BDM Compared with P_i and Low Ca^{2+} in the Cross-Bridge Reaction Initiated by Flash Photolysis of Caged ATP

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ABSTRACT Using flash photolysis of caged ATP in skinned muscle fibers from rat psoas, we examined the inhibitory effects of 2,3-butanedione monoxime (BDM) on the contraction kinetics and the rate of ATP hydrolysis of the cross-bridges at ~10°C. The hydrolysis rate was estimated from the stiffness records. The effects of BDM were compared with those of orthophosphate (P_i) and of reduction in [Ca^{2+}] (low Ca^{2+}), and it was found that i) BDM and low Ca^{2+} inhibited ATPase activity to the same extent as they inhibited the steady tension, whereas P_i inhibited ATPase activity much less than tension; ii) BDM and P_i decreased tension per stiffness during the steady contraction more than did low Ca^{2+}; iii) neither BDM nor low Ca^{2+} affected the initial relaxation of the fiber on release of ATP, but P_i slightly slowed it; and iv) BDM hardly influenced the rate of contraction development after relaxation, although P_i and low Ca^{2+} accelerated it. We concluded that BDM inhibits the Ca^{2+}-regulated attachment of the cross-bridges and force-generation of the attached cross-bridges.

INTRODUCTION

The cross-bridge in muscle cell is a complex of actin and myosin, which hydrolyzes ATP and produces the mechanical work of contraction. Although the detailed mechanism of the cross-bridge reaction has not been clarified, its general framework is assumed to be the following (Dantzig et al., 1991; Kawai and Halvorson, 1991). When the nucleotide-free cross-bridge binds ATP (AM + ATP), actin is substantially dissociated from myosin (A + M·ATP), and ATP is rapidly hydrolyzed into ADP and P_i (M·ADP·P_i). After the hydrolysis, myosin reattaches firmly to actin (AM·ADP·P_i), releasing P_i (AM*·ADP + P_i) to produce the contractile force, and later liberates ADP (AM + ADP) before it enters the next cycle with another ATP molecule.

Caged ATP is a photolabile synthetic precursor of ATP that releases ATP on exposure to near-UV light (Kaplan et al., 1978). With the technique of flash photolysis of this compound, the mechanical aspects of the ATP-induced cross-bridge reaction can be examined in muscle fibers without limitation from diffusion delay on application of ATP (Goldman et al., 1984a). The observations in such photolysis experiments have been valuable for the study of the cross-bridge reaction (review by Goldman, 1987).

2,3-Butanedione monoxime (BDM) is an inhibitor of muscle contraction that directly inhibits the cross-bridges, at least under certain conditions (Horiuti et al., 1988). To understand the detailed mechanism of the action of BDM on the cross-bridges, we in the present study examined the effects of BDM on the mechanical properties of the cross-bridges that can be probed in experiments with flash photolysis of caged ATP. We paid particular attention to the following two points. First, the contractile reaction of the cross-bridges is inhibited by millimolar concentrations of P_i (Cooke and Pate, 1985) and by reduction of the concentration of the regulatory Ca^{2+}. Thus, we compared the effect of BDM with those of P_i and low Ca^{2+}. Second, we measured the rate of ATP hydrolysis of the cross-bridges concomitantly in the same mechanical experiments with the same muscle fibers. Previously we proposed a simple method for estimating the myofibrillar ATPase activity from the time course of muscle stiffness after photolysis of caged ATP (Horiuti et al., 1993). We gave this method further consideration and applied it to the present study.

Our results indicated that BDM inhibits transition of the cross-bridges to the force-generating state that occurs just before the rate-limiting step of the cross-bridge reaction. Preliminary reports of this study have appeared elsewhere (Kagawa et al., 1993, 1994).

MATERIALS AND METHODS

The setup, preparations, and procedures were generally the same as in our previous studies (Horiuti et al., 1992, 1993, 1994b). Single muscle fibers from rat psoas were treated with a detergent (1% Triton X-100 for 30–60 min) in a relaxing solution to remove the cytoplasmic and internal membranes (Kurebayashi and Ogawa, 1991). A fiber segment was mounted on the apparatus for the isometric measurements of tension and stiffness at the sarcomere length of ~2.8 μm. The fiber length was sinusoidally oscillated (0.1–0.2% at 500 Hz), and the magnitude of the in-phase component of the resultant tension oscillation is the "muscle stiffness" in this paper. Several experimental trials of contraction (or relaxation) were carried out with each muscle fiber. Every experiment was repeated with different fibers and a different order of trials, and the means ± SEM with the numbers (n) of the examined fibers will be presented in the Results. Statistical differences among the data sets were examined by Student's t-test, where the differences were taken to be significant when the p-values were less than 0.05.

In any experimental trials, the fiber in the relaxing solution was first put into the rigor state by washing it with ATP-free (rigor) solutions. The fiber in rigor was then immersed in a photolysis solution that contained caged ATP (P^3-1-(2-nitro)phenylethyl ATP; ATP + ADP < 0.01% caged ATP). In the trials with BDM, P_i, or ADP, the agent to be tested was added to the photolysis solutions. The standard photolysis solution contained both

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$CaCl_2$ and EGTA at 10 mM (pCa ≈ 4.5), but in the low Ca^{2+} trials [$CaCl_2$] was appropriately reduced, assuming the apparent stability constant of Ca·EGTA to be $10^{6.264}$ M^{-1} under our experimental conditions (10 mM $MgCl_2$, pH 7.0, 9–12°C, ionic strength of 0.20 M). It should be noted that no ATP-regenerating system was used for measurement of ATPase activity (see below).

After 30–60 s equilibration in the photolysis solution, the fiber was transferred to a photolysis trough filled with a silicone oil. This was done to exclude the bulk solution and to release ATP only in the muscle fiber. The oil had been saturated with a solution that had the same constituents as the photolysis solution except for caged ATP. (Without this precaution, the contraction after the later photolysis would be significantly small, probably because the fiber dries in the oil.) After 30–60 s in oil, a photolytic UV flash was applied to the fiber, through a flexible light guide from a 200-J xenon lamp. The digitized data of tension and stiffness on photolysis were compressed logarithmically (Horiuti et al., 1989) and stored in a personal computer for later analysis.

ATPase activity was not to be measured in the relaxation experiments (Figs. 8 and 9), so that the experimental protocol there was slightly different from the above. The oil was not used, and the photolysis trough was filled with the caged ATP solution. Apyrase (0.16 mg/ml, grade V; Sigma Chemical, St. Louis, MO) was added in the photolysis solution to eliminate possible effects of the contaminating ATP and ADP (Thirlwell et al., 1994). The ATPase and ADPase activities were on the order of 10^{-1} mM/min in the apyrase solutions for photolysis. (No apyrase was used in the contraction experiments.) The fibers were stretched by ~1%, 5 s before photolysis, to increase the rigor tension before release of ATP.

The fractions of caged ATP photolyzed to ATP were examined by chromatographic analyses (Fig. 1). When the muscle experiments were carried out without oil, the caged ATP solutions (4–5 μl) after photolysis were directly collected in the muscle experiments. In the case of experiments with oil, the samples were obtained separately from the muscle experiments as follows. The trough was filled with a photolysis solution instead of the oil, the same UV pulse as used in the muscle experiments was applied to the trough, and then the photolysis solution was collected for analysis. For matching the UV absorption by caged ATP, its concentration in the trough was set to be ~5 times lower than in the muscle experiments, as the photolysis trough was 0.5 mm wide but the muscle fibers were ~0.1 mm thick.

