In Vivo Assessment of Acceleration of Motor Activity Associated With Acetylcholine Release via 5-Hydroxytryptamine$_4$ Receptor in Dog Intestine

Noriaki Makimoto$^1$, Yasuko Sakurai-Yamashita$^2$, Akira Furuichi$^1$, Shunsuke Kawakami$^1$, Akihito Enjoji$^1$, Takashi Kanematsu$^1$ and Kohtaro Taniyama$^2$*

Departments of $^1$Surgery and $^2$Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8523, Japan

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ABSTRACT—Effect of mosapride, a benzamide, on the motor activity associated with the release of endogenous acetylcholine (ACh) from enteric neurons was examined in the ileum of anesthetized dogs using an in vivo microdialysis method and compared with the effect of 5-hydroxytryptamine (5-HT). Intraarterial administration of 5-HT accelerated intestinal motor activity and increased the concentration of dialysate ACh, and the responses were inhibited by SB204070, a specific 5-HT$_4$-receptor antagonist, but were apparently not affected by methiothepin, ketanserin and granisetron. Intraarterial administration of mosapride, a prokinetic benzamide, accelerated intestinal motor activity and the concentration of dialysate ACh increased. The effects of mosapride were antagonized by SB204070. Specific $[^{125}\text{I}]$SB207710 binding was observed in the myenteric and submucosal plexuses and muscle layers of dog ileum by in vitro receptor autoradiography. High densities of $[^{125}\text{I}]$SB207710 binding sites were detected in the myenteric and submucosal plexuses. Mosapride as well as SB204070 inhibited $[^{125}\text{I}]$SB207710 binding. Thus, in the whole body of dogs, 5-HT and mosapride accelerated the intestinal motor activity due to the increases in ACh release mediated by stimulation of the 5-HT$_4$ receptor.

Keywords: In vivo microdialysis, Receptor autoradiography, Mosapride, SB204070

5-Hydroxytryptamine (5-HT) locally regulates gastrointestinal motility via multiple 5-HT receptor subtypes. In in vitro experiments, 5-HT$_1$ and 5-HT$_3$ receptors have been shown to be opposite in functions related to mechanical activities of the intestine; activation of 5-HT$_1$ receptor inhibits contractile responses due to inhibition of ACh release (1) and activation of 5-HT$_4$ receptor potentiates the contractile response due to stimulation of excitatory neurons such as cholinergic neurons and tachykinin-containing neurons (2–7). Mechanical responses via the 5-HT$_4$ receptor also differ, depending on the species and anatomical region (8–11). In isolated preparations from rats, activation of the 5-HT$_4$ receptor relaxes the esophagus (12–14) and ileum (13). In isolated preparations from guinea pigs, the 5-HT$_4$ receptor participates in excitatory responses, contractions of stomach (7, 15), ileum (2, 4, 5) and colon (3, 6). In isolated human colonic preparations, activation of the 5-HT$_4$ receptor relaxes circular muscles (13, 16, 17), as is the case in rats.

The gastrointestinal prokinetic benzamides, such as metoclopramide and cisapride, interact with 5-HT receptors (8, 18). In vitro experiments using isolated intestinal preparations have shown that cisapride stimulates the release of acetylcholine (ACh) from enteric cholinergic neurons, mainly via 5-HT$_4$ receptors (4) and accelerates intestinal motility (4, 19, 20). Mosapride has 5-HT$_4$ receptor agonist properties, expresses gastrokinetic actions similar to those seen with cisapride (21, 22), and increases the electrically-evoked cholinergic contractions of the isolated guinea pig ileum (21).

Application of the microdialysis method to the gastrointestinal tissue may have a potential advantage over traditional methods using isolated preparations, as mechanisms underlying effects induced by bioactive substances and drugs are determined by measuring simultaneously functions and solute concentration within the region of gastrointestinal tissues in which a dialysis probe has been placed.

*Corresponding author. FAX: +81-95-849-7048
E-mail: taniyama@net.nagasaki-u.ac.jp
We have shown that the intestinal motor activity was associated with ACh release from enteric nerves in the whole body of dogs using the in vivo microdialysis method (23, 24).

