Contractile Properties of the Cultured Vascular Smooth Muscle Cells
The Crucial Role Played by RhoA in the Regulation of Contractility

Abstract—Vascular smooth muscle cells (VSMCs) have a remarkable degree of plasticity and in response to vascular injury, they can change to a dedifferentiated state that can be typically seen in cell cultures. Recently, Y27632, a Rho kinase inhibitor, has been reported to preferentially correct hypertension in a hypertensive rat model. We thus tested the hypothesis that the contraction of the cultured VSMCs might be more dependent on the function of RhoA than the VSMCs in fresh tissue. For this purpose, a tissue-like ring preparation was made using the cultured porcine coronary artery SMCs (CASMCs) and collagen gel (reconstituted ring: R-ring). The R-ring developed an isometric tension on stimulation by high external K⁺ or various receptor agonists. The phorbol ester (a protein kinase C (PKC) activator)-induced contraction of the intact R-ring was greatly inhibited, while the GTPγS (an activator of RhoA)-induced and Ca²⁺-independent contraction of permeabilized R-ring was greatly enhanced, in comparison to the fresh coronary artery ring. An immunoblot analysis showed the expression levels of RhoA and myosin phosphatase subunits (MYPT1 and PP1cδ) to be up-regulated, while the levels of CPI-17 (PKC-potentiated protein phosphatase-1 inhibitory protein), h1-calponin and PKC isoforms were downregulated in cultured CASMCs. The knock down of RhoA by RNA interference decreased the contractility of the cultured CASMCs. It is concluded that the contractility of the cultured VSMCs thus appears to be much more dependent on the function of RhoA than VSMCs in fresh tissue. The expression level of RhoA thus plays a crucial role in regulating the contractility of cultured VSMCs. (Circ Res. 2005; 96:890-897.)

Key Words: vascular smooth muscle □ contraction □ RhoA □ CPI-17 □ RNA interference □ vascular disease

Contraction is a primary function of vascular smooth muscle cells (VSMCs) and those within adult blood vessels proliferate at an extremely low rate. However, in response to vascular injury, VSMCs dramatically increase their proliferation rate and become dedifferentiated due to their remarkable plasticity, which induces changes in their contractile properties. It is thus of great interest to elucidate the changes in the contractile properties of the proliferating dedifferentiated VSMCs. We considered the most suitable model of cells to investigate this to be cultured VSMCs, because in vitro culturing of VSMCs mimics the dedifferentiation induced by vascular injury. The contractility of cultured VSMCs had been indirectly evaluated by measuring the cell shape, cell shortening, or cell area. However, new methods to directly measure an isometric tension development of the VSMCs, SMCs,9 even of the nonmuscle cells such as fibroblasts8 have recently been reported. As a result, it is now possible to measure the isometric tension development of cultured VSMCs.

The contraction of VSMCs is primarily regulated by the transient changes in the intracellular Ca²⁺ concentration ([Ca²⁺]) and the subsequent phosphorylation of the myosin light chain (MLC) catalyzed by Ca²⁺/calmodulin-dependent MLC kinase (MLCK). However, the Ca²⁺ sensitivity of the contractile apparatus has been demonstrated to change during various types of stimulation. Recent research on the mechanism regulating the increase in Ca²⁺ sensitivity showed two major pathways to be involved in this mechanism, namely, the RhoA-Rho kinase pathway and the protein kinase C (PKC)-CPI-17 (PKC-potentiated protein phosphatase-1 inhibitory protein) pathway. Especially the RhoA-Rho kinase pathway has recently been implicated in the pathogenesis of the abnormal contraction of the VSMCs in vascular diseases, thus indicating that this pathway might play an important role in cultured VSMCs.

