Contractile Deactivation and Uncoupling of Crossbridges
Effects of 2,3-Butanediione Monoxime on Mammalian Myocardium

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We investigated the effects of 1 and 3 mM 2,3-butanediione monoxime (BDM, diacetyl monoxime) on excitation and contraction of cardiac muscle in several types of preparations at various levels of organization. We selected a concentration of BDM that was not expected to affect sarcoplasmic calcium flux and action potential duration in cardiac tissue. Two indicators were used to record intracellular calcium. Aequorin, a bioluminescent calcium indicator, was used in studies with ferret papillary muscle preparations, and fura-2, a fluorescent calcium indicator, was used in studies with guinea pig cardiac myocytes. In both cases, addition of BDM resulted in a reduction of peak intracellular calcium released from the sarcoplasmic reticulum and a reduction of peak twitch force. The duration of the action potential of isolated myocytes was slightly abbreviated in the presence of BDM. In studies on the calcium current in the myocytes, addition of BDM was associated with reduced calcium current at any potential. Peak calcium current was reduced by 7.9±1% in the presence of BDM. In tetanized ferret papillary muscles, BDM reduced maximal calcium-activated force by 30±5% and increased the calcium ion concentration required for half-maximal force by 0.1±0.01 μM. The Hill coefficient was reduced from 5.00±0.11 to 3.40±0.20. Maximal shortening velocity of ferret papillary muscles was increased in the presence of BDM from 1.55±0.24 to 2.04±0.33 mm/sec. Ca2+ binding to troponin C in skinned fiber preparations from guinea pig, bovine, and canine hearts was unaffected by addition of up to 10 mM BDM. Our results indicate that BDM affects both calcium availability and responsiveness of the myofilaments to Ca2+. Uncoupling of contractile activation from excitation may also result from altered crossbridge kinetics. (Circulation Research 1991;69:1280–1292)

A n agent that apparently uncouples cardiac excitation from contraction,1 2,3-butanedi-one monoxime (BDM, diacetyl monoxime) has been used as an experimental tool to investigate contractile and regulatory systems in normal and dystrophic cardiac tissue,2 contracture and calcium paradox,3 intracellular calcium mobilization,4 and crossbridge kinetics5–7 and to inhibit contractile function.8 BDM has been shown to affect force–pCa relations in chemically skinned fiber preparations in which the sarcoplasmic reticulum (SR) and sarcotemy has been extracted.9,10 There are conflicting reports as to whether BDM affects slow inward calcium current (Ica), thereby inducing a negative inotropic effect.10–12 Previous studies have been unable to rule out possible effects of BDM on SR calcium handling.2,10 Because the SR is the predominant intracellular organelle that supplies calcium for activation of force in cardiac muscle, the effect of BDM on this organelle is pivotal in potentially explaining its mechanism of action.

Studies using this compound rely on a full understanding of its mechanism of action, yet the mechanism by which BDM produces force inhibition remains unclear. This study was designed to provide greater insight into the mechanisms of contractile deactivation in the presence of BDM. We investi-
gated the effects of BDM on peak twitch force and peak SR calcium release. To avoid comparisons of peak twitch force–peak calcium relations, which have been shown not to reflect changes in calcium responsiveness at the level of the myofilaments, we investigated steady-state force–calcium relations by tetanizing the muscles in the presence of ryanodine. Steady-state force–calcium relations were considered to be measures of myofilament Ca\textsuperscript{2+} responsiveness in the presence and absence of BDM. We used aequorin, a bioluminescent calcium indicator, in intact papillary muscles and fura-2, a fluorescent calcium indicator, in single isolated myocytes. Further, we addressed the question as to whether BDM could be affecting sarcolemmal calcium flux with resultant reduced SR release and/or loading and negative inotropic effect. Troponin C (TnC) calcium binding studies were performed to determine whether BDM directly affects TnC calcium binding affinity and/or stoichiometry. Results of our studies support the idea that, even at relatively low concentrations, BDM affects force development by reducing transsarcolemmal I\textsubscript{Ca} and intracellular SR calcium release and decreasing myofilibrillar calcium responsiveness. Further, BDM does not affect force development by altering TnC calcium binding affinity or stoichiometry but affects crossbridge kinetics and actin–myosin interaction.

Materials and Methods

Chemicals

All chemicals used in these experiments were purchased from Sigma Chemical Co., St. Louis, Mo. Aequorin was purchased from Dr. J.R. Blinks, Rochester, Minn. Ryanodine was generously supplied by Merck Sharp & Dohme, West Point, Pa. Dr. John R. Blinks provided the generous supply of buprenorphine.

