

Smooth Muscle Myosin Cross-bridge Interactions Modulate Actin Filament Sliding Velocity In Vitro

David M. Warshaw, Janet M. Desrosiers, Steven S. Work, and Kathleen M. Trybus*

Department of Physiology and Biophysics, University of Vermont, College of Medicine, Burlington, Vermont 05405; and
*Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254

Abstract. Although it is generally believed that phosphorylation of the regulatory light chain of myosin is required before smooth muscle can develop force, it is not known if the overall degree of phosphorylation can also modulate the rate at which cross-bridges cycle. To address this question, an in vitro motility assay was used to observe the motion of single actin filaments interacting with smooth muscle myosin copolymers composed of varying ratios of phosphorylated and unphosphorylated myosin. The results suggest that un-

phosphorylated myosin acts as a load to slow down the rate at which actin is moved by the faster cycling phosphorylated cross-bridges. Myosin that was chemically modified to generate a noncycling analogue of the "weakly" bound conformation was similarly able to slow down phosphorylated myosin. The observed modulation of actin velocity as a function of copolymer composition can be accounted for by a model based on mechanical interactions between cross-bridges.

SMOOTH muscle generates comparable or greater force per cross-sectional area than skeletal muscle while consuming 300 times less energy (i.e., ATP) (Paul et al., 1976; Siegelman et al., 1980). The molecular basis for this economy of force production may depend on how the most basic contractile structure, the myosin cross-bridge, interacts with neighboring actin filaments (Murphy, 1980; Fay et al., 1981; Hellstrand and Paul, 1982). Although the cross-bridge cycle in smooth and skeletal muscle is qualitatively similar, these muscles differ in their mode of regulation which may account for the unique contractile capabilities of the smooth muscle cell.

Phosphorylation of the 20-kD myosin light chain is required to initiate contraction and rapid cross-bridge cycling in smooth muscles (for review see Kamm and Stull, 1985). In addition to acting simply as an "on-off" switch, Murphy and co-workers (Dillon et al., 1981) proposed that the degree of light chain phosphorylation may also modulate the rate at which cross-bridges cycle. This modulatory role was based on the observation that both light chain phosphorylation and shortening velocity peaked early in a contraction and then fell during the period of force maintenance (Dillon et al., 1981). To account for the temporal correlation between these two parameters, Murphy and co-workers suggested that two mechanically distinct cross-bridge populations can interact: a population of rapidly cycling phosphorylated cross-bridges and a slower or noncycling population of latchbridges that are dephosphorylated by phosphatases (Dillon et al., 1981). As more cross-bridges are dephosphorylated during the period of force maintenance, they would impose a load on the faster cycling phosphorylated cross-bridges, thus reduc-

ing the muscle's shortening velocity. Alternatively, although light chain phosphorylation and shortening velocity are correlated in time, a mechanism other than light chain phosphorylation could modulate the cycling rate of the entire cross-bridge population (Butler and Siegelman, 1985).

The hypothesis that populations of cross-bridges can mechanically interact to modulate shortening velocity was tested by an in vitro motility assay (Kron and Spudich, 1986). The rate at which actin slides over smooth muscle myosin filaments containing varying proportions of unphosphorylated and phosphorylated myosin was measured. The results suggest that unphosphorylated cross-bridges impose a significant load on normally cycling phosphorylated cross-bridges. The extent of this load was estimated indirectly by studying actin filament motion on myosin copolymers containing smooth and either unmodified or modified skeletal muscle myosins. Based on these observations, a mechanistic model was developed that describes the modulation of the rate of actin movement by mechanical interactions between cross-bridges.

Materials and Methods

Contractile Protein Isolation and Preparation

Unphosphorylated smooth muscle myosin was isolated from turkey gizzards (Sellers et al., 1981). Myosin was completely thiophosphorylated upon addition of myosin light chain kinase, calmodulin, calcium, and ATP- γ -S (Trybus and Lowey, 1984), as determined by glycerol/acrylamide gels (Perrie and Perry, 1970). Skeletal muscle myosin was prepared from chicken pectoralis (Margossian and Lowey, 1982). The purified myosins were stored in monomeric form at -20°C in a 50% glycerol solution.

