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RESEARCH PAPER

Emodin inhibits tonic tension through suppressing PKCδ-mediated inhibition of myosin phosphatase in rat isolated thoracic aorta

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BACKGROUND AND PURPOSE

Dysregulated tonic tension and calcium sensitization in blood vessels has frequently been observed in many cardiovascular diseases. Despite a huge therapeutic potential, little is known about natural products targeting tonic tension and calcium sensitization.

EXPERIMENTAL APPROACH

We screened natural products for inhibitory effects on vasoconstriction using the rat isolated thoracic aorta and found that an anthraquinone derivative, emodin, attenuated tonic tension. Organ bath system, primary vascular smooth muscle cells, confocal microscopy and Western blot analysis were employed to demonstrate the suppressive effects of emodin on PKCδ-mediated myosin phosphatase inhibition.

KEY RESULTS

Emodin, an active ingredient of *Polygonum multiflorum* extract, inhibited phenylephrine-induced vasoconstriction in rat isolated thoracic aorta, and inhibited vasoconstriction induced by 5-HT and endothelin-1. It also generally suppressed vasoconstrictions mediated by voltage-operated, store-operated calcium channels and intracellular calcium store. However, emodin did not affect agonist-induced calcium increases in primary smooth muscle cells. In contrast, post-treatment with emodin following phenylephrine stimulation potently suppressed tonic tension in rat aortic rings. Western blot analysis revealed that emodin inhibited phenylephrine-induced phospho-myosin light chain (pMLC) and the phosphorylation of myosin-targeting subunit and C-kinase-activated protein phosphatase-1 inhibitor (CPI-17). This was mediated by selective inhibition of PKCδ, whereas PKCα was not involved.

CONCLUSION AND IMPLICATIONS

Emodin attenuates tonic tension through the blockade of PKCδ and CPI-17-mediated MLC-phosphatase inhibition. This new mode of action for the suppression of tonic tension and structural insights into PKCδ inhibition revealed by emodin may provide new information for the development of modulators of tonic tension and for the treatment of hypertension.

Abbreviations

DAB, 3,3′-diaminobenzidine; ET-1, endothelin-1; ICS, intracellular calcium store; KR solution, Krebs-Ringer solution; MLC_{20}, myosin light chain; MYPT1, myosin-targeting subunit; NCEs, new chemical entities; PDBu, phorbol 12,13-dibutyrate; pMLC, phospho-MLC; ROCK, RhoA–Rho-associated PK; SOCC, store-operated calcium channel; TCA, trichloroacetic acid; VOCC, voltage-operated calcium channel; VSMC, vascular smooth muscle cell

Table of Links

TARGETS	LIGANDS
PKCδ	Phenylephrine
PKCα	5-HT
VOCC	Endothelin-1
MLCK	
ROCK	

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b).

Introduction

Hypertension is a major risk factor for acute and chronic heart diseases. High arterial blood pressure can also increase the mortality and morbidity from many other life-threatening cardiovascular diseases such as stroke, renal failure and atherosclerosis (Roger *et al.*, 2012). The prevalence of hypertension continually increases, reaching over 25–35% of the adult population, and 60–70% of the elderly population aged beyond 70 (Hajjar *et al.*, 2006; CDC, 2011). To tackle this deadly disease, many molecular targets have been explored to develop effective anti-hypertensive agents that include diuretics, adrenoceptor agonists/antagonists, calcium channel blockers, angiotensin converting enzyme (ACE) inhibitors, vasodilators and renin inhibitors/receptor blockers. Indeed, these drugs have helped to save millions of lives. However, there are still a significant number of patients with hypertension, as many as 56% of all treated, whose blood pressure cannot be controlled with currently available drugs (Lloyd-Jones *et al.*, 2010), underlining a urgent demand for novel approaches and more effective new anti-hypertensive drugs.

The vascular smooth muscle cell (VSMC) and its contractile machinery has been a subject of intense research for the discovery of novel anti-hypertensive drugs. VSMC contracts in response to various physiological contractile agonists and maintains vascular tension for a certain time to provide an adequate blood flow to tissues. Primary tension generation, that is phasic tension, is initiated by rapid and high cytosolic calcium increase that is provided from receptor-mediated, store-operated or voltage-gated calcium channels (Aksoy *et al.*, 1983). Subsequently, calcium-calmodulin activated myosin light chain kinases phosphorylate myosin light chain (MLC_{20}), ultimately generating contractile force. Concomitantly with phasic tension, receptor-mediated activation of PKC-CPI-17 (C-kinase-activated protein phosphatase-1 inhibitor) (Kitazawa *et al.*, 2000) and RhoA–Rho-associated PK (ROCK) pathways inhibit MLC-phosphatase via the phosphorylation of a regulatory subunit, MYPT1, sustaining MLC phosphorylation for a certain time even after the initial calcium peak dissipates. This phenomenon is called calcium sensitization and the maintenance of the tension generated is described as tonic tension (de Godoy and Rattan, 2011). While most conventional anti-hypertensive drugs have focused on the control of phasic tension, recent interest is being transferred to the regulation of calcium sensitization and tonic tension (Christ and Wingard, 2005). Augmented calcium sensitization has been frequently observed in patients with cardiovascular diseases and in many animal models of hypertension (Satoh *et al.*, 1994; Shimokawa *et al.*, 1999; Seko *et al.*, 2003; Chiba *et al.*, 2005), indicating the importance of regulating calcium sensitization for the treatment of hypertension.

