Glutamate is an essential mediator in glutamine-amplified insulin secretion

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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 b-cells MIN6-K8, wild-type B6 mouse islets, glutamate dehydrogenase (GDH) knockout clonal b-cells (Glud1KOCL), and glutamate-oxaloacetate transaminase 1 (GOT1) knockout clonal b-cells (Got1KOCL) 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 Ca2+ concentration ([Ca2+]i) was also measured.

Results: Glutamine dose-dependently amplified insulin secretion in the presence of high glucose in both MIN6-K8 cells and Glud1KOCL. 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 [Ca2+]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 [Ca2+]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 b-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 b-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 inputs1,2. Glucose is the primary stimulator of insulin secretion from pancreatic b-cells, and a series of intracellular events is involved: inhibition of ATP-sensitive K+ (KATP) channels through the production of ATP by glucose metabolism induces membrane depolarization, which leads to opening of the voltage-dependent Ca2+ channels and influx of extracellular Ca2+, thereby triggering insulin secretion3. Glucose-induced insulin secretion (GICS) 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,
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 charge, which is possibly K<sub>ATP</sub> channel-dependent action. The effect of glutamine on insulin secretion has long been investigated. However, most of these studies used a combination with leucine in their examinations. 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 fuel.

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. 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 neurons. 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 secretion. 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 secretion. The circulation level of glutamine is ~0.6 mM in healthy subjects, but is lower in diabetic patients, 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<sup>2+</sup> 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 dimethylster hydrochloride (dm-Glu) was purchased from Tokyo Chemical Industry (Tokyo, Japan). [U-13C]-Glucose, [U-13C]-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-Kb and MIN6-m1 were previously reported. Clonal β-cell lines deficient for GDH (Gls<sup>−/−</sup>) were generated by CRISPR/Cas9 nickase system as described previously, using sgRNA pair purchased from GeneCopoeia (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, Nuaillé, France) and 1 mM sodium pyruvate at 37°C with 95% air and 5% CO<sub>2</sub>.

**Animal care**

Male C57BL/6Jcl mice were obtained from CLEA Japan (Tokyo Japan). Animals were maintained under specific pathogen-free conditions at 23 ± 2°C and 55 ± 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/6Jcl mice at 12 weeks of age were euthanized by sodium pentobarbital overdose. Pancreatic islets were isolated by collagenase digestion method as described previously. Isolated islets were cultured in RPMI-1640 medium (Sigma-Aldrich) at 37°C with 95% air and 5% CO<sub>2</sub>.

**Insulin secretion**

Insulin secretion experiments were performed as described previously. 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 GtIKOβCL 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 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, and analyzed by LCMS-8060 (Shimadzu, Kyoto, Japan) and CE7100-G6224A TOFMS system (Agilent, Santa Clara, CA, USA) as described previously.31,12

**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 ΔCT and normalized to endogenous Gapdh as internal control. Probe details: Gls, Mm 01257297_m1; Glk2, 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, Millpore, 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, Llitle Chalfont, UK) and detected by Image Quant LAS 4000 mini (GE Healthcare).

**Ca²⁺ assay**

Cells were loaded with 5 μM 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’s, Tukey’s, or Sidak’s test as indicated in the figure legends using GraphPad Prism Version 8.4.2. Data are presented as means ± 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 (Glud1KOβCL) from MIN6-K8 cells by genome editing using the CRISPR/Cas9 system, 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 GDH, to confirm the functional deficiency of GDH enzymes. BCH stimulated insulin secretion at 2.8 mM glucose in MIN6-K8 cells but not in Glud1KOβCL (Figure 1c lower), indicating...
that GDH is functionally absent in Glud1KOβCL. We found that glutamine dose-dependently amplified GIIS in Glud1-KOβCL 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 glutamate-amplifying effect on GIIS by ~50%, and ~60%, respectively (Figure 1g). CB-839, a specific inhibitor of GLS2, 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 glycolysis. As glutamine-amplifying effect of GIIS in 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). These results indicate that elevation of GIIS 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 MIN6-K8 cells (Figure 2b lower right). These results indicate that 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. 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.

**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 changes 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 Glud1KOβCL (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 production from glutamine directly increases [Ca2+]i. In fact, glutaminase dimethylster (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.

