Calcium Regulation of Skeletal Muscle Thin Filament Motility In Vitro

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ABSTRACT Using an in vitro motility assay, we have investigated Ca^{2+} regulation of individual, regulated thin filaments reconstituted from rabbit fast skeletal actin, troponin, and tropomyosin. Rhodamine-phalloidin labeling was used to visualize the filaments by epifluorescence, and assays were conducted at 30°C and at ionic strengths near the physiological range. Regulated thin filaments exhibited well-regulated behavior when tropomyosin and troponin were added to the motility solutions because there was no directed motion in the absence of Ca^{2+}. Unlike F-actin, the speed increased in a graded manner with increasing $[Ca^{2+}]$, whereas the number of regulated thin filaments moving was more steeply regulated. With increased ionic strength, Ca^{2+} sensitivity of both the number of filaments moving and their speed was shifted toward higher $[Ca^{2+}]$ and was steepest at the highest ionic strength studied (0.14 M $\Gamma/2$). Methylcellulose concentration (0.4% versus 0.7%) had no effect on the Ca^{2+} dependence of speed or number of filaments moving. These conclusions hold for five different methods used to analyze the data, indicating that the conclusions are robust. The force-pCa relationship ($pCa = -\log_{10}[Ca^{2+}]$) for rabbit psoas skinned fibers taken under similar conditions of temperature and solution composition (0.14 M $\Gamma/2$) paralleled the speed-pCa relationship for the regulated filaments in the in vitro motility assay. Comparison of motility results with the force-pCa relationship in fibers suggests that relatively few cross-bridges are needed to make filaments move, but many have to be cycling to make the regulated filament move at maximum speed.

INTRODUCTION

Calcium regulates contraction in striated muscle through its binding to troponin-C (TnC) of the troponin-tropomyosin (Tn-Tm) complex on the thin filament (Ebashi and Endo, 1968; Grabarek et al., 1992; Tobacman, 1996). Molecular structural information suggests that calcium binding causes a conformational change in TnC, which in turn alters the position of Tm on the thin filament and thereby modulates the interaction between actin and myosin (Lehman et al., 1994). This results in calcium activation of ATPase activity and force and/or shortening in skinned fibers and intact muscle cells (Ebashi and Endo, 1968). It is not clear what aspects of the actin-myosin interaction are regulated by calcium at the molecular level to control contraction.

The in vitro motility assay is a powerful tool for bridging the gap in knowledge between the molecular structures of contractile proteins and filaments and the cellular physiology of muscle (Harris and Warshaw, 1993; Homsher et al., 1992; Kron et al., 1991; Sellers et al., 1993; Uyeda et al., 1990; Warrick et al., 1993). This assay allows study of the function of small numbers of purified proteins. It also makes it possible to test and compare specific isoforms or mutant proteins. The in vitro motility assay brings the same benefits to the study of regulation.

Calcium has been shown to regulate the in vitro sliding speed of muscle thin filaments reconstituted from F-actin and Tm-Tn, but there is disagreement on important aspects of this regulation. Honda and Asakura (1989), Harada et al. (1990), and Sata et al. (1995a,b, 1996) found that control is in an on-off manner, with speed being zero below a threshold pCa ($pCa = -\log_{10}[Ca^{2+}]$), switching to a maximum above this threshold (within 0.1–0.2 pCa units). This on-off regulation implies that either the entire thin filament activates as a unit (Brandt et al., 1984, 1987) or that the thin filament speed is independent of the level of activation (Huxley, 1957). In contrast, others (Fraser and Marston, 1995; Homsher et al., 1996; Kellermayer and Granzier, 1996) found that filament speed is a continuous function of $[Ca^{2+}]$. Graded activation implies a different mechanism in which speed is limited by the load on the filaments (Metzger and Moss, 1988; Moss, 1986; Moss et al., 1995), cross-bridge duty cycle (Harris and Warshaw, 1993; Uyeda et al., 1990), or direct Ca^{2+} control of cross-bridge cycling (Brenner, 1988). Furthermore, there are differences in whether filaments slide in the absence of $[Ca^{2+}]$, with some investigators (Harada et al., 1990; Homsher et al., 1996; Honda and Asakura, 1989; Sata et al., 1995a,b, 1996) finding no directed motion in the absence of $[Ca^{2+}]$, and others (Fraser and Marston, 1995; Kellermayer and Granzier, 1996) finding motion, even in the absence of $[Ca^{2+}]$.

These contrasting results could be due to differences in data analysis, in specific protein isoforms, or in experimental conditions such as surface preparation, temperature, and ionic strength. There were variations in the regulatory proteins used (cardiac or skeletal), but there was no correlation between the Tm-Tn isoform and whether regulation was graded or on-off. Differences in ionic strength appeared to be more important, as all studies showing on-off regulation were done at very low ionic strength (less than 50 mM), whereas those showing graded activation were all at some-

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what higher ionic strength, but still much below the physiological range. Ionic strength is an important variable because it affects Ca^{2+} binding to TnC through ionic screening. Furthermore, ionic strength modulates protein-protein interactions, including regulatory protein interactions with F-actin and F-actin interactions with myosin. In fact, at low ionic strength, activation can even occur in the absence of Ca^{2+} (Gordon et al., 1973; Gulati and Podolsky, 1981; Head et al., 1995).

Thus this study was undertaken to better understand the control of individual, regulated skeletal muscle thin filaments over a broad range of $[Ca^{2+}]$ and to understand how $[Ca^{2+}]$ regulates both the number of filaments moving and their speed at ionic strengths near the physiological range. To gain further insight into Ca^{2+} regulation of filament motion under steady-state conditions, the results of in vitro motility assays (0.14 M Γ/2) were additionally compared to force-pCa data obtained with skinned fibers under conditions comparable to the motility experiments. Preliminary results from these experiments have been described in abstract form (Gordon et al., 1996).

MATERIALS AND METHODS

Protein preparations

Myosin and heavy meromyosin

Myosin was prepared from rabbit back muscles according to the methods of Homsher et al. (1996), Margossian and Lowey (1982), Pan et al. (1989), and Regnier et al. (1996) and either used immediately or stored at −20°C in 50% glycerol for up to 7 weeks (typically up to 3 weeks). Heavy meromyosin (HMM) was prepared by chymotryptic digestion of myosin as described by Kron et al. (1991) and was stored on ice for up to 5 days. The MW and extinction coefficients used to assay myosin concentration were 520,000 and 0.53 cm^{-1} at 280 nm; the corresponding values for HMM were 350,000 and 0.6 cm^{-1} at 280 nm. For the five preparations of myosin used in these experiments, K-EDTA and Ca^{2+} ATPases (Margossian and Lowey, 1982) were 17.4 ± 1.6 and 4.0 ± 0.6 s^{-1} per S1 (mean ± SD; N = 5), respectively. For 5 preparations of HMM out of a total of 11 preparations of HMM used (all were made from this myosin), K-EDTA and Ca^{2+} ATPases were measured and were 12.6 ± 1.9 and 3.6 ± 0.5 s^{-1} per S1 (mean ± SD; N = 5), respectively. Myosin, HMM, and all other protein preparations were also examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1).

Each experimental day, ATP-insensitive heads were removed from an aliquot of HMM as described by Kron et al. (1991). F-actin was added (1.5-fold molar excess of actin monomers over S1 heads) to HMM, followed by 1 mM ATP and centrifugation to sediment actin and irreversibly bound HMM. Centrifugation at 4°C was either in an airfuge (Beckman model AF-IM-5) at 180,000 × g or in a tabletop ultracentrifuge (Beckman model TL-120.2 or TL-100) at 541,000–672,000 × g.

Actin and rhodamine-phalloidin F-actin filaments

F-actin was prepared from rabbit back and leg muscle ether powder (Potter, 1982) by the procedure of Pardee and Spudich (1982) and was stored for up to 4 weeks on ice. After 2 weeks, F-actin was rejuvenated by recycling, which consisted of depolymerization and then repolymerization (Kron et al., 1991). The MW and extinction coefficient used for actin were 42,000 and 0.62 cm^{-1} at 290 nm. For visualization by fluorescence microscopy, F-actin was labeled with rhodamine-phalloidin (RhPh) (Molecular Probes, Eugene, OR) according to the method of Kron et al. (1991) and maintained in a low-light environment until used. Before infusion into motility flow cells, Rh-Ph F-actin was diluted 1:100 in actin buffer (AB) (25 mM KCl, 25 mM imidazole, 4 mM $MgCl_2$ 1 mM EGTA, 1 mM dithiothreitol (DTT), pH 7.4) (Kron et al., 1991).