Natural resources have helped to enrich the pharmacotherapeutic arsenal for the treatment of hypertension by providing important insights into pharmacophore, molecular targets and structural properties for the development of new anti-hypertensive drugs. According to the analysis by Newman *et al.* (2003), 49 of 75 new chemical entities (NCEs) in the anti-hypertensive category have been derived from the knowledge gained from a study of natural products, highlighting the importance of natural products in development of new anti-hypertensive drugs. Recently, Seok *et al.* (2008) demonstrated that isoflavones can attenuate vascular contraction through inhibition of RhoA/ROCK-mediated calcium sensitization, providing an important line of evidence supporting the possibility that the anti-hypertensive effect of natural products may stem from the regulation of tonic tension. Incidentally, to the best of our knowledge there has been no report regarding natural products that target PKC-CPI-17-dependent tonic tension.

In the present study we demonstrated that emodin, an anthraquinone derivative widely present in herbal medicines

(Srinivas *et al.*, 2007), can modulate agonist-induced vasoconstriction. The decreased contraction induced by emodin resulted from the inhibition of tonic tension and calcium sensitization. Notably, this was induced by selective blockade of PKCδ, a novel calcium-independent PKC isoform in VSMCs (Salamanca and Khalil, 2005), and subsequent suppression of CPI-17-mediated MLC-phosphatase inhibition and contractile force generation.

Methods

Herb extracts

Herb extracts were provided by the National Centre for Standardization of Herbal Medicines in Korea. Dry powders of herbs (*Achyranthes japonica*, *Atractylodes japonica*, *Aucklandia lappa*, *Foeniculum vulgare*, *Lycium chinense*, *Kalopanax pictus*, *Polygonatum stenophyllum*, *Polygonum multiforum*, *Rehmannia glutinosa*) were extracted with 70% ethanol at 70–80°C for 3 h. The extraction was repeated three times. After filtration and concentration under reduced pressure, extracts were lyophilized and the resultant powder was stored at –20°C. For experiments extracts were dissolved in DMSO.

Animals

The entire animal protocol was approved by the Ethics Committee of the Animal Service Centre at Seoul National University. Male Sprague-Dawley rats (SamTako., Seoul, Korea) weighing 250–300 g were used (except for the primary smooth muscle cell culture assay which used animals weighing 150–170 g). Before the experiments, animals were acclimatized for 1 week in the laboratory animal facility maintained at constant temperature (22 ± 2°C) and humidity (55 ± 5%) with a 12 h light/dark cycle. Food and water were provided *ad libitum*. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Measurement of vasoconstriction in isolated aortic rings

After rats were humanely decapitated and exsanguinated, the thoracic aorta was carefully isolated and cut into ring segments in lengths of 3–4 mm on ice. Aortic rings without endothelium were prepared by gently rubbing the intimal surface of the aortic rings with a cotton swab. Complete removal of endothelium was checked by the absence of relaxation response to ACh challenge. The rings were then mounted in organ baths filled with Krebs-Ringer solution (KR solution; 115.5 mM NaCl, 4.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11.1 mM glucose, pH 7.4) continuously saturated with 95% O_2/5% CO_2 mixture gas and maintained at 37°C. The change in tension was measured with Grass FT03 force transducers (Grass Instrument Co., Quincy, MA, USA) and recorded using the AcqKnowledge III (BIOPAC Systems Inc., Goleta, CA, USA). To investigate the effect on vasoconstriction, the aortic rings were pretreated with emodin or vehicle (DMSO) and vasoconstriction was initiated by the cumulative addition of phenylephrine, 5-HT, ET-1 or PDBu. In order to examine whether emodin might affect the stage of tension maintenance, that is, the tonic tension, emodin was treated 10 min after induction of vasoconstriction by phenylephrine (10^{-5} M).

Measurement of lactate dehydrogenase leakage

To examine the non-specific cytotoxicity of emodin, the extent of LDH leakage from aortic rings was measured. After incubation with emodin for 2 h and lysophosphatidylcholine for 24 h, aliquots were collected. Fifty microlitres of aliquot was added to 1 mL of Tris-EDTA-NADH buffer (56 mM Tris(hydroxymethyl)aminomethane, 5.6 mM EDTA, 0.17 mM β-NADH, pH 7.4) and then incubated for 10 min at 37°C. After incubation, 100 μL of prewarmed 14 mM pyruvate solution 37°C was added. The reduction in absorbance at 340 nm by the conversion of NADH to NAD^+ was measured for 1 min to evaluate LDH activity in the aliquots.

TUNEL staining of aortic rings and histopathological assessment

Aortic rings were placed in MEM containing 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin and emodin, monomethylarsonous acid or DMSO was added and the rings incubated in a 95% air/5% CO_2 incubator for 24 h at 37°C. After this incubation, aortic rings were fixed in buffered formalin solution (10%) and embedded in paraffin. The TUNEL assay was performed using a commercial kit according to the manufacturer's instruction (Chemicon International, Temecula, CA, USA). The embedded tissue was cut into sections, 4 μm thick, and placed on an adhesive slide. The sections were deparaffinized by washing with xylene following serial dehydration with ethanol (100, 95, 80 and 70%). Dehydrated sections were treated with 0.3% H_2O_2 to quench endogenous peroxidase activity followed by 20 μg·mL^{-1} DNase-free proteinase K to retrieve antigenic epitopes. After that, the samples were treated with terminal deoxynucletidyl transferase enzyme reagent for 1 h at 37°C to label free 3′-OH termini with digoxigenin-dUTP. To detect incorporated digoxigenin-conjugated nucleotides, HRP-conjugated antidigoxigenin antibody and 3,3′-diaminobenzidine (DAB) was used. The samples were treated with anti-digoxigenin-peroxidase for 30 min at room temperature followed by DAB development. They were then counterstained with Mayer's haematoxylin. The dehydrated samples were cleared in xylene and mounted.

