Full Paper

Myosin Light Chain Kinase / Actin Interaction in Phorbol Dibutyrate–Stimulated Smooth Muscle Cells

Sean E. Thatcher¹, Mike E. Fultz², Hideyuki Tanaka³, Haruo Hagiwara³, Hou-Li Zhang^{4,6}, Ying Zhang^{4,6}, Kohichi Hayakawa⁴, Shinji Yoshiyama⁴, Akio Nakamura⁴, Hong Hui Wang⁴, Takeshi Katayama⁴, Masaru Watanabe⁵, Yuan Lin⁶, Gary L. Wright¹, and Kazuhiro Kohama^{1,4,7,*}

¹Department of Pharmacology, Physiology, and Toxicology, The Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25704, USA

²Department of Biology, Morehead State University, Morehead, KY 40351, USA

³Department of Health Sciences, Gunma University, Maebashi, Gunma 371-8511, Japan

⁴Department of Molecular and Cellular Pharmacology, Gunma University, Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

⁵Department of Frontier Health Sciences, Graduate School of Human Health Sciences, Tokyo Metropolitan University, Tokyo 116-8551, Japan

⁶Department of Pharmacology, Dalian Medical University, Dalian, 116-044, PR China

⁷Cell Differentiation and Development Center, Marshall University, Huntington, WV 25755-1090, USA

Received November 21, 2010; Accepted March 24, 2011

Abstract. Previous work has suggested that in addition to its kinase activity, myosin light chain kinase (MLCK) exhibits non-kinase properties within its N-terminus that could influence cytoskeletal organization of smooth muscle cells (A. Nakamura et al. *Biochem Biophys Res Commun.* 2008;369:135–143). Myosin ATPase activity measurements indicate that the 26–41 peptide of MLCK significantly decreases ATPase activity as the concentration of this peptide increases. Sliding velocity of actin-filaments on myosin and stress responses in skinned smooth muscle tissue are also inhibited. Peptide-mediated uptake and the microinjection technique in cells indicate that the peptide was necessary for actin-filament stabilization. Fluorescence resonance energy transfer analysis indicated that in the presence of MLCK, α -actin but not β -actin remodeled during phorbol 12,13-dibutyrate (PDBu)-induced contractions. PDBu also induced podosomes in the cell. When MLCK expression was down-regulated by introduction of RNAi for MLCK by lentivirus vector into the cells, we failed to observe the podosome induction upon PDBu stimulation. Rescue experiments indicate that the non-kinase activity of MLCK plays an important role in maintaining actin stress fibers and in the PDBu-induced reorganization of actin-filaments in smooth muscle cells.

Keywords: actin-remodeling, podosome, phorbol 12,13-dibutyrate (PDBu), FRET analysis, myosin light chain kinase (MLCK)-deficient cell

Introduction

Regulation of smooth muscle contraction is a major subject of pharmacological investigations. The well-established pathway of regulation is initiated by increases in intracellular Ca^{2+} [Ca^{2+}]_i (see ref. 1, for review). How-

ever, we know of examples in which contraction is not subject to [Ca^{2+}]_i increase regulation (ref. 2, for review), such as the contraction induced by phorbol 12,13-dibutyrate (PDBu) (3).

Myosin light chain kinase (MLCK), a well-established regulatory protein for smooth muscle contraction (for a review, see ref. 1), is a serine/threonine kinase important in the regulation of smooth muscle contraction, and isoforms of MLCK have been identified (for a review, see ref. 4). The larger 210-kDa isoform is thought to serve primarily in cytokinesis and cell division and interacts

*Corresponding author (affiliation #4).

kohamak@med.gunma-u.ac.jp

Published online in J-STAGE on May 3, 2011 (in advance)

doi: 10.1254/jphs.10296FP

not only with actin but with microtubules as well (5). Down-regulation of the smaller isoform has suggested that this kinase is directly involved in smooth muscle contraction (6, 7). In the presence of the Ca^{2+} /calmodulin (CaM) complex, MLCK phosphorylates the serine-19 residue of the 20-kDa myosin light chain (MLC), activating myosin ATPase activity with subsequent force development (8). In addition to its site of kinase activity located in the central region of the molecule, MLCK has multiple actin binding sites at its N-terminal (9) and myosin binding activity at its C-terminal (10). Although research interest has centered on the kinase properties of MLCK, there are some evidences to suggest the enzyme is a multifunctional protein. For example, it is proposed that when myosin is fully phosphorylated, MLCK exerts an inhibitory effect through its actin-binding domain and that MLCK binding of myosin could contribute to sustained force maintenance in smooth muscle (11).

Podosomes are abundantly formed in several cell types in culture including smooth muscle (for a review, see ref. 12). Smooth muscle cells can form podosomes in response to PDBu stimulation which is thought to play an important role in the PDBu-stimulated contraction (13). The structure of the podosome of smooth muscle cells is determined by actin-polymerization that can form rings with a wide inner cavity. They contain actin-binding proteins of myosin II, tropomyosin, and calponin (for a review, see ref. 14). However, we do not know if MLCK is a component of the podosome.

