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Blebbistatin extends culture life of adult mouse cardiac myocytes and allows efficient and stable transgene expression

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Kabaeva Z, Zhao M, Michele DE. Blebbistatin extends culture life of adult mouse cardiac myocytes and allows efficient and stable transgene expression. *Am J Physiol Heart Circ Physiol* 294: H1667–H1674, 2008. First published February 22, 2008; doi:10.1152/ajpheart.01144.2007.— The characterization of cellular phenotypes of heart disorders can be achieved by isolating cardiac myocytes from mouse models or genetically modifying wild-type cells in culture. However, adult mouse cardiac myocytes show extremely low tolerance to isolation and primary culture conditions. Previous studies indicate that 2,3-butanedione monoximine (BDM), a nonspecific excitation-contraction coupling inhibitor, can improve the viability of isolated adult mouse cardiac myocytes. The mechanisms of the beneficial and unwanted nonspecific actions of BDM on cardiac myocytes are not understood. To understand what contributes to murine adult cardiac myocyte stability in primary culture and improve this model system for experimental use, the specific myosin II inhibitor blebbistatin was explored as a media supplement to inhibit mouse myocyte contraction. Enzymatically isolated adult mouse cardiac myocytes were cultured with blebbistatin or BDM as a media supplement. Micromolar concentrations of blebbistatin significantly increased the viability, membrane integrity, and morphology of adult cardiac myocytes compared with cells treated with previously described 10 mM BDM. Cells treated with blebbistatin also showed efficient adenovirus gene transfer and stable transgene expression, and unlike BDM, blebbistatin does not appear to interfere with cell adhesion. Higher concentrations of BDM actually worsened myocyte membrane integrity and transgene expression. Therefore, the specific inhibition of myosin II activity by blebbistatin has significant beneficial effects on the long-term viability of adult mouse cardiac myocytes. Furthermore, the unwanted effects of BDM on adult mouse cardiac myocytes, perhaps due to its nonspecific activities or action as a chemical phosphatase, can be avoided by using blebbistatin.

2,3-butanedione monoximine; gene transfer; adenovirus

THE EXPLOSION OF MUTANT MODELS and the accessibility of gene targeting and transgenic techniques within the mouse have significantly increased the use of the mouse to study the genetic basis of heart disease and to identify important genes underlying the basic mechanisms of cardiovascular physiology. Currently, there are several established techniques available to characterize a mouse heart on the organ and tissue level, whereas extending experiments to the cellular level in the mouse has been more difficult. Primary adult cardiac myocyte culture is a valuable system to address questions on cellular mechanisms of cardiac disorders. Cardiac muscle cells from many species can be isolated enzymatically in their fully differentiated state and studied in the absence of the complex neural and hormonal homeostatic control that is required for the life of the animal in vivo. In disease models, isolated adult cardiac myocytes can provide insight into the direct alterations in myocyte cellular function that occur without the confounding secondary functional changes in the myocardium due to cardiac remodeling. Primary cultured adult myocytes can be used in range of experimental settings including cell contractility assessment, membrane excitability, and cell-signaling studies. Perhaps most importantly, recombinant viral vectors show great efficacy for gene transfer to adult cardiac myocytes in vitro, allowing genetic manipulation to directly test the molecular mechanism in vitro.

Although many laboratories have been successful in employing adult mouse cardiac myocytes in acute studies performed within the same day of isolation (29, 32), maintaining adult mouse cardiac myocytes in primary culture for gene transfer or other long-term experiments has proved to be challenging due to a rapid decline in cell viability that begins almost immediately after isolation from the heart. As we know from our experience and other studies, this is in contrast with cardiac myocytes isolated from other species, such as the rat and the rabbit that can be cultured for 5 to 7 days in vitro (18, 21). The mechanism for this difference in the ability to maintain adult cardiac myocyte viability in vitro, even between fairly closely related rodent species, is currently unclear.

The increased interest in using the mouse as a model system for studying cardiovascular physiology and cardiovascular disease has led to a number of efforts to define reproducible protocols for improving both the viability and long-term stability of adult cardiac myocytes in primary culture (20, 25, 32, 34). One major factor that may influence the difference in long-term viability between myocytes from different species is the property of rat and rabbit cardiac myocytes to remain quiescent when isolated, whereas mouse cardiac myocytes show spontaneous contractile activity. Although the mechanism is unknown, it is possible this spontaneous activity increases either the metabolic demands of cardiac myocytes, or effects their stable connections to extracellular matrix, that cannot be supported by current culture media formulations or culture substrata. In support of this idea, adult mouse cardiac myocytes appear to show increased viability in culture media supplemented with 2,3-butanedione monoximine (BDM) at a concentration that inhibits cardiac myocyte contraction. BDM was previously known to inhibit cardiac muscle cell contraction and has been proposed as an additive to improve viability

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in cardioplegia protocols. The addition of BDM to both the isolation buffer and culture media resulted in an increase of yield of viable cells as well as an extension of the culture life of the adult mouse cardiac myocyte (23, 25, 32, 34). The mechanism of the protective effect of BDM action is not yet completely understood, although there are studies indicating that BDM inhibits skeletal muscle myosin II ATPase (9, 24). However, BDM also acts as a chemical phosphatase (27) and may cause the uncoupling of excitation contraction by affecting cell contractility through a number of different mechanisms including inhibiting voltage-gated calcium channels (5), transient outward potassium channels (5), sodium calcium exchange (31), and blocking gap junctional communication (30).

