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Methylisogermabullone isolated from radish roots stimulates small bowel motility via activation of acetylcholinergic receptors

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Abstract

We have previously reported that extract of radish roots exhibits an increase in gastrointestinal motility through the activation of muscarinic acetylcholine (ACh) receptors. Based on the stimulatory activity-guided fractionation on rat ileal segments, this study isolated methylisogermabullone (MIGB, $C_{23}H_{31}O_5NS$, MW 433) from methanol extracts of radish roots. MIGB caused a significant increase of the isolated rat ileal contraction in a concentration-dependent manner (23–693 μM), and the pattern of MIGB-induced ileal contraction was different in the time course to that produced by ACh. The EC50 value of MIGB, to produce 50% maximum ileal contraction, was estimated to be 45.5 μM. MIGB (230 μM)-induced ileal contractions were enhanced by pretreatment of segments with ACh (0.1 μM). Ileal contractions produced by MIGB (230 μM) or ACh (0.1 μM) at submaximal concentration were partially inhibited by pretreatment of hexamethonium (0.1 mM), a ganglionic blocker, whereas they were almost completely abolished by atropine (10 μM). Oral administration of MIGB to mice stimulated the small intestinal transit of charcoal in a dose-dependent manner (10–100 mg kg^{-1}), and MIGB (100 mg kg^{-1})-induced stimulation of small intestinal transit was significantly attenuated by co-administration of atropine (50 mg kg^{-1}). Taken together, these results demonstrate that MIGB isolated from radish roots stimulates the small bowel motility through the activation of ACh receptors. These findings suggest that MIGB may become a potential regulatory agent for therapeutic intervention in dysfunction of gastrointestinal motility.

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Introduction

In the gastrointestinal tract, acetylcholine (ACh) receptors, metabotropic muscarinic (mAChR) and ionotropic nicotinic receptors (nAChRs) play important physiological roles in the stimulation of spontaneous phasic and tonic contraction in the fasting and postprandial states (Wood 1984; Shi & Sarna 1997). In mammals, the main subtypes of mAChR present in the gastrointestinal tract are M_2 and M_3, with a ratio of four to one (Stadelmann et al 1998; Unno et al 2003). M_2 receptors act predominantly via inhibition of the adenylate cyclase activity and hence decrease the intracellular levels of adenosine 3′,5′ cyclic monophosphate, and M_3 receptors cause activation of phospholipase C and subsequent hydrolysis of phosphatidyl inositol bisphosphate into inositol triphosphate and diacylglycerol. These biochemical reactions are generally considered to be a principal mechanism involved in the regulation of gastrointestinal motility. ACh, acting at nAChRs, is the principal excitatory neurotransmitter in the myenteric plexus. Recent immunohistological studies of myenteric neurons have suggested that nAChRs may also be located at presynaptic sites (Kirchgessner & Liu 1998; Obaid et al 1999). Indeed, functional evidence for presynaptic nAChRs within the enteric nerve system has been reported for myenteric excitatory motor neurons innervating longitudinal smooth muscle (Delbro & Lange 1997; Galligan 1999).

In a previous report (Jung et al 2000), we have demonstrated that extract of radish roots stimulates the spasmodic activity of the gastrointestinal tract through regulation

of post-junctional muscarinic receptors in-vitro and in-vivo. In this regard, we have tried to purify the bioactive phytochemical(s) contained in radish roots, and stimulatory activity-guided fractionation of radish extracts on the isolated rat ileal segments led to identification of methylisogermabullone (MIGB). To assess the pharmacological property of MIGB on the spasmogenic activity of the gastrointestinal tract, this study was undertaken to examine whether MIGB can modulate the contractility of the isolated ileal segments in-vitro and the small intestinal transit in-vivo. The results obtained in this study clearly indicate that MIGB stimulates small bowel motility through activation of the acetylcholinergic pathway.

Materials and Methods

Chemicals and reagents

ACh, atropine and hexamethonium were obtained from Sigma Chemicals (St Louis, MO). All other chemicals were of the highest grade from commercial sources.

