GLP1- and GIP-producing cells rarely overlap and differ by bombesin receptor-2 expression and responsiveness

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Abstract

The incretin hormones glucagon-like peptide-1 (GLP1) and glucose-dependent insulino-tropic polypeptide (GIP) are secreted from intestinal endocrine cells, the so-called L- and K-cells. The cells are derived from a common precursor and are highly related, and co-expression of the two hormones in so-called L/K-cells has been reported. To investigate the relationship between the GLP1- and GIP-producing cells more closely, we generated a transgenic mouse model expressing a fluorescent marker in GIP-positive cells. In combination with a mouse strain with fluorescent GLP1 cells, we were able to estimate the overlap between the two cell types. Furthermore, we used primary cultured intestinal cells and isolated perfused mouse intestine to measure the secretion of GIP and GLP1 in response to different stimuli. Overlapping GLP1 and GIP cells were rare (~5%). KCl, glucose and forskolin + IBMX increased the secretion of both GLP1 and GIP, whereas bombesin/neuromedin C only stimulated GLP1 secretion. Expression analysis showed high expression of the bombesin 2 receptor in GLP1 positive cells, but no expression in GIP-positive cells. These data indicate both expressional and functional differences between the GLP1-producing 'L-cell' and the GIP-producing 'K-cell'.

Key Words

- ▶ BB2
- ▶ neuromedin C
- ▶ forskolin/IBMX
- ▶ perfused mouse intestine
- ▶ incretin hormones

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Introduction

The incretin hormones, glucagon-like peptide-1 (GLP1) and glucose-dependent insulino-tropic polypeptide (GIP) strongly potentiate postprandial insulin secretion and are therefore important regulators of glucose homeostasis. They are secreted from so-called enteroendocrine L- and K-cells of the intestinal mucosal epithelium. Traditionally,

K-cells have been described to be primarily located in the proximal small intestine, whereas GLP1-producing L-cells are located more distally in the small intestine and colon (Sjolund *et al.* 1983, Eissele *et al.* 1992), but a recent study showed that similar amounts of GLP1 are secreted from the proximal and distal small intestine (Svendsen *et al.*

2015). In addition, immunohistochemical studies have indicated that there may be co-localisation of the two hormones in endocrine cells of the proximal gut (Mortensen *et al.* 2003, Theodorakis *et al.* 2006), and cells isolated on the basis of their production of one of the hormones often show co-expression of the other hormones (Egerod *et al.* 2012, Habib *et al.* 2012, Sykaras *et al.* 2014). Furthermore, a transgenic mouse model with diphtheria toxin-induced knockdown of proglucagon-producing cells showed reduced number of GLP1 positive as well as GIP positive cells (Egerod *et al.* 2012). Comparisons of expression profiles have indicated that proximal L-cells (defined as cells producing proglucagon-derived peptides) are more similar to K-cells than to the distal L-cells (Habib *et al.* 2012). On the other hand, another study showed that knocking down proglucagon-producing cells with diphtheria toxin in transgenic mice resulted in decreased expression of proglucagon, cholecystokinin (CCK), neurotensin and peptide YY (PYY), but no change in GIP expression. Likewise, knockdown of proGIP did not decrease GLP1 expression (Pedersen *et al.* 2013). Furthermore, a recent immunohistochemical study in rats showed co-localisation of GIP and GLP1 in only ~10% of GLP1-producing cells (Svendsen *et al.* 2015). Previous studies employing several experimental models including single K- and L-cells have shown that the hormones are secreted in response, for example, to glucose, which after entry via the sodium-glucose transporter-1 results in membrane depolarization and exocytosis secondary to calcium influx in both cell types (Reimann *et al.* 2008, Parker *et al.* 2009). Other studies have shown that stimuli activating the cAMP system may also result in both GLP1 and GIP secretion (Simpson *et al.* 2007, Moss *et al.* 2012, Hauge *et al.* 2014, Svendsen *et al.* 2015).

In order to further investigate the relationship between GLP1- and GIP-secreting cells, we now decided to investigate colocalization using transgenic mice in which GIP and GLP1 cells express different fluorescent markers. In addition, we used several experimental models to investigate the presence or absence of co-secretion of the two hormones, including single cells isolated from transgenic mice in which GLP1- and GIP-producing cells can be identified on the basis of their expression of cell markers under the control of the proGIP or proglucagon promoters, as well as isolated intestinal crypts and isolated perfused proximal mouse small intestine. Mice were chosen to allow comparisons between the models. If the chosen stimuli invariably stimulated the secretion of both hormones, this would provide a strong support for a

functional similarity and developmental relationship between K- and L-cells. It could arise by simultaneous secretion from two separate cell types, or might suggest some degree of co-expression of either hormone from two otherwise distinct cell-types, or even from a distinct cell-type showing co-expression of both hormones, a so-called K/L cell (Theodorakis *et al.* 2006). The stimuli we used included bombesin, neuromedin C, KCl, glucose and forskolin + 3-isobutyl-1-methylxanthine (IBMX). Because the results suggested differential responses to bombesin and neuromedin C, we mapped the expression of bombesin (BB) receptors (1–3) in single cells isolated on the basis of fluorescent markers expressed under the control of GLP1 or GIP promoters (Habib *et al.* 2012).

