Islet β-cell secretion determines glucagon release from neighbouring α-cells

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Homeostasis of blood glucose is maintained by hormone secretion from the pancreatic islets of Langerhans. Glucose stimulates insulin secretion from β-cells but suppresses the release of glucagon, a hormone that raises blood glucose, from α-cells[1]. The mechanism by which nutrients stimulate insulin secretion has been studied extensively: ATP has been identified as the main messenger and the ATP-sensitive potassium channel as an essential transducer in this process[2]. By contrast, much less is known about the mechanisms by which nutrients modulate glucagon secretion. Here we use conventional pancreas perfusion and a transcriptional targeting strategy to analyse cell-type-specific signal transduction and the relationship between islet α- and β-cells. We find that pyruvate, a glycolytic intermediate and principal substrate of mitochondria, stimulates glucagon secretion. Our analyses indicate that, although α-cells, like β-cells, possess the inherent capacity to respond to nutrients, secretion from α-cells is normally suppressed by the simultaneous activation of β-cells. Zinc released from β-cells may be implicated in this suppression. Our results define the fundamental mechanisms of differential responses to identical stimuli between cells in a microorgan.

Pancreatic islets have an essential role in glucose homeostasis and contain four types of endocrine cell, namely α-, β-, δ- and F-cells. In general the cells have the following distribution: α-cells, 21%; β-cells, 68%; δ-cells, 5%; F cells, 6% (ref. 3). Although studies of the β-cell, the predominant cell type of the islet, have made great progress, α-cell research has been hampered by a lack of methodology for analysing this minor cell type within the heterogeneous cellular populations.

We initially re-evaluated the effect of various nutrients on the modulation of glucagon and insulin secretion in isolated rat pancreas (Fig. 1a–f). Glucagon secretion was decreased and insulin release was increased when the concentration of glucose in the perfusate was raised. Like glucose, monomethyl-succinate (mmSuc), a membrane-permeant mitochondrial substrate, induced a biphasic secretion of insulin and caused a decrease in glucagon secretion.

As expected[4] pyruvate, another mitochondrial substrate, provoked only a minor and transient stimulation of insulin secretion, but unexpectedly it evoked a sustained secretion of glucagon. We characterized this pyruvate-stimulated secretion of glucagon further (Fig. 1g, h). Sodium azide, an inhibitor of mitochondrial cytochrome *c* oxidase, and diazoxide, an opener of the ATP-sensitive potassium (K_{ATP}) channel, completely and reversibly suppressed the pyruvate-stimulated secretion of glucagon. These results indicate that pyruvate metabolism in the mitochondria is implicated in the initiation of glucagon secretion. The mode of action seems to involve the closure of K_{ATP} channels in α-cells, a notion that is supported by the findings that rat islet α-cells express functional K_{ATP} channels[5] and voltage-dependent calcium channels[6].

We used molecular biological techniques to study the mechanisms by which glucose and the two mitochondrial substrates pyruvate and mmSuc evoke distinct patterns of hormone release in islet. As this approach requires the use of isolated islets, we also examined the effects of the nutrients in this preparation. The results in isolated islets were qualitatively similar to those in perfused pancreas: glucagon secretion was induced by pyruvate, but not by glucose or mmSuc (Fig. 1i, j). The inhibition of pyruvate-stimulated glucagon release by sodium azide or diazoxide also occurred in isolated islets (data not shown).

To study the coupling between metabolism and secretion in α- and β-cells in isolated islets, we used a transcriptional targeting technique based on recombinant adenoviruses (rAds) incorporating promoters specific for the two types of cell[7]. This method is particularly suitable for detecting cell-type-specific events in heterogeneous cellular populations without destroying organ architecture; it is important to preserve this architecture because of the cell-contact-dependent and extracellular communication pathways that exist between the different cells[8,9]. We found, however, that whereas the activity of the insulin I promoter was sufficient, the activity of the glucagon promoter was below the detection limit of our recording system (data not shown).

We therefore used a dual rAd strategy (ref. 10 and Supplementary Information Fig. 1) that incorporated regulator and target rAds: the regulator rAd produces nuclear-localized Cre recombinase (NCre) from cell-type-specific promoters, whereas the target rAd expresses the desired genes from the potent CAG promoter unit[11] after excision of the intervening sequence by NCre.

