Amino acids differ in their capacity to stimulate GLP-1 release from the perfused rat small intestine and stimulate secretion by different sensing mechanisms

© Ida Marie Modvig,1,2 Rune Ehrenreich Kuhre,1,2 Sara Lind Jepsen,1,2 Stella Feng Sheng Xu,1,2 Maja Storm Engelstoft,1 Kristoffer Lihme Egerod,1 Thue Walther Schwartz,1 Cathrine Ørskov,2 Mette Marie Rosenkilde,2 and Jens Juul Holst1,2

1Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark and 2Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Abstract

The aim of this study was to explore individual amino acid-stimulated GLP-1 responses and the underlying stimulatory mechanisms, as well as to identify the amino acid-sensing receptors involved in amino acid-stimulated GLP-1 release. Experiments were primarily based on isolated perfused rat small intestines, which have intact epithelial polarization allowing discrimination between luminal and basolateral mechanisms as well as quantitative studies of intestinal absorption and hormone secretion. Expression analysis of amino acid sensors on isolated murine GLP-1 secreting L-cells was assessed by qPCR. We found that L-valine powerfully stimulated GLP-1 secretion but only from the luminal side (2.9-fold increase). When administered from the vascular side, L-arginine and the aromatic amino acids stimulated GLP-1 secretion equally (2.6- to 2.9-fold increases). Expression analysis revealed that Casr expression was enriched in murine GLP-1 secreting L-cells, whereas Gpr35, Gprc6a, Gpr142, Gpr93 (Lpar5), and the umami taste receptor subunits Tas1r3 and Tas1r1 were not. Consistently, activation of GPR35, GPR93, GPR142, and the umami taste receptor with specific agonists or allosteric modulators did not increase GLP-1 secretion (P > 0.05 for all experiments), whereas vascular inhibition of CaSR reduced GLP-1 secretion in response to luminal infusion of mixed amino acids. In conclusion, amino acids differ in their capacity to stimulate GLP-1 secretion. Some amino acids stimulated secretion only from the intestinal lumen, whereas other amino acids exclusively stimulated secretion from the vascular side, indicating that amino acid-stimulated GLP-1 secretion involves both apical and basolateral (postabsorptive) sensing mechanisms. Sensing of absorbed amino acids involves CaSR activation as vascular inhibition of CaSR markedly diminished amino acid stimulated GLP-1 release.

NEW & NOTEWORTHY Using isolated perfused rat small intestines, we show that amino acids differ in their mechanisms and capacity of stimulating GLP-1 release. Furthermore, we demonstrate that sensing by GPR142, GPR35, GPR93, and the umami taste receptor (Tas1R1/Tas1R3) are not involved in amino acid stimulated GLP-1 release. In contrast to previous studies, this experimental model allows discrimination between the luminal and the vascular side of the intestine, which is essential when studying mechanisms of amino acid-acid-stimulated GLP-1 secretion.

amino acids; CaSR; GLP-1 secretion; GPCR sensing; L-cells

INTRODUCTION

Ingestion of dietary proteins and amino acids has long been known to stimulate the secretion of the appetite-inhibiting and blood glucose lowering gut hormone, glucagon-like peptide 1 (GLP-1) (1–7). Ingested protein thereby contributes to postprandial metabolic and appetite control in animals and humans. Currently, GLP-1 mimetics are being used for the treatment of obesity and type 2 diabetes (8–10). An alternative approach would be to enhance the endogenous GLP-1 secretion (as seen after gastric bypass surgery) to mimic the beneficial effects of GLP-1, potentially with fewer side effects. As dietary proteins are believed to elicit the greatest appetite-suppressive effect of all macronutrients (11), possibly by stimulating the release of various anorectic hormones including peptide YY (PYY), cholecystokinin (CCK), and GLP-1 (6, 7, 12–15), and as a recent study has shown that a protein preload was able to improve the efficacy of the dipeptidyl peptidase-4 (DPP-4) inhibitor to increase incretin concentrations, slow gastric emptying, and reduce
postprandial glycaemia in patients with type 2 diabetes (16), strategies based on the mechanisms underlying these effects could be valuable in the treatment of type 2 diabetes and obesity. In this context, a detailed knowledge of the mechanisms involved in the sensing of dietary peptides and amino acids by the enteroendocrine cells is essential. However, our knowledge of the mechanisms whereby the individual amino acids stimulate GLP-1 secretion is limited.

Sensing of amino acids by enteroendocrine cells is believed to occur by three modes of actions: 1) by electrogenic amino acid uptake by different amino acid transporters, 2) by intracellular metabolism, which may result in cell depolarization by closure of ATP-sensitive potassium channels, or 3) by activation of different nutrient receptors, including G-protein coupled receptors (GPCRs), which then initiate intracellular signaling cascades leading to release of calcium from intracellular stores or increased cyclic adenosine monophosphate (cAMP) production, both of which may result in hormone release (Fig. 1).

Some amino acids are known to be transported in a sodium-coupled manner, which might depolarize cell membranes, followed by voltage-dependent calcium entry (Fig. 1). In addition, several GPCRs including the calcium-sensing receptor (CaSR), GPR142, GPR93, GPR35, GPRC6A (GPCR, Class C, group 6, subtype A), and the umami taste receptor (Tas1R1/Tas1R3) have been demonstrated in various static in

Figure 1. Sensing of amino acids by enteroendocrine L-cells. Sensing of amino acids by enteroendocrine L-cells is believed to occur by three modes of action: 1) through electrogenic amino acid and peptide uptake, which may result in cell depolarization leading to opening of voltage-gated calcium channels ($Ca^{2+}_{V}$) and hormone secretion (as indicated by red arrows), and 2) intracellular metabolism of amino acids and peptides may lead to closure of ATP-sensitive potassium channels ($K_{ATP}$), which depolarizes the cell membrane again leading to opening of voltage-gated calcium channels, calcium influx, and hormone release (indicated by green arrows), or 3) absorbed amino acids may stimulate nutrient receptors, including G-protein coupled receptors (GPCRs), which then initiate intracellular signaling cascades leading to release of calcium from intracellular stores or increased cyclic adenosine monophosphate (cAMP) production, both of which may result in hormone release (indicated by blue arrows).
vitro models to bind and respond to dietary peptides and amino acids accompanied by release of gastrointestinal hormones like CCK, glucose-dependent insulinotropic polypeptide (GIP), GLP-1, and PYY (17–25).

Several amino acids have been demonstrated to be potent stimulators of GLP-1 release in vivo and in vitro (4, 5, 26–30), however, detailed characterizations of relative efficacy of the individual amino acids are rare, and important aspects of the responsible mechanisms remain unknown, since neither studies in vivo nor conventional in vitro studies can discriminate between stimulation of GLP-1 secretion from the luminal or vascular side of the gut. In addition, further identification of the repertoire of receptors responsible for sensing of the individual amino acids is needed. The aim of this study was therefore to identify amino acid-sensing receptors involved in GLP-1 secretion, and to assess the relative stimulatory potential of the individual amino acids as well as to elucidate the responsible stimulatory mechanisms. We hypothesized that the underlying mechanisms would differ with some amino acids stimulating GLP-1 secretion from the luminal side and others from the vascular side of the intestine. We also speculated that the aromatic amino acids would be the most powerful secretagogues, as these have been shown to activate both CaSR and GPR142, which both stimulate secretion of GLP-1 in vivo and in vitro (18, 25, 31).