Several isoforms of actin have been identified in the smooth muscle cell and based on their mobility in isoelectric focusing have been classified as α -, β -, and γ -actins. α -Actin is the predominant muscle actin isoform (15, 16). We previously reported that remodeling of these actin isoforms was remarkably different in response to PDBu-stimulation. α -Actin was transported to podosomes, with loss of the isoform from stress fibers. By comparison, remodeling of β -actin was less obvious (17).

In the present study, we examined the role of MLCK in α -actin remodeling using a variety of experimental methods. We evaluated the influence of the MLCK N-terminal, actin-binding site on actin cytoskeletal structure in resting and contracted smooth muscle cells. We then utilized MLCK RNA interference (RNAi) to down-regulate the expression of MLCK, a mutant that does not form podosomes during PDBu-stimulation. Podosome formation was rescued by the transfections of various constructs of mutant MLCK cDNA. The results suggest that the actin binding properties of MLCK play an important role in determining actin cytoskeletal organization.

Materials and Methods

Biochemical procedures

Myosin (myosin II isoform) was extracted from chicken gizzard and purified according to the method of Ebashi (18). Actin was extracted from the acetone powder of chicken skeletal muscle and purified using the method of Spudich and Watt (19) with slight modifications (20) and was used as actin-filament after polymerization. MLCK was purified from chicken gizzard by the method of Adelstein and Klee (21) with slight modification (22). CaM of bovine brain was purchased from Sigma-Aldrich (St. Louis, MO, USA). After the purification, myosin was unphosphorylated and analyzed by urea-glycerol polyacrylamide gel electrophoresis (PAGE), which was originally described by Perrie and Perry (23) and modified by Kishi et al. (6). Part of the myosin was phosphorylated by incubation with MLCK in the presence of Ca^{2+} /CaM for 10 min at 25°C as described by Okagaki et al. (24) and used as phosphorylated myosin. We checked the purity (> 95%) of actin and myosin by SDS-PAGE using the method of Laemmli (25) with slight modifications (26). The actin-activated ATPase activity was assayed at 25°C for 10 min with the unphosphorylated and phosphorylated myosins in the presence of actin-filament at 2 μM as converted into the concentration of monomeric actin and various concentrations of the 26–41 peptide (see below). The reaction was terminated by adding trichloroacetic acid, and the liberated phosphate was quantified by the malachite green method of Kodama et al. (27). The effect of the 26–41 peptide on the ATP-dependent movement of actin-filaments was analyzed by means of an *in vitro* motility assay (24). In short, movement of 3 nM actin-filaments labeled with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) was observed under a fluorescence microscope equipped with a video camera and a recorder. The velocity of the movement of each actin-filament was determined using MT rack 2 plug-in of WCIF Image J. Protein concentrations were determined with bovine serum albumin as the standard using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Tissue contraction

Contraction of skinned smooth muscle was measured as described previously (28). In short, taenia caeci of guinea pig were isolated and a small muscle layer strip was connected to a force transducer (BG-10; Kulite Semiconductor Products, Leonia, NJ, USA) and was subjected to the skinning procedures with 0.2% Triton X-100. The contraction was induced in a bubble-plate system consisting of 9 wells at $25.0 \pm 1.0^\circ\text{C}$. The wells

contained artificial intracellular solution containing 0 – 500 μM 26 – 41 peptide. The ionic strength of the solution was kept at 200 mM by adding potassium methanesulfonate, and the solution was adjusted with 20 mM 1,4-piperazine-bis(2-ethanesulphonic acid) (PIPES) in potassium hydroxide to achieve a pH of 7.0. In addition, the solution contained 0.85 mM Mg^{2+} , 2.1 mM $\text{Mg}(\text{methanesulfonate})_2$, 1 mM MgATPNa_2 (1.35 mM total ATP), 2 mM creatine phosphate Na_2 , 10 mM Ca-EGTA buffer, and 1 μM CaM. Ca^{2+} concentrations were calculated using an apparent dissociation constant of $10^{6.4} \text{ M}^{-1}$. When the active tension increased to a maximal steady state, the preparation was fixed by immersing in ice cold trichloroacetic acid (TCA) at 10% containing 10 mM dithioethiol (DTT). At the end of the measurement, the preparation was subjected to urea-glycerol PAGE (see above) and Western blots (see below) were performed as described to evaluate myosin phosphorylation.

Cell culture of vascular smooth muscle cells

A7r5 cells derived from embryonic rat thoracic aorta and purchased from ATCC (Manassas, VA, USA) and Gba-SM4 cells derived from the brain basal artery of guinea pig (29) were utilized. Cells were plated on 75 cm^2 flasks and grown to approximately 85% confluence at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Sigma, St. Louis, MO, USA). Medium was changed every other day, and cells were passaged at least once a week. Passaging of cells was accomplished by addition of a trypsin/EDTA solution (Invitrogen) in phosphate-buffered saline (PBS), and cells were collected by centrifugation.

