

Glutamate is an essential mediator in glutamine-amplified insulin secretion

Guirong Han^{1,2,3}, Harumi Takahashi^{2*} , Naoya Murao² , Ghupurjan Gheni², Norihide Yokoi^{2,4} , Yoshiyuki Hamamoto³ , Shun-ichiro Asahara⁵, Yutaka Seino³ , Yoshiaki Kido^{1,5} , Susumu Seino^{2*}

¹Division of Metabolism and Disease, Department of Biophysics, Kobe University Graduate School of Health Sciences, Kobe, Japan, ²Division of Molecular and Metabolic Medicine, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe, Japan, ³Kansai Electric Power Medical Research Institute, Kobe, Japan, ⁴Laboratory of Animal Breeding and Genetics, Graduate School of Agriculture, Kyoto University, Kyoto, Japan, and ⁵Division of Diabetes and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

Keywords

Glutamine, Glutamate, Insulin secretion

*Correspondence

Susumu Seino or Harumi Takahashi
Tel.: +81-78-304-6061
Fax: +81-78-304-6064
E-mail addresses:
seino@med.kobe-u.ac.jp (S.S.) or
htakahashi@puppy.kobe-u.ac.jp (H.T.)

J Diabetes Investig 2021; 12: 920–930

doi: 10.1111/jdi.13497

ABSTRACT

Aims/Introduction: Glutamine is the most abundant amino acid in the circulation. In this study, we investigated cell signaling in the amplification of insulin secretion by glutamine.

Materials and Methods: Clonal pancreatic β -cells MIN6-K8, wild-type B6 mouse islets, glutamate dehydrogenase (GDH) knockout clonal β -cells (*Glud1KO* β CL), and glutamate-oxaloacetate transaminase 1 (GOT1) knockout clonal β -cells (*Got1KO* β CL) were studied. Insulin secretion from these cells and islets was examined under various conditions, and intracellular glutamine metabolism was assessed by metabolic flux analysis. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was also measured.

Results: Glutamine dose-dependently amplified insulin secretion in the presence of high glucose in both MIN6-K8 cells and *Glud1KO* β CL. Inhibition of glutaminases, the enzymes that convert glutamine to glutamate, dramatically reduced the glutamine-amplifying effect on insulin secretion. A substantial amount of glutamate was produced from glutamine through direct conversion by glutaminases. Glutamine also increased $[\text{Ca}^{2+}]_i$ at high glucose, which was abolished by inhibition of glutaminases. Glutamic acid dimethylester (dm-Glu), a membrane permeable glutamate precursor that is converted to glutamate in cells, increased $[\text{Ca}^{2+}]_i$ as well as induced insulin secretion at high glucose. These effects of glutamine and dm-Glu were dependent on calcium influx. Glutamine also induced insulin secretion in clonal β -cells MIN6-m14, which otherwise exhibit no insulin secretory response to glucose.

Conclusions: Glutamate converted from glutamine is an essential mediator that enhances calcium signaling in the glutamine-amplifying effect on insulin secretion. Our data also suggest that glutamine exerts a permissive effect on glucose-induced insulin secretion.

INTRODUCTION

Insulin secreted from pancreatic β -cells is a key hormone that regulates glucose homeostasis and impaired insulin secretion causes diabetes mellitus. Insulin secretion is regulated by nutrients including glucose, fatty acids, and amino acids as well as hormonal and neural inputs^{1,2}. Glucose is the primary stimulator of insulin secretion from pancreatic β -cells, and a series of

intracellular events is involved: inhibition of ATP-sensitive K^+ (K_{ATP}) channels through the production of ATP by glucose metabolism induces membrane depolarization, which leads to opening of the voltage-dependent Ca^{2+} channels and influx of extracellular Ca^{2+} , thereby triggering insulin secretion³. Glucose-induced insulin secretion (GIIS) is modulated by various amino acids. Regulation of insulin secretion by amino acids has been studied for years. While an admixture of amino acids is well known to strongly stimulate insulin secretion, only alanine,

Received 23 December 2020; revised 4 January 2021; accepted 5 January 2021

leucine, and arginine have been found to individually induce insulin secretion: alanine and leucine do so through generation of ATP in β -cells while arginine acts through calcium influx by depolarization primarily due to its positive charge⁴, which is possibly K_{ATP} channel-dependent action⁵. The effect of glutamine on insulin secretion has long been investigated^{6–8}. However, most of these studies used a combination with leucine in their examinations^{8,9}. Under this condition, glutamine transported into β -cells is initially converted to glutamate by glutaminase, and further converted to α -ketoglutarate (α -KG) by glutamate dehydrogenase (GDH), which is activated by leucine and then supplied to the TCA cycle as fuel¹⁰.

We previously reported that cytosolic glutamate in β -cells acts as a key signal linking glucose metabolism to incretin/cAMP action in incretin-induced insulin secretion. Cytosolic glutamate is produced mainly from α -KG by glutamate-oxaloacetate transaminase 1 (GOT1), a component of the malate-aspartate shuttle in glucose metabolism^{11,12}. However, it is unclear whether the cytosolic glutamate derived from glutamine acts as a signal in insulin secretion. Glutamine is the most abundant amino acid in mammalian plasma, and plays an essential role as fuel for cell metabolism in a variety of tissues and cells such as kidney, intestine, liver, heart, and neurons¹³. Pancreatic β -cells consume a substantial amount of glutamine and utilize it for essential cellular processes including synthesis of protein, pyrimidine, and purine, in addition to protection of β -cells against oxidative stress as well as insulin secretion⁴. Glutamine transported into β -cell likely supplies also γ -aminobutyric acid, which plays as a paracrine and/or an autocrine mediator for intra-islet regulation of hormone secretion¹⁴. The circulation level of glutamine is ~ 0.6 mM in healthy subjects¹⁵, but is lower in diabetic patients¹⁶, suggesting a permissive role of glutamine in maintaining glucose homeostasis.

In this study, we investigate intracellular signaling in the amplification of insulin secretion by glutamine. We find that two important steps are required for the glutamine-amplifying effect: (1) conversion of glutamine to glutamate by glutaminases and (2) enhancement by glutamate of intracellular Ca^{2+} signaling, which triggers insulin secretion. Glutamate thus functions as an essential mediator in glutamine-amplified insulin secretion.

METHODS

Materials

Glutamine was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Glutamic acid dimethylester hydrochloride (dm-Glu) was purchased from Tokyo Chemical Industry (Tokyo, Japan). $[U-^{13}C]$ -Glucose, $[U-^{13}C]$ -Glutamine and 2-amino-2-norbornane carboxylic acid (BCH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CB-839 was from Selleck chemicals (Houston, TX, USA). Small interfering RNAs for mouse *Gls* and *Gls2* were purchased from Horizon Discovery (Cambridge, UK). Fura-2 acetoxymethyl ester (Fura-2 AM) was from Dojindo (Kumamoto, Japan). Anti-GLUD1/2 antibody was purchased from Santa Cruz Biotechnology

(Dallas, TX, USA). Anti-actin antibody was from Calbiochem, Merck KGaA (Darmstadt, Germany).

