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Research Paper

Signalling pathways responsible for the methylisogermabullone-induced contraction of ileal longitudinal muscles

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Abstract

Objectives We have previously reported that methylisogermabullone (MIGB) stimulates small bowel motility through activation of acetylcholinergic receptors. This study investigated the cellular signalling pathways implicated in the regulation of ileal contractility by MIGB.

Methods The ileal longitudinal muscles prepared from rats were treated with MIGB isolated from radish roots, and muscle contractility and protein expression were measured by force transducer and Western blot, respectively.

Key findings MIGB at 30 μM induced a sustained phasic contraction of ileal longitudinal muscles. Acetylcholine (ACh, 0.5 μM) and MIGB stimulated translocation of protein kinase C (PKC) to cell membrane of ileal longitudinal muscles, and these stimulatory effects were remarkably attenuated by atropine (0.5 μM). ACh and MIGB induced phosphorylation of ERK 1/2 and p38 MAPKs in ileal longitudinal muscles, and they also phosphorylated the caldesmon and 20-kDa regulatory light chain of myosin (MLC_{20}). Additionally, PD-98058 (10 μM), a selective ERK 1/2 MAPK inhibitor, and SB-203580 (10 μM), a selective p38 MAPK inhibitor, significantly reduced the MIGB-induced contraction of ileal longitudinal muscles.

Conclusions The muscarinic receptor activated by MIGB translocates the PKC to cell membrane which phosphorylates the ERK 1/2 and p38 MAPKs, resulting in subsequent phosphorylation of caldesmon and MLC_{20}. These cellular events likely converge on the contraction of ileal longitudinal muscles in rats.

Keywords ileal longitudinal muscle; MAPK; methylisogermabullone; MLC_{20}; muscarinic receptor

Introduction

The peristaltic motility of the gastrointestinal tract is a dynamic and highly regulated process consisting of activation of ascending excitatory and descending inhibitory reflex pathways, which produces a synchronous oral contraction and anal relaxation of both the longitudinal and circular muscles, respectively.[1] In fact, it is generally accepted that acetylcholine (ACh) released from the enteric postsynaptic cholinergic neurons is the primary excitatory neurotransmitter for contractile activity of gastrointestinal smooth muscles and that muscarinic M_2 and M_3 receptors are primarily responsible for excitatory mechanisms of gastrointestinal smooth muscles.[2,3]

Regarding the cellular and molecular pharmacology, the muscarinic M_3 receptors coupled to $G_{q/11}$ contribute to contraction of gastrointestinal smooth muscles through activation of phospholipase C and protein kinase C (PKC) and intracellular $Ca^{[2+]}$ mobilization,[4,5] and the muscarinic M_2 receptors act through $G_{i/o}$ to regulate the activity of adenylyl cyclase and sensitization of the contractile proteins to calcium.[6,7] In addition to the muscarinic M_2 receptor-mediated adenylyl cyclase inhibition, other downstream signalling pathways including extracellular-regulated protein kinase 1/2 (ERK 1/2) and p38 mitogen-activated protein kinases (MAPKs) are possibly implicated in the muscarinic M_2 receptor-induced smooth muscle contractions.[8–10]

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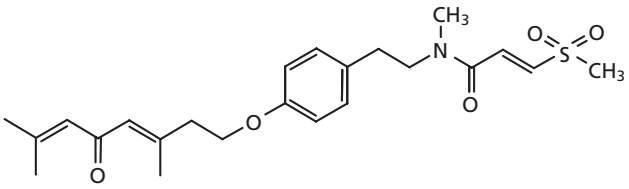

Figure 1 Chemical structure of methylisogermabullone isolated from the rhizome of radish ($C_{23}H_{31}O_5NS$, MW 433).

On the other hand, we have identified methylisogermabullone (MIGB, $C_{23}H_{31}O_5NS$, MW 433) from radish roots (Figure 1), and its pharmacological property has been reported for the first time.[11] MIGB exhibits a stimulatory effect on the small bowel motility through activation of the acetylcholinergic muscarinic receptors, likely the M_2 and M_3 subtypes. However, our understanding of the signalling mechanisms underlying the MIGB-induced contraction of ileal smooth muscles is entirely limited. Therefore, in addition to the pharmacological effects of MIGB on the regulation of small bowel motility, this study investigated the signalling pathways responsible for the MIGB-induced contraction of ileal smooth muscles and found that MIGB contracts the ileal longitudinal muscles through phosphorylation of caldesmon and MLC_{20}, which is mediated by activation of PKC-ERK 1/2 and p38 MAPKs pathways.

