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Insights into GLP-1 and insulin secretion mechanisms in pasireotide-induced hyperglycemia highlight effectiveness of Gs-targeting diabetes treatment

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Pasireotide frequently causes severe hyperglycemia; however, its detailed mechanism remains unknown. There are no published guidelines regarding the optimal management of pasireotide-induced hyperglycemia based on its pathophysiology. Herein, we successfully switched a patient with acromegaly from a dipeptidyl peptidase-4 (DPP-4) inhibitor to a glucagon-like peptide-1 (GLP-1) analog due to pasireotide-induced deterioration of glycemic control, and we examined the underlying mechanism for glycemic control. An in vitro study was conducted using pancreatic β-cell line, MIN-6, stably expressing GLP-1R (GLP-1R-MIN-6 cells) and intestinal L-cell line, GLUTag. High glucose levels and Gs-coupled receptor stimulation synergistically triggered insulin and GLP-1 secretion. Gs-coupled receptor stimulation primarily triggered GLP-1 secretion, which was amplified by high glucose levels in GLUTag cells. Pasireotide drastically inhibited GLP-1 secretion induced by Gs-coupled receptor stimulation through SSTR5-Gi-dependent inhibition of cAMP levels, suggesting that the main pathway was completely blocked. Furthermore, administering GLP-1 partially overcame the inhibitory effect of pasireotide in GLP-1R-MIN-6 cells, leading to a partial recovery of insulin secretion. The drastic inhibition of GLP-1 secretion via shutdown of the main pathway is the primary cause of pasireotide-induced hyperglycemia. GLP-1 analogs, rather than DPP-4 inhibitors, can spare pasireotide-induced depletion of endogenous GLP-1 and restore insulin secretion.

Keywords GLP-1, GLP-1 analog, GPCRs, Insulin, L-cells, Pasireotide

Several physiological functions, including those of the endocrine and metabolic systems, depend on G-protein-coupled receptor (GPCR) signaling[1–6]. In many types of endocrine cells, coordination between the accelerating and braking actions of GPCR-G protein signaling physiologically regulates hormone synthesis and secretion[4,7–11]. Physiologically, the secretion of growth hormone (GH) in somatotrophs is stimulated mainly by growth hormone-releasing hormone receptor-Gs signaling and partially by ghrelin receptor-Gq signaling, whereas somatostatin inhibits GH secretion by activating somatostatin receptor (SSTR)-Gi signaling[12,13]. Pathophysiologically, excessive Gs signaling results in excess GH, leading to acromegaly.

Dopamine analogs and somatostatin analogs (SSAs) have been used for medical adjuvant therapies to suppress excessive Gs signaling as activating the Gi/o-coupled dopamine 2 receptor (D2R) or SSTRs, respectively[14]. SSTRs, particularly SSTR2 and SSTR5, are highly expressed on somatotrophs in GH-secreting pituitary tumors, in which SSAs inhibits GH secretion by impeding adenylyl cyclase and decreasing cAMP levels[15,16].

Pasireotide frequently causes severe hyperglycemia, probably by activating somatostatin receptor 5 expressed in the pancreases and intestines[17,18]. Furthermore, SSTRs, particularly SSTR5, are highly expressed in intestinal L-cells that secrete glucagon-like peptide-1 (GLP-1) and K-cells that secrete glucose-dependent insulinotropic

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polypeptide (GIP). SSTR5 activation strongly inhibits GLP-1 or GIP secretion by activating Gi signaling[19,20]. Pasireotide long-acting release (PAS-LAR) has also been reported to lower serum GLP-1 and GIP levels in phase 1 studies conducted on healthy volunteers[21]. By targeting the intestine, PAS-LAR can cause or exacerbate diabetes mellitus by strongly inhibiting GLP-1 or GIP secretion.

There are no published guidelines regarding the optimal management of pasireotide-induced hyperglycemia in patients with acromegaly[22], but the most currently recommended treatment is metformin, followed by dipeptidyl peptidase-4 (DPP-4) inhibitors, GLP-1 analogs, and then insulin[22–24]. Several studies have suggested that DPP-4 inhibitors and GLP-1 analogs are more effective treatments for pasireotide-induced hyperglycemia than metformin or insulin therapy[25,26]. However, there must be a significant difference between the effect GLP-1

(a) after the improvement of glycemic control		
Time (min)	**0**	**120**
Plasma glucose levels (mg/dL)	74	211
CPR (ng/mL)	< 0.2*	0.4
(b) 3 days after dulaglutide administration		
Time (min)	**0**	**120**
Plasma glucose levels (mg/dL)	138	154
CPR (ng/mL)	0.3	1.0

Table 1. Mixed-meal tolerance test (MMTT) was performed (a) after the improvement of glycemic control. MMTT was performed with 2250 mg daily of metformin, 150 mg daily of miglitol, 5 mg daily of dapagliflozin, 25 mg daily of alogliptin and 16 units/day of insulin glargine. Insulin glargine was given in the morning. Abbreviation: CPR, C-peptide immunoreactivity. < 0.2* mg/dl: less than sensitivity. (b) 3 days after dulaglutide administration. MMTT was performed in 3 days after 25 mg daily of alogliptin was changed to 0.75 mg weekly of dulaglutide, leaving the other antidiabetic drugs and insulin glargine unchanged.

a

Genes	MIN-6 cells	GLUTag cells
Sstr1	0.1256	0
Sstr2	0	8.821
Sstr3	35.09	0.92
Sstr4	0	0
Sstr5	5.26	16.74

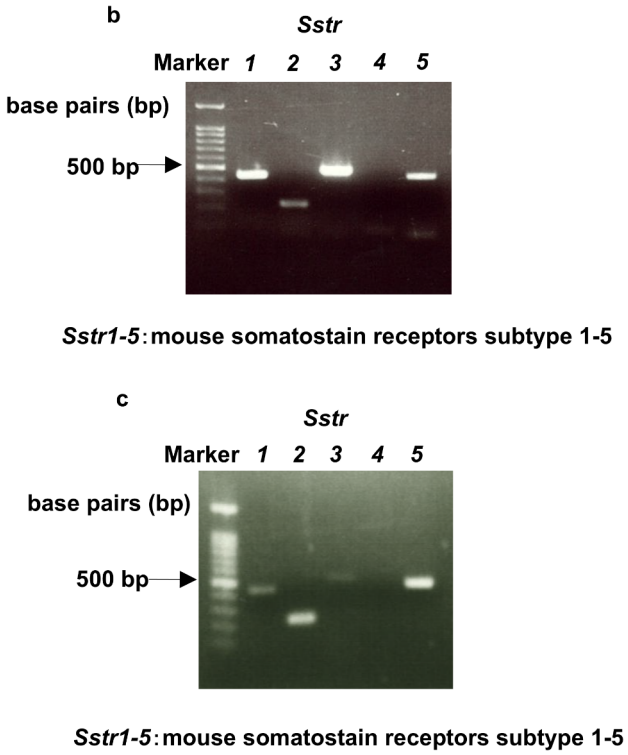


Fig. 1. mRNA expression analysis of mouse SSTR (mSSTR) in GLP-1R-MIN-6 cells and in GLUTag cells by RNA-seq and RT-PCR. (a) mRNA analysis of mouse SSTR subtypes 1–5 (*Sstr1-5*) in MIN-6 and GLUTag cells was conducted using RNA-Seq. A read count analysis using StringTie was performed to determine the relative abundance of genes. (b) mRNA expression analysis of *Sstr1-5* in GLP-1R-MIN-6 cells was performed using RT-PCR. (c) mRNA expression analysis of *Sstr1-5* in GLUTag cells was also performed using RT-PCR.

