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Effects of serotonin 5-HT_3 receptor antagonists on CRF-induced abnormal colonic water transport and defecation in rats

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ABSTRACT

The effects of corticotropin releasing factor (CRF) and serotonin (5-HT)$_3$ receptor antagonists on intestinal water transport are not well understood. Hence, we established a CRF-induced abnormal water transport model in rat colon, and evaluated the effects of 5-HT_3 receptor antagonists including ramosetron, alosetron, and cilansetron, and the antidiarrheal agent loperamide, in this model. In addition, the effects of 5-HT_3 receptor antagonists and loperamide on abnormal defecation induced by CRF in rats were examined. Colonic water transport was measured in colonic loops in conscious rats. Centrally administered CRF (3–30 μg/kg) markedly decreased colonic fluid loss, whereas oral administration of ramosetron (3, 30 μg/kg), alosetron (300 μg/kg), cilansetron (300 μg/kg), or loperamide (3 mg/kg) significantly inhibited it. Ramosetron (1–10 μg/kg), alosetron (10–100 μg/kg), cilansetron (10–100 μg/kg), or loperamide (0.3–3 mg/kg) also showed dose-dependent inhibition of CRF-induced defecation in rats. These results suggest that 5-HT_3 receptors are involved in both abnormal colonic water transport and defecation induced by CRF, and that the inhibitory effects of 5-HT_3 receptor antagonists on CRF-induced abnormal defecation partly result from their ameliorating action on colonic water transport.

1. Introduction

Irritable bowel syndrome is a functional disease with persistent gastrointestinal symptoms, mainly abdominal pain/discomfort and abnormal defecation, not accompanied by an organic disease (Longstreth et al., 2006). The pathogenesis of irritable bowel syndrome is not fully understood, but various psychogenic stresses which excite the descending nerve to induce abnormal motility and gut perception are considered to be major pathogenic factors (Grundy et al., 2006). It has been reported that stress-induced abnormal gut function including the stimulation of defecation and delay of gastric emptying was mimicked by exogenous corticotropin releasing factor (CRF) and inhibited by CRF receptor antagonists (Tache et al., 2004), suggesting that CRF plays an important role in stress-induced gut abnormalities. In addition, abnormal colonic transit and defecation induced by either stress or exogenous CRF were inhibited by serotonin (5-HT)$_3$ receptor antagonists (Miyata et al., 1998; Nakade et al., 2007). Recently, several 5-HT_3 receptor antagonists have been reported to improve the symptoms of irritable bowel syndrome with diarrhea, including abnormal bowel habits and abdominal pain, in clinical settings (Camilleri et al., 2000; Caras et al., 2001). These reports indicate that endogenous CRF, 5-HT, and 5-HT_3 receptors are involved in the pathogenesis of irritable bowel syndrome.

It is well known that the intestine is a major organ for water/electrolyte transport and that diarrhea is induced by an imbalance between water absorption and secretion across intestinal mucosa as well as by acceleration of colonic transit (Spiller, 2006). The 5-HT_3 receptors have been reported to regulate not only intestinal peristalsis and pain transmission but also colonic water/electrolyte transport. This was demonstrated by reports showing that 5-HT-induced increases in the short circuit current of isolated rat colon were inhibited by 5-HT_3 receptor antagonists (Kiso et al., 1997) and that a selective 5-HT_3 receptor agonist increased short circuit current (Kiso et al., 2001). Although it has been shown that stress caused abnormal water transport in rat intestine (Burdick et al., 1994; Empey and Fedorak, 1989), the influence of CRF on intestinal water/electrolyte transport is not well understood. Further, Funatsu et al. (2007) have reported that the inhibitory effect of 5-HT_3 receptor antagonists on stress-induced abnormal defecation is attributable to the ameliorating effects of such antagonists on stress-enhanced colonic transit in rats, but the effects of 5-HT_3 receptor antagonists on abnormal water/electrolyte transport induced by stress or CRF are, again, poorly understood.

In the present study, we therefore evaluated the influence of centrally administered CRF on colonic water transport in rats. Moreover, the effects of 5-HT_3 receptor antagonists including ramosetron, alosetron, and cilansetron on CRF-induced abnormal colonic water transport were examined in rats. We also explored the

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effects of these drugs on CRF-induced defecation in rats, and compared these effects with those of the antidiarrheal agent loperamide.

2. Materials and methods

2.1. Animals

Animals were housed in a temperature-controlled environment (22±2 °C) under a 12 h:12 h light/dark cycle, and were given food and water *ad libitum*. All experimental procedures were approved by the Animal Ethical Committee of Astellas Pharma Inc. (Tokyo, Japan).

