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Comparison of the effects of 2,3-butanedione monoxime on force production, myosin light chain phosphorylation and chemical energy usage in intact and permeabilized smooth and skeletal muscles

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Summary

The primary goal of this study was to determine the utility of 2,3-butanedione monoxime as a tool for determining and separating the chemical energy usage associated with force production from that of force-independent, or 'activation' processes in smooth and skeletal muscles. We determined the effects of 2,3-butanedione monoxime on force production, myosin light chain phosphorylation and high energy phosphate usage in intact and permeabilized smooth (rabbit taenia coli) and skeletal (mouse extensor digitorum longus) muscles. In the intact taenia coli, 2,3-butanedione monoxime depressed the tonic phase of the tetanus, contractures evoked by high potassium (90 mM) and by carbachol (10^{-5} M) and the small force response evoked by these agonists after treatment with D-600 (10^{-5} M). In the electrically stimulated intact taenia coli 2,3-butanedione monoxime (0–20 mM) caused a proportional inhibition of tetanic force output, myosin light chain phosphorylation and high energy phosphate usage ($ED_{50} \sim 7$ mM for all these parameters). At 20 mM 2,3-butanedione monoxime, force and energy usage fell to near zero and the degree of myosin light chain phosphorylation decreased to resting values, indicating a shut-down of both force-dependent and force-independent energy usage at high concentrations of 2,3-butanedione monoxime. In permeabilized taenia coli, 2,3-butanedione monoxime had little or no depressant effects on force production, ATPase activity or calcium sensitivity. 2,3-butanedione monoxime had a very modest inhibitory effect on the *in vitro* motility of unregulated actin filaments interacting with thiophosphorylated myosin. In solution, 2,3-butanedione monoxime inhibited myosin light chain kinase, but not the phosphatase (SMP-IV). These results suggest that the major effect of 2,3-butanedione monoxime is not on the contractile proteins themselves, but rather on calcium delivery during excitation, thereby reducing the degree of activation of myosin light chain kinase and subsequent activation of myosin by light chain phosphorylation. Thus, 2,3-butanedione monoxime is not useful for the determination of the energetics of activation processes in smooth muscle because of its inhibition of both force-dependent and force-independent processes. In contrast, in the intact mouse extensor digitorum longus, 2,3-butanedione monoxime inhibits tetanic force production ($ED_{50} \sim 2$ mM) to a much greater extent than myosin light chain phosphorylation. When 2,3-butanedione monoxime was used to manipulate force production in muscles at L_o, it was found that ~60% of the total energy usage was force-independent and the remainder was force-dependent. In the permeabilized extensor digitorum longus treated with 12 mM 2,3-butanedione monoxime, there was a decrease in calcium-activated force production and a decrease in calcium sensitivity. The effects of 2,3-butanedione monoxime were considerably greater in the intact than in the permeabilized mouse extensor digitorum longus. At 2,3-butanedione monoxime concentrations that block force production in the intact muscle, the effects on *in vitro* motility were small, yet far greater than those on smooth muscle myosin. These results suggest that 2,3-butanedione monoxime has a direct effect on the contractile proteins, but what cannot be ignored is the decrease in myosin light chain phosphorylation in the skeletal muscle, which, like the decreased force output, may result from a reduction in calcium release from the sarcoplasmic reticulum. For these reasons, the use of 2,3-butanedione monoxime to probe the components of energy usage during the contraction of skeletal muscle requires considerable caution and a full definition of its actions.

Introduction

During muscle contraction, the energy released from the hydrolysis of ATP by actomyosin ATPase is transduced into mechanical work or heat. Simultaneously, there are other energy-consuming processes in which ATP is split by other ATPases, such as those involved in calcium pumping by the sarcolemma and sarcoplasmic reticulum. A requirement for determining the energy usage associated with force production is the separation of its energy cost from force-independent processes, or 'activation' processes.

The nucleophilic oxime, 2,3-butanedione monoxime (BDM) has been found to inhibit contraction of cardiac (Wiggins *et al.*, 1980; Blanchard *et al.*, 1990; Li *et al.*, 1985) and skeletal muscles (Mulieri & Alpert, 1984; Fryer *et al.*, 1988a; Hui & Maylie, 1988; Maylie & Hui, 1988). Mulieri and Alpert (1984) and Blanchard and colleagues (1984) found that BDM had no significant effect on excitation-contraction coupling at concentrations that markedly reduced force output in frog skeletal and mammalian cardiac muscles. This enabled them to develop a method for partitioning the initial heat production during isometric twitches into force-dependent and force-independent components (Alpert *et al.*, 1989). Providing its actions were well defined, BDM, had the potential for being a useful tool for energetics studies on muscle under a variety of mechanical conditions.

The actions of BDM may be complex, depending on concentration, species and muscle type. Numerous studies of the electrophysiological properties, excitation-contraction coupling and contractile properties of permeabilized cardiac fibres suggest that BDM may depress force production through an effect on the sarcolemma, intracellular calcium mobilization, or the contractile proteins directly (Wiggens *et al.*, 1980; Li *et al.*, 1985; Sada *et al.*, 1985; Gwathmey *et al.*, 1991). 2,3-butanedione monoxime has an inhibitory effect on the action potential, charge movement and sarcoplasmic reticulum Ca-ATPase at high concentrations (10 mM) in frog skeletal muscles (Hui & Maylie, 1988; Maylie & Hui, 1988; Horiuti *et al.*, 1988), but these effects are seen even at low concentrations (< 1 mM) in mammalian muscles (Fryer *et al.*, 1988b). A general observation in both types of striated muscle is that of a primary effect on the calcium-activated force production in chemically permeabilized fibre preparations lacking a functional sarco-lemma and sarcoplasmic reticulum. Specifically, there is a decrease in maximal calcium-activated force development and a decreased sensitivity to calcium (Fryer *et al.*, 1988b; Higuchi & Takemori, 1989). These effects have been attributed to a modification of crossbridge kinetics and actin-myosin interaction (Higuchi & Takemori, 1989; Yagi *et al.*, 1992) in the striated muscles. Recent evidence suggests that BDM interacts with myosin (M) in the M-ADP-Pi state, and decreases the rate of release of Pi (Higuchi & Takemori, 1989; Lenart *et al.*, 1989) and increases the equilibrium constant of the cleavage step M-ATP⇌MADP-Pi (Hermann *et al.*, 1992). The decrease in force caused by BDM could be explained by a decrease in the number of crossbridges in the strong binding state (AM-ADP) and an increase in the number in the weak binding state (AM-ADP-Pi). Bagni and colleagues (1992) found that BDM decreases the rate constant for crossbridge attachment and reduces the force per crossbridge in frog skeletal muscle.

Work from this laboratory showed that BDM depresses force production in the intact taenia coli of the rabbit (Warren *et al.*, 1985), and this has been confirmed for rat anococcygeus (Wendt & Lang, 1987), canine trachealis (Packer *et al.*, 1988), and guinea-pig taenia coli (Osterman *et al.*, 1993) and portal vein (Watanabe, 1993). Taking into account what has been learned about the actions of BDM since the original reports of Alpert and colleagues, we thought it interesting to investigate the utility of BDM as a tool for determining force-independent energy usage in mammalian smooth and skeletal muscle and to compare the actions of this agent in intact and permeabilized muscle preparations. A preliminary report on aspects of this work was presented earlier (Warren *et al.*, 1985).

In the present studies, a direct measure of high energy phosphate utilization was used to examine the effects of BDM on isometric force production and its energy cost in intact smooth and skeletal muscles. We also measured the degree of myosin light chain phosphorylation (MyLCP), in part as an indicator of changes in myoplasmic calcium concentration during treatment with BDM. We found that in the electrically stimulated intact taenia coli of the rabbit, BDM inhibited both force-dependent and force-independent processes and had little or no effect on calcium-activated force production or calcium sensitivity in the permeabilized preparation. Comparable studies on the mouse extensor digitorum longus (EDL) showed that BDM decreases force production and associated energy usage more than it affects activation processes, and appears to interfere with crossbridge cycling through a mechanism not present in smooth muscle.

Materials and methods

PREPARATION AND TREATMENT OF SMOOTH MUSCLES

The taenia coli muscle was isolated from the caecum of immature female rabbits (New Zealand strain, white) weighing 1.5–2.5 kg, which had been killed by overdose with Nembutal (50 mg kg^{-1}). The taenia coli was freed of adhering connective tissue by blunt dissection and divided transversely into three segments about 15 mm long × 2 mm wide with a wet weight of ~15 mg.

Intact muscles

The muscle segments were mounted for measurement of isometric force and length was adjusted to L_0, the optimum length for active production, as described previously (Siegman

et al., 1980). The muscles were bathed in a flowing Krebs-bicarbonate solution at 20°C and supra-maximally stimulated in a transverse field with platinum-platinum chloride electrodes and 10 V rms, 60 Hz AC pulses (Gordon & Siegman, 1971). Force was measured with a DSC-6 force transducer (Kistler-Morse) and recorded on a Brush 440 or 2200 recorder (Gould Instruments).