One of the major advantages of using cultured cells is that we can knock down the expression of a gene of interest in these cells by the use of RNA interference (RNAi). RNAi is a strategy of sequence-specific posttranscriptional gene silencing, and it has been successfully applied for the disruption of a gene transcript in plants and caenorhabditis elegans. Gene silencing by RNAi had been accomplished in mamma-
lian cells. Recently, RNAi has been successfully applied to the VSMCs. As a result, it is now possible to knock down a gene of interest in the cultured VSMCs.

In the present study, we investigated the contractile properties of cultured porcine coronary artery SMCs (CASMCs) and compared them with those of fresh coronary artery rings (F-CA-rings). The obtained results indicated that the contractions of the cultured CASMCs are more dependent on the RhoA-mediated pathway than on the PKC-mediated pathway, in comparison to the F-CA-rings. The expression levels of RhoA, MYPT1 (a large subunit of MLC phosphatase, MLCP), and PP1cα (a catalytic subunit of MLCP) in the cultured CASMCs were upregulated, whereas the levels of CPI-17, Rac1, two isoforms of Rho kinase, MLCK, h1-calponin, PKCa, and PKCe were downregulated. The knockdown of RhoA induced a decrease in the contractility of the cultured CASMCs. These results indicated that the expression level of RhoA thus plays a critical role in the regulation of contractility in the dedifferentiated VSMCs.

Materials and Methods

Cell Culture

The cultured porcine CASMCs were prepared as previously described, with minor modifications. Briefly, the media layer of the right coronary artery was treated with 1.0 mg/mL collagenase and 10 U/mL elastase for 60 minutes. The primary cultured cells were prepared by the collagen gel embedded method, using a collagen gel matrix (CellMatrix Type I-P, Nitta gelatin) and a Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The primary cultured cells were subcultured in Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cultured CASMCs were collected by trypsinization and mixed with the culture medium (107 cells/mL) containing 0.6 mg/mL type I collagen (Cellmatrix Type I-P). The mixture (100 μL, 105 cells) was placed around a silicone column (10 mm). After the collagen gel became solid, the culture medium was added and cultured for 24 to 48 hours. The ring preparation so obtained was used for measurements of isometric tension development.

Production of a Reconstituted Ring

The cultured CASMCs were collected by trypsinization and mixed with the culture medium (107 cells/mL) containing 0.6 mg/mL type I collagen (CellMatrix Type I-P). The mixture (100 μL, 105 cells) was placed around a silicone column (ϕ=2 mm), which was attached on the center of the silicone disc (ϕ=8 mm). After the collagen gel became solid, the culture medium was added and cultured for 24 to 48 hours. The ring preparation so obtained was used for measurements of isometric tension development.

Measurement of Tension in the Intact or α-Toxin Permeabilized Preparations

Both the tension measurements and α-toxin permeabilization were performed as described previously. The F-CA-rings (ϕ=2 mm, 1-mm long) were prepared from porcine right coronary artery after removing the adventitial tissue and endothelium. For the tension measurements, the ring preparations (F-CA or reconstituted ring, R-rings) were mounted onto two tungsten wires by passing the wires through the lumen of the rings. When needed, the rings were permeabilized with 5000 U/mL Staphylococcus α-toxin (α-hemolysin, Sigma) for 60 minutes in a solution composed of (in mmol/L) 10 EGTA, 100 potassium methanesulphonate, 3.38 MgCl2, 2.2 Na2ATP, 10 creatine phosphate, and 20 Tris-maleate; pH 6.8. Measurement of isometric tension was performed at 24 to 25°C.

MLC Phosphorylation

The phosphorylation of MLC was determined as described previously. The permeabilized R-rings were treated with pCa >8 solution alone or with pCa >8 plus 10 μmol/L Y27632 or with pCa >8 plus 10 μmol/L GTPγS. The sample was subjected to a urea-glycerol gel electrophoresis and a subsequent immunoblot analysis using anti-MLC (Sigma) or anti–phospho-MLC at Ser19 (generously provided by Dr M. Ikebe, University of Massachusetts Medical School, Worcester, Mass) antibody.