Adenoviral gene transfer technology has become an attractive tool of genetic manipulation in isolated cardiac myocytes from a number of species including rats and rabbits (16, 21). The combination of viral gene delivery to myocytes from mice with targeted genetic mutations would be an important model system for gene complementation to attempt to restore cellular phenotypes and test molecular mechanisms of gene function. However, the application of adenovirus gene transfer techniques to adult mouse cardiac myocytes has been limited by the low viability of these cells in culture beyond 24 h even in the presence of BDM. Our observations of cells cultured in the presence of BDM showed decreasing viability after 24 h, and the small number of remaining rod-shaped cardiac myocytes after 48 h appeared to become mechanically rigid cell "ghosts," having lost membrane integrity and the ability to contract even in response to direct mechanical stimulation.

Therefore, this study sought to understand what the mechanisms by which BDM appears to partially protect adult mouse cardiac myocytes in primary culture are and whether these mechanisms could be more specifically targeted to improve adult mouse cardiac myocyte primary culture long-term stability and viability. Blebbistatin is a recently discovered cell motility inhibitor that specifically and preferentially binds to the ADP-P_i complex of myosin II (2, 12) and inhibits myosin cycling. Here we report that switching from BDM to blebbistatin allowed culturing of viable adult mouse cardiac myocytes for >72–96 h in primary culture and performing high efficiency adenoviral gene transfer experiments. Therefore, the specific inhibition of myosin activity and myocyte contraction by blebbistatin results in the marked improvement of the viability of adult cardiac myocyte primary culture and its use as a genetically modifiable cell culture model of the cardiovascular system.

MATERIALS AND METHODS

Cardiac myocyte primary culture. The procedures used in this study were approved by the University of Michigan Committee on the Use and Care of Animals. Animal care was provided by the University of Michigan Unit for Laboratory Animal Medicine in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* (10).

Adult cardiac myocytes were isolated from 9–13-wk-old C57BL/6 mice with a protocol worked out based on previous studies (25, 32, 34). Mice were injected intraperitoneally with heparin (100 USP units/mouse), followed by a mixture of 100 μg/g ketamine and 8 μg/g xylazine. After 20 min, the heart was removed from the chest, cannulated via the ascending aorta, and mounted on a modified Langendorff perfusion apparatus. The heart was perfused for 4 min with myocyte buffer (Table 1) at 37°C. The initial perfusion rate of 2 to 3 ml/min was an indicator of successful cannulation. After myocyte buffer, the perfusion was switched to an enzyme solution that was 75 ml of myocyte buffer plus 620 U/ml collagenase type 2 (Worthington, Lakewood, NJ), 0.104 U/ml protease XIV (Sigma), and 0.015 mg/ml DNase I grade 2 (Roche, Indianapolis, IN). After 2 min of perfusion, the enzyme solution was supplemented with 40 μl of 100 mM $CaCl_2$, and perfusion was continued for another 8 to 10 min until the flow became very fast. Enzyme solution flow through was collected in a sterile beaker, and 5 ml of it were transferred to a petri dish, into which the heart was then taken down for mechanical disaggregation (the rest of the enzyme solution was reused for a second heart if needed). The atria and aorta were removed; the ventricles were cut into two pieces, disaggregated with Dumont No. 5/45 forceps, and gently triturated five to six times with a sterile 10-ml tissue culture pipette. The resulting tissue-cell suspension was transferred to a 15-ml conical tube, incubated for 3–5 min to allow further digestion, and triturated again five to six times. Five milliliters of stopping buffer (myocyte buffer plus 2.5% BSA and 0.1 mM $CaCl_2$) were then added to the suspension, bringing its total volume to 10 ml. After the tissue-cell suspension was gently mixed by inversion, tissue chunks were allowed to settle down for ~10–15 s and the cell-containing supernatant was transferred to a new 15-ml tube. The cells were pelleted at 18 *g* for 3 min and resuspended in 10 ml of fresh stopping buffer that was then titrated to 0.5 mM Ca^{2+} by the addition of 10 μl of 100 mM $CaCl_2$ in four steps over 20 min. Five minutes after the last $CaCl_2$ addition, the cardiac myocytes were pelleted at 18 *g* for 3 min and resuspended in plating media. The cells were plated in this media for 2 h to allow attachment. Plating was performed on 22-mm^2 glass coverslips (Corning, Corning, NY) coated with 100 μl/coverslip of 56-nM mouse laminin (Invitrogen, Grand Island, NY) for 30–40 min. The plating density was ~60 cells/mm^2 with total of 20,000 cells/coverslip.

After 2 h, serum-containing plating media was switched to culture media. The culture media (Table 2) contained 10–30 mM BDM or 1–100 μM blebbistatin. (S)-(−)- blebbistatin and BDM were purchased from Toronto Research Chemicals (Cat. No. B592500; North York, ON, Canada) and Sigma (St. Louis, MO), respectively. To obtain plating media, culture media containing 10 mM BDM was supplemented with 5% fetal bovine serum (Invitrogen). The culture media was changed daily.

