Deletion of CDKAL1 Affects Mitochondrial ATP Generation and First-Phase Insulin Exocytosis

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Abstract

Background: A variant of the CDKAL1 gene was reported to be associated with type 2 diabetes and reduced insulin release in humans; however, the role of CDKAL1 in β cells is largely unknown. Therefore, to determine the role of CDKAL1 in insulin release from β cells, we studied insulin release profiles in CDKAL1 gene knockout (CDKAL1 KO) mice.

Principal Findings: Total internal reflection fluorescence imaging of CDKAL1 KO β cells showed that the number of fusion events during first-phase insulin release was reduced. However, there was no significant difference in the number of fusion events during second-phase release or high K+ -induced release between WT and KO cells. CDKAL1 deletion resulted in a delayed and slow increase in cytosolic free Ca2+ concentration during high glucose stimulation. Patch-clamp experiments revealed that the responsiveness of ATP-sensitive K+ (KATP) channels to glucose was blunted in KO cells. In addition, glucose-induced ATP generation was impaired. Although CDKAL1 is homologous to cyclin-dependent kinase 5 (CDK5) regulatory subunit-associated protein 1, there was no difference in the kinase activity of CDK5 between WT and CDKAL1 KO islets.

Conclusions/Significance: We provide the first report describing the function of CDKAL1 in β cells. Our results indicate that CDKAL1 controls first-phase insulin exocytosis in β cells by facilitating ATP generation, KATP channel responsiveness and the subsequent activity of Ca2+ channels through pathways other than CDK5-mediated regulation.

Introduction

Type 2 diabetes is a multifactorial disease characterized by decreased insulin secretion and decreased insulin action at target tissues. While the primary molecular defects in type 2 diabetes remain largely unknown, genetic factors in combination with environmental factors are thought to be involved in the onset and development of the disease. Recent genome-wide association studies have identified CDKAL1 (cyclin-dependent kinase 5 regulatory subunit associated protein 1-like 1) as a susceptibility gene for type 2 diabetes, which has been replicated in several populations [1–5]. The CDKAL1 gene is located on chromosome 6p22.3 and encodes a 65-kDa protein (CDKAL1). The expression of CDKAL1 mRNA has been detected in human tissues including pancreatic islets, skeletal muscle and brain [1,4]. Although the function of CDKAL1 is still unclear, CDKAL1 is similar to cyclin-dependent kinase 5 regulatory subunit-associated protein 1 (CDK5RAP1), which is expressed in neuronal tissues, and inhibits cyclin-dependent kinase 5 (CDK5) activity by binding to the CDK5 activator p35 [6]. CDK5 was previously implicated in islet function [7–9], suggesting that CDKAL1 plays a role in β cell function by inhibiting CDK5 kinase activity [1–4].

Genome-wide association studies have shown that several single-nucleotide polymorphisms (SNPs) in intron 5 of the CDKAL1 gene are associated with type 2 diabetes [1–4]. An association between SNPs and insulin release has also been suggested [2,3]. Recently, Groenewoud et al. [10] and Stancakova et al. [11] reported that a CDKAL1 variant (rs7754840) decreased first-phase insulin secretion but not second-phase insulin secretion during hyperglycemic clamps and intravenous glucose tolerance tests, respectively. Furthermore, the gene variants were not associated with the insulin sensitivity index [10,11], suggesting that CDKAL1 variants influence the risk of type 2 diabetes by impairing first-phase insulin secretion. However, the molecular
mechanisms through which CDKAL1 modulates insulin release in pancreatic β cells are unknown.

To understand the role of CDKAL1 in β cells, we studied the phenotype of mice deficient in CDKAL1 expression, which was established using a gene-trap technique (CDKAL1 KO mice). Our data demonstrate that CDKAL1 has a role in first-phase insulin exocytosis in β cells by facilitating ATP generation, K<sub>ATP</sub> channel responsiveness and subsequent Ca<sup>2+</sup> channel activity; surprisingly, these effects were independent of CDK5 activity.

