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Addition of phosphate to active muscle fibers probes actomyosin states within the powerstroke

Edward Pate[1] **and Roger Cooke**[2]

[1] Department of Mathematics, Washington State University, Pullman, WA 99164, USA
[2] Department of Biochemistry and Biophysics, and the CVRI, University of California, San Francisco, CA 94143, USA

Abstract. We have measured the effect of phosphate (P_i) on the tension and maximum shortening velocity of permeable rabbit psoas fibers. Work in a number of laboratories has established that addition of phosphate (0–25 mM) to active muscle fibers at physiological MgATP concentrations decreases isometric tension with little effect on the maximum shortening velocity. Here we extend these results to a wider range of P_i concentrations and to low MgATP concentrations. Low levels of P_i (approx. 150 μM–200 μM) were obtained by using sucrose phosphorylase and sucrose to reduce contaminating P_i in the solutions used to activate the fiber, and high levels (52–73 mM) were obtained by replacing acetate with P_i as the principal anion. In an activating solution containing either 50 μM or 4 mM MgATP, pH 6.2 or 7.0, isometric tension declines linearly with the logarithm of P_i concentration. Although the isometric tension decreases with increasing concentrations of H^+ or MgATP, the slope of relative isometric tension as a function of log[P_i] is the same at the two values of pH and [MgATP]. At pH 7 and 4 mM MgATP, the velocity of contraction increased slightly as P_i increased from 0.2 to 52 mM. At 50 μM MgATP the velocity decreased slightly as P_i increased from 0.2 to 10 mM with a substantial decrease as P_i increased from 10 to 52 mM. These results are discussed in terms of models of cross-bridge energetics. The observation that force declines linearly with the logarithm of [P_i] is compatible with models in which a major force producing state occurs subsequent to P_i release. The inhibition of shortening velocity by P_i at low concentration of MgATP can be explained by a competition between MgATP and P_i at the end of the cross-bridge powerstroke.

Key words: Skeletal muscle – Cross-bridge energetics – Isometric tension – Contraction velocity – Phosphate – Sucrose phosphorylase

Introduction

In functioning muscle, one molecule of MgATP binds to a site on myosin and is hydrolyzed to MgADP and orthophosphate (P_i) during each force-producing cycle of the actomyosin interaction. Of the two products, P_i is the first to be released after the hydrolysis step, with release being a readily reversible transition (Webb et al, 1986; Sleep and Hutton 1980; Ulbrich and Ruegg 1977). Inhibition of a reversible enzyme reaction by the end-products is a commonly observed phenomenon, and elevated levels of phosphate have been found to alter several parameters of contraction of permeable muscle fibers. Isometric force declines by about 30% as P_i is raised from 1 to 25 mM (Ruegg et al. 1971; Hibberd et al. 1985; Kentish 1986; Nosek et al. 1987a; Chase and Kushmerick 1988; Brozovich et al. 1988; Cooke et al. 1988). The ATPase activity is inhibited to a lesser extent than is tension (Kawai et al. 1987; Cooke et al. 1988). Photogeneration of P_i inside fibers has provided additional evidence that changes in P_i concentration are accompanied by rapid changes in tension (Dantzig et al. 1987). The rate of tension transients following step changes in length or MgATP concentration are increased with increasing [P_i] (Herzig et al. 1981; Hibberd et al. 1985), however, little effect on either the maximum shortening velocity or the shape of the force-velocity relation has been found at physiological MgATP concentrations (Altringham and Johnson 1985; Cooke and Pate 1985; Chase and Kushmerick 1988). The oscillatory work produced by vertebrate fibers is enhanced and shifted to a higher frequency, an effect that is more pronounced in insect flight muscle (Abbott and Mannherz 1970; Kawai 1986; Kawai et al. 1987; White and Thorson 1972). Whether $H_2PO_4^-$ or HPO_4^{2-} is the biochemically and physiologically active form of phosphate remains unresolved (Nosek et al. 1987a; Chase and Kushmerick 1988).

Studies of the actomyosin interaction in solution have been used to infer the energies of many of the intermediate states. Although the properties of this interaction are most probably altered by the steric constraints imposed by the filament lattice, the free energy of a state defined in solution is widely regarded to be a reasonable measure of the free energy minimum that occurs for the corresponding cross-bridge state during the interaction in the fiber. The observation that the M.D.P_i complex binds weakly to actin in solution has thus suggested models in which an A.M.D.P_i complex (A, actin; M, myosin; D, ADP) is a weakly-bound, low-force cross-bridge state occurring near the beginning of the working portion of the powerstroke (Eisenberg et al. 1980; Hibberd and Trentham 1986). In the functioning filament array, the phosphate release steps are thought to be involved in the transition to a more strongly-bound, high-force state (Hibberd et al. 1985; Webb et al. 1986). Increasing concentrations of phosphate decrease the free energy of hydrolysis of MgATP. As this occurs, the free energies of those states that precede phosphate release decrease in free energy relative to those that follow the release step. From the perspective of cross-bridge energetics, one can expect an increasing net transfer of cross-bridges from force-produc-

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ing states back into the A.M.D.P_i and M.D.P_i states with increasing concentrations of P_i (Hill 1977; Hibberd and Trentham 1986; Webb et al. 1986), and the quantitative nature of the changes in the mechanical properties of contracting fibers as [P_i] increases can serve as a probe the properties of states that are involved in force production during the cross-bridge powerstroke.