## MATERIALS AND METHODS

### Ethical Considerations

Studies were conducted with permission from the Danish Animal Experiments Inspectorate (2018-15-0201-01397) and the local ethical committee (EMED, P18-336) in accordance with the guidelines of Danish legislation governing animal experimentation (1987) and the National Institute of Health.

### Animals

Male Wistar rats (∼220 g) were obtained from Janvier (Le Genest-Saint-Isle, France), housed two to four rats per cage, and kept on a 12:12-h light/dark cycle with ad libitum access to water and standard chow. Rats were allowed at least 1 wk of acclimatization before the experiments.

GLU-Venus mice were derived from an in-house breed, originally generated at University of Cambridge (32). They were housed 2–8 per cage with free access to standard rodent chow under a 12:12-h light/dark cycle.

### Isolation and Perfusion of the Proximal Rat Small Intestine

Nonfasted rats (∼220 g) were anesthetized with a subcutaneous injection of hypnorm/midazolam (0.0158 mg fentanyl citrate + 0.5 mg fluanisone + 0.25 mg midazolam/100 g). The proximal part of the small intestine was surgically isolated by ligation of the blood supply and removal of the colon and the distal half of the small intestine as described previously (23). A plastic tube was inserted into the lumen of the intestine and the intestine was gently flushed with heated (37°C) isotonic saline to remove luminal contents. A catheter was placed into the superior mesenteric artery and the intestine was perfused with heated (37°C) and oxygenated (95% O₂ and 5% CO₂) perfusion buffer (pH 7.4) at a flow rate of 7.5 mL/min using a single-pass perfusion system (UP100, Hugo Sachs Harvard Apparatus, Germany).

The venous effluent was collected every minute via a draining catheter inserted into vena porta. As soon as proper flow was apparent, the rats were euthanized by perfusion of the diaphragm. The intestine was perfused for 25 min before initiation of the experimental protocol for stabilization. Each protocol started with a baseline period followed by addition of various test substances applied either into the intestinal lumen or intra-arterially through the tube inserted in the lumen or through the catheter in the upper mesenteric artery. Effluent samples were immediately placed on ice and stored at −20°C until analysis.

### Test Compounds

All test substances used for perfused intestine studies were purchased from Sigma Aldrich (Brøndby, Denmark) unless otherwise stated. Test stimulants included the following compounds infused to reach the following final perfusate concentrations: 10 μmol/L Compound 10 (GPR35 agonist, Cat. No. SML0174), 25 μmol/L NPS2143 (CaSR inhibitor, Cat. No. SML0362), 10 mmol/L inosine 5′-monophosphate (IMP, potentiator of TasiRI/TasIR3, Cat. No. 57510), 10 μmol/L oleoyl-L-α-lysophosphatidic acid (LPA, GPR93 agonist, Cat. No. L77260), 10 mmol/L 2-amino-2-norbornecarboxylic acid (BCH, amino acid analogue, Cat. No. A7902), 10 μmol/L U73122 (PLC inhibitor, Cat. No. B4272), 50 mmol/L dipeptiven (L-alanyl and L-glutamine, Cat. No. H6034), L-ornithine (Cat. No. 75469), L-histidine (Cat. No. P5607), L-threonine (Cat. No. T8441), and L-tryptophan (Cat. No. G5792), L-glutamic acid (Cat. No. G8415), L-cysteine (Cat. No. C7880), L-methionine (Cat. No. M5308), L-phenylalanine (Cat. No. P5482), L-arginine (Cat. No. A6969), L-proline (Cat. No. F5607), L-ornithine (Cat. No. 75469), L-histidine (Cat. No. H6034), L-threonine (Cat. No. T8441), and L-tryptophan (Cat. No. T8941).

Perfusion buffer was a modified Krebs–Ringer bicarbonate buffer supplemented with 3.5 mmol/L glucose, 0.1% (w/v) bovine serum albumin (Cat. No. 1.2018.0500, Merck, Denmark), 5% (w/v) dextran T-70 (Pharmacosmos, Denmark), 5 mmol/L of each glutamate, pyruvate, and fumarate (Sigma Aldrich, Brøndby, Denmark), and 10 μmol/L 3-isobutyln-L-methylxanthine (IBMX, Cat. No. S879, Sigma Aldrich). Luminal test solutions were prepared in isotonic saline whereas vascular test solutions were prepared in perfusion buffer.

### Hormone and L-Amino Acid Measurement

Total GLP-1 (the sum of 7–36NH₂, 9–36NH₂, and potential mid-sequence cleaved fragments) was measured with
an in-house radioimmunoassay employing a C-terminal specific antibody targeting amidated forms of GLP-1 (code no. 89390) (33).

Total amino acid concentrations were measured using a colorimetric 1-amin0 acid assay kit from Abcam (Cat. No. ab65347, Cambridge, UK) following provided instructions. The sensitivity of the assay is reported to be 40 pmol/L. The assay detects all L-amino acids except glycine.

**Isolation of Murine L-Cells and Quantitative PCR Expression Analysis**

L-cells were isolated from intestinal segments of 30 male C57BL/6JR transgenic GLU-Venus mice expressing the fluorescent Venus protein driven by the proglucagon promoter (32) as previously described (34). Cells were sorted by fluorescence-activated cell sorting (FACS) using a BD FACSARia II (BD Biosciences, Palo Alto, CA) yielding a GLU-Venus positive or GLU-Venus negative fraction of cells. The RNA was extracted using NucleoSpin RNA XS kit (Cat. No. 740902, Macherey-Nagel, Germany). The cDNA was generated using QuantiTect Whole Transcriptome kit (Cat. No. 207043, Qiagen, Germany). Expression was analyzed using custom-designed 384-well quantitative PCR plates from Lonza, Qiagen, Germany). Expression was analyzed using qRT-PCR (qPCR) (3). The sequences used for the receptors and housekeeping genes are published elsewhere (35). Expression of Gpr6ca, Casr, Gpr35, and Gpr142, 10 mice were pooled in each run of quantitative PCR (qPCR) (n = 3). The sequences used for the receptors and housekeeping genes are published elsewhere (35). Expression of Gpr6ca, Casr, Gpr35, and Gpr142 is shown as an expression relative to a mean of three housekeeping genes: 18S ribosomal RNA (Rn18s), tyrosine 3-monooxygenase (YWHAZ), and hypoxanthine phosphoribosyltransferase 1 (HPRT1). Relative expression of Lpar5, Tas1r1, and Tas1r3 was analyzed from another dataset published elsewhere (36). The relative copy number was calculated according to the formula: Relative copy number = (2 \(^{-}\Delta C_{T}}\text{Target} - \Delta C_{T}\text{DNK}/NF) \times C$, where $C_{T}\text{Target}$ is the CT value of the receptor in the cDNA sample. $C_{T}\text{DNA}$ is the CT value of the receptor in a genomic DNA sample containing all assayed genes. NF is a GeNorm derived normalization factor using all genes with CT values below 35 in all samples. C is an arbitrary constant dependent on the DNA concentration, in this case $C = 11,585$, consequently CT values of 35 are on average equal to one relative copy. Undetectable targets were assigned a CT value of 40 (35).

