Stimulatory Effects of HSR-803 on Ileal Motor Activity

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ABSTRACT—Stimulatory effects of HSR-803 on intestinal motor activity in vitro were studied in guinea pig ileum. HSR-803 (1 × 10⁻⁶-1 × 10⁻⁴ M) increased the amplitude of longitudinal muscle contractions and increased the frequency of peristalsis in isolated segments of guinea pig ileum. The stimulatory effect in amplitude and not frequency was abolished by 1 × 10⁻⁶ M atropine. In the Magnus method with ileal segments, HSR-803 (1 × 10⁻⁷-1 × 10⁻⁴ M) produced contractions concentration-dependently, which were inhibited by atropine (1 × 10⁻⁸ and 3 × 10⁻⁸ M) and 3 × 10⁻⁷ M tetrodotoxin (TTX). In the [³H]-quinuclidinyl benzilate (QNB) binding experiment with ileal smooth muscle, HSR-803 had low affinity for acetylcholine (ACh) receptors (pKᵢ=4.47±0.04). In addition, HSR-803 failed to increase the spontaneous release and the electrical stimulation-induced [³H]ACh release in ileal smooth muscle. On the other hand, HSR-803 (1 × 10⁻⁵ M) enhanced contractions induced by ACh, but had no effect on contractions induced by carbachol, which is not hydrolyzed by acetylcholinesterase (AChE). In conclusion, HSR-803 stimulated ileal motor activity. However, HSR-803 had low affinity for ACh receptors and had no influence on ACh release. It is likely that HSR-803 stimulated motor activity mainly due to prevention of ACh hydrolysis.

Keywords: Ileum (guinea pig), Peristalsis, HSR-803, [³H]Acetylcholine release, [³H]Quinuclidinyl benzilate binding

HSR-803 (N-[4-[2-(dimethylamino)ethoxy]benzyl]-3,4-dimethoxybenzamide hydrochloride) is a newly synthesized gastroprokinetic agent. It was shown to stimulate gastric motor activity in conscious dogs (1), and HSR-803 also accelerated not only gastric emptying in dogs and rats but also accelerated small intestinal transit in mice (2). In conscious dogs, HSR-803 reversed the dopamine-induced inhibition of gastric motility and enhanced acetylcholine (ACh)-induced gastric contractions (1). In in vitro studies, the antagonistic nature of HSR-803 toward dopamine D₂-receptors was suggested by receptor binding assays with rat striatum membranes, and HSR-803 inhibited the activity of acetylcholinesterase (AChE) derived from electric eel (3). These data suggested that HSR-803 stimulated gastric motility through dopamine-D₂-receptor blocking and AChE inhibition. Sakaguchi et al. (4) have already reported that the anti-AChE activity of some newly synthesized benzamide derivatives, including HSR-803, was closely related to the motor-stimulating activity in the ileum. Recently, we (5) have been able to study the steady peristalsis of the ileum for a few hours in vitro by a procedure based on the method of Bülbir and Lin, with modifications (6). In the present study, we used this method to examine whether HSR-803 stimulates peristaltic movement. Furthermore, we studied the mechanisms through which HSR-803 stimulates intestinal motor activity by the Magnus method, receptor binding assay and the measurement of [³H]ACh release.

MATERIALS AND METHODS

Effect of HSR-803 on peristalsis in isolated guinea pig ileum

Male guinea pigs weighing 300–500 g were killed by a blow on the head and 20-cm segments of the ileum approximately 15 cm proximal to the ileoceleal junction were taken. After the mesentery was carefully removed, a portion of the ileum (3–4 cm) was horizontally held in a 40-ml organ chamber. Continuous peristalsis was induced according to our previously reported method (5).

The oral and anal ends of the ileum were connected to the inflow silicon tube and the outflow T-shaped glass can-
nula, respectively. To the connected point of the oral end, the tip of a stainless rod was tied, and the other tip was tied to a strain-gauge (Shinkoh, Nagano) to record the longitudinal muscle contraction. One end of the T-shape glass cannula was connected to a pressure transducer (Nihon Kohden, Tokyo) to record the intraluminal pressure.

