Biomolecules & Therapeutics, **18(4)**, 442-447 (2010)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2010.18.4.442

The Inhibitory Effect of Eupatilin on the Intestinal Contraction Induced by Carbachol

Hyun Dong JE[1,a], Jong-Min LEE[1,a], and Hyen-Oh LA[2,*]

[1]*Department of Pharmacology, College of Pharmacy, Catholic University of Daegu, Gyeongbuk 712-702,*
[2]*Department of Pharmacology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea*

(Received July 27, 2010; Revised August 25, 2010; Accepted August 25, 2010)

Abstract − This study was conducted to determine whether treatment with the anti-inflammatory eupatilin influences intestinal smooth muscle contraction stimulated by carbachol and, if so, to investigate the related mechanism. Denuded ileal or colonic muscles from Sprague-Dawley rats were used for the study and measurements of isometric contractions were obtained using a computerized data acquisition system; this data was also combined with results from molecular experiments. Eupatilin from Artemisia asiatica Nakai significantly decreased carbachol-induced contractions in both ileal and colonic muscles. Interestingly, eupatilin decreased carbachol-induced phosphorylation of ERK1/2 more significantly than that of MYPT1 at Thr855 in ileal and colonic muscles. However, eupatilin significantly decreased phosphorylation of MYPT1 at Thr855, but only in ileal muscle. Therefore, thin filament regulation, including MEK inactivation and related phospho-ERK1/2 decrease, is mainly involved in the eupatilin-induced decrease of intestinal contraction induced by carbachol. In conclusion, this study provides the evidence and a possible related mechanism concerning the inhibitory effect of the flavonoid as an antispasmodic on the agonist-induced contractions in rat ileum and colonic muscles.

Keywords: Colon, ERK, Eupatilin, Ileum, MYPT1, Smooth muscle

INTRODUCTION

The coordinated contractions and relaxations of intestinal smooth muscles produce the motor activities of gastrointestinal tracts and are important functions for the optimal transport and digestion of the ingested foods. Regulation of intestinal motility is mediated by several different pathways including extrinsic autonomic nerves, intrinsic neurons and intestinal hormones, in addition to the central nerve system (Mizuta *et al.*, 2006). These regulatory mechanisms can be classified according to their main activities such as stimulation and inhibition of intestinal motility. The excitatory neurotransmitters specifically interact with their neuronal receptors, and ligand-receptor interactions cause depolarization of cellular membranes and activation of voltage-dependent Ca^{2+} channels, resulting in contractions of intestinal smooth muscles (Caulfield and Birdsall, 1998; Murthy, 2006). Acetylcholine- and serotonin-mediated and signaling pathways are well known as excitatory stimulatory mechanisms for intestinal motility. Among five different subtypes of acetylcholinergic muscarinic receptors, the M_2 and M_3 receptors are predominantly distributed in the smooth muscles throughout gastrointestinal tracts and primarily induce the excitation of gastrointestinal smooth muscles by acetylcholine (Ehlert, 2003; Unno *et al.*, 2005). However, the stable acetylcholine analogue carbamoylcholine (carbachol) is commonly used in experiments, since acetylcholine itself is unstable.

The smooth muscle contractile system is basically regulated by myosin light chain (MLC) phosphorylation, which is driven by the balance between MLCK activity and myosin phosphatase activity. During receptor agonist-induced contractions, contractile elements are sensitized to Ca^{2+} and induce a greater MLC phosphorylation and greater force at a given cytosolic Ca^{2+} level (Somlyo and Somlyo, 2000; Pfitzer, 2001), referred to as Ca^{2+} sensitization. Recent studies have shown that a small GTP-binding protein, RhoA, and RhoA-dependent coiled-coil serine/threo-

*Corresponding author
Tel: +82-2-2258-7853 Fax: +82-2-2258-7859
E-mail: hola@catholic.ac.kr
[a]These authors contributed equally to this work.

nine kinases play major roles in Ca^{2+} sensitization. The activated RhoA activates the Rho-kinase, which in turn phosphorylates a noncatalytic subunit of myosin phosphatase (MYPT1), inactivating the myosin phosphatase activity. PKC is also activated by receptor stimulation. The activated PKC phosphorylates MEK, which in turn phosphorylates ERK1/2, attenuating the effect of caldesmon. As a result, MLCK-induced MLC phosphorylation is sustained or augmented to induce a greater contraction at a given concentration of cytosolic Ca^{2+}.

