RESEARCH COMMUNICATION

Mitochondrial priming modifies Ca²⁺ oscillations and insulin secretion in pancreatic islets

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Increases in mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) have recently been reported to cause long-term alterations in cellular ATP production [Jouaville, Bastianutto, Rutter and Rizzuto (1999) Proc. Natl. Acad. Sci. U.S.A. **96**, 13807–13812]. We have determined the importance of this phenomenon for nutrient sensing in pancreatic islets and β-cells by imaging adenovirally expressed Ca^{2+} and ATP sensors (aequorin and firefly luciferase). $[Ca^{2+}]_m$ increases provoked by KCl or tolbutamide evoked an immediate increase in cytosolic and mitochondrial free ATP concentration ($[ATP]_c$ and $[ATP]_m$ respectively) at 3 mM glucose. Subsequent increases in [glucose] (to 16 or 30 mM) then caused a substantially larger increase in $[ATP]_c$ and $[ATP]_m$ than in naïve cells, and pre-stimulation with tolbutamide led to a larger secretory response in response to glucose. Whereas pre-challenge of islets with KCl altered the response to high [glucose] of $[Ca^{2+}]_m$ from periodic oscillations to a sustained elevation, oscillations in $[ATP]_c$ were observed neither in naïve nor in stimulated islets. Hence, long-term potentiation of mitochondrial ATP synthesis is a central element in nutrient recognition by pancreatic islets.

Key words: aequorin, ATP, calcium, luciferase, mitochondria.

INTRODUCTION

Oxidation of glucose-derived metabolites by islet β-cell mitochondria is crucial for the activation of insulin secretion by the sugar [1,2]. Closure of ATP-sensitive K^+ (K_{ATP}) channels [3], increases in cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_c$) and subsequent uptake of Ca^{2+} by mitochondria [4–6] may then activate mitochondrial metabolism [7–11], further enhancing the synthesis of ATP [12].

Recent studies in cells which are not normally responsive to nutrients [13,14] demonstrated that increased intramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) leads to a stable increase in mitochondrial ATP production ('mitochondrial potentiation'). Here we image adenovirally expressed recombinant aequorin [4,15] and firefly luciferase [12,14] to show that this phenomenon is elicited by a transient elevation of $[Ca^{2+}]_m$ in both MIN6 β-cells and intact islets. Mitochondrial potentiation is shown to be important in the dynamics of oscillations in $[Ca^{2+}]_m$ and free ATP concentration in the cytosol ($[ATP]_c$).

EXPERIMENTAL

Isolation and culture of islets and MIN6 cell culture

Islets were isolated from male Wistar rats, and cultured for 16 h as described in [16]. MIN6 cells (passage nos. 24–30) were continuously cultured as described in [16]. In each case, [glucose] was lowered to 3 mM for 16 h before experiments.

