Detachment of low-force bridges contributes to the rapid tension transients of skinned rabbit skeletal muscle fibres

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1. To probe the cross-bridge cycle and to learn more about the cardioplegic agent BDM (2,3-butanedione monoxime), its effects on the force–velocity properties and tension transients of skinned rabbit muscle fibres were studied at 1–2 °C and pH 7·0.

2. Three millimolar BDM decreased isometric force by 50%, velocity by 29%, maximum power by 73%, and stiffness by 25%, so that the relative stiffness (stiffness/force ratio) increased by 50% compared with reference conditions in the absence of BDM.

3. Tension transients obtained under the reference condition (0 BDM) could be represented by three components whose instantaneous stiffness accounted for the initial (Phase 1) force deviation and whose exponential recoveries caused the rapid, partial (Phase 2) force recovery following the step. The fastest component had non-linear extension–force properties that accounted for about half the isometric stiffness and it recovered fully. The two slower components had linear extension–force properties that together accounted for the other half of the sarcomere stiffness. These components recovered only partially following the step, producing the intermediate (T_2) level which the force approached during Phase 2.

4. Matching the force transients obtained under test conditions (3 mM BDM) required three alterations: (1) reducing the amplitude of the two slower components by 50%, in proportion to isometric force, (2) adding a non-relaxing component and (3) decreasing the amplitude of the rapidly recovering component by 12·5% so that its relative amplitude (amplitude/isometric force) was increased by 75%. The non-recovering component and the increase in relative amplitude of the rapid component were responsible for the increase in relative stiffness of the fibres produced by BDM. The rapidly recovering component had the same time constant and step-size-dependent recovery rates as the fastest of the three mono-exponential components isolated from the tension transient response under the reference condition. BDM therefore appeared to augment the fastest component of the tension transient under the reference condition.

5. The results suggest that BDM detains cross-bridges in low-force, attached states. Since these bridges are attached, they contribute to sarcomere stiffness. Since they are detained, relaxation or reversal of their immediate responses is probably due to bridge detachment rather than to their undergoing the power stroke. The observation that a portion of the test response matched the fastest component of the reference response when the amplitude of the fastest component was increased suggests that a part of the normal rapid, transient tension recovery following a release step is due to detachment of low-force bridges moved to negative-force positions by the step.

BDM (2,3-butanedione monoxime) was used to probe the cross-bridge cycle of skinned rabbit muscle fibres. It has been shown that BDM decreases isometric force by inhibiting myosin attachment to actin (Horiuti, Higuchi, Umazume, Konishi, Okazaki & Kurihara, 1988; Higuchi & Takemori, 1989; Hui & Maylie, 1991). Although BDM also inhibits excitation–contraction coupling (Edery, 1959; Wiggins, Reiser, Fitzpatrick & Bergey, 1980; Fryer, Neering & Stephenson, 1988; Fryer, Gage, Neering, Dulhunty & Lamb, 1988; Gwathmey, Hajjar & Solaro, 1991), force can be inhibited in intact fibres with concentrations of BDM so low as to have almost no effect on

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the resting membrane potential, the action potential (Horiuti *et al.* 1988; Hui & Maylie, 1991), or the calcium transient (Blanchard, Smith, Allen & Alpert, 1990; Steel & Smith, 1993). Thus, BDM can be used to probe the cross-bridge cycle of the intact muscle. Because its effects are reversible, BDM is also a useful cardioplegic agent (Mulieri, Hasenfuss, Ittleman, Blanchard & Alpert, 1989).

Recent studies of the effects of BDM on actomyosin interactions in skeletal muscle suggest that the oxime detains cross-bridges in low-force states (Herrmann, Wray, Travers & Barman, 1992; Bagni, Cecchi, Colomo & Garzella, 1992; Zhao & Kawai, 1994; Zhao, Naber & Cooke, 1995; Regnier, Morris & Homsher, 1995). The present study supports this conclusion and further shows that the detained bridges respond to a sudden change of length with an exaggeration of the response obtained in the absence of BDM. It therefore suggests that part of the transient tension response to a step change of sarcomere length is not due to the movements of attached cross-bridges first described by Huxley & Simmons (1971), but to rapid detachment of bridges from an initially attached, low-force state first postulated by Huxley (1973).

METHODS

The methods were identical to those used in our earlier study (Seow & Ford, 1993) with minor modifications. One modification was that the ends of the fibres were fixed with glutaraldehyde using the method of Chase & Kushmerick (1988) before the fibres were clipped. Another was the use of a 15 kHz force transducer. This transducer was similar to our earlier photoelectric transducer (Chiu, Asayama & Ford, 1982) except that the phototransistors were replaced with a dual photodiode (part no. 3367; Hamumatsu Photonics, Hamumatsu City, Japan). The reduced noise in these photosensors allowed the stiffness of the moving element to be increased substantially without a decrease in the signal-to-noise ratio. Details of these modifications have been described previously (Seow & Ford, 1997).

Preparation

Muscle tissue was obtained from the carcasses of animals used in other experiments. Adult New Zealand White rabbits of either sex weighing 2–3 kg were pre-anaesthetized with 5 mg kg^{-1} xylazine and 0·2 mg kg^{-1} atropine. After 10 min they were anaesthetized with 30–50 mg kg^{-1} ketamine and 1 mg kg^{-1} acepromazine. Tracheostomy was performed after anaesthesia was achieved and the animals were ventilated with room air at a respiratory rate of 43 breaths min^{-1} and tidal volume of 25–30 ml until they were killed by removal of their hearts, which were used in other experiments. Bundles of muscle fibres were then dissected from the psoas muscle, skinned, and prepared for experiments as previously described (Seow & Ford, 1997).

Solutions

Solution composition was calculated using a computer program kindly given to us by Dr R. E. Godt and using stability constants previously compiled by Godt (1974), Godt & Lindly (1982), Godt & Maughan (1988), Godt & Nosek (1989) and Andrews, Maughan, Nosek & Godt (1991). All solutions contained 5 mM MgATP, 1 mM free Mg^{2+}, 20 mM creatine phosphate, 10 mM imidazole, 56 g l^{-1} Dextran T-70, and sufficient potassium propionate to yield an ionic strength of 210 mM. Relaxing solution contained 5 mM EGTA. Activating solution contained calcium buffered with 5 mM EGTA to pCa 4·5, a level shown to produce full activation in both the presence and absence of BDM (Higuchi & Takemori, 1989). A rinse solution containing 0·1 mM EGTA was used to lower the EGTA concentration immediately before each activation. The pH of the solutions was adjusted to 7·0 at the temperature used, 1–2 °C.

Protocols

Fibres were activated five to nine times, alternately in the presence and absence of BDM, conditions termed 'test' and 'reference' respectively. Two types of experiments were done. In the first type, steadily contracting fibres were released to nine different isotonic loads in each activation and a single force–velocity point was determined from each release, as described previously (Seow & Ford, 1992). In these experiments the test conditions were always bracketed by reference conditions, so that each series began and ended with a reference activation. All the data points obtained with each condition, test or reference, for a single fibre were grouped together and fitted with the Hill (1938) hyperbola. Differences in the fitted parameters of the hyperbola were used to describe changes in the steady-state contractile properties. For analytical purposes, the test parameters from each fibre were divided by the reference parameters in the same fibre to determine the relative effect of the test condition in that fibre.

In the second type of experiment the steadily contracting fibres were subjected to sudden changes of sarcomere length, using sarcomere length detector and servomotor capable of imposing steps complete within 200–250 μs. Again, five to nine activations, alternating test and reference conditions, were studied in each fibre and nine length steps were applied in each activation. Unlike the isotonic experiments in which the reference conditions bracketed the test conditions, the test and reference conditions were studied in sequential pairs. When both members of a pair of steps met the criteria for inclusion (described below), both were included for signal averaging and analysis. The analysis consisted of comparisons of the signal-averaged records obtained under the test and reference conditions.

