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ORIGINAL PAPER

Luminal trypsin induces enteric nerve-mediated anion secretion in the mouse cecum

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Abstract Proteases play a diverse role in health and disease. An excessive concentration of proteases has been found in the feces of patients with inflammatory bowel disease or irritable bowel syndrome and been implicated in the pathogenesis of such disorders. This study examined the effect of the serine protease, trypsin, on intestinal epithelial anion secretion when added to the luminal side. A mucosal-submucosal sheet of the mouse cecum was mounted in Ussing chambers, and the short-circuit current (I_{sc}) was measured. Trypsin added to the mucosal (luminal) side increased I_{sc} with an ED_{50} value of approximately 10 μM. This I_{sc} increase was suppressed by removing Cl^- from the bathing solution. The I_{sc} increase induced by 10–100 μM trypsin was substantially suppressed by tetrodotoxin, and partially inhibited by a neurokinin-1 receptor antagonist, but not by a muscarinic or nicotinic ACh-receptor antagonist. The trypsin-induced I_{sc} increase was also significantly inhibited by a 5-hydroxytryptamine-3 receptor ($5\text{-}HT_3$) antagonist and substantially suppressed by the simultaneous addition of both $5\text{-}HT_3$ and $5\text{-}HT_4$ receptor antagonists. We conclude that luminal trypsin activates the enteric reflex to induce anion secretion, 5-HT and substance P playing important mediating roles in this secreto-motor reflex. Luminal proteases may contribute to the cause of diarrhea occurring with some intestinal disorders.

Keywords Intestine · Irritable bowel syndrome · Inflammatory bowel disease · 5-hydroxytryptamine · Substance P

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Introduction

Intestinal fluid secretion is mainly derived from electrogenic anion secretion that accompanies Na^+ and water [1]. Under physiological conditions, it is important for surface lubrication in order to enable the luminal contents to pass through smoothly and also for maintaining an appropriate level of luminal fluidity for the digestion and absorption of nutrients. It also plays a role in diseased conditions by flushing out noxious luminal agents [2, 3]. Intestinal anion secretion is regulated by a variety of luminal and subepithelial substances (or by such a condition as mechanical distortion) that may have been exogenously derived or originated from the host itself. They may directly affect the epithelium, but their effects may also be mediated in the endocrine, paracrine and neurocrine fashion. The enteric nervous system, particularly the submucosal neuron, plays a central role in the neurocrine regulation of intestinal anion secretion [2, 3].

Proteases are not merely protein-degrading enzymes but are now viewed as signaling molecules that have vital roles in a variety of physiological processes and are also associated with multiple disease conditions [4, 5]. The

signaling functions of proteases are often mediated by the G-protein-coupled proteinase-activated receptors (PAR) [6–8]. The role of proteases and PARs in regulating intestinal anion secretion has been previously studied in vitro in an Ussing chamber. Trypsin or thrombin, both serine proteases, added to the serosal side modulated anion secretion, and the response was at least partly mediated by PARs on the epithelial cells or on the enteric nervous system [9–20]. In contrast to the effects of protease when added to the serosal side, those when applied to the luminal surface on intestinal anion secretion are relatively little known [9, 14]. It is well known, however, that the intestinal lumen is rich in proteases derived either from secretions from the gastrointestinal tract or from intestinal microflora [21–28]. In addition, an excessive concentration of proteases has been found in the feces of patients with inflammatory bowel disease or irritable bowel syndrome and have been implicated in the pathogenesis of such disorders [29–36].

We have previously reported in the mouse cecum in vitro that the serine protease, trypsin, when applied from the serosal side induced anion secretion by activating the enteric secreto-motor nerves [19, 20]. This response was partly initiated by activating PAR_1 on the enteric nerves. The purpose of this present study was, by using the same preparation, to examine the effect of trypsin when applied from the luminal side. We found that luminal trypsin activated the enteric reflex to induce anion secretion, and that 5-hydroxytryptamine (5-HT) played an important mediating role in this reflex.

Materials and methods

Tissue preparation

All procedures used in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka, and were approved by the University of Shizuoka Animal Usage Ethics Committee.

Male mice (30–40 g, Std: ddY; Japan SLC, Hamamatsu, Japan) were fed with standard food and water ad libitum until the time of the experiments. The animals were then killed by cervical dislocation and the cecum was excised. The resulting tissue was opened into a flat sheet, and the musculature was removed by blunt dissection. The tissue was divided into four pieces of approximately equal size. One of them was used for determining the trypsin-induced response under control conditions, and the others for determining the trypsin-induced response under various treatments.

Each piece was then mounted vertically between Ussing-type chambers that provided an exposed area of 0.2 cm^2. The volume of the bathing solution on each side was 5 ml, and the solution temperature was maintained at 37 °C in a water-jacketed reservoir. The bathing solution at pH 7.4 contained (mM): NaCl, 119; $NaHCO_3$, 21; K_2HPO_4, 2.4; KH_2PO_4, 0.6; $CaCl_2$, 1.2; $MgCl_2$, 1.2; glucose,10. A Cl–free solution was provided by respectively using 119 mM Na-gluconate, 1.2 mM Mg-$(gluconate)_2$ and 8 mM Ca-$(gluconate)_2$ in place of 119 mM NaCl, 1.2 mM $MgCl_2$ and 1.2 mM $CaCl_2$. Each solution was bubbled with 95 % O_2/5 % CO_2.

Electrical measurements

The experiments were performed under short-circuit conditions. The short-circuit current (I_{sc}) and transmucosal conductance (G_t) were measured by using an automatic voltage-clamping device (CEZ9100; Nihon Kohden, Tokyo, Japan) that compensates for the solution resistance between the potential measuring electrodes as previously described [37]. The inhibitors were administered to the indicated side 20 min before administering trypsin to the mucosal side.

