BDM drives protein dephosphorylation and inhibits adenine nucleotide exchange in cardiomyocytes

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Stapleton, Mary T., Claudia M. Fuchsbaeur, and Ashley P. Allshire. BDM drives protein dephosphorylation and inhibits adenine nucleotide exchange in cardiomyocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1260–H1266, 1998.—Contractile dysfunction plays a key role in injury sustained by ischemic myocardium at reperfusion, whereas interventions that impede hypercontracture enhance recovery. In permeabilized adult rat cardiomyocytes, the negative inotrope 2,3-butanedione monoxime (BDM; 10–50 mM) inhibited rigor at low MgATP concentration but stimulated net ATP hydrolysis. Hydrolysis was attenuated by H-7, kaempferol, chelerythrine, and genistein. Evidently, BDM opposes phosphorylation of both serine/threonine and tyrosine kinase target proteins, either directly or by enhancing protein phosphatase activity, in a futile cycle of ATP hydrolysis independent of cross-bridge cycling. Although 20 mM BDM did not affect the onset of rigor contraction in permeabilized cells at low MgATP, in intact cells exposed to the metabolic inhibitors cyanide and 2-deoxyglucose rigor onset was accelerated, indicating that BDM increases ATP depletion in quiescent cardiomyocytes. Conversely, in cells exposed to the mitochondrial uncoupler carbonyl cyanide p-trifluoro-methoxyphenylhydrazone, BDM delayed the onset of contracture and hence ATP depletion, consistent with an inhibition of adenine nucleotide movements across the mitochondrial inner membrane. Such effects will limit the value of BDM as a cardioprotective agent at physiological temperature.

rigor; myofibrillar adenosine 5′-triphosphatase; ischemia; reperfusion; adenosine 5′-diphosphate

AS POWERFUL NUCLEOPHILES, 2,3-butanedione monoxime (BDM) and related oximes act as “chemical phosphatases” and so may exert biological effects by altering protein phosphorylation. The ability of BDM to impede calcium-dependent contraction of cardiac (35) and other muscle is now well documented, although the mechanism remains controversial (reviewed in Ref. 27). Inhibitory effects have been described both on the calcium transient through impaired ion flux across the sarcoplasmic reticulum and sarcolemma and directly on the myofilaments. However, the primary effect of BDM in muscle appears to vary with concentration, temperature, and species.

In calcium-activated myofibrils, BDM shifts the equilibrium distribution of cross bridges from “strong” to “weak” attachment states (15, 20) and reduces myofibrillar ATPase activity (16), consistent with a stabilization of the M**.ADP.Pi intermediate state of the cross-bridge cycle (15). This negative inotropic effect has been exploited to suppress acute injury arising from myocardial hypercontracture at reperfusion (10, 26), reduce cutting injury (22), and lengthen the storage time for hearts in cardioplegic solutions (31). However, deleterious effects of BDM on cardiomyocyte recovery were evident in an in vitro model of reperfusion (3).

As cytosolic ATP levels fall to a threshold, rigor complex formation activates cross-bridge cycling and myosin ATPase independent of calcium (5), whereas, at cell level, contraction coincides with abrupt depletion of residual cytosolic ATP (4). A positive feedback loop whereby rigor activation of actomyosin accelerates ATP depletion has been suggested to produce the striking temporal association observed between contracture and erosion of transsarcomemal sodium and calcium gradients in individual poisoned or hypoxic cardiomyocytes (1). On this basis inhibition of rigor should conserve ATP and delay or attenuate loss of ion homeostasis. However, although BDM indeed attenuates ATP and phosphocreatine depletion in working myocardium during metabolic inhibition (13), hypoxia (23), or ischemia (33), this may be secondary to reduced energy demand. When ATP resynthesis was limited in arrested heart during ischemia (14, 32) or in quiescent cardiomyocytes during hypoxia or metabolic inhibition (3, 18, 29) at physiological temperature, high-energy phosphate levels declined at least as rapidly in the presence of BDM as in its absence. Thus either rigor inhibition conferred no benefit or, alternatively, that benefit was offset by an ATP-wasting effect of BDM. Therefore, we studied in parallel how BDM affects rigor and myofibrillar ATPase activity in isolated quiescent cardiomyocytes at 37°C and investigated its impact on ATP metabolism and cell survival during metabolic inhibition.

