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Diacylglycerol kinase ζ is a positive insulin secretion regulator in pancreatic β-cell line MIN6

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ABSTRACT

Some isoforms of diacylglycerol (DAG) kinase (DGK), an enzyme converting DAG into phosphatidic acid, i.e., DGKα, γ and δ, have been reportedly involved in the regulation of pancreatic β-cell function. DGKζ has also been reported to be expressed in rat pancreatic β-cells. However, its function in pancreatic β-cells remains unknown. The present study aimed to elucidate the function of DGKζ in pancreatic β-cells. The expression of DGKζ was detected in the β-cell line MIN6B and mouse pancreatic islets and in the cytoplasmic fraction from MIN6B cells. The knockdown of DGKζ with siRNA significantly decreased glucose-induced insulin secretion in MIN6B cells. The induction of DGKζ expression in MIN6CEon1 cells with a doxycycline-inducible stable expression system significantly increased glucose-induced insulin secretion. In contrast, glucose-induced insulin secretion was not changed when a kinase-dead DGKζ mutant (G356D) was overexpressed in MIN6CEon1 cells, indicating that a mechanism dependent on its kinase activity mediates the facilitatory effect of DGKζ on glucose-induced insulin secretion. Additionally, we revealed that DGKζ overexpression exhibited no effect on cell cycle of MIN6 cells. These results suggest that DGKζ plays a facilitatory role in insulin secretion in pancreatic β-cells.

1. Introduction

Pancreatic β-cell dysfunction is a major cause of both type 1 and type 2 diabetes. Understanding insulin secretion regulation is, therefore, important for the treatment of diabetes. However, the mechanism of insulin secretion regulation has not been fully elucidated. Diacylglycerol kinase (DGK) is the enzyme that metabolizes diacylglycerol (DAG) into phosphatidic acid (PA). DAG and PA are both important lipid signal messengers, and each controls various protein kinase activities. Therefore, DGK is likely to act as a switch from DAG signaling to PA signaling. Ten DGK isoforms have been identified and classified into type I (DGKα, β, and γ), type II (δ, η, and κ), type III (ε), type IV (ζ and ι), and type V (θ) based on their characteristic domain structures [1–4]. Each DGK isoform may exhibit a different function depending on tissue or subcellular distribution and substrate specificity. In pancreatic β-cells, DGKα and γ, which are expressed in the cytoplasm, facilitate insulin secretion [5,6] and DGKδ, which is abundantly expressed in the nucleus, suppresses proliferation [7].

DGKζ, classified as type IV DGK, involves two C1 domains, a MARCKS homology domain, a catalytic domain, four ankyrin repeats, and a PDZ binding domain from the N-terminal [1,2,8]. DGKζ was reported to be localized in the nucleus in rat β-cells and translocated into the cytoplasm under stress conditions [9,10]. Additionally, a study using conventional DGKζ knock-out mice fed high-fat diets has revealed the involvement of DGKζ in insulin sensitivity in skeletal muscle [11]. Furthermore, the involvement of DGKζ in the membrane translocation of glucose transporter 4 (GLUT4) has been shown in adipocytes [12]. These findings suggest that DGKζ is important in controlling blood glucose levels, obesity, and diabetes. However, the role of DGKζ in pancreatic β-cell remains unclear. The present study investigated the role of DGKζ in the regulation of β-cell function.

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2. Materials and methods

2.1. Animals

Male C57BL/6J mice (SLC, Hamamatsu, Japan) were housed under a 12-h light-dark cycle and fed a standard diet (rodent diet CE-2, CLEA Japan, Tokyo, Japan) and water ad libitum. All animal experiments adhered to European Community guidelines and were conducted under the protocols approved by the Institutional Animal Care and Use Committee of the University of Shizuoka (approval numbers: 206434 and 236606).

2.2. Cell culture

MIN6B cells (provided by Professor J. Miyazaki, Osaka University, Osaka, Japan), a mouse pancreatic β-cell line, were cultured in Dulbecco's Modified Eagle's Medium (D-MEM; Fujifilm WAKO, Osaka, Japan or Nacalai Tesque, Kyoto, Japan) with 100 units/mL of penicillin G, 100 μg/mL of streptomycin (Meiji Seika Pharma, Tokyo, Japan), and 15 % fetal bovine serum (FBS) at 37 °C under 95 % air/5 % CO_2 conditions. MIN6CEon1 cells are based on MIN6c4 cells subcloned from MIN6 cells and involve a platform for easily generating stable cell lines by genetic recombination with Flp recombinase (recombinase-mediated cassette exchange [RMCE]) [13,14]. The culture conditions for MIN6-CEon1 cells were the same as those for MIN6B cells.

2.3. Fractionation

MIN6B cells were incubated on ice for 5 min in hypotonic buffer (10 mM of Tris-HCl at pH of 7.4, 2 mM of $MgCl_2$, 5 μg/mL of phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of leupeptin, 1 μg/mL of aprotinin, and 1 mM of $NaVO_4$), and the cell suspension was transferred to a tube. Triton X-100 was added to a final concentration of 0.1 % and incubated for 30 min $MgCl_2$ was added to a final concentration of 5 mM and centrifuged at 400×*g* for 5 min. The supernatant was used as the cytoplasmic fraction. The pellet was resuspended in the hypotonic buffer. The sonicated pellet was employed as the nuclear fraction. Each fraction was mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer (125 mM of Tris-HCl at pH of 6.8, 4 % of SDS, 20 % of glycerol, 10 % of 2-mercapto ethanol, and 0.001 % of bromophenol blue) and boiled at 95 °C for 5 min.

2.4. Transfection of siRNA or plasmid DNA by electroporation

Using CLB-Transfection™ Device (Lonza, Basel, Switzerland) with a specific pulse (CLB-Transfection™ Pulse: CELL 8), MIN6B cells were transfected with siRNA and MIN6CEon1 cells were stably transfected with plasmid DNA (*vide infra*). MIN6B or MIN6CEon1 cells were used at 2.5×10^6 cells per sample. Further, siRNA for Dgkz knockdown was as follows: sense: 5′-CCAAGAUUCAGGACCUGAAACCGCA-3′ and antisense: 5′-UGCGGUUUCAGGUCCUGAAUCUUGG-3' (Thermo Fisher Scientific, MA, USA). AllStars Negative control siRNA (QIAGEN, Hilden, Germany) was used as a control.

