Blebbistatin inhibits the chemotaxis of vascular smooth muscle cells by disrupting the myosin II-actin interaction

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1Department of Molecular and Cellular Pharmacology, Gunma University Graduate School of Medicine, 2Department of Research Science, Gunma University School of Health Sciences, Gunma, 3Department of Bioresources, Me University, Tsu, Japan; 4Department of Biochemistry, College of Life Sciences, Nankai University, Tianjin, Peoples’ Republic of China; and 5Department of Physiology, The Joan Edwards School of Medicine, and 6Department of Biological Sciences, Laboratory of Molecular Pharmacology, Marshall University, Huntington, West Virginia

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Wang HH, Tanaka H, Qin X, Zhao T, Ye LH, Okagaki T, Katayama T, Nakamura A, Ishikawa R, Thatcher SE, Wright GL, Kohama K. Blebbistatin inhibits the chemotaxis of vascular smooth muscle cells by disrupting the myosin II-actin interaction. Am J Physiol Heart Circ Physiol 294: H2060–H2068, 2008. First published February 22, 2008; doi:10.1152/ajpheart.00970.2007.—Blebbistatin is a myosin II-specific inhibitor. However, the mechanism and tissue specificity of the drug are not well understood. Blebbistatin blocked the chemotaxis of vascular smooth muscle cells (VSMCs) toward sphingosylphosphorylcholine (IC50 = 26.1 ± 0.2 and 27.5 ± 0.5 μM for GbaSM-4 and A7r5 cells, respectively) and platelet-derived growth factor BB (IC50 = 32.3 ± 0.9 and 31.6 ± 1.3 μM for GbaSM-4 and A7r5 cells, respectively) at similar concentrations. Immunofluorescence and fluorescent resonance energy transfer analysis indicated a blebbistatin-induced disruption of the actin-myosin interaction in VSMCs. Subsequent experiments indicated that blebbistatin inhibited the Mg2+-ATPase activity of the unphosphorylated (IC50 = 12.6 ± 1.6 and 4.3 ± 0.5 μM for gizzard and bovine stomach, respectively) and phosphorylated (IC50 = 15.0 ± 0.6 μM for gizzard) forms of purified smooth muscle myosin II, suggesting a direct effect on myosin II motor activity. It was further observed that the Mg2+-ATPase activities of gizzard myosin II fragments, heavy meromyosin (IC50 = 14.4 ± 1.6 μM) and subfragment 1 (IC50 = 5.5 ± 0.4 μM), were also inhibited by blebbistatin. Assay by in vitro motility indicated that the inhibitory effect of blebbistatin was reversible. Electron-microscopic evaluation showed that blebbistatin induced a distinct contractile change (i.e., swelling) of the myosin II head. The results suggest that the site of blebbistatin action is within the S1 portion of smooth muscle myosin II.

Bovdenn chamber; fluorescence resonance energy transfer; ATPase; in vitro motility assay; electron microscopy

CELL MIGRATION IS DRIVEN through complex processes, such as extension of the leading membrane edge, with formation of adhesive contacts and stress fibers (26, 52). Myosin II is widely believed to be one of the main components producing the forces required in this process (12). However, the details of the regulatory mechanism of myosin II in cell migration remain to be established. The general understanding is that cell migration is regulated not only by pathways of signal transduction involving myosin light chain (MLC) phosphorylation, but also by MLC phosphorylation-independent pathways (2, 18, 28, 30). The exact role of myosin II in cell migration and the mechanism whereby myosin II contributes force generation for cell migration remains unanswered.