The photolysis samples were loaded on an FPLC mono-Q column (Pharmacia, Sweden), and the nucleotides were eluted on a linear NaCl gradient (20 mM Tris-HCl at pH 8.0, 0–1 M NaCl) under a monitor of absorbance at 254 nm (Fig. 1, top). The fraction of photolyzed caged ATP was calculated from the areas under the nucleotide peaks on the elution pattern, taking account of the ratio of 259 nm absorbance of caged ATP to that of ATP, 1.27 (Goldman et al., 1984a). As shown in Fig. 1 (top), a small amount of ADP was always detected in the analysis, even when the photolysis was performed without muscle fibers. It was reported that caged ADP was negligible in our caged ATP. Thus, the detected ADP seemed to be due to hydrolysis during the analysis of the photoreleased ATP. The sum of the peak areas of ADP and ATP was taken as that of photoreleased ATP. In the analysis of the apyrase-containing solution, it was assumed that AMP + ADP + ATP = released ATP.

In the typical experiments, [caged ATP] in the photolysis solutions was 4.0–5.6 mM, and the single 200-J flashes photolyzed 22–35% of the caged ATP, yielding 1.1–1.4 mM ATP. To change [photoreleased ATP], we kept [caged ATP] constant in the photolysis solution, and varied the electrical energy for the flash discharge. As shown in Fig. 1 (bottom), the photolysis efficiency was approximately proportional to the flash energy.

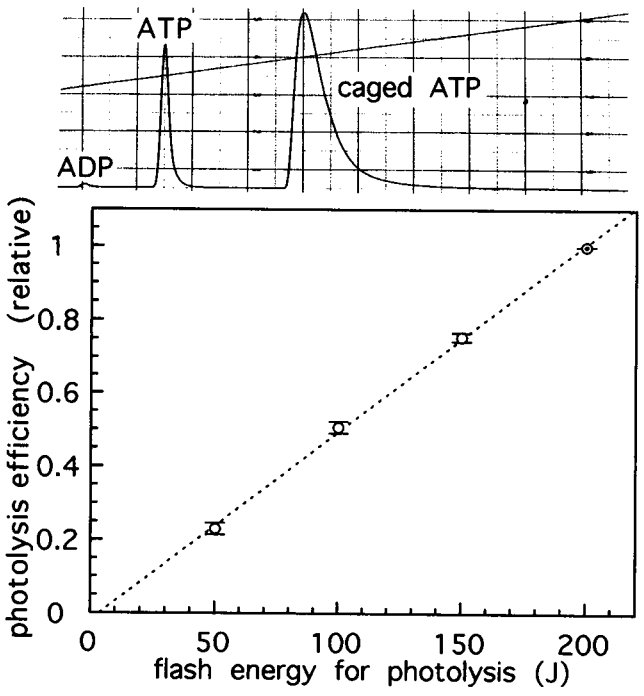


FIGURE 1 (*Top*) An FPLC elution profile at 254 nm of a photolysis sample. The peaks were identified as labeled. The oblique line crossing the peaks shows the [NaCl] gradient (~0.3–0.5 M). The photolysis efficiency was estimated to be 23%, which was calculated as $(A_{ATP} + A_{ADP})/(A_{ATP} + A_{ADP} + A_{caged\text{-}ATP}/1.27)$, where A represents the areas under the labeled peaks. (*Bottom*) The relation between the flash energy and photolysis efficiency. The efficiencies are normalized to that at 200 J, which is 22 ± 1% (mean ± SEM; n = 8) in this particular experiment. The dotted line was obtained by linear regression analysis.

Analysis of the sub-second mechanical reaction

Fig. 2 shows a stiffness record obtained on photorelease of ATP (1.2 mM) in the presence of Ca^{2+} (pCa ≈ 4.5). In such records, the muscle first relaxes for several tens of milliseconds, and then starts to contract, reaching a steady state in less than a second (Fig. 2, bottom). This time course has been interpreted as a two-step reaction (rigor + ATP → detachment →

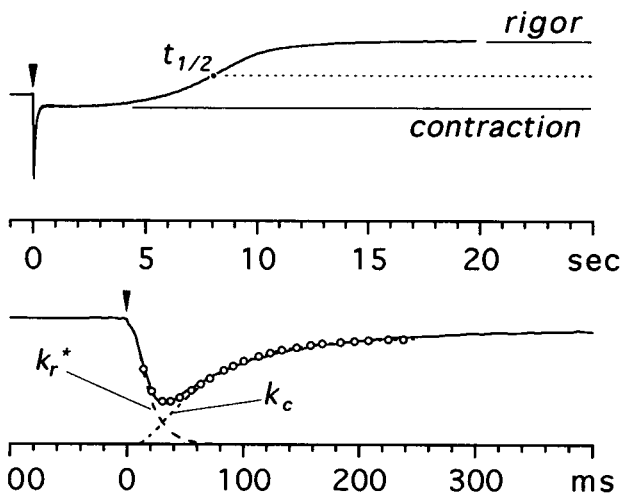


FIGURE 2 A typical record of muscle stiffness, on two different time bases. ATP (1.2 mM) was released at time 0 (*arrowheads*) under the control conditions (no BDM or P_i at pCa ≈ 4.5). (*Top*) At the point labeled $t_{1/2}$, the stiffness is half way between the level during the steady contraction and the final level in rigor. (*Bottom*) The sub-second reaction was analyzed by curve fitting (*circles*), and the rates (k_r^* and k_c) of initial relaxation (*broken line*) and after contraction (*dotted line*) were extracted (see text for details).

contraction) with an initial delay, so that short segments of the records were analyzed by fitting the following function (Goldman et al., 1984a):

$$F(t) = F_0 \cdot \exp(-k_r \cdot t') + F_\infty \cdot \left\{1 - \frac{k_c}{k_c - k_r} \cdot \exp(-k_r \cdot t') - \frac{k_r}{k_r - k_c} \cdot \exp(-k_c \cdot t')\right\},$$

where $t' = t - t_d$, and t is time after release of ATP. On the right-hand side of the expression, the first term represents the initial relaxation from the rigor level (F_0) with a delay (t_d) and a rate constant (k_r), and the second term represents the subsequent contraction to the steady level (F_∞) with another rate constant (k_c). The segments to be fitted were chosen from the records (circles in Fig. 2, bottom) so that they began after the initial sigmoid phase of relaxation and ended before the late creep phase of contraction. We converted the above extracted t_d and k_r into a single, apparent rate constant $k_r^* = \ln(2)/[t_d + \ln(2)/k_r]$ to characterize the overall time course of the initial relaxation (Goldman et al., 1984a; Horiuti et al., 1993).

The rate k_c obtained from tension records was about 30% slower than that from the simultaneously obtained stiffness records, as in the previous study (Horiuti et al., 1992). However, as regards the fractional changes induced by BDM, P_i, and low Ca^{2+}, there was no great difference between the k_c from tension and that from stiffness. The data of k_c in Results (Fig. 7) are only those from stiffness records to avoid redundancy. The rate k_r^* from the tension records was always similar to that from the stiffness records, and the data of k_r^* in Results (Figs. 7 and 9) are also from the stiffness records.

