Facilitation of Acetylcholine Release by SK-951, a Benzofuran Derivative, via the 5-Hydroxytryptamine\textsubscript{4} Receptor in Guinea Pig Stomach

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ABSTRACT—Facilitation of acetylcholine (ACh) release by SK-951 ((−)4-amino-N-[2-(1-azabicyclo[3.3.0]octan-5-yl)ethyl]-5-chloro-2,3-dihydro-2-methylbenzo[b]furan-7-carboxamide hemifumarate), a benzofuran derivative, via the 5-hydroxytryptamine (5-HT\textsubscript{4}) receptor in guinea pig stomach was examined by in vitro receptor autoradiography and functional studies. \textsuperscript{[\textsuperscript{125}I]}SB207710 binding was detected in the myenteric plexus of the gastric corpus. High densities of binding sites were observed in the myenteric plexus and a moderate density in the muscle layer. SK-951 inhibited the binding of \textsuperscript{[\textsuperscript{125}I]}SB207710, a specific 5-HT\textsubscript{4} receptor ligand, as in the case of SB204070, a specific 5-HT\textsubscript{4}-receptor antagonist, thus indicating the presence of 5-HT\textsubscript{4} receptors in guinea pig stomach. SK-951 as well as 5-HT enhanced the electrically stimulated twitch contractions of gastric corpus strips, which were sensitive to tetrodotoxin and atropine, and enhanced electrically stimulated release of ACh from corporal strips, which was tetrodotoxin-sensitive and Ca\textsuperscript{2+}-dependent. The enhancements of twitch contractions and ACh release by SK-951 were antagonized by GR113808, a selective 5-HT\textsubscript{4}-receptor antagonist. Thus, SK-951 binds to 5-HT\textsubscript{4} receptors of the guinea pig gastric corpus and may accelerate gastric motility due to facilitation of ACh release.

Keywords: SK-951, \textsuperscript{[\textsuperscript{125}I]}SB207710 binding, Acetylcholine release, 5-Hydroxytryptamine\textsubscript{4} receptor, Guinea pig stomach

Stimulation of 5-hydroxytryptamine (5-HT\textsubscript{4}) receptors accelerates the motility of the gastrointestinal tract in vivo, while this stimulation shows excitatory or inhibitory effects on isolated preparations, depending on the species and the anatomical region examined (1). The inhibitory response appears to be induced by stimulation of the 5-HT\textsubscript{4} receptors mainly located on the smooth muscle cells (2), while excitatory responses are mediated by stimulation of receptors mainly located on the excitatory neurons such as cholinergic neurons and tachykinin-containing neurons (3–8). In the stomach, 5-HT\textsubscript{4} receptors seem to have an excitatory effect on motility both in vivo and in vitro (8, 9). To obtain gastrointestinal prokinetic drugs with higher potency than currently available benzamide derivatives, we previously synthesized and evaluated a series of compounds (10, 11). SK-951((−)4-amino-N-[2-(1-azabicyclo[3.3.0]octan-5-yl)ethyl]-5-chloro-2,3-dihydro-2-methylbenzo[b]furan-7-carboxamide hemifumarate), a benzofuran derivative that is structurally similar to benzamides, was found to have 5-HT\textsubscript{4}-receptor agonist properties, and it was shown to have gastrokinetic effects similar to those of cisapride (12). This agent increases the electrically evoked cholinergic contractions of the isolated guinea pig ileum mediated by stimulation of 5-HT\textsubscript{4} receptors similar to the effects of benzamide derivatives (12). Whether SK-951 can act as a 5-HT\textsubscript{4}-receptor agonist in the stomach remains to be elucidated. Thus, the present study was performed to examine the presence of 5-HT\textsubscript{4} receptors in the guinea pig stomach, to determine the binding affinity of SK-951 to 5-HT\textsubscript{4} receptors located on the myenteric neurons by in vitro receptor autoradiography, and the effects of this agent on contractility and acetylcholine (ACh) release in the guinea pig gastric corpus.

