Cardiovascular Research (2011) **91**, 162–170
doi:10.1093/cvr/cvr043

Enhanced contractility and myosin phosphorylation induced by Ca^{2+}-independent MLCK activity in hypertensive rats

Young-Eun Cho[1], Duck-Sun Ahn[1], Kathleen G. Morgan[2], and Young-Ho Lee[1]*

[1]Department of Physiology, College of Medicine, BK 21 Project for Medical Sciences, Yonsei University, CPO Box 8044, Seoul 120-752, Korea; and [2]Health Sciences Department, Boston University, 635 Commonwealth Avenue, Boston, MA 02215, USA

Received 1 September 2010; revised 27 January 2011; accepted 2 February 2011; online publish-ahead-of-print 4 March 2011

Time for primary review: 26 days

Aims The role of Ca^{2+} sensitization induced by a Ca^{2+}-independent myosin light chain kinase (MLCK) in hypertension has not been determined. The aim of this study was to clarify the role of possible Ca^{2+}-independent MLCK activity in hypertension.

Methods and results We compared increases in contractile force and phosphorylation of myosin light chain (MLC) evoked by calyculin A, a phosphatase inhibitor, in β-escin-permeabilized mesenteric arteries at pCa 9.0 between spontaneously hypertensive rat (SHR) and Wistar Kyoto rat (WKY). We found that there was no detectable phosphorylation of MLC at pCa 9.0, but that the administration of 1 μM calyculin A gradually increased force and mono- and di-phosphorylation of MLC. This contraction was inhibited by staurosporine but not by wortmannin, Y-27632, or calphostin-C. The calyculin A-induced contraction was significantly greater in the SHR than in the WKY and was associated with an increase in mono- and di-phosphorylation of MLC. SM-1, a zipper-interacting protein kinase (ZIPK)-inhibiting peptide, significantly inhibited the amplitude of the calyculin A-induced contraction and di-phosphorylation. Total ZIPK expression (54 + 32 kDa) was greater in the SHR than in the WKY. Phosphorylation of myosin phosphatase target subunit at Thr^{697}, but not at Thr^{855}, was consistently stronger in the SHR than in the WKY in calyculin A-treated tissues at pCa 9.0.

Conclusions Our results suggest that Ca^{2+}-independent MLCK activity is enhanced in the SHR, and that ZIPK plays, at least in part, an important role as a candidate for this kinase in rat mesenteric arteries.

Keywords ZIPK • Hypertension • Phosphorylation of MLC • Phosphorylation of MYPT1 • Calyculin A

1. Introduction

Smooth muscle contraction is activated primarily by phosphorylation at Ser^{19} of the 20 kDa regulatory light chains of myosin II (RLC_{20}),[1] which is controlled by the opposing activities of myosin light chain kinase (MLCK)[2] and myosin light chain phosphatase (MLCP).[3] The amplitude of contraction can be affected by any change in the ratio of MLCK:MLCP activity. MLCK activity is dependent on Ca^{2+}-calmodulin; hence, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the primary determinant of smooth muscle contraction.[4] However, both contractile force development and RLC_{20} phosphorylation in smooth muscle can be elicited without corresponding changes in $[Ca^{2+}]_i$. The increase in sensitivity of smooth muscle contractile response to $[Ca^{2+}]_i$ (i.e. Ca^{2+} sensitization) may be caused by inhibition of MLCP activity[4] or by activation of extracellular-regulated kinase-mediated caldesmon phosphorylation.[5] Inhibition of MLCP can occur either directly by phosphorylation of the myosin phosphatase target subunit (MYPT1) of MLCP[6] or indirectly via phosphorylation of a protein kinase C (PKC)-potentiated phosphatase inhibitor protein of 17 kDa (CPI-17).[7]

Another possible mechanism for Ca^{2+} sensitization also exists. Ca^{2+}-independent MLCK activity has been identified. When microcystin-LR, a type 1 and 2A phosphatase inhibitor, is applied to permeabilized smooth muscle strips in Ca^{2+}-free media, a Ca^{2+}-independent sustained contraction is observed, which cannot be attributed to traditional MLCK activity.[8–10] This Ca^{2+}-independent contraction is accompanied by di-phosphorylation of RLC_{20} at Ser^{19} and Thr^{18}.[10] Similar results have been obtained with a number of different smooth muscle tissues and with other type 1 and 2A phosphatase inhibitors, okadaic acid, and calyculin A,[11–13] suggesting a

* Corresponding author. Tel: +82 2 2228 1708; fax: +82 2 393 0203, Email: yhlee@yuhs.ac

common mechanism. Since MLCK is absolutely dependent on Ca^{2+} and, at physiological levels of the kinase, is specific for phosphorylation at Ser^{19}, the kinase responsible is clearly not MLCK itself.[14] Previous reports have suggested several candidates for this Ca^{2+}-independent MLCK, including rho-associated kinase (ROCK),[15] PKC,[16] mitogen-activated protein kinase (MAPK)–activated protein kinase-2,[17] MAPK-activated protein kinase-1b,[18] Integrin-linked kinase (ILK),[19] and ZIPK.[8] Phosphorylation of RLC_{20} by ROCK, MAPK-activated protein kinase-2, MAPK-activated protein kinase-1b is restricted to Ser^{19}, and PKC phosphorylates neither Ser^{19} nor Thr^{18}. Furthermore, among these kinases, only ROCK[15], ILK[19], and ZIPK[8] have been shown to induce RLC_{20} phosphorylation and contraction of smooth muscle. Taken together, only ILK and/or ZIPK have emerged as practical candidates to fill the role of Ca^{2+}-independent MLCK for the di-phosphorylation of RLC_{20} and contraction of smooth muscle.[20]

Earlier reports have shown that myosin in di-phosphorylated RLC_{20} has a higher actin-activated Mg^{2+}-ATPase activity than mono-phosphorylated RLC_{20}[21,22], and di-phosphorylation of the RLC_{20} enhances the tension acting on stress fibres in fibroblasts.[23] While RLC_{20} mono-phosphorylation is most commonly observed in smooth muscle tissue in response to physiological contractile stimuli, RLC_{20} di-phosphorylation is more commonly observed in pathological situations such as vasospasm.[24,25] These observations have led to the suggestion that di-phosphorylation of RLC_{20} is associated with hypercontractility of vascular smooth muscle. Furthermore, augmented Ca^{2+} sensitization has been reported in hypertensive animal models.[26–28]

Although pathological alterations to the sensitivity of the smooth muscle contractile response to Ca^{2+} have been hypothesized to underlie many diseases, such as hypertension[29,30] and vasospasm,[31] the role of Ca^{2+} sensitization induced by a Ca^{2+}-independent MLCK in hypertension has not been determined and the precise identity of the Ca^{2+}-independent MLCK remains controversial. In the present study, we determined that an increased contractile force and di-phosphorylation of RLC_{20} caused by a Ca^{2+}-independent MLCK occurs in a model of hypertension and, furthermore, that the subcellular mechanism likely involves ZIPK-mediated phosphorylation of MYPT1.

2. Methods

This investigation confirms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

2.1 Tissue preparation and organ bath study

Male spontaneously hypertensive rat (SHR) and Wistar Kyoto rat (WKY) aged 16–18 weeks were purchased from Japan SLC, Inc., Inasa Production Facility. The mean systolic blood pressures of the SHR and WKY rats measured by the tail-cuff method were 202 ± 10 and 145 ± 10.5 mmHg, respectively.

