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RESEARCH PAPER

Activation of submucosal 5-HT$_3$ receptors elicits a somatostatin-dependent inhibition of ion secretion in rat colon

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Background and purpose: 5-Hydroxytryptamine (5-HT) is a key regulator of the gastrointestinal system and we have shown that submucosal neuronal 5-HT$_3$ receptors exerted a novel inhibitory effect on colonic ion transport. The aim of the present study was to investigate the precise mechanism(s) underlying this inhibitory effect.
Experimental approach: Mucosa/submucosa or mucosa-only preparations from rat distal colon were mounted in Ussing chambers for measurement of short-circuit current (I_{sc}) as an indicator of ion secretion. Somatostatin release was determined with radioimmunoassay. Intracellular cAMP content was measured with enzyme-linked immunoadsorbent assay (ELISA). Immunohistochemical techniques were used to study the expression of 5-HT$_3$ receptors, somatostatin and somatostatin receptors in colonic tissue.
Key results: In rat distal colonic mucosa/submucosa preparations, pretreatment with 5-HT$_3$ receptor antagonists enhanced 5-HT-induced increases in I_{sc}. However, in mucosa-only preparations without retained neural elements, pretreatment with 5-HT$_3$ receptor antagonists inhibited 5-HT-induced ΔI_{sc}. Pretreatment with a somatostatin-2 (sst$_2$) receptor antagonist in mucosa/submucosa preparations augmented 5-HT-induced ΔI_{sc}. Combination of sst$_2$ and 5-HT$_3$ receptor antagonists did not cause further enhancement of 5-HT-induced ΔI_{sc}. Moreover, both sst$_2$ and 5-HT$_3$ receptor antagonists enhanced 5-HT-induced increase in intracellular cAMP concentration in the mucosa/submucosa preparations. 5-HT released somatostatin from rat colonic mucosa/submucosa preparations, an effect prevented by pretreatment with 5-HT$_3$ receptor antagonists. Immunohistochemical staining demonstrated the presence of 5-HT$_3$ receptors on submucosal somatostatin neurons and of sst$_2$ receptors on colonic mucosa.
Conclusion and implications: Activation of neuronal 5-HT$_3$ receptors in the submucosal plexus of rat colon suppressed 5-HT-induced ion secretion by releasing somatostatin from submucosal neurons.
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Keywords: 5-HT$_3$ receptor; somatostatin; colonic ion transport

Abbreviations: DMSO, dimethyl sulphoxide; ELISA, enzyme-linked immunoadsorbent assay; ENS, enteric nervous system; GI, gastrointestinal; I_{sc}, short-circuit current; K-HS, Krebs-Henseleit solution; PBS, phosphate buffer solution; RIA, radioimmunoassay; sst$_2$ receptor, somatostatin receptor-2; TTX, tetrodotoxin

Introduction

5-Hydroxytryptamine (5-HT) is an important biogenic regulator, widely distributed in the gastrointestinal (GI) tract. 5-HT mediates responses as diverse as nausea, vomiting, secretion, peristalsis, and has been implicated in many GI dysfunctions such as irritable bowel syndrome and inflammatory bowel disease (Hicks *et al.*, 2002; Spiller, 2008). More than 95% of the GI 5-HT is present in the enterochromaffin cells, which are widely dispersed over the intestinal mucosa. In addition, 5-hydroxytryptaminergic neurons also exist in the enteric nervous system (ENS), but the amount of 5-HT in enteric neurons is very low in comparison with the amount in enterochromaffin cells (Racke *et al.*, 1996). 5-HT secreted by enterochromaffin cells or enteric neurons acts on 5-HT receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$ and 5-HT$_7$; nomenclature follows Alexander *et al.*, 2009) located on smooth muscles,

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enterocytes and nerves to regulate GI motility, secretion and sensation (O'hara *et al*., 2004; Moskwa and Boznanska, 2007).

In vitro studies suggested that 5-HT-evoked intestinal secretion can be described by two pathways: neural and non-neural (Budhoo *et al*., 1996; Hansen and Skadhauge, 1997; Kiso *et al*., 1997). The non-neural pathway involves activation of the 5-HT$_4$ receptors residing at the level of the colonocyte, while the neural pathway is mainly mediated by 5-HT$_3$ receptors (Ning *et al*., 2004; King *et al*., 2004a,b). 5-HT$_4$ receptors belong to G protein-coupled receptor superfamily that stimulate cAMP production in response to 5-HT (Ning *et al*., 2004). Unlike other 5-HT receptor subtypes, the 5-HT$_3$ receptor is a ligand-gated cation channel and usually exerts excitatory influences within the GI tract (Camilleri *et al*., 1999; Crowell, 2001). Activation of 5-HT$_3$ receptors promotes GI motility, secretion and sensation (Rahimi *et al*., 2008). As a possible therapeutic target in bowel disorders, 5-HT$_3$ receptors have become a centre of attention (Budhoo *et al*., 1996; Kiso *et al*., 1997; Glatzle *et al*., 2002). 5-HT$_3$ receptor antagonists have been shown to slow down colonic transit and increase fluid absorption, thus improving symptoms in some diarrhoea-predominant irritable bowel syndrome patients (Camilleri *et al*., 1999; 2001). Understanding the 5-HT$_3$ receptor regulatory pathways in GI secretion is likely to provide mechanistic insight into the aetiology and treatment of GI disorders or diseases.

Our recent study demonstrated an inhibitory effect on colonic ion secretion mediated by activation of 5-HT$_3$ receptors in the submucosal plexus (Yang *et al*., 2008). This finding provides novel insights into the role of 5-HT$_3$ receptors in GI secretion. However, the mechanism(s) underlying the inhibitory effect of 5-HT$_3$ receptors is not fully understood.

Somatostatin is an important regulatory peptide in the mammalian GI tract, where it inhibits a wide variety of processes, including cell proliferation, motility, release of digestive enzymes/regulatory peptides and electrogenic ion secretion (Warhurst *et al*., 1993; Samson *et al*., 2000). The effects of somatostatin are mediated by five G protein-coupled high affinity receptors (sst$_1$ to sst$_5$) (Hope *et al*., 2001). *In vitro* studies suggest that somatostatin-induced inhibition of basal or forskolin/carbachol-stimulated ion secretion in the rat isolated distal colonic mucosa is mediated by somatostatin receptor-2 (sst$_2$) (Mckeen *et al*., 1995). Moreover, regulation of somatostatin release by 5-HT has been reported in the GI tract (Koop and Arnold, 1984; Racke *et al*., 1996). These results suggest that somatostatin might be involved in the inhibitory action of 5-HT$_3$ receptor on rat colonic ion transport. Thus, the aim of the present study was to test the hypothesis that activation of the neuronal 5-HT$_3$ receptors in the submucosal plexus inhibits colonic secretion through somatostatin release.

Methods

Animal care and tissue preparation

The animal care and experimental protocols were approved by the Animal Care and Use Committee of Capital Medical University and met NIH guidelines. Adult male Sprague-Dawley rats (Laboratory Animal Services Center, Capital Medical University) ranging 200–300 g had free access to standard rodent laboratory food and water until the day of experiments. The animals were killed by cervical dislocation. The distal colon was removed and defined as the ~7 cm long segment proximal to the lymph node (typically situated 3 cm from the anus). The distal colon was then divided into four segments, termed DC$_1$ (adjacent to the lymph node), DC$_2$, DC$_3$ and DC$_4$ respectively. Preliminary results indicated that the responses to 5-HT were different in the four segments (Yang *et al*., 2006); however, the responses in DC$_3$ and DC$_4$ were more reliable and consistent. Thus, DC$_3$ and DC$_4$ segments were used in the present study. Each segment was cut along the mesenteric border into a flat sheet and flushed with ice-cold oxygenated Krebs-Henseleit solution (K-HS) containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2{\cdot}6H_2O$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 2.5 $CaCl_2{\cdot}2H_2O$. The tissue was pinned flat with the mucosal side down in a Petri dish containing ice-cold oxygenated K-HS. The serosa and the muscle layers were carefully removed under a dissecting microscope to obtain the mucosa/submucosa preparation. For the mucosa preparation, the submucosal plexus was also removed.

