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Physiological characterization of the human EndoC-βH1 β-cell line

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ABSTRACT

In the new human EndoC-βH1 β-cell line, a detailed analysis of the physiological characteristics was performed. This new human β-cell line expressed all target structures on the gene and protein level, which are crucial for physiological function and insulin secretion induced by glucose and other secretagogues.

Glucose influx measurements revealed an excellent uptake capacity of EndoC-βH1 β-cells by the Glut1 and Glut2 glucose transporters. A high expression level of glucokinase enabled efficient glucose phosphorylation, increasing the ATP/ADP ratio along with stimulation of insulin secretion in the physiological glucose concentration range. The EC_{50} value of glucose for insulin secretion was 10.3 mM. Mannoheptulose, a specific glucokinase inhibitor, blocked glucose-induced insulin secretion (GSIS). The nutrient insulin secretagogues L-leucine and 2-ketoisocaproate also stimulated insulin secretion, with a potentiating effect of L-glutamine. The Kir 6.2 potassium channel blocker glibenclamide and Bay K 8644, an opener of the voltage-sensitive Ca^{2+} channel significantly potentiated GSIS. Potentiation of GSIS by IBMX and forskolin went along with a strong stimulation of cAMP generation.

In conclusion, the new human EndoC-βH1 β-cell line fully mirrors the analogous physiological characteristics of primary mouse, rat and human β-cells. Thus, this new human EndoC-βH1 β-cell line is very well suited for physiological β-cell studies.

1. Introduction

Over many years permanent insulin-producing cell lines of mouse (e.g. MIN6 cells) [1] and rat (e.g. INS1/INS1E cells) [2] origin have served as valuable tools for exploring the mechanisms of physiological insulin secretion and of cytokine toxicity leading to β-cell dysfunction and loss under diabetic conditions.

However, a reliable β-cell line of human origin has not been available. This changed recently with the EndoC-βH1 cell line [3,4], which can be permanently propagated in tissue culture [3]. This is a great progress. This new permanent human model β-cell line with high insulin content and proper insulin secretory responsiveness [3,4] allows for the first time detailed comparative studies of the physiological characteristics.

We show that this new permanent human β-cell line has physiological characteristics closer to primary β-cells than any permanent β-cell line of rodent origin. We consider the results obtained in the present study with respect to the usefulness of this new EndoC-βH1 β-cell line as a tool for the analysis of physiological β-cell function and the regulatory mechanisms of insulin secretion in response to glucose and other physiological and pharmacological stimuli. In addition we will consider whether its characteristics make the EndoC-βH1 β-cells a suitable substitute for primary pancreatic β-cells.

Techniques for disintegration and separation of β-cells, which are established for isolated rat pancreatic islets [5–7], are rather stressful for the cells. Analogous techniques for isolation of human β-cells are not readily available because of complex technical problems associated with the isolation procedure [8]. Thus a good permanent human β-cell line with physiological characteristics equivalent to those of primary β-cells may be a valuable new tool and a solution to the problem of the limited availability of human isolated islets for research purposes. Such a cell line may also obviate the need for increasing the availability of isolated human islets for research purposes [9], which potentially compete with the need of human islets for insulin replacement transplantation therapy in patients with diabetes [10].

Abbreviations: GK, glucokinase; GLUT, glucose transporter; GLP1R, glucagon like peptide 1 receptor; GRP, glucokinase regulatory protein; HK, hexokinase; IBMX, 3-isobutylmethylxanthine; Kir 6.2, K(ATP) channel subunit; Pdx1, pancreatic and duodenal homeobox 1; Sur1, sulfonylurea receptor.

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2. Materials and methods

2.1. Reagents

Chemicals were obtained from: dNTP mixture (PromoCell, Heidelberg,Germany), Biotherm™ *Taq* polymerase (GeneCraft,Münster,Germany), SuperScript II RT™ reverse transcriptase, primers and tissue culture materials (Invitrogen, Karlsruhe,Germany), antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), D-glucose, L-leucine, 2-ketoisocaproate, L-glutamine, pyruvate, L-lactate, IBMX, and forskolin (Sigma-Aldrich,Taufkirchen, Germany), mannoheptulose (Bujno Synthesis, Warsaw, Poland), glibenclamide (Santa Cruz Biotechnology), Bay K8644 (Alomone Labs, Jerusalem,Israel). All other reagents were from Sigma or Merck. Human primers were obtained from Invitrogen.