**Immunohistochemistry**

CaSR and GLP-1 immunoreactivity was tested in archival paraffin-embedded specimens of human small intestine (jejunum, n = 3). The tissue blocks were sectioned on a microtome (section thickness 5 μm) and sections were placed on glass slides. The sections were dewaxed and subjected to antigen retrieval by boiling for 15 min in citrate buffer of pH 6. Next, sections were pretreated with 2% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS), all washing steps were carried out in PBS. The sections were then incubated overnight at 4°C with a mixture of the primary antibodies against GLP-1 and CaSR. Antibodies were in-house rabbit GLP-1 antibody 2135 and mouse CaSR antibody (19347, Abcam, Cambridge, UK), both diluted 1:2,500 in PBS with 2% BSA. On day 2, slides were incubated with a mix of Alexa 488-labeled donkey anti-mouse antibody (1:200) and Alexa 568-labeled donkey anti-rabbit (1:200) for 1 h, washed, mounted with Dako fluorescence mounting medium (Agilent, Santa Clara, CA), and coverslipped. The slides were then examined using an Axioscope 2 plus microscope (Zeiss, Jena, Germany) and images were taken using a CoolSNAP camera (Photometrics, Tucson, AZ).

**Calculations and Statistical Analysis**

Hormone concentrations (pmol/L to fmol/mL) and amino acid concentrations (μmol/L) in the venous effluents are presented as means ± SE. As perfusion flow was constant throughout the experiments, the actual hormone secretion rate (fmol/min) can be calculated by multiplying with the flow rate (7.5 mL/min).

To test for statistical significance, mean values from the test period (based on 10 consecutive minutes) were compared with mean values from the baseline period (10 min before administration of test stimulant) using Student’s t test or one-way ANOVA followed by Dunnett multiple comparisons test, as indicated in figure legends. Statistical testing and construction of graphs was done in GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA). Adobe CC software (San Francisco, CA) was used for illustrations. P values <0.05 were considered statistically significant.

**RESULTS**

**Individual Amino Acid-Stimulated GLP-1 Secretion**

To assess potential differences in amino acid-stimulated GLP-1 secretion, we initially administered individually all amino acids except L-tyrosine into the lumen of the perfused rat’s small intestine at a concentration of 50 mmol/L (1-tyrosine was not soluble at the desired concentration), and measured GLP-1 concentrations in the venous effluent (each graph can be seen in Supplemental Fig. S1; see https://doi.org/10.6084/m9.ﬁgshare.13607333, n = 4 for all groups). For these experiments, bombesin (BBS) was used as a positive control for GLP-1 secretion and was administered by the end of the experiments (Supplemental Fig. S1).

The branched chain amino acid, L-valine, and the aromatic amino acid, L-phenylalanine, increased GLP-1 secretion 2.9-fold and 1.9-fold (P < 0.05, Fig. 2A), with L-valine being the most powerful luminal stimulator of all administered amino acids. As L-glutamine is unstable at physiological pH (37, 38), we infused a stable dipeptide isoform of L-glutamine, alanyl-L-glutamine (Dipeptiven), to ensure the stability of L-glutamine in our preparation. Dipeptiven likewise increased GLP-1 secretion 1.9-fold (P < 0.05, Fig. 2A), L-Leucine, L-serine, L-isoleucine, L-glutamine, and L-glutamic acid also increased GLP-1 secretion, but the responses were transient and nonsignificant (Fig. 2A).

We next infused the same amino acids intra-arterially (at 20 mmol/L) (each experiment can be seen in Supplemental Fig. S1, n = 4 for all groups). In these experiments, L-arginine served as a positive control for GLP-1 secretion, as vascular infused L-arginine powerfully stimulated GLP-1 secretion in pilot studies (data not shown, n = 6). Thus, the L-cell amino acid responses thereby intentionally could be compared with that induced by L-arginine.
Figure 2. Individual amino acid-stimulated glucagon-like peptide-1 (GLP-1) secretion from the perfused rat intestine. A and B, B1: fold changes of GLP-1 levels (pmol/L) relative to baseline levels (before amino acid administration) in the venous effluent. Amino acids were administered intraluminally at a concentration of 50 mmol/L and intravascularly at a concentration of 20 mmol/L, n = 4 male Wistar rats/group. Total amino acid concentration in the venous effluent (μmol/L) following intraluminal administration of L-valine (C), L-phenylalanine (D), sialyl-L-glutamine (dipeptiven) (E), L-glutamine (F), L-arginine (G), and L-tryptophan (H) between minute 11 and 25, n = 4 male Wistar rats/group. I and J, L-valine was intraluminally infused between minute 11 and 25 and between minute 46 and 60. An amino acid analog, 2-amino-2-norbornanecarboxylic acid (BCH; 10 mmol/L), was intraluminally infused between minute 30 and 60 to block the absorption of L-valine, n = 6 male Wistar rats. I, total GLP-1 concentrations (pmol/L) in venous effluent and mean baseline-subtracted GLP-1 concentrations in response to L-valine 2 stimulation and L-valine 2 stimulation with the addition of BCH. J, total amino acid concentrations (μmol/L) in venous effluent and mean baseline-subtracted amino acid concentrations in response to L-valine 2 stimulation and L-valine 2 stimulation with the addition of BCH. Data are shown as means ± SE. NS, nonsignificant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by a One-way ANOVA with correction for multiple testing (Dunnett).
Compared with intraluminal administration, more amino acids increased GLP-1 secretion when infused intra-arterially, and the GLP-1 responses observed were in general greater, suggesting that amino acids predominantly stimulate GLP-1 secretion by basolateral activation of the intestinal L-cells. Of particular note, vascular but not luminal infusion of l-arginine and l-tryptophan resulted in 2.9- and 2.7-fold increases in GLP-1 secretion \((P < 0.05, \text{Fig. 2BI, } n = 4)\), indicating that these amino acids stimulate GLP-1 secretion mainly through postabsorptive mechanisms. This stands in contrast to l-valine (and l-glutamine), which only stimulated GLP-1 secretion when given intraluminally (\text{Fig. 2BI, } n = 4). Thus, amino acids stimulate secretion both by activation of luminal sensors/uptake mechanisms and by postabsorptive mechanisms (i.e., by basolateral uptake or receptor activation).

l-Phenylalanine stimulated GLP-1 secretion from both sides of the intestine \((P < 0.05, \text{Fig. 2, A and B})\), but the vascular response was considerably higher (2.6- vs. 1.9-fold higher than baseline, \(P = 0.0005\) vs. \(P = 0.0145\)) (\text{Fig. 2, A and B, } n = 4). As the luminally induced GLP-1 response may reflect a postabsorptive “intra-arterial” response, we decided to evaluate the absorption rate of luminal infused amino acids.

**Luminally infused l-valine is efficiently absorbed in contrast to l-arginine and l-tryptophan.**

To evaluate the absorption rate of luminally infused amino acids, the total amino acid concentrations in the venous effluent during luminal amino acid administration was measured (Supplemental Fig. S2; see https://doi.org/10.6084/m9.figshare.13607501). Luminally infused l-valine rapidly appeared in the venous effluent, reflecting a rapid and efficient absorption (baseline: 355.9 \pm 30.4 vs. l-valine: 1,858 \pm 143.3 \mu\text{mol/L, } P < 0.05, n = 4) (\text{Fig. 2C}). l-Phenylalanine was likewise efficiently absorbed (baseline: 300.5 \pm 54.9 vs. l-phenylalanine: 1,138 \pm 189.4 \mu\text{mol/L, } P < 0.05, n = 4) (\text{Fig. 2D}), however, the concentration in the vascular effluent was lower than that of l-valine, although infused at similar concentrations (baseline subtracted; l-valine: 1,502 \pm 166 \mu\text{mol/L vs. l-phenylalanine: 838 \pm 160 \mu\text{mol/L, } P < 0.05}).

