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Glutamine-induced signaling pathways via amino acid receptors in enteroendocrine L cell lines

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Abstract

Glucagon-like peptide-1 (GLP-1), secreted by gastrointestinal enteroendocrine L cells, induces insulin secretion and is important for glucose homeostasis. GLP-1 secretion is induced by various luminal nutrients, including amino acids. Intracellular Ca^{2+} and cAMP dynamics play an important role in GLP-1 secretion regulation; however, several aspects of the underlying mechanism of amino acid-induced GLP-1 secretion are not well characterized. We investigated the mechanisms underlying the L-glutamine-induced increase in Ca^{2+} and cAMP intracellular concentrations ($[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$, respectively) in murine enteroendocrine L cell line GLUTag cells. Application of L-glutamine to cells under low extracellular $[\text{Na}^+]$ conditions, which inhibited the function of the sodium-coupled L-glutamine transporter, did not induce an increase in $[\text{Ca}^{2+}]_i$. Application of G protein-coupled receptor family C group 6 member A and calcium-sensing receptor antagonist showed little effect on $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$; however, taste receptor type 1 member 3 (TAS1R3) antagonist suppressed the increase in $[\text{cAMP}]_i$. To elucidate the function of TAS1R3, which forms a heterodimeric umami receptor with taste receptor type 1 member 1 (TAS1R1), we generated TAS1R1 and TAS1R3 mutant GLUTag cells using the CRISPR/Cas9 system. TAS1R1 mutant GLUTag cells exhibited L-glutamine-induced increase in $[\text{cAMP}]_i$, whereas some TAS1R3 mutant GLUTag cells did not exhibit L-glutamine-induced increase in $[\text{cAMP}]_i$ and GLP-1 secretion. These findings suggest that TAS1R3 is important for L-glutamine-induced increase in $[\text{cAMP}]_i$ and GLP-1 secretion. Thus, TAS1R3 may be coupled with G_s and related to cAMP regulation.

Key Words

- ▶ cell signaling
- ▶ G protein-coupled receptor
- ▶ calcium
- ▶ cyclic AMP (cAMP)
- ▶ hormone
- ▶ CRISPR/Cas9

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Introduction

Glucagon-like peptide-1 (GLP-1) is a member of the incretin family and is secreted by the gastrointestinal endocrine L cells (Harada *et al.* 2017, 2018a,b, Drucker 2018). GLP-1 regulates the increase in insulin secretion from the pancreatic β cells (Lamont *et al.* 2012, Smith *et al.* 2014)

and suppresses glucagon secretion from the pancreatic α cells, which helps control blood glucose concentration (de Heer *et al.* 2008, Richards *et al.* 2014). Thus, GLP-1 receptor agonists and GLP-1 protease inhibitors are used for achieving glycemic control, especially in patients

with type 2 diabetes mellitus (T2DM) (Eng *et al.* 1992, Villhauer *et al.* 2003, Drucker *et al.* 2017). Indeed, a genome-wide association study identified a significant missense variant of GLP-1 receptor in patients with T2DM ($P=6.10\times 10^{-14}$, 36,614 cases and 155,150 controls of Japanese ancestry) (Suzuki *et al.* 2019). Furthermore, neural transmission initiated by GLP-1 signaling reduces food intake (Rodríguez de Fonseca *et al.* 2000, Hsu *et al.* 2018). Despite the importance of GLP-1 for appetite regulation and treatment of T2DM, the precise mechanism of GLP-1 secretion remains unclear.

Recent studies have shown that luminal nutrients (including glucose, fatty acids, and amino acids) induce GLP-1 secretion from the L cells (Tolhurst *et al.* 2012, Feng *et al.* 2013, Oya *et al.* 2013). Glucose-induced GLP-1 secretion depends on the depolarization triggered by Na^+ influx via the sodium-dependent glucose transporter-1 (SGLT-1) and K^+ efflux via ATP-sensitive K^+ (K_{ATP}) channels (Reimann *et al.* 2008). The depolarization triggers the opening of voltage-gated Ca^{2+} channels and induces GLP-1 secretion (Gribble *et al.* 2003, Reimann *et al.* 2008, Diakogiannaki *et al.* 2012). Indeed, the glucose-induced Ca^{2+} response and GLP-1 secretion are impaired in *Sglt-1*-knockout mice. The sweet receptor is another regulator of glucose-induced GLP-1 secretion (Margolskee *et al.* 2007). This receptor is a heterodimer composed of taste receptor type 1 member 2 (TAS1R2) and member 3 (TAS1R3). Fatty acid-induced GLP-1 secretion is regulated by the pathways of G protein-coupled receptors (GPCRs) (Janssen & Depoortere 2013). Increase in the intracellular concentrations of Ca^{2+} ($[\text{Ca}^{2+}]_i$) and cAMP ($[\text{cAMP}]_i$) triggered via G_q proteins and G_s proteins, respectively, is the key mechanism of nutrient-induced GLP-1 secretion.