Permeabilized muscles

The muscles were divided into segments that were, on average, 5 mm long, 1–1.5 mm wide, and 150 μm thick. The muscle segments were mounted on stainless steel holders and bathed in Ca^{2+}-free Krebs-bicarbonate solution (2 mM EGTA) for 1 h followed by incubation in the permeabilizing solution at 4°C for 48 h (Haeberle *et al.*, 1985). The muscles and permeabilizing solution were then frozen and stored at −76°C for up to 1 month. Before an experiment, the muscles were thawed, washed in a rigor solution for 10 min, and then incubated in a similar solution which also contained 1% Triton X-100 for 30 min. The muscles were again washed in two changes of rigor solution for at least 10 min before use in a specific protocol.

Some muscles were permeabilized by the freeze-drying technique of Gagelmann and colleagues (1984). Following treatment with Ca^{2+}-free Krebs solution, as described above, the muscles were blotted and placed in a desiccator and freeze-dried at 2°C for 24 h. The dried muscles were stored in the sealed desiccator at −5°C for up to 1 month. Prior to use, the muscles were thawed and hydrated in a rigor solution and treated as described above for freeze-glycerinated muscles.

Treatment of intact smooth muscles with metabolic inhibitors

For the determination of high energy phosphate usage during contraction, it was necessary to prevent the resynthesis of ATP through glycolysis and oxidative phosphorylation (Butler *et al.*, 1978). This was accomplished by treating intact smooth muscles with iodocacetic acid (IAA) and sodium fluoroacetate (FAA) under anaerobic conditions. Under the specific conditions used, such treatment has been shown to preserve the chemical content of the muscles as well as the mechanical responses. After equilibration in normal Krebs solution and adjustment of muscle length to L_0 (the optimum length for active force production), the bathing medium was replaced by the IAA-FAA-BDM medium at 5°C for 30 min. The muscles were then rapidly rewarmed to 18°C for 3.5 min prior to a 60 s stimulus, or, in the case of controls, further incubation for the same duration without stimulation, and then freeze-clamped in clamps pre-cooled in liquid N_2 and prepared for chemical analysis.

Measurement of ATPase activity in permeabilized muscles

ATPase activity was determined using a fluorescence device and myograph developed by Guth and colleagues (Guth & Junge, 1982; Guth & Wojciechowski, 1986). In this method, ADP formed from ATP splitting is resynthesized to ATP by reactions coupled to a change in NADH concentration using pyruvate kinase and lactate dehydrogenase. Fluorescence was measured from a cuvette with a 1 mm^2 cross-section that surrounded the isometrically held muscle bathed in the appropriate solutions. The solutions were refreshed at 20 s intervals by pulsed injection into the cuvette. The decrease in NADH fluorescence, which is proportional to the ATP utilized, was monitored for the 20 s period between injections. Muscle segments for these experiments were necessarily very slender, with a width of ~200 μm and thickness of ~100 μm, in order to reduce diffusion distance and equilibration time in the various bathing solutions.

PREPARATION AND TREATMENT OF SKELETAL MUSCLES

The EDL was dissected from 24–35-day-old male mice (Charles River, CD-1) which had been killed by decapitation. The main body of the EDL was exposed by removal of the anterior tibialis muscle. The proximal and distal tendons were carefully tied with cotton suture, and the EDL was excised with tendons intact. During the dissection the muscles were kept moist with oxygenated Krebs solution and were allowed to recover for at least 1 h in Krebs solution at 20°C.

Intact muscles

In general, the muscles were treated according to the methods described in detail by Barsotti and Butler (1984). Briefly, the pair of EDL muscles obtained from each animal was mounted in a myograph fitted with a hammer-freeze device similar to that described by Kretzschmar and Wilkie (1969). One end of each muscle was attached to a force transducer (Grass Instruments, Quincy, Massachusetts, FT-10C) and the other end was attached to a rack and pinion device for the adjustment of muscle length. Preliminary length-force determinations showed that passive force was approximately 3.0 gm at L_0, and on this basis muscle length was set to L_0. After a 10 min equilibration period in oxygenated Krebs solution at 20°C, a 2 s supra-maximal stimulus yielding a fused tetanus (square pulses, 2 ms, 20 V, 66 Hz) was applied in air via platinum wire electrodes directly in contact with the main body of the muscle. The active force produced was taken as P_0. After a 10 min recovery in Krebs solution, the muscles were incubated for 30 min either in IAA-CN Krebs solution or in IAA-CN Krebs solution containing BDM. After a 7 s stimulus, the muscles were rapidly frozen. Resting control muscles were treated in an identical fashion but not stimulated during the final 7 s.

Permeabilized muscles

Extensor digitorum longus muscles were permeabilized according to the freeze-drying technique of Gagelmann and colleagues (1984), and, except for the brief exposure to Triton X-100, were treated as described above for smooth muscle.

SOLUTIONS

Bathing media

(1) Normal Krebs solution contained the following (in mM): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.9 $CaCl_2$, and 11 glucose. The solution was gassed with a mixture of 95% O_2 − 5% CO_2 (pH 7.4). (2) Calcium-free Krebs solution was similar except that no $CaCl_2$ was added, and it contained 2 mM ethylene glycol-bis(b-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). (3) High potassium Krebs solution was the same as normal Krebs solution, except that the KCl concentration was increased to 98 mM by isosmotic replacement of NaCl. (4) IAA-FAA Krebs solution: Similar to normal Krebs solution but without glucose and containing 0.5 mM iodoacetic acid (IAA, Sigma) and 5.0 mM sodium

fluoroacetate (FAA). 2,3-butanedione monoxime was added to this solution as required by the particular protocol. The solution was gassed with a mixture of 95% N_2 and 5% CO_2. (5) IAA-CN Krebs solution: Similar to normal Krebs solution but containing 2 mM NaCN and 0.5 mM iodoacetic acid and no glucose, and bubbled with a mixture of 95% N_2 − 5% CO_2.

Normal relaxing and activating solutions

The normal relaxing solution for the permeabilized muscles contained 30 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 2 mM EGTA, 1 mM MgATP (Calbiochem), 20 mM phosphocreatine, 3 mM free Mg^{2+}, 0.5 mM leupeptin, 1 mM dithiothreitol, 1μM calmodulin (Sigma), and 1 mg ml^{-1} creatine phosphokinase (Sigma). 1,6 diaminohexane-N,N,N',N'-tetra-acetic acid (HDTA) was added to bring the ionic strength to 200 mM, and the pH was adjusted to 6.8. In the activating solutions, the total EGTA was kept at 2 mM, but pCa was adjusted to 4.5 by the addition of Ca-EGTA. In some cases, the muscles were incubated in a relaxing solution containing 0.1 mM EGTA for 5 min before exposure to activating solution with or without added BDM. This calcium 'jump' procedure (Stephenson & Williams, 1981) resulted in more rapid activation of the muscles. The composition of the solutions was calculated by means of a computer program provided by Dr Yale Goldman.

Permeabilizing solution

The permeabilizing solution used for freeze-glycerination was made of an equal volume of glycerol and of relaxing solution with 3 mM ATP. The solution also contained streptomycin sulfate (0.3 mg ml^{-1}) and penicillin G (125 U ml^{-1}).

Other solutions for permeabilized muscles

Rigor solutions contained 0.2 mM P1,P5-di(adenosine-5')pentaphosphate (Ap5A) but no ATP, creatine phosphokinase or phosphocreatine. The solutions bathing the muscle for ATPase measurements were similar to those described above for permeabilized muscles, except that phosphocreatine was excluded and they contained 1.0 mM phosphoenolpyruvate, 0.24 mM NADH, 50 U ml^{-1} pyruvate kinase and 50 U ml^{-1} lactate dehydrogenase.

CHEMICAL ASSAYS

For intact muscle studies, the bath was rapidly drained and the muscle was either frozen in a clamp between two stainless steel plates (taenia coli) or hammer-frozen (EDL). In both cases the freezing apparatus was pre-cooled in liquid nitrogen. The frozen muscles were extracted with 0.5 N $HClO_4$, neutralized and stored at −76°C as described previously (Butler *et al.*, 1978; Siegman *et al.*, 1980; Barsotti & Butler, 1984). Total creatine (Ct) and free creatine of the muscle extracts were assayed according to the method of Eggleton and colleagues (1943). Phosphoryl-creatine (PCr) was estimated as the difference between total and free creatine, with correction for creatine formation during acid hydrolysis. ATP, ADP, and AMP were assayed with a Perkin-Elmer high-performance liquid chromatograph. The nucleotides were isocratically eluted from a reverse-phase column (μ Bondapak C18, Alltech Associates) with a mobile phase of 0.05 M ammonium phosphate + 10 mM EDTA. The effluent was monitored at 259 nm with a Perkin-Elmer Model LC-75 liquid chromatography detector. The chromatograms were recorded and integrated with a Perkin-Elmer Sigma 10 Data Station. The areas of the ATP, ADP and AMP absorbance peaks were compared to those of standards.

CALCULATION OF CHEMICAL ENERGY USAGE

The chemical energy usage in the smooth muscle was determined using a 60 s isometric contraction after treatment with metabolic inhibitors that prevented resynthesis of ATP. The chemical energy usage was taken as the sum of the differences in ATP and PCr contents of stimulated and unstimulated muscles. For the skeletal muscle, chemical energy usage was determined during a 7 s isometric contraction in IAA-CN Krebs solution. The equation $\Delta PCR + 2 \times \Delta ATP + \Delta ADP$ was used to calculate the energy usage, taking into account any net ATP breakdown or resynthesis by myokinase. In both types of muscle, the phosphagen contents were normalized to the total creatine content of the muscle, which is taken as a measure of muscle mass (Butler *et al.*, 1978). Previous experiments showed that the total creatine contents of the rabbit taenia coli and the mouse EDL were 2.7 and 19 μmol g^{-1} wet wt., respectively (Butler *et al.*, 1978; Barsotti & Butler, 1984).

