Blebbistatin and blebbistatin-inactivated myosin II inhibit myosin II-independent processes in *Dictyostelium*

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Blebbistatin, a cell-permeable inhibitor of class-II myosins, was developed to provide a tool for studying the biologic roles of myosin II. Consistent with this use, we find that blebbistatin inhibits three myosin II-dependent processes in *Dictyostelium* (growth in suspension culture, capping of Con A receptors, and development to fruiting bodies) and does not inhibit growth on plates, which does not require myosin II. As expected, macropinocytosis (myosin I-dependent), contractile vacuole activity (myosin V-dependent), and phagocytosis (myosin VII-dependent), none of which requires myosin II, are not inhibited by blebbistatin in myosin II-null cells, but, unexpectedly, blebbistatin does inhibit macropinocytosis and phagocytosis by cells expressing myosin II. Expression of catalytically inactive myosin II in myosin II-null cells also inhibits macropinocytosis and phagocytosis. Both blebbistatin-inhibited myosin II and catalytically inactive myosin II form cytoplasmic aggregates, which may be why they inhibit myosin II-independent processes, but neither affects the distribution of actin filaments in vegetative cells or actin and myosin distribution in dividing or polarized cells. Blebbistatin also inhibits cell streaming and plaque expansion in myosin II-null cells. Our results are consistent with myosin II being the only *Dictyostelium* myosin that is inhibited by blebbistatin but also show that blebbistatin-inactivated myosin II inhibits some myosin II-independent processes and that blebbistatin inhibits other activities in the absence of myosin II.

phagocytosis | macropinocytosis | cell streaming | development

Blebbistatin (1), a cell-permeable inhibitor of myosin II ATPase activity, promises to be a very useful tool for identifying and studying myosin II-dependent processes in cells. As for any pharmacologic agent, however, extended use of blebbistatin will depend on the specificity and generality of its inhibition of class-II myosins. Thus far, blebbistatin's effect has been tested *in vitro* (1, 2) on only a few representatives of four of the 18 myosin classes that, together, contain >200 myosins. Blebbistatin inhibits myosin II from striated muscle, vertebrate nonmuscle cells, and *Dictyostelium* by $\approx 95\%$, with an IC_{50} of 0.5–5 μM . Smooth muscle and *Acanthamoeba* myosin II are only incompletely inhibited at blebbistatin concentrations as high as 160 μM , with IC_{50} values of $\approx 80 \mu M$, and blebbistatin does not inhibit either rat myosin Ib, *Acanthamoeba* myosin IC, mouse myosin V, or bovine myosin X. It is not practical to test the blebbistatin sensitivity of all of the myosins or to determine whether blebbistatin might inhibit enzymes other than myosins, whether some functions of myosin II might not require enzymatic activity (and, therefore, not be inhibited by blebbistatin), or whether enzymatically inactive myosin II might directly or indirectly affect any non-myosin II-dependent cell function in all cells.

The social amoeba *Dictyostelium discoideum* provides a useful system for assessing some of these variables. *Dictyostelium* contains a limited number of myosins: seven class-I, one class-II, two class-V, one class-VII, and two myosins (myoG and myoM) that do not belong to any established class (<http://dictybase.org>).

At least some of the functions of *Dictyostelium* class-I, -II, -V, and -VII myosins, but not of myoG and myoM, are known and myosin II-null cells have been extensively studied. If blebbistatin is specific for myosin II in *Dictyostelium*, (i) blebbistatin should have no effect on myosin II-null cells, (ii) blebbistatin should inhibit all of the myosin II-dependent functions of cells expressing myosin II, and (iii) blebbistatin-inhibited wild-type cells should be essentially identical to myosin II-null cells, unless the presence of enzymatically inactive myosin II affects the phenotype, which can be evaluated by expressing enzymatically inactive myosin II in myosin II-null cells.

After confirming that blebbistatin inhibits the basal and actin-activated ATPase activities of purified *Dictyostelium* myosin II, we evaluated the effects of blebbistatin on three cellular activities known to require myosin II [growth in suspension culture, capping of Con A receptors, and development to fruiting bodies (3–7)]; on five processes that do not require myosin II (8) [growth on plates, cell streaming, macropinocytosis, which involves class-I myosins (9, 10), contractile vacuole function, which involves class-V myoJ (11), and phagocytosis in suspension, which is myosin VII-dependent (12, 13)]; and on the organization of the actomyosin cytoskeleton. In all of the experiments, we compared the effects of blebbistatin on myosin II heavy chain-null (HS1) cells, HS1 cells expressing wild-type myosin II heavy chain (WT), and HS1 cells expressing an enzymatically inactive myosin II heavy chain mutant (E476K) (14).

