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ACTION OF 2,3-BUTANEDIONE MONOXIME ON CALCIUM SIGNALS IN FROG CUT TWITCH FIBRES CONTAINING ANTIPYRYLAZO III

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SUMMARY

1. The effects of 2,3-butanedione monoxime (BDM) on the optical retardation and myoplasmic Ca^{2+} signal were studied in twitch fibres of *Rana temporaria*. The myoplasmic Ca^{2+} transient in response to action potential stimulation was monitored in cut fibres containing Antipyrylazo III under current clamp in a double Vaseline-gap chamber.

2. In fibres not stretched adequately to suppress all the contraction, BDM blocked the movement-related intrinsic optical signal at 810 nm very effectively.

3. In fibres stretched to sarcomere lengths $\geqslant 4\ \mu m$ to reduce the contraction to below detectable levels, the effect of BDM on the Ca^{2+}–Antipyrylazo III signal was studied after correcting for the instrinsic signal unrelated to movement. With increasing concentrations of BDM, the peak of the Ca^{2+}–Antipyrylazo III signal was suppressed progressively. Concomitantly, the half-width was prolonged somewhat. On average, 5, 10 and 20 mM-BDM reduced the peak amplitude to 88, 78 and 54% of control, respectively.

4. BDM had no effect on the rising phase or the peak amplitude of the retardation signal measured at 720 nm, but suppressed the undershoot in the decay phase of the signal in a dose-dependent manner. BDM also had no effect on the late pedestal level of the signal.

5. During repetitive stimulation by a train of ten action potentials, 10 mM-BDM suppressed the second to the tenth peaks of the Ca^{2+} signal and of the retardation signal more effectively than the first peak. Twenty millimolar BDM almost completely suppressed the later peaks of both signals such that the signals decayed with a time course similar to that elicited by a single action potential.

6. The effect of BDM on the Ca^{2+}–Antipyrylazo III signal was also studied in fibres under voltage clamp; 10 mM-BDM lowered the threshold for the Ca^{2+}–Antipyrylazo III transient by a few millivolts and reduced the steepness of the peak amplitude *versus* voltage plot near threshold.

7. Based on a model used by Baylor, Chandler & Marshall (1983) to estimate the net Ca^{2+} release from the sarcoplasmic reticulum, 10 and 20 mM-BDM were found to reduce the peak release to 75 and 52%, to prolong the half-width of the release

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waveform to 118 and 147%, and to reduce the peak uptake to 76 and 54% of control values, respectively.

8. It is concluded that BDM affects the optical retardation and myoplasmic Ca^{2+} signal monitored with Antipyrylazo III in a dose-dependent manner. However, its suppression of the optical signals is much smaller than of twitch tension.

9. It is suggested that a low concentration (a few millimolar) of BDM could be a useful contraction blocker for skeletal muscle fibres when used in conjunction with moderate stretch of the fibres.

INTRODUCTION

The preceding paper (Hui & Maylie, 1991) described the effects of 2,3-butanedione monoxime (BDM) on twitch tension, resting potential, action potential, and charge movement in frog twitch fibres. It was found that BDM affects every step in the excitation–contraction coupling sequence under study in a dose-dependent manner, although the suppressing effects on the earlier steps are much smaller than those on twitch tension. In view of these multiple actions of BDM, there is a distinct possibility that the drug might also affect Ca^{2+} release from the sarcoplasmic reticulum (SR). If this is true, the action of BDM in blocking contraction in skeletal muscle is far more complicated than simply interfering with cross-bridge formation, as originally suggested by Mulieri & Alpert (1984). The goal of this paper is to investigate the extent to which Ca^{2+} release is affected by BDM so as to clarify further the action of the drug and to evaluate whether the drug is useful as a contraction blocker in future experiments on Ca^{2+}–indicator signals. A preliminary report of this work has appeared (Maylie & Hui, 1988).

METHODS

Experiments were carried out at Yale University with the optical set-up described in Irving, Maylie, Sizto & Chandler (1987).

Muscle and fibre preparation

All experiments were performed on twitch fibres from English frogs (*Rana temporaria*) that were cold-adapted at around 4 °C. Animals were killed by decapitation and pithing the spinal cord. Cut fibres, originally used by Hille & Campbell (1976), were dissected from semitendinosus muscles and mounted in a double Vaseline-gap chamber similar to the procedure used by Kovacs, Rios & Schneider (1983) and Irving *et al.* (1987). Briefly, a stretched muscle bundle was exposed to a Ca^{2+}-free, high-K^+ relaxing solution (solution I in Table 1 of Hui & Maylie, 1991) which caused a transient contraction. A 6–12 mm length of a single fibre was isolated and mounted in a double Vaseline-gap chamber. The ends of the muscle segment were affixed to movable clips adhered to the bottoms of the two end-pools to allow for adjustment of sarcomere length. To facilitate Antipyrylazo III entry, the outer membranes of the fibre in the end-pools were permeabilized by a 2 min exposure to 0·01% saponin followed by thorough rinsing with solution I. The end-pool solutions were then replaced with the internal solution (either solution J or K). The centre-pool solution was changed to either normal Ringer (solution E) or an isotonic tetraethylammonium (TEA) solution (solution G).

Solutions

Solutions are given in Table 1 of the preceding paper (Hui & Maylie, 1991). Antipyrylazo III (ICN K & K Laboratories, Inc., Plainview, NY, USA) or Arsenazo III (Sigma Chemical Co., St Louis, MO, USA) was introduced into the fibre by dissolving it in the internal solution and allowing it to diffuse into the fibre from the end-pools.

Optical measurements

Most experiments were carried out with Antipyrylazo III, except for a few which were carried out with Arsenazo III. Details concerning the experimental method and processing of optical records are given in Irving *et al.* (1987) and Maylie, Irving, Sizto & Chandler (1987*a*, *b*). Briefly, optical measurements were made with a 50 μm diameter spot of light focused on the middle of the fibre in the centre-pool. Since three wavelengths are required to accurately describe the Ca^{2+}–indicator signal in muscle (Baylor, Chandler & Marshall, 1982*a*), the transmitted light was separated into three beams with two beam-splitting cubes. Each beam was made quasi-monochromatic by passing through an interference filter. For Antipyrylazo III, interference filters with peak transmission wavelengths at 550 nm (10 nm bandwidth), 720 nm (30 nm) and 810 nm (30 nm) were placed in the appropriate positions in the apparatus. For Arsenaso III, interference filters with peak transmission wavelengths at 570 nm (10 nm), 660 nm (30 nm) and 750 nm (30 nm) were used. Each beam was further split into two beams of orthogonal, linear polarizations with polarizing beam-splitting cubes. The intensities of the transmitted light at three different wavelengths and two planes of polarization were measured simultaneously with the membrane potential and current.

For the measurements of absorbance and linear dichroism (mode 1 recording; Irving *et al.* 1987) the longitudinal axis of the fibre was oriented parallel to the optical axis of the apparatus. The absorbance at each wavelength is given as a 1:2 average of the absorbance of light linearly polarized along the optical axis (A_0) and the absorbance of light linearly polarized perpendicular to the optical axis (A_{90}). Linear dichroism is taken as the difference $A_0 - A_{90}$. For the measurement of absorbance and retardation (mode 2 recording) a linear polarizer was placed in the incident light path and the fibre was rotated 45 deg with respect to the optical axis. The absorbance and retardation signals were then calculated as described in Irving *et al.* (1987).

