Calcium Regulation of Thin Filament Movement in an In Vitro Motility Assay

Earl Homsher,* Bibiana Kim,* Alyona Bobkova,* and Larry S. Tobacman‡
*Department of Physiology, School of Medicine, Center for Health Sciences, UCLA, Los Angeles, California 90025, and ‡Departments of Medicine and Biochemistry, University of Iowa, Iowa City, Iowa 52242 USA

ABSTRACT The ability of calcium to regulate thin filament sliding velocity was studied in an in vitro motility assay system using cardiac troponin and tropomyosin and rhodamine-phalloidin-labeled skeletal actin and skeletal heavy meromyosin to propel the filaments. Measurements showed that both the number of thin filaments sliding and their sliding speed (S_f) were dependent on the calcium concentration in the range of pCa 5 to 9. Thin filament motility was completely inhibited only if troponin and tropomyosin were added at a concentration of 100 nM to the motility assay solution and the pCa was more than 8. The filament sliding speed was dependent on the pCa in a noncooperative fashion (Hill coefficient = 1) and reached maximum at 5 μm/s at a pCa of 5. The number of filaments moving uniformly decreased from >90% at pCa 5–6 to near zero in less than 1 pCa unit. This behavior may be explained by a hypothesis in which the regulatory proteins control the number of cross-bridge heads interacting with the thin filaments rather than the rate at which they individually hydrolyze ATP or translocate the thin filaments.

INTRODUCTION

In 1957 A. F. Huxley hypothesized that the unloaded shortening velocity (V_u) in muscle was set by a balance of forces, i.e., the force produced by cross-bridges propelling thin filaments toward the center of the sarcomere (positive force producing cross-bridges) balanced by attached cross-bridges resisting thin filament movement (drag stroke cross-bridges). In this formulation, a cross-bridge initially attaches to the thin filament and produces a positive force, which declines toward zero as shortening proceeds. With continued filament sliding, some cross-bridges are dragged into a negative force-generating region, consequent to a limited rate of cross-bridge detachment from the thin filament. Huxley's formulation of the cross-bridge mechanism was particularly significant in that it successfully accounted for a variety of known mechanical and energetic features of contraction. Among these, his theory successfully predicted that the unloaded shortening velocity (V_u) would be independent of the number of cross-bridges attached to the thin filament (the sarcomere length) (Gordon et al., 1966).

According to interpretations of x-ray diffraction studies, regulation of muscular contraction is accomplished by limiting cross-bridge access to "strong" hydrophobic binding sites on actin by the position of tropomyosin, which is regulated itself by calcium binding to troponin (Haselgrove, 1972; Huxley, 1972). Such a "recruitment" model, together with Huxley's view of V_u, predicts that V_u will be independent of the calcium concentration. However, evidence on this point is conflicting. In skinned muscle fibers, several groups have reported that V_u is independent of pCa (the negative log of $[Ca^{+2}]$) (Thames et al., 1974; Podolin and Ford, 1986), whereas others have shown that V_u increases as pCa is decreased (Julian, 1971; Julian and Moss, 1981). Moss (1991) has suggested this discrepancy may be reconciled by the presence of an internal load associated with a C-protein limitation of cross-bridge head displacement during shortening.

The in vitro motility assay offers a method of directly testing Huxley's concept of thin filament regulation and may itself offer clues to unraveling the contradictory results obtained using skinned muscle fibers. Honda and Asakura (1989) and Harada et al. (1990), using skeletal regulatory proteins, reported that the sliding speed of thin filaments was regulated in an "on and off" fashion predicted by Huxley's model; i.e., at pCa > 5.7–6.0 the filaments bound to the myosin-coated surface and did not move. However, when pCa was lowered further, at a pCa of 5.7 (Honda and Asakura, 1989) or 6.0 (Harada et al., 1990), filaments began to move at maximum speed. A recent study by Sata et al. (1995) confirmed these observations by using cardiac regulatory proteins. Unfortunately, these studies did not characterize filament movement in detail, did not define the methods used to compute the filament movement, did not define the filament selection criteria, or specify the protein constituency of the filaments (actin:Tm:Tn). Recently, Fraser and Marston (1995) successfully addressed some of these issues, by varying the amount of regulatory proteins attached to the thin filament and by randomly selecting filaments for analysis. They concluded that calcium regulated the number of filaments moving and that regulation was an "all-or-none" process. However, the regulation was incomplete in that even at calcium concentrations of $<10^{-7}$ M, 20% of the filaments moved at speeds near maximum.

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Address reprint requests to Dr. Earl Homsher, Department of Physiology, School of Medicine, Center for Health Sciences, UCLA, Los Angeles, CA 90024. Tel.: 310-825-6976; Fax: 310-206-6551; E-mail: ehomsher@physiology.medsch.ucla.edu.

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In the study below we used native thin filaments isolated from bovine cardiac muscle as well as reconstituted thin filaments composed of cardiac tropomyosin and troponin bound to skeletal actin to characterize thin filament activation with regard to the number, speed, and quality of filament movement. We find that the inclusion of additional tropomyosin/troponin (100 nM) in the motility assay was necessary to ensure complete regulation of the thin filaments. Furthermore, at ionic strengths of 50 mM (in the presence and absence of methyl cellulose) and at 100 mM in the presence of methyl cellulose, calcium regulates both the fraction of moving filaments and the filament sliding speed (S_f). However, control experiments suggest that the fall in filament sliding speed at low [Ca^{+2}] is a consequence of a reduction in the number of myosin heads strongly attached to the thin filaments. These results suggest that filament sliding speed per se is not modulated by calcium concentration.

MATERIALS AND METHODS

Proteins

Rabbit skeletal myosin was prepared as described by Margossian and Lowey (1982) and used immediately or stored in 50% (w/v) glycerol at −20°C. The stored myosin was used within 4 weeks of preparation. Actin was prepared as described by Pardee and Spudich (1982) from acetone powder processed from the residues of the rabbit muscle after myosin extraction. The F-actin was stored on ice and could be used for up to 2 months after preparation. Bovine ventricular tropomyosin and troponin were purified as previously described (Tobacman and Adelstein, 1986; Tobacman and Lee, 1987). These proteins were aliquoted and stored in a low-salt solution containing 10 mM Tris (pH 7.5), 1 mM dithiothreitol (DTT), 0.01% NaN_3, 5 mg/ml each of L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK) and Nα-p-tosyl-L-lyrine chloromethyl ketone (TLCK), and 0.3 mM phenylmethylsulfonyl fluoride (PMSF) for up to 6 months at −70°C. Bovine ventricular native thin filaments (NTFs) were made as described by Tobacman and Sawyer (1990) and were stored for up to a month on ice in 4 mM imidazole (pH 7.4 at 25 C), 3 mM $MgCl_2$, 2 mM K_2EGTA, 3 mM NaN_3, 5 mg/liter of TLCK and TPCK. Heavy meromyosin (HMM) was prepared by chymotryptic digestion as described by Kron et al. (1991) and was used in the assay for 3–5 days. On the day of an assay, a fraction of the HMM solution (typically 300 μg/ml in a low-salt buffer) was reacted with a stoichiometrically equivalent concentration of F-actin in the presence of 2 mM MgATP and centrifuged in a Beckman airfuge at 180,000 × g at 4°C for 15 min to remove inactive HMM molecules unable to dissociate from the actin. For these studies, the molecular masses for myosin, HMM, actin, troponin, and tropomyosin were taken as 480, 350, 42, 77, and 68 kDa, respectively. The corresponding extinction coefficients (mg/ml) used in this work were $E_{280} = 0.52\ cm^{-1}$, $E_{280} = 0.60\ cm^{-1}$, $E_{280} = 1.15\ cm^{-1}$, $E_{276} = 0.45\ cm^{-1}$, and $E_{280} = 0.33\ cm^{-1}$ (Tobacman and Lee, 1987). Reconstitution of thin filaments from actin, troponin, and tropomyosin was done by mixing the proteins at a concentration of 2 μM F-actin, 0.5 μM tropomyosin, and 0.48–0.5 μM troponin in F-actin buffer (4 mM imidazole, pH 7.1 at 25°C), 2 mM $MgCl_2$, 0.5 mM ATP, 3 mM NaN_3, 1 mM DTT). The solution was allowed to incubate overnight before use.

Fluorescent labeling of the actin

Rhodamine phalloidin (RP) (60 μl, 3.3 μM; Molecular Probes, Eugene, OR) in methanol was centrifuged under vacuum to dryness. Actin buffer (90 μl) was then added to the RP residue and sonicated for 1 h, after which 10 μl of 20 μM actin or 20 μM actin/5 μM tropomyosin/5 μM troponin was added to the solution and allowed to incubate overnight. The labeled actin can be used for 1–2 weeks with satisfactory results.