Artemisia asiatica Nakai (family Asteraceae) is an herbal medicine used as a hepatoprotective, antioxidative, anti-inflammatory, and antibacterial agent (Kalemba *et al.*, 2002; Song *et al.*, 2008). Eupatilin, a flavone or a type of flavonoid, is isolated from Artemisia asiatica Nakai and used in the treatment of acid-related disorders. We investigated the possible influence and related mechanisms of the anti-inflammatory eupatilin on gastrointestinal smooth muscle contractility to develop a better gastrointestinal modulator or antispasmodic. Denuded ileal or colonic rings from male Sprague-Dawley rats were used and isometric contractions were recorded using a computerized data acquisition system. These data were combined with molecular experiments.

It is possible that contractions induced by carbachol involve the participation of the RhoA/Rho-kinase or MEK pathways. However, there are no reports as to whether these pathways are inhibited in the eupatilin-induced decrease of intestinal smooth muscle contraction by carbachol in ileal or colonic rings. Therefore, the purpose of the present study was to elucidate a possible role for Rho-kinase inhibition or MEK inhibition in the eupatilin-induced decrease of contractile response to carbachol in isolated rat ileum or colon muscles using an organ culture system and molecular experimentation.

MATERIALS AND METHODS

Tissue preparation

Male Sprague-Dawley rats, weighing 320-350 g, were acclimated to the laboratory environment for several days before entering the study. During the experimental period, animals were housed in standard plastic cages with wood chip bedding and were kept in temperature-controlled (22 ± 2°C), relative humidity-controlled (55 ± 15%), and 12-h light/dark cycle-controlled rooms with free access to food and water. Animals were anesthetized using sodium pentobarbital (50 mg/kg i.p., supplemented if required) followed by cervical dislocation, in agreement with procedures approved by the Institutional Animal Care and Use Committee. The intestine, including ileum and colon, was quickly removed and immersed in oxygenated (95% O_2/5% CO_2) physiological saline solution composed of (mM): 118.0 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 24.0 $NaHCO_3$, 1.2 KH_2PO_4 and 11.0 dextrose (pH 7.4). The intestine was then cleaned of all adherent connective tissue, and the mucosa was removed by gentle abrasion with a cell scraper.

Contraction measurements

Intestinal ileal or colonic circular strips (8 mm wide) were prepared, suspended in a water-jacketed organ bath (10 ml) maintained at 37°C, and aerated with a mixture of 95% O_2 and 5% CO_2. The strips were attached to a force transducer and passively stretched by applying an optimal resting tension of 1.0 g, which was maintained throughout the experiment. Strips were allowed to equilibrate at 37°C for at least 1 h and then challenged with a depolarizing solution containing 72 mM KCl. Muscle strips were then washed and allowed to equilibrate for another 1 h before beginning the experiment. To study pretreatment effects, eupatilin was applied 30 min before the addition of 10 μM carbachol.

Western blot analysis

Muscle strips were quick frozen by immersion in a dry-ice/acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Muscles were also washed in an acetone/DTT mixture and stored at −80°C until use. Samples were brought to room temperature and homogenized in a buffer containing 20 mM mops, 4% SDS, 10% glycerol, 10 mM DTT, 20 mM β-glycerophosphate, 5.5 μM leupeptin, 5.5 μM pepstatin, 20 KIU aprotinin, 2 mM Na_3VO_4, 1 mM NaF, 100 μM $ZnCl_2$, 20 μM 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF) and 5 mM EGTA. Protein-matched samples (modified Lowry protein assay, DC Protein Assay Kit, Bio-Rad) were electrophoresed on SDS-PAGE (Protogel, National Diagnostics), transferred to PVDF membranes and subjected to immunostaining and densitometry using the appropriate antibody. The success of protein matching was confirmed by Naphthol Blue Black staining of the membrane and densitometry of the actin band. Any mismatch of lane loading was corrected by normalization to actin staining. Each set of samples from an individual experiment was run on the same gel and densitometry was performed on the same film. The densitometry was performed using KODAK Molecular Imaging Software, Version 4.0 (Woodbridge, CT, USA) and corrected by normalization to background intensity.

