RESEARCH PAPER

Galanin inhibits GLP-1 and GIP secretion via the GAL1 receptor in enteroendocrine L and K cells

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BACKGROUND AND PURPOSE
Galanin is a widely expressed neuropeptide, which in the gut is thought to modulate gastrointestinal motility and secretion. We aimed to elucidate the poorly characterised mechanisms underlying the inhibitory effect of galanin and the potential involvement of G-protein coupled inwardly rectifying potassium, Kir3, (GIRK) channels in glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinoactive polypeptide (GIP) secretion.

EXPERIMENTAL APPROACH
Purified murine L and K cells were analysed for expression of galanin receptors and GIRK subunits. Hormone secretion was measured from primary murine intestinal cultures. Intracellular cAMP was monitored in primary L cells derived from mice expressing the Epac2camps sensor under the control of the proglucagon promoter.

KEY RESULTS
Galanin receptor 1 (GAL1, Galr1) and GIRK channel 1 (Kirs.3.1, Kcnj3) and 4 (Kirs.3.4, Kcnj4) mRNA expression was highly enriched in K and L cells. Galanin and a selective GAL1 receptor agonist (M617) potently inhibited GLP-1 and GIP secretion from primary small intestinal cultures. In L cells, galanin significantly inhibited the forskolin-induced cAMP response. The GIRK1/4 activator ML297 significantly reduced glucose-stimulated and IBMX-stimulated GLP-1 secretion but had no effect on GIP. The GIRK blocker tertiapin-Q did not impair galanin-mediated GLP-1 inhibition.

CONCLUSIONS AND IMPLICATIONS
Galanin, acting via the GAL1 receptor and Gi-coupled signalling in L and K cells, is a potent inhibitor of GLP-1 and GIP secretion. Although GIRK1/4 channels are expressed in these cells, their activation does not appear to play a major role in galanin-mediated inhibition of incretin secretion.

Abbreviations
CFP, cyan fluorescent protein; FRET, Förster resonance energy transfer; GIP, glucose-dependent insulinoactive polypeptide (gastric inhibitory polypeptide); GIRK, G-protein coupled inwardly rectifying potassium channel, Kirs.3.x; GLP-1, glucagon-like peptide 1; TPN-Q, tertiapin-Q; YFP, yellow fluorescent protein
Galanin inhibits GLP-1 and GIP via GAL1 receptor

Introduction

Galanin is a 29–30-amino acid neuropeptide, synthesised in both the central and peripheral nervous systems including the enteric nervous system (Tatemoto et al., 1983). Galanin has been shown to regulate a wide variety of physiological and pathophysiological processes (reviewed by Lang et al., 2015). Within the intestine, galanin mRNA is most abundant in the distal small intestine and colon (Kaplan et al., 1988). The upper small intestine harbours enteroendocrine L and K cells responsible for secreting the gut hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), respectively, which have established roles in gastrointestinal function, glucose homeostasis and satiety (Campbell et al., 2015). However, our understanding of the neuro-hormonal regulation of GLP-1 and GIP secretion are currently unknown. Therefore, this study aimed to (i) characterise the expression profiles of galanin receptors and GIRK (Kir3) channel subunits in purified enteroendocrine L and K cells, (ii) determine the mechanism underlying galanin-mediated inhibition of incretin release and (iii) investigate the role of GIRK (Kir3) channels in GLP-1 and GIP secretion. We demonstrated that galanin, acting via the GAL1 receptor and GIRK3 channel subunits, inhibits GLP-1 and GIP secretion. We found no evidence to suggest a role for GIRK1/4 (Kir3.1/3.4) channels in galanin-mediated inhibition of gut hormone secretion.

Methods

Animal welfare and ethical statements
All animal care and experimental procedures conformed to the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039) and were approved by the local ethical review committee and. The work was performed under the UK
Creation of proglucagon promoter-driven Epac2camps expressing transgenic mice

To express yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP)-based CAMP-Förster resonance energy transfer (FRET)-sensor Epac2camps (Nikolaev et al., 2004), a kind gift by Martin Lohse (Institute of Pharmacology and Toxicology, University of Würzburg, Germany), under the control of the proglucagon promoter, we replaced the sequence between the proglucagon start codon in exon 2 and stop codon in exon 6 in the murine-based BAC RP23-343C17 (Children’s Hospital Oakland Research Institute, Oakland, CA, USA) by the Epac2camps sequence using Red/ET recombination technology (Genebridges, Heidelberg, Germany) (Figure 3A).