We attempted to examine the mechanism underlying the acceleration of motor activity induced by 5-HT and mosapride in the whole body of dogs, in relation to ACh release using an in vivo microdialysis method.

MATERIALS AND METHODS

Animal preparation

The study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Nagasaki University, as adopted and promulgated by the notification of the Director-General of the Science and International Affairs Bureau of the Japanese Ministry of Education, Culture, Sports, Science and Technology, Japan. Healthy, mature, mongrel dogs of either sex, weighing between 8 and 15 kg were anesthetized with pentobarbital Na (30 mg/kg, i.v.), and surgical procedures were done under aseptic conditions. After exposing the abdominal cavity by a low ventral laparotomy, a 3-French Disposable catheter (ATOM Corp., Tokyo) connected to a injection-syringe was inserted into the intestinal marginal artery for the intraarterial injection of drugs. The area of arterial supply was defined by flushing with 1 ml of Ringer solution (147.0 mM Na\(^{+}\), 2.3 mM Ca\(^{2+}\), 155.6 mM Cl\(^{-}\) and 4.0 mM K\(^{+}\)). The animals were intubated and ventilated with room air mixed with oxygen. The intravenous injections of pentobarbital Na (30 mg/kg) were given as needed throughout the experiments to maintain anesthesia.

Procedure for recording of mechanical response

The animal preparation for mechanical response recording was carried out by the method of Daniel and Kostolanska (25). A strain gauge force transducer was sutured to the serosa of a defined area of the small intestine, and contractility in the circular muscle direction was recorded isometrically.

Procedure for microdialysis

A dialysis probe (O-P-100-10; EICOM, Kyoto) implanted in the wall of the small intestine was gently inserted tangentially into the wall of the small intestine, part of the dialysis membrane of the probe was passed through the longitudinal muscle layer, myenteric plexus and the circular muscle layer, and then the probe was sutured to the surface of the intestine at approximately the site of the transducer. The active site of the dialyzer was 10-mm-long, with a 0.2-mm inner diameter and a 0.22-mm outer diameter, and a weight cutoff value of 5 kDa. At the end of the experiment, the tissue from around the probe was dissected and the exact position of the probe was verified histologically. We assessed only data from experiments in which the probe had been implanted correctly.

Measurement of ACh concentration

The dialysis probe was connected to a perfusion pump (EP-60, EICOM) and to the injection valve of the set-up of high performance liquid chromatography with electrochemical detector (HPLC-ECD) system by means of polyethylene tubing. The motor-driven injection valve of an autoinjector (AS-10, EICOM) was controlled by an adjustable electronic timer. One stainless steel cannula was connected to the perfusion pump by a polyethylene tube, and the outlet of the other cannula was connected to the injection valve. In the present case, the internal standard, ethylhomocholine (EHC), delivered by the perfusion pump, was fed into the perfusate tube proximal to the injection valve. Preliminarily, a dialysis probe was exposed to 37°C Ringer solution containing 0.2 mM physostigmine with a constant ACh concentration (10\(^{-7}\) M), and dialysate samples were collected at various flow rates (1–8 \(\mu\)l/min), and then a flow rate of 2 \(\mu\)l/min was used for in vivo experiments. Under conditions of the flow rate of 2 \(\mu\)l/min, the in vitro recovery for ACh was fairly steady at 51.0 ± 1.6% with different ACh concentrations in the testing solution. Thus, the dialysis probe was continually perfused at a flow rate of 2 \(\mu\)l/min with Ringer solution containing 0.2 mM physostigmine. In vitro recovery was reported to be higher than in vivo recovery (26); however, for our experiments the in vitro value was considered to approximate the in vivo value. The dialysate was collected every 15 min in the sample loop of the automated sample injector, which was set up on line with the HPLC-ECD system. Since analysis of choline and ACh was complete within 15 min, the sample loop was set to be held in the load position for 15 min and was automatically switched to the injection position for 60 s, after which the cycle was repeated. The space between the dialysis membrane and detector was measured at the start of each experiment, and the lag time to expression of the drug effect was taken into account.