Intraluminal perfusion was performed at the rate of 0.3–0.4 ml/min. The bathing solution which contained 3 × 10−10 M pirenzepine to enhance the peristalsis (7) in the organ chamber was also continuously exchanged at the rate of 2 ml/min. The perfusion solution and the bathing solution were Krebs solution (120 mM NaCl, 2.0 mM CaCl2, 1.0 mM MgCl2, 20.0 mM NaHCO3, 5.0 mM HEPES, 1.0 mM NaHPO4 and 14.0 mM glucose). When the effects of HSR-803 or physostigmine were investigated, the drugs were added to the organ chamber, and the bathing solution was switched to the drug-containing one. The experiments were done at 35°C.

Effect of HSR-803 on the contractions of isolated guinea pig ileum in the Magnus method

A segment of guinea pig ileum about 15 mm in length was suspended with a 0.5-g counterweight in a 10-ml organ chamber containing Krebs Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.55 mM CaCl2, 1.18 mM MgSO4, 1.18 mM KH2PO4, 24.88 mM NaHCO3 and 11.1 mM glucose). Krebs Henseleit solution was maintained at 37°C and gassed with 95% O2 and 5% CO2. Contractile responses were recorded by means of an isotonic transducer (Nihon Kohden or Medical Electronics Commercial Co., Tokyo). The preparations were allowed to equilibrate for at least 30 min until spontaneous contractions became constant. After the equilibration period, the response to 1 × 10−6 M ACh was recorded, to be taken as 100%, and then the preparation was washed 3 times within 15 min. In the experiments to study the effects of HSR-803 or ACh alone, a single dose of HSR-803 or ACh was applied 30 min after the last washout. Dose-response curves for HSR-803 or ACh were obtained in the presence or absence of HSR-803 or physostigmine in 3 × 10−7 M TTX-treated ileum on the same schedule (n = 7) as described above. In all experiments, the single dose experiment was performed only once in one preparation.

Effect of HSR-803 on the specific binding of [3H]quintulin/clidinium benzilate (QNB) to ACh receptors on guinea pig ileum membranes

Binding assays were performed using smooth muscle membranes of guinea pig ileum. The mucosal layers were removed from the isolated ileum, and the muscle strips were homogenized in 20 volumes of sucrose solution (0.32 M sucrose, 0.05 M Tris-HCl, pH 7.5) in a Polytron™ (Kinematica, Luzern, Switzerland), at a setting of 7 with three 15-sec bursts. The homogenates were centrifuged (49,000 × g) for 10 min at 4°C. Hard foam on the supernatant and the pellets were discarded. The supernatant was adjusted to 50 volumes of assay buffer (20 mM HEPES, 10 mM Tris-HCl, 5 mM KCl and 10 mM NaCl, pH 7.4) and centrifuged (49,000 × g) for 10 min at 4°C (Hitachi, Tokyo). The pellets were collected and homogenized again in 20 volumes of assay buffer in a Polytron (setting 7, 15 sec × 2). After the homogenates were centrifuged (49,000 × g) for 10 min at 4°C, the resulting pellets were resuspended in 50 volumes of buffer in the Polytron. The membrane preparation was kept at −80°C until used. The binding assay was performed by a modification of the method of Yamamura and Snyder (8).

In briefly, aliquots (2.3 ml) of membrane preparation (2.5–5.5 μg/ml) were added to tubes containing 0.1 ml of 1-[3H]QNB (1639.1 GBq/mmol, New England Nuclear, Boston, MA, U.S.A., final concentration 58–108 pM) and 0.1 ml of test compounds or 0.1 ml of atropine (final concentration 1 μM) to define the nonspecific binding. Incubation was carried out for 60 min at 37°C. The Kd and Bmax values were examined by Scatchard analysis, and the pKb value was calculated by the method of Cheng and Prusoff (9). Proteins were assayed by the method of Bradford (10). The binding assay was carried out in duplicate.