Results

Expression and histological examination of CDKAL1 KO mice

To explore the potential role of CDKAL1 in β cells, we studied CDKAL1 KO β cells. CDKAL1 KO mice did not express CDKAL1 mRNA in pancreatic islets or the brain (Fig. 1A). In immunoblot analysis (Fig. 1B), CDKAL1 protein was not detected in KO islets or KO brain, whereas it was more highly expressed in WT islets than in WT brain. We next morphologically examined the pancreatic islets. Immunostaining of pancreatic sections with insulin antibodies revealed no significant difference in islet architecture, including β cell area per pancreas, between WT and KO mice (Fig. 2A-B). Electron microscopy of the pancreatic β cells also revealed that cell size, total number of granules per section and mean granule diameter were similar in WT and KO mice (Fig. 2C-F). Furthermore, pancreatic insulin content, islet DNA content and islet insulin content of CDKAL1 KO mice were similar to those of WT mice (Fig. 2G-I). Thus, CDKAL1 KO β cells displayed specific CDKAL1 protein depletion but their characteristics were similar to those of WT cells.

Effects of CDKAL1 on glucose-induced biphasic insulin exocytosis

We next investigated the effects of CDKAL1 KO on glucose-induced insulin release from β cells. Batch experiments showed that 16.7 mM glucose-induced insulin release (for 30 min) from CDKAL1 KO β cells was decreased (~19%) relative to that from WT β cells (P<NS) (Fig. 3A). To evaluate in detail the effects of CDKAL1 deficiency on glucose-evoked biphasic insulin exocytosis, the dynamic motion of individual insulin granules tagged with green fluorescent protein (GFP) was observed under total internal reflection fluorescence (TIRF) microscopy. As previously reported [12], stimulation with 22 mM glucose evoked biphasic insulin granule exocytosis through fusion of two types of granules (previously docked granules and newcomers) in WT β cells (Fig. 3B, top). During the first phase (within 5 min), the fusion events mostly occurred from the previously docked granules with some of the fusion events from newcomers while continuous fusion was observed thereafter, mostly from newcomers during the second phase (over 15 min). In contrast, CDKAL1 KO β cells showed abnormalities in biphasic insulin granule exocytosis (Fig. 3B, bottom) because the number of fusion events during the first phase was significantly reduced in KO cells [WT (n=16) vs. KO (n=14), 43.0±6.2 vs. 12.7±3.8, P<0.001]. In contrast, during the second phase, the number of fusion events tended to be increased but there was no significant difference [WT (n=16) vs. KO (n=14), 87.5±6.4 vs. 105.6±8.0, P=NS].

On the other hand, high K<sup>+</sup>-induced granule fusion was normal in KO cells (Fig. 3C), suggesting that the exocytotic steps after the elevation of intracellular calcium concentration ([Ca<sup>2+</sup>]) were not impaired in KO cells. In fact, the number of morphologically docked insulin granules observed by TIRF and electron microscopy (Fig. 4A and B), and fusion kinetics (data not shown) were not altered in CDKAL1 KO β cells. In addition, the expression levels of SNARE proteins (syntaxin1A, SNAP25 and VAMP2), important mediators of insulin granule docking and fusion [12], were similar in WT and KO β cells (Fig. 4C). These results indicate that granule docking and Ca<sup>2+</sup>-triggered fusion are not impaired in CDKAL1 KO β cells.

Changes in the [Ca<sup>2+</sup>]<sup>i</sup> increase in KO cells

To determine whether the impairments in glucose-induced first-phase insulin exocytosis were associated with changes in the [Ca<sup>2+</sup>]<sup>i</sup> increase, we monitored the time-course of the glucose-induced [Ca<sup>2+</sup>]<sup>i</sup> increase using Fura-2 methods. CDKAL1 KO resulted in a delayed and slow increase in [Ca<sup>2+</sup>]<sup>i</sup>, during high glucose stimulation with a similar time-course to insulin exocytosis in KO cells. In contrast, a rapid and marked [Ca<sup>2+</sup>]<sup>i</sup> rise was observed in WT cells (Fig. 5A). The mean lag time (Δ time from stimulation to onset) and rise time (Δ time from onset to peak) were slower in KO cells than in WT cells [WT vs. KO (n=12) each lag time: 1.2±0.1 min vs. 3.0±0.1 min, P<0.0001; rise time: 3.0±0.2 min vs. 4.1±0.2 min, P<0.0001]. The magnitude of the [Ca<sup>2+</sup>]<sup>i</sup> rise was not different between KO and WT cells. Furthermore, there was no difference in the high K<sup>+</sup>-induced [Ca<sup>2+</sup>]<sup>i</sup> rise between KO and WT cells (Fig. 5B). These data suggest that specific steps in glucose-induced early signal transduction that precede the [Ca<sup>2+</sup>]<sup>i</sup> rise, were impaired in KO cells.