Intracellular and extracellular Ca^{2+} pathways

To examine the effect of emodin on the contraction mediated by release of the intracellular Ca^{2+} store, vasoconstriction was induced in Ca^{2+}-free conditions. After the aortic rings without endothelium had been treated with emodin or vehicle for 2 h, the KR solution was replaced with Ca^{2+}-free KR solution (120.0 mM NaCl, 5.9 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 2 mM EGTA and 11.5 mM glucose, pH 7.4) containing emodin, and phenylephrine (10^{-5} M) was added to initiate vasoconstriction. For selective activation of SOCC, the aortic rings without endothelium were pretreated with 1 μM thapsigargin (an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase) for 90 min in Ca^{2+}-free KR solution to

deplete the intracellular Ca^{2+} store. Aortic rings were then treated with emodin or vehicle for 2 h and the bath solution was exchanged for KR solution containing 2.5 mM Ca^{2+} to induce SOCC-mediated contraction. To investigate the effect of emodin on the contraction induced by an influx of extracellular Ca^{2+}, Bay K8644, an L-type Ca^{2+} channel opener was used. Bay K8644 was added cumulatively in 15 mM K+ buffer solution (105 mM NaCl, 15 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.026 mM EDTA and 11.1 mM glucose, pH 7.4) to initiate vasoconstriction.

Calcium measurement in live cells

The intracellular calcium level was measured using a fluorometric method, which employed fura-2 and digital imaging as described previously (Lee *et al.*, 2006). After the endothelium and adventitia were removed, aortic rings were chopped finely and smooth muscle cells were liberated from the tissue using collagenase and elastase (Worthington Biochemical Corp., Lakewood, NJ, USA). Vascular smooth muscle cells grown on coverslips were treated with emodin for 2 h. To load fura-2, cells were incubated in physiological salt solution (PSS; 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 11.5 mM glucose and 10 mM HEPES, pH 7.4) containing 1 μM fura-2/AM and 1% BSA for 60 min. Coverslips were mounted in a superfusion chamber on the microscope stage and were superfused with PSS (2 mL·min^{-1}). All experiments were performed at 33°C. Cells were imaged with a Nikon Eclipse Ti-U inverted microscope equipped with a S Fluor 40X (N.A. 1.30, oil) objective lens (Nikon, Melville, NY, USA) and an evolve EMCCD camera (Photometrics, Tucson, AZ, USA). Illumination was provided by a Sutter DG-4 filter changer (Sutter Instruments, Novato, CA, USA). Excitation and emission wavelengths used for fura-2 were 340/380 and 535 nm respectively. Images were acquired and analysed with a Meta Imaging System (Molecular Devices, West Chester, PA, USA).

Western blotting

After the aortic rings without endothelium had been treated with emodin or vehicle for 2 h, 10^{-5} M phenylephrine was added for 2 min and the reaction was terminated by addition of acetone containing 10% TCA and 10 mM DTT precooled to –80°C. The aortic rings were washed three times with ice-cold acetone containing 10 mM DTT and then lyophilized at –80°C overnight. Aortic rings were homogenized in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS, pH 7.6) containing 1% Halt protease and a phosphatase inhibitor cocktail and lysed at 4°C for 1 h with frequent vortexing. The protein extracts (20 μg) were subjected to Western blot analysis using SDS-PAGE and antibodies specific against MLC, pMLC, MYPT1, pMYPT1 (for phosphorylation site at Thr^{835}), CPI-17, pCPI-17, PKCα, pPKCα, PKCδ and pPKCδ. The bands were detected and analysed with ChemidocTM XRS system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All the data are shown as mean ± SEM and were subjected to one-way ANOVA followed by Duncan's multiple-ranged tests to determine which means were significantly different from the control. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software (SPSS, Inc., Chicago, IL, USA). In all cases, a P value <0.05 was taken to indicate significant difference.

Reagents

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA): emodin, phenylephrine, endothelin-1 (ET-1), 5-HT creatinine sulfate, NADH, pyruvate, (-)-(S)-Bay K8644, thapsigargin, phorbol 12,13-dibutyrate (PDBu), DMSO, trichloroacetic acid (TCA), DTT. Fura-2/AM was obtained from Molecular Probes (Eugene, OR, USA), and reagents and media used in cell culture were purchased from Gibco Co. (Calsbad, CA, USA). MLC antibody, phospho-myosin light chain (pMLC) antibody, MYPT1 antibody, pMYPT1 antibody, PKCα antibody, pPKCδ antibody and HRP-conjugated anti-rabbit secondary antibody were from Cell Signaling Technology, Inc. (Danvers, MA, USA). pPKCα antibody and PKCδ antibody were from Abcam (Cambridge, UK). Antibody to CPI-17 was from Upstate Biotechnology, Inc. (Charlottesville, VA, USA) and pCPI-17 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Halt protease and phosphatase inhibitor cocktail and RIPA buffer were from Thermo Scientific (Rockford, IL, USA). All other reagents used were of the highest purity available.