In the present paper we have reinvestigated the effect of phosphate on the isometric force and shortening velocity of permeable, fast skeletal muscle fibers. Using enzymes to carefully define the concentration at low levels of phosphate, and replacing acetate with phosphate as the principal anion at high levels of phosphate, we have significantly extended the range of concentrations explored in previous investigations. We find that force declines linearly with the logarithm of [P_i], and we show that this observation is compatible with models in which P_i release precedes the major portion of the powerstroke. Measurement of the effect of P_i on contraction velocity also provides information on the energetics of those states involved in force production. At the maximum shortening velocity, the work done by those cross-bridges in the working portion of the powerstroke is exactly balanced by the drag of myosin heads that enter regions of negative force (Huxley 1957). Phosphate can affect this process at two points. At higher concentrations of P_i, the work performed in the powerstroke is diminished and the negative work performed can be increased by competition between P_i and MgATP, which retards the dissociation of the myosin head from actin at the end of the powerstroke. Thus the effect of P_i on the velocity of lightly loaded fibers allows further characterization of the states within the powerstroke.

Materials and methods

Thin strips of psoas muscle, 2–3 mm in diameter were dissected from a rabbit and tied to wooden sticks with surgical silk. Fibers were glycerinated at 0°C for 24 h in a buffer containing 0.24 M potassium acetate (KAc), 10 mM EGTA, 40 mM n-tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES), 10 mM magnesium acetate ($MgAc_2$) diluted 50:50 (v/v) with glycerol, pH 7. The fibers were then transfered to fresh glycerinating solution and stored at −20°C for up to 4 months. Mechanical measurements in the absence of added P_i were performed using a relaxing buffer containing 6 mM $MgAc_2$, 1 mM EGTA, 1 mg/ml creatine phosphokinase (CK), 20 mM creatine phosphate (CP), 10 mM sucrose, 0.15 units/ml sucrose phosphorylase, 4 mM or 50 μM MgATP, 40 mM TES (experiments at pH 7.0) or 40 mM 2(N-morpholino)ethanesulfonic acid (MES) (experiments at pH 6.2), and added KAc (approximately 100 mM) resulting in a final ionic strength of 210 mM. The concentrations of the various ionic species were calculated using standard binding constants and a Fortran computer program which solves the appropriate, full, nonlinear system of balance equations by a Newton iteration technique. For experiments performed in the presence of P_i, K_2HPO_4 and KH_2PO_4 were added to the above buffers as described below with the pK of P_i taken as 6.75. Na_2ATP, CP, CK, and sucrose phosphorylase (partially purified powder) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sucrose phosphorylase was dialyzed for 24 h at 0°C against a 1000-fold excess of buffer with three changes of dialysis solution prior to use.

We found that commercially available CP and ATP contained small amounts of contaminating free phosphate which amounted to up to 0.2–0.5 mM in our activating solutions, a problem also identified by Kentish (1986). As a primary goal of these experiments is to define the mechanical properties of fibers over as large a range of P_i concentrations as possible, sucrose and sucrose phosphorylase were added to reduce P_i contamination. Sucrose phosphorylase catalyzes the reaction:

$$\text{Sucrose} + P_i \longleftrightarrow \alpha\text{-D-glucose-1-phosphate} + \text{D-fructose}.$$

In order to eliminate contaminating phosphate, relaxing buffers containing 0.15 units/ml sucrose phosphorylase and 10 mM sucrose were incubated at room temperature (20°C) for 2 h prior to experimentation. (One unit of sucrose phosphorylase converts 1 μM sucrose per minute at 10°C.) Using an equilibrium constant of 0.06 (Silverstein et al. 1967), 10 mM sucrose, and an upper limit of 1 mM contaminating P_i, one can calculate that less than 10 μM residual free phosphate would remain in our solutions at equilibrium. These solutions, scavenged for P_i, were used to activate fibers at a minimal P_i concentration, the only P_i arising from the fiber ATPase activity. Higher concentrations of P_i, up to 20 mM, were achieved by addition of an aliquot of the above relaxing solution containing either 52 mM (pH 7.0) or 73 mM (pH 6.2) P_i, with KAc concentration adjusted to maintain ionic strength of 210 mM, and omitting sucrose phosphorylase. Phosphate concentrations higher than 20 mM were obtained by changing solutions. Final concentrations of the individual ionic components are not linear functions of the added species. The above method of forming solutions at intermediate P_i concentrations results in a maximum 0.5% change in either ionic strength or substrate concentration at 10 mM and 20 mM P_i. No effort was made to adjust for this small change.

For mechanical measurements, single fibers were dissected from a small bundle of glycerinated fibers on a cold stage and mounted between a solid state force transducer and an arm connected to a rapid motor as described previously (Cooke et al. 1988). The fiber was first immersed in a relaxing buffer for 5 min to allow diffusion of creatine kinase into the fiber, and was then fully activated by addition of $CaCl_2$ using stock solutions containing 33 mM $CaCl_2$, 205 mM TES, pH 7.5 for experiments at pH 7.0 or 25 mM $CaCl_2$, 175 mM MES, pH 6.5 for experiments at pH 6.2. At full activation, these additions resulted in changes of final pH in the experimental buffer of less than 0.05 pH units, as determined by direct measurement. Ca^{2+} sensitivity of glycerinated fibers varies with MgATP concentration (Brozovich et al. 1988), P_i concentration (Kentish 1986), and pH (Donaldson and Hermansen 1978). The effective binding of Ca^{2+} to EGTA is also pH dependent (Martell and Smith 1974). As we are measuring changes in tension as a function of [P_i], pH, and [MgATP], extreme care was taken in all experiments to insure that fibers were fully activated. Addition of Ca^{2+} buffer resulted in a calculated 1–2 mM increase in ionic strength.

