Changes in cytoplasmic ATP concentration parallels changes in ATP-regulated K^+-channel activity in insulin-secreting cells

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Abstract Changes in cytoplasmic ATP concentration were monitored in intact insulin-producing cells and correlated to changes in the activity of ATP-sensitive K^+-channels (K_{ATP} channels). Luciferase was introduced into HIT M2.2 cells and whole pancreatic islets by transient expression of firefly (*Photinus pyralis*) luciferase cDNA. In transfected cells, extracellular addition of luciferin increased the luminescence signal to a maximum within 50–120 s. Addition of 1 μM of the mitochondrial uncoupler FCCP decreased the luminescence, an effect partly reversed upon withdrawal of the compound. High concentrations of glucose increased cytoplasmic free ATP concentration. Changes in the luminescence signal were accompanied by changes in activity of the ATP-sensitive K^+-channel. Transfection per se did not deteroriate cell function, as verified by experiments showing similar changes in cytoplasmic free Ca^{2+}-concentration, $[Ca^{2+}]_i$, in both transfected and non-transfected cells. By measuring the cytoplasmic ATP concentration and K_{ATP} channel activity under similar experimental conditions, it was possible to establish, for the first time, a direct relationship between these two parameters. This indeed suggests that the cytoplasmic ATP concentration has a crucial role in the regulation of K_{ATP} channel activity under physiological conditions.

Key words: Luciferase; Cytoplasm; Transfection; Microscopy; ATP

1. Introduction

The pancreatic β-cell regulates glucose homeostasis by combining a glucose sensing function with the capacity to secrete insulin [1]. Glucose is rapidly taken up and metabolized in the β-cell. This leads to an increase in the intracellular ATP/ADP ratio and thereby closure of the K_{ATP} channel [2]. This results in membrane depolarization and thereby opening of voltage-dependent L-type Ca^{2+} channels, leading to a rise in $[Ca^{2+}]_i$ and subsequent stimulation of insulin release [3]. Thus, ATP has a key role in the β-cell stimulus-secretion coupling and it has been speculated that an inadequate ATP production, due to a defective glucose metabolism, is responsible for the deranged stimulus-secretion coupling prevailing in non-insulin dependent diabetes mellitus (NIDDM) [4]. Therefore, measurements of the cytoplasmic ATP concentration are of major importance for evaluation of a direct coupling between glucose metabolism and exocytosis of insulin. For ATP measurements, sensitive assays based on the North American firefly (*Photinus pyralis*) luciferase (EC 1.13.12.7) have been used [5–8]. This method was used to determine ATP levels in extracts, but luciferase has also been injected into *Limulus* photoreceptors [9], cardiomyocytes [10] and hepatocytes [11], to monitor changes in intracellular ATP in living cells. We have previously shown that ATP can be monitored in insulin-secreting cells transiently expressing luciferase [12]. Recently an insulin-producing luciferase expressing cell line was developed and used for the same purpose [13].

The objective of the present study was to use transient expression of firefly luciferase in combination with microscopic luminescence detection to measure cytoplasmic free ATP levels in intact insulin-producing cells and whole pancreatic islets and to correlate these levels to the activity of the K_{ATP} channel.

2. Materials and methods

2.1. Materials

Cell culture media were from Flow Laboratories, Scotland, UK. All other cell culture reagents were purchased from Gibco (Life Technologies, Gaithersburg, MD, USA). Plastic materials were from Costar (Costar, Cambridge, MA, USA). FCCP and D-luciferin were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

The insulin-secreting cell line HIT M2.2 was cultured at 37°C in 5% CO_2/95% air in DMEM medium supplemented with 2 mM L-glutamine, 10% fetal calf serum and 50 μg/ml gentamycin. At half-confluency, cells were detached from culture flasks by trypsinization and subjected to transfection as described below.

Whole pancreatic islets were isolated from 3–4 months old C57BL/KsJ-db/+ mice as previously described [14].