Materials and Methods

Cells. HS1 cells, which lack the endogenous copy of myosin II heavy chain (15), were cultured at 21°C in HL5 medium supplemented with 60 $\mu g/ml$ penicillin and streptomycin (16). *Dictyostelium* WT, GFP-WT, and inactive myosin II heavy chain mutant GFP-E476K cDNAs were electroporated into HS1 cells by using a Bio-Rad gene pulser (17). Individual clones were selected and maintained in the presence of 12 $\mu g/ml$ geneticin (G418) sulfate in HL5 medium containing 60 $\mu g/ml$ each penicillin and streptomycin.

Drug Treatment. S-(–)-blebbistatin (lot no. 5-SHG-28-2) was purchased from Toronto Research Chemicals (Downsview, ON, Canada); (–)-blebbistatin (lot no. B62655 and (+)-blebbistatin (lot no. 62052) were purchased from EMD Biosciences (San Diego, CA). Blebbistatin was dissolved in DMSO to make a 100-mM stock solution, and cells were treated with 50, 100, or 150 μM blebbistatin, as stated, or the same volume of DMSO. S-(–)-blebbistatin from Toronto Research Chemicals was used in all experiments except where otherwise stated. In all of the biological experiments, flasks containing cells were kept in the dark and/or covered with aluminum foil to prevent photoinac-

Abbreviations: HS1, myosin II heavy chain-null; WT, myosin II heavy chain-null HS1 cells expressing wild-type myosin II heavy chain; E476K, HS1 cells expressing an enzymatically inactive myosin II heavy chain mutant.

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Fig. 1. Blebbistatin inhibits *Dictyostelium* myosin II. (A) *S*-(−)-blebbistatin (Toronto Research Chemicals) was used except for data indicated by + and −, which were (+)- and (−)-blebbistatin (EMD Biosciences), respectively. Myosin head concentration, 160 nM; F-actin concentration, 25 μM (filled circles); no F-actin (open circles). (B) Myosin head concentration, 160 nM; blebbistatin concentration, 8 μM . K_{actin} is the concentration of F-actin at half-maximal ATPase activity.

pure (−)-blebbistatin was slightly less active than the mixture of (−)- and (+)-blebbistatin (Fig. 1A).

Inhibition by blebbistatin is due entirely to a reduction in k_{cat} with no change in K_{actin} (Fig. 1B). These results are consistent with blebbistatin binding to the myosin-ADP-Pi complex, inhibiting the rate of release of P_i and trapping myosin in an F-actin weak-binding state, as has been proposed from more detailed studies of skeletal muscle myosin II (25) and nonmuscle myosin IIB (26).

Effect of Blebbistatin on Myosin II-Dependent Activities. Like myosin II-null (HS1) and E476K cells, blebbistatin-inhibited WT cells do not divide in suspension culture (Fig. 2A) resulting in large, multinucleated cells (Fig. 2B); 100 μM blebbistatin is required for complete inhibition. Blebbistatin also inhibits both the development of WT cells beyond the mound stage (Fig. 3) and the ability of WT cells to cap Con A receptors (Fig. 4A). In both assays, blebbistatin-inhibited WT cells resemble HS1 and E476K cells (Figs. 3 and 4). When added after completion of Con A-capping, blebbistatin inhibits the redistribution of myosin II that normally is complete in <20 min (Fig. 4B). *S*-(−)-blebbistatin from Toronto Research Chemicals was used in these

Fig. 2. Blebbistatin inhibits growth and cytokinesis of *Dictyostelium* in suspension culture. (A) Growth in suspension culture of WT cells and WT cells in the presence of 50 and 100 μM blebbistatin (Bb) compared with HS1 cells and HS1 E476K cells. (B) Representative images of nuclei-stained cells and the number of nuclei per cell after 3 days. (Bar, 100 μm .)

experiments but control experiments with pure (−)- and (+)-blebbistatin showed that only the (−)-isomer is active (Fig. 11, which is published as supporting information on the PNAS web site).

