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Luminal 5-HT-evoked colonic HCO3− secretion

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RESEARCH PAPER

Luminal 5-HT stimulates colonic bicarbonate secretion in rats

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BACKGROUND AND PURPOSE

The bioactive monoamine 5-HT, implicated in the pathogenesis of functional gastrointestinal disorders, is abundantly synthesized and stored in rat proximal colonic mucosa and released to the gut lumen and subepithelial space. Despite much data regarding its expression and function, the effects of luminal 5-HT on colonic anion secretion have not been fully investigated.

EXPERIMENTAL APPROACH

We measured short-circuit current (I_{sc}) as an indicator of ion transport in mucosa-submucosa or mucosa-only preparations of rat proximal colon. Total CO_2 output was measured in vitro and in vivo. Immunohistochemistry was performed to investigate the localization of 5-HT$_4$, NOS1 and NOS2.

KEY RESULTS

Luminal 5-HT gradually increased the amplitude and sustained the elevation of I_{sc}. Luminal 5-HT-evoked ΔI_{sc} was acetazolamide sensitive and HCO_3^- dependent, consistent with cytosolic carbonic anhydrase-dependent electrogenic HCO_3^- secretion, while not affected by tetrodotoxin (TTX), atropine or indomethacin. Pretreatment with the selective 5-HT$_4$ antagonist GR113808, but not antagonists for 5-HT$_3$, 5-HT$_6$ or 5-HT$_7$, inhibited luminal 5-HT-evoked ΔI_{sc}. Furthermore, luminal cisapride and tegaserod increased I_{sc} to the same extent as did 5-HT in the presence of indomethacin and TTX. Removal of the submucosa or pretreatment with NOS inhibitors enhanced luminal 5-HT-evoked ΔI_{sc}, suggesting that NO synthesized in the submucosa suppresses mucosal anion secretion. NOS1 and NOS2 were immunostained in the submucosal neurons and glial cells respectively. Luminal 5-HT-evoked HCO_3^- secretion was confirmed in vivo, inhibited by co-perfusion of GR113808, but not by ondansetron.

CONCLUSIONS AND IMPLICATIONS

A novel apical 5-HT$_4$-mediated HCO_3^- secretory pathway and an NO-dependent inhibitory mechanism are present in the proximal colon. Luminal 5-HT-evoked HCO_3^- secretion may be important for the maintenance of mucosal integrity by regulating luminal pH.

Abbreviations

EC, enterochromaffin; GI, gastrointestinal; L-NIO, N-iminoethyl-L-ornithine; NPA, N^ω-propyl-L-arginine; SCFA, short-chain fatty acid; SERT, 5-HT uptake transporter; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; SSRI, selective serotonin reuptake inhibitor; TPH, tryptophan hydroxylase; TTX, tetrodotoxin.

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TARGETS
Ligand-gated ion channels[a]
5-HT3 receptor
GPCRs[b]
5-HT$_4$ receptor
5-HT$_6$ receptor
5-HT$_7$ receptor
Ion channels[c]
CFTR anion channel
ENaC sodium channel
Transporters[d]
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Amiloride
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GR113808
Indomethacin
L-NAME
NO
Ondansetron
SB258719
Tegaserod
TTX

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson et al., 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d,e]Alexander et al., 2013a,b,c,d,e).

Introduction

5-HT (serotonin) is one of the most important signalling molecules affecting gastrointestinal (GI) function due to its many contributions to physiological and pathophysiological conditions through its seven receptor subtypes and reuptake transporters (Gershon and Tack, 2007). More than 98% of 5-HT in humans is synthesized by enterochromaffin (EC) cells by the rate-limiting enzyme tryptophan hydroxylase (TPH)-1, followed by L-amino acid decarboxylase, whereas the remaining 2% is produced in CNS by TPH-2. The highest percentage of enteroendocrine cells is composed of EC cells (Sjölund et al., 1983), with EC cell density highest in the proximal colon in rats (Glisic et al., 2006). Luminal release of 5-HT in relation to increased luminal pressure and peristalsis was first reported in 1958 in guinea pig and rabbit small intestine (Bülbring and Lin, 1958). Moreover, mucosal stroking, luminal acid or vagal nerve stimulation released 5-HT from EC cells into the lumen more than into the circulation (Ahlman et al., 1981a,b; Kellum et al., 1983; 1999), suggesting that luminal 5-HT release is functionally regulated. In the rat colon, luminal 5-HT concentration is elevated by luminal perfusion with short-chain fatty acids (SCFAs) or by an increase in luminal pressure (Fukumoto et al., 2003; Tsukamoto et al., 2007). Yet, despite these many observations, the effects of luminal 5-HT on colonic ion secretion have rarely been investigated. In the mouse distal colon, the 5-HT$_4$ receptor is expressed in colonic epithelial cells; luminal application of the 5-HT$_4$ agonist tegaserod increased short-circuit current (I_{sc}), inhibited by tetrodotoxin (TTX) or the 5-HT$_4$ antagonist GR113808 (Hoffman et al., 2012). As 5-HT is also biosynthesized in plants as a signal transmitter from tryptophan by non-mammalian pathways (Kang et al., 2007), some common foodstuffs such as tomatoes, corn, rice and bananas contain considerable amounts of 5-HT (30–140 µg·g^{-1}) (Badria, 2002; Ly et al., 2008), suggesting that the GI mucosa is frequently exposed to measurable endogenous and exogenous concentrations of 5-HT. As 5-HT is implicated in the generation of functional GI symptoms (Cremon et al., 2011), a knowledge of the actions of luminal 5-HT is important for understanding GI physiology and functional disorders.

NO is a gaseous signal molecule produced by a variety of cell types with three NOS isozymes: NOS1 (also known as brain NOS or neuronal NOS), NOS2 (inducible NOS) or NOS3 (endothelial NOS). Released NO affects mucosal secretory function either as a pro- or anti-secretory modulator. Serotonergic Cl$^-$ secretion is partially mediated by NO-dependent pathways (Kadowaki et al., 1996; Stoner et al., 2000), involved in the stimulation of anion secretion via neural and COX activation, as studied in Ussing-chambered rat colon (Tamai and Gaginella, 1993; Wilson et al., 1993). Nevertheless, cAMP-dependent secretion is attenuated by NOS2-dependent NO accumulation (Asfaha et al., 1999; Freeman and MacNaughton, 2000; MacEachern et al., 2011). Furthermore, nicotinergic Cl$^-$ secretion is suppressed by endogenous NO, which is tonically derived from myenteric ganglia in mouse distal colon (MacEachern et al., 2011).

Here, we show that luminal 5-HT increased electrogenic HCO$_3^-$ secretion via 5-HT$_4$ receptors in rat proximal colon in vivo and in vitro. The luminal 5-HT-induced I_{sc} increase was independent of neural or COX activity, and was enhanced by removal of the submucosal layer or by NOS inhibition. Our

findings are consistent with the presence of a novel apical 5-HT$_4$-mediated HCO_3^- secretory pathway and with an NO-dependent anti-secretory mechanism for Cl^- secretion in rat proximal colon.

Methods

Animals

All animal care and experimental studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 166 animals were used in the experiments described here. Male Sprague-Dawley rats weighing 200–250 g (Harlan, San Diego, CA, USA) were fed with a pellet diet and water *ad libitum*. Rats were fasted overnight in wire-bottomed cages with free access to water before the experiments.

Tissue preparation for Ussing chamber studies

Rats were anaesthetized by isoflurane and killed by exsanguination. Mucosa-submucosa preparations were fashioned from proximal, mid and distal colon as described previously (Kaji *et al.*, 2012). The colonic segments, opened along the mesenteric border, were stripped off the muscle layers with fine forceps under a stereomicroscope in ice-cold Krebs–Ringer buffer. Submucosal layers were further removed for some experiments. Two preparations were made from each segment by dividing longitudinally, and mounted between two hemichambers with an aperture with area = 0.3 cm^2 (Physiologic Instruments, San Diego, CA, USA). A matched pair of preparations was used for the control and experimental groups, with combinations of three or more groups used for multiple comparisons. Removal of muscle layers or submucosal layers was confirmed by histological examination of frozen sections obtained from chambered mucosa after completion of the Ussing chamber studies.

I_{sc} and total CO_2 output measurements in Ussing chambers

The chambers were bathed with serosal and luminal bathing solutions in a volume of 4 mL each, maintained at 37°C with a water-recirculating heating system. The bathing Krebs–Ringer solution contained (in mM) 117 NaCl, 4.7 KCl, 1.2 MgCl$_2$, 1.2 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 25 NaHCO$_3$, 11 glucose, and bubbled with 95% O$_2$-5% CO$_2$ to maintain pH at 7.4. For HCO_3^--free conditions, NaHCO$_3$ was replaced with NaCl and acetazolamide (0.2 mM) was added into the serosal and luminal baths bubbled with 100% O$_2$. For Cl$^-$-free solutions, NaCl, KCl and CaCl$_2$ were replaced with sodium gluconate, potassium gluconate and 8 mM calcium gluconate respectively. The tissues were short-circuited by a voltage clamp (Physiologic Instruments) at zero potential automatically with compensation for solution resistance. I_{sc} and tissue conductance (G_t) were determined every 2 s and recorded by the DataQ system (Physiologic Instruments). Positive values for I_{sc} indicate a negative electrical charge flux from serosal →

luminal bath indicating anion secretion or cation absorption. Tissues were stabilized for 50 min before 5-HT or other drugs were added. DMSO (<0.3%) in the bathing solution did not affect basal I_{sc}.