A recent study investigated the effect of various amino acids on GLP-1 secretion using mouse primary cultured small intestinal cells; the authors found that L-glutamine had the greatest effect GLP-1 secretion (Tolhurst *et al.* 2011). However, the precise molecular mechanism of L-glutamine-induced GLP-1 secretion remains unclear. In the present study, we used GLUTag cell lines derived from mouse gastrointestinal endocrine L cells and found that L-glutamine, among various other amino acids, increased both $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$, and induced GLP-1 secretion.

To elucidate the molecular mechanisms of L-glutamine-induced GLP-1 secretion, we first examined the contribution of sodium-coupled L-glutamine transporters. We found that the L-glutamine-induced $[\text{Ca}^{2+}]_i$ increase was suppressed. We next investigated the relationship between L-glutamine-induced GLP-1 secretion and amino acid-sensitive GPCRs

(Wellendorph *et al.* 2009), such as G protein-coupled receptor class C group 6 member A (GPC6A), calcium-sensing receptor (CaSR), TAS1R1, and TAS1R3. We found no significant involvement of GPCR6A and CaSR in the increase of $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$. Furthermore, a TAS1R3 antagonist partially suppressed the increase in $[\text{cAMP}]_i$, suggesting the role of an unknown signaling pathway downstream of TAS1R3. To confirm the contribution of TAS1R3 on $[\text{cAMP}]_i$ elevation, we generated and analyzed TAS1R3-homozygous mutant GLUTag cells using the CRISPR/Cas9 system. Two of the three TAS1R3 mutant GLUTag cell lines showed no significant L-glutamine-induced $[\text{cAMP}]_i$ elevation. These findings indicate that L-glutamine-induced GLP-1 secretion from GLUTag cells was triggered by the increase in intracellular cAMP via TAS1R3.

Materials and methods

Chemicals

L-glutamine, L-asparagine monohydrate, L(-)-phenylalanine, L-leucine, and L-arginine were purchased from Nacalai Tesque (Kyoto, Japan). L-glutamate potassium monohydrate, L-alanine, L-tryptophan, NPS-2143, and lactisole were purchased from Sigma-Aldrich. Glycine, L(+)-lysine monohydrochloride, forskolin (Fsk), and BSA were purchased from FUJIFILM Wako Pure Chemical Institute (Osaka, Japan).

Cell culture and transfection

GLUTag cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 1 g/L glucose, L-glutamine, sodium pyruvate, 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Sigma-Aldrich), at 37°C in an atmosphere of 5% CO_2 . For imaging experiments, the cells were trypsinized and $1\text{--}1.5\times 10^5$ cells were added to poly-L-lysine (Sigma-Aldrich)-coated glass coverslips in 35 mm dishes. Two days after plating, the cells were transfected with 1.5 μg of Flamindo2 plasmid using 2 μL of X-treme GENE HP DNA Transfection Reagent (Roche) according to the manufacturer's protocol. The medium was exchanged 8 h after transfection and the cells were cultured at $30\text{--}32^\circ\text{C}$ for 2 days until imaging. For establishment of mutant GLUTag cell lines, 2.5×10^5 cells were plated in the wells of a six-well plate 1 day prior to transfection. The cells were transfected with 1.5 μg of single guide RNA-*Streptococcus pyogenes* Cas9-green fluorescent protein (sgRNA-SpCas9-GFP)

all-in-one vectors using 3.0 μ L of Lipofectamine 2000 (Thermo Fisher Scientific).

RNA isolation and RT-PCR analysis

Total RNA from GLUTag cells and mouse brain was isolated using the RNeasy Mini Kit (QIAGEN). After DNase treatment using RNase-Free DNase Set (QIAGEN), cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). The synthesized cDNA was amplified using the EmeraldAmp PCR Master Mix (TaKaRa Bio). For PCR amplification of *Casr* (NM_013803), the forward primer 5'-AGCAGGTGACCTTCGATGAGT-3' and the reverse primer 5'-ACTTCCTTGAACACAATGGAGC-3' were used. For *Gprc6a* (NM_153071), the forward primer 5'-CGGGATCCAGACGACCACAAATCCAG-3' and the reverse primer 5'-CCAAGCTTGATTCATAACTCACC TGTGGC-3' were used. For *Tas1r1* (NM_031867), the forward primer 5'-GCAGAGAGATCTTCGCAACCA-3' and the reverse primer 5'-TACTTATCGCTGGGGATGGTG-3' were used. For *Tas1r3* (NM_031872), the forward primer 5'-GAACATGTGATGGGGCAACG-3' and the reverse primer 5'-GTAGGGTGTGTGAAGGGCT-3' were used. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NM_001289726), the forward primer 5'-CCGGTGCTG AGTATGTCGTGGAGTCTAC-3' and the reverse primer 5'-CTTCCAGAGGGGCCATCCACAGTCTTC-3' were used.