Isolated Ferret Papillary Muscle Studies

Hearts were quickly excised from ferrets after deep chloroform anesthesia and placed in an oxygenated physiological salt solution at room temperature. Suitable right ventricular papillary muscles (n=47) were dissected free with mean fiber diameters of 1.1±0.05 mm and lengths of 4.6±0.25 mm. The composition of the salt solution was as follows (mM): NaCl 120, KCl 5.9, glucose 11.5, NaHCO\textsubscript{3} 25, NaH\textsubscript{2}PO\textsubscript{4} 1.2, MgCl\textsubscript{2} 1.2, and CaCl\textsubscript{2} 2.5. Muscles were placed into temperature-regulated organ baths. The solution was bubbled with 95% O\textsubscript{2}–5% CO\textsubscript{2} to a pH of 7.4. Experiments were performed at 30°C. Each muscle was attached to a Kulite force transducer (Gould, Inc., Cleveland, Ohio) to record isometric tension development and stimulated to contract at 0.33 Hz, unless otherwise noted. Square wave pulses of 5-msec duration were used to deliver threshold voltage through a punctate platinum electrode located at the base of the muscle. After maximum active tension was obtained by stretching the muscle to the muscle length at which maximal active force developed (L\textsubscript{max}), the muscle was allowed to equilibrate for 1 hour. Calcium concentration–effect curves, in the presence and absence of BDM (3 mM), were generated (0.5–24 or 1–20 mM) in the absence of phosphate to avoid calcium precipitation.

Other papillary muscles were placed in a specially designed light-collecting apparatus and loaded with aequorin as previously described. Force measurements were obtained using an ergometer (Cambridge Technology, Cambridge, Mass.). Aequorin luminescence was converted to [Ca\textsuperscript{2+}], using the method of fractional luminescence with an in vitro calibration curve derived at 30°C. To address the question of possible muscle rundown, a group of muscles from the preparations was exposed to varying [Ca\textsuperscript{2+}], and washed to normal calcium, and the protocol was repeated. This was done for both isometric twitch force and tetanus. There was no significant difference between the first and second calcium concentration–response relation. Therefore, differences observed in the presence of BDM reflect effects of this agent on force development.

Tetanization of Ferret Papillary Muscles

Muscles were exposed to 1 µM ryanodine for 1 hour. Muscle tetani were induced by stimulating intact muscles at 15–20 Hz with pulse durations of 50 msec for −4 seconds. Tetani were separated by an interval of at least 3 minutes, during which muscles were stimulated at 0.33 Hz. Tetani were elicited at varying [Ca\textsuperscript{2+}]\textsubscript{0} (0.5–24 mM) in the presence or absence of BDM. Steady-state force versus [Ca\textsuperscript{2+}] curves derived in aequorin-loaded preparations were fit to a modified Hill function:

\[
F = F_{\text{max}} \frac{[\text{Ca}^{2+}]^n}{[\text{Ca}^{2+}]_{50\%}^n + [\text{Ca}^{2+}]^{100\%}}
\]

where F is developed force, F\textsubscript{max} is the maximal force developed, n is the Hill coefficient, and [Ca\textsuperscript{2+}]\textsubscript{50%} is the [Ca\textsuperscript{2+}] for 50% force activation.

Quantitative Measurement of Intracellular Calcium

To translate light signals from muscle preparations into absolute [Ca\textsuperscript{2+}]\textsubscript{s}, the luminescence signals (L in amperes) were normalized by the maximal rate of light emission (L\textsubscript{max}) determined by lysing the cell membranes with a 2% solution of the detergent Triton X-100 in phosphate-free physiological salt solution containing 10 mM Ca\textsuperscript{2+} at 30°C. This allows all the aequorin to be instantaneously exposed to a saturating solution of Ca\textsuperscript{2+}. Exposure of muscles loaded chemically with aequorin to the solution resulted in a sudden burst of light emission; light returned to baseline slowly (2–5 hours). After subtracting the contribution of background light, the integral of the area encompassed by the light signal during exposure to Triton X-100 was multiplied by the rate constant for aequorin consumption in the presence of saturating Ca\textsuperscript{2+} in order to calculate L\textsubscript{max}. The rate constant was measured to be 1.96/sec at 30°C.
The normalized light signal termed the fractional luminescence (L/L_{max}) could then be used to obtain \([Ca^{2+}]_i\) by referring to an in vitro calibration curve. The \(Ca^{2+}\) concentration–effect curve was determined under the same conditions of temperature, ionic strength, and \(Mg^{2+}\) intracellularly. The in vitro solution contained (mM) KCl 154, MgCl\(_2\) 2, and PIPES 5, pH 7.1 at 30°C.

The relation was described by Yue et al\(^{13}\):

\[
\frac{L}{L_{max}} = \frac{1 + K[Ca^{2+}]_i}{1 + K_{TR} + K[R][Ca^{2+}]_i}^3
\]

where \(K_{TR}\) and \(K_R\) are constants.

**Velocity of Unloaded Shortening**

The velocity of unloaded shortening was determined using the slack test as described by Edman\(^{18}\) in both intact and skinned muscles. In intact preparations, when the muscles reached a plateau during the tetanus, an electromagnetic puller (Cambridge 510) was used to produce a quick (<1.0 msec) length shortening. In skinned preparations, muscles were skinned with saponin (250 mg/ml) as described by Hajjar et al.\(^{19}\) The muscles were then maximally activated at pCa 4.0 with a buffer solution containing (mM) EGTA 10, \(Mg^{2+}\) 3.2, MgATP 3.2, ATP 10, and phosphocreatine 12, along with 15 units/ml creatine phosphokinase, pH 7.1 at 20°C. The activated muscles were then subjected to varying release steps that were performed from the same starting point. The relationship between the amplitude of the steps (\(\Delta L\)) and the duration of the unloaded shortening (\(\Delta t\)) was fitted with a regression line using the least-squares method. The slope of this line provided a measure of the shortening velocity at zero load.