Cell viability. With the use of ×20 objective of the Olympus CKX31 inverted microscope (Olympus, Tokyo, Japan), three fields were counted and averaged in three to seven independent experiments. Cells were counted before and after the Trypan blue test at 0-, 24-, 48-, 72-, and 96-h time points. The *0 time point* was immediately counted after changing plating media to culture media.

The Trypan blue test was performed as follows. Media was aspirated from culture dishes, and cells were incubated with 0.04% Trypan blue (Alfa Aesar, Heysham, Lancashire, UK) for 10 min. It was followed by three washes with Dulbecco's phosphate-buffered

Table 1. *Myocyte buffer composition, pH 7.4*

Reagent	Stock Solution, M	Final Concentration, mM
NaCl	1.0	113
KCl	0.5	4.7
$MgSO_4$	0.1	1.2
KH_2PO_4	0.1	0.6
NaH_2PO_4	0.5	0.6
HEPES	Fresh	10
2,3-butanedione monoximine	Fresh	10
$NaHCO_3$	Fresh	1.6
Taurine	Fresh	30
Glucose	Fresh	20

Table 2. *Culture media composition, pH 7.4 to which 2,3-butanedione monoximine or blebbistatin was added*

Reagent	Final Concentration
MEM, Invitrogen	1×
Insulin (μg/ml)-transferrin (μg/ml)-selenium (ng/ml)	1-0.55 -0.5
Penicillin (U/ml)-streptomycin (μg/ml)	100-100
Glutamine, mM	2
$NaHCO_3$, mM	4
HEPES, mM	10
Bovine serum albumin, %	0.2

saline (PBS; Invitrogen). Rod-shaped morphology and the exclusion of Trypan blue were the criteria for identifying viable cells, whereas either the round-shaped appearance or the uptake of Trypan blue (positive Trypan blue test) was taken as the criteria for nonviable cells. Viability was defined as a ratio of nonblue rod-shaped and total cells.

One-way ANOVA with Newman-Keuls posttest was performed (unless otherwise stated) using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). A P value <0.05 was considered significant.

Recombinant adenovirus preparation and transduction. A recombinant adenoviral vector encoding the CMV promoter and GFP (AdGFP) was a kind gift from Dr. Joseph Metzger at the University of Michigan. AdGFP was amplified in HEK293 cells and purified using a cesium chloride gradient as described (1). The titer of the virus was assessed by a plaque assay and was $1–2.4 \times 10^9$ plaque-forming units/1 ml. After 2 h of attachment, cardiac myocytes were transduced with 200–480 multiplicity of infection AdGFP in 200 μl of BDM or blebbistatin culture media for 1 h. The media was then switched to fresh BDM or blebbistatin culture media.

At study time points, cells were fixed in 3% paraformaldehyde for 15 min, washed three times with PBS, and mounted on glass slides with PermaFluor (Thermo Electron, Pittsburgh, PA). The expression of GFP was visualized using an Olympus BX51 microscope (Olympus).

Western blot analysis. At 24-, 48-, and 72-h time points, the cells were washed with PBS and scraped in equal amounts of a lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 3% SDS, and protease inhibitors (0.6 mM benzamidine, 0.4 mM PMSF, 0.5 μg/ml pepstatin, 2 kallikrein inhibitor units/ml aprotinin, and 1 μg/ml leupeptins). Phosphatase inhibitors (in mM) 50 sodium fluoride, 10 sodium pyrophosphate, and 1 sodium orthovanadate were added to the lysis buffer for insulin signaling experiments. The samples were sonicated for 10 s and centrifuged at 14,000 rpm for 2 min to pellet cell debris. All of the resulting cell lysates was loaded, or volumes were normalized to the total protein content determined using DC Protein Assay (Bio-Rad, Hercules, CA). Proteins were separated by a 3–15% gradient SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The gels were afterward stained with Coomassie blue. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing (in mM) 150 NaCl and 20 Tris (pH 7.5). As a primary antibody, we used GFP (B2), which is a mouse monoclonal anti-GFP antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-mouse IgG conjugated to horseradish peroxidase (1:1,000 dilution; Jackson ImmunoResearch, West Grove, PA) was used as a secondary antibody. The membranes were developed using an enhanced chemiluminiscence assay (Pierce, Rockford, IL).

RESULTS

With the protocol reported here, we obtained a consistent cell yield ($7.1 \pm 2.7 \times 10^5$ cells/heart, $n = 20$) and a high percentage of rod-shaped cardiac myocytes ($68 \pm 6\%$, $n = 20$). The withdrawal of BDM from the isolation buffer resulted in a more than twofold decrease in the percentage of rod-shaped cells immediately after isolation (74% with BDM and 32% without BDM). Therefore, contractile inhibition during isolation is critical to cell viability.

