Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts

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1Department of Structural Analysis, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565; 2Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma, Nara 630-0101; 3Department of Cell Pharmacology, Nagoya University School of Medicine, Showa, Nagoya, Aichi 466-8550, Japan; and 4Center for Cardiovascular Research, University of Rochester, Rochester, New York 14642

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Katoh, Kazuo, Yumiko Kano, Mutsuki Amano, Kozo Kaibuchi, and Keigi Fujiwara. Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. Am J Physiol Cell Physiol 280: C1669–C1679, 2001.—To understand the roles of Rho-kinase and myosin light chain kinase (MLCK) for the contraction and organization of stress fibers, we treated cultured human foreskin fibroblasts with several MLCK, Rho-kinase, or calmodulin inhibitors and analyzed F-actin organization in the cells. Some cells were transfected with green fluorescent protein (GFP)-labeled actin, and the effects of inhibitors were also studied in these living cells. The Rho-kinase inhibitors Y-27632 and HA1077 caused disassembly of stress fibers and focal adhesions in the central portion of the cell within 1 h. However, stress fibers located in the periphery of the cell were not severely affected by the Rho-kinase inhibitors. When these cells were washed with fresh medium, the central stress fibers and focal adhesions gradually reformed, and within 3 h the cells were completely recovered. ML-7 and KT5926 are specific MLCK inhibitors and caused disruption and/or shortening of peripheral stress fibers, leaving the central fibers relatively intact even though their number was reduced. The calmodulin inhibitors W-5 and W-7 gave essentially the same results as the MLCK inhibitors. The MLCK and calmodulin inhibitors, but not the Rho-kinase inhibitors, caused cells to lose the spread morphology, indicating that the peripheral fibers play a major role in keeping the flattened state of the cell. When stress fiber models were reactivated, the peripheral fibers contracted before the central fibers. Thus our study shows that there are at least two different stress fiber systems in the cell. The central stress fiber system is dependent more on the activity of Rho-kinase than on that of MLCK, while the peripheral stress fiber system depends on MLCK.

Rho-kinase; stress fiber; contraction; myosin regulatory light chain; myosin light chain kinase

STRESS FIBERS are seen as bundles of actin filaments and are an actin-myosin-based contractile system. We have recently developed a method for isolating stress fibers from cultured cells and reactivating them (11, 12). The method is based on sequentially extracting cells with low ionic strength and then detergent (Triton X-100) solutions. The stress fibers isolated by the procedure contained actin, myosin, α-actinin, calmodulin, and myosin light chain kinase (MLCK). A rapid contraction was achieved by treating isolated stress fibers with Mg-ATP and Ca2+. We concluded that this contraction was regulated by the Ca2+-dependent calmodulin/MLCK system.

Rho (Ras homology) proteins are GTPases involved in signal transduction. Activation of Rho proteins is known to modulate the organization of actin filaments in cells, including formation of stress fibers and focal adhesions (1, 19). Several proteins are known to be targets of activated Rho, such as Rho-kinase (ROKα/ROCK II) (8, 17, 18), myosin binding subunit (MBS) of myosin phosphatase (15), p140mDia (24), protein kinase N (4), and phospholipase D (21). It is now known that actomyosin contraction can be regulated by Rho-kinase in two ways. One way is by phosphorylating myosin regulatory light chain (MRLC) at serine-19 of smooth muscle (3, 16) and fibroblast (2, 7). Interestingly, serine-19 is the residue phosphorylated by MLCK. The other way contraction can be regulated by Rho-kinase is by inhibiting the myosin phosphatase activity via phosphorylation of MBS of myosin phosphatase (15).