We were unable to simply introduce proglucagon gene specific 3’ and 5’ sequences through PCR amplification of Epac2camps with the primers mGLP002 and mGLP006, presumably because of duplication of the 5’ YFP sequence and the 3’ CFP sequence within the FRET sensor. Using a combination of PCR amplification and fragment subcloning of in-house plasmids containing 5’ and 3’ proglucagon sequence around a YFP-variant (Venus) insert (Reimann et al., 2008), a construct containing 364 bp 5’ to the gcg start codon and 663 bp 3’ to the gcg stop codon in which the gcg coding sequence is replaced by the Epac2camps sequence was created and amplified with the primer pair FRGLU008/mGLP005 (see oligonucleotides tabulated below). Homologous recombination was achieved upon co-transforming, an rpsLneo-modified BAC (Reimann et al., 2008) containing Escherichia coli DH10B clone with this 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 Epac2camps sequence was confirmed by direct sequencing. BAC-DNA for microinjection was purified using the large-construct Maxi-Prep kit (Qiagen, Manchester, UK) and dissolved at ~1–2 ng·μl⁻¹ in injection buffer containing (mmol·l⁻¹): 10 Tris–HCl pH 7.5, 0.1 EDTA, 100 NaCl, 0.03 spermine and 0.07 spermidine. Pronuclear injection into ova derived from C57BL6/CBA F1 parents and reimplantation of embryos into pseudopregnant females was performed by the Central Biomedical Services at Cambridge University. The DNA of pups was isolated from ear clips by proteinase K digestion and screened for the transgene by PCR using the following primer pairs: mGLP013/Epac_in1, GF002/003 (and RM41/42, which amplifies the β-catenin sequence used as a DNA quality control). We initially created 12 founders, of which 10 passed on the transgene. Two lines GLU-Epac20 and GLU-Epac21 were selected based on the brightness of intestinal L cells expressing the sensor and the observable responses of Epac2camps-positive L cells to a test stimulus [forskolin (Fsk)/IBMX 10 μM each or taurolithocholate 10 μM] and were backcrossed onto C57BL6 for at least seven generations. Correct expression of Epac2camps in intestinal L cells was confirmed by immunohistochemistry in the GLU-Epac20 and GLU-Epac21 mouse lines (Figure 3B (upper small intestine), Supporting Information Table 2 and Supporting Information Figure 1 (colon)). We observed that, on average, >80% of all proglucagon expressing cells also express the sensor, and >90% of all Epac2camps-expressing cells also express proglucagon, without obvious differences in the two lines and in line with previous findings in GLU-Venus mice carrying a similar transgene (Habib et al., 2012).

Expression analysis

L and K cells were isolated by FACS from GLU-Venus (Reimann et al., 2008) and GIP-Venus (Parker et al., 2009) transgenic mice, respectively, as previously described. Briefly, the small intestine (upper or lower 10 cm) or colon was digested as described in the succeeding texts for primary cultures, but using 1 mg·ml⁻¹ in HBSS (Sigma, Poole, UK), to obtain single cells. Cell suspensions were separated using a MoFlo Cytomation sorter (Beckman Coulter, High Wycombe, UK) (488 nm excitation) to obtain populations of fluorescent cells, as assessed by ELISA whereas intestinal L cells expressing the sensor and GLU-Venus-positive or control Venus-negative cells, which were collected directly into lysis buffer for mRNA extraction. Purified K and L cell populations were over 95% pure as assessed by fluorescence microscopy and were enriched more than 2000-fold for GIP and GLP-1 concentrations, respectively, compared with non-fluorescent cells, as assessed by ELISA (Parker et al., 2009; Reimann et al., 2008).

