

## Glucagon-like peptide-1 mobilizes intracellular $\text{Ca}^{2+}$ and stimulates mitochondrial ATP synthesis in pancreatic MIN6 $\beta$ -cells

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Glucagon-like peptide-1 (GLP-1) is a potent regulator of glucose-stimulated insulin secretion whose mechanisms of action are only partly understood. In the present paper, we show that at low (3 mM) glucose concentrations, GLP-1 increases the free intramitochondrial concentrations of both  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_m$ ), and ATP ( $[\text{ATP}]_m$ ) in clonal MIN6  $\beta$ -cells. Suggesting that cAMP-mediated release of  $\text{Ca}^{2+}$  from intracellular stores is responsible for these effects, increases in  $[\text{ATP}]_m$  that were induced by GLP-1 were completely blocked by the Rp isomer of adenosine-3',5'-cyclic monophosphothioate (Rp-cAMPS), or by chelation of intracellular  $\text{Ca}^{2+}$ . Furthermore, inhibition of  $\text{Ins}(1,4,5)\text{P}_3$  ( $\text{IP}_3$ ) receptors with xestospongins C, or application of ryanodine, partially inhibited GLP-1-induced  $[\text{ATP}]_m$  increases, and the simultaneous blockade of both  $\text{IP}_3$  and ryanodine receptors (RyR) completely eliminated the rise in  $[\text{ATP}]_m$ . GLP-1 appeared

to prompt  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release through  $\text{IP}_3$  receptors via a protein kinase A (PKA)-mediated phosphorylation event, since ryanodine-insensitive  $[\text{ATP}]_m$  increases were abrogated with the PKA inhibitor, H89. In contrast, the effects of GLP-1 on RyR-mediated  $[\text{ATP}]_m$  increases were apparently mediated by the cAMP-regulated guanine nucleotide exchange factor cAMP-GEFII, since xestospongins C-insensitive  $[\text{ATP}]_m$  increases were blocked by a dominant-negative form of cAMP-GEFII (G114E,G422D). Taken together, these results demonstrate that GLP-1 potentiates glucose-stimulated insulin release in part via the mobilization of intracellular  $\text{Ca}^{2+}$ , and the stimulation of mitochondrial ATP synthesis.

Key words: aequorin, insulin, islet, luciferase, mitochondria.

### INTRODUCTION

Insulin secretion is regulated by many intracellular signals, including nutrients, hormones and neurotransmitters [1,2]. Glucose is believed to act principally to stimulate mitochondrial ATP synthesis, leading to the closure of ATP-sensitive  $\text{K}^+$  channels ( $\text{K}_{\text{ATP}}$ ) [3], cell depolarization and  $\text{Ca}^{2+}$  influx [4]. However, additional  $\text{K}_{\text{ATP}}$ -independent mechanisms have also been proposed to have a role in the responses to glucose [5,6].

Released from gut L-cells, glucagon-like peptide-1 (GLP-1) is a physiologically important potentiator of glucose-induced insulin secretion [7]. The peptide causes an elevation of intracellular cAMP concentrations and the activation of cAMP-dependent protein kinase A (PKA), a potent stimulator of insulin release [8–10]. However, whereas the activation of PKA is a well-accepted mechanism by which late exocytotic events can be stimulated in  $\beta$ -cells [11], the impact of changes in intracellular cAMP concentration on glucose metabolism and  $\text{Ca}^{2+}$  homeostasis is less well defined [7].

It has recently been proposed that GLP-1 induces elevations in cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) by a cAMP-dependent modulation of the activity of intracellular  $\text{Ca}^{2+}$  release channels [12–14]. Whereas the type II isoform of ryanodine receptor (RyR) channels [15,16] has been implicated in the effects of cAMP, PKA is also believed to phosphorylate and activate Type I

[17], and possibly also Type III,  $\text{Ins}(1,4,5)\text{P}_3$  ( $\text{IP}_3$ ) receptors (the predominant isoform in the  $\beta$ -cell) [18]. Furthermore, GLP-1 may also elevate  $[\text{Ca}^{2+}]_c$  via cAMP-induced opening of a non-selective cation channel, leading to depolarization by sodium influx and subsequent opening of voltage-dependent calcium channels [19,20]. Finally, the cAMP-binding protein cAMP-GEFII (cAMP-regulated GTP exchange factor II) [21] and Rim2, a target of the small G-protein Rab3 [22] may also be critical for the potentiation of insulin secretion by GLP-1 [23].

The activation of  $\text{Ca}^{2+}$  influx elicited by cell depolarization [24] or glucose [25,26] leads to rapid increases in mitochondrial free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_m$ ) in  $\beta$ -cells. Our recent studies using recombinant targeted luciferases [27–30] have shown that this leads to a stimulation of mitochondrial metabolism and increases in the free concentration of ATP in both the mitochondrial matrix ( $[\text{ATP}]_m$ ) and cytosol ( $[\text{ATP}]_c$ ). In contrast, the effects of mobilizing intracellular  $\text{Ca}^{2+}$  on mitochondrial oxidative metabolism and insulin release are more poorly characterized.

The above observations have led us to explore the possibility that changes in intracellular cAMP concentration elicited by GLP-1 may have a direct role in the regulation of mitochondrial ATP synthesis. To address this question, we have used dynamic bioluminescence imaging to record ATP and  $\text{Ca}^{2+}$  concentrations in real time during challenge of MIN6  $\beta$ -cells with GLP-1 and other stimuli. We show that GLP-1 evokes  $\text{Ca}^{2+}$  release from intracellular stores in a cAMP-dependent manner and that the

Abbreviations used:  $[\text{Ca}^{2+}]_c$ ,  $[\text{ATP}]_c$ ,  $[\text{Ca}^{2+}]_m$ ,  $[\text{ATP}]_m$ , cytosolic or mitochondrial matrix free  $\text{Ca}^{2+}$  or ATP concentration; cAMP-GEFII, cAMP-regulated guanine nucleotide exchange factor II; CICR,  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release; cytLuc, cytosolic-luciferase-expressing adenovirus; DMEM, Dulbecco's modified Eagle's medium; GLP-1, glucagon-like peptide-1; hGH, human growth hormone;  $\text{IP}_3$ ,  $\text{Ins}(1,4,5)\text{P}_3$ ;  $\text{K}_{\text{ATP}}$ , ATP-sensitive  $\text{K}^+$  channel; mitAeq, mitochondrial-aequorin-expressing adenovirus; mitLuc, mitochondrial-luciferase-expressing adenovirus; PKA, protein kinase A; Rp-cAMPS, Rp isomer of adenosine-3',5'-cyclic monophosphothioate; RyR, ryanodine receptor.

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released  $\text{Ca}^{2+}$  may then lead to an increase in  $[\text{Ca}^{2+}]_m$  and a stable increase in ATP synthesis. The present paper provides evidence for a new mechanism by which increases in intracellular cAMP may be coupled to enhanced electrical activity and insulin secretion in the  $\beta$ -cell. In particular, we demonstrate that  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) and stimulation of mitochondrial metabolism are likely to have a central role in the response of  $\beta$ -cells to GLP-1.

## EXPERIMENTAL

### Materials

Dulbecco's modified Eagle's medium (DMEM) was from Sigma or Invitrogen. LIPOFECTAMINE 2000<sup>TM</sup> was from Invitrogen. Human growth hormone (hGH) ELISA kit was from Roche. Beetle luciferin was from Promega and coelenterazine was from Molecular Probes. GLP-1 (7–36) amide was from Sigma. Ryanodine [high purity (> 99%)], xestospongion C, H-89 and the Rp isomer of adenosine-3',5'-cyclic monophosphothioate (Rp-cAMPS) were from Calbiochem. Other reagents were from Sigma or BDH.

### MIN6 cell culture

MIN6  $\beta$ -cells [31] were used between passages 19 and 30, and were grown in DMEM containing 15% (v/v) heat-inactivated foetal bovine serum (Life Technologies), 25 mM glucose, 5.4 mM KCl, 2 mM glutamine, 50  $\mu\text{M}$  2-mercaptoethanol, 100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cells were cultured in a humidified atmosphere at 37 °C with 5%  $\text{CO}_2$ .

### Plasmids

For  $[\text{ATP}]_c$  and  $[\text{ATP}]_m$  measurements, we used adenoviruses that expressed cytosolic (untargeted; cytLuc) and mitochondrially targeted luciferase (mitLuc) respectively [27,29]. To measure  $[\text{Ca}^{2+}]_m$ , an adenovirus that expressed mitochondrial aequorin (mitAeq) was used [27]. Infection of cells was as described in [28]. Plasmid pcDNA3-hGH was a gift from Professor R. D. Burgoyne (Department of Physiology, University of Liverpool, U.K.) [32]. Wild-type cAMP-GEFII expression vector pSR $\alpha$ -cAMP-GEFII and mutant cAMP-GEFII (G114E,G422D) were provided by Dr Susumu Seino (Chiba University, Japan) [22].

### Microinjection

Intranuclear microinjection was performed using an Eppendorf 5171/5246 micromanipulator. Plasmids were dissolved at the concentrations indicated in 2 mM Tris/HCl, pH 8.0, and 0.2 mM sodium EDTA [33,34]. At 1 day after microinjection and culture under the conditions given, photon-counting imaging of firefly luciferase activities was performed in single living cells (see below). Individual experiments involved injection of 100–200 separate cells per condition.

