Original

Blebbistatin, a myosin II inhibitor, suppresses Ca²⁺-induced and "sensitized"- contraction of skinned tracheal muscles from guinea pig

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Abstract

Blebbistatin, a potent inhibitor of myosin II, has inhibiting effects on Ca^{2+}-induced contraction and contractile filament organization without affecting the Ca^{2+}-sensitivity to the force and phosphorylation level of myosin regulatory light chain (MLC_{20}) in skinned (cell membrane permeabilized) taenia cecum from the guinea pig (Watanabe et al., Am J Physiol Cell Physiol. 2010; 298: C1118–26). In the present study, we investigated blebbistatin effects on the contractile force of skinned tracheal muscle, in which myosin filaments organization is more labile than that in the taenia cecum. Blebbistatin at 10 µM or higher suppressed Ca^{2+}-induced tension development at any given Ca^{2+} concentration, but had little effects on the Ca^{2+}- induced myosin light chain phosphorylation. Also blebbistatin at 10 µM and higher significantly suppressed GTP-γS-induced "sensitized" force development. Since the force inhibiting effects of blebbistatin on the skinned trachea were much stronger than those in skinned taenia cecum, blebbistatin might directly affect myosin filaments organization.

Key words: airway smooth muscle, skinned preparations, contractile filaments, blebbistatin

Introduction

Blebbistatin was found as an inhibitor of myosin II by Sellers and his colleagues (1, 2). This agent strongly inhibited most vertebrate striated muscle- and non-muscle myosin II ATPase activities (2) as well as vertebrate smooth muscle myosin (SMM) ATPase activity (3, 4). Several groups, including us, have also found that blebbistatin inhibited the smooth muscle preparations and smooth muscle cell contraction at around 10 µM (3–7). The inhibitory mechanism on the actin-myosin interaction has been thought to be due to inhibition of myosin

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ATPase resulting in interference of cross-bridge cycling (1, 2). Also previous studies have indicated that conformational change of SMM by blebbistatin spatially interferes with the actin-myosin interaction of smooth muscle cells (4, 5, 7). Blebbistatin simultaneously inhibited F-actin-SMM interaction, force development and organization of contractile filaments in skinned smooth muscles of the guinea pig taenia cecum (5).

Myosin filament lability is thought to be different between different smooth muscle preparations (8, 9). The amount of myosin filaments was changed during contraction-relaxation cycles in several types of smooth muscle including airway muscles, but not in taenia cecum (9–11). Therefore, we hypothesized that the action of blebbistatin on the skinned muscle contraction in airway preparations might be different from those in taenia cecum preparations. To test this hypothesis, we examined the effects of blebbistatin on the contraction of guinea pig tracheal skinned preparations. Also we investigated the effects of this agent on the GTP-γS-induced "sensitized" skinned tracheal preparations, since the sensitizing mechanisms through G-protein coupled pathways are major mediators of contraction of airway smooth muscles physiologically and patho-physiologically (12, 13).

A preliminary report of this study has been submitted in an abstract form (14).

