Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 319 (2004) 95–102
BBRC
www.elsevier.com/locate/ybbrc

Translocation and association of ROCK-II with RhoA and HSP27 during contraction of rabbit colon smooth muscle cells

Suresh B. Patil,[a] Yasuhiro Tsunoda,[b] Mercy D. Pawar,[a] and Khalil N. Bitar[a,*]

[a] *Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109, USA*
[b] *Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA*

Received 19 March 2004

Abstract

The focus of the paper is to understand the role of HSP27 in mediating the association of RhoA with ROCK-II in sustained contraction of smooth muscle cells from the rabbit colon. In circular smooth muscle cells; acetylcholine-induced contraction (10^{-7} M) was associated with translocation of ROCK-II to the particulate fraction, which remained sustained at 4 min after stimulation ($135.1 \pm 8.1\%$ increase, $P \leqslant 0.05$). There was also an increased association of ROCK-II with RhoA particulate fraction (147.46 ± 9.31 and 148.22 ± 9.41, $n = 3$, $P \leqslant 0.05$) and with HSP27 ($155.6 \pm 10.7\%$ increase, $P \leqslant 0.05$) in the particulate fraction. Pre-incubation of cells with Y27632 resulted in the inhibition of the association of ROCK-II with RhoA in the particulate fraction. Acetylcholine (10^{-7} M) induced sustained phosphorylation of MLC ($122.75 \pm 9.97\%$, $P \leqslant 0.05$ and $174.65 \pm 28.36\%$, $P \leqslant 0.05$ increase in the di phospho-MLC at 30 s and 4 min, respectively), which was inhibited upon pre-incubation with Y27632. Results suggest that ROCK-II undergoes a translocation to the particulate fraction with RhoA and with HSP27, suggesting that translocation and association of ROCK-II with RhoA is mediated by HSP27. Maintenance of the functional association of RhoA with ROCK-II in the particulate fraction mediated by HSP27 appears to be important to retain MLC in the phosphorylated state and hence the sustained contraction.

Keywords: HSP27; ROCK-II; RhoA; MLC; Phosphorylation; Smooth muscle; Contraction

It is widely accepted that the degree of myosin light chain (MLC) phosphorylation is the essential factor that determines the extent to which smooth muscle contracts. MLC phosphorylation promotes smooth muscle contraction whereas MLC dephosphorylation results in muscle relaxation [1]. MLC phosphorylation by myosin light chain kinase (MLCK) is the predominant process in the initiation of smooth muscle contraction. Although this mode of action is well established, there are reports of smooth muscle contraction that is not associated with MLCK activity [2]. The MLCK-independent MLC phosphorylation and contraction is suggested to be mediated by RhoA [2,3].

The small GTPase RhoA, a member of subfamily of Ras superfamily GTPases, exhibits both GDP/GTP binding activity and GTPase activity cycling between inactive GDP-bound state and active GTP-bound state. RhoA is suggested to act by inhibiting myosin phosphatase (MYPT) activity [4]. In addition, RhoA is suggested to play a crucial role in Ca^{2+} sensitization of smooth muscle contraction that is mediated by effector, Rho-kinase (ROCK-II). In addition, CPI-17, a PKC activated molecule, is also shown to activate ROCK-II and increase its MYPT inhibiting activity [5]). Thus, it is apparent that a pathway that is alternative or parallel to MLCK activation of MLC exists in smooth muscle contraction that helps in sustenance of myosin phosphorylation and therefore, contraction.

We have previously shown that RhoA co-localizes with the actin binding protein, HSP27, on the membrane as observed under confocal microscope [6]. We have also previously shown a role for RhoA in regulating smooth muscle contraction through cytoskeletal reorganization

* Corresponding author. Fax: 1-734-647-9703.
E-mail address: bitar@umich.edu (K.N. Bitar).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.04.159

of HSP27 [6,7]. We have also shown that acetylcholine induces association of HSP27 with RhoA in the particulate fraction of smooth muscle cells from the rabbit colon [8]. We have also shown that HSP27 phosphorylation increases its association with RhoA in the particulate fraction [7]. Furthermore, RhoA associates directly with HSP27 [7]. Therefore, we postulate that association of translocated ROCK-II with RhoA and with HSP27 would facilitate the activation of ROCK-II during agonist-induced contraction.

Rho-kinase is a Ser/Thr protein kinase that was identified as a GTP Rho binding protein and was named as ROKα and ROCK-II. A pyridine derivative, Y-27632, has been shown to selectively inhibit smooth muscle contraction by inhibiting Ca^{2+} sensitization by selectively inhibiting ROCK-II [9]. In the present studies we used this compound to selectively inhibit ROCK-II and examine the role of RhoA activation of ROCK-II during agonist-induced contraction of smooth muscle cells from the rabbit colon. Results suggest that ROCK-II associates with HSP27 and with RhoA during acetylcholine-induced contraction. However, inhibition of ROCK-II resulted in (a) decrease of acetylcholine-induced smooth muscle contraction and (b) decrease in the association and translocation of ROCK-II with RhoA and with HSP27. Thus, it appears that HSP27-mediated translocation and association of RhoA with ROCK-II is important for sustenance of MLC phosphorylation and contraction of smooth muscle cells.

Materials and methods

Materials

Y-271362 was kindly supplied by Yoshitomi Pharmaceutical Industries. (Osaka, Japan). ML-7 and MLCK substrate (chicken gizzard MLC11-23) were purchased from Biomol. Acetylcholine chloride was purchased from Sigma Chemical. Collagenase type II was from Worthington. Protein G–Sepharose was from Pharmacia Biotech (Uppsala, Sweden). Polyvinyl fluoride (PDVF) membranes were from Bio-Rad. Enhanced chemiluminescence detection reagents were from Amersham. Mouse monoclonal anti-RhoA antibody was from Cytoskeleton. Rabbit polyclonal ROCK-II (H-85) was from Santa Cruz Biotechnology. Mouse monoclonal anti-HSP27 (2B4-123) was previously described [10]. Peroxidase-conjugated anti-mouse secondary antibody or anti-rabbit secondary antibody was purchased from Bio-Rad, Irvine, CA.