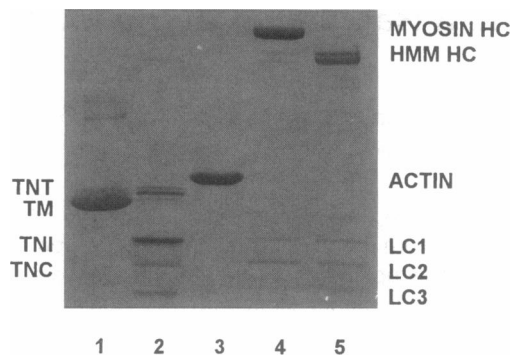

FIGURE 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of rabbit skeletal muscle tropomyosin (Tm; *lane 1*), troponin (TnT, TnI, and TnC; *lane 2*), actin (*lane 3*), myosin (*lane 4*), and HMM (*lane 5*). The gel is a 4–20% gradient minigel (Novex, San Diego, CA) and was stained with Coomassie blue.

Tropomyosin

Tm was made from rabbit muscle ether powder, as described by Smillie (1982). The MW and extinction coefficient used for Tm were 68,000 and 0.29 cm^{-1} at 276 nm. After purification, Tm was aliquoted, freeze-dried, and stored at −20°C.

Troponin

The method of Potter (1982) was used to make Tn from rabbit muscle ether powder. The MW and extinction coefficient used for troponin were 70,000 and 0.45 cm^{-1} at 276 nm. In most preparations, Tn was stored on ice for up to 1 week; however, 3 of 14 preparations were frozen before use. In most preparations (10 of 14), the final DEAE-52 Sephadex chromatography step was omitted, as it rendered Tn less stable (i.e., the amount of Tn necessary to obtain complete Ca^{2+} regulation, as described in Results, increased each day after the chromatography procedure) and led to filament bundling. All of the stable preparations of Tn used in this study met the criteria for maintaining well-regulated motility when incorporated into regulated thin filaments (see Results), and thus all of the data were combined for analysis.

Regulated rhodamine-phalloidin-labeled F-actin

Regulated actin filaments were made by incubating Rh-Ph-labeled F-actin (400 nM) with Tm (100 nM) and Tn (80 nM) on ice in regulated filament buffer (RB) (100 mM KCl, 25 mM imidazole, 6 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, pH 7.4) for at least 12 h before use. As with unregulated F-actin, Rh-Ph F-actin-TnTm was diluted 1:100 in RB before infusion in the flow cell; however, it was necessary to add Tn and Tm (4:5 molar ratio to minimize the inhibitory effects of free troponin) to this dilution of Rh-Ph F-actin-TnTm to retain complete Ca^{2+} regulation (see Results) in the motility assay (Homsher et al., 1996).

Solutions

Motility assays were carried out either with RhPh F-actin (unregulated) or with RhPh F-actin-TnTm (regulated), under four ionic strength (Γ/2) conditions: AB solution (0.045 M Γ/2); 0.085 M Γ/2; 0.115 M Γ/2; and

0.14 M Γ/2. In all motility assay buffers, 3 mg ml^{-1} glucose, 100 μg ml^{-1} glucose oxidase (Sigma, St. Louis, MO), 18 μg ml^{-1} catalase (Boehringer-Mannheim, Indianapolis, IN), and 40 mM DTT (BioRad, Hercules, CA) were added to minimize photooxidation and photobleaching (Kron et al., 1991). In motility buffers other than AB, the conditions were (mM): 2 MgATP, 10 EGTA, and 2.6 Mg^{2+}, with 3-(*N*-morpholino)propanesulfonic acid and K-propionate (KPr) added to adjust ionic strength (28–70 mM and 65 mM, respectively, at 0.085 M Γ/2; 88–128 mM and 100 mM, respectively, at 0.115 M Γ/2; and 155–194 mM and 133 mM, respectively, at 0.14 M Γ/2). The pH was 7.0 at 30°C, and appropriate amounts of $CaPr_2$ were added to obtain pCa's between 9.2 and 4.0 (Martyn and Gordon, 1988). Methylcellulose (MC) was added to assay buffers (0.4% MC, typically, or 0.7% MC for control experiments at 0.085 M Γ/2; 0.6% or 0.7% MC at 0.115 M Γ/2; and 0.7% MC at 0.14 M Γ/2) to prevent F-actin diffusion from the assay surface at elevated Γ/2 (Homsher et al., 1996; Kron et al., 1991; Uyeda et al., 1990); MC was prepared as a 2% wt/vol concentrated stock solution by dialysis against 1.5 mM NaN_3 (Homsher et al., 1996), and volumes of this highly viscous solution were measured using a positive displacement micropipette (Labindustries, Berkeley, CA) to ensure accuracy. Motility assay solutions other than AB were mixed as 2× concentrated stocks and were stored as frozen aliquots until used; immediately before each assay, MgATP, antibleaching agents, MC, TnTm (where required), and an appropriate volume of water were added to dilute the motility buffer to the correct concentration.

Slide preparation and fluorescence microscopy

Flow cells were constructed on microscope slides from no. 1 coverslips resting on no. 1-1/2 thickness glass spacers held in place with silicone high-vacuum grease (Dow Corning, Midland, MI) (Kron et al., 1991). Coverslips were coated with a thin layer of nitrocellulose (Ernest Fullam, Latham, NY) freshly diluted to 0.1% in amyl acetate, and were used the same day (Kron et al., 1991). Total chamber volume was typically 40–50 μl.

Solutions were added to the flow cell in an order similar to that described by Homsher et al. (1996). First, HMM was added for 1 min, followed by AB plus 0.5 mg ml^{-1} bovine serum albumin for 1 min to block nonspecific protein binding. After the chamber was washed with AB, unlabeled F-actin (1 mg ml^{-1}; sheared by about 15 rapid passages through a 23-gauge needle) was added for 1 min. The chamber was washed with AB, AB with 0.5 mM ATP, and again with AB. This procedure uses unlabeled F-actin to block ATP-insensitive heads on HMM that either were not removed by centrifugation (see Myosin and Heavy Meromyosin Preparation) or were formed when HMM bound to the nitrocellulose surface (Kron et al., 1991; Sellers et al., 1993). Diluted (1:100) RhPh F-actin or RhPh F-actin-TnTm was added to the chamber for 20 s or 1 min, respectively, and was washed with either AB alone or RB plus appropriate concentrations of Tn and Tm (see Results), respectively. Finally, the assay buffer was infused into the flow cell and the slide was transferred to the microscope stage.

Fluorescence microscopy was carried out on a Diastar upright microscope (Leica, Deerfield, IL) equipped with a 100-W Hg arc lamp (Howard et al., 1993). The flow cell temperature was maintained at about 30°C by circulating water through a copper coil wrapped around the 100× objective (Hunt et al., 1994). RhPh F-actin and RhPh F-actin-TnTm filaments were imaged with a SIT camera (model VE 1000; Dage-MTI, Michigan City, IN) and recorded with a time-date generator signal (model WJ-810; Panasonic, Secaucus, NJ) on VHS videocassettes (VCR model AG7350; Panasonic).

Data acquisition and analysis

Analysis of speed and fraction of moving filaments

Filament speeds were obtained from analysis of centroids using hardware and Expert Vision software from Motion Analysis Systems (Santa Rosa, CA) (Homsher et al., 1992, 1996; Sellers et al., 1993). Data were acquired both in real time during experiments and from videocassette recordings, although the numbers reported here were obtained exclusively from analysis of recorded images. For most assays, data were sampled by the Motion Analysis system at 30 frames per second (fps) for 20–60 s, and individual filament paths were retained only when the filament centroid could be unambiguously tracked for a minimum of 2 s. Individual filaments that were undergoing nontranslational motion (e.g., spirals or "tail-wagging," where one end of the filament was attached and the rest was in solution) or diffusing in solution rather than interacting with the surface (i.e., filaments that were out of the focal plane, yet were still detected by the Motion Analysis system) were removed from further analysis, using the "choose" procedure in the Expert Vision software, if this population of filaments was >5% of the total. Initially, a three-point moving average filter (equal weights) was applied to each path, and a subsample of the data was retained to yield an effective sampling rate of 10 fps. Examples of filament centroids on identified paths obtained using the Motion Analysis system are shown for three different $[Ca^{2+}]$ in Fig. 2.