Materials and Methods

Animal experiments were performed at Tokyo Medical University and Tokyo Metropolitan University. Animal 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 regulations of the animal studies committee of Tokyo Medical University and the research ethics committee of Tokyo Metropolitan University. In addition, Tokyo Medical University and Tokyo Metropolitan University approved all procedures involving animals. Hartley guinea pigs weighing from 200 to 500 g were sacrificed under deep anesthesia with diethyl ether. A small muscle layer strip (1–2 mm wide and 3 mm long) was prepared by cutting off the tracheal cartilage and stripping connective tissue from the specimen. The preparation 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, USA) to measure isometric tension (15, 16). A bubble plate system with eight wells (0.135 ml each) was used to change the solution quickly (17). The skinning (cell membrane permeabilization) procedure was described elsewhere (15, 18, 19). Briefly, an intact tracheal muscle preparation was treated for 20 min with 200 µM β-escin (Sigma, St. Louis, MO, USA) and for 10 min with 20 µM Ca ionophore A23187 (Sigma) in the relaxing solution. To prevent serious deterioration of the skinned preparations and precipitation of blebbistatin (Toronto Research Chemicals Inc, North York, ON, Canada) from the solution, the experimental temperature was maintained at 30.0 ± 1.0 °C (5). 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 (Roche, Indianapolis, IN, USA), 20 mM phosphocreatine (Nacalai Tesque, Kyoto, Japan), and 10 mM ethylene glycol-bis(2-aminoethyl) tetraacetic acid(EGTA, Nacalai). After the passive tension reached a steady level (basal tension; ~10 µN), the preparation was immersed in 10 µM Ca^{2+} to elicit the maximal Ca^{2+}-induced contraction (control contraction). When the active tension attained a maximal steady level (the maximal Ca^{2+}-induced tension), the preparation was relaxed by quickly lowering the Ca^{2+} concentration with the relaxing solution and then reactivated with various concentrations of Ca^{2+} in the absence or presence of blebbistatin (test contraction). In some experiments, we applied 1 mM GTP-γS (Roche) to the reactivating solutions to test the effects of blebbistatin on the sensitization of the contractile elements. Also tautomycin (Wako Pure chemicals, Osaka, Japan), a myosin phosphatase inhibitor, was used to produce irreversible activation of the skinned preparations.

Artificial intracellular solutions for skinned preparations were prepared according to the method of Horiuti (17). Solutions of various Ca^{2+} 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 addition of 1 μM calmodulin (Wako). The apparent dissociation constant of Ca^{2+}-EGTA was assumed to be $10^{6.4}$ /M.

Data analysis of the mechanical properties

The developed tension levels of the test contraction of skinned preparations were expressed as; relative tension = (an observed tension of the test contraction – the basal tension)/(the maximal tension of the control contraction – the basal tension)

To estimate blebbistatin concentration for half maximal effect of the active tension (ED_{50}), data were fitted to a modified Hill equation with the program Kaleida Graph (Synergy Software, Reading, PA, USA) using the Levenberg-Marquardt algorithm:

Relative tension = $F_{min} + (F_0 - F_{min}) \times [blebbistatin]^n/([blebbistatin]_{50}^n + [blebbistatin]^n)$, where F_0, F_{min} and $[blebbistatin]_{50}$ denote an active tension level in the absence of blebbistatin and the minimal tension level by blebbistatin induced force suppression, and blebbistatin concentration for half maximal inhibition of active tension respectively. The Hill coefficient (n) is a measure of the slope.

Ca^{2+} sensitivity for the Ca^{2+}-induced contraction of skinned preparations was also estimated by data fitting to the Hill equation; Relative tension = $F_{max-Ca^{2+}} \times [Ca^{2+}]^n/([Ca^{2+}]_{50}^n + [Ca^{2+}]^n)$, where $F_{max-Ca^{2+}}$ is the tension level of the maximal Ca^{2+}-induced contraction, and $[Ca^{2+}]_{50}$ denotes Ca^{2+} concentration for the half maximal Ca^{2+} activated tension.

Measurement of myosin regulatory light chain (MLC_{20}) phosphorylation

After Ca^{2+} exposure for 10 min, the skinned- preparations, which were still attached to the tungsten wires of the force measurements apparatus, were quickly fixed by immersion in iced-cold trichloroacetic acid (TCA; Wako) at 10% in acetone containing 10 mM dithiothreitol (DTT; Wako) for 15 min. Then the preparations were removed from the force measurements apparatus, and then washed out three times to remove the TCA with acetone containing 10 mM DTT. The dried preparations were then incubated in urea-sample buffer (containing 20 mM Trizma base, 10 mM DTT, 8 M urea, and 0.1% bromophenol blue) for 8 h at 4 °C. The extracts were subjected to glycerol-PAGE coupled with Western blot (4, 20, 21). MLC_{20} was detected with anti-MLC_{20} antibody (gift of Dr. S. Yoshiyama of Gunma University) and visualized with horseradish peroxidase-conjugated anti rabbit IgG (GE Healthcare UK, Buckinghamshire, UK) and enhanced chemiluminescence (ECL) Western blotting detection system (ECLplus, GE Healthcare, Buckinghamshire, England). The contents of un-, mono-, and doubly-phosphorylated MLC_{20} were quantitatively measured using a densitometer (Densitograph®, ATTO Co, Tokyo, Japan). The phosphorylation level of MLC_{20} was calculated as follows: Phosphorylation level = (T – U)/T, where T and U are densities of total- and unphosphorylated-MLC_{20}, respectively.