Although regulation of MLCK and Rho-kinase activities is well characterized, each separately by in vitro analyses, it is not easy to determine the distinct roles of MLCK and Rho-kinase in living cells. In this study, we have made an attempt at examining different effects of MLCK and Rho-kinase on the organization of stress fibers. Our study has revealed that there are two stress fiber systems: 1) a thick peripheral stress fiber system that is more sensitive to MLCK and calmodulin inhibi-
itors than to Rho-kinase inhibitors and 2) a thin central stress fiber network that is more sensitive to Rho-kinase inhibitors than to the other two types. Focal adhesions associated with the central stress fibers were also sensitive to Rho-kinase inhibitors. In vitro contraction of peripheral stress fibers occurred before central stress fibers began to shorten. These observed differences indicate that not all stress fibers are regulated in the same way. We suggest that the formation, maintenance, and contraction of the central stress fibers depend largely on the activity of Rho-kinase, while the Ca\(^{2+}\)-dependent calmodulin/MLCK system plays a major role in regulating the peripheral stress fibers.

**METHODS**

**Cell culture.** Human foreskin fibroblasts (FS-133), bovine endothelial cells, and human lung fibroblast (WI-38) cells were cultured with the use of a 1:1 mixture of Dulbecco's modified Eagle medium and a nutrient mixture of F-12 (GIBCO, Grand Island, NY), pH 7.4, containing 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin, and 10% fetal bovine serum (Salmond Smith Biolab, New Zealand) (21). The cells were maintained at 37°C in a humidified, 5% CO\(_2\) atmosphere.

**Antibodies.** The following monoclonal antibodies were purchased: anti-\(\alpha\)-smooth muscle actin (Sigma, St. Louis, MO), anti-pan-myosin (Amersham, Amersham, UK), anti-MLCK (Sigma), anti-calmodulin (Upstate Biotechnology, Lake Placid, NY), and anti-vinculin (Sigma). Rhodamine-labeled phalloidin was also purchased for F-actin staining (Molecular Probes, Eugene, OR). A rabbit affinity-purified polyclonal antibody against the glutathione-S-transferase (GST)-bound MBS peptide (GST-MBS-N; 1–706 amino acids) was described previously (14).

**Inhibitors.** The MLCK inhibitors ML-7 (Seikagaku, Tokyo, Japan), ML-9 (Seikagaku), and wortmannin (Wako, Osaka, Japan) were purchased. The calmodulin inhibitors W-5 (Biomol, Plymouth Meeting, PA), W-7 (Biomol), and KT5926 (Calbiochem, La Jolla, CA) were also purchased. The Rho-kinase inhibitors Y-27632 (23) and HA1077 (22, 23) were kindly provided by Yoshitomi Pharmaceutical Industry (Osaka, Japan) and Asahi Chemical Industry (Shizuoka, Japan), respectively.

**Isolation of stress fibers.** Stress fibers were isolated by a modified method described in our previous report (11, 12). Briefly, FS-133 cultured for 3–4 days in culture dishes (150 mm in diameter; Greiner, Frickenhausen, Germany) were quickly washed in ice-chilled phosphate-buffered saline (PBS) and then extracted for 20–30 min with gentle agitation in a low-ionic strength solution consisting of 2.5 mM triethanolamine (2,2\(^9\),2\(^{10}\)-nitrilotriethanol; TEA) (Wako), 20 \(\mu\)g/ml Trasylol (Bayer, Leverkusen, Germany), 1 \(\mu\)g/ml leupeptin (Peptide Institute, Osaka, Japan), and 1 \(\mu\)g/ml pepstatin (Peptide Institute), pH 8.2 at 4°C. At this stage, most cells

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**Fig. 1.** Morphology of peripheral and central stress fibers. **a:** video-enhanced phase-contrast image of a control living fibroblast showing the central (arrowheads) and peripheral (arrows) stress fibers. Electron micrographs show isolated peripheral (b) and central (c) stress fibers. Low-magnification micrographs are included so that the peripheral location of the stress fiber (b) can be clearly shown. Boxed areas in b and c are enlarged in insets. Note that the peripheral fiber is thicker than the central stress fibers. **d:** control fibroblast fixed and stained with rhodamine-labeled phalloidin.
Fig. 2. Effects of Rho-kinase (Y-27632), myosin light chain kinase (MLCK; ML-7) and calmodulin (W-7) inhibitors on peripheral and central stress fibers of fibroblasts. a–c: cell treated with Y-27632 and stained with rhodamine-phalloidin showing reduced number of central stress fibers. Peripheral fibers are present. d–f: cell treated with ML-7. Rhodamine-phalloidin staining shows peripheral stress fibers more severely affected than central fibers. g–i: fibroblast treated with W-7 and stained with rhodamine-phalloidin. Like the cell treated with the MLCK inhibitor, peripheral but not central stress fibers were severely affected. The cells treated with ML-7 (d–f) or W-7 (g–i) lost their spread morphology.

