Muscle Force and Stiffness during Activation and Relaxation

Implications for the Actomyosin ATPase

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ABSTRACT Isolated skinned frog skeletal muscle fibers were activated (increasing [Ca^{2+}]) and then relaxed (decreasing [Ca^{2+}]) with solution changes, and muscle force and stiffness were recorded during the steady state. To investigate the actomyosin cycle, the biochemical species were changed (lowering [MgATP] and elevating [$H_2PO_4^-$]) to populate different states in the actomyosin ATPase cycle. In solutions with 200 μM [MgATP], compared with physiological [MgATP], the slope of the plot of relative steady state muscle force vs. stiffness was decreased. At low [MgATP], cross-bridge dissociation from actin should be reduced, increasing the population of the last cross-bridge state before dissociation. These data imply that the last cross-bridge state before dissociation could be an attached low-force-producing or non-force-producing state. In solutions with 10 mM total P_i, compared to normal levels of MgATP, the maximally activated muscle force was reduced more than muscle stiffness, and the slope of the plot of relative steady state muscle force vs. stiffness was reduced. Assuming that in elevated P_i, P_i release from the cross-bridge is reversed, the state(s) before P_i release would be populated. These data are consistent with the conclusion that the cross-bridges are strongly bound to actin before P_i release. In addition, if Ca^{2+} activates the ATPase by allowing for the strong attachment of the myosin to actin in an $A \cdot M \cdot ADP \cdot P_i$ state, it could do so before P_i release. The calcium sensitivity of muscle force and stiffness in solutions with 4 mM [MgATP] was bracketed by that measured in solutions with 200 μM [MgATP], where muscle force and stiffness were more sensitive to calcium, and 10 mM total P_i, where muscle force and stiffness were less sensitive to calcium. The changes in calcium sensitivity were explained using a model in which force-producing and rigor cross-bridges can affect Ca^{2+} binding or promote the attachment of other cross-bridges to alter calcium sensitivity.

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INTRODUCTION

Eisenberg and Green (1980) recently proposed a model for the states in the hydrolysis of MgATP by actomyosin involving two types of actin-myosin states: weakly and strongly bound, with the state depending on the nucleotide bound to myosin. During ATP hydrolysis, a set of weakly bound states is proposed during which ATP is bound to myosin and hydrolyzed to ADP·P_i with or without actin attached. A set of strongly bound force-producing states (A·M·ADP and A·M) are thought to exist after actin binding and subsequent P_i release. The weakly bound states, which exist in a rapid equilibrium between bound and unbound states, have been observed in relaxed fibers at low ionic strength as an increase in muscle stiffness (Brenner et al., 1982; Schoenberg et al., 1984) as well as an increase in the fluorescence of a fluorescent ATP analogue (Nagano and Yanagida, 1984). Calcium is proposed to change the kinetics of the ATPase by enhancing the rate of P_i release (Greene and Eisenberg, 1980), in contrast to allowing actin and myosin interaction, as in the steric hindrance model (Haselgrove and Huxley, 1973; Parry and Squire, 1973). The cross-bridge is thought to change from the weakly bound, non-force-producing state(s) to the strongly bound force-producing state(s) after P_i release from the A·M·ADP·P_i complex. The association of P_i release with force production is consistent with the results of many investigators, who have found that an elevation of the free $[H_2PO_4^-]$ causes a decrease in the maximally Ca^{2+}-activated muscle force (Herzig et al., 1981; Altringham and Johnson, 1985; Cooke and Pate, 1985; Godt et al., 1985; Hibberd et al., 1985; Dawson et al., 1986; Kawai, 1986; Kentish, 1986; Kawai et al., 1987; Nosek et al., 1987).

The next step, after force production and cross-bridge movement, is thought to be the release of ADP. Siemankowski et al. (1985) provided arguments that ADP release limits the maximal speed of shortening. This is consistent with the observations that elevated free [ADP] decreases muscle V_{max} (Cooke and Pate, 1985; Luney and Godt, 1986) and elevates the maximally calcium-activated muscle force (Cooke and Pate, 1985; Godt et al., 1985). Finally, binding of ATP to the A·M complex rapidly dissociates myosin from actin with a second-order rate constant of $\sim 10^5$ $M^{-1}s^{-1}$ (Goldman et al., 1984*a*), returning the cross-bridge to a weakly bound state.

To investigate the mechanical properties of several of the proposed cross-bridge states, a skinned fiber preparation can be employed. Given the proposed biochemical pathway of the actomyosin ATPase, the cross-bridge can be "forced" into certain biochemical states by varying the proportions of substrates and/or end products used in the actomyosin ATPase pathway. Dissociation of myosin from actin should be slowed by lowering the [MgATP], and the population of cross-bridges in the state(s) just before and including dissociation should be increased. This would then allow one to investigate the consequences of this shift in the proportions of cross-bridge states on muscle force and stiffness. If these attached cross-bridges allowed others to attach in a cooperative manner (Bremel and Weber, 1972), one might observe that with low [MgATP] and decreasing levels of free Ca^{2+}, a given $[Ca^{2+}]$ might produce a higher force and stiffness during relaxation than during activation. This might be seen as a

hysteresis in the muscle force and stiffness vs. $[Ca^{2+}]$ relations (Ridgway et al., 1983). If cross-bridges ended their cycle in an attached non- or low-force-producing state before detachment, populating this state by lowering [MgATP] would be expected to decrease muscle stiffness more than force, and as such, the relative force per cross-bridge should decrease.

Similarly, the consequences on muscle force and stiffness of preferentially populating other cross-bridge states can be probed by elevating free P_i. At elevated free $[H_2PO_4^-]$, the population of cross-bridges in the state before P_i release should be elevated. If this is the only effect of elevated P_i and if actin and myosin are weakly bound in this state ($A \cdot M \cdot ADP \cdot P_i$ and $A \cdot M \cdot ATP$), the cross-bridge should produce neither force nor stiffness (at normal ionic strength), and muscle force and stiffness should be depressed to the same extent. However, if a strongly bound cross-bridge state exists before P_i release, muscle force would be depressed to a larger extent than muscle stiffness.

Thus, force and stiffness vs. $[Ca^{2+}]$ relations were compared during activation (increasing the free $[Ca^{2+}]$) and relaxation (decreasing the free $[Ca^{2+}]$) in solutions with physiological levels of MgATP, with lower [MgATP], or with elevated free $[H_2PO_4^-]$, to investigate the consequences of preferentially populating different states of the actomyosin ATPase.

METHODS

Fiber Preparation

Single fibers (~1,300 × 75 μm) from the IV lumbrical of the hindfoot of male frogs, *Rana temporaria,* were first isolated in chilled Ringer's solution. Then the fibers were transferred to relaxing solution ($[Ca^{2+}] = 10^{-8}$ M, [MgATP] = 4 mM), where small aluminum-foil T-clips (Ford et al., 1977) were attached near the myotendon junction. Next, the fibers were mounted horizontally in the experimental chamber on long (~2 cm) stainless-steel hooks between a force transducer (AME 801, Aksjeselskapet Mikroelektronik, Horton, Norway; resonant at 10 kHz) and a length driver (step response of 500 μs). Fibers were then chemically skinned for 30 s in relaxing solution with 0.5% added CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), a detergent.