Chemicals and antibodies

Carbachol, potassium chloride, sodium bicarbonate, D-(+)-glucose and eupatilin were purchased from Sigma (St Louis, MO, USA). Dithiothreitol, trichloroacetic acid and acetone were obtained from Fisher Scientific (Hampton, NH, USA). Enhanced chemiluminescence (ECL) kits were obtained from Pierce (Rockford, IL, USA). Antibodies against phospho-myosin phosphatase targeting subunit 1 (phospho-MYPT1) at Thr855 (1:5,000), MYPT1, ERK or phosphoERK at Thr202/Tyr204 were purchased from Upstate Biotechnology (Lake Placid, NY, USA), BD Biosciences (San Jose, CA, USA) or Cell Signaling Technology (Danvers, MA, USA) to determine levels of RhoA/Rho-kinase activity (Wooldridge *et al.*, 2004; Wilson *et al.*, 2005) or MEK activity. Anti-mouse IgM (goat) and anti-rabbit IgG (goat), conjugated with horseradish peroxidase, were used as secondary antibodies (1:2,000, 1:2,000, respectively, Upstate, Lake Placid, NY). All other chemicals or reagents were of highest commercial grade available, purchased from Sigma or Fisher Scientific and used without further purification.

Statistics

The data were expressed as mean ± standard error of the mean (SEM). The student's unpaired *t* test was used to determine the statistical significance of the means between two groups using SPSS 12.0 (SPSS Inc., Chicago, Illinois, U.S.A.). *p*-values < 0.05 were regarded as statistically significant.

RESULTS

The effect of eupatilin on carbachol-induced contraction in denuded ileum

The addition of carbachol (10 μM) produced contraction in rat ileum with mucosa denuded. Pretreatment with eupatilin produced no significant effect on ileal basal tension (data not shown), but significantly decreased carbachol-induced contraction in ileal muscles, regardless of mucosal function (Fig. 1A).

The effect of eupatilin on carbachol-induced contraction in denuded colon

The addition of carbachol (10 μM) produced contraction in rat colon with mucosa denuded. Pretreatment with eupatilin showed no significant effect on colonic basal tension (data not shown), but significantly decreased carbachol-induced contraction in colonic muscles regardless of mucosal function (Fig. 1B).

The effect of eupatilin on the levels of phospho-ERK1/2

To confirm a role for eupatilin in thin filament regulation, including MEK inactivation and related phospho-ERK1/2 decreases in the decrease of smooth muscle contraction, we measured levels of ERK1/2 and phospho-ERK1/2 in muscles quick frozen after 30 minutes of exposure to 10 μM carbachol. When compared with vehicle-treated rat ileum or colon muscles, a significant decrease in carbachol-induced ERK 1/2 phosphorylation was produced by eupatilin in quick frozen eupatilin (0.1 mM)-treated ileum or colon denuded of mucosa (Fig. 2, Fig. 4). These findings show that thin or actin filament regulation, including

