Blebbistatin, a myosin II inhibitor, suppresses contraction and disrupts contractile filaments organization of skinned taenia cecum from guinea pig

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BLEBBISTATIN WAS FOUND as an agent to inhibit cytokinesis (31) and characterized as an inhibitor of myosin II by Sellers and colleagues (15, 16). The agent strongly inhibited most vertebrate striated muscle and nonmuscle myosin II ATPase activities at around 1 μM (16). Blebbistatin also suppressed the activities of myosin ATPase from vertebrate smooth muscles at around 10 μM (4, 13), although the agent was less sensitive to inhibiting turkey gizzard myosin ATPase activity (16). We, along with other investigators, also have found that blebbistatin inhibited the tissue and cell smooth muscle contraction at around 10 μM (4, 5, 13, 34). The inhibitory mechanisms of blebbistatin on the actin-myosin interaction have been thought to inhibit enzymatic activity of myosin heavy chain, resulting in interference of cross-bridge cycling (15, 16). In addition, our previous study showed that blebbistatin induced distinct conformational change of chicken gizzard myosin II and heavymeromyosin (HHM) irrespective of phosphorylation of myosin regulatory light chain (MLC20) at around 20 μM (13). The results indicated a possibility that conformational change of smooth muscle myosin (SMM) by blebbistatin spatially interferes with actin-myosin interaction of smooth muscle cells. In the present study, we thus hypothesized that conformational change of SMM by blebbistatin would induce disruption of the arrangement and structure of contractile filaments and inhibition of the ATPase activity of SMM simultaneously, causing suppression of the smooth muscle contraction without affecting the phosphorylation level of MLC20. To test the hypothesis, we studied the effects of blebbistatin on the mechanical properties, phosphorylation level of MLC20, and structure of the contractile filaments of skinned (cell membrane permeabilized) preparations from guinea pig taenia cecum. Blebbistatin at 10 μM or higher suppressed Ca2+-induced tension development at any given Ca2+ concentration but had little effects on the Ca2+-induced myosin light chain phosphorylation. Blebbistatin also suppressed the 10 and 2.75 mM Mg2+-induced, “myosin light chain phosphorylation-independent” tension development at more than 10 μM. Furthermore, blebbistatin induced conformational change of smooth muscle myosin (SMM) and disrupted arrangement of SMM and thin filaments, resulting in inhibition of actin-SMM interaction irrespective of activation with Ca2+. In addition, blebbistatin partially inhibited Mg2+-ATPase activity of native actomyosin from guinea pig taenia cecum at around 10 μM. These results suggested that blebbistatin suppressed smooth muscle contraction through disruption of structure of SMM by the agent.

smooth muscle; skinned preparations; contractile filaments; adenosinetriphosphatase

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All experimental procedures conformed to the “Guidelines for Proper Conduct of Animal Experiments” approved by the Science Council of Japan and were carried out under the “Rules and Regulation of the Animal Studies Committee of Tokyo Medical University” and “The Principles of Animal Care and Experimental Committee of Gunma University.” In addition, the institutional Animal Care and Use Committee of Tokyo Medical University and Gunma University approved all procedures involving animals. Male Hartley guinea pigs weighing from 250 to 500 g were euthanized under deep anesthesia with diethyl ether, and then the taenia cecum was removed.

Measurements of mechanical responses using skinned muscle preparations. A small muscle layer strip (100–200 μm wide and 3.0–4.0 mm long) was attached to a pair of tungsten wires with silk thread monofilaments, one of which was connected to a force transducer (BG-10; Kulite Semiconductor Products, Leonia, NJ) to measure isometric tension (36). To change the solution quickly, we used a bubble plate system with eight wells (0.135 ml each) (9). An intact muscle preparation was attached to the experimental chamber and then stretched to produce a passive tension of ~30 μN in physiological saline solution [PSS; 150 mM NaCl, 4 mM KCl, 2 mM Ca(methanesulfonate)2, 1 mM Mg(methanesulfonate)2, 10 mM glucose, and 5 mM HEPES, and pH was adjusted with Tris to pH 7.45 at 25°C] with 50 μM/μl insulin (Sigma, St. Louis, MO) to keep the preparations healthy (8, 29). The skinned (cell membrane permeabilization) procedure has been described elsewhere (19, 27, 38). Briefly, an intact preparation was treated with 200 μM β-escin (Sigma) for 20 min and

