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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 \(\mu\)m; 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, Baghi 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, Lyster & Stephenson 1995). 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 canine 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 Pi, 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$_2$HPO$_4$ + NaH$_2$PO$_4$, 2.0; pH, 7.0. BDM-Ringer solution: standard Ringer + BDM either 1.0, 1.8 or 2.0 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 x400 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 off- ($k_s$) and on- ($k_a$) rate constants for the Ca$^{2+}$-fluo-3 complex following the procedure described by Caputo et al. (1994). The numerical values of $k_s$ and $k_a$ 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 ($f_i$) and dissociation ($g_i$ and $g_s$) 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:

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

(1)

where $P$ is force, $V$ velocity of shortening and $P_0$ isometric force. The quantity of $\phi$ is given by the ratio $(f_i + g_i)/s$, where $s$ is sarcomere length and $b$ is the range at which cross-bridge attachment can occur. Instead of 10 nm (Huxley 1957), $b$ 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 $(f_i + g_i)/g_s$. $P_0$ in Eqn (1) can be expressed as:
\[ P_0 = \epsilon \frac{f_1}{f_1 + g_1} \]  

(2)

where \( \epsilon \) 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 \( \epsilon \) 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 ± 1.5% (\( n = 6 \)), 10.7 ± 0.8% (\( n = 7 \)) and 5.1 ± 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 ± 0.9% (\( n = 6 \), \( P < 0.01 \)), 91.8 ± 0.9% (\( n = 7 \), \( P < 0.01 \)) and 89.1 ± 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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.
restored. Figure 5 summarizes the effects of BDM (1.0 and 1.8 mM) on maximum force and peak amplitude of the Ca\textsuperscript{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\textsuperscript{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
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 increased by 22.3 ± 3.9% (n = 6) and 31.1 ± 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\(^{2+}\)]\textsubscript{i} during the linear phase of relaxation as illustrated in Figure 6b. The time for [Ca\(^{2+}\)]\textsubscript{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 ± 2.5% (n = 6; \(P > 0.05\), non-significant) and 10.5 ± 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 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 $V_{\text{max}}$ 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 $g_2/(f_1 + g_2)$, and $P_0$ and $V_{\text{max}}$ 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 $V_{\text{max}}$. 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.
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 (Kovacs & Szucs 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

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

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 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 subfragment-1 ATPase have suggested that BDM slows the release of P, 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 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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