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Mechanism of action of 2, 3-butanedione 2-monoxime on contraction of frog skeletal muscle fibres

K. HORIUTI, H. HIGUCHI, Y. UMAZUME, M. KONISHI, O. OKAZAKI and S. KURIHARA

Department of Physiology, Jikei University School of Medicine, Tokyo, 105, Japan

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Summary

The mechanism of the inhibitory effect of 2, 3-butanedione 2-monoxime (BDM) on contraction of frog skeletal muscles was studied using skinned fibres and aequorin-injected intact fibres. The tension development of skinned fibres directly activated with calcium was strongly inhibited by BDM. This agent also had effects on the sarcoplasmic reticulum in the skinned preparations, suppressing the calcium pump function and enhancing the activity of the 'calcium-induced calcium release' mechanism. In electrically stimulated intact fibres, although BDM slightly suppressed the elevation of the intracellular calcium ion concentration, this effect was so weak that it would not explain the strong inhibitory effect of the agent on the tension development by the intact fibres. It was concluded that the tension reducing effect of BDM on intact fibres was due mainly to its direct action on the contractile system. The mode of this action of BDM was further examined with skinned fibres in view of its effects on the maximum shortening speed and isometric tension in low MgATP environments.

Introduction

2, 3-butanedione 2-monoxime (BDM; CH_3C: NOH-$COCH_3$) has been reported to have an inhibitory effect on muscle contraction (Mulieri & Alpert, 1984; Blanchard *et al.*, 1984; Kometani, 1985) and Mulieri and his colleagues have suggested the possibility that the agent could be used as a selective inhibitor for the crossbridge function of contractile system of muscles.

We have studied the effects of BDM on the contractile apparatus and on the calcium control system in frog skeletal muscles using skinned fibres as well as intact single fibres injected with a calcium indicator, aequorin. Our results indicated that, although BDM could affect the calcium movements to and from the sarcoplasmic reticulum (SR), the inhibitory effect of the agent on contraction of living muscles was due mainly to its direct action on the contractile system provided that the agent concentration was low.

Methods

Measurement of aequorin luminescence and tension in intact muscle fibres

Single muscle fibres were dissected from m. tibialis anterior of *Rana temporaria* stored at 4° C and mounted horizontally in a narrow channel of the experimental chamber, one end of the fibre being attached to the lever of a tension transducer (AE801, AME, Norway) with the other end fixed to a hook. The average sarcomere length was adjusted to 2.4×10^{-6} m by observing its laser diffraction. When the fibre was to be stimulated, 0.5 ms square wave pulses at $\times$ 1.5 threshold were applied to the fibre through a pair of platinum black wires placed parallel to it.

A glass micropipette filled with a solution containing 150 mM KCl, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate, pH 7.0, adjusted with KOH) and 2 mg ml^{-1} aequorin, was used for the pressure injection of aequorin. The fibre condition was checked before and after the aequorin injection by observing tetanic contractions, and only fibres that showed complete tetanus with a sustained plateau were used for experiments.

The aequorin light signal from the muscle fibre was detected by a photomultiplier (EMI 9789A, UK) and its intensity was expressed as the photomultiplier current. The light and tension data were stored in an FM tape recorder and analysed later. A microcomputer was used for smoothing the light signal data. See Allen & Kurihara (1982) for further details of the experimental apparatus and procedure.

The Composition of the normal Ringer solution was (in mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; NaH_2PO_4, 0.85; Na_2HPO_4, 2.15; pH 7.1. The temperature of the Ringer solutions was kept at 18° C.

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The direct effect of 10 mM BDM on the relation between the aequorin luminescence and free calcium ion concentration was examined *in vitro* in a manner similar to that described by Blinks *et al.* (1982), and no appreciable effect was detected.

Experiments with skinned fibres

Most of the skinned fibres were prepared from *Rana catesbeiana* but some from *R. temporaria* and one from *Xenopus laevis*. Single fibres were isolated from m. semitendinosus, sartorius or iliofibularis muscles of the frogs, and their cell membranes were removed mechanically in a relaxing solution. When properties of the contractile system were to be examined, fibres were treated with detergent (0.5% Brij-58 or 1% Triton X-100) for ¼ h in the relaxing solution to destroy the SR in the fibres. The completeness of destruction was confirmed with six skinned fibres by using the 'caffeine method' (see below) as a technique to assay the calcium content of the SR. Some fibres were not skinned mechanically and their cell membranes were permeabilized only with the detergent treatment. Parts of the skinned fibres, several millimetres in length, were cut for use in the experiments.

One end of the skinned segment was tied to the extension of a tension transducer (UL2, Shinkoh, Japan or AE801, AME, Norway) with the other end fixed, and its average sarcomere lengths was adjusted to 2.5–2.8 × 10^{-6} m in the relaxing solution. Fibres were bathed in solutions in small wells (0.5 ml in volume) which were bored on aluminium or brass plates. The mounted skinned fibre was dipped in one of the solutions and, when the solution was to be changed, the plate was moved so that the fibre could be immersed in the next solution. The surface of the well wall of the metal plates had been coated with fluorine-containing polymer (Flonwax, NRK, Japan). By circulating chilled water just underneath the plates, solutions in the plate wells were kept at a constant, low temperature of 0–5° C.

