

The Inhibitory Effect of Apigenin on the Agonist-Induced Regulation of Vascular Contractility via Calcium Desensitization-Related Pathways

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Abstract

Apigenin, a natural flavonoid found in a variety of vegetables and fruits, has been shown to possess many biological functions. The present study was undertaken to investigate the influence of apigenin on vascular smooth muscle contractility and to determine the mechanism involved. Denuded aortic rings from male rats were used and isometric contractions were recorded and combined with molecular experiments. Apigenin significantly relaxed fluoride-, thromboxane A₂ mimetic- or phorbol ester-induced vascular contraction, which suggests that apigenin could be an anti-hypertensive that reduces agonist-induced vascular contraction regardless of endothelial nitric oxide synthesis. Furthermore, apigenin significantly inhibited fluoride-induced increases in pMYPT1 levels and phorbol ester-induced increases in pERK1/2 levels, which suggests the mechanism involving the inhibition of Rho-kinase and MEK activity and the subsequent phosphorylation of MYPT1 and ERK1/2. This study provides evidence regarding the mechanism underlying the relaxation effect of apigenin on agonist-induced vascular contraction regardless of endothelial function.

Key Words: Apigenin, ERK1/2, Fluoride, MYPT1, Phorbol ester, Rho-kinase

INTRODUCTION

Apigenin (4',5,7-trihydroxyflavone, Fig. 1) is a natural flavonoid that is found abundantly in parsley, peppermint, lemon, perilla, berries, and fruits (Peterson and Dwyer, 1998). Apigenin has been reported to have beneficial effects which include anti-inflammatory (Nicholas *et al.*, 2007) and anti-carcinogenic effects for skin, and free radical scavenging properties in many *in vitro* systems (Han *et al.*, 2012). Studies on anti-carcinogenic effects have shown that apigenin possesses growth inhibitory properties against many human cancer cell lines, for examples, breast, colon (Wang *et al.*, 2000), skin (Caltagirone *et al.*, 2000), thyroid, leukemia cells, and solid malignant tumor cells (Fotsis *et al.*, 1998). Although the influence of endothelial nitric oxide synthesis is well established, we investigated the possible influence and related mechanisms of the anti-inflammatory apigenin on vascular smooth muscle contractility to develop a better antihypertensive. Denuded aortic rings from male Sprague-Dawley rats were used and isometric contrac-

tions were recorded using a computerized data acquisition system and combined with molecular experiments.

Alterations in the arterial tone are frequently associated with cardiovascular diseases constituting an important cause of morbidity and mortality in humans, one of which is hypertension that is a multifactorial disorder that involves many mechanisms including endothelial dysfunction and leading to risk factors for cardiovascular diseases. Besides endothelial dysfunction, it is generally accepted that vascular smooth muscle contractility is predominantly controlled by Ca²⁺ sig-

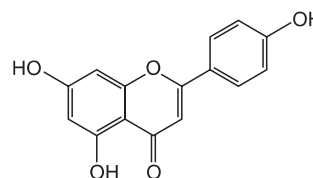


Fig. 1. The chemical structure of apigenin (4',5,7-trihydroxyflavone).

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nalizing involving Ca^{2+} influx, release or sensitization and regulating a Ca^{2+} -dependent increase in the phosphorylation of a 20 kDa myosin light chain (MLC_{20}) (Somlyo and Somlyo, 1994). The extent of MLC_{20} phosphorylation or force of contraction induced by agonist stimulation is usually higher than that caused by an increase in the cytosolic Ca^{2+} concentration referred to as Ca^{2+} sensitization (Somlyo and Somlyo, 1994). Subsequent studies suggested that the inhibition of MLC phosphatase by Rho-kinase (Kitazawa *et al.*, 1991; Uehata *et al.*, 1997; Somlyo and Somlyo, 1998; Sakurada *et al.*, 2003) or thin filament regulation including the activation of protein kinase C (PKC), mitogen-activated protein kinase kinases (MEK) and extracellular signal regulated kinase (ERK) 1/2, and phosphorylation of the actin binding protein caldesmon (Wier and Morgan, 2003) may be major components of the pathway that facilitates in Ca^{2+} sensitization.

