presence of extracellular Ca^{2+} and DZ, as indicated on the top of each panel. B, D, E, dotted lines represent $^{45}\text{Ca}^{2+}$ efflux measured in the presence of 0.5 mmol.L^{-1} glucose throughout the experiment. F, effects of DZ and removal of extracellular Ca^{2+} on $[\text{Ca}^{2+}]_i$ in islets perfused with a medium containing G0.5. Data are means $\pm$ S.E. for 4-5 independent experiments.

Figure 5 : Role of endoplasmic reticulum Ca^{2+} stores in the elevation of $[\text{Ca}^{2+}]_i$ in High-glucose versus Control islets. A-C, after culture, furaPE3-loaded islets were perfused with a medium containing G0.5 for 60 min (C), or were stimulated with G10 after 30 min in G0.5

before sequential removal of extracellular Ca^{2+} (Ca^{2+} omission + 2 mmol.L⁻¹ EGTA, $\text{Ca}^{2+}\emptyset$) and addition of 100 $\mu\text{mol.L}^{-1}$ acetylcholine (ACh) (A,B). B, the islets were pretreated with 1 $\mu\text{mol.L}^{-1}$ thapsigargin for the last hour of the furaPE3-loading period. A, the dotted lines are extrapolations (based on data in panel C) of $[\text{Ca}^{2+}]_i$ if a control solution with G0.5 had been used throughout. Data are means $\pm$ S.E. for 4-5 (A,B) or 2 (C) independent experiments.

D, after loading with $^{45}\text{CaCl}_2$ for the last 18 h of culture and eventual addition of 1 $\mu\text{mol.L}^{-1}$ thapsigargin for the last one hour (Tg), Control and High-glucose islets were rinsed in the presence of La^{3+} to block Ca^{2+} efflux. The islet $^{45}\text{Ca}^{2+}$ content is expressed as a percentage of the content of untreated Control islets, after normalization for minor differences in islet DNA content and in total radioactivity of the loading medium within each experiment. Data are means $\pm$ S.E. for 9 batches of islets from 3 independent experiments. *, $p < 0.001$ vs all other groups.