2.2. CRF-induced abnormal colonic water transport in rats

Male Wistar rats (9 weeks old, Clea Japan, Inc., Tokyo, Japan) were used in this study. Rats were anesthetized by i.p. administration of pentobarbital sodium (50 mg/kg) and placed in a stereotaxic apparatus (Korf Instruments, Tujunga, CA, USA). The musculature on the skull was removed and the skull exposed. After a hole was drilled through the skull with a hand-operated drill (VIVA-MATE 3, Nakanishi Inc, Tochigi, Japan), a cannula for i.c.v. injection of drug was inserted perpendicular to the right lateral ventricle (coordinates: 0.8 mm caudal to bregma, 1.5 mm lateral from midline, 3.5 mm ventral from dura) (Miyata et al., 1998) and fixed to the skull with resin (REPAIRSIN, GC, Tokyo, Japan). Experiments were performed at least five days after the surgery. After the completion of an experiment, successful i.c.v. injection of CRF or the vehicle was confirmed by injection of 2 mg/ml of Evans blue solution i.c.v. If an i.c.v. injection was thought to have failed, the data obtained from that animal were excluded from the study.

Rats were fasted for 24 h and then lightly anesthetized with diethyl ether. The animals were kept warm using a thermostatic sheet and a lamp and the intestinal tract was exposed using a midline incision. A loop of approximately 13 cm was prepared by ligating the colon about 2 cm distal to the cecal-colon junction and about 2 cm proximal to the anus (Kobayashi et al., 2001). Physiological saline (2 ml) warmed to 37 °C was injected into the colonic loop immediately before ligation and the abdominal incision was then sutured. CRF or vehicle was then injected i.c.v. just after preparation of the colonic loop. Two hours after CRF injection, the colonic loop was removed under ether anesthesia to measure total weight. The loop was opened, washed, and wiped to remove residual contents, and the weight and length of each loop without intraluminal contents were then measured. The volume of residual intraluminal fluid was calculated as follows:

Residual fluid volume (μl/cm)=(isolated loop weight with intraluminal contents−isolated loop weight without intraluminal contents)/loop length

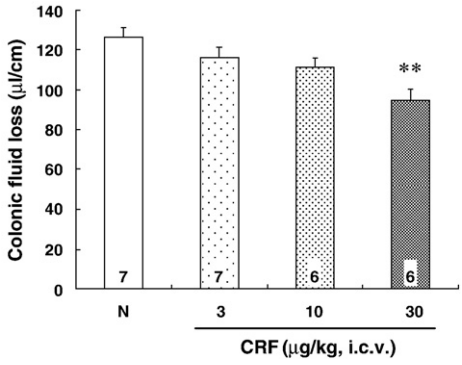

Fig. 1. Effects of CRF administered i.c.v. on colonic water transport in rats. Each column represents the mean±S.E.M. for six or seven animals. Number of animals used for each group is indicated within the column. ** $P<0.01$ compared to normal rats treated with saline i.c.v. using Dunnett's multiple range test. *N*: normal.

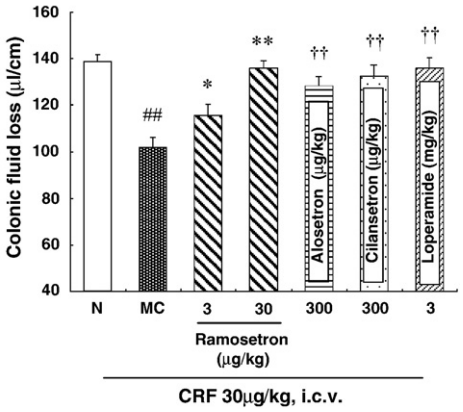

Fig. 2. Effects of ramosetron, alosetron, cilansetron, and loperamide on the CRF-induced decrease in colonic fluid loss in rats. Each column represents the mean±S.E.M. for 12 animals. ## $P<0.01$ compared to normal rats using Student's *t*-test. * $P<0.05$ and ** $P<0.01$ when ramosetron-treated rats were compared to the control group using Dunnett's multiple range test. †† $P<0.01$ when rats receiving alosetron, cilansetron, or loperamide were compared to the control group using Student's *t*-test. *N*: normal.

