Force and Stiffness in Glycerinated Rabbit Psoas Fibers

Effects of Calcium and Elevated Phosphate

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ABSTRACT   Force (F) and stiffness (K) were measured in glycerinated psoas fibers at various calcium levels with 0, 10, 20, and 30 mM orthophosphate (Pi) added to the bathing solutions. The concentrations of bathing solution constituents were as follows: 110 mM potassium, 40 mM sodium, 4 mM MgATP, 10 mM total EGTA, and variable amounts of MOPS (pH buffer). The pH was 7.0, the ionic strength was 200 mM, and the temperature was 10°C. Calcium levels were established by adding various amounts of $CaCl_2$. All solutions contained 4% Dextran T-500. Fiber K was measured by imposing sinusoidal length changes (0.03–0.1%) at 1 kHz and by applying rapid steps in length and measuring the resulting F changes. At all [Pi] tested, K was more sensitive to calcium than F. Elevating bathing solution [Pi] caused a decrease in the calcium sensitivity of both F and K, while the slopes of F-calcium and K-calcium relations increased. In maximally activating calcium, raising [Pi] caused a continuous decrease in F over the range tested, while from very low to 10 mM Pi K remained constant. Above 10 mM Pi K declined, but to a lesser extent than did F. The results suggest that under our experimental conditions strongly attached crossbridges can exist in both force-producing and non–force-producing states, and that the relative population of these states may be calcium dependent.

INTRODUCTION

Several lines of evidence suggest that the myosin crossbridge binding to actin in skeletal muscle fibers proceeds through a number of steps before force generation. For example, fiber stiffness leads force during the rise of force in a tetanus (Ford et al., 1986; Cecchi et al., 1987), as do changes in actin layer lines and changes in the equatorial x-ray diffraction pattern (Kress et al., 1986; Wakabayashi et al., 1985). The binding of calcium to thin filament regulatory proteins mediates myosin crossbridge attachment in skeletal muscle fibers (Ebashi and Endo, 1968). If the transition from

relaxation to force generation involves a number of attached states, with varying degrees of attachment and force-generating capability, it is possible that calcium may not only regulate initial attachment in a low force state, but may also influence subsequent steps in the cycle. For example, transition from low force states early in the crossbridge cycle to force-generating states appears to involve the release of orthophosphate (Pi) from actomyosin (Hibberd et al., 1985), and release of Pi from actomyosin is thought to be regulated by calcium (Chalovich et al., 1981; Rosenfeld and Taylor, 1987).

There are currently two views of how calcium binding to troponin C (TnC) regulates contraction. In one view calcium binding causes conformational changes in tropomyosin which "unblock" the myosin binding sites on actin (Haselgrove and Huxley, 1973). This model is supported by the observation that conformational changes in tropomyosin appear to occur before either crossbridge attachment (as evidenced by changes in the equatorial x-ray diffraction pattern) or force production (Kress et al., 1986). Furthermore, these thin filament structural changes occurred at lengths beyond myofilament overlap and thus without crossbridge attachment (Kress et al., 1986).

Biochemical evidence suggests an alternative mechanism. It has been proposed that calcium regulates the transition from weakly to strongly attached crossbridge states by regulating Pi dissociation from the AM-ADP-Pi complex (Chalovich et al., 1981). In support of this idea it was found that calcium binding to TnC in regulated thin filaments has relatively little effect on myosin binding when compared with the effect on actin–myosin ATPase activity (Chalovich et al., 1981). Similarly, evidence in skinned fibers indicates the presence of a rapid equilibrium of actin–myosin attachment and detachment, as observed in both stiffness measurements (Brenner et al., 1982) and x-ray diffraction studies on relaxed fibers (Brenner et al., 1984) at low ionic strengths. Additionally, Pi release from acto-S1-ATP appears to be regulated by calcium (Rosenfeld and Taylor, 1987). These observations support the hypothesis that the regulatory proteins exert their influence by affecting the transitions between attached crossbridge states, rather than by steric blocking. However, recent evidence suggests that Pi release in skinned fibers is not calcium dependent, but that a weak to strong transition before Pi release may be calcium sensitive (Millar and Homsher, 1990).

In this study we have investigated the effects of both altered calcium activation and elevated Pi on the distribution of myosin crossbridges between attached crossbridge states. Elevation of bathing solution Pi should shift the population of attached crossbridges toward low force-generating states (Hibberd et al., 1985). Fiber stiffness is taken as an indicator of changes in the degree of actin–myosin interaction and crossbridge attachment (Ford et al., 1981). We have chosen experimental conditions (200 mM ionic strength) in which the contribution of weak crossbridge attachment to the stiffness measurements should be minimal (Schoenberg, 1988). While expanding the range of tested calcium and phosphate levels we, like others (Hibberd et al., 1985; Kawai et al., 1987; Brozovich et al., 1988), found that elevated Pi causes a greater decrease of force than stiffness. This observation is consistent with Pi release being coupled with transition to a force-generating state and that the transition is between two strongly attached states. In addition, we have found that crossbridge

attachment, as evidenced by stiffness, is more sensitive to calcium than is force. This finding suggests either that calcium regulates more than one step in the crossbridge cycle or that there are cooperative interactions between strongly attached crossbridge states (Bagni et al., 1988).

METHODS

Preparation

The surface membrane of rabbit psoas muscle fibers was rendered permeable to bathing solution constituents by chemical skinning in glycerinating solutions. Bundles of rabbit psoas muscle were isolated, tied to wooden sticks, and stored at $-20°C$ in a skinning solution which contained (mM): 100 KCl, 9.0 $MgCl_2$, 4.0 MgATP, 5.0 K_2EGTA, 10 MOPS, pH 7.0, and 50% vol/vol glycerol. Fibers were used 1 wk after preparation.

Single fibers were isolated and attached at one end to a length changer and at the other to a force transducer. Fiber segment lengths ranged from 3 to 5 mm. The fiber was attached to a fine wire hook (which had been treated with abrasive to prevent slippage) by wrapping the fiber several times around the hook. There was no tendency for the fibers to slip on the hooks during activation, as the sarcomere length (SL) before and after contraction was the same. Some fibers were attached to the apparatus with aluminum foil T clips. In later experiments, to promote maximum stiffness at the point of attachment, the ends of fibers were chemically fixed by the local controlled application of 5% (vol/vol) gluteraldehyde before being placed in the clips (Chase and Kushmerick, 1988*b*). In this technique the fiber is placed in a solution of 50% relaxing solution plus glycerol, with 10 mg/ml bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO). The BSA presumably minimizes diffusion of the 5% gluteraldehyde solution away from the point of application. Gluteraldehyde was applied with an apparatus that consisted of a small 23-gauge needle through which the gluteraldehyde flowed under a small pressure and a 21-gauge needle which aspirated solution away from the fiber. With this device a small, steady stream of gluteraldehyde could be set up between the two needles and applied to the ends of the fiber segment. This technique produces a very noncompliant attachment of the fiber to the apparatus. The transition between fixed and unfixed regions of the fiber occurred within 5–10 sarcomeres. The length of the fixed portion of the fiber was determined at the end of an experiment by allowing the fiber to shorten in maximum calcium and measuring the obvious unshortened portions of the fibers, with sharp sarcomere structure, near the clip junctions. The attached fiber was placed in a narrow chamber made of plexiglass with a glass bottom, which was mounted on the moveable stage of an inverted microscope. The volume (0.5 cm^3) of bathing solution in the chamber could be rapidly changed. The chamber was cooled to 10°C by circulating coolant through the hollow chamber walls. Fiber length (FL) was controlled with a linear motor in a length feedback servo control loop and force was measured with a silicon semiconductor strain gauge (AME 801) with a resonant frequency of 7 kHz.