2.3. Transient expression of firefly luciferase

Transient expression was obtained by means of electroporation (Gene Pulser transfection apparatus, Bio-Rad, Richmond, CA, USA) or adenovirus transfection. In optimization of the transient expression of luciferase, the Promega luciferase assay system (Promega, Madison, WI, USA) was used for cell lysis and determination of luciferase activity in cell extracts with and without the metabolic inhibitors. Light emittance from this luciferase assay was detected with

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Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; K_{ATP} channel, ATP-sensitive K^+-channel; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; NIDDM, non-insulin dependent diabetes mellitus; CAT, chloramphenicol acetyltransferase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); CCD, charge coupled device

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a liquid scintillation analyzer (Packard 1900 TR, Packard, Meriden, CT, USA). Extracts from untransfected cells, or the reagent buffer without addition of cell extract, did not result in any increased light emittance over background. CAT assays were performed as described [16]. For electroporation, 6×10^6 cells were suspended in 800 μl of PBS supplemented with 50 μg of the plasmid pRSVL [17] in 0.4-cm cuvettes (Bio-Rad) kept on ice. Capacitance setting was 960 μF and the electric field was 1.05 kV/cm, resulting in a time constant of approximately 15 ms. Electroporation procedure caused more than 90% cell death. After electroporation, cells were plated on 25-mm round glass cover slips in 35-mm Petri dishes. Luciferase activity was detected in situ 44 h after transfection. Intact pancreatic mouse islets were transfected with a luciferase adenovirus construct (AdCMVluc) by incubation in 1 ml of 5.3×10^9 pfu/ml for 44–55 h prior to the experiment.

2.4. Measurements of luminescence in microscope

The medium used for luminescence microscopic experiments (here denoted 'Buffer A') was a HEPES buffer (pH 7.4) containing (in mM): NaCl 125, KCl 5.9, $CaCl_2$ 2.56, $MgCl_2$ 1.2, HEPES 25 and glucose 11. Bovine serum albumin was added to a concentration of 1 mg/ml.

Cells attached to coverslips were superfused in a custom built open chamber with a flow rate of 0.1 ml/min. The chamber was mounted on an inverted microscope (Zeiss Axiovert 135TV, Zeiss, Germany) equipped with a photon-counting photometer tube, chilled to between −30 and −25°C with a custom made water cooled peltier element. Lens was Zeiss Fluar 40×/1.30 Oil. Data acquisition was performed with hardware from Spex Industries (Edison, NJ, USA). Integration time was 5 s per time point and background (dark) signal was on average 2.5 counts per second (cps). Temperature during experiments was 33°C.

2.5. Measurements of $[Ca^{2+}]_i$ in luciferase expressing cells

The $[Ca^{2+}]_i$ response in cells expressing luciferase was measured in the same microscope system as described above for the luminescence measurements, using the lens Zeiss Fluar 40×/1.30 Oil. 'Buffer A' (see above) was used with different glucose concentrations and additions during the experiments. Cells were incubated with Fura-2/AM (2 μM) and 3 mM glucose for 30–40 min at 37°C. Thereafter the cells on the coverslip were transferred to the perifusion chamber. After addition of 0.1 mM luciferin, luminescence was detected using an intensified integrating CCD camera (Luminescence Imager, Photonic Science, East Sussex, UK) and integration for 5 min. Image acquisition and camera control were done from a PC with the framegrabber Matrox Meteor (Matrox Graphics, Québec, Canada) and the software Image-Pro Plus (Media Cybernetics, MD, USA). The luciferase expressing cells were localized by overlaying the luminescence image onto the corresponding brightfield image. When luminescence was clearly detected from any cell/cells in the field, the glucose concentration was changed to 0.1 mM for some minutes. Thereafter changes in $[Ca^{2+}]_i$ were detected in response to 20 mM glucose and addition of 10 mM leucine with an imaging system (Inovision, Durham, NC, USA) with the same camera as above. Fura-2 was excited by light from the SPEX fluorolog-2 CM1T11I system at the wavelengths 340 and 380 nm, and fluorescence was selected by a 500–530-nm bandpass filter. $[Ca^{2+}]_i$ was monitored with the software Ratiotool (Inovision).