Visualization of $[Ca^{2+}]_i$ and cAMP dynamics

For Ca^{2+} imaging, GLUTag cells plated on coverslips for 2 days were loaded with 2.5 μ M Fluo-3 AM (Dojindo, Kumamoto, Japan) in modified Ringer Buffer (RB: 140 mM NaCl, 3.5 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, 2 mM $NaHCO_3$) containing 5 mM glucose. After incubation for 20 min at 37°C in an atmosphere of 5% CO_2 , the cells were washed twice, added to RB containing 0.1 mM glucose, and mounted on a stage maintained at 37°C. For cAMP imaging, GLUTag cells expressing Flamingo2 were washed and imaged in RB.

Imaging was performed using an inverted microscope (ECLIPSE Ti-E; Nikon) equipped with an oil-immersion objective lens (CFI Plan Fluor, 40 $\times$, numerical aperture=1.30; Nikon) and an EM-CCD camera (iXon, Andor, Belfast, UK), whose exposure was controlled by MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Images were acquired using a mercury lamp (Nikon) using a filter set with 465–495 nm excitation filter, 505 nm dichroic mirror, and 515–555 nm emission filter (Nikon). Images were acquired every 5 s for 10 min, and L-glutamine

stimulation was performed with a pipette 180 s after the initiation of image acquisition. High $[K^+]$ treatment was performed by perfusing a solution containing 83.6 mM NaCl, 50 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, 2 mM $NaHCO_3$, and 0.1 mM glucose.

The acquired images were aligned using 'Stackreg' plugin in ImageJ (National Institutes of Health), and the fluorescence intensity of the cells was quantified using the MetaMorph software. The fluorescence intensity in the background of acquired images was calculated as zero. After subtracting the background, basal fluorescence intensity, normalized to 100%, was calculated as the average fluorescence intensity between 150 and 180 s after initiation of image acquisition. For Ca^{2+} imaging, cells which showed excessive oscillation by over 40% of basal fluorescence intensity (before stimulation) were excluded from further analysis, and the peak amplitude of normalized fluorescence intensity after stimulation was compared. For cAMP imaging, cells that constantly exhibited an increase or decrease in fluorescence intensity before stimulation were excluded from further analysis. To distinguish between transient decrease in fluorescence intensity caused by pH changes and long-lasting decrease in fluorescence intensity caused by cAMP elevation, the area under the curve (AUC) was calculated as the summary of normalized fluorescence intensity between 185 and 600 s after the initiation of image acquisition. To assess whether the basal fluorescence intensity of Fluo3 and Flamingo2 affects the kinetics of the indicators, we investigated the correlation between the basal fluorescence intensity and the response to L-glutamine and found no significant correlation (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article).

Enzyme-linked immunosorbent assay

GLUTag cells were plated in six-well plates at 0.5×10^6 cells per well. Two days after plating, cells were washed twice with RB containing 0.1 mM glucose and 0.5% (w/v) BSA (FUJIFILM Wako Pure Chemical Institute). The washed cells were incubated in 1 mL of RB with 5 mM glucose, 0.5% (w/v) BSA, 1% (v/v) dipeptidyl peptidase 4 inhibitor (Merck Millipore), and 500 μ M L-glutamine for 3 h at 37°C in an atmosphere of 5% CO_2 . After centrifugation at 1000 g for 10 min at 4°C, the amount of GLP-1 in the supernatant was measured using the Glucagon-Like Peptide-1 (Active) ELISA Kit (Merck Millipore) and a microplate reader (Varioskan LUX; Thermo Fisher Scientific).

Inhibition experiments

Low $[Na^+]$ treatment was performed using a solution containing 140 mM N-methyl-D-glucamine, 3.5 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, 2 mM $NaHCO_3$, and 0.1 mM glucose. BIM-46187 was applied at 25 μ M in serum-free medium for 2 h prior to imaging. NPS-2143 was applied simultaneously with glutamine at 3 μ M. Lactisole was applied at 3 mM in RB for 30 min before the experiments, and constantly applied in the RB during imaging. The same concentration of 0.1% (v/v) dimethyl sulfoxide was added in the control RB upon application of NPS-2143 and lactisole.

Plasmid construction

We generated the sgRNA-SpCas9-GFP all-in-one vectors using pSpCas9(BB)-2A-GFP (PX458) plasmid, as described in our previous work (Nakamura *et al.* 2018). The sequence of inserted sgRNA was as follows: the forward primer, 5'-caccgCGTCATACAGTTCATACCCC-3'; the reverse primer, 5'-aaacGGGTATGAACTGTATGACGc-3' for *Tas1r1* and the forward primer, 5'-caccgTCTAGTCTGGCCAATGCACG-3'; the reverse primer, 5'-aaacCGTGCATTGGCCAGACTAGAc-3' for *Tas1r3*. The genome cleavage activity of the vectors was validated by the Surveyor assay using the Surveyor Mutation Detection Kit (Integrated DNA Technologies, Coralville, IA, USA). PCR fragments were amplified by the PCR conditions shown in Supplementary Table 1A using TaKaRa LA Taq (TaKaRa Bio).