Effect of HSR-803 on [3H]ACh release in longitudinal muscle-myenteric plexus preparations

This experiment was performed according to the method of Kilbinger and Wessler (11). Longitudinal muscle-myenteric plexus preparations were prepared from the segments of guinea pig ileum. The preparations of about 3 cm in length were mounted vertically under a tension of 0.5 g in an organ chamber. Each preparation was incubated for 1 hr in Krebs Henseleit solution containing [3H]-choline (1 μM; 5 μCi/ml, 87.8 Ci/mM, New England Nuclear) at 37°C. In the latter half of this period, electrical stimulation (4 V, 0.2 Hz, 1 msec duration × 360 pulses) was applied in order to label ACh stores. After the labeling period, the preparation was washed out with Krebs Henseleit solution containing 10 μM hemicholinium-3 to prevent reuptake of choline. After 1-hr washing, the superfusate was collected in 1-min fractions. The preparations were stimulated by field stimulation (4 V,
1 Hz, 1 msec duration \times 30 \text{ pulses}) through the platinum electrodes. Electrical stimulation was applied at 10 and 40 min after the beginning of the superfusate collection, named S1 and S2, respectively. Drug application was initiated 15 min before S2 by switching the superfusate to Krebs Henseleit solution containing drugs, and the superfusion with drugs was continued for 30 min. Tritium in the superfusate samples was measured by liquid scintillation spectrometry (Packard, Meriden, CT, U.S.A. or Aloka, Tokyo). The outflow of tritium produced by electrical stimulations or each drug (HSR-803 or 5-hydroxytryptamine (5-HT)) was obtained from the difference between the total tritium outflow of 10-min collections and the estimated spontaneous outflow. The effects of drugs on ACh release were examined by calculating the ratios of tritium outflow produced by drugs or S2 to tritium outflow produced by S1.

**Drugs**

HSR-803 was synthesized by Central Research Laboratories, Hokuriku Seiyaku Co., Ltd., Katsuyama, Japan. Acetylcholine chloride (Dai-ich Seiyaku Co., Ltd., Tokyo), carbachol (carbamylcholine chloride, Sigma, St. Louis, MO, U.S.A.), physostigmine (Sigma), neostigmine (Nacalai Tesque, Kyoto), atropine sulfate monohydrate (Wako Chemical Industries Ltd., Osaka), pirenzepine dihydrochloride (Sigma), 5-hydroxytryptamine hydrochloride (Sigma) and hemicholinium-3 (Sigma) were purchased. [3H]QNB and [3H]choline were purchased from New England Nuclear/DuPont.

**Statistical analysis**

Student’s t-test (non-paired) or the Aspin-Welch method was used to assess statistical significance. Significance was accepted at \( P < 0.05 \).

**RESULTS**

**Effect of HSR-803 on peristalsis in isolated guinea pig ileum**

An adequate loading pressure (2 – 3 cmH2O) produced peristalsis almost constantly in the isolated guinea pig ileum (Fig. 1). As shown in Fig. 1B, a cycle of peristalsis consisted of the repetitive phasic contractions of longitudinal muscle (LC) accompanied by a gradual increase in tonic tension, and the propagating constriction of the circular muscle accompanied by a transient rise in the intraluminal pressure (P). The constriction of the circular muscle initiated at the oral end at the threshold pressure, and propagated toward the anal end, thereby ejecting the intraluminal fluid. Accompanying the ejection, the longitudinal muscle was elongated, and then the next cycle of peristalsis was started.

Figure 1A shows the effects of increasing concentrations of HSR-803 on the peristalsis. HSR-803 at \( 1 \times 10^{-7} \text{ M} \) did not influence the peristaltic movements, but HSR-803 at concentrations of \( 1 \times 10^{-6} \text{ M} \) or more enhanced the contractions of the longitudinal muscle and increased the frequency of peristalsis. The pressure rise during the ejection was reduced by HSR-803. The onset of the action of HSR-803 was rapid, and the full effects developed in a few min after the application of HSR-803. The action persisted, although somewhat weakened in time, as long as HSR-803 was present in the bathing solution. At its peak effect, \( 1 \times 10^{-5} \text{ M} \) HSR-803 increased the longitudinal...
muscle contractions by 31.1±7.2% (mean ±S.E., n=15, P<0.01), and increased the frequency from 15.2±1.1 times per 10 min to 18.6±1.3 times per 10 min (P<0.01).