MATERIALS AND METHODS

Isolation of rat ventricular myocytes. Ventricular myocytes were isolated from male Wistar rats (200–300 g) by Langendorff perfusion with collagenase (Warthington Biochemical, NJ) as described previously (2). The isolate contained predominantly rod-shaped cells (>60%) and was maintained at 37°C in medium 199 (ICN/F low, Thame, UK) under 5% CO2 in air for use within 8 h.

Solutions. Experiments with permeabilized cells were conducted in a standard buffer comprising (in mM) 30 MOPS, 3.5 EGTA, and 1 dithiothreitol (DTT). ATP (sodium salt), MgCl2, and KCl were added to give defined MgATP concentrations, 0.5 mM free magnesium, 3 mM free calcium, and 180 mM total ionic strength after pH had been adjusted to 7.10 at 37°C with KOH (6). Chemicals were of the highest quality commercially available from BDH (Pole, UK), Calbiochem (Nottingham, UK), or Sigma (St. Louis, MO). All solutions were prepared in fresh double-distilled water purified by reverse osmosis (Millipore), except for digitonin, fura 2, and some inhibitor stocks dissolved in DMSO.
Rigor and myofibrillar ATPase activity in suspensions of permeabilized cardiomyocytes. Cells (2 x 10^6) harvested at 20 g for 40 s from 1.5-ml aliquots of the cardiomyocyte suspensions were resuspended in an equal volume of intracellular-type medium (ICM-1) preequilibrated to 37°C, which comprised (in mM) 164 KCl, 3.5 EGTA, 1.02 MgCl2, and 30 MOPS-KOH, pH 7.10. After 3 min, suspensions were cooled to 2°C over the next 5 min with occasional gentle trituration to prevent settling, and then cells were transferred to ice-cold ICM-1 supplemented with digitonin (2 µmol/mg protein, 0.1% wt/vol) for a further 5 min. Finally, permeabilized cells were washed free of excess detergent in four changes of an equal volume of fresh, cold ICM-1 and were allowed to settle before the supernatant was aspirated.

Immediately after a 20-µl sample of the slurry of permeabilized cells had been fixed and stained by addition to an equal volume of 1% (wt/vol) each of glutaraldehyde and trypan blue for later analysis, the Microfuge tube containing the remainder of the cell pellet was transferred from ice to a 37°C water bath. Twenty seconds later the experiment was begun by resuspending the pellet in 750 µl of thermoequilibrated buffer with defined MgATP concentration. After 10 and 30 s, 250-µl samples of the cell suspension were removed for measurement of ATPase activity using the malachite green assay for P_i (7). Finally, at 40 s a 20-µl sample was fixed and stained for confirmation of cell permeabilization and rigor frequency. Rigor was defined as a cell length-to-width ratio < 3 (3), and frequency was normalized as a percentage of elongated cells present at the start of the experiment.