A constant temperature was maintained by passing cooled water through the aluminum block surrounding the fiber chamber and monitored by a small thermistor mounted adjacent to the force transducer. The experimental buffer was stirred by taking up and ejecting 10 μl of solution at a frequency of approximately 2 Hz. Mounted fiber length was approximately 5 mm. Fiber diameters were determined at

X100 magnification at three to five locations along the fiber and the values averaged to determine cross-sectional area. Initial sarcomere lengths varied between 2.4 and 2.6 μm as determined by laser light diffraction. Load clamps to low loads were performed using a protocol to eliminate drifts in baseline force described by Cooke et al. (1988). The load was clamped to 10% of isometric tension for 40 ms, followed by a step release of 1–2% of fiber length to determine the absolute value of zero force. The fiber was reextended, and this value of zero force was used in a subsequent clamp to 5% of isometric tension.

Determination of internal [P_i]

As noted previously, P_i contamination in experimental buffers was reduced by addition of sucrose phosphorylase and sucrose. In an actively contracting fiber, P_i is also produced as a direct consequence of the fiber ATPase, and then accumulates in the fiber due to the finite rate for diffusion out into the surrounding buffer. The activity of sucrose phosphorylase used in these experiments is too low to adequately buffer the buildup of phosphate within the fiber, which is produced at a rate of approximately 1 per second per myosin head. (Kawai et al. 1987; Cooke et al. 1988). This accumulation of P_i limits the lower range of P_i concentrations we have been able to consider. Internal P_i concentrations were determined by solving the appropriate diffusion equation. Letting $C(r)$ be the concentration of P_i at radius r from the center of a fiber of radius a, then $C(r)$ must satisfy

$$D\left[\frac{d^2C(r)}{dr^2}+\frac{1}{r}\frac{dC(r)}{dr}\right]+\frac{VS\varrho}{K_m+S}=0 \quad 0<r<a,$$

$$\frac{dC(0)}{dr}=0,$$

$$C(a)=C_0,$$

with solution

$$C(r)=C_0+\frac{(a^2-r^2)\,VS\varrho}{4D(K_m+S)}.$$

Here V is the maximum ATPase rate, ϱ is the concentration of myosin heads, S is the concentration of MgATP, K_m is the Michaelis constant for the ATPase, C_0 is the buffer P_i concentration, and D is the diffusion coefficient of P_i. The bracketed term in the first equation indicates the diffusion of P_i, the second term models the production of P_i by the ATPase. The diffusion coefficient for P_i was taken as 2.1×10^{-6} cm^2/s, the value determined in frog muscle by Yoshizaki et al (1982) at 25°C adjusted for the change in the viscosity of water at 10°C. V_{max} for the ATPase was taken to be 1.3 s^{-1} per head at pH 7.0, and 1.1 s^{-1} per head at pH 6.2 (Cooke et al. 1988). Myosin head concentration was assumed to be 220 μM; the K_m for the ATPase was assumed to be 20 μM (Sleep and Glyn 1986); and C_0 was taken to be 0. Average fiber radius was 35.0 ± 0.9 μm (mean ± SEM, 25 obs.). Averaging P_i concentration across the fiber, the following values were obtained. At 4 mM MgATP, pH 7.0 and 6.2, P_i concentrations were 0.20 mM and 0.17 mM respectively. At 50 μM MgATP, pH 7.0 and 6.2, P_i concentrations were 0.14 mM and 0.12 mM. These values define the lower limit of P_i concentrations that are available by the methods employed in these studies. The upper limit is reached by replacing the principal buffer anion, acetate, with phosphate at an equivalent ionic strength.

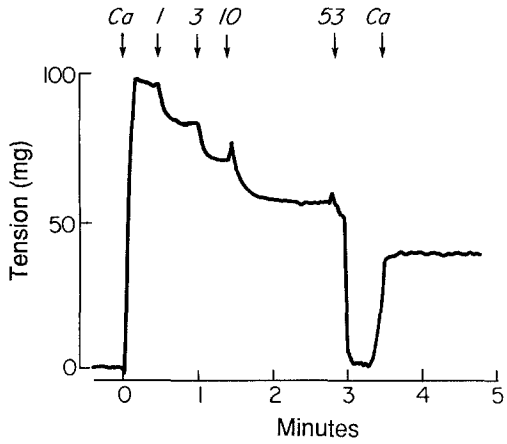

Fig. 1. The isometric force of a single glycerinated rabbit psoas fiber is shown as a function of time. The fiber was initially incubated in a relaxing solution and maximally activated at the first *arrow* by addition of Ca until full activation was achieved. Addition of aliquots of a phosphate activating solution, in which 52 mM potassium phosphate replaced 120 mM KAc, increased the phosphate concentration to 1 mM, 3 mM and 10 mM at the next three *arrows* respectively. At the last *arrow* the solution was removed and replaced with a 52 mM phosphate relaxing solution and then reactivated by addition of $CaCl_2$. The brief rise in tension at the fourth *arrow* is a temperature artifact. The fiber had a diameter of 60 μm and a length of 4.7 mm. The temperature was 10°C

