CARDIOVASCULAR SYSTEM

Blebbistatin: use as inhibitor of muscle contraction

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Abstract Blebbistatin (BLEB) is a recently discovered
compound that inhibits myosin-II ATPase activity. In this
study, we tested BLEB in intact and skinned isolated rat
cardiac trabeculae, rat intact myocytes, and single rabbit
psoas myofibrils. BLEB (10 μM) reduced twitch force and
cell shortening that was reversed by exposure to light.
BLEB treatment of skinned trabeculae in the dark (1 hr)
reduced Ca^{2+}-activated force ($EC_{50} = 0.38 \pm 0.03$ μM).
Rapid (<5 ms) BLEB application in Ca^{2+}-activated rabbit
myofibrils reduced force proportional to [BLEB]. Two-
photon Indo1-AM ratio-metric confocal line-scan micros-
copy revealed no impact of BLEB on the cytosolic Ca^{2+}
transient. BLEB reduced contractile force in skinned
trabeculae without affecting tension-dependent myofila-
ment ATPase activity. We conclude that BLEB specifically
uncouples cardiac myofilament activation from Ca^{2+}
activation without affecting EC coupling or cross-bridge
cycling parameters. This agent could be useful to uncouple
myofilament contractility from electrical events that lead to
sarcoplasmic reticulum Ca^{2+} release in the cardiac myocyte
(uncoupling agent) However, the compound is very
sensitive to light, a property that limits its application to
mechanistic physiological studies.

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Introduction

The ability to carefully and precisely uncouple the
excitation–contraction cycle has been of much interest to
muscle physiologists. Of the many compounds tested one in
particular, 2,3-butanedione monoxime (BDM)—a chemical
phosphatase—has been widely used by as an uncoupling
agent in various muscle types [2, 3, 7, 12, 20, 22, 29, 35,
37, 38]. However, although this drug is effective in
inhibiting myofilament force, it is also recognized that
BDM has side effects. For example, application of BDM at
concentrations greater than 10 mM induces a reduction in
calcium handling [7, 12, 21, 23, 28, 36], alters action
potential, as well as induces a depletion of internal
adenosine triphosphate (ATP) stores [33].

Blebbistatin, recently discovered by means of high
throughput drug screening, is an ATPase inhibiting agent
specific for myosin II with no appreciable reactivity for the
other members of the myosin superfamily (I, V, and X) [1,
4, 15, 25, 26, 34]. The drug exhibits stereo specificity, with
the (−) isoform being the active compound as determined
by in vitro motility assay and by its ability to prevent cell
division [27, 32].

Because of its specificity, Blebbistatin has the promise to
become a useful and widely used uncoupling agent in
cardiovascular physiology. However, to date, only few
studies have addressed the impact of application of
Blebbistatin on myocardial excitation–contraction coupling
and myofilament function [14, 19]. Accordingly, we studied
the impact of Blebbistatin on contractile function in intact
and permeabilized isolated muscle. We first determined the

impact of Blebbistatin application on intact electrically stimulated isolated rat cardiac trabeculae and found that Blebbistatin induced a dose-dependent decrease in twitch force within 10–30 min. A similar experiment in electrically stimulated single isolated rat cardiac myocytes revealed that Blebbistatin application irreversibly reduced cell shortening at a rate that was dependent on the Blebbistatin concentration. Inhibition of cell shortening was reversed by exposure to white light. The rate of myofilament force inhibition by Blebbistatin, as measured in single rabbit psoas myofibrils, was proportional to the concentration of the drug. Because Blebbistatin's effect was slow, skinned cardiac trabeculae were exposed overnight to the drug so as to ensure steady-state inhibition; these experiments revealed that Blebbistatin inhibits myofilament force with $EC_{50}=0.4$ μM. To be useful as an uncoupling agent, Blebbistatin should not affect the cytosolic calcium transient. Because the agent is light sensitive, we employed two-photon line-scan confocal microscopy in isolated cardiac myocytes. The fluorescent indictor Fluo-4 could not be used as a Ca^{2+} probe because of a specific interaction between this dye and Blebbistatin. Two-photon Indo-1 ratio-metric Ca^{2+} transient showed that Blebbistatin inhibited cell shortening without impact on the cytosolic Ca^{2+} transient. Finally, measurement of myofilament ATPase activity in skinned cardiac trabeculae revealed that Blebbistatin did not affect cross-bridge cycling kinetics. That is, Blebbistatin binding to myosin effectively removes that cross-bridge from the pool of available cross-bridges without impacting on the cycling kinetics of the remaining drug-free cross-bridges.

Materials and methods

All experiments were performed according to institutional guidelines concerning the care and use of experimental animals and the NH guide for the Care and Use and Use of Laboratory Animals. Male rats (LBNF-1; 250–350 g) received intraperitoneal injections of 50 mg/kg sodium pentobarbital and 1.5 mL heparin. Rabbits were killed by intravenous administration of pentobarbital (120 mg/kg) through the marginal ear vein. Next, both psoas muscles were excised, cut into 5 mm strips, which were tied to wooden sticks, and stored in a glycerol-relax solution at −20°C for up to 2 months [11].

Isolated trabeculae

Under deep anesthesia, rat hearts were excised and perfused retrograde with a modified Krebs–Henseleit solution also containing 0.2 mM $CaCl_2$ and 20 mM BDM to inhibit spontaneous contractions (25°C; pH=7.4) [17, 18]. Un-

branched right ventricular trabeculae were dissected and were either mounted intact on the stage of an inverted microscope (see below) or placed in standard relaxing solution with 0.1% Triton-X 100 added to chemically permeabilize the preparation. For the skinned trabeculae dose–response experiments, the muscles were permeabilized for 4 h at 4°C; next, aluminum T-clips were wrapped around the ends of the preparation, and the muscles were transferred to small wells containing 100 μL of relaxing solution with various amounts (0.01–10 μM) of (−) Blebbistatin added to incubate overnight in the dark at 4°C. For skinned trabeculae to be used for the force/ATPase experiments, the muscles were permeabilized overnight at 4°C.

