BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01561.x
www.brjpharmacol.org

RESEARCH PAPER

Molecular mechanisms underlying bile acid-stimulated glucagon-like peptide-1 secretion

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Keywords
GLP-1; L-cells; GPBA; TGR5; cAMP

Received
16 March 2011
Revised
31 May 2011
Accepted
2 June 2011

BACKGROUND AND PURPOSE

The glucagon-like peptides GLP-1 and GLP-2 are secreted from enteroendocrine L-cells following nutrient ingestion. Drugs that increase activity of the GLP-1 axis are highly successful therapies for type 2 diabetes, and boosting L-cell secretion is a potential strategy for future diabetes treatment. The aim of the present study was to further our understanding of the bile acid receptor GPBA (TGR5), an L-cell target currently under therapeutic exploration.

EXPERIMENTAL APPROACH

GLUTag cells and mixed primary murine intestinal cultures were exposed to bile acids and a specific agonist, GPBAR-A. Secretion was measured using hormone assays and intracellular calcium and cAMP responses were monitored using real-time imaging techniques.

KEY RESULTS

Bile acid-triggered GLP-1 secretion from GLUTag cells was GPBA-dependent, as demonstrated by its abolition following *tgr5* siRNA transfection. Bile acids and GPBAR-A increased GLP-1 secretion from intestinal cultures, with evidence for synergy between the effects of glucose and GPBA activation. Elevation of cAMP was observed following GPBA activation in individual GLUTag cells. Direct calcium responses to GPBAR-A were small, but in the presence of the agonist, a subpopulation of cells that was previously poorly glucose-responsive exhibited robust glucose responses. *In vivo*, increased delivery of bile to more distal regions of the ileum augmented L-cell stimulation.

CONCLUSIONS AND IMPLICATIONS

GPBA signalling in L-cells involves rapid elevation of cAMP, and enhanced calcium and secretory responses to glucose. Modulation of this receptor therapeutically may be an attractive strategy to enhance GLP-1 secretion and achieve better glycaemic control in diabetic patients.

Abbreviations

CDCA, chenodeoxycholic acid; CFP, cyan fluorescent protein; DCA, deoxycholic acid; Epac, cAMP-guanine nucleotide exchange factors; fsk/IBMX, forskolin plus 3-isobutyl-1-methylxanthine; GLP-1, -2, glucagon-like peptide-1, -2; K_{ATP}, ATP-sensitive potassium channel; LCA, lithocholic acid; PYY, peptide YY; TLCA, taurolithocholic acid; YFP, yellow fluorescent protein

Introduction

The hormones glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) are secreted from enteroendocrine L-cells, which are found scattered throughout the intestinal epithelium, particularly in the ileum and colon. GLP-1 is best known as an incretin hormone causing enhanced postprandial insulin secretion, but it also has a range of additional effects including reduced gastric motility and appetite suppression, which indirectly impact on glucose metabolism *in vivo*. For these reasons, newly introduced GLP-1-based therapies, which either mimic GLP-1 or slow its clearance, are highly successful agents for the treatment of type 2 diabetes (Drucker and Nauck, 2006). GLP-2, on the other hand, is implicated in the growth and repair of the intestinal mucosa and gut motility, and is under therapeutic evaluation for the treatment of gut disorders such as short bowel syndrome (Wallis *et al.*, 2009). Identifying strategies to target the secretion of these peptides *in vivo* offers an attractive prospect for developing alternative treatments for type 2 diabetes, obesity and intestinal disorders.

Secretion from L-cells *in vivo* is stimulated by the arrival and digestion in the intestinal lumen of nutrients such as carbohydrates, fats and proteins. However, other luminal components, such as bile acids, which are released into the intestine from the gall bladder after lipid ingestion, have also been shown to stimulate GLP-1 secretion. Thus, glucagon immunoreactivity was shown to increase in canine ileal loops and human colon following infusion of bile acids (Namba *et al.*, 1983; Adrian *et al.*, 1993) and GLP-1 levels were found to increase in portal effluent following the luminal perfusion of bile into perfused rat colon preparations (Plaisancie *et al.*, 1995). More recently, intrarectal infusion of taurocholic acid has been shown to increase plasma GLP-1 and peptide YY (PYY) levels in obese, type 2 diabetic subjects (Adrian *et al.*, 2010).

Bile acids are released into the duodenum in response to meal ingestion and are actively absorbed back into the portal circulation in the terminal ileum, with only a small fraction spilling into the colon (Hofmann, 2009). It has been hypothesized that postprandial variations of bile acids in the gut lumen and circulation could be important in signalling to various organs that a meal has been ingested (Houten *et al.*, 2006). Beneficial effects of bile acids on whole body metabolism are suggested by a number of findings, including that diversion of bile away from the duodenum in dogs reduced the insulin response to orally ingested glucose (Gomez *et al.*, 1987) and that dietary supplementation with bile acids efficiently attenuated diet-induced obesity in mice (Watanabe *et al.*, 2006). Altering bile acid delivery to the distal gut using bile acid sequestrants has been found to increase plasma GLP-1 levels in humans and rodents (Chen *et al.*, 2010; Shang *et al.*, 2010; Garg *et al.*, 2011). It has also been proposed that direct access of bile acids to L-cell-rich regions of the intestine and enhanced circulating bile acid concentrations may contribute to the rapid remission of type 2 diabetes after gastric bypass surgery (Nakatani *et al.*, 2009; Patti *et al.*, 2009).

While bile acids are recognized to signal via specific nuclear hormone receptors (Hylemon *et al.*, 2009), there is also a cell surface bile acid-sensitive GPCR known as TGR5 (Maruyama *et al.*, 2002) or GPBA (receptor nomenclature as in Alexander *et al.*, 2011). GPBA (TGR5) is believed to couple to $G_{\alpha s}$ proteins and activation of cAMP signalling pathways in a number of tissues including brain, liver, skeletal muscle and brown adipose tissue (Watanabe *et al.*, 2006; Keitel *et al.*, 2007; 2010). In the enteroendocrine cell lines, STC-1 and NCI-H716, its activation results in enhanced GLP-1 secretion (Katsuma *et al.*, 2005; Thomas *et al.*, 2009), although the signalling mechanisms remain incompletely characterized. Downstream pathways that have been proposed include elevation of intracellular cAMP and Ca^{2+} concentrations and closure of ATP-sensitive potassium (K_{ATP}) channels (Thomas *et al.*, 2009).