MYOSIN LIGHT CHAIN PHOSPHORYLATION

For smooth muscles, two-dimensional gel electrophoresis as previously described by Butler and colleagues (1983) was performed on the acid-insoluble material remaining after extraction of the muscle. Briefly, the precipitate was dissolved in a solution containing 9.0 M urea, 5% (wt/vol) 2-mercaptoethanol, and 1.5% (pH 5–7) and 0.5% (pH 7–9) ampholines (LKB, Rockville, MD, USA). This solution was subjected to isoelectric focusing followed by second dimension electrophoresis in sodium dodecyl sulfate (SDS). The relative areas of the peaks corresponding to unphosphorylated and phosphorylated forms of the 20 000 dalton light chains (LCs) were determined.

For skeletal muscle, the acid-insoluble precipitate was subjected to isoelectric focusing as described by Barsotti and Butler (1984). Following staining with Coomassie Blue, the relative areas of the bands corresponding to the phosphorylated and unphosphorylated forms of the 18 000 dalton LCs were determined.

SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE AND PHOSPHATASE ACTIVITIES

Myosin light chain kinase (MLC kinase) was prepared from frozen turkey gizzard (Ikebe *et al.*, 1987a) and calmodulin was prepared from bull testes (Walsh *et al.*, 1983). The 20 000 dalton LC of myosin was prepared from gizzard myosin as described previously (Ikebe *et al.*, 1987b). Myosin light chain phosphatase (SMP-IV) was prepared from frozen gizzard according to Pato and Kerc (1985).

Myosin light chain kinase activity was measured at 25°C using isolated 20 000 dalton LC (0.1 mg ml^{-1}) as a substrate in 30 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 μg ml^{-1} calmodulin, 1 μg ml^{-1} MLC kinase, 50 mM NaCl, 200 μM ATP containing γ-^{32}P-ATP (~40 000 cpm $nmol^{-1}$) and various concentrations of BDM. The incorporated ^{32}P was quantitated according to Walsh and colleagues (1983). The MLC kinase activity was estimated from the initial time course of the phosphorylation reaction.

For the phosphatase assay, the LC (1 mg ml^{-1}) was first phosphorylated by MLC kinase (15 μg ml^{-1}) for 20 min in the solution described above, except 10 μg ml^{-1} calmodulin and

0.2 M NaCl were used. The phosphorylated LC was precipitated by adding 5% TCA and the pellets were collected by centrifugation (3000 × g for 5 min). The pellets were washed twice with 5% TCA then dissolved with 0.1 M $NaHCO_3$. The phosphorylated LC was dialysed overnight at 4°C against 30 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol and 50 mM NaCl and used as a substrate for the phosphatase. Phosphatase activity was measured at 25°C using the phosphorylated LC (0.1 mg ml^{-1}) as a substrate in 30 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.5 mM EGTA, 50 mM NaCl and various concentrations of BDM.

IN VITRO MOTILITY ASSAY

The isolation and preparation of contractile proteins and assay procedures were performed as previously described (Warshaw *et al.*, 1990, 1991). Monomeric chicken gizzard smooth muscle myosin (thiophosphorylated) or chicken pectoralis skeletal muscle myosin, at a concentration of 250 μg ml^{-1}, was adhered to a nitrocellulose coated coverslip. The coverslip was part of a microchamber (30 μl) that was placed on the stage of an inverted microscope equipped for rhodamine epifluorescence and low light level video imaging. Unregulated actin filaments from chicken pectoralis were labelled with tetramethylrhodamine (TRITC)-phalloidin and allowed to interact with the myosin surface in the presence of 0–40 mM BDM and 1 mM MgATP at low ionic strength (25 mM KCl). The effects of varying BDM concentration were studied in separate microchambers. All experiments were performed at 30°C. The velocity of actin filament movement was determined by computer analysis of digitized video images as previously described (Warshaw *et al.*, 1990; Work & Warshaw, 1992).

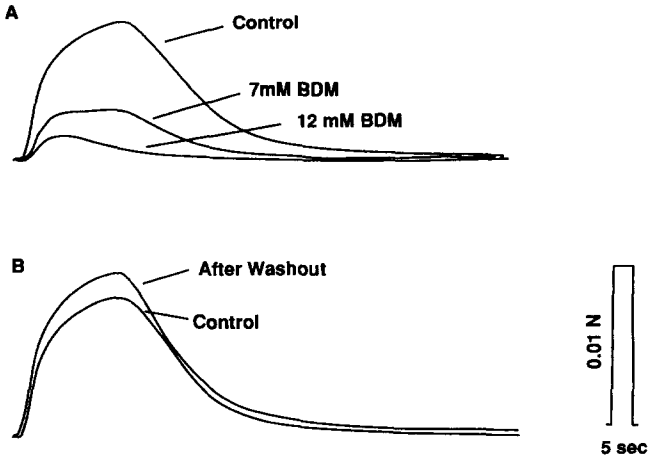

Fig. 1. Effect of BDM on tetanic force production in the intact taenia coli of the rabbit. Panel A shows original trace of a control 25 s tetanus in normal Krebs solution and tetani evoked following 15 min treatment with 7 mM and 12 mM BDM. Muscle was supra-maximally stimulated at L_0 with 60 Hz AC, 10 V rms pulses in a transverse field. Panel B shows the reversibility of the effect of BDM in the same muscle. After treatment with 12 mM BDM, the muscle was washed repeatedly with normal Krebs solution during a 15 min period and then re-stimulated. The original control trace is shown for purposes of comparison.

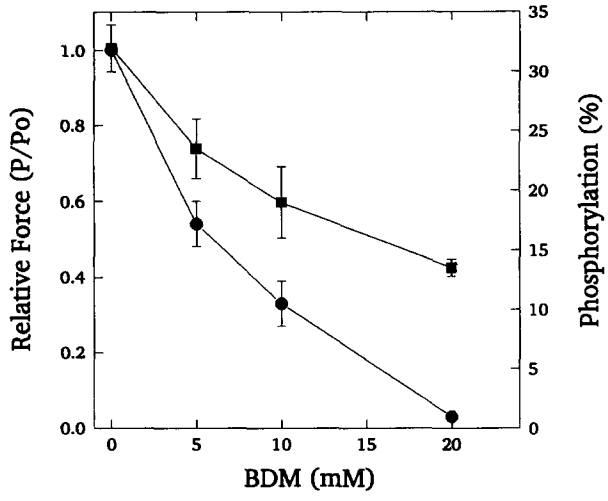

Fig. 2. Effect of BDM on maximum active force production (●) and the degree of MyLCP (■) in the intact taenia coli of the rabbit. Muscles were tetanically stimulated (60 Hz AC, 10 V rms) at L_0 for 25 s in Krebs solution containing 0, 5, 10, or 20 mM BDM. At the end of stimulation the muscles were rapidly frozen and extracted for the determination of the degree of MyLCP. Data are means ± SEM, $n = 6$ for each concentration of BDM. Data for 0 mM BDM are from Siegman and colleagues (1984).

STATISTICS

All data are reported as means ± SE. Comparisons are made using Student's *t*-test.

Results

Force production, chemical energetics and myosin phosphorylation in intact taenia coli of the rabbit

The effects of BDM on the time course of a 25 s isometric tetanus in the electrically stimulated taenia coli are shown in Fig. 1A. There is a dose-dependent depression of force production in the presence of 7 mM and 12 mM BDM, manifest primarily on the tonic phase of force production. These effects of BDM were completely reversed by thorough washing of the muscle in normal Krebs solution for 15 min (Fig. 1B).

A summary of the concentration-dependent inhibition of force production by BDM during a 25 s tetanus is shown in Fig. 2. 2,3-butanedione monoxime caused a dose-dependent reduction in active force production, with a 50% reduction in force occurring at about 7 mM BDM. Myosin light chain phosphorylation is thought to play a major role in promoting crossbridge cycling in smooth muscle. If BDM were acting only at the crossbridge, then an effect on the degree of MyLCP would not be expected.

Also shown in Fig. 2 are the corresponding effects of BDM on the degree of MyLCP. The degree of MyLCP in the resting muscle is about 10% and increases to about 32% after stimulation for 25 s in normal Krebs solution

(Siegman *et al.*, 1984). In stimulated muscles treated with BDM, there was a decrease in MyLCP, with essentially resting levels of phosphorylation occurring with 20 mM BDM, where force output was less than 5% of P_0. In order to determine whether these actions of BDM could be prevented by pretreatment with high calcium, the calcium concentration of the bathing medium was increased prior to exposure to 10 mM BDM. Specifically, muscles were incubated in Krebs solution containing 4.5 mM calcium for 1 h and tetanically stimulated for 25 s. The force response was not different from that in normal Krebs solution containing 1.9 mM calcium ($n = 4$, see also Siegman *et al.*, 1976, 1984). After relaxation, the bathing medium was replaced by another which included 10 mM BDM. The force response after BDM treatment was 22 ± 3% ($n = 4$) of the response in high calcium alone. In control muscles not exposed to BDM, the force response to a second stimulus was 90 ± 4% of the initial. Therefore, the actions of BDM could not be prevented by pretreatment with high calcium, and, surprisingly, the inhibition of force production was similar to that seen in the normal calcium medium.