RNA was extracted using a microscale RNA isolation kit (Applied Biosystems, Warrington, UK) as previously described (Parker et al., 2009; Reimann et al., 2008). For hybridisation to mouse 430 2.0 arrays GeneChips (Affymetrix, High Wycombe, UK), RNA underwent two rounds of amplification based on in vitro translation from a T7 promoter induced during oligo dT-priming (two-cycles cDNA synthesis Kit, Affymetrix; MEGAscript T7 kit, Ambion, Austin, TX, USA), and expression levels of each probe were determined by robust multichip average analysis.

Quantitative RT-PCR

Extraction of RNA from purified intestinal cells was performed using an RNeasy Micro Kit (Qiagen). The appropriate amount of first-strand cDNA template was mixed with specific TaqMan primers (Applied Biosystems, Foster City, CA, USA), RNase-free water and PCR Master Mix (Applied Biosystems, Foster City). Quantitative RT-PCR was conducted using a 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City). All
Primary murine intestinal cultures

C57BL6 mice aged 15–21 weeks were culled, and the intestines were collected into ice-cold Leibovitz (L-15) medium (Sigma). Mixed intestinal cultures were produced, as previously described (Reimann et al., 2008). Briefly, following the removal of the muscle layers, the intestine was opened longitudinally, rinsed in PBS and cut into 1–2 mm² pieces. Duodenal cultures contained tissue from the top 10 cm of the small intestine distal to the stomach, and ileal cultures contained tissue from the bottom 10 cm of the small intestine, proximal to the caecum. Tissue was digested with 0.3 mg mL⁻¹ Collagenase (Sigma), centrifuged at 100× g and resuspended in DMEM (24 mM glucose) supplemented with 10% FBS, 2 mM glutamine, 100 U mL⁻¹ of penicillin and 0.1 mg mL⁻¹ of streptomycin. Intestinal cell/crypt suspensions were plated onto 24-well plates coated with 1% v/v Matrigel (BD Biosciences, Oxford, UK) and incubated overnight at 37°C in 5% CO₂. Each intestinal segment (duodenum, ileum or colon) of an individual mouse gave rise to a single 24-well plate, and each 24-well plate only contained tissue isolated from one mouse.

Secretion studies

Secretion studies on primary intestinal cultures were performed approximately 24 h after plating. Cultures were washed thoroughly and incubated with test reagents in saline solution (see Solutions and chemicals) supplemented with 0.1% fatty acid-free BSA for 2 h at 37°C. Supernatant and lysate samples were collected. Samples were assessed for GLP-1 content using a total GLP-1 assay (Meso Scale Discovery, Gaithersburg, MD, USA). GIP was measured using a total GIP ELISA kit (Millipore, Billerica, MA, USA). GIP and GLP-1 were measured from the same samples. Hormone secretion was calculated as a percentage of total hormone content per well, to account for an unknown number of L cells per well in the mixed primary cell cultures. Statistical analysis was carried out on the % hormone secretion values. Each graph represents five independent experiments (cultures/24-well plates).

cAMP FRET measurements

Single-cell measurements of cAMP levels were made using intestinal cultures from transgenic mice expressing the FRET-based sensor Epac2camps (Nikolaev et al., 2004) under the control of the proglucagon promoter. Three transgenic mice were used to obtain cells for the FRET experiments. The protocol used was based on the one previously described for GLUTag cells (Moss et al., 2012). Briefly, primary duodenal cells, continuously perfused with saline solution with or without test reagents, were visualised with a ×40 oil immersion objective on an inverted microscope (Olympus IX71, Southend on Sea, UK). Excitation at 435 nm was achieved using a xenon arc lamp coupled to a monochromator (Cairn Research, Faversham, UK) controlled by MetaFluor software (Molecular Devices, Wokingham, UK). CFP emission at 470 nm and YFP emission at 535 nm were monitored using an Optosplit II beam splitter (Cairn Research) and an Orca-ER digital camera (Hamamatsu, Welwyn Garden City, UK) and expressed as the CFP/YFP fluorescence ratio (Friedlander et al., 2011; Moss et al., 2012). Data were smoothened with a sliding average across 30 s. Maximum ratios were determined at baseline (30 s period prior to test condition) and following test reagent application, and calculated increments were normalised to baseline.