In the relaxation experiments in the absence of Ca^{2+} (Figs. 8 and 9), we directly measured the relaxation half-time ($t_{1/2}$) on the raw records and converted it to k_r^* ($= \ln(2)/t_{1/2}$). From the relaxation $t_{1/2}$ we further estimated the second-order rate constant of the ATP binding (Fig. 9) by using a model proposed by Goldman et al. (1984a). In the model, a concentration ($[ATP]_o$) of ATP is released from caged ATP with a first-order rate (k_p) and binds to the cross-bridges with the second-order rate (k_{+T}) to relax them. If we know k_p and the cross-bridge concentration ([M]), we can calculate the time course and $t_{1/2}$ of relaxation at a given set of k_{+T} and $[ATP]_o$. In this study, we assumed that $k_p = 40\ s^{-1}$ at our experimental temperature (~10°C) (Goldman et al., 1984a) and [M] = 0.15 mM (Ferenczi et al., 1984; Horiuti et al., 1993), and searched for the value of k_{+T} that best fitted the data of $t_{1/2}$ at various $[ATP]_o$.

RESULTS

Estimation of rate of ATP hydrolysis

When muscle fibers were activated by photorelease of ATP in the presence of Ca^{2+}, the fibers consumed the released ATP during the contraction and eventually returned to rigor because they were in oil and no ATP-regenerating system was present. Development of this rigor state is clearly seen on the stiffness tracing in Fig. 2 (top). The stiffness signal on the plateau of steady contraction sigmoidally elevated in seconds to the higher level in rigor. To estimate the time required for the fiber to deplete the released ATP, we defined the half-time ($t_{1/2}$) to rigor as the length of time from photorelease of ATP to the half-development of the rigor stiffness (Fig. 2, top, $t_{1/2}$). In some cases, especially at high [added P_i] (see Fig. 5 *B*), the plateau of the steady contraction was short, and both tension and stiffness significantly crept up, even in the very early phase. Thus, we fitted a line on the record of the early phase (1–2 s after ATP release), extrapolated this back to time 0, and defined its height as the level during the steady contraction.

As illustrated in Fig. 3, the $t_{1/2}$ to rigor was reasonably shortened by reduction in the concentration of photoreleased ATP ($[ATP]_o$), and the relation between $[ATP]_o$ and $t_{1/2}$ was linear in the range we examined. We do not know the exact amount of the ATP remaining at the time of half-rigor ($t = t_{1/2}$). However, if it is quite small compared to the amount of initially photoreleased ATP, the slope ($\Delta[ATP]_o/\Delta t_{1/2}$) of the $t_{1/2}$-$[ATP]_o$ relation would represent the rate of ATP hydrolysis during the steady contraction. The ATPase activities in Results refer to the values of $\Delta[ATP]_o/\Delta t_{1/2}$. In the Discussion we will further consider the validity of our method.

As shown in Fig. 4, the $t_{1/2}$-$[ATP]_o$ relations were also linear in the test experiments with BDM, P_i, and low Ca^{2+}. It was notable that the intercepts of the lines on the $[ATP]_o$ axis in these tests were approximately the same as in the control (Fig. 3 *B*), 0.15–0.2 mM. For the argument in the Discussion, we examined the effect of ADP on the $t_{1/2}$ to rigor. The $t_{1/2}$-$[ATP]_o$ relation was linear with a constant $[ATP]_o$-intercept also in the ADP experiments (data not shown). ADP at 1.2 and 4.8 mM increased the $t_{1/2}$ in the 200

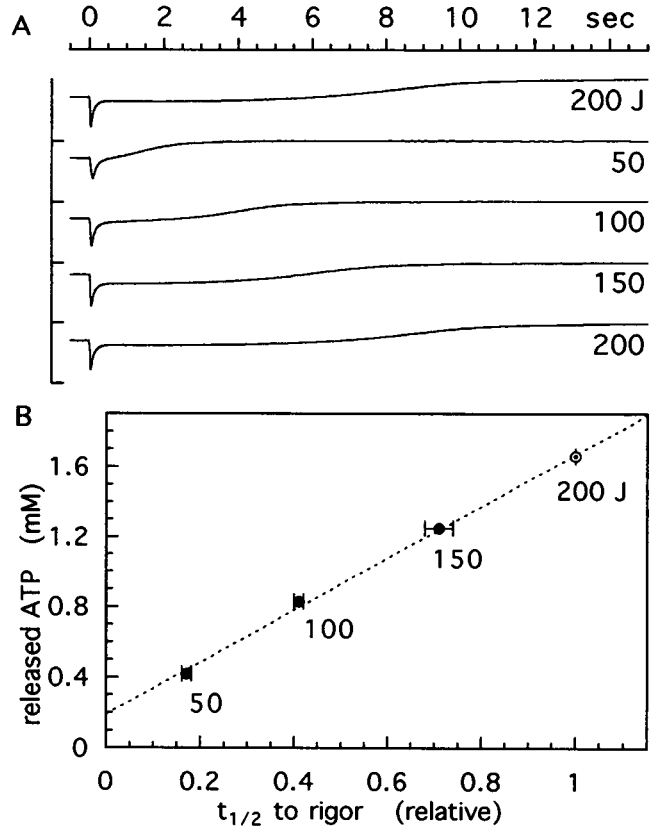


FIGURE 3 (*A*) A set of stiffness records obtained in successive trials with a fiber segment. The flash energy was varied as labeled on the records. 1.2 mM ATP was released at 200 J from 5.6 mM caged ATP. The levels of zero stiffness are marked with the ticks on the vertical axis. The order of the trials were from top to bottom. (*B*) The relation between the halftime ($t_{1/2}$) to rigor and [released ATP] ($[ATP]_o$). The $t_{1/2}$ are expressed as fractions of that at 200 J, which was 12 ± 1 s (n = 6). 1.7 mM ATP was released at 200 J from 6.2 mM caged ATP.

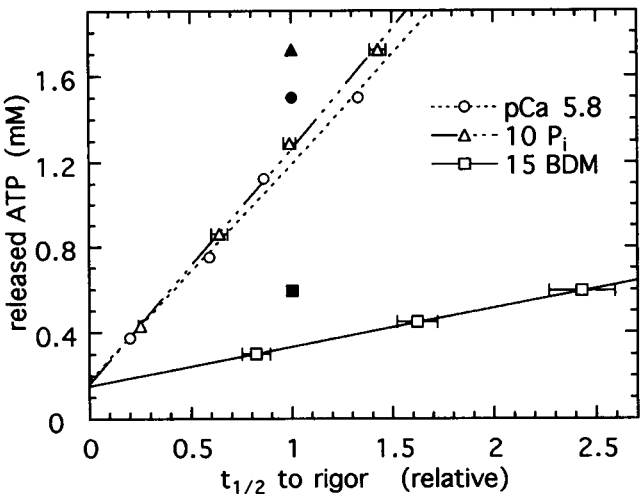


FIGURE 4 Relations between $t_{1/2}$ to rigor and $[ATP]_o$ under the conditions with BDM (15 mM; □), P_i (10 mM; △), and low Ca^{2+} (pCa 5.8; ○). Caged ATP in the photolysis solution was 2.7 (□), 6.2 (△), and 4.6 (○) mM. The filled symbols represent the data obtained under the control conditions with the 200 J flash. The $t_{1/2}$ are normalized to these control values, which were 1.8 ± 0.1 (■), 16 ± 3 (▲), and 10 s (●), in the experiments with BDM, P_i, and low Ca^{2+}, respectively. Four fibers were used for BDM, five for P_i, and three for low Ca^{2+}. SEMs were not calculated for the low Ca^{2+} data.