Activation of ERK1/2 cannot only regulate vascular contractility but also is connected with pathologic hypertrophy, hyperplasia, hypertension and atherosclerosis (Xu *et al.*, 1996; Touyz *et al.*, 1999). ERK1/2 is activated by threonine and tyrosine phosphorylation by the specific kinase MEK activated by Raf. In various smooth muscles, fluoride, phorbol ester or thromboxane A_2 mimetic has been shown to induce contractions, which may be due to primarily enhanced Ca^{2+} sensitivity or partially increased Ca^{2+} concentration only in thromboxane A_2 mimetic. ERK1/2 activation was induced by the phorbol ester, phorbol 12,13-dibutyrate (PDBu). The stimulus PDBu triggers ERK1/2 dependent cytoskeletal remodeling and formation of podosomes inducing ERK1/2 activation (Gu *et al.*, 2007). On the other hand, it is possible that the contractions induced by fluoride or thromboxane A_2 mimetic involve the RhoA/Rho-kinase pathway (Jeon *et al.*, 2006). However, it has not been reported as to whether this pathway is inhibited during apigenin-induced vascular smooth muscle relaxation in aortic rings precontracted with Rho-kinase activator fluoride or MEK activator phorbol ester. Therefore, the aim of the present study was to investigate the possible roles of Rho-kinase or MEK inhibition on Ca^{2+} desensitization during the apigenin-induced relaxation of isolated rat aortas by using RhoA/Rho-kinase activators fluoride or thromboxane A_2 mimetic or a MEK activator phorbol ester excluding endothelial nitric oxide synthesis.

MATERIALS AND METHODS

Tissue preparation

Male Sprague-Dawley rats weighing 200-300 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.) as subjected to cervical dislocation, in accord with the procedures approved by the Institutional Animal Care and Use Committee at our institutions. Thoracic aortas were quickly removed and immersed in oxygenated (95% O_2 /5% CO_2) physiological saline solution composed of (mM): 115.0 NaCl, 4.7 KCl, 2.5 CaCl_2 , 1.2 MgCl_2 , 25.0 NaHCO_3 , 1.2 KH_2PO_4 , and 10.0 dextrose (pH 7.4). They were then freed of adherent connective tissue, and aortic endothelia were removed by gentle abrasion using a cell scraper.

Contraction measurements

Two stainless-steel triangles were inserted through each vessel ring and each aortic ring was then suspended in a wa-

ter-jacketed organ bath (10 ml) maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2 . One triangle was anchored to a stationary support, and the other was connected to an isometric force transducer (Grass FT03C, Quincy, Mass., USA). The rings were stretched passively by applying an optimal resting tension of 2.0 g, which was maintained throughout the experiment. Each ring was equilibrated in the organ bath solution for 60 min before contractile responses to 50 mM KCl were measured. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, AD Instruments, Castle Hill, NSW, Australia).

The direct effect of apigenin was determined by addition of it after KCl (50 mM), thromboxane A_2 mimetic (U46619, 0.1 μM), phorbol ester (1 μM) or fluoride (6 mM) induced contractions had plateaued in normal Krebs' solution.

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 stored at -80°C until use. Tissues were brought up to room temperature in a dry ice/acetone/TCA/DTT mixture and then 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 sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE (Protogel, National Diagnostics), transferred to polyvinylidene fluoride PVDF membranes, and subjected to immunostaining and densitometry using appropriate antibodies. The success of protein matching was confirmed by Naphthol Blue Black staining of the membrane and by densitometry of the actin band. Lane loading variations were corrected by normalization versus β -actin.