In the first set of experiments, the influence of BDM and blebbistatin on the rod-shaped morphology of adult mouse cardiac myocytes in primary culture was tested and compared at different time points. The BDM concentration of 10 mM was initially selected based on previous reports using BDM in adult mouse cardiac myocytes (25, 29, 32, 34). The blebbistatin concentration of 25 μM was initially chosen based on trial experiments as well as studies that have used this reagent on other cell lines (8, 11, 26, 33). In a serial observation of the cultures under the microscope, as expected, untreated adult cardiac myocytes showed a rapid decline in culture viability as early as 24 h postisolation. Cardiac myocytes cultured in 10 mM BDM appeared to be stabilized for the initial 24 h, whereas cardiac myocytes cultured with 25 μm blebbistatin retained their rod-shaped appearance without a marked decline in cell number up to 72–96 h postisolation (Fig. 1). The quantification of these observations shown in Fig. 2 demonstrated that when no contraction inhibitor was used, cardiac myocytes showed a significant loss of rod-shaped morphology within the first 24 h of primary culture. The cardiac myocytes cultured with BDM started rounding up as early as after 24 h, and by 48 h in culture, the number of rods in the BDM-

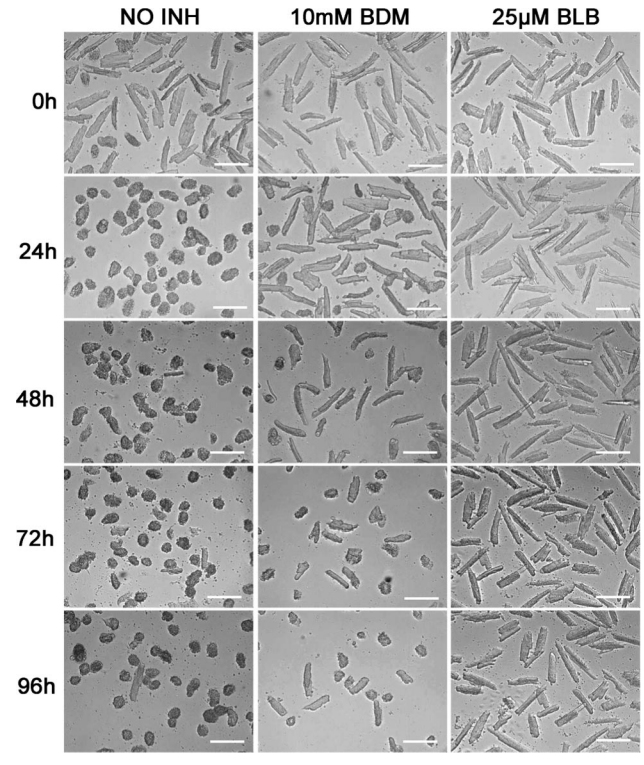

Fig. 1. Light microscope images of adult mouse cardiac myocytes cultured with no inhibitors (No Inh), 10 mM 2,3-butanedione monoximine (BDM), or 25 μM blebbistatin (BLB). In the presence of No Inh, cardiac myocytes lose their rod-shaped morphology within the first 24 h. Addition of 10 mM BDM preserves rod-shaped cells for 24 h, and 25 μM BLB preserves cardiac myocyte morphology through 96 h. Scale bar, 100 μM.

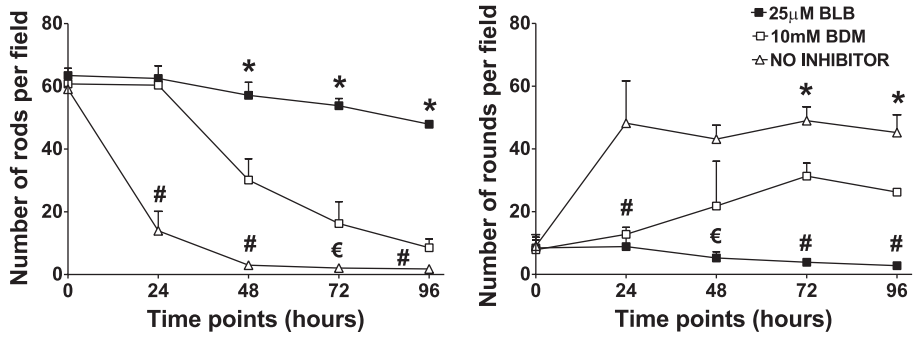

Fig. 2. Cell number dynamics of adult mouse cardiac myocytes cultured with No Inh, 10 mM BDM, or 25 μM BLB. The graphs show a number of rod-shaped (*left*) or round (*right*) cardiac myocytes per field. Each condition had a same starting number of rod-shaped cells plated. At 24 h, there was no difference in the number of rod-shaped cells in BDM- and BLB-cultured cardiac myocytes. However, by 48 h in culture, the number of rod-shaped myocytes in the presence of BDM dropped dramatically (~50%) and continued to decline over the next few days, whereas myocytes in the presence of BLB showed a relatively mild gradual decline of rod-shaped morphology. When no contraction inhibitor was used, the number of rods declined dramatically by 24 h. The number of round cells had opposite dynamics to that of rod-shaped cells. *$P < 0.05$ for 25 μM BLB vs. 10 mM BDM; #$P < 0.05$ for No Inh vs. 10 mM BDM and 25 μM BLB; €$P < 0.05$ for No Inh vs. 25 μM BLB. Data are means ± SE of 3 experiments.

containing media was significantly and markedly decreased (~50%) and continued to decline over the next 2 days (Fig. 2). In contrast, the cells cultured with blebbistatin showed significantly improved maintenance of rod-shaped morphology after 48 h and up to 96 h of postisolation. Interestingly, in the presence of untreated media, even though myocytes rapidly lose their rod-shaped morphology, a relatively high number of round-shaped cells remained attached throughout the course of the study, suggesting that BDM may also inhibit cellular attachment to the culture substrata (Figs. 1 and 2, *left*).