Experimental Chamber

The experimental chamber was made of Plexiglas and constructed in such a way that cold water was circulated through channels near the muscle bath to keep the temperature constant (T = 5–8°C). Hooks were passed through small holes in the ends of the muscle bath, which connected to the length driver and force transducer. An additional channel in the chamber allowed for chilled solutions (T = 5–8°C) to be injected into the muscle chamber and to wash the previous solution out the end of the force transducer side. The assembly was mounted on the movable stage of an inverted microscope (Carl Zeiss, Inc., New York, NY) with the force transducer position adjustable so that the initial fiber position and length could be set.

Sarcomere Length Imaging System

Sarcomere length was determined using a phase-lock loop system. This device has been described elsewhere (Myers et al., 1982), but briefly, the fiber was transilluminated and the striation image was focused with a Zeiss 40× water immersion lens (0.75 NA) onto

a linear photodiode array (RE 128, Reticon, Sunnyvale, CA). The array was scanned sequentially every 256 μs and its output, a quasi-sinusoidal signal, was used as an input for the phase-lock loop, which computed the period of the sine wave corresponding to the striation image. The voltage output was proportional to the sarcomere length. Before experiments, the system was calibrated using etched gratings of known spacing (American Holographic, Boston, MA) and the output was linear from 1.97 to 3.67 μm.

Experimental Protocol

Skinned fibers were set at an initial sarcomere length of 2.2–2.6 μm and, with solution changes, activated by increasing free $[Ca^{2+}]$ and then relaxed by decreasing free $[Ca^{2+}]$. Data were not routinely taken for pCa values ($-\log_{10}[Ca^{2+}]$) below the value of maximal activation (determined from several fibers) because of the deleterious effects of prolonged activation at high $[Ca^{2+}]$ on the striation pattern. Before each activation/relaxation sequence, the fiber was slackened and the zero force and slack stiffness levels were measured. These levels were subtracted from those observed in relaxing solution to determine the resting muscle force and stiffness, and experimental values were measured above those of the slackened fiber. To measure the force and stiffness at a particular $[Ca^{2+}]$ for increasing and decreasing $[Ca^{2+}]$, $[Ca^{2+}]$ was increased in steps to maximal activation and then decreased in steps to relaxation solution.

During the solution changes, the observed sarcomere lengths changed. However, since the fiber moved laterally in the chamber, different sections of the fiber, possibly with different sarcomere lengths, were brought into the optical section. Contractions were assumed to occur isometrically if the changes in sarcomere length were less than the variations at rest (±0.2 μm), and the recorded changes in length did not continuously decrease during activation and then increase during relaxation. During the course of an experiment, maximally calcium-activated force would decline slightly from one activation/relaxation sequence to the next. However, the decrement was usually small (<5%). Data were not used if the maximum force in a control contraction declined to <80% of the level reached in the first control contraction.

After force reached a steady state ($t > 10$ s), stiffness was measured using a bandpass filtering technique (response time = 1 ms) and was found to be at steady state. This technique, described by Kawai and Brandt (1976, 1977), measured the magnitude of the force change produced by small (<1%), 1-kHz sinusoidal oscillations of muscle length. These measurements were checked by dividing the magnitude of the force change by the amplitude of the length oscillation (to yield muscle stiffness × 10^5 N/m^2 at maximal calcium activation, ~100–155; at full relaxation, ~0–5 range; most records showed no measurable stiffness).

For isometric contractions, the data of relative force and stiffness vs. pCa were fitted using a nonlinear least squares method with the Hill equation:

$$\text{relative force or stiffness} = A_0 + A_1\{1/(1 + 10^{n[\text{pCa-pK}]})\},$$

where A_0 is a constant, A_1 is the maximum predicted relative force or stiffness, pCa is the $-\log_{10}[Ca^{2+}]$, pK is the pCa at the half-maximal force level of the Hill fit, and n is the Hill coefficient, a measure of the slope of the curve at the pK. In these fits of the data, the predicted maximum force was not constrained to be that measured at maximal calcium activation, removing any bias in choosing both the baseline and maximum force. This did not affect the calculated pK's, but the n values were slightly increased. To further avoid biasing the results, the steady state muscle force and stiffness (as a percent of the maximum), recorded in sequential contractions at each pCa, were averaged for each

fiber. The data of average relative steady state force and stiffness vs. pCa were fitted to obtain a pK and n value for each fiber, which were then averaged to yield pK and n values for muscle force and stiffness vs. pCa for all fibers during activation and relaxation.

The slack test was used to measure the unloaded, maximal muscle shortening velocity, V_{max} (Edman, 1979). Fibers were maximally activated, and then were shortened using a length step large enough to drop force to zero (slacken the fiber). The force dropped coincident with the length step, oscillated around zero for a short time, and then, as the fiber shortened to take up the slack, force redeveloped. The slope of the regression line of the length step size vs. the time taken to shorten and begin to redevelop force was defined as V_{max}.

TABLE I

Binding Constants, and pH, Ionic Strength, and Temperature Corrections

EGTA	
H^+, Ca^{2+}, and Mg^{2+} binding	Martell and Smith (1974, 1982)
Ca^{2+} and Mg^{2+} ionic strength corrections	Robinson and Stokes (1965)
H^+ ionic strength corrections	Ellis and Morrison (1982)
Temperature corrections	Martell and Smith (1974, 1982)
ATP	
H^+, Ca^{2+}, Mg^{2+}, and K^+ binding	O'Sullivan and Smithers (1979)
H^+ ionic strength corrections	Phillips et al. (1963)
Ca^{2+} and Mg^{2+} ionic strength corrections	Phillips et al. (1966)
Temperature corrections	Martell and Smith (1974, 1982)
P_i	
H^+ and K^+ binding	Martell and Smith (1974, 1982)
Ca^{2+} and Mg^{2+} binding	O'Sullivan and Smithers (1979)
Ca^{2+}, Mg^{2+} and H^+ ionic strength corrections	Phillips et al. (1963)
Temperature corrections	Martell and Smith (1974, 1982)
CP	
H^+ binding	Martell and Smith (1974, 1982)
Ca^{2+} and Mg^{2+} binding	O'Sullivan and Smithers (1979)
MOPS	
H^+ binding	Ellis and Morrison (1982)
Temperature and ionic strength corrections	Ellis and Morrison (1982)
H^+ concentration	
H^+ from pH	Khoo et al. (1977)
Temperature and ionic strength corrections	Khoo et al. (1977)

Solutions

Solutions were mixed according to an iterative computer program that calculates, for specified free ion concentrations, the amount of solution constituents that must be added. The binding constants of the various ionic species present were corrected for both temperature and ionic strength (Table I). The ionic strength of all solutions was 0.2 M, and MOPS (morpholinopropane sulfonic acid) was used to correct for differences in ionic strength and as a buffer to control pH at 7.0. The composition of the solution

constituents was as follows: 10^{-5}–10^{-8} M Ca^{2+}, 15 mM EGTA, 3 mM Mg^{2+}, 0.13 M K^+, 15 mM phosphocreatine, and >15 mM MOPS. Approximately 20 U/ml creatine kinase was added daily before experimentation. The major anion was propionate. The total amounts of each solution constituent for representative integer pCa values are given in Table II.