In standard group, the volume of residual intraluminal fluid was measured immediately after the preparation of colonic loop. Colonic fluid loss for 2 h was determined as follows:

Colonic fluid loss (μl/cm)=residual fluid volume in standard group (average)−residual fluid volume in each treatment group (individual value)

In the first experiment, four groups (six or seven rats per treatment) were included to evaluate the effects of CRF on colonic water transport: saline (i.c.v.), CRF (3, 10, 30 μg/kg, i.c.v.). The second experiments to evaluate the effects of test drugs on CRF-induced abnormal colonic transport involved the following treatments (12 rats per group): 0.5% (w/v) methylcellulose (MC) solution without CRF (normal group), 0.5% (w/v) MC (control group), ramosetron (3, 30 μg/kg), alosetron (300 μg/kg), cilansetron (300 μg/kg), and loperamide (3 mg/kg). The test drugs were administered orally 1 h before i.c.v. injection of CRF or vehicle.

2.3. CRF-induced defecation in rats

This experiment was performed according to the method of Miyata et al. (1998). Male Wistar rats (8 weeks old, Clea Japan, Inc.) were attached to cannula for i.c.v. injections by the method described above. At least five days after surgery, each rat (without fasting) was placed into an individual observation cage after i.c.v. administration of CRF. Preliminary experiments showed that CRF at doses of 1–30 μg/kg i.c.v. increased defecation, whereas the effect reached a plateau at doses of 10 μg/kg or more (data not shown). Further, the effect of CRF on defecation was sustained for 2 h (data not shown). Therefore, the inhibitory activity of a test drug was evaluated 2 h after i.c.v. administration of CRF at a dose of 10 μg/kg. Stools were collected 2 h after CRF injection to determine total wet weights. The experiment included six treatment groups (eight rats per group): 0.5% (w/v) MC without CRF (normal group), 0.5% (w/v) MC (control group), ramosetron (1, 3, 10 μg/kg), alosetron (10, 30, 100 μg/kg), cilansetron (10, 30, 100 μg/kg), and loperamide (0.3, 1, 3 mg/kg). Test compounds were administered orally 1 h before the injection of CRF or vehicle.

2.4. Drugs

Ramosetron hydrochloride, alosetron hydrochloride, cilansetron hydrochloride (all from Astellas Pharma Inc.), loperamide hydrochloride (Sigma-Aldrich Japan, Tokyo, Japan), and CRF (human, rat) (Peptide Institute, Inc., Osaka, Japan) were used in this study. Ramosetron, alosetron, and cilansetron were dissolved in distilled water and diluted with 0.5% (w/v) MC solution. Loperamide was suspended in and diluted with 0.5% (w/v) MC solution. CRF was

dissolved in and diluted with saline. In this study, all test compounds were used in their salt forms.

2.5. Statistical analysis

All results were statistically analyzed using the Statistical Analysis System version 8.2 (SAS Institute Japan Ltd., Tokyo, Japan). All data were described after rounded off to two significant figures. The mean ± S.E.M. of colonic fluid losses and stool weights were calculated for each treatment group. Statistical significances of differences were evaluated using Student's *t*-test and Dunnett's test for comparisons between pairs of treatment groups and amongst multiple treatment groups, respectively, with the significance level set to 5% ($P<0.05$). To determine drug inhibitory potencies on defecation, the ED_{50} values with 95% confidence limits (CLs) for each compound were estimated by linear regression analysis.

3. Results

3.1. CRF-induced abnormal colonic water transport in rats

In normal rats, the fluid in colonic loop was markedly decreased for 2 h after i.c.v. injection of saline, and the estimated colonic fluid loss was 130 ± 3.8 μl/cm (Fig. 1). Injection of CRF at doses of 3, 10, and 30 μg/kg, i.c.v. decreased colonic fluid loss in a dose-dependent manner, with a net fluid loss of 120 ± 5.3, 110 ± 4.5, and 95 ± 5.5 μl/cm, respectively, and a significant difference from the saline-treated group was observed at a dose of 30 μg/kg (Fig. 1). Therefore, the effects of test drugs on abnormal colonic water transport were evaluated in rats treated with CRF at a dose of 30 μg/kg, i.c.v.

Ramosetron (3 or 30 μg/kg) significantly improved the CRF-induced decrease in colonic fluid loss, and restored the fluid loss to the normal level at a dose of 30 μg/kg (Fig. 2). Similarly, alosetron (300 μg/kg), cilansetron (300 μg/kg), and loperamide (3 mg/kg) significantly improved the decreases in colonic fluid losses induced by CRF (Fig. 2).