Methods

Isolation of smooth muscle cells from rabbit colon. Smooth muscle cells from rabbit recto sigmoid were isolated as described earlier [11]. Briefly, the internal anal sphincter from anesthetized New Zealand rabbits, consisting of the most distal 3 mm of the circular muscle layer, ending at the junction of skin and mucosa, was removed by sharp dissection. A 5-cm length of the recto sigmoid, orad to the junction of jejunum, was dissected and digested with collagenase to yield isolated smooth muscle cells. The tissue was incubated for two successive 1-h periods at 31 °C in 15 ml Hepes (pH 7.4) (in mM): 115 NaCl, 5.7 KCl, 2.0 KH_2PO_4, 24.6 Hepes, 1.9 $CaCl_2$, 0.6 $MgCl_2$, and 5.6 glucose, containing 0.1% (wt/vol) collagenase (150 U/mg, Worthington CLS type II), 0.01 (wt/vol) soybean trypsin inhibitor, and 0.184 (wt/vol) DMEM. After the end of second enzymatic incubation period, the medium was filtered through 500-μm Nitex. The partially digested tissue left on the filter was washed four times with 10 ml of collagenase-free buffer solution. Tissue was then transferred into 15 ml of fresh collagenase-free buffer solution, and cells were gently dispersed. After a hemocytometric cell count, the harvested cells were re-suspended in collagenase-free Hepes buffer (pH 7.4). Each recto sigmoid yielded 10–20×10^6 cells.

Measurement of contraction. Aliquots consisting 2.5×10^4 cells in 0.5 ml medium were added to 0.1 ml of a solution containing the test agents. In kinetic experiments the reaction was interrupted at various time intervals (30 s to 4 min) by the addition of 0.1 ml acrolein at a final concentration of 1% (vol/vol). Individual cell length was measured by computerized image microscopy. The average length of cells in the control state or after addition of test agents was obtained from 50 cells encountered randomly in successive microscopic fields. The contractile response is defined as the decrease in the average length of the 50 cells and is expressed as the absolute change or the percent change from control length [12].

Particulate fractions. Isolated smooth muscle cells were counted on a hemocytometer and diluted with HEPES buffer as needed. Cells were then treated with agonists and/or antagonists for the indicated periods. After the treatment, the particulate fractions were prepared as described earlier [7]. Briefly, the cells were washed twice with buffer A (in mM: 150 NaCl, 16 Na_2HPO_4, 4 NaH_2PO_4, and 1 sodium orthovanadate, pH 7.4) and sonicated in buffer B (1 mM sodium orthovanadate, 1 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 1 mM Na_4MoO_4, 1 mM dithiothreitol, 20 mM NaH_2PO_4, 20 mM Na_2HPO_4,, 20 mM $Na_4P_2O_7{\cdot}10H_2O$, 50 μg/ml DNase–RNase, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml antipain, pH 7.4). The cell sonicates were centrifuged at 100,000*g* for 60 min. The supernatant material from the high-speed centrifugation was collected as soluble cytosolic fraction. The pellet material was resuspended by sonication twice for 30 s in lysis buffer plus 1% Triton X-100 and collected as soluble particulate fraction. The protein content was determined using Bio-Rad protein assay reagent.

Immunoprecipitation using RhoA, ROCK-II, and HSP27 antibodies. Smooth muscle cells were diluted in PBS buffer (in mM): 150 NaCl, 16 Na_2HPO_4, and 4 NaH_2PO_4 at pH 7.4, containing 1 mM sodium orthovanadate. The cells were then disrupted by sonication in buffer B and centrifuged for 15 min at 14,000*g*. Protein G–Sepharose was washed two times with buffer B to make a 50% suspension. Lysate containing 200 μg proteins in a total 500 μl buffer B was prepared with 50 μl of protein G–Sepharose bead slurry by rocking at 4 °C for 30 min. The mixture was spun at 14,000*g* for 5 min at 4 °C, and 1–2 μg of mouse-anti-RhoA antibody, rabbit ROCK-II antibody, or mouse monoclonal anti-HSP27 antibody was added to the resultant supernatant. The mixture was rocked at 4 °C for 1 h followed by addition of 50 μl of protein G–Sepharose bead slurry. The mixture was further rocked at 4 °C for 2 h and spun at 14,000*g* for 5 min, and the supernatant was aspirated off. The pellet was washed three times with buffer A and resuspended in 25 μl of 2× sample buffer and boiled for 5 min.

Western immunoblotting of lysates and immunoprecipitates. Lysates (~80 μg) or immunoprecipitates of RhoA, ROCK-II or HSP27 were size separated by SDS–PAGE and electrophoretically transferred to PDGF membranes. Immunoblotting was performed using a monoclonal anti-RhoA antibody, a polyclonal anti-ROCK-II antibody, or a monoclonal anti-HSP27 antibody as primary antibody. The membrane was reacted with peroxidase-conjugated goat anti-mouse IgG antibody or anti-rabbit antibody (1:3000) for 1 h at 24 °C. The enzymes in the membrane were detected with luminescent substrates.

Measurement of MLC phosphorylation. Freshly isolated cells were washed with PBS (pH 7.4) three times and the cells were dissolved in IEF buffer containing 4% Chaps, 7 M urea, 2 M thiourea, 10 mg/mL

dithiothreitol, and 1% carrier ampholytes (pH 3–10) (Pharmalytes; Amersham–Pharmacia Biotech, Uppsala, Sweden). The resulting samples were centrifuged at 4000 rpm and the insoluble material was discarded. The lysates were mixed with sample loading buffer (50% glycerol) and applied onto mini IEF slab gels that contained a mixture of 1:4 ampholytes of pH 3–10 and pH 5–8. The samples were run at 10 V for 15 min, 200 V for 15 min, and overnight (~10 h) at 450 V. They were then electrophoretically transferred onto PVDF membrane. The membranes were Western blotted with a monoclonal anti-myosin light chain antibody (1:2000) followed by peroxidase-conjugated anti-mouse secondary antibody and detected with luminescent (ECL) substrates.

Data analysis. Bands were quantified using a densitometer (model GS-700, Bio-Rad), and band volumes (absorbance units × mm^2) were calculated and expressed as a percentage of the total volume. Blotting data are within the linear range of detection for each antibody used.