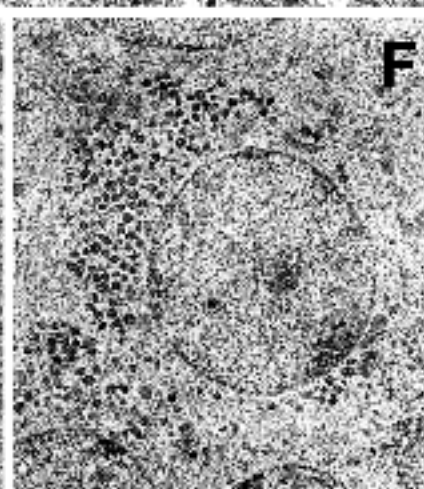
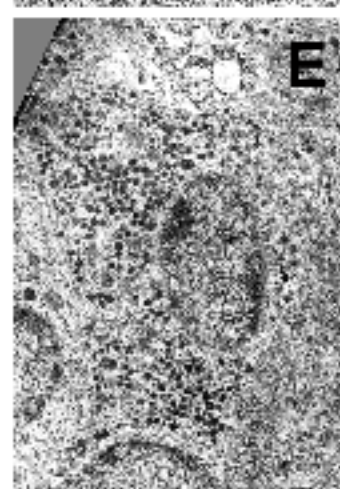
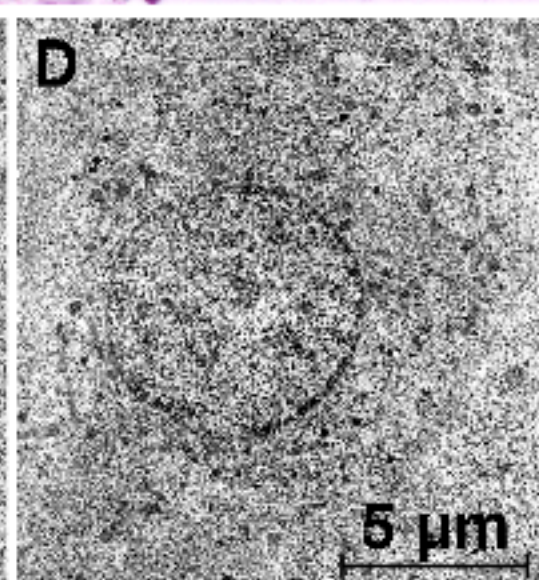
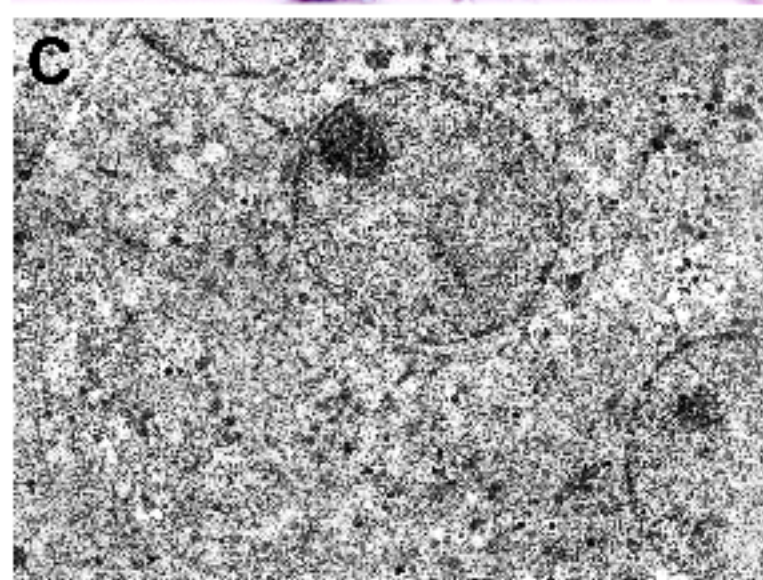
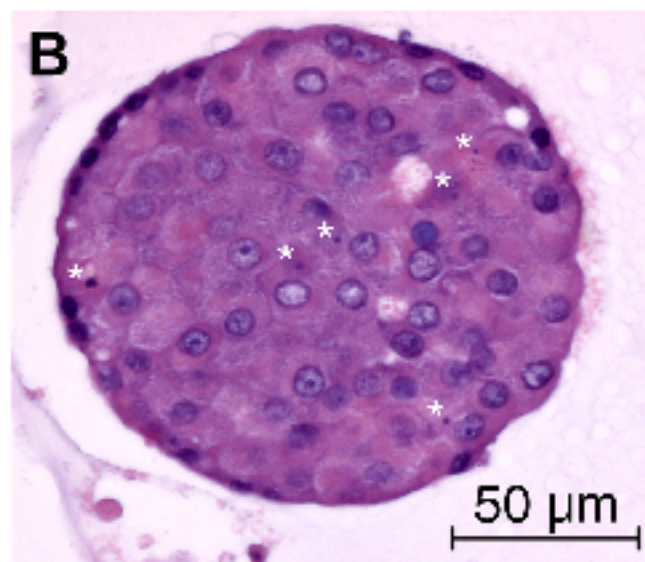
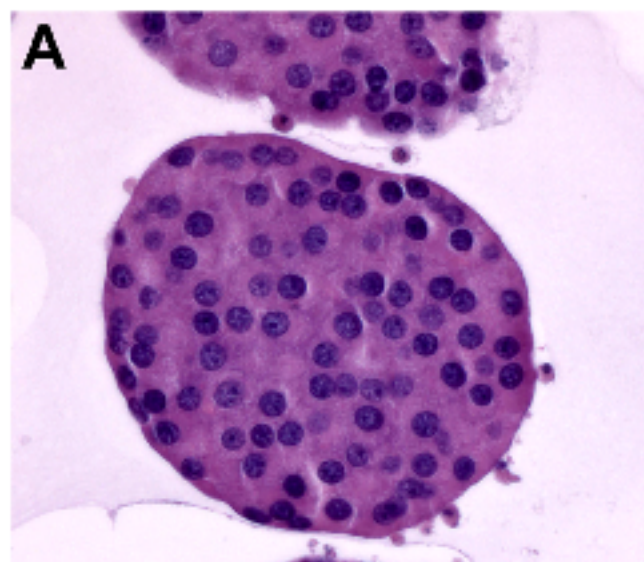