Generation of adenoviruses

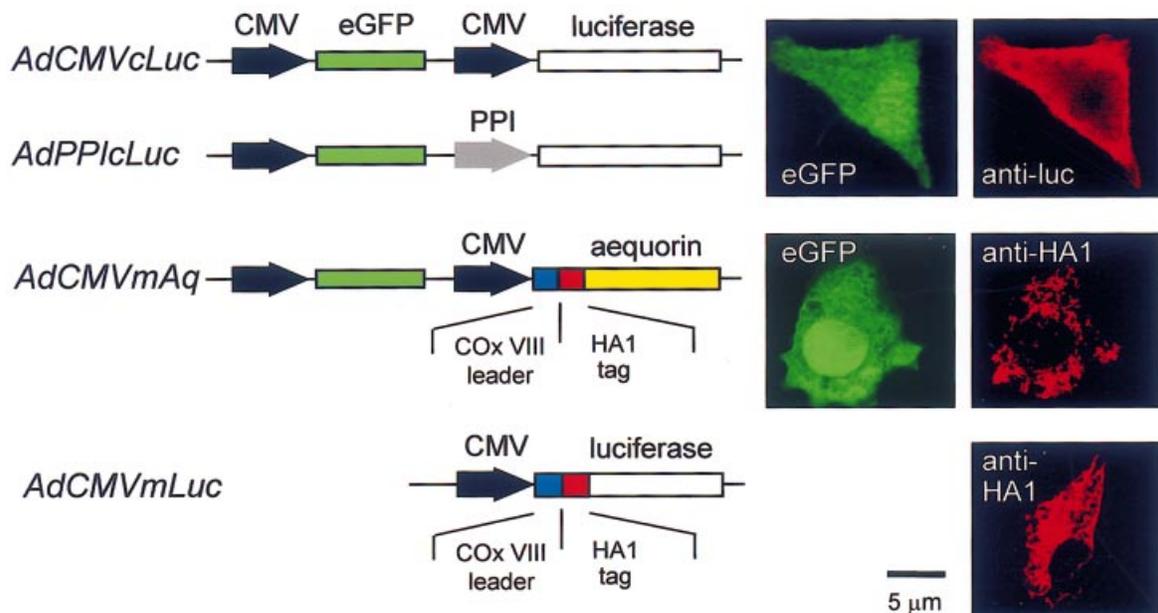Adenoviruses (Figure 1) were constructed and amplified using the pAdEasy system [17] (http://www.coloncancer.org) as de-scribed in [18]. To generate virus encoding cytoplasmic luciferase under cytomegalovirus (CMV) promoter control (*AdCMVcLuc*), plasmid cLuc [12] was digested using *Bgl*II and *Sal*I and the 1.7 kbp fragment containing the CMV promoter, and luciferase cDNA/polyadenylation site was ligated into plasmid pAdTrack. Adenovirus encoding luciferase under the promoter (-260 to $+1$ nt) of the human preproinsulin gene (*AdPPIcLuc*) was similarly generated using a *Pvu*II/*Sal*I fragment [19]. Adenovirus encoding mitochondrial luciferase, under CMV promoter control (*AdCMVmLuc*), was generated using the fragment from plasmid mluc [12] in which the *Nar*I/*Sal*I fragment had been replaced with the *Nar*I/*Sal*I fragment from humanized luciferase cDNA. Mitochondria-targeted luciferase cDNA (*Pvu*I/*Sal*I fragment) was subcloned across the *Pvu*I and *Xho*I sites in the multiple cloning region of pEGFP-N1 (ClonTech). The *Sal*I and *Xba*I fragment was inserted with the correct orientation into plasmid pShuttle-CMV [17]. Adenovirus expressing mitochondrial aequorin (*AdCMVmAq*) was constructed from plasmid mtAEQ [15] by inserting an *Eco*RI fragment into the multiple cloning site of pcDNA 3 (Invitrogen) and a correctly orientated *Kpn*I/*Xho*I fragment was inserted into vector pAdTrackCMV. Adenoviral generation from the recombinant shuttle vectors was performed, and infection of cells and islets was as described in [18].

Immunocytochemistry

Immunocytochemistry was performed as previously described [12]. Cytoplasmic luciferase was revealed using a rabbit polyclonal anti-luciferase primary antibody (Promega) and mitochondrially targeted aequorin and luciferase were stained with a monoclonal mouse anti-(haemagglutinin HA1 tag) primary antibody (Roche). Tetramethylrhodamine-conjugated anti-rabbit and anti-mouse Ig antibodies (Sigma) were used as secondary antibodies. Images were obtained by using a confocal imaging

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Figure 1   Construction and expression of adenoviruses expressing targeted luciferase and aequorin

Schematic representation of the constructs used in adenoviral generation. Images show enhanced green-fluorescent-protein (eGFP) expression and localization of luciferase and aequorin in infected MIN6 cells, revealed by immunocytochemistry. Identical luciferase localization was observed for *AdCMVcLuc* and *AdPPIcLuc*.

spectrophotometer system (TCS-SP) running on a DM/IRBE inverted microscope ($\times 63$ objective) and analysed using Leica TCS software (Leica Microsystems GmbH, Mannheim, Germany).

Microinjection

Microinjection was performed as previously described [9,16]. Calcium Crimson–dextran (Molecular Probes, Eugene, OR, U.S.A.) was microinjected at $0.5\,\mu$M and imaged (568 nm excitation; Kr laser; 580–640 nm emission) after 1 h using an inverted-optics Leica DM/IRBE confocal system [20] in Krebs–Ringer bicarbonate (KRB) medium (37 °C) comprising 125 mM NaCl, 3.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM KH_2PO_4, 2.5 mM $NaHCO_3$, 10 mM sodium Hepes, pH 7.4, containing, initially, 3 mM glucose, and equilibrated with O_2/CO_2 (19:1).