The responsiveness of the K<sub>ATP</sub> channel current to glucose was impaired in CDKAL1 KO β cells

We next investigated the function of K<sub>ATP</sub> channels in CDKAL1 KO cells. As shown in Fig. 6A, there were no differences in the expression of the K<sub>ATP</sub> channel components Kir6.2 and SUR1 between WT and KO islets. The K<sub>ATP</sub> channel currents evoked by the voltage ramp from −100 to −50 mV in WT and KO mice are expressed as the current density determined as the calculated conductance (pS) divided by the cell capacitance (pF). The K<sub>ATP</sub> currents were determined in the presence of 2.8 and 8.3 mM...
glucose in WT and KO mice (Fig. 6B and C). The current densities at 2.8 mM glucose were 392.9 ± 121.6 pS/pF in WT (n = 6) and 639.5 ± 145.1 pS/pF in KO cells (n = 8) (P > 0.05). In WT cells, the K<sub>ATP</sub> current density decreased significantly by increasing the glucose concentration from 2.8 to 8.3 mM (Fig. 6D; P < 0.02), while there was a relatively small, non-significant decrease in the current density in KO mice (Fig. 6E; P > 0.05).

In addition, the time to the most inhibited level of K<sub>ATP</sub> currents after exposing β cells to 8.3 mM glucose tended to be longer in KO cells (4.73 ± 1.16 min, n = 5) compared with that in WT cells (3.11 ± 0.48 min, n = 6; P > 0.05). Thus our data indicate that the K<sub>ATP</sub> channel responsiveness to glucose stimulation was blunted in CDKAL1 KO cells compared with WT cells.

On the other hand, we did not observe any differences in the ATP sensitivity of the channel in studies using inside-membrane patches. The half-maximal concentration of channel inhibition for ATP was 2.6 μM (n = 9) in CDKAL1 KO mice (data not shown), which is less than those previously reported in control mice [13,14]. These results suggest that changes in K<sub>ATP</sub> channel activity in KO cells were not responsible for the reduced ATP sensitivity of these channels.

ATP generation was impaired in KO cells

It is conceivable that the reduced K<sub>ATP</sub> channel responsiveness to glucose in KO cells might be due to a defect in ATP production following glucose metabolism. Therefore, we measured ATP content in islets from WT and KO mice after incubation with 2.2 and 22 mM glucose using high-performance liquid chromatography (HPLC) with a reverse-phase column. As shown in Fig. 7A, ATP content was significantly increased in WT islets by stimulation with 2.2 and 22 mM glucose (P < 0.05 and P < 0.01, respectively). In KO cells, ATP content was increased by incubation with 2.2 mM glucose (P < 0.05), but not with 22 mM glucose (P > 0.05). These results suggest that reduced ATP generation may be responsible for the impaired K<sub>ATP</sub> channel activity in KO cells.

Figure 2. Pancreatic histology and insulin content in CDKAL1 KO mice. A. CDKAL1 KO mice have normal islet architecture. Pancreatic sections were peroxidase stained for insulin. Scale bar: 100 μm. B. Relative area occupied by β cells (percentage of total pancreatic area). Random sections of the entire pancreas from WT and CDKAL1 KO mice were immunostained (as shown in A) and analyzed (60 sections from each of three mice per group). C-F. Electron micrographs of pancreatic tissue sections. C. Representative sections (scale bar: 5 μm). D. β cell size. E. Total number of granules per cell section, and F. Mean granule diameter in ultra-thin sections (100 nm) (n = 20 cells per group) of CDKAL1 KO and WT β cells. G-I. Insulin content in CDKAL1 KO mice. G. Pancreatic insulin content measured in acid-ethanol extracts from WT and KO mice by ELISA (n = 6 per group). H. DNA content per islet and I. Islet insulin content per DNA from WT and KO mice (n = 6 per group). Results are means ± SEM. doi:10.1371/journal.pone.0015553.g002
with 22 mM glucose (P<0.001; n = 7). In contrast, the ATP levels in the KO islets showed a small but not statistically significant increase, indicating impaired glucose metabolism in KO islets.