The procedures described in Maylie *et al.* (1987*a*, *b*) were used to subtract the contributions due to the intrinsic optical changes in the fibres. Ca^{2+}–indicator signals were elicited either by action potentials (in the current-clamp mode) or by depolarizing pulses (in the voltage-clamp mode). To avoid repetitive use of words, the term 'Ca^{2+} signal' will be used to refer to the change in absorbance of the Ca^{2+}–indicator complexes. $\Delta A(\lambda)$ and $\Delta R(\lambda)$ will be used to refer to the change in absorbance and the change in retardation at wavelength λ.

Based on cuvette calibration, we found that BDM has no appreciable effect on the Ca^{2+} difference spectrum of the Ca^{2+}–Antipyrylazo III complex.

RESULTS

Effect of BDM on myoplasmic Ca^{2+}–indicator signals elicited by action potentials

In optical experiments, cut fibres were generally stretched to a sarcomere length of about 4 μm to reduce movement-related optical signals which can be observed at shorter sarcomere lengths. Figure 1*A* shows optical signals, following action potential stimulations, from a fibre with a sarcomere length of 3·7 μm. Changes in light intensities expressed as absorbance changes were measured at 550, 720 and 810 nm. The absorbance signals at all three wavelengths were large and were characteristic of movement-related optical signals in fibres that were not well stretched. The signals at 550 and 720 nm had an early absorbance change that is consistent with Ca^{2+} complexation with Antipyrylazo III plus a large signal similar in waveform to that at 810 nm, a wavelength at which light is not absorbed by Antipyrylazo III. Based on the idea that the 550 and 720 nm signals are linear combinations of a Ca^{2+}-related signal and an intrinsic signal represented by the 810 nm signal, a procedure was developed by Maylie *et al.* (1987*a*) for correcting changes in intrinsic absorbance at 550 and 720 nm. Applying this procedure, a linear combination of the 810 and 720 nm signals was fitted to the 550 nm signal to determine a scaling factor, which was then applied to the 810 nm signal and

subtracted from the 720 nm signal. The resulting signal is shown at the bottom of Fig. 1*A* and labelled 'corrected 720'. This trace shows that, in the presence of large movement-related artifacts, the Ca^{2+} signal was poorly corrected.

Figure 1*B* shows the effect of the addition of 10 mM-BDM to the external solution in the centre-pool. The large intrinsic signals seen in the traces of Fig. 1*A* were

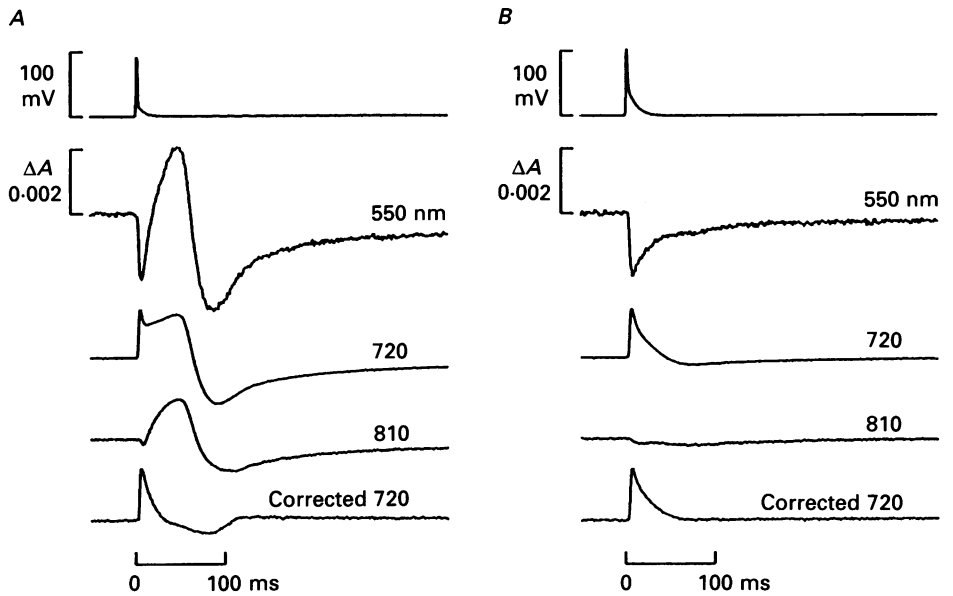

Fig. 1. Effects of 10 mM-BDM on optical changes following action potential stimulation in a cut fibre containing Antipyrylazo III. *A*, control records taken 75 min after saponin treatment of the end-pool segments. The Antipyrylazo III concentration was 0·61 mM. The top trace shows the action potential, attenuated by the 0·625 kHz Bessel filter. The actual amplitude, measured on a storage oscilloscope, was 128 mV. The next three traces show the uncorrected signals at 550, 720 and 810 nm. The bottom trace shows the corrected 720 nm signal obtained by subtracting the estimated intrinsic signal, given by the 810 nm trace scaled by $(720/810)^{-1\cdot83}$, from the third trace. *B*, test records taken 2 min after the addition of 10 mM-BDM to the Ringer solution in the central pool or 78 min after saponin treatment of the end-pool segments. The Antipyrylazo III concentration was 0·63 mM. The amplitude of the action potential, measured on the oscillosocpe, was 124 mV. The corrected 720 nm trace at the bottom was obtained after subtracting the estimated intrinsic signal, given by the 810 nm trace scaled by $(720/810)^{-1\cdot83}$. Fibre identification 63251; sarcomere length 3·7 μm; diameter 89 μm; temperature 17·5 °C; gap factor, defined by $r_e/(r_i+r_e)$ (see Irving *et al.* 1987), 0·99.

abolished by BDM at all three wavelengths. The effect was observed within 2 min after the addition of BDM and was fully reversible within 2 min following the removal of BDM from the bath. The signal remaining at 810 nm was characteristic of the intrinsic signal measured in cut fibres with a sarcomere length greater than 4 μm. The corrected 720 nm signal in Fig. 1*B* was also characteristic of the Ca^{2+} signals measured in highly stretched fibres in the absence of BDM. This result showed that BDM can reduce movement-related signals that mask or contaminate the Ca^{2+} signal.