Immunocytochemistry

Cells were seeded on ethanol-flamed 22 $\times$ 22 mm coverslips (Fisher Inc., Rockford, IL, USA) and allowed to grow for 24 – 48 h. Cells were stimulated with PDBu (Sigma) for a period of 30 min. After stimulation, cells were fixed and permeabilized with ice-cold acetone for a period of 1 min. Cells were then washed 3 times with PBS / 0.5% Tween-20 (PBS-T), pH 7.5, followed by incubation in blocking solution containing 5% nonfat dry milk in PBS for 1 h. MLCK staining was accomplished by incubation of cells in a 1:100 dilution of monoclonal MLCK clone K36 (Sigma), overnight at 4°C. Subsequently, cells were rinsed 3 times in PBS-T followed by an Alexa 488 anti-mouse IgG secondary antibody (Molecular Probes) at a 1:100 dilution for 1 h. Cells were also stained for actin by incubation with α -actin clone

1A4 or β -actin clone AC-15 for 1 h at a 1:100 dilution (Sigma). Cells were rinsed 3 times in PBS-T followed by an Alexa 568 anti-mouse IgG secondary antibody (Molecular Probes) at a 1:100 dilution for 1 hour. Finally, cells were rinsed 3 times in PBS-T and mounted in Gel Mount medium (Biomedica Inc., Foster City, CA, USA).

Effects of MLCK N-terminus peptides on cultured smooth muscle cells

We examined the effects of peptides containing the N-terminal actin binding site of MLCK on actin cytoskeletal structure in unstimulated A7r5 cells. Peptides containing the 1 – 25 (NH_2 -MDFRANLQRQVKPKTLSEERKVVH-G-COOH), 26 – 41 (NH_2 -PQQVDFRSVLAKKGTP-COOH), and 1 – 41 N-terminal amino acid sequences of MLCK were conjugated to a peptide (NH_2 -CRQIKIWFQNRRMKWKK-COOH) derived from *Drosophila* antennapedia homeodomain protein, which has been shown to facilitate cellular uptake of peptides (30). The conjugated peptides were dissolved in DMEM at a concentration of 2.0 mg/mL (375 μM for 1 – 25 peptide shown in Fig. 4 a, b; 487 μM for 26 – 41 peptide shown in Fig. 4 c, d; and 282 μM for 1 – 41 peptide shown in Fig. 4 e, f) and added to the cells for 30 min. The cells were then rinsed and fixed with ice cold acetone for immunostaining and imaging. In a second study, the MLCK peptides were introduced directly into the cell by microinjection. Cells were injected with a Pneumatic Pico Pump (World Precision Instruments, Sarasota, FL, USA) at a pressure setting of 5 and time constant of 2 s using glass pipettes pulled with a Flaming Brown Micropipette Puller (total volume injected was 10 – 50 nL) (Sutter Instruments Co., Novato, CA, USA) according to the manufacturer's protocol. Peptide was dissolved (25 mg/mL, 3.5 mM) in 200 mM K^+ injection buffer (10 mM Alexa 594) while the control cells received vehicle only. Following injection, cells were returned to the incubator for a 2-h recovery period prior to fixation and staining.

Confocal/FRET microscopy

Immunostained cells were mounted on a Nikon Diaphot microscope (Nikon, Tokyo) and confocal microscopy was performed with a BioRad Model 1024 (Bio-Rad Laboratories, Richmond, CA, USA) laser confocal microscope equipped with a krypton/argon laser. For FRET analysis, we used the BioRad Model 1024 confocal microscope. MLCK was labeled with Alexa 488 and served as the donor component of the system. α -Actin and β -actin were labeled with Alexa 568 and served as the acceptor molecules. The donor molecule of MLCK was directly excited and the resulting emission was obtained with a 522DF32 bandpass filter. However, a portion of the energy of emission was transferred to neighboring