Table 1. Effects of various inhibitors on FS-133 cells

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<th>Inhibitors</th>
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MLCK, myosin light chain kinase; SF, stress fibers. Results indicate whether cell characteristics are present or not greatly reduced (+) or absent or greatly reduced (−) in response to inhibitors.
lost their dorsal side and nuclei, but some still had these parts of the cell, which could be removed by gentle shearing under a phase-contrast microscope with a stream of extraction buffer. The material attached to culture dishes was gently washed by Triton X-100-based extraction buffer [0.05% Triton X-100 (Wako), 20 μg/ml Trasylol, 1 μg/ml leupeptin, and 1 μg/ml pepstatin in PBS, pH 7.4] for 3–30 min. Still attached to coverslips were mainly stress fibers, and such preparations were used for protein localization and contraction studies.

**Immunofluorescence microscopy.** Cells and stress fiber models were fixed with 1% paraformaldehyde in PBS for 30–60 min and treated with 10% normal goat serum for 1 h at room temperature. They were then stained with anti-MLCK (1:100), anti-calmodulin (1:100), anti-MBS (1:100), anti-myosin (1:100), or anti-vinculin (1:400) for 60 min. After being washed in PBS, samples were incubated with fluorescein (Cappel, Durham, NC), rhodamine (Cappel), or Texas red (EY-Lab, San Mateo, CA)-labeled goat anti-rabbit or anti-mouse IgG. Some specimens were double-stained with one of the antibodies (and the appropriate secondary antibody) and rhodamine-labeled phalloidin.

Specimens were observed using a Zeiss Axiophot (Carl Zeiss, Germany) epifluorescence microscope with an apochromat ×63 [numerical aperture (N.A.) 1.4, oil-immersion] objective lens. Fluorescent images were photographed by using Kodak T-Max 400 film (Eastman Kodak, Rochester, NY).

**Electron microscopy.** For thin-section electron microscopy, isolated stress fiber models on coverslips were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature. Fixed stress fibers were washed for 30 min in 0.1 M sodium cacodylate buffer and postfixed for 1 h with 1% OsO₄ in the same buffer at 4°C. Samples were dehydrated through a graded series of ethanol (50, 65, 75, 85, 95, 99, 100%) and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate. Samples were examined with a 2000FX electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

**Contraction of stress fibers.** Isolated stress fiber models in a perfusion chamber were first immersed in a wash solution (10 mM imidazole, 100 mM KCl, and 2 mM EGTA) and perfused with an Mg-ATP solution (0.1 mM ATP, 3 mM MgCl₂, 1 mM EGTA, 75 mM KCl, and 20 mM imidazole, pH 7.2) with 1 mM CaCl₂. Contraction was observed under a phase-contrast microscope (Zeiss Axiophot with a plan-neofluar ×63, N.A. 1.25, oil-immersion, antiflex objective lens; Carl Zeiss) equipped with a video-enhanced imaging system for 5–20 min. The video system consisted of a high-resolution charge-coupled device camera (C2400–77;
Hamamatsu Photonics, Hamamatsu, Japan) and a digital image processor (Image Sigma-II; Nippon Avionics, Tokyo, Japan), and images were recorded by a high-resolution laser video disk recorder (LV-250H; TEAC, Tokyo, Japan).

Green fluorescent protein-labeled actin expression and inhibitor experiments. Human foreskin fibroblasts (FS-133) were transfected with the pEGFP-actin vector obtained from Clontech (Palo Alto, CA) using the Tfx-50 reagents (Promega, Madison, WI). Transfected cells were cultured as described in Cell culture. For time-lapse confocal laser-scanning microscopy, green fluorescent protein (GFP)-actin-transfected cells were plated on a culture dish (5 cm in diameter) and placed on a temperature-controlled stage at 37°C (Tokai, Shizuoka, Japan). Culture medium was exchanged every 20 min for long-term observations.