Isolated myocytes

Rat myocytes were isolated as previously described [8]. Briefly, under deep anesthesia, rat hearts were removed rapidly and perfused retrograde through the coronary arteries for 35 min with a Ca^{2+}-free 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (see below) that also contained 20 mM taurine bubbled with O_2 at 37°C. This was followed by perfusion with the same solution containing 1.3 mg/mL collagenase (Type IV, Worthington) for 30–35 min. Ventricles were then removed and minced into small pieces and gently massaged through a nylon mesh, and dissociated cardiac myocytes were washed with collagenase-free washing solution. Subsequently, Ca^{2+} was increased in a graded manner to a concentration of 1 mM. Cells were kept in this solution at 37°C until used in the experiments within 4 h using the same solution at $[Ca^{2+}]=1$ mM. The percentage of Ca^{2+}-tolerant cells was ~60%.

Isolated myofibrils

Single myofibrils or bundles of two to three myofibrils were prepared from fast skeletal muscle by homogenization of glycerinated rabbit psoas muscles, as previously described [11]. All solutions to which the myofibrils were exposed contained a cocktail of protease inhibitors including leupeptin (10 μM), pepstatin (5 μM), and phenylmethylsulphonyl fluoride (200 μM), as well as NaN_3 (500 μM) and 10 mM dithiothreitol. Myofibril preparations were stored at 4°C and used for up to 3 days after preparation.

Intact trabeculae force measurement

Trabeculae were mounted in an experimental chamber between a silicon strain gauge (Sensonor) and a motor (Cambridge) positioned on an inverted microscope (Olympus MT-2) as previously described [9]. Briefly, trabeculae

were stimulated via platinum electrodes running parallel to the muscle (2 ms; 50% above threshold; 1 Hz). Resting sarcomere length, as measured by laser diffraction techniques, was set to 2.10 μm. Blebbistatin was added to the modified Krebs–Henseleit solution in increasing concentrations from a 100-mM stock solution in dimethyl sulfoxide (DMSO); $[Ca^{2+}]$ in the perfusion medium was kept at 1.0 mM. Exposure to white light was limited as much as possible, albeit not entirely, by use of a red filter.

Skinned trabeculae force and ATPase measurement

Two separate series of experiments were performed on the skinned trabeculae. In a first series, maximum Ca^{2+}-activated force development was measured as previously described [18] on preparations that were pretreated overnight in the dark with varying concentrations of Blebbistatin. Experiments were performed under conditions of dimmed red light (Kodak darkroom light). Resting sarcomere length was set at 2.0 μm by laser diffraction; all solutions (relaxed, preactivating, and activating) contained the same [Blebbistatin] as used in the overnight incubation with the drug. Force was normalized to cross-sectional area as determined during the dissection of the muscle. Note that this experimental design required separate muscles to be used for each [Blebbistatin] tested. In a second series of experiments, Ca^{2+} activated force and ATPase activity were measured simultaneously using an enzyme-coupled assay as previously described [9, 13]. Briefly, in this assay, adenosine diphosphate produced by cycling cross-bridges is stoichiometrically coupled to the consumption of NADH in the measurement cuvet by the enzymes pyruvate kinase and lactate dehydrogenase; [NADH] is measured by UV light absorption. The rate of ATP hydrolysis thus measured is plotted vs isometric force development over a range of activation levels by varying free $[Ca^{2+}]$ in the solution; the slope of this relationship, tension cost, is a direct measure of cross-bridge cycling kinetics [9, 13]. Brief exposure to UV light (up to 60 s), by design, is unavoidable in this type of measurement. Therefore, we employed a relatively high concentration of Blebbistatin (15 μM) that was present in all solutions, for at least 10 min. In a separate series of experiments, BDM was used instead of Blebbistatin to directly compare the two uncoupling reagents.

Single myofibril measurement of Blebbistatin force inhibition kinetics

An aliquot of myofibril suspension in rigor solution was injected into a chamber filled with ~3 mL relaxing solution that was mounted on the stage of an inverted microscope (Olympus IX-70) [11]. Myofibrils selected for use (~50 μm long) were mounted horizontally between two glass micro-

tools: a calibrated cantilever force probe and a length-control motor using Nomarski optics; sarcomere length was determined by video microscopy and set to 2.5 μm. Experiments were performed at 15°C. Isometric force was measured by the deflection of the bright field shadow of the force probe that was projected onto a split photodiode (~50× magnification, 1–3 nm/nN, ~5 kHz resonance frequency). Activated myofibrils were exposed to Blebbistatin by rapid switching of two continuous solution streams flowing by gravity from a double-barreled glass pipette placed within 0.5–1 mm of the preparation (~200 μL/min, flowing past the myofibril at ~2 cm/s). Solution changes occurred with a time constant of 2–4 ms and were complete within 10 ms. For the current experiments, both barrels contained maximum activating solution (pCa=3.5) and one barrel contained additional Blebbistatin at 1, 5, or 10 μM. Experiments were performed in red light. Activation was initiated by starting the solution flow from both barrels. Blebbistatin application was accomplished by rapidly switching to the barrel containing activating solution plus Blebbistatin; switching back initiated rapid washout of the drug. Relaxation was induced by turning off both solution flows (the bulk bath solution contained relaxing solution). Note that this protocol precluded estimation of Ca^{2+} activation kinetics, relaxation kinetics, or resting force after the activation cycle because of contaminant Ca^{2+}-activating solution and Blebbistatin in the bulk bath solution after activation.

Intact myocyte cell shortening

An aliquot of myocyte suspension in HEPES-buffered physiological solution was placed in a laminin-coated dish positioned on an inverted microscope (Nikon Diaphot TMD) and observed using a 40× oil immersion objective. Myocytes were paced via two platinum electrodes positioned on either side of the cell via a micromanipulator (2 ms; 20 V) at 0.5 Hz. Blebbistatin, dissolved in the same solution as the bulk bath solution, was applied via a perfusion pipette positioned close to the myocyte; flow was ~200 μL/s. Red light was used to illuminate the cell. Myocyte images were recorded at 240 Hz, and sarcomere length was analyzed in real time using IonOptix equipment and software (IonOptix, Milton, MA).

