THE EFFECTS OF ADP AND PHOSPHATE ON THE CONTRACTION OF MUSCLE FIBERS

ROGER COOKE* AND EDWARD PATE‡
*Department of Biochemistry and Biophysics and Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143; ‡Department of Pure and Applied Mathematics, Washington State University, Pullman, Washington 99164

ABSTRACT The products of MgATP hydrolysis bind to the nucleotide site of myosin and thus may be expected to inhibit the contraction of muscle fibers. We measured the effects of phosphate and MgADP on the isometric tensions and isotonic contraction velocities of glycerinated rabbit psoas muscle at 10°C. Addition of phosphate decreased isometric force but did not affect the maximum velocity of shortening. To characterize the effects of ADP on fiber contractions, force-velocity curves were measured for fibers bathed in media containing various concentrations of MgATP (1.5–4 mM) and various concentrations of MgADP (1–4 mM). As the [MgADP]/[MgATP] ratio in the fiber increases, the maximum velocity achieved by the fiber decreases while the isometric tension increases. The inhibition of fiber velocities and the potentiation of fiber tension by MgADP is not altered by the presence of 12 mM phosphate. The concentration of both MgADP and MgATP within the fiber was calculated from the diffusion coefficient for nucleotides within the fiber, and the rate of MgADP production within the fiber. Using the calculated values for the nucleotide concentration inside the fiber, observed values of the maximum contraction velocity could be described, within experimental accuracy, by a model in which MgADP competed with MgATP and inhibited fiber velocity with an effective K_i of 0.2–0.3 mM. The average MgADP level generated by the fiber ATPase activity within the fiber was ~0.9 mM. In fatigued fibers MgADP and phosphate levels are known to be elevated, and tension and the maximum velocity of contraction are depressed. The results obtained here suggest that levels of MgADP in fatigued fibers play no role in these decreases in function, but the elevation of both phosphate and H^+ is sufficient to account for much of the decrease in tension.

INTRODUCTION

The force of muscle contraction is the result of a cyclic interaction involving actin, myosin, and nucleotides. Studies of this interaction in solution have defined a number of states and have measured many of the transition rates between them (reviewed: Taylor, 1979; Eisenberg and Greene, 1980). Actin and myosin form a strong complex in the absence of nucleotides, which can be dissociated by the binding of MgATP to a site on the myosin head. The MgATP is subsequently cleaved by myosin, and the products of hydrolysis are released after the myosin has rebound to actin. One present goal is to understand how this cycle operates in the organized filament array of the muscle fiber. The results obtained in solution suggest that in an active muscle the products of MgATP hydrolysis, MgADP and phosphate (P_i), are released when the myosin is bound to actin during the power-stroke portion of the cycle in which force is produced. An increased concentration of the products should inhibit the transitions in which they are released, and the nature of this inhibition will help define the energetic and kinetic parameters of these transitions.

Previous work has shown that millimolar concentrations of MgADP inhibit the ATPase activity of actomyosin in solution (White, 1977). However, in fibers MgADP causes an increase in the isometric tension, presumably because it inhibits the release of attached myosin heads (Abbott and Mannherz, 1970). MgADP has been reported to both inhibit and elevate the ATPase activity of isometric fibers (Abbott and Mannherz, 1970; Sleep, 1984). The effects of phosphate on the mechanics of fiber contraction have been studied extensively in insect flight muscle (White and Thorson, 1972; Pybus and Tregear, 1975; Ruegg et al., 1971). Addition of phosphate in the millimolar range causes a decrease in the tension generation and an increase in the frequency for the optimal generation of oscillatory work. In vertebrate muscle elevated concentrations of P_i cause a decrease in the isometric tension, an increase in the fiber ATPase activity, and an increase in the rates of the transient changes in tension that follow a step change in length (Herzig et al., 1981). A systematic study of the effects of MgADP and P_i on the steady state contractions of vertebrate muscle has not been performed previously. In the case of MgADP this is at least partly due to the problems that arise because the levels of both MgATP and MgADP cannot be rigorously controlled inside the fiber. To surmount this problem, we incubated fibers in high

concentrations of both MgATP and MgADP, and calculated the actual nucleotide concentrations in the fiber.

One reason for investigating the effect of products on the mechanics of fiber contraction is to help elucidate the process of muscle fatigue. When a muscle fiber maintains a high level of activity for an extended time a number of metabolites accumulate including MgADP, P_i, H^+, lactate, etc. (Barsotti and Butler, 1984; Dawson et al., 1980; Edstrom et al., 1982; Nassar-Gentina et al., 1978). The effects of prolonged activity on the muscle are complex. The strength of isometric tension, the velocity of isotonic contractions, the rate of substrate use, and the rate of both tension development and relaxation all decrease (Crow and Kushmerick, 1982; Dawson et al., 1978; Godt et al., 1984; Sahlin et al., 1981). At present the mechanisms that account for these effects are not fully understood. For instance the decrease in force could be due to a decrease in the level of activation, or it could be due to some direct action of decreased substrates and increased products on the actomyosin interaction (Edman and Mattiazzi, 1981). These mechanisms can be better defined by measuring the influence of the products, MgADP, P_i, and H^+ on the mechanics of contraction.

We have measured the effects of MgADP, P_i, and H^+ on the force-velocity relation of chemically skinned, mammalian, muscle fibers. Our results show that elevated concentrations of MgADP increase isometric tension, in agreement with previous work, and decrease the velocity of isotonic contractions. These results are consistent with a model in which MgADP is released at the end of the power stroke. The measured inhibition of contraction velocity is sufficiently weak that it is unlikely that the increased concentrations of MgADP in fatigued fibers play a role in the mechanisms of fatigue. Both phosphate and H^+ cause a decrease in the isometric tension, but no change in the maximum velocity of contraction. The effect of phosphate can be adequately described by models of cross-bridge kinetics in which phosphate is released during the power stroke. Elevated phosphate and H^+ levels in fatigued fibers could account for a large part of the observed decrease in tension.