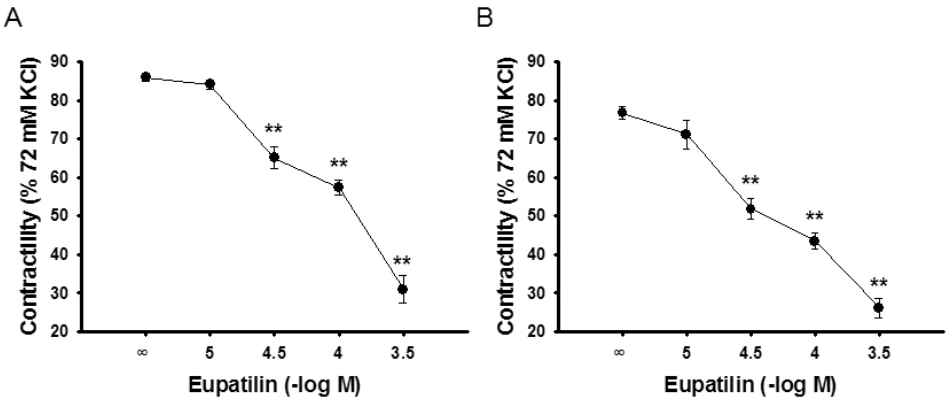

Fig. 1. Effect of eupatilin pretreatment on ileal or colonic contraction induced by carbachol. (A) Effect of eupatilin pretreatment on ileal contraction induced by carbachol. (B) Effect of eupatilin pretreatment on colonic contraction induced by carbachol. Carbachol was added to elicit tension in the presence or absence of eupatilin in the ileal or colonic rings with mucosa denuded. Developed tension is expressed as a percentage of the maximum contraction produced by 72 mM KCl. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. $^{**}p < 0.01$, presence versus absence of eupatilin.

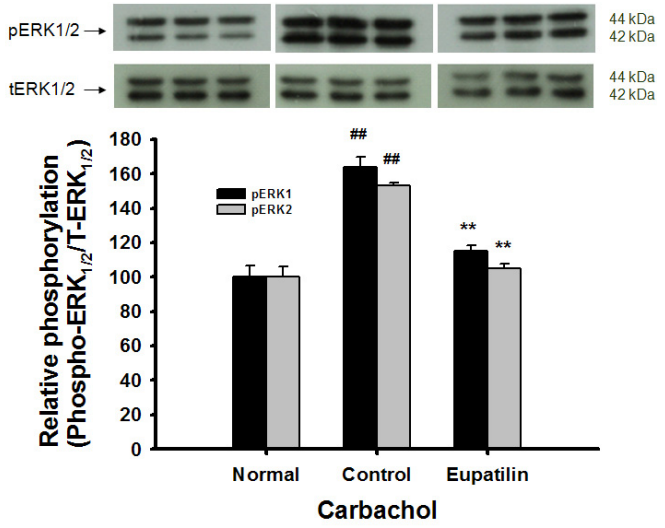

Fig. 2. Effect of eupatilin on carbachol- induced increases in ileal phospho-ERK1/2 levels. The phospho-ERK1/2 protein levels were significantly decreased in quick frozen eupatilin-treated rat ileum in the absence of mucosa, compared to the vehicle-treated rat ileum contracted with carbachol. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-ERK1/2. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. $^{**}p<0.01$, $^{\#\#}p<0.01$, versus control or normal group respectively. Eupatilin: 0.1 mM; carbachol 10 μM.

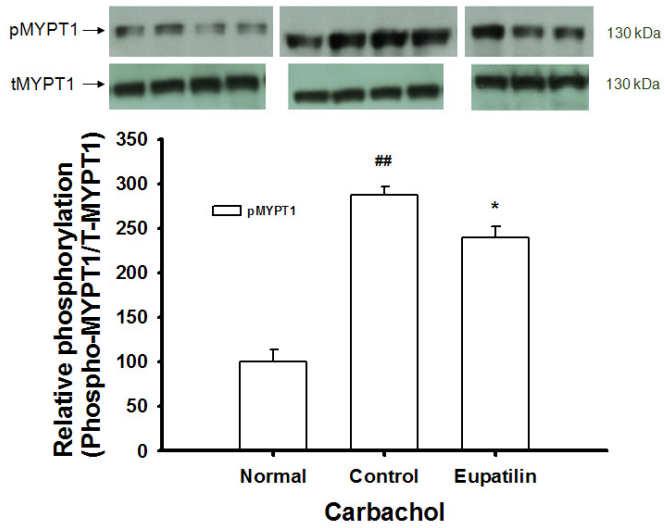

Fig. 3. Effect of eupatilin on carbachol-induced increases in ileal phospho-MYPT1 levels. The phospho-MYPT1$_{Thr855}$ protein levels were partially decreased in quick frozen eupatilin-treated rat ileum in the absence of mucosa, compared to the vehicle-treated rat ileum contracted with carbachol. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-MYPT1. Data are expressed as the means of 3-5 experiments with vertical bars representing SEMs. $^{*}p<0.05$, $^{\#\#}p<0.01$, versus control or normal group respectively. Eupatilin: 0.1 mM; carbachol 10 μM.

