Activation of Colonic Mucosal 5-HT$_4$ Receptors Accelerates Propulsive Motility and Inhibits Visceral Hypersensitivity

JILL M. HOFFMAN,* KARL TYLER,‡ SARAH J. MACEACHERN,§ ONESMO B. BALEMBA,* ANTHONY C. JOHNSON,‡ ELICE M. BROOKS,* HONG ZHAO,¶ GREG M. SWAIN,¶ PETER L. MOSES,# JAMES J. GALLIGAN,‖ KEITH A. SHARKEY,§ BEVERLEY GREENWOOD–VAN MEERVELD,‡ and GARY M. MAWE*

*Department of Anatomy & Neurobiology, University of Vermont; #Department of Medicine, Division of Gastroenterology and Hepatology, University of Vermont, Burlington, Vermont; ‡VA Medical Center and Oklahoma Center for Neuroscience, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma; §Hotchkiss Brain Institute & Snyder Institute of Infection, Immunity and Inflammation, Department of Physiology & Pharmacology, University of Calgary, Alberta, Canada; ‖Department of Pharmacology & Toxicology, ¶Department of Chemistry, Michigan State University, East Lansing, Michigan

BACKGROUND & AIMS: 5-hydroxytryptamine receptor (5-HT$_4$R) agonists promote gastrointestinal motility and attenuate visceral pain, but concerns about adverse reactions have restricted their availability. We tested the hypotheses that 5-HT$_4$ receptors are expressed in the colonic epithelium and that 5-HT$_4$R agonists can act intraluminally to increase motility and reduce visceral hypersensitivity. **METHODS:** Mucosal expression of the 5-HT$_4$R was evaluated by reverse-transcriptase polymerase chain reaction and immunohistochemical analysis of tissues from 5-HT$_4$R(BAC)-enhanced green fluorescent protein mice. Amperometry, histology, and short-circuit current measurements were used to study 5-HT, mucus, and Cl^- secretion, respectively. Propulsive motility was measured in guinea pig distal colon, and visceromotor responses were recorded in a rat model of colonic hypersensitivity. 5-HT$_4$R compounds included cisapride, tegaserod, naronapride, SB204070, and GR113808. **RESULTS:** Mucosal 5-HT$_4$ receptors were present in the small and large intestines. In the distal colon, 5-HT$_4$ receptors were expressed by most epithelial cells, including enterochromaffin and goblet cells. Stimulation of 5-HT$_4$Rs evoked mucosal 5-HT release, goblet cell degranulation, and Cl^- secretion. Luminal administration of 5-HT$_4$R agonists accelerated propulsive motility; a 5-HT$_4$R antagonist blocked this effect. Bath application of 5-HT$_4$R agonists did not affect motility. Oral or intracolonic administration of 5-HT$_4$R agonists attenuated visceral hypersensitivity. Intracolonic administration was more potent than oral administration, and was inhibited by a 5-HT$_4$R antagonist. **CONCLUSIONS: Mucosal 5-HT$_4$ receptor activation can mediate the prokinetic and antinociceptive actions of 5-HT$_4$R agonists. Colon-targeted, intraluminal delivery of 5-HT$_4$R agonists might be used to promote motility and alleviate visceral pain, while restricting systemic bioavailability and resulting adverse side effects.**

Keywords: Constipation; ATI-7505; Peristaltic Reflex; Cavitation.

5-hydroxytryptamine (5-HT, serotonin) is an important gastrointestinal (GI) signaling molecule involved in motor, secretory, and sensory functions.[1,2] These actions are mediated by a large family of serotonin receptors located within the neural circuitry and on a variety of other cell types in the gut.[3] Of the 5-HT receptors expressed in the intestines, the 5-HT$_4$ receptor (5-HT$_4$R) has been one of the most widely studied in regards to GI function, and 5-HT$_4$R agonists have been developed for the treatment of constipation and visceral pain.[4] Despite clinical effectiveness, the 5-HT$_4$R agonists tegaserod and cisapride were removed from the market because of concerns related to the possibility of adverse cardiovascular effects.[4]