Immunoblot Analysis

Proteins were extracted from cultured CASMCs or F-CA and subjected to immunoblot analysis as previously described. Regarding the tissue samples, freshly dissected right coronary artery was rapidly frozen in liquid nitrogen and then was shattered by hammering. Thirty micrograms of total protein was separated with SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated with the indicated antibodies. Antigen detection was performed with an ECL plus detection kit (Amersham). The following antibodies were used: Anti-RhoA, anti-MLCK, anti–h1-calponin (Santa Cruz Biotechnology), anti-Rac1, anti-ROCK I, anti-ROCK II, anti-PKCa (BD Biosciences, San Jose, CA), anti–CPI-17, anti–PKCe, anti–PP1cα (Upstate Biotechnology), and anti-MYPT1 (Babco).

RhoA Translocation

RhoA translocation was determined using the methods described by Massey et al. The α-toxin permeabilized F-CA-rings or R-rings were lysed with ice-cold hypotonic buffer, after they were treated with pCa >8 solution with or without 10 μmol/L GTPγS. After the membrane and cytosolic fractions were separated by differential centrifugation, equal amounts of proteins (15 μg) were applied to SDS-PAGE. The RhoA protein was detected by an immunoblot analysis as described. The membrane to total RhoA ratio was considered to represent RhoA translocation.

Knockdown of RhoA by RNAi

The single-stranded sense and antisense RNAs, based on the pig RhoA mRNA, and their inverted sequences (control) were synthesized by Hokkaido System Sciences. The sequences were 5'-GUGGAUGGAAACAGGAGATTT-3' (RhoA sense), 5'-CUCUACCUCUUCCAUCCCTT-3' (RhoA antisense), 5'-GAGAUGGACGAAGGUGUGTGT-3' (RhoA inverted sense), 5'-CACCUCUCCUUUCGCUACUUCCT-3' (RhoA inverted antisense). The annealing of RNAs and transfection were performed using the method described by Elbashir et al. Cationic lipid complexes containing 200 pmol of indicated small interfering RNA (siRNA) and 6 μL oligofectamine (Invitrogen) were added to a dish. The cells were exposed to Opti-MEMI (Invitrogen) containing 25 mmol/L RNA for 40 to 45 hours and then the cells were used for the experiments.

Measurement of [Ca2+]i in the CASMCs

The changes in [Ca2+]i of the cells cultured on 35-mm dishes were monitored with front-surface fluorometry using fura-2 as previously described. The [Ca2+]i levels were expressed as a percentage, assigning those obtained at rest and with 25 μmol/L ionomycin to be 0% and 100%, respectively.

Data Analysis

The data were expressed as the mean±SEM along with the number of observation (=n). Statistical differences between the sets of data were determined using either the unpaired or paired Student t tests.

Results

Contractility of the R-Ring Made of CASMCs

In order to examine the contractility of the cultured VSMCs, we first established a method to measure the isometric tension development of the cultured CASMCs. We added sodium ascorbate to the culture medium to keep the cultured CASMCs. We added sodium ascorbate to the culture medium to keep the cultured CASMCs. We added sodium ascorbate to the culture medium to keep the cultured CASMCs.
cell–populated collagen gel fiber described by Oishi et al.24

As shown in Figure 1, we could determine the isometric tension development of the R-ring made of these CASMCs. The R-ring responded to a high external K+] solution (44.1 ± 4.2 mg, n = 7) as well as the various receptor agonists, including bradykinin (90.9 ± 12.3 mg, n = 7), histamine (91.9 ± 14 mg, n = 7), and endothelin-1 (8.6 ± 1.9 mg, n = 7; Figure 1A through 1D). Furthermore, the R-ring relaxed after the addition of sodium nitroprusside, as shown in Figure 1E.