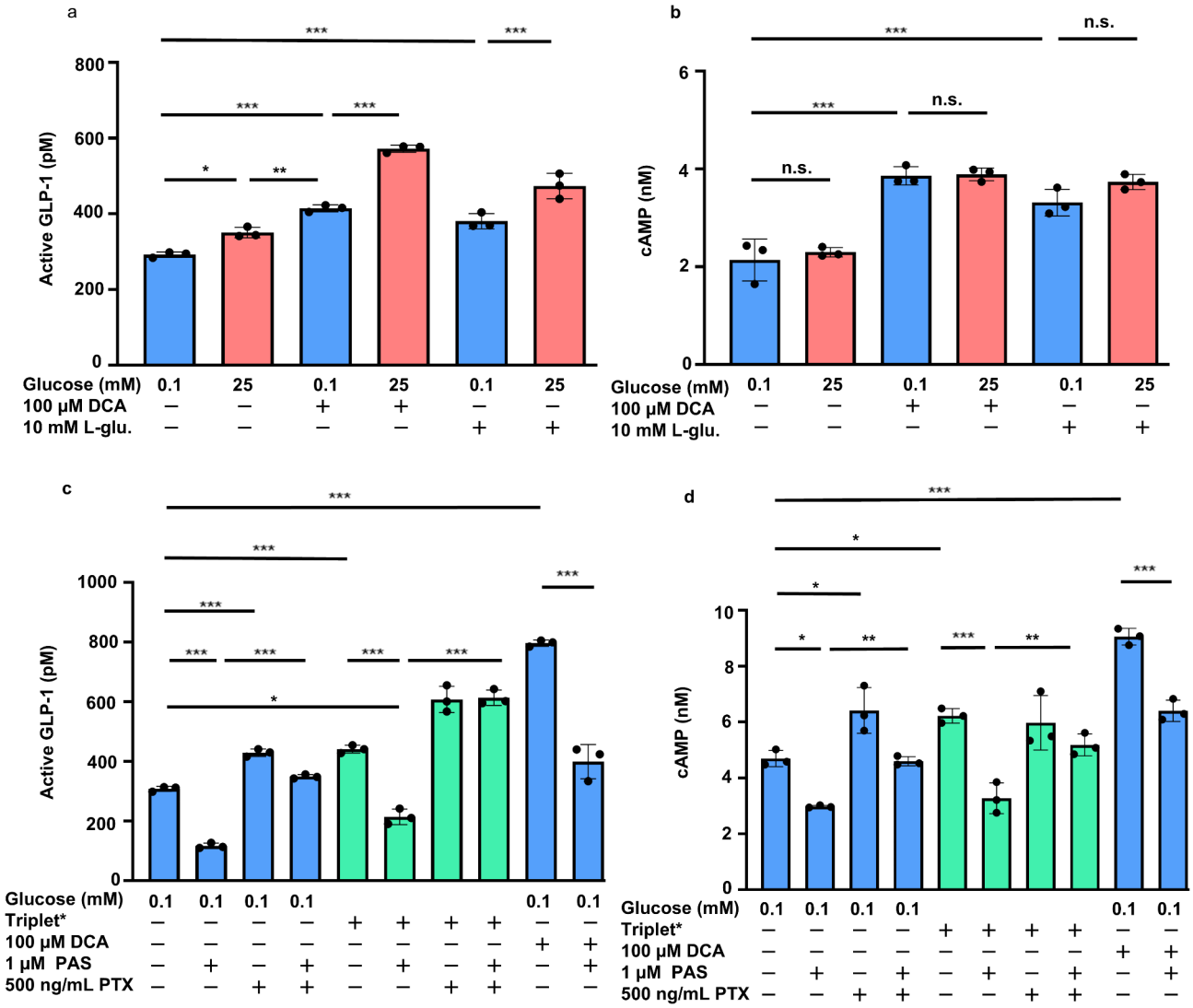
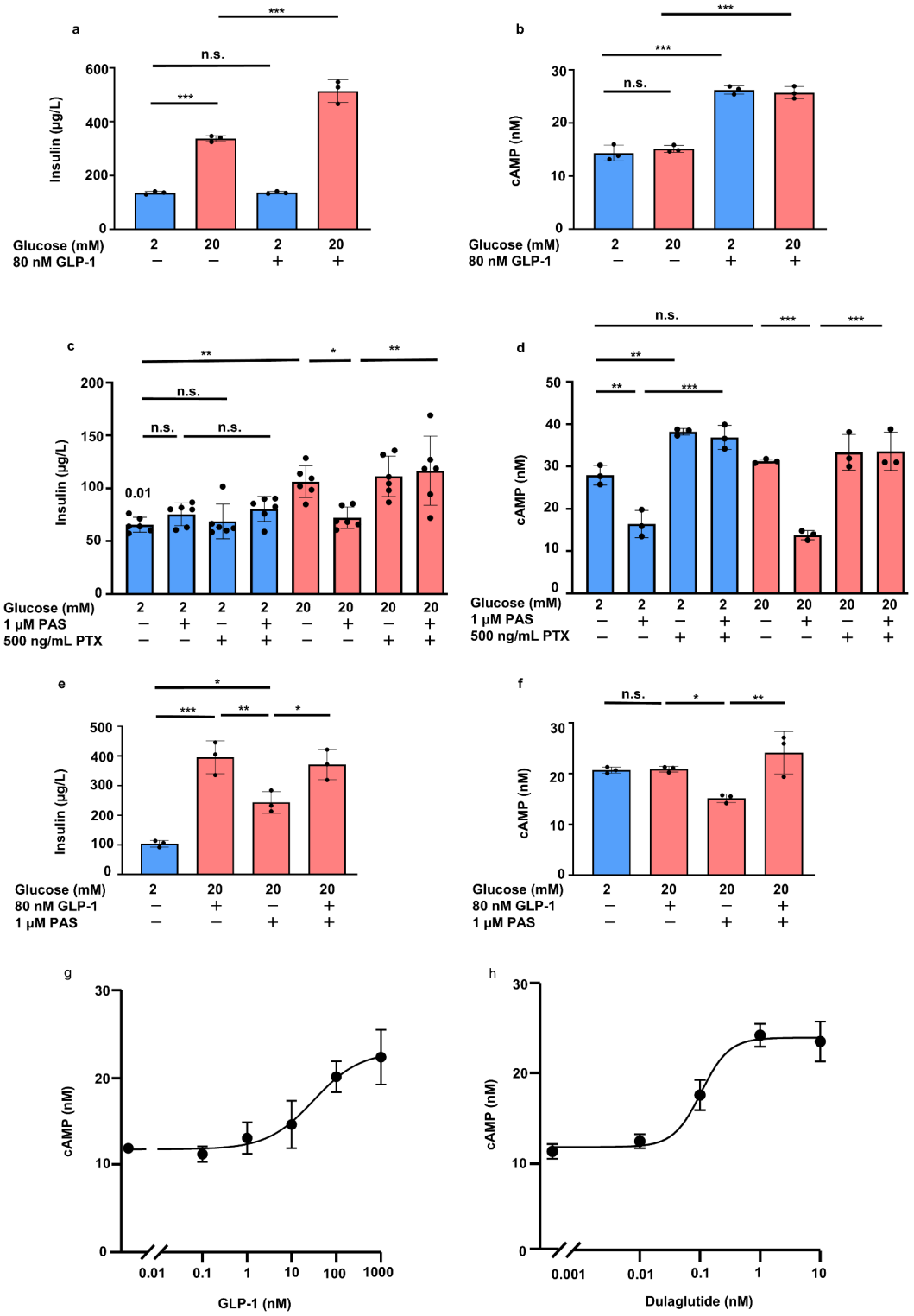