METHODS

Thin bundles of rabbit psoas fibers, 1–2 mm in diameter, were dissected and tied to thin wooden rods with surgical thread. The bundles were incubated in 50% glycerol, 50% 0.15 M KCl, 5 mM $MgCl_2$, 10 mM EGTA, and 2 mM 2-[Tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES), pH 7.0. After 24 h at 0°C the solution bathing the fibers was exchanged for fresh solution and the fibers were stored at −20°C until use. Storage lasted typically from 2 wk to 2 mo and did not appear to affect mechanical properties.

For mechanical measurements, single fibers were dissected on a cold stage and glued to the motor arm and force transducer using Duco (Dupont Chemical Co., Wilmington, DE) diluted 1:10 in acetone. Initial sarcomere lengths varied from 2.3 to 2.5 μm as determined by diffraction. Fiber diameters were measured at three different positions along the fiber using a dissecting microscope and the values averaged. Measurements of fiber mechanics were made using the experimental apparatus described by Crowder and Cooke (1984). The muscle was mounted between a solid state force transducer (801; Akers, Horten, Norway) and a light, stiff beryllium arm connected to a rapid motor (General Scanning Inc, Watertown, MA) for changing muscle length. The length of the mounted fiber was 1 cm. The frequency response of the transducer with fiber loaded was 2 KHz. Tension was monitored by a Compupro Computer (Hayward CA) using Tecmar A/D boards (Tecmar Co., Cinncinnati OH). For isotonic contractions the tension of the fiber was maintained at a desired level by a feedback loop. Fiber tension was compared with a reference tension by the computer and a signal to either lengthen or shorten the fiber was sent to the motor. This feedback loop operated at a cycle rate of 1 KHz. Stirring of the solution bathing the fibers was achieved by taking up and ejecting ~25 μl of solution at a frequency of 10 Hz. The velocity of the stirred solution could be adjusted by altering the volume of solution taken up.

All activating solutions were obtained by addition of the required concentrations of ATP, ADP, $MgCl_2$, K_2HPO_4, and KH_2PO_4 to a basic rigor buffer containing 0.15 M potassium acetate (KAc), 5 mM $MgCl_2$, 1 mM EGTA, 0.95 mM $CaCl_2$, 20 mM TES, pH 7.0, pCa ≃ 5. Stability constants used for ATP and ADP binding to Mg^{++} were $1.2 \times 10^4 M^{-1}$ and $1.5 \times 10^3 M^{-1}$, respectively (Smith and Martell, 1975). A free Mg^{++} concentration of 5 mM was maintained in all experiments. Ionic strength of each activating solution was adjusted to a constant, equal to that of the basic rigor solution, by decreasing the KAc concentration. In all experiments involving MgADP, the activity of adenylate kinase was inhibited by addition of 100 μM diadenosine pentaphosphate (AP_5A). Rigor buffers containing 2 mM EGTA and 5 mM EGTA yielded identical results indicating adequate buffering of Ca^{++} near the fiber. In some experiments, 20 mM creatine phosphate (CP) and 30 μM creatine kinase (CK) were added to the contraction buffer to serve as a feeder system to maintain the level of MgATP inside contracting fibers (Cooke and Bialek, 1979). Creatine kinase was prepared by the method of Kuby (1954) or purchased from Sigma Chemical Co., St. Louis, MO).

Measuring the effect of MgADP on fiber mechanics presents difficulties. The competition between MgATP and a ligand with which it competes is most evident when the MgATP concentration is maintained constant and low. This was achieved by the use of a creatine kinase-creatine phosphate, ATP-regenerating system in a previous study of the effects of ligand, competition on contracting fibers (Pate and Cooke, 1985). However, the equilibrium constant for the reaction catalyzed by creatine kinase strongly favors the formation of MgATP and creatine from MgADP and creatine phosphate (Kenyon and Reed, 1983), and attempts to buffer both MgATP and MgADP in the fiber were not successful. Efforts to measure the concentrations of MgATP and MgADP in a contracting fiber using a luciferin-luciferase system were also unsuccessful. We thus elected to measure fiber mechanics using a wide variety of relatively high concentrations of both MgATP and MgADP. In this approach we calculated the internal concentrations of MgATP and MgADP using known physical and kinetic parameters, and we obtained data under a sufficient variety of conditions to determine the parameters that were required to specify these nucleotide concentrations.

The steady state mechanics of fiber contraction can be largely characterized by measuring the velocity of contraction during load clamps as the applied load is varied from isometric tension (P_o) to zero. These data determine the relationship between force and contraction velocity. One of the observable parameters that is most easily analyzed in terms of cross-bridge kinetics is the contraction velocity at zero load, $V(0)$. $V(0)$ was determined by measuring the force-velocity relation at loads between P_o and 0.1 P_o, fitting this data with Eq. 2 as described below, and then extrapolating to zero load.

Force velocity data were analyzed using a variation of the Hill equation (Hill, 1938). Let:

$$(a + P)V(P) = (P_o - P)b, \qquad (1)$$

where $V(P)$ = velocity at tension P, P_o = isometric force, and a, b are parameters. At $P = 0$, the maximum contraction velocity is $V(0) = bP_o/a$.

