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Mucosal glucagon-like peptide 1 (GLP-1) responses are mediated by calcitonin gene-related peptide (CGRP) in the mouse colon and both peptide responses are area-specific

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Abstract

Background: Glucagon-like peptide (GLP)-1 is an incretin hormone and its mimetics are proven antidiabetic and antiobesity drugs. GLP-1 exerts antimotility and mucosal proliferative activities but its epithelial ion transport effects are uncharacterized and these may contribute to the gastrointestinal (GI) disturbance, i.e., diarrhea experienced with some GLP-1 mimetics. Our aim was to establish GLP-1 agonist mechanisms and identify potential mucosal mediator(s) in the colonic tissue from C57BL/6J mice.

Methods: A tissue survey of GLP-1 responses (using exendin 4, Ex4) and α-calcitonin gene-related peptide (αCGRP) was undertaken, dividing the mouse colon into eight adjacent mucosal-submucosal preparations. Each preparation was voltage-clamped and changes in short-circuit current (Isc) measured. The involvement of submucosal neurons in GLP-1 agonism was tested using Ex(9-39) and tetrodotoxin (TTX), and CGRP receptors were blocked with BIBN4094.

Key Results: Ex4 responses along the length of the colon were inhibited by the GLP-1 antagonist, Ex(9-39) or TTX, indicating neural mediation in all colonic regions. In the ascending colon, Ex4 increased Isc levels that were abolished by 10 nM BIBN4096, while in the descending colon it reduced Isc levels that were again BIBN4096-sensitive, but at 1 μM. The latter αCGRP response was dependent on epithelial Cl^- conductance and Na^+/K^+-ATPase, and was partially (~25%) peptide YY-mediated, but was not nitrergic, somatostatin sst_2, or α_2-adrenoceptor-mediated.

Conclusions and Inferences: GLP-1 modulates epithelial ion transport indirectly by activating CGRP-containing submucosal enteric neurons in the mouse colon. This GLP-1-CGRP response was area-specific and could potentially contribute to the diarrheal side effect of certain GLP-1R therapeutics.

Abbreviations: AC, ascending colon; AMY_1, amylin 1 receptor; ATC, ascending-transverse colon; BIBO3304, *N*-[(1*R*)-1-[[[[4-[[(Aminocarbonyl)amino]methyl]phenyl]methyl]amino] carbonyl]-4-[(aminoiminomethyl)amino] butyl]-α-phenyl-benzeneacetamide ditrifluoroacetate; BIIE0246, *N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl) ethyl]amino] carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide; BIBN4096, 1-[3,5-Dibromo-*N*-[[4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-1-piperidinyl]carbonyl]-D-tyrosyl-L-lysyl]-4-(4-pyridinyl)-piperazine; CaSR, calcium-sensing receptor; CFTR, cystic fibrosis transmembrane regulator; CGRP, calcitonin gene-related peptide; COX2, cyclooxygenase 2; CYN, CYN154806; DAMGO, [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin; DC, descending colon; DMSO, dimethyl sulphoxide; DPPIV, dipeptidyl peptidase IV; ENaC, epithelial sodium channel; Ex4, exendin 4; GI, gastrointestinal; GIP, glucose-dependent insulinotropic peptide; GPCR, G protein-coupled receptor; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; KH, Krebs Henseleit; Isc, short-circuit current; MC_4, melanocortin 4 receptor; NKCC1, Na-K-Cl symporter; NOS, nitric oxide synthase; PYY, peptide YY; SGLT1, sodium-dependent glucose cotransporter 1; sst_2, somatostatin type 2 receptor; SRIF, somatostatin release inhibitory factor-14; TDC, transverse-descending colon; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide.

KEYWORDS
calcitonin gene-related peptide, enteric submucosal neurons, glucagon-like peptide 1, mouse colon mucosa

1 | INTRODUCTION

Glucagon-like peptide (GLP)-1 is probably the best-known gut-derived incretin hormone and its mimetics are proven antidiabetic and antiobesity drugs. GLP-1 activates the GLP-1 receptor (GLP-1R) to cause insulin secretion from pancreatic β cells, improving glucose tolerance in rodents and humans via a combination of peripheral and central mechanisms.[1,2] Nutrient ingestion releases GLP-1 primarily from enteroendocrine L cells that occur with the highest frequency in distal GI tract.[3,4] Here GLP-1 can be co-secreted with peptide YY (PYY), GLP-2 and oxyntomodulin, however in the proximal small intestine GLP-1 can be released with glucose-dependent insulinotropic peptide (GIP) and neurotensin[5] depending on the nutrient or metabolite stimulus.[6,7] These L cell peptides exert similar functions that include slower gastric emptying, reduced gastrointestinal (GI) motility and satiety.[2]

Two of the most highly expressed G protein-coupled receptors (GPCRs) on L cells rather than their surrounding epithelia are the acylethanolamine receptor GPR119[8] and the melanocortin 4 receptor (MC_4). Activation of these receptors are glucose-sensitive and result in PYY release (with consequent Y_1 receptor-mediated epithelial cell antisecretory effects)[9,10] together with GLP-1, mechanisms that persist in GI mucosae from diabetic rodents.[11] Although GPR119 and MC_4 receptors are G_s-coupled receptors that elevate $cAMP_i$ levels, amino acids such as L-glutamine activate G_q-coupled pathways via the calcium-sensing receptor (CaSR), which in rat small intestinal L cells causes Ca^{2+}-mediated co-release of PYY, GLP-1, and GIP,[12] whereas in the mouse colon involves endogenous PYY and, to a lesser extent, GLP-1.[13] Preliminary studies show that responses to exendin 4 (Ex4; the more stable GLP-1 agonist) increase distally along the mouse GI tract and that a degree of endogenous GLP-1R tone exists, particularly in the colon.[13] Given the growing importance of nutrient-sensing mechanisms mediated by GLP-1, notwithstanding its rapid degradation by DPP-IV,[14] we undertook a survey of GLP-1R responses along the length of the mouse colon.