2.5. Plasmid construction for RMCE

Each plasmid for RMCE was constructed based on pF3HygSTreGFw^r, which was developed with mutant FRT (F3), hygromycin resistance gene (Hyg R), splice acceptor site of rabbit β-globin (SpA), tetracycline response element (Tre), β-globin polyA region (GpA), and inverted mutant FRT (Fwr). A DGKζ cDNA fragment with a restriction site was amplified by polymerase chain reaction (PCR) using pEGFP-C3-DGKζ as a template. pEGFP-C3-DGKζ construct was created by amplifying the coding region of the mouse Dgkz2 gene from a mouse muscle cDNA library and inserting it into the *Eco*RI and *Bam*HI restriction sites of the pEGFP-C3 plasmid (Clontech, CA, USA). The DGKζ cDNA was inserted into the *Sal*I-*Eco*RI site between Tre and GpA of pF3HygTreFw^r to develop a plasmid for generating DGKζ-overexpressed MIN6 cells (pF3HygSTreDGKzGFw^r) (Fig. S1A). The primers for PCR were as follows: sense: 5′-TTAAGTCGACCCACCATGGAGCCGCGGGACCC-3′ and antisense: 5′-ATTAGAATTCCTACACAGCTGTCTCCTGG-3.' The RMCE plasmid for DGKζ G356D mutant-overexpressed MIN6 cells was constructed with pF3HygSTreDgkzGFw^r as a template using the KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan) (pF3Hyg-STreDgkzG356DGFw^r) (Fig. S1B). The primers for PCR were as follows: sense: 5′- GACACGGTTGGCTGGATTCTCTCCA-3′ and antisense: 5′-ATCACCCCCGCAAGCCAGAA-3'.

2.6. Generation of stable cell line

As describe above, 2.5×10^6 MIN6CEon1 cells were transfected with 2.5 μg of plasmid DNA for RMCE and 2.5 μg of pCAG-Flpe, which was a gift from Connie Cepko (Addgene plasmid # 13787; http://n2t.net/addgene:13787; RRID: Addgene_13787) [15]. Approximately two weeks after transfection, drug selection was performed for 2–3 weeks by culturing in a medium with hygromycin B (200 μg/mL, Tokyo Chemical Industry, Tokyo, Japan) and, if necessary, ganciclovir (10 μg/mL, Fujifilm WAKO). Colonies were combined and used as stable cell lines after drug selection. DGKζ overexpression was induced by treating the cells with doxycycline (DOX; Tokyo Chemical Industry).

2.7. Isolation of pancreatic islets

Pancreatic islets were isolated from mice using a collagenase digestion method as previously described [16]. HEPES-Krebs-Ringer bicarbonate buffer (HK buffer, 119 mM of NaCl, 4.75 mM of KCl, 5 mM of $NaHCO_3$, 2.54 mM of $CaCl_2$, 1.2 mM of $MgSO_4$, 1.2 mM of KH_2PO_4, and 10 mM of HEPES (pH of 7.4 adjusted with NaOH) with 2.8 mM of glucose was used for isolation.

2.8. Measurement of insulin secretion

MIN6B cells or DGKζ-overexpressed MIN6 cells were seeded at 5.0×10^5 or 2.5×10^5 cells/well, respectively, in a 24-well plate and cultured for 3 days. DGKζ-overexpressed MIN6 cells were treated with 0.3 μg/mL of DOX for 2 days to induce DGKζ overexpression. The cells were pre-incubated for 1 h in HK buffer with 2.8 mM of glucose and 0.1 % bovine serum albumin (BSA) fraction V (Roche, Basel, Switzerland). They were then incubated for 1 h in HK buffer containing various concentrations of glucose and 0.1 % BSA. The incubation medium was collected, and a mouse/rat insulin kit (Morinaga Institute of Biological Science, Yokohama, Japan) was used to quantify insulin in the medium. Cells were collected after the insulin secretion experiment, and protein content was quantified using a microspectrophotometer (DS-11; DeNovix, DE, USA) and used to adjust for cell number differences.

2.9. Measurement of insulin content

DGKζ-overexpressed MIN6 cells were seeded at 2.5×10^5 cells/well in a 24-well plate and cultured for 3 days. The cells were treated with 0.3 μg/mL of DOX for 2 days to induce DGKζ overexpression, cultured in D-MEM with low glucose (#D6046, Sigma-Aldrich, MO, USA) for 1 day. Cellular insulin was extracted using acid ethanol (95 % of ethanol+5 % of 12 N HCl) and quantified by a mouse/rat insulin kit (Morinaga Institute of Biological Science). Protein content in the lysate was quantified using a microspectrophotometer (DS-11; DeNovix) and used to adjust for cell number differences.

2.10. RNA extraction and reverse transcription (RT)-PCR

Pancreatic islets isolated from mice were preserved in RNAlater (QIAGEN), and RNeasy Total RNA (QIAGEN) was used to extract the

total RNA of islets. NucleoSpin RNA (MACHEREY-NAGEL, Düren, Germany) was used to extract the total RNA of MIN6 cells. A microspectrophotometer (DS-11, DeNovix) was used to quantify the concentration of extracted RNA. The RT-PCR Kit (QIAGEN) was used for RT-PCR. The amplified PCR fragments were separated and detected by 2 % agarose gel electrophoresis. The primers used were as follows:

Dgkz, sense: 5′-ATGCACGACTACGAGGCTCT-3′ and antisense: 5′-GCATCCAGGAAACACCACTT-3’; Dgkz1, sense: 5′-CAGGAGGGAAATGAGACAGC-3′ and antisense: 5′-CGTCGATAGTGACCATGCAG-3’; Dgkz2, sense: 5′-TTTGGGCACAGGAAAGCCAT-3′ and antisense: 5′-GCTGACTCACTCCAGTCCAC-3’; Actin Beta (Actb), sense: 5′-AGCCATGTACGTAGCCATCC-3′ and antisense: 5′-CTCTCAGCTGTGGTGGTGAA-3’.

2.11. Quantitative PCR (qPCR)

RNA extracted from MIN6 cells was used, as described above. The StepOnePlus™ Real-Time PCR System (Applied Biosystems, MA, USA) with the One Step TB Green® PrimeScript PLUS RT-PCR Kit (Takara Bio, Shiga, Japan) was used for qPCR. Actb was used as an internal control. The primers used were as follows: Dgkz, sense: 5′-ATGCACGACTACGAGGCTCT-3′ and antisense: 5′-GCATCCAGGAAACACCACTT-3’; Dgka, sesne: 5′-CTGGGCACTGGAAATGATCT-3′ and antisense: 5′-TCATGAGATGGAATCGGTCA-3’; Dgkg, 5′-GAGCTGAAATGCTGTGTCCA-3′ and antisense: 5′-GGTGCCTGGTTTTTGTGAGT-3’; Dgkd, sense: 5′-TGGATTGCAGCACTCAAGAC-3′ and antisense: 5′-ACCCCTGACAAAACTTCACG-3’; Dgke, sense: 5′-GCCTCAAGAAGGTCGACAAG-3′ and antisense: 5′-AATGCACCTGTAATCGCACA-3’; Dgkh, sense: 5′-GCCCACCTTCTGTAACGTGT-3′ and antisense: 5′-ACTGCACACTTGGCACTCCAC-3’; Dgkq, sense: 5′-GCCCATGGACTGACAAAGTT-3′ and antisense: 5′-AACCCTTCTGGGCACTAGGT-3’; Ins1, sense: 5′-GGACCCACAAGTGGAACAAC-3′ and antisense: 5′-GGTGGGCCTTAGTTGCAGTA-3’; Ins2, sense: 5′-TTTGTCAAGCAGCACCTTTG-3′ and antisense: 5′-GGTCTGAAGGTCACCTGCTC-3’; Actb, sense: 5′-AGCCATGTACGTAGCCATCC-3′ and antisense: 5′-CTCTCAGCTGTGGTGGTGAA-3’.