Fiber stiffness (K) was measured by applying either sinusoidal or step changes in FL and recording the corresponding changes in force (F). For sinusoidal K measurements 1-kHz length changes of 0.03–0.1% FL were imposed. 1 kHz was chosen as the oscillation frequency as it was well above the frequency at which the amplitude and relative phase of the resulting F oscillations became independent of frequency. The F signal was fed to a band pass rectifier circuit which was tuned to 1 kHz. The resulting DC output voltage was verified to be linearly related to the amplitude of the 1-kHz component of the F signal and therefore to fiber K (for a constant amplitude length oscillation). For step K measurements rapid stretches and releases were used. Length changes of 30 μm were complete in 750 μs. K is defined as the slope of the

relationship between changes in either FL or SL and the corresponding changes in F. For SL K measurements, the peak change in F following the length change was plotted against the peak change in SL that occurred during the FL step; because of filtering of the SL signal (see below) there was a ~200-μs delay between the F step and the SL step. Absolute K values were transformed to Young's modulus (Y = force/fiber cross-sectional area × initial length/length change = N/mm^2). FL changes are expressed as a fraction of the initial FL and SL changes are expressed as nanometers per half sarcomere (nm/hs). F and the output of the bandpass rectifier (K) were recorded on a chart recorder. F and length changes were also sampled at a rate of 12.5 kHz and stored digitally with 14-bit A/D resolution by a computer. K measured from the digitized signals agreed with those from the bandpass rectifier. Active F and K are determined by subtracting the values obtained in a relaxed fiber from the total value obtained during activation. Data were rejected if maximum F or K in control contractures (no added Pi) declined by >15%.

SL was measured by laser diffraction and the position of the first order diffraction maxima was detected with a 256-element photodiode array (Reticon RL256C; Reticon Corp., Sunnyvale, CA; Iwazumi and Pollack, 1979). The Reticon array was scanned every 256 μs and the resulting SL signal was filtered with a 4-pole Bessel filter, which had a corner frequency of 2 kHz. Initial SL was set at 2.5–2.7 μm. Fibers were rejected if the resting sarcomere diffraction pattern became indistinct after a series of contractures.

For comparison, F and K at each calcium concentration are expressed as a fraction of the maximum value obtained at maximally activating calcium concentration (pCa 4.0). The relationship of F and K to calcium concentration was obtained by nonlinear least-squares fitting of the Hill equation to the data, as follows:

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

pK is the pCa at half-maximum F or K and n reflects the slope of the calcium dependence.

When mean values (±SEM) of F or K were calculated, data were obtained from no fewer than five fibers for any experimental condition. Where means were compared, the Student's t test was used and differences were considered significant at the 95% confidence level. Standard error bars are not shown for figure symbols if the error bar is smaller than the symbol.

Solutions

The concentrations of solution constituents were as follows: 110 mM potassium, 40 mM sodium, 4 mM MgATP, 1 mM free magnesium, 15 mM creatine phosphate, 20 U/ml creatine phosphokinase, 10 total calcium buffer (EGTA), and variable amounts of pH buffer. The solution pH was 7.0 and MOPS was the pH buffer; to insure adequate buffering, the concentration of free MOPS was always greater than 12 mM. Potassium was added primarily as the propionate salt. Ionic strength was 200 mM. Phosphate replaced proprionate to maintain constant ionic strength. Based on assays provided by Sigma Chemical Co., the contaminating levels of phosphate in our solutions come from the creatine phosphate stock solution and probably were no more than 1.0 mM, although they were not measured directly. Similar levels of Pi contamination have been found by others (Pate and Cooke, 1989a). Concentrations of added phosphate were 0, 10, 20, or 30 mM. To test for the effect of contaminating Pi on F and K measurements, 10 mM sucrose was added to pCa 4.0 activating solutions containing the tested added Pi concentrations. Sucrose phosphorylase (0.20 U/ml) was then added only to a set of solutions with no added Pi 2 h before an experiment to further depress Pi levels within the activated fiber to ~200 μM (Pate and Cooke, 1989a; Millar and Homsher, 1990). Free calcium was varied by adding different amounts of calcium proprionate ($CaCl_2$). The appropriate amount of all constituents was determined with a computer program utilizing the binding constants of all ionic species present to solution constituents and the desired free concentra-

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tions of the specified ions, as previously described (Martyn and Gordon, 1988). To compensate for different amounts of $CaCl_2$, added ionic strength was held constant, at constant potassium, by varying the amount of pH buffer. Changes in pH buffer concentration were small over the range of pCa's at which fibers developed F.

Because chemical skinning causes the myofilament lattice spacing to swell by ~20%, fibers were osmotically compressed with 4% Dextran T-500 in all solutions (Maughan and Godt, 1979). This amount of compression returns lattice spacing back to in vivo levels and minimizes the lattice spacing decrease observed in skinned fibers during contraction (Brenner and Yu, 1985; Matsubara et al., 1985). Experiments were also done without Dextran T-500 in the bathing solutions to test for the effect of osmotic compression on fiber K.

RESULTS

F-calcium and K-calcium relations were measured in solutions with no added Pi and with elevated Pi concentrations. In each experiment fibers were exposed to a relaxing solution containing 10 mM EGTA and no calcium. Before activation with calcium the fibers were exposed to a relaxing solution containing 100 μM EGTA. This procedure significantly increased the rate of tension development during subsequent calcium activation. The fibers were then exposed to increasing levels of calcium. At each calcium level F was allowed to reach a steady state before K was measured. The cycle was ended by relaxing the fiber in high EGTA relaxing solution. The sequence of solution changes had no effect on the results. Representative traces of F and K (measured with sinusoids) for a range of calcium levels (pCa) are shown in Fig. 1, with zero (A) and 30 mM added Pi (B). Maximally calcium-activated F (no added Pi) was typically 0.17 N/mm².

The data in Fig. 1 illustrate three principal findings: (1) at either no added Pi or 30 mM added Pi, K exhibits a greater sensitivity to calcium than F; (2) elevated Pi causes a greater depression of F than K; and (3) elevated Pi causes a shift of F-calcium and K-calcium relations to higher calcium concentrations. In 30 mM Pi the ratio of maximum F to that in no added Pi is 0.71, while the corresponding ratio for maximum K is 0.83. Therefore, the data suggest that both decreased calcium concentration (below maximum) and elevated Pi cause a greater decrease in F than K. Since the difference in calcium sensitivity between F and K did not depend on the presence of added Pi, the results suggest that lowered [Ca] and elevated [Pi] exert similar influences on F and K, but by different mechanisms.

The K and F data shown in Fig. 1 were obtained from fibers that were attached to the apparatus by wrapping them around the abraided hooks. Some sarcomere shortening was observed in these fibers (the laser beam typically monitored the central 100 μm of the fiber), which could be as much as 0.2 μm for maximally activated fibers. This internal shortening could result from uneven activation of the fiber from one region to the next or from compliance at the attachment to the hooks. It is unlikely that slipping of the fibers at the hooks occurred because SL returned to the initial value upon relaxation. Increased inhomogeneity of SL was also indicated by a broadening and decrease in intensity of the first order diffraction maxima during maximum activation. These observations lead us to question if the difference in F and K calcium sensitivity could be due to an underestimation of maximum fiber K, either because of compliant attachment at the ends of the fibers or excessive SL inhomogeneity.