Extraction and separation of methylisogermabullone

The freeze-dried roots of radish (377 g) were extracted three times with MeOH (2 L) for 21 days at room temperature, and the MeOH extracts (40.3 g) were washed with Et_2O. The MeOH extracts were concentrated (35.1 g) and chromatographed on Sephadex-LH 20 using $CHCl_3$ and MeOH (10:1 $\rightarrow$ 3:1) as solvents (each fraction was checked by TLC) to give 6 fractions (F1–F6). Fraction 2 (3.04 g) retained the activity, and was thus fractionated further by silica gel column chromatography (3×50 cm; stepwise gradient of 10%, 20% and 50% (v/v) $CHCl_3$ in MeOH, followed by 300 mL of MeOH; collecting 50 mL fraction). A portion (275 mg) of the active fraction was subjected to the recycling preparative HPLC JAIGEL-GS 310 column (21.5×500 mm, MeOH over 58 min, 3.5 mL min^{-1}, detection at 210 nm) to yield compound **1** (129 mg, 0.03% w/w). The chemical structure of the isolated compound (Figure 1) was determined by analysis of 1D NMR and 2D NMR data and in comparison with literature values (Greger et al 1994): ^{1}H NMR (600 MHz, CD_3OD): δ 7.78 (1H, d, $J = 16.8$ Hz, H-2″), 7.67 (1H, d, $J = 16.8$ Hz, H-3″), 7.46 (2H, d, $J = 8.6$ Hz, H-2,6), 6.61 (2H, d, $J = 8.6$ Hz, H-3,5), 6.38 (1H, s, H-6′), 6.30 (1H, s, H-4′), 4.05 (2H, m, H-1′), 3.64 (2H, m, H-8), 2.80 (3H, s, -N-CH_3), 2.77 (3H, s, =S-CH_3), 2.70–2.65 (2H, m, H-7), 2.36 (2H, m, H-2′), 1.50 (6H, s, H-8′, 9′), 1.31 (3H, s, H-10′); ^{13}C NMR (150 MHz, CD_3OD): δ 178.4 (C-5′), 174.2 (C-1″), 162 (C-4), 158 (C-3′), 152.9 (C-7′), 131.8 (C-2, 6), 130.9 (C-2″), 129.8 (C-3″), 123.9 (C-4′, 6′), 123.7 (C-1), 117.6 (C-3, 5), 68.5 (C-1′), 51.6 (C-8), 39.2 (C-2′), 38.4 (S-CH_3), 29.6 (N-CH_3), 25.3 (C-8′), 18.1 (C-9′), 16.9 (C-10′). Copies of the original spectra are obtainable from the corresponding author.

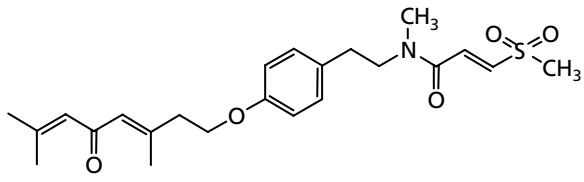

Figure 1 Chemical structure of methylisogermabullone isolated from radish roots ($C_{23}H_{31}O_5NS$, MW 433).

Animals

Male Sprague-Dawley rats, 180–200 g, were used for examining gastrointestinal motility in-vitro, and male ICR mice, 25–30 g, were used to test small intestine transit of charcoal in-vivo. All animals were handled in accordance with standards established by the Korean Association for Laboratory Animal Science. The study was begun after acclimatization of animals for 1 week under a temperature of 22 ± 2°C, a relative humidity of 50–60% and 12-h dark–light cycle. Food was withheld for 16 h before the experiments, but there was free access to drinking water.