Materials

The saline solution contained (in mM) 138 NaCl, 4.5 KCl, 4.2 NaHCO₃, 1.2 NaH₂PO₄, 2.6 CaCl₂, 1.2 MgCl₂ and 10 HEPES (±10 glucose, as indicated). Saline with 10 mM glucose was used in all secretion experiments for GIP analysis. Unless otherwise stated, all drugs and chemicals were obtained from Sigma UK. Where possible, drugs were made up as a 1000× stock as per manufacturer instructions. Galanin (1–29), the GAL1 receptor agonist M617 (Lundstrom et al., 2005) and TPN-Q (Jin et al., 1999a; Jin & Lu, 1999b) were obtained from Tocris Bioscience (Abingdon, UK). The GIRK1/4 (K₃.1/K₃.4) channel activator ML297 was obtained from Abcam Biochemicals (Cambridge, UK).

Data analysis and statistical procedures

Quantitative RT-PCR. All experiments were performed on three independently isolated cDNA samples (n = 3 mice). However, in line with Curtis et al. (2015), as n < 5, no statistical analysis has been presented. Data are expressed as geometric mean (2^ΔCT) ± SEM.

Secretion studies. Hormone secretion data (%GLP-1, %GIP) were analysed by linear regression using cluster–robust standard error estimation (Huber, 1967), to account for non-independence of observations made on each culture (mouse). Each graph represents data from five independent experiments/cultures (24-well plates). Statistical analysis was carried out using the statistical software STATA® 14 (College Station, Texas, USA). The threshold for significance was P < 0.05. Data are expressed as mean ± SD.

Imaging. For CFP/YFP emission ratio data, linear regression using cluster–robust standard error estimation was used to estimate the mean of the paired differences. The threshold for significance was P < 0.05. Data are expressed as mean ± SD.

Results

Galar1 mRNA is enriched in enteroendocrine K and L cells

To identify the galanin receptor expressed by primary incretin-secreting enteroendocrine cells, FACS-purified L and K cells from GLU-Venus and GIP-Venus mice, respectively, along with non-fluorescent control cells were analysed
by microarray. The only galanin receptor expressed at meaningful levels and enriched in the enteroendocrine populations was the GAL1 receptor (Figure 1A). Quantitative qPCR using mRNA from additional FACS-sorted cells also demonstrated marked enrichment of the GAL1 receptor in enteroendocrine L and K cells (Figure 1B).

Galanin and a GAL1 receptor agonist inhibit GLP-1 and GIP secretion

GIP secretion from K cells in mixed primary small intestinal cultures was significantly stimulated (~3.5-fold) by incubation with the non-specific PDE inhibitor IBMX (100 μM) (Figure 2A). IBMX also significantly stimulated GLP-1 secretion from L cells in duodenal and ileal cultures (~2- to 3-fold, Figure 2B and C). Galanin (100 nM) and the GAL1 receptor agonist M617 (100 nM) significantly inhibited IBMX-stimulated GIP and GLP-1 secretion from duodenal and ileal cultures (Figure 2). Galanin and M617 also suppressed basal GLP-1 secretion from the duodenum; however, this effect did not achieve statistical significance (Figure 2B).

Galanin significantly reduces forskolin-stimulated intracellular CAMP response in L cells

Forskolin, a potent activator of adenylyl cyclase, stimulates GLP-1 secretion via an increase in intracellular CAMP ([cAMP]i) in duodenal cultures (Figure 3C–E). L cells within duodenal cultures derived from GLU-Epac2camps mice were imaged to determine the effect of galanin on the forskolin-induced [cAMP]i response. In the presence of galanin, the increase in [cAMP]i stimulated by forskolin was significantly inhibited (Figure 3C–E).

GIRK1/4 (Kir3.1/Kir3.4) subunits are enriched in enteroendocrine K and L cells

To determine whether GIRK (Kir3) channels are expressed by primary incretin-secreting enteroendocrine cells, FACS-

Figure 1

Galt1 mRNA is enriched in enteroendocrine K and L cells. (A) Mean microarray robust multichip average (RMA) intensities for probes against Galt1, Galt2 and Galt3 in K cells (K+ cells), small intestinal and colonic L cells (L+ and CL+ respectively) and non-fluorescent control cells from the same tissue preparations (K-, L- and CL- respectively) (n = 2–3 each, as indicated). (B) Relative expression of Galt1 mRNA relative to β-actin assessed by RT-PCR in FACS-sorted cell populations derived from the upper and lower small intestine (SI) and colon. Data are presented as the geometric mean and upper SEM (n = 3 mice each).