### Transfection

For hGH-secretion assays, MIN6  $\beta$ -cells seeded in 12-well plates were co-transfected with 1.0  $\mu\text{g}$  of pcDNA3-hGH using LIPOFECTAMINE 2000<sup>TM</sup>, according to the manufacturer's instructions.

### Measurement of secreted hGH

MIN6  $\beta$ -cells were transfected with a plasmid bearing hGH cDNA and cultured in DMEM containing 25 mM glucose for 24 h, then 3 mM glucose for 16 h. Cells were then washed in PBS and incubated in Krebs–Hepes bicarbonate medium (132.5 mM NaCl, 3.6 mM KCl, 0.5 mM  $\text{NaH}_2\text{PO}_4$ , 0.5 mM  $\text{MgSO}_4$ , 1.5 mM  $\text{CaCl}_2$ , 10 mM Hepes, 2 mM  $\text{NaHCO}_3$ ), also containing 3 mM or 30 mM glucose for 20 min at 37 °C in a shaking water bath. hGH released into the incubation medium was assayed using an hGH ELISA kit, according to the manufacturer's instructions [32]. Expressed hGH immunoreactivity closely co-localized with insulin in MIN6  $\beta$ -cells [33], demonstrating efficient sorting to the regulated secretory pathway.

### Measurement of $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$

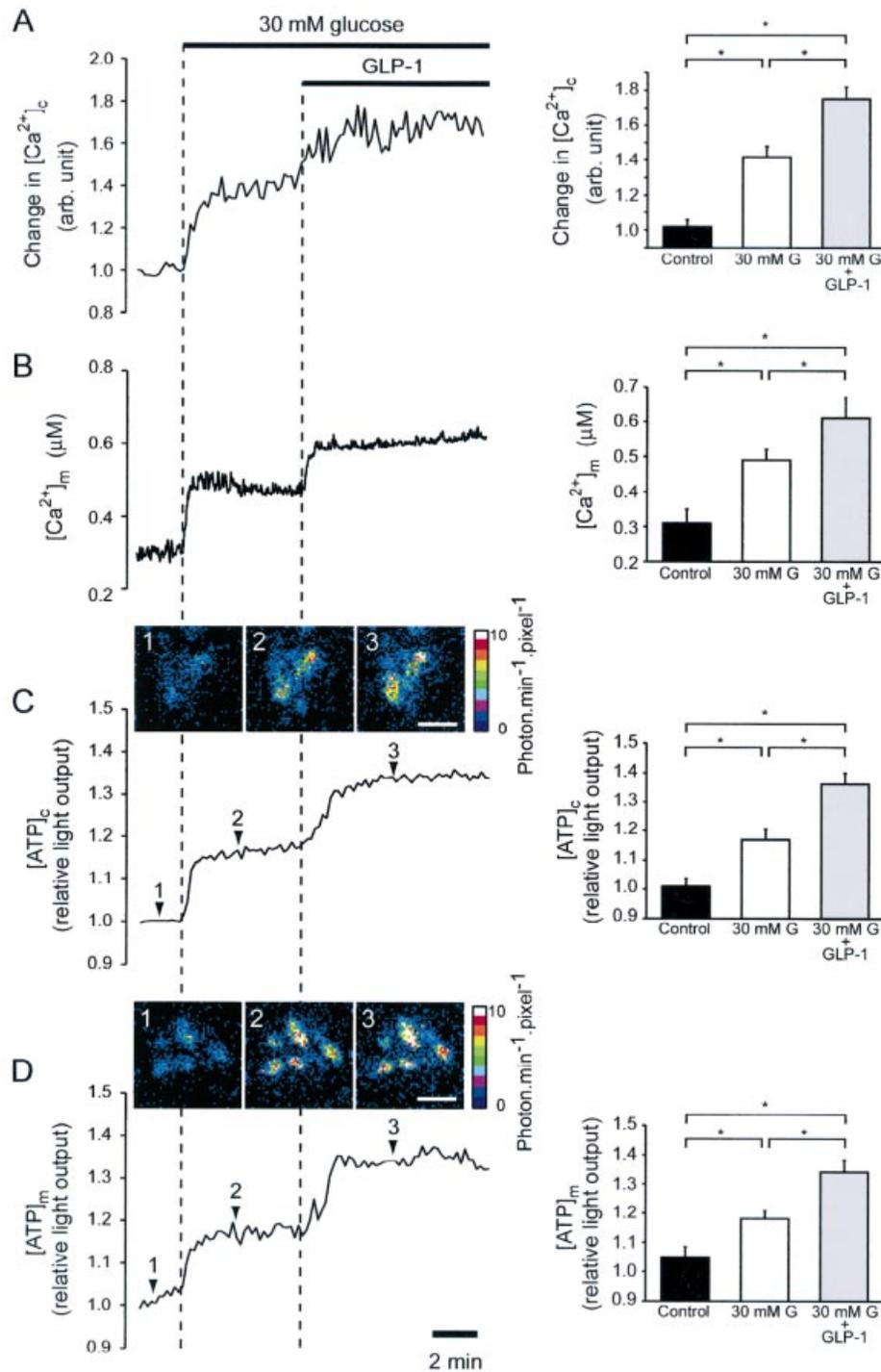
Changes in  $[\text{Ca}^{2+}]_c$  were measured using fluo-3 acetoxymethyl-ester (fluo-3; Sigma). Cells on coverslips (24 mm diameter) were loaded with 5  $\mu\text{M}$  fluo-3 for 40 min at 37 °C in Krebs–Ringer bicarbonate medium, pre-equilibrated with 95:5  $\text{O}_2/\text{CO}_2$ , pH 7.4, containing various glucose concentrations as indicated in the Figure legends. Cells were then mounted in a chamber, placed on the stage of an Olympus IX-70 inverted microscope ( $\times 40$  objective lens, NA = 1.35; Olympus UK). Fluo-3-loaded cells were excited at 480 nm at 6 s intervals using a Till photonics monochromator (Munich, Germany), and emission signals at 515 nm were detected with an Imago SensiCam<sup>TM</sup> cooled charge-coupled device camera. Changes in  $[\text{Ca}^{2+}]_m$  were measured 2 days after cell infection with mitAeq virus. Aequorin luminescence was monitored continuously using a photomultiplier tube (ThornEMI, Electron Tubes, Ruislip, U.K.), during perfusion in Krebs–Ringer bicarbonate medium [24]. Light output was recorded at 1 s intervals.

### Measurement of $[\text{ATP}]_c$ and $[\text{ATP}]_m$

Changes in  $[\text{ATP}]_c$  and  $[\text{ATP}]_m$  were measured after cell infection with cytLuc or mitLuc adenoviruses [27,29] by single cell photon-counting imaging on an Olympus IX-70 microscope fitted with a  $\times 10$  objective and a Photek ICCD218<sup>TM</sup> intensified camera (Lewes, U.K.) [27].

### Measurement of cAMP content

MIN6  $\beta$ -cells were seeded in 24-well plates and used at > 70% confluence on the experimental day. At 1 h before the experiment, the cell culture medium was replaced with 0.5 ml of Krebs–Ringer bicarbonate medium. At 15 min before agonist addition, 100  $\mu\text{M}$  of the phosphodiesterase type IV inhibitor Ro201724 was added to each well to prevent degradation of the cAMP generated in the assay. In pre-incubation studies, forskolin and GLP-1 were added to wells 10 min before agonist addition. Agonist or vehicle was added to each well at zero time and the cell plate was incubated in the water bath. After incubation, 20  $\mu\text{l}$  of 100% trichloroacetic acid was added to terminate the signalling reaction. To assay whole cell cAMP accumulation in each well, 50  $\mu\text{l}$  of the supernatant was added to 50  $\mu\text{l}$  of 1 M NaOH and 200  $\mu\text{l}$  of TE buffer (50 mM Tris/HCl, 4 mM EDTA, pH 7.4). A 50  $\mu\text{l}$  aliquot of this solution was then added to LP4 tubes containing 100  $\mu\text{l}$  of TE buffer, 100  $\mu\text{l}$  of [<sup>3</sup>H]cAMP in TE buffer (approx. 20000 c.p.m.) and 100  $\mu\text{l}$  of binding protein in TE buffer prepared from bovine adrenal cortex (final concentration approx.



**Figure 1** Effect of glucose on GLP-1-induced  $[Ca^{2+}]_m$  and  $[ATP]_m$  increases

Effect of glucose (30 mM) on (A)  $[Ca^{2+}]_c$  and (B)  $[Ca^{2+}]_m$  increases in the presence or absence of 100 nM GLP-1. Effect of glucose (30 mM) on (C)  $[ATP]_c$  and (D)  $[ATP]_m$  in the presence or absence of 100 nM GLP-1. The initial glucose concentration was 3 mM in each case. Typical traces from 6–8 separate experiments are shown. Bars show means  $\pm$  S.E.M., \* $P < 0.005$ . The images in (C) and (D) show the monitored luminescence changes in a typical field of cells, imaged during 60 s at the corresponding time points (1–3). The scale bar represents 50  $\mu m$ .