The rats were killed by decapitation. All mesenteric arteries were excised and placed in a HEPES-Tyrode solution composed of the following (in mmol/L): NaCl, 134; KCl, 5.6; $CaCl_2$, 2.5; $MgCl_2$, 1; HEPES, 10; glucose, 10. The HEPES-Tyrode solution was continuously aerated with 100% O_2. The second or third branches of the mesenteric arteries were dissected and ring segments (~1 mm in length) were prepared. Fat and adventitia were removed mechanically under a binocular microscope, and the endothelium of the arterial ring was removed by gently rubbing the endothelial surface with the edge of a forceps. Removal of endothelium was confirmed by observing the absence of relaxation on challenge with acetylcholine when 70 mmol K^+ solution (equimolar substitution of Na^+ with K^+)-induced contraction had reached a plateau.

Force was measured as we have previously described.[32]

2.2 Permeabilization with β-escin and force recording

Force in permeabilized preparations was measured as we have previously described.[32,33] Briefly, 1 μM calyculin A and several inhibitors were treated after stable responses were obtained at pCa 9.0. All experiments, except the experiments concerned with the effect of several protein kinase inhibitors and SM-1 peptide on calyculin A-induced contraction, were conducted in 10 μM wortmannin (inhibitor of Ca^{2+}-dependent MLCK and phosphatidylinositol 3-kinase)-treated tissues to eliminate the possible contribution of Ca^{2+}-dependent MLCK.

2.3 RLC_{20} phosphorylation measurements

Phosphorylation of the RLC_{20} in the SHR and WKY was measured using glycerol-urea minigels as described previously.[34] The vessels were frozen when the contractile response became stable at pCa 9.0, pCa 4.5, and pCa 9.0 with calyculin A.

2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting

The expression levels of proteins in non-stretched SHR and WKY mesenteric arteries, the effects of calyculin A on the mono- and di-phosphorylation of the RLC_{20}, Thr^{697}, and Thr^{855} phosphorylation of MYPT1, and CPI-17 phosphorylation were measured by using western blot as described previously.[32]

2.5 Immunofluorescence

The expression of protein in the first or second mesenteric branches and di-phosphorylated forms of RLC_{20} in the SHR and WKY were measured by the immunofluorescence method using a confocal laser scanning microscope. Non-stretched arteries and vessels after a functional study were fixed with paraformaldehyde embedded in an optimal cutting temperature (Sakura Fine Tec Inc., Torrance, CA, USA) compound and frozen in liquid nitrogen. The frozen sections (10 μm) were cut onto gelatin-coated slides, air-dried, and then washed with phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10.14 mmol/L Na_2HPO_4, 1.76 mmol/L KH_2PO_4, 0.05% Tween 20). After a blockade with blocking solution composed of PBS containing 3% bovine serum albumin and 0.5% Triton X-100, the sections were incubated with primary antibodies in blocking solution overnight in a humidified chamber at room temperature. The specific primary antibodies used are as follows: ZIPK (1:50, Santa Cruz), total MLC (1:100, Sigma), β-actin (1:500, Abcam), and ppMLC (1:100, Cell Signaling, MA, USA). After washing with PBS, sections were incubated with the secondary antibodies for 1 h in the dark. Immunofluorescent signals were viewed using a confocal laser scanning microscope (Carl Zeiss, LSM 510) with oil immersion lens.

The specificity of the immunostaining was evaluated by omission of the primary antibody (negative control) and processed as above.

2.6 Drugs

The following drugs were used: calyculin A (Enzo Life Science; Farmingdale, NY, USA), wortmannin (Enzo Life Science), *trans*-4-[(1R)-1-Aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide (Y-27632; Enzo Life Science), calphostin-C (Sigma), staurosporine (Sigma), SM-1 peptide (sequence: AKKLSKDRMKKYMARRKWQKTG, Bio Basic Inc. and Thermo scientific), and scramble SM-1 peptide (sequence: KWARMKDKAMRYKTKGRLQKSK, Bio Basic Inc. and Thermo scientific). The general laboratory reagents used were analytical grade or better.

2.7 Statistics

All values given in the text are expressed as mean ± SEM and were analysed by ANOVA, followed by Tukey's test. Significant differences were taken at the $P < 0.05$ level. Force was expressed as a relative percentage of the amplitude of the contraction at pCa 4.5.

3. Results

3.1 Calyculin A induces a Ca^{2+}-independent contraction of β-escin-permeabilized rat mesenteric arterial smooth muscle

Figure 1A depicts a typical calyculin A-induced contraction of β-escin-permeabilized rat mesenteric arterial smooth muscle in the absence of Ca^{2+}. β-escin-permeabilized tissue contracted in response to an increase in $[Ca^{2+}]$ (pCa 4.5) and relaxed upon return to pCa 9.0. The administration of 1 μM calyculin A to the permeabilized smooth muscle in pCa 9.0 elicited a gradual increase in force, reaching a plateau 20–30 min after administration (*Figure 1A*). To clarify which kinase is responsible for the calyculin A-induced Ca^{2+}-independent contraction, we examined the effects of various protein kinase inhibitors on the calyculin A-induced contraction at pCa 9.0 (*Figure 1B–F*). The compounds evaluated included inhibitors of: MLCK (10^{-5} M wortmannin), ROCK (10^{-5} M Y-27632), PKC (5×10^{-7}M calphostin-C), and a non-specific, broad-spectrum protein kinase inhibitor (10^{-5} M staurosporine). As shown in *Figure 1*, wortmannin, Y-27632, and calphostin-C were ineffective, whereas staurosporine inhibited the calyculin A-induced contraction. Staurosporine decreased the calyculin A-induced contraction from 59.9 ± 5.2% ($n = 7$, contraction normalized to maximal pCa 4.5-induced contraction) to 6.7 ± 6.7% ($n = 4$, $P < 0.01$).