GLP-1Rs are expressed by sensory afferent neurons originating in nodose ganglia[15] and vagal terminals innervating the hepatic portal vein.[16] Vagal afferents in the intestine also express GLP-1R, as do intrinsic enteric neurons, specifically fibers and cell bodies of myenteric and submucosal neurons,[17,18] the latter innervating the lamina propria close to GLP-1-positive L cells.[18] Notably, a proportion of GLP-1R-positive enteric neurons in culture co-stained for calretinin and calcitonin gene-related peptide (CGRP)[18] implicating the involvement of intrinsic sensory neurons[19] in GLP-1's mucosal effects, there being no discernible epithelial GLP-1R expression.[18] Recent ultrastructural studies show a direct relationship between enteric neurons and L cells[20] and this may not only provide a monosynaptic pathway for nutrient-sensing but could also provide a peripheral mechanism that contributes to the diarrheal side effects of certain GLP-1 therapeutics.[21] Given the apparent juxtaposition between GLP-1 and its neural receptor, we hypothesized that the majority of GLP-1's effects on ion transport in mucosal preparations (that include intact submucosal innervation) could be indirect and mediated by a sensory neurotransmitter such as CGRP acting on an as yet uncharacterized epithelial CGRP receptor type in the mouse colon.

Key points

- Glucagon-like peptide 1 (GLP-1) is released from enteroendocrine cells; however, we do not fully understand how GLP-1 signals within the intestine. Here we analyzed GLP-1 responses in the colon, a tissue that contains significant GLP-1-receptor levels and we elucidated its mechanisms of action.
- GLP-1-epithelial responses, although variable, were consistently mediated by enteric neuron-derived calcitonin gene-related peptide.
- We describe a new direct link between two intestinal peptides that could explain the diarrheal side effect of some GLP-1 therapeutics.

2 | MATERIALS AND METHODS

2.1 | Materials

BIBO3304, BIIE0246, BIBN4096, CYN154806, ouabain, amiloride, phloridzin, and bumetanide were purchased from Tocris (Bristol, UK). Stock solutions of BIBO3304, BIIE0246, and BIBN4096 were dissolved in 10% dimethyl sulphoxide (DMSO, at 1 mM), whereas CYN154806 and bumetanide were dissolved in water and all were stored at −20°C. Of the peptides used in this study, somatostatin (sst) and αCGRP were from Bachem (Bubendorf, Switzerland), whereas all other peptides were from Cambridge Bioscience (Cambridge, UK) and their stock aliquots were stored at −20°C, undergoing a single freeze-thaw cycle. Tetrodotoxin (TTX) was from Abcam (Cambridge, UK) and other agents not mentioned above were purchased from Sigma (Poole, UK).

2.2 | Methods

All mice were on the same C57BL/6-129/SvJ background and had free access to standard chow and water ad libitum. Animals were

housed under controlled conditions (12:12 h light/dark cycle, lights on 07.00 h, 22±2°C) and their care and experimental procedures complied with the Animals (Scientific procedures) Act 1986. Full-length colonic specimens were placed in fresh Krebs-Henseleit buffer (KH; in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, D-glucose 11.1, pH 7.4). Mucosal preparations devoid of overlying smooth muscle and myenteric innervation but with intact submucosal innervation were dissected swiftly as described previously.[9,13,22] Adjacent pieces of mucosae from either the cecal junction (ascending colon, AC1-AC3), the transverse colonic regions (designated ascending-transverse colon, ATC; and transverse-descending colon, TDC), or descending colon (DC3-DC1, the latter immediately proximal to the rectum) were prepared by cutting the colon into eight equally sized pieces. Each mucosal preparation was then placed in an Ussing chamber and voltage-clamped at 0 mV (DVC1000; WPI UK, Hitchin, Herts, UK) aerating with 95% O_2/5% CO_2 at 37°C. Mucosal short-circuit current (Isc) levels were allowed to stabilize (within 20 min) before initial drug additions. The GLP-1 antagonist, Ex(9-39) (1 μM), TTX (100 nM), or the CGRP receptor antagonist, BIBN4096 (10 nM-1 μM) were added to naive mucosae 20 min prior to addition of a single agonist concentration, either Ex4 (100 nM) or αCGRP (10 nM). At the end of some experiments control secretory responses (vasoactive intestinal polypeptide, VIP 30 nM) followed by an antisecretory agent (either 10 nM PYY or 1 μM UK14,304) were used to increase or lower the Isc, respectively.