2.2. Human EndoC-βH1 β-cell line culture

β-cells of the human EndoC-βH1 cell line (ENDOCELLS SARL, Paris, France) were cultured onto coated dishes or plates in a cell culture medium without serum at 37 °C in a humidified atmosphere of 5% CO_2 as described earlier [3]. When used for purpose of comparison rat RINm5F insulin-producing cells were cultured as described [11].

2.3. Glucose influx

Human EndoC-βH1 β-cells as well as RINm5F control cells and RINm5F cells overexpressing either human GK (RINm5F-hGK) or human GLUT2 (RINm5F-hGLUT2) [12] were seeded on glass coverslips (150,000 cells) and grown for 48 h. Cells were transduced at a multiplicity of transfection of 10 for 2-h with an adenoviral FLII^{12}Pglu-700μ-δ6 construct and fluorescence microscopic analysis was performed 72 h after transduction as described [13].

2.4. Insulin secretion and content

Cells were seeded (150,000 cells/well) onto 6-well coated plates 3-days before test components were added. After an overnight starvation, insulin secretion was determined after a 1-h incubation period with glucose or other stimulators or inhibitors as given in Fig. 3 legend. Thereafter supernatants were collected for measurement by radioimmunoassay with human insulin as standard. Insulin values were normalized to the DNA content of the incubated cells measured by the PicoGreen assay (Promega, Mannheim,Germany).

2.5. ATP and ADP concentration measurements

ATP content was determined using the ATPlite Detection Assay System (Perkin Elmer Life Sciences, Hamburg,Germany), as described previously [14]. Cells were seeded (200,000 cells/well) in 6-well plates 72 h before the addition of chemical compounds. Cells were starved overnight and incubated for 1-h with glucose and/or chemical compounds. After lysis ATP and ADP concentration measurements were performed. For determination of ADP content, the sum of ATP and ADP was measured by conversion of ADP to ATP with pyruvate kinase and phosphoenolpyruvate for 15-min.

2.6. cAMP concentration measurements

The cAMP concentration was measured by the cAMP-Glo™ assay kit according to the manufacturer's instructions (Promega) as described earlier [14].

2.7. RNA isolation, cDNA preparation and real-time RT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). After quality control, 1 μg of RNA was reverse transcribed. The QuantiTect SYBR Green™ technology (Qiagen) was employed as described earlier [15]. The values for the genes of interest were normalized to the house-keeping gene β-actin.

2.8. Immunofluorescence

For immunofluorescence staining cells were seeded onto coated glass chamber slides and incubated as described above following an overnight fixation with 4% paraformaldehyde in PBS. After fixation, wash and blocking, slides were incubated with primary antibodies as described before [16]. Nuclear counterstaining was performed with 300 nM 4-,6-diamidino-2-phenylindole (DAPI), followed by mounting with Mowiol (Merck) plus 0.6% Dabco (Sigma). Images were captured and analyzed using a Cell^R/Olympus IX81 inverted microscope system (Olympus, Hamburg, Germany).

2.9. Western blotting

Cells were homogenized in ice-cold PBS including protease inhibitors (Roche) using short bursts (Braun-Sonic 125 Homogenizer, Quigley-Rochester, Rochester, NY, USA). Protein content was determined by the BCA assay (Pierce). For Western blotting, 50 μg (for GK and HK, or IL-1β) or 20 μg (for GLUT1 and GLUT2) of total protein was resolved by SDS polyacrylamide gel electrophoresis and then electroblotted onto membranes. Immunodetection was performed using specific primary antibodies as described [15]. Pictures were captured by the INTAS^R chemiluminescence detection system (Intas Science Imaging Instruments,Göttingen,Germany). The intensity of the bands was quantified through densitometry with the Gel-Pro Analyzer 4.0 program (Media Cybernetics, Silver Spring,MD, USA).