Figure 2

Inhibition of IBMX-stimulated GIP and GLP-1 secretion by galanin (Gal) and GAL1 receptor agonism. (A) GIP secretion from primary duodenal cultures treated with IBMX (100 μM) with or without Gal (100 nM) and the GAL1 receptor agonist M617 (100 nM) in the presence of 10 mM glucose. (B, C) GLP-1 secretion from primary duodenal (B) and ileal (C) cultures treated with IBMX (100 μM) with or without Gal (100 nM) and the GAL1 receptor agonist M617 (100 nM) in the absence of glucose. Data represent mean % hormone secretion ± SD. The number of wells that contributed to the column mean is displayed above each column; the number of independent cultures/experiments/mice is found in brackets. Statistical significance was assessed by linear regression using cluster–robust standard error estimation. *P < 0.05, **P < 0.01.
Galanin (Gal) reduces the Fsk-stimulated cAMP and GLP-1 responses in primary duodenal L cells of GLU-Epac2camps mice. (A) Schematic diagram of genetic alteration of the GLU-Epac2camps transgene. (B) Correct expression of Epac2camps in upper small intestinal L cells of the GLU-Epac lines was confirmed by immunohistochemistry and confocal microscopy. Representative photomicrograph demonstrating co-localisation of glucagon (GLP-1, red fluorescence) and GFP (Epac2camps, green fluorescence). Nuclei were visualised with Hoechst staining (blue). (C) Changes in cAMP concentration in response to GAL1 receptor/Gαi activation. Primary duodenal L cells were perfused with Fsk (2 μM) with or without Gal (100 nM), as indicated. CFP and YFP emission was monitored in response to excitation with 435 nm. An example trace representing the CFP/YFP ratio of a single cell is depicted. (D) All individual cAMP increases relative to baseline (36 cells) in response to Fsk/Gal and Fsk alone are shown. (E) Mean changes in the CFP/YFP emission ratio in response to application of Fsk with or without Gal in experiments performed as in (C). Data are means ± SD (36 cells, from five cultures/independent experiments/mice). Linear regression using cluster–robust standard error estimation was used to estimate the mean of the paired differences. ***P < 0.001 compared with Fsk alone. (F) GLP-1 secretion from primary murine duodenal cultures treated with Fsk (2 μM) with or without Gal (100 nM) and the GAL1 receptor agonist M617 (100 nM). Data represent mean % hormone secretion ± SD. The number of wells that contributed to the column mean is displayed above each column; the number of independent cultures/experiments/mice is found in brackets. Statistical significance was assessed by linear regression using cluster–robust standard error estimation. *P < 0.05, **P < 0.01.
purified L and K cells from GLU-Venus and GIP-Venus mice, respectively, along with non-fluorescent control cells were analysed by microarray. The only GIRK channel subunits expressed at meaningful levels and enriched in the enteroendocrine populations of the small intestine were GIRK1/4 (Kir3.1/Kir3.4), which are known to co-assemble (Chan et al., 1996; Duprat et al., 1995; Krapivinsky et al., 1995; Spauschus et al., 1996) (Figure 4A). Quantitative qPCR using non-amplified mRNA from additional FACS-sorted cells also demonstrated marked enrichment of Kcnj3 and Kcnj5 in small intestinal enteroendocrine L and K cells (Figure 4).

**GIRK (Krer3) activation inhibits basal and stimulated GLP-1 secretion**

GIRK1/4 (Kir3.1/Kir3.4) channels were activated using ML297, a potent GIRK activator (EC50 for GIRK1/4 ≈ 1 μM; Kaufmann et al., 2013). Incubation of duodenal cultures with ML297 (100 μM) significantly inhibited glucose-stimulated and IBMX-stimulated GLP-1 secretion in duodenal cultures (Figure 5B). However, ML297 had no effect on basal or IBMX-induced GIP secretion (Figure 5A).

