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The Effect of Galangin on the Regulation of Vascular Contractility via the Holoenzyme Reactivation Suppressing ROCK/CPI-17 rather than PKC/CPI-17

Hyuk-Jun Yoon[1,†], Won Pill Jung[1,†], Young Sil Min[2], Fanxue Jin[3], Joon Seok Bang[4], Uy Dong Sohn[5] and Hyun Dong Je[1,*]

[1]*Department of Pharmacology, College of Pharmacy, Daegu Catholic University, Gyeongsan 38430,*
[2]*Department of Pharmaceutical Science, Jungwon University, Goesan 28024,*
[3]*Department of Pharmacology, Kyungpook National University School of Medicine, Daegu 41944,*
[4]*College of Pharmacy, Sookmyung Women's University, Seoul 04310,*
[5]*Department of Pharmacology, College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea*

Abstract

In this study, we investigated the influence of galangin on vascular contractibility and to determine the mechanism underlying the relaxation. Isometric contractions of denuded aortic muscles were recorded and combined with western blot analysis which was performed to measure the phosphorylation of phosphorylation-dependent inhibitory protein of myosin phosphatase (CPI-17) and myosin phosphatase targeting subunit 1 (MYPT1) and to evaluate the effect of galangin on the RhoA/ROCK/CPI-17 pathway. Galangin significantly inhibited phorbol ester-, fluoride- and thromboxane mimetic-induced vasoconstrictions regardless of endothelial nitric oxide synthesis, suggesting its direct effect on vascular smooth muscle. Galangin significantly inhibited the fluoride-dependent increase in pMYPT1 and pCPI-17 levels and phorbol 12,13-dibutyrate-dependent increase in pERK1/2 level, suggesting repression of ROCK and MEK activity and subsequent phosphorylation of MYPT1, CPI-17 and ERK1/2. Taken together, these results suggest that galangin-induced relaxation involves myosin phosphatase reactivation and calcium desensitization, which appears to be mediated by CPI-17 dephosphorylation via not PKC but ROCK inactivation.

Key Words: CPI-17, Fluoride, Galangin, MYPT1, Phorbol ester, ROCK

INTRODUCTION

Galangin (3,5,7-trihydroxyflavone, Fig. 1) is a bioflavonoid derived primarily from propolis, a natural compound produced by honeybee and rhizome of *Alpinia officinarum*, and it has various pharmacological activities such as anti-oxidant, anti-inflammatory, antidiabetic and anti-carcinogenic activities (Kim *et al*., 2013; Zha *et al*., 2013; Devadoss *et al*., 2018) including the inhibition of proliferation, induced apoptosis and promoted autophagy in hepatocellular and esophageal carcinoma cells (Zhang *et al*., 2012; Ren *et al*., 2016) and cell invasion by suppressing the epithelial-mesenchymal transition (Cao *et al*., 2016). The scientific rationale on experimental design was to investigate the influence of galangin nonspecifically most potent on vascular contractility and determine the mechanism involved using denuded muscles from male rats and recording isometric contractions combined with molecular experiments.

The vascular contractility is is regulated via both Ca^{2+}-dependent and Ca^{2+} sensitization mechanisms (Kuriyama *et al*., 2012; Sasahara *et al*., 2015; Liu and Khalil, 2018) and dysregulated contractility and Ca^{2+} sensitization in blood vessels is observed in many cardiovascular diseases. The mechanism responsible for Ca^{2+} sensitization involves repression of myosin phosphatase, leading to the phosphorylation of 20-kDa myosin light chain (MLC) and subsequent enhanced contractility. The inhibition of myosin phosphatase in vascular smooth muscle is mediated by phosphorylation of either the phosphorylation-dependent inhibitory protein of myosin phosphatase (CPI-17) or the myosin phosphatase targeting subunit 1 (MYPT1) via either Rho-kinase (ROCK) or protein kinase C (PKC), which leads to attenuated dephosphorylation of MLC_{20}. Inhibition of myosin phosphatase in smooth muscle

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***Corresponding Author**
E-mail: hyundong@cu.ac.kr
Tel: +82-53-850-3615, Fax: +82-53-359-6734
[†]The first two authors contributed equally to this work.