Cell culture

Mouse clonal pancreatic β -cells MIN6-K8¹⁷ and MIN6-m14¹⁸, and a clonal β -cell line deficient for GOT1 (*Got1KO* β CL)¹² were previously reported. Clonal β -cell lines deficient for GDH (*Glud1KO* β CL) were generated by CRISPR/Cas9 nickase system as described previously¹², using sgRNA pair purchased from GeneCopia (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Sigma-Aldrich) containing 10% heat-inactivated Fetal Bovine Serum (FBS) (Biowest, Nuaille, France) and 1 mM sodium pyruvate at 37°C with 95% air and 5% CO₂^{12,17}.

Animal care

Male C57BL/6J mice were obtained from CLEA Japan (Tokyo Japan). Animals were maintained under specific pathogen-free conditions at $23 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ relative humidity with 12-h light-dark cycle, and were provided with water and commercially obtained CE-2 diet (CLEA Japan) at the Animal Facility of Kobe Biotechnology Research and Human Resource Development Center of Kobe University. All animal experiments were approved by the Institutional Animal Care and Use Committee (Permission number: 27-04-01-R3, 2020-06-05) and carried out according to the Kobe University Animal Experimentation Regulations.

Pancreatic islets isolation

For islet isolation, male C57BL/6J mice at 12 weeks of age were euthanized by sodium pentobarbital overdose. Pancreatic islets were isolated by collagenase digestion method as described previously¹⁹. Isolated islets were cultured in RPMI-1640 medium (Sigma-Aldrich) at 37°C with 95% air and 5% CO₂.

Insulin secretion

Insulin secretion experiments were performed as described previously¹¹. Briefly, cells were pre-incubated for 30 min in HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.1% bovine serum albumin (H-KRB) with 2.8 mM glucose. Cells were then incubated for 30 min in H-KRB containing various stimuli indicated in the figures. After stimulation, the buffer in each well was collected for measurements of released insulin. Cells were lysed with 0.1 % Triton-X100 in H-KRB for measurements of insulin content. For islet batch incubation, after 30 min preincubation in H-KRB with 2.8 mM glucose, 5 size-matched islets were collected in each well, and then incubated for 30 min in H-KRB containing stimuli indicated in the figures. The incubation medium was collected for measurements of insulin release. Islets were lysed as described above for measurements of insulin contents. Released insulin and insulin contents were measured by homogeneous time-resolved fluorescence assay (HTRF) using Insulin Ultrasensitive HTRF Assay kit (PerkinElmer, Waltham, MA, USA). The

amount of insulin secretion was normalized by insulin content.

Measurements of glutamate content

For metabolic flux analysis, MIN6-K8 cells and *Glut1*KO β CL were pre-incubated in H-KRB containing 2.8 mM glucose for 60 min and then stimulated for 30 min with [U-¹³C]-glucose or [U-¹³C]-glutamine at concentrations indicated in the figures. For the determination of glutamate under the inhibition of glutaminases, siRNA-transfected cells were stimulated with glutamine with or without CB-839 as indicated in the figures. After incubation, cells were collected with the isotonic buffer [0.27 M Sucrose in 10 mM MOPS-Tris (pH 6.8)] and homogenized with 21 G and 25 G needle and syringe. The cell lysates were centrifuged for 35 min at 27,000 g, 4°C and supernatant was collected as cytosolic fraction. Metabolites were extracted by adding extraction buffer (67.5% methanol, 25% water, 7.5% chloroform) to the fraction. The aqueous layer was collected and analyzed by LCMS-8060 (Shimadzu, Kyoto, Japan) and CE7100-G6224A TOFMS system (Agilent, Santa Clara, CA, USA) as described previously^{11,12}.

Quantitative PCR analysis

Total RNA was extracted from MIN6-K8 cells and mouse islets using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared by reverse transcription of total RNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). mRNA expression levels were determined by TaqMan Gene Expression Assays using StepOnePlus Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, MA, USA). Relative abundance of mRNAs was calculated by Δ CT and normalized to endogenous *Gapdh* as internal control. Probe details: *Gls*, Mm 01257297_m1; *Gls2*, Mm 01164862_m1; *Gapdh*, Mm 99999915_g1.

Knockdown experiments

MIN6-K8 cells were transfected with siRNAs using DharmaFECT2 transfection reagent (Horizon Discovery) according to the manufacturer's instruction. After 3-day culture, cells were used for insulin secretion experiments, Ca²⁺ assay, measurements of glutamate content, and qPCR analysis.

Western blot analysis

Cells were lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and Complete, a protease inhibitor Cocktail (Roche, Basel, Switzerland)]. Proteins were separated by SDS-PAGE and electrophoretically transferred onto PVDF membrane (Immobilon P, Millipore, Billerica, MA, USA). The membrane was blocked in 0.5% skim milk for 60 min and incubated with anti-GLUD1/2 antibody (1:2,000) or anti-actin antibody (1:5,000) overnight at 4°C. The following morning, membranes were washed with Tris-buffered saline [20 mM Tris-HCl (pH 7.5), 150 mM NaCl] containing 0.1% of Tween 20 (TBS-T) and

incubated with anti-Rabbit IgG-HRP or anti-goat IgG-HRP secondary antibodies for 60 min at room temperature and washed with TBS-T. Immunoreactivity was visualized with ECL Prime detection reagents (GE Healthcare, Little Chalfont, UK) and detected by Image Quant LAS 4000 mini (GE Healthcare).

Ca²⁺ assay

Cells were loaded with 5 μ M of Fura-2 AM for 20 min in H-KRB with 2.8 mM glucose, and then incubated in H-KRB containing various stimuli indicated in the figures. Fluorescent intensity of Fura-2 was measured by a dual-excitation wavelength method (340/380 nm) with a fluorometer, Fluoroskan (Thermo Fisher Scientific)²⁰.

Measurement of ATP production

Cells were pre-incubated for 60 min in H-KRB with 2.8 mM glucose and then stimulated with H-KRB containing various stimuli indicated in the figures. The ATP content was determined by an EnzyLight ATP assay kit (Bio Assay Systems, Hayward, CA, USA).

Statistical analysis

Statistical analyses were performed by one-way ANOVA or two-way ANOVA followed by Dunnett, Tukey's, or Sidak's test as indicated in the figure legends using GraphPad Prism Version 8.4.2. Data are presented as means \pm SEM. $P < 0.05$ was regarded as statistically significant.

RESULTS

Amplification of insulin secretion by glutamine and its suppression by inhibition of glutaminases

We examined the acute effects of glutamine on insulin secretion from insulin-secreting clonal β -cells MIN6-K8 and isolated mouse islets. In MIN6-K8 cells, glutamine dose-dependently enhanced insulin secretion in the range of 1–10 mM in the presence of 8.8 mM glucose (Figure 1a). In mouse islets, 2 mM glutamine significantly amplified glucose-induced insulin secretion (GIIS) (Figure 1b). It has been known that glutamine is transported into β -cells and then metabolized to glutamate by glutaminase and that the glutamate enhances insulin secretion induced by leucine or arginine through a further conversion to α -KG by glutamate dehydrogenase (GDH, gene symbol *Glud1*)⁹. We therefore examined whether the conversion of glutamate to α -KG by GDH is required for the amplification of GIIS by glutamine. To this end, we generated clonal β -cell lines deficient for GDH (*Glud1*KO β CL) from MIN6-K8 cells by genome editing using the CRISPR/Cas9 system¹², and established two lines (G16 and G17) in which the knockout of GDH protein was confirmed by western blot analysis (Figure 1c upper). The G17 line was used throughout this study. We used BCH (2-amino-2-norbornane carboxylic acid), an activator of GDH¹⁰, to confirm the functional deficiency of GDH enzymes. BCH stimulated insulin secretion at 2.8 mM glucose in MIN6-K8 cells but not in *Glud1*KO β CL (Figure 1c lower), indicating

that GDH is functionally absent in *Glud1*KO β CL. We found that glutamine dose-dependently amplified GIIS in *Glud1*-KO β CL as was found in MIN6-K8 cells (Figure 1d). These results indicate that glutamine amplifies GIIS through mechanisms not involving the conversion of glutamate to α -KG by GDH.