To assess the viability and membrane integrity of cells cultured with either BDM or blebbistatin, Trypan blue exclusion was used as a marker. Culturing cells in the presence of 25 μM blebbistatin resulted in a marked and significant improvement of cell viability up to 72 h compared with culturing cells in the presence of 10 mM BDM (Fig. 3*A*). The Trypan blue test involves a number of serial washes of the cultured cells with media to remove the dye from the experiment, and it was noted that a number of cells cultured in the presence of BDM appeared to be easily detached during these washes. Overall, the use of blebbistatin markedly improved the attachment of cells to culture substrata at 24 h as well as at later time points compared with cells cultured in the present of BDM (Fig. 3*B*). Interestingly, the percentage of cells in blebbistatin that remained firmly attached actually increased from 0 to 24 h, whereas the number of cells firmly attached in BDM decreased. Although we do not have a clear explanation for the lower attachment at 0 h compared with 24 h with blebbistatin, this could reflect the inherent attachment kinetics of adult mouse cardiac myocytes in primary culture. Alternatively, because BDM was used in the initial plating media, it is possible BDM has direct effects on cell attachment that can be removed or reversed by switching the cells to blebbistatin-containing media, leading to the improvement in attachment by 24 h. Regardless, the data suggest that adult mouse cardiac myocytes switched to blebbistatin do become more firmly attached over time in culture, and identically prepared cardiac myocytes fail to become firmly attached when cultured in the presence of BDM (Fig. 3*B*). One potential explanation for the improved effect of blebbistatin on the viability and attachment of adult mouse cardiac myocytes in primary culture was that perhaps 25 μM blebbistatin more strongly inhibited cardiac myocyte contraction than the 10 mM BDM. However, no differences were observed in the number of residual beating

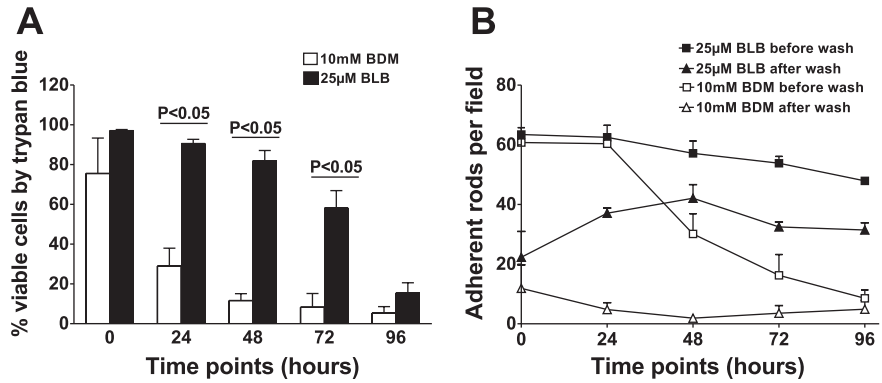

Fig. 3. Cell viability as assessed by Trypan blue (TB) test. *A*: a much higher percentage of rod-shaped cells remained TB negative (e.g., viable) up to 72 h when cultured with 25 μm BLB compared with 10 mM BDM. Percentage of TB negative rod cells out of total cells per field is plotted. Data are means ± SE of 3 experiments. *B*: the BDM condition showed remarkably greater loss of cardiac myocytes during the TB test procedure, suggesting a weak attachment. Rods were counted before and after the TB test. $P < 0.05$ for 10 mM BDM after TB test vs. 25 μM BLB after TB test at 24, 48, 72, and 96 h; $P < 0.05$ for 10 mM BDM before TB test vs. 25 μM BLB before TB test at 48, 72, and 96 h; $P < 0.05$ for 10 mM BDM before vs. after TB test at 0, 24, and 48 h; $P < 0.05$ for 25 μM BLB before vs. after TB test at 0, 24, 48, 72, and 96 h. Data are means ± SE of 3 experiments; Student's *t*-test was used to compare means.

cells in either condition (data not shown). In addition, a separate set of experiments was designed to test whether increasing the BDM concentration to 30 mM in the culture media would improve adult mouse myocyte viability compared with a media supplemented with a range of blebbistatin concentrations. Figure 4 shows that the BDM concentration of 30 mM was significantly worse than 25 μM blebbistatin, and already after 24 h in culture, the viability of myocytes cultured in the presence of 30 mM BDM dropped below 50%. Regarding different blebbistatin concentrations, these experiments show that the effects of blebbistatin on the viability of cardiac myocyte are dose dependent (Fig. 4). There was an improvement in viability with an increase in the dose from 1 to 25 μM. However, raising the dose up to 100 μM did not bring any additional improvement. Thus beneficial effects of blebbistatin can be achieved with a concentration range of 10–25 μM that is also relatively cost efficient. Using BDM in the isolation buffer and introducing blebbistatin at the later time point also reduce the total cost using this method, and the effects of BDM appear to be tolerated and reversible in cardiac myocytes after exposure for a short period of time (30).