Unregulated actin was purified from chicken pectoralis acetone powder

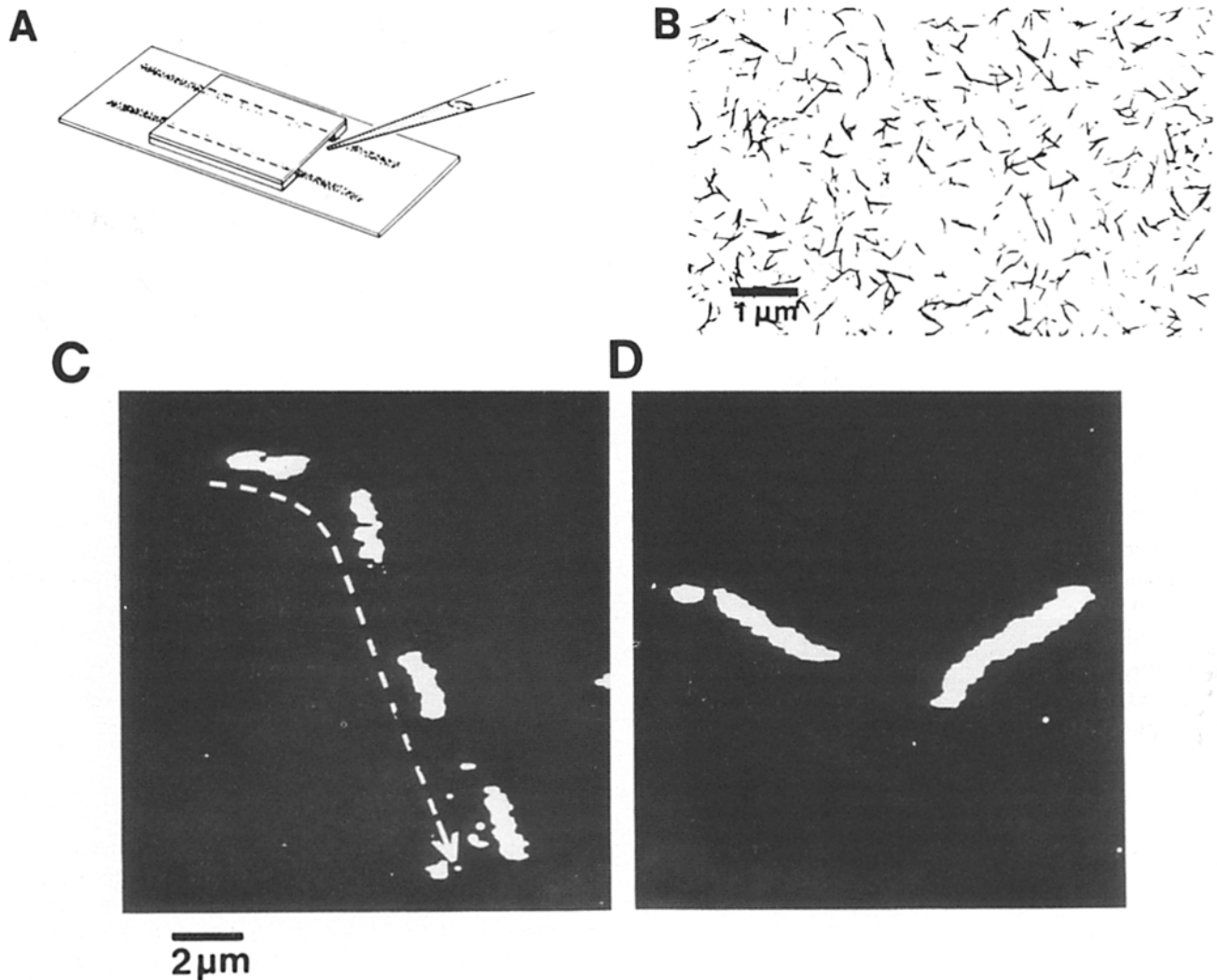


Figure 1. (A) Experimental flow-through microchamber. A coverslip, coated with nitrocellulose, was placed on two small support pieces of coverslip with the coated surface facing the microscope slide. The edges of the chamber were sealed with grease (Apiezon M) (stippled area). (B) Electron micrograph of negatively stained phosphorylated smooth muscle myosin filaments on a nitrocellulose coated coverslip at $300 \mu\text{g/ml}$. See text for details of filament preparation for electron microscopy. (C) Movement of a single fluorescently labeled actin filament in the presence of phosphorylated smooth muscle myosin. The image is a composite of four video snapshots taken 10 s apart. The average velocity of the filament in 1 mM MgATP assay buffer was $0.4 \mu\text{m/s}$. The arrow indicates the direction of motion. (D) Two individual actin filaments in the presence of unphosphorylated smooth muscle myosin. The image is a composite of three video snapshots spaced 10 s apart. There was no detectable motion since the first and last actin images were superimposable.

by the methods of Pardee and Spudich (1982) and stored in filamentous form at 4°C . Before a motility experiment, actin filaments were fluorescently labeled overnight with TRITC phalloidin (Sigma Chemical Co., St. Louis, MO) (Kron and Spudich, 1986). Protein concentrations were determined at $\lambda = 280 \text{ nm}$ using the following extinction coefficients (1 mg/ml): myosin, 0.5; actin, 1.1.

Preparation of Chemically Modified Myosin

Skeletal muscle myosin was chemically modified to produce analogues of the "weak" and "strong" actin binding conformations. Myosin that binds weakly to actin was prepared by reacting 2 mg/ml myosin with a 1.5-fold molar excess of *N,N'*-*p*-phenylenedimaleimide (pPDM)¹ in 20 mM imidazole, pH 7.5, 0.1 mM ADP, 0.2 mM MgCl_2 for 75 min on ice (Chalovich et al., 1983). The reaction was stopped by addition of 2 mM DTT. Un-

modified myosin was removed by pelleting the reaction mixture in the presence of actin without MgATP. Approximately 60% of the myosin remained in the supernatant and was assumed to be modified and in the weak binding state. Myosin that binds strongly to actin both in the presence and absence of MgATP was formed by extensive sulfhydryl modification with *N*-ethylmaleimide (NEM). Myosin (2 mg/ml) in 20 mM imidazole (pH 7.5) was reacted with 0.5–1.0 mM NEM for 40 min at room temperature, and then stopped by addition of 25 mM DTT (Pemrick and Weber, 1976). The percentage of modified myosin ($\sim 60\%$ of the total) was determined from the amount of myosin that pelleted with actin in the presence of MgATP.

Myosin Polymerization

Filamentous myosin was formed by a single step dilution of monomeric myosin in 0.3 M KCl standard buffer (25 mM imidazole, pH 7.4, 4 mM MgCl_2 , 1 mM EGTA, and 1 mM DTT) into 0.1 M KCl standard buffer. To form copolymers of either unphosphorylated and phosphorylated smooth muscle myosin or smooth and skeletal muscle myosins, the desired monomers were first mixed in 0.3 M KCl standard buffer and then diluted in 0.1 M

1. Abbreviations used in this paper: NEM, *N*-ethylmaleimide; pPDM, *N,N'*-*p*-phenylenedimaleimide.

KCl standard buffer to a final concentration of 250 $\mu\text{g/ml}$. The myosin filaments that were formed were $\sim 0.5 \mu\text{m}$ long (Fig. 1 B).

Copolymers containing unphosphorylated smooth muscle myosin can be formed at low ionic strength, but addition of MgATP depolymerizes the filament (Trybus and Lowey, 1984). Therefore, a recently developed monoclonal antibody specific for the smooth muscle myosin rod (LMM.1; Trybus and Henry, 1989; Trybus, 1989) was used to block filament depolymerization upon addition of nucleotide. After mixtures of monomers containing unphosphorylated smooth muscle myosin were polymerized, antibody LMM.1 was added in a threefold molar excess. These antibody stabilized filaments were then applied to the nitrocellulose-coated coverslip.

Actin-activated ATPase Activity

Inorganic phosphate was determined colorimetrically (Taussky and Shorr, 1953) after the reaction was stopped with SDS as described by White (1982). Rates were obtained from the average slope through three time points taken during the initial 30% of the reaction. The activity of copolymers and homopolymers of phosphorylated and unphosphorylated smooth muscle myosin was determined in 20 mM imidazole, pH 7, 75 mM KCl, 3 mM MgSO_4 , 1 mM EGTA, 37°C at V_{max} (20 μM actin, 5 μM gizzard tropomyosin) in the presence of antibody LMM.1. The actin-activated ATPase of smooth/skeletal muscle myosin copolymers was determined in 20 mM imidazole, pH 7.5, 25 mM KCl, 4 mM MgCl_2 , 1 mM EGTA, 37°C with 20 μM actin in the presence of antibody LMM.1.

Electron Microscopy

The appearance and distribution of myosin filaments on the nitrocellulose-coated coverslip (Fig. 1 B) were determined by transmission electron microscopy. Thin copper grids (300 mesh) were sandwiched between the nitrocellulose film and glass coverslip and then carbon coated (Kron and Spudich, 1986). The coverslip, containing the copper grids, was then used to construct a flow cell. As in an experiment, myosin (250 $\mu\text{g/ml}$), in 0.1 M KCl standard buffer, was perfused into the flow cell. However, after 60 s, 1% uranyl acetate was flushed through the flow cell at room temperature, negatively staining the filaments. The coverslip was then removed from the flow cell and allowed to dry on filter paper. The grids were removed and examined in a Zeiss CA-10 electron microscope operated at 60 kV. For higher magnification images of unphosphorylated smooth and skeletal muscle myosin copolymers (Fig. 4), copolymers (25–50 $\mu\text{g/ml}$) were applied directly to a carbon-coated grid, negatively stained with 1% uranyl acetate, and then viewed in a Philips EM301 electron microscope operated at 80 kV.