Materials and methods

Primary small intestinal crypt culture

All animal procedures were approved by local ethical review committees and conformed to UK Home Office regulations. Three- to six-month-old C57B6 male and female mice weighing 25–30 g were used in the study. They were housed in groups of 3–5/cage in a SPF-maintained facility and had *ad libitum* access to food and drinking water. Small intestinal crypts were isolated and cultured as previously described (Reimann *et al.* 2008). In summary, mice were killed by cervical dislocation and the duodenum (10 cm distal to the stomach) was excised. Luminal contents were flushed thoroughly with PBS and the outer muscle layer removed. Tissue was minced and digested with Collagenase Type XI and the cell suspension plated onto Matrigel (BD Bioscience, Oxford, UK) pre-coated 24-well plates for GLP1 and GIP secretion experiments or on 35 mm glass bottomed dishes (MatTek Corporation, Ashland, MA, USA) for calcium imaging.

GLP1 and GIP secretion assay

Eighteen to 24 h after plating, cells were washed and incubated for 2 h with or without bombesin (100 nM) and glucose (10 mM). Test agents made up in saline buffer (in mM 138 NaCl, 4.5 KCl, 4.2 NaHCO₃, 1.2 NaH₂PO₄, 2.6 CaCl₂, 1.2 MgCl₂, and 10 HEPES, pH adjusted to 7.4 with NaOH) supplemented with 0.1% BSA for 2 h at 37 °C. At the end of the 2-h incubation, supernatants were collected and centrifuged at 2000 *g* for 5 min and snap-frozen on dry ice. Cells were lysed with lysis buffer and centrifuged at 10 000 *g* for 10 min and snap-frozen. GLP1 and GIP concentrations were assayed in supernatant

fractions and cell extracts from primary intestinal cultures using a total GLP1 assay (Meso Scale Discovery, Gaithersburg, MD, USA) and GIP Total ELISA kit (Millipore, Billerica, MA, USA). Each test agent was tested in three or four wells per 24-well plate on four cultures independently established from one mouse each. Results were expressed as a percentage of the total hormone content (secreted + lysate) per well. Hormone secretion was compared with baseline or between conditions by ANOVA with subsequent Bonferroni's test.

Transgenic mouse models

Transgenic mice were generated and bred in Cambridge with the approval of the Local Ethical Review Committee and in accordance with UK Home Office guidelines. K- and L-cells for transcriptomic analysis were purified from GIP-Venus and GLU-Venus strains (Reimann *et al.* 2008, Parker *et al.* 2009). For calcium imaging, transgenic mice were engineered to express the fluorescence-based calcium sensor GCaMP3 in either L- or K-cells. L-cell specificity was achieved by crossing the GLU-Cre strain with ROSA26-GCaMP3 (Parker *et al.* 2012).

To label K-cells, we generated a new mouse strain GIP-Cre. To express Cre-recombinase *iCre* (a kind gift from Rolf Sprengel) under the control of the *gip* promoter, we replaced the sequence between the *gip* start codon in exon 2 and stop codon in exon 6 in the murine-based bacterial artificial chromosome (BAC) RP24-298N3 (mouse based) (Children's Hospital Oakland Research Institute) by the *iCre* sequence using Red/ET recombination technology (GeneBridges). In summary, *iCre* sequence was amplified by PCR adding *gip* gene-specific 3' and 5' sequences and homologous recombination was achieved upon co-transforming an rpsLneo-modified BAC containing E. coli DH10B clone with the PCR product and the plasmid pSC101-BAD-gbaA, which provides the recombination enzymes (GeneBridges). Positive recombinants were isolated using appropriate antibiotic selection and characterised by PCR and restriction analysis. Identity and correct positioning of the introduced *iCre* sequence were confirmed by Sanger sequencing. BAC-DNA for micro-injection was purified using the large-construct Maxi-Prep kit (Qiagen) and dissolved at ~1–2 ng/μl in injection buffer containing (mmol/l) 10 Tris-HCl pH 7.5, 0.1 EDTA, 100 NaCl, 0.03 spermine and 0.07 spermidine. Pronuclear injection into ova derived from C57B6/CBA F1 parents and re-implantation of embryos into pseudo-pregnant females was performed by the Central Biomedical Services at Cambridge University. DNA of pups was isolated from

ear clips by proteinase K digestion and screened for the transgene by PCR using primer pairs against *gip*, *iCre*, reporter genes and beta-catenin, which served as a DNA quality control. Mice were back-crossed into C57B6 for at least seven generations. We initially received three founders (mGIP-Cre-117, -122 and -125), all of which faithfully passed on the transgene (estimated transgene copy numbers were 1; 1 and 2) in a Mendelian fashion with no gender bias. mGIP-Cre-117 was used in all experiments, and is referred to hereon as GIP-Cre.