Two-photon confocal line-scan myocyte Ca^{2+} transients

Images were recorded with a Zeiss LSM 510-META laser scanning microscope. To image electrically stimulation-evoked Ca^{2+} transients, myocytes were loaded either with Fluo4-AM or Indo1-AM in a HEPES-buffered physiological solution that contained 1 mM Ca^{2+} at 37°C (Invitrogen; 4 μM; 20 min) and then washed with fresh buffer. As

above, an aliquot of myocyte suspension was placed in a recording dish positioned on an inverted microscope (Zeiss Axiovert 200 M) and observed using a 40× water immersion objective (NA 1.2). Myocytes were paced via two platinum electrodes positioned on either side of the cell via a micromanipulator (2 ms; 20 V) at 0.5 Hz. Confocal lines scans (512 samples; 3.07-ms repeat frequency) were obtained by excitation with a mode-locked Ti/Sapphire laser (Chameleon, Coherent), which generated a train of 140 fs pulses at a repetition rate of 90 MHz. The laser was tuned to a center wavelength of 790 (Fluo-4) or 740 nm (Indo-1). Fluorescence emission was recorded using an appropriate dichroic beam-splitting mirror, the Zeiss META detector set to a single 500–700 nm emission channel for Fluo-4 detection, and two emission channels (380–410 and 450–570 nm) for Indo-1 detection. Line-scan images were collected on disk for offline processing; images from ten twitches were digitally averaged.

Solutions

(−) Blebbistatin was obtained from Sigma. Several solutions were used for the various experimental protocols in this study. The standard solution used for intact twitching trabeculae was composed of (in mM): Na^+ 143, K^+ 5, Cl^- 128, Mg^{2+} 1.2, $H_2PO_4^-$ 2.0, SO_4^{2-} 1.2, HCO_3^- 21, glucose 10, and Ca^{2+} 1 and equilibrated with 95% O_2/5% CO_2 (25.0±0.1°C) [9]. For the isolated single myocyte experiments, a HEPES-buffered physiological solution was used. This solution contained (in mM): NaCl 117, KCl 5.7, $NaHCO_3$ 4.4 $MgCl_2$ 1.7, KH_2PO_4 1.5, HEPES 21, glucose 11, and Ca^{2+} as indicated. The pH was adjusted to 7.4 (cell studies) or 7.2 (cell isolation) with NaOH. The compositions of the solutions used in skinned trabecula experiments are summarized in Table 1 [9, 13]. For the single fast

skeletal single myofibril experiments, the solutions contained (in mM): total ethylene glycol-bis(β-amino-ethyl ether)-N,N,N,N-tetra acetic acid 10, MgATP 5, free Mg^{2+} 1, MOPS 10; pH=7.0 adjusted with KOH [13]. Contaminant phosphate (around 170 μM in standard solutions) was reduced to less than 5 μM by a phosphate-scavenging enzyme system (purine-nucleoside phosphorylase with substrate 7-methyl-guanosine). The ionic compositions of all skinned muscle and myofibril solutions were calculated using the methods of Fabiato and Fabiato [16].

Data analysis

Statistical significance was tested by one- or two-way analysis of variance; $p < 0.05$ was considered significant. Data are expressed as mean±SEM. Sigmoidal relationships were fit to a modified Hill equation [13]:

$$P(x) = P_{max}[x]^{nhill} \Big/ \left([x]^{nhill} + EC_{50}^{nhill} \right) \quad (1)$$

Where $P(x)$ represents the dependent parameter of interest, P_{max} is the maximum value of P, EC_{50} is the $[x]$ at which P is 50% of P_{max} (a compound index of sensitivity), and nhill is the Hill coefficient (an index of the level of cooperativity). Commercially available software was used for all statistical analyses (SYSTAT, Evanston, IL); KaleidoGraph (Synergy Software, Reading, PA, USA). In skinned trabeculae, ATP hydrolysis rate as function of steady state force over a range of free $[Ca^{2+}]$ was fit by linear regression, the slope of which represents tension-dependent myofilament ATP consumption rate (tension cost parameter; a direct measure of average cross-bridge cycling rate). The rate of Ca^{2+} decay was determined by a nonlinear mono-exponential fit; Blebbistatin-induced loss of force in single myofibrils was determined by linear regression.

Table 1 Ionic composition of the skinned muscle solutions

	EGTA	CaEGTA	HDTA	Na$_2$H$_2$ATP	MgCl$_2$	KPROP	PCr	PEP
Skinned trabeculae force measurement (15°C)								
Relaxing	20	0	0	6.54	6.52	40.8	10	0
Activating	0	20	0	6.70	6.20	41.0	10	0
Preactivating	0.5	0	19.5	6.54	6.31	41.2	10	0
Skinned trabeculae ATPase measurement (20°C)								
Relaxing	20	0	0	6.32	7.14	23.4	0	10
Activating	0	20	0	6.43	6.59	24.1	0	10
Preactivating	0.5	0	19.5	6.32	6.71	24.1	0	10

Ionic strength was maintained at 200 mM wih potassium propionate (KPROP). In addition, all solutions contained (in mM): N,N-bis[2 hydroxyethyl]-2-aminoethanesulphonic acid (BES) 100, pH=7.0 adjusted with KOH, leupeptin 0.01, pepstatin 0.001, PMSF 0.01, and DTT 1. Solutions used for ATPase measurement additionally contained NaN$_3$ 5, A$_2$P$_5$ 0.01, oligomycin 0.01, pyruvate kinase 0.5 mg/mL (386 U/mg), and lactate dehydrogenase 0.05 mg/mL (880 U/mg). The ionic compositions of all skinned muscle and myofibril solutions were calculated using the methods of Fabiato and Fabiato [16]; free MgATP was calculated as 5 mM and free Mg^{2+} as 1 mM.
EGTA Ethylene glycol-*bis*(β-amino-ethyl ether)-N,N,N,N-tetra acetic acid, *HDTA* hexamethylenediamine tetraacetic acid (Fluka), *PCr* phosphocreatine, *PEP* phosphoenolpyruvate.