Preparation of conditioned culture medium

The supernatant of the medium used to grow confluent GLUTag cells was collected and centrifuged for 5 min at 500 g to exclude contaminants, followed by sterilization using a 0.22- μ m filter (Merck Millipore). The sterilized supernatant was diluted 10-fold using the culture medium.

Establishment of TAS1R1- or TAS1R3- Δ C GLUTag cells using CRISPR/Cas9

We previously reported the detailed protocol for establishing mutant cell lines using the CRISPR/Cas9 system (Nakamura *et al.* 2018). Briefly, the transfected cells in 6-well plates were trypsinized and collected by a suspension buffer for fluorescence-activated cell sorting in 2% FBS, 0.02 M glucose, and 1 $\times$ penicillin and streptomycin diluted with 1 $\times$ phosphate buffered saline. The collected

cells were exposed to 7-amino actinomycin D (7-AAD; BD Biosciences, San Jose, CA, USA) to distinguish dead cells (5 μ L per 1.0×10^6 cells). GFP (+) and 7-AAD (–) GLUTag cells were single cell-sorted into wells of 96-well plates containing 200 μ L of conditioned culture medium using a FACSaria apparatus (BD Biosciences).

After single cell sorting (day 0), the plates were placed in an incubator (37°C and 5% CO_2) and incubated statically until day 5. At that time, growth of a few cells was evident. One hundred microliters of conditioned medium were added to each well. The medium was changed every 4–5 days until adequate growth of the colonies. On day 20, the colonies were split by pipetting and transferred to new 96-well plates. Upon achieving confluent growth, the cells were transferred to wells of a 24-well plate for scale-up and to wells of a 96-well plate for genotyping. Each clone was grown to confluence in 10 cm dish and frozen with 1 mL of Cell Banker 1 Plus (NIPPON ZENYAKU KOGYO, Fukushima, Japan).

Crude PCR for genotyping of mutant GLUTag cells

GLUTag cells cultured in wells of 96-well plates were exposed to 50 μ L of lysis buffer for crude PCR (10 mM Tris-HCl (pH 8.0), 0.1% (v/v) Triton-X, 10 mM EDTA, and 1/100 diluted proteinase K) at 37°C overnight. One microliter aliquots of the ten-fold diluted crude cell lysates were added to 10 μ L scale of PCR mix containing LA Taq polymerase (TaKaRa Bio). PCR fragments were amplified by the same conditions used for the Surveyor assay and sequenced using primer No.1 (*Tas1r3*) and primer No.3 (*Tas1r3*) (Supplementary Table 1A). Off-target mutation analyses were performed using the PCR conditions shown in Supplementary Table 1B using PrimeSTAR GXL DNA polymerase (TaKaRa Bio). We extracted genome DNA of the mutant GLUTag cells using DNeasy Blood & Tissue Kits (QIAGEN), according to manufacturer's protocol, to validate the insertion of Cas9 plasmid. The primers used to amplify GFP region were as follows: forward primer, 5'-GACGACGGCAACTACAAGAC-3'; reverse primer, 5'-GTGCTCAGGTAGTGGTTGTC-3'.

Owing to the detection of GFP bands in several lines, we assessed those cells for the presence or absence of GFP fluorescence. Three days after plating onto glass coverslips, the cells were examined with an inverted microscope (IX-71, Olympus) equipped with an objective lens (UPlanApo, 20 $\times$, NA=0.70, Olympus) and an EM-CCD camera (Evolve, Photometrics, Tucson, AZ, US). Images were acquired using a xenon lamp, 460–495 nm excitation filter, 505 nm dichroic mirror, and 510–555 nm emission filter

(Olympus). The exposure time of the EM-CCD camera was controlled by the MetaMorph software.

As positive control, cells were transfected with 1.5 µg of pEGFP-C1 or 3 µg of pSpCas9(BB)-2A-GFP plasmids using 3 µL of Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) 2 days after plating, as recommended by the manufacturer. The medium was exchanged 8 h after transfection and the cells were cultured for 1 day until imaging.

Statistical analyses

Between-group differences were assessed using the two-sided Welch's *t* test with Bonferroni correction or one-way ANOVA with Dunnett or Tukey's multiple comparison test. The GraphPad Prism 6 software (GraphPad Software) was used for all statistical analyses.

Results

L-glutamine increases both intracellular Ca^{2+} and cAMP levels, and induces GLP-1 secretion