All peptides, TTX (100 nM), ouabain (100 μM), bumetanide (50 μM), and various GPCR antagonists were added to the basolateral compartment only. The cyclooxygenase 2 (COX2) inhibitor, piroxicam, was added to apical and basolateral reservoirs (at 5 μM), whereas the epithelial sodium channel (ENaC) inhibitor, amiloride (50 μM) and SGLT1 inhibitor, phloridzin (50 μM) were added apically.

Changes in Isc to Ex4 or αCGRP were recorded within 20 min and where initial increases (designated the 1° phase) in Isc occurred prior to decreases in Isc (2° phase) then the peaks or troughs (from the extrapolated Isc baseline) were recorded and pooled separately to provide a mean±1 SEM for each phase. Other agonist responses were also pooled to provide a mean±1 SEM from at least five different colons. When comparing the effect of an antagonist in an adjacent mucosal preparation the control and experimental agonist responses were compared using Student's *t* test. When more than one pretreatment was compared (e.g., after different receptor antagonists) then one-way ANOVA with Dunnett's post-test was applied. *P* values ≤.05 were statistically significant.

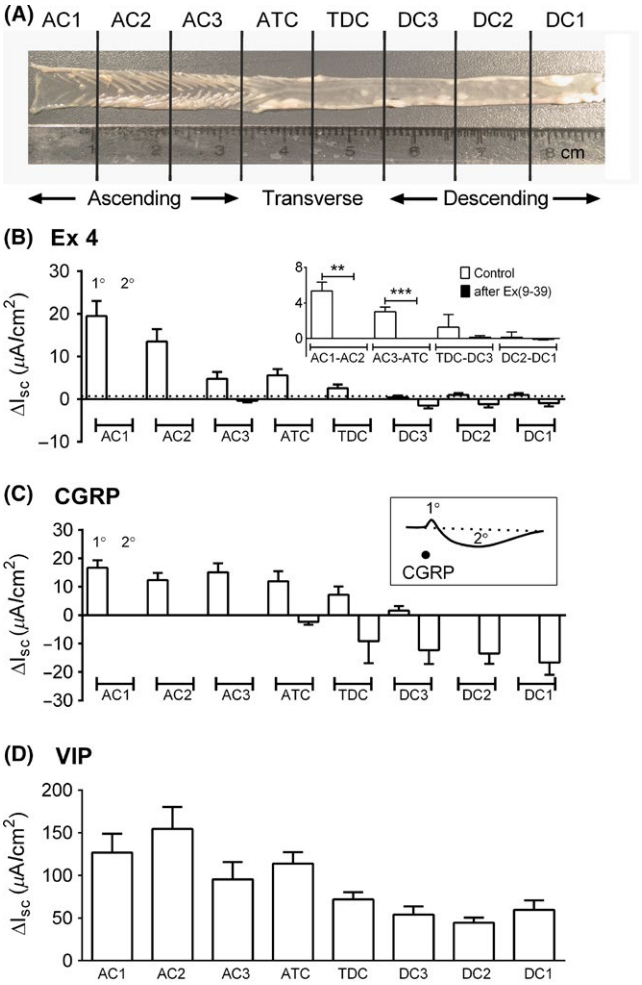

FIGURE 1 Variability of peptide responses along the colonic length. (A) Photograph illustrating the designated areas of a mouse colon, separated into equal-sized eighths. From the left: ascending colon (AC) 1, 2, and 3 (i.e., from the cecal junction to the end of the striated proximal region); ascending-transverse colon (ATC); transverse-descending colon (TDC) and descending colon (DC) regions (DC) 1, 2, and 3. (B) GLP-1R activation by Ex4 (100 nM) in each colon segment, showing primary (1°) increases, followed by secondary (2°) decreases in baseline I_{sc}, with H_2O vehicle control (dashed line). (Inset) Abolition of Ex4 responses in adjacent pairs of preparations by the GLP-1 antagonist, Ex(9-39) along the colon length. (C) Variation of αCGRP (10 nM) colonic responses with a representative trace (inset) illustrating 1° and 2° components of this peptide response in the transverse-descending colon (TDC). (D) VIP (30 nM) peak responses in the same colonic preparations. All bars represent the mean±1 SEM from five observations. $^{**}P<.01$, $^{***}P<.001$ compared controls with Ex(9-39)-preteated tissues using Student's *t* test

3 | RESULTS

3.1 | Exendin 4 (Ex4) and αCGRP responses vary along the colon length

Responses to a basolateral addition of the GLP-1 agonist, Ex4 varied along the length of the mouse colon (Figure 1A, B). In the distal colon Ex4 responses were small and biphasic, comprising of an initial transient rise in Isc (1°; Figure 1B) followed by a slower small Isc reduction (2°) below the original baseline Isc. In ascending colon mucosae, larger monophasic increases in Isc occurred (Figure 1B) and these secretory responses are potentially physiologically relevant and could result in diarrhea. The GLP-1 antagonist, Ex(9-39), blocked all aspects of the Ex4 response along the length of the colon (Figure 1B, inset). Additionally, Ex(9-39) alone decreased basal Isc (data not shown) indicating a degree of endogenous GLP-1 tone (as observed

previously).[13] As the GLP-1R is expressed in enteric nerves[16,17] and αCGRP-containing submucosal fibers have been identified close to the epithelial layer with integral L cells,[18] we next surveyed mucosal αCGRP responses along the length of the colon (Figure 1C). We observed αCGRP responses that changed in an area-specific manner and broadly resembled the pattern observed for Ex4 sensitivity. In ascending colon mucosa, αCGRP increased Isc levels, while in the transverse colon, biphasic responses were apparent (1° increases followed by 2° decreases in Isc, see inset in Figure 1C) compared with monophasic Isc reductions in the distal colon. Such variations in peptide response have not been observed in adjacent mucosal preparations to date. VIP responses were in contrast, always sustained secretory responses (note the different y axes scales in Figure 1) and the peak increases of these epithelial responses are shown for comparison (Figure 1D), reducing in size (~40%) in the descending colon, but this was not statistically significant. Subsequent PYY (10 nM) responses were also consistent but were monophasic reductions in Isc, the size of which declined (~40%) from the most proximal to distal colon areas (data not shown).