Measurement of ileal contractility

The contractile responses of the isolated ileal segments were measured as described previously (Jung et al 2000). In brief, rats were slaughtered by decapitation, and segments of ileum (approximately 1.5 cm) were carefully removed in a rostral direction, starting at a point approximately 10 cm from the caecum, and intraluminal contents were flushed out using Krebs–Ringer bicarbonate buffer (KRB buffer in mM: NaCl 112, KCl 5.9, $CaCl_2$ 2, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.5, pH 7.4). Ileal segments were longitudinally mounted in a 5-mL organ bath containing KRB buffer and kept at 37°C and oxygenated with 95% O_2 and 5% CO_2. The segments were washed and allowed to equilibrate for the first 30 min with a washout every 10 min, and tension of 0.5 g was then slowly applied to the tissues. The ileum was washed and allowed to rest minimally for 10 min between each test condition, and ileal contractions were measured for approximately 8–13 min. ACh was employed as a positive control to compare the effects of MIGB on the ileal contraction. Changes in ileal contractions were isometrically measured using a force-displacement transducer (Grass) and biological recording system (PowerLab 2/25, AD Instruments). All contractile responses were expressed as mass equivalents (i.e., g) minus resting tension.

Evaluation of gastrointestinal transit

To examine the effects of MIGB on intestinal motility in-vivo, the small intestinal transit in mice was measured by a slight modification of the method described previously (Jung et al 2000). For examining the competitive effect of atropine on MIGB-induced gastrointestinal transit, atropine

($50\,mg\,kg^{-1}$) was subcutaneously injected 5 min before administration of MIGB. Several doses ($10–100\,mg\,kg^{-1}$) of MIGB were orally administered 10 min before treatment with marker (0.2 mL/mouse, 10% charcoal suspension in 5% Arabic gum). Mice were slaughtered by cervical dislocation at 15 min after administration of charcoal marker, and the small intestine was quickly removed avoiding stretching. The gastrointestinal transit was then evaluated by comparing the distance travelled by the charcoal meal from the pyloric sphincter with the total length of the small intestine from the pyloric sphincter to the ileo-caecal junction. Control mice received the drug vehicle by the oral route at the same time. The distance travelled by the marker was expressed as a percentage of the total length of small intestine.

Statistical analysis

Values obtained in this study were expressed as mean ± s.e. of seven experiments. The significance of differences was evaluated by Student's *t*-test or one-way analysis of variance followed by post-hoc analysis by Student–Newman–Keuls test. $P < 0.05$ was considered significant. The EC50 value (concentration producing 50% maximal contraction) was interpolated from concentration–response curves by means of personal computer programs (GraphPad Prism and InStat).

Results

Contraction of ileal segments

There is, to our knowledge, no information suggesting whether MIGB pharmacologically modulates gastrointestinal motility in-vitro or in-vivo. Therefore, this study firstly examined the effects of MIGB on gastrointestinal motility using isolated rat ileal segments. It was found that MIGB reproduced typical patterns of ileal contractility (Figure 2A): a decreased tension was transiently recorded from the ileal segments immediately after treatment with MIGB, and then recovery to resting levels occurred within 20 s. Ileal contraction reached a maximal level 1.5–2 min after adding MIGB to the organ bath, and sustained contraction was then observed up to the 2 h tested here. These patterns in time course and magnitude markedly differed from those produced by ACh, which caused a tonic contraction immediately after treatment of agonist, followed by a gradual decline to the resting level. MIGB produced ileal contractility (mean and maximum) in a dose-dependent manner (23–693 μM), and its EC50 value in producing ileal contraction was estimated to be 45.5 μM (Figure 2B). Based on these results, a submaximum effective concentration (230 μM) of MIGB was used in subsequent experiments to evaluate the possible pharmacological mechanism involved in MIGB-induced stimulation of isolated ileal segments.

Combinatory effects of ACh and MIGB

This study investigated the combined effects of MIGB and ACh on the contractility of isolated rat ileal segments by