Motility assay

The movement of the RP-labeled thin filaments over HMM-covered nitrocellulose surfaces in reaction chambers was observed by video epifluorescence microscopy, as described by a number of investigators (Kron and Spudich, 1986; Homsher et al., 1992). The nitrocellulose coverslips were prepared as described by Umemoto and Sellers (1990) and were used within 5 h. Typically, HMM (200–300 μg/ml) was injected into the chamber and allowed to incubate for 2 min. In several experiments (indicated in the text) this concentration was reduced to 30 μg/ml. This was followed by 50 μl of 0.5 mg/ml of bovine serum albumin (Sigma Chemical Co., St. Louis, MO), which incubated for 1 min. The slide chamber was them washed with two 50-μl aliquots of assay buffer (20 mM KCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.4 at 25°C), 2 mM $MgCl_2$, 1 mM DTT). Next a 1 μM solution of sheared unlabeled actin filaments (produced by rapid flow of the actin solution through a 23-gauge syringe needle) was introduced onto the surface for 1 min, followed by two 50-μl aliquots of assay buffer containing 1 mM ATP. This procedure acts to block any "dead" S-1 heads. Next the chamber was washed twice with assay buffer to clear the chamber of any ATP. A 20–40 nM solution (50 μl) of the RP-labeled thin filaments or regulated thin filaments (made by dilution of the 2 μM labeled actin solution immediately before its use) was then introduced into the chamber and incubated for 1 min. Finally, the chamber was rinsed twice with assay buffer, and the activating solution was introduced. All activation solutions contained 3 mg/ml glucose, 100 μg/ml glucose oxidase, 10 μg/ml catalase, and 10 mM DTT to minimize photobleaching of the fluorescent label. The solutions used in these experiments were at 50 or 100 mM ionic strength and calcium concentrations (or pCa), which were calculated using a solution program written in QuickBasic by E. Homsher and N. Millar based on the equations of Fabiato and Fabiato (1979). The composition of the 50 mM ionic strength solution was 25 mM MOPS (pH 7.4), 25 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA (with varying ratios of CaKEGTA or K_2EGTA), 1 mM Na_2-ATP, and the glucose/oxidase/catalase system to slow photobleaching. The 100 mM ionic strength solutions contained 140 mM MOPS (pH 7.4), 2 mM $MgCl_2$, 10 mM DTT, and the appropriate ratios of K_2EGTA and CaKEGTA as well as the enzymes. In some solutions up to 0.4% methyl cellulose (MW ~90,000; Sigma Chemical Co.) was added to the solutions to reduce lateral diffusion of the filaments away from the surface (Uyeda et al., 1990).

Motility measurements

The motility chamber was placed on a temperature-controlled stage of a Leitz epifluorescence microscope. The light from a 200 W/4 Hg arc lamp was passed through an N-14 filter cube to excite fluorescence after it had passed through a temperature-controlled 60× 1.3 NA objective. The fluorescence signal was imaged on a DAGE 68 SIT camera, whose image was recorded without enhancement on a Mitsubishi VCR and recorded on videotape. Typically 8–10 sites on a surface were observed, and each was recorded for 20–30 s, during which photobleaching was insignificant. The recorded sites were then played back through a Motion Analysis Expert Vision System (Santa Rosa, CA.). This system makes it possible to track the movement of each filament in the field of view, using frame grabbing rates of 0.1 to 30/s. In these experiments data were acquired at 1–10 frames/s, depending on filament sliding speed (the faster the filament movement, the faster the frame grabbing rate). The data were acquired and analyzed as previously described in detail (Homsher et al., 1992). Use of these techniques allowed determination of the number of filaments moving in the field of view and computation of the frame-to-frame speed for each filament and each filament's average speed. As previously, filaments were classified as belonging to one of three categories: 1) filaments not moving (i.e., having an average speed of <0.8 μm/s when acquired at 5 frames/s

or <0.32 μm/s when acquired at 1 frame/s [the apparent speed produced by arc wander and mechanical vibration] as measured in filaments bound to the surface in the absence of ATP); 2) filaments moving at uniform speed (the standard deviation of the frame-to-frame speed for a filament was <0.5 of the filament's mean speed); and 3) erratically moving filaments (those whose frame-to-frame SD was >0.5 of the filament's mean speed). This methodology removes the bias that may arise from experimenter selection of filaments to track. To qualify as a filament the filament must be tracked for at least 2 s at an acquisition rate of 5 frames/s and for 4 s for 1 frame/s. Typically the speed reported as a mean in this work is the average of >150 different filaments. Plots of the mean speed or fraction of uniformly moving filaments moving against pCa were fitted to a Hill plot using a nonlinear curve-fitting routine using NFIT (Island, Inc., Galveston, TX). Data are reported as mean ± SD.

RESULTS

Control experiments

To define the extent to which filament speed and number of filaments moving varied from one slide to another, motility assays were replicated to define the intrinsic variability of the system. For these studies, five separate motility assays were prepared using the same proteins and solutions under identical conditions. In the analysis of the records, nine different representative sites on each assay were chosen for analysis, and filament movement at those sites was recorded for 15–20 s. At each site 10-s segments of the videotaped filament movement were analyzed; the results are tabulated in Table 1. It can be seen that the total number of filaments observed in the nine fields in a slide varied about a mean of 360 filaments with a coefficient of variation of 23%. The average filament sliding speed (S_f) in a particular slide was more reproducible, yielding a mean (with this preparation of HMM) and a coefficient of variation of 4%. An analysis of variance testing the null hypothesis that the variance and means recorded in these studies were identical revealed that neither null hypothesis could be accepted, as there were at least two slides that varied significantly from the others at the $p < 0.001$ level. Pairwise t-tests for independent samples with unequal variance of this data revealed that only slides 3 and 4 and slides 3 and 5 were not significantly different from each other. Thus even in this sample, in which the S_f group means differed by less than 10% from each other, there is significantly different motion of the filaments. This difference, albeit small, is real and does not stem from the selection criteria, definition of nonmoving filaments, or duration of observation because the difference remains if these criteria are dropped. It is unlikely that the differences occur as a result of differences in illumination (e.g., arc wander or light intensity), because the former is of short duration and the consistency of the gray threshold over different slides argues against the latter possibility. The difference cannot stem from temperature variation because it is regulated. The difference is not caused by the analysis procedure, because a 10% difference in filament sliding velocity is discernible by casual observers as well. It is more likely that the differences derive from subtle differences in the surface quality. Thus when small differences are found between surfaces, one must carefully examine the data or perform a series of identical assays to avoid falsely concluding that a difference exists when none does.

TABLE 1 Replicate samples of motility assays

Slide no.	No. of filaments	Mean velocity (μm/s)	SD (μm/s)
1	279	4.7	0.9
2	293	4.9	0.8
3	417	5.1	0.7
4	339	5.1	0.7
5	473	5.0	0.7
Mean	360	5.0	
SD	83	0.2	

Because the experiments described below assume that the movement of filaments at differing pCa is a consequence of changes produced by the regulatory proteins we also tested the effect of $[Ca^{+2}]$ on filament sliding speed at pCa's ranging from 9 to 5.0 in unregulated thin filaments. The data in Fig. 1 indicate there are no significant differences in filament sliding velocity, fraction of smoothly moving filaments, or number of moving filaments. Thus any changes observed stem from the presence of regulatory proteins. Similar results have been reported by Fraser and Marston (1995).

Native thin filaments

The first type of regulated filament movement examined was "native" thin filaments (NTFs), thin filaments directly isolated from muscle by washes of homogenized muscle in low ionic strength, in which it is assumed that the thin filaments have a "normal" complement of regulatory proteins (Tobacman and Sawyer, 1990). Gels of these filaments were similar to those reported earlier

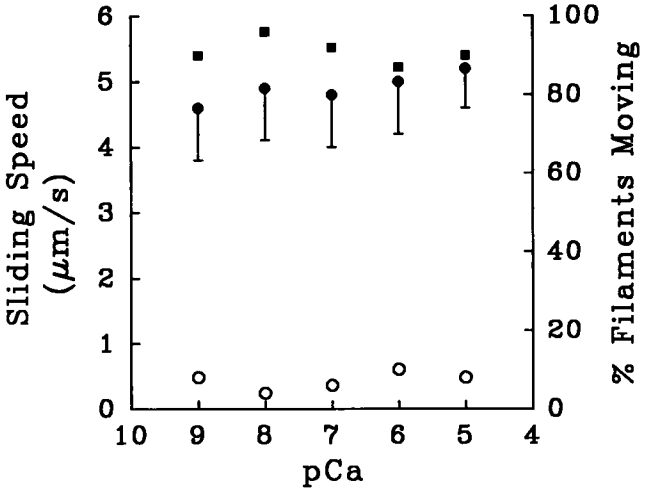

FIGURE 1 S_f (●) and percentage of unregulated thin filaments moving at uniform speed (■) and moving erratically (○) at various pCa at 25°C and 50 mM ionic strength. The vertical line on the S_f data indicates one SD. The number of filaments analyzed at pCa 9, 8, 7, 6, and 5 were 197, 74, 153, 167, and 106, respectively.

