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RESEARCH PAPER

Endogenous PYY and GLP-1 mediate L-glutamine responses in intestinal mucosa

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BACKGROUND AND PURPOSE

L-glutamine (Gln) is an energy source for gastrointestinal (GI) epithelia and can stimulate glucagon-like peptide 1 (GLP-1) release from isolated enteroendocrine L-cells. GLP-1 and peptide YY (PYY) are co-secreted postprandially and both peptides have functional roles in glucose homeostasis and energy balance. The primary aim of this project was to establish the endogenous mechanisms underpinning Gln responses within intact GI mucosae using selective receptor antagonists.

EXPERIMENTAL APPROACH

Mouse mucosae from different GI regions were voltage-clamped and short-circuit current (I_{sc}) was recorded to Gln added to either surface in the absence or presence of antagonists, using wild-type (WT) or PYY-/- tissues. The glucose sensitivity of Gln responses was also investigated by replacement with mannitol.

KEY RESULTS

Colonic apical and basolateral Gln responses (at 0.1 and 1 mM) were biphasic; initial increases in I_{sc} were predominantly GLP-1 mediated. GLP-1 receptor antagonism significantly reduced the initial Gln response in the PYY-/- colon. The slower reductions in I_{sc} to Gln were PYY-Y1 mediated as they were absent from the PYY-/- colon and were blocked selectively in WT tissue by a Y1 receptor antagonist. In jejunum mucosa, Gln stimulated monophasic I_{sc} reductions that were PYY-Y1 receptor mediated. Gln effects were partially glucose sensitive, and Calhex 231 inhibition indicated that the calcium-sensing receptor (CaSR) was involved.

CONCLUSION AND IMPLICATIONS

Gln stimulates the co-release of endogenous GLP-1 and PYY from mucosal L-cells resulting in paracrine GLP-1 and Y1 receptor-mediated electrogenic epithelial responses. This glucose-sensitive mechanism appears to be CaSR mediated and could provide a significant therapeutic strategy releasing two endogenous peptides better known for their glucose-lowering and satiating effects.

Abbreviations

Ala, L-alanine; Asn, L-asparagine; BIBO3304, (R)-N-[[4-(aminocarbonylaminomethyl)-phenyl]methyl]-N^2-(diphenylacetyl)-argininamide trifluoroacetate; BIIE0246, (S)-N^2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6H)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide; Ca_i^{2+}, intracellular calcium; Calhex 231, 4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl] amino]cyclohexyl]-benzamide; CaSR, calcium-sensing receptor; ENaC, epithelial sodium channel; GI, gastrointestinal; Gln, L-glutamine; GLP-1, glucagon-like peptide 1; I_{sc}, short-circuit current; KH, Krebs Henseleit; NPY, neuropeptide Y; Phe, L-phenylalanine; PYY, peptide YY; PYY-/-, PYY knockout; R568, 2-chloro-N-[(1R)-1-(3-methoxyphenyl)ethyl]-benzenepropanamine hydrochloride; SGLT1, sodium glucose co-transporter 1; T2D, type 2 diabetes; VIP, vasoactive intestinal polypeptide; WT, wild-type

Introduction

Enteroendocrine L-cells that co-express and co-release glucagon-like peptides (GLPs) and peptide YY (PYY) are present along the length of the mammalian gastrointestinal (GI) tract, but they are more frequently observed in the distal ileum and colon (Böttcher *et al.*, 1984; Ku *et al.*, 2003). Glucagon-like peptide 1 (GLP-1) and PYY both mediate incretin activity and slow gastric emptying and intestinal transit, resulting in reduced postprandial glycaemia in rodents (Boey *et al.*, 2007; Holst, 2007) and in lean as well as diabetic subjects (Greenfield *et al.*, 2009; Samocha-Bonet *et al.*, 2011). The capacity of L-cells to sense luminal contents has become apparent recently, aided by the ability to investigate the signalling mechanisms in enriched preparations of these relatively scarce cells, using transgenic mice expressing cell-specific fluorescence driven by a chosen gut pro-hormone promoter (Reimann *et al.*, 2008; Egerod *et al.*, 2012; Habib *et al.*, 2012). These studies have identified GPCRs that are enriched selectively in different enteroendocrine cell types. A range of nutrients, including proteins, fatty acids and some of their metabolites, can stimulate L-cells to co-secrete their peptide products (Engelstoft *et al.*, 2008; Reimann *et al.*, 2012), while loss of L-cells has been shown recently to result in impaired glucose homeostasis (Pedersen *et al.*, 2012). L-glutamine (Gln) is not only a widely available nutritional supplement, a major source of epithelial metabolic energy and contributor to protein synthesis in GI mucosa (particularly post-injury; Wilmore, 2001), but it is also a GLP-1 secretagogue in primary colonic cultures and murine endocrine GLUTag cells (Reimann *et al.*, 2004; Tolhurst *et al.*, 2011). However, to date, few studies have investigated the effects of Gln or other amino acids on intact GI mucosa where the functional consequences of endogenous GLP-1 and PYY release can be investigated pharmacologically.

GLP-1 enhances insulin release, which is beneficial in type 2 diabetes (T2D), and with PYY, these two peptides have crucial roles in slowing upper and lower GI transit (Savage *et al.*, 1987; Drucker, 2005; Tough *et al.*, 2011) and in signalling satiety (Anini *et al.*, 1999; Holst, 2013). GLP-1 mimetics have been licensed to treat T2D for some time now and are additionally weight-losing (Astrup *et al.*, 2012; Holst, 2013). Therapeutic developments are now including intestinal-sensing mechanisms and strategies that promote co-release of GLPs and PYY by activating GPCRs found to be enriched in L-cells, for example, GPR119, which is also expressed on pancreatic beta cells (Overton *et al.*, 2008; Oshima *et al.*, 2013). However, our understanding of the repertoire of GLP's and PYY's incretin mechanisms lags somewhat behind that of their pancreatic mechanisms.

