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Original Paper

Presenilin-1 Established ER-Ca^{2+} Leak: a Follow Up on Its Importance for the Initial Insulin Secretion in Pancreatic Islets and β-Cells upon Elevated Glucose

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Key Words
Mitochondria • Endoplasmic reticulum • Ca^{2+} spiking • Insulin secretion

Abstract
Background/Aims: In our recent work, the importance of GSK3β-mediated phosphorylation of presenilin-1 as crucial process to establish a Ca^{2+} leak in the endoplasmic reticulum and, subsequently, the pre-activation of resting mitochondrial activity in β-cells was demonstrated. The present work is a follow-up and reveals the importance of GSK3β-phosphorylated presenilin-1 for responsiveness of pancreatic islets and β-cells to elevated glucose in terms of cytosolic Ca^{2+} spiking and insulin secretion. ***Methods:*** Freshly isolated pancreatic islets and the two pancreatic β-cell lines INS-1 and MIN-6 were used. Cytosolic Ca^{2+} was fluorometrically monitored using Fura-2/AM and cellular insulin content and secretion were measured by ELISA. ***Results:*** Our data strengthened our previous findings of the existence of a presenilin-1-mediated ER-Ca^{2+} leak in β-cells, since a reduction of presenilin-1 expression strongly counteracted the ER Ca^{2+} leak. Furthermore, our data revealed that cytosolic Ca^{2+} spiking upon administration of high D-glucose was delayed in onset time and strongly reduced in amplitude and frequency upon siRNA-mediated knock-down of presenilin-1 or the inhibition of GSK3β in the pancreatic β-cells. Moreover, glucose-triggered initial insulin secretion disappeared by depletion from presenilin-1 and inhibition of GSK3β in the pancreatic β-cells

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and isolated pancreatic islets, respectively. ***Conclusion:*** These data complement our previous work and demonstrate that the sensitivity of pancreatic islets and β-cells to glucose illustrated as glucose-triggered cytosolic Ca^{2+} spiking and initial but not long-lasting insulin secretion crucially depends on a strong ER Ca^{2+} leak that is due to the phosphorylation of presenilin-1 by GSK3β, a phenomenon that might be involved in the development of type 2 diabetes.

Introduction

The elevation of blood glucose is sensed by pancreatic β-cells that take up and subsequently metabolize glucose, resulting in an increased mitochondrial production of ATP that, in turn, inhibits K_{ATP} channels yielding plasma membrane depolarization resulting in activation of L-type Ca^{2+} channels [1]. This stimulated Ca^{2+} entry triggers the exocytosis of insulin-containing secretory granules [2]. In the coupling between mitochondrial ATP production to elevated glucose and insulin secretion (i.e. glucose stimulated insulin secretion; GSIS) mitochondrial Ca^{2+} uptake has been found to play a crucial role [3–5]. However, all these reports convincingly demonstrate that mitochondrial Ca^{2+} uptake in response to the entering Ca^{2+} facilitates mitochondrial ATP production, most likely by Ca^{2+}-mediated activation of mitochondrial dehydrogenases [6–9], thus, facilitating citric acid cycle and the subsequent ATP production. Notably, such effect boosts mitochondrial ATP formation in a second phase, when mitochondria sequester the Ca^{2+} that entered via L-type channels, thus, this effect follows a mitochondrial ATP production in response to elevated glucose.

In our recent work, we reported a β-cell-specific phenomenon of Ca^{2+}-mediated pre-activation of mitochondria already under basal (i.e. resting glucose) conditions [10]. In particular, we described a large ER Ca^{2+} leak in pancreatic β-cell lines and freshly isolated mouse pancreatic islets [10]. Mechanistically, this leak is established by the phosphorylation of ER-localized presenilin-1 by glycogen synthase kinase 3 beta (GSK3β) and installs a unidirectional Ca^{2+} flux from the ER towards the mitochondrial matrix yielding enhanced resting mitochondrial Ca^{2+} levels and, subsequently, enhanced resting respiratory activity and mitochondrial ATP levels [10]. In extension to the Ca^{2+}-facilitating function on mitochondrial ATP production in the second phase as described above, these data point to a pre-sensitization of β-cell mitochondria. Accordingly, one might hypothesize that such pre-sensitizing of β-cell mitochondria might facilitate the responsiveness of β-cell mitochondria to elevated glucose and, thus, for the initial insulin secretion.

The present work is designed to challenge this hypothesis and to elaborate the importance of GSK3β-mediated phosphorylation of presenilin-1 for responsiveness of β-cells to elevated glucose and GSIS. The two clonal pancreatic β-cell lines, INS-1 [11] and MIN-6 [12] and freshly isolated pancreatic islets were used and the importance of presenilin-1 and its phosphorylation by GSK3β for glucose triggered cytosolic Ca^{2+} spiking and consecutive insulin secretion was investigated.

Materials and Methods

Reagents

Cell culture materials were purchased from Greiner Bio-One (Kremsmünster, Austria). The selective GSK3β inhibitor 6-[[2-[[4-(2, 4-Dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino] ethyl] amino]-3-pyridinecarbonitrile (CHIR99021; PubChem CID: 9956119) was obtained from Tocris (Bristol, UK). The selective JNK inhibitor (SP600125; PubChem CID: 8515) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Acetyloxymethyl 2-[6-[bis [2-(acetyloxymethoxy)-2-oxoethyl]amino]-5-[2-[2-[bis [2-(acetyloxymethoxy)-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-1-benzofuran-2-yl]-1, 3-oxazole-5-carboxylate (Fura-2/AM; PubChem CID: 3364574) was from MoBiTec GmbH (Göttingen, Germany). Carbachol (PubChem CID: 5831), Thapsigargin (PubChem CID: 446378) and 2, 5-Di-Tert-

Butylhydroquinone (BHQ; PubChem CID: 2374) were obtained from Sigma Aldrich (Vienna, Austria). All other buffer salts were from Carl Roth (Karlsruhe, Germany).