TABLE II

Solution Constituents for Representative Integer pCa's

	4 mM [MgATP]		
	pCa 5	pCa 6	pCa 8
Ca^{2+}	1.46×10^{-2}	1.17×10^{-2}	5.14×10^{-4}
Mg^{2+}	8.91×10^{-3}	9.16×10^{-3}	1.01×10^{-2}
K^+	1.30×10^{-1}	1.30×10^{-1}	1.30×10^{-1}
EGTA	1.50×10^{-2}	1.50×10^{-2}	1.50×10^{-2}
MOPS	4.00×10^{-2}	5.37×10^{-2}	1.06×10^{-1}
ATP	4.17×10^{-3}	4.16×10^{-3}	4.16×10^{-3}
CP	1.68×10^{-2}	1.68×10^{-2}	1.68×10^{-2}
KOH	5.29×10^{-2}	5.32×10^{-2}	5.42×10^{-2}
	200 μM [MgATP]		
	pCa 5	pCa 6	pCa 8
Ca^{2+}	1.46×10^{-2}	1.17×10^{-2}	5.14×10^{-4}
Mg^{2+}	5.07×10^{-3}	5.32×10^{-3}	6.31×10^{-3}
K^+	1.30×10^{-1}	1.30×10^{-1}	1.30×10^{-1}
EGTA	1.50×10^{-2}	1.50×10^{-2}	1.50×10^{-2}
MOPS	7.90×10^{-2}	9.29×10^{-2}	1.46×10^{-2}
ATP	2.08×10^{-4}	2.08×10^{-4}	2.08×10^{-4}
CP	1.68×10^{-2}	1.68×10^{-2}	1.68×10^{-2}
KOH	6.13×10^{-2}	6.17×10^{-2}	6.26×10^{-2}
	10 mM P_i		
	pCa 4	pCa 5	pCa 8
Ca^{2+}	1.52×10^{-2}	1.46×10^{-2}	5.26×10^{-2}
Mg^{2+}	1.16×10^{-2}	1.17×10^{-3}	1.29×10^{-3}
K^+	1.38×10^{-1}	1.38×10^{-1}	1.38×10^{-1}
EGTA	1.50×10^{-2}	1.50×10^{-2}	1.50×10^{-2}
MOPS	2.33×10^{-2}	2.66×10^{-2}	9.30×10^{-2}
ATP	4.24×10^{-3}	4.17×10^{-4}	4.16×10^{-4}
CP	1.68×10^{-2}	1.68×10^{-2}	1.68×10^{-2}
KOH	5.41×10^{-2}	5.47×10^{-2}	5.80×10^{-2}
P_i	1.00×10^{-2}	1.00×10^{-2}	1.00×10^{-2}
$H_2PO_4^-$	3.52×10^{-3}	3.54×10^{-3}	3.54×10^{-3}

The three sets of solutions used to probe the mechanical states of the actomyosin ATPase had different [MgATP] or [$H_2PO_4^-$]. In one set of solutions, [MgATP] was set at 4 mM. In another, [MgATP] was lowered to 200 μM (both with zero $H_2PO_4^-$). In the final set, [MgATP] was 4 mM with a total P_i of 10 mM (the form [$H_2PO_4^-$] was ~3.2 mM but, varied slightly [<1%] with [Ca^{2+}]). $H_2PO_4^-$ appears to be the active form of P_i for depressing muscle force (Dawson et al., 1986; Godt et al., 1986; Lacktis and Homsher, 1986; Nosek et al., 1987). In any case, the effects of P_i may be complex, but the major one is a reversal of the P_i relationship (Hibberd et al., 1985; Webb et al., 1986).

Fibers were isolated in chilled Ringer's solution. The composition of the dissecting solution was as follows (millimolar): 111 NaCl, 1.8 KCl, 2.3 $NaHCO_3$, 0.18 NaH_2PO_4, 1.08 $CaCl_2$, with the final pH adjusted to 7.4.

Statistical Analysis

Data from each fiber in solutions with physiological levels of MgATP were used as their own control for data obtained in low [MgATP] or elevated P_i. Thus, comparisons between means were made using the t test for paired data unless otherwise noted (see Table III). Slopes and intercepts from linear regression as well as values of pK and n from Hill fits were compared using covariance analysis. Values in the text are given as means ± SD, and any differences were considered significant at the $p < 0.05$ level.

RESULTS

The preparations used in these studies were different from the traditional skinned fiber. Single whole skeletal muscle fibers were first isolated, enabling us to chemically skin the entire muscle fiber from tendon to tendon, rather than a piece of the fiber. Therefore, the natural attachment of the fiber, the tendon,

TABLE III

Relative Muscle Force and Stiffness in Solutions with 200 μM [MgATP] and 10 mM Total P_i Relative to 4 mM [MgATP]

	Relative force	Relative stiffness
200 μM [MgATP]	1.0±0.1	1.0±0.1
10 mM P_i	0.7±0.2	1.0±0.3

Average (± SD) ratio of maximally calcium-activated muscle force or stiffness in 200 μM [MgATP]/4 mM [MgATP] (both at pCa 5, in the same fiber) and in the same fiber at 10 mM P_i (pCa 4.6)/4 mM [MgATP] (pCa 5), expressed relative to the maximum in 4 mM [MgATP]. Comparisons between means were made using the Wilcoxon matched-pairs signed-ranks nonparametric test. The muscle force in solutions with 10 mM P_i was significantly ($p < 0.05$) depressed compared with its control in solutions with physiologic levels of MgATP. The number of fibers was 10–14.

was used to mount the preparation. Fibers prepared in this manner would contract more than eight times with up to 12 solution changes per contraction. When precautions were taken to minimize the time spent in solutions at maximal calcium activation, the fibers maintained an excellent striation image without significant deterioration in the maximally activated muscle force. Further evidence of the integrity of fibers prepared in this manner was their mechanical performance. Maximal calcium-activated forces were between 1 and 2×10^5 N/m^2 and the V_{max} (3.0–3.5 fiber lengths/s) was comparable to that measured using the slack test in isolated single intact fibers (Edman, 1979; Brozovich et al., 1983).

