Glucose-Stimulated Oscillations in Free Cytosolic ATP Concentration Imaged in Single Islet β-Cells

Evidence for a Ca^{2+}-Dependent Mechanism

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Normal glucose-stimulated insulin secretion is pulsatile, but the molecular mechanisms underlying this pulsatility are poorly understood. Oscillations in the intracellular free [ATP]/[ADP] ratio represent one possible mechanism because they would be expected to cause fluctuations in ATP-sensitive K^+ channel activity and hence oscillatory Ca^{2+} influx. After imaging recombinant firefly luciferase, expressed via an adenoviral vector in single human or mouse islet β-cells, we report here that cytosolic free ATP concentrations oscillate and that these oscillations are affected by glucose. In human β-cells, oscillations were observed at both 3 and 15 mmol/l glucose, but the oscillations were of a longer wavelength at the higher glucose concentration (167 vs. 66 s). Mouse β-cells displayed oscillations in both cytosolic free [Ca^{2+}] and [ATP] only at elevated glucose concentrations, both with a period of 120 s. To explore the causal relationship between [Ca^{2+}] and [ATP] oscillations, the regulation of each was further investigated in populations of MIN6 β-cells. Incubation in Ca^{2+}-free medium lowered cytosolic [Ca^{2+}] but increased [ATP] in MIN6 cells at both 3 and 30 mmol/l glucose. Removal of external Ca^{2+} increased [ATP], possibly by decreasing ATP consumption by endoplasmic reticulum Ca^{2+}-ATPases. These results allow a model to be constructed of the β-cell metabolic oscillator that drives nutrient-induced insulin secretion. *Diabetes* 51 (Suppl. 1): S162–S170, 2002

Pancreatic islets and β-cells display oscillations in insulin secretion (1,2) and components of the glucose-sensing mechanism (3–8). These oscillations appear to be important for the normal regulation of insulin secretion and may be disrupted in type 2 diabetes (9,10). However, the molecular nature of the underlying "oscillator" is poorly understood.

Nutrient-induced increases in the ratio of cytosolic free ATP concentration ($[ATP]_{cyt}$)/[ADP] (11) are likely to stimulate the release of insulin by inhibition of ATP-sensitive K^+ (K_{ATP}) channels and activation of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (12) or by a more direct mechanism (13,14). It has thus been proposed that metabolic oscillations, driven by the intrinsic pulsatility of the glycolytic pathway (15), may lead to oscillations in $[ATP]_{cyt}$ and hence pulsatile insulin release. However, another likely influence on $[ATP]_{cyt}$ in the β-cell are the increases in intramitochondrial [Ca^{2+}] after elevations of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (16–18). Such increases are likely to lead to the activation of citrate cycle dehydrogenases (19,20) and the stimulation of ATP synthesis (21–23), potentially leading to a feed-forward loop. By contrast, larger increases in mitochondrial [Ca^{2+}] may inhibit oxidative metabolism (24,25) under some circumstances by opening the mitochondrial permeability transition pore (26). Furthermore, the fact that many ATP-consuming reactions, such as the exocytosis of insulin, plasma membrane Ca^{2+}-ATPase, and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) activities, may be sensitive to $[Ca^{2+}]_{cyt}$ means that large increases in $[Ca^{2+}]_{cyt}$ may exert a negative effect on $[ATP]_{cyt}$.

Using adenovirally driven expression of recombinant firefly luciferase (27), we have now monitored $[ATP]_{cyt}$ in real time in isolated human and mouse pancreatic β-cells. $[ATP]_{cyt}$ was found to oscillate with a frequency similar to that of $[Ca^{2+}]_{cyt}$ oscillations (2,8,28) in both cell types. We have also investigated *1*) the possibility that a Ca^{2+}-dependent mechanism drives the oscillations in $[ATP]_{cyt}$ and *2*) the contribution of Ca^{2+}-sensitive ATP production (by mitochondrial activation) and consumption (by SERCA [29]) to changes in $[ATP]_{cyt}$. Taken together, the results lead to a possible model to explain glucose-induced oscillations in $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$.

RESEARCH DESIGN AND METHODS

Islet and β-cell isolation. Islets of Langerhans were isolated from 12- to 16-week-old male CD1 strain mice using collagenase digestion (30). Briefly, the pancreases were injected in situ via the bile duct with a solution of 0.5 mg/ml collagenase (Serva, Heidelberg, Germany) in serum-free Dulbecco's modified Eagle's medium (DMEM) (Sigma, Poole, Dorset, U.K.). The pancreases were removed, finely chopped, and further digested in the above collagenase solution for 20 min at 37°C. The pancreases were transferred to DMEM containing 10% fetal bovine serum to stop the digestion and then vigorously shaken and filtered through a nylon mesh with an approximate pore size of 500 μm. The samples were washed three times in serum-containing (10% vol/vol) medium before hand-picking of islets. Islets were incubated overnight in DMEM containing 10% (vol/vol) fetal bovine serum and 11 mmol/l glucose before dissociation into individual cells.

Islets were dissociated by incubation in 25 mg/ml trypsin (Sigma) for 20 min at 37°C. After pelleting by brief centrifugation (1,000*g* for 3 min), the cells were resuspended in serum-containing medium and spotted onto glass coverslips that had been precoated with 0.35 mg/ml Cell-Tak cell adhesion

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$[ATP]_{cyt}$, cytosolic free ATP concentration; $[Ca^{2+}]_{cyt}$, cytosolic free Ca^{2+} concentration; CPA, cyclopiazonic acid. DFT, discrete Fourier transformation; DMEM, Dulbecco's modified Eagle's medium; K_{ATP} channel, ATP-sensitive K^+ channel; KRB, Krebs Ringer bicarbonate; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

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protein (Collaborative Biomedical Products, Bedford, MA) in 50 μmol/l $NaHCO_3$. The cells were maintained in culture medium (11 mmol/l glucose) for 1–2 days before incubating in medium containing 3 mmol/l glucose 12–16 h before use.

Human islets were obtained from Dr. Roger F.L. James (University of Leicester, Leicester, U.K.) and were dissociated after overnight incubation as described above. The islets were then fixed to plastic Petri dishes as opposed to glass coverslips. Dissociated islet cells were infected with adenovirus and treated before experimentation as described for mouse cells.