When infusing the dipeptide, alanyl-l-glutaminate (dipeptiven), l-α-lanyl, and l-glutaminate rapidly appeared in the venous effluent (baseline: 231 \pm 17.2 vs. dipeptiven: 1,184 \pm 95.3 \mu\text{mol/L, } P < 0.05, n = 4) (\text{Fig. 2E}), similar to infusion of luminal l-glutamine (baseline: 333.9 \pm 40.8 vs. l-glutamine: 1,524 \pm 345.3 \mu\text{mol/L, } P < 0.05, n = 4) (\text{Fig. 2F}), indicating that l-glutamine was stable in this experimental setup. In contrast, l-arginine was poorly absorbed (baseline: 248 \pm 40.8 vs. l-arginine: 547.8 \pm 71.1 \mu\text{mol/L, } P < 0.05, n = 4) (\text{Fig. 2G}), and l-tryptophan did not appear to be absorbed at all (baseline: 419 \pm 39.2 vs. l-tryptophan: 368.5 \pm 20.3 \mu\text{mol/L, } P > 0.05, n = 4) (\text{Fig. 2H}), illustrating important differences in the absorptive capacity of individual amino acids from the proximal rat small intestine.

To validate whether these amino acids were all detected by the l-amino acid assay to a similar extent, we tested the recoveries of the individual amino acids used in the perfusion experiments. As in the perfusion experiments, amino acids were prepared in perfusion buffer. At a concentration range between 0.375 and 3 mmol/L, the recovery of all amino acids was measured (Supplemental Fig. S2). At a concentration of 3 mmol/L, the majority of amino acids could be detected with a recovery close to 100%, however, some amino acids could not be detected at this concentration (serine, threonine, glutamate, aspartate, proline, cysteine, and lysine). At a concentration range lower than 3 mmol/L, the recoveries were far from 100% except for histidine (Supplemental Fig. S2).

**Inhibition of L-type amino acid transporter 2 does not affect the absorption of l-valine or l-valine-mediated GLP-1 secretion.**

As we hypothesized that luminal l-valine stimulates GLP-1 secretion through mechanisms directly linked to the absorption, we infused l-valine intraluminally together with 2-amino-2-norbornanecarboxylic acid (BCH; 10 mmol/L), an amino acid analog inhibiting transport by system L-transporters, which transport neutral amino acids like l-valine, (\text{Fig. 2I, } n = 6). At the concentration employed, BCH is reported to inhibit transport by all members of the L-type amino acid transporter (LAT) family (system L transporters) but also the apical sodium-coupled amino acid transporters, B\text{A}T1 and B\text{AT}2, which likewise transport neutral amino acids like l-valine (39–41). Infusion of BCH in combination with l-valine did not affect the secretory GLP-1 response to luminally infused l-valine (baseline-subtracted values; l-valine 2: 4.5 \pm 0.7 vs. l-valine + BCH: 6.6 \pm 2.3 \mu\text{mol/L, } P = 0.4102, n = 6) (\text{Fig. 2I}), presumably because the absorption of l-valine was also unaffected (baseline-subtracted values; l-valine 2: 418.5 \pm 49.5 vs. l-valine + BCH: 386.5 \pm 94 \mu\text{mol/L, } P = 0.7701, n = 6) (\text{Fig. 2J}), suggesting that l-valine is being absorbed through other transporters, or that the BCH used was inactive in our experimental setting.

As inhibiting amino acid transport was unsuccessful in this experimental setup, we next continued investigating the amino acid sensors involved in GLP-1 secretion.

**Expression of Amino Acid Sensors in Murine and Human Enteroendocrine L-Cells**

To examine which receptors are involved in sensing of amino acids by the intestinal L-cells, we investigated the expression of selected amino acid sensors in enteroendocrine L-cells. Intestinal cells obtained from duodenum, jejunum, and ileum of transgenic GLU-Venus mice were sorted using fluorescence-activated cell sorting (FACS) giving rise to GLU-Venus positive (GLP-1 producing L-cells) and GLU-Venus negative (intestinal cells not expressing the proglucagon promoter) cell populations. In all intestinal segments (duodenum, jejunum, and ileum), the expression of CaSR was enriched in GLP-1 secreting L-cells compared with neighboring intestinal cells (GLU-Venus negative cells) (\text{Fig. 3, A–C}), supporting that CaSR may be involved in stimulation of GLP-1 secretion. \text{Gpr}35 and \text{Gpr}142 were likewise expressed in GLP-1 secreting L-cells (GLU-Venus positive cells), however, the expression was not enriched compared with neighboring cells in neither of the intestinal segments examined (\text{Fig. 3, A–C}). \text{Gpr}6ca1 was not detected in either GLU-Venus negative or GLU-Venus positive cells, suggesting that this receptor is not involved in sensing of amino acids by intestinal cells in duodenum, jejunum, and ileum of mice. \text{Gpr}93 (\text{Lpar}5) and umami taste receptor subunit, \text{Tas}3r3, were both expressed in GLP-1 secreting L-cells (GLU-Venus positive cells), however, the
expression was not enriched compared with neighboring GLU-Venus negative cells in the upper part (~10 cm) of the proximal mouse small intestine (32) (Fig. 3D). The expression of the other subunit of the umami taste receptor, Tas1r1, was very low both in GLP-1 secreting L-cells as well as in the neighboring intestinal cells (GLU-Venus negative cells) (Fig. 3D).

To examine whether CaSR was likewise expressed on human endocrine L-cells, human jejunal tissue was immunohistochemically co-stained for CaSR and GLP-1. CaSR and GLP-1 frequently colocalized with few GLP-1 positive cells without CaSR and many CaSR positive cells without GLP-1 (Fig. 3E, n = 3), suggesting that CaSR is also involved in other functions in the gastrointestinal tract.

**Amino Acid Sensors Involved in the Stimulation of GLP-1 Secretion**

To further investigate which receptors are involved in amino acid-stimulated GLP-1 secretion, as well as their localization (apical or basolateral) on the intestinal L-cells, we stimulated the perfused intestine with specific candidate receptor agonists both intra-arterially and intraluminally.

Stimulation of GPR35, a GPCR reported to bind mono- or dicarboxylic acids (42) as well as tryptophan and tyrosine metabolites (21), with the specific agonist, Compound 10 (10 μmol/L), did not increase GLP-1 secretion either when infused luminally or vascularily (baseline 1: 9.7 ± 1.5 vs. luminal: 11.1 ± 1.2 pmol/L, P = 0.2492, and baseline 2: 12.9 ± 1.5 vs. vascular: 12.1 ± 1.7 pmol/L, P = 0.7929, n = 6) (Fig. 4A).

Stimulation of GPR93 (LPAR5), a GPCR previously demonstrated to be involved in dietary peptide-stimulated CCK secretion (19, 43), with the specific agonist oleoyl-1-alpha-lysophosphatidic acid (LPA, 10 μmol/L), also did not increase GLP-1 secretion either when infused intraluminally or intra-arterially (baseline 1: 14.9 ± 0.9 vs. luminal: 16.4 ± 1.6 pmol/L, P = 0.8143, and baseline 2: 19.8 ± 1.4 vs. vascular: 18.6 ± 1.7 pmol/L, P = 0.8758, n = 6) (Fig. 4B).