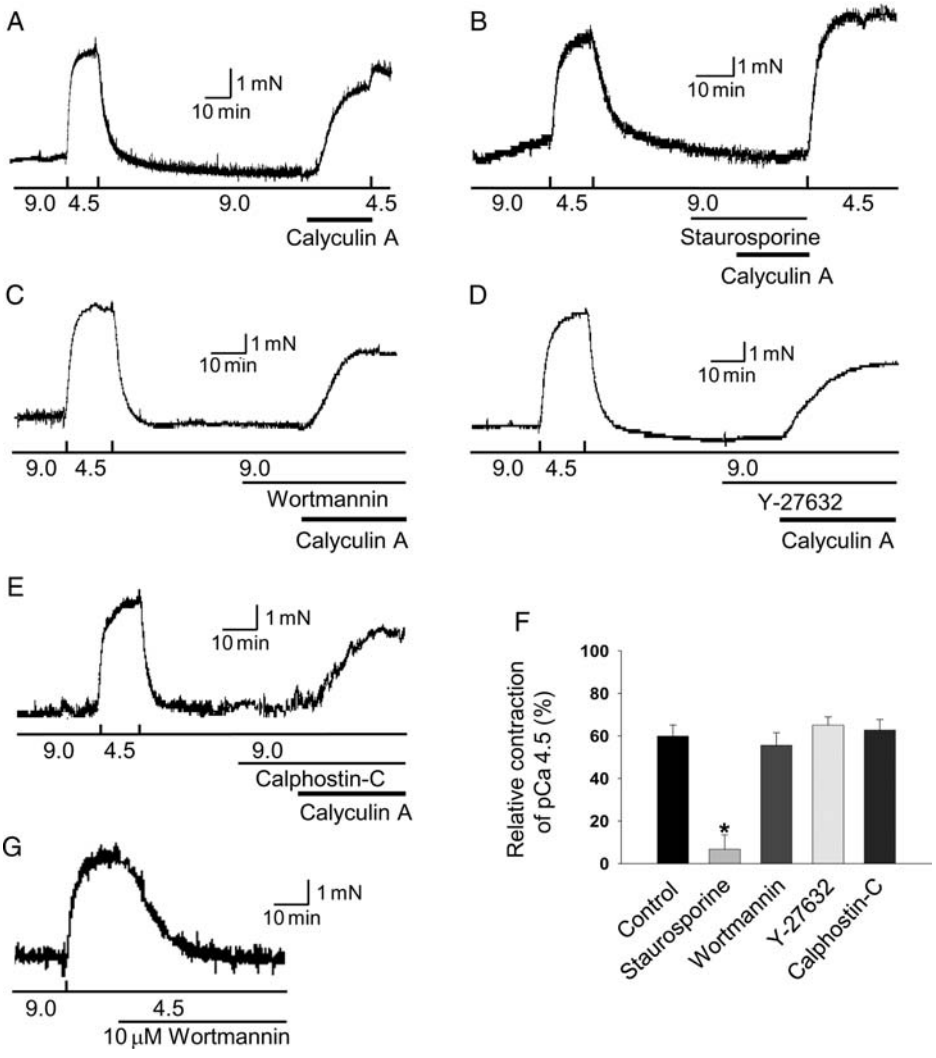

Figure 1 Characterization of protein phosphatase inhibitor, calyculin A-induced contraction in β-escin-permeabilized rat mesenteric arterial smooth muscle. (*A*) Typical recording for the effect of 1 μM calyculin A in β-escin-permeabilized rat mesenteric arteries at pCa 9.0. (*B–E*) Typical recording for the effects of protein kinase inhibitors on the calyculin A-induced contraction at pCa 9.0. 10^{-5} M staurosporine (*B*), 10^{-5} M wortmannin (*C*), 10^{-5} M Y-27632 (*D*), and 5×10^{-7} M calphostin-C (*E*) were applied 15 min before administration of calyculin A. (*F*) Mean data for calyculin A-induced contraction with protein kinase inhibitors at pCa 9.0. The magnitude of contraction was normalized by the response of pCa 4.5 (100%). Data are a summary of 4–15 independent experiments and are expressed as mean ± SEM. *, $P < 0.01$. (*G*) Control data for Ca^{2+} (pCa 4.5)-induced contraction.

3.2 The Ca^{2+}-independent, calyculin A-induced contraction and RLC_{20} phosphorylation are augmented in the SHR

To clarify whether the Ca^{2+}-independent, calyculin A-induced contraction increases in a hypertensive model, we compared the calyculin A-induced contraction in permeabilized rat mesenteric arterial smooth muscle of SHR and WKY at pCa 9.0. As shown in *Figure 2A*, the Ca^{2+}-independent, calyculin A-induced contraction was concentration dependent. The maximum response was obtained at 1 μM in the SHR and the WKY, but the amplitude of the contraction was greater in the SHR ($79.0 \pm 7.8\%$, $n = 9$) than in the WKY ($53.6 \pm 4.1\%$, $n = 7$; *Figure 2A*). We further compared the effect of calyculin A on RLC_{20} phosphorylation in the permeabilized rat mesenteric arterial smooth muscle of SHR and WKY. We first examined the level of RLC_{20} phosphorylation with a total RLC_{20} antibody using urea/glycerol gel electrophoresis that separates un-phosphorylated and phosphorylated forms of RLC_{20}. As shown in *Figure 2B*, in permeabilized tissue, during application of pCa 4.5, un- and mono-phosphorylated forms of RLC_{20} were detected in both SHR and WKY tissues, but the signal for mono-phosphorylated forms of RLC_{20} was stronger in the SHR than in the WKY. Di-phosphorylated forms of RLC_{20} were not detected in pCa 4.5. In the case of pCa 9.0 without calyculin A, the total RLC_{20} antibody recognized only un-phosphorylated forms of RLC_{20} in control SHR and WKY tissues. However, in the presence of calyculin A at pCa 9.0, three bands, un-, mono-, and di-phosphorylated forms of RLC_{20} appeared in both SHR and WKY. The amounts of mono- and di-phosphorylated RLC_{20} were larger in the SHR than in the WKY.

We also compared agonist-induced di-phosphorylation in intact arterial smooth muscle of SHR and WKY to clarify if the agonist can induce di-phosphorylation and whether agonist-induced di-phosphorylation is augmented in a hypertensive animal model. As shown in *Figure 2C*, phenylephrine-induced di-phosphorylation of RLC_{20} and di-phosphorylated forms of RLC_{20} were augmented in the SHR compared with the WKY in intact tissue. We also compared the levels of RLC_{20} mono- and di-phosphorylation induced by Ca^{2+}-independent, calyculin A in permeabilized rat mesenteric arterial smooth muscle of SHR and WKY using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) with antibodies specific for RLC_{20} phosphorylated exclusively at Ser^{19} or both Ser^{19} and Thr^{18}. As shown in *Figure 3*, the results obtained are similar to those seen with urea/glycerol gel electrophoresis. In SHR and WKY, Ser^{19}-phosphorylated RLC_{20} (*Figure 3A*) was increased in pCa 4.5 alone and in pCa 9.0 with calyculin A, but Ser^{19}- and Thr^{18}-

