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Continuous monitoring of ATP levels in living insulin secreting cells expressing cytosolic firefly luciferase

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Abstract The second messenger role of ATP in insulin secretion was investigated in living INS-1 insulinoma cells. ATP-dependent luminescence was monitored in cells expressing high levels of firefly luciferase under the control of the tetracycline-dependent transactivator. The calibration of luminescence in permeabilized cells yielded similar ATP levels as those obtained in cell extracts with a conventional ATP assay. Stimulation of insulin secretion by glucose or methyl-succinate was correlated with rises of cellular ATP in simultaneous measurements. ATP generation was decreased by inhibition of the ADP-ATP translocase. This approach demonstrates the feasibility of defining the dynamic relationship between ATP and other parameters involved in metabolism-secretion coupling.

Key words: ATP; Luciferase; Insulin secretion; INS-1 cell; Tetracycline transactivator; ADP-ATP translocase

1. Introduction

The measurement of cellular ATP levels in living cells is crucial for assessing the metabolic state. In the pancreatic β-cell glucose undergoes a complex metabolic cascade leading to adequate insulin secretion. A rise in cytosolic ATP concentration is a key event in the closure of K_{ATP} channels and the following membrane depolarization [1,2]. This results in the opening of voltage-dependent L-type Ca^{2+} channels and the subsequent increase in cytosolic Ca^{2+} is the main trigger for exocytosis [3]. The ATP/ADP ration has been reported to oscillate in synchrony with cytosolic [Ca^{2+}] in glucose stimulated β-cells [4]. Furthermore, ATP is also required in the distal events governing exocytosis [3].

Determinations of cellular ATP levels are usually performed in cell extracts but have also been assessed in cells injected with the ATP-dependent luminescent protein firefly luciferase [5,6]. In pancreatic β-cells the measurement of cytosolic ATP levels in cell extracts is biased by the high ATP content of the insulin-containing secretory granules [7]. The present study describes the establishment of an insulin secreting cell line (INS-1) expressing cytosolic luciferase (EC 1.13.12.7) derived from the firefly *Photinus pyralis* [8]. Using these cells in a photon detection apparatus we have monitored cellular ATP changes simultaneously with the stimulation of insulin secretion by glucose and the mitochondrial substrate methyl-succinate.

2. Materials and methods

2.1. Materials

Luciferin and rabbit anti-luciferase immunoglobulin were obtained from Promega (Madison, WI, USA); bovine serum albumin, doxycycline, methyl-succinate, firefly lantern extract, FCCP and atractyloside from Sigma (St. Louis, MO, USA); anti-insulin antibody from (Linco, St Charles, MO, USA); G418 and hygromycin from Calbiochem (San Diego, CA, USA).

2.2. Cell culture and establishment of luciferase expressing cells

INS-1 cells were cultured in RPMI1640 medium as previously described [9]. The inducible luciferase expressing INS-1 sub-line was established by two-stage transfection using the Ca^{2+}-phosphate-DNA co-precipitation method [10]. The first-step stable cell line expressing the reverse tetracycline-dependent transactivator was generated as previously reported [11] by transfection of INS-1 cells with plasmid PUHG17-1 [12] plus RSVNeo. Resistant colonies were isolated with cloning rings after selection with 350 μg/ml G418 for 3 weeks. Clones positive for expression of reverse tetracycline-dependent transactivator were screened out by transient transfection with plasmid PUHC13-3 encoding the luciferase gene driven by a minimal cytomegalovirus promoter complemented with multiple copies of the tetracycline operator DNA element [12,11]. Positive clones that exhibited very high tetracycline-inducible luciferase activity and barely detectable basal luciferase activity were chosen for the next round of stable transfection with plasmid PUHC13-3 plus TK-hygromycin (a kind gift from Dr. Nancy Quintrell, University of California, San Francisco, CA). After 3 weeks selection with 200 μg/ml hygromycin, resistant colonies were cloned and screened for high luciferase activity after 16 h of cell culture with 1 μg/ml doxycycline. The clone used for the current study (INS-r3-LUC7) exhibited several thousand-fold tetracycline-inducible luciferase activities. The cells were maintained in long-term culture with G418 (150 μg/ml) and hygromycin (100 μg/ml).