Cell culture. MIN6 cells (31) were cultured essentially as described (27) and were used between passage 25 and 32. Briefly, cells were routinely cultured in DMEM containing 25 mmol/l glucose and 15% fetal bovine serum. For experimental use, cells were grown on poly-L-lysine–treated glass coverslips and incubated in medium containing 3 mmol/l glucose overnight before measurements.

Single-cell imaging of [ATP] with expressed recombinant luciferase. Luciferase expression was achieved by infection of cells with an adenoviral vector encoding cytoplasmic (*AdCMVcLuc*)-targeted luciferase under the control of the cytomegalovirus major-late promoter (18). Infection was carried out by incubation for 4 h with a multiplicity of infection of 100 viral particles per cell for isolated β-cells and 30 particles per cell for MIN6 cells.

Time-resolved photon-counting imaging of individual cells expressing cytoplasmic or mitochondrial luciferases was performed during static incubation in Krebs-Ringer bicarbonate (KRB) medium. The medium comprised 125 mmol/l NaCl, 3.5 mmol/l KCl, 1.5 mmol/l $CaCl_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l KH_2 PO_4, 2.5 mmol/l $NaHCO_3$, and 10 mmol/l Hepes-Na^+, pH 7.4, containing initially 3 mmol/l glucose and equilibrated with 95:5 O_2/CO_2 and supplemented with 1 mmol/l luciferin on the thermostated (37°C) stage of an Olympus IX70 inverted microscope (×10 objective, 0.4 NA) linked to a three-stage intensified charge-coupled device camera (Photek; Lewes, East Sussex, U.K.) as previously described (27,32).

For perifusion of MIN6 cell populations, luciferase luminescence was monitored continuously using a photomultiplier tube (ThornEMI Electron Tubes, Ruislip, U.K.) and perifusion system (33) in KRB medium (37°C) plus 5 μmol/l luciferin and other additions as given (18). Bioluminescence was recorded at 1-s intervals and averaged over 5-s intervals. Light output was normalized to that observed under basal conditions (i.e., 3 mmol/l glucose) to give the traces shown in Figs. 2–4. Where EGTA (0.1 mmol/l) was added, $CaCl_2$ was omitted from KRB.

Measurement of $[Ca^{2+}]_{cyt}$. Changes in $[Ca^{2+}]_{cyt}$ were measured dynamically at 37°C with entrapped fura-2 (16,32) using a Leica DM-IRBE inverted optics epifluorescence microscope (×40 objective) and a Hamamatsu C4742-995 charge-coupled device camera driven by OpenLab (Improvision, Coventry, U.K.) software. Primary β-cells were loaded by incubation for 20 min in KRB medium supplemented with 2 μmol/l fura-2-AM (Sigma) and 0.05% Pluronic F-127 (BASF, Mount Olive, NJ). MIN6 cells were loaded similarly by incubation for 40 min with 5 μmol/l fura-2-AM and 0.05% Pluronic F-127. Changes in $[Ca^{2+}]_{cyt}$ are reported as changes in the ratio of the fluorescence emission (510 nm) during excitation with light at 340 or 380 nm (32).

Oscillator analysis/statistical analysis. Three separate statistical tests were performed on the data from the single-cell luciferase light output and fura-2 fluorescence assays to identify oscillatory behavior in $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$, respectively. Discrete Fourier transformation (DFT) was performed on the data to highlight the principal resonant frequencies present and the approximate amplitude of the oscillations at those frequencies. Mathematical analysis was carried out with a Microsoft Visual Basic script written in-house. Calculations were based on those described by Briggs and Henson (34). The presence of oscillations at a specific frequency was implicated by a high value for the amplitude of the Fourier transform at that frequency.

Autocorrelation of data were performed as described (35). The Pearson product-moment correlation coefficient was determined for the data set paired with itself but was offset by a specific time period. If the data displayed oscillations, then it was expected that a positive correlation would be found when the offset time period was equal to the wavelength of oscillation. Conversely, a negative correlation would be expected when the data were offset by half the wavelength of oscillation. The significance of the correlation coefficient was tested using an *F* test (36).

The fit of the data to a sine curve was assessed by nonlinear least-squares regression analysis. The amplitude, frequency, and phase were fitted by minimizing the sum of squares of the residuals using the "Solver" Add-in within Microsoft Excel, and the mean value of the sine curve was determined as the mean of the data set. The significance of the fit of the sine curve was determined from an *F* test comparing the residual sum of squares from the curve-fit to the total sum of squares. In effect, this tested the significance of the improvement of fit for the sine curve compared with a single mean value for the data set.

Data and images were generated from a 20-s integration of the light output. This did not enable reliable detection of oscillations of periods <60 s. Reanalysis of the data from individual cells with a 10-s integration window gave better temporal resolution, yet in most cases, gave essentially the same best-fit sine curve. However, the significance of fit was reduced in this case because of increased noise.

The significance of a difference between two data sets was assessed using a *t* test. Data are given as means ± SE. All calculations and statistical tests were carried out in Microsoft Excel 1997 running under Microsoft Windows NT, version 4.0.

RESULTS

Evidence for $[ATP]_{cyt}$ oscillations in primary β-cells. The existence of oscillations in $[ATP]_{cyt}$ in pancreatic β-cells was investigated using primary isolated β-cells expressing recombinant firefly luciferase (27,37). An adenoviral vector (18) was used here to introduce luciferase cDNA into the primary cells because this achieved both efficient transformation and a high level of luciferase expression in individual cells. Whereas we did not attempt to correct for the presence of non–β-cells in these cultures, β-cells could usually be identified by their morphology (being somewhat larger than the majority of non–β-cells).

The response of a typical single human islet β-cell to a stepped increase in glucose concentration is shown in Fig. 1*A*. An increase in light output occurred upon incubation in high (15 mmol/l) glucose concentrations compared with incubation in low (3 mmol/l) glucose concentrations, equivalent to 9.0% of the average light output under basal conditions for the cell in Fig. 1*A*. $[ATP]_{cyt}$ was observed to oscillate in human β-cells at both high and low glucose concentrations, albeit around a raised average point at 15 mmol/l glucose (Fig. 1*A*). Images of the light output from the cell at the peak and trough of these oscillations at 3 and 15 mmol/l glucose are shown in Fig. 1*B*.