To correlate HCO_3^- secretion with I_{sc} changes, total CO$_2$ content in the luminal bathing solutions was measured using a CO$_2$ electrode (Lazar Research Laboratories, Los Angeles, CA, USA) as previously described (Akiba *et al.*, 2001). As luminally applied 5-HT hydrochloride (100 µM) and acetazolamide (0.2 mM) are acidic, the pH-stat method with normal saline in the luminal bath cannot accurately measure titratable alkalinity, total CO$_2$ content changes in the luminal bath were measured as luminal HCO_3^- output. The proximal colonic tissues were mounted in Ussing chambers as described above, with pH 7.4 Krebs buffer containing (in mM) 136 NaCl, 2.6 KCl, 1.8 CaCl$_2$, 10 HEPES and 10 glucose in the luminal bath with 100% O$_2$ bubbling. Luminal pH was continuously monitored with a pH electrode (Radiometer Analytical, Lyon, France). After ~30 min to ensure I_{sc} stabilization, time was set as $t = 0$ min. One-half millilitres of the luminal bathing solution was collected and replaced with pre-warmed, O$_2$ bubbled Krebs buffer every 5 min. Each sample was added to 4.5 mL of 0.1 M citrate buffer (pH 4.5) to convert free HCO_3^- to CO$_2$, which was measured by a CO$_2$ electrode (Akiba *et al.*, 2001). As the secreted HCO_3^- was accumulated in the luminal solution, basal total CO$_2$ content averaged from basal period for 10 min was subtracted from total CO$_2$ content at each time point. Luminal HCO_3^- output change was expressed as Δtotal CO$_2$ output (µmol·min^{-1}·cm^{-2}).

Effect of 5-HT on colonic HCO_3^- secretion in vivo

Colonic loops were prepared and perfused according to the modified method for duodenal perfusion (Akiba *et al.*, 2007; 2009). Under isoflurane anaesthesia (2%), the abdomen was incised, the caecum and proximal colon were exposed, and a 0.5 cm incision was made in the proximal colonic wall 0.5 cm caudal to the caecum using a thermal cautery. A polyethylene tube (outer diameter 5 mm, inner 3 mm) was inserted through the incision and positioned until it was 0.5 cm caudal to the incision, where it was secured with a nylon ligature. The distal portion of the proximal colon was ligated proximal to the mid colon followed by filling the colonic loop with 1 mL saline pre-warmed at 37°C. The distal portion was then incised, and another polyethylene tube (outer 7 mm, inner 5 mm) was inserted through the incision and sutured into place. To prevent colonic wall ischaemia, sutures were placed between serosal arteries and branches of mesenteric arteries. The resultant closed proximal colonic loop (perfused length 2 cm) was perfused with pH 7.0 Krebs buffer by using a peristaltic pump at 1 mL·min^{-1}. The perfusate, bubbled with 100% O$_2$, was kept at 37°C using a heated stirrer. To eliminate buffering by added compounds, which would over- or underestimate the pH-stat titration volume, flow-through pH and CO$_2$ electrodes (Lazar Research Laboratories) were connected to the perfusion loop, where pH and CO$_2$ concentration ([CO$_2$]) were simultaneously and continuously measured. As the input (perfusate) [CO$_2$] is ≈ 0, the effluent [CO$_2$] and pH were used to calculate the total CO$_2$

output equivalent to the secreted HCO_3^-. After stabilization following continuous perfusion of pH 7.0 Krebs buffer for about 30 min, the time was set as $t = 0$. The colonic loop was then perfused with pH 7.0 Krebs buffer from $t = 0$ min to $t = 10$ min (basal period). The perfusate was then changed to pH 7.0 Krebs buffer containing 5-HT (pH 7.0) from $t = 10$ to 35 min (experiment period), with or without inhibitors. At $t = 10$ min, the system was gently flushed so as to rapidly change the composition of the perfusate. Colonic HCO_3^- secretion was expressed as total CO_2 output ($\mu mol \cdot min^{-1} \cdot cm^{-1}$) calculated from the measured pH and $[CO_2]$ in the effluent solution as previously reported (Akiba et al., 2007).

Real-time RT-PCR and immunohistochemistry

Expression of 5-HT$_3$ and 5-HT$_4$ receptors in the colonic mucosa or submucosa was analysed by real-time RT-PCR as previously described (Akiba et al., 2009). The PCR primers of rat 5-HT$_3$ were sense (5′-tgctgaggctgtcagatcac) and antisense (5′-ccacgtccacaaactcattg), giving rise to a predicted 285 bp PCR product; the primers of 5-HT$_4$ were sense (5′-gagaccaaagcagccaagac-3′) and antisense (5′-aggaaggcacgtctgaaaga-3′), a predicted 212 bp product. β-actin was used as an internal control. The expression level was described as fold induction per 10^3 copies of β-actin by ΔC_t method.

In order to investigate the localization of 5-HT$_4$ receptors in the colonic mucosa, the proximal colonic segments were fixed with methanol for 10 min, followed by Zamboni's fixative overnight. Cryostat sections (up to 8 μm thick) were reacted with rabbit anti-5-HT$_4$ receptor antibody against extracellular epitope at 1:300 (Alomone Labs, Jerusalem, Israel), followed by incubation with the corresponding Alexa 488 secondary antibody (Molecular Probes, Grand Island, NY, USA). Negative controls were processed identically with the omission of the primary antibody or by incubation with primary antibody pre-absorbed with the immunizing peptide (30 μg·mL^{-1}) overnight at 4°C.

Whole-mount preparations of submucosal plexus were made in order to determine the location of NOS isoforms in the proximal colon. The submucosal layers of rat proximal colon were isolated and pinned on a silicone-filled Petri dish and immersed in Zamboni's fixative overnight at 4°C. The fixed submucosal preparations were washed in PBS to remove picric acid. Non-specific immunoreactions were blocked by preincubation with 0.5% normal donkey serum in PBS for 1 h at room temperature. Pre-blocked tissues were reacted with primary antibodies for NOS1 (ab72428; Abcam, Cambridge, MA, USA), NOS2 (sc650; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PGP9.5 (ab8189; Abcam) or glial fibrillary acidic protein (GFAP; GFAP-Go-Af1000; Frontier Institute Co., Ltd, Sapporo, Japan) diluted at 1:200 by blocking solution for 24 h at 4°C. After washing in PBS, fluorescence-conjugated antibodies were incubated for 2 h at room temperature. The tissues were counterstained with 4′,6-diamidino-2-phenylindole and mounted with EverBrite mounting medium (Biotium, Hayward, CA, USA). Immuno-fluorescence was imaged and captured using a confocal laser microscope (LSM710; Carl Zeiss GmbH, Jena, Germany).

Data analysis

Values are expressed as mean ± SE, with n indicating the number of animals. Statistical analysis was performed in GraphPad Prism 6 (La Jolla, CA, USA) using Student's t-test between two groups. In three or more groups, one- or two-way ANOVA was used based on the experimental design, followed by multiple comparisons with Fischer's least significant difference post hoc test. Differences were considered significant when P values < 0.05.

Materials

1400W was purchased from Cayman Chemical (Ann Arbor, MI, USA). GR113808, fluoxetine, S-nitroso-N-acetylpenicillamine (SNAP), BGC20-761, SB258719, N-iminoethyl-L-ornithine (L-NIO) and N^ω-propyl-L-arginine (NPA) were from Tocris Bioscience (Ellisville, MO, USA). 5-HT, ondansetron and other chemicals were from Sigma Chemical (St. Louis, MO, USA). 5-HT was dissolved in distilled water before each experiment. For stock solutions, indomethacin was dissolved in ethanol; TTX, 1400W and N^G-nitro-L-arginine methyl ester (L-NAME) were dissolved in distilled water; GR113808, ondansetron, fluoxetine, bumetanide and acetazolamide were dissolved in DMSO. Chemical concentrations used in this study were typically chosen based on concentrations used in comparable published studies.

Results

Effect of luminal 5-HT on I_{sc} in rat proximal colon

In Ussing-chambered mucosa-submucosa preparations, luminal application of 5-HT (100 μM) increased I_{sc}, accompanied by G_t increase, peaking within 5 min and sustained for more than 30 min (Figure 1A). Pretreatment with indomethacin (10 μM) significantly decreased basal I_{sc} from 59.0 ± 8.55 to 43.6 ± 3.53 μA·cm^{-2} ($P < 0.05$ vs. untreated tissues), but did not alter the luminal 5-HT-evoked I_{sc} increase. The combination of indomethacin and TTX (1 μM) further decreased the basal I_{sc} to 32.6 ± 2.21 μA·cm^{-2} ($P < 0.05$ vs. untreated tissues), but did not affect the response to 5-HT. Pretreatment with atropine (10 μM) did not change basal and luminal 5-HT-evoked ΔI_{sc}, indicating that COX-dependent, cholinergic or TTX-sensitive pathways were not involved in the increase in I_{sc} in response to luminal 5-HT (Figure 1B). To minimize the effects of endogenous prostaglandins and of neural reflexes, subsequent experiments were performed in the presence of indomethacin and TTX.

Ionic basis of luminal 5-HT-evoked I_{sc} increases

We next investigated the ionic components of the I_{sc} response to 5-HT with the inhibitor of the NKCC1 ion transporter, bumetanide, and the carbonic anhydrase inhibitor, acetazolamide. As shown in Figure 2A, the I_{sc} response to luminal 5-HT was decreased by serosal addition of bumetanide (0.1 mM) and further decreased by the addition of acetazolamide (0.2 mM) into the serosal and luminal baths. Bumetanide and acetazolamide decreased the I_{sc} response to 5-HT by 19 and
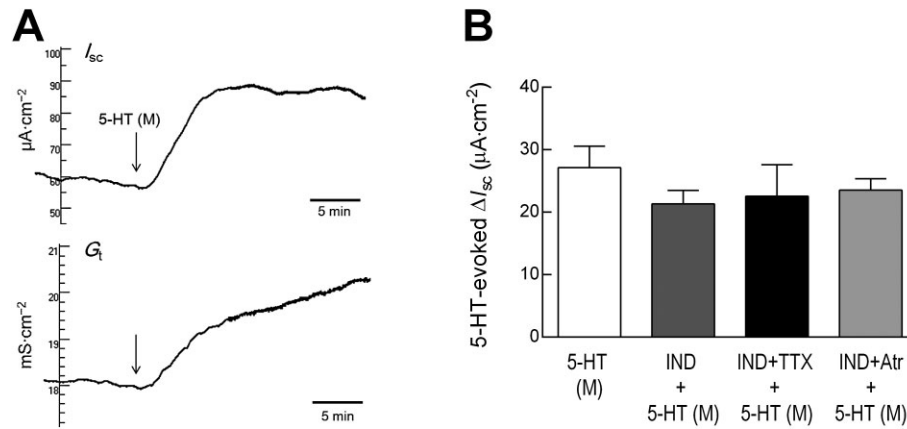
Figure 1

Luminal 5-HT-evoked increases in I_{sc} in rat proximal colon. (A) Representative I_{sc} and G_t traces illustrated that luminal (M) 5-HT (100 µM) gradually and simultaneously increased I_{sc} and G_t. (B) Luminal 5-HT-evoked ΔI_{sc} was determined in the presence or absence of indomethacin (IND, 10 µM) added to the serosal and luminal bathing solutions with TTX (1 µM) or atropine (Atr, 10 µM) added to the serosal bath. Inhibitors were added immediately after mounting tissues in the Ussing chamber and incubated during stabilization period. There was no significant difference between groups as determined by one-way ANOVA ($F = 0.55$, $P = 0.66$). $n = 5$.