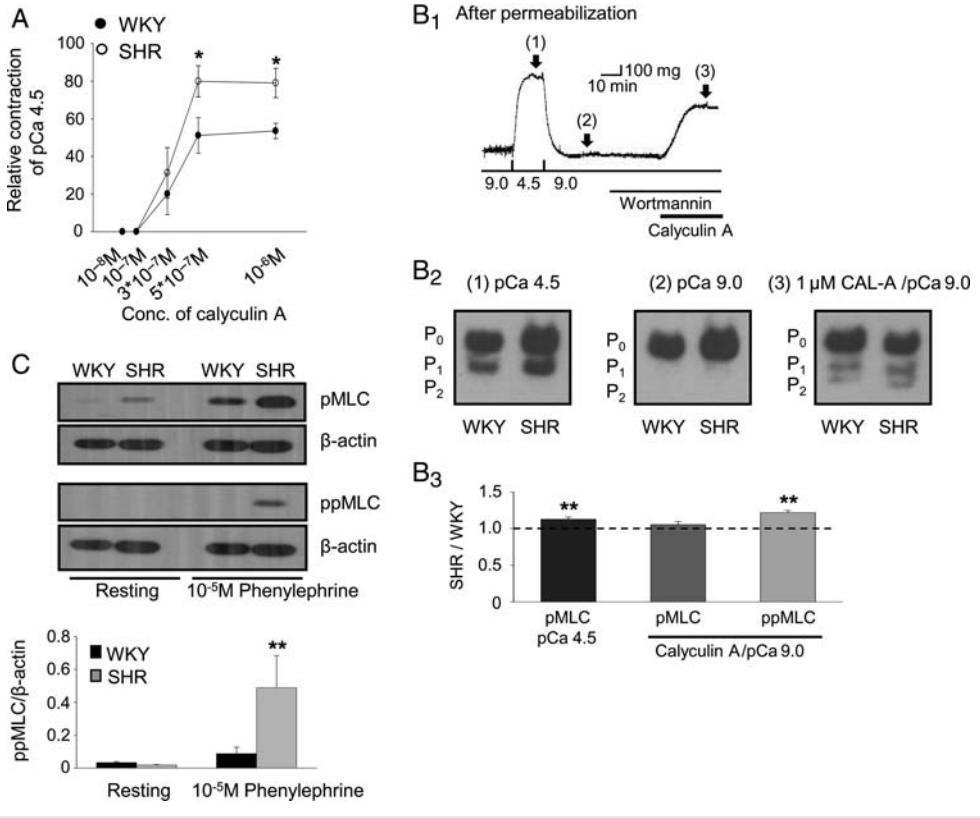

Figure 2 Changes in Ca^{2+}-independent, calyculin A-induced contractions and RLC_{20} phosphorylation between SHR and WKY. (A) Concentration-dependent effect of Calyculin A in β-escin-permeabilized mesenteric arteries of SHR ($n = 9$) and WKY ($n = 7$) at pCa 9.0. *, $P < 0.01$. (B_1–B_3) After β-escin permeabilization, RLC_{20} phosphorylation levels at various conditions. These results are representative of four independent experiments. The vessels are collected at peak contraction induced by pCa 4.5 (1), at pCa 9.0 without calyculin A (2), and at peak contraction induced by 1 μM calyculin A at pCa 9.0 (3). Un- (P_0), mono- (P_1), and di-(P_2) phosphorylated forms of RLC_{20} were separated by urea/glycerol gel electrophoresis. The densitometric sum of the bands in each category was normalized to that seen in the WKY (B_3). (*C*) Effect of phenylephrine on RLC_{20} di-phosphorylation in intact arteries of WKY and SHR. **, $P < 0.05$. WKY, Wistar Kyoto rat; SHR, spontaneously hypertensive rat; CAL-A, calyculin A.

di-phosphorylated RLC_{20} (*Figure 3B*) was increased only in pCa 9.0 with calyculin A. Quantification of the immunoblots shows that Ser^{19}-phosphorylated RLC_{20} at pCa 4.5 and at pCa 9.0 with calyculin A are stronger in the SHR than in protein-matched samples from the WKY. However, Ser^{19}- and Thr^{18}- di-phosphorylated forms of RLC_{20} are stronger in the SHR than in protein-matched samples from the WKY only at pCa 9.0 with calyculin A, but not with pCa 4.5 alone. Finally, we confirmed the expression levels of di-phosphorylated forms of RLC_{20} using immunostaining and image analysis with confocal microscopy. As shown in *Figure 3C*, in SHR and WKY, di-phosphorylated RLC_{20} was not detected at pCa 9.0, whereas di-phosphorylated RLC_{20} was significantly increased at pCa 9.0 with calyculin A. These results indicate that the contraction induced by calyculin A at pCa 9.0 was accompanied by an increase in mono- and di-phosphorylation of RLC_{20}.

3.3 Identification of the protein kinase involved in the Ca^{2+}-independent, calyculin A-induced contraction

To identify the nature of protein kinase involved in Ca^{2+}-independent, calyculin A-induced contraction and RLC_{20} di-phosphorylation, we studied the effects of SM-1, a ZIPK-inhibiting peptide, on the calyculin A-induced contraction and di-phosphorylation of RLC_{20} in permeabilized rat mesenteric arterial smooth muscle. As shown in *Figure 4A*, SM-1 (100 μM) significantly inhibited the amplitude of contraction ($31.1 \pm 9.3\%$, $n = 5$) induced by calyculin A at pCa 9.0 (*Figure 4A*$_2$) compared with the control group ($57.9 \pm 3.6\%$, $n = 9$; *Figure 4A*$_1$), but scrambled SM-1 had no effect on the calyculin A-induced contraction ($57.4 \pm 7.4\%$, $n = 3$; *Figure 4A*$_3$). SM-1 also significantly inhibited the amount of di-phosphorylation induced by calyculin A (*Figure 4B*). To confirm the presence of ZIPK in mesenteric arterial smooth muscle, we studied the double immunofluorescence staining of ZIPK and actin or myosin light chain (MLC) in rat mesenteric arterial smooth muscle. As shown in *Figure 4C*, ZIPK immunoreactivity was verified by co-staining with smooth muscle-specific actin or MLC (*Figure 4C*). The negative control obtained without primary antibodies gave no detectable signal. To determine whether expression of ZIPK changes in SHR animals, we measured the expression of ZIPK in the rat mesenteric artery of SHR and WKY using western blot. We also compared the expression levels of ROCK, CPI-17, and ILK. As shown in *Figure 4D*, anti-ZIPK detected a prominent band of both 32 kDa and 54 kDa in SHR and a weaker band in protein-matched WKY tissues. Quantification of the immunoblots showed that total (54 + 32 kDa) ZIPK is 1.3 ± 0.04-fold ($n = 23$) more abundant in the SHR than in protein-matched samples from the WKY. ROCK (180 kDa) was detected in both SHR and WKY tissues, and the signal was consistently stronger in the SHR than in the WKY. CPI-17 and ILK were detected in both SHR and WKY tissues at equal expression levels.

3.4 Contribution of MYPT1 and CPI-17 phosphorylation to MLCP inhibition during calyculin A-induced contraction in SHR and WKY

We examined whether calyculin A modifies phosphorylation of specific sites on MYPT1 and whether differences in MYPT1 phosphorylation induced by calyculin A exist between SHR and WKY. As shown in *Figure 5A*, Thr^{697} (numbering for rat isoform) phosphorylation of MYPT1 was significantly increased by treatment of SHR and WKY tissue with calyculin A at pCa 9.0 compared with pCa 9.0 alone, and the signal was consistently stronger in the SHR than in the WKY. Quantification of immunoblots for calyculin A