We constructed two regulator rAds, rAdGluPNCre and rAdInsPNCre, for expressing NCre in α-cells and β-cells, respectively. The target rAds, rAdCAGlxSTlxEGFP (Fig. 2a, b) and rAdCAGlxSTlxLuc, expressed enhanced green fluorescent protein (EGFP) and luciferase, respectively. This approach yielded roughly 5–15-fold higher expression than that achieved by single rAds with cell-type-specific promoters (data not shown).

As is common to almost all exocytotic events, calcium and ATP are required for the secretion of glucagon[12]. We therefore compared the production of ATP in α- and β-cells challenged with glucose and mmSuc (Fig. 2c–f). As expected, islet β-cells responded to glucose and mmSuc with an increase in ATP concentration (+47.2 ± 3.7%, $n = 9$, and +34.1 ± 3.2%, $n = 9$, respectively). α-Cells also showed an increase in ATP concentration in response to mmSuc that was similar to that of β-cells (+38.1 ± 4.0%, $n = 5$). By contrast, glucose increased the ATP concentration in α-cells less efficiently (+14.2 ± 3.2%, $n = 6$) than in β-cells. Unfortunately, we could not measure the change in ATP in response to pyruvate because intracellular acidification by pyruvate uptake affects the luciferase reaction and might mask pyruvate-stimulated ATP generation.