The mean speed along each path was calculated using the Motion Analysis algorithm, and the mean speed for a condition (combining information from all filament paths recorded on one slide) was obtained in

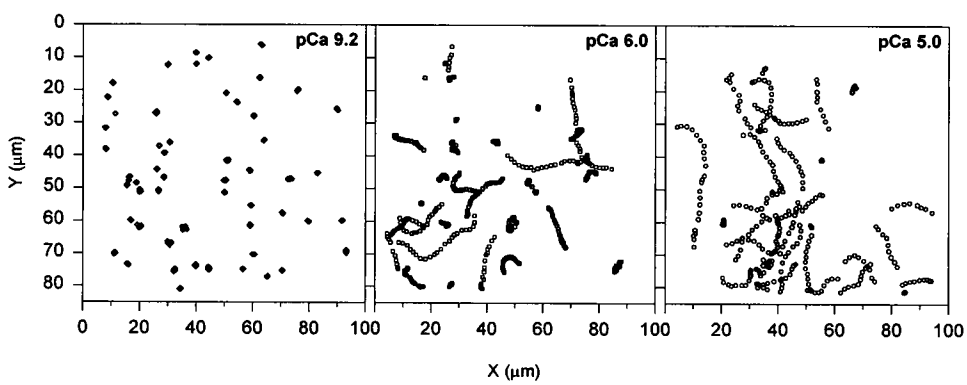

FIGURE 2 Centroid positions of well-regulated, rhodamine-phalloidin labeled F-actin at three calcium concentrations: pCa 9.2 (◇; *left*); pCa 6.0 (□; *center*); and pCa 5.0 (○; *right*). Conditions were 100 nM Tm, 80 nM Tn, 0.085 M Γ/2, and 0.4% MC (also see Materials and Methods). The data were obtained using Motion Analysis hardware and Expert Vision software (see Materials and Methods). The unfiltered data were acquired at a constant sampling rate (5 fps), thus spacing between adjacent points (i.e., centroids indicating position of the same filament in sequential frames digitized) corresponds to distance traveled per unit time. Total sampling time was 15 s (*left*); 6 s (*center*); or 3 s (*right*). Note that as $[Ca^{2+}]$ increases (comparing panels from left to right), there is an increasing proportion of centroids that have been translocated between frames and that the spacing between centroids is typically greater, indicating more moving filaments, at faster speeds.

several different ways. We used several methods to analyze our data because a variety of methods have been used previously to analyze motility data (Introduction), and we were concerned that this may have contributed to apparent differences in results with regulated actin filaments. We were also concerned that motility at intermediate pCa's (see Figs. 2 and 3) may not have been optimally characterized by previous methods.

The Motion Analysis algorithm uses a finite difference approximation to obtain frame-to-frame estimates of speed for individual filament paths, as shown in Fig. 2. A first-order central difference is used to compute interior points, and a first-order forward (backward) difference to compute the first (last) point. After this smoothing to obtain frame-to-frame speeds, the mean speed for an individual filament path was equivalent to the total distance traveled divided by the total time.

In method A, the speed (V_A) was obtained from the unweighted mean of all of the path speeds. In method B, filaments whose paths had a ratio of speed standard deviation to mean speed that exceeded a threshold (0.5) were removed, and the mean speed (V_B) was calculated from the remaining paths (Homsher et al., 1992, 1996; Sellers et al., 1993). Because of various sources of noise in the instrumentation, filaments in rigor exhibit a speed distribution that yields a nonzero average speed. To remove paths of nonmoving filaments (i.e., those that were indistinguishable from filaments in rigor), three additional methods were used. In method C, a decaying exponential function ($y = ae^{-bV}$) was fit to the rigor histogram data. To remove the nonmoving filaments from the slide histogram data with a given pCa, $y = ae^{-bV}$ was subtracted from the slide histogram data. The "a" was scaled to the maximum near the origin, and the "b" was taken from the rigor file with the same effective frame grabbing rate as the slide data. Remaining speeds greater than the rigor average were then used to calculate V_C. In method D, nonmoving filaments were removed by including only paths with average speeds greater than a cutoff speed (equal to the rigor mean speed) in calculating V_D. Finally, in method E, the cutoff speed used was the rigor mean speed plus one standard deviation. Methods D and E had the disadvantage of removing from analysis paths of filaments that were in fact moving very slowly, whereas method C did not.

The apparent mean speed and speed distribution of rigor filaments depended on the frame grabbing rate and averaging process. For rigor data acquired at 30 fps, smoothing and effective frame rate reduction gave a mean apparent speed of 2.2 $\mu m\ s^{-1}$ at 10 fps, 1.2 $\mu m\ s^{-1}$ at 5 fps, and 1.1 $\mu m\ s^{-1}$ at 2 fps. Rigor data acquired directly at 5 fps gave a mean apparent speed of 2.0 $\mu m\ s^{-1}$. For rigor data acquired at 5 fps, smoothing and effective frame rate reduction gave a mean apparent speed of 1.5 $\mu m\ s^{-1}$ at 1.6 fps. The speed distribution, which is a reflection of all of the combined noise sources in the system, could be fit very well by a simple exponential decay, $y = ae^{-bV}$. The different frame grabbing rates yielded rigor speed histograms with decay constants ranging from 3.2 to 12 $(\mu m/s)^{-1}$, with the lower values corresponding to the higher frame grabbing rates. If the subtraction of the exponential function yielded a negative count in a given bin, this bin was set to zero.

With respect to optimizing the signal-to-noise ratio, the most appropriate sampling rate for a given data set depends on the speed of the filaments (Homsher et al., 1992, 1996; Sellers et al., 1993). We therefore reanalyzed filament speed data that were initially characterized by predominantly slow movement, as determined from the analyses described thus far, after examination of the data to ensure that truly fast motility was not masked. When the average speed of a slide was $<5\ \mu m\ s^{-1}$, the 30 fps data were reanalyzed by more extensive filtering and using slower apparent frame rates. For average speeds of 2–5 $\mu m\ s^{-1}$, a five-point moving average filter was applied, and every sixth point was retained to yield an effective sampling rate of 5 fps. Similarly for data with an average speed of $<2\ \mu m\ s^{-1}$, a 15-point moving average filter was used, and every point was retained, for an effective sampling rate of 2 fps.

The fraction of filaments moving could then be calculated using the definitions of methods B, C, D, and E to identify "moving" paths and dividing their number by the total number of paths (f_B, f_C, f_D, and f_E, respectively). Note that all paths are included in method A, so there is no corresponding calculation for fraction of moving filaments by this method.

Where motility data are shown, the speeds (Figs. 4 *B*, 5 *B*, 7, and 8 *B*, and Table 1) are those calculated from method A (V_A), whereas the fraction of moving filaments (Figs. 4 *A*, 5 *A*, 6, 8 *A*, and Table 1) was obtained using method C (f_C). We chose V_A to quantify speed because it is most representative of the entire population of filament paths under all conditions; other methods excluded a substantial fraction of filaments from the mean calculation in some experimental conditions. We chose f_C to quantify the fraction of moving filaments because method C used truly nonmoving filaments (rigor) as a representative, standard population for identifying nonmoving filaments in other experimental conditions. As discussed in detail below (Results, Discussion) and as seen by comparison of Table 1 (V_A and f_C) with Table 2 (V_B and f_B), the qualitative results were independent of the method used to calculate mean speed or fraction moving.

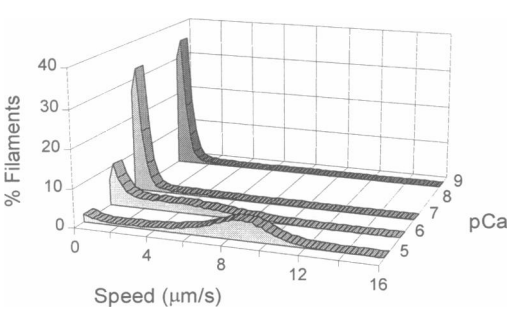

FIGURE 3 Speed histograms for motility of well-regulated, rhodamine-phalloidin labeled F-actin at four calcium concentrations: pCa 9.2; pCa 7; pCa 6; and pCa 5. Conditions as in Fig. 2. Data were acquired at 5 fps (pCa 9.2, pCa 7, and pCa 6) or 10 fps (pCa 5), as in Fig. 2. Each histogram represents data from one slide and thus includes combined information from ≥192 filament paths. Note that, as pCa was decreased ($[Ca^{2+}]$ was increased), there was a sharp decrease in the fraction of observations at near-zero speed and a graded redistribution of individual speed measurements toward greater speeds.

Regression analysis

Nonlinear least-squares regression (SigmaPlot software version 3.0; Jandel Scientific, San Rafael, CA) was used to obtain parameter estimates and standard errors of the parameter estimates for the Hill equation:

$$y = \frac{y_{max}}{1 + 10^{n(pCa-pK)}} + y_0, \quad (1)$$

where y can represent either filament speed (V, in $\mu m\ s^{-1}$), fraction moving (f), or normalized force at a given pCa; y_0 and y_{max} are the limits of high and low pCa (low and high $[Ca^{2+}]$), respectively; pK is equal to the pCa at the midpoint of the relationship (i.e., where $y = y_0 + y_{max}/2$); and n is related to the steepness of the transition from y_0 to y_{max}, where a higher n indicates a steeper transition. In terms of Eq. 1, increased or enhanced Ca^{2+} sensitivity indicates a relative shift of the Hill equation toward higher pK (i.e., lower $[Ca^{2+}]$ at the midpoint), and, conversely, reduced Ca^{2+} sensitivity indicates a shift in the opposite direction toward lower pK (i.e., higher $[Ca^{2+}]$).

