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2,3-Butanedione monoxime increases speed of relaxation in single muscle fibres of frog

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ABSTRACT

The effects of 2,3-butanedione monoxime (BDM) on intracellular Ca^{2+} transient and cross-bridge function were studied in frog single fibres from the anterior tibialis muscle of *Rana temporaria* (sarcomere length, 2.2 μm; temperature, 2–4 °C). The fluorescent dye fluo-3 was used to monitor the intracellular free calcium concentration ($[Ca^{2+}]_i$) during isometric contractions. BDM (1–5 mM) reduced the amplitude of the Ca^{2+} transient during twitches, but this effect was too small to explain the marked inhibition of BDM on twitch force. $[Ca^{2+}]_i$ reached at the end of 1-s tetanic stimulation was not significantly affected by BDM (1.0 and 1.8 mM) while the maximum tetanic tension was substantially reduced. The rate of relaxation during isometric tetanus was increased by BDM whereas the rate of decay of the Ca^{2+} transient was reduced in the presence of BDM. The results strongly suggest that BDM, under the experimental conditions used, mainly affects the contractile machinery resulting in altered performance of the cross-bridges. These effects of BDM were evaluated in terms of the cross-bridge model of Huxley (1957) which was fitted to the experimental force–velocity data in the presence and absence of BDM.

Keywords BDM, Ca^{2+} transient, crossbridges, force, force–velocity relation, skeletal muscle.

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2,3-Butanedione monoxime (BDM) has been found to inhibit contraction of both cardiac (Alpert *et al.* 1989, Gwathmey *et al.* 1991, Backx *et al.* 1994) and skeletal muscles (Fryer *et al.* 1988, Horiuti *et al.* 1988, Higuchi & Takemori 1989, Hui & Maylie 1991, Bagni *et al.* 1992, Yagi *et al.* 1992, McKillop *et al.* 1994, Sun *et al.* 1995). The evidence obtained so far suggests that BDM exerts a direct inhibitory action on the contractile machinery and also reduces the release of calcium from the sarcoplasmic reticulum (SR) in skeletal muscle fibres (Fryer *et al.* 1988, Horiuti *et al.* 1988, Maylie & Hui 1991, Lyster & Stephenson 1995). There is also evidence that these actions of BDM may vary from one animal species to another. Studies on frog skeletal muscle fibres have demonstrated that BDM in low concentrations (<10 mM) markedly depresses force without significantly affecting the release of calcium from the SR and that BDM only in high concentrations (10 mM and higher) suppresses the release of calcium from SR (Horiuti *et al.* 1988, Maylie & Hui 1991). By contrast, in mammalian skeletal muscles the release of calcium from SR is very sensitive to low concentrations

of BDM (e.g. 0.5 mM) (Fryer *et al.* 1988) while at higher concentrations (>2 mM) there is a direct action of BDM on the contractile apparatus (Fryer *et al.* 1988, McKillop *et al.* 1994). A recent study by Lyster & Stephenson (1995) has shown that the effects of BDM on the cane toad muscle appear to be intermediate between those of mammalian and frog muscles.

Because muscle contraction is associated with hydrolysis of ATP by myosin ATPase, the effects of BDM on the myosin ATPase activity are therefore of particular interest. Higuchi & Takemori (1989) have reported that BDM inhibits the ATPase activity of myofibrils and of heavy meromyosin in solution. More recent experiments suggest that BDM inhibits the myosin ATPase activity by slowing the release of P_i from its binding site (Herrmann *et al.* 1992). These findings provide evidence that BDM directly affects the cross-bridge kinetics.

Our previous experiments on frog muscle fibres (Sun *et al.* 1995) have demonstrated that BDM, in addition to reducing the rate of rise of force and the peak amplitude of the isometric tetanus, also markedly

increases the relaxation rate. The present study was undertaken to investigate further the cellular basis of the effects of BDM on force development and force relaxation. Evidence will be presented to show that both these effects of BDM are indeed explainable by assuming that BDM reduces the rate of attachment of the cross-bridges.

MATERIALS AND METHODS

Preparation and mounting

Single muscle fibres were dissected from the anterior tibialis muscle of *Rana temporaria*. Frogs were killed by decapitation followed by destruction of the spinal cord. After dissection the fibres were mounted horizontally in a thermostatically controlled Perspex chamber between a force transducer (AE801, Aksjeselskapet Mikroelektronikk, Horten, Norway) and a stainless steel hook fixed to the bottom of the experimental chamber or, in certain experiments, to a servo-controlled electromagnetic motor (Edman & Reggiani 1984). Clips of aluminium foil were attached to the tendons, and the side parts of the clips were tightly folded around the hooks on the force transducer and the opposite attachment site. The setting of the clips was carefully adjusted to minimize any lateral, vertical or twisting movements of the fibre during contractile activity.