Ussing chamber experiments

Flat sheets of colonic mucosa-only or mucosa/submucosa preparations were mounted in modified Ussing chambers with a cross-sectional area of 0.5 cm^2. The mucosal and serosal surfaces of the tissue were bathed with 5 mL K-HS circulated from a reservoir maintained at 37°C and bubbled with 95% O_2 and 5% CO_2 to maintain the pH at 7.4. Drugs were added directly to the basolateral sides of the samples, and responses were recorded continuously. Transepithelial potential difference for every colonic sample was measured with Ag/AgCl reference electrodes (Physiologic Instruments, San Diego, CA, USA; P2020S) connected to a preamplifier that was in turn connected to a voltage-clamp amplifier (Physiologic Instruments, San Diego, CA, USA; VCC MC6). The change in short-circuit current (I_{sc}) was calculated as the difference between before and after drug application. I_{sc} was normalized as current per unit area of epithelium ($\mu A{\cdot}cm^{-2}$), which allowed the area under the curve for 15 min to be calculated ($\mu A{\cdot}min$).

Immunofluorescence

Frozen sections. Segments of rat distal colon were fixed for 10 min at room temperature in acetone and then washed (3 × 10 min) in phosphate buffer solution (PBS). The segments were embedded in optimum cutting temperature medium (McCormick, St. Louis, MO, USA) and cut at 8 μm thickness with a Cryostat microtome (Leica CM1850, St. Gallen, Switzerland). The sections were air dried for 1 h at room temperature, permeabilized with 0.3% Triton X-100 overnight at 4°C, blocked with 10% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature, followed by primary antibody incubation (4°C, overnight). Then, sections were washed in PBS (3 × 10 min) and incubated in the secondary antibody diluted in 0.3% Triton X-100 PBS for 1 h. After the final wash, the tissue sections were coverslipped and observed under a fluorescence microscope (Leica DM LB2, St. Gallen, Switzerland).

Table 1 Primary antibodies

Antibody	*Immunizing antigen*	*Host species*	*Dilution*	*Source/catalogue no.*
5-HT$_3$ receptor	A synthetic peptide (SLEKRDEMREVARD) corresponding to amino acids 444–457 of rat 5-HT$_3$ receptor, conjugated to KLH	Rabbit	1:1000	Calbiochem/PC347
Somatostatin	Somatostatin conjugated to KLH using glutaraldehyde method	Sheep	1:4000	American research Products/13-2366
sst$_2$ receptor	Epitope corresponding to amino acids 320–369 mapping at the C-terminus of sst$_2$a human receptor	Rabbit	1:500	Santa Cruz/sc-25676
HuC/D	12 amino acid synthetic peptide representing amino acids 240–251 from human HuD (monoclonal 16AI1)	Mouse	1:50	Mol. Probes/A21271

5-Hydroxytryptamine; Hu, antihuman neuronal protein; sst$_2$, somatostatin receptor-2.

Whole-mount preparations. For preparing whole-mount submucosal plexus, the distal colon segments were opened along the mesenteric border, stretched tautly, pinned out flat with mucosa side up onto a Sylgard-coated Petri dish. Preparations were fixed in Zamboni's fixative (4% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) for 3 h at room temperature. Whole mounts of the submucosal plexuses were dissected from these segments under a stereoscope. The mucosa was removed with fine-tip dissecting forceps, and the submucosal plexus was cut off using a pair of microscissors (Liu S *et al*., 2008; Xue *et al*., 2009). Whole-mount preparations were permeabilized with 0.3% Triton X-100 in PBS or 10 min and blocked with 10% normal donkey serum (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature. The preparations were then incubated with the primary antibody for 5-HT$_3$ receptors for 48 h at 4°C. After washing in PBS, the tissues were incubated in FITC-labelled donkey anti-rabbit IgG at room temperature for 1 h. The tissue was washed in PBS and coverslipped with VECTASHIELD Mounting Medium (Vector lab, Burlingame, CA, USA). Fluorescence labelling was examined under the fluorescence microscope to ensure the quality of the labelling. The tissues were then washed in PBS and subsequently incubated with the appropriate primary and secondary antibodies for somatostatin or Hu C/D with intermittent washes. After a thorough rinse, the tissues were coverslipped with VECTASHIELD mounting medium and examined and photographed on a Nikon EclipseTE300 microscope using a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Preabsorption of the 5-HT$_3$ receptor antibodies with their corresponding immunizing peptides (Calbiochem, San Diego, CA, USA) was carried out to determine if this would abolish the immunostaining. Immunostaining was also performed by omitting either the primary or the secondary antibody, and the antibodies were replaced by the solution for antibody dilution. Information on primary and secondary antibodies used in this study is summarized in Tables 1 and 2.

Table 2 Secondary antibodies

Secondary antibody	*Conjugation*	*Dilution*	*Source/catalogue no.*
Goat anti-rabbit IgG	FITC	1:200	Sigma/F9887
Donkey anti-sheep IgG	Cy3	1:500	Jackson/713-166-147
Donkey anti-rabbit IgG	FITC	1:100	Jackson/713-095-147

Cy3, indocarbocyanin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G.

Antibody characterization

Information on primary antibodies used in this study is summarized in Table 1. Further details regarding the specificity of each of the primary antibodies are given below.

The anti-5-HT$_3$ receptor antibody was raised against to a synthetic peptide corresponding to amino acids 444–457 of rat 5-HT$_3$ receptor. No Western blotting data are available for this antibody. Nevertheless, as detailed in *Results*, the 5-HT$_3$ receptor antibody displayed the same pattern of cellular morphology and distribution as previously published for the rat ENS (Glatzle *et al*., 2002). Specificity of the 5-HT$_3$ receptor antibody was also tested by preadsorption of the 5-HT$_3$ receptor antibody with the corresponding blocking peptide provided by the manufacturers and by omitting the secondary antibody. Both abolished immunostaining in the submucosal plexus (not shown).

The sheep somatostatin antibody was raised against a synthetic somatostatin peptide conjugated to KLH. No Western blotting data are available for this antibody. Nevertheless, the somatostatin antibody displayed the same pattern of cellular morphology and distribution as previously published for the ENS (Liu *et al*., 2005).

The anti-sst$_2$ receptor antibody was raised against the C-terminus of the sst$_2$a receptor of human origin (amino acids 320–369). This antibody identified a single band of 54 kDa by Western blotting in the rat cardiomyocytes (Bell *et al*., 2008). Ablation of sst$_2$ receptor immunoreactivity (IR) by omitting the primary antibody of sst$_2$ receptor or by preadsorption with an excess of the corresponding synthetic peptide confirmed the specificity of the antibody (data not shown).

Anti-human neuronal protein C/D (HuC/D) monoclonal (clone 16A11) antibody labelled neuronal cell nuclei and perikarya (Marusich *et al*., 1994). The staining pattern of cellular morphology and distribution in the present work was the same as previously described for the ENS (Lin *et al*., 2002).

cAMP measurement

Rat colonic mucosa/submucosa samples (about 150 mg) were incubated in K-HS solution for 30 min for equilibration. The samples were pretreated with vehicle (saline), 5-HT$_3$ receptor antagonists or sst$_2$ receptor antagonist for 5 min before adding 5-HT (10 μM, 5 min). All the samples were frozen in

liquid nitrogen immediately, then homogenized on ice in saline (0.9% NaCl) and centrifuged (10 620× *g*, 5 min). Intracellular levels of cAMP were determined by enzyme-linked immunoadsorbent assay (ELISA) according to the manufacturer's instructions (Sigma). The stimulation of cAMP formation was occurring in enterocytes and that none of the changes in cAMP were related to ganglion cells in the submucosal plexus. This is because activation of the neuronal 5-HT$_3$ receptor, which is a type of ligand-gated ion channel, does not stimulate cAMP formation.

Somatostatin release

Rat colonic mucosa/submucosa tissues (about 150 mg) were incubated in tubes containing 500 μL K-HS solution for 30 min for equilibration. The tissues were pretreated with vehicle (0.9% NaCl), 5-HT$_3$ receptor antagonists or sst$_2$ receptor antagonist for 5 min before adding 5-HT (10 μM, 5 min). At the end of the incubation, all samples in the tubes were frozen in liquid nitrogen immediately, then homogenized on ice in saline (0.9% NaCl) and centrifuged (10 620× *g*, 5 min). Somatostatin levels in the supernatant were measured by a commercial radioimmunoassay (RIA) kit (Beijing Sinouk Institute of Biological Technology, Beijing, China).

Statistical analysis

The data were expressed as means ± SEM (standard error of mean); *n* refers to the number of rats/tissues used. Comparisons between groups of data were made via Student's paired or unpaired *t*-test as appropriate. A *P*-value of less than 0.05 was considered statistically significant.