For inhibitor experiments, FS-133, WI-38, and endothelial cells as well as GFP-actin-transfected FS-133 cells were treated for 60 min with Y-27632 (10 μM), HA1077 (30 μM), wortmannin (100 nM), ML-7 (25 μM), ML-9 (100 μM), KT5926 (15 μM), W-5 (100 μM), or W-7 (100 μM). Each of these inhibitors was added to culture medium. Samples were observed under a confocal laser-scanning fluorescence microscope (Fluoview with a LUMPlanFl ×60, N.A. 0.90, water-immersion lens; Olympus, Tokyo, Japan) or a video-enhanced phase-contrast microscope. Some samples were fixed with paraformaldehyde and stained simultaneously with anti-vinculin (Sigma) and rhodamine-labeled phalloidin as described in Immunofluorescence microscopy. Recovery experiments were done by treating cells with one of these inhibitors for 1 h and washing them with fresh medium, and the process of recovery was recorded under time-lapse confocal microscope or a video-enhanced phase-contrast microscope.

RESULTS

Effect of various inhibitors on living fibroblasts. Stress fibers consist of bundles of actin filaments and can be best observed in various types of cultured cells. Despite its excellent detectability, fluorescence microscopy cannot provide accurate size information for stained structures. Phase-contrast microscopy, on the other hand, reveals the size of the structure. Because compactness of the actin filaments in stress fibers causes enough refractive index difference between the stress fibers and the surrounding cytoplasm, one can observe the stress fibers by phase-contrast microscopy. Figure 1a is a video-enhanced phase-contrast image of a living fibroblast and shows stress fibers in the basal part of the cell (basal stress fibers; see Ref. 13). There were many thin stress fibers located in the central (i.e., away from the lateral cell border) portion of the cell (Fig. 1a, arrowheads). In addition, there were thicker phase dense structures associated with the gently arched smooth parts of the lateral cell border (Fig. 1a, arrows). Because they may also be stress fibers, we investigated the structure in more detail by electron microscopy. The stress fiber isolation procedure we had reported earlier enabled us to stabilize stress fibers while other cellular structures were effectively removed (12). Figure 1b shows a structure, found at the lateral border of a cell, that contains parallel microfilaments in a bundle. This ultrastructure is identical, even at a high magnification, to that of stress fibers.

Fig. 4. Effects of Y-27632 on living fibroblasts expressing green fluorescent protein (GFP)-labeled actin. The drug was added at time 0, and the cell was imaged by confocal time-lapse microscopy using GFP-actin fluorescence. Time of drug treatment (in hours:minutes) is indicated for each plate. Note that central stress fibers are gradually lost, while peripheral fibers (arrowheads) remain. Note also that new cell extensions (arrow) are being formed in the presence of the Rho-kinase inhibitor.
located in the central part of the cell (Fig. 1c). The only difference between the two was their widths; the peripheral stress fibers were significantly thicker than the central fibers. Stress fibers stained with rhodamine-labeled phalloidin also revealed the peripheral and central stress fibers (Fig. 1d). Note that, in general, the stress fibers at the cell periphery stain more strongly than those in the center. The staining intensity of the cell cortex was much weaker compared with that of peripheral stress fibers. Thus there are two types of basal stress fibers, those located in the central region of the cell (central stress fibers; Fig. 1a, arrowheads) and those associated with the lateral cell border (peripheral stress fibers; Fig. 1a, arrows).

We wondered whether the two types have different properties other than morphological. Because it is now known that stress fiber formation and maintenance are regulated by Rho and Rho-kinase, presumably by controlling the level of MRLC phosphorylation (1, 3), we thought that the two stress fiber types might respond differently to various inhibitors that affected MRLC phosphorylation. Living fibroblasts were treated with Rho-kinase inhibitors, MLCK inhibitors, or calmodulin inhibitors, and cells treated with these drugs have shown clear and specific reduction in peripheral stress fibers, leaving the central type unaffected (Fig. 2, g–i). As was the case for cells treated with the MLCK inhibitors, the calmodulin inhibitors also induced cell rounding. These observations suggest that the loss of peripheral stress fibers causes cell rounding. Effects of various inhibitors on FS-133 cells are summarized in Table 1. These effects were reproducible and were observed in almost all of the cells treated. To ascertain that these drug effects were not limited to FS-133 cells, we also treated bovine endothelial and WI-38 cells in the same way. We observed the identical drug effects in these cells, suggesting that our findings are not cell type-specific events.