Results

Effect of P_i on isometric force

Single fibers were mounted between a tension transducer and a fast motor, incubated in a relaxing solution containing no added P_i, and activated by addition of $CaCl_2$ as shown in Fig. 1. The P_i concentration bathing the fiber was increased by addition of aliquots of an activating solution containing 52 mM P_i instead of KAc, pH 7, bringing the added P_i concentration successively to 1 mM, 3 mM, and 10 mM. Total fiber P_i concentration is then equal to the added P_i plus the P_i accumulation inside the fiber due to the fiber ATPase, as discussed above. Two additional factors could alter total P_i concentration in the protocol of Fig. 1. Upon addition of an aliquot of P_i containing buffer, the buffer bathing the fiber would still contain sucrose phosphorylase and sucrose, and P_i concentration will decrease with time. This would be most noticable at 1 mM P_i. Using Michaelis constants of 2.5 mM and 1.8 mM for sucrose and phosphate, respectively, and a Ping-Pong scheme for the enzymatic kinetics (Silverstein et al. 1967), the P_i concentration would decrease by approximately 40 μM in 1 min, a rate which is too small to alter our conclusions. We ignore this effect. Additionally, from Eq. (2) it is clear that buildup of P_i in the solution bathing the fiber due to phosphate generated by the fiber ATPase could possibly alter the results. With an ATPase of 1.3 s^{-1} per head, a fiber 0.5 cm in length would increase the P_i concentration in 150 μl of stirred, activating buffer, by only about 0.5 nM/min. Thus we can ignore this effect as well. As shown in Fig. 1, concentrations of P_i greater than 20 mM were obtained by changing

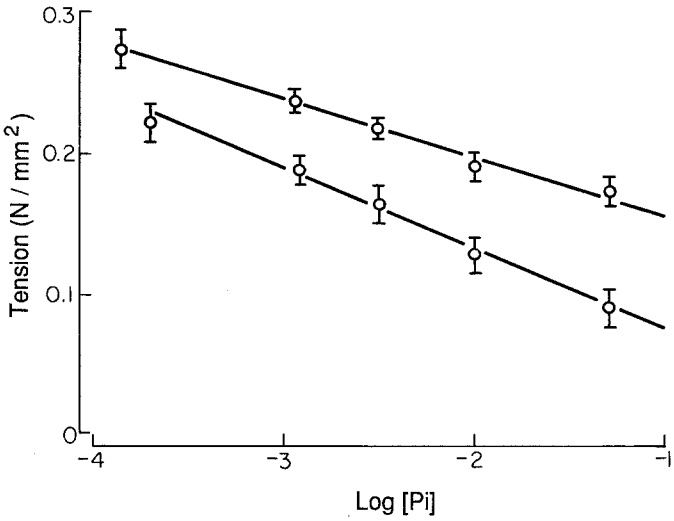

Fig. 2. Isometric force measured as described in Fig. 1 is shown as a function of the logarithm of the phosphate concentration at pH 7.0, 50 μM MgATP (*upper curve*) and 4 mM MgATP (*lower curve*). The background P_i due to the fiber ATPase was calculated to be 0.20 mM at 4 mM MgATP or 0.14 mM at 50 μM MgATP, as described in the text. *Solid lines* are least-squares, linear fits. The *error bars* show the standard error of the mean for 5—8 measurements

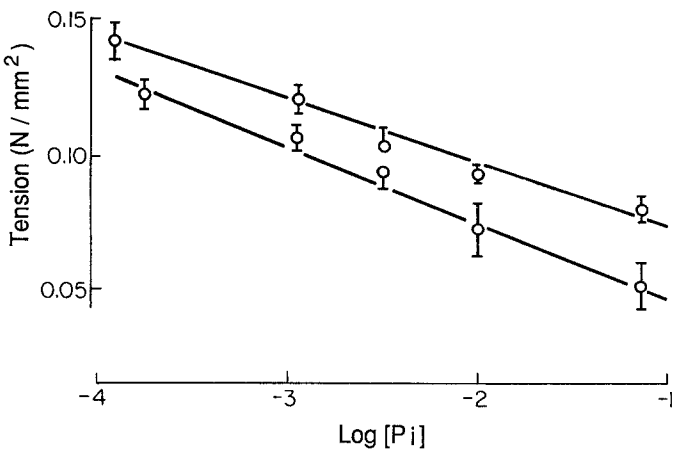

Fig. 3. Isometric force measured as described in Fig. 1 is shown as a function of the logarithm of the phosphate concentration at pH 6.2, 50 μM MgATP (*upper curve*) and 4 mM MgATP (*lower curve*). Background P_i concentration due to the fiber ATPase was calculated to be 0.12 mM at 50 μM MgATP or 0.17 mM at 4 mM MgATP as described in the text. Solid lines are least-squares, linear fits. The *error bars* show the standard error of the mean for 5—8 measurements

the solution bathing the fiber and then reactivating the fiber. The replacement solution did not contain sucrose phosphorylase, but did contain 10 mM sucrose.

Under some conditions the isometric tension of the fibers decreased with time at constant P_i concentration. The mechanisms responsible for this decline in force are probably complex, including increasing sarcomere heterogeniety, etc. However, the decline in tension was more evident at the lower P_i concentrations where the fibers generate more tension. Above 10 mM P_i, the fibers would maintain stable tensions for several minutes. This complication was addressed using several protocols. In some experiments phosphate was raised directly from the lowest to the highest concentration, and in other experiments the fiber was shifted from a high P_i concentration to a lower one. In some cases the fiber was relaxed and reactivated at a new P_i concentration. All of these protocols gave similar results, showing that the effects measured were due to changes in tension resulting from differing phosphate concentrations and not due to a decline in fiber function.