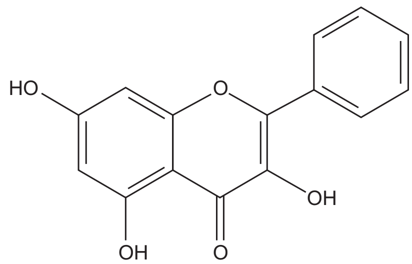

Fig. 1. The chemical structure of galangin (3,5,7-trihydroxyflavone).

is mediated by phosphorylation of myosin phosphatase target subunit via ROCK, which leads to sustained phosphorylation of MLC_{20}. PKC is an important kinase involved in increasing the contractile filament sensitivity to calcium. Ca^{2+} antagonist-insensitive forms of hypertension and coronary vasospasm require other treatment modalities that target other pathways such as ROCK and PKC. PKC inhibits myosin phosphatase activity by activating CPI-17, a myosin phosphatase inhibitor when it is phosphorylated at Thr38 by PKC or ROCK, resulting in increased levels of MLC phosphorylation (Kim *et al*., 2012; Yang *et al*., 2018). CPI-17 (Thr38) and MLC phosphorylation levels coordinately correspond with smooth muscle contraction during many physiological processes within smooth muscles and other cell types. Extracellular signal regulated kinase (ERK) 1/2 and its activator mitogen-activated protein kinase kinase (MEK) have been shown to be activated via PKC-mediated phosphorylation in various cell types (Ansari *et al*., 2009). Thromboxane A_2 mimetics, fluoride, and phorbol esters have been shown to induce contractions of vascular muscles, which may be due to enhanced Ca^{2+} sensitivity or partially due to an increased Ca^{2+} concentration. Activation of ERK1/2 induced by a thromboxane A_2 mimetic (Gallet *et al*., 2003) or phorbol ester triggers ERK1/2-dependent cytoskeletal remodeling and relieves the inhibitory action of caldesmon increasing the affinity between myosin and actin and cross-bridge cycling (Hedges *et al*., 2000).

However, the specific protein kinases and associated cellular pathways primarily responsible for increased calcium desensitization in response to galangin remain unknown. Therefore, the purpose of this study was to investigate the specific protein kinase and associated cellular signaling pathways responsible for myosin phosphatase reactivation and calcium desensitization induced by galangin.

MATERIALS AND METHODS

Preparation of aorta

Male Sprague-Dawley rats (210-240 g) were anesthetized with etomidate (0.3 mg/kg i.v.) and euthanized by thoracotomy and exsanguination according to the guidance approved by the Institutional Committee at Chung-Ang University and Daegu Catholic University (IACUC-2016-040). After euthanasia performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals, the thoracic aorta was carefully and rapidly isolated and placed in oxygenated physiological saline solution consisting (mM) of 115.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, and 10.0 glucose. The aorta was separated from the surrounding connective tissue and the endothelia were cleaned by gentle abrasion using a pipette tip and N^G-monomethyl-L-arginine (L-NMMA) if necessary.

Evaluation of vascular contraction

To examine functional changes of the muscle in response of a vasoconstrictor, each muscle was incubated with the vasoconstrictor *ex vivo* in a water-jacketed organ bath aerated with gas mixture. Muscles were stretched until an optimal resting tension of 2.0 g was enforced, and changes in their tension were analyzed using a force-displacement transducer (FT03C, Grass, Quincy, MA, USA) linked to a PowerLab recording system (AD Instruments, Castle Hill, NSW, Australia). After equilibration (for 60 min), arterial integrity was examined by contracting the rings with 50 mM KCl or 1 μM phenylephrine, followed by relaxation with acetylcholine (1 μM).

The relaxation effect of galangin was determined by its application after KCl- (50 mM), phenylephrine- (1 μM), thromboxane A_2- (0.1 μM), phorbol ester- (1 μM) or fluoride- (6 mM) evoked contractions had plateaued in normal Krebs' solution.