Perifusion of MIN6 cell populations, measurement of luciferase and aequorin luminescence and insulin secretion

Luciferase luminescence was monitored continuously using a photomultiplier tube (ThornEMI Electron Tubes, Ruislip, Middx., U.K.) [15,21], during perifusion in KRB plus $5\,\mu$M luciferin and other additions as given in [14]. Light output was recorded at 1 s intervals and averaged over 5 s intervals to give the traces in Figures 2 and 3. Typically, light output from a coverslip (1 cm²) of virally infected MIN6 cells was 30 000–150 000 counts/s versus a 'dark' value of 10 counts/s. The same system was used to record luminescence in cells transfected (LIPoFECTAMINE™; Promega, Poole, Dorset, U.K.) with cDNAs encoding targeted aequorin [15], reconstituted as described in [4]. Insulin released during perifusion with KRB supplemented with 0.1 % BSA was quantified by RIA (Linco Research™, St Charles, MO, U.S.A.).

Imaging of luciferase and aequorin in islets and single cells

Time-resolved photon-counting imaging of luciferase bioluminescence was performed at 1 mM (static incubation; single cells micro-injected with luciferase cDNA) or $5\,\mu$M luciferin (perifusion; virally transfected islets), with an intensified charge-coupled device camera (Photek, Lewes, East Sussex, U.K.) [12,18]. Islets were immobilized in an in-house-built perifusion cell with 500 μm nylon mesh (Lockertex, Warrington, U.K.). The same system was used to image virally transfected islets expressing recombinant aequorin.

Statistics

Results are expressed as means $\pm$ S.E.M. for the number of separate experiments or cells given. Statistical significance was calculated by Student's *t* test unless stated otherwise.

RESULTS

Mitochondrial potentiation in single MIN6 cells

Elevations in [glucose] from 3 to 30 mM caused a monophasic increase in $[ATP]_c$ in MIN6 cell populations, which decayed slowly upon lowering the glucose concentration (Figure 2a). To determine whether glucose-induced increases in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ may be involved in this stable increase in $[ATP]_c$, we pre-stimulated MIN6 cells with 70 mM KCl (Figure 2b). After return of both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ to prestimulatory levels (within 90 s of removal of the stimulus; Figures 2d and 2e), subsequent increase of glucose to 30 mM resulted in an increase in light output of 37 ± 3 % ($n = 7$) and 21 ± 1 % ($n = 3$) for cytosolic and mitochondrial luciferases respectively (calculated from fitted exponential curves). Naïve cells displayed a significantly smaller increase in light output in response to glucose from both the cytosolic (25 ± 3 %, $n = 7$; $P < 1$ %) and mitochondrial (13 ± 1 %, $n = 3$; $P < 5$ %) luciferases. These luminescence
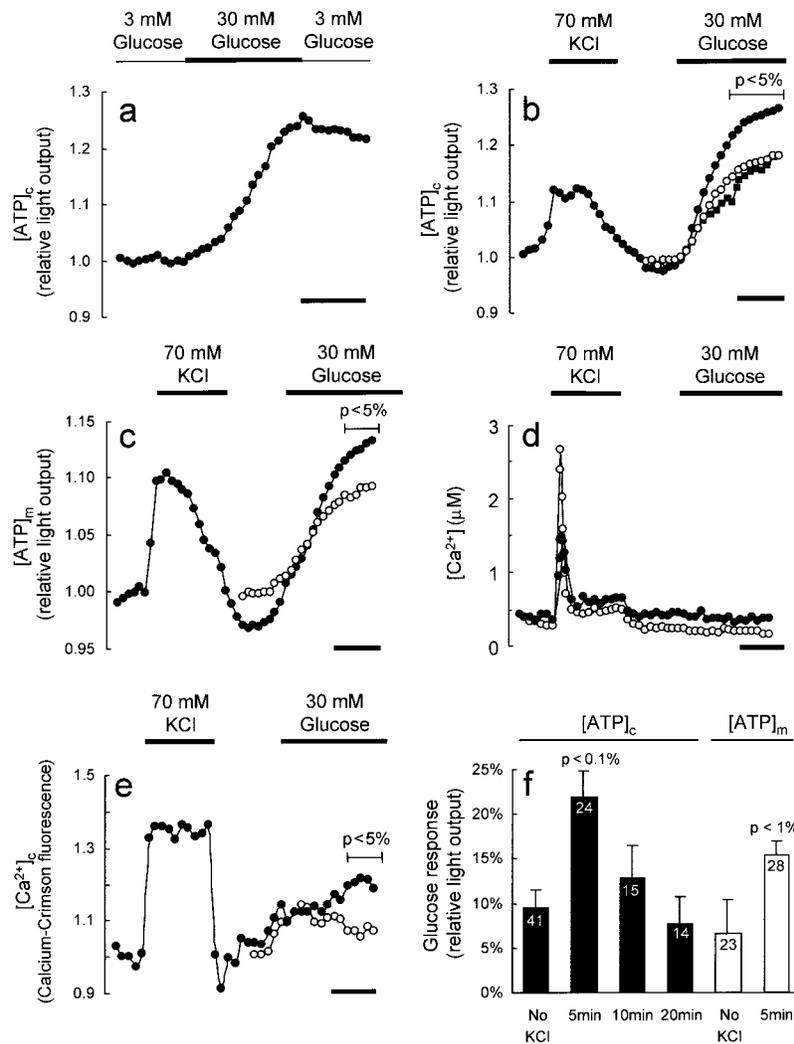
Fig. 2   Changes in ATP synthesis evoked by glucose and imposed increases in [Ca^{2+}]$_m$ in MIN6 cells