2.12. Immunoblotting

Isolated islets or MIN6 cells were lysed in lysis buffer (20 mM of Tris at pH of 7.4, 2 mM of ethylenediaminetetraacetic acid, 10 % of glycerol, 2 % of SDS, 50 μg/ml of PMSF, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 5 % of 2-mercaptoethanol, and 0.02 % of bromophenol blue) and boiled at 95 °C for 5 min. Protein samples were separated on 8 % polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with TBS-T (10 mM of Tris, 137 mM of NaCl, and 0.1 % of Tween 20) containing 5 % skim milk and incubated with primary antibodies overnight at 4 °C. Bands were visualized with Amersham™ ECL™ Prime (Cytiva, Tokyo, Japan) or ImmunoStar LD (Fujifilm WAKO) using Amersham™ Imager 600 (Cytiva) or FUSION SOLO.6S.EDGE (Vilber, Marne-la-Vallée, France) after further incubation with secondary antibodies for 1 h at room temperature. Primary antibodies included rabbit anti-DGKζ antibody (1:1000, ab239081; Abcam, Cambridge, UK), mouse anti-β-actin antibody (1:10000, A5441; Sigma-Aldrich), rabbit anti-GAPDH antibody (1:1000, #5174; Cell Signaling Technology, MA, USA), or rabbit anti-vinculin antibody (1:10000, ab129002; Abcam). Secondary antibodies included goat antirabbit IgG, HRP-linked antibody (1:2500, #7074; Cell Signaling Technology), or goat anti-mouse IgG (whole molecule)–peroxidase antibody produced in goat (1:4000, A4416; Sigma-Aldrich).

2.13. Immunostaining

MIN6 cells were seeded on poly-$_L$-lysine-coated glass-bottom dishes. The cells were fixed with ice-cold methanol, blocked with 3 % BSA/phosphate buffered saline (PBS) at room temperature for 30 min, and then incubated with a primary antibody overnight at 4 °C. The cells were mounted with DAPI-containing Fluorokeeper (DAPI-Fluoromount-G®, Southern Biotechnology Associates Inc.) after further incubation with a secondary antibody for 1 h at room temperature. Primary antibodies include rabbit anti-DGKζ antibody (1:100, ab239081; Abcam) or guinea pig antiinsulin antibody (1:500, 70R-10659; Fitzgerald Industries, MA, USA). Secondary antibodies include Alexa Flour 488-conjugated goat antirabbit IgG antibody (1:200, # A-11034; Thermo Fisher Scientific) or Alexa Flour 555-conjugated goat antirabbit IgG antibody (1:200, #A-21435; Thermo Fisher Scientific). An all-in-one fluorescence microscopy (BZ-X700 or BZ-X810; KEYENCE, Osaka, Japan) was used to observe these samples.

2.14. Flow cytometry

MIN6 cells were seeded at 8.0×10^5 cells/well in a 6-well plate and cultured for 24 h. Then, DGKζ-overexpressed MIN6 cells were treated with 1.0 μg/mL of DOX for 2 days to induce DGKζ overexpression. S661 (500 nM), an insulin receptor antagonist, was added at the same time as DOX to eliminate influence of secreted insulin. In the experiments where the cell cycle was synchronized, MIN6 cells were seeded at 6.0×10^5 cells/well in a 6-well plate and cultured for 24 h, treated with 1.0 μg/mL of DOX for 24 h, and incubated in serum-free medium containing DOX for 24 h followed by in serum- and DOX-containing medium for 24 h. Non-synchronized and synchronized cells were harvested with 0.25 % trypsin and fixed with ice-cold 70 % ethanol. They were incubated with Cell Cycle Assay Solution Blue (Dojindo, Kumamoto, Japan) at 37 °C for 15 min after washing with PBS. BD FACSCelesta™ (BD Biosciences, NJ, USA) and FlowJo software (BD Biosciences) were then used for cell analyses.

2.15. Statistics

Results are presented as mean ± standard error of the mean. Comparisons were conducted using an unpaired *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression and role of DGKζ in MIN6 cells and mouse pancreatic islets

We initially investigated the expression and intracellular localization of DGKζ in β-cells. RT-PCR revealed the expression of DGKζ1 and ζ2 in MIN6B cells and mouse pancreatic islets (Fig. 1A and Fig. S2). Western blotting analysis demonstrated the expression of DGKζ in the cytoplasm of MIN6B cells (Fig. 1B). In DGKζ-knockdown MIN6B cells by siRNA, which was confirmed by reverse transcription quantitative PCR (RT-qPCR) and Western blotting (Fig. 1C and D), insulin secretion stimulated by 16.7 mM of glucose was significantly reduced (Fig. 1E).

3.2. Glucose-induced insulin secretion in DGKζ-overexpressed MIN6 cells

The involvement of DGKζ in glucose-induced insulin secretion was further examined with DGKζ-overexpressed MIN6 cells, which had a platform for generating stable cell lines by RMCE. The present study revealed that DGKζ2, which was dominant in MIN6B cells and mouse islets (Fig. 1B–D, and S2), was overexpressed in MIN6CEon1 cells. MIN6CEon1 cells were then transfected with pF3HygSTreDGKζGFw^r and pCAG-Flpe and treated with hygromycin and ganciclovir to select the cells with proper cassette exchange. DGKζ-overexpressed MIN6 cells were named MIN6CEon1oeDgkz. Treatment with various concentrations of DOX for 2 days increased DGKζ expression concentration-dependently (Fig. S3A). DGKζ2 expression was increased in MIN6CEon1oeDgkz cells treated with 0.3 μg/mL of DOX (Fig. 2A and B). The increase in DGKζ expression was observed in both the cytoplasm and