Materials

5-HT, indomethacin, MDL72222 and tetrodotoxin (TTX) were obtained from Sigma Chemical Company. CYN 154806, tropanyl-3, 5-dimethylbenzoate and 2-methyl-5-HT hydrochloride were purchased from Tocris Cookson Inc. (Ellisville, MO, USA). The agonists and antagonists concentrations were chosen based upon previous reports. Stock solutions of some chemicals (indomethacin, MDL72222, GR113808, tropanyl-3, 5-dimethylbenzoate) were dissolved in dimethyl sulphoxide (DMSO), with final DMSO concentrations less than 0.1% (vol/vol). Preliminary experiments indicated that the vehicle did not alter any baseline electrophysiological parameters.

Results

Effects of 5-HT$_3$ receptor antagonists on 5-HT-induced ΔI_{sc} in colonic mucosa/submucosa preparations

Previous studies have reported that 5-HT produced a concentration-dependent increase in I_{sc} with an EC_{50} of 5.4 ± 0.8 μM in the rat colon (Budhoo and Kellum, 1994). Thus, 10 μM was chosen as the concentration for 5-HT in the present study. As in our previous findings, addition of 5-HT (10 μM) to the basolateral side of the colonic mucosa/submucosa preparations elicited an increase in I_{sc}, while apical application of 5-HT had a negligible effect (Ning *et al*., 2004). Therefore, in the present study, 5-HT and 5-HT receptor agonists/antagonists were added only to the basolateral side of the tissue. After equilibration for 30 min, the baseline I_{sc} and the transepithelial resistance (*R*t) of the mucosa/submucosa preparations of rat distal colon were 45.2 ± 4.6 μA·cm^{-2} and 39.6 ± 4.0 Ω·cm^2 (n = 17).

The cyclooxygenase (COX) pathway plays a major role in mediation of the secretory response to exogenous 5-HT *in vitro* (King *et al*., 2004a). Indomethacin (10 μM), a COX inhibitor, was routinely used to abolish endogenous prostaglandin production involved in this process (Gierse *et al*., 1995). Basolateral application of indomethacin resulted in a significant reduction of baseline I_{sc} values by 26.3 ± 3.7 μA·cm^{-2} (n = 17).

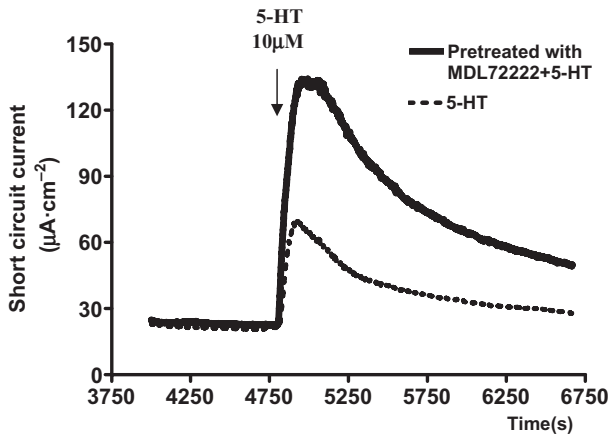
To investigate the role of 5-HT$_3$ receptors in the submucosal plexus in 5-HT-induced colonic secretion, 5-HT$_3$ receptor antagonists or agonists were added to the basolateral side of the preparations. Pretreatment with 5-HT$_3$ receptor antagonists MDL72222 (0.1 μM; Figure 1A) or tropanyl-3, 5-dimethylbenzoate (0.1 μM; Figure 1B) for 5 min did not inhibit, but increased 5-HT (10 μM)-induced ΔI_{sc} (P < 0.01). Effects of different doses of MDL72222 (0.001, 0.01, 0.1, 1 and 10 μM) on the 5-HT (10 μM)-induced ΔI_{sc} in rat mucosa/submucosa preparations were also tested. Pretreatment with MDL72222 at lower doses (0.01 and 0.1 μM) significantly enhanced 5-HT-evoked ΔI_{sc} (Figure 1C). The enhancing effect of MDL72222 on 5-HT-evoked ΔI_{sc} became weaker when its concentration increased to 1.0 and 10 μM. In more than half of the preparations, pretreatment with MDL72222 at 1.0 and 10 μM inhibited, instead of enhancing, 5-HT-induced I_{sc} response (Figure 1C).

Effects of tetrodotoxin

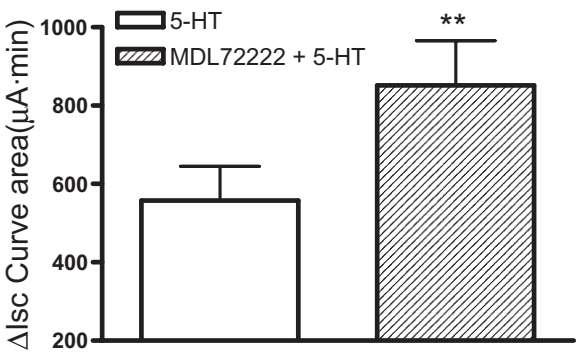
5-HT$_3$ receptors are found not only on neurons, but also on epithelial cells (Day *et al*., 2005). To determine the origin of the inhibitory effect on colonic ion secretion mediated by activating the 5-HT$_3$ receptors, TTX (1 μM), a voltage-gated Na^+ channel blocker, was applied to the basolateral side to block the neurally mediated colonic ion secretion in the mucosa/submucosa preparations (Pérez-navarro *et al*., 2005). TTX pretreatment did not significantly change the baseline I_{sc} or the *R*t. In the presence of TTX, 5-HT (10 μM)-induced ΔI_{sc} was significantly inhibited by the 5-HT$_3$ antagonist, MDL72222 (0.1 μM) (P < 0.01; Figure 2A). These results, combined with the results in the above section, suggested that the neuronal 5-HT$_3$ receptor in the submucosal plexus mediated an indirectly inhibitory action on colonic secretion, while the non-neuronal 5-HT$_3$ receptors on the epithelium mediated a directly stimulatory action on colonic secretion.

To further test our hypothesis, rat colonic mucosa-only preparations (submucosa was removed) were also used. After 30 min equilibration, the baseline I_{sc} and *R*t of the rat distal colonic mucosa were 54.2 ± 6.0 μA·cm^{-2} and 37.9 ± 4.3 Ω·cm^2 (n = 10) respectively. To avoid the effects of any remaining enteric neurons and endogenous prostaglandins, both TTX (1 μM) and indomethacin (10 μM) were applied to the basolateral side of the mucosa-only preparations. Treatment with indomethacin resulted in a significant reduction of the baseline I_{sc} to 30.6 ± 2.6 μA·cm^{-2} (n = 10) and TTX did not

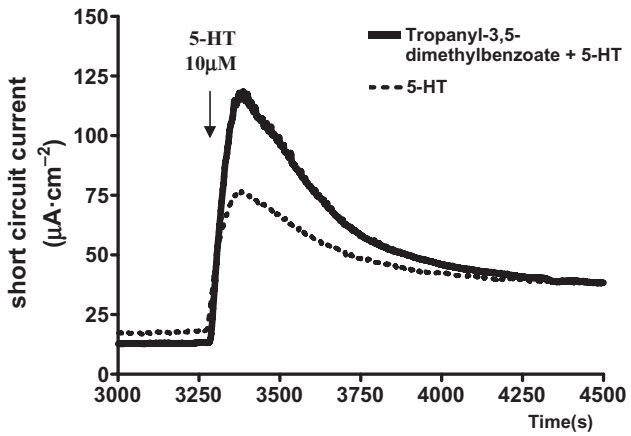
A1



A2



B1



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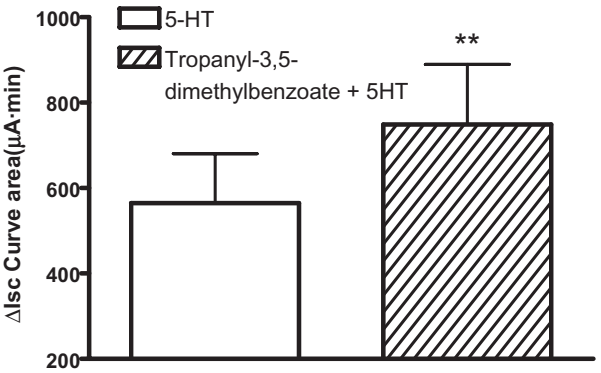