Materials and Methods

Drugs and antibodies

MIGB was purified from radish roots (*Brassica oleraceae*) as described in a previous report,[11] and its chemical structure and purity (≥96%) were determined by 1D and 2D NMR. ACh (Sigma, St Louis, MO, USA) and atropine (Sigma) were dissolved in distilled water. MIGB, PD-98059 (Tocris), SB-203580 (Tocris) and phorbol 12,13-dibutyrate (PDBu; Sigma) were dissolved in dimethyl sulfoxide (DMSO) and diluted with KHB. The final concentration of DMSO used in experiments was less than 0.05%. Mouse monoclonal anti-caldesmon (C21), goat anti-phospho-caldesmon (Ser^{789}), mouse monoclonal anti-p38, rabbit anti-phospho-p38 (Thr^{180}/Tyr^{182}) and HRP-conjugated goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-MLC_{20} and anti-β-actin were purchased from Sigma (St Louis, MO, USA). Mouse monoclonal anti-ERK1/2 and mouse monoclonal anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) were received from Cell Signaling Technology (Beverly, MA, USA). The PKC activity assay kit was obtained from Stressgen Bio Reagents Tech (San Diego, CA, USA). All other chemicals and reagents were of the highest grade from commercial sources.

Experimental animals

Male Sprague–Dawley rats, 7-weeks old, were received from Samtako BioKorea (Kyungki, Korea) and acclimatized for one week until use. The rats were kept in a controlled environment (temperature 23 ± 2°C, relative humidity 50–60% and 12-h dark–light cycle) and allowed free access to standard rodent chow (Samyang, Kyungki, Korea) and water. Animal experiments were conducted in accordance with the institutional guideline established by the Wonkwang University Committee for the Care and Use of Laboratory Animals.

Preparation of ileal longitudinal muscles

Ileal longitudinal muscle strips from rats were isolated as described earlier.[11,12] In brief, after rats were sacrificed by CO_2 asphyxiation and cervical dislocation, the ileum was excised approximately 5 cm from the ileo-caecal junction and the lumen was flushed with Krebs–Henseleit buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.6 $CaCl_2$, 25 $NaHCO_3$ and 11.5 D-glucose, pH 7.4) to remove any intestinal debris. To prepare the isolated ileal longitudinal muscles, the ileum was opened along the mesenteric border, pinned to the base of a Sylgard-coated dish and viewed under a dissecting microscope. The mucosa, submucosa and circular muscles of ileum were removed using small forceps and ophthalmological trabecula scissors, and the isolated ileal longitudinal muscles (approximately 1 × 0.5 cm) were obtained.

Measurement of muscle contraction

The contractility of ileal longitudinal muscles was measured using the intact strips (1 cm length) mounted in the longitudinal direction in a 10-ml organ bath containing Krebs–Henseleit buffer. One edge of each strip tied with suture silk was fixed to the bottom of the organ bath, and the other edge was connected to the force transducer (Grass Technologies, West Warwick, RI, USA). The ileal strips were allowed to equilibrate for 30–60 min with a washout every 10 min and oxygenated with 95% O_2 and 5% CO_2 at 37°C. Tension (0.5 g) was slowly applied to the tissues before treating with drugs. Contractility outputted from the force transducer was measured by a biological recording system equipped with amplifier (PowerLab 4/25; AD Instruments, Colorado Springs, CO, USA). The spontaneous contractility (tension and amplitude) recorded over a10-min period before delivering any drugs was considered as a basal control, and ACh (0.5 μM)-induced and MIGB (30 μM)-induced contractile responses were measured for 10 min. After ileal strips were pretreated with PD-98059 (10 μM) or SB-203580 (10 μM) for 20 min, ACh-induced and MIGB-induced contractile responses were recorded over 10-min periods. Relative change of drug-induced contractile responses (average tension and amplitude) to the basal control was calculated as percentage.

Measurement of protein kinase C activity

The particulate fraction from the ileal longitudinal muscles was prepared as described previously,[13] and PKC activity was measured according to the manufacturer's instruction (Stressgen Bio Reagents Tech) based on a solid phase enzyme-linked immuno-absorbent assay. The isolated ileal longitudinal muscles were pretreated with or without atropine (0.5 μM) for 5 min and subsequently incubated with ACh (0.5 μM) or MIGB (30 μM) for 5 min. The tissues were homogenized in lysis buffer (in mM: 20 Tris/HCl, 1 Na_3VO_4, 1 NaF, 2 phenylmethylsulfonyl fluoride (PMSF), 5 EDTA, 1 dithiothreitol (DTT), 20 NaH_2PO_4 and 20 Na_2HPO_4 with 10 μg/ml of aprotinin, leupeptin, pepstatin A and antipain-HCl, pH 7.4) and centrifuged at 100 000*g* for 1 h at 4°C. The supernatant was collected as a cytosolic fraction. The pellets were

re-suspended in lysis buffer containing 1% Triton X-100 and sonicated twice for 30 s each time and particular (membrane) fractions were collected by centrifugation at 100 000*g* for 30 min.