The fast recordings shown in Fig. 1B demonstrate that in the presence of 1×10⁻⁵ M HSR-803, the longitudinal muscle contractions were enhanced during the accumulation of Krebs solution in the lumen, and that the threshold pressure to trigger the ejection was not significantly affected by HSR-803. The latter fact may indicate that the increase in peristalsis frequency can not be ascribed to the decreased threshold. Possibly, due to the enhanced contractions of longitudinal muscle, the period for the intraluminal pressure to reach the threshold was shortened, leading to the frequency increase.

HSR-803 reduced the ejection pressure (Fig. 1, A and B). However, it was visually observed that during the ejection, the ileal segment became thinner in the presence of HSR-803 than in the absence of the drug, indicating that HSR-803 did not inhibit the circular muscle contraction. Since the ejection pressure is thought to depend not only on the strength of constriction of the circular muscle but also on the volume of intraluminal fluid, the reduction in the ejection pressure may be due to the reduced amount of Krebs solution accumulated between the ejections when the peristalsis cycle length was shortened. Similarly, physostigmine at 1×10⁻⁷ M enhanced the longitudinal contractions, increased the frequency, but decreased the ejection pressure (Fig. 1C).

It has been reported that the guinea pig ileum developed the peristalsis even in the presence of atropine (12, 13). Under the present experimental conditions, the atropine-resistant peristalsis occurred soon after the application of 1×10⁻⁶ M atropine as shown in Fig. 2. HSR-803 at 1×10⁻⁵ M did not affect the atropine-resistant peristalsis (Fig. 2). Similar results were obtained in 4 ileal segments.

Effect of HSR-803 on the contractions of isolated guinea pig ileum in the Magnus method

HSR-803 stimulated contraction in a concentration-dependent manner in isolated guinea pig ileum (Fig. 3). The stimulatory effect of HSR-803 was observed at 1×10⁻⁷ M or more, and its maximum effect was observed at 1×10⁻⁴ M (Fig. 3). The effect of ACh was observed at 1×10⁻⁸ M or more, and its maximum effect was observed at 1×10⁻⁵ M (data not shown). ACh (1×10⁻⁶ M) induced a rapid increase in tonic tension accompanied by small phasic contractions. On the other hand, HSR-803 (1×10⁻⁵ M) induced a gradual increase in tonic tension superimposed by phasic contractions.

Atropine (1×10⁻⁸ and 3×10⁻⁸ M) pretreatment shifted the dose-response curves for HSR-803 to the right and decreased the maximal responses; and at 1×10⁻⁷ M, atropine almost abolished the contractile responses to HSR-803, showing noncompetitive inhibition (Fig. 3). The dose-response curve for ACh was shifted to the right in a parallel way by 1×10⁻⁴ and 1×10⁻⁷ M atropine (data not shown). Atropine (1×10⁻⁵ M) rapidly and significantly inhibited both contractile responses induced by HSR-803 (1×10⁻⁵ M) and ACh (1×10⁻⁶ M), but TTX (3×10⁻⁷ M) inhibited only the HSR-803-induced contraction (data not shown). Physostigmine (1×10⁻⁷ M) also induced contractions, which were inhibited by atropine and TTX (data not shown). Thus these data suggest that the ACh released from neurons was necessary to the HSR-803-induced contraction.

Effect of HSR-803 on the specific binding of [³H]QNB to ACh receptors on guinea pig ileum membranes

Atropine potently displaced [³H]QNB binding, and its

![Fig. 2. Effects of 1×10⁻⁵ M HSR-803 on the atropine-resistant peristalsis. Even after the application of 1×10⁻⁶ M atropine, the isolated segment (3-4 cm) of guinea pig ileum constantly developed peristalsis (atropine-resistant peristalsis), although the frequency was much reduced. HSR-803 was applied in the presence of atropine. LC, P: same as in Fig. 1.](image)

![Fig. 3. Stimulatory effects of HSR-803 in guinea pig ileum in the absence or presence of atropine. HSR-803 produced contractions in a concentration-dependent manner. Atropine shifted the concentration-response curves for HSR-803 to the right and decreased the maximal responses. Symbols indicate control responses (○, n=4-6) or responses in the presence of atropine (□: 1×10⁻⁸ M, n=5-7; △: 3×10⁻⁴ M, n=5-6; ◇: 1×10⁻⁷ M, n=7).](image)
pK₁ was 9.66±0.30. The pK₁ values of physostigmine and neostigmine were 4.14±0.04 and 4.67±0.33, respectively. HSR-803 also displaced [³H]QNB binding only in high concentrations; and its pK₁ was 4.47±0.04, suggesting that the affinity of HSR-803 for ACh receptors was low.