Results and discussion

Results

Effect of pre-incubation with Y-27632 on acetylcholine-induced contraction of colonic smooth muscle cells

Smooth muscle cells from the rabbit colon exhibited a sustained contraction in response to acetylcholine (10^{-7} M) 33.23. ± 2.3% decrease in cell length ($n = 3$) at 30 s and 29.99 ± 0.27% decrease in cell length at 4 min ($n = 3$) (Fig. 1). Preincubation of cells with Rho-kinase inhibitor Y-27632 (10 μM) for 20 min partially inhibited acetylcholine-induced contraction (20.18 ± 0.09% and 18.8 ± 2.43% decrease in cell length, respectively, at 30 s and 4 min), thus suggesting that ROCK-II is involved in acetylcholine-induced smooth muscle contraction.

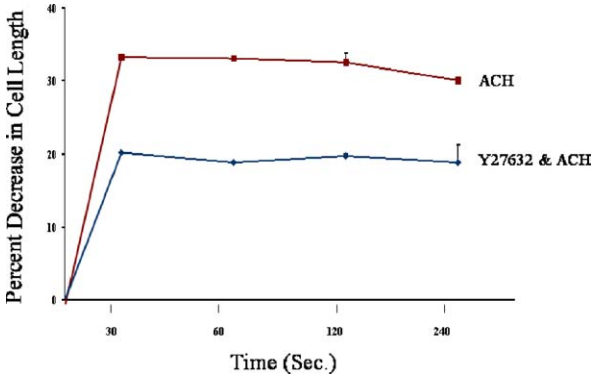

Fig. 1. Effect of pre-incubation with Y-27632 on acetylcholine-induced contraction of colonic smooth muscle cells. Smooth muscle cells from the rabbit colon were stimulated with acetycholine (10^{-7} M) for 30 s and 4 min and the reaction was arrested by 1% acrolein and the cells were measured under microscope. Smooth muscle cells exhibited contraction in response to acetylcholine (10^{-7} M) (33.23. ± 2.3% decrease in cell length ($n = 3$) at 30 s) and the contraction remained sustained (29.99 ± 0.27% decrease in cell length at 4 min ($n = 3$). Sustained contraction was greatly inhibited (% inhibition) in cells preincubated with the Rho-kinase inhibitor Y-27632 (20.18 ± 0.09% and 18.8 ± 2.43% decrease in cell length, respectively, at 30 s and 4 min. Data are means ± SE from three separate experiments.

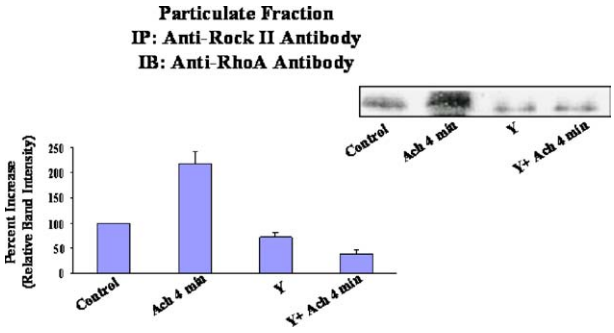

Fig. 2. Effect of pre-incubation with Y-2763 acetylcholine-induced association of ROCK-II with RhoA. Particulate fractions from smooth muscle cells from rabbit colon stimulated with acetylcholine (10^{-7} M) were immunoprecipitated with anti-ROCK-II antibody separated by SDS–PAGE, and Western blotted with anti-RhoA antibody. Stimulation of cells with acetylcholine resulted in a significant and sustained increase in the association of ROCK-II with RhoA in the particulate fraction, at 4 min (216.32 ± 24.11, $n = 4$, $P \leqslant 0.05$) compared to control. Pre-incubation of the cells with the Rho-kinase inhibitor Y-27632 (10 mM) for 20 min inhibited acetylcholine-induced increase (71.84 ± 8.7 Y alone and 38.44 ± 8.4 Y + Ach 4 min, $P \leqslant 0.05$, $n = 4$) in the association of ROCK-II with RhoA in the detergent-soluble particulate fraction.

Acetylcholine-induced association of ROCK-II with RhoA

Stimulation with the contractile agonist, acetylcholine (10^{-7} M), resulted in a significant and sustained increase in the association of ROCK-II with RhoA in the particulate fraction, at 4 min (216.32 ± 24.11, $n = 4$, $P \leqslant 0.05$) compared to control (Fig. 2). Preincubation of the cells with the Rho-kinase inhibitor Y-27632 (10 μM) for 20 min inhibited acetylcholine-induced association in the particulate fraction of ROCK-II with RhoA (71.84 ± 8.7 Y alone and 38.44 ± 8.4 Y + Ach 4 min percent increase $P \leqslant 0.05$, $n = 4$).

Acetylcholine-induced translocation of RhoA

We have previously shown that acetylcholine induces translocation of RhoA and its association with HSP27 in the particulate fraction. This was inhibited by preincubation of cells with C3 exoenzyme [6]. To investigate if inhibition of Rho-kinase affected acetylcholine-induced translocation of RhoA, particulate fractions of smooth muscle cells stimulated with acetylcholine in the presence or absence of Y-27632 were subjected to SDS–PAGE and followed by Western blot against anti-RhoA antibody. Acetylcholine induced an increase in the translocation of RhoA to the particulate fraction (147.46 ± 9.31 and 148.22 ± 9.41, $n = 3$, $P \leqslant 0.05$) (Fig. 3). Pre-incubation of cells with the Rho-kinase inhibitor resulted in inhibition of acetylcholine-induced translocation of RhoA. There was no change in the amount of RhoA in the particulate fraction in cells treated with Y-27632 (103.05 ± 1.14 control un-stimulated cells vs. 98.4 ± 0.57 in the presence of acetylcholine