2.10. Data analysis

Analyses of the real-time RT-PCR data were performed using the Opticon Monitor v. 1.07 (MJ Research, Waltham, MA, USA). All data are expressed as means ± SEM. Statistical analyses were performed using the Prism analysis program (GraphPad, San Diego, CA, USA); p-values of <0.05 were considered significant.

3. Results

3.1. Gene and protein expression analysis in human EndoC-βH1 β-cells

Marker genes characteristic of the β-cell phenotype were expressed in the human EndoC-βH1 β-cells (Table 1). These were the genes for insulin, the transcription factor PDX1, the KIR 6.2 ATP-dependent potassium channel and the SUR1 sulphonylurea receptor as well as the GLP1 receptor (Table 1). Vice versa the genes for albumin and the glucokinase regulatory protein (GRP), which are characteristic for a hepatocyte phenotype, were not expressed in the EndoC-βH1 cells (Table 1). Amylase, a marker of the exocrine pancreas, was also not expressed (Table 1).

In the EndoC-βH1 β-cells the expression of the GLUT2 glucose transporter both on the gene and protein level was lower ($p < 0.01$) than that of the high affinity GLUT1 glucose transporter (Table 2 and Fig. 1).

Similarly, the expression of the low affinity hexokinase glucose phosphorylating isoenzyme (HK3), both on the gene and protein level, was very much lower ($p < 0.01$) than that of the high affinity

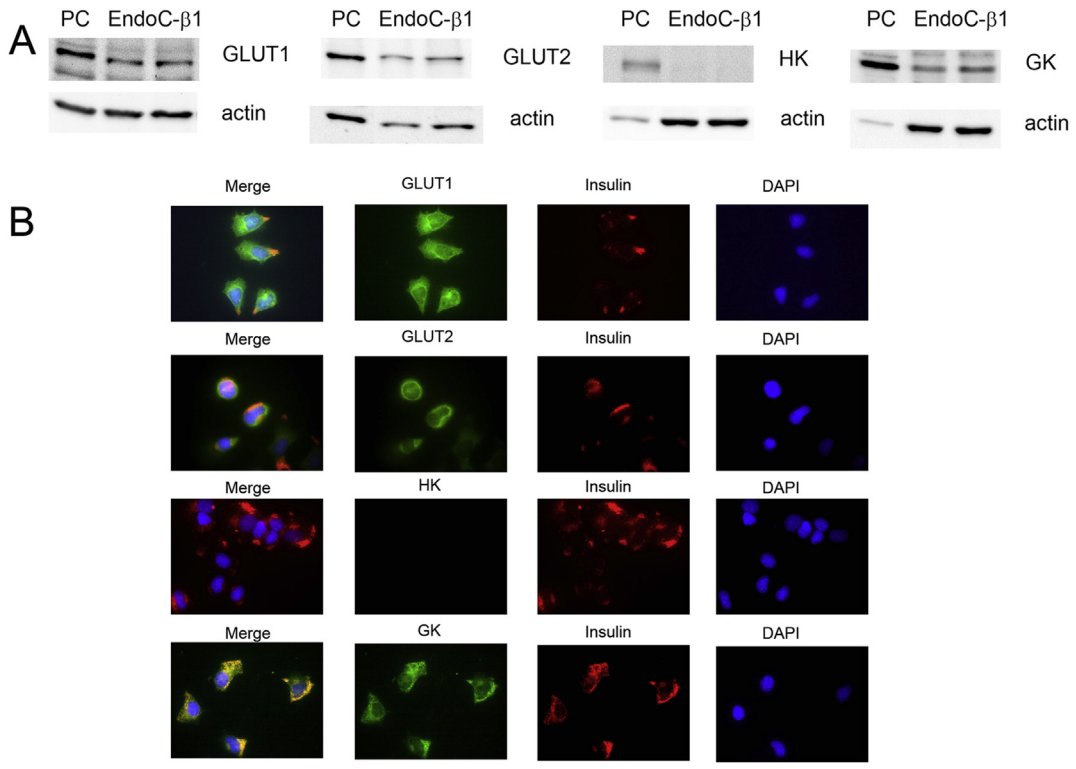

Fig. 1. Beta cell protein expression of glucose handling structures in human EndoC-βH1 β-cells. EndoC-βH1 cells were seeded at least 3 days before samples were collected. Shown are (A) a representative Western blot of 4–6 independent experiments. As a loading control β-actin was used. Two samples of EndoC-βH1 cells from two different passages and collected on two different days are shown. PC: positive control, for Glut1 and HK rat liver, for GK RINm5F-GK cells, for Glut2 RINm5F-Glut2 cells (B) Immunofluorescence, green: protein of interest, red: insulin, blue (DAPI): nuclei. Images were captured and analyzed using an Olympus fluorescence microscope, using a 60× oil objective. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