(**a**) The response to 30 mM glucose of [ATP]$_c$; (**b, c**) response to 30 mM glucose (added 5 min after lowering [KCl]) of [ATP]$_c$ and [ATP]$_m$ in populations of cells pre-stimulated with 70 mM KCl for 5 min (●) or naïve cells (○). Data are the means for seven independent experiments in each case. ■, Cells pre-incubated in Ca^{2+}-free KRB during KCl stimulation ($n = 2$). (**d**) Corresponding changes in [Ca^{2+}]$_c$ (closed symbols) or [Ca^{2+}]$_m$ (open symbols) in cell populations expressing recombinant aequorin targeted to the cytosol and mitochondria respectively; typical traces from three separate experiments are shown. (**e**) Changes in [Ca^{2+}]$_c$ monitored by Calcium Crimson fluorescence in similarly pre-stimulated (closed symbols) and naïve (open symbols) single cells (mean for 11 cells each, from three independent experiments). (**f**) Apparent [ATP]$_c$ and [ATP]$_m$ increases in single MIN6 cells in response to 30 mM glucose, added at the time indicated after [KCl] normalization (mean + S.E.M. for the number of cells given within bars). The time bar represents 200 s.

increases underestimate the increase in [ATP]$_c$ and mitochondrial free ATP concentration ([ATP]$_m$) by a factor of about 2, at the resting free ATP concentration in each compartment ($\approx$ 1 mM) [12]. The half-time for the exponential increase was unaffected by pre-stimulation ([ATP]$_c$ = 98 ± 27 s, and [ATP]$_c$ = 108 ± 27 s), indicating an increased capacity for ATP production. Quantified in either the cytosol or the mitochondria of individual cells (Figure 2f), the potentiation of the [ATP]$_c$ increase was clearly evident 5 min after normalization of [K$^+$], but then declined to basal levels after 20 min. Furthermore, pre-stimulated cells displayed an elevated [Ca^{2+}]$_c$ compared with naïve cells in response to 30 mM glucose (Figure 2e) when imaged using the sensitive dye Calcium Crimson. No significant response to glucose was seen when using aequorin (Figure 2d), presumably due to the limited sensitivity of this probe at low [Ca^{2+}] [21].