Myofibrillar ATPase was taken as cell-dependent ATP hydrolysis in the presence of ouabain, oligomycin, and cyclosporin A (50 µM each) to inhibit other major ATPases, namely the Na+-K+-ATPase, the mitochondrial F$_0$F$_1$-ATPase, and the sarcoplasmic reticulum Ca$_{2+}$-ATPase, on the basis that it could be abolished by a peptide mimetic of the troponin I (TnI) switch sequence, TnI$_{137–148}$ (30). Cells were exposed to ATPase inhibitors and 1 mM DTT at 2°C for 10 min and then to the inhibitors alone during the subsequent period at 37°C. When BDM and kinase inhibitors were used, the latter were present from 2 min before the start of the experiment. Kaempferol, chelerythrine, and genistein stocks were dissolved to 10 mM in ICM-2. Kaempferol was used at a final concentration of 100 µM in ICM-2, supplemented with digitonin (2 µmol/mg protein, 0.1% wt/vol) for a further 5 min. Digitonin was added to 0.01% (wt/vol), and then, after 1 min, cells were washed three times in an equal volume of ICM-2 without detergent. A portion of this suspension (6 x 10^6 cells) was transferred to a thermoregulated chamber (volume 125 µl) maintained at 37°C on the stage of an inverted microscope (Nikon), and the permeabilized cardiomyocytes were allowed to attach to the untreated glass floor for 2 min before superfusion was begun. Rigor was then induced by reducing concentrations of ATP and phosphocreatine in the ICM-2 superfusate to 10 µM and 2 mM, respectively. The x100 image of the cells (5–10 cells per field of view) was relayed via charge-coupled device camera to a video recorder and TV monitor so that shortening could be tracked off-line relative to initial cell length as marked on the monitor screen. Onset and duration of rigor was measured for cells individually. When BDM was used, it was added to the cells 2 min before induction of rigor and its effects were compared with same-day controls.

Rigor and cytosolic free calcium in intact, attached cardiomyocytes. Cells were transferred to ECM supplemented with glucose (10 mM) and calcium (1 mM), exposed to 2 µM fura 2 (Molecular Probes, Eugene, OR) in the dark for 15 min at 37°C, and then transferred to dye-free medium for 30 min to allow fura 2 deesterification to reach completion. Fura 2-loaded cells were placed in the superfusion chamber on the microscope stage at 37°C and allowed to attach for 2 min before superfusion was begun. Cytosolic free calcium was measured at 33 Hz in individual, rod-shaped cardiomyocytes with a dual-excitation microfluorimeter (Cairn, Sittingbourne, UK) by exciting intracellular dye at 340 and 380 nm (bandwidth 10 nm), measuring fluorescence at 400–470 nm with a low-noise photomultiplier (Thorn EMI, Ruislip, UK), and calculating free calcium by a standard method (11). The cell was simultaneously imaged under red light. After stable resting calcium was measured from the cell over 5 min, glucose in the superfusate was replaced with 2-deoxyglucose (5 mM) and either cyanide (2 mM) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 2 µM) to inhibit or uncouple mitochondrial respiration, respectively.

BDM. BDM (50 mM) was freshly dissolved in ICM-2 on the day of use, the pH readjusted, and the solution protected from light. Consistent with the observation that BDM affects osmotic strength only marginally (27), we found that replacement of BDM with equimolar sucrose or mannitol caused none of the parameters measured to deviate significantly from control values.

Protein. Protein content of cell suspensions was measured against bovine serum albumin standards using the Bio-Rad assay.

Statistical analysis. Data sets were compared with the use of Wilcoxon-Mann-Whitney and unpaired t-tests by using Astute software (DDU Software, University of Leeds, Leeds, UK) and taking significance as P < 0.05.

RESULTS

BDM inhibition of rigor. BDM (50 mM) impeded development of rigor in cardiomyocytes as measured after 40 s at 37°C over a range of MgATP concentrations (Fig. 1). The effect was dose dependent (IC$_{50}$ ~13 mM at 56 µM MgATP; Fig. 1, inset). At the individual cell level, this inhibition was expressed as a slowing of the shortening process (Fig. 2) and a reduction in its extent (Fig. 3A). However, even at 50 mM, the agent did not reverse established rigor in cells that had already undergone contracture after 40 s at 32 µM MgATP (data not shown).