Results

The overall goal of the current investigation was to evaluate Blebbistatin as an uncoupling agent for the study of cardiac excitation–contraction coupling.

Intact electrically stimulated rat cardiac trabeculae

Application of Blebbistatin reduced contractile force in intact electrically stimulated rat cardiac trabeculae in a dose-dependent manner. Force reduction occurred in 10–30 min after application of Blebbistatin at a high concentration of the drug. Figure 1 shows the impact of Blebbistatin on twitch force (left panel) and the dose–response relationship obtained in four trabeculae (right panel). The EC_{50} for inhibition of twitch force by Blebbistatin was 3.9±0.7 μM, a value similar to ~2 μM previously reported in murine isolated papillary muscle [14] but higher than ~0.5 μM found in solution biochemistry studies [25, 26, 34]. It should be noted that in these experiments, exposure to light was not entirely avoided (see below). In comparison, the application of BDM inhibited twitch force in rat cardiac trabeculae within 10 s ($EC_{50} = 4.0 \pm 0.9$ mM; results not shown).

Inhibition of single isolated cardiac myocyte cell shortening by Blebbistatin

Isolated myocyte experiments were performed entirely in the dark, except for dimmed red light used for locating a cell and for measurement of myocyte sarcomere length by online video microscopy. Figure 2 shows typical examples of rat cardiac myocytes exposed to perfusion pipette to 1 μM (top) or 10 μM (bottom) Blebbistatin. Like in trabeculae, Blebbistatin induced a reduction in sarcomere shortening that eventually resulted in complete cessation of
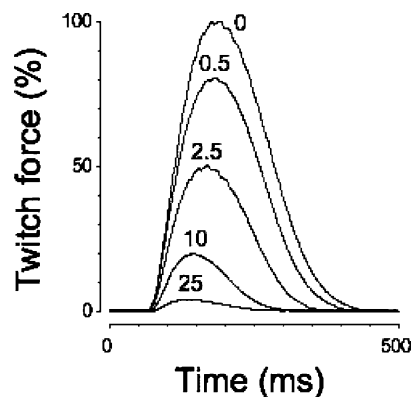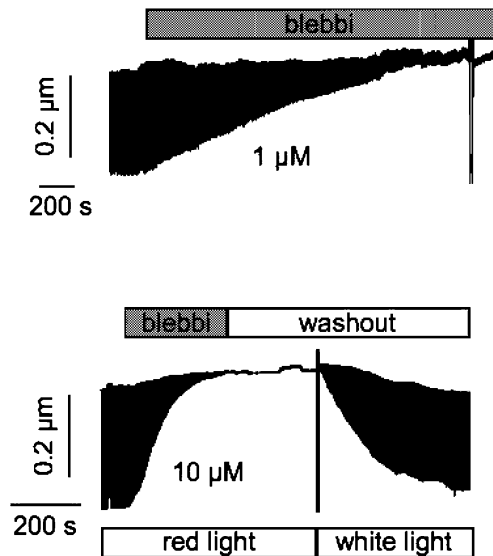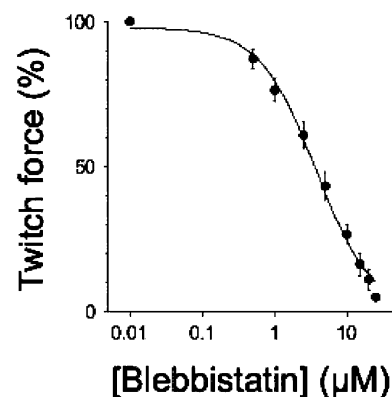

Fig. 2 Blebbistatin inhibition of sarcomere shortening (SL) in an isolated rat cardiac myocyte. Sarcomere length was measured by video microscopy. Experiments were performed in the dark, except for red microscope illumination. Blebbistatin, 1 (*top panel*) or 10 μM (*bottom*) was applied via a perfusion pipette at the time indicated by the *shaded bar on top* (followed by a washout period in the *bottom panel*). Blebbistatin inhibited SL shortening with a relatively slow onset; the rate of inhibition depended on the concentration of the drug; washout of the agent did not reverse this effect (note difference of time scale between the panels). Removal of the red filter in the microscope illumination path exposed the cell to white light that resulted in recovery of SL shortening (*bottom panel*). HEPES-buffered physiological solution, $[Ca^{2+}]_o = 1.0$ mM, 0.5-Hz stimulation rate, room temperature

sarcomere shortening. The rate of contractile inhibition by Blebbistatin was faster at higher concentrations of the drug. Attempts to obtain a steady-state dose–response relationship in these experiments were unsuccessful; inhibition of sarcomere shortening at the lower concentrations of the drug did not reach steady state within ~60 min. Moreover,

Fig. 1 Blebbistatin inhibition of twitch force in intact rat cardiac trabeculae. Resting sarcomere length 2.10 μm; $[Ca^{2+}]_o = 1.0$ mM, 1-Hz stimulation rate, 25°C. Blebbistatin was added to the modified Krebs–Henseleit solution in increasing concentrations from a 100-mM stock solution in DMSO; exposure to white light was limited as much as possible, albeit not entirely, by use of a red filter. *Left panel*, inhibition of twitch force in response to an increasing dose of Blebbistatin at the indicated concentrations (in μM). *Right panel*, average dose–response relationship; $EC_{50} = 0.39 \pm 7$ μM Blebbistatin; $n=4$

washout of Blebbistatin did not reverse the action of the drug for up to 30 min. Finally, we found that Blebbistatin is highly sensitive to light exposure, as has been reported previously [32]. This phenomenon is also illustrated in Fig. 2 (bottom panel). Removal of the red light filter in the microscope illumination pathway immediately initiated recovery of sarcomere length shortening to near control levels. It should also be noted that exposure to UV light resulted in only partial recovery of sarcomere length shortening that invariably was followed by pronounced arrhythmias and cell death. It has been reported that destruction of Blebbistatin by UV light yields free radicals that may have been damaging to the cardiac myocyte [32].

