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Pancreas

Calcium signaling in a genetically engineered human pancreatic β -cell line

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Abstract:	<p>Objectives: The use of primary human β-cells for studying Ca^{2+} signaling is limited by the scarcity of human pancreatic islets. Rodent insulinoma cell lines are widely used but it is difficult to extrapolate results obtained from rodent cells to human. Recently a genetically engineered human β-cell line, EndoC-BH1 has been developed. We have examined whether the EndoC-BH1 cells could be used as a model for studying Ca^{2+} signaling in the β-cells.</p> <p>Methods: We used microscope-based fluorometry to measure cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) from fura-2 loaded single EndoC-BH1 cells cultured on glass coverslips. Ca^{2+} responses to different agonists of insulin secretion were studied. Insulin secretion was measured by radioimmunoassay.</p> <p>Results: EndoC-BH1 cells secreted insulin in response to glucose in a dose dependent manner and the secretion was enhanced by GLP-1. Glucose, potassium chloride, carbachol, L-arginine, and tolbutamide increased $[\text{Ca}^{2+}]_i$ in the EndoC-BH1 cells. We found that GLP-1 was essential for Ca^{2+} response to glucose and tolbutamide.</p> <p>Conclusions: We conclude that the EndoC-BH1 cells can be used as model cells to study Ca^{2+} signaling and stimulus secretion coupling in the human β-cells.</p>

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Calcium signaling in a genetically engineered human pancreatic β -cell line

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Conflicts of Interest and Source of Funding

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Abstract

Objectives: The use of primary human β -cells for studying Ca^{2+} signaling is limited by the scarcity of human pancreatic islets. Rodent insulinoma cell lines are widely used but it is difficult to extrapolate results obtained from rodent cells to human. Recently a genetically engineered human β -cell line, EndoC-BH1 has been developed. We have examined whether the EndoC-BH1 cells could be used as a model for studying Ca^{2+} signaling in the β -cells.

Methods: We used microscope-based fluorometry to measure cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) from fura-2 loaded single EndoC-BH1 cells cultured on glass coverslips. Ca^{2+} responses to different agonists of insulin secretion were studied. Insulin secretion was measured by radioimmunoassay.

Results: EndoC-BH1 cells secreted insulin in response to glucose in a dose dependent manner and the secretion was enhanced by GLP-1. Glucose, potassium chloride, carbachol, L-arginine, and tolbutamide increased $[\text{Ca}^{2+}]_i$ in the EndoC-BH1 cells. We found that GLP-1 was essential for Ca^{2+} response to glucose and tolbutamide.

Conclusions: We conclude that the EndoC-BH1 cells can be used as model cells to study Ca^{2+} signaling and stimulus secretion coupling in the human β -cells.

Key words

Calcium signaling, islets of Langerhans, human β -cell line, insulin, stimulus secretion coupling, and EndoC-BH1 cells.

List of abbreviations

$[\text{Ca}^{2+}]_i$ - Cytoplasmic free Ca^{2+} concentration.

GLP-1- Glucagon like peptide 1

K_{ATP} - ATP sensitive potassium channels

PI-PLC - phosphatidylinositol-specific phospholipase C

VGCC - Voltage gated Ca^{2+} channels

Introduction

Ca^{2+} signaling is essential for stimulus-secretion coupling in the pancreatic β -cells. In these cells, nutrients like glucose, increase the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) that triggers the exocytosis of insulin. Different agonists increase the $[\text{Ca}^{2+}]_i$ in the β -cells by different mechanisms that include the participation of different ion channels, and different intracellular second messengers. Glucose increases $[\text{Ca}^{2+}]_i$ mainly by depolarizing the plasma membrane potential, and thereby activating the voltage-gated Ca^{2+} channels (VGCC). This process requires metabolism of the hexose, increase in the cytoplasmic ATP/ADP ratio, and closure of the ATP-sensitive potassium (K_{ATP}) channels. Closure of the K_{ATP} channels increases the input resistance of the β -cells so that small inward depolarizing currents can further depolarize the membrane potential to the threshold for activation of the VGCCs¹. Sulphonylurea drugs used in the treatment of diabetes also stimulate insulin secretion by

closing the K_{ATP} channel. On the other hand, neurotransmitters like acetylcholine increase the $[Ca^{2+}]_i$ by the inositol 1,4,5-trisphosphate-mediated release of Ca^{2+} from the endoplasmic reticulum, and subsequent entry of Ca^{2+} through the store-operated Ca^{2+} channels.