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with 20 μM Ca\(^{2+}\) ionophore A-23187 (Sigma) for 10 min in the relaxing solution. Note that the skinning procedure using β-escin might not critically affect functional or structural characterization of the SMM of the preparations, since even treatment with much higher concentrations of β-escin did not elute large cellular molecules such as myosin heavy chain from the smooth muscle preparations (36). To prevent serious deterioration of the skinned preparations and precipitation of blebbistatin in the solution, we maintained the experimental temperature at 30.0 ± 1.0°C. The skinned preparation was stretched in a relaxing solution [115 mM K(methanesulfonate), 1.2 mM Mg(methanesulfonate)\(^2\), 1.35 mM Na\(_2\)-ATP, 20 mM creatine phosphate, and 10 mM EGTA]. After the passive tension reached a steady level (resting tension, ~10 μN), the preparation was immersed in 10^{-4.55} M Ca\(^{2+}\) to elicit the maximal Ca\(^{2+}\)-induced contraction (control contraction). When the active tension increased to the maximal steady level, the preparation was relaxed by quickly lowering Ca\(^{2+}\) concentration within the relaxing solution and then reactivated with various concentrations of Ca\(^{2+}\) or 10 or 2.75 mM Mg\(^{2+}\) in the absence (vehicle, 1% DMSO) or presence of blebbistatin (test contraction).

Solutions and chemicals. Artificial intracellular solutions for skinned preparations were prepared according to the method of Horiuti (9). Solutions of various Ca\(^{2+}\)-containing concentrations were prepared by mixing the relaxing solution that contained 10 mM EGTA and the solution for maximal contraction that contained 10 mM EGTA and 10 mM Ca(methanesulfonate)\(^2\) in the appropriate proportion with the addition of 1 μM calmodulin (Wako Pure Chemicals, Osaka, Japan). The ionic strength was kept at 200 mM by adjusting the K(methanesulfonate) concentration, and pH was set to 7.0 with 20 mM PIPES and KOH at 30°C. All solutions contained 0.85 mM free Mg\(^{2+}\) and 1.0 mM MgATP (8, 40). Mg\(^{2+}\) and MgATP concentrations were chosen based on those measured in the intact taenia cecum (20, 33). To keep the MgATP supply sufficient, we chose concentrations of creatine phosphate (20 mM; Ref. 8) about 10 times higher than that in the intact taenia cecum preparations (20). The apparent dissociation constant of Ca\(^{2+}\)-EGTA was assumed to be 10^{-6.4} M. The 10 and 2.75 mM Mg\(^{2+}\) and MgCTP solutions, which were used for MLC\(_{20}\) phosphorylation-independent contraction, contained 10 and 2.75 mM Mg\(^{2+}\), respectively. Also, the solutions contained 1.0 mM MgCTP (1.4 mM total Na-CCTP; Sigma), 20 mM creatine phosphate, and 10 mM EGTA (38), since MgCTP is a poor substrate for several kinase reactions including MLC\(_{20}\) phosphorylation but strongly activates myosin ATPase (2, 6). S(-)-blebbistatin was purchased from Toronto Research Chemicals (North York, ON, Canada) and used as an active form of blebbistatin. According to Shu et al. (30), S(-)-blebbistatin is a mixture of 59% (-)-enantiomer and 41% (+)-enantiomer (30). It is known that the active isomer of blebbistatin is (-)-blebbistatin, and (+)-blebbistatin had no effects on ATPase activities of various types of myosin II (4, 30, 31, 34), cell migration of cultured smooth muscles (34), or contraction of intact smooth muscles (4). All other chemicals were reagent grade.