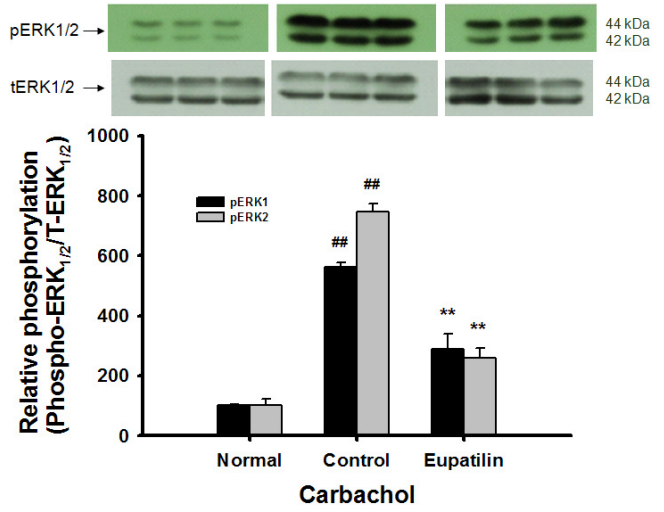

Fig. 4. Effect of eupatilin on carbachol-induced increases in colonic phospho-ERK1/2 levels. The phospho-ERK1/2 protein levels were significantly decreased in quick frozen eupatilin-treated rat colon in the absence of mucosa, compared to the vehicle-treated rat colon contracted with carbachol. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-ERK1/2. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. $^{**}p<0.01$, $^{\#\#}p<0.01$, versus control or normal group respectively. Eupatilin: 0.1 mM; carbachol 10 μM.

ERK1/2 phosphorylation via MEK activation, might be of great importance in the decreased contractility induced by eupatilin throughout the intestine.

The effect of eupatilin on the level of phospho-MYPT1 at Thr855

To confirm the effect of eupatilin on thick filament regulation, including Rho-kinase inactivation and related phospho-MYPT1 decrease in the decrease of smooth muscle contraction, we measured the levels of myosin phosphatase targeting subunit 1 (MYPT1) and phospho-MYPT1 in the muscles quick frozen after 30 min exposure to 10 μM carbachol. Interestingly, there was a significant decrease in the 10 μM carbachol-induced MYPT1 phosphorylation at the site of Thr855 (Wilson *et al.*, 2005; Jeon *et al.*, 2006; Tsai and Jiang, 2006) in quick frozen, eupatilin-treated rat ileum denuded of mucosa, compared to the vehicle-treated rat ileum (Fig. 3). However, no significant decrease in carbachol-induced MYPT1 phosphorylation was produced by eupatilin in quick frozen eupatilin (0.1 mM)-treated colons denuded of mucosa (Fig. 5). Therefore, thick or myosin filament regulation, including myosin phosphatase activation through RhoA/Rho-kinase inactivation, might be partially involved in decreased contractility of eupatilin-treated rat ileum.

