Bioluminescence imaging of nuclear calcium oscillations in intact pancreatic islets of Langerhans from the mouse

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Abstract
The stimulus-secretion coupling for insulin secretion by pancreatic β-cells in response to high glucose involves synchronic cytosolic calcium oscillations driven by bursting electrical activity. Calcium inside organelles can regulate additional functions, but analysis of subcellular calcium signals, specially at the single cell level, has been hampered for technical constrains. Here we have monitored nuclear calcium oscillations by bioluminescence imaging of targeted aequorin in individual cells within intact islets of Langerhans as well as in the whole islet. We find that glucose generates a pattern of nuclear calcium oscillations resembling those found in the cytosol. Some cells showed synchronous nuclear calcium oscillations suggesting that the islet of Langerhans may also regulate the activation of Ca2+-responsive nuclear processes, such as gene transcription, in a coordinated, synchronic manner. The nuclear Ca2+ oscillations are due to bursting electrical activity and activation of plasma membrane voltage-gated Ca2+ channels with little or no contribution of calcium release from the intracellular Ca2+ stores. Irregularities in consumption of aequorins suggests that depolarization may generate formation of steep Ca2+ gradients in both the cytosol and the nucleus, but further research is required to investigate the role of such high [Ca2+] microdomains.

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1. Introduction
Pancreatic β-cells within the islet of Langerhans are responsible for insulin secretion after an increase of blood glucose and their dysfunction causes diabetes mellitus. The stimulus-secretion coupling process involves glucose metabolism, which provokes the closure of ATP-dependent potassium channels (KATP), responsible for the resting membrane potential [1]. As a result, the plasma membrane depolarizes and causes the opening of voltage dependent Ca2+ channels and a rise of the cytosolic Ca2+ concentration ([Ca2+][cyt]) [2]. Glucose-induced Ca2+ signals in all the β-cells within the same islet of Langerhans have a synchronous and homogeneous [Ca2+]cyt oscillatory pattern. This is a consequence of the bursting pattern of electrical activity characteristic of pancreatic β-cells [3,4] which drives Ca2+ oscillations, and the characteristic gap junctional-mediated coupling among the β-cell population [5]. Consequently, insulin secretion is pulsatile, coinciding with the peaks of [Ca2+]cyt [6–8].

In addition to its role in insulin secretion, Ca2+ mediates other processes, such as gene transcription, proliferation and apoptosis. The ability of Ca2+ to elicit different cell responses depends on its route of entry, localization and code of amplitude or frequency of Ca2+ oscillations. The role of local subplasmalemmal gradients in the control of exocytosis is well known [9]. In contrast, information on the role of local Ca2+ signals in subcellular organelle is scarce. It is well estab-
lished that the nucleoplasmic concentration of free calcium ([Ca\(^{2+}\)\(_{\text{nuc}}\)] regulates gene transcription [10], but how nucleoplasmic Ca\(^{2+}\) signals are generated is still unclear.

In pancreatic β-cells, nutrients, such as glucose and certain fatty acids, modulate the expression of several candidate immediate early genes (IEGs), most of them being involved in many aspects of β-cell function including differentiation, proliferation and apoptosis. The action of these nutrients is specific for some IEGs and occurs at the transcriptional level in a Ca\(^{2+}\)-dependent manner [11–13]. Additionally, Ca\(^{2+}\) ions are thought to participate in the transcriptional control of insulin [14,15]. Thus, regulation of Ca\(^{2+}\) dynamics in the nucleus might be important for β-cell function. In pancreatic β-cells there seems to be no Ca\(^{2+}\) barrier between the cytosol and the nucleoplasm [16], but the nuclear envelope contains functional ATP-dependent K\(^+\) channels that may link glucose metabolism, nuclear Ca\(^{2+}\) signaling and gene expression [17]. Therefore Ca\(^{2+}\) signals in nucleus and cytosol may be differentially regulated. Current evidence suggests independent regulation of nuclear calcium in some cell types [18–22].

The aim of the present work is to study nuclear Ca\(^{2+}\) signals in β-cells within intact islets by bioluminescence imaging of targeted aequorins. We find that [Ca\(^{2+}\)\(_{\text{nuc}}\)] was increased in response to stimulation with high glucose. Some individual cells of the islet showed synchronous [Ca\(^{2+}\)\(_{\text{nuc}}\)] oscillations which resemble those triggered by electrical activity in cytosol. [Ca\(^{2+}\)\(_{\text{nuc}}\)] oscillations were dependent of Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels at the plasma membrane but were not affected by emptying of intracellular Ca\(^{2+}\) stores. The existence of this nuclear synchronization indicates that β-cells within each intact islet may also behave as a syncytium in terms of nuclear Ca\(^{2+}\) signals and the regulation of nuclear Ca\(^{2+}\)-responsive processes. This is suggested by the fact that gap-junction-mediated coupling between β-cells is a requirement for an appropriate gene expression [25].