The application of 10 μmol/L sodium nitroprusside during the contraction induced by 118 mmol/L K+] solution reduced the level of tension to 3.8 ± 1.8% (n = 7). To examine the involvement of PKC in the contraction of the cultured contractile CASMCs, we applied phorbol-12,13-dibutyrate (PDBu), a PKC activator, as shown in Figure 2A. Although 0.3 μmol/L PDBu induced a contraction of the R-ring, it was much smaller than the 118 mmol/L K+] solution–induced contraction (23 ± 5%, n = 5). We next examined the involvement of the G protein (presumably RhoA)–mediated pathway in the contraction of the cultured CASMCs. For this purpose, we performed experiments using α-toxin–permeabilized preparation. In the presence of Ca2+ (pCa = 6), the enhancement of contraction induced by 10 μmol/L GTPγS, a nonhydrolyzable GTP analog that is known to activate G protein, in R-rings (26 ± 3% of the 10 μmol/L Ca2+–induced contraction, n = 3) was significantly (P < 0.05) greater than that in F-CA-rings (17 ± 2%, n = 3, traces not shown). However, the most marked difference could be seen in the nominally Ca2+ free medium containing 10 mmol/L EGTA (pCa > 8). As shown in Figure 2A, the permeabilized preparation contracted at pCa > 8 with the addition of 10 μmol/L GTPγS. The level of this contraction reached 31 ± 7% (n = 7) of the maximal 10 μmol/L Ca2+–induced contraction. Figure 2B shows the effects of 0.3 μmol/L PDBu or 10 μmol/L GTPγS on the contractions of the F-CA-rings. The PDBu-induced contractions relative to the 118 mmol/L K+ solution–induced contraction (129 ± 7.2%; n = 4) in the F-CA-rings were significantly (P < 0.01) greater than those in the R-rings, whereas inactive phorbol (4α-phorbol-12,13-didecanoate; Sigma) had no effect (trace not shown). On the other hand, the GTPγS–induced contractions at pCa > 8 relative to the 10 μmol/L Ca2+–induced maximum contractions (5.7 ± 1.5%; n = 5) in the permeabilized F-CA-rings were significantly (P < 0.01) smaller than those in the R-rings.

In order to further characterize the GTPγS–induced contraction, we measured the MLC phosphorylation. As shown in Figure 2C, the level of MLC phosphorylation at pCa > 8 induced by 10 μmol/L GTPγS was 21 ± 4% (n = 7) of the maximal phosphorylation level at pCa > 8 (P < 0.01, n = 8). MLC was monophosphorylated at Ser 19, and no diphosphorylated MLC could be detected.
in Figure 2C, GTPγS-induced Ca^{2+}-independent contraction of R-ring accompanied the increased level of MLC phosphorylation that was revealed to be at Ser 19 determined by the anti–phospho-MLC at Ser19 antibody. No diphosphorylated MLC could be detected. In addition, the relatively high level of MLC phosphorylation (20\% n=8) at rest (pCa 8) decreased to 5\% (n=3, P<0.01) in the presence of 10 μmol/L Y27632.

**Change in the Expression of the Selected Proteins in Cultured CASMCs**

These results indicated that the contraction of the cultured VSMCs might be more dependent on the function of RhoA, but less dependent on the function of PKC, than that of the normal VSMCs. We thus next examined the expression levels of the selected proteins in cultured CASMCs and compared them with those in F-CA. As shown in Figure 3, the expression levels of RhoA, MYPT1, and PP1cδ increased in the cultured CASMCs in comparison to those in F-CA. On the other hand, the expression levels of Rac1, CPI-17, ROCK I, ROCK II, h1-Calponin, MLCK, PKCα, and PKCε were downregulated (all P<0.01). In Figure 2C, GTPγS-induced Ca^{2+}-independent contraction of R-ring accompanied the increased level of MLC phosphorylation that was revealed to be at Ser 19 determined by the anti–phospho-MLC at Ser19 antibody. No diphosphorylated MLC could be detected. In addition, the relatively high level of MLC phosphorylation (20±2\% n=8) at rest (pCa >8) decreased to 5±1\% (n=3, P<0.01) in the presence of 10 μmol/L Y27632.