We took several approaches to minimizing effects due to SL inhomogeneity and fiber end compliance. First, the ends of fibers were chemically fixed and attached to the apparatus with aluminum T clips. Second, to stabilize the SL pattern in high calcium solutions and to minimize SL inhomogeneity, fibers were cycled with brief periods of unloaded shortening (about four fiber lengths per second and 12–15% of rest length in amplitude), as described by Brenner (1983). We also measured K using step changes in FL as well as sinusoids, and measured the resulting changes in SL. SL measurement effectively removes the contribution of end compliances to the step K measurements.
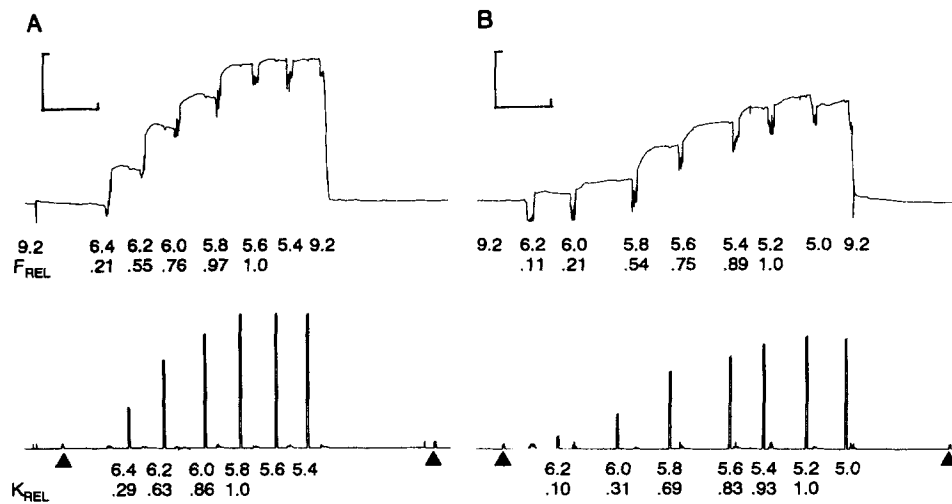

FIGURE 1. Traces of F (*top*) and K (*bottom*) at various calcium concentrations are shown for experiments in 0 (*A*) and 30 mM (*B*) added Pi. Fibers were exposed to a low EGTA relaxing solution (9.2 E.1) before activation and subsequently relaxed in a high EGTA (8 mM) relaxing solution (9.2). Dips in the F records result from changing solution in the chamber. The pCa to which the solution was changed is indicated below the F traces. Below each pCa is the relative force (F_{rel}) achieved at that calcium level. In each pCa the FL was briefly oscillated ~0.1% FL at 1 kHz for 2 s, and the resulting output of the bandpass rectifier is shown in the bottom traces of *A* and *B*. The pCa at which each K measurement was made is indicated below the corresponding K trace and below the pCa is the corresponding relative K obtained (K_{rel}). The arrows mark resting K measurements. Vertical calibration bars for F are 1 mN and horizontal time calibration bars are 30 s.

Figs. 2 and 3 show data obtained from a fiber using these approaches. As in Fig. 1, lower calcium and elevated Pi caused a greater depression of F than K. The fibers were first exposed to low calcium (pCa 6.4), then to maximal calcium (pCa 4.0, no added Pi), and finally to maximal calcium with 20 mM Pi. K was measured both with sinusoids (Fig. 2 *A*) and by applying step changes in FL (Fig. 3). The FL, SL, and F changes recorded during the sinusoidal K tests are shown in Fig. 2 *B*. Sinusoidal K measurements using the bandpass rectifier indicate that in pCa 6.4 relative F was 43% of maximum, while relative K was 54%. In pCa 4.0 plus 20 mM Pi, relative F and K were 68 and 73% of maximum, respectively. Therefore, the data in Figs. 1 and 2
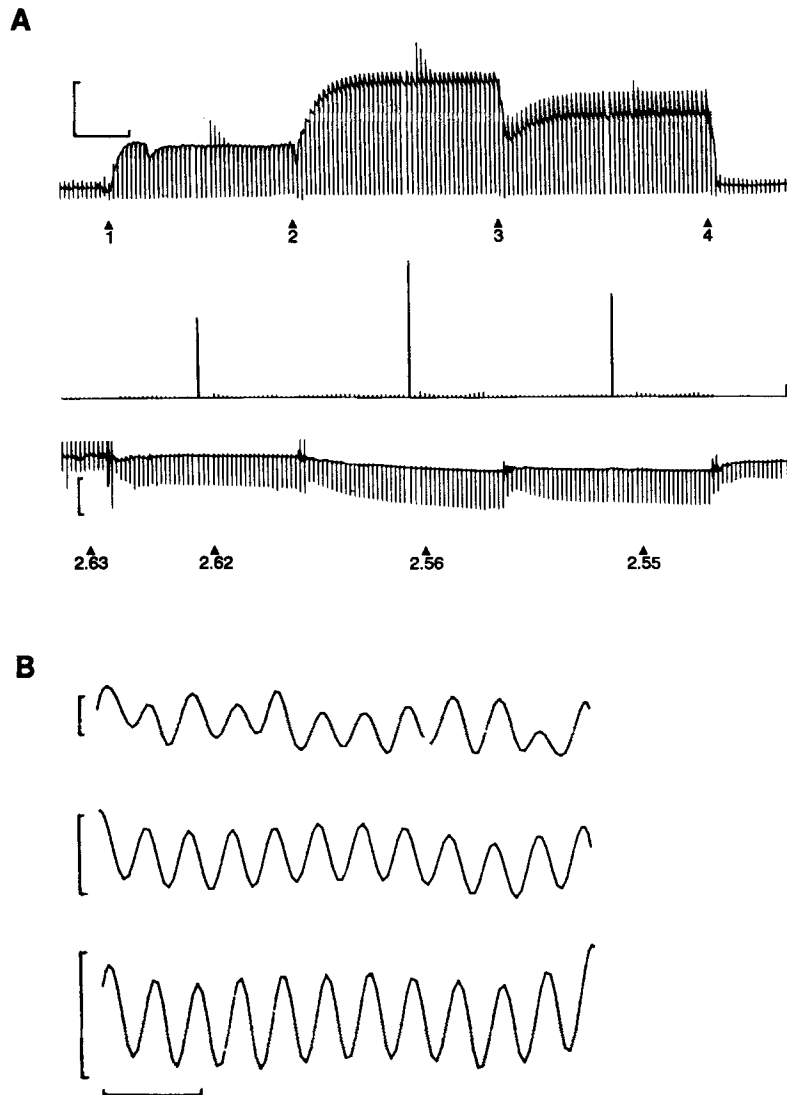
A