3.2 | Neurotoxin TTX and BIBN4096 sensitivity of αCGRP and GLP-1R responses

In the ascending colon mucosa (AC1-3) αCGRP raised basal Isc with an EC_{50} of 3.8 nM (Figure 2A) and these responses were not TTX-sensitive, unlike Ex4 responses that were clearly TTX-sensitive (Figure 2B) and thus neuronal. Both αCGRP and Ex4 responses were blocked by the CGRP receptor antagonist (BIBN4096)[23] (Figure 2C) showing that GLP-1 (and αCGRP) responses are CGRP receptor-mediated in the mouse ascending colon.

The biphasic responses to αCGRP in the descending colon were also concentration-dependent. The 1° phase although small, appeared maximal at 100 nM αCGRP with an EC_{50} of ~3 nM, while the 2° component appeared not to reach a maxima between 100 and 300 nM (Figure 3A). As observed in the ascending colon, αCGRP responses were TTX-insensitive, while the smaller biphasic Ex4 responses appeared to be partially sensitive to the neurotoxin (Figure 3B). The small size of control Ex4 Isc responses limited statistical analyses of potential mediators but nevertheless descriptive comparisons were made. The CGRP receptor antagonist, BIBN4096[23] abolished both αCGRP and Ex4 responses but only at a 100× higher antagonist concentration than was required to block ascending colon αCGRP responses (Figure 3C). In summary, both ascending and descending colon GLP-1 activity appears to involve CGRP and this sensory peptide's responses were TTX-insensitive, and therefore potentially epithelial.

3.3 | Investigation of the potential mechanisms mediating αCGRP responses in the descending colon

As αCGRP responses in the descending colon (DC1-DC3) were TTX-insensitive, this implicated either direct peptide-epithelial action(s) or the involvement of mediators from TTX-insensitive cells within the lamina propria, eg, subepithelial leukocytes. If CGRP receptors are expressed by epithelial cells, their activation may couple preferentially

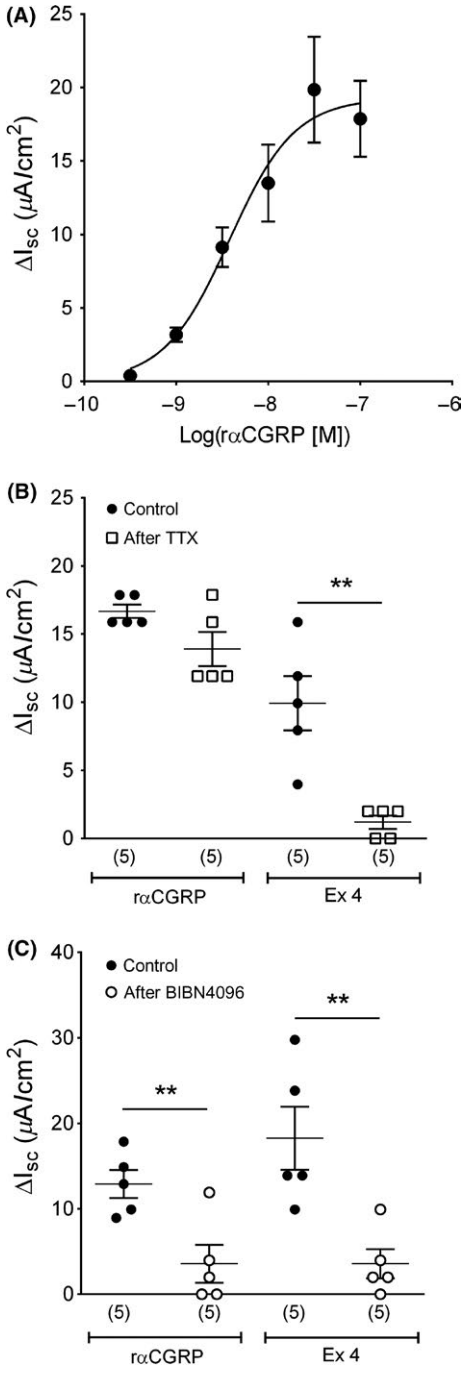

FIGURE 2 Monophasic secretory αCGRP responses are TTX-insensitive in the mouse ascending colon (AC1-3 only). (A) Concentration-response curve for αCGRP in the ascending colon. Each point was pooled from single peptide additions per preparation. (B) TTX-insensitivity (100 nM) of αCGRP (10 nM) responses, but not Ex4 (100 nM) responses. (C) Both αCGRP and Ex4 responses were inhibited significantly by the CGRP receptor antagonist, BIBN4096 (10 nM). All points in A are the mean±1 SEM from five observations. Statistical significance was analyzed between groups using Student's *t* test, $^{**}P<.01$, comparing controls with TTX (in B) or BIBN4096 (in C)

to the G_s-linked pathway with resultant increases in epithelial $cAMP_i$ and consequently raised Isc levels (similar to 1° phase observed in ascending colon) via increased Cl^- secretion through apical cystic