During the past decades, investigators have used a variety of rodent insulinoma cell lines for studying the basic mechanisms of Ca^{2+} signaling and stimulus-secretion coupling in the β -cells. Such cell lines include the rat insulinoma RINm5F cells and INS-1 cells, the hamster insulinoma HIT-T15 cells, and the mouse insulinoma β -TC3 cells and MIN6 cells². It is convenient to use such cells for Ca^{2+} studies because these cells can be cultured and propagated in vitro rather easily, and they provide a ready source of pure β -cells. These cells can be grown on glass cover slips, can be loaded with the acetoxymethyl ester forms of different fluorescent Ca^{2+} indicators, and $[Ca^{2+}]_i$ can be measured by microscope-based fluorometry from single cells.

While rodent insulinoma cells have been extensively used for the study of Ca^{2+} signaling, it has always been a concern that results obtained from such cells cannot be extrapolated to the human β -cells. Over the years, it has proved extremely difficult to develop human insulinoma cell lines. Recently, a genetically engineered human β -cell line has become available³. These cells secrete insulin in response to glucose in a dose dependent manner. Ca^{2+} signaling in these human insulinoma cells has not been reported. In this study, we have measured $[Ca^{2+}]_i$ from the fura-2 loaded single human insulinoma EndoC-BH1 cells by microscope-based fluorometry. We have found that the EndoC-BH1 cells respond to the agonists that increase $[Ca^{2+}]_i$ in the rodent insulinoma cells, and the primary β -cells by an increase of $[Ca^{2+}]_i$.

Materials and methods

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), HEPES, fetal calf serum (FCS), 2-mercaptoethanol, penicillin-streptomycin, sodium pyruvate, Hank's balanced salt solution (HBSS), PBS, fura-2 acetoxymethyl ester (fura-2 AM) and trypsin-EDTA, were purchased from Life technologies, Sweden. Human transferrin, sodium selenite, bovine serum albumin (BSA) fraction V, nicotinamide, extracellular matrix gel, fibronectin from bovine plasma, GLP-1 (7-37), tolbutamide, D-glucose, and carbachol, were from Sigma-aldrich, Sweden.

Culture of the human insulinoma EndoC-BH1 cells

EndoC-BH1 cells were a generous gift from Philippe Ravassard and Raphael Scharfmann, ICM, Biotechnology & biotherapy, France. The cells were grown in culture flasks containing DMEM (Glucose 1 g/L), BSA fraction V (2 % w/v), 2-mercaptoethanol (50 μ M), nicotinamide (10 mM), transferrin (5.5 μ g/ml), sodium selenite (6.7 ng/ml), penicillin (100 units/ml) and streptomycin (100 μ g/ml). The cells were cultured at 37 °C in a humidified incubator in 5 % CO₂. The cells were passaged once every second week. We found that these cells were more difficult to culture, compared to, for instance, the rat insulinoma, INS-1E cells.

Cell culture support

The EndoC-BH1 cells were grown on matigel/fibronectin-coated flasks with DMEM containing high glucose (4.5 g/L), penicillin /streptomycin (1 %), fibronectin (2 μ g/ml), and ECM (1 % V/V). The coating volume was 2.5 ml for a 25 cm² flask. The culture flask was

coated with the culture support, and incubated for one hour in 5 % CO₂ at 37 °C, before the cells were seeded.

Sub-culture of the EndoC-BH1 cells

The cells were rinsed with PBS and then treated with trypsin-EDTA for 3 min in 5 % CO₂ at 37 °C in an incubator. A neutralization solution containing PBS (80 % V/V) and FCS (20 % V/V) was then added to stop the trypsin action. The cell suspension was then centrifuged for five minutes at 700 g and the supernatant aspirated. The cells were re-suspended in fresh culture medium, and were seeded at a density of ~70000 cells/cm² on the coated cell support.

Measurement of insulin secretion from the EndoC-BH1 cells

The EndoC-BH1 cells were cultured in a 24 well plate that were first coated with the cell culture support. The cells were seeded at a density of 226 000 cells/cm². After seeding, the cells were grown for 48 h in DMEM medium with glucose (5.5 mM), BSA (2% w/v), 2-mercaptoethanol (50 µM), nicotinamide (10 mM), transferrin (5.5 µg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml). After that, the cells were incubated overnight in the glucose-starving medium containing DMEM with low glucose (2.8 mM), BSA (2% w/v), 2-mercaptoethanol (50 µM), nicotinamide (10 mM), transferrin (5.5 µg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml). On the day of the experiments, the cells were incubated in KRBH buffer supplemented with glucose (0.5 mM) for one hour, and then with an increasing concentrations of glucose (3, 11, 16.7 and 25 mM glucose). In each experiment three wells were dedicated for each concentration of glucose. The cells were incubated in 37°C at 5% CO₂ for one hour. After the incubation, the supernatant was removed and stored at -20 °C. Total insulin content was measured after extraction of insulin overnight at 4 °C in acid-ethanol (70 % v/v).