Results

To find natural compounds with anti-hypertensive activities, rat isolated aortic rings were pretreated with 70% ethanol extracts of eight herbs, that included *Achyranthes japonica*, *Atractylodes japonica*, *Aucklandia lappa*, *Foeniculum vulgare*, *Lycium chinense*, *Kalopanax pictus*, *Polygonatum stenophyllum*, *Polygonum multiflorum*, *Rehmannia glutinosa*, at 250 μg·mL^{-1} for 30 min and a contractile agonist, phenylephrine, was cumulatively added to induce contraction. As shown in Figure 1A, the extract of *Polygonum multiflorum* exhibited strong inhibitory activities on phenylephrine-induced vasoconstriction. To identify the active ingredient for this effect, known components of *Polygonum multiflorum* were tested and the vasoconstriction was examined. Among the six compounds, emodin, an anthraquinone derivative, was the most effective (Figure 1B); it completely inhibited phenylephrine-induced vasoconstriction at a relatively low concentration, 10 μM (Figure 1C). The phenylephrine-induced vasoconstriction was inhibited by emodin in concentration- and time-dependent manner (Figure 1C and D), while the vehicle had no effect up to 2 h. We examined the effects of emodin on the vasoconstriction induced by other contractile agonists. As shown in Figure 2A and B, 5-HT- and ET-1-induced vasoconstrictions were also suppressed by emodin suggesting that it affects common contractile pathways following the activation of adrenoceptors, $5\text{-}HT_{2A}$ and ET_A receptors. These effects were not from non-specific cytotoxic or apoptotic effectss (Figure 2C and D). In addition, a role for reactive oxygen species (ROS) could be excluded by the failure of ROS-removal, using catalase and superoxide dismutase, to reverse the effects of emodin (data not shown).

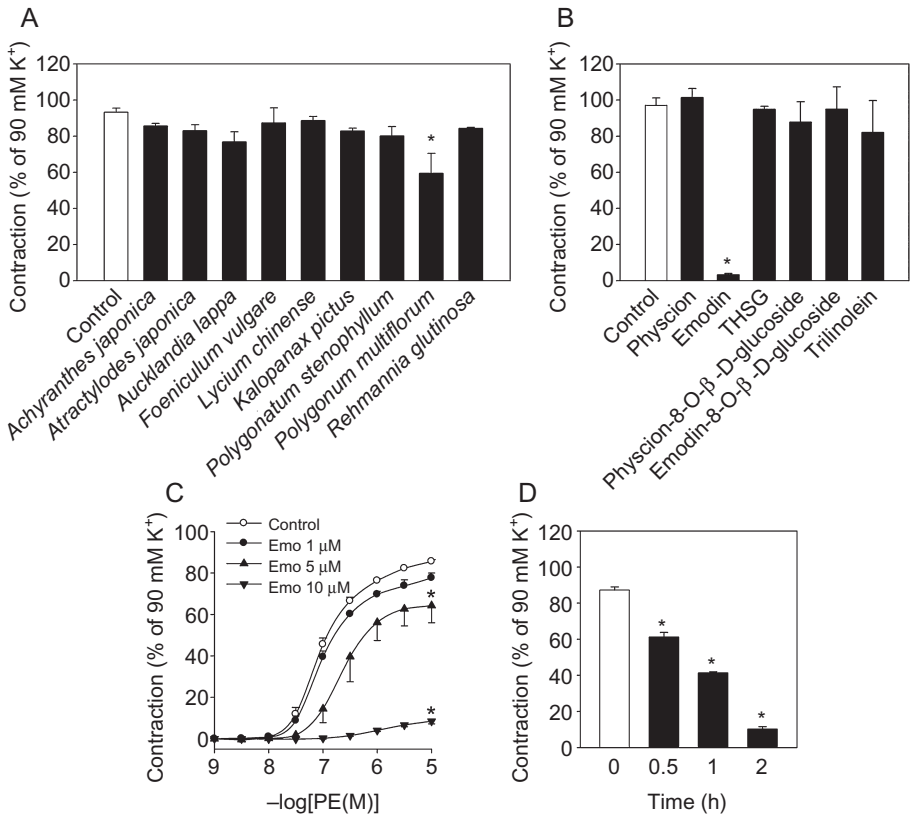

Figure 1

Effects of herbal extract on phenylephrine (PE)-induced vasoconstriction in aortic rings with intact endothelium. (A) After 250 μg·mL^{-1} of herbal extracts were treated to aortic rings with endothelium for 30 min, phenylephrine-induced contraction was measured. (B) Components of *Polygonum multiforum* (100 μM) were treat to aortic rings with endothelium for 30 min and phenylephrine-induced contraction were measured. THSG; 2,3,5,4-tetrahydroxystilbene-2-O-β-D-glucoside. *Represents significant difference from control (Student's *t*-test, $P < 0.05$). After emodin was treated to aortic rings with endothelium, (C) concentration- (with 2 h incubation) and (D) time-dependent effects (at 10 μM) on phenylephrine-induced vasoconstriction were obtained. Values are mean ± SEM of three to six independent experiments. *Represents significant differences from control (ANOVA and Duncan's *post hoc* analysis, $P < 0.05$).

Vasoconstriction is achieved by the balanced sum of the vasodilator and contractile responses of endothelial cells and smooth muscle cells constituting the blood vessel. Inhibition of vasoconstriction by emodin was retained in endothelium-denuded aortic rings suggesting that it mainly affects the contractile function of vascular smooth muscle cells (Figure 3A). Contraction of vascular smooth muscle is accomplished in two distinct phases: phasic tension and tonic tension. Phasic tension, the generation of contractile force, is initiated by a rapid increase in cytosolic calcium, which is supplied from an intracellular calcium store (ICS), voltage-operated (VOCC) or store-operated calcium channels (SOCC). Emodin inhibited phasic tension without affecting ICS, VOCC and SOCC activation (Figure 3B), suggesting that emodin affected the contractile events downstream of calcium increases. Further supporting this, emodin did not affect agonist-induced increases in intracellular calcium in primary vascular smooth muscle cells (Figure 3C).