Immunohistochemistry

Cecal tissue was taken from the mice perfused with Zamboni's fixative (2 % paraformaldehyde and 0.2 % picric acid in a 0.1 M phosphate buffer at pH 7.4), and was further immersed overnight in Zamboni's fixative at 4 °C. The fixed tissues were washed three times in PBS for 10 min, and then stored at 4 °C in PBS containing 0.1 % sodium azide, changing the PBS solution each day for 3 days. After washing, the tissues were next stored at 4 °C in PBS containing 30 % sucrose and 0.1 % sodium azide. The cryoprotected tissues were finally rapidly frozen by liquid nitrogen with a Tissue-Tek® optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan), and cut into 10-μm-thick sections with a cryostat. The sections on glass slides were dried and then washed three times in PBS for 10 min to remove the OCT compound. The sections were next incubated for 30 min with 10 % donkey normal serum and 0.3 % Triton X-100 in PBS at room temperature to suppress non-specific binding of the antibodies, and incubated overnight at 4 °C with the goat anti-5-HT antibody (×8,000 dilution; ImmunoStar, Hudson, WI, USA) with 0.3 % Triton X-100. The sections were then washed three times in PBS for 10 min, and incubated with donkey anti-goat IgG-Alexa Fluor 594 (×500 dilution; Molecular Probes, Eugene, OR, USA) and 0.1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) for 1 h at room temperature. After washing three times in PBS for 10 min, the sections were coverslipped with a mounting medium (DakoCytomation,

Glostrup, Denmark). The immunoreactivity was then visualized and captured by using an Axio Observer Z1 fluorescence microscope, an AxioCam cooled CCD digital camera and AxioVision digital imaging software (Hallbergmoss, Germany).

Reagents

Bumetanide, 3-tropanyl-3,5-dichlorobenzoate (bemesetron), SB-204070 hydrochloride, atropine, hexamethonium, indomethacin, nordihydroguaiaretic acid (NDGA), 5-hydroxytryptamine hydrochloride, pyrilamine, thrombin from bovine plasma (T4648), trypsin from porcine pancreas (T0303), a soybean trypsin inhibitor (T9003) and protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatinA, E-64, bestatin, leupeptin and aprotinin, in DMSO) were purchased from Sigma (St. Louis, MO, USA.). Tetrodotoxin (TTX) was purchased from Calbiochem (La Jolla, CA, USA). L-703,606 and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were purchased from Research Biochemical International (Natick, MA, USA). SKF-525A was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Indomethacin was dissolved in 21 mM $NaHCO_3$ (used by $\times$100 dilution). Bumetanide, bemesetron, NDGA and SKF-525A were dissolved in dimethyl sulfoxide before being administered to the bathing solution, the final concentration of dimethyl sulfoxide being 0.1 %. The administration of 10 and 100 μM of trypsin used a bathing solution respectively containing 100 and 1 mM trypsin, replacing with 500 μl of the mucosal solution ($\times$10 dilution; note that chamber volume was 5 ml). The other chemicals were each applied from an aqueous stock solution ($\times$100–1,000 dilution).

Statistics

Results are expressed as a percentage of the control response determined for each animal. Each value is presented as the mean ± SE, with n representing the number of animals. Statistical comparisons were made by Student's paired t test, significance being accepted at $P < 0.05$.

Results

Electrical response to mucosal trypsin

Adding 100 μM (2.34 mg/ml) trypsin[1] to the mucosal solution resulted in changes to the I_{sc} and G_t values (Fig. 1a). The I_{sc} value started to increase 2–3 min after the addition, reached its peak 20–30 min later, and then gradually decreased. The G_t value initially increased slightly after adding trypsin in some but not all cases, and, after 20–30 min, the second increasing G_t change became apparent in some but not all preparations. The second G_t increase was presumably a reflection of the epithelial disintegration caused by the proteolytic activity of trypsin. Figure 1b shows the relationship between the concentration of mucosal trypsin and the resulting I_{sc} and G_t changes (ΔI_{sc}, peak increases; ΔG_t, initial increases before the second increase were apparent). Trypsin produced a distinct I_{sc} response from a concentration of 0.1 μM, and a maximal I_{sc} response at about 100 μM. The trypsin-induced increase in G_t was generally correlated with the increase in I_{sc} value at low-to-medium concentrations of trypsin, although the increase at 100 μM was smaller than that at 10 μM. This suggests that mucosal trypsin, particularly at 100 μM, had a decreasing effect on G_t, in addition to the increasing effect (see later).

Ionic basis for the mucosal trypsin-induced I_{sc} increase

We then examined whether the mucosal trypsin-induced I_{sc} increase was due to the activation of electrogenic Cl^- secretion (Fig. 2). Bumetanide, an Na/K/2Cl co-transporter inhibitor, added to the serosal side (100 μM), substantially suppressed the trypsin-induced I_{sc} increase. In addition, Cl^--removal from both the mucosal and serosal bathing solutions almost removed the trypsin-induced I_{sc} increase. Furthermore, mucosal 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 100 μM), an anion channel/transporter blocker, substantially suppressed the trypsin-induced I_{sc} increase. The trypsin-induced increase in G_t value was not affected by bumetanide, but was significantly suppressed by Cl^- removal and NPPB (data not shown). Accordingly, the trypsin-induced increases in I_{sc} and G_t values are likely to have been mainly due to the activation of electrogenic Cl^- secretion [1, 2].

Role of the proteinase-activated receptor

Proteinase-activated receptors (PAR_1–PAR_4) belong to a family of G protein-coupled receptors that are activated by a variety of serine proteases. In order to determine whether PARs were present on the apical membrane and responsible for the mucosal trypsin-induced I_{sc} increase, we applied PAR_1-activating peptide TFLLRN-NH_2 (300 μM), PAR_2-activating peptide SLIGRL-NH_2 (300 μM), or PAR_4-activating peptide GYPGKF-OH (500 μM) to the mucosal side along with the protease inhibitor cocktail (1,000-fold dilution; to prevent a possible degradation of peptides by tissue proteases). However, they failed to

[1] The product information for trypsin (T0303) provided by Sigma describes that "this enzyme is soluble in 1 mM HCl (1 mg/ml)" which corresponds to 43 μmol/l. There is thus a possibility that trypsin was not completely soluble at 100 μmol in 1 liter of the present experimental solution.