Western blot analysis

Protein expression was quantified using immunoblotting, as reported previously (Jeon *et al*., 2006; Je and Sohn, 2009). Aortic tissues were quick-frozen in a dry ice/acetone slurry including 10 mM dithiothreitol (DTT) and 10% trichloroacetic acid (TCA), washed several times in room temperature with the washing buffer including acetone and DTT, and homogenized with the homogenization buffer including antioxidants. Protein-matched samples were subjected to sodium dodecyl sulfate-polyacrylamide denaturing gel electrophoresis (Protogel, National Diagnostics, Atlanta, GA, USA), transferred to polyvinylidene difluoride or nitrocellulose membranes, and subjected to immunostaining incubating with primary and secondary antibodies. Lane loading variations were corrected by normalization with beta-actin. Sets of samples produced during individual experiments were conducted on the same gel and the densitometry was performed on the same image.

Chemicals and antibodies

Sodium chloride, potassium chloride, sodium fluoride, acetylcholine, galangin, U46619 and phorbol 12,13-dibutyrate were obtained from Sigma (St. Louis, MO, USA). DTT, TCA and acetone were purchased from Fisher Scientific (Hampton, NH, USA). Enhanced chemiluminescence (ECL) kits were purchased from Pierce (Rockford, IL, USA). Antibodies against phospho-myosin phosphatase targeting subunit 1 (phospho-MYPT1) at Thr855 (1:5,000), MYPT1, phospho-phosphorylation-dependent inhibitory protein of myosin phosphatase (phospho-CPI-17) at Thr38 (1:1,000), CPI-17, adducin or phospho-adducin at Ser662, ERK or phosphoERK at Thr202/Tyr204 (Upstate Biotechnology, Lake Placid, NY, USA or Cell Signaling Technology, Danvers, MA, USA) were used to determine levels of RhoA/ROCK activity (Kitazawa *et al*., 2000; Wooldridge *et al*., 2004; Wilson *et al*., 2005) or MEK activity. Anti-rabbit IgG (goat) and anti-mouse IgM (goat) conjugated with horseradish peroxidase were used as secondary antibodies (1:2,000 dilutions for both, Upstate B.). A specific MLC_{20} antibody (1:1,500, Sigma) and anti-mouse IgG (goat) conjugated with horseradish peroxidase (1:2,000, Upstate B.) were used to determine the level of myosin light chain (LC_{20})

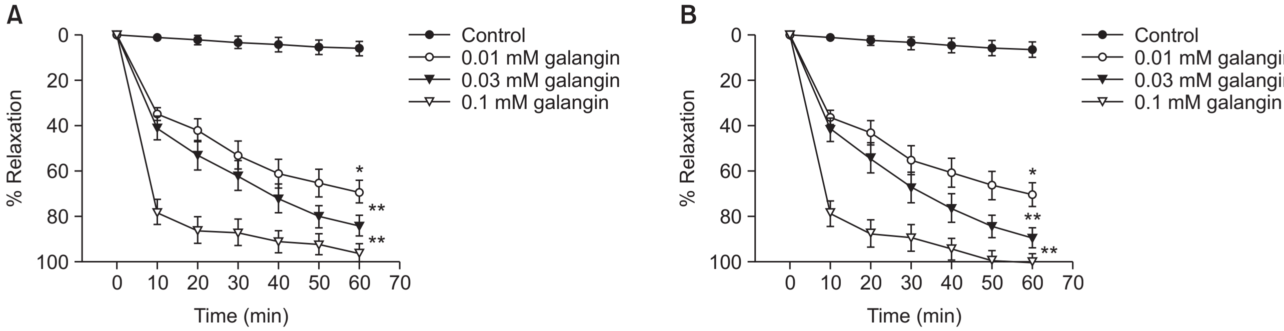

Fig. 2. Effect of galangin on fluoride-dependent vascular contractions in denuded (A) or intact (B) muscles. Each muscle was equilibrated in the organ bath solution for 40-50 min before relaxation responses to galangin were measured. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. *p<0.05, **p<0.01, presence versus absence of galangin.