Isolation of murine pancreatic islets

For all experiments, age-matched male C57BL/6 mice purchased from Jackson Laboratory (Bar Harbor, ME) between 3-4 months of age were used. Mice were fed regular chow diet (11.9% caloric intake from fat; Altromin 1324, Lage, Germany) and maintained in a 12:12-h light-dark cycle in a temperature-controlled environment. All animal experiments were carried out in accordance with the guidelines set by the Division of Genetic Engineering and Animal Experiments and were approved by the Austrian Federal Ministry of Science, Research, and Economy (Vienna, Austria). Murine islets were isolated as described [13]. Briefly, mice were euthanized by cervical dislocation. The common bile duct was cannulated using a 27G needle, and 2 ml of ice-cold Liberase II (Roche Diagnostics, Basel, Switzerland) was injected immediately. The perfused pancreas was dislodged from the intestine, spleen, and stomach. To facilitate complete digestion, the pancreas was further incubated in a water bath preheated at 37°C for predetermined time intervals (batch variation, average time 12 min and 30 s). After digestion, islets were purified by filtration and gradient separation. Islets were maintained in RPMI (GIBCO; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin (GIBCO) for 24 h, and healthy islets were hand-picked for experiments. Islet GSIS was determined from ~200 hand-picked islets.

Cell culture and transfection

INS-1 832/13 (INS-1) cells were a generous gift from Prof. Dr. Claes B. Wollheim and Dr. Françoise Assimacopoulos-Jeannet (University Medical Center, Geneva, Switzerland). INS-1 cells were cultured in RPMI 1640 containing 11 mM glucose (PubChem CID: 5793) supplemented with 10 mM HEPES (PubChem CID: 23831), 10% fetal calf serum (FCS), 1 mM sodium pyruvate (PubChem CID: 23662274), 50 µM β-mercaptoethanol (PubChem CID: 1567), 50 µg of penicillin (PubChem CID: 5904) and 100 µg of streptomycin (PubChem CID: 19646). MIN-6 cells (ATCC® CRL-11506™) were cultured in DMEM supplemented with 25 mM glucose, 10 mM HEPES, 10% FCS, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 50 µg penicillin and 100 µg streptomycin. Origin of cells was confirmed by STR-profiling by the cell culture facility of ZMF (Graz).

Buffers

2CaNa buffer: 2 mM $CaCl_2$, 138 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM Hepes, 10 mM D-glucose, pH 7.4. 0 CaNa buffer: same as 2CaNa buffer but without $CaCl_2$, plus 10 mM EGTA (Ca^{2+} chelator), pH 7.4. Experimental Buffer (EB): 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 2.6 mM $NaHCO_3$, 440 µM KH_2PO_4, 340 µM Na_2HPO_4, 10 mM D-glucose, 0.1% vitamins, 0.2% essential amino acids, and 1% penicillin-streptomycin (all v/v); pH adjusted to 7.4. Hank's buffered salt solution (HBSS): 114 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM Hepes, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.2% BSA, 3 mM D-glucose.

Transfection with siRNAs and plasmids

Cells seeded on 30 mm glass coverslips (for Ca^{2+} measurements) or in 6-well plates were transiently transfected at 60 - 80% confluency with 100 nM siRNA using 2.5 µl TransFast transfection reagent (Promega, Madison, WI, USA) in 1 ml of serum- and antibiotic-free medium. Cells were maintained in a humidified incubator (37°C, 5% CO_2, 95% air) for 16-20 h. Thereafter, the transfection mix was replaced by full culture medium. Knock-down efficiency for presenilin-1 is shown in the Supplementary Information to our previous work [10]. For inhibition of GSK3β or JNK cells were incubated in their respective media containing 2.5 µM CHIR99021 or 10 – 100 µM SP600125, respectively. All experiments were performed 24 - 48 h after transfection or treatment. siRNAs were obtained from Microsynth (Balgach, Switzerland) or Thermofisher Scientific (Vienna, Austria).

mRNA Isolation and qRT-PCR

24 h after treatment of INS-1 cells with different concentrations of SP600125 or DMSO control, total RNA isolation, reverse transcription, PCR and real-time-PCR were performed according to our recently published protocols [18]. Relative expression of specific genes was normalized to rat GAPDH, as a housekeeping gene. Primers for rat presenilin-1 and GAPDH were obtained from Qiagen (QuantiTect Rn_PSEN1 and QuantiTect Rn_GAPDH).

Cytosolic Ca^{2+} imaging in cultured β-cells

ER Ca^{2+} leak experiments. 24 h after treatment with 100 µM SP600125, cells were loaded with 2 µM Fura-2/AM (TEFLabs) in EB for 40 min as previously described [5] and were alternately illuminated at 340 and 380 nm, whereas fluorescence emission was recorded at 510 nm. Results of Fura-2/AM measurements are shown as the ratio of F_{340}/F_{380}. For measurements of cytosolic Ca^{2+} to indirectly visualize ER Ca^{2+} leak, after Fura-2/AM loading, cells were incubated for the indicated times (1 min or 20 min) in an experimental buffer without Ca^{2+} (0CaNa buffer) and subsequently stimulated with 100 µM of IP_3-generating agonist carbachol in the presence of 15 µM of the SERCA inhibitor BHQ.

L-type Ca^{2+} channel and thapsigargin experiments. As indicated in the respective graphs different combinations of 2CaNa, 0CaNa buffers with or without 1 µM thapsigargin (non-competitive SERCA inhibitor) have been used. To evaluate the maximal Ca^{2+} uptake via L-type Ca^{2+} channels, cells were depolarized with a high K^+ buffer (30 mM K^+), where 25 mM NaCl (2 CaNa recipe) were substituted with KCl to reach a final K^+ concentration of 30 mM.

Glucose-induced Ca^{2+} oscillations. After Fura-2/AM loading, cells were stabilized by a 20 min incubation in glucose free buffer (0G; 10 mM mannitol substitute the glucose) before imaging, to minimize the cytosolic oscillations and to improve the signal upon exposure to high glucose. On the microscope, cells were perfused with 0G buffer for 2 min before switching to 16 mM glucose (16G) during acquisition. Cells were alternately illuminated at 340 and 380 nm, whereas fluorescence emission was recorded at 510 nm. Results of Fura-2/AM measurements are shown as the ratio of $F_{340}/F_{380.}$ To evaluate the maximal Ca^{2+} uptake via L-type Ca^{2+} channels, cells were depolarized with a high K^+ buffer, where 25 mM NaCl were substituted with KCl.