MATERIALS AND METHODS

Preparation of stomach strips
Male Hartley guinea pigs weighing 300 to 450 g were decapitated, and the stomach was dissected out. Circular strips were immediately cut from the body of the stomach. The mucosa was carefully removed from the tissue, and muscle layers and the intramural plexus were left intact.
In vitro receptor autoradiography

The mucosa-free preparations were immediately immersed in isopentane at –30°C. The frozen tissues were cut into sections 20-μm-thick on a cryostat, thaw-mounted onto gelatin-coated glass slides, and then stored overnight under vacuum at 4°C. Tissue sections were incubated with 25 pM [3H]5HT, a specific 5-HT₆-receptor ligand (13), in 50 mM Tris-HCl buffer (pH 7.4) containing 4 mM MgCl₂, 10 μM pargyline, 0.3% bovine serum albumin and 0.2 mM ascorbic acid for 2 h at 23°C in the presence or absence of various concentrations of SK-951 following pre-incubation in the same buffer for 30 min. Nonspecific binding was determined in adjacent sections, incubated with 25 pM [3H]SB207710, in the presence of 1 μM SB204070, a specific 5-HT₆-receptor antagonist (14). The labeled sections were then washed three times (for 1 min each) at 4°C in 50 mM Tris-HCl buffer (pH 7.2), tapped in ice-cold distilled water and dried under a stream of cold air. The radioligand binding sites in the related tissue sections were quantified using the computerized radioluminographic imaging-plate system (Bio-imaging analyzer BAS 5000; Fuji Photo Film Co., Tokyo). The dry labeled sections were exposed to a radioluminographic imaging plate with calibrated [3H]Istandards ([3H]micro-scales; Amersham, Buckinghamshire, UK) and the results are expressed as means ± S.E.M. in fmol/mg, and the specific binding and its inhibition by SK-951 were calculated. To obtain autoradiograms of a higher resolution, the dry labeled sections were opposed against Hyperfilm-3H (Amersham), which was then developed using a D19 developer (Eastman Kodak, Rochester, NY, USA) for 7 min at 4°C.

Measurement of [3H]ACh release

Strips from the guinea pig gastric corpus were preincubated with [3H]choline for 60 min at a final concentration of 2 x 10⁻⁷ M in oxygenated Krebs' solution. After washing in fresh medium for 60 min, the strips were mounted between two parallel platinum electrodes and superfused at 34–36°C at a flow rate of 1 ml/min with Krebs' solution gassed with 95% O₂ and 5% CO₂ containing 10⁻⁴ M hemicholinium-3 to prevent the uptake of [3H]choline. Experiments were started 90 min after superfusion of tritium had reached a plateau. Two parallel platinum electrodes were used to stimulate the intramural nerves. The strips were stimulated electrically for 2 min with pulses 1 ms in duration, intensity of 8 V and frequency of 2 Hz. Drugs were applied 10 min before stimulation. The superfusate was collected every 2 min and the radioactivity of the samples was determined by counting in a liquid scintillation spectrometer (Beckman, Fullerton, CA, USA).

The validity of assuming total tritium as a measure of [3H]ACh release under the present experimental conditions was documented in our previous study (16). The release of [3H]ACh was represented as the fractional rate obtained by dividing the amount of tritium in the superfusate by that in the tissue. The tritium content of the tissue at each period was calculated by adding the amount of tritium in each fraction to the tritium content of the tissue at the end of the experiment. From each of the release curves obtained by plotting the fractional release of tritium against time, the stimulated release was estimated as the amount of tritium in the fraction after application of stimulation, and thus the stimulated release of [3H]ACh was calculated as increase over the basal release.

Calculations and statistics

Results are expressed as means ± S.E.M. Data were analyzed by one-way analysis of variance followed by Fisher’s protected least significant difference procedure. The pEC₅₀ values were calculated by fitting linear regression to the concentration-effect curve. The E₅₀max values were calculated graphically.

Drugs and chemicals

Drugs and chemicals used were as follows: [3H]choline chloride (81.0 Ci/mmol) (New England Nuclear Research Products, Boston, MA, USA); [3H]SB207710 ((1-n-butyl-4-piperidinyl)methyl-8-amin-7-iodo-1,4-benzodioxane-5-carboxylate, 74 TBq/mmol) (Amersham); SK-951, GR-113808 ([1-[2-(methylsulfonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate) and granisetron (Sanwa Kagaku Kenkyusho Co., Ltd., Mie); 5-hydroxy-
tryptamine HCl (Sigma Chemical Co., St. Louis, MO, USA); methysergide maleate and ketanserin tartrate (Research Biochemicals Incorporated, Natick, MA, USA); atropine sulfate, tetrodotoxin and hexamethonium chloride (Wako Pure Chemical Industries Ltd., Osaka); and hemicolinium-3 (Aldrich Chemical Co., St. Louis, MO, USA). SB204070 ((1-n-butyl-4-piperidinyl)methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate) was generously provided by Smith Kline Beecham, Worthing, UK. Other chemicals used were of reagent grade.