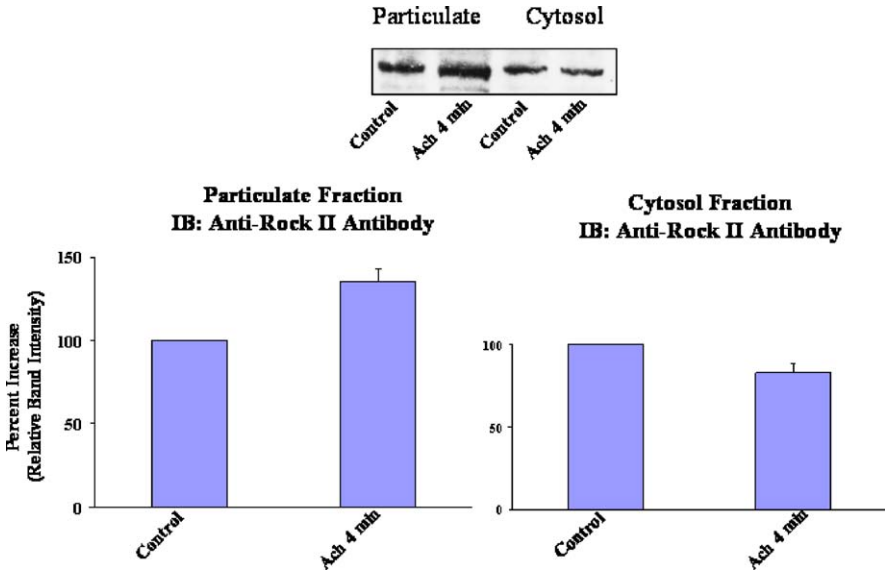

Fig. 3. Effect of pre-incubation with Y-27632 acetylcholine-induced translocation of RhoA. Particulate fractions from smooth muscle cells from rabbit colon stimulated with acetylcholine (10^{-7} M) were separated by SDS–PAGE, and Western blotted with anti-RhoA antibody. Acetylcholine induced an increase in the translocation of RhoA to the particulate fraction (147.46 ± 9.31 and 148.22 ± 9.41, $n = 3$, $P \leqslant 0.05$). Pre-incubation of cells with the Rho-kinase inhibitor resulted in inhibition of acetylcholine-induced translocation of RhoA. (103.05 ± 1.14 control un-stimulated cells vs. 98.4 ± 0.57 in the presence of acetylcholine (10^{-7} M) at 30 s and 98.96 ± 0.059 at 4 min, respectively, $P \leqslant 0.05$, $n = 4$.)

(10^{-7} M) at 30 s and $98.96 \pm .059$ at 4 min, respectively, $P \leqslant 0.05$, $n = 4$).

Acetylcholine-induced translocation of ROCK-II to the particulate fraction

Stimulation of smooth muscle cells with acetylcholine (10^{-7} M) resulted in a significant and sustained increase in the translocation of ROCK-II to the particulate fraction, at 4 min (135.12 ± 8.07, $n = 9$ $P \leqslant 0.01$) compared to control (Fig. 4). There was a concomitant decrease in ROCK-II in the cytosolic fraction at 4 min after stimulation with acetylcholine. Preincubation of the cells with the Rho-kinase inhibitor Y-27632 (10 μM) for 20 min inhibited acetylcholine-induced increase (82.83 ± 5.7 compared to control $P \leqslant 0.01$, $n = 10$). The data suggest that there is a basal level of association of RhoA-bound ROCK-II in the particulate fraction in the resting non-contracted smooth muscle cells (needs to be discussed). In addition, Y-27632 not only inhibited the association binding of RhoA/ROCK-II, but also resulted in a lowering of the basal levels of RhoA/ROCK-II, suggesting a possible dissociation of RhoA/ROCK-II immunocomplex.

Acetylcholine-induced association and translocation of ROCK-II with HSP27

Stimulation of isolated smooth muscle cells with acetylcholine (10^{-7} M) resulted in a significant and sustained increase in the association of ROCK-II with HSP27 in the particulate fraction (190.40 ± 6.92 and 155.63 ± 10.70, respectively, at 30 s and 4 min $n = 3$ $p \leqslant 0.05$) compared to control (Fig. 5). Pre-incubation of

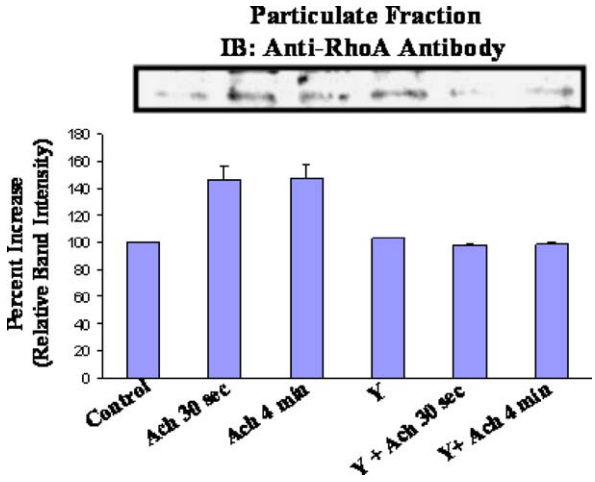

Fig. 4. Acetylcholine-induced association and translocation of ROCK-II with HSP27. Particulate fractions from smooth muscle cells from rabbit colon stimulated with acetylcholine (10^{-7} M) were immunoprecipitated with anti-ROCK-II antibody separated by SDS–PAGE, and Western blotted with anti-HSP27 antibody. Stimulation with the contractile agonist, acetylcholine, (10^{-7} M) resulted in a significant and sustained increase in the association of ROCK-II with HSP27 in the detergent-soluble particulate fraction, at 4 min (155.63 ± 10.70, $n = 3$ $P \leqslant 0.05$) compared to control. Pre-incubation of the cells with the Rho-kinase inhibitor Y-27632 (10 mM) for 20 min, inhibited acetylcholine-induced increase (49.98 ± 12.26 and 40.16447 ± 7.56 Y alone and at Ach 4 min, respectively, $P \leqslant 0.05$, $n = 4$ compared to control) in the association of ROCK-II with HSP27 in the detergent-soluble particulate fraction.

the cells with Y-27632 (10 μM) for 20 min inhibited acetylcholine-induced increase (49.98 ± 12.26 Y alone, and 44.28 ± 17.81 at 30 s 40.16 ± 7.56 at 4 min, respectively,