We examined the mitochondrial calcium concentration in α- and

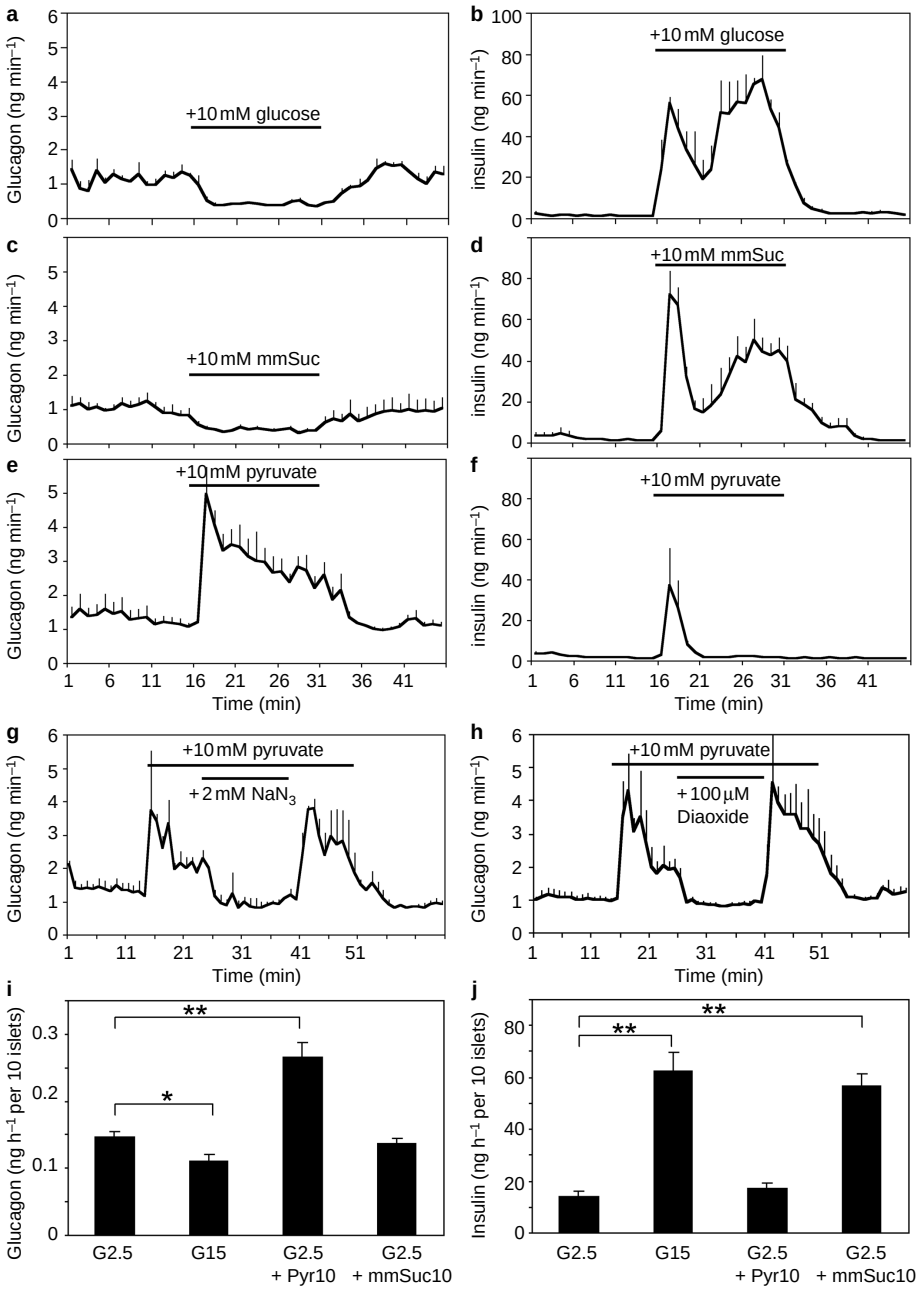

Figure 1 **Nutrient-stimulated hormone release from perfused pancreas and isolated islets. a–f**, Isolated pancreata were perfused with 2.8 mM glucose and then challenged with 10 mM glucose (n = 3), mmSuc (n = 5) or pyruvate (n = 4). **g**, **h**, Isolated pancreata were stimulated with pyruvate and then challenged with 2 mM sodium azide (n = 3) or 100 μM diazoxide (n = 4). **i**, **j**, Isolated islets were pre-incubated with 2.5 mM glucose and then challenged for 60 min with 2.5 mM glucose (n = 20), 15 mM glucose (n = 7), 2.5 mM glucose plus 10 mM pyruvate (n = 13) or 2.5 mM glucose plus 10 mM mmSuc (n = 9). Data are the mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$.

β-cells as an indicator of intracellular calcium homeostasis. The mitochondrial calcium concentration in β-cells increased in response to both glucose (peak 653 ± 104 nM, n = 7) and mmSuc (616 ± 87 nM, n = 5), whereas it barely changed in α-cells after challenge with these nutrients (Fig. 2g–j). By contrast, α-cells responded to pyruvate with an increase in mitochondrial calcium (409 ± 47 nM, n = 5) (Fig. 3e) whereas β-cells did not (248 ± 44 nM, n = 5; Fig. 3c). These data indicate that there are differences in the coupling of ATP generation and calcium homeostasis between α- and β-cells.

On the basis of β-cell physiology, there are two alternative explanation for these differences: either the increase in ATP concentration fails to depolarize the plasma membrane, or ATP-mediated depolarization can not induce calcium influx in α-cells. To examine this issue, we investigated the change in cytosolic calcium concentrations in response to plasma membrane depolarization in the two types of cell. The rise in cytosolic calcium in response to different potassium concentrations was almost identical in α- and β-cells (Fig. 2k, l). The mitochondrial calcium response also showed a similar increase in the two types of cell (Supplementary Information Fig. 2). These data indicate that