Data analysis of the mechanical properties. The developed tension levels of the test contraction of skinned preparations are expressed as follows:

\[
\text{relative tension} = \left(\frac{\text{observed tension of test contraction}}{\text{maximum tension of control contraction}}\right) \times \left(\frac{\text{resting tension}}{\text{resting tension}}\right)
\]

To estimate blebbistatin concentration for the half-maximal effect of the relative tension level of the test contraction (ED\(_{50}\)), we carried out nonlinear least-squares fitting by a modified Hill equation with the program Kaleida Graph (Synergy Software, Reading, PA) using the Levenberg-Marquardt algorithm:

\[
\text{relative tension} = F_{\text{min}} + \left(\frac{F_0 - F_{\text{max}}}{[\text{blebbistatin}]^{n_s}/([\text{blebbistatin}]^{n_s} + [\text{blebbistatin}]^{n_s} + [\text{blebbistatin}]^{n_s})}\right)
\]

where \(F_0\), \(F_{\text{min}}\), and \([\text{blebbistatin}]^{n_s}\) denote a relative tension level of the test contraction in the absence of blebbistatin, the maximally suppressed relative tension level of the test contraction by blebbistatin, and the blebbistatin concentration for half-maximal inhibition of the relative tension, respectively. The Hill coefficient \(n_s\) is a measure of the slope.

Ca\(^{2+}\) sensitivity for the Ca\(^{2+}\)-induced contraction of skinned preparations was also estimated by data fitted to the Hill equation:

\[
\text{relative tension} = F_{\text{max}} \times \left(\frac{[\text{Ca}^{2+}]^{n_s}/([\text{Ca}^{2+}]^{n_s} + [\text{Ca}^{2+}]^{n_s})}{F_{\text{max}} + [\text{Ca}^{2+}]^{n_s}}\right)
\]

where \(F_{\text{max}}\) is the relative tension level of the maximal Ca\(^{2+}\)-induced contraction of the test contraction and \([\text{Ca}^{2+}]^{n_s}\) denotes the Ca\(^{2+}\) concentration for half-maximal relative tension level of the test contraction activated with Ca\(^{2+}\).
antibody and FITC(488)-phalloidin (fluorescein; Invitrogen) targeted to SMM and F-actin, respectively, and then treated with fluorescent probe Alexa 546 IgG (Invitrogen) secondary antibody to designate F-actin (donor) and SMM (acceptor) as the components of the FRET system. Initially, F-actin was imaged with a 522DF32 band-pass filter at 3% laser power. The acceptor, SMM, was photobleached with 100% laser power using the 568-nm laser line for 45–60 min until the signal was lost. A second image was recorded using the same donor setting to evaluate protein interaction. Cell pixel intensity data were collected using ImageJ software and analyzed with PeakFit software version 4.11 (SPSS Science, Chicago, IL) for the before and after photobleaching image. Paint Shop Pro software version 7.0 (Corel, Ottawa, ON, Canada) was used to show the difference between before and after photobleaching (FRET response). Relative increases in fluorescence for vehicle- or blebbistatin-treated preparations were presented as indexes of the FRET responses.

Electron microscopic observation. After mechanical measurements were made, the skinned preparations were prefixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 5 min at 4°C, and then the preparations were removed from the force measurement apparatus and immersed in the same solution at 4°C for 5 min. After being washed with 10% sucrose in cacodylate buffer (pH 7.3), the preparations were postfixed with 10% sucrose and 1% OsO4 in the cacodylate buffer (pH 7.3) for 2 h at 4°C. The preparations were then stained with 0.5% aqueous uranyl acetate and Epon 812 (Nissin, Tokyo, Japan). Thin sections (~100 nm thick) of the stained preparations were cut with a diamond knife (Micro Star Technologies, Huntsville, TX) and further stained with 0.5% uranyl acetate and 0.4% lead citrate. Finally, the stained preparations were observed with an electron microscope (JEM-1010; JEOl, Tokyo, Japan). To measure the size of the dot-like structures, we used the image analysis software Scion Imaging (Scion, Frederick, MD).