Insulin measurements

For determination of insulin secretion rate INS-1 cells were routinely plated on 6-well plates and transfected at a confluency of 70 to 80% with either scrambled control siRNA or siRNA against presenilin-1 or treated with 100 µM JNK inhibitor (SP600125) or DMSO. Islets were treated with either 2.5 µM CHIR99021 or DMSO control for 24 h. 48 h after transfection or 24 h after treatment cells or islets were washed twice with HBSS buffer containing 3 mM D-glucose, followed by a 30 min incubation in this buffer to record basal insulin secretion. For stimulation of insulin secretion cells and islets were stimulated with HBSS buffer containing high D-glucose concentrations to reach a final concentration of 16 mM D-glucose. Samples were taken for the next 2 to 45 min. Samples were stored on ice and centrifuged at 3000 rpm for 5 min to remove residual cells. Supernatant was transferred into fresh tubes, stored at -80°C and assayed with Mercodia Rat Insulin ELISA (Mercodia, Uppsala, Sweden). Insulin secretion rate was calculated as described previously [5] following a standard procedure [14].

Statistical analyses

Data shown represent the mean ± SEM. 'n' values refer to the number of individual experiments performed. For life cell imaging numbers indicate the numbers of cells/independent repeat. If applicable analysis of variance (ANOVA) was used for data evaluation and statistical significance of differences between means was estimated by Bonferroni post hoc test or two-tailed Student's t-test assuming unequal variances was used, where applicable using GraphPad Prism 5.0f (GraphPad Software, La Jolla, CA, USA).

Data availability

Original data are available from the corresponding author upon request.

Results

Reduced presenilin-1 expression by treatment with the JNK inhibitor SP600125 counteracts ER Ca^{2+} leak in pancreatic β-cells

In order to corroborate our previous findings of the presenilin-1-mediated ER Ca^{2+} leak in β-cells, we used another approach to reduce presenilin-1 expression by treating INS-1 cells with the selective c-Jun N-terminal kinase (JNK) inhibitor SP600125. Notably, besides its multiple substrates [15], amongst other in pancreatic β-cells [16], inhibition of JNK was shown to reduce presenilin-1 expression in the neuroblastoma cell line SK-N-SH [17]. Accordingly, we treated INS-1 cells with different concentrations of the inhibitor for 24 h and evaluated presenilin-1 expression on mRNA level. Presenilin-1 expression was reduced at high concentrations of the JNK inhibitor (i.e. 100 µM) (Fig. 1A). Next, we performed ER Ca^{2+} leak experiments according to our ER Ca^{2+} leakage protocol [10]. Extracellular Ca^{2+} was removed from the cells and the ER Ca^{2+} content was indirectly measured as cytosolic Ca^{2+} increase upon ER Ca^{2+} mobilization with an inositol1, 4,5-trisphosphate- (IP_3) generating agonist (100 µM carbachol) in the presence of the reversible SERCA inhibitor tert-butylhydroxyquinone (BHQ) to avoid refilling of the ER. Under control conditions a massive ER Ca^{2+} loss was detected after 20 min pre-incubation in Ca^{2+}-free buffer (Fig. 1B, D). After 24 h treatment with the JNK inhibitor this ER Ca^{2+} loss was strongly reduced (Fig. 1C, D). These data indicate that JNK-

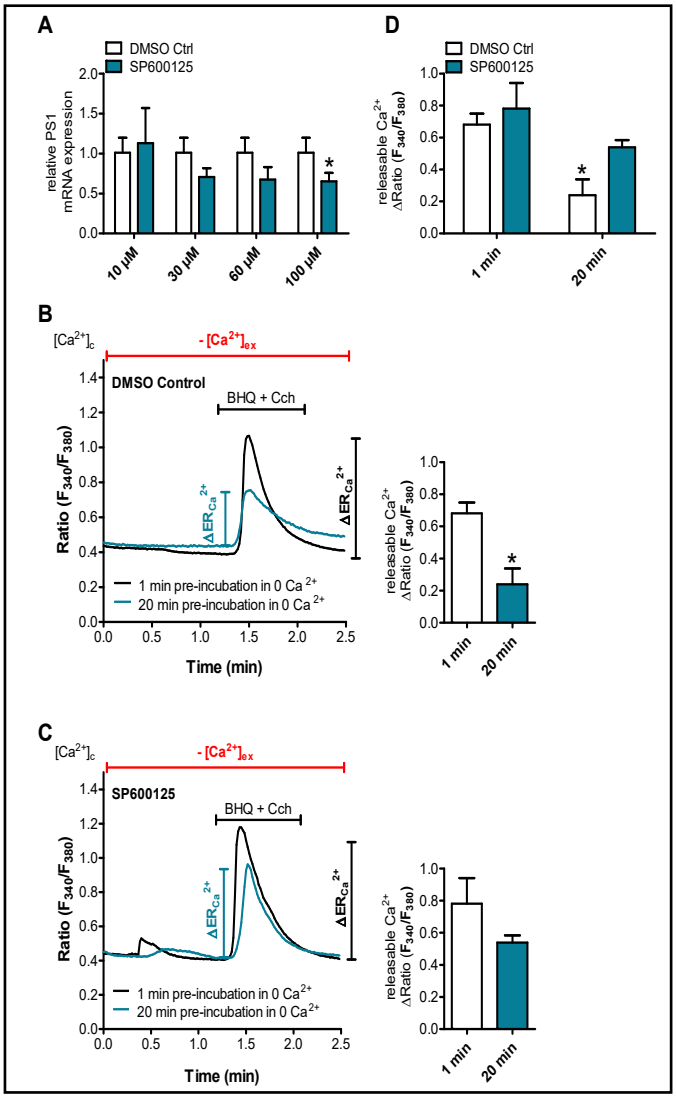

Fig. 1. JNK inhibitor SP600125 reduces presenilin-1 expression and counteracts ER Ca^{2+} leak. (A) Quantification of mRNA expression levels of presenilin-1 in INS-1 cells treated with different concentrations of the JNK-inhibitor SP600125 or DMSO control for 24 h via qRT-PCR. GAPDH was used as housekeeping gene. Bars indicate mean ± SEM (DMSO Control: n=6; SP600125: n=6). *p<0.05. (B&C) After loading INS-1 cells with the cytosolic Ca^{2+} indicator Fura-2/AM, untreated cells (B) and cells pretreated for 24 h with 100 µM SP600125 (C) were pre-incubated in Ca^{2+}-free EGTA containing buffer for either 1 or 20 min. For evaluation of ER Ca^{2+} content, ER Ca^{2+} stores were fully depleted by applying IP_3-generating agonist (100 µM carbachol) together with 15 µM of the SERCA inhibitor BHQ. The maximal ER store depletion was measured as maximal releasable ER Ca^{2+} in the cytosol whereas the ER is considered as fully filled at the one minute time point. Bars on the right represent corresponding statistics, mean ± SEM (B: DMSO Control 1 min: n=164/8; DMSO Control 20 min: n=206/8; C: SP600125 1 min: n=126/6; SP600125 20 min: n=196/8). *p<0.05 tested with unpaired Student's t-test.

inhibitor-mediated reduction of presenilin-1 expression yields reduction of the ER Ca^{2+} leak in pancreatic β-cells, and, thus, strongly supporting our previous findings [10].