Flow cytometry

The upper small intestine (10 cm distal to the gastric pylorus) of GIP-Cre/ROSA26-RFP mice was stripped of the muscle layer and digested to single cells using 1 mg/ml collagenase XI in calcium free-Hanks' buffered salt solution. Cell suspensions were fixed using 4% paraformaldehyde in PBS for 15–30 min at room temperature, permeabilised with 0.1% v/v Triton X-100 for 30 min at room temperature, blocked with 10% goat serum in PBS for 30 min, and incubated with primary antibody (GLP; Santa Cruz; sc-13091; 1:200. GIP; antiserum #80867 from JJ Holst (Lindgren *et al.* 2011); 1:1000) in PBS-10% goat serum at room temperature for 3 h.

Cells were rinsed ×3 in PBS and then incubated with secondary antibody (Alexa-Fluor 488, Invitrogen; 1:300). After three washes with PBS, cells were analysed using a five-laser LSRFortessa analyser (BD Biosciences, San Jose, CA, USA) and FlowJo 7.6 software (Ashland, OR, USA). Events with very low side and forward scatter or with high pulse width were excluded to eliminate debris and cell aggregates. Presented mean data is the average from three to seven independent preparations, with each preparation derived from a different mouse.

Immunohistochemistry

Freshly dissected intestines from GIP-Cre/ROSA26-GCaMP3 mice (*n*=3) were cleaned using PBS and dropped into 4% paraformaldehyde in PBS and left at 4 °C to fixate overnight. The intestines were dehydrated in 15 and 30% sucrose solutions and frozen in OCT embedding media (CellPath, UK). Cryostat-cut sections (10–12 μm) were mounted directly onto poly-lysine covered glass slides.

The sections were incubated with a blocking solution of PBS containing 10% donkey serum, 0.1% BSA and 0.05% Tween20 (VWR, Lutterworth, UK) for 1 h. Blocking solution was replaced with 10%-PBS serum solution with 0.1% BSA and primary antibodies (GIP; as used for flow cytometry. GFP; Abcam, Cambridge, UK; Ab5450; 1:1000)

and left overnight. The following day, the slides were rinsed three times in PBS and incubated with a 10%-PBS serum solution containing secondary antibodies (Alexa-Fluor 488 and 555; Invitrogen; 1:300) and Hoechst diluted to 1:1300, for 1 h. The sections were rinsed three times in PBS to remove unconjugated antibodies. All slides were mounted with Prolong Gold (Life Technologies, Paisley, UK) prior to confocal microscopy on a Zeiss LSM510. Control slides were stained with secondary antibodies only.

Calcium imaging

Intracellular calcium concentrations were monitored from crypt cultures prepared from the duodenum of GLU-Cre/ROSA26-GCaMP3/ROSA26-tdRFP or GIP-Cre/ROSA26-GCaMP3/ROSA26-tdRFP mice. K- and L-cells were identified by Red Fluorescent Protein (RFP) fluorescence and changes in intracellular calcium levels were represented by a change in the intensity of GCaMP3 fluorescence excited at 488 ± 10 nm. Imaging was performed using an Olympus IX71 microscope with a $40\times$ oil immersion objective, fitted with a monochromator (Cairn Research, Kent, UK) and OrcaER camera (Hamamatsu, Shizuoka, Japan). Images were acquired at 1 Hz and analysed, after background subtraction, using MetaFluor software (Molecular Devices, Sunnyvale, CA, USA). Solutions were perfused continuously at a rate of ~ 1 ml/min and responses to test reagents were calculated by determining the mean intensity at 488 nm during perfusion of the test reagent divided by the average of the baseline 488 intensity taken before and after test reagent application. Test reagents were tested on 10–20 individual cells from four independent crypt cultures established from four GIP-Cre and four GLU-Cre mice respectively.

