Blebbistatin specifically inhibits actin-myosin interaction in mouse cardiac muscle

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Blebbistatin specifically inhibits actin-myosin interaction in mouse cardiac muscle. Am J Physiol Cell Physiol 293: C1148–C1153, 2007. First published July 5, 2007; doi:10.1152/ajpcell.00551.2006.—Blebbistatin is a powerful inhibitor of actin-myosin interaction in isolated contractile proteins. To examine whether blebbistatin acts in a similar manner in the organized contractile system of striated muscle, the effects of blebbistatin on contraction of cardiac tissue from mouse were studied. The contraction of paced intact papillary muscle preparations and shortening of isolated cardiomyocytes were inhibited by blebbistatin with inhibitory constants in the micromolar range (1.3–2.8 μM). The inhibition constants are similar to those previously reported for isolated cardiac myosin subfragments showing that blebbistatin action is similar in filamentous myosin of the cardiac contractile apparatus and isolated proteins. The inhibition was not associated with alterations in action potential duration or decreased influx through L-type Ca2+ channels. Experiments on permeabilized cardiac muscle preparations showed that the inhibition was not due to alterations in Ca2+ sensitivity of the contractile filaments. The maximal shortening velocity was not affected by 1 μM blebbistatin. In conclusion, we show that blebbistatin is an inhibitor of the actin-myosin interaction in the organized contractile system of cardiac muscle and that its action is not due to effects on the Ca2+ influx and activation systems.

MYOSIN IS THE MAIN MOTOR PROTEIN in many muscle organs, including the heart, and its interaction with actin is a key process in the cardiac pump function. Specific inhibitors of this interaction can provide insight into the molecular mechanism of cardiac and muscle function, as well as providing possible novel therapeutic approaches. One of these compounds, 2,3-butanedione monoxime (BDM), was originally introduced as an inhibitor of actin-myosin interaction in cardiac muscle (6), and it has also been applied in studies on skeletal and smooth muscle (13, 17). BDM has been used in several experimental approaches in cardiology, e.g., to determine the nonmechanical component of cardiac oxygen consumption or heat production (2, 25) and in more clinical applications, e.g., in myocardial preservation (23). However, it was early recognized that BDM, in addition to inhibiting actin-myosin interaction, also affects several other steps in the cardiac activation process, including Ca2+ release from the sarcoplasmic reticulum, Ca2+ currents, action potentials, and the Ca2+ sensitivity of the contractile filaments (12).

With the use of a screening approach, N-benzyl-p-toluene sulfonamide (BTS) was recently identified as an inhibitor of myosin II. This compound weakens myosin interaction with actin and is selective for fast skeletal myosin; its effects on cardiac and slow skeletal muscle are small (9). With the use of a similar approach, another small molecular myosin inhibitor blebbistatin was identified (22). This compound exhibits a selectivity for myosin II isoforms in biochemical analyses (15). In the concentration range 0.5–5 μM, it effectively inhibits the actin-activated myosin Mg-ATPase activity of several striated muscle and nonmuscle myosins. Kinetic analysis has shown that blebbistatin binding to the myosin-ADP-Pi complex with high affinity, interferes with the phosphate release process, and traps the myosin in a state with low actin affinity (14, 18). Furthermore, structural studies on Dictyostelium discoideum myosin II have shown that blebbistatin binds to a hydrophobic pocket at the apex of the 50-kDa cleft and keeps the cleft partially open, which is suggested to inhibit the transition of myosin into force-generating states (1). Blebbistatin exhibits an interesting specificity for myosin type when the actin-activated ATPase activity is examined. Several striated muscle myosins are inhibited with inhibition constants in the micromolar range, whereas smooth muscle myosin and some nonconventional myosins (I, V, and X) have been reported to be little influenced, with almost 100-fold higher inhibition constants. Blebbistatin is cell permeable and has been used to inhibit myosin function during cell division and motility in different cell types (5, 20, 22). It has been used to inhibit contraction of invertebrate muscle (8) and mammalian muscle preparations containing nonmuscle myosin (10, 19). Although blebbistatin potently inhibits actin-myosin interaction in biochemical studies, very little is known regarding its action in the organized contractile system of cardiac and skeletal muscles. The structural arrangement of contractile filaments and mechanical constraints imposed on the myosin crossbridges during force development and shortening might influence the blebbistatin action. To address these issues, with a focus on the cardiac striated muscle system, we have examined the effects of blebbistatin on intact and skinned mouse cardiac muscle preparations and on isolated cardiac myocytes.

METHODS AND MATERIALS

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and was approved by the local animal ethical committee.