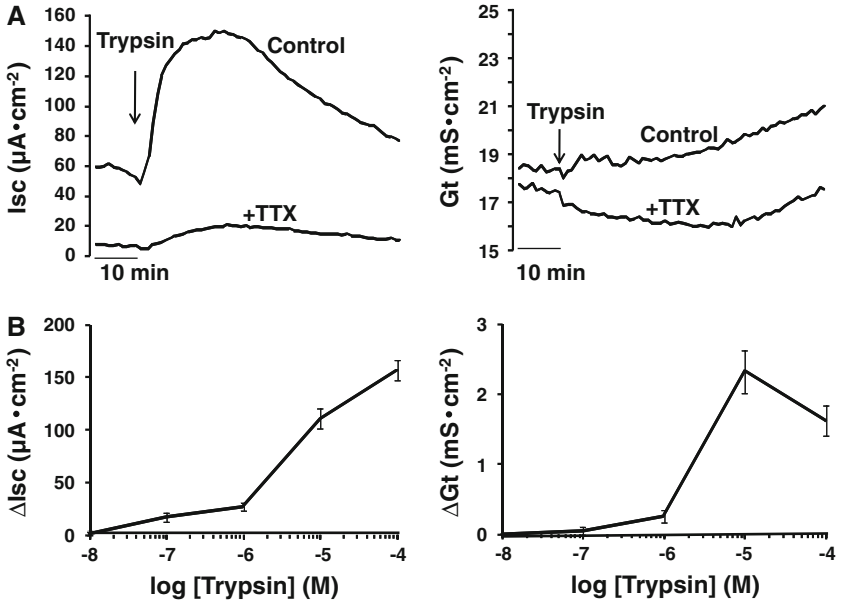

Fig. 1 Changes in the short-circuit current (I_{sc}) and transmucosal conductance (G_t) induced by mucosal trypsin and the effect of the tetrodotoxin (*TTX*) pretreatment. **a** Time-course characteristics of the changes in I_{sc} and G_t induced by 100 μM trypsin which was added to the mucosal side at the *arrowed* time in the absence and presence of TTX (300 nM, serosal side). A progressively increasing G_t change was apparent 20–30 min after starting the trypsin treatment with some but not all preparations. Mean values are presented, $n = 6$. **b** Concentration dependence of the trypsin-induced changes in I_{sc} (ΔI_{sc}, peak increases) and G_t (ΔG_t, initial increases before the second increase were apparent). Each data value is presented as the mean ± SE, with 5–7 animals being used

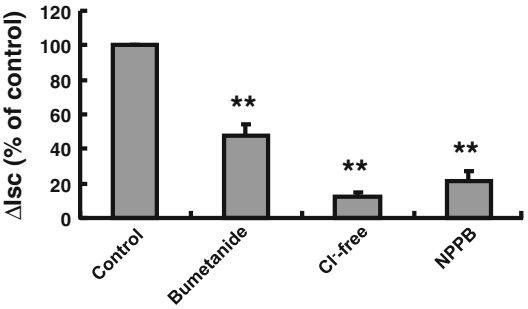

Fig. 2 Ionic basis for the I_{sc} increase induced by 100 μM of mucosal trypsin. Bumetanide (100 μM, serosal) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (*NPPB*; 100 μM, mucosal) were added, or Cl^- was removed (from both the mucosal and serosal bathing solutions) 20 min before adding 100 μM trypsin to the mucosal side. The maximum values for the increase in I_{sc} (ΔI_{sc}) induced by trypsin were determined and are expressed as a percentage of the control response obtained in the adjacent tissue. Each data value is presented as the mean ± SE, with $n = 5$. $^{**}P < 0.01$, compared with the control response using the paired *t* test

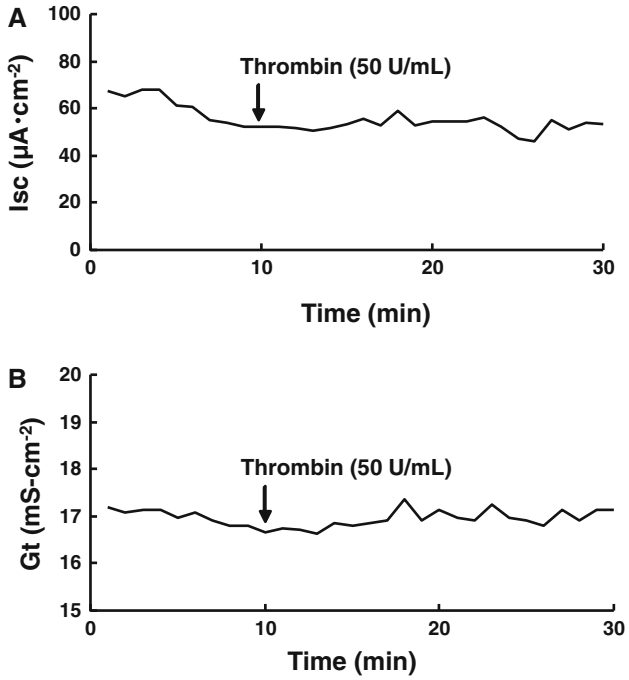

Fig. 3 Typical time-course characteristics of the changes I_{sc} (**a**) and G_t (**b**) induced by thrombin (50 U/ml) added to the mucosal side at the arrowed time. One of three similar observations is shown

evoke any noticeable change in the I_{sc} and G_t values ($n = 2$ for each peptide). The PAR_3 agonist was not available. In addition, the mucosal addition of thrombin (50 U/ml), an activator of PAR_1, PAR_3 and PAR_4, but not of PAR_2 [6], had hardly any effect on the I_{sc} and G_t values (Fig. 3). Since PAR-activating peptides and thrombin were applied at more than sufficient doses to activate PARs, it is unlikely, although cannot be entirely excluded, that PARs were present on the epithelial apical membrane and played a role in activating the anion secretion induced by mucosal trypsin [17, 19].