Rapid application of Blebbistatin by solution switching in single myofibrils

We measured the rate of Blebbistatin-induced force inhibition in Ca^{2+}-activated single rabbit psoas myofibrils in red light as illustrated in Fig. 3. Upon activation with saturating Ca^{2+}, myofibrils generated ~1 μN force. Rapid (<10 ms) application of Blebbistatin reduced myofibrillar

force with a dose-dependent rate. On average, the rate for force inhibition was 0.28 ± 0.05, 2.5 ± 0.6, and $5.9\pm0.5\%/s$ at 1, 5, and 10 μM Blebbistatin application, respectively ($n=4$).

Steady-state Blebbistatin dose–response in skinned rat cardiac trabeculae

The single myofibril data indicates that inhibition of force by Blebbistatin is slow (<0.3%/s) at concentrations close to the EC_{50} reported for solution biochemistry studies (~0.5 μM) [25, 26, 34]. To test this hypothesis, we applied Blebbistatin over a wide concentration range (0.01–10 μM) in skinned rat cardiac trabeculae overnight in complete darkness; next, these muscles were activated in dimmed red light at maximum Ca^{2+} saturation. The average results are summarized in Fig. 4. This experimental approach revealed an EC_{50} value for Blebbistatin of 0.38 ± 0.03 μM, a value close to that found in solution biochemistry studies and significantly lower than the EC_{50} we found in electrically stimulated trabeculae under conditions where white light exposure was not altogether avoided (cf. Fig. 1; 3.9 ± 0.7 μM). Hence, prolonged exposure to the drug in the dark is essential to obtain an accurate assessment of the Blebbistatin dose–response relationship.

Two-photon confocal line-scan Ca^{2+} transients

The sensitivity of Blebbistatin to light exposure complicates assessment of the impact of the drug on the intracellular Ca^{2+} transient using conventional fluorescent indicator
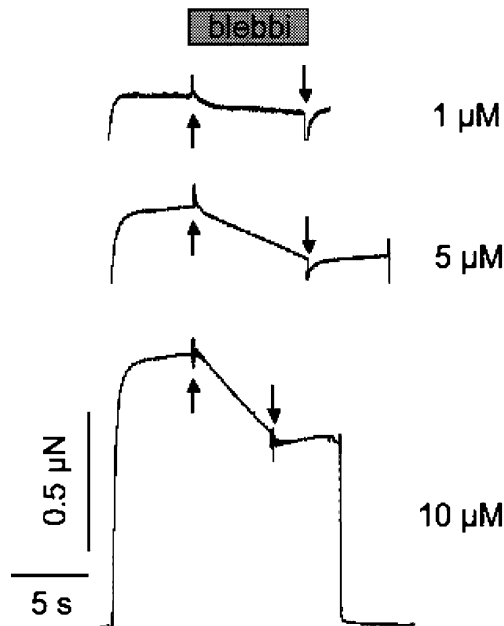

Fig. 3 Blebbistatin inhibition of force in single rabbit psoas myofibrils. Single myofibrils were activated by exposure to saturating Ca^{2+} from a double-barreled perfusion pipette (flow ~200 μL/min). At the time indicated by the *up arrow*, perfusion was rapidly (<10 ms) switched to activating solution containing Blebbistatin (1 μM, *top panel*; 5 μM, *middle panel*; 10 μM *bottom panel*). At the time indicated by the *down arrow*, perfusion was rapidly switched back to the activating solution without Blebbistatin (note: the slow apparent recovery force was likely due to solution-switching artifact). Subsequently, both solution streams were stopped resulting in relaxation of the muscle. Blebbistatin inhibited force at a dose-dependent rate. Activating solution pCa=3.5, 15°C, calibrations as indicated
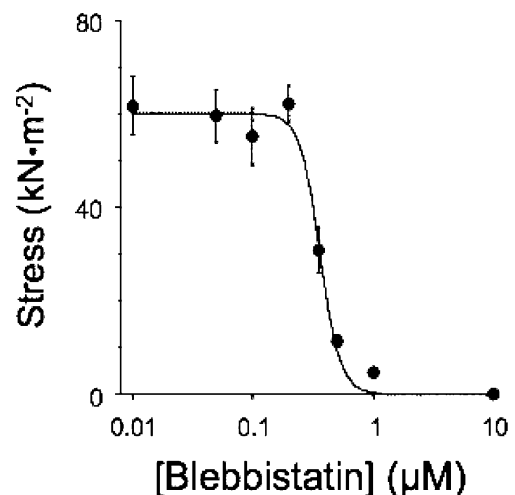

Fig. 4 Blebbistatin inhibition of force in skinned rat cardiac trabeculae exposed to a range of Blebbistatin concentrations overnight at 4°C in the dark (separate muscles were used for each [Blebbistatin]; $n=5$–7 per data point). Muscles were activated at saturating Ca^{2+} (pCa=4.3). Developed force is normalized to cross-sectional area determined during dissection of the muscle. Blebbistatin $EC_{50}=0.38+0.03$ μM; sarcomere length=2.0 μm

techniques. To overcome this problem, we employed two-photon confocal excitation laser scanning fluorescence microscopy in line-scan mode, a technique whereby excitation of the fluorescent indicator is accomplished with near-infrared high-intensity pulsed laser light. Blebbistatin (0.5 μM) was applied to intact isolated myocytes loaded with a fluorescent indicator for 1 h in the dark. Exposure to Blebbistatin in this manner virtually arrested cell shortening. In a first series of experiments, line-scan image Ca^{2+} transients were recorded using Fluo-4 fluorescence (cf. Fig. 5). Although Blebbistatin did not appreciably affect the Ca^{2+} transient amplitude, we did observe elevation of diastolic Fluo-4 fluorescence, which consequently affected the amplitude value of the calcium transient when expressed, normalized to baseline fluorescence. A similar increase in resting fluorescence has also been observed recently with another nonratio-metric indicator, Fluo5 [19]. This result could be interpreted to indicate that Blebbistatin elevates resting Ca^{2+} levels. To further investigate this, we measured two-photon fluorescence emission from Fluo-3 K-salt in relaxing solution (30 μM), i.e., a solution similar to the intracellular ionic milieu, both in the presence (Fig. 5a, right panel, trace a) and absence (trace b) of