Influence of presenilin-1 depletion on plasma membrane Ca^{2+} uptake via L-type Ca^{2+} channels

In order to validate whether or not the depletion of presenilin-1 affects the Ca^{2+} entry pathways existing in pancreatic β-cells, the Ca^{2+} entry due to Ca^{2+} store (ER) depletion (store-operated Ca^{2+} entry, SOCE) and depolarization (L-type Ca^{2+} channels) was assessed in control and presenilin-1 depleted cells. Therefore, cells were treated in the absence of extracellular Ca^{2+} with the irreversible SERCA inhibitor thapsigargin to empty the ER followed by the re-addition of extracellular Ca^{2+}. The Ca^{2+} upon the re-addition of extracellular Ca^{2+} reflects the SOCE activity. Our experiments did not reveal any effect of the knock-down of presenilin-1 in SOCE activity in pancreatic β-cells (Fig. 2A).

In order to investigate a possible influence of presenilin-1 knock-down on the activity of voltage-gated L-type Ca^{2+} channels, we measured cytosolic Ca^{2+} in the presence of extracellular Ca^{2+} and depolarized the pancreatic β-cells with extracellular 30 mM K^+. Knock-down of presenilin-1 failed to affect the increases in cytosolic Ca^{2+} upon cell depolarization (Fig. 2B), thus, indicating that the lack of presenilin-1 did not affect the activity of L-type Ca^{2+} channels.

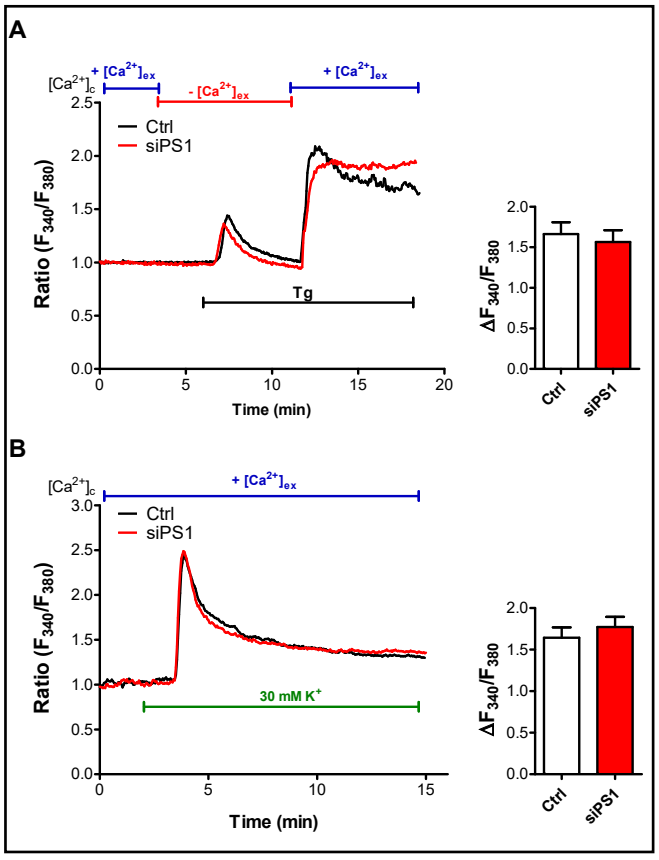

Fig. 2. Influence of presenilin-1 on plasma membrane Ca^{2+} uptake via L-type Ca^{2+} channels. Representative curves (left panels) and respective statistics (right panels) of cytosolic Ca^{2+} measurements in INS-1 cells transfected with either control siRNA or siRNA against presenilin-1. Cells were loaded with the cytosolic Ca^{2+} indicator Fura-2/AM and perfused and imaged on the microscope under different conditions. (A) INS-1 cells were perfused with Ca^{2+}-containing buffer ($+[Ca^{2+}]_{ex}$) until the baseline was stable before switching to Ca^{2+}-free EGTA-containing buffer ($-[Ca^{2+}]_{ex}$) as indicated in the graphs. 1 µM of the SERCA inhibitor thapsigargin (Tg) was applied under Ca^{2+}-free conditions leading to an increase in cytosolic Ca^{2+}. Afterwards, extracellular Ca^{2+} was re-added under the presence of Tg. Bars on the right represent the cytosolic Ca^{2+} increase after Ca^{2+} re-addition, mean ± SEM (Control: n=275/9; siPS-1: n=277/9). (B) INS-1 cells were perfused with Ca^{2+}-containing buffer ($+[Ca^{2+}]_{ex}$) and depolarized with 30 mM K^+-containing buffer. Bars on the right represent the cytosolic Ca^{2+} increase after depolarization with 30 mM K^+, mean ± SEM (Control: n=273/9; siPS-1: n=281/9).

Presenilin-1 is prerequisite for robust Ca^{2+} responsiveness of β-cells to elevated glucose

Because D-glucose-stimulated Ca^{2+} spiking is known to link mitochondrial activity with insulin secretion in β-cells, we examined the putative involvement of presenilin-1 on glucose-triggered cytosolic Ca^{2+} spiking in the two pancreatic β-cell lines INS-1 and MIN-6 cells under control conditions and after siRNA-mediated depletion from presenilin-1. β-cells were