In the skinned-fiber preparation, we examined the muscle speed of contraction using dynamic stiffness spectra.\(^{20,21}\) When the skinned muscles achieved maximal activation, they were subjected to sinusoidal perturbations (0.1–50 Hz at 1% of \(L_s\)). The dynamic stiffness was defined as the ratio of the amplitude of the force oscillation to the amplitude of the muscle length oscillation. The stiffness was fairly constant at low frequencies; it decreased to a minimum at an intermediate range and then increased steeply. This frequency of minimum stiffness has been shown to be an index of the crossbridge cycling rate.\(^{20,21}\)

**Isolated Guinea Pig Ventricular Myocytes**

The method of cell isolation has been described in detail elsewhere.\(^{22}\) Briefly, guinea pigs (200–250 g) were anesthetized with sodium pentobarbitone (75 mg/kg i.p.) after anticoagulation (4,000 units/kg i.p. heparin). The heart was quickly excised, and a retrograde perfusion of the isolated heart was performed. Isolated cells were stored in Tyrode’s solution containing 2 mM \([Ca^{2+}]_i\) at room temperature. The composition of Tyrode’s solution used in these experiments was as follows (mM): NaCl 135, dextrose 10, HEPEs 10, KCl 4, MgCl\(_2\) 1, Na\(_2\)HPO\(_4\) 0.33, and \(Ca^{2+}\) 2, pH 7.3.

The pipette-filling solution contained (mM) NaCl 12, MgCl\(_2\) 2, KCl 140, Mg\(_2\)ATP 2, and HEPES-KOH 10, along with 70 \(\mu\)M fura-2-salt. Experiments were performed at room temperature. The cells were loaded with fura-2 (penta-potassium salt) by internal perfusion with glass micropipettes (2–3 M\(\Omega\)). The apparatus for recording and calibration of fluorescence has been previously described.\(^{22}\) Fura-2 fluorescence was recorded from voltage-clamped guinea pig ventricular myocytes.

In all cases, test pulses were preceded by a train of eight conditioning pulses from a holding potential of −45 mV with a 45-mV step for 300 msec to inactivate sodium currents and allow loading of the SR. The stimulation interval was 1.2 seconds. At the end of the prepulses, the membrane potential was voltage-clamped at −45 mV for 1 second and then stepped from −40 to +80 mV in 10-mV steps. In some experiments, only membrane currents were measured. In these experiments the pipette solution did not contain fura-2 and had 10 mM EGTA added to suppress mechanical activity. Experiments were performed in the absence and presence of 1 mM BDM.

**Aequorin and Fura-2**

BDM was tested in an in vitro calibration device for possible effects on aequorin luminescence. BDM did not affect peak aequorin luminescence or time course.\(^{9}\) Fura-2 fluorescence\(^{8}\) has been shown not to be affected by BDM.

**Action Potentials in Isolated Guinea Pig Myocytes**

Action potentials were recorded at a stimulus frequency of 1 Hz in single isolated guinea pig ventricular myocytes in the presence and absence of BDM. At least 10 action potentials were recorded to obtain steady-state measurements. Resting membrane potential, amplitude, and time to 80% repolarization were measured.

**Troponin C Binding in Skinned Guinea Pig, Canine, and Bovine Heart**

Detergent extracted fibers and myofibrils were prepared from guinea pig, canine, and bovine hearts as described previously.\(^{23}\) With the aid of \(^{45}\)Ca and \(^{[\text{H}]\text{glucose}}\) as a solvent space marker, Ca\(^{2+}\) binding to TnC in these preparations was measured at pH 7.0, ionic strength 0.15, and 23°C with and without the addition of various concentrations of BDM. Other conditions were 60 mM imidazole, 2 mM Mg\(^{2+}\), 5 mM MgATP\(^{2-}\), 12 mM creatine, 1 unit/ml creatine kinase, 1 mM D-glucose, 0.3 Ci/ml \(^{45}\)Ca, and 0.3 Ci/ml \(^{[\text{H}]\text{glucose}}\). Our results were the same whether the experiments were done in MOPS or imidazole. Compensations were made for BDM effects on the protein concentration assay. Details of the methods are described in Pan and Solaro\(^{23}\) and Blanchard and Solaro.\(^{24}\)
FIGURE 1. Left panels: Graphs showing the effect of 3 mM 2,3-butanedione monoxime (BDM) on peak twitch force (n=6). Right panels: Graphs showing the effect of 3 mM BDM on peak twitch force in the presence of ryanodine (n=6). Isometric twitch is expressed as a percent of the maximal response (F_{max}) in the absence of BDM. The maximal absolute value is 25.0±7.0 and 41.5±3.3 mN/mm² in the presence and absence of ryanodine, respectively. The lower panels show the effect of BDM on twitch force in the absence and presence of ryanodine as a percent of F_{max} in the presence of BDM (n=6).