58% respectively (Figure 2B). Benzolamide (Akiba *et al.*, 2006), an ecto-carbonic anhydrase inhibitor, had no effect on 5-HT-evoked I_{sc} changes (data not shown), suggesting that the inhibitory effect of acetazolamide on the I_{sc} response to 5-HT is due to the inhibition of cytosolic carbonic anhydrase activity. Bumetanide and acetazolamide inhibited the I_{sc} response independently of their order of application, indicating that the 5-HT-evoked increase in I_{sc} is due to anion secretion and that cytosolic HCO_3^- production is the principal component (as compared with basolateral NKCC-mediated Cl^- uptake) of the 5-HT-evoked I_{sc} increase in rat proximal colon.

Removal of Cl^- from both bathing solutions decreased basal I_{sc} and G_t by 32.9 ± 5.45 µA·cm^{-2} and 12.0 ± 1.52 mS·cm^{-2}, respectively ($n = 5$), indicating that the basal I_{sc} is generated by electrogenic Cl^- secretion in rat proximal colon. The decrease of basal I_{sc} ($\Delta I_{sc} = -9.7 \pm 3.92$ µA·cm^{-2}) and G_t ($\Delta G_t = -1.1 \pm 0.38$ mS·cm^{-2}) in HCO_3^--free conditions was significantly smaller than in Cl^--free buffer ($P < 0.05$, $n = 5$), indicating that electrogenic HCO_3^- transport contributed less to basal I_{sc} and G_t. The absence of Cl^- or HCO_3^- significantly reduced the I_{sc} responses to luminal 5-HT by 62 or 68% respectively (Figure 2C). These results suggest that the increase of I_{sc} in response to 5-HT is due either to electrogenic HCO_3^- or Cl^- secretion. Nonetheless, increased I_{sc} in response to carbachol was due mostly to Cl^- rather than HCO_3^- secretion, consistent with the known Ca^{2+}-mediated Cl^- secretory pathway in response to cholinergic stimuli (Figure 2D). These results suggest that 5-HT-evoked I_{sc} is principally generated by HCO_3^- secretion, in comparison to the predominant Cl^- secretion generated by carbachol.

Effect of luminal 5-HT on HCO_3^- secretion in parallel with increase in I_{sc}

In order to confirm HCO_3^- secretion in response to luminal 5-HT, parallel measurements of I_{sc} and total CO_2 output were performed as shown in Figure 3. With HCO_3^--free HEPES buffer in the luminal bath, luminal 5-HT increased I_{sc} as the

same extent as Krebs–Ringer buffer described in Figure 1. The Δtotal CO_2 output was increased commensurately with the increase of I_{sc}. Serosal addition of bumetanide slightly decreased I_{sc}, with no effect on Δtotal CO_2 output, whereas luminal and serosal addition of acetazolamide dramatically decreased I_{sc}, concomitant with remarkable decrease of Δtotal CO_2 output. These results confirmed that the luminal 5-HT-induced increase in I_{sc} was equivalent to luminal HCO_3^- secretion, and that acetazolamide-sensitive I_{sc} changes following luminal 5-HT-induced increase of I_{sc} represented the acetazolamide-sensitive HCO_3^- secretion into the lumen.

Effect of 5-HT receptor antagonists on luminal 5-HT-evoked anion secretion

In rat colon, serosal 5-HT-evoked secretion is mediated by a ligand-gated ion channel 5-HT$_3$ receptor and a G$_s$-coupled 5-HT$_4$ receptor (Bunce *et al.*, 1991; Ning *et al.*, 2004; Day *et al.*, 2005; Yang *et al.*, 2010). We therefore examined the effect of the selective 5-HT$_3$ antagonist ondansetron (10 µM) or the selective 5-HT$_4$ antagonist GR113808 (1 µM) on luminal 5-HT-evoked secretion. Pretreatment with luminal ondansetron or GR113808 did not change the basal I_{sc} or G_t. GR113808, but not ondansetron, significantly reduced the response to luminal 5-HT by 73% (Figure 4A). The 5-HT$_6$ and 5-HT$_7$ receptor subtypes are also identified as G$_s$-coupled receptors (Baker *et al.*, 1998; Zhang *et al.*, 2003). The selective 5-HT$_6$ antagonist BGC20-761 (1 µM) or the selective 5-HT$_7$ antagonist SB258719 (10 µM) did not affect 5-HT-evoked secretion (Figure 4A). As K$_i$ values of BGC20-761 and SB258719 are 20 and 30 nM, respectively, and SB258719 is 100-fold more selective for 5-HT$_7$ than for other receptor subtypes (Forbes *et al.*, 1998), these concentrations are likely to be ideal for selective inhibition of each target receptor. We also confirmed that GR113808 inhibited the response to luminal 5-HT in a dose-dependent manner (Figure 4B), indicating that luminal 5-HT-evoked anion secretion is mainly mediated by luminal 5-HT$_4$ receptors.
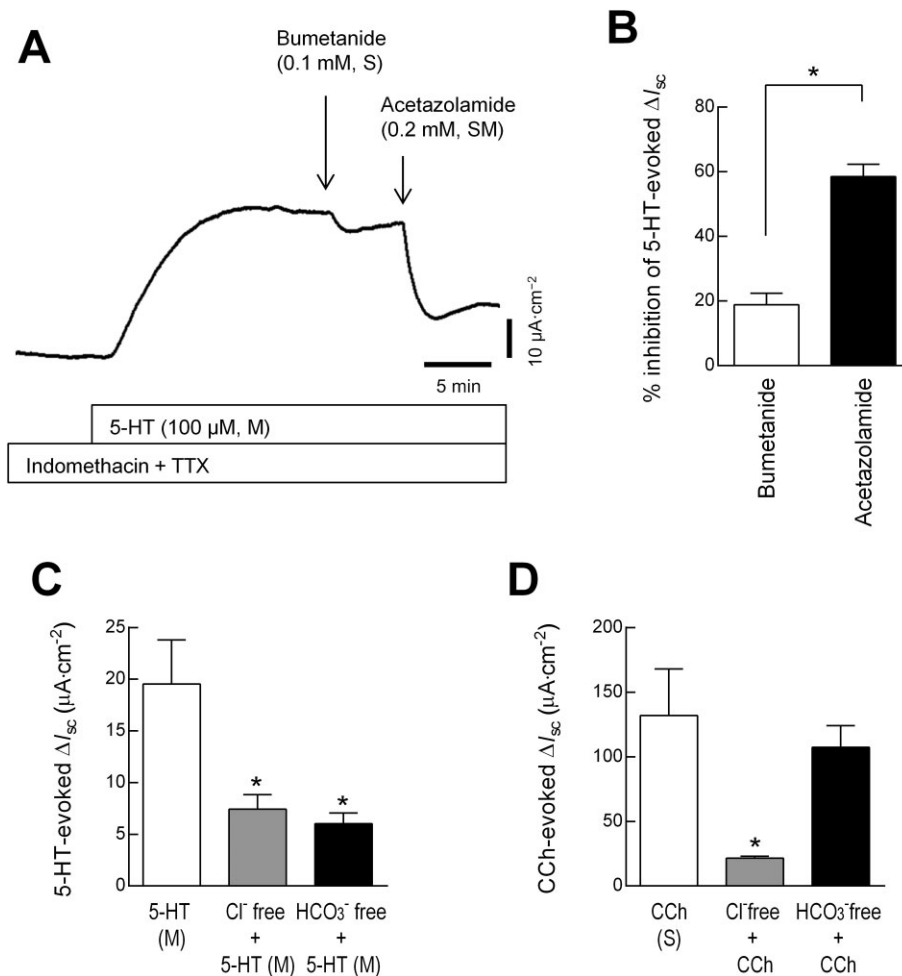
Figure 2

Contribution of anion secretion to luminal 5-HT-evoked I_{sc} increase. (A) A representative time course of I_{sc} illustrated that luminal 5-HT increased I_{sc} over a sustained period. Further serosal addition (S) of bumetanide followed by acetazolamide into the serosal and luminal baths (SM) decreased 5-HT-evoked I_{sc}. (B) Inhibitory rate of 5-HT-evoked ΔI_{sc} by bumetanide or acetazolamide. The application order of inhibitors was randomized. *$P < 0.05$. $n = 6$. (C, D) The effect of Cl^- or HCO_3^- depletion on 5-HT-evoked ΔI_{sc} (C) and on CCh-evoked ΔI_{sc} (D). After the stabilization period, the serosal and luminal bathing solutions were replaced with Cl^--free or HCO_3^--free buffers. Fifteen minutes after the replacement, 5-HT (100 µM) was added to the luminal bath followed by CCh (10 µM) into the serosal bath. *$P < 0.05$ versus each control group (open bar) determined by one-way ANOVA (C, $F = 7.82$; D, $F = 6.36$); $n = 5$.