The effect of BDM on the Ca^{2+} signal following action potential stimulation can be more clearly seen in a highly stretched cut fibre in which BDM has little, if any, effect

on the 810 nm intrinsic signal. A typical experiment is shown in Fig. 2. The top pair of traces show action potentials in the absence (thin trace) and in the presence (thick trace) of 10 mM-BDM. The drug had no effect on the peak of the action potential but elevated the after-potential. The bottom pair of traces show that the drug had no

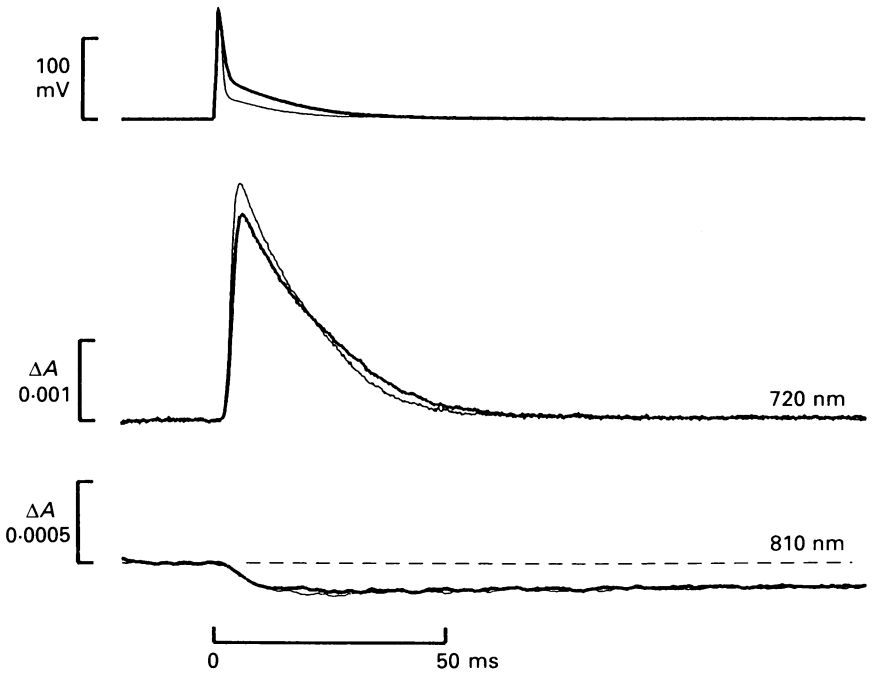

Fig. 2. Effects of 10 mM-BDM on optical changes following action potential stimulation in a highly stretched cut fibre containing Antipyrylazo III. Thin traces were recorded 94 min after saponin treatment of the end-pool segments and the Antipyrylazo III concentration was 0·84 mM. Thick traces were recorded 7 min after the addition of 10 mM-BDM to the Ringer solution in the central pool or 103 min after saponin treatment of the end-pool segments. The Antipyrylazo III concentration was 0·82 mM. Top traces show action potentials. Middle traces show the corrected 720 nm optical signals after subtracting the corresponding intrinsic signals, given by the 810 nm traces (bottom traces) scaled by $(720/810)^{-1}$. All signals were digitized at 6·25 kHz after being filtered by 10 kHz eight-pole Bessel filters. The 810 nm traces were additionally filtered with a 0·1 kHz Gaussian digital filter. The action potentials were not attenuated by the 10 kHz Bessel filter and had an amplitude of 136 mV in both traces. Fibre identification 63271; sarcomere length 4·1 μm; diameter 107 μm; temperature 16·0 °C; gap factor 0·99.

effect on the intrinsic signal. The middle pair of traces show that the drug decreased the amplitude of the Ca^{2+} signal from 0·00301 to 0·00259 absorbance units and increased the half-width from 15·4 to 17·8 ms.

Figure 3 shows an experiment to study the effects of different concentrations of BDM on the Ca^{2+} signal and intrinsic signal in a fibre stretched to minimize but not completely eliminate the movement-related intrinsic signal. The top traces show that the half-width of the action potential was prolonged progressively with increasing concentrations of BDM. The middle traces show that the corrected 720 nm Ca^{2+} signal was reduced in a concentration-dependent manner. The half-width of the Ca^{2+} signal was also reduced slightly in BDM. This latter reduction was not due to a direct

effect of BDM on the half-width of the signal, which is in the opposite direction (see below), but suggested a poor correction of the intrinsic signal in the absence of BDM, as evidenced by the small pedestal at the end of the transient. The bottom traces show that BDM reduced the delayed positive-going absorbance change in the 810 nm

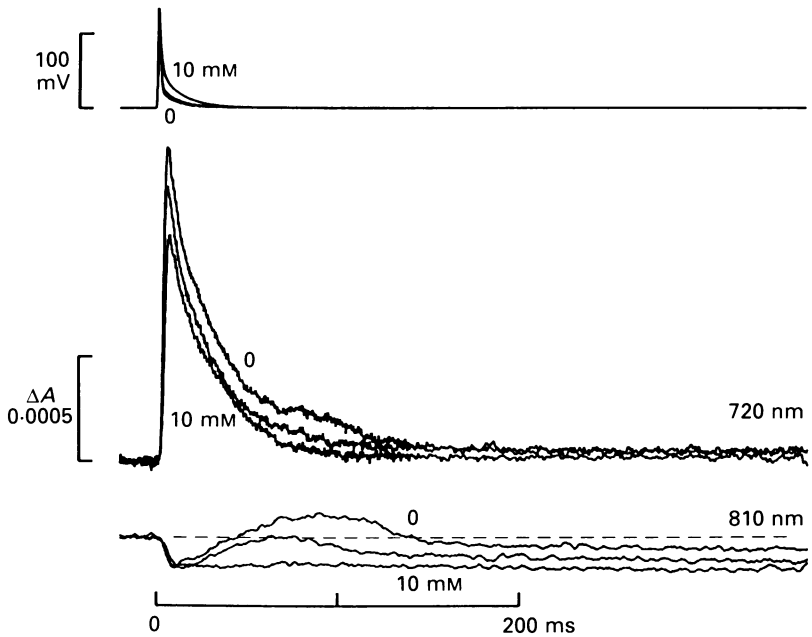

Fig. 3. Concentration dependence of the effects of BDM on the action potential, Ca^{2+}–indicator signal and intrinsic optical signal. Top traces show superimposed records of action potentials in the absence and presence of 2 and 10 mM-BDM. The traces taken without and with 10 mM-BDM are labelled correspondingly, but those taken with 2 mM-BDM are not labelled. The control action potential has the smallest after-potential and that in 10 mM-BDM has the most depolarized after-potential. The middle traces show the Ca^{2+}–indicator signals given by the 720 nm absorbance changes after the subtraction of the corresponding intrinsic signals, given by the 810 nm traces (bottom traces) scaled by $(720/810)^{-n}$, with $n = 4{\cdot}5$, 2·27 and 1·47 for the 0, 2 and 10 mM traces, respectively. All signals were digitized at 6·25 kHz after being filtered by 10 kHz eight-pole Bessel filters. The 810 nm traces were additionally filtered with a 0·1 kHz Gaussian digital filter. The action potentials were not attenuated by the 10 kHz Bessel filter and had an amplitude of 137 mV (0 BDM), 137 mV (2 mM-BDM) and 134 mV (10 mM-BDM). Records were taken 156–167 min after saponin treatment of the end-pool segments and the Antipyrylazo III concentration was between 0·46 and 0·45 mM. Fibre identification 66242; sarcomere length 4·0 μm; diameter 124 μm; temperature 16·0 °C; gap factor 0·98.

intrinsic signal in a concentration-dependent manner. The early decrease in absorbance appeared unaffected. The intrinsic signal with 10 mM-BDM was similar in magnitude and waveform to that observed in a more stretched fibre without BDM (Fig. 2).