The perfused mouse intestine

Eight male C57BL/6J mice (10 weeks, purchased from Taconic, Denmark) were used for perfusion of the proximal intestine. The perfusion experiments conformed to international guidelines and were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark. Animals were housed five animals to a each cage and kept on a ratio of 12 h light:12 h darkness cycle with free access to standard rat chow and water, and allowed to acclimatize for 1 week before use. The animals were anaesthetized with i.p. injection of ketamine/xylazine (Ketamine 90 mg/kg (Ketaminol Vet.; MSD Animal Health, Madison, NJ, USA) + Xylazine 10 mg/kg (Rompun Vet.; Bayer Animal Health, Leverkusen,

Germany). A catheter (0.7 mm) was placed in the aorta for inflow of the perfusion medium and perfusion of the intestine via the superior mesenteric artery at a flow rate of 2.2 ml/min. The stomach, kidneys, spleen, colon and distal small intestine were tied off and removed to prevent perfusion, thus only the proximal small intestine was perfused (~ 10 cm). The venous effluent was collected in 1-min periods via a catheter (0.9 mm) inserted into the portal vein, now exclusively draining the perfused segment. The intestinal lumen was perfused with 37 °C saline at a flow rate of 0.04 ml/min.

The perfusion medium (a modified Krebs Ringer bicarbonate buffer containing, in addition, 0.1% BSA (Merck KGaA), 5% Dextran T-70 (Dextran Products Limited, Scarborough, Canada), 3.5 mmol/l glucose, and 5 mmol/l of each of pyruvate, fumarate, and glutamate) was gassed with a 95% O₂/5% CO₂ mixture to achieve pH 7.3–7.4 and maintained at 37 °C during the experiment. Perfusion pressure remained relatively constant throughout each experiment (~ 50 mmHg). After the intestinal perfusion was established, the animals were exsanguinated and the intestine was allowed to stabilise for ~ 30 min before the experiment was started. Test substances included bombesin (10 nM; Bachem), Neuromedin C (10 nM), forskolin (10 μ M) + IBMX (10 μ M), and KCl (70 mM) infused through a sidearm with a syringe infusion pump (0.11 ml/min) for 5–10 min followed by a resting period of 20 min. Chemicals were obtained from Sigma Aldrich, unless otherwise stated. For these experiments, GLP1 concentrations in perfusion effluent samples were measured using RIA (antiserum #89390 (Orskov *et al.* 1994)) and GIP was measured with Millipore total rat GIP ELISA kit according to the manufacturer's instructions. Responses to a given stimulus were analysed by comparing the mean total outputs during the 5 min and the total output during the 5 min of infusion, using paired t-tests.

Single cell expression analysis

Single cell suspensions were prepared from the first 10 cm of the proximal small intestine from GLU-Venus or GIP-Venus mice and separated into fluorescence-positive and fluorescence-negative pools of cells using fluorescence-activated cell sorting (FACS) as described (Habib *et al.* 2012). The cells were sorted directly into lysis buffer (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) and the RNA was purified using the RNAqueous-Micro micro-scale RNA isolation kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was prepared using Superscript III (Invitrogen, Thermo Fisher

Scientific, Waltham, MA, USA). The expression of 379 G protein-coupled receptors was tested using custom-designed 384 well qPCR plates from Lonza. Primer target sequences have been published before (Engelstoft *et al.* 2013). A genomic DNA sample was used as a calibrator and relative copy numbers were calculated as described by Engelstoft *et al.* (2013).

Results

Hormone secretion from primary cultures

We investigated the secretion of GLP1 and GIP from primary cultures of the upper small intestine (Fig. 1). Cultures were incubated for 2 h in the presence and absence of glucose (10 mM) and bombesin (100 nM), and supernatants and cell extracts were then analysed for their contents of both GLP1 and GIP. Glucose-enhanced secretion of both GLP1 and GIP 2.5 fold (Fig. 1) compared with baseline. Bombesin further increased GLP1 release ~2–2.5 fold in the absence of glucose, and ~1.5 fold in the presence of glucose. GIP release was unaffected by bombesin under either condition.

Figure 1

GLP1 and GIP secretion from primary murine duodenal cultures. Mixed epithelial cultures established from the top 10 cm of the small intestine in 24 well plates were incubated for 2 h with or without bombesin (BB; 100 nM) and glucose (Glc; 10 mM) as indicated. GLP1 (A) and GIP (B) contents of the supernatant and cell lysates were measured, and results calculated as a percentage of the hormone content (secreted plus intracellular) per well. Data are from four independent cultures established from one mouse each and represent the mean and s.e.m. of the number of wells indicated above each bar. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with baseline, or between conditions.

Low overlap of L- and K-cells in transgenic mouse models

As the secretion results suggested that GIP and GLP1 secretion are regulated differentially, we generated a new transgenic mouse model GIP-Cre to label K-cells with fluorescent markers or reporters of intracellular signalling events (Fig. 2). Upper small intestinal cell suspensions from GIP-Cre/ROSA26-tdRFP mice were immunostained for GIP and GLP, and analysed by flow cytometry to determine the cell specificity of K-cell labelling and the overlap between K- and L-cells. GIP staining was detected in >90% of tdRFP-labelled cells, confirming the K-cell specificity of Cre expression. The proportion of GIP-stained cells that were labelled with the fluorescent reporter was ~60% in this model, compared with ~30% in GIP-Venus mice (Fig. 2). Only ~5% of tdRFP positive cells in GIP-Cre/ROSA26-tdRFP mice stained for GLP, indicating only a limited overlap of K- and L-cell populations.