2.3. Immunofluorescence

INS-1 cells were grown on polyornithine treated glass coverslips for 3 days prior to overnight treatment with 1 μg/ml doxycycline to induce luciferase expression. The cells were then washed, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline, 1% BSA (PBS-BSA). The preparation was then blocked with PBS-BSA before staining using rabbit anti-luciferase immunoglobulin followed by rhodamine-labelled goat anti-rabbit IgG as second antibody. Cells were viewed using a Zeiss laserscan confocal 410 microscope.

2.4. ATP measurements in cell extracts

According to the protocol of Stanley and Williams [13], cells were stimulated for 10 min at 37°C and scraped into 1 ml 0.4 N $HClO_4$ to terminate the reaction. Following neutralization with 2 N K_2CO_3, cell extracts were incubated with a luciferin-luciferase mixture in arsenate buffer (0.1 M $Na_2HAsO_4 \cdot 7H_2O$ pH 7.4, 20 mM $MgSO_4 \cdot 7H_2O$) and the resultant luminescence measured.

2.5. Permeabilization of cells

Attached cells were grown on coverslips coated with an extracellular matrix which was generated by confluent A431 cells detached with 1% Triton X-100 [14]. After a 4–5 day culture period, cells were first

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Abbreviations: met-Suc, methyl-succinate; KRBH, Krebs-Ringer bicarbonate HEPES buffer; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone

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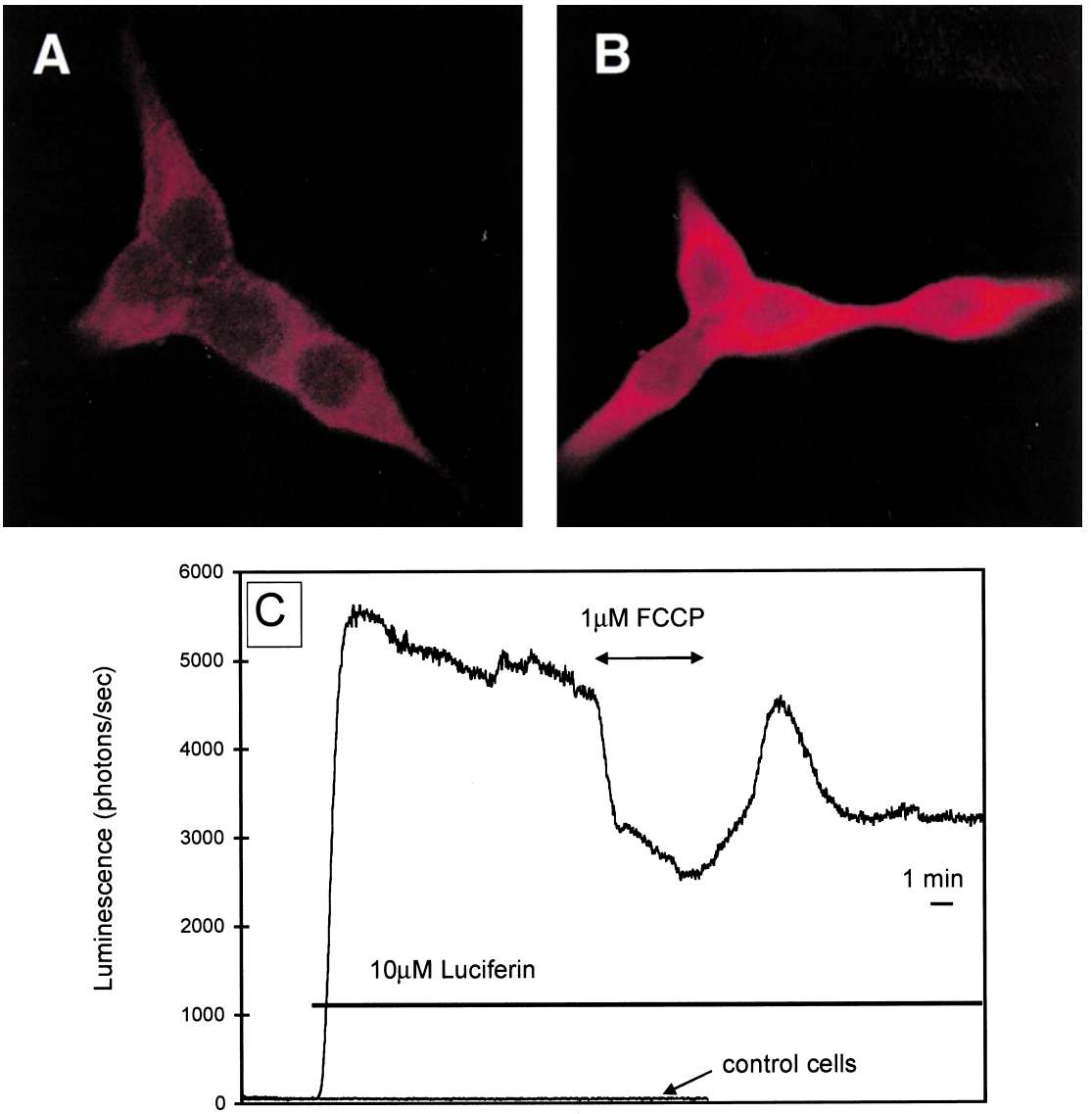