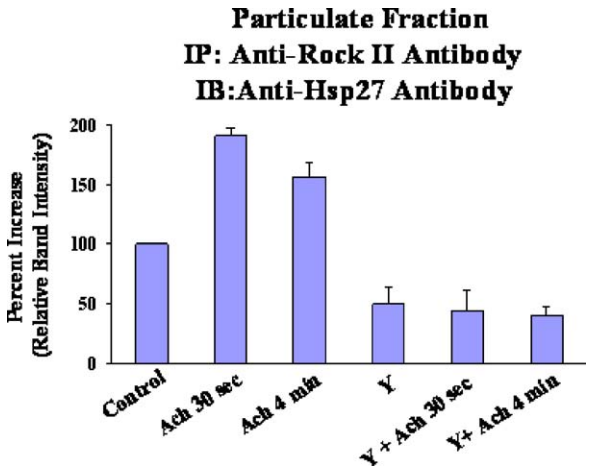

Fig. 5. Acetylcholine-induced translocation of ROCK-II to the particulate fraction. Particulate fractions from smooth muscle cells from rabbit colon stimulated with acetylcholine (10^{-7} M) were separated by SDS–PAGE, and Western blotted with anti-ROCK-II antibody. Stimulation with acetylcholine (10^{-7} M) resulted in a significant and sustained increase in the translocation of ROCK-II to the particulate fraction at 4 min (135.12 ± 8.07, $n = 9$ $P \leqslant 0.001$) compared to control. There was a concomitant decrease in ROCK-II in the cytosolic fraction at 4 min after stimulation with acetylcholine. Pre-incubation of the cells with the Rho-kinase inhibitor Y-27632 (10 mM) for 20 min inhibited acetylcholine-induced increase (82.83 ± 5.7 compared to control $P \leqslant 0.01$, $n = 10$).

$P \leqslant 0.05$, $n = 4$ compared to control) in the association of ROCK-II with HSP27 in the particulate fraction.

MLC phosphorylation

Stimulation with the contractile agonist, acetylcholine (10^{-7} M), resulted in a significant and sustained increase in the phosphorylation of MLC_{20} in the smooth muscle cells at 30 s and at 4 min ($122.75 \pm 9.97\%$, $P \leqslant 0.001$ and $174.65 \pm 28.36\%$, $P \leqslant 0.001$ increase in the diphospho-MLC_{20} at 30 s and 4 min, respectively), compared to control (Fig. 6). Pre-incubation of the cells with the Rho-kinase inhibitor Y-27632 (10 μM) for 20 min inhibited acetylcholine-induced increase in MLC_{20} phosphorylation (67.24 ± 30.12 Y alone, and 50.37 ± 12.78, 112.53 ± 16.35 at 30 s and 4 min $P \leqslant 0.05$, $n = 3$ compared to control).

Discussion

Two different contractile pathways have been identified in smooth muscle cells. A transient contraction, which is calmodulin-dependent mediated by inositol 1,4,5-trisphosphate-dependent calcium release [13]; and a sustained contraction [14,15] that is brought about by PKC activation of a cascade of signals that includes calmodulin kinase and MLCK. PKC phosphorylates an endogenous 17-kDa protein (CPI-17) resulting in inhibition of myosin phosphatase [5,16] thereby increasing MLC phosphorylation.

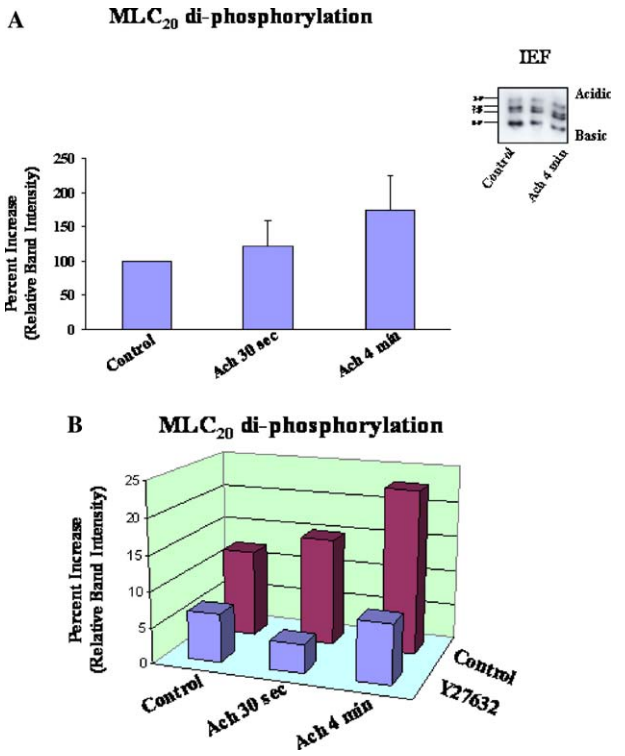

Fig. 6. (A) MLC phosphorylation. Cell lysates from smooth muscle cells from rabbit colon stimulated with acetylcholine (10^{-7} M) were separated urea-acrylamide gels and Western blotted with anti-MLC antibody. Stimulation with acetylcholine (10^{-7} M) resulted in a significant and sustained increase in the phosphorylation of MLC_{20} in the smooth muscle cells at 30 s and at 4 min ($122.75 \pm 9.97\%$, $P \leqslant 0.001$ and $174.65 \pm 28.36\%$, $P \leqslant 0.001$ increase in the di-phospho-MLC_{20} at 30 s and 4 min, respectively), compared to control. (B) Preincubation of the cells with the Rho-kinase inhibitor Y-27632 (10 mM) for 20 min inhibited acetylcholine-induced increase (67.24 ± 30.12 Y alone, and 50.37 ± 12.78, 112.53 ± 16.35 at 30 s and 4 min, $P \leqslant 0.05$, $n = 3$ compared to control) in the MLC_{20} phosphorylation.

It is generally accepted that phosphorylation of 20 kDa myosin light chain (MLC) by myosin light chain kinase (MLCK) is the major mechanism responsible for contraction in smooth muscles. Phosphorylation of MLC by MLCK is counterbalanced by its de-phosphorylation by myosin light chain phosphatase (MLCP) [17]. Maintenance of the MLC state of phosphorylation is a complex process of signal transduction. Ca^{2+} and myosin light chain (MLC) phosphorylation are the key regulators of the dynamic reorganization of actin filaments. Because the contraction-to-Ca^{2+} ratio is not always proportional, the Ca^{2+}- and calmodulin-dependent myosin light chain kinase (MLCK) pathway cannot solely account for the Ca^{2+} sensitivity [18,19]. Several lines of evidence have indicated that the Ras-related small GTP binding protein Rho is another important signaling element that mediates various actin-dependent cytoskeletal functions, including smooth muscle contraction [18,19]. RhoA belongs to the superfamily of Ras-related proteins [20]. These proteins function by