Tolhurst *et al.* (2011) showed that Gln is a GLP-1 secretagogue acting via two independent signalling pathways; firstly, an increased cell excitability stimulated by a Na^+-dependent elevation of intracellular calcium (Ca_i^{2+}) and, secondly, an amplifying mechanism that occurred via elevated intracellular cAMP (another potent L-cell stimulus). Additionally, L-cells were shown to express a variety of amino acid transporters, which could varyingly contribute to the Na^+-dependent electrogenic entry of Gln across the plasma membrane and thus trigger depolarization and GLP-1 release (Reimann *et al.*, 2004; Tolhurst *et al.*, 2011). L-cell activity has also been shown to be involved in amino acid-induced GLP-1 and PYY release *in vivo*, and that the calcium-sensing receptor (CaSR; receptor nomenclature throughout follows Alexander *et al.*, 2011) is required for this response (Mace *et al.*, 2012). We therefore hypothesized that in intact mucosa, Gln was likely to initiate electrogenic epithelial responses as a consequence of endogenous peptide co-release, and that if both GLP-1 and PYY were involved functionally, then their respective GPCR signalling via G_s and G_i coupling in epithelia would result in secretory and anti-secretory responses respectively.

Functional studies utilizing mouse GI and colonic mucosae have characterized fully the Y receptors involved in the long-lasting anti-secretory responses induced by PYY and neuropeptide Y (NPY), an accepted inhibitory enteric neurotransmitter. The colon appears to be most sensitive to these Y peptides and their shorter (3–36) products (Cox *et al.*, 2001). In both the mouse and the human colon, the effects of exogenous and endogenous PYY are predominantly mediated by epithelial Y1 receptors, while NPY's effects are a combination of neuronal Y2- and epithelial Y1-mediated responses (Hyland *et al.*, 2003; Cox & Tough, 2002, respectively; Tough *et al.*, 2011). Notably, the cellular pharmacology of these peptide responses is the same in mouse and human colon mucosa, and thus, the mouse intestine is a valid model for testing PYY/NPY-Y receptor mechanisms further.

The primary aim of this project was therefore to investigate the mechanisms of action of Gln in intact mucosal preparations from selected areas of the mouse GI tract and to establish whether these responses were glucose sensitive. Several amino acids have been shown to secrete GLP-1 (Tolhurst *et al.*, 2011) and these were also tested, as was the sidedness of the Gln response. The GLP-1 sensitivity of mucosae from different GI regions was included as a prelude to determining the relative contributions provided by endogenous GLP-1 and PYY to Gln responses in wild-type (WT) tissue using proven, selective GLP-1 and Y1 receptor antagonists, and comparing these Gln responses with those from the PYY-/- colon.

Methods

Tissue preparation

All experimental procedures complied with the Animals (Scientific Procedures) Act 1986 and were approved by the local ethical committee. WT and PYY null male adult mice lacking the entire PYY coding sequence (PYY-/-; on aC57Bl6-129/SvJ background; Boey *et al.*, 2006; Karl *et al.*, 2008) were age-matched (11–20 weeks) and had free access to standard chow and water. Mice were killed by CO_2 asphyxiation or cervical dislocation and GI tissues were excised and placed immediately in fresh Krebs Henseleit (KH) with the following composition (in mM): 117 NaCl, 24.8 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$ and 11.1 D-glucose. Mucosae primarily from the jejunum or descending colon (but also other GI regions for GLP-1 sensitivity studies) were obtained by dissection under a microscope, removing the overlying smooth muscle and associated myenteric innervation, as described previously (Cox *et al.*, 2010).

Measurement of short-circuit current (I_{sc}) across mucosae and drug pretreatments

Adjacent mucosal preparations from a given GI area were placed individually between two halves of an Ussing chamber (exposed area 0.14 cm^2) and bathed both sides with 5 mL oxygenated (95% O_2, 5%CO_2) KH (pH 7.4) maintained at 37°C. Mucosae from the distal colon or jejunum were voltage-clamped at 0 mV and the resulting short-circuit current (I_{sc}, measured as $\mu A \cdot cm^{-2}$) was recorded continuously as described previously (Cox *et al.*, 2010; Tough *et al.*, 2011). Following tissue equilibration (20 min), mucosae were pretreated with 10 nM vasoactive intestinal polypeptide (VIP, an optimal secretory pretreatment upon which epithelial G_i-coupled mechanisms can be readily discerned; Cox *et al.*, 2001, 2010; Tough *et al.*, 2011). Once VIP-elevated I_{sc} levels had stabilized, Gln (0.1 or 1 mM), other amino acids or glucosamine (1 mM) were added to either the basolateral or apical reservoirs, and changes in I_{sc} were monitored for a further 20 min. All peptides, plus Y and GLP-1 receptor antagonists were added basolaterally. In pharmacological studies, selective antagonists were added prior to VIP addition, at their optimal blocking concentrations throughout, that is, 300 nM BIBO3304 {(*R*)-*N*-[[4-(aminocarbonylaminomethyl)-phenyl]methyl]-N^2-(diphenylacetyl)-argininamide trifluoroacetate}, 1 μM BIIE0246 {(*S*)-N^2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[*b*,*e*]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide} (Tough *et al.*, 2006; Cox *et al.*, 2001) or 1 μM exendin (9–39) (Cox *et al.*, 2010). Finally, PYY (10 nM) was added as an internal Y1 plus Y2 receptor agonist control. PYY-/- mouse colonic mucosa was used to determine whether the absence of this L-cell derived peptide resulted in a loss of Gln function in the absence or presence of GLP-1 blockade with 1 μM exendin (9–39). These PYY-/- mice were the same line used extensively in previous functional studies (Boey *et al.*, 2006; Karl *et al.*, 2008; Cox *et al.*, 2010; Tough *et al.*, 2011).