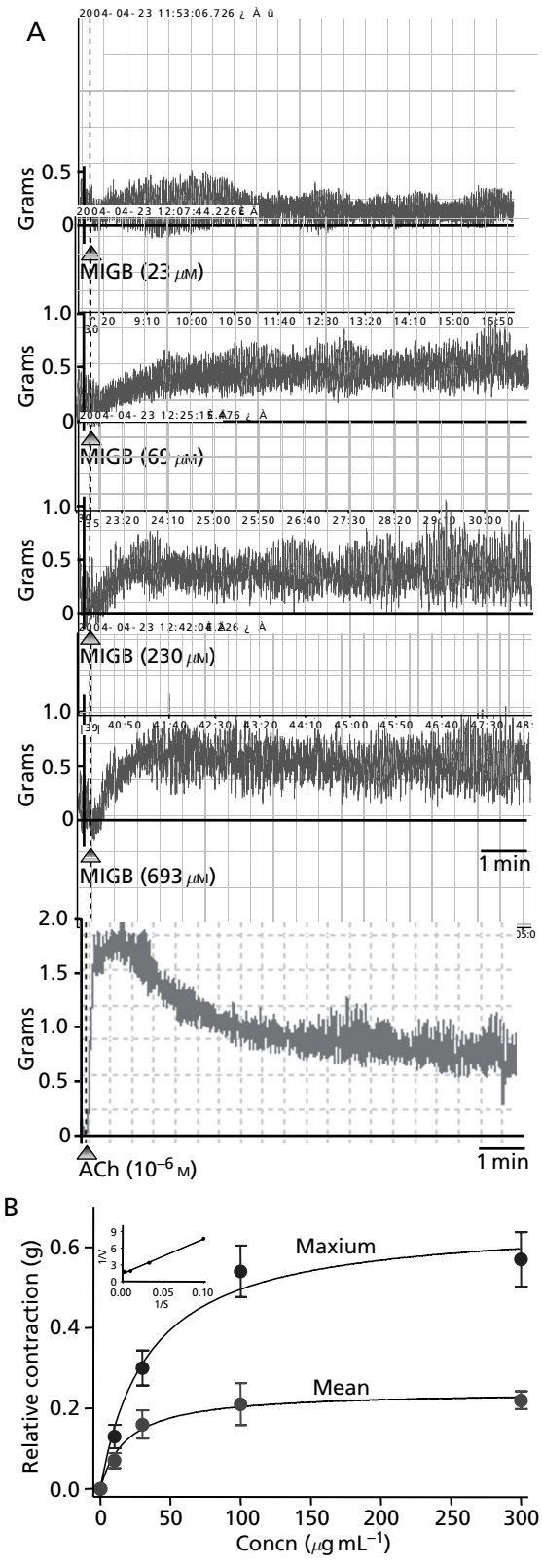

Figure 2 Representative traces (A) and concentration–response curves (B) for methylisogermabullone (MIGB)-induced contraction of rat isolated ileal segments. Each point represents the mean ± s.e. for seven rats. Molar units of MIGB: $10\,\mu g\,mL^{-1} = 23\,\mu M$, $30\,\mu g\,mL^{-1} = 69\,\mu M$, $100\,\mu g\,mL^{-1} = 230\,\mu M$, $300\,\mu g\,mL^{-1} = 693\,\mu M$.

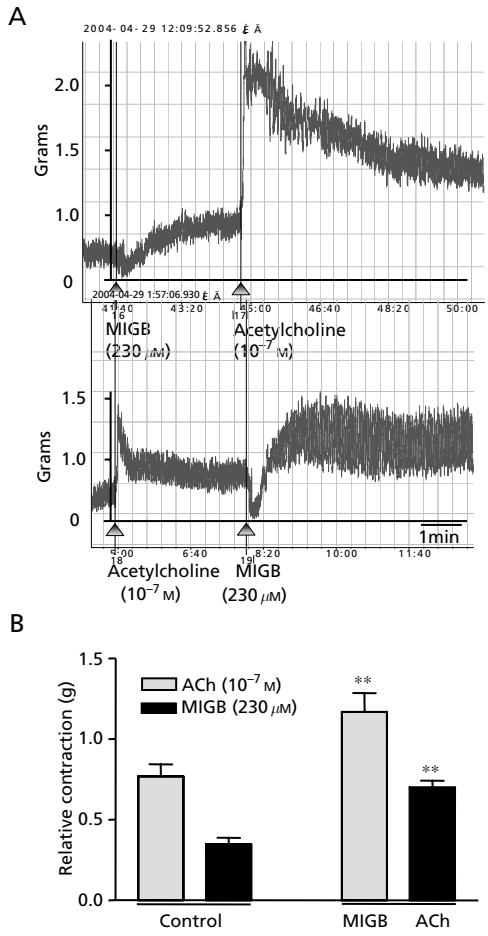

Figure 3 Synergic effect of methylisogermabullone (MIGB) and acetylcholine (ACh) on rat isolated ileal segments. Ileal segments were pretreated with ACh (10^{-7} M) or MIGB (230 μM) for 3 min, followed by combining treatment. Each column represents the mean ± s.e. of seven rats. $^{**}P < 0.01$ vs control.