The effect of 0, 7, and 12 mM BDM on chemical energy usage during a 60 s isometric tetanus in the taenia coli is shown in Fig. 3. We have previously shown that treatment with metabolic inhibitors, which is essential for the determination of chemical energy usage in smooth muscle, does not compromise force production (Butler *et al.*, 1978). 2,3-butanedione monoxime causes a proportional decrease in both active force output and the associated chemical energy usage. When force production was nearly completely blocked with 12 mM BDM, there was no significant energy usage. In order to see how the changes in MyLCP relate to force production, the data shown in Fig. 2 were replotted as a function of force in Fig. 3. In addition to the decrease in energy usage, BDM also caused a decrease in MyLCP which was proportional to the decrease in force output. Resting values of both LC phosphorylation and energy usage were obtained when force production was nearly completely blocked. This suggests that there is a shutdown of all major energy requiring processes, both force-dependent and force-independent, at high concentrations of BDM in smooth muscle.

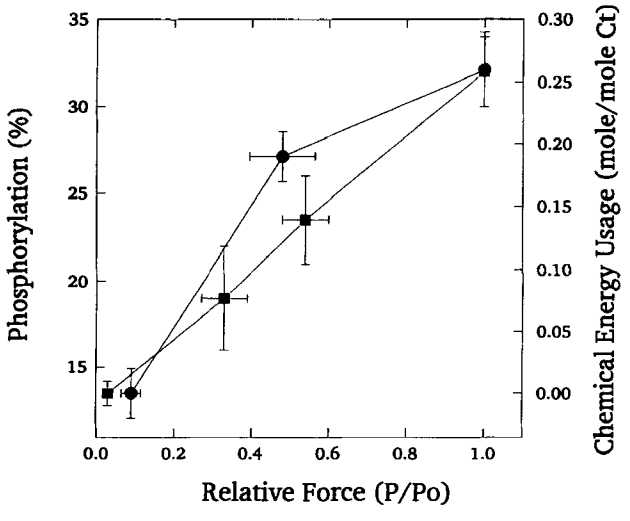

Fig. 3. Effect of BDM on total chemical energy usage (●), degree of MyLCP (■) and force production in the intact taenia coli of the rabbit. For determination of chemical energy usage, muscles were tetanically stimulated (60 Hz AC, 10 V rms) for 60 s under anaerobic conditions in glucose-free, IAA-FAA Krebs solution; the medium also contained 0, 7 or 12 mM BDM. At the end of stimulation the muscles were rapidly frozen and extracted for the determination of ATP, ADP and PCr contents. Data are means ± SEM, $n = 5$–8. The data for MyLCP, in which 0, 5, 10 or 20 mM BDM were used to vary force production, are replotted from Fig. 2.

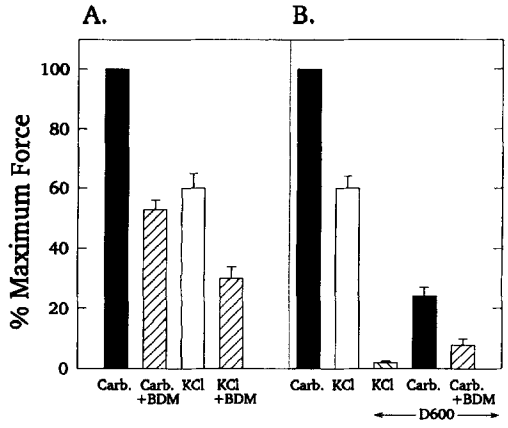

Fig. 4. Effect of BDM on the contractile responses of the intact taenia coli of the rabbit to high potassium (98 mM) and carbachol (10^{-5} M). Panel A shows control responses to carbachol and high potassium, and the corresponding responses in the presence of 7 mM BDM. Responses are normalized to the control response to carbachol. Data are means ± SEM, $n = 4$. Panel B shows the control responses in another set of muscles. Voltage-dependent Ca^{2+} channels were blocked with D600 (10^{-5} M) prior to re-activation with high K^+ or carbachol in the absence and presence of 7 mM BDM ($n = 4$).

In order to gain some insight regarding the site of action of BDM, its effect on activation through electromechanical and pharmaco-mechanical coupling pathways were tested by activating the muscle with high potassium or carbamylcholine (carbachol). In these experiments, the activation of voltage-dependent calcium channels was accomplished with high potassium or with carbachol, whereas carbachol also activated the pathways of calcium release from internal stores. It was expected that from the effects of BDM on the force responses to these agonists alone and following blockage of voltage-dependent calcium channels, the effects of BDM on pharmacomechanical coupling would be revealed. As shown in Fig. 4, panel A, compared to control responses with carbachol (10^{-5} M), force production was depressed to 52% in the presence of 7 mM BDM. The control response to high

potassium (98 mM KCl) was 60% of the carbachol responses, and this was reduced to 30% in the presence of BDM. In another group of muscles, control responses to carbachol and high potassium were again evoked (Panel B). Following blockade of voltage-dependent calcium channels with D-600 (10^{-5} M), the response to carbachol was reduced to 20% of control, and following treatment with 7 mM BDM, the carbachol response was further reduced to 10% of control. These results suggest that BDM has multiple sites of action. The ability of BDM to inhibit force production in potassium-stimulated muscles points to a plasmalemmal site of action, and the persistence of some force production in response to carbachol in the face of blockade of voltage-dependent plasmalemmal channels suggests that BDM also acts at intracellular sites of calcium release, or perhaps directly on the regulatory and/or contractile proteins.

Force production, chemical energetics and myosin phosphorylation in intact EDL of the mouse

The effects of BDM on isometric tetani in the mouse EDL are illustrated in Fig. 5A. In these experiments, muscles were stimulated for 1 s at 20°C. After recovery and 30 min treatment in Krebs solution containing BDM, the muscles were re-stimulated. Force decreased in a dose-dependent manner, and almost fully recovered following washout and return to normal Krebs solution for 30 min (Fig. 5B).

The effects of various concentrations of BDM on force production during a 7 s tetanus are summarized in Fig. 6. During this period of stimulation, steady force is maintained. At a BDM concentration of 2 mM, force output was decreased, on average, by 25%. At 7.5 mM BDM, force was reduced to 15% P_0 and did not decrease further when the BDM concentration was increased to 15 mM. It was also of interest to determine the effects, if any, of BDM on the degree of MyLCP. Although not obligatory for contraction in mouse fast-twitch skeletal muscle, as a calcium-calmodulin-dependent process, LC phosphorylation might serve as an index of changes in intracellular calcium concentration, even though the increased activity of MLC kinase outlasts the tetanus (Manning & Stull, 1979; Barsotti & Butler, 1984).

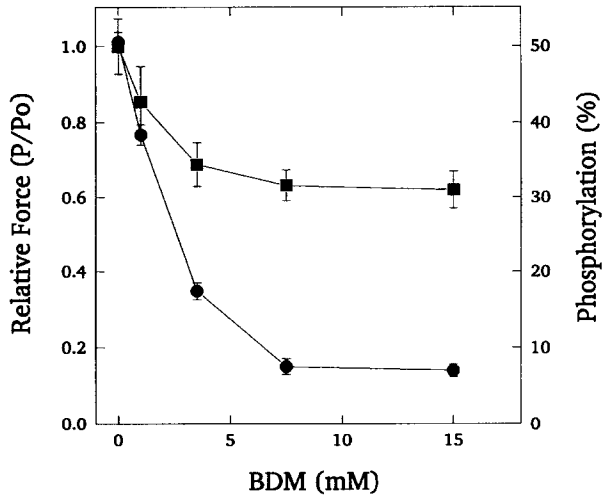

Fig. 6. Effect of BDM on maximum active force production (●) and the degree of MyLCP (■) during a 7 s tetanus in the intact EDL of the mouse. Muscles were freeze-clamped upon cessation of stimulation, and extracted for determination of the degree of MyLCP. Data are means ± SEM, $n = 8$–9 for each BDM concentration.

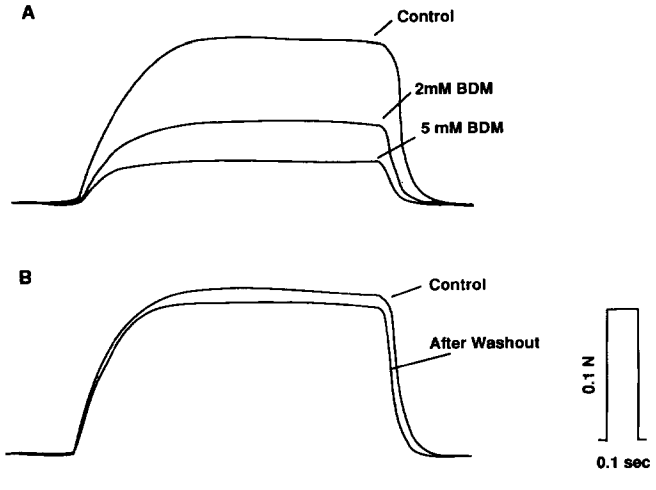

Fig. 5. Effect of BDM on tetanic force production in the intact EDL of the mouse. In Panel A the muscle was tetanically stimulated (square pulses, 2 ms, 20 V, 66 Hz) at L_0. The muscle was then treated with 2 or 5 mM BDM for 30 min prior to re-stimulation. Panel B shows the reversibility of the effect of BDM in the same muscle. After treatment with 5 mM BDM, the muscle was washed repeatedly with normal Krebs solution during a 30 min period and then re-stimulated. The original control trace is shown for purposes of comparison.