**Association of contractile proteins with drug-resistant stress fibers.** Stress fibers are not just bundles of actin filaments but contain a host of actin and myosin binding proteins (5, 6, 12). Whether or not the drug-resistant stress fibers were still associated with such proteins, we used the Y-27632-resistant stress fibers and investigated whether certain of the known stress fiber component proteins were associated with the remaining fibers. Because contraction and/or tension production is the major function of stress fibers, we have selected proteins that are related to stress fiber contraction and its regulation.

Fig. 5. Recovery of stress fibers and focal adhesions in cells being washed after 1 h of Y-27632 treatment. Untreated control cells (a), cells treated with Y-27632 for 1 h (b), and cells washed with fresh medium for 1 (c) or 3 h (d) were fixed and then double-stained with rhodamine-phalloidin (red) and anti-vinculin (green). Yellow staining indicates overlap of the two colors where F-actin and vinculin colocalize. Before drug treatment, cells had well-developed stress fibers and large focal adhesions in both peripheral and central cell regions (a). After 1 h of Y-27632 treatment, cells had peripheral stress fibers but only a few central stress fibers and small focal adhesions at the cell periphery (b). After cells were washed with fresh medium, stress fibers and focal adhesions were gradually reorganized at the central portion. Cells recovered for 1 (c) or 3 h (d) are shown.
Fibroblasts treated with Y-27632 for 1 h were fixed and stained with anti-MLCK (Fig. 3a) or anti-myosin (Fig. 3b) together with rhodamine-phalloidin for actin visualization. The double-labeled cells (Fig. 3, a and b) showed that the remaining peripheral stress fibers contained myosin and MLCK. Calmodulin was also associated with the stress fibers (data not shown). Thus the remaining peripheral stress fibers still contain the MLCK/calmodulin-based actin-myosin contractile system even after Y-27632 treatment. The tight appearance of the remaining peripheral stress fibers suggests that they may indeed be under tension.

Fig. 6. Effects of ML-7 on living fibroblasts expressing GFP-actin. The drug was added at time 0, and the cell was imaged by confocal time-lapse microscopy using GFP-actin fluorescence. Time of drug treatment (in hours:minutes) is indicated for each plate. Peripheral stress fibers were gradually shortened and/or disrupted. Although the number of central stress fibers decreased slightly, they persisted throughout the observation period. Note that the cells are more rounded and that motile activities at the cell border are inhibited by the drug.
The same observation was made in cells treated with HA1077, another Rho-kinase inhibitor (data not shown). Thus, from the standpoint of molecular composition, the remaining stress fibers are not different from those in control cells.

Time-lapse observation of living cells treated with inhibitors. The effects of inhibitors on stress fibers were examined using living cells. Fibroblasts transfected with GFP-actin were observed using a confocal time-lapse recording system for up to 1 h while they...
were treated with either a Rho-kinase or an MLCK inhibitor. These continuous observations allowed us to ascertain the origin of remaining stress fibers. They also allowed us to study closely how each type of stress fiber was affected by the drugs. Many cells were studied, and the most prevalent types of stress fiber responses to various inhibitors are described here. Fibroblasts expressing GFP-actin developed stress fibers and exhibited the cell shape indistinguishable from those of nontransfected cells. Figure 4 shows a series of fluorescence images of the same cell being treated with Y-27632. Although overexpressed GFP-actin gave a considerable level of general background fluorescence, both the central and the peripheral stress fibers were clearly recognized when the Rho-kinase inhibitor was added (Fig. 4, time 00:00). With time, however, the central stress fibers gradually disappeared (Fig. 4). On the other hand, the peripheral stress fibers were not lost (Fig. 4, arrowheads). Interestingly, the cell continued to exhibit membrane-associated activities such as formation of new lamellipodia (Fig. 4, arrow) and extension of processes at the periphery.