The standard Ringer solution had the following composition (mM): NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; $Na_2HPO_4 + NaH_2PO_4$, 2.0; pH, 7.0. BDM-Ringer solution: standard Ringer + BDM either 1.0, 1.8 or 5.0 mM. The pH of these solutions did not change when BDM was added. All solutions were pre-cooled before entering the muscle chamber.

The temperature of the bathing fluid varied between 2 and 4 °C in the different experiments but was constant to within 0.2 °C during any given experiment.

The sarcomere length of the resting fibre was set to 2.2 μm by direct microscopy at ×400 magnification. The fibres were stimulated by passing rectangular current pulses (0.2 ms duration) between two platinum plate electrodes placed symmetrically on either side of the fibre. The stimulus strength was 15–20% above the threshold.

Estimation of the intracellular Ca^{2+} transients

The intracellular Ca^{2+} transients were monitored by using the Ca^{2+}-sensitive fluorescent indicator, fluo-3 (Minta *et al.* 1989). The loading procedure and the approach used for recording the fluo-3 signal have been described previously (Caputo *et al.* 1994). In brief, the fibre was immersed in Ringer solution containing about 20 μM fluo-3 AM (Molecular Probes, Eugene, OR,

USA) for about 45 min at room temperature. The fibre was thereafter immersed in ordinary Ringer solution for at least 20 min before experimentation. The muscle chamber was mounted on the stage of a Zeiss inverted microscope (Axiovert 35) equipped with an epi-fluorescence attachment. The light source was a 100-W Hg lamp driven by a stabilized power supply. The set of filters used for fluo-3 was (excitation/dichroic/barrier) 450–490/510/520 nm. A manual shutter was used to illuminate the fibre only during recording of the light signal. The light signal was collected from an area with a diameter of about 1 mm which was kept constant during the experiment.

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the fluo-3 signal by taking account of the on- (k_+) and off- (k_-) rate constants for the Ca^{2+}-fluo-3 complex following the procedure described by Caputo *et al.* (1994). The numerical values of k_- and k_+ for fluo-3 in the myoplasm were chosen as described by Sun *et al.* (1996).

Analysis of force-velocity data based on Huxley's (1957) cross-bridge model

Force–velocity data were obtained using a load-clamp technique which was performed by rapidly changing the mode of operation of the puller from fibre-length control to force control, as described previously (Edman 1988, Sun *et al.* 1995). The switch-over to force control occurred on the plateau of an isometric tetanus and the force-control mode was maintained for the remainder of the tetanus period.

The effects of BDM on the force–velocity relation were evaluated in terms of Huxley's (1957) two-state cross-bridge model. The numerical values of the rate constants for association (f_1) and dissociation (g_1 and g_2) of the cross-bridges were obtained by fitting Eqn (1) to the force–velocity data using non-linear regression analysis. According to Huxley's theory (Huxley 1957) the force–velocity relation can be given by:

$$P = P_0 \left(1 - \frac{V}{\phi} \left(1 - \exp\left(-\frac{\phi}{V} \right) \right) \left(1 + \frac{d^2 V}{2\phi} \right) \right)$$

(1)

where P is force, V velocity of shortening and P_0 isometric force. The quantity of ϕ is given by the ratio $(f_1 + g_1)h/s$, where s is sarcomere length and h is the range at which cross-bridge attachment can occur. Instead of 10 nm (Huxley 1957), h is taken to be 27 nm (Woledge *et al.* 1985) according to the observation that a moderately quick release of half this distance is sufficient to drop the tension to zero (Ford *et al.* 1977). The quantity of d is given by ratio $(f_1 + g_1)/g_2$. P_0 in Eqn (1) can be expressed as:

$$P_0 = c \frac{f_1}{f_1 + g_1} \qquad (2)$$

where c is a constant, which was assumed to have the same numerical value in the presence and absence of BDM. Thus, any change in P_0 induced by BDM was attributed to alteration of the ratio $f_1/(f_1 + g_1)$. In the computation, the ratio $f_1/(f_1 + g_1)$ was taken as 13/16 in the normal Ringer solution (Huxley 1957), and the numerical value of c could be derived from Eqn (2).

Recording and analysis of data

The optical signals, the output of the force transducer and the stimulation signals were fed into a data acquisition and analysis system (Asystant+, Asyst Software Technologies, Rochester, NY, USA). The data were collected at a sampling rate of 1 kHz and were stored on diskettes for later analysis.

All statistics are given as mean ± SE. Student's paired *t*-test was used for determination of statistical significance. Probability <0.05 (two-tailed) was considered as statistically significant.