750  $\mu g$  of protein per ml). The solution was then vortex mixed. The unknown cAMP content in the samples was estimated from a standard curve (0.125–40 pmol cAMP in 50  $\mu l$ ). After 1 h incubation at 4  $^{\circ}C$ , excess radiolabel was removed by addition of 200  $\mu l$  of TE buffer containing charcoal (Norit GXS; final concentration 50 mg/ml) and BSA (2 mg/ml final concentration) for 15 min. The tubes were then centrifuged at 3600 g for 12 min

at 4  $^{\circ}C$ . The resulting supernatant was transferred into vials for liquid scintillation counting.

#### Statistical analysis

Data are given as means  $\pm$  S.E.M. Comparisons between means were performed using a one-tailed Student's *t* test for paired

data with Origin software (Microcal, Northampton, MA, U.S.A.).

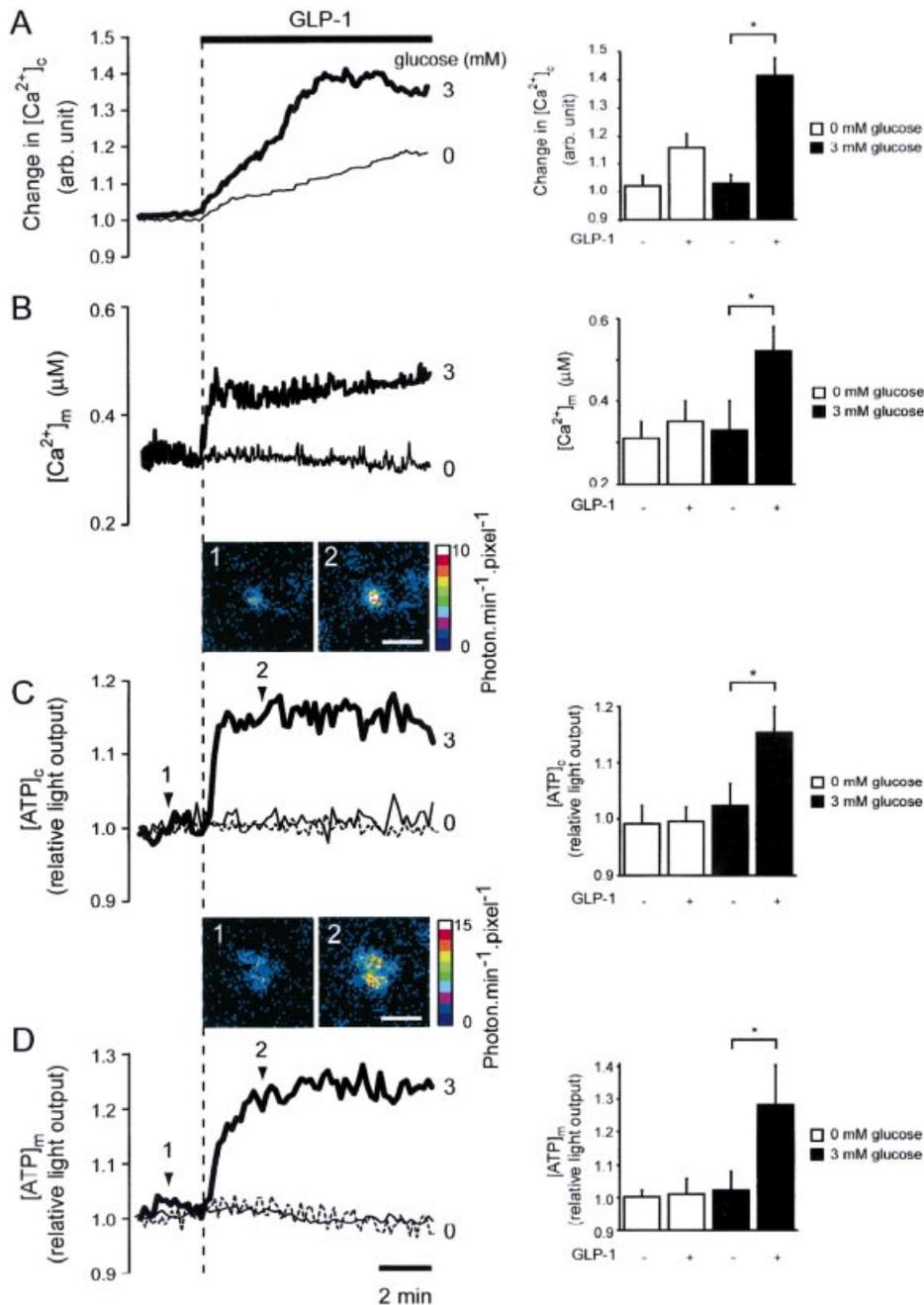
## RESULTS

### Effect of glucose and GLP-1 on $[Ca^{2+}]_c$ and $[ATP]_m$ in MIN6 cells

The challenge of MIN6 cells [31] with glucose (30 mM versus 3 mM) elicited sustained increases in both  $[Ca^{2+}]_c$ , measured using fluo-3 (Figure 1A;  $142.5 \pm 12.6\%$  of the initial value,  $n = 40$  cells

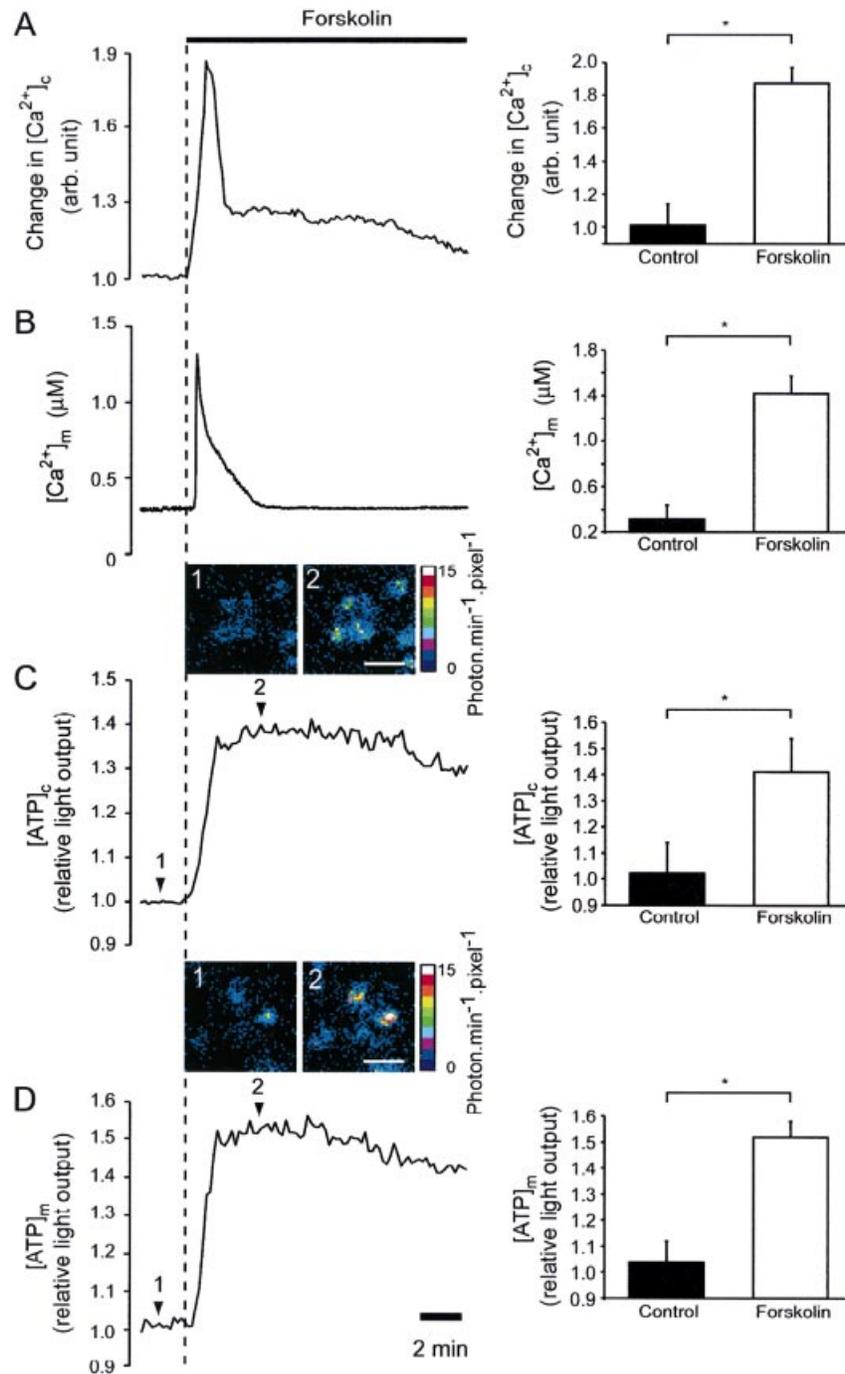
from eight trials) and in  $[Ca^{2+}]_m$  (Figure 1B;  $0.51 \pm 0.16 \mu\text{M}$ ,  $n = 6$  separate experiments) assessed using a mitAeq [24,35]. Similarly, 30 mM glucose promoted a rapid increase in both  $[ATP]_c$  (Figure 1C;  $117.4 \pm 5.8\%$  of the initial value,  $n = 6$ ) and  $[ATP]_m$  (Figure 1D;  $118.2 \pm 6.4\%$  of the initial value,  $n = 6$ ), measured *in situ* after adenoviral expression of targeted firefly luciferases [27,29,30].