using submaximum concentrations of MIGB (230 μM) and ACh (10^{-7} M). When isolated rat ileal segments were pre-incubated with MIGB for 3 min, followed by subsequent treatment with ACh, the ACh-induced ileal contractility was significantly increased to 51.9% of ACh alone (Figure 3). When ileal segments were treated with MIGB after treatment with ACh for 3 min, it was found that pretreatment with ACh dramatically enhanced the MIGB-induced ileal contractility compared with that produced by MIGB alone (about 2-fold increment).

Effects of acetylcholinergic antagonists

To corroborate the observation shown in Figure 3, suggesting that the contractile response elicited to MIGB is possibly a result of cholinergic ACh neurotransmitter, we made an attempt to use a non-selective muscarinic antagonist, atropine, and a ganglionic blocker, hexamethonium (Delbro & Lange 1997; Vianna-Jorge et al 2000). After the isolated rat ileal segments were pre-incubated with 10 μM

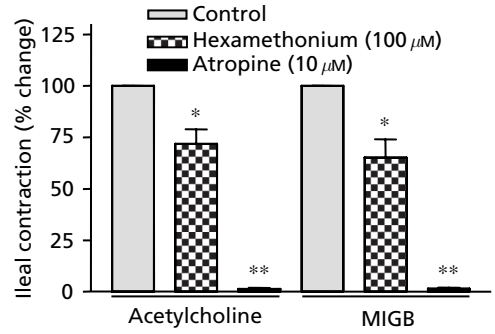

Figure 4 Antagonistic effect of acetylcholinergic receptor blockers on methylisogermabullone (MIGB)-induced contraction of rat isolated ileal segments. Rat ileal segments were pretreated with hexamethonium (100 μM) or atropine (10 μM) for 5 and 3 min, respectively, followed by treatment with ACh (10^{-7} M) or MIGB (230 μM). Each column represents the mean ± s.e. of seven rats. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control.

of atropine for 3 min or 100 μM of hexamethonium for 5 min, ACh (10^{-6} M) or MIGB (230 μM) was added. Atropine produced an obvious relaxation of ileal segments, but hexamethonium slightly reduced the spontaneous contractility of ileal segments (data not shown). As shown in Figure 4, both ACh- and MIGB-induced ileal contractions were almost completely inhibited by pretreatment of ileal segments with atropine, and these were partially, but significantly, blocked by hexamethonium to 71.9% and 65.2% of control, respectively.

Small intestinal transit

To evaluate the pharmacological property of MIGB on gastrointestinal motility in-vivo, this study examined the effects of MIGB on the small intestinal transit of charcoal suspension in mice. In vehicle-administered mice, the distance travelled by the charcoal suspension accounted for 35.7% of the total length of small intestine (Figure 5). Atropine (50 mg kg^{-1}) caused a significant inhibition of normal small intestinal transit. In contrast to the traversed distance of marker in vehicle-treated mice, MIGB produced a significant enhancement of small intestinal transit in a dose-dependent manner (10–100 mg kg^{-1}). MIGB (100 mg kg^{-1})-induced stimulation of small intestinal transit was significantly antagonized by co-administration of atropine (50 mg kg^{-1}).