2.6. Electrophysiological recordings

We used the cell-attached configuration of the patch-clamp technique [18] to record single K_{ATP} channel activity. Pipettes were prepared from borosilicate glass capillary tubes, isolated near the tips and fire-polished. Their resistance was 2–6 MΩ. The extracellular solution was a HEPES buffer (pH 7.4) containing (in mM): NaCl 138, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 5 and glucose 3. The pipette contained the extracellular solution but without glucose. All experiments were done at 32°C.

Single K_{ATP} channel currents were recorded at a membrane potential of −70 mV. K_{ATP} channel activity was identified by its sensitivity to the sulfonylurea drug tolbutamide [19], as well as on the basis of its single channel amplitude (1.0–1.2 pA). The current signal was recorded using an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Pending analysis, the signal was stored on video tape using a VR-100A digital recorder (Instrutech, Elmont, NY, USA) and a video cassette recorder (JVC, Japan). Channel records were filtered at 100 Hz (−3 dB value) through an 8-pole Bessel filter (Frequency Devices, Haverhill, MA, USA) and displayed according to the convention, where upward deflections denote outward currents.

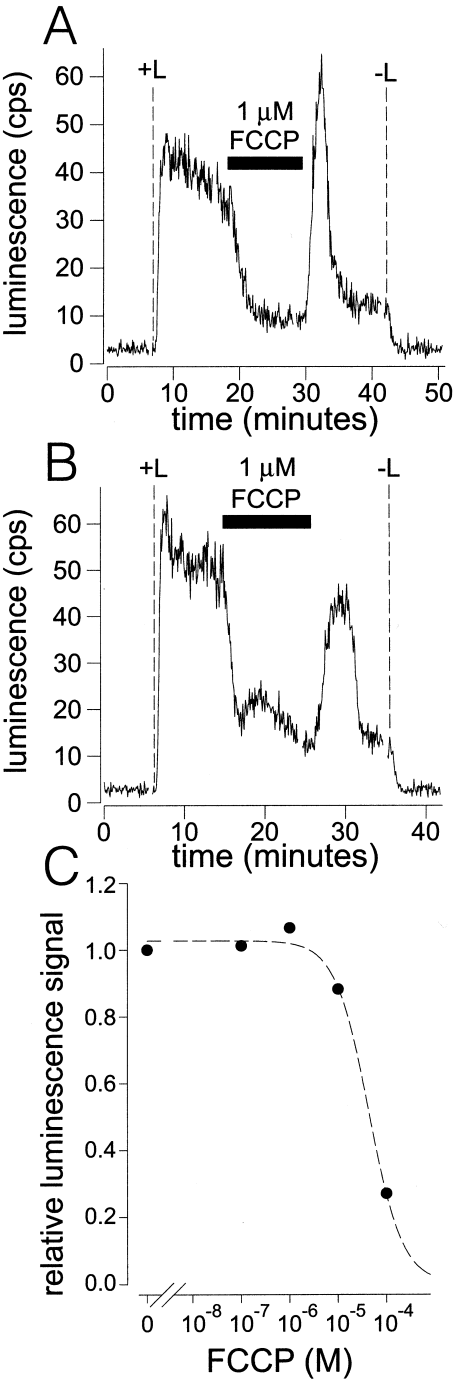

Fig. 1. Luminescence from luciferase expressing HIT M2.2 cells and luciferase inhibition by FCCP. A and B: Luminescence detected from groups of luciferase expressing HIT M2.2 cells (20–30 cells in the measuring field), during exposure to 0.1 mM D-luciferin added to the extracellular medium. Addition of 1 μM FCCP, as indicated in these recordings, was accompanied by a decrease in luminescence. Two representative recordings are shown ($n=12$). Due to light artifacts, short segments of the trace were excluded when changing perifusion buffers. '+L' denotes addition of D-luciferin and '−L' denotes withdrawal of D-luciferin. C: Direct inhibition of the luciferase bioluminescence by FCCP, as monitored by the luminescence from extracts of luciferase expressing HIT M2.2 cells with a luciferase assay (Promega). Every point represents the mean of at least two measurements.