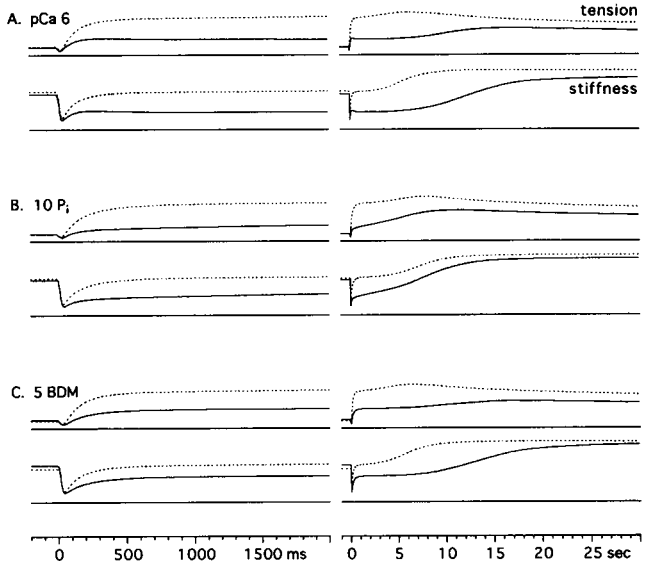


FIGURE 5 The records of tension (*top*) and stiffness (*bottom*) at 200-J flashes, on fast (*left*) and slow (*right*) time bases. Dotted lines were those in the control condition, and solid lines were those at pCa 6.0 in (*A*), with 10 mM P_i in (*B*), and with 5 mM BDM in (*C*). $[ATP]_o$ was 1.2, 1.4, and 1.1 mM in (*A*), (*B*), and (*C*), respectively. The tracings are averages of 5 records obtained from 3 fibers in (*A*), 6 records from 6 fibers in (*B*), and 11 records from 8 fibers in (*C*). We also took the 100-J records (not shown) in each experiment to estimate ATPase activity.

J trials (1.2 mM ATP) by 6% (±1; $n = 5$) and 26% (±4; $n = 5$), respectively.

Effects of BDM, P_i, and low Ca^{2+} on the steady contraction

Fig. 5 shows examples of the tension and stiffness records, which were obtained in the photolysis trials where relatively large amounts of ATP (1.1–1.4 mM) were released by the 200 J flashes. The experiments in Fig. 5, *A*, *B*, and *C*, were carried out to examine the inhibitory effects of low Ca^{2+} (pCa 6.0), P_i (10 mM), and BDM (5 mM), respectively. In each panel, the solid lines represent the test records, and the dotted lines represent the records from the same muscle fibers in the control condition. Note that the same sets of records are shown on two different time bases (Fig. 5, left and right).

Experiments similar to the above were performed while varying the concentrations of BDM (5–15 mM), P_i (4–10 mM), and Ca^{2+} (pCa 5.8–6.0). Fig. 6 *A* shows the relations between the relative magnitude of tension and that of stiffness during those steady contractions that were inhibited by BDM, P_i, and low Ca^{2+}. In this plot, the slopes of the three lines connecting the data points are less than unity, suggesting that tension per stiffness during the inhibited contraction was smaller than during the control contraction. It should be noted that the ATP-induced contractions at the intermediate $[Ca^{2+}]$ below pCa 6.0 were transient (Horiuti et al., 1994a) and thus were inappropriate for the measurement of ATPase activity.

We summarized the data at 5 mM BDM, 4 mM P_i, and pCa 6.0 in Table 1, where the steady tension was about half that of the control in any of the three sets of data. Tension per stiffness (Table 1) was significantly ($p < 0.001$) decreased in all of the test experiments. Compared to the small decrease by low Ca^{2+} (−9%), the decrease by BDM was relatively large (−27%), and that by P_i was intermediate (−19%) between the above two ($p < 0.001$). Although we were the first to compare the three directly, the decreased tension per stiffness at low Ca^{2+} has been observed by Martyn and Gordon (1992); that with BDM by Higuchi and Takemori (1989), Bagni et al. (1992), Zhao and Kawai (1994), and Zhao et al. (1995); and that with P_i by Hibberd et al. (1985), Kawai et al. (1987), Brozovich et al. (1988), Horiuti et al. (1992), and Martyn and Gordon (1992).

The relations between tension and ATPase activity are shown in Fig. 6 *B*. ATPase activity was estimated from the $t_{1/2}$ to rigor in the 200 and 100 J trials ($\Delta[ATP]_o/\Delta t_{1/2}$). The rate of ATP hydrolysis under the control conditions was 0.21 mM s^{-1} (±0.02; $n = 39$), which was 1.4 s^{-1} per cross-bridge if the cross-bridge concentration was 0.15 mM. As in Fig. 6 *B*, ATPase activity seemed to decrease in parallel with the steady tension in the experiment with BDM, and the same was the case in the low Ca^{2+} experiment.

When we added P_i to inhibit the contraction, ATPase activity was not effectively suppressed, although tension was markedly decreased. Thus, tension per ATPase in the presence of P_i was much smaller than in the control. The

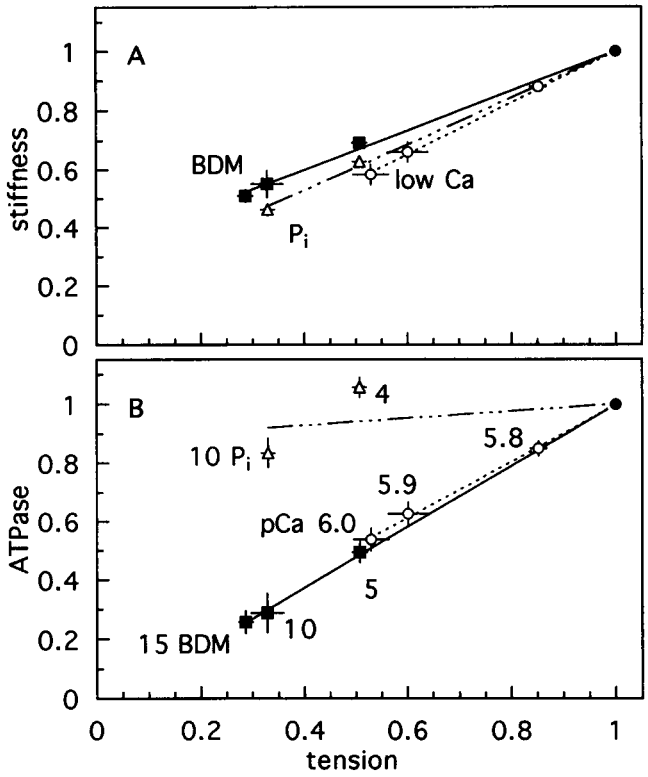


FIGURE 6 Stiffness (*A*) and ATPase activity (*B*) as functions of tension during the steady contractions that were influenced by BDM (■; n = 5–19), P_i (△; n = 9–15), or low Ca^{2+} (○; n = 6–13). The magnitudes of tension, stiffness, and ATPase activity were expressed as fractions of those in the control condition (●). The labels attached to the symbols represent the concentrations of BDM (mM), P_i (mM), and Ca^{2+} (pCa).

dissociation of ATPase activity from tension during the P_i-affected contraction is evident, even in the raw records of stiffness in Fig. 5 *B* (*right*): P_i at 10 mM decreased the steady tension to a third of the control, but its effect on the $t_{1/2}$ to rigor was very small. The low tension per ATPase in the presence of P_i has been repeatedly reported (Webb et al., 1986; Kawai et al., 1987; Cooke et al., 1988), but our result was much more prominent than the others. In our experiments, ATPase activity was more tolerant and tension was more sensitive to P_i than in the previous studies.

Statistical analyses of the data of tension per ATPase in Table 1 revealed a highly significant decrease in the ratio by P_i ($p < 0.001$), but no detectable changes by BDM or low Ca^{2+} ($p > 0.05$). The result with low Ca^{2+} was consistent with the previous report by Brenner (1988).