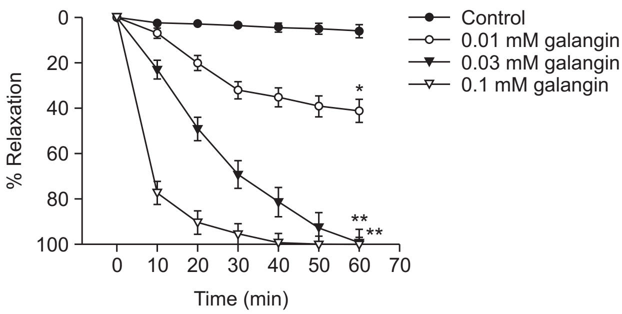

Fig. 3. Effect of galangin on thromboxane mimetic-dependent vascular contractions in denuded muscles. Each muscle was equilibrated in the organ bath solution for 40-50 min before relaxation responses to galangin were measured. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. *p<0.05, **p<0.01, presence versus absence of galangin.

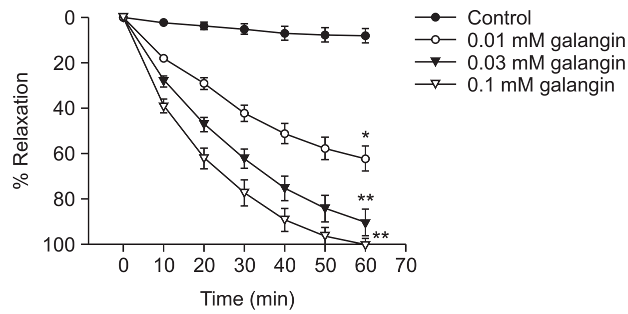

Fig. 4. Effect of galangin on phorbol ester-dependent vascular contractions in denuded muscles. Each muscle was equilibrated in the organ bath solution for 40-50 min before relaxation responses to galangin were measured. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. *p<0.05, **p<0.01, presence versus absence of galangin.

phosphorylation. Galangin was prepared in dimethyl sulfoxide (DMSO) as a 0.1 M stock solution and frozen at –20°C for later use.

Statistics

The data are presented as mean ± standard error of the mean (SEM). Statistical evaluations between two groups were performed using student's unpaired t-test or ANOVA. These statistical analyses were made using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when p<0.05.

RESULTS

Effect of galangin on contractions of endothelium-denuded muscles induced by a full RhoA/ROCK activator fluoride

Removal of endothelium, the regulator of vascular homeostasis, was achieved by gently rubbing with a pipette tip and N_G-mono-methyl-L-arginine (L-NMMA) to identify the relaxation effect of galangin on vascular smooth muscle. The absence of endothelium was identified by a lack of relaxation after treating contracted muscle segments with acetylcholine (1 μM). Galangin had no observable effect on basal tension (data not shown), but it significantly inhibited the contraction evoked by a ROCK activator fluoride, regardless of the absence of endothelial nitric oxide synthesis in denuded (Fig. 2A) or intact (Fig. 2B) muscles. This suggests that the relaxation mechanism of galangin might include the repression of ROCK activity and myosin phosphatase reactivation besides endothelial nitric oxide synthesis and the activation of guanylyl cyclase.

Effect of galangin on contractions of denuded aortas induced by the dual ROCK and MEK activator thromboxane A_2

Galangin inhibited thromboxane A_2 mimetic U46619-induced contraction in denuded muscles (Fig. 3), suggesting that the mechanism includes repression of ROCK activity and myosin phosphatase reactivation and a dual activator (thromboxane mimetic) acts similar to a full activator targeting ROCK.

Effect of galangin on contractions of denuded muscles induced by a MEK activator phorbol 12, 13-dibutyrate

Phorbol esters are primarily MEK activators and partial ROCK activators (Goyal *et al*, 2009; Je and Sohn, 2009). Interestingly, phorbol 12,13-dibutyrate (PDBu)-induced contraction was inhibited by galangin, regardless of endothelial nitric oxide synthesis in denuded muscles (Fig. 4), which suggested that thin filament regulation including MEK/ERK was inhibited.