C1

Concentration of MDL 72222 (paired n) / 5-HT induced Δ *Isc*		*Isc* ↑ (n)	*Isc* ↓ (n)	*Isc* → (n)
0.001μ M	(n=6)	2	1	3
0.01μ M**	(n=12)	10	2	0
0.1μ M*	(n=11)	9	1	1
1.0μ M	(n=14)	4	7	3
10.0μ M	(n=12)	3	6	3

C2

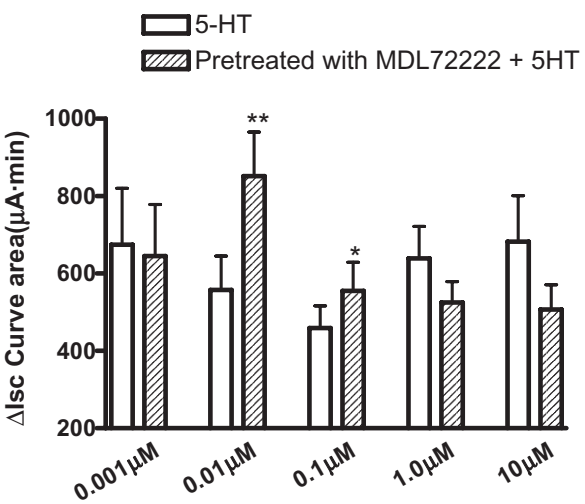

Figure 1 Effect of 5-HT$_3$ receptor antagonists on 5-HT-induced I_{sc} responses in rat distal colon mucosa/submucosa preparations. (A1) Representative I_{sc} recording with arrow indicating the basolateral application of 5-HT (10 μM) to the mucosa/submucosa preparations pretreated with MDL72222 (0.1 μM). (A2) Summary of the effects of MDL72222 (0.1 μM) on 5-HT-induced ΔI_{sc}. (B1) Representative I_{sc} recording showing the effect of tropanyl-3, 5-dimethylbenzoate (0.1 μM) on 5-HT-induced I_{sc} responses. (B2) Summary of the effects of Tropanyl-3, 5-dimethylbenzoate (0.1 μM) on 5-HT-induced ΔI_{sc}. Values are means ± SEM; $^{**}P < 0.01$; $n = 12$. (C1) Effects of different concentrations of MDL72222 on 5-HT-induced I_{sc} responses in rat distal colonic mucosa/submucosa preparations. Numbers in the table indicate the number of tissues demonstrating the variable effect on I_{sc} at different doses of MDL72222. (C2) Summary of the effects of different doses of MDL72222 on 5-HT-induced ΔI_{sc}. Values are means ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $n = 6$ (0.001 μM), $n = 12$ (0.01 μM), $n = 11$ (0.1 μM), $n = 14$ (1.0 μM), $n = 12$ (10 μM). 5-HT, 5-Hydroxytryptamine; I_{sc}, short-circuit current.

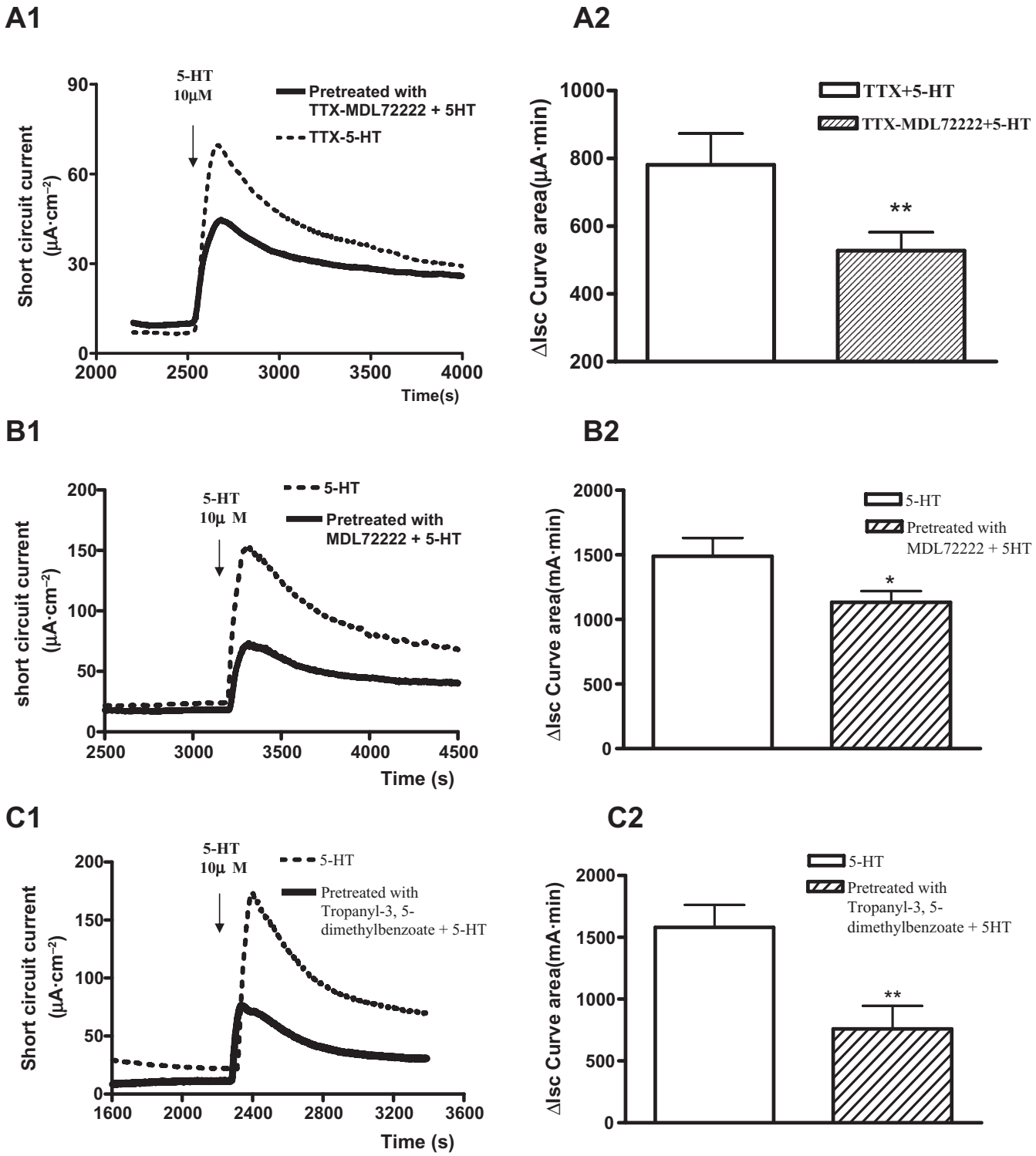

Figure 2 Effects of 5-HT$_3$ receptor antagonist on 5-HT-induced I_{sc} responses in rat colonic mucosa/submucosa and mucosa-only preparations in the presence of TTX. (A1) Representative I_{sc} recording with arrow indicating the basolateral application of 5-HT (10 μM) to the mucosa/submucosa preparations pretreated with MDL72222 (0.1 μM) plus TTX (1 μM). (A2) Summary of the effects of MDL72222 (0.1 μM) plus TTX (1 μM), on 5-HT-induced ΔI_{sc}. Values are means ± SEM; $^{**}P < 0.01$; $n = 9$. (B1/C1) Representative I_{sc} recording with arrows indicating the basolateral application of 5-HT (10 μM) to rat mucosa-only preparations pretreated with MDL72222 (0.1 μM, B1) or tropanyl-3, 5-dimethylbenzoate (0.1 μM, C1) plus TTX (1 μM) respectively. (B2/C2) Summary of the effects of MDL72222 (0.1 μM, B2) and tropanyl-3, 5-dimethylbenzoate (0.1 μM, C2) on 5-HT-induced ΔI_{sc}. Values are means ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $n = 5$ respectively. 5-HT, 5-Hydroxytryptamine; I_{sc}, short-circuit current; TTX, tetrodotoxin.