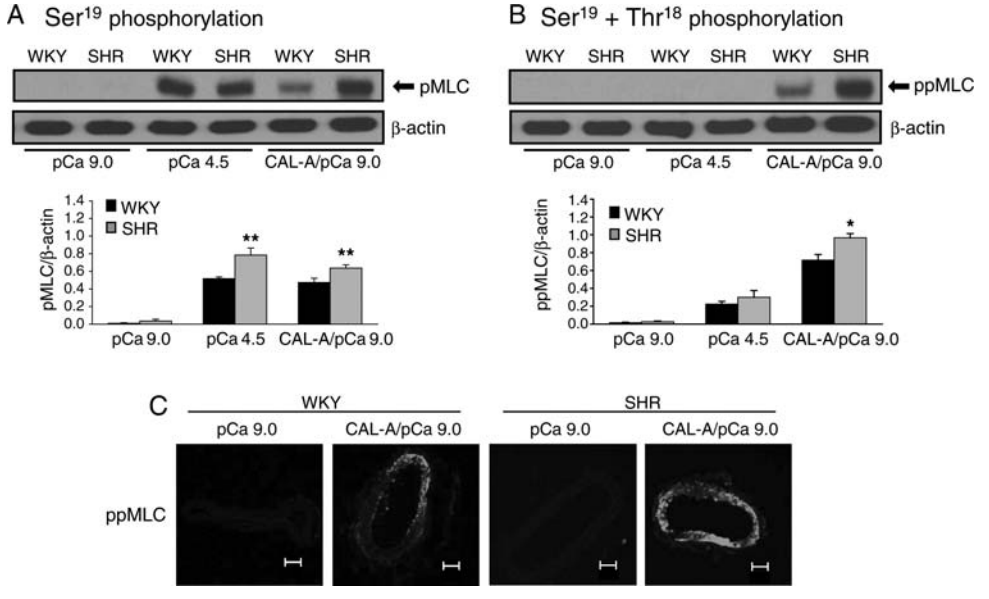

Figure 3 RLC_{20} phosphorylation at Ser^{19} and Thr^{18} is increased in the SHR. (*A* and *B*) Ser^{19}-(pMLC) and both Ser^{19} and Thr^{18} phosphorylated (ppMLC) RLC_{20} levels in SHR and WKY at pCa 9.0, pCa 4.5, and pCa 9.0 with calyculin A using SDS/PAGE gel electrophoresis with antibodies specific for RLC_{20} phosphorylated exclusively at Ser^{19} (*A*) or both Ser^{19} and Thr^{18} (*B*). The magnitude of phosphorylation was expressed as a pMLC/actin (lower panel of *A*) or ppMLC/actin (lower panel of *B*). *, $P < 0.01$; **, $P < 0.05$ ($n = 7$). (C) Immunofluorescence staining for di-phosphorylated forms (ppMLC) of RLC_{20}. Data were obtained with confocal microscopy. Scale bar: 50 μm.

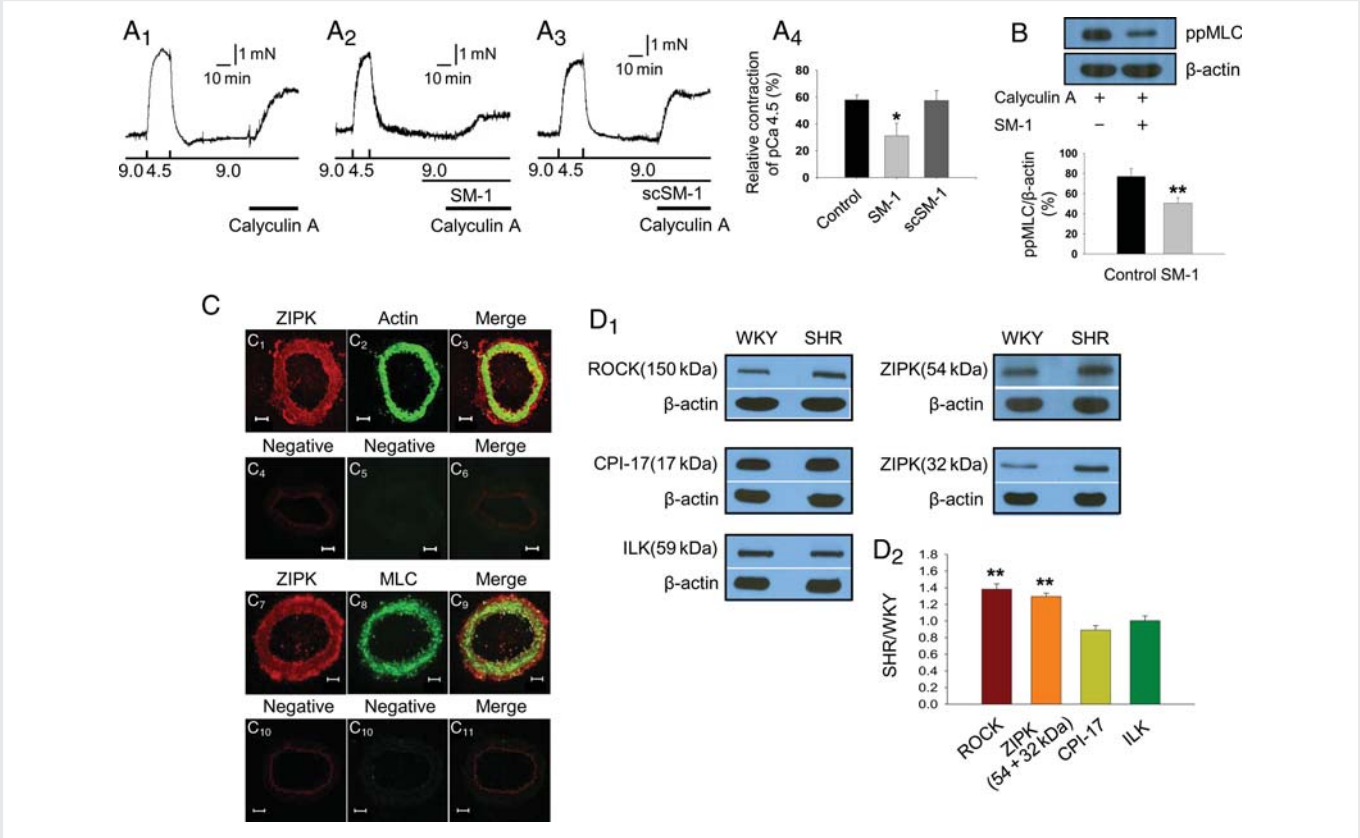

Figure 4 Effect of SM-1 on Ca^{2+}-independent, calyculin A-induced contraction and expression of various protein kinases in SHR and WKY. (*A*) Effect of SM-1 on Ca^{2+}-independent, calyculin A-induced contraction of β-escin-permeabilized rat mesenteric arteries. Contraction induced by 1 μM calyculin A in the absence of peptide (A_1) and in the presence of 100 μM SM-1 (A_2) or scrambled SM-1 (scSM-1, A_3). (A_4) Mean data for the effect of SM-1 (or scSM-1) on calyculin A-induced contraction at pCa 9.0. The magnitude of the contraction was normalized by the response of pCa 4.5 (100%). **, $P < 0.05$ ($n = 3–9$). (*B*) Effect of SM-1 on calyculin A-induced di-phosphorylation of β-escin-permeabilized rat mesenteric arteries. (C) Double immunofluorescence staining for ZIPK and actin or myosin light chain (MLC) in rat mesenteric arterial smooth muscle. Localization of ZIPK immunoreactivity (C_1 and C_7), actin (C_2), or MLC (C_8), and (C_3 and C_9) their co-localization in smooth muscle tissues. C_{4-6} and C_{10-12}. The negative controls consisted of omission of the primary antibody. Scale bar: 50 μm. (D_1) Immunoblots of ROCK, CPI-17, ILK, and ZIPK in rat mesenteric arteries of SHR and WKY. (D_2) The magnitude of expression was expressed as a SHR/WKY ratio. **. $P < 0.05$ ($n = 12–23$).

and pCa 9.0 gave a Thr^{697} phosphorylation/total MYPT1 ratio of 1.68 ± 0.3 ($n = 7$) in SHR and 1.06 ± 0.1 ($n = 7$) in WKY. Thr^{855} (numbering for rat isoform) phosphorylation of MYPT1 was significantly increased by treatment of SHR and WKY tissue with calyculin A in addition to pCa 9.0 conditions, but the phosphorylation levels increased by calyculin A did not differ significantly between SHR and WKY (*Figure 5B*).