Fig. 2. The main pathway for GLP-1 secretion is via cAMP signaling, and pasireotide inhibits GLP-1 secretion via SSTRs-Gi activation in GLUTag cells. **(a**, **b)** Effects of low glucose (0.1 mM) level or high glucose (25 mM) levels, DCA with low or high glucose levels, and L-glutamine (L-glu.) with low or high glucose levels on active GLP-1 secretion and cAMP accumulation in GLUTag cells. **(c**, **d)** Effects of low glucose level, triplet*, and DCA with or without PAS and/or PTX on active GLP-1 secretion and cAMP accumulation in GLUTag cells. PAS: pasirotide. PTX: pertussis toxin, an irreversible Gi inhibitor. The term "triplet*" refer to a high glucose (25 mM) level combined with 10 mM L-glutamine and 5 mM sodium butyrate, as indicated by the green square patterns. The blue and red square patterns indicate low glucose (0.1 mM) and high glucose (25 mM) levels, respectively. Values represent the mean ± SEM of triplicate experiments. Each set of results is representative of at least two additional experiments. The Y-axis shows the pM in the culture medium for active GLP-1 **(a**, **c)** and nM in the culture medium for cAMP **(b**, **d)** on the graphs. Data are expressed as means (SEM). The Tukey–Kramer multiple comparison test was conducted for statistical analysis. n.s., not significant; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

analogs and those of DPP-4 inhibitors based on the pathophysiology of pasireotide-induced hyperglycemia. As DPP-4 inhibitors may not be sufficient in the presence of suppressed GLP-1 secretion form intestinal L-cells, we and others assume that GLP-1 analogs are superior for the management of pasireotide-induced hyperglycemia[27]. Furthermore, GLP-1 analogs may restore insulin secretion by activating the GLP-1 receptor (GLP-1R)-Gs signals because GLP-1 analogs overcome SSTR5-Gi signals in pancreatic β-cells under the condition of suppressed insulin production and spare suppressed incretin secretion from intestinal L-cells[27]. Herein, we successfully switched a patient with acromegaly from a DPP-4 inhibitor to a GLP-1 analog due to pasireotide-induced deterioration of glycemic control. Our experience led to a proof-of-concept study in the insulin-secreting pancreatic β-cell line, MIN-6, and the GLP-1-secreting intestinal L-cell line, GLUTag.


a
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Dulaglutide (nM)

◀**Fig. 3**. Pasireotide inhibits insulin secretion via SSTRs-Gi activation in GLP-1R-MIN-6 cells, and GLP-1 reverses the inhibitory action of pasiretoide on insulin secretion. **(a**, **b)** Effects of low glucose (2 mM) levels, high glucose (20 mM) levels, and GLP-1 with low or high glucose levels on insulin secretion and cAMP accumulation in GLP-1R-MIN-6 cells. **(c**, **d)** Effects of low glucose levels or high glucose levels with or without PAS and/or PTX on insulin secretion and cAMP accumulation in GLP-1R-MIN-6 cells. **(e**, **f)** The effects of GLP-1 on insulin secretion or cAMP accumulation in GLP-1R-MIN-6 cells incubated with high glucose levels and PAS. **(g**, **h)** Dose-dependent cAMP accumulation stimulated by GLP-1 and dulaglutide in GLP-1R-MIN-6 cells. The EC50 values were 31.0 nM in GLP-1 and 0.10 nM in dulaglutide. PAS: pasirotide. PTX: pertussis toxin, an irreversible Gi inhibitor. Values represent the mean ± SEM of $n = 3$ or $n = 6$ experiments. Each set of results is representative of at least two additional experiments. The Y-axis shows μg/L in the culture medium for insulin **(a**, **c**, **e)** and the nM in the culture medium for cAMP **(b**, **d**, **f**, **g**, **h)** on the graphs. Data are expressed as means (SEM). The Tukey–Kramer multiple comparison test was conducted for statistical analysis. n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Results

Case report

A 35-year-old female with GH-secreting pituitary tumors was treated with surgery and medical therapy using the first-generation SSA, octreotide, and the D2 agonist, cabergoline. As she was resistant to conventional therapy (GH 17.5 ng/mL, IGF-1 947 ng/mL), we introduced PAS-LAR. Despite the improvement in GH/IGF-1 levels (GH 10.6 ng/mL, IGF-1 462 ng/mL), her glycemic control drastically deteriorated for 4 months (Hb1Ac 8.2–12.0%). Her glycemic control improved after administration of intensive insulin therapy, oral agents such as, dapagliflozin, metformin, alogliptin, and miglitol, and dietary restriction; however, a mixed-meal tolerance test (MMTT) revealed unsatisfactory postprandial insulin secretion (Table 1a). Based on our hypothesis that GLP-1 analogs might be superior to DPP-4 inhibitors for pasireotide-induced hyperglycemia, we switched from alogliptin 25 mg daily to dulaglutide 0.75 mg weekly, while other antidiabetic drugs remained unchanged. Considering the adherence of this patient, we chose dulaglutide, a once-weekly GLP-1 receptor agonist, instead of the once-daily GLP-1 receptor agonists. At the time this clinical data was collected in 2017, dulaglutide was the only once-weekly GLP-1 receptor agonist available. Regarding the dosage of dulaglutide, it is typically administered to adults in Japan at a starting dose of 0.75 mg. Within 3 d after the switch, an MMTT suggested recovery of basal and postprandial insulin secretion (Table 1b). In this second experiment, the basal glucose level increased (Table 1b). Although the cause remains unclear, one possible explanation is that the blood glucose level before sleep on the day prior to the second experiment was 82 mg/dL, prompting the consumption of three biscuits, which may have elevated the basal glucose level on the day of the second experiment. After switching from alogliptin 25 mg daily to dulaglutide 0.75 mg weekly, and reducing IDeg/Asp from 36 to 24 units/day while maintaining other antidiabetic drugs, glycemic control was effectively maintained during the subsequent hospitalization. Fasting blood glucose levels ranged from 101 to 139 mg/dL, and postprandial glucose levels ranged from 130 to 157 mg/d. A dosage of 0.75 mg of dulaglutide was highly effective in this clinical case. Thus, this case of acromegaly supports our hypothesis that GLP-1 analogs are more effective than DPP-4 inhibitors for pasireotide-induced hyperglycemia.

mRNA expression analysis of mouse SSTR (mSSTR) in GLP-1R-MIN-6 cells and in GLUTag cells by RNA-seq and RT-PCR.

A proof-of-concept study was further planned. First, an insulin-secreting β-cell line MIN-6 cells stably expressing GLP-1R (GLP-1R-MIN-6 cells) was prepared, as we intended to observe a stronger effect of GLP-1R activation than that of endogenous GLP-1R. A GLP-1-secreting intestinal L-cell line, GLUTag cells, were also prepared. RNA-sequencing analysis revealed abundant expressions of *Sstr1*, *Sstr3*, and *Sstr5* in MIN-6 cells and abundant expression of *Sstr2* and *Sstr5* in GLUTag cells (Fig. 1a). These results were confirmed by RT-PCR analysis (Fig. 1b and c).

The main pathway for GLP-1 secretion is via cAMP signaling, and pasireotide inhibits GLP-1 secretion via SSTRs-Gi activation in GLUTag cells.