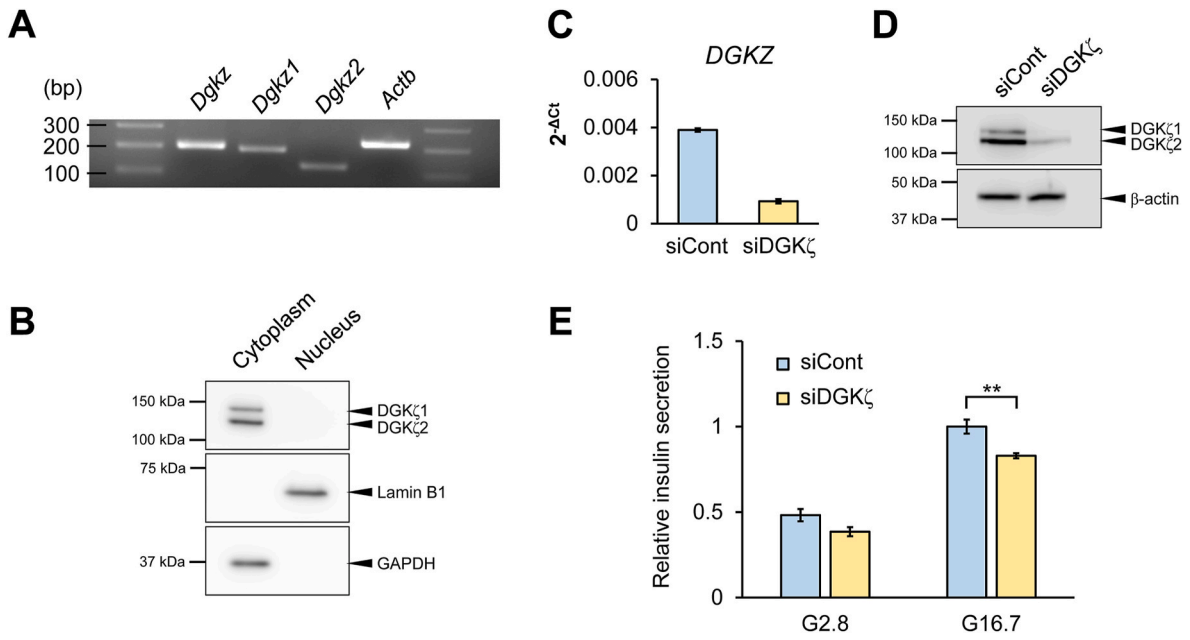

Fig. 1. DGKζ expression and DGKζ knockdown effect on glucose-induced insulin secretion in MIN6B cells. (A) DGKζ mRNA, including *Dgkz1* and *Dgkz2*, expression in MIN6B cells analyzed by RT-PCR. (B) DGKζ expression in fractions from MIN6B cells analyzed by Western blotting. Cytoplasm (GAPDH) and nucleus (Lamin B1) markers were used to check the quality of fractionation. DGKζ knockdown by siRNA confirmed by RT-qPCR (C) and Western blotting (D). (E) Effect of DGKζ knockdown by siRNA on glucose-induced insulin secretion. The graph illustrates the mean ± standard error of the mean (SEM) of four experiments. $^{**}p < 0.01$.

nucleus (Fig. 2C and 3C). In addition, DGKζ overexpression had no effect on the expression of other DGK isoforms (Fig. S3B). Insulin secretion in response to glucose concentrations except at 2.8 mM was significantly increased in DGKζ-overexpressed MIN6 cells (Fig. 2D). In DGKζ-overexpressed MIN6 cells, no change in insulin gene expression was observed, whereas insulin content was significantly increased (Fig. 2E and F).

3.3. Kinase activity involvement in the effect of DGKζ on insulin secretion

We generated MIN6CEon1 cells overexpressing DGKζ G356D mutant, in which GxGxxG, an ATP-binding motif of the protein kinase [17], is changed to GxDxxG to lose its kinase activity [18,19] and ability to produce phosphatidic acid [18]. The same method as for MIN6CEon1oeDgkz was used to generate DGKζ G356D-overexpressed MIN6 cells named MIN6CEon1oeDgkz-G356D cells. Treatment with 0.3 μg/mL of DOX for 2 days increased DGKζ G356D expression in MIN6CEon1oeDgkz-G356D (Fig. 3A and B). Following the results by Western blotting (Fig. 1B), wild type DGKζ was primarily expressed in the cytoplasm in MIN6CEon1oeDgkz cells (Fig. 3C). The localization of DGKζ G356D was almost the same as that in the wild type (Fig. 3C). However, the increased insulin secretion in the DGKζ-overexpressed MIN6 cells was not observed in the DGKζ G356D-overexpressed MIN6 cells (Fig. 3D). These results indicate that the facilitatory effect of DGKζ on glucose-induced insulin secretion depends on its kinase activity.

3.4. Possible involvement of DGKζ in MIN6 cell cycle regulation

Nuclear DGKζ has been reported to suppress the cell cycle in COS7 [20] and C2C12 cells [18]. Therefore, we investigated the effect of DGKζ on the cell cycle in MIN6 cells. The DGKζ-overexpressed MIN6 cells were used for cell cycle analysis by flow cytometry. The experiments were conducted under conditions of treatment with the insulin receptor antagonist peptide S661 to exclude the influence of insulin released from MIN6 cells. DGKζ overexpression with or without S661 demonstrated no effect on the cell cycle (Fig. 4). Furthermore, DGKζ overexpression also had no effect on the cell cycle in MIN6 cells synchronized with serum starvation (Figs. S3C and D). These results indicate that DGKζ is not involved in cell cycle regulation in MIN6 cells.

4. Discussion

DAG metabolism by DGK is an important factor in regulating insulin secretion in pancreatic β-cells. We previously revealed that DGKα and γ are present in mouse β-cells and participate in facilitation of insulin secretion [5] and that the inhibition of these DGK isoforms in MIN6 β-cells causes a dual regulation of insulin secretion, i.e., protein kinase C (PKC)-mediated facilitation and PKC-independent suppression when inhibited weakly and strongly, respectively [6]. The present study revealed that similar to DGKα and γ, DGKζ also acts as a positive regulator of insulin secretion, where its kinase activity is involved.

The knockdown and overexpression of DGKζ significantly reduced and increased glucose-induced insulin secretion in MIN6 β-cells, respectively, indicating that DGKζ is a positive regulator of glucose-induced insulin secretion. In contrast, DGKζ overexpression exhibited no effect on the cell cycle of MIN6 β-cells, indicating less involvement of DGKζ in the cell cycle regulation. In other cell types, DGKζ has been reported to be localized in the nucleus and play as a negative regulator of the cell cycle [19,20]. Nuclear localization of DGKζ has also been shown in rat pancreatic β-cells, although its function was not investigated [9]. In contrast, the present study revealed that DGKζ is primarily expressed in MIN6 β-cell cytoplasm and does not affect the cell cycle. We previously reported that DGKδ, a type II DGK, is localized in the nucleus of mouse β-cells and is a negative regulator of proliferation [7]. Thus, lower nuclear expression of DGKζ may be responsible for its different roles in β-cells.

DGK phosphorylates DAG to produce PA. A decrease in DAG and/or an increase in PA would be responsible for the effect because the effect of DGKζ on insulin secretion was dependent on its kinase activity. DAG activates PKC and Munc13-1, which both increase insulin secretion [21–23]. Therefore, a decrease in DAG is unlikely responsible for the effect of DGKζ on insulin secretion, and increased PA is more likely involved. PA binds to mTOR and activates mTOR complex 1 and 2 (mTORC1/2), which may have been involved in the regulation of insulin