(Tobacman and Sawyer, 1990) and contained actin, Tm, TnI, TnT, and TnC. A drawback of studies using NTFs is that the filaments are only about 1.1 μm long, which corresponds to 4–6 pixels. Consequently in the analysis, noise should be minimized and the movement of specific filaments carefully compared to the paths computed by the filament movement analysis program. The experiments were done at 50 mM ionic strength. In preliminary studies the filament mean sliding velocity at pCa 5 was 5.1 ± 0.9 μm/s (mean ± SD), and 92.0% of the filaments (565) were moving at uniform speeds. At pCa 9, 43.3% of the 808 filaments analyzed did not move, 43.3% moved erratically, and 13.3% continued to move at a velocity of 3.7 ± 1.4 μm/s (mean ± SD). This result, which is similar to that of Fraser and Marston (1995), suggests that the regulatory proteins can dissociate from the filaments at the low concentrations of actin (20 nM) used in the motility assay. To prevent such dissociation, various concentrations of Tm/Tn ranging from 10 to 100 nM were added to the motility assay solution to ensure that regulatory proteins remained bound to the thin filaments. Although addition of 10 nM Tm/Tn to the motility buffer had little effect on the filament motion at pCa 9, addition of 100 nM Tm/Tn produced an essentially complete inhibition of filament movement at pCa 9 (less than 5% of the filaments showed any movement at all), and there was no effect of the added Tm/Tn on the filament sliding at pCa 5. Experiments with 1 μM Tm/Tn were not significantly different from those using 100 nM Tm/Tn.

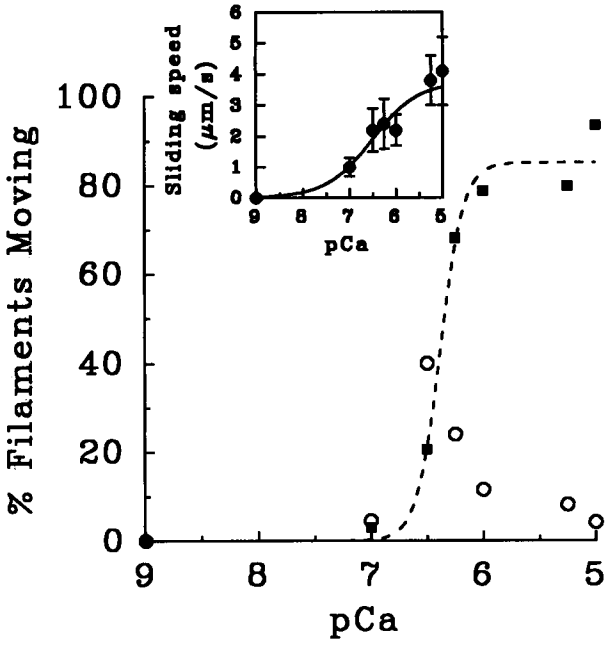

FIGURE 2 S_f (●) and fractions of native thin filaments moving at uniform speed (■) and moving erratically (○) at various pCa. Least-squares fits to the Hill equation for S_f gave the maximum S_f as 3.7 ± 0.4 μm/s, a Hill coefficient of 0.9 ± 0.4, and a pCa_{50} of 6.5 ± 0.2; comparable values for the percentage of filaments uniformly moving were 91%, Hill coefficient of 4.5 ± 1.3, and pCa_{50} of 6.2 ± 0.03 (means ± SD).

Thus all subsequent motility solutions contained 100 nM added Tm/Tn. Fig. 2 illustrates the effects of variation of pCa on filament motion under these conditions (for each data point 402–953 filaments moving for at least 2 s were analyzed). The data show nearly complete calcium regulation of the filament movement. At pCa 9 none of the filaments move. At pCa 7 less than 0.6% of the filaments are moving at uniform speed (4.5% show erratic movement), and 94.9% do not move at all. Those that are moving do so at a speed of 1 μm/s, 20% of the maximum filament velocity. When $[Ca^{+2}]$ rises from pCa 7 to 6, more than 74.5% of the filaments move smoothly and do so at an average speed of 2.2 μm/s, and at a pCa of 5m filament sliding speed is maximum at 4.1 μm/s and 93.7% of the filaments are moving at a uniform speed. Fits of the data in Fig. 2 using the Hill equation yield a Hill coefficient for the fraction of smoothly moving filaments of 4.5, whereas that for S_f is only 0.9. Furthermore, the pCa_{50} for the fraction of smoothly moving filaments is 6.2 and that for the S_f is 6.5. Thus in native thin filaments there is a calcium-dependent gradation of both the number of filaments moving and their speed that has not previously been reported.

Reconstituted thin filaments at 50 mM ionic strength

Experiments were performed using filaments reconstituted from rabbit skeletal muscle F-actin and bovine cardiac tropomyosin and troponin as described in Materials and Methods. These filaments are longer and more easily visualized than NTF, thus simplifying motion analysis. Fig. 3 summarizes the results of experiments using reconstituted filaments under the conditions used for native thin filaments. The results are similar to those obtained using native thin filaments. At pCa 9 numerous filaments bind (n = 308), and none move smoothly or erratically; as the calcium concentration is increased, filaments begin moving (pCa 8.0) at a slow speed (<1.0 μm/s), with less than 1% of the filaments (5) moving at a uniform speed and a small fraction moving erratically (<1%). At still higher calcium concentrations (pCa 7.0–6.0), 31–96% of the filaments move at uniform and high filament speed (~3.5–5.4 μm/s). Maximum velocity is reached at pCa 5 (5.8 μm/s), with 95% of all filaments moving smoothly (n = 686). The fraction of erratically moving filaments peaks (37%) at pCa 7 and then falls to 4% at pCa 5. Fig. 3 shows that reconstituted thin filaments, like NTFs, exhibit a Hill coefficient for S_f (0.7) that is less than that for the fraction of filaments moving uniformly (1.1). Finally, the pCa_{50} for S_f is slightly greater (7.1) than that for NTF (6.5). The difference is comparable to that previously noted between native and reconstituted thin filaments in the K_{app} for myosin S-1 ATPase (Tobacman and Sawyer, 1990). In that study, the respective K_{app} were 5.4 and 5.7–5.9.

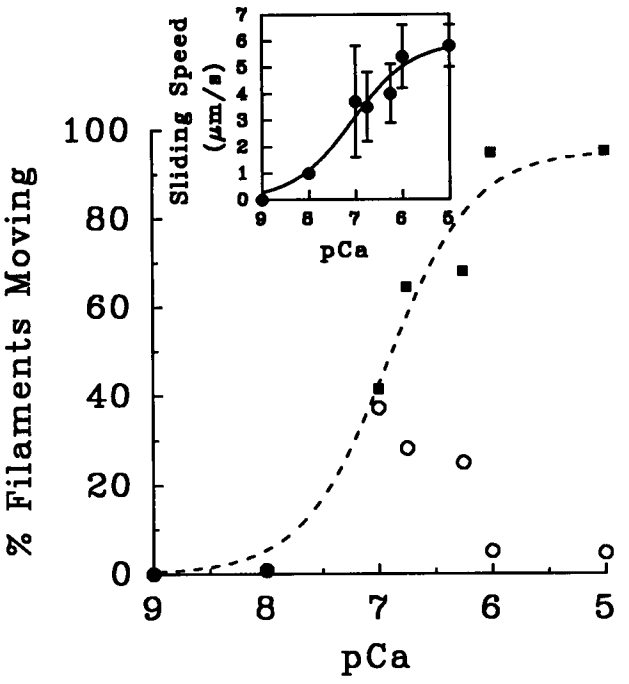

FIGURE 3 S_f (●) and fractions of reconstituted thin filaments at 50 mM ionic strength moving at uniform speed (■) and moving erratically (○) at various pCa. Least-squares fits to the Hill equation for S_f gave the maximum S_f as 5.8 ± 0.7 μm/s, a Hill coefficient of 0.7 ± 0.3, and a pCa_{50} of 6.9 ± 0.2; comparable values for the percentage of filaments uniformly moving were 97%, Hill coefficient of 3.9 ± 0.9, and pCa_{50} of 6.9 ± 0.06 (means ± SD). The number of filaments analyzed at pCa 9, 8, 7, 6.75, 6.25, 6, and 5 were, respectively, 308, 601, 398, 929, 172, 390, and 721.

