

Tension Transients Initiated by Photogeneration of MgADP in Skinned Skeletal Muscle Fibers

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ABSTRACT Addition of MgADP to skinned skeletal muscle fibers causes a rise in Ca^{2+} -activated isometric tension. Mechanisms underlying this tension increase have been investigated by rapid photogeneration of ADP within skinned single fibers of rabbit psoas muscle. Photolysis of caged ADP (P^2 -1(2-nitrophenyl)ethyladenosine 5'-diphosphate) resulted in an exponential increase in isometric tension with an apparent rate constant, k_{ADP} , of $9.6 \pm 0.3 \text{ s}^{-1}$ (mean \pm SE, $n = 28$) and an amplitude, P_{ADP} , of $4.9 \pm 0.3\% \text{ P}_o$ under standard conditions (0.5 mM photoreleased MgADP, 4 mM MgATP, pH 7.0, pCa 4.5, 0.18 M ionic strength, 15°C). P_{ADP} depended upon the concentration of photoreleased MgADP as well as the concentration of MgATP. A plot of $1/P_{\text{ADP}}$ vs. $1/[\text{MgADP}]$ at three MgATP concentrations was consistent with competition between MgADP and MgATP for the same site on the crossbridge. The rate of the transient, k_{ADP} , also depended upon the concentration of MgADP and MgATP. At both 4 and 1 mM MgATP, k_{ADP} was not significantly different after photorelease of 0.1–0.5 mM MgADP, but was reduced by 28–40% when 3.5 mM MgADP was added before photorelease of 0.5 mM MgADP. k_{ADP} was accelerated by about twofold when MgATP was varied from 0.5 to 8 mM MgATP. These effects of MgATP and MgADP were not readily accounted for by population of high force-producing states resulting from reversal of the ADP dissociation process. Rather, the results suggest that competition between MgADP and MgATP for crossbridges at the end of the cycle slows detachment leading to accumulation of force-generating crossbridges. Elevation of steady-state P_i concentration from 0.5 to 30 mM caused acceleration of k_{ADP} from 10.2 ± 0.5 to $27.8 \pm 1.8 \text{ s}^{-1}$, indicating that the tension rise involved crossbridge flux through the P_i dissociation step of the cycle.

INTRODUCTION

Muscle contraction results from cyclic interactions between myosin heads in the thick filament and actin in the thin filament. These interactions are driven by the hydrolysis of ATP (H. E. Huxley, 1969; A. F. Huxley, 1974; Taylor, 1979), which occurs in four major biochemical steps: (1) the binding of ATP to acto-myosin (AM), (2) the cleavage of ATP, (3) the release of inorganic phosphate (P_i), and (4) the

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release of adenosine 5'-diphosphate (ADP) (Taylor, 1979; Eisenberg and Hill, 1985; Hibberd and Trentham, 1986; Goldman, 1987). It is of interest to define how these specific biochemical steps are coupled to mechanical crossbridge transitions.

P_i has been shown to reduce isometric tension and accelerate rates of force development (Hibberd, Dantzig, Trentham, and Goldman, 1985, and references therein), leading to the idea that force-producing conformational changes in the crossbridge are coupled to P_i dissociation (Hibberd and Trentham, 1986; Pate and Cooke, 1989*b*). Isotope exchange measurements have demonstrated that P_i dissociation is readily reversible in isometrically contracting skinned fibers (Webb, Hibberd, Goldman, and Trentham, 1986; Bowater and Sleep, 1988; Pate and Cooke, 1989*b*). This reversibility was exploited in caged P_i measurements, which permitted the kinetics of P_i dissociation and associated force transitions to be examined in some detail (Dantzig, Goldman, Laktis, Millar, and Homsher, 1992; Walker, Lu, and Moss, 1992).

MgADP has been viewed as a competitive inhibitor of MgATP-induced crossbridge detachment in order to explain its ability to reduce the maximum velocity of muscle shortening (Cooke and Pate, 1985; Pate and Cooke, 1989*a*). MgADP has also been shown to increase active isometric force in skinned fibers (Cooke and Pate, 1985; Hoar, Mahoney, and Kerrick, 1987), although the mechanism of this effect is unknown. Recent evidence has suggested that MgADP alters mechanical properties of crossbridges, including an increase in both the average lifetime and the average force borne by crossbridges during isometric contraction (Dantzig, Hibberd, Trentham, and Goldman, 1991; Seow and Ford, 1992; Lu, Z., J. M. Metzger, R. L. Moss, and J. W. Walker, manuscript in preparation).

The goal of this work was to determine whether caged ADP photolysis can be used to probe the kinetics of ADP dissociation in actively contracting muscle fibers. ADP dissociation from force-generating crossbridges must be somewhat reversible for this to be possible, but there is no clear evidence to date that ADP release steps are reversible (Sleep and Hutton, 1980; Dantzig and Goldman, 1985). Photolysis of caged ADP was used to create rapid and uniform changes of MgADP within contracting fibers, and rates and amplitudes of the resultant tension transients were evaluated. The dependence of the amplitude of the tension transients on the concentration of MgADP and MgATP indicated that these nucleotides compete for binding to crossbridges at the end of the cycle. However, the observed dependence of the rate on the concentration of MgADP and MgATP was complex. Acceleration of the caged ADP tension transient by P_i suggested the involvement of P_i release steps in the pathway of force development in response to rapid elevation of MgADP.

Some of the results have been presented in preliminary form to the Biophysical Society (Lu, Walker, and Moss, 1991).

METHODS

Muscle Preparation

Male New Zealand rabbits (2.5–3.5 kg body weight) were killed by cervical dislocation. Psoas muscles were quickly excised, dissected into small bundles, and tied with surgical silk to capillary tubes. Fiber bundles were chemically permeabilized (i.e., skinned) in a 50% (vol/vol)

glycerol solution containing (mM): 100 KCl, 5.6 MgCl₂, 5 ATPNa₂, 5 EGTA, and 20 imidazole, pH 7.00 ± 0.01 for 24 h at 4°C, and then stored at -20°C in the skinning solution for 2–21 d. Each bundle was bathed for 20 min in cold relaxing solution containing 0.5% (wt/vol) Brij 58 to eliminate functional sarcoplasmic reticulum before use. Single fibers were then pulled free from the end of the bundle and tied to the force transducer as described (Moss, Giulian, and Greaser, 1985). The physiological apparatus for carrying out isometric force measurements in conjunction with pulse photolysis has been described (Walker et al., 1992). Fiber segments were 2.0–2.5 mm long and 60–100 μm in diameter, and sarcomere length was adjusted to 2.5–2.6 μm and measured by photography.

Solutions

Biochemicals including creatine kinase (type I, rabbit muscle) and P¹,P⁵-di(adenosine-5')penta-phosphate (AP₅A) were purchased from Sigma Chemical Co. (St. Louis, MO). Caged ADP (P²-1(2-nitrophenyl)ethyladenosine 5'-diphosphate) and caged AMP (P-1(2-nitrophenyl)ethyladenosine 5'-monophosphate) were synthesized according to the method of Walker, Reid, McCray, and Trentham (1988), and purified by DEAE-cellulose chromatography using a linear gradient of 0–0.5 M triethylammonium bicarbonate. Contamination of caged ADP by free ADP or caged AMP by free AMP was <1 mol% as judged by analytical HPLC (Walker, Reid, and Trentham, 1989).

Activating solutions had the following composition (mM): 5 ATP (4 MgATP), 9 creatine phosphate, 5.6 MgCl₂ (1 free Mg²⁺), 12 EGTA, 100 N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), and sufficient KCl to obtain a final ionic strength of 180 mM. Solution pH was adjusted to 7.00 with KOH. pCa 4.5 solution contained 12 mM CaCl₂ and 40 mM KCl, and pCa 9 solution contained no added CaCl₂ and 66 mM KCl. Stability constants for metal and proton binding to creatine phosphate, phosphate, ADP, ATP, and EGTA were taken from Fabiato (1988). The apparent stability constant for Ca-EGTA after correction for temperature (15°C), ionic strength, and pH was $2.39 \times 10^6 \text{ M}^{-1}$.