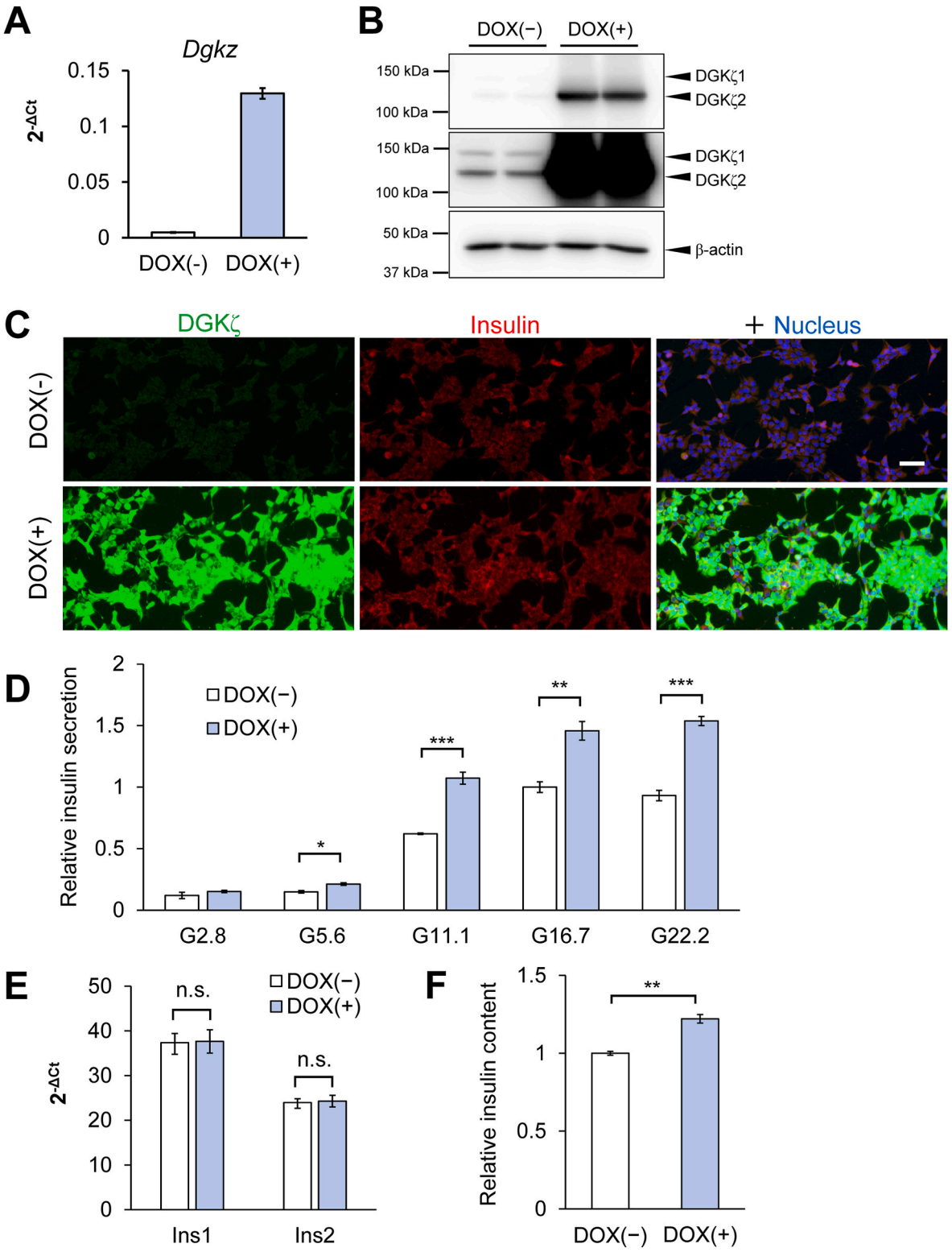

Fig. 2. Effect of DGKζ overexpression on glucose-induced insulin secretion in MIN6 cells. DGKζ overexpression induced by doxycycline (DOX; 0.3 μg/mL) confirmed by RT-qPCR (A) and Western blotting (B). Endogenous DGKζ was detected by long exposure in the middle image in panel B using the same membrane as in the upper image. (C) DGKζ overexpression induced by DOX confirmed by immunostaining (Green: DGKζ, Red: insulin, Blue: nuclear counterstaining with DAPI). The scale bar indicates 50 μm. (D) Effect of DGKζ overexpression on insulin secretion induced by various concentrations of glucose. (E) The expression of insulin 1 and insulin 2 mRNA in MIN6CEon1oeDgkz cells analyzed by RT-qPCR. (F) Effect of DGKζ overexpression on insulin content. The graph shows the mean ± SEM of three experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{*}p < 0.05$, n.s.: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

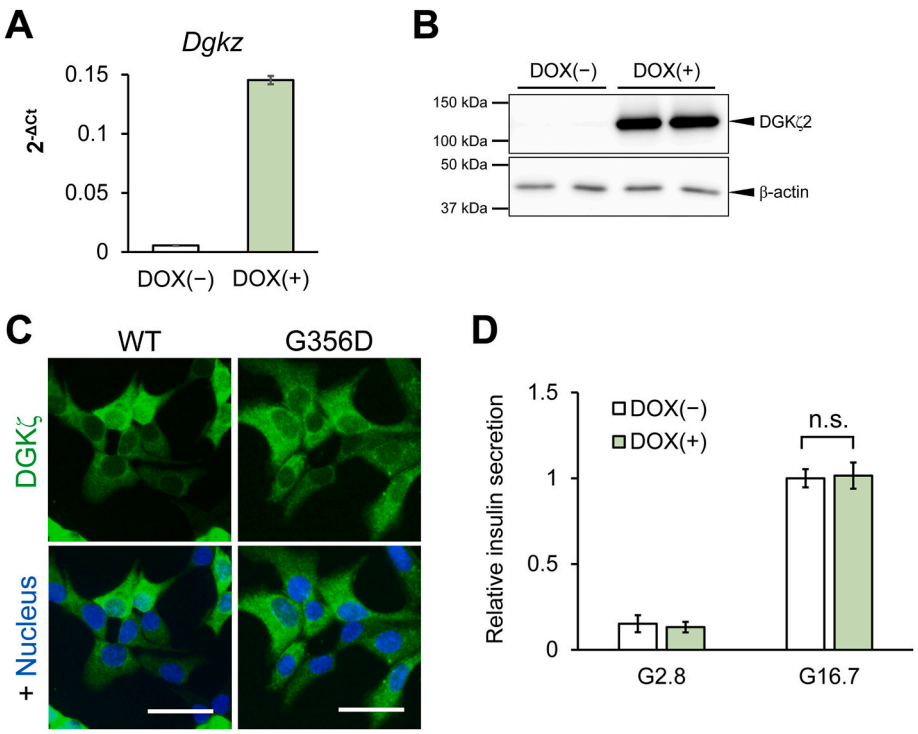

Fig. 3. Effect of DGKζ G356D mutant overexpression on glucose-induced insulin secretion in MIN6 cells. DOX-induced DGKζ G356D mutant overexpression confirmed by RT-qPCR (A) and Western blotting (B). (C) Localization of wild type DGKζ and G356D mutant confirmed by immunostaining (Green: DGKζ, Blue: nuclear counterstaining with DAPI). The scale bar indicates 30 μm. (D) Effect of DGKζ G356D overexpression induced by DOX on glucose-induced insulin secretion. The graph illustrates the mean ± SEM of three experiments. n.s.: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