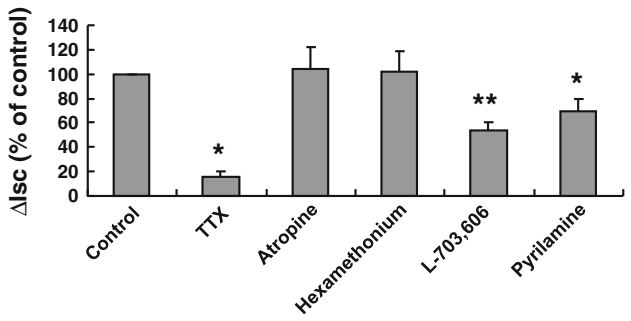

Fig. 4 Role of enteric submucosal neurons in the 100 μM mucosal trypsin-induced Cl^- secretion. Tetrodotoxin (*TTX*, 300 nM), atropine (10 μM), hexamethonium (10 μM), L-703,606 (10 μM), and pyrilamine (10 μM) were added to the serosal side 20 min before adding trypsin (100 μM) to the mucosal side. The maximum values for the increase in I_{sc} (ΔI_{sc}) induced by trypsin were determined and are expressed as a percentage of the control response obtained in the adjacent tissue. Each data value is presented as the mean ± SE, with $n = 4$ for L-703,606, and $n = 5$ for the others. *$0.01 < P < 0.05$ and **$P < 0.01$, compared with the control response by using the paired *t* test

Role of enteric nerves and other mediators

We next explored the role of the enteric nervous system in trypsin-induced Cl^- secretion. The I_{sc} and G_t increases induced by mucosal trypsin were largely suppressed by serosal TTX, indicating that they were mediated, if not totally, by the activation of secreto-motor neurons within the enteric submucosal nervous system (Figs. 1, 4) [3].

It was notable that 100 μM mucosal trypsin clearly decreased G_t in the presence of TTX, at least in some tissues. Our previous study on the mouse cecum has reported that trypsin added to the serosal side also decreased G_t (cf. Fig. 2 of [19]). A serine protease-induced decrease in paracellular permeability and tightening of the epithelial barrier has been shown in other epithelial systems [38, 39].

Both acetylcholine (ACh)-containing neurons and substance P-containing neurons have been reported to be present in the mouse large intestine [40, 41]. Accordingly, we next elucidated the involvement of ACh and substance P in trypsin-induced Cl^- secretion (Fig. 4). Neither atropine (a muscarinic ACh-receptor antagonist) nor hexamethonium (a nicotinic ACh receptor antagonist) suppressed the trypsin-induced I_{sc} increase, indicating that trypsin-induced Cl^- secretion was not mediated by ACh. In contrast, treating the tissue with L-703,606, a neurokinin-1 (NK_1) receptor antagonist, partially suppressed the trypsin-induced increase in I_{sc}, suggesting that the response was partially mediated by a release of substance P, resulting in activation of the NK_1.

A previous report has shown that histamine caused an I_{sc} increase in the mouse cecum which was partially inhibited by TTX and almost completely suppressed by pyrilamine, a histamine H_1 receptor antagonist [42]. We therefore tested the effect of pyrilamine on the trypsin-induced anion secretion (Fig. 4). Pyrilamine slightly but significantly suppressed the trypsin-induced increase in I_{sc} value, indicating that histamine via the histamine H_1 receptor was partially involved in the trypsin-induced Cl^- secretion.

To explore the role of arachidonate metabolites in mucosal trypsin-induced Cl^- secretion, we next examined the effects of pretreating the tissue with indomethacin (an inhibitor for cyclooxygenase), nordihydroguaiaretic acid (NDGA, an inhibitor for lipoxygenases), and SKF-525A (an inhibitor for cytochrome P450 monooxygenases) on mucosal trypsin-induced anion secretion [43]. The I_{sc} increase induced by trypsin was not significantly affected by either indomethacin (10 μM) added to both the mucosal and serosal sides or NDGA (50 μM, serosal side), but was slightly but significantly inhibited by SKF-525A (30 μM, serosal side) (data not shown). We have previously reported that SKF-525A inhibited the anion secretion induced by serosal trypsin [19]. However, there was no further information provided about epithelial regulation by the arachidonate metabolites through the cytochrome P450 monooxygenase pathway in the intestines [43].

Involvement of 5-HT

In the intestinal mucosa, 5-HT is mainly present in epithelial enterochromaffin cells and could be released to the serosal side by a variety of mucosal and serosal stimulation [44, 45]. Indeed, 5-HT-containing cells, probably enterochromaffin cells, were studded among epithelial cells in the mouse cecal mucosa (Fig. 5). The apical membrane of the 5-HT-containing cells appeared to access the lumen with a fine process.

To characterize the role of 5-HT in regulating the anion secretion in the mouse cecum, we applied 5-HT (1 μM) to the serosal side, and found that it caused increases in the I_{sc} and G_t values (Fig. 6). These 5-HT-induced responses were partly inhibited by TTX, indicating that 5-HT receptors were present on both the neuronal and non-neuronal cells. The 5-HT-induced responses were almost completely suppressed in the presence of both the 5-HT_3 receptor antagonist, bemesetron, and the 5-HT_4 receptor antagonist, SB-204070.

We then examined whether 5-HT played a role in mediating the trypsin-induced stimulation of anion secretion. Figure 7 shows that treating the tissue with bemesetron or SB-204070 significantly suppressed the mucosal trypsin- induced increase in I_{sc}. The simultaneous presence of both antagonists further suppressed the I_{sc} increase induced by trypsin. Therefore, 5-HT played a major role in mucosal trypsin-induced anion secretion through activation of both the 5-HT_3 and 5-HT_4 receptors.

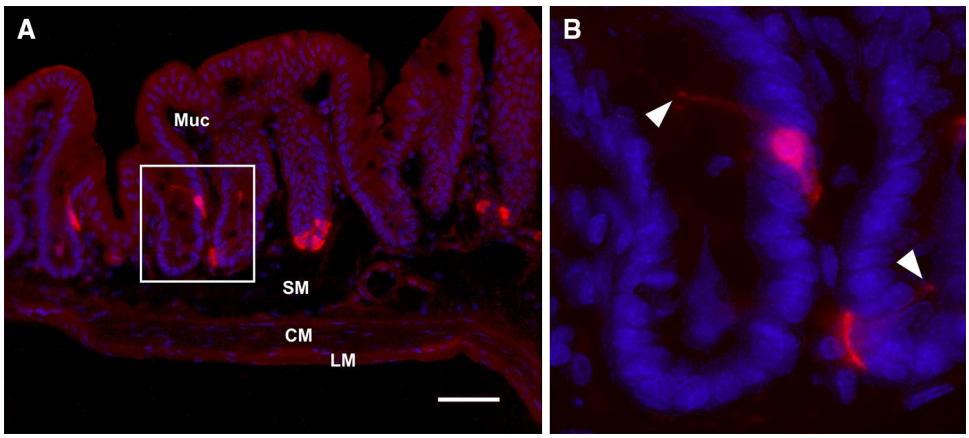

Fig. 5 5-Hydroxytryptamine (5-HT)-containing enterochromaffin cells in the mouse cecal mucosa. Ten-μm-thick sections of the mouse cecum were stained by using the goat anti-5-HT antibody and Alexa594-labeled anti-goat IgG antibody as the secondary antibody (*red*). 4′,6-Diamidino-2-phenylindole (1 μg/mL) was used with the secondary antibody for staining the nuclei (*blue*). **a** Images of whole layers of the mouse cecal wall, including mucosa (*Muc*), submucosa (*SM*), circular muscle (*CM*) and longitudinal muscle (*LM*) were taken with a ×20 objective lens (*bar* 20 μm). **b** Magnified image of the rectangular region in image A taken with a ×40 objective lens. *Arrowheads* indicate the apical plasma membrane of the 5-HT-immunoreactive enterochromaffin cells accessing the lumen with a fine process (color figure online)