We then examined whether production of cellular glutamate from glutamine is required for the amplification of GIIS by glutamine. We focused on glutaminase, the enzyme that converts glutamine to glutamate. Quantitative RT-PCR analysis showed that MIN6-K8 cells express both kidney-type (GLS, gene symbol *Gls*) and liver-type (GLS2, gene symbol *Gls2*) glutaminases (Figure 1e). We therefore examined the effects of inhibition of GLS and GLS2 on insulin secretion. The mRNA expressions were suppressed by ~60% for *Gls* and by ~80% for *Gls2* by knockdown (KD) in MIN6-K8 cells (Figure 1f). KD of *Gls* and *Gls2* reduced the glutamine-amplifying effect on GIIS by ~50%, and ~60%, respectively (Figure 1g). CB-839, a specific inhibitor of GLS²¹, reduced the amplifying effect to a level similar to that in *Gls* KD (Figure 1h left). We used the combination of CB-839 and *Gls2* KD for double inhibition of GLS and GLS2 as a high concentration of total siRNAs (70 nM) for double transfection affects cellular conditions. Double inhibition of GLS by CB-839 and GLS2 by KD more potently reduced insulin secretion (Figure 1h right).

Direct conversion of glutamine to glutamate by glutaminases

We previously reported that high glucose promotes cytosolic glutamate production through the malate-aspartate shuttle linked to glycolysis¹¹. As glutamine-amplified insulin secretion is glucose dependent, we attempted to identify the origin of the cellular glutamate produced from glutamine that is required for amplification of GIIS. For this purpose, we performed metabolic flux analysis. Cells were stimulated with [¹³C] glucose or [¹³C]-glutamine and the subsequently produced glutamate isotopomers were determined by mass spectrometry. Isotopomers M+2 to M+5 are derived from [¹³C] glucose or [¹³C]-glutamine. Using [¹³C]-glucose, we confirmed that high glucose (8.8 mM) produced cytosolic glutamate through the malate-aspartate shuttle as M+2 to M+5 glutamate isotopomers, which were increased in MIN6-K8 cells (Figure 2a left), were not increased in clonal β -cells deficient for glutamate-oxaloacetate transaminase 1 (*Got1*KO β CL), a critical enzyme for the production of cytosolic glutamate from α -KG associated with the malate-aspartate shuttle (Figure 2a right). This is consistent with our previous findings¹². By exposure of MIN6-K8 cells to 2 mM [¹³C]-glutamine, M+5 isotopomers were markedly increased at both 2.8 mM and 8.8 mM glucose, indicating that cytosolic glutamate is produced from [¹³C]-glutamine (Figure 2b lower left). Similar results were obtained in *Got1*KO β CL (Figure 2b lower right). These findings indicate that cytosolic glutamate is produced from extracellular glutamine independently of the malate-aspartate shuttle. Double inhibition of GLS by CB-839 and GLS2 by KD significantly

reduced cytosolic glutamate production by glutamine treatment at 8.8 mM glucose (Figure 2c).

Enhancement of intracellular Ca²⁺ by glutamine and its dependency on Ca²⁺ influx

As intracellular Ca²⁺ is an essential signal in insulin secretion, we examined the effects of glutamine on change in intracellular Ca²⁺ concentration ([Ca²⁺]_i) in MIN6-K8 cells loaded with Fura 2-AM. Glutamine at 2 mM enhanced a rise in [Ca²⁺]_i induced by 8.8 mM glucose in both MIN6-K8 cells and *Glud1*KO β CL (Figure 3a), while glutamine showed no effect on [Ca²⁺]_i at 2.8 mM glucose (Figure 3a left). We also found that cellular ATP content was increased by glucose but was not further increased by glutamine at 8.8 mM glucose in MIN6-K8 cells (Figure 3b). This suggests that glutamine does not amplify insulin secretion through ATP production under the experimental conditions used in the present study.

Enhancement of the rise in [Ca²⁺]_i by glutamine was almost completely abolished by double inhibition of glutaminases by CB-839 and *Gls2* KD (Figure 3c), suggesting that glutamate produced from glutamine directly increases [Ca²⁺]_i. In fact, glutamic acid dimethylester (dm-Glu), a membrane permeable glutamate precursor that is converted to glutamate by cytosolic esterase in cells, enhanced the rise in [Ca²⁺]_i as well as GIIS (Figure 3d), supporting direct enhancement of the glucose-induced rise in [Ca²⁺]_i by glutamate.

To determine whether the rise in [Ca²⁺]_i by glutamine is related to Ca²⁺ influx into β -cells, we investigated the effect of nifedipine, a blocker of L-type Ca²⁺ channels, on the change in [Ca²⁺]_i by glutamine. We found that nifedipine significantly reduced elevation of [Ca²⁺]_i by glutamine (Figure 4a left) and that the glutamine-amplifying effect on insulin secretion was abolished by nifedipine (Figure 4a right). In addition, the increment of [Ca²⁺]_i as well as glutamine- and dm-Glu-amplified insulin secretion was almost completely abolished when cells were stimulated with Ca²⁺-free KRB buffer (Figure 4b,c). These results indicate that elevation of [Ca²⁺]_i by glutamine is dependent on influx of extracellular Ca²⁺ through voltage-dependent Ca²⁺ channels.

Improvement of insulin secretion in a glucose-unresponsive β -cell line and enhancement of effectiveness of glibenclamide by glutamine

We then examined the effect of glutamine on sulfonylurea-induced insulin secretion, and found that glutamine significantly amplified insulin secretion induced by 100 nM glibenclamide with increasing [Ca²⁺]_i (Figure 5a). To investigate the role of glutamine in dysfunctional β -cells, we used MIN6-m14, a β -cell line that shows unresponsiveness to glucose in insulin secretion and [Ca²⁺]_i¹⁸. Insulin secretion at basal state (2.8 mM glucose) was elevated, but no GIIS was detected in MIN6-m14, as was reported previously. Interestingly, the addition of 2 mM glutamine markedly induced a rise in [Ca²⁺]_i as well as glucose-responsive insulin secretion in MIN6-m14 cells (Figure 5b).