In Vitro Motility Assay

The in vitro motility assay has been described in detail previously (Kron and Spudich, 1986; Harada et al., 1987; Toyoshima et al., 1987; Kron et al., 1990). In brief, a 30- μl flow-through chamber was created by supporting a nitrocellulose-coated (1.0%) coverslip (18 mm², No. 1 glass) on a microscope slide by two pieces of coverslip material (Fig. 1 A). By using a micropipetter, various test solutions were perfused through the chamber. To begin an experiment, 30 μl of filamentous myosin (250 $\mu\text{g/ml}$) in 0.1 M KCl standard buffer were perfused into the chamber and the filaments allowed to adhere to the nitrocellulose for 60 s. The adherent myosin filaments were uniformly distributed in a random orientation on the coverslip (Fig. 1 B). Then 60 μl of 0.1 M KCl standard buffer containing BSA (0.5 mg/ml) were perfused through to wash out any unbound myosin and to coat any exposed nitrocellulose. Next, 30 μl of fluorescently labeled filamentous actin (0.5 ng/ml) in assay buffer (25 mM KCl standard buffer with 0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase, and 2.3 mg/ml glucose) were added twice to the flow cell and the actin filaments allowed to bind to myosin for 30 s each time. Then 60 μl of assay buffer were perfused through to wash out any free actin. Finally, actin motion was observed after 90 μl of assay buffer containing 1 mM MgATP were perfused through the chamber (Fig. 1 C). All experiments were performed at room temperature unless noted.

Detection and Analysis of Actin Filament Motion

Actin filament motion was observed using an inverted microscope (Zeiss IM) equipped for epifluorescence with a 100 W mercury lamp, and rhodamine filter set. Given the low level fluorescence, a high numerical aperture objective (63X Zeiss planapochromat, NA 1.4) and image intensified video camera (Dage 66 SIT) were used to record actin images on videotape (Sony SL-4FR70 VCR). An oxygen scavenger system was used (glucose oxidase

and catalase) to reduce fluorescence photobleaching, allowing actin filaments to be visualized continuously for at least 3 min (Kishino and Yanagida, 1988).

To determine the velocity of actin movement, video images were digitized into a 480 \times 512 pixel array by a video grabber card (Oculus 200; Coreco Inc., Ville St.-Laurent, Quebec, Canada) in a laboratory computer (IBM PC-XT). A previously developed computer program (Work and Warsaw, 1988) was modified to take video snapshots at either 3–5 or 0.3 s between images for smooth or skeletal muscle myosins, respectively (Fig. 1 C). The investigator, using a mouse, located an actin filament's leading edge in successive snapshots, thus allowing the computer to calculate a mean actin filament velocity. Given the discreet nature of the digitized video image and the time between snapshots, the actin filament velocity resolution was 0.30 $\mu\text{m/s}$ for 0.3 s and 0.035 $\mu\text{m/s}$ for 3.0 s snapshots, respectively. Only those filaments that moved continuously for at least 3 μm were included in the data set. Under conditions where no filament motion was detected, a velocity equal to the lowest velocity resolution was assigned. The actin filament velocities are presented as the mean and standard deviation of the means of at least 10 filaments for any experimental condition.

Results

Parameters Affecting Actin Sliding Velocity

Actin filament motion was highly dependent on the constituents in the assay solution. In the absence of myosin, actin floated freely in solution and did not bind to the nitrocellulose-coated coverslip. With myosin present on the coverslip, actin became rigidly bound. Upon addition of 1 mM MgATP, the actin moved in a directed and continuous fashion for distances up to 20 μm with average velocities of 0.2–0.4 $\mu\text{m/s}$ with phosphorylated smooth muscle myosin (Fig. 1 C). The motion of actin was always unidirectional; filaments never stopped to reverse their motion. Within a given visual field, >80% of the actin filaments moved.

The velocity of actin sliding was independent of the concentration of filamentous phosphorylated smooth muscle myosin between 62–1,000 $\mu\text{g/ml}$. Below this concentration (i.e., low filament density), the increased spacing between myosin filaments did not allow actin to undergo continuous motion over long (>3 μm) distances. The velocity of movement was also independent of actin filament length which varied between 0.5 and 4 μm (Fig. 2 A). Velocity was sensitive to MgATP concentration, however, as might be expected if actin filament motion reflects actomyosin interactions. At high MgATP concentrations (>1 mM), phosphorylated smooth muscle myosin moved actin filaments at $0.303 \pm 0.030 \mu\text{m/s}$ ($n = 10$), an order of magnitude slower than skeletal muscle myosin. The relationships between actin filament velocity and MgATP concentration were similar for both myosins when normalized to their respective maximum velocities (Fig. 2 B), with half maximal velocity occurring at 29 μM MgATP.

Actin velocity increased with temperature: at 22°C, the average velocity was 0.254 $\mu\text{m/s}$ compared with 0.546 $\mu\text{m/s}$ at 32°C. The apparent Q_{10} of 2.1 suggests that actin motion was caused by an active biochemical process and not simple diffusion, which would be expected to have a $Q_{10} < 1.4$. The similarity between the bell shaped pH dependence of actin filament velocity (Fig. 2 C) and the in vitro skeletal muscle actomyosin ATPase activity (Stone and Prevost, 1973) is additional evidence that actin movement is related to this enzymatic process. Between pH 7.0 and 7.5, actin filament velocity was maximal and thus all experiments were performed at pH 7.4. An ionic strength dependence of velocity was also