**Effect of HSR-803 on [³H]ACh release in the longitudinal smooth muscle-myenteric plexus preparations**

Two consecutive electrical stimulations (S₁ and S₂) produced [³H]-outflow. During the superfusion with 3×10⁻⁷ M TTX, S₂ produced no [³H]ACh release, suggesting that [³H]ACh was released from neurons (Table 1). On the other hand, the superfusion with 1×10⁻⁵ M 5-HT produced a transient but significant increase in [³H]ACh release (Table 1), as previously reported by Kilbinger and Pfeuffer-Friederich (14). The superfusion of 1×10⁻⁵ M HSR-803 did not increase a spontaneous [³H]ACh release or an electrical stimulation-induced [³H]ACh release (Table 1). These data suggested that HSR-803 had no effect on the ACh release.

**Enhancing effects of HSR-803 on the contractions induced by ACh or carbachol in guinea pig ileum**

This experiment was performed to study whether the AChE inhibition of HSR-803 was able to influence the ACh-induced contractions or not. As previously described, HSR-803 alone produced contractions in the ileum (Fig. 3). Thus in the presence of 3×10⁻⁷ M TTX, we have studied the effects of HSR-803 on exogenous ACh and carbachol. The contractile responses to ACh were significantly enhanced by 1×10⁻⁵ M HSR-803, which stimulated peristalsis (Fig. 4A). However, HSR-803 (1×10⁻⁵ M) failed to enhance the contractile responses to carbachol in the presence of TTX (Fig. 4A). The same result was obtained by 1×10⁻⁵ M physostigmine, which stimulated peristalsis (Fig. 4B). These data suggest that ACh hydrolysis was an important factor in the action of HSR-803.

**DISCUSSION**

Non-ulcer dyspepsia, one of the gastrointestinal disorders, with such symptoms as discomfort in the epigastrium, a feeling of fullness in the abdomen, nausea and emesis is mainly due to delayed gastric emptying. Metoclopramide and domperidone, which are known to

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**Table 1. Effects of HSR-803, 5-HT and TTX on [³H]ACh release in longitudinal muscle-myenteric plexus preparations**

| Drug (M) | D/S₁ | S₂/S₁ |
|----------|------|-------|
| Control | 0.01±0.01 | 0.71±0.04 |
| HSR-803 | 0.08±0.06 | 0.71±0.06 |
| 5-HT | 0.79±0.17* | 0.67±0.12 |
| TTX | 0.08±0.09 | 0.03±0.05** |

D/S₁: Effects of drugs on spontaneous release of [³H]ACh. S₂/S₁: Effects of drugs on the electrical stimulation-induced release of [³H]ACh. The effects on the spontaneous release were estimated from the data collected for 10 min immediately after the drug perfusion. The effects on the electrical stimulation-induced release were estimated from the data collected for 10 min immediately after each electrical stimulation. The data represent the means±S.E. Numerals in parentheses are the number of experiments. *P<0.05 vs. control, **P<0.01 vs. control.

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**Fig. 4. Effects of HSR-803 (A) and physostigmine (B) on contractile responses to acetylcholine (ACh) and carbachol (CCh) in guinea pig ileum. HSR-803 at a concentration of 1×10⁻⁵ M significantly enhanced only the acetylcholine-induced contractions. Each column and vertical bar represent the mean and S.E. for 7 experiments. All experiments were performed in the presence of 3×10⁻⁷ M tetrodotoxin. *P<0.05, **P<0.01 vs. the contractile responses in the absence of HSR-803.**
be dopamine-D₂-receptor antagonists (15), are used in the treatment of non-ulcer dyspepsia, because they stimulate gastric emptying. HSR-803 is a novel gastroprokinetic agent with a benzamide structure in its molecule. HSR-803 was reported to stimulate gastric contractile activity (1), gastric emptying and small intestinal transit (2). HSR-803 also has a dopamine D₂ blocking action like the above gastroprokinetics (3).