Force-pCa relationship in single, skinned fibers

Force-pCa relations were determined in single, skinned fibers from rabbit psoas muscle under conditions as similar to the 0.14 M Γ/2 in vitro motility measurements as possible, except that an ATP regenerating system (phosphocreatine and creatine phosphokinase; PCr + CK) was included, and the anti-photooxidation and antibleaching agents were omitted. These solutions thus contained (in mM) 2 MgATP, 15 PCr, 2.6 Mg^{2+}, 10 EGTA, 20 3-(*N*-morpholino)propanesulfonic acid, 1 DTT, Ca^{2+} to give the desired pCa, $K^+ + Na^+$ to give 0.14 M Γ/2 (about 100), and 250 units ml^{-1} CK.

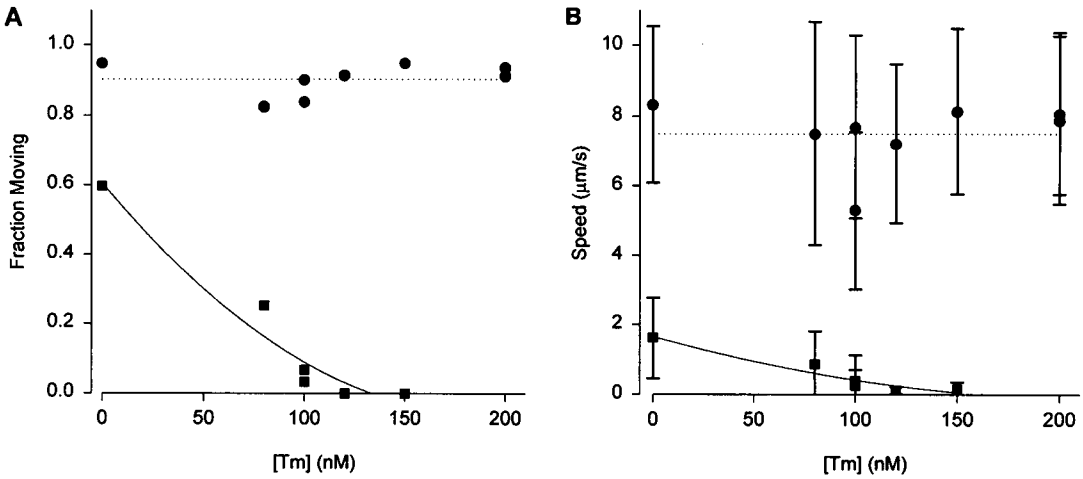

FIGURE 4 Establishing conditions for well-regulated motility: effects of excess Tm and Tn ([Tn]:[Tm] = 4:5) on the motility of regulated, rhodamine-phalloidin labeled F-actin (RhPh F-actin-TnTm) at pCa 9.2 (■) and pCa 5.0 (●). Tn and Tm were added to all solutions that RhPh F-actin-TnTm was exposed to (Materials and Methods; note that only [Tm] is indicated in the figure) and the fraction moving, f_C (*A*), and mean speed, V_A (± SD; *B*), were measured in the motility assay (Materials and Methods). Conditions were 0.085 M Γ/2 and 0.7% MC (also see Materials and Methods). Each point represents summary information from a single slide (≥80 filament paths). Lines correspond to the average of all measurements (·····; pCa 5) or the linear least-squares regression fit to a second-order polynomial (——; pCa 9.2). Note that excess Tn and Tm must be added to motility assay solutions to avoid significant motility in the absence of Ca^{2+}; also note that there was little or no effect of excess Tn and Tm on motility at pCa 5 over the concentration range examined in this experiment.

Skinned fiber measurements were made essentially as described by Martyn et al. (1993), with the following modifications. Because these experiments were performed at 30°C to match the conditions of the motility assays, we used a protocol described by Pate and co-workers (1994, 1995) to maintain fiber performance throughout the experiment. This involved using two sets of solutions at each pCa, one maintained on ice while the second was maintained at 30°C. For each pCa, the fiber was transferred first into the cold solution until steady-state force was achieved. Then the fiber was transferred into the 30°C solution (at the same pCa), steady-state force was recorded on a chart recorder, and the fiber was rapidly returned to a cold solution trough. This protocol was repeated at consecutively higher [Ca^{2+}]'s until a maximum force was achieved at pCa 4.5. Steady-state, Ca^{2+}-activated force was measured in the 30°C solutions as the deviation from the force baseline in relaxing solution (pCa 9.2) and was expressed as a fraction of the maximum force obtained at pCa 4.5 and 30°C. These normalized, steady-state force-pCa data were fit to the Hill equation (Eq. 1), except that the maximum force (y_{max} in Eq. 1) was constrained to be 1.0 and the minimum (y_0 in Eq. 1) was constrained to be 0.

TABLE 1 Nonlinear least-squares regression parameter estimates (±SE) for calcium dependence of fraction moving (f_C; Fig. 6) and filament speed (V_A; Fig. 7) data at each of three Γ/2 conditions fit to the Hill equation (Eq. 1)

Speed (V_A) vs. pCa			
Γ/2 (M)	0.085	0.115	0.140
pK	5.87 ± 0.16	5.77 ± 0.05	5.65 ± 0.07
n	1.12 ± 0.40	1.53 ± 0.26	2.09 ± 0.62
V_{max} (μm s^{-1})	6.75 ± 1.14	6.97 ± 0.49	5.42 ± 0.61
V_0 (μm s^{-1})	0.36 ± 0.41	0.26 ± 0.23	0.09 ± 0.40
Fraction of filaments moving (f_C) vs. pCa			
Γ/2 (M)	0.085	0.115	0.140
pK	6.17 ± 0.07	6.08 ± 0.07	5.80 ± 0.02
n	2.27 ± 0.73	1.35 ± 0.31	4.57 ± 1.04
f_{max}	0.85 ± 0.08	0.88 ± 0.08	0.78 ± 0.04
f_0	0.05 ± 0.05	0.05 ± 0.05	0.04 ± 0.03

RESULTS

Unregulated F-actin

The speed of RhPh F-actin (V_A) at 30°C in AB conditions was 4.9 ± 1.0 μm s^{-1} (mean ± SD; N = 19 slides, which represents a total of 17,682 filament paths recorded), in agreement with previous reports (Fraser and Marston, 1995; Homsher et al., 1992, 1996; Toyoshima et al., 1990). In these assays, the fraction of moving filaments (f_C = 93 ± 1%; mean ± SD; N = 19 slides) indicates a very low proportion of ATP-insensitive "dead-heads" that, if present, would impede filament sliding.

TABLE 2 Nonlinear least-squares regression parameter estimates (±SE) for calcium dependence of fraction moving (f_B) and filament speed (V_B) at each of three Γ/2 conditions fit to the Hill equation (Eq. 1)

Speed (V_B) vs. pCa			
Γ/2 (M)	0.085	0.115	0.140
pK	6.09 ± 0.16	5.94 ± 0.04	5.77 ± 0.06
n	0.98 ± 0.34	1.73 ± 0.29	2.19 ± 0.54
V_{max} (μm s^{-1})	7.06 ± 1.07	7.35 ± 0.41	6.22 ± 0.56
V_{min} (μm s^{-1})	0.67 ± 0.46	0.73 ± 0.24	0.52 ± 0.42
Fraction of filaments moving (f_B) vs. pCa			
Γ/2 (M)	0.085	0.115	0.140
pK	6.00 ± 0.11	5.59 ± 0.21	5.69 ± 0.05
n	1.40 ± 0.50	0.86 ± 0.24	4.29 ± 1.73
f_{max}	0.77 ± 0.10	0.81 ± 0.16	0.51 ± 0.05
f_{min}	0.07 ± 0.05	0.12 ± 0.04	0.14 ± 0.04

As in Table 1, the data are from the experiments shown in Figs. 6 and 7, except that filament speed and fraction moving were estimated by selecting only paths of smoothly moving filaments (path speed SD/mean speed < 0.5; Materials and Methods) (Homsher et al., 1992, 1996; Sellers et al., 1993).