Alexa 568 fluorophore, resulting in emission that was captured on a second channel with a HQ598/40 bandpass filter. Subsequently, the sample was excited at the 568-nm laser line at 100% power to photobleach the acceptor of the α - or β -actin molecule and a second image of the cell was acquired again at the 488-nm laser line with the multichannel set to obtain MLCK fluorescence (522DF32) and to verify the absence of acceptor label 568 emission (HQ598/40). An intensity profile was generated for each image (Image J Software, NIH, Bethesda, MD, USA) and the resulting plot was analyzed with Peakfit V4.11 software (SPSS Science, Chicago, IL, USA) to obtain the area under the curve. The values were then used to calculate the increase in fluorescence intensity after photobleaching. Because resonance energy transfer can only occur if the donor and acceptor molecules are sufficiently close to one another for efficient transfer, the resulting values were analyzed by comparing the treated cells with the control cells as an index of the association between MLCK and either α - or β -actin. To evaluate the responsiveness of the FRET system as presently employed, a series of control experiments were performed. As a positive control, cells were incubated with either α - or β -actin specific antibodies labeled with both donor and acceptor fluorophores. In these experiments, it was expected that the increased availability of closely associated binding sites would result in a significant increase in the FRET effect compared to the MLCK/actin evaluations. As a negative control, FRET analysis was performed on the association of α - and β -actin with the α -subunit of the L-type calcium channel protein located in the plasma membrane. In this case, it was expected that the FRET effect would be negligible. In addition, FRET analysis of MLCK/actin was conducted in the absence of acceptor fluorophore or donor fluorophore to evaluate the effects of autofluorescence and signal bleed-through on the results. Inclusion of nuclear staining of MLCK in the A7r5 cell did not affect the significance of the results presented herein (data not shown). The quantification by FRET using this experimental protocol was described previously (31, 32).

Time lapse phase-contrast microscopy

A7r5 cells were seeded in 35-mm culture dishes. Cells were rinsed and incubated with CO₂-Independent DMEM (Invitrogen) supplemented with 1X GlutaMax-I (Invitrogen), pen-strep, and 10% FCS. Culture dishes were placed in a DH-35 Culture Dish Heater attached to a TC-324B Heat Controller (Warner Instruments, Hamden, CT, USA) and incubated at 37°C. Cells were then treated with PDBu with contractions monitored every 10 min for a total time of 140 min. Phase-contrast images were obtained with a 10 $\times$ objective and a Nikon D70 SLR

camera attached to a microscope.

Down-regulation of endogenous MLCK and over-expression of kinase-dead MLCKs

Lentiviral siRNA silencing of endogenous MLCK named RNAi was generated according to the protocol published recently (33). Gba-SM-4 cells were incubated with virus-containing condition media (titer = 2.8×10^5 transducing unit/mL) supplemented with polybrene (6 μ g/mL) for 14 days. After the blasticidin-resistant colonies were isolated and expanded, the expressions of MLCK isoforms were evaluated by Western blot analysis. The Gba-SM-4 stable cell lines constitutively expressing RNAi for guinea-pig MLCK, and thus lacking the expression of both isoforms MLCK, were used as the MLCK-deficient cells (for the silencing mechanism, see Fig. 4 of ref. 33). For over-expression of kinase-deficient MLCK cells, we constructed a wild-type (WT) plasmid using the full length bovine stomach cDNA, pUC 118/BS 155K, as previously described (33). The kinase inactive-MLCK named dATP was generated by the mutagenesis of the ATP-binding site. We then deleted the N-terminal 1 – 41 sequence from the kinase-inactive MLCK, which was named dNdATP. Transfection of these constructs into RNAi cells was performed using Nucleofector (Lonza, Basel, Switzerland). Because the codon usage of the MLCK target sequence is different, these exogenous MLCKs of bovine stomach expressed in Gba-SM-4 cells do not become a cellular target for the siRNA against endogenous guinea-pig MLCK.

Statistics

Differences between treatment groups were evaluated by Student's *t*-tests using Sigma Stat V.2.03 (SPSS Science, Inc.). MLC phosphorylation was evaluated by a One-Way ANOVA followed by a Student-Newman-Keuls test. Differences were considered significant at $P < 0.05$ or better.

Results

The N-terminal 26 – 41 peptide of MLCK was synthesized and incubated at various concentrations in the presence of unphosphorylated or phosphorylated myosin (Fig. 1). ATPase activity measurements indicated a significant 65% decrease in the activity for phosphorylated myosin with the 26 – 41 peptide of MLCK. The inhibitory effect on the activity for unphosphorylated myosin was significantly less (approximately 20%). These data indicate that the 26 – 41 peptide of MLCK can inhibit myosin ATPase activity preferentially for the phosphorylated myosin. In order to understand the effect of the 26 – 41 peptide on the myosin-actin interaction, actin-

Fig. 1. Effect of 26–41 peptide on actin-activated ATPase activity. The peptide was synthesized from the Pro²⁶–Pro⁴¹ sequence of chicken smooth muscle MLCK. The ATPase activities of smooth muscle myosin in the phosphorylated (filled symbols) and unphosphorylated (open symbols) forms were determined in the presence of various concentrations of the peptide plotted on the abscissa. The assays were conducted in 60 mM KCl, 5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM ATP, and 20 mM Tris-HCl (pH 7.5) at 25°C for 10 min. Values are expressed as the mean $\pm$ S.D. * P < 0.05 and ** P < 0.001.

filaments labeled by rhodamin-phalloidin was co-incubated with phosphorylated myosin and the ATP-dependent sliding velocity of the filaments was measured. The 26–41 peptide created a leftward shift causing an inability for myosin–actin interaction (Fig. 2). The 26–41 peptide (10 μ M) significantly decreased sliding velocity, and this effect was enhanced as the concentration of the peptide increased. These data indicate that the 26–41 peptide of MLCK has an important role in actin-filament binding and myosin ATPase activity.