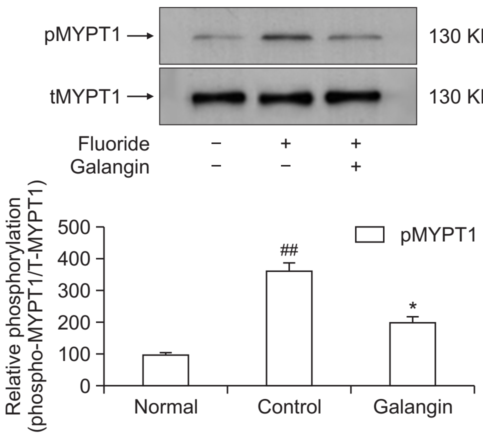

Fig. 5. Effect of galangin on fluoride-dependent increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were decreased in rapidly-frozen galangin-treated muscles in the absence of endothelium compared to vehicle-treated muscles precontracted with fluoride. Upper panel indicates a typical blot, and lower panel indicates average densitometric results for relative levels of phospho-MYPT1. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. ##p<0.01, *p<0.05, versus normal or control group respectively. Galangin: 0.1 mM galangin; Fluoride: 6 mM sodium fluoride.

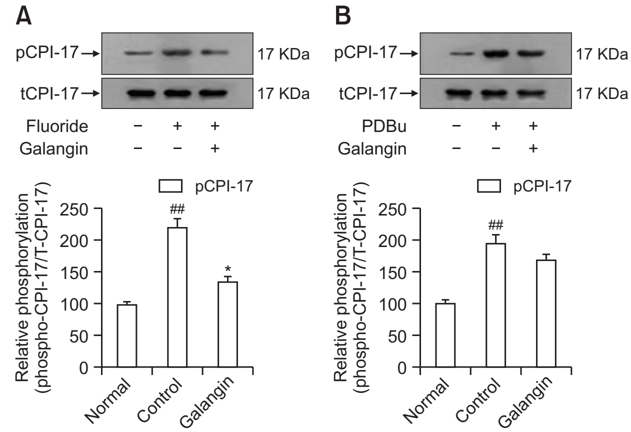

Fig. 7. Effect of galangin on fluoride (A) or phorbol ester (B)-dependent increases in phospho-CPI-17 levels. Phospho-CPI-17 protein levels were decreased in rapidly-frozen flavonol-treated muscles in the absence of endothelium compared to vehicle-treated muscles precontracted with fluoride. Upper panel indicates a typical blot, and lower panel indicates average densitometric results for relative levels of phospho-CPI-17. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. ##p<0.01, *p<0.05, versus normal or control group respectively. Galangin: 0.1 mM galangin; Fluoride: 6 mM sodium fluoride; PDBu: 1 μM phorbol 12,13-dibutyrate.

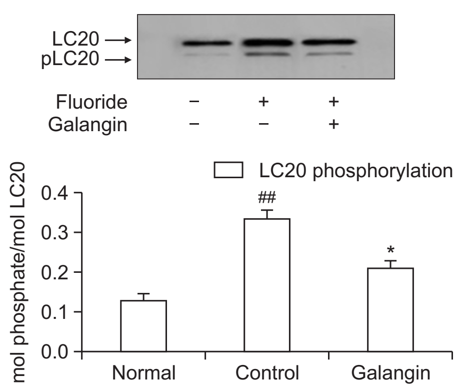

Fig. 6. Effect of galangin on fluoride-induced increase in phospho-MLC_{20} level. Phospho-MLC_{20} levels expressed as a percentage of total MLC_{20} were decreased in rapidly-frozen galangin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with fluoride (6 mM). Data are presented as the means of 3-5 experiments with a vertical line indicating SEM. ##p<0.01, *p<0.05, versus normal or control group respectively. Galangin: 0.1 mM galangin; Fluoride: 6 mM sodium fluoride.

Effect of galangin on levels of MYPT1 phosphorylation at Thr-855

To identify the role of galangin on thick filament regulation of vascular contractibility, we measured levels of myosin phosphatase targeting subunit 1 (MYPT1) and phospho-MYPT1 in aortas quick-frozen after a 60-min exposure to galangin for equilibration. Each relaxing muscle was contracted with 6 mM fluoride. This work was conducted using quick frozen galangin (0.1 mM)-treated muscles in the absence of endothelium, and levels were compared to those of vehicle-treated muscles (Fig. 5). A significant decrease in fluoride-induced MYPT1 phosphorylation at Thr855 in response to galangin treatment was observed (Fig. 5). Furthermore, a decrease in fluoride-induced LC_{20} phosphorylation was found in response to galangin treatment (Fig. 6). Therefore, thick filament regulation, including myosin phosphatase reactivation via RhoA/ROCK inactivation might be involved in the decreased contractility of galangin-treated rat aortas.