Figure 2 shows isometric tension as a function of the logarithm of the average fiber phosphate concentration for maximally activated fibers at 50 μM (upper) and 4 mM (lower) MgATP, pH 7.0. The solid lines represent least-squares, linear fits to the data showing that isometric tension is well approximated as linear in the logarithm of $[P_i]$ over the range which we have been able to examine. Although fibers activated at 4 mM MgATP generate less tension, as has been observed previously (Cooke and Bialek 1979; Ferenczi et al. 1984), tensions decrease in parallel as $[P_i]$ increases. The slope of the linear fit is slightly less for the lower concentration of MgATP, however the differences in slopes are not statistically significant. Accepting the above linearity, an alternative scale for the horizontal axis in Fig. 2 might be average logarithm of fiber phosphate concentration instead of the more intuitive logarithm of the average phosphate concentration, which we have used. Any difference between these two scales would be most noticable at the lowest phosphate concentrations considered. Analysis of the data at the lowest concentrations in terms of the alternative scale yields a horizontal shift on the concentration axis of only 0.1 unit. This is much too small to be distinguished by our data. Thus we used the physically more intuitive logarithm of average $[P_i]$. As pH is lowered, the fraction of phosphate in the diprotonated, singly-charged form increases relative to the doubly-charged species. Thus at a given ionic strength, higher P_i concentrations are possible at lower pH. Figure 3 shows least-squares, linear fits to isometric tension at pH 6.2, with P_i concentration extending from approximately 0.12 mM to 73 mM. Again tension is linear in $\log[P_i]$, with no statistically significant difference in the slopes obtained at 50 μM and 4 mM MgATP. Although the fibers exert less tension at pH 6.2 than at pH 7.0 (Donaldson and Hermansen 1978), the relative slopes, i.e. percent decrease in tension per tenfold increase in $[P_i]$, are identical. We note, however, that at 50 μM MgATP for both pH 6.2 and pH 7.0, as $[P_i]$ rises from 10 mM to the highest $[P_i]$ considered, the tension decrease is slightly less than expected from the least-squares fits. Thus the effect of P_i may be leveling off at sufficiently high $[P_i]$ and low [MgATP].

Effect of P_i on shortening velocity

Previous investigators, working at high substrate concentrations, have reported that P_i has little effect on the maximum contraction velocity (Cooke and Pate 1985; Altringham and Johnston 1985; Chase and Kushmerick 1988; Cooke et al. 1988). The velocity of contraction at a low load, 5% of P_o, was measured in load clamps as shown in Fig. 4. At high substrate concentration addition of P_i causes a small but measurable increase in the velocity of contraction. At low substrate concentration P_i causes a significant decrease in velocity. These effects were reversible, and in Fig. 4, for each case, the conditions resulting in the greater velocity followed those producing the lower velocity.

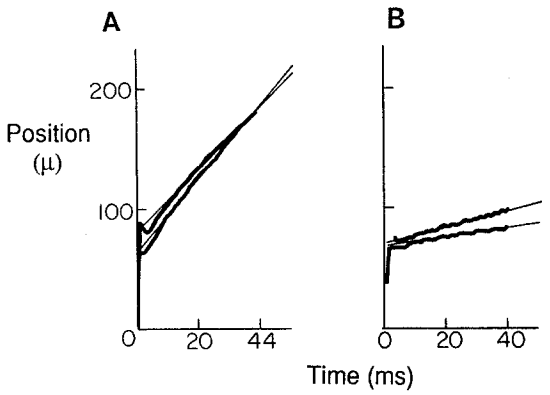

Fig. 4 A, B. The fiber length during a series of load clamps is shown as a function of time in the clamp. Fibers were activated isometrically in one solution and at peak tension they were released to 5% of isometric tension. The solution was then exchanged and after approximately 20 s a second load clamp to 5% of isometric tension was performed. In **A** the MgATP concentration was 4 mM, the inital P_i concentration was 1 mM (*upper trace*) and the final P_i concentration was 52 mM (*lower trace*). In **B**, the MgATP concentration was 50 μM, the inital P_i concentraion was 52 mM (*lower trace*) and the final P_i concentration was 1 mM (*upper trace*). The pH was 7, and the fiber length was 3.7 mm. In each trace the change in the slope is proportional to the change in velocity. The intercept also changes due to the decreased tension generated at higher $[P_i]$

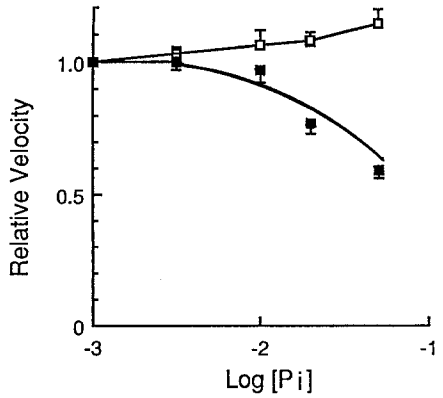

Fig. 5. The velocity of contraction during load clamps to 5% of P_o is shown as a function of the logarithm of the phosphate concentration. The data were obtained as described in Fig. 4. The velocity is shown relative to that obtained in 1 mM P_i. The MgATP concentration was 4 mM (□) or 50 μM (■). The fiber velocity at 1 mM P_i was 1.2 lengths/s (4 mM MgATP) or 0.34 lengths/s (50 μM MgATP). *Lower solid curve* is fit for competitive inhibition as described in text. The *error bars* show the standard error of the mean for 5–8 measurements

We note also in Fig. 4 that although the decrease in P_o at higher $[P_i]$ was of the magnitude reported in the previous section, the zero intercept of the least-squares fits to the data changed to a smaller degree, however, due to the nonlinear nature of the elastic element. The data shown in Figs. 4 and 5 were obtained in load clamps to 5% of P_o. The value of 5% was chosen to allow rapid collection of data. Previous observations that the curvature of the force-velocity relation is not altered by P_i (Cooke et al. 1988), as well as extrapolation of a series of clamps 2–10% of P_o, indicate that a similar effect occurs at the maximum velocity obtained by extrapolation. Figure 5 shows that there is a slight increase in velocity at 4 mM MgATP as P_i increases from 0.2 mM to 52 mM (14 ± 6%, mean ± SEM). Previous investigations had failed to observe this small effect on velocity (Altringham and Johnston 1985; Cooke et al. 1988) due to the more limited phosphate range which those studies employed.