We examined if emodin could affect tonic tension. After vasoconstriction was induced with phenylephrine, emodin or vehicle was added. As shown in Figure 4A, phenylephrine induced a long-lasting contraction of aortic rings, but the treatment with emodin shortened it significantly, suggesting that emodin may affect tonic tension. To confirm this, pMLC was measured in the presence and absence of emodin (10 μM). Emodin treatment significantly reduced the level of pMLC (Figure 4B). In the tonic tension, the pMLC level is regulated by the phosphorylation of MYPT1, which is the inhibitory regulatory subunit for MLC-phosphatase. A decreased pMYPT1 results in insufficient inhibition of MLC-phosphatase and an increase in pMLC. We observed that in the presence of emodin, phosphorylation of MYPT1 was almost completely prevented (Figure 4C), suggesting that emodin affects MYPT1 phosphorylation. MYPT1 phosphorylation is regulated by PKC-dependent CPI-17. Next, we examined the effects of emodin on CPI-17. Phenylephrine induced a robust activation of CPI-17, which was significantly attenuated by emodin, suggesting that emodin affects the PKC-CPI-17 pathways (Figure 4D).

To confirm that emodin affects PKC-CPI-17 pathways PDBu, a PKC activator, was introduced. Emodin significantly inhibited the vasoconstriction and CPI-17 phosphorylation induced by PDBu (Figure 5A and B). To identify the PKC isoform affected by emodin, we examined the activation of

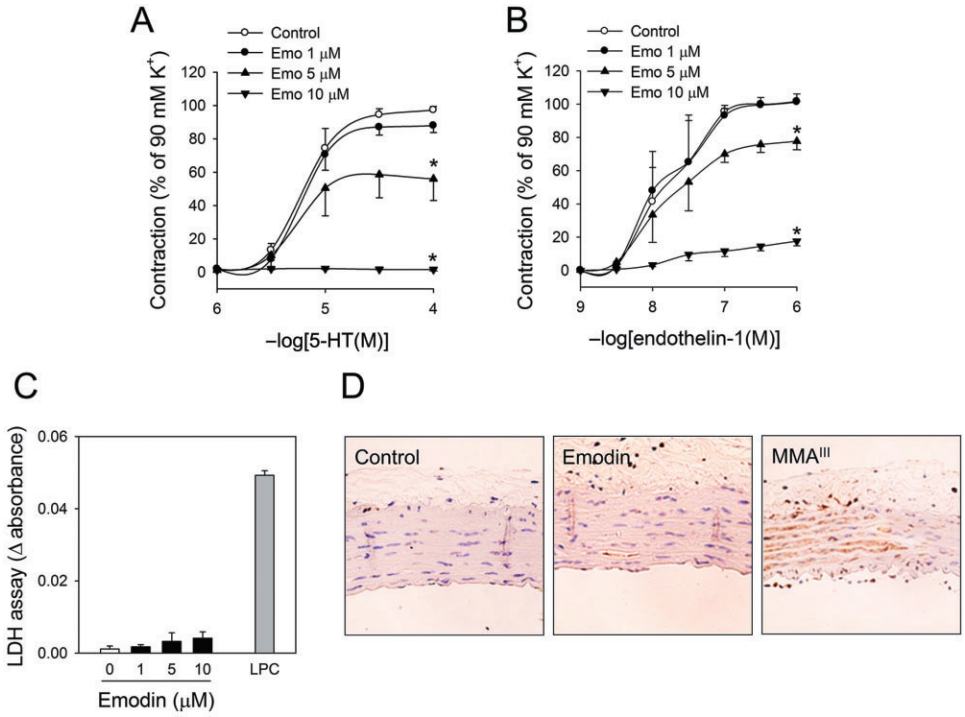

Figure 2

Effects of emodin on agonist-induced vasoconstriction and nonspecific cytotoxicities in aortic rings. Aortic rings with endothelium were pretreated with emodin for 2 h, then (A) 5-HT- and (B) ET-1-induced contractions were measured. (C) Cytotoxicity was determined by LDH leakage in emodin-treated aortic rings. Lysophosphatidylcholine (LPC; 100 μM) was used as positive control. (D) Aortic rings with endothelium were treated with emodin for 24 h, apoptosis was measured using TUNNEL assay. Monomethylarsonous acid (MMA, 5 μM) was used as positive control. Values are mean ± SEM of three to four independent experiments. *Represents significant differences from control (ANOVA and Duncan's *post hoc* analysis, $P < 0.05$).

PKC isoforms. Conspicuously, while a calcium-dependent typical PKC isoform, PKCα was not affected, emodin selectively suppressed the activation of a novel calcium-independent PKC isoform, PKCδ; this represents a potential new mode of action for the regulation of tonic tension and anti-hypertensive agents (Figure 5C and D, Supporting Information Figure S1).

Discussion

Here we demonstrated that a natural anthraquinone derivative, emodin attenuates tonic tension and suppresses calcium sensitization through the blockade of PKCδ- mediated MLC-phosphatase inhibition. Importantly, emodin did not affect agonist-stimulated cytosolic calcium increase or phasic tension, but selectively inhibited the phosphorylation of CPI-17 and MYPT1, so preventing MLC-phosphatase activities, which resulted in a decreased level of pMLC. With this distinct mode of action, emodin inhibited phenylephrine-, ET-1- and 5-HT-induced vasoconstriction; thus revealing a new therapeutic target for the development of anti-hypertensive agents.