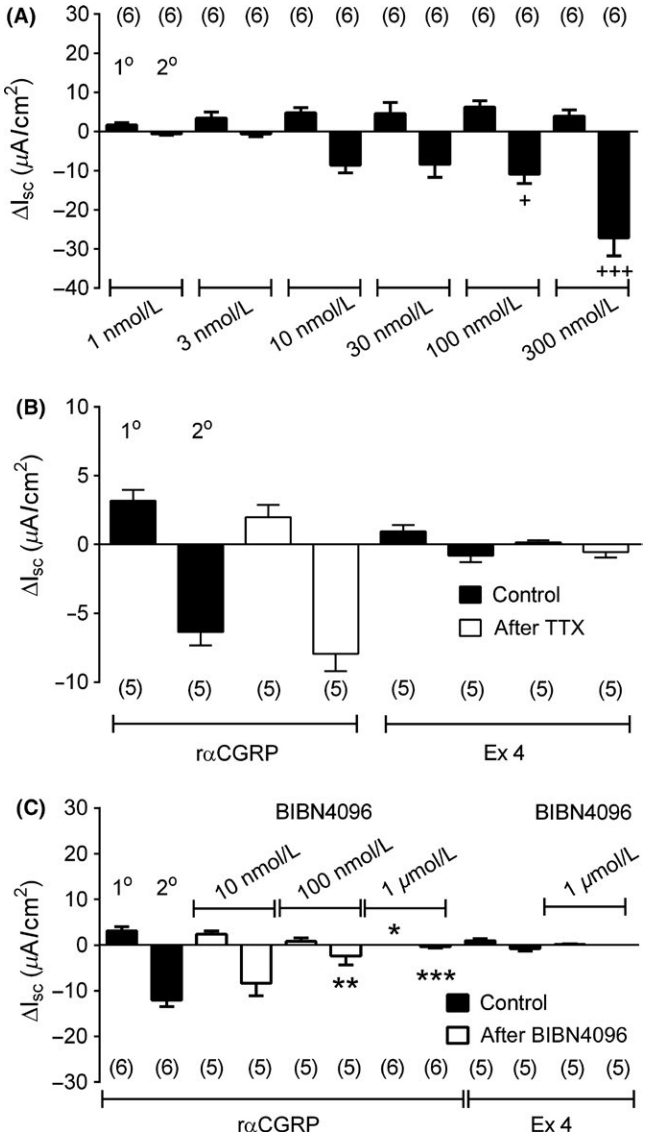

FIGURE 3 Biphasic αCGRP responses in the descending colon (DC1-3 only) are TTX-insensitive while GLP-1 responses are TTX-sensitive and CGRP receptor-mediated. (A) Concentration-dependent αCGRP relationship for the two Isc phases (primary [1°] and secondary [2°]) in descending colon. Each bar was derived from individual αCGRP concentrations per mucosal preparation. (B) TTX did not alter either phase of the αCGRP (10 nM) response but appears to abolish the small Ex4 responses. (C) BIBN4096 (10 nM-1 μM) inhibition of αCGRP (10 nM) and Ex4 (100 nM) responses. All bars are the mean±1 SEM with n values shown in parenthesis. $^{+}P<.05$, $^{+++}P<.001$ (in A) compared to αCGRP 2° responses at 1 nM. In C, $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ compare control responses with BIBN4096-preteated response using one-way ANOVA with Dunnett's post-test for αCGRP, and Student's *t* test for Ex4 data

fibrosis transmembrane regulator (CFTR). Inhibition of CFTR conductance, resulting for example from G_i-coupled reductions in epithelial $cAMP_i$, would reduce Isc, reminiscent of the 2° αCGRP response in descending colon. In the absence of efficacious CFTR blockers (Cox et al., unpublished data), we inhibited Cl^- transport indirectly by blocking basolateral Cl^- entry via NKCC1 using bumetanide. The loop diuretic significantly attenuated the 2° αCGRP reduction in Isc (Figure 4A). Apical Na^+ conductances were then blocked using previously optimized concentrations of amiloride (an ENaC inhibitor) or phloridzin (a SGLT1 inhibitor) neither of which significantly altered the αCGRP response (Figure 4A). Only after a combination of NKCC1, ENaC, and Na^+/K^+-ATPase inhibition (with bumetanide, amiloride, and ouabain, respectively), were subsequent αCGRP responses abolished (Figure 4A) implicating epithelial Cl^- and Na^+/K^+ electrogenic transport processes. SGLT1 inhibition (phloridzin) had no effect on the αCGRP response (Figure 4A) and when combined with the three other transport inhibitors, αCGRP 2° responses were again significantly inhibited.