To investigate the stimulation mechanism of GLP-1 secretion, we examined the response of intestinal GLUTag cells to glucose stimulation and Gs activation by deoxycholic acid (DCA) through G-protein coupled bile acid receptor 1 (GPBAR1) and L-glutamine, which are prevalent in daily ingested proteins. It has been shown that L-glutamine stimulates GLP-1 secretion by activating Gs through Gs-coupled receptors like taste receptor type 1 member 3 (TAS1R3) in intestinal L-cells[28]. A high glucose (25 mM) level promoted GLP-1 secretion in GLUTag cells, but did not increase the cAMP levels (Fig. 2a, b). In a low-glucose (0.1 mM) state, Gs activation by 100-μM DCA or 10-mM L-glutamine promoted GLP-1 secretion. GLP-1 secretion induced by Gs activation with100-μM DCA or 10-mM L-glutamine were further enhanced by high glucose (25 mM) stimulation (Fig. 2a). A high glucose level did not increase the cAMP levels stimulated by 100-μM DCA or 10-mM L-glutamine, suggesting that high glucose potentiates Gs activation-stimulated GLP-1, but not via cAMP in GLUTag cells (Fig. 2a, b). To explore the mechanism by which pasireotide acts on intestinal L-cells, we investigated the impact of DCA, or a nutrient mimicking our daily meals, on GLP-1 secretion from GLUTag cells. In the low-glucose (0.1 mM) state, DCA-stimulated GLP-1 secretion was completely inhibited by 1-μM pasireotide treatment, paralleling the cAMP levels in GLUTag cells (Fig. 2c, d). Similarly, a high glucose level (25 mM) with 10 mM L-glutamine and 5 mM sodium butyrate, major physiological nutrients, promoted GLP-1 secretion and cAMP accumulation, both completely inhibited by 1-μM pasireotide treatment in GLUTag cells (Fig. 2c, d). Treatment with 500 ng/

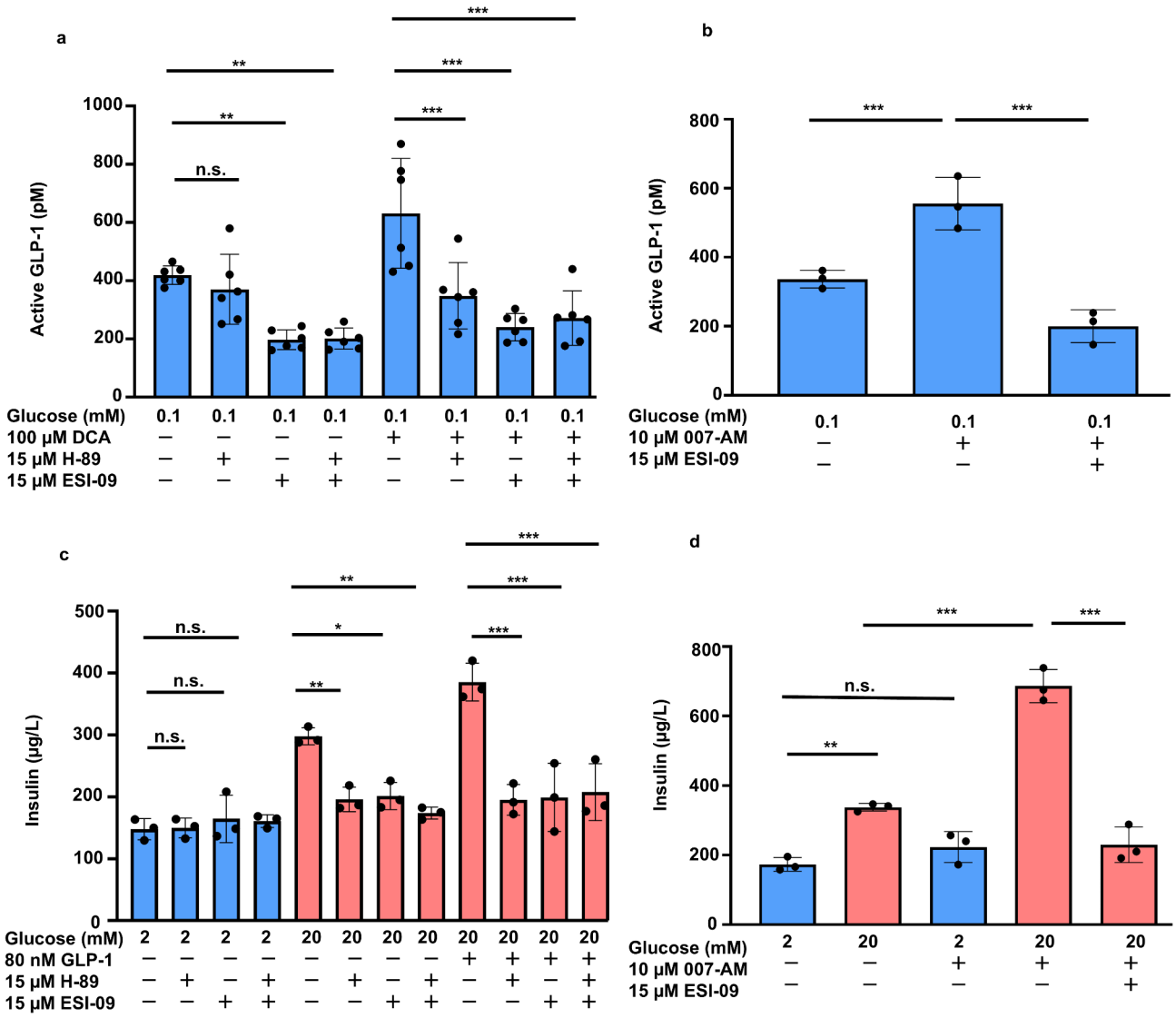


Fig. 4. PKA and EPAC signals via Gs activation partly increase GLP-1 secretion in GLUTag cells and insulin secretion in GLP-1R-MIN-6 cells.**(a)** Effects of DCA with or without H-89 and/or ESI-09 in the low-glucose (0.1 mM) state on active GLP-1 secretion in GLUTag cells. **(b)** Effects of 007-AM or 007-AM with ESI-09 in the low-glucose (2 mM) state on active GLP-1 secretion in GLUTag cells. **(c)** Effects of low (2 mM) or high glucose (20 mM) levels with or without GLP-1, H-89, and/or ESI-09 on insulin secretion in GLP-1R-MIN-6 cells. **(d)** Effects of 007-AM with low or high glucose levels, and the effect of ESI-09 on insulin secretion in GLP-1R-MIN-6 cells. H-89, is an inhibitor of protein kinase A. ESI-09, is an inhibitor of EPAC1 and EPAC2. 007-AM is a selective EPAC activator. The blue and red square patterns indicate low glucose (0.1 or 2 mM) and high glucose (20 mM) levels, respectively. Values represent the mean ± SEM of $n = 3$ or $n = 6$ determinations. Each set of results is representative of at least two additional experiments. The Y-axis shows pM in the culture medium for active GLP-1 **(a, b)** and μg/L in the culture medium for insulin **(c, d)** on the graphs. Data are expressed as means (SEM). The Tukey–Kramer multiple comparison test was conducted for statistical analysis. n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

mL PTX reversed the inhibition of pasireotide on GLP-1 secretion and cAMP accumulation (Fig. 2c, d). These findings suggest that DCA- or nutrient-induced GLP-1 secretion was inhibited by pasireotide via SSTRs-Gi-dependent inhibition of cAMP levels. Furthermore, the GLP-1 secretion, which was inhibited by pasireotide in response to a high glucose level with L-glutamine and sodium butyrate stimulation, was significantly lower than the "basal" GLP-1 secretion in the low-glucose (0.1 mM) state in GLUTag cells (Fig. 2c). In a state where Gs activation is inhibited by pasireotide, a high glucose level could not stimulate GLP-1 secretion. This result suggests that the cAMP signaling is the main pathway, while the glucose-induced signaling pathway serves as the amplification pathway in GLUTag cells. In addition, the basal GLP-1 secretion was significantly inhibited by 1-μM pasireotide treatment in GLUTag cells, and the inhibitory effect of pasireotide was reversed by PTX treatment (Fig. 2c, d). These findings suggest that cAMP signaling is the main pathway for GLP-1 secretion in

GLUTag cells, with glucose-induced signaling amplifying it. This suggests that the drastic inhibition of GLP-1 secretion by shutdown of the main pathway via the SSTRs-Gi axis is the primary cause of pasireotide-induced hyperglycemia.

Pasireotide inhibits insulin secretion via SSTRs-Gi activation in GLP-1R-MIN-6 cells, and GLP-1 reverses the inhibitory action of pasiretoide on insulin secretion.