Movement of reconstituted thin filaments at higher ionic strengths

Motility assays are often performed at ionic strengths of 50 mM or less because at higher ionic strengths the binding to the "weak" binding sites is reduced by the electrostatic "screening." Furthermore, as the ionic strength is reduced below 50 mM the filament sliding speed declines (Homsher et al., 1992), the K_m for actin activated ATPase declines (Wagner and Weeds, 1979), and the rate of ATP cleavage by myosin S-1 heads declines (Stein et al., 1979). To determine whether the relationships seen in the preceding studies applied to conditions in which weak binding was reduced, motility assays were conducted at an ionic strength of 100 mM. At 100 mM ionic strength the motility solution must contain MeCel to prevent filaments from diffusing from the surface by Brownian motion. The methylcellulose concentration at 50 and 100 mM was 0.4%. The lower ionic strength was used to compare the behavior of the filaments to those in the absence of MeCel as a control for the effect of MeCel itself. The data obtained in this comparison are shown in Table 2 at pCa of 5, 6, and 7. There is no significant difference in S_f, the fraction of uniformly moving filaments, or the fraction of erratically moving and nonmoving filaments. This result is consistent with similar lack of effect of MeCel on S_f reported earlier (Uyeda et al., 1990; Homsher et al., 1992). Fig. 4 shows the results of studies of the effect of pCa at 100 mM ionic strength. These data show first that there is no qualitative difference between the filament sliding behavior seen in the presence of MeCel and that in its absence. Second, at an ionic strength of 100 mM, the typical dependence of the fraction of smoothly moving filaments, the number of nonmoving filaments, and S_f on pCa is observed. The Hill coefficient for S_f is 1.2 and that for the fraction of moving filaments is 4.1. In this preparation the pCa_{50} for the fraction of smoothly moving filaments is greater (7.1) than that for S_f (6.8). Thus, the intrinsic calcium dependence of the fraction of filaments moving uniformly and S_f are not qualitatively altered by the ionic strength. As at lower ionic strengths, as the pCa is increased from 5, the fraction of erratically moving filaments increases (reaching a maximum near pCa 7) and then declines toward zero as pCa is further increased. This behavior is illustrated in Fig. 5, which shows the centroid path of filaments moving at a pCa of 9 (Fig. 5 *A*), 5 (Fig. 5, *B-I* and *B-II*), and 7 (Fig. 5, *C-I* and *C-II*) during a representative 10-s segment of videotape. Panel I in each section shows the path followed by the filaments during the 10-s period of analysis. Each filament in Panel I was assigned a letter. These data show that there is essentially no move-

TABLE 2 Comparison of filament motion in the presence and absence of methylcellulose at 50 mM ionic strength

		pCa		
		5.0	6.0	7.0
S_f (μm/s)	50 mM	5.8 ± 0.8 (127)	5.4 ± 1.2 (309)	3.7 ± 2.1 (398)
	50 mM + MeCel	6.6 ± 0.8 (721)	5.4 ± 1.0 (390)	2.5 ± 0.9 (398)
% filaments uniform	50 mM	95.1	94.9	41.5
	50 mM + MeCel	94.4	96.0	31.0
% filaments nonmoving	50 mM	4.7	5.1	37.2
	50 mM + MeCel	5.6	4.2	46.8
% filaments erratic	50 mM	0.2	0.0	21.4
	50 mM + MeCel	0.0	0.0	22.2

S_f is given as mean ± SD, and the numbers in parentheses correspond to the total number of filaments analyzed.

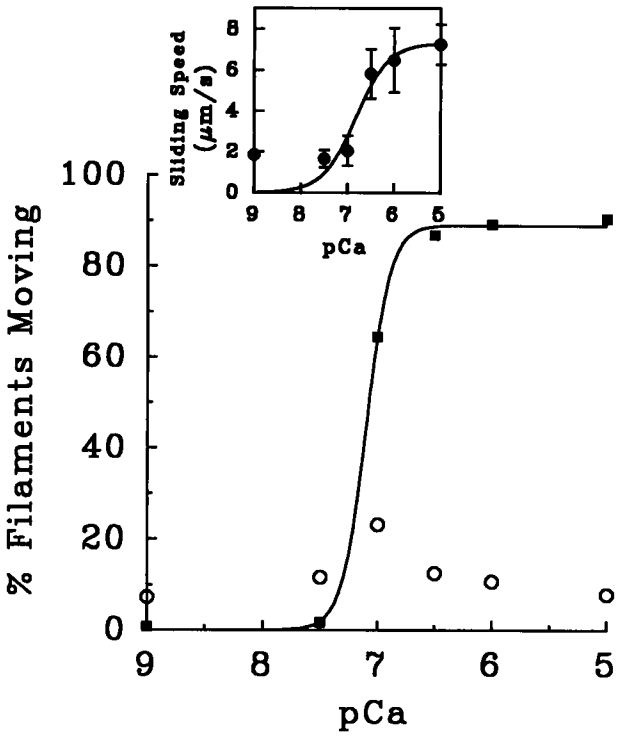

FIGURE 4 S_f (●) and fractions of reconstituted thin filaments at 100 mM ionic strength in the presence of methyl cellulose moving at uniform speed (■) and moving erratically (○) at various pCa. Least-squares fits to the Hill equation for S_f gave the maximum S_f as 7.4 ± 0.7 μm/s, a Hill coefficient of 1.2 ± 0.4, and a pCa_{50} of 6.8 ± 0.2; comparable values for the percentage of filaments moving uniformly were 89%, Hill coefficient of 4.1 ± 0.6, and pCa_{50} of 7.1 ± 0.02 (means ± SD). The speed observed at pCa 9 is from two filaments of 246 observed at pCa 9. The number of filaments analyzed at pCa 9, 7.5, 7, 6.5, 6, and 5 were 246, 630, 347, 568, 464, and 670, respectively.

ment at pCa 9, aside from noise associated with arc wander and mechanical vibration. At pCa 5 (Fig. 5 *B-I*) the filament paths are long compared those seen at pCa 7 (Fig. 5 *C-I*), indicating a greater S_f for pCa 5 than for pCa 7. In Fig. 5, *B-II* and *C-II*, the frame-to-frame location of each filament's centroid is designated by a tic mark. The numbers at the beginning and end of a filament centroid path indicate the frame in the sequence at which the filament entered the tracking field (the lower number) and the frame at which filament tracking ceased because the filament left the field, the data acquisition stopped, or the filament size dropped below the number of pixels (6) needed to qualify as a filament (the greater number). At pCa 5 the frame grabbing rate was 5/s and at pCa 7 it was reduced to 2/s, so that the pattern of frame-to-frame filament centroid movement could better be seen. At pCa 5 (Fig. 5 *B-II*) each of the eight filaments exhibits uniform movement because the distance between successive tick marks is relatively constant. By contrast, filament movement at pCa 7 is rather erratic. Filaments A, H, and J show uniform movement in that successive tick marks are uniformly spaced (a distance roughly similar to that in Fig. 5 *B-II*, indicating that at pCa 7 the velocity is roughly half that at pCa 5). On the other hand, filaments B, C–G, I, and K have very erratic paths, with periods of uniform movement interspersed with little or no movement. These data, with those in Figs. 2 and 3, show that both the fraction of moving filaments and filament sliding speed are dependent on pCa. Furthermore, the fact that at pCa near 9, large numbers of filaments are bound to the surface indicates that "weakly attached" cross-bridges can bind filaments to the surface. Fig. 6 contains Gaussian fits describing the number of filaments sliding at a different mean speeds as a function of the pCa. It might be that the reduced velocities observed at high pCa were a consequence of alternating periods of "stop and go" sliding. If so, there would be a significant broadening of the standard deviation and the appearance of slower moving subpopulations for given means. As pCa is increased beyond 5, mean filament sliding speed declines, the number of moving filaments decreases markedly, but no subpopulations of slower moving filaments appear. A similar histogram analysis of the frame-by-frame speeds at pCa 6 and 7 show that the fall in speed is not caused by filaments moving smoothly at high velocity for a period of time and then ceasing to move for increasing periods of time as pCa rises. We have also increased the frame grabbing rate to 30/s and find no evi-

TABLE 3 Summary of calcium sensitivity of regulated thin filaments

Protein	Ionic strength (mM)	Filament sliding speed			Fraction uniformly moving		
		S_{fmax} (μm/s)	Hill Coef.	pCa_{50}	Max.	Hill Coef.	pCa_{50}
NTF‡-12/94*	50	3.7 ± 0.4	0.9 ± 0.2	6.5 ± 0.2	0.91 ± 0.04	4.5 ± 1.3	6.2 ± 0.03
CRTF§-7/95	31	1.3 ± 0.1	0.6 ± 0.2	6.5 ± 0.2	0.84 ± 0.03	2.9 ± 0.9	6.6 ± 0.1
CRTF-2/95	50	4.0 ± 0.7	1.2 ± 0.7	6.6 ± 0.2	0.88 ± 0.03	5.6 ± 0.9	6.2 ± 0.03
CRTF¶-12/94	50	5.8 ± 0.6	0.7 ± 0.3	7.1 ± 0.2	0.95 ± 0.1	1.3 ± 0.4	6.9 ± 0.1
CRTF-3/95	50	6.9 ± 0.9	0.7 ± 0.4	6.9 ± 0.2	0.97 ± 0.04	3.9 ± 2.4	6.9 ± 0.06
CRTF‖-3/95	100	7.4 ± 0.7	1.2 ± 0.4	6.8 ± 0.2	0.89 ± 0.01	4.1 ± 0.6	7.1 ± 0.02

* Month/year of filament preparation and measurement.
* Data given as mean ± SD for fitted values.
‡ Data from Fig. 2.
§ Data from Fig. 7.
¶ Data from Fig. 3.
‖ Data from Fig. 4.