Immuno-electron microscopy. Analysis of immuno-electron microscopic images was carried out using the methods described by Tanaka et al. (32). Briefly, the skinned preparations that had been used for mechanical measurements were fixed with 2% paraformaldehyde and then frozen with liquid nitrogen. Sections of the fixed preparations were cut and placed on acrylamide-coated glass slides. After incubation in PBS with 5% BSA for 3 h at 4°C, the sections on the glass slides were reacted with anti-SMM antibody (Sigma) as the primary antibody and then incubated with secondary antibody conjugated with 6-nm gold particles (Jackson ImmunoResearch). The immunogoldstained sections on acrylamide sheets were peeled from the glass slides and then processed for electron microscopic observation.

Measurement of Mg2+-ATPase activity of native actomyosin from guinea pig taenia cecum. A Ca2+-sensitive native actomyosin (myosin B) preparation, which contained a calmodulin-myosin light chain kinase and phosphatase system, was prepared from guinea pig taenia cecum in accordance with Ozaki et al. (21). Briefly, intact taenia cecum (~500 μg) was homogenized and blended with four volumes of solution containing 400 mM KCl, 20 mM Tris·HCl (pH 7.5), 5 mM ATP, and 0.1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (p-ABSF; Wako) to inhibit protease activities. The material was centrifuged at 10,000 g for 5 min. ATP (10 mM) was added to the supernatant solution, which was recentrifuged at 80,000 g for 30 min. The supernatant was then dialyzed against a ninefold solution containing 1 mM NaHCO3, 1 mM MgCl2, 1 mM NaN3, 1 mg/ml DTT, and 0.1 mM p-ABSF for 4 h. Dialyzed material was centrifuged at 10,000 g for 5 min. Native actomyosin preparation was obtained after being washed twice in 50 mM KCl, 1 mM MgCl2, and 1 mM NaHCO3. The Mg2+-ATPase reaction of native actomyosin was carried out at 30°C in a medium containing 1 mg protein/ml actomyosin, 50 mM KCl, 10 mM MgCl2, 20 mM Tris-maleate (pH 6.8), and Ca2+-EGTA buffer for 3 min. Inorganic phosphate liberated during a 3-, 5-, or 10-min incubation was determined with the EnzChek phosphatase assay kit (Invitrogen). Blebbistatin concentration for the half-maximal effect on actomyosin ATPase activity was estimated by data-fitting analysis to the modified Hill equation described above.

Statistical analysis. Results are means ± SE. Statistical hypotheses on the differences between means were tested with Student’s t-test for paired samples or one-way ANOVA with post hoc analysis using Kaleida Graph 3.6J. The null hypotheses were rejected when P was <0.05.

RESULTS

Effects of blebbistatin on the Ca2+-induced contraction of skinned preparations. Figure 1A presents typical tension records of the β-escin skinned preparations of taenia cecum from guinea pig. When a smooth muscle preparation was activated with 2 μM Ca2+ and 1 μM calmodulin, the active tension quickly developed and reached a sustained level within 2 min. In the presence of blebbistatin at a concentration of 10 μM or higher, the active tension development was irreversibly suppressed. On the other hand, blebbistatin did not affect the resting (passive) tension at all, even when treated at 100 μM (data not shown). Therefore, blebbistatin seemed to interfere with the actin-myosin interaction of the contractile filaments, rather than changing the function and/or structure of the cytoskeleton and the other elastic components of the skinned taenia cecum. Blebbistatin at 10 μM or higher also suppressed the active tension when the agent was applied after the developed force reached the sustained level (data not shown).

Figure 1B shows the blebbistatin effects on the relationship between Ca2+ concentration and the active tension. Blebbistatin partially reduced the Ca2+-induced tension development at any given concentration of Ca2+. Data fitting to the Hill equation indicated that blebbistatin inhibited Fmax without changing the Hill coefficient nH and [Ca2+]50, indexes of the Ca2+ sensitivity for the active tension (Table 1). The estimated ED50 value for inhibition of Fmax was 6.94 ± 3.60 μM.