Calcium Sensitivity of Force and Stiffness in Different Biochemical States

A recording of muscle force and stiffness for a single skinned muscle fiber during activation (increasing free [Ca^{2+}]) and relaxation (decreasing free [Ca^{2+}]) in solutions with 4 mM [MgATP], 200 μM [MgATP], and 10 mM total P_i is dis-

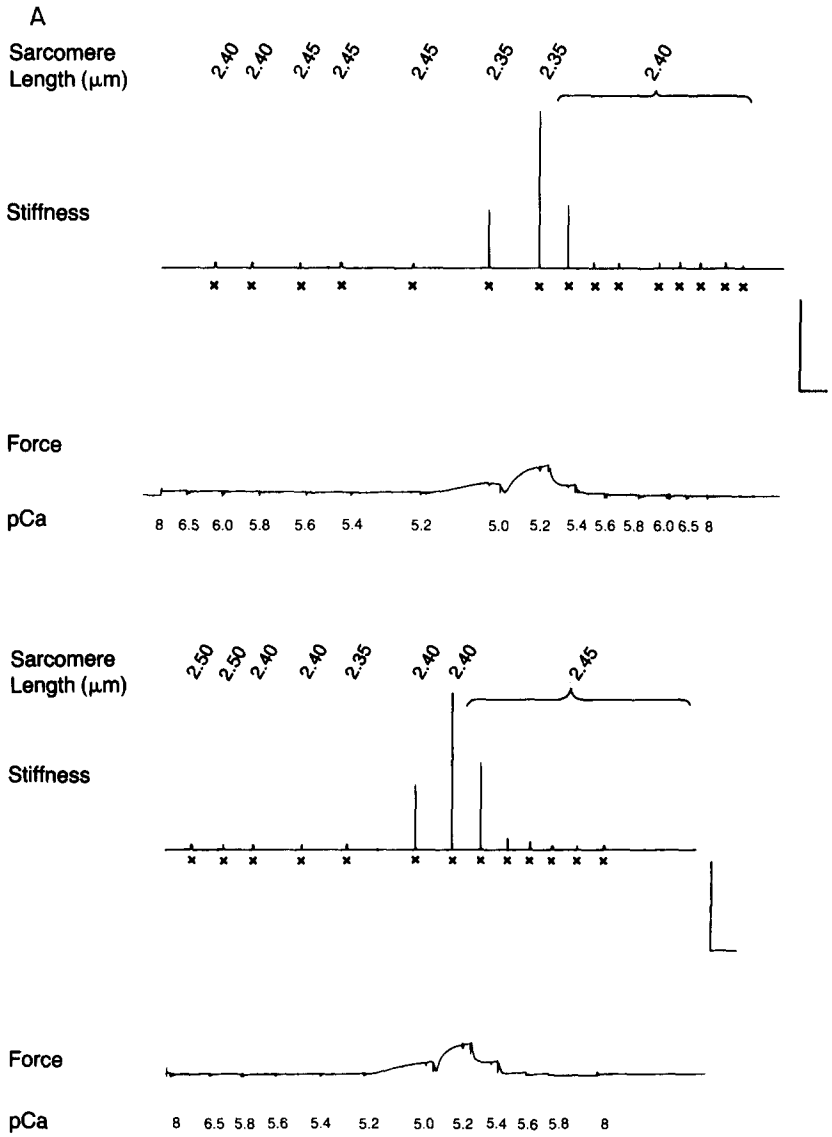
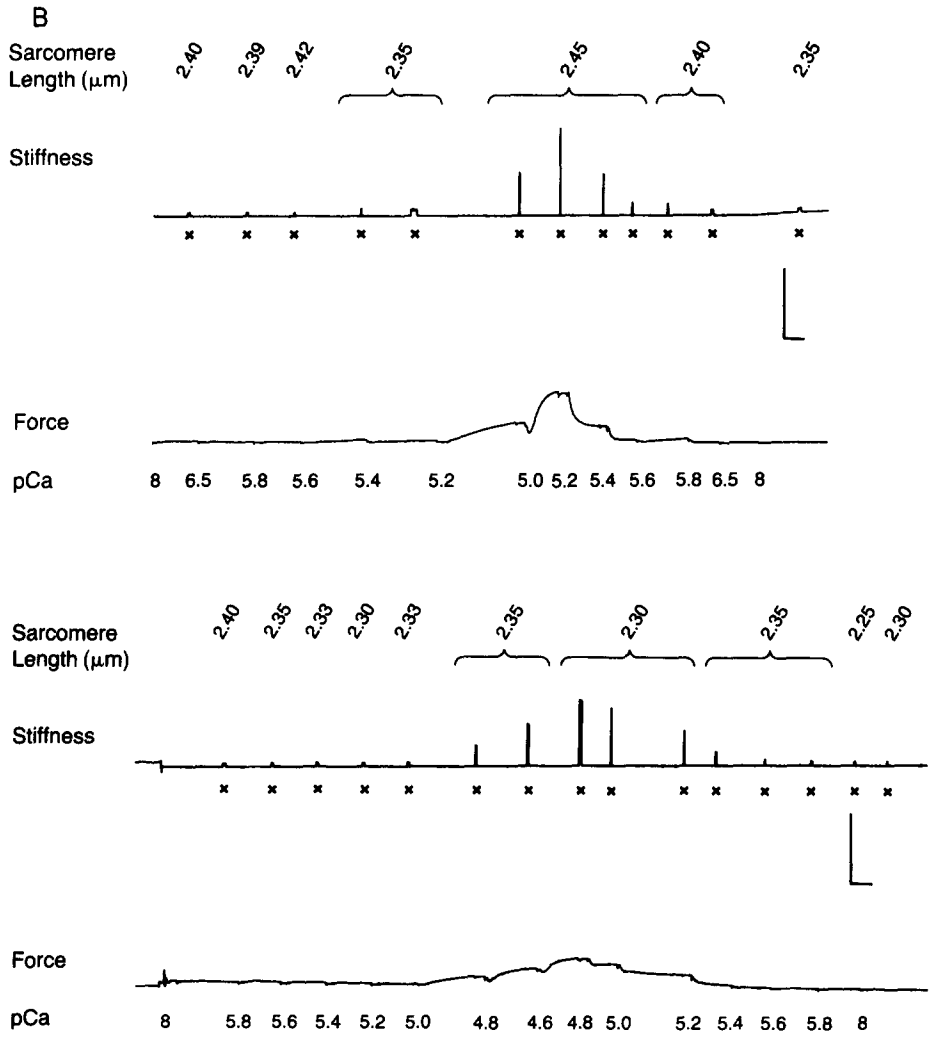


FIGURE. 1. Example of force and stiffness recorded during activation (increasing $[Ca^{2+}]$) and relaxation (decreasing $[Ca^{2+}]$) in successive contractions of a single skinned skeletal muscle fiber in solutions with: (*A*) 4 mM [MgATP] (upper trace) and 200 μM [MgATP] (lower trace); (*B*) 4 mM [MgATP] without (upper trace) and with (lower trace) 10 mM P_i. Force and stiffness (recorded with a 1-kHz, 7-μm [0.56%] oscillation of muscle length) measurements were taken at the points marked by an × and the sarcomere length recorded during the force steady states is displayed. Fiber dimensions: 1,250 × 75 μm (both fibers). Maximal activated force in 4 mM [MgATP], assuming a circular fiber cross-section, was $\sim 1.89 \times 10^5$ N/m^2 (*A*) and 1.75×10^5 N/m^2 (*B*) and maximum stiffness was 130×10^5 N/m^2 (*A*) and 100×10^5 N/m^2 (*B*). Vertical and horizontal calibration bars represent 100 mg and 10 s, respectively.