We also examined the possibility that calyculin A-induced contractions could be a reflection of the CPI-17 phosphorylation status. However, as shown in *Figure 5C*, phosphorylation levels of CPI-17 increased by calyculin A were not significantly different between SHR and WKY.

4. Discussion

The present study demonstrates that, in a model of hypertension, there is a significant increase in Ca^{2+}-independent, calyculin A-induced contractions. This type of contraction is significantly greater in the SHR than in the WKY, and the augmentation of contractions in the SHR is associated with an increase in mono- and di-phosphorylation of RLC_{20}. We also show that ZIPK appears to be the Ca^{2+}-independent MLCK involved. We provide evidence that SM-1, a ZIPK-inhibiting peptide, significantly inhibits the contraction and di-phosphorylation of RLC_{20} induced by calyculin A at pCa 9.0, that ZIPK is expressed in the mesenteric artery, and that the expression level of ZIPK is higher in the SHR than in the WKY. Finally, we show that the increase in Thr^{697} phosphorylation of MYPT1, likely caused by the higher expression levels of ZIPK in SHR, plays an important role in the enhanced calyculin A-induced contraction and RLC_{20} phosphorylation in SHR. Taken together, these results suggest that Ca^{2+}-independent MLCK activity is enhanced in the SHR and that the increase in the expression level of ZIPK in SHR appears to play an important role in the enhanced calyculin A-induced contraction and RLC_{20} phosphorylation.

To investigate the existence of Ca^{2+}-independent contraction and di-phosphorylation of RLC_{20} in rat mesenteric arterial smooth muscle, we first measured the effects of calyculin A on the contractility of β-escin-permeabilized arterial smooth muscle at pCa 9.0. MLCK is absolutely dependent on Ca^{2+} and calmodulin for its activity, and has no activity under these experimental conditions. Thus, in the absence of Ca^{2+} (pCa 9.0), we found that there is no phosphorylation of MLC in this system (*Figure 2*). However, we also showed that

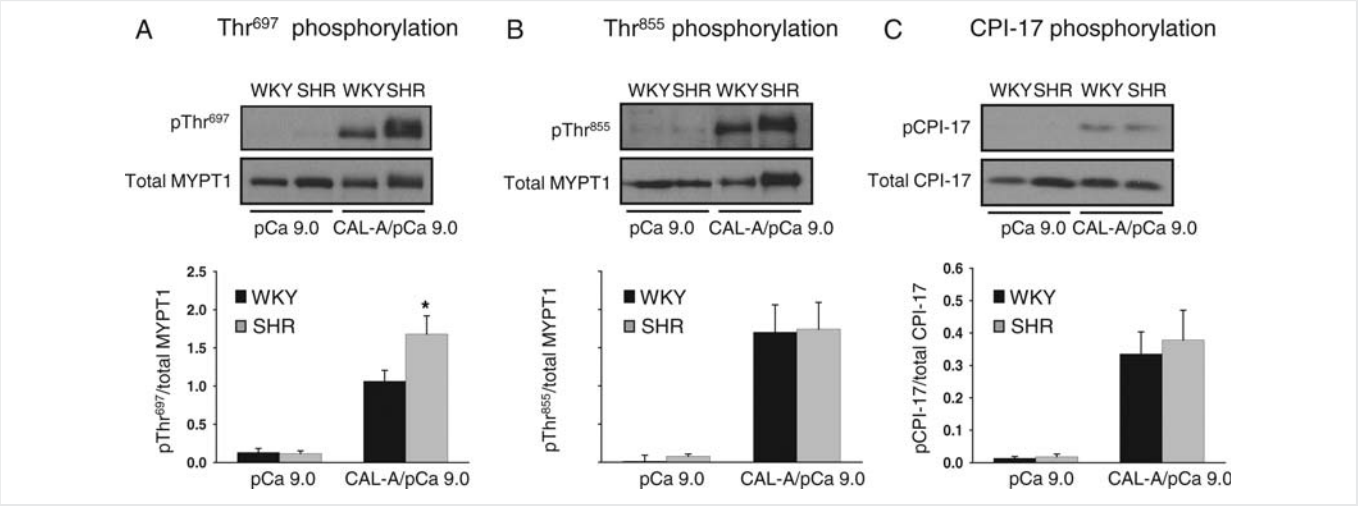

Figure 5 Analysis of MYPT1 and CPI-17 phosphorylation contribution to Ca^{2+}-independent, calyculin A-induced contraction in SHR and WKY. (*A* and*B*) Changes of Thr^{697} (*A*) and Thr^{855} (*B*) phosphorylation of MYPT1 by treatment of calyculin A at pCa 9.0 (CAL-A/pCa 9.0) in β-escin-permeabilized rat mesenteric arteries of SHR and WKY. (*C*) Changes of phosphorylation of CPI-17 by treatment of calyculin A at pCa 9.0 (CAL-A/pCa 9.0) in β-escin-permeabilized rat mesenteric arteries of SHR and WKY. Data are expressed as signal-intensity ratios for phosphorylated ($pThr^{697}$ and $pThr^{855}$ or pCPI-17): total (Total MYPT1 or Total CPI-17) MYPT1 or CPI-17. *, $P < 0.01$ ($n = 7$).

calyculin A induces a gradual increase in force and mono- and di-phosphorylation of RLC_{20} in permeabilized rat mesenteric arterial smooth muscle at pCa 9.0. We also showed that the calyculin A-induced contraction is insensitive to treatment with wortmannin, Y-27632, and calphostin-C, ruling out the involvement of traditional MLCK, ROCK, or PKC in this Ca^{2+}-independent contraction. These results are consistent with previous reports[8,11–13,35] and suggest that a Ca^{2+}-independent MLCK activity is unmasked in our experimental conditions.

To clarify whether the Ca^{2+}-independent MLCK activity might be increased in hypertension, we used a model of hypertension, the SHR, and compared Ca^{2+}-independent, calyculin A-induced contractions in permeabilized rat mesenteric arterial smooth muscle of SHR and WKY at pCa 9.0. We showed that the amplitude of calyculin A-induced contraction is greater in the SHR than in the WKY. Consistent with this finding, we also showed that in the presence of calyculin A at pCa 9.0, the prevalence of mono- and di-phosphorylated forms of RLC_{20} is stronger in SHR than in WKY. In the present study, we measured RLC_{20} phosphorylation using three different methods: urea/glycerol gel electrophoresis with a total RLC_{20} antibody, SDS/PAGE gel electrophoresis with antibodies specific for RLC_{20} phosphorylated exclusively at Ser^{19} or both Ser^{19} and Thr^{18}, and immunostaining and image analysis with confocal microscopy. Consistent results were obtained with all three methods, and the results indicate that an increase in the amplitude of calyculin A-induced contraction in the SHR is associated with elevations in the mono- and di-phosphorylated forms of RLC_{20}.