We first examined the effect of exposure of GLUTag cells to various amino acids (500 µM) on the $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$ levels. Live cell imaging analysis was performed using the Ca^{2+} -sensitive dye, Fluo3, and the genetically encoded cAMP indicator, Flamindo2 (Odaka *et al.* 2014). Flamindo2 is an intensimetric cAMP indicator; it exhibits a decrease in fluorescence intensity upon binding to cAMP. A previous study has used the FRET-based cAMP indicator epac2-camps in GLUTag cells (Friedlander *et al.* 2011). The basal CFP/YFP ratio was approximately 1.0, which corresponds to 400 nM of cAMP (Nikolaev *et al.* 2004). The EC_{50} value of Flamindo2 is 3.2 µM; therefore, it is appropriate for monitoring L-glutamine-induced increase in cAMP level in GLUTag cells. L-glutamine, L-asparagine, L-alanine, L-glycine, and L-arginine induced an increase in the fluorescence intensity of Fluo3 (Fig. 1A). Only L-glutamine induced a decrease in the area under curve (AUC) of the 10-min kinetics in the fluorescence intensity of Flamindo2, whereas L-glutamate, L-arginine, and L-lysine increased the AUC (Fig. 1B). Because the fluorescence intensity of Flamindo2 decreases upon binding to cAMP, the data indicated that L-glutamine increases $[\text{cAMP}]_i$, whereas L-glutamate, L-arginine, and L-lysine decrease $[\text{cAMP}]_i$. Based on these results, we concluded that L-glutamine increased both $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$ in GLUTag cells. Higher concentration of L-glutamine induced a greater increase in $[\text{Ca}^{2+}]_i$

and $[\text{cAMP}]_i$ levels (Supplementary Fig. 2). As compared to positive controls (high $[\text{K}^+]$ solution which induces membrane depolarization for Ca^{2+} , and 10 µM of adenylyl cyclase activator forskolin (Fsk) for cAMP), L-glutamine induced a weaker response (Supplementary Fig. 3). To validate whether L-glutamine induces GLP-1 secretion, we quantified GLP-1 after L-glutamine treatment using ELISA. L-glutamine significantly increased the amount of GLP-1 secreted by GLUTag cells (Fig. 1C).

Sodium-coupled L-glutamine transporter and amino acid-sensitive GPCRs are involved in L-glutamine-induced increase in $[\text{Ca}^{2+}]_i$

Several putative Ca^{2+} sources have been implicated in L-glutamine-induced GLP-1 secretion. Uptake of L-glutamine by the cytosol together with Na^+ via sodium-coupled L-glutamine transporters causes an increase in intracellular Na^+ levels, which induces membrane depolarization and Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs). To examine the involvement of sodium-coupled L-glutamine transporters, we applied L-glutamine to GLUTag cells in a solution containing low $[\text{Na}^+]$. Treatment with low $[\text{Na}^+]$ significantly suppressed the L-glutamine-induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 1D and E). However, $[\text{cAMP}]_i$ increase was not inhibited but enhanced by treatment with low $[\text{Na}^+]$ (Fig. 1F and G); this was due to the lower recovery of the Flamindo2 signal in the low $[\text{Na}^+]$ condition after 400 s. We speculated that the low $[\text{Na}^+]$ condition did not alter $[\text{cAMP}]_i$ generation but did alter cAMP turnover. These results suggested the contribution of sodium-coupled L-glutamine transporters to the L-glutamine-induced increase in $[\text{Ca}^{2+}]_i$ level.

In addition to sodium-coupled L-glutamine transporters, L-glutamine can activate amino acid-sensitive GPCRs and can mobilize Ca^{2+} from the endoplasmic reticulum via the G_q signaling pathway. We thus examined the expression profile of putative amino acid-sensitive GPCRs. Reverse-transcription PCR (RT-PCR) was performed to assess the mRNA expressions of *Casr*, *Gprc6a*, *Tas1r1*, and *Tas1r3* (Supplementary Fig. 4A). First, we applied the pan-G protein inhibitor, BIM-46187, with L-glutamine (Ayoub *et al.* 2009) and found that BIM-46187 significantly suppressed the L-glutamine-induced increase in both $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$ levels (Supplementary Fig. 4B, C, D and E). We next utilized NPS-2143 (an antagonist for CaSR and GPRC6A) with L-glutamine. However, NPS-2143 did not suppress the L-glutamine-induced increase in $[\text{Ca}^{2+}]_i$ but showed a mild effect on $[\text{cAMP}]_i$ (Fig. 2A, B, C and D). These findings suggested that the

Figure 1

Effect of L-glutamine on intracellular Ca^{2+} and cAMP dynamics in GLUTag cells and the involvement of sodium-dependent L-glutamine transporters. (A) Peak amplitude calculated from the fluorescence intensity (FI) of Fluo3 after application of various amino acids; $N \geq 17$ cells from ≥ 3 independent experiments. One-way ANOVA and Dunnett's multiple comparison test. (B) Area under the curve (AUC) calculated from the FI of Flamindo2 using various amino acids; $N \geq 17$ cells from ≥ 3 independent experiments. One-way ANOVA and Dunnett's multiple comparison test. (C) Results of ELISA showing the amount of GLP-1 secreted by GLUTag cells after application of 500 μ M L-glutamine. The data are from seven trials from two experiments; Welch's t test. (D) Typical time course of FI of Fluo3 during application of 500 μ M L-glutamine in low $[Na^+]$ -containing solution. (E) Peak amplitude calculated from the FI of Fluo3 by application of 500 μ M L-glutamine in low $[Na^+]$ -containing solution; $N \geq 23$ cells from three independent experiments; Welch's t test. (F) Typical time course of FI of Flamindo2 during application of 500 μ M L-glutamine in low $[Na^+]$ -containing solution. (G) Area under curve (AUC) calculated from the FI of Flamindo2 by application of 500 μ M L-glutamine in low $[Na^+]$ -containing solution; $N \geq 17$ cells from three independent experiments; Welch's t test. Data presented as mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

L-glutamine-induced increase in $[Ca^{2+}]_i$ was independent of G_q -coupled CaSR and GPRC6A.