Stimulation of GPR142, a GPCR reported to bind aromatic amino acids leading to insulin, GIP and GLP-1 release (25), with a specific agonist (LY3201143, 10 μmol/L, kindly provided by Eli Lilly) likewise had no effect on GLP-1 secretion from the perfused intestine either when infused intraluminally or intra-arterially (baseline 1: 15 ± 2.5 vs. luminal: 16.3 ± 2.6 pmol/L, P = 0.6994, and baseline 2: 19.5 ± 2.9 vs. vascular: 19.8 ± 3.4 pmol/L, P = 0.9951, n = 6) (Fig. 4C), suggesting that neither GPR35, GPR93, nor GPR142 is involved in stimulation of GLP-1 secretion from the perfused proximal rat intestine.

The potency of the specific GPR142 agonist used was validated in transfected HEK293 cells of transiently expressing

---

**Figure 3.** Expression of amino acid sensors in murine and human enteroendocrine L-cells. A–C: scattergram of the expression of Gprc6a, Gpr42, Gpr35, and Casr in intestinal cells obtained from duodenum, jejunum, and ileum of GLU-Venus mice. The expression levels are shown in relative to copy numbers in GLU-Venus-positive cells (y-axis) and in GLU-Venus-negative cells (x-axis). Dotted lines indicate 1-, 10-, and 100-fold enrichment of expression in GLU-Venus-positive vs. neighboring cells. In gray, the noise area for the analysis is indicated. Data are presented as expression levels relative to a mean of three reference genes: Rn18s, YWHAH42, and HPRT1, n = 3, 10 mice pooled/qPCR run. D: scattergram of the expression of Lpar5, Tas1r3, and Tas1r1 in intestinal GLU-Venus positive and GLU-Venus negative cells obtained from the upper proximal mouse small intestine (SI). The expression levels are shown in relative to copy numbers in GLU-Venus-positive cells (y-axis) and in GLU-Venus-negative cells (x-axis). Dotted lines indicate 1-, 10-, and 100-fold enrichment of expression in GLU-Venus-positive vs. neighboring cells, n = 3. E: immunohistochemical colocalization of calcium-sensing receptor (CaSR) and glucagon-like peptide-1 (GLP-1) in human jejunal tissue, n = 3. Yellow arrows indicate overlap, white arrows indicate CaSR positive cells, and blue arrows indicate GLP-1 positive cells.
rat GPR142 (Supplemental Fig. S3; see https://doi.org/10.6084/m9.figshare.13607537, n = 4), demonstrating that the agonist used potently activated rat GPR142 with an EC₅₀ of 3 nmol/L.

**Potentiation of Tas1R1/Tas1R3 does not increase L-glutamic acid-stimulated GLP-1 secretion.**

To investigate the potential involvement of the umami taste receptor (Tas1R1/Tas1R3), a GPCR reported to primarily respond to L-glutamic acid and L-aspartic acid in humans and to all amino acids in mice (44, 45), we infused inosine 5'-monophosphate (IMP, a positive allosteric modulator of Tas1R1/Tas1R3) in combination with L-glutamic acid (44). This did, however, not lead to a further increase in L-glutamic acid-stimulated GLP-1 secretion (baseline-subtracted values; luminal (−IMP): 2.1 ± 1.2 vs. luminal (+IMP): 5.3 ± 0.6 pmol/L, P = 0.1347, vascular (−IMP): 9.6 ± 2.5 vs. vascular (+IMP): 10 ± 2.6 pmol/L, P = 0.9996, n = 6) (Fig. 4D).
However, further studies with an inhibitor of Tas1R1, like Gurmarin, are needed to further clarify the involvement of Tas1R1/Tas1R3 in L-glutamic acid-stimulated GLP-1 secretion.

**Inhibition of calcium-sensing receptor with NPS2143 decreases the GLP-1 response to mixed amino acids but not to L-arginine.**

As we recently showed that basolaterally located CaSR is essential for protein-stimulated GLP-1 secretion (23), we next studied whether CaSR also mediates the GLP-1 response to luminal infusion of mixed amino acids (Vamin; 51 mg/mL). Inhibition of CaSR with a vascularily infused negative allosteric modulator, NPS2143 (25 μmol/L), decreased the amino acid-stimulated GLP-1 response (baseline-subtracted values; Vamin 2: 11.2 ± 2.6 vs. Vamin 2 + NPS2143: 4.2 ± 2 pmol/L, P = 0.058, n = 6) (Fig. 4E), indicating that CaSR is indeed involved in basolateral amino acid-stimulated GLP-1 secretion.

As L-arginine was the most powerful stimulator of GLP-1 release from the vascular side, and as CaSR seemed to play an important role in amino acid stimulated GLP-1 secretion, we next studied whether L-arginine stimulated GLP-1 secretion through activation of CaSR by infusing NPS2143 (25 μmol/L), while stimulating with vascular infused l-arginine (20 mmol/L) (Fig. 4F). Inhibition of CaSR had no effect on L-arginine-stimulated GLP-1 secretion (baseline-subtracted values; arginine 2: 24.2 ± 2.8 vs. arginine 2 + NPS2143: 31.6 ± 1.7 pmol/L, P = 0.0495, n = 6) (Fig. 4F), demonstrating that vascular l-arginine does not stimulate GLP-1 secretion through CaSR-mediated mechanisms.

**Opening of ATP-sensitive potassium channels does not affect amino acid-induced GLP-1 secretion.**

As a large amount of absorbed amino acids will be used by the intestinal cells for metabolism, a mechanism of amino acid sensing could also involve increased intracellular ATP/ADP ratio leading to closure of ATP-sensitive potassium channels (K$_{ATP}$-channels) and subsequently membrane depolarization (Fig. 1). To examine this, we administered the K$_{ATP}$-channel opener, diazoxide (250 μmol/L), while stimulating with luminal infused amino acids (Vamin; 51 mg/mL) (Fig. 4G). Opening of K$_{ATP}$-channels neither appeared to affect the basal GLP-1 secretion nor the GLP-1 response to mixed amino acids (baseline-subtracted values; Vamin 2: 11.2 ± 2.6 vs. Vamin 2 + diazoxide: 11.7 ± 1.3 pmol/L, P = 0.9161, n = 6) (Fig. 4G), suggesting that amino acids do not stimulate GLP-1 secretion through intracellular metabolism followed by closure of K$_{ATP}$-channels.

**Inhibition of phospholipase C decreases the GLP-1 response to mixed amino acids.**

To investigate further the intracellular pathway involved in amino acid-induced GLP-1 secretion, we infused a phospholipase C (PLC) inhibitor (U73122; 10 μmol/L), while stimulating with a mixture of luminal infused amino acids (Vamin). Inhibition of PLC decreased amino acid induced GLP-1 secretion significantly when subtracting baseline secretion (baseline-subtracted values; Vamin 2: 11.2 ± 2.6 vs. Vamin 2 + U73122: 2.2 ± 2.7 pmol/L, P < 0.05, n = 6) (Fig. 4H), suggesting that activation of PLC leading to formation of diacylglycerol and inositol triphosphate is involved in the mechanisms of amino acid stimulated GLP-1 release. This furthermore supports the involvement of CaSR in amino acid stimulated GLP-1 secretion, as CaSR is mainly found to be Gα$_{q}$-coupled, thereby leading to activation of PLC (46, 47).