To confirm that the apparent oscillations in luciferase luminescence reported genuine oscillations in $[ATP]_{cyt}$, three different statistical tests were performed. This was considered important, given the intrinsically low signal-to-noise ratio entailed by the low output of photon events (typically 3–10 photons $\cdot$ s^{-1} $\cdot$ $cell^{-1}$) from the luciferase enzyme expressed in a single cell (27). DFT of the data (34) was carried out first to highlight the likely frequency with which $[ATP]_{cyt}$ may be oscillating. The results of this analysis of the data in Fig. 1*A* are shown in Fig. 1*C*. From comparison of the amplitudes of the DFT over the frequency range, this analysis suggested that $[ATP]_{cyt}$ displayed oscillations of an approximate period of 67 s (from the amplitude peak at a frequency of 15 mHz) at 3 mmol/l glucose and 133 s (7.5 mHz) at 15 mmol/l glucose, both with an amplitude of ~9%.

Autocorrelation was used as a second indication of the approximate wavelength of oscillations (see RESEARCH DESIGN AND METHODS) (35). Autocorrelation of the data (Fig. 1*D*) obtained at 3 mmol/l glucose demonstrated a significant negative correlation when the data were offset by either 40 or 120 s ($P < 5\%$; *F* test) and a positive correlation when the data were offset by 80 s (consistent with oscillation of a wavelength of ~80 s [12.5 mHz]). Similar analysis of the data during incubation of this cell at 15 mmol/l glucose showed a significant negative correlation when offset by 60 s ($P < 1\%$; *F* test) and a positive

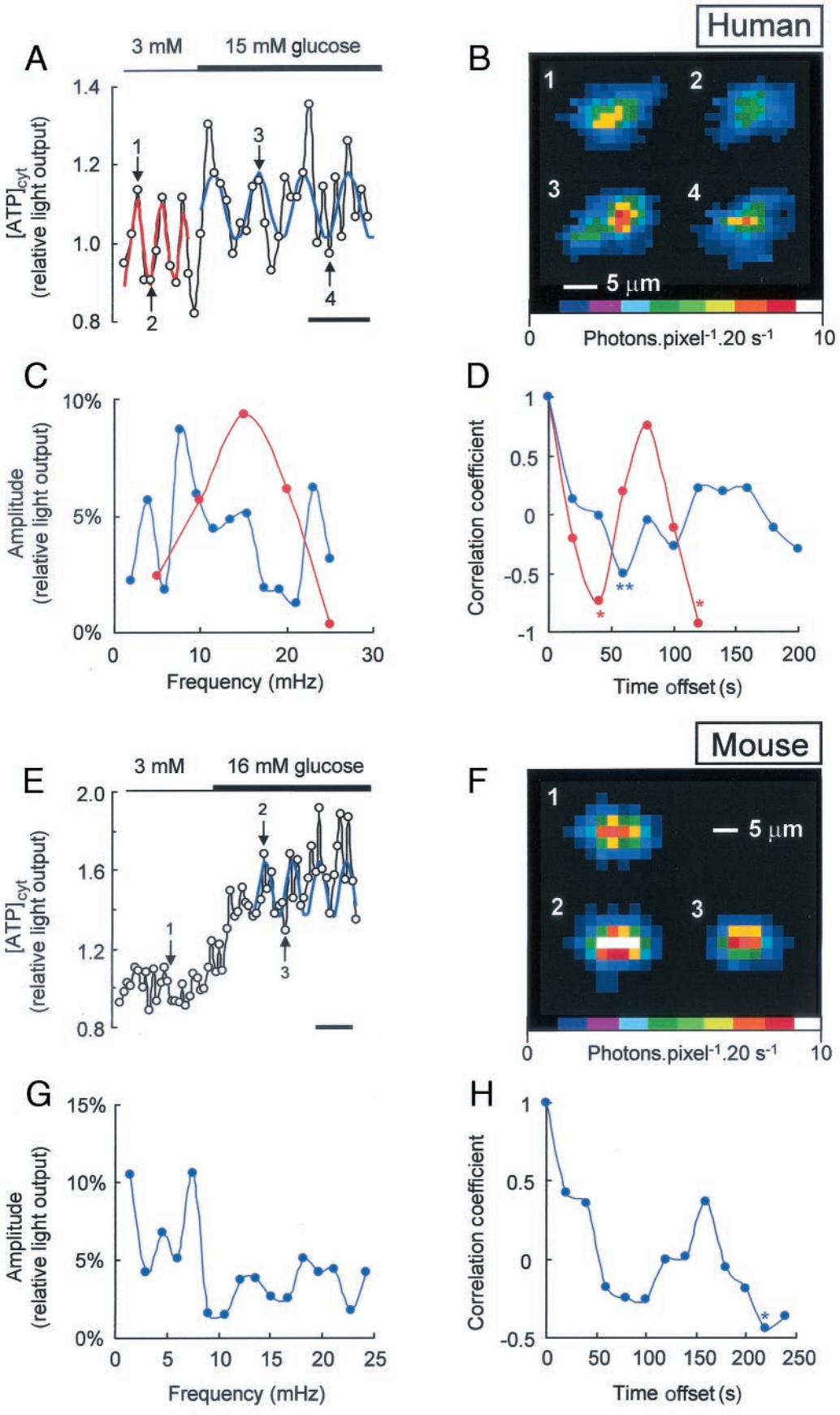

FIG. 1. Oscillations in $[ATP]_{cyt}$ in human and mouse pancreatic β-cells. Dynamic oscillations in $[ATP]_{cyt}$ were monitored in primary β-cells. *A*: Response of a single human β-cell during incubation at 3 and 15 mmol/l glucose. The red and blue traces show the least squares–fitted sine curve to the data obtained at 3 and 15 mmol/l glucose, respectively (see RESULTS). *B*: Pseudo-color images of the same cell during oscillations in $[ATP]_{cyt}$ at the time points indicated in *A*. DFT (*C*) and autocorrelation (*D*) of the light output data during incubation with 3 mmol/l glucose (red) and 15 mmol/l glucose

correlation when offset by ~140 s (consistent with oscillation of a wavelength of ~140 s [7.1 mHz]).

