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Osmotic induction of calcium accumulation in human embryonic kidney cells detected with a high sensitivity FRET calcium sensor

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Abstract

Calcium serves as a second messenger in glucose-triggered insulin secretion of pancreatic cells. Less is known about sugar signaling in non-excitabile cells. Here, the high sensitivity FRET calcium sensor TN-XXL was used to characterize glucose-induced calcium responses in non-excitabile human embryonic kidney HEK293T cells. HEK293T cells responded to perfusion with glucose with a sustained and concentration-dependent increase in cytosolic calcium levels. Sucrose and mannitol triggered comparable calcium responses, suggesting that the increase of the calcium concentration was caused by osmotic effects. HEK293T cells are characterized by low endogenous glucose uptake capacity as shown with a high sensitivity glucose sensor. Consistently, when glucose influx was artificially increased by co-expression of GLUT glucose transporters, the glucose-induced calcium increase was significantly reduced. Neither calcium depletion, nor gadolinium or thapsigargin were able to inhibit the calcium accumulation. Taken together, membrane impermeable osmolytes such as sucrose and mannitol lead to an increase in calcium levels, while the effect of glucose depends on the cell's glucose uptake capacity and will thus vary between cell types in the body that differ in their glucose uptake capacity.

Keywords

GLUT; glucose transporter; calcium homeostasis; osmotic

1. Introduction

Blood sugar levels are kept within tight limits to ensure adequate supply to organs such as the brain that are fully dependent on external supply and to prevent accumulation to toxic levels. To achieve this tight control, uptake of glucose from the blood stream into muscle cells and the release of glucose from the liver are regulated in a sugar-level dependent manner. Two major pathways are involved in this process: glucose-induced insulin release from pancreatic cells and insulin-triggered induction of glucose transporter activity in muscle cells. Pancreatic cells and neurons continuously measure glucose content in blood

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using a glucose-derived signaling cascade that leads to activation of K_{ATP} channels. Activation of ATP-sensitive potassium K_{ATP} channels in the hypothalamus is sufficient to lower blood glucose levels through inhibition of hepatic gluconeogenesis [1]. In both cases, activation of the channels leads to calcium influx, triggering insulin secretion. Several studies suggest that cells outside the pancreas and the brain also respond to glucose with a calcium change [2, 3]. Calcium signaling in turn then modulates glucose uptake, e.g. in muscle cells [4].

A recent study used the calcium dye Fura-2 combined with electrophysiological analyses to show that human embryonic kidney cells accumulate calcium when glucose levels drop [5]. Calcium levels increased with decreasing glucose levels. The calcium accumulation appeared to be mediated by a novel signaling pathway since it was insensitive to a wide spectrum of calcium channel inhibitors.

Recently, a suite of highly sensitive genetically encoded FRET sensors had been developed, including sensors for ions such as calcium [6, 7] and phosphate [8], as well as for sugars [9] and amino acids [10, 11]. Here we used the troponin C-based calcium FRET sensor TN-XXL [7] and a highly sensitive FRET sensor for glucose [9] to measure the calcium responses elicited by increasing rather than decreasing glucose levels in the medium.

TN-XXL reported sustained and glucose concentration-dependent accumulation of calcium in the cytosol of human embryonic kidney HEK293T cells. The response was readily reversible when glucose was removed. Quantitatively comparable responses were observed for glucose, sucrose and mannitol, demonstrating that the calcium accumulation was osmotically induced. Co-expression of the human glucose transporter GLUT1 lead to increased glucose uptake activity as evidenced by the glucose FRET sensor FLII¹²Pglu-700 μ 86. The increased glucose uptake capacity lead to a reduction in the glucose-induced calcium accumulation, suggesting that the uptake capacity of a given cell will determine whether a cell accumulates calcium when blood glucose levels change. No evidence for calcium spiking was found. Similar as in the case of decreasing glucose levels, the calcium accumulation was insensitive to a wide range of inhibitors.

2. Methods

2.1. Cells, DNA constructs and Reagents

HEK293T cells were obtained from the Craig Garner lab at Stanford University. The troponin C-based calcium sensor TN-XXL and the high-sensitivity glucose sensor FLII¹²Pglu-700 μ 86 have been described previously [7, 9]. To express GLUT1 and GLUT2 in HEK293T cells, the ORFs were amplified by RT-PCR from human liver total RNA (Clontech). GLUT1 was subcloned into pcDNA3.2/v5-DEST (Invitrogen) by LR reaction (Invitrogen) from GLUT1-pENTR-TOPO [12]. GLUT2 was subcloned into pIRES (Clontech). The GLUT expression plasmids as well as the expression vector expressing the glucose sensor are available from Addgene (www.addgene.org).