The 5-HT$_4$R is a G-protein–coupled receptor that promotes activation of the adenylate cyclase/cyclic adenosine monophosphate (cAMP)/protein kinase A pathway, and can affect various cellular functions including facilitation of neurotransmitter release.[3] Stimulation of presynaptic 5-HT$_4$Rs on myenteric cholinergic nerve terminals enhances fast excitatory synaptic inputs to neurons and increases neurogenic muscle contractions in the intestines.[5,6] As a result, presynaptic facilitation within the peristaltic reflex circuitry is thought to be responsible for the prokinetic actions of 5-HT$_4$R agonists. It also is possible that 5-HT$_4$R agonists act via a mucosal site of action. Luminal application of 5-HT$_4$R agonists promotes propulsive motility and enhances the ascending contractile and descending relaxatory limbs of the peristaltic reflex.[7,8] However, the existence and distribution of 5-HT$_4$Rs in the mucosal layer of the intestines have not been investigated directly.

The aim of this investigation was to explore the hypothesis that 5-HT$_4$Rs are expressed in the colonic mucosa and their activation promotes motility and/or alleviates visceral pain. 5-HT$_4$Rs have been identified in the GI tracts of a number of species, including human beings. In these studies, we used assays that have been validated previously in mouse, rat, and guinea pig to evaluate functional responses to 5-HT$_4$R activation. The expression pattern of 5-HT$_4$Rs in the GI epithelium was determined by quanti-

Abbreviations used in this paper: BAC, bacterial artificial chromosome; CRD, colorectal distension; EC, enterochromaffin; eGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GI, gastrointestinal; 5-HT, 5-hydroxytryptamine or serotonin; 5-HT$_4$R, 5-hydroxytryptamine receptor; I_{SC}, short-circuit current; RT-PCR, reverse-transcription polymerase chain reaction; SW, Swiss Webster; TTX, tetrodotoxin; VMR, visceromotor response.

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tative reverse-transcription polymerase chain reaction (RT-PCR) and by evaluation of green fluorescent protein (GFP) immunoreactivity in 5-HT_4R(BAC)-enhanced GFP (eGFP) transgenic mice. Epithelial responses were evaluated by measuring 5-HT_4R agonist–induced 5-HT release, mucus secretion, and ion transport. Colonic propulsive motility was measured in response to luminal vs serosal administration of 5-HT_4R agonists. Finally, we evaluated the effects of luminal administration of 5-HT_4R agonists on the visceromotor response (VMR) to colorectal distension (CRD) in a colonic hypersensitivity model. Our findings indicate that 5-HT_4Rs are expressed in the epithelial layer of the colon, and suggest that targeted activation of these receptors has prokinetic and antinociceptive effects.

Materials and Methods

See Supplementary Materials and Methods section for details of immunohistochemistry protocols, strains and sources of animals used, and physiological solution recipes and reagents.

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of Vermont, Oklahoma City VA Medical Center and University of Oklahoma Health Sciences Center, and the University of Calgary Animal Care Committee. In all cases, animals were euthanized by isoflurane and exsanguination or cervical dislocation.

Human Biopsies

Human tissue biopsy specimens were obtained from patients of the Division of Gastroenterology and Hepatology using protocols approved by the University of Vermont Institutional Review Board. Individuals provided informed consent before their scheduled screening procedures. Mucosal samples were obtained using standard biopsy forceps. Samples were immediately placed in RNA stabilization solution (RNA*later*; Ambion, Austin, TX).