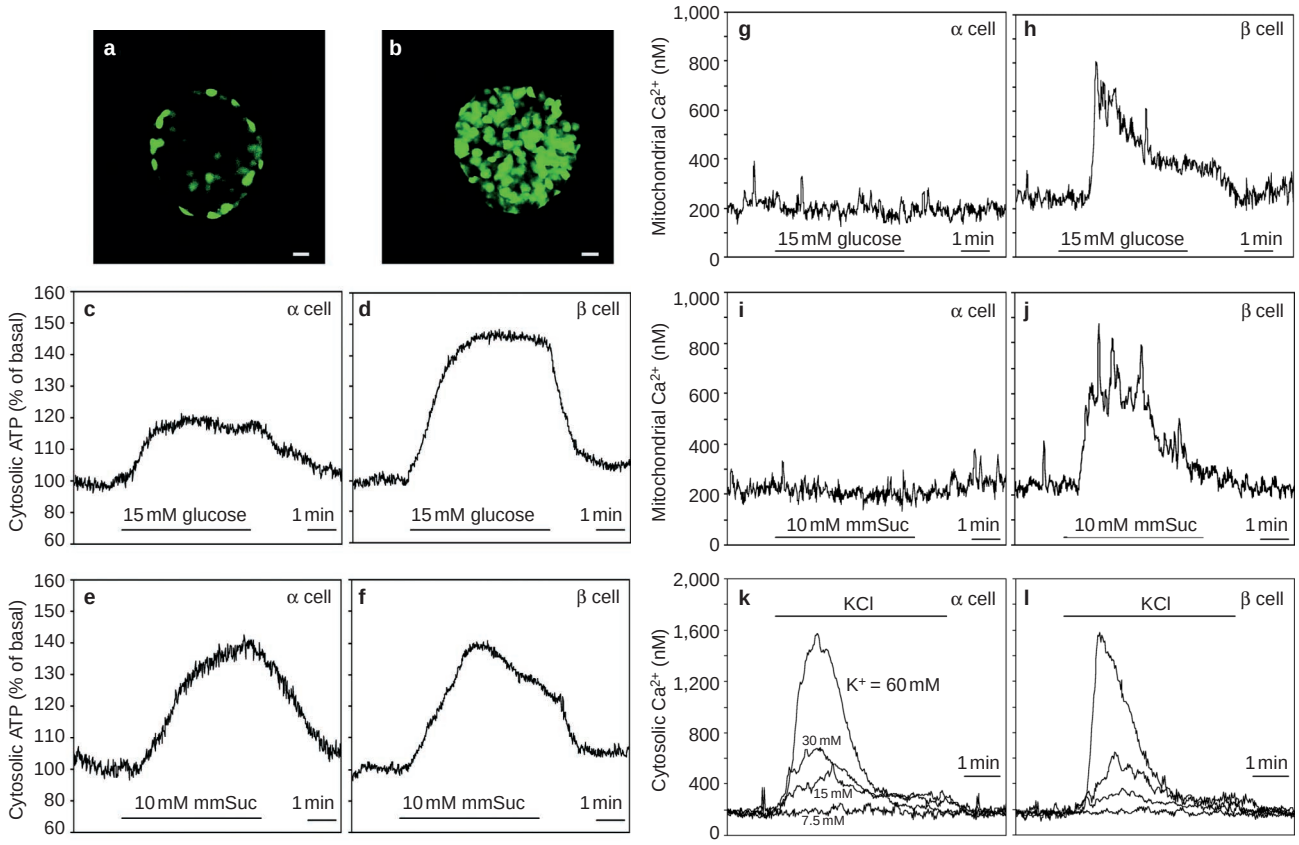

Figure 2 **Different signal transduction in islet α- and β-cells. a**, **b**, Islets infected with rAdCAGlxSTlxEGFP plus either rAdGluPNCre (**a**) or rAdInsPNCre (**b**) show EGFP-expressing cells mainly in the peripheral region or throughout almost the whole islet area, corresponding to the localization of α-cells or β-cells, respectively. Scale bars, 10 μm. **c–f**, Islets infected with rAdCAGlxSTlxLuc plus either rAdGluPNCre (**c**, **e**) or rAdInsPNCre (**d**, **f**) were challenged with 15 mM glucose (**c**, **d**) or 10 mM mmSuc (**e**, **f**). **g–j**, Islets infected with rAdCAGlxSTlxmAQ plus either rAdGluPNCre (**g**, **i**) or rAdInsPNCre (**h**, **j**) were challenged with 15 mM glucose (**g**, **h**) or 10 mM mmSuc (**i**, **j**). **k**, **l**, Islets infected with rAdCAGlxSTlxcAQ plus either rAdGluPNCre (**k**) or rAdInsPNCre (**l**) were challenged with 7.5, 15, 30 or 60 mM KCl.

mmSuc does not depolarize the α-cell plasma membrane, despite the generation of ATP.

How might ATP generation be uncoupled from membrane depolarization in α-cells challenged with mmSuc? As pyruvate but not mmSuc could stimulate glucagon secretion, we considered that the simultaneous activation of β-cells might inhibit membrane depolarization and glucagon secretion in α-cells. Because pyruvate is inefficient in activating normal β-cells owing to a low expression of monocarboxylate transporters (MCTs)[4], we overexpressed MCT-1 in β-cells to examine whether β-cell activity could affect α-cell function. When islets were infected with rAdInsPMCT-1, β-cells responded to pyruvate by increasing their secretion of insulin (Fig. 3b). The imposed alteration in membrane transport in β-cells suppressed the pyruvate-stimulated secretion of glucagon from neighbouring α-cells (Fig. 3a).