figure 2

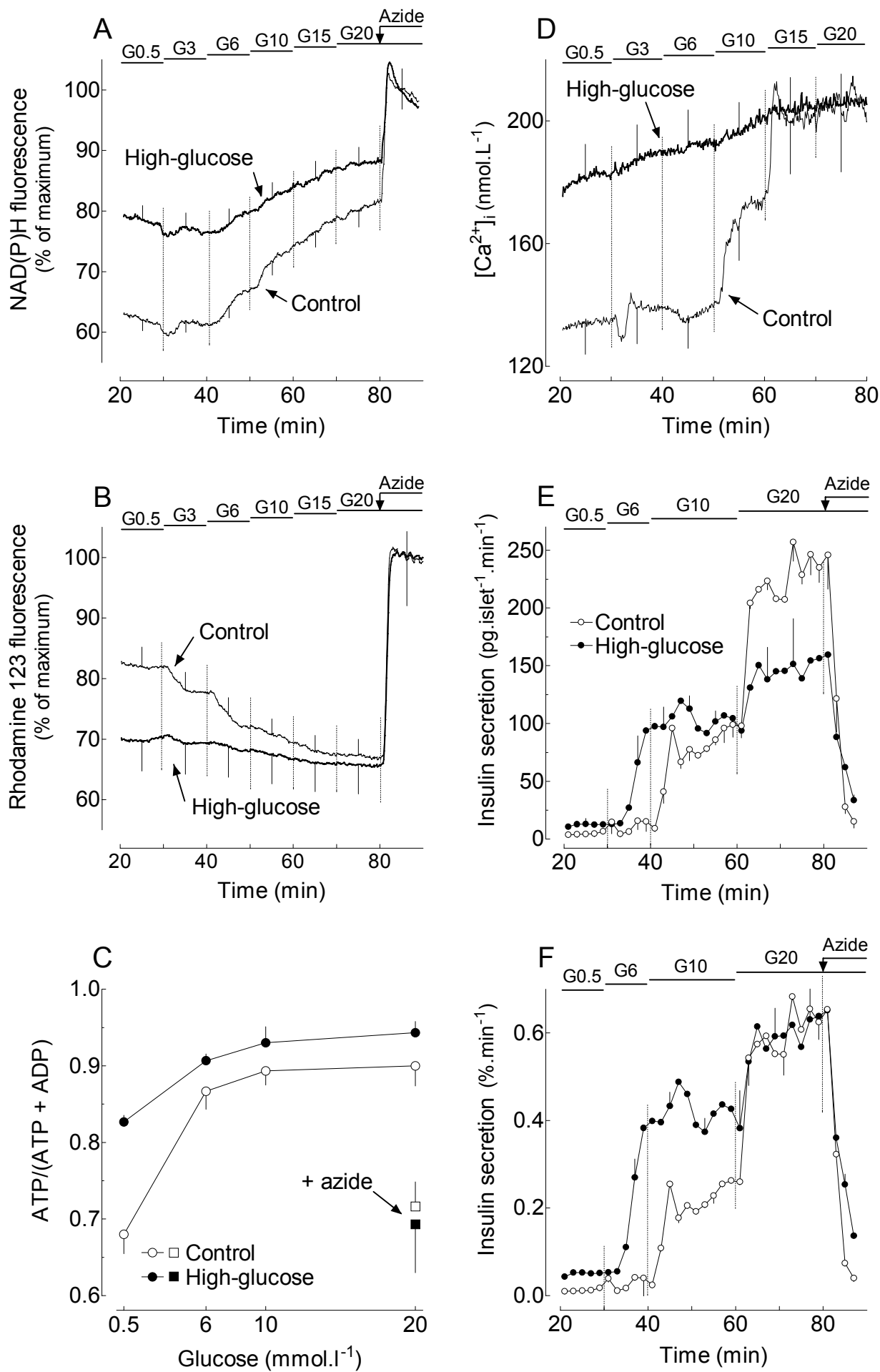