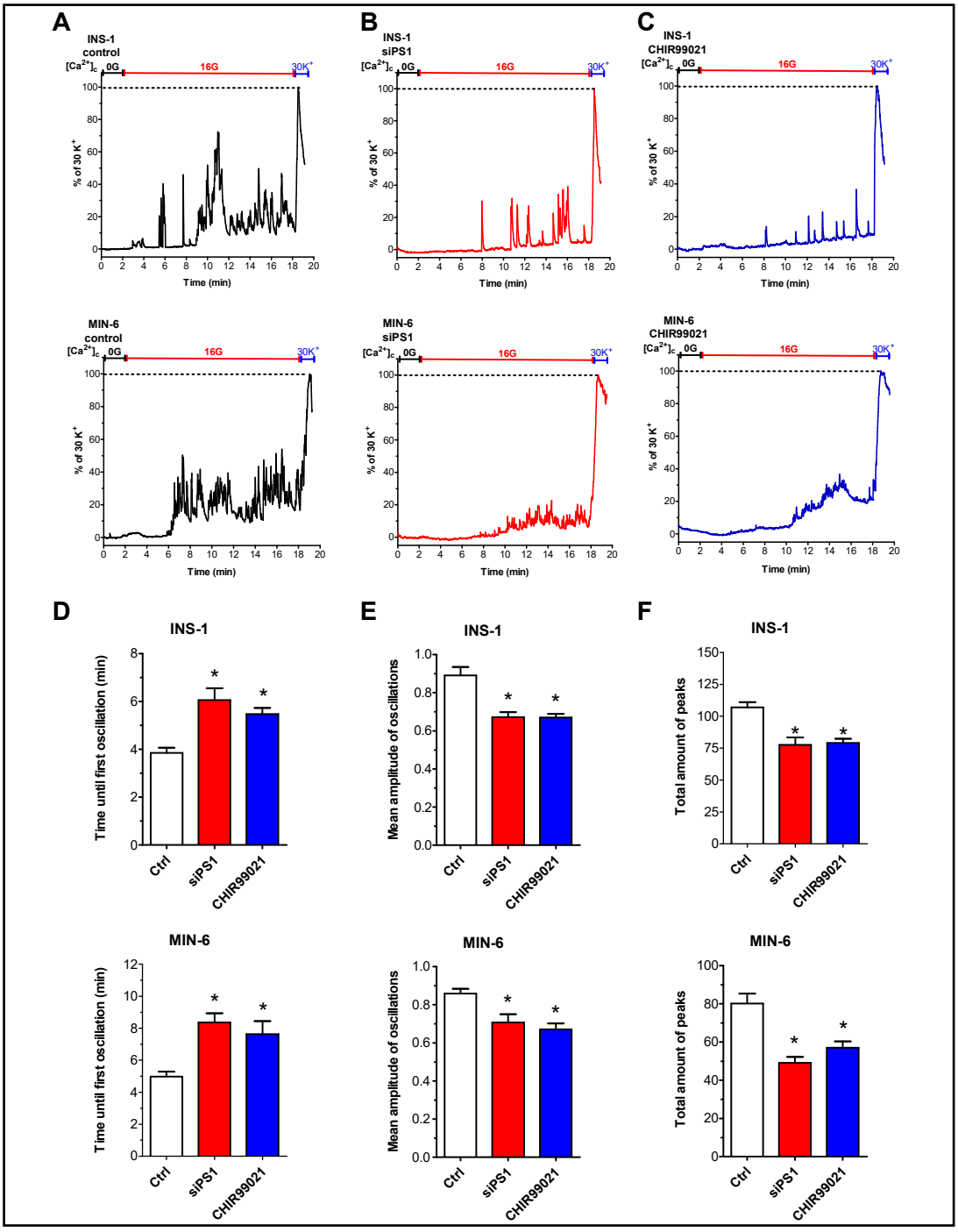

Fig. 3. ER Ca^{2+} leak is essential for the responsiveness of β-cells. (A-C) Representative traces showing glucose-induced cytosolic Ca^{2+} oscillations in INS-1 cells (upper panels) and in MIN-6 cells (lower panels) under control conditions (A), after knock-down of presenilin-1 with specific siRNAs (B) or after treatment with GSK3β inhibitor CHIR99021 (2.5 μM) (C). Cells were loaded with Fura-2/AM in experimental buffer (EB) for 40 min, followed by a 20 min incubation in glucose-free buffer (0G) before imaging. As bars indicate, cells were perfused with EB without glucose (0G) for 2 min prior switching to 16 mM glucose-containing EB (16G) during acquisition, followed by depolarization with 30 mM K^+ to evaluate the maximum Ca^{2+} signal. Corresponding statistical analyses showing (D) time until first oscillation, (E) mean amplitude of the oscillations and (F) total amount of peaks under control conditions (white bars), after knock-down of presenilin-1 (red bars) or after treatment with GSK3β inhibitor CHIR99021 (2.5 μM) (blue bars). *p<0.05 versus control using one-way ANOVA (n≥126/6).

exposed to elevated glucose to trigger cytosolic Ca^{2+} transients, which were observed in both β-cell lines (Fig. 3A), demonstrating their normal responsiveness. Depletion of presenilin-1 by siRNA-mediated knock-down reduced the Ca^{2+} responses of the β-cells to elevated glucose (Fig. 3B). Statistical analyses of the kinetics of glucose-induced Ca^{2+} transients revealed that in both β-cell lines the depletion of presenilin-1 delayed the response times until cytosolic Ca^{2+} elevations occurred (Fig. 3D), reduced mean amplitudes (Fig. 3E) and numbers of individual Ca^{2+} transients (Fig. 3F).

GSK3β activity is essential for the initiation of Ca^{2+} spiking to elevated glucose in β-cells

Since in our previous work presenilin-1 is essentially needed to be phosphorylated by GSK3β in order to establish ER Ca^{2+} leak that yields pre-activation of mitochondria in the β-cells, we next examined the effect of an inhibition of GSK3β with its inhibitor CHIR99021 [18] on glucose-triggered cytosolic Ca^{2+} spiking in the two pancreatic β-cell lines. Compared with controls (Fig. 3A), in cells treated with CHIR99021 glucose-induced Ca^{2+} spiking was strongly attenuated (Fig. 3C). Hence, the statistical analyses of the kinetics of glucose-induced Ca^{2+} transients showed that inhibition of GSK3β delayed cytosolic Ca^{2+} spiking (Fig. 3C), lowered mean amplitudes (Fig. 3D) and the count of Ca^{2+} transients (Fig. 3E).