To substantiate this finding, we studied the response in mitochondrial calcium in these cells. Pyruvate raised mitochondrial calcium marginally in the β-cells of control islets (Fig. 3c–f). By contrast, the concentration of mitochondrial calcium increased to 620 ± 51 nM, ($n = 4$) after overexpression of MCT-1 in β-cells. Control islets showed a rise in mitochondrial calcium (409 ± 47 nM, $n = 5$) in α-cells in response to pyruvate, but this rise was suppressed (253 ± 31 nM, $n = 5$) by overexpressing MCT-1 in β-cells. These data suggest that simultaneous activation of β-cells by pyruvate suppresses the rise in mitochondrial calcium and glucagon secretion in α-cells.

To explore further the possible influence of the β-cell activity on α-cell function, we eliminated the β-cell population by infecting islets with an rAd (rAdCAGATGlxSTlxΔN-DTA) that would express the diphtheria toxin A (DTA) chain on co-expression of NCre (Supplementary Information Fig. 3). When islets were infected with rAdCAGATGlxSTlxΔN-DTA and rAdInsPNCre (hereafter referred to as Δβ-islets) and cultured for 5 d, the insulin content decreased by 60% (126 ± 17 and 51 ± 13 ng per islet, control and Δβ-islets, respectively, $n = 6$), whereas the glucagon content did not change significantly (2.07 ± 0.34 and 1.95 ± 0.41 ng per islet, $n = 6$). Insulin secretion from Δβ-islets was reduced by about 70% in response to glucose (63.4 ± 3.4 and 20.9 ± 2.3 ng h^{-1} per 10 islets, $n = 6$) or mmSuc (55.8 ± 3.1 to 22.3 ± 2.8 ng h^{-1} per 10 islets, $n = 6$). Notably, mmSuc stimulated the secretion of glucagon in Δβ-islets (Fig. 3g). In addition, glucagon secretion at 15 mM glucose was significantly higher in Δβ-islets than in control islets.

What substances are released from β-cells that could suppress glucagon secretion? Insulin has been proposed to inhibit the release of glucagon[13]. In theory, substances found in insulin granules, such as islet amyloid polypeptide, zinc, ATP and possibly glutamate, could all be candidates for inhibiting glucagon release. In addition, GABA (γ-amino butyric acid), released from distinct vesicles, can inhibit glucagon secretion[14]. It has been also reported that insulin signalling through the phosphoinositide 3-kinase pathway is enhanced by zinc in 3T3-L1 adipocytes[15], and zinc can exert a strong modulatory effect on synaptic function[16]. We therefore tested the effects of zinc on glucagon secretion.