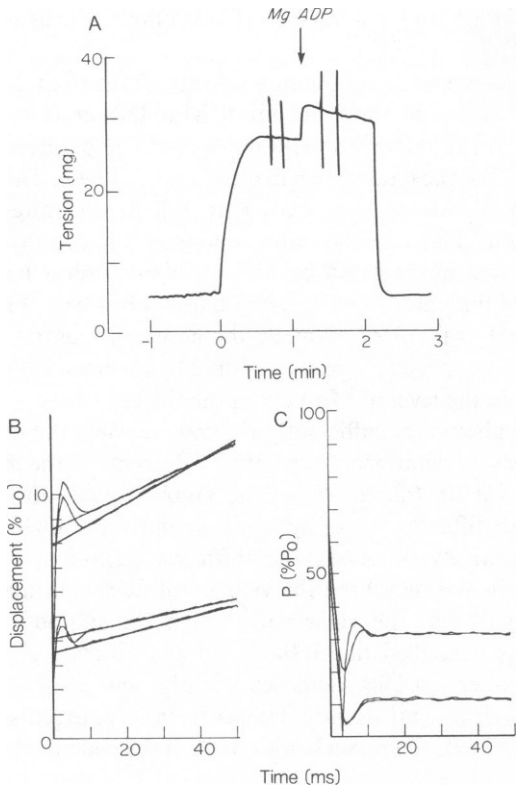

FIGURE 1 (*A*) Time course of isometric tension development in a single contracting glycerinated psoas muscle fiber in activating solution initially containing 4 mM MgATP is shown as a function of time. At the arrow 4 mM MgADP and $MgCl_2$ were added to the solution in a volume that maintained a constant ionic strength. Deviations represent four isotonic releases for measurements of contraction velocity. Force drops at the end when the fiber is broken mechanically by grasping with forceps and a check is made to insure that there has been no drift in the base line of the force transducer. Displacement (*B*) and tension (*C*) are shown for four isotonic releases. Fiber tension was clamped at 0.12 and 0.35 of isometric tension both before and after additions of MgADP. Following each release the fiber was extended to its original length (1 cm) at a constant velocity of 1 mm/s. (*B*) Decrease in fiber length as a function of time for isotonic releases. The *upper* two traces are for releases at 0.12 P_o and the lower traces are for releases of 0.35 P_o; for each pair the *upper* trace with *lower* slope and greater intercept on the vertical axis is obtained following addition of MgADP. The increase in the intercept is due to a greater extension of series elastic elements by the increased tension in the presence of MgADP. Straight lines represent least-squares linear fits to data; slopes give velocities of contraction. (*C*) Force (*P*) divided by the isometric tension at the beginning of the tension clamp (P_o) as a function of time for contractions shown in *left* panel. The fiber diameter was 60 μm and length was 10 mm.

Setting $\alpha = a/P_o$, the equation can easily be rewritten to give

$$V(P) = \alpha V(0)(1 - P/P_o)/(\alpha + P/P_o). \qquad (2)$$

Data analysis is simplified by use of the form in Eq. 2. The two parameters, $V(0)$ and α, were determined by a nonlinear least-squares fit of the individual data points to Eq. 2 using a Gauss-Newton type iteration (Dahlquist et al., 1974). A grid search for starting values of α and $V(0)$ was used in an attempt to insure the global minimum sum of the squared residuals. All data points were weighted equally. A similar iteration scheme was used for the nonlinear least-squares fits to $V(0)$ used to determine a value of K_i for MgADP.

To determine nucleotide concentrations from Eqs. 3 and 6, the differential equation was approximated using second-order, centered, finite differences. Thirty mesh points were used in all simulations. The singularity at $r = 0$ is removed by modifying the diffusion operator and diffusion coefficient at the corresponding mesh point (Pate and Odell, 1981). The resulting nonlinear system of equations for the concentrations at the mesh points was solved by a Newton iteration (see Discussion). Numerical calculations were performed using an Amdahl 470V/8 computer (Amdahl Corp., Sunnyvale CA) or a VAX 11/750 Computer (Digital Equipment Corp., Malboro, MA) Computer code was written in double precision FORTRAN 77 (15 decimal digits) or used the Statistical Analysis Systems routines (Release 82.4, SAS Institute, SAS Circle, Box 8000, Cary, NC).

RESULTS

In all protocols the fiber was immersed in relaxing solution and activated only once by addition of $CaCl_2$. Under some conditions the isometric tension decreased and the velocity of isotonic contractions increased with time following activation. These changes were most evident when MgATP levels were low and MgADP levels were high. To more easily minimize the problems introduced by the time dependence of the mechanical behavior of glycerinated fibers, two experimental protocols were used to measure the mechanics of fiber contraction. In Protocol 1, used to make consistent measurements of isotonic velocities, fibers were activated in solutions containing selected concentrations of nucleotides and isotonic velocities were measured at set times quickly (<60 s) following activation. The order of measurement was randomized to eliminate systematic biases in the data. A second protocol (Protocol 2), shown in Fig. 1 was used to determine relative isometric tensions and also to measure the change in isotonic velocity produced by an addition of a small concentration of MgADP to a fiber contracting in 4 mM MgATP. Because the measurement of fiber diameters introduces the largest error into determination of the isometric tension, the change in fiber tension

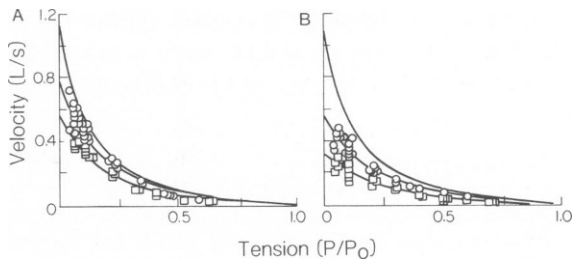

FIGURE 2 Force-velocity relationship for glycerinated rabbit psoas fibers. The medium bathing the fibers contained activating buffer plus 4mM MgATP (○) or 4 mM MgATP and 4 mM MgADP (□) in *A*, and activating buffer with 12 mM phosphate plus 2mM MgATP (○) or 2 mM MgATP and 4 mM MgADP (□) in *B*. Fibers were immersed directly into the appropriate activating solution and two to four load clamps were applied as described in Fig. 1. The results from at least six fibers are shown for each condition. Fiber diameters were 60–70 μm. The solid lines are fits of the Hill equation (Eq. 2) to the data. In each figure the upper solid line is the curve obtained with 4 mM MgATP plus creatine kinase and creatine phosphate in the respective activating buffers; the data shown in Fig. 6.

following addition of MgADP or P_i to the solution was measured and isometric tensions are expressed relative to that in a reference activating solution. The reference solution contained 4 mM MgATP in the presence of an MgATP regeneration system and no added MgADP or P_i.