Statistical Analysis

Results are presented as the mean ± standard error (SEM). Statistical hypotheses on the differences between means were tested with Student's t-test for paired samples unless noted otherwise. The null hypotheses were rejected when P was less than 0.05.
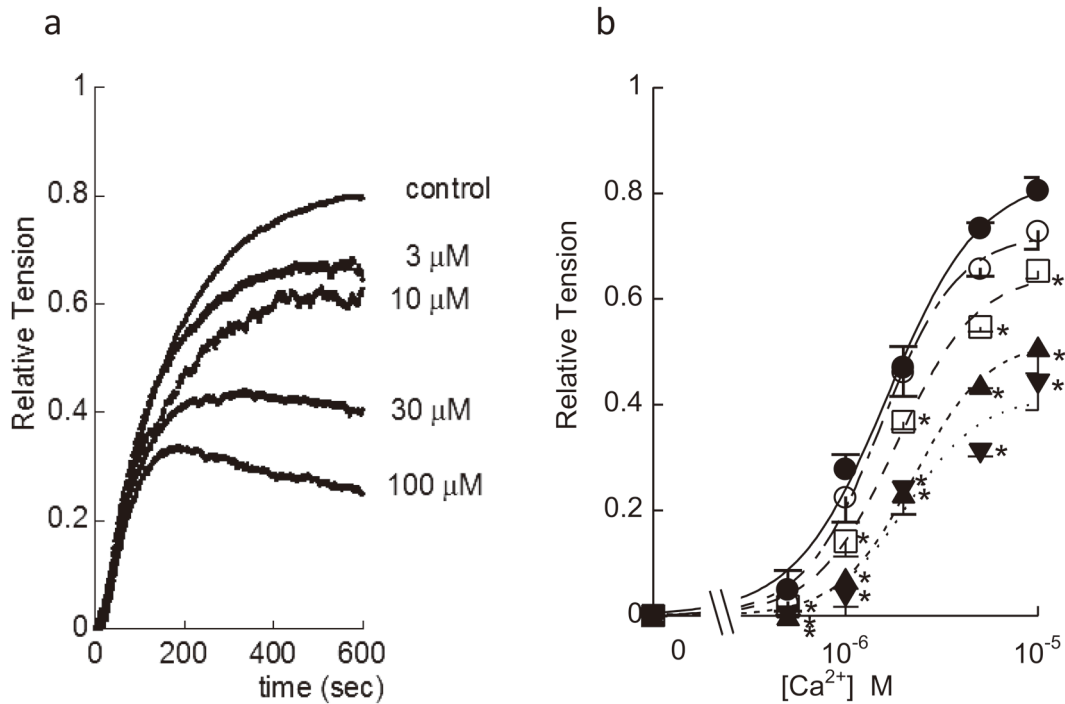
Fig. 1. a: Typical tension traces of skinned tracheal muscle preparations. Each preparation was activated with 10 μM Ca^{2+} and 1 μM calmodulin. Blebbistatin at 10 μM or higher clearly suppressed the Ca^{2+}-induced tension development. 30.0 ± 1.0 °C. b: Effects of blebbistatin on the Ca^{2+} concentration-relative tension relationship. Blebbistatin was applied for 3 min before activation and for 10 min during subsequent activation with Ca^{2+}. 1% DMSO (control; ●), or 3 μM (○), 10 μM (□), 30 μM (▲) or 100 μM (▼) blebbistatin was added to the artificial intracellular solutions. Data were fitted to the modified Hill equation (straight or dotted lines). Values are the mean ± SEM of 6–7 experiments. Asterisk indicates a significant difference of the active force compared with that of control, where P values are less than 0.05.