Fig. 1. A: typical tension traces of skinned taenia cecum. The preparation was activated with 2 μM Ca2+ and 1 μM calmodulin. Blebbistatin at 10 μM or higher clearly suppressed the Ca2+-induced tension development at 30.0 ± 1.0°C. B: effects of blebbistatin on the Ca2+-concentration-relative tension relationship. Blebbistatin was applied for 3 min before activation and for 10 min during subsequent activation with Ca2+. Data were fitted to the modified Hill equation (straight or dotted lines). Values are means ± SE of 6–7 experiments. *P < 0.05, significant difference of the active force compared with control.
Table 1. Effects of blebbistatin on the F_{max}, Hill coefficient, and [Ca^{2+}]_{50}

<table>
<thead>
<tr>
<th>F_{max}</th>
<th>Vehicle</th>
<th>3 μM</th>
<th>10 μM</th>
<th>30 μM</th>
<th>100 μM</th>
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<tr>
<td>0.826 ± 0.055</td>
<td>0.827 ± 0.049</td>
<td>0.646 ± 0.031*</td>
<td>0.539 ± 0.084*</td>
<td>0.614 ± 0.028*</td>
<td></td>
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<tr>
<td>n_H</td>
<td>3.17 ± 1.32</td>
<td>2.44 ± 1.19</td>
<td>4.77 ± 0.64</td>
<td>5.21 ± 2.01</td>
<td>4.82 ± 0.79</td>
</tr>
<tr>
<td>[Ca^{2+}]_{50}, μM</td>
<td>0.60 ± 0.15</td>
<td>0.82 ± 0.07</td>
<td>0.69 ± 0.10</td>
<td>0.85 ± 0.03</td>
<td>0.83 ± 0.09</td>
</tr>
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Relation between Ca^{2+} concentration and Ca^{2+}-induced tension was fitted to the Hill equation. F_{max}, tension level of the maximal Ca^{2+}-induced contraction; n_H, Hill coefficient; [Ca^{2+}]_{50}, Ca^{2+} concentration for the half-maximal Ca^{2+}-activated tension. Vehicle was 1% DMSO. *P < 0.05, significant difference of parameters compared with control.

To determine whether blebbistatin affects MLC_{20} phosphorylation/dephosphorylation processes, we measured the MLC_{20} phosphorylation level of the skinned preparations used for the force measurement. No phosphorylated MLC_{20} was detected when the concentration of Ca^{2+} in the solution was 10^{-7.0} M or lower, as previously shown (8), and the increase of the MLC_{20} phosphorylation level was dependent on Ca^{2+} concentration (data not shown). Blebbistatin did not significantly change the MLC_{20} phosphorylation level at any given Ca^{2+} concentration (P > 0.34).

Effects of blebbistatin on the high Mg^{2+}-induced contraction of skinned preparations. Pharmacological concentrations of Mg^{2+} are known to induce force development (12, 18, 25, 38) without changing the phosphorylation level of MLC_{20} (12, 18). As shown in Fig. 2A, 10 mM Mg^{2+} with 1.0 mM Mg_{CTP} induced an increase in the tension with a short time lag (~45 s). On the other hand, 10 mM Mg^{2+} did not induce phosphorylation of MLC_{20} at all, irrespective of blebbistatin (data not shown). Blebbistatin significantly suppressed the 10 mM Mg^{2+}-induced tension development at concentrations higher than 10 μM (Fig. 2B), and the estimated ED_{50} for the relative force inhibition was 12.5 μM (Fig. 2B). Blebbistatin did not affect the short time lag (Fig. 3A). Blebbistatin at 30 μM also significantly suppressed very slow tension development induced by much lower concentrations of Mg^{2+} (Fig. 4), indicating that the agent suppressed not only the steady-state cross-bridge cycling but also the initial rate of the force development.