Figure 5 shows that the behavior seen at 50 μM MgATP is dramatically different from that observed at 4 mM MgATP. Little change in velocity occurs until the P_i concentration increases to 10 mM, and then above 10 mM, a substantial decrease is observed, with the highest $[P_i]$ giving a value for velocity of 59 ± 4%, (mean ± SEM) that obtained at the lowest $[P_i]$. Phosphate competes weakly with creatine phosphate in the creatine phosphokinase reaction (Nihei et al. 1961). To insure that the observed decrease in contraction velocity was not a result of inadequate buffering of MgATP levels inside the fiber, an effect which would be most noticable at low MgATP concentrations, additional experiments were done with the CK concentration increased to 10 mg/ml. The change in velocity and tension was the same as with the standard CK concentration of 1 mg/ml indicating adequate buffering of substrate.

Both $H_2PO_4^-$ and HPO_4^{2-} bind magnesium. At 50 μM MgATP and the highest phosphate concentrations used in these experiments, the total concentration of magnesium phosphate complexes is in excess of 2 mM, raising the possibility that phosphate complexed to magnesium is the effective species. To investigate this possibility, the $MgAc_2$ concentration was raised to 12 mM, effectively doubling the concentration of magnesium phosphate complexes. However, no difference was detected in either the change in isometric tension or velocity between the two $MgAc_2$ concentrations suggesting that P_i, not magnesium phosphate, is the active species.

Discussion

As noted in the Introduction, the work of a number of investigators has shown that increased concentrations of phosphate cause a decrease in isometric tension in contracting skeletal muscle fibers, that the phosphate release step is readily reversible in active fibers, and that phosphate increases the rate of a tension producing reaction. Due to an apparent, pivotal position that the step(s) involved in phosphate release play in force generation in contracting muscle, we have extended previous analyses of the effects of P_i on contracting fibers to encompass as large a variation in $[P_i]$ as possible. By using an enzymatic system to reduce contaminating P_i levels in order to accurately define a minimum concentration, and using phosphate as the principal anion in contraction buffers to attain a maximum concentration, we have been able to accurately vary $[P_i]$ over more than 2.5 orders of magnitude. This, coupled with extension of experimental analyses to low MgATP concentrations allows us to explore new aspects of the chemomechanical cycle.

The free energy of hydrolysis of MgATP, ΔG^{ATP}, can be expressed as

$$\Delta G^{ATP} = \Delta G^0 + RT \ln([ATP]/[ADP]\,[P_i])$$

where ΔG^0 is the standard free energy. Thus raising the concentration of P_i from 0.2 to 52 mM will lower the free energy of hydrolysis of MgATP by 5.6 RT, which will of

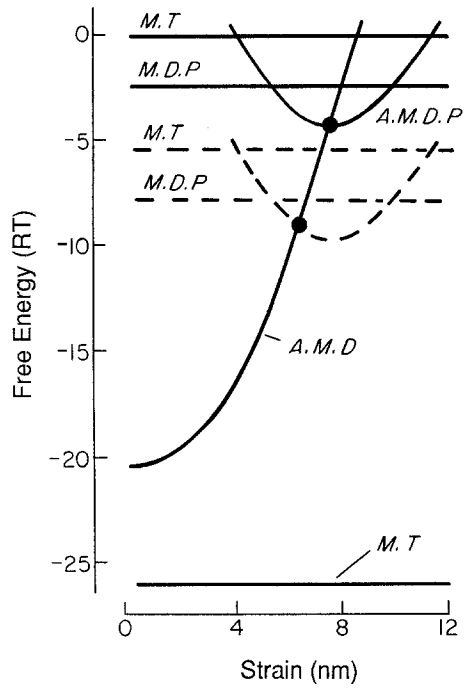

Fig. 6. A model of the cross-bridge states involved in the analysis of phosphate release is shown. The free energy of the states is plotted as a function of the strain, x, which measures the position of an actin site relative to a myosin cross-bridge, A = actin, M = myosin, T = ATP, D = ADP. The *solid lines* show the states at a low concentration of P_i and the *dashed lines* at a much higher concentration. When $[P_i]$ increases the free energy of MgATP hydrolysis, i.e. the distance between two M.T states decreases, and the free energies of the states that precede P_i release fall relative to those states that follow release. The two sets of states are drawn for 0.2 mM P_i (*solid lines*) and 52 mM P_i (*dashed lines*)

necessity decrease the ability of the muscle to perform work. This decrease in the free energy of MgATP hydrolysis goes linearly with the logarithm of the phosphate concentration. Our experimental observations indicate a similar linear relationship between isometric tension and $\log[P_i]$. We hasten to point out that although this is apparently the first explicit recognition that a logarithmic transformation can produce linearity of tension with respect to P_i concentration, other data consistent with this observation currently exist in the literature. In particular, the data on isometric tension of Kawai (1986) using rabbit psoas fibers and the data of Kentish (1986) from rat heart fibers prove linear in the logarithm of phosphate concentration. One exception, however, is Nosek et al. (1987a) who suggest that tension falls linearly with $[P_i]$. We have no explanation for the discrepancy between this work and the data reported here and cited above.