Calcium responses in primary L- and K-cells

To monitor intracellular Ca^{2+} in identified primary upper small intestinal L- and K-cells, we crossed the ROSA26-GCaMP3 reporter mouse with either GLU-Cre (expressing cre recombinase under proglucagon promoter control) or GIP-Cre mouse strains. L-cells (Fig. 3A and B) showed robust elevations of GCaMP3 fluorescence in response to 100 nM bombesin, 10 mM glucose and 70 mM KCl, indicative of an elevation of intracellular Ca^{2+} with all three stimuli, and consistent with previous reports that bombesin receptor 2 (BB₂; GRP-receptor) activation recruits G_q proteins, triggering Ca^{2+} release from intracellular stores. K-cells (Fig. 3C and D) exhibited good responses to glucose and KCl, but did not respond to bombesin, mirroring the absence of bombesin-triggered GIP secretion in both perfused intestine and primary cultures.

Hormone secretion from perfused mouse small intestine

We next investigated whether the secretion results from the primary cultures were mirrored in secretion studies performed on the perfused mouse small intestine (Fig. 4). GLP1 secretion from the perfused proximal small intestine was significantly increased by vascular infusion of both bombesin (from 92 ± 14 to 493 ± 59 fmol/5 min; 5.7 fold; $P < 0.01$) and neuromedin C (114 ± 40 to 467 ± 7 fmol/5 min; 5.1 fold; $P < 0.05$), as well as forskolin + IBMX (from 162 ± 19 to 505 ± 80 fmol/5 min; 3.1 fold;

Figure 2

Characterisation of GIP-Cre/ROSA26-tdRFP mice. Single-cell suspensions from the upper small intestine of GIP-Cre/ROSA26-RFP mice were stained with antibodies against GIP (A) or GLP (B) and a green secondary and analysed by FACS. Frequency histograms represent the green fluorescence of all RFP-positive cells (dark shading) or all strongly green fluorescent cells (light shading). Y-axes represent frequency of cell detection on an arbitrary scale. (C) Mean data from experiments performed as in panels A and B, representing the percentage of RFP-positive cells that stained positive for GIP or GLP (red bars). Results obtained from GIP-Venus mouse are also

shown for comparison (green bars). (D) Mean data from experiments performed as in panels A and B, representing the percentage of green fluorescent (antibody stained) cells that were also positive for RFP (red bars). GIP-Venus data are presented alongside as a comparator (green bars). Data represent mean $\pm$ s.e.m. of the number of preparations indicated. (E) Intestines taken from GIP-Cre/ROSA26-GCaMP3 mice ($n=3$) were fixed, sectioned and stained with antibodies against GIP (red) and GFP (green). A representative image of a double positive cell located in the duodenum is shown.

$P<0.05$) and KCl (113 ± 6 to 433 ± 92 fmol/5 min; 4.0 fold; $P<0.05$). Secretion of GIP was increased by infusion of forskolin+IBMX (39 ± 9 to 129 ± 32 fmol/5 min; 3.6 fold; $P<0.05$) and KCl (77 ± 2 to 249 ± 62 fmol/5 min;

2.9 fold; $P0.05$) but no significant effect was observed after infusion of either bombesin (27 ± 4 to 44 ± 4 fmol/5 min) or neuromedin C (87 ± 21 to 118 ± 14 fmol/5 min) (Fig. 4).

Figure 3

[Ca²⁺] imaging in mouse primary duodenal L-cells and K-cells. GFP fluorescence (reflecting [Ca²⁺]_i) was utilized to record changes in intracellular [Ca²⁺]_i changes following the addition of 100 nM bombesin (BB), 10 mM Glucose (Glc) and 70 mM KCl. (A) and (C) Representative trace showing the change in GCaMP3 fluorescence before, during and after the application to a primary duodenal GLP1 cell cultured from GLU-Cre/

ROSA26-GCaMP3 mice (A) or a primary duodenal GIP cell cultured from GIP-Cre/ROSA26-GCaMP3 mice (C). (B) and (D) GFP intensity in the presence of the test agent was normalised to the mean background of each cell measured pre and post application of the test compound in GLP1 cells (B) and GIP cells (D). Data represent the mean and s.e.m. of the number of cells indicated above each bar. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with baseline.

Figure 4

Secretion of GLP1 and GIP from the perfused mouse proximal small intestine. The intestine was stimulated with vascular infusion of (A) and (B) bombesin (10 nM) and forskolin/IBMX (10 μ M); and (C) and (D) neuromedin

C (10 nM) and KCl (70 mM). (B) and (D) Total hormone outputs from 5 min before and during infusion of test substances are compared; mean $\pm$ S.E.M., $n=4$. * $P<0.05$, ** $P<0.01$.