0.5 μM Blebbistatin. In the presence of Blebbistatin, Fluo-3 fluorescence increased (29.9±0.2%); exposure to white light completely eliminated this "extra" fluorescence (0.5±0.2%). In contrast, the presence of 0.5 μM Blebbistatin alone (i.e., without Fluo-3) did not result in increased fluorescence above the dark current (−2.0±0.2%; trace c). These results suggest a specific interaction between Blebbistatin and this fluorescent indicator. A similar test using Indo-1 fluorescence revealed no such interaction. Therefore, to unambiguously assess the impact of Blebbistatin on myocyte Ca^{2+} signaling, we employed the ratio-metric fluorescent indicator Indo-1. Figure 6a shows representative recordings using two-photon line scan of an Indo-1 loaded myocyte before (left panels) and after 1 h exposure to 0.5 μM Blebbistatin (right panels). While application of Blebbistatin inhibited sarcomere shortening, it did not affect the computed Ca^{2+} transient. The average results of ~30 cells are summarized in Fig. 6b. Neither diastolic Ca^{2+}, Ca^{2+} transient amplitude, nor the rate of Ca^{2+} decay were affected by 1 h application of 0.5 μM Blebbistatin. Cell shorting, in contrast, decreased from 7.0±0.5% cell length before drug application to 0.7± 0.3% after exposure to Blebbistatin ($n=31$; myocytes isolated from five different rat hearts).
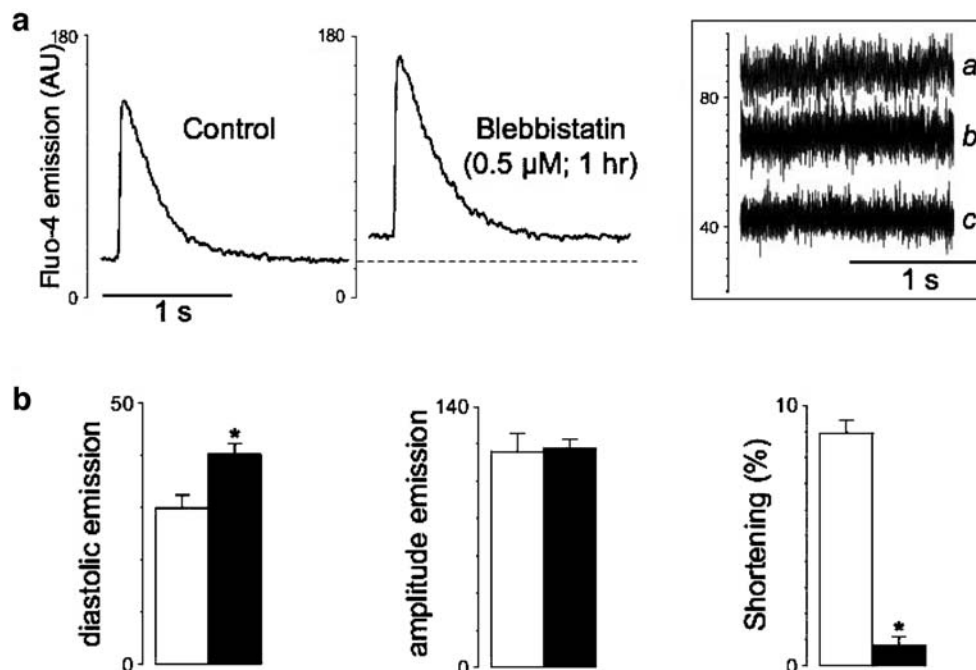