Photolysis efficiency was measured by photolyzing different concentrations of caged ADP with a single flash in the physiological apparatus and analyzing for percent of conversion of caged ADP to free ADP using analytical HPLC (Walker et al., 1989). The relationship between moles of ADP and moles of total caged ADP (0–5 mM caged ADP) was approximately linear with a slope of 0.25 (i.e., 25% photolysis per flash). The final concentration of MgADP achieved after photolysis was calculated from the free Mg²⁺ concentration, the concentration of photogenerated ADP, and a MgADP stability constant of $6.24 \times 10^2 \text{ M}^{-1}$. Thus, for photogeneration of MgADP, 1.0, 2.6, and 5.2 mM caged ADP were required to obtain 0.10, 0.25, and 0.50 mM MgADP, respectively. Caged ADP was assumed to bind Mg²⁺ with the same stability constant as caged ATP, $3.3 \times 10^2 \text{ M}^{-1}$ (Walker et al., 1988). Uncertainty in the Mg-caged ADP stability constant may cause up to a 20% error in the calculated values for MgADP. This was determined by assuming that the outside limits of this stability constant were 1 and $6.24 \times 10^2 \text{ M}^{-1}$.

The free MgADP before photolysis in contracting fibers was estimated as follows. If the creatine kinase reaction is near equilibrium, then $[\text{MgADP}] = [\text{MgATP}][\text{creatine}]/[\text{creatine phosphate}][\text{K}_{\text{eq}}] = (4 \text{ mM})(2 \text{ mM})/(7 \text{ mM})(140) = 0.01 \text{ mM}$ (Meyer, Kushmerick, and Brown, 1982). This reaction could be significantly displaced from equilibrium by the fiber ATPase activity estimated at 1.8 s^{-1} myosin head⁻¹ or 270 μM/s at 12°C (Ferenczi, Homsher, and Trentham, 1984). The steady-state concentrations of MgADP and MgATP in the core of the fiber can be estimated using the equation $C(r) = C(0) + (r^2)k/4D$, where $C(0) = 0.01 \text{ mM}$, $r = 0.003\text{--}0.005 \text{ cm}$ (fiber radius), $k = 0.27 \text{ mM/s}$ (ATPase rate), and $D = 2 \times 10^{-7} \text{ cm}^2/\text{s}$ (diffusion coefficient) (Cooke and Pate, 1985). Without creatine phosphate and creatine kinase, MgADP concentration can reach 3–4 mM within the fiber, with a considerable concentration gradient across the fiber. Addition of creatine kinase at 165 U/ml will reduce MgADP at 2

mM/s, suggesting adequate buffering of MgADP within the fiber. If creatine kinase reduces the net rate of MgADP accumulation by 100-fold or greater, then the MgADP concentration in the fiber core will be <0.10 mM. If creatine kinase reduces the net MgADP accumulation rate by only 10-fold, then MgADP in the core could build up to 0.3–0.9 mM. Thus, added creatine kinase is important to keep MgADP concentration low before photolysis; a conservative estimate of MgADP concentration in the fiber core without added MgADP is 0.5 mM. A gradient will also exist with MgADP approaching 0.01 mM at the fiber edge.

When high levels of MgADP were added to solutions (Figs. 3 and 4) we found it possible to keep creatine phosphate in the solutions. Two opposing factors come into play here: MgADP that is diffusing in from the surrounding solution will tend to be reduced by endogenous creatine kinase, and MgADP will build up in the innermost fiber interior due to fiber ATPase. Calculation of these factors separately gives MgADP diffusion gradients of 1–2 mM in opposite directions. Thus, addition of 2–4 mM MgADP appears to be a reasonable way to increase MgADP before photolysis, especially at 4 mM MgATP concentrations. This method may be less reliable at 1 mM MgATP.

To maintain an ionic strength of 180 mM, contributions to ionic strength from caged ADP (M^+ , A^{2-} ; Mg-caged ADP has no net charge), glutathione (M^+ , A^-), and in some experiments P_i ($\sim 1.5 M^+$, $A^{1.5-}$) were taken into account. 10 mM glutathione was included to protect cellular proteins from photolysis by-products. The rigor caged ADP solution had the following composition (mM): 5.2 caged ADP, 12 EGTA, 12 $CaCl_2$, 2.1 $MgCl_2$, 100 TES, pH 7.0, 0.1 mM AP_5A , pCa 4.5, and nominally 1.6 free Mg^{2+} . Data collection and analysis were essentially as described previously (Walker et al., 1992). Rate constants of tension transients were obtained by nonlinear least-squares fits to a single exponential [$P_t = P_o[1 - \exp(-k \times t)]$] or a double exponential [$P_t = P_1[1 - \exp(-k_1 \times t_1)] + P_2[1 - \exp(-k_2 \times t_2)]$]. Error bars represent mean \pm standard error.

RESULTS

ADP versus MgADP

Activating solutions were initially prepared with different ratios of ADP to Mg^{2+} to examine whether tension increases were due to ADP or to the MgADP complex. A solution calculated to contain 1 mM MgADP, 4 mM free Mg^{2+} , and 0.4 mM free ADP resulted in a $14 \pm 3\%$ increase in tension at pCa 4.5 compared with control solutions (4 mM Mg^{2+} , no added ADP). By comparison, a solution containing 1 mM MgADP (same as the first solution), 1 mM free Mg^{2+} , and 1.6 mM free ADP (fourfold greater than the first solution) resulted in a similar tension increase ($11 \pm 2\%$). Variations in free Mg^{2+} over the range 1–4 mM did not alter tension significantly. Thus, MgADP is likely to be the relevant species at least for tension increases at pCa 4.5. A similar conclusion was reached by Hoar et al. (1987) in studies of rabbit soleus muscle fibers.

Caged ADP Photolysis in Actively Contracting Fibers

Illustrated in Fig. 1, trace *b*, is a representative tension transient resulting from the photolysis of 5.2 mM caged ADP in a single rabbit psoas fiber maximally activated at pCa 4.5. Before photolysis, tension was allowed to develop to a steady level. The fiber and surrounding solution were then irradiated with a 1-ms flash of near-UV light (300–350 nm), which increased MgADP concentration by 0.5 mM. Isometric tension increased by 5% P_o with an approximately exponential time course and an apparent rate constant (k_{ADP}) of $9.7 s^{-1}$ (Fig. 1, trace *b*). The new tension level was maintained

for several seconds, indicating that the ATP regenerating system reduced MgADP in the fiber and surrounding solution quite slowly relative to the caged ADP tension transient. 1 s after photolysis the fiber was released by 10% of its length, allowed to shorten for several milliseconds, and then restretched to its original length (Fig. 1, trace *b*). This permitted measurements of steady-state tension and the rate of tension redevelopment. The mean values of k_{ADP} and the rate constant of tension redevelopment, k_{tr} , measured sequentially in the same fiber were 9.6 ± 0.3 and $10.7 \pm 0.5 \text{ s}^{-1}$, respectively. Sarcomere nonuniformity may be slowing the time course of tension development in these measurements (Brenner and Eisenberg, 1986). Caged ADP tension transients typically were exponential for >95% of their time courses, and k_{tr} measurements with sarcomere length control (Metzger, Greaser, and Moss, 1989) were <50% greater than values reported here, suggesting that internal shortening did not greatly influence the results.

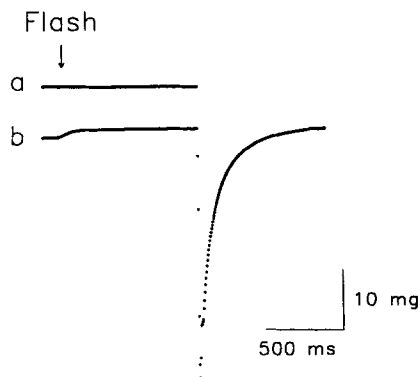


FIGURE 1. Pulse photolysis of (*a*) 5.2 mM caged AMP, or (*b*) 5.2 mM caged ADP within a skinned fiber at full Ca^{2+} activation. 1 s after photolysis the fiber was released, allowed to shorten, and then restretched for a measurement of tension redevelopment kinetics (Metzger et al., 1989). In *a*, 1.2 mM AMP was released; in *b*, 1.3 mM ADP was released, resulting in 0.5 mM MgADP. The increase in tension after photogeneration of MgADP was 5% P_{o} , and rate constants obtained from single exponential fits were $k_{\text{ADP}} = 9.7 \text{ s}^{-1}$ and $k_{\text{tr}} = 10.4 \text{ s}^{-1}$.