To investigate the involvement of the CaSR in mucosal Gln responses, we used the CaSR positive allosteric modulator R568 {2-chloro-*N*-[(1*R*)-1-(3-methoxyphenyl)ethyl]-benzenepropanamine hydrochloride} (Nemeth *et al.*, 1998; Petrel *et al.*, 2004) added apically, at a concentration shown previously to increase GLP-1 and PYY secretion from perfused rat small intestine (20 μM; Mace *et al.*, 2012). We also used the CaSR antagonist Calhex231 (Petrel *et al.*, 2004) at 10 μM, a concentration shown previously to selectively inhibit intestinal GLP-1 and PYY secretion (Mace *et al.*, 2012) to determine whether it would inhibit Gln responses in colonic mucosa.

Amiloride (1 μM) and phloridzin (50 μM) were only added apically as their targets, epithelial sodium channel (ENaC) and sodium glucose co-transporter 1 (SGLT1), respectively, are selectively expressed on apical membranes. Changes in I_{sc} following each drug addition were monitored and their effects on subsequent Gln responses were recorded.

Regional variation in mucosal GLP-1 responses

Here, GI mucosae from selected areas of the mouse upper and lower GI tract were prepared as described above but were not pretreated with VIP. Instead, the effect of GLP-1 agonist exendin 4 alone (100 nM, added basolaterally) or following addition of the GLP-1 antagonist, exendin (9–39) (1 μM), was measured under basal conditions. Changes in I_{sc} to the antagonist alone, and then to exendin 4 ± antagonism, were compared in mucosae from the duodenum, jejunum, terminal ileum and the ascending and descending colon from WT mice.

Glucose sensitivity of apical and basolateral Gln responses in mouse colon mucosa

To test whether Gln responses were glucose sensitive, colonic mucosae were bathed with KH buffer containing either glucose (11.1 mM)on both sides or by replacing glucose for mannitol (11.1 mM) on one side only, as described previously (Cox *et al.*, 2010). Apical or basolateral addition of Gln (1 mM) after VIP pretreatment resulted in changes in I_{sc} that were compared with controls (KH on both sides) and subsequent responses to PYY (10 nM) and the SGLT-1 inhibitor, phloridzin (50 μM), were also compared across experimental groups.

Data and statistical analysis

GraphPad Prism v5.0 (GraphPad Prism Inc., La Jolla, CA, USA) was used to analyse all the data presented. Single comparisons were performed using Student's unpaired *t*-test, while multiple comparisons utilized one-way ANOVA with Dunnett's or Bonferroni's post-tests where applicable. Statistical significance was defined as a *P*-value ≤0.05.

Materials

PYY and VIP were obtained from Bachem Laboratories Inc. (St Helen's, UK), and aliquots were frozen and stored at −20°C, undergoing a single freeze-thaw cycle only. Gln, L-phenylalanine (Phe), L-asparagine (Asn), L-alanine (Ala), phloridzin, amiloride, dimethyl sulphoxide, D-glucose, mannitol and UK14,304 [5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine] were all purchased from Sigma (Poole, UK). BIBO3304, BIIE0246 and R568 {2-chloro-*N*-[(1*R*)-1-(3-methoxyphenyl)ethyl]-benzenepropanamine hydrochloride} were from Tocris Bioscience (Bristol, UK). Calhex 231 {4-chloro-*N*-[(1*S*,2*S*)-2-[[(1*R*)-1-(1-naphthalenyl)ethyl]amino]cyclohexyl]-benzamide} was from Santa Cruz (Heidelberg, Germany) and glucosamine was purchased from Alfa Aesar (Heysham, UK).

Results

Basal resistance and I_{sc} levels, as well as secretory responses to VIP (10 nM pretreatment, unless otherwise stated) in small intestine and colonic mucosae, were within the ranges published previously (Cox *et al.*, 2001; 2010; Tough *et al.*, 2011). Neither VIP nor Gln (at 0.1 or 1 mM) altered mucosal resistances during the course of these experiments (data not shown).

Y_1 receptor activation mediates the Gln-induced reductions in I_{sc}

Following VIP pretreatment of colonic mucosa Gln added to either the apical or basolateral reservoir resulted in a biphasic

change in I_{sc}; an initial small, transient elevation in I_{sc} followed by a slower, longer-lasting decrease in I_{sc} [Figure 1A, labelled as a primary (1°) or secondary (2°) component, respectively], while in jejunum mucosa, Gln triggered monophasic reductions in I_{sc} only (Figure 1B). The Y1 receptor antagonist BIBO3304 (Wieland *et al.*, 1998) increased I_{sc} levels in colonic and jejunal mucosa as seen previously, indicating endogenous Y1 tone that was significant in the former tissue (Figure 1C). In Y1-blocked colonic mucosa, the 2° Gln response was abolished compared with vehicle-treated controls (***$P < 0.001$ for apical and *$P < 0.05$ for basolateral Gln responses; Figure 1D). Irrespective of the surface to which Gln was added, there was no change in the size of the 1° response ± BIBO3304 (Figure 1D). In the jejunum, apical and basolateral Gln responses were simpler, monophasic I_{sc} reductions that were attenuated significantly by Y1 antagonism (Figure 1E). Following Gln addition, maximally effective PYY (10 nM) reduced I_{sc} levels in a BIBO3304-sensitive manner in both colonic and jejunum mucosae (Figure 1F, G respectively).

Colonic I_{sc} responses to different L-amino acids added apically or basolaterally

Three L-amino acids (each added at 1 mM), namely Gln, Phe and Asn, exhibited similarly sized biphasic I_{sc} responses in colonic mucosa, while Ala (1 mM) responses were smaller (but not significantly, compared with Gln). The amino sugar, glucosamine was inactive, significantly so after apical and basolateral additions (Figure 2A,B). Subsequent control PYY responses were no different from those observed without prior amino acid addition (data not shown). The relative activity we observed with these five amino acids was similar to their ability to release GLP-1 from primary colonic cultures (Tolhurst *et al.*, 2011).