P_0 denotes the maximum tetanic force and is used in several diagrams as a standard.

RESULTS

Effects of BDM on force and intracellular Ca^{2+} transient during isometric twitches

Figure 1 illustrates the isometric twitch force and the Ca^{2+} transient obtained at different concentrations of BDM from a frog single muscle fibre. The peak twitch force can be seen to decrease monotonically with increasing BDM concentration, while the amplitude of the Ca^{2+} transient is only slightly reduced. The peak twitch force was decreased to $18.7 \pm 1.5\%$ ($n = 6$), $10.7 \pm 0.8\%$ ($n = 7$) and $5.1 \pm 0.8\%$ ($n = 5$) of the control value in the presence of 1.0, 1.8, 5.0 mM BDM, respectively. By contrast, under the same conditions,

the peak amplitude of the Ca^{2+} transient was merely reduced to $95.9 \pm 0.9\%$ ($n = 6$, $P < 0.01$), $91.8 \pm 0.9\%$ ($n = 7$, $P < 0.01$) and $89.1 \pm 3.3\%$ ($n = 5$, $P < 0.05$) of the value recorded in the control Ringer solution.

In addition to reducing the peak amplitude of the twitch force, BDM also affected the time course of the twitch response. Thus, BDM consistently abbreviated the isometric twitch response. This is clearly seen in Figure 2, which shows typical records of twitch force and Ca^{2+} transient in control Ringer solution and in the presence of 1.8 mM BDM. The BDM trace has been scaled to the same amplitude as the control in Figure 2c and d. BDM decreased the time to peak twitch force and also markedly decreased the duration of twitch response (Fig. 2c). The changes in the time course of the twitch response did not correlate with the effects of BDM on the Ca^{2+} transient. BDM did not significantly affect the rising phase of the Ca^{2+} transient but reduced the rate of decay of $[Ca^{2+}]_i$ causing a moderate widening of the Ca^{2+} transient (Fig. 2d). The effects of BDM on twitch response and Ca^{2+} transient are summarized in Figure 3. It is evident that the most prominent effect of BDM was depression of the peak twitch force.

#### Effects of BDM on tetanic force and Ca^{2+} transient

Figure 4 shows results of force and Ca^{2+} transient obtained during tetanic stimulation under control conditions and in the presence of 1.0, 1.8 and 5.0 mM BDM. It can be seen that the maximal tetanic force (P_0) was markedly depressed in 1.0 and 1.8 mM BDM while the $[Ca^{2+}]_i$ reached at the end of stimulation was very little affected. In the presence of 5.0 mM BDM, the tetanic force and the Ca^{2+} transient both exhibited irregularities during the stimulation volley, suggesting that some of the stimuli failed to activate the fibre. After the removal of BDM from the bathing solution the tetanic force and the Ca^{2+} transient were both fully