Possible Artifacts of the Photolysis Approach

Pulse photolysis of 5.2 mM caged AMP under similar conditions produced the same photolysis by-products as caged ADP, but caused no significant change in tension (Fig. 1, trace *a*), indicating that the increase in force in trace *b* was due to MgADP and not to the photolysis procedure or by-products. The amplitude of the tension transient, $4.9 \pm 0.3\% P_{\text{o}}$, was similar to the effect of adding 0.5 mM MgADP to the activating solution, $4.3 \pm 0.6\% P_{\text{o}}$ (in the absence of creatine phosphate, with appropriate ionic strength and Mg^{2+} adjustments). This similarity may be fortuitous because MgADP is expected to build up substantially within the fiber interior when the ATP regenerating system is removed (as it must be when adding low levels of MgADP to the solution). Diffusion gradients of MgADP across the fiber are also likely (Cooke and Pate, 1985). Introducing MgADP by photolysis of caged ADP was advantageous because it permitted the use of an ATP regenerating system to minimize build-up of MgADP before photolysis. At 9 mM creatine phosphate, 4 mM MgATP, and 165 U/ml creatine kinase, the preflash MgADP concentration was estimated to be below $\sim 0.5 \text{ mM}$ (see Methods). Photolysis also permitted the effects

of MgADP to be examined during a single contraction without solution changes, and increases in MgADP were probably more uniform within the fiber, at least immediately after photolysis. The importance of these factors is indicated by the finding that it was difficult to measure significant MgADP effects on tension below 0.5 mM MgADP without the use of caged ADP. The caged ADP compound itself at 5.2 mM had no detectable effects on the steady-state tension-pCa relationship or the response to 0.5 mM MgADP (data not shown).

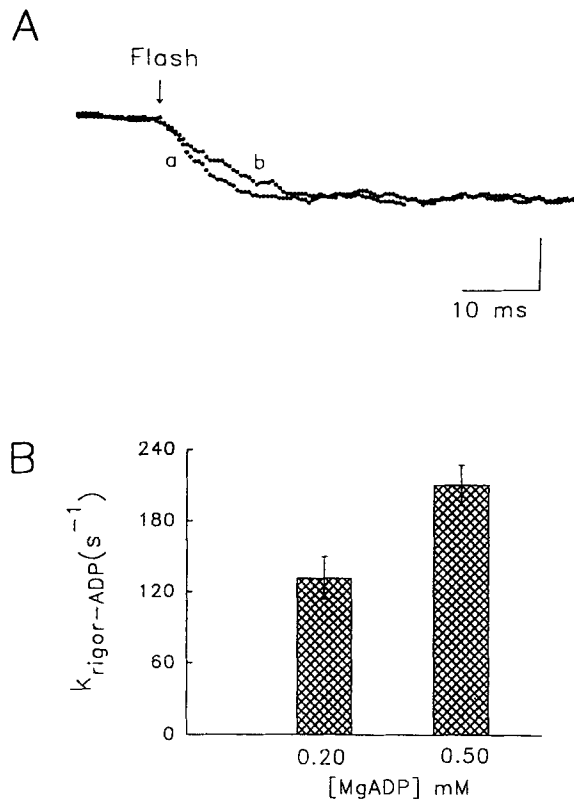


FIGURE 2. Tension transients after photolysis of caged ADP in fibers in rigor. (A) Time courses of rigor tension decline induced by (a) 0.5 mM MgADP and (b) 0.2 mM MgADP resulting from caged ADP photolysis. Vertical calibration bar represents 1.75 and 1.50 mg for a and b, respectively. (B) The apparent first-order rate constant, $k_{\text{rigor-ADP}}$, presented at the two MgADP concentrations. Data points are mean \pm SE of four measurements.

Caged ADP Photolysis in Fibers in Rigor

For comparison, tension responses resulting from photolysis of caged ADP in fibers in rigor were studied. Rigor contraction of fibers was achieved by incubation in pCa 4.5 solution without ATP or creatine phosphate, but containing 0.1 mM di(adenosine-5')pentaphosphate (AP₅A) to inhibit myokinase. Photolysis of caged ADP induced a rapid decline in rigor tension by $9.7 \pm 0.9\%$ at 0.2 mM and 0.5 mM MgADP (Fig. 2A) (percent of tension decline was normalized to the maximum active tension, P_0). Apparent rate constants for these rigor caged ADP transients ($k_{\text{rigor-ADP}}$) estimated by single exponential fits increased from 131.8 ± 18.2 to $210.8 \pm 16.9 \text{ s}^{-1}$ ($P < 0.05$,

two-tailed student's *t* test) as MgADP concentration was raised from 0.2 to 0.5 mM (Fig. 2 B). These rates and magnitudes of tension transients in rigor fibers are consistent with similar measurements made by Tanner, Vallette, Thomas, and Goldman (1989) and Dantzig et al. (1991). The rate constant, $k_{\text{rigor-ADP}}$ (measured in rigor) is at least an order of magnitude faster than k_{ADP} (measured in actively contracting fibers), indicating that (a) k_{ADP} is not limited by slow dark reactions in the photolysis of caged ADP, and (b) k_{ADP} and $k_{\text{rigor-ADP}}$ are probably measures of fundamentally different mechanical transitions.

Effects of Varying MgADP and MgATP

Fig. 3 A shows caged ADP transients at three concentrations of MgADP. The observed rate constant (k_{ADP}) at 0.5 mM MgADP was $9.6 \pm 0.3 \text{ s}^{-1}$ and was not significantly different at 0.1 mM MgADP (Fig. 4 A). However, the amplitude of the transient (P_{ADP}) increased by about threefold as MgADP was increased from 0.1 to 0.5 mM (Fig. 3). Thus, the amplitude but not the rate of the caged ADP transient depended on the MgADP concentration in this range (cf. Lactis and Homsher, 1987). This was observed whether the final MgADP was varied by altering the initial concentration of caged ADP (over a fivefold range as in Fig. 3 A) or by varying the near-UV light intensity (over a threefold range; not shown). This supports the earlier contention that caged ADP itself had no effect on steady-state force levels or on the caged ADP transient, because caged ADP concentrations were different in these two approaches.

To investigate a larger range of MgADP concentrations, the final MgADP was increased by addition of MgADP before photorelease of 0.5 mM MgADP. A small but significant decrease in k_{ADP} was observed at 2 and 4 mM MgADP using this method (Fig. 4 A). Moreover, the amplitudes of these caged ADP transients were reduced, suggesting that the MgADP effects on tension were approaching apparent saturation near 4 mM.

To examine the possibility of competition between MgADP and MgATP for binding to crossbridges, the MgATP concentration was varied and caged ADP transients were obtained at various MgADP concentrations. As shown in Fig. 3, A and B, amplitudes of caged ADP transients after photorelease of 0.1 or 0.5 mM MgADP were larger when MgATP was lowered to 1 mM. The amplitude of the transient (P_{ADP}) vs. MgADP concentration was plotted in Fig. 3 C in the form of a double reciprocal plot. The slope of these plots depended on the MgATP concentration (Fig. 3 C, *inset*), consistent with competition between MgADP and MgATP for the same site on myosin (Schoenberg and Eisenberg, 1987). It should be noted that the amplitude of the transient also depended on the addition of creatine kinase when the MgATP concentration was low (Fig. 3 B). This illustrates the importance of maintaining an adequate MgATP regenerating system in the face of MgATP hydrolysis to MgADP as well as diffusion gradients across the fiber.