The maximum shortening speed of skinned fibres was determined using a slack method similar to that described by Edman (1979). In these experiments, the compliance of the tension measurement system (with an element AE801) was 1.5 mm N^{-1} and the resonance frequency of the force transducer was 2.5 kHz. The quick length change (by 10–25% preparation length) on the skinned fibres was carried out by using a scanner motor (G100PD) and its control unit (CCX101, General Scanning Inc., US), the motor arm being connected to the end of the fibre which was not attached to the transducer. The length change could be completed within a millisecond.

Solutions used for skinned fibres were basically the same as those described by Horiuti (1986) but with some modifications. The solution was buffered with 20 mM PIPES (piperazine-*N*,*N*'-bis(ethylenesulphonate)) and adjusted with KOH to pH 7.0. The calculated value of free magnesium ion concentration was 1.5 mM, and that of MgATP 3.5 mM (ATP: adenosine-5'-triphosphate), where the following stability constants were assumed in accordance with Smith & Martell (1974–1982): log MgATP/Mg·ATP = 4.00, log CaATP/Ca·ATP = 3.78, log HATP/H·ATP = 6.63 and log KATP/K·ATP = 1.0. The ionic strength was 0.2 M, being adjusted with K-methanesulphonate. Unless otherwise indicated, free calcium ions were buffered with 10 mM-EGTA (ethyleneglycol-bis(β-aminoethylether)-*N*,*N*'-tetraacetate), where the apparent stability constant of the Ca–EGTA complex was taken as $10^{6.39}$ M^{-1} at pH 7.0 (Smith & Martell).

In some series of experiments, solutions contained 10 mM creatinephosphate and 15 units/ml creatine phosphokinase as an ATP regenerating system. In these solutions, ionic strength was 0.15 M, and the main anion was Cl^- instead of methanesulphonate.

When MgATP-deficient solutions were prepared, total ATP concentration was fixed to 1 mM and free magnesium ion concentration was varied using 10 mM EDTA (ethylenediaminetetraacetate) as the buffer. In these solutions, the following stability constants were assumed in accordance with Smith & Martell: log HEDTA/H·EDTA = 10.36, log H_2EDTA/H·HEDTA = 6.28, log MgEDTA/Mg·EDTA = 8.79 and log CaEDTA/Ca·EDTA = 10.69. The main anion was Cl^- and ionic strength 0.17 M in these magnesium-deficient solutions.

The calcium of the SR in the skinned fibres was assayed using the 'caffeine contracture method'. A detailed explanation and the validity of this method were described and discussed by Horiuti (1986). Briefly, the fibre was immersed in a low-EGTA relaxing solution (solution G0.7 in Horiuti, 1986) and then transferred to an assay solution (mG0.7caf) which contained a high concentration of caffeine (40 mM) and a low concentration of free magnesium ions (< 0.1 mM). Since this assay solution had a potent action of releasing calcium from the SR, on its application the fibre contracted transiently due to the released calcium. We estimated the amount of calcium from the relative magnitude of the caffeine contracture of the preparation. The assay solution was also used for emptying the SR when it was required for the initialization, just before loading with calcium.

2,3-Butanedione 2-monoxime (BDM) was purchased from Nakarai Chemicals, Ltd. (Japan) and stored in darkness. Once this crystal was dissolved in solutions, they were used within 1–2 days. On addition of BDM, the pH of the solutions became more acidic and it was readjusted appropriately if the change was greater than 0.01 pH units.

Dr S. Takemori in our laboratory has shown experimentally (personal communication) that BDM had no detectable effect on the apparent stability constant of Ca-EGTA that was estimated using a potentiometric method (Moisescu & Thieleczek, 1979).

Results

Effects of BDM on tension development and calcium transient in intact fibres

As reported by Mulieri & Alpert (1984), the twitch contraction was remarkably inhibited by addition of BDM in Ringer solution, and the effect was reversible (Fig. 1a). Figure 1b shows the fast sweep records of the tension and the simultaneously obtained light signals of aequorin which was injected into the sarcoplasm. The suppressive effect of BDM on the aequorin signal seemed to be much less prominent