SDS-PAGE and Western blot

MIGB-induced phosphorylation of ERK 1/2 and p38 MAPKs and caldesmon was examined using the isolated ileal longitudinal muscles. After tissues were pretreated with or without atropine (0.5 μM) for 5 min and sequentially incubated with ACh (0.5 μM) or MIGB (30 μM) for 5 min, and they were quickly frozen in liquid nitrogen and kept at −70°C. Frozen tissues were thaw at 4°C and homogenized in the lysis buffer (composition in mM: 20 Tris/HCl, 150 NaCl, 2 Na_3VO_4, 2.5 Na_4PO_7, 10 NaF, 1% Nonidet P-40, 5 EGTA, 1 DTT, 20 sodium β-glycerophosphate, 5 ethylene-bis(oxyethylenenitrilo)-tetracaetic acid and protease inhibitors, pH 7.4). Cell lysates were centrifuged at 13 000*g* for 15 min at 4°C. The supernatants were recovered and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein content was measured by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Samples (20 μg protein/well) were boiled at 95°C for 5 min with SDS-PAGE loading buffer (4% SDS, 1% β-mercaptoethanol, 0.01% bromophenol blue, 20% glycerol and 125 mM Tris/HCl, pH 6.8) and subjected to SDS-PAGE. The separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA), and nonspecific binding sites of membranes were blocked with TBS (5% non-fat dry milk and 0.1% Tween-20 in Tris-buffered saline, pH 7.4) for 1 h. The membranes were then briefly washed and incubated for overnight at 4°C in TBS containing one of the following primary antibodies: mouse monoclonal anti-caldesmon, goat anti-phospho-caldesmon (Ser^{789}), mouse monoclonal anti-ERK 1/2, mouse monoclonal anti-phospho-ERK 1/2 (Thr^{202}/Tyr^{204}), mouse monoclonal anti-p38, rabbit anti-phospho-p38 (Thr^{180}/Tyr^{182}) or anti-β-actin. After washing three times for 30 min in TBS, the membrane was incubated in TBS containing HRP-conjugated secondary antibody for 1 h and washed three times for 30 min in TBS. Immunoreactive bands were detected by an ECL Plus detection kit (Amersham Pharmacia Biotech, Aylesbury, UK).

Measurement of MLC_{20} phosphorylation

Phosphorylation of the 20-KDa regulatory light chain of myosin (MLC_{20}) was measured by a previously reported method with a slight modification.[14] In brief, the ileal longitudinal muscles were pretreated with or without atropine (0.5 μM) for 5 min and incubated with ACh (0.5 μM) or MIGB (30 μM) for 5 min. Tissues were quickly frozen with 10% trichloroacetic acid (TCA) in acetone-dry ice containing 10 mM DTT and allowed to reach room temperature, followed by removal of the residual TCA. Proteins, including MLC_{20}, were extracted with 10 volumes of urea–glycerol PAGE sample buffer (composition: 8 M urea, 20 mM Tris/HCl, 25 mM glycine, 10 mM DTT, 2% Nonidet P-40, 0.004% bromophenol blue and saturated sucrose, pH 8.6) for 60 min. Phosphorylated and nonphosphorylated MLC_{20} was separated by urea–glycerol PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). Nonspecific binding sites of membranes were blocked with 5% non-fat dry milk-containing TBS (0.1% Tween-20 in Tris-buffered saline, pH 7.4) for 1 h, and the membrane was incubated with anti-MLC_{20} overnight at 4°C. After washing three times for 30 min in TBS, the membrane was incubated in TBS containing HRP-conjugated goat anti-mouse IgG for 1 h and washed three times for 30 min in TBS. Proteins were detected by an ECL Plus detection kit (Amersham Pharmacia Biotech, Aylesbury, UK). The relative amounts of nonphosphorylated and phosphorylated MLC_{20} were quantified by LumiImager F1 (Roche Diagnostic, Germany), and MLC_{20} phosphorylation was expressed as a percentage of total MLC_{20}.

Data analysis

The obtained results are expressed as means ± SE. Statistical differences between means were determined by Student's *t*-test. Differences between multiple groups were tested using analysis of variance for repeated measures and checked for significance using Scheffe's *F*-test. $P < 0.05$ was considered statistically significant.

Results

Methylisogermabullone-induced ileal longitudinal muscle contraction

Consistent with the previous report,[11] MIGB at 30 μM produced a distinct contractile pattern in ileal longitudinal muscles: a gradual increase of tension and amplitude, followed by sustained contraction (Figure 2a). The contractile patterns were remarkably different to those caused by 0.5 μM of ACh (data not shown). The magnitude of MIGB-induced ileal longitudinal muscle contraction (both tension and amplitude) reached a maximal level at 5 min after treatment with MIGB (Figure 2b). Based on these results, ileal longitudinal muscles were treated with 30 μM of MIGB for 5 min in the subsequent experiments.