Fig. 1 shows the basic phenomena that accompany addition of MgADP to a contracting fiber: the isometric tension increases and the velocity of isotonic shortening decreases. The dependence of contraction velocity on force for different experimental conditions is shown in Fig. 2. Omission of the CK-CP, MgATP regenerating system, causes a decrease in the velocity of contraction, which is more evident at 2 mM MgATP than at 4 mM MgATP. This decrease is mainly due to elevated concentrations of MgADP, because in the presence of a regenerating system the contraction velocity does not begin to decrease until the MgATP concentration is <1 mM (Cooke and Bialek, 1979; Ferenczi et al., 1984). Addition of 4 mM MgADP to the fibers causes an additional decrease in the contraction velocity. The decrease is relatvely constant as a function of the load; however, at loads above 0.5 P_o the contraction velocity was too slow to make accurate comparisons. The isometric tensions and maximum contraction velocities observed under a variety of conditions are listed in Table I.

If the decrease in contraction velocity observed at 2 mM MgATP is due to the build up of MgADP, or P_i in the fiber, then the velocity should depend on the diameter of the fiber. For the data shown in other figures and in Table I the fiber diameters were chosen to fall in the range of 60–70 μm. The effect of fiber diameter on contraction velocity was investigated by picking fibers with a larger variety of diameters and by using bundles of fibers. Fig. 3 shows that as the fiber diameter increases, the contraction velocity does indeed decrease as would be expected from an increase in the level of MgADP in the thicker fibers.

In the absence of sufficiently vigorous stirring, the levels of ligands in solution immediately adjacent to the fiber surface will be different from the ambient concentration due to the diffusive flux of ligands into and out of the fiber. This effectively increases the diffusion path for either nucleotide. We measured the velocity of fiber contraction as a function of the vigor with which the solution was stirred as described in Methods. In the absence of any stirring, fiber tensions increased slightly, and contraction velocities decreased slightly. However, in any stirred solution examined, fiber mechanics were independent of the

TABLE I
MEASURED AND CALCULATED CONTRACTION PARAMETERS AS A FUNCTION OF NUCLEOTIDE CONCENTRATIONS

Conditions*				Results		Calculated velocities	
MgATP	MgADP	P_i	CK-CP	Tension‡	$V(0)$§	$V(0)$‖	
						I	II
mM	*mM*	*mM*					
4	0	0	+	1.00 ± 0.03	1.22 ± 0.08	1.22	1.0
4	0	0	–	1.1 ± 0.03	0.85 ± 0.05	0.92	0.85
4	4	0	–	1.35 ± 0.02	0.61 ± 0.05	0.56	0.60
2.75	0	0	–	1.18 ± 0.02	0.76 ± 0.06	0.78	0.77
2.75	4	0	–	1.27 ± 0.03	0.45 ± 0.04	0.42	0.46
2	0	0	–	1.19 ± 0.02	0.66 ± 0.07	0.63	0.64
2	4	0	–	1.25 ± 0.01	0.32 ± 0.04	0.31	0.32
1.5	0	0	–	1.24 ± 0.02	0.45 ± 0.06	0.47	0.46
1.5	4	0	–	1.41 ± 0.02	0.19 ± 0.03	0.23	0.19
4	0	12	+	0.70 ± 0.01	1.24 ± 0.04	—	—
2	0	12	–	0.88 ± 0.02	0.62 ± 0.03	—	—
2	4	12	–	1.10 ± 0.02	0.33 ± 0.03	—	—

*Force-velocity curves were obtained for single fibers in an activating buffer that contained the additions shown in the first four columns (the composition of the solutions is described in Methods).

‡The isometric tension of single fibers in various activating solutions was determined relative to the tension in 4 mM MgATP plus a CK-CP regeneration system, as described in Results. Fiber tension is shown relative to that obtained in the activating buffer described in the first row, which was 0.18 N/mm^2. Each entry is the mean and SEM taken from 3–5 fibers.

§The maximum contraction velocities $V(0)$ were obtained by measuring force-velocity curves, fitting the curves with Eq. 2 and extrapolating to zero tension. All velocities are given in muscle lengths per second. Between 24 and 66 force-velocity data points were used for each fit (average = 35). Each entry gives the extrapolated value and the standard error, both determined from the least-squares fit to the data.

‖The maximum velocity was calculated for a model in which MgADP was a competitive inhibitor of both fiber velocity and fiber ATPase activity, as described in the Discussion. Column I gives the velocities found when the value of K_i was the only independent variable in this calculation, and column II gives the values found when seven parameters were allowed to vary.

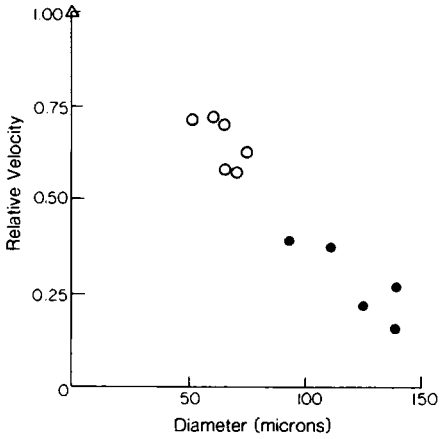

FIGURE 3 The effect of fiber diameter on the maximum velocity of contraction. The medium contained activating buffer plus 2 mM MgATP. The maximum velocity was determined by recording isotonic velocities between 0.8 P_o and 0.1 P_o, fitting these data with the Hill equation (Eq. 2), and extrapolating to zero force. The extrapolated velocities are shown relative to the maximum velocity in 4 mM MgATP with a CK-CP regeneration system. The data are plotted as a function of the diameter of single fibers (open symbols) or fiber bundles (closed symbols). The point plotted at zero diameter (△) is the velocity observed in the presence of the CK-CP system to regenerate MgATP. This is the velocity that would be observed in the absence of a regeneration system as the diameter approached zero and the nucleotide concentrations approached their values in the solution.