In line with glucose metabolism, we measured changes in the mitochondrial membrane potential in WT and KO cells during glucose stimulation using tetramethyl rhodamine ethyl ester.
Figure 4. *CDKAL1* KO did not affect the number of morphologically docked granules or granule fusion kinetics. **A.** Total internal reflection fluorescence (TIRF) microscopy of insulin granules morphologically docked to the plasma membrane. (top) Typical TIRF images of docked insulin granules in WT and *CDKAL1* KO β cells. The surrounding lines represent the outline of cells attached to the cover glass. Scale bar: 5 μm. Pancreatic β cells were prepared from WT and KO mice, fixed, and immunostained for insulin. (bottom) Number of insulin granules morphologically docked to the plasma membrane. Individual fluorescent spots shown in the TIRF images were manually counted per 200 μm² in 15 cells per group. **B.** Electron micrograph of β cell sections. (top) Typical images of the plasma membrane area facing the blood capillary (C) of WT and KO β cells (B). Bar: 500 nm. (bottom) Number of morphologically docked insulin granules per 10 μm of the plasma membrane. Granules at their shortest distance of <10 nm from the plasma membrane were defined as morphologically docked granules (red arrowheads). Results are means ± SEM. **C.** Expression of SNARE proteins in wild-type (WT) and KO islets by immunoblotting. Equal amounts of islet protein (30 μg) were separated by SDS-PAGE and immunoblotted. β-actin was used as a loading control.

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Figure 5. Effects of *CDKAL1* KO on changes in intracellular free calcium ([Ca²⁺]i). **A.** 22 mM glucose- and **B.** 40 mM high K⁺-induced changes in [Ca²⁺]i in WT and *CDKAL1* KO β cells. Changes in [Ca²⁺]i were measured by Fura-2 acetoxymethyl (2 μM). Time 0 indicates when the stimulants were added. The fluorescence ratio (340/360) at time 0 was taken as 1. Results are means ± SEM (n = 12 cells per group).

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Hyperpolarization of the mitochondrial membrane potential is evident by an increase in the fluorescent intensity of TMRE [15]. CDKAL1 KO cells exhibited a significantly reduced membrane potential response to glucose stimulation compared with WT cells (Fig. 7B). The reduced mitochondrial membrane potential response was further confirmed using α-ketoisocaproate (KIC) (Fig. 7C), which is a direct substrate for the mitochondrial TCA cycle and bypasses glycolysis [16]. Thus, it seems reasonable to conclude that the defective ATP generation in KO cells is due to impaired mitochondrial function, without changes in mitochondrial number or morphology in KO cells on electron microscopy (data not shown).

CDK5/p35 protein kinase activity in CDKAL1 KO islets

CDKAL1 is homologous to CDK5RAP1, which inhibits CDK5 activity by binding to the kinase activator p35 [6]. To test whether CDK5-mediated regulation is involved in the function of CDKAL1 in β cells, we examined the expression and protein kinase activity of CDK5 in CDKAL1 KO islets. CDK5 and p35 were expressed in KO islets as much as in WT islets (Fig. 8A). Surprisingly, we found no difference in CDK5/p35 kinase activity between CDKAL1 KO and WT islets, when using histone H1 as a substrate after immunoprecipitation with a p35-specific antibody (Fig. 8B). The specificity of this activity was confirmed using the CDK5 inhibitor roscovitine. Thus, it seems likely that CDK5 kinase does not contribute to CDKAL1 function in β cells.

Subcellular localization of CDKAL1 in β cells

Finally, we examined the subcellular localization of CDKAL1 using a biochemical subcellular fractionation of MIN6 β cells. In the fractionation procedure [17], the postnuclear S1 fraction was separated by differential centrifugation into an S2 soluble cytosolic/microsomal fraction and a pellet membrane fraction. Then, the S2 fraction was separated into the S3 soluble cytosolic fraction. Endogenous proteins were used as markers for cytosolic (MEK-1/2), endoplasmic reticulum (ER) (BIP/GRP78), mitochondrial (Bcl2), and plasma membrane proteins (SNAP25). CDKAL1 immunoreactivities were detected in the S2 and pellet fractions, but not in the S3 soluble cytosolic fraction (Fig. 9A), suggesting that CDKAL1 seems to associate with microsomes or plasma membrane.

We next performed immunocytochemistry to confirm the subcellular localization of CDKAL1 in β cells. The CDKAL1 antibody cross-reacted with a non-specific protein band (Fig. 1B).
and Fig.9A), suggesting that the antibody is not suitable for immunostaining. In fact, in immunostaining experiments with the CDKAL1 antibody, we could not detect any specific signal in WT cells (data not shown). Therefore, we expressed the CDKAL1 protein as a fusion with enhanced green fluorescent protein (EGFP) and the intracellular localization was examined under a confocal microscope immunostaining with organelle markers or with MitoTracker staining. As shown in Fig. 9B, the fluorescence of the CDKAL1–EGFP protein was colocalized with the ER protein calnexin, but not with MitoTracker Red (a mitochondria marker). These results suggest that CDKAL1 is predominantly localized to the ER.