B

FIGURE 2.   (*A*) F (*top*), K (*middle*), and SL (*bottom*) traces are shown for a fiber that was first activated submaximally in pCa 6.4 (*1*), then maximally in pCa 4.0 (*2*), exposed to a pCa 4.0 solution containing 20 mM Pi (*3*), and finally relaxed (*4*). The K trace shows the output of the bandpass rectifier circuit used to monitor sinusoidal fiber K. Initial SL was 2.63 μm (filled triangles indicate the SL at the indicated time), FL was 2.88 mm, and diameter was 60 μm. Temperature was 10°C. The calibration bars for F and SL are 0.3 mN and 0.2 μm, respectively. The time marker is 1 min. (*B*) SL (*top*), FL (*middle*), and F (*bottom*) traces are shown for sinusoidal K measured in pCa 4.0 for the fiber in *A*. The amplitude of the sinusoidal FL oscillations were 1.4 μm (0.05% FL). Calibration marks represent 1 nm/hs (*top*), 2 μm (*middle*), and 0.15 mN (*bottom*). The time axis marker is 2 ms. The SL signal has not been corrected for a 180-μs delay caused by the fourth order Bessel filter. There is a ~36-μs delay in F relative to the FL oscillations (when corrected for the A/D conversion delay between data channels).
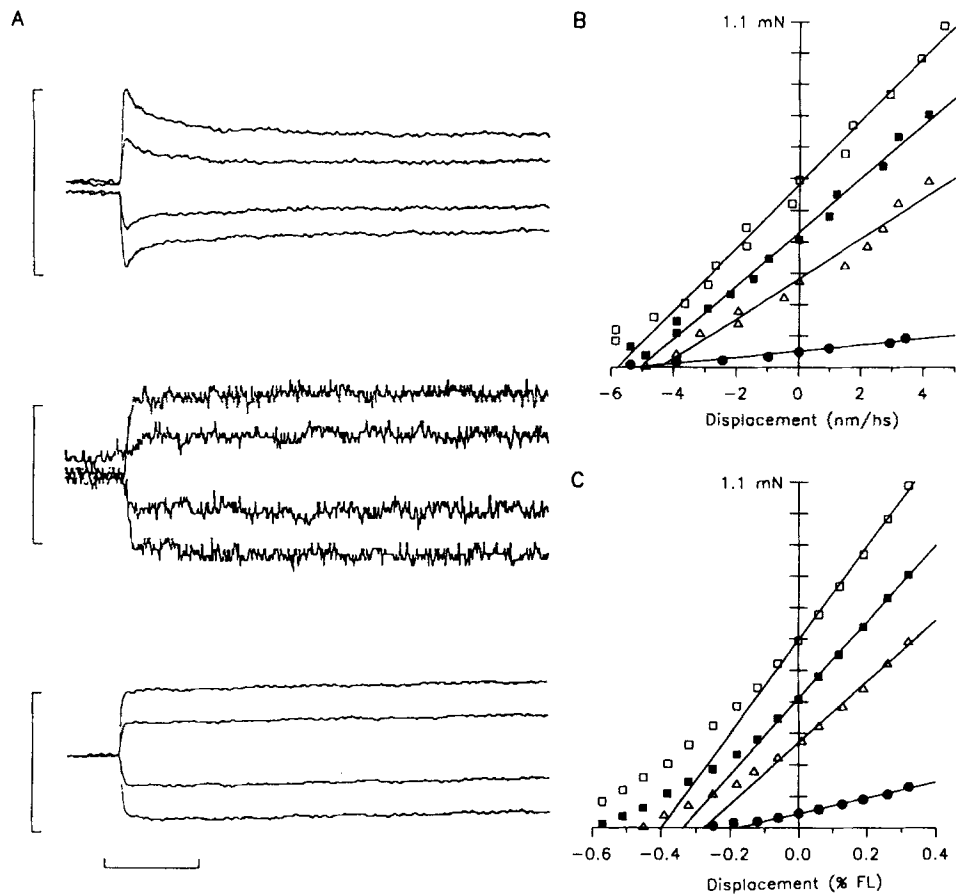
FIGURE 3. (A) F (top), SL (middle), and FL (bottom) changes are shown to step K measurements made in pCa 4.0 from the fiber shown in Fig. 3 A. The calibration bar for F ranges from 0.4 to 0.9 mN. The bars for SL and FL are 5.0 nm and 8.0 μm, respectively. The time scale calibration mark is 10 ms. (B) Data from the determination of K using a series of step stretches and releases in FL from A are shown. The symbols correspond to data obtained in pCa 4.0 with no added Pi (□), pCa 4.0 plus 20 mM Pi (■), pCa 6.4 (△), and relaxing solution (●). The rapid change in F resulting from the change in length is plotted against the corresponding change in SL. The linear portions of the F-length change relationship between −4.0 and +4.0 nm were fit by least-squares linear regression and the relationship was extrapolated to zero F (y_o). Young's modulus for the corresponding conditions are 46.5 (□), 39.1 (■), 28.5 (△), and 4.9 (●) N/mm^2. The corresponding SL axis intercepts are −5.79 (□), −5.06 (■), −4.60 (△), and −4.93 (●) nm/hs. (C) The changes in F are plotted against the change in muscle length corresponding to the SL data in B. The change in muscle length is expressed as a percentage of the initial muscle length. The slopes and FL axis intercepts were obtained by least-squares linear regression of the data between −0.30 and +0.30% FL. Young's moduli for F/FL relations are 53 (□), 42.5 (■), 37.2 (△), and 9.3 (●) N/mm^2. The corresponding length axis intercepts are −0.40 (□), −0.34 (■), −0.28 (△), and −0.18 (●) percent FL.

show that similar results are obtained whether the fibers were attached to the apparatus by wrapping around the attachment hooks or by using foil clips and chemical fixation of the fiber segment ends (in addition to Brenner cycling); this suggests that the greater depression of F than of K at lowered calcium or elevated Pi was not due to fiber inhomogeneity or compliance.

For comparison with the sinusoidal K measurements, K was also measured with step changes in length in fibers with chemically fixed ends and T clips. Fig. 3 *A* shows representative traces of F (top), SL (middle), and FL (bottom) obtained from the step K protocol for the fiber shown in Fig. 2. The entire series of step changes in SL (Fig. 3 *B*) and FL (Fig. 3 *C*) is plotted against the corresponding changes in F. The slope of the relationship between F and length is the fiber K for each condition (Huxley and Simmons, 1971). The values of Young's modulus are given in the figure legend. The linear portion of each data set (from +4 nm/hs to −4 nm/hs for SL and +0.3 to −0.1% FL) is extrapolated to zero F in order to determine the length change necessary to rapidly decrease F to zero (y_0). y_0 was found to be smaller when F was

TABLE I

pCa	Added Pi	F_{rel}	$K_{l\,rel}$	$K_{s\,rel}$	y_0
	mM				*nm/hs*
4.0	0	1.0	1.0	1.0	−8.33 ± 1.02
6.4	0	0.40 ± 0.05	0.59 ± 0.10	0.49 ± 0.07	−6.4 ± 0.63 ($P < 0.01$)
4.0	20	0.71 ± 0.02	0.91 ± 0.10	0.79 ± 0.04	−5.83 ± 1.1 ($P < 0.01$)

F_{rel}, F relative to that obtained in pCa 4.0 with no added Pi. $K_{l\,rel}$, the ratio of K determined from step K tests relative to pCa 4.0 with no added Pi. $K_{s\,rel}$, the ratio of K determined from sinusoidal tests relative to pCa 4.0 with no added Pi. The difference in the means of $K_{l\,rel}$ and $K_{s\,rel}$ at pCa 6.4 was not significantly different ($P > 0.05$), while at pCa 4.0 plus 20 mM Pi the difference was just significant ($P = 0.05$). y_0, the amplitude of SL decrease necessary to decrease F to zero, as determined by linear extrapolation of F/SL relations. The means of y_0 at pCa 6.4 and 4.0 plus 20 mM Pi are compared with pCa 4.0 with no added Pi by Student's *t* test.
Data were obtained from five fibers, with means ± standard errors given.

decreased by either low calcium or elevated Pi. The value for y_0 maximal calcium is similar to that found at low levels of activation (Goldman and Simmons, 1986), while the value in elevated Pi is similar to that found for intact frog fibers (Ford et al., 1981). y_0 obtained from the data in Fig. 3 *B* for each condition are: −5.79 nm/hs (pCa 4.0), −4.6 nm/hs (pCa 6.4), and −5.06 nm/hs (pCa 4.0 plus 20 mM Pi). If F and K had changed proportionately, y_0 would have remained constant. A decrease in y_0 indicates that the K per unit F has increased at low calcium and at elevated Pi.