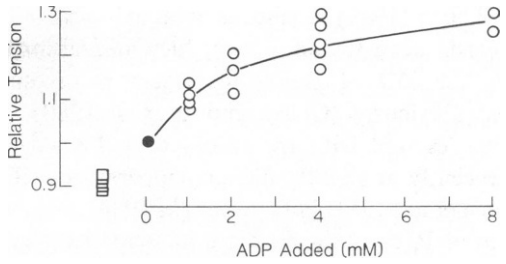

FIGURE 4 The isometric tension of single glycerinated psoas fibers is shown as a function of the MgADP added to the medium (open circles). The activating solution initially contained 4 mM MgATP and the increase in tension was determined following addition of MgADP and $MgCl_2$ to the medium as described in Results. The tension is plotted relative to the tension obtained in the initial solution (given by the closed circle). The isometric tension obtained following addition of CP (20 mM) and CK (0.8 mg/ml) to the initial activating solution is given by the squares, and is plotted assuming that the MgADP level in the fibers in the initial activating solution was 1 mM.

velocity of stirring indicating that in stirred solutions the unstirred layer surrounding the fiber is too small to influence our results.

The dependence of the isometric tension on the MgADP concentration is shown in Fig. 4. The isometric tension relative to that in an activating solution containing 4 mM MgATP was obtained by addition of MgADP to the solution following Protocol 2 described above. MgADP was added so that the ionic strength and the free Mg^{++} concentration are unchanged, as described in Methods. Even in the absence of added ADP the MgADP concentration is elevated inside the fiber because no ATP regenerating system is present. To relate the tension measured in the absence of the regenerating systems to that obtained in high concentrations of MgATP and low concentrations of MgADP, CP and CK were added to fibers contracting in 4 mM MgATP and the change in tension recorded. The tension observed in the presence of the regenerating system is plotted in Fig. 4 to the left of zero added MgADP as if the internal MgADP concentration for fibers in an activating solution containing 4 mM MgATP was 1 mM as determined from the inhibition of contraction velocity as described in the Discussion.

One of the most accurate methods of determining the ability of MgADP to compete with MgATP is to measure the change in velocity induced by addition of a small concentration of MgADP to a fiber contracting in a high concentration of MgATP. 1 mM MgADP was added to fibers contracting in 4 mM MgATP and the changes in velocities were observed for load clamps at 0.1 and 0.3 P_o. Addition of 4 mM MgADP was observed to produce similar changes in both $V(0)$ and in the velocities at these lower loads suggesting that similar estimates of inhibition can be obtained at these loads. Addition of 1 mM MgADP caused a decrease in velocity of 12% ± 1% (six fibers, mean ± sem) at 0.1 P_o and 13% ± 1% (five fibers) at 0.3 P_o.

The above data, as well as the data of Table I, can be used to obtain a measure of the K_i for the inhibition of velocity by MgADP as described in the Discussion. These analyses require a value of the K_m for the dependence of velocity on substrate. To determine K_m, force-velocity measurements were made at several concentrations of MgATP in the presence of a CK-CP regenerating system and the data fit by Eq. 2. The extrapolated values obtained for $V(0)$, in muscle lengths/second, were found to be 1.22 at 4 mM MgATP, 0.97 at 500 μM MgATP, 0.70 at 200 μM MgATP, and 0.51 at 100 μM MgATP. For these fits, 40, 18, 24, and 14 force-velocity data, points were used, respectively. A least-squares linear fit of $1/V(0)$ vs. 1/MgATP for these data gives a value of ~150 μM for K_m. This value is a little lower than the value of 225 μM obtained in previous work in this laboratory under similar conditions (Cooke and Bialek, 1979; Pate and Cooke, 1985). The lower value is due to the lower value of $V(0)$ obtained at high levels of MgATP. This value was not well specified in the previous work due to limitations in the design of the experimental apparatus. This lower value for $V(0)$ agrees well with that obtained by Moss (1982).

To determine the elevation of MgADP and depletion of MgATP in the contracting fibers one has to know the magnitude of the ATPase activity inside the fibers. Several (three to six) single fibers were mounted on the experimental apparatus and the amount of phosphate generated during a 5-min incubation in an activating solution containing 4 mM MgATP was measured using the method of

Martin and Doty (1949). The value obtained was 0.9 ± 0.1 s^{-1} per myosin head (mean ± sem, four measurements), assuming that 45% of the fiber protein is myosin as determined previously (Cooke and Franks, 1980). This value compares well with the values of 1–1.8 s^{-1}/head obtained recently at slightly higher temperatures (Sleep, 1984; Chaen et al., 1981; Ferenczi et al., 1984).

Addition of P_i causes a decrease in isometric force as described by previous workers (White and Thorson, 1972; Pybus and Tregear, 1975; Herzig et al., 1981). The decrease in force is shown as a function of the P_i concentration at two different values of pH in Fig. 5. To relate the data obtained at two values of pH, the effect of pH on isometric tension was also investigated by directly measuring the pH of the activating solution with an electrode and changing it during the contraction by addition of KOH or HCl. For these experiments the concentration of the buffer TES was decreased to 5 mM. As the pH was lowered from 7 to 6.5, the tension decreased reversibly in an approximately linear fashion by 30%. Similar results have been reported by Bolitho Donaldson and Hermansen (1978). In Fig. 5 the tensions are plotted relative to the isometric tension at pH 7.0 in the absence of added phosphate. The absolute drop in force is roughly the same at both values of pH. At pH 7, 20 mM P_i results in an ~35% drop in tension with the half-maximal tension drop at 3 mM P_i. Surprisingly, as shown in Fig. 6 and in Table I, 12 mM P_i did not affect maximum contraction velocity. This is in marked contrast to insect flight muscle where P_i modifies the frequency for optimal generation of oscillatory work (White and Thorson, 1972). Lowering the pH from 7 to 6.5 did not affect $V(0)$. The effect of P_i on the inhibition of