Using the information gained from DFT and autocorrelation, it was possible to refine the fit of a sine curve to the data set using nonlinear least-squares regression analysis (see RESEARCH DESIGN AND METHODS). The refined sine curves, fitted to the data obtained during incubation at 3 or 15 mmol/l glucose in Fig. 1*A*, are shown as bold (blue or red) traces. Both fitted sine curves showed a statistical improvement of fit compared with a horizontal line that was equal to the mean of the data ($P = 1.5\%$ for both traces, *F* test). For the data obtained during incubation in 3 mmol/l glucose, the fitted sine curve had a wavelength of 78 s (12.8 mHz) and amplitude of 11%. The corresponding data during incubation with 15 mmol/l glucose had a wavelength of 141 s (7.1 mHz) and amplitude of 9%. Thus, it can be seen that these parameters are fully consistent with both the DFT and autocorrelation analyses (above) and confirm the presence of oscillations in $[ATP]_{cyt}$.

Using the above analyses, significant $[ATP]_{cyt}$ oscillations (i.e., $P < 5\%$ for fitted sine curve) were observed in 4 of 20 human β-cells during incubation at 3 mmol/l glucose and 9 (of 20) cells during incubation at 15 mmol/l glucose (separate experiments on islet cells from two different donors). The mean period of $[ATP]_{cyt}$ oscillation showed a significant increase from 66 ± 6 to 167 ± 32 s upon elevation of glucose ($P < 5\%$, unpaired *t* test). However, the amplitude of oscillations was unchanged from 13 ± 2 to 11 ± 1% at low and high glucose, respectively.

Oscillations in free $[ATP]_{cyt}$ were also seen in primary β-cells isolated from mouse islets. In contrast to human islet cells, oscillations were not observed at 3 mmol/l glucose, i.e., only one cell displayed significant oscillatory behavior ($P < 5\%$) of 27 cells analyzed (five separate experiments). During incubation at 16 mmol/l glucose, however, 9 of 27 cells gave significant oscillations. The normalized light output during incubation at 3 and 16 mmol/l glucose for a typical cell is given in Fig. 1*E*, and pseudo-color images of the cell at low glucose and during an oscillation are given in Fig. 1*F*. As with human (above) and rat (27) islet β-cells, the elevation of glucose concentration caused a clear increase in the average light output from mouse cells and hence in $[ATP]_{cyt}$. DFT and autocorrelation analyses of the data obtained at 16 mmol/l glucose are given in Fig. 1*G* and *H*, respectively. Least-squares fitting of a sine curve to the data gave a wavelength of 147 s (6.8 mHz) and amplitude of 15% (bold trace, Fig. 1*E*; $P = 0.1\%$, *F* test). The average wavelength of the seven cells displaying significant oscillations was 112 ± 9 s, and the amplitude was 14 ± 1%. Oscillations of similar frequency were observed in cells expressing mitochondrially targeted luciferase (27) expressed via an adenoviral vector (18) (not shown).

$[Ca^{2+}]_{cyt}$ oscillations in primary mouse β-cells. We then explored the temporal relationship between oscillations in $[ATP]_{cyt}$ and oscillations in free $[Ca^{2+}]_{cyt}$ to assess whether the two may be causally linked. In these experiments, the response to glucose of $[Ca^{2+}]_{cyt}$ was imaged in single β-cells using the ratiometric dye fura-2. Similar to the oscillations in $[ATP]_{cyt}$, oscillations in $[Ca^{2+}]_{cyt}$ were observed in 6 of 22 cells analyzed at 16 mmol/l glucose (eight separate experiments), whereas no significant $[Ca^{2+}]_{cyt}$ oscillations were seen during incubation at 3 mmol/l glucose. The mean period of the oscillations observed in $[Ca^{2+}]_{cyt}$ was 113 ± 17 s—the same period as that typically seen for oscillations in $[ATP]_{cyt}$ (Fig. 1*B*). However, the oscillations in $[Ca^{2+}]_{cyt}$ appeared with a delay of 217 ± 29 s after elevation of glucose (assessed as the time to the beginning of the first down-slope of the oscillation). In contrast, the time of onset for oscillations in $[ATP]_{cyt}$ was significantly shorter than that of the first $[Ca^{2+}]_{cyt}$ oscillations, at 136 ± 17 s ($P < 5\%$; two-tailed unpaired *t* test).

Relationship between intracellular free $[Ca^{2+}]_{cyt}$ and $[ATP]_{cyt}$. The next series of experiments investigated the control that $[Ca^{2+}]_{cyt}$ exerted over free $[ATP]_{cyt}$ and examined whether oscillations in $[Ca^{2+}]_{cyt}$ could form part of a feed-forward/feedback mechanism that could contribute to oscillations in $[ATP]_{cyt}$ (8,38). To achieve improved temporal resolution and sensitivity compared with that possible when imaging single primary β-cells, the response of $[ATP]_{cyt}$ to various regulators of $[Ca^{2+}]_{cyt}$ was measured in populations of the murine β-cell line MIN6. In this approach, MIN6 cells (~300,000 per experiment) were infected with *AdCMVcLuc* and perifused with luciferin (5 μmol/l) to enable changes in light output (and hence $[ATP]_{cyt}$ or mitochondrial [ATP]) to be detected at a level of <1% (18).

Increasing the glucose concentration from 3 to 30 mmol/l caused a time-dependent increase in intracellular free $[Ca^{2+}]_{cyt}$, measured in single fura-2–loaded MIN6 cells (Fig. 2*A* and *E*). In cells infected with *AdCMVcLuc* (Fig. 2*B* and *F*), this step in glucose concentration caused a clear increase in luminescence, similar to that previously reported in single MIN6 and primary rat β-cells microinjected with the corresponding cDNAs (27). The onset of this increase in $[ATP]_{cyt}$ occurred within 5 s of the increase in glucose concentration and before any detectable increase in $[Ca^{2+}]_{cyt}$ (not detectable before 30 s). At 3 mmol/l glucose, depolarization of the plasma membrane with 70 mmol/l KCl caused a dramatic biphasic increase in $[Ca^{2+}]_{cyt}$ and an increase in apparent $[ATP]_{cyt}$, which was stable for ~100 s, but then decreased with time (Fig. 2*D*). Little or no time lag was observed between the initiation of these two changes upon KCl addition (Fig. 2*C* and *D*). By contrast, addition of 70 mmol/l KCl to cells in which $[Ca^{2+}]_{cyt}$ had previously been elevated by incubation for 5 min in the presence of 30 mmol/l glucose caused a brief "spiking" in light output followed by a clear decrease in $[ATP]_{cyt}$ (Fig. 2*F*).