Conventional anti-hypertensive agents have focused on the modulation of phasic tension, but many cardiovascular diseases stem from the dysregulation of tonic tension and calcium sensitization (de Godoy and Rattan, 2011). Abnormally prolonged vasoconstriction or excessively sensitive vasoconstrictor responses are frequently observed in many cardiovascular diseases, like pulmonary artery hypertension, vasospasm and right ventricular hypertrophy, suggesting that the modulation of calcium sensitization may lower high blood pressure in these diseases. Indeed, modulators of calcium sensitization like the ROCK inhibitor, fasudil, are successfully going through clinical trials for the treatment of pulmonary hypertension (Fukumoto *et al.*, 2005) and cerebral vasospasm (Zhao *et al.*, 2006), supporting the fact that modulation of tonic tension may provide new therapeutic treatments for refractory hypertensive diseases.

At a similar intracellular calcium level, agonists induce a higher contraction than that achieved by simple membrane depolarization (DeFeo and Morgan, 1985). This phenomenon, agonist-induced force enhancement, has been coined as calcium sensitization (Kitazawa *et al.*, 1991; Uehata *et al.*, 1997). Importantly, there is increasing evidence suggesting that calcium sensitization has broad pathological implications in muscle-related gastrointestinal, respiratory and cardiovascular diseases. Dysregulation of calcium sensitization is involved in the pathogenesis of rectoanal incontinence, certain forms of constipation, recurrent anal fissures, haemorrhoids (de Godoy and Rattan, 2011), erectile dysfunction, asthma, vasospasm and congestive heart failure (Brozovich,

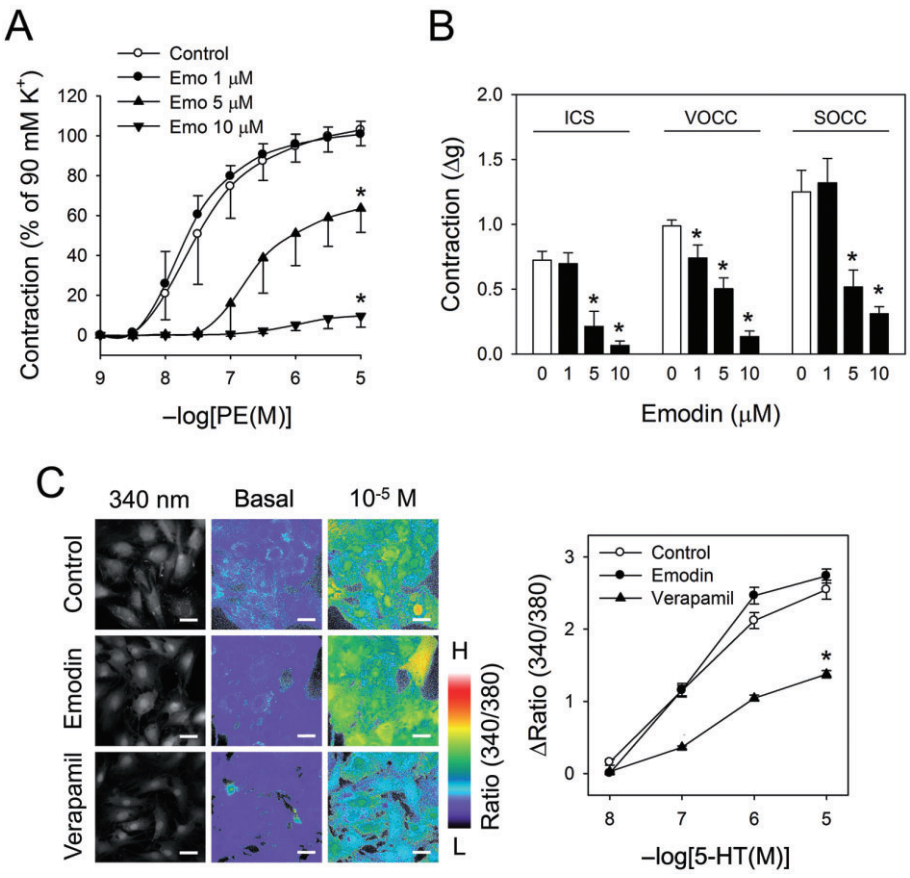

Figure 3

Effects of emodin on calcium mobilization and vasoconstriction. (A) Aortic rings without endothelium were pretreated with emodin for 2 h, phenylephrine-induced contraction was measured. (B) Effects of emodin on ICS-, VOCC- and SOCC-mediated vasoconstriction were measured in aortic rings without endothelium. (C) Effects of emodin (10 μM, 2 h) on calcium were observed in primary smooth muscle cells with a fluorometric method employing fura-2/AM. Verapamil was used as positive control. Values are mean ± SEM of three to four independent experiments. *Represents significant difference from control (ANOVA and Duncan's *post hoc* analysis, $P < 0.05$).

2002), underlining the therapeutic importance of modulators of calcium sensitization. We believe that it would be interesting to examine the effects of emodin in these diseases in the future.

Calcium sensitization is mainly accomplished by the inhibition of MLC-phosphatase. MLC-phosphatase modulates the level of pMLC and tension generation. The inhibition of MLC-phosphatase activity generates a stronger contractile force, while its stimulation attenuates the vasoconstriction. MLC-phosphatase is composed of three subunits; a small ~20-kDa subunit with unidentified function, a ~38-kDa catalytic subunit (protein phosphatase 1c), and a large regulatory subunit, myosin-targeting subunit (MYPT1) of 110–133 kDa (Brozovich, 2002). Phosphorylation of MYPT1 inhibits the catalytic activities of MLC-phosphatase (Ichikawa *et al.*, 1996). Phosphorylation of MYPT1 and resultant inhibition of MLC-phosphatase occurs through two pathways, Rho-ROCK and PKC-CPI-17. Translocation of Rho A from the cytosol to the cell membrane leads to spatial activation of ROCK, which phosphorylates MYPT1. PKC activation following contractile agonist-binding to Gq-coupled receptors results in the phosphorylation of CPI-17 at Thr^{38}, which can phosphorylate MYPT1 potently. Many PKC isoforms including PKCα, PKCδ and PKCε are involved in the phosphorylation of CPI-17, but PKCδ is known to be threefold more specific for CPI-17 than PKCα or ROCK suggesting that PKCδ has an important role in the phosphorylation of CPI-17 (Eto *et al.*, 2001). In addition, the PKC-CPI-17 pathway is considered to be dominant in phenylephrine-induced tonic tension in blood vessels (Kitazawa and Kitazawa, 2012).