RT-PCR

RNA was extracted from human biopsies and murine full-thickness preparations or mucosal scrapings using the RNeasy Mini Kit (Qiagen, Valencia, CA) and complementary DNA (cDNA) was generated by reverse-transcriptase reaction (Promega, Madison, WI). An Applied Biosystems 7500 Fast Real-time PCR System was used with Fast Universal PCR Master Mix and validated TaqMan Gene Expression Assays for human 5-HT_4R (Hs00410577_m1), mouse 5-HT_4R (Mm00434129_m1), human HPRT1 (Hs99999909_m1), and mouse HPRT1 (Mm00446968_m1) (Applied Biosystems, Foster City, CA). Resulting data were calculated using the standard curve method and the level of 5-HT_4R expression was normalized to HPRT1. HPRT1 expression was consistent across the regions studied. To ensure that mucosal samples did not contain neuronal cell bodies, a subset of human samples were immunostained for anti-human neuronal protein HuC/D and neuron-specific enolase, and no neurons were observed. Furthermore, HuC/D transcript was not detected in cDNA from mouse mucosal samples.

Immunohistochemistry/BAC Transgenic Mice

Tissue samples from 5-HT_4(BAC)-eGFP mice with a Swiss Webster (SW) strain genetic background (kindly provided by Eric Schmidt and Nathaniel Heintz, Rockefeller University) were fixed with 4% paraformaldehyde, paraffin-embedded, and sectioned at 10 μm. The eGFP signal was amplified by GFP immunostaining, which yielded similar, but more intense, fluorescence than emitted by eGFP alone. Sections were examined on an Olympus AX70 fluorescence microscope (Olympus America, Inc, Melville, NY), and some sections were double-stained for 5-HT or mucin 2. Images of microscopic fields were captured with an Optronics MagnaFire digital camera (Optronics, Goleta, CA) using identical exposure settings.

Amperometry

Boron-doped diamond microelectrodes were used for continuous amperometric recordings.[9,10] The holding potential for the electrode was set at 700–750 mV with an Axoclamp-2B amplifier (Axon Instruments, Union City, CA). This potential was determined previously to oxidize 5-HT at a mass transfer limited rate. Electrical signals were acquired using a MiniDigi 1A (Axon Instruments) interfaced with pClamp software (Molecular Devices, Sunnyvale, CA) on an iMac computer (Apple, Cupertino, CA). Experiments on guinea pigs were performed with the colon bathed in oxygenated (95% O_2, 5% CO_2) Krebs solution at room temperature to minimize muscle contractions (flow rate 2 mL min-1). Mouse experiments were conducted at 37°C. After a 30-minute or longer equilibration period and confirmation of basal 5-HT release, recordings were obtained with the electrode placed 50 μm above the mucosal surface.

Mucus Release

Full-thickness colonic segments were equilibrated for 30 minutes in oxygenated, 37°C Krebs solution, followed by 30 minutes of an experimental condition. Tissues were rinsed and fixed in 10% formalin overnight at 4°C. Preparations were paraffin-embedded and sectioned, and stained with periodic acid–Schiff and Alcian Blue. The percentage of goblet cells that were cavitated was evaluated blindly by counting cavitated and non-cavitated goblet cells at 400× magnification.

Measurement of Ion Transport

Full-thickness preparations were mounted in Ussing chambers bathed in oxygenated Krebs solution warmed to 37°C.[11,12] The tissue was voltage-clamped at 0 mV. Short circuit current (I_{SC}, $\mu A/cm^2$) responses were measured as the maximum increase occurring within 10 minutes of drug application. At the end of each experiment, forskolin (10 μmol/L) was added to confirm tissue viability.

Motility Analysis

A Gastrointestinal Motility Monitor (Catamount Research and Development, Inc, St. Albans, VT) was used to record and analyze the rate of propulsive motility in guinea pig colonic segments.[13] A segment of distal colon was pinned onto a Sylgard-lined (Dow Corning Co, Midland MI) organ bath perfused with recirculating warmed (37°C) and oxygenated Krebs solution at a flow rate of 10 mL min^{-1}. Intraluminally delivered compounds were delivered at a flow rate of 0.25 mL min^{-1}. An epoxy-coated pellet was inserted into the oral end of an isolated segment, and the motility pattern of the pellet was tracked with a digital camera coupled to the Gastrointestinal Motility Monitor computer analysis software. After a 30-minute equilibration period, at least 3 trials were recorded, with a 5-minute recovery between each trial.