Immunoreactive insulin was measured by radioimmunoassay using polyclonal insulin antibodies (Fitzgerald Inc, USA), and human insulin as standard.

Measurement of $[Ca^{2+}]_i$ by microfluorometry

EndoC-BH1 cells were incubated for 40 min in 2 ml of the “loading buffer” consisting of DMEM containing low glucose (1 g/L), fura-2 AM (1 μ M), and BSA (2 %). The coverslips were then incubated for another 15 min in modified Krebs-Ringer Bicarbonate (KRB) buffer containing NaCl (116 mM), KCl (5 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (24 mM), HEPES (10 mM, pH 7.4), BSA (0.2 %), and glucose (3 mM), to let the endogenous esterases hydrolyze the AM ester. A coverslip was then mounted on an open perfusion chamber, which was then placed on the stage of an inverted microscope (CK 40, Olympus Inc, Japan). Solutions were perfused by a peristaltic pump. A water bath and a thermistor connected to the perfusion chamber were used to control the temperature of the solution in the perfusion chamber. For measurement of $[Ca^{2+}]_i$ from single EndoC-BH1 cells, the large and round cells that looked like differentiated β -cells were chosen. The fluorescence was measured by dual wavelength excitation ratiometric fluorometry. A monochromator (PhotoMed DeltaRam) generated two excitation wavelengths that were directed onto the cells by a dichroic mirror. The emitted light chosen by a 510 nm filter was detected by the photomultiplier tube detector, and monitored by the Felix32 software (Photon Technology International, Inc). The cells loaded with fura-2 were excited at 340 nm and 380 nm alternately. The ratios between the emitted fluorescence intensities upon excitations at 340 nm and 380 nm (F340/F380) were calculated by the Felix32 software (Photon Technology International, Inc, Germany). One F340/F380 data point per second was recorded. The background fluorescence was measured by removing the cell from the area of the study. The background was subtracted from the traces before calculation of the $[Ca^{2+}]_i$. For calibration, R_{min} and R_{max} were determined from external standards containing fura-2 free acid and

sucrose (2 M). The K_d for Ca^{2+} -fura-2 was taken as 225 nM. $[\text{Ca}^{2+}]_i$ was calculated from F340/F380, $R_{\min}$ and $R_{\max}$ as described before⁴.

Statistical Analysis

Results were expressed as mean $\pm$ SEM. Chi-square test was used to test the significance. P -value < 0.05 was considered as significant.

Results

Insulin secretion from the EndoC-BH1 cells in response to glucose.

The EndoC-BH1 cells secreted insulin in response to glucose in a dose dependent manner. The insulin contents of the cells used in the experiments were $24970 \pm 778 \mu\text{U}$ per 10^5 cells ($n = 10$). The cells incubated with glucose (11 mM) secreted ~5 fold more insulin compared to the cells incubated with basal glucose (3 mM) ($n = 2$) (Fig 1A). The cells stimulated with glucose (16.7 mM) in the presence of GLP-1 (50 nM) secreted ~2 fold more insulin compared to the cells stimulated with glucose (16.7 mM) ($n = 2$) (Fig 1B).

Plasma membrane depolarization increased $[\text{Ca}^{2+}]_i$ in the EndoC-BH1 cells.

It is known that plasma membrane depolarization increases $[\text{Ca}^{2+}]_i$ in the β -cells by activating the VGCCs. High concentration of extracellular KCl depolarizes the plasma membrane. Application of KCl (25 mM) to the fura-2 loaded EndoC-BH1 cells increased $[\text{Ca}^{2+}]_i$ in 18 out of 20 cells examined (Fig 2A). In these and the remaining experiments, a $> 50 \text{ nM}$ increase in the $[\text{Ca}^{2+}]_i$ from the baseline was considered a positive $[\text{Ca}^{2+}]_i$ response. The $[\text{Ca}^{2+}]_i$ response consisted of an initial rapid ($< 2 \text{ min}$) increase to a peak followed by a plateau phase. The

average maximal $[Ca^{2+}]_i$ increase was 220 ± 32 nM ($n = 18$). The $[Ca^{2+}]_i$ returned to the baseline after washout of the KCl.