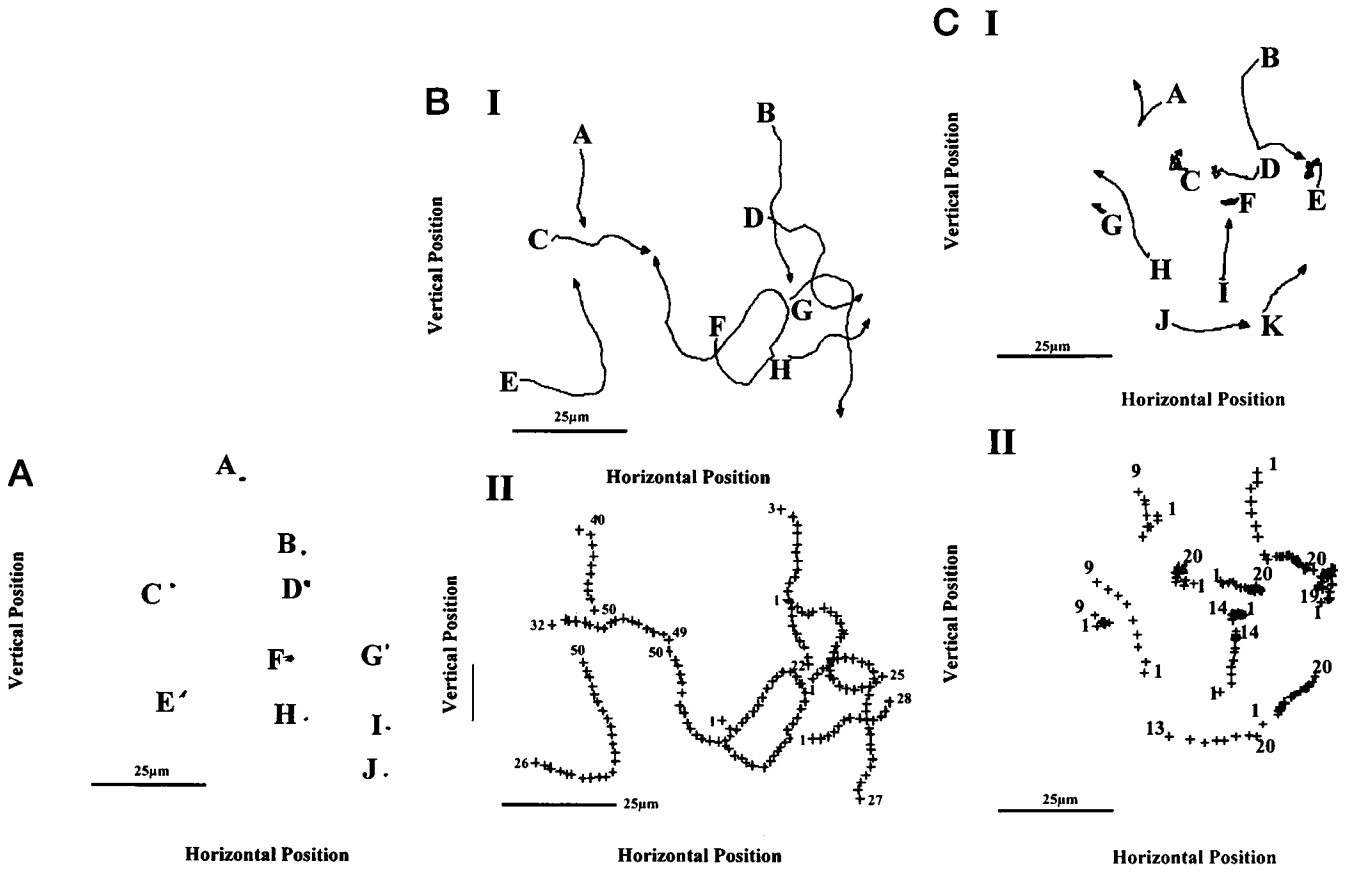

FIGURE 5 Paths followed by filaments moving at pCa 9 (*A*), pCa 5 (*B-I* and *B-II*), and pCa 7 (*C-I* and *C-II*). In the panels labeled *I* (*B-I* and *C-I*), the path is traced for filaments designated by letters. Panels *II* (*B* and *C*) show, by the tic marks, the frame-to-frame centroid position of each filament as the time filament motion was recorded for speed computations. In *B*, data were acquired at 5 frames/s, and in *C* data were acquired at 2 frames/s.

dence of gross "stop-and-go" filament speeds. Thus the change in velocity is not an artifact of the method of analysis. This conclusion does not preclude the possibility that there is microscopic stop-and-go behavior beyond the limit of our resolution.

The relation between sliding speed and thin filament ATPase

Tobacman's group has measured the rate of actomyosin ATPase activity as a function of pCa in reconstituted cardiac thin filaments in several papers (Tobacman and Sawyer, 1990; Tobacman and Lee, 1987). The results of these studies showed that the actomyosin ATPase rate had a pCa_{50} of 5.7–6.0 and a Hill coefficient of 1.7–2 at 25°C, pH 7.06, and ionic strength of 31 mM. Our results to this point have been obtained at a higher ionic strength (50–100 mM) and pH (7.4). To determine the extent to which the measured ATPase corresponded to the filament sliding speed, we measured filament sliding velocity under conditions essentially identical to those used in previous ATPase studies (Tobacman and Sawyer, 1990). The results in Fig. 7 show three significant features. First, the sliding speed is substantially reduced at lower pH and reduced ionic strength, two factors that have previously been shown to depress filament sliding speed (Homsher et al., 1992). Second, both the fraction of filaments moving and filament sliding speed exhibit a pattern similar to that seen in studies at higher pH and ionic strength (a pCa_{50} of 6.5–6.9 and a higher Hill coefficient (2.2) for the fraction moving than that for filament sliding speed). Thus S_f has little correlation with the actomyosin ATPase-pCa curve. The relationship of fraction of filaments sliding smoothly to pCa is shifted to the left by about 1 pCa unit compared to the actomyosin ATPase-pCa curve. The data thus suggest that the filaments will be sliding at a nearly maximum speed when the observed ATPase is less than 20% of maximum.

Table 3 contains a summary of the variation of velocity and fraction of moving filaments as a function of pCa at various ionic strengths and preparations. The number of filaments analyzed for each of these preparations was greater than 1273. The results are qualitatively consistent with each other.

Calcium dependence of filament sliding speed

The reduction in filament sliding speeds at very low calcium concentrations in an apparently calcium-dependent fashion

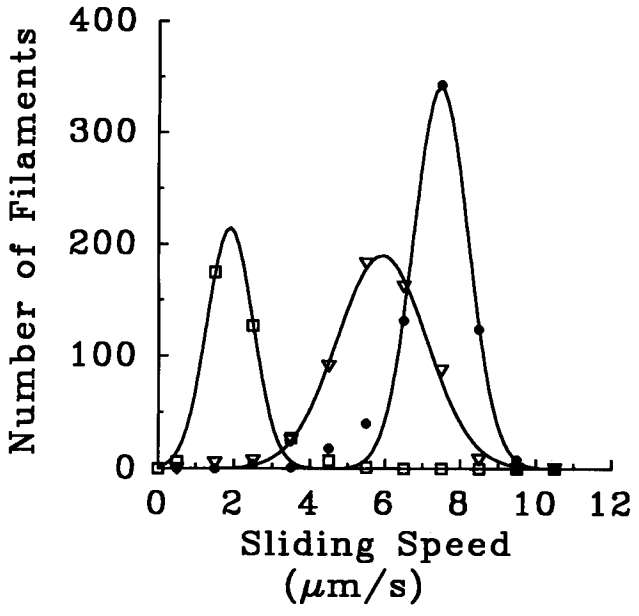

FIGURE 6 Histogram of filaments moving at specific speeds during motility assays at pCa 5 (●), pCa 6 (▽), and pCa 7 (□). The solid lines are least-squares fits to Gaussian distribution for the data. The Gaussian fits (mean, number of observations, and SD) were not significantly different from the means and SD computed from the filament velocity.