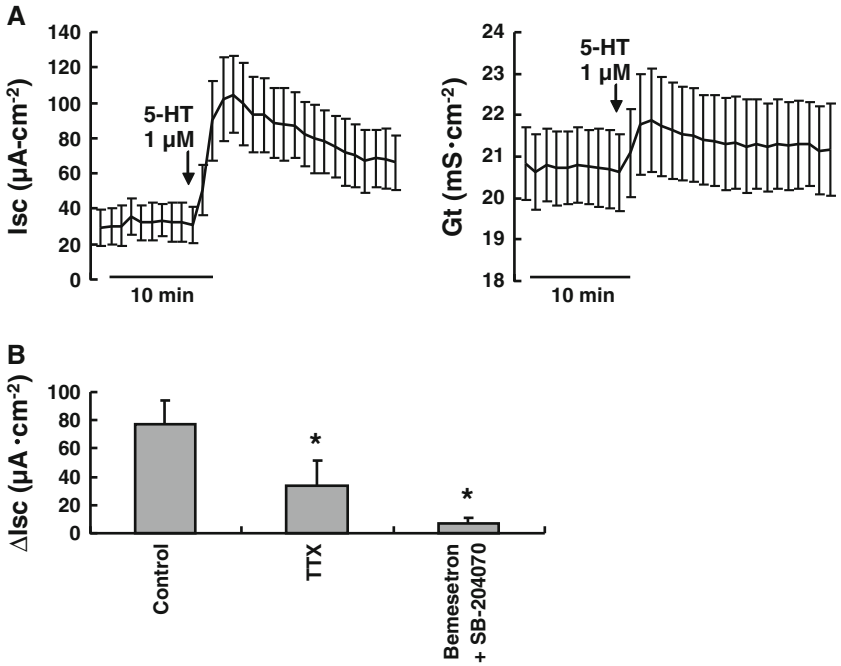

Fig. 6 Changes in I_{sc} and G_t induced by 5-HT. **a** Time-course characteristics for the I_{sc} and G_t changes induced by 5-HT (1 μM) added to the serosal side ($n = 8$). **b** Effects of TTX (300 nM), or the 5-HT_3 and 5-HT_4 receptor antagonists (20 μM bemesetron and 10 μM SB-204070, respectively) on the I_{sc} induced by 5-HT. Each inhibitor was added to the serosal side 20 min before adding 5-HT. Values for the maximum increase in I_{sc} (ΔI_{sc}) induced by 5-HT were compared in the presence and absence of the inhibitors. Six animals were used for TTX, and three for the 5-HT receptor antagonists. *$0.01 < P < 0.05$ compared with the control response that was determined in the adjacent tissue by using the paired *t* test

Similarity of the characteristics of the I_{sc} responses to 10 and 100 μM of mucosal trypsin

We used 100 μM of trypsin in all of the inhibitor experiments just described. The solution containing this concentration of trypsin became foamy when moderately bubbled which might have caused experimental errors. In addition, there is a possibility that trypsin was not completely soluble at this concentration (see footnote in "Electrical response to mucosal trypsin"). We therefore

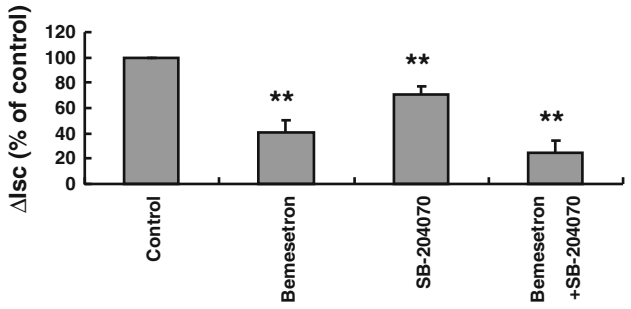

Fig. 7 Effects of inhibitors of 5-HT receptors type 3 and type 4 on the I_{sc} increase induced by 100 μM of mucosal trypsin. The 5-HT_3-receptor antagonist, bemesetron (20 μM; $n = 5$), or 5-HT_4-receptor antagonist, SB-204070 hydrochloride (10 μM; $n = 7$), or both ($n = 3$) were added to the serosal side 20 min before adding trypsin (100 μM) to the mucosal side. Each data value is presented as the mean ± SE. $^{**}P < 0.01$, compared with the control response using the paired t test

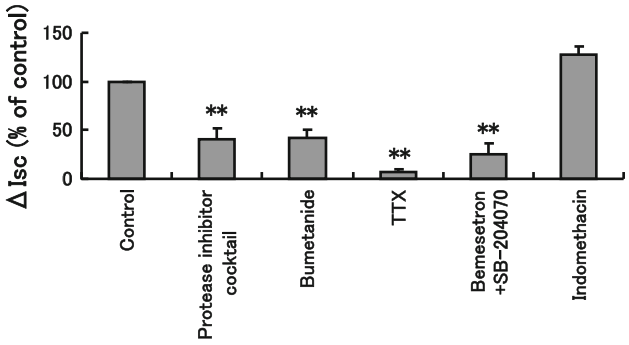

Fig. 8 Effect of inhibitors on the I_{sc} changes induced by 10 μM of mucosal trypsin. A protease inhibitor cocktail was added to the mucosal solution with a 1,000-fold dilution. The other inhibitors were treated as described in the foregoing experiments using 100 μM of mucosal trypsin. Each data value is presented as the mean ± SE, with $n = 4$ for the protease inhibitor cocktail, and $n = 5$ for the others. $^{*}0.01 < P < 0.05$ and $^{**}P < 0.01$, compared with the control response that had been determined in the adjacent tissue by using the paired t test

repeated some of the key experiments with 10 μM of trypsin. Figure 8 shows that the I_{sc} increase induced by 10 μM trypsin added to the mucosal side was partially inhibited by serosal bumetanide and almost completely so by serosal TTX. In addition, the I_{sc} response to 10 μM trypsin was substantially suppressed in the presence of both bemesetron and SB-204070, while not being inhibited by indomethacin. It is therefore likely that the mechanisms involved in stimulating the I_{sc} increase induced by trypsin were essentially similar whether 10 or 100 μM of trypsin was used. Of particular note is that the G_t decrease in the presence of TTX was not apparent by 10 μM of mucosal trypsin, in contrast to 100 μM of trypsin (data not shown).