2. Material and methods

2.1. Materials

Twelve-weeks old, male Balb/c mice were kept at the university animal facilities under 12 h light–12 h dark conditions and fed ad libitum. Culture medium, sera and antibiotics were obtained from Gibco. Fura-2 and fluo-3 were purchased from Molecular Probes Europe (Leiden, The Netherlands). Nuclear and cytosolic aequorin cDNAs were obtained from Molecular Probes and cloned in the pHSVpUC plasmid as previously reported [26].

2.2. Pancreatic islet culture and expression of aequorins

Experiments were conducted under the guidelines of the Commission d’Ethique d’Experimenteration Anneule of the University of Louvain School of Medicine. Islets were isolated by collagenase digestion of the pancreas followed by selection by hand [27]. Islets were infected with herpes simplex virus type 1 (HSV1) delivering the targeted aequorins (1–3 x 10\(^3\) infectious virus particles per islet) [26]. Infected islets were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics for 24 h. Packaging and titration of the pHSVnuAEQ (nuclear) and pHSVcytAEQ (cytosolic) viruses was performed as reported previously [26].

2.3. Fura-2 imaging of cytosolic calcium

Cultured pancreatic islets were incubated with fura-2/AM (4 µM) for about 1 h at room temperature in standard medium of the following composition (mM): NaCl, 145; KCl, 5; MgCl\(_2\), 1; CaCl\(_2\), 1; HEPES, 10; pH 7.4; glucose, 10. Then, islets were washed with the same medium and placed in a chamber thermostated at 37 °C in the stage of an inverted microscope (Nikon Diaphot). Islets were then perfused with prewarmed (37 °C) bicarbonate-buffered solution of the following composition (in mM): NaCl, 120; KCl, 4.8; CaCl\(_2\), 2.5; MgCl\(_2\), 1.2; NaHCO\(_3\), 24; glucose, 3. The solution was continuously gassed with 94% O\(_2\)–6% CO\(_2\) to maintain pH at 7.4. Islets were epi-illuminated alternately at 340 and 380 nm, and light emitted above 520 nm was measured using a Magic Image Processor (Applied Imaging, Newcastle, UK). Pixel by pixel ratios of consecutive frames were produced and [Ca\(^{2+}\)], was estimated from these ratios by comparison with fura-2 standards. Other details have been reported earlier [28].

2.4. Confocal microscopy of cytosolic calcium

Swiss albino OF1 male mice (8–10 weeks old) were killed by cervical dislocation according to national guidelines. Pancreatic islets of Langerhans were isolated by collagenase digestion as previously described [27] and loaded with 5 µM Fluo-3 AM for at least 1 h at room temperature. Loaded islets were kept in a medium containing (mM): NaCl, 115; NaHCO\(_3\), 25; KCl, 5; MgCl\(_2\), 1.1; NaH\(_2\)PO\(_4\), 1.2; CaCl\(_2\), 2.5; HEPES, 2.5; bovine serum albumin, 1%; α-glucose, 5 mM. This solution was kept at pH 7.35 by continuous gassing with 95% O\(_2\)–5% CO\(_2\). Islets were perfused with a modified Ringer solution containing (mM): 120 NaCl, 5 KCl, 25 NaHCO\(_3\), 1.1 MgCl\(_2\) and 2.5 CaCl\(_2\); pH 7.35, when gassed with 95% O\(_2\) and 5% CO\(_2\). Experiments were performed at 34 °C. Calcium measurements were performed in individual cells with a Zeiss Pascal 5 confocal microscope equipped with
a $\times 40$ oil immersion objective (N.A., 1.3). Images were collected at 2 s intervals and treated with the Zeiss LSM software package. Results are expressed as the change in fluorescence $\Delta F$ expressed as the percentage of the basal fluorescence ($F_0$) observed in absence of stimulus, $\alpha$ and $\beta$-cells within the islets were identified by their $[Ca^{2+}]_i$ oscillatory pattern in 3 and 11 mM glucose, respectively [29].