In the presence of 30 mM glucose, 100 nM GLP-1 promoted further increases in  $[Ca^{2+}]_c$  (Figure 1A;  $174.8 \pm 4.2\%$  of the initial value,  $n = 40$  cells from eight trials) and  $[Ca^{2+}]_m$  (Fig-



**Figure 2** GLP-1-induced  $[Ca^{2+}]_m$  and  $[ATP]_m$  increases in MIN6  $\beta$ -cells

Effect of GLP-1 (100 nM) on (A)  $[Ca^{2+}]_c$  and (B)  $[Ca^{2+}]_m$  changes in the presence (thick trace) or absence (thin trace) of glucose (3 mM) in MIN6  $\beta$ -cells. Effect of GLP-1 (100 nM) on (C)  $[ATP]_c$  and (D)  $[ATP]_m$  in the presence (thick trace) or absence (thin trace) of glucose. Broken traces in (C) and (D) indicate that the effect of oligomycin ( $5 \mu\text{M}$ ) on GLP-1 induced  $[ATP]_c$  and  $[ATP]_m$  increases respectively. Measurement of  $[Ca^{2+}]_m$ ,  $[ATP]_c$  and  $[ATP]_m$  were performed in parallel batches of cells transiently infected with mitAeq, cytLuc and mitLuc respectively. Each trace is a mean of 5–8 independent experiments. Bars show means  $\pm$  S.E.M., \* $P < 0.005$ . Images in (C) and (D) show light output during 60 s, as in Figure 1. The scale bar represents  $50 \mu\text{m}$ .

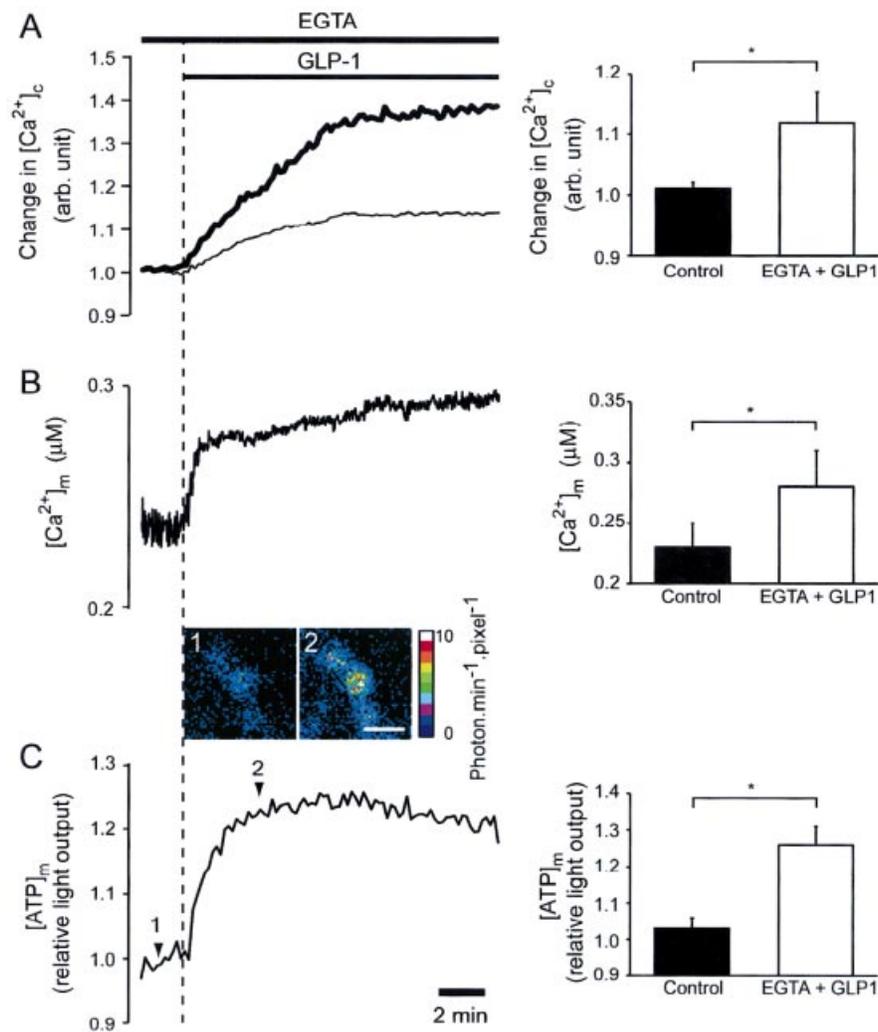


**Figure 3** Effect of forskolin on  $[Ca^{2+}]_m$  and  $[ATP]_m$  increases in MIN6  $\beta$ -cells

Effect of forskolin (1  $\mu M$ ) on (A)  $[Ca^{2+}]_c$  and (B)  $[Ca^{2+}]_m$  changes in MIN6  $\beta$ -cells. Effect of forskolin (1  $\mu M$ ) on (C)  $[ATP]_c$  and (D)  $[ATP]_m$ . Glucose concentration was at 3 mM in all cases. Each trace is a mean of 5–8 independent experiments. Bars show means  $\pm$  S.E.M., \* $P < 0.005$ . Images in (C) and (D) show light output during 60 s, as in Figure 1. The scale bar on the images represents 50  $\mu m$ .

ure 1B;  $0.61 \pm 0.18 \mu M$ ,  $n = 6$ ). Under these conditions, GLP-1 also caused further increases in  $[ATP]_c$  (Figure 1C;  $132.3 \pm 6.2\%$  of the initial value,  $n = 6$ ) and  $[ATP]_m$  (Figure 1D;  $134.1 \pm 9.2\%$  of the initial value,  $n = 6$ ). Essentially identical effects were also observed using lower concentrations of GLP-1 (in the physiological range). Thus, at 30 mM glucose, 1 nM GLP-1

promoted increases in  $[Ca^{2+}]_c$  ( $163.2 \pm 6.4\%$  of the initial value),  $[Ca^{2+}]_m$  ( $0.56 \pm 0.22$  mM,  $n = 3$ ),  $[ATP]_c$  ( $126.3 \pm 5.4\%$  of the initial value,  $n = 3$ ) and  $[ATP]_m$  ( $127.8 \pm 6.4\%$  of the initial value,  $n = 3$ ). Taken together, these data indicate that GLP-1-induced increases in  $[Ca^{2+}]_m$  may act to enhance mitochondrial ATP synthesis.



**Figure 4** Effect of EGTA on GLP-1-induced  $[Ca^{2+}]_m$  and  $[ATP]_m$  increases

(A) Effect of GLP-1 (100 nM) on  $[Ca^{2+}]_c$  changes in the presence (thin trace) or absence (thick trace) of EGTA (2 mM) in the presence of 3 mM glucose. (B) Pre-treatment of EGTA (2 mM) reduced the GLP-1-induced  $[Ca^{2+}]_m$  increases in the presence of 3 mM glucose. (C) Pre-treatment of EGTA did not inhibit the GLP-1-induced  $[ATP]_m$  in the presence of 3 mM glucose. Typical traces from 5–7 separate experiments are shown. Bars show means  $\pm$  S.E.M., \* $P < 0.005$ . Images in (C) show light output during 60 s, as in Figure 1. The scale bar represents 50  $\mu m$ .