played in Fig. 1. In *A*, sequential contractions for a fiber bathed in solutions containing 200 μM or 4 mM [MgATP] show that force and stiffness reached the same absolute maximum level (Table III), without an increase in the baseline stiffness. In Fig. 1 *B*, the sequential contractions for another fiber in solutions with 4 mM [MgATP] with and without added 10 mM total P_i demonstrate that the baseline stiffness was unchanged. In this record, 10 mM total P_i reduced muscle force by 40%, while muscle stiffness fell by only 20%. There was some variability in the absolute amount by which 10 mM total P_i affected muscle force and stiffness; however, muscle force was always reduced more than stiffness. On the average, 10 mM total P_i reduced the maximally calcium-activated muscle force to 70% of its level in solutions with 4 mM [MgATP] without added P_i. Stiffness remained at approximately the same level ($p > 0.05$) with or without

added P_i (Table III). This reduction in force was repeatable, reversible, and independent of how the solutions were sequenced.

The relationship between free $[Ca^{2+}]$ and relative force and stiffness was analyzed with the Hill equation (Fig. 2). Within each solution condition used, no systematic statistical difference was observed ($p > 0.05$) between the activation or relaxation sequences for the curves of force and stiffness (Table IV). Thus, both force and stiffness levels from each solution set were the same at any pCa, both going up and coming down in calcium.

Decreasing [MgATP] from 4 mM to 200 μM increased the calcium sensitivity of muscle force and stiffness by ~0.10 pCa unit. This significant shift was similar to the increase in the pK of the calcium sensitivity of muscle force in skeletal muscle fibers (Reuben et al., 1971; Brandt et al., 1972; Godt, 1974; Fabiato and

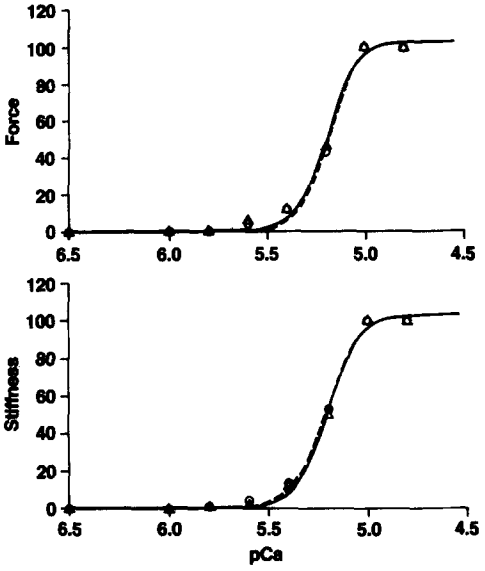


FIGURE 2. Muscle force and stiffness vs. pCa during activation and relaxation. Curves were fitted with the Hill equation (see Methods) by nonlinear least squares regression. For solutions with 4 mM [MgATP], force during activation (Δ) has pK 5.19 and $n = 5.5$ and during relaxation (O) has pK 5.18 and $n = 5.7$. Stiffness during activation (Δ) has pK 5.20 and $n = 5.3$, and during relaxation (O) has pK 5.21 and $n = 5.0$. 100% force represents 1.89×10^5 N/m^2, while stiffness is 130×10^5 N/m^2. Similar fits were obtained in solutions with low [MgATP] and elevated free P_i.

Fabiato, 1975). With the addition of 10 mM P_i to the 4 mM [MgATP] solutions that bathed the fibers, the steady state muscle force and stiffness became less sensitive to $[Ca^{2+}]$: the pK's decreased by ~0.3 pCa units. This is similar to the results reported in skinned skeletal muscle fibers (Brandt et al., 1982; Godt et al., 1985). Further analysis demonstrates that the Hill coefficient (n) of muscle force and stiffness, an indicator of cooperativity, was the same ($p > 0.05$) for fibers bathed in solutions with 5 mM and 200 μM [MgATP] during both activation and relaxation (Table IV, Fig. 3). However, for fibers bathed in solutions containing 10 mM total P_i, the n values of muscle force and stiffness during activation and relaxation were significantly less ($p < 0.05$) than the n values in either 4 mM or 200 μM [MgATP].

The plot of relative muscle force vs. stiffness during activation and relaxation over the entire range of calcium activation in all three solutions appeared to be

TABLE IV

Calcium Sensitivity of Muscle Force and Stiffness in Solutions with 4 mM [MgATP], 200 μM [MgATP], and 10 mM Total P_i

	Muscle force				Muscle stiffness			
	Activation		Relaxation		Activation		Relaxation	
	pK	*n*	pK	*n*	pK	*n*	pK	*n*
4 mM [MgATP]	5.19	6.6	5.17	6.2	5.20	7.0	5.21	5.8
(13 fibers)	(0.02)	(1.6)	(0.03)	(1.3)	(0.05)	(1.5)	(0.04)	(1.6)
200 μM [MgATP]	5.27	5.8	5.26	5.4	5.29	6.7	5.29	5.7
(24 fibers)	(0.09)	(1.8)	(0.07)	(1.5)	(0.08)	(2.8)	(0.07)	(2.3)
10 mM P_i	4.92	3.9	4.92	4.0	4.91	4.2	4.97	3.8
(10 fibers)	(0.08)	(0.8)	(0.09)	(1.5)	(0.10)	(0.3)	(0.10)	(0.5)

Mean (±SD) pK and *n* values of the contractions in solutions with 4 mM [MgATP], 200 μM [MgATP], and 10 mM P_i for the Hill fits of steady state muscle force and stiffness vs. pCa during activation (increasing $[Ca^{2+}]$) and relaxation (decreasing $[Ca^{2+}]$). Differences between pK and *n* values were tested using the Student's *t* test and there were no significant differences between pairs of values in a single solution that were consistent from solution to solution, at the $p < 0.05$ level. Muscle force and stiffness during activation and relaxation at 200 μM [MgATP] were significantly to the left of those at 4 mM [MgATP] ($p < 0.05$). Also, the curves at 10 mM P_i were significantly shifted to the right of 4 mM [MgATP] ($p < 0.05$). The *n* values at both 4 mM and 200 μM [MgATP] were the same ($p > 0.05$), but were significantly greater than those at 10 mM P_i ($p < 0.05$).

linear. The slope of this plot is thought to represent the relative force per cross-bridge (Ford et al., 1981). The plot of relative steady state muscle force vs. stiffness had a slope of 0.95 for fibers during activation, in 4 mM [MgATP] solutions, which decreased to 0.85 ($p < 0.05$) for fibers in 200 μM [MgATP] solutions and decreased even further to 0.68 ($p < 0.05$) for fibers in solutions containing 10 mM total P_i (Fig. 4). In contrast, the results obtained during the relaxation solution sequence had a relative force per cross-bridge of 0.85 for fibers in 4 mM [MgATP] solutions, which was lowered to 0.80 ($p < 0.05$) for fibers in 200 μM [MgATP] solutions and reduced even further to 0.64 ($p < 0.05$) for fibers in solutions containing 10 mM total P_i (see legend to Fig. 4).