Presenilin-1 is essential for the initial insulin secretion upon elevated glucose in β-cells

We obtained a time course of GSIS in the pancreatic β-cell lines INS-1 that were transfected with scrambled siRNA or specific siRNA against presenilin-1 to prevent the ER Ca^{2+} leak as previously shown [10]. In control cells, the administration of elevated glucose triggered an initial burst of GSIS within 6-8 min of glucose exposure followed by a sustained phase of insulin secretion (Fig. 4A). However, in cells that were depleted of presenilin-1 the initial burst of GSIS was absent while the sustained phase of insulin secretion was unaffected by the presenilin-1 knockdown (Fig. 4A). Notably, the absolute amount of insulin secreted remained delayed over the entire 50 min observation time while insulin secretion after

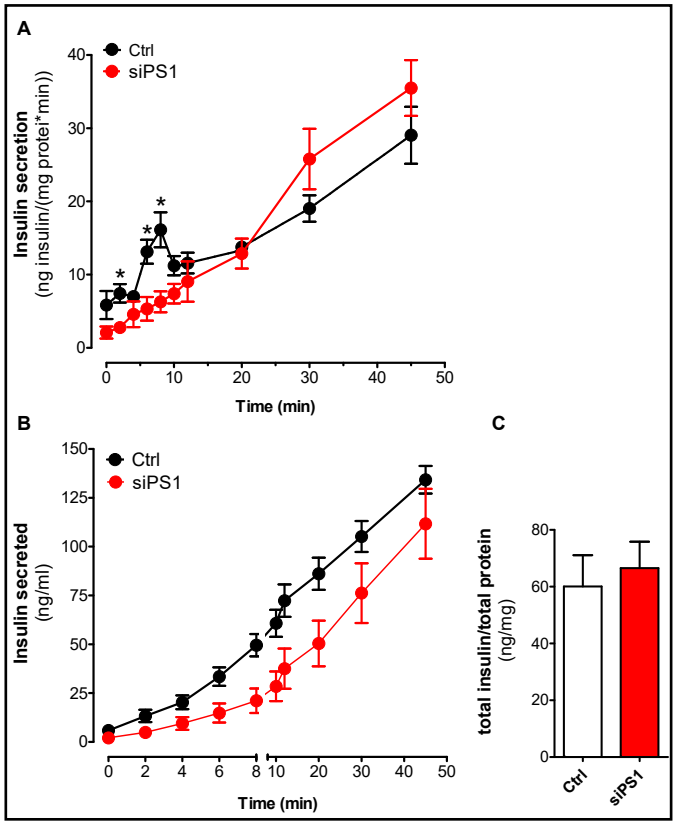

Fig. 4. The presenilin-1 established ER Ca^{2+} leak is essential for a proper biphasic insulin secretion upon elevated glucose in pancreatic β-cells. (A) GSIS is shown as insulin secretion rate in β-cells (INS-1). Lines and points represent insulin secretion rate under control conditions (Ctrl; black) and knock-down of presenilin-1 (β-cells; red). (B) Cumulative total concentration of secreted insulin at the indicated time points from β-cells (INS-1) calculated in ng/ml either under control conditions (black) or knock-down of presenilin-1 (INS-1; red). To equilibrate the insulin secretion, cells were kept on HBSS buffer containing 3 mM glucose for 30 min. Afterwards medium was changed to HBSS buffer containing 16 mM glucose and medium was sampled at the indicated time points. (C) Total insulin content in β-cells (INS-1) transfected with siRNA control (Ctrl) or siRNA against presenilin-1 (siPS1). *p<0.05 versus control using the unpaired Student's t-test (n=5).

10 min glucose administration was comparable in control cells and that depleted from presenilin-1 (Fig. 4B). Notably, depletion of presenilin-1 did not affect the insulin content in the INS-1 cells (controls: 60.1 ± 10.9 ng insulin/mg protein; presenilin-1 depleted cells: 66.5 ± 9.3 ng insulin/mg protein; n=5) (Fig. 4C).

GSK3β activity is crucial for the initial insulin secretion upon elevated glucose in pancreatic β-cells and pancreatic islets

Moreover, experiments were conducted using INS-1 cells and in freshly isolated pancreatic islets that were treated with the GSK3β inhibitor CHIR99021 to avoid the enhanced ER Ca^{2+} leak recently described in freshly isolated pancreatic islets [10]. Similar to our findings in the INS-1 cells where presenilin-1 expression was reduced by siRNA-mediated knock-down, the inhibition of ER Ca^{2+} leak by administration of the GSK3β inhibitor prevented the initial GSIS (Fig. 5A, D) but not the long-lasting insulin secretion (Fig. 5B, E) in response to elevated