Figure 4*A* shows the dose–response relation of the effect of BDM on the intrinsic signal. The magnitude of the intrinsic signal blocked by BDM was estimated from the difference between the peak of the early negative deflection and the peak of the

delayed positive deflection (see inset in the figure). These results show that the reduction in the changes in the intrinsic signal was concentration dependent with a half-blocking concentration of approximately 3 mM.

Figure 4*B* shows the dose–response relation of the effect of BDM on the Ca^{2+} signal. The Ca^{2+} signal was suppressed by BDM but to a lesser extent than was the

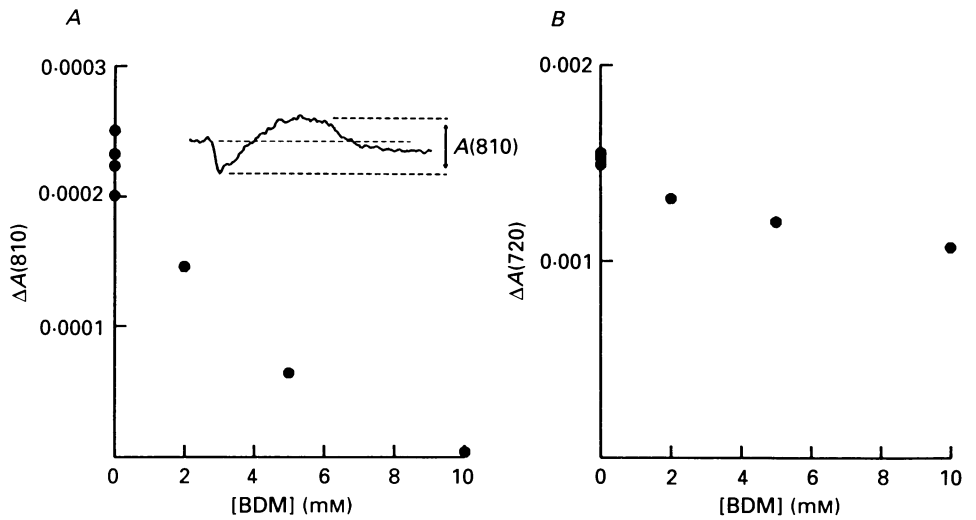

Fig. 4. Dose–response curves of the effects of BDM on the intrinsic optical signal and the peak of Ca^{2+}–indicator signal. *A*, plot of the change in the intrinsic optical signal at 810 nm, which was measured as indicated in the inset and described in the text, as a function of BDM concentration. *B*, plot of the peak of the corrected $\Delta A(720)$ signal as a function of BDM concentration. Same experiment as in Fig. 3.

810 nm intrinsic signal. In this fibre, BDM reduced the peak of the Ca^{2+} signal following an action potential to 84% in 2 mM, 79% in 5 mM, 68% in 10 mM and 21% in 20 mM (point not shown) BDM. These values (except for that in 2 mM-BDM) are listed in the last row of the last three columns of Table 1. Thus, the half-blocking concentration of BDM for the Ca^{2+}-related signal was higher than that for the movement-related intrinsic signal.

Similar experiments were performed on eleven other fibres and the results are also listed in Table 1. On average, 5, 10 and 20 mM-BDM reduced the peak of the Ca^{2+} signal elicited by an action potential to 88, 79 and 52% of control, respectively. There was substantial fibre-to-fibre variation in this reduction, particularly with 20 mM-BDM. The origin of the variability is unknown. Twenty millimolar BDM also prolonged the half-width of the signal by about 25% (data not shown), though lower concentrations had no noticeable effect on the half-width.

Effect of BDM on retardation signals elicited by action potentials

The early retardation signal may reflect an increase in myoplasmic free $[Ca^{2+}]$ (Suarez-Kurtz & Parker, 1977). It is, therefore, of interest to investigate whether BDM affects this signal. Figure 5 shows an experiment in which the changes in

absorbance and retardation were measured simultaneously. Panel *A* shows the effects of 10 mM-BDM (thick traces) on the action potential, the $\Delta R(720)$ retardation signal, the corrected $\Delta A(720)$ Ca^{2+} signal and the $\Delta A(810)$ intrinsic signal. As the fibre was stretched to a sarcomere length of 4·1 μm, the $\Delta A(810)$ intrinsic signal in the absence of BDM (thin trace) did not show any movement artifact and was almost

TABLE 1. Effects of various concentrations of BDM on the peak amplitude of the Ca^{2+}–Antipyrylazo III signal in cut fibres

Fibre reference (1)	Sarcomere length (μm) (2)	Peak Ca^{2+}–indicator signal (% of control) 5 mM (3)	10 mM (4)	20 mM (5)
63251	3·7	—	95	82
63252	4·1	—	76	—
63271	4·1	89	90	87
66061	4·1	97	87	—
66062	3·9	—	—	52
66191	4·1	—	63	35
66201	4·0	—	75	63
66202	4·3	—	76	46
66211	3·9	—	77	67
66212	4·1	—	—	54
66231	3·9	—	86	19
66242	4·1	79	68	21
Mean		88	79	52
S.E.M.		5	3	7

Columns (1) and (2) give the fibre references and the sarcomere lengths. Columns (3)–(5) give the fractions of the peak Ca^{2+}–indicator signal remaining in the presence of 5, 10 and 20 mM-BDM.

identical to that taken with 10 mM-BDM. Ten millimolar BDM enhanced the after-potential of the action potential and decreased the peak amplitude of the Ca^{2+} signal, similar to the results shown in previous figures.

The reduction in the peak Ca^{2+} signal, however, was not accompanied by a reduction in the peak retardation signal. Rather, the time course of the inverted retardation signal in Fig. 5*A* was altered and its half-width prolonged. The waveform of the control inverted retardation signal, which is typical of that recorded in the absence of an indicator (Maylie *et al.* 1987*a*), had several phases. After the peak change in retardation, the signal transiently undershot the baseline and slowly returned to an elevated pedestal that lasted for several seconds. The undershoot phase was suppressed by 10 mM-BDM, leaving the pedestal unaltered. Five millimolar BDM also reduced the undershoot phase, but to a lesser extent (not shown).

The difference between the inverted retardation signal measured without and with BDM is shown in Fig. 5*B*. This difference reflects the BDM-sensitive component of the retardation signal and has a waveform similar to the dichroic signal measured with Arsenazo III (Baylor, Chandler & Marshall, 1982*b*; Maylie *et al.* 1987*a*). The half-width of the difference signal is 32 ms, which is shorter than the half-width of the dichroic signal measured with $> 0{\cdot}2$ mM-Arsenazo III at similar times after

saponin treatment (Maylie *et al.* 1987*a*). However, for concentrations of Arsenazo III < 0·1 mM, the half-width of the dichroic signal is ~ 40 ms (Maylie *et al.* 1987*a*), which is not much larger than the half-width of the difference signal in Fig. 5*B*.

The effect of BDM on the dichroic signal at 570 nm was measured in three fibres containing Arsenazo III. The magnitude of the dichroic signal was reduced to 0·65 ± 0·03 (S.E.M.) of control in 10 mM-BDM and 0·32 ± 0·05 (S.E.M.) in 20 mM-BDM.