Removal of external Ca^{2+} ions with EGTA (39,40) decreased the $[Ca^{2+}]_{cyt}$ and also the magnitude of the $[ATP]_{cyt}$ increase after exposure to 30 mmol/l glucose to <4% over basal (Fig. 3*A* and *B*) compared with a mean increase of 14.2 ± 0.9% ($n = 12$ separate experiments) seen in untreated cells. Thus, increases in $[Ca^{2+}]_{cyt}$ appeared to be necessary for effective glucose metabolism.

(blue). *E–H*: corresponding data for a single mouse β-cell to that shown in *A–B*. Data obtained during incubation at 16 mmol/l glucose are analyzed in *G* and *H*. Time bars in *A* and *E* represent 200 s. The cells shown are representative of the ~30% of cells that displayed significant ($P < 5\%$ for curve fit) oscillations.

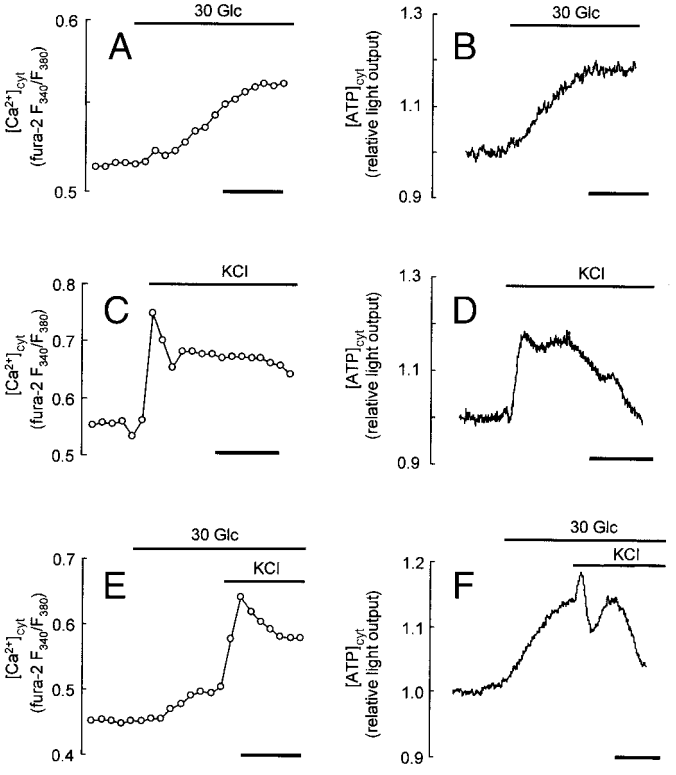

FIG. 2. Effect of glucose on the relationship between $[Ca^{2+}]_{cyt}$ and $[ATP]_{cyt}$ in MIN6 β-cell populations. Changes in $[Ca^{2+}]_{cyt}$ and $[ATP]_{cyt}$ were monitored (see RESEARCH DESIGN AND METHODS) in response to stimulation with 30 mmol/l glucose (30 Glc) (*A* and *B*), 70 mmol/l KCl (*C* and *D*), and 30 mmol/l glucose followed by 70 mmol/l KCl (*E* and *F*). Traces are typical of at least three independent experiments. Time bars represent 200 s. F_{340}/F_{380} represents the fluorescence intensity of fura-2 upon excitation at 340 and 380 nm.

However, in cells incubated at either 3 or 30 mmol/l glucose, addition of EGTA alone led to a drop in $[Ca^{2+}]_{cyt}$ (Fig. 3*A* and *C*) but caused a significant increase in luciferase luminescence (Fig. 3*B* and *D*). Thus, the final increase in $[ATP]_{cyt}$ over basal achieved when glucose was increased to 30 mmol/l before the addition of EGTA was 60% compared with 33% when EGTA was added before the increase in glucose. Identical results were obtained by closure of L-type Ca^{2+} channels with verapamil as opposed to EGTA treatment (not shown).

Contribution of extramitochondrial Ca^{2+}-ATPases to Ca^{2+}-stimulated ATP consumption. The observation (Fig. 3*B* and *D*) that the lowering of $[Ca^{2+}]_{cyt}$ by incubation in Ca^{2+}-free medium resulted in an ~20% increase in light output from unstimulated MIN6 cells (Fig. 2*B* and *F*) indicated that lowering $[Ca^{2+}]$ suppressed basal ATP consumption. Under these conditions, changes in the activity of ATP-consuming processes, rather than in mitochondrial ATP synthesis, seem likely to underlie the increase in $[ATP]_{cyt}$ because the change in $[Ca^{2+}]_{cyt}$ upon the blockade of Ca^{2+} influx occurred below the range (i.e., <100 nmol/l) at which mitochondrial oxidative metabolism is sensitive to $[Ca^{2+}]_{cyt}$ (19).

We considered the SERCA pumps as a principal candidate for the Ca^{2+}-sensitive ATP-consuming processes (29). The contribution of these Ca^{2+} pumps to cellular ATP turnover was investigated by measuring the effect of their inhibition with 10 μmol/l cyclopiazonic acid (CPA) on light output in luciferase-expressing MIN6 cells.

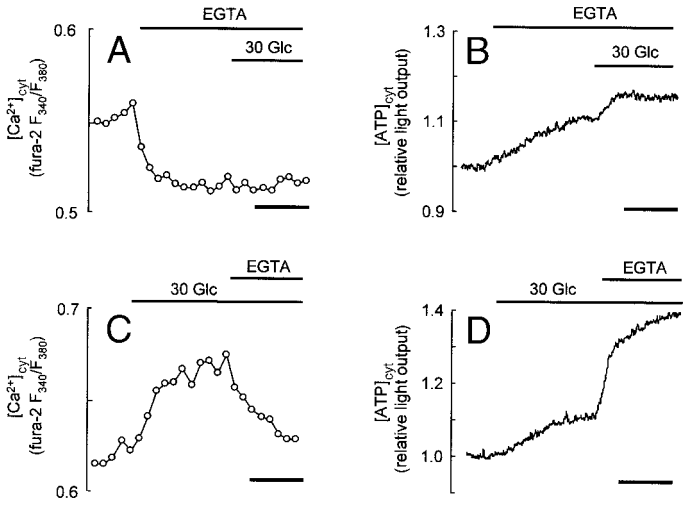

FIG. 3. Effect of the removal of external Ca^{2+} and L-type Ca^{2+} channel blockade on the response to glucose of $[ATP]_{cyt}$ in MIN6 cells. Changes in $[Ca^{2+}]_{cyt}$ (*A* and *C*) and $[ATP]_{cyt}$ (*B* and *D*) in response to a challenge with 0.1 mmol/l EGTA in Ca^{2+}-free KRB and subsequently 30 mmol/l glucose (30 Glc) (*A* and *B*) are shown, as are the effects of 30 mmol/l glucose and subsequently 0.1 mmol/l EGTA in Ca^{2+}-free KRB (*C* and *D*). Traces are typical of at least two independent experiments. Time bars represent 200 s.