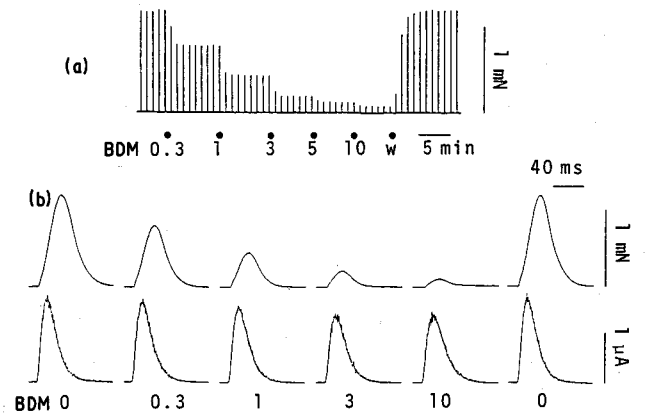

Fig. 1. (a) Effect of BDM on the twitch tension of an intact fibre. The fibre was electrically stimulated at intervals of 1 min. BDM was added to Ringer's solution at the times indicated with dots in the figure; the concentration (mM) of the agent is labelled at each dot. At the point labelled w, BDM was washed out with the normal Ringer. Temperature: 18° C.
(b) Fast sweep traces of the tension (upper row) and the simultaneously obtained light signal (lower row). Both were an average of five signals recorded at the steady state before and after application or washing out of BDM. The number under each pair of traces is the BDM concentration in mM.

when compared with that on the twitch tension. With three other fibres, similar results were obtained. However, it should be noted that because of the transient nature of the twitches it is difficult to assess quantitatively the time course of the sarcoplasmic free calcium level at the twitch contractions: the aequorin light signal lags behind the free calcium level with a time constant of several milliseconds (Blinks *et al.*, 1982). To overcome this difficulty we performed the following experiment, where muscle fibres were stimulated repetitively to elicit steady tetanic contractions.

Figure 2 shows the effect of the agent on tetanus triggered by 50 Hz stimulation for 1 s. BDM inhibited the peak developed tension and markedly delayed the tension rise. In contrast to this, even if the BDM concentration was increased up to 5 mM, the agent brought about no remarkable change in the light intensity of aequorin at least at the end of the tetanic stimulation. It should be noted that the aequorin light signal is approximately proportional to the 2.5th power function of the free calcium ion concentration in the steady state (Blinks *et al.*, 1982). In the earlier phase of the light signal, BDM slightly suppressed the transient peak and widened the following dip. However, this inhibitory effect of the agent on the calcium transient would not fully explain the marked delay in the tension development, since in the absence of BDM the tension reached almost maximum even at such a low calcium level as was observed in the earlier phase with BDM. The result in Fig. 2 therefore suggests the existence of a site of BDM action in the contractile apparatus. Results similar to that in Fig. 2 were obtained with two other fibres.

At 10 mM, BDM remarkably suppressed the elevation of the intracellular calcium ion concentration at tetanus. As shown in the bottom traces of Fig. 2, the degree of suppression was always abruptly intensified during the train of stimulations, and it seemed as if some of the stimulations had been made completely ineffective. Repeated tetanus stimulations of a muscle fibre in the continuous presence of 10 mM BDM revealed that the appearance of this abrupt effect depended on the time after the start of each tetanus stimulation, but not on the duration of incubation of the fibre with the agent. This time-dependent all-or-nothing action suggested that this particular effect of BDM was due to the agent making the membrane action potential abortive, in a manner similar to a local anaesthetic. However, as we were not interested in such an action of BDM, we did not pursue its mechanism further in this study.

Figure 3 shows the relation between the dose of BDM and the peak tension of twitch and tetanus,

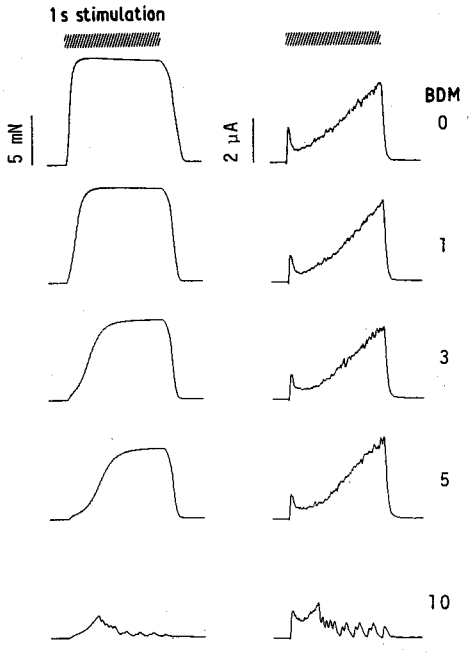

Fig. 2. Tension and light signal in tetanus (50 Hz, 1 s) in the absence or presence of various concentrations of BDM. The BDM concentration (mM) is indicated next to the traces. The abortive contraction at 10 mM BDM was not due to an irreversible damage to the fibre since it could show a normal tetanus contraction after washing out the agent (not shown). The same fibre that was used in the experiment in Fig. 1. Temperature: 18° C.