The magnitude of the change in relative K for step measurements is determined by taking the ratio of the slopes of the F/SL change relations to the slope obtained in pCa 4.0 with no added Pi. The K ratio determined from the linear regression analysis of step SL/F changes in Fig. 3 *B* for pCa 6.4 is 0.62, while the corresponding F ratio is 0.43. For pCa 4.0 plus 20 mM Pi the K and F ratios are 0.83 and 0.63, respectively. The relative K values from sinusoidal measurements were 0.54 (pCa 6.4) and 0.73 (pCa 4.0 plus 20 mM Pi). Pooled data from five fibers are shown in Table I and indicate that at low calcium and elevated [Pi] F is depressed more than K, and that qualitatively and quantitatively similar results were obtained with either step or

sinusoidal K tests. Analysis of K using the measured step changes in FL leads to similar results. The relation between step changes in FL and corresponding changes in F for the data in 2 A is shown in Fig. 3 C. The ratios of FL K relative to pCa 4.0 for pCa 6.4 and pCa 4.0 plus 20 mM Pi are 0.57 and 0.76, respectively. These ratios are slightly lower than those found with SL measurements but still greater than the corresponding changes in F. The values of FL y_0 (as the percentage of initial FL) are −0.50 (pCa 4.0), −0.36 (pCa 6.4), and −0.44 (pCa 4.0 plus 20 mM Pi).

To further illustrate that F is suppressed more than K over a range of calcium concentrations, K was measured by steps and sinusoids and the individual data points from six fibers are shown in Fig. 4. F and K were measured at pCa 6.4, 6.2, and 6.0 (O, ●). For comparison, similar data for pCa 4.0 plus 20 mM Pi (△, ▲) are included. Both F and K are expressed relative to pCa 4.0 with no added Pi. Filled symbols correspond to K ratios determined from step tests and open symbols represent ratios determined from sinusoidal tests. All experiments were done with gluteraldehyde
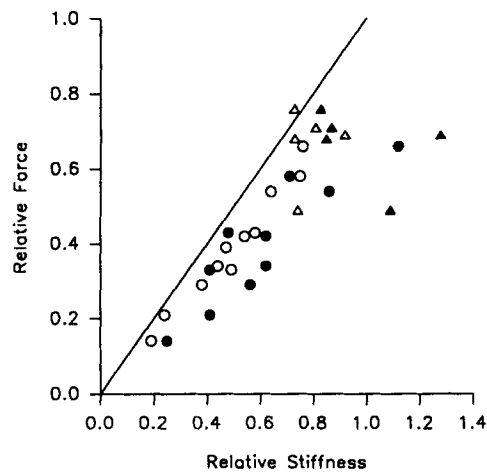

FIGURE 4. F is plotted against K for individual trials for data from six fibers. F and K are both expressed as a fraction of the value obtained at pCa 4.0 with no added Pi. K was determined when F was changed by changing the calcium concentration (O, ●) and adding 20 mM Pi to the pCa 4.0 bathing solution (△, ▲). The filled symbols represent K measurements obtained by step length change analysis (see Fig. 5) and the open symbols indicate data obtained by sinusoidal analysis, with both protocols being performed on the same fibers. There is a tendency for K to be higher when measured by step analysis.

fixation of the fiber ends and T clips. The decrease in F relative to K (measured by either technique) which was observed for low calcium or elevated Pi are similar to those observed from fibers with sinusoidal K measurements, which were simply wrapped around the attachment hooks (Fig. 1). If the K of individual attached crossbridges is unaffected by varying concentrations of Pi or calcium, so that K is a measure of attachment, the results would imply that these conditions decreased F production by the attachment population of crossbridges to a greater extent than the degree of attachment.

Our observation that K is more sensitive to calcium (Figs. 2 and 5) is at odds with the observation by others that F and K generally change in direct proportion when calcium concentration is altered (Herzig et al., 1981; Goldman and Simmons, 1984; Brozovich et al., 1988). However, our experiments differ because we osmotically compressed the fibers by adding 4% Dextran T-500 to all bathing solutions. This causes a 15% decrease in fiber diameter and has been shown to reverse the

myofilament lattice swelling observed with skinning (see Methods). Since it has been shown that the myofilament lattice spacing is inversely related to F in uncompressed skinned fibers (Brenner and Yu, 1985) and that K is lower at greater lattice spacing (Goldman and Simmons, 1986), we compared the effects of calcium on F and K in the same fibers under uncompressed (no Dextran T-500) and compressed (4% Dextran T-500) conditions. K was measured in three fibers by step tests and results from a representative experiment are shown in Fig. 5. The symbols correspond to experiments done at pCa 4.0 (no added Pi) with (■) and without (□) Dextran T-500 and at pCa 6.2 with Dextran (▲) and at pCa 6.0 without Dextran (△). Values for Young's modulus and y_0 intercepts are given in the figure legend. Osmotic compression had little effect on K and y_0 in pCa 4.0, as might be expected since radial
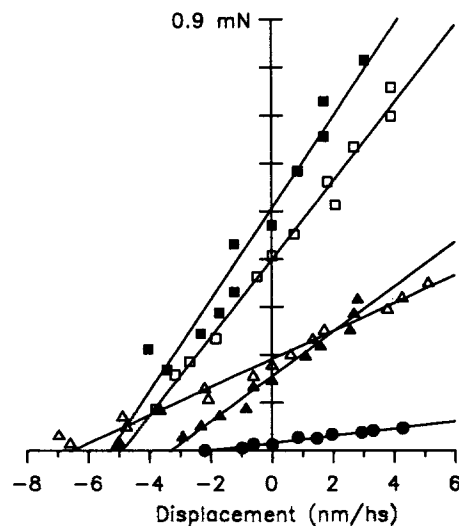

0.9 mN

Displacement (nm/hs)

FIGURE 5. K was obtained by step analysis from a single representative fiber and is compared when the fiber was osmotically compressed with 4% Dextran T-500 in all bathing solutions and when there was no osmotic compression with Dextran T-500. With Dextran T-500 data were obtained at pCa 4.0 (■), pCa 6.2 (▲), and in relaxing solution (●). The corresponding values of Young's modulus and y_0 obtained were 29.9 N/mm² and −5.29 nm/hs (■), 15.3 N/mm² and −3.31 nm/hs (▲), and 2.41 N/mm² and −2.1 nm/hs (●), respectively. Without Dextran T-500 data were obtained at pCa 4.0 (□) and 6.0 (△). The corresponding values of Young's modulus and y_0 are 28.2 N/mm² and −4.90 nm/hs (□), and 18.1 N/mm² and −6.53 nm/hs (△). Resting K was negligible in zero Dextran T-500. For the uncompressed condition the ratio of submaximal to maximal F was 0.48, while the corresponding K ratio was 0.40. With osmotic compression the ratio of submaximal to maximal F was 0.31 and the K ratio was 0.45. With 4% Dextran T-500 K was corrected by subtracting the resting K. FL was 2.40 mm, cross-sectional area was 3.85 × 10⁻³ mm², and initial SL was 2.63 μm.

crossbridge F would be expected to decrease the lattice spacing without Dextran T-500 (Brenner and Yu, 1985). However, at lower calcium concentrations (comparable forces) y_0 was greater when the fiber was not compressed with Dextran T-500, and comparable to the value obtained at pCa 4.0. To monitor relative changes in myofilament lattice spacing we observed fiber diameter during contractions. With no osmotic compression, fiber diameter decreased with activation and was 74% of the relaxed value in pCa 4.0 (○) and 88% of relaxed diameter in pCa 6.0 without Dextran T-500 (△). With 4% Dextran T-500 fiber diameter was not altered relative to the relaxed value during activation and remained at 83% of the relaxed, zero Dextran T-500 value. For pooled data from the three fibers where F and K (measured with steps) were compared at similar F levels, without Dextran T-500 the

average submaximal F was 48% (±0.06) of maximum and the corresponding average K was 52% (±0.07, 11 observations); the difference in mean F and K was not significant ($P > 0.05$). In the presence of 4% Dextran T-500 the corresponding values of relative F and K at comparable F levels from the same fibers were 43% (±0.07) for submaximal F and 55% (±0.08, nine observations) for K; at submaximal F levels osmotic compression leads to a relative K greater than relative F ($P < 0.05$). These results suggest that, without some osmotic compression of fibers, K measurements may be affected both by the degree of F activation and activation-dependent changes in myofilament lattice spacing.