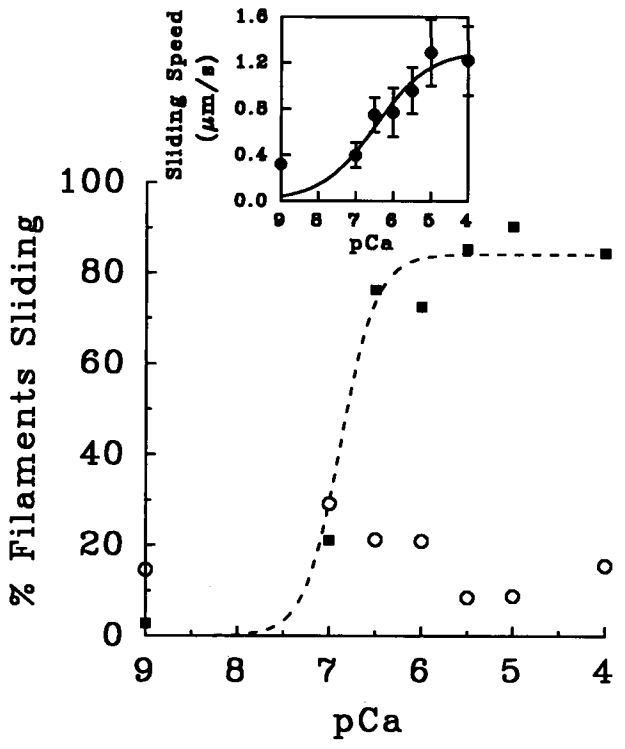

FIGURE 7 S_f (●) and fractions of reconstituted thin filaments at an ionic strength of 31 mM moving at uniform velocity (■) and moving erratically (○) at various pCa. Least-square fits to the Hill equation for S_f gave the maximum S_f as 1.3 ± 0.65 μm/s, a Hill coefficient of 0.6 ± 0.2, and a pCa_{50} of 6.5 ± 0.2; comparable values for the percentage of filaments uniformly moving were 84%, Hill coefficient of 2.9 ± 0.9, and pCa_{50} of 6.6 ± 0.1. The sliding speed at pCa 9 is the mean from four smoothly moving filaments of 137 observed. The number of filaments observed at pCa 9, 7, 6.5, 6, 65.6, and 4 were, respectively, 137, 137, 325, 153, 199, 225, and 97.

is not predicted by the notion of cross-bridge recruitment and seems better fit by the idea of kinetic regulation of the cross-bridge cycle (Julian, 1971; Brenner, 1988). There are at least four hypotheses that could account for S_f's dependence on pCa. First, it may be that weakly bound cross-bridges contribute to a drag force, slowing filament movement. As $[Ca^{+2}]$ declines, fewer cross-bridges are strongly attached and pulling on the thin filaments, and the number of weakly attached cross-bridges increases. Thus at low calcium concentrations the "drag" force of weakly attached cross-bridges might constitute a retarding force, opposing force-generating cross-bridges pulling the thin filaments axially (the "weak binding drag" hypothesis). A second possibility is that at low calcium concentrations so few cross-bridges attach and pull on the thin filament that the filament simply cannot move at the maximum rate. This idea assumes a modest average cross-bridge throw (~10–20 nm) and a short duty cycle (the fractional time during which a cross-bridge is attached to and pulling on the thin filament per ATP split [2–10 ms/ATP split]). If a single cross-bridge can attach and pull on the thin filament 25 times per second, then one cross-bridge will move the filament at 250–500 nm/s; two cross-bridges acting independently would propel the filament at twice this velocity (500–1000 nm/s), three at ~750–1500 nm/s, and so on. This hypothesis might be called the "limited cross-bridge number" hypothesis and can be described by equations derived by Uyeda et al. (1990). A third possibility is that the cross-bridge cycle rate (the number of times per second an S-1 molecule can attach, pull, and detach from a thin filament) is regulated by $[Ca^{+2}]$. According to this "cross-bridge cycle rate" hypothesis, the reduction in S_f is a reflection of calcium control of the cross-bridge cycling rate, not the number of cross-bridges interacting with the thin filaments. The fourth possibility is that the regulatory proteins bound to the thin filament may interact with proteins bound to the nitrocellulose surface in a fashion that provides a "drag" or "load" against which the cycling cross-bridges must work. This hypothesis might be called the "regulatory protein drag" hypothesis.

These hypotheses can be experimentally distinguished from each other. If the nitrocellulose-covered motility surface were coated with HMM at a concentration 10 times less than that used in the previous studies, the four hypotheses described above would make the following predictions about the change in the pCa-S_f curve. According to the "weak binding drag" hypothesis, both the number of weak binding (retarding) and pulling (propelling) cross-bridges will be reduced by the same extent, so that the pCa-S_f curve would not be affected by the reduction in cross-bridge density. The "cross-bridge cycling rate" hypothesis also predicts no change in the sliding speed-pCa curve if it is only the cross-bridge cycling rate that is regulated by the

pCa. The "limited cross-bridge number" hypothesis predicts that the pCa-S_f curve will be shifted to the right at the reduced HMM concentration because as the pCa rises, the point at which the number of cross-bridges needed to pull the filaments at a specific velocity would be reached is at a higher [Ca^{+2}] or lower pCa. This assumes that the reduction in HMM surface density is not sufficient to produce a fall in the maximum velocity (Uyeda et al. (1990) find no change in the maximum velocity for filaments >1 μm in length until the [HMM] is reduced to less than 20 μg/ml). The "regulatory protein drag" hypothesis also predicts a rightward shift in the pCa-S_f curve and possibly a reduction in the sliding speed at low pCa. These hypotheses were tested by measuring the fraction and rate of thin filaments sliding on a surface equilibrated with HMM at 300 μg/ml and 30 μg/ml. The results of these experiments are shown in Figs. 8 and 9. In Fig. 8 the fraction of filaments moving is plotted as a function of the pCa. At a pCa of 5 the fraction of filaments moving on the surface is same in the two differently coated surfaces. However, as the pCa is progressively raised, the number of moving filaments on the less densely covered surface declines, reaching 50% at a pCa of 6.5, whereas that of the more densely covered surface required a rise of pCa of 7.2 to achieve a comparable decline. A similar, although less pronounced behavior is seen in the plots of filament sliding speeds (Fig. 9); i.e., the maximum sliding speed is unaffected by the dilution of the HMM surface, but the pCa-S_f curve is shifted to the right by about 0.2 pCa units on the surfaces containing the reduced density of HMM. Such behavior is consistent with the "limited cross-bridge number" hypothesis. This result is similar to a cross-bridge "recruitment" hypothesis for the regulation of muscle contraction. The fact that the fraction of moving filaments shows a reduced Hill coefficient and weaker pCa_{50} when the HMM surface concentration is reduced may suggest that strongly bound cross-bridges play a role in turning on the thin filaments.

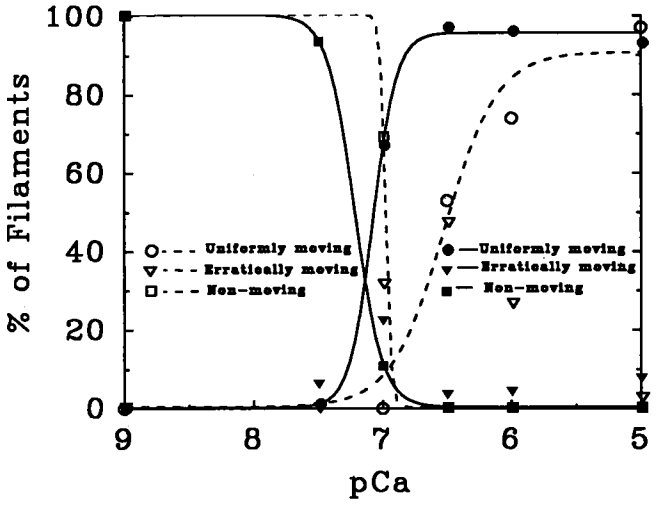

FIGURE 8 Fraction of filaments moving (uniformly, not moving, and erratically moving) at 100 mM ionic strength, 300 μg HMM/ml (●, ▼, ■) or 30 μg/mol (○, ▽, □). On decreasing the HMM from 300 to 30 μg/ml, the fraction of smoothly moving filaments as a function of the pCa (from least-squares fits to the data) declined from 95 ± 1 to 90 ± 8, the Hill coefficient from 4.8 ± 1.5 to 2.2 ± 0.9, and the pCa_{50} from 7.1 ± 0.02 to 6.5 ± 0.1 (means ± SD).