Discussion

In this study, we found that pancreatic β cells from mice null for CDKAL1, which was identified as a susceptibility gene for type 2 diabetes in humans, have a reduced first-phase insulin release in vitro. TIRF imaging revealed that CDKAL1 KO caused a marked reduction in the number of fusion events during first-phase release (Fig. 3), but not in second-phase release. The impaired glucose-induced first-phase insulin exocytosis was associated with a delayed and slow increase in $\left[\text{Ca}^{2+}\right]$ during glucose stimulation in KO cells (Fig. 5). This may be due to the blunted responsiveness of K_ATP channels to glucose stimulation in KO cells (Fig. 6). Indeed, ATP production in KO cells was low (Fig. 7) and may lead to blunted glucose-induced K_ATP channel responsiveness followed by impaired $\text{Ca}^{2+}$ channel activity.

The target molecules for CDKAL1 are not yet known, although our data indicate that CDKAL1 KO influences glucose-induced K_ATP channel responsiveness through reduced ATP generation. Since CDKAL1 is similar to CDK5RAP1, we originally speculated that the function of CDKAL1 is mediated by CDK5, and is probably directly associated with increased $\text{Ca}^{2+}$ channel activity, as observed in p35 KO mice [8], or with insulin gene expression [7,9]. However, there was no relationship between CDKAL1 and CDK5 in this study. Therefore, CDK5/p35-mediated regulation does not seem to be involved in CDKAL1 regulation of insulin release. Our data suggest that CDKAL1 is instead related to glucose metabolism, which is associated with ATP generation, K_ATP channel activity and $\text{Ca}^{2+}$ channels activity.

The failure of glucose-induced ATP-generation may be due to defects at several stages of glucose metabolism including cytosolic glycolysis and mitochondrial metabolism. In CDKAL1 KO cells, the glucose-stimulated mitochondrial membrane potential response was blunted (Fig. 7B). In addition, KIC, which is a direct substrate for the TCA cycle, bypassing glycolysis, also reduced the mitochondrial membrane potential response (Fig. 7C). Therefore, blunted mitochondrial membrane potential response is likely to be a key defect, resulting in a reduced ATP generation, K_ATP channel responsiveness and $\text{Ca}^{2+}$ channel activity, and eventually leading to impaired insulin granule exocytosis in KO cells. Indeed, it has
been reported that the selective impairment of glucose-induced insulin release in diabetic Goto-Kakizaki rats is associated with reduced sensitivity of KATP channels to glucose by deficient oxidative metabolism of glucose in mitochondria [18,19].

In KO cells, cytosolic free \([\text{Ca}^{2+}]_i\) induced by glucose gradually reached the same level observed in WT cells. We assume that the increase of \([\text{Ca}^{2+}]_i\) during second-phase may reflect suppression of \(\text{Ca}^{2+}\) clearance mechanism. As for other animal cells, four mechanisms remove \(\text{Ca}^{2+}\) from cytosol of \(\beta\) cells: sarcoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (SERCA) pumps, plasma membrane \(\text{Ca}^{2+}\)-ATPase (PMCA), \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX), and the calcium uniporter of mitochondria [20,21]. In mouse and rat \(\beta\) cells, the SERCA pump is the dominant \(\text{Ca}^{2+}\) clearance mechanism [20,21], thus it is conceivable that reduced ATP generation impairs the \(\text{Ca}^{2+}\) clearance function via the SERCA pump, resulting that there is no difference of \([\text{Ca}^{2+}]_i\) in second-phase between KO and WT cells.

As shown in Fig. 9, CDKAL1 is likely to localize to the ER in \(\beta\) cells. The relationship between CDKAL1 deletion in the ER and reduced mitochondrial ATP generation remains unknown. However, it has been reported that the ER and mitochondria are in close contact, which supports communication between these two organelles [22]. It is possible that ER dysfunction caused by CDKAL1 KO may affect mitochondrial function in the KO cells. Such ER–mitochondria communication has been shown to involve the ER stress pathway [22,23], even in \(\beta\) cells [24,25].