Blebbistatin was recently identified as a specific inhibitor of myosin II-dependent cell processes (49). Its membrane-permeable characteristic and effect on various myosin II isoforms make this agent an invaluable tool in research of myosin II-involved cellular events, including cell motility (5, 23), cell shape maintenance (50), muscle contraction (7, 15), and cytokinesis (49). Blebbistatin has been shown to preferentially bind to the myosin-ADP-phosphate complex with high affinity and prevent phosphate release (24, 37), resulting in inhibition of the actin-myosin interaction. Blebbistatin also inhibited the ability of skeletal muscle and nonmuscle myosin II to move actin filaments in the in vitro motility assay (27, 41). The mechanism of inhibition by blebbistatin has been investigated in kinetic studies of several classes of myosin (27). Although the effect of the inhibitor on skeletal muscle myosin II and nonmuscle myosin Ib was remarkable (IC50 = 0.4–5 μM), this was not the case for smooth muscle myosin II (IC50 = 80 μM) (27). In light of the conserved structure of myosin II heads, the relative insensitivity of smooth muscle myosin II to blebbistatin is unexpected (3). In particular, the residues with which blebbistatin has been shown to form a complex with ADP-vanadate (24) are highly conserved among various class II myosins, including skeletal muscle myosin II, smooth muscle myosin II, and nonmuscle myosin II (3).

Previous work in our laboratory found that blebbistatin was able to inhibit contraction of cultured vascular smooth muscle with an IC50 of ~23 μM (15), which is much lower than that previously reported for the inhibition of smooth muscle myosin II (27). Eddinger et al. (7) recently reported that the ATPase activity of smooth muscle myosin was inhibited by blebbistatin at a concentration low enough to explain our data (15) and aroused our interest in the effect of blebbistatin on the migration of vascular smooth muscle cells (VSMCs). In the present study, we analyzed the mechanism of inhibition of blebbistatin on VSMC migration on the basis of the motor activity of smooth muscle myosin II.

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**MATERIALS AND METHODS**

**Cell culture.** GbaSM-4 cells, a VSMC line derived from the brain basilar artery of guinea pigs (33) and characterized elsewhere (15), were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. A7r5 cells, a VSMC line derived from embryonic rat aorta, were purchased from American Type Culture Collection and then cultured in the same way as GbaSM-4 cells. These VSMCs were used for the examination of chemotaxis. The presence of smooth muscle myosin II was described by Firulli et al. (8) for A7r5 cells and by Oishi et al. (33) for GbaSM-4 cells. These VSMCs were used for the examination of chemotaxis. The presence of smooth muscle myosin II was described elsewhere (15), basilar artery of guinea pigs (33) and characterized elsewhere (15), skeletal muscle myosin II was purified from rabbit skeletal muscle as described elsewhere (29). Actin was purified from the actin monomer of chicken skeletal muscle according to the method of Spudich and Watt (48) with slight modifications (21) and used as F-actin. MLC kinase (MLCK) was purified from chicken gizzard smooth muscle by the method of Adelstein and Klee (1) with slight modifications (11). Smooth muscle myosin II was phosphorylated with MLCK in the presence of Ca2+/calmodulin as described by Ogakagi et al. (35) and used as phosphorylated myosin II. Calmodulin from bovine brain, glucose oxidase, and catalase were purchased from Sigma. (+)-Blebbistatin (active form) was purchased from Toronto Research Chemicals and (-)-blebbistatin (inactive form) from Calbiochem. They were dissolved in DMSO, which was also used as vehicle in the experiments.

**Migration assay.** Migration of VSMCs was assayed by the Boyden chamber method as previously described (19). Briefly, VSMCs suspended in DMEM containing 0.2% BSA and various concentrations of blebbistatin or vehicle were incubated for 1 h at 37°C and then applied to the upper compartments of the Boyden chamber (Neuro Probe), which was equipped with an 8-µm-pore polycarbonate membrane coated with type I collagen. DMEM containing 0.2% BSA and various concentrations of blebbistatin or vehicle in the presence of platelet-derived growth factor (PDGF)-BB (10 ng/ml) or 1 µM sphingosylphosphorylcholine (SPC) was introduced into the lower compartments of the Boyden chamber. The cells that had migrated to the lower surface of the membrane were counted under the microscope, and the sum of the numbers of cells from five randomly chosen microscopic fields (magnification ×400, area = 0.14 mm²) was determined as the number of migrating cells in each well. Percentages of the numbers of migrating cells at various blebbistatin concentrations relative to that with vehicle were plotted against inhibitor concentrations. Experiments were performed in quadruplicate and repeated on three separate occasions, and results are expressed as means ± SD.