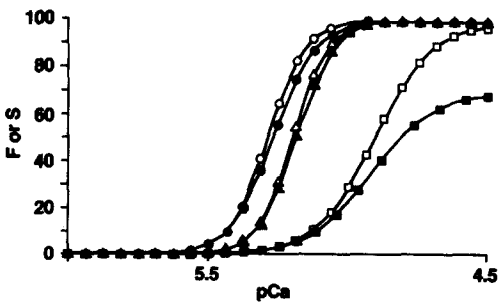


FIGURE 3. Predicted curves for average relative steady state muscle force (filled symbols) and stiffness (open symbols) vs. pCa during activation. The curves are represented for 200 μM [MgATP] (circles), 4 mM [MgATP] (triangles), and 10 mM P_i (squares). The pK and *n* values for force and stiffness are the averages given in Table IV. Similar fits were obtained during relaxation.

Controls

For the stiffness measured using changes in muscle length to represent the true stiffness of the active fiber, the length change must be distributed uniformly along the fiber. To check this, the sarcomere length was measured both at rest and during contraction in several fibers during 500-Hz oscillations in muscle length. In fibers rejected because the sarcomere length decreased by >0.2 μm during the activation sequence, the recorded changes in sarcomere length were less than the muscle length changes. However, in fibers that met the criteria for data acceptance, the recorded oscillations in sarcomere length when recorded were similar to the muscle length change (within 10%). Thus, muscle length

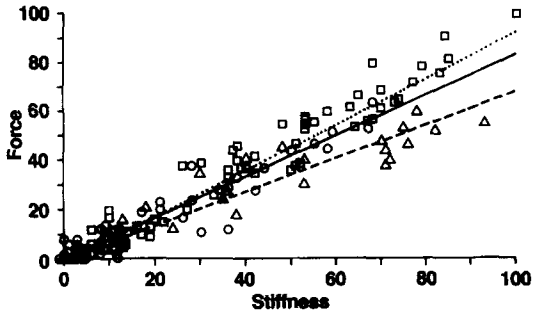


FIGURE 4. Plots of relative percent steady state muscle force vs. relative percent steady state muscle stiffness and lines of best fit for the fibers during activation: (□) 4 mM [MgATP]: percent force = (0.95 ± 0.02); (percent stiffness) + (−2.37 ± 0.64); r^2 = 0.935. (○) 200 μM [MgATP]: percent force = (0.85 ± 0.02); (percent stiffness) + (−0.61 ± 0.38); r^2 = 0.935. (Δ) 10 mM P_i: percent force = (0.68 ± 0.02); (percent stiffness) + (−0.35 ± 0.65); r^2 = 0.941. In all cases, force and stiffness are expressed relative to the maximum value in solutions with 4 mM [MgATP] and no P_i. Similar fits were obtained during relaxation with regression lines denoted by: 4 mM [MgATP]: percent force = (0.85 ± 0.03); (percent stiffness) + (−1.62 ± 0.84); r^2 = 0.873; 200 μM [MgATP]: percent force = (0.80 ± 0.02); (percent stiffness) + (−0.88 ± 0.45); r^2 = 0.910; 10 mM P_i: percent force = (0.64 ± 0.01); (percent stiffness) + (−2.78 ± 0.50); r^2 = 0.970. Note that in the fits of the data in solutions with 4 mM and 200 μM [MgATP] there are actually 256 data points, and in solutions with 10 mM P_i there are 128 data points, but because of data overlay, they are not all displayed. The slopes of these curves are significantly ($p < 0.05$) different from one another when compared with covariance analysis.

changes were not taken up in compliant ends of the fibers but rather were distributed relatively uniformly along the fiber. Thus, the stiffness measured was characteristic of the active fiber and not of the compliant ends.

It was assumed that muscle stiffness measures the number of attached cross-bridges (Ford et al., 1981). The oscillatory method of measuring muscle stiffness will tend to underestimate muscle stiffness during the release phase because of force recovery while overestimating muscle stiffness during the stretch because cross-bridges are stiffer for stretch than release (Bressler and Dusik, 1984; Ford et al., 1985). These two effects will tend to cancel each other (Ford et al., 1985). However, using the oscillatory technique of measuring stiffness, the frequency

of oscillation must be high enough so that the measured stiffness is independent of the frequency and the phase angle between length and force is low and independent of frequency (Julian and Sollins, 1975; Kawai and Brandt, 1980). We verified that both of these conditions were satisfied. Muscle stiffness was measured during the force steady states at intermediate and maximal levels of calcium activation at various frequencies of muscle length oscillation up to 1 kHz. For solutions with both 4 mM and 200 μM [MgATP], muscle fiber stiffness was constant at frequencies >500 Hz. In contrast, in 10 mM total P_i, the measured muscle stiffness was not constant until frequencies >650 Hz. However, in all the solutions, the muscle stiffness, measured at an oscillation frequency of 1 kHz, was independent of frequency. Measurements in intact fibers from the same preparation under identical circumstances showed that the stiffness was independent of the frequency of oscillation above 500 Hz and the phase angle was very small (unpublished observations). The stiffness measured is a good estimate of the series elastic element and therefore of the number of attached cross-bridges.

The stiffness of skinned fibers has been shown to be 2–2.5 times less than that of intact fiber preparations. This is thought to be due to the increased filament separation in skinned fibers since the majority of the increase in fiber compliance could be reversed by osmotic compression of the fiber lattice (Goldman and Simmons, 1986). Since we did not compress our fibers, their stiffness would be expected to be somewhat smaller than that of intact fibers, but still a good estimate of cross-bridge attachment (Goldman and Simmons, 1986).

To investigate the mechanical properties of the fibers and the relative maximal turnover rates under the various biochemical conditions, the maximum unloaded shortening velocity of these fibers was determined using the slack test. For fibers in solutions with 4 mM [MgATP], the slope of the regression line of the plot of change in muscle length vs. time to redevelop force, or V_{max}, averaged 3.5 $\pm$ 0.2 muscle lengths/s (N = 8), while in 200 μM [MgATP] solutions, it decreased to 0.80 $\pm$ 0.13 muscle lengths/s (N = 5), and in 10 mM free phosphate solutions, fiber V_{max} was 3.2 $\pm$ 0.6 muscle lengths/s (N = 4). The intercept of this plot was 0.10 $\pm$ 0.01, 0.13 $\pm$ 0.03, and 0.08 $\pm$ 0.02 muscle lengths for fibers bathed in solutions with 4 mM [MgATP], 200 mM [MgATP], and 10 mM free phosphate, respectively. These intercepts are not good measurements of series elasticity because of the oscillations in the force record seen with large releases, the possible curvature of the shortening-time record, and the difficulty in determining when the force first redevelops. In addition, the series elasticity in skinned fibers should be more compliant under our conditions of a swollen filament lattice, particularly for large releases (Goldman and Simmons, 1986).