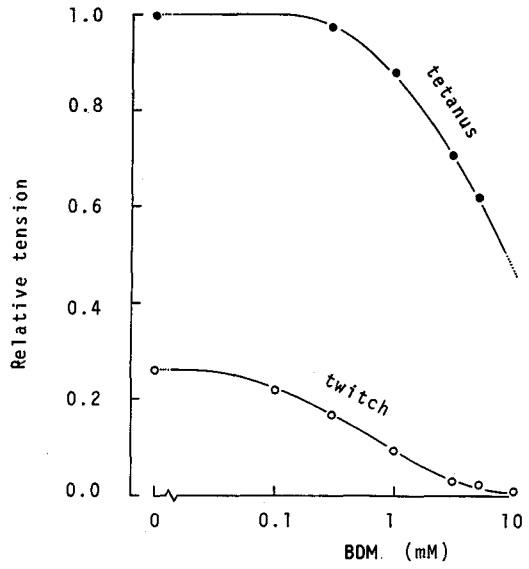

Fig. 3. Relation between the BDM concentration and the peak tension at twitch (○) and tetanus (●). Tension was normalized to that of tetanus observed in the absence of BDM. Average of four sets of data from four single intact fibres. Temperature: 18° C.

which was determined with four intact fibres. It can be seen from this figure that the inhibitory effect of the agent was less prominent on tetanus than on twitch, when it was assessed by suppressed fraction of the control tension.

Effects of BDM on contractile apparatus of skinned fibres

The action of BDM on the contractile apparatus was directly tested with skinned fibres, but at a lower temperature (0–5° C) because skinned fibre experiments at room temperature are difficult due to damage to the preparations caused by the contractions. The tension trace in Fig. 4 evidently shows that the agent greatly inhibited the tension development of the skinned fibre which was directly activated by calcium ions, and this effect could be quickly reversed when the agent was washed out. Figure 5 shows another set of data on the effect of the agent on the relation between the steady-state isometric tension and the calcium ion concentration in the bathing solution. The agent reduced the maximum tension and elevated the calcium ion concentration required for the half-maximal tension. Figure 6 shows that BDM suppressed the maximal isometric tension to ½ at ~ 1 mM and to less than ⅕ at 10 mM. These results with skinned fibres supported the view that the BDM effect on intact fibres could be ascribed to its direct action on the contractile system.

Although the dose-effect relation for the tetanus of intact fibres was similar to that for the maximum contraction for skinned fibres (compare Fig. 3 and Fig. 6), BDM appeared to be a little less potent in intact fibres than in skinned fibres. This could be explained by the difference of temperature between the two experiments: 18° C with intact fibres and 0–5° C with skinned fibres. In fact, it was found that the BDM effect appeared to be greater at lower temperature in both intact and skinned fibres from *R. temporaria* (for skinned fibres see legend for Fig. 6, filled symbols) and at 4–5° C the two sets of data (not shown) were consistent with each other.

We then examined further if this agent influenced the maximal shortening velocity of skinned fibres,

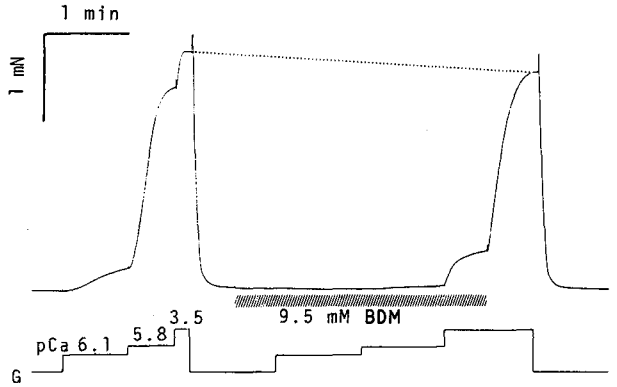

Fig. 4. Tension traces obtained from a skinned fibre. The fibre was activated with various calcium solutions with or without 9.5 mM BDM. Sequence of the solution application is schematically illustrated below the tension trace, where the numbers indicate the pCa value of the solutions and G represents the relaxing solution containing 10 mM EGTA. The negative slope of the dotted line indicates natural decline of the maximal tension of the preparation due to the prolonged calcium applications. Sarcomere length: 2.8 × 10^{-6} m and 2° C. Preparation dissected from *R. temporaria*.

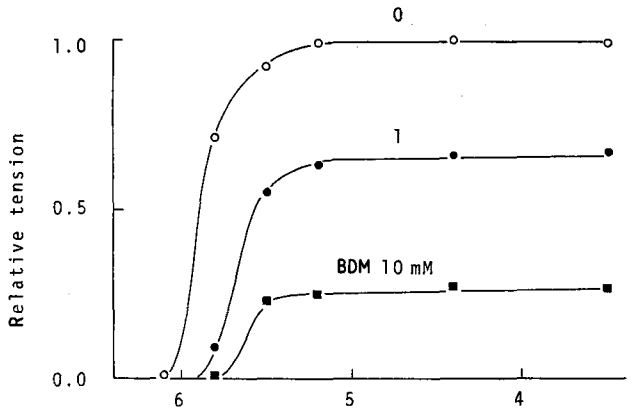

Fig. 5. Effect of BDM on the relation between the steady isometric tension developed by skinned fibres and the free calcium ion concentration in the bathing solution. Concentration of agent is labelled on each graph. The tension was normalized to that observed at pCa 4.4 without BDM. Three skinned preparations from *R. catesbeiana* were used. Sarcomere length: 2.5 × 10^{-6} m; temperature 1° C.