We finally examined the effect of a protease inhibitor cocktail (5 μl) to corroborate that the trypsin-induced I_{sc} increase was due to the proteolytic activity of trypsin. Although the I_{sc} increase induced by trypsin (100 μM) was not suppressed by the protease inhibitor cocktail, the 10 μM trypsin-induced I_{sc} increase was partially suppressed (Fig. 8). Furthermore, pretreating the tissue with a soybean trypsin inhibitor (9.6 mg/ml on the mucosal side) completely removed the effect of both 10 and 100 μM mucosal trypsin ($n = 2$ for each trypsin concentration). The stimulatory effect of mucosal trypsin on I_{sc} therefore required the proteolytic activity of trypsin.

Discussion

The present study has demonstrated with the mouse cecum in vitro that the serine protease, trypsin, evoked an increase in the I_{sc} value when added to the mucosal (luminal) side in an Ussing chamber. This I_{sc} increase induced by trypsin was substantially or partially inhibited in the absence of Cl^- from the medium, or in the presence of mucosal NPPB or serosal bumetanide, suggesting that the I_{sc} increase was mainly due to the increased anion secretion [1, 2]. In addition to this anion secretion, the large intestine has been shown to have mechanisms for Na^+ and Cl^- absorption, K^+ absorption and secretion, and short-chain fatty acid absorption [46]. The comprehensive features of the changes in nutrient and ion transport induced by luminal trypsin therefore remain elusive. Nevertheless, the stimulation of anion secretion may at least result in fluid secretion, and thereby an increase in the fluid content of the intestinal lumen [1, 2].

The present results suggest that the activation of anion secretion by mucosal trypsin was mainly mediated by a reflex pathway involving submucosal secreto-motor neurons, since the responses were substantially suppressed by the nerve conduction blocker, TTX (Figs. 1, 4). In addition, the response was significantly inhibited by a 5-HT_3 antagonist, 5-HT_4 antagonist, NK_1 antagonist, and histamine H_1 antagonist, but not by a muscarinic ACh receptor antagonist or a nicotinic ACh receptor antagonist (Figs. 4, 7). These results suggest that the reflex pathway was mediated by 5-HT, substance P, and histamine, but not by ACh. 5-HT and substance P are possibly responsible as neurotransmitters, since both have been demonstrated to be present as neurotransmitters in the enteric nervous system [41, 47], and that the exogenous application of 5-HT (Fig. 6) and substance P (Fig. 4 of [17]) to the chamber-mounted mouse cecum have been shown to evoke an increase in the I_{sc} value. On the other hand, the precise source (enteric nerves vs. intestinal mast cells) and the release mechanism of histamine involved in the trypsin-induced reflex are not clear, although a previous report has shown that histamine in the mouse cecum can cause a TTX- and pyrilamine-sensitive I_{sc} increase [42]. It is intriguing that intestinal mast cells has been reported to be

closely associated with enteric nerves, suggesting that they closely communicate with each other to regulate a variety of intestinal functions [48, 49].

What could be the target of mucosal trypsin, whose activation eventually leads to the secretory reflex? One possible candidate is proteinase-activated receptors. PARs have been shown to be present in the intestinal mucosal tissues, and trypsin has been a strong activator of some PAR subtypes [6, 7]. However, we failed to reproduce the mucosal trypsin-induced I_{sc} changes by applying a high concentration of the PAR_1-, PAR_2-, or PAR_4-activating peptide to the mucosal side. In addition, thrombin, an activator of PAR_1, PAR_3, and PAR_4, but not of PAR_2 [6], had hardly any effect on the I_{sc} and G_t values. These findings suggest, although it cannot be entirely excluded, that PARs were not involved in the trypsin-induced secretory reflex. The initial target molecule of trypsin acting from the luminal side remains to be elucidated.

We could not determine whether trypsin added to the mucosal side acted on the luminal border of the epithelium or on the subepithelial tissue after it had penetrated across the epithelium. In the latter case, trypsin may well stimulate anion secretion, since we have previously shown that subepithelial trypsin could induce the secreto-motor reflex at a concentration two orders of magnitude lower than mucosal trypsin [19]. In the former case, a component may have been required that transduced the activation of the putative receptor on the luminal border into the subepithelial secreto-motor reflex. One possible candidate is enteroendocrine cells, particularly 5-HT-containing enterochromaffin cells [45, 50]. Indeed, 5-HT-containing enterochromaffin cells were studded among other epithelial cells with the apical plasma membrane accessing the lumen in the present mouse cecal tissue (Fig. 5). Previous studies have shown that a variety of luminal stimuli could release 5-HT from enterochromaffin cells [44, 45, 50, 51]. In addition, morphological and functional evidence has indicated that $5\text{-}HT_3$ was present on the enteric sensory nerve and that a $5\text{-}HT_3$ agonist could induce TTX-sensitive intestinal secretion [34, 52–56]. Whether 5-TH release from the enterochromaffin cells is stimulated by trypsin remains to be confirmed in the future.