Impact of BDM on cell energy status and cytosolic free calcium. Intact cells exposed to 2-deoxyglucose and either cyanide or FCCP underwent contractures of similar extent (Fig. 3A), followed by similar rises in cytosolic free calcium (data not shown), but showed different rates of deenergization, as indicated by onset of contracture (Fig. 3B). We could not measure temporal changes of cytosolic ATP directly in individual cells because BDM interferes with the only method available, namely microinjection with firefly luciferase (4).
However, because BDM did not affect onset of rigor in permeabilized cells superfused with low concentrations of ATP (not shown), onset of rigor in intact cells could be used as an index of ATP depletion to a threshold value. On this basis it was evident that BDM accelerated ATP depletion in cells poisoned with cyanide but attenuated ATP depletion in cells uncoupled with FCCP (Fig. 3B). The essential difference between these conditions is that cyanide prevents mitochondrial respiration and hence oxidative phosphorylation, whereas FCCP, by uncoupling mitochondria, activates a powerful intramitochondrial ATP sink as the F$_0$F$_1$-ATP synthase reverts to an H$^+$-pumping ATPase to counter depolarization of the inner membrane.

With this approach taken a stage further, Fig. 4 shows the time course of ATP depletion in individual cells treated with 50 mM BDM, as indicated by rigor contracture. Prompt removal of BDM before onset of
rigor did not affect the extent of cell shortening, suggesting that the agent had not modified the contractile apparatus irreversibly during the preceding exposure period (~4 min). Similarly, BDM removal as late as halfway through the contracture event allowed rigor to proceed to the same extent as in control cells (Fig. 4, lower dashed line). However, later removal of BDM permitted only partial cell shortening so that, by no more than 20 s after shortening was complete, cytosolic ATP was evidently too low to support further rigor at removal of BDM (Fig. 4, upper dashed line). Furthermore, the minimum time interval between development of full rigor (up to 35 s after rigor onset) and no development of further rigor on BDM removal (80 s after rigor onset) was similar to the duration of the shortening event in control cells. Thus, although BDM reduced the extent of cell shortening, cytosolic ATP in these cells was depleted just as quickly to levels too low to support cross-bridge cycling.

BDM effect on ATP hydrolysis in permeabilized cells. In parallel with measurements of cardiomyocyte rigor at low ATP concentration, we studied how BDM affected ATP hydrolysis in permeabilized cells. At 37°C, but not 2°C, cell-dependent ATP hydrolysis in the presence of inhibitors of major non-myosin ATPases was stimulated by BDM (Fig. 5), although BDM neither interfered with the Pi assay nor dephosphorylated ATP directly. However, the effect was abolished when ATP was replaced with adenosine 5'-O-(3-thiotriphosphate) (ATPγS; data not shown), suggesting that the ATP hydrolysis induced by BDM involved protein dephosphorylation to which thiophosphate esters were resistant. The possibility that BDM enhanced net ATP hydrolysis by opposing kinase-mediated protein phosphorylation was tested directly using the general serine/threonine kinase inhibitor H-7 and the tyrosine kinase inhibitor genistein. Together, H-7 and genistein returned net Pi release essentially to control values in the presence of BDM (Fig. 6), despite having no effect in its absence. More specific inhibitors of myosin light-chain kinase and protein kinase C (PKC), kaempferol and chelerythrine, respectively, also inhibited BDM-dependent ATP hydrolysis significantly. Finally, neither kinase inhibitors nor ATP replacement with ATPγS affected inhibition of rigor by BDM.

**DISCUSSION**

BDM inhibits actin-myosin interaction (15, 20, 24, 35) and reduces myofilament sensitivity to calcium (12). Although the agent decreases the efficiency of energy coupling in cardiac contraction (8), it has been used successfully as a negative inotrope to prevent tissue hypercontracture at reoxygenation or reperfusion until calcium has been restored to diastolic levels (10, 26). The present study addressed the impact of BDM on energy status of cardiomyocytes at 37°C. Besides inhibiting development of rigor, BDM showed three other major effects: 1) it accelerated deenergization under conditions that restrict ATP regeneration, 2) it promoted promiscuous dephosphorylation of phosphoproteins at serine/threonine and tyrosine residues, and 3) it inhibited adenine nucleotide movement across the mitochondrial inner membrane. Although BDM impeded hypercontracture of cardiomyocytes at re-
mation of metabolic blockade, it provided no ATP-
sparking effect nor postponed ionic derangements.