Effect of galangin on the level of CPI-17 phosphorylation at Thr-38

The myosin phosphatase inhibitor CPI-17 is phosphorylated by ROCK or PKC. CPI-17 phosphorylation is usually increased during contraction as it is one mechanism that increases myofilament Ca^{2+} sensitivity. Fluoride or phorbol 12,13-dibutyrate was used as a control for CPI-17 phosphorylation as it directly activates ROCK or PKC producing a significant increase in CPI-17 phosphorylation. To confirm the role of galangin in thick or thin filament disinhibition of smooth muscle contractility, we measured levels of CPI-17 and phospho-CPI-17 in aortas quick-frozen after a 60-min exposure to galangin for equilibration. Each relaxing muscle was precontracted with 6 mM fluoride or 1 μM phorbol ester. This work was conducted using quick frozen flavonol (0.1 mM)-treated muscles in the absence of endothelium, and levels were compared to those of vehicle-treated muscles (Fig. 7). Interestingly, a significant decrease in fluoride-induced CPI-17 phosphorylation at Thr-38 in response to galangin treatment was observed (Fig. 7A). The decrease in CPI-17 phosphorylation with galangin during fluoride stimulation suggests that ROCK is inactivated in the galangin-induced decrease in contraction and MLC phosphorylation, and myosin phosphatase reactivation.

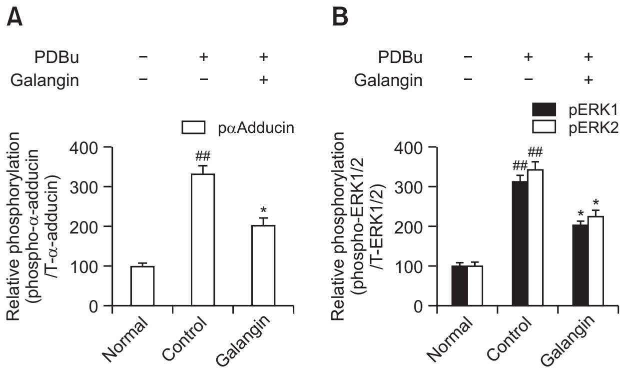

Fig. 8. Effect of galangin on phorbol ester-dependent increases in phospho-α-adducin (A) and phospho-ERK1/2 levels (B). Phospho-ERK1/2 protein levels were decreased in rapidly-frozen galangin-treated muscles in the absence of endothelium compared to vehicle-treated muscles precontracted with phorbol ester. The panel indicates average densitometric results for relative levels of phospho-ERK1/2. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. ##p<0.01, *p<0.05, versus normal or control group respectively. Galangin: 0.1 mM galangin; PDBu: 1 μM phorbol 12,13-dibutyrate.

Effect of galangin on the level of adducin phosphorylation at Ser662 and ERK1/2 phosphorylation at Thr-202/Tyr-204

To identify the role of galangin on thin filament disinhibition of vascular contractibility, we measured levels of adducin and phospho-adducin and ERK1/2 and phospho-ERK1/2 in aortas quick frozen after 60 minutes of exposure to galangin for equilibration. Each relaxing muscle was precontracted with 1 μM phorbol 12,13-dibutyrate. As compared with vehicle-treated muscles, a decrease in adducin and ERK 1/2 phosphorylation at Ser662 and Thr202/Tyr204 was observed in galangin (0.1 mM)-treated muscles in the absence of endothelium (Fig. 8); significant relaxation (Fig. 4) and thin filament regulation were observed. These findings represent that thin filament regulation, including adducin and ERK1/2 phosphorylation via PKC and MEK activation, plays a role in galangin-induced relaxation.