**Inhibition of GIRK (Krer3) channels or KATP (Ker6) channels does not abolish galanin-mediated inhibition of GLP-1**

TPN-Q is a high-affinity stable derivative of tertiapin from honey bee venom and selectively inhibits GIRK1/4 and ROMK1 channels with nanomolar affinities (Jin & Lu, 1999b). ROMK1 (Kcnj1) is not expressed in enteroendocrine L and K cells according to our microarray data (data not shown). Pretreatment (30 min) and incubation of duodenal cultures with TPN-Q (10 μM) did not affect the ability of galanin to inhibit IBMX-stimulated or glucose-stimulated GLP-1 secretion (Figure 6). Inhibition of KATP channels using tolbutamide and gliclazide, at established concentrations (Proks et al., 2013; Reimann et al., 2008), also did not prevent galanin from inhibiting GLP-1 secretion in response to IBMX (Supporting Information Figure 2).

**Discussion and conclusions**

We have demonstrated that galanin potently inhibits both GLP-1 and GIP secretion from primary small intestinal cultures by acting on the GAL1 receptor, which we have identified as the only galanin receptor highly expressed and enriched in enteroendocrine L and K cells. Using a novel transgenic mouse strain, GLU-Epac2camps, which expresses the YFP/CFP-based

**Figure 4**

Expression of GIRK channels in enteroendocrine K and L cells. (A) Mean microarray robust multichip average (RMA) intensities for probes against Kcnj3 (GIRK1, Kir3.1) and Kcnj5 (GIRK4, Kir3.4) in K cells (K+ cells), small intestinal and colonic L cells (L+ and CL+ respectively) and non-fluorescent control cells from the same tissue preparations (K-, L- and CL- respectively) (n = 2–3 each as indicated). (B) Relative expression of Kcnj3 and Kcnj5 mRNA relative to β-actin assessed by RF-PCR in FACS-sorted cell populations derived from the upper and lower small intestine (SI) and colon. Data are presented as the geometric mean and upper SEM (n = 3 mice each).

**Figure 5**

Inhibition of IBMX-stimulated GLP-1 but not GIP secretion by ML297, an activator of GIRK channels. (A) GIP secretion from primary duodenal cultures treated with IBMX (100 μM) with or without galanin (Gal) (100 nM) and the GIRK channel agonist ML297 (100 μM) in the presence of 10 mM glucose. (B) GLP-1 secretion from primary duodenal cultures treated with IBMX (100 μM) with or without Gal (100 nM) and the GIRK channel agonist ML297 (100 μM) in the presence of 10 mM glucose. Data represent mean % hormone secretion ± SD. The number of wells that contributed to the column mean is displayed above each column; the number of independent cultures/experiments/mice is found in brackets. Statistical significance was assessed by linear regression using cluster-robust standard error estimation. **P < 0.01.
Galanin inhibits GLP-1 and GIP via GAL1 receptor

Figure 6
Inhibition of IBMX-stimulated GLP-1 secretion by galanin (Gal) is not affected by GIRK channel inhibition. (A) GLP-1 secretion from primary duodenal cultures treated with IBMX (100 μM) and Gal (100 nM) in the presence or absence of the GIRK channel blocker TPN-Q (10 μM) and following a 30 min pretreatment with TPN-Q (10 μM). (B) GLP-1 secretion from primary duodenal cultures treated with glucose (10 mM) and Gal (100 nM) in the presence or absence of the GIRK channel blocker TPN-Q (10 μM) and following a 30 min pretreatment with TPN-Q (10 μM). Data represent mean % hormone secretion ± SD. The number of wells that contributed to the column mean is displayed above each column; the number of independent cultures/experiments/mice is found in brackets. Statistical significance was assessed by linear regression using cluster–robust standard error estimation. *P < 0.05, **P < 0.01.

cAMP-FRET-sensor Epac2camps under the control of the proglucagon promoter, we showed that galanin significantly inhibits the intracellular accumulation of cAMP triggered by Fsk in primary L cells. This finding is consistent with the G<sub>i</sub> coupling of the GAL1 receptor. L and K cells were also shown to express GIRK1/4 (Kir3.1/Kir3.4) channels, which have previously been associated with GAL1 receptor signalling. However, we were unable to demonstrate a role for GIRK activation in the inhibition of incretin secretion by galanin.