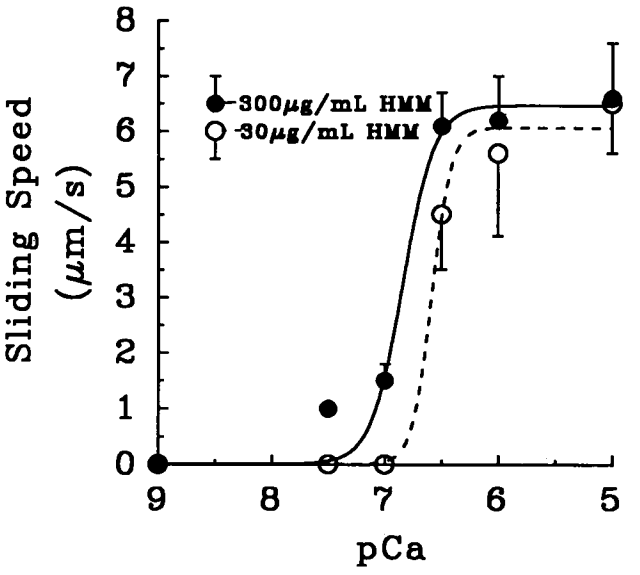

FIGURE 9 Speed of filament movement at ionic strength of 100 mM, 300 μg HMM/ml, or 30 μg/ml. On decreasing the HMM from 300 to 30 μg/ml, the fitted maximum filament speed increased from = 6.4 ± 0.40 to 6.5 ± 0.4 μm/s, the Hill coefficient changed from 1.5 ± 0.7 to 1.3 ± 0.3, and the pCa_{50} changed from 6.85 ± 0.08 to 6.7 ± 0.08 (means ± SD).

DISCUSSION

Four primary observations made in these experiments were:

1. Exogenous Tm/Tn (at 100 nM) must be added to the motility assay buffer to secure complete calcium regulation.
2. The fraction of regulated filaments moving is a highly calcium-dependent aspect of thin filament movement. The fraction of filaments moving uniformly decreases from >90% at pCa 5 and 6 to near zero within 0.5–1.0 pCa unit.
3. S_f is a function of pCa, and the dependency is noncooperative (Hill coefficient = 1.0).
4. Thin filament motion still occurs under conditions in which the calcium occupancy of the TnC regulatory sites is relatively low (Tobacman and Sawyer, 1990).

Fraser and Marston (1995) have varied the relative concentrations of actin, Tm, and Tn to examine their effects on calcium regulation of S_f. They found that a mixture of actin:Tm:Tn (100 nM:100 nM:50 nM) yielded clear-cut thin filament calcium regulation when the filaments were added to the motility assay at 1/10 of their original composition. They also showed in centrifugation studies that about 85% of the regulatory proteins remained bound to the thin filament pellet even after dilution. However, the fact that considerable thin filament motility remained in Fraser and Marston's (1995) thin filaments and in our native thin filament and reconstituted thin filament preparations in the

absence of exogenous free regulatory proteins suggests that it is likely that the regulatory proteins dissociate from the thin filaments at low concentrations existing in the motility chamber during the washing and assay solutions. That regulatory proteins are still functional in our preparations is shown by the presence of regulatory thin filaments attached to HMM-coated slides, which exhibited incomplete regulation in the absence of added regulatory proteins. However, to obtain complete regulation we found it necessary to provide Tm and Tn in the motility solution at 100 nM to optimize the likelihood of regulatory protein binding to the thin filaments bound to the slide. Studies of Tm/Tn binding to actin (Dahiya et al., 1994) indicate that 100–200 nM Tm/Tn is required to saturate the thin filament with the regulatory proteins, at least under the high ionic strength (0.3 M KCl) conditions of these earlier experiments. The observed binding was very cooperative, and little Tm/Tn was bound when the concentration was 10 nM, as evidenced by the incomplete regulation. Other work indicates that the affinity of Tm/Tn for actin increases as the ionic strength is lowered (Butters et al., 1993), so it could be anticipated that the present experiments, performed at a much lower ionic strength, would require a Tm/Tn concentration of less than 200 nM. The suppression of all movement at pCa 9 in 100 nM Tm/Tn thus becomes a functional test of the adequacy of the exogenous protein regulation of the thin filaments.

In these studies the fraction of smoothly moving filaments is dependent on the calcium concentration, an observation also made by Fraser and Marston (1995) in regulated skeletal filaments but not previously noted by others (Honda and Asakura, 1989; Harada et al., 1990; Sata et al., 1995). If the 18% of the filaments moving at pCa 9 in Fraser and Marston's work (1995) are taken as unregulated and moving at all pCa, then the fraction of uniformly moving filaments can be fitted to a Hill plot with a Hill coefficient of 1.2, a pCa_{50} of 6.0, and a maximum fraction of moving filaments of 76%. These data differ from the results presented here in that the average Hill coefficient observed above was 4.1 and the average pCa_{50} was 6.7 for the results in Figs. 2, 3, 6, and 7. This behavior is more in line with the reports of Honda and Asakura (1989) and Harada et al. (1990), who reported steep relationships between the filament sliding speed and pCa (if filaments are moving at maximum speed at pCa 6.0 and not moving at all at 6.5, the number of moving filaments must subject to substantial cooperativity). This behavior stands in contrast to the more gradual decline in acto-S1 or acto-HMM ATPase rates, which decline from maximum values from pCa 4.5 to near zero at 6.5 (Tobacman and Lee, 1987). This result suggests, as would be predicted from Huxley's view of unloaded shortening velocity (1957), that the unloaded shortening velocity and hence the number of filaments sliding in our assay would be relatively independent of the number of cross-bridges attached and cycling. This result is not a consequence of our "definition" of a uniformly moving filament as that having a ratio of SD/mean of <0.5, for two reasons. First, if the definition of uniformly moving is relaxed to include filaments whose SD/mean is <2, the same basic observation about the fraction of smoothly moving filaments and the pCa is made. Second, that more than 80% of the filaments in our assay systems move at a uniform speed at $pCa < 6$ implies that the preparation itself is uniform and little affected by normalization idiosyncrasies.

The variation of the filament sliding velocity with pCa has not been observed by others, aside from Fraser and Marston (1995), who attribute the variation in filament velocity to a "separate Ca^{+2} dependent process," i.e., a process separate from the control of the number of filaments moving. It may be that their observation of a variation of filament velocity was made possible by the fact that they objectified the filament speed analysis by selecting, before the fact, the specific filaments whose movement would be analyzed and used only those sections of the recordings that showed continuous movement. This procedure is akin to the methods used in this work. However, the difference in our observations and those of Honda and Asakura (1989), Harada et al. (1990), and Sata et al. (1995) is more fundamental than a simple difference in data analysis. The slowing of filament sliding speed at higher pCa is obvious without analysis and is clearly identified in Fig. 5. The reason that this variation in sliding speed was not noted by others may be a consequence of the ionic strength or not including exogenous Tm/Tn used in the various assays using reconstituted regulated thin filaments. In Table 3 we summarize our experience with the regulation of filament sliding velocity at various ionic strengths. One could speculate that at lower ionic strengths, such as 50 mM or less, the number of filaments moving decreases steeply with pCa (average Hill coefficient = 3.6), and at a pCa at which the number of filaments moving is large (pCa 6), the filament sliding speed is nearly maximum (see Figs. 4 and 9). However, when the filament sliding speed does drop significantly, such as at pCa 6.5, the fraction of filaments moving uniformly has dropped to 3–12%, so one could conclude that the filaments that were still moving were only partially regulated or were subject to diffusional movement. Only when these filaments are tracked carefully does one find that they are moving at a slower but uniform speed. At higher ionic strength (100 mM; Fig. 8), this change is clearly seen, because at a pCa at which significant numbers of filaments are still moving (pCa 7; >60%), the sliding velocity has declined to 30–50% of that seen under full calcium activation. The calcium dependence of S_f is also seen in NTF at low ionic strength, although in this case the data were more scattered and the number of erratically moving filaments was rather high (20–40%) at intermediate pCa. The experiments at reduced cross-bridge head concentrations suggest that the reduction in sliding velocity is a consequence of a reduction in the number of cross-bridge heads per unit thin filament length needed to produce maximum sliding velocity as opposed to an actual modulation of the cross-bridge cycling per se. Fig. 9 also raises the possibility of a cross-bridge-mediated cooperativity in the regulation of the number of thin filaments moving, by virtue of the reduction of the Hill coefficient from 4.76 at higher

HMM density as compared to the Hill coefficient of 2.15 at the lower head density.