Figure 4*A* and *B* show the responses of $[Ca^{2+}]_{cyt}$ and $[ATP]_{cyt}$ to incubation with CPA of MIN6 cells under basal (3 mmol/l glucose) conditions. The responses to CPA during stimulation with either 30 mmol/l glucose or 70 mmol/l KCl are shown in Fig. 4*C* and *D*. CPA caused a transient elevation of $[Ca^{2+}]_{cyt}$ (Fig. 4*A*), presumably due to the release of Ca^{2+} from stores in the endoplasmic reticulum and a large increase in $[ATP]_{cyt}$ even under basal conditions (Fig. 4*B*). Increases in $[ATP]_{cyt}$ were also evident upon CPA incubation in the presence of both high KCl and glucose concentrations. Overall, these results suggest that the SERCA pump is very active even in

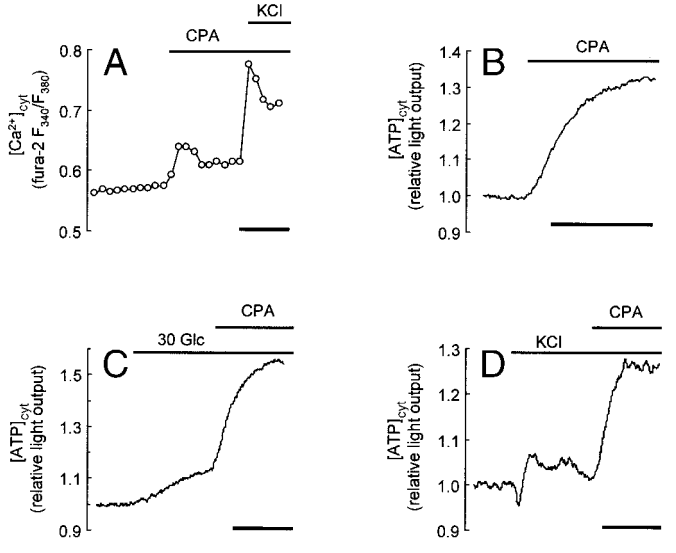

FIG. 4. Role of SERCA pump activity in controlling $[ATP]_{cyt}$. $[Ca^{2+}]_{cyt}$ (*A*) and $[ATP]_{cyt}$ (*B–D*) were monitored after the inhibition of SERCA pump activity with 10 μmol/l CPA. Cells were prestimulated with 30 mmol/l glucose (30 Glc) (*C*) or 70 mmol/l KCl (*D*). Traces are typical of at least two independent experiments. Time bars represent 200 s.

unstimulated cells, representing an important Ca^{2+}-dependent ATP-consuming process.

DISCUSSION

Properties of $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$ oscillations in islet β-cells. Using adenovirus-driven expression of the ATP-dependent bioluminescent enzyme, firefly luciferase (27,37), we were able to image oscillations in free $[ATP]_{cyt}$ in individual primary β-cells from mouse and human islets. In both cases, $[ATP]_{cyt}$ displayed oscillatory changes that were affected by glucose concentration (Fig. 1). In human β-cells, $[ATP]_{cyt}$ oscillations were evident during incubation at either low (3 mmol/l) or high (15 mmol/l) glucose concentrations, whereas in mouse β-cells, $[ATP]_{cyt}$ oscillations were only seen at elevated glucose concentrations. The origin of this difference may lie in the respective sensitivities of β-cells from these two species to glucose. The normal blood glucose concentration in humans (4–5 mmol/l) is substantially lower than that in mice (8–10 mmol/l) and thus, 3 mmol/l glucose may be below a threshold for which oscillations occur in mouse β-cells but above the threshold for human β-cells. In contrast to mouse and human β-cells (this study), we have previously reported that oscillations in $[ATP]_{cyt}$ were not apparent in dissociated rat islet β-cells (27) or whole islets (18) at either high or low glucose concentrations, consistent with the failure of others (41) to detect $[Ca^{2+}]_{cyt}$ oscillations in rat islets (although low-frequency oscillations in mitochondrial $[Ca^{2+}]$ were evident in our own studies) (18). These observations suggest that important differences exist in the mechanisms responsible for secretory oscillations in different mammalian species.

Upon elevation of glucose concentration, the period of $[ATP]_{cyt}$ oscillations in human β-cells displayed a clear increase from 60 to 170 s, but there was no apparent change in amplitude of the oscillations. Interestingly, these oscillations are much faster than those of insulin secretion measured in living subjects (12–15 min) (9,35), suggesting that some entrainment of the $[ATP]_{cyt}$ pulses may occur in the context of the intact islet, or within the pancreas, by neural or other means. Nevertheless, a small number of cells displayed oscillations in $[ATP]_{cyt}$ at 3 mmol/l glucose, as has been reported for $[Ca^{2+}]_{cyt}$ oscillations measured in dissociated human islet β-cells ex vivo (42). Furthermore, the increase in wavelength of $[ATP]_{cyt}$ oscillations in response to elevated glucose is similar to the behavior of $[Ca^{2+}]_{cyt}$ oscillations observed in human islets (28). However, it must be appreciated that isolation and culture conditions can profoundly influence the glucose-induced oscillatory behavior of β-cells (43).

In mouse β-cells, the period of $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$ oscillations at elevated glucose concentrations was identical, at ~120 s, in cells from identical preparations. The period of these oscillations in $[ATP]_{cyt}$ was compatible with the "slow wave" oscillations seen in islet $[Ca^{2+}]_{cyt}$ and insulin secretion (1,2). Thus, it appears that the structure of the β-cell oscillator is inherent to individual cells and is not a property of the islet, although the organization of cells within the islet may influence oscillatory behavior (1).