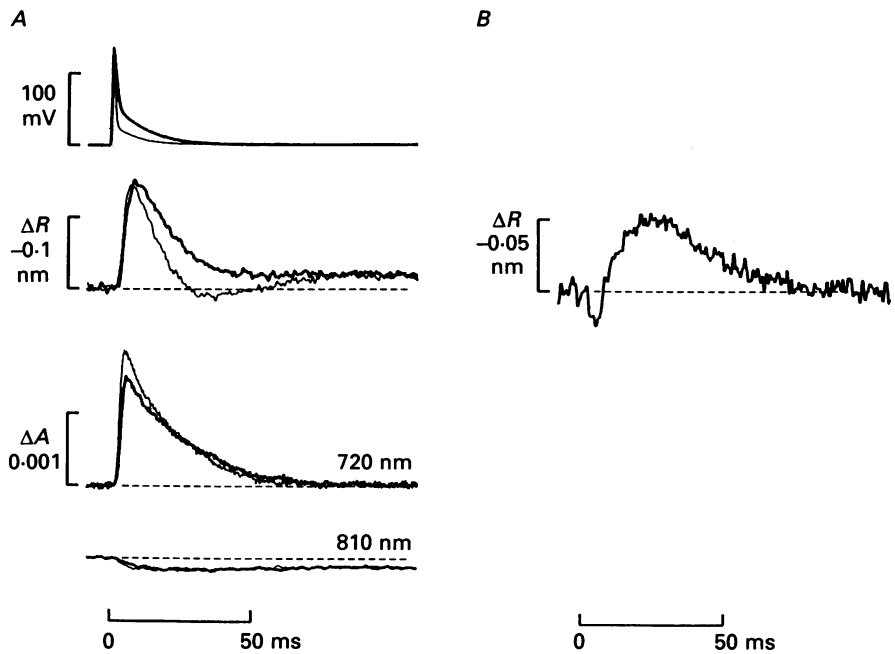

Fig. 5. Effects of 10 mM-BDM on the retardation and Ca^{2+}–indicator signals following action potential stimulation. *A*, thin traces were recorded 158 min after saponin treatment of the end-pool segments and the Antipyrylazo III concentration was 0·69 mM. Thick traces were recorded 3 min after the addition of 10 mM-BDM to the Ringer solution in the central pool or 163 min after saponin treatment of the end-pool segments and the Antipyrylazo III concentration was 0·67 mM. Top pair of traces show action potentials. The next three pairs show the retardation signals at 720 nm (ΔR, inverted), the corrected 720 nm Ca^{2+}–indicator signals after subtracting the 810 nm intrinsic signals scaled by $(720/810)^{-1}$, and the 810 nm intrinsic signals. All signals were digitized at 6·25 kHz after being filtered by 10 kHz eight-pole Bessel filters. The 810 nm traces were additionally filtered with a 0·1 kHz Gaussian digital filter. The action potentials were not attenuated by the 10 kHz Bessel filter and had an amplitude of 137 mV. *B*, BDM-sensitive component of retardation signal from the difference between the ΔR traces with and without BDM in panel *A*. Fibre identification 63271; sarcomere length 4·1 μm; diameter 107 μm; temperature 16·0 °C; gap factor 0·99.

Effects of BDM on retardation and Ca^{2+} signals during repetitive stimulation

Figure 6*A* shows that the Ca^{2+} and early retardation signals did not summate during repetitive stimulation, as the later peaks of either signal reached a maintained level lower than the first peak (Quinta-Ferreira, Baylor & Hui, 1984; Maylie *et al.* 1987*b*). Ten millimolar BDM slightly reduced the first peak of both signals, made the

later peaks less prominent and suppressed the maintained level of the later peaks substantially (Fig. 6*B*); 20 mM-BDM completely abolished the later peaks of both signals such that the waveform of either signal was similar to that following a single action potential (Fig. 6*C*).

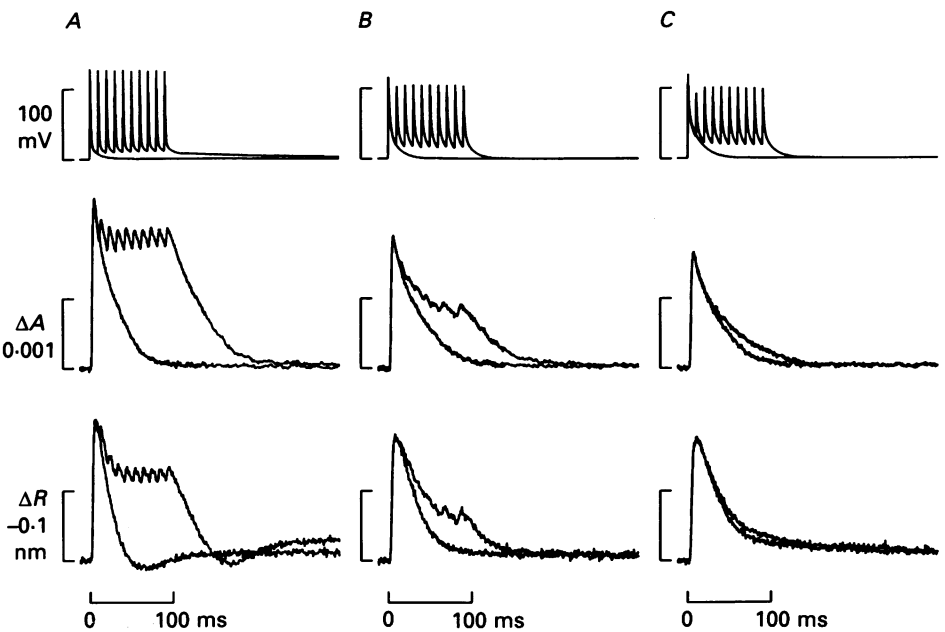

Fig. 6. Effects of BDM on the Ca^{2+} and retardation signals elicited by one and ten action potentials. Traces were recorded without BDM (*A*), with 10 mM-BDM (*B*) and with 20 mM-BDM (*C*) 100–127 min after saponin treatment and the Antipyrylazo III concentration was 0·82–0·84 mM. In each panel, the upper pair of superimposed traces shows the action potentials; the middle pair shows the corrected $\Delta A(720)$ signals and the bottom pair shows the inverted $\Delta R(720)$ retardation signals. All signals were digitized at 6·25 kHz after being filtered by 10 kHz eight-pole Bessel filters; the 810 nm trace was aditionally filtered with a 0·1 kHz Gaussian digital filter. Fibre identification 66202; sarcomere length 3·9 μm; diameter 99 μm; temperature 15·8 °C; gap factor 0·97.

The pedestal of the retardation signal, measured after the recovery of the Ca^{2+} signal, did summate during repetitive stimulation (Fig. 6*A*). The summation of the pedestal was suppressed by 10 and 20 mM-BDM, as was the undershoot of the recovery phase of the retardation signal (Fig. 6*B* and *C*). Similar findings were observed in three other fibres.