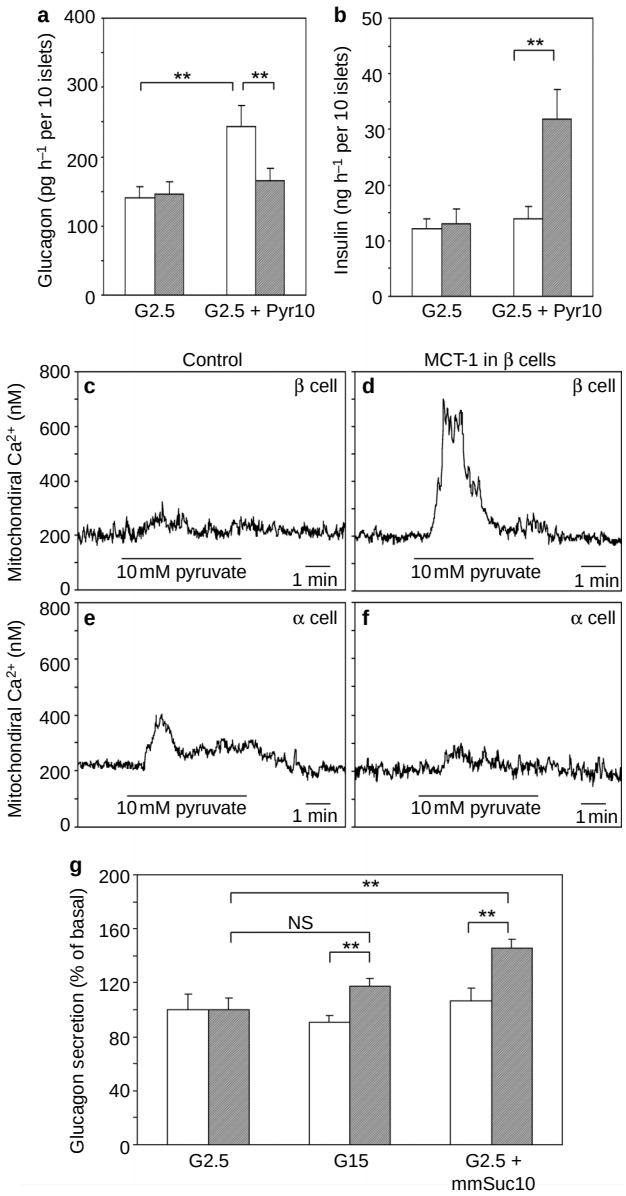

Figure 3 **β-cell activation inhibits the α-cell secretory response. a**, **b**, Islets infected with rAdCAGlacZ (open bars) or rAdInsPMCT-1 (filled bars) were stimulated with 10 mM pyruvate. Data are the mean ± s.e.m. of four experiments. $^{**}P < 0.01$. **c**, **d**, Islets infected with rAdCAGlacZ (**c**) or rAdInsPMCT-1 (**d**) plus rAdCAGlxSTlxmAQ and rAdInsPNCre were challenged with 10 mM pyruvate. **e**, **f**, Islets infected with rAdCAGlacZ (**e**) or rAdInsPMCT-1 (**f**) plus rAdCAGlxSTlxmAQ and rAdGluPNCre were challenged with 10 mM pyruvate. **g**, Islets infected with rAdCAGATGlxSTlxΔN-DTA plus either rAdInsPEGFP (open bars) or rAdInsPNCre (filled bars) were challenged with 2.5 mM glucose, 15 mM glucose or 2.5 mM glucose plus 10 mM mmSuc. Data from six experiments are expressed as the mean percentage of the basal secretion. Values at 2.5 mM glucose were 133 ± 16 and 135 ± 11 pg h^{-1} per ten islets for control and Δβ-islets, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$; NS, not significant.

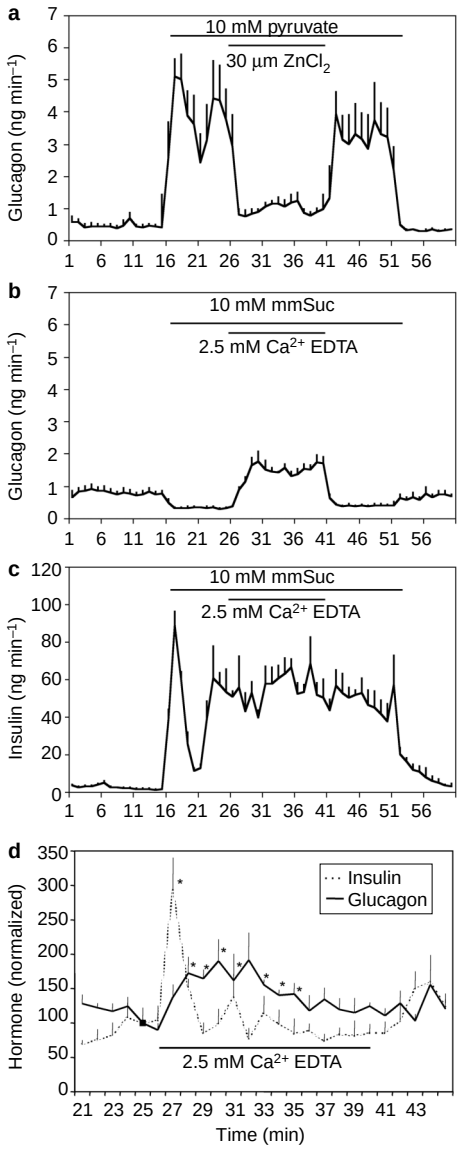

Figure 4 **Effects of zinc on glucagon secretion. a**, Isolated pancreata ($n = 3$) were perfused with 2.8 mM glucose and exposed to 10 mM pyruvate before 30 μM zinc chloride was infused. **b**–**d**, Pancreata ($n = 4$–5) were exposed to 10 mM mmSuc (**b**, **c**) or 12.8 mM glucose (**d**), and then infused with 2.5 mM calcium EDTA. Hormone release is expressed in ng min^{-1} (**b**, **c**) or as a percentage of the glucose-evoked responses (**d**). $^{*}P < 0.05$ versus 25-min timepoint.