Force in intact papillary muscles. Adult female C57Bl/6 mice (weight 20–30 g, 12–20 wk old) were euthanized by cervical dislo-

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cation. Left ventricular papillary muscles were dissected in oxygenated Krebs-Henseleit solution (in mM): 119 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 11.1 glucose, and 0.026 MgCl₂. The preparations were mounted horizontally by using surgical silk thread in an organ bath between a fixed hook and an AE801 force transducer (Capto as, Horten, Norway). Force was recorded using an AD converter (PowerLab, ADInstruments). The bath was perfused with Krebs-Henseleit solution at room temperature. The muscles were stimulated at 1 Hz via platinum electrodes at supramaximal voltage (0.5-ms pulse duration), using a Grass S48 stimulator. The preparations were stretched to a length at which active force development was maximal and were allowed to equilibrate for 30 min. Thereafter, they were exposed to blebbistatin (dl-blebbistatin, EMD Biosciences) at different concentrations (1–100 μM) or to solvent (DMSO) control during continued stimulation and force recording for 20 min. Then time course and extent of the force decay were determined.

**Force, Ca²⁺ sensitivity, and maximal shortening velocity of chemically permeabilized preparations.** Strips of mouse cardiac papillary muscle and trabeculae were permeabilized for 24 h at 4°C in a solution containing (in mM) 5 ATP, 4 EGTA, 30 imidazole (pH 7.0), 5 MgCl₂, 2 dithioerythritol (DTE), 0.5 leupeptin, 50% glycerol, and 1% Triton-X100, essentially as described in Ref. 16. Thereafter, the preparations were prepared at −20°C in a solution as above but without Triton. Thin muscle strips were dissected and mounted between a fixed pin and an extended arm of an AE801 force transducer using aluminum foil clips in a small solution bubble with continuous stirring at room temperature. The preparations were incubated in a relaxing solution containing (in mM) 30 imidazole, 5 EGTA, 11.61 Mg-acetate, 10 ATP, 20 K-methanesulfonate, 12.5 phosphocreatine, 1 DTE, and 0.5 mg/ml creatine kinase. The contraction solution was made by replacing EGTA with Ca-EGTA. The pH was adjusted to 7.0 with KOH. To determine the Ca²⁺ sensitivity, the preparations were first activated with contraction solution and then relaxed and incubated in relaxing solution with 10 or 3 μM blebbistatin dissolved in DMSO for 20 min followed by exposure to solutions with increasing Ca²⁺ concentrations, obtained by varying the ratio of Ca-EGTA-to-EGTA. Control experiments were performed in solutions containing adequate amounts of solvent (DMSO). For determination of the dose dependence and the inhibition constant, the preparations were first activated in contraction solution and then preincubated in relaxing solution with different concentrations of blebbistatin (1–100 μM) for 20 min and subsequently exposed to a contraction solution containing the same concentration of blebbistatin. Force values are normalized to the initial force determined in the absence of blebbistatin.

The maximal shortening velocity (Vₘₐₓ) of permeabilized mouse cardiac preparations was determined with the isotonic quick release method by using an apparatus as previously described (4, 16). In these experiments the preparations were mounted using aluminum clips at low passive tension between a force transducer and an isotonic lever. The muscle was activated at high Ca²⁺ concentration (pCa 4.3) in the contraction solution above, and a series of releases to different afterloads were imposed at the plateau of contraction. The muscle was then relaxed and activated a second time at pCa 4.3 with or without 1 μM blebbistatin. For comparison we also performed the second contraction at 1 mM ADP and 1 mM ATP in the absence of phosphocreatine and creatine kinase. The velocity was determined at 100 ms after release, and the Vₘₐₓ was determined by fitting a hyperbolic equation to the force and velocity data and extrapolating to zero force.

**Cardiac myocyte isolation.** Mice were euthanized by cervical dislocation, and the hearts were rapidly removed and perfused through the aorta with a perfusion buffer containing (in mM) 113 NaCl, 4.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄, 12 NaHCO₃, 10 KHCO₃, 10 HEPES, 30 taurine, 5.5 glucose, and 10 BDM, pH 7.4, at 37°C for 5 min followed by perfusion buffer containing 12.5 μM Ca²⁺ and 2 mg (547 U/ml) collagenase type II (Invitrogen) for 15 min. The ventricles were then removed, and cells were isolated following the protocols developed by the Alliance for Cellular Signaling (http://www.signaling-gateway.org; Procedure Protocol ID PP00000125).