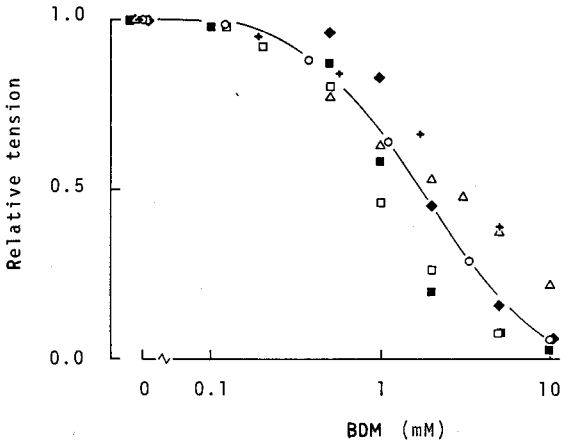

Fig. 6. Relation between BDM concentration and maximum isometric tension developed by skinned fibres at 0–5° C. Tension was normalized to that observed in the absence of BDM. Free calcium ion concentration was $10^{-4.4}$ (or 10^{-4}) M in the activating solution. Sarcomere length: 2.5 – 2.8 × 10^{-6} m. The results shown were obtained from 5 independent series of experiments. (□, ■, ◆) Solutions contained an ATP-regenerating system, main anion Cl^-. (+, ○, △) Without the regenerating system, main anion methane sulphonate. (■, ◆) Paired sets of data obtained with *R. temporaria*, the former at 2° C and the latter at 5° C. Excepting these paired experiments, skinned fibres were all prepared from *R. catesbeiana*.

using a slack method. Skinned fibres, of average sarcomere length 2.8 × 10^{-6} m, were activated isometrically at pCa 4.4 in the absence or presence of BDM. After a steady-state tension level was achieved, the fibre was quickly slackened and the time required to redevelop tension was measured. Carrying out this test with two slack lengths of about ¼ and ⅐ the fibre length, the maximum shortening speed at no load was calculated from the difference (l) in the length and that (t) in the time as being l/t.

Tension traces in Fig. 7a were obtained from such slack tests, and reveal that the 2 mM BDM had a less prominent effect on the maximum speed in contrast to its marked inhibitory effect on the isometric tension development. Similar experiments were carried out using various BDM concentrations, and the results are shown in Fig. 7b. The effect on the maximum speed was generally minimal when the agent's concentration was lower than 3 mM.

When MgATP in the bathing medium is appropriately reduced, skinned fibres develop active tension even in the absence of free calcium ions (Reuben *et al.*, 1971). We aimed to find out if BDM affected this particular contraction, and the results are compiled in Fig. 8.

In the absence of BDM, the tension–MgATP

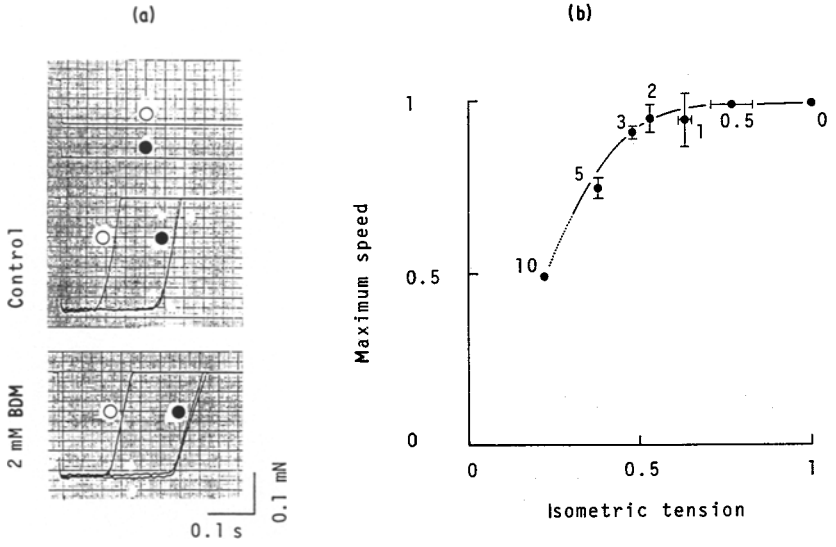

Fig. 7. (a) Part of tension records obtained in a series of slack tests with a skinned preparation from *R. catesbeiana*. The skinned fibre was activated with a calcium solution (pCa 4.4) in the absence or presence of 2 mM BDM, and quickly slackened in the midst of the full activation. This chart represents the magnified records at the slack, where the record(s) at the slack length of 13.2% and those at 23.0% are superimposed to clearly show the difference in time courses of the tension redevelopment between the two test lengths. Tension was scaled out in this chart when too large. Isometric tension level before the slack was 2.90 mN in the absence of BDM, and 1.55 mN in its presence. Uppermost traces represent the time courses of the changes in the end-to-end distance of the preparation. Initial sarcomere length: 2.8 × 10^{-6} m; total length of the preparation: 4.5 mm. Temperature: 0° C.