In both types of experiments the fibres were subjected to a sudden shortening at the end of each step. This sudden shortening had two purposes: (1) to zero the force transducer and (2) to provide a period of unloaded shortening from which the fibre was rapidly restretched, to maintain sarcomere homogeneity (Brenner, 1983).

Calibration of the sarcomere length sensor

The accuracy of the sarcomere length detector was affected by variable amounts of background light scattered by the fibres and possibly other factors. To account for this variability, the detector was calibrated using a functional test at the beginning of each activation used for tension transients. The muscle was released to an isotonic load of about 20% of isometric force and the ratio of the sarcomere length change to the overall muscle length change (S/L ratio) was determined over a 75 ms period beginning 75 ms after the release, a period when the damped series elastic recoil has been shown to have died away (Seow & Ford, 1992). This calibration factor was used to determine the size of the subsequent sarcomere length steps in that activation; the measured change in sarcomere length was divided by the S/L ratio.

Criteria for data inclusion

Our prior force–velocity experiments have shown substantial scatter in the velocity measurements made at isotonic loads below

0·01 mN, equivalent to 1–3% of isometric force (Ford, Nakagawa, Desper & Seow, 1991; Seow & Ford, 1993). To reduce variation in the fitted curves, force–velocity points were excluded when the isotonic force was less than the minimum acceptable value. The force–velocity data presented below were derived from 1846 separate data points obtained from 209 activations of 25 fibres. An additional 35 data values (1·9%) were excluded because the isotonic force fell below 0·01 mN. No force–velocity data were excluded for any other reason.

The criteria for selection of records for inclusion in the analysis of the tension transients was done in two parts. Pairs of sequential activations under test and reference conditions were accepted only if the S/L ratios for both activations differed by less than 2%. If a pair of activations met this criteria, the individual records were examined for speed of step and glitches. If one member of the pair was found to have an unacceptably slow step or a large glitch, both members were excluded. The results from a fibre were included only if at least one pair of records for each step size was available for averaging. The data presented were obtained from 666 pairs of steps, in 74 pairs of activations, in 30 fibres. An additional 17 pairs of steps (2·6%) were excluded because the records from one or both members of the pair were judged deficient.

Mathematical description of the transient responses

Exact descriptions of the force responses to shortening steps were obtained using a three-step process extended from a method developed by Ford, Huxley & Simmons (1977). In the first step, a mathematical description of the reference records was obtained by dividing the responses into several components. The rapid components of force recovery were separated from the slower components of the response by fitting a straight line to an inflection in force recovery (Phase 3 of Huxley & Simmons, 1973). The value of line at each time, $T_a(t)$, was defined as:

$$T_a(t) = T_a(0) + kt, \quad (1)$$

where t is time after the mid-point of the step, k is the slope and $T_a(0)$ is the value of the line at $t = 0$. The components of the recovery, obtained by subtracting the early parts of the record (Phase 2) from the fitted line, were scaled in both the amplitude and time dimensions to make them superimpose in a 'standard curve' that was fitted with three exponentials. The amplitude, A_i, and rate constant, B_i, of each component were used in later reconstructions of the records. The time scaling factor was defined as the inverse of their initial half-time, $1/t'_{½}$, for each stripped record. To obtain the rate constant of recovery for the separate processes at each length, the interpolated value of this scale factor was multiplied by the rate constants of the standard curve. The values of A_i were assumed to be invariant for each condition studied.

In the second step of the process, the procedure was reversed to reproduce the reference response. The instantaneous response of each component was calculated using a fourth-order Runge-Kutta integration of each exponential and the digitized sarcomere length record as the driving function. The rate of change of the instantaneous tension deviation from isometric value in each component (T_i) was defined by:

$$dT_i/dt = S_i \times dy/dt - (B_i/t'_{½}) \times (T_i - (A_i \times T_{pre} - T_{ai}(t))), \quad (2)$$

where y is the deviation of half-sarcomere length from its isometric position, S_i is the slope of the stress–strain relationship for the ith component at each length, T_{pre} is the isometric force before the step, and $T_{ai}(t)$ is the intermediate force level approached by the ith component during the rapid, early force recovery. The S_i values were initially determined as a constant sarcomere stiffness multiplied by A_i. The value of $T_{ai}(t)$ was defined by:

$$T_{ai}(t) = T_{ai}(0) + k_i t. \quad (3)$$

Initially the values of $T_{ai}(0)$ and k_i were apportioned among the values of $T_a(0)$ and k according to the relative amplitudes of the separate components, A_i. The values of $T_a(0)$, k and $t'_{½}$ at each instantaneous length were interpolated from the values measured after the end of the different sizes of step. The computer program that solved eqn (2) summed the responses for the individual components and superimposed this response on the experimental records. The individual parameters of the separate components could then be adjusted to obtain the best match to the reference records, as judged by eye.

The third step in the process used the same program to make adjustments to the parameters of the reference responses to superimpose them on the records obtained under test conditions. The values of S_i, $T_{ai}(0)$, and k_i were allowed to vary independently at this stage of curve fitting. The matches were judged by eye and the change in parameters was used to define the alteration caused by the test intervention.

Signal averaging

The tension transient experiments were analysed both by comparing the digitized records directly and by comparing the parameters of mathematical functions fitted to the records. To allow finer distinctions in these comparisons noise was reduced by signal averaging. When more than one pair of steps of a given size from a single fibre were found acceptable for inclusion, the individual records for the same condition and step size were first signal averaged and this average record was included as a single record in the final average.

The records obtained from thirty fibres comprise the data set for the tension transient experiments. This large number of fibres, which greatly reduced the noise in the averaged records, was derived from the inclusion of the control experiments described immediately below.

Control observations

Lack of effect of overstretch. Homogeneity of sarcomere spacing was maintained in these experiments by the Brenner (1983) protocol of rapidly shortening and restretching the fibres. The results presented below show that there was an extra component of relative stiffness in the presence of BDM, as manifest by a greater change in force relative to the isometric force during the step, and that some of the extra force change decayed rapidly following a step. This observation raised the question of whether the rapid restretch used in the Brenner protocol resulted in long-lasting, highly stretched bridges that contributed to the initial stiffness and then contributed to the rapid force recovery by detaching following a release. To test this possibility, the effects of BDM were measured in two sets of fifteen fibres each. In the first set (non-overstretched), the fibre was stretched back to the original isometric length after a brief period of rapid shortening (Brenner, 1983). The protocol for the second set was identical to the first except that the fibres were subjected to a brief overstretch. The overstretch and the subsequent release back to the original isometric length was equivalent to 20 nm per half-sarcomere, a distance that is likely to require all bridges to shorten beyond their normal range of attached motion when settling to the final isometric length. The overstretch lasted 5 ms.

No significant difference was found between the two protocols. The two sets of data were therefore signal-averaged together to examine the effects of BDM on the transients. The resulting decrease in noise permitted a more precise examination.

RESULTS

Effects of BDM

Steady-state force–velocity properties

All of the results presented here were obtained with series of five to nine activations at sarcomere length 2·5 μm, alternating test conditions in the presence of BDM with reference conditions in its absence. Thus, the differences between the two represent the fully reversible effects of BDM. The effect of different concentrations of BDM was first assessed to determine a concentration suitable for tension transient measurement. As shown in Fig. 1, isometric force declined with increasing BDM concentration, reaching 50% of reference at 3 mM. A similar dependence of force on BDM concentration has been reported previously (Freyer *et al.* 1988; Horiuti *et al.* 1988; Higuchi & Takemori, 1989; Regnier *et al.* 1995; Zhao *et al.* 1995). The effect of BDM has been found to vary among preparations (Freyer *et al.* 1988), and is greater at low temperatures (Higuchi & Takemori, 1989). The results shown in Fig. 1 are very similar to those of Higuchi & Takemori (1989) for rabbit psoas fibres at 1–2 °C.