### GLP-1-stimulated increases in $[Ca^{2+}]_m$ and $[ATP]_m$ are dependent upon glucose

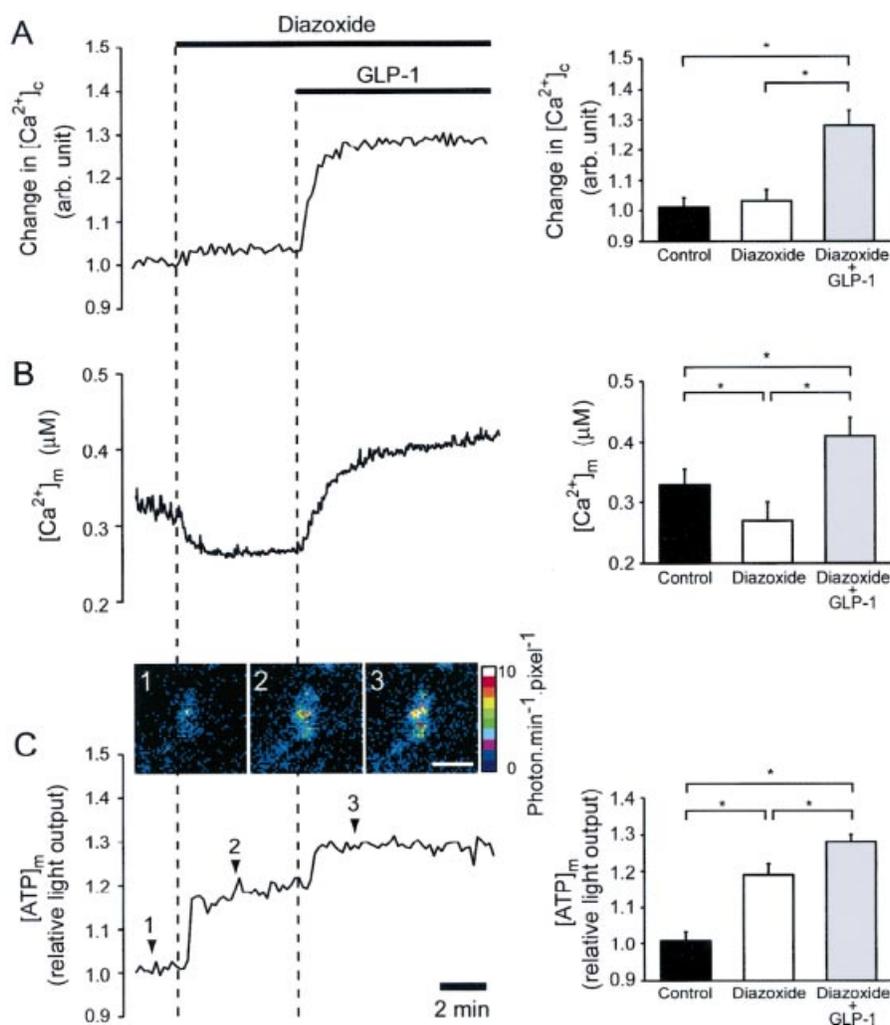
In order to determine whether or not the effects of GLP-1 required the presence of glucose, we next measured the responses to the hormone of cytosolic and mitochondrial concentrations of  $Ca^{2+}$  and ATP, either in the complete absence of the sugar or at a glucose concentration (3 mM) which is just below the threshold for insulin release ([31] and see below). As shown in Figure 2,  $[Ca^{2+}]$  increased both in the cytosol and mitochondria in response to 100 nM GLP-1 when 3 mM glucose was present, but not in the complete absence of the sugar (Figure 2). Similarly, in the presence of 3 mM glucose, 100 nM GLP-1 promoted increases in both  $[ATP]_c$  (Figure 2C;  $115.4 \pm 8.5\%$ ,  $n = 8$ ) and  $[ATP]_m$  (Figure 2D;  $128.3 \pm 12.1\%$ ,  $n = 8$ ). In contrast, GLP-1 failed to cause any increase in  $[ATP]_c$  (Figure 2C; thin trace,  $99.6 \pm 2.5\%$ ,  $n = 5$ ) and  $[ATP]_m$  (Figure 2D; thin trace,  $101.1 \pm 4.6\%$ ,  $n = 5$ ) at 0 mM glucose.

These observations suggest that GLP-1 may act to increase intracellular ATP levels by enhancing glucose metabolism. To

confirm that the effects of GLP-1 on  $[ATP]_c$  and  $[ATP]_m$  were due to an increase in the mitochondrial metabolism of glucose, rather than to a stimulation of glycolysis, we next examined the effect of pre-treatment of cells with oligomycin (5  $\mu M$ ), an inhibitor of the mitochondrial  $F_0F_1$  ATP synthase, on GLP-1-induced  $[ATP]_c$  and  $[ATP]_m$  increases. Oligomycin completely inhibited GLP-1-induced  $[ATP]_c$  (Figure 2C; broken trace,  $99.6 \pm 2.4\%$ ,  $n = 4$ ) and  $[ATP]_m$  increases (Figure 2D; broken trace,  $99.1 \pm 3.8\%$ ,  $n = 5$ ) respectively. Taken together, these results indicate that GLP-1-induced  $[Ca^{2+}]_m$  signals are likely to be responsible for subsequent increases in the mitochondrial oxidation of glucose carbon, and thus the observed increases in  $[ATP]_m$  and  $[ATP]_c$ .

### Effects of increases in intracellular cAMP on intracellular $Ca^{2+}$ homeostasis and mitochondrial metabolism

To determine whether the above effects of GLP-1 might be explained by an increase in intracellular cAMP levels, we next determined whether forskolin, a potent activator of adenylate



**Figure 5** Effect of diazoxide on GLP-1-induced  $[Ca^{2+}]_m$  and  $[ATP]_m$  increases

Effect of diazoxide (100  $\mu$ M) on (A)  $[Ca^{2+}]_c$ , (B)  $[Ca^{2+}]_m$  and (C)  $[ATP]_m$  increases in the presence of 3 mM glucose. Typical traces from 5–6 separate experiments are shown. Bars show means  $\pm$  S.E.M., \* $P < 0.005$ . Images in (B) show light output during 60 s, as in Figure 1. The scale bar represents 50  $\mu$ m.

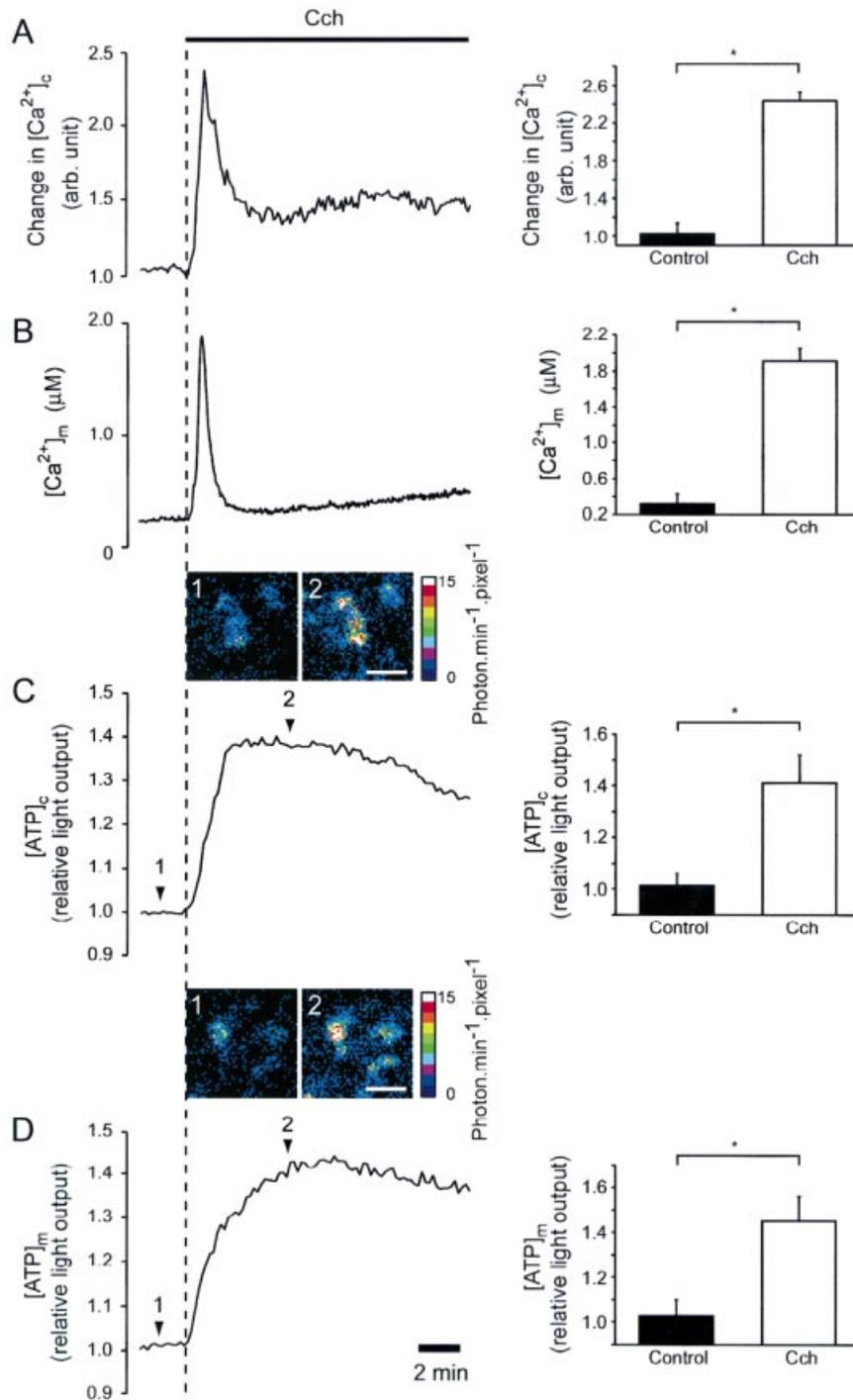
cyclase, could mimic the effects of the incretin. Both GLP-1 and forskolin, added sequentially, caused large ( $> 2$ -fold; results not shown) increases in cAMP content. Like GLP-1, forskolin caused large, but more transient, increases in  $[Ca^{2+}]_c$  and  $[Ca^{2+}]_m$  (Figures 3A and 3B). These changes were followed by sustained increases in  $[ATP]_c$  and  $[ATP]_m$  (Figures 3C and 3D), possibly reflecting a long-term activation of mitochondrial metabolism by  $Ca^{2+}$  [27,30,36].