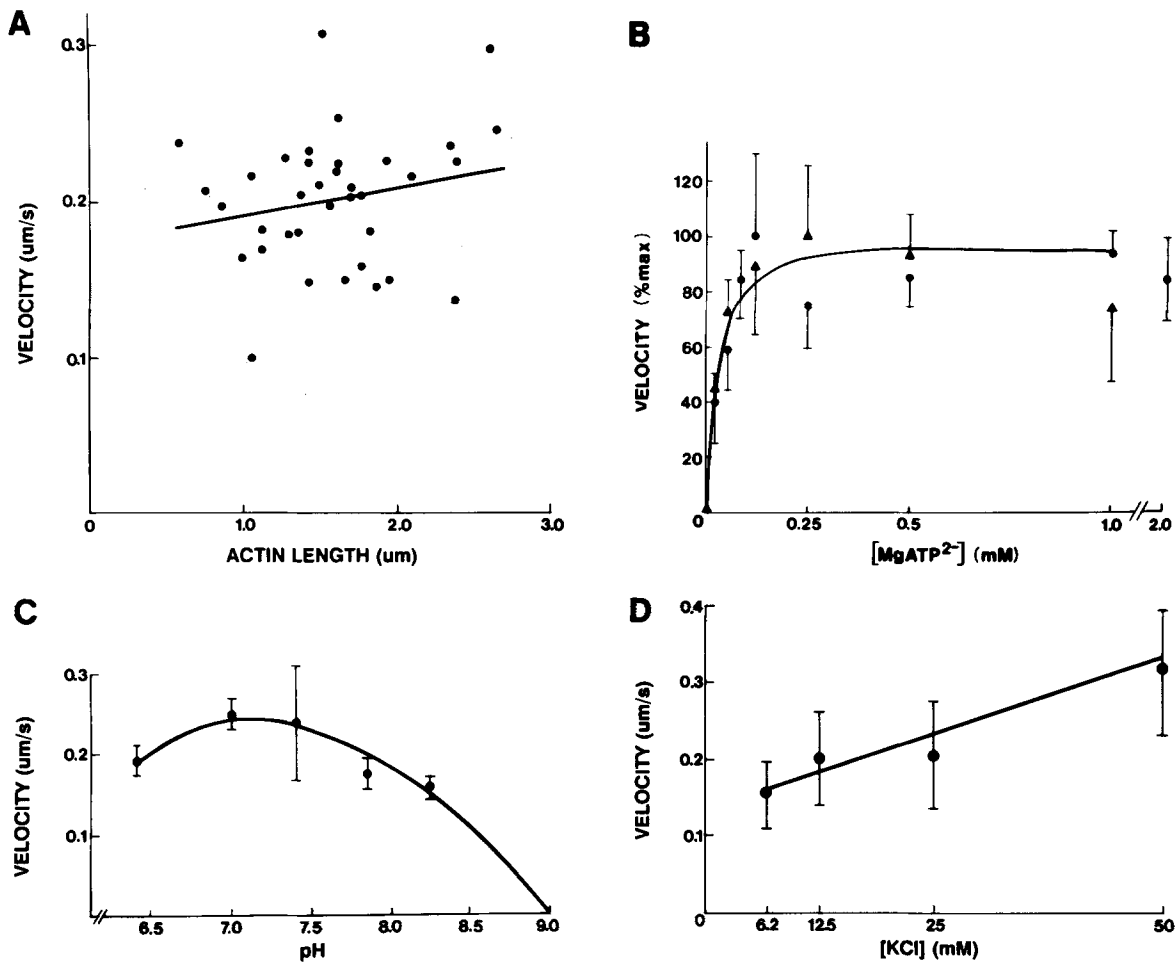


Figure 2. Parameters affecting the rate of actin movement. (A) Actin filament velocity versus actin filament length. Velocities were obtained in the presence of fully phosphorylated smooth muscle myosin, 1 mM MgATP assay buffer at 22°C. The linear regression was described by: velocity = $0.173 \mu\text{m/s} + (0.019/\text{s}) \text{ length}$; $r = 0.22$. The slope of the regression was not significantly different than zero ($P > 0.05$). Therefore actin filament velocity was independent of actin filament length. (B) Actin filament velocity versus MgATP concentration. Actin filament velocities were obtained in the presence of either fully phosphorylated smooth muscle myosin (\bullet) or skeletal muscle myosin (\blacktriangle). The velocities are normalized to their respective maximums (smooth = $0.3 \mu\text{m/s}$; skeletal = $3.25 \mu\text{m/s}$). Data points are the means and standard deviations of the means for 10 filaments. The curve was fit by eye. (C) Actin filament velocity versus pH. Curve fit by eye. (D) Actin filament velocity versus KCl concentration.

observed: the average velocity increased from $0.145 \mu\text{m/s}$ at 6 mM KCl to $0.326 \mu\text{m/s}$ at 50 mM KCl (Fig. 2 D). Above 60 mM KCl, experiments could not be done because actin binding was greatly diminished.

Unphosphorylated Smooth Muscle Myosin Inhibits Actin Movement by Phosphorylated Myosin

The rate at which actin moved over copolymers of unphosphorylated and phosphorylated smooth muscle myosin was determined to assess whether the relative amount of phosphorylated myosin within a filament affected the velocity of actin sliding. In all experiments, monoclonal antibody LMM.1 was added to prevent filaments containing unphosphorylated myosin from depolymerizing upon addition of MgATP (Trybus and Henry, 1989; Trybus, 1989). Fully phosphorylated myosin filaments moved actin at $0.21 \pm 0.05 \mu\text{m/s}$ ($n = 9$) (Fig. 3 A) in the presence and absence of antibody, suggesting that antibody LMM.1 does not alter acto-

myosin interactions. Actin also bound to unphosphorylated myosin filaments, but no movement was detected (Fig. 1 D). The rigid appearance of the bound actin was similar to that observed when actin bound to myosin in the absence of MgATP, i.e., rigor. As the percentage of phosphorylated myosin in the copolymer decreased from 100 to 50%, actin filament velocity remained constant (Fig. 3 A). At 40% phosphorylated myosin, however, the velocity fell precipitously with a half-maximal rate at 25% phosphorylated myosin. This sigmoidal relationship was observed at ionic strengths varying between 25 and 60 mM KCl. If unphosphorylated myosin had no effect on phosphorylated myosin, then actin filament velocity would have been independent of the concentration of phosphorylated myosin.

The modulation of actin filament velocity by light chain phosphorylation could be due to a change in the enzymatic activity of the myosin heads. This phenomenon, however, was not observed by measuring the solution actin-activated ATPase activity of phosphorylated/unphosphorylated co-

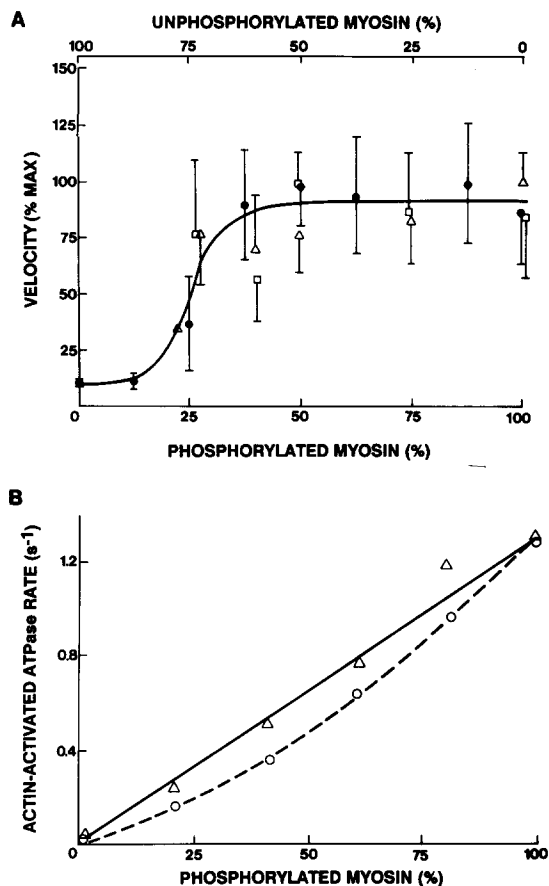


Figure 3. (A) Actin filament velocity versus the percentage of either phosphorylated (*lower axis*) or unphosphorylated (*upper axis*) smooth muscle myosin in the copolymer. Velocities are normalized to the maximums at a given KCl concentration (0.24 $\mu\text{m/s}$ at 25 mM, \bullet ; 0.32 $\mu\text{m/s}$ at 40 mM, Δ ; 0.48 $\mu\text{m/s}$ at 60 mM, \square). Curve was fit by eye through all the data. (B) Actomyosin ATPase activity versus percentage of phosphorylated smooth muscle myosin. ATPase activity was obtained for copolymers (\circ) of unphosphorylated and phosphorylated myosin and for mixtures of unphosphorylated and phosphorylated smooth muscle myosin homopolymers (Δ).

polymers. The actin-activated activity was almost linearly proportional to the concentration of phosphorylated heads within a filament and quite similar to the ATPase activity of mixtures of homopolymers where no cooperative interactions would be expected (Fig. 3 B). These data suggest that in solution, myosin heads within a filament act independently and the ATPase activity of a phosphorylated head does not change depending on the neighboring molecules. The modulation of actin filament velocity seen in the motility assay may therefore reflect a mechanical impedance that unphosphorylated cross-bridges impose on the faster cycling phosphorylated cross-bridges.