Methylisogermabullone-induced protein kinase C activation

Subcellular distribution of PKC activity was measured using PDBu (1 μM), a selective PKC activator, as a positive control drug, and the obtained results are shown in Figure 3. The basal PKC activity distributed in the membrane and cytosolic fractions was 6.8 ± 0.58 (32.6% of total activity) and 14.1 ± 0.93 (67.4% of total activity) pmol/mg protein/min, respectively. PDBu significantly increased the PKC activity in the membrane fractions, but caused a significant reduction of PKC activity in the cytosolic fractions. ACh (0.5 μM) and MIGB (30 μM) significantly increased the PKC activity of membrane fractions, and this was accompanied by simultaneous decreases of PKC activity present in the cytosolic fractions. These stimulatory effects were remarkably attenuated by atropine (0.5 μM).

Methylisogermabullone-induced ERK 1/2 and p38 MAPKs phosphorylation

Phosphorylation of ERK 1/2 and p38 MAPKs was examined to understand the signalling pathways implicated in the MIGB-induced contraction of ileal longitudinal muscles.

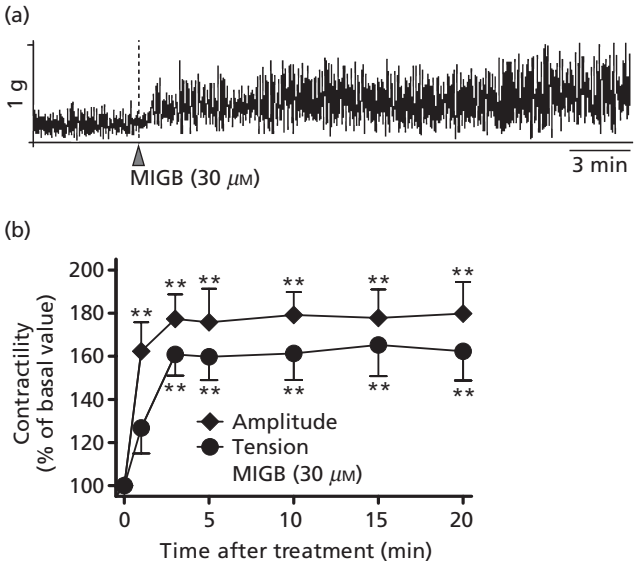

Figure 2 Representative tracing (a) and time-course curve (b) for the methylisogermabullone (MIGB)-induced contraction of rat ileal longitudinal muscles. After ileal longitudinal muscle strips were stabilized, MIGB (30 μM) was applied to organ bath at the point indicated by arrow. The average tension and amplitude were measured by isometric force transducer before (basal, 10 min) and after (1, 3, 5, 10, 15 and 20 min) treatment with MIGB. Each point represents the mean ± SE of 7 strips. $^{**}P < 0.01$ vs basal contractility.

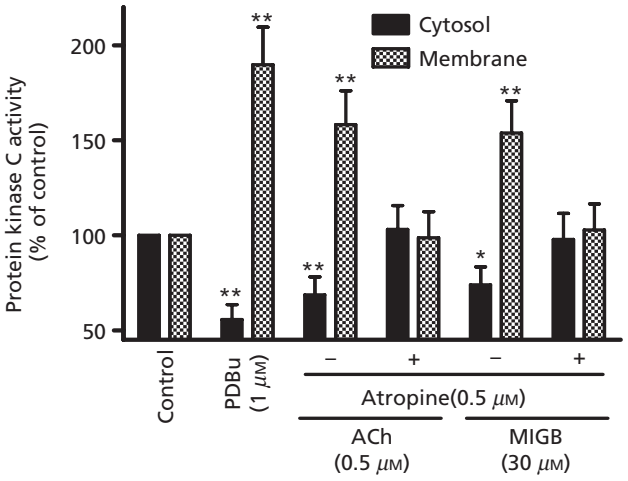

Figure 3 Effect of methylisogermabullone (MIGB) on the subcellular distribution of protein kinase C (PKC) in rat ileal longitudinal muscles. The ileal longitudinal muscles were pretreated with or without atropine (0.5 μM) for 5 min, followed by treatment with phorbol 12,13-dibutyrate (1 μM), acetylcholine (0.5 μM) or MIGB (30 μM) for 5 min. After preparation of cytosolic and particular (membrane) fractions as described in Materials and Methods, PKC activity was measured by a solid phase enzyme-linked immuno-absorbent assay. Each point represents the mean ± SE of 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control.

Treatment of ileal longitudinal muscles with ACh (0.5 μM) or MIGB (30 μM) significantly increased the phosphorylation of ERK 1/2 at Thr^{202}/Tyr^{204} (Figure 4a) and p38 MAPK at Thr^{180}/Tyr^{182} (Figure 3b). Pretreatment of the ileal longitudinal muscles with atropine (0.5 μM) remarkably reduced the ACh-induced and MIGB-induced phosphorylation of ERK 1/2 (Figure 4a) and p38 (Figure 4b) MAPKs.