Regional variation in GLP-1 mediated increases in I_{sc} in mouse GI tract mucosae

In order to determine whether exogenous GLP-1 activated epithelial I_{sc} responses directly (prior to investigating whether the 1° Gln effects were mediated by endogenous GLP-1), we used the stable GLP-1 agonist, exendin 4 (100 nM), in the absence or presence of the GLP-1 receptor antagonist, exendin (9–39) (1 μM). The antagonist alone (added basolaterally) reduced basal I_{sc} levels with an onset of 1 min and maximal effect within 5 min and in all GI regions tested, indicating endogenous GLP-1 activity along the length of the mouse GI tract (Figure 3). Exendin 4 added basolaterally to naive tissue increased I_{sc} levels (presumably via epithelial G_s-linked GLP-1 receptors) with the largest responses in ascending colon and all exendin 4 responses were abolished by pretreatment with exendin (9–39) (Figure 3).

Glucose sensitivity of Gln response

GPCR mechanisms in L-cells are often glucose sensitive, while epithelial GPCR-mediated responses are not (e.g. L-cell GPR119 agonism is glucose sensitive, while consequent PYY-Y1 receptor-mediated epithelial responses are glucose insensitive; Cox *et al.*, 2010). In keeping with these different mechanisms, apical replacement of glucose by mannitol resulted in a significant inhibition of the 2° Gln response, and although there was also ~60% inhibition of Gln's 1° response, this was not statistically significant (Figure 4A). Basolateral glucose removal had no significant effect on either phase of the apical Gln response (Figure 4A). Interestingly, when basolateral glucose was removed, the basolateral Gln response (specifically the 2° phase) was significantly reduced, while the initial small increases in I_{sc} were apparently glucose insensitive (Figure 4B). Subsequent basolateral PYY responses were not glucose sensitive (Figure 4C). Finally, the SGLT1 inhibitor, phloridzin, was used as an internal control to confirm selective loss of apical SGLT1 activity when glucose had been replaced by mannitol in this reservoir. Here, apical mannitol rendered phloridzin significantly less effective as shown by the smaller phloridzin-induced reductions in I_{sc} compared with SGLT1 block in control conditions or after basolateral mannitol (Figure 4D).

Involvement of the CaSR but not ENaC in colonic Gln responses

To test whether CaSR mechanisms were involved in the amino acid responses observed in colonic mucosa, we utilized R568, a CaSR positive allosteric modulator (Nemeth *et al.*, 1998) and the selective CaSR antagonist, Calhex 231 (Petrel *et al.*, 2004), at optimal concentrations previously shown to enhance or inhibit, respectively, GLP-1 and PYY secretion from rat small intestine (Mace *et al.*, 2012). Firstly, the 1° and 2° components of colonic Gln responses were enhanced significantly by pretreatment with R568 (Figure 5). Secondly, Calhex 231 pretreatment reduced the size of both components of the Gln response, but only the reductions in I_{sc} were significantly inhibited (Figure 5). Neither CaSR modulator altered I_{sc} levels *per se* at these concentrations (data not shown).

As Na^+ absorption via apical ENaC is significant in colon mucosa and may account for a proportion, particularly of the Gln 1° response, we tested whether the ENaC inhibitor amiloride affected either of the components of the apical Gln response (1 mM). Apical amiloride (1 μM) reduced I_{sc} levels *per se* as expected (–8.3 ± 2.1 μA·cm^{-2}, $n = 3$), after which, the Gln response was 2.6 ± 0.9 μA·cm^{-2} ($n = 3$ for the 1° component) and –2.0 ± 0.8 μA·cm^{-2} ($n = 3$ for the 2° component) compared with control Gln responses (1°, 6.1 ± 1.5 μA·cm^{-2}, $P = 0.19$; 2°, –3.1 ± 0.7 μA·cm^{-2}, $n = 7$, $P = 0.44$). There were no statistical differences between either of the components of the Gln response.

Loss of Gln function in PYY-/- colon mucosa treated with a GLP-1 antagonist

Having established that Y1 receptor antagonism blocked the Gln-induced reductions in I_{sc} in jejunal and colonic mucosae, we next set out to confirm that colonic Gln responses involved a combination of endogenous PYY and GLP-1. We did this by comparing Gln responses in WT and PYY-/- tissue in the absence or presence of the GLP-1 antagonist, exendin (9–39). In WT colon, Gln induced 1° responses were reduced by exendin (9–39); however, this was not statistically significant (Figure 6A; Student's *t*-test, $P = 0.15$), while in PYY-/- tissue, the 1° response was abolished by exendin (9–39) (***$P < 0.001$) indicating significant mediation by endogenous GLP-1 (Figure 6B). Notably, apical and basolateral