These effects were unrelated to changes in the medium osmolarity, since pre-stimulation with 70 mM KCl in the presence of

EGTA (Figure 2b, ■) resulted in no potentiation of ATP synthesis. Demonstrating that the observed potentiation was not due to differences in insulin secretion (and thus ATP consumption), neither clonidine (an α_2-adrenergic agonist) nor EGTA, which inhibited secretion by > 80 % or > 95 % respectively, altered the effects of prestimulation with KCl (results not shown).

Effect of pre-challenge with tolbutamide on ATP production and insulin secretion

Prestimulation with 200 μM tolbutamide potentiated the subsequent [ATP]$_c$ increase in response to 30 mM glucose (Figure 3a) and also augmented glucose-induced insulin secretion significantly (Figure 3b). Indeed, a lower concentration of tolbutamide (20 μM; Figure 3c), as well as lower [KCl] (15 mM), also led to the potentiation of glucose-induced [ATP]$_c$ increases (Figure 3d).
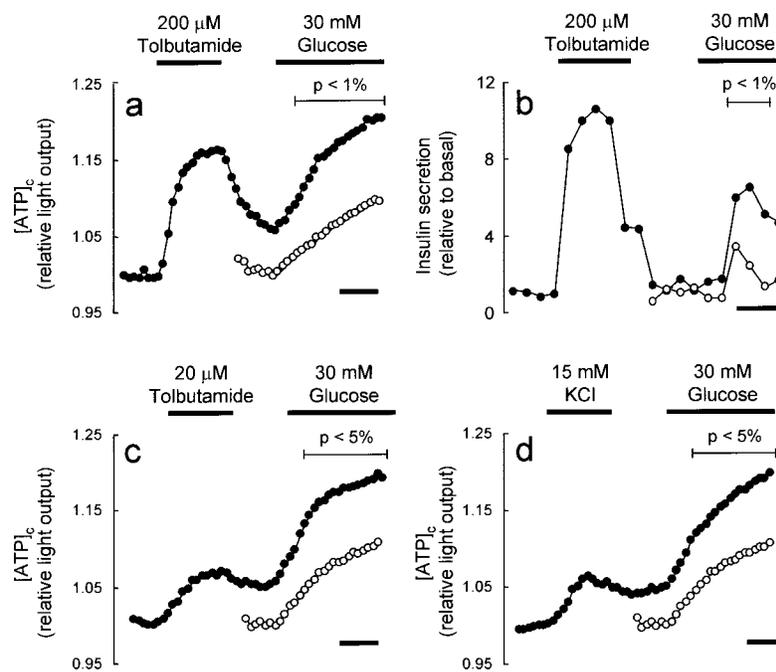
Figure 3   Effects of tolbutamide and low [KCl] on mitochondrial ATP synthesis and insulin secretion in MIN6 cells

[ATP]$_c$ was monitored in cells transduced with *AdCMVcLuc*, exactly as in Figure 2, but with: (**a**, **b**) 200 μM tolbutamide, (**c**) 20 μM tolbutamide or (**d**) 15 mM KCl. In (**b**), secreted insulin was measured in the perifusate as given in the Experimental section. Data are means for three experiments. The time bars represent 200 s.

Potentiation of mitochondrial ATP synthesis in intact islets and impact on [Ca^{2+}]$_m$ oscillations

Luciferase was selectively expressed in the β-cells of intact rat islets using the adenovirus *AdPPIcLuc*. As observed with MIN6 cells, elevation of glucose to 16 mM (optimal for rat islets) caused a monophasic increase in photon production by $6.0 \pm 1.3 \%$ (n = five islets; Figure 4a). Essentially identical data were obtained with *AdCMVcLuc* (results not shown). Stimulation of islets with 35 mM KCl caused a dramatic increase in luminescence ($\approx 40 \%$), which was quickly reversed on removal of K$^+$ (Figure 4a). Subsequent elevation of glucose to 16 mM, 5 min after the withdrawal of high [K$^+$], resulted in a potentiation of the [ATP]$_c$ increase compared with naïve islets, to $16.2 \pm 3.9 \%$ (n = five islets; $P < 5 \%$ compared with naïve islets) above basal light output.