At the three ionic strengths used (0.085, 0.115, and 0.14 M, and thus in the presence of 0.4, 0.6, or 0.7% MC, respectively, used to obtain motility in the three ionic strength conditions), we observed no substantial change in speed of moving RhPh F-actin filaments, but a small decrease in the fraction moving when compared to paired measurements in AB. More importantly, there was no effect of $[Ca^{2+}]$ (pCa 9.2 to 5.0) on the movement of RhPh F-actin, in agreement with previous observations (Homsher et al., 1996; Honda and Asakura, 1989; Sata et al., 1995a,b). This result shows that there is little or no Ca^{2+}-dependent modulation of skeletal muscle filament sliding in vitro in the absence of Tn-Tm, in accord with observations of unloaded shortening velocity in skinned skeletal fibers during Ca^{2+}-independent activation (Martyn et al., 1994).

Regulated F-actin

Establishing conditions for maintaining well-regulated motility of RhPh F-actin-TnTm

Well-regulated, reconstituted actin filament motility should mimic muscle contraction such that force and/or directed motion should be at minimum in the absence of Ca^{2+} (pCa > 8) and should be at maximum (or nearly so) at pCa 5. To obtain complete cessation of filament movement in the absence of Ca^{2+} (pCa 9.2), we found it necessary to add Tn and Tm to all solutions that RhPh F-actin-TnTm was exposed to (Fig. 4; Materials and Methods). This result is similar to that found for RhPh F-actin-TnTm reconstituted with cardiac Tn and Tm (Homsher et al., 1996). In these experiments, Tn and Tm were always added at a 4:5 molar ratio to ensure that Ca^{2+}-dependent inhibitory effects of free Tn were minimized (Ebashi and Endo, 1968; Leavis and Gergeley, 1984). In most titrations, we also found that excessive amounts of skeletal Tn and Tm (>200 nM) inhibited the motility of a fraction of RhPh F-actin-TnTm at pCa 5, although this is not shown in the experiment in Fig. 4, where TnTm was varied over only a narrow range of concentrations. Thus it was necessary to titrate the amount of Tn and Tm added to assay buffers at both pCa 9 and pCa 5 to determine appropriate concentrations for calcium regulation of motility. There were slight variations in the exact concentrations of Tn and Tm that yielded well-regulated motility related to different protein preparations and age of Tn preparation, and between assay conditions. The amount of Tm (Tn) added was 100 nM (80 nM) at 0.085 M Γ/2, 60–200 nM (48–160 nM) at 0.115 M Γ/2, or 40–50 nM (32–40 nM) at 0.14 M Γ/2, which is generally consistent with the increased affinity of Tm for F-actin at higher ionic strengths (Heeley et al., 1989; Kellermayer and Granzier, 1996; but see Butters et al., 1993). All of the preparations used to construct Figs. 5-8 met the criteria adopted to define well-regulated motility: the concentration of Tn and Tm added was just sufficient to inhibit motility at pCa 9.2 without affecting motility at pCa 5.

At the beginning of the experiment, we also titrated the amount of MC necessary to keep filaments from diffusing away from the surface. For experiments at higher Γ/2, more MC was needed (0.4% at 0.085 M; 0.6% or 0.7% at 0.115 M; and 0.7% at 0.14 M Γ/2). MC was maintained at the lowest possible concentration to minimize optical scattering and thus maintain image quality. As discussed later, we found that MC in this 0.4–0.7% range did not affect the sliding speed of regulated or unregulated filaments or the $[Ca^{2+}]$ regulation of F-actin-TnTm filaments. After optimal MC and TnTm conditions were established at pCa 9 and pCa 5 for Ca^{2+} regulation at a given Γ/2, we performed

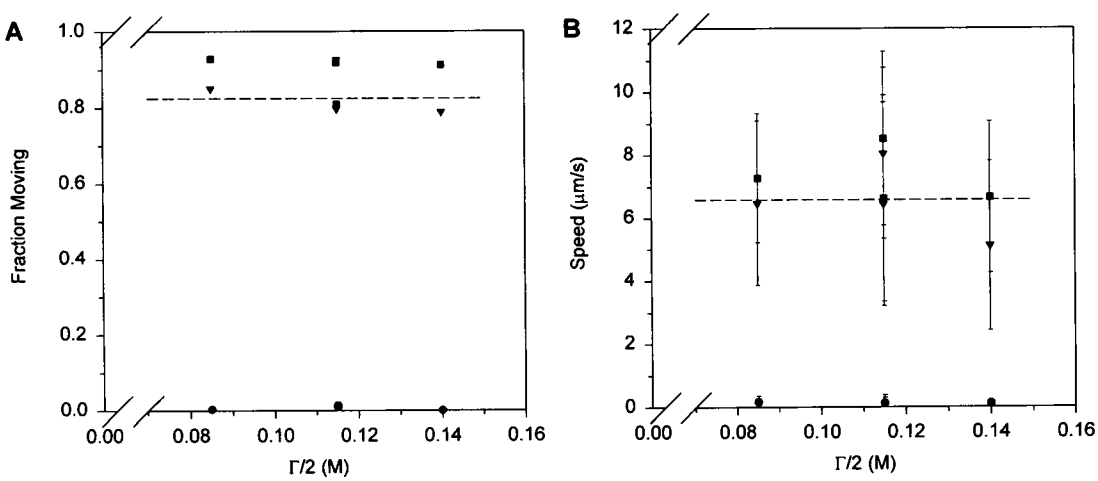

FIGURE 5 Ionic strength (Γ/2) dependence of fraction moving, f_C (*A*), and mean speed, V_A (± SD; *B*), for motility of well-regulated, rhodamine-phalloidin labeled F-actin at three calcium concentrations: pCa 4.6 (■); pCa 5.0 (▼); and pCa 9.2 (●). Conditions were: 0.7% MC (all Γ/2); 100 nM Tm and 80 nM Tn (0.085 M Γ/2); 50–75 nM Tm and 40–60 nM Tn (0.115 M Γ/2); and 50 nM Tm and 40 nM Tn (0.14 M Γ/2) (Materials and Methods). Each point represents summary information from a single slide (≥112 filament paths). Lines were drawn according to the average of pCa 5 data for fraction moving (*A*) and speed (*B*). Note that there was little or no effect of Γ/2 on maximum Ca^{2+}-activated motility over the range examined in this experiment.

assays at intermediate and lower pCa's to determine the mode of calcium regulation of motility.

Calcium regulation of RhPh F-actin-TnTm movement in vitro

The main question addressed in this study is, what qualitative and quantitative changes in RhPh F-actin-TnTm motility are effected by Ca^{2+}, particularly at near-physiological ionic strength? The answer to this question for well-regulated motility is particularly important for defining the mechanism of control for actomyosin interactions and thus muscle contraction.

The maximum speed obtained for RhPh F-actin-TnTm was not greatly affected by $\Gamma/2$ over the range of 0.085–0.14 M (Fig. 5, *A* and *B*). Although there was a statistically significant difference in the maximum speeds between observations made with the same protein preparation at pCa 5.0 ($p < 0.01$; analysis of variance; Fig. 5 *B*), this was in due part to decreased Ca^{2+} sensitivity at 0.14 M $\Gamma/2$ (Fig. 7, Table 1). Because pCa 5.0 solutions did not produce a maximum speed, a pCa 4.6 was required (Fig. 5 *B*); however, there may be a small decrease in speed at the higher ionic strength consistent with the effect of ionic strength on the actomyosin ATPase (see references in Gordon et al., 1973). A minor $\Gamma/2$ dependence of maximum sliding speed in vitro is consistent with a lack of $\Gamma/2$ dependence of maximum speed of shortening of muscle fibers over a similar ionic strength range (Gulati and Babu, 1984; Gulati and Podolsky, 1981; Julian and Moss, 1981).

In these experiments we consistently found that the maximum Ca^{2+}-activated speed for RhPh F-actin-TnTm was faster than for RhPh F-actin. This was found by using RhPh F-actin and RhPh F-actin-TnTm filaments made from the same actin preparation and measured under the same solution conditions. The elevated speed is apparently related to the presence of Tn and Tm. Such differences in maximum speed for RhPh F-actin-TnTm compared with RhPh F-actin are in accord with data previously reported by Fraser and Marston (1995), although this phenomenon was not observed in the data of Kellermayer and Granzier (1996), Homsher et al. (1996), and Sata et al. (1995a,b). Our data are also in accord with those of Wang et al. (1993), who observed a similar enhancement of filament speed by Tm (although in the absence of Tn in their experiments), using *Limulus* muscle proteins recombined with Tm from a variety of species. This elevation of speed in the motility assay is also in accord with the biochemistry, as the MgATPase activity can be enhanced in the presence of Ca^{2+} for regulated thin filaments over that for F-actin (Bremel et al., 1972; Bremel and Weber, 1972; Lehrer and Morris, 1982).