2.5. Bioluminescence imaging of islets expressing targeted aequorin

Islets expressing apoaequorins were incubated for 1–2 h at room temperature in standard medium with 1 mM coelenterazine. Then the islets were placed into an incubation chamber thermostated to 37 °C mounted in a Zeiss Axiovert 100 TV microscope and perfused at 5–10 ml/min with the same bicarbonate-buffered medium containing high glucose (11 mM), high K+ (30 mM) or other test solutions, prewarmed at 37 °C. At the end of each experiment, cells were permeabilized with 0.1 mM digitonin in 10 mM CaCl$_2$ to release all the residual aequorin counts. Bioluminescence images were taken with a Hamamatsu VIM photon counting camera handled with an Argus-20 image processor and integrated for 10 s periods. Photons/cell in each image were quantified using the Hamamatsu Aquacosmos software. Data were first quantified as rates of photoluminescence emission/total counts remaining at each time and divided by the integration period ($L_{TOTAL}$ in s$^{-1}$). Emission values of less than 4 c.p.s or 40 c.p.s for individual cells and whole islets respectively, were not used for calculations. Calibrations for $[Ca^{2+}]_i$ are shown with the Figures. More details about bioluminescence imaging of aequorin have been reported previously [24]. For calculation of oscillation indexes the differences (in absolute value) between each $L/L_{TOTAL}$ value and the following one were added and divided by the total number of measurements during the integration period. This parameter is sensitive to both the amplitude and the frequency of oscillations [20,24,30].

2.6. Batch luminescence measurements

In some experiments batch measurements using 2–5 islets were performed in order to increase sensitivity. For these purposes, islets expressing nuclear aequorin were incubated with coelenterazine for 2 h at room temperature. Then 2–5 islets were placed into the incubation chamber of a luminescence monitor constructed by Cairn Research. The islets were covered with a 70 µm mesh nylon net to keep them in place and they were perfused with prewarmed (37 °C) standard medium and photoluminescence emission measured using a photon-counting phototube. Counts were integrated at 1 s intervals and $[Ca^{2+}]_{nuc}$ or $[Ca^{2+}]_{cyt}$ was estimated as previously reported [20,31]. For $[Ca^{2+}]_{cyt}$ measurements, islets were infected with a virus delivering an aequorin construct fused with the luciferase structural gene. The resulting fusion protein locates at the cytosol, but is excluded from the nucleus (data not shown).

3. Results

Adequate expression of the targeted aequorins requires 24 h of culture. Therefore, we investigated first whether the infection and the subsequent culture for 1 day in vitro (DIV) influences the normal cytosolic calcium responses of pancreatic islets. For this end, islets were infected with the virus delivering targeted probes and cultured for 24 h. Then, they were loaded with fura-2 or fluo-3 and subjected to either conventional fluorescence microscopy (Fig. 1A) or confocal microscopy (Fig. 1B). Fig. 1A shows the cytosolic calcium responses imaged in a single fura-2 loaded islet. Increasing the glucose (GLU) concentration from 3 to 11 mM (GLU) induced, after a 2–4 min lag, a rapid increase of $[Ca^{2+}]_{cyt}$ followed by a plateau that was sometimes associated with oscillations occurring over the plateau. When imaged with confocal microscopy, these $[Ca^{2+}]_{cyt}$ oscillations were more apparent in some individual cells (Fig. 1B) with clear signs...
of synchronization among them, as previously documented [4,29]. Return to low glucose concentration restored resting \([Ca^{2+}]_{cyt}\) values. A second stimulation with glucose provoked a second, \([Ca^{2+}]_{cyt}\) increase, which was often associated with slow \([Ca^{2+}]_{cyt}\) oscillations (Fig. 1A). The dihydropyridine VOCC blocker furnidipine prevented the effects of stimulation with high glucose (Fig. 1A). Removal of extracellular calcium had the same effect (not shown). Depolarization with high \(K^+\) provoked larger \([Ca^{2+}]_{cyt}\) increases (Fig. 1A and B), which were also prevented by \(Ca^{2+}\)-free medium or furnidipine (not shown). Taken together, these data indicate that islets cultured for 1 DIV show the same behavior in terms of \([Ca^{2+}]_{cyt}\) responses to glucose and depolarization than freshly obtained pancreatic islets.