TABLE 1 Effects of BDM, P_i, and low Ca^{2+} on tension, stiffness, and ATPase activity during steady contraction

Conditions	Tension	Tension per stiffness	Tension per ATPase	n*
Controls	100%	100%	100%	13–19
5 mM BDM	51 ± 1%	73 ± 1%	111 ± 7%	19
4 mM P_i	51 ± 1%	81 ± 2%	48 ± 1%	15
pCa 6.0	53 ± 3%	91 ± 1%	104 ± 9%	13

The means ± SEMs are shown.
* Number of observations. Each test was paired with a control.

Effects on the initial mechanical reaction

The sub-second reactions such as shown in Fig. 5 (left) were analyzed by the curve-fitting procedure described in Materials and Methods, and the results are shown in Fig. 7. Consistent with the analysis in the above section, the tension during the steady contraction (T_∞) was suppressed to ~50% by any of 5 mM BDM, 4 mM P_i, or *p*Ca 6.0. BDM at this concentration, however, had no significant effect on the rate (k_r^*) of the relaxation immediately after release of ATP or on the rate (k_c) of the following contraction. P_i or low Ca^{2+} did not greatly affect k_r^*, like BDM, but they significantly increased k_c, unlike BDM. Similar results were obtained at different concentrations of BDM (10–15 mM), P_i (10 mM), and Ca^{2+} (*p*Ca 5.8–5.9). The results with P_i and low Ca^{2+} were consistent with those in the previous reports (Hibberd et al., 1985; Horiuti et al., 1992, 1994a).

In the above experiments at pCa ≈ 4.5, 10 mM P_i appeared to slow k_r^* slightly, although the effect was not statistically significant. However, the previous short report by Lenart et al. (1989) suggested that BDM accelerated the cross-bridge detachment in the absence of Ca^{2+}. Zhao and Kawai (1994) also suggested that BDM increased the affinity of ATP to the cross-bridges. Thus, we conducted an experiment in the absence of Ca^{2+}, where the ATP-induced reaction showed no large contraction after the relaxation (Fig. 8), so that we could directly measure the relaxation $t_{1/2}$ without fitting curves.

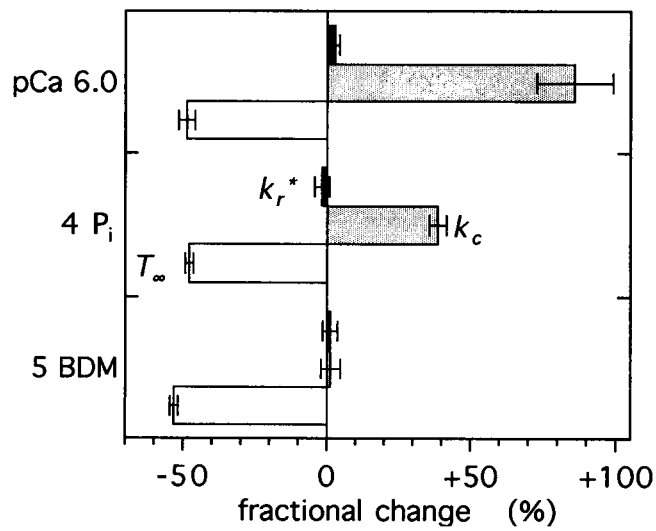


FIGURE 7 Effects of 5 mM BDM (n = 13), 4 mM P_i (n = 9), and pCa 6.0 (n = 7) on the rate of the initial relaxation (k_r^*; *filled bars*), the rate of the following contraction (k_c; *shaded bars*), and the magnitude of tension during the steady contraction (T_∞; *open bars*). The bars indicate the fractional changes from the paired controls. The data of k_r^* and k_c are those extracted from the stiffness records. ATP (1.1–1.4 mM) was released from caged ATP (4.0–5.6 mM) by single 200-J flashes, and the control values of k_r^* and k_c were 34–38 s^{-1} and 11–13 s^{-1}, respectively.

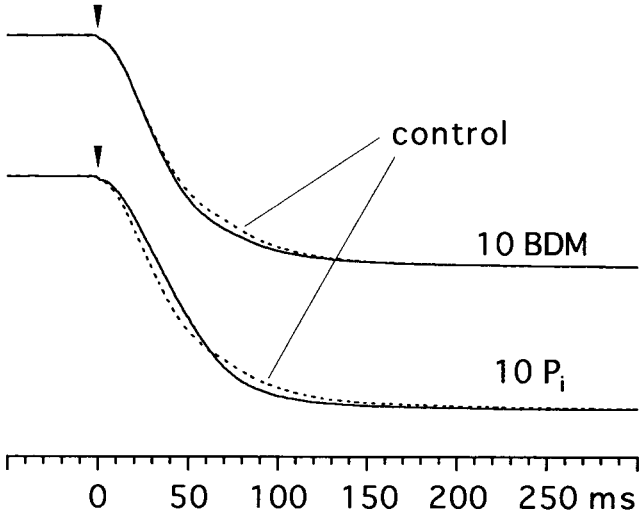


FIGURE 8 The tracings of stiffness, one (*top*) in the presence of 10 mM BDM and the other (*bottom*) in the presence of 10 mM P_i. The two dotted curves represent the same control record obtained in the absence of BDM and P_i. 0.6 mM ATP was released by the 100-J flash from 4.1 mM caged ATP at time 0 (*arrowheads*) in the absence of Ca^{2+}. The tracings are averages of 4 records from 4 fibers. Although the sizes of stiffness before flash were slightly different from each other (±2%), the tracings in this figure were scaled so that they have the same size before flash.

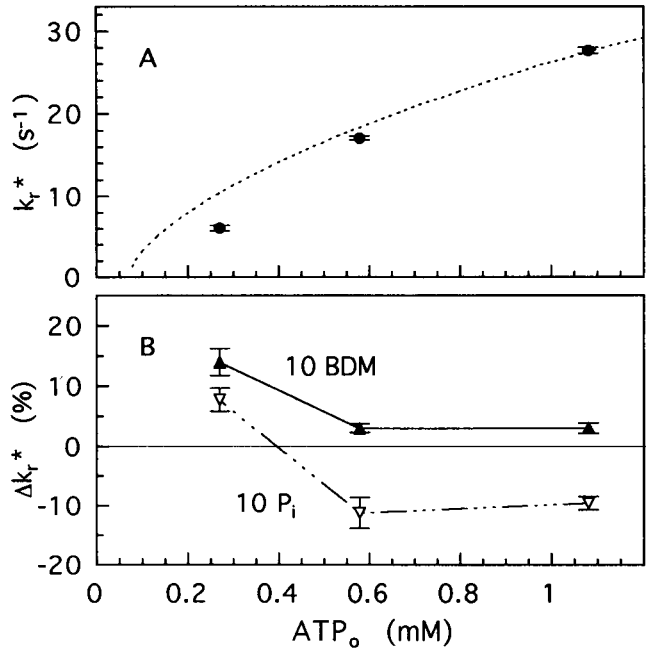


FIGURE 9 ATP dependence of the rate of relaxation in the absence of Ca^{2+}. The ordinate in (*A*) represents the apparent rate (k_r^*) of relaxation, which was derived from the $t_{1/2}$ of the stiffness relaxation under the control conditions, and that in (*B*) represents its relative change (Δk_r^*) induced by 10 mM BDM or 10 mM P_i. The abscissa indicates the concentration of the photoreleased ATP ($[ATP]_o$). The experiments (n = 4–10) were performed with 4.1 mM caged ATP. The dotted curve in (*A*) represents the data calculated from a model with $k_{+T} = 7.5 \times 10^4$ M^{-1} s^{-1} (see text for details).