Fig. 1. Expression and cellular localization of luciferase in INS-1 cells. Immunofluorescence using anti-luciferase antibody was performed in control untransfected INS-1 cells (A) and cells expressing tetracycline inducible luciferase (B) after an overnight treatment with 1 μg/ml doxycycline. C: Doxycycline induced cells were placed in a termostatted chamber for photon collection. After a few minutes of perifusion, luciferin (10 μM) was added to the KRBH buffer. The mitochondrial uncoupler FCCP (1 μM) was added for 5 min to the cells expressing luciferase.

washed with a Ca^{2+}-free HEPES balanced Krebs-Ringer bicarbonate buffer (KRBH) prior to permeabilization with *Staphylococcus aureus* α-toxin [15,16] (1 μg/coverslip, i.e. per $4–5\times10^5$ cells) at 37°C for 8 min in 100 μl of an intracellular-type buffer as described elsewhere [16].

2.6. Measurements of luminescence and insulin secretion

Luciferase expressing cells were seeded on 13 mm diameter coverslips 4–5 days prior to analysis and maintained in the same medium as above except for the omission of G418 and hygromycin. For intact cell experiments, cells were seeded on plastic polyornithine treated coverslips at a density of 4×10^5 cells/ml. For permeabilized cell experiments, cells were seeded at 2×10^5 cells/ml on A431 extracellular matrix coated coverslips as described above. Prior to luminescence measurements, cells were maintained in glucose- and glutamine-free RPMI1640 plus 10 mM HEPES for 2–5 h at 37°C. Luminescence was measured by placing the coverslip in a 0.5 ml termostatted chamber at 37°C approximately 5 mm from the photon detector. We used a photomultiplier apparatus (EMI 9789, Thorn-EMI, England) and data were collected every second on a computer-photon counting board (EMI C660). The cells were perifused at a rate of 1 ml/min and, where indicated, 1 min fractions of the effluent were collected for insulin measurements. Intact cells were perifused with KRBH (135 mM NaCl, 3.6 mM KCl, 10 mM HEPES, pH 7.4, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 2.8 mM glucose) plus 10 μM beetle luciferin where mentioned. Permeabilized cells were perifused with an intracellular-type buffer with different ATP concentrations (1–10 mM) and a free Ca^{2+} concentration of about 500 nM (140 mM KCl, 5 mM NaCl, 7 mM $MgSO_4$, 20 mM HEPES, pH 7.0, 10.2 mM EGTA, 6.67 mM $CaCl_2$). For insulin secretion experiments, 0.1% of bovine serum albumin was added to buffers as carrier and insulin was measured by radioimmunoassay using rat insulin as standard [16].

3. Results and discussion

3.1. Expression of luciferase in INS-1 cells

Expression and cellular localization of luciferase in INS-1 cells was examined by immunofluorescence. In untransfected cells only the background due to non-specific binding of the antibody can be seen (Fig. 1A). In INS-1 cells expressing tetracycline-inducible luciferase (INS-r3-LUC7) the immuno-