**Detection of MLC phosphorylation.** GbaSM-4 and A7r5 cells cultured as described above were treated with 40 µM blebbistatin or vehicle for 60 min and then stimulated with 1 µM SPC or 10 ng/ml PDGF-BB for 2 min. Phosphorylation of MLC was detected with glyceraldehyde-PAGE followed by Western blotting as previously described (19). VSMCs were fixed with 5% TCA and 10 mM DTT in acetone, scraped from the dishes, and washed three times each with 1 ml of acetone containing 10 mM DTT. After centrifugation, the pellets were resuspended in loading buffer (20 mM Tris base, 23 mM glycine, 10 mM DTT, 8 M urea, and 0.04% bromphenol blue) and incubated for 2 h at room temperature. The samples were then loaded on glyceraldehyde-PAGE for separation of unphosphorylated, monophosphorylated, and diphosphorylated forms of MLC. After the samples were blotted to polyvinylidene difluoride (PVDF) membranes (Millipore), MLC was visualized with monoclonal antibody to MLC (MY-21, Sigma). Densitometric quantification of MLC phosphorylation was determined by using WClF ImageJ. Data (means ± SD) are presented as percentage of total MLC.

**Immunofluorescence.** VSMCs on collagen-coated coverslips were fixed with 1.5% paraformaldehyde in PBS for 5 min, washed with PBS, and permeabilized with 1% Triton X-100 in PBS. After incubation with 5% BSA in PBS for 1 h, cells were stained with FITC-phalloidin (Molecular Probes) and a mouse monoclonal antibody against smooth muscle myosin II heavy chain (clone HSM-V, Sigma) followed by a RedX-conjugated mouse secondary antibody (Jackson Immuno-Research). After they were washed with 1% BSA in PBS, cells were incubated with 20% (wt/vol) Mowiol 4-88 (Cali Biochem) in PBS and observed by laser-scanning confocal microscopy (model MRC-1024, Bio-Rad Laboratories).

**Myosin II isoform detection.** To determine the smooth muscle myosin II content of total myosin II in VSMCs, GbaSM-4 and A7r5 cells were extracted with SDS sample buffer as previously described (28). The heavy chain of myosin II isoforms was separated by SDS-PAGE and transferred to a PVDF membrane. To quantitate smooth muscle myosin II and all types of myosin II in the GbaSM-4 and A7r5 cells, various amounts of bovine stomach myosin, together with the extracts of GbaSM-4 and A7r5 cells, were subjected to SDS-PAGE in duplicate. After the transfer to PVDF membranes, the membranes were incubated with a monoclonal antibody specific for smooth muscle myosin II (clone HSM-V, Sigma) or a monoclonal antibody specific for all types of myosin II (pan-myosin antibody, clone C5C.S2, Covance). The blots were visualized using Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore). For the loading control, they were examined by a monoclonal antibody specific for β-actin (clone AC-15, Sigma). Standard curves for smooth muscle myosin II and total myosin II were made by the densitometric quantification of the blots as described for MLC, and the ratios of smooth muscle myosin II to total myosin II in GbaSM-4 and A7r5 cells were calculated.