We asked next whether bioluminescence imaging of targeted aequorins would enable monitoring dynamics of \([Ca^{2+}]_{nuc}\) in pancreatic islets. Fig. 2A shows the changes of \([Ca^{2+}]_{nuc}\) in an islet stimulated first with high glucose and then with high \(K^+\). Four representative images of photonic emissions superimposed to the bright field image of the pancreatic islet are also shown. In the resting condition the rate of photon emission was very low (Resting in Fig. 2A). Increasing glucose concentration from 3 to 11 mM induced, after a 2–3 min lag, a rise in the rate of photonic emissions (Glucose) that returned to resting values when glucose was again decreased to 3 mM. Depolarization with high \(K^+\) induced a much larger increase in the rate of photonic emissions (High \(K^+\)). Finally, all the remaining aequorin was burned by permeabilization of the islet with digitonin and exposure to excess (10 mM) \(Ca^{2+}\) (Digitonin). Integration of the whole aequorin counts is required for calibration of the signal [31]. This maneuver also revealed all the (infected) individual cells showing large levels of photonic emissions, thus enabling analysis of individual cells within the islet (see supplementary movie). Fig. 2B compares the average changes of \([Ca^{2+}]_{nuc}\) in the whole islet and in four representative individual cells within the same islet. At the single cell level the response to high glucose include \([Ca^{2+}]_{nuc}\) oscillations that were not apparent either before increasing glucose concentration or after returning to low glucose concentration. The oscillatory activity remained as long as perfusion with high glucose was maintained. In Fig. 2C the oscillatory activity has been quantified using the oscillation index, a parameter which reflects both the amplitude and frequency of oscillations [24,30]. The oscillation index increased about 20 fold on stimulation with high glucose and returned back near the resting levels when glucose was decreased to the low concentration (Fig. 2C).

It has been established previously that high glucose induces \([Ca^{2+}]_{cyt}\) oscillations that are synchronized among pancreatic \(\beta\)-cells of the same islet [4,29], and we have shown here that this behavior is preserved in islets cultured during 1 DIV (Fig. 1). We asked then whether the glucose-induced \([Ca^{2+}]_{cyt}\) oscillations could also be synchronized. Fig. 3 shows an experiment in which \([Ca^{2+}]_{nuc}\) was monitored simultaneously in two individual islets (a and b) that became attached during culture. The effects of two consecutive stim-
Fig. 3. Synchronization of nuclear calcium oscillations. (A) Effects of two sequential stimulation with high glucose on nuclear calcium dynamics in two islets (a and b). The traces corresponding to the average of each islet and to five individual cells is shown. (B) Detailed comparison of [Ca$^{2+}$]$_{\text{nuc}}$ oscillations induced by high glucose in two individual cells. Results are representative of three independent experiments.

ulations with glucose in both islets and in several individual cells within them (a1–a3 and b1–b2) are shown. The bright field (left) and photonic emissions (right) images are shown on top. The first stimulation with glucose induced a biphasic increase of [Ca$^{2+}$]$_{\text{nuc}}$ in both islets whereas the second stimulation induced a [Ca$^{2+}$]$_{\text{nuc}}$ increase composed of three slow waves. The behavior of the individual cells illustrates further the synchronization among cells of the same islet. Notice that the slow oscillations occurring during second glucose stimulation were also observed at the level of the cytosol (Fig. 1A).

Fig. 3B illustrates further a quite extensive (though not absolute) synchronization among two cells within the same islet. It has been established that high glucose-induced cytosolic calcium oscillations are due to electrical activity and opening of voltage-gated L-type Ca$^{2+}$ channels (VOCC) [2–4].

We next investigated the source of Ca$^{2+}$ for the [Ca$^{2+}$]$_{\text{nuc}}$ changes induced by high glucose. Fig. 4 shows that removal of extracellular Ca$^{2+}$ abolished the effects of high glucose on [Ca$^{2+}$]$_{\text{nuc}}$ and that these effects resumed on re-addition of Ca$^{2+}$. The effects of glucose on [Ca$^{2+}$]$_{\text{nuc}}$ were also inhibited by furoxipine but not by emptying the intracellular calcium stores with thapsigargin (Fig. 4), suggesting that the intracellular Ca$^{2+}$ stores do not participate in the generation of the [Ca$^{2+}$] responses.

Fig. 5 compares the [Ca$^{2+}$]$_{\text{nuc}}$ responses to sequential stimulation with high glucose and with high K$^+$. Two consecutive applications of high glucose provoked a reproducible aequorin consumption of about 5% (Fig. 5A). In contrast, the first high K$^+$ stimulus consumed much more aequorin than the following ones (25% versus 5%; Fig. 5A). As a consequence,
Fig. 4. The high-glucose-induced [Ca\textsubscript{2+}]\text{nucl} increase is due to activation of voltage-operated calcium channel and not to release of calcium from intracellular stores. Pancreatic islets were infected, cultured and handled as in Fig. 2. The effects of furnidipine (1 μM), Ca\textsuperscript{2+} removal and thapsigargin pre-treatment (1 μM during 1 h) are shown.