Enteroendocrine secretion is known to be under the influence of several peptides derived from the enteric nervous system (Psichas et al., 2015) such as members of the bombesin/gastrin-releasing peptide family and calcitonin gene-related peptide, which trigger GLP-1 secretion in various settings (Dumoulin et al., 1995; Herrmann-Rinke et al., 1995; Plaisancie et al., 1994). However, despite the likely importance of inhibitory influences in fine-tuning or terminating incretin secretion, they have received relatively little attention. In humans, there is some evidence to suggest that galanin reduces gastrointestinal motility and suppresses the initial postprandial rise in plasma PY and GLP-1 but not GIP (Baue et al., 1989). Furthermore, galanin has been shown to dose-dependently inhibit GLP-1 secretion from isolated vascularly perfused rat ileum (Herrmann-Rinke et al., 1996) and cholecystokinin secretion from STC-1 cells (Chang et al., 1995) in response to a variety of stimulants including bombesin and Fsk. We demonstrated that galanin is a potent inhibitor of both GLP-1 and GIP secretion from primary murine small intestinal cultures.

Investigation into the mechanisms underlying the inhibitory effect of galanin on gut hormone secretion has been limited. Chang et al. (1995) and Saflia et al. (1998) demonstrated that the inhibitory mechanism was sensitive to pertussis toxin in rat ileal cells and STC-1 cells, respectively, suggesting the recruitment of a G<sub>i</sub>-coupled pathway. However, the identity of the galanin receptor and the nature of the inhibition, whether direct or indirect, had not been determined. We demonstrate here for the first time that mRNA for the G<sub>i</sub>-coupled galanin 1 receptor (Galr1) is highly enriched in enteroendocrine L and K cells. Moreover, incubation of primary small intestinal cultures with the selective GAL1 receptor agonist M617 significantly inhibited GLP-1 and GIP secretion in the presence of IBMX. In accordance with galanin activating a G<sub>i</sub>-coupled pathway, the presence of galanin significantly inhibited the increase in intracellular cAMP mediated by Fsk in primary L cells, as measured using cultures from mice expressing the Epac2camps sensor under the control of the proglucagon promoter.

Galanin receptor activation has also been associated with the opening of GIRK (K<sub>G</sub>3.1/K<sub>G</sub>3.4) channels, which act as inward rectifiers play a fundamental role in maintaining the resting membrane potential (Counts et al., 2002; de Weille et al., 1989; Parsons et al., 1998; Smith et al., 1998). GIRK subunits released from pertussis toxin-sensitive G<sub>i</sub> proteins are thought to bind directly to GIRK channels resulting in permeability to K<sup>+</sup> ions and consequent hyperpolarisation (Huang et al., 1995; Logothetis et al., 1987; Pfaffinger et al., 1985; Reuveny et al., 1994; Wickman et al., 1994). We demonstrate here for the first time that mRNA for the GIRK subunits 1 and 4 (K<sub>G</sub>3.1/K<sub>G</sub>3.4) is enriched in enteroendocrine L and K cells. Furthermore, incubation of primary duodenal cultures with the GIRK channel activator ML297 is capable of inhibiting GLP-1 secretion in the presence of IBMX and/or glucose. Conversely, ML297 did not inhibit GIRK secretion. The reasons for this are unclear but might reflect a failure of GIRK mRNA translation in K cells or a higher non-GIRK resting potassium conductance in K cells compared with L cells. In addition, it is possible that at 100 μM, ML297 is exhibiting off-target effects on voltage-gated ether-a-go-go-related gene (Kcnh2, Kv11.1) potassium channels (Kaufmann et al., 2013), which are expressed in L cells but not K cells (data not shown). Therefore, we sought to determine whether galanin may activate GIRK1/4 channels as part of its mechanism of action in L cells. However, incubating with the GIRK blocker TPN-Q did not affect the ability of galanin to inhibit either IBMX-stimulated or glucose-stimulated GLP-1 secretion. TPN-Q was used at a concentration ~1000-fold higher than the K<sub>i</sub> for GIRK1/4. However, the lack of a positive control for TPN-Q activity in L cells prevents us from conclusively discounting a role for these channels in galanin-mediated inhibition of GLP-1 secretion.