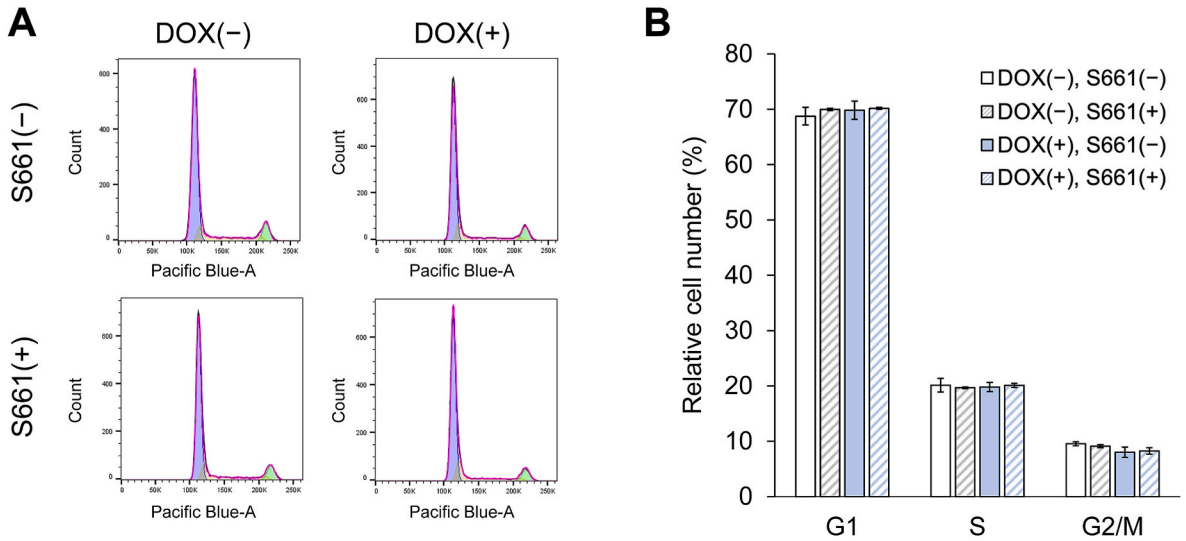

Fig. 4. Effect of DGKζ overexpression on cell cycle in MIN6 cells. (A) Typical plots of cell cycle in MIN6 cells analyzed by flow cytometry. DGKζ overexpression was induced by DOX in the absence and presence of S661 (500 nM), which is an insulin receptor antagonist. Blue: G1 phase, yellow: S phase, and green: G2/M phase. (B) The percentage of cells in each cell cycle phase. The graph illustrates the mean ± SEM of three experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

secretion via various pathways, including insulin synthesis [24–26]. PLD1, which produces PA through a pathway different from DGK, has been shown to increase insulin secretion via mTOR activation [27,28]. Additionally, PA activates PIP5K and c-Raf, which increase insulin secretion through MEK/ERK signaling [29–31]. Further, PA promotes trafficking and membrane association of small GTPase Rac1, a positive insulin secretion regulator [32,33], in the INS-1 β-cell line [34]. In the present study, we found that DGKζ overexpression did not alter insulin gene expression, but significantly increased insulin content. These results are likely consistent with a previous report having shown that the activation of the mTOR signaling by increased PA is involved in the processing of proinsulin to insulin in β-cell [26]. Therefore, DGKζ may promote insulin secretion through PA-mediated signaling.

DGKζ generally exerts its function by phosphorylating DAG. However, DGKζ is also likely to exhibit physiological functions independently of its kinase activity. For example, DGKζ has been shown to

regulate RhoA activation as a scaffold protein [35] and the degradation of p53 and IκBα in a kinase-independent manner [36]. In addition, the N-terminal region of DGKζ containing the C1 domain has been reported to be responsible for the interaction with IRS-1 and PIP5K1α, which enhances GLUT4 translocation independently of its kinase activity [12]. In the present study, however, no facilitatory effect on glucose-induced insulin secretion was observed in MIN6CEon1oeDgkz-G356D cells, a kinase-dead DGKζ G356D mutant, indicating that the kinase activity is required for the insulinotropic action of DGKζ.

The present study revealed that similar to DGKα and γ, DGKζ is localized in the cytoplasm of β-cells and facilitates insulin secretion. However, DGKζ may exhibit different roles from that of the type I DGKs. Intracellular Ca^{2+} concentration changes are well known to occur chronologically and locally in the cytoplasm during glucose stimulation in β-cell. Type I DGKs are activated in the presence of Ca^{2+}, and the activity of type I DGKs is rapidly regulated by intracellular Ca^{2+} concentration. Moreover, a type I DGK inhibitor decreases intracellular Ca^{2+} concentration in MIN6 cells [5]. Thus, the effect of the type I DGKs is likely closely associated with changes in intracellular Ca^{2+} concentration. In contrast, DGKζ has no Ca^{2+}-binding site. This could make the difference between DGKζ and type I DGKs [1,2,8]. Although we confirmed that the expression level of DGKs other than DGKζ was not changed in DGKζ-overexpressed MIN6 cells, the possibility still remains that DGKζ overexpression affects the activity and function of other DGKs and facilitates insulin secretion in a cooperative manner.

Sakane et al. have recently revealed that each DGK isoform metabolizes DAG with a different fatty chain [37]. Moreover, Ware et al. have indicated that DGKζ has a selectivity to C16:0_C22:6 DAG in HEK293 cells, which varies from other DGK [38]. Fatty acids with different acyl chains may have a distinct cellular function in β-cells. Several studies have revealed that long-chain saturated fatty acid species, such as palmitic acid (C16:0) and stearic acid (C18:0), are involved in β-cell lipotoxicity, whereas myristic acid (C14:0) and octanoic acid (C8:0) are not, and pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), and monounsaturated fatty chains, including palmitoic acid (C16:1) and oleic acid (C18:1), are rather cytoprotective [39–41]. Moreover, the deletion of the long-chain fatty acid elongase Elovl6, which converts C16 fatty acids into C18 species, in *db/db* mice has been shown to improve glucose tolerance via enhancing glucose-induced insulin secretion and increasing β-cell mass [42]. Thus, the involvement of fatty acid species in β-cell function and the development of diabetes is considered important for a proper understanding of the development of diabetes. Furthermore, PKC isoform activation has reported to depend on the molecular species of DAG [43,44]. Therefore, the substrate specificity of each DGK isoform may be responsible for its effect on β-cell function.

5. Conclusion

In conclusion, DGKζ is expressed in pancreatic β-cells and promotes insulin secretion by phosphorylating DAG to generate PA. The switching from the DAG signaling to the PA signaling by DGKζ may become a novel target for improving insulin secretion defects in type 2 diabetes.