Figure 1 also shows the effects of 1 and 3 mM BDM on isometric force, maximum shortening velocity, maximum power and stiffness. The values of the reference parameters are listed in the figure legend. As shown, 3 mM BDM decreased maximum shortening velocity by 29%, maximum power by 73%, isometric force by 50%, and stiffness by 25%, so that the stiffness/force ratio was increased by 50%. As discussed below, these results suggest that BDM inhibits a transition from a low-force attached cross-bridge state and thereby detains a fraction of the attached bridges in low-force states. This conclusion leads to the rationale for the analysis of the transients described below. Three millimolar BDM was used to study tension transients because the data in Fig. 1 indicated a substantial depression of cross-bridge function with adequate force remaining for mechanical studies.

Tension transients

The effects of 3 mM BDM on the transients are shown in Fig. 2. The test and reference records for each size of step were superimposed after being scaled to match their respective isometric forces. A selection of records for different step sizes are shown in the upper panels (Fig. 2*A*) and the difference (reference minus test) for all nine pairs of records is shown in the lower panels (Fig. 2*B*). The rationale for scaling and subtracting the records derives from the consideration that the overall transient is the sum of several populations of bridges, some of which are affected by the test conditions and some of which are not. If BDM detains bridges in low-force states, the isometric force would be generated by non-detained bridges that function normally. To a first approximation, therefore, the difference records shown in Fig. 2*B* should represent the response of the detained bridges, at least in the short term during and following the step.

The increased stiffness/force ratio caused by BDM is shown as a greater deviation of the force record during and immediately following the step. In the difference records (Fig. 2*B*) the increased relative stiffness is seen as a force difference that develops during the step. This difference could be divided into two distinct components, one that remained nearly constant over the entire recording period and another that relaxed rapidly with a time constant that varied directly with the size of release, in a manner similar to the remainder of the tension transient.

Mathematical description of the tension transients

Descriptions of the transients were obtained in three stages; the first used a method developed by Ford *et al.* (1977) for separating the reference records into several components,

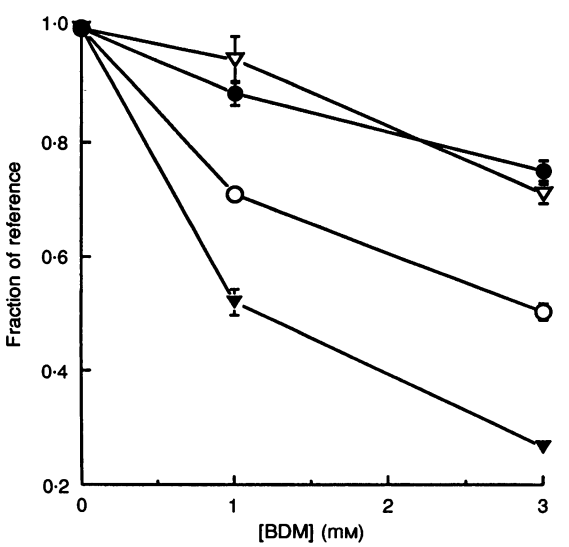

Figure 1. Maximum velocity, stiffness, isometric force and maximum power plotted against BDM concentration

The values plotted have been normalized to their reference values measured in the absence of BDM. The means of these reference values were 1·07 μm s^{-1} × half-sarcomere for maximum velocity, 0·32 T_{pre} × half-sarcomere nm^{-1} for stiffness (T_{pre} is isometric force), 176 kPa for isometric force, and 13·1 mW g^{-1} for maximum power. Values plotted are means ± S.E.M. ▽, velocity; ●, stiffness; ○, force; ▼, power.

the second recombined and modified slightly the individual components to reconstruct the reference records (Ford *et al.* 1977), and the third altered the amplitudes and rate constants of these fitted components to match the test records. Stretches were not included in this analysis both because stretch may produce instabilities of sarcomere length and because no Phase 3 inflection could be distinguished in some of the reference records following stretch, so that the early transients could not be distinguished from the later responses.

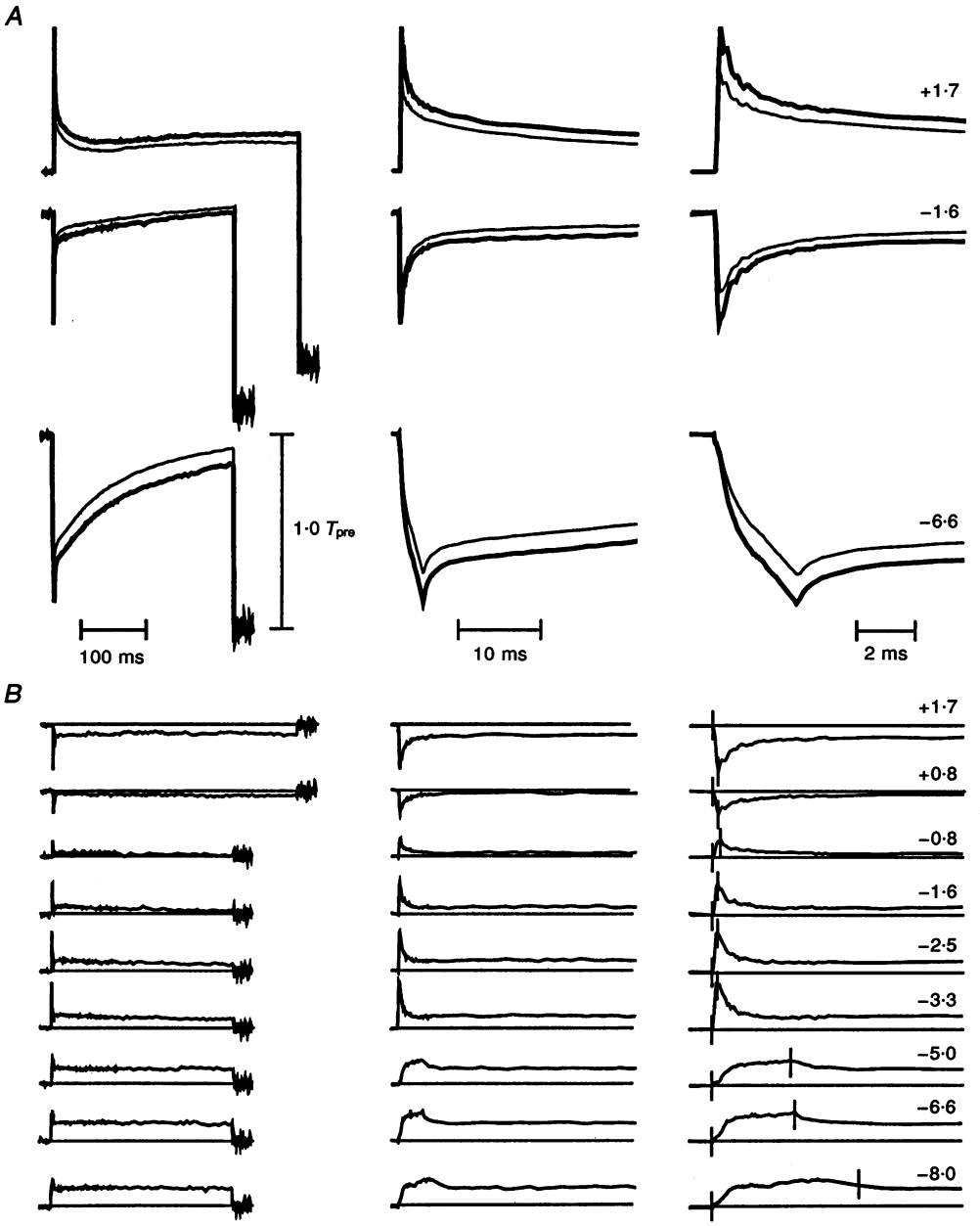

Figure 2. Tension transients in the presence and absence of BDM

A, tension transients normalized to the isometric force. Traces represent the signal averages from 30 fibres. The records obtained in the presence (thick traces showing greater force deviation from the isometric) and absence (thin traces) of 3 mM BDM are superimposed for each size of step. The traces on the left are shown on a slow time base to display the entire record. The time base in the middle and right-hand panels have been expanded to resolve the events associated with the step. The numbers above the right-hand end of the traces indicate step size (in nm per half-sarcomere). The large steps are slowed to avoid making the muscle go slack while sarcomere length was being servocontrolled. A large step in length control was applied to make the fibre go slack at the end of the recording period. A selection of three steps is shown to illustrate the general time course of the transient. *B*, difference records (reference minus test) for all nine steps in the series. The two vertical cursors on the fastest records (right-hand traces) mark, respectively, the time of onset of the step and the time near the end of the step when the extreme tension deviation was reached.