figure 3

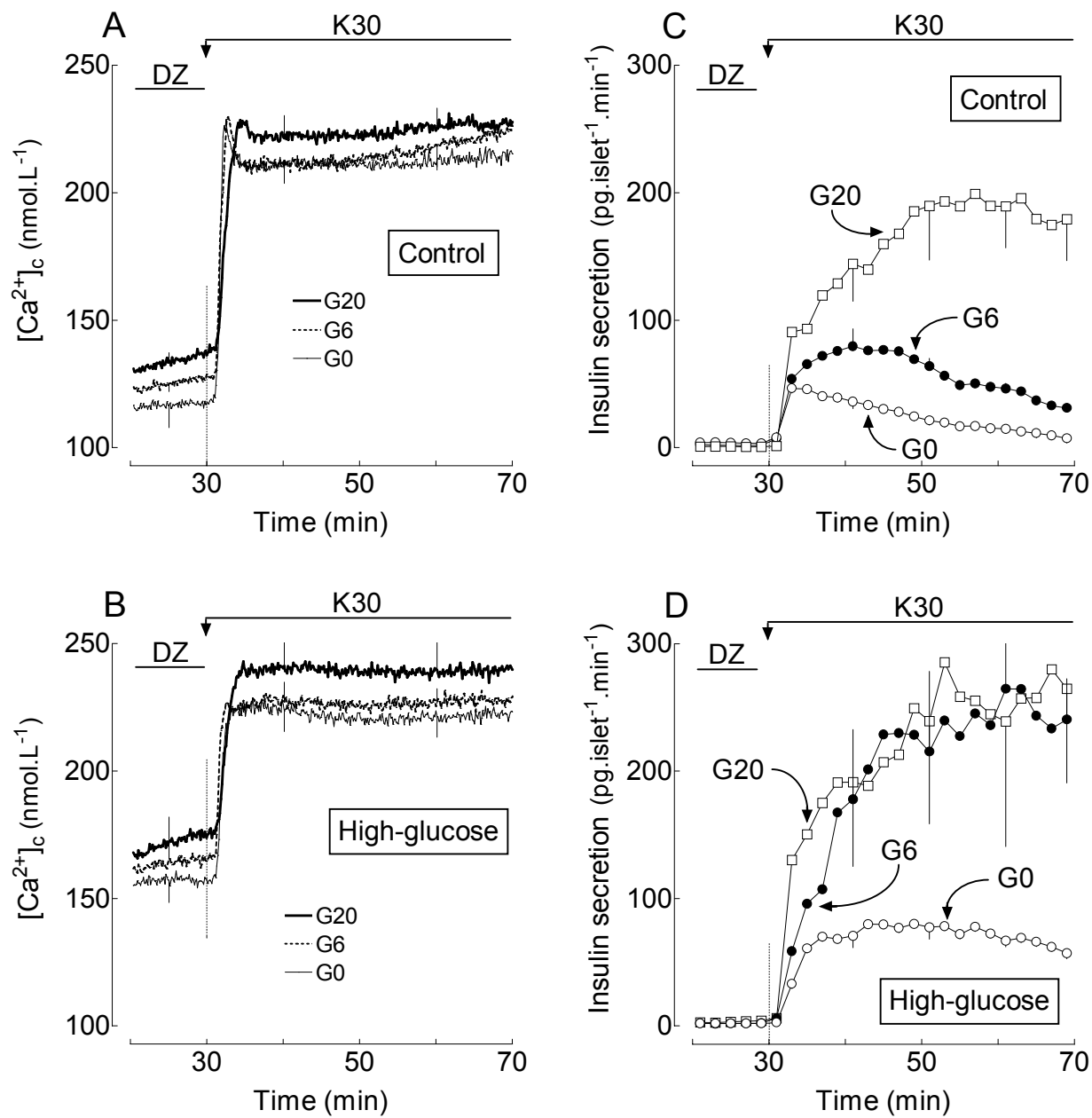


figure 4