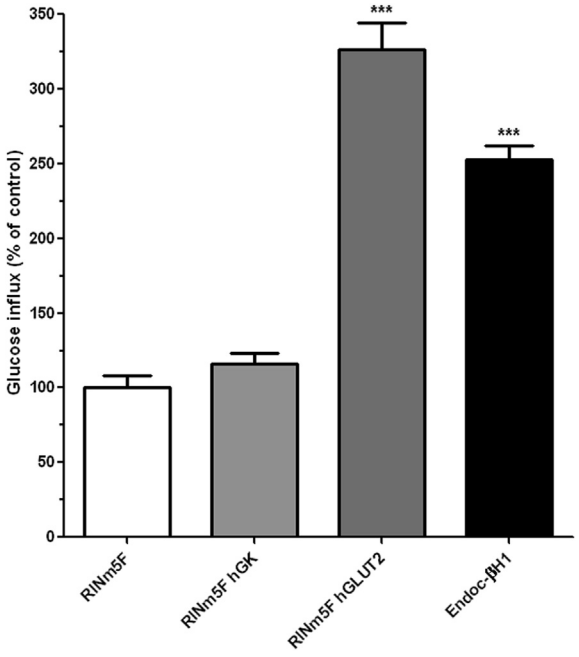

Fig. 2. Quantification of glucose uptake by human EndoC-βH1 β-cells and in comparison to various clonal rat insulin-secreting cell lines. Glucose influx rates were determined in EndoC-βH1 cells, RINm5F control cells and RINm5F cells over-expressing either human GK (RINm5F-hGK) or human GLUT2 (RINm5F-hGLUT2). Glucose influx was calculated from the initial change in the citrine$_{em}$/ECFP$_{em}$ ratio upon changing the concentration in the perifusion medium from 0 to 10 mM glucose. RINm5F control cells were set as 100%. Mean values ± SEM of 10–40 individual cell traces are shown. ***$p < 0.001$ vs. RINm5F; ANOVA followed by Dunnett.

glucokinase glucose phosphorylating isoenzyme (GK) in EndoC-βH1 cells (Table 2 and Fig. 1). Of note is also the observation, that a gene expression of the other two hexokinase isoenzymes (HK1 and HK2) was not detectable (data not shown).

3.2. Glucose influx into human EndoC-βH1 β-cells and a comparison with different clones of the RINm5F insulin-producing rat cell line

Human EndoC-βH1 β-cells displayed a 2.5fold higher influx capacity of glucose when glucose exposure was increased from 0 to 10 mM concentration (Fig. 2). A comparison was performed with RINm5F insulin-producing rat tissue culture cells, which do not express the GLUT2 glucose transporter; these cells showed no such increase (Fig. 2). RINm5F-hGK cells overexpressing human glucokinase showed also no changes of glucose influx (Fig. 2). However, in RINm5F-hGLUT2 cells overexpressing the human GLUT2 glucose transporter, glucose influx was increased by 3.2fold (Fig. 2). These comparative studies in insulin-producing RINm5F tissue culture cells of rat origin with a GLUT2 gene expression achieved through genetic modification prove that the human EndoC-βH1 β-cells possess a functionally relevant level of albeit low GLUT2 glucose transporter expression.