MLC_{20} Phosphorylation

Muscle strips were quick-frozen by immersion in acetone containing 10% TCA and 10 mM DTT precooled to -80°C. Tissues were brought to room temperature in acetone/TCA/DTT, then ground with glass pestles, and washed with ether to remove TCA. Tissues were extracted in a urea sample buffer as previously described and run on 10% polyacrylamide gels. Proteins were transferred to PVDF membranes and subjected to immunoblot with a specific MLC_{20} antibody (1:1500, Sigma). Anti-mouse IgG (Goat) conjugated with horseradish peroxidase was used as a secondary antibody (1:2000, Upstate). Bands were detected with enhanced chemiluminescence (ECL) visualized on films and then analyzed by a molecular imager.

Chemicals and antibodies

Drugs and chemicals were obtained from the following sources. Sodium fluoride, KCl, acetylcholine, apigenin, U46619 and phorbol 12,13-dibutyrate were purchased from Sigma (St. Louis, MO, USA). DTT, TCA and acetone were obtained from Fisher Scientific (Hampton, NH, USA). Enhanced chemiluminescence (ECL) kits were from Pierce (Rockford, IL, USA). Antibodies against phospho-myosin phosphatase targeting subunit protein 1 (phospho-MYPT1) at Thr855 (1:5,000), MYPT1, ERK or phosphoERK at Thr202/Tyr204 were purchased from

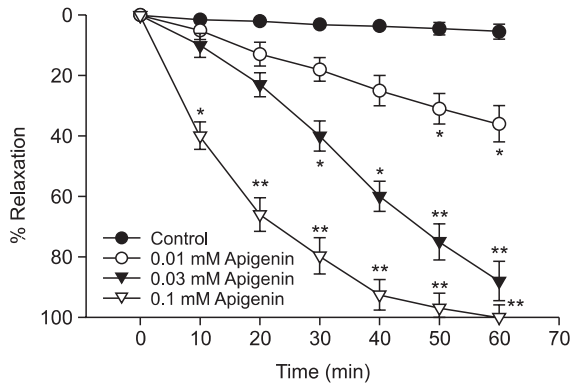


Fig. 2. Effect of apigenin on fluoride-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to apigenin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. * $p < 0.05$, ** $p < 0.01$, presence versus absence of apigenin.

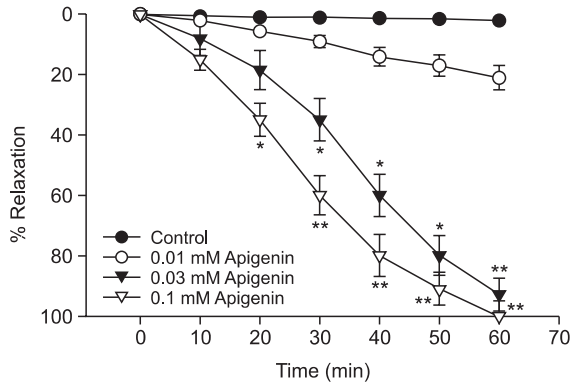


Fig. 3. Effect of apigenin on thromboxane A_2 mimetic-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to apigenin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. * $p < 0.05$, ** $p < 0.01$, presence versus absence of apigenin.

Cell Signaling Technology (Danvers, MA, USA) or Upstate Biotechnology (Lake Placid, NY, USA) to determine levels of RhoA/Rho-kinase activity (Wilson *et al.*, 2005; Wooldridge *et al.*, 2004) or MEK activity. Anti-mouse IgM (goat) and anti-rabbit IgG (goat), conjugated with horseradish peroxidase, were used as secondary antibodies (1:2,000 and 1:2,000, respectively, Upstate, Lake Placid, NY). Apigenin was prepared in dimethyl sulfoxide (DMSO) as a 100 mM stock solution and frozen at -20°C for later use. DMSO alone had no observable effect at concentrations used (data not shown).