Expression

GIP-venus and GLP1-venus positive cells were FACS-purified from single-cell preparations of mucosal cells generated from the proximal small intestine of transgenic GIP-venus (Parker *et al.* 2009) or GLU-venus reporter mice (Reimann *et al.* 2008). cDNA from the purified cell populations was analysed for bombesin receptor expression by a qPCR array targeting 379 non-odorant 7TM receptors (Fig. 5) as previously reported (Panaro *et al.* 2014). None of the three bombesin receptors was expressed above background levels in GIP cells (Fig. 5A). In contrast, the BB₂ receptor was expressed in GLP1 cells – as the only one of the bombesin receptors (Fig. 5B). Grpr mRNA was enriched 37-fold in the GLP1 cells compared to the surrounding epithelial cells.

Discussion

In this study, we tried to analyze more closely the relationship between the cells secreting the two incretin hormones GIP and GLP1, by investigating both

co-localisation and co-secretion of the hormones. As already alluded to, the classical concept has it that the GIP-producing cells are located proximally and the GLP1-producing cells more distally, suggesting that GIP is the primary incretin, while GLP1 would function more as an 'ileal brake' hormone, providing a stop signal with respect to appetite and upper gastrointestinal function, as well as facilitating further deposition of already absorbed nutrients. However, the rapid postprandial increase in the plasma concentration of GLP1 generally parallels GIP responses (Knop *et al.* 2007). Both neural and endocrine mechanisms have been proposed to account for this (Plaisancie *et al.* 1994, Rocca & Brubaker 1999, Hansen & Holst 2002), but a simpler explanation has now been provided with the demonstration that proximal L-cells are capable of providing similar responses to nutritional and other stimuli as those elicited from the distal small intestine (Svendsen *et al.* 2015). In addition, there is strong evidence that the small intestinal cells may be more closely related and more promiscuous in terms of secretory

Figure 5

Expression of bombesin receptors in GIP and GLP1 cells. Scatter plots showing qPCR expression of the three bombesin receptors (annotated red dots) among 379 7TM receptors (grey plus red dots) examined in FACS-purified cells from the proximal small intestine from transgenic (A) GIP-Venus, and (B) GLP1-Venus reporter mice (Y-axis) vs expression in non-fluorescent mucosal cells (X-axis). The 45°-angled lines indicate the enrichment of expression in the fluorescent FACS-purified enteroendocrine cells vs the neighbouring enterocytes. The grey shaded area in each of the scatter plots is considered as noise level.

products than hitherto believed, showing a high degree of co-expression of several gut hormones (Habib *et al.* 2012, Egerod *et al.* 2012, Pedersen *et al.* 2013, Sykaras *et al.* 2014). In particular, the relationship between the GIP- and the

GLP1-secreting cells has attracted interest, resulting in proposals that a 'K/L' cell type secreting both hormones might exist (Mortensen *et al.* 2003, Theodorakis *et al.* 2006). In this analysis, we examined the simultaneous secretion of GIP and GLP1 from the isolated upper small intestine from mice, using stimuli meant to engage different signalling pathways, namely i) depolarizing concentrations of potassium chloride, expected to cause an opening of calcium channels, thereby providing a secretory stimulus; ii) forskolin/IBMX providing a strong amplification of the cAMP system by stimulating adenylate cyclase and inhibiting cAMP degradation and iii) bombesin and neuromedin C, agents that via activation of Gq would increase intracellular calcium concentrations by mobilising intracellular depots in response to inositol trisphosphate (IP3) signalling (Snow *et al.* 1994). In addition to studying the secretion of the hormones from the integrated but isolated endocrine organ, the gut, capable of responding in a manner identical to that expected in the intact animal *in vivo*, we were able to study the responses of primary cultured enteroendocrine cells with active promoters for either proGIP or proglucagon (i.e., GIP- and GLP1-producing cells) identified and isolated on the basis of this promoter activity. For the secretion experiments, we found it essential to study mice, since the studies regarding the 'promiscuity' of the gut endocrine cells and the primary cell studies were all performed in mice. This required the development of a procedure for isolating and perfusing the mouse upper small intestine, which turned out to be feasible on the basis of experience with the perfused rat small intestine (Svendsen *et al.* 2015). Histological appearance of the perfused segments was completely normal, and measures of respiration and motility patterns all indicated a viable preparation. The findings indicated that GLP1 and GIP secretion were both stimulated by forskolin/IBMX promoting the cAMP pathways and by KCl depolarization. In contrast, neuromedin C and bombesin exclusively stimulated the secretion of GLP1. The patterns of secretion also showed differences – in response to KCl, the GLP1 response was short-lived, whereas the GIP response was more prolonged. In the isolated cells, glucose-stimulated secretion from both proglucagon- and proGIP-expressing cells, but bombesin enhanced secretion further only in the proglucagon cells.