Results

Effects of blebbistatin on the Ca^{2+}-induced contraction of skinned preparations

Fig. 1a represents typical tension record of β-escin skinned preparations of tracheal smooth muscle from the guinea pig. When a muscle preparation was activated with 10 μM Ca^{2+} and 1 μM calmodulin, the active tension gradually developed and reached a sustained level within 600 sec. 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 basal (passive) tension at all, even when treated with 100 μM (data not shown). 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).

Fig. 1b shows the effects of blebbistatin on the relationship between Ca^{2+} concentration and active tension. Blebbistatin partially reduced the Ca^{2+}-induced tension development at any given concentration of Ca^{2+}. Data fitting to the Hill equation indicated that blebbistatin inhibited $F_{max-Ca^{2+}}$ without changing the Hill coefficient n and $[Ca^{2+}]_{50}$, indices of the Ca^{2+} sensitivity for the active tension (Table 1). The estimated $[blebbistatin]_{50}$ (ED_{50} for blebbistatin) value for inhibition of $F_{max-Ca^{2+}}$ was 11.6 ± 2.58 μM and F_{min} (the minimal active tension level by blebbistatin induced force suppression) /F_0 (active tension level in the absence of blebbistatin) was 0.53 ± 0.13.
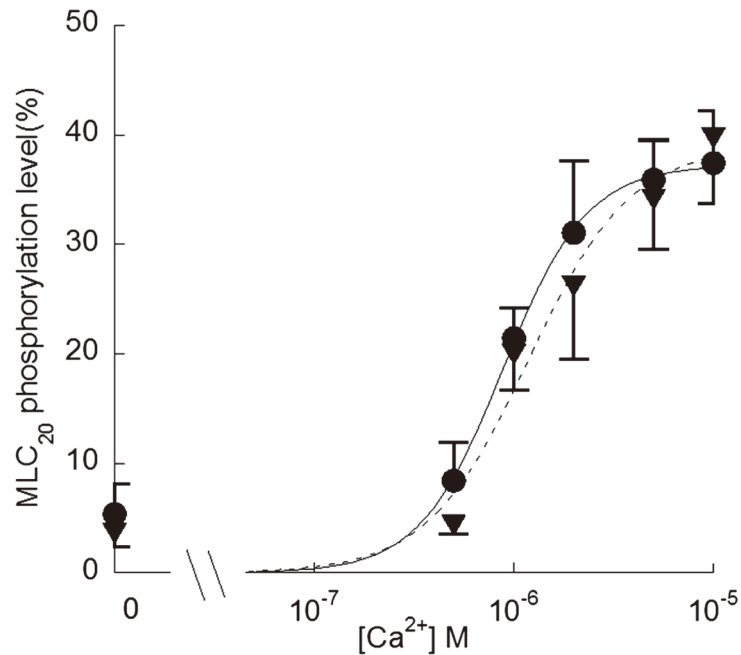
Fig. 2. The relationship between Ca^{2+} and MLC_{20} phosphorylation levels of the skinned tracheal muscle preparations in the presence ($\blacktriangledown$) or absence ($\bullet$) of 100 μM blebbistatin. Blebbistatin did not change the myosin light chain (MLC_{20}) phosphorylation level at any concentrations of Ca^{2+}. The extracts of muscle strips were subjected to 15% glycerol-PAGE coupled with Western blot. MLC_{20} was detected with anti-MLC_{20} antibody and visualized with horserad-ish peroxidase-conjugated anti-rabbit IgG. For more details, see Materials and Methods. The contents of unphosphorylated and phosphorylated MLC_{20} were quantitatively measured and analyzed using a computer-based densitometer system. The percentage of MLC_{20} phosphory-lation was determined by the ratio of phosphorylated MLC_{20} to the total MLC_{20} densitometri-cally determined. Values are expressed as the mean ± SEM of 5–7 experiments. 30.0 ± 1.0 °C.