Fig. 8 shows a set of relaxation records in the 100 J trials. BDM at 10 mM slightly accelerated the late phase of the sigmoid relaxation but did not influence its early phase. On the other hand, 10 mM P_i accelerated the late phase and slowed the early phase. The effect of P_i on the early phase would be an actual effect on the time course of relaxation. However, those of P_i and BDM on the late phase could be spurious: even in the absence of Ca^{2+}, there remains some contractile activity, which becomes more distinct at lower $[ATP]_o$ (Goldman et al., 1984a; Sakoda and Horiuti, 1992). P_i and BDM probably suppressed those small "low ATP" contractions, resulting in the apparently accelerated relaxation.

The rate of relaxation (k_r^*) was calculated as ln(2)/(relaxation $t_{1/2}$) in the Ca^{2+}-free experiment, and the summary of the results is illustrated in Fig. 9. The rate k_r^* decreased with reduction in $[ATP]_o$ (Fig. 9 *A*). Using the model described in Materials and Methods, we also estimated the second-order rate constant for ATP binding (k_{+T}) from the $t_{1/2}$-$[ATP]_o$ relation, and the best-fit k_{+T} was 7–8 × 10^4 M^{-1} s^{-1} (in the control condition). (In our estimation we did not use the data at 0.27 mM ATP in Fig. 9 *A*. Relaxation at this $[ATP]_o$ was too slow for the model to be fitted to, which could be due to a significant size of the low ATP contraction.) According to the argument by Sleep et al. (1994), our k_{+T} would be an underestimate by a factor of ~3, because of the binding of caged ATP to the cross-bridges.

As in Fig. 9 *B*, 10 mM BDM increased the k_r^* derived from $t_{1/2}$ at all three $[ATP]_o$, and it was greatest at the lowest $[ATP]_o$. This effect of BDM could be due to suppression of such small low ATP contractions as mentioned above. P_i at 10 mM slowed k_r^* by ~10% at relatively high $[ATP]_o$, and this decrease in k_r^* corresponded to an 18% decrease in k_{+T} in the model. The opposite effect of P_i at the lowest $[ATP]_o$ was likely related to the low ATP contraction again.

DISCUSSION

In this section we first argue the validity of our method for measurement of the ATPase activity in skinned muscle fibers and then discuss the action of BDM on the cross-bridges, comparing with those of P_i and low Ca^{2+}.

Measurement of ATPase activity

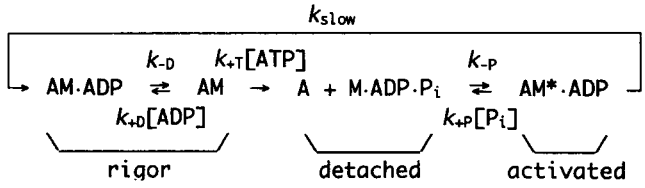
We varied [ATP] at t = 0 ($[ATP]_o$) in the muscle fiber to measure the $t_{1/2}$ to rigor, and took the slope ($\Delta[ATP]_o/\Delta t_{1/2}$) of the $t_{1/2}$-$[ATP]_o$ relation as the rate of the steady-state ATP hydrolysis. Adoption of this method implicates the following assumptions: 1) The concentration of the ATP remaining at the half-rigor ($[ATP]_{t1/2}$) is negligibly low compared with that of the initially released ATP, and 2) the rate of ATP hydrolysis near rigor ($t \leqq t_{1/2}$) is little affected by the ADP and P_i that are produced from the initial ATP.

Assumption 2 seemed to be appropriate in the present study. Because $[ATP]_o \approx 1.7$ mM at most, the concentra-

tions of products (ADP and P_i) at $t \leqq t_{1/2}$ must be less than that. Inhibition of the myofibrillar ATPase by such [ADP] and [P_i] is small, according to the previous reports (Hoar et al., 1987; Kawai et al., 1987). This was supported by the very linear $t_{1/2}$-$[ATP]_o$ relation in Fig. 3 *B*: because more products are accumulated at higher [ATP], the slope of the $t_{1/2}$-$[ATP]_o$ relation would have been less steep at higher $[ATP]_o$ if the product inhibition had been substantial. In fact, 1.2 mM ADP added in the photolysis solution increased the $t_{1/2}$ only by ~6%, and 4 mM added P_i showed no detectable effect on the $t_{1/2}$. It should be noted that the effect of the product inhibition on the measured ATPase activity is in the direction opposite that of the other effect of ADP, which will be discussed below.

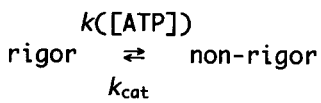
Was the other assumption (1) also reasonable? ADP, the hydrolysis product, would compete with ATP (Dantzig et al., 1991; Horiuti et al., 1993), so that $[ATP]_{t1/2}$ might have been substantially elevated by the accumulated ADP when $[ATP]_o$ was relatively high.

The following scheme represents a simplified cycle of the cross-bridge reaction (Dantzig et al., 1991; Kawai and Halvorson, 1991):



Scheme I

where A and M represent actin and myosin, $k_{\pm X}$ are the rate constants for association and dissociation of the ligand X (T = ATP, D = ADP, and P = P_i). k_{slow} is the rate of isomerization of the ADP-bound cross-bridges, which limits the rate (k_{cat}) of the overall cycle at supramaximal [ATP]. In a situation where the photoreleased ATP is being depleted and the fiber is entering the rigor states, we may lump the above cross-bridge states into two states with different stiffnesses, i.e., rigor (AM·ADP + AM) and non-rigor (detached + activated):



Scheme II

Generally, the two-step reaction $[A_1 \overset{k_1}{\longleftrightarrow} A_2 \overset{k_{+2}}{\longrightarrow} \cdots]$ has two relaxation rates, $\Sigma k \cdot [1 \pm \sqrt{\{1 - 4k_{+1}k_{+2}/(\Sigma k)^2\}}]/2 \approx \Sigma k$ and $k_{+1}k_{+2}/\Sigma k$, where $\Sigma k = k_{+1} + k_{-1} + k_{+2}$. The smaller one ($k_{+1}k_{+2}/\Sigma k$) of the two approximates would correspond to the apparent, first-order rate constant for the outflow from [A_1 plus A_2] on a slow time base. Applying this kinetics to [AM · ADP ↔ AM → · · ·] in Scheme I, we find the ATP dependence of the apparent k in Scheme II as the following:

$$k([ATP]) \approx \left\{\frac{1}{k_{-D}} + \frac{1 + [ADP]/K_D}{k_{+T} \cdot [ATP]}\right\}^{-1}, \quad (1)$$

where $K_D = k_{-D}/k_{+D}$, $[ADP] = [ATP]_o - [ATP]$.

At the time of half-rigor in Scheme II, [rigor] ≈ [non-rigor], and d[rigor]/dt > 0. Therefore, $k_{cat} > k([ATP])$ at $t = t_{1/2}$, because d[rigor]/dt = k_{cat} · [non-rigor] − $k([ATP])$ · [rigor]. Replacing $k([ATP])$ with the right-hand side of Eq. 1, one obtains an upper limit of $[ATP]_{t1/2}$:

$$[ATP]_{t_{1/2}} < \frac{[ATP]_o + K_D}{1 + K_D \cdot k_{+T}\,(1/k_{cat} - 1/k_{-D})} < \frac{[ATP]_o + K_D}{K_D \cdot k_{+T}/k_{cat}},$$

and thus,

$$\frac{[ATP]_{t_{1/2}}}{[ATP]_o} < k_{cat}\left\{\frac{1}{K_D \cdot k_{+T}} + \frac{1}{k_{+T} \cdot [ATP]_o}\right\}. \quad (2)$$

Note that $1/k_{cat} \gg 1/k_{-D}$ because $k_{cat} < 2\ s^{-1}$ (see below) and $k_{-D} \approx 30\ s^{-1}$ under our conditions (Horiuti et al., 1993, 1994b).