(b) Effect of BDM on isometric tension and the maximum shortening speed of skinned fibres fully activated at pCa 4.4. With each fibre, the speed and the tension were measured in both the absence and presence of BDM; those in its presence were normalized to those in its absence. The numbers attached to the plotted points indicate BDM concentration (mM) and each point represents the mean of serveral sets of data, error bars being the S.E. of the mean, but there is only one set of data at 10 mM BDM. Nine skinned preparations from *R. catesbeiana* were used. Temperature: 0° C. Average maximum speed 0.78±0.08 (mean±S.D.; n=8) muscle length s^{-1} without BDM.

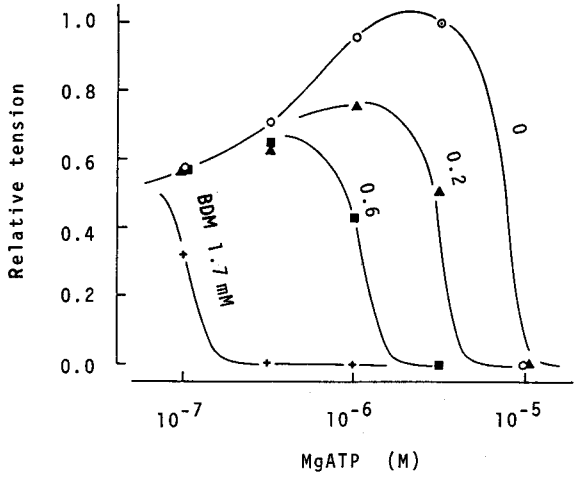

Fig. 8. Effect of BDM on the tension development by skinned fibres in MgATP-deficient solution. Skinned fibres were firstly transferred from the relaxing solution into a MgATP-free solution (10 mM EDTA and no added magnesium salts) to achieve a 'rigor state' of the fibres, and then slackened a little to expel the passive tension developed in the course of the achievement of the rigor state. After this conditioning, the fibres were immersed in various MgATP-deficient solutions with or without BDM, and the steady level of the active tension developed by the fibres in the solutions was plotted here, being normalized to that observed at $10^{-5.5}$M-MgATP without BDM, which was revealed to be 0.53 – 0.54 of the maximum tension of the fibres in the normal pCa 4 solution. Number at each point show the concentration of BDM (mM). $[EDTA]_{total}$: 10 mM; $[ATP]_{total}$: 1 mM; ionic strength: 0.17 M. Cl was the main anion in the solutions. Temperature: 2° C. *R. catesbeiana*.

relation showed a bell shape, as has been reported previously (Reuben *et al.*, 1971). Although the right-hand side of this bell-shaped curve was truncated by BDM, its left side showed tolerance to the agent: the active tension at sufficiently low MgATP was scarcely influenced at an agent concentration under 0.6 mM, and this tendency could be observed even at 1.7 mM BDM. In this sense, the crossbridge formation induced by low MgATP appeared to be more tolerant than that by calcium ions since, as shown in Fig. 5, the suppression of the tension induced by calcium could not be surmounted even by sufficient elevation of calcium ion concentration. We could detect no effect of 4–10 mM BDM on the stiffness of fibres incubated in a MgATP-free solution, where the stiffness was measured from the change in tension to the stretch of 0.3–0.8% of the fibre length and the obtained values were some 0.05 of the maximum tension per 1 nm per half sarcomere.

Effects of BDM on the calcium pump and on the 'calcium-induced calcium-release' mechanism of the SR

Since the SR is known to play a central role for controlling the cytoplasmic calcium ion concentration, we also tested BDM for effects on SR functions with skinned fibres.

Skinned fibres with empty SR were immersed in calcium-containing solutions for various periods with or without BDM and then the calcium content of the SR was assayed in the absence of the drug using the 'caffeine method' (see Methods for detail). Time courses of the calcium accumulation in the SR determined in this manner are shown in Fig. 9. BDM reduced the rate of calcium accumulation at both pCa 6.75 and pCa 5, and this could be interpreted as an inhibitory effect of the agent on the calcium pump of the SR. This effect was evident at 1 mM BDM and at 10 mM its extent was so great that the calcium content of the SR which was loaded at pCa 6.75 for 3 min was made to be a half of the control.

Another series of experiments was also performed to examine whether BDM affected the 'calcium-induced calcium release mechanism (Endo *et al.*, 1970) of the SR. The experimental technique was similar to that described by Endo (1981; see also Horiuti, 1986). In brief, we measured the rate of calcium release from the SR in skinned fibres that were immersed in solutions containing various concentrations of free calcium ions. Apart from calcium ions, these test solutions contained 1.5 mM free magnesium ions and 10 mM AMP. The substitution of AMP for ATP was required to make the calcium-pump ATPase inactive in order that the pure activity of the calcium release mechanism could be observed.