Measurement of myosin regulatory light chain (MLC_{20}) phosphorylation

To determine whether blebbistatin affects MLC_{20} phosphorylation/dephosphorylation processes, we mea-sured the MLC_{20} phosphorylation level of the skinned preparations used for the force measurement. When the concentration of Ca^{2+} in the solution was 100 nM or lower, the phosphorylation level was about 5% irrespec-tive of the presence of blebbistatin, and MLC_{20} phosphorylation level was increased in a Ca^{2+} concentration dependent manner. Blebbistatin at 100 μM and lower had little effects on the MLC_{20} phosphorylation level of skinned tracheal preparations at any given concentration of Ca^{2+} (Fig. 2).

Effects of blebbistatin on the tautomycin induced contraction

Tautomycin, a potent myosin phosphatase inhibitor, induced irreversible tension development and MLC_{20} phosphorylation (22) in skinned smooth muscle preparations even in the absence of Ca^{2+}. In β-escin skinned tracheal preparations, 1 μM tautomycin slowly developed active tension and reached to a steady level within 15 min. Blebbistatin at 100 μM significantly suppressed the tautomycin-induced tension development (Fig. 3).

Table 1. Effects of blebbistatin on the $F_{max-Ca^{2+}}$, Hill coefficient, and $[Ca^{2+}]_{50}$

Trachea	control	3 μM	10 μM	30 μM	100 μM
$F_{max-Ca^{2+}}$	0.828 ± 0.026	0.819 ± 0.088	0.649 ± 0.016*	0.515 ± 0.046*	0.436 ± 0.042*
Hill's N	1.974 ± 0.234	2.442 ± 0.480	2.467 ± 0.249	3.816 ± 0.988	3.463 ± 0.759
$[Ca^{2+}]_{50}$ μM	1.625 ± 0.189	2.444 ± 0.940	1.782 ± 0.083	2.238 ± 0.229	2.583 ± 0.680
Trachea GTPγS	control	3 μM	10 μM	30 μM	100 μM
$F_{max-Ca^{2+}}$	1.094 ± 0.040	1.340 ± 0.207	0.850 ± 0.034*	0.787 ± 0.066*	0.579 ± 0.032*
Hill's N	3.032 ± 0.610	2.632 ± 0.833	4.454 ± 0.963	3.777 ± 1.029	3.330 ± 0.721
$[Ca^{2+}]_{50}$ μM	1.750 ± 0.137	4.516 ± 2.119	1.808 ± 0.132	2.651 ± 0.412	2.702 ± 0.424

The relationship between Ca^{2+} concentration and Ca^{2+}-induced tension development in the absence and presence of 1 mM GTP-γS was fitted to the Hill equation. $F_{max-Ca^{2+}}$, tension level of the maximal Ca^{2+}-induced contraction; Hill's n, Hill coefficient; $[Ca^{2+}]_{50}$, Ca^{2+}-concentration for the half-maximal Ca^{2+}-activated tension. *P<0.05, significant difference of parameters compared with control.
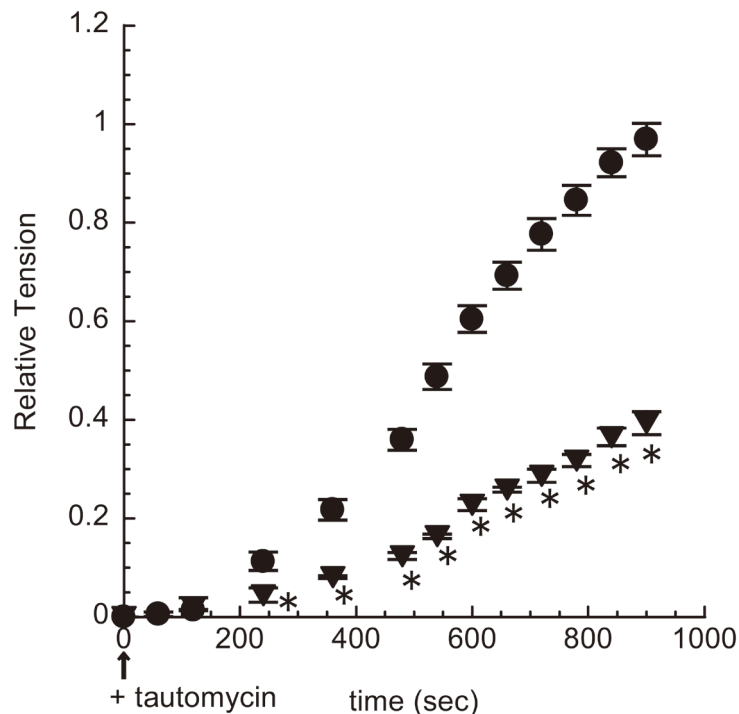