In summary, the present results for the mouse cecum have demonstrated that luminal protease could induce fluid secretion through the activation of submucosal secreto-motor neurons (Fig. 9). Certain levels of protease activities derived from digestive secretion as well as from enteric microflora are likely to be maintained under normal conditions in the lumen of the large intestine [21–28]. The fluid secretion induced by these luminal proteases could serve to maintain the level of luminal fluidity necessary for certain physiological functions. Otherwise, it may contribute to host defense by eliminating the aggravating proteases from the lumen. The protease activity in the lumen of the large intestine has been reported to increase under such pathological conditions as inflammatory bowel

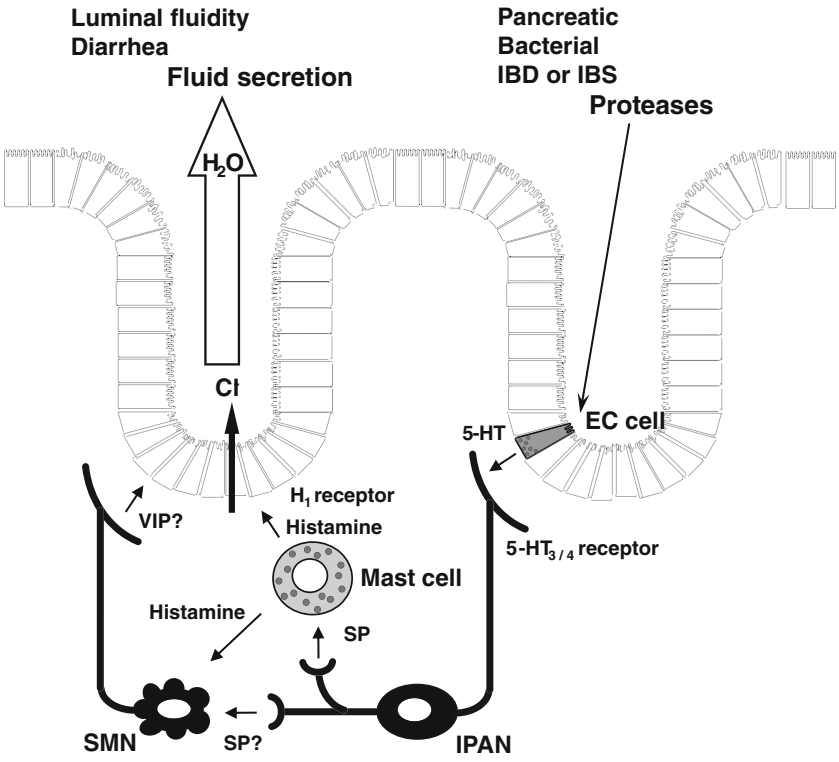

Fig. 9 Schematic drawing showing the neural loop involved in anion secretion induced by luminal proteases. Modified from Karaki and Kuwahara [57]. See the text and [57] for details. *IBD* inflammatory bowel disease, *IBS* irritable bowel syndrome, *EC cell* enterochromaffin cell, *IPAN* intrinsic primary afferent neuron, *SMN* secretomotor neuron, *SP* substance P, *VIP* vasoactive intestinal polypeptide. Most of the non-cholinergic secretomotor neurons have been suggested to be VIP-containing neurons [3]

disease, irritable bowel syndrome, and infectious colitis [29–36]. It is therefore likely, although it remains to be determined, that the secretory reflex induced by luminal proteases contributed to the diarrhea observed under these diseased conditions. Additionally, the diarrhea observed under certain antibiotic treatments could be partially due to the increased level of trypsin resulting from abating the bacterial breakdown of pancreatic trypsin in the large intestine [21, 22, 26].

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Frizzell RA, Hanrahan JW (2012) Physiology of epithelial chloride and fluid secretion. Cold Spring Harb Perspect Med 2(6):a009563
2. Field M (2003) Intestinal ion transport and the pathophysiology of diarrhea. J Clin Invest 111(7):931–943
3. Bornstein JC, Gwynne RM, Sjoeval H (2012) Enteric neural regulation of mucosal secretion. In: Johnson LR (ed) Physiology of the gastrointestinal tract, 2012. Elsevier, London, pp 769–790
4. Turk B (2006) Targeting proteases: successes, failures and future prospects. Nat Rev Drug Discov 5(9):785–799
5. Lopez-Otin C, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283(45):30433–30437
6. Ossovskaya VS, Bunnett NW (2004) Protease-activated receptors: contribution to physiology and disease. Physiol Rev 84(2):579–621
7. Adams MN et al (2011) Structure, function and pathophysiology of protease activated receptors. Pharmacol Ther 130(3):248–282
8. Ramachandran R et al (2012) Targeting proteinase-activated receptors: therapeutic potential and challenges. Nat Rev Drug Discov 11(1):69–86
9. Kong W et al (1997) Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. Proc Natl Acad Sci USA 94(16):8884–8889
10. Vergnolle N et al (1998) Proteinase-activated receptor 2 (PAR2)-activating peptides: identification of a receptor distinct from PAR2 that regulates intestinal transport. Proc Natl Acad Sci USA 95(13):7766–7771
11. Green BT et al (2000) Intestinal type 2 proteinase-activated receptors: expression in opioid-sensitive secretomotor neural circuits that mediate epithelial ion transport. J Pharmacol Exp Ther 295(1):410–416
12. Buresi MC et al (2001) Protease-activated receptor-1 stimulates $Ca^{(2+)}$-dependent $Cl^{(-)}$ secretion in human intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 281(2):G323–G332
13. Cuffe JE et al (2002) Basolateral PAR-2 receptors mediate KCl secretion and inhibition of Na^+ absorption in the mouse distal colon. J Physiol 539(Pt 1):209–222
14. Kunzelmann K et al (2002) Ion transport induced by proteinase-activated receptors (PAR2) in colon and airways. Cell Biochem Biophys 36(2–3):209–214
15. Mall M et al (2002) Activation of ion secretion via proteinase-activated receptor-2 in human colon. Am J Physiol Gastrointest Liver Physiol 282(2):G200–G210
16. Buresi MC et al (2005) Activation of proteinase-activated receptor-1 inhibits neurally evoked chloride secretion in the mouse colon in vitro. Am J Physiol Gastrointest Liver Physiol 288(2):G337–G345
17. Ikehara O et al (2010) Proteinase-activated receptors-1 and 2 induce electrogenic Cl^- secretion in the mouse cecum by distinct mechanisms. Am J Physiol Gastrointest Liver Physiol 299(1): G115–G125
18. Mueller K et al (2011) Activity of protease-activated receptors in the human submucous plexus. Gastroenterology 141(6):2088 e1–2097 e1
19. Ikehara O et al (2012) Subepithelial trypsin induces enteric nerve-mediated anion secretion by activating proteinase-activated receptor 1 in the mouse cecum. J Physiol Sci 62(3):211–219
20. Suzuki Y et al (2012) Subepithelial trypsin stimulates enteric nerve-mediated anion secretion in the mice cecum. J Physiol Sci 62(Supplement 1):S197
21. Borgstrom A, Genell S, Ohlsson K (1977) Elevated fecal levels of endogenous pancreatic endopeptidases after antibiotic treatment. Scand J Gastroenterol 12(5):525–529
22. Norin KE, Gustafsson BE, Midtvedt T (1986) Strain differences in faecal tryptic activity of germ-free and conventional rats. Lab Anim 20(1):67–69
23. Corthier G et al (1989) Interrelationships between digestive proteolytic activities and production and quantitation of toxins in pseudomembranous colitis induced by *Clostridium difficile* in gnotobiotic mice. Infect Immun 57(12):3922–3927
24. Gibson SA et al (1989) Significance of microflora in proteolysis in the colon. Appl Environ Microbiol 55(3):679–683
25. Bustos D et al (1994) Colonic proteolysis following pancreatic duct ligation in the rat. Int J Pancreatol 16(1):45–49
26. Ramare F et al (1996) Inactivation of tryptic activity by a human-derived strain of *Bacteroides distasonis* in the large intestines of gnotobiotic rats and mice. Appl Environ Microbiol 62(4): 1434–1436
27. Koshikawa N et al (1998) Expression of trypsin by epithelial cells of various tissues, leukocytes, and neurons in human and mouse. Am J Pathol 153(3):937–944
28. Wen JY et al (2002) Inhibition of proteolysis in luminal extracts from the intestine of the brushtail possum. J Pharm Pharmacol 54(10):1365–1372
29. Hansen KK et al (2005) A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. Proc Natl Acad Sci USA 102(23):8363–8368
30. Cenac N et al (2007) Role for protease activity in visceral pain in irritable bowel syndrome. J Clin Invest 117(3):636–647
31. Roka R et al (2007) A pilot study of fecal serine-protease activity: a pathophysiologic factor in diarrhea-predominant irritable bowel syndrome. Clin Gastroenterol Hepatol 5(5):550–555
32. Roka R et al (2007) Colonic luminal proteases activate colonocyte proteinase-activated receptor-2 and regulate paracellular permeability in mice. Neurogastroenterol Motil 19(1):57–65
33. Gecse K et al (2008) Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. Gut 57(5):591–599
34. Buhner S et al (2009) Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. Gastroenterology 137(4):1425–1434
35. Demaude J et al (2009) Acute stress increases colonic paracellular permeability in mice through a mast cell-independent mechanism: involvement of pancreatic trypsin. Life Sci 84(23–24):847–852