PKCδ is a representative member of novel PKC isoforms (PKCδ, ε, ϕ, η). It is ubiquitously expressed and plays a critical role in cytoskeleton maturation, cellular growth, differentiation and apoptosis (Wu-Zhang *et al.*, 2012). A recent study demonstrated that PKCδ is important in angiotensin II-mediated extracellular matrix collagen synthesis and cardiac fibrosis (Chintalgattu and Katwa, 2009), suggesting that it may also be widely involved in other cardiovascular diseases. In contrast to classic PKCs (PKCα, βI, βII, γ), PKCδ is calcium-independent, but is activated by DAG from receptor-mediated hydrolysis of membrane inositol phospholipids (Steinberg, 2004). Direct activation of PKCδ by stimulation of

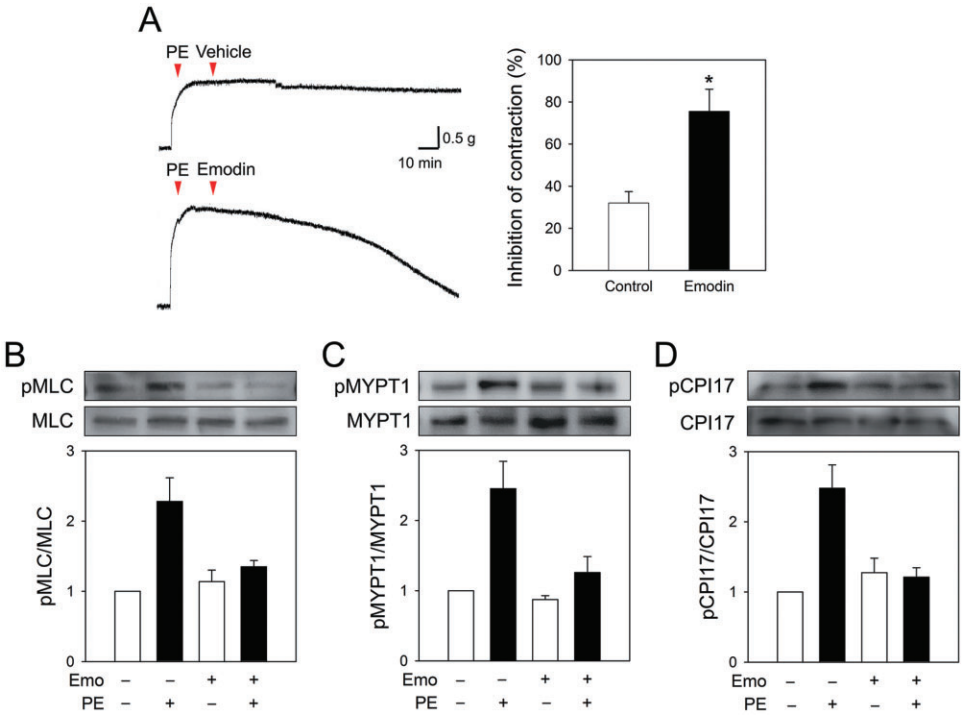

Figure 4

Effects of emodin on tonic tension, MLC phosphorylation, MYPT1 phosphorylation and CPI-17 phosphorylation in aortic rings without endothelium. (A) After aortic rings without endothelium were precontracted with phenylephrine (10^{-5} M, 15 min), they were treated with emodin (10 μM) and the change in contractility was measured after 2 h. After aortic rings without endothelium were treated with emodin (10 μM) for 2 h, aortic rings were stimulated with phenylephrine (10^{-5} M) for 2 min, and protein was extracted from aortic tissues. (B) MLC phosphorylation, (C) MYPT1 phosphorylation and (D) CPI-17 phosphorylation were determined by Western blotting. Values are mean ± SEM of three to four independent experiments. *Represents significant difference from control (Student's *t*-test, $P < 0.05$).

the insulin receptor in skeletal muscle was also observed (Braiman *et al.*, 2001), suggesting that PKCδ is regulated by a distinct mode of action.

In contrast to the remarkable advance in the drug development of ROCK inhibitors, few PKCδ inhibitors are currently under active development. This may be due to a lack of information on the pharmacophore or selective inhibitors for PKCδ, which might be rectified by insights gained from the studies on natural products. Rottlerin, which is a natural compound isolated from *Mallotus philippinensis*, was originally identified as a specific inhibitor of PKCδ (Gschwendt *et al.*, 1994), but recent reports showed that rottlerin was not effective at inhibiting PKCδ activity (Soltoff, 2007). Instead, it was suggested that rottlerin may have off-target effects and just elicit cellular changes mimicking those caused by the direct inhibition of PKCδ. Emodin inhibited CPI-17 and MYPT1 phosphorylation induced by PDBu as well as that by phenylephrine without affecting PKCα or RhoA activities (data not shown). Considering that PDBu binds to the phorbol ester-binding domain of PKC, causing direct activation of PKC catalytic domain, emodin appears to directly inhibit the catalytic activity of PKCδ, rather than modulating the receptor-binding domain or Gq-mediated DAG production. However, further studies employing purified PKC isoforms are needed to elucidate the selectivity and mode of inhibition of emodin against PKCδ.