Our data show that central stress fibers disassemble in cells treated with Y-27632 and that 1 h of treatment is sufficient to remove practically all of them. Concomitant with the loss of central stress fibers, focal adhesions were also lost (compare Fig. 5, a and b). These drug effects were reversible. When cells incubated with the Rho-kinase inhibitor for 1 h were washed with fresh culture medium and incubated, central stress fibers as well as focal adhesions were gradually reorganized (Fig. 5c). Complete recovery was achieved in ~3 h (Fig. 5d).

We similarly analyzed the effect of ML-7, a specific inhibitor for MLCK, using fibroblasts transfected with GFP-actin (Fig. 6). Our earlier experiments (Fig. 2, d–f) indicated that ML-7 affected the peripheral stress fiber more severely than the central type. As shown in Fig. 6, the length of peripheral stress fibers gradually shortened, many of them within 40 min. Fibroblasts also rounded up during the treatment with ML-7, presumably due to the loss of peripheral fibers. However, in contrast to the Y-27632 treatment that caused disassembly of the central stress fiber (Fig. 5), cells treated with ML-7 for 40 min to 1 h exhibited many central stress fibers, albeit they appeared thinner and less in number. Cells being treated with ML-7 did not show peripheral membrane activities, such as membrane ruffles and lamellipodia/filopodia extension. This is quite contrary to the effect of the Rho-kinase inhibitor shown in Figs. 3–5.

Recovery of cells from ML-7 treatment was also studied. Fibroblasts treated with ML-7 for 1 h were washed with fresh medium at time 0 and observed with a video-enhanced phase-contrast microscope for 1 h (Fig. 7a). The most obvious response was rapid extension of lamellipodia, followed by cell spreading. To observe stress fibers and focal adhesions, we fixed recovering cells and stained them simultaneously with rhodamine-phalloidin and anti-vinculin. After being treated with ML-7 for 1 h, cells were rounded and had no obvious arch terminal stress fibers at the cell periphery (Fig. 7b, time 0). The lateral cell border receded so that it presumably rested on straight central stress fibers. Interestingly, ML-7 treatment did not appear to affect focal adhesions, because many anti-vinculin staining plaques were present and their size and morphology were comparable to those in control cells (Fig. 5a). Cells allowed to recover for 45 min had well-developed arched peripheral stress fibers and began to spread (Fig. 7b, 45 min). Note the presence of filopodia in this cell. By 2 h of recovery, cells looked normal with the fully developed stress fiber system (Fig. 7b, 120 min).

Our data show that the peripheral and central stress fiber systems have different drug sensitivity. The peripheral type was more sensitive to MLCK inhibitors than to Rho-kinase inhibitors, while the central stress fibers were more sensitive to Rho-kinase inhibitors than to MLCK inhibitors. These results indicate that there may be some physiological differences between the two types of stress fibers. Using a contractile stress fiber system, we investigated whether the mode of contraction was different between these stress fibers.

**Contraction of isolated peripheral and central stress fibers.** Stress fiber models were made by extracting cells with Triton X-100 (12). These models contracted when Mg-ATP was added in the presence of Ca^{2+}. To observe the mode of contraction of stress fibers, we used a video-enhanced phase-contrast microscope and time-lapse recording. Contraction of peripheral stress fibers (Fig. 8, arrowheads) occurred before the central fibers (Fig. 8, arrows) began to contract. In fact, the central fibers began to contract when the peripheral fibers had almost finished contacting. The speed of contraction of each type of stress fibers was almost identical (for the speed of isolated stress fiber contraction, see Ref. 12). These observations indicate that the onset of contraction is differently regulated between the two types of stress fibers.