Very recently, Arragain et al. reported that CDKAL1 is a methylthiotransferase involved in the biosynthesis of 2-methylthio-N6-threonylcarbamoyladenosine in transfer RNA (tRNA) [26]. The methylthio modification of adenosine in tRNA appears to be essential for efficient and highly accurate protein translation by the ER ribosome. However, it is still unclear how the role of CDKAL1 in tRNA may be linked to ER function or ATP generation in mitochondria. Thus, it is tempting to speculate that either ER dysfunction or ER stress may affect the mitochondrial membrane potential in KO cells.

It is particularly interesting to note that our TIRF imaging data showed that CDKAL1 deletion only impaired the first-phase, but not the second-phase, of glucose-induced insulin exocytosis. Our data are in good agreement with the results of Groenewoud et al. [10] and Stancakova et al. [11], who reported that CDKAL1 variants decreased first-phase insulin secretion but not second-phase insulin in humans with type 2 diabetes. Thus, the altered expression of CDKAL1 is probably associated with reduced
glucose-induced first-phase insulin exocytosis and thus confers an increased risk for diabetes.

Although our data showed a critical role of CDKAL1 in insulin exocytosis in vivo, the CDKAL1 KO mice had almost normal glucose homeostasis (N. Kato, in submission). The reason for the normal glucose homeostasis in vivo, despite reduced first-phase insulin release in vitro, is unknown, but there are several possibilities to be considered. First, the magnitude of the reduction in first-phase insulin release is not sufficient to cause impaired glucose tolerance in mice at this stage we examined. In fact, Stancakova et al. [11] reported that the SNP rs7754840 of CDKAL1 is associated with impaired insulin secretion in non-diabetic offspring of type 2 diabetic subjects and in a large sample of men with normal glucose tolerance. Second, the role of CDKAL1 on the regulation of insulin exocytosis may have a limited influence on whole-body glucose metabolism. Therefore, additional genetic and environmental factors may be needed to cause impaired glucose tolerance.

In summary, we have provided the first report documenting a role of CDKAL1 in β cells. Our study using CDKAL1 KO mice shows that CDKAL1 controls first-phase insulin exocytosis in β cells by facilitating ATP generation, glucose-induced KATP channel responsiveness and subsequent Ca2+ channel activity. Defects in this process caused by decreased CDKAL1 expression levels may confer an increased risk of diabetes.

Methods

Generation of CDKAL1 KO mice

CDKAL1 KO mice were generated by the gene-trapping method [27–29] at TransGenic Inc. (Kobe, Japan) and thereafter established as an experimental model at the National Center for Global Health and Medicine. Male mice were used at the age of 10–14 weeks. Animal experiments were approved by the Animal Care and Use Committee of NCGM Research Institute (permit number: 22-TG-35) and the Kyorin University Animal Care Committee (permit number: 65-2).

Islets and pancreatic β cells

Pancreatic islets of Langerhans were isolated from male WT and CDKAL1 KO mice by collagenase digestion, as previously described [30]. The isolated islets were then dispersed and cultured [30]. To label the insulin secretory granules, pancreatic β cells were infected with recombinant adenovirus Adex1ICA insulin–GFP [30].

RT-PCR

Total RNA was extracted using an Illusta kit (GE healthcare, Piscataway, NJ, USA), and the full-length CDKAL1 was amplified by RT-PCR using specific primers (forward primer: cttctcagttcg-gacagattcatcttcaaggaa; reverse primer: gcttgatctgtgctaggtc-caggg; product 2 kb). The PCR product was separated on agarose gels.

Immunoblotting

Proteins were extracted from mouse pancreatic islets and whole brain and immunoblotted [12,31]. We used antibodies targeting the following proteins: CDKAL1 (ab68045, Abcam, Cambridge, UK), p35 (C19, Santa Cruz, CA, USA), CDK5 (DC17, Santa Cruz), syntaxin 1A (a gift from Dr. T. Fujiwara, Kyorin University School of Medicine, Tokyo, Japan), SNAP25 (Wako Pure Chemical Industries Ltd., Osaka, Japan), VAMP2 (StressGen Biotechnologies Corp, Victoria, BC, Canada), Kir6.2 (Santa Cruz), SUR1 (Santa Cruz), MEK-1/2 (Cell Signaling Technology, Beverly, MA, USA), BIP/GPR78 (BD Transduction Laboratories, Lexington, KY, USA), Bel-2 (BD Transduction Laboratories) and β-actin (Sigma-Aldrich, St. Louis, MO, USA).