We first examined the effects of zinc (30 μM) on pyruvate-stimulated glucagon secretion from perfused rat pancreas (Fig. 4a). Glucagon secretion evoked by pyruvate (48.2 ± 14.2 ng, $n = 3$, from 16 to 25 min) was inhibited by 80% when zinc was added during exposure to the nutrient (10.3 ± 0.9 ng, $n = 3$, from 31 to 40 min). In addition, when the zinc chelator calcium EDTA (2.5 mM) was added during mmSuc infusion, mmSuc was converted from an inhibitory (−31.2 ± 6.7%, $n = 5$, $P < 0.05$, as compared with 2.8 mM glucose) to a stimulatory agent for glucagon secretion (+103.1 ± 12.6%, $n = 5$, $P < 0.01$; Fig. 4b) without affecting insulin output (Fig. 4c). Application of calcium EDTA during glucose challenge also caused a sustained increase in glucagon secretion with only a transient rise in insulin release (Fig. 4d). These results are compatible with the idea that zinc from a β-cell origin has an inhibitory action on glucagon secretion.

Taken together, our data indicate that rat α-cells share common features with β-cells. Both types of cell can transduce a metabolic signal (ATP) into ionic signals (membrane potential and calcium) in the same direction. Our results indicate that it is the simultaneous activation of β-cells that inhibits calcium influx and glucagon secretion in rat α-cells. It is well established that membrane depolarization stimulates glucagon secretion in this

species[17]. In the mouse, different mechanisms have been postulated to regulate glucagon secretion in α-cells. For example, it has been proposed that membrane depolarization owing to the closure of K_{ATP} channels leads to the inhibition of glucagon secretion by causing a voltage-dependent inactivation of ion channels involved in generating action potentials[18]. A reason for this discrepancy might be species differences in the density and composition of ion channels in α-cells: mouse α-cells express roughly 100 times fewer K_{ATP} channels than do rat α-cells[5,19]. It has also been reported that the closure of K_{ATP} channels with sulphonylurea activates the secretory mechanism in rat[19] but not mouse[20] α-cells. In pancreatic β-cells, zinc is concentrated in insulin granules in the form of zinc–insulin crystals with an estimated concentration of about 20 mM (ref. 21). Free zinc will be produced in the islet when the insulin crystals are dissolved on exocytosis of β-cells[22]. The plasma concentration of zinc in the rat is 15 μM, of which 5 μM is free[23]. Here we used 30 μM zinc chloride and 0.25% bovine serum albumin (BSA), yielding a free zinc concentration of about 15 μM, which could be attained during β-cell stimulation. Further work is needed to clarify the precise mechanism by which zinc released from β-cells inhibits glucagon secretion. □

Methods

Construction of recombinant adenoviruses

We constructed the recombinant adenovirus rAdInsPMCT-1 by placing hamster MCT-1 cDNA under the control of the rat insulin I promoter (410 base pairs). The rat glucagon promoter (1.6 kilobase pairs) was used to construct rAdGluPNCre. The *loxP*–STOP–*loxP* cassette from pBS302 (ref. 24; Life Technologies, Basel, Switzerland) was used to construct the rAdCAGlxSTlx series: rAdCAGlxSTlxEGFP (expressing EGFP), rAdCAGlxSTlxLuc (luciferase), rAdCAGlxSTlxcAQ (cytosolic aequorin) and rAdCAGlxSTlxmAQ (mitochondrial aequorin). To construct an expression unit of DTA that would be completely silent under normal conditions but activated by expression of NCre, the stop cassette was modified as follows: a CCACCATGGCA sequence for the initial methionine codon and a Kozak consensus were introduced just before the first *loxP* sequence by polymerase chain reaction. Amplified DNA was ligated to the *loxP*–STOP–*loxP* sequence and then inserted at the *Bam*HI site of DTA[25], resulting in ATGlxSTlxΔN–DTA. The procedures for rAd construction and measuring virus quantities have been described[26]. We purified viruses by caesium chloride equilibrium centrifugation.