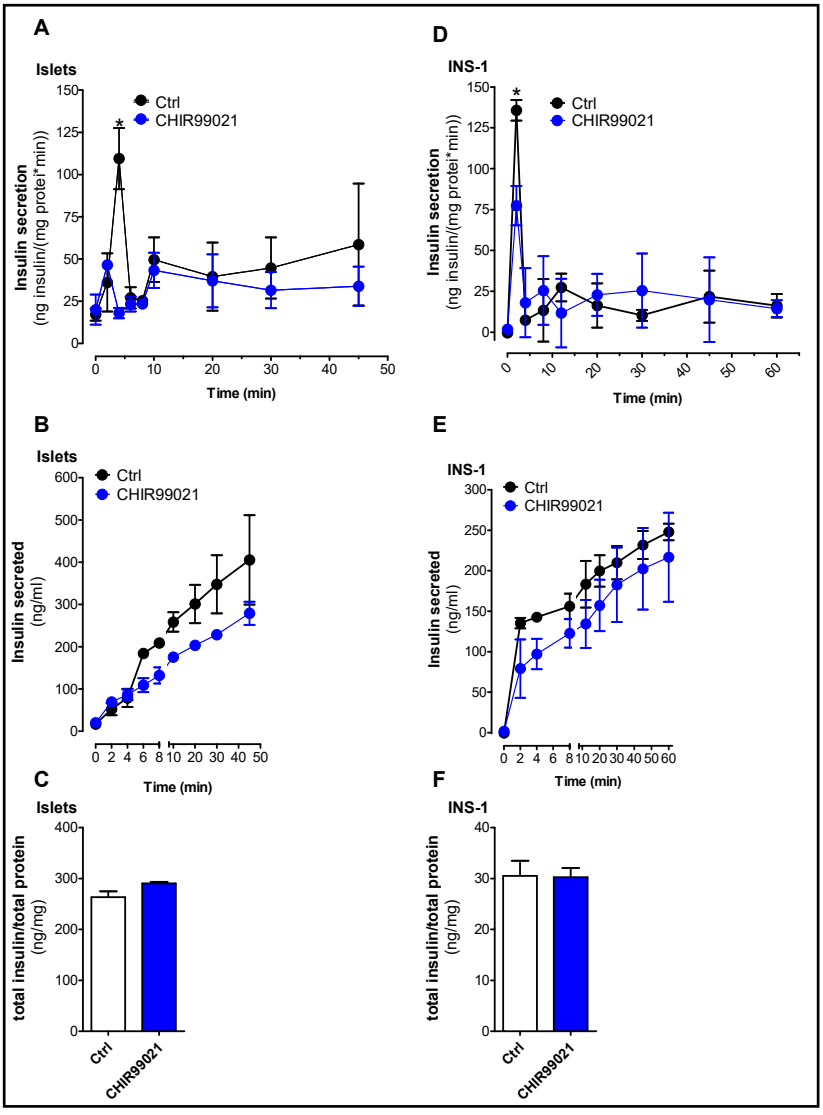

Fig. 5. The activity of GSK3β is prerequisite for the initial insulin secretion upon elevated glucose in pancreatic islets and in pancreatic β-cells. GSIS is shown as insulin secretion rate in isolated murine pancreatic islets (A) and β-cells (INS-1) (D). Lines and points represent insulin secretion rate under control conditions (Ctrl; black) and after pretreatment with 2.5 µM of the GSK3β inhibitor CHIR99021 for 24h (CHIR99021; blue). Prior to experiments, freshly isolated pancreatic islets were kept on HBSS buffer containing 3 mM glucose for 30 min. Islets were challenged by 16 mM glucose in HBSS buffer and samples were collected at the given time points. Cumulative insulin secretion at the indicated time points from isolated pancreatic islets (B) and pancreatic β-cells (INS-1; E) calculated as increased concentration (ng/ml) either under control conditions (black) or after pre-treatment for 24 h with 2.5 µM CHIR99021 (blue). Total insulin content of isolated murine pancreatic islets (C) and pancreatic β-cells (INS-1; F) pre-treated with 2.5 µM CHIR99021 for 24 h. *p<0.05 versus control using the unpaired Student's t-test (pancreatic islets: n=3; INS-1 cells: n=5).

glucose. Despite an inhibition of GSK3β by CHIR99021 the insulin content in INS-1 cells and the freshly isolated pancreatic islets remained only marginally affected (islets: controls: 263.6 ± 11.1 ng insulin/mg protein; CHIR99021: 290.7 ± 2.8 ng insulin/mg protein INS-1 cells: controls: 30.5 ± 2.9 ng insulin/mg protein; CHIR99021: 30.3 ± 1.8 ng insulin/mg protein) (Fig. 5C, F).

Discussion

The predominant function of pancreatic β-cells is the control of blood glucose levels by secretion of sufficient amounts of insulin. Insulin production and release are tightly controlled by mainly glucose, several peptide hormones, neurotransmitters and other nutrients under physiological conditions [3, 19]. Importantly, after glucose metabolization and ATP formation, the inhibition of K_{ATP} channels yields opening of L-type Ca^{2+} channels and, subsequently, Ca^{2+} influx into the β-cell as the final trigger for insulin secretion [20–22]. Notably, during the cytosolic Ca^{2+} elevations, the sequestration of these ions into the mitochondrial matrix via MCU [23]/MICU1 [5]-dependent pathway is fundamental for GSIS [3, 24–26]. While the crucial role of mitochondrial Ca^{2+} has been clearly presented [4, 5,23], these reports focus on the importance of mitochondrial Ca^{2+} uptake of entering Ca^{2+}, thus, describing the later phase that succeeds mitochondrial ATP production, membrane depolarization by ATP-mediated inhibition of K_{ATP} channels, and the subsequent opening of L-type voltage-gated Ca^{2+} channels. Notably, in our recent work [10], elevated resting mitochondrial Ca^{2+} levels were described in pancreatic β-cells that builds on an increased ER Ca^{2+} leak due to GSK3β-mediated phosphorylation of presenilin-1 [10]. Our present findings that inhibition of JNK kinase that decreases the expression of presenilin-1 [35] reduced the ER Ca^{2+} leak further support our previous hypothesis on the crucial role of presenilin-1 on the ER Ca^{2+} leak in pancreatic β-cells. These findings shed a light on the resting situation of mitochondria in pancreatic β-cells and reveal a cell specific setting of enhanced basal mitochondrial activity under resting conditions. The present work represents a follow-up work that explores the physiological consequences of such elevated basal mitochondrial activity in terms of the β-cell most prominent function: the glucose-stimulated insulin secretion (GSIS).

Matrix Ca^{2+} is a major determinant for mitochondrial activity [4]. According to the current concept of GSIS, glycolysis-derived pyruvate fuels matrix citric acid cycle required for mitochondrial ATP production. The ATP inhibits plasma membrane K_{ATP} channels resulting in plasma membrane depolarization and opening of L-type Ca^{2+} channels. Subsequently, entering Ca^{2+} initiates fusion of insulin-containing granules with the cell membrane, thus, releasing insulin [2]. Notably, mitochondrial Ca^{2+} increase is known to serve as a key trigger for insulin release in β-cells [27], because of its stimulatory effect on the matrix dehydrogenases of the citric acid cycle [6, 22, 28]. Missing from the current concept, however, is the exact time point of the contribution of mitochondrial Ca^{2+} to GSIS. In theory, a mitochondrial Ca^{2+} increase would occur upon Ca^{2+} entry due to the opening of L-type Ca^{2+} channels, however, this would be already downstream of mitochondrial activation by glycolysis-derived pyruvate and ATP production and hence be of questionable importance in triggering GSIS. Since an increase in mitochondrial Ca^{2+} stimulates Ca^{2+}-dependent dehydrogenases of the citric acid cycle [28], we envisaged that a ER Ca^{2+} leak evoked pre-stimulation of β-cell mitochondrial dehydrogenases, which are ultimately responsible for the necessary ATP production, would be of metabolic advantage by ensuring a fast and precise insulin response solely controlled by glucose.