**Knockdown of RhoA by RNAi**

To reduce the expression level of RhoA, we used RNAi. As shown in Figure 5A, the expression of RhoA of the CASMCs transfected by siRNA targeting RhoA was much lower than that of the control cells (Figure 5B; P<0.01) that were transfected with siRNA of the reversed sequence. On the other hand, there was no difference in the expression levels of Rac1, ROCK I, ROCK II, and CPI-17 between RhoA knocked-down and control cells. Figure 5B shows a summary of the data obtained from three experiments done in a similar manner as in Figure 5A. These results indicated that RhoA protein could be selectively knocked down by RNAi technology. In addition, there was no significant difference in [Ca^{2+}].

**Figure 4.** RhoA translocation induced by GTPγS in F-CA-rings and R-rings at pCa >8. α-Toxin permeabilized F-CA-rings or R-rings were treated with pCa >8 solution with or without 10 μmol/L GTPγS. After the membrane (M) and cytosolic (C) fractions were separated, equal amounts of proteins (15 μg) were applied to SDS-PAGE. Representative photograph and a summary (n=4 in F-CA-rings and n=3 in R-rings) are shown. Ratio of membrane-bound to total RhoA (M/T) significantly (P<0.05) increased in the GTPγS-treated R-rings, but not in the F-CA-rings.

**Figure 5.** Immunoblot of the RhoA knocked-down CASMCs by RNAi. A, At 48 hours after transfection, equal amounts of lysate proteins (30 μg) of control and RhoA knocked-down (KD) cells were analyzed on an immunoblot with an antibody against RhoA, Rac1, ROCK I, ROCK II, or CPI-17. In case of CPI-17, the sample from F-CA was also run as a positive control. B, Summary of the densitometric analysis of the three independent experiments performed in a similar manner as in A. Expression levels of the indicated proteins in the RhoA knocked-down cells are shown, with those in the control cells set at 100%.
transients induced by 100 mmol/L K⁺-depolarization, bradykinin, or histamine between RhoA knocked-down and control cells, as shown in Figure 6. On the other hand, RhoA knocked-down cells showed a very low contractility in comparison to the control cells. The contractions induced by 118 mmol/L K⁺-depolarization as well as the various receptor agonists, including bradykinin, histamine, and endothelin-1 became significantly smaller than those of the control (Figure 7), whereas the contractions of the RhoA knocked-down cells induced by 1 μmol/L calyculin A, a membrane permeant Type 1 and 2A phosphatase inhibitor, remained unchanged (104±44% of the control, n=4, P>0.05). Figure 8 shows the GTPγS-induced contraction of the α-toxin–permeabilized preparation made of control or RhoA knocked-down cells at pCa >8. The GTPγS-induced contraction of the RhoA knocked-down cells (42±3%; n=7; Figure 8B) was significantly (P<0.05 by paired Student t test) smaller than that of the control cells (46±4%; n=7; Figure 8A), although the GTPγS-induced Ca²⁺-independent contraction of the RhoA knocked-down cells could not be completely inhibited. In addition, the maximal contractions induced by 10 μmol/L Ca²⁺ solution in the RhoA knocked-down cells were also much smaller than those in the control cells (75±17 versus 42±16 mg; P<0.05), thus indicating a possible involvement of RhoA in the Ca²⁺-induced contraction of R-rings. As shown in Figure 8C, the addition of 1 μmol/L Y27632 inhibited the 10 μmol/L Ca²⁺-induced contraction of the R-rings to 11.1±1.4% (n=3) of this contraction. However, 1 μmol/L Y27632 had no effect on the 10 μmol/L Ca²⁺-induced contraction of the F-CA-rings (Figure 8D; 97±2%, n=3; P<0.01 compared with the R-rings).