To explore pasireotide's mechanisms on pancreatic β-cells, we examined its effect on insulin release from GLP-1R-MIN-6 cells. Gs activation by 80-nM GLP-1 significantly potentiated insulin secretion triggered by high glucose (20 mM), suggesting a synergistic effect of glucose and Gs-coupled receptor stimulation (Fig. 3a, b). GLP-1 strongly promoted cAMP accumulation but did not stimulate insulin secretion in low-glucose (2 mM) state, indicating glucose-induced signaling as the main pathway (Fig. 3a, b). High glucose (20 mM)-induced insulin secretion was inhibited by 1 μM pasireotide treatment, comparable with the cAMP levels (Fig. 3c, d). PTX treatment reversed this inhibition, suggesting that insulin secretion is inhibited by pasireotide via SSTRs-Gi-dependent cAMP inhibition (Fig. 3c, d). Furthermore, we discovered that insulin secretion in the low-glucose (2 mM) state was not significantly inhibited by pasireotide, even though cAMP accumulation was significantly inhibited by pasireotide (Fig. 3c, d). This result is consistent with the finding that cAMP signaling serves as the amplification pathway in pancreatic β-cells. Additionally, we found that high glucose (20 mM)-induced insulin secretion, inhibited by 1 μM pasireotide, was higher than insulin secretion in the low-glucose (2 mM) state. This suggests that high glucose can independently stimulate insulin secretion even when Gs activation is suppressed by pasireotide in GLP-1R-MIN-6 cells. We found that administering 80-nM GLP-1 significantly counteracted the inhibitory effect of pasireotide on high glucose (20 mM)-induced insulin production, aligning with the cAMP levels in GLP-1R-MIN-6 cells. This suggests that GLP-1R-Gs activation rescues insulin secretion

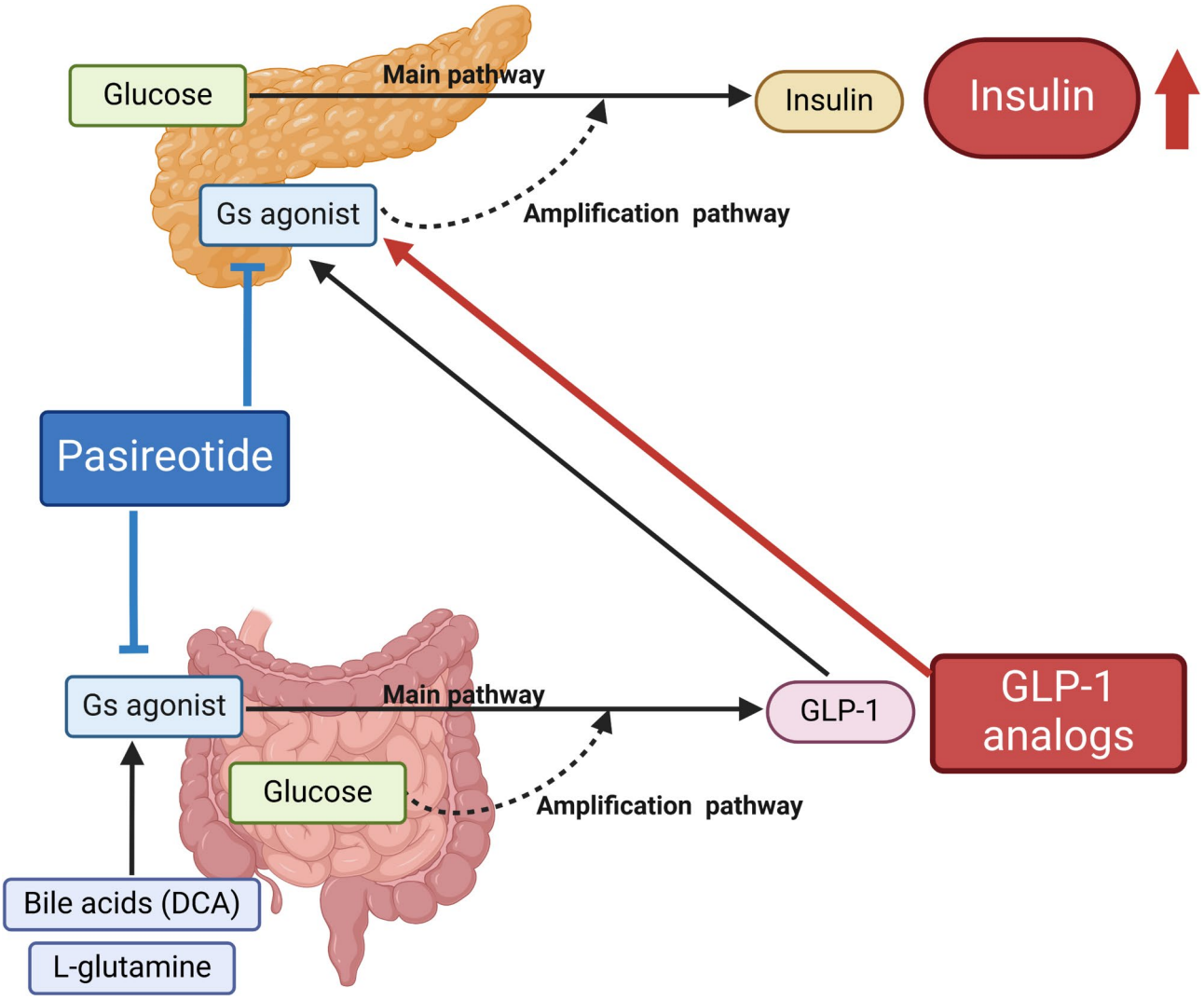
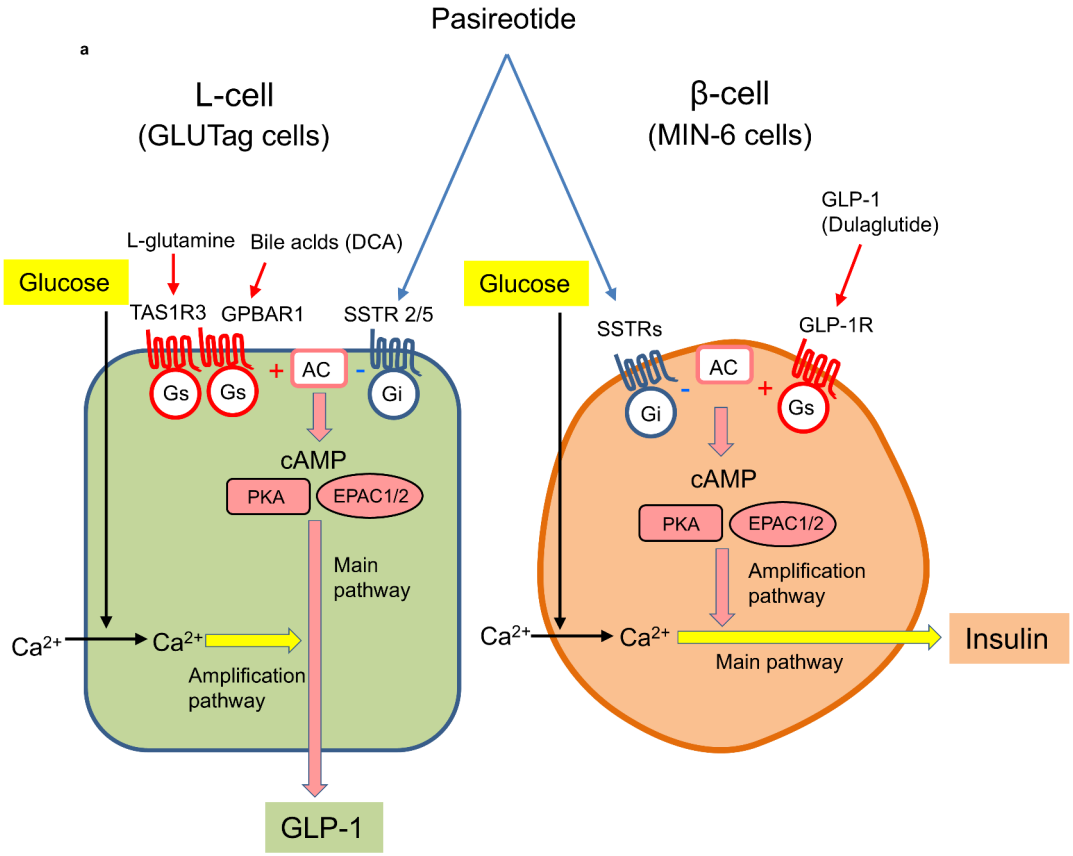