It is well known that phosphorylation of RLC_{20} at Ser^{19} markedly increases actin-activated Mg^{2+}-ATPase activity of smooth muscle myosin,[36] and this is known to be further enhanced by additional phosphorylation at Thr^{18}.[21] In vascular smooth muscle tissues, RLC_{20} di-phosphorylation has been observed in pathological conditions such as vasospasm.[24,25] In addition, we previously reported that the $[Ca^{2+}]_i$-induced contraction is larger in SHR than in WKY, and the $[Ca^{2+}]_i$-force curve is significantly shifted to the left in SHR when compared with WKY.[33] These observations have led to the suggestion that di-phosphorylation of RLC_{20} is associated with hypercontractility in vascular smooth muscle. Our results indicate that Ca^{2+} sensitization by Ca^{2+}-independent MLCK activity might play an important role in the hypercontractility of vascular smooth muscle of SHR.

Since Ca^{2+}-independent, microcystin-LR-induced contraction and RLC_{20} phosphorylation were first demonstrated by Kureishi *et al.*[35] a number of different Ca^{2+}-independent kinases have subsequently been shown to phosphorylate RLC_{20} *in vitro*.[20] However, ILK and ZIPK have emerged as the most likely candidates for Ca^{2+}-independent di-phosphorylation of RLC_{20} at Ser^{19} and Thr^{18} in smooth muscle.[20] In the present study, to identify the nature of the protein kinase causing the Ca^{2+}-independent, calyculin A-induced contraction and RLC_{20} di-phosphorylation, we studied the effects of SM-1, a ZIPK-inhibiting peptide, on the calyculin A-induced contraction and di-phosphorylation of RLC_{20}. SM-1 significantly inhibited the amplitude of contraction and di-phosphorylation of RLC_{20} induced by calyculin A at pCa 9.0; in contrast, scrambled SM-1 had no effect. We also showed that ZIPK is expressed in the rat mesenteric artery, in both the 32 kDa and 54 kDa forms of ZIPK, and that more ZIPK is present in the SHR than in the WKY. It has been shown that a 32 kDa form of ZIPK, expressed in the smooth muscle, co-localizes with actin filaments and plays a role in the regulation of smooth muscle contraction.[37] A 54 kDa form of ZIPK is also expressed in smooth muscle and activated by prostaglandin $F_{2\alpha}$ stimulation.[38] Taken together, our results suggest that the increased expression levels of ZIPK in SHR induce augmentation of the calyculin A-induced contraction and RLC_{20} phosphorylation. However, in the present study, calyculin A-induced residual contraction in the presence of SM-1, a ZIPK-inhibiting peptide, still exists despite the fact that we used the concentration of SM-1 which is the maximal effective dose. Therefore, ILK might be another possible candidate for Ca^{2+}-independent MLCK, although it was detected at equal expression levels in both SHR and WKY tissues in the present study.

We found the expression level of ROCK to be stronger in the SHR than in the WKY. Because ROCK can activate ZIPK, ROCK also plays an important role for Ca^{2+}-independent MLC phosphorylation. However, because the ROCK inhibitor Y-27632 had no inhibitory effect on the calyculin A-induced contraction, ROCK might not be a candidate for the Ca^{2+}-independent MLCK.

Interestingly, we also found evidence for an effect of ZIPK to phosphorylate MLCP in addition to its apparent direct effect to phosphorylate RLC_{20}. It is known that ZIPK can mediate inhibition of MLCP[39,40] either directly, via phosphorylation of the MYPT1, or indirectly, via phosphorylation of a CPI-17.[41] Therefore, augmentation of di-phosphorylation by calyculin A in the SHR may be due to inhibition of MLCP, either directly, through enhanced phosphorylation of the MYPT1 of MLCP, or indirectly, through enhanced phosphorylation of a CPI-17. To clarify this possibility, we examined the differences of MYPT1 phosphorylation induced by calyculin A between SHR and WKY. We showed that both Thr^{697} and Thr^{855} phosphorylation of MYPT1 was significantly increased by the treatment of SHR and WKY tissue with calyculin A at pCa 9.0, but the signal of phosphorylation at Thr^{697}, and not at Thr^{855}, was consistently stronger in the SHR than in the WKY. These results are consistent with the previous finding that ZIPK directly regulates MYPT activity through phosphorylation of Thr^{696} (numbering for human isoform and equivalent to Thr^{697}).[42] We also showed that the phosphorylation of CPI-17 was significantly increased by treatment of SHR and WKY tissue with calyculin A at pCa 9.0, and that the increase caused by calyculin A was similar in both SHR and WKY. Taken together, our results suggest that the increase in both Thr^{697} phosphorylation of MYPT1 and direct phosphorylation of RLC_{20}, driven by the higher expression levels of ZIPK in SHR, plays an important role in the enhanced calyculin A-induced contraction and RLC_{20} phosphorylation in this model of hypertension.

In the present study, the SHR have been employed as animal models of human primary (essential) hypertension. Although SHR are widely used as a model to investigate the mechanisms underlying essential hypertension, there are some differences between SHR and human hypertension. Therefore, further study is needed to elucidate the role of Ca^{2+} sensitization induced by Ca^{2+}-independent MLCK in hypertension using other hypertensive animal models, such as deoxycorticosterone acetate salt-induced hypertensive rats. Investigation is also necessary into whether the increased contractility and di-phosphorylation of RLC_{20} induced by Ca^{2+}-independent MLCK in hypertension is a causative factor or a consequence of hypertension development.

In summary, our results suggest that contraction and phosphorylation of RLC_{20} induced by Ca^{2+}-independent MLCK activity are further enhanced in the SHR than in the WKY, and ZIPK is a key candidate for causing this effect. Furthermore, the increase in the expression level of ZIPK in SHR appears to play an important role in enhanced calyculin A-induced contraction and RLC_{20} phosphorylation.

Conflict of interest: none declared.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2009-0072278), and the National Institute of Health Heart & Lung Institute (HL80003).