**Recording of electrophysiological properties and myocyte shortening.** Whole cell voltage-clamp experiments were performed at room temperature using an Axon2B (Molecular Devices). Patch pipettes had a resistance of 2–5 MO. To record action potentials, the pipette solution contained (in mM) 150 KCl, 5.4 KCl, 10 HEPES, and 2 MgCl₂. The pH was adjusted to 7.2 (with KOH). The bath solution contained (in mM) 150 NaCl, 5.4 KCl, 10 HEPES, 2 MgCl₂, 10 glucose, and 1.5 CaCl₂. The pH was adjusted to 7.4 (with NaOH). Action potentials were elicited by a 3-ms current injection of suprathreshold intensity (3). Ca²⁺ current (I_Ca) was recorded in a bath solution containing (in mM) 135 TEA-Cl₂, 15 4-aminopyridine (4-AP), 10 HEPES, 2 MgCl₂, 1.5 CaCl₂, and 10 glucose. The pipettes were filled with (in mM) 140 CsCl, 10 HEPES, and 10 EGTA. A holding potential of −40 mV was used. I_Ca was elicited by imposing a series of 300-ms steps of different amplitude to a maximal potential of +40 mV. The I_Ca amplitude was estimated as the difference between peak I_Ca and the current level at the end of the pulse. The decay of I_Ca with time (t) was best fitted by the sum of two exponential components with amplitudes A and time constants τ using the following formula: I_Ca = A_fast·exp(−θ·τ_fast) + A_slow·exp(−θ·τ_slow). The effects of blebbistatin on action potential duration and I_Ca were...
examined in cells treated with 10 μM blebbistatin or DMSO control for 5 min. The effects of blebbistatin on cardiomyocyte shortening was determined in separate experiments using field stimulation via platinum electrodes at 1 Hz (0.5 ms duration, supramaximal voltage) as described previously (24).

Statistics. All data are reported as means ± SE, with the number of observations given within parenthesis. Statistical analysis (Student’s t-test for unpaired data, with Bonferroni corrections when more than two means were compared) and curve fitting were performed using SigmaPlot software (SPSS Science, Chicago, IL).
RESULTS

Effects of blebbistatin on cardiac muscle force and cardiomyocyte shortening. Figure 1 shows the time course of blebbistatin (10 μM) inhibition of papillary muscle force (Fig. 1A) and cardiomyocyte shortening (Fig. 1B). The maximal shortening responses of the isolated cardiomyocytes were about 5% of cell length as shown previously (24). In both preparations, blebbistatin inhibited contraction to near zero levels with a half-time for the effect of <3 min. Control contractions in solvent (DMSO) were not inhibited. Figure 2 shows the dose dependence of the blebbistatin inhibition in intact papillary muscles (Fig. 2A) and in maximally activated (pCa 4.3) permeabilized trabecular preparations (Fig. 2B). The inhibition was dose dependent, and when the force and concentration (c) data were fitted by a hyperbolic inhibition function (Force = h × Vmax/[c + IC50]), the inhibition constant (IC50) was determined to 1.3 μM in the intact and 2.8 μM in the permeabilized muscle. The h values were 0.9 and 0.7 in the intact and permeabilized tissues, respectively.

Effects of blebbistatin on Ca2+ sensitivity of contraction and Vmax. The effects of blebbistatin on Ca2+ sensitivity of force were examined in chemically permeabilized preparations. Figure 3 shows summarized data for active force at different free [Ca2+] in control (DMSO) and in two different concentrations of blebbistatin (3 and 10 μM), which inhibit force by 61 and 73%, respectively. The force and Ca2+ concentration (c) data were analyzed using a hyperbolic equation [Force = c/Vmax/(c + EC50)]. The relation between force and [Ca2+] was not altered at low concentrations (3 μM) of blebbistatin (EC50 in pCa units: 5.62 ± 0.06 in DMSO and 5.54 ± 0.03 in blebbistatin). At higher blebbistatin concentrations (10 μM), the relation was shifted toward higher Ca2+ concentrations (EC50 in pCa units 5.78 ± 0.07 in DMSO and 5.44 ± 0.05 in blebbistatin, P < 0.05). The h values were in the range of 1.6–1.9.

In each skinned cardiac preparation we determined an initial control force-velocity relationship during the first contraction at pCa 4.3. The subsequent force and velocity data were related to this initial control. Thereafter, force-velocity relationships were determined during the second contraction in blebbistatin-free, blebbistatin-containing, and, for comparison, in ADP-containing solution. The force and Vmax of the second contraction in the absence of blebbistatin were 94 ± 3% and 92 ± 1% of the initial contraction, respectively. In the presence of 1 μM blebbistatin the force was lowered to 66 ± 5% (P < 0.01 compared with blebbistatin-free group) and the Vmax was 89 ± 11%, showing that blebbistatin inhibited force with minor effect on shortening velocity. For comparison we performed contractions at low ATP, high ADP, which also inhibited force (to 69 ± 5%, P < 0.01 compared with blebbistatin-free group) but significantly reduced the Vmax to 53 ± 8% (P < 0.05 compared with the blebbistatin-free and blebbistatin-containing groups).