Figure 1 Effects of BDM on twitch force and Ca^{2+} transient in a single fibre of the frog. Upper panel, twitch responses obtained under control conditions (a), in the presence of varying concentrations of BDM (b, 1.0 mM; c, 1.8 mM; d, 5.0 mM) and after return to standard Ringer solution (e). Lower panel, Ca^{2+} transients corresponding to the force records shown in upper panel. Note that BDM greatly inhibits the twitch force while only slightly affecting the Ca^{2+} transient.
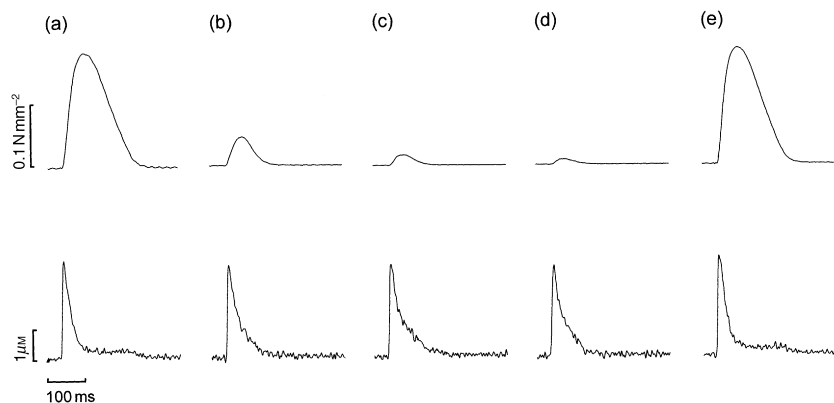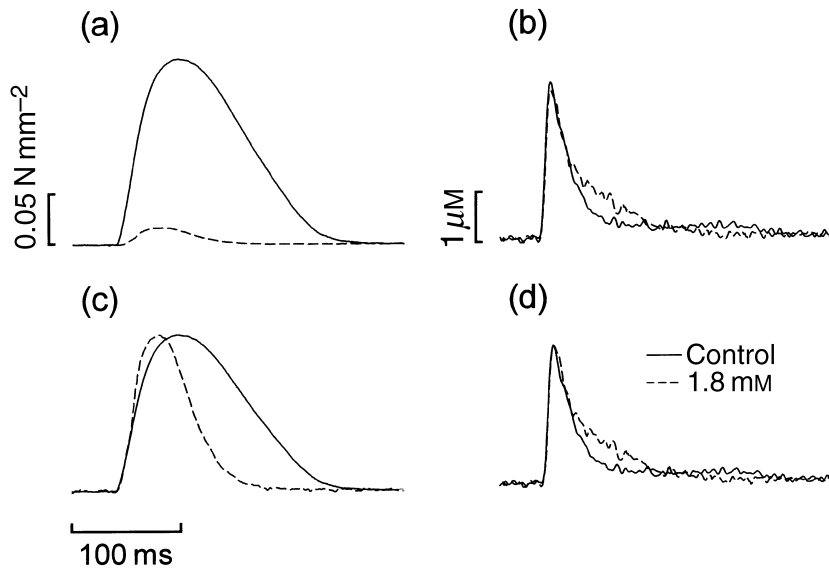
Figure 2 Effects of BDM on the time course of twitch force (a and c) and Ca^{2+} transient (b and d). Results from the same fibre as in Figure 1. The BDM traces in (c) and (d) (1.8 mM, dashed) have been scaled to obtain the same amplitude as the controls (continuous). Note the different effects of BDM on the time course of the mechanical response and of the Ca^{2+} transient.
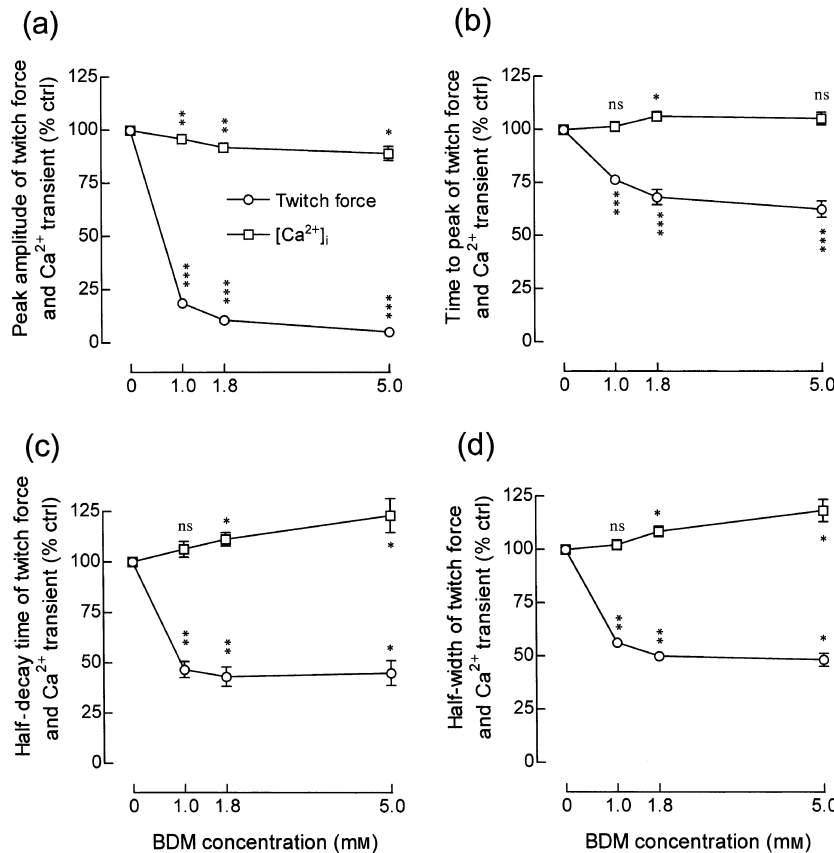

Figure 3 Effects of BDM on peak amplitude (a), time to peak (b), half-decay time (c) and half-width (d) of the twitch force (open circles) and of the Ca^{2+} transient (open squares). Each point represents the mean of 5–7 fibres. Vertical bars indicate s.e.m. if exceeding the size of the symbol.

restored. Figure 5 summarizes the effects of BDM (1.0 and 1.8 mM) on maximum force and peak amplitude of the Ca^{2+} transient during 1-s tetanic stimulation of 6–7 single fibres. It can be seen that BDM reduced the maximum amplitude of tetanic tension with no significant change of the peak Ca^{2+} concentration reached during tetanic stimulation. No

data are shown for 5.0 mM BDM because of the inconsistency of the results at this high concentration of BDM as described above.