Effect of luminal 5-HT$_4$ receptor agonists on I_{sc} in rat proximal colon

To further confirm the contribution of 5-HT$_4$ receptors to colonic anion secretion, we investigated the effects of the selective 5-HT$_4$ agonists cisapride and tegaserod in the presence of indomethacin and TTX. Luminal application of cisapride (1–100 µM) dose dependently increased I_{sc}, temporally resembling the response to luminal 5-HT. Pretreatment with luminal GR113808 (1 µM) completely abolished the response to luminal cisapride (100 µM) (Figure 5A). Luminal application of tegaserod (0.1–10 µM) also dose-dependently increased and sustained I_{sc} (Figure 5B).

Effect of a selective 5-HT uptake inhibitor (SSRI) on 5-HT-evoked secretion

The 5-HT transporter (SERT), expressed in intestinal epithelial cells, locally affects the mucosal actions of 5-HT (Wade et al.,

1996). To test the hypothesis that inhibition of SERT would modulate luminal 5-HT-evoked secretion, we used the fluoxetine, which inhibits 5-HT uptake in the intestinal Caco2 cell line with IC$_{50}$ = 0.02 µM (Martel et al., 2003). Luminal or serosal pretreatment with fluoxetine (0.1 µM) enhanced 5-HT-evoked anion secretion (Figure 6), consistent with the presence of the 5-HT transporter on the apical and basolateral membrane of colonocytes.

Secretory response to luminal 5-HT in mucosal preparations

In muscle- and submucosa-stripped mucosal preparations, which excluded submucosal and myenteric plexuses, the serosal addition of TTX (1 µM) decreased basal I_{sc} by 6.7 ± 1.63 µA·cm^{-2} ($n = 5$), indicating that neural reflexes remain in the lamina propria, consistent with previous reports (Bridges et al., 1986). In the presence of indomethacin and TTX, basal
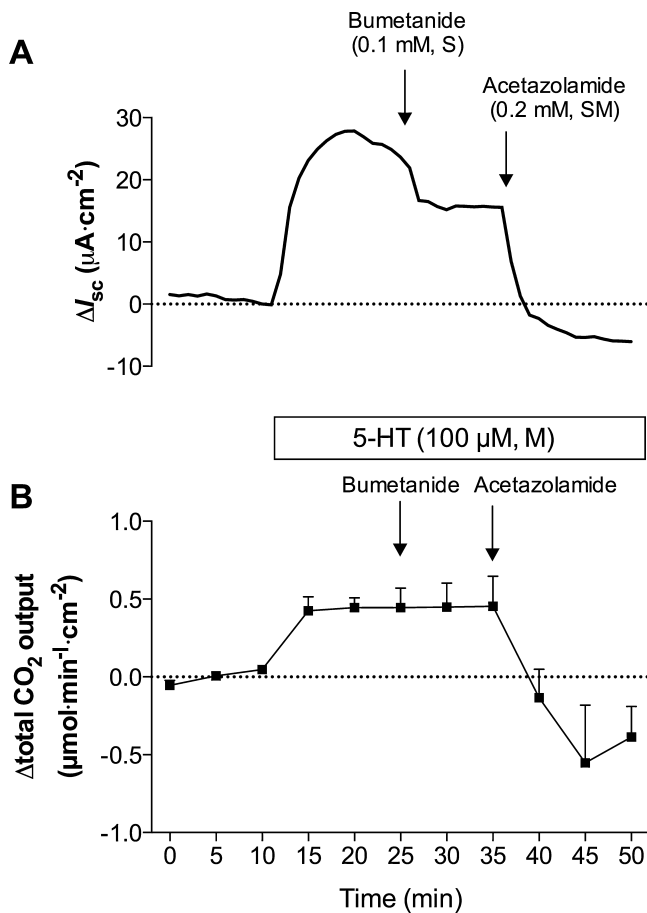
Figure 3

Effect of 5-HT, bumetanide and acetazolamide on HCO_3^- secretion in Ussing chambers. Changes of I_{sc} and total CO_2 output into the luminal bath were simultaneously measured and results were described as the differences (Δ) from the averaged values at $t = 0–10$ min. After 10 min basal period ($t = 0–10$ min), 5-HT (100 µM) was added to luminal bath at $t = 10$ min, followed by bumetanide (0.1 mM) to serosal bath at $t = 25$ min and acetazolamide (0.2 mM) to luminal and serosal baths at $t = 35$ min. A representative time course of ΔI_{sc} (A) and means ± SEM of Δtotal CO_2 output (B) showed that luminal 5-HT increased I_{sc} and total CO_2 output in parallel, in an acetazolamide-sensitive manner. $n = 6$.

I_{sc} (37.7 ± 2.18 µA·cm^{-2}, $n = 33$) and G_t (25.3 ± 1.38 mS·cm^{-2}) were similar to those in mucosa-submucosa preparations (I_{sc}, 30.2 ± 1.75 µA·cm^{-2} and G_t, 22.6 ± 1.29 mS·cm^{-2}; $n = 39$), with no significant difference in basal potential difference and resistance. The increase in I_{sc} induced by luminal 5-HT was significantly larger in mucosal preparations than that in mucosa-submucosa preparations at all 5-HT doses tested (Figure 7A). Luminal 5-HT-evoked I_{sc} in mucosal preparations was sustained, and rapidly decreased by addition of bumetanide (0.1 mM) followed by acetazolamide (0.2 mM) (Figure 7B). The acetazolamide-sensitive I_{sc} component (38%) was significantly smaller, whereas the bumetanide-sensitive I_{sc} component of the response to 5-HT in mucosal preparations (44%) was larger than that in mucosa-submucosa preparations (Figure 7C). Thus, the calculated ΔI_{sc} value of the
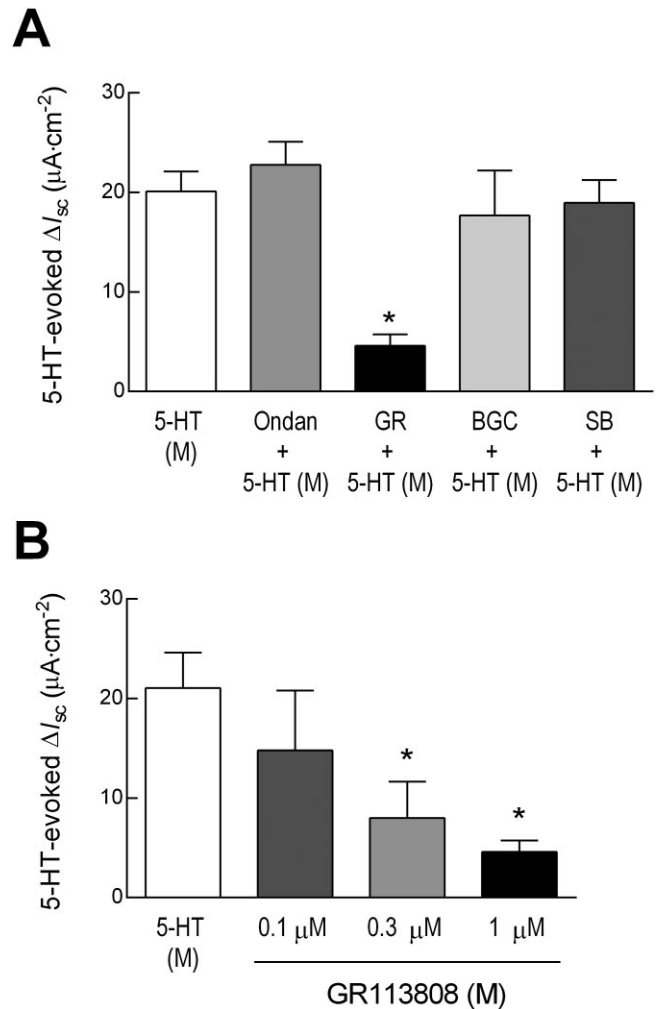

Figure 4

Effect of selective 5-HT receptor antagonists on 5-HT-evoked I_{sc} increase. (A) Luminal 5-HT-evoked ΔI_{sc} was measured in the presence or absence of the antagonist for 5-HT$_3$ ondansetron (10 µM), for 5-HT$_4$ GR113808 (GR, 1 µM), for 5-HT$_6$ BGC20-761 (BGC, 1 µM) or for 5-HT$_7$ SB 258719 (SB, 10 µM). The antagonist was added into the luminal bath 10 min before the luminal application of 5-HT (100 µM). $n = 5$ for each inhibitor and $n = 8$ for control. *$P < 0.05$ versus 5-HT group by one-way ANOVA ($F = 6.81$). (B) Dose-dependent inhibition of luminal 5-HT$_4$ antagonist of the response to 5-HT. Each concentration of GR113808 was individually added into the luminal bath 10 min before the addition of 5-HT (100 µM). $n = 6$. *$P < 0.05$ versus 5-HT group by one-way ANOVA ($F = 3.37$).

bumetanide-sensitive component corresponded to the difference of the responses to 5-HT between the mucosal and mucosa-submucosal preparations (Figure 7D). There was no difference in the magnitude of the acetazolamide-sensitive components between the mucosal preparations and the mucosa-submucosa preparations.