L-arginine depolarizes the plasma membrane by the cationic charges carried by it. In our experiments, L-arginine (20 mM) increased $[Ca^{2+}]_i$ in all the EndoC-BH1 cells examined ($n = 4$). The maximal $[Ca^{2+}]_i$ increase by L-arginine was 170 ± 15 nM ($n = 4$), the magnitude of which was comparable to that obtained by the depolarization with KCl (Fig 2B). However, the rate of $[Ca^{2+}]_i$ increase by L-arginine was slower compared to that obtained by the depolarization by KCl. Repeated stimulations of the same cell by L-arginine elicited repeated $[Ca^{2+}]_i$ responses of similar magnitude. We also tested the effect of GLP-1 on the L-arginine induced $[Ca^{2+}]_i$ increase, but GLP-1 did not alter the magnitude of the $[Ca^{2+}]_i$ increase or time to the peak $[Ca^{2+}]_i$ increase by L-arginine.

Activation of receptors coupled to the phospholipase C pathway increased $[Ca^{2+}]_i$ in the EndoC-BH1 cells

Carbachol is a long acting analogue of the neurotransmitter acetylcholine, which increases $[Ca^{2+}]_i$ in the β -cells by binding to the muscarinic receptors, and thereby activating the phosphatidyl-inositol-specific phospholipase C (PI-PLC) leading to the formation of inositol 1,4,5 tris phosphate (IP3). Carbachol (100 μ M) increased $[Ca^{2+}]_i$ in 13 out of 15 cells examined. $[Ca^{2+}]_i$ increase by carbachol consisted of an initial rapid increase followed by a plateau phase that persisted during the time of exposure to carbachol (Fig 2C). The maximal $[Ca^{2+}]_i$ increase was 243 ± 57 nM ($n = 13$). $[Ca^{2+}]_i$ returned back to the baseline after removal of carbachol.

The sulphonylurea drug tolbutamide increased $[Ca^{2+}]_i$ in the EndoC-BH1 cells in a GLP-1 dependant manner.

Single EndoC-BH1 cells were stimulated by high concentration of tolbutamide (500 μ M), but none of the cells examined responded by an increase in the $[Ca^{2+}]_i$ ($n = 7$) (Fig 3A). We then tested whether GLP-1 could make the cells responsive to tolbutamide. We tested the effect of GLP-1 alone in the presence of 3 mM glucose and found that GLP-1 did not increase $[Ca^{2+}]_i$ by itself (data not shown). We then stimulated the cells with tolbutamide (100 μ M) in the presence of GLP-1 (50 nM), and found that in the presence of GLP-1 all the cells examined responded to tolbutamide even though the concentration of tolbutamide was lower than 500 μ M ($n = 5$) (Fig 3B). Peak $[Ca^{2+}]_i$ increase by tolbutamide (100 μ M) was $(144 \pm 11 \text{ nM})$ ($n = 5$). After washout of tolbutamide, $[Ca^{2+}]_i$ returned to the baseline. There was a significant difference between the number of cells responding to tolbutamide by an increase in $[Ca^{2+}]_i$ when stimulated in the presence of or in the absence of GLP-1 ($\chi^2 = 12.00$, $df = 1$, $P = 0.0005$).

$[Ca^{2+}]_i$ response of the EndoC-BH1 cells to glucose is dependent on GLP-1

High concentration of glucose (16.7 mM) did not increase $[Ca^{2+}]_i$ in any of the EndoC-BH1 cells examined ($n = 10$) (Fig 4A). We then tested whether GLP-1 could make the EndoC-BH1 cells responsive to glucose. We stimulated the cells with glucose (16.7 mM) in the presence of GLP-1 (50 nM) and it increased $[Ca^{2+}]_i$ in 11 out of 17 cells examined although the peak $[Ca^{2+}]_i$ was relatively low $(103 \pm 11 \text{ nM})$ ($n = 11$) (Fig 4B). There was, thus, a significant difference between the number of cells responding to glucose by an increase in $[Ca^{2+}]_i$ when stimulated by the sugar in the presence of or in the absence of GLP-1 ($\chi^2 = 10.92$, $df = 1$, $P = 0.001$).