K<sub>ATP</sub> channels (K<sub>ATP</sub>6) have also been implicated in the inhibition of gut hormone secretion mediated by galanin (Saflia et al., 1998). We have previously reported that K<sub>ATP</sub> channel subunits are expressed in primary enteroendocrine L and K cells (Parker et al., 2009; Reimann et al., 2008). However, using the well-characterised sulfonylureas tolbutamide and gliclazide to block K<sub>ATP</sub> channels in duodenal cultures, we found no evidence to suggest involvement of these channels in the suppression of GLP-1 secretion by galanin.

Considerable effort has been made to identify stimulants of incretin secretion. However, our understanding of the
physiological regulation of gut hormone secretion is incomplete without knowledge of the contribution of inhibitory neuropeptides and hormones acting simultaneously to prevent, dampen down or terminate secretion. We have used an in vitro primary cell model to investigate the inhibitory effect of galanin on gut hormone secretion. As with all in vitro models, there are several inherent limitations including the loss of innervation, blood supply and polarity of cells in culture. It is difficult to ascertain the potential impact of these artificial conditions. However, as the inhibitory effects of galanin have been previously demonstrated in both the ex vivo and in vivo settings (Bauer et al., 1989; Herrmann-Rinke et al., 1996) with similar results, we are confident that our primary cell culture model is a valid tool and, in conjunction with novel transgenic mouse models, has provided important mechanistic insight.

Previous studies revealed that galanin-immunoreactive nerve fibres could be readily detected in the vicinity of L cells (Herrmann-Rinke et al., 1996). However, the general consensus was that a space of several micrometres separated nerve fibres and enteroendocrine cells. Thus, it was argued that enteroendocrine cells were targeted by neuropeptides via diffusion, a method of neurotransmission commonly employed by the enteric nervous system, rather than by synaptic transmission. Recent work (Bohorquez et al., 2014; Bohorquez et al., 2015) now suggests that enteroendocrine cells possess a prominent cytoplasmic process referred to as a “neuropod,” which is capable of direct contact with nerve fibres. Therefore, it is evident that we do not fully understand the interaction between efferent neurotransmission and the fine-tuning of gut hormone secretion and nutrient responsiveness. Further work is warranted in this emerging field.

In conclusion, we have shown in primary cells that galanin inhibits GLP-1 and GIP secretion via the GAL1 receptor and activation of G_i-coupled signalling leading to a reduction in intracellular cAMP. Enteroendocrine L cells also express GIRK1/4 (K_γ3.1/K_γ3.4) channels, and the GIRK-agonist M1297 inhibited GLP-1 secretion, presumably opening a potassium conductance. However, we found no evidence to suggest that GIRK1/4 or K ATP channel activation plays a role in galanin-mediated inhibition of GLP-1 secretion.

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Author contributions

A. P., L. L. G. and F. R. performed the research. A. P., F. R. and F. M. G. designed the research study. A. P. and S. J. S. analysed the data. A. P., F. R. and F. M. G. wrote and edited the paper.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

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Table S1 Oligonucleotides used to create and verify GLU-Epac2camps mice.

Table S2 Immunohistochemical characterization of GLU-Epac2camps mice (small intestine, n=3 mice per strain; colon, n=2 mice per strain).

Figure S1 Epac2camps in colonic L cells from a Glu-Epac2camps mouse. Fixed colonic slices were co-immunostained for GFP (representing Epac2camps) together with glucagon (Gcg, GLP-1). Nuclei were visualised with Hoechst staining.

Figure S2 Inhibition of IBMX-stimulated GLP-1 secretion by galanin is not affected by K_ATP (K_6) channel inhibition. GLP-1 secretion was measured from primary duodenal cultures treated with IBMX (100μM) and Gal (100nM) in the presence or absence of the K_ATP channel blockers Tolbutamide (100μM) and Gliclazide (100nM), in the absence of glucose. Data represent mean % hormone secretion ± SD. The number of wells that contributed to the column mean is displayed above each column; the number of independent cultures/experiments/mice is found in brackets. Statistical significance was assessed by linear regression using cluster-robust standard error estimation. *P<0.05, **P<0.01.