Primary culture of adult mouse cardiac myocytes has a number of cells that retain spontaneous contractile activity after isolation and become suppressed once contraction inhibitor agents are added to media. To gain insight into the reversibility of the effects of blebbistatin, we counted cells in blebbistatin-containing media as well as 2 h after replacing with blebbistatin-free media. The increase in the number of spontaneously beating cells was taken as a marker of reversibility. At 24 h, the number of beating rods increased from 7% to 31% with 10 μM blebbistatin, whereas with 100 μM blebbistatin, it increased from 0.5% to 7%. These data suggest that the reversibility of blebbistatin is fairly rapid but may be dose dependent. Another possibility for fewer beating cells following the removal of 100 μM blebbistatin is that this dose of blebbistatin better stabilizes cells and renders them quiescent. With all concentrations of blebbistatin, there was a decrease in the number of rods recovering their spontaneous activity after blebbistatin removal at 48 h (data not shown).

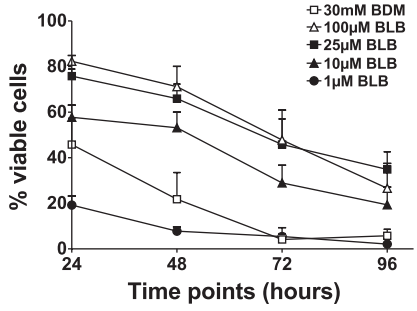

Fig. 4. Effect of 30 mM BDM and 1–100 μM BLB on cell viability. Increase of BDM concentration to 30 mM did not improve cell viability; it dropped below 50% already after 24 h in culture. Effects of BLB on cell viability are dose dependent. Comparable numbers of blue-negative rod cells were observed with 100 μM or 25 μM BLB, and results were better than with 10 μM BLB. The BLB concentration of 1 μM did not seem to improve the cell viability. $P < 0.05$ for 30 mM BDM vs. 25 and 100 μM BLB at 24 h; $P < 0.05$ for 10 μM vs. 100 μM BLB at 24 h; $P < 0.05$ for 1 μM BLB vs. rest at 24 h; $P < 0.05$ for 30 mM BDM and 1 μM BLB vs. 10, 25, and 100 μM BLB at 48 h; $P < 0.05$ for 30 mM BDM and 1 μM BLB vs. 25 and 100 μM BLB. Data are means ± SE of 3–7 experiments.

Although this may suggest that reversibility decreases with long-term exposures, it could also be explained by an inherent cell membrane remodeling occurring in myocyte culture that prevents spontaneous excitability even in the absence of any additive (13, 19).

Recombinant adenoviral gene transfer is an effective tool to address hypotheses on cellular mechanisms of cardiac disorders. The application of this technique in adult mouse cardiac myocytes requires viable cells that in the first place are able to express a transgene as well as last in primary culture for enough time to allow a sufficient amount of the transgene to be produced. In the next set of experiments, cells were transduced with adenoviral vector to test how the presence of a contraction inhibitor would influence transgene expression. Cardiac myocytes cultured with 25 μM blebbistatin showed highly efficient adenoviral transduction and GFP expression with nearly 100% of the cells expressing GFP following treatment with AdGFP (Fig. 5). This transduction was comparable with that of 10 mM BDM cultured myocytes (25) and previous results in other species (16–18, 25). These data suggest that blebbistatin does not adversely affect viral gene transfer efficiency or protein expression, and cells are viable enough to express a transgene. As Fig. 5 shows, there was an increase in the fluorescence intensity of individual cells from 24 to 72 h with both conditions; however, the population of rod-shaped cells decreased by 48 h in the presence of 10 mM BDM. At 72 h, most of the cells in 10 mM BDM media detached and were washed off during media change and fixation procedures. This also resulted in reduced amounts of total GFP on the Western blot as discussed further (supplemental Fig. 1; note: all supplementary material can be found with the online version of this article). Interestingly, cells cultured without inhibitors did show GFP expression as well, despite losing their rod-shaped morphology within the first 24 h.

Similar to the above described adverse effects of increasing the BDM concentration on culture viability, 30 mM BDM exhibited an adverse effect on GFP expression (Fig. 6*A*). At 24 h, a number of cardiac myocytes showed very weak or no GFP expression when cultured in the presence of 30 mM BDM, compared with the high-efficiency GFP expression in parallel-transduced blebbistatin-cultured cardiac myocytes from identical cell preparations. By 48 h, nearly all myocytes cultured in 30 mM BDM detached and the few remaining cells did not show any GFP expression, suggesting a toxicity of BDM at this concentration (Fig. 6*A*). One possible explanation is that higher doses of BDM compromise cell membrane integrity resulting in the loss of unattached cytoplasmic proteins. Alternatively, the prolonged treatment with higher concentrations of BDM rapidly shuts down protein transcription or translation in a relatively stochastic manner.