Smooth Muscle Myosin Slows the Movement of Actin by Skeletal Muscle Myosin

If unphosphorylated cross-bridges impose an internal load on faster cycling cross-bridges, it may be possible to estimate this load indirectly by copolymerizing smooth and skeletal

muscle myosins. These experiments should show if two mechanically distinct cross-bridge populations can interact to modulate actin velocity. To insure that the interactions between unphosphorylated smooth and skeletal muscle myosin cross-bridges occurred within the same filament, the copolymers were again stabilized by antibody LMM.1 to prevent depolymerization of unphosphorylated smooth muscle myosin.

The formation of copolymers between these two species was confirmed by electron microscopy. Negatively stained, antibody stabilized, smooth muscle myosin filaments (Fig. 4 C) were distinctly different from the much longer skeletal muscle myosin homopolymers (Fig. 4 D). Notice the intense striped appearance of smooth muscle myosin filaments caused by binding of antibody LMM.1 specific for smooth muscle myosin (Fig. 4 C). When copolymers of smooth and skeletal muscle myosin were formed and then decorated with antibody, all filaments showed some "striping," suggesting that smooth muscle myosin was present in all polymers. In addition, long antibody-free skeletal muscle myosin filaments were never observed in copolymer solutions (Fig. 4 A) but were always seen in mixtures of smooth and skeletal muscle myosin homopolymers (Fig. 4 B). It was not readily apparent if the copolymers were of the bipolar or sidepolar morphology.

Control experiments confirmed that antibody LMM.1 did not affect the ability of skeletal muscle myosin to move actin. When unphosphorylated smooth muscle myosin was copolymerized with skeletal muscle myosin, however, the presence of only 12.5% unphosphorylated smooth muscle myosin caused a significant reduction in velocity (Fig. 5 A). Note that much more unphosphorylated myosin was required to slow phosphorylated smooth muscle myosin (Fig. 3 A). With further addition of unphosphorylated myosin, the rate of actin movement continued to decrease and was completely inhibited once the proportion of unphosphorylated myosin reached 50%. A similar relationship between actin filament velocity and skeletal muscle myosin content was observed for copolymers of phosphorylated smooth and skeletal muscle myosins (Fig. 5 B). Once the percentage of phosphorylated myosin increased to 50%, the velocity of actin filament movement was similar to that of phosphorylated smooth muscle myosin homopolymers.

The actin-activated ATPase activity of smooth/skeletal muscle myosin copolymers was measured to determine if modulation of actin filament velocity merely reflected changes in intrinsic ATPase activity. This was not the case because the actomyosin ATPase activity was approximately a weighted average of the activities of the two myosin species. Smooth and skeletal muscle cross-bridges therefore hydrolyze MgATP independently of one another within the copolymer.

Movement of Actin over Copolymers Containing Analogues of Weak and Strong Actin Binding States

Skeletal muscle myosin can be chemically modified to produce cross-bridge states that no longer hydrolyze MgATP but bind weakly (pPDM-myosin) or strongly (NEM-myosin) to actin (Chalovich et al., 1983; Pemrick and Weber, 1976). These modified myosins were copolymerized with either phosphorylated smooth or unmodified skeletal muscle myosin to assess how cross-bridges with different binding strengths to actin can modulate the velocity of actin movement.