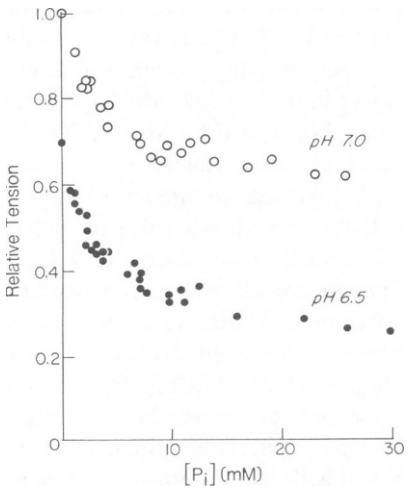

FIGURE 5 The isometric tension of single fibers was measured before and after addition of various concentrations of inorganic phosphate to the activating solution as described in Results. Results were obtained at two values of pH and plotted relative to the tension generated at pH 7.0 in the absence of added P_i. The initial activating solution consisted of rigor buffer with 4 mM MgATP, 20 mM CP, and 0.8 mg/ml CK. The ionic strength was maintained constant by adjusting the concentration of KAc as described in Methods.

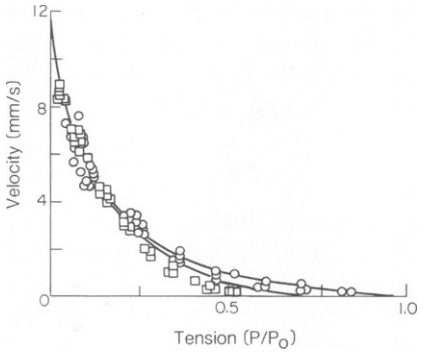

FIGURE 6 The effect of 12 mM phosphate on the force velocity relation is shown. Velocity of contraction is plotted as a function of force (relative to isometric in the absence of P_i) in the presence (squares) and absence (circles) of 12 mM P_i. Fibers were immersed in the appropriate activating solution containing 4 mM MgATP and a CK-CP regenerating system and allowed to stabilize; typically this took 30 s. Then two to six load clamps were applied as described in Fig. 1. The results of six to eight fibers are shown for each condition. The solid lines are fits of the Hill equation (Eq. 2) to the data as described in Methods.

velocity by MgADP was investigated, and similar velocities were obtained in the presence and absence of P_i so that the effects of P_i and MgADP on fiber velocities and isometric tensions are independent of each other, as shown in Table I.

DISCUSSION

Elevation of MgADP inside a contracting fiber decreases the contraction velocity and increases the isometric tension. Both of these effects are consistent with models in which the binding of MgADP prevents dissociation of the myosin head by MgATP. A more quantitative analysis of these phenomena requires that we know the exact concentrations of both MgATP and MgADP inside the fiber. The concentrations of these nucleotides are altered by the fiber ATPase activity and as discussed previously, they are difficult to control or to measure directly. We can, however, calculate them from known nucleotide diffusion rates and kinetic constants. The concentration of a diffusing ligand that is either consumed or produced at a constant rate within an object having cylindrical symmetry can be obtained from the diffusion equation.

$$D[d^2C(r)/dr^2 + r^{-1}\,dC(r)/dr] + k = 0 \qquad 0 < r < a$$
$$dC(0)/dr = 0$$
$$C(a) = C_o, \tag{3}$$

where $C(r)$ is the concentration of the ligand at radius r, a is the radius of the cylinder, D is the diffusion coefficient of the ligand, k is its rate of production or consumption (k is negative for consumption) and C_o is the concentration of ligand in the solution surrounding the cylinder. This differential equation can be solved (Carslaw and Jaeger, 1959) to obtain

$$C(r) = C_o + (a^2 - r^2)k/4D. \tag{4}$$

Previous work has shown that the velocity of contraction is inhibited by the binding of the ligands MgAMPPNP and $MgPP_i$ to the myosin site and that this inhibition follows the classic competitive form described by

$$V = V_{max}C_s/[K_m(1 + C_L/K_i) + C_s], \quad (5)$$

where K_i is the inhibition constant of the ligand, C_L and C_S are the concentrations of the ligand and substrate, respectively, K_m is the Michaelis constant for the substrate, V is the velocity of the reaction, and V_{max} is the velocity extrapolated to infinite substrate concentration (Pate and Cooke, 1985). An equation similar to Eq. 5 also exists for the fiber ATPase

$$V^A = C_sV^A_{max}/(K^A_m[1 + C_L/K^A_i) + C_s]. \quad (6)$$

As the MgATP concentration, C_s, is lowered or the MgADP concentration, C_L, is raised both the fiber velocity and the fiber ATPase are inhibited. The relevant constants for the fiber ATPase are denoted with a superscript A. Our approach is to first calculate the nucleotide concentrations inside the fiber using Eq. 3 assuming that the fiber ATPase activity is inhibited according to Eq. 6. Using these calculated nucleotide concentrations, we then determine whether the values of $V(0)$ listed in Table I can be described by the equation for pure competitive inhibition, Eq. 5. Thus an adequate description of the data in Table I as a function of MgATP and MgADP concentrations requires two values for the Michaelis constant of the substrate, one, K_m, for the contraction velocity and one, K^A_m, for the ATPase activity, two corresponding values for the inhibition constant, K_i and K^A_i, and two corresponding values for the maximum velocities, V and V^A. In addition, Eq. 3 and 4 require specification of the diffusion coefficient of the nucleotides through the fiber and the fiber radius. We have measured K_m to be 150 μM, V_{max} to be 1.22 1/s and V^A_{max} to be 0.9 s^{-1}/head as described in Results. The value of K^A_m has been measured to be <10–20 μM, considerably less than that for contraction velocity (Chaen et al., 1981; Sleep, 1984), and K^A_i is equal to 200 μM (Sleep, 1984). The coefficient for diffusion of MgATP into fibers has been measured by two groups who both found values of 1–2 $\times$ 10^{-7} cm^2/s (Mannherz, 1968; Marston, 1973). Average fiber radius was 33 μm. Thus the only unknown parameter is the K_i for contraction velocity.