Discussion

Our previous work has demonstrated that extract of radish roots modulates gastrointestinal motility in-vitro and in-vivo, and that its pharmacological activity is, at least, mediated by activation of muscarinic acetylcholinergic mechanism (Jung et al 2000). Based on this report, we hypothesized that radish roots might contain a potent cholinomimetic phytochemical that could modulate gastrointestinal motility. To examine this hypothesis, this study was designed to identify the cholinomimetic substance contained in radish roots and to characterize its pharmacological

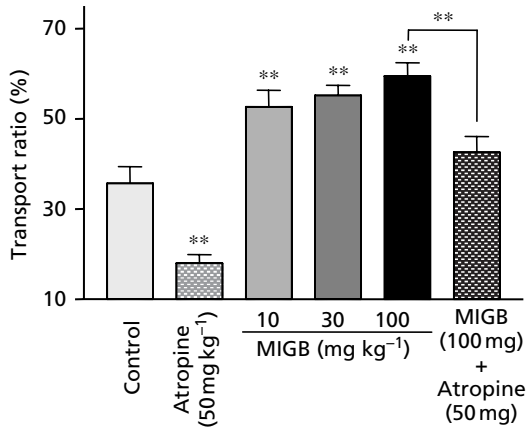

Figure 5 Effect of methylisogermabullone (MIGB) on the small intestinal transit of charcoal in mice. Atropine was subcutaneously injected 5 min before oral administration of MIGB, and charcoal suspension was administered 10 min after MIGB treatment. Small intestinal transit of charcoal was observed for 15 min after marker administration. Each column represents the mean ± s.e. of seven mice. $^{**}P < 0.01$ *vs.* control.

property on gastrointestinal motility in-vitro and in-vivo. Based on the stimulatory activity-guided fractionation on the isolated rat ileal segments, we purified MIGB from radish roots, and this phytochemical exhibited a potent spasmodic activity on small intestine through regulation of acetylcholinergic mechanism.

Since cholinomimetic mechanism related to the excitatory action of ACh may be principally involved in the regulation of gastrointestinal motility (Eglen et al 1996; Caulfield & Birdsall 1998), the spasmogenic activity of MIGB in small intestine was compared with that of ACh. MIGB produced a significant increase in the contraction of the isolated rat ileal segments, with a micromolar EC50 value, and these effects were comparable with the ACh-induced contractility of rat ileal segments. Interestingly, MIGB induced a transient relaxation of the rat ileum immediately after treating the ileal segments, followed by a gradual increase of phasic and tonic contraction. These contractile patterns were obviously different to those produced by ACh, which indicated that ACh did not cause a relaxation of the isolated rat ileal segments. Although biochemical mechanisms involved in the MIGB-mediated relaxation of ileal segments were not examined in this study, we carefully consider that this may be related to the hyperpolarization of cellular membranes in rat ileum. To clarify the biochemical mechanism involved in the MIGB-induced ileal relaxation, future investigations, such as electropharmacological study, may be needed. Moreover, MIGB produced a sustained contraction of rat ileal segments up to 2 h tested in this study, whereas ACh-induced ileal contraction gradually declined to the resting levels after reaching maximum contraction. In the preliminary experiment, we also observed that MIGB at 1 mg mL^{-1} did not change the acetylcholinesterase (AChE) activity (data not shown). These results lead us to consider that MIGB may be resistant to the AChE activity, because a gradual decrease in the ACh-induced contraction of isolated intestinal segments may be a result of enzymatical degradation of agonist by AChE (Barlow 1978; Ennis et al 1986), and MIGB-induced ileal contraction may not be mediated by inhibition of AChE activity.