**Discussion**

In the present study, we investigated the contractility of the cultured CASMCs by measuring the isometric tension development of the R-rings and then compared it with that of the F-CA-rings. The contraction induced by PDBu was much smaller than that induced by high K⁺ in R-rings, although it was much greater than that by high K⁺ in F-CA-rings. In contrast, GTPγS induced a significant contraction of the permeabilized R-rings at pCa >8 compared to 10 μmol/L Ca²⁺-induced contraction, whereas it induced only slight contractions in F-CA-rings under the same condition.

**Figure 6.** Effects of the RhoA knockdown on [Ca²⁺]i of cultured CASMCs. A, Changes in [Ca²⁺]i during stimulation by 100 mmol/L K⁺ depolarization, 1 μmol/L bradykinin (BK), and 100 μmol/L histamine (His) in the RhoA knocked-down CASMCs (RhoA KD) and the control CASMCs (Control). Level of [Ca²⁺]i in the presence of 25 μmol/L ionomycin was designated as 100%. B, Summary of the results obtained from 4 independent experiments performed in a similar manner as in A. There was no significant difference in the [Ca²⁺]i elevation induced by 100 mmol/L K⁺ depolarization, bradykinin, or histamine between RhoA knocked-down CASMCs and the control CASMCs.

**Figure 7.** Effect of RhoA knockdown on the contractility. A, Contractions induced by 118 mmol/L K⁺, 1 μmol/L bradykinin (BK), 100 μmol/L histamine (His), and 1 μmol/L endothelin-1 (ET-1) of the RhoA knocked-down CASMCs (RhoA KD) were smaller than those of the control CASMCs (Control). B, Summary of the effects of RhoA knockdown on the contractility. Data are the mean±SEM (n=4). Contractions of the RhoA knocked-down CASMCs were significantly (P<0.05) smaller than the control not only with agonist stimulation but also with 118 mmol/L K⁺ depolarization. Level of contraction induced by 118 mmol/L K⁺ depolarization in the control was designated as 100%. Statistical differences between the sets of data were determined using paired Student t tests.
An immunoblot analysis revealed that the expressions of PKC isoforms and CPI-17 decreased, whereas those of RhoA, MYPT1, and PP1cβ increased in the cultured CASMCs, compared with those of the F-CA. The knockdown of RhoA by RNAi markedly decreased the contractility. These results indicated that the contraction of the cultured CASMCs might be more dependent on the function of RhoA, but less dependent on the function of PKC, than fresh tissue. The expression level of RhoA appeared to be crucial in the regulation of contractility of the cultured VSMCs.

Although the cultured VSMCs have been reported to lose their contractility,6 L’Heureux et al6 recently reported a method to obtain the contractile cultured VSMCs that displayed a differentiated phenotype as demonstrated by the reexpression of VSMC-specific markers and actual tissue contraction in response to physiological stimuli. Using this method, we could also obtain the contractile cultured CASMCs that expressed VSMC-specific marker, h1-calponin, and functionally expressed cGMP-dependent protein kinase (PKG). Because it has been reported that the synthetic and proliferative phenotype cells do not express PKG,27 these observations (contractility, h1-calponin expression, and functional expression of PKG) supported the notion that the CASMCs used in the present study are more differentiated, compared with the conventional synthetic cultured VSMCs.