Infection of Islets with rAds

Islets were isolated from male Wister rats (300–350 g) by digestion with collagenase (Serva Electrophoresis, Heidelberg, Germany) and hand picking. On the evening of the isolation day, we infected 100–200 islets in 1 ml of RPMI 1640 medium for 1 h at a multiplicity of infection (MOI) of 100–500, as specified below, assuming that one islet contains 3,000 cells[3]. In islets infected with rAd at an MOI of up to 500 in 1 ml of medium, the glucose- or arginine-stimulated secretion of insulin and pyruvate- or arginine-stimulated secretion of glucagon did not differ from that in untreated islets (data not shown). Islets were usually cultured for 2–3 d in RPMI 1640 medium supplemented with 10% fetal calf serum before experiments. For the experiments using rAdCAGATGlxSTlxΔN?DTA, 5 d of culture after infection was needed to obtain sufficient effects.

Isolated pancreas perfusion

Pancreata were isolated from male Wister rats (300–350 g) as described[27]. The basal perfusate was KRBH (135 mM NaCl, 3.6 mM KCl, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 10 mM HEPES; pH 7.4) containing 2.8 mM glucose, 0.25% BSA (fraction V; Sigma, St. Louis, MO) and 4% dextran T-40 (Amersham Pharmacia, Dubendorf, Switzerland). The flow rate was set at 5 ml min^{-1}. We altered the composition of the perfusate through a side-arm syringe at a flow rate of 0.05 ml min^{-1}. The whole apparatus was kept at 37 °C. At the beginning of each experiment, the basal medium was perfused for 20 min to obtain baseline values for hormone secretion. We collected samples of the effluent at 1-min intervals into tubes containing benzamidine to a final concentration of 50 mM. The immunoreactive insulin concentration was determined as described[4] and glucagon concentration was measured using a glucagon radioimmunoassay kit (Linco Research, St Charles, MO).

Hormone release from isolated islets

Islets were either not treated or infected with rAdCAGlacZ, rAdCAGLDH-A or rAdInsPMCT-1 at an MOI of 100. In some experiments, islets were infected with rAdCAGATGlxSTlxΔN–DTA at an MOI of 100 and rAdInsPNCre at an MOI of 300. Cultured islets (ten per tube) were incubated over a period of 60 min at 37 °C in KRBH containing 2.5 mM glucose, 0.4% BSA and the indicated stimulators.

Immunocytochemistry

We infected roughly 100 islets with rAdCAGlxSTlxEGFP at an MOI of 100 together with either rAdInsPNCre or rAdGluPNCre at an MOI of 300. Half of the islets were then treated with trypsin and dispersed cells were seeded on polyornithine-coated glass coverslips. After 3 d, we viewed intact islets directly by confocal microscopy (Zeiss laserscan confocal microscope 410). Dispersed islet cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized for 10 min with 0.2% Triton X-100 in PBS. Nonspecific binding was blocked by treatment with 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA). We incubated the cells with antisera as described[28] and then secondary antibodies against guinea-pig or rabbit IgG conjugated to Texas Red (1:400 dilution; Jackson ImmunoResearch).

Measurement of bioluminescence from isolated islets

Islets were infected with rAdCAGlxSTlxLuc, rAdCAGlxSTlxmAQ or rAdCAGlxSTlxcAQ at an MOI of 100 together with rAdInsPNCre or rAdGluPNCre at an MOI of 300. In some experiments, islets were further infected with rAdCAGlacZ or rAdInsPMCT-1 at an MOI of 100. We placed the infected islets on plastic coverslips coated with extracellular matrix for 3 d. To measure the ATP concentration, we perfused the islets with KRBH containing 2.5 mM glucose, 10 μM beetle luciferin (Promega, Madison, WI) and stimulatory substances at 37 °C and counted the emitted photons with a photomultiplier apparatus (Thorn-EMI Electron Tubes, UK). To measure the cytosolic and mitochondrial calcium concentrations, the infected islets were pre-incubated with 2.5 μM coelenterazine (Calbiochem, SanDiego, CA) for 2–3 h before perfusion with KRBH containing 2.5 mM glucose and stimulators, and photons were counted as above.

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Supplementary Information accompanies the paper on www.nature.com/naturecellbiology
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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.