Our new mouse model, GIP-Cre, enabled us to reassess the overlap between K- and L-cells and to monitor calcium responses in primary living K-cells. FACS analysis confirmed previous findings from GLU-Venus and GIP-Venus mice (Habib *et al.* 2012) that only a small

proportion (<10%) of K-cells stain positive for GLP1. Also by calcium imaging, it was clear that L-cells, but not K-cells, responded to bombesin, although both cell types exhibited similar responses to KCl and glucose.

Finally, in the expression profiling studies on isolated cells, it was evident that while the BB₂ receptor, thought to be the main target for bombesin and neuromedin C (i.e., the C-terminal fragment of the neuropeptide, gastrin-releasing peptide) (Erspamer & Melchiorri 1976), was highly enriched in proglucagon-expressing cells, whereas proGIP cells exhibited a very low expression level.

Taken together, these studies indicate that the secretion of GLP1 and GIP must derive from separate cell types that, probably among other things, at least can be distinguished by their differential expression of the BB₂ receptor. The results are consistent with the cell ablation studies that showed that knock-down of one of the two incretin-producing cell types with diphtheria toxin, although accompanied by reduced expression of several other gut hormones, did not influence the expression of the other hormone (Pedersen *et al.* 2013). The results are also consistent with recent studies in upper rat small intestine where immunohistochemical co-localisation of GLP1 and GIP was a rare phenomenon (Svendsen *et al.* 2015). Therefore, in spite of many similarities in terms of localisation and function, the GIP cells and the GLP1 cells of the proximal gut of mice seem mainly to be separate entities. Our results imply that co-localisation does not invariably lead to co-secretion of the gut hormones. Rather, mechanisms for release must be characterised for each of the hormones separately.

Declaration of interest

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

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References

- Egerod KL, Engelstoft MS, Grunddal KV, Nohr MK, Secher A, Sakata I, Pedersen J, Windelov JA, Fuchtbauer EM, Olsen J *et al.* 2012 A major lineage of enteroendocrine cells coexpress CCK, secretin, GIP, GLP1, PYY, and neurotensin but not somatostatin. *Endocrinology* **153** 5782–5795. (doi:10.1210/en.2012-1595)
- Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R & Goke B 1992 Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *European Journal of Clinical Investigation* **22** 283–291. (doi:10.1111/j.1365-2362.1992.tb01464.x)
- Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK *et al.* 2013 Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. *Molecular Metabolism* **2** 376–392. (doi:10.1016/j.molmet.2013.08.006)
- Erspamer V & Melchiorri P 1976 Proceedings: Amphibian skin polypeptides active on the gut. *Journal of Endocrinology* **70**(Suppl 3) 12P–13P (available at: http://joe.endocrinology-journals.org/content/70/3_Suppl.toc)
- Habib AM, Richards P, Cairns LS, Rogers GJ, Bannon CA, Parker HE, Morley TC, Yeo GS, Reimann F & Gribble FM 2012 Overlap of endocrine hormone expression in the mouse intestine revealed by transcriptional profiling and flow cytometry. *Endocrinology* **153** 3054–3065. (doi:10.1210/en.2011-2170)
- Hansen L & Holst JJ 2002 The effects of duodenal peptides on glucagon-like peptide-1 secretion from the ileum. A duodeno-ileal loop? *Regulatory Peptides* **110** 39–45. (doi:10.1016/S0167-0115(02)00157-X)
- Hauge M, Vestmar MA, Husted AS, Ekberg JP, Wright MJ, Di SJ, Weinglass AB, Engelstoft MS, Madsen AN, Luckmann M *et al.* 2014 GPR40 (FFAR1) - combined Gs and Gq signaling *in vitro* is associated with robust incretin secretagogue action *ex vivo* and *in vivo*. *Molecular Metabolism* **4** 3–14. (doi:10.1016/j.molmet.2014.10.002)
- Knop FK, Vilsboll T, Larsen S, Hojberg PV, Volund A, Madsbad S, Holst JJ & Krarup T 2007 Increased postprandial responses of GLP1 and GIP in patients with chronic pancreatitis and steatorrhea following pancreatic enzyme substitution. *American Journal of Physiology. Endocrinology and Metabolism* **292** E324–E330. (doi:10.1152/ajpendo.00059.2006)
- Lindgren O, Carr RD, Deacon CF, Holst JJ, Pacini G, Mari A & Ahren B 2011 Incretin hormone and insulin responses to oral versus intravenous lipid administration in humans. *Journal of Clinical Endocrinology and Metabolism* **96** 2519–2524. (doi:10.1210/jc.2011-0266)
- Mortensen K, Christensen LL, Holst JJ & Orskov C 2003 GLP1 and GIP are colocalized in a subset of endocrine cells in the small intestine. *Regulatory Peptides* **114** 189–196. (doi:10.1016/S0167-0115(03)00125-3)
- Moss CE, Marsh WJ, Parker HE, Ogunnowo-Bada E, Riches CH, Habib AM, Evans ML, Gribble FM & Reimann F 2012 Somatostatin receptor 5 and cannabinoid receptor 1 activation inhibit secretion of glucose-dependent insulintropic polypeptide from intestinal K cells in rodents. *Diabetologia* **55** 3094–3103. (doi:10.1007/s00125-012-2663-5)
- Orskov C, Rabenhøj L, Wettergren A, Kofod H & Holst JJ 1994 Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. *Diabetes* **43** 535–539. (doi:10.2337/diab.43.4.535)
- Panaro BL, Tough IR, Engelstoft MS, Matthews RT, Digby GJ, Møller CL, Svendsen B, Gribble F, Reimann F, Holst JJ *et al.* 2014 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 Metabolism* **20** 1018–1029. (doi:10.1016/j.cmet.2014.10.004)
- Parker HE, Habib A, Rogers G, Gribble F & Reimann F 2009 Nutrient-dependent secretion of glucose-dependent insulintropic polypeptide from primary murine K cells. *Diabetologia* **52** 289–298. (doi:10.1007/s00125-008-1202-x)
- Parker HE, Adriaenssens A, Rogers G, Richards P, Koepsell H, Reimann F & Gribble FM 2012 Predominant role of active versus facilitative