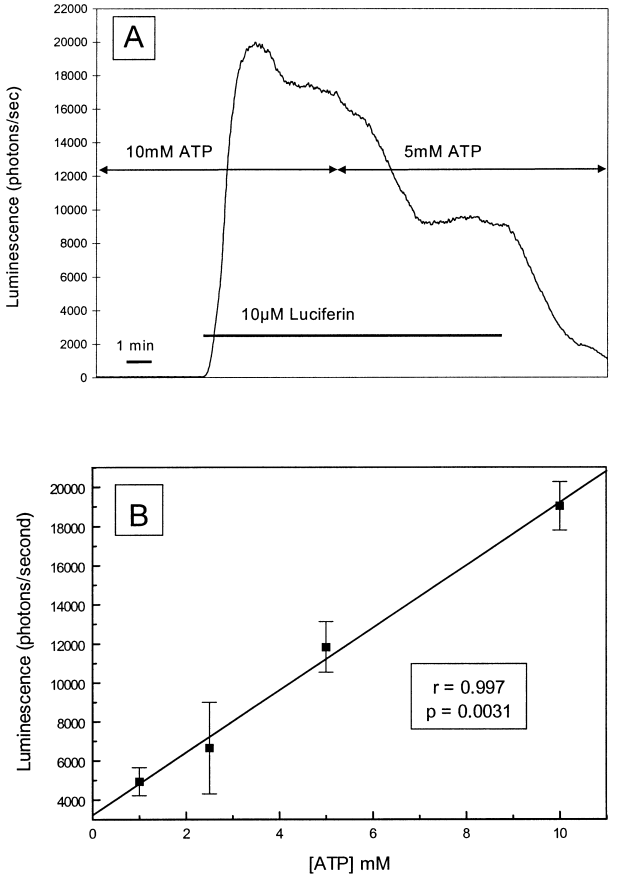


Fig. 2. Cellular ATP calibration in permeabilized cells. Luciferase expression was induced by doxycycline treatment before permeabilization with α-toxin and perifusion with an intracellular-type buffer containing different ATP concentrations ranging from 1 to 10 mM. A: Typical experiment of luminescence monitoring when cells were perifused first with 10 mM then with 5 mM ATP in the presence of 10 μM luciferin. B: Linear regression analysis for the correlation between ATP concentration and luciferin-luciferase-dependent luminescence.

fluorescence revealed a homogeneous cytosolic expression of the protein (Fig. 1B). The luciferase expression is under the control of a reverse tetracycline-dependent transactivator and was therefore induced by the tetracycline derivative doxycycline (1 μg/ml of culture medium for 20–60 h). This permits a very high expression of the transfected gene (around 1000-fold) at the time of the experiment [17,11].

Changes in luminescence were monitored on attached cells maintained at 37°C. In non-transfected control cells only the background emission (approximately 30 photons/s) was measured even in the presence of 10 μM luciferin (Fig. 1C). In luciferase expressing cells the addition of 10 μM luciferin raised the baseline to a plateau of approximately 5000 photons/s. Impairment of mitochondrial function by the uncoupling protonophore FCCP (1 μM) rapidly decreased the signal. This latter effect was not fully reversible, probably indicative of some mitochondrial damage. We have previously observed morphological changes in INS-1 cell mitochondria treated for 30 min with FCCP [18].

3.2. Cellular ATP calibration in permeabilized cells

Luciferase expressing cells were permeabilized with α-toxin and perifused with an intracellular-type buffer containing different ATP concentrations ranging from 1 to 10 mM. In a typical experiment, perifusion first of 10 mM then of 5 mM ATP resulted in a plateau of photon emission of approximately 17 500/s and 9500/s respectively (Fig. 2A). The transient peak above the plateau just after the addition of luciferin is likely to reflect the transition to a new equilibrium binding state of luciferase with its two substrates [19]. There was a linear relationship between ATP perfused into permeabilized cells and photon emission from 1 to 10 mM ATP (Fig. 2B). The constants for the linear regression, $y = ax+b$, were $a = 1600$ and $b = 3209$, $r = 0.997$ and $P = 0.0031$.