**DISCUSSION**

Approximately 95% of dietary protein is absorbed in the small intestine (48). Of these, a considerable amount is used by the intestinal cells for metabolism (e.g., oxidation and protein synthesis), while the remainder will be released into the portal system (49, 50). As amino acids that are not metabolized by the intestinal cells will be transported to the interstitium to diffuse into the circulation, the local concentration of amino acids at the basolateral surface of the intestinal cells may reach high levels after a protein-rich meal.

In this study, the absorption of amino acids in the proximal rat small intestine was measured when stimulating with luminal infused amino acids. When administering individual amino acids intraluminally at a concentration of 50 mmol/L (corresponding to the total intraluminal concentration in the upper small intestine after a high protein meal) (51–53), the amino acid concentration measured in the venous effluent increased from 0.5 mmol/L to ~2 mmol/L (except for L-tryptophan and l-arginine). In humans, plasma total amino acid

**Figure 4.** Amino acid sensors involved in stimulation of glucagon-like peptide-1 (GLP-1) secretion. A: GPR35. Total GLP-1 concentrations (μmol/L) in venous effluent and mean GLP-1 concentrations in response to GPR35 agonist infusion (Compound 10; 10 μmol/L). The GPR35 agonist was intraluminally infused between minute 11 and 25 and intravasically infused between minute 46 and 60, n = 6 male Wistar rats. B: GPR93. Total GLP-1 concentrations (μmol/L) in venous effluent and mean GLP-1 concentrations in response to GPR93 agonist infusion (lyso-phosphatidic acid, LPA; 10 μmol/L), n = 6 male Wistar rats. C: GPR42. Total GLP-1 concentrations (μmol/L) in venous effluent and mean GLP-1 concentrations in response to GPR42 agonist infusion (LY320143; 10 μmol/L), n = 6 male Wistar rats. D: Tas1R1/Tas1R3. Total GLP-1 concentrations (μmol/L) in venous effluent and mean baseline-subtracted GLP-1 concentrations in response to L-glutamate infusion alone (red curve) or L-glutamate infusion (20 mmol/L) in combination with inosine 5’-monophosphate (IMP; 10 μmol/L) (black curve), n = 6 male Wistar rats/group. E: calcium-sensing receptor (CaSR). Total GLP-1 concentrations (μmol/L) in venous effluent and mean baseline-subtracted GLP-1 concentrations in response to luminal Vamin infusion (51 mg/mL alone (Vamin 1 and 2; black curve) or in combination with vascular infusion of NPS2143, a negative allosteric modulator of calcium-sensing receptor (CaSR) (NPS2143; 25 μmol/L), during Vamin 2 infusion (red curve), n = 6 male Wistar rats/group. F: CaSR. Total GLP-1 concentrations (μmol/L) in venous effluent and mean baseline-subtracted GLP-1 concentrations in response to vascular L-arginine infusion (20 mmol/L) alone (Arginine 1 and 2; black curve) or in combination with vascular infusion of 25 μmol/L of L-glutamate (Arginine 2 infusion; red curve), n = 6 male Wistar rats/group. G: ATP-sensitive potassium channels (K$_{ATP}$-channel) opener. Total GLP-1 concentrations (μmol/L) in venous effluent and mean baseline-subtracted GLP-1 concentrations in response to luminal Vamin infusion (51 mg/mL alone (Vamin 1 and 2; black curve) or in combination with vascular infusion of U73122, a phospholipase C inhibitor (U73122; 10 μmol/L), during Vamin 2 infusion (red curve), n = 6 male Wistar rats/group. Data are shown as means ± SE. NS, nonsignificant, P > 0.05. *P < 0.05 by a one-way ANOVA with correction for multiple testing (Dunnett). BBS, bombesine.

DOI: 10.1152/ajpendo.00026.2021 • www.ajpendo.org
concentration at fasting is around 2 mmol/L and may increase up to 5 mmol/L after meal intake (51, 54).

The finding that l-tryptophan and l-arginine were not efficiently absorbed could explain why these amino acids did not stimulate GLP-1 secretion when infused intraluminally (Fig. 2A), although both powerfully stimulated GLP-1 release from the vascular side of the gut (Fig. 2B). The underlying reason of the differences in absorption rates of the individual amino acids warrants further investigation, but it is likely to be influenced by differences in absorption capacity of different transporters involved and/or differences in transporter expression along the small intestine as well as detection issues. However, in a physiological setting, regional differences in transporter abundance are presumably of less importance for the total rate of amino acid absorption as uptake of di- and tripeptides through peptide transporter 1 (PepT1) mainly is responsible for the amino acid absorption, since PepT1 transports several amino acid monomers for each turnover of the transporter (55). The dipeptides and tripeptides are thought to be cleaved into single amino acids intracellularly, and will be transported as such across the basolateral membrane (56).

The mechanisms of l-arginine- and l-tryptophan-stimulated GLP-1 release most likely involve postabsorptive sensing mechanisms, as vascularly infused l-arginine and l-tryptophan powerfully stimulated GLP-1 release. The sensing mechanisms of l-arginine-stimulated GLP-1 release remains to be established, but is unlikely to involve CaSR activation as inhibition of CaSR had no effect on vascular l-arginine-stimulated GLP-1 secretion.

The fact that l-phenylalanine both stimulated GLP-1 secretion when infused into the intestinal lumen and when infused through the vascular supply indicate that l-phenylalanine is a powerful stimulator of GLP-1 release. However, the luminally induced response could also reflect a postabsorptive basolateral response, as l-phenylalanine was efficiently absorbed (reaching a vascular concentration of 1 mmol/L). Furthermore, since l-phenylalanine is reported to activate CaSR (18, 31, 57), the stimulatory mechanism possibly involves postabsorptive CaSR-mediated sensing.

An important finding in this study was that l-valine is a powerful stimulator of GLP-1 release when infused into the lumen but not when administered intra-arterially. This suggests that an apical sensing mechanism, possibly coupled to sodium uptake, is involved. However, we were not able to identify the specific mechanism as blocking l-type amino acid transporters (SLC7A5, SLC7A8, SLC43A1, and SLC43A2) with BCH did not affect l-valine absorption, highlighting the complexity of the amino acid transporter systems with several transporters capable of transporting the same amino acids (perhaps some unknown as well).

Limitations of this study include that we were unable to include l-tyrosine in our analysis, since it was insoluble in isotonic saline and in our perfusion buffer at the desired concentrations. Furthermore, we did not test whether specific activation of GPRC6A, which preferentially binds the basic amino acids l-arginine and l-ornithine (58, 59), affected GLP-1 secretion from the perfused rat intestine like previously demonstrated in the murine colonic GLUTag cell line (24). However, as recent findings in mice in vivo questions the involvement of GPRC6A in GLP-1 secretion (27), and as Gprc6a was not expressed in murine intestinal cells (either in GLU-Venus positive or GLU-Venus negative cells), GPRC6A is most likely not involved in amino acid stimulated GLP-1 release. Moreover, the antagonists currently available for GPRC6A are the CaSR positive allosteric modulator, Calindol, and the CaSR negative allosteric modulator, NPS2143, which both have a 30-fold higher potency of binding to CaSR than GPRC6A (60, 61), making it difficult to differentiate between GPRC6A- and CaSR-mediated responses.