- glucose transport for glucagon-like peptide-1 secretion. *Diabetologia* **55** 2445–2455. (doi:10.1007/s00125-012-2585-2)
- Pedersen J, Ugleholdt RK, Jorgensen SM, Windelov JA, Grunddal KV, Schwartz TW, Fuchtbauer EM, Poulsen SS, Holst PJ & Holst JJ 2013 Glucose metabolism is altered after loss of L cells and alpha-cells but not influenced by loss of K cells. *American Journal of Physiology. Endocrinology and Metabolism* **304** E60–E73. (doi:10.1152/ajpendo.00547.2011)
- Plaisancie P, Bernard C, Chayvialle JA & Cuber JC 1994 Regulation of glucagon-like peptide-1-(7-36) amide secretion by intestinal neurotransmitters and hormones in the isolated vascularly perfused rat colon. *Endocrinology* **135** 2398–2403. (doi:10.1210/endo.135.6.7988423)
- Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ & Gribble FM 2008 Glucose sensing in L cells: a primary cell study. *Cell Metabolism* **8** 532–539. (doi:10.1016/j.cmet.2008.11.002)
- Rocca AS & Brubaker PL 1999 Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion. *Endocrinology* **140** 1687–1694. (doi:10.1210/endo.140.4.6643)
- Simpson AK, Ward PS, Wong KY, Collord GJ, Habib AM, Reimann F & Gribble FM 2007 Cyclic AMP triggers glucagon-like peptide-1 secretion from the GLUTag enteroendocrine cell line. *Diabetologia* **50** 2181–2189. (doi:10.1007/s00125-007-0750-9)
- Sjolund K, Sanden G, Hakanson R & Sundler F 1983 Endocrine cells in human intestine: an immunocytochemical study. *Gastroenterology* **85** 1120–1130. (doi:10.1016/S0016-5085(83)80080-8)
- Snow ND, Prpic V, Mangel AW, Sharara AI, McVey DC, Hurst LJ, Vigna SR & Liddle RA 1994 Regulation of cholecystokinin secretion by bombesin in STC-1 cells. *American Journal of Physiology* **267** G859–G865.
- Svendsen B, Pedersen J, Albrechtsen NJ, Hartmann B, Torang S, Rehfeld JF, Poulsen SS & Holst JJ 2015 An analysis of cosecretion and coexpression of gut hormones from male rat proximal and distal small intestine. *Endocrinology* **156** 847–857. (doi:10.1210/en.2014-1710)
- Sykaras AG, Demenis C, Cheng L, Pisitkun T, McLaughlin JT, Fenton RA & Smith CP 2014 Duodenal CCK cells from male mice express multiple hormones including ghrelin. *Endocrinology* **155** 3339–3351. (doi:10.1210/en.2013-2165)
- Theodorakis MJ, Carlson O, Michopoulos S, Doyle ME, Juhaszova M, Petraki K & Egan JM 2006 Human duodenal enteroendocrine cells: source of both incretin peptides, GLP1 and GIP. *American Journal of Physiology. Endocrinology and Metabolism* **290** E550–E559. (doi:10.1152/ajpendo.00326.2004)

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