Fig. 5. The detailed mechanism of pasireotide-induced hyperglycemia and its optimal management in relation to pathophysiology. Drastic inhibition of GLP-1 secretion through shutdown of the main pathway is the primary cause of pasireotide-induced hyperglycemia. GLP-1 analogs, rather than DPP-4 inhibitors, spare pasireotide-induced depletion of endogenous GLP-1 and restore insulin secretion.


a
Pasireotide
L-cell
(GLUTag cells)
β-cell
(MIN-6 cells)
GLP-1
(Dulaglutide)
L-glutamine
Bile acids (DCA)
Glucose
TAS1R3
GPBAR1
SSTR 2/5
Glucose
SSTRs
GLP-1R
AC
Gs
Gs
Gi
Gi
Gs
cAMP
PKA
EPAC1/2
Main
pathway
Amplification
pathway
Ca2+
Ca2+
Ca2+
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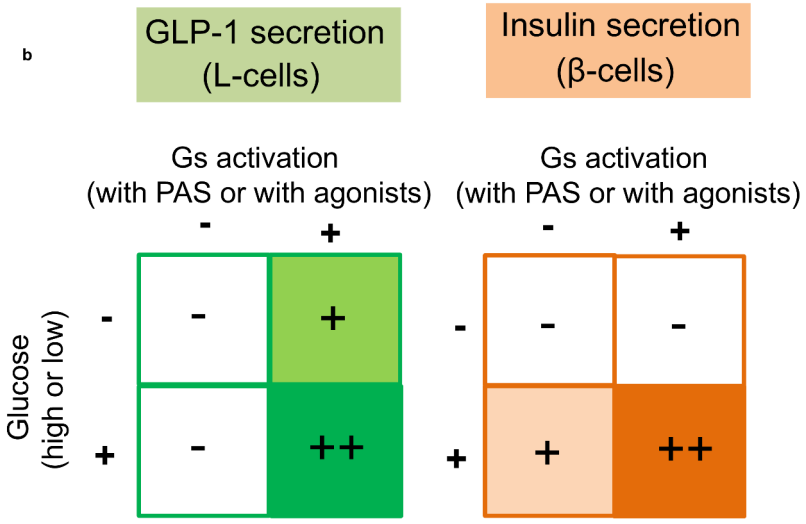
Amplification
pathway
Main pathway
Insulin
GLP-1


b
GLP-1 secretion
(L-cells)
Insulin secretion
(β-cells)
Gs activation
(with PAS or with agonists)
Gs activation
(with PAS or with agonists)
Glucose
(high or low)
-
+
-
+
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+
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+
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+
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◀**Fig. 6**. (**a**) Model of the molecular mechanisms involved in GLP-1 secretion from intestinal L-cells and insulin secretion from pancreatic β-cells. A schematic summary of the results. We demonstrated the commonality that GLP-1 from intestinal L-cells and insulin from pancreatic β-cells are synergistically regulated by Gs signaling and glucose. We also highlighted that the main pathways of GLP-1 and insulin secretion are different; Gs signaling in L-cells and glucose in β-cells. In intestinal L-cells, bile acids (acting on GPBAR1) and L-glutamine (acting on TAS1R3) stimulate GLP-1 secretion via protein kinase A and EPAC1/2 (the main pathway). A glucose-induced signal amplifies GLP-1 secretion (the amplification pathway). Suppression of adenylyl cyclase (AC) activity via SSTR2,5-Gi activation by pasireotide drastically inhibits GLP-1 secretion by shutting down the main pathway. In pancreatic β-cells, high glucose stimulates insulin secretion (the main pathway). GLP-1-amplified insulin secretion via protein kinase A and EPAC1/2 (the amplification pathway). Suppression of adenylyl cyclase (AC) activity via SSTRs-Gi activation by pasireotide inhibits insulin secretion, which can be overcome by GLP-1 analogs (**b**) Synergy between glucose and Gs-coupled GPCR agonist for GLP-1 and insulin secretion. We demonstrated that the main pathway can independently stimulate hormone secretion, even if the amplification pathway is suppressed (Gs activation is suppressed by PAS or in a low glucose state). In contrast, the amplification pathway cannot stimulate hormone secretion when the main pathway is significantly suppressed (Gs activation is suppressed by PAS or in a low glucose state). However, it can potentiate hormone secretion induced by the main pathway. PAS: pasireotide. Intestinal L-cells (left , drawn in green) . Gs-coupled GPCR agonists, such as bile acids or L-glutamine, can independently stimulate GLP-1 secretion in the low-glucose (0.1 mM) state (+, upper-right column). A high glucose (25 mM) level can stimulate GLP-1 secretion under "basal" Gs activation, that is, Gs-coupled receptor activation without agonists; however, it cannot stimulate GLP-1 secretion when Gs activation is significantly suppressed by pasireotide (−, lower-left column). However, a high glucose level can potentiate Gs-coupled GPCR-stimulated GLP-1 secretion (++, lower-right column). Pancreatic β-cells (right , drawn in orange) . A high glucose (20 mM) level can independently stimulate insulin secretion even when Gs activation is significantly suppressed by pasireotide (+, lower-left column). In contrast, Gs-coupled GPCR agonists such as GLP-1 cannot independently stimulate insulin secretion in the low-glucose (2 mM) state (−, upper-right column), but they can potentiate insulin secretion stimulated by high glucose levels (++, lower-right column).

inhibited by pasireotide through the activation of SSTRs-Gi in GLP-1R-MIN-6 cells (Fig. 3e, f). We used GLP-1 instead of dulaglutide in this experimentas we aimed to explore the class effect of GLP-1. We consider that 80 nM of GLP-1 is comparable to the effective serum concentration of dulaglutide used in our case (Fig. 3g and h).

PKA and EPAC signals via Gs activation partly increase GLP-1 secretion in GLUTag cells and insulin secretion in GLP-1R-MIN-6 cells.

cAMP signaling through Gs-coupled receptors activates protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC) signals to potentiate insulin or GLP-1 secretion in pancreatic β-cells[29]or intestinal L-cells[30,31]. The effects of these signals on GLP-1 or insulin secretion were investigated. In GLUTag cells, DCA-stimulated GLP-1 secretion was significantly inhibited by H-89, a PKA inhibitor, and/or ESI-09, inhibitors of EPAC1, and EPAC2, in the low-glucose (0.1 mM) state (Fig. 4a). These results suggest that protein kinase A and EPAC are involved in GLP-1 secretion from GLUTag cells. The basal GLP-1 secretion in the low-glucose (0.1 mM) state was significantly inhibited by ESI-09, or ESI-09 with H-89, and was stimulated only by 007-AM, a selective EPAC activator (Fig. 4a, b). These results suggest that GLP-1 secretion depends primarily on Gs-EPAC signals in GLUTag cells. In GLP-1R-MIN-6 cells, a high glucose (20 mM) level in the basal Gs activation state or 80 nM GLP-1 with a high glucose level stimulated insulin secretion, which was inhibited by H-89 and/or ESI-09. Insulin secretion in the high-glucose (20 mM) state was potentiated by 007-AM, and 007-AM-potentiated insulin secretion was inhibited by ESI-09 (Fig. 4c, d). These results suggest that basal Gs activation or GLP-1-stimulated Gs activation enhances insulin secretion, relying on protein A kinase and EPAC signals in GLP-1R-MIN-6 cells. Insulin secretion in the low-glucose (2 mM) state was neither inhibited by H-89 and/or ESI-09 nor stimulated by 007-AM (Fig. 4c, d). These results further support the concept that cAMP signaling serves as the amplification pathway in GLP-1R-MIN-6 cells.