#### Requirements for intra- and extracellular $Ca^{2+}$ of GLP-1-induced $[ATP]_m$ increases

To determine whether  $Ca^{2+}$  influx was essential for GLP-1-induced  $[Ca^{2+}]_m$  and  $[ATP]_m$  increases, we examined the effect of chelation of extracellular  $Ca^{2+}$ . Addition of 2 mM EGTA inhibited GLP-induced increases in both  $[Ca^{2+}]_c$  (to  $31.6 \pm 7.3\%$  of the Figure 2A control value,  $n = 28$  cells from 7 trials) and  $[Ca^{2+}]_m$  (to  $0.27 \pm 0.11 \mu$ M of the Figure 2A control value,  $n = 6$ ; Figures 4A and 4B). However, the addition of extracellular EGTA had a much smaller effect on GLP-1-induced  $[ATP]_m$

increases, which remained at  $93.8 \pm 10.6\%$  ( $n = 6$ ; Figure 4C) of the control value (Figure 2D, thick trace).

Since the complete removal of external  $Ca^{2+}$  may lead to some depletion of internal  $Ca^{2+}$  stores, we next examined the effect on GLP-1-induced  $[ATP]_m$  increases of inhibiting voltage-dependent  $Ca^{2+}$  influx with diazoxide. By opening  $K_{ATP}$  channels, this agent hyperpolarizes the plasma membrane, thus preventing the opening of L-type  $Ca^{2+}$  channels [37]. At 3 mM glucose, diazoxide (100  $\mu$ M) slightly inhibited GLP-1-induced increases in  $[Ca^{2+}]_c$  (to  $74.6 \pm 8.7\%$  of the control value in Figure 2A,  $n = 32$  cells from 6 trials; Figure 5A). In contrast, diazoxide caused a small decrease in the resting  $[Ca^{2+}]_m$  values (to  $73.4 \pm 9.7\%$  of the control value,  $n = 6$ ), but had no impact on the subsequent increase in  $[Ca^{2+}]_m$  in response to GLP-1 ( $116.4 \pm 8.9\%$  of the control value,  $n = 6$ ; Figure 5B). Interestingly, application of diazoxide increased basal  $[ATP]_m$  levels (by  $121.3 \pm 8.6\%$  of the initial value,  $n = 5$ ), but did not prevent the GLP-1-induced increase in  $[ATP]_m$  (to  $130.6 \pm 6.8\%$  of the initial value,  $n = 5$ ; Figure 5C). These, and the results shown in Figure 4, indicate that influx of external  $Ca^{2+}$  is not necessary for GLP-1 to raise intramitochondrial  $Ca^{2+}$  concentrations or to stimulate mitochondrial ATP synthesis.



**Figure 6** Effect of carbachol on  $[Ca^{2+}]_m$  and  $[ATP]_m$  increases

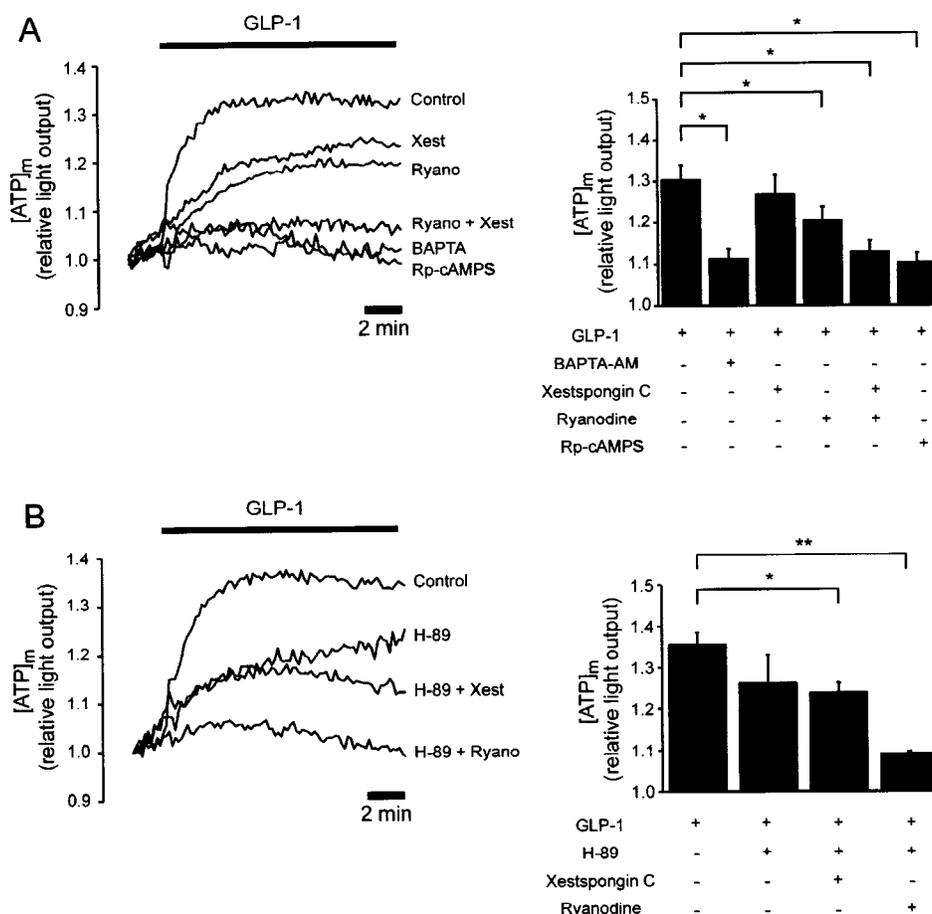
Effect of carbachol (Cch; 100  $\mu M$ ) on (A)  $[Ca^{2+}]_c$  and (B)  $[Ca^{2+}]_m$ , (C)  $[ATP]_c$  and (D)  $[ATP]_m$  increases in the presence of 3 mM glucose. Typical traces from 6–8 separate experiments are shown. Bars show means  $\pm$  S.E.M., \* $P < 0.005$ . Images in (C) and (D) show light output during 60 s, as in Figure 1. The scale bar represents 50  $\mu m$ .

We also examined the effect of depletion of endoplasmic reticulum  $Ca^{2+}$  with the inhibitor of the sarco(endo)plasmic reticulum  $Ca^{2+}$ -ATPase, thapsigargin [38]. Addition of 1  $\mu M$  thapsigargin inhibited GLP-1-induced increases in both  $[Ca^{2+}]_c$  (to  $18.2 \pm 6.7\%$  of the control value in Figure 2A,  $n = 10$  cells from 3 trials) and  $[Ca^{2+}]_m$  (to  $0.23 \pm 0.11 \mu M$  of the control value in Figure 2A,  $n = 3$ ). Furthermore, thapsigargin treatment strongly reduced GLP-1-induced  $[ATP]_m$  increases, to

$32.4 \pm 8.9\%$  ( $n = 3$ ) of the control value of Figure 2(D) (bold trace).

#### Effect of changes in intracellular $[Ca^{2+}]$ on GLP-1-induced $[ATP]_m$ increases

To explore further the hypothesis that mobilization of  $Ca^{2+}$  from intracellular stores contributes to the stimulation of mito-



**Figure 7** Effect of IP<sub>3</sub> and RyR inhibitors on GLP-1-induced [ATP]<sub>m</sub> increases

(A) Effect of Rp-cAMPS on GLP-1-induced [ATP]<sub>m</sub> increases. MIN6  $\beta$ -cells treated with 3 mM glucose together with GLP-1 (100 nM) alone, GLP-1 plus bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA; 10  $\mu$ M), GLP-1 plus xestospongin C (Xest; 10  $\mu$ M), GLP-1 plus ryanodine (Ryano; 10  $\mu$ M), GLP-1 plus xestospongin C plus ryanodine or Rp-cAMPS (200  $\mu$ M). Bars show means  $\pm$  S.E.M., \* $P$  < 0.005. (B) Effect of H-89 on GLP-1-induced [ATP]<sub>m</sub> increases. MIN6  $\beta$ -cells treated with 3 mM glucose together with GLP-1 (100 nM) alone, GLP-1 plus H-89 (10  $\mu$ M), GLP-1 plus H-89 plus xestospongin C (10  $\mu$ M) or GLP-1 plus H-89 plus ryanodine (10  $\mu$ M). Bars show means  $\pm$  S.E.M., \* $P$  < 0.005, \*\* $P$  < 0.0001.

chondrial ATP synthesis in response to GLP-1, we sought to demonstrate that mobilization of intracellular Ca<sup>2+</sup> by other mechanisms could also increase [ATP]<sub>c</sub> and [ATP]<sub>m</sub>. Supporting this view, activation of muscarinic acetylcholine receptors with carbachol [39], and thus the production of IP<sub>3</sub>, led to a robust, but transient, increase in [Ca<sup>2+</sup>]<sub>c</sub> and [Ca<sup>2+</sup>]<sub>m</sub>, and sustained increases in [ATP]<sub>c</sub> and [ATP]<sub>m</sub> (Figure 6).

In order to determine whether GLP-1-induced [Ca<sup>2+</sup>]<sub>m</sub> increases were essential to trigger a rise in [ATP]<sub>m</sub>, we next examined the effect of chelation of intracellular Ca<sup>2+</sup> with bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM). Under these conditions, the ability of GLP-1 to augment [Ca<sup>2+</sup>]<sub>c</sub> and [Ca<sup>2+</sup>]<sub>m</sub> (results not shown) and to increase [ATP]<sub>m</sub> (Figure 7A) was completely abolished. These results suggest that the mobilization of Ca<sup>2+</sup> from an intracellular Ca<sup>2+</sup> store is essential for the subsequent elevation of [Ca<sup>2+</sup>]<sub>m</sub> and [ATP]<sub>m</sub> by GLP-1.