3.3. Insulin secretory responsiveness of human EndoC-βH1 β-cells to stimulation by glucose and other insulin secretagogues

Glucose induced a significant concentration-dependent stimulation of insulin secretion from EndoC-βH1 β-cells in the concentration range between 0.5 mM and 30 mM. The EC_{50} concentration of glucose for half maximal induction of glucose-induced insulin

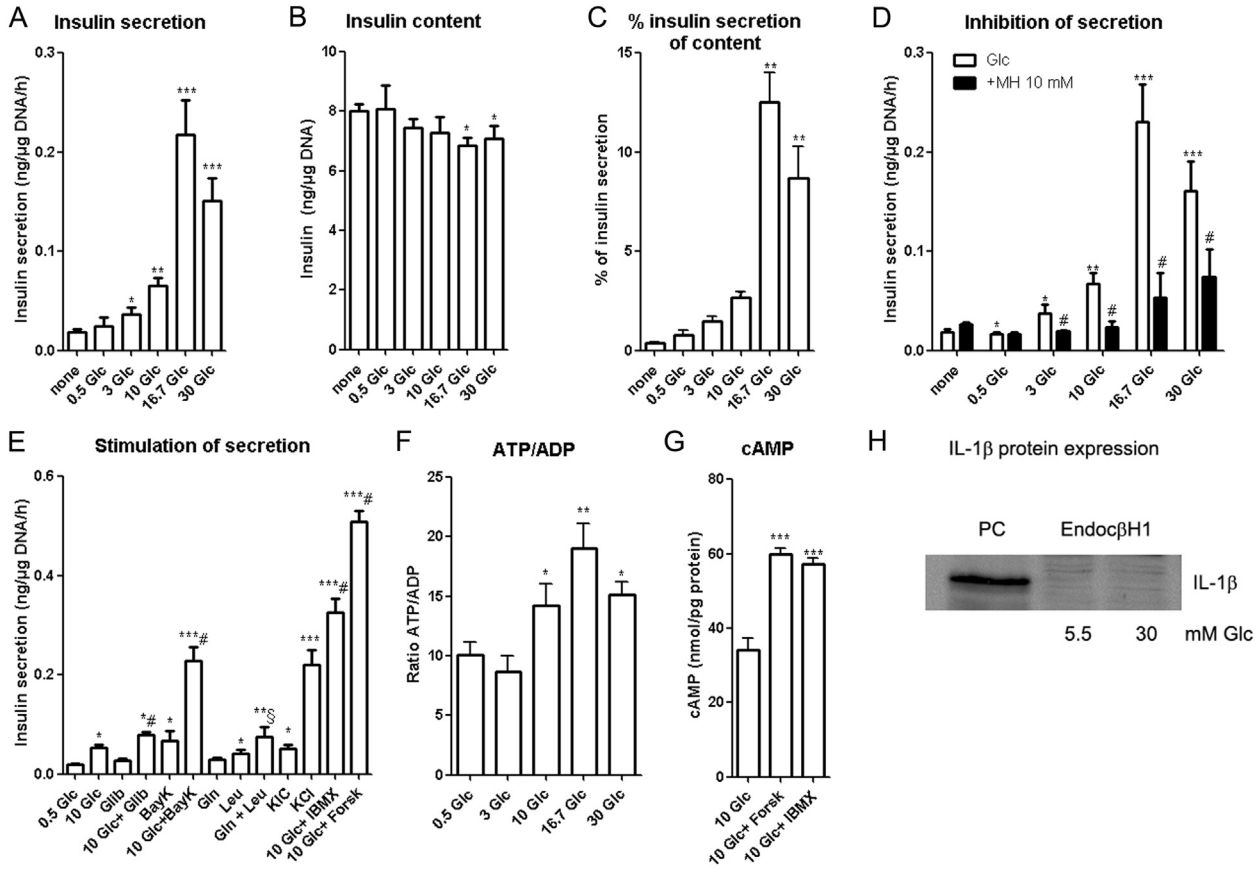

Fig. 3. Effects of different test compounds on insulin secretion and content in human EndoC-βH1 β-cells. Insulin secretion by EndoC-βH1 cells was analyzed after 1-h incubation with test compounds. Insulin was determined by RIA and normalized to the DNA content. Shown are mean values of 4–6 independent experiments. Compounds used: glucose (Glc, 0.5–30 mM), BayK 86444 (BayK, 1 µM), glibenclamide (Glib, 10 µM), glutamine (Gln, 2 mM), leucine (Leu, 20 mM), 2-ketoisocaproate (KIC; 20 mM), potassium chloride (KCl, 25 mM), IBMX (IBMX, 0.1 mM), forskolin (Forsk, 5 µM). (A) insulin secretion; (B) insulin content; (C) % of insulin release; (D) inhibition of insulin secretion; (E) stimulation of insulin secretion; (F) ATP/ADP ratio; (G) cAMP concentration; (H) protein expression of IL-1β after a 24 h-incubation in the presence of 5.5 or 30 mM Glc, PC (positive control) was cell extract of human-293-cells transfected with human IL-1β. *p < 0.05 vs. 0.5 mM Glc; **p < 0.01 vs. 0.5 mM Glc, ***p < 0.001 vs. 0.5 mM Glc; #p < 0.05 vs. the same Glc concentration; §p < 0.05 vs. Leu. ANOVA followed by Bonferroni.