Our data show for the first time, that emodin may be a selective and potent inhibitor of PKCδ without affecting PKCα activation. Emodin is widely available in many herbal plants, such as *Polygonum multiflorum*, *Rheum Senna obtusifolia*, *Fallopia japonica* and *Rheum palmatum*. Various pharmacological effects of emodin have been reported, including anti-diabetic (Feng *et al.*, 2010), anti-tumour (Muto *et al.*, 2007), neuroprotective (Kuo *et al.*, 2009) and anti-inflammatory effects (Meng *et al.*, 2010). These therapeutic activities have been suggested to result from inhibition of 11β-hydroxysteroid dehydrogenase type 1, JAK2 or NF-κB activation. In the present study, we demonstrated that emodin can inhibit PKCδ activity, providing a new mode of action for its therapeutic effects.

Conclusion

In conclusion, our study demonstrated that emodin suppresses tonic tension by suppressing the activity of PKCδ and CPI-17-mediated MLC-phosphatase inhibition (see Figure 6). We believe that this new mode of action for the suppression of tonic tension and structural insights into PKCδ inhibition revealed by emodin may be instrumental for the development of new modulators of tonic tension and for the treatment of hypertensive diseases.

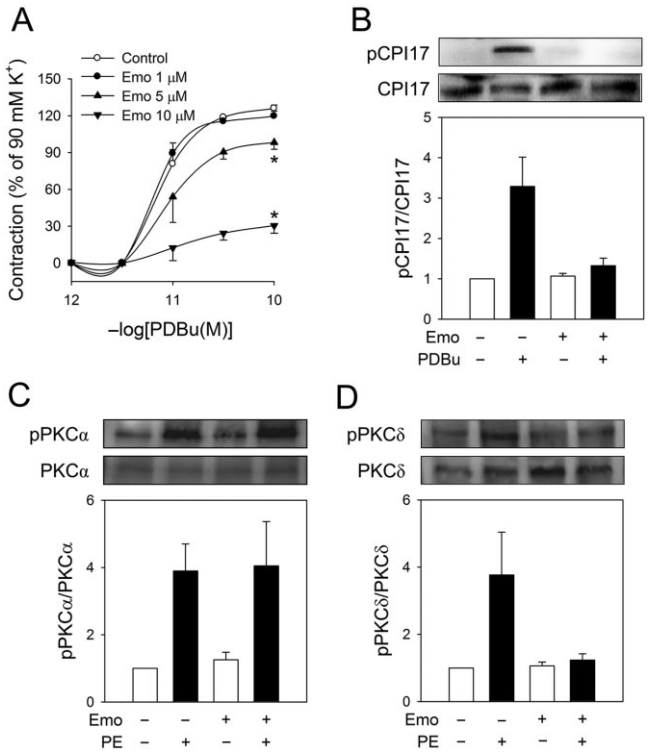

Figure 5

Effects of emodin on PKCδ activation in aortic rings without endothelium. (A) Aortic rings without endothelium were pretreated with emodin for for 2 h, PDBu-induced contraction was then measured. (B) Aortic rings without endothelium were pretreated with emodin (10 μM) for 2 h, aortic rings were stimulated with PDBu (10^{-10} M) for 2 min and protein was extracted from the aortic tissues. CPI-17 phosphorylation was determined by western blotting. (C, D) Aortic rings without endothelium were treated with emodin (10 μM) for 2 h, they were then stimulated with phenylephrine (10^{-5} M) for 2 min, and protein was extracted from aortic tissues. (C) PKCα and (D) PKCδ activation was determined by PKC phosphorylation through Western blotting. Values are mean ± SEM of three to four independent experiments. *Represents significant differences from control (ANOVA and Duncan's *post hoc* analysis, $P < 0.05$).

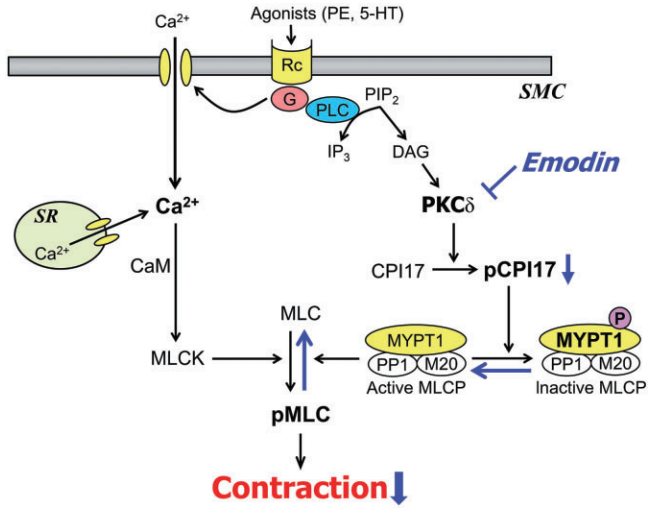

Figure 6

Suggested mechanism for the inhibitory effect of emodin on agonist-induced vasoconstriction.

Conflict of interest

None.

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Author contributions

K.M.L., J.H.K. and K.K. designed the experiments, analysed the data and wrote the paper; J.Y.N. and S.K. performed additional experiments for the revision; J.M.P and M.Y.L. performed calcium experiments in live cells; O.N.B. analysed the data and edited the manuscript. J.H.C. supervised the study.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12804

Figuer 1 Effects of emodin on phenylephrine-induced activation of PKCα and PKCδ. Phosphorylation of PKCα was determined after aortic rings were treated with or without emodin and phenylephrine. To confirm the specificity of antibodies against PKCα and PKCδ, the whole blot was obtained with molecular weight markers.