Nitric oxide is inhibitory in the GI tract and its potential involvement in αCGRP's effects was tested using the nitric oxide synthase (NOS) inhibitor, L-NNA. This abolished L-arginine (200 μM) responses, which were small increases in Isc, but had no significant effect on the αCGRP response in the descending colon (Figure 4B). Next, a number of selective antagonists of known antisecretory epithelial GPCRs were tested. Antagonism of sst_2 (CYN154806), PYY via Y_1 (BIBO3304) and Y_2 (BIIE0246) receptors, or α_2 adrenoceptor (UK14,304) activities had no effect on the reductions in Isc elicited by αCGRP (Figure 4C). Only the combination of optimal PYY-Y_1 and Y_2 blockade (Figure 4D) inhibited the 2° αCGRP response by 24.8% (although this was not statistically significant), indicating a minor contribution by L cell-derived PYY to this reduction in Isc by αCGRP. Neither the sst_2 antagonist CYN154806 nor α_2 antagonist, yohimbine had any significant effect on the αCGRP response, so these G_i-coupled receptors do not appear to be involved in αCGRP responses in descending colon (Figures 4C and 4D). The μ-opioid antagonist naloxone, and cholinoceptor blockade (which can be epithelial and inhibited by hexamethonium and atropine)[24] also had no effect on αCGRP responses despite abolishing control agonism at μ-opioid (DAMGO, 10 μM), and cholinergic responses (nicotine, 10 μM, and carbachol, 10 μM), respectively (data not shown). The COX2 inhibitor piroxicam had no effect on αCGRP 2˚ responses (controls; −1.7±0.5 μA/cm^2 [n=4] vs plus piroxicam −1.6±0.4 μA/cm^2 [n=5]).

To summarize, in the descending colon ~50% of the 2° αCGRP response is due to epithelial ion transport processes, together with a degree (~25%) of PYY-Y_1/Y_2 receptor involvement. What mediates the remainder of αCGRP's electrogenic responses in this distal region of the colon is unknown currently, but does not apparently involve cholinergic, nitrergic, somatostatin-sst_2 receptor, α_2-adrenoceptor, or μ-opioid mechanisms, or endogenous cytokines generated within the lamina propria via COX2 activity.

4 | DISCUSSION

4.1 | Regional variations in the GLP-1R response within the mouse colon

This study established a graded decline in secretory responses to Ex4 (GLP-1R) agonism in a descending direction (i.e., from cecum to rectum). In addition, αCGRP responses also changed along the colon length, starting as monophasic secretory responses in ascending colon

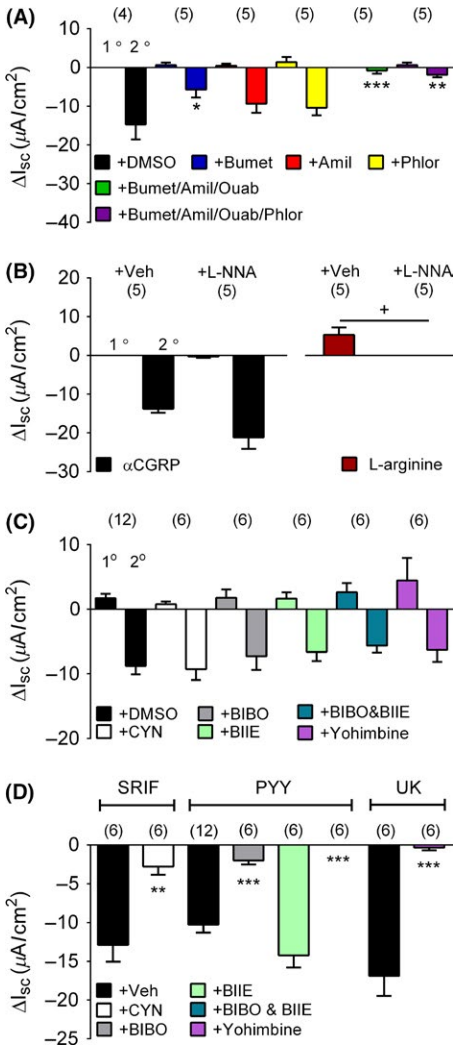

FIGURE 4 Pharmacological investigation of αCGRP responses in the descending colon (DC1-3 only). (A) The αCGRP responses (10 nM throughout) were compared after vehicle (0.1% DMSO, black bars) or bumetanide (100 μM bl; +Bumet), amiloride (50 μM ap; +Amil) or phloridzin alone (50 μM ap; +Phlor), then bumetanide, amiloride, and ouabain (100 μM bl; +Bumet/Amil/Ouab), or all four transport inhibitors together (+Bumet/Amil/Ouab/Phlor). (B) NOS inhibition by L-NNA (1 mM) of αCGRP responses (black bars) and internal control responses to L-arginine (200 μM bl). (C) αCGRP responses were also investigated in the presence of the relevant vehicle (+Veh) or the sst_2 receptor antagonist, CYN154806 (1 μM; CYN), PYY-Y_1 antagonist BIBO3304 (BIBO, 300 nM), Y_2 antagonist BIIE0246 (BIIE, 1 μM), or BIBO3304 and BIIE0246 combined (+BIBO&BIIE), or the α_2 adrenoceptor antagonist, yohimbine (10 μM). Vehicles were H_2O for CYN154806 and yohimbine, 0.03% DMSO for BIBO and BIIE individually, and 0.06% DMSO for BIBO & BIIE. (D) Agonist controls were SRIF (100 nM), PYY (10 nM), or the α_2 adrenoceptor agonist, UK14,304 (1 μM; UK) in the absence (black bars) or presence (colored bars) of different antagonist(s). Each bar is the mean±1 SEM with n values in parentheses. Levels of statistical significance were; $^+$ or * $P<.05$, ** $P<.01$, *** $P<.001$ comparing vehicle controls with respective inhibitor-preteated tissues, using Student's *t* test for L-arginine data in (B) and SRIF and UK14,304 data in (D), otherwise one-way ANOVA with Dunnett's post-test was used