The observation that force decreases with increasing P_i, is predicted by simple models of cross-bridge energetics which are derived from studies of the actomyosin interaction. Such models have been discussed by Eisenberg et al (1980), Webb et al. (1986), and by Hibberd and Trentham (1986), and we will show that many models of this class predict a linear relation between force and $\log[P_i]$. Figure 6 shows the free energies of the cross-bridge states of one such model. In the model, detached M.T and M.D.P_i states are of constant free energy and do not produce force. Initial attachment is in a weakly-bound, low-force A.M.D.P_i complex as suggested from studies carried out in solution (for review, see Cooke 1986; Hibberd and Trentham 1986). Phosphate release is accompanied by a transition to a strongly-bound, highly-strained A.M.D state as suggested by Hibberd et al. (1985). As shown in Fig. 6, isometric tension in the model is primarily generated by those cross-bridges in the A.M.D state with strains between 0 and 8 nm. As the P_i concentration is raised, those states preceding P_i release decrease in free energy relative to those states that follow P_i release. This is indicated by the solid and dashed lines in Fig. 6. Due to the relative shifts in free energies between states, as $[P_i]$ concentration increases, some of the highly-strained cross-bridges that were bound in the A.M.D state at lower $[P_i]$, now find it energetically favorable to remain in the A.M.D.P_i state where they produce less force. The fact that fiber stiffness is decreased by P_i to a lesser extent than is tension, suggests that cross-bridges shifted into the A.M.D.P_i state are less stiff or that some fraction of these are then detached (Hibberd et al. 1985; Brozovich et al. 1988.)

The relation between isometric tension and phosphate is most easily visualized by considering the cross-bridges that make the transition from the A.M.D state to the A.M.D.P_i state. These lie roughly between the intersections of the A.M.D and A.M.D.P_i states shown by the two solid circles in Fig. 6. Letting m be the number of myosin/cm^3, s the sarcomere length, and c the separation of actin sites along the thin filament, the force/cm^2 predicted by the model, F, is given by

$$F = \frac{ms}{2c}\int_x \left[n(x)\frac{dG(x)}{dx} \right] dx.$$

where $n(x)$ is the proportion of cross-bridges bound in the high-force state as a function of strain, x, and the integral is taken over all values of strain. The derivative of free energy, $dG(x)/dx$, equals the force produced by a cross-bridge with strain x (Huxley 1957; Hill 1977). In this expression we have ignored the small force generated by the weakly-bound state. Although this still results in a complex expression, the result of added phosphate and the transition from the A.M.D state to the A.M.D.P_i state can be approximated rather simply. To evaluate the effect of this transition on the tension, we assume $n(x)$ is a constant. Then $n(x)$ can be removed from the integral and the expression for the change in force becomes

$$\Delta F = \frac{ms}{2c}\left[n\int dG \right] = \frac{ms}{2c}[n(G_2 - G_1)].$$

Note that this expression depends upon two factors, n, the number of cross-bridges attached in the state per unit length, and $G_2 - G_1$, the difference in free energy between the beginning and end of the region considered, i.e. the region in Fig. 6 bounded by the two solid circles. The expression is independent of the actual slope of the free energy with respect to strain. The difference between G_1 and G_2 is the difference between $\log[P_i]$ at the two concentrations. To a good approximation, the force becomes

$$F = F_o - \alpha \log[P_i]/[P_{io}]$$

where F is the force at concentration $[P_i]$, F_o is the force at concentration $[P_{io}]$ (~ 0.2 mM), and the constant α involves n and other constant factors from the original integral for force. As is evident, the model predicts that isometric force

decreases linearly with the logarithm of phosphate concentration.

Several simplifying assumptions were made in the above analysis in order to determine an analytical approximation to force with varying $[P_i]$. A more rigorous treatment, however, involving the complete numerical solution of a 5-state cross-bridge model and including the contributions to isometric force from all of the states in Fig. 6 shows that tension does fall linearly with $\log[P_i]$ for P_i concentrations less than approximately 1 M (Pate and Cooke 1989). The critical assumptions in development of the model appear to be: (1) a low-force P_i state that precedes a state in which substantial force is produced, and (2) the population in the second state is approximately constant in the region in which it is affected by the descending P_i state. Our conclusions would appear of general applicability to such models.

There has been considerable interest in the relationship between changes in the free energy of hydrolysis of MgATP and changes in isometric tension. As noted previously, ΔG^{ATP} changes with alterations in concentration of MgATP, MgADP, and P_i. Although the data presented here indicate a linear relationship between changes in P_o and changes in ΔG^{ATP} when $[P_i]$ is altered, we stress that this effect does not occur with MgADP or MgATP. Indeed, increasing the concentration of either P_i or MgADP will result in a decrease in ΔG^{ATP}, while increasing [MgADP] actually increases isometric tension in glycerinated fibers, contrary to the case for P_i, (Abbott and Mannherz 1970; Cooke and Pate 1985). A similarly contradictory result holds for MgATP (Cooke and Bialek 1979; Ferenczi et al 1984). Additionally, maintaining constant ΔG^{ATP} by simultaneous changes in both [MgADP] and $[P_i]$ fails to yield a simple relationship between P_o and ΔG^{ATP} (Kentish 1986). A thermodynamically consistent (Hill 1977) model for cross-bridge function has been presented (Pate and Cooke 1989) reconciling the fact that ΔG^{ATP} and P_o change linearly with $\log[P_i]$, while changes in [MgADP] and [MgATP] fail to give similar relationships.

As the concentration of MgATP decreases, the force exerted by a fiber increases and the maximum velocity of contraction decreases. Both of these effects have been attributed to an increase in the population of A.M cross-bridge states due to the slower net rate of binding of MgATP (Cooke and Bialek 1979; Ferenczi et al. 1982). The data of Figs. 2 and 3 show that at low concentrations of MgATP, the addition of 10 mM P_i has an effect on tension that is very similar to that produced by addition of phosphate at high concentrations of MgATP. In the context of the model shown in Fig. 6 this would indicate that the population of the force-producing A.M.D state in the vicinity of the A.M.D.P_i state at low MgATP is approximately the same as that at high MgATP.