Statistics

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

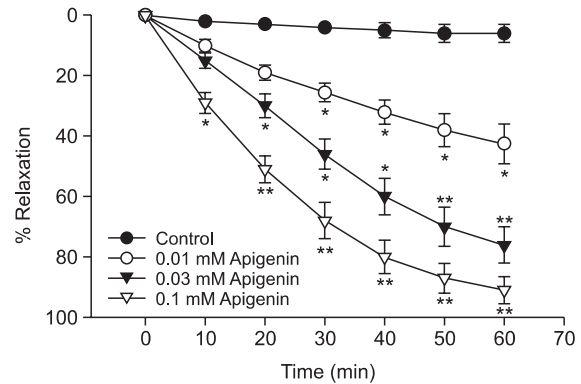


Fig. 4. Effect of apigenin on phorbol ester-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to apigenin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. * $p < 0.05$, ** $p < 0.01$, presence versus absence of apigenin.

RESULTS

Effect of apigenin on contractions of endothelium-denuded aortas induced by a full RhoA/Rho-kinase activator fluoride or thromboxane A_2 mimetic

Endothelium was removed by gentle abrasion with a cell scraper to identify the direct effect of apigenin on vascular smooth muscle. The absence of endothelium was confirmed by a lack of relaxation after treating precontracted ring segments with acetylcholine ($1 \mu\text{M}$). Apigenin showed no significant effect on basal tension (data not shown), and significantly inhibited the contraction induced by a Rho-kinase activator fluoride regardless of endothelial nitric oxide synthesis (Fig. 2). This suggests that the relaxation mechanism of apigenin might involve the inhibition of Rho-kinase activity in addition to endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase. On the other hand, apigenin at the same concentration significantly inhibited thromboxane A_2 mimetic U46619-induced contraction in denuded muscles (Fig. 3) suggesting that thromboxane A_2 mimetic acts similarly with fluoride where Rho-kinase activation was the main pathway.

Effect of apigenin on the contractions of denuded aortas induced by a MEK activator phorbol ester

Phorbol esters used have been proved to be MEK activators and partial RhoA/Rho-kinase activators (data not shown). Interestingly, phorbol 12,13-dibutyrate-induced contraction was significantly inhibited by apigenin at a low concentration regardless of endothelial nitric oxide synthesis (Fig. 4), which suggested that thin or actin filament regulation including MEK/ERK activation were significantly inhibited.

Effect of apigenin on levels of ERK1/2 phosphorylation at Thr-202/Tyr-204

To confirm the role of apigenin on thin filament regulation of smooth muscle contractility, we measured levels of ERK1/2 and phospho-ERK1/2 in muscles quick frozen after 60 minutes of exposure to apigenin for the equilibration. Each relaxing ring was precontracted with $1 \mu\text{M}$ phorbol ester (phorbol 12,13-dibutyrate). As compared with vehicle-treated rat aortas,

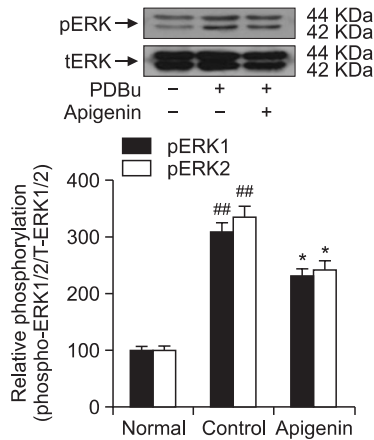


Fig. 5. Effect of apigenin on phorbol ester-induced increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels were decreased in quick frozen apigenin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with phorbol ester. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-ERK1/2. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. **p*<0.05, ^{##}*p*<0.01, versus control or normal group respectively. Apigenin: 0.1 mM apigenin; PDBu: 1 μM phorbol 12,13-dibutyrate.