The time course of the tetanus response was also affected by BDM consistent with the effects on the isometric twitch. Figure 4 demonstrates that the rate of rise of tetanic force was markedly reduced by
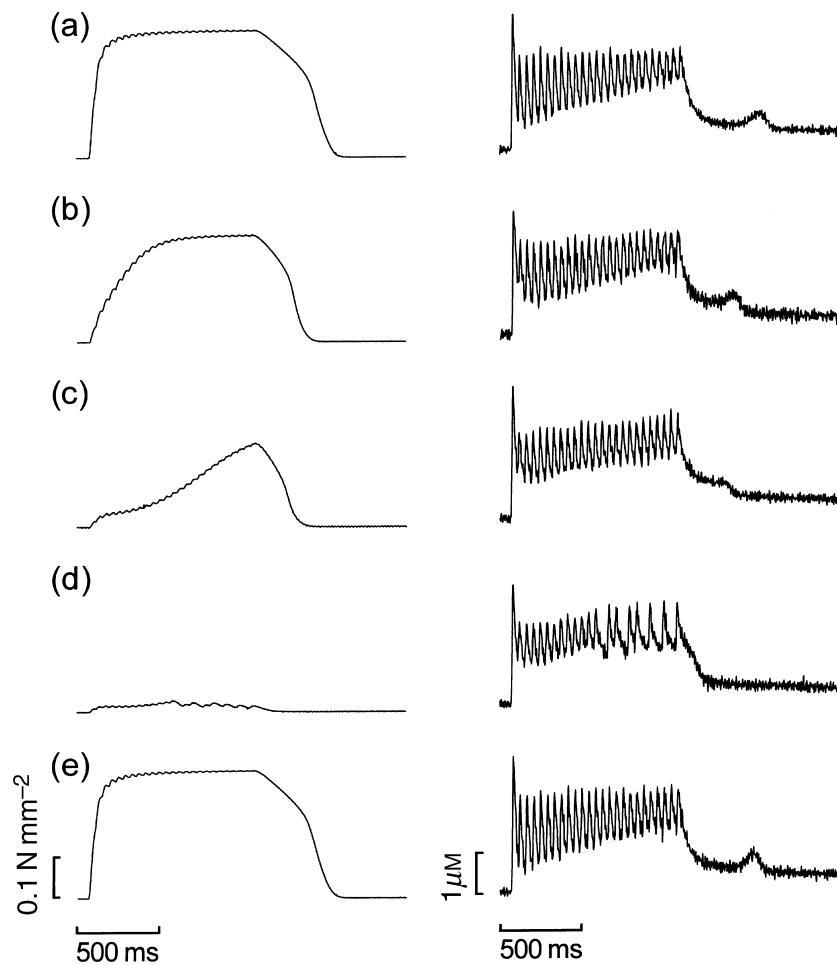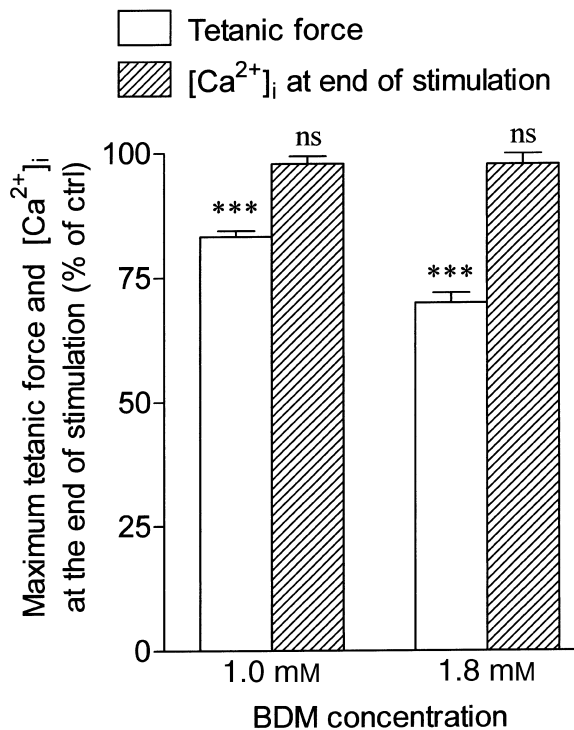
Figure 4 Effects of BDM on tetanic force and Ca^{2+} transient in a single muscle fibre. The left panel shows tetanic responses obtained under control conditions (a), in the presence of various concentrations of BDM (b, 1.0 mM; c, 1.8 mM; d, 5.0 mM) and after return to standard Ringer solution (e). The right panel shows the corresponding Ca^{2+} transients. Note the progressive decrease of the tetanic force with increasing bath concentrations of BDM. Also note the complete reversibility of the depressant effect after return to normal Ringer solution (e).