The expression of Gpr142, Gpr35, Gpr93 (Lpar5), Tas1r1, and Tas1r3 was not enriched in murine GLP-1 secreting L-cells (GLU-Venus positive cells) compared with neighboring intestinal cells (GLU-Venus negative cells) in line with the finding that activation of these receptors with specific agonists or allosteric modulators did not increase GLP-1 secretion from the perfused rat small intestine. However, the involvement of GPR142, GPR35, GPR93, and Tas1r1/Tas1r3 in secretion of other gut hormones, like GIP and CCK, deserves further investigation, as previous studies have demonstrated an important role e.g., for GPR142 in stimulation of GIP secretion in mice (25) and GPR93 in stimulation of CCK secretion from murine intestinal STC-1 cells (19).

### CONCLUSIONS

Amino acids differ markedly in their capacity to stimulate GLP-1 release and the mechanism of stimulation differs between the individual amino acids. Some amino acids only stimulated GLP-1 secretion when infused through the vascular supply (l-arginine and l-tryptophan), whereas others only stimulated secretion when infused intraluminally (l-valine and l-glutamine), suggesting that amino acids and therefore protein meals stimulate GLP-1 secretion both through apical absorptive and through postabsorptive mechanisms (by activation of basolateral CaSR and perhaps unidentified receptors). Amino acid sensing by GPR35, GPR93, GPR142, and Tas1r1/Tas1r3 does not seem to be involved in GLP-1 release (at least in the perfused proximal rat small intestine, which is the relevant part of the intestine to study as digested protein is rapidly absorbed before reaching the more distal part of the small intestine). Furthermore, closure of ATP-sensitive potassium channels as a result of intracellular amino acid metabolism does not seem to be involved in amino acid-stimulated GLP-1 release. Rather, activation of PLC may be involved in the mechanisms of amino acid-stimulated GLP-1 release as inhibition of PLC decreased mixed amino acid-induced GLP-1 release.

Finally, our findings suggest that dietary supplementation with l-valine may serve as a potential strategy to increase GLP-1 secretion.

### ACKNOWLEDGMENTS

We thank Ruth E. Gimeno, Eli Lilly, Indianapolis, Indiana for providing the GPR142 agonist, (LY3201143).

### GRANTS

The study was supported by a grant from the European Research Council Grant no. 695069 (to J. J. Holst) and an unrestricted grant to J. J. Holst from the Novo Nordisk Foundation Center for Basic Metabolic Research (Novo Nordisk Foundation,
Denmark). R.E. Kuhre was supported by a postdoctoral grant from Lundbeck foundation (Lundbeckfonden, R264-2017-3492).

DISCLOSURES
R.E. Kuhre is employed at Novo Nordisk, but worked exclusively at University of Copenhagen (Denmark) when this study was conceived, designed and the manuscript was drafted. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS
I.M.M., R.E.K., and J.J.H. conceived and designed research; I.M.M., S.L.J., M.S.E., K.L.E., and C.O. performed experiments; I.M.M., S.L.J., S.F.S.X., M.S.E., and C.O. analyzed data; I.M.M., R.E.K., S.L.J., M.S.E., K.L.E., T.W.S., C.O., M.M.R., and J.J.H. interpreted results of experiments; I.M.M. prepared figures; I.M.M. drafted manuscript; I.M.M., R.E.K., S.L.J., S.F.S.X., M.S.E., K.L.E., T.W.S., C.O., M.M.R., and J.J.H. edited and revised manuscript; I.M.M., R.E.K., S.L.J., S.F.S.X., M.S.E., K.L.E., T.W.S., C.O., M.M.R., and J.J.H. approved final version of manuscript.