The estimate of the [Ca\textsuperscript{2+}]\text{nucl} response (shown as \text{L}/\text{L}_{\text{TOTAL}} in Fig. 5B) was much larger for the first high-K\textsuperscript{+} stimulus than for the two following. Conversely, both fura-2 (Fig. 1A) and fluo-3 (Fig. 1B) reported reproducible [Ca\textsuperscript{2+}]\text{cyt} responses to repetitive stimulations with high K\textsuperscript{+}. Fig. 5C compares the estimates of [Ca\textsuperscript{2+}]\text{cyt} and of [Ca\textsuperscript{2+}]\text{nucl} for the first and the second high K\textsuperscript{+} stimulus obtained in several experiments. [Ca\textsuperscript{2+}]\text{cyt} was measured using both approaches, fura-2 and aequorin. Measurements using aequorins reported that the first response to K\textsuperscript{+} was about four times larger than the following ones. Despite these differences between consecutive K\textsuperscript{+} pulses, Ca\textsuperscript{2+} values in the cytosol and in the nucleus were similar for each stimuli, suggesting that changes of [Ca\textsuperscript{2+}]\text{cyt} are faithfully transmitted to the nucleus (see Section 4).

4. Discussion

Calcium signals within cells are sculpted in time and space in the form of calcium oscillations, waves and microdomains. This allows specific regulation of different cell functions, for example, regulated exocytosis in the subplasmalemmal region, respiration in mitochondria or gene transcription in nucleus. Measurements of subcellular calcium concentration inside discrete organelles or functional domains has been hampered by technical constrains, but development of targeted (protein) probes is paving the way for this analysis. For example, the bioluminescent probe aequorin has been used to monitor calcium changes in different organelles including the nucleus [23], even though single-cell imaging of targeted aequorin in discrete organelles has been a very difficult task achieved only in a few cases [20,24,32]. Here we have used this novel approach to gain new insights into the homeostasis of nuclear calcium in whole pancreatic islets and its regulation by glucose. As demonstrated by in vivo studies [33], intact whole islets constitute a preparation closer to the physiological situation than isolated single pancreatic cells or islet-derived cell lines, especially when considering glucose-induced Ca\textsuperscript{2+} signals.

We find that stimulation by high glucose produced an increase of [Ca\textsuperscript{2+}]\text{nucl}, which often took the form of oscillations that were synchronized in numerous single cells within the islet of Langerhans. This [Ca\textsuperscript{2+}]\text{nucl} response pattern resembles the [Ca\textsuperscript{2+}]\text{cyt} response, synchronous and oscillatory, responsible for the pulsatile secretion of insulin [6–8]. Thus, our results suggest that the cytosolic calcium...
Ca\textsuperscript{2+} signals and intracellular Ca\textsuperscript{2+} release sites in spatial studies, spot confocal microscopy, excels in measuring local reasons. First, while the technique used in these previous studies, spot confocal microscopy, excels in measuring local signals of different amplitude in the cytosol and the nucleus of isolated pancreatic \( \beta \)-cell [12-15]. Thus, the nuclear calcium oscillatory pattern reported here could lead to a pulsatile pattern of oscillations found here.

Whether nuclear and cytosolic calcium signaling are differentially regulated is a matter of controversy. Depending on the cell type studied or the technical approach employed, differences supporting either one view or the other have accumulated [18-22]. In the case of the pancreatic \( \beta \)-cell, a previous study concluded that glucose induced parallel changes in both [Ca\textsuperscript{2+}]\textsubscript{nuc} and [Ca\textsuperscript{2+}]\textsubscript{cyt} of isolated individual cells from the ob/hh mouse [16]. However, it has been reported recently that activation of K\textsubscript{ATP} channels located at the nuclear envelope could lead to changes of [Ca\textsuperscript{2+}]\textsubscript{nuc} that may modulate nuclear function [17]. In addition, some nutrients may induce Ca\textsuperscript{2+} signals of different amplitude in the cytosol and the nucleus of isolated pancreatic \( \beta \)-cells [43]. Here we have found in intact islets that high glucose induces a similar pattern of [Ca\textsuperscript{2+}]\textsubscript{nuc} oscillations in the cytosol and in the nucleus. In addition, the response to glucose was dependent on Ca\textsuperscript{2+} entry through VOCCs in both the cytosol and the nucleus and not affected by emptying intracellular calcium stores with thapsigargin.

Thus, our observations using targeted aequorins support the hypothesis of glucose dependent electrical activity and ATP-regulated K\textsuperscript{+} currents in isolated mouse pancreatic beta-cells, FEBS Lett. 261 (1990) 187-190.


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