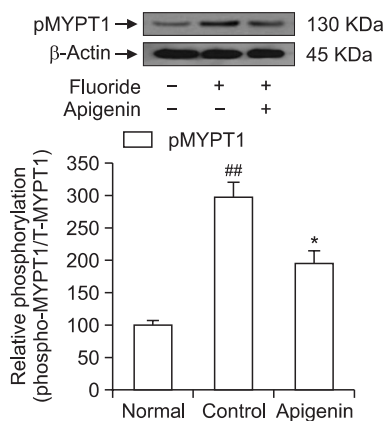


Fig. 6. Effect of apigenin on fluoride-induced increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were significantly decreased in quick frozen apigenin-treated rat aorta in the absence of endothelium compared to vehicle-treated rat aorta precontracted with fluoride. The upper panel shows 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 lines representing SEMs. **p*<0.05, ^{##}*p*<0.01, versus control or normal group respectively. Apigenin: 0.1 mM apigenin; Fluoride: 6 mM sodium fluoride.

a significant decrease in ERK 1/2 phosphorylation at Thr202/Tyr204 was led by apigenin in these apigenin (0.1 mM)-treated rat aortas in the absence of endothelium (Fig. 5) showing full vasorelaxation (Fig. 4) and thin filament regulation. These findings show that thin or actin filament regulation including ERK1/2 phosphorylation via MEK activation might be of importance in the decreased contractility induced by apigenin.

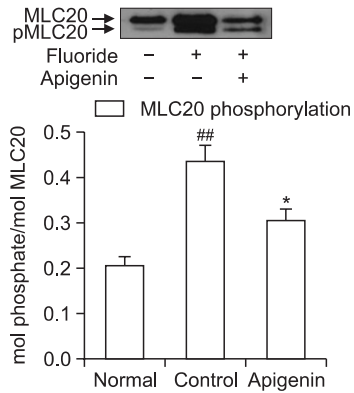


Fig. 7. Effect of apigenin on fluoride-induced increases in phospho-MLC₂₀ levels. Phospho-MLC₂₀ levels expressed as a percentage to the total MLC₂₀ were significantly decreased in quick frozen apigenin-treated rat aorta in the absence of endothelium compared to vehicle-treated rat aorta precontracted with fluoride. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. **p*<0.05, ^{##}*p*<0.01, versus control or normal group respectively. Apigenin: 0.1 mM apigenin; Fluoride: 6 mM sodium fluoride.

Effect of apigenin on the level of MYPT1 phosphorylation at Thr-855

To confirm the role of apigenin on the thick filament regulation of smooth muscle contractility, we measured levels of myosin phosphatase targeting subunit 1 (MYPT1) and phospho-MYPT1 in muscles quick frozen after 60 min exposure to apigenin for the equilibration. Each relaxing ring was precontracted with 6 mM fluoride. This work was done using quick frozen apigenin (0.1 mM)-treated rat aortas in the absence of endothelium and the levels were compared to those of vehicle-treated rat aortas (Fig. 6). Interestingly, a significant decrease in fluoride-induced MYPT1 phosphorylation at Thr855 was found to be led by apigenin (Fig. 6). Thus, thick or myosin filament regulation including myosin phosphatase activation via RhoA/Rho-kinase inactivation might be involved in the reduced contractility of apigenin-treated rat aorta.

Effect of apigenin on the level of MLC₂₀ phosphorylation

In order to investigate whether apigenin affect fluoride-induced MLC₂₀ phosphorylation, aortic rings were treated with apigenin after the addition of fluoride. Each relaxing ring was precontracted with 6 mM sodium fluoride. This work was done using quick frozen apigenin (0.1 mM)-treated rat aortas in the absence of endothelium and the levels were compared to those of vehicle-treated rat aortas (Fig. 7). Interestingly, a significant decrease in fluoride-induced LC₂₀ phosphorylation was found to be led by apigenin (Fig. 7). Thus, thick or myosin filament regulation including calcium immobilization and MLCK inactivation might be involved in the reduced contractility of apigenin-treated rat aorta.