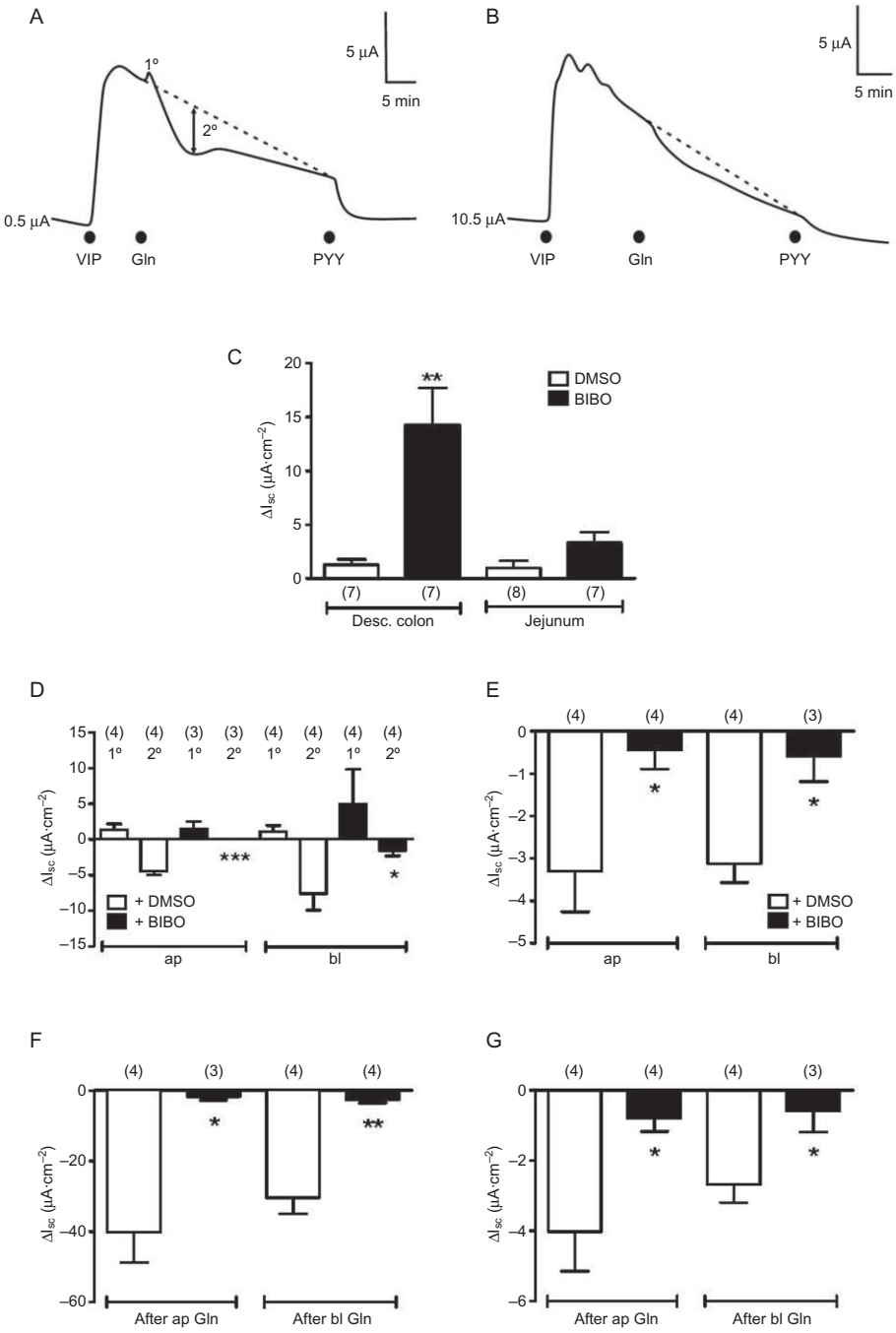

Figure 1

Gln-induced changes in I_{sc} in mucosae from mouse colon and jejunum. In (A) and (B) are traces showing basolateral (bl) Gln (1.0 mM) responses (biphasic in colon, in A) and monophasic reductions in I_{sc} in jejunum (in B). Gln responses were recorded after the addition of a near-maximal VIP concentration (10 nM) and were followed by basolateral addition of a maximal PYY concentration (10 nM). In (C), the increases in I_{sc} observed on the addition of vehicle [dimethyl sulphoxide (DMSO), 10%] or Y1 antagonist BIBO3304 (300 nM) to mucosae from the descending colon or jejunum, revealing significant Y1 tonic activity in the former. In (D), pooled data from colonic mucosa of primary (1°) and secondary (2°) response components to Gln [apical (ap) and bl, 0.1 mM] showing significant inhibition of the reductions in I_{sc} by pretreatment with BIBO3304 (***$P < 0.001$ and *$P < 0.05$ respectively). In (E), jejunal Gln(0.1 mM) responses were monophasic reductions in I_{sc} that were inhibited significantly by Y1 antagonism (+ BIBO, 300 nM; *$P < 0.05$). In (F) and (G), reductions in I_{sc} to exogenous PYY(10 nM, bl) show clear Y1 receptor sensitivity (plus 300 nM BIBO3304; **$P < 0.01$ and *$P < 0.05$) in colonic (F) and jejunal mucosae (G) respectively. Values are the mean ± SEM from the number of observations shown in parentheses.

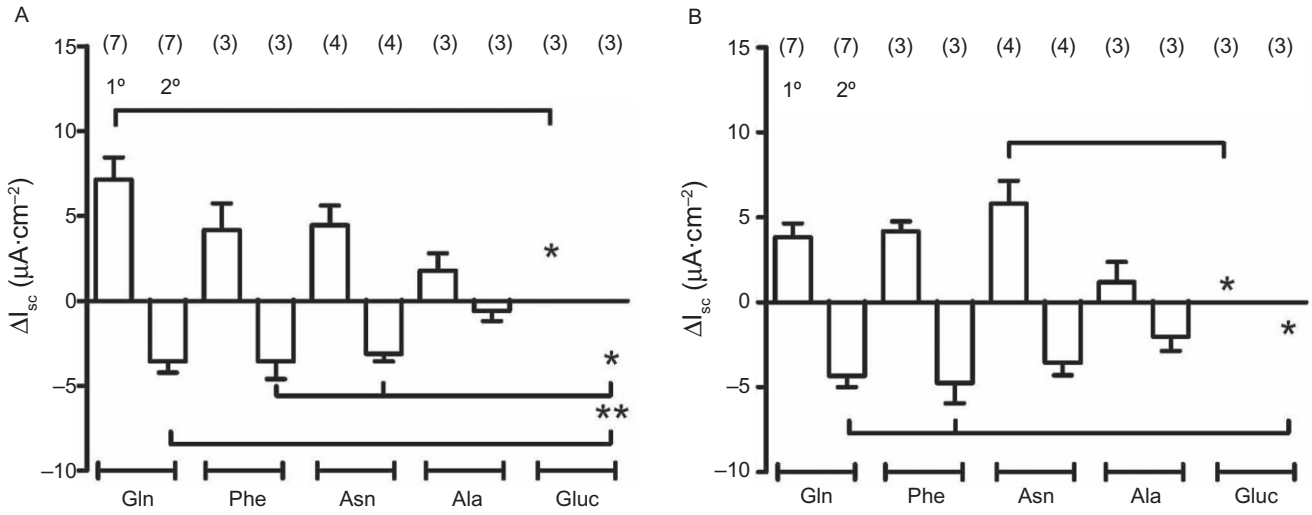

Figure 2

Responses to different amino acids and glucosamine (1 mM throughout) added either apically (in A) or basolaterally (in B) to mouse descending colon mucosa. Each amino acid induced biphasic changes in I_{sc} with the exception of glucosamine (Gluc, *$P < 0.05$ or **$P < 0.01$, compared with specific responses as indicated). Values are the mean ± SEM from the number of observations shown in parentheses.