Fig. 5 a Two-photon confocal (790-nm excitation) of Fluo-4 Ca^{2+} transient in cardiac myocytes in the absence (left panel) and presence (middle panel; dotted line indicates diastolic fluorescence measured in the left panel) of Blebbistatin (0.5 μM; 1 h exposure). The myocyte was electrically stimulated at the time indicated by the arrows (0.5 Hz). The right panel shows increased fluorescence emission in the presence (trace a) and absence (trace b) of 0.5 μM Blebbistatin of 30 μM Fluo-3-K-salt in an intracellular solution. White light exposure eliminated this "extra" fluorescence (trace b overlaid). Blebbistatin (0.5 μM) alone did not exhibit two-photon fluorescence emission (trace c). b Average Fluo-4 Ca^{2+} transient parameters obtained in rat cardiac myocytes before (open bars) and after application of Blebbistatin (0.5 μM, 1 h exposure, closed bars). Blebbistatin application increased apparent diastolic Ca^{2+} (left panel) but not Ca^{2+} transient amplitude (middle panel) and almost completely inhibited cell shortening (right panel). HEPES-buffered physiological solution, $[Ca^{2+}]_o=1.0$ mM, room temperature, $n=41–46$, five hearts. Calibrations as indicated; fluorescent emission scale: AU arbitrary units
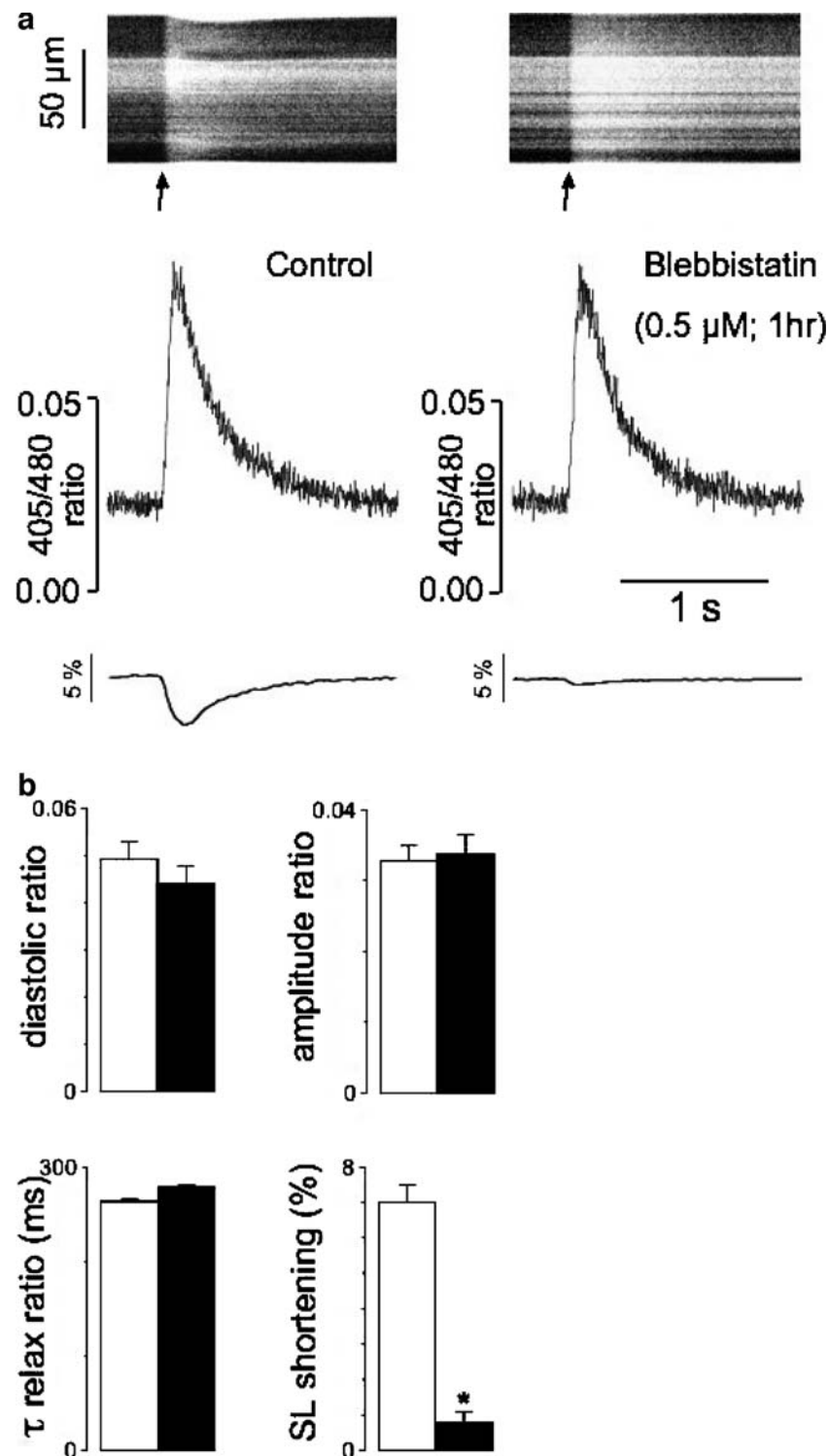
Fig. 6 a Two-photon confocal analysis (730 nm excitation) of Indo-1 fluorescence in cardiac myocytes in the absence (*left panels*) and presence (*right panels*) of Blebbistatin (0.5 μM, 1 h exposure). The myocyte was electrically stimulated at the time indicated by the *arrows* (0.5 Hz). *Top panels* show repeated line scans of Indo-1 fluorescence emission (3.07-ms scan frequency, average of ten twitches, inverted 450–570 band signal). *Middle panels* show the computed ratio-metric 405/480 Ca^{2+} transients. *Bottom panels* show cell shortening. Blebbistatin virtually blocked sarcomere shortening without affecting the Ca^{2+} transient; calibrations as indicated. **b** Average ratio-metric 405/480 Ca^{2+} transient parameters obtained in rat cardiac myocytes before (*open bars*) and after application of Blebbistatin (0.5 μM, 1 h exposure, *closed bars*). Blebbistatin application did not affect diastolic Ca^{2+} (*top left*), Ca^{2+} transient amplitude (*top right*), or rate of Ca^{2+} relaxation (*bottom left*). Blebbistatin reduced SL shortening (*bottom right*). HEPES-buffered physiological solution, $[Ca^{2+}]_o = 1.0$ mM, room temperature, $n = 31$–39, five hearts
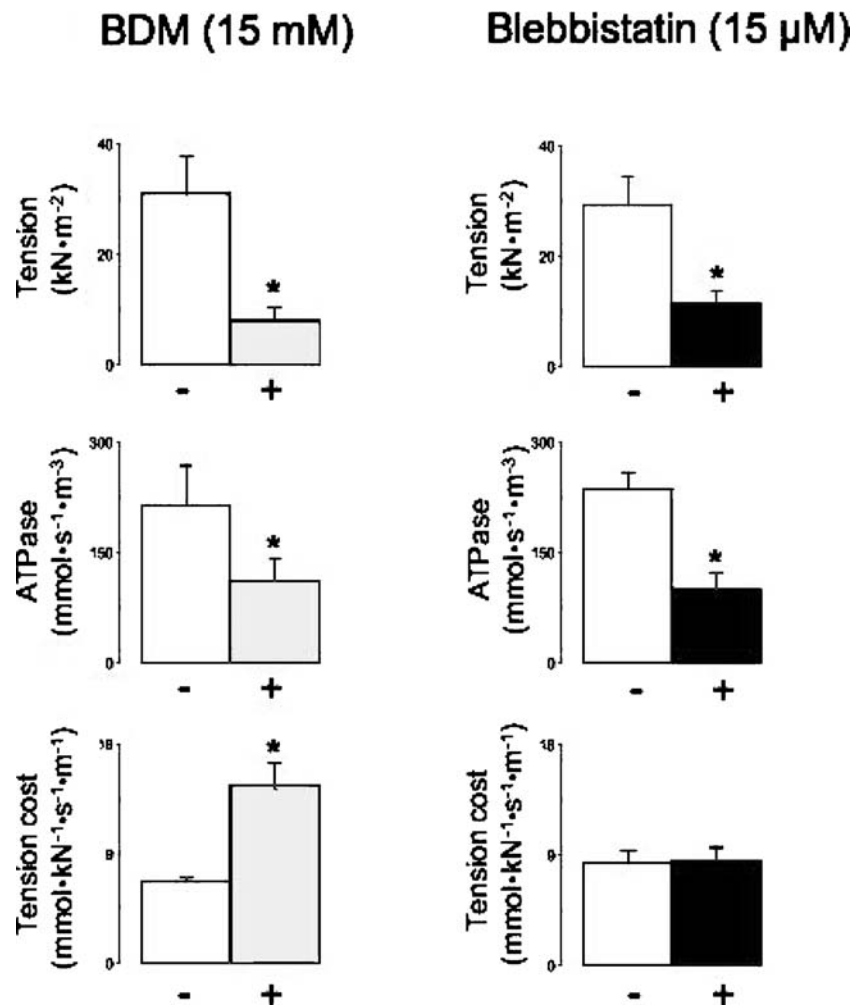
Fig. 7 Impact of BDM (*left panels*) and Blebbistatin (*right panels*) in skinned rat cardiac trabeculae on developed force normalized to cross-sectional area (*Tension*; *top*), ATPase activity (*middle*), and tension cost (*bottom*; index of cross-bridge cycling rate). *Open bars* ("−") represent control conditions, while *closed bars* ("+") represent treatment with the drug. Force and ATPase activity were measured simultaneously using an enzyme coupled absorbance assay. Blebbistatin (15 μM) and BDM (15 mM) both reduced active force development and ATPase activity. BDM application reduced tension to a greater extent than ATPase activity, indicative of increased tension cost. In contrast, Blebbistatin did not affect tension cost. *Asterisk*, $p < 0.05$, 20°C