Description of the reference (0 BDM) records

For each release, the inflection in force recovery (Phase 3 of the transient) was fitted with a straight line (Fig. 3) described by eqn (1) and the rapid components of the transient were isolated by subtracting the force value at each instant from the line. These isolated rapid responses, shown in Fig. 4*A*, are difficult to distinguish because all the records cross each other; the recovery from the larger steps is both faster and larger than that from the smaller steps. The records could, however, be made to superimpose by the two-step procedure shown in Fig. 4*B* and *C*.

The amplitudes of the stripped records were first normalized to their relative values by dividing each point in the record by its theoretical maximum, T_1', determined as a linear extrapolation of the T_1 value for the largest stretch extended through the isometric point. This theoretical maximum assumes that the sarcomere stiffness is linear and that the curvature in the measured T_1 curves results from progressively greater truncation of the force response by rapid recovery during the larger releases. As shown in Fig. 4*B*, this normalization ordered the records so that they did not cross. They were then made to superimpose by scaling the time from the mid-point of step for each record (Fig. 4*C*). The time unit used to define these curves is the initial half-time, plotted as the abscissa in Fig. 4*C* and *D*, and the scaling factor for each record, described as the inverse of the initial half-time ($1/t'_{½}$), is plotted in Fig. 5*A*. A 'standard curve' describing the force recovery was obtained by averaging the superimposed records (continuous curve in Fig. 4*D*). This standard curve was then fitted with three exponentials (dotted curve in Fig. 4*D*) defined by their rate constants, B_i, and relative amplitudes, A_i. The rate constants for a standard curve having an initial half-time of 1 ms were 3·65, 0·95 and 0·32 ms^{-1}. The corresponding relative amplitudes of the three exponentials were 0·44, 0·39 and 0·17. These three exponentials served as the starting point for further analysis.

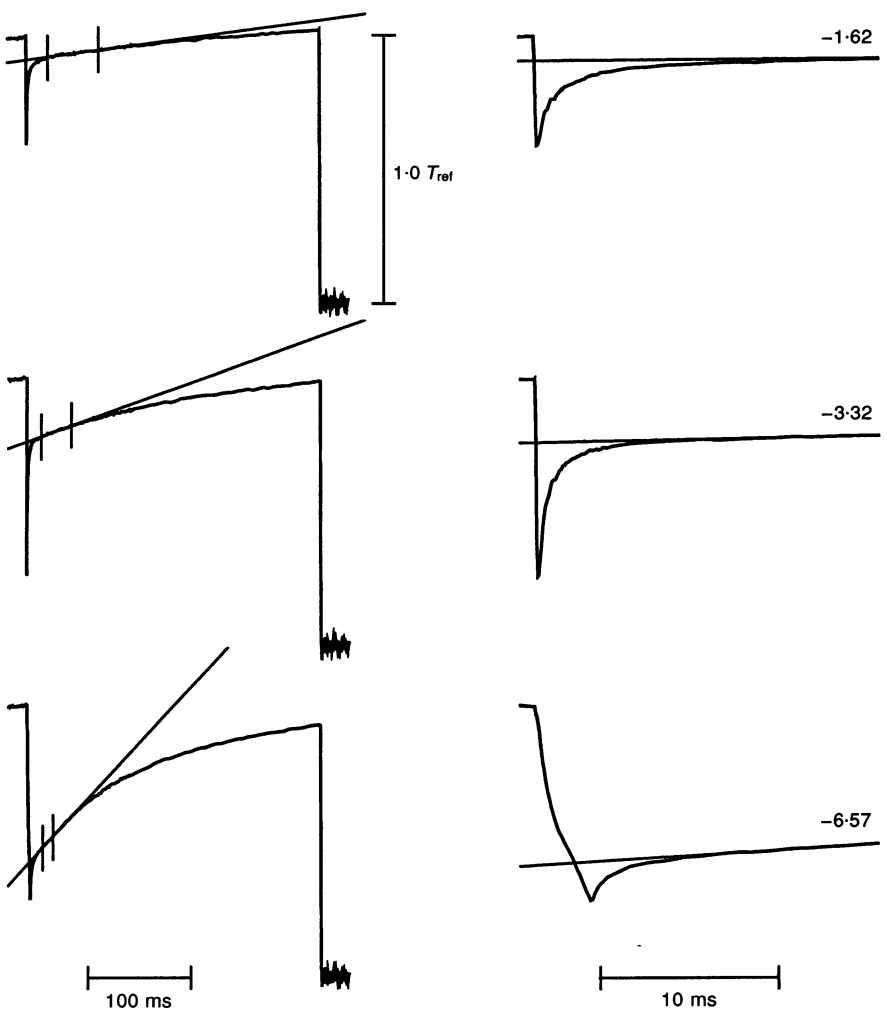

Figure 3. Isolation of the rapid transients

Isolation of the rapid transients by fitting a line to the Phase 3 inflection in the force recovery and subtracting the recorded force from this line. The value of the line at the time of the mid-point of step, $T_a(0)$, is used to indicate the T_2 level. T_{ref}, reference isometric force. The numbers above the right-hand end of the traces indicate step size (in nm per half-sarcomere).

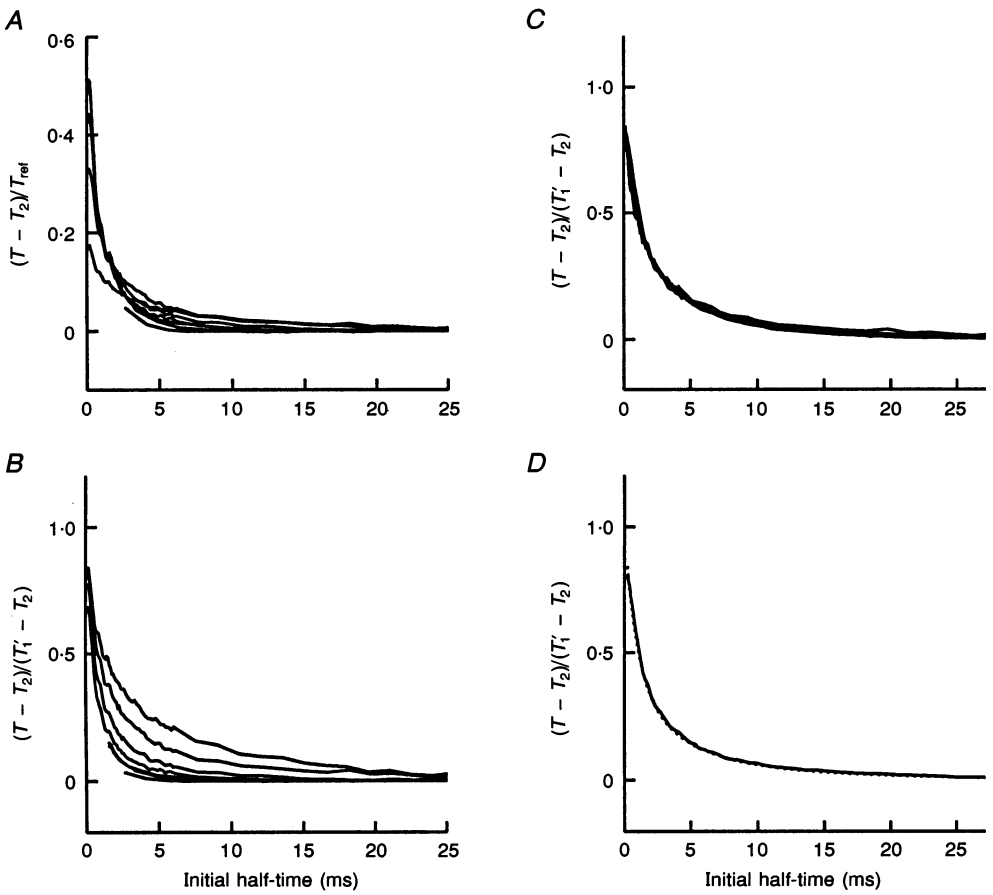

Figure 4. Derivation of the 'standard curve'

A, the difference record obtained by the subtraction of $T_a(t)$ (Fig. 3). *B*, difference record normalized to T_1'. *C*, time from mid-point of step scaled to superimpose records. Time is plotted in units of initial half-time. *D*, records averaged (continuous curve) and fitted with three exponentials (dotted curve).