We also investigated the effect of bumetanide on basal I_{sc} in mucosa-submucosa or mucosal preparations. After the stabilization period, the serosal application of bumetanide (0.1 mM) did not change in the mucosa-submucosa preparations, whereas basal I_{sc} was decreased in the mucosal
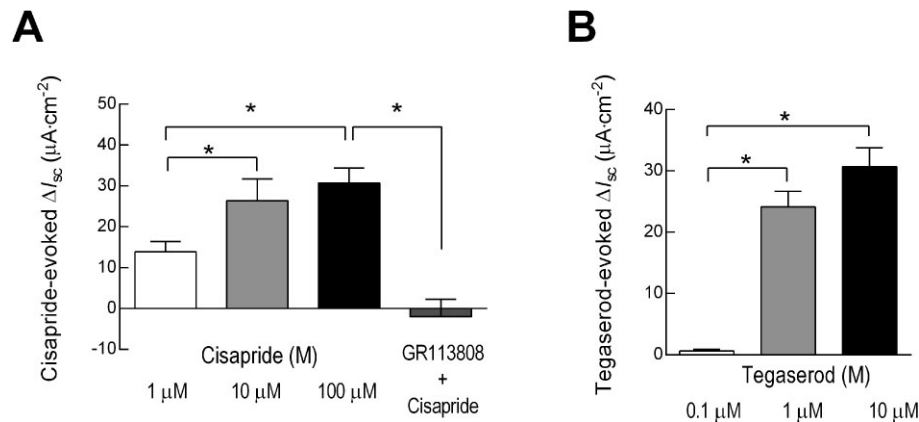
A

B

Figure 5
Effect of luminal 5-HT$_4$ receptor agonists on I_{sc}. (A, B) Each concentration of cisapride (A) or tegaserod (B) was individually added into the luminal bath (M) and I_{sc} changes were measured in the presence of TTX and indomethacin. Both cisapride ($F = 13.2$ by one-way ANOVA) and tegaserod ($F = 47.4$) increased I_{sc} in a dose-dependent manner. Pretreatment with luminal GR113808 (1 μM) abolished the response to cisapride (100 μM). *$P < 0.05$. $n = 5$.
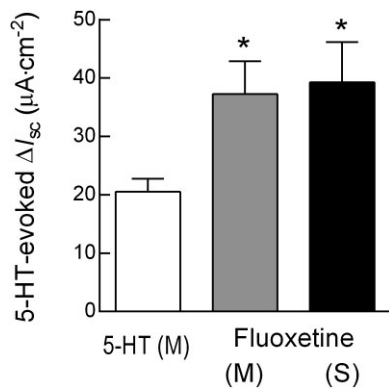

Figure 6
Effect of an SSRI on 5-HT-evoked I_{sc} increase. Fluoxetine (0.1 μM) was added into the luminal (M) or serosal (S) bath 10 min before the luminal application of 5-HT (100 μM). $n = 8$. There were significant differences among groups ($F = 3.84$ by one-way ANOVA). *$P < 0.05$ versus 5-HT group.

preparations (ΔI_{sc}, -8.0 ± 2.9 μA·cm^{-2}; $n = 6$). These results support the hypothesis that the submucosa suppresses basal and luminal 5-HT-evoked NKCC1-sensitive Cl$^-$ secretion.

Involvement of NO signalling in 5-HT-evoked anion secretion

We hypothesized that NO may mediate the secretory inhibition attributable to the submucosa. Serosal pretreatment with the non-selective NOS inhibitor L-NAME (0.1 mM) did not alter basal I_{sc} either in mucosa-submucosa or mucosa-only preparations. L-NAME significantly enhanced 5-HT-evoked secretion in mucosa-submucosa preparations (Figure 8A), to the same extent as that in mucosal preparations (Figure 8B). Conversely, L-NAME treatment did not alter the response to

luminal 5-HT in mucosa-only preparations (Figure 8B), suggesting that NO generated in the submucosa principally suppresses 5-HT-evoked Cl$^-$ secretion.

The serosal addition of the NO donor SNAP increased basal I_{sc}, whereas the luminal addition of SNAP (0.1 or 1 mM) decreased I_{sc} in mucosa-submucosa preparations, consistent with NO inhibiting anion secretion in proximal colonic mucosa rather than in the submucosa (Figure 8C). Luminal 5-HT-evoked I_{sc} gradually returned to basal values after the luminal addition of SNAP in a dose-dependent manner (Figure 8D). SNAP (1 mM) treatment decreased 5-HT-evoked I_{sc} by 48%. These results suggest that submucosa-derived NO suppresses luminal 5-HT-evoked anion secretion.

Selective inhibitors for NOS1 (NPA, 10 μM) and NOS2 (1400W, 10 μM), and the relatively selective inhibitor for NOS3 (L-NIO, 100 μM), were used to identify the predominant anti-secretory NOS isoform. The inhibitor concentrations used were chosen based on the concentrations used in earlier studies conducted in comparable preparations (MacEachern *et al.*, 2011). Serosal NPA or 1400W, but not L-NIO, significantly enhanced the response to luminal 5-HT (Figure 8E), indicating that submucosal NOS1 and NOS2 primarily affected 5-HT-evoked anion secretion.

The downstream pathway of NO was investigated to confirm the inhibitory effect of NO signalling on luminal 5-HT-evoked secretion in mucosa-submucosa preparations. We used the soluble GC (sGC) inhibitor NS-2028 (10 μM) and the cGMP-dependent PK (PKG) inhibitor KT-5823 (10 μM), which inhibits NO- and cGMP-induced ion transport, respectively, at this concentration (Cho *et al.*, 1999; Stoner and Kellum, 2001). Luminal application of NS-2028 did not alter basal I_{sc}, but significantly enhanced the response to luminal 5-HT (Figure 8F). The basal I_{sc} or luminal 5-HT-evoked I_{sc} increase was not altered by pretreatment with KT-5823 in the serosal and luminal baths (Figure 8G). These results suggest that NO suppresses the luminal 5-HT-evoked secretion via sGC activation, but independent of PKG activation.
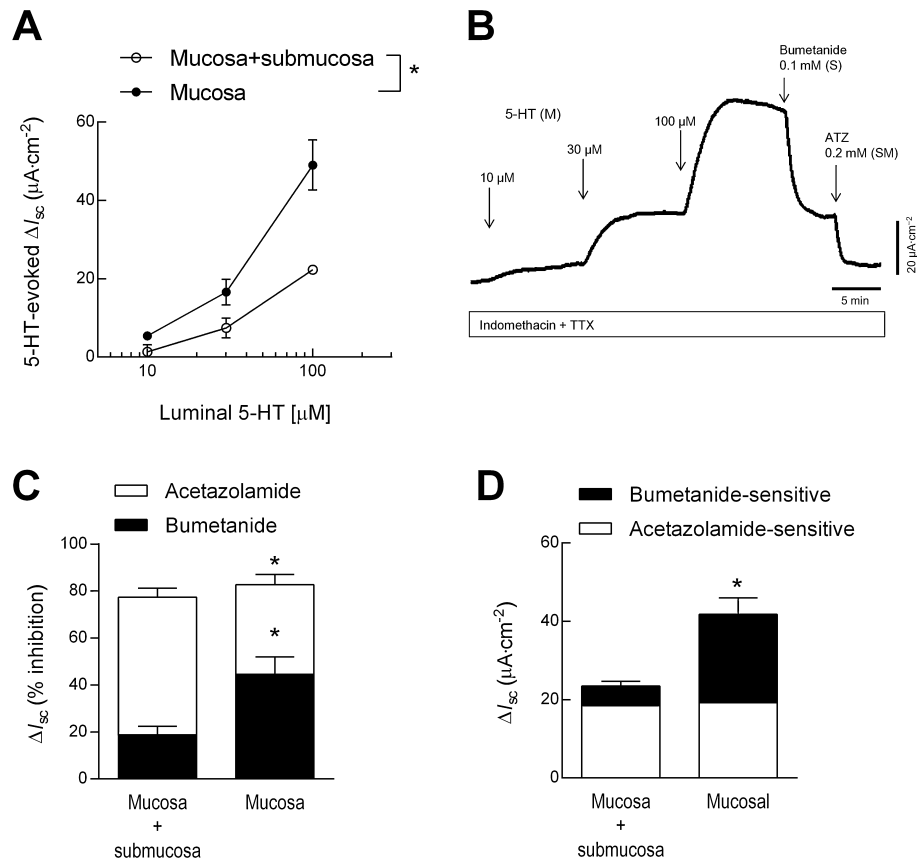
Figure 7

Comparison of luminal 5-HT-evoked increase in I_{sc} with or without submucosa present. (A) Dose dependency of 5-HT-evoked ΔI_{sc} in mucosa-submucosa or mucosal preparations. 5-HT was cumulatively added into the luminal bath and ΔI_{sc} was determined as the difference from basal I_{sc}. There were significant differences between the two types of preparation ($F = 53.3$) and among 5-HT concentrations ($F = 25.4$) by two-way ANOVA. $n = 5$. *$P < 0.05$. (B) A representative trace of I_{sc} in the mucosal preparation illustrated that luminal application of 5-HT increased I_{sc} in a stepwise manner. Further addition of bumetanide followed by acetazolamide returned I_{sc} to basal level. (C) Inhibitory rates of 5-HT (100 μM)-evoked ΔI_{sc} by bumetanide and acetazolamide. (D) Bumetanide- or acetazolamide-sensitive I_{sc} portions of luminal 5-HT-evoked ΔI_{sc}. $n = 6$. *$P < 0.05$ versus mucosa-submucosa preparations.

Segmental differences in effect of luminal 5-HT in rat colon

As the secretory response to luminal compounds varies by colonic segment (Nobles *et al.*, 1991; Kaji *et al.*, 2012), we next investigated the segmental differences in the response to luminal 5-HT and the contribution of NO. In the presence of indomethacin and TTX, luminal 5-HT (100 μM) increased I_{sc} in all colonic segments with a decreasing gradient from proximal to distal (Figure 9A). Unlike the proximal colon, L-NAME treatment failed to enhance the response to luminal 5-HT in the mid or distal colon (Figure 9B), indicating that NO-sensitive secretory pathway was restricted to the proximal colon.

Effect of amiloride on luminal 5-HT-evoked I_sc in rat colon

As an I_{sc} increase could be also generated by cation absorption, we next investigated the involvement of the ENaC channel on luminal 5-HT-evoked I_{sc} increases using the semi-selective ENaC inhibitor amiloride (100 μM). Pretreatment

with luminal amiloride decreased basal I_{sc} by 3.2 ± 0.6 μA·cm^{-2} ($n = 5$) in the distal colon, but had no effect in the mid and proximal colon. Luminal 5-HT (100 μM)-evoked I_{sc} increases were significantly enhanced by amiloride in all colonic segments with no segmental difference, rather than inhibiting 5-HT-evoked I_{sc} increase (Figure 9C), suggesting that 5-HT-evoked I_{sc} increase is not due to Na$^+$ absorption, whereas inhibition of ENaC enhances 5-HT-induced anion secretion by uncertain mechanisms.