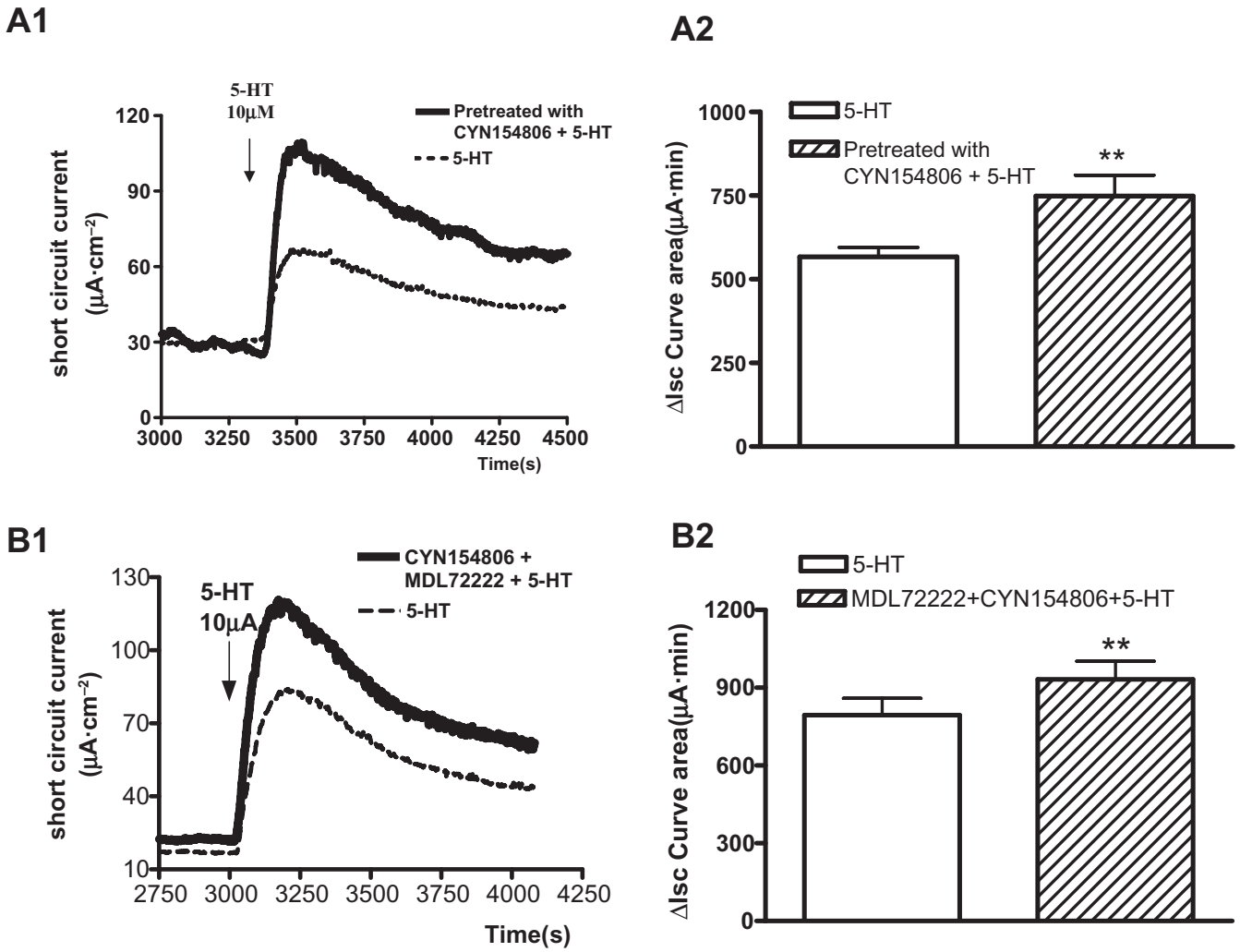

Figure 3 Effect of the sst$_2$ receptor antagonist CYN 154806 on 5-HT-induced I_{sc} responses in rat distal colon mucosa/submucosa preparations. (A1) Representative I_{sc} recording with arrow indicating the basolateral application of 5-HT (10 μM) to the mucosa/submucosa preparations pretreated with CYN 154806 (0.5 μM). (A2) Summary of the effect of CYN 154806 (0.5 μM) on 5-HT-induced ΔI_{sc}. Values are means ± SEM; $^{**}P < 0.01$; $n = 7$. (B1) Representative I_{sc} recording with arrow indicating the basolateral application of 5-HT (10 μM) to the mucosa/submucosa preparations pretreated with CYN 154806 (0.5 μM) and MDL72222 (0.1 μM). (B2) Summary of the effect of CYN 154806 (0.5 μM) and MDL72222 (0.1 μM) on 5-HT-induced ΔI_{sc}. Values are means ± SEM; $^{**}P < 0.01$; $n = 4$. 5-HT, 5-Hydroxytryptamine; I_{sc}, short-circuit current; sst$_2$, somatostatin receptor-2.

have distinct influence on *R*t or baseline I_{sc}. Basolateral pretreatment with MDL72222 (0.1 μM) or tropanyl-3, 5-dimethylbenzoate (0.1 μM) significantly inhibited 5-HT (10 μM)-induced increase in I_{sc} ($P < 0.05$, Figure 2B) and ($P < 0.01$, Figure 2C) respectively. Similar to a previous reported by Day *et al.* (Day *et al.*, 2005), basolateral addition of 5-HT$_3$ receptor agonist, 2-methyl-5-HT (100 μM), to the mucosa-only preparations induced an increase in I_{sc} (294.5 ± 84.5 μA·min; $n = 7$), which was blocked by pretreatment with the 5-HT$_3$ receptor antagonist, MDL72222 (0.1 μM, $n = 4$). 2-methyl-5-HT, given at 10 μM, failed to induce an increase in $I_{sc.}$ (Ning *et al.*, 2004).

Effects of the sst$_2$ receptor antagonist, CYN 154806

Somatostatin is expressed in the submucosal plexus and has been known to decrease basal as well as forskolin/carbachol-induced Cl^- secretion in rat colonic mucosa via activation of sst$_2$ receptors (Mckeen *et al.*, 1995). The sst$_2$ receptor antagonist CYN 154806, when used at 0.1–1 μM, was able to inhibit somatostatin-induced decrease in I_{sc} (Holliday *et al.*, 2007). In the present study, basolateral application of CYN 154806 (0.5 μM) significantly increased 5-HT-induced ΔI_{sc} in rat mucosa/submucosa preparations ($P < 0.01$, Figure 3A). Comparing with CYN 154806 (0.5 μM) or MDL72222 (0.1 μM) alone, the combination of CYN 154806 (0.5 μM) and MDL72222 (0.1 μM) did not cause any further enhancement of 5-HT-induced I_{sc} response (Figure 3B). The similar enhancing effects on 5-HT-induced ΔI_{sc} by 5-HT$_3$ receptor antagonists and sst$_2$ receptor antagonist suggest that somatostatin might be involved in the inhibitory effect on colonic secretion through activation of the neuronal 5-HT$_3$ receptors.

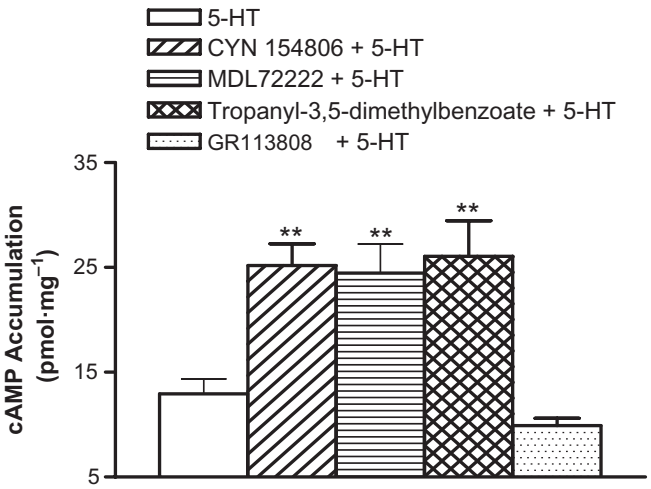

Figure 4 Intracellular cAMP measurement. Summary of the effects of pretreatment with the sst$_2$ receptor antagonist, CYN 154806 (0.5 μM), 5-HT$_3$ receptor antagonists, MDL72222 (0.1 μM) and tropanyl-3, 5-dimethylbenzoate (0.1 μM), and 5-HT$_4$ receptor antagonists, GR113808 (0.5 μM) on 5-HT (10 μM)-stimulated intracellular cAMP production in the rat colonic mucosa/submucosa preparations. $^{**}P < 0.01$; $n = 6$ respectively. 5-HT, 5-Hydroxytryptamine; sst$_2$, somatostatin receptor-2.