Myosin light chain phosphorylation in the resting, untreated muscle was 5% and increased to 50 ± 4% ($n = 8$) at 7 s of stimulation. As active force production decreased with increasing concentrations of BDM, there was a statistically significant decrease in the degree of LC phosphorylation at BDM concentrations of 4 mM and greater (Fig. 6). In the presence of 15 mM BDM, force was reduced by 85% whereas MyLCP was inhibited by about 40%. Therefore, the calcium-dependent activation of the MLC kinase is affected by BDM to a lesser extent than is active force production. In order to determine whether there was some causal relationship between these processes, conditions were sought in which the degree of phosphorylation could be independently increased. To this end, we took advantage of the observation of Barsotti and Butler (1984) that application of consecutive tetani in the mouse EDL resulted in an increase in the degree of MyLCP without affecting force production. Experiments were done in which three consecutive tetani of 7 s duration separated by intervals of 2 s were applied in the presence of 15 mM BDM; the muscles were frozen

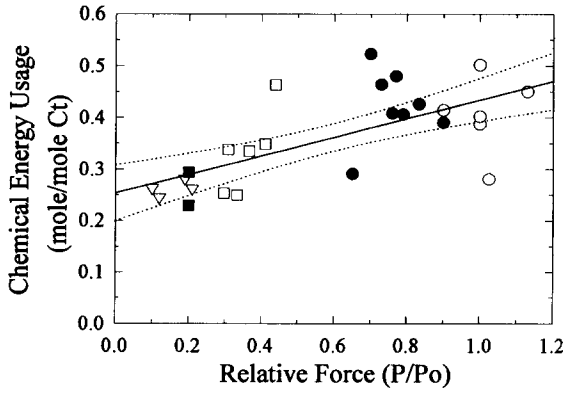

Fig. 7. The relationship between chemical energy usage and active force production under isometric conditions in mouse EDL muscles. Force production was varied by treatment of the muscles with BDM (in mM): (●), 1; (□), 3.5; (■), 7.5; (▽), 15. Untreated muscles are shown as (○). Each point represents an individual muscle. The solid line is the regression line calculated from all the individual data points. The dotted lines are the confidence level at 95%.

at the end of the third tetanus. Although the degree of LC phosphorylation in these muscles had increased to 79 ± 2% ($n = 7$), force production was only 15% of P_0. These results suggest that at the calcium concentration achieved during a tetanus in the intact EDL, the decrease in force production with BDM is primarily due to a direct effect on activation of crossbridge function rather than the consequence of a decrease in the degree of MyLCP. Both effects could result from a lower than normal increase in cytosolic calcium upon stimulation.

The mechanism of action of BDM on skeletal muscle was further investigated by determination of the chemical energy usage associated with force production in the presence of different concentrations of BDM. Muscles were electrically stimulated for 7 s at L_0, as usual, prior to freezing. Figure 7 shows the chemical energy usage and force production in the mouse EDL following a 7 s isometric tetanus in the presence of 0–15 mM BDM. In the absence of BDM, both force output (1.022 ± 0.034 P_0, $n = 6$) and chemical energy usage (0.416 ± 0.031 mole ~ P/mole Ct, $n = 6$) are high. With increasing concentrations of BDM, force progressively decreased as did chemical energy usage. The solid line in Fig. 7 is the regression line ($r = 0.69$). If BDM were blocking both force-dependent and force-independent energy usage, as was the case for smooth muscle, extrapolation of this line to zero force would intercept at the origin, indicating a complete shut-down of energy usage. This is not the case, however, and the remaining energy usage in the EDL can be ascribed to some force-independent processes which are unaffected by BDM. This force-independent energy usage is about 61% of the total energy usage in the EDL in normal Krebs solution at 20°C.

Force production of the permeabilized taenia coli

The effects of BDM on calcium-activated force was studied in permeabilized strips of taenia coli. Two methods of permeabilization were used: freeze-drying (Gagelmann *et al.*, 1984) and freeze-glycerination (Haeberle *et al.*, 1985). For freeze-dried muscles, after equilibration in relaxing solution for 30 min, the muscles were initially maximally activated at pCa 4.5, and allowed to fully relax before re-activation at a given calcium concentration. For muscles treated with BDM, this agent was added to the relaxing solution prior to re-activation. Force responses at a given pCa were normalized to the maximum force produced at pCa 4.5 during the initial activation. Treatment of several segments from a single muscle in this way enabled the determination of the relationship between calcium concentration and force. In this permeabilized preparation, 12 mm BDM had no effect on force production at any calcium concentration (Fig. 8). Because of uncertainties about the efficacy of this permeabilization method for mammalian smooth muscle, which had heretofore been reported only for molluscan catch muscles (Gagelmann *et al.*, 1984), and the paucity of information regarding the actions of BDM on permeabilized smooth muscles, the effects of BDM were also tested using muscles permeabilized by freeze-glycerination.

Freeze-glycerinated muscles were sequentially bathed in a series of solutions of increasing calcium concentration and then allowed to relax fully before the sequence was repeated in the same solutions or in others containing 7.5 or 15 mm BDM. The force responses during the initial exposure to pCa 5 and pCa 4.5 were not significantly different. Therefore, response to the initial exposure to pCa 4.5 was taken as maximum to which all other responses were normalized. In this way, any change in force production due to the re-activation of contraction

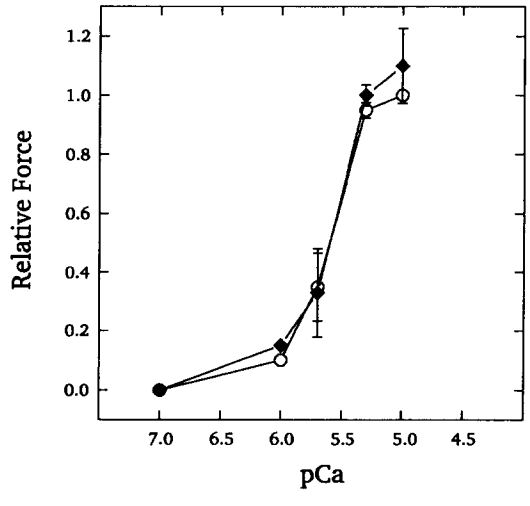

Fig. 8. Effect of BDM on calcium-activated force production in the rabbit taenia coli permeabilized by freeze-drying. Untreated control muscles are shown as (○), and muscles treated with 12 mM BDM are shown as (◆). See text for details of protocol.

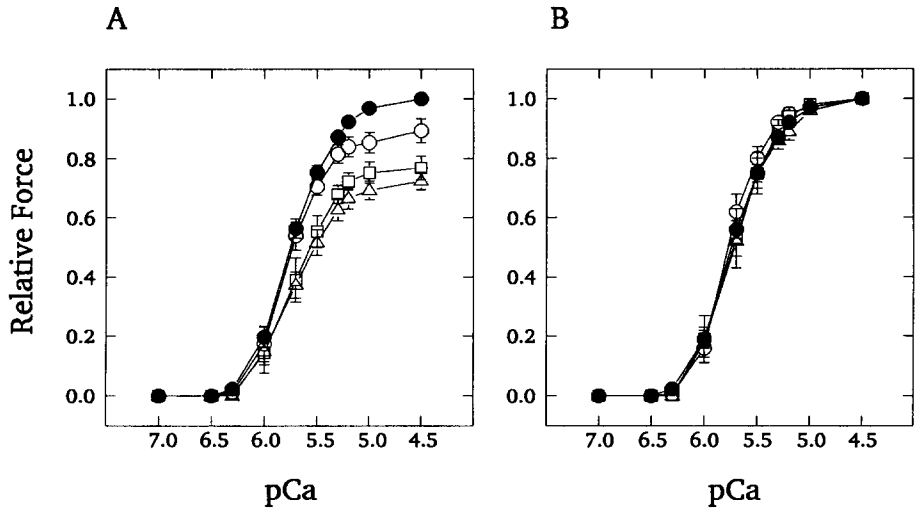

Fig. 9. Effect of BDM on calcium-activated force production in the rabbit taenia coli permeabilized by freeze-glycerination. Panel A shows force production during initial activation and re-activation at various pCa as (●) (○), respectively, $n = 25$. Muscles re-activated in the presence of 7.5 or 15 mM BDM are shown as (□) and (△), respectively, $n = 7$ for each concentration. Force responses were normalized to the maximum force developed during the initial exposure to pCa 4.5. See text for details of protocol. In Panel B, force responses were normalized to the maximum force produced at pCa 4.5 in 0, 7.5, or 15 mM BDM.

in the various calcium solutions could be accounted for. The results are shown in Fig. 9a

Re-activation of the muscles caused a significant reduction in force production at calcium concentrations of pCa 5.2–pCa 4.5. Treatment with 7.5 mm BDM caused a further reduction in force production at calcium concentrations in the range pCa 5.3–pCa 4.5, and with 15 mm BDM, force production was decreased in the range pCa 5.7–pCa 4.5. However, there were no significant differences between the force responses in 7.5 and 15 mm BDM at any of the calcium concentrations. Thus, taking the effect of re-activation into account, in muscles sequentially activated in increasing calcium concentrations, the effect of BDM was to reduce force production in the range pCa 5.7–pCa 4.5 on average, by about 17%.