Calcium-Selective Electrode

Free calcium content of the Kreb's physiological salt solution with calcium concentrations of 0.5–24 mM was measured in the presence and absence of 3 mM BDM at 30°C using a calcium ion-selective electrode with a sensitivity range of 10⁻⁵–10⁻¹ M (Corning pH meter and ion-selective electrode 240, Fisher Scientific, Pittsburgh, Pa.).

It has been suggested that BDM is a calcium chelator. BDM (n=6) was found to not significantly reduce the free ionized calcium concentration. Previous studies have shown that BDM does not affect the binding of Ca²⁺ by EGTA.

Statistical Analysis

All data were analyzed using Student's t test for paired observations or by analysis of variance (ANOVA). For steady-state force–[Ca²⁺]₀ relations, each experiment was fit to the Hill equation. Pooled Hill parameters were then computer-fit to a curve as suggested by Brandt et al. When comparing the force–[Ca²⁺]₀ relations, which were fit to the Hill equation, differences between means of the half-maximally activating [Ca²⁺]₀ and Hill coefficient were tested for significance using one-way ANOVA. Computer fits were derived by inputting the mean values of the Hill parameters. A value of p≤0.05 was considered significant.

Results

Effect of BDM on Peak Isometric Force and Peak Intracellular Calcium Transients

A concentration of BDM (3 mM) was selected that has been reported not to affect the sarcolemma or action potential parameters. Maximal twitch force calcium concentration–effect curves were generated (n=5). [Ca²⁺]₀ was varied from 0.5 to 24 mM or from 1.0 to 20 mM. At 16 mM [Ca²⁺]₀, maximal twitch force was attained. BDM reduced peak isometric twitch force at all levels of [Ca²⁺]₀ (Figure 1). Figure 1 illustrates the reduction in peak isometric twitch force seen in the presence of BDM when normalized to maximal control response in the absence of BDM. Data normalized to the maximal peak response in the presence of BDM showed no shift in the force–[Ca²⁺]₀ relations for peak twitch force. In aequorin-loaded preparations (n=6), BDM reduced the amplitude of the peak Ca²⁺ transient (Figure 2). Experiments performed in the presence of bupranol, a β-receptor antagonist, did not alter these results (n=3) (data not shown).
Effect of BDM on Peak Twitch Force in the Presence of Ryanodine

Peak force–peak calcium relations may not solely reflect changes at the level of the myofilaments.13-15 To investigate the effect of BDM on twitch force in the absence of a normally functioning SR, ryanodine (1 μM) was added to the bath. A 1-μM concentration of ryanodine has been shown to abolish the calcium transient that predominantly reflects SR calcium uptake and release.26 Exposure to 1 μM ryanodine reduced peak isometric twitch force by ~70%.

BDM resulted in a 70% decrease in maximum peak twitch force. In the presence of ryanodine, BDM decreased maximum peak twitch force by 30%. This reduction in maximum peak twitch force of ~30% was similar to the depression of maximum force obtained during tetani (see below). It is possible that the depression of the maximum twitch force in ryanodine-treated muscles reflects largely the decline in myofilament sensitivity to Ca2+, whereas the larger percent depression in control may reflect the additional influence of BDM on other processes involved in activation of the myofilaments (e.g., Ca2+ current or SR Ca2+). The effect of BDM on isometric twitch time course in the absence and presence of ryanodine is summarized in Table 1.

Effect of BDM on Steady-State Force–[Ca2+]i Relations and Troponin C

Myofilament calcium interactions can directly affect force production in cardiac tissue. Many investigations using aequorin have attempted to make inferences about force–[Ca2+]i relations in intact muscle preparations using peak force and peak calcium measurements obtained during isometric twitches.27-29 We have recently shown that, under some circumstances, such relations during twitches cannot be used to examine the sensitivity of the myofilaments to Ca2+.13,14,15 Therefore, we avoided such comparisons and used steady-state force–calcium relations to address alterations at the level of the myofilaments. Figure 3 demonstrates the reduction in peak steady-state force in the presence of BDM when normalized to peak response in the absence of BDM. The reduction in tetanus force was similar to the reduction in maximal twitch force in the presence of ryanodine. After normalization to peak response in the presence of BDM, the steady-state force–[Ca2+]i relation in the presence of BDM remained shifted to the right at lower levels of [Ca2+]i. This most likely reflects the decline in myofilament sensitivity.

Shifts in the steady-state force–[Ca2+]i relation have been shown to reflect changes at the level of the myofilaments.13-15 Steady-state force–[Ca2+]i relations were derived in intact muscles loaded with aequorin (n=5) in the presence of 1 μM ryanodine. Simultaneous recordings of steady-state force and calcium are demonstrated in 16 mM [Ca2+]o in the presence and absence of BDM (Figure 4). In the presence of BDM the force–[Ca2+]i relation was shifted to higher [Ca2+]i by 0.1 μM (p<0.01), indicating a decrease in sensitivity of the myofilaments to Ca2+. Further, Fmax was diminished by 30±5%.