Recent technological developments now enable studies on enteroendocrine cell lines to be validated in primary cells and whole animals. Indeed, global knockout of GPBA has been shown to affect glucose tolerance, fat deposition and GLP-1 levels in mice (Thomas *et al.*, 2009). Our own group has developed tools for studying the expression profile and function of primary mouse L-cells, identified by their cell-specific expression of a yellow fluorescent protein (YFP) derivative, Venus, in a transgenic mouse model GLU-Venus (Reimann *et al.*, 2008). Transcriptomic analysis of FACS-purified L-cells from GLU-Venus mice demonstrated that GPBA mRNA is highly enriched in L-cells compared with neighbouring epithelial cells throughout the gastrointestinal tract (Reimann *et al.*, 2008). The validation of intracellular probes for monitoring cAMP levels in model L-cells (Friedlander *et al.*, 2010) also provides a tool for assessing dynamic responses to activation of $G_{\alpha s}$-coupled receptors in GLP-1-secreting cells. The aim of this current study was to investigate the effect of GPBA stimulation on L-cell secretion in the model cell line GLUTag (Drucker *et al.*, 1994) using real-time intracellular calcium and cAMP imaging techniques (Friedlander *et al.*, 2010), and to validate the findings in mixed primary intestinal cultures from adult mice (Reimann *et al.*, 2008) and in an *in vivo* model of biliary tract diversion in rats.

Methods

Animals models

All animal care and experimental procedures were approved by the local ethics committee and conformed to UK Home Office regulations.

Duodenal transposition in a rodent model

Sixteen adult male Wistar rats were randomized to either sham operation or duodenal transposition. Rats were anaesthetized with a mixture of Hypnorm® (0.35 mL) and diazepam (0.35 mL), which was injected i.m. before the procedure. Duodenal transposition involved excision of the segment of duodenum containing the hepatopancreatic ampulla, which allows drainage of both bile and pancreatic juices into the gut, followed by the anastomosis of the transected segment to the ileum, 10 cm proximal to the caecum. The sham operation consisted of transection of the proximal and distal parts of the duodenum followed by re-anastomosis in order for the rats to have the same surgical insult and time under anaesthesia. Rats were housed indi-

vidually and received a normal diet of chow and water *ad libitum*. Animals were killed 4 weeks post-operatively. Mixed arterial and venous blood was collected into lithium-heparin tubes containing 5000 kallikrein inhibitor units aprotinin, immediately spun down at 1600× *g* and stored at –70°C until analysis.

Primary murine intestinal cultures

The 2- to 6-month-old C57BL6 mice were killed by cervical dislocation and the gut collected into ice-cold Leibovitz-15 (L-15) medium. The intestine was opened longitudinally, rinsed in PBS, and chopped into 1–2 mm pieces. Upper small intestinal cultures contained tissue from the top 10 cm of the gut distal to the stomach and colon cultures consisted of tissue distal to the ileocolic junction. Tissue was digested with 0.4 mg·mL^{-1} Collagenase XI, centrifuged at 300× *g*, and resuspended in Dulbecco's modified Eagle's medium (DMEM; 25 mM glucose) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U·mL^{-1} penicillin and 0.1 mg·mL^{-1} streptomycin. Aliquots of tissue were plated and incubated at 37°C, 5% CO_2.

Cell line culture

GLUTag cells were cultured in DMEM (5.6 mM glucose) supplemented with 10% (v/v) FBS, 10 µg·mL^{-1} penicillin/streptomycin and 2 mM glutamine. They were incubated at 37°C, 5% CO_2 and the culture medium was changed every 3 days. When reaching ~70% confluence (~every 5 days), cells were trypsinized with 1X trypsin-EDTA, diluted and reseeded.

siRNA transfection

GLUTag cells plated on 6 cm Matrigel-coated plastic plates were transfected with either scrambled siRNA (control) or siRNA targeting murine GPBA (Qiagen, Crawley, UK) for 24 h. Transfection was performed using OptiMEM (Invitrogen, Paisley, UK) media, 12 µL Lipofectamine 2000 (Invitrogen) and 50 nM siRNA as instructed by the manufacturer. GLUTag cells were re-seeded on 24-well plates and allowed to recover for 24 h before being used in secretion experiments.

GLP-1 and GLP-2 secretion assay

Secretion studies on GLUTag and primary intestinal cultures were performed 24–36 h after plating on 24-well culture plates coated with Matrigel (BD Bioscience, Oxford, UK). Cultures were washed thoroughly and incubated with test reagents in saline solution (see below) containing 0.1% fatty acid-free BSA for 2 h at 37°C. Media was then collected and centrifuged to remove contaminating cells. In primary cell secretion experiments, the plated cells were treated with lysis buffer containing: 50 mM Tris-HCl, 150 mM NaCl, 1% IGEPAL-CA 630, 0.5% deoxycholic acid (DCA) and one tablet of complete EDTA-free protease inhibitor cocktail (Roche, Burgess Hill, UK). Plates were freeze-thawed and mechanically disrupted to extract intracellular peptides.

GLP-1 was assayed in the supernatant collected from GLUTag cells and in both the supernatant and cell extracts from primary intestinal cultures using an ELISA specific for GLP-1 (7–36) amide and GLP-1 (7–37) (GLP-1 active ELISA-kit, Millipore, Billerica, MA, USA).

Total GLP-2 was measured in supernatant collected from GLUTag experiments and in rat plasma by radioimmunoassay using antiserum FT-17 which cross-reacts 100% with human and rat GLP-2, less than 0.1% with GLP-1 and not with any other known gastrointestinal or pancreatic hormone. The assay measures GLP-2 (1–33) and its degradation product GLP-2 (3–33) and major proglucagon fragment (Feltrin *et al.*, 2006).

For the primary cell experiments, GLP-1 secretion in each well was expressed as a fraction of the total of that hormone measured in the same well, and was normalized to the basal secretion measured in parallel on the same day. For GLUTag cell experiments, secretion was normalized to the basal measured in parallel on the same day.

RNA extraction and qRT-PCR

Total RNA from FACS-sorted cells and GLUTag cultures was isolated using a micro scale RNA isolation kit (Ambion, Warrington, UK) and Tri-Reagent, respectively. A total of 100–150 ng of RNA was reverse-transcribed according to standard protocols using a Peltier Thermal Cycler-200 (MJ Research, Waltham, MA, USA). Quantitative RT-PCR was performed with 7900 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). PCR reactions mix consisted of first-strand cDNA template, primers (TaqMan gene expression assays, Applied Biosystems) and PCR Master mix (Applied Biosystems). In all cases, expression was compared with that of β-actin measured on the same sample, in parallel, on the same plate, giving a cycle threshold difference (ΔCT) for β-actin minus the test gene. Mean, SEM and statistics were obtained for the ΔCT data, and only converted to relative expression levels (2^ΔCT) for presentation.

Ca^{2+} measurements

Cells were plated in Matrigel-coated glass bottom dishes (MatTek, Ashland, MA, USA) 1–3 days prior to use and loaded with fura-2 by incubation in 2 µM of the acetoxymethyl ester (Molecular Probes, Leiden, the Netherlands) for 30 min in saline solution containing 1 mM glucose at room temperature. Cells were then washed, and dishes mounted on an inverted fluorescence microscope (Olympus IX71, Southall, UK) with a ×40 oil immersion objective. Excitation at 340 and 380 nm was achieved using a 75 W xenon arc lamp with a monochromator (Cairn Research, Faversham, UK) controlled by MetaFluor software (Universal Imaging; Cairn Research) and emission was recorded with a charged-coupled device camera (Orca ER, Hammamatsu; Cairn Research). Background-subtracted fluorescence was normalized to a baseline average measured before application of the first test reagent and expressed as a 340/380 nm ratio, and the response to test reagents was defined as the maximum concentration (averaged over 20 s) achieved during their application.

cAMP FRET measurements

Single cell measurements of cAMP levels were made using the FRET-based sensor, Epac2-camps kindly donated by M. Lohse (Nikolaev *et al.*, 2004). GLUTag cells were seeded on 6 cm Matrigel-coated plastic dishes and when 80–90% confluent transfected with 3 µg of Epac2-camps DNA probe and 15 µL Lipofectamine 2000 for 24 h. Cells were trypsinized and

re-seeded on Matrigel-coated 35 mm glass-bottomed dishes for experiments 24–48 h later. Before each experiment, cells were washed with saline solution and dishes mounted on an inverted microscope and continually perfused with standard saline solution with or without test reagents. Cells were visualized with a ×40 oil immersion objective and excitation at 435 nm was achieved using a CFP/YFP filter set and Xenon Arc Lamp coupled to a monochromator (Cairn Research) and controlled by MetaFlour software (Universal Imaging; Cairn Research). Cyan fluorescent protein (CFP) emission was acquired at 470 nm and YFP emission was acquired at 535 nm using an Optosplit II image splitter and Hamamatsu Orca-ER digital camera (Cairn Research). For the analysis, all data were normalized to an average baseline FRET measured before application of the first test reagent. Average fluorescence ratios were determined over 10 s periods, before addition of a test agent, during its perfusion, and during perfusion with forskolin (fsk)/IBMX (10 μM of each). The maximal response of each cell to a test reagent was expressed as a fraction of the maximal response achieved with fsk/IBMX measured in the same cell at the end of each experiment, where 0 represents no response and 1 is the maximal response. Cells in which the response was less than 5% of the fsk/IBMX response were classed as non-responders and excluded from subsequent data analysis.

Single cells were monitored as either taurolithocholic acid (TLCA) or GPBAR-A was perfused into the bath chamber. With this probe, addition of either reagent resulted in a decreased FRET signal, consistent with an increase in intracellular cAMP.

Solutions and chemicals

The saline solution contained (in mM) 128 NaCl, 5.6 KCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES. For experiments performed at a high potassium concentration, KCl was increased to 70 mM and NaCl decreased to 72.5 mM to maintain osmolarity. Unless stated, all drugs and chemicals were obtained from Sigma (Poole, UK). Where possible, drugs were made up as 1000× stock. The bile acids were tested at both 10 and 30 μM in the initial secretion studies, as indicated, and as both stimulated a similar response the results were pooled. For all other experiments thereafter, bile acids were used at 10 μM. The GPBA agonist, referred to as GPBAR-A, is ((4-[[3,5-bis(trifluoromethyl)phenyl]methyl]-6-(2-fluorophenyl)-4,5-dihydro-pyrido[3,2-f]-1,4-oxazepin-3(2H)-one) (Keitel *et al.*, 2009) and was used at 3 μM.

Statistical analysis

Comparisons between conditions were made using ANOVA followed by Student's one-sample or two-sample *t*-tests, or by χ^2-test (Microsoft Excel), as indicated, with a threshold for significance of $P = 0.05$. All data are expressed as mean ± SEM.

Results

Bile acid stimulated GLP-1 and GLP-2 secretion from GLUTag cells

We first investigated whether GLUTag cells are responsive to bile acids, as has been reported previously for the enteroendocrine cell lines STC-1 and NCI-H716. In secretion studies, we found that both GLP-1 and GLP-2 release from this cell line were responsive to a range of bile acids, including TLCA, lithocholic acid (LCA) and DCA (Figure 1A,B). Consistent with the involvement of the bile acid receptor, GPBA, GLP-1 release was only marginally responsive to the primary bile acid chenodeoxycholic acid (CDCA), but was stimulated by the specific GPBA agonist, GPBAR-A (Figure 1A,B). Responses to LCA, DCA and GPBAR-A were evident both in the absence and presence of glucose (Figure 1A,C). The involvement of GPBA was further examined by knockdown experiments using *gpba*-specific siRNA. Quantitative RT-PCR analysis showed that siRNA treatment significantly reduced *gpba* expression in GLUTag cells by 64% compared with the negative control siRNA ($P < 0.01$, $n = 4$) (Figure 1D). The *gpba*-specific siRNA abolished the GLP-1 secretory response to TLCA, and significantly reduced GPBAR-A-triggered GLP-1 secretion from 2.2-fold ($P < 0.001$, $n = 6$) to 1.5-fold ($P < 0.001$, $n = 6$) (Figure 1E).

Bile acids and a specific GPBA agonist stimulate GLP-1 secretion from primary intestinal cultures

Bile acid responses were further studied in mixed murine primary intestinal cultures, a model developed to mimic more closely the physiology of the native adult L-cell (Reimann *et al.*, 2008; Friedlander *et al.*, 2010; Rogers *et al.*, 2010; Tolhurst *et al.*, 2011). In primary colonic cultures, TLCA, LCA and DCA promoted GLP-1 secretion by 1.8-fold ($P < 0.001$, $n = 24$), 1.8-fold ($P < 0.001$, $n = 12$) and 2.4-fold ($P < 0.001$, $n = 9$), respectively (Figure 2A). The response to CDCA did not reach statistical significance. GPBAR-A stimulated a substantial, 4.2-fold ($P < 0.001$, $n = 21$), increase in GLP-1 release from colonic cell cultures. Upper small intestinal cultures exhibited a smaller, 2.6-fold ($P < 0.01$, $n = 7$), GLP-1 response to GPBAR-A, and were not responsive to TLCA in the absence of added nutrient (Figure 2B). In 10 mM glucose, however, TLCA triggered an additional 1.5-fold increment in secretion, suggesting a synergistic interaction between glucose and bile acid sensing pathways in these cells.

GPBA mediated cAMP response

We employed a FRET-based cAMP sensor, Epac2-camps (Nikolaev *et al.*, 2004), recently validated in our laboratory for use in GLUTag cells (Friedlander *et al.*, 2010), to monitor the time course and relative magnitude of cAMP changes in individual GLP-1-producing cells following addition of either TLCA or GPBAR-A. Figure 3A shows an example of a FRET trace, in which the CFP/YFP ratio acts as a readout of cAMP. GPBAR-A and TLCA triggered an elevation of cAMP concentrations within a few minutes of their addition to the perfusion solution, as exemplified in Figure 3A. The response to TLCA was 38% ($P < 0.05$, $n = 11$) of the maximal response to fsk/IBMX and, consistent with the secretion results, GPBAR-A stimulated a greater increase in cAMP with a mean elevation to 57% ($P < 0.001$, $n = 11$) of the maximum (Figure 3B).

GPBA mediated $Ca^{2+}{}_i$ response

To investigate whether GPBA activation, and the associated more modest cAMP response, would affect intracellular calcium levels, we monitored the 340/380 nm fluorescence ratio in GLUTag cells loaded with the calcium indicator, fura-2 (Figure 4A). GLUTag cells were selected in preference to

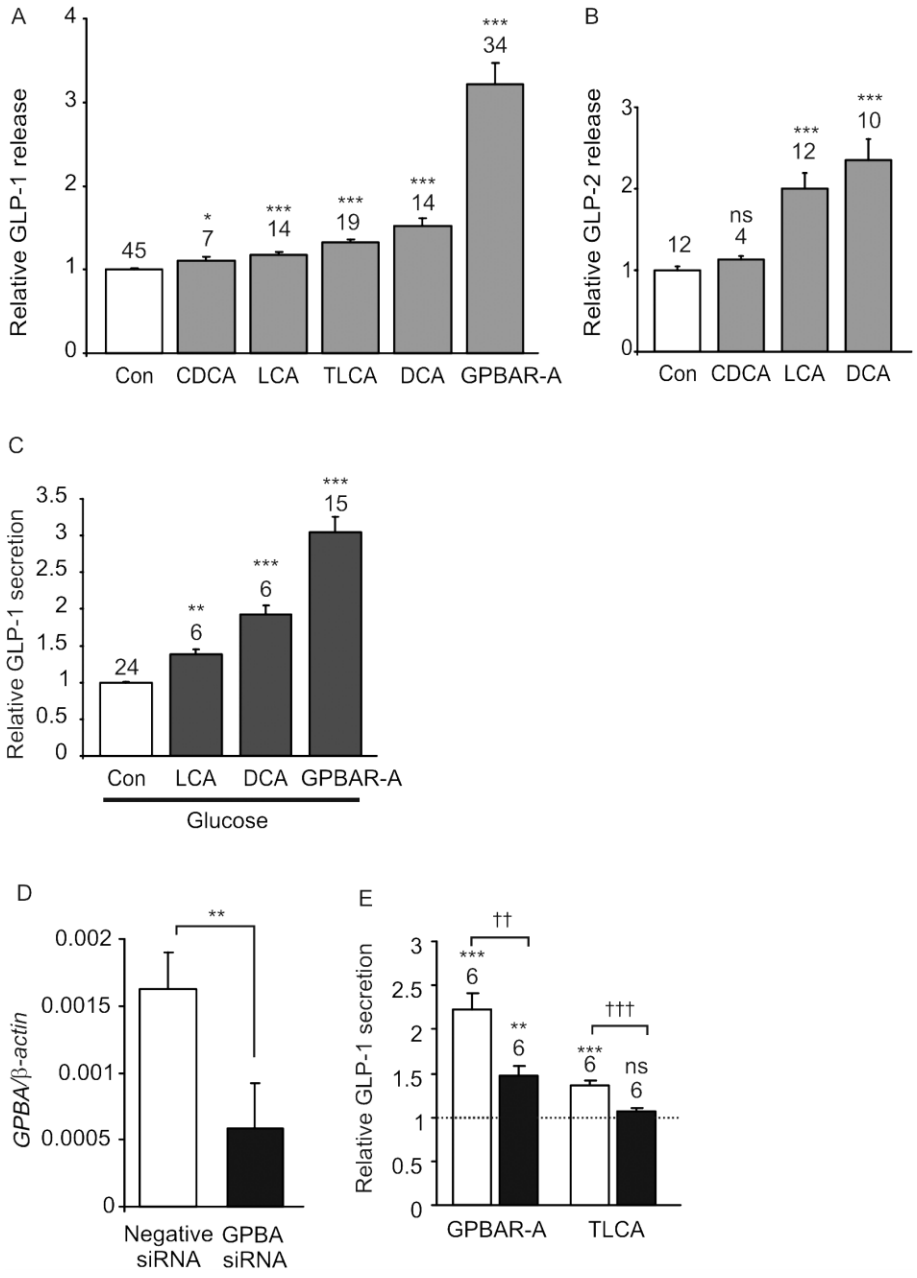

Figure 1

GLP-1 and GLP-2 secretion from GLUTag cells and GPBA siRNA knockdown. (A) GLP-1 secretion from GLUTag cells in response to the bile acids CDCA, LCA, TLCA and DCA (10–30 μM) along with GPBAR-A (3 μM). (B) GLP-2 secretion from GLUTag cells in response to CDCA, LCA, DCA (30 μM). (C) Secretory responses measured from GLUTag cells incubated with LCA, DCA (both at 30 μM) and GPBAR-A (3 μM) in the presence of glucose (10 mM). (D) Histograms showing the relative expression of GPBA mRNA as determined by qRT-PCR. mRNA was extracted for analysis 24 h post-transfection. Expression is shown relative to that of β-actin mRNA measured in the same sample. mRNA from four experiments were analysed for each column. Data are presented as geometric mean and upper SE calculated from the log (base 2) data. Significance was analysed by Student's *t*-test on the non-transformed ΔCT data, $^{**}P < 0.01$. (E) Effect of GPBA knockdown on GPBAR-A- (3 μM) and TLCA- (10 μM) stimulated GLP-1 secretion. Secretion experiment performed 48 h post-transfection with siRNA. In A, B, C and E, secretion was measured from GLUTag cells cultured for 2 h under the conditions indicated. Secretion was normalized to basal levels measured in parallel on the same day and the number of wells is indicated above each column. Data were collated from 2–11 separate experiments. Statistical significance was evaluated using ANOVA followed by Student's *t*-test, to compare to baseline: ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and to compare responses to different reagents, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$.

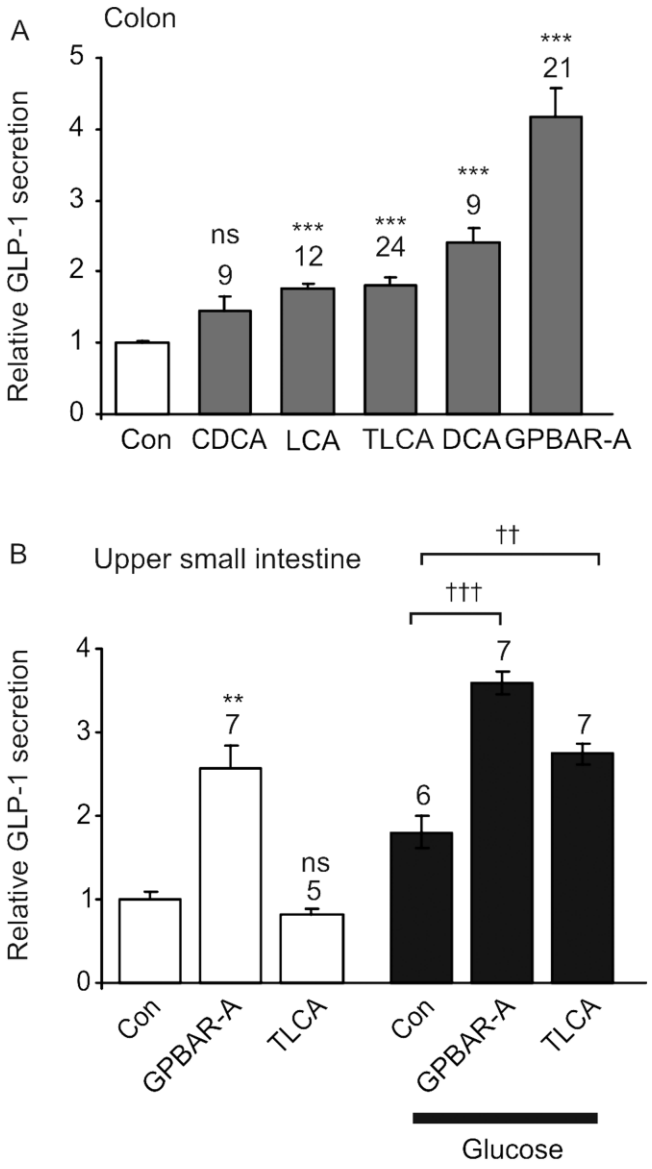

Figure 2

GLP-1 secretion from primary murine cultures. (A) Mixed murine intestinal colon cultures were incubated in saline solution containing bile acids (10 or 30 μM) or GPBAR-A (3 μM). (B) Mixed murine upper intestinal cultures were incubated with TLCA (10 μM) or GPBAR-A (3 μM) in the presence (solid columns) or absence (open columns) of glucose (10 mM). GLP-1 was measured in the supernatant and cell extracts and secretion expressed as a percentage of total GLP-1 relative to basal secretion measured in parallel on the same day. Error bars represent 1 SEM; the number of wells is shown above each column, and significance is shown relative to baseline, using ANOVA followed by a single factor *t*-test: ns, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$; $^{††}P < 0.01$, $^{†††}P < 0.001$. Data were collated from two to nine separate experiments.

primary cells for this experiment, because of our previous experience that a relatively large cell number would be required to dissect the effects of cAMP. Using the definition of a calcium response as an increase in the fluorescence ratio of at least 1.2-fold, we found that GPBAR-A increased calcium in

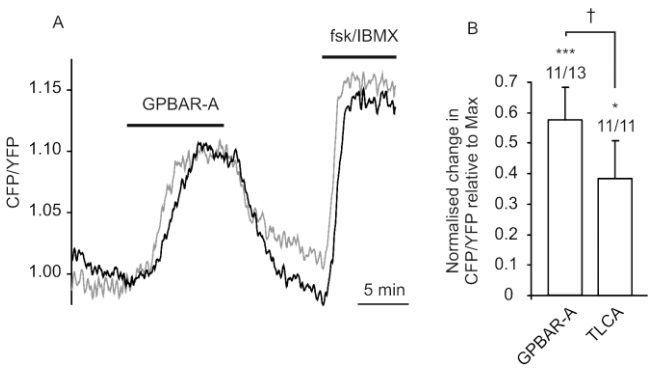

Figure 3

Intracellular cAMP changes in response to GPBA activation. (A) GLUTag cells transfected with a cytosolic Epac2-based FRET probe were perfused with GPBAR-A (3 μM), followed by forskolin (10 μM)/IBMX (10 μM) as indicated, while CFP and YFP emission was monitored. Each trace represents the CFP/YFP ratio of a single cell normalized to the starting value. (B) Mean changes in the CFP/YFP emission ratio in response to GPBAR-A or GPBA relative to the maximal response to fsk/IBMX, where 0 represents no change in the emission ratio and 1 is the maximal change. The number of cells that responded out of the total number of cells monitored is indicated above each column. The mean was calculated from responding cells only, from three to five separate experiments. Statistical significance was tested using Student's *t*-test; versus basal $^{*}P < 0.05$,$^{***}P < 0.001$, and GPBAR-A versus TLCA $^{†}P < 0.05$. Error bars represent 1 SEM.

56/149 GLUTag cells, with mean response of 1.3-fold across all cells ($P = 0.01$, $n = 149$). Glucose, by contrast, triggered an elevation in calcium in 132/149 cells, with a mean 2.2-fold increment above basal ($P = 2.0 \times 10^{-8}$, $n = 149$). When glucose was added in the presence of GPBAR-A, 148/149 cells responded ($P = 0.0001$ by χ^2-test vs. glucose alone), and the mean calcium response was 2.6-fold above basal ($P = 2.1 \times 10^{-40}$, $n = 149$).

To examine the explanation for the synergistic calcium responses triggered by glucose and GPBAR-A, we stratified the cells into quartiles based on the size of their initial glucose response (Figure 4B). Analysed in this way, it is evident that cells that were initially unresponsive to glucose became glucose-responsive in the presence of GPBAR-A. Thus, in the 25% least (non-) glucose-responsive cells, in which the mean initial glucose response was only 1.2-fold ($P < 0.001$, $n = 37$), the response to glucose in the presence of GPBAR-A was increased to 1.7-fold ($P < 0.001$, $n = 37$) relative to GPBAR-A. The second quartile of responders similarly showed an enhanced glucose response in GPBAR-A. By contrast, cells that exhibited a robust initial response to glucose (top 50% of glucose responders), on average, showed no significant enhancement of the glucose response in the presence of GPBAR-A (third quartile 2.3-fold vs. 2.4-fold, $P = 0.1$, $n = 38$ and fourth quartile 3.6-fold vs. 3.2-fold, $P = 0.07$, $n = 37$).

GPBA-mediated GLP-1 secretion is not dependent on K_{ATP} channel closure

To investigate the role of K_{ATP} channel closure as a critical event downstream of GPBA activation, we tested the effects of TLCA and GPBAR-A on primary colonic cultures in the presence of

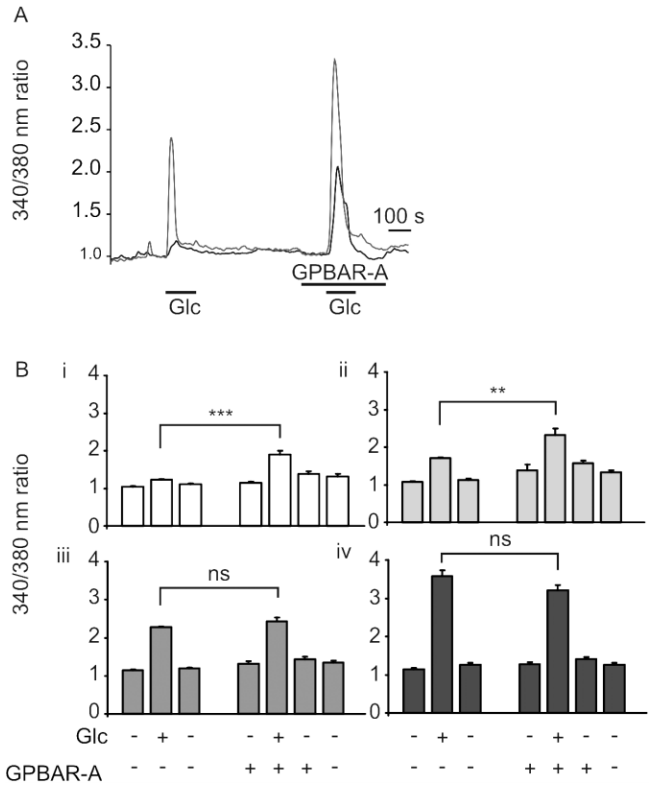

Figure 4

Intracellular calcium responses to GPBA activation in GLUTag cells. Ca^{2+} monitored as the ratio of fluorescence at 340 and 380 nm in individual GLUTag cells loaded with fura-2. (A) Representative traces of the response observed to the application of GPBAR-A (3 μM) and glucose (Glc, 10 mM) added as indicated by the horizontal bars. (B) Mean 340/380 nm ratio averaged over 10 s periods (n = 149 cells from five separate experiments) divided into quartiles based upon the size of the initial glucose response. (i) The 25% least glucose-responsive cells, (ii) the next 25% least glucose-responsive, (iii) the third quartile of glucose responders and (iv) the top 25% of glucose-responsive GLUTag cells. Error bars indicate 1 SE. ns, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

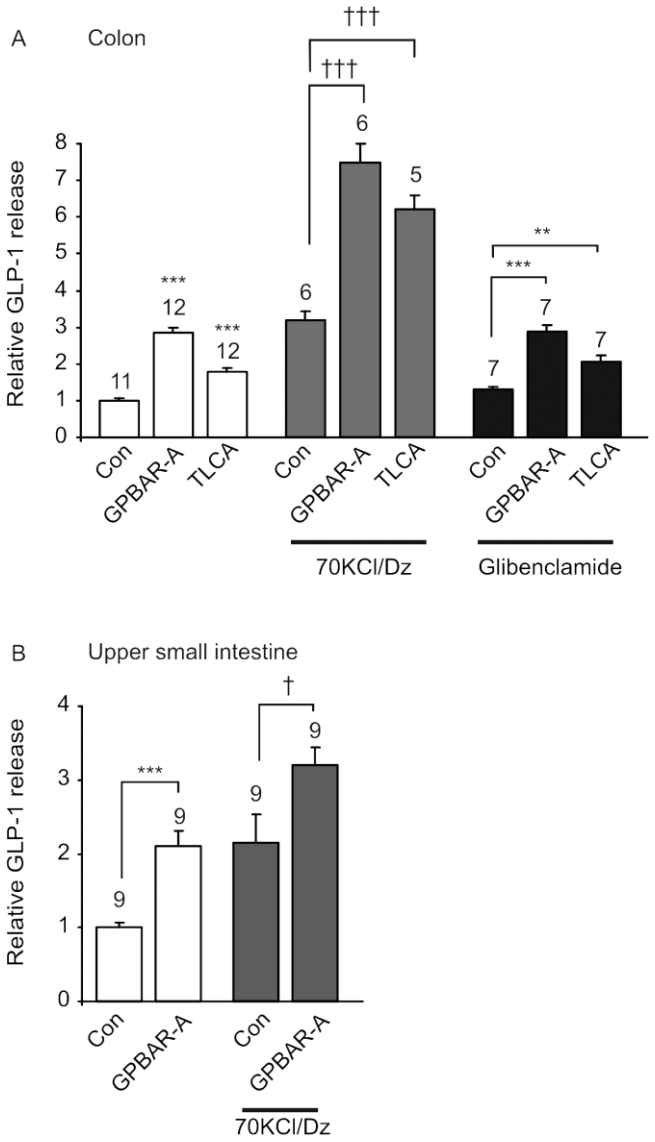

Figure 5

GLP-1 secretion from primary murine cultures in response to GPBA activation in the presence of glibenclamide and under depolarizing conditions. (A) Mixed murine intestinal colon cultures were incubated in saline solution containing GPBAR-A (3 μM) or TLCA (10 μM) in either standard saline solution (open columns), high KCl (70 mM) saline solution containing diazoxide (Dz, 340 μM) or with glibenclamide (100 nM) in standard saline solution. (B) GLP-1 response from primary cultures of murine upper small intestine incubated with GPBAR-A (3 μM) or TLCA (10 μM) in either standard saline solution (open columns) or a high KCl (70 mM) saline solution containing diazoxide (340 μM). GLP-1 was measured in the supernatant and cell extracts and secretion expressed as a percentage of total GLP-1 relative to basal secretion measured in parallel on the same day. Error bars represent 1 SEM; the number of wells is shown above each column, and significance is shown relative to baseline and between conditions calculated by ANOVA followed Student's t-tests: ns, not significant; $^{\dagger}P < 0.05$; $^{**}P < 0.01$, $^{***/\dagger\dagger\dagger}P < 0.001$. Data were collated from two to four separate experiments.

the sulphonylurea glibenclamide at a concentration predicted to maximally close K_{ATP} channels (100 nM). While glibenclamide itself triggered GLP-1 release, consistent with the known expression of K_{ATP} channel subunits in L-cells (Reimann *et al.*, 2008), it had no effect on the ability of GPBAR-A and TLCA to stimulate GLP-1 secretion (Figure 5A). Similarly, both TLCA and GPBAR-A enhanced GLP-1 secretion in the presence of 70 mM KCl and the K_{ATP} channel opener diazoxide (340 μM), a combination predicted to depolarize the cell membrane towards the Nernst-potential for K^+ and thus activate voltage-gated Ca^{2+} channels. In small intestinal and colonic cultures, 70 mM KCl + diazoxide triggered the release of GLP-1 by 2.1-fold ($P < 0.05$, $n = 9$) and 3.2-fold ($P < 0.001$, $n = 6$), respectively (Figure 5A, B), and GPBAR-A increased the GLP-1 secretory response by a further 1.5-fold ($P < 0.05$, $n = 9$) in small intestine cultures and 2.4-fold ($P < 0.001$, $n = 6$) in the colon. TLCA stimulated secretion a further 2.0-fold ($P < 0.001$, $n = 5$) under these depolarizing conditions in the colon.

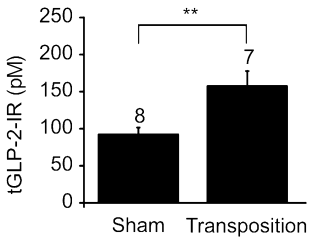

Figure 6

Duodenal transposition in rats. Total plasma GLP-2 concentrations measured 4 weeks post duodenal transposition versus sham-operated controls. The number of animals is shown above each column and significance between conditions was tested using Student's two-tailed *t*-test, $^{**}P < 0.01$.

Duodenal transposition elevates plasma GLP-2 levels

The ability of bile to stimulate secretion from primary L-cells *in vivo* was examined by transposing bilio-pancreatic drainage to the ileum in rats. The increased delivery of bile to more distal regions of the gut resulted in an approximately twofold elevation of fasting GLP-2 plasma levels compared with sham-operated controls ($P = 0.04$) (Figure 6).

Discussion

We show here that bile acids acutely stimulate GLP-1 and GLP-2 secretion from GLUTag cells, mirroring previous studies using alternative immortalized enteroendocrine cell lines (Katsuma *et al.*, 2005; Thomas *et al.*, 2009). The response was largely attributable to GPBA activation, as demonstrated by the effects of a small molecule GPBA agonist and *gpba*-specific siRNA-mediated knockdown. Importantly, the profile of bile acid- and GPBA agonist-triggered GLP-1 release was reproduced in mixed primary cultures of the adult mouse colon and small intestine, thus validating the use of GLUTag and other cell lines for assessing bile acid-triggered GLP-1 secretory mechanisms. The results support findings from a recent study that showed elevated and blunted *in vivo* GLP-1 responses to bile acids in GPBA-overexpressing and -knockout mice, respectively (Thomas *et al.*, 2009).