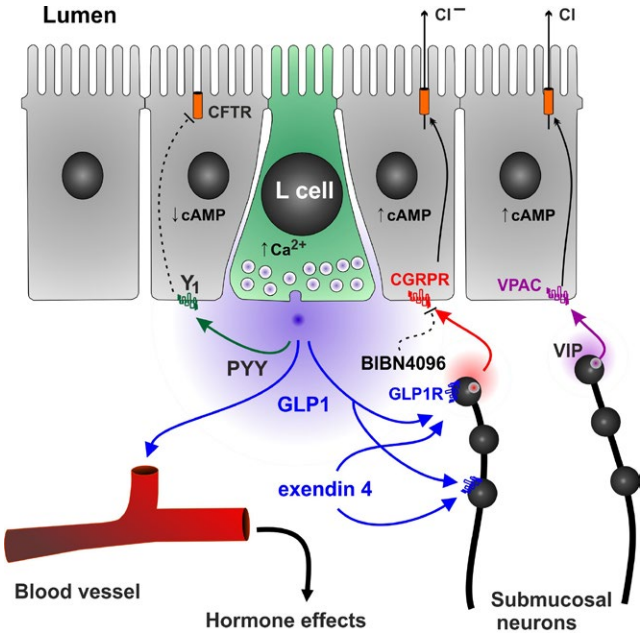

FIGURE 5 A schematic figure showing GLP-1 (Ex 4-mediated) and αCGRP mechanisms together with PYY and VIP activities in the mouse ascending colon mucosa

converting to predominantly antisecretory responses in the most distal regions of the colon. This variability is not mimicked by other secretory or antisecretory peptides (eg, VIP or PYY, respectively) and is therefore noteworthy because it occurs in the most commonly used mouse model, C57BL/6J. The secretory responses observed to Ex4 and αCGRP particularly in the ascending colon (a region that is pro-absorptive) suggest that GLP-1 and CGRP receptor signaling in this region may contribute to the diarrheal side effects observed with longer acting GLP-1 mimetics.[25]

In the ascending colon, GLP-1R agonism was neuronal and primarily CGRP-mediated (Figure 5) and this proximal colonic region exhibited greater GLP-1 sensitivity than the descending colon (as seen previously[13]). In both regions, GLP-1 responses were CGRP receptor-mediated. Submucosal neurons that express CGRP are conventionally thought to be sensory in function; however, in a previous study we observed that all the cholinergic submucosal neurons in the distal colon co-stained for CGRP.[26] We also found significantly fewer numbers of neurons in the submucous plexi of descending (equivalent to DC1-2 used in the present study) compared to the ascending colon (equivalent to AC1-2 here). In the most distal region, ~80% of all submucosal neurons were non-cholinergic/VIP-positive compared with the ~20% that were ChAT/CGRP-positive. It is likely that these regional differences in submucosal-mucosal innervation underlie the functional variations we observed, particularly for the TTX-sensitive GLP-1R responses. However, differing tissue sensitivity includes variation of receptor expression and to date there is little information on the patterns of GLP-1R localization or for that matter, CGRP receptor

localization in mouse or rodent gut, primarily because of the lack of selective receptor antibodies. However, a *glp*1r-Cre mouse crossed with a fluorescent reporter has recently allowed clarification and identification of the major sites of *glp*1r expression.[18] The localization of a GLP-1R-positive endocrine cell (within the otherwise GLP-1R-negative mucosal layer) near to a *glp*1r-fluorescent submucosal nerve fiber, initiated this functional study. The additional observation that *glp*1r-positive nerves in culture stained for CGRP provided additional impetus to investigate whether endogenous CGRP mediated GLP-1R activity in the mouse colon.

4.2 | GLP-1R ion transport responses utilize neuronal CGRP in mouse colon

To our knowledge, very little has been published on GLP-1 induced ion transport effects,[13] with only marginally more information on CGRP activities in rat and guinea-pig GI mucosae[27–29] and human colon epithelial lines,[30] and no studies that we could find for CGRP in murine GI mucosae. We confirmed that the mucosal GLP-1R activities were consistently sensitive to the GLP-1R antagonist, Ex(9-39). However, unexpected, but clear differences in GLP-1R activities were observed, even between adjacent mucosal areas. Ascending colon mucosal Ex4 responses were secretory (Figure 5), and a step-wise decline in the peak of this Isc response was seen with each cm of colon traversed, resulting in very small, biphasic Isc changes in the descending colon. Ex4 secretory responses were consistently TTX-sensitive and therefore neuronal, while the small changes in Isc in descending colon were too small to analyze statistically, but indicated at least partial sensitivity to the neurotoxin. We conclude that GLP-1R agonism involves submucosal neurons in mouse ascending colon and that this indirect mechanism appears to prevail in the descending colon. By describing these patterns using designated colonic areas, we highlight the need to identify the region of colonic tissue used in future functional and morphological studies and the requirement for careful interpretation of data derived from mouse colon.