The data in Figs. 1 and 2 indicate that Pi reduced maximum F, but reduced K to a lesser extent. The depression of maximum F by phosphate is consistent with that
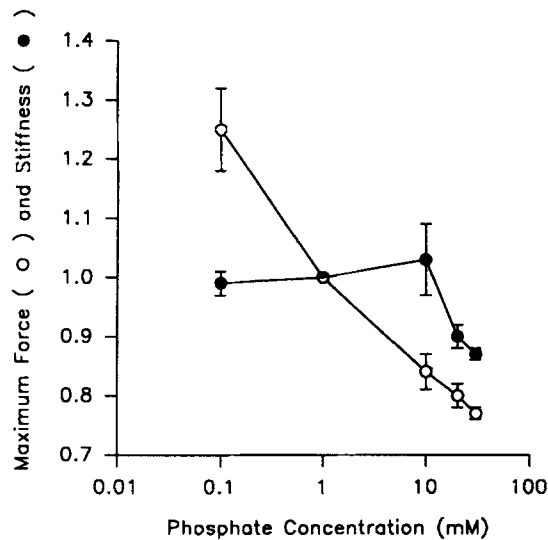

FIGURE 6. The effect of phosphate on maximally activated F (O) and K (●). F and K are normalized to the values obtained at maximum activation with no phosphate added to the solutions. Data at 0.20 mM Pi were obtained when 10 mM sucrose and sucrose phosphorylase were added to the activating solution (five fibers); the value of Pi present was not measured directly (see Methods). The concentration of Pi present in the zero added solutions is represented as 1 mM (see Pate and Cooke, 1989a). The points represent the means (±SEM) of data obtained from 5 (no added Pi with sucrose and sucrose phosphorylase), 15 (no added Pi), 6 (10 mM Pi), 8 (20 mM Pi), and 6 (30 mM Pi) fibers.

observed by others in skeletal (Godt et al., 1986; Brozovich et al., 1988; Pate and Cooke, 1989a; Millar and Homsher, 1990) and cardiac muscle (Kentish, 1986). Fig. 6 illustrates the effect of 0 (~1 mM Pi; see Methods), 10, 20, and 30 mM added phosphate on maximally activated F and K. Also, Pi was further depressed (to ~200 μM; see Pate and Cooke, 1989a; Millar and Homsher, 1990) by adding 10 mM sucrose and sucrose phosphorylase to the solutions with no added Pi. K was measured with 1-kHz sinusoidal oscillation of fiber length. The suppression of maximum F by Pi is similar to that seen by Pate and Cooke (1989a) but less than that observed by Millar and Homsher (1990) at similar Pi levels. Inclusion of sucrose and sucrose phosphorylase elevated F 25 ± 0.07% relative to pCa 4.0 solutions with no added Pi, with no change in K, indicating that our solutions with no added Pi contained
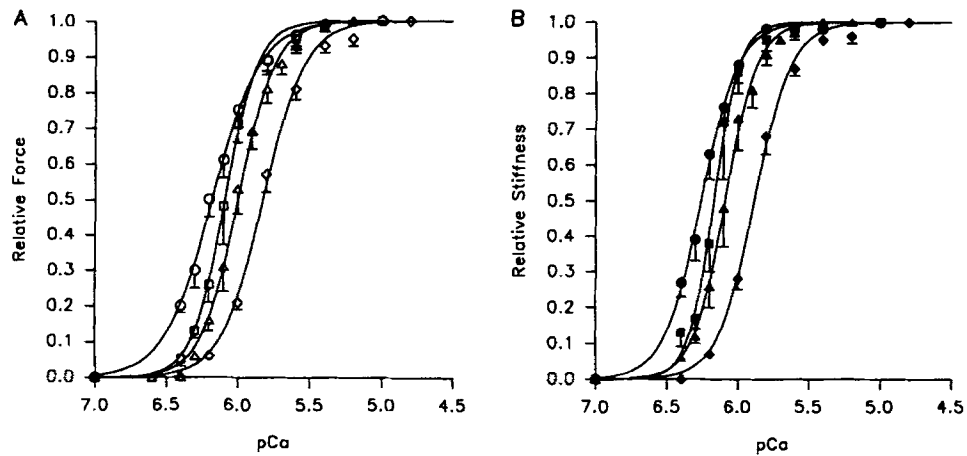
FIGURE 7. (*A*) The calcium dependence of F is shown for 0 (○), 10 (□), 20 (△), and 30 (◇) mM added phosphate. Data points represent the means (±SEM) of values obtained from 15, 6, 8, and 6 fibers, respectively. Values have been normalized to the maximum F obtained at each phosphate concentration. The solid lines were obtained by fitting the data with the Hill equation and the values of pK and *n* included in Table II. (*B*) K was measured for the same fibers under the same conditions shown in Fig. 8. The filled symbols correspond to the same Pi concentrations. As in Fig. 8, K at each Ca level was normalized to the maximum values obtained at each Pi concentration and the data (means ± SEM) were fit with the Hill equation (solid lines). The values of pK and *n* are included in Table II for comparison with the corresponding F data.

contaminating Pi. Elevated Pi caused a decrease of maximum F at all phosphate levels, while K was unaffected by very low Pi levels to 10 mM added phosphate and was relatively less depressed than F at levels > 10 mM. Similar results from no added Pi to 10 mM Pi have been obtained in detergent skinned frog fibers (Brozovich et al., 1988).

TABLE II

No. of fibers	[Pi]	Dependent variable	pK	*n*
	mM			
6	0	F	6.18 ± 0.01	2.68 ± 0.11
6	0	K	6.26 ± 0.04	3.37 ± 0.14
7	10	F	6.10 ± 0.01	4.08 ± 0.17
7	10	K	6.17 ± 0.01	5.00 ± 0.39
5	20	F	6.01 ± 0.01	3.6 ± 0.16
5	20	K	6.10 ± 0.01	4.0 ± 0.18
6	30	F	5.84 ± 0.01	3.21 ± 0.16
6	30	K	5.89 ± 0.01	3.58 ± 0.17

Data shown were obtained from nonlinear regression of F-calcium and K-calcium relations with best fit values ± standard error given.

In addition to suppressing maximum F relative to K, elevated Pi also caused a decrease in calcium sensitivity of both F and K (Fig. 7, *A* and *B*). F-calcium and K-calcium relations were determined at added Pi concentrations of 0, 10, 20, and 30 mM. K was measured with 1-kHz oscillations of fiber length. The solid curves in both figures were obtained by fitting the data with the Hill equation. The values of pK and *n* for each condition are included in Table II. The decrease in calcium sensitivity of both F and K, relative to zero added Pi, was significant ($P < 0.01$ at all added Pi levels). At each Pi level K was more sensitive to calcium than F ($P < 0.01$). Also, K-calcium relations were steeper than for F at all Pi levels ($P < 0.01$). In addition to decreasing calcium sensitivity elevated Pi also caused the slopes of both F-calcium and K-calcium relations to increase ($P < 0.01$ for all added Pi levels), although the increases were less at 30 mM Pi. The data in Fig. 7 were obtained from fibers that had been attached by wrapping to attachment hooks and K was obtained by the sinusoidal method.