Fig. 3. Effects of blebbistatin on the tautomycin induced contraction. Values are expressed as the mean ± SEM of 5 experiments. 30.0 ± 1.0 °C. Tautomycin at 1 μM gradually developed force in skinned tracheal muscle preparations in the presence (▼) and absence of 100 μM blebbistatin (●). Note that Ca^{2+} in the tautomycin containing solution was almost completely chelated with 10 mM EGTA. Asterisk indicates the significant difference of the active force compared with that of control, where P values are less than 0.05.

Effects of blebbistatin on the GTP-γS induced "sensitized" contraction of skinned tracheal preparations in the presence of Ca^{2+}

GTP-γS is known to "sensitize" contractile elements through activation of G-protein coupling signal transduction irreversibly (23–25), resulting in enhancement of the Ca^{2+}-induced contractile force in the smooth muscle preparations skinned with α-toxin or β-escin. In β-escin skinned tracheal preparations, GTP-γS at 1 mM enhanced the active tension of Ca^{2+} induced contraction by 50%, but did not affect the basal tension level
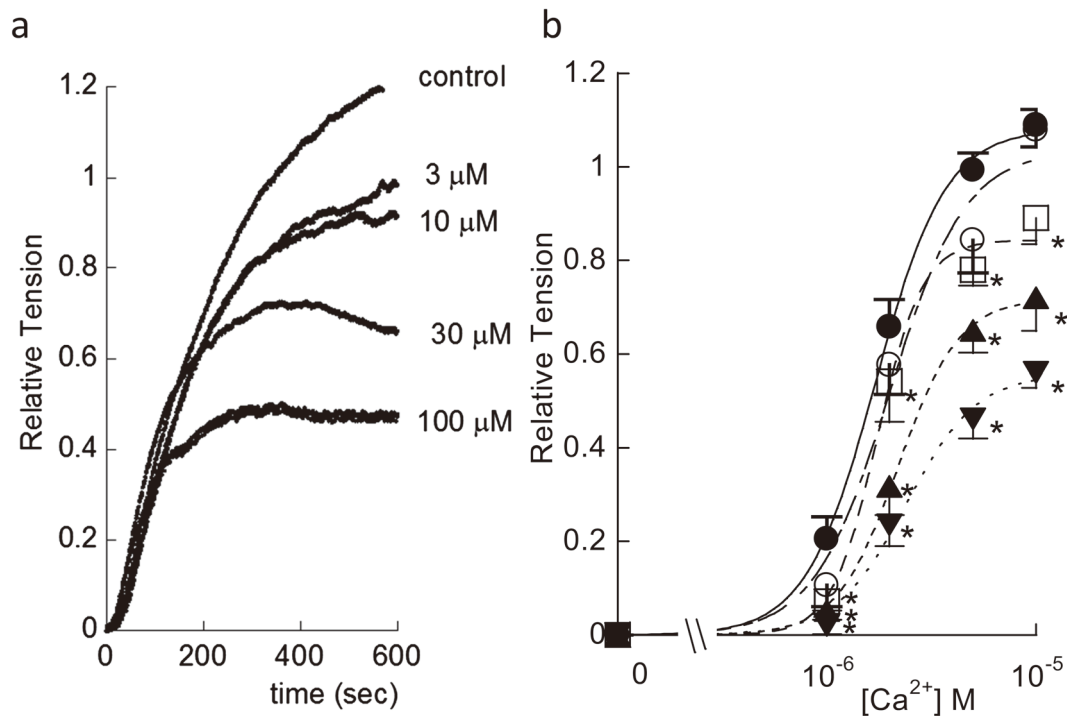
Fig. 4. a: Typical tension traces of skinned tracheal muscle preparations in the presence of 1 mM GTP-γS. Each preparation was activated with 10 μM Ca^{2+} and 1 μM calmodulin. Blebbistatin at 10 μM or higher clearly suppressed the Ca^{2+}-induced tension development. 30.0 ± 1.0 °C. b: Effects of blebbistatin on the Ca^{2+} concentration-relative tension relationship in the presence of GTP-γS at 1 mM. Blebbistatin was applied for 3 min before activation and for 10 min during subsequent activation with Ca^{2+}. 1% DMSO (control; ●), or 3 μM (○), 10 μM (□), 30 μM (▲) or 100 μM (▼) blebbistatin was added to the artificial intracellular solutions. Data were fitted to the modified Hill equation (straight or dotted lines). Values are expressed as the mean ± SEM of 6–7 experiments. Asterisk indicates a significant difference of the active force compared with that of control, where P values are less than 0.05.