On the other hand, HSR-803 has an anti-AChE activity (1, 16). Sakaguchi et al. (4) studied the effects of many HSR-803 analogues on AChE and the contraction of ileum, and it was found that there was a significant correlation between the AChE inhibition and the contractile response of the ileum. In conscious dogs, HSR-803 enhanced the gastric contractions induced by exogenous ACh but not the bethanechol-induced contractions, suggesting that AChE inhibition was one of the mechanisms through which HSR-803 stimulated gastric motility.

In the present study, HSR-803 increased the frequency of peristalsis and the amplitude of contractions in the direction of the longitudinal muscle in guinea pig ileum. Similar stimulation of peristalsis in the ileum was observed following the application of the AChE inhibitor physostigmine. In addition, the stimulatory effect of HSR-803 was not observed in atropine-treated preparations. Thus these data suggested that the stimulation by HSR-803 was exerted through cholinergic mechanisms.

In the following experiments, action mechanisms through which HSR-803 stimulated ileal peristalsis were studied. In the Magnus method, HSR-803 (1 × 10⁻⁷ -1 × 10⁻⁴ M) produced contractions in guinea pig ileum in a concentration-dependent manner, and atropine (1 × 10⁻⁸ -1 × 10⁻⁷ M) inhibited these responses. As mentioned above, HSR-803 failed to stimulate peristalsis in the presence of atropine. All these data suggest that the stimulation of HSR-803 was exerted through cholinergic mechanisms. In addition, the HSR-803-induced contraction in the ileum was strongly suppressed by 3 × 10⁻⁷ M TTX, which did not affect the ACh-induced contraction at all. ACh seems to be always synthesized and released from the cholinergic neurons in the isolated guinea pig ileum. The fact that HSR-803 produced no contraction in the presence of TTX strongly suggests that the released ACh is responsible for the HSR-803-induced contraction. It is unlikely that the action of HSR-803 depends on ACh receptors on the smooth muscle. The results of the [³H]-QNB binding study support that HSR-803 did not activate ACh receptors directly.

Another possible mechanism was the increase in ACh release. Gastroprokinetic benzamides, metoclopramide and cisapride were reported to facilitate ACh release (17, 18). Therefore we examined the effects of HSR-803 on the [³H]ACh release in longitudinal muscle-myenteric plexus preparations. These preparations responded to 1 × 10⁻⁵ M 5-HT, resulting in an increase in spontaneous ACh release as previously reported (14, 19). However, HSR-803 alone did not increase spontaneous [³H]ACh release from neurons and did not enhance the electrical stimulation-induced [³H]ACh release. Thus these data suggest that HSR-803 failed to increase ACh release. The last possible mechanism was the prevention of ACh hydrolysis. In the presence of TTX, HSR-803 enhanced ACh-induced contractions, but not carbachol-induced contractions like physostigmine. Because carbachol is not hydrolyzed by AChE, it is clear that the enhancement was produced by the inhibition of ACh hydrolysis. This in vitro result was consistent with the data in conscious dogs demonstrating that HSR-803 enhanced the ACh-induced gastric contractions and not the bethanechol-induced contractions. Thus it was considered that HSR-803 inhibited both AChE located on the smooth muscle membrane and AChE secreted from neurons (20), and then ACh was accumulated around ACh receptors, so that smooth muscle motor activity was increased.

On the other hand, HSR-803 has a dopamine-D₂-blocking action (3). In the guinea pig stomach, dopamine inhibited ACh release through dopamine-D₂-receptors located on cholinergic neurons, and a dopamine-D₂-receptor antagonist restored the ACh release (21, 22). In this study, HSR-803 did not influence ACh release in the ileum, even though contractile responses to HSR-803 were dependent on the neural ACh. Thus it was likely that the action of HSR-803 in the ileum was independent of dopamine D₂-receptors.

In conclusion, HSR-803 stimulated intestinal motor activity. However, HSR-803 had low affinity for ACh receptors and did not affect ACh release. It is likely that HSR-803 stimulated intestinal motor activity mainly due to prevention of ACh hydrolysis.

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