Experimental procedures

Concentrations of dialysate ACh collected at 15-min intervals remained at a fairly steady state level in each experiment from 60 to 240 min after probe implantation; therefore, the samples from the first to 4th fractions after probe implantation were discarded and 4 fractions (15-min dialysates) of the 5th to 8th fractions were determined to be the mean basal concentration of dialysate ACh. Intraarterial administrations of saline at the flow rate of less than 0.5 ml/min did not alter the concentration of dialysate...
ACh; therefore, saline containing substances was infused at the flow rate of 0.5 ml/min. When effects of intraarterial administration of 5-HT were examined regarding motor activity and concentration of dialysate ACh, 4 fractions of 15-min control dialysates were collected, and then 5-HT was infused into the marginal artery at a flow rate of 0.5 ml/min for 10 min. Effects of methiothepin, ketanserin, granisetron and SB204070 were examined by simultaneous infusion of these substances and 5-HT. Mosapride was infused into the marginal artery at a flow rate of 0.5 ml/min for 10 min. The concentrations of dialysate ACh in the presence of substance were given as the percentage of the basal concentration of dialysate ACh (before application of substance), in each experiment. 5-HT, methiothepin, granisetron and SB204070 were dissolved in saline immediately before use, and mosapride was dissolved in 1% lactic acid to the given concentrations.

In vitro receptor autoradiography

Five animals were used for receptor autoradiographic experiments. The small intestine was excised and immediately immersed in isopentane at −30°C. Frozen tissues were cut into 20-μm-thick sections on a cryostat, thaw-mounted onto gelatin-coated glass slides and stored overnight under vacuum at 4°C. After preincubation in buffer of the following composition: 50 mM Tris-HCl buffer (pH 7.4) containing 4 mM MgCl₂, 0.3% bovine serum albumin, 0.2 mM ascorbic acid, and 10 mM pargyline, at 23°C for 30 min, tissue sections were incubated in 2 ml of buffer containing [125I]SB207710 at the concentration of 10⁻¹¹ M at 23°C for 2 h. Consecutive tissue sections were labelled to characterise [125I]SB207710 binding in the presence of unlabelled 10⁻⁶ M SB204070 and 10⁻⁸ M mosapride. Then, the labelled sections were washed three times (for 1 min each) at 4°C in 50 mM Tris-HCl buffer (pH 7.2) and ice-cold distilled water and then dried under a stream of cold air. To obtain autoradiograms of a higher resolution, the dry-labelled sections were apposed against Hyperfilm-[^H] (Amersham, Buckinghamshire, UK) for 1 week and the films were developed using a D19 developer (Eastman Kodak, Rochester, NY, USA) for 7 min at 4°C. Cholinesterase in consecutive tissue sections was stained to verify the anatomical location of the myenteric plexus.

Statistics

A statistical analysis between the control and substance-treated group was made with Dunnett’s test or the Mann-Whitney U test (MUSCOT Statistical Analysis Program; Yukms Co., Ltd., Tokyo). A probability (P) value of <0.05 was considered to be statistically significant.

Drugs and chemicals

Substances used were as follows: [125I]SB207710 ((1-n-butyl-4-piperidinyl)methyl-8-amino-7-iodo-1,4-bezoxodioxane-5-carboxylate, 74 TBq/mmol) (Amersham); physostigmine (eserine) sulfate and tetramethylammonium chloride (Wako Pure Chemical Industries, Osaka); 1-decanesulfonic acid sodium salt (Tokyo Kasei Organic Chemicals, Tokyo); ethylhomocholine (EICOM); choline chloride (Naclalai Tesque, Kyoto); acetylcholine perchlorate, 5-hydroxytryptamine (5-HT) creatinine sulfate and ketanserin tartrate (Sigma, St. Louis, MO, USA); methiothepin mesylate (Funakoshi Chemical Co., Ltd., Tokyo). Mosapride was generously provided by Dainippon Pharmaceutical Co., Ltd. (Osaka). Granisetron and SB204070 ((1-n-butyl-4-piperidinyl)methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate) were generously provided by SmithKline Beecham (Worthing, UK). Other chemicals used were of reagent grade.