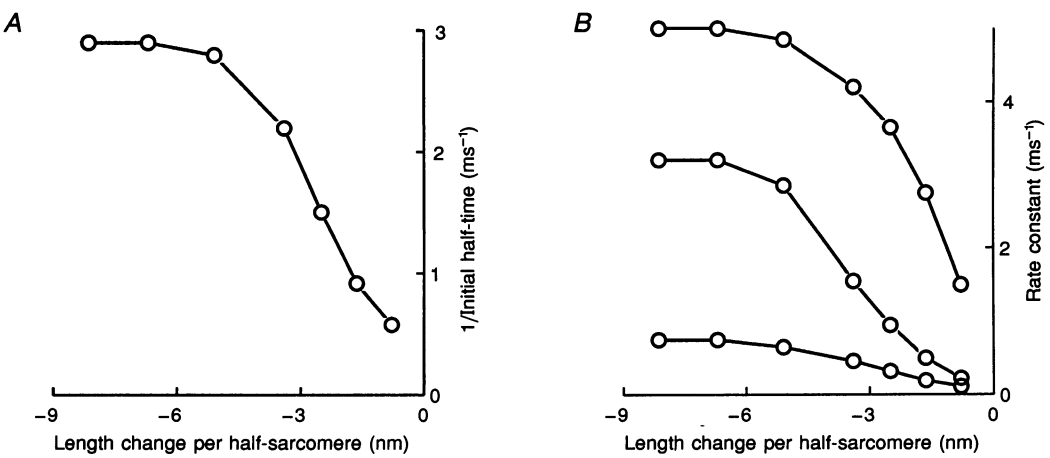

Figure 5. Scale factor and rate constants

A, the scale factor used to superimpose the records in Fig. 4*C*, expressed as the inverse of initial half-time. *B*, rate constants of the separate components used to fit records in Figs 6–8. The three curves, from top to bottom, represent the step-size-dependent rate constants of the three fitted components, from fast to slow rates of recovery.

Reconstruction of the reference record. The tension transients were calculated using a fourth-order Runge-Kutta integration of the force responses with the digitized sarcomere length records as the driving function (see Methods). Each component was described (eqn 2) as a force deviation caused by the length change and an exponential recovery towards an intermediate level. Solutions were made individually for each of the three exponential processes and summed. A computer program displayed the calculated response on the experimental record so that the parameters could be adjusted to obtain the best match.

The program assumed that a length change caused force to deviate from its isometric value according to the stiffness of the component and then recover towards an intermediate level with a time constant that varied with instantaneous deviation of sarcomere length. The stiffness (S_i) and the intermediate levels toward which force recovered (T_{ai}) and rate constants (B_i) could be adjusted separately for each component and each step. The instantaneous values at any length were calculated by interpolation between the values for the different steps. The intermediate force towards which the component recovered, T_{ai}, varied linearly with time according to eqn (3). The values of the relative amplitudes of each component, A_i, were assumed to be invariant for each condition.

As a first step in the reconstruction of the reference records the procedures used to isolate and superimpose the individual records were simply reversed. A linear stiffness was assumed for all components and both the stiffness and the $T_{ai}(0)$ values were apportioned according to the relative amplitudes, A_i, of the individual components. The parameters derived for the individual components were substituted in the model and the fits shown in Fig. 6 were obtained. As in the original description of Ford *et al.* (1977),

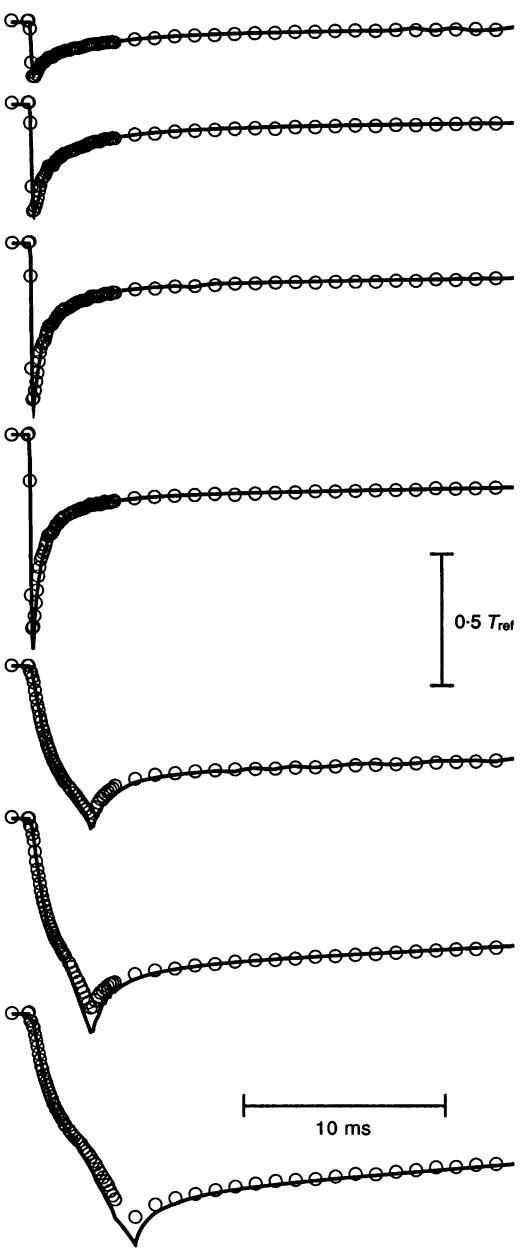

Figure 6. Fit of the reference records by reversal of the procedures shown in Figs 3–5

Radius of circles indicating the digitized data points in this and subsequent figures is equivalent to 2·5% T_{ref}. Continuous line is the calculated response.

the overall sarcomere stiffness (S) had to be increased to match the amplitudes of the deviations. The fitted curves superimposed on the reference data in Fig. 6 show that this method gave a close description of the records for the small, rapid steps. The lower records in Fig. 6 also show, however, that the calculated force would fall substantially below the experimental record following large releases. It was therefore necessary to assume some non-linearity of the stiffness.

The methods used up to this point are the same as Ford *et al.* (1977) except that: (1) only three and not four exponential components were required, (2) the relative amplitudes of the components were not readjusted and (3) none of the small corrections to the records required by inertial factors were applied. Most of the corrections in the earlier study were necessitated by the much greater inertia caused by the 4-fold greater length of the intact fibres. In the present study only the correction for the force transducer resonance would have been required, and this was not possible because of the longer digitization interval (60 instead of 20 μs) and higher frequency of the present transducer. If applied, this correction would have been no more than a few per cent of isometric force, mainly at the onset of the rapid steps.

A close match to the large, slowed steps might have been obtained by assuming a uniform non-linear stiffness for all three components, but a different approach was suggested by comparisons with the test records. These comparisons suggested that all the records could be fitted by assuming that only the component with the fastest time constant had a non-linear stiffness.