DISCUSSION

Our observations indicate that: (*1*) fiber K, as measured with both sinusoidal and step changes in length, is more sensitive to calcium than is F; (*2*) elevated Pi causes a greater decrease in maximum F than K; and (*3*) elevated Pi causes a decrease in the calcium sensitivity of both F and K. As described above, all experimental approaches gave similar results, suggesting that the depression of F relative to K at lower calcium and elevated concentrations Pi cannot be attributed to an under estimation of fiber or sarcomere K. These results are discussed in relation to previous observations, in terms of the known mechanochemistry of the crossbridge cycle and in relation to the mechanisms by which calcium regulates the crossbridge cycle.

K Is More Sensitive to Calcium Than Is F: Implications for Calcium Regulation

Two general models have been proposed for the calcium regulation of F: (*1*) the steric hindrance model and (*2*) calcium regulation of a weak to strongly attached crossbridge transition. Both models for calcium activation predict that K and F should have similar calcium dependences. This is because K should be proportional to the total attached population of crossbridges (weakly and strongly bound), while those bridges producing F are a subset of that population. However, our results indicate that steady K is more sensitive to calcium than F.

The observation that steady-state K is more sensitive to calcium than F (irrespective of Pi level) has several potential interpretations which depend in part on whether the K measurements reflect only strong crossbridge attachment or a combination of weakly and strongly attached states. For example, relaxed K was very low at 200 mM ionic strength, implying either that (*1*) there was very little weak attachment and crossbridges spent most of their time detached from actin, or (*2*) there was significant weak binding, but the equilibrium between attached and detached states was too rapid to be effectively detected at the speeds of length changes we were able to generate (Brenner et al., 1982, 1986). We believe that we have detected primarily strong or strengthened crossbridge attachment for the following reasons. While our apparatus is fast enough (ramp or step time ~ 750 μs) to detect weak crossbridges at

low ionic strength, the detectable fraction of weak attachments is probably ~10%, assuming detachment rate constants appropriate for low ionic strength conditions (10^4 s^{-1}; Schoenberg, 1991). At 200 mM ionic strength the attachment–detachment rate constants would be expected to be even higher and the detectable fraction of weakly bound bridges correspondingly smaller (Brenner, 1986; Brenner et al., 1986; Schoenberg, 1988). Therefore, if there were significant undetected weak binding in the relaxed fiber, an increase in K relative to F upon activation implies that the strength of binding (as evidenced by K) to a zero or low F state was regulated by calcium. This is consistent with calcium regulating a weak to strong transition, as biochemical evidence suggests; but in this case the transition does not lead immediately to F production. However, x-ray diffraction evidence suggests that at high ionic strength myosin is detached from actin, indicating that weak attachment is decreased (Brenner and Yu, 1985). If there is little weak attachment in a relaxed fiber, an increase of K relative to F is consistent with calcium regulating strong attachment of crossbridges to actin in a low F state which is mechanically detectable at high ionic strengths—an idea consistent with the steric hindrance model. By either mechanism calcium binding leads directly to a more strongly attached low F crossbridge state.

A different calcium sensitivity of F and K could have several plausible explanations. If calcium regulated more than one step in the crossbridge cycle (perhaps stronger attachment and Pi release), the only requirement would be that the two steps have different calcium sensitivities. This kind of scheme would be consistent with the large body of biochemical evidence which indicates that calcium regulates Pi release or some step leading to Pi release. Another possible explanation for the difference in calcium sensitivity of F and K is suggested by the idea that each head (S1) of a single rigor crossbridge may see a different effective actin concentration, with attachment of only one head per crossbridge needed for full K, at least in rigor (Pate and Cooke, 1988). Although it has not been established that this is true for cycling crossbridges, or if attachment of the second S1 head leads to F generation in the presence of ATP (see Pate and Cooke, 1988), the results could be consistent with the attachment of the first head giving K while the attachment of the second head at higher calcium concentration would lead to F. If attachment of both heads of a crossbridge are necessary for F, with only one head needed for K, changes in K would still indicate of strong crossbridge attachment, as described above. Another possibility is that calcium binding to myosin light chain 2 (LC2) might alter the relative K of crossbridges. However, recent evidence suggests that although LC2 extraction altered contraction velocity, the relationship between F and K was unaltered (Hofman et al., 1990).

While our results are consistent with calcium regulating more than one step in the crossbridge cycle, they are also consistent with a model for the cooperative transition of crossbridges between strongly attached states (Bagni et al., 1988). According to the model, the equilibrium distribution of strongly attached crossbridges between high and low force-producing states depends on the number of attached crossbridges, being shifted toward the high F state as strong crossbridge attachment increases. Therefore, a difference in the calcium dependence of F and K occurs because at low calcium levels there are fewer attached crossbridges with a greater proportion in low F states (so K increases to a greater extent than F), while as calcium increases more crossbridges attach, favoring a relative shift to the high F state.

Our observation that K is more sensitive to calcium is at odds with observations made by others in frog skeletal muscle fibers (Herzig et al., 1981; Goldman and Simmons, 1984; Brozovich et al., 1988) and glycerinated rabbit fibers (Brenner, 1986). There are several possible explanations for this discrepancy. The range of calcium levels used in previous studies on frog fibers was limited, with few points in the middle of the calcium sensitivity curve. Also, there could be a species difference between frog and rabbit skeletal fibers in the kinetics of transitions within the crossbridge cycle (see Brenner et al., 1986). A further important difference between the other studies and ours is that we osmotically compressed the myofilament lattice with 4% Dextran T-500. Both mechanical and chemical skinning cause the myofilament lattice to swell by ~20% (Maughan and Godt, 1979; Matsubara et al., 1984, 1985), and lattice swelling has been shown to cause a significant decrease in skinned fiber K (Goldman and Simmons, 1986). We used 4% Dextran T-500 since it has been shown to restore the myofilament lattice back toward in vivo dimensions and minimize lattice shrinkage during active and rigor contractions (Matsubara et al., 1985). On the other hand, in uncompressed, skinned fibers lattice spacing would be greater at low calcium and F levels, leading to an underestimation of crossbridge K, while as F increased at higher calcium levels lattice spacing would decrease (Brenner and Yu, 1985) and the apparent K of crossbridges would rise. Therefore, in uncompressed fibers F and K might change proportionately with calcium only because relative crossbridge K is lower at low F and greater lattice spacing. The idea is supported by the evidence in Fig. 6, which indicates that if the filament lattice is not compressed with Dextran T-500 the increased fiber diameter (and lattice spacing) at low calcium results in relative K being comparable to that in maximum calcium. A recent relevant observation indicates that while F and K change in direct proportion to each other in skinned rat slow and fast twitch fibers at pH 7.0 with no osmotic compression, when pH was lowered to 6.2 relative K increased to a greater extent than F with increasing activation (Metzger and Moss, 1990). Lower pH has been shown to cause a decrease in both myofilament lattice spacing and fiber diameter (Umazume et al., 1986; Martyn and Gordon, 1988). Therefore, it is possible that lowered pH established the conditions necessary for unmasking the relationship between F and K; that is, decreased pH leads to decreased myofilament lattice spacing, which may result in reduced change during activation. Thus, for the results in the literature which indicate that F and K have similar calcium sensitivities, one must question whether changing lattice spacing with developed F was not masking the true calcium dependence of K.