### Table 1. Influence of 2,3-Butanediene Monoxime on the Isometric Contraction Duration in the Presence and Absence of Ryanodine

<table>
<thead>
<tr>
<th></th>
<th>2 mM [Ca2+]o</th>
<th>16 mM [Ca2+]o</th>
<th>p</th>
<th>2 mM [Ca2+]o</th>
<th>16 mM [Ca2+]o</th>
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<tr>
<td></td>
<td>Control</td>
<td>BDM</td>
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<td>Control</td>
<td>BDM</td>
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<tr>
<td>Ryanodine present</td>
<td></td>
<td></td>
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<tr>
<td>TPT (msec)</td>
<td>263±7</td>
<td>255±7</td>
<td>NS</td>
<td>248±11</td>
<td>304±10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T50T (msec)</td>
<td>129±7</td>
<td>131±7</td>
<td>NS</td>
<td>213±14</td>
<td>185±10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ryanodine absent</td>
<td></td>
<td></td>
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<tr>
<td>TPT (msec)</td>
<td>160±5</td>
<td>120±5</td>
<td>&lt;0.001</td>
<td>150±12</td>
<td>110±16</td>
<td>0.002</td>
</tr>
<tr>
<td>T50T (msec)</td>
<td>160±36</td>
<td>74±15</td>
<td>0.03</td>
<td>230±18</td>
<td>130±22</td>
<td>0.01</td>
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Values are mean±SEM (n=21). BDM, 2,3-butanediene monoxime; TPT, time to peak tension; T50T, time from peak tension to 50% relaxation. Values of p are control vs. BDM.
(\(p<0.01\)). There was a decrease in the Hill coefficient from 5.00±0.11 to 3.40±0.20 (\(p<0.01\)), indicating reduced cooperativity between thick and thin myofilaments (Figure 4).

To investigate the question of the site responsible for the decreased responsiveness of the myofilaments to Ca\(^{2+}\), we measured Ca\(^{2+}\) binding to TnC directly in skinned guinea pig, canine, and bovine ventricular myocardium (\(n=5\) per group). Results of the experiments on guinea pig myofibrils with 3 mM BDM are shown in Figure 5. Concentrations of BDM up to 10 mM were studied and had no significant effects on myofibrillar TnC Ca\(^{2+}\) binding. The same results were obtained with canine and bovine heart myofibrils.

**Effect of BDM on the Unloaded Velocity of Shortening**

After a steady-state force level was achieved, muscles were quickly slackened and allowed to redevelop tension at the new length. The release caused a rapid fall in force to zero, and the force remained at zero level until the muscle shortened to take up the slack. Four amplitudes of release (10%, 15%, 20%, and 25% of \(L_s\)) were used, with the smallest step being more than sufficient to slacken the muscle. The time required to redevelop force was measured for each amplitude. Force tracings in Figure 6 were obtained from one such experiment and revealed that 3 mM BDM shortened the time of force recovery, whereas after washout, the time to recovery became longer. The velocity of unloaded shortening, determined as described earlier in “Materials and Methods,” was 1.55±0.24 mm/sec (\(n=4\)) in the control solution and 2.04±0.33 mm/sec (\(n=4\)) in the presence of BDM. This significant increase in the velocity of unloaded shortening (\(p<0.02\)) occurred in the presence of 3 mM BDM. In skinned fibers, 3 mM BDM decreased maximal Ca\(^{2+}\)-activated force by 18±6% (\(n=3\)). BDM shifted the frequency of minimum stiffness from 1.29±0.04 to 1.56±0.08 Hz (\(n=2\)), indicating an increase in the speed of crossbridge cycling rate. In the presence of 3 mM BDM, there was a decrease in stiffness at higher frequencies, indicating a decrease in the number of force-generating crossbridges.

**Effect of BDM on Intracellular Calcium, Transsarcolemmal Calcium Current, and Action Potential Parameters in Isolated Guinea Pig Myocytes**

The linearity of the response obtained with fura-2 may more truly reflect the spatially averaged intracellular calcium.\(^{15,30}\) In isolated myocytes loaded with the fluorescent indicator fura-2 (\(n=10\)), the addition
of 1 mM BDM resulted in diminished amplitude of the intracellular calcium transient (921±158 versus 650±175 nM, p=0.06) with prolongation of its time course (Figure 7). Resting intracellular calcium was increased in most cases (109±11 versus 131±11 nM, p>0.05). This most likely reflects SR calcium "leak." There were no differences in peak amplitude of the calcium transient or I_{Ca} between recordings done in sequence in the absence of BDM. The addition of BDM resulted in a decrease in the peak amplitude of the calcium transient (n=6) and I_{Ca} (n=10) (Figure 7). The transmembrane I_{Ca} was reduced by 7.9±1.0% in the presence of BDM (p<0.001). I_{Ca} for the second control was 99.5±0.4% (p>0.1) compared with the first control recording (n=5). In two experiments, three control recordings, timed to simulate an experiment with addition of BDM, showed no significant difference in peak current (p>0.1). These data suggest that the effects seen in the presence of BDM cannot be explained on the basis of I_{Ca} or calcium transient rundown. An unsubtracted current-voltage relation in the absence and presence of BDM is also shown in Figure 7. The peak I_{Ca} was decreased by 7.9±1% without any apparent shift in the voltage relation (n=13) (Figure 7).