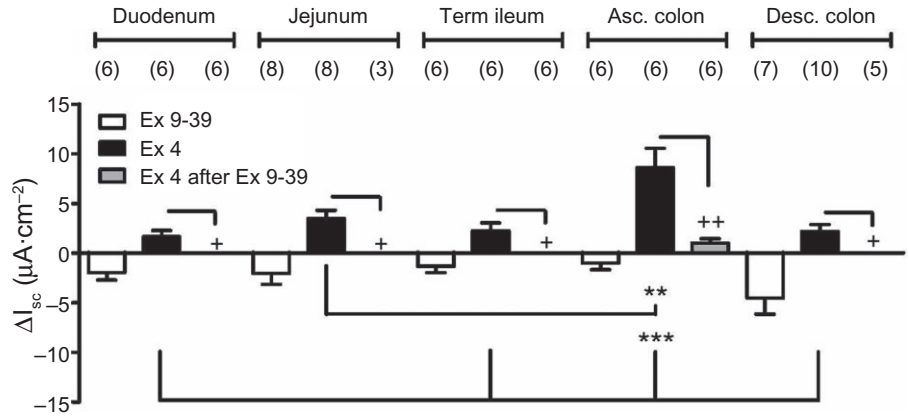

Figure 3

Regional GLP-1 responses to basolateral addition of the GLP-1 antagonist [exendin (9–39), 1 μM] alone; to exendin 4 (Ex 4, 100 nM) in the absence or presence of exendin (9–39) (1 μM). Values are the mean ± SEM from the number of observations shown in parentheses. In all GI areas tested, Ex 4-induced increases in I_{sc} were significantly inhibited by the GLP-1 receptor antagonist (Ex 9–39) (++$P < 0.01$ and + $P < 0.05$, Student's *t*-test). Multiple comparisons showed the greatest GLP-1 responses in ascending colon mucosa compared with other GI areas (***$P < 0.001$ and **$P < 0.01$).

Gln-induced reductions in I_{sc} were absent from PYY-/- colon (significantly so compared with WT tissue) and indicating the requirement for PYY in this component of the Gln response.

Discussion

The reductions in I_{sc} observed to apical or basolateral addition of Gln in jejunal and colonic mucosae were Y1 receptor mediated and epithelial in origin, based on their Y1 antagonist, BIBO3304 sensitivity. Previous functional studies have established that PYY-mediated Y1 receptor responses are direct and epithelial, while Y2 responses are indirect and neuronally mediated (Hyland *et al.*, 2003; Tough *et al.*, 2006; 2011). The biphasic changes in I_{sc} stimulated by Gln addition (0.1 and 1.0 mM) to colonic preparations were replicated by other L-amino acids (at 1 mM). Plasma concentrations of Gln are within the 0.1–1.0 mM range and an oral dose (30 g) of Gln raises plasma levels after 60 min to 1.3–2.7 mM in lean humans with co-incident elevations in plasma GLP-1 (Greenfield *et al.*, 2009). As a major product of protein digestion and a widely used nutritional supplement, similar luminal Gln concentrations have been measured postprandially (1.0–4.0 mM; Rhoads and Wu, 2009), so the use of a 1.0 mM Gln in the present study is physiologically relevant. Phe, Asn and, to a lesser degree, Ala mimicked Gln effects,