REFERENCES
1. Carr RD, Larsen MO, Winzell MS, Jelic K, Lindgren O, Deacon CF, Ahren B. Incretin and islet hormonal responses to fat and protein ingestion in healthy men. Am J Physiol Endocrinol Metab 295: E779–E784, 2008. doi:10.1152/ajpendo.90233.2008.
2. Dumoulin V, Moro F, Barcelo A, Dakka T, Cuber JC. Peptide YY, glucagon-like peptide-1, and neuropeptide responses to luminal factors, the isolated vascularly perfused rat ileum. Endocr J 39: 3780–3786, 1998. doi:10.1210/endo.139.9.6202.
3. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V. Glucagon-like peptide-1 (7–37) amide and glucose-dependent insulino-tropic polypeptide secretion in response to nutrient ingestion in normal and type 2 diabetic patients. Am J Physiol Gastrointest Liver Physiol 304: G271–G282, 2013. doi:10.1152/ajpgi.00074.2012.
4. Lejeune MPGM, Westerterp KR, Adam TC, Luscombe-Marsh ND, Westerterp-Plantenga MS. Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and measured in a respiration chamber. Am J Clin Nutr 83: 99–113, 2006. doi:10.1093/ajcn/83.1.99.
5. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368: 1696–1705, 2006.
6. Pl-Sunyer X, Astrup A, Fujikoa K, Greenway F, Halpern A, Krempf M, Lau DCW, Luedtke CW, Violante Ortiz R, Jensen CB, Wilding JPH. A randomized, controlled trial of 3.0 mg of liraglutide in weight management. N Engl J Med 373: 11–22, 2015. doi:10.1056/NEJMoa1411892.
7. Zander M, Madsbad S, Madsen JL, Holst JH. Effect of 6-week course of glucagon-like peptide 1 on glycemic control, insulin sensitivity, and β-cell function in type 2 diabetes: a parallel-group study. Lancet 359: 824–830, 2002. doi:10.1016/S0140-6736(02)07952-7.
8. Bensaid A, Tomé D, Gietzen D, Even P, Morens C, Gausseres N, Fromentin G. Protein is more potent than carbohydrate for reducing appetite in rats. Physiol Behav 75: 577–582, 2002. doi:10.1016/s0031-9384(02)00646-7.
9. Batterham RL, Heffron H, Kapoor S, Chivers JE, Chandarana K, Herzog H, Le Roux CW, Thomas EL, Bell JD, Withers DJ. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. Cell Metab 4: 223–233, 2006. doi:10.1016/j.cmet.2006.08.001.
10. Belza A, Ritz C, Serensen MQ, Holst J, Rehfeld JF, Astrup A. Contribution of gastrointestinal pancreatic hormones to protein-induced satiety. Am J Clin Nutr 97: 980–989, 2013. doi:10.3945/ajcn.112.047563.
11. Blom WA, Luch A, Staflieu A, Vinoy S, Holst J, Schaafisma G, Hendriks HF. Effect of a high-protein breakfast on the postprandial ghrelin response. Am J Clin Nutr 83: 211–220, 2006. doi:10.1093/ajcn/83.2.211.
12. Brennan IM, Luscombe-Marsh ND, Seimon RV, Otto B, Horowitz M, Wishart JM, Feinle-Bisset C. Effects of fat, protein, and carbohydrate and protein load on appetite, plasma cholecystokinin, peptide YY, and ghrelin, and energy intake in lean and obese men. Am J Physiol Gastrointest Liver Physiol 303: G129–G140, 2012. doi:10.1152/ajpgi.00478.2011.
13. Wu T, Littie TJ, Bound MJ, Borg M, Zhang X, Deacon CF, Horowitz M, Jones KL, Rayner CK. A protein preload enhances the glucose-lowering efficacy of vildagliptin in type 2 diabetes. Diabetes Care 39: 511–517, 2016. doi:10.2337/dc15-2238.
14. Acar I, Cetinkaya A, Lay I, Ileri-Gurel E. The role of calcium sensing receptors in GLP-1 and PYY secretion after acute intraduodenal administration of l-tryptophan in rats. Nutr Neurosci 23: 481–489, 2020. doi:10.1080/20411391.2018.1521906.
15. Alshamah A, Sprechley E, Norton M, Kinsey-Jones JS, Amin A, Ramgulam A, Cao Y, Johnson R, Salek H, Akalestou E, Malik Z, Gonzalez-Abuin N, Nomad A, Amarsi R, Moola A, Sargent PR, Gray GW, Bloom SR, Murphy KG. L-Phenylalanine modulates gut hormone release and glucose tolerance, and suppresses food intake through the calcium-sensing receptor in rodents. Int J Obses (Lond) 41: 1693–1701, 2017. doi:10.1038/ijobes.2017.164.
16. Choi S, Lee M, Shiu AL, Yo SJ, Haldgren A, Aponte GW. GPR39 activation by protein hydrolysate induces CCK transcription and secretion in STC-1 cells. Am J Physiol Gastrointest Liver Physiol 292: G1366–G1375, 2007. doi:10.1152/ajpgi.00516.2006.
17. Daly K, Al-Rammahi M, Moran A, Marcello M, Ninomiya Y, Shirazi-Beechey SP. Sensing of amino acids by the gut-expressed taste receptor T1R1–T1R3 stimulates CCK secretion. Am J Physiol Gastrointest Liver Physiol 304: G271–G282, 2013. doi:10.1152/ajpgi.00074.2012.
18. Deng H, Hu H, Fang Y. Multiple tyrosine metabolites are GPR35 agonists. Sci Rep 2: 373, 2012. doi:10.1038/srep00373.
19. Mackenzie AE, Lappin JE, Taylor DL, Nicklin SA, Milligan G, GPR35 as a novel therapeutic target. Front Endocrinol (Lusanne) 2: 68, 2011. doi:10.3389/fendo.2011.00068.
20. Modvig IM, Kuhre RE, Holst JJ. Peptone-mediated glucagon-like peptide-1 secretion depends on intestinal absorption and activation of basolaterally located calcium-sensing receptors. Physiol Rep 7: e4056, 2019. doi:10.1484/phy2.104056.
21. Oya M, Kitaguchi T, Piais R, Reimann F, Gribble F, Tsaiboi T. The G protein-coupled receptor family C group 6 subtype A (GPRC6A) receptor is involved in amino acid-induced glucagon-like peptide-1 secretion from GLUTag cells. J Biol Chem 288: 4513–4521, 2013. doi:10.1074/jbc.M112.402677.
22. Rudenko O, Shang J, Munk A, Ekberg JP, Petersen N, Engelsted MS, Egerod KL, Hjorth SA, Wu M, Feng Y, Zhou YP, Mokrosinski J, Thams P, Reimann F, Gribble F, Rehfeld JF, Holst JJ, Treebak JT, Howard AD, Schwartz TW. The aromatic amino acid sensor GPR42 controls metabolism through balanced regulation of pancreatic and gut hormones. Mol Metab 19: 49–64, 2020. doi:10.1016/j.molmet.2018.10.012.
23. Amin A, Neophytou C, THEIN S, Martin NM, Alshamah A, Sprechley E, Bloom SR, Murphy KG. L-Arginine increases postprandial circulating GLP-1 and PYY levels in humans. Obesity 26: 1721–1726, 2018. doi:10.1002/oby.22323.
24. Chemmensen C, Jergensen CV, Smajlovic S, Brauner-Osbome H. Robust GLP-1 secretion by basic l-aminos acids does not require the GPRC6A receptor. Diabetes Obes Metab 19: 599–603, 2017. doi:10.1111/dom.12845.
Glutamine triggers and potentiates glucagon-like peptide-1 (GLP-1) secretion by raising cytosolic Ca²⁺ and cAMP. Endocrinology 152: 405–411, 2011. doi:10.1210/en.2010-0956.

Conigrave AD, Quinn SJ, Brown EM. L-Amino acid sensing by the extracellular Ca²⁺-sensing receptor. Proc Natl Acad Sci USA 97: 4814–4819, 2000. doi:10.1073/pnas.97.9.4814.

Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ, Gribble FM. Glucose sensing in L cells: a primary cell study. Cell Metab 8: 532–539, 2008. doi:10.1016/j.cmet.2008.11.002.

Orskov C, Raben H, Wettergren A, Kofod H, Holst JJ. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide 1 in humans. Diabetes 43: 535–539, 1994. doi:10.23736/S0012-1878.94.03435-5.

Jepsen SL, Grunddal KV, Wewer Albrechtsen NJ, Engelstoft MS, Gabe MBN, Jensen EP, Ørskov C, Poulsen SS, Rosenkilde MM, Pedersen J, Gribble FM, Reimann F, Deacon CF, Schwartz TW, Christ AD, Martin RE, Holst JJ. Paracrine crosstalk between intestinal L- and D-cells controls secretion of glucagon-like peptide 1 in mice. Am J Physiol Endocrinol Metab 317: E1081–E1093, 2019. doi:10.1152/ajpendo.00239.2019.

Engelstoft MS, Park W-M, Sakata I, Kristensv L, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM, Schwartz TW. Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2: 376–392, 2013. doi:10.1016/j.molmet.2013.08.006.

Panaro BL, Tough IR, Engelstoft MS, Matthews RT, Digby GJ, Møller CL, Svensden B, Gribble F, Reimann F, Holst JJ, Holst B, Schwartz TW, Cox HM, Cone RD. The melanocortin-4 receptor is expressed in enteroendocrine L cells and regulates the release of peptide YY and glucagon-like peptide 1 in vivo. Cell Metab 20: 1018–1029, 2014. doi:10.1016/j.cmet.2014.10.004.

Jagúš M, Forcić D, Bigles M, Kutle L, Sántak M, Jergović M, Kotarski L, Bendelja K, Halassy B. Stability of minimum essential medium functionality despite L-glutamine decomposition. Cytootechnology 68: 1171–1183, 2016. doi:10.1007/s10583-015-9875-8.

Khan K, Elia M. Factors affecting the stability of L-glutamine in solution. Clin Nutr 10: 186–192, 1991. doi:10.1016/S0143-6104(91)80037-D.

Broer S, Fairweather SJ. Amino acid transport across the mammalian intestine. Comp Physiol 9: 343–373, 2018.

Jando J, Camargo SMR, Herzog B, Verrey F. Expression and regulation of the neutral amino acid transporter B0AT1 in rat small intestine. PLoS One 12: e0184845, 2017. doi:10.1371/journal.pone.0184845.

Wang Q, Holst J. L-type amino acid transport and cancer: targeting the mTORC1 pathway to inhibit neoplasia. Am J Cancer Res 5: 1281–1294, 2015.

Zhao P, Lane TR, Gao HGL, Hurst DP, Kotsikourou E, Le L, Brailoiu E, Reggio PH, Abood ME. Crucial positively charged residues for ligand activation of the GPR35 receptor. J Biol Chem 289: 3625–3638, 2014. doi:10.1074/jbc.M113.508382.

Choi S, Lee M, Shiu AL, Yo SJ, Aponte GW. Identification of a protein hydrolase responsive G protein-coupled receptor in enterocytes. Am J Physiol Gastrointest Liver Physiol 292: G98–G112, 2007. doi:10.1152/ajpgi.00295.2006.