**DISCUSSION**

Stress fibers are commonly thought of as cytoskeletal structures found along the basal plasma membrane. However, they are also found in other parts of the cell. In addition to the basal stress fibers located in the basal portion of the cell, we have shown the presence of apical stress fibers that run along the apical plasma membrane (13). The two types of stress fibers are also found in situ cells (10). The ends of stress fibers are anchored to the plasma membrane. Both ends of basal stress fibers are anchored to focal adhesions. Our previous study revealed that one end of the apical stress fiber is anchored to the apical plasma membrane via a structure we termed the apical plaque (13) and that the other end is anchored to the basal membrane, presumably to the focal adhesion. In cells forming a monolayer, such as endothelial cells in situ, we have found that apical stress fibers run between the apical plaque and the lateral cell-cell adhesion site (9). Furthermore,
we have some preliminary data indicating the presence of stress fibers spanning between the lateral cell adhesion and the focal adhesion (Kano and Katoh, unpublished observation). The categorization discussed here is solely based on morphological features of stress fibers. Whether or not there are differences in their molecular composition, contractility, stability, and other features is not known.

In the present study, we show different responses among basal stress fibers to various inhibitors. As summarized in Table 1, the central stress fiber is more sensitive to Rho-kinase inhibitors than to MLCK and calmodulin inhibitors. On the other hand, the peripheral stress fiber is more susceptible to the same set of MLCK and calmodulin inhibitors than to the Rho-kinase inhibitors. These inhibitor studies indicate that activities of kinases that regulate myosin function are necessary to maintain the stress fiber system in the cell and that two kinase systems control two different sets of stress fibers. Thus it appears that cells have a sophisticated system for maintaining this cytoskeletal structure.

The reason for cells to use different kinase systems for the peripheral and the central stress fibers is not clear. It is likely that the difference reflects the structural and/or functional differences of these stress fibers. Besides the fact that the peripheral stress fibers are generally thicker than the central ones, there is no other obvious structural difference between the two types. To our knowledge, there has been no study focused on the molecular compositions of the two stress fiber types. This study provides a rationale for carefully analyzing the composition of various types of stress fibers.

Our present study has revealed that the two types of stress fibers have different contractile properties. When stress fiber models were reactivated, peripheral stress fiber contraction was almost complete when the central ones began to shorten. Because the components, such as ATP and Ca\(^{2+}\), in the reactivation solution should be equally accessible to all the stress fibers in the model, the difference in their reactivation time might be an indication that the contraction of the two stress fiber systems is regulated differently. In a separate study, we found that isolated stress fibers could be reactivated by activating either MLCK or Rho-kinase and that MLCK initiated contraction faster and more extensively than Rho-kinase (10a). It is possible that the contraction of peripheral stress fibers that are sensitive to MLCK inhibitors is regulated largely by MLCK, while the contraction of central stress fibers that are sensitive to Rho-kinase inhibitors depend mainly on Rho-kinase. This hypothesis predicts delayed reactivation time for central stress fibers, and our data are in agreement with this. The hypothesis also predicts that the contraction of the peripheral and the central stress fibers may be inhibited by MLCK and Rho-kinase inhibitors, respectively, and that the two kinases may have different localization patterns. However, we observed that the contraction of both stress fiber types was inhibited by either type of inhibitor alone (10a), and our localization studies so far showed that both kinases were present in both stress fiber types without any clear-cut differences. These results were obtained from both fixed cells and isolated stress fibers as well as different cell types. Thus, at present,
there is no easy explanation for the observed difference in the stress fiber reactivation times.

Our data show that Rho-kinase activity is necessary for maintaining the organization of the central stress fibers, including the focal adhesions associated with them. However, because there are multiple targets of Rho-kinase in the cell, it is not easy to determine the mechanism for the observed effects of Rho-kinase inhibitors. It is possible that the target of Rho-kinase is not a component of the stress fiber but is a component of the focal adhesion. This could cause stress fibers to lose tension needed to maintain their structure, making them disassemble. Our study also showed that the peripheral stress fibers and the formation of lamellipodia and filopodia were severely affected by MLCK but not Rho-kinase inhibitors. Activities of MLCK were also needed to maintain the spread morphology of the cell. Because MLCK is a specific kinase for the regulatory light chain of myosin, our results indicate that actomyosin contraction is necessary to maintain cell spreading and to form lamellipodia and filopodia. How contraction is required for developing cell extensions is an interesting puzzle that needs to be resolved.

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REFERENCES