RESULTS

Effects of SK-951 on the $[^{125}]$I/SB207710 binding

$[^{125}]$I/SB207710 binding sites were detected in the muscle layer including the myenteric plexus of the guinea pig gastric corpus (Fig. 1A). High densities of binding sites were observed in the myenteric plexus (indicated by arrows in Fig. 1A) and a moderate density was found in the muscle layer. Specific binding of $[^{125}]$I/SB207710 at a concentration of 25 pM was calculated as 0.232 ± 0.003 fmol/mg in the myenteric plexus and 0.106 ± 0.006 fmol/mg in the muscle. Binding was abolished by addition of 1 μM SB204070 (Fig. 1C). SK-951 also inhibited $[^{125}]$I/SB207710 binding; this agent at 1 μM decreased the density in the myenteric plexus by approximately 80% and that in the muscle layer by approximately 66% (Fig. 1B). The inhibition was dependent on the concentration of SK-951 in both the myenteric plexus (Fig. 2) and the muscle layer (data not shown).

Effects of SK-951 on electrically stimulated twitch contractions

Application of electrical stimulation (ES) with a single pulse (1 ms, 8 V) to the muscle layer preparations attached to the intramural plexus caused twitch contractions. ES-evoked twitch contractions were inhibited by application of $3 \times 10^{-7}$ M tetrodotoxin, $10^{-7}$ M atropine or removal of Ca$^{2+}$ from the Krebs’ solution, but $3 \times 10^{-3}$ M hexamethonium showed no effect (data not shown). 5-HT ($10^{-10} - 3 \times 10^{-7}$ M) enhanced ES-evoked twitch contractions in a concentration-dependent manner, and the enhancement by 5-HT was completely antagonized by GR113808 (a selective 5-HT$_2$-receptor antagonist) (Fig. 3). SK-951 ($3 \times 10^{-10} - 10^{-6}$ M) also enhanced the ES-evoked twitch contractions in a concentration-dependent manner, and the enhancement by SK-951 was completely antagonized by GR113808 (Fig. 3). The pEC$_{50}$ values for 5-HT and SK-951 were 8.89 and 7.88, respectively, and the Emax values for 5-HT and SK-951 were 45.8% and 57.0%, respectively (Table 1).

Effects of SK-951 on electrically stimulated outflow of $[^{3}H]$ACh

The spontaneous outflow of $[^{3}H]$ACh from guinea pig stomach preparations preloaded with $[^{3}H]$choline reached a steady level at 90 min after the start of superfusion. ES (1 ms, 8 V) at 0.2 Hz for 2 min produced an increase in the outflow of $[^{3}H]$ACh. The ES-evoked outflow of $[^{3}H]$ACh was abolished by application of $3 \times 10^{-7}$ M tetrodotoxin or by removal of Ca$^{2+}$ from the superfusate (data not shown). SK-951 at $10^{-6}$ M significantly enhanced the ES-evoked outflow of $[^{3}H]$ACh without affecting the spontaneous outflow. The enhancement induced by SK-951 was completely antagonized by pretreatment with $10^{-6}$ M GR113808 (Fig. 4).
Table 1. pEC<sub>50</sub> and E<sub>max</sub> values for 5-HT<sub>4</sub> receptors mediating electrical stimulation evoked twitch response in guinea pig stomach

<table>
<thead>
<tr>
<th></th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt; [95% Confidence limit]</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (%) [95% Confidence limit]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>8.89 [8.78 – 9.28]</td>
<td>45.8 [16.6 – 75.0]</td>
</tr>
<tr>
<td>SK-951</td>
<td>7.88 [7.83 – 7.96]</td>
<td>57.0 [46.9 – 67.2]</td>
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Values are calculated as the mean of 7 preparations.