**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+]i. 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 Glud1K0bCLs (G16 and G17) (n = 4 in lower). (d) Dose-dependent effect of glutamine on insulin secretion from Glud1K0bCL (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 ± 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-13C]-glucose in MIN6-K8 cells (left) and Got1K0bCL (right) (n = 3). Cells were stimulated with 2.8 mM or 8.8 mM [U-13C]-glucose for 30 min. M+2 to M+5, glutamate isotopomers. (b) Upper, a scheme of production of glutamate isotopomers from exogenous [U-13C]-glutamine in the cells. M+0 to M+5, glutamate isotopomers. GLS, glutaminase. Lower, cytosolic glutamate content derived from [U-13C]-glutamine in MIN6-K8 cells (left) and Got1K0bCL (right) (n = 3). Cells were stimulated with [U-13C]-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 ± SEM. **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).
In the present study, we demonstrate that glutamine amplifies GHS 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 \(\alpha\)-KG by GDH, which is activated by leucine and supplied to the TCA cycle as fuel to produce ATP, thereby stimulating insulin secretion\(^9\). Although it has been reported that the activity of GDH is inhibited at high glucose due to the elevated ATP level\(^9\), 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 \(\text{Glud1 KO}_{\text{βCL}}, \) indicating that glutamine amplifies insulin secretion at a high concentration of glucose through a different pathway from that via conversion to \(\alpha\)-KG. 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 identified\(^26\). 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 | Ca\(^{2+}\) influx-dependency of the glutamine-effect on [Ca\(^{2+}\)]. (a) Effect of nifedipine on rise in [Ca\(^{2+}\)], (left) and insulin secretion (right) in MIN6-K8 cells. (b) Effect of depletion of extracellular Ca\(^{2+}\) on [Ca\(^{2+}\)], (left) and amplifying effect of glutamine on insulin secretion (right). Cells were stimulated in normal KRB or Ca\(^{2+}\)-free KRB. (c) dm-Glu-induced increase in [Ca\(^{2+}\)] and insulin secretion were almost completely abolished when cells were stimulated with Ca\(^{2+}\)-free KRB. G, glucose. Values are means with ± 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.
converted to glutamate by glutaminase in the cytosol. 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 proteins. It has been reported that in rat pancreatic islets, GLS is expressed predominantly in $\alpha$-cells, while GLS2 is expressed predominantly in $\beta$-cells. Considering that GLS2 has lower $K_m$ for phosphate than that of GLS, glutaminase activity might well be retained at high glucose in $\beta$-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 action. 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 $\beta$-cells, 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. 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 $Ca^{2+}$ dynamics. 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.
without conversion to glutamate in certain conditions. Indeed, it
has been reported that the intracellular Ca\(^{2+}\) rise induced by 10 mM glutamate was not suppressed in the presence of 6-di-
azo-5-oxo-1-norleucine (DON), a glutaminase inhibitor, in
SUR1 (Abcc8) knockout islets\(^2\). In GLUTag cells, a clonal intestinal
L cell line, glutamate has been shown to evoke a rise in
[Ca\(^{2+}\)], and stimulate secretion of glucagon-like peptide-1\(^3\). This
effect was retained even in the presence of DON, indicating that
glutamine metabolism is not required\(^3\). Although the detailed
mechanism by which glutamine increases [Ca\(^{2+}\)], 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\(^{2+}\)], found in the second phase.

Interestingly, we find that glutamine enhanced insulin secre-
tion (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 addi-
tion, we find that insulin secretion induced by glibenclamide is
markedly augmented in the presence of 2 mM glutamine (Fig-
ure 5a), suggesting that glutamine might enhance the effective-
ness of sulfonylureas. Given that glutamine is the most abundant
amino acids in the circulation\(^1,3\), 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 supple-
mentation of glutamine has been shown to reduce body weight
and attenuate hyperglycemia in mice fed a high fat diet\(^3\) and to
improve glucose tolerance in combination with sitagliptin
treatment in T2DM patients\(^39\). Together with our current data,
these findings suggest that glutamine supplementation might well
ameliorate β-cell function as well as increase incretin secretion
from intestine\(^40\) to ameliorate glucose intolerance.

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DISCLOSURE
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