Effect of Blebbistatin on Myosin II-Independent Activities: Growth on Plates, Cell Streaming, and Plaque Expansion. As expected, blebbistatin does not inhibit growth or cytokinesis of WT or HS1 cells on plates, and E476K cells grow as well as WT and HS1 cells (Fig. 5A). The cells are of similar size and mostly mononucleate (Fig. 5B).

Although HS1 and E476K cells plated in non-nutrient medium stream somewhat less well than WT cells (Fig. 6A), HS1 and E476K cells form essentially normal mounds (Fig. 4). However, streaming of all three cell lines is severely affected by blebbistatin (Fig. 6A); in the presence of blebbistatin, the cells form many more streams leading to very much smaller mounds (not shown) than in the absence of blebbistatin.

Similarly, in the plaque expansion assay, HS1 cells form somewhat smaller, and E476K cells very much smaller, plaques

Fig. 6. Blebbistatin inhibits cell streaming and plaque expansion in the absence of myosin II. (A) Streams formed by WT, HS1, and E476K cells in the absence and presence of 100 μ M blebbistatin 7 h after plating in starvation buffer. (B) Plaques of WT, HS1, and E476K cells imaged 5 days after cells were plated with a suspension of dead *K. aerogenes* in HL-5 medium in the absence or presence of 150 μ M blebbistatin. (Bar, 400 μ m.)

8); endogenous myosin II in WT cells is affected identically as the GFP-myosin (not shown). Blebbistatin does not inhibit myosin II polymerization into filaments *in vitro*, nor does it cause the filaments to aggregate (Fig. 8). The effects of pure (–)-isomer were identical to those of the enantiomeric mixture and pure (+)-isomer had no effect (Fig. 11). Even though blebbistatin-inactivated myosin II is mostly aggregated in vegetative cells, it

Fig. 7. Effects of blebbistatin on macropinocytosis, contractile vacuole activity, and phagocytosis. (A) Blebbistatin (Bb) inhibits macropinocytosis of rhodamine-labeled dextran by WT but not by HS1 cells. Fluorescence is in arbitrary units normalized to protein concentration. (B) Blebbistatin (Bb) does not inhibit contractile vacuole activity as assessed by survival of cells in distilled water. (C) Blebbistatin (Bb) severely inhibits phagocytosis of latex beads by WT cells. (D) Confocal images show that blebbistatin (Bb) does not inhibit adherence of latex beads to the cell surface. Blebbistatin concentration: 150 μ M. (Bar, 3 μ m.)

Fig. 8. Blebbistatin-inhibited myosin II and E476K mutant myosin II form cytoplasmic aggregates. (Upper) Confocal images of GFP-myosin in WT cells, GFP-WT cells incubated for 1 h with 100 μ M blebbistatin (Bb), and HS1 cells expressing GFP-E476K myosin. (Bar, 5 μ m.) (Lower) Filaments of WT myosin II in the absence and presence of 100 μ M blebbistatin (Bb). (Bar, 100 nm.)

does localize properly to the rear of polarized cells and to the cleavage furrow of dividing cells (Fig. 9). Blebbistatin does not interfere with the localization of F-actin in the cortex of vegetative cells or at the leading edge of motile and dividing cells (Fig. 9).

Discussion

Although blebbistatin has been, and will continue to be, a useful tool for elucidating at least some of the biological roles of class-II myosins, one should be cautious in reaching conclusions simply from the effect or lack of effect of blebbistatin on a cellular activity. First, the myosin II in question should be shown by *in vitro* assays to be essentially completely inhibited by blebbistatin. For example, *Acanthamoeba* myosin II is only $\approx 60\%$ inhibited by 160 μ M blebbistatin, which is about as high a concentration as can be attained, and smooth muscle myosin II is only $\approx 50\%$ inhibited by 95 μ M blebbistatin (1, 2). Because we find that 50 μ M blebbistatin, which inhibits *Dictyostelium* myosin II by 85%, does not completely inhibit any of the processes that are inhibited by 100 μ M blebbistatin, myosin II functions in *Acanthamoeba* and smooth muscle might be expected to be incompletely, if at all, inhibited by blebbistatin. In the original report on blebbistatin (1), the (+)-blebbistatin enantiomer was said to

Fig. 9. Blebbistatin does not inhibit F-actin or myosin II localization in polarized and dividing cells. Confocal images of F-actin (red) and endogenous myosin II (green) in vegetative, polarized (motile), and dividing WT cells in the absence and presence of 100 μ M blebbistatin (Bb). (Bar, 10 μ m.)