secretion was 10.3 ± 0.1 mM (n = 4) (Fig. 3A). High rates of insulin release at the high concentrations of 16.7 and 30 mM glucose went along with a significant decrease of the insulin content (Fig. 3B). Consequently, the percentage of insulin released by the EndoC-βH1 cells in relation to the insulin content was significantly increased at these higher glucose concentrations (Fig. 3C). High glucose (30 mM) did not induce the expression of IL-1β when compared with a basal concentration of 5.5 mM (Fig. 3H).

The specific glucokinase inhibitor mannoheptulose (10 mM) strongly reduced glucose-induced insulin secretion at all glucose concentrations starting at 3 mM (Fig. 3D). The extent of this reduction increased with increasing glucose concentrations, which is in harmony with the increasing contribution of glucokinase to the mediation of glucose-induced insulin secretion.

Pyruvate (10 mM) and lactate (10 mM) did not stimulate insulin secretion (data not shown).

The K_{ATP} channel blocker glibenclamide, a sulfonylurea drug, as well as the Ca^{2+} channel activator Bay K 8644 strongly potentiated insulin secretion in the presence of glucose (10 mM) (Fig. 3E).

The nutrient insulin secretagogues L-leucine (20 mM) and 2-ketoisocaproate (20 mM) also stimulated insulin secretion significantly. The secretory potency was larger in the presence of L-glutamine (2 mM) than in its absence (Fig. 3E). KCl (25 mM), which

Table 1
Expression of the genes of interest in human EndoC-βH1 β-cells.

Gene	Expression
Insulin	0.472 ± 0.080 (4)
Pdx1	0.177 ± 0.038 (4)
Sur1	0.021 ± 0.005 (3)
Kir6.2	0.004 ± 0.001 (5)
GRP	0 (4)
GLP1R	0.408 ± 0.153 (5)
Albumin	0 (4)
Amylase	0 (4)

Real-time RT-PCR was performed. The expression of the gene of interest was normalized to the house-keeping gene β-actin. Shown are mean values ± SEM with the number of experiments in the parentheses.

Table 2
Expression of the cellular structures important for insulin secretory function of human EndoC-βH1 β-cells.

Cellular structure	Gene expression	Protein expression
GLUT1	0.205 ± 0.043 (4)	1.60 ± 0.17 (8)
GLUT2	0.029 ± 0.009 (4)	0.38 ± 0.07 (10)
HK	0.001 ± 0.0004 (4)	0.15 ± 0.07 (4)
GK	0.031 ± 0.006 (4)	1.12 ± 0.27 (7)

Real-time RT-PCR was performed. Western blots (representative blots shown in Fig. 1) were quantified by GelProAnalyzer software. Expression was normalized to β-actin. Shown are mean values ± SEM with the number of experiments in the parentheses.

depolarises the plasma membrane, also stimulated insulin secretion significantly (Fig. 3E). The adenylate cyclase activators IBMX (0.1 mM) and forskolin (5 μM) strongly potentiated glucose-induced insulin secretion in the presence of glucose (10 mM) (Fig. 3E).

3.4. ATP and ADP content in human EndoC-βH1 β-cells

The ATP content at 3 mM glucose was 330 ± 5 nmol/mg protein, the ADP content 39 ± 6 nmol/mg protein. Increasing glucose concentrations in the culture medium raised the ATP/ADP ratio significantly (Fig. 3F).