We use the following approach to estimate K_i for the inhibition of fiber velocity by MgADP using the data of Table I. The nucleotide concentration within the fiber is first determined by solving the diffusion equation taking into account fiber ATPase. Eq. 4 shows that parabolic profiles for MgADP result when the ATPase rate is a constant as a function of MgADP concentration. This occurs only when the MgATP concentration, C_s, is $>K^A_m(1 + C_L/K^A_i)$, and k in Eq. 4 becomes equal to V^A_{max} (see Eq. 6). As this may not be the case in all conditions we instead solve Eq. 3 with k replaced with the right-hand side of Eq. 6 and C_o equal to the concentration of MgADP in the contraction buffer. Fig. 7 shows the nucleotide levels expected inside a fiber bathed in 2.75 mM MgATP, 4 mM MgADP using parameters listed in the legend. Inside the fiber, the concentration of MgADP increases above that in solution in an approximately parabolic fashion with its maximum at the fiber center. The concentration of MgATP decreases as one approaches the fiber center so that the sum of the MgADP and MgATP concentrations always equals 6.75 mM. From the solution of Eq. 3 and 6 average concentrations of MgADP and MgATP can be determined. The average nucleotide concentrations were inserted into Eq. 5, and a least-squares nonlinear fit of this equation to the experimentally measured velocities in Table I establishes a value for K_i. Using K_m = 150 μM, K^A_m = 20 μM, V_{max} = 1.22 1/s, V^A_{max} = 0.9 s^{-1} per head, D = 2 $\times$ 10^{-7} cm^2/s and K^A_i = 200 μM, the best fit, shown in column I, on the right-hand side of Table I, was found for K_i = 200 μM ± 32 μM (95% confidence limit). A second approach to determine K_i was to fit the data of Table I with the protocol outlined above with the exception that seven parameters in the model were allowed to vary in the nonlinear least-squares fit. The seven parameters were K_m, K^A_m, K_i, K^A_i, D, V_{max}, and V^A_{max}. In this approach we have included some known parameters as independent variables in order to verify that our approach converged to reasonable values, and in order to determine whether alterations in the values of some parameters would alter our basic conclusions. The best fit, shown in column II of Table I,

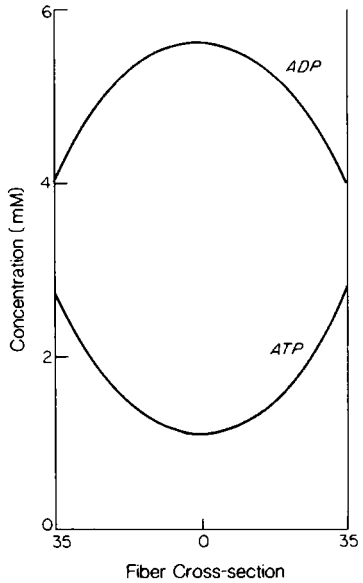

FIGURE 7 The nucleotide concentrations across a fiber of diameter 70 μm are shown for a fiber bathed in 2.75 mM MgATP, 4 mM MgADP. Concentrations were determined by solving Eq. 3 and 6 as described in Methods. Due to the fiber ATPase activity, the MgATP concentration decreases across the fiber reaching a minimum at the fiber center, where MgADP concentration is greatest. Parameters: D = 2 $\times$ 10^{-7} cm^2/s, V^A_{max} = 0.9 s^{-1}/head, K^A_m = 20 μM, K^A_i = 200 μM, fiber diameter = 70 μm.

was for $K_m = 120\ \mu M$, $K_m^A = 9\ \mu M$, $K_i = 320\ \mu M$, $K_i^A = 310\ \mu M$, $V_{max} = 1.0$ 1/s, $V_{max}^A = 1.5\ s^{-1}$/head, $D = 4 \times 10^{-7}$ cm^2/s. The calculated values of $V(0)$ are in excellent agreement with the experimentally measured values; this is not surprising since seven parameters are varying for the fit. The important observation is that the values of the parameters, which give the best fit to the data, all agree to within a factor of two with the experimental values, and the value of K_i agrees quite well with that found in the previous calculation. Our basic conclusion is not modified by reasonable variations in the parameters.

An alternative method for determining K_i, which avoids or minimizes several problems associated with the above approach is to measure the change in contraction velocity at fixed tension when a known amount of MgADP is added to the contraction buffer. We now consider this approach. Both of the calculations described above show that in the inhibition of contraction velocity, MgADP acts as a simple competitive inhibitor as described in Eq. 5. Expanding Eq. 5 in a Taylor Series, it is straightforward to show that the fractional change in velocity, $\Delta V/V$, resulting from a change in ADP concentration, ΔADP, is given by

$$\Delta V/V = \sum_{n=1}^{n=\infty} (-V \cdot K_m \cdot \Delta\text{ADP})^n / [V_m(\text{P}) \cdot K_i \cdot C_s]^n, \quad (7)$$