Effect of BDM on myoplasmic Ca^{2+} signals elicited by voltage-clamped pulses

The effect of BDM on the Ca^{2+} signal was also studied in voltage-clamped fibres. Figure 7 shows the effect of 10 mM-BDM on the Ca^{2+} signal recorded near threshold. Panel *A* shows the corrected 720 nm traces in the absence (thin traces) and in the presence (thick traces) of 10 mM-BDM. At −66 mV (bottom pair), the peak of the Ca^{2+} signal was larger with 10 mM-BDM, whereas at −57 mV (middle pair), it was smaller with BDM. The subsequent oscillations at −57 mV, routinely observed in

control fibres (Maylie *et al.* 1987*b*), were also abolished by BDM. The peaks of the Ca^{2+} signals from these traces (and other not shown) are plotted as a function of membrane potential in Fig. 7*B*. Ten millimolar BDM shifted the voltage threshold for the first detectable Ca^{2+} transient by 3–5 mV to more negative potentials. The

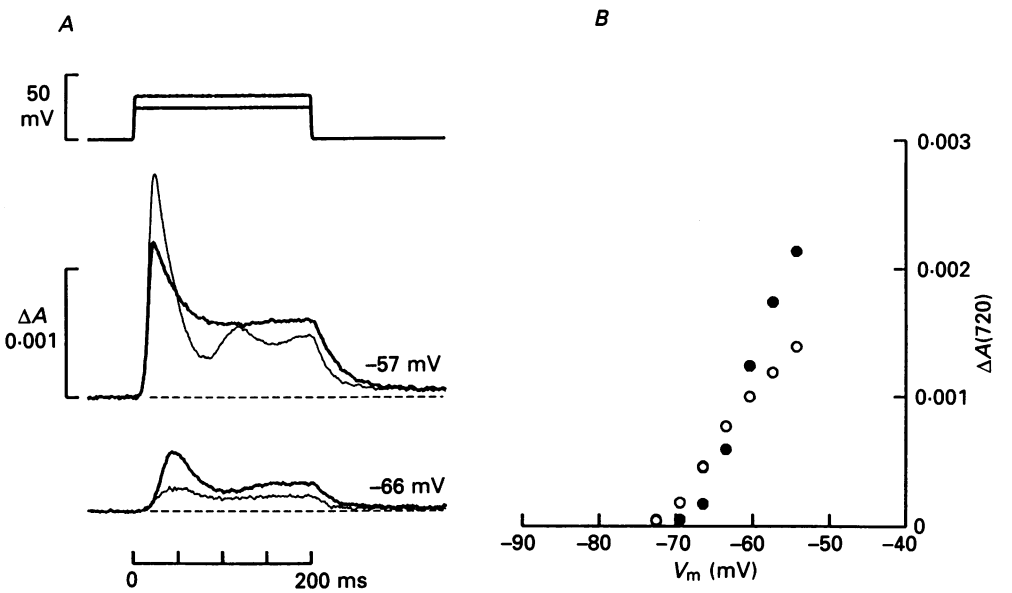

Fig. 7. Effect of BDM on the Ca^{2+}–indicator signal recorded near threshold under voltage clamp. *A*, top traces show two depolarizing voltage pulses from a holding potential of −90 mV to −57 and −66 mV. The command pulse was rounded with a 0·5 ms time constant. The corrected 720 nm traces recorded at −57 and −66 mV are shown in the middle and at the bottom, respectively. Thin and thick traces were recorded without and with 10 mM-BDM in the TEA solution, respectively. The control records were taken 73–82 min after saponin treatment of the end-pool segments and the Antipyrylazo III concentration was between 0·80 and 0·87 mM. The records in BDM were taken 88–92 min after saponin treatment of the end-pool segments and the Antipyrylazo III concentration was between 0·87 and 0·92 mM. All signals were digitized at 2·5 kHz after being filtered by 0·625 kHz eight-pole Bessel filters; the 810 nm trace was additionally filtered with a 0·1 kHz Gaussian digital filter. *B*, plot of the peak of the corrected 720 nm signal as a function of the potential during the pulse. Filled and open circles correspond to the values in the absence and in the presence of BDM, respectively. Fibre identification 64191; sarcomere length 4·1 μm; diameter 78 μm; temperature 17·4 °C; gap factor 0·99.

steepness of the voltage dependence of the peak Ca^{2+} transient near threshold was also reduced by 10 mM-BDM, with the e-fold factor increasing from 2·4 mV in control to 3·4 mV in 10 mM-BDM.

The effect of BDM on the Ca^{2+} signal elicited by larger depolarizing pulses was examined in three fibres. Figure 8 shows a representative experiment in which 20 mM-BDM greatly affected the waveform of the Ca^{2+} signal associated with a depolarization to −30 mV. The early peak of the Ca^{2+} signal in the absence of BDM was suppressed and the Ca^{2+} signal rose monotonically to a plateau that was approximately double that observed without BDM. BDM slowed the rate of rise of

the Ca^{2+} signal and the rate of decay to the baseline following repolarization. In this fibre, the time constant of recovery of the Ca^{2+} signal following repolarization was ~14 ms in control and ~25 ms in BDM.

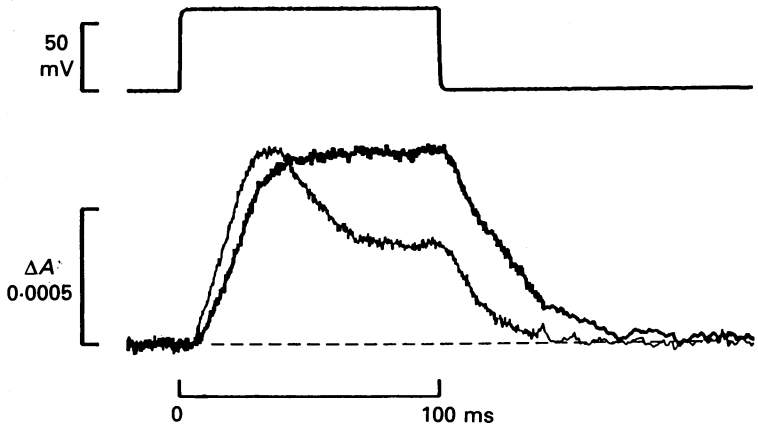

Fig. 8. Effect of BDM on the Ca^{2+}–indicator signal recorded at −30 mV under voltage clamp. Top trace shows the depolarizing voltage pulse from a holding potential of −90 mV to −30 mV. Bottom pair of traces show the corrected $\Delta A(720)$ signals in the absence (thin trace) and presence of 20 mM-BDM (thick trace). The control record was taken 123 min after saponin treatment of the end-pool segments and the Antipyrylazo III concentration was 0·45 mM. The record in BDM was taken 7 min later and the Antipyrylazo III concentration was 0·45 mM. All signals were digitized at 2·5 kHz after being filtered by 0·625 kHz eight-pole Bessel filters. The 810 nm traces were additionally filtered with a 0·1 kHz Gaussian digital filter. Fibre identification 66241; sarcomere length 4·1 μm; diameter 90 μm; temperature 15·3 °C; gap factor 0·99.