TAS1R3 is involved in L-glutamine-induced cAMP production

TAS1R1 and TAS1R3 act as a heterodimer and form the umami receptor. To investigate the role of TAS1R1 and TAS1R3, GLUTag cells were treated with lactisole. This TAS1R3 inhibitor was recently shown to inhibit human and murine TAS1R3 (Hamano *et al.* 2015). Interestingly, lactisole had little effect on the $[Ca^{2+}]_i$ but significantly suppressed L-glutamine-induced increase in $[cAMP]_i$

(Fig. 2E, F, G and H). Because the umami receptor is coupled with gustducin, which inhibits cAMP production, this result suggested the existence of an uncanonical signaling pathway via TAS1R1 or TAS1R3.

To explore this, we established homozygous TAS1R1- or TAS1R3- C terminal deletion (Δ) GLUTag cell lines using CRISPR/Cas9 (Fig. 3). First, we checked the insertion of the Cas9-GFP plasmid into genome DNA to exclude the possibility of contamination of Fluo3 fluorescence by the GFP fluorescence of the plasmid. We amplified the EGFP fragment by PCR using genomic DNA derived from each mutant clone and detected amplification of EGFP bands in several clones (Supplementary Fig. 5A). However,

Figure 2

Role of CaSR and GPRC6A, and TAS1R1 or TAS1R3 in L-glutamine-induced increase in $[Ca^{2+}]_i$ and $[cAMP]_i$. (A) Typical time course of fluorescence intensity (FI) of Fluo3 during application of 500 μM L-glutamine with 3 μM NPS-2143. (B) Peak amplitude calculated from the FI of Fluo3 by application of 500 μM L-glutamine with 3 μM NPS-2143; $N \geq 30$ cells from three independent experiments; Welch's *t* test. (C) Typical time course of FI of Flamingo2 during application of 500 μM L-glutamine with 3 μM NPS-2143. (D) Area under curve (AUC) calculated from the FI of Flamingo2 by application of 500 μM L-glutamine with 3 μM NPS-2143. $N \geq 13$ cells from three independent experiments; Welch's *t* test. (E) Typical time course of FI of Fluo3 during application of 500 μM L-glutamine with 3 mM lactisole. (F) Peak amplitude calculated from the FI of Fluo3 by application of 500 μM L-glutamine with 3 mM lactisole; $N \geq 26$ cells from three independent experiments; Welch's *t* test. (G) Typical time course of FI of Flamingo2 during application of 500 μM L-glutamine with 3 mM lactisole. (H) Area under curve (AUC) calculated from the FI of Flamingo2 by application of 500 μM L-glutamine with 3 mM lactisole; $N \geq 22$ cells from ≥ 3 independent experiments; Welch's *t* test. Data presented as mean $\pm$ S.E.M. N.S., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

we did not find any EGFP signals in any of the clones that showed EGFP bands by imaging analysis (Supplementary Fig. 5B). We thus investigated L-glutamine-induced GLP-1 secretion by TAS1R1- or TAS1R3-ΔC GLUTag cells using ELISA. L-glutamine-induced GLP-1 secretion was significantly lower in TAS1R3-ΔC GLUTag cells (Fig. 4A). However, mutant TAS1R1 had no effect on L-glutamine-induced GLP-1 secretion.

Next, we investigated L-glutamine-induced increase in $[Ca^{2+}]_i$ and $[cAMP]_i$ using other additional TAS1R1- or TAS1R3-ΔC GLUTag cell lines (Supplementary Figs 6 and 7). As expected, L-glutamine induced an increase in both $[Ca^{2+}]_i$ and $[cAMP]_i$ in TAS1R1-ΔC GLUTag cell lines (Fig. 4B, C and Supplementary Fig. 8). Of note, the $[Ca^{2+}]_i$ elevation was impaired in one TAS1R3-ΔC cell line (#2), which was inconsistent with the result of lactisole treatment. The $[cAMP]_i$ elevation was impaired in two of the three TAS1R3-ΔC GLUTag cell lines (#1 and #2). These findings suggested that TAS1R3 is important for the $[cAMP]_i$ increase, which is consistent with the results of lactisole treatment; however, the dynamics of $[Ca^{2+}]_i$ and $[cAMP]_i$ were different in each TAS1R3-ΔC cell line.