in the absence of Ca^{2+} (Fig. 4a). Blebbistatin significantly suppressed the GTP-γS-induced "sensitized" force at any given concentration of Ca^{2+} (Fig. 4b). Data fitting to the Hill equation indicated that blebbistatin inhibited $F_{max-Ca^{2+}}$ without changing the Hill coefficient n and $[Ca^{2+}]_{50}$, indices of the Ca^{2+} sensitivity for the active tension (Table 1). The estimated $[blebbistatin]_{50}$ value for inhibition of $F_{max-Ca^{2+}}$ was 10.5 ± 1.35 μM and F_{min} (the minimal tension level by blebbistatin induced force suppression) /F_0 (active tension level in the absence of blebbistatin) was 0.55.

GTP-γS also increased MLC_{20} phosphorylation level in the presence of Ca^{2+} (Fig. 5). Blebbistatin did not affect the GTPγS-induced increase in MLC_{20} phosphorylation level at any concentrations of Ca^{2+} (Fig. 5).

Discussion

The present study showed that, in β-escin skinned tracheal smooth muscle preparations from guinea pigs, blebbistatin at 10 μM and higher significantly suppressed the Ca^{2+}-calmodulin induced active tension development, but had little effects on the MLC_{20} phosphorylation level. The agent significantly suppressed the contractile response to tautomycin which irreversibly inhibits myosin phosphatase activity (22). Our previous results using β-escin skinned taenia cecum from the guinea pig indicated that blebbistatin at 10 μM and higher
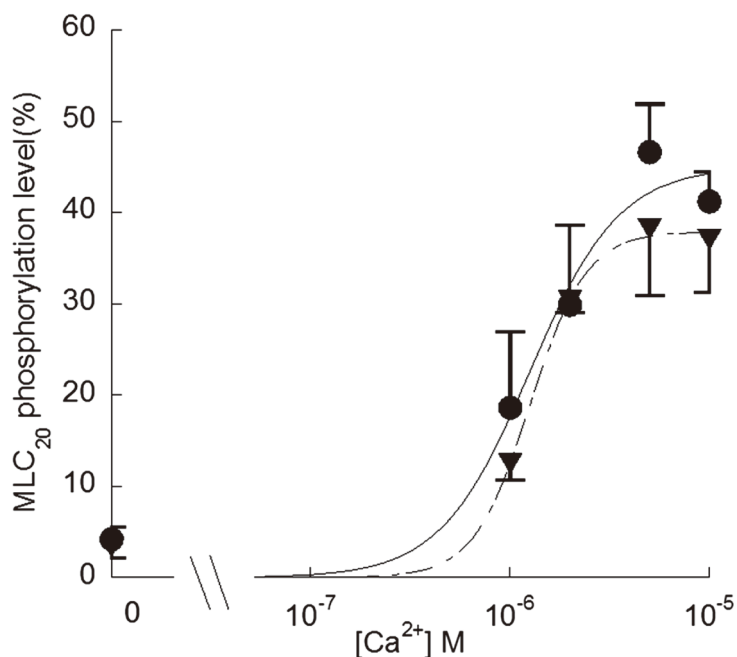
Fig. 5. The relationship between Ca^{2+} and myosin light chain (MLC_{20}) phosphorylation level of the skinned tracheal muscle preparations treated with GTP-γS at 1 mM. Skinned tracheal muscle (●) and skinned tracheal muscle in the present of 100 mM blebbistatin (▼). Blebbistatin did not change the phosphorylation level. The extracts of muscle strips were subjected to 15% glycerol-PAGE coupled with Western blot. MLC_{20} was detected with anti-MLC_{20} antibody and visualized with horseradish peroxidase-conjugated anti-rabbit IgG. For more details, see Materials and Methods. The contents of unphosphorylated and phosphorylated MLC_{20} were quantitatively measured and analyzed using a computer-based densitometer system. The percentage of MLC_{20} phosphorylation was determined by the ratio of phosphorylated MLC_{20} to the total MLC_{20} densitometrically determined. Values are expressed as the mean ± SEM of 5–7 experiments. 30.0 ± 1.0 °C.