Description of the BDM records

The rate constants and amplitudes of force recovery for the difference records in Fig. 2*B* are similar to those of the fastest of the three components fitted to the transients in Fig. 7. If the difference records were to be fitted with a

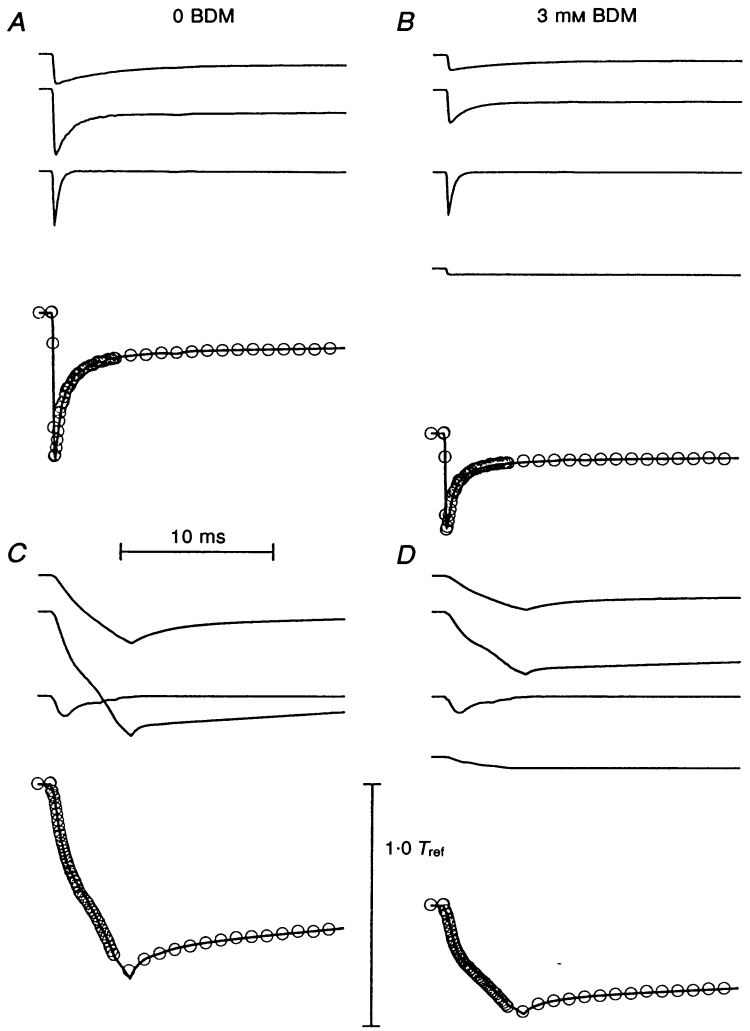

Figure 7. Fit of selected records

Each panel shows the separate components of the fit in the upper traces and the sum of the components (continuous curve) superimposed on the data (○). The upper panels (*A* and *B*) are for a 2·5 nm release, the largest fast release that did not cause force in the test record to approach zero. Lower panels (*C* and *D*) are for the largest (8·1 nm) release. Note that the fourth component is absent under the reference condition.

function that relaxed with a single time constant, however, they required a non-linear stiffness. This observation suggested that the fastest component might have been due to low-force bridges and that BDM might have increased the fraction of attached bridges in low-force states without otherwise altering their properties. To test this hypothesis, the calculations were redone using a non-linear stiffness to match only the fastest component. To match the test records, the amplitudes of the two slower components were halved, the amplitude of the fastest component was reduced by 12·5% and a non-relaxing, non-linear fourth component was added to account for the constant deviation of the difference records (Fig. 2*B*). The rationale for halving of the two slower components was the consideration that these two components were due to non-detained bridges that contributed to isometric force, which was reduced by 50% by 3 mM BDM. The fourth component was adjusted to match the constant portion of the difference record and the rate constants of the three components (Fig. 5*B*) were then adjusted to give the best match to both the reference and test record. The fitted curves (continuous curves) are superimposed on the digitized records (○) in Figs 7 and 8.

In each panel of Fig. 7 the upper traces show the responses of the separate monoexponential components of the fitted record and the trace superimposed on the circles shows the sum of these individual responses superimposed on the experimental record. Two responses to two sizes of step are shown for each condition, the largest fast release that did not bring force to near zero in the BDM experiment and the largest slowed release. Figure 8 shows the fitted record superimposed on all seven releases. In both figures, the left-hand panels show the responses under reference conditions and the right-hand panels show the responses in 3 mM BDM. Figure 7 plots the forces in absolute units, normalized to T_{ref}, to show the changes in amplitude of the individual components. Figure 8 plots force normalized to the immediate isometric force, to enlarge the test responses and facilitate comparison of the fitted record and the data. In both figures, the size of the circles representing the individual data points was chosen to be ±2·5% of the

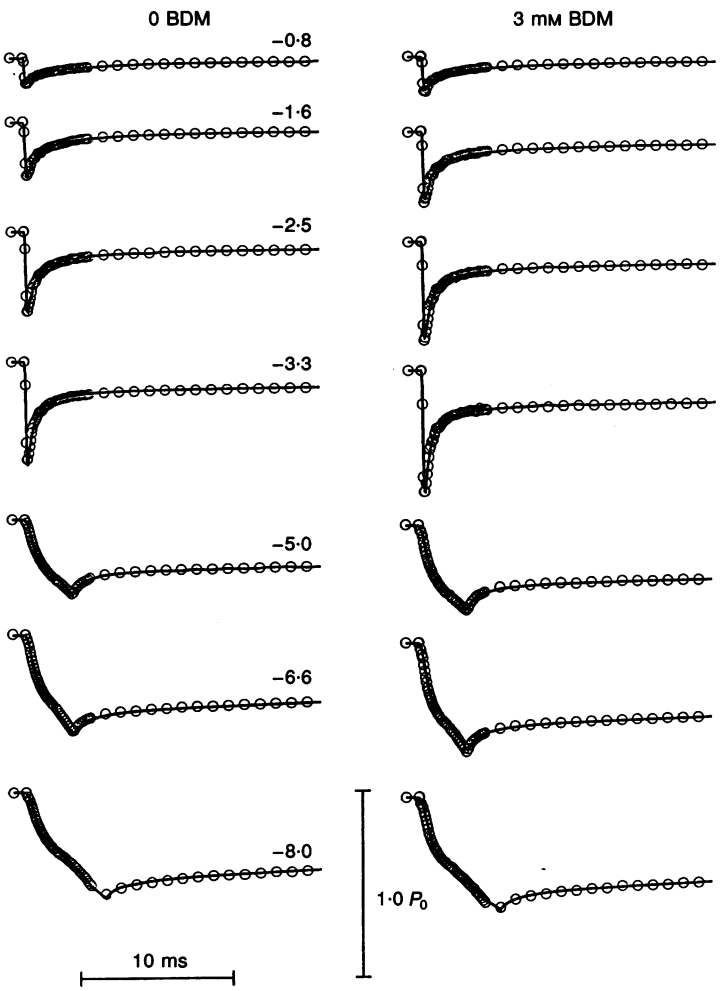

Figure 8. Calculated responses superimposed on the experimental records for all 7 releases
Numbers above the reference records indicate the size of release (in nm per half-sarcomere). All forces are normalized to isometric force.

reference isometric force. As indicated, the fitted traces fell well within the circles except at the end of the largest fast steps, where force approached zero.

Extension–force relations

The extension–force properties of several components of the responses are plotted in Fig. 9. The forces have been normalized to the isometric force, so that the relative values are plotted. The relative T_1 and T_2 curves are plotted in Fig. 9*A*, with the open symbols representing the reference values and the closed symbols the test values. The values for individual components are plotted separately in Fig. 9*B*. The values for the two slower components are shown as deviating from the isometric point and the values for the other two components are shown as deviating from the origin (Fig. 9*B*). This presentation illustrates the interpretation that only the two slow components contribute to isometric force and that the other two components are due to detained, low-force bridges. The combined, instantaneous stiffness of the two slow components (diamonds) was linear and extrapolated to zero force at ~6 nm per half-sarcomere shortening. The T_2 values for the two slower components (○, Fig. 9*B*) are the same as the T_2 values for the overall response under reference conditions plotted in Fig. 9*A*. The difference in the T_2 values obtained in BDM was accounted for by the addition of the non-relaxing, fourth component (●, Fig. 9*B*). The slope of the extension–force curve for the fastest relaxing component (□), representing its stiffness, was decreased by about half as step size increased. The component itself was assumed to recover fully to zero; that is, the T_2 curve for this component is the horizontal zero baseline (dotted line, Fig. 9*B*). To match the test record the relative amplitude of this component was increased by 75%.