BDM while there was no corresponding change of the Ca^{2+} transient. In contrast to the slow development of force during tetanic stimulation, the rate of relaxation was increased in the presence of BDM (Fig. 6a). In an attempt to quantify the rate of relaxation of the tetanus, a straight line was fitted to the force recorded in the interval 95–75% of maximum tetanic tension using the least squares method. This regression thus covered the 'linear phase' of relaxation, i.e. the phase preceding the tension shoulder of the isometric myogram (Fig. 6a). The rate of relaxation, indicated by the linear regression, was 0.89 ± 0.08 P_0/s ($n = 7$) under control conditions and was increased by $22.3 \pm 3.9\%$ ($n = 6$) and $31.1 \pm 5.1\%$ ($n = 7$) in the presence of 1.0 and 1.8 mM BDM, respectively. Both changes were statistically significant at the 1% level. There were no corresponding changes of the Ca^{2+} transient. On the contrary, BDM tended to reduce the rate of decay of $[Ca^{2+}]_i$ during the linear phase of relaxation as illustrated in Figure 6b. The time for $[Ca^{2+}]_i$ to decline from its maximum value after the last stimulus to half this value was used as an index of the rate of decay

of the Ca^{2+} transient. This time was increased by $4.1 \pm 2.5\%$ ($n = 6$; $P > 0.05$, non-significant) and $10.5 \pm 4.8\%$ ($n = 7$; $P < 0.05$) in the presence of 1.0 and 1.8 mM BDM, respectively.
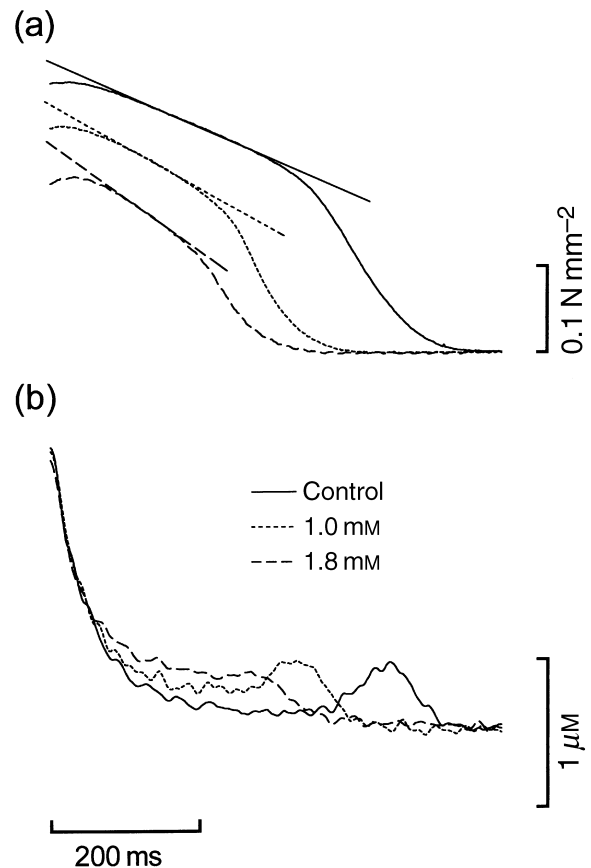
Effects of BDM on cross-bridge function

The above results suggest strongly that the action of BDM on the isometric force is not attributable to changes in the Ca^{2+} transient. It appears more probable therefore that BDM, under the experimental conditions used, exerts its principal effect on the contractile machinery itself. To evaluate the effects of BDM on cross-bridge function, Huxley's (1957) cross-bridge model was fitted to force–velocity data in the presence and absence of BDM. In this model the attachment of myosin cross-bridges is described by the rate constant f_1 and the detachment of bridges by the rate constants g_1 and g_2. Here g_1 refers to positively strained cross-bridges and g_2 to cross-bridges exerting negative or zero tension. By fitting Eqn (1) (see Materials and Methods) to experimental force–velocity data, it was possible to estimate the effects of BDM on the constants f_1, g_1 and g_2 in the model.

Figure 5 Bar diagram illustrating the effects of BDM (1.0 and 1.8 mM) on tetanic force and $[Ca^{2+}]_i$ at the end of 1-s stimulation, which was measured as the average $[Ca^{2+}]_i$ level between last two stimuli. Each column represents the mean ± SEM of six or seven experiments. Statistical significance (two-tailed pair-observation) is indicated by: [n.s.], $P > 0.05$; ***, $P < 0.001$. Note that BDM markedly reduced the peak amplitude of the tetanic tension without significantly affecting the $(Ca^{2+})_i$ reached during tetanic stimulation.