In order to understand the influence of the 26–41 peptide on the contraction of smooth muscle, taenia coli in which the membrane was destroyed was connected to a strain gauge and contracted with various concentrations of calcium in the absence or presence of the 26–41 peptide (Fig. 3). At basal calcium levels (pCa approximately 8) there was no effect with the peptide at various concentrations. However, with calcium increase (pCa approximately 4), there was an observable decrease in tension production at 500 μ M of the peptide (Fig. 3).

Fig. 2. Inhibition of 26–41 peptide on velocity of the actin-filament sliding on smooth muscle myosin. Fluorescent-labeled actin-filament in the presence of the peptide at various concentrations was perfused through the flow chamber over a glass slide coated with phosphorylated smooth muscle myosin, and sliding velocities of actin-filaments were measured. The assay solution contains the peptide at various concentrations, 50 mM KCl, 4 mM MgCl₂, 1 mM ATP, 0.1 mM EGTA, 25 mM dithiothreitol, and 30 mM Tris-HCl (pH 7.5). Glucose oxidase (0.2 mg/mL), catalase (0.04 mg/mL), and glucose (4.5 mg/mL) were added to the above solution to prevent photobleaching of rhodamine. A–F: sliding velocities of actin-filaments (n = 100). Lines, actual distribution of actin-filaments in the absence of the peptide (A) and in the presence of 10 μ M peptide (B), 50 μ M peptide (C), 100 μ M peptide (D), 200 μ M peptide (E), or 500 μ M peptide (F). G) Velocities of actin-filament movement derived from panels A–F. Values are means $\pm$ S.D. ** P < 0.001.

Fig. 3. Effect of 26–41 peptide on the contraction of the skinned fiber of smooth muscle. A strip of taenia caeci of guinea pig treated with Triton-X100 to permeabilize smooth muscle membrane was connected to the strain gage, and contraction was induced by increasing Ca^{2+} concentrations of the contraction solution. The peptide was mixed with the solution at various concentrations. The solution contained 200 mM K^+ , 0.85 mM Mg^{2+} , 1 mM MgATP, 2 mM creatine phosphate, 1 μM CaM, calcium-EGTA buffer of various concentrations, and PIPES buffer (pH 7.0). For adjusting constant K^+ concentration in the solution, see the text. Contraction data are normalized to results from the same cool preparations at $\text{pCa} = 5.7$. Inset: the effect of 500 μM 26–41 peptide on MLC phosphorylation, as detected by the urea-glycerol-PAGE followed by Western blots with the antibody against MLC.

MLC phosphorylation measured at basal and activated calcium levels showed little change from control levels even at the highest concentration of the 26–41 peptide (Fig. 3). These data suggest that the 26–41 peptide affects the tension development of smooth muscle cells. However, this effect is independent of MLC phosphorylation.

The effects of the 26–41 peptide on the actin-myosin interaction are variable according to the assay methods (Figs. 1–3), raising the question of whether it is effective in the living cell. To answer this question, we cultured A7r5 cells in the presence of the peptides of which entry into the cells was facilitated by the *Drosophila* antennapedia peptide. After uptake for 30 min, we fixed the cells and subjected them to confocal microscopy (Fig. 4). We found that those A7r5 cells incubated with either the 26–41 (c, d) or the 1–41 (e, f) peptide showed a marked loss of actin-fiber organization. However, we observed no effect of the 1–25 peptide (a, b) on actin organization, as shown in the control of the right panel. Microinjection of the 1–41 peptide was also performed and also found to cause dissolution of the actin fibers in the perinuclear region where the 1–41 peptide was localized (Fig. 4), supporting the results with the *Drosophila* antennapedia-linked peptides.

FRET is a powerful tool to quantitatively assess the interactions between two different proteins in cells (33). Therefore, the FRET interaction between MLCK and

Fig. 4. Effect of N-terminal peptides on the actin-cytoskeleton of A7r5 cells. Left panel: Peptide-induced uptake into A7r5 cells of 1–25 (a, b), 26–41 (c, d), and 1–41 (e, f) peptides of the N-terminus of MLCK. These peptides were conjugated to a peptide derived from *Drosophila* antennapedia homeodomain protein to facilitate uptake and incubated with cells for 30 min. An α -actin FITC antibody was used for visualization. Scale bar represents 40 μm . Right panel: Effects of the microinjected 1–41 peptides without antennapedia. Control cells were injected with vehicle + Alexa 594 tracer in the injection buffer solution, while experimental cells included the 1–41 peptide. The white bar represents 20 μm . In both panels, arrows represent areas where the actin dissolution occurred, and the experiments were performed in duplicate.