3. Results

To monitor changes in cytoplasmic ATP concentration in intact and viable cells, we registered luciferase luminescence in HIT M2.2 cells, an SV-40 transformed hamster insulinoma cell line [15,20], as well as in whole pancreatic islets from mouse. For optimization of transfection in HIT cells, luciferase activity was quantified in extracts from transfected cells using a liquid scintillation counter, as described in Section 2. Electroporation in combination with the RSV-driven luciferase construction (pRSVL) produced the highest and most reproducible expression in HIT cells [12].

To investigate the correlation between changes in ATP concentration and changes in luminescence, we exposed cells to 1 μM of the mitochondrial uncoupler FCCP. We then compared the responses in luminescence with another method of ATP detection, namely K_{ATP} channel activity. The direct concentration dependent effect of FCCP on the bioluminescence reaction ($IC_{50} = 0.04$ mM) was investigated (Fig. 1C). FCCP at a concentration of 1 μM did not affect the bioluminescence reaction. The luminescence from 10–40 transfected cells, in the same field of view, was measured in the microscopic system (Fig. 1A and B). Addition of 0.1 mM D-luciferin to the extracellular medium resulted in an increase in signal, which reached a maximal value within 50–120 s. Thereafter luminescence decayed, with slightly varying kinetics in the different experiments. In response to addition of 1 μM FCCP ($n = 12$) a decrease of the luminescence signal was recorded. This decrease was partly reversed following withdrawal of FCCP. In response to a change in glucose concentration from 3 mM to 20 mM a clear increase in the luminescence signal could be seen in β-cell containing pancreatic islets transfected with luciferase adenovirus (AdCMVluc) ($n = 4$) (Fig. 2).

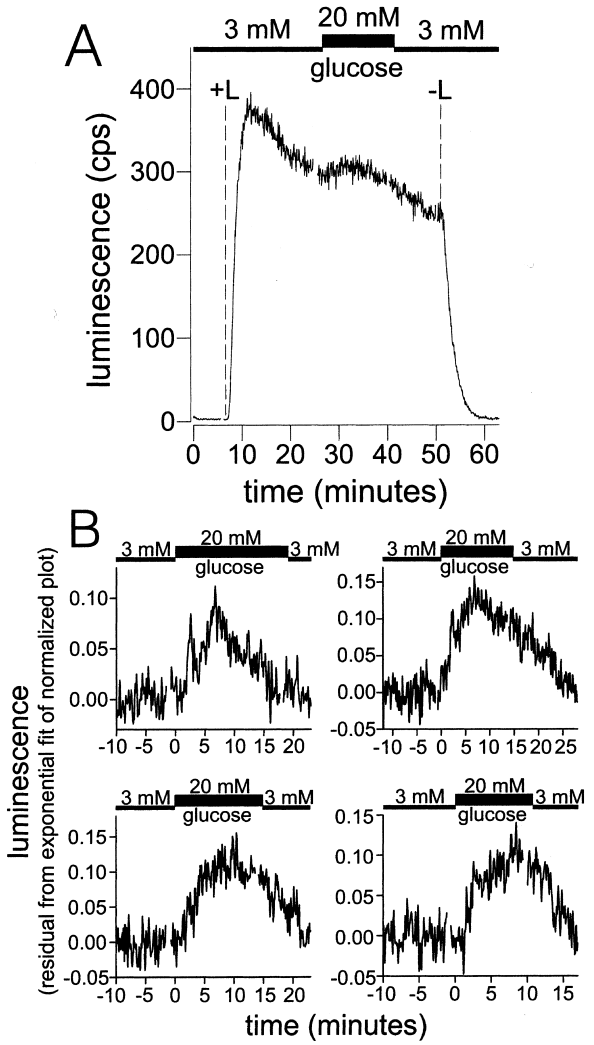

Fig. 2. Luminescence from AdCMVluc transfected mouse islets in response to 20 mM glucose as indicated. Due to light artifacts, short segments of the trace were excluded when changing perifusion buffers. '+L' denotes addition of D-luciferin and '−L' denotes withdrawal of D-luciferin. A: One typical recording out of four. B: Glucose effects in the four individual islets visualized by the residuals from exponential fit to the luminescence before and after the effect. The recordings were filtered by a moving average procedure. Time was changed so that time = 0 means start of glucose stimulation.