RESULTS

Basal concentration of dialysate ACh

As the basal concentration of ACh in the dialysate collected at 15-min intervals decreased over 60 min after probe implantation, subsequently reaching a fairly steady state level, the experiments were started 60 min after implantation of the probe. Four samples (dialysates collected at 15-min intervals) of the first to 4th fractions were determined as a mean basal concentration of dialysate ACh. Basal concentration of dialysate ACh was 0.733 ± 0.380 pmol/15 min (n = 15), a value that remained constant until 240 min from 60 min after perfusion; there was little variation among different dogs. Intraarterial infusion of saline also did not alter the concentration of dialysate ACh or the motor activity (data not shown).

Effect of 5-HT on motor activity and the concentration of dialysate ACh

After 4 fractions of dialysates had been collected as a control, 5-HT was infused into the marginal artery at a flow rate of 0.5 ml/min for 10 min. 5-HT at the doses of 10⁻⁹ and 10⁻⁸ mol accelerated both the motor activity and concentration of dialysate ACh, in a dose-dependent manner (Fig. 1). Concentrations of dialysate ACh were increased to approximately 1.3 times and 1.5 times over the basal concentration in the first 15-min fraction in the case of 5-HT at 10⁻⁹ and 10⁻⁸ mol, respectively, and reverted to the basal concentration with time.

When SB204070, a specific 5-HT₂ antagonist at 10⁻¹⁰ mol was simultaneously infused with 5-HT at 10⁻⁹ mol for 10 min, the motor activity and concentration of dialysate ACh did not accelerate (Fig. 2). Infusion of SB204070 at 10⁻¹⁰ mol alone decreased slightly, but not significantly, the motor activity and concentration of ACh in the dialysate.

Methiothepin, a 5-HT₁ antagonist; ketanserin, a 5-HT₂ antagonist; ketanserin, a 5-HT₂ antagonist.
antagonist; and granisetron, a 5-HT₁ antagonist, did not affect the 5-HT-induced acceleration of motor activity and concentration of dialysate ACh (Fig. 3).

**Effect of mosapride on motor activity and concentration of dialysate ACh**

Since mosapride was dissolved in 1% lactic acid, the effect of this vehicle was also examined. Intraarterial administration of 1% lactic acid accelerated the motor activity by approximately 4 g and the concentration of ACh in the dialysate by approximately 30%. Intraarterial administration of mosapride at 10⁻¹⁰ mol significantly accelerated the motor activity and concentrations of ACh in the dialysate, as compared to the case of induction with 1% lactic acid.
acid. Since mosapride (10^{-7} mol)-induced accelerations of motor activity and the ACh release were maintained over 60 min after injection of this drug, SB204070 or saline was administered 30 min after the injection of mosapride. The mosapride (10^{-7} mol)-induced effects were represented as the effects obtained by subtracting the lactic acid effects from the effects of mosapride dissolved in lactic acid. SB204070 at 10^{-9} mol or saline was infused into the marginal artery 30 min after the infusion with mosapride at 10^{-7} mol at a flow rate of 0.5 ml/min for 10 min. Concentration of dialysate ACh was calculated taking the basal concentration of dialysate ACh (before application of mosapride) as 100%. Each value represents the mean ± S.E.M. from 5 animals. *Significantly different from the basal value and #significantly different from the value in the infusion of saline after infusion of mosapride (P<0.05).