two different actin isoforms, α - and β -actin, was examined with unstimulated and PDBu-stimulated A7r5 smooth muscle cells. We performed positive and negative controls with our FRET technique and found that α/β -actin stained with both donor and acceptor fluorophores showed higher responses than our experimental groups (Table 1). Negative controls using the α -subunit of the L-type Ca^{2+} -channel protein indicated an abolishment of the FRET response (Table 1). Earlier work has shown that α - and β -actin remodel differently in the A7r5 cell under PDBu-stimulation (17). FRET interaction between MLCK and α/β -actin were not significantly different from one another under basal conditions, but there was a significant enhancement of the interaction between MLCK and α -actin under PDBu-stimulated conditions (Table 1, Fig. 5). The increase in the interaction was not present between MLCK and β -actin (Table 1) under PDBu-stimulated conditions. However, we found podosomes (13) in the periphery of the cells (Fig. 5). These data suggest that MLCK preferentially binds with α -actin in a PDBu-stimulated cell and that this interaction may be involved in podosome formation.

Regulatory proteins such as calponin stabilize podosome structure (for a review, see ref. 14). To examine the involvement of MLCK in the stabilization of podosomes, we used the Gba-SM-4 cultured smooth muscle cells, in

which MLCK was down-regulated by expressing its siRNA continuously. The results from these cells were then compared to those from rescue experiments in which the transient expression of MLCK cDNA was induced as indicated in Materials and Methods. Figure 6 shows the

Fig. 5. Capture of donor emission before and after acceptor photobleaching in untreated and PDBu (10^{-7} M)-stimulated A7r5 cells. α -Actin was the acceptor and MLCK was the donor. Note the appearance of fibers and increase in intensity of emission at podosomes after photobleaching, indicating significant quenching of donor emission by the acceptor. The white bar represents 20 μm .

Table 1. FRET values for % increase in donor fluorescence following photobleaching of acceptor molecules

% Increase in fluorescence	
a. Actin/MLCK	
Control cells	
α -actin (N = 20)	13.2 ± 1.3
β -actin (N = 20)	14.9 ± 1.4
PDBu-treated cells (10^{-7} M)	
α -actin (N = 20)	$24.7 \pm 1.8^*$
β -actin (N = 20)	12.9 ± 0.9
b. Positive control	
α -actin (N = 15)	82.5 ± 3.1
β -actin (N = 5)	43.9 ± 4.1
c. Negative control	
α -actin (N = 10)	-1.5 ± 5.2
β -actin (N = 10)	-3.0 ± 5.2

An asterisk indicates a significant difference between the control vs. PDBu-treated cells, $P < 0.05$. Values are means $\pm$ S.E.M. Note: a) Values indicating actin/MLCK association in control and PDBu-treated A7r5 cells. b) Positive control in which both donor and acceptor fluorophores were labeled to anti- α - or anti- β -actin antibodies. c) Negative control in which α - or β -actin interaction with a plasma membrane-bound calcium-channel protein was evaluated.

Fig. 6. Podosomes were induced by the various concentrations of PDBu in the control Gba-SM-4 cells (open circles). The filled circles were for a MLCK-deficient Gba-SM-4 cell line of which MLCK expression was silenced by RNAi as described in Materials and Methods (33). Both cell lines were stained by MLCK and α -actin antibodies. The podosome-positive cells were identified to contain ten or more bright dots and/or their assembly, i.e., rosettes according to the reviews (12, 14). One hundred cells were counted under each concentration.

Fig. 7. Rescue experiments using MLCK-deficient Gba-SM-4 cells. A) Untreated Gba-SM-4 cells stained for α -actin (green) and MLCK (red). Colocalization is depicted in the third panel in yellow. B) PDBu (10⁻⁵ M)-stimulated Gba-SM-4 cells. C) MLCK-deficient cells of which MLCK expression was silenced by RNAi, as described in the Fig. 6 legend. D) The cells in panel C were stimulated by 10⁻⁵ M PDBu. E) Silencing of MLCK followed by rescue with full length (WT) MLCK, i.e., MLCK-deficient cells that were rescued by WT-MLCK. F) The MLCK-deficient cells rescued by WT-MLCK were treated with 10⁻⁵ M PDBu. G) The MLCK-deficient cells rescued by the kinase-inactive mutant MLCK. H) The MLCK-deficient cells rescued by the kinase-inactive mutant MLCK were treated by 10⁻⁵ M PDBu. I) The MLCK-deficient cells rescued by the double mutant MLCK where both kinase activity and the N-terminal actin-binding site were lost. J) The MLCK-deficient cells rescued by the double mutant were treated by 10⁻⁵ M PDBu. Note that we could observe little, if any, podosomes. Circle graphs denote the percentage of the cells with podosomes after 10⁻⁵ M PDBu stimulation in blue. They were generated by counting about 100 cells under the criteria described in the legend to Fig. 6. Scale bar represents 2 μ m.

dose-response curve of the cells that contained podosomes after stimulation by PDBu. The number of untreated, control Gba-SM-4 cells that contained podosomes increased with the increase in a PDBu concentrations, indicating that a PDBu concentration of 10^{-5} M was sufficient to stimulate the cells. However, MLCK-deficient cells showed little podosome formation even at concentrations of 10^{-6} – 10^{-4} M of PDBu.