To examine the effect on glucose-induced oscillations in [Ca^{2+}]$_m$, we expressed mitochondrially targeted aequorin via an adenoviral vector (*pAdCMVmAq*) under CMV promoter control. This virus seems likely to report changes largely in β-cells, given the similarity of the results with *pAdCMVcLuc* and *pAdPPIcLuc* viruses (see above). At 3 mM glucose, light output from individual islets was extremely low (Figures 4b and 4c), and calibrated resting [Ca^{2+}]$_m$ was ≈ 200 nM. Increasing [glucose] to 16 mM caused the onset of baseline oscillations (Figure 4b). In five out of six islets from two preparations, glucose caused an initial spike in [Ca^{2+}]$_m$ (to 900 ± 70 nM), followed by further spikes, with a period of 1–2 min. Challenge of islets, maintained at 3 mM glucose, with 35 mM KCl, caused an immediate and large increase in [Ca^{2+}]$_m$ to ≈ 800 nM, followed by a plateau at ≈ 500 nM (Figure 4c). After normalization of [KCl], subsequent challenge with 16 mM glucose now provoked a small, but sustained, increase in [Ca^{2+}]$_m$ (to ≈ 500 nM), but did not cause

oscillations in [Ca^{2+}]$_m$ in any of the five islets imaged (three preparations; $P < 1 \%$, χ^2 test).

DISCUSSION

We show here that imposition of [Ca^{2+}]$_m$ increases enhances the metabolic and secretory response of β-cells to subsequent challenge with a fuel secretagogue. This behaviour is closely similar to that which we recently described in a variety of non-nutrient-sensitive cell types [14]. Indeed, increases in [Ca^{2+}]$_m$ appear to act as a particularly potent regulator of ATP production, as they are able to provoke a greater increase in [ATP]$_c$ than provision of glucose alone to islets (Figure 4a). In addition, it now seems likely that mitochondrial potentiation may contribute to the well-known effects of elevated glucose concentrations [22,23], or phospholipase C-linked agonists [24], to potentiate the subsequent secretory responses of islets to glucose.

Mitochondrial potentiation is likely to have important roles in the response of islets to glucose. First, the phenomenon may well play a role in the cephalic phase of insulin secretion, which occurs after ingestion of food, but prior to increases in blood glucose concentrations, and may contribute to the potentiation of insulin secretion by sulphonylureas (Figures 3a–3c). Secondly, the induction of memory is likely to play a part in orchestrating the pattern and dynamics of oscillations in [Ca^{2+}]$_m$ (Figures 4b and 4c) [25] and secretion [26]. Since these oscillatory patterns are diminished in Type 2 diabetes [27], alterations in the ability of mitochondria to acquire a memory of prestimulation may impact on insulin release. Potentiation led to a change in the response to glucose of [Ca^{2+}]$_m$ in islets from transient elevation to a more sustained increase. One possible explanation is that, under these conditions, the [Ca^{2+}]$_c$ oscillations may occur at too high a rate to elicit parallel oscillation within mitochondria.
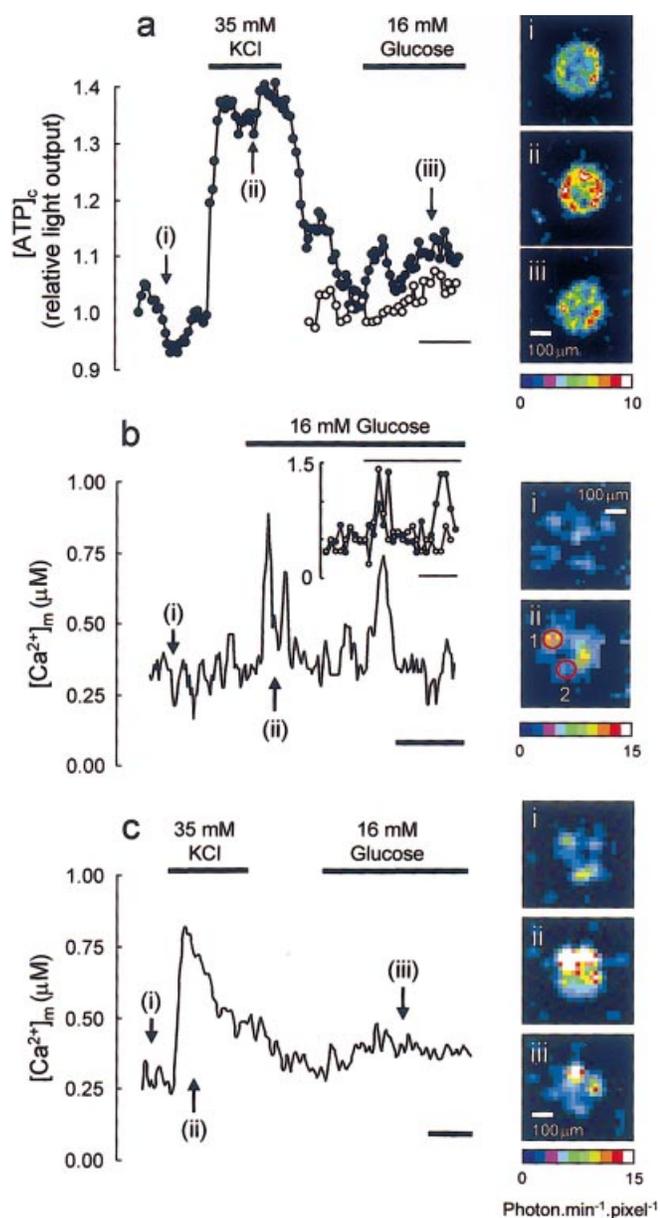
Figure 4   Imaging changes in free [ATP]$_c$ and [Ca^{2+}]$_m$ in single intact islets