Effects of luminal 5-HT on colonic HCO_3^- secretion in vivo

In order to confirm our assumption that luminal 5-HT stimulates colonic HCO_3^- secretion, colonic HCO_3^- secretion was measured with pH and CO_2 electrodes in a proximal colonic loop in anaesthetized rats. Colonic HCO_3^- secretion expressed as total CO_2 output was stable during perfusion with pH 7.0 Krebs buffer (Figure 10). Luminal perfusion of 5-HT (100 μM) significantly increased the rate of total CO_2 output, abolished by co-perfusion with GR113808 (10 μM), but not by ondansetron (10 μM).
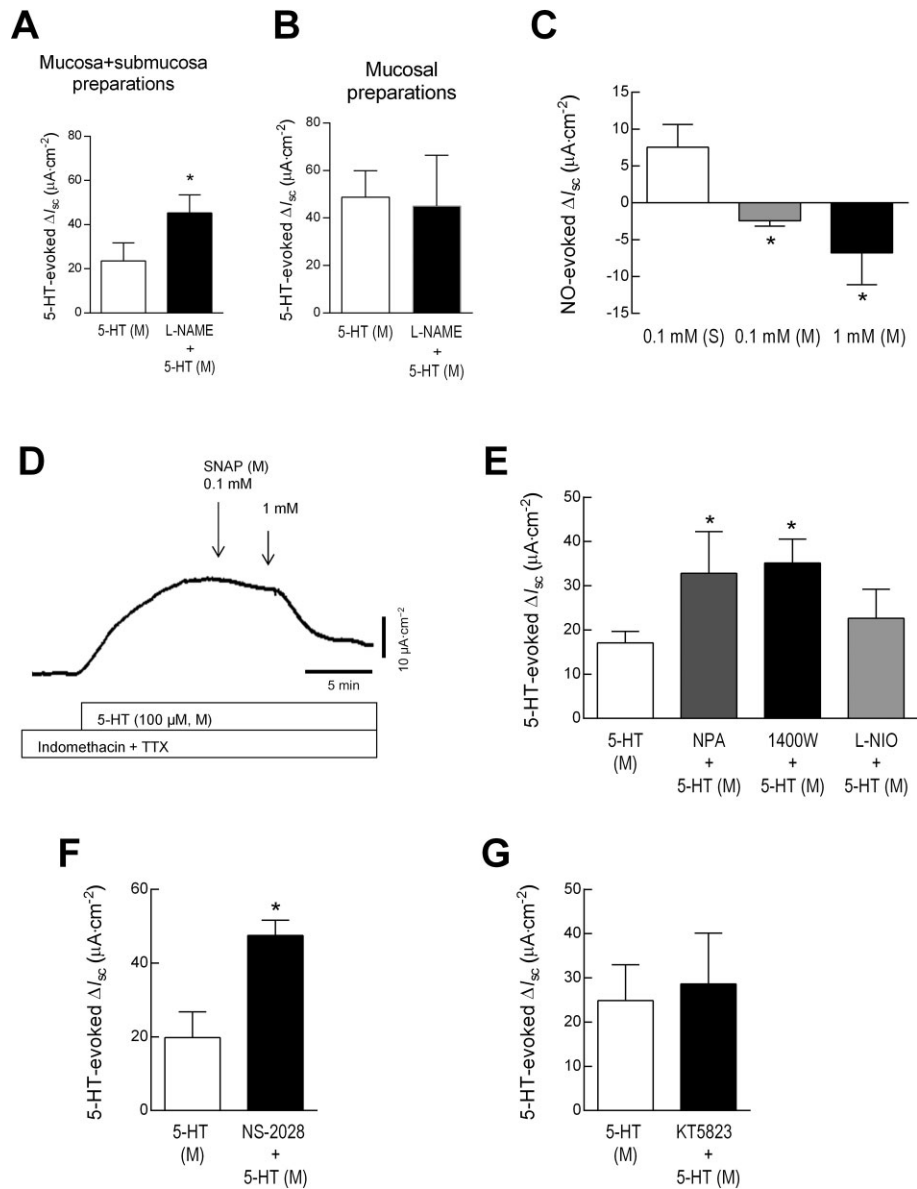
Figure 8

Inhibition of 5-HT-evoked I_{sc} increases by NO signalling. (A, B) Effect of L-NAME on luminal 5-HT-evoked ΔI_{sc} in mucosa-submucosa (A) or mucosal (B) preparations. L-NAME was added to the serosal bath 20 min before the application of 5-HT (100 µM). $n = 5$. *$P < 0.05$ versus 5-HT group. (C) Effects of SNAP on basal I_{sc} in the presence of TTX and indomethacin in mucosa-submucosa preparations. There were significant differences among the groups ($F = 5.61$ by one-way ANOVA). $n = 5$. *$P < 0.05$ versus 0.1 mM (S). (D) A representative I_{sc} trace in mucosa-submucosal preparation illustrated that luminal 5-HT increased I_{sc} and further addition of luminal SNAP decreased the 5-HT-evoked I_{sc}. (E) Effects of selective NOS isozyme inhibitors on luminal 5-HT-evoked ΔI_{sc} in mucosa-submucosa preparations. The selective inhibitor for NOS1 NPA (10 µM), for NOS2 1400W (10 µM) or for NOS3 L-NIO (100 µM) was added into the serosal bath 20 min before the application of 5-HT (100 µM). There were significant differences among groups ($F = 3.24$ by one-way ANOVA). $n = 6$ for inhibitors, $n = 15$ for control. *$P < 0.05$ versus 5-HT group. (F) Effect of the sGC inhibitor on response to luminal 5-HT. NS-2028 (10 µM) was added into the luminal bath 10 min before the application of 5-HT (100 µM). $n = 5$. *$P < 0.05$ versus 5-HT group. (G) Effect of PKG inhibitor on response to luminal 5-HT. KT-5823 (10 µM) was added into the serosal and luminal baths 10 min before the application of 5-HT (100 µM). $n = 6$.

Expression of 5-HT$_3$ and 5-HT$_4$ receptors in the colonic mucosa

We tested the hypothesis that the segmental heterogeneity in luminal 5-HT-induced I_{sc} changes would reflect the heterogeneity of 5-HT$_4$ receptor expression, and that 5-HT$_4$ receptors are localized on the apical membrane of rat proximal colon.

Real-time RT-PCR results confirmed that 5-HT$_4$ receptors were predominantly expressed in the colonic mucosa, compared with the expression of 5-HT$_3$ receptors, with significantly higher expression in the proximal colonic mucosa than in the mid or distal colonic mucosa (Figure 11A). In contrast, 5-HT$_3$ receptors were highly expressed in the submucosal
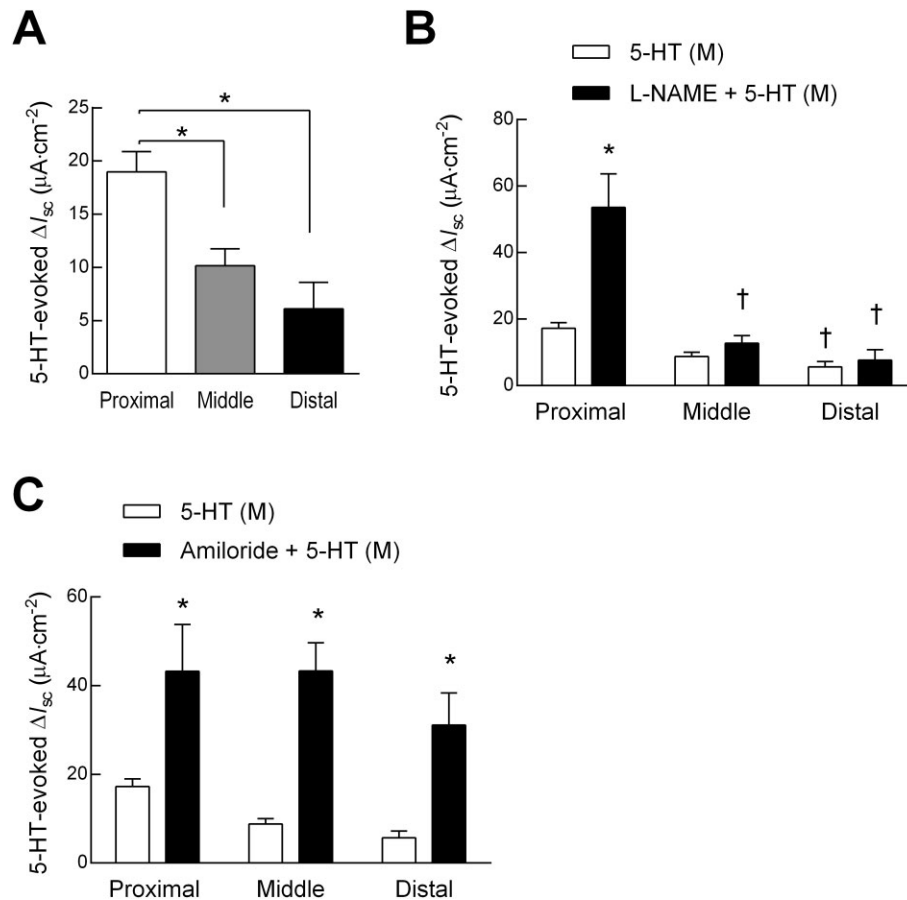
Figure 9

Segmental heterogeneity of 5-HT-evoked I_{sc} increases in rat colon. (A) 5-HT (100 μM) was added to the luminal bath with ΔI_{sc} measured in mucosa-submucosa preparations of proximal, mid or distal colon. There were significant differences ($F = 10.5$ by one-way ANOVA). $n = 5$. *$P < 0.05$ versus proximal colon. (B, C) Luminal 5-HT-evoked ΔI_{sc} was measured in the presence or absence of L-NAME (0.1 mM, B) or amiloride (0.1 mM, C). L-NAME was added into the serosal bath, whereas amiloride was added into the luminal bath 20 min before the application of 5-HT (100 μM). There were significant differences among segments (B: $F = 27.7$, C: $F = 3.78$) and between treatment groups (B: $F = 14.0$, C: $F = 61.1$) as detected by two-way ANOVA. $n = 5$. *$P < 0.05$ versus 5-HT group in each segment, +$P < 0.05$ versus proximal colon in each treatment group.

layer without segmental differences (Figure 11B). 5-HT_4 receptors were also equally expressed in the submucosa across the segments. These results support our hypothesis that segmental differences in the 5-HT_4-mediated secretory response to luminal 5-HT correspond to segmental differences in 5-HT_4 receptor expression.