For all three ionic strength conditions used, both the fraction of moving filaments (Fig. 6) and the mean filament speed (Fig. 7) increased, from near-zero levels to a maximum, as $[Ca^{2+}]$ was increased. Each point in Figs. 6–7 corresponds to data obtained from one slide and thus summarizes information from 18–1552 filament paths; a total of

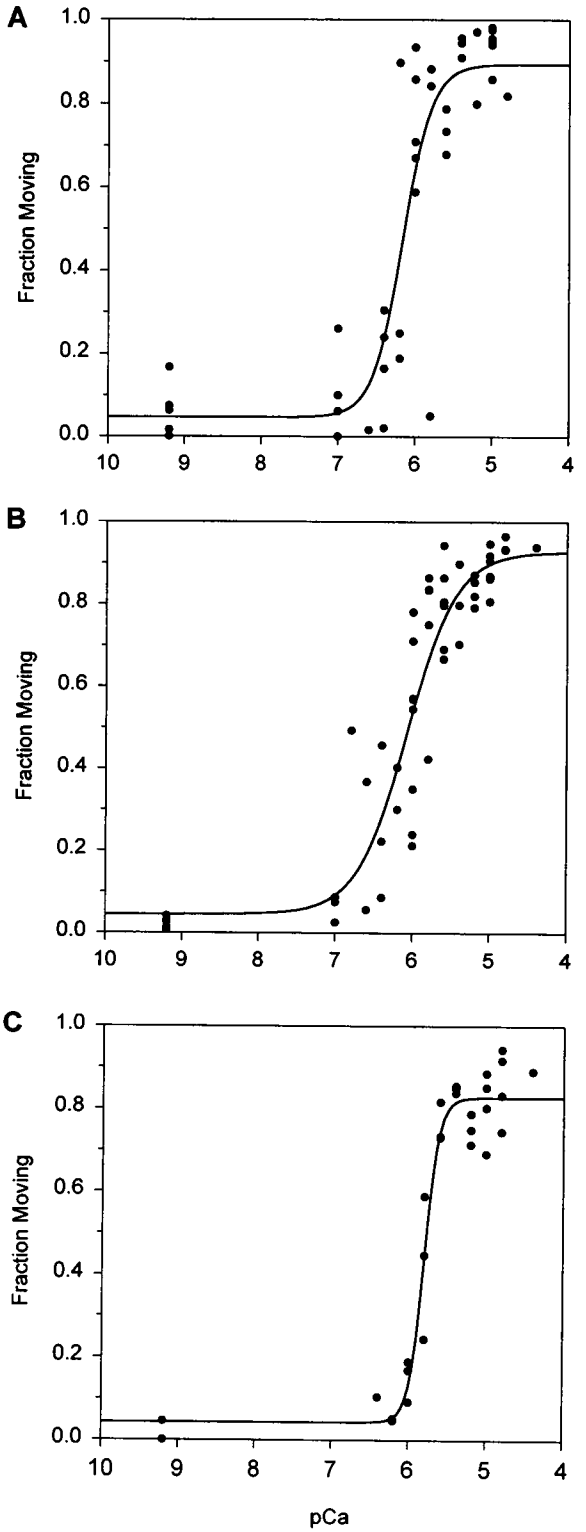

FIGURE 6 Calcium dependence of fraction moving (f_C) for motility of well-regulated, rhodamine-phalloidin labeled F-actin at three ionic strengths ($\Gamma/2$): 0.085 M (*A*); 0.115 M (*B*); and 0.14 M (*C*). For conditions, see Materials and Methods. Each point represents summary information from a single slide (18–1552 filament paths). Lines were drawn according to the nonlinear least-squares regression fit to the Hill equation (Eq. 1); parameters are given in Table 1.

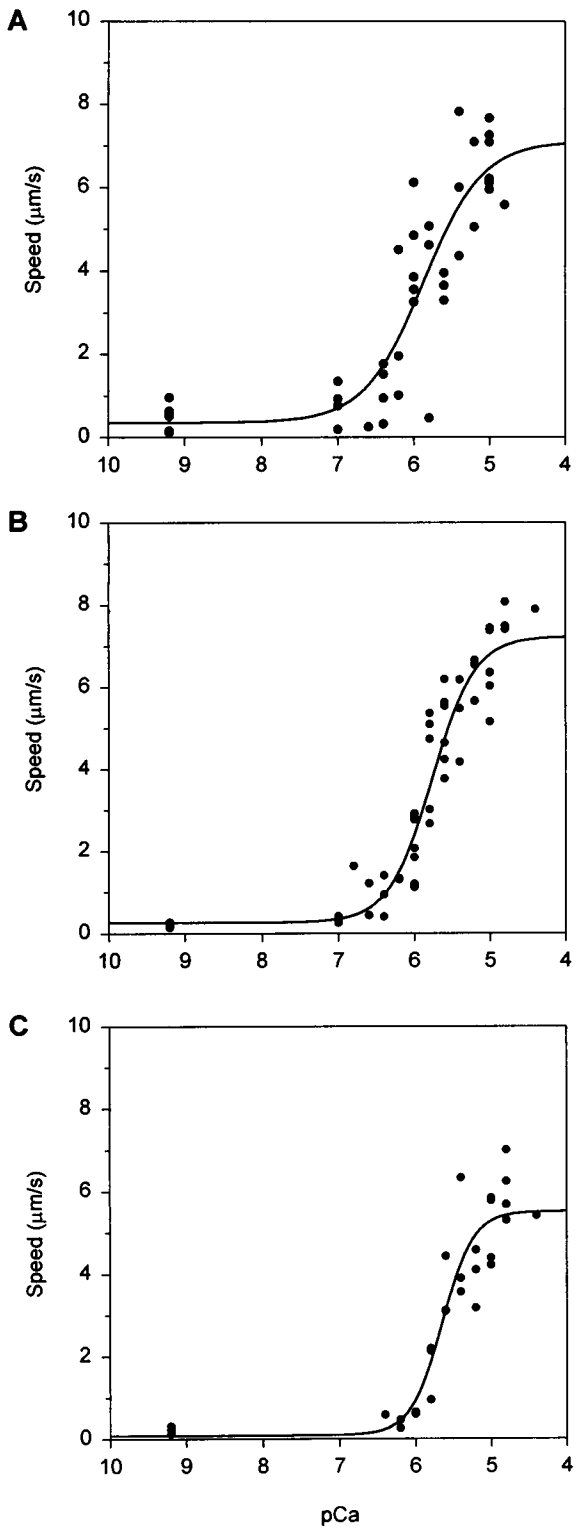

FIGURE 7 Calcium dependence of mean speed (V_A) for motility of well-regulated, rhodamine-phalloidin labeled F-actin at three ionic strengths (Γ/2): 0.085 M (*A*); 0.115 M (*B*); and 0.14 M (*C*). Each point represents summary information from a single slide corresponding to fraction moving data in Fig. 6; error bars were omitted for clarity and are comparable to those in Figs. 4 *B*, 5 *B*, and 8 *B*. Lines were drawn according to the nonlinear least-squares regression fit to the Hill equation (Eq. 1); parameters are given in Table 1. Note the gradation of speed with [Ca^{2+}] at all three conditions examined.

65,879 filament paths were examined, and the average number of filament paths obtained from each slide was 511 ± 299 (mean ± SD; N = 129 slides). The mean filament speed (V_A) is clearly graded with pCa (Fig. 7). The fraction of moving filaments (f_C) exhibits a steeper dependence on [Ca^{2+}] (Fig. 6, Table 1), which in two of the three cases approaches on-off behavior (Fig. 6, *A* and *C*). The control of both the fraction moving and average filament speed is also clearly seen in the original video images (not shown), individual filament paths (Fig. 2), and compiled frame-to-frame speed histograms (Fig. 3). In particular, Fig. 3 illustrates that the distribution of individual speed measurements at submaximum [Ca^{2+}] (pCa 7 and pCa 6), even for brief time intervals, is not simply the weighted sum of two populations equivalent to those found at pCa 9.2 and pCa 5.0. Thus our results are qualitatively consistent with previous data obtained using RhPh F-actin-TnTm containing cardiac Tn and Tm (Homsher et al., 1996), but differ from those of others (Fraser and Marston, 1995; Harada et al., 1990; Honda and Asakura, 1989; Kellermayer and Granzier, 1996; Sata et al., 1995a,b, 1996). The similarity of our results with those of Homsher et al. (1996) suggests that the mechanism of regulation of motility is basically similar for RhPh F-actin-TnTm reconstituted with either skeletal or cardiac regulatory proteins. On the other hand, differences with the other studies imply that the experimental methods used to measure regulation of motility can significantly influence the results.