K_{ATP} channel activity is regulated by cytosolic ATP [21,22] and this channel may thus serve as a biosensor of the ATP concentration in the submembrane space. We used the cell-attached patch configuration of the patch-clamp technique to monitor changes in K_{ATP} channel activity in response to 1 μM FCCP (Fig. 3). In the presence of 3 mM glucose, little channel activity was observed, whereas the number of channel openings significantly increased upon exposure to 1 μM FCCP. This effect was reversed by withdrawal of the metabolic inhibitor. The electrophysiological experiments showed a similar temporal and quantitative response to FCCP as the luminescence measurements. A similar effect has been shown previously using 3 mM azide [19,12].

To verify that signal-transduction in cells expressing luciferase was intact compared to cells not expressing luciferase, changes in $[Ca^{2+}]_i$ in response to glucose and leucine were monitored. There was no difference in $[Ca^{2+}]_i$ response between cells expressing or not expressing luciferase (Fig. 4).

4. Discussion

The aim of the present study was to monitor dynamic changes in the free cytoplasmic ATP concentration in intact insulin-producing cells and to correlate these changes to changes in K_{ATP} channel activity. This is essential when trying to understand how metabolism is coupled to the molecular events governing the release of insulin. Luciferase monitoring is commonly used for determinations of ATP levels in extracts from non-transfected cells and reporter gene analysis in extracts from transfected eukaryotic cells. Recently, detection of luciferase in intact cells has been used in reporter gene studies [23]. Changes in intracellular ATP have also been monitored in intact luciferase injected cells [9–11] in cells transiently expressing luciferase [12] and in a luciferase expressing cell line [13].

In transfected cells, luciferase is targeted to peroxisomes by a target sequence in the C-terminus close to the active center of the enzyme [24–26]. To overcome possible limitations due to peroxisomal targeting, a luciferase lacking the targeting sequence, but with retained enzymatic activity, would be needed. At present, we have no such recombinant luciferase available. Nevertheless, the fast response to FCCP in our experiments indicates that either a large proportion of luciferase was present in the cytosol or that ATP in the peroxisomal lumen is effectively equilibrated with cytosolic ATP. Image analysis did not show any clear subcellular compartmentaliza-

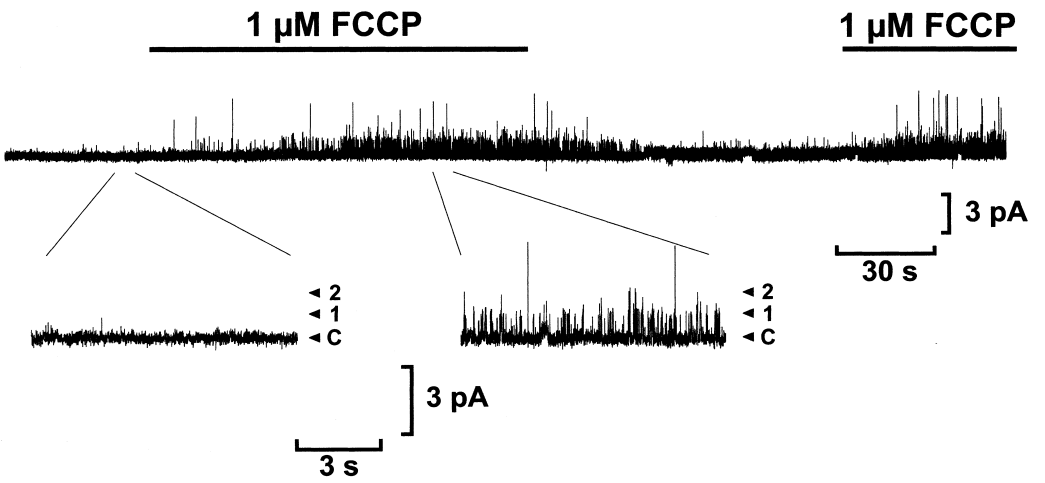

Fig. 3. Effects of FCCP on K_{ATP} channel activity in HIT M2.2 cells recorded in the cell-attached patch configuration. The figure shows a recording from one cell in the presence of 3 mM glucose and exposed to FCCP as indicated in the figure ($n = 3$). C, 1 and 2 denote numbers of open K_{ATP} channels. Occasional openings of a channel with amplitude and kinetics similar to those of the large conductance K^+ channel (K_{BK}) could also be observed in the presence of FCCP.