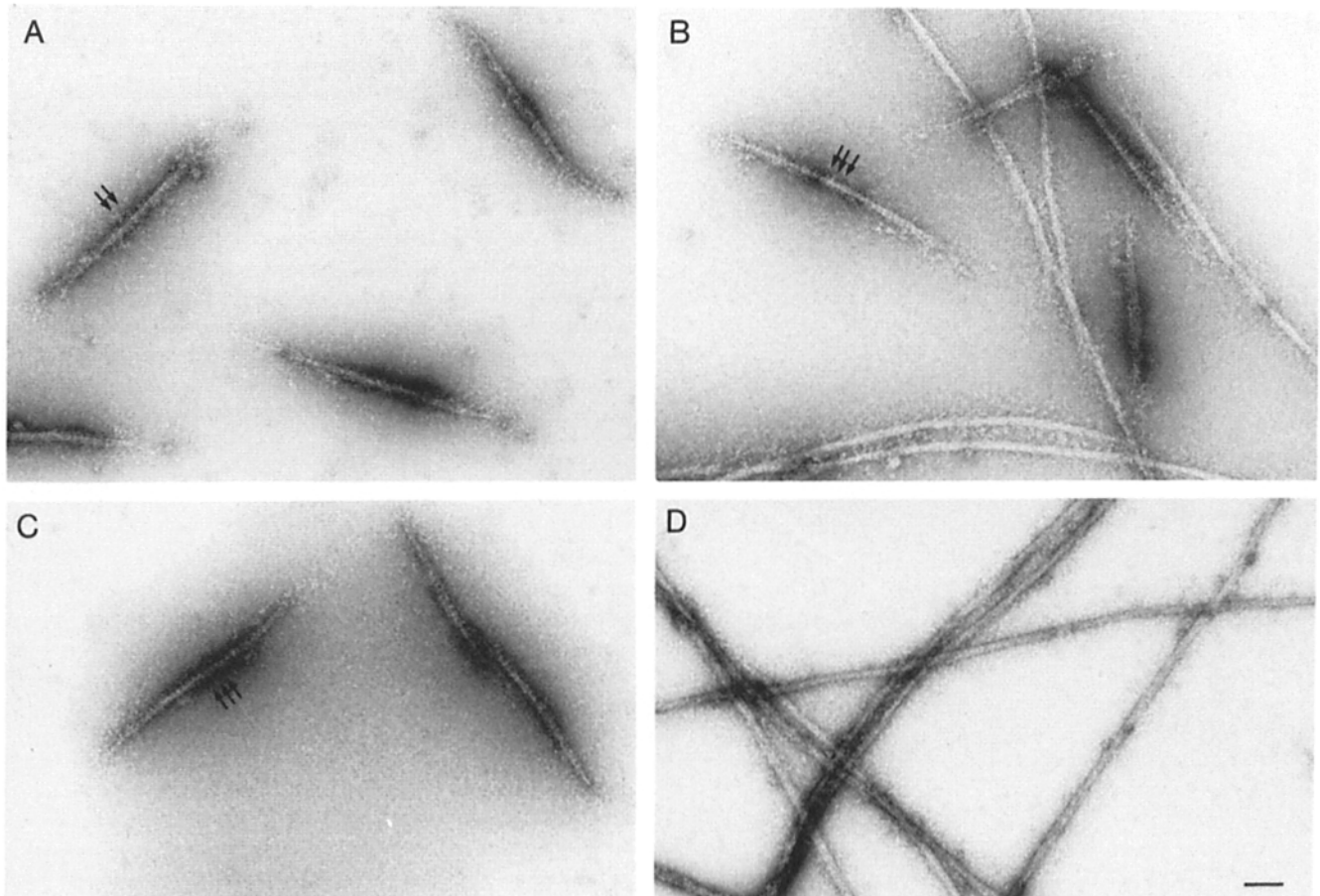


Figure 4. Electron micrographs of negatively stained myosin filaments in the presence of antibody LMM.1. (A) Copolymers of unphosphorylated smooth and skeletal muscle myosin (equal proportions). (B) Homopolymers of unphosphorylated smooth and skeletal muscle myosin (equal mixtures). (C) Unphosphorylated smooth muscle myosin homopolymers. (D) Skeletal muscle myosin homopolymers. Arrowheads identify 15-nm periodic antibody binding. Bar, 0.1 μm .

The presence of as little as 1.0% of the strong binding analogue (NEM-myosin) in a copolymer was sufficient to completely inhibit movement of actin by either phosphorylated smooth or unmodified skeletal muscle myosin. Once the proportion of NEM-myosin within the copolymer was reduced to <1.0%, actin moved with a velocity equal to that observed for homopolymers of phosphorylated smooth or unmodified skeletal muscle myosin.

The effect of the weak binding myosin analogue (pPDM-myosin) on actin velocity was more gradual (Fig. 6). 10% pPDM-myosin copolymerized with unmodified skeletal muscle myosin reduced actin filament velocity below that observed for skeletal muscle myosin homopolymers. The velocity was further reduced as the proportion of pPDM-myosin within the copolymer increased until actin filament motion was completely inhibited in the presence of 50% pPDM-myosin. In contrast, a greater proportion of pPDM-myosin (i.e., 75%) was needed to affect the movement of actin by phosphorylated smooth muscle myosin. The pattern of modulation of actin filament velocity by the weak binding analogue is strikingly similar to that observed when unphosphorylated smooth muscle myosin was copolymerized with phosphorylated smooth or skeletal muscle myosin.

Discussion

Smooth muscle's high economy of force maintenance during prolonged isometric contractions is in part due to a reduction in the cross-bridge cycling rate with time of contraction (Siegman et al., 1980; Dillon et al., 1981). One mechanism that could account for a lower cycling rate during force maintenance is a mechanical interaction between two cross-bridge populations within the same myosin filament, i.e., rapid cycling phosphorylated and slowly or noncycling dephosphorylated cross-bridges (Dillon et al., 1981). This hypothesis was tested here by observing the rate of movement of fluorescently labeled actin filaments on phosphorylated/unphosphorylated smooth muscle myosin copolymers. The results suggest that the apparent modulatory role of light chain phosphorylation is a consequence of mechanical interactions between cross-bridges having different cycling rates and strengths of binding to actin.

Key Features of the Motility Assay

Although the *in vitro* movement of actin by skeletal muscle myosin has been well characterized (Kron and Spudich, 1986; Harada et al., 1987), a detailed study of actin filament

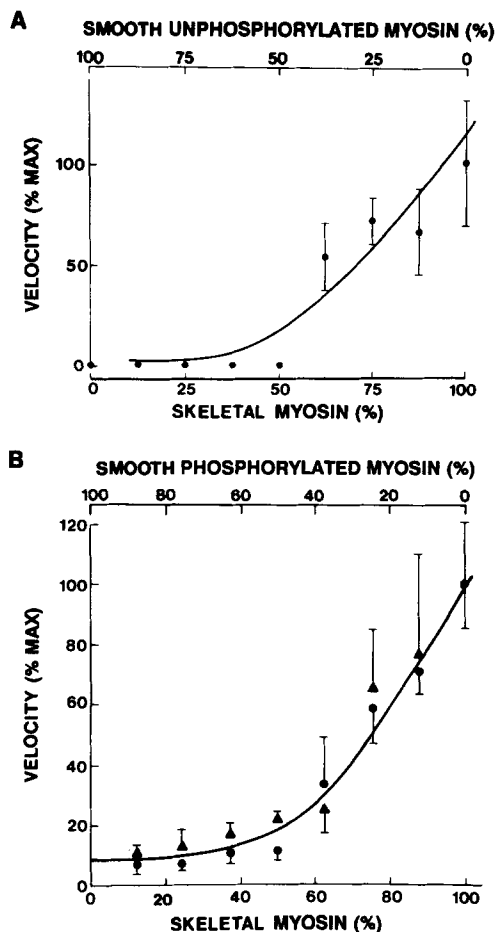


Figure 5. (A) Actin filament velocity versus percentage of unphosphorylated smooth (*upper axis*) or skeletal muscle myosin (*lower axis*) in the copolymer. Copolymers were stabilized by antibody against the depolymerizing effect of MgATP. Since no motion was detected at proportions of unphosphorylated smooth muscle myosin that exceeded 50%, the minimum resolvable velocity was assigned. Velocities are normalized to the maximum for skeletal muscle myosin (2.17 $\mu\text{m/s}$). (B) Actin filament velocity versus percentage of phosphorylated smooth (*upper axis*) or skeletal muscle myosin (*lower axis*) in the copolymer. Different symbols represent two different experiments. Velocities are normalized to the maximum for skeletal muscle myosin (\bullet , 3.87 $\mu\text{m/s}$; \blacktriangle , 2.38 $\mu\text{m/s}$). Curves were fit by eye.

motion on smooth muscle myosin has not been reported. Some of the key features are described below. The velocity at which actin moves over phosphorylated smooth muscle myosin is independent of actin filament length (Fig. 2 A), as observed previously for skeletal muscle myosin (Kron and Spudich, 1986; Harada et al., 1987). These data suggest that although increased actin filament lengths provide more opportunity for cross-bridge interactions, actin velocity is independent of cross-bridge number. This may be analogous to the observation that in intact skeletal muscle, the maximum shortening velocity is independent of the extent of actin and myosin filament overlap within the sarcomere (i.e., number of cycling cross-bridges) (Edman, 1979).