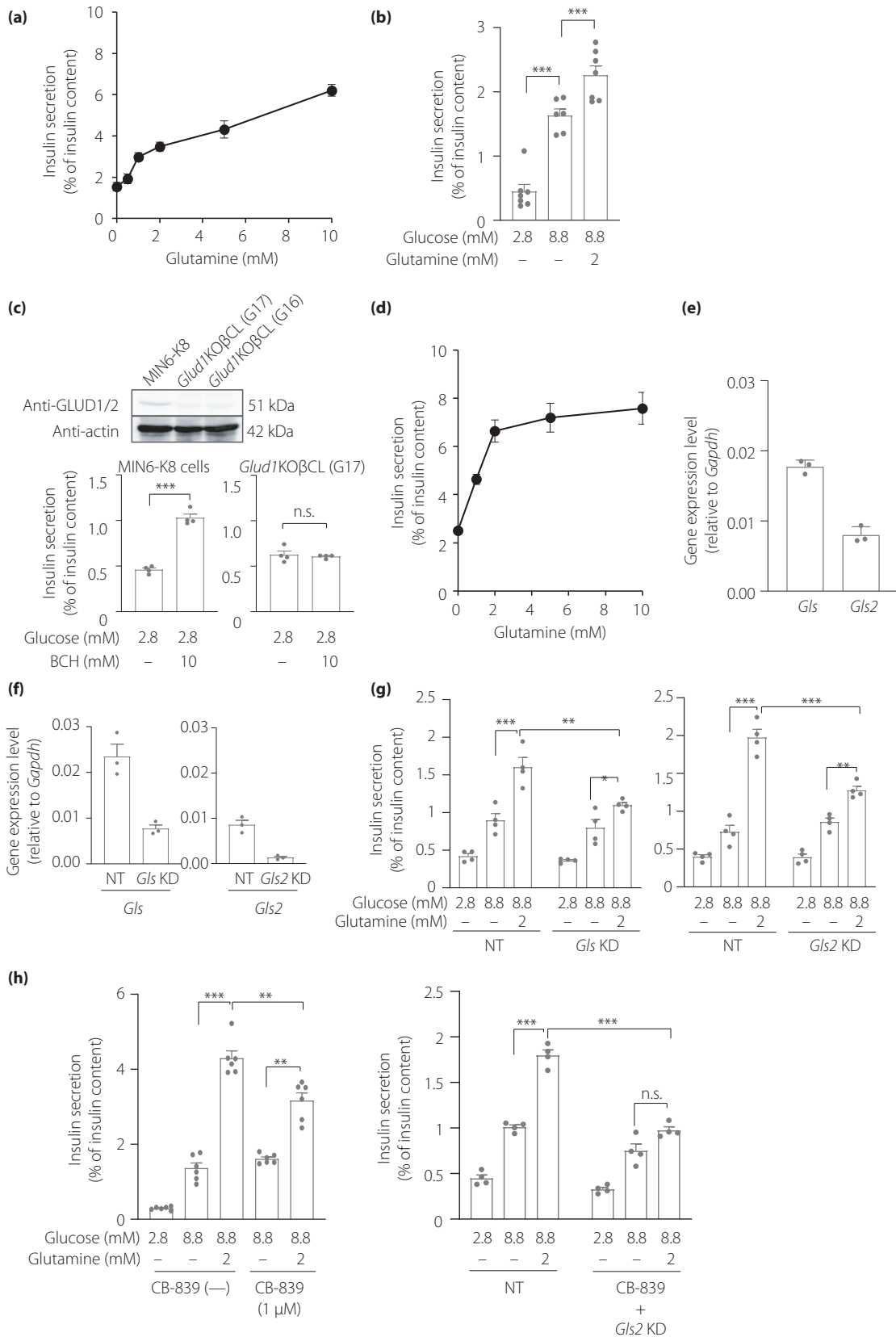


Figure 1 | Amplifying effects of glutamine on insulin secretion and its suppression by glutaminase inhibitions. (a) Dose-dependent effect of glutamine on insulin secretion from MIN6-K8 cells at 8.8 mM glucose ($n = 4$). (b) Effect of 2 mM glutamine on insulin secretion from mouse isolated islets ($n = 6-7$). (c) Confirmation of knockout of GDH protein by western blot analysis (upper) and functional deficiency of GDH enzyme using BCH, a leucine analog that activates GDH (lower) in *Glud1*KO β CLs (G16 and G17) ($n = 4$ in lower). (d) Dose-dependent effect of glutamine on insulin secretion from *Glud1*KO β CL (G17) at 8.8 mM glucose ($n = 4$). (e) mRNA expression levels of glutaminase (*Gls*) and glutaminase 2 (*Gls2*) in MIN6-K8 cells ($n = 3$). (f) Efficiency of knockdown for *Gls* and *Gls2* in MIN6-K8 cells ($n = 3$). NT, non-targeting control siRNA. *Gls* KD, knockdown for *Gls*. *Gls2* KD, knockdown for *Gls2*. (g) Effect of individual knockdown of *Gls* and *Gls2* on glutamine-amplified insulin secretion in MIN6-K8 cells ($n = 4$). (h) Effect of CB-839, a specific inhibitor for GLS (left) ($n = 6$), and double inhibition of glutaminases by CB-839 and *Gls2* KD (right) ($n = 4$) on glutamine-amplified insulin secretion in MIN6-K8 cells. Values are means \pm SEM. $**P < 0.01$, $***P < 0.001$. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparisons test.