Intracellular cAMP measurement

It has been reported that somatostatin inhibits colonic ion secretion through activation of sst$_2$ receptors on epithelia cells and inhibition of intracellular cAMP accumulation (Warhurst *et al.*, 1996). The 5-HT$_3$ receptor is a ligand-gated cation channel that does not affect the intracellular cAMP concentration. In order to determine which neurotransmitter directly inhibits 5-HT-evoked colonic ion transport, we tested the effects of 5-HT$_3$ antagonists and an sst$_2$ receptor antagonist on 5-HT-stimulated intracellular cAMP accumulation in the colonic mucosa/ submucosa preparations. The basal level of cAMP was 6.9 ± 1.1 pM·mg^{-1} ($n = 6$). Pretreatment with the sst$_2$ receptor antagonist, CYN 154806 (0.5 μM), augmented the 5-HT (10 μM)-induced increase in intracellular cAMP concentration ($P < 0.01$; Figure 4). Consistent with our hypothesis, after pretreatment with 5-HT$_3$ antagonists, MDL72222 (0.1 μM) or tropanyl-3, 5-dimethylbenzoate (0.1 μM), 5-HT (10 μM)-stimulated intracellular cAMP production was similarly increased ($P < 0.01$; Figure 4). Pretreatment with the 5-HT$_4$ receptor antagonist GR113803 (1 μM), which has been shown to totally block 5-HT-induced increase in I_{sc} in rat colon mucosa preparations at this concentration (Ning *et al.*, 2004), did not significantly inhibit 5-HT-induced accumulation of intracellular cAMP concentration.

Expression of 5-HT$_3$ receptor on colonic submucosal somatostatin neurons

In order to provide morphological evidence supporting the release of somatostatin from submucosal plexus as an inhibitory neurotransmitter following activation of the neuronal 5-HT$_3$ receptors, rabbit anti-5-HT$_3$ receptor and sheep anti-somatostatin were used to double-label the 5-HT$_3$ receptor- and somatostatin-immunoreactive neurons in the submucosal plexus of the rat distal colon. In frozen sections of the rat distal colon, 5-HT$_3$ receptor IR was observed in neurons in close proximity to mucosa and was weakly expressed in the crypts (Figure 5A). 5-HT$_3$ receptor IR was observed mostly in somatostatin-positive neurons in the submucosal ganglia (Figure 5B,C). In whole-mount submucosal plexus of the rat distal colon, 5-HT$_3$ receptor IR was detected in fibres as well as neuronal cell bodies (Figure 5D). The staining pattern, cellular morphology and distribution of 5-HT$_3$ in the present study were the same as previously described for the rat ENS (Glatzle *et al.*, 2002). Omission of the primary antibody or preadsorption of the primary antibody with the corresponding immunogen peptide abolished the immunostaining in the distal colon (data not shown). Double labelling with a pan-neuronal marker anti-human neuronal protein HuC/D (Figure 5E) revealed that 5-HT$_3$ was localized to enteric neurons and did not appear in neighbouring glial cells (Figure 5F). Double labelling immunohistochemistry in the whole mounts demonstrated that almost all of the 5-HT$_3$-immunopositive submucosal neurons contained somatostatin IR (Figure 5G–I).

Expression of sst$_2$ receptors in colonic mucosa

Experiments with antibody to sst$_2$ receptors showed IR in the rat distal colonic mucosa and that this was widely located in the colonic crypts (Figure 6A and B). Ablation of sst$_2$ receptor IR by omitting the primary antibody of the sst$_2$ receptor and preadsorption with an excess of the corresponding synthetic peptide confirmed the specificity of the antibody (Figure 6C).

5-HT-induced somatostatin release

To further test the hypothesis that somatostatin is involved in 5-HT$_3$-mediated inhibition of colonic ion secretion, RIA was used to measure somatostatin release following 5-HT$_3$ receptor activation in the rat mucosa/submucosa preparations (Figure 7). In samples without any stimulation, that is, basal conditions, there was a low level of somatostatin in the supernatant of rat colonic mucosa/submucosa preparations (Figure 7). Incubation of the rat colonic mucosa/submucosa preparations with 5-HT (10 μM) caused an increase in the concentration of somatostatin in the supernatant ($P < 0.01$). Pretreatment with the 5-HT$_3$ antagonists MDL72222 (0.1 μM) or tropanyl-3, 5-dimethylbenzoate (0.1 μM) significantly inhibited the 5-HT-induced somatostatin release to about basal values ($P < 0.05$). The sst$_2$ receptor antagonist, CYN 154806 (0.5 μM), did not affect 5-HT-induced somatostatin release.

Discussion

The usual explanation of 5-HT action in the gut is that this amine modulates intestinal secretion predominantly by direct activation of the epithelia 5-HT$_4$ receptors (Budhoo and Kellum, 1994; Ning *et al.*, 2004; Fang *et al.*, 2008), which are G protein-coupled receptors stimulating intracellular cAMP accumulation. An indirect pathway mediated by the 5-HT$_3$ receptors, which are non-selective cationic channels on the intrinsic neural plexuses of the gut, has also been proposed

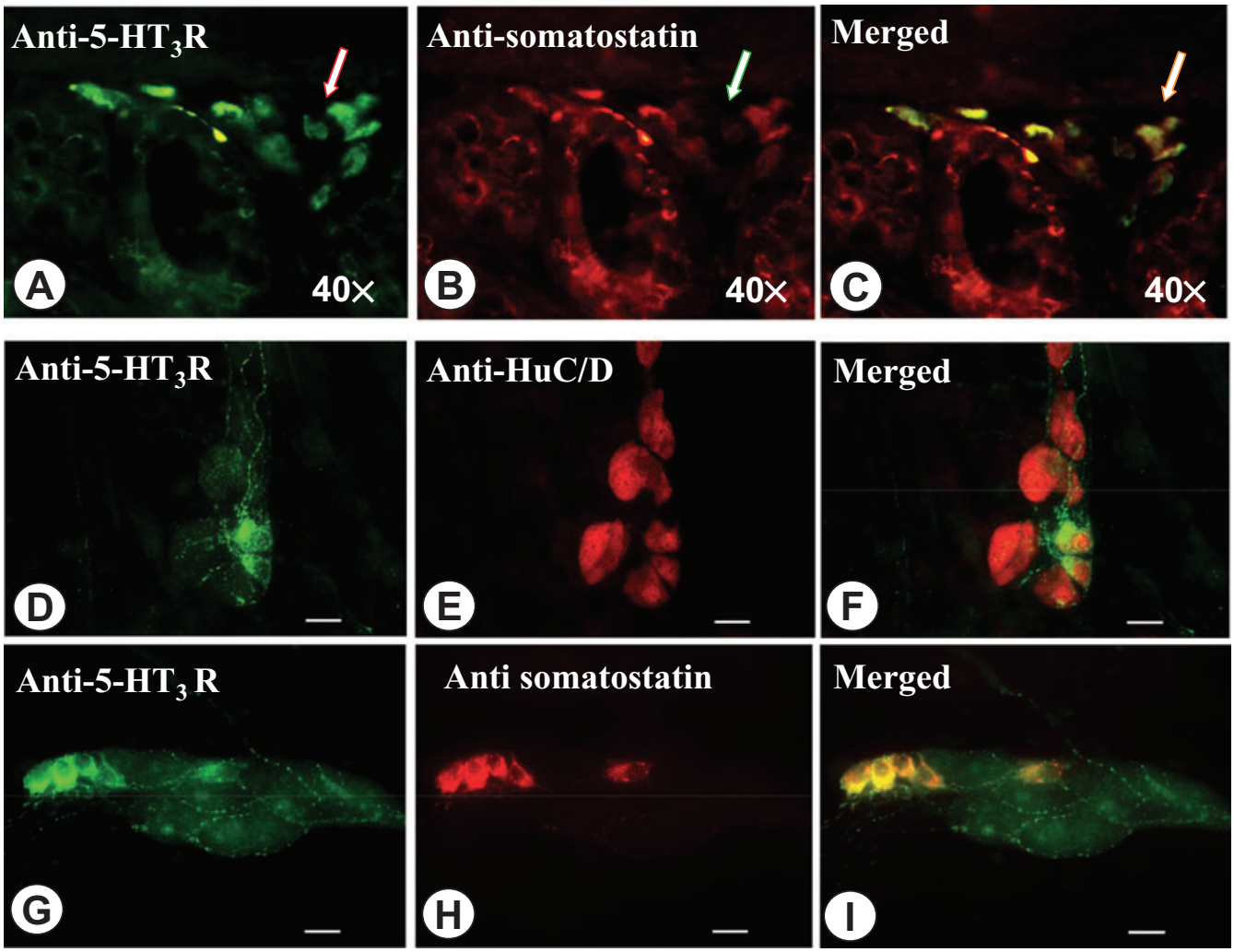

Figure 5 Expression of 5-HT$_3$ receptor in somatostatin neurons of the rat colonic submucosal plexus. (A–C) Immunoreactivity of 5-HT$_3$ receptor (A) and somatostatin (B) in the frozen sections of the rat distal colon. (D–F) Immunoreactivity of 5-HT$_3$ receptor in whole-mount preparations of the rat distal colon. 5-HT$_3$ receptor (green) was expressed in two neurons of a submucosal ganglion and nerve fibres. Neuronal phenotype was confirmed using a pan-neuronal marker HuC/D (red). (G–I) 5-HT$_3$ receptor (green) and somatostatin (red) was colocalized in neurons in whole-mount preparations of the submucosal plexus. Scale bars, 20 μm. 5-HT, 5-Hydroxytryptamine.