inhibited active tension development through direct inhibition in SMM conformation and/or ATPase activity, since blebbistatin disrupted SMM organization in the preparations (5) without significant effects on MLC_{20} phosphorylation level irrespective of presence of Ca^{2+}. Force inhibiting effects of blebbistatin on the β-escin skinned tracheal muscles are similar to those on the skinned taenia cecum, therefore, we suggest that blebbistatin inhibited tracheal force development through direct inhibition of SMM conformation and/or ATPase activity, although we have measured neither tracheal SMM ATPase activity, nor SMM organization in the skinned preparations.

Interestingly, force inhibiting effect of blebbistatin seems to be more effective in tracheal smooth muscle preparations (F_{min}/F_0; 0.53) than those from the taenia cecum (0.75, see Table 1 of [5]). Since ED_{50} values for blebbistatin in inhibiting force development of tracheal smooth muscle and taenia cecum preparations were 11.6 ± 2.58 μM and 6.94 ± 3.60 μM (5) respectively, the blebbistatin sensitivity for SMM of tracheal smooth muscle preparations does not appear to be different from that of taenia cecum preparations. In the taenia cecum, myosin filament organization is known to be robust rather than that in several types of smooth muscle including airway muscles in which myosin filament number changed as a result of contraction-relaxation cycles (8–11). Therefore, it might be possible that the difference in efficacy of the force inhibiting effects of blebbistatin between trachea and taenia cecum is attributable to the difference in lability of myosin filaments between these two types of smooth muscle. Further morphological studies are necessary to determine whether

blebbistatin disrupts the organization of muscle myosin filaments more effectively in tracheal muscle preparations than in those of the taenia cecum.

In the present study, we also investigated the effects of blebbistatin on the GTP-γS-induced "sensitized" contraction in β-escin skinned tracheal smooth muscle preparations, as airway smooth muscle contraction might be mediated by MLC_{20} phosphorylation due to G-protein coupled inhibition of myosin phosphatase as well as Ca^{2+}-induced activation of myosin light chain kinase (12, 13). Efficacy and sensitivity for the force inhibiting effects of blebbistatin on the GTP-γS-induced contraction were quite similar to those on the Ca^{2+}-induced contraction without GTP-γS (Table 1). These results indicate that blebbistatin did not affect the "G-protein coupled myosin phosphatase regulatory pathway", but acted on SMM directly, resulting in force suppression in the tracheal smooth muscle preparations. Since the increment of the contractility of airway smooth muscle by "sensitization" is thought to be a major cause of airway hyper-responsiveness (12, 13), and several mechanisms including Rho-A mediated- and CPI-17 mediated-pathways are thought to contribute "sensitization"(12, 13), blebbistatin, a potent inhibitor of the downstream end of the smooth muscle contraction, will be considered as a therapeutic agent for airway hyper-responsiveness.

Acknowledgements

Conflict of interest

No conflict of interest are declared by the authors.

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