Methylisogermabullone-induced caldesmon and MLC_{20} phosphorylation

Ttreatment of the ileal longitudinal muscles with ACh (0.5 μM) significantly increased caldesmon phosphorylation at Ser,[789] and a similar extent of phosphorylation was also observed in the ileal longitudinal muscles treated with MIGB (30 μM) (Figure 5a). Additionally, ACh and MIGB also stimulated phosphorylation of MLC_{20} (Figure 5b). ACh-induce and MIGB-induced stimulation of caldesmon and MLC_{20} phosphorylation was remarkably reduced by pretreatment of ileal longitudinal muscles with 0.5 μM of atropine (Figure 5a and 5b).

Effects of MAPK inhibitors on the MIGB-induced muscle contraction

Using ERK 1/2 and p38 MAPK inhibitors, this study examined involvement of ERK 1/2 and p38 MAPKs in the MIGB-induced contraction of ileal longitudinal muscles. Pretreatment of ileal longitudinal muscles with PD-98059 (10 μM), a selective ERK 1/2 MAPK inhibitor, significantly reduced the ACh (0.5 μM)-induced and MIGB (30 μM)-induced contraction (tension and amplitude) of ileal longitudinal muscles (Figure 6a). SB-203580 (10 μM), a selective p38 MAPK inhibitor, also significantly attenuated the ACh-induced and MIGB-induced contraction of ileal longitudinal muscles (Figure 6b).

Discussion

The purpose of this study was to evaluate the signalling pathways implicated in the MIGB-induced contraction of ileal longitudinal muscles. The results lead us to consider that muscarinic receptors activated by MIGB cause phosphorylation of ERK 1/2 and p38 MAPK through translocation of PKC