Discussion

We demonstrated the mechanisms of the L-glutamine-induced increase in $[Ca^{2+}]_i$ and $[cAMP]_i$ related to GLP-1 secretion (Fig. 5). L-glutamine induced GLP-1 secretion from GLUTag cells and triggered an increase in both

Figure 3

Establishment of homozygous TAS1R1- and TAS1R3-ΔC GLUTag cell lines. (A and B) Genome sequences of TAS1R1- (A) and TAS1R3- (B) ΔC GLUTag cells. PAM, protospacer adjacent motif sequence; sgRNA, single guide RNA; bp, base pairs; del, deletion; ins, insertion.

Figure 4

Examination of the involvement of TAS1R1 and TAS1R3 in GLP-1 secretion using homozygous TAS1R1- or TAS1R3-ΔC GLUTag cells. (A) Results of ELISA showing fold-change of secreted GLP-1 in genome-edited GLUTag cells after the application of 500 μM L-glutamine. The data are from seven trials from two experiments; One-way ANOVA multiple comparison test. (B) Peak amplitude calculated from the fluorescence intensity (FI) of Fluo3 by 500 μM L-glutamine in control, TAS1R1-ΔC, and TAS1R3-ΔC GLUTag cells; $N \geq 20$ cell from ≥ 4 independent experiments. Welch's *t* test. The *P* values were calculated by Bonferroni correction. (C) Area under curve (AUC) calculated from the FI of Flamingo2 by application of 500 μM L-glutamine in control, TAS1R1-ΔC, and TAS1R3-ΔC GLUTag cells; $N \geq 13$ cells from ≥ 4 independent experiments; Welch's *t* test. The *P* values were calculated by Bonferroni correction. Data presented as mean $\pm$ S.E.M. N.S., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

$[Ca^{2+}]_i$ and $[cAMP]_i$. We suggest that sodium-coupled L-glutamine transporters regulate the L-glutamine-induced increase in $[Ca^{2+}]_i$ via VDCCs following Na^+ influx. The pharmacological experiments revealed that

the L-glutamine-induced increase in $[Ca^{2+}]_i$ and $[cAMP]_i$ levels was regulated via amino acid-sensitive GPCRs including TAS1R3.

In a previous study, human TAS1R3 were shown to exhibit sensitivity to lactisole in contrast to murine TAS1R3. This was attributable to specific amino acid residues in mouse and rat TAS1R3 that are critical for lactisole insensitivity; when those residues are mutated to human ones, they acquire lactisole sensitivity (Jiang *et al.* 2005). Another animal study showed that lactisole has little effect on sweet receptor, a heterodimer of TAS1R2 and TAS1R3 (Sclafani & Pérez 1997). In this study, even higher concentration of lactisole had little effect on sucrose preference in rats. Conversely, although the sensitivity of murine TAS1R3 to lactisole was less than that of human TAS1R3, murine TAS1R3 was sensitive to high concentration of lactisole (Hamano *et al.* 2015). Furthermore, the present study provides evidence of the sensitivity of murine TAS1R3 to lactisole.

We speculate that the mouse TAS1R3 is insensitive to lactisole when combined with TAS1R2, typically in the tongue. Jiang *et al.* and Winning *et al.* found no inhibitory effect of lactisole on HEK293 cells with equal expressions of murine TAS1R2 and TAS1R3 (Jiang *et al.* 2005, Winnig *et al.* 2005). Sclafani *et al.* also showed the lack of sensitivity of the sweet receptor to lactisole (Sclafani & Pérez 1997). On the other hand, Hamano *et al.* (Hamano *et al.* 2015) and the present study used the pancreatic β cell line MIN6 and GLUTag cells, respectively, which dominantly express endogenous murine TAS1R3 compared to TAS1R2.

Figure 5

Schematic model of L-glutamine-induced secretion of GLP-1 by GLUTag cells. L-glutamine promotes the secretion of GLP-1 from GLUTag cells via elevation of $[Ca^{2+}]_i$ and $[cAMP]_i$ levels. Uptake of L-glutamine through sodium-dependent L-glutamine transporters causes membrane depolarization and induces Ca^{2+} influx via voltage-dependent Ca^{2+} channels (VDCCs). cAMP is produced by G_s signaling pathway via TAS1R3, which may act as a homodimer or a heterodimer with an unknown GPCR without conjugation to TAS1R1 as the umami receptor.

As mentioned below, TAS1R3 of MIN6 and GLUTag cells may form a homodimer or a heterodimer with other GPCRs and become at least partially sensitive to lactisole, unlike TAS1R3 in HEK293 cells and rats shown in the previous reports (Sclafani & Pérez 1997, Jiang *et al.* 2005, Winnig *et al.* 2005). Further studies are required to validate this hypothesis.