Impact of Blebbistatin on tension cost

It has been established that Blebbistatin binding to myosin locks the cross-bridge in a weak-binding state [1, 25]. Therefore, Blebbistatin could also be useful as a reagent to uncouple cross-bridge formation from thin-filament Ca^{2+} activation [10, 24, 31]. We tested this hypothesis by measuring force and ATPase activity in skinned rat trabeculae. The ratio between ATPase activity and force development, tension cost, is a measure of cross-bridge cycling kinetics [13]. An ideal uncoupling reagent should reduce the number of strongly bound cycling cross-bridges without affecting their kinetics. The summarized data in Fig. 7 contrast the impact of Blebbistatin (15 μM) to an equally effective concentration of BDM (15 mM) on force (top panels), ATPase activity (middle panels), and tension cost (bottom panels). BDM and Blebbistatin reduced force to 26 and 39%, respectively. However, unlike BDM, Blebbistatin did not affect myofilament tension cost, in line with the proposed molecular mechanism of myosin inhibition of Blebbistatin [1, 25]. The increase in tension cost upon BDM application is consistent with the notion that BDM accelerates specific reaction steps in the cross-bridge cycle [5, 33] as opposed to simply inhibiting formation of strongly bound cross-bridges.

Discussion

We found that Blebbistatin reduces contractile force in a dose-dependent manner with relatively slow kinetics at low yet fully inhibitory concentrations. Additionally, Blebbistatin application did not affect myocyte Ca^{2+} handling or tension cost. However, the compound exhibits light

sensitivity that complicates its use and possibly reduces its usefulness as an uncoupling agent.

Although the molecular mechanisms by which Blebbistatin inhibits myosin ATPase activity has been well established [1, 25–27, 32, 34], there have been only a few studies investigating the impact of Blebbistatin of myocardial excitation–contraction coupling parameters or cardiac myofilament function. Encouraging results were recently reported by Dou et al. [14], who showed that application of Blebbistatin resulted in reduced contractile force in murine isolated papillary muscles, isolated myocytes, and skinned myocardium. In addition, these investigators found no impact of Blebbistatin on the slow inward Ca^{2+} current in isolated myocytes as well as no effect on the maximum velocity of shortening in partially inhibited skinned myocardium. However, Blebbistatin is very sensitive to light exposure, a confounding factor that was not considered in that study. Fedorov et al. recently reported the impact of Blebbistatin on excitation–contraction coupling in rat and rabbit myocardium [19]. These investigators showed reduction of contractile force upon application of Blebbistatin without effects on the electrocardiogram, electrical conduction, and action potential. In addition, application of Blebbistatin did not affect the magnitude of the intracellular calcium transient as assessed by the nonratio-metric fluorescent indicator Fluo-5F. However, diastolic fluorescence was significantly elevated upon application of Blebbistatin. Because Fluo-5F is a low-affinity Ca^{2+} dye indicator, these results could indicate an effect of Blebbistatin on resting Ca^{2+} levels, or it could mean that Blebbistatin itself contributes to fluorescence emission. In this study, we found that Blebbistatin by itself does not emit two-photon elicited fluorescence when excited at 790 nm. However, we did find interaction between Fluo-3 and Blebbistatin that altered Fluo-3 fluorescence emission. Indo-1, on the other hand, did not suffer from this shortcoming. Therefore, to circumvent this confounding factor, we chose here to use this ratio-metric indicator and found no effect of Blebbistatin on Ca^{2+} homeostasis in isolated rat myocytes at a concentration that was otherwise sufficient to block contraction.

Unlike BDM, we found that Blebbistatin reduced myofilament force without affecting tension cost. That is, our results suggest that Blebbistatin exhibits an all-or-nothing effect where binding of the drug to an individual myosin head inhibits force production by that particular cross-bridge without affecting cycle kinetics of the remaining drug-free cross-bridges. Hence, Blebbistatin could be used to probe thin-filament Ca^{2+} signaling without altering actin–myosin interactions. This property could be utilized in structural studies using light scattering techniques such as X-ray diffraction [18] or with fluorescent probes positioned within the sarcomere by protein exchange

techniques [6], provided that Blebbistatin's light sensitivity does not prevent such measurements [32]. Of note, in preliminary studies, we did not find evidence of Blebbistatin inactivation by irradiation with synchrotron-derived X-rays.

Recently, a nitro-derivative of Blebbistatin has been described, a modification that renders Blebbistatin much less light sensitive without altering its ability to inhibit the myosin ATPase activity [30]. This derivative of Blebbistatin may be a superior myofilament-uncoupling reagent should this compound, as Blebbistatin, prove to be without side effects on parameters such as Ca^{2+} handling and myofilament function.

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