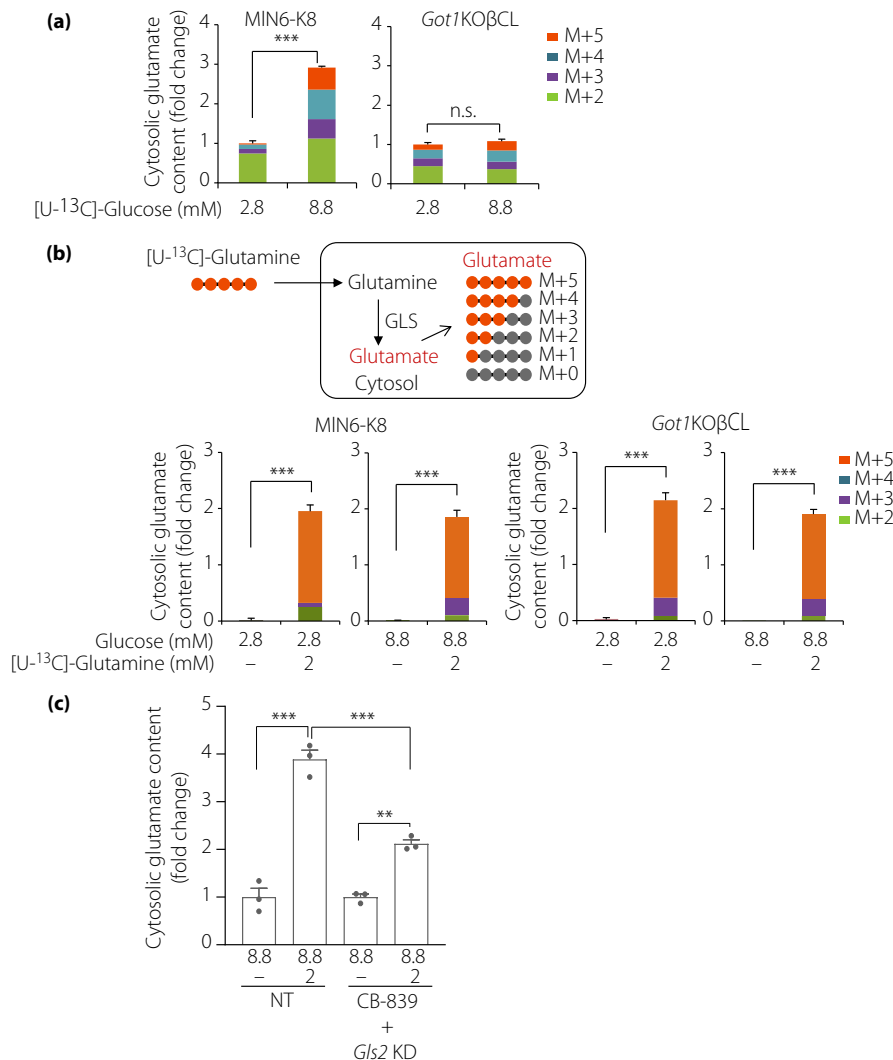


Figure 2 | Glutamate production by glutamine in clonal β -cells. (a) Cytosolic glutamate content derived from $[U-^{13}C]$ -glucose in MIN6-K8 cells (left) and *Got1*KO β CL (right) ($n = 3$). Cells were stimulated with 2.8 mM or 8.8 mM $[U-^{13}C]$ -glucose for 30 min. M+2 to M+5, glutamate isotopomers. (b) Upper, a scheme of production of glutamate isotopomers from exogenous $[U-^{13}C]$ -glutamine in the cells. M+0 to M+5, glutamate isotopomers. GLS, glutaminase. Lower, cytosolic glutamate content derived from $[U-^{13}C]$ -glutamine in MIN6-K8 cells (left) and *Got1*KO β CL (right) ($n = 3$). Cells were stimulated with $[U-^{13}C]$ -glutamine at 2.8 mM or 8.8 mM for 30 min. (c) Effect of double inhibition of GLS and GLS2 by CB-839 and *Gls2* KD, respectively, on production of cytosolic glutamate converted from glutamine in MIN6-K8 cells ($n = 3$). NT, non-targeting control siRNA. Values are means \pm SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s., not significant. Statistical analyses were performed by Student's *t*-test for (a) and (b), one-way ANOVA followed by Sidak's multiple comparisons test for (c).

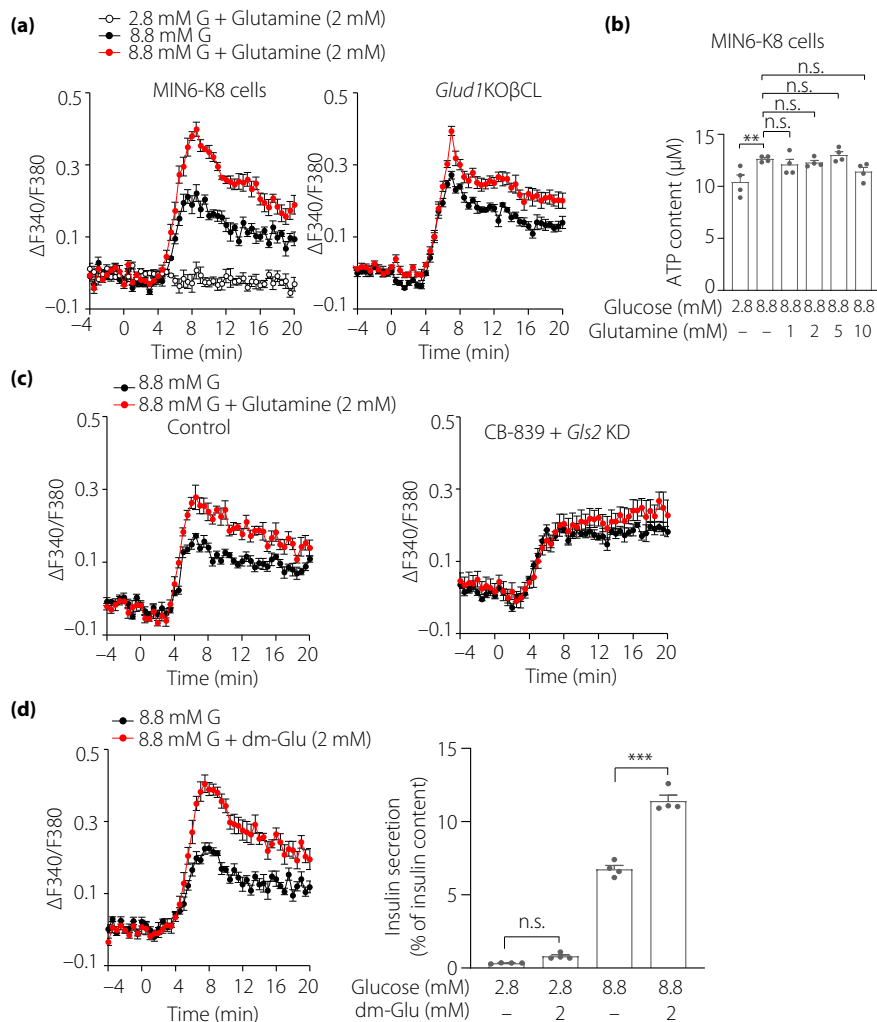


Figure 3 | Enhancement of rise in $[Ca^{2+}]_i$ by glutamate converted from glutamine. (a) Effect of 2 mM glutamine on rise in $[Ca^{2+}]_i$ induced by 8.8 mM glucose in MIN6-K8 cells (left) and *Glud1KOβCL* (right) ($n = 6$ for each point). (b) Effect of glucose and glutamine on ATP content in MIN6-K8 cells ($n = 4$). (c) Effect of 2 mM glutamine on rise in $[Ca^{2+}]_i$ induced by 8.8 mM glucose in control cells (left) and double-inhibited cells (right) ($n = 6$ for each point). (d) Effect of 2 mM glutamic acid dimethylester (dm-Glu) on rise in $[Ca^{2+}]_i$ (left, $n = 6$ for each point) induced by 8.8 mM glucose and on insulin secretion (right, $n = 4$) in MIN6-K8 cells. G, glucose. Values are means \pm SEM. $**P < 0.01$, $***P < 0.001$, n.s., not significant. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparisons test for (b) and Student's *t*-test for (d).

DISCUSSION

In the present study, we demonstrate that glutamine amplifies GIIS through its conversion to glutamate, which enhances Ca^{2+} signaling. Effects of glutamine on insulin secretion have been reported in many studies in the past^{7,9,22}. However, most of these studies investigated the effects of glutamine on insulin secretion in combination with leucine at a low concentration of glucose. Under this condition, glutamine is converted to α -KG by GDH, which is activated by leucine and supplied to the TCA cycle as fuel to produce ATP, thereby stimulating insulin secretion⁹. Although it has been reported that the activity of GDH is inhibited at high glucose due to the elevated ATP level⁹, several studies have shown the amplifying effect of

glutamine on insulin secretion in high glucose condition^{23–25}. In the present study, we show that glutamine amplifies glucose-induced insulin secretion in both MIN6-K8 and *Glud1KOβCL*, indicating that glutamine amplifies insulin secretion at a high concentration of glucose through a different pathway from that via conversion to α -KG by GDH. Glutamine is converted to glutamate by glutaminase after being transported into the cell. There are two isoforms of glutaminase, kidney-type glutaminase (GLS) and liver-type glutaminase (GLS2), so far identified²⁶. Their expressions differ among tissues. Both isoforms are localized predominantly on the inner mitochondrial membrane, but many have been shown to exert activity toward the cytoplasmic side; hence, most glutamine transported into the cell is