To quantitatively assess viral-delivered transgene expression and how cell viability decline in BDM media affects protein expression, a time course study was performed where cells were collected at 24-, 48-, and 72-h time points after AdGFP treatment and blotted for GFP. As supplemental Fig. 1 shows, a total amount of GFP remained stable from 48 to 72 h in myocytes cultured in the presence of 25 μM blebbistatin. In contrast, there was a reduction in the total level of GFP at 72 h when myocytes were cultured with 10 mM BDM. This decrease in GFP levels can be mostly accounted for by a decrease in the amount of total cellular protein due to the loss of cells

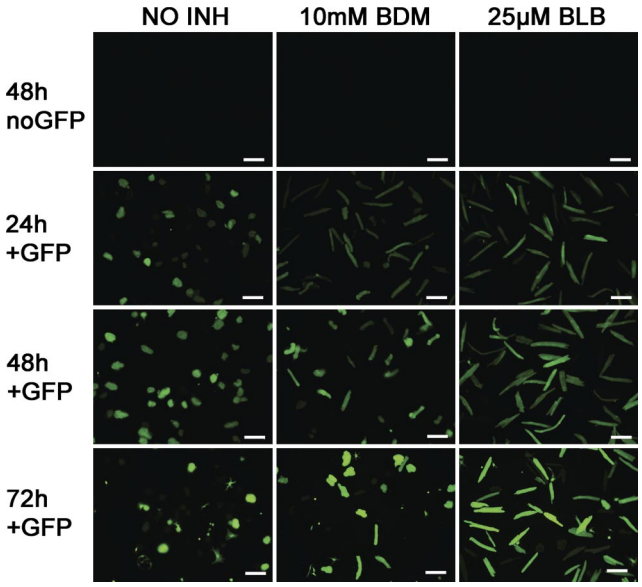

Fig. 5. Immunofluorescent images of adult mouse cardiac myocytes transduced with adenoviral vector carrying GFP and cultured with or without contraction inhibitors. Cardiac myocytes cultured with 25 μM BLB showed comparable levels of GFP expression as those cultured with 10 mM BDM; however, total yield of cells was better with 25 μM BLB. Interestingly, cells with No Inh lost their rod-shaped morphology but were still able to express GFP. Scale bar, 100 μM.

treated with 10 mM BDM. However, when the BDM concentration was increased to 30 mM, a marked reduction in the GFP protein levels was already observed at 48 h (Fig. 6*B*), suggesting additional toxic effects of BDM at higher concentrations on protein machinery in addition to cell attachment effects observed with a lower dose of BDM.

Because BDM has been reported to have a phosphatase activity, we hypothesized that the difference between blebbistatin and BDM might be explained by the interference with the insulin/Akt signaling pathway shown to be important for cell survival. To test this hypothesis, we cultured cardiac myocytes in the presence of BDM/blebbistatin for 18 h and treated them with 50 nM insulin for 15 min. Insulin was then washed out, and levels of phosphorylated Akt were assessed in cell lysates collected at 0, 5, 15, 30, and 120 min postwash (supplemental Fig. 2). The increase in Akt phosphorylation immediately after the insulin treatment (0 min) and a gradual drop-off at later time points in the presence of 25 μM blebbistatin was comparable with that of 10 mM BDM. These data suggest that neither the enhanced activation of Akt by insulin nor the prevention of its dephosphorylation is the primary mechanism of the beneficial effects of blebbistatin compared with BDM. However, these results do also show that cells cultured with blebbistatin show improved viability and can be used for cell signaling studies, likely for even longer periods than has been previously shown using BDM (22).

DISCUSSION

In this study, the contractile inhibitor BDM was replaced with the specific myosin II inhibitor blebbistatin in primary cultures of adult mouse cardiac myocytes to improve this model system for use in the systematic study of genetically modified mice. Blebbistatin significantly improved cell viability, rod-shaped morphology, cell attachment, and protein expression from an adenovirus-delivered transgene in adult mouse cardiac myocytes when compared with myocytes cultured in the presence of the conventional BDM. Therefore, the specific inhibition of myosin II activity by blebbistatin has significant beneficial effects on the long-term use of adult mouse cardiac myocytes in primary culture. Furthermore, the unwanted effects of BDM on adult mouse cardiac myocytes, perhaps due to its nonspecific activities or known action as a chemical phosphatase, can be avoided by using blebbistatin.