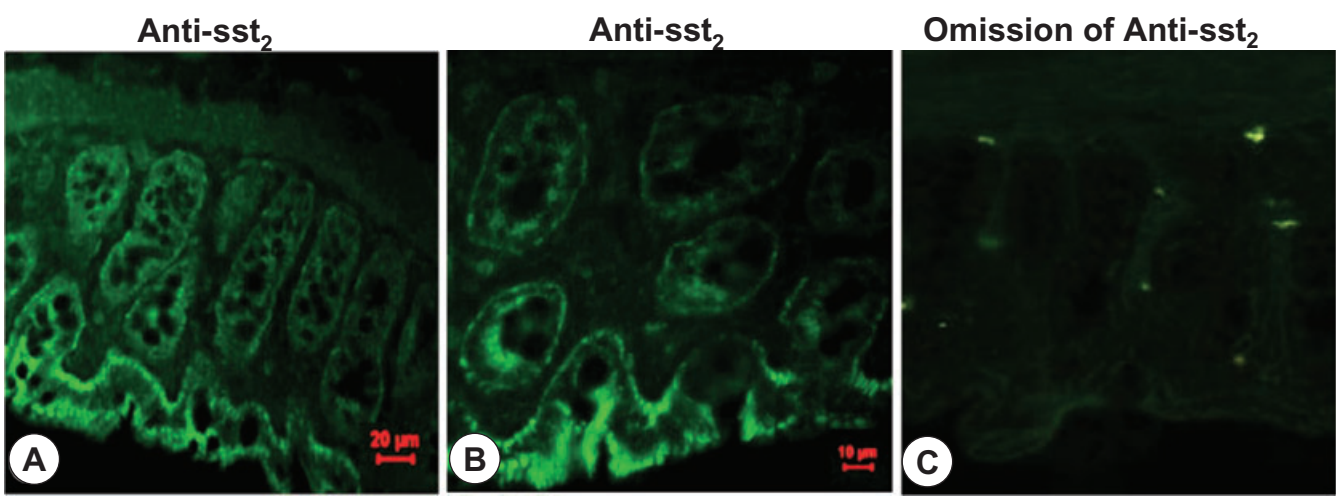

Figure 6 Expression of sst$_2$ receptors in the epithelium of the rat distal colonic mucosa. sst$_2$ receptor immunoreactivity was found to be abundantly expressed in the crypts of the rat distal colonic mucosa (A. lower magnification; B. higher magnification). (C) Omission of the primary antibody of sst$_2$ receptors resulted in no immunostaining. sst$_2$, somatostatin receptor-2.

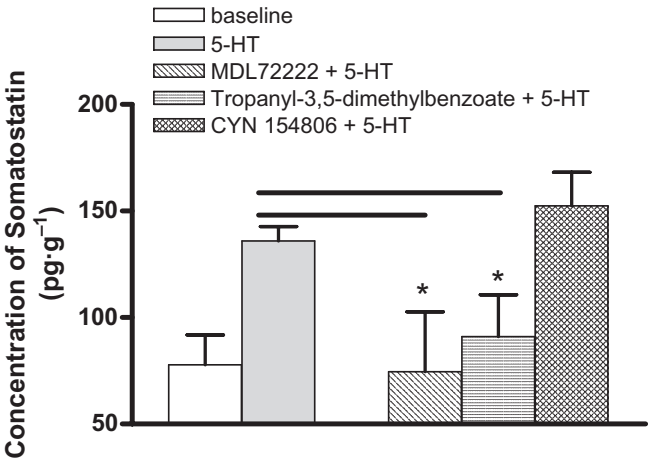

Figure 7 Radioimmunoassay of somatostatin release in rat colonic mucosa/submucosa preparations. A summary of the effects of pretreatment with different concentrations of 5-HT$_3$ antagonist, MDL72222 (0.1 μM, n = 6) and tropanyl-3, 5-dimethylbenzoate (0.1 μM, n = 5), as well as the sst$_2$ receptor antagonist, CYN 154806 (0.5 μM, n = 5) on the 5-HT (10 μM, n = 6)-stimulated somatostatin release. n = 7. *P < 0.05 compared with 5-HT treatment alone. 5-HT, 5-Hydroxytryptamine; sst$_2$, somatostatin receptor-2.

(Cooke, 2000; Kozlowski *et al*., 2000). Recently, a non-neuronal 5-HT$_3$ receptor-mediated chloride secretion was also reported in the rat distal colonic mucosa (Day *et al*., 2005). A combined blockade of 5-HT$_3$ and 5-HT$_4$ receptors completely inhibited 5-HT-induced diarrhoea in mouse and rat (Nagakura *et al*., 1997). Most recently, we found a novel dual role of 5-HT$_3$ receptors in 5-HT-induced ion transport in rat distal colon (Yang *et al*., 2008). Besides the well-known stimulatory effect on intestinal secretion, activation of the submucosal neuronal 5-HT$_3$ receptors also inhibits ion secretion. In the present study, we further investigated the mechanism(s) underlying the inhibitory effect mediated by the neuronal 5-HT$_3$ receptors.

Submucosal 5-HT$_3$ receptor-mediated inhibition of colonic ion secretion

Our results demonstrated that blocking 5-HT$_3$ receptors increased, rather than decreased, 5-HT-induced ΔI_{sc} in the rat distal colonic mucosa/submucosa preparations. This enhancing effect was abolished by the neuronal blocker TTX, suggesting that activation of neuronal 5-HT$_3$ receptors in the submucosal plexus inhibits colonic ion transport in the mucosa/submucosa preparations. Similarly, when the submucosal plexus was removed, the enhancing effect of 5-HT$_3$ antagonists on 5-HT-induced secretory response was diminished. These data confirm that 5-HT$_3$ receptor-mediated inhibition of colonic ion secretion requires the presence of the submucosal plexus.

Submucosal plexus contains a variety of neurotransmitters involved in regulation of ion secretion. Activation of the neuronal 5-HT$_3$ receptors in the submucosal plexus is expected to cause the release of one or more types of neurotransmitters, which in turn inhibit colonic secretion. Among these, somatostatin has been known to inhibit basal and stimulated chloride secretion in colonocytes, both by a direct action on the colonocytes and by inhibiting other enteric neurons (Mckeen *et al*., 1995; Warhurst *et al*., 1996; Cooke *et al*., 2003). Somatostatin is present in about 15–20% of secretomotor neurons in the submucosal plexus (Furness, 2000) and also in D cells of the gastric and duodenal epithelia (Booth *et al*., 2001). Once released, somatostatin is believed to function as an inhibitory neurotransmitter to inhibit colonic secretion by binding to the sst$_2$ receptors on epithelial cells to reduce intracellular cAMP (Mckeen *et al*., 1995; Warhurst *et al*., 1996) and/or by hyperpolarizing the membrane potential and suppressing excitability of secretomotor neurons in the submucosal plexus (Shen and Surprenant, 1993; Liu *et al*., 2000) (Figure 8A). Several lines of evidence support our hypothesis that somatostatin is involved in neuronal 5-HT$_3$-mediated inhibition of colonic ion secretion. First, as do 5-HT$_3$ receptor antagonists, the sst$_2$ receptor antagonist increased 5-HT-induced I_{sc} responses in the colonic mucosa/submucosa preparations, suggesting that the neural 5-HT$_3$ receptor and somatostatin in the submucosal plexus mediate inhibition of 5-HT-induced transepithelial ion transport. Second, pretreatment of rat submucosa/mucosa preparations with the sst$_2$ receptor antagonist or 5-HT$_3$ receptor antagonists further enhanced 5-HT-induced intracellular cAMP accumulation in the colonic mucosa/submucosa preparations. As 5-HT$_3$ receptors are a ligand-gated cation channels and have no direct effect on intracellular cAMP concentration, other neurotransmitter(s)/neuromodulator(s) (e.g. somatostatin) must be involved. Moreover, immunohistochemical staining demonstrated the expression of 5-HT$_3$ receptor on submucosal somatostatin neurons and sst$_2$ receptors in colonic mucosa, providing histological evidence. Finally, 5-HT induced somatostatin release from the colonic mucosa/submucosa preparations, which was blocked by 5-HT$_3$ receptor antagonists. All of these evidence suggest that activation of the 5-HT$_3$ receptors at the level of the submucosal plexus inhibits colonic ion secretion by stimulating somatostatin release from submucosal neurons (Figure 8A). Once released, somatostatin activated sst$_2$ receptors in intestinal epithelial cells to reduce intracellular cAMP concentration and inhibit ion secretion.