**DISCUSSION**

The binding of [<sup>125</sup>I]SB207710, a specific 5-HT<sub>4</sub>-receptor ligand (13), in the myenteric plexus and muscle layer was abolished by SB204070, a specific 5-HT<sub>4</sub>-receptor antagonist (14). The presence of specific [<sup>125</sup>I]SB207710 binding sites indicated the presence of 5-HT<sub>4</sub> receptors. Thus, the present study demonstrated that 5-HT<sub>4</sub> receptors are present in the guinea pig stomach. [<sup>125</sup>I]SB207710 binding was much more dense in the myenteric plexus than in the muscle layer, suggesting that the 5-HT<sub>4</sub> receptors may be predominantly located on the nerve cell body/dendrites. In the muscle layer, however, it remains unclear whether the receptors are located on the smooth muscle cells and/or nerve terminals innervating smooth muscle cells. SK-951 also inhibited the binding of [<sup>125</sup>I]SB207710, similarly to SB204070. Therefore, SK-951 was shown to bind to the 5-HT<sub>4</sub> receptors located on the nerve cell body/dendrites in
the guinea pig stomach.

To determine the function of SK-951 binding to 5-HT₄ receptors within the myenteric plexus, the effects of SK-951 were examined in relation to the activity of cholinergetic neurons in mucosa-free preparations. The electrically stimulated twitch contractions of the muscle layer preparations attached to intramural plexus were tetrodotoxin-sensitive and atropine-sensitive. Therefore, the twitch contractions are mediated by stimulation of cholinergetic neurons. SK-951 as well as 5-HT caused enhancement of the twitch contractions in a concentration-dependent manner, and the enhancement was completely prevented by GR113808, a specific 5-HT₄-receptor antagonist (15). It has been reported that the maximum response of ES-evoked twitch contraction in guinea pig stomach by 5-HT in the presence of methysergide, ketanserin and granisetron was partially depressed by SDZ 205-557, a 5-HT₄-receptor antagonist (8). Buchheit et al. (17) showed that SDZ 205-557 is a 5-HT₄-receptor antagonist of 5-HT-evoked contraction in guinea pig ileum with a pA₂ value of 7.4. The pKₐ value for SDZ 205-557 in stomach was 7.5, but this antagonism was not a simple competitive one (8). Gale et al. (15) showed that GR113808 was a competitive antagonist and had a pA₂ value of 9.2 in guinea pig colon. This antagonism of the response to 5-HT may reflect the depression of the maximum response. In longitudinal muscle with myenteric plexus preparations from guinea pig ileum, the 5-HT- and SK-951-induced enhancement of ES-evoked contractions were competitively antagonized by GR113808 (12). Furthermore, both SK-951 and 5-HT enhanced the electrically stimulated Ca²⁺-dependent and tetrodotoxin-sensitive release of ACh from preparations of muscle layers attached to the intramural plexus in a concentration-dependent manner, and the enhancement was prevented by GR113808. These results indicated that both SK-951 and 5-HT facilitate release of ACh from the cholinergic nerve terminals due to stimulation of the 5-HT₄ receptors and then cause contraction of the guinea pig gastric corpus. Stimulation of 5-HT₄ receptors has been shown to evoke the release of ACh from the guinea pig stomach (8) and to cause cholinergic and non-cholinergic contraction of the rat gastric fundus (18). Thus, SK-951 as well as 5-HT may express its functions through 5-HT₄ receptors located at least on the cholinergic enteric nerve cell bodies/dendrites in the myenteric plexus of the guinea pig gastric corpus, as demonstrated by autoradiographic analysis of the receptor.

In the stomach, the 5-HT₄ receptors have contractile functions in the human gastric fundus, corpus and antrum (19); rat gastric fundus (18); and guinea pig gastric corpus (8, 9) and antrum (20). These responses appear to be mediated via 5-HT₄ receptors located on the cholinergic neurons in the guinea pig gastric corpus (8) and the non-cholinergetic neurons in the rat gastric fundus (16). SK-951 was found to bind to 5-HT₄ receptors and act as an agonist in the myenteric plexus of the gastric corpus. Stimulation of 5-HT₄ receptors enhances gastric emptying (21, 22), and thus SK-951 may act to accelerate gastric motility as a gastromotoric.

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