In a recent study, treatment of MIN6 cells with lactisole was shown to suppress artificial sweetener-induced increase in $[Ca^{2+}]_i$ level but not that of $[cAMP]_i$ (Hamano *et al.* 2015). However, we found that treatment of GLUTag cells with lactisole suppressed L-glutamate-induced increase in $[cAMP]_i$ but not that of $[Ca^{2+}]_i$ (Fig. 2). We speculate that MIN6 and GLUTag cells have variable dimerization patterns of TAS1R3. While Hamano *et al.* proposed that TAS1R3 forms a homodimer acting as a G_q -coupled sweet receptor, we speculate that TAS1R3 in GLUTag cells acts as a L-glutamine receptor, probably in combination with TAS1R3 itself or an unknown GPCR. The expression of *Tas1r3* in intestinal cells is reported to be higher than that of *Tas1r1* and *Tas1r2*, which form heterodimers with TAS1R3 (Reimann *et al.* 2008). Therefore, it is likely that TAS1R3 forms a homodimer or an unknown heterodimer that includes TAS1R3 coupled with G_s . Further investigation into the characteristics of TAS1R3, especially the dimerization kinetics, will help understand the significance of sweetener and amino acid receptors.

We observed that the increase in $[Ca^{2+}]_i$ was inhibited by treatment with low $[Na^+]_i$, but not by treatment with NPS-2143 (Figs 1D and 2A). These results suggest that sodium-coupled L-glutamine transporters play an important role in evoking the increase in $[Ca^{2+}]_i$. We also found that NPS-2143 and lactisole did not suppress basal $[Ca^{2+}]_i$ and $[cAMP]_i$, or the high K^+ -induced increase in $[Ca^{2+}]_i$ and Fsk-induced increase in $[cAMP]_i$, respectively; this suggests a specific inhibitory effect of lactisole on $[cAMP]_i$ (Supplementary Fig. 3).

We developed TAS1R1- and TAS1R3-ΔC GLUTag cells using the CRISPR/Cas9 system and found that the increase in $[Ca^{2+}]_i$ and $[cAMP]_i$ were not impaired in TAS1R1-ΔC GLUTag cells (Fig. 4B). TAS1R1 and TAS1R3 form a heterodimer (umami receptor) coupled with gustducin, which decreases $[cAMP]_i$. Because the $[cAMP]_i$ dynamics of TAS1R1-ΔC GLUTag cells were not impaired, we suggest that the umami receptor is not important for the L-glutamine-induced increase in $[cAMP]_i$. On the other hand, two of the three TAS1R3-ΔC GLUTag cell lines did not exhibit an increase in $[cAMP]_i$ (Fig. 4C). Therefore, we suggest that TAS1R3 is important for the

L-glutamine-induced increase in $[cAMP]_i$ and that a homodimer of TAS1R3 or a heterodimer of TAS1R3 and an unknown receptor might be coupled with G_s protein. However, one of the three TAS1R3-ΔC cell lines exhibited cAMP response to L-glutamine; this suggests the existence of alternative mechanisms of increase in $[cAMP]_i$ in GLUTag cells, which are independent of TAS1R3.

The TAS1R3-ΔC GLUTag cell line in the present study displayed varied $[Ca^{2+}]_i$ and $[cAMP]_i$ responses to L-glutamine stimulation. Assuming that this variability may be attributable to off-target mutations due to CRISPR/Cas9 system, we investigated the sequences of the top five candidate loci of off-target mutations predicted by gRNA design tools. However, we did not find any off-target mutations in TAS1R1- or TAS1R3-ΔC GLUTag cells (Supplementary Figs 9 and 10). The diversity may be attributable to other unexpected off-target mutations. We previously reported unexpected off-target mutations in a genome-edited mouse line generated by the CRISPR/Cas9 system (Nakajima *et al.* 2016). Furthermore, several cell lines that were cloned, displayed different characteristics owing to the single cell cloning step; this may be another possible reason for the diversity of TAS1R3-ΔC GLUTag cells. Further analyses are necessary using other cloned cell lines to validate the reproducibility of the $[Ca^{2+}]_i$ and $[cAMP]_i$ responses in TAS1R3-ΔC GLUTag cells.

Unexpectedly, we found that the Cas9-GFP plasmids may have possibly been inserted into genomic DNA in 4 of 9 GLUTag cell lines treated with CRISPR/Cas9. Such unexpected insertions of plasmids may damage unrelated genes; therefore, due caution should be exercised while interpreting the results of genetically modified cell lines. Hence, we emphasize the need to check for potential genomic insertion of plasmids during establishment of mutant cell lines by single cell cloning using CRISPR/Cas9.

In the present study, we could not investigate L-glutamine-induced GLP-1 secretion by ELISA under conditions of pharmacological experiments; this was because prolonged exposure of GLUTag cells to antagonists, which is a necessary step for ELISA, may affect the ability of cells to secrete GLP-1. The mechanisms of L-glutamine-induced GLP-1 secretion have not been investigated *in vivo*. Thus, further studies are needed to fully elucidate the mechanisms of GLP-1 secretion.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0260>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K O, Tet K, and T T designed the general concept. T N and K H wrote the original draft. K O, Tet K, and T T edited and completed the manuscript. T N designed and performed the experiments to generate mutant cell lines. K H conducted the imaging analysis of mutant cell lines. Tai K performed the pharmacological experiments and RT-PCR to validate the expression of each GPCR. M T measured the amount of GLP-1 by ELISA. J K and M G provided BIM-46187. K N and Tad K supervised the experiment to generate mutant cell lines.

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