**Fluorescence resonance energy transfer analysis by confocal microscopy.** GbaSM-4 cells were treated with vehicle or 20 µM blebbistatin for 1 h. Antibody-based fluorescence resonance energy transfer (FRET) was used to measure a number of different protein-protein interactions (17). GbaSM-4 cells were labeled with primary antibodies targeted to β-actin (clone AC-15, Sigma) and pan-myosin II (clone C5C.S2, Covance) with fluorescent probes Alexa 488-IgG and Alexa 546-IgM secondary antibodies to designate β-actin (donor) and myosin II (acceptor) as the components of the FRET system. Initially, β-actin was imaged with a 522DF32 band-pass filter at 3% laser power. The acceptor, myosin II, was photobleached at 100% laser power using the 568-nm laser line for 30–45 min until the signal was lost. A second image was recorded using the same donor settings used to evaluate protein interaction. Cell pixel intensity data were collected using ImageJ and analyzed by PeakFit software version 4.11 (SPSS Science) for before- and after-photobleaching images. Paint Shop Pro version 7.0 was used to show the difference between before and after photobleaching (FRET response). Data for increase in fluorescence for vehicle- and blebbistatin-treated cells are expressed as FRET response.

**Assay for ATPase activity.** Phosphorylated smooth muscle myosin II (0.1 µM) or skeletal muscle myosin II (0.1 µM), unphosphorylated smooth muscle myosin II (1 µM), HMM (0.2 µM), or S1 (0.2 µM) was mixed with various concentrations of blebbistatin from vehicle (DMSO) up to 100 µM in 60 mM KCl, 5 mM MgCl₂, 0.2 mM EGTA, 0.75 mM DTT, and 20 mM Tris- HCl (pH 7.5) at 25°C for 60 min. After addition of 0.5 mM ATP (and 6 µM F-actin in some experiments), solutions were further incubated for 10 min at 25°C, and the amount of liberated phosphate in the solutions was measured colorimetrically using malachite green as previously described (20).

**In vitro motility assay.** The effect of blebbistatin on F-actin sliding on smooth muscle myosin II was examined by the in vitro motility assay (53) with slight modification (35). Briefly, F-actin (3 nM)
labeled with rhodamine-phalloidin (Molecular Probes) was introduced into the flow cells constructed between a glass slide and a coverslip coated with phosphorylated smooth muscle myosin II in motility assay buffer: 60 mM KCl, 40 mM HEPES (pH 7.5), 1 mM MgCl₂, 2 mM ATP, 25 mM DTT, and 1 mM EGTA. Glucose oxidase (0.2 mg/ml; Sigma), catalase (0.04 mg/ml; Sigma), and glucose (4.5 mg/ml) were added to the motility buffer to prevent photobleaching of rhodamine. To examine the reversibility of the effect, blebbistatin that remained in the flow cell was washed out three times with motility assay buffer, and the flow cell was perfused with F-actin in vehicle, i.e., in the absence of inhibitor. The movement of actin filaments was recorded under a fluorescence microscope equipped with a silicone intensifier target camera. The average velocity was determined using the MTrack2 plug-in of WCIF ImageJ.

**Electron microscopy.** Gizzard smooth muscle myosin II and its HMM were phosphorylated by MLCK as described above and mixed with 20 μM blebbistatin in 20 mM Tris-HCl (pH 7.5) and 0.3 M KCl. After 20 min of incubation, the mixture was sandwiched between freshly cleaved mica sheets. The mica sheet was washed three times with 30% glycerol in 0.1 M ammonium acetate, stained with 2% aqueous uranyl acetate, and then washed three times. The top mica sheet was removed and rotary replicated with platinum at low angles in a BAF 060 rotary shadowing system. The replicated specimen was placed on a specimen grid and observed with a JEM-1010 electron microscope.

**Other procedures.** Protein concentrations were determined using a protein assay kit (Bio-Rad) with BSA as a standard (4). SDS-PAGE
was performed by the method of Laemmli (25) with slight modification (14) to confirm the purity of the proteins. Statistical significance was determined by Student’s t-test or one-way ANOVA followed by Student-Newman-Keuls test using Sigma Stat version 3.1. P < 0.003 or P < 0.001 was considered statistically significant.