3.2. CRF-induced defecation in rats

The total weight of stools excreted for 2 h after i.c.v. injection of saline was 0.47 ± 0.11 g in normal rats. In contrast, i.c.v. injection of CRF at a dose of 10 μg/kg significantly increased stool excretion to 3.8 ± 0.47 g over 2 h in control rats (Fig. 3). Oral administration of ramosetron (1, 3, and 10 μg/kg) showed dose-dependent inhibition of CRF-induced defecation and a significant difference from the control group was observed at a dose of 10 μg/kg (Fig. 3), with an ED_{50} value (CLs) of 7.7 (4.4–31) μg/kg (Table 1). Similarly, alosetron (10, 30, and 100 μg/kg), cilansetron (10, 30, and 100 μg/kg), and loperamide (0.3, 1, and 3 mg/kg) dose-dependently inhibited CRF-induced defecation (Fig. 3), with ED_{50} values (CLs) of 63 (44–110), 74 (39–610), and 1900 (1100–8900) μg/kg, respectively (Table 1). The potencies of alosetron, cilansetron, and loperamide relative to ramosetron were 1/8.2, 1/9.6, and 1/250, respectively (Table 1).

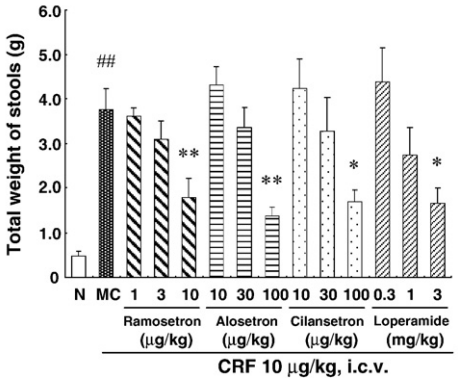

Fig. 3. Effects of ramosetron, alosetron, cilansetron, and loperamide on CRF-induced defecation in rats. Each column represents the mean ± S.E.M. for eight animals. ## $P<0.01$ compared to normal rats using Student's *t*- test. * $P<0.05$ and ** $P<0.01$ compared to 0.5% (w/v) MC-treated control rats using Dunnett's multiple range test. *N*: normal.

Table 1
Potencies of ramosetron, alosetron, cilansetron, and loperamide on inhibition of CRF-induced defecation in rats

	ED50 [95% confidence limits] μg/kg
Ramosetron	7.7 [4.4–31] (1)
Alosetron	63 [44–110] (1/8.2)
Cilansetron	74 [39–610] (1/9.6)
Loperamide	1900 [1100–8900] (1/250)

Values in parentheses represent potencies relative to ramosetron. The ED_{50} values with 95% confidence limits, which refer to the hydrochloride forms of test compounds, were estimated by linear regression analysis from eight animals.

4. Discussion

The present study revealed for the first time that CRF caused abnormal colonic water transport in rats. In addition, 5-HT_3 receptor antagonists inhibited both CRF-induced abnormal colonic water transport and defecation in rats. These results indicate that the inhibitory effect of 5-HT_3 receptor antagonists on CRF-induced abnormal defecation is, at least in part, due to an ameliorating action on colonic water transport, and that 5-HT_3 receptors are involved in stress-induced abnormal colonic water transport and defecation.

The colon performs a crucial function in water absorption and secretion. Because previous reports showed that stimulation of intestinal water secretion resulted in acceleration of intestinal transit and increase in stool frequency (Andresen et al., 2007; Camilleri et al., 2006), and that diarrhea was induced by an imbalance between water absorption and secretion across intestinal mucosa, intestinal water transport was considered to be closely related to defecation. It has been previously shown that stress caused a decrease in colonic fluid loss in rats (Burdick et al., 1994; Empey and Fedorak, 1989), and this phenomenon is thought to be one of the key processes in stress-induced diarrhea. CRF is well known to be released from hypothalamus in response to various stress, and play a major role in the stress-induced abnormal gut function (Tache et al., 2004). Centrally administered CRF mimics stress-induced gut abnormalities, such as delay of gastric emptying and stimulation of colonic motility, transit, and stool excretion, and peripheral and central administration of a CRF antagonist prevented stress-induced defecation (Tache et al., 2004). In contrast, the influence of CRF on colonic water/electrolyte transport has been less evaluated. In this study, we clearly showed for the first time that i.c.v. administration of CRF dose-dependently caused abnormal colonic water transport in rats. Thus, our present results suggest that CRF may be involved in stress-induced abnormal colonic water transport as well as abnormal gut motility.

The stimulatory effects of CRF on colonic motor function are mediated by the activation of parasympathetic and serotonergic pathways (Tache et al., 2004). Miyata et al. (1998) have shown that 5-HT_3 receptor antagonists significantly inhibit defecation induced by both stress and CRF in rats. These data are well consistent with the results of this paper, indicating that CRF, 5-HT, and 5-HT_3 receptors are involved in stress-induced abnormal defecation. As with CRF, 5-HT has been reported to act as a mediator of stress-induced colonic dysfunction, with symptoms including an increase in stool excretion, diarrhea, and abdominal pain (Crowell, 2004). It has been reported

that a 5-HT-induced increase in the short circuit current of isolated rat colon was inhibited by 5-HT_3 receptor antagonists (Kiso et al., 1997). Further, a selective 5-HT_3 receptor agonist has been shown to increase short circuit current (Kiso et al., 2001). From these results, we consider that 5-HT stimulates intestinal water secretion, as well as intestinal motility and pain transmission, via the activation of 5-HT_3 receptors. Taken together, these findings suggest that central CRF, administered exogenously or secreted in response to stress, enhances the release of endogenous 5-HT which, in turn, acts on the 5-HT_3 receptor, resulting in abnormal colonic water transport in addition to intestinal motility and defecation.

Funatsu et al. (2007) reported that conditioned-fear stress caused the acceleration of colonic transit with abnormal defecation in rats, and that 5-HT_3 receptor antagonists inhibited both of these processes in the same dose ranges, suggesting that the inhibitory effects of 5-HT_3 receptor antagonists on stress-induced abnormal defecation might be explained by the ameliorating effects of such antagonists on stress-enhanced colonic transit. A clinical trial has also shown that alosetron delayed colonic transit in patients suffering from irritable bowel syndrome at a dose which improved the symptoms of irritable bowel syndrome (Houghton et al., 2000). The present study is the first to show, however, that all 5-HT_3 receptor antagonists tested significantly inhibited CRF-induced abnormal colonic water transport in dose ranges almost identical to those significantly inhibiting abnormal defecation induced by CRF. Furthermore, it has been previously shown that all 5-HT_3 receptor antagonists used in this study significantly inhibited stress-induced abnormal defecation at almost the same doses with those to inhibit CRF-induced abnormal colonic water transport (Funatsu et al., 2007; Hirata et al., 2007). Thus, the inhibitory effect of 5-HT_3 receptor antagonists on CRF- and stress-induced abnormal defecation is considered to be attributable to their ameliorating action not only on abnormal colonic transit but also on water transport.

Loperamide, an antidiarrheal agent, is widely prescribed for patients suffering from irritable bowel syndrome with diarrhea, and has been reported to improve both the abnormal frequency of defecation and the stool form in clinical settings (Cann et al., 1984). In our present study, loperamide significantly inhibited CRF-induced abnormal colonic water transport and defecation. The pharmacological mechanism of action of loperamide has been shown to be primarily an agonistic effect on opioid μ-receptors (Awouters et al., 1993) and secondarily a direct inhibitory effect on calcium channels in intestinal mucosa, decreasing intracellular calcium concentration (Reynolds et al., 1984). Nishiwaki et al. (2000) reported that the activation of opioid μ-receptors on the myenteric plexus decreased the release of acetylcholine from nerve endings. Additionally, intracellular calcium regulates intestinal electrolyte/water transport, and low concentrations of intracellular calcium stimulate intestinal absorption (Berridge, 1983; Reynolds et al., 1984). Taking these data together, it appears that loperamide may slow CRF-accelerated intestinal transit by exerting an inhibitory effect on acetylcholine release via the activation of opioid μ-receptors, and ameliorate CRF-induced abnormal water transport by stimulating intestinal absorption via the inhibition of calcium channels, resulting in the reduction of intracellular calcium, in intestinal epithelial cells.

In conclusion, our present study revealed for the first time that CRF caused abnormal colonic water transport in rats. Moreover, 5-HT_3 receptor antagonists inhibited both the abnormal colonic water transport and defecation induced by CRF in rats at almost the same doses. These results indicate that 5-HT_3 receptors are involved in CRF-induced abnormal colonic water transport, as well as in abnormal defecation induced by CRF, and that the inhibitory effects of 5-HT_3 receptor antagonists on CRF-induced abnormal defecation are, at least in part, due to ameliorating actions on colonic water transport. Taken together, the data suggest that 5-HT_3 receptor antagonists will be promising therapeutic agents for irritable bowel syndrome with diarrhea in clinical settings.

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