Our findings are in opposition to the epithelial GLP-1R localization described by Kedees et al.[31] in the CD-1 mouse ileum and colon. In this mouse strain GLP-1R expression was observed in mucosa and in enteric neurons of submucosal and myenteric ganglial networks (as we, and others have seen respectively[27,28]) but a confounding issue may have been the non-specificity of the GLP-1R antibody used.[31] Our functional studies more closely match the observations described by Richards et al. utilizing the fluorescent reporter *glp*1r-Cre mouse.[18] GLP-1R were located on vagal afferent fibers[16] that innervate upper GI areas and extend distally to the transverse colon using traditional immunohistochemistry (although this antibody was withdrawn by Abcam, subsequently). The patterns of GLP-1R expression revealed by the transgenic mouse are the most revealing to date, showing extensive innervation of gastric pylorus (some of which were inhibitory and NOS-positive), less frequent neuronal fibers and cell bodies along the small intestine and colon that were distinguished by whole cell patch-clamp as either secretomotor or sensory in character. A small proportion of GLP-1R fluorescent cells in culture, co-stained for calretinin, calbindin, or importantly CGRP,[18] and it is these that we propose are involved functionally in GLP-1R agonism in mouse colon mucosa.

The secretory CGRP responses of murine ascending colon were similar in profile and potency to those we described previously in rat descending colon.[27] However, in the latter we observed biphasic CGRP responses that were quite different, highlighting another species difference. In mouse colon, we consistently observed αCGRP-induced increases in Isc prior to decreased Isc levels, whereas in the rat low nM concentrations of αCGRP elicited long-lasting decreases in Isc that converted to increases in Isc at higher αCGRP concentrations.[27] The latter were direct effects (ie, TTX-insensitive, presumably epithelial) and exhibited an EC_{50} (~10 nM) very similar to that seen here for αCGRP in mouse ascending colon. Somatostatin was suspected as the antisecretory mediator of higher αCGRP concentrations in rat colon,[27] but in the mouse descending colon this was not the case, as a sst_2 antagonist that abolished somatostatin activity had no effect on αCGRP responses.

4.3 | BIBN4096 inhibits Ex4 and αCGRP responses in mouse ascending and descending colon mucosa

The competitive antagonist BIBN4096 is selective for CGRP receptors with very low, if any affinity for calcitonin, amylin, or adrenomedullin receptors in native tissues.[23] However, in recombinant COS-7 cells expressing different calcitonin receptors and receptor-activity modifying proteins (RAMPs), this antagonist exhibited low affinity (at least 2 orders of magnitude lower) for the calcitonin-/amylin-preferring AMY_1 receptor that includes RAMP1.[32] It is possible therefore, that BIBN4096 may inhibit CGRP and AMY_1 receptors, but the latter only at significantly higher antagonist concentrations. This complements the sensitivity of peptide responses we observed in mouse colon, where αCGRP (and Ex4) responses were virtually abolished by 10 nM BIBN4096 in ascending mucosa, but 1 μM antagonist was required to abolish responses in descending colon. As no commercially available AMY_1 antagonists exist to our knowledge, this receptor's involvement in CGRP or indeed GLP-1R activities remains to be determined. Our priority in this study was to determine whether endogenous CGRP-mediated GLP-1R-agonism and this appears to be the case, but it is possible that the calcitonin receptors in these two colonic areas are different in the mouse.

4.4 | Mechanisms involved in descending colon CGRP responses

Inhibiting transepithelial Cl^- secretion significantly reduced αCGRP responses in descending colon (in agreement with Esfandyari et al.[29]) and blockade of Cl^-, Na^+, and K^+ vectorial ion transport using a combination of transport inhibitors, eradicated αCGRP's activity, confirming epithelial involvement in the CGRP receptor response. No other pharmacological intervention significantly altered the albeit small electrogenic αCGRP responses in mouse descending colon mucosa, including nitrergic, cholinergic, α_2-adrenoceptor, sst_2, or μ-opioid receptor blockade; or inhibition of COX2 activity (in agreement with a number of discounted mediators in rat colon).[29] Only PYY-Y1 and Y2

agonism combined contributed ~25% toward the αCGRP antisecretory effect in the mouse descending colon.

In conclusion, we describe unexpected differences in Ex4 and αCGRP mucosal signaling from proximal to distal colon of the mouse. Despite the gradation in these two peptides' responses, GLP-1R agonism was consistently neuronal and involved CGRP released from enteric submucosal neurons, possibly intrinsic sensory neurons. The increasing clinical relevance of GLP-1R agonism in the treatment of T2D[1,2] with coincident diarrheal side effects,[20] necessitate a more thorough understanding of the underlying GLP-1R mucosal activities, especially in widely used mouse models. Endogenous GLP-1 released from enteroendocrine L cells into the underlying lamina propria has the potential to rapidly activate CGRP-containing intrinsic enteric neurons and initiate local gut reflexes that lead to hypersecretion, particularly in the proximal half of the mouse colon. It is unlikely that CGRP retains activity sufficiently long to circulate and contribute to the reductions in gastric emptying[33] and other antimotility and antiobesity effects of GLP-1 agonism,[34] but our study describes for the first time, a CGRP-dependent pro-secretory mechanism that may contribute to the diarrhea experienced by 10%-20% of patients taking GLP-1 mimetics.

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CONFLICT OF INTEREST

The authors have no competing interests.

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