**RESULTS**

**Effect of blebbistatin on VSMC migration.** We chose two chemoattractants for induction of migration, PDGF-BB and SPC. The former is known to induce VSMC migration without promoting increased phosphorylation levels of MLC (2, 18); the latter induced migration with phosphorylation of MLC (28). Migration of GbaSM-4 cells induced by PDGF-BB gradually decreased with increasing concentrations of blebbistatin with IC50 = 32.3 ± 0.9 μM (Fig. 1A). Similar inhibition was observed when SPC was used as a chemoattractant (IC50 = 26.1 ± 0.2 μM), indicating that blebbistatin inhibited MLC phosphorylation-dependent and -independent cell migration. We also examined the effect of blebbistatin using A7r5 cells. Migration of A7r5 cells toward PDGF-BB and SPC was inhibited with IC50 = 31.6 ± 1.3 and 27.5 ± 0.5 μM, respectively.

Next, we examined whether blebbistatin affected the phosphorylation level of MLC. As previously reported, the phosphorylation level was unchanged when cells were treated with PDGF-BB (Fig. 1B), whereas it was significantly increased by treatment with SPC compared with vehicle (P < 0.001; Fig. 1B). In both cases, pretreatment with blebbistatin did not affect the phosphorylation level of MLC (Fig. 1B), indicating that blebbistatin inhibits smooth muscle myosin II without changing the phosphorylation level of MLC.

**Dissociation of smooth muscle myosin II from actin filaments in GbaSM-4 cells after treatment with blebbistatin.** Figure 2, A and B, shows staining of actin-filaments and smooth muscle myosin, respectively, in GbaSM-4 cells in vehicle. Merging of these images in Fig. 2, C and G, shows distribution of smooth muscle myosin II along the actin stress fiber in a patchy pattern. We estimated the amount (percentage) of smooth muscle myosin II as revealed by the smooth muscle myosin antibody relative to total myosin II, including non-muscle myosin II, as revealed by the pan-myosin antibody. A typical estimation shown in Fig. 2I indicates that the content of smooth muscle myosin was 80% in GbaSM-4 cells and 68% in A7r5 cells.

To address whether blebbistatin inhibits the actin-myosin II interaction, we examined the distribution of actin and myosin II in GbaSM-4 cells after treatment with blebbistatin (Fig. 2, D–F). The cells extended spiky protrusions (cf. Fig. 2, A and G), as reported by Kolega (23). Furthermore, actin stress fibers disappeared and fragmented. Kinky actin bundles were observed. These changes suggest that the activity of myosin II is necessary to maintain actin stress fibers. The patchy structure of myosin II, however, persisted (Fig. 2, E and H) but was not colocalized with actin, indicating that blebbistatin causes the dissociation of smooth muscle myosin II from actin structures.

**FRET analysis of the association of β-actin and smooth muscle myosin II.** In GbaSM-4 cells, α-actin associates with β-actin (28). Because β-actin plays a major role in cell migratory activity (28), we stained the cells with the β-actin antibody. The actin-myosin interaction was quantitated by confocal imaging with FRET analysis, which showed clear β-actin filament structure with myosin II and protein interaction, after the myosin II was photobleached (Fig. 3). On average, the...
FRET response as expressed by the increase in β-actin fluorescence after photobleaching was 157 ± 12.5%. By comparison, the FRET response of the β-actin-myosin II interaction in blebbistatin-treated cells was only 112 ± 3.0%. The difference in the FRET response was significant (P < 0.003, n = 10). The loss of β-actin filaments was evident in all the blebbistatin-treated cells, which is consistent with disruption of the contractile apparatus in the GbaSM-4 cell (Fig. 2).