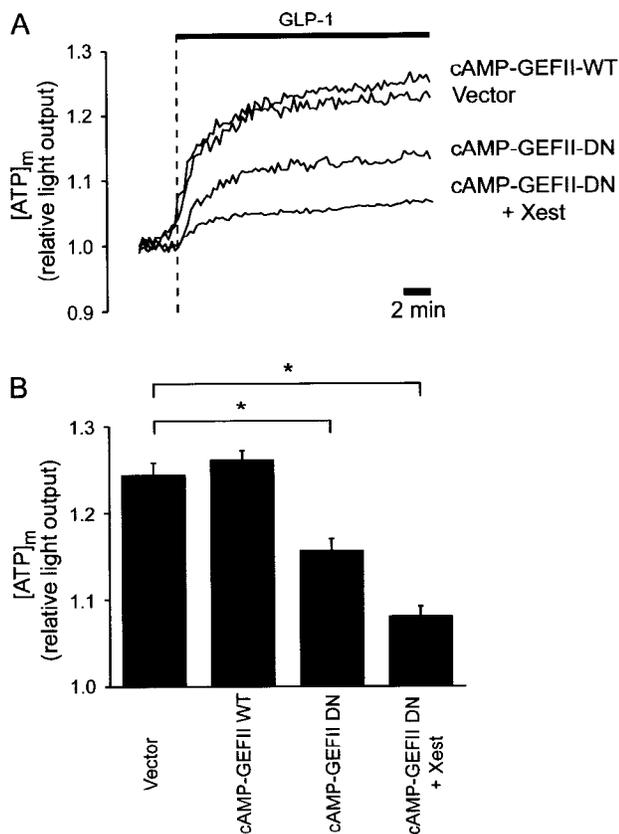
To explore the mechanisms involved in the GLP-1-induced activation of mitochondrial ATP synthesis in more detail, we next used the membrane-permeable IP<sub>3</sub> receptor antagonist, xestospongin C [40,41] and the RyR inhibitor, ryanodine. Added separately, xestospongin C (10  $\mu$ M) or ryanodine (10  $\mu$ M) slightly reduced GLP-1-induced [ATP]<sub>m</sub> increases (Figure 7A), whereas

the simultaneous addition of both inhibitors completely blocked [ATP]<sub>m</sub> increases. As expected, pre-treatment of MIN6 cells with the cAMP inhibitor Rp-cAMPS (200  $\mu$ M) completely inhibited the GLP-1-induced [ATP]<sub>m</sub> increases (Figure 7A).

In order to determine whether GLP-1-induced increases in intracellular cAMP concentration acted via PKA, a well-characterized PKA inhibitor, H-89 [42], was used. The action of GLP-1 was only partially reduced by pre-treatment with 10  $\mu$ M H-89 (Figure 7B), either in the presence or absence of the IP<sub>3</sub> receptor antagonist, xestospongin C. In contrast, co-application of ryanodine and H-89 completely inhibited GLP-1-induced [ATP]<sub>m</sub> increases. Taken together, these results suggest that GLP-1 acts to increase [ATP]<sub>m</sub> by sensitizing IP<sub>3</sub> receptors via PKA-dependent phosphorylation and by gating RyRs via a cAMP-dependent, PKA-independent, pathway, as reported previously in INS-1 cells [43].

#### Involvement of cAMP-GEFII in GLP-1-induced [ATP]<sub>m</sub> increases

The above observations are consistent with recent findings [14] that demonstrate that increases in cAMP may gate RyRs in the  $\beta$ -cell through the action of cAMP-GEFII/Epac2. If the gating of endoplasmic reticulum RyR channels is necessary for the



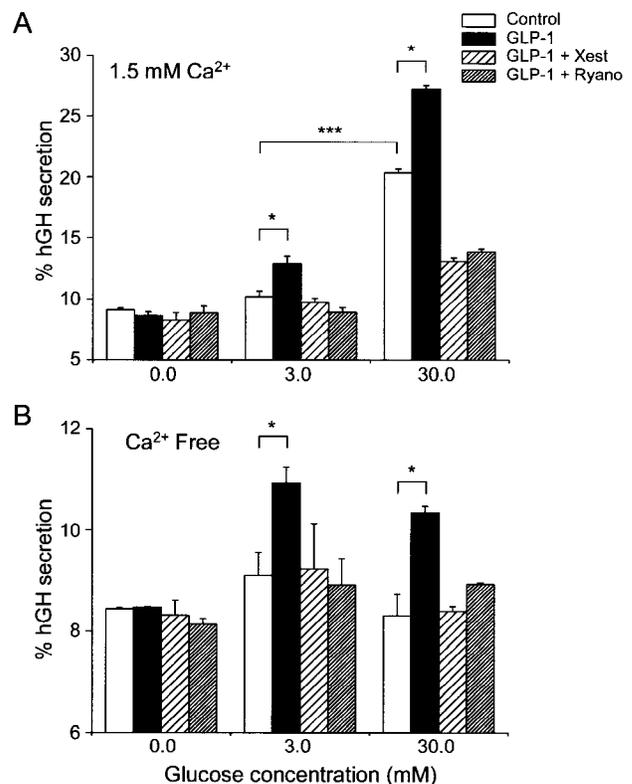
**Figure 8** Inhibition of GLP-1-induced  $[ATP]_m$  increases by dominant-negative cAMP-GEFII

(A) Effect of null vector, wild-type or dominant-negative isoforms of cAMP-GEFII on GLP-1-induced  $[ATP]_m$  increases in the presence of 3 mM glucose. (B) Mean data from six independent experiments as shown in (A), \* $P < 0.005$ .

stimulation of mitochondrial ATP synthesis by GLP-1, it might be expected that changes in the activity of cAMP-GEFII should also alter the ability of the incretin to raise  $[ATP]_m$ . Consistent with this view, cells that were microinjected with a null vector showed the typical sustained increases in  $[ATP]_m$  when challenged with GLP-1, and this response was similar to that of cells injected with a plasmid that encoded wild-type cAMP-GEFII (Figure 8A). However, when cells were microinjected with an expression plasmid that encoded a dominant-negative form of cAMP-GEFII (G114E,G422D) [23], the action of GLP-1 was partially (approx. 50%) inhibited. These findings are summarized in Figure 8(B) and suggest that GLP-1-induced  $[ATP]_m$  increases are mediated in part by changes in cAMP-GEFII activity.

#### Effects of GLP-1 on insulin secretion in MIN6 $\beta$ -cells

Preliminary experiments revealed that GLP-1 had no detectable effect on insulin release from MIN6  $\beta$ -cells incubated at 3 mM glucose, but potentiated the stimulatory effect of 30 mM glucose by approx. 20% (results not shown). In order to examine the effects of GLP-1 on glucose-stimulated exocytosis with greater precision, we used a transfection approach involving hGH, which is efficiently packaged and co-secreted with insulin [32,33] (Figure 9A). Secretion of hGH was strongly (approx. 2-fold) stimulated by 30 mM, but not 3 mM glucose, in the presence of



**Figure 9** Effect of GLP-1 on glucose-regulated secretion

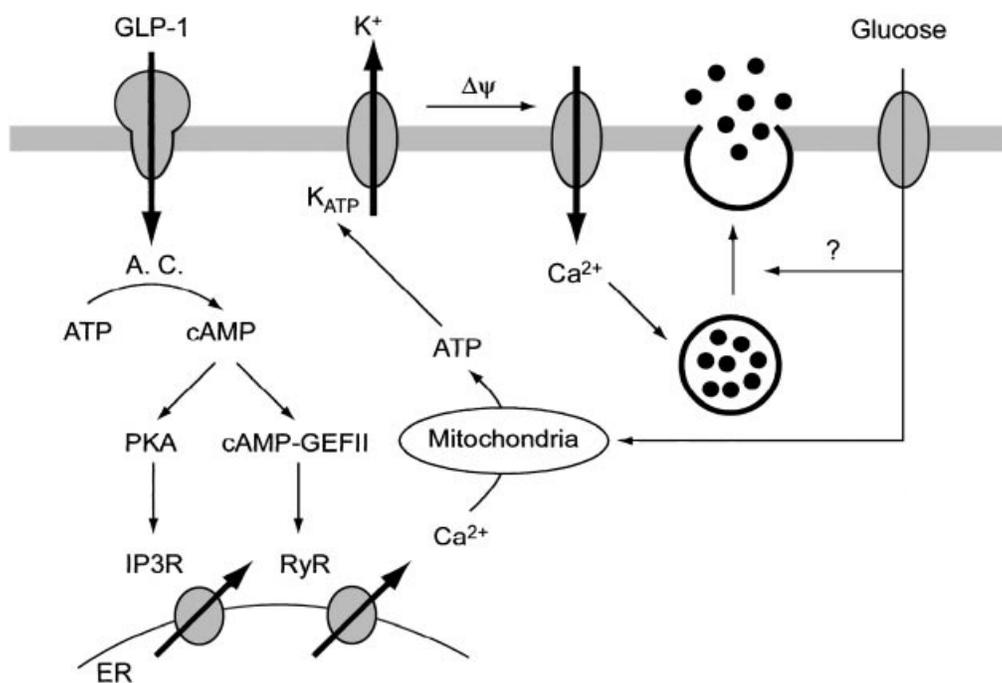
MIN6  $\beta$ -cells transfected with plasmid pcDNA3.hGH were incubated for 20 min in Krebs–Ringer bicarbonate medium at the indicated glucose concentrations, together with GLP-1 (100 nM) alone, GLP-1 plus xestospongin C (Xest; 10  $\mu$ M) or GLP-1 plus ryanodine (Ryano; 10  $\mu$ M). hGH release was measured in the presence (A) or complete absence (B) of external  $Ca^{2+}$  (1.5 mM EGTA). Released and total cellular hGH were measured by ELISA assay as described in the Experimental section. Bars show means  $\pm$  S.E.M., \* $P < 0.05$  for the effect of GLP-1 and \*\*\* $P < 0.05$  for the effect of glucose.