3.5. cAMP generation by human EndoC-βH1 β-cells

Rising concentrations of glucose did not induce cAMP generation in the EndoC-βH1 β-cells (data not shown). Incubation of EndoC-βH1 β-cells with 5 μM forskolin or 0.1 mM IBMX, however, led to a strong cAMP increase (Fig. 3G).

4. Discussion

In 2011 Ravassard, Scharfmann and colleagues published the first description of the novel human β-cell line EndoC-βH1, which they created by genetic engineering in their laboratory [3]. Insulin content and glucose-responsiveness of this new human β-cell line allowed reversal of chemically-induced diabetes in mice [3].

Our detailed characterization of the physiological features of the human EndoC-βH1 cell line shows that this new human β-cell line expresses all the crucial biological structures on the gene and protein level which constitute the phenotype of a typical pancreatic β-cell (Fig. 4). The high glucokinase/hexokinase expression ratio guarantees an insulin secretory responsiveness to glucose stimulation in the physiological concentration range [17–21] with a half-maximal effective glucose concentration of around 10 mM as observed in the present study. The stimulatory effects of rising glucose concentrations on insulin secretion were accompanied by parallel increases of the ATP/ADP ratio, as known also from mouse and rat β-cells as well as human islets [18,22–25].

Because the incubation of Endoc-βH1 β-cells with high glucose (30 mM) did not induce the expression of IL-1β, the observed glucose responsiveness was not influenced by IL-1β. This is at variance from previous observations of a stimulation of IL-1β production by glucose in β-cells [26,27] but in agreement with [28].

On the other hand, though rather low, HK expression in EndoC-βH1 β-cells is apparently adequate to supply sufficient energy at low glucose concentrations to maintain β-cell replication and allow cell survival. The cells also express the glucose transporters of the GLUT1 and GLUT2 type required for efficient glucose uptake as shown in the present glucose influx experiments. As reported before for primary human β-cells [29,30], the expression of the GLUT2 glucose transporter was lower than that of the GLUT1 glucose transporter in EndoC-βH1 β-cells, when compared to rodent β-cells [29].

The relatively high expression of the low affinity GLUT1 glucose transporter should allow sufficient glucose funnelling into the EndoC-βH1 cells at concentrations around the K_m value (4–6 mM) of human glucokinase [20,31,32] thereby allowing efficient glucose phosphorylation in the postprandial state. Hexokinase alone, due to its low expression level, would not generate sufficient metabolic signal to secure a half-maximal insulin secretion rate at around 10 mM as observed in the present study. Thus EndoC-βH1 β-cells like primary human β-cells [29,30] can afford a low expression level of the GLUT2 glucose transporter.