The contraction of the VSMCs is primarily regulated by the level of \([\text{Ca}^{2+}]_i\). The increase in \([\text{Ca}^{2+}]_i\), causes the calmodulin-mediated activation of MLCK, which phosphorylates the MLC and thereby induces contraction. A decrease in \([\text{Ca}^{2+}]_i\), inactivates MLCK and permits the dephosphorylation of MLC by MLCP, thus causing relaxation. However, the extent of \([\text{Ca}^{2+}]_i\) elevation has been reported to not always be parallel to the extent of contraction,12,28 thus suggesting that a secondary regulatory pathway might play a role in the control of smooth muscle contractility.10,11 Such secondary pathways may include the PKC-CPI-17–mediated or RhoA–Rho kinase–mediated inhibition of MLCP.12 However, the contraction induced by PDBu was much smaller than that induced by high \(K^+\) in R-rings, although it was much greater than that by high \(K^+\) in F-CA-rings. In support of this physiological observation, the immunoblot analysis revealed the expression levels of CPI-17 and PKC isoforms (PKCα and PKCe) to be downregulated. From these results, the PKC-mediated pathway was considered to possibly not be a major pathway for the contraction of cultured CASMCs.

GTPγS is known to increase \(\text{Ca}^{2+}\) sensitivity of myofilament of VSMCs, but the presence of intracellular \(\text{Ca}^{2+}\) is thought to be a prerequisite condition for GTPγS-induced contraction of the permeabilized preparation.29 Consistent with this, the \(\text{Ca}^{2+}\) sensitizing effects by GTPγS could be observed in both R-rings and F-CA-rings in the presence of \(\text{Ca}^{2+}\) (pCa=6). However, the \(\text{Ca}^{2+}\) sensitizing effects by GTPγS were greater in R-rings than in F-CA-rings. This difference could be clearly seen at pCa >8. The permeabilized preparation of the R-rings significantly contracted even at pCa >8 with the addition of 10 μmol/L GTPγS, in comparison to the 10 μmol/L \(\text{Ca}^{2+}\)–induced contraction, whereas only little contraction could be obtained in F-CA-rings under the same condition. In good agreement with this physiological observation, the immunoblot analysis revealed that the expression of RhoA in cultured CASMCs increased and the membrane-bound active form of RhoA of the R-rings was also increased during activation with GTPγS. In order to further confirm the role of RhoA, we next examined the effect of the RhoA knockdown on the contractility of the cultured CASMCs. We first checked the validity of the method of RNAi. The transfection of the siRNA targeting RhoA selectively knocked down the expression of RhoA. The R-ring preparation made of RhoA knocked-down cells showed markedly decreased contractility, compared with the control. This decrease in contractility of the RhoA knocked-down cells was not due to the impaired function of the \(\text{Ca}^{2+}\) mobilization, because there was no difference in \([\text{Ca}^{2+}]_i\),
transient between RhoA knocked-down and control cells. To further exclude this possibility, we also performed the experiments using the permeabilized preparation. The RhoA knockdown inhibited not only the GTP\(^\gamma\)-S-induced Ca\(^{2+}\)-independent contractions, but also the 10 \(\mu\)mol/L Ca\(^{2+}\)-induced contraction. These results supported the idea that the contractility of the cultured CASMCs may be mainly dependent on the function of RhoA.

Because the GTP\(^\gamma\)-S-induced Ca\(^{2+}\)-independent contractions of R-rings accompanied the increased level of MLC phosphorylation, we examined the kinases that were responsible for MLC phosphorylation. This increased MLC phosphorylation was found out to be monophosphorylation at Ser19 (Figure 2C). We thus considered it unlikely that ZIP-like kinase and/or integrin-linked kinase are involved in this contraction, because these kinases were reported to be nonselective to Ser19 and Thr18.\(^{30,31}\) On the other hand, Rho kinase has been shown to directly phosphorylate MLC at Ser19 to induce contraction in a Ca\(^{2+}\)-independent manner.\(^{32,33}\) It is thus possible that this mechanism might play a role in cultured VSMCs because of the increased expression of RhoA in the cultured CASMCs. Consistent with this speculation, the relatively high level of MLC phosphorylation at rest (pCa >8) could be inhibited in the presence of 10 \(\mu\)mol/L Y27632.