Effects of blebbistatin on the myofilament localization of skinned taenia cecum. Since several myosin II inhibitors, such as 2,3-butanedione monoximine (BDM) or N-benzyl-N-nitroso-p-toluenesulfonamide (BTS), disrupt the organization and filamentous structure of myosin and actin in the striated myotube (10, 23), we examined the effects of blebbistatin on the localization of contractile filaments of skinned smooth muscle preparations of taenia cecum (Fig. 4). In the control experiments, F-actin and anti-SMM antibody labeled with anti-mouse IgG conjugated with rhodamine red-X and FITC-phalloidin, respectively, were almost entirely colocalized when the preparation was activated with Ca^{2+} (Fig. 4F). On the other hand, in the presence of 30 μM blebbistatin, F-actin and anti-SMM antibody were only partially colocalized even when activated with Ca^{2+} (Fig. 4L). These results indicate that, in the presence of 30 μM blebbistatin at which Ca^{2+}-activated contraction was maximally inhibited, most of the SMM and F-actin in the skinned taenia cecum were separately localized even when activated with Ca^{2+}. Note that neither nonmuscle myosin IIA (NMM-A) nor IIB (NMM-B) was detected by immunostaining irrespective of Ca^{2+} (data not shown).

Fig. 2. A: typical tension traces of 10 mM Mg^{2+}-induced contraction. To prevent activation of phosphorylation-mediated cellular mechanisms, including MLC_{20} phosphorylation, ATP was substituted for CTP. B: effects of blebbistatin on the relative tension level of 10 mM Mg^{2+}-induced contraction. Blebbistatin was applied for 3 min before activation and then for 10 min during activation with 10 mM Mg^{2+}. Data were fitted to the modified Hill equation (dotted line). ED_{50}, blebbistatin concentration for the half-maximal effect of the relative tension level of the test contraction. Values are means ± SE of 6 experiments. *P < 0.05, significant difference of the force compared with control.

Fig. 3. Effects of blebbistatin on the slow tension developments induced by 2.75 mM Mg^{2+}. Values are means ± SE of 5 experiments. To prevent activation of phosphorylation-mediated cellular mechanisms, including MLC_{20} phosphorylation, ATP was substituted for CTP. *P < 0.05, significant difference of the force compared with control.
FRET analysis of association of F-actin and smooth muscle-myosin. To determine whether actin-SMM interaction in skinned smooth muscles was really interfered with by blebbistatin treatment, we analyzed confocal images of immuno-histochemically stained skinned taenia cecum using FRET (14, 34), which showed a clear increment of fluorescent intensity of F-actin after photobleaching of SMM only when the SMM interacted with the F-actin (Fig. 5). FRET, on average, showed that contracted preparations increased F-actin fluorescence (175.7 ± 2.1%). By comparison, FRET values of relaxed, relaxed and blebbistatin-treated, and Ca²⁺-activated and blebbistatin-treated preparations were 110.9 ± 1.2, 106.0 ± 0.9,

Fig. 4. Representative confocal images of skinned taenia cecum preparations. After mechanical measurements were made, relaxed (A, B, C, G, H, and I) and 5 µM Ca²⁺-activated skinned taenia cecum preparations (D, E, F, J, K, and L) were fixed and then stained with FITC-phalloidin (A, D, G, and J) and anti-smooth muscle myosin (anti-SMM) antibodies (B, E, H, and K). C, F, I, and L are superimposed images of A and B, D and E, G and H, and J and K, respectively. A–F: vehicle without blebbistatin (1% DMSO). G–L: 30 µM blebbistatin. Scale bar, 2 µm.