CRediT authorship contribution statement

Naoya Watanabe: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Yukiko K. Kaneko:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Hisamitsu Ishihara:** Writing – review & editing, Resources, Methodology. **Ryota Shizu:** Writing – review & editing, Resources, Methodology. **Kouichi Yoshinari:** Writing – review & editing, Supervision. **Momoka Yamaguchi:** Writing – review & editing, Supervision. **Toshihide Kimura:** Writing – review & editing, Supervision. **Tomohisa Ishikawa:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] M.K. Topham, R.M. Epand, Mammalian diacylglycerol kinases: molecular interactions and biological functions of selected isoforms, Biochim. Biophys. Acta Gen. Subj. 1790 (2009) 416–424, https://doi.org/10.1016/j.bbagen.2009.01.010.
[2] F. Sakane, S. Imai, M. Kai, S. Yasuda, H. Kanoh, Diacylglycerol kinases: why so many of them? Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1771 (2007) 793–806, https://doi.org/10.1016/j.bbalip.2007.04.006.
[3] I. Mérida, A. Avila-Flores, E. Merino, Diacylglycerol kinases: at the hub of cell signalling, Biochem. J. 409 (2008) 1–18, https://doi.org/10.1042/BJ20071040.
[4] K. Goto, Y. Hozumi, H. Kondo, Diacylglycerol, phosphatidic acid, and the converting enzyme, diacylglycerol kinase, in the nucleus, Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1761 (2006) 535–541, https://doi.org/10.1016/j.bbalip.2006.04.001.
[5] Y. Kurohane Kaneko, Y. Kobayashi, K. Motoki, K. Nakata, S. Miyagawa, M. Yamamoto, D. Hayashi, Y. Shirai, F. Sakane, T. Ishikawa, Depression of type I diacylglycerol kinases in pancreatic β-cells from male mice results in impaired insulin secretion, Endocrinology 154 (2013) 4089–4098, https://doi.org/10.1210/en.2013-1356.
[6] T. Sawatani, Y.K. Kaneko, T. Ishikawa, Dual effect of reduced type I diacylglycerol kinase activity on insulin secretion from MIN6 β-cells, J. Pharmacol. Sci. 140 (2019) 178–186, https://doi.org/10.1016/j.jphs.2019.06.001.
[7] T. Sato, C. Ishiwatari, Y.K. Kaneko, Y. Ishikawa, Y. Kimura, N. Watanabe, I. Aoshima, Y. Matsuda, T. Nakayama, R. Chiba, T. Fujinuki, K. Iwata, Q. Lu, T. Usuki, F. Sakane, T. Ishikawa, Diacylglycerol kinase δ functions as a proliferation suppressor in pancreatic β-cells, Faseb. J. 35 (2021) e21420, https://doi.org/10.1096/fj.202001279RR.
[8] M. Bunting, W. Tang, G.A. Zimmerman, T.M. McIntyre, S.M. Prescott, Molecular cloning and characterization of a novel human diacylglycerol kinase ζ (*), J. Biol. Chem. 271 (1996) 10230–10236, https://doi.org/10.1074/jbc.271.17.10230.
[9] Y. Hozumi, T. Nakano, T. Tanaka, K. Goto, Localization of diacylglycerol kinase ζ in rat pancreatic islet cells under normal and streptozotocin-induced stress conditions, Arch. Histol. Cytol. 76 (2016) 23–33, https://doi.org/10.1679/aohc.76.23.
[10] H. Ali, T. Nakano, S. Saino-Saito, Y. Hozumi, Y. Katagiri, H. Kamii, S. Sato, T. Kayama, H. Kondo, K. Goto, Selective translocation of diacylglycerol kinase ζ in hippocampal neurons under transient forebrain ischemia, Neurosci. Lett. 372 (2004) 190–195, https://doi.org/10.1016/j.neulet.2004.09.052.
[11] B. Benziane, M.L. Borg, R.Z. Tom, I. Riedl, J. Massart, M. Björnholm, M. Gilbert, A. V. Chibalin, J.R. Zierath, DGKζ deficiency protects against peripheral insulin resistance and improves energy metabolism, J. Lipid Res. 58 (2017) 2324–2333, https://doi.org/10.1194/jlr.M079723.
[12] T. Liu, B. Yu, M. Kakino, H. Fujimoto, Y. Ando, F. Hakuno, S.-I. Takahashi, A novel IRS-1-associated protein, DGKζ regulates GLUT4 translocation in 3T3-L1 adipocytes, Sci. Rep. 6 (2016) 35438, https://doi.org/10.1038/srep35438.
[13] A. Furukawa, A. Tanaka, S. Yamaguchi, M. Kosuda, M. Yamana, A. Nagasawa, G. Kohno, H. Ishihara, Using recombinase-mediated cassette exchange to engineer MIN6 insulin-secreting cells based on a newly identified safe harbor locus, Journal of Diabetes Investigation 12 (2021) 2129–2140, https://doi.org/10.1111/jdi.13646.