ACh receptor subtypes are abundantly expressed in the longitudinal muscle layers in the gastrointestinal tract (Candell et al 1990; Eglen et al 1996; Galligan 1999). Preparations of circular and longitudinal smooth muscle from the small intestine of rats and guinea-pigs contain mACh receptor subtypes (Stadelmann et al 1998; Unno et al 2003) and nAChRs are also present on myenteric excitatory motor nerve fibres near sites of neurotransmitter release to the circular smooth muscle (Nakamura et al 1998; Schneider et al 2000). Activation of the mACh M_3 receptor subtype elicits PIP_2 hydrolysis and muscle contraction and there are presynaptic nAChRs on nerve terminals that release ACh to cause contraction of longitudinal smooth muscle (Chiang et al 1991; Seya et al 1998). These observations highlight the central role of ACh receptors in the control of gastrointestinal motor function. Regarding the localization and physiological role of cholinergic ACh receptors in ileal segments, it is conceivable that cholinomimetic mechanisms participate in the regulation of spasmogenic activity mediated by MIGB in the isolated rat ileal segments. Therefore, pharmacological strategies were employed to examine the involvement of cholinomimetic mechanisms in MIGB-induced ileal contraction. When isolated rat ileal segments were simultaneously treated with MIGB and ACh at submaximum concentration, the amplitude of MIGB- or ACh-stimulated ileal contraction was synergically enhanced. These results indicate a sensitization of ileal segments by simultaneous treatment with both substances and may suggest that MIGB-induced stimulation of isolated ileal segments is possibly mediated by acetylcholinergic mechanism, because this study was carried out by using submaximum concentration of MIGB and ACh. In addition, MIGB- and ACh-induced ileal contractions were almost completely inhibited by pretreatment with atropine, and hexamethonium partially, but significantly, blocked the agonist-induced ileal contraction. These results may indicate that MIGB stimulates the contraction of the isolated rat ileal segments through activating ACh receptors, and the large inhibitory effects of hexamethonium suggest that the contractile response to MIGB and ACh in ileal segments may be a result of released ACh acting on postjunctional muscarinic ACh receptors (Chiang et al 1991; Galligan 1999; Seya et al 1998). Therefore, it is conceivable that MIGB may have ACh-like pharmacological properties, and further studies may be needed to examine the pharmacological affinity between MIGB and ACh receptor subtypes.

Although the intestinal transit of charcoal is not a quantitative method in the sense that the radioisotopic method is (Purdon & Bass 1973), it is widely used as a useful visible marker to estimate gastrointestinal motility in-vivo (Gaginella et al 1994; Rao et al 1997). Davis et al (2000) suggested that the pig can be considered a suitable animal model for the evaluation of the performance of

oral pharmaceutical products, and that the rat and mouse are also considered to be good animal models for studying gastrointestinal motility mediated by pharmaceutical substances (Jung et al 2000; Bajad et al 2001; Martinez et al 2004). Using the mouse model, this study observed that the leading front of charcoal progressed further in MIGB-treated mice than in control mice. This response may be due to the pharmacological effects of MIGB on the stomach or gastrointestinal tract. Moreover, atropine itself significantly inhibited the normal travel of charcoal in the small intestine, and MIGB-induced enhancement of charcoal transit was dramatically reduced by co-administration of atropine. It has been suggested that atropine prolongs gastric empting by gastroparesis in man, and this may occur by an inhibitory effect of atropine on muscarinic ACh receptors distributed in the smooth muscle of the gastrointestinal tract (Botts et al 1985). This suggestion may lead us to explain the pharmacological mechanisms involved in the inhibitory effects of atropine on MIGB-induced stimulation of small-intestinal charcoal transit. MIGB increases small intestinal transit of charcoal through activation of muscarinic ACh receptors, and this may be, at least, attenuated by gastroparesis, which is mediated by atropine-induced inhibition of muscarinic ACh receptors. Therefore, the results obtained in this study may demonstrate the oral effect of MIGB on gastrointestinal motility in-vivo, indicating that MIGB stimulates the motility of the gastrointestinal tract in mice, and this response may be mediated, at least in part, by cholinomimetic mechanisms localized in the enteric nerve system of the gastrointestinal tract. This suggestion is supported by the findings of previous reports (Eglen et al 1996; Delbro & Lange 1997; Caulfield & Birdsall 1998), indicating that pre- and post-junctional cholinergic ACh receptors play an important role in the regulation of physiological excitation in gastrointestinal fundic strips.

Conclusions

This study provides a pharmacological link between MIGB and gastrointestinal motility, indicating that MIGB stimulates the spasmodic activity of the gastrointestinal tract in rats and mice, and these effects may be, at least, mediated by regulation of cholinergic ACh receptors. Therefore, MIGB may be considered as a useful pharmaceutical substance to improve or treat decreased gastrointestinal motility. Based on our knowledge, the findings of this study may be the first evidence demonstrating that MIGB has an ACh-like pharmacological property.

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