Discussion

The development of genetically engineered human insulinoma cell lines that can be cultured and propagated has opened up new possibility for studying signal transduction in the β -cells. Here, we show for the first time that $[\text{Ca}^{2+}]_i$ can be measured from these cells by using the Ca^{2+} indicators like fura-2 and microscope-based fluorometry. We demonstrate that several physiological or pharmacological agonists that are known to increase $[\text{Ca}^{2+}]_i$ in the primary β -cells also increase $[\text{Ca}^{2+}]_i$ in the EndoC-BH1 cells. These observations prove that the ion channels and the signaling molecules that are essential for the regulation of the membrane potential, and Ca^{2+} signaling in the primary β -cells are present also in the EndoC-BH1 cells. The cells responded to the sulphonylurea drug tolbutamide indicating that they possess functional K_{ATP} channels, and the voltage-gated Ca^{2+} channels. They also responded to the cholinergic agent carbachol indicating that the PI-PLC-IP3 pathway for Ca^{2+} signaling is intact in these cells.

We found that, GLP-1 was essential for eliciting Ca^{2+} response of the EndoC-BH1 cells to tolbutamide or glucose. These observations are consistent with the fact that GLP-1 makes β -cells competent to glucose⁵. However, we found that in the EndoC-BH1 cells, the Ca^{2+} response to glucose, even in the presence of GLP-1 was small compared to that reported in the primary human β -cell⁶. It should be noted that most of the rodent insulinoma cells also respond to glucose only poorly if at all. In our study, tolbutamide alone did not increase $[\text{Ca}^{2+}]_i$ in the EndoC-BH1 cells. Apparently, closure of the K_{ATP} channels alone is not enough to depolarize the EndoC-BH1 cells to the threshold for the activation of the VGCCs. GLP-1,

presumably, by triggering inward depolarizing current enables tolbutamide to depolarize the membrane potential to the threshold for the activation of the VGCCs.

Conclusion

In the past, because of lack of availability of human insulinoma cells, numerous investigators have used rodent insulinoma cells in their experiments. Now that a human insulinoma cell line has become available, we think that more and more investigators could use these new cells for the study of Ca^{2+} -signaling and stimulus-secretion coupling in the β -cells.

Author contributions

Krishnan K, planned and performed most of the experiments and assisted in writing the manuscript, Ma Z, performed radioimmunoassay and tutored insulin secretion experiments, Björklund A was involved in the planning of the insulin secretion studies and Islam M S designed the study and revised the manuscript. All authors have reviewed and approved the manuscript.

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Figure legends

Fig.1. Glucose induced insulin secretion from the human insulinoma cells. (A) The EndoC-BH1 cells stimulated by glucose for 60 minutes secreted insulin in a dose dependent manner (n =2). (B) Insulin secretion upon stimulation by glucose (16.7 mM) plus GLP-1 (50 nM) (black bar) was ~2 fold higher compared to that by glucose (16.7 mM) alone (white bar) (n =2).

Fig. 2. Increase in $[Ca^{2+}]_i$ in response to various pharmacological tools. (A) Stimulation of EndoC-BH1 cells with KCl (25 mM) increased $[Ca^{2+}]_i$ (n =18). (B) The trace shows the changes in $[Ca^{2+}]_i$ in a single EndoC-BH1 cell stimulated first with arginine (20 mM) plus GLP-1 (50 nM), and after washout, with arginine (20 mM) alone (n = 4). (C) The trace shows a characteristic pattern in $[Ca^{2+}]_i$ increase induced by carbachol (100 μ M) (n = 13).

Fig. 3. Tolbutamide increased $[Ca^{2+}]_i$ only in the presence of GLP-1. (A) Stimulation of EndoC-BH1 cells with high concentration of tolbutamide (500 μ M) did not increase $[Ca^{2+}]_i$ (n = 7). (B) The trace shows increase in $[Ca^{2+}]_i$ by tolbutamide (100 μ M) plus GLP-1 (50 nM), (n = 5).

Fig. 4. Glucose increased $[Ca^{2+}]_i$ only in the presence of GLP-1. (A) Stimulation of EndoC-BH1 cells with glucose (16.7 mM) alone did not increase $[Ca^{2+}]_i$ (n =10). (B) The trace shows increase in $[Ca^{2+}]_i$ by glucose (16.7 mM) plus GLP-1 (50 nM). KCl (25 mM) was used as a positive control. (n = 11).

FIG 1

Figure 2
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FIG 2

FIG 3

FIG 4

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To

Editor-in-Chief
Pancreas

Dear Editor-in-Chief

I am sending you our manuscript entitled "Calcium signaling in a genetically engineered human pancreatic β -cell line" authored by Islam MS et al. The manuscript contains original data that has not been submitted elsewhere. All the authors have been involved in planning and execution of the experiments described in the manuscript, and have read and approved the manuscript. The authors declare no conflicts of interest.

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