In R-rings, all the contractions induced by depolarization, 10 \(\mu\)mol/L Ca\(^{2+}\), and agonists, except for those induced by calyculin A, were inhibited by RhoA knockdown, indicating that RhoA-mediated pathway is thus likely involved in all three types of contractions. Consistent with this, it has recently been reported that membrane depolarization by 60 mmol/L KCl as well as noradrenaline (3 \(\mu\)mol/L) stimulation induced similar time-dependent, sustained increases in the amount of an active GTP-bound form of RhoA.\(^{34}\) This notion was also supported by the observations that the 118 mmol/L Ca\(^{2+}\)-induced contraction (Figure 1F), as well as the 10 \(\mu\)mol/L Ca\(^{2+}\)-induced contraction (Figure 8C), could be effectively inhibited by 1 \(\mu\)mol/L Y27632 in R-rings. In contrast, 10 \(\mu\)mol/L Ca\(^{2+}\)-induced contraction of F-CA-rings could not be inhibited by 1 \(\mu\)mol/L Y27632 (Figure 8D). It was thus indicated that the contractions are more dependent on RhoA-Rho kinase pathway in R-rings than in F-CA-rings. This was thought to be the major reason why the GTP\(^\gamma\)-S-induced contraction relative to that induced by 10 \(\mu\)mol/L Ca\(^{2+}\) could not be completely inhibited after RhoA knockdown, because both contractions were equally inhibited by RhoA knockdown that cannot completely inhibit the expression of RhoA.

Concerning the mechanism for the greater dependency of contraction on RhoA-Rho kinase pathway in R-rings than in F-CA-rings, the increased MYPT1 and PP1\(^\alpha\) expressions and the decreased MLCK and h-\(\alpha\)-calponin expressions were thought to be involved, in addition to the increased RhoA expression. In R-rings, the Ca\(^{2+}\)-MLCK pathway may not effectively induce contraction, because the increased MYPT1 and PP1\(^\alpha\) expressions should be coupled with the increase in the MLCP activity. In contrast, RhoA-Rho kinase pathway may be more effective in inducing contraction, because this pathway also inhibits MLCP.\(^{12}\) The h-\(\alpha\)-calponin could be another possible candidate for the increase in GTP\(^\gamma\)-S-induced contraction of the cultured CASMCs, because calponin has been shown to inhibit the myosin ATPase.\(^{35}\)

In conclusion, we measured the isometric tension development of the cultured CASMCs. The excitation-contraction coupling of the cultured CASMCs was much different from that of the fresh tissue, in terms of the dependency on Ca\(^{2+}\), G protein, or PKC, possibly due to the upregulation of RhoA, MYPT1, and PP1\(^\alpha\) and/or downregulation of PKC isoforms, CPI-17 and h-\(\alpha\)-calponin. We thus were tempted to speculate that similar changes in the contractile properties might be involved in the abnormal contractility seen in various vascular diseases. In good agreement with this speculation, Uehata et al\(^{36}\) reported that Y27632 dramatically corrected hypertension in several hypertensive rat models, although it did not change the blood pressure of the nonhypertensive rats. If there was no difference in the contractile mechanism of VSMCs between hypertensive and normal rats, Y27632 should have similar effects on the blood pressure of the normal rats.

**Acknowledgments**

This study was supported in part by the grant from the 21st Century COE Program and Grants-in-Aid for Scientific Research (nos. 15590758, 16590695) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Dr M. Ikebe (University of Massachusetts Medical School, Worcester, Mass) for supplying the anti-phospho-MLC at Ser19 antibody. We also thank Brian Quinn for linguistic comments.

**References**


Contractile Properties of the Cultured Vascular Smooth Muscle Cells: The Crucial Role Played by RhoA in the Regulation of Contractility
Dan Bi, Junji Nishimura, Naohisa Niiro, Katsuya Hirano and Hideo Kanaide

Circ Res. 2005;96:890-897; originally published online March 17, 2005;
doi: 10.1161/01.RES.0000163018.66460.85
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/96/8/890

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/