BDM has been reported to affect action potential duration, with resultant negative inotropism.10 We recorded action potentials in single isolated myocytes in the presence and absence of BDM (Figure 8). Action potential parameters are listed in Table 2. BDM sometimes tended to increase the resting membrane potential (p=NS) but did not significantly affect action potential amplitude or time course. There was a slight abbreviation of the action potential in the presence of BDM, as previously reported in papillary muscle preparations.11 In a few experiments with 2 and 3 mM BDM added to the bath, action potential durations were significantly abbreviated (data not shown).

**Discussion**

**Effect of BDM on Electrical Properties, I_{Ca} and [Ca^{2+}]_{o}**

BDM is a nucleophilic agent that under some circumstances acts as a phosphatase.11 BDM has
been reported to produce its concentration-dependent negative inotropic effect via dephosphorylation of calcium channels\textsuperscript{11,12} and alteration of action potential parameters in a variable and concentration-dependent way.\textsuperscript{10} Several arguments have been put forward to support the hypothesis that BDM acts through an inhibition of Ca\textsuperscript{2+} influx via the slow inward channel. First, BDM causes a concentration-dependent decrease in the amplitude and duration of the action potential in Purkinje fibers.\textsuperscript{12,31} Second, BDM blocks different (i.e., Na\textsuperscript{+}-free and elevated potassium plus isoproterenol) models of slow-response action potentials.\textsuperscript{11,31} This point has been disputed.\textsuperscript{10} Third, BDM produces a concentration-dependent negative inotropic effect in ventricular myocardiun. These observations combined together have been used to suggest that BDM exerts its negative inotropic effect via dephosphorylation of intracellular sites and reduced voltage-dependent I\textsubscript{Ca}.\textsuperscript{11,31}

A potential mechanism whereby BDM exerts its negative inotropic effect is by reducing [Ca\textsuperscript{2+}], in the bathing medium via a calcium-chelating effect with resultant diminished intracellular calcium and SR loading. Although it has been reported that high concentrations of BDM (30 mM) chelate [Ca\textsuperscript{2+}], this finding should be reevaluated in light of the highly hydrophobic nature of BDM and potential calcium electrode resin interaction. In our experiments, BDM at the concentration studied did not significantly affect calcium electrode measurements. Further, BDM did not affect aequorin luminescence in vitro in the presence of a low calcium concentration and background bioluminescence. Subsequent addition of 1 \( \mu \)M EDTA, a known calcium chelator, reduced the background bioluminescence.

At the sarcolemmal level it has been suggested that BDM, via its phosphatase activity, dephosphorylates cardiac sarcolemmal calcium channels,\textsuperscript{11,12,31} thereby reducing voltage-dependent I\textsubscript{Ca}. BDM has been demonstrated to affect membrane electrical properties in frog twitch fibers.\textsuperscript{32} Because we wanted to study the effects of the compound in a relatively physiological state, we did not remove sodium or replace potassium with cesium in the bath or pipette. In the presence of BDM, transmembrane I\textsubscript{Ca}s were reduced at all test potentials. This finding has recently been confirmed in rat cardiac myocytes, in which 1 mM [BDM] reduced by 10% the peak amplitude of the I\textsubscript{Ca} with little effect on current kinetics.\textsuperscript{33} The reduction in transsarcolemmal calcium flux would result in a lesser trigger for calcium-induced calcium release and reduced SR calcium loading.\textsuperscript{34}

The calcium-induced-calcium release mechanism might be expected to predict that the release of Ca\textsuperscript{2+} from the SR would be related linearly to the peak I\textsubscript{Ca} because, until the initiation of release, the maximal
FIGURE 7. Top panels: Tracings of intracellular calcium transients. The reduced noise in the second calcium tracing reflects continued fura-2 loading. Middle panels: Membrane calcium currents (I\textsubscript{Ca}) in an isolated cell. I\textsubscript{Ca} has been illustrated so that peak currents can be distinguished. Bottom panel: Graph showing membrane current-voltage (IV) relation in the absence and presence of 1 mM BDM in another isolated myocyte in which calcium measurements were not performed.

\[ \text{Ca}^{2+} / \Delta t \] should be proportional to the peak current.22 Beuckelmann and Wier22 investigated the relations between voltage and [Ca\textsuperscript{2+}], transient, voltage and peak I\textsubscript{Ca} and voltage and Ca\textsuperscript{2+} entry during depolarization and repolarization. They found the decline in normalized [Ca\textsuperscript{2+}], transient roughly paralleled the normalized total Ca\textsuperscript{2+} entry. Callewaert et al\textsuperscript{33} reported a similar relation between I\textsubscript{Ca} and the Ca\textsuperscript{2+} transient amplitude. Taken together, these reports suggest that the Ca\textsuperscript{2+} transient should be reduced by \(-8\%\), similar to the reduction in peak I\textsubscript{Ca} in the presence of 1 mM BDM in isolated cardiac myocytes, which is not that dissimilar to our findings. Therefore, the reduction in the peak calcium transient in voltage-clamped myocytes reflects, in part, reduction of the I\textsubscript{Ca} with reduced calcium-induced calcium release. Similarly, in papillary muscles exposed to 3 mM BDM, one would predict a decrease of \(-21\text{--}28\%\) in peak calcium transient. This idea is supported by a recently reported BDM concentration-dependent decrease in the amplitude of the I\textsubscript{Ca}.