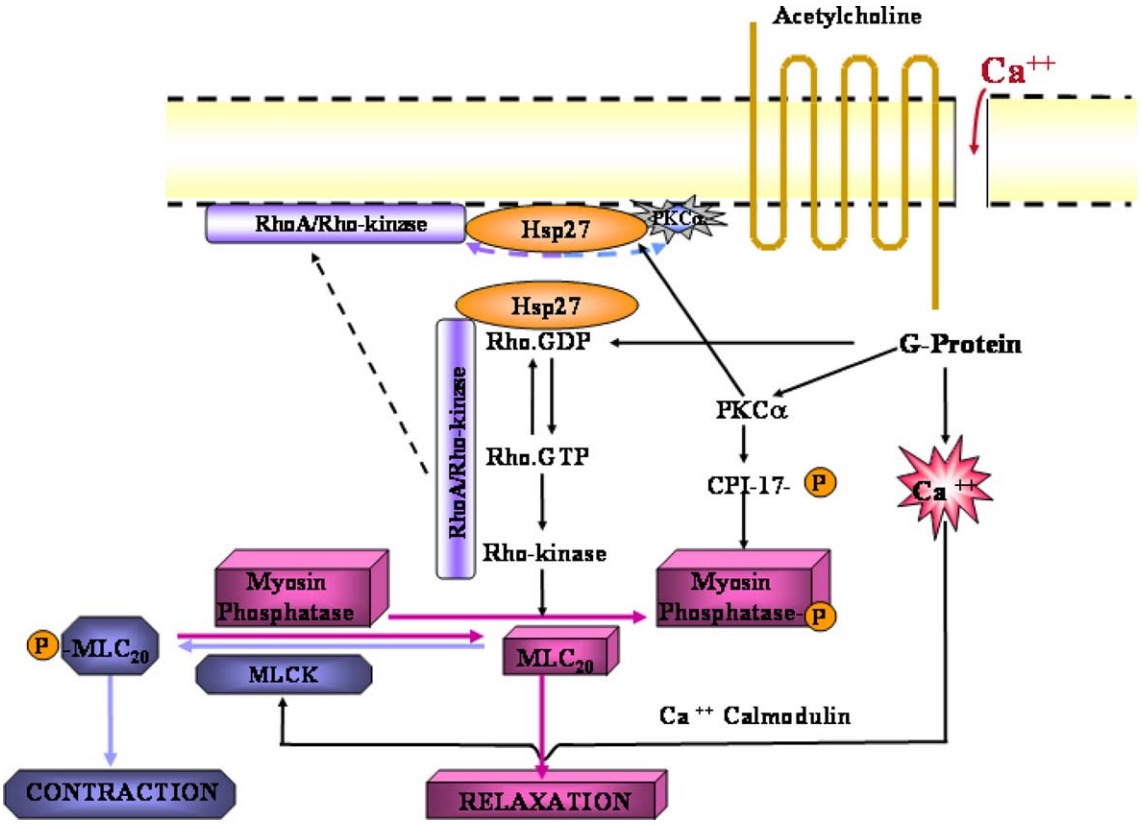

Fig. 7. Proposed mechanism of signaling during acetylcholine-induced contraction of rabbit colon smooth muscle. In acetylcholine-induced smooth muscle contraction, PKCα and RhoA/Rho-kinase appear to work simultaneously to phosphorylate myosin light chain phosphatase (MLCP). HSP27 coordinates the association and translocation of RhoA/Rho-kinase (ROCK-II), and of PKCα. Although, MLCK and other kinase(s) phosphorylate MLC directly, it is possible that myosin phosphatase de-phosphorylates the phosphorylated MLC. In a parallel mechanism, PKCα phosphorylates CPI-17 which in turn enhances its ability to phosphorylate MLCP inhibiting its activity and thus maintains the phosphorylated state of MLC_{20}. Rho activated ROCK-II also inhibits myosin phosphatase activity and thus helps MLC to maintain a phosphorylated state. HSP27, together with Rho and ROCK-II, translocates to the membrane and forms a complex thus helping for activation of ROCK-II for its activation and sustains the contraction of the smooth muscle cell.

utilizing a guanine nucleotide-binding and -hydrolyzing cycle [21,22]. The evidences indicate that Rho regulates the cytoskeletal system, particularly actin-dependent functions, such as cell motility [23], formation of stress fibers and focal adhesions [24], and smooth muscle contraction [25,26]. We have also shown that transfection of negative dominant RhoA inhibited translocation of HSp27 using confocal microscopy [6]. However, the mode of action of RhoA in reorganization of the cytoskeleton has not been clearly defined. The most intensively studied RhoA targets are two highly related Ser/Thr kinases, ROCK-I and ROCK-II, which are involved in regulating actomyosin-based contractility via increased phosphorylation of MLC_{20} [27–30]. In addition to regulating MLC_{20}, ROCK can phosphorylate a number of other proteins that may be relevant to the function of RhoA in regulating cell morphology. These include α-adducin, a protein that promotes the binding of spectrin to actin filaments; glial fibrillary acidic protein (GFAP), an intermediate filament protein; the Na^+/H^+ exchanger NHE1, which has been implicated in stress-fiber formation; and ezrin/radixin/moesin (ERM) proteins [31]. It is intriguing to note that these proteins (α-adducin and ERM) are also substrates for myosin binding subunit (MBS). Therefore, ROCK can directly phosphorylate these substrates as well.

The first implication that the RhoA/ROCK-II pathway may be involved in the smooth muscle contraction arose from the use of selective inhibitors of ROCK-II. Ueheta et al. [9] developed a series of potent relaxants of vascular and bronchial smooth muscles, of which, Y-27632 is a representative. Similar relaxant effects in other smooth muscle tissue have been reported, including inhibition of angiotensinII-induced contraction of rat internal anal sphincter (IAS) [32], inhibition of thromboxane analogue-induced contraction of bovine arterial muscle [33]. Previous studies from our laboratory have shown that RhoA plays a role in cytoskeletal reorganization of rabbit colon smooth muscle cells [6,8]. Present results suggest that Y-27632 inhibited acetylcholine-induced MLC phosphorylation and contraction (Fig. 1) suggesting a role for ROCK-II in the acetyl-

choline-induced contraction in rabbit colon smooth muscle cells.