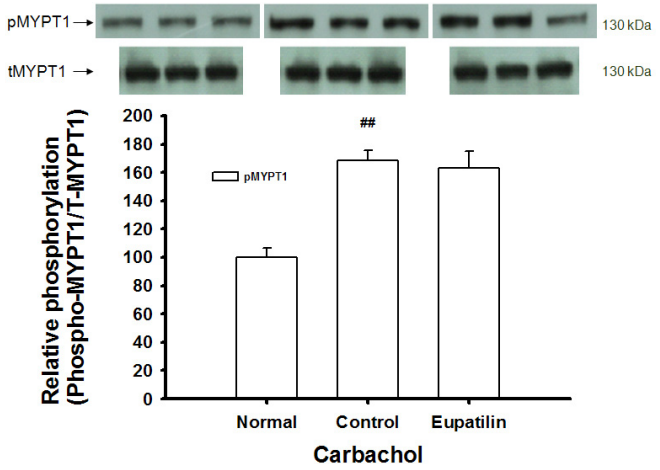

Fig. 5. Effect of eupatilin on carbachol-induced increases in colonic phospho-MYPT1 levels. The phospho-MYPT1$_{Thr855}$ protein levels were sustained in quick frozen eupatilin-treated rat colon in the absence of mucosa, compared to the vehicle-treated rat colon contracted with carbachol. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-MYPT1. Data are expressed as the means of 3-5 experiments with vertical bars representing SEMs. $^{\#\#}p<0.01$, versus normal group. Eupatilin: 0.1 mM; carbachol 10 μM.

DISCUSSION

Separate from cardiac and skeletal muscle, smooth muscle is clearly defined as a distinct muscle group (Webb, 2003) whose contraction is regulated by autonomic neural innervations as well as hormones, autocrine/paracrine agents, and other local chemical signals relying on Ca^{2+}-dependent activation of MLCK and LC_{20} phosphorylation to initiate cross-bridge cycling between actin and myosin filaments (Gerthoffer, 2005). Gastrointestinal motility is also regulated by complex and mutual interactions among neurotransmitters, hormones and spontaneous muscle contractility. Following the interaction at muscarinic M_2 or M_3 receptors, smooth muscle contraction is induced by calcium and related myosin phosphorylation, which is regulated by the balance between myosin light chain kinase activity and myosin phosphatase activity (Somlyo and Somlyo, 2003). In intestinal smooth muscles, as in other types of smooth muscle, myosin phosphatase activity can be modulated by phosphorylation of the myosin phosphatase regulatory subunit MYPT1 or CPI-17. Myosin light chain kinase activity can be modulated by Ca^{2+} or calmodulin, and actomyosin interaction can be regulated by PKC, MEK or ERK1/2. Further investigation is necessary to clarify this point. Therefore, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributes to the eupatilin-induced decrease in contraction by a muscarinic carbachol in mucosal denuded rat ileum or colon by measuring the phospho-substrate levels.

Eupatilin has been previously recognized for its anti-inflammatory activity. The ileal or colonic muscle strips pretreated with eupatilin showed a significant and dose-dependent decrease of contraction induced by carbachol (Fig. 1). Based on physiological results from carbachol-induced gastrointestinal contraction studies, a candidate eupatilin may be useful as an anti-spasmodic agent, regardless of mucosal function. For simplicity, we will discuss only the roles of ERK1/2 and MYPT1, but also recognize that other proteins in related signaling pathways exert similar functions. Eupatilin more significantly inhibited the carbachol-induced increase of pERK1/2 levels rather than pMYPT1 levels in ileal or colonic contraction (Fig. 2, Fig. 4). However, inhibition of pMYPT1 is partially involved in the eupatilin-induced decrease of ileal contraction (Fig. 3), indicating that Rho-kinase or the downstream myosin phosphatase activity is more important in ileum than in the colon. Therefore, the mechanism of the relaxation effect of eupatilin seems to involve inhibition of MEK and the subsequent phosphorylation of ERK at the site of Thr202/Tyr204, or partial inhibition of Rho-kinase activity and the subsequent phosphorylation of MYPT1, depending on the type of tissue. The most important substrate of Rho-kinase is MYPT1, which is a subunit of myosin phosphatase. Therefore, the level of pMYPT1 directly reflects Rho-kinase activity in the thick filament regulation, including MLCK or MP activation. Moreover, the ERK1/2 is a substrate of MEK, reflecting MEK activity, and is believed to be one of the most important regulators in the thin filament regulation including PKC, MEK, ERK or caldesmon activation. Therefore, calcium sensitivity is closely related to MEK activation or Rho-kinase activation, in efficient use of calcium. This study supports the roles of thin filament regulation, partial thick filament regulation and the related decrease of sensitivity to calcium as the inhibitory mechanisms underlying the effect of eupatilin on carbachol-induced intestinal contraction.