In histological sections of proximal colon, the 5-HT_4 receptor was immunolocalized to the epithelial cells, especially the apical membrane (Figure 11C), whereas preabsorption of 5-HT_4 antibody by immunized peptide abolished the staining (Figure 11D). These results suggest the functional expression of 5-HT_4 receptor on the apical membrane, consistent with luminal 5-HT-induced signal inhibited by luminal 5-HT_4 receptor antagonism.

Expression of NOS isozymes in the submucosal layer

Immunohistochemistry of whole-mount submucosal preparations of rat proximal colon localized NOS-1-positive neurons to the submucosal plexus, confirmed with the neural marker PGP9.5 (Figure 12A–C). In contrast, NOS-2 was located in GFAP-positive glial cells surrounding submucosal neurons (Figure 12D–F). No colocalization of NOS1 with NOS2 was observed in the submucosal preparations (Figure 12G–I).

Discussion

We have demonstrated that luminal 5-HT-evoked anion secretion, which was predominantly HCO_3^- secretion in the proximal colon, was mediated by 5-HT_4 receptor activation, independent of neural and COX pathways. Furthermore, 5-HT-evoked anion secretion was enhanced by the removal of submucosa or by NOS inhibition. NO release followed by sGC activation suppressed luminal 5-HT-evoked Cl^- secretion. I_{sc} responses to luminal 5-HT occurred predominantly in the proximal colon rather than in the mid and distal colon. Our findings indicate that a novel luminal 5-HT_4-mediated HCO_3^- secretory pathway and a submucosal inhibitory pathway are present in the rat proximal colon.
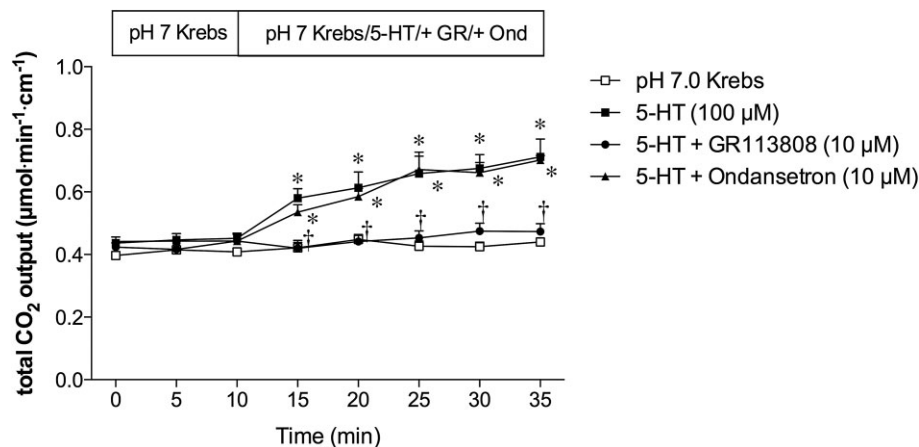
Figure 10

Effect of luminal perfusion of 5-HT on colonic HCO_3^- secretion *in vivo*. HCO_3^- secretory rate was measured as total CO_2 output by flow-through pH and CO_2 electrodes. The proximal colonic loop was perfused with 5-HT from $t = 10$ min with or without ondansetron or GR113808. $n = 6$. *$P < 0.05$ versus pH 7.0 Krebs group, †$P < 0.05$ versus 5-HT group. There were significant differences among time points ($F = 39.8$) and between treatment groups ($F = 14.6$) as detected by two-way ANOVA.

One of the well-studied 5-HT receptor subtypes, 5-HT_4, which is ubiquitously and abundantly expressed in the plasma membrane of colonocytes, has protective functions, including the attenuation of visceral hypersensitivity and the augmentation of mucus and anion secretion (Hoffman *et al.*, 2012), consistent with our data. Immunolocalized to the apical membrane of the proximal colonic epithelial cells, 5-HT_4 receptors mediated HCO_3^- secretion *in vitro* (in Ussing chamber preparations) and *in vivo*, in response to luminal 5-HT, cisapride or tegaserod. These studies indicate that functional 5-HT_4 receptors are present on the apical membrane of the epithelial cells lining rat proximal colon, whereas other colonic segments were less responsive to luminal 5-HT, paralleled by higher expression of 5-HT_4 receptor in the proximal in comparison with the mid and distal colonic segments according to real-time RT-PCR. Although the contribution of 5-HT_3 receptors to GI function is well known, the expression of 5-HT_3 receptors was relatively low in scraped colonic mucosa in all segments, but high in the submucosal layer. These results suggest disparate functions for the 5-HT_4 and 5-HT_3 receptors in the mucosa and in the submucosa respectively.

Luminal 5-HT- or 5-HT_4 agonist-evoked I_{sc} increases were not affected by TTX or by atropine in muscle-stripped preparations of rat proximal colon. In contrast, luminal tegaserod in whole-mounted mouse distal colon increased I_{sc} via a TTX-sensitive pathway (Hoffman *et al.*, 2012), suggesting that the myenteric plexus might be involved in luminal 5-HT-evoked anion secretion. The rat caecum is a major site of bacterial fermentation, producing high luminal concentrations of SCFAs to which the cecal mucosa is constantly exposed, although the pH of luminal content is maintained at ~7 throughout the caudad large intestine (Annison *et al.*, 2003). Our data thus support the hypothesis that luminal pH is maintained by HCO_3^- secretion in the proximal colon. Proximal region-specific electrogenic HCO_3^- secretion is likely to be mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel, which is expressed in the caecum and proximal colon, but not in the distal colon (Jakab *et al.*, 2011). Yet, none of the available CFTR inhibitors, such as $CFTR_{inh}$-172, glibenclamide or PPQ102, had any effect on 5-HT-evoked I_{sc} increases in the Ussing chamber studies (data not shown). Because Cl^- depletion reduced the response to luminal 5-HT to a greater extent than NKCC1 inhibition, a Cl^--dependent HCO_3^- secretion pathway may be present. The anion exchanger SLC26A3 (down-regulated in adenoma; DRA) is likely to contribute to colonic HCO_3^- secretion (Talbot and Lytle, 2010). Still, DRA, an exchanger whose electrogenicity has not been confirmed in intact tissues, is highly expressed in the distal, but not in the proximal colon. Therefore, the identity of the 5-HT-induced electrogenic HCO_3^- transporter or channel remains to be clarified.

Our results are also consistent with the presence of a negative feedback system provided by SERT expressed on the luminal and serosal poles of enterocytes and on subepithelial tissues. The presence of a 5-HT reuptake mechanism on the luminal pole underscores the importance of luminal 5-HT signalling, perhaps for luminal pH homeostasis, at least in the proximal colon. Similarly, 5-HT is released into the duodenal lumen in response to acid exposure (Kellum *et al.*, 1983; Smith *et al.*, 2006), suggesting that luminal 5-HT signalling may protect the mucosa from acid injury.

We demonstrated that L-NAME treatment increased luminal 5-HT-evoked anion secretion in mucosa-submucosa preparations, consistent with the anti-secretory effect of NO. The prosecretory effect of luminal 5-HT and the inhibitory effect of NO were significantly greater in the proximal colon compared with mid and distal colon, again indicating the segmental heterogeneity of colonic HCO_3^- secretion. Furthermore, luminal addition of the NO donor SNAP decreased basal and 5-HT-evoked secretion in the presence of TTX and indomethacin. Consistent with our results, cAMP-dependent anion secretion is attenuated by endogenous NOS2-derived NO, accumulated in the presence of inflammation or radiation (Asfaha *et al.*, 1999; Freeman and MacNaughton, 2000; MacEachern *et al.*, 2011). In the mouse distal colon, myen-
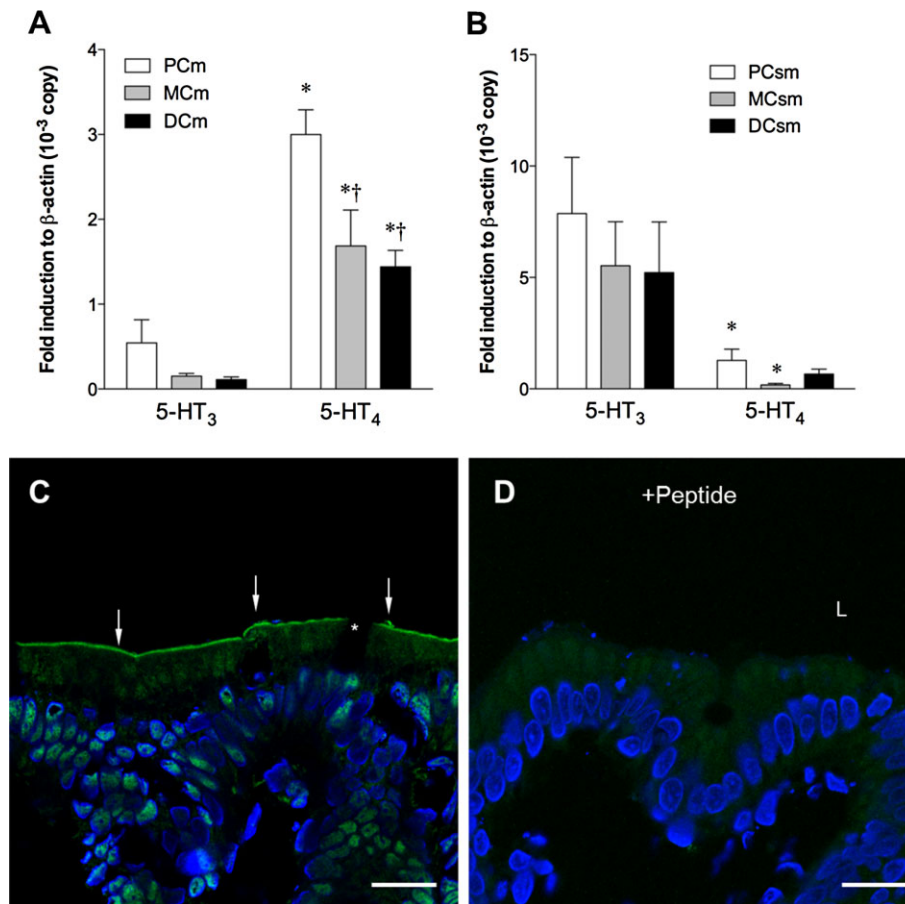
Figure 11