where $V_m(P)$ is the velocity at load P measured at saturating substrate concentrations, and the other symbols have been defined above. The velocities V, $V_m(P)$, and ΔV can be measured accurately at low load as outlined in Results. K_m has also been measured to be 150 μM and the preceding calculations indicate that at the MgATP concentration used, 4 mM, the average MgATP concentration, C_s, is ~3.1 mM. At 0.1 P_o, upon addition of 1 mM MgADP (ΔADP) the contraction velocity decreased from 0.47 1/s by 12%. At 0.1 P_o, the fit to the data in Fig. 6 give a value for V_m (0.1) of 0.6 1/s. Substituting into Eq. 7, keeping two terms on the right-hand side, and solving for the unknown, K_i, yields a value $K_i = 280\ \mu M$. A similar calculation for the data obtained at 0.3 P_o produces an almost identical value for K_i of 290 μM. This method for determining K_i is probably more accurate than the fit to the data of Table I for several reasons. An accurate estimate of the internal MgADP concentration is not required, because only the change in MgADP concentration enters into Eq. 7. The uncertainties in the MgATP concentration introduce only a small error because this concentration is relatively high compared with its expected depletion within the fiber. Furthermore the data involve a comparison of velocities before and after a small perturbation, eliminating fiber to fiber variability. These values also do not require the extrapolation to zero tension. The fact that this value agrees with those obtained by a fit to the data of Table I argues that MgADP is acting as a pure competitive inhibitor over a large range of concentrations. This agreement also suggests that the gradients in nucleotide concentrations across the fiber do not greatly influence the data. In all of the above calculations, C_s and C_L have been taken as their average values. This is a fairly reasonable approximation at high concentrations of nucleotides but at low concentrations the relative change in concentration across the fiber is large. As an additional test of this approximation, values of K_i for MgADP were determined by least-squares fits to the velocity data in Table I using data from only the three highest MgATP concentrations (4 mM, 2.75 mM, 2 mM) and independently from the three lowest concentrations (2.75 mM, 2 mM, 1.5 mM). The values of K_i obtained were 202 and 193 μM, respectively, and differ from the value of K_i determined using data from all MgATP concentrations by <4%. The agreement between the data and Eq. 5 thus suggests that over the concentration range used in these experiments, the fiber response is adequately described by using the average nucleotide concentrations.

The observation that the inhibition of velocity by MgADP is independent of the concentration of P_i suggests that in the fiber the two products are released in separate steps as has been found in solution studies (Taylor, 1979). The lack of an effect of P_i on the maximum contraction velocity shows that P_i does not inhibit the binding of MgATP and subsequent detachment of the myosin head from actin at the end of the power-stroke. These observations are most consistent with the release of P_i occurring at some point within the working portion of the powerstroke, as has been proposed by some theorists (Eisenberg et al., 1980). In this type of model a major effect of increasing the concentration of P_i is to decrease the free energy available within the power-stroke. Calculations of internal ligand concentrations, similar to those described above, show that the internal concentration of P_i in the absence of added P_i is ~0.2 mM. Thus raising the internal concentration of P_i from 0.2 to 20 mM will drop the available free energy by 12 kJ/mol. To maintain reasonable efficiency of contraction, models for cross-bridge function suggest that the free energy drop within the power-stroke is ~40 kJ/mol at pH 7.0 (Eisenberg et al., 1980; Cooke and Bialek, 1979). Then the fractional drop in the free energy available in the power stroke is approximately equal to the fractional drop in tension. An analysis of the model of Eisenberg et al. as a function of P_i was made by determining the solution of the relevant ordinary differential equations by using Gears method (Pate E. F., and R. Cooke, manuscript submitted for publication). It was found that increasing phosphate concentrations from 0.2 to 20 mM would decrease force by 15%, without affecting the maximum contraction velocity, in qualitative agreement with the data. The net effect of increasing P_i in this model is to reduce the energy available in the power-stroke by the same amount that the free energy of MgATP is decreased. In the model the addition of P_i does not affect the value of V_{max}, because the ADP · P_i

state equilibrates rapidly with the MgADP state in a region where the former is not generating much tension.

The observation that MgADP inhibits the velocity of contraction suggests that it binds to a state near the end of the power-stroke. If one assumes that the K_i found here represents the strength of binding of MgADP to the protein complex, then the binding constant 3–5 $\times$ $10^3 M^{-1}$ is similar to that found for the binding of MgADP to acto-S-1 in solution (Greene and Eisenberg, 1980; Highsmith, 1976). Thus the data are consistent with a model in which MgADP competes with MgATP at the end of the power-stroke and the actomyosin complex in this region resembles that found for acto-S-1 in solution. This is in contrast to the results found for MgAMPPNP and $MgPP_i$ where the measured values of K_i's for inhibition of contraction velocity are much weaker than the dissociation constant measured for the binding of the ligand to fibers or to acto-S-1 (Pate and Cooke, 1985).

Analyses of motility in another cross-bridge system, the dynein-microtubule system, have been influenced by our understanding of the actomyosin interaction. The chemomechanical cycles appear to share some basic characteristics (Gibbons and Gibbons, 1974; Takahashi and Tonomura, 1978; Johnson, 1983). The K_i for the inhibition of maximum contraction velocity by MgADP, we measured here is very close to the value of 400 μM reported for the competitive inhibition of beat frequency in demembranated sea urchin sperm flagella (Okuno and Brokaw, 1979). Furthermore, P_i is a very poor inhibitor of beat frequency, with an inhibition constant of 60 mM. These observations provide additional evidence for similarities in the two motility systems.

The data presented here can help define the mechanisms responsible for the depressed contractile response of fatigued fibers. At a point where both tension and contractile velocity have been decreased by approximately a factor of two, the concentration of MgATP remains almost unaltered at 3–5 mM, the concentration of MgADP has risen to only ~200 μM and the concentration of P_i may have reached 10–15 mM (Barsotti and Butler, 1984; Dawson et al., 1980; Edstrom et al., 1982; Nassar-Gentina et al., 1978). The value of K_i and the measured tension increases found here show that although in fatigued fibers MgADP may have increased more than 10-fold over resting fibers, it will have a negligible effect on either the tension or the contraction velocity. Elevation of P_i from 1–2 to 10–15 mM, however, would cause a significant decrease in tension. A decrease in pH from 7.0 to 6.5 has been shown to occur in fatigued muscle (Dawson et al., 1978, 1980), and along with the increase in P_i this could explain most of the decrease observed in force. Surprisingly, the decrease in velocity, however, cannot simply be explained by an accumulation of hydrolysis products or the decrease in pH. Additional factors in the process of fatigue must be involved.

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