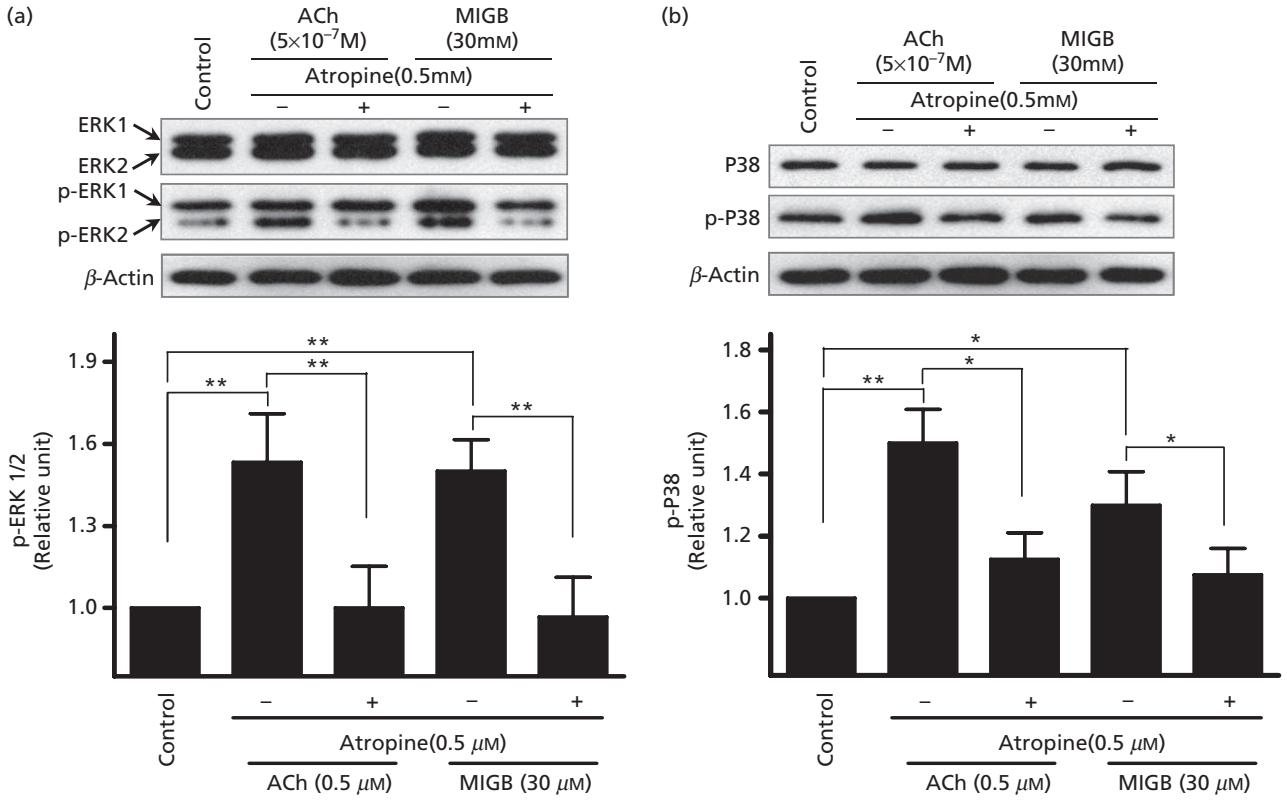

Figure 4 Effect of methylisogermabullone (MIGB) on the ERK 1/2 (a) and p38 (b) MAPK phosphorylation in rat ileal longitudinal muscles. The ileal longitudinal muscles were pretreated with or without atropine (0.5 μM) for 5 min and sequentially treated with acetylcholine (0.5 μM) or methylisogermabullone (30 μM) for 5 min. Proteins extracted from the tissues were subjected to SDS-PAGE and transferred to PVDF membrane for Western blot probed with mouse monoclonal anti-ERK1/2, mouse monoclonal anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), mouse monoclonal anti-p38, rabbit anti-phospho-p38 (Thr^{180}/Tyr^{182}) and anti-β-actin. Relative changes in ERK 1/2 and p38 MAPKs phosphorylation were estimated by comparing with nonphosphorylated proteins. Each point represents the mean ± SE of 6 experiments. $*P < 0.05$, $**P < 0.01$.

to the cell membranes, followed by a subsequent phosphorylation of caldesmon and MLC_{20}, These cellular events eventually converge on the contraction of ileal longitudinal muscles. The results obtained in this study possibly explain the signalling pathways responsible for MIGB-induced small bowel motility, which was suggested by our previous report,[11] and provide a new insight into the cellular and molecular pharmacological mechanisms involved in the activation of muscarinic receptors coupled to MIGB.

The ACh released from the postsynaptic cholinergic neurons of the enteric nerve system is, in fact, the primary excitatory neurotransmitter for contractile activity of gastrointestinal smooth muscles, and ACh-induced gastrointestinal smooth muscle contraction is mainly mediated by activation of the muscarinic M_2 and M_3 receptors.[15] In this study, we show that MIGB produces a sustained contraction of ileal longitudinal muscles, and maximum contractile responses are reached at 5 min after treatment with MIGB. These findings are well accorded with those of previous reports,[11] suggesting that MIGB enhances the spasmodic activity of small bowel in both *in vitro* and *in vivo*. Additionally, we have also suggested that stimulatory effects of MIGB on the contractility of ileal smooth muscles are likely mediated, at least, by activation of acetylcholinergic muscarinic, possibly the M_2 and M_3 subtypes, receptors. However, no evidence of intracellular signalling mechanism involved in the MIGB-induced small bowel motility has been reported.

In many types of mammalian cells, including gastrointestinal smooth muscle cells, cellular signalling after binding of ACh to the odd-numbered muscarinic M_1, M_3 and M_5 receptors is associated with an increase in diacylglycerol and inositol 1,4,5-triphosphate (IP_3) through stimulating phospholipase C activity, resulting in PKC activation and smooth muscle contraction. The PKC activation is closely associated with translocation of the cytosolic enzyme to the cell membrane. For example, PKC_α and PKC_ε are translocated and activated in the cell membrane in response to stimulation with ACh in colonic and oesophageal smooth muscles, respectively.[16,17] In this study it was observed that ACh and PDBu used as positive agonistic drugs significantly elevated the PKC activity in membrane fractions, and the stimulatory effects were dramatically blocked by atropine. These findings are in accordance with those of previous reports,[5,13,18] suggesting that ACh induces a sustained phosphorylation and translocation of PKC to the cell membranes, and these cellular responses are implicated in the regulation of sustained MLC_{20} phosphorylation and smooth muscle contraction. In this study, we also found that MIGB stimulated the atropine-sensitive translocation of PKC to the membrane fractions. Taken together, the results are suggestive of the fact that MIGB translocates and activates