DISCUSSION

Calcium signals in frog skeletal muscle have been studied in both single intact fibres (Baylor *et al.* 1982*a*; Baylor, Quinta-Ferreira & Hui, 1983) and cut fibres (Kovacs *et al.* 1983; Maylie *et al.* 1987*a*, *b*). The cut-fibre preparation was chosen for this study primarily for the ease of dissection and indicator application. This preparation, however, is not without complications. Maylie *et al.* (1987*b*) reported that the Ca^{2+} signal is stable in a freshly cut fibre, but broadens monotonically as a function of time in the later phase of the experiment. It is easy to imagine that the fibres could be losing some vital endogenous regulatory constituents through the saponized membranes of the muscle segments in the end-pools. Hence, in comparing the results from this paper with those in the preceding paper (Hui & Maylie, 1991) readers should be aware of the differences in fibre preparation in some of the experiments.

Effect of BDM on the Ca^{2+} signal

Figure 2 shows that BDM suppressed and broadened the Ca^{2+} signal slightly. To our knowledge, the effect of BDM on the Ca^{2+} signal monitored with metallochromic indicators has not been studied. Horiuti, Higuchi, Umazume, Konishi, Okazaki &

Kurihara (1988) studied the effect of BDM on the Ca^{2+} transient in frog skeletal muscle with aequorin. Because of the difference in characteristics between the Ca^{2+}–Antipyrylazo III reaction and the Ca^{2+}–aequorin reaction, it is difficult to compare the effect of BDM on the kinetics of the Ca^{2+} signal when different indicators are used. Nonetheless, they concluded that BDM had minimal effect on the Ca^{2+} transient which agreed qualitatively with the results here. Fryer, Gage, Neering, Dulhunty & Lamb (1988) and Fryer, Neering & Stephenson (1988) also studied the effect of BDM on the Ca^{2+} signal in rat skeletal muscle with aequorin. They observed a larger suppression of the aequorin signal in rat muscle than that of the Antipyrylazo III signal reported in this paper or of the aequorin signal reported by Horiuti *et al.* (1988) in frog muscle. Whether this difference can be attributed to the difference in animals remains to be clarified.

Effect of BDM on the retardation signal

Suarez-Kurtz & Parker (1977) first suggested that the retardation signal in skeletal muscle is the consequence of the myoplasmic free Ca^{2+} transient. This idea was supported by the finding that the time-to-half-peak of the retardation signal following action potential stimulation lags that of the Ca^{2+} signal by 0·69 ms (Maylie *et al.* 1987*b*). It is thus likely that the binding of Ca^{2+} to some myoplasmic factor gives rise to a change in retardation.

The shapes of the retardation and Ca^{2+} signals are also different. The retardation signal following action potential stimulation in a highly stretched fibre appears to contain at least three components: an early transient component having a time course similar to the Ca^{2+} signal, a delayed transient component having a reversed polarity and a time course similar to the Arsenazo III dichroic signal (Baylor *et al.* 1982*b*), and a maintained pedestal (Fig. 5). BDM appeared to suppress the delayed transient component, unmasking the decay phase of the early transient component. This was accompanied by a broadening of the Ca^{2+} signal and a reduction in the peak of the Ca^{2+} signal. In the presence of BDM, the retardation and Ca^{2+} signals decayed with similar time courses (Fig. 5).

An intriguing possibility for the action of BDM is that it reduces SR Ca^{2+} uptake, thereby slowing the rate of relaxation and decreasing the amount of Ca^{2+} available for release in the SR. In support of this possibility, Horiuti *et al.* (1988) showed that SR loading of Ca^{2+} in skinned frog skeletal fibres was reduced to half of control by 10 mM-BDM. Such a possibility would explain partially the much greater decrease in the final levels of Ca^{2+} during repetitive stimulation compared to the decrease in peak Ca^{2+} following a single action potential (Fig. 6). One interpretation of the delayed transient component of the retardation signal that is suppressed by BDM is that this component reflects a change in the orientation of the SR ATPase protein following Ca^{2+} binding, as suggested by Oetliker (1982).

Modelling

To further study the effect of BDM on the Ca^{2+} release waveform and to investigate the possibility that SR Ca^{2+} uptake is reduced by BDM, the net flux of Ca^{2+} from the SR, release minus uptake, was computed according to the procedure described by Baylor *et al.* (1983) and Maylie *et al.* (1987*b*). The procedure utilized the

free [Ca^{2+}] transient derived from the measured absorbance change to compute the time course and the extent of Ca^{2+} binding to the major myoplasmic Ca^{2+} buffers, including the Ca^{2+}-regulatory sites on troponin, the Ca^{2+}–Mg^{2+} binding sites on parvalbumin and the Ca^{2+} indicator. The time course of the total myoplasmic Ca^{2+} is