Morphometric analysis of islets

To analyze islet size and β cell mass, paraffin-embedded pancreas sections (10 μm) were labeled with anti-insulin antibodies and detected by an avidin-biotin-peroxidase technique (Vector Laboratories, Burlingame, CA, USA). Sections were collected at 500 μm intervals from tissue blocks, and all islets in the sections were analyzed to determine islet area as a percentage of total pancreatic area. Images were acquired with an Olympus microscope IX70 equipped with a charge-coupled device camera, and analyzed with Metamorph software (Molecular Devices, Downingtown, PA, USA).

Electron microscopy

Electron microscopy was carried out using previously described methods [12]. Tissues were fixed in phosphate-buffered 2.5% glutaraldehyde (pH 7.4), postosmicated, dehydrated with graded alcohols, and embedded in Epon 812. After staining with uranyl acetate and lead citrate, ultra-thin sections were examined under a transmission electron microscope (TEM-1010C, JEOL, Akishima, Tokyo, Japan). In electron microscopy, granules at their shortest distance of <10 nm from the plasma membrane were defined as morphologically docked granules.

Insulin content

To measure insulin content in the whole pancreas, the excised pancreata were frozen in liquid nitrogen and disrupted with Cryopress (Microtech Nichion, Funabashi, Japan). The resulting powder was then suspended in cold acid-ethanol, and insulin was extracted overnight at 4°C [32]. The supernatants were diluted and subjected to enzyme-linked immunosorbent assays (ELISA) as described previously [12]. Insulin content per isolated islet was measured by ELISA [12]. Hoechst-33258 staining of sonicated islets was performed to determine the islet DNA content.

Insulin release in batch incubated β cells

Batch incubation experiments for insulin release have been described elsewhere [33]. Briefly, after plating WT and KO cells on 96-well plates, the cells were preincubated for 30 min in Krebs-Ringer buffer (KRB) containing (in mM): 110 NaCl, 4.4 KCl, 1.45 KH2PO4, 1.2 MgSO4, 2.3 calcium gluconate, 4.8 NaHCO3, 2.2 glucose, 10 HEPES, pH 7.4, and 0.3% bovine serum albumin (BSA), and then challenged with 2.2 mM glucose or 22 mM glucose for 30 min. At the end of the stimulation period, the cells were disrupted by sonication and aliquots of media and cell extracts were analyzed by ELISA. The released insulin is expressed as a percentage of total cellular content.

TIRF microscopy

The Olympus total internal reflection system with a high-aperture objective lens was used as described previously [12]. Briefly, primary β cells expressing insulin–GFP on the glass cover slip (Olympus, Tokyo, Japan) were mounted in an open chamber and incubated for 30 min with KRB. Cells were then transferred to a thermostat-controlled stage (37°C) and stimulated with 22 mM glucose (final) and 40 mM KCl (final). To evaluate the number of docked insulin granules by TIRF microscopy, wild-type and CDKAL1 knockout cells were cultured on high refractive index glass, fixed, permeabilized with 4% paraformaldehyde/0.1% Triton X-100, and processed for immunohistochemistry [12].
Cells were labeled with anti-insulin antibodies (Sigma-Aldrich) and processed with goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular probes, Eugene, OR, USA). Immunofluorescence was detected by TIRF microscopy.

Measurements of intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(se\))

Fura-2 acetoxyethyl ester (Fura-2 AM; Molecular Probes) was loaded into cultured \(\beta\) cells and the ARGUS/HiSCA system (Hamamatsu, Japan) was used for measurement, as previously described [12].

Patch-clamp experiments in mouse single \(\beta\) cells

Perforated whole-cell currents were recorded using a pipette solution containing amphotericin B (200 \(\mu\)g/ml) dissolved in 0.1% DMSO [34]. Membrane currents were recorded using an amplifier (200B; Axopatch, CA) and stored online in a computer running pCLAMP10.2 software. The voltage clamp in perforated mode was considered to be adequate when the series resistance was <20 \(\Omega\). Patch pipettes were pulled from glass (Narishige, Tokyo, Japan); the resistances of the pipettes ranged from 4 to 7 \(\Omega\). When filled with the pipette solution containing 40 mM K\(_2\)SO\(_4\), 50 mM KCl, 5 mM MgCl\(_2\), 0.5 mM EGTA and 10 mM HEPES at pH 7.2 with KOH. To record the \(K\)\(_{ATP}\) channel current, the \(\beta\) cells were voltage-clamped to the holding potential of −70 mV, stepped to −100 mV to apply the voltage ramp from −100 to −50 mV at a speed of 5 mV/100 ms, and stepped back to −70 mV every 10 s. Electrophysiological experiments were performed at room temperature (25°C).