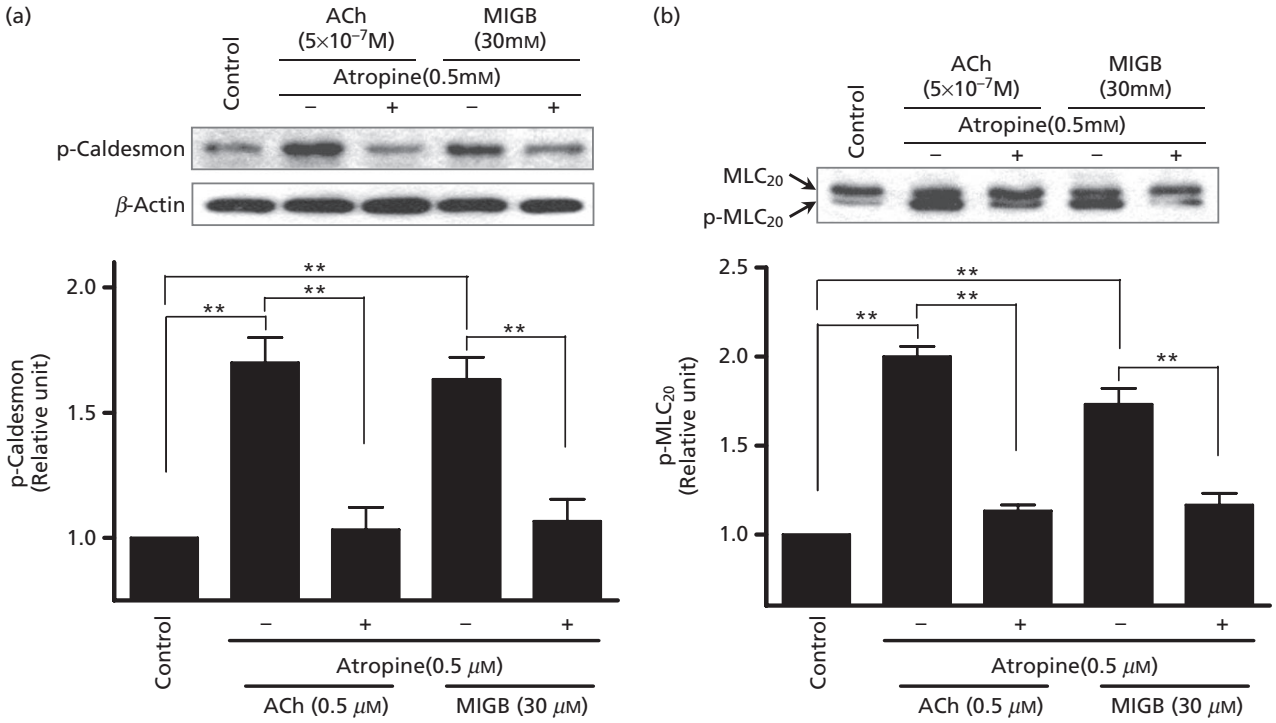

Figure 5 Effect of methylisogermabullone on the caldesmon (a) and MLC_{20} (b) phosphorylation in rat ileal longitudinal muscles. The ileal longitudinal muscles were pretreated with or without atropine (0.5 μM) for 5 min and sequentially treated with acetylcholine (0.5 μM) or MIGB (30 μM) for 5 min. Proteins extracted from the tissues were subjected to SDS-PAGE and urea/glycerol PAGE for caldesmon and MLC_{20}, respectively. The separated proteins were transferred to PVDF or nitrocellulose membrane for Western blot probed with mouse monoclonal anti-caldesmon, goat anti-phospho-caldesmon (Ser^{789}), mouse monoclonal anti-MLC_{20} and anti-β-actin. Relative changes in caldesmon and MLC_{20} phosphorylation were estimated by comparing with β-actin and nonphosphorylated proteins, respectively. Each point represents the mean ± SE of 6 experiments. $^{**}P < 0.01$.

PKC enzymes in the membrane of ileal longitudinal muscle cells, and these cellular and molecular responses may act as an up-stream regulator of cellular signalling pathways responsible for the MIGB-induced contraction of ileal longitudinal muscles.

It is known that activation of PKC allows phosphorylation of ERK 1/2 and p38 MAPKs, and they then phosphorylate the caldesmon, resulting in sequential molecular and cellular responses such as no longer inhibition of acto-myosin interaction, conformational change of cytoskeletal proteins and contraction of smooth muscles.[9,19] Many reports have demonstrated that ACh-induced contraction of gastrointestinal smooth muscles is dependent on PKC activity, and PKC-dependent contraction of smooth muscles is mediated by ERK 1/2 and p38 MAPKs.[17,18,20] Based on these suggestions, we have examined whether ERK 1/2 and p38 MAPKs are regulated by MIGB-induced PKC activation in the ileal longitudinal muscles and observed that ACh and MIGB phosphorylated the ERK 1/2 (Thr^{202}/Tyr^{204}) and p38 (Thr^{180}/Tyr^{182}) MAPKs in the ileal longitudinal muscles, and the phosphorylation effects were remarkably reduced by atropine. Therefore, it is considered that ERK 1/2 and p38 MAPKs likely act as down-stream mediators of PKC activated by the muscarinic receptors coupled with MIGB. This speculation can be also supported by the findings of previous reports,[17,18,20] indicating that ACh-induced contractile response of gastrointestinal smooth muscles depends on activation of the ERK 1/2 and/or p38 MAPKs.

It has been established that caldesmon and MLC_{20} play an important role in the regulation of smooth muscle contractility. Phosphorylation of MLC_{20} at $Thr^{[18]}/Ser^{[19]}$ occurs in several different types of smooth muscle tissues stimulated with carbachol.[21] In gastrointestinal smooth muscles treated with carbachol, the initial contraction reflects stimulation of phospholipase C activity, resulting in Ca^{2+}/calmodulin-dependent activation of MLC kinase and transient MLC_{20} phosphorylation and contraction, and the sustained contraction reflects the activation of PKC-mediated signalling pathways, resulting in sustained MLC_{20} phosphorylation and contraction.[5,9] Additionally, stimulation of the muscarinic receptors appears to be critical for caldesmon phosphorylation at Ser^{789} through phosphorylation of ERK 1/2 and p38 MAPKs in the smooth muscle cells of distal colon and airway.[8,20] In this study, we have examined whether MIGB produces a sustained contraction of ileal longitudinal muscles through phosphorylation of caldesmon or MLC_{20}, which may be mediated by activation of PKC-ERK 1/2 and p38 MAPK pathways.[17,18,22] We show a significant increase in phosphorylation of caldesmon and MLC_{20} in the ileal longitudinal muscles treated with ACh or MIGB, and their phosphorylation was remarkably reduced by blocking the muscarinic receptors with atropine. These results are comparable to previous findings,[4,8,9] indicating that in gastrointestinal smooth muscle cells, activation of muscarinic receptors with ACh produces PKC-dependent caldesmon and MLC_{20} phosphorylation. We have also observed that MIGB phosphorylates