[14] A. Tanaka, M. Kosuda, M. Yamana, A. Furukawa, A. Nagasawa, M. Fujishiro, G. Kohno, H. Ishihara, A large-scale functional analysis of genes expressed differentially in insulin secreting MIN6 sublines with high versus mildly reduced glucose-responsiveness, Sci. Rep. 13 (2023) 5654, https://doi.org/10.1038/s41598-023-32589-2.
[15] T. Matsuda, C.L. Cepko, Controlled expression of transgenes introduced by in vivo electroporation, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 1027–1032, https://doi.org/10.1073/pnas.0610155104.
[16] Y. Kaneko, Y. Kimura, H. Kimura, I. Niki, L-cysteine inhibits insulin release from the pancreatic beta-cell: possible involvement of metabolic production of hydrogen sulfide, a novel gasotransmitter, Diabetes 55 (2006) 1391–1397, https://doi.org/10.2337/db05-1082.
[17] S.K. Hanks, A.M. Quinn, T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains, Science 241 (1988) 42–52, https://doi.org/10.1126/science.3291115.
[18] C. Evangelisti, P.L. Tazzari, M. Riccio, R. Fiume, Y. Hozumi, F. Falà, K. Goto, L. Manzoli, L. Cocco, A.M. Martelli, Nuclear diacylglycerol kinase-zeta is a negative regulator of cell cycle progression in C2C12 mouse myoblasts, Faseb. J. 21 (2007) 3297–3307, https://doi.org/10.1096/fj.07-8336com.
[19] N. Kobayashi, Y. Hozumi, T. Ito, T. Hosoya, H. Kondo, K. Goto, Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells, Eur. J. Cell Biol. 86 (2007) 433–444, https://doi.org/10.1016/j.ejcb.2007.05.002.
[20] M.K. Topham, M. Bunting, G.A. Zimmerman, T.M. McIntyre, P.J. Blackshear, S. M. Prescott, Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta, Nature 394 (1998) 697–700, https://doi.org/10.1038/29337.
[21] A.J. Trexler, J.W. Taraska, Regulation of insulin exocytosis by calcium-dependent protein kinase C in beta cells, Cell Calcium 67 (2017) 1–10, https://doi.org/10.1016/j.ceca.2017.07.008.
[22] E.P. Kwan, L. Xie, L. Sheu, C.J. Nolan, M. Prentki, A. Betz, N. Brose, H.Y. Gaisano, Munc13-1 deficiency reduces insulin secretion and causes abnormal glucose tolerance, Diabetes 55 (2006) 1421–1429, https://doi.org/10.2337/db05-1263.
[23] L. Kang, Z. He, P. Xu, J. Fan, A. Betz, N. Brose, T. Xu, Munc13-1 is required for the sustained release of insulin from pancreatic β cells, Cell Metabol. 3 (2006) 463–468, https://doi.org/10.1016/j.cmet.2006.04.012.
[24] H. Zhou, Y. Huo, N. Yang, T. Wei, Phosphatidic acid: from biophysical properties to diverse functions, FEBS J. 291 (2024) 1870–1885, https://doi.org/10.1111/febs.16809.
[25] J.-S. You, H.C. Lincoln, C.-R. Kim, J.W. Frey, C.A. Goodman, X.-P. Zhong, T. A. Hornberger, The role of diacylglycerol kinase ζ and phosphatidic acid in the mechanical activation of mammalian target of rapamycin (mTOR) signaling and skeletal muscle hypertrophy, J. Biol. Chem. 289 (2014) 1551–1563, https://doi.org/10.1074/jbc.M113.531392.
[26] M. Blandino-Rosano, R. Barbaresso, M. Jimenez-Palomares, N. Bozadjieva, J. P. Werneck-de-Castro, M. Hatanaka, R.G. Mirmira, N. Sonenberg, M. Liu, M. A. Rüegg, M.N. Hall, E. Bernal-Mizrachi, Loss of mTORC1 signalling impairs β-cell homeostasis and insulin processing, Nat. Commun. 8 (2017) 16014, https://doi.org/10.1038/ncomms16014.
[27] W.E. Hughes, Z. Elgundi, P. Huang, M.A. Frohman, T.J. Biden, Phospholipase D1 regulates secretagogue-stimulated insulin release in pancreatic β-cells, J. Biol. Chem. 279 (2004) 27534–27541, https://doi.org/10.1074/jbc.M403012200.
[28] W. Ma, S.-Y. Park, J.-S. Han, Role of phospholipase D1 in glucose-induced insulin secretion in pancreatic Beta cells, Exp. Mol. Med. 42 (2010) 456–464, https://doi.org/10.3858/emm.2010.42.6.047.
[29] J. Zhang, R. Luo, H. Wu, S. Wei, W. Han, G. Li, Role of Type Iα phosphatidylinositol-4-phosphate 5-kinase in insulin secretion, glucose metabolism, and membrane potential in INS-1 β-cells, Endocrinology 150 (2009) 2127–2135, https://doi.org/10.1210/en.2008-0516.
[30] B.T. Andresen, M.A. Rizzo, K. Shome, G. Romero, The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade, FEBS (Fed. Eur. Biochem. Soc.) Lett. 531 (2002) 65–68, https://doi.org/10.1016/S0014-5793(02)03483-X.
[31] Y.M. Ikushima, M. Awazawa, N. Kobayashi, S. Osonoi, S. Takemiya, H. Kobayashi, H. Suwanai, Y. Morimoto, K. Soeda, J. Adachi, M. Muratani, J. Charron, H. Mizukami, N. Takahashi, K. Ueki, MEK/ERK signaling in β-cells bifunctionally regulates β-cell mass and glucose-stimulated insulin secretion response to maintain glucose homeostasis, Diabetes 70 (2021) 1519–1535, https://doi.org/10.2337/db20-1295.
[32] J. Li, R. Luo, A. Kowluru, G. Li, Novel regulation by Rac1 of glucose- and forskolin-induced insulin secretion in INS-1 β-cells, Am. J. Physiol. Endocrinol. Metabol. 286 (2004) E818–E827, https://doi.org/10.1152/ajpendo.00307.2003.
[33] Z. Wang, D.C. Thurmond, Mechanisms of biphasic insulin-granule exocytosis – roles of the cytoskeleton, small GTPases and SNARE proteins, J. Cell Sci. 122 (2009) 893–903, https://doi.org/10.1242/jcs.034355.
[34] P. McDonald, R. Veluthakal, H. Kaur, A. Kowluru, Biologically active lipids promote trafficking and membrane association of Rac1 in insulin-secreting INS 832/13 cells, Am. J. Physiol. Cell Physiol. 292 (2007) C1216–C1220, https://doi.org/10.1152/ajpcell.00467.2006.
[35] R. Ard, K. Mulatz, H. Abramovici, J.-C. Maillet, A. Fottinger, T. Foley, M.-R. Byham, T.A. Iqbal, A. Yoneda, J.R. Couchman, R.J. Parks, S.H. Gee, Diacylglycerol kinase ζ regulates RhoA activation via a kinase-independent scaffolding mechanism, Mol. Biol. Cell 23 (2012) 4008–4019, https://doi.org/10.1091/mbc.E12-01-0026.
[36] T. Tanaka, R. Tsuchiya, Y. Hozumi, T. Nakano, M. Okada, K. Goto, Reciprocal regulation of p53 and NF-κB by diacylglycerol kinase ζ, Advances in Biological Regulation 60 (2016) 15–21, https://doi.org/10.1016/j.jbior.2015.09.009.
[37] J.C. Bozelli Jr., J. Yune, D. Takahashi, F. Sakane, R.M. Epand, Membrane morphology determines diacylglycerol kinase α substrate acyl chain specificity, Faseb. J. 35 (2021) e21602, https://doi.org/10.1096/fj.202100264R.
[38] T.B. Ware, C.E. Franks, M.E. Granade, M. Zhang, K.-B. Kim, K.-S. Park, A. Gahlmann, T.E. Harris, K.-L. Hsu, Reprogramming fatty acyl specificity of lipid kinases via C1 domain engineering, Nat. Chem. Biol. 16 (2020) 170–178, https://doi.org/10.1038/s41589-019-0445-9.
[39] H.J. Welters, M. Tadayyon, J.H.B. Scarpello, S.A. Smith, N.G. Morgan, Mono-unsaturated fatty acids protect against β-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure, FEBS (Fed. Eur. Biochem. Soc.) Lett. 560 (2004) 103–108, https://doi.org/10.1016/S0014-5793(04)00079-1.
[40] K. Maedler, J. Oberholzer, P. Bucher, G.A. Spinas, M.Y. Donath, Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic β-cell turnover and function, Diabetes 52 (2003) 726–733, https://doi.org/10.2337/diabetes.52.3.726.
[41] P. Thomas, K.A. Leslie, H.J. Welters, N.G. Morgan, Long-chain saturated fatty acid species are not toxic to human pancreatic β-cells and may offer protection against pro-inflammatory cytokine induced β-cell death, Nutr. Metab. 18 (2021) 9, https://doi.org/10.1186/s12986-021-00541-8.
[42] H. Zhao, T. Matsuzaka, Y. Nakano, K. Motomura, N. Tang, T. Yokoo, Y. Okajima, S.-I. Han, Y. Takeuchi, Y. Aita, H. Iwasaki, S. Yatoh, H. Suzuki, M. Sekiya, N. Yahagi, Y. Nakagawa, H. Sone, N. Yamada, H. Shimano, Elovl6 deficiency improves glycemic control in diabetic db/db mice by expanding β-cell mass and increasing insulin secretory capacity, Diabetes 66 (2017) 1833–1846, https://doi.org/10.2337/db16-1277.
[43] M. Schuhmacher, A.T. Grasskamp, P. Barahtjan, N. Wagner, B. Lombardot, J. S. Schuhmacher, P. Sala, A. Lohmann, I. Henry, A. Shevchenko, Ü. Coskun, A. M. Walter, A. Nadler, Live-cell lipid biochemistry reveals a role of diacylglycerol side-chain composition for cellular lipid dynamics and protein affinities, Proc. Natl. Acad. Sci. USA 117 (2020) 7729–7738, https://doi.org/10.1073/pnas.1912684117.
[44] Y. Kamiya, S. Mizuno, S. Komenoi, H. Sakai, F. Sakane, Activation of conventional and novel protein kinase C isozymes by different diacylglycerol molecular species, Biochem Biophys Rep 7 (2016) 361–366, https://doi.org/10.1016/j.bbrep.2016.07.017.