Figure 6 The relaxation phase of the mechanograms (a), and the corresponding Ca^{2+} transients (b), shown in Figure 4a–c are super-imposed starting from the last peak of the Ca^{2+} transient in each case. Continuous traces, control; dotted traces, 1.0 mM BDM; dashed traces, 1.8 mM BDM. Lines in (a) indicate the slope of the linear phase of relaxation. The Ca^{2+} transient baseline is indicated by the lower end of the calibration bar. Note increased speed of relaxation as the concentration of BDM is increased. Also note that the decay of the Ca^{2+} transient becomes slower with increasing BDM concentration.

Figure 7 illustrates force–velocity data and a computer simulation. In accordance with our previous results (Sun *et al.* 1995), in the presence of BDM, P_0 and V_{max} were suppressed markedly while the curvature of the force–velocity relation was increased. According to Huxley's model the curvature of the force–velocity relation is dependent on $g_2/(f_1 + g_1)$, and P_0 and V_{max} are proportional to $f_1/(f_1 + g_1)$ and g_2, respectively. The changes of these parameters derived from the computer simulation are summarized in Table 1. The results show that f_1 and g_2 were both markedly reduced in the presence of BDM. The numerical value of g_1, on the other hand, was slightly increased. However, only the change of g_1 caused by 1.0 mM BDM was statistically significant. The changes of f_1 and g_1 lead to a decrease of the ratio $f_1/(f_1 + g_1)$ (Table 1), which determines the number of attached cross-bridges during isometric contraction. This decrease of $f_1/(f_1 + g_1)$ corresponds to the depression of P_0 observed in the presence of BDM while the decrease of g_2 is consistent with the depression of V_{max}. It can also be seen from Table 1 that there is an increase of the ratio $g_2/(f_1 + g_1)$, corresponding to the increased curvature of the force–velocity relation induced by BDM.

DISCUSSION

Effects of BDM on Ca^{2+} transient and force development

The results obtained in this study demonstrate that BDM, in 1.0–1.8 mM concentrations, exerts its inhibitory effect on the mechanical performance of frog skeletal muscle mainly at the level of the myofilament system at a temperature of 2–4 °C. Although BDM somewhat reduced the amplitude of the Ca^{2+} transient during twitch contraction, this effect was far too small to explain the marked inhibition of BDM on twitch tension. A similar conclusion was reached by Horiuti *et al.* (1988) in an earlier study performed at 18 °C in which aequorin was used as a calcium indicator.
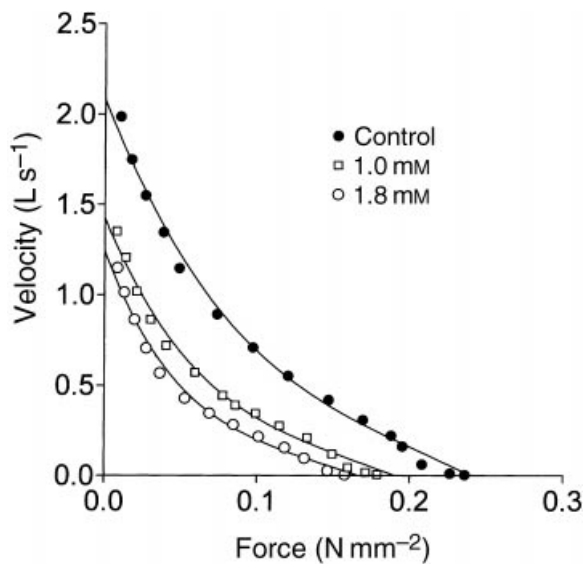
Figure 7 Representative force–velocity data derived from a single muscle fibre in Ringer solution (●) and in the presence of 1.0 mM (□) and 1.8 mM (○) BDM. The continuous curves are fittings of Eqn (1) (see Materials and Methods) to the experimental points.

In addition to depressing the peak twitch force, BDM was found to reduce the duration of the twitch response (Fig. 3). The shorter time to peak tension and the faster relaxation of the twitch in the presence of BDM were not, however, associated with an abbreviation of the Ca^{2+} transient. On the contrary, the decay of the Ca^{2+} transient was prolonged by BDM without any significant change of the rising phase of the transient. The prolongation of the Ca^{2+} decay time may be the result of an inhibitory effect of BDM on the calcium reuptake by SR (Horiuti *et al.* 1988).