References

1. Somlyo AP, Somlyo AV. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G-proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;**83**:1325–1358.
2. Taylor DA, Stull JT. Calcium dependence of myosin light chain phosphorylation in smooth muscle cells. *J Biol Chem* 1988;**263**:14456–14462.
3. Hartshorne DJ, Ito M, Erdodi F. Role of protein phosphatase type 1 in contractile functions: myosin phosphatase. *J Biol Chem* 2004;**279**:37211–37214.
4. Kanaide H. Measurement of $[Ca^{2+}]_i$ in smooth muscle strips using front-surface fluorimetry. *Methods Mol Biol* 1999;**114**:269–277.
5. Kim HR, Appel S, Vetterkind S, Gangopadhyay SS, Morgan KG. Smooth muscle signaling pathways in health and disease. *J Cell Mol Med* 2008;**12**:1–16.
6. Hartshorne DJ, Ito M, Erdodi F. Myosin light chain phosphatase: subunit composition, interaction and regulation. *J Muscle Res Cell Motil* 1998;**19**:325–341.
7. Li L, Eto M, Lee MR, Morita F, Yazawa M, Kitazawa T. Possible involvement of the novel CPI-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle. *J Physiol* 1998;**508**:871–881.
8. Niiro N, Ikebe M. Zipper-interacting protein kinase induces Ca^{2+}-free smooth muscle contraction via myosin light chain phosphorylation. *J Biol Chem* 2001;**276**:29567–29574.
9. Gong MC, Cohen P, Kitazawa T, Ikebe M, Masuo M, Somlyo AP *et al*. Myosin light chain phosphatase activities and the effects of phosphatase inhibitors in tonic and phasic smooth muscle. *J Biol Chem* 1992;**267**:14662–14668.
10. Weber LP, Van Lierop JE, Walsh MP. Ca^{2+}-independent phosphorylation of myosin in rat caudal artery and chicken gizzard myofilaments. *J Physiol* 1999;**516**:805–824.
11. Obara K, Takai A, Ruegg JC, de Lanerolle P. Okadaic acid, a phosphatase inhibitor, produce a Ca^{2+} and calmodulin-independent contraction of smooth muscle. *Pflugers Arch* 1989;**414**:134–138.
12. Ishihara H, Ozaki H, Sato K, Hori M, Karaki H, Watabe S *et al*. Calcium-independent activation of contractile apparatus in smooth muscle by calyculin-A. *J Pharmacol Exp Ther* 1989;**250**:388–396.
13. Suzuki A, Itoh T. Effects of calyculin A on tension and myosin phosphorylation in skinned smooth muscle of the rabbit mesenteric artery. *Br J Pharmacol* 1993;**109**:703–712.
14. Ikebe M, Hartshorne DJ, Elzinga M. Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. *J Biol Chem* 1986;**261**:36–39.
15. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T *et al*. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* 1996;**271**:20246–20249.
16. Ikebe M, Hartshorne DJ, Elzinga M. Phosphorylation of the 20,000-daton light chain of smooth muscle myosin by the calcium-activated, phospholipid-dependent protein kinase. Phosphorylation sites and effects of phosphorylation. *J Biol Chem* 1987;**262**:9569–9573.
17. Komatsu S, Hosoya H. Phosphorylation by MAPKAP kinase 2 activates Mg^{2+}-ATPase activity of myosin II. *Biochem Biophys Res Commun* 1996;**223**:741–745.
18. Suizu F, Ueda K, Iwasaki T, Murata-Hori M, Hosoya H. Activation of actin-activated MgATPase activity of myosin II by phosphorylation with MAPK-activated protein kinase-1b (RSK-2). *J Biochem* 2000;**128**:435–440.
19. Deng JT, Van Lierop JE, Sutherland C, Walsh MP. Ca^{2+}-independent smooth muscle contraction. a novel function for integrin-linked kinase. *J Biol Chem* 2001;**276**:16365–16373.
20. Wilson DP, Sutherland C, Borman MA, Deng JT, MacDonald JA, Walsh MP. Integrin-linked kinase is responsible for Ca^{2+}-independent myosin diphosphorylation and contraction of vascular smooth muscle. *Biochem J* 2005;**392**:641–648.
21. Ikebe M, Hartshorne DJ. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J Biol Chem* 1985;**260**:10027–10031.
22. Ikebe M, Koretz J, Hartshorne DJ. Effects of phosphorylation of light chain residues threonine 18 and serine 19 on the properties and conformation of smooth muscle myosin. *J Biol Chem* 1988;**263**:6432–6437.
23. Mizutani T, Haga H, Koyama Y, Takahashi M, Kawabata K. Diphosphorylation of the myosin regulatory light chain enhances the tension acting on stress fibers in fibroblasts. *J Cell Physiol* 2006;**209**:726–731.
24. Harada T, Seto M, Sasaki Y, London S, Luo Z, Mayberg M. The time course of myosin light-chain phosphorylation in blood-induced vasospasm. *Neurosurgery* 1995;**36**:1178–1183.
25. Katsumata N, Shimokawa H, Seto M, Kozai T, Yamawaki T, Kuwata K *et al*. Enhanced myosin light chain phosphorylation as a central mechanism for coronary artery spasm in a swine model with interleukin-1β. *Circulation* 1997;**96**:4357–4363.
26. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T *et al*. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;**389**:990–994.
27. Satoh S, Kreutz R, Eilm C, Ganten D, Pfitzer G. Augmented agonist-induced Ca^{2+}-sensitization of coronary artery contraction in genetically hypertensive rats. *J Clin Invest* 1994;**94**:1397–1403.

28. Shaw LM, Ohanian J, Heagerty AM. Calcium sensitivity and agonist-induced calcium sensitization in small arteries of young and adult spontaneously hypertensive rats. *Hypertension* 1997;**30**:442–448.
29. Lee DL, Webb RC, Jin L. Hypertension and RhoA/Rho-kinase signaling in the vasculature: highlights from the recent literature. *Hypertension* 2004;**44**:796–799.
30. Seko T, Ito M, Kureishi Y, Okamoto R, Moriki N, Onishi K *et al.* Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ Res* 2003;**92**:411–418.
31. Miwa K, Fujita M, Sasaysma S. Recent insights into the mechanisms, predisposing factors, and racial differences of coronary vasospasm. *Heart Vessels* 2005;**20**:1–7.
32. Choi SK, Ahn DS, Lee YH. Comparison of contractile me*chanisms* of sphingosylphosphorylcholine and sphingosine-1-phosphte in rabbit coron*ary artery*. *Cardiovasc Res* 2009;**82**:324–332.
33. Ahn DS, Choi SK, Kim YH, Cho YE, Shin HM, Morgan KG *et al.* Enhanced stretch-induced myogenic tone in the basilar artery of spontaneously hypertensive rats. *J Vasc Res* 2007;**44**:182–191.
34. Yeon DS, Kim JS, Ahn DS, Kwon SC, Kang BS, Morgan KG *et al.* Role of protein kinase C- or RhoA-induced Ca^{2+} sensitization in stretch-induced myogenic tone. *Cardiovasc Res* 2002;**53**:431–438.
35. Kureishi Y, Ito M, Feng J, Okinaka T, Isaka N, Nakano T. Regulation of Ca^{2+}-independent smooth muscle contraction by alternative staurosporine-sensitive kinase. *Eur J Pharmacol* 1999;**376**:315–320.
36. Walsh MP. Calcium-dependent mechanisms of regulation of smooth muscle contraction. *Biochem Cell Biol* 1991;**69**:771–800.
37. Ihara E, Edwards E, Borman MA, Wilson DP, Walsh MP, MacDonald JA. Inhibition of zipper-interacting protein kinase function in smooth muscle by a myosin-light chain kinase pseudosubstrate peptide. *Am J Physiol* 2007;**292**: C1951–C1959.
38. Ihara E, MacDonald JA. The regulation of smooth muscle contractility by zipper-interacting protein kinase. *Can J Physiol Pharmacol* 2007;**85**:79–87.
39. MacDonald JA, Borman MA, Muranyi A, Somlyo AV, Hartshorne DJ, Haystead TA. Identification of the endogenous smooth muscle myosin phosphatase-associated kinase. *Proc Natl Acad Sci USA* 2001;**98**:2419–2424.
40. Borman MA, MacDonald JA, Muranyi A, Hartshorne DJ, Haystead TA. Smooth muscle myosin phosphatase-associated kinase induces Ca^{2+} sensitization via myosin phosphatase inhibition. *J Biol Chem* 2002;**277**: 23441–23446.
41. MacDonald JA, Eto M, Borman MA, Brautigan DL, Haystead TA. Dual Ser and Thr phosphorylation of CPI-17, an inhibitor of myosin phosphates, by MYPT-associated kinase. *FEBS Lett* 2001;**493**:91–94.
42. Hagerty L, Weitzel DH, Chambers J, Fortner CN, Brush MH, Loiselle D *et al.* ROCK1 phosphorylates and activates zipper-interacting protein kinase. *J Biol Chem* 2007;**282**: 4884–4893.