Measurement of islet ATP content

The ATP content of pancreatic islets was measured by HPLC using a modified version of previously described methods [35,36]. Experiments were performed on islets from WT and KO mice after incubation overnight in RPMI containing 11 mM glucose. Groups of 25 islets were hand-picked and preincubated in KRB containing 2.2 mM glucose for 60 min. After preincubation, the islets were incubated for 5 min in KRB containing 2.2 or 22 mM glucose. Then, ATP was extracted with 0.4 M perchloric acid and assayed by HPLC with a reversed-phase SUPELCOSIL column (LC-18-T, 3 \(\mu\m\), 15×4.6 mm, Sigma-Aldrich) according to the manufacturer’s instructions. ATP levels were standardized to protein concentrations.

Mitochondrial membrane potential

WT and KO cells cultured on cover glass were loaded with 10 nM TMRE (Molecular Probes) [15] for 45 min in KRB containing 2.2 mM glucose and washed and incubated for a further 15 min with KRB. We obtained TMRE fluorescence images from multiple individual cells before and after increasing the extracellular glucose concentration from 2.2 mM to 22 mM or with 10 mM KIC (Nakarai Tesque Inc, Kyoto Japan) using confocal fluorescent microscopy (FV-1000, Olympus) [543-nm laser line and emission filter set (BA 560–620)].

Immunoprecipitation and detection of the histone H1 kinase activity of CDK5

The kinase activity of CDK5/p35 was measured after immunoprecipitation with anti-p35 antibody, as previously described [31]. Briefly, the isolated islets were sonicated in MOPS buffer (20 mM MOPS, pH 6.8, 1 mM EGTA, 0.1 mM EDTA, 0.3 M NaCl, 1 mM MgCl\(_2\), 0.5% Nonident P-40, 0.2 mM Pefabloc SC, 1 mg/ml leupeptin, and 1 mM DTT), and centrifuged at 20,000 \(\times\)g for 30 min to collect the supernatant. The kinase activity of CDK5-p35 immunoprecipitated with anti-p35 antibody (C19, Santa Cruz) from an equal protein amount of islet extract was measured using histone H1 as a substrate in the absence or presence of 20 \(\mu\)M roscovitine.

Subcellular localization of CDKAL1 in \(\beta\) cells

Fractionation by differential centrifugation was performed with minor modification as described previously [17]. MIN6 \(\beta\) cells (a gift from Dr. J.-I. Miyazaki, Osaka University, Osaka, Japan) were homogenized in sucrose buffer (5 mM HEPES (pH 7.5), 0.32 M sucrose, and protease inhibitors). The homogenates were centrifuged at 8,900 \(\times\)g for 10 min to obtain the postnuclear supernatant (S1). S1 was centrifuged at 9,900 \(\times\)g for 15 min to separate the soluble cytosolic/microsomal fraction and precipitate. The precipitate was resuspended in sucrose buffer, and centrifuged at 54,000 \(\times\)g for 2 h, to yield the pellet (membrane fraction). The S2 fraction was centrifuged for 1 h at 540,000 \(\times\)g for 2 h, yielding a soluble cytosolic fraction (S3). Equal amounts (2 \(\mu\)g protein) of the fractions were separated by SDS-PAGE and immunoblotted with specific antibodies.

EGFP-CDKAL1 was generated using a cDNA fragment encoding a full open reading frame of murine CDKAL1 (Dnafoum, Kanagawa, Japan). N-terminally tagged with EGFP, and subcloned into the HindIII/KpnI site of a pcDNA3 vector (Invitrogen). For immunohistochemical localization of CDKAL1, MIN6 \(\beta\) cells were transfected with EGFP-CDKAL1 using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions and cultured for 2 days. The intracellular localization was examined under a confocal microscope with MitoTracker (Red CM-H2XRos, Molecular probes) staining (100 nM for 20 min) or immunostaining (fixed and permeabilized with 4% paraformaldehyde/0.1% Triton X-100) with anti-calnexin antibody.

Author Contributions

Conceived and designed the experiments: MOI SN. Performed the experiments: MOI MY KA TS HT YA YN MK. Analyzed the data: CN HK SH. Contributed reagents/materials/analysis tools: TO RTY NK. Wrote the paper: MOI SN.

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