**DISCUSSION**

Exogenously applied 5-HT accelerated the intestinal motor activity associated with ACh release via 5-HT_{4} receptors. In our previous study, ACh in the dialysate was determined to originate from the enteric cholinergic nerve terminals, based on the findings that intraarterial administration of tetrodotoxin inhibited spontaneous motor ac-

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**Fig. 4.** Mosapride-induced increases in motor activity and concentration of dialysate ACh. A: Representative pattern of motility. B: Time-course of dialysate ACh concentration. The mosapride (10^{-7} mol)-induced effects were represented as the effects obtained by subtracting the lactic acid effects from the effects of mosapride dissolved in lactic acid. SB204070 at 10^{-9} mol or saline was infused into the marginal artery 30 min after the infusion with mosapride at 10^{-7} mol at a flow rate of 0.5 ml/min for 10 min. Concentration of dialysate ACh was calculated taking the basal concentration of dialysate ACh (before application of mosapride) as 100%. Each value represents the mean ± S.E.M. from 5 animals. *Significantly different from the basal value and #significantly different from the value in the infusion of saline after infusion of mosapride (P<0.05).

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**Distribution of specific binding of [^{125}I]SB207710 in the small intestine**

Figure 5 shows typical receptor autoradiograms of [^{125}I]SB207710 binding sites in the dog small intestine. The [^{125}I]SB207710 binding was visible in the intestine (Fig. 5A) and was abolished by the addition of unlabelled 10^{-6} M SB204070 (Fig. 5B). When comparing with the consecutive cholinesterase-stained sections (Fig. 5C), specific [^{125}I]SB207710 binding sites were seen in the myenteric and submucosal plexuses, muscle layers and mucosal layer. The dense binding sites of [^{125}I]SB207710, indicated by arrow heads and arrows, corresponded to findings with the myenteric plexus and submucosal plexus in the section stained with cholinesterase (Fig. 5C), respectively. The density was higher in the circular muscle layer than in the longitudinal muscle layer. Specific [^{125}I]SB207710 bindings were abolished by 10^{-6} M mosapride (Fig. 5D).
vity and Ach release (24) and the nerve-stimulated contrac-
tions and increases in Ach release (23). The released
Ach, which was associated with motility of circular
muscle, was suggested to originate mainly from the cholin-
ergic nerve terminals in the deep muscular plexus of the
circular muscle layer (27, 28).

5-HT infused into the marginal artery accelerated the
motor activity and increased the dialysate Ach concen-
trations in the small intestine of the dog. The acceleration
by 5-HT was inhibited by SB204070, a selective 5-HT4-
receptor antagonist (29), but not by 5-HT1, 5-HT2 and
5-HT3 antagonists; therefore, 5-HT acted at 5-HT4 recep-
tors located on cholinergic neurons, and the stimulation of
5-HT4 receptors increased the release of Ach from enteric
cholinergic nerve terminals. The 5-HT3 receptor has been
shown to participate in stimulation of the propulsive ac-
vity of guinea pig colon (30), yet in the whole body of
dog, the 5-HT3 receptor does not appear to participate in
the acceleration of motor activity. These findings may
explain the mechanism underlying the 5-HT4 recep-
tor-mediated stimulation of gut motility detected in the in vivo
studies (9, 31, 32). In isolated tissues, stimulations of
5-HT4 receptors were seen to modulate the motility of the
gastrointestinal tract, in either an excitatory or an inhibitory
manner, depending on species and anatomical regions
(8, 10, 13, 16, 17). In the colon, 5-HT4 agonists applied to
mucosa have been shown to induce the release of calcitonin
gene-related peptide (CGRP) due to the activation of
5-HT4 receptors located on CGRP-containing sensory
nerves, and CGRP causes release of vasoactive intestinal
peptide (VIP) caudad and substance P (SP) release oral to
the site of stimulation, with the release of VIP and SP being
accompanied by circular muscle relaxation and contraction,
respectively (29, 33). It has been proposed that the 5-HT4
receptors located on myenteric neurons and on CGRP-
containing sensory nerves may play an integral part in
initiating the peristaltic reflex, by releasing excitatory
substances and CGRP (11). On the other hand, an inhibi-
tory 5-HT receptor, such as the 5-HT1 receptor, was
reported to be present in the intestine (1, 30). 5-HT applied
exogenously binds to both inhibitory 5-HT1 receptor and
excitatory 5-HT4 receptor, and in the whole body of dogs,
the excitatory 5-HT4 receptors were found to function
predominantly.