![Graph showing membrane current-voltage (IV) relation](image)

FIGURE 8. Recordings of action potentials in single isolated myocytes in the presence and absence of 2,3-butanedione monoxime (BDM). See Table 2 for action potential parameters.

<table>
<thead>
<tr>
<th>Table 2. Action Potential Parameters</th>
<th>APA (mV)</th>
<th>RMP (mV)</th>
<th>APD\textsubscript{80} (msec)</th>
<th>APD (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-BDM</td>
<td>126±7</td>
<td>-81±0.6</td>
<td>586±30.0</td>
<td>621±29.0</td>
</tr>
<tr>
<td>3 mM BDM</td>
<td>121±9</td>
<td>-78±3.0</td>
<td>391±138</td>
<td>541±120</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=5). APA, action potential amplitude; RMP, resting membrane potential; APD\textsubscript{80}, action potential duration at 80% repolarization; APD, total action potential duration; BDM, 2,3-butanedione monoxime. Measurements were made at room temperature.
BDM may increase SR calcium “leak.” This is supported by our observed increase in resting calcium in the presence of BDM in isolated myocytes. We recently reported that postrest twitch potentiation induced by caffeine and high \([Ca^{2+}]_o\), was decreased from 341±42% to 73±10% by BDM.36 Our findings support previous findings that BDM affects the SR, directly resulting in a decreased amount of calcium released.9,37

Others have proposed that BDM’s negative inotropic effect is due solely to abbreviation of action potential duration and reduced slow inward current.11 In this study, although the action potential tended to be abbreviated, this finding did not attain statistical significance at the 1 mM BDM concentration.

**Sarcoplasmic Reticulum Calcium Handling**

The SR has been shown to be the predominant intracellular organelle that supplies calcium for activation of the myofilaments, resulting in myocardial contraction. In previous studies, the potential effect of BDM on intracellular calcium supplied by the SR could not be dismissed. In our studies we have used both aequorin, a nonlinear calcium indicator, and fura-2, a linear fluorescent indicator. Peak intracellular calcium was diminished in aequorin-loaded preparations, indicating that SR calcium release is reduced by BDM at relatively low concentrations compared with what has been reported in the literature.5,6 These findings are similar to those reported in skeletal muscle38 but are in contradiction to those previously reported in cardiac tissue.4,39 Blanchard et al39 recently reported that BDM did not affect SR Ca2+ release, but their data clearly showed that the aequorin light signal was decreased by BDM in a concentration-dependent way. In this study by Blanchard et al, a relatively high concentration of propranolol, a nonselective β-receptor antagonist, which has been demonstrated to have both agonistic as well as membrane-stabilizing effects40,41 was used with BDM. Propranolol, at the concentration used, not only affects peak twitch force but also affects the peak intracellular calcium transient. We performed experiments in the presence of bupranolol, a β-blocker, which does not have significant agonistic properties or membrane-stabilizing effects40,41 and obtained similar results as those in its absence (e.g., a decrease in peak intracellular calcium). Since our experiments clearly show that BDM has membrane effects, this might explain the differences in light output and the different results obtained by Blanchard et al.39

It should be pointed out that relatively small changes in peak intracellular calcium can result in significant changes in peak isometric twitch force, because twitch force lies on the relatively steep portion of the force–[Ca2+] relation.14,42 Our experiments demonstrate that BDM has a greater effect on peak twitch force as opposed to steady-state force, consistent with this idea. The small shift in [Ca2+] of 0.1 µM cannot solely explain the effect of BDM on peak twitch force. One has to take into account that aequorin is a nonlinear indicator. There is a tendency to incorrectly interpret the significance of a decrease in the aequorin light signal because true \([Ca^{2+}]_o\), is approximately proportional to \(\sqrt{\text{Normalized Light}}\). Since \([Ca^{2+}]_o\) values are <1 M, a small decrease in light would reflect a much larger decrease in \([Ca^{2+}]_o\). Therefore, diminished SR release could explain a significant portion of BDM’s negative inotropic effect.

**Sensitivity of the Myofilaments to Calcium**

Studies with skinned-fiber preparations indicate that BDM induces a decrease in the \([Ca^{2+}]_o\) sensitivity of the myofilaments.2,10 We studied the effects of BDM in intact preparations on steady-state force–calcium relations. We have shown that ryanodine, in ferret ventricular myocardium, does not affect the sensitivity of the myofilaments to calcium in both tetanized and skinned-fiber preparations.14,17 We have also shown that steady-state force–[Ca2+] relations reflect changes at the level of the myofilaments.14,17 In the presence of BDM, the steady-state force–[Ca2+] relation shifted to higher \([Ca^{2+}]_o\) by 0.12 pCa, similar to what has been reported in cardiac skinned fibers (0.08 pCa) and skeletal muscle (0.1 pCa),2 indicating a decrease in Ca2+ sensitivity of the myofilaments.2,9,10 Similar to findings in smooth muscle, \(P_{\text{max}}\) was reduced in the presence of BDM.7 As suggested, the reduction of \(P_{\text{max}}\) probably reflects a decrease in the number of crossbridges, most likely because of the action of BDM on a step in the signal transduction or contractile protein regulation before crossbridge formation.2,5,9 Unlike results in skinned cardiac fibers from the mouse, we found a reduction in the Hill coefficient.2 In contrast, BDM has been shown to reduce the Hill coefficient in fast-twitch fibers in the rat and in rabbit skeletal muscle.37,43 Our finding suggests that BDM alters the cooperativity between thick and thin myofilaments.