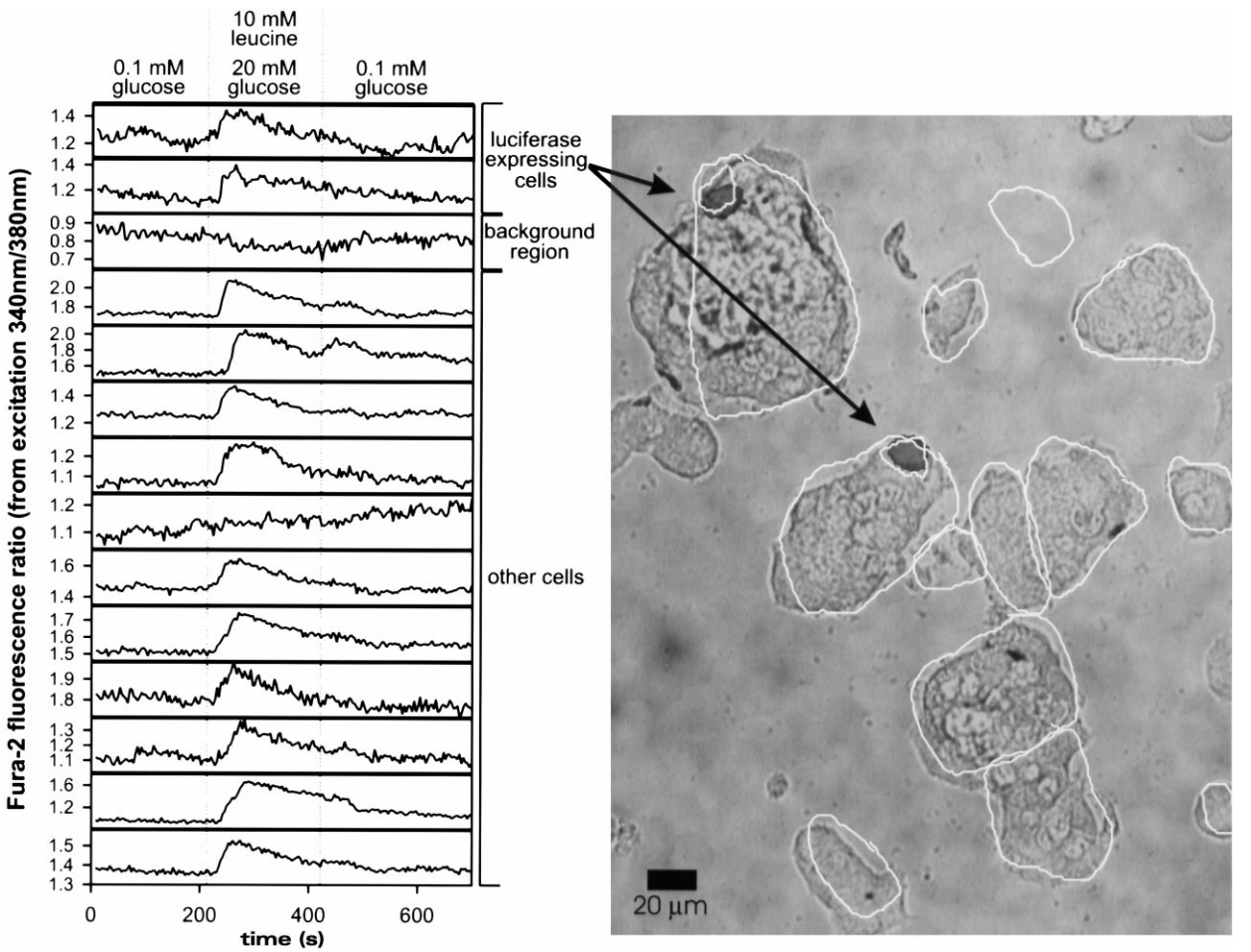

Fig. 4. Increase in $[Ca^{2+}]_i$ in response to 20 mM glucose and 10 mM leucine. The right part shows a transmission image of a field of view of cells and luminescence integrated during 5 min of exposure to 0.1 mM D-luciferin (shown as darker cells). The white lines indicate regions with cells where we monitored $[Ca^{2+}]_i$ using Fura-2/AM. The left part shows $[Ca^{2+}]_i$ recordings from these regions, where the luciferase expressing cells are shown on top. One typical experiment out of three.

tion [12], but this question may be better addressed by parallel immunohistochemical staining of luciferase and peroxisomal markers. Microinjection of luciferase would be an alternative solution to this potential problem, but small cells, like the pancreatic β-cell, are difficult to microinject and hence damage caused by microinjection often results in death of a high percentage of the cells. It is also possible to transfect cells with modified gene constructs targeted to specific organelles, like mitochondria. Furthermore, as opposed to microinjection of luciferase, transfection may provide not only expression in a large number of cells simultaneously but also stable expression of the enzyme.

The reaction catalyzed by luciferase requires the passage of the substrate luciferin from the culture media through the cell membrane. The delivery of luciferin may be a limiting factor, as the molecule is negatively charged at physiological pH [27]. However, this is apparently not the case, since luciferin at a concentration of 0.1 mM enters the cells almost as well as its ester derivatives [27], although a 10–20-fold gradient across the cell membrane is reported [28]. In our transfected insulin-secreting cells, luciferase activity appeared to be widely and homogeneously distributed throughout the cell [12]. Moreover, light emission reached peak values within 2 min, demonstrating that luciferin rapidly entered the cellular compartment containing luciferase.

Similar time response to addition and withdrawal of FCCP in luminescence and K_{ATP} channel activity measurements, indicate that the recorded luminescence indeed reflects cytoplasmic free ATP concentration. However, the kinetics of the reaction catalyzed by luciferase raises the question whether changes in luciferase luminescence and cytosolic ATP concentration