Discussion

We successfully switched an acromegaly patient with pasireotide-induced deterioration of glycemic control from a DPP-4 inhibitor to a GLP-1 analog. To investigate the underlying mechanism, we conducted a proof-of-concept study using model cell lines. The results revealed that the drastic inhibition of GLP-1 secretion, caused by the shutdown of the main pathway via the SSTRs-Gi axis, is the main cause of pasireotide-induced hyperglycemia. Therefore, GLP-1 analogs are superior to DPP-4 inhibitors for managing pasireotide-induced hyperglycemia (Fig. 5). Additionally, we discovered that a high glucose level and Gs-coupled receptor stimulation synergistically triggered GLP-1, involving PKA/Epac pathway. This suggests that GLP-1 and insulin secretions have striking commonalities in the synergistic release and signaling mechanisms. At the same time, however, we demonstrated that Gs-coupled receptor stimulation mainly triggers GLP-1 secretion, which is further amplified by a high glucose level in GLUTag cells (Fig. 6a, b).

It is well known that the glucose-stimulated pathway, involving an increase in Ca^{2+} influx via a glucose transporter (GLUT), is the main pathway for insulin secretion. This process is amplified by Gs activation through Gs-coupled GLP-1 and GIP receptors, leading to an increase in intracellular cAMP levels in pancreatic β-cells[29]. Contrary to pancreatic β-cells, the mechanism of GLP-1 stimulation-secretion coupling in intestinal L-cells is poorly understood[32]. Commonalities regarding the mechanism of GLP-1 and insulin secretions, such

as the sharing of glucose-sensing machinery, including Gs activation and glucose stimulation[32,33], have been reported[6,34]. Glucose is transported into intestinal L-cells by sodium-glucose cotransporters, and the resultant increase in Ca^{2+}influx triggers GLP-1 secretion[32]. Furthermore, Gs activation can both enhance the Ca^{2+}signal and increase the efficacy of the secretory machinery in intestinal L-cells[35]. However, their synergic effects on GLP-1 secretion are still unknown. In the present study we discovered that GLP-1 secretion was primarily stimulated by Gs-coupled receptors, such as DCA-stimulated GPBAR1 or L-glutamine-stimulated TAS1R3 and amplified by a high glucose level in GLUTag cells. On the other hand, insulin secretion was primarily stimulated by high glucose and amplificated by Gs-coupled receptors such ss GLP-1-stimulated GLP-1 receptor in GLP-1R-MIN-6 cells. These results suggest that Gs-cAMP signals are the main pathways for GLP-1 secretion in intestinal L-cells, in contrast to insulin secretion in pancreatic β-cells (Fig. 6a, b). Pasireotide could drastically suppress GLP-1 secretion induced by Gs-cAMP signals through SSTR5-Gi-dependent inhibition of cAMP levels, effectively blocking the main pathways. This novel concept could explain why the GLP-1 analog might be superior to a DPP-4 inhibitor for the management of pasireotide-induced hyperglycemia. Furthermore, this novel concept suggests that the GLP-1 analog could spare the pasireotide-induced decrease in endogenous GLP-1 concentrations and recover insulin secretion suppressed by pasireotide through GLP-1R-Gs activation counteracting the SSTRs-Gi axis. Alternatively, DPP-4 inhibitors could not restore sufficient GLP-1 levels (Fig. 5). It might be challenging to compare DPP-4 inhibitors and GLP-1 receptor agonists in pasireotide-induced hyperglycemia. However, in an in vitro study using pasireotide, we considered the possibility of endogenous DPP-4 being present in GLUTag cells and added a DPP-4 inhibitor. Nevertheless, under the administration of a DPP-4 inhibitor, pasireotide drastically inhibited GLP-1 secretion. This could indirectly suggest that DPP-4 inhibitors may be less effective in pasireotide-induced hyperglycemia.

It has been reported that nutrients such as amino acids, fatty acids, and glucose stimulate GLP-1 secretion[36,37]. In GLUTag cells, we demonstrated that a high glucose level with L-glutamine and sodium butyrate, which are major physiological nutrients, stimulated GLP-1 secretion and increased cAMP accumulation. These effects were completely inhibited by pasireotide. This suggests that nutrient-induced GLP-1 secretion was inhibited by pasireotide via SSTR-Gi-dependent inhibition of cAMP levels. Among several amino acids, L-glutamine is the most effective in promoting the release of GLP-1 via intracellular cAMP and Ca^{2+}increases in GLUTag cells[38]. In fact, in GLUTag cells, we showed that L-glutamine enhanced GLP-1 secretion and cAMP accumulation, suggesting that L-glutamine stimulates GLP-1 secretion by activating Gs-coupled receptors such as TAS1R3 [28]. We demonstrated that in GLUTag cells, a high glucose level with L-glutamine stimulates GLP-1 secretion through a synergistic effect rather than cAMP amplification, and that a high glucose level potentiates L-glutamine-Gs activation in GLP-1 secretion.

The implications of commonalities and differences in insulin and GLP-1 secretions would provide deep insights into the coordination between blood glucose levels and the maintenance of body homeostasis. Throughout evolution, Gs-cAMP or glucose-Ca^{2+}signaling pathways have been preserved for hormone secretion as classic cellular signals. These pathways have simultaneously developed for "organ-specific adaptation" to meet the physiological demands specific to each organ, sensing glucose in pancreatic β-cells for insulin secretion, and sensing various nutrients like amino acids, fatty acids, and glucose for GLP-1 secretion in intestinal L-cells[32]. Pancreatic β-cells respond to changes in blood glucose levels through insulin secretion, whereas intestinal L-cells respond to various nutrients, such as amino acids, fatty acids, glucose, and bile acids, through incretin secretion under physiological conditions[35]. A recent study demonstrated that consuming fish or meat before rice can aid in managing postprandial glucose levels in both patients with type 2 diabetes and healthy individuals by increasing incretin secretion and delaying gastric emptying[39]. This may support the hypothesis that the main pathway in intestinal L-cells is Gs-cAMP signaling.

Several aspects remained undetermined in this study. First, the understanding of the mechanisms of insulin secretion regulation in pancreatic β-cells has made significant progress, especially in recent years[40]. However, regarding the mechanisms of insulin secretion regulation, particularly the potentiation mechanism of the main pathway, there are still unresolved aspects that need to be elucidated. Furthermore, regarding the mechanisms of GLP-1 secretion regulation in intestinal L-cells, including the potentiation mechanism of the main pathway, many unresolved aspects remain to be elucidated.

Second, we did not explore Gq signaling in either cell line. The processes behind the release of insulin and GLP-1 are significantly influenced by Gq signaling[29,33]. Recent research demonstrated that the distinct insulinotropic potential of incretins in diabetes is highlighted by a Gs/Gq signaling shift in pancreatic β-cells chronically exposed to high glucose[41]. Elucidating the detailed mechanisms of Gq signaling in insulin and GLP-1 secretions may contribute to the development of new treatment strategies for diabetes.