Pi Effects on the Calcium Dependence of F and K

Elevated bathing solution Pi causes a decrease in the calcium sensitivity of both F and K (Fig. 7, Table II). The decrease in calcium sensitivity of F (Brandt et al., 1982; Kentish, 1986; Brozovich et al., 1988; Millar and Homsher, 1990) and K (Brozovich et al., 1988) is consistent with that observed by others and suggests that Pi decreases the apparent level of thin filament activation. Pi might affect calcium sensitivity by altering the distribution between states which have differing affects on thin filament activation (Millar and Homsher, 1990), by direct effects on filament charge, or by altering regulatory calcium binding. For example, Pi could affect calcium binding if

elevated Pi caused a change in myofilament charge density, perhaps by binding to sites of positive charge. However, because the myofilaments have a net negative charge at pH 7.0 (Collins and Edwards, 1971) Pi binding to charged sites would increase myofilament charge density and lead to an increase in calcium sensitivity, rather than the observed decrease (Martyn and Gordon, 1988). Recent evidence suggests that elevated Pi does not affect calcium binding to troponin (Kentish and Palmer, 1989).

Strong crossbridge attachment has been shown to affect thin filament activation either by cooperatively promoting crossbridge binding (Hill et al., 1980) or by increasing calcium binding to regulated thin filaments (Bremel and Weber, 1972; Grabarek et al., 1983). It has also been suggested that elevated Pi leads to a decrease in strong crossbridge attachment, causing a decrease in both maximum F and pK of F-calcium relations (Millar and Homsher, 1990). Millar and Homsher (1990) also suggested that at decreased calcium sensitivity cooperative strong crossbridge attachment dominates thin filament activation, so the slope of F-calcium relations increases. However, our results indicate that a strongly attached, low force-producing state exists before Pi release (see below for discussion). Additionally, strong crossbridge attachment (as evidenced by K) remains constant over a range of Pi concentrations at which maximum F and the calcium sensitivities of F and K are decreased (Fig. 7, Table II). Thus, a strongly attached AM-ADP-Pi low force-producing state may have less of an effect on thin filament activation than does AM-ADP. This idea is supported by the observation that elevated ADP increases both F (Cooke and Pate, 1985; Pate and Cooke, 1989b; but see Chase and Kushmerick, 1988a) and calcium sensitivity (Hoar et al., 1987).

Pi Depresses Maximum F More Than K: Implications for the Crossbridge Cycle

We have found that elevated Pi depresses maximal calcium activated F in skinned skeletal fibers (Fig. 6), as has been observed by others for skeletal (Brandt et al., 1982; Cooke and Pate, 1985; Hibberd et al., 1985; Godt et al., 1986; Hoar et al., 1987; Brozovich et al., 1988; Pate and Cooke, 1989b; Millar and Homsher, 1990) and cardiac muscle (Herzig et al., 1981; Godt et al., 1986; Kentish, 1986). Presumably, elevated Pi reverses the AM-ADP-Pi to AM-ADP reaction, where AM-ADP is probably a force-producing crossbridge state, and one of the AM-ADP-Pi states is not (Eisenberg and Greene, 1980). That maximum F decreases linearly as the log [Pi] increases (Fig. 6) has been observed by others (Millar and Homsher, 1990; Pate and Cooke, 1989a) and is consistent with the idea that Pi release is associated with transition to a force-producing crossbridge state (see Pate and Cooke, 1989b for a comprehensive discussion of this point). The data in Fig. 6 also indicate that elevated Pi has a different effect on fiber K; K remains constant from very low to 10 mM Pi and then declines less than F at higher Pi concentrations. This result suggests that the transition between Pi-bound and Pi-released crossbridge states may include a step between strongly attached states.

A potential complication for our interpretation of the K data is that both low F (Bagni et al., 1988) and elevated Pi (Hibberd et al., 1985; Kawai et al., 1987) have been shown to increase the mechanical rate constants within the crossbridge cycle. However, if this is the case then the use of sinusoidal length changes to measure fiber

K should underestimate, not overestimate, the true fiber K and tend to decrease apparent K more than F. For K determined from step changes in length our measurements probably underestimate K because the length changes are relatively slow (completed in ~750 μs; see Ford et al., 1981). If steps involved in the rapid redevelopment phase of F recovery following a step (Huxley and Simmons, 1971) are faster in high Pi (as for sinusoidal tests), the step measurements will underestimate fiber K under those conditions. Thus, changes in rate constants cannot explain higher relative K in elevated Pi and the increased calcium sensitivity of K over F (Table II).

The observation that elevated Pi causes a depression of F with less effect on maximum K (Fig. 6) is consistent with recent observations by others (Hibberd et al., 1985; Kawai et al., 1987; Brozovich et al., 1988) and is at odds with the idea that the Pi release step of the crossbridge cycle involves a transition between a weakly and a strongly attached state (Chalovich et al., 1981); in the latter case elevated Pi would be expected to decrease F and K to the same degree, at least under the conditions present when we measured them. However, our interpretation depends on the measured K reflecting only strong crossbridge attachment. While it is possible that weak crossbridge attachment could contribute to our measurements of K, the ionic strength of the bathing solutions should significantly reduce weak binding and the step times and oscillatory frequency used for K measurements should underestimate weak binding (see above). Therefore, if our K measurements contained a significant component from weak attachment, K should decline continuously as Pi is elevated and the weakly attached Pi bound state is populated; Fig. 6 suggests that this is not the case.

Our results (Fig. 6) indicate that the Pi release step within the crossbridge cycle involves a transition between two strongly attached states, with the state before Pi release producing less F than the state subsequent to release. The existence of a strongly attached non–force-generating state is supported by other evidence. For example, K leads F during the rising phase of a tetanus in intact fibers (Ford et al., 1986; Cecchi et al., 1987). Also, crossbridges that bind the analogue ATP[γS] produce no F but exhibit a calcium-dependent K (Dantzig et al., 1988). In addition, aluminofluoride, which is a slowly dissociating analogue of Pi, also inhibits F to a greater extent than K in skinned fibers (Chase et al., 1991). Our results do not exclude the possibility that a transition from a weakly to a strongly attached state exists within the crossbridge cycle at normal ionic strengths, but only that this transition takes place at some step before Pi release. For example, recent evidence suggests that a step before Pi release may be rate limiting during F development (Millar and Homsher, 1990).

We have assumed that our K measurements monitor strong crossbridge attachment, whether or not Pi is bound, and that F declines as Pi is elevated because crossbridges with Pi bound do not produce F. However, our interpretation may be too simplistic because we did not measure changes in Pi binding to AM-ADP. Our observations do not allow us to strictly equate a change in F with a reistribution of crossbridges between Pi-bound and Pi-dissociated crossbridge states because we cannot exclude the possibility that Pi-bound crossbridges could produce some F (see Millar and Homsher, 1990); but we can conclude that they produce less F than the Pi

released state(s). Also, there is evidence that Pi may preferentially bind to high force-producing states (Hibberd et al., 1985; Webb et al., 1986), thereby causing a disproportionate decrease in F relative to K. Under conditions where K is unchanged and F depressed (from very low to 10 mM Pi; Fig. 6), the results do allow us to conclude that Pi-bound crossbridge states do not produce less F simply because the number of strongly attached crossbridges has decreased, as monitored by K.

Increasing Pi above 10 mM causes a decrease in both F and K, with the decrease in relative K being less than that in F (Fig. 7). A possible interpretation of this result would be that elevated Pi shifts the crossbridge population to a low force-producing, strongly attached Pi-bound state, as would be expected if the Pi release step is reversible (Hibberd et al., 1985). As Pi is further elevated, the Pi bound states are further populated so both F and K decrease as strong crossbridge attachment is reversed, resulting in detachment or transition to a weakly attached, low apparent K state (perhaps as AM-ATP or AM-ADP-Pi).

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