3.3. Generation of ATP and insulin secretion

After the doxycycline treatment period for induction of luciferase expression, cells were placed in the termostatted chamber, which permits photon detection and collection of the effluent for insulin measurements. The water volume of INS-1 cells is assumed to be approximately 1 μl per 10^6 cells, a value established for RINm5F cells which are derived from the same rat insulinoma [20]. Using this value and the calibration described above the basal ATP level was 7.9 mM (mean photons/s at basal for 43 traces: 15 845 ± 1577). This value falls into the same range as the ATP concentration measured in a luminometer on cell extracts from non-stimulated cells (6.6 mM or 52.4 ± 2.8 nmol/mg protein). When cells were stimulated for 10 min with 12.8 mM glucose the ATP concentration in cell extracts was increased to 63.6 ± 3.0 nmol/mg protein (+21%, $P < 0.05$, $n = 5$). When monitored by photon emission glucose (12.8 mM) rapidly raised the ATP signal with a plateau around +16% above baseline (Fig. 3A) (mean of +18 ± 2%, $n = 11$ from five experiments). As measured simultaneously, glucose stimulated insulin secretion correlated well with ATP generation but the onset lagged at least 30 s behind that of the ATP signal (Fig. 3B). Methyl-succinate is a precursor of the tricarboxylic acid cycle intermediate succinate and it has been shown to be an insulin secretagogue in pancreatic islets [21] and INS-1 cells [16]. The addition of this mitochondrial substrate (5 mM methyl-succinate) to luciferase expressing cells increased the cellular ATP by +38% measured as luminescence (Fig. 3C) (mean of +40 ± 3%, $n = 13$ from seven experiments). Insulin secreted from the same cell preparation was stimulated 5-fold above baseline (Fig. 3D). In this case the two parameters increased almost simultaneously after a 1 min lag. This lag probably reflects deesterification and transport into the mitochondrial matrix of succinate.

3.4. Inhibition of mitochondrial ATP transport

Atractyloside was used to block the mitochondrial ADP-ATP translocase which transports ADP into the matrix in exchange for ATP [22,23]. The presence of 10 μM atractyloside markedly attenuated the cytosolic increase in ATP concentration induced by methyl-succinate (Fig. 4A). It should be noted that the inhibitor had no effect on basal luminescence (see Fig. 4A first 5 min of pretreatment) nor on luciferase activity as assessed directly in cell lysates up to 100 μM (data not shown). When atractyloside was added 5 min after the beginning of stimulation with methyl-succinate, the signal was reduced only partially (Fig. 4B). After the removal of the inhibitor, and the continuous stimulation with methyl-succinate, the cytosolic ATP levels rapidly rose again. The diminished efficacy of atractyloside in the second compared to the