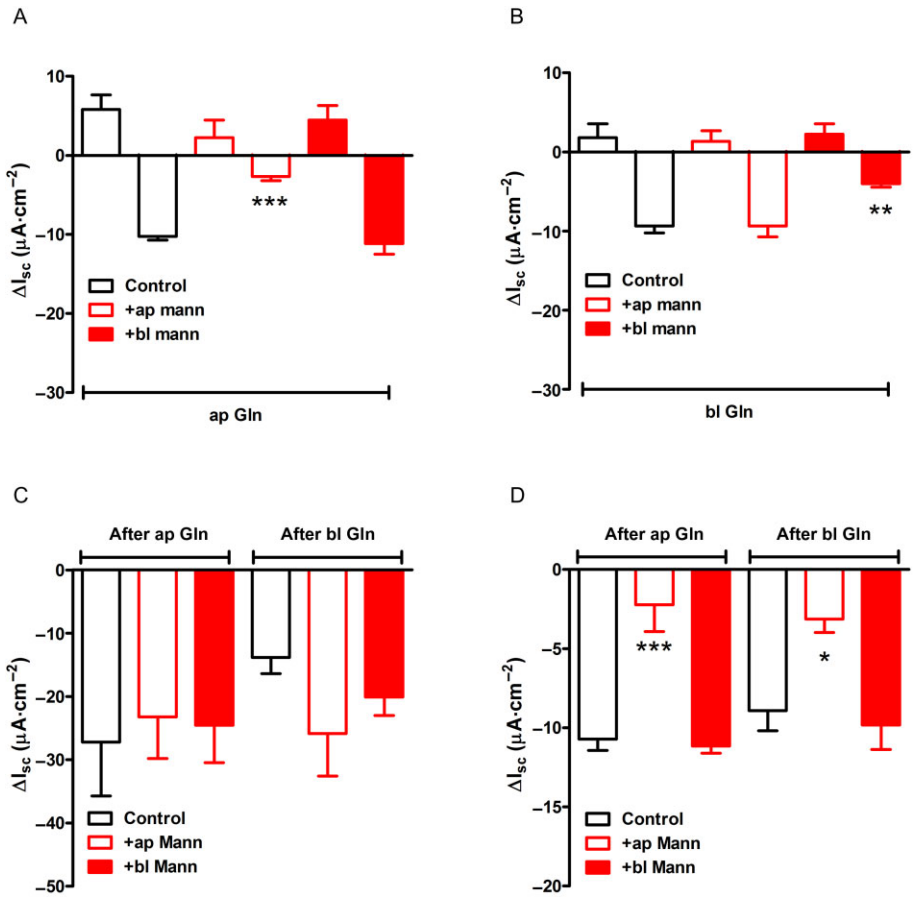

Figure 4

Glucose sensitivity of Gln but not PYY responses in mouse colon mucosa. In (A) and (B), the effect of replacing glucose for mannitol in the apical (+ap mann) or basolateral (+bl mann) reservoirs on responses to Gln added to either the apical (A) or basolateral (B) reservoir. The absence of glucose from either side did not appear to alter the size of the small 1° increases in I_{sc}, but it did reduce the Gln-induced reductions in I_{sc} significantly (***$P < 0.001$ and **$P < 0.01$). Subsequent PYY (10 nM) responses were not glucose sensitive (in C), while in (D), SGLT1 inhibition by phloridzin (50 μM, in all bars) was significantly reduced when apical glucose was replaced by mannitol (***$P < 0.001$ and *$P < 0.05$, compared with controls). Values are the mean ± SEM from four observations throughout.

while the amino sugar, glucosamine, was inactive (on both sides) and this activity series resembled their relative GLP-1 secretory capacities (Tolhurst *et al.*, 2011). Glucosamine can additionally be transported directly into L-cells via the facilitative transporter glucose transporter 2 (Reimann *et al.*, 2008), and because the amino sugar is uncharged, this process remains electroneutral and would thus not alter I_{sc} levels *per se*.

Tolhurst *et al.* (2011) found that Gln induced L-cells to release GLP-1 with an EC_{50} value of ~0.1 mM and E_{max} of ~1 mM, and we provide evidence that apical and basolateral Gln has the capacity to stimulate rapid changes in I_{sc} across mucosal preparations at the same low mM concentrations. The involvement of GLP-1 in the initial transient increases in I_{sc} seen in colonic mucosa was most clearly revealed in PYY-/- tissue, where with GLP-1 receptor blockade, subsequent Gln responses were abolished. In WT colon, the GLP-1 antagonist exendin (9–39) reduced the initial I_{sc} elevation seen after apical Gln but not significantly, and we conclude that another electrogenic mechanism may contribute to this albeit small electrogenic response. It is notable that certain Na^+-dependent neutral amino acid transporters are highly enriched in L-cells (e.g. SNAT2 and b^{0}AT1; Tolhurst *et al.*, 2011), but whether these or alternative epithelial-derived processes contribute to the initial transient Gln I_{sc} response in colon remains to be resolved. Using exendin (9–39), we observed low, but significant levels of tonic GLP-1-mediated secretory activity present along the length of the mouse small and large intestine, and marginally greatest in the descending colon mucosa. To our knowledge, this is the first evidence of such tonic activity. Responses to the addition of a stable GLP-1 mimetic exendin 4 were significantly larger in ascending colon reflecting a higher GLP-1 receptor expression in this region. In all the GI areas tested, these responses were abolished by GLP-1 receptor blockade. Few studies have determined whether GLP-1 has the capacity to elicit epithelial transport responses directly, but the present study provides evidence that it can in mouse colon, and a previous study showed similar GLP-1 tonic activity was also present in human colon mucosa (and more obvious when Y receptors

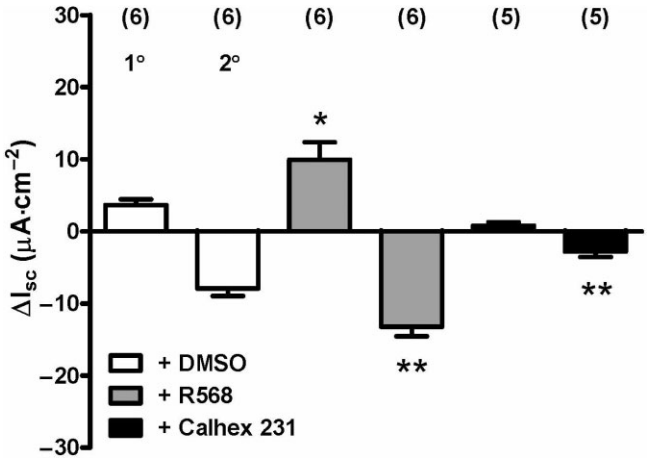

Figure 5

The effect of CaSR modulators R568 (20 μM) and Calhex 231 (10 μM) on Gln (1 mM) responses in mouse descending colon mucosa. All additions were made to the apical reservoir only and values are the mean ± SEM from the observation numbers shown in parentheses. Statistical comparisons compared with the vehicle (≤0.2% DMSO) controls revealed significant differences, *$P < 0.05$ for the 1° component of the Gln response, and **$P < 0.01$ for comparison of control and pretreated 2° component Gln responses.