The force responses were normalized to the maximum force produced at pCa 4.5 in the presence of 0, 7.5 or 15 mm BDM, and plotted as shown in Fig. 9b. It is apparent that the small change in the pCa-force relationship during re-activation of contraction is not due to a change in calcium sensitivity, nor does this relationship change as a result of treatment with BDM. As estimated from these curves, the calcium concentration required to produce 50% of the maximum force response was about pCa 5.75 in both control and BDM treated muscles.

In another set of experiments, freeze-glycerinated muscles were sequentially activated in pCa 6 and pCa 4.5 and then allowed to relax fully prior to wash in three changes of rigor solution. One group of muscles was treated with 15 mm BDM in the third rigor wash, and reactivated in BDM-containing calcium solution, whereas another was subjected to the same protocol but without BDM. Comparisons were made of the force produced in the second contraction to that in the first. For control muscles, the ratio of the responses in pCa 6 and pCa 4.5 and 1.07 ± 0.04 and 1.00 ± 0.04, respectively, and in muscles treated with 15 mm BDM, the corresponding ratio were 0.92 ± 0.07 and 0.93 ± 0.03 ($n = 4$ for each group). There were no significant differences in the forces, either in magnitude or calcium sensitivity, that could be attributed to the presence of 15 mm BDM.

ATPase activity of the permeabilized taenia coli

The effect of BDM on the ATPase activity of smooth muscle permeabilized by freeze-glycerination was determined. These experiments were particularly difficult to design because of the fact that a time-dependent decrease in ATPase activity occurs even after activation of the muscle at a constant calcium concentration. Specifically, during force development, and for a varying period thereafter, there is a high ATPase rate, which then diminishes to some steady rate. Such transient behaviour has been reported for ATPase activity in permeabilized preparations of guinea-pig taenia coli (Kuhn *et al.*, 1990) and rabbit portal vein (Butler *et al.*, 1990, see Fig. 3). In the *Staphylococcus aureus* α-toxin permeabilized mesenteric artery activated by calcium, norepinephrine and GTP, an early transient in MyLCP occurs which is not accompanied by any change in shortening velocity (Moreland *et al.*, 1992b). We have found that the consequences of a temporal slowing of the ATPase rate impact on interpretation of results obtained in at least two types of protocols. In the first, a muscle may be activated to a steady level of force in a given calcium solution, which is then followed by the same solution, either with or without BDM. In the second, the muscle is activated by cumulative additions of calcium, and the force and ATPase activity measured at steady-state. Under these

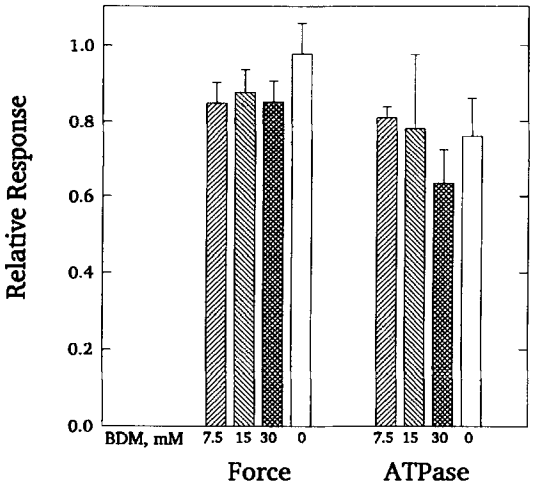

Fig. 10. Effect of BDM on force production and suprabasal ATPase activity of freeze-glycerinated taenia coli. Initial determinations were made in pCa 4.5 and, following relaxation, during re-activation in the presence of 0 ($n = 5$), 7.5 ($n = 6$), 15 ($n = 3$), or 30 ($n = 5$) mM BDM.

conditions, the ATPase activity may have decreased maximally in the control solutions, and show no further change whether or not exposed to BDM, or, decrease while force production is actually increasing. Clearly, the period of the contraction during which measurements are made will influence the results. We found that it was imperative that the development of force be brisk, thereby reducing the duration of the transient, and that measurements be made during the earliest period of constant force maintenance. Of course, limitations on the resolution of changes in ATPase rate are posed by the method itself (e.g., injection frequency, equilibration time with new solutions).

For the above reasons, it was necessary to forego the possibility of obtaining cumulative or complete dose-response relationships from the ATPase activity and force under the influence of BDM. Rather, the effects of BDM at a given calcium concentration were tested. To this end, the force and ATPase activity during an initial contraction at pCa 4.5 were compared with those obtained during re-activation at the same calcium concentration or in the presence of 7.5, 15, or 30 BDM. In these experiments, the duration of exposure to the various solutions was rigorously controlled. Muscles initially in rigor solution were equilibrated in relaxing solution for 10 min, treated with 'jump' solution for 5 min, activated at some calcium concentration for 10 min, allowed to relax for 20 min, and the procedure repeated. Only those ATPase values occurring between 5 and 10 min during the plateau of force production were used. The same protocol was followed for muscles treated with BDM, except that the agent was included in the 'jump' relaxing solution and calcium activating solution used for re-activation.

In order to assess any effects of BDM on the resting ATPase, comparisons were made of the resting ATPase activity when the muscle was bathed in relaxing solution and in the 'jump' relaxing solutions prior to the first and second contractions (ratio of initial : second measurement). In the BDM protocol, 7.5 mm BDM was included in the 'jump' relaxing solution prior to the second contraction. The ratio of the ATPase activities of the muscles in relaxing solution was 1.14 ± 0.09, $n = 8$). The ratio of the ATPase activity in the 'jump' relaxing solution for control muscles was 1.05 ± 0.05, $n = 7$, and in the presence of 7.5 mm BDM was 1.03 ± 0.04, $n = 6$. Therefore, BDM had no effect on the resting ATPase activity.

The effect of BDM on force and suprabasal ATPase activity at pCa 4.5 are shown in Fig. 10. The force

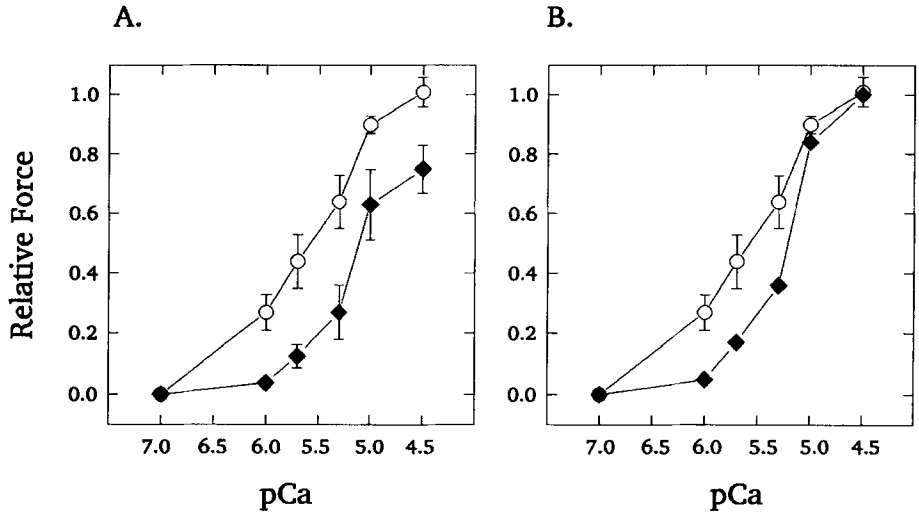

Fig. 11. Effect of BDM on calcium-activated force production in the mouse EDL permeabilized by freeze-drying. Panel A shows force production during the initial activation at various pCa (○), and during re-activation at various pCa in the presence of 12 mM BDM (◆), all relative to the maximum initial response at pCa 4.5. In Panel B, force responses in the absence of BDM are plotted along with those for BDM-treated muscles normalized to the maximum response at pCa 4.5.

produced on re-activation in calcium solutions alone was unchanged, and the response of muscles reactivated in the presence of 7.5, 15, or 30 mm BDM showed no significant dependence on the concentration of BDM used. Force production was inhibited by about 15%, and these results are of the same magnitude as those shown in Fig. 9. Taken together, it is apparent that BDM has a very small inhibitory effect on force production in the permeabilized smooth muscle.

Although the force response did not change upon re-activation in calcium solutions alone, there was a decrease in the ratio of ATPase activity of the first and second contractions to 0.76 ± 0.10 ($0.025 < p < 0.05$). In the presence of 7.5, 15, or 30 mm BDM, the ratios of the ATPase activity upon reactivation were reduced, and not significantly different from each other or from the ATPase activity of the untreated, control muscles. Thus, BDM had no effect on the ATPase activity. It is interesting that the ATPase activity decreased during re-activation in the face of unchanged maximal force. Clearly the relationship between force and ATPase was not further perturbed by treatment with BDM. It is conceivable that the ATPase rate during the second contraction may have decreased maximally, so that BDM had no apparent effect. Along these lines, in a limited number of trials, the BDM concentration was suddenly increased from 7.5 or 15 to 30 mm during the plateau of force maintenance, with no effect on force or ATPase (data not shown).

Force production of the permeabilized EDL

For purposes of comparison, the effect of BDM on Ca^{2+}-activated force was studied in skeletal muscles permeabilized by freeze-drying. The protocol was identical to that described for freeze-dried smooth muscles. The results are shown in Fig. 11a.