Phosphorylated smooth muscle myosin, like skeletal myosin, propels actin filaments at a rate dependent on MgATP

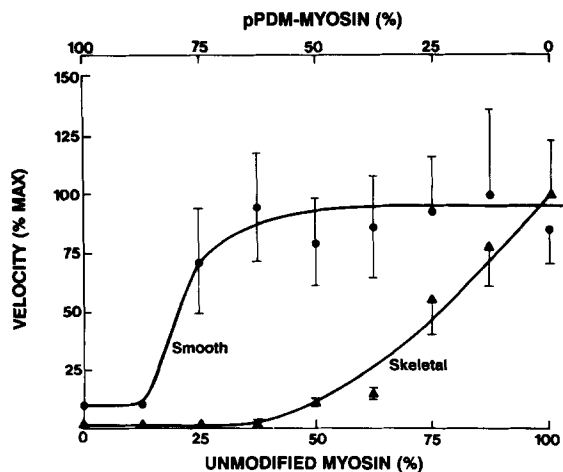


Figure 6. Actin filament velocity versus the percentage of pPDM-modified myosin copolymerized with either unmodified phosphorylated smooth or skeletal muscle myosin. Velocities are normalized to their respective maximums (smooth, 0.23 $\mu\text{m/s}$; skeletal, 2.46 $\mu\text{m/s}$). Curves fit by eye.

concentration (Fig. 2 B) (Kron and Spudich, 1986; Harada et al., 1987). The MgATP concentration (29 μM) for half-maximal velocity in the motility assay is, however, 10 times greater than the K_m for the actomyosin ATPase of isolated proteins in solution (Moos, 1973). A possible explanation for this difference is that while a small amount of rigor cross-bridges can impede the movement of actin by cycling cross-bridges (Kron and Spudich, 1986; Harada et al., 1987), a small population of rigor cross-bridges does not greatly affect the overall turnover of MgATP in solution. If this interpretation is correct, then the motility assay provides additional information about mechanical interactions between cross-bridges which can not be obtained from solution biochemistry.

Modulation of the Velocity of Actin Movement

If changes in the state of myosin light chain phosphorylation act only as a switch to turn rapid cross-bridge cycling on and off, then the velocity of actin movement should be independent of the percentage of phosphorylated cross-bridges within the myosin filament. The observation that actin velocity depended on the ratio of phosphorylated to unphosphorylated myosin suggests that these two cross-bridge populations can also interact mechanically. The experiments described here also established that small amounts of smooth muscle myosin can inhibit movement of actin by the much faster cycling skeletal muscle myosin.

A somewhat similar, but less steep relationship between actin velocity and the percentage of phosphorylated myosin in smooth muscle myosin copolymers was obtained by Sellers et al. (1985) using an earlier version of the motility assay. In this assay, myosin-coated polystyrene beads moved on actin cables in the dissected wall of the algae, *Nitella*. The smooth muscle myosin copolymers, in this earlier study, were not stabilized to disassembly by nucleotide, thus the initial proportion of phosphorylated and unphosphorylated cross-bridge heads within a myosin filament probably were not maintained throughout the assay. Here, a monoclonal an-

tibody was used to block filament disassembly. In addition, each bead in the *Nitella* assay was coated with a thick matrix of aggregated myosin filaments (see Fig. 2 in Sellers et al., 1985). Here, an even distribution of filaments was laid onto nitrocellulose (Fig. 1 B). Therefore the observed bead velocity did not result from as simplified a contractile protein system as in the present study where ~50 cross-bridge heads produce motion of a single actin filament.

Based on biochemical evidence, it is surprising that unphosphorylated smooth muscle myosin cross-bridges can cause such a large resistance to actin movement. The rate-limiting step for MgATP hydrolysis by unphosphorylated smooth muscle myosin is the release of inorganic phosphate from the complex of actomyosin and the products of ATP hydrolysis (Sellers, 1985). This step in the cross-bridge cycle is believed to be associated with the transition from a weakly to a strongly bound cross-bridge state. Therefore, unphosphorylated smooth muscle myosin cross-bridges should exist predominantly in a weakly bound state, rapidly attaching and detaching from actin (Eisenberg et al., 1980). Experimentally, unphosphorylated filaments bind strongly enough to actin in the presence of MgATP to prevent the actin from freely floating into solution. One might have assumed, however, that such a rapid attachment and detachment from actin (>1,000/s) would offer little resistance to actin movement. This assumption is based on mechanical studies in skinned skeletal muscle fibers which show that in relaxed muscle, cross-bridges at physiological ionic strength exist predominantly in a weakly bound state that offers little resistance to stretch. When the ionic strength of the bathing solution is lowered, the cross-bridges bind more tightly and only then can the weakly bound cross-bridges be detected through measurements of fiber stiffness (Brenner et al., 1982). Because the *in vitro* motility assay is performed at low ionic strength, the apparent load that unphosphorylated smooth muscle myosin cross-bridges place on faster cycling phosphorylated cross-bridges may be higher than at physiological ionic strength. Smooth muscle myosin shows less of an ionic strength dependence of binding to actin than skeletal muscle myosin (Greene et al., 1983), however, such that unphosphorylated smooth muscle myosin cross-bridges may exert a drag to movement even at physiological ionic strength.

Given this interpretation, how can the relaxed state in smooth muscle be achieved? One possibility is that the smooth muscle cell contains a protein that prevents unphosphorylated myosin from attaching to actin in the relaxed state. Actin binding proteins such as caldesmon may serve this regulatory role as suggested by their ability to inhibit actomyosin ATPase activity *in vitro* (Clark et al., 1986). Alternatively, Siegman et al. (1976) have reported calcium-dependent resistance to stretch in relaxed taenia coli, suggesting that significant numbers of presumably unphosphorylated cross-bridges may be attached in relaxed muscle, in agreement with these motility data.

In an attempt to show by another approach that biochemically defined weakly bound cross-bridges are capable of inhibiting fast cycling cross-bridges, skeletal muscle myosin was modified with pPDM to produce an analogue of a weakly bound noncycling cross-bridge (Chalovich et al., 1983). Copolymers containing either phosphorylated smooth or skeletal muscle myosin and the weakly bound cross-bridge

analogue showed reduced velocities as the proportion of pPDM-myosin within the myosin filament increased, confirming that weakly bound noncycling cross-bridges have the ability to impede faster cycling cross-bridges. Both the qualitative and quantitative similarities in the modulation of actin filament velocity caused by unphosphorylated smooth muscle myosin and pPDM-myosin suggest that these two cross-bridge species are mechanically indistinguishable. *In vitro*, therefore, unphosphorylated smooth muscle myosin cross-bridges most likely exist in a state that is weakly bound to actin (Sellers, 1985).

How can weakly bound cross-bridges exert a load to faster cycling cross-bridges? One possible explanation is that the rates of attachment and detachment are strain dependent (Somlyo et al., 1988). Any weakly bound cross-bridges that are attached to actin could be strained as the filament moves. This could result in altered kinetics such that the weakly bound cross-bridges become more tightly bound and then retard the faster cycling cross-bridges.

The most profound effect on actin filament velocity was observed when the strong binding myosin analogue (NEM-myosin) was present in a copolymer. Only 1.0% NEM-myosin was needed to completely inhibit actin motion. Given that the dissociation constant for this strongly bound cross-bridge analogue is very low, it is not surprising that these cross-bridges can anchor actin to the myosin copolymer. A similar reduction in the rate of actin movement occurs when nonsaturating concentrations of MgATP are used in the motility assay, and small amounts of rigor bridges are present.

Model for Cross-bridge Mechanical Interactions

To explain the observed relationships between actin velocity and myosin filament composition, the following cross-bridge interaction model was developed (see Appendix for details). The model's basic premise is that if a filament contains myosins that have different cycling rates and affinities for actin, then the mechanical interaction between these cross-bridges determines the overall cycling rate of the entire cross-bridge population.