Control Gba-SM-4 cells developed robust podosome formation upon stimulation with 10^{-5} M PDBu (Fig. 7: A and B), whereas MLCK-deficient cells did not (Fig. 7: C and D). These results indicate that MLCK is important in podosome development in the PDBu-stimulated smooth muscle cells. Furthermore, the cells were rescued with a WT·MLCK cDNA of bovine stomach by transient transfection (Fig. 7: E and F). These cells showed strong podosome development with PDBu stimulation (compare Fig. 7: B and F). Site-directed mutants expressing MLCK in which glycine residues were substituted for alanine at the ATP-binding sites to kill the kinase activity of MLCK (31) were transfected into Gba-SM-4 cells devoid of MLCK. This kinase-inactive mutant displayed podosomes under PDBu stimulation (Fig. 7: G and H), indicating that the kinase activity of MLCK is not necessary for podosome formation. We also transfected the double mutant of MLCK (dNdATP), where the 1-41 of N-terminal actin-binding domain of this kinase-inactive mutant MLCK was deleted. In these cells, we found only little if any podosomes upon PDBu-stimulation (compare Fig. 7: I and J). Taken together, this data would indicate that MLCK is necessary for PDBu to induce podosome development, and that this ability resides in the N-terminal region.

So far we did not describe whether PDBu-induced contraction was associated with changes in MLC phosphorylation. In Fig. 4, we interpreted the inhibitory effect of 200 and 500 μ M of 26 – 41 peptide as not being associated with changes in MLC phosphorylation, although we did not perform a statistical examination. When culture cells of A7r5 were stimulated by PDBu, they contracted slowly as shown in Fig. 8. The MLC phosphorylation was not increased in comparison to unstimulated cells. Because the effects of PDBu are independent of increases in intracellular calcium (34), we interpreted the effect of PDBu as being independent of MLC phosphorylation.

Discussion

In this study, we examined α -actin remodeling, the actin-myosin interaction, and podosome formation in smooth muscle using PDBu as a pharmacological tool. We used both A7r5 cells of rat origin and Gba-SM-4

Fig. 8. Phase contrast images of cultured A7r5 cells in the presence of 10^{-7} M PDBu. The bar graph shows MLC phosphorylation in % for 12 assays. Bars represent the mean $\pm$ S.E.M.

cells of guinea pig origin. The former is good for the study of the stress fiber and the latter, for the podosome. In A7r5 cells, PDBu stimulation is not an absolute requirement for podosome formation (see Fig. 2 of ref. 14). However, podosomes in Gba-SM-4 cells were PDBu-dependent, providing a good assay for the podosome (Fig. 6). Nakamura et al. (35) have previously reviewed that MLCK consists of multiple domains. Our present results further suggest that the actin-binding domain of MLCK is important in podosome formation.

The 26 – 41 sequence peptide of MLCK did show a dose-dependent response on myosin ATPase activity in the phosphorylated state, suggesting that this peptide does change the cycling head rate on actin-activated myosin. This effect was also seen with sliding filament velocity as the 26 – 41 peptide hindered filament velocity significantly at a low micromolar concentration. These data would suggest that a specific peptide sequence on the MLCK protein is important in the actin-myosin interaction in a calcium-independent manner.

It is known that calcium induces MLC phosphorylation and subsequently causes myosin-actin crossbridges to form and induce cellular contraction (see ref. 1 for a review). Skinned taenia caeci of guinea pigs were utilized to look at the effects of the 26 – 41 peptide on calcium-induced contraction (Fig. 3). Stress responses under basal conditions indicated that the 26 – 41 peptide had no effect. Upon activation of the myosin-actin cross-bridges

by calcium, we observed a decrease in tension development at two different peptide concentrations and this response was independent of MLC phosphorylation.

A7r5 and Gba-SM-4 cells display a smooth muscle cell phenotype and contain MLCK (Figs. 5 and 7). These cells also display the ability to form podosomes in a PDBu-stimulated manner. A7r5 cells were incubated with either 1–25, 26–41, or 1–41 antennapedia protein-conjugated peptides, and the 26–41 and 1–41 peptide sequences of MLCK were found to cause actin dissolution in the perinuclear region of the cell. The dissolution was confirmed using microinjection techniques. These results indicate that the 26–41 region of MLCK is important for actin-filament structure, especially in the perinuclear region where actin polymerization is critical for cellular contraction (see ref. 14 for a review).