Both blebbistatin and BDM have been used as contraction/motility inhibitors on different cell lines (8, 11, 26, 33). The mechanism of BDM action is not yet completely clear, although there are studies indicating that BDM is a largely nonspecific agent, which has properties of a chemical phosphatase (4, 28). BDM markedly suppresses muscle contraction by at least the following two described mechanisms: *1*) inhibiting skeletal muscle myosin II ATPase (24) and *2*) uncoupling excitation and contraction due to impaired calcium transients on multiple levels (7, 9). In addition, BDM has been shown to alter the activity of ATP-sensitive K^+ channels and proteins of gap junctions in cardiac muscle (3, 30). A number of previous studies have used BDM in the concentration of 10 mM to aid in maintaining adult mouse cardiac myocytes in culture (23,

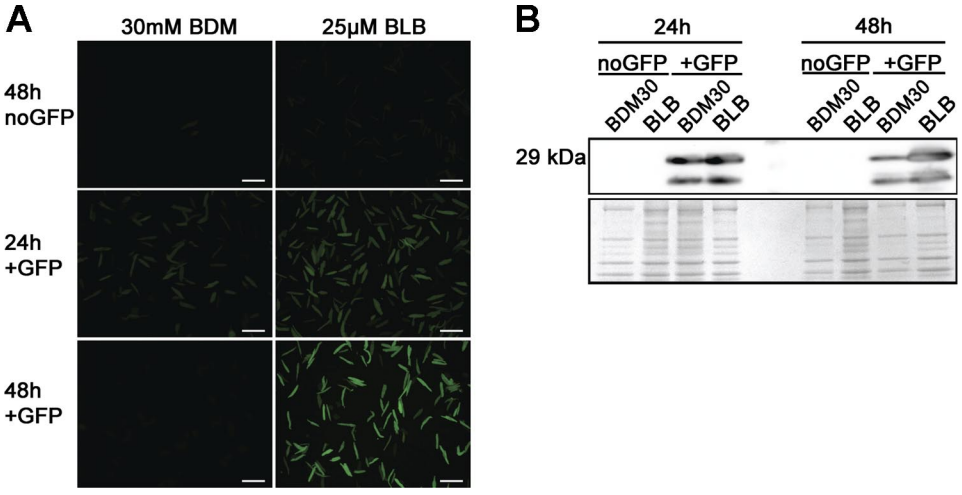

Fig. 6. BDM concentration of 30 mM exhibited an adverse effect on GFP expression. *A*: at 24 h, the level of GFP expression in cardiac myocytes cultured with 30 mM BDM was considerably less than that in BLB cultured cardiac myocytes. By 48 h, nearly all cells in 30 mM BDM detached and few remaining cells did not show any GFP expression, suggesting a toxicity of BDM at this concentration. Scale bar, 100 μM. *B*, *top*: a Western blot of cells cultured with 30 mM BDM (BDM30) or 25 μM BLB and probed with anti-GFP antibody. *B*, *bottom*: a Coomassie blue-stained gel. Increase of BDM concentration to 30 mM resulted in reduced levels of GFP expression at 48 h, suggesting cytoxicity. Loading volumes were normalized to the total protein content.

25, 34). Our experiments demonstrate that this conventional dose of BDM is relatively well tolerated by cardiac myocytes but results in decreased viability and a negative effect on cell attachment compared with blebbistatin. Because cell attachment has been shown to be important for rat cardiac myocytes survival (14), it is possible that the beneficial effects of blebbistatin relate to the improved cell attachment compared with those of BDM. Although the exact mechanism of compromised viability, cell attachment, and protein expression caused by BDM is unknown, each of these parameters was improved by the use of blebbistatin.

In contrast to BDM, blebbistatin is a more specific contraction inhibitor that binds with high affinity to the ADP-P_i complex of myosin II as shown by both functional and structural studies (2, 12). The other advantageous property of blebbistatin is that it blocks myosin in the actin-detached state, preventing rigid acto-myosin cross-linking (2, 12). The specificity of blebbistatin may be a basis for its superiority to BDM in our side-to-side experiments. Blebbistatin, being relatively recently characterized as a contraction inhibitor, has been less studied, and so far most published reports have evaluated it as a specific myosin II inhibitor (2, 6, 12). In a recent study by Dou et al. (6), 10 μM blebbistatin inhibited papillary muscle force and cardiomyocyte shortening but showed no effects on Ca^{2+} currents via L-type channels and the duration of action potential. This is in contrast with the following effects observed in the presence of BDM: exposure to BDM induces a dose-dependent shortening of action potential and progressively inhibits the current through L-type Ca^{2+} channels (4). In the same study by Dou et al. (6), the Ca^{2+} sensitivity of permeabilized papillary muscle was not influenced by a lower dose of blebbistatin (3 μM). However, the calcium sensitivity was slightly lowered with 10 μM blebbistatin, although the authors acknowledge that with a higher degree of force inhibition as observed with the 10-μM dose, the Ca^{2+} sensitivity is more difficult to determine. Furthermore, the changes in calcium sensitivity induced by blebbistatin could be a secondary consequence of its direct effect on myosin cross-bridge cycling, since alterations in myosin isoform composition have been shown to affect calcium sensitivity (15). Therefore, calcium desensitization through a noncross-bridge-mediated phenomenon is a possible limitation of blebbistatin that may be avoided by using minimal effective concentrations of blebbistatin.

In summary, this study demonstrates that the use of a specific myosin II inhibitor blebbistatin as a contraction inhibitor markedly improves cell viability and the efficiency of adenovirus-mediated gene transfer in cultured adult mouse cardiac myocytes compared with conventional methods using BDM. Therefore, the use of blebbistatin should now allow studies of genetically modifiable adult cardiac myocytes from normal, diseased, and gene-targeted mouse models.

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