BDM slowed and reduced the extent of rigor contrac-
ture in intact cells, whether they had been subjected to
respiratory inhibition or uncoupling. BDM also slowed
rigor induced in low ATP concentration in the myofil-
ament array of permeabilized cells but could not reverse
contracture that had already taken place. Inhibition of
development, but not reversal of contracture, by BDM
(13, 18, 24) is consistent with stabilization by BDM of
weakly bound cross bridges that contain ATP hydroly-
sis products at the expense of strongly bound (force-
producing) states (13, 20).

BDM exacerbates the rate of decline of ATP in cyanide-
treated cells. Although cytosolic free ATP cannot be
measured directly in individual cardiomyocytes ex-
posed to BDM, onset of contracture marks ATP deple-
tion to the threshold at which rigor is initiated. On this
basis we found that BDM at high concentration (50 mM
but not 20 mM) accelerated deenergization in cells in
which ATP resynthesis was prevented. This is consis-
tent with the observation that BDM greatly reduced
both extramitochondrial and mitochondrial ATP in
ischemic guinea pig heart at 35°C, but not at 4°C (14),
indicating that ATP depletion promoted by BDM is not
only concentration dependent but also strongly tempera-
ture sensitive.

BDM promotes protein dephosphorylation. BDM acts
as a noncompetitive inhibitor in preparations of myosin
ATPase from skeletal muscle (15, 20) and reduces
myosin ATPase activity in cardiac muscle (8). However,
although it attenuated rigor in permeabilized cardio-
myocytes, ATP hydrolysis was increased considerably
(Fig. 5) unless ATP was replaced with ATP-γ-S. This
suggested that promotion of cell-dependent ATP hydro-
ysis by BDM might involve phosphatase activity
against which protein thiosteres generated by kinases
were resistant. Such an interpretation is strongly sup-
ported by our observation that kinase inhibitors reduce
or prevent BDM-dependent P1 release from ATP. Both
serine/threonine kinase and tyrosine kinase inhibitors
were effective and, in combination, restored ATP hydro-
ysis in the presence of BDM to control levels. Thus it
would appear that at 37°C, but not at 2°C, BDM
dramatically shifts the equilibrium between protein
phosphorylation and dephosphorylation in cardiomyo-
cytes, eliciting a compensatory increase in kinase activ-
ity at the expense of ATP. This might contribute to the
ATP depletion produced by BDM in ischemic heart (14).

Protein dephosphorylation may also underlie the os-
motic fragility (3) and susceptibility to hypercontract-
ture (19) induced in cardiomyocytes by BDM against
which phosphatase inhibitors afford protection. Never-
thless, we found no evidence that putative changes in
protein phosphorylation affected inhibition of rigor by
BDM.

It was outside the scope of this study to identify the
proteins dephosphorylated in the presence of BDM.
However, kaempferol and chelerythrine each strongly
attenuated BDM-dependent ATP hydrolysis at concen-
trations expected to produce specific inhibition of myo-
sin regulatory light chain kinase and PKC, respec-
tively. Furthermore, chelerythrine was effective at 3 nM
free calcium, consistent with activity of calcium-
dependent or novel δ and ε isoforms of PKC, the latter
of which is known to interact with myofilaments in
cardiomyocytes (17). As a strong nucleophile, BDM has
repeatedly been suggested to act as a phosphatase in
solution (3, 16, 35). Direct measurements in rat cardiomyo-
cytes showed that 5 mM BDM dephosphorylated myo-
sin regulatory light chain extensively, troponin T, tropo-
myosin, and C protein less so, and TnI not at all (34).
However, data presented here do not allow us to
distinguish whether the agent dephosphorylated pro-
teins directly or indirectly. Prolonged exposure of cardio-
myocytes to high concentrations of BDM has been
reported to cause dephosphorylation of TnI and phos-
pholamban, evidently not directly but rather through
stimulation of protein phosphatase because okadaic
acid prevented the effect (36). In contrast, in vitro
measurements at 25°C showed no effect of BDM on
myosin light-chain phosphatase from smooth muscle
but showed an inhibition of myosin light-chain kinase
(28). Hence, this question remains open. Overall, the
central role of protein phosphorylation in signal trans-
duction makes it likely that BDM will elicit diverse
effects.