DISCUSSION

In order to understand these observations in the context of cycling cross-bridges and the actomyosin ATPase, a set of assumptions must first be introduced. Cross-bridges will be considered to be independent and the two-headed nature of myosin will initially be ignored (the implications for a two-headed model will

be discussed later). For any condition, decreases in the force per cross-bridge during an isometric contraction reflect a process in which the cross-bridges spend more time in low- or non-force-producing states. Changes in calcium sensitivity may represent either (*a*) changes in calcium binding to activating sites, (*b*) cooperativity in calcium binding owing to attached bridges and their effect on the regulatory proteins, or (*c*) cooperativity in binding of cross-bridges themselves. The material that follows will discuss the data using these assumptions and Eisenberg and Greene's (1980) model of the actomyosin ATPase (see also Eisenberg and Hill, 1985).

Implications for the ATPase

In solutions containing 200 μM [MgATP], the relative force per cross-bridge (as determined by the slope of the force-stiffness plot) was reduced during both activation and relaxation as compared to that observed with physiologic levels of MgATP. The lower substrate concentration slows cross-bridge dissociation (indicated by the depressed V_{max}), preferentially populating the state before dissociation, i.e., A·M·ADP and A·M. However, no significant difference was observed between the maximum force or stiffness between 4 mM and 200 μM [MgATP] so that under conditions of full calcium activation, the average force per cross-bridge is not changed. These data, combined with the fact that in solutions with 200 μM [MgATP], the stiffness-pCa curve had a slightly greater Hill coefficient than the force-pCa curve, indicate that the relationship between force and stiffness is not linear, especially at force or stiffness levels above the pK (Fig. 4). Because of the greater number of points below maximum calcium activation in these plots of stiffness vs. force, the average force per cross-bridge would be weighted by points for which the average force per cross-bridge is low. This would in turn decrease the value of the force per cross-bridge. However, below maximum calcium activation, the decreased average force per cross-bridge implies that within the state being measured, there is a state, such as A·M, that produces little or no force but contributes to stiffness. The same conclusion about the existence of a low-force state during relaxation was reached on the basis of a delay in the decline of muscle stiffness relative to muscle force during relaxation (Schoenberg and Wells, 1984; Bressler and Dusik, 1984; our unpublished data).

One explanation of the reduced force per cross-bridge is that both heads of myosin may not need to be attached to produce stiffness (Brunsvold et al., 1986; Pate and Cooke, 1986). These investigators, using either AMPPNP and ethylene glycol, or glycerol and pyrophosphate, produced a myosin state intermediate between rigor and relaxation. By electron spin resonance, it was demonstrated that muscle stiffness was constant until the percentage of the myosin heads in the rigor configuration fell by 50%. Below this value, stiffness declined in a nonlinear manner with decreasing number of attached heads. Their data imply that the relationship between the number of attached S1 heads and stiffness is nonlinear and is consistent with a model in which attachment of only one of the two S1 heads per myosin is necessary to maintain cross-bridge stiffness. If these measurements on rigor attachments are applicable to cycling cross-bridges, it may

imply that attachment of a single head will produce stiffness, while attachment of both myosin heads is required to produce force. The decreased relative force per cross-bridge could then be due to an increase in the proportion of cross-bridges with only one attached head at low ATP concentrations. The detachment of one of the myosin heads could decrease force but produce an attached non-force-producing state with sustained stiffness.

Adding 10 mM P_i to 4 mM [MgATP] solutions reduced the maximally calcium-activated muscle force. This effect has been reported by others for skinned fibers from skeletal muscle (Brandt et al., 1982; Altringham and Johnson, 1985; Cooke and Pate, 1985; Godt et al., 1985; Hibberd et al., 1985; Dawson et al., 1986; Chase and Kushmerick, 1986; Kawai, 1986; Lacktis and Homsher, 1986; Kawai et al., 1987; Nosek et al., 1987) and cardiac muscle (Herzig et al., 1981; Godt et al., 1985; Kentish, 1986). Muscle stiffness, on the other hand, at maximal calcium activation, reached approximately the same level of stiffness observed with solutions containing 4 mM [MgATP]. The results of other investigators (Herzig et al., 1981; Altringham and Johnson, 1985; Kawai, 1986) reported that elevations of P_i decreased muscle force and stiffness by equal amounts. Our results agree with those of Hibberd et al. (1985), Chase and Kushmerick (1986), and Kawai et al. (1987), who reported that P_i decreased isometric force but increased the ratio of muscle stiffness to force. One difference between these studies is the method used to measure stiffness; two of the former studies used step length changes, while all the latter (including Kawai, 1986, and Kawai et al., 1987, in both groups) used sinusoidal length oscillations. Since phosphate approximately doubles the rate of force recovery (Altringham and Johnson, 1985; Hibberd et al., 1985), this could lead to an underestimation of the stiffness using the force response to a rapid change in muscle length. Stiffness measured by sinusoidal length changes is also sensitive to this change, but is also affected by any asymmetry in the response to stretch and release. However, it appears from the decrease in the slope of the force-stiffness plot that in the presence of elevated P_i there may be a cross-bridge state that produces little or no force.

An elevation in P_i should preferentially populate the actomyosin step before P_i release, namely, $A \cdot M \cdot ADP \cdot P_i$, $A \cdot M \cdot ATP$, $M \cdot ATP$, and $M \cdot ADP \cdot P_i$ (Hibberd et al., 1985; see also Hibberd and Trentham, 1986). The addition of P_i not only reverses the release of P_i, but reverses the actomyosin ATPase in fibers back to $A \cdot M \cdot ATP$ and $A + M \cdot ATP$ (Hibberd et al., 1985; Webb et al., 1986; Lund et al., 1987). If the states $A \cdot M \cdot ATP$ and $A \cdot M \cdot ADP \cdot P_i$ were weakly bound, as implied by Eisenberg and Greene (1980), then elevated P_i should be expected to decrease the muscle stiffness along with force. The average stiffness does not change to the extent that force does in the presence of P_i. The fiber variability observed in stiffness measurements cannot exclude the possibility that muscle stiffness was reduced slightly. In any case, muscle force was always depressed more than stiffness.