According to the previous studies (15–20°C) with various methods (Ferenczi et al., 1984; Glyn and Sleep, 1985; Kawai et al., 1987; Potma et al., 1994), k_{cat} at our experimental temperature (~10°C) would be $<2\ s^{-1}$ (consistent with the 1.4 s^{-1} we obtained in this study). [$K_D \cdot k_{+T}$] has been estimated from the effect of ADP (Dantzig et al., 1991), being 20–30 s^{-1} under our experimental conditions (Horiuti et al., 1993, 1994b; Kagawa and Horiuti, unpublished observation). k_{+T} is 5–8 × $10^4\ M^{-1}\ s^{-1}$ at ~10°C in the presence of caged ATP (Horiuti et al., 1994b; this study). Evaluating Eq. 2 with these values, we find that $[ATP]_{t1/2}/[ATP]_o$ is definitely less than 14% and 12% at $[ATP]_o$ = 1 and 2 mM, respectively, in our control experiments. In the test experiments with BDM, P_i, or low Ca^{2+}, k_{cat} must be even slower than in the control, and the nucleotide binding step does not seem to be greatly affected (this study). Thus, the same argument would be also applicable to the test contractions.

Effects of BDM compared with P_i and low Ca^{2+}

We examined the effects of BDM, comparing with those of P_i and low Ca^{2+}, on tension, stiffness, and ATPase activity during the steady contraction, and on the ATP-induced mechanical transitions. Although reduction in the steady tension was common to all the three, their modes of action were different from each other.

Tension per ATPase during the steady contraction

Both BDM and low Ca^{2+} decreased tension and ATPase activity, keeping their ratio approximately constant (Fig. 6 *B*, Table 1). It has been assumed in Scheme I that the AM*·ADP cross-bridges in the activated state support the

contractile force, and that the slow isomerization (k_{slow}) from this to the next state (AM*·ADP → AM·ADP) is the rate-limiting step of the overall ATPase cycle (Dantzig et al., 1991; Kawai and Halvorson, 1991). Therefore, the simplest interpretation of the above observations is as follows: both BDM and low Ca^{2+} somehow reduce the AM*·ADP population, resulting in the proportional decrease in the flow through the k_{slow} step.

In Scheme I, P_i accelerates the backward rate ($k_{+P} \cdot P_i$) of the step between the detached and activated states. This would decrease the activated cross-bridges and thus could reduce the ATPase rate, like BDM and low Ca^{2+}. However, in the experiments with P_i, suppression of the ATPase rate was much less than that of tension. P_i at 4 mM decreased tension per ATPase to about half that of the control (Fig. 6 *B*, Table 1).

The chemo-mechanical model of Pate and Cooke (1989b) explains, at least to some extent, the discrepancy between tension and ATPase in the presence of P_i. Their model takes account of distortion (x) of the cross-bridges from the elastic equilibrium point. The P_i-releasing transition for activation (A + M·ADP·P_i → AM*·ADP + P_i) is fast when the x value is large, but the ATP-induced detachment (ATP + AM*·ADP → A + M·ADP·P_i + ADP) is fast when x is small or negative, so that the cross-bridges with large x properly produce large force with small ATP consumption. Addition of P_i in the system suppresses formation of these high-force low-ATPase cross-bridges, so that it reduces tension per ATPase in the model.

Although the above explanation for the tension-ATPase dissociation is reasonable, we should not overlook possible side effects of P_i on steps other than the P_i-releasing one. In fact, as will be discussed later, P_i seems to compete with ATP under low [ATP] conditions. Furthermore, P_i must have another side effect at high [ATP], because the shortening velocity at very low loads is increased by P_i at 4 mM ATP (Pate and Cooke, 1989a). It would be even possible that P_i somehow inhibits the force production by the individual, activated cross-bridges.

Tension per stiffness

When the steady contraction was suppressed, tension per stiffness was decreased (Fig. 6 *A*, Table 1). If the myofilaments were much stiffer than the cross-bridges, the overall stiffness of the muscle fiber would be proportional to the number of the attached, force-generating cross-bridges. However, recent x-ray diffraction studies (Huxley et al., 1994; Wakabayashi et al., 1994) indicate that the myofilaments are as compliant as the cross-bridges in maximally activated fibers. When the number of the attached cross-bridges is decreased by half, the fiber stiffness may remain at ~⅔ (Goldman and Huxley, 1994), tension per stiffness being as low as ~¾.

The different tensions per stiffness among BDM, P_i, and low Ca^{2+}, however, are not explained by the filament compliance. In our study, if tension was inhibited to the same extent (Table 1), the decrease in tension per stiffness was the smallest with low Ca^{2+}, intermediate with P_i, and the largest with BDM. Even if low Ca^{2+} may only induce detachment of the cross-bridges, P_i and BDM seem to affect tension or stiffness of the cross-bridges that are left attached.

To explain the low tension per stiffness in the presence of P_i, it has been suggested that there is an attached state with little force, which is located immediately before the P_i-releasing step (Brozovich et al., 1988; Pate and Cooke, 1989b; Horiuti et al., 1992; Martyn and Gordon, 1992). In the model with such an attached state (e.g., Scheme III, below), P_i increases its population relative to that in the next, force-generating state, resulting in a low tension per stiffness. Because BDM was likely to reduce the population in the force-generating state, it would be plausible that this agent, like P_i, reduced tension per stiffness by shifting backward the equilibrium of the P_i-releasing step (such as c in Scheme III). It is not clear why the effect of BDM on tension per stiffness was even larger than that of P_i.

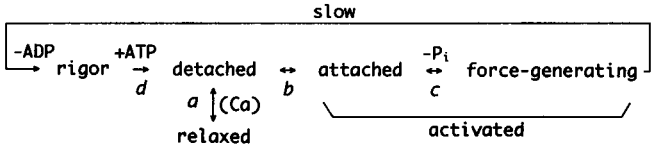
Rate of contraction in the ATP-induced reaction

The rate constant (k_c) for the contraction development after ATP release was increased by P_i (Fig. 7), which is consistent with previous studies (Hibberd et al., 1985; Horiuti et al., 1992). In Scheme I, k_c is equal to $\{k_{-P} + k_{+P}[P_i]\}$, so that the acceleration of k_c by P_i has been taken as evidence supporting the model (Hibberd et al., 1985).

The reduction in [Ca^{2+}] resulted in a twofold increase in k_c (Fig. 7), which is consistent with the results in our previous study (Horiuti et al., 1994a). However, this effect of low Ca^{2+} cannot be easily explained in Scheme I, because low Ca^{2+} is supposed to slow the release of P_i (Millar and Homsher, 1990), and because the tension development induced by Ca^{2+} jumps is indeed slower at lower [Ca^{2+}] (Moisescu, 1976; Ashley et al., 1991; Araujo and Walker, 1994).

The different effects of low Ca^{2+} on the rates of ATP- and Ca^{2+}-induced contractions have been interpreted in two ways (Horiuti et al., 1994a). One is a "cooperative" interaction among the cross-bridges in Scheme I. If the cross-bridges in the rigor state accelerate the Ca^{2+}-sensitive, P_i-releasing transition from the detached to the activated state (Goldman et al., 1984b; Millar and Homsher, 1990), this cooperative mechanism will work in the initial phase of the ATP-induced contraction in the low Ca^{2+} condition, resulting in a k_c higher than that without the cooperativity.