Calcium-activated force production decreased in the presence of 12 mm BDM. This concentration was chosen because of its ability to maximally, albeit not totally, depress force production in the intact, electrically stimulated muscle. Force production decreased significantly at every calcium concentration tested. Further, the calcium sensitivity of the contractile apparatus decreased (Fig. 11b). The calcium concentration required to produce 50% of the maximum force response increased from pCa 5.6 to pCa 5.2 in the presence of 12 mm BDM. It is interesting that in the range 7.5–15 mm BDM, tetanic force production in the intact muscle was reduced by about 85%. Thus, it appears that the effects of BDM are greater in the intact than in the permeabilized mouse EDL.

In vitro motility

The effects of BDM at the level of the contractile apparatus were directly assessed using an *in vitro* motility assay. In this assay, the movement of fluorescently labelled actin filaments are observed as they are propelled by monomeric myosin adhered to a glass surface. Contractile proteins isolated from smooth and skeletal

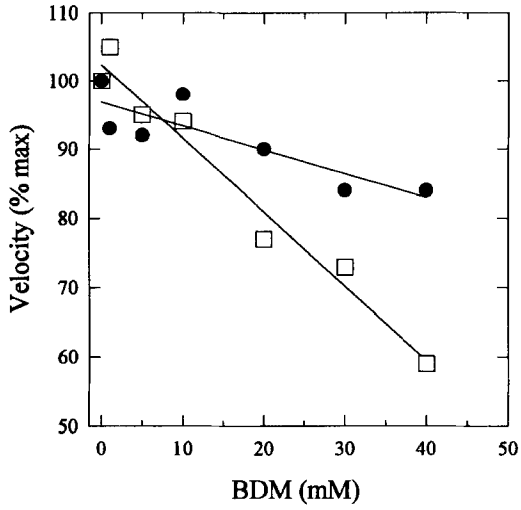

Fig. 12. Effect of BDM on actin filament velocity over skeletal (□) and thiophosphorylated smooth muscle (●) myosin. Actin filament velocity at various BDM concentrations was normalized to the velocity in the absence of BDM (V_{max}). The velocity of at least ten filaments were determined for each BDM concentration and the mean value calculated. The experiment was repeated twice and the data shown represents the mean of the mean velocities for each BDM concentration. The solid lines are the linear regressions through the data. The mean velocities at 0 BDM were 3.98 μm^{-1} for skeletal muscle myosin, and 0.54 μm^{-1} for thiophosphorylated smooth muscle myosin.

muscles were compared. For smooth muscle, the myosin was maximally thiophosphorylated. As shown in Fig. 12, the presence of BDM resulted in the slowing of actin filament motility in a dose-dependent manner up to the highest BDM concentration studied (40 mm). There was no apparent effect on actin filament binding to the myosin surface, given that the number and percentage of moving actin filaments was unchanged in the presence of BDM (data not shown). Therefore, the primary effect was a reduction in actin filament velocity. This effect was more pronounced with skeletal muscle myosin, where, at 40 mm BDM, a 41% reduction in actin filament velocity was observed, as compared to only a 16% reduction with smooth muscle myosin. However, at concentrations of BDM that maximally inhibit force production in intact smooth muscle, the effect of BDM was very small. A similar comparison for skeletal muscle myosin also showed a rather modest effect.

Regulation by smooth muscle myosin light chain kinase and phosphatase

The profound inhibitory effects of BDM on force production and the degree of MyLCP in intact smooth muscle raises the question of whether these changes are due to direct effects on the regulatory enzymes, MLC kinase and MLC phosphatase. That is, BDM may reduce the net phosphorylation of the LCs by inhibiting the kinase and/or by stimulating the phosphatase. Also, BDM is known to act as a phosphatase on channel proteins

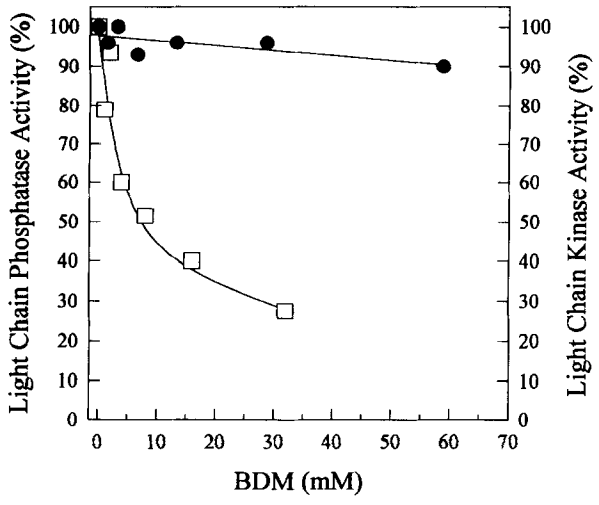

Fig. 13. Effects of BDM on smooth muscle MLC kinase and phosphotase (SMP-IV) activities. Turkey gizzard MLC kinase (□) and phosphatase (●) activities, in the presence of various concentrations of BDM, were determined as described in Materials and Methods.

(Huang & McArdle, 1992) but there is no comparable information regarding its direct effects on the enzymes acting on MLCs. Therefore, direct determinations were made of the effects of a wide range of BDM concentrations (1–55 mm) on smooth muscle MLC kinase and phosphatase (SMP-IV) activities. The results are shown in Fig. 13. 2,3-butanedione monoxime, at concentrations of 1–32 mm, markedly inhibited MLC kinase activity, but at concentrations up to 55 mm, had practically no effect on SMP-IV activity.

Discussion

Heretofore, the utility of BDM as a tool for partitioning force-dependent and force-independent energy usage has only been shown from measurements of heat production in cardiac muscle (Alpert *et al.*, 1989). It is well established that there is a tight correlation between mechanical output (crossbridge cycling) and force-dependent heat production (Woledge, 1971; Homsher *et al.*, 1972; Gibbs & Loiselle, 1978). This relation is linear and extrapolates to the origin when force production is zero. Alpert and colleagues (1989) found that within the range 2–5 mm, BDM reduced initial heat by selectively decreasing crossbridge cycling and the accompanying heat production. Under these conditions, the relationship between mechanical output and heat production was linear; at higher concentrations of BDM, the linearity was lost. On these grounds, it was concluded that BDM, within the limits stated, could be used to estimate force-independent energy usage. Moreover, the estimates obtained agreed well with those from studies in which both heat and high energy phosphate usage were measured in muscles stimulated at long lengths, where filament overlap was nil (Homsher *et al.*, 1972). The latter manoeuvre proved to be impractical in many smooth muscles because of the very high passive tension at long muscle lengths and the irreversible reduction in force production that occurs following return to L_0 (Peterson & Paul, 1974; unpublished observations from this laboratory). For this reason, BDM was an attractive probe. In the present study, we have made direct measurements of high energy phosphate utilization in order to test the effects of BDM. In addition to measurements of force production and high energy phosphate utilization, the degree of MyLCP was determined. The rationale for measuring the latter was that as a calcium-dependent process with very different roles in smooth and skeletal muscle, it would, nevertheless, provide some index of the fate of calcium within the cell upon stimulation. We reasoned that, if BDM selectively blocks the force-dependent energy utilization, both the force-independent energy usage and the calcium-calmodulin-dependent process of MyLCP would be expected to remain constant as force production is inhibited by increasing concentrations of BDM.

Effects of BDM on smooth muscle

In the intact, electrically-stimulated taenia coli, BDM caused a proportional, dose-dependent decrease in active force output, MyLCP, and chemical energy usage, with an ED_{50} of about 7 mm for all. There was a rather linear relationship between force output and chemical energy usage, and at high concentrations of BDM, force and energy usage fell to near zero. Under the same conditions MyLCP decreased to resting levels. These results suggest that there is a shut-down of all energy-requiring processes (both force-dependent and force-independent) at high concentrations of BDM. These effects of BDM were similar to those which occurred when the calcium concentration of the bathing medium was reduced (Butler & Siegman, 1983), and point to an effect of BDM on calcium translocation during excitation.

In searching for the site(s) of action of BDM in the rabbit taenia coli, processes related to excitation-contraction coupling were probed with respect to force production. A major portion of the contractile response to high potassium was blocked by BDM, an observation consistent with those for other smooth muscles such as the rat anococcygeus (Wendt & Lang, 1987) and guinea-pig taenia coli, in which BDM blocks inward Ca^{2+} currents through voltage-activated channels (Lang & Paul, 1991). Also consistent with these results was the pronounced depression of the tonic phase of the tetanus in the intact electrically stimulated muscle. Further, a major portion of the force produced by carbachol was blocked by BDM, as was the much smaller portion of the response that persisted after blockade of voltage-activated calcium channels at the surface membrane, a response that relies on pharmaco-mechanical coupling mechanisms.