The results obtained in this study furthermore demonstrate that the intracellular Ca^{2+} concentration reached at the end of 1-s tetanic stimulation was very little affected by BDM (at 1.0 and 1.8 mM concentrations) in spite of the fact that the maximal tetanic force was markedly depressed by BDM. There is furthermore evidence that BDM does not change the binding of calcium to troponin C (Yagi *et al.* 1992, Martyn *et al.* 1999). These results suggest that the contractile system is fully activated during tetanic stimulation in the

presence of BDM. In line with this finding, our previous study (Sun *et al.* 1995) has shown that caffeine (0.5 mM), which is known to increase the myofibrillar Ca^{2+} concentration in response to stimulation (Kovács & Szücs 1983, Delay *et al.* 1986, Klein *et al.* 1990), has virtually no effect on the maximal isometric tension after depression of the tetanic force by BDM.

In accordance with previous work (Horiuti *et al.* 1988, Bagni *et al.* 1992, Sun *et al.* 1995), the results obtained in this study show that BDM reduces the rate of rise of force. It has been reported that BDM has direct actions on myosin molecules, i.e. BDM inhibits the myosin ATPase activity by slowing the release of inorganic phosphate, P_i (Higuchi & Takemori 1989, Herrmann *et al.* 1992). These results suggested that BDM inhibits the transition of the cross-bridges from weak to strong binding states leading to accumulation of weak attachments, which may largely account for the reduced rate of force development in the presence of BDM.

The present study further demonstrates that the rate of relaxation during isometric tetanus was increased by BDM. At the same time, however, the rate of decay of the Ca^{2+} transient was reduced in the presence of BDM concentration. Taken together the present results provide additional strong evidence that BDM, under the experimental conditions used, mainly affects the contractile machinery resulting in altered performance of the cross-bridges.

Effects of BDM on cross-bridge function

Our previous work has shown that BDM produces significant changes of the force–velocity relation in frog skeletal muscle fibres (Sun *et al.* 1995). The isometric force (P_0) and the maximum velocity of shortening (V_{max}) were both decreased, and the curvature of the force–velocity relation was increased (Sun *et al.* 1995, see also Bagni *et al.* 1992). These results further emphasize the direct action of BDM on cross-bridge function.

The effects of BDM on cross-bridge kinetics were evaluated in terms of Huxley's (1957) two-state cross-bridge model (see Materials and Methods). According

Table 1 Effects of BDM on the rate constants in Huxley's (1957) cross-bridge model

	f_1 (s^{-1})	g_1 (s^{-1})	g_2 (s^{-1})	$f_1/(f_1 + g_1)$	$g_2/(f_1 + g_1)$
Control	62.5 ± 2.3	14.4 ± 0.5	154.7 ± 4.2	0.813	2.02 ± 0.06
1.0 mM BDM	26.8 ± 1.6*	17.8 ± 1.1†	105.6 ± 4.5*	0.601 ± 0.011*	2.41 ± 0.14‡
1.8 mM BDM	15.3 ± 1.7*	15.7 ± 1.7§	91.2 ± 6.6*	0.490 ± 0.014*	3.12 ± 0.31†

Significance levels of Student's *t*-test (paired observation) vs. control: *$P < 0.001$; †$P < 0.01$; ‡$P < 0.05$; §Non-significant.
Mean values (±SE) of eight experiments. The numerical values of the rate constants for association (f_1) and dissociation (g_1 and g_2) of the cross-bridges were obtained by fitting Eqn (1) to the force-velocity data (see Materials and Methods).

to this analysis BDM reduces both f_1 and g_2 (Table 1). The numerical value of g_1, on the other hand, was slightly increased at 1.0 mM of BDM and was not significantly affected ($P > 0.05$) at 1.8 mM BDM (Table 1).

Studies of the action of BDM on the myosin sub-fragment-1 ATPase have suggested that BDM slows the release of P_i while BDM has little effect on the kinetics of binding of ATP and of release of ADP from myosin (Herrmann *et al.* 1992, McKillop *et al.* 1994). It is generally thought that the force-generating step is initiated by the dissociation of P_i from myosin and this step corresponds to the cross-bridge attachment governed by f_1 in Huxley's (1957) two-state model. The rate constants for detachment of cross-bridges (g_1 and g_2) are dependent on the release of ADP from myosin and the subsequent binding of ATP. On these grounds BDM would be expected to have a depressant effect on f_1 but little effect on g_1. These expectations are thus in accord with the present results which indicate a marked reduction of f_1 in the presence of BDM. Such a depression of BDM on the rate of cross-bridge association (f_1) of BDM may largely account for the reduced rate of force development by BDM. Because reattachment of cross-bridges may probably occur during relaxation (Goldman *et al.* 1982, 1984), the increased rate of isometric relaxation in the presence of BDM may thus be a consequence of the decrease in f_1.

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