The other interpretation of the effects of Ca^{2+} is the presence of a side branch for the Ca^{2+} regulation (Horiuti et al., 1994a):



Scheme III

(Although an attached state with little force is inserted for the above argument about tension/stiffness, this is not essential for the current argument about k_c.) In the Ca^{2+}-induced contraction, the relaxed cross-bridges reach the activated state through the path regulated by Ca^{2+}. On the other hand, in the ATP-induced contraction, the path for the rigor cross-bridges to the activated state is not directly regulated by Ca^{2+}. If [Ca^{2+}] is low at the ATP activation, some of the cross-bridges initially in the detached state come down to the relaxed state in time through the Ca^{2+}-regulated step, and this results in an increased k_c (Horiuti et al., 1994a).

An interesting observation in the present study was that k_c was little changed by 5 mM BDM(Fig. 7), which appears to be in contrast to previous studies. In the study by Fryer et al. (1988) with mammalian skinned fibers, 10 mM BDM slowed the Ca^{2+}-activated contraction by a factor of 2–4. In the study by Bagni et al. (1992), 3 mM BDM slowed the rate of tension redevelopment 4–5 times after a large shortening of tetanized frog muscle fibers. It should be noted that in all three studies tension was inhibited to about the same extent, to half that of the control.

Judging from the effects of BDM that we observed in the steady contraction, the slowing of the Ca^{2+}-activated contraction by BDM seems to be due to its inhibition of the P_i-related steps in Schemes I or III. However, this is not readily compatible with the very small effect of BDM on k_c in our study. Here we discuss the contradictory effects of BDM using the modified models that we mentioned in the above.

1) Under the hypothesis of the cooperative interaction, the following would be inferred. The Ca^{2+}-induced contraction is slowed by BDM because of its inhibitory effect on the P_i-releasing, force-generating step. If the cooperative interactions had not accelerated this step, the rate (k_c) of the ATP-induced contraction would have also been slowed by BDM in our experiments. Thus, the fact that BDM did not slow k_c is a support for the cooperativity hypothesis.

2) In the case of the model in Scheme III: The effect of BDM on the ATP-induced contraction was different from that on the Ca^{2+}-induced one. Among the related reaction steps (*d*, *a* and *b* + *c*), the ATP-binding step (*d*) from rigor to detached state does not seem to be influenced by BDM (see below), and the transition (*b* + *c*) from the detached to the activated state is common to the two contractions. Therefore, the Ca^{2+}-regulated transition (*a*) from the relaxed to the detached state must have been influenced (or slowed) by BDM. If BDM had only this effect, k_c was probably increased by BDM, as by low Ca^{2+}. Consequently, it is likely that BDM also slowed the transition (*b* + *c*) from the detached to the activated state.

Rate of relaxation immediately after release of ATP

The rate of relaxation (k_r^*) observed on photolysis of caged ATP would be smaller than k_{+T}[ATP] in Scheme I, because the release of ATP from caged ATP is not quite fast (~40 s^{-1} under our conditions; Goldman et al., 1982) and because caged ATP binds to the cross-bridges, competing with ATP ($K_i \approx 1.6$ mM; Sleep et al., 1994). However, k_r^* can still be an index of the rate of binding of ATP to the cross-bridges. In fact, this rate is dependent on $[ATP]_o$ (Fig. 9) and is decreased by added ADP (Dantzig et al., 1991; Horiuti et al., 1994b).

When we derived k_r^* from the contraction records in the presence of Ca^{2+}, this did not seem to be affected by BDM, P_i, or low Ca^{2+} (Fig. 7). However, the experiment in the absence of Ca^{2+} revealed that 10 mM P_i reduces k_r^* slightly but significantly (by about 10%; Fig. 9). This effect of P_i cannot be explained by the action of P_i on the P_i-releasing step in Scheme I or III. Pate and Cooke (1989a) inferred competition of P_i with ATP at the ATP-binding step from their result that P_i decreased the shortening velocity at low [ATP] (50 μM). Their idea may be consistent with the observation by Endo et al. (1989) that the isometric tension is slightly but significantly enhanced by an appropriate amount of P_i. The slowdown of k_r^* by P_i found in this study may be because of the P_i action on the ATP-binding step.

BDM showed no significant effect on the ATP-induced detachment. Although it appeared that BDM slightly accelerated k_r^* in the absence of Ca^{2+} (Fig. 9), this was likely a spurious effect from suppression of the remnant "low-ATP" contraction. Our result appears to be inconsistent with a conclusion in the previous study by Zhao and Kawai (1994). They found in the sinusoidal analysis that the ATP-dependent, fastest component of the mechanical transient ("process D") is markedly accelerated by BDM and interpreted this result as evidence for the action of BDM on the binding of ATP to the cross-bridges. Their observations do not conflict with ours; they did not directly examine the ATP-induced reaction, whereas we did not examine the very fast mechanics in process D.

Relation of the present results with the biochemical data on BDM

In this study, we examined effects of BDM on the ATP-induced mechanical reaction of the cross-bridges. If we take Scheme III to interpret the results, the reaction steps supposed to be inhibited by BDM are *a*, *b*, and *c*. If we take Scheme I, BDM is supposed to inhibit the P_i-releasing step. In any case, the overall effect of BDM is the shift of the cross-bridge population in the state of active force generation back to the non-force-generating state(s) after the binding of ATP. This would be the reason for the proportional decreases in ATPase activity and tension under the isometric condition.

Higuchi and Takemori (1989) examined the effects of BDM on the heavy-meromyosin ATPase from rabbit white muscles. BDM slowed the steady-state rate of the ATPase ($M + ATP \rightarrow M + ADP + P_i$) but did not affect the size of its initial P_i burst ($M + ATP \rightarrow M{\cdot}ADP{\cdot}P_i$). The authors referred to the possibility that a stabilization of $M{\cdot}ADP{\cdot}P_i$

was the main action of BDM for its inhibitory effect on the myofibrillar contraction. Herrmann et al. (1992) studied the rapid kinetics of the myosin subfragment-1 ATPase in the presence of BDM:

$$M + ATP \overset{k}{\rightarrow} M{\cdot}ATP$$

$$\overset{K_3}{\leftrightarrow} M{\cdot}ADP{\cdot}P_i \overset{k_4}{\rightarrow} M{\cdot}ADP + P_i \overset{k_6}{\rightarrow} M + ADP + P_i,$$

where K_3 was increased and k_4 was decreased. The same authors also examined the Ca^{2+}-activated myofibrillar ATPase and found that BDM little affected the amplitude of its initial burst ($AM + ATP \rightarrow A/M{\cdot}ADP{\cdot}P_i$).

Although there are few biochemical data on the rapid kinetics of myofibrillar or actomyosin ATPase in the presence of BDM, it is true that the hypothesis of $M{\cdot}ADP{\cdot}P_i$ stabilization is generally consistent with our mechanical results: BDM had little effect on the ATP binding kinetics but slowed the P_i-related reactions to inhibit the ATPase rate. It should be noted, however, that it is still unclear why tension per stiffness in the presence of BDM was lower than that in the presence of P_i or low Ca^{2+}. According to the electron paramagnetic resonance study by Zhao et al. (1995), BDM induced a population of cross-bridges that were attached but with little force and whose orientation was disordered, as in the relaxing muscle. It would be interesting to compare BDM, P_i, and low Ca^{2+} with each other in such electron paramagnetic resonance experiments.

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