Our data on the enhanced basal respiratory activity in β-cells [10] support such new perspective on GSIS that involves a continuous priming of β-cells based on a weak mitochondrial stimulation to ensure accurate responsiveness to elevated blood glucose sensing [29]. This conclusion was previously supported by our recent findings that inhibition of ER Ca^{2+} leak, either by knock-down of presenilin-1 expression or an inhibition of GSK3β by CHIR99021 [18] abolished enhanced mitochondrial resting metabolic activity and

elevation in basal mitochondrial ATP production [10]. The importance of GSK3β/presenilin-1-provided ER Ca^{2+} for the responsiveness of pancreatic islets and β-cells to elevated glucose is further illustrated by our present experiments measuring glucose-induced cytosolic Ca^{2+} spiking and GSIS, the ultimate readout for pancreatic islet/β-cell function. Notably, the lack of ER Ca^{2+} leak yielded a delayed onset time of cytosolic Ca^{2+} oscillations, reduced their mean amplitude and total number of spikes upon elevated glucose in the β-cells, thus, pointing to a reduced responsiveness of the cells to elevated glucose. Our findings, that the depletion of presenilin-1 did neither affect the depolarization-induced Ca^{2+} entry nor the store-operated Ca^{2+} entry, let us speculate that the reduced Ca^{2+} spiking in presenilin-1 depleted cells is not due to a direct effect on the existing Ca^{2+} entry pathways. Hence these findings are in line with our previous experiments [10] and support our hypothesis that the presenilin-1 established ER Ca^{2+} leak is fundamental for glucose sensing/metabolism to ensure proper β-cell responsiveness to elevated glucose. Moreover, our present data reveal that in isolated pancreatic islets and in the two β-cell lines used for this study the GSK3β/presenilin-1-induced ER Ca^{2+} leak is a prerequisite for the initial insulin release that occurs 10 min after exposure to elevated glucose. Notably, the inhibition of enhanced ER Ca^{2+} leak had no effect on the second phase of insulin secretion after 12 min of exposure to elevated glucose. These data point to a delayed responsiveness to elevated glucose of pancreatic β-cells and pancreatic islets that lack the enhanced ER Ca^{2+} leak while the long-lasting insulin secretion remains unaffected. Several studies showed that a loss of the first phase of the biphasic insulin response [30] is associated with a delayed β-cell response/insulin secretion [31] as early irregularity in type 2 DM and to worsening post-prandial hyperglycemia [32]. Therefore, the lack of the initial phase of insulin secretion serves as predictive marker for the risk of developing DM [33]. Our data showing that a decreased ER Ca^{2+} leak ultimately results in reduced mitochondrial metabolism [10] as well

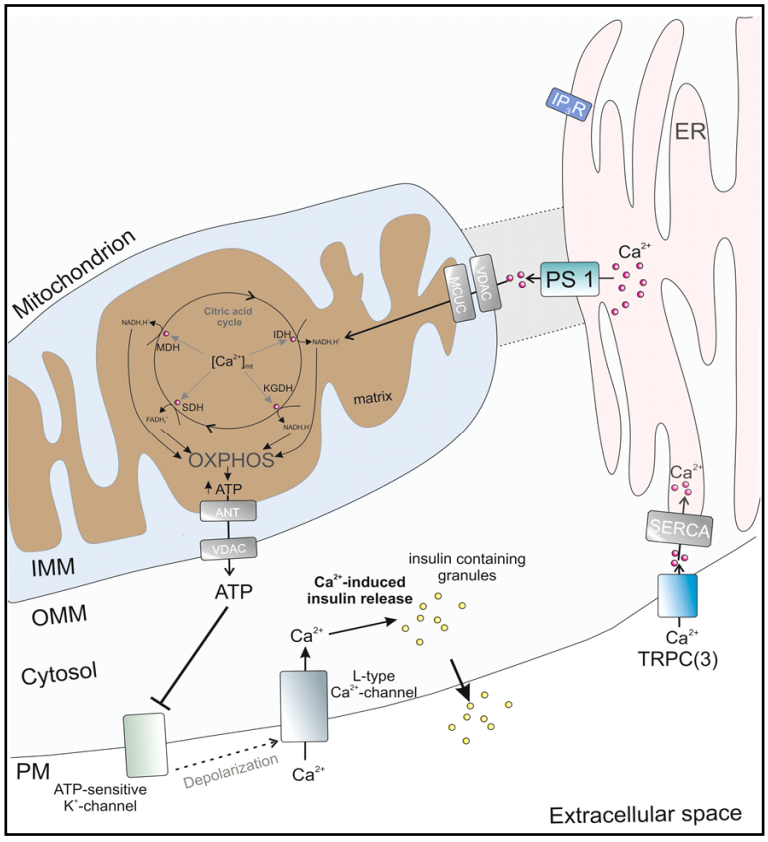

Fig. 6. Schematic representation of the consequences of presenilin-1 mediated ER Ca^{2+} leak on pancreatic islets and pancreatic β-cells. The presenilin-1-mediated ER Ca^{2+} leak is directly sequestered by mitochondria, leading to increased basal matrix Ca^{2+} levels that yields enhanced resting activity of mitochondria in the pancreatic β-cells due to pre-stimulation the Ca^{2+}-dependent dehydrogenases of the citric acid cycle. Upon elevation of glucose, glucose is metabolized and the pre-activated citric cycle in the mitochondria efficiently converts glucose metabolism to activation of the respiratory chain (OXPHOS) and, subsequently, fast ATP production, thus, ensuring a fast, initial insulin secretion within 10 minutes of exposure to elevated glucose.

as reduced glucose induced cytosolic Ca^{2+} oscillations (this study), demonstrate the molecular basis for such loss of the first phase insulin response. In line with our findings, recent studies point to the importance of ER-mitochondrial Ca^{2+} transfer for an adequate insulin release [34–38]. Accordingly, we hypothesize that disturbances in the presenilin-1 dependent ER Ca^{2+} leak in pancreatic islets/β-cells hamper the cell's fast and precise first insulin response which, in turn, may contribute to postprandial hyperglycemia and the development of type 2 DM (Fig. 6).

Conclusion

Our present data together with our previous report [10] unveil an important phenomenon that builds on a GSK3/presenilin-1-established ER Ca^{2+} leak that yields basal activation of mitochondria as prerequisite for responsiveness of pancreatic β-cells to elevated glucose (i.e. GSIS). Besides its physiological importance for GSIS, disturbances of this very β-cell-specific mechanism might be of pathological importance because its possible contribution to the development of type 2 DM.

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Authors' contributions

C.K., G.Z. M.W.-W. and M.R.D. performed cytosolic, mitochondrial and endoplasmic reticulum calcium measurements. C.K. and CTMS performed oscillation experiments as well as immunosorbent assay and expression evaluation. B.G. performed analysis of cytosolic Ca^{2+} oscillation data. V.S., M.D.-M. and D.K. isolated the murine pancreatic islets, and, R.R. was responsible for cell preparation, culture and transfection. W.F.G. supervised research and project planning, and together with R.M., T.M., D.K. and J.H. performed data interpretation and prepared the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

Disclosure Statement

The authors declare no competing financial interests.

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