Based on our present understanding of the cross-bridge mechanism in muscle, the model's most crucial assumptions are the following. (a) Cross-bridges act independently. (b) The ability of a cycling cross-bridge to generate force and move an actin filament is described by a hyperbolic force versus velocity relationship similar to that observed in the whole muscle (see Fig. 7 C). (c) As fast cycling cross-bridges propel an actin filament, the compression (i.e., negative strain) of attached, slower cycling or weakly bound cross-bridges results in an internal load that opposes the faster cycling cross-bridges; (d) The internal load (i.e., negative force) and the velocity at which the slower or weakly bound cross-bridges are being compressed are described by a force/compression velocity relationship. For cycling cross-bridges, this relationship may be continuous with the force/velocity relationship defined for positive forces (evidence for this is based on the report by Edman (1979) in whole skeletal muscle); (e) Viscous drag on the moving actin filament is negligible (Sheetz and Spudich, 1983).

To implement the model, force/velocity relationships must be specified for the various myosins. Since these relation-

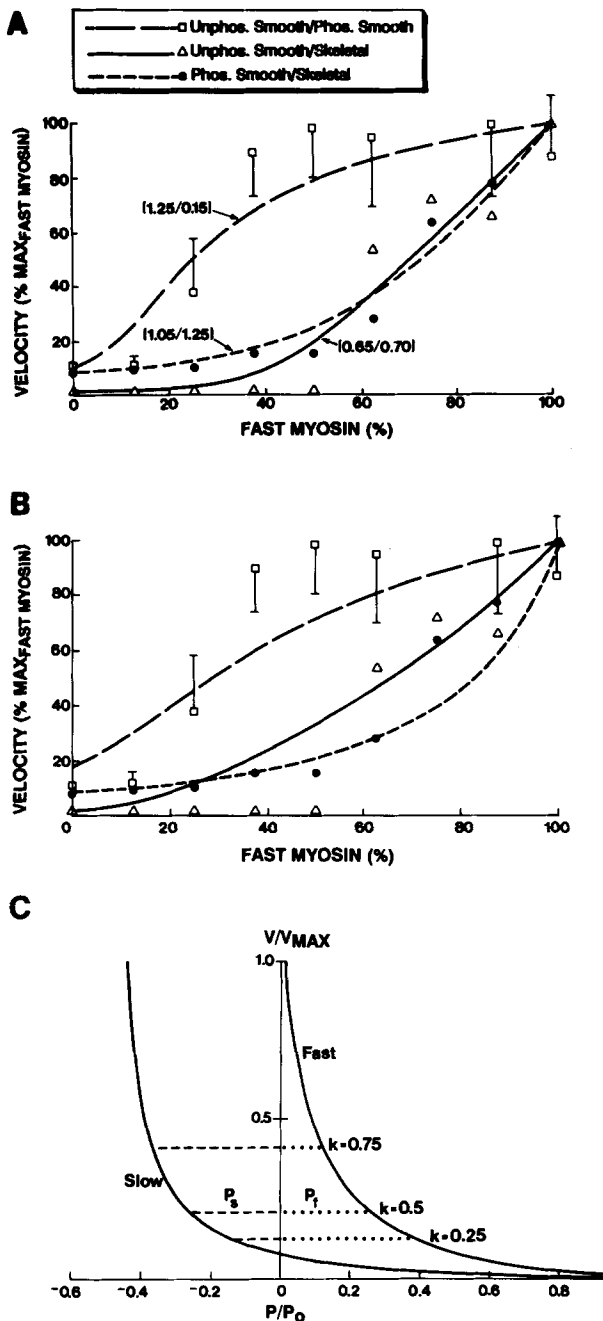


Figure 7. Predicted relationships for actin filament velocity versus myosin copolymer composition using a model for crossbridge mechanical interactions (see Appendix). (A) Observed data (open and solid symbols) from individual copolymer mixtures were optimally fitted (solid and dashed curves) using hypothetical crossbridge force/velocity relationships having a/P_0 values in parentheses next to each curve [(fast a/P_0)/(slow a/P_0)]. (Observed data taken from Fig. 3 A at 25 mM KCl, 5A and 5B). Standard deviation bars were omitted for the smooth/skeletal copolymer data for clarity. (B) Copolymer data were simultaneously fitted using a single set of force:velocity relationships. (a/P_0 ; phosphorylated smooth, 0.60; unphosphorylated smooth, 0.15; skeletal, 0.10.) With this more stringent test of the model, the curvature of the predicted relationships follow the data, but the actual fits are somewhat poorer than in A, particularly for the smooth/skeletal copolymers. (C) Hypothetical cross-bridge force/velocity relationships for fast and slow myosins. The predicted actin velocities for myosin copolymers having 25, 50, or 75% fast myosin are graphically displayed. For exam-

ships were not available for the myosins studied, force/velocity relationships were assumed. The curves were, however, constrained by curvatures (i.e., hyperbolic constant of a/P_0) reported in the literature for smooth and skeletal muscles (Hellstrand and Paul, 1982; Woledge et al., 1985) and by measured maximum actin filament velocities in the motility assay. With these constraints, the observed relationship between actin filament velocity and myosin filament composition (Fig. 7 A) could be adequately fit to within one standard deviation of the actual data for any given copolymer. However, when all the various smooth and skeletal muscle myosin copolymer data were simultaneously fitted with a single set of force/velocity relationships, the general shapes of the predicted relationships were appropriate but the fits were not as good (see Fig. 7 B). The poorer fit for this stringent test might indicate that other phenomena, such as cooperativity between neighboring cross-bridges, may be a factor in determining actin filament velocity. Another possibility is that the force/velocity relationship from whole muscle is too macroscopic a relationship to adequately describe mechanical events in the motility assay. A critical test of the model would be to measure actin filament velocities using myosins from muscles that have well characterized force/velocity relationships. Nevertheless, this simple model, in which slowly cycling or weakly bound cross-bridges mechanically oppose the propulsive forces of faster cycling cross-bridges, does serve an important first step in explaining the myosin copolymer data.

If the model's assumptions are correct, then unphosphorylated smooth muscle myosin cross-bridges can sustain negative forces. This would support the earlier contention of Murphy and co-workers that dephosphorylated cross-bridges present an internal load to the cycling phosphorylated population (Dillon et al., 1981). However, Hai and Murphy (1988) have more recently suggested that the overall cycling rate for a cross-bridge population containing both dephosphorylated and phosphorylated bridges is merely a weighted average of the two populations rather than a mechanical interaction between heads. This modification to the latchbridge hypothesis would accommodate the findings of Butler et al. (1986) who argue against an internal load based on energetic considerations. These motility data, however, strongly suggest that in vitro, cross-bridges do mechanically interact.

Conclusions

The ability of unphosphorylated smooth muscle myosin to impede skeletal muscle myosin to a greater extent than it does phosphorylated smooth muscle myosin may indicate that smooth muscle cross-bridges spend a greater fraction of their cycle time in the strongly bound, high-force producing state (i.e., greater duