Brief Report
Mechanisms controlling glucose-induced GLP-1 secretion in human small intestine.

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Running Title: Glucose-induced GLP-1 secretion in human

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This article has supplementary data online at: xxxx

Word count: 2555
Figures: 3
Tables: 1
Abstract
Intestinal glucose stimulates secretion of the incretin hormone glucagon-like peptide 1 (GLP-1). The mechanisms underlying this pathway have not been fully investigated in humans. In this study, we showed that a 30 minute intraduodenal glucose infusion activated half of all duodenal L cells in humans. This infusion was sufficient to increase plasma GLP-1. With an *ex vivo* model using human gut tissue specimens, we showed a dose-responsive GLP-1 secretion in ileum at 200mM glucose or above. In *ex vivo* tissue from duodenum and ileum, but not colon, 300mM glucose potently stimulated GLP-1 release. In ileum, this response was independent of osmotic influences and required delivery of glucose via GLUT2 and mitochondrial metabolism. Requirement of voltage-gated Na\(^+\) and Ca\(^{2+}\) channel activation indicates that membrane depolarization occurs. K\(_{\text{ATP}}\) channels do not drive this, as tolbutamide did not trigger release. The sodium glucose co-transporter 1 (SGLT1) substrate, α-MG, induced secretion, and the response was blocked by the SGLT1 inhibitor phlorizin or by replacement of extracellular Na\(^+\) with NMDG. This is the first report of the mechanisms underlying glucose-induced GLP-1 secretion from human small intestine. Our findings demonstrate a dominant role of SGLT1 in controlling glucose-stimulated GLP-1 release in human ileal L cells.
Introduction

The incretin hormone glucagon-like peptide 1 (GLP-1) is secreted postprandially by enteroendocrine L cells to enhance glucose-dependent insulin release from pancreatic β-cells. The two incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), together account for 50–70 % of insulin secretion following oral glucose administration (1). Glucose-lowering effects of GLP-1 also occur through increasing insulin-independent glucose disposal (2). The use of GLP-1-based anti-diabetic agents, namely dipeptidyl-peptidase 4 inhibitors and GLP-1 receptor agonists, highlights the clinical relevance of GLP-1 in maintaining glucose homeostasis. Furthermore, there is increasing acceptance the metabolic benefits of gastric bypass surgeries are partly attributable to elevation of GLP-1 levels (3).

L cells predominantly reside in the epithelia of distal small intestine and colon; however, the early phase of postprandial GLP-1 secretion is likely mediated by smaller populations of L cells dispersed along the duodenum (4). Luminal nutrient exposure to potently stimulates GLP-1 release and the underlying mechanisms have been investigated in various experimental models (5). Seminal studies using primary murine intestinal mixed cell cultures (6-8), in vivo transgenic mouse models (9) and an ex vivo rat models (10), have shown that glucose induces GLP-1 release through the sodium glucose co-transporter, SGLT1, and to a lesser extent, the glucose transporter, GLUT2. Intracellular glucose metabolism and subsequent closure of K<sub>ATP</sub> channels are also implicated. In addition, sweet taste receptor (STR) signalling is involved in glucose-induced GLP-1 release in animals (11), and possibly in humans (12).
However, similar mechanistic examinations in human L cells are lacking. In this study, we examine the mechanisms controlling glucose-induced GLP-1 release in humans. We showed marked L cell activation upon intraduodenal glucose infusion in humans. We then used an \textit{ex vivo} static secretion model in human gut intestinal mucosa to show that glucose potently triggered GLP-1 release in duodenal, ileal, but not colonic, mucosae at concentrations equivalent to luminal, but not postprandial, blood glucose levels. SGLT1 is predominantly responsible for this glucose-induced GLP-1 secretion from human ileal mucosae.

\textbf{Research Design and Methods}

The detailed protocols of this study are available online in the supplementary material. Briefly, healthy subjects (n = 8) were fasted overnight before the endoscopic studies commenced. Mucosal biopsies were collected using standard biopsy forceps before and 30 minutes after an intraduodenal glucose infusion, immediately placed in 4 \% paraformaldehyde for 2 hours for immunohistochemistry. Immunoreactivity was detected using a polyclonal GLP-1 primary antibody (C-17, 1:400, SC-7782 Santa Cruz Biotechnology) and a polyclonal phospho-Ca$^{2+}$-calmodulin-dependent protein kinase II primary antibody (pCaMKII, 1:400, AB32678 Abcam) by sequential labelling.

For \textit{ex vivo} secretion experiments, morphologically normal ileal and colonic tissue specimens were collected with consent from patients undergoing bowel resections for cancer or stoma reversal (Supplementary Table 1). The mucosae were isolated and transferred to 96-well plates for static incubations of 15 minutes in different stimulants (37\degree C, 95\% O$_2$/ 5\% CO$_2$). GLP-1 (active) content in the supernatants was assayed with a commercial ELISA kit according to manufacturer’s instructions (EGLP-35K, Merck Millipore). GLP-1 secretion was normalized to basal secretion measured in parallel from the same sample. All statistical
analysis was conducted as paired analyses, comparing responses in tissues obtained from the same individual to relevant control conditions. A paired ratio Student’s t-test was used for single comparisons and a paired one-way ANOVA with Fishers Least Significant Difference post-hoc test used for multiple comparisons. Statistical significance was P < 0.05. All data are shown as mean ± SEM.

Results

Intraduodenal glucose infusion activated duodenal L cells

Subjects tolerated the study well and their characteristics are listed in Table 1. Immunolabelling for GLP-1 was reliably detected in dispersed duodenal epithelial cells (N = 8, Figure 1A, D). Immunopositive cells were most abundant in villi, and showed cytoplasmic labelling with basal predominance. Immunolabelling for the activation marker pCaMKII was present throughout the cytoplasm of single dispersed cells in the duodenal villi and crypts following intraduodenal glucose infusion (Figure 1B, E) and under basal conditions (Supplementary Figure 1). The density of duodenal mucosal cells expressing pCaMKII increased 1.54-fold after 30 minutes intraduodenal glucose infusion (P < 0.05; Figure 1G). The proportion of duodenal L-cells co-expressing pCaMKII increased 1.74-fold following intraduodenal glucose (27 ± 2 % vs. 45 ± 3 %, P < 0.001; Figure 1H). Thus, almost half of all L cells are activated by glucose exposure. This glucose infusion in these individuals was sufficient to cause a significant (P < 0.05) increase in plasma active GLP-1 levels (Figure 1I).

Glucose triggers GLP-1 release in the duodenum and ileum

To test the glucose range that L cells respond to, we tested a range of glucose concentrations in ileal epithelial tissue (n = 5, Figure 2A). Release was only triggered once concentrations
reached 200 mM, and 300 mM gave the highest GLP-1 secretion of the glucose concentrations tested. From all the samples in which we tested 300 mM glucose, we observed a significant increase in GLP-1 secretion in ileum (Figure 2B, n=19) and duodenum (Figure 2C, n=6). In contrast, 300 mM glucose did not trigger GLP-1 release from colonic epithelial tissue (n = 24; Figure 2D). This data from ileum and colon was pooled from both non-diabetes and type 2 diabetes individuals as we observe no difference in basal, stimulated release or fold change release between either group (Supplementary Figure 2). 300 mM D-mannitol did not increase GLP-1 release (n = 8, Supplementary Figure 3), indicating osmotic stress did not drive the observed glucose response. All ex vivo mechanistic analysis was conducted in ileum as this was the glucose-responsive section of the GI tract most available to us in this study, and the size of biopsy samples, such as from duodenum, severely limits the number of ex vivo secretion experiments possible in a single patient sample.

**Mechanism regulating glucose-induced GLP-1 secretion**

The SGLT1 inhibitor, phlorizin (1 mM, n = 9), and the GLUT2 inhibitor, phloretin (1 mM, n = 9), both blocked glucose-induced GLP-1 secretion (Figure 3A). The non-metabolizable SGLT1 substrate, α-MG (300 mM) induced GLP-1 secretion (n = 8, P < 0.05) but was less potent than glucose (P < 0.01). The blockade of SGLT1 with phlorizin (1 mM) attenuated α-MG-induced GLP-1 release (n = 8, Figure 3A). Replacement of external Na⁺ with N-Methyl-D-Glucamine (NMDG) blocked the response to 300 mM glucose in ileal tissue (n=5, Supplementary Figure 4), further demonstrating the need for Na⁺ transport via SGLT1 for glucose-induced GLP-1 release. Glucose failed to induce GLP-1 secretion in the presence of the K<sub>ATP</sub> channel opener, diazoxide (500 µM, n = 9) or the ATP synthesis inhibitor, 2,4-DNP (100 µM, n = 8). The K<sub>ATP</sub> channel antagonist tolbutamide (500 µM, n = 9) did not stimulate GLP-1 release (Figure 3B). The voltage-gated Na⁺ channel blocker, lignocaine (100 µM, n =
9) and L-type Ca^{2+} channel blockade by nifedipine (10 µM) both returned GLP-1 secretion to basal level in the presence of 300 mM glucose (n = 9, Figure 3C). The non-caloric sweetener, sucralose, stimulated GLP-1 release in the absence of high glucose (n = 9, P < 0.05), indicating a role for sweet taste receptors in GLP-1 secretion. Glucose-stimulated GLP-1 release remained in the presence of the STR antagonist lactisole (n = 9, P < 0.05, Figure 3D), indicating that glucose-induced GLP-1 secretion is independent of this pathway.

**Discussion**

This study is the first to demonstrate that approximately half of all duodenal L cells in humans are activated acutely by intraduodenal glucose infusion. We further established that levels of glucose, equivalent to that seen postprandially in plasma, did not stimulate small intestinal GLP-1 release in our human *ex vivo* model. Rather, glucose concentrations equivalent to dietary intraluminal glucose were capable of triggering *ex vivo* GLP-1 secretion from human ileum, but not colon. Finally, we identified the mechanisms by which this glucose response occurs in human ileal L cells and that SGLT1 is central to this pathway.

While it has previously been reported that GLP-1 is secreted in response to duodenal glucose infusions at even lower doses than tested in this study (13), our *in vivo* investigation provides the first quantification of the proportion of L cells which are activated by glucose infusion. Our *ex vivo* preparations also demonstrate duodenal responsiveness to high glucose, similar to that observed in ileal tissue. This finding is consistent with a role of the duodenal L cell in the increased plasma GLP-1 levels we observed upon duodenal glucose infusion in our study. It needs to be noted that this assay using biopsy tissue from subjects administered glucose is not necessarily a means by which to detect direct effects of glucose at L cells, as it could be
that pCaMKII is regulated by factors delivered from adjacent non-L cells, or by extra-intestinal factors.

Our *ex vivo* model demonstrates that exposure of human ileal mucosa to glucose triggers GLP-1 secretion, independent of secondary influences such as neural inputs or gut contraction. We were able to pool our data in ileum and colon from both non-diabetic and type 2 diabetic individuals, as secretion was similar in both groups. Furthermore, glucose-stimulated GLP-1 release was not secondary to osmotic stress, as equimolar amounts of mannitol failed to induce secretion. Our data are similar to responses in perfused rat small intestine, where luminal, but not vascular, infusions of high glucose triggered substantial GLP-1 secretion (10). We also showed that L cells in the human duodenum, but not colon, are glucose-sensitive in our assay. The result in colon contrasts the *in vitro* glucose-induced GLP-1 release observed at very low glucose ranges in fluorescently-tagged L cells from mouse colon (6; 8). Whether these differences are species-, experiment- or preparation-dependent remain unknown. This data supports clinical findings that resection of the L cell-rich distal colon did not affect glucose-induced GLP-1 release, and that rectally administered glucose did not trigger GLP-1 release (14). One limitation of our study was that we were unable to acquire total GLP-1 content from our samples to observe whether this was altered in these two groups.

We defined pivotal roles of electrogenic and facilitative glucose transport via SGLT1 and GLUT2, respectively, in GLP-1 release from the human ileum. The significant GLP-1 release triggered by equimolar amounts of the non-metabolizable SGLT1 substrate, α-MG, reversal of α-MG-induced GLP-1 release by phlorizin and blockade of the glucose response by substituting external Na⁺ with NMDG, all support a central role of SGLT1 in driving this
glucose response. Thus, our results confirm the critical role of electrogenic sodium-dependent glucose uptake by SGLT1 in causing membrane depolarization and the subsequent GLP-1 release in human L cells, similar to that shown in various rodent models (7-10; 15). Blockade of GLUT2 by phloretin was also sufficient to block glucose-stimulated GLP-1 secretion. This may be a species-specific pathway as it is also observed in ex vivo rat small intestine perfusion models (10; 16), but not in mouse in vivo and in vitro studies using Glut2 knockout mice (9) and primary murine small intestine cell cultures (8). Such differences further highlight the need for caution when translating findings in animal models to human. GLUT2 may be important in human L cells by facilitating glycolytic and/or mitochondrial metabolism so that metabolism-dependent, but $\text{K}_{\text{ATP}}$ channel-independent, glucose-induced GLP-1 release can occur. In addition to this, GLUT2 function is also implicated in mediating $\text{K}_{\text{ATP}}$ channel-independent GLP-1 secretion by other secretagogues such as lipids and bile acids (16).

It has been proposed that glucose induces GLP-1 release through glucose internalization, production of ATP via oxidative phosphorylation to close $\text{K}_{\text{ATP}}$ channels, and subsequent membrane depolarization (5). We demonstrate that the $\text{K}_{\text{ATP}}$ channel opener, diazoxide, potently reduced glucose-induced GLP-1 secretion in humans, consistent with in vitro (6; 17) and ex vivo (10) rodent data. Inhibiting intracellular ATP synthesis with the proton ionophore 2,4-DNP, abolished the stimulatory effect of glucose on GLP-1 secretion in the human small intestine, consistent with results in rat small intestine (10). In contrast to that same study and other in vitro experiments (6), we did not observe increased GLP-1 secretion with tolbutamide, consistent with the finding that sulfonylureas are ineffective in triggering GLP-1 secretion in human in vivo (18; 19). Diazoxide increases $\text{K}^+$ permeability and subsequently clamps membrane potential below the $\text{K}^+$ equilibrium potential. This hyperpolarization must
override any membrane depolarization induced by the inward \( \text{Na}^+ \) current associated with SGLT1 activity and block what is normally a \( K_{\text{ATP}} \) channel-independent, SGLT1-dependent glucose-induced GLP-1 secretion.

A role for \( \text{Na}^+ \)-dependent action potentials and voltage gated L-type \( \text{Ca}^{2+} \) currents in mediating basal and stimulated GLP-1 release has been reported in an \textit{in vitro} study of mouse L cells (17). Our study supports the role of both of these channels in glucose-induced GLP-1 release from the human ileum. Blockade of voltage-gated \( \text{Na}^+ \) channels after intravenous lignocaine administration failed to attenuate glucose-induced GLP-1 secretion in the perfused rat small intestine model (10). Such differing results may highlight a potential shortcoming of the approach used in our study, as cell polarisation is lost upon dissection of the epithelial tissue. A major limitation of our study was the inability to differentiate between pathways derived from apical and basolateral membranes. This could potentially also explain the lack of glucose response in human colonic tissue previously observed in mice (6). Experiments using human vascularly perfused tissue, or using Ussing chambers, could mitigate these shortcomings but were not possible in our present study.

Intestinal sweet taste receptors are potential regulators of gut hormone secretion. GLP-1 co-localizes with the STRs T1R2 and T1R3 and its signal transduction protein \( \alpha \)-gustducin in human small intestine and the STR antagonist, lactisole, dose-dependently inhibits glucose-stimulated GLP-1 release \textit{in vivo} in humans (12; 20). GLP-1 secretion from primary murine small intestine mixed cell cultures increased upon stimulation by the artificial sweetener, sucrrose (6). In contrast to results in a clinical study (21) and in a rat \textit{ex vivo} perfusion model (10), we observed significant sucralse-induced GLP-1 secretion from human ileal mucosae. However, blockade of the STR by lactisole did not significantly reduce glucose-stimulated
GLP-1 secretion. This could be due to the fact that sucralose is a more potent activator of the STRs at this dose, or that a small portion, if any, of the glucose-stimulated GLP-1 secretion is mediated by this pathway.

It should be noted that L cells within the ileum are not likely to be exposed to significant, if any, levels of ingested glucose under normal physiological conditions. Likely it would be the duodenal, and the more prevalent jejunal L cells involved in this nutrient response. Due to tissue from those sites not being readily available to us in large quantities for this study, we can only assume at this stage that the mechanisms underlying glucose-induced GLP-1 secretion are the same across these different areas of the GI tract. The ability to respond to ingested glucose by ileal L cells may therefore represent a back-up system utilised under certain pathophysiological conditions, rather than being a primary physiological response system.

Our study highlights the importance of species differences in studying L cell physiology. While several pathways shown to govern GLP-1 secretion in rodents were implicated in our study, some were not. Our data demonstrates that glucose-induced GLP-1 secretion in human small intestine is mediated by the electrogenic activity of SGLT1. It additionally involves a component reliant on intracellular glucose metabolism and is dependent on voltage-gated Na\(^+\) and Ca\(^{2+}\) channels. While sweet taste receptors can also regulate GLP-1 release in human ileum, these receptors do not appear to be involved in glucose-induced GLP-1 release.
RLY designed the clinical study while EWS and DJK designed the *ex vivo* experiments. Recruitment of patients for the clinical study was performed by RLY, CKR and AMD while the clinical experiments were performed by CKR and AMD at the Royal Adelaide Hospital. Immunohistochemistry analysis was performed by RLY. DAW, DF, PR, PH, LS and SLD performed surgeries and provided tissue for the *ex vivo* experiments. EWS performed the *ex vivo* experiments. EWS and DJK undertook statistical analyses and wrote the manuscript. All authors critically reviewed the manuscript and have approved the final version. DJK is the guarantor of this work and had access to all study data and takes responsibility for the integrity of the data and the data analysis.

**Acknowledgements**

This work was supported by the Australian Research Council (LP150100419) and the National Health and Medical Research Council (APP1088737). The authors declare no potential conflicts of interest regarding the contents of this manuscript. The authors are grateful to the voluntary participants who made this work possible. The authors wish to thank staff of the Gastrointestinal Investigation Unit, Royal Adelaide Hospital and Discipline of Surgery, Flinders Medical Centre, for their assistance with the study.
Figure Legends

Figure 1: Functional activation of duodenal cells after intraduodenal glucose infusion in healthy subjects. (A, D) GLP-1 immunopositive cells in the duodenal mucosa. (B, E) pCaMKII immunopositive cells in duodenum after 30 min intraduodenal glucose infusion, highlighted by blue arrows. (C) Composite images of a pCaMKII immunopositive L-cell, and (F) separate pCamKII and L-cells. (G) Increase in density of duodenal pCaMKII immunopositive cells after glucose infusion in healthy subjects; *P < 0.05. (H) Increased proportion of duodenal L-cells co-expressing pCaMKII in healthy subjects after glucose infusion; **P < 0.01. (I) Increased plasma GLP-1 following a duodenal glucose infusion of 30 minutes in these individuals; *P < 0.05. Scale bar in A = 20 µm for all images. Data are mean ± SEM.

Figure 2: GLP-1 secretion upon glucose stimulation in human gut mucosae. (A) Concentration-response curve for GLP-1 secretion in response to increasing glucose in human ileum tissue (n=5. *P<0.05). 300 mM glucose potently triggered GLP-1 secretion from L cells in human (B) ileum (n = 19), (C) duodenal (n=6) and (D) colon mucosae (n = 24). Bar graph data are mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 compared to respective control groups.

Figure 3: Mechanisms controlling glucose-induced GLP secretion in human ileal L cells. (A) SGLT1 and GLUT2 blockade by phlorizin and phloretin, respectively abolished stimulatory effect of high glucose on GLP-1 secretion. The non-metabolizable SGLT1 substrate, α-MG caused significant GLP-1 secretion but was less potent than equimolar glucose and its stimulatory effect was reversed by the phlorizin. (B) The K\textsubscript{ATP} channel opener and ATP synthesis inhibitor, diazoxide and 2,4-DNP, respectively, completely abolished the effect of
high glucose on GLP-1 secretion but the $K_{\text{ATP}}$ channel closer, tolbutamide, did not cause significant GLP-1 secretion from basal levels. (C) Blockade of voltage-gated $\text{Na}^+$ and $\text{Ca}^{2+}$ channels by lignocaine and nifedipine, respectively, significantly inhibited the stimulatory effect of high glucose. (D) Sweet taste receptor activation by the non-caloric artificial sweetener, sucralose caused significantly GLP-1 secretion from basal levels but the sweet taste receptor blocker, lactisole did not attenuate the stimulatory effect of high glucose. Bar graph data are mean ± SEM, *P < 0.05, **P < 0.01, ****P < 0.0001 compared to respective control groups (n = 7 – 9).

Table 1: Characteristics and blood glucose responses of subjects for intraduodenal glucose infusion study. BMI – body mass index, BGL – blood glucose level, M – male, F – female. Data are mean ± SEM.

|                  | Control          |
|------------------|------------------|
| **N**            | **8**            |
| **Age**          | **41 ± 6**       |
| **Gender**       | **7M : 1F**      |
| **BMI (kg/m^2)** | **28 ± 2**       |
| **HbA1c (%)**    | **5.7 ± 0.1**    |
| **Fasting BGL (mmol/L)** | **5.9 ± 0.2** |
| **T=30 BGL (mmol/L)** | **9.0 ± 0.7**   |
| **BGL AUC30 (mmol/L/min)** | **85 ± 6**     |
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Supplementary Material

**Supplementary Table 1:** Characteristics of specimen donors for *ex vivo* experiments. BMI – body mass index, BGL – blood glucose level, M – male, F – female. Data are mean ± SEM.

|               | ileum specimen | colon specimen |
|---------------|----------------|----------------|
| N             | 19             | 24             |
| Age           | 71 ± 4         | 65 ± 2         |
| Sex           | 7M : 12F       | 13M : 11F      |
| BMI (kg/m$^2$) | 28 ± 1         | 29 ± 1         |
| History of type 2 diabetes (yes/no) | 4/ 15 | 7/ 17 |

**Supplementary Figure 1**

pCaMKII immunopositive cells in the duodenal mucosa under basal conditions, highlighted by blue arrow. Scale bar = 20 µm.

**Supplementary Figure 2**

GLP-1 release is not different in non-diabetes (ND) and type 2 diabetes (T2D) tissue samples. Ileum (A) basal release, (B) in response to 300 mM glucose and (C) fold change in response to high glucose (n=19). Colon (D) basal release, (E) in response to 300 mM glucose and (F) fold change in response to high glucose.

**Supplementary Figure 3**

Glucose response in ileum is not due to osmotic pressure. 300 mM mannitol causes no GLP-1 secretion in ileum while equimolar glucose causes significant GLP-1 release (**P<0.01, n=8 paired experiments**).

**Supplementary Figure 4**
Glucose response requires external Na\(^+\). Replacement of external Na\(^+\) with N-Methyl-D-Glucamine (NMDG) blocks the response to 300 mM glucose in ileal tissue (n=5).

**Human tissue collection:**

For endoscopic duodenal biopsies, healthy control subjects (N=8, BMI < 30 kg/m\(^2\), Table 1) were recruited to these studies in the Gastrointestinal Investigation Unit of the Royal Adelaide Hospital (RAH). Subjects were excluded if aged less than 18 years, pregnant, known to have diabetes, had a contraindication to endoscopy, had a history of surgery on the stomach, duodenum or small intestine, or were at increased risk of bleeding due to platelet or coagulation disorders or medications. The female subject was studied during the follicular phase of her menstrual cycle. Protocols were approved by the Human Research Ethics Committee of the Royal Adelaide Hospital and were conducted in accordance with the Declaration of Helsinki as revised in 2000. Each subject provided written informed consent.

Subjects fasted overnight, and after attending the endoscopy unit at 0900 an intravenous cannula was inserted into a forearm vein for blood sampling. A small diameter video endoscope (GIF-XP160, Olympus, Tokyo, Japan) was then passed via an anesthetised nostril or mouth into the second part of the duodenum, where mucosal biopsies were collected using standard biopsy forceps and immediately placed in 4% paraformaldehyde (PFA) for 2 hours. An intraduodenal (ID) infusion containing 30 g glucose in 150 mL was then commenced at T=0 via the biopsy channel of the endoscope, and continued for 30 min (1 g/min; 4 kcal/min). Three further biopsies were then collected at T=30.

For *ex vivo* secretion experiments, patients gave consent for tissue donation at Flinders Medical Centre and Flinders Private Hospital approved by the Southern Adelaide Clinical
Human Research Ethics Committee. Ileal and colonic tissue specimens were collected from patients undergoing bowel resection for cancer or stoma reversal. In the case of resection specimens, samples were obtained from sites at least 10 centimetres proximal to the tumour location. Specimens from patients with clinical or macroscopic evidence of inflammatory bowel disease were excluded from this study. Characteristics of the patient cohort are listed in Supplementary Table 1. For duodenal ex vivo experiments, a total of three patient samples were collected as biopsy tissue. Different tissue from each individual was used at two different sites on the same day. The specimens were immediately placed in iced-cold Krebs buffer (in mM, NaCl 138, KCl 4.5, CaCl$_2$ 2.6, NaHCO$_3$ 4.2, MgCl$_2$ 1.2, NaH$_2$PO$_4$ 1.2, HEPES 10, Glucose 5) and transported to the laboratory for dissection within 15 minutes. The specimens were rinsed with iced-cold Krebs buffer to remove any luminal content and dissected clear of adipose, muscular and connective tissue. The mucosae were gently dissected off from the submucosae as intact sheets using a stainless steel spatula, cut into 5 mm pieces and weighed individually. The mucosal pieces were then transferred to a 96-well plate for secretion assays.

**Immunohistochemistry:**

Fixed tissues were cryoprotected (30% sucrose in phosphate-buffered saline, PBS), embedded in cryomolds, and frozen, before being sectioned at 10 µm (Cryocut 1800, Leica Biosystems, Nussloch, Germany) and thaw-mounted onto gelatin-coated slides. Immunoreactivity was detected using a polyclonal GLP-1 primary antibody (C-17, 1:400, SC-7782 Santa Cruz Biotechnology) and a polyclonal phospho-Ca$^{2+}$-calmodulin-dependent protein kinase II primary antibody (pCaMKII, 1:400, AB32678 Abcam) by sequential labelling. Immunoreactivity for GLP-1 and pCaMKII were visualised using species-specific
secondary antibodies conjugated to Alexa Fluor dyes (1:200 in PBST) as previously described (21; 22).

**Imaging and cell counts:**

Nucleated mucosal cells immunopositive for GLP-1, pCaMKII or both markers were counted per square millimetre of duodenal mucosal area under high power field in an observer-blind manner. Counts were averaged over at least 10 intact transverse sections per subject.

**Secretion experiments:**

Mucosal pieces were incubated with 250µL of buffer (control) or buffer containing test agents in a 96-well plate for 15 minutes. Each well received a single treatment, and secretion was compared to that under control conditions in tissue from the same patient. The buffer was a modified Krebs buffer described above with the addition of sitagliptin 1 µM and 0.1 % fatty acids-free bovine serum albumin (BSA, A1595, Sigma Aldrich) at pH 7.4. Following incubation at 37 °C in 95 % O₂/5 % CO₂, supernatants were collected and stored in aliquots at -20 °C. Active GLP-1 levels were quantitated using a commercially available ELISA kit, according to manufacturer’s instructions (EGLP-35K, Merck Millipore). All compounds for *ex vivo* studies were purchased from Sigma Aldrich. Hormone secretion was normalized, as indicated, to the basal secretion measured in parallel from the same sample on the same day.

**Statistical analysis:**

All statistical analysis was conducted as paired analyses, comparing responses in tissues obtained from the same individual to relevant control conditions. A paired ratio Student’s t-test was used for single comparisons. As secretion experiments did not always include a full sequence of all conditions used in this study, a paired one-way ANOVA with Fishers Least
Significant Difference post-hoc test was used for multiple comparisons. Statistical significance was $P < 0.05$. All data are shown as mean $\pm$ SEM.
Supplementary Figure 1
pCaMKII immunopositive cells in the duodenal mucosa under basal conditions, highlighted by blue arrow. Scale bar = 20 µm.
Supplementary Figure 2

GLP-1 release is not different in non-diabetes (ND) and type 2 diabetes (T2D) tissue samples. Ileum (A) basal release, (B) in response to 300 mM glucose and (C) fold change in response to high glucose (n=19). Colon (D) basal release, (E) in response to 300 mM glucose and (F) fold change in response to high glucose.
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