Fig. 4. Action potential recorded from isolated mouse cardiac myocytes before and 5 min after exposure to blebbistatin 10 μM (A). A: data presented as the duration to 25, 50, 75, and 90% repolarization levels. B: representative L-type Ca2+ currents evoked at 0 mV from a holding potential of −40 mV before (control) and 5 min after exposure to 10 μM blebbistatin. C: mean peak calcium current (Icalc) for each data set at each voltage potential. The inactivation phase of the Icalc evoked at 0 mV from a holding potential of −40 mV was fitted by a double exponential function. D: fast (τfast) and slow (τslow) time constants and their related amplitudes (Afast and Alsow).
did not cause rundown of $I_{\text{Ca}}$ amplitude. Figure 4B shows $I_{\text{Ca}}$ traces under control conditions and in the presence of blebbistatin, and Fig. 4C shows summarized data for the current-voltage relationship determined in cells before and after blebbistatin. In Fig. 4D, the time constants and amplitudes of the current decay are shown. These data show that blebbistatin does not affect $\text{Ca}^{2+}$ influx via the L-type $\text{Ca}^{2+}$ channels, although it significantly decreased shortening of the cardiac myocytes.

**DISCUSSION**

We show that blebbistatin directly inhibits shortening of cardiomyocytes and contraction of muscle preparations from the mouse heart. Previous studies have reported that blebbistatin inhibits the actin-activated Mg-ATPase in biochemical experiments on isolated contractile proteins (14, 15) and the cellular motility in different motile cells (5, 20, 22). Our results demonstrate that blebbistatin also acts in the organized contractile system in cardiac muscle tissue. The compound is cell permeable, and obviously diffusion into this tissue is comparatively fast, since the time required for inhibition was not markedly slower in intact papillary muscle compared with the time required for inhibition in isolated cells. The presence of cell membrane did not affect the blebbistatin sensitivity since the calculated inhibition constant was not lower in permeabilized compared with intact cardiac preparations. The inhibition constants (1.3–2.8 $\mu$M) in the mouse cardiac muscle are in the same range as the value reported for isolated porcine S1 fragments of $\beta$-cardiac muscle myosin (1.2 $\mu$M) (15), showing that the action of blebbistatin is not dramatically different between the isolated cardiac myosin fragment and the whole myosin in the thick filaments of cardiac muscle. Blebbistatin is considered to inhibit actomyosin ATPase via stabilization of a state preceding phosphate release and force generation (1). As shown by the control experiments at high ADP, inhibition of reactions immediately preceding ATP binding and cross-bridge dissociation reduces both $V_{\text{max}}$ and active force, which is not the case in blebbistatin. We show that blebbistatin inhibits force to a larger extent than it affects shortening velocity, which suggests that blebbistatin specifically inhibits force-generating cross-bridge transitions in organized contractile system. Although the main effects on cardiac contractility can be related to blebbistatin inhibition of cardiac myosin, it should be noted that blebbistatin also inhibits other myosin types, e.g., nonmuscle myosin. It is therefore possible that additional effects on cardiac cell function can be introduced by blebbistatin via inhibition of other cellular processes, e.g., on translocation of cellular components (21).

Contraction of cardiac muscle involves several cellular processes, in addition to the actin-myosin ATPase, and blebbistatin could, similarly to BDM, inhibit contraction via effects upstream of the contractile protein interaction. We find that action potential duration and $\text{Ca}^{2+}$ influx through L-type channels are not influenced by blebbistatin in a concentration that significantly inhibits cardiomyocyte shortening. The $\text{Ca}^{2+}$ sensitivity of force determined in permeabilized muscle was not influenced by a lower dose of blebbistatin (3 $\mu$M), which inhibited force by about 60%. This shows that the primary inhibitory action of blebbistatin is not by lowering $\text{Ca}^{2+}$ sensitivity. At a higher dose of blebbistatin (10 $\mu$M), which inhibits force by about 70%, the $\text{Ca}^{2+}$ dose-response curve was slightly shifted toward higher concentrations. At these lower forces, the $\text{Ca}^{2+}$ sensitivity is more difficult to determine, but the results could suggest a decreased sensitivity to $\text{Ca}^{2+}$ at higher blebbistatin concentrations. The nature of this $\text{Ca}^{2+}$-desensitizing effect is presently unknown. Attachment of crossbridges greatly enhances $\text{Ca}^{2+}$ affinity of thin filaments in striated muscle (7, 11). It is therefore possible that decreased cross-bridge cycling or lower cross-bridge binding to thin filaments in the presence of higher doses of blebbistatin alters the $\text{Ca}^{2+}$ sensitivity of the thin filaments. Based on this hypothesis, blebbistatin could tentatively be used to assess the extent of cooperative effects of cross-bridge binding on $\text{Ca}^{2+}$ sensitivity in different striated muscles.

In conclusion, we show that blebbistatin is a significant inhibitor of actin-myosin interaction in the organized contractile system of cardiac muscle and that its action is not due to effects on the $\text{Ca}^{2+}$ influx and activation systems.

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