Fig. 5. Fluorescence resonance energy transfer (FRET) analysis of the association of F-actin and SMM. Relaxed (A, B, C, G, H, and I) and 5 µM Ca²⁺-activated skinned taenia cecum preparations (D, E, F, J, K, and L) were imaged under identical donor settings using the 488-nm laser line before (A, D, G, and J) and after (B, E, H, and K) photobleaching of SMM. Differences between before and after photobleaching are depicted in C, F, I, and L. A–F: vehicle (1% DMSO). G–L: 30 µM blebbistatin. Scale bar, 2 µm.
and 106.0 ± 0.9%, respectively. Statistical analysis performed using Dunn’s procedure for multiple comparison showed that the average responses of these three conditions were significantly lower than that of the Ca²⁺-activated condition (P < 0.0001, n = 10), but there were no significant differences among the three conditions (P > 0.09). These results suggest that the interaction of actin and SMM by Ca²⁺-induced activation was clearly interfered with by the blebbistatin treatment.

Effects of blebbistatin on the filamentous structure of skinned taenia cecum preparations. We also examined the blebbistatin effects on the filamentous structure and organization of the contractile filaments of skinned taenia cecum. Figure 6 shows typical electron microscopic images of longitudinal section of the skinned taenia cecum. There are dot-like structures and filamentous structures in the images. Immunogold particles conjugated with anti-SMM bound to dot-like structures (Fig. 6F), suggesting that most of the dot-like structures were heads of SMM. On the other hand, the filamentous structures seemed to be thin filaments because of their size. In the resting states, the orientation of the dots and thin filaments was not so regular (Fig. 6A). During contraction, the dots and the thin filaments orientated regularly compared with those in the resting states (Fig. 6C). However, in the presence of blebbistatin at 30 μM, the orientation of the thin filaments and the dots was still irregular even during activation (Fig. 6D). In addition, the dot size of the skinned preparations treated with 30 μM blebbistatin was significantly larger than that of those treated with vehicle (1% DMSO) irrespective of Ca²⁺ (Fig. 7).

Effects of blebbistatin on the actomyosin ATPase activity. Figure 8 shows a relationship between blebbistatin concentrations and relative Mg²⁺-ATPase activity of native actomyosin from taenia cecum. Blebbistatin partially inhibited actomyosin ATPase activity in the presence of 20 μM Ca²⁺, and the estimated ED₅₀ value for the inhibition by blebbistatin was...
and disrupted organization of the contractile filaments and inhibited active tension development, changed SMM structure, ATPase activities of native actomyosin (data not shown). 10 min) did not affect blebbistatin effects on the relative blebbistatin effects among the studies have been discussed (4, have been studied by several groups, and large differences of susceptibility of the blebbistatin effects on the smooth muscle blebbistatin on the smooth muscle contraction, we must note the idea that animal and/or tissue species and type of smooth muscle cells are the major determinants of the sensitivity for blebbistatin effects on the smooth muscle contractile function, whereas straight-tail 6S SMM monomers, which assemble SMM filaments easily, release these products (3). There-fore, blebbistatin-induced force suppression of skinna taenia cecum seemed to at least partially arise from the spatial separation of the thick and thin filaments by the disruption of the organization of the contractile filaments (Figs. 4 and 6). In fact, analysis of FRET by immunohistochemistry of stained preparations indicated that interaction of F-actin and SMM during activation with Ca2+ was clearly inhibited by treatment with blebbistatin (Fig. 5).

There are several possible mechanisms for disruption of the thick filaments’ organization of skinned taenia cecum by blebbistatin. Generally, stabilization of SMM filaments is correlated to the phosphorylation level of MLC20 (28). Although the thick filaments abundantly exist in living and skinned smooth muscle cells even in the resting states (7, 37, 39), further formation of the thick filaments in the intact and skinned preparations during activation was observed in several types of smooth muscle, such as porcine and canine trachea and rat anococcygeus (7, 28, 37, 39). In fact, Qi et al. (22) showed that MLC20 phosphorylation at least partially regulated the thick filament formation in living smooth muscles. However, blebbistatin-induced disruption of the thick filament arrangement in skinned taenia cecum (Fig. 6) seemed to be independent of MLC20 phosphorylation/dephosphorylation, since blebbistatin had no significant effects on MLC20 phosphorylation level at any concentration of Ca2+ (data not shown).