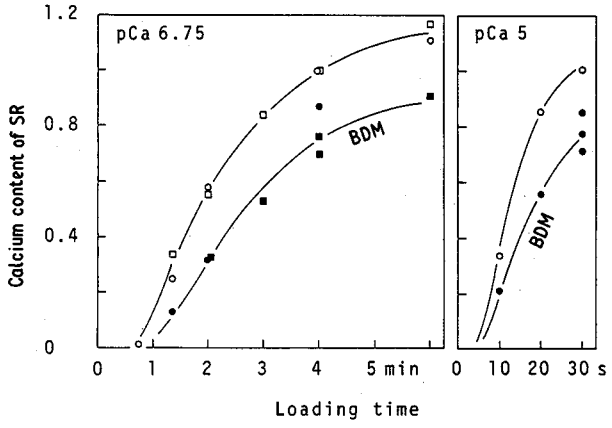

Fig. 9. Effect of BDM on calcium accumulation in the SR at pCa 5 (right panel) and 6.75 (left panel). Filled symbols, in the presence of 5.7 mM BDM; open symbols, in its absence. The calcium content of the SR was assayed using the caffeine method (see Methods for detail) and plotted here as a relative value to that at pCa 6.75 and 4 min without BDM. Due to the non-linearity of this assay system, the initial part of the uptake curves appear to be a little truncated. Two fibres from *R. catesbeiana* were used, the circle and square in this graph representing these two fibres, respectively.

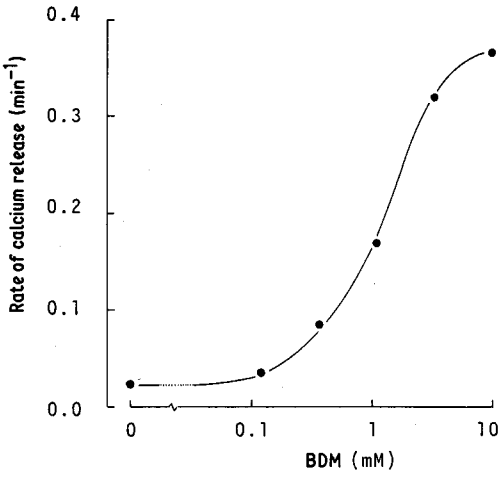

Fig. 10. Dose-effect relation for the action of BDM to potentiate the calcium release at pCa 5.5 in the presence of 10 mM AMP and 1.5 mM free magnesium ions. Three fibres, two from *R. catesbeiana* and one from *X. laevis*, were used and the average data are shown here.

BDM at 5.7 mM had a potentiating effect on the calcium release mechanism, and this effect was quite clear when the calcium-ion concentration was so low (pCa 6.5–5.5) that the activity of the calcium-induced calcium release was weak, but it was obscure when the calcium ion was sufficient (pCa 5–4.4) for maximum activation of the release mechanism. This mode of action of BDM was very different from those of other potentiators for the calcium-induced calcium release such as caffeine (Endo, 1981), halothane (Endo *et al.*, 1983), adenine (Ishizuka & Endo, 1983), adenosine monophosphate (Endo, 1981) or β, γ-methylene adenosine triphosphate (Matsumura, Horiuti & Endo, unpublished observation). These were all known to potentiate the calcium release machanism at high as well as low calcium ion concentrations. We could not find any effect of BDM on the calcium release in the absence of calcium ions (10 mM EGTA) — the 'leakage' of calcium from the SR.

The relation between the BDM concentration and the calcium release rate was determined at pCa 5.5; the result is displayed in Fig. 10. Although the BDM effect on the calcium release was still evident at concentrations under 1 mM, its maximum extent was relatively small in comparison with the potency of caffeine (see Horiuti, 1986).

Discussion

In this study we confirmed the inhibitory effect of 2,3-butanedione 2-monoxime (BDM) on contraction of intact muscle fibres, and showed that this agent strongly inhibits tension development of the contractile apparatus which was directly activated by calcium ion application. However, this agent was found to have some other side effects.

Effects of BDM on the SR

BDM inhibited the calcium pump and potentiated the activity of the 'calcium-induced calcium release' mechanism in skinned fibres. It is possible that both effects would result in an increase in the amount of calcium released from the SR in physiological contraction of intact muscle fibres. However, BDM scarcely potentiated the calcium transient in intact cells, and rather showed a tendency to suppress it. Possible explanations for this apparent contradiction could be:

1. Since the physiological calcium release from the SR is elicited through an unknown mechanism distinct from that of the calcium-induced calcium release (Endo, 1977), the stimulatory effect on the latter mechanism may not manifest itself in intact cells.
2. As the removal of calcium by the SR from the calcium receptor of the contractile system, troponin, is partly aided by the slow calcium binding to parvalbumin as well as by calcium pumping by the SR (Baylor *et al.*, 1983), the suppressive affect on the latter may not seriously affect calcium removal.
3. Some inhibitory effects of BDM on the physiological calcium release mechanism might mask the inverse effects resulting from the action on the pump and the calcium-induced calcium release. For example, we cannot deny that the agent might affect the shape of the action potential of the surface membrane, because at extreme doses it seemed to have a local anaesthetic-like effect (see Results).
4. It is also possible that the effect of BDM we found with skinned fibres was minimal in intact fibres. In fact, the artificial milieu for skinned fibres is quite different from the physiological sarcoplasm.