(**a**) A rat islet infected with *pAdPPlcLuc* was trapped and perifused as described in the Experimental section. Changes in [ATP]$_c$ upon pre-stimulation with 35 mM KCl (closed symbols) for 5 min, and 16 mM glucose (5 min post-KCl removal) compared with the response to 16 mM glucose of a single paired (i.e. from same preparation and identically infected) naïve islet (open symbols). (**b**) Effect of 16 mM glucose on [Ca^{2+}]$_m$ in islets infected with *AdCMVmAq*; inset, the response of the two subregions, '1' (closed symbols) and '2' (open symbols), outlined. (**c**) Impact of pre-stimulation with 35 mM KCl on oscillations in [Ca^{2+}]$_m$. In each case images are pseudocolour representations of light output from the islet (100 s integration of photon release) stimulated at the time points indicated on the main traces, and are representative traces from five individual islets from three separate preparations. The time bars represent 200 s.

Interestingly, this is analogous to the impact on metabolic oscillations of increasing agonist concentrations in isolated hepatocytes [28].

Relationship between oscillations in intracellular [Ca^{2+}] and [ATP]

Many studies have demonstrated the existence of glucose-induced oscillations in cytosolic [Ca^{2+}]$_c$ in the islet [28–30]. We dem-

onstrate here that oscillations in mitochondrial [Ca^{2+}] also occur in response to elevations in glucose, presumably tracking oscillations in [Ca^{2+}]$_c$. [Ca^{2+}]$_m$ oscillations displayed similar periodicity to typical 'slow-wave' [Ca^{2+}]$_c$ oscillations [28], but were of slightly higher amplitude (typical peak [Ca^{2+}]$_c$ values $\approx$ 300 nM) [31]. Spatial heterogeneity of [Ca^{2+}]$_m$ oscillations was also observed within islets (Figure 4b, inset). Indeed, regions corresponding to 20–50 cells appeared to show an enhanced response, suggesting that these cells could have a 'pacemaker' role.

In contrast with [Ca^{2+}]$_m$, neither [ATP]$_c$ nor [ATP]$_m$ oscillated in response to glucose, when imaged throughout the islet (Figure 4a) or in islet subregions (results not shown). This result was surprising, given reports of metabolic oscillations as measured via oscillations in both O$_2$ and glucose consumption throughout the islet [30]. However, the present observations are consistent with the absence of changes in K$_{ATP}$ channel current between the plateau phase and the inter-burst interval in mouse islets [32] and in islets from mice with defective K$_{ATP}$ channel function [33], as well as with the failure to observe oscillations in mitochondrial NAD(P)H in intact islets [34,35]. One possible explanation is that the time-dependent potentiation of mitochondrial function renders ATP synthesis partially independent of Ca^{2+}.

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