Expression of 5-HT$_3$ and 5-HT$_4$ receptors in rat colonic mucosa. (A) mRNA expression levels of 5-HT$_3$ and 5-HT$_4$ in the scraped colonic mucosa were compared among proximal (PCm), mid (MCm) and distal (DCm) segments. There were significant differences among colonic segments ($F = 9.25$) and between 5-HT$_3$ and 5-HT$_4$ ($F = 75.3$) as detected by two-way ANOVA. *$P < 0.05$ versus 5-HT$_3$ in the corresponding segment, †$P < 0.05$ versus proximal colon in each receptor group. (B) mRNA expression levels of 5-HT$_3$ and 5-HT$_4$ in the submucosa were compared among proximal (PCsm), mid (MCsm) and distal (DCsm) segments. There was no significant difference among colonic segments ($F = 0.72$) whereas there was significant difference between 5-HT$_3$ and 5-HT$_4$ ($F = 17.3$) as detected by two-way ANOVA. *$P < 0.05$ versus 5-HT$_3$ in the corresponding segment. (C) Immunoreactivity for 5-HT$_4$ receptor (green, arrows) was localized on the apical membrane of surface epithelial cells in the proximal colonic mucosa, but not of the goblet cells (*). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). (D) Lack of immunoreactivity with pre-incubated 5-HT$_4$ antibody with antigen peptide. L, lumen. Bar = 20 μm.

teric ganglia decrease cholinergic secretion via NOS1- and NOS2-derived NO (MacEachern *et al.*, 2011). In contrast, serosal 5-HT-evoked secretion is partly mediated by NO in Ussing-chambered colon (Kadowaki *et al.*, 1996; Stoner *et al.*, 2000). Serosal NO, exogenously generated by sodium nitroprusside (SNP) stimulated colonic anion secretion depending on neural and COX activation pathways (Tamai and Gaginella, 1993; Wilson *et al.*, 1993). As the removal of the submucosa mimicked the effect of NOS inhibition, endogenous NO is probably released by the submucosal compartment under basal conditions. We provided data supporting the distinct expression of NOS1 and NOS2 in the submucosal plexus of rat proximal colon. Consistent with previous reports, NOS1 was localized to submucosal neurons (Ekblad *et al.*, 1994) with NOS2 localized to the enteric glia (Green *et al.*, 2004). NOS3 is normally ubiquitously expressed in the

vascular endothelium of submucosa (Takahashi *et al.*, 1997), although a NOS3 inhibitor had no effect on luminal 5-HT-evoked anion secretion. Taken together, these results suggest that the effects of NO will vary according to cell type, location and physiological/pathological concentrations.

Although inhibition of sGC enhanced luminal 5-HT-evoked secretion, the inhibition of its downstream enzyme PKG had no effect, indicating that NO suppressed anion secretion via cGMP production without PKG activation. Incubation with SNP increases intracellular cGMP in colonic enteric neurons and in the subepithelial compartment, but not in the epithelial cells (Young *et al.*, 1993; Wilson *et al.*, 1996). These reports indicate that NO-sensitive sGC is predominantly present in the neurons and subepithelial tissues, not in the colonocytes. Therefore, submucosa-derived NO might increase cGMP via sGC in the neurons or in the sub-
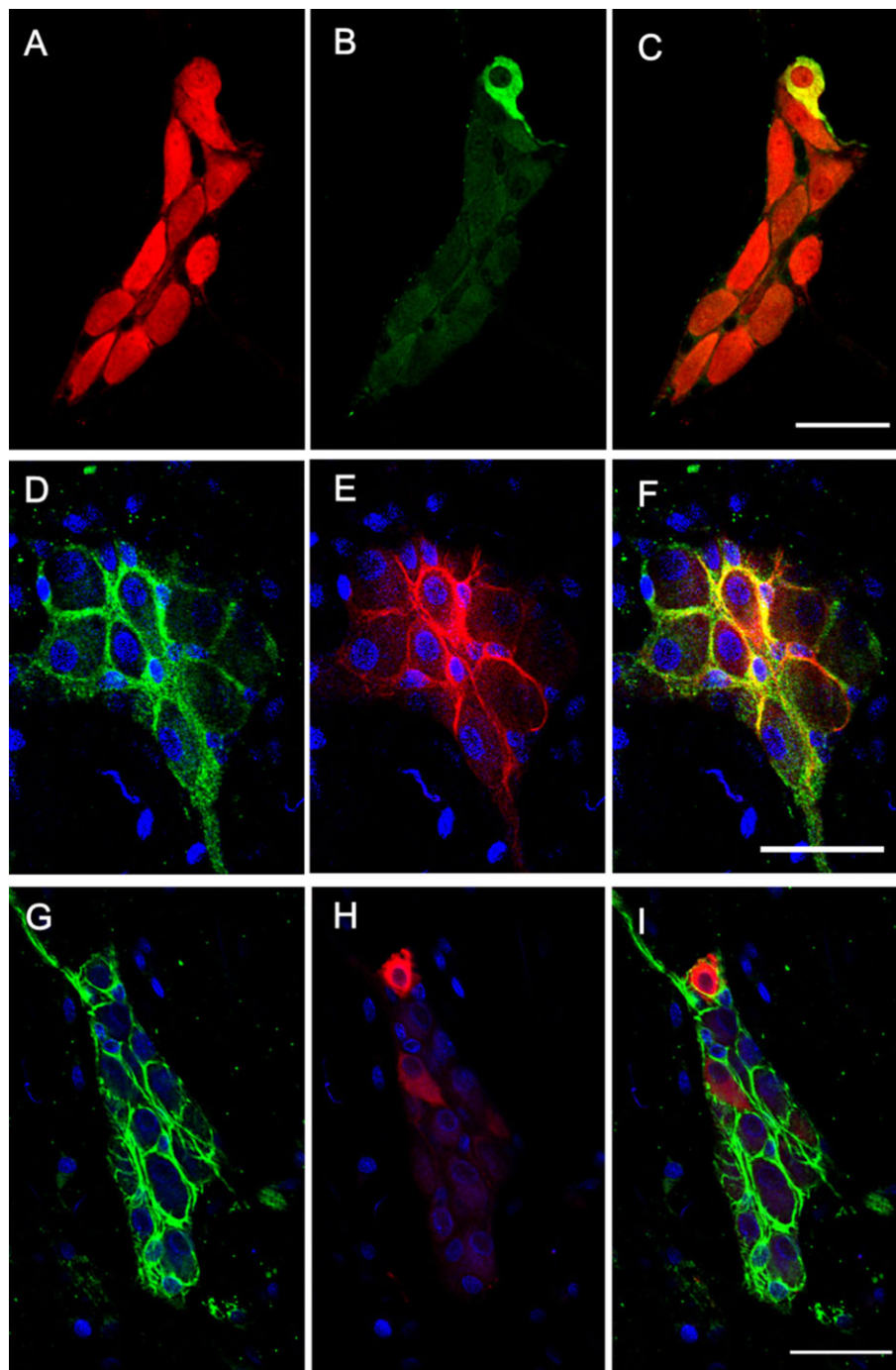
Figure 12

Localization of NOS isoforms in submucosa of rat proximal colon. (A–F) Double immunostaining for a neural marker PGP9.5 (red, A) with NOS1 (green, B), or a glial marker GFAP (green, D) with NOS2 (red, E) in whole-mount preparations of submucosa. Colocalization appeared as yellow (C, F). (G–I) Double immunostaining for NOS2 (green, G) and NOS1 (red, H) revealed lack of colocalization (I). Bar = 50 μm.

epithelial tissues, inhibiting luminal 5-HT-evoked Cl⁻ secretion independently of PKG. ENaC inhibition with amiloride also enhanced luminal 5-HT-evoked anion secretion in all colonic regions, suggesting that ENaC activity might 'mask' anion secretion-dependent I_{sc} changes. These findings suggest that luminal 5-HT-evoked secretory responses may be modulated by a variety of epithelial and subepithelial signal transmitters which balance the physiological and pathological states.

In conclusion, luminal 5-HT stimulated HCO_3^- secretion via activation of apical 5-HT₄ receptors, whereas 5-HT-induced Cl⁻ secretion was suppressed by NO generated in the submucosa of the proximal colon. Our findings suggest that luminal 5-HT release due to luminal acidic pH as a result of

high SCFA concentrations followed by HCO_3^- secretion may be important for maintenance of mucosal integrity by regulating luminal pH near 7.

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Author contributions

I. K. and Y. A. were responsible for the design, acquisition and analysis of data, and preparation of the manuscript. H. S. and K. N. contributed to the acquisition of additional data. I. K., Y. A and J. D. K. contributed to the interpretation of data and to the editing of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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