Table 1 summarizes the parameters for the Hill fits (Eq. 1) to the data shown in Figs. 6 (f_C) and 7 (V_A) at all three ionic strengths. These fits were done, allowing not only the pK and n to vary, but also the maximum and minimum speed or fraction moving (Eq. 1). In general, the trend is for pK to decrease as Γ/2 increases (indicating a shift toward higher [Ca^{2+}]) and for n to increase (indicating a steeper Ca^{2+} dependency). Table 2 summarizes the fit parameters when method B (Homsher et al., 1992, 1996; Sellers et al., 1993) was used to analyze the speed (V_B) and fraction of filaments moving (f_B). This analysis involves considering only the smoothly moving filaments with a speed SD/average speed ratio of <0.5 (Materials and Methods). The actual fits give somewhat higher average maximum and minimum speeds and higher Ca^{2+} sensitivities, and no changes in the steepness of the relationships (n), but the same trend in all parameters with changes in Γ/2.

Because the concentration of MC covaried with Γ/2, we tested the possibility that variations in [MC] (rather than Γ/2) might be responsible for changes observed in the speed-pCa and fraction moving-pCa relationships (Figs. 6 and 7, Table 1). As seen in Fig. 8, *A* and *B*, there was no significant change in the fraction moving-pCa or speed-pCa relationships determined at 0.085 M Γ/2 using either 0.4 or 0.7% MC; comparison of these two concentrations of MC was only possible at 0.085 M Γ/2, because motile filaments would diffuse away from the surface when 0.4% MC was used at higher Γ/2 (0.115 M or 0.14 M in this study; Materials and Methods). Therefore we conclude that Γ/2

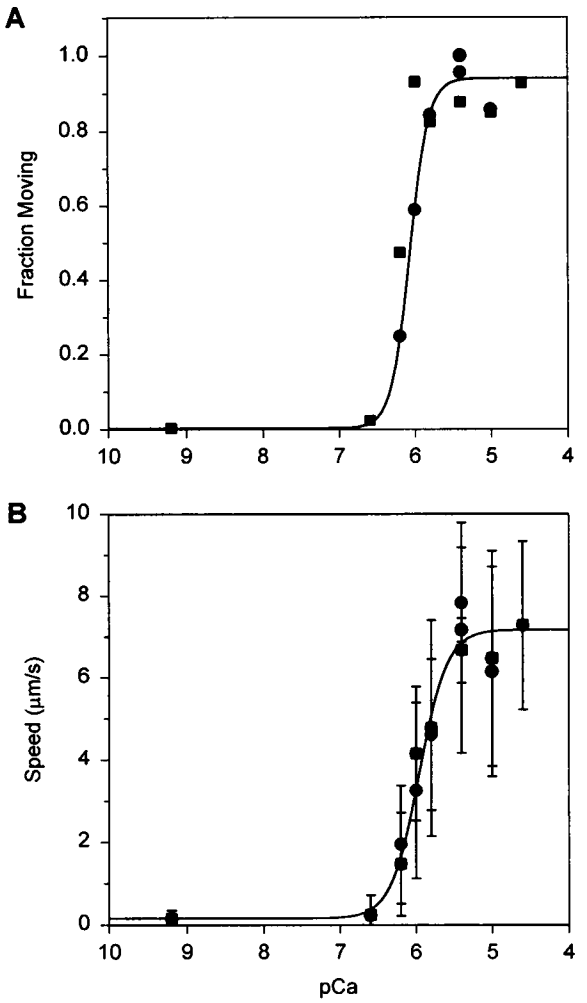

FIGURE 8 Lack of effect of methylcellulose concentration (MC; 0.4%, ●, versus 0.7%, ■) on calcium dependence of fraction moving, f_C (*A*), or mean speed, V_A (± SD; *B*), for motility of well-regulated, rhodamine-phalloidin-labeled F-actin. Conditions were: 0.085 M Γ/2, 100 nM Tm, and 80 nM Tn (also see Materials and Methods). Each point represents summary information from a single slide (4–632 filament paths). Lines were drawn according to the nonlinear least-squares regression fit to the Hill equation (Eq. 1) using the following parameters: pK, 6.1 ± 0.04 (*A*) and 6.0 ± 0.04 (*B*); *n*, 3.49 ± 0.84 (*A*) and 2.43 ± 0.55 (*B*); f_{max}, 0.94 ± 0.07 (*A*); v_{max}, 6.7 ± 0.3 μm s^{-1} (*B*). Because of the limited number of data points at each [MC], we reduced the number of free parameters in the regression equation by applying the constraints f_{min} = 0.003 (*A*) and v_{min} = 0.16 (*B*). Note that there were no significant differences between regression parameter estimates for 0.4% MC data compared with 0.7% MC data in either *A* or *B*.

was the dominant factor responsible for shifts in Ca^{2+} sensitivity observed in Figs. 6 and 7.

Force-pCa relationship in single, skinned fibers under conditions comparable to motility experiments

To determine how closely Ca^{2+} regulation of motility correlates with Ca^{2+} regulation of force production in fibers, we investigated the force-pCa relationship in single, skinned rabbit psoas skeletal muscle fibers under conditions comparable to those used in the motility experiments. To reliably measure force at 30°C in fibers, we adopted the data collection technique of Pate et al. (1994, 1995) described in Materials and Methods. Fig. 9 shows the results for the steady-state force-pCa data for six fibers at 30°C and Γ/2 of 0.14 M. The Hill equation fit to the data is shown with a pK ($pCa_{1/2}$) of 5.40 ± 0.023 and an *n* of 1.93 ± 0.20. For comparison, the Hill fits to the 0.014 M Γ/2 motility data for speed-pCa and percentage of moving filaments-pCa shown in Figs. 6 *C* and 7 *C* were replotted in Fig. 9 as dotted and dashed lines, respectively. The Ca^{2+} sensitivity of force appears to be less than that of either filament speed or percentage moving in the motility assay, as judged by the pK values (Fig. 9 and Table 1). The question is whether this is a real or apparent shift in Ca^{2+} sensitivity between the fiber and motility assays. There is some uncertainty in the pK's for the motility data, because these absolute numbers depend somewhat on the method of analysis (see Tables 1 and 2). In addition, the solutions and conditions of the two assays were as similar as possible, but were not identical (see Materials and Methods). Fiber solutions contained an ATP regenerating system (PCr and CK) to ameliorate nucleotide gradients within fibers (Chase and Kushmerick, 1995; Cooke and Pate, 1985). PCr and CK should not have an intrinsic effect (Chase and Kushmerick, 1995), but are associated with reduced [MgADP] and with increased amounts of inorganic phosphate (P_i) (contamination typically <1 mM), both of which would decrease Ca^{2+} sensitivity in the skinned fiber assay (Godt and Nosek, 1989; Martyn and Gordon, 1992). In addition, the in vitro motility

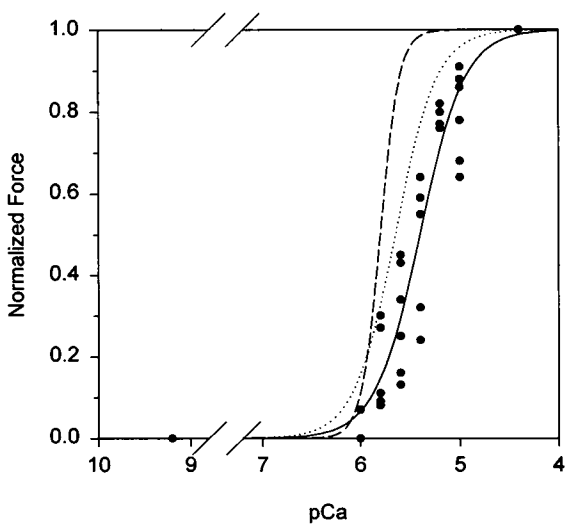

FIGURE 9 Calcium dependence of steady-state isometric force of glycerinated fiber segments from rabbit psoas muscle at 0.14 M ionic strength (Γ/2) and 30°C. See Materials and Methods for complete details on conditions. Each point represents a single determination. The nonlinear least-squares regression fit of the Hill equation (Eq. 1) to the combined data from six fibers (——) yielded pK = 5.40 ± 0.02 and *n* = 1.93 ± 0.20. For comparison, regression fits to motility data at 0.14 Γ/2 were redrawn from Fig. 6 *C* (– – –; fraction moving) and Fig. 7 *C* (·····; filament speed).

regulated filaments contain Ph, which has been shown to enhance Ca^{2+} sensitivity (Bukatina and Fuchs, 1994; Bukatina et al., 1995). The MgADP, P_i, and Ph would contribute to the difference in Ca^{2+} sensitivity between the skinned fiber force and in vitro motility filament speed, so that the difference is less than that shown (Fig. 9) and thus may not be significant. However, the number of filaments moving appears to be even more sensitive than force, with a much steeper dependence on $[Ca^{2+}]$.