DISCUSSION

This is the study to suggest that galangin attenuates tonic tension and suppresses Ca^{2+} sensitization through the blockade of not PKC-mediated CPI-17 phosphorylation but ROCK-mediated CPI-17 phosphorylation. Pharmacological activators of ROCK (fluoride), MEK (phorbol 12,13-dibutyrate) or both (thromboxane mimetic) were used to determine their involvement in suppressed contraction. The CPI-17-mediated and calcium-sensitized contraction, elicited by various agonists, was enhanced consistently. Galangin attenuates tonic tension and suppresses calcium sensitization through the blockade of ROCK-mediated myosin phosphatase inhibition. Importantly, galangin did not affect PDBu-stimulated phosphorylation of CPI-17, but selectively inhibited fluoride-stimulated phosphorylation of CPI-17 and MYPT1, so preventing myosin phosphatase activities, which resulted in a decreased level of LC phosphorylation. With this distinct mode of action, galangin inhibited fluoride, phorbol 12,13-dibutyrate and thromboxane mimetic-induced vasoconstriction; thus revealing a novel therapeutic target for the development of novel antihypertensive agents.

Activation of ROCK or PKC, phosphorylation of CPI-17 or MYPT1, and subsequent inhibition of myosin phosphatase are part of the Ca^{2+} sensitization pathway that promotes increased MLC phosphorylation without requiring an increase in Ca^{2+} influx or release. ROCK phosphorylates myosin phosphatase, which inhibits phosphatase activity and leads to an accumulation of phosphorylated MLCs (Johnson *et al*., 2009; Qi *et al*., 2009; Qiao *et al*., 2014) and phosphorylates MLCs directly and independently of myosin light chain kinase and phosphatase activity (Amano *et al*., 1996). ROCK was reported to be involved in vascular contractions induced by fluoride, phorbol ester or thromboxane A_2 (Wilson *et al*., 2005; Jeon *et al*., 2006; Tsai and Jiang, 2006).

The present study demonstrates that galangin ameliorates contractions induced by vasoconstrictors (phorbol ester or fluoride) in an endothelium-independent manner (Fig. 2-4), and that the mechanism included the PKC/MEK/ERK and RhoA/ROCK pathways. Galangin attenuated the fluoride-evoked phosphorylation of CPI-17 at Thr38, suggesting that CPI-17 included in fluoride-induced contraction is a downstream effector activated by ROCK. Furthermore, galangin significantly decreased the contraction and the phosphorylation of MYPT1 at Thr855 and CPI-17 at Thr-38 evoked by fluoride (Fig. 5, 7A) with the full relaxation (Fig. 2) and α-adducin and ERK 1/2 phosphorylation at Ser662 and Thr202/Tyr204 induced by a phorbol ester (Fig. 8), suggesting that inhibition of PKC/MEK or ROCK activity is a major mechanism underlying the effects of galangin on smooth muscle contractility. Activation of ROCK by fluoride decreases the activity of myosin phosphatase through phosphorylation of MYPT1 and CPI-17, resulting in an increase in MLC_{20} phosphorylation and contractions (Sakurada *et al*., 2003; Somlyo and Somlyo, 2003; Wilson *et al*., 2005) inhibited by galangin (Fig. 6). Therefore, thick or myosin filament regulation including pCPI-17 inactivation or myosin phosphatase activation through RhoA/ROCK inactivation rather than PKC/CPI-17 might be involved in galangin-induced inhibition of vascular contractility.

In summary, galangin without newly reported adverse effects (Aloud *et al*., 2018) significantly attenuates the RhoA/ROCK activator fluoride-induced contractions decreasing CPI-17 phosphorylation and inhibits phorbol ester-induced contraction due to PKC/MEK activation. Thus, the mechanism underlying the flavonol-evoked relaxation of phorbol ester- or fluoride-induced contractions involves inhibition of PKC/MEK and ROCK activity. Inhibition of ROCK activity and subsequent CPI-17/MYPT1 phosphorylation evoked by galangin during fluoride-induced contraction suggests that ROCK/CPI-17 rather than PKC/CPI-17 inactivation is required for myosin phosphatase reactivation and relaxation.

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