The fact that force declined more than stiffness in the presence of elevated P_i indicates that there exist bound cross-bridge states that have a reduced force per cross-bridge. The major force-producing state, $A \cdot M \cdot ADP$ (Webb et al.,

1986), may have a rate constant that depends upon cross-bridge strain (A. F. Huxley, 1957; Eisenberg and Hill, 1985). Since the rate constant for the binding of P_i to A·M·ADP may increase with strain (Webb et al., 1986), P_i would preferentially bind to a population of cross-bridges (A·M·ADP) that exist in a high-strain state, shifting them to A·M·ADP·P_i and A·M·ATP. If stiffness measures only the cross-bridge states of A·M·ADP and A·M, then P_i binding to those A·M·ADP states having high force would reduce the force more than stiffness and would result in a decrease in the average force per cross-bridge. An alternative explanation would be that stiffness measures A·M·ADP·P_i in addition to states of A·M·ADP and A·M. Stiffness would then not decrease as much, since there would be a proportion of cross-bridges shifted out of the A·M·ADP state through P_i binding included in the A·M·ADP·P_i state. The oxygen exchange data of Lund et al. (1987) using insect muscle fibers suggest that A·M·ADP·P_i is not only an attached state but a force-producing one as well. They further proposed an expansion of the ATPase scheme to include two A·M·ADP·P_i states, one a binding state and the other a force-generating state. The presence of bound A·M·ADP·P_i implies that this must be preceded by a relatively weak binding intermediate (Trybus and Taylor, 1980; Murray et al., 1982; Rosenfeld and Taylor, 1987). The fact that the P_i release step may be rate limiting in the isometric case (Lund et al., 1987) further suggests that, in the presence of P_i, a buildup of this intermediate would occur. The data of Dantzig and Goldman (1985), using the P_i analogue vanadate, provide additional evidence that A·M·ADP·V_i in rabbit muscle is a strongly attached state in the presence of Ca^{2+}, but not in its absence. The existence of an attached low- or non-force state prior to force production can also be inferred from stiffness measurements during the rising phase of muscle force, which demonstrates that muscle stiffness leads force (Cecchi et al., 1982; Schoenberg and Wells, 1984; Ford et al., 1986). Thus, our data are consistent with a model in which there is at least one strongly bound actomyosin state before P_i release that produces little or no force.

Some authors have suggested that elevations in free P_i decrease the time during which cross-bridges are bound to actin to explain their observed equal depression of muscle force and stiffness (Herzig et al., 1981; Altringham and Johnson, 1985). Others have reported that P_i increases the optimal frequency of oscillatory work (White and Thorsen, 1972; Chase and Kushmerick, 1986; Kawai, 1986; Kawai and Guth, 1986; Kawai et al., 1987), or shifts the frequency at which stiffness becomes independent of frequency (this article), or increases the rate of relaxation and force redevelopment from rigor (Hibberd et al., 1985). All of these authors imply that the kinetics of the cross-bridges are changed. However, the rate-limiting step for shortening is not affected since, both in this study and others, the maximal velocity of muscle fiber shortening is the same with or without added P_i (Altringham and Johnson, 1985; Cooke and Pate, 1985; Chase and Kushmerick, 1986; Luney and Godt, 1986). By preferentially populating the state before phosphate release, P_i might be expected to change the rate of force production. This could explain the former observations, but could not explain why the oscillatory work increases with P_i (Kawai et al., 1987). To explain this, Kawai et al. (1987) hypothesized another cycle within

the cross-bridge cycle. Their parallel cycles could be consistent with our data if, in solutions with elevated P_i, stiffness were maintained by cross-bridges that produced little force but could produce oscillatory work. The possibility also exists that there are two or more effects from different forms of P_i influencing muscle force, the calcium sensitivity, and rate constants of the ATPase (Kawai, 1986; Nosek et al., 1987).

Implications for the Calcium Sensitivity of Muscle Force and Stiffness

The increase in the calcium sensitivity of muscle force and stiffness in solutions with 200 μM [MgATP], along with the decrease in calcium sensitivity of muscle force and stiffness observed in 10 mM P_i, may be interpreted by a hypothesis in which force-producing and rigor cross-bridges affect calcium sensitivity. This hypothesis is supported by the observation that the apparent binding constant of calcium to the regulated thin filament increases in the presence of active cross-bridges (Grabarek et al., 1983). Furthermore, it has been proposed that rigor cross-bridges both promote the attachment of other cross-bridges (Bremel and Weber, 1972; Goldman et al., 1982, 1984*a, b*) and increase the apparent affinity of the thin filament for calcium (Bremel and Weber, 1972). In lowering the [MgATP], cross-bridge dissociation from actin will be slowed, allowing more cross-bridges to populate the rigor-like state prior to dissociation and thereby increasing the calcium sensitivity of muscle force and stiffness. In contrast, increasing the free P_i would reverse phosphate release from the actomyosin complex or possibly increase oscillatory work. This would preferentially shift the populations of bound cross-bridges from force-producing states to either a state before phosphate release or to oscillatory work states, both of which may not decrease the number of bound cross-bridges (see above). Thus, if the shift in calcium sensitivity is due to cross-bridge attachment, this implies that the bound state before force production is less effective in shifting the Ca^{2+} sensitivity than the states following force production. This is further supported by the observation that the Hill coefficient of both force and stiffness was decreased in the presence of P_i, which indicates a decreased cooperativity of either calcium or cross-bridge binding. However, an effect of P_i on sites other than cross-bridges in affecting calcium sensitivity has not been ruled out (Nosek et al., 1987).

Implications for Activation

During the activation sequence (increasing free $[Ca^{2+}]$), no difference was observed in fiber calcium sensitivity of force and stiffness in solutions containing physiological levels of MgATP. Also, a plot of the relative steady state muscle force vs. relative stiffness was linear, with a slope of $\sim$1 during activation. Our study extends the observation of Goldman and Simmons (1984) over the entire range of calcium activation, implying for physiological levels of MgATP that cross-bridge binding and force production are regulated by Ca^{2+} binding to a single class of sites. If there are two classes of Ca^{2+}-binding sites (possibly thin filament and thick filament), they must be cooperatively coupled such that they act as a single site. As discussed previously, at 200 μM [MgATP], it would be difficult to exclude a curvilinear portion of the plot of relative muscle force vs.

stiffness at high levels of Ca^{2+} activation. Thus, this conclusion may not apply for all levels of MgATP.

The relative force per cross-bridge was reduced to the largest extent by elevations of P_i. These data imply that when the fiber is activated, there is at least one strongly bound actomyosin state prior to phosphate release that produces little or no force. The existence of an attached low- or non-force-producing state prior to force production can also be inferred from stiffness measurements (Cecchi et al., 1982; Schoenberg and Wells, 1984; Ford et al., 1986), X-ray diffraction studies (H. E. Huxley, 1979; Amemiga et al., 1980; H. E. Huxley and Faruqi, 1985), and studies using optical depolarization (Burton et al., 1986). Therefore, these data are most consistent with a model of contraction in which the cross-bridge assumes a more strongly bound, but low- or non-force-producing, state prior to P_i release.

Our data are consistent with a model in which one of the cross-bridge states before P_i release is a strongly attached, low- or non-force-producing state. If Ca^{2+} activates the actomyosin ATPase by controlling the attachment of cross-bridges in strongly bound states, it must do so before P_i release, possibly by decreasing the off rate of actin for this intermediate and thereby increasing the strength of binding (see Adelstein and Eisenberg, 1980).

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