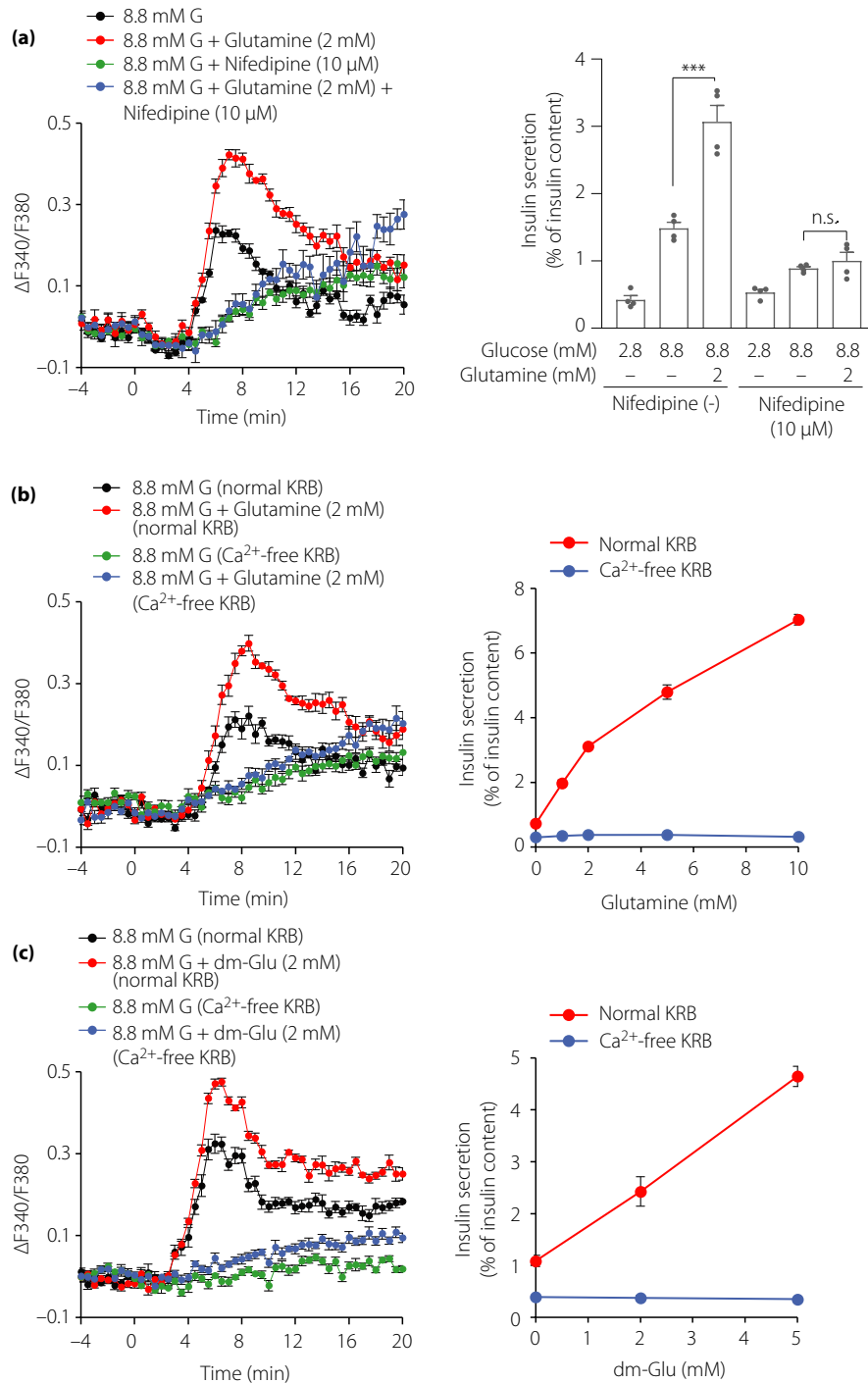


Figure 4 | Ca²⁺ influx-dependency of the glutamine-effect on [Ca²⁺]_i. (a) Effect of nifedipine on rise in [Ca²⁺]_i (left) and insulin secretion (right) in MIN6-K8 cells. (b) Effect of depletion of extracellular Ca²⁺ on [Ca²⁺]_i (left) and amplifying effect of glutamine on insulin secretion (right). Cells were stimulated in normal KRB or Ca²⁺-free KRB. (c) dm-Glu-induced increase in [Ca²⁺]_i and insulin secretion were almost completely abolished when cells were stimulated with Ca²⁺-free KRB. G, glucose. Values are means with \pm SEM; $n = 6$ for each point in (a)–(c) left, $n = 4$ – 6 for (a)–(c) right. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant. Statistical analyses were performed by two-way ANOVA followed by Tukey's multiple comparisons test.

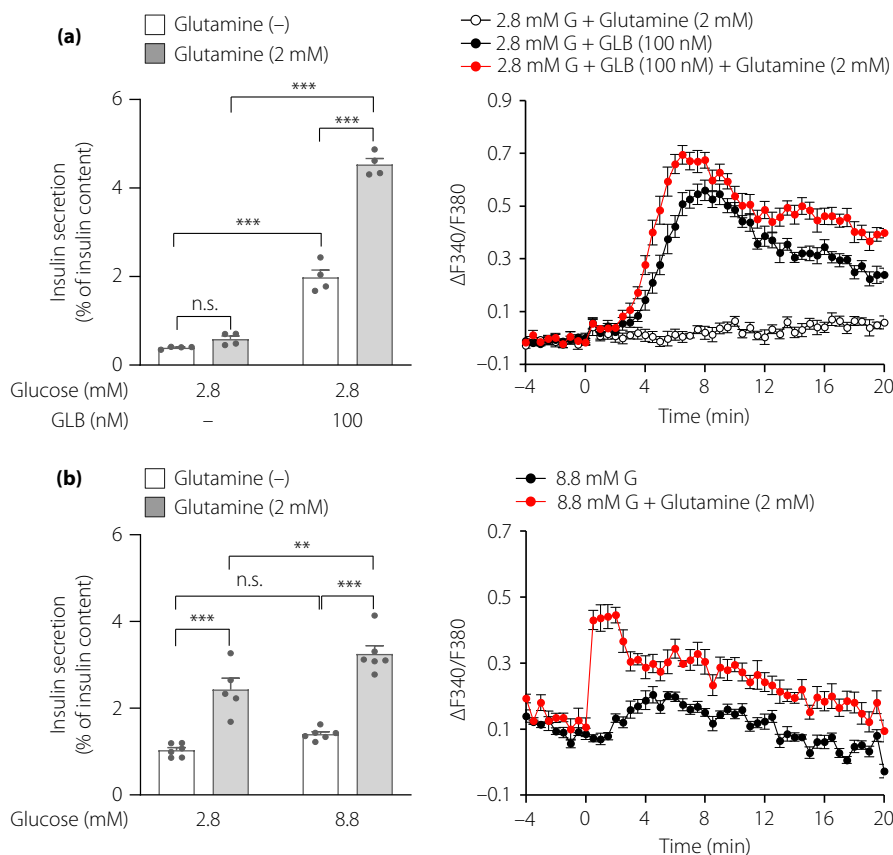


Figure 5 | Enhancement of glibenclamide effect on insulin secretion and rise in $[Ca^{2+}]_i$ and improvement of insulin secretion in a glucose-unresponsive β -cell line. (a) Effect of glutamine on insulin secretion (left, $n = 4$) and rise in $[Ca^{2+}]_i$ (right, $n = 6$ for each point) induced by glibenclamide (GLB) in MIN6-K8 cells. (b) Effect of glutamine on insulin secretion (left, $n = 4$) and $[Ca^{2+}]_i$ (right, $n = 6$ for each point) in MIN6-m14, a glucose-unresponsive clonal β -cell line. G, glucose. Values are means \pm SEM. ** $P < 0.01$, *** $P < 0.001$, n.s., not significant. Statistical analyses were performed by two-way ANOVA followed by Sidak's multiple comparisons test.

converted to glutamate by glutaminase in the cytosol²⁷. Because of the phosphate-dependency of glutaminase, its activity might be inhibited in the high glucose condition in which the cellular phosphate level is reduced by its consumption for the phosphorylation of various proteins⁹. It has been reported that in rat pancreatic islets, GLS is expressed predominantly in α -cells, while GLS2 is expressed predominantly in β -cells^{28,29}. Considering that GLS2 has lower K_m for phosphate than that of GLS³⁰, glutaminase activity might well be retained at high glucose in β -cells. Our result shows that the increment of cytosolic glutamate level by glutamine treatment is reduced by glutaminase inhibition at high concentrations of glucose (Figure 2c), suggesting a contribution of GLS2 to glutamate production, particularly at high glucose.