The precise mechanism by which MLCK cascades and RhoA/ROCK-II pathways independently cause MLC_{20} phosphorylation and contraction remains to be determined. A model has been proposed in which RhoA regulates MLC_{20} phosphorylation through its effectors, Rho-associated kinase (Rho-kinase ROCK-II), and MBS [26,34,35]. GTP-bound Rho interacts with both Rho-kinase and myosin binding subunit (MBS) of myosin phosphatase, resulting in activation of Rho-kinase. Activation of ROCK-II by RhoA phosphorylates MBS, thereby inactivating myosin phosphatase [35]. Constitutively active RhoA induces contraction of vascular smooth muscle cells and MLC phosphorylation in the absence of MLCK activity [36]. Recently, Murthy et al. [2] reported that MLCK activity declines within about 1 min after stimulation with acetylcholine. Whether MLC phosphorylation and sustained contraction can completely be achieved in the absence of MLCK thus remains debatable. However, data suggest that ROCK-II appears to regulate MLC_{20} phosphorylation downstream of RhoA in non-muscle as well as in muscle cells [35]. Therefore, the mechanism by which RhoA activates ROCK-II and helps in sustained contraction remains to be elucidated.

The present results indicate that Y-27632 inhibited acetylcholine-induced translocation of ROCK-II to the particulate fraction. Our results also suggest that there is increased association of ROCK-II with RhoA in the particulate fraction of smooth muscle cells treated with acetylcholine for 30 s or 4 min. Independent approaches have shown that activated RhoA translocates to the plasma membrane [37–39]. We have previously shown that RhoA translocates and associates with HSP27 in the particulate fraction upon stimulation with contractile agonists [8]. Recently, we have shown that there was a direct association of RhoA with HSP27 and that there is increased association and translocation of RhoA with phosphorylated HSP27 [7]. In the present studies we have shown that acetylcholine-induced increases in the translocation of ROCK-II and its association with RhoA. There is a concomitant decrease of ROCK-II in the cytosolic fraction indicating the translocation of ROCK-II to the membrane. Immunocytochemical appraoches have shown that ROCK-II translocates to the membrane upon stimulation with contractile agonists [38]. Association and translocation of RhoA with ROCK-II is suggestive of the fact that ROCK-II is activated by RhoA at the plasma membrane [40,41]. Furthermore, MYPT1 has also been shown to translocate to membrane in vascular smooth muscle cells treated with PGF2a and the contraction remained sustained [42]. Shin et al. [42] also showed that Y27632 inhibited the translocation of both ROK and MYPT1 to the membrane. Thus, it is apparent that translocation of ROCK to the membrane is crucial in sustenance of the contraction. Our present results suggest that Y-27632 inhibits acetylcholine-induced increases in the association of HSP27 with ROCK-II and with RhoA. This suggests that the translocation and association of RhoA with ROCK-II may be mediated by HSP27. Therefore, it appears that maintenance of sustained MLC phosphorylation is mediated by activation of ROCK-II and its association with RhoA. Maintenance of the functional association of RhoA with ROCK-II in the particulate fraction mediated by HSP27 appears to be important to retain MLC in the phosphorylated state and the sustained contraction (Fig. 7). We have recently shown that smooth muscle cells transfected with non-phosphorylated form of HSP27 failed to exhibit sustained contraction and also that the translocation of RhoA was diminished in these cells. Thus, the present results also imply that HSP27-mediated association and translocation of RhoA with ROCK-II are crucial in maintenance of sustained contraction.

Acknowledgments

This study was supported by NIH Grant 2 RO1 DK-42876. We thank Dana Thomas and Kelly Smid for their assistance with technical editing and figure preparation.

References

[1] M. Walsh, The Ayerst Award Lecture 1990. Calcium-dependent mechanisms of regulation of smooth muscle contraction, Biochem. Cell Biol. 69 (1991) 771–800.

[2] K.S. Murthy, H. Zhou, J.R. Grider, D.L. Brautigan, M. Eto, G.M. Makhlouf, Differential signalling by muscarinic receptors in smooth muscle: m2-mediated inactivation of myosin light chain kinase via Gi3, Cdc42/Rac1 and p21-activated kinase 1 pathway, and m3-mediated MLC20 (20 kDa regulatory light chain of myosin II) phosphorylation via Rho-associated kinase/myosin phosphatase targeting subunit 1 and protein kinase C/CPI-17 pathway, Biochem. J. 374 (2003) 145–155.

[3] Y. Fukata, M. Amano, K. Kaibuchi, Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells, Trends Pharmacol. Sci. 22 (2001) 32–39.

[4] D.J. Hartshorne, M. Ito, F. Erdodi, Myosin light chain phosphatase: subunit composition interactions and regulation, J. Muscle Res. Cell Motil. 19 (1998) 325–341.

[5] M. Eto, L. Wong, M. Yazawa, D.L. Brautigan, Inhibition of myosin/moesin phosphatase by expression of the phosphoinhibitor protein CPI-17 alters microfilament organization and retards cell spreading, Cell Motil. Cytoskeleton 46 (2000) 222–234.

[6] P. Wang, K.N. Bitar, Rho A regulates sustained smooth muscle contraction through cytoskeletal reorganization of Hsp27, Am. J. Physiol. 275 (1998) G1454–G1462.

[7] S.B. Patil, M.D. Pawar, K.N. Bitar, Phosphorylated HSP27 essential for acetylcholine-induced association of RhoA with PKCa, Am. J. Physiol. 286 (2004) G635–G644.

[8] K.N. Bitar, A. Ibitayo, S.B. Patil, HSP27 modulates agonist-induced association of translocated RhoA and PKC-alpha in muscle cells of the colon, J. Appl. Physiol. 92 (2002) 41–49.