2.2. Cell culture and transfection

HEK293T cells were grown in high glucose DMEM (Invitrogen) with 10 % fetal calf serum, 50 U/ml penicillin and 50 μ g/ml streptomycin (Invitrogen). Cells were cultured at 37°C and 5 % CO₂. For FRET analysis, transfected cells were cultured on collagen coated round cover glasses (2.5 mm diameter) in 6-well plates. The cells were transiently transfected at 50 %–70 % confluence using Lipofectamine 2000 Reagent (Invitrogen) in Opti-MEM I reduced serum medium (Gibco). After transfection, cells were cultured for ~6 h in Opti-DMEM. On day 2 after transfection, the medium was replaced and cells were analyzed. Cover glasses

with the attached cells were mounted in a perfusion-chamber (Vacu-Cell™ perfusion chamber, VC-MPC-TW; C&L Instr., Hershey, PA).

2.3. Image acquisition and FRET analysis

FRET analysis was performed on an inverted fluorescence microscope (DMIRE2, Leica) with an on-chip multiplication gain QuantEM camera (Roper Scientific) and a 40x oil immersion objective (NA1.25-0.75, IMM HCxPL Apo CS, Leica). Dual emission intensity ratios were simultaneously recorded using a DualView (Optical Insights) unit with a dual eCFP/eYFP-ET filter set (high transmission modified Magnetron sputter-coated filter sets ET470/24m (470 indicates emission wavelength in nm, 24 indicates bandwidth) and ET535/30 (Chroma, USA) and Slidebook 4.2 software (Intelligent Imaging Innovations). Excitation (excitation filter; eCFP ET430/24x, eYFP ET500/20x; Chroma) was provided by a Lambda DG4 (Sutter Instruments) using 100% Xenon lamp output. Images were acquired within the linear detection range of the camera at intervals of 5 sec. Quantitative data were derived by pixel-by-pixel integration of regions defined in the ratiometric images. The fluorescence intensity (in arbitrary units A.U.) for the eCFP and Citrine emission channels was monitored both at eCFP and Citrine excitation. The FRET index ratio, F_c/D for sensitized emission was calculated from the peak intensity ratios (emission of Citrine/CYP at CFP_{ex}) using background and fluorescence bleed-through corrections [13]. The FRET index $F_c = F_f - D_f \times (F_d/D_d) - A_f \times (F_a/A_a)$; F_f is the sensitized emission (eCFP_{ex}/Citrine_{em}), D_f is the CFP emission from the FRET sensor (eCFP_{ex}/eCFP_{em}), A_f is the apparent YFP emission from the FRET sensor (Citrine_{ex}/Citrine_{em}); $F_d/D_d = 77.3 \pm 0.1\%$, $F_a/A_a = 5.30 \pm 0.07\%$. Depending on the expression level of the nanosensor, exposure times were varied between 100-200 msec, the EM gain was varied between 300 and 600, software binning was set to 1. Perfusions were performed with Hanks' buffered saline (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 1.26 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM MgSO₄, pH 7.4) using a computer-controlled 8-channel gravity flow system equipped with a perfusion pencil (AutoMate Sci., Berkeley) attached to the Vacu-Cell perfusion chamber at a flow rate of 1.0 ml/min. The baseline of the recordings was corrected using a 3rd order polynomial fit of the FRET index measured in the absence of glucose in Matlab (the script was programmed by Dr. Oliver Schweissgut, <http://www.uni-siegen.de/fb11/simtec/software/fret/>). Changes in the FRET index for sensors expressed were measured in at least double independent perfusion experiments, each set of data comprised 5-26 cells in the field of view. TXXXL responses were calibrated using ionomycin [6]. R_{min} and R_{max} were 5.1 ± 0.01 and 1.0 ± 0.003 , respectively. Cell area was quantified from fluorescence images using ImageJ software.

3. Results

3.1. Sugar-induced sustained and inhibitor-insensitive calcium accumulation in HEK293T cells

A variety of studies found that glucose induces calcium spikes [14]. To characterize the calcium responses in more detail, the high sensitivity FRET calcium sensor TN-XXL was expressed in the cytosol of HEK293T cells. Perfusion with a bolus of 40 mM glucose induced a slow ($F_c/D \sim 0.2 \text{ min}^{-1}$) and sustained accumulation of calcium (Fig. 1A). The response reversed slowly when glucose was removed. The glucose-induced calcium response corresponded to ~3% of the maximal range of the sensor, and thus to only a small absolute increase in cytosolic calcium (data not shown). To test whether calcium enters the cell from the medium or is released from intracellular stores, cells were exposed to gadolinium, which blocks Gd³⁺-sensitive, stretch-activated cation channels (SAC; Fig. 1B), nifedipine, a dihydropyridine calcium channel blocker (Fig. 1C), and thapsigargin, an inhibitor of the ER Ca²⁺-ATPase (Fig. 1D and E). None of the inhibitors had a significant