DISCUSSION

The present study demonstrates that apigenin can modulate the vascular contractility in an agonist-dependent manner. Interestingly, the mechanism involved seems to be not

only endothelium-dependent but also to involve the nonspecific inhibition of MEK and Rho-kinase activity. Apigenin has been previously recognized for its anti-inflammatory, antioxidant activity or endothelial vasorelaxation (Zhang *et al.*, 2000). Therefore, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributes to apigenin-induced vascular relaxation in rat aortas denuded and precontracted by a RhoA/Rho-kinase activator fluoride or by a MEK activator phorbol ester.

The mechanism by which phorbol ester activates MEK/ERK has been established (Kordowska *et al.*, 2006; Gu *et al.*, 2007). On the other hand, previous studies that examined the mechanisms underlying arterial contractions induced by fluoride or thromboxane A₂ mimetic have reported variable findings with regard to the contraction triggered by calcium entry and Rho-kinase activation (Wilson *et al.*, 2005; Tsai and Jiang, 2006). These findings are consistent with the notion that apigenin can decrease phorbol ester or fluoride-induced contraction by inhibiting MEK or Rho-kinase activity.

The mechanisms by which MEK activation causes vascular contraction is an area of intense study, and several possibilities exist. The phosphorylation of caldesmon by MEK/ERK appears to regulate smooth muscle contractility (Kordowska *et al.*, 2006). In this process MEK/ERK is activated by PKC which in turn can be stimulated by phorbol esters or GPCR receptor agonists.

The present study demonstrates that apigenin ameliorates the maximal or submaximal contraction induced by vasoconstrictor fluoride or phorbol ester endothelium-independently (Fig. 2, 4), and that this ameliorative mechanism involves the MEK/ERK and RhoA/Rho-kinase pathway. Previously, most vasodilation was believed to be caused by endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase (Zhang *et al.*, 2000). Zhang *et al.* reported apigenin-induced endothelium-dependent relaxation and related pathway involving cGMP production. In the present study, apigenin at a low concentration significantly inhibited phorbol ester- or fluoride-induced contraction regardless of endothelial function (Fig. 2, 4). Furthermore, apigenin decreased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 induced by phorbol ester (Fig. 5) and significantly decreased the phosphorylation of MYPT1 at Thr855 induced by fluoride (Fig. 6) with full relaxation (Fig. 2) suggesting the inhibition of Rho-kinase or MEK activity as a major mechanism.

MLC₂₀ is known to be phosphorylated by both MLCK and Rho-kinase (Somlyo and Somlyo, 1994). The activation of Rho-kinase by U46619 or fluoride inhibits the activity of myosin light chain phosphatase through the phosphorylation of MYPT1, leading to an increased MLC₂₀ phosphorylation as well as contractions (Sakurada *et al.*, 2003; Wilson *et al.*, 2005). Coincidentally, fluoride phosphorylated MLC₂₀, which was inhibited by apigenin (Fig. 7).

In summary, apigenin significantly attenuates the contractions induced by a MEK activator phorbol ester regardless of endothelial function. Furthermore, a Rho-kinase activator fluoride-induced contraction was significantly inhibited by apigenin at this low concentration. Thus, the mechanism underlying the relaxation induced by apigenin in phorbol ester or fluoride-induced contractions involves the nonspecific inhibition of MEK activity and Rho-kinase activity. Interestingly, during fluoride-induced contraction, the inhibition of Rho-kinase activity and subsequent MYPT1 phosphorylation induced by

apigenin suggest that Rho-kinase inactivation is required for relaxation. In conclusion, in addition to endothelial nitric oxide synthesis (Zhang *et al.*, 2000), both MEK and Rho-kinase inhibition make a major contribution to the mechanism responsible for apigenin-induced vasorelaxation in the denuded muscle and the decrease of intracellular calcium level which should be elucidated directly may be involved as well (Rotondo *et al.*, 2009).

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