[9] M. Ueheta, T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, S. Narumiya, Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension, Nature 389 (1997) 990–994.
[10] K.N. Bitar, M.S. Kaminski, N. Hailat, K.B. Cease, J.R. Strahler, Hsp27 is a mediator of sustained smooth muscle contraction in response to bombesin, Biochem. Biophys. Res. Commun. 181 (1991) 1192–1200.
[11] A.I. Ibitayo, J. Sladick, S. Tuteja, O. Louis-Jacques, H. Yamada, G. Groblewski, M. Welsh, K.N. Bitar, Hsp27 in signal transduction and association with contractile proteins in smooth muscle cells, Am. J. Physiol. 277 (1999) G445–G454.
[12] K.N. Bitar, HSP27 phosphorylation and interaction with actin-myosin in smooth muscle contraction, Am. J. Physiol. 282 (2002) G894–G903.
[13] K.N. Bitar, C. Hillemeier, P. Biancani, K.J. Balazovich, Differential regulation of smooth muscle contraction in rabbit internal anal sphincter by substance P and bombesin, Life Sci. 47 (1990) 2429–2434.
[14] K.N. Bitar, C. Hillemeier, P. Biancani, K.J. Balazovich, Regulation of smooth muscle contraction in rabbit internal anal sphincter by protein kinase C and Ins(1,4,5)P_3, Am. J. Physiol. 260 (1991) G537–G542.
[15] A.I. Ibitayo, Y. Tsunoda, F. Nozu, C. Owyang, K.N. Bitar, Src kinase and PI 3-kinase as a transduction pathway in ceramide-induced contraction of colonic smooth muscle, Am. J. Physiol. 38 (1998) G705–G711.
[16] L. Li, M. Eto, M.R. Lee, F. Morita, M. Yazawa, T. Kitazawa, Possible involvement of the novel CP1-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle, J. Physiol. 508 (1998) 871–881.
[17] M.C. Gong, A. Fuglsang, D. Alessi, S. Kobayashi, P. Cohen, A.V. Somlyo, A.P. Somlyo, Arachidonic acid inhibits myosin light chain phosphatase and sensitizes smooth muscle to calcium, J. Biol. Chem. 267 (1992) 21492–21498.
[18] S. Narumiya, A. Sekine, M. Fujiwara, Substrate for botulinum ADP-ribosyltransferase, Gb, has an amino acid sequence homologous to a putative rho gene product, J. Biol. Chem. 263 (1988) 17255–17257.
[19] A. Hall, Small GTP-binding proteins and the regulation of the actin cytoskeleton, Ann. Rev. Cell Biol. 10 (1994) 31–54.
[20] Y. Takai, T. Sasaki, K. Tanaka, H. Nakanishi, Rho as a regulator of the cytoskeleton, Trends Biochem. Sci. 20 (1995) 227–231.
[21] H.R. Bourne, D.A. Sanders, F. Mccormick, The GTPase superfamily: conserved structure and molecular mechanism, Nature 349 (1991) 117–127.
[22] A. Hall, The cellular functions of small GTP-binding proteins, Science 249 (1990) 635–640.
[23] M.J. Stasia, A. Jouan, N. Bourmeyster, P. Boquet, P.V. Vignais, ADP-ribosylation of a small size GTP-binding protein in bovine neutrophils by the C3 exoenzyme of Clostridium botulinum and effect on the cell motility, Biochem. Biophys. Res. Commun. 180 (1991) 615–622.
[24] H.F. Paterson, A.J. Self, M.D. Garrett, I. Just, K. Aktories, A. Hall, Microinjection of recombinant p21Rho induces rapid changes in cell morphology, J. Cell Biol. 111 (1990) 1001–1007.
[25] K. Hirata, A. Kikuchi, T. Sasaki, S. Kuroda, K. Kaibuchi, Y. Matsuura, H. Seki, K. Saida, Y. Takai, Involvement of Rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction, J. Biol. Chem. 267 (1992) 8719–8722.
[26] A.P. Somlyo, A.V. Somlyo, Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II, J. Physiol. 522 (2000) 177–185.
[27] T. Leung, X.Q. Chen, E. Manser, L. Lim, The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton, Mol. Cell. Biol. 16 (1996) 5313–5327.
[28] K. Kimura, M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, K. Kaibuchi, Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase) [see comment], Science 273 (1996) 245–248.
[29] M. Amano, Y. Fukata, H. Shimokawa, K. Kaibuchi, Purification and in vitro activity of Rho-associated kinase, Methods Enzymol. 325 (2000) 149–155.
[30] M. Amano, M. Ito, K. Kimura, Y. Fukata, K. Chihara, Phosphorylation and activation of myosin by Rho-associated kinase, J. Biol. Chem. 271 (1996) 20246–20249.
[31] A. Hall, GTPases, Oxford University Press, New York, 2000.
[32] S. Rattan, R.N. Puri, Y. Fan, Involvement of Rho and Rho-associated kinase in sphincteric smooth muscle contraction by angiotensin II, Exp. Biol. Med. (2003) 972–981.
[33] Y. Maeda, K. Hirano, J. Nishimura, T. Sasaki, H. Kanaide, Rho-kinase inhibitor inhibits both myosin phosphorylation-dependent and -independent enhancement of myofilament Ca^{2+} sensitivity in the bovine middle cerebral artery, Br. J. Pharmacol. 140 (2003) 871–880.
[34] M. Amano, K. Chihara, N. Nakamura, Y. Fukata, T. Yano, Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase, Genes Cells 3 (1998) 177–188.
[35] K. Kimura, M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, K. Kaibuchi, Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase), Science 273 (1996) 245–248.
[36] Y. Kureishi, S. Kobayashi, M. Amano, K. Kimura, H. Kanaide, T. Nakano, K. Kaibuchi, M. Ito, Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation, J. Biol. Chem. 272 (1997) 12257–12260.
[37] M.C. Gong, H. Fujihara, A.V. Somlyo, A.P. Somlyo, Translocation of rhoA associated with Ca^{2+} sensitization of smooth muscle, J. Biol. Chem. 272 (1997) 10704–10709.
[38] M.J. Taggart, Y.H. Lee, K.G. Morgan, Cellular redistribution of PKCalpha, rhoA, and ROKalpha following smooth muscle agonist stimulation, Exp. Cell Res. 251 (1999) 92–101.
[39] K. Miyazaki, T. Yano, D.J. Schmidt, T. Tokui, M. Shibata, L.M. Lifshitz, S. Kimura, R.A. Tuft, M. Ikebe, Rho-dependent agonist-induced spatio-temporal change in myosin phosphorylation in smooth muscle cells, J. Biol. Chem. 277 (2002) 725–734.
[40] K. Sward, M. Mita, D.P. Wilson, J.T. Deng, M. Susnjar, M.P. Walsh, The role of RhoA and Rho-associated kinase in vascular smooth muscle contraction, Curr. Hypertens. Rep. 5 (2003) 66–72.
[41] A.P. Somlyo, A.V. Somlyo, Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase, Physiol. Rev. 83 (2003) 1325–1358.
[42] H.M. Shin, H.D. Je, C. Gallant, T.C. Tao, D.J. Hartshorne, M. Ito, K.G. Morgan, Differential association and localization of myosin phosphatase subunits during agonist-induced signal transduction in smooth muscle [see comment], Circ. Res. 90 (2002) 546–553.