Some of the effects reported here may be due to an interaction of P_i at a site other than the nucleotide site on myosin. Several observations, however, suggest that they are due primarily to the binding of P_i at the myosin ATPase site. Studies of isotope exchange between water and P_i, and between P_i and MgATP have shown that P_i binds reversibly to this site with subsequent incorporation into MgATP (Ulbrich and Ruegg 1977; Sleep and Hutton 1980; Webb et al. 1986). The linearity of tension as a function of $\log[P_i]$ at high substrate, which is compatible with model predictions, argues that even at high concentrations P_i does not have a large nonspecific effect on these parameters. Kentish (1986) showed that another divalent anion, creatine phosphate, has only a small effect on tension. In addition the attachment of other groups, phenyl or methyl, to the phosphate inhibits its effect on tension (Nosek et al. 1987b.) These observations indicate that the effect on tension requires a functional phosphate, and is not a nonspecific effect of divalent anions. Although all of the above argue that the effects of P_i on contraction arise from binding to the nucleotide site, none of them can rigorously rule out some additional nonspecific effect of P_i influencing contractile properties at another location.

We find that the slope of the relative tension versus $\log[P_i]$ is the same at pH 7 and pH 6.2. This corrects previous observations by ourselves (Cooke and Pate 1985) which suggested the same absolute drop in tension with increasing P_i. Unlike the present experiments, in those previous experiments phosphate concentrations were not adequately controlled. The present observations are also in agreement with Kentish (1986) and Chase and Kushmerick (1988) who have reported that phosphate has a similar relative effect at two different values of pH. There has been considerable speculation that the inhibition of tension by P_i is due only to the diprotonated form of P_i. Our data suggest that this question cannot be answered by measuring the effect of P_i on tension, if tension depends on the logarithm of P_i concentration. Similar slopes are expected irrespective of whether the inhibition of tension is due to total P_i concentration or to either the diprotonated or monoprotonated species alone. Although the concentration of the diprotonated species is greater at the lower pH, its concentration will always increase by the same factor as does the total P_i concentration. That is to say, if the total P_i is increased by a factor of ten, that of the diprotonated species will also increase by a factor of ten at any pH. If the data of Figs. 2 and 3 are replotted as a function of the logarithm of the $H_2PO_4^-$ concentration, the data obtained at pH 6.2 still lie below those obtained at pH 7.0, eliminating the possibility that the diprotonated form of P_i is the only inhibitor, and that the sole effect of pH is to shift more P_i into this form. A determination of the species of P_i involved requires measurement of a parameter that depends directly on $[P_i]$ and not on $\log[P_i]$.

The effect of phosphate on shortening velocity depends strongly on the MgATP concentration. At high concentrations of MgATP there is a very marginal increase in shortening velocity as $[P_i]$ increases from 0.2 mM to 52 mM. Models of cross-bridge kinetics in which P_i release involves a transition from a weakly-bound state to a strongly-bound, high-force state, such as shown in Fig. 6 predict that P_i should very slightly inhibit the contraction velocity. In a more complete analysis of such a model, the velocity at high [MgATP] was found to decrease by only 1% as P_i increased from 1 mM to 20 mM (Pate and Cooke 1988). Although the model predicts change in a different direction from experiment, both changes are extremely small. As shown in Fig. 5, at low concentration of MgATP, there is little change in velocity until the concentration of P_i exceeds 10 mM, with significant inhibition at higher $[P_i]$. The inhibition of velocity can be explained by competition between P_i and MgATP for the myosin nucleotide site at the end of the cross-bridge powerstroke. Binding of P_i to the nucleotide site would then prevent dissociation of the myosin head from actin by MgATP. Heads that were not dissociated would generate negative work slowing fiber velocity. At low [MgATP], the data of Fig. 5 can be fit with an expression for pure competitive inhibition:

$$V = \frac{V_m[ATP]}{K_m(1 + [P_i]/K_i) + [ATP]}.$$

Here V is the fiber velociy as a function of [MgATP], V_m is the fiber velocity at saturating [MgATP], K_m is the [MgATP] for half-maximal velocity (K_m = 150 μM, Cooke and Pate 1985), and K_i describes the inhibition of V by P_i. The fit to the data shown by the lower line in Fig. 5 defines a value for K_i of 80 mM, showing that P_i binds only weakly to the nucleotide site. The binding of MgATP to myosin has been shown to be competitively inhibited by P_i, with a value for K_i of 1.5 mM (Bagshaw and Trentham 1974). Thus the binding of P_i to myosin in the absence of bound MgADP is weakened by a factor of about 50 by the binding of actin. Actin is also known to weaken the binding of P_i to myosin-ADP during steady-state hydrolysis, suggesting that P_i may be competing with MgATP by binding to the position usually occupied by the gamma phosphate, and that actin affects this bond even in the absence of bound MgADP.

The simple model discussed above can explain the effects of added P_i on the steady-state responses of muscle fibers. A more complete analysis of the model would be required to determine whether it could also explain the responses of fibers that are oscillated sinusoidally, or whether a more complex model, with two different cross-bridge cycles is required as suggested by Kawai et al. (1987).

In summary, we find that isometric tension decreases linearly with the logarithm of phosphate concentration. This observation is compatible with a wide class of models of cross-bridge function in which a tension producing step is coupled to phosphate release. Additionally, the powerstroke distributions appear similar at both high and low [MgATP]. Maximum shortening velocity, on the other hand displays differing behavior as a function of phosphate concentration at high and low concentrations of substrate. The inhibition of velocity observed at low substrate concentration is probably due to competition between P_i and MgATP, preventing dissociation of the myosin head from actin at the end of the powerstroke.

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