DISCUSSION

The finding that BDM slows maximum velocity indicates that it slows cross-bridge cycling. To the extent that stiffness changes reflect changes in the number of attached bridges (Bressler & Clinch, 1975; Ford, Huxley & Simmons, 1981), the greater decrease in force than stiffness further suggests a lower average force per attached bridge. Together these findings suggest that the intervention detains bridges in low-force states. A similar conclusion about BDM has been

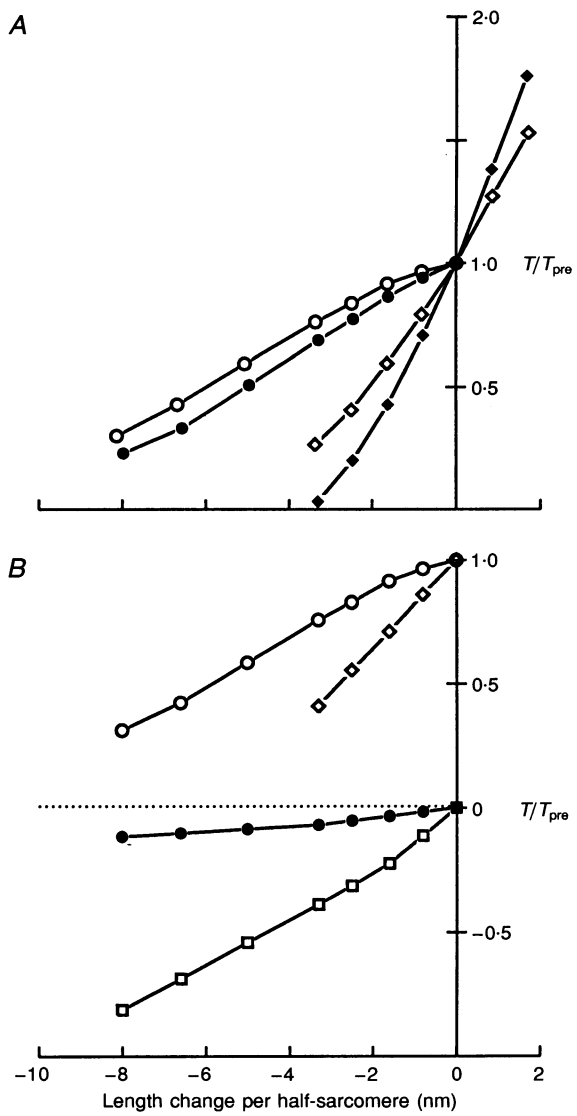

Figure 9. Components of the force responses normalized to the isometric force

Open symbols represent reference responses, closed symbols test responses. *A*, T_1 (diamonds) and T_2 ($T_a(0)$, circles) curves. *B*, $T_a(0)$ values are shown for the two slower components combined (○). Instantaneous extension–force relations are shown for two slow components combined (◇), for the fastest relaxing component (□), and for the non-relaxing component (●). The effect of BDM was to add the non-relaxing component and increase the relative amplitude of the fastest relaxing component by 75%, without changing any other properties of the responses.

reached in skinned fibre studies by Higuchi & Takemori (1989), Bagni *et al.* (1992), Zhao & Kawai (1994), Regnier *et al.* (1995) and Zhao *et al.* (1995), and in isolated proteins by Herrmann *et al.* (1992). The present work extends this conclusion to suggest that BDM causes bridges to accumulate in at least two low-force states and defines the nature of these states.

An important conclusion suggested by these results is that part of the early, rapid force recovery following a length step is not due the cross-bridge movement originally proposed by Huxley & Simmons (1971) but to rapid detachment of bridges from negative-force states. This conclusion follows from the observation that the increased stiffness produced by BDM appears to be due to an increase in the fraction of attached bridges detained in a low-force state and that much of the force decline resulting from these bridges relaxes rapidly following a release. Since the bridges are in low-force states during the isometric condition, they will resist shortening with a negative force during and following a release. Since they are detained, it seems unlikely that they can move to a less negatively strained attached state following a release, particularly with the speed observed for the fastest component. On the other hand, rapid detachment of these negatively strained bridges will contribute to the recovery of positive force. The observation that an extra component of stiffness produced by BDM behaves in a manner similar to a major component seen in the reference condition further suggests that some of the rapid, Phase 2 recovery seen under the reference condition results from the same rapid detachment of low-force bridges.

While the proposed mechanism offers a partial alternative to the original explanation of the tension transients offered by Huxley & Simmons (1971), it is in keeping with Huxley's (1973) later proposal of strain-dependent, rapid detachment of bridges from an initial, low-force state, as discussed below.

Experimental evidence for the existence of negatively strained bridges comes from the observation that the T_1 curve intersects the abscissa at a steep angle (Ford *et al.* 1977). The angle becomes much less steep when the lateral filament spacing is increased (Goldman & Simmons, 1986), because bridges under this condition are less able to resist compressive strain (Goldman, 1987).

The similarity of the detained state created by BDM to the fast component of the reference transient was somewhat unexpected. It is expected that BDM has its effect by binding to the cross-bridges, creating a different class of bridges. The results suggest that these different bridges detach from their initially attached state with kinetics similar to the normal bridges. Kinetic modelling by Regnier *et al.* (1995) suggests that BDM detains cross-bridges in a low-force state along the normal pathway. The present work suggests that the BDM bridges pass through states with normal kinetics for detachment.

The finding of an additional, non-relaxing or very slowly relaxing component in the responses obtained in the presence of BDM suggests that the intervention causes bridges to accumulate in two separate low-force states, one from which they relax very quickly following a release and one from which they relax very slowly. A possible explanation is that BDM does not prevent bridge attachment or the kinetics of bridge detachment from an initially attached state, but inhibits a later low-force transition, such that bridges accumulate in two low-force states that precede the inhibited transition.

Properties of the cross-bridge states

Undetained bridges

Two transitions appeared to vary in amplitude with isometric force, without otherwise altering their kinetics. These were assumed to be due to bridges that undergo the normal power stroke with kinetics similar to those postulated by Huxley & Simmons (1971). The diminution in force, and the fraction of bridges in the states that undergo the power stroke, is believed to be due to the sequestration of BDM bridges in low-force states where they do not generate force or participate in the power stroke. The responses of the non-BDM bridges are recognized both by the slower exponential processes following the rapid steps and by the partial recovery during the slowed, ramp steps. The increase in rate constants for these processes with intermediate releases (Fig. 5) caused substantial curvature in the force records during ramp shortening. This curvature was sufficient to account for the distinctive shape of the experimental records obtained with slow-speed ramps. It was not necessary to assume that the bridges had a diminished stiffness with large releases in order to reproduce the records. It was also not necessary to assume any change in the relative T_2 values in fitting the test records because it was possible to accommodate such changes entirely with the non-relaxing component.

Rapidly relaxing state

The rapidly relaxing component of the responses was attributed to rapid detachment of initially attached bridges. The rate constants for the recovery of this component reflect the rate constants for detachment of negatively strained bridges and therefore reflect one side of the detachment function. While these rates seem high, it should be remembered that these are the rates for negatively strained bridges. The rates for unstrained or positively strained bridges were not assessed directly.

The proposed state is identical to one proposed by Huxley (1973) to account for Hill's (1964) finding that shortening heat was less during rapid shortening than during shortening at intermediate loads and velocities. In his original cross-bridge model, Huxley (1957) proposed that the rate of attachment of bridges, and therefore their cycling rate and heat rate, would increase in proportion to shortening velocity. This scheme accounted for Hill's 1938 finding that the rate of total energy liberation, heat rate

plus work rate, increased in proportion to velocity. Hill (1964) later found that this relationship applied only at lower velocities, and that the heat rate fell at higher velocities. Huxley (1973) then proposed a two-stage attachment model to accommodate the later data. The initial attachment rate increased with shortening velocity, as before, but bridges in this state had to make a transition to a second state before they became committed to ATP hydrolysis. The bridges in the first state would detach rather than make the second transition if the muscle were shortened rapidly, in a manner similar to that proposed here for the initially attached state. Dantzig *et al.* (1988) have provided experimental evidence for the rapidly detaching zero-force cross-bridges. The rapid rate of detachment ensures that these initially attached bridges do not impose a load on the shortening muscle.