effect on calcium accumulation; in the case of thapsigargin no effect was detectable even when cells were exposed to the drug for extended periods (15 min and 1 hr). Also, Ca^{2+} -depletion did not lead to a significant effect of the Ca^{2+} response to glucose (30 min incubation in medium without Ca^{2+} ; Fig. 1F). The sustained response and the drug insensitivity suggest that calcium accumulation was osmotically induced.

3.2 Calcium accumulation is negatively correlated with glucose transport activity

To test whether sugars that can not enter the cells such as sucrose would induce comparable responses to glucose, cells expressing TN-XXL were exposed to boluses of sucrose and glucose (Fig. 2). Both sucrose and glucose induced a comparable, concentration dependent accumulation of calcium. The simplest explanation was that HEK293T cells have only a limited capacity to take up glucose or sucrose, and thus both sugars act osmotically. To test directly for the ability of HEK293T cells to accumulate glucose, steady state glucose levels and their change after addition or removal of glucose from the medium was quantified in cells expressing the high sensitivity FRET glucose sensor FLII¹²Pglu-700 μ 86 [9] (Fig. 3A). In contrast to HepG2 cells, which readily accumulate glucose with a $K_{0.5}$ of about 1.54 mM [15], glucose accumulation was not detectable in the HEK293T cells. However, high rates of glucose accumulation could be induced by co-expression of the human glucose transporters GLUT1 or GLUT2 (Fig. 3B and data not shown), confirming that glucose uptake capacity of HEK293T cells is limiting. As predicted, the calcium increase was diminished in cells that co-expressed GLUT1 or GLUT2 with the calcium sensor TN-XXL (Fig. 4A and B). The response to sucrose, which is not taken up effectively by the cells, was unaffected in cells overexpressing GLUTs.

3.3. Volume changes may at least in part explain the increase in calcium levels

Visual inspection of the images from the time series shown in Figure 2B suggested that addition of sugars led to a decrease in cell size (Fig. 5). Quantitative analysis of the fluorescence demonstrates a glucose concentration-dependent loss in cell area; with a decrease by 10.2 ± 2.8 % for cells that had been perfused with 40 mM glucose. Calcium levels were determined by epifluorescence microscopy, thus the decrease in volume cannot be inferred directly, however a 10% decrease is expected to lead to a corresponding increase in calcium concentration. Thus the reduction in volume due to osmotic changes can at least in part explain the increase in cytosolic calcium levels.

3.4. Calcium accumulation at elevated base levels of glucose

In order to observe maximal calcium responses, the base level of glucose in the medium was 0 mM in all experiments shown above. To test, whether qualitatively similar responses can be detected at more physiological conditions, cells were perfused with medium containing 4 mM glucose and boluses of 10, 25 and 40 mM glucose were given (Fig. 5B). Calcium showed also a sustained accumulation in response to glucose addition, but was reduced corresponding to the lower differential across the cell membrane (compare Fig. 2A). This finding suggests that cells that have a limited ability to accumulate glucose respond to the osmotic challenge caused by a glucose bolus with sustained accumulation of calcium.

Discussion

Sugars are known to act as signals, e.g. for insulin secretion in pancreatic cells or for controlling sugar uptake capacity in yeast. Signaling is mediated by sugar receptors such as $\text{G}\alpha$, or transceptors such as SNF3 [16, 17]. Alternatively signaling can occur intracellularly, e.g. hexokinase is thought to act as both glucose-phosphorylating enzyme and as a receptor [18]. Furthermore, downstream metabolites, e.g. ATP levels, can be detected by the cell and lead to inactivation of K^+ channels as in the case of pancreatic sugar signaling [19]. In

addition, sugars can affect the osmotic status of a cell triggering osmotic acclimation responses.

The data presented here show that glucose boluses can lead to calcium accumulation in human embryonic kidney cells. The glucose response is concentration-dependent and readily reversible with no signs of calcium spiking. The comparatively low noise at 5 sec acquisition intervals indicates that calcium spiking is absent under the conditions tested. Boluses of 10mM glucose trigger calcium accumulation in cells kept at either 0 or 4 mM basic glucose levels.