We have previously reported that cytosolic glutamate produced through the malate-aspartate shuttle in glucose metabolism acts as a key signal in incretin/cAMP action¹¹. In the present study, we find that glutamate converted from glutamine by glutaminases is an essential mediator in glutamine-amplified insulin secretion (Figure 6). Our results show that enhancement

of rise in $[Ca^{2+}]_i$ by glutamine and dm-Glu is dependent on influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels. The $[Ca^{2+}]_i$ increment is crucial for the glutamine-amplifying effect on insulin secretion. It is not yet clear whether this effect is due to direct activation of VDCCs or is secondary to mobilization of Ca^{2+} from Ca^{2+} stores resulting from Ca^{2+} influx. Indeed, little is known about the effect of intracellular glutamate on intracellular Ca^{2+} signaling. However, glutamate has been shown to inhibit protein phosphatase activity in β -cells³¹, and this could contribute to enhancement of the Ca^{2+} signaling by increasing phosphorylation levels of proteins related to Ca^{2+} influx and mobilization of Ca^{2+} from Ca^{2+} stores^{32–34}. It has been reported that intracellular glutamate interacts with voltage-dependent anion channels (VDAC) and modulates the mitochondrial permeability transition pore, which may induce changes in cytosolic Ca^{2+} dynamics³⁵. The present results show that inhibition of glutaminase resulted in rather slight increase in $[Ca^{2+}]_i$ in the second phase and in insulin secretion induced by glucose alone (Figure 3c), suggesting that glutamine itself might affect intracellular Ca^{2+} dynamics and insulin secretion

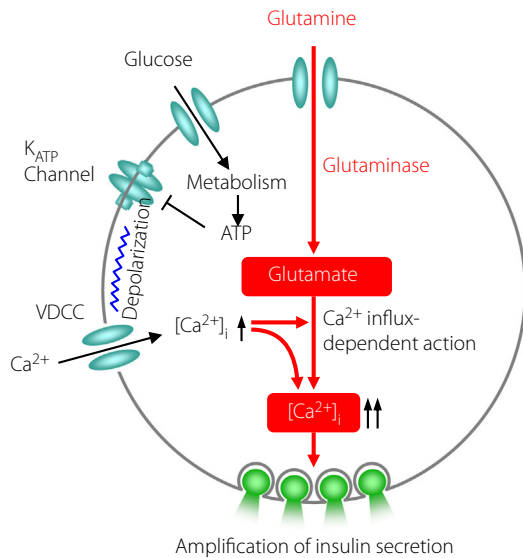


Figure 6 | Glutamate as an essential mediator in glutamine-amplified insulin secretion. Glutamate converted from glutamine by glutaminases amplifies insulin secretion by increasing $[Ca^{2+}]_i$. Action of glutamate on the enhancement is dependent on Ca^{2+} influx through voltage-dependent calcium channels (VDCCs). K_{ATP} channel, ATP-sensitive K^+ channel.

without conversion to glutamate in certain conditions. Indeed, it has been reported that the intracellular Ca^{2+} rise induced by 10 mM glutamine was not suppressed in the presence of 6-di-azo-5-oxo-L-norleucine (DON), a glutaminase inhibitor, in SUR1 (*Abcc8*) knockout islets⁶. In GLUTag cells, a clonal intestinal L cell line, glutamine has been shown to evoke a rise in $[Ca^{2+}]_i$ and stimulate secretion of glucagon-like peptide-1³⁶. This effect was retained even in the presence of DON, indicating that glutamine metabolism is not required³⁶. Although the detailed mechanism by which glutamine increases $[Ca^{2+}]_i$ without conversion to glutamate in GLUTag cells has not been reported, β -cells may well employ the same process, which would underlie the sustained increase in $[Ca^{2+}]_i$ found in the second phase.

Interestingly, we find that glutamine enhanced insulin secretion (GIIS) and Ca^{2+} signaling, and that it induces glucose responsiveness to some extent in glucose-unresponsive MIN6-m14 cells (Figure 5b). These results suggest that glutamine might improve glucose responsiveness in dysfunctional β -cells. In addition, we find that insulin secretion induced by glibenclamide is markedly augmented in the presence of 2 mM glutamine (Figure 5a), suggesting that glutamine might enhance the effectiveness of sulfonylureas. Given that glutamine is the most abundant amino acids in the circulation^{13,37}, it is likely that glutamine exerts a permissive effect on GIIS in the physiological state. It has been reported that plasma glutamine levels are reduced in patients with type 2 diabetes mellitus (T2DM)¹⁶. Chronic supplementation of glutamine has been shown to reduce body weight and attenuate hyperglycemia in mice fed a high fat diet³⁸ and to improve glucose tolerance in combination with sitagliptin

treatment in T2DM patients³⁹. Together with our current data, these findings suggest that glutamine supplementation might well ameliorate β -cell function as well as increase incretin secretion from intestine⁴⁰ to ameliorate glucose intolerance.

ACKNOWLEDGMENTS

We thank K. Shimomura (Fukushima Medical University) for valuable comments for this study. This study was supported by JSPS KAKENHI Grant number JP19K09026 (G.G.), JP19K07400 (H.T.), and JP18H02864 (S.S.). N.M. was supported by a fellowship grant from Novo Nordisk, Denmark, and the Research Fellowship of JSPS for Young Scientist DC1. The Department of Molecular and Metabolic Medicine is supported by MSD K.K., Novo Nordisk Pharma Ltd., Japan, Kowa Co. Ltd., and Taisho Pharmaceutical Holdings Co. Ltd.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

- Henquin JC. Regulation of insulin secretion: a matter of phase control and amplitude modulation. *Diabetologia* 2009; 52: 739–751.
- Prentki M, Matschinsky FM, Madiraju SR. Metabolic signaling in fuel-induced insulin secretion. *Cell Metab* 2013; 18: 162–185.
- Rorsman P, Ashcroft FM. Pancreatic beta-cell electrical activity and insulin secretion: of mice and men. *Physiol Rev* 2018; 98: 117–214.
- Newsholme P, Cruzat V, Arfuso F, et al. Nutrient regulation of insulin secretion and action. *J Endocrinol* 2014; 221: R105–R120.
- Miki T, Minami K, Shinozaki H, et al. Distinct effects of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 on insulin secretion and gut motility. *Diabetes* 2005; 54: 1056–1063.
- Li C, Buettinger C, Kwagh J, et al. A signaling role of glutamine in insulin secretion. *J Biol Chem* 2004; 279: 13393–13401.
- Sener A, Somers G, Devis G, et al. The stimulus-secretion coupling of amino acid-induced insulin release. Biosynthetic and secretory responses of rat pancreatic islet to L-leucine and L-glutamine. *Diabetologia* 1981; 21: 135–142.
- Malaisse WJ, Sener A, Malaisse-Lagae F, et al. The stimulus-secretion coupling of amino acid-induced insulin release. Metabolic response of pancreatic islets of L-glutamine and L-leucine. *J Biol Chem* 1982; 257: 8731–8737.
- Li C, Najafi H, Daikhin Y, et al. Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets. *J Biol Chem* 2003; 278: 2853–2858.
- Sener A, Malaisse WJ. L-leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. *Nature* 1980; 288: 187–189.
- Gheni G, Ogura M, Iwasaki M, et al. Glutamate acts as a key signal linking glucose metabolism to incretin/cAMP action to amplify insulin secretion. *Cell Rep* 2014; 9: 661–673.