The rapid step applied in the present study, however, forces some of the bridges over to the negative-force range, despite their rapid detachment rate. The subsequent detachment of the negative-force bridges is responsible for the fastest component of the Phase 2 force recovery. This conclusion is further supported by the observation that BDM, which detains bridges in low-force states, augments this fast component.

Non-recovering component

The difference records (Fig. 2*B*) showed an initial spike that decayed within a few milliseconds to a level that remained very nearly constant for the remainder of the recording period. The constant difference between the test and reference records developed almost immediately with the length steps. Thus, it would appear that this component is due to structures that increase sarcomere stiffness during the step and do not change their force after the step ends. The most easily explained structure is a cross-bridge that is immobilized. As explained below, the non-linearity of this component suggests that these bridges detach more rapidly than we could detect when shortened by some threshold amount but are otherwise immobilized in their attached positions.

The reason for proposing an immobilized attached bridge rather than some other mechanism, such as the detachment of bridges into an inactive state that did not contribute to subsequent force recovery, is the flatness of the difference records. If the bridges responsible for the initial stiffness increase were to make a force altering transition, their movements would have to be balanced exactly by transitions elsewhere in the cycle.

Changes in velocity

Since the two slower components of the response vary in all respects with isometric force, it is unlikely that these two components contributed to the decreases in velocity seen with BDM. The very rapid and complete recovery of the fastest component of the response further suggests that this component would not have imposed a substantial load on the shortening muscle. The decline in velocity thus appears to be due entirely to the the fourth component added by the BDM imposing an internal load during shortening.

Non-linear stiffness

Although much of the non-linearities in force records (Fig. 8) observed during the slowed steps could be matched by constant stiffness and increasing rate constants for the different recovery processes, some non-linear stiffness was required for a good fit. We chose to place all of the non-linear stiffness in the fastest component because of the finding that the amplitude of the spikes in the difference records (Fig. 2*B*) varied in a non-linear way with step size and resembled a component of the reference transients (Fig. 7). If the individual bridges had the same linear compliance in different states, as expected, then the apparent non-linearity of the stiffness would require another explanation. One possible explanation is that the compliance is linear but that the bridges detach very rapidly when shortened beyond some critical distance. This rapid detachment of bridges would then produce the apparent non-linearity of stiffness. Rapid reattachment of detached bridges would also produce a force difference that remained nearly constant with steps larger than a certain size, as with the component added by BDM. This type of strain relief by cross-bridge detachment and rapid reattachment has been invoked to explain the flattening of the force response to stretches (Brenner, Schoenberg, Chalovich, Greene & Eisenberg, 1982; Lombardi & Piazzesi, 1990).

Rapid detachment of bridges causing an apparent non-linearity of stiffness should produce a component of the response that deviates initially and then recovers rapidly to an intermediate level. Thus, it might be expected that the component of the difference record that did not recover should be fitted with a linear stiffness that relaxed very rapidly and only partially. A possible explanation of the lack of need for such a rapid component is that the recovery was so rapid that it could not be detected with our fastest digitization rate. As explained below, Ford *et al.* (1977) found a fourth, very rapidly relaxing component in their tension transients that was not seen here.

Methods of analysis

The two methods of analysis used here were greatly facilitated by digital recording. With the first, the records are scaled, shifted and subtracted, a method that would be impossibly onerous without digitized records. This method is predicated on the belief that the tension transients are the sum of stochastic responses of the individual bridges and that some of the bridges respond normally while others are influenced by the test intervention. When an intervention detains bridges in low-force states, as found here, the isometric force is attributed to attached bridges which have moved beyond the inhibited transition. These bridges are assumed to be able to respond normally to the length step, at least in the short term. Scaling the reference records to the isometric force and then subtracting the scaled record

from the test response is therefore expected to yield a difference record which reflects the responses of the abnormal, detained bridges. This method has the advantage that it makes no assumption about the form of the response and as a consequence is a useful way of making an initial investigation of the effects of an intervention. It is important to emphasize two limitations of this method. The first is that the bridges will become redistributed as the response progresses; some of the normally responding bridges will move to detained states while detained bridges will move past the inhibited transition and behave normally. Thus, the differences in the early responses may be a more reliable indicator of the effects of an intervention than the later differences. The second limitation is that it is possible that some interventions will alter the isometric distribution of bridges that are functioning normally, so that the difference record indicates both the response of abnormal bridges and the response of normal bridges that are abnormally distributed.

The second method of analysis uses mathematical descriptions to define the effects of interventions. The advantage of this method is that it provides a mathematical description of the experimental result. For some purposes, such as the quantification of instantaneous stiffness, this precise description is useful even if the theory leading to the description is inappropriate. It should also be emphasized that while the close fit of these functions to the experimental records suggests that the descriptions are accurate, there is nothing to indicate whether they are appropriate. Thus, as always, interpretation of the experimental findings depends as much on intuition as on the goodness of fit.

Relationship to studies of intact frog fibres

The present data required only three exponential components for description in contrast to four required by Ford *et al.* (1977). Fitting of earlier experiments required a very rapidly relaxing component not seen here. As described above, it is possible that the fourth component was present but relaxed more rapidly than could be detected with the relatively slow digitization rate used here. Alternatively, there might have been a genuine absence of one component, perhaps due to the species difference or to fibre skinning, with concomitant loss of some soluble substance. Finally, it is possible that two components might be indistinguishable because their rate constants were more nearly equal than in frog fibres.

The sarcomeres are substantially stiffer in the preparation studied here than in intact frog fibres at similar temperatures (cf. Ford *et al.* 1977). The present results suggest that some of the increased stiffness is due to detention of bridges in an initially attached, low-force state. The relative amplitude of the fastest component in the present study was substantially larger than either of the two fastest components observed by Ford *et al.* but the same size as the two combined, so that a conclusion about differences in the distribution of bridges among components cannot be made with certainty.

A strong dependence of stiffness on temperature was described in the earlier studies. The present results suggest that some of this temperature dependence might be due to a large temperature dependence of the transition from the initial attached state. The consideration that the preparation studied here evolved to work at a fixed warm body temperature while the frog fibres usually work at lower temperatures suggests that the two preparations might become more similar if the present fibres were studied at a slightly warmer temperature.

The earlier study examined the effect of making the T_1 stiffness non-linear and concluded that the fit was not improved by this added freedom. It was definitely required in the present study, but only when the fit was extended to the larger, slowed releases, that were not fitted in the earlier study. Thus, we cannot be certain whether the requirement of a non-linear stiffness is a genuine difference in the preparations or simply due to the larger size of step fitted.

The analysis of the data from intact fibres had the rate constants of the several components of the response all varying in the same proportion with step size. This analysis presumes that the several components of the response derive from similar mechanisms. In the present analysis all the rate constants varied with step size, but that of the fastest component varied in a manner that was different from the other two. If this component is due to a different mechanism, as suggested here, it is reasonable to assume that its rate constant will vary in a different manner. The variation with step size in this case would be due to a rate function for detachment of low-force bridges that increased with compressive strain of the bridges.

Conclusion

The recent three-dimensional structural determination of the myosin–actin complex has been interpreted as showing that myosin attaches to actin in several stages (Rayment *et al.* 1993). The present study identifies a separate low-force transition that must correlate with separate states of the cross-bridge cycle. Together with other work, it suggests the existence of multiple low-force states through which the bridges pass before they generate force. The present work further suggests that detachment of negative-force bridges contributes to the recovery of positive force following a step reduction in sarcomere length.

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