Contrary to our present finding in the rat distal colon, Cooke *et al*. (2003) have shown that activation of the neuronal 5-HT$_3$ receptors in the submucosal plexus of the guinea pig distal colon stimulates colonic Cl^- secretion, which is mainly mediated by the cholinergic neuronal pathway. Although this discrepancy is hard to resolve, species and regional differences may contribute to the divergent findings on the effect of 5-HT *in vitro* on electrolyte transport and I_{sc}. In our previous study, we found that even within the same species, epithelial ion transport induced by stimulants demonstrated regional heterogeneity in the rat distal colon (Yang *et al*., 2006). The 5-HT uptake, metabolism and neuronal systems have need noted as potential sources of variation between species and between the regions of the same species (Chetty *et al*., 2006).

Epithelial 5-HT$_3$ receptor-mediated stimulation of colonic ion secretion

5-HT$_3$ receptors are not only expressed in enteric neurons, but also in non-neuronal cells, such as intestinal epithelium. In

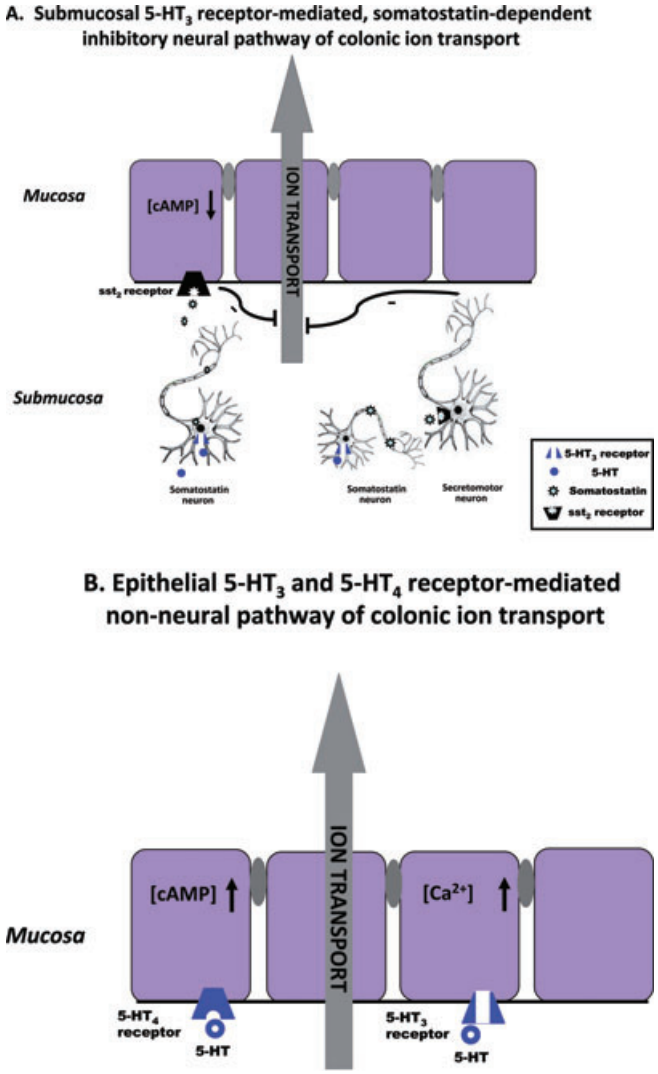

Figure 8 An illustration of the working model of 5-HT$_3$ and 5-HT$_4$ receptors in rat colonic submucosal plexus and mucosa. (A) Submucosal 5-HT$_3$ receptor-mediated somatostatin-dependent inhibitory neural pathway. Activation of 5-HT$_3$ receptors in the submucosal plexus leads to the release of somatostatin. Somatostatin binds with to the sst$_2$ receptors on the colonic epithelium and reduces the intracellular cAMP concentration, which leads to a decrease of ion secretion in the rat colon. Somatostatin also inhibits secretomotor neuron activity, which leads to a decrease of ion secretion. (B) Epithelial non-neuronal pathway mediated by 5-HT$_3$ and 5-HT$_4$ receptors. In this pathway, 5-HT binds to 5-HT$_3$ and 5-HT$_4$ receptors in the rat colonic epithelium and stimulates ion secretion in rat colon. 5-HT, 5-Hydroxytryptamine; sst$_2$, somatostatin receptor-2.

the rat distal colonic mucosa preparations without submucosal neural elements, the 5-HT$_3$ receptor antagonists suppressed the 5-HT-induced secretory responses. Furthermore, the 5-HT$_3$ receptor agonist, 2-methyl-5-HT, induced an increase in I_{sc} in mucosa-only preparations, which was abolished by the 5-HT$_3$ receptor antagonists. This observation is consistent with the report by Day *et al*. (2005) and suggests that the 5-HT$_3$ receptors located at the colonic mucosa level stimulate intestinal secretion (Figure 8B). The exact cellular location and function of these non-neuronal 5-HT$_3$ receptors are not known. Day *et al*. (2005) suggested that the epithelial 5-HT$_3$ receptors may exist on the enterochromaffin cells as an autocrine receptor in releasing 5-HT.

Our pharmacological experiments with 5-HT$_3$ antagonists did not demonstrate a concentration-dependent relationship on the enhancement of 5-HT-induced ion transport in the colonic mucosa/submucosa preparations (Figure 1C). A possible reason for this result could be related to the opposite effects by activating the 5-HT$_3$ receptors at different locations within the GI tract. At lower concentrations, the 5-HT$_3$ antagonists have easy access to the 5-HT$_3$ receptors on submucosal neurons as the drugs were added to the basolateral side of the preparations. Blockade of the neuronal 5-HT$_3$ receptors and enhancing 5-HT-induced ion transport would be the predominant effect. When the concentrations of the 5-HT$_3$ antagonists were raised, the drugs would not only block the 5-HT$_3$ receptors on the submucosal neurons, but also antagonize the 5-HT$_3$ receptors on the colonic epithelial cells that would reduce 5-HT-induced colonic ion secretion. The weakening of the 5-HT-evoked responses by higher concentrations of MDL72222 might also reflect a non-selective local anaesthetic action on secretomotor neurons (Fozard, 1984). However, the exact mechanism still need to be clarified in future studies.

In summary, this study provides evidence that activation of the neuronal 5-HT$_3$ receptor in the submucosal plexus suppressed colonic ion secretion by causing somatostatin release from submucosal neurons. Somatostatin activates sst$_2$ receptors either on non-somatostatin submucosal neurons, which inhibit excitability of secretomotor neurons (Shen and Surprenant, 1993; Liu *et al*., 2000), or on colonocytes to decrease intracellular cAMP levels; both would lead to the inhibition of colonic ion secretion. Activation of the non-neuronal 5-HT$_3$ receptor on the epithelium stimulates colonic ion secretion. These findings provide novel insights into the role of 5-HT signalling system in gut function and may also help to explain the complicated symptoms caused by the disturbance of the 5-hydroxytryptaminergic system in the gut.

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Conflict of interest

The authors have no financial, consultant, institutional and other relationships that might lead to bias or a conflict of interest.

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