**Blebbistatin inhibition of the Mg$^{2+}$-ATPase activity of smooth muscle myosin II.** We purified smooth muscle myosin II from chicken gizzard and examined the effects of blebbistatin on the Mg$^{2+}$-ATPase activity of smooth muscle myosin II. The Mg$^{2+}$-ATPase activities of unphosphorylated smooth muscle myosin II (IC$_{50}$ = 12.6 ± 1.6 μM) and phosphorylated myosin II (IC$_{50}$ = 15.0 ± 0.6 μM) decreased with increasing concentrations of blebbistatin (Fig. 4A). Conforming results were obtained when HMM (IC$_{50}$ = 14.4 ± 1.6 μM) and S1 (IC$_{50}$ = 5.5 ± 0.4 μM) were used. When bovine smooth muscle myosin II was tested, IC$_{50}$ was even lower (IC$_{50}$ = 4.3 ± 0.5 μM for the unphosphorylated form). Actin-activated Mg$^{2+}$-ATPase activity of myosin II was also examined (Fig. 4B). Blebbistatin inhibited the activities of unphosphorylated (IC$_{50}$ = 17.5 ± 0.7 and 10.1 ± 0.7 μM for gizzard and bovine smooth muscle myosin II, respectively) and phosphorylated (IC$_{50}$ = 23.5 ± 2.8 μM for gizzard smooth muscle myosin II) forms of myosin II. These results suggest that blebbistatin inhibits the activity of smooth muscle myosin II at lower concentrations than previously reported (27) and agrees with the result of Eddinger et al. (7).

**Effect of blebbistatin on the motor activity of smooth muscle myosin II.** To confirm the effect of blebbistatin on the actin-myosin interaction, we examined the in vitro motility of Fig. 5. Blebbistatin inhibition of F-actin sliding on smooth muscle myosin II. Fluorescent-labeled F-actin in the presence of blebbistatin at various concentrations was perfused through the flow chamber over a glass slide coated with phosphorylated smooth muscle myosin II, and sliding velocities of F-actin filaments at the surface of the chamber were measured. A–F: sliding velocities of F-actin (n = 100). Dotted lines, actual distribution of F-actin in the absence of blebbistatin (A), in the presence of 5 μM blebbistatin (B), and in the presence of 20 μM blebbistatin (C). Blebbistatin in the flow chamber in C was washed out, and the chamber was perfused with F-actin in the absence of inhibitor (D) and in the presence of 50 μM blebbistatin (E). Blebbistatin in the flow chamber in E was washed out, and the chamber was perfused with F-actin in the absence of inhibitor (F). G: velocities of F-actin derived from A–F. Values are means ± SD. **P < 0.001.
F-actin on phosphorylated smooth muscle myosin II. The number of fast-sliding filaments on phosphorylated smooth muscle myosin II gradually decreased at increasing concentrations of blebbistatin (Fig. 5, A, B, C, and E). The average velocities of the filaments dropped by 27% in the presence of 20 µM blebbistatin and by 76% in the presence of 50 µM blebbistatin (P < 0.001; Fig. 5G). The average velocities recovered when blebbistatin was washed out (Fig. 5, D, F, and G), indicating that the inhibition of myosin II was a reversible reaction.

**Structure of smooth muscle myosin II treated with blebbistatin.** On the basis of the structures of myosin II and blebbistatin, the binding site of blebbistatin for myosin II is predicted to be on the 50-kDa cleft of the myosin II head (3). Therefore, we observed the rotary-shadowed images of smooth muscle myosin II by electron microscopy after treatment with blebbistatin. Compared with controls, the structure of myosin II (Fig. 6A) heads in blebbistatin-treated samples was altered to form single “ball-like” structures. A similar result was observed when HMM was used (Fig. 6, C and D). These results are consistent with the idea that blebbistatin binds to the head of myosin II (3) and indicate that blebbistatin binding results in a significant conformational change in structure. The ball-like structure formed by blebbistatin treatment was also observed with unphosphorylated myosin and HMM (data not shown).