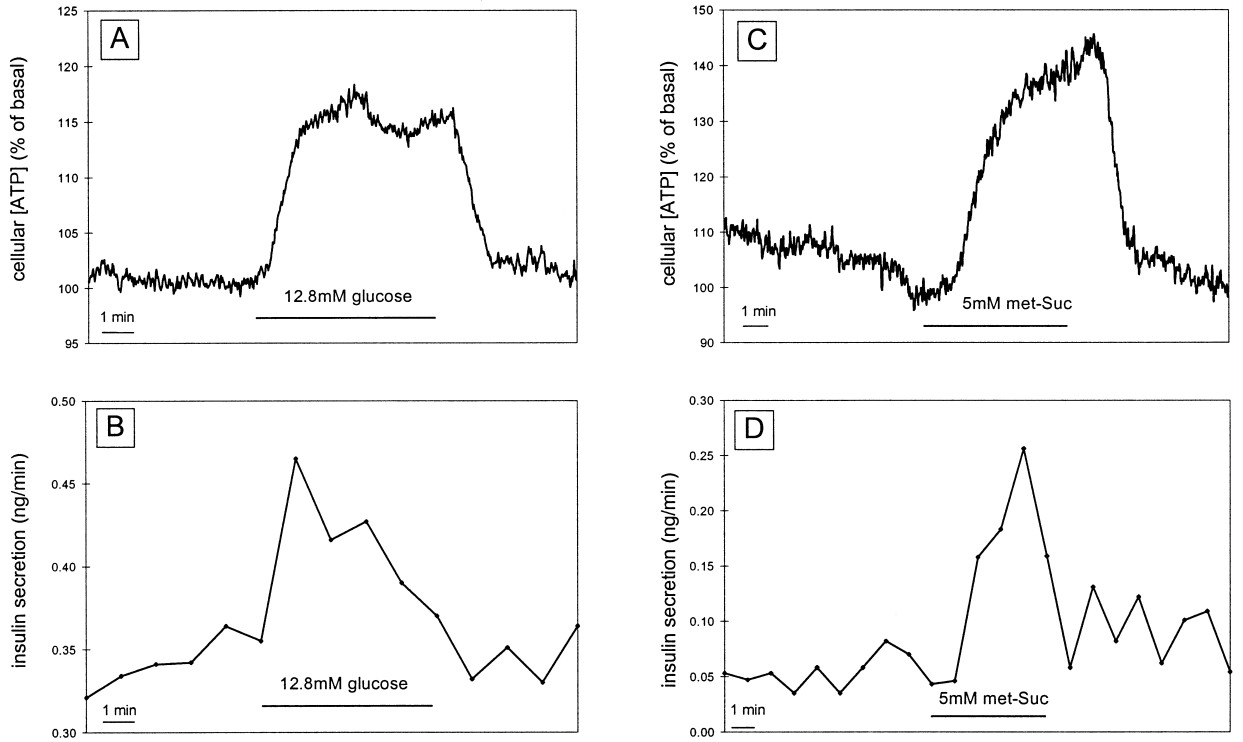
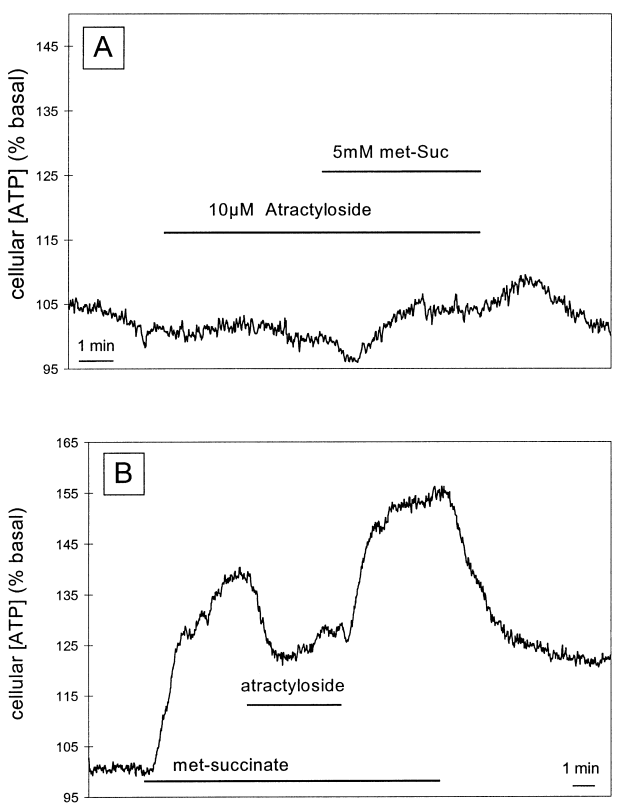


Fig. 3. Nutrient stimulated generation of ATP and insulin secretion. After the doxycycline treatment period for induction of luciferase expression, cells were placed in the termostatted chamber, which permits photon detection and collection of the effluent for insulin measurements. A–B: After baseline perifusion at 2.8 mM glucose, cells were stimulated for 5 min with 12.8 mM glucose and the effects on cellular ATP (A) and insulin secretion (B) were monitored on the same cell preparation. C–D: The same protocol was used for stimulation with 5 mM methyl-succinate.



first protocol may be explained by the accumulation of ADP in the cytosol favoring the production of ATP+AMP by adenylate kinase [24].

The present report shows that cells expressing a high level of luciferase can be used to monitor cytoplasmic ATP changes in living cells. This approach permitted the establishment of the dynamic correlation between ATP levels and insulin secretion in nutrient stimulated cells. Such luciferase expressing cell lines will be a useful tool for the further elucidation of the role of ATP in metabolism-secretion coupling in the pancreatic β-cell. In this context, it will also become necessary to target luciferase to the mitochondria and other cellular compartments.

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Fig. 4. Effect of inhibition of mitochondrial ATP transport on ATP generation. Atractyloside (10 μM) was used to block the mitochondrial ADP-ATP translocase. After induction of luciferase expression by doxycycline treatment, cells were placed in the termostatted chamber to monitor luminescence in the presence of 10 μM luciferin. A: Effect of atractyloside added 5 min prior to methyl-succinate (5 mM) stimulation. B: Effect of atractyloside added for 5 min during the continuous stimulation by 5 mM methyl-succinate.

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