Finally, the pCa_{50} for both the number of moving filaments and filament velocity averages 6.7 for all of the data. The data previously published regarding the effect of calcium on ATPase rate and pCa indicated a K_{app} of 5.4, a difference of 1.3 pCa units (Tobacman and Sawyer, 1990). A similar difference is seen in force-pCa curves of bovine ventricular tissue in which the pCa50 is 5.35 (Barth et al., 1995). These differences can be explained by our interpretation of the variation of filament sliding velocity with pCa. A 1-μm length of actin filament contains ~360 g-actin monomers with which the S-1 heads could interact. If one assumes that the cross-bridge throw (d) is 10 nm (Uyeda et al., 1990), that the cross-bridge stays attached during the power stroke for 2 ms (t_s), and the maximum ATPase rate at 25°C is 25 s^{-1} (k_{cat}) per S-1 (White and Taylor, 1976), then the equation relating the S_f to the number of attached cross-bridges (N) (Uyeda et al., 1990) is

$$S_f = d/t_s \times (1 - (1 - t_s \times k_{cat})^N)$$

Solution of this equation for differing numbers of cross-bridges attached to the thin filament shows that the speed is 0.95 of the maximum value (5 μm/s for this case) when 58 actin monomers (17% of those/μm thin filament) are occupied by the S-1, is 0.5 when occupied by 14 cross-bridges (4% of those actins/μm of actin), and is 0.25 when occupied by six cross-bridges (2% of the actins/μm of thin filament). This equation for S_f predicts that relatively few cross-bridges are needed to propel the filament at nearly maximum velocities. This stands in contrast to the fact that the ATPase rate and isometric force would be directly proportional to the number of S-1 cross-bridge heads free to interact with actin. Thus one expects that the variation of velocity with calcium would begin to decline at pCa greater than that seen when measuring the ATPase rate. Indeed, using a Hill coefficient for the binding of calcium to regulated thin filaments of 1.16 and a K_{app} of 4×10^5 M^{-1} (Tobacman and Sawyer, 1990), the relationship between S_f and the number of actin monomers activated (N, taken as proportional to the fraction of Tn binding calcium), one can calculate (using the equation relating S_f, d, t_s, k_{cat}, and N) that the observed relationship between S_f and pCa would be one with a Hill coefficient of 1.26 and a pCa_{50} of 7.05, values similar to those observed in these experiments. An important model of regulation has been proposed by McKillop and Geeves (1993), suggesting that a local region of the thin filament can be in one of three states (block, opened, or closed), that Ca^{2+} and myosin influence the transitions between these states, and that the open state is required for force production. Our results have not included measurements of myosin or Ca^{2+} binding to the thin filament and thus do not provide a test of this model.

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REFERENCES

Barth, Z., J. Strauss, S. Heyder, J. Van Eyk, R. Wiesner, and J. Ruegg. 1995. Ca^{2+} sensitizing effects of EMD 53998 after troponin replacement in skinned fibers from porcine atria and ventricles. *Pflugers Arch-Eur. J. Physiol.* 430:220–229.

Brenner, B. 1988. Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. *Proc. Natl. Acad. Sci. USA.* 85:3265–3269.

Butters, C., K. Willadsen, and L. S. Tobacman. 1993. Cooperative interactions between adjacent roponin-tropomyosin complexes may be transmitted through the actin filament. *J. Biol. Chem.* 268:15565–15570.

Dahiya, R., C. Butters, and L. Tobacman. 1994. Equilibrium linkage analysis of cardiac thin filament assembly. *J. Biol. Chem.* 269: 29457–29461.

Fabiato, A., and F. Fabiato. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle fibers. *J. Physiol. (Paris).* 75: 463–505.

Fraser, I., and S. Marston. 1995. In vitro motility analysis of actin-tropomyosin regulation by troponin and calcium. *J. Biol. Chem.* 270: 7836–7841.

Gordon, A., A. F. Huxley, and F. Julian. 1966. Tension development in highly stretched vertebrate muscle fibers. *J. Physiol.* 184:143–169.

Harada, Y., K. Sakurada, T. Aoki, D. Thomas, and T. Yanagida. 1990. Mechanochemical coupling in actomyosin energy transduction studies by in vitro movement assay. *J. Mol. Biol.* 216:49–68.

Haselgrove, J. 1972. X-ray evidence for a conformational change in the actin containing filaments of vertebrate striated muscle. *Cold Spring Harb. Symp. Quant. Biol.* 37:341–352.

Hofmann, P., M. Greaser, and R. Moss. 1991. C-protein limits shortening velocity of rabbit skeletal muscle fibers at low levels of calcium activation. *J. Physiol.* 349:701–715.

Homsher, E., F. Wang, and J. Sellers. 1992. Factors affecting movement of f-actin filaments propelled by skeletal muscle heavy meromyosins. *Am. J. Physiol.* 262:C714–C723.

Honda, H., and S. Asakura. 1989. Calcium triggered movement of regulated actin in vitro. *J. Mol. Biol.* 205:677–683.

Huxley, A. F. 1957. Muscle structure and theories of contraction. *Prog. Biophys. Biophys. Chem.* 7:255–318.

Huxley, H. E. 1972. Structural change in the actin and myosin containing filaments during contraction. *Cold Spring Harbor Symp. Quant. Biol.* 37:361–376.

Julian, F. 1971. The effect of calcium on the force-velocity relation of briefly glycerinated frog muscle fibers. *J. Physiol.* 218:117–145.

Julian, F., and R. Moss. 1981. Effects of calcium and ionic strength on shortening velocity and tension development in frog skinned muscle fibers. *J. Physiol.* 311:179–199.

Kron, S., and J. Spudich. 1986. Fluorescent actin filaments move on myosin fixed to a glass surface. *Proc. Natl. Acad. Sci. USA.* 83: 6272–6276.

Kron, S., Y. Toyoshima, T. Ueyda, and J. Spudich. 1991. Assays for actin sliding movement over myosin-coated surfaces. *Methods Enzymol.* 197: 403–414.

Margossian, S., and S. Lowey. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85:55–71.

McKillop, D., and M. Geeves. 1993. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys. J.* 65:693–701.

Moss, R. L. 1991. Ca^{2+} regulation of mechanical properties of striated muscle. *Circ. Res.* 70:865–884.

Pardee, J., and J. Spudich. 1982. Purification of muscle actin. *Methods Cell Biol.* 24:271–289.

Podolin, R., and L. Ford. 1986. Influence of partial activation on force-velocity properties of frog skinned muscle fibers in millimolar magnesium ion. *J. Gen. Physiol.* 87:607–631.

Sata, M., S. Suguira, H. Yamashita, H. Fujita, S. Momomura, and T. Serizawa. 1995. MCI-154 increases Ca^{2+} sensitivity of reconstituted thin filament. *Circ. Res.* 76:626–633.

Stein, L., R. Schwarz, B. Chock, and E. Eisenberg. 1979. Mechanism of actomyosin adenosine triphosphatase. *Biochemistry.* 18:3895–3909.

Thames, M., L. Teichholz, and R. Podolsky. 1974. Ionic strength and the contraction kinetics of skinned muscle fibers. *J. Gen. Physiol.* 63:509–530.

Tobacman, L., and R. Adelstein. 1986. Mechanism of regulation of cardiac actin-myosin subfragment 1 interaction by troponin-tropomyosin. *Biochemistry.* 25:798–802.

Tobacman, L., and R. Lee. 1987. Isolation and functional comparison of bovine cardiac troponin T isoforms. *J. Biol. Chem.* 262:4059–4064.

Tobacman, L., and D. Sawyer. 1990. Calcium binds cooperatively to the regulatory sites of the cardiac thin filament. *J. Biol. Chem.* 265:931–939.

Umemoto, S., and J. Sellers. 1990. Characterization of in vitro motility assays using smooth muscle and cytoplasmic myosins. *J. Biol. Chem.* 265:14864–14869.

Uyeda, T., S. Kron, and J. Spudich. 1990. Myosin step size. *J. Mol. Biol.* 214:699–710.

Wagner, P., and A. Weeds. 1979. Determination of the association of myosin subfragment 1 with actin in the presence of ATP. *Biochemistry.* 18:2260–2266.

White, H., and E. Taylor. 1976. Energetics and mechanism of actomyosin adensoine triophosphatase. *Biochemistry.* 15:5818–5826.