Fig. 5. Typical receptor autoradiographic localization of [125I]SB207710 binding sites and cholinesterase staining in the dog small intestine. Consecutive, 20-μm-thick sections were labeled with 10−11 M [125I]SB207710 in the absence (total binding) (A) and presence of 10−6 M SB204070 (non-specific binding) (B) and 10−6 M mosapride (D), in vitro. C: Histochemical staining of cholinesterase. Arrow heads and arrows indicate the myenteric plexus and submucosal plexus, respectively.
Mosapride, a prokinetic benzamide, accelerated intestinal motor activity in parallel with increases in the ACh release within the dog small intestine in the whole body. As the mosapride-induced acceleration of motor activity and increase in ACh release were antagonized by SB204070, mosapride may act at the 5-HT₄ receptor and accelerating effects would ensue. Mosapride has been shown in in vitro experiments to enhance the electrically-stimulated contractions of isolated longitudinal muscles with attached myenteric plexus of the guinea pig ileum via the 5-HT₄ receptor (21). Our present study using the intact whole body of dogs revealed that mosapride-induced acceleration of intestinal motor activity was associated with ACh release from enteric cholinergic neurons via the 5-HT₄ receptor. The excitatory effect of mosapride lasted markedly longer than that of 5-HT. We have no clear explanation for this event, although the dissociation constant of mosapride for the 5-HT₄ receptor may contribute to the long-acting effect of mosapride, while exogenously applied 5-HT may be metabolized by endogenous enzyme. Furthermore, the acceleration of motor activity and ACh release induced by mosapride were greater than those induced by 5-HT. Mosapride may act at the excitatory 5-HT₄ receptor, without acting at the inhibitory 5-HT₁ receptor, as shown by receptor binding assay (34), while 5-HT may act at both excitatory and inhibitory 5-HT receptors.

Specific binding sites of [¹²⁵I]SB207710 were detected in the dog small intestine. [¹²⁵I]SB207710 is a radioligand of high specific activity and selectivity for 5-HT₄ receptors (35), and [¹²⁵I]SB207710 binding was abolished by the addition of unlabelled SB204070; therefore, specific [¹²⁵I]SB207710 binding sites may reveal the 5-HT₄ receptors. A comparison with cholinesterase stained tissue demonstrated that the dense binding sites of [¹²⁵I]SB207710 detected in the myenteric plexus and submucosal plexus may indicate that receptors locate on the nerve cell body/dendrite. With regard to the 5-HT₁ receptors detected in the muscle layer, it remains to be determined if the receptors are located on smooth muscle cells and/or nerve terminals innervating smooth muscle cells. The motor activity of circular muscle was proposed to be associated with ACh released from cholinergic nerve terminals located mainly in the deep muscular plexus of the circular muscle layer (26, 27). The deep muscular plexus is an aggregation of nerve fibers and terminals that originate from cell bodies of the myenteric ganglia (36). The densities of 5-HT₄ receptors were markedly higher in the myenteric and submucosal plexuses than in the muscle layers; therefore, 5-HT₁ receptors located in high density on the myenteric plexus may mainly participate in the motor activity of circular muscle. Mosapride also inhibited specific [¹²⁵I]SB207710 binding. Mosapride has binding affinity for 5-HT₄ receptor, with no binding affinity for the dopamine D₂ receptor, 5-HT₁ receptor, 5-HT₂ receptor, and α-adrenoceptor (21, 34), being different from cisapride, a prokinetic benzamide; therefore, mosapride may be a selective 5-HT₄ agonist. Thus, mosapride was confirmed to express 5-HT₄ receptor-mediated functions, as indicated in physiological experiments (21).

Mechanisms of actions of many substances in vivo have been determined by extrapolating data on in vitro studies. The present studies using an in vivo microdialysis technique within the wall of dog gastrointestinal tissues, revealed that 5-HT, receptor-mediated excitatory effects on intestinal motor activity were associated with increases in ACh release. Mechanisms underlying the stimulatory effects of mosapride on intestinal motor activity are apparently 5-HT₄ receptor-mediated increases in ACh release.

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