are directly proportional. There is a clear decay of luminescence over time in most experiments. The reason for this decay is at the moment not clear. It may partly be accounted for by the luciferase/luciferin reaction, by formation of a catalytically inactive luciferase-oxyluciferin complex and by thermal inactivation of luciferase [28]. After withdrawal of FCCP a high and prolonged peak in luminescence was observed, where the signal eventually declined to a level close to the level obtained in the presence of FCCP. Presently, we have no explanation for this phenomenon. The corresponding effect in K_{ATP} channel activity was not observed within the time of the recordings. In order to verify that transfection per se did not interfere with cell function, changes in $[Ca^{2+}]_i$ were recorded in cells expressing luciferase. In this context an increase in $[Ca^{2+}]_i$ reflected a well-functioning glucose metabolism with subsequent membrane depolarization and opening of voltage-gated L-type Ca^{2+}-channels. Since we obtained a normal $[Ca^{2+}]_i$ response in the transfected cells, this clearly demonstrates the usefulness of this type of technique in measurements of cytoplasmic ATP in insulin-releasing cells.

We also wanted to verify that the luciferase method could report physiological increases in ATP concentration subsequent to glucose stimulation. For this purpose we used normal β-cell containing pancreatic islets that we transfected with an adenovirus construct. Adenovirus transfection has been demonstrated to transfect virtually all cells throughout the islet [29]. It is clear that glucose caused a significant increase in luminescence under these conditions.

We have shown that changes in cytoplasmic free ATP concentration have their immediate counterpart in changes in K_{ATP} channel activity. We have also shown that physiological increases in cytoplasmic free ATP concentration in response to glucose stimulation can be detected when β-cells are located within their physiological milieu, the intact islet of Langerhans. The next step will be to monitor ATP at different locations in the insulin secreting cell, like in close vicinity of the K_{ATP} channel and the site for exocytosis. This is possible using luciferase expressed with different tags directed to specific sites.

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