The permeabilized muscle was used as a model for testing the effects of BDM on the contractile and

regulatory proteins under conditions of constant calcium concentrations. There appears to be a disparity in the calcium-activated force responses obtained when muscles permeabilized by freeze-glycerination were re-activated following cumulative increases in calcium concentration (Fig. 9), compared to activation and re-activation at a single calcium concentration (Fig. 10). For example, in the former protocol, but not in the latter, there was a significant decrease in force production upon re-activation at pCa 4.5. When the maximum force production upon re-activation at pCa 4.5 from the two protocols are compared, however, no significant differences are noted (0.89 ± 0.04, $n = 5$ vs. 0.98 ± 0.08, $n = 25$). In the former protocol, but not in the latter, force production was decreased further following BDM treatment at a wide range of calcium concentrations. But when the magnitude of the differences in responses after treatment with 7 and 15 mm BDM are compared to the re-activated force at pCa 4.5 in each protocol, these are not significantly different. In muscles permeabilized by freeze drying, BDM was without effect (Fig. 8). Taken together, we conclude that the effect of BDM on force production in the permeabilized muscle is, at best, very small, and may fall within the error of the measurement. In the same preparations, no matter what protocol was followed, BDM had no effect on the ATPase rate at rest or during activation. The very modest inhibitory effect of BDM on the motility of single actin filaments interacting with thiophosphorylated smooth muscle myosin suggests that there is no major direct effect of BDM on the contractile proteins.

In solution, BDM had a marked inhibitory effect on MLC kinase activity, but no effect on the LC phosphatase (SMP-IV). It is interesting that BDM, which has phosphatase-like activity in other systems (BDM dephosphorylates cholinesterase poisoned with organophosphorus groups, Wilson & Ginsberg, 1955; Green & Saville, 1956; and causes dephosphorylation of calcium channel proteins, Huang & McArdle, 1992), does not itself act as a phosphatase nor does it affect the LC phosphatase (SMP-IV) of smooth muscle. On the basis of the observed decrease in the activity of the LC kinase, it is somewhat surprising that there was no decrease in calcium sensitivity of force production in the permeabilized muscles. However, Moreland and colleagues (1992a) noted that various peptides which were potent inhibitors of MLC kinase in solution, had no significant effect on force development or MyLCP levels in smooth muscles permeabilized with glycerol, saponin, or Triton X-100. As was the case for the peptides, the absence of an effect of BDM on myosin in the permeabilized muscle might be attributed to differences in protein structure and conformation, differences in access of the inhibitor, or differences in binding affinities in the isolated proteins and the structured permeabilized system.

The observations of a complete blockade of force production and energy usage and the concomitant decrease in phosphorylation to resting levels in the intact muscle with little effect on the permeabilized muscle, suggest that the primary target of BDM action in smooth muscle is calcium translocation during activation. This conclusion is supported by a report by Lang and Paul (1991), who found that BDM inhibited inward calcium current in isolated smooth muscle cells of guinea-pig taenia coli, and more recently, from studies of calcium translocation during excitation using Fura-2 fluorescence measurements in intact guinea-pig taenia coli (Osterman *et al.*, 1993), portal vein (Watanabe, 1993) and isolated smooth muscles cells from uterine artery (Otun *et al.*, 1993).

The absence of a significant effect of BDM on force production of permeabilized taenia coli in this study are in agreement with those of Watanabe (1993) on guinea-pig portal vein permeabilized with *Staphylococcus aureus* α-toxin. However, our results on permeabilized rabbit taenia coli differ from those of Osterman and colleagues (1993) on permeabilized guinea-pig taenia coli. Whereas we saw small significant effects of BDM on force production and no effect on calcium sensitivity, in the latter study, BDM caused a decrease in both these parameters. Further, we observed no effect on ATPase activity, but Osterman and colleagues found a decrease in maximum shortening velocity. These results were attributed to a possible decreased level of MyLCP, although the site of action of BDM, e.g., MLC kinase or phosphatase, was not demonstrated. Notwithstanding species differences, the differences in the results from the two studies are difficult to explain, but may be due to the permeabilization methods employed and/or the calmodulin concentrations used: freeze-glycerination, 1 μm calmodulin (present study) vs. Triton X-100 and 0.5 μm calmodulin (Osterman *et al.*, 1993). Such factors may also account for the differences in time courses of contraction during calcium activation. In the present study, maximum steady force was achieved within 10 min at pCa 4.5, whereas in the Triton X-100 permeabilized guinea-pig taenia coli, force continued to creep upward even after 60 min. Despite these technical differences, there is concurrence in the conclusion that the major inhibitory effect of BDM on smooth muscle, based on other evidence, appears to be related to calcium translocation.

Effects of BDM on skeletal muscle

In the skeletal muscle, BDM inhibits force production ($ED_{50} \sim 2$ mm) to a much greater extent than MyLCP. Force production was maximally reduced by about 85% in 7.5 mm BDM, when phosphorylation was reduced by only 36%. This shows that the calcium dependent activation of MLC kinase is affected to a much small extent than is crossbridge interaction. In addition, chemical energy usage was not reduced to the same extent as force production upon treatment with increasing concentrations of BDM. Thus, as the number of force-generating crossbridges progressively decreases, energy usage remains relatively high. This would be expected if

the force-independent energy usage remained constant in the face of a decreased force-dependent energy usage.

Division of the total chemical energy usage into force- dependent (actin-myosin interaction) and force-independent (primarily calcium pumping by the sarcoplasmic reticulum) components has been made in this muscle by varying filament overlap. In this way, Barsotti and Butler (1984) estimated that the force-independent energy usage was 40–50% of the total. In the current study, using BDM to chemically manipulate force production in muscles at L_0, 61% of the total energy usage is force-independent and the remainder is force-dependent. From this analysis, BDM does not seem to have a major inhibitory effect on the fraction of the total energy ascribed to force-independent processes in skeletal muscle. When the mouse EDL is stretched to lengths exceeding L_0, there is a decrease in the degree of LC phosphorylation following a 7 s tetanus; at a length where force was reduced to 25% P_0 the degree of phosphorylation was 20% lower than at L_0 (60% MyLCP at L_0, Barsotti & Butler, 1984). In the present studies, when muscles were treated with BDM at L_0, a reduction in tetanic force to 25% P_0 was accompanied by a decrease in the degree of LC phosphorylation of about 36% (from $49.9 \pm 3.6\%$, $n = 8$ to about 32%, see Fig. 6). It is possible that both manoeuvres result in a decrease in calcium release. Homsher and Kean (1978) have argued that the length-dependent reduction in calcium release (Frank & Winegrad, 1976; Blinks *et al.*, 1978) may represent only 5% of the total amount of calcium released. Such a small change then, might not be reflected by a measurable change in energy usage.

It is interesting that the inhibition of force production by BDM in intact EDL muscles of the mouse far exceeds that of permeabilized muscles, results that are similar to those of Fryer and colleagues (1988b) for rat EDL muscles. On average, there was a 30% reduction in maximum force production at high calcium, and a somewhat lower reduction in the force produced at the other calcium concentrations tested. Therefore, BDM may directly inhibit the maximum number of crossbridges that can be activated as well as the number turned on at any calcium concentration. This is consistent with the decrease in calcium sensitivity, for the proportion of crossbridges that are affected is larger at low calcium concentrations. The decrease in calcium sensitivity is probably not manifest primarily through a LC phosphorylation-dependent mechanism, because force production was markedly inhibited in the face of a high degree of LC phosphorylation, a condition which would be expected to enhance calcium sensitivity (Sweeney & Stull, 1986). Moreover, the time course of phosphorylation is very slow compared to the time course of force production during a tetanus in the EDL, indeed the kinase activity outlasts the mechanical event (rat EDL, Manning & Stull, 1979; rat and mouse EDL, Barsotti & Butler, 1984).

In summary, the results support the idea that the primary effect of BDM on smooth muscle is not on the contractile proteins themselves. 2,3-butanedione monoxime most likely causes changes in calcium translocation and the observed inhibition of force output in the intact muscle may result from a decrease in the extent to which the muscle is activated. That is, in the presence of BDM, the free myoplasmic calcium concentration achieved upon stimulation is diminished, leading to both a decreased activation of MLC kinase and subsequent activation of myosin by LC phosphorylation. This is supported by recent direct measurements of calcium transients (Osterman *et al.*, 1993; Watanabe, 1993). Under these conditions, both force-dependent and force-independent energy requiring processes would be decreased by BDM, thereby precluding its use for the determination of the energetics of activation processes in smooth muscle.

In contrast, the effects of BDM on skeletal muscle are much more consistent with a direct effect of BDM on the contractile proteins. Distinctive inhibitory effects of BDM can be seen in both permeabilized muscles and in the *in vitro* motility assay. It is somewhat puzzling, however, from a quantitative view, as to how relatively low concentrations of BDM cause a large effect on force output in intact muscles when the effects on both the skinned fibres and *in vitro* motility assay at similar concentrations are relatively modest. There are, of course, some indications that BDM does have other actions. The fact that there is a significant decrease in the degree of MyLCP suggests that there is at least some decrease in calcium release from the sarcoplasmic reticulum, which may also contribute to the decrease in force output. The quantitative effect of BDM on the energy requirements of calcium pumping is difficult to assess, but it would, in general, result in an underestimate of the force-independent energy usage. It is interesting that the force-independent energy usage appears to be about 60% of the total energy expenditure in these experiments. This is somewhat higher, rather than lower, than other estimates on skeletal muscle (Homsher *et al.*, 1972; Homsher & Kean, 1978; Barsotti & Butler, 1984). It is also possible that BDM might decrease relative force more than it decreases myosin ATPase activity. Although such an effect of BDM on the crossbridge is speculative, it has been shown that increasing concentrations of inorganic phosphate, which decrease force output, do result in an increase in the tension cost of permeabilized skeletal muscles (Kawai *et al.*, 1987). Taking all of these results into account, the use of BDM as a probe of the components of energy usage during the concentration of smooth muscle is not possible, and such a use of skeletal muscle must be made with great caution.

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