extracellular  $Ca^{2+}$  ions (Figure 9A), and 30 mM (but not 3 mM) glucose weakly, but significantly, stimulated exocytosis in the complete absence of extracellular  $Ca^{2+}$  (Figure 9B). GLP-1 (100 nM) potentiated hGH secretion in a glucose-concentration-dependent manner, as reported previously in islets [23] and in both the presence (Figure 9A) and absence (Figure 9B) of external  $Ca^{2+}$  ions.

## DISCUSSION

### Effects of GLP-1 on mitochondrial oxidative metabolism: role in the potentiation of insulin release

The mechanisms by which GLP-1 potentiates glucose-induced insulin secretion are only partly resolved [7]. It is generally accepted that GLP-1 binds to  $G_s$ -protein coupled receptors on the  $\beta$ -cell membrane [44], activating adenylate cyclase, and thus elevating intracellular cAMP levels [18]. However, Cullinan et al. [18] also reported that GLP-1 increased  $[Ca^{2+}]_c$  at glucose concentrations of 3 mM or above, in *ob/ob* mouse pancreatic islets. This increase was attributed, at least in part, to the mobilization of intracellular  $Ca^{2+}$  stores, since it was still apparent, though of smaller magnitude, in the presence of an L-type  $Ca^{2+}$ -channel blocker. Moreover, ryanodine was later found to inhibit GLP-1-induced  $[Ca^{2+}]_c$  increases in a clonal  $\beta$ -cell line [12]. However, the latter authors also reported that GLP-1-induced



**Figure 10** Model to illustrate the potential mechanisms by which GLP-1 may potentiate glucose-stimulated insulin secretion

A.C. represents adenylate cyclase. For other details, see the text.

$\text{Ca}^{2+}$  increases were blocked by chelation of extracellular  $\text{Ca}^{2+}$  with EGTA, and therefore proposed that the release of intracellular  $\text{Ca}^{2+}$  occurred as a consequence of  $\text{Ca}^{2+}$  influx and CICR [7].

Several hypotheses exist concerning the mechanisms of cAMP-dependent  $\text{Ca}^{2+}$  release from internal stores, notably the endoplasmic reticulum, in pancreatic  $\beta$ -cells. Thus it has been proposed that the relevant  $\text{Ca}^{2+}$ -release channels targeted by cAMP correspond to  $\text{IP}_3$  receptors [45]. However, evidence for a major role of type II RyRs, the major isoform in  $\beta$ -cells [46], has also been provided [15,47]. Importantly, both RyR and  $\text{IP}_3$   $\text{Ca}^{2+}$ -release channels are sensitized by cAMP and PKA [17,48–50]. Recently, we [51] and others [13] have demonstrated the presence of RyRs on insulin-containing vesicles, suggesting a possible role for  $\text{Ca}^{2+}$  release from these organelles.

In the present study, we have used the well-differentiated clonal  $\beta$ -cell line, MIN6 [31], which appears to represent a good model of the behaviour of primary  $\beta$ -cells. Importantly, these cells, in common with pancreatic islets, display a robust exocytotic response to glucose over the physiological concentration range [28,31], and this response is potentiated by GLP-1 (Figure 9A). These results lead us to propose a new model whereby GLP-1 may enhance glucose-stimulated insulin secretion (Figure 10). Thus, acting via an increase in intracellular cAMP concentration, GLP-1 initially appears to cause PKA-dependent phosphorylation and opening of  $\text{IP}_3$ -sensitive stores. At the same time, RyRs (probably class II) are opened as a result of the direct activation of cAMP-GEFII by cAMP [23]. Given that neither channel is gated directly through changes in phosphorylation status (nor through the action of cAMP-GEFII in the case of RyR), we suspect that each channel may already be slightly opened as a result of a small elevation of  $[\text{Ca}^{2+}]_c$  at 3 mM, but not 0 mM, glucose. Thus the main action of GLP-1 may be to potentiate the effect of this elevated  $\text{Ca}^{2+}$  concentration. Indeed,

such a phenomenon might explain why a threshold concentration of glucose is required to observe effects of GLP-1 on mitochondrial ATP synthesis (Figure 1A). However, we should stress that any such increase of  $\text{Ca}^{2+}$  concentration by 3 mM, but not 0 mM, glucose must be extremely small (and may be highly localized) since (i) it is very difficult to detect using fluorescent or luminescent  $\text{Ca}^{2+}$  probes (T. Tsuboi and G. A. Rutter, unpublished work) and (ii) does not lead to a significant stimulation of secretion under control conditions (Figure 9A).

Next, we propose that the released  $\text{Ca}^{2+}$  is taken up by mitochondria [35,36], activating mitochondrial dehydrogenases, and subsequently ATP synthesis [27,29]. The consequent increases in  $[\text{ATP}]_c$  may then lead to the closure of  $\text{K}_{\text{ATP}}$  channels [1,3], enhancing  $\text{Ca}^{2+}$  influx through voltage-gated channels and the activation of insulin release. Interestingly, neither mitochondrial  $\text{Ca}^{2+}$  accumulation nor increases in  $[\text{ATP}]_m$  were significantly affected by a substantial reduction in  $[\text{Ca}^{2+}]_c$  when  $\text{Ca}^{2+}$  influx was blocked, consistent with the existence of local domains' high  $\text{Ca}^{2+}$  concentration at intracellular release sites that are in close apposition to sites of mitochondrial  $\text{Ca}^{2+}$  uptake [36,52].

#### Role of $\text{K}_{\text{ATP}}$ -independent mechanisms in glucose-stimulated insulin secretion

An unexpected finding of the present study was that, when added with 3 mM glucose, GLP-1 raised cytosolic and mitochondrial ATP and  $\text{Ca}^{2+}$  concentrations to almost the same extent as 30 mM glucose (compare Figures 1 and 2). In contrast, GLP-1 stimulated the release of insulin (results not shown) or of transfected hGH (Figure 9A) much more weakly than did 30 (versus 3) mM glucose. Thus, whereas 30 mM glucose stimulated hGH release by approx. 2-fold, secretion was stimulated by only approx. 30% by GLP-1 under identical conditions (Figure 9A).

One possible explanation of these findings is that addition of 30 mM glucose, in contrast with GLP-1, acts principally to provoke  $\text{Ca}^{2+}$  influx, and thus to raise  $\text{Ca}^{2+}$  concentration preferentially beneath the plasma membrane, i.e. at the site of exocytosis [53]. However, an alternative hypothesis to explain the greater efficacy of the sugar to stimulate insulin release is that glucose acts not only to increase intracellular  $\text{Ca}^{2+}$  concentration, but also by additional mechanisms which sensitize the exocytotic machinery to ambient  $\text{Ca}^{2+}$  concentration, as proposed by Henquin and colleagues [54].

A further important finding of the present work is that, even in the complete absence of external  $\text{Ca}^{2+}$  ions, elevated glucose concentrations are still able to significantly stimulate exocytosis from  $\beta$ -cells in the presence of GLP-1 (Figure 9B). However, this activation was observed already at 3 mM glucose, consistent with a marked left-shift in the glucose-dose response in the presence of the incretin [23]. This result extends earlier findings in rat islets [55], where glucose was shown to stimulate insulin release in  $\text{Ca}^{2+}$ -free conditions, but only in the combined presence of both a cAMP-raising agent, forskolin, and the phorbol ester, PMA. Taken together with the earlier results, the present findings therefore provide further evidence for the existence of a  $\text{K}_{\text{ATP}}$ -channel independent 'potentiating' pathway for glucose-induced insulin secretion [56,57].

## Conclusion

These results demonstrate that GLP-1 enhances intracellular  $\text{Ca}^{2+}$  release in  $\beta$ -cells by prompting CICR through both  $\text{IP}_3$  and RyR  $\text{Ca}^{2+}$ -release channels. The resulting transfer of  $\text{Ca}^{2+}$  into mitochondria, and accelerated ATP synthesis, may then lead to the closure of plasma-membrane  $\text{K}_{\text{ATP}}$  channels,  $\text{Ca}^{2+}$  influx and insulin secretion.

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