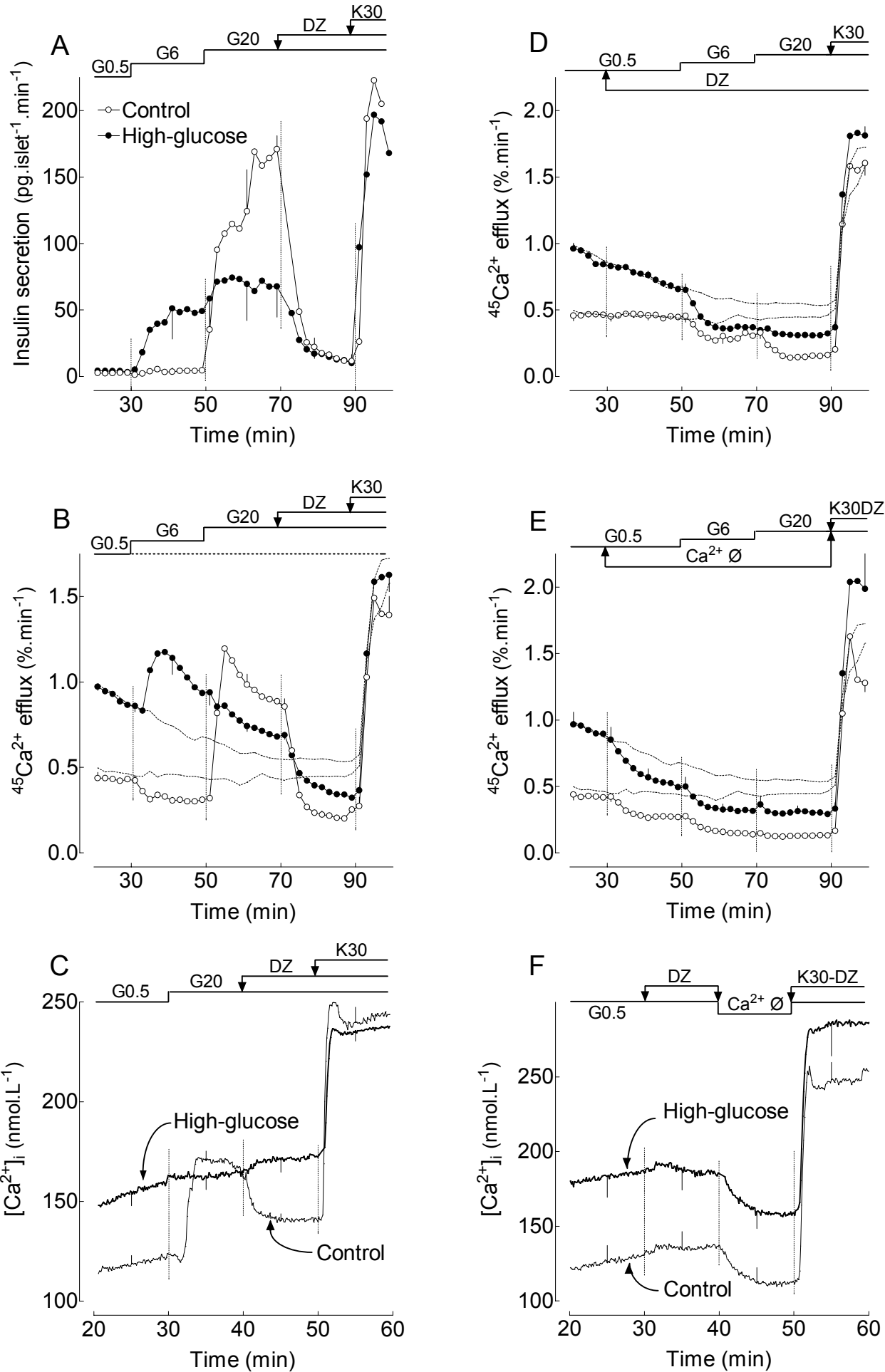


figure 5