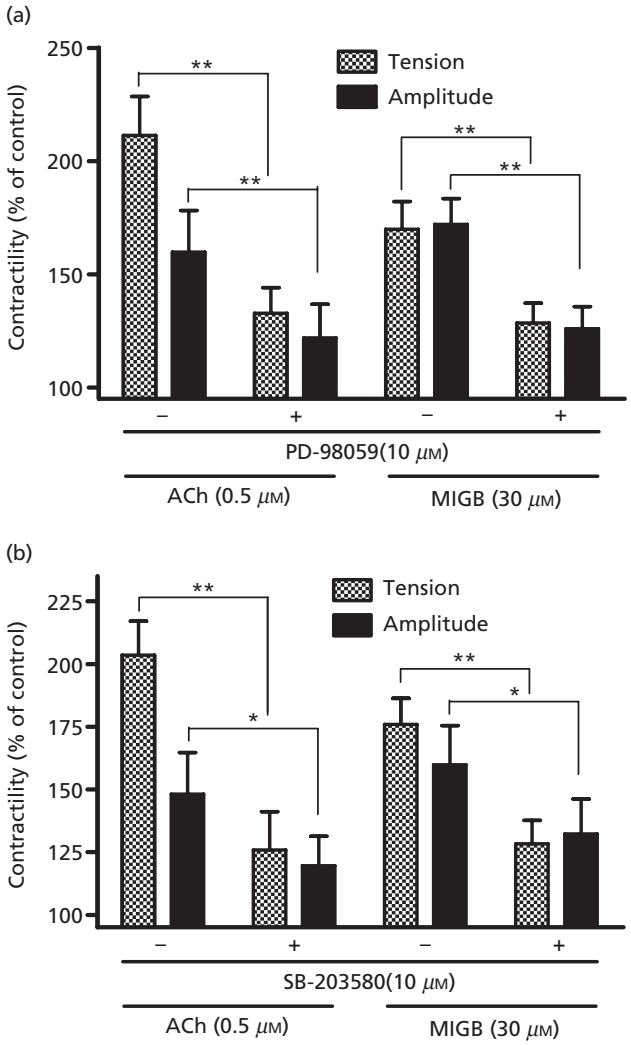

Figure 6 Effect of ERK 1/2 and p38 MAPKs inhibitors on the methylisogermabullone-induced contraction of rat ileal longitudinal muscles. After ileal longitudinal muscle strips were stabilized, the strips were pretreated with PD-98059 (10 μM) or SB-203580 (10 μM) for 20 min, and subsequently treated with acetylcholine (0.5 μM) or MIGB (30 μM) 10 min. The average tension and amplitude were measured by isometric force transducer before (basal, 10 min) and after (response, 10 min) treatment with each agonist. Each point represents the mean ± SE of 7 strips. $*P < 0.05$, $**P < 0.01$.

caldesmon at Ser^{789} and MLC_{20} at $Thr^{[18]}/Ser^{[19]}$ through activation of PKC-dependent ERK 1/2 and p38 MAPK pathways in the ileal longitudinal muscles.

In addition to cellular and molecular aspects, this study also functionally demonstrated involvement of ERK 1/2 and p38 MAPK pathways in the MIGB-induced contraction of ileal longitudinal muscles. Inhibition of ERK 1/2 and p38 MAPKs with PD-98059 and SB-203580, respectively, attenuated the ACh-induced and MIGB-induced contraction of ileal longitudinal muscles, and these findings were closely related with previous suggestions,[8,18,20] indicating that inhibiting the ERK 1/2 and p38 MAPKs blocks the ACh-induced caldesmon and MLC_{20} phosphorylation and ileal longitudinal muscle contraction. Therefore, it is considered that ERK 1/2 and p38 MAPKs mediate the MIGB-induced contraction of ileal longitudinal muscles.

Conclusions

In summary, these results suggest the signalling pathways underlying the MIGB-induced contraction of ileal longitudinal muscles. Acetylcholinergic muscarinic receptors coupled with MIGB induce translocation of PKC to the cell membranes, and these cellular events sequentially phosphorylate the ERK 1/2 and p38 MAPKs, resulting in caldesmon and MLC_{20} phosphorylation and ileal longitudinal muscle contraction. Further studies are needed to elucidate whether Rho kinase and MLC phosphatase are implicated in the regulation of MIGB-induced contraction of ileal longitudinal muscles.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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