Despite the high levels of luciferase expression achieved with the adenoviral vector, it was not possible to observe dynamic changes in light output with sufficient temporal resolution to differentiate rapid oscillations, of a period of 5–20 s, as seen in $[Ca^{2+}]_{cyt}$ and oxygen consumption (43,44). However, the present results demonstrate the coherent behavior of oscillations in both $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$ in the β-cell and the likely interdependence of each.

Regulation of ATP turnover by Ca^{2+}. Given the strong regulatory influence that ATP exerts over Ca^{2+} influx into the β-cell (by modulating K_{ATP} channel activity), it is possible that an oscillator that solely governed ATP production, such as a proposed glycolytic oscillator (45), would also be able to drive oscillations in $[Ca^{2+}]_{cyt}$. However, it is widely acknowledged that, in a variety of cell types, $[Ca^{2+}]_{cyt}$ is a potent activator of mitochondrial metabolism (19,21). It therefore seems more likely that oscillations in β-cell $[Ca^{2+}]_{cyt}$ influence ATP production and thus form part of the basis of the β-cell oscillatory mechanism.

To explore the regulation by Ca^{2+} of mitochondrial ATP production, we investigated the effect of modulations in $[Ca^{2+}]_{cyt}$ on $[ATP]_{cyt}$ in populations of MIN6 cells during stimulation with high glucose concentrations. Elevation of glucose concentration led to increases in $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$ in MIN6 cells. An increase in $[Ca^{2+}]_{cyt}$ at low glucose concentrations (3 mmol/l) in response to membrane depolarization with KCl also led to a transient elevation of $[ATP]_{cyt}$ (Fig. 3*A–D*). Furthermore, blockade of Ca^{2+} influx with verapamil, or removal of external Ca^{2+}, inhibited the activation of ATP generation in response to 30 mmol/l glucose by >75% (Fig. 4*B* and *F*). Thus, these data demonstrate the requirement for Ca^{2+} influx for the full activation of glucose metabolism, as previously suggested by measurements of NAD(P)H fluorescence in mouse islets (40). Full $[ATP]_{cyt}$ increases in response to subsaturating concentrations of two substrates of mitochondrial metabolism, leucine and methylsuccinate, also required Ca^{2+} influx (results not shown). Elevated $[Ca^{2+}]_{cyt}$ increased the apparent sensitivity of mitochondrial oxidation for both substrates but not the maximal apparent ATP production (not shown). The requirement for maintained intracellular Ca^{2+} concentrations for the metabolism of these substrates, as well as glucose, therefore probably reflects the activation of mitochondrial ATP production by Ca^{2+} (19,23,46).

In contrast, in cells where $[Ca^{2+}]_{cyt}$ (and hence mitochondrial ATP synthesis) had already been elevated by incubation with 30 mmol/l glucose, a further dramatic increase in $[Ca^{2+}]_{cyt}$, imposed by cell depolarization with KCl, led to a fall in $[ATP]_{cyt}$ (Fig. 2*F*). This result, which is consistent with previous measurements of total cellular ATP and ADP in intact islets (38), indicates that excessive accumulation of Ca^{2+} by mitochondria exerts either no effect or an inhibitory effect (24,25,47) on mitochondrial ATP synthesis, i.e., the ATP production subsystem is effectively saturated with Ca^{2+}. Interestingly, such an inhibitory effect seems unlikely to involve opening of the mitochondrial permeability transition pore (26) because it was unaffected by the potent inhibitor of mitochondrial permeability transition pore opening, cyclosporin A (not shown).

In addition to the above effects on mitochondrial func-

tion, the $[Ca^{2+}]_{cyt}$ rise induced by KCl in the presence of an elevated glucose concentration is also likely to stimulate ATP-consuming events in the cytosol. These events seem likely to include Ca^{2+} pumping into the endoplasmic reticulum (Fig. 4) and possibly out of the cell. We have previously considered the possibility that the activation of insulin synthesis, as well secretory vesicle movement (48) and exocytosis (49), may contribute significantly to the consumption of ATP in response to increases in $[Ca^{2+}]_{cyt}$. Surprisingly, however, inhibition of secretion with clonidine (an α_2-adrenoreceptor agonist) (18) or diazoxide (50) had little effect on $[ATP]_{cyt}$, indicating that ion pumping may represent that the more important ATP sink under these conditions. Whatever the mechanism(s) responsible for the observed $[ATP]_{cyt}$ changes, the ability of an increase in $[Ca^{2+}]_{cyt}$ (and/or mitochondrial $[Ca^{2+}]$) to reduce $[ATP]_{cyt}$ supports the proposal of Detimary et al. (38) and Krippeit-Drews et al. (47) that increases in $[Ca^{2+}]_{cyt}$ may act in a feedback loop to decrease $[ATP]_{cyt}$. This is then likely to lead to the opening of K_{ATP} channels and thus initiate the down-stroke of electrical and $[Ca^{2+}]_{cyt}$ oscillations in islet β-cells.

Overall, Ca^{2+}-regulated processes, including Ca^{2+} ion pumping, appear to contribute a considerable proportion of total cellular ATP turnover. Thus, lowering cytosolic $[Ca^{2+}]$ apparently diminishes mitochondrial ATP synthesis to a much smaller extent than the decrease in extramitochondrial ATP consumption, giving a net increase in $[ATP]_{cyt}$ (Fig. 3). It should be pointed out that the lowering in basal $[Ca^{2+}]_{cyt}$ by verapamil in MIN6 cells may not be typical of primary β-cells (51) and may reflect partially altered Ca^{2+} homeostasis in the derived cell line. The fact that the increase in $[ATP]_{cyt}$ upon Ca^{2+} removal was greater after incubation with 30 mmol/l glucose (Fig. 3*D* vs. 3*B*) may be indicative of the increased activity of the Ca^{2+}-dependent ATP-consuming reactions during elevation of $[Ca^{2+}]_{cyt}$ by glucose (although it should be stressed that this cannot be proven by measurement of $[ATP]_{cyt}$ alone).

We propose that the negative regulation of $[ATP]_{cyt}$ by Ca^{2+} may be due to SERCA pump activity, since we found the latter to be a major ATP-consuming process in these cells judged by the large positive effect of CPA on $[ATP]_{cyt}$ and the fact that treatment with verapamil severely diminished the effect of CPA on $[ATP]_{cyt}$ (not shown). In contrast, vanadate ions, at a concentration reported to inhibit plasma membrane Ca^{2+}-ATPase (10 μmol/l) (52), was without effect on $[ATP]_{cyt}$ at resting and glucose-stimulated conditions. Only under KCl stimulation did vanadate evoke an increase in $[ATP]_{cyt}$ (not shown), suggesting that under most conditions, the plasma membrane Ca^{2+}-ATPase does not significantly control $[ATP]_{cyt}$. However, it should be mentioned that the efficacy of vanadate in inhibiting plasma membrane Ca^{2+}-ATPases has been questioned in the β-cell type (53).