Action of BDM on the contractile apparatus

In the experiment with intact fibres, although we found an inhibitory effect of BDM on the calcium transient at tetanus, this effect was not so great that it would explain the suppression of the tetanus contraction even if its concentration was increased up to 5 mM. We should therefore consider that the tension suppressing effect of BDM in intact fibres was mainly a result of the direct action of BDM on the contractile proteins. In fact, this agent strongly inhibited the tension development of skinned fibres which were activated directly with calcium, and the dose-tension relation was similar to that observed in tetanic contraction of intact fibres.

Effect of BDM on the maximum shortening speed

Mulieri & Alpert (1984) reported that 7.5 mM BDM depressed the maximum shortening speed of intact fibres. In the present study with skinned fibres, we found that lower doses of the agent had only a very small effect on the maximum speed even when it had the prominent inhibitory effect on the isometric tension. From the viewpoint of Huxley's sliding-filament theory (1957), our results with low doses of BDM could be interpreted as showing that the instantaneous number of working crossbridges at isometric condition was reduced by the agent, but the breakdown rate of the formed crossbridge at free shortening was kept normal. Probably the breakdown rate in the isometric state is also preserved even with the agent, because Kometani (1985) reported that the heat production at the isometric steady state was suppressed by BDM only in a linear relationship with the depression of the isometric tension.

If the instantaneous number of the working crossbridges was actually reduced in the presence of BDM and if the breakdown rate was unchanged at the time, the cause of this reduction must have been either (a) reduction in the second-order rate constant of the crossbridge formation, or (b) decreases in the effective concentration of the contractile proteins in muscle, or both. From this view of crossbridge kinetics, it is interesting to see whether this agent affects the curvature of the force-velocity curve, because in the case of (a) this curvature is expected to be accentuated in Huxley's model, but it would not be affected in (b). Mulieri & Alpert (1984) have reported that 7.5 mM BDM markedly increased the curvature but their concentration of the agent was too large to preserve the maximum shortening speed. Further studies with low doses of BDM are required.

Slow tension rise at tetanus in the presence of BDM

As was above referred to, when BDM concentration was relatively low, the agent did not seem to affect the breakdown rate of the crossbridges. However, such low doses of BDM applied to intact fibres not only suppressed the peak tetanus tension but also delayed the tension rise. This has also been reported by Kometani (1985) as the lengthening of the time to the half-maximal tension development. The slower tension development caused by BDM may be due to the possible reduction of the crossbridge formation rate (case (a) in the above section). However, this can also be explained by the following two effects of this agent:

1. The decreasing of the apparent calcium sensitivity of the contractile system, i.e. the right shift of the pCa– tension curve;
2. The slight but evident suppression of the earlier part of the elevation of the sarcoplasmic calcium level.

These two factors would have worked synergistically so as to delay the tension rise at tetanus.

Greater effect of BDM on twitch than on tetanus

The tension-depressing effect of BDM was more prominent on twitch than on tetanus. Since this reflects the slow tension development discussed above, we may be able to consider the inhibitory effect of BDM on twitch as a result of the agent's action on the calcium release, as well as a result of that on the contractile apparatus. However, the latter action cannot be a minor factor since dantrolene sodium — a muscle relaxant which has been known to inhibit calcium release from the SR without any effect on the contractile apparatus (Brocklehurst, 1975) — at 2×10^{-6} (w/w) suppressed the peak of the aequorin light transient at twitch down to 40%, while it inhibited the twitch tension only to 50% (Konishi & Kurihara, unpublished observation). This contrasts with the result with BDM in the present study.

Site of action of BDM in contractile apparatus

The physiological activation on contractile apparatus by calcium ions is brought about in sequential steps as follows: troponin binds calcium to remove the suppressive effect of the troponin–tropomyosin complex on actin, and in turn actin binds to myosin to produce the sliding force. Which step did BDM inhibit? As mentioned above, it seems that this agent reduced the crossbridge number but preserved the breakdown rate. This may suggest that the site of action of BDM was in some step preceding the force generation by actin and myosin. The relative tolerance of the crossbridge formation by low MgATP in comparison with that by high calcium ion concentration may support the above idea. Since the inhibitory effect of BDM on the normal calcium contraction could not be surmounted even by sufficient elevation of calcium ion concentration, the action site was probably not in the step of the calcium binding by troponin, but in a step following it. Biochemical studies with purified and reconstructed contractile proteins are required to definitely determine the site of the action of BDM.

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