GPBA is considered a $G_{\alpha s}$-coupled receptor, as bile acids have been shown to raise cAMP content in extracts from cell lines that either naturally or heterologously overexpress GPBA (Maruyama *et al.*, 2002; Kawamata *et al.*, 2003; Katsuma *et al.*, 2005). These previous measurements were taken from single time points in cells treated with the global PDE inhibitor IBMX, so cannot represent the physiological time course of cAMP changes following GPBA activation. Using a FRET-based cAMP sensor, previously validated for use in GLUTag cells (Friedlander *et al.*, 2010), we showed here that cAMP levels become elevated within minutes of GPBA activation in individual GLUTag cells, even in the absence of PDE inhibition. Consistent with its greater efficacy in secretion assays using both primary and GLUTag cells, the previously reported GPBA-specific agonist GPBAR-A elevated cAMP levels to a greater extent than TLCA within individual cells. Although the use of higher concentrations of bile acids in secretion assays is restricted by their hydrophobicity and detergent effects, it is notable that all the bile acids we tested, which included both conjugated and unconjugated forms, were less effective than GPBAR-A in triggering GLP-1 release. It is therefore possible that GPBAR-A either differs from natural bile acids in its mode of interaction with GPBA, or exhibits additional GPBA-independent activity. The latter possibility is suggested by the finding that *gpba* siRNA abolished bile acid-triggered GLP-1 release, but impaired GPBAR-A-triggered secretion by only ~50%.

In both primary cultures and GLUTag cells, glucose (10 mM) further enhanced GPBAR-A- or bile acid-stimulated secretion, indicating at least an additive, if not a potential synergistic interaction between the two signalling pathways. This mirrors our previous observation that elevating intracellular cAMP levels in GLUTag cells, using a combination of forskolin and IBMX, enhanced the response to depolarizing stimuli (Simpson *et al.*, 2007). Part of this synergism in GLUTag cells could be attributed to the depolarizing action of cAMP, which is mediated by the activation of hyperpolarization-activated cyclic nucleotide-gated cation channels and inhibition of an unidentified potassium conductance distinct from the K_{ATP} channel (Simpson *et al.*, 2007). In skeletal and smooth muscle, activation of GPBA has been reported to increase type 2 iodothyronine deiodinase (D2) activity and oxygen consumption, resulting in the modulation of energy expenditure (Watanabe *et al.*, 2006). An increase in oxygen consumption has also been proposed to link GPBA signalling to GLP-1 secretion, mediated via changes in the ATP/ADP ratio and closure of K_{ATP} channels (Thomas *et al.*, 2009). However, our current finding that GPBA activation was similarly effective at triggering GLP-1 secretion from primary cultures under conditions in which K_{ATP} currents were either closed (100 nM glibenclamide) or open (340 μM diazoxide) sheds doubt on the idea that K_{ATP} channels play a role in the key pathways linking GPBA activation to GLP-1 release.

L-cells in primary culture are electrically active and employ voltage-gated Ca^{2+} channels to link membrane potential changes to alterations in secretion (Rogers *et al.*, 2010). GPBAR-A stimulates GLP-1 release both in the absence of added nutrient and when intracellular Ca^{2+} is raised by KCl and diazoxide, suggesting that elevated cAMP levels enhance secretion over a range of physiological Ca^{2+} concentrations. Its effectiveness even in the absence of added nutrient may be because GLP-1 release from primary cultures is maintained under these conditions by ongoing electrical activity and Ca^{2+} entry, as demonstrated by inhibition of basal GLP-1 secretion by the K_{ATP} channel opener diazoxide, the Na^+ channel blocker tetrodotoxin or the L-type Ca^{2+} channel blocker nifedipine (Rogers *et al.*, 2010). In GLUTag cells, GPBAR-A itself had little effect on intracellular Ca^{2+}, as monitored by the fura-2 ratio, but it increased Ca^{2+} responses to glucose. The enhanced glucose response was manifest as both an increase in the number of active cells and the magnitude of responses in individual cells, a pattern similar to that reported previously following cAMP elevation by forskolin/IBMX (Simpson *et al.*, 2007). Thus, in GLUTag cells, GPBA stimulation appears both to amplify intracellular Ca^{2+} responses and to enhance the secretory response to elevated Ca^{2+}, probably as a downstream consequence of elevated cAMP levels.

The observed greater effectiveness of TLCA and GPBAR-A in colon cultures compared with small intestinal cultures is likely to reflect the previously observed higher expression of GPBA in distal versus proximal L-cells (Maruyama *et al.*, 2006). Enhanced secretory responses have also been reported following GPBA overexpression in the STC-1 cell line (Katsuma *et al.*, 2005) or *in vivo* (Thomas *et al.*, 2009). The elevated GLP-2 concentrations observed after duodenal transposition in rats are therefore likely to reflect the increased delivery of bile acids to the distal ileum, where L-cells both occur at a higher density and express greater levels of the bile acid receptor. This is also in agreement with a recent paper that showed elevated PYY levels following biliary diversion to the ileum in dogs (Sato *et al.*, 2010). Indeed, the improvement of glucose tolerance and elevated GLP-1 levels that have been observed to follow gastric bypass surgery in obese patients, along with the recently reported increased crypt cell proliferation and elevated GLP-2 levels in rat models of gastric bypass (le Roux *et al.*, 2010), may be linked to the increased delivery of bile acids to distal gut regions, as well as to the conversion by colonic bacteria of primary to secondary bile acids which have increased potency on GPBA (Kawamata *et al.*, 2003).

There is a prevailing hope that understanding the dramatic beneficial effects of bypass surgery on glucose metabolism will lead to the development of new drugs for the treatment of diabetes and obesity. Our data suggest that bile acid stimulation of GPBA receptors on distal L-cells could provide one explanation for the observed high GLP-1 levels following bypass surgery, and provide support for ongoing strategies to target GPBA in L-cells as a therapeutic avenue.

Acknowledgements

The authors would like to thank Martin Lohse for the gift of the Epac-based cAMP sensors and the GLUTag cells were kindly provided by Dr D Drucker (Toronto). We also thank Professors MA Ghatei and SR Bloom for the GLP-2 assays. This work was supported by grants from the Wellcome Trust (#WT088357, #WT084210), and an MRC PhD studentship. ClR was funded by a NIHR clinician scientist award. Mouse work was supported by MRC-CORD.

Conflict of interest

The authors have nothing to disclose.

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