Another possibility is that changes in kinetics of ATP binding and/or releasing of its hydrolyzing substances in SMM by blebbistatin induced disruption of the thick filament organization. When MLC20 is unphosphorylated, ATP binding to purified SMM is known to lead to changes in SMM conformation from the tail-extended 6S form to the tail-folded 10S form. Folded-tail 10S SMM traps ADP and inorganic phosphate, whereas straight-tail 6S SMM monomers, which assemble SMM filaments easily, releases these products (3). Therefore, ATP binding and release of ADP and inorganic phosphate in SMM is closely related to the conformation of the SMM molecule. Interestingly, blebbistatin specifically binds to the ATP binding region of myosin II heads and blocks the release of inorganic phosphate from the myosin heads; hence, it inhibits force generation of striated muscles (15). Furthermore, our previous study presented that blebbistatin induced conformational changes in the myosin heads structure of purified chicken gizzard irrespective of MLC20 phosphorylation (34).
In fact, the present study showed that blebbistatin significantly increased the size of the dot-like structure irrespective of Ca^{2+} concentration (Figs. 6 and 7). Since conformational change of the myosin head by blebbistatin is thought not to enlarge the size of the myosin head itself (34), in skinned taenia cecum, blebbistatin might bend myosin molecules as observed in vitro (Fig. 6; Ref. 34) or induce similar conformational changes of the myosin molecules. Therefore, blebbistatin-induced disruption of the arrangement of the thick filaments of skinned taenia cecum preparations (Figs. 4 and 6) seems to result from SMM bending or similar conformational changes of the SMM molecules.

Interestingly, the maximal inhibitory effects of blebbistatin treatment on the active force of skinned preparations and the Mg^{2+}-ATPase activity of native actomyosin was much smaller than those on the Mg^{2+}-ATPase activity of purified smooth muscle (34). Also, in the absence of actin, blebbistatin effects on the myosin ATPase activity were much more potent than those in the presence of actin (Fig. 4; Ref. 34). Actin filaments and their binding proteins, such as caldesmon, are known to have stabilizing effects on the myosin filaments in vitro (1, 11, 17). Thus the inhibitory effects of blebbistatin on the mechanical force of the skinned preparations and also actomyosin ATPase activity are at least partially due to the disturbance of the thick filament organization as discussed above; it is therefore likely that the thin filaments interfered with the disruption of the thick filaments’ organization of skinned preparations by blebbistatin treatment, resulting in relatively less potent effects of the agent on the mechanical activity of the skinned preparations.

Blebbistatin disrupted not only the arrangement of myosin heads but also the thin filament organization both in the resting states and during Ca^{2+} activation (Fig. 6). Earlier reports using the striated myotube indicated that BTS- and BDM-induced disruption of the thick and thin filament organization during myogenensis was due to a failure of cross-bridge cycling (10, 23), since BDM and BTS are thought to have little effect on thin filaments directly. However, in the present study, disruption of the thin filament orientation was still observed even in the resting states in which actin and myosin should not interact strongly, suggesting that mechanisms other than interference with cross-bridge cycling concerned the blebbistatin-induced disruption of the thin filament organization. Because blebbistatin, as well as BDM and BTS, might have little effect on thin filaments, it is possible that some regulatory proteins (e.g., caldesmon, calponin, and myosin light chain kinase), which can bind with both actin and myosin in the physiological conditions, transmit mechanical and/or chemical information of disruption of the myosin filament structure induced by blebbistatin into the thin filaments. Further studies are necessary to explore precise mechanisms of disruption of the thin filament organization by blebbistatin treatment.

In conclusion, in skinned smooth muscles of taenia cecum, blebbistatin simultaneously inhibited F-actin-SMM interaction, force development, and organization of contractile filaments. Since remodeling of contractile filaments is thought to be a key regulatory mechanism of smooth muscle contraction (28), blebbistatin may be a useful tool to understand smooth muscle contractile mechanisms.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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