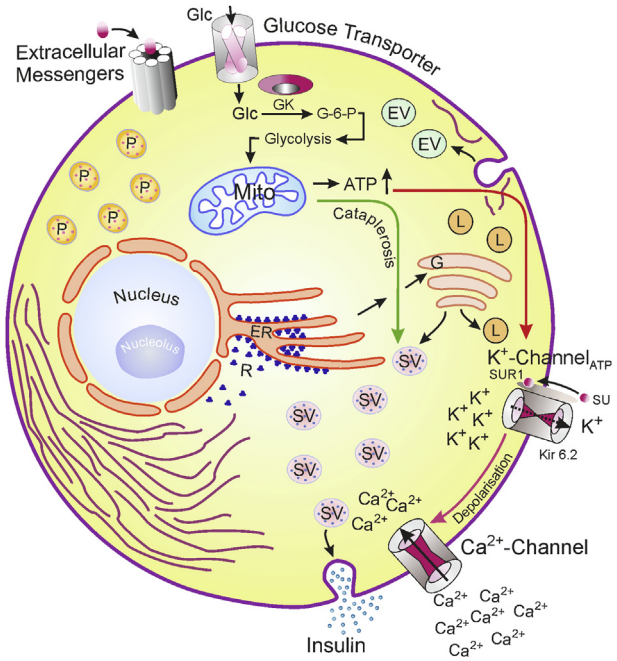

Fig. 4. A scheme of the cellular structures important for physiological regulation of insulin secretion in human EndoC-βH1 β-cells. Shown are: nucleus, mitochondrion (Mito), peroxisomes (P), lysosomes (L), endoplasmic reticulum (ER), Golgi apparatus (G) and secretory granules (SV). Elevated postprandial blood glucose increases the Glc-concentration within β-cells via rapid equilibration through Glc-transporters in the plasma membrane. Glc is quickly phosphorylated by glucokinase (GK). This step leads to the production of glucose-6-phosphate (G6P) and determines the rate of glycolysis and pyruvate generation. The elevated glycolytic flux and mitochondrial metabolism stimulate the ATP production. An increase in the cytosolic ATP/ADP ratio causes closure of ATP-sensitive K^+ (K_{ATP}) channels and depolarizes the plasma membrane. This is called the "initiating pathway" (red arrow). The K_{ATP} channel is a complex of four pore-forming Kir6.2 subunits and four regulatory SUR1 subunits. The SUR1 subunit can react with blood glucose-lowering sulfonylurea drugs (SU) to initiate insulin secretion. The closure of K_{ATP} channels results in the opening of voltage-sensitive Ca^{2+} channels and leads to Ca^{2+} influx into the cell. This stimulates exocytosis of insulin-containing secretory granules. The second mechanism enabling glucose-induced insulin secretion potentiation is called the "amplifying pathway" (green arrow). This mechanism is based on the cataplerosis (providing intermediates of tricarboxylic acid cycle) without involvement of K_{ATP}. Many hormones, small peptides and extracellular messengers can also potentiate glucose-induced insulin secretion through binding to their plasma membrane receptors and activation of intracellular signalling cascades, typically G-protein mediated (here not depicted). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A higher expression level of the low affinity GLUT2 glucose transporter, as observed in mouse and rat β-cells [29] would rather be undesirable since this would allow easy access of glucose at supraphysiological blood glucose levels above 10 mM into the cell. In a sense, the predominance of the high GLUT1 glucose transporter expression in combination with a predominance of the low affinity glucose phosphorylating enzyme glucokinase prevailing in EndoC-βH1 β-cell line thus represents a safety device to protect human β-cells at supraphysiologically high glucose concentrations against glucotoxicity.

The inhibition of glucose-induced insulin secretion by the specific glucokinase inhibitory sugar mannoheptulose [33] documents clearly that glucose-induced insulin secretion is glucokinase-mediated in the EndoC-βH1 β-cells.

The EndoC-βH1 β-cell is also responsive to other classical insulin secretory stimuli, namely L-leucine and 2-ketoisocaproic acid [34]. Insulin secretion is strongly increased also by the sulfonylurea drug glibenclamide, a Kir 6.2 potassium channel blocker that exerts its effect via interaction with the sulfonylurea receptor SUR1 [35]. Both

structures are expressed [36] in the same way as the voltage-sensitive Ca^{2+} channel [37]. BayK 86444, which strongly potentiated glucose-induced insulin secretion, mediates its potentiating effect on insulin secretion through opening of this channel [38]. This is in line with earlier observations in human islets [39].

The classical adenylate cyclase activators IBMX and forskolin [40] also strongly potentiated glucose-induced insulin secretion in EndoC-βH1 β-cells. This is in accordance with earlier observations by Ravassard et al. [3] showing strong potentiating effects of exendin-4 and IBMX. We show here that this strong potentiation is the result of a large increase of cAMP, as has been observed also in mouse and rat β-cells [18].

Thus in conclusion the insulin secretory characteristics of the new EndoC-βH1 β-cell line to glucose and other stimuli mirror characteristics analogous to those observed in primary human β-cells (Fig. 4). At variance from the different clones of the permanent rat INS1 tissue culture cell line, which differ from primary β-cells through a non-physiological insulin release in response to pyruvate and lactate [41,42], the EndoC-βH1 β-cell does not respond to pyruvate and lactate.

This new human EndoC-βH1 β-cell line, in contrast to human islet preparations with their variable quality [43], provides reproducible results. It makes this new human β-cell line very well suited for physiological studies. Since the EndoC-βH1 β-cell line also mirrors nicely the physiological characteristics of primary human β-cells, this cell line may represent a good substitute for isolated human pancreatic islets, where the interpretation of the results is always hampered by the risk of false interpretation of data due to the admixture of different other islet cell types, which cannot be separated easily from the β-cells in the case of human islets [8].

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.072.

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