In summary, our results indicate that the anti-inflammatory, eupatilin, decreased carbachol-induced contraction in rat ileum and colonic muscles by inhibiting MEK or Rho-kinase activity, regardless of mucosal function. Thin filament regulation via MEK inactivation and related phospho-ERK1/2 decreases is involved in the decrease of intestinal contraction. In conclusion, this study provides evidence concerning the inhibitory effect and the related mechanism of a flavonoid (eupatilin) as a gastrointestinal

modulator or antispasmodic affecting contraction in rat ileal or colonic rings, regardless of mucosal function.

REFERENCES

Caulfield, M. P. and Birdsall, N. J. M. (1998). International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* **50**, 279-290.

Ehlert, F. J. (2003). Contractile role of M_2 and M_3 muscarinic receptors in gastrointestinal, airway and urinary bladder smooth muscle. *Life Sci.* **74**, 355-366.

Gerthoffer, W. T. (2005). Signal-transduction pathways that regulate visceral smooth muscle function. III. Coupling of muscarinic receptors to signaling kinases and effector proteins in gastrointestinal smooth muscles. *Am. J. Physiol. Gastrointest. Liver Physiol.* **288**, G849-G853.

Jeon, S. B., Jin, F., Kim, J. I., Kim, S. H., Suk, K., Chae, S. C., Jun, J. E., Park, W. H. and Kim I. K. (2006). A role for Rho kinase in vascular contraction evoked by sodium fluoride. *Biochem. Biophys. Res. Commun.* **343**, 27-33.

Kalemba, D., Kusewicz, D. and Swiader, K. (2002). Antimicrobial properties of the essential oil of Artemisia asiatica Nakai. *Phytother. Res.* **16**, 288-291.

Murthy, K. S. (2006). Signaling for contraction and relaxation in smooth muscle of the gut. *Annu. Rev. Physiol.* **68**, 345-374.

Mizuta, Y., Shikuwa, S., Isomoto, H., Mishima, R., Akazawa, Y., Masuda, J., Omagari, K., Takeshima, F. and Kohno, S. (2006). Recent insights into digestive motility in functional dyspepsia. *J. Gastroenterol.* **41**, 1025-1040.

Somlyo, A. P. and Somlyo, A. V. (2003). Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.* **83**, 1325-1358.

Somlyo, A. P. and Somlyo, A. V. (2000). Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol. (Lond.)* **522**, 177-185.

Song, H. J., Shin, C. Y., Oh, T. Y. and Sohn, U. D. (2008). The protective effect of eupatilin on indomethacin-induced cell damage in cultured feline ileal smooth muscle cells: involvement of HO-1 and ERK. *J. Ethnopharmacol.* **118**, 94-101.

Tsai, M. H. and Jiang, M. J. (2006). Rho-kinase-mediated regulation of receptor-agonist-stimulated smooth muscle contraction. *Pflugers. Arch.* **453**, 223-232.

Unno, T., Matsuyama, H., Sakamoto, T., Uchiyama, M., Izumi, Y., Okamoto, H., Yamada, M., Wess, J. and Komori, S. (2005). M_2 and M_3 muscarinic receptor-mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice. *Br. J. Pharmacol.* **146**, 98-108.

Webb, R. C. (2003). Smooth muscle contraction and relaxation. *Adv. Physiol. Educ.* **27**, 201-206.

Wilson, D. P., Susnjar, M., Kiss, E., Sutherland, C. and Walsh, M. P. (2005). Thromboxane A_2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca^{2+} entry and Ca^{2+} sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem. J.* **389**, 763-774.

Wooldridge, A. A., MacDonald, J. A., Erdodi, F., Ma, C., Borman, M. A., Hartshorne, D. J. and Haystead, T. A. (2004). Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of Serine 695 in response to cyclic nucleotides. *J. Biol. Chem.* **279**, 34496-34504.