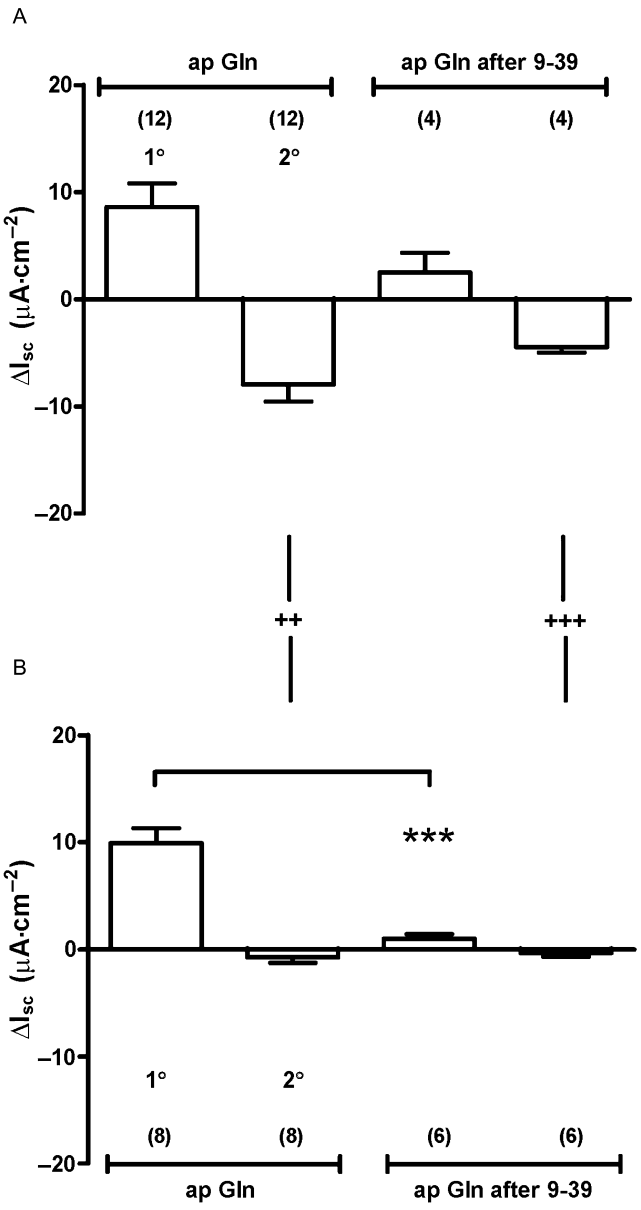

Figure 6

The effect of GLP-1 receptor inhibition with exendin (9–39) (1 μM) and absence of PYY upon colonic responses to apical Gln. Comparison of biphasic Gln responses showing 1° increases followed by 2° decreases in I_{sc} in WT (A) and PYY-/- tissue (B) shows that GLP-1 antagonism significantly reduced the 1° response compared with untreated Gln responses (*** $P < 0.001$, in B). In WT tissue (A), the 1° increase in I_{sc} to Gln was partially sensitive to GLP-1 receptor blockade but this was not statistically significant ($P = 0.15$, Student's *t*-test). PYY ablation results in loss of the 2° component of control ap Gln responses (++$P < 0.01$) and after GLP-1 blockade (+++$P < 0.001$). Values are the mean ± SEM from observation numbers shown in parentheses.

were blocked; Cox *et al.*, 2010), and we presume these increases in I_{sc} occur via G_s signalling in epithelial cells because they are rapid in onset and thus unlikely to be indirect.

In colonic mucosa, the reductions in I_{sc} (PYY-Y1 mediated) to apical Gln were glucose sensitive, while the smaller initial Gln response appeared to be partially glucose sensitive. If a proportion of the latter was mediated by a non-GLP-1, electrogenic transport mechanism, then that could result in the sensitivity pattern we observed with apical Gln. It was also interesting to note that basolateral Gln reduced I_{sc} in a partially glucose-sensitive manner and this is unlikely to be SGLT1 mediated as the transporter is located apically. Instead, a basolateral glucose-sensitive electrogenic mechanism appears to be involved here and may be one of the transporters or exchangers highlighted by Tolhurst *et al.* (2011). Further studies with selective inhibitors or sided Na^+-replacement studies may possibly help clarify this, as well as the smaller initial glucose-insensitive basolateral Gln response.

Mucosal Gln responses appear to be mediated by the CaSR, a class C GPCR member proposed to function as an L-amino acid sensor in the gut. Our *in vitro* findings in native tissue complement those of Mace *et al.* (2012) who showed that several L amino acids, including Gln, cause release of PYY and GLP-1 from rat perfused small intestine, which was sensitive to the selective CaSR antagonist Calhex 231, while K-cell derived gluco-insulinotropic peptide release was unaffected. Although widely expressed, CaSR are present notably in pancreatic beta cells (Rasschaert and Malaisse, 1999) and also gastrin-secreting antral G-cells (Ray *et al.*, 1997), implicating functional roles in insulin and gastrin secretion. In normal human colon, immunohistochemical studies have shown CaSR immunoreactivity on L-cells (Sheinin *et al.*, 2000) and CaSR can mediate L-Phe stimulated CCK release from mouse duodenal I-cells (Liou *et al.*, 2011), but these sensing receptors do exhibit a wider, epithelial expression pattern in certain species (Sheinin *et al.*, 2000; Geibel and

Hebert, 2009), making mechanistic studies challenging. CCK does not alter I_{sc} levels significantly in mouse small intestine mucosa (Cox *et al.*, unpublished), and although Gln responses were small in this gut area compared with colonic responses, they were blocked by BIBO3304 and thus were PYY-Y1 receptor mediated. Therefore, we conclude that I-cell derived CCK does not have a significant role to play in the electrogenic mucosal responses we measured to Gln.

We provide evidence for the first time that Gln-induced CaSR responses on either mucosal surface were glucose sensitive (whereas the subsequent PYY-Y1 effects were not) and this appears to be a common feature of L-cell GPCR mechanisms. Glucose sensitivity is exhibited by the acylethanolamine receptor GPR119, which is enriched on L-cells (Chu *et al.*, 2008) and upon stimulation in mouse and human colon mucosa results in PYY-release and Y1 receptor-mediated paracrine activity with evidence of coincident GLP-1-mediated mucosal responses (Cox *et al.*, 2010). These L-cell-specific GPCR activities, in combination with GPCR-induced pancreatic endocrine function (Engelstoft *et al.*, 2008), raise their profile as targets for future development of novel anti-diabetic and anti-obesity therapeutics. In addition, PYY and GLP-1 appear to contribute significantly to the weight loss observed following Roux-en-Y bariatric surgery (Chronaiou *et al.*, 2012; Dirksen *et al.*, 2013), and thus, dual agonism afforded by upstream stimulation of, for example, GPR119 or similarly enriched L-cell GPCRs, should offer improved therapeutic potential as treatments for T2D and potentially as 'knifeless' alternatives to bariatric surgery. Thus, understanding the detailed mechanisms by which Gln (and other amino acids) mediate the release of endogenous incretins, such as GLP-1 and PYY in native mucosae, could help validate the use of Gln supplementation as an adjunct therapy for treating T2D and obesity.

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Conflicts of interests

None.

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