The inhibitory effect of BDM on the maximum contractile activation is rapid and fully reversible, consistent with its binding and inhibiting of the independent force generators. Unlike previous studies, we found that BDM increased the velocity of unloaded shortening at a concentration at which it had prominent inhibitory effects on isometric tension. Our results, with low concentrations of BDM, could be interpreted as showing that the instantaneous and steady-state number of force-generating crossbridges was reduced by BDM but that the rate at which crossbridges were reacting with actin at zero load was increased. This suggests that BDM affects the biochemical states of the crossbridges during their working cycle, resulting in a reduction of the number of crossbridges in a force-generating state. In the presence of BDM, the overall cycle time of the crossbridges is shorter, allowing the muscle to redevelop force faster at zero load. Since the crossbridges are spending less time in a force-generating state, this provides an explanation of the inhibitory effect of...
BDM on force development. The energy cost of tension maintenance would thus be increased by BDM. Yet, Alpert et al. have shown that heat production and isometric tension are depressed proportionately in the presence of BDM. The data of Alpert et al. thus suggest that the reduction of force is mainly due to a reduced number of crossbridges and not to the shorter time the crossbridges spend in the force-producing state. The effects of BDM on the contractile proteins can be explained with reference to a scheme whereby the crossbridges have three states as shown on Figure 9. As indicated in the scheme in Figure 9, we propose two sites of action for BDM. BDM first inhibits the conversion of the detached state into the weakly attached state, thus reducing the number of attached crossbridges. Since \( F_{\text{max}} \) reflects the number of force-generating crossbridges, it will be reduced by the action of BDM on this site. BDM's second site of action is the facilitation of \( P_i \) release in the conversion of the weakly attached state into the force-generating state. This will increase the cycling rate of the crossbridges and could result in a rightward shift of the force-[Ca\(^{2+}\)] relation. According to the scheme, the combined effect of BDM on both sites will result in the observed experimental effect, an increase in [Ca\(^{2+}\)]\(_{\text{SER}}\) and a reduction in \( F_{\text{max}} \). In this scheme, we would expect the rate of ATP hydrolysis to be decreased by BDM, which has been shown to be the case in previous reports.

The calcium occupancy of TnC is largely determined by the amount of Ca\(^{2+}\) released from the SR. The reduced SR calcium release seen in the presence of BDM in conjunction with no change in TnC affinity or stoichiometry would explain, in part, the marked negative inotropic effect seen on twitch force. It is clear that, with twitches, the calcium transient is short and the force-generating process is interrupted long before it reaches maximum. Twitch tension, therefore, is much smaller than tetanic force, where the force is high as is the occupancy of TnC by calcium. This would also explain the greater effect of BDM on twitch force compared with tetanus. In frog skeletal muscle, a similar differential effect on twitch versus tetanus force has been reported.

As illustrated in Figure 6, on washout of BDM the crossbridge cycling rate is lower. This occurs at a time when peak force is restored and precedes the return to pre-BDM levels of [Ca\(^{2+}\)]. Contrary to what has previously been proposed, shifts in the steady-state force-[Ca\(^{2+}\)] relation may reflect changes at the level of TnC or distal to it. This study emphasizes the importance of considering the effects of crossbridge kinetics on shifts in the force-[Ca\(^{2+}\)] relation and \( F_{\text{max}} \) as derived from steady-state measurements.

**Conclusions**

To investigate the effects of BDM at varying steps in the excitation–contraction cascade, we used a multifaceted approach to address the question, “How does BDM exert its negative inotropic effect in intact cardiac muscle preparations?” Voltage-clamp experiments revealed that BDM concentrations as low as 1 mM reduced \( I_{\text{Ca}} \) at all test potentials without affecting the current–voltage relation. Using two calcium indicators, aequorin (a bioluminescent indicator) in multicellular preparations and fura-2 (a fluorescent indicator) in isolated cardiac myocytes, we report that the peak Ca\(^{2+}\) transient was reduced. Further, BDM resulted in a reduction in the amount of SR calcium available for release and force production. Steady-state force-[Ca\(^{2+}\)] relations indicated a rightward shift on the force–[Ca\(^{2+}\)] axis toward higher [Ca\(^{2+}\)], suggesting a decrease in the sensitivity of the myofilaments. However, TnC calcium binding studies indicated that there was no change in calcium binding affinity or stoichiometry. BDM did, however, increase the crossbridge cycling rate as detected with maximal velocity of shortening and dynamic stiffness determinations. We conclude that the combination of 1) reduced \( I_{\text{Ca}} \) with resultant reduced calcium-induced calcium release, 2) diminished loading of the SR, 3) abbreviation of the action potential, 4) increased crossbridge cycling rates, and 5) uncoupling of force generating crossbridges causes the negative inotropic effects seen with BDM. BDM appears to be a nonspecific agent with effects on numerous cellular systems.

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**KEY WORDS**

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- troponin C
- crossbridge kinetics
- action potentials
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