As shown with the help of FRET glucose sensors, HEK293T cells are characterized by very low endogenous glucose uptake capacity. Co-expression of the glucose transporter GLUT1 largely eliminates the calcium response since expression of GLUT1 allows glucose to cross the membrane; thus glucose can not act an osmoticum. Other sugars that can not cross the cell membrane such as mannitol or sucrose induce quantitatively comparable calcium responses as glucose in HEK293T cells. This suggests that the calcium response will be very different depending on whether a given cell type has transporters sufficiently active to prevent osmotic calcium accumulation. The calcium response was insensitive to a wide range of calcium channel blockers, also depletion of calcium from the stores by extended exposure to calcium-free medium did not alter the glucose-induced calcium response. It is conceivable, that besides an increase in calcium concentration caused by shrinkage, the normal Ca^{2+} extrusion mechanisms ($\text{Na}^+/\text{Ca}^{2+}$ exchanger or plasma membrane Ca^{2+} pump) are osmotically inhibited [20].

In summary, at least in non-excitable HEK293T cells, no signs of glucose-induced calcium signaling were observed; and the observed calcium accumulation can, at least in part, be attributed to osmotically induced volume changes.

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Abbrev.

FLIP	fluorescent indicator protein
FRET	Förster (fluorescence) resonance energy transfer
gluc	glucose

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Figure 1.

FRET analysis of intracellular calcium level with TN-XXL in HEK293T cells. Cells were perfused with 40 mM glucose (A-F). Open bars with gray columns indicate the bolus of external glucose during perfusion with glucose-free Hanks' balanced buffer. Quantitative data were derived by pixel-by-pixel integration of the ratiometric images. The fluorescence intensity in arbitrary units (A.U.) for individual eCFP (ET470/24m) and Citrine (ET535/30m) emission channels were monitored with eCFP excitation (ET430/24x) and the FRET index for Venus and eCYP was determined (Y-axis corresponds to sensitized fluorescence F_c (background and bleed-through corrected using Citrine excitation (ET500/20x)) normalized to donor emission; note that peak intensities were used and that only the short wavelength emission peak of eCFP was considered for donor emission). FRET images were acquired every 5 sec and cytosolic calcium levels were analyzed. HEK293T cells were preincubated with 50 μM gadolinium (30 min) (B), 10 μM nifedipine (30 min) (C), 1 μM thapsigargin (15 min; D, 30 min; E) or no calcium (30 min) (F) without

glucose. When steady-state calcium values were reached, a bolus of 40 mM glucose was added for 2 min. Data are shown as mean $\pm$ SD (n = 5-12).

Figure 2.

Sugar-induced calcium accumulation in HEK293T cells. Red (sucrose) and blue (glucose) bars indicate the loading time for external sugars (5, 25, and 40 mM) during continuous perfusion with glucose-free Hanks' balanced buffer (A). FRET images were acquired and data were analyzed as described in Fig. 1. Data are shown as mean $\pm$ SD ($n = 8$). Bleed-through corrected ratio images are shown in pseudo-color ranging from blue (low) to red (high) (B). Bar corresponds to 10 μ m.

Figure 3.

Cytosolic glucose levels in HEK293T cells using FLII¹²Pglu-700μδ6. Cells were perfused with different external glucose concentrations. Blue bars indicate the exposure time for a given bolus of external glucose (5, 25, and 40 mM) during perfusion with glucose-free Hanks' balanced buffer in the absence (A) and in the presence of GLUT2 (B). FRET images were acquired and data were analyzed as described for Fig. 1. Data are shown as mean ± SD (n = 18-26). The bleed-through corrected ratio images are shown in pseudo-color ranging from blue (low) to red (high) (C). Bar is 10 μm.

Figure 4.

Calcium levels in HEK293T cells expressing GLUT1 or GLUT2 determined by TN-XXL. Bars indicate the loading time for external sugars (sucrose and glucose; 5, 25, and 40 mM, mannitol 40 mM) during perfusion with glucose-free Hanks' balanced buffer in the presence of GLUT1 (A) or GLUT2 (B). FRET images were acquired and data were analyzed as described for Fig. 1. Data are shown as mean $\pm$ SD ($n = 7-8$).

Figure 5.

Sugar-induced volume change measurement in HEK293T cells expressing GLUT2 and analysis of calcium accumulation in HEK293T cells at physiologically relevant base glucose levels. (A) HEK293T cells expressing TN-XXL were perfused with different glucose concentrations (data from Figure 2). Four images corresponding to four time points from each bolus were analyzed. The outline of the cells was determined from black and white images and the cell area was determined by pixel counting using ImageJ. (B) HEK293T cells expressing TN-XXL were perfused with a base glucose concentration of 4 mM and glucose boluses of 10, 25, and 40 mM were applied. Blue (glucose) bars indicate the loading time of external sugar during continuous perfusion with 4 mM glucose in Hanks' balanced buffer.