12. Murao N, Yokoi N, Honda K, *et al.* Essential roles of aspartate aminotransferase 1 and vesicular glutamate transporters in beta-cell glutamate signaling for incretin-induced insulin secretion. *PLoS One* 2017; 12: e0187213.
13. Curi R, Lagranha CJ, Doi SQ, *et al.* Molecular mechanisms of glutamine action. *J Cell Physiol* 2005; 204: 392–401.
14. Jenstad M, Chaudhry FA. The amino acid transporters of the glutamate/GABA-glutamine cycle and their impact on insulin and glucagon secretion. *Front Endocrinol (Lausanne)* 2013; 4: 199.
15. Cruzat V, Macedo Rogero M, Noel Keane K, *et al.* Glutamine: metabolism and immune function, supplementation and clinical translation. *Nutrients* 2018; 10: 1564.
16. Menge BA, Schrader H, Ritter PR, *et al.* Selective amino acid deficiency in patients with impaired glucose tolerance and type 2 diabetes. *Regul Pept* 2010; 160: 75–80.
17. Iwasaki M, Minami K, Shibasaki T, *et al.* Establishment of new clonal pancreatic beta-cell lines (MIN6-K) useful for study of incretin/cyclic adenosine monophosphate signaling. *J Diabetes Investig* 2010; 1: 137–142.
18. Minami K, Yano H, Miki T, *et al.* Insulin secretion and differential gene expression in glucose-responsive and -unresponsive MIN6 sublines. *Am J Physiol Endocrinol Metab* 2000; 279: E773–E781.
19. Wollheim CB, Meda P, Halban PA. Isolation of pancreatic islets and primary culture of the intact microorgans or of dispersed islet cells. *Methods Enzymol* 1990; 192: 188–223.
20. Minami K, Yokokura M, Ishizuka N, *et al.* Normalization of intracellular Ca(2+) induces a glucose-responsive state in glucose-unresponsive beta-cells. *J Biol Chem* 2002; 277: 25277–25282.
21. Gross MI, Demo SD, Dennison JB, *et al.* Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. *Mol Cancer Ther* 2014; 13: 890–901.
22. Henquin JC, Dufrane D, Nenquin M. Nutrient control of insulin secretion in isolated normal human islets. *Diabetes* 2006; 55: 3470–3477.
23. Liu Z, Jeppesen PB, Gregersen S, *et al.* Dose- and glucose-dependent effects of amino acids on insulin secretion from isolated mouse islets and clonal INS-1E beta-cells. *Rev Diabet Stud* 2008; 5: 232–244.
24. McClenaghan NH, Barnett CR, O'Harte FP, *et al.* Mechanisms of amino acid-induced insulin secretion from the glucose-responsive BRIN-BD11 pancreatic B-cell line. *J Endocrinol* 1996; 151: 349–357.
25. Modi H, Cornu M, Thorens B. Glutamine stimulates biosynthesis and secretion of insulin-like growth factor 2 (IGF2), an autocrine regulator of beta cell mass and function. *J Biol Chem* 2014; 289: 31972–31982.
26. Aledo JC, Gomez-Fabre PM, Olalla L, *et al.* Identification of two human glutaminase loci and tissue-specific expression of the two related genes. *Mamm Genome* 2000; 11: 1107–1110.
27. Kvamme E, Torgner IA, Roberg B. Kinetics and localization of brain phosphate activated glutaminase. *J Neurosci Res* 2001; 66: 951–958.
28. Baglietto-Vargas D, Lopez-Tellez JF, Moreno-Gonzalez I, *et al.* Segregation of two glutaminase isoforms in islets of Langerhans. *Biochem J* 2004; 381: 483–487.
29. Inagaki N, Kuromi H, Gono T, *et al.* Expression and role of ionotropic glutamate receptors in pancreatic islet cells. *FASEB J* 1995; 9: 686–691.
30. Kovacevic Z, McGivan JD. Mitochondrial metabolism of glutamine and glutamate and its physiological significance. *Physiol Rev* 1983; 63: 547–605.
31. Lehtihet M, Webb DL, Honkanen RE, *et al.* Glutamate inhibits protein phosphatases and promotes insulin exocytosis in pancreatic beta-cells. *Biochem Biophys Res Commun* 2005; 328: 601–607.
32. Larsson O, Barker CJ, Sjöholm A, *et al.* Inhibition of phosphatases and increased Ca²⁺ channel activity by inositol hexakisphosphate. *Science* 1997; 278: 471–474.
33. Ammala C, Eliasson L, Bokvist K, *et al.* Activation of protein kinases and inhibition of protein phosphatases play a central role in the regulation of exocytosis in mouse pancreatic beta cells. *Proc Natl Acad Sci USA* 1994; 91: 4343–4347.
34. Haby C, Larsson O, Islam MS, *et al.* Inhibition of serine/threonine protein phosphatases promotes opening of voltage-activated L-type Ca²⁺ channels in insulin-secreting cells. *Biochem J* 1994; 298(Pt 2): 341–346.
35. Gincel D, Shoshan-Barmatz V. Glutamate interacts with VDAC and modulates opening of the mitochondrial permeability transition pore. *J Bioenerg Biomembr* 2004; 36: 179–186.
36. Tolhurst G, Zheng Y, Parker HE, *et al.* Glutamine triggers and potentiates glucagon-like peptide-1 secretion by raising cytosolic Ca²⁺ and cAMP. *Endocrinology* 2011; 152: 405–413.
37. Newsholme P, Procopio J, Lima MM, *et al.* Glutamine and glutamate—their central role in cell metabolism and function. *Cell Biochem Funct* 2003; 21: 1–9.
38. Opara EC, Petro A, Tevrizian A, *et al.* L-glutamine supplementation of a high fat diet reduces body weight and attenuates hyperglycemia and hyperinsulinemia in C57BL/6J mice. *J Nutr* 1996; 126: 273–279.
39. Samocho-Bonet D, Chisholm DJ, Gribble FM, *et al.* Glycemic effects and safety of L-Glutamine supplementation with or without sitagliptin in type 2 diabetes patients – a randomized study. *PLoS One* 2014; 9: e113366.
40. Reimann F, Williams L, da Silva XG, *et al.* Glutamine potently stimulates glucagon-like peptide-1 secretion from GLUTag cells. *Diabetologia* 2004; 47: 1592–1601.