Whole cell FRET analysis, as presently employed, has been used successfully to evaluate protein–protein interactions in smooth muscle cells (32, 33, 36). Because the donor/acceptor must be within 10-nm distance from each other for efficient energy transfer (37, 38), this technique provides a measure of protein–protein distances compatible with molecular interaction. FRET analysis indicated dynamic changes in the α -actin/MLCK contractile protein during the course of PDBu-stimulated contraction and provided clearer resolution of actin/MLCK-associated structure compared to that obtained with colocalization imaging. As expected, the labeling of actin with both donor and acceptor fluorophores resulted in a significantly higher FRET index than obtained for actin/MLCK, while analysis of the association of actin with the protein located in the plasma membrane show no FRET effect. These results suggest that whole cell FRET analysis is responsive to changes in protein–protein associations and can be used as a tool for assessing these interactions in fixed samples.

As previously reported, PDBu-stimulated contraction resulted in the reorganization of α -actin into podosomes, whereas β -actin was retained in stress fibers (39). In these cells, MLCK was observed in association with α -actin in podosomes. Most notably, FRET analysis indicated an approximate 87% increase in the α -actin/MLCK complex, while β -actin/MLCK association was unchanged in contracted cells (Table 1). Taken together, these results indicated significant levels of actin/MLCK interaction in specific structures and that recruitment of α -actin/MLCK interaction may be important in the PDBu-induced contraction and actin reorganization in A7r5 cells.

Inhibitory concentrations 50% (IC_{50}) varied among the assay methods. They were as follows: approximately 30 μ M (for in vitro motility assay, Fig. 2), ≤ 200 μ M (for ATPase activity assay, Fig. 1), and 200–500 μ M (for the

skinned smooth muscle contraction). We also noticed that the actin concentration relative to the myosin concentration increased in this order. We interpreted this increase as an effect of the N-terminal peptide via its inhibition on actin. When the smooth muscle cell is alive, that is, when it has intact cell membrane, the estimation of its intracellular concentration is difficult. To our knowledge, how much of the peptide with the antennapedia is incorporated into the cell is not known. If 10%–100% of the peptide can penetrate the membrane, the intracellular peptide concentration will be in the working range of the assays shown in Figs. 1 and 2. Concerning the injection experiments, the concentrations of the intracellular concentrations were much lower than those shown in Fig. 2, if we assume that the injected peptide diffuses freely. These cell studies indicate that the actual concentrations of peptide remain to be investigated in future experiments.

Peptides containing the 26–41 N-terminal sequence have been successfully utilized to competitively inhibit MLCK at its actin binding site and to inhibit the actin–myosin interaction when Ca^{2+} /CaM is absent (for a review, see ref. 11). The kinase active site, lentiviral constructs were established to understand the role of the kinase domain with and without the N-terminus of MLCK. RNAi was used as a tool to silence MLCK expression in Gba-SM-4 cells and this technique has been utilized in previous investigations (33). However, it has not been studied with PDBu-stimulated conditions. Silencing of MLCK expression significantly changed podosome-formation in Gba-SM-4 cells. After silencing MLCK, a MLCK construct was transfected into Gba-SM-4 cells and shown to rescue actin-filament structure and induce podosome formation. These data indicate that MLCK is necessary for actin structure and is an important component of the podosome. Use of additional constructs (dNdATP) lacking both the actin-binding domain at the N-terminus (dN) and the functional kinase domain (dNdATP) indicated that the N-terminus of MLCK is necessary for podosome formation. However, it should be noted that our peptide studies in cells showed a much more pronounced effect on actin structure than the lentiviral constructs, when we compared these data (Fig. 7) with those with peptides (Fig. 4). It is possible that the concentrations of the 26–41 peptide in microinjection and conjugated peptide studies were significantly higher than achieved in our lentiviral studies.

Growing evidence suggests that the ability to reorganize the contractile apparatus and supporting cytoskeleton plays a central role in determining the contractile properties of smooth muscle (40–43). However, the exact nature of this remodeling and the mechanisms regulating cytoskeletal reorganization in contracting smooth muscle

are not certain. Early work indicated that blockade of actin polymerization depressed force development (43, 44), suggesting filament elongation or the generation of new filaments is an important aspect of contractile remodeling. The present results further suggest that actin-filament crosslinked by MLCK may play an important role in stabilizing actin structure in the precontracted cell and could contribute to actin reorganization during calcium-independent contraction. Based on these new findings, in addition to activation of ATPase activity, MLCK may serve to both maintain actin contractile structure in the resting cell and release filaments for movement by myosin and force development. In the absence of this crosslinking activity, the loss in actin organization in the resting cell inhibits cell contraction and contractile remodeling.

Acknowledgments

Work was supported with Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to K.K., A.N., H.H., and M.W. and a grant from the Smoking Research Foundation to K.K. Work was also supported by NIH Grant #P20 RR-16481 from the IDeA Networks of Biomedical Research Excellence (INBRE) Program of the National Center for Research Resources to M.E.F. and by the grant from National Natural Science Foundation of China # 30772601 to Y.L.

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