36. Steck N et al (2012) Bacterial proteases in IBD and IBS. Gut 61(11):1610–1618
37. Koyama K et al (1999) Induction of epithelial Na^+ channel in rat ileum after proctocolectomy. Am J Physiol 276(4 Pt 1):G975–G984
38. Swystun VA et al (2009) Serine proteases decrease intestinal epithelial ion permeability by activation of protein kinase Czeta. Am J Physiol Gastrointest Liver Physiol 297(1):G60–G70
39. Buzza MS et al (2010) Membrane-anchored serine protease matriptase regulates epithelial barrier formation and permeability in the intestine. Proc Natl Acad Sci USA 107(9):4200–4205
40. Sang Q, Young HM (1998) The identification and chemical coding of cholinergic neurons in the small and large intestine of the mouse. Anat Rec 251(2):185–199
41. Shimizu Y et al (2008) Tachykinins and their functions in the gastrointestinal tract. Cell Mol Life Sci 65(2):295–311
42. Homaidan FR et al (1997) Regulation of ion transport by histamine in mouse cecum. Eur J Pharmacol 331(2–3):199–204
43. Ferrer R, Moreno JJ (2010) Role of eicosanoids on intestinal epithelial homeostasis. Biochem Pharmacol 80(4):431–438
44. Hansen MB, Witte AB (2008) The role of serotonin in intestinal luminal sensing and secretion. Acta Physiol (Oxf) 193(4): 311–323
45. Bertrand PP, Bertrand RL (2010) Serotonin release and uptake in the gastrointestinal tract. Auton Neurosci 153(1–2):47–57
46. Kunzelmann K, Mall M (2002) Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiol Rev 82(1):245–289
47. Neal KB, Parry LJ, Bornstein JC (2009) Strain-specific genetics, anatomy and function of enteric neural serotonergic pathways in inbred mice. J Physiol 587(Pt 3):567–586
48. Buhner S, Schemann M (2012) Mast cell-nerve axis with a focus on the human gut. Biochim Biophys Acta 1822(1):85–92
49. Schemann M, Camilleri M (2013) Functions and imaging of mast cell and neural axis of the gut. Gastroenterology 144(4):698 e4–704 e4
50. Bertrand PP (2009) The cornucopia of intestinal chemosensory transduction. Front Neurosci 3:48
51. Kidd M et al (2008) Luminal regulation of normal and neoplastic human EC cell serotonin release is mediated by bile salts, amines, tastants, and olfactants. Am J Physiol Gastrointest Liver Physiol 295(2):G260–G272
52. Kiso T, Ito H, Miyata K (1997) Effect of ramosetron on short-circuit current response in rat colonic mucosa. Eur J Pharmacol 320(2–3):187–192
53. Glatzle J et al (2002) Expression of 5-HT3 receptors in the rat gastrointestinal tract. Gastroenterology 123(1):217–226
54. Liu MT et al (2002) Expression and function of 5-HT3 receptors in the enteric neurons of mice lacking the serotonin transporter. Am J Physiol Gastrointest Liver Physiol 283(6):G1398–G1411
55. Mazzia C, Hicks GA, Clerc N (2003) Neuronal location of 5-hydroxytryptamine3 receptor-like immunoreactivity in the rat colon. Neuroscience 116(4):1033–1041
56. Michel K et al (2005) Serotonin excites neurons in the human submucous plexus via 5-HT3 receptors. Gastroenterology 128(5): 1317–1326
57. Karaki SI, Kuwahara A (2004) Regulation of intestinal secretion involved in the interaction between neurotransmitters and prostaglandin E2. Neurogastroenterol Motil 16(Suppl 1):96–99