Model of Ca^{2+}-dependent oscillations in $[ATP]_{cyt}$. A possible cycle of events that may lead to oscillations in both $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$ is outlined in Fig. 5. In this scheme, which was developed from the schemes of Jung et al. (8) and Detimary et al. (38), an increase in glucose concentration leads to a limited increase in $[ATP]_{cyt}$, which then triggers K_{ATP} channel closure and a small

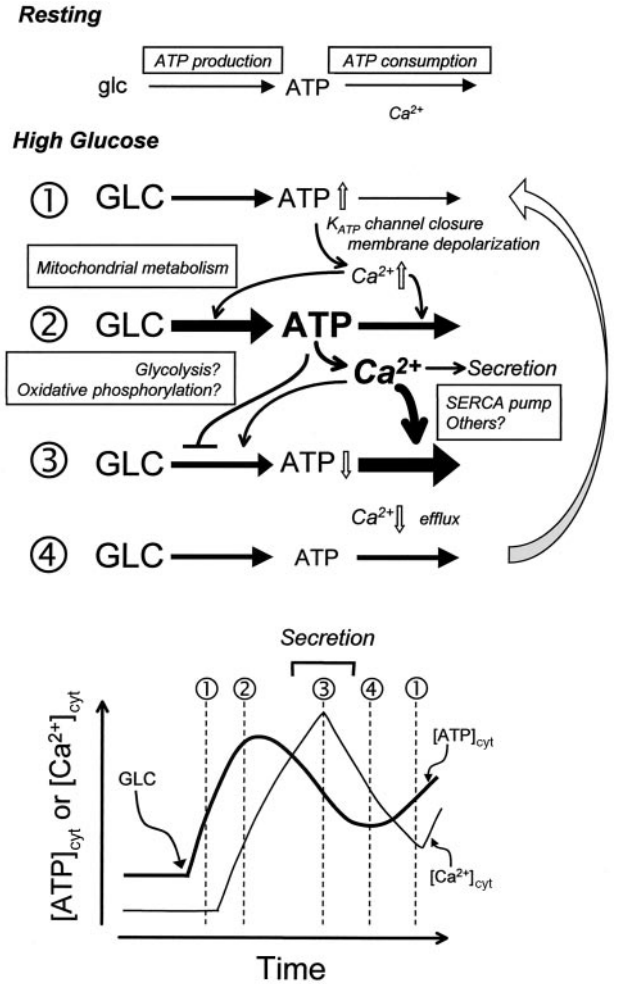

FIG. 5. Proposed cycle of events during Ca^{2+}-dependent oscillations in $[ATP]_{cyt}$. For further details, see DISCUSSION. The lower figure shows a theoretical oscillation in $[ATP]_{cyt}$ and $[Ca^{2+}]_{cyt}$ initiated by an elevation of glucose (GLC, glc) concentration generated by a simple simulation model encompassing the regulatory mechanisms shown.

influx of Ca^{2+} into the cell (1 in Fig. 5). The resulting increase in $[Ca^{2+}]_{cyt}$ begins to activate mitochondrial metabolism, permitting the accelerated metabolism of glucose carbon atoms by mitochondria, thus creating a feed-forward loop that further enhances the increase in $[ATP]_{cyt}$ (2 in Fig. 5). As $[Ca^{2+}]_{cyt}$ increases still further, insulin secretion will be stimulated but the activation of mitochondrial ATP production is saturated. At this point, further influx of $[Ca^{2+}]_{cyt}$ results in a decrease in $[ATP]_{cyt}$ due to increased ATP consumption (3 in Fig. 5). This will result in $[ATP]_{cyt}$ falling to sufficiently low levels that Ca^{2+} influx is reduced, and $[Ca^{2+}]_{cyt}$ decreases because of net Ca^{2+} efflux (4 in Fig. 5). The now-reduced activity of Ca^{2+}-dependent ATP-consuming reactions means that ATP production outweighs ATP consumption, leading to increasing $[ATP]_{cyt}$, and the cycle starts again.

We propose that the critical decrease in $[ATP]_{cyt}$ during oscillations is due to increased ATP consumption activated by Ca^{2+}. In agreement with Detimary et al. (38), we suggest that increased ATP consumption is due to increased ion cycling rather than secretion alone. Ion cycling involving the SERCA pumps is a major contributor to ATP consumption (Fig. 4*B*) and is therefore a strong

candidate to be the Ca^{2+}-dependent ATP-consuming process capable of driving $[ATP]_{cyt}$ oscillations. Nevertheless, it must be stressed that the above conclusion does not preclude the contribution of other cytosolic ATP-consuming processes nor the contribution of changes in cytosolic [ATP]/[ADP] to the regulation of glycolysis (8,15). However, a simple glycolytic oscillator, of the type proposed by Tornheim (15), would seem alone insufficient to explain the metabolic oscillations that presumably underlie pulsatile insulin secretion.

After reviewing the mechanism outlined in Fig. 5, we propose that oscillations in $[ATP]_{cyt}$ and $[Ca^{2+}]$ are out of phase with each other, as suggested by others (8), on the basis of measurements of oxygen and glucose consumption by intact islets. Crucially, we observed that the appearance of an $[ATP]_{cyt}$ increase in response to elevated glucose concentration preceded that of $[Ca^{2+}]_{cyt}$ by approximately half the wavelength of the oscillations in single mouse β-cells. This finding is consistent with earlier measurements of total extractable adenine nucleotides (38), measurements of the onset of NAD(P)H increases and $[Ca^{2+}]_{cyt}$ oscillations (6,40), and K_{ATP} channel activity (54). Further investigations will require the development of new techniques to investigate the relative phase of oscillations in $[Ca^{2+}]$ and $[ATP]_{cyt}$, as well as oxygen and glucose consumption, and insulin secretion in the same single β-cell or islet. Such studies should be particularly instructive in assessing the relative strengths of the regulatory mechanisms described here and thus to provide a fuller picture of the β-cell oscillator.

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