**DISCUSSION**

In the present study, we show that chemotaxis of VSMCs by MLC phosphorylation-dependent and -independent mechanisms was effectively inhibited by blebbistatin. Active myosin II-based motors appear to be crucial for regulation of the contractile forces in eukaryotic cell migration, in which the regulation of MLC phosphorylation is widely believed to be essential (12). Although upstream factors may vary, cell migration is controlled by spatial and temporal factors, such as cdc42, Rac, and RhoA, which control contractile forces through effectors such as Rho kinase and p21-activated kinase (51). These signaling pathways help regulate MLC phosphorylation. In contraction, MLC phosphorylation is directly controlled by activation of MLCK, which is sensitive to intracellular Ca\(^{2+}\) concentration, and inhibition of MLC phosphatase, which is insensitive to intracellular Ca\(^{2+}\) concentration and regulated by a variety of upstream factors (10, 45, 47). The generation of contractile force by MLC phosphorylation in the migration of cells is thought to be similar to that regulating contraction (46) and may be activated by agonists such as lysophosphatidic acid and SPC (2, 28, 30). However, migration or contraction may also occur via an MLC phosphorylation-independent pathway (43). Contraction of smooth muscle induced by oxytocin (34), phorbol ester (9, 42), serum (31), or PDGF (44) is uncoupled from phosphorylation of MLC. Conversely, relaxation of contraction may not be strictly related to the decrease of MLC phosphorylation (22, 32). Ai et al. (2) confirmed that an increase in MLC phosphorylation is not required in PDGF-induced smooth muscle cell migration as reported earlier by Kishii et al. (18). In addition, smooth muscle cell migration toward SPC indicated that actin remodeling was dissociated from MLC phosphorylation (28). Hence, overwhelming evidence indicates that cell migration may be regulated not only by an MLC phosphorylation-dependent pathway, but also by an MLC phosphorylation-independent pathway. Because the effect of blebbistatin was similar in both kinds of migration (Fig. 1, A and B), our data suggest that the inhibitory mechanism of the drug did not involve interference with phosphorylation of MLC.

Blebbistatin was reported to inhibit only weakly the ATPase activity of smooth muscle myosin II with IC\(_{50}\) = 80 µM (27). However, inhibition of contraction has been reported in smooth muscle at significantly lower concentrations of blebbistatin (7, 15, 39). Because IC\(_{50}\) for nonmuscle myosin II is 1–5 µM (27), the increased inhibition in these studies might be explained in terms of the nonmuscle myosin II (7, 39). The content of nonmuscle myosin II in the primary culture of vascular smooth muscle tissues increased with the subculture (16). However, nonmuscle myosin II was not the major component in GbaSM-4 and A7r5 cells (see below). Probably, these cells

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**Fig. 6.** Electron micrographs of smooth muscle myosin II and HMM. Chicken gizzard smooth muscle myosin II and HMM were treated with 20 µM blebbistatin, subjected to the rotary-shadowing process, and observed with an electron microscope. A: myosin II with DMSO treatment (P-myo); B: myosin II + blebbistatin (Ble); C: HMM with DMSO (P-HMM); D: HMM + blebbistatin. Scale bars, 10 nm.
keep the phenotype of vascular smooth muscle, which is consistent with the reports that GbaSM-4 (33) and A7r5 (8) cells express calponin, caldesmon, and/or SM-22. Our finding of blebbistatin inhibition at lower IC50 (~15 μM for gizzard smooth muscle myosin II and 5 μM for bovine stomach myosin II; Fig. 4) indicates that an alternate explanation is needed to describe the effects of blebbistatin on smooth muscle myosin II and the inhibition of smooth muscle contraction. Furthermore, as described below, it is possible that myosin II inhibition by blebbistatin fails to maintain appropriate actin organization for cell migration.