BDM impedes adenine nucleotide transfer across the
mitochondrial inner membrane. A crucial observation
in this study is that BDM accelerated ATP depletion in
cardiomyocytes subject to respiratory inhibition but
conserved ATP in cells poisoned with respiratory uncou-
pler. Mitochondrial uncoupling increases ATP hydroly-
sis by F0F1-ATPase in opposition to the fall in mitochon-
drial membrane potential. In the absence of BDM, FCCP
induced rigor much faster than did cyanide,
indicating that if respiratory inhibition caused mito-
chondrial depolarization (25) under our conditions,
then it resulted in far less F0F1-mediated ATP hydroly-
sis than did FCCP. However, these rates of cyanide-
and FCCP-induced deenergization were equalized in
the presence of BDM, although the agent has no direct
effect on F0F1-ATPase (21). The most direct explanation
is that BDM restricted access of cytosolic ATP to
F0F1-ATPase, consistent with the suggestion that ad-
enine nucleotide exchange across the mitochondrial
inner membrane is inhibited (21). Thus, in the presence
of BDM, nonmitochondrial sinks for ATP predomi-
nated, potentially including futile cycling of protein
phosphorylation. On the basis of its simultaneous,
mutually reinforcing effects on ATP synthesis and
expenditure, BDM at high concentration would there-
fore be expected to hasten deenergization of cardiomyo-
cytes under hypoxia and hinder their reenergization at
reoxygenation, with the latter probably masked by low
energy demand during contractile paralysis. This is
consistent with what was observed during metabolic
inhibition (Fig. 4); although BDM reduced contracture,
it rapidly depleted cell energy reserves. This also
explains why BDM does not postpone or attenuate the
rise in cytosolic free calcium. We conclude that, in
quiescent cardiomyocytes at 37°C, BDM initiates ATP
wastage that negates any energy-sparing effect of rigor inhibition.

BDM use in cardioplegia. BDM is likely to be far more specific at low temperature because its negative inotropic action increases (16), whereas its effects on mitochondrial ATP synthesis and protein phosphorylation may diminish. We observed no BDM-induced ATPase activity in permeabilized cells at 2°C, and cold storage of rat hearts with the general kinase inhibitor staurosporine in addition to BDM did not enhance survival (9). In contrast to the situation at physiological temperature, the energy-sparing effect of BDM also outweighs inhibition of ATP resynthesis at 4°C (14). BDM has been found to improve myocardial survival of ischemia during cold storage (9, 31) and of reperfusion at 37°C (10). However, the data presented here suggest that prolonged exposure of myocardium to BDM at physiological temperature should be avoided under circumstances in which the capacity of the tissue to resynthesize ATP is limited, for example, as in bypass surgery.

In conclusion, the negative inotrope BDM accelerates rather than slows ATP depletion in quiescent, metabolically poisoned cardiomyocytes at 37°C. This may reflect both redundant cycling of protein dephosphorylation-rephosphorylation and impaired access of adenine nucleotides to the mitochondrial matrix. Hence, prolonged exposure of warm heart muscle to BDM is likely to be detrimental during surgical procedures that constrain myocardial capacity to regenerate high-energy phosphates.

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REFERENCES