Third, intestinal K-cells and pancreatic islet cells, including α-cells, may also contribute to pasireotide-induced hyperglycemia[17,21,42]. In the present study, by focusing on intestinal L-cells and pancreatic β-cells, we discovered the commonalities and differences between insulin and GLP-1 secretions, using GLUTag cells and MIN-6 cells. However, since they are tumor-derived cells, they are not perfect models and represent a limitation of this study. In the future, the use of primary intestinal cells and islets should be considered.

Fourth, as the clinical data in this study was collected in 2017, semaglutide had not yet been available at that time. The potential effects of semaglutide on pasireotide-induced hyperglycemia are highly intriguing and warrant further investigation. In the future, semaglutide is likely to emerge as a more suitable treatment option for pasireotide-induced hyperglycemia.

Conclusion

We have found that insulin and GLP-1 secretions share striking commonalities in signaling, yet each exhibits different unique properties that may have developed evolutionarily and purposefully. Gs-coupled receptor stimulation primarily induces GLP-1 secretion, which is amplified by high glucose levels in GLUTag cells. In

contrast, high glucose levels primarily trigger insulin secretion, which is amplified by GLP-1 receptor stimulation in GLP-1R-MIN-6 cells. We hypothesize that the drastic inhibition of GLP-1 secretion via the shutdown of the main pathway through the SSTRs-Gi axis is the primary cause of pasireotide-induced hyperglycemia. We propose that GLP-1 analogs are superior to DPP-4 inhibitors for managing pasireotide-induced hyperglycemia because GLP-1 analogs, unlike DPP-4 inhibitors, spare pasireotide-induced depletion of endogenous GLP-1 and restore insulin secretion suppressed by pasireotide through GLP-1R-Gs activation, counteracting the SSTRs-Gi axis. We believe that Gs-coupled GPCRs in intestinal L-cells, the "main pathway for GLP-1 secretion," could serve as novel drug targets by acting as primary signals for GLP-1 secretion. This understanding provides a basis for the "eat protein first" strategy.

Methods

Cell culture, expression constructs, and transfection

MIN-6 (purchased from ATCC) cells and GLUTag cells (obtained from Takashi Tsuboi, the university of Tokyo, Japan) were cultured in DMEM with 10% (v/v) heat-inactivated fetal bovine serum. An expression construct encoding a human FLAG-tagged GLP-1R in pcDNA3.1, along with a hygromycin-resistant gene, was created by FLAG for subcloning a PCR product-tagged human GLP-1R into a cytomegalovirus promoter vector. MIN-6 cells were transfected with constructs encoding the human FLAG-tagged GLP-1R in the hygromycin-resistant vector pcDNA3.1 with Lipofectamine 2000 (Thermo Fisher Scientific, Massachusetts, USA)[3,8,43]. As previously stated, clones were selected in a medium with 0.4 mg/mL hygromycin[8,44]. MIN-6 cells, GLUTag cells and MIN-6 cells stably expressing GLP-1R (GLP-1R-MIN-6 cells) were routinely tested and found to be negative for mycoplasma.

cAMP assay

In 96-well plates, $2.0–3.0 \times 10^5$MIN-6 stably expressing GLP-1R (GLP-1R-MIN-6 cells) or GLUTag cells were seeded. Two days later, GLP-1, dulaglutide, DCA, forskolin, L-glutamine, sodium butyrate, and 007-AM (8-pCPT-2-O-Me-cAMP-AM), as well as glucose in Hank's Balanced Salt Solution (HBSS) containing 0.01% (v/v) bovine serum albumin (BSA), 5-mM HEPES, and 0.5-mM 3-isobutyl-1-methylxanthine (IBMX), were applied to the cells at a volume of 50 µL per well and incubated for 60 min. When investigating the effects of pasireotide and pertussis toxin (PTX), the cells were preincubated with pasireotide for 30 min and PTX for 4 h, respectively[10,11]. The cells were then permeabilized in the presence of 1% (v/v) Triton X-100, and the supernatants of the cells were collected. The LanceUltracAMP Kit (Perkin Elmer, Massachusetts, USA), was used to measure cAMP according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay for the measurement of insulin

In the 96-well plates, MIN-6 cells stably expressing GLP-1R (GLP-1R-MIN-6 cells) were plated at a density of $2.0–3.0 \times 10^5$ cells per well. Two days after plating, various concentrations of glucose, GLP-1, and 007-AM in the modified Krebs-Ringer bicarbonate HEPES buffer (KRBH: 129-mM NaCl, 5-mM $NaHCO_3$, 4.8-mM KCl, 1.2-mM KH_2PO_4, 1.2-mM $MgSO_4$, 10-mM HEPES, 1-mM $CaCl_2$) containing 0.1% (v/v) BSA and 0.1-mM glucose were administered to the cells, which were then incubated for 1 h. To investigate the effects of pasireotide, ESI-09, H-89, and PTX were examined; the cells were preincubated with pasireotide for 1.5 h, PTX for 4–6 h, H-89, and ESI-09, respectively. The supernatants were analyzed using the Mouse Insulin ELISA Kit (Mercodia, Sweden).

Enzyme-linked immunosorbent assay for measuring active GLP-1

In 24-well plates, GLUTag cells were seeded at a density of $1.0–2.0 \times 10^5$ cells per well. Two days after plating, various concentrations of glucose, DCA, triplete, and 007-AM in modified Ringer buffer (RB: 140 mM NaCl, 3.5 mM KCl, 0.5 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 10-mM HEPES, and 2-mM $NaHCO_3$) containing DPP-4 inhibitor (25 µM) and 0.5% (v/v) BSA were administered to the cells, which were then incubated for 2 h. To investigate the effects of pasireotide, H-89, ESI-09, and PTX, the cells were preincubated with pasireotide for 2.5 h, PTX for 4–6 h, H-89, and ESI-09, respectively. The supernatants were analyzed using the Glucagon-Like Peptide-1 (Active) ELISA Kit (Millipore Sigma, Saint Louis, MO, USA)[28]. The term "triplet*" refer to a high glucose (25 mM) level with 10 mM L-glutamine and 5 mM sodium butyrate.

RT-PCR analysis

The RNeasy Protect Mini Kit was used to extract total RNA (Qiagen, KJ Venlo, The Netherlands). ReverTraR Ace qPCR RT Master Mix with gDNA Remover and Blend TaqR was utilized to produce cDNA and to amplify the synthesized cDNA (Toyobo Co., Ltd., Science Department, Osaka, Japan).

RNA-seq analysis

RNA-seq was performed for Macrogen Japan Incorporated (Kyoto, Japan). StringTie was used to determine the relative abundance of genes on read counts, and statistical analysis was conducted using the estimates of abundance for each gene in samples to identify differentially expressed genes.

Statistics

Two-sided Tukey–Kramer multiple comparison test was used for statistical analysis[10,45]. The JMP Pro 17 software was utilized to conduct all analyses. Data are expressed as means (SEM). Error bars reflect the SEM unless otherwise noted, and the data are averaged across more than three different experiments. For all analyses, $P < 0.05$ was considered statistically significant. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant.

MMTT was performed in 3 days after 25 mg daily of alogliptin was changed to 0.75 mg weekly of dulaglutide, leaving the other antidiabetic drugs and insulin glargine unchanged.

Data availability

The data sets during the current study are available upon reasonable request to the corresponding authors.

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Author contributions
Junichiro SATO, Hirofumi HORIKOSHI, Maho TAGUCHI, and Noriko MAKITA performed the experiments and/or acquired the data. Junichiro SATO, Katsunori MANAKA, Taro IIRI and Noriko MAKITA analyzed the data. Junichiro SATO, Taro IIRI, and Noriko MAKITA. designed the study and wrote the manuscript. All authors discussed the manuscript and approved the final version. Noriko MAKITA and Taro IIRI are the quarantors of this work and, as such, had full access to all the data in the study and takes responsibility for integrity of the data and the accuracy of the data analysis.

Declarations

Competing interests
The authors declare no competing interests.

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