Similar to the inhibition of contraction, migration of VSMCs was effectively inhibited by a concentration (Fig. 1A) much lower than the IC50 of blebbistatin shown to inhibit ATPase activity of smooth muscle myosin II (27). Immunostaining of smooth muscle myosin II heavy chain and FITC-phalloidin labeling of actin indicated dissociation of myosin II and actin filaments after treatment with blebbistatin (Fig. 2, F and H). Furthermore, FRET analysis also indicated that treatment with blebbistatin disrupted the contractile apparatus in the cell (Fig. 3). The migratory activity of VSMCs is associated with the remodeling of the contractile apparatus (28). Its disruption found in the present study (Fig. 2) provides an example of the remodeling and indicates that smooth muscle myosin II is the site of blebbistatin inhibition. According to the assumption that pan-myosin antibody detects total myosin II, the differences of smooth muscle myosin from pan-myosin would be nonmuscle myosin II. Thus the contents of nonmuscle myosin in GbaSM-4 and A7r5 cells would be 20% and 32%, respectively. Furthermore, under the conditions of Fig. 4, the ATPase activity of nonmuscle myosin II would be inhibited by blebbistatin (27). Taken together, nonmuscle myosin should be partly responsible for the inhibition of migration by blebbistatin.

Unconventional myosin may play a partial role in the migration toward PDGF-BB and SPC, in addition to smooth muscle and nonmuscle myosin II (38). In the presence of 80 μM blebbistatin, the residual 20–30% of VSMCs migrated toward both chemoattractants (Fig. 1A). Because unconventional myosins were not inhibited by 80 μM blebbistatin (27), the residual migration should be attributed to the blebbistatin-resistant nature of unconventional myosin.

It is difficult to understand the discrepancy in sensitivity to inhibition by blebbistatin of cell functions such as contraction (7, 15), migration (Fig. 1A), and ATPase activity (7). However, a likely explanation is that use of purified myosin II protein in Mg2+-ATPase assays may, in some fashion, enhance the access of blebbistatin to its binding site. Moreover, because the structure of class II myosins is highly conserved in the head domain, the inner sphere residues of the speculated blebbistatin interacting site is thought to be essentially identical in smooth and skeletal muscle myosin II (3). Hence, the relative insensitivity of smooth muscle myosin II to blebbistatin observed in previous studies (3) is unexpected. We investigated the dose dependency of inhibition of the ATPase activity of unphosphorylated myosin II, phosphorylated myosin II, HMM, and S1. Interestingly, basic and actin-activated ATPase activities of smooth muscle myosin II were inhibited with a much lower concentration of blebbistatin than shown in the previous report (27) (Fig. 4, A and B), which is consistent with the report of Eddinger et al. (7). Such an effective inhibition was confirmed by actin filament motility assays (Fig. 5A). We also noticed that the IC50 for smooth muscle myosin II from bovine stomach smooth muscle is much lower than that for chicken smooth muscle myosin II (Fig. 4). The recombinant smooth muscle myosin II expressed from cDNA of rabbit smooth muscle myosin II (40) is inhibited by blebbistatin at an IC50 similar to that of bovine smooth muscle (7). It is possible to conclude that smooth muscle myosin II derived from mammalian smooth muscle should be highly sensitive to blebbistatin.

The blebbistatin used in the present study was (±)-blebbistatin, which contained (+)-blebbistatin as an inactive form. We compared effects of (+)-blebbistatin on migration and Mg2+-ATPase activities with the effects of (±)-blebbistatin. As shown in Fig. 7A, the migratory activity was not affected by (+)-blebbistatin, although (±)-blebbistatin inhibited the migration. These data are compatible with those shown in Fig. 1A. Figure 7B also shows that the effect of (+)-blebbistatin on the smooth muscle myosin ATPase activities was absent, confirming the data shown in Fig. 4A.
Finally, our electron-microscopic observations show a marked alteration in the myosin II head after blebbistatin treatment. We interpret this to indicate that the site of blebbistatin action is within the heavy chain that comprises the myosin II head domain, which contains nucleotide and actin-binding sites and confers ATPase activity. We speculate that blebbistatin inhibits ATPase activity through an alteration of the conformation of the motor domain of myosin II, which also inhibits the interaction of myosin II with actin filaments and blocks actomyosin-involved cell motility.

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