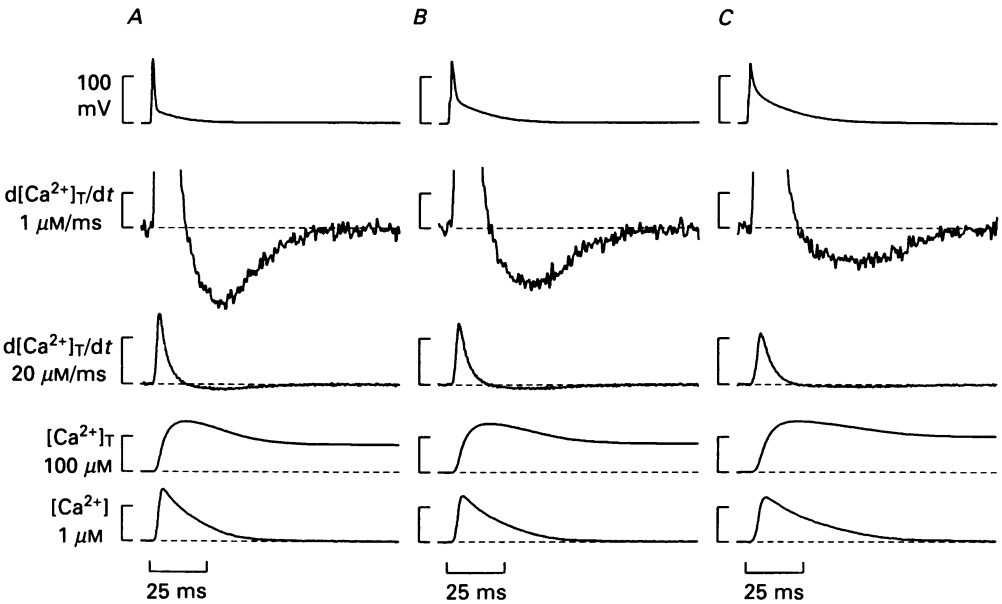

Fig. 9. Effect of BDM on the SR Ca^{2+} release estimated from modelling of Ca^{2+} movements in the myoplasm after one action potential. *A*, control; *B*, with 10 mM-BDM, and *C*, with 20 mM-BDM. In each panel, the top trace shows the action potential. The free [Ca^{2+}] (bottom trace) was calculated from the $\Delta A(720)$ signal (Maylie *et al.* 1987*b*), assuming a stoichiometry of 1:2 for the Ca^{2+}–Antipyrylazo III complex and that all the indicator was available to react with Ca^{2+}. The apparent K_D for Ca^{2+} with 1 mM [Mg^{2+}] in the internal solution was taken as $3{\cdot}4 \times 10^{-8}$ M^2 and the resting level of free Ca^{2+} was set to 0·02 μM. The calculation of net total Ca^{2+} released (4th trace labelled $[Ca^{2+}]_T$) was based on model 2 of Baylor *et al.* (1983) with free [Mg^{2+}] set at 1 mM; the myoplasmic concentration of Ca^{2+}-regulatory sites on troponin was taken as 240 μM, the K_D as 2 μM and the backward rate constant for Ca^{2+} as 115 s^{-1}; the concentration of Ca^{2+}–Mg^{2+} sites on parvalbumin was taken as 1 mM, the K_D for Ca^{2+} as 4 nM (forward rate constant $1{\cdot}25 \times 10^8$ M^{-1} s^{-1} and backward rate constant 0·5 s^{-1}) and K_D for Mg^{2+} as 91 μM (forward rate constant $3{\cdot}3 \times 10^4$ M^{-1} s^{-1} and backward rate constant 3·0 s^{-1}). The net SR release signal, given by the time derivative of the $[Ca^{2+}]_T$ trace, is shown in the 3rd trace and at an increased gain in the 2nd trace to show the negative deflection. Same experiment as in Fig. 2.

given by the sum of the free [Ca^{2+}] transient and [Ca^{2+}] bound to troponin, parvalbumin and the indicator. The net flux of Ca^{2+} from the SR minus uptake is given by the time derivative of the computed total myoplasmic Ca^{2+} waveform.

Figure 9 shows the effects of 10 and 20 mM-BDM on the computed SR release signal (third trace in each panel). The release signal is characterized by two phases: the initial rapid positive phase corresponding to the net movement of Ca^{2+} from the SR into the myoplasm which represents the release of SR Ca^{2+} and a small negative phase corresponding to a net movement of Ca^{2+} out of the myoplasm which, under

TABLE 2. Effects of various concentrations of BDM on the $\Delta[Ca^{2+}]$ and Ca^{2+} release waveforms in cut fibres under the assumption that all indicator molecules can react with Ca^{2+}

		$\Delta[Ca^{2+}]$ waveform			Ca^{2+} release waveform			
Fibre reference	[BDM] (mM)	Peak (%)	$t_{p/2}$ (ms)	$\Delta T_{1/2}$ (%)	Peak (%)	$t_{p/2}$ (ms)	$\Delta T_{1/2}$ (%)	Minimum (%)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
63251	20	75	1·17	153	75	1·11	—	—
	10	90	0·61	136	89	0·53	—	—
63252	10	73	0·37	111	78	0·23	122	71
63271	5	83	0·05	101	87	0·08	101	90
	10	90	0·37	115	88	0·28	112	82
	20	80	1·52	154	72	1·34	145	58
66061	5	82	0·06	99	87	0·05	—	—
	10	81	0·41	103	83	0·38	—	—
66062	20	44	2·66	117	43	2·78	139	66
66191	20	30	1·32	145	33	1·08	192	72
	10	65	0·58	93	69	0·65	114	86
66201	10	73	1·25	105	76	0·33	105	102
	20	58	0·82	128	61	0·73	122	48
66202	10	71	0·19	105	74	0·18	114	76
	20	64	0·82	122	65	0·79	126	57
66211	10	75	0·20	105	76	0·15	113	76
	20	66	1·03	156	61	0·88	149	26
66212	20	48	1·68	112	51	1·61	140	80
66231	20	18	0·48	135	22	0·25	162	28
	10	51	0·60	115	52	0·38	146	38
66242	20	39	1·89	119	38	1·63	—	—
	5	76	0·08	89	79	0·11	—	—
	2	84	−0·10	88	88	−0·05	—	—
	10	66	0·45	99	70	0·45	—	—
Mean	5	80	0·06	96	84	0·08	101	90
S.E.M.		2	0·01	4	3	0·02	—	—
	10	74	0·47	109	75	0·35	118	76
		4	0·10	4	3	0·05	5	7
	20	52	1·34	134	52	1·22	147	54
		6	0·20	5	6	0·22	8	7

Columns (1) and (2) give the fibre references and the BDM concentrations. For each fibre the rows are arranged in the order the concentrations of BDM were applied. From the Ca^{2+}–indicator signals in the absence and in the presence of BDM, the free $\Delta[Ca^{2+}]$ and the Ca^{2+} release waveforms were computed as described in the text. Columns (3)–(5) show the effect of a particular concentration of BDM on the free $\Delta[Ca^{2+}]$ waveform and columns (6)–(9) show the effect on the Ca^{2+} release waveform. For peak amplitude, half-width ($\Delta T_{1/2}$) and negative peak amplitude (minimum), the changes are expressed in percentages of control. For time to half-peak ($t_{p/2}$), the change is expressed as relative increase in milliseconds with respect to the control value. Same fibres as in Table 1.

the constraints of the model, represents SR uptake (enlarged in the second trace). BDM decreased the peak of the initial release and increased the duration or half-width of release. It also suppressed the negative phase of the release signal, i.e. uptake. The changes in the values of these parameters from this and other experiments (not shown) are listed in Table 2. On average, 10 and 20 mM-BDM reduced the peak release to 75 and 52%, prolonged the half-width of the release to 118 and 147%, and reduced the peak uptake to 76 and 54% of control values, respectively.

The results in Fig. 9 and Table 2 were obtained under the assumption that all indicator molecules can react with Ca^{2+}. We have repeated the calculations by assuming that only freely diffusible indicator molecules can react with Ca^{2+}, similar to the analysis of Maylie *et al.* (1987*b*). The absolute values of the parameters describing the $\Delta[Ca^{2+}]$ and Ca^{2+} release waveforms were somewhat altered, but the relative values of the parameters, i.e. the value in the presence of BDM divided by the control value, remained practically unchanged (data not shown). Hence, under the constraints of the model used, it is clear that BDM affects both release and uptake. What is not clear is whether the effect of BDM on release is the result of decreased loading of the SR or a direct effect on the SR release channel, in addition to its effect on the voltage sensor (Hui & Maylie, 1991).

Usefulness of BDM as a contraction blocker

We have shown multiple actions of BDM on different steps in excitation–contraction coupling leading to tension generation. However, the effects of BDM on the action potential, charge movement and the Ca^{2+} signal were much smaller than its effect on twitch tension. The effect on the action potential can be avoided in experiments in which signals such as charge movement and the Ca^{2+} signal are elicited by depolarizing pulses under voltage-clamp conditions. In this case, the effect of BDM on charge movement or the Ca^{2+} signal was found to be minimal, as long as the concentration of BDM was kept at < 10 mM. It is thus reasonable to conclude that BDM might still be a useful contraction blocker for studying the early steps in excitation–contraction coupling. An optimal concentration to minimize movement artifact is probably around a few millimolar when coupled with moderate stretch of the fibre. This could be particularly valuable when other means for blocking contraction cannot be applied.

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