DISCUSSION

The main results of these experiments are:

1. Ca^{2+} control of regulated thin-filament sliding is complete under appropriate conditions; i.e., regulated thin filaments do not show directed motion in the absence of Ca^{2+}.
2. Unlike F-actin, the speed and number of regulated filaments moving increase with increasing $[Ca^{2+}]$.
3. With increased ionic strength, the Ca^{2+} sensitivity of both the number of filaments moving and their speed is decreased, but at high ionic strength the relationship is quite steep. These observations are independent of the specific method of analysis.
4. The filament speed was found to be graded with $[Ca^{2+}]$ and parallels the gradation of force in skinned fibers.

We will discuss the significance of each of these results below and other implications, starting with the increased confidence in the results engendered by the use of different methods of analysis.

Methods of analysis

Processing the images of the fluorescently labeled filaments with the Motion Analysis system enables one to analyze the motion of many thousands of filaments, yielding a representative sample of the filament population. Using the Motion Analysis system (Homsher et al., 1992, 1996; Sellers et al., 1993) makes it possible to easily apply a number of different techniques to analyze the motion of large numbers of regulated actin and F-actin filaments (Materials and Methods); this is highly beneficial because various analyses used previously could have contributed to variability in previous results (Introduction; Materials and Methods). However, we found that each analysis led to the same basic conclusions, although the specific numbers varied somewhat, based on whether the speed and fraction of filaments moving were analyzed using the "unweighted" (V_A) and "nonrigor" (f_C) methods, respectively (Figs. 6 and 7 and Table 1), the "smoothly moving" filament criteria (V_B, f_B; Table 2), or the other "moving" filament criteria discussed in Materials and Methods. In comparing the various criteria, the "unweighted" mean speeds plotted (V_A; Fig. 7) are found to be somewhat lower than those obtained with the other criteria, particularly the "smoothly moving" filaments (V_B) at lower $[Ca^{2+}]$. Using the "nonrigor" criteria to determine the fraction of filaments moving (f_C) underestimates the fraction somewhat compared to the "moving" filament criteria (particularly f_D, and to a much lesser degree f_E); f_C also underestimates the fraction moving compared to the "uniformly moving" criteria (f_B) at lower $[Ca^{2+}]$, but overestimates at higher $[Ca^{2+}]$. Because the four main results given above were obtained with all methods of analysis, the conclusions are robust.

Ca^{2+} regulation of in vitro motility assay

Because muscles are relaxed at low $[Ca^{2+}]_i$ and skinned fibers generate no force at very low $[Ca^{2+}]$ (Fig. 9), it was expected that regulated filaments should not exhibit directed motion at very low $[Ca^{2+}]$. Thus it was reassuring that regulated thin-filament motility could be "turned off" completely in the absence of added Ca^{2+} (Fig. 4). The need to add additional Tm-Tn to motility solutions to achieve this degree of regulation implies that Tm-Tn dissociates from RhPh F-actin-TnTm in the absence of sufficient added free proteins. This is consistent with the reported affinity of actin for Tm-Tn (Hill et al., 1992). In the present experiments, regulated filaments were formed with 100 nM Tm and 80 nM Tn added to 400 nM F-actin (Materials and Methods), but these were diluted 1:100 for the in vitro motility assay (Materials and Methods), thus diluting the free Tm to less than 1 nM. With the affinities given by Hill et al. (1992), one must add additional Tm to ensure that all binding sites are occupied. Because the filaments are probably effectively unloaded in the in vitro motility assay, the presence of only a few "bare" regions (i.e., regions missing Tm, thus allowing binding of HMM) on the otherwise "regulated" thin filaments would permit motility in the absence of Ca^{2+}.

Implications for Ca^{2+} regulation of muscle contraction

A major result of these studies is that $[Ca^{2+}]$ grades the filament speed and more steeply modulates the number of filaments moving (Figs. 6 and 7 and Table 1). This would appear to be due to the regulatory proteins and not to a direct effect on myosin (HMM), because there is no $[Ca^{2+}]$ dependence of the speed of sliding of F-actin filaments (see Results: Unregulated F-actin). Analysis of the histograms of the speeds of regulated filaments at different pCa's (Fig. 3) shows that the intermediate speeds at intermediate pCa's are not due to the existence of two distributions, one at near-zero speeds and the other centered about the maximum speed, with pCa changing the relative number of filaments in each distribution and thus the mean speed.

The data taken at higher ionic strength shows that Ca^{2+} grades the sliding speed even for $\Gamma/2$ near the physiological range (Fig. 7 *C*). Thus this graded activation is not an artifact of working at low $\Gamma/2$, with stronger ionic interactions and possibly different control features (Head et al., 1995). Elevated $\Gamma/2$ shifts the Ca^{2+} sensitivity of both the speed and fraction of filaments moving (Figs. 6 and 7), as it

does the force-pCa relationship in skinned fibers (Fink et al., 1986). In fact, the observed shift (~0.1 ΔpCa/25 mM Γ/2) is similar to that reported by Fink et al. (1986). These observations could be attributed to the effects of Γ/2 and $[K^+]$ on Ca^{2+} binding to TnC, but there may also be contributions from Γ/2 effects on cross-bridge binding to actin. These results show convincingly that regulation in the in vitro motility assay can occur at Γ/2 in the physiological range and that, although the number of filaments moving depends steeply on $[Ca^{2+}]$, speed is graded less steeply by $[Ca^{2+}]$.

In considering the cause of the graded speed of the regulated filaments with increasing $[Ca^{2+}]$, we should first consider the comparison with the force-pCa data obtained under similar solution conditions in skinned fibers (Fig. 9). Even though there are some uncertainties in comparing the pCa's and conditions between the skinned fiber and in vitro motility (Results), increase in speed with Ca^{2+} (V_A; Figs. 7 *C* and 9) parallels the increase in force (Fig. 9), but is possibly a little more sensitive (<0.25 pCa units; Fig. 9 and Table 1). Parallel increases of filament speed and number of force-generating cross-bridges seen here are comparable to previous observations of maximum Ca^{2+}-activated force and unloaded shortening velocity of skinned fibers (Martyn et al., 1994; Metzger and Moss, 1988; Moss, 1986). This implies that the gradation of speed is an intrinsic property of regulation at the level of single filaments and is therefore not due to sarcomeric constraints. On the other hand, the fraction of filaments moving (f_C; Fig. 6 *C*) begins to increase at about the same pCa as force, but increases steeply, so that it reaches maximum at a pCa where force is ~50% of maximum (Fig. 9). This suggests that only a moderate percentage of cross-bridges need to generate force to get all of the filaments moving.

Homsher et al. (1996) suggested four different hypotheses for the speed variation with Ca^{2+}: 1) a "weak binding drag" hypothesis in which weakly attached cross-bridges provide a Ca^{2+}-independent drag that would provide a load, making speed dependent on the number of active cross-bridges and thus $[Ca^{2+}]$; 2) a "limited cross-bridge number" hypothesis in which if cross-bridges have a short duty cycle and modest throw, the speed will be increased with each recruited cross-bridge for the first few recruited by Ca^{2+}, as suggested by Uyeda et al. (1990); 3) a "cross-bridge cycling rate" or intrinsic control of Ca^{2+} over the cross-bridge cycle; and 4) a Ca^{2+}-dependent drag due to secondary interactions of the regulatory proteins with components of the motility assay, in addition to primary binding to the actin filament. The studies of Moss and colleagues (Metzger, 1996; Metzger and Moss, 1988; Moss, 1986) on skinned fibers suggest a fifth hypothesis, that under partial activation conditions, there is an increased likelihood of cross-bridges becoming negatively strained, thus providing an internal resistance to shortening.

Our data help us decide among these alternative mechanisms for gradation of speed. Hypothesis 3 is unlikely, because the speed of unregulated filaments was not affected by $[Ca^{2+}]$ (see Results: Unregulated F-actin) (Homsher et al., 1996; Honda and Asakura, 1989; Sata et al., 1995a,b). Furthermore, our data suggest that hypothesis 1 is unlikely because speed was graded, even at the highest ionic strengths, where the weak cross-bridge interactions with actin are minimal (Brenner et al., 1986; Schoenberg, 1991). In contrast, the observation that elevated Γ/2 greatly steepens the relationship between the number of filaments moving and pCa (Fig. 6 *C* and Table 1) suggests that weakly attached cross-bridges may play a role in keeping filaments from moving in the absence of Ca^{2+}. As discussed above, motion would occur when enough force-generating cross-bridges had attached to overcome this resistance. Distinguishing between the remaining hypotheses requires measurements with improved spatial and temporal resolution.

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