ADP generated on photolysis was reduced more rapidly when creatine kinase was added. In this case, the amplitude, P_{ADP} , was taken at the peak of the tension response, and k_{ADP} was evaluated over the rising phase of the transient. This practice could lead to underestimates of the amplitude and possibly overestimates of the rate

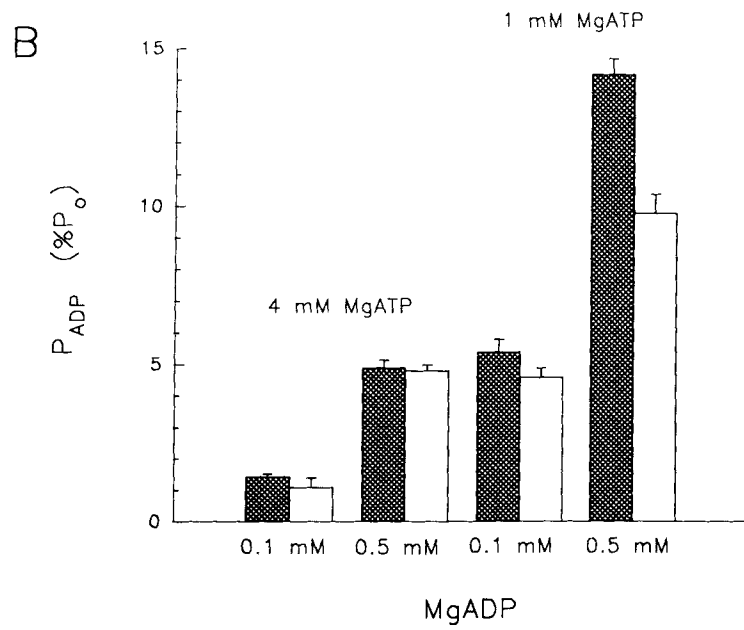
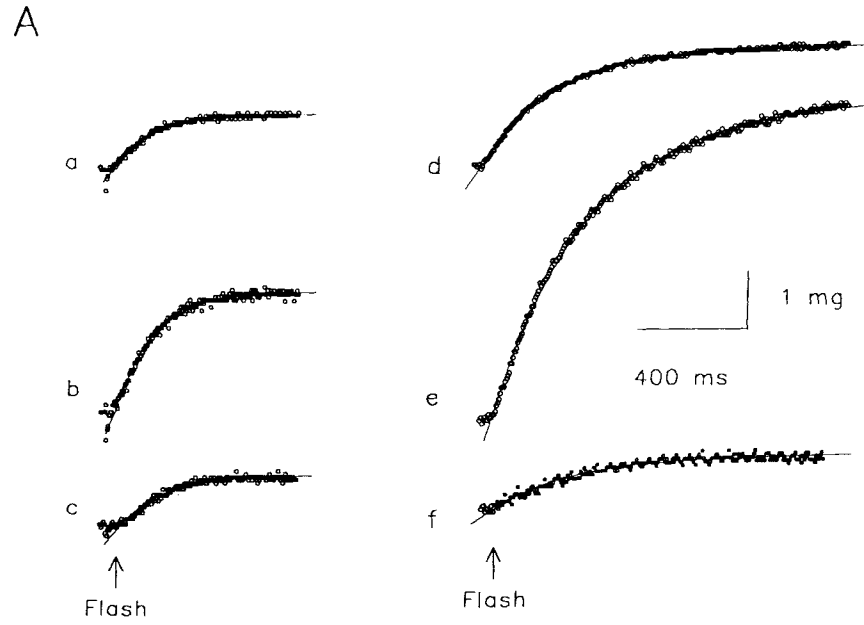
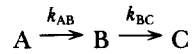


FIGURE 3

constant. The magnitudes of these errors were estimated by performing simulations of



with values of $k_{AB} = 10 \text{ s}^{-1}$ taken from the rising phase of a caged ATP transient and $k_{BC} = 0.4\text{--}0.5 \text{ s}^{-1}$ taken from the falling phase in the presence of creatine kinase.

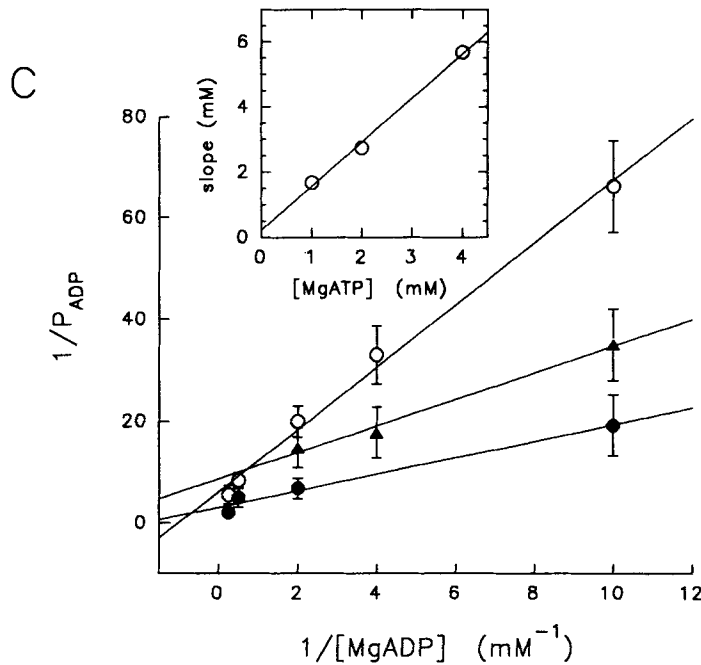
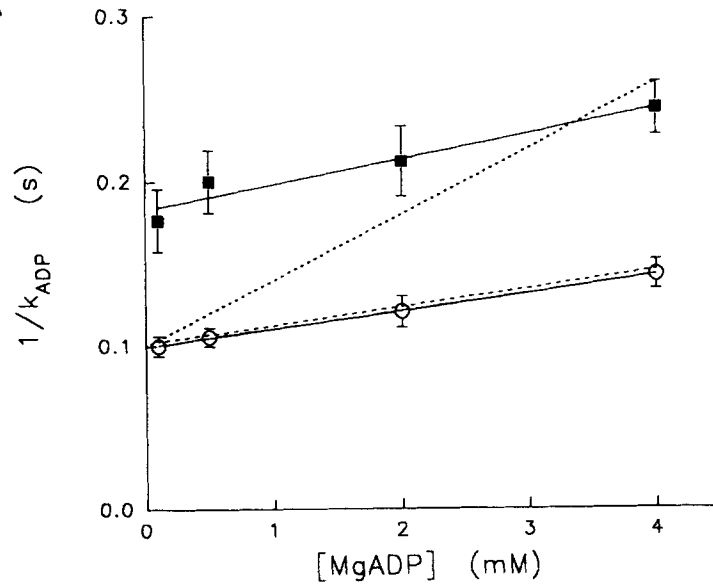


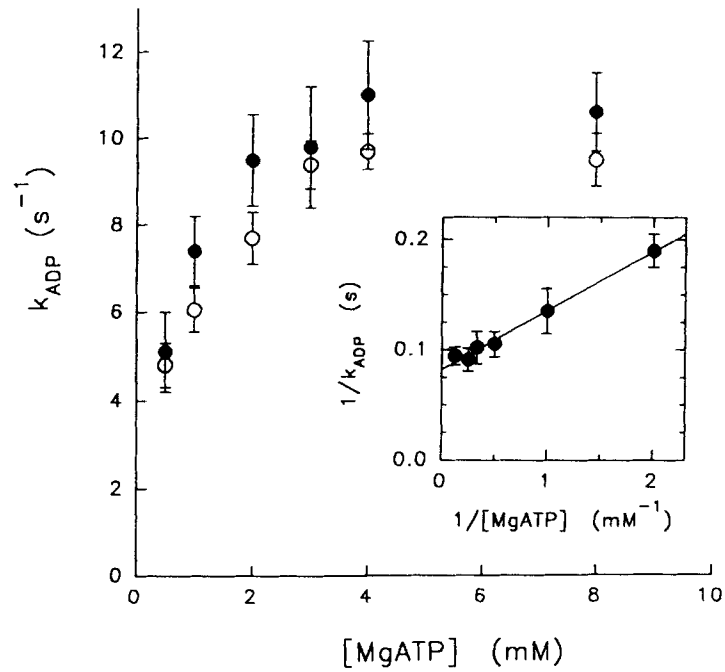
FIGURE 3. Dependence of the amplitude of the tension rise on MgADP and MgATP concentration. (A) Original records of tension transients at (a) 0.1 mM MgADP, 4 mM MgATP; (b) 0.5 mM MgADP, 4 mM MgATP; (c) 4 mM MgADP, 4 mM MgATP; (d) 0.1 mM MgADP, 1 mM MgATP; (e) 0.5 mM MgADP, 1 mM MgATP; and (f) 4 mM MgADP, 1 mM MgATP. Lines show single exponential fits. (B) Amplitudes of tension increases (P_{ADP}) normalized to a reference tension (P_0) at 4 mM MgATP, no added ADP, pCa 4.5. Filled and open bars illustrate amplitudes obtained in the presence and absence, respectively, of creatine kinase. (C) Double reciprocal plots of P_{ADP} (normalized as in B, in the presence of creatine kinase) vs. [MgADP]. (○) 4 mM MgATP, (▲) 2 mM MgATP, (●) 1 mM MgATP. Data for [MgADP] > 1 mM represent the tension increase due to added plus photogenerated MgADP. (Inset) Slope of regression lines vs. [MgATP]. Data from seven fibers.

Simulations showed that the amplitude of the transient was reduced to 85–86% and the observed rate constant (obtained by ignoring the falling phase) was increased by 11–34% compared with simulations with $k_{BC} = 0$. These are relatively small errors and are consistent with what was observed experimentally. There was a tendency for k_{ADP} to be faster by up to 20% in the presence of creatine kinase (Fig. 4 B), and at

A



B



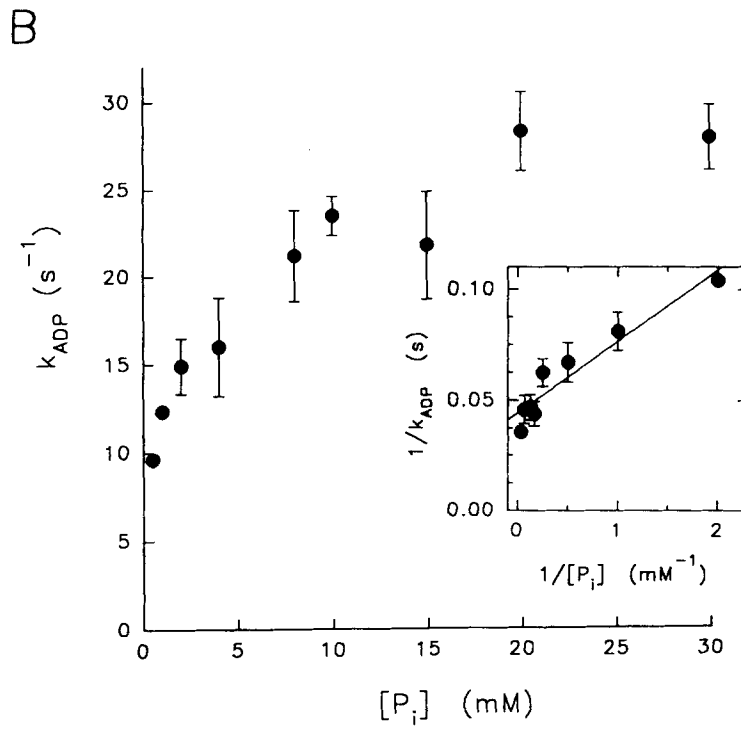
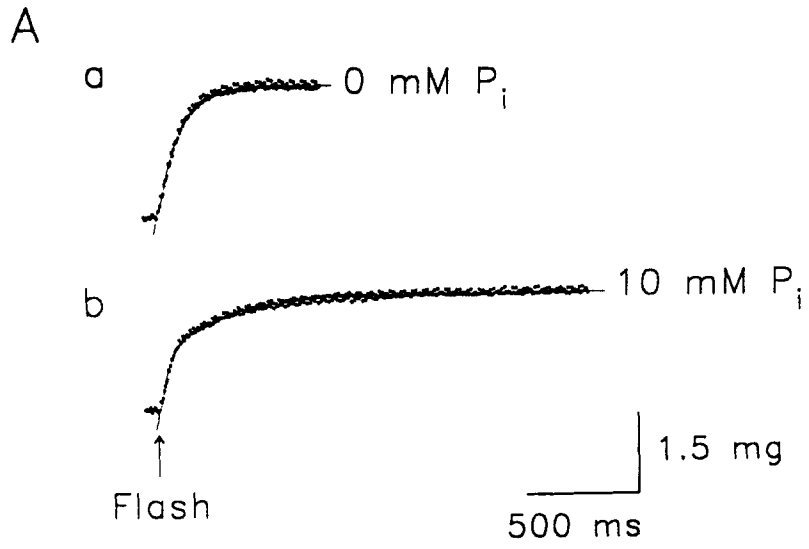
high MgATP levels amplitudes were not significantly different with and without added creatine kinase. As mentioned above, amplitudes at low MgATP (1 mM) were rather dependent on added creatine kinase, but because the amplitude increased with creatine kinase this effect is probably not an artifact of our method of estimating amplitudes.

Fig. 4 summarizes the effects of variations in MgADP and MgATP on the apparent rate constant of the caged ADP transient, k_{ADP} . At two different MgATP concentrations, 1 and 4 mM, the effects of MgADP concentration on k_{ADP} were similar, although at 1 mM MgATP, k_{ADP} was about twofold slower at all MgADP levels (Fig. 4 A). Thus, the largest effect of altering MgATP concentration was on the intercept of the MgADP dependence rather than on its slope. Of these two parameters, the intercept is the most reliable because it is largely defined by measurements at 0.1 and 0.5 mM MgADP carried out in the presence of the MgATP regenerating system, which afforded a measure of control over MgATP and MgADP levels. When the concentration of photoreleased MgADP was kept constant at 0.5 mM, variations in MgATP concentration from 0.5 to 4 mM increased k_{ADP} from 5.1 ± 0.9 to 10.4 ± 1.2 (Fig. 4 B), while higher MgATP concentrations had no further effect. The dependence of k_{ADP} on MgATP was not significantly altered by the addition of creatine kinase (Fig. 4 B). Thus, the large change in k_{ADP} with MgATP was not likely to be due to build-up or underestimates of MgADP concentration in the low concentration range. The less reliable measurements were those in which MgADP was added before photogeneration of additional MgADP (Fig. 3 A, traces *c* and *f*). In these experiments, the creatine phosphate was kept at 9 mM but no creatine kinase was added. The actual levels of MgADP and MgATP within the fiber could be influenced by myosin ATPase, diffusion gradients, and residual creatine kinase within the fibers. Calculations show that MgADP and MgATP levels could vary by as much as 2 mM across the fiber (see Methods); clearly, such variation would be more problematic at low MgATP concentrations.

Acceleration of k_{ADP} by P_i

To explore the possibility that the P_i dissociation step was involved in the MgADP-induced tension rise, the influence of P_i on k_{ADP} was examined. Photorelease of 0.5 mM MgADP increased tension at all levels of P_i examined, but above 6 mM P_i the transients were more adequately described by double exponentials than by single exponentials (Fig. 5 A). The faster phase accounted for at least 50% of the amplitude of the transient with the rate constant (still referred to as k_{ADP}) increasing from $10.2 \pm 0.5 \text{ s}^{-1}$ (no added P_i) to $27.8 \pm 1.8 \text{ s}^{-1}$ in 30 mM P_i and was half-maximal at 6 mM P_i (Fig. 5 B). Apparent rate constants for the slower phase, which was observed only at

FIGURE 4 (*opposite*). Dependence of apparent rate constant (k_{ADP}) on MgADP and MgATP concentration. (A) Elevating MgADP decreased k_{ADP} by 28% at 4 mM MgATP (○) and by 40% at 1 mM MgATP (■). Solid lines show linear regressions: (○) slope = 10.7 s M^{-1} , intercept = 0.10 s; (■) slope = 14.7 s M^{-1} , intercept = 0.19. Dashed lines illustrate the relationship predicted by scheme 1, case 2 (see Appendix). (B) k_{ADP} , measured by photorelease of 0.5 mM MgADP, in the presence (●) and absence (○) of creatine kinase. (*Inset*) Double reciprocal plot of data collected in the presence of creatine kinase. Slope = $5.2 \times 10^{-5} \text{ s M}$, intercept = 0.08 s.



the elevated P_i concentrations, increased from $2.7 \pm 0.6 \text{ s}^{-1}$ at 10 mM P_i to $3.8 \pm 0.3 \text{ s}^{-1}$ at 30 mM P_i . The nature of transitions underlying the slow phase is unknown, but it is worth noting that similar slow phases have been observed with caged ATP transients in the presence of 10 mM P_i (Hibberd et al., 1985). The existence of a slow phase and its variability made it difficult to analyze the effects of P_i on the amplitude of the caged ADP transient. However, in the presence of 10 mM P_i , tension decreased to $\sim 60\%$ of its value in the absence of added P_i , and the amplitude of the fast phase of the caged ADP transient decreased to a similar extent (Fig. 5A).

DISCUSSION

MgADP was rapidly introduced within skinned skeletal muscle fibers by photolysis of caged ADP. When the fibers were initially in rigor, tension decreased rapidly with a rate that increased with MgADP concentration. The apparent second-order association rate constant for MgADP binding to rigor crossbridges was $5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. These results are consistent with previous experiments that have shown that MgADP can form complexes with crossbridges in rigor (Schoenberg and Eisenberg, 1987; Tanner et al., 1989; Dantzig et al., 1991). Most importantly, the results indicate that MgADP can bind rapidly to nucleotide-free crossbridges.

When the fibers were actively contracting (in the presence of ATP and Ca^{2+}), the result of elevating MgADP was a 10-fold slower increase in isometric tension. The rate of the tension rise was further slowed by increasing MgADP concentration. Thus, tension responses to MgADP were very different during active contraction than during rigor contraction. Possible reasons for this include that the MgADP complexes formed in active and rigor fibers are different or that the increase in tension requires crossbridge cycling. The presence of MgATP was certainly a major difference between active contractions and rigor contractions. Evidence for competition between MgADP and MgATP for binding to crossbridges was obtained from the dependence of the amplitude of tension rise on MgATP and MgADP concentrations. The observation that the changes in force in response to changes in MgADP and MgATP concentration could be adequately described by a simple double reciprocal plot argues that, to a first approximation, force increases in proportion to population of an $\text{AM} \cdot \text{ADP}$ complex. However, this amplitude analysis does not provide insight into the mechanism of the force rise (i.e., the pathway of crossbridge flux). The mechanism of the MgADP effect on active isometric tension generation was explored by examining the dependence of the rate of tension rise on the concentrations of MgADP, MgATP, and P_i .

Other investigators have reported increases in isometric force in the presence of

FIGURE 5 (opposite). Effects of P_i . (A) Tension transients after photo-release of 0.5 mM MgADP in the absence (a) and presence (b) of 10 mM P_i added to the photolysis solution. Lines show fits to (a) a single exponential, $k_{\text{ADP}} = 8.9 \text{ s}^{-1}$, $P_{\text{ADP}} = 4.3\% P_o$ and (b) a double exponential, $k_{\text{ADP}} = 21 \text{ s}^{-1}$, $P_{\text{ADP}} = 2.6\% P_o$, $k_{\text{slow}} = 3 \text{ s}^{-1}$, $P_{\text{slow}} = 1.5\% P_o$. (B) Dependence of k_{ADP} on $[P_i]$ between 0.5 mM (contaminating level) and 30 mM. The half-maximal acceleration of k_{ADP} was observed at $\sim 6 \text{ mM } P_i$. 4–14 measurements were made at each $[P_i]$ in eight fibers. (Inset) Double reciprocal plot with slope = $5 \times 10^{-5} \text{ M s}$ and intercept = 0.04 s.

MgADP (Abbott and Mannherz, 1970; Cooke and Pate, 1985; Hoar et al., 1987; Seow and Ford, 1992). Interestingly, perturbation of the crossbridge cycle by sinusoidal length changes has provided evidence for competition between MgADP and MgATP in actively isometrically contracting fibers (Kawai and Halvorson, 1989), but these investigators have not detected a significant increase in force in the presence of MgADP.

Dependence of Rates on MgADP Concentration

As already mentioned, the rate constant (k_{ADP}) decreased as MgADP concentration was elevated from 0.1 to 4 mM. That this is the appropriate concentration range to examine MgADP effects on k_{ADP} is supported by the observation that the amplitude of the transient changes in this range (Fig. 3 B) and by measurements of MgADP dissociation constants using a variety of approaches that give values of 60–360 μM (Kawai and Halvorson, 1989; Pate and Cooke, 1989a; Dantzig et al., 1991; and references therein). The decrease in k_{ADP} at high concentrations of MgADP indicates several things. First, it suggests quite strongly that the observed tension increase is not the immediate result of MgADP binding to nucleotide-free crossbridges (AM) to form AM · ADP. If there was a significant change in force on binding MgADP to AM, then the rate of force development would be expected to increase with MgADP concentration (see Eq. 1 in the Appendix). The force change might be difficult to detect if the proportion of crossbridges in AM is very small. Increasing the proportion of AM by reducing MgATP concentration did not reveal an additional phase of force development (or decline), nor did it alter the dependence of k_{ADP} on MgADP concentration. Thus, there is no evidence that a state is being rapidly populated that generates a different amount of force than AM. Second, the absence of an increase in k_{ADP} with increases in MgADP concentration is consistent with the idea that detached crossbridges are not readily reversed into an AM state that can bind MgADP and produce force (i.e., the end of the crossbridge cycle does not operate in reverse). Third, ADP binding and dissociation are probably not separated from ATP binding (and crossbridge detachment) by slow steps (see Appendix, case 3), but these nucleotides probably bind to the same AM state in the cycle.

The decrease of k_{ADP} with an increase in MgADP concentration can be explained by competition between MgADP and MgATP. This possibility is analyzed for a simple case (see Appendix, case 2), namely, that MgADP is perturbing one or a few crossbridge transitions at the end of the cycle that are kinetically isolated from early steps. This analysis shows that simple competition models can account for a decrease in k_{ADP} when MgADP concentration is elevated. However, such models also predict that lowering the MgATP concentration should enhance the effect of MgADP on k_{ADP} especially at high MgADP concentrations. In other words, the slope of the MgADP dependence should increase while the intercept should remain unchanged when MgATP is reduced, but this was not observed (Fig. 4 A). The inability to resolve a fast phase of force development is also difficult to rationalize for this model (see Appendix, case 2).

Dependence of Rates on MgATP Concentration

The large effect of MgATP on the intercept of Fig. 4A is an important aspect of the data and significantly constrains kinetic models. To explain this, the ATP-induced detachment step must limit the rate of force development under conditions of low MgATP (<2 mM). Work from several laboratories has provided estimates of the bimolecular rate constant for ATP-induced detachment: $0.2\text{--}1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ based on caged ATP measurements in rigor fibers (Goldman, Hibberd, and Trentham, 1984), $0.6\text{--}2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ based on [MgATP] dependence of shortening velocity (Cooke and Bialek, 1979; Ferenczi, Goldman, and Simmons, 1984), and $6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ from the MgATP dependence of force oscillations (Kawai and Halvorson, 1989). A process this fast would not be expected to limit k_{ADP} unless only a very small proportion of crossbridges is in the AM state. In order for AM to be so sparsely populated, the steps preceding it in the cycle (i.e., ADP dissociation) must be slow.

This situation is illustrated by a simple kinetic model in which the rate constants for P_i release and ATP-induced detachment steps are rapid compared with ADP dissociation (Appendix, case 1). As shown in Fig. 4B, such a model explains the MgATP dependence of k_{ADP} . The bimolecular rate constant for ATP-induced detachment of AM obtained from the data is $10^5 \text{ M}^{-1} \text{ s}^{-1}$, which is close to previous measurements (see above). This simple analysis is based on slow ADP release as defined in scheme 1 (Appendix, case 1). In this case, rate constants for ADP release fall out of the analytical expressions, and so the model does not explicitly account for the reduction of k_{ADP} with elevated MgADP. However, if MgADP further reduces the proportion of crossbridges in AM by forming $\text{AM} \cdot \text{ADP}$, then it can be expected that the ATP-induced detachment rate and, in turn, k_{ADP} will be further reduced when MgADP is increased. Thus, the model described in case 1 is at least qualitatively compatible with the dependence of k_{ADP} on MgADP concentrations as well as on MgATP concentration.

As with any model that invokes competition between MgADP and MgATP, the effects of MgADP should be more pronounced at low MgATP concentrations. This expectation was not realized in the data (Fig. 4A); it is likely that the data points at 1 mM MgATP and 2 and 4 mM MgADP are the least reliable points on the curve because control of MgADP/MgATP ratios is least certain when MgATP is low and when creatine kinase is not included. The results at each MgATP concentration were consistent with competition between MgADP and MgATP, but it may be difficult to compare results at different MgATP levels.

Dependence on P_i Concentration

The rate constant of the caged ADP transient, k_{ADP} , increased threefold when P_i was varied from 0.5 mM (contaminating level) to 30 mM. In general, the P_i sensitivity of k_{ADP} means that the increase in force results from population of an ADP-bound state that can react with P_i , such as $\text{AM}' \cdot \text{ADP}$ (Goldman, 1987). In support of this interpretation, the half-maximal acceleration of k_{ADP} by P_i was observed at 6 mM P_i , which is in the range of dissociation constants reported previously for P_i binding to the active site of cycling crossbridges (Bowater and Sleep, 1988; Kawai and Halvor-

son, 1991; Dantzig et al., 1992; Walker et al., 1992). The P_i dependence of caged ADP transients can be explained by the kinetic model of scheme 1 (Appendix, case 1) in which the rate of tension development is dictated by flux through ATP-induced crossbridge detachment and P_i release steps. The value of the apparent P_i association rate constant obtained from the P_i dependence of k_{ADP} (Fig. 5 B, *inset*) was $2.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. This value was similar to the apparent rate constant for P_i binding derived from caged P_i measurements under similar conditions ($k_{-1} \times 1/K_2 = 2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; Walker et al., 1992). Overall, the P_i effects on k_{ADP} fit well with the same simple kinetic model that accounted for MgATP and MgADP dependencies, and derived rate constants were comparable with other measurements.

Another explanation for the data is that binding and dissociation steps for all three ligands (MgATP, MgADP, and P_i) are kinetically coupled (i.e., not isolated by slow steps). Computer simulations of models of this nature, including scheme 1 and more complex models, were not satisfactory for at least two reasons: (a) magnitudes of the effects of MgADP, MgATP and P_i on k_{ADP} were not adequately accounted for, and (b) apparent ligand binding rate constants that fit the data were considerably less ($< 10^4 \text{ M}^{-1}\text{s}^{-1}$) than those measured independently. For these reasons the simpler model of scheme 1, case 1 is preferred. Kinetic models of the crossbridge cycle that feature kinetic coupling of all ligand binding steps make predictions about the effects of MgATP, MgADP, and P_i on mechanical transitions such as force development rate and shortening velocity, and these predictions will be further tested in future studies.

Implications and Limitations of the Kinetic Model

The kinetic model that most adequately accounted for our data was based on the assumption that the caged ADP tension transient is dominated by flux of crossbridges through the P_i release step of the crossbridge cycle (scheme 1, case 1, Appendix). The model accounts for the effects of MgATP on k_{ADP} (Fig. 4 B) by ensuring that AM is not significantly populated; this is a consequence of the ADP dissociation steps being much slower than ATP-induced detachment. Decreases in k_{ADP} with elevated MgADP (Fig. 4 A) were explained by competition between MgADP and MgATP for very low steady-state levels of AM. Acceleration of k_{ADP} by P_i (Fig. 5) was also well accounted for. One significant implication of this interpretation is that caged ADP photolysis in contracting fibers does not probe ADP release from force-generating states and therefore does not provide information about these steps (other than that they are much slower than ATP binding, crossbridge detachment, and P_i release steps).

Scheme 1 must be considered an oversimplified model of the crossbridge cycle. Several well-characterized crossbridge states (Hibberd and Trentham, 1986; Goldman, 1987) have been left out of scheme 1 in order to simplify the analysis. It is important to note, however, that the main features of the kinetic data were accounted for by this simple model. Of course, considering additional states may be necessary to completely rationalize the data. For example, if MgADP binding to AM during active contractions is slow ($k_{-2}[\text{MgADP}] \leq 1 \text{ s}^{-1}$ as required by case 1), then what causes the perturbation that results in tension development at $\sim 10 \text{ s}^{-1}$? This can be rationalized if MgADP binds rapidly to a state not shown in scheme 1 (perhaps $\text{AM} \cdot \text{ADP}$), which itself does not result in a measurable change in force but promotes an increase in force by reducing the AM detachment rate.

Isotope exchange experiments have indicated the existence of two AM states bound by MgADP both in solution (Sleep and Hutton, 1980) and in fibers (Dantzig and Goldman, 1985). One MgADP state ($AM \cdot ADP$) was obtained by addition of MgADP to acto-S1 (or rigor fibers), and a different MgADP state ($AM' \cdot ADP$) was formed by ATP hydrolysis and subsequent P_i dissociation. $AM' \cdot ADP$ is thought to be a major force-generating biochemical state during active contractions (Dantzig and Goldman, 1985; Hibberd and Trentham, 1986), but the importance of $AM \cdot ADP$ is unknown. This work indicates that addition of MgADP to actively contracting fibers does not give an immediate increase in force but may increase force indirectly by slowing crossbridge detachment. It further suggests that rapid MgADP binding populates a state that generates approximately the same level of force as does AM.

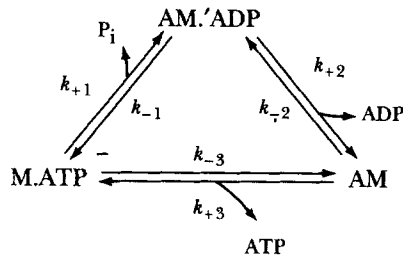
An important limitation of the kinetic modeling is that it did not include possible changes in crossbridge strain in response to changes in ligand concentrations. In this regard, scheme 1 must again be considered an oversimplified explanation for the effects of MgADP on force development because several investigators have observed that MgADP increases force more than stiffness in isometrically contracting fibers (Dantzig et al., 1991; Seow and Ford, 1992; Lu, Z., J. M. Metzger, R. L. Moss, and J. W. Walker, manuscript in preparation). Proposed mechanisms for such changes have invoked interactions between the two heads of myosin and preferential MgADP binding to highly strained AM bridges, leaving less strained bridges to detach and recycle (Dantzig et al., 1991). The present results do not provide insight into the precise mechanisms of such a change in crossbridge mechanics (i.e., force per crossbridge), but the results do favor the interpretation that cycling crossbridges contribute to the increase in force. Changes in crossbridge strain could also be occurring when MgATP is reduced or when P_i is elevated, and such changes could influence the rate constants of force-generating transitions (Huxley and Simmons, 1971; Eisenberg and Hill, 1985; Homsher and Millar, 1991). Determining how much these factors influence the kinetics of tension development must await detailed tension and stiffness measurements in the presence of different concentrations of MgADP, MgATP, and P_i .

Conclusions

The increase in active isometric tension observed in the presence of MgADP is likely to be the result of formation of complexes between MgADP and AM (nucleotide-free crossbridges). Apparent competition between MgADP and MgATP was observed when amplitudes of the caged ADP tension transients were analyzed. The main result of this competition is a decrease in the detachment rate of crossbridges at the end of the cycle. It is unlikely that reversal of ADP dissociation steps followed by a redistribution of ADP-bound states caused the tension rise because tension development was adequately described by a single exponential, with a rate that decreased at high MgADP concentration. The dependence of the rate on the concentrations of MgATP and P_i was most compatible with slow dissociation of MgADP from crossbridges and with net crossbridge flux occurring through ATP hydrolysis and P_i release steps of the crossbridge cycle.

APPENDIX

Scheme 1 illustrates a three-state cyclic model that was used as a framework for interpreting the dependency of k_{ADP} on MgADP, MgATP, and P_i .



Scheme 1

While scheme 1 is undoubtedly too simple to precisely account for all aspects of crossbridge kinetics, a significant advantage is that it can be solved analytically. According to the principles of chemical kinetics outlined by Bernasconi (1976), a three-state cyclic model will have two relaxation times, although both may not be readily detected. For scheme 1:

$$k_{fast} = k_{+1} + k_{-1}[P_i] + k_{+2} + k_{-2}[ADP] + k_{+3}[ATP] + k_{-3} \quad (1)$$

and,

$$k_{slow} = \frac{k_{+1}k_{+2} + k_{-2}[ADP](k_{+1} + k_{-1}[P_i]) + k_{+3}[ATP](k_{-1}[P_i] + k_{+2} + 1)}{k_{+1} + k_{-1}[P_i] + k_{+2} + k_{-2}[ADP] + k_{+3}[ATP] + k_{-3}} \quad (2)$$

assuming that $[ADP]$, $[ATP]$, and $[P_i]$ are constant during the tension transient (Bernasconi, 1976). Since the caged ADP tension transients were well fit by single exponentials (except in high P_i), the fast phase is either too small in amplitude to detect, not resolved (in time) from the slow process, or not associated with a detectable change in tension. The kinetic model and values for rate constants that accounted for most of the data will be presented first.

Case 1

(a) The rate constants for MgADP binding (k_{-2}) and release (k_{+2}) were assumed to be slow. (b) P_i dissociation (k_{+1}) and the bimolecular rate constants for ATP binding (k_{+3}) and P_i binding (k_{-1}) were assumed to be fast. (c) k_{-3} was assigned a value of zero based on the likelihood from thermodynamic considerations that once crossbridges are detached by MgATP, they cannot reattach and generate force by release of ATP and rebinding of ADP because of the absence of ATP hydrolysis. If P_i release and ATP-induced detachment steps are considered to be much faster or otherwise kinetically isolated from ADP dissociation steps, and $k_{+3}[ATP] > k_{+1}$, $k_{-1}[P_i] \gg k_{+2}$, $k_{-2}[ADP]$, then k_{slow} can be simplified to:

$$k_{slow} = k_{+3}[ATP]k_{-1}[P_i]/(k_{+3}[ATP] + k_{+1} + k_{-1}[P_i]) \quad (3)$$

which can be linearized to:

$$1/k_{slow} = (1/[ATP])(k_{+1}/(k_{-1}[P_i]k_{+3}) + 1/k_{+3}) + 1/k_{-1}[P_i] \quad (4)$$

Thus, if $k_{slow} = k_{ADP}$, then a plot of $1/k_{ADP}$ vs. $1/[ATP]$ will permit evaluation of k_{+1} , k_{-1} , and k_{+3} . Values for rate constants were obtained from the data in Fig. 4 B (*inset*) using the relationship slope = $1/k_{+3}(1 + k_{+1}/k_{-1}[P_i])$ and intercept = $1/k_{-1}[P_i]$, giving $k_{+3} = 1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 2.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, and $k_{+1} = 50 \text{ s}^{-1}$. The value of k_{-1} was obtained from the slope of the P_i

dependence in Fig. 5 B. k_{fast} may be undetectable because of the low level of AM that MgADP reacts with, or because AM and $\text{AM}' \cdot \text{ADP}$ support similar amounts of tension. The effects of MgADP concentration on k_{ADP} are explained by a reduction in the steady-state level of AM, which reduces the effective rate at which ATP can detach crossbridges. A more detailed analysis of the consequences of such a competition between MgADP and MgATP for binding to AM is presented next.

Case 2

(a) Bimolecular rate constants for MgADP binding (k_{-2}) and MgATP binding (k_{+3}) were assumed to be fast based on measurements in rigor fibers (Goldman et al., 1984; Dantzig et al., 1991; this work). (b) k_{+2} was further assumed to be faster than k_{+1} and $k_{-1}[\text{P}_i]$. (c) Again, $k_{-3} = 0$ (see above). If the ADP release and ATP-induced detachment steps are much faster than, or otherwise kinetically isolated from, P_i release steps, and $k_{+2}, k_{-2}[\text{ADP}], k_{+3}[\text{ATP}] \gg k_{+1}, k_{-1}[\text{P}_i]$, then k_{slow} above can be simplified to:

$$k_{\text{slow}} = k_{+3}[\text{ATP}]k_{-1}[\text{P}_i]/(k_{+3}[\text{ATP}] + k_{-2}[\text{ADP}] + k_{+2}) \quad (5)$$

which can be linearized to:

$$1/k_{\text{slow}} = (k_{-2}[\text{ADP}]/k_{+2}k_{+3}[\text{ATP}]) + (1/k_{+3}[\text{ATP}] + 1/k_{+2}) \quad (6)$$

Thus, if $k_{\text{slow}} = k_{\text{ADP}}$, then a plot of $1/k_{\text{ADP}}$ vs. $[\text{ADP}]$ will permit $k_{-2}, k_{+2},$ and k_{+3} to be evaluated from the slope and intercept. A plot of $1/k_{\text{ADP}}$ vs. $[\text{MgADP}]$ should have a slope equal to $k_{-2}/k_{+2}k_{+3}[\text{MgATP}]$ and an intercept of $(k_{+3}[\text{MgATP}])^{-1} + (k_{+2})^{-1}$. Data collected at 4 mM MgATP (Fig. 4 A, open circles) were consistent with such a model with $k_{+3} = 1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-2} = 4.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, and $k_{+2} = 10 \text{ s}^{-1}$ (Fig. 4 A, dotted line). However, the data (Fig. 4 A, filled squares) and model predictions (dotted lines) did not match at 1 mM MgATP. To account for the twofold reduction of k_{ADP} when MgATP was lowered from 4 to 1 mM, values for rate constants in scheme 1 became $k_{+2} = 12 \text{ s}^{-1}$, $k_{-2} = 5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, and $k_{+3} = 1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, which are dramatically different than values obtained at 4 mM MgATP. The model prediction of a fourfold increase in the slope of Fig. 4 A was also not observed. Another major drawback of this case is that explaining away the lack of a fast phase is difficult. AM is not sparsely populated, nor can we assume AM and $\text{AM}' \cdot \text{ADP}$ support the same amount of tension, because a slow phase dictated by k_{+2} would also not be observed under these circumstances.

For completeness, a model is developed in which ATP-induced detachment is slow and the kinetics are determined by rapid ADP binding/release and rapid P_i binding/release.

Case 3

If the ADP and P_i release steps are faster than, or otherwise kinetically isolated from, ATP-induced detachment (i.e., $k_{+1} + k_{-2}[\text{ADP}] > k_{-1}[\text{P}_i]$, $k_{+2} \gg k_{+3}[\text{ATP}], k_{-3}$), then k_{slow} can be simplified to:

$$k_{\text{slow}} = k_{+1}k_{-2}[\text{ADP}]/(k_{+1} + k_{-2}[\text{ADP}]) \quad (7)$$

which can be linearized to:

$$1/k_{\text{slow}} = 1/k_{-2}[\text{ADP}] + 1/k_{+1} \quad (8)$$

Thus, if $k_{\text{slow}} = k_{\text{ADP}}$, then a plot of $1/k_{\text{slow}}$ vs. $1/[\text{ADP}]$ should have a positive slope. This is not compatible with the observed decrease in k_{ADP} with an increase in $[\text{MgADP}]$ (Fig. 4 A). In fact, any arrangement of $k_{+1}, k_{-1}[\text{P}_i], k_{+2},$ and $k_{-2}[\text{ADP}]$, as long as they are $\gg k_{+3}[\text{ATP}], k_{-3}$, would predict a similar dependence of $1/k_{\text{slow}}$ on $1/[\text{ADP}]$, which is incompatible with the data.

The observation that tension transients became biphasic in the presence of P_i could not be

accounted for by the simple kinetic models. Since k_{ADP} was slowed by increasing MgADP concentrations, k_{ADP} must represent the slow phase predicted by the three-state model. The appearance of an even slower process, which occurs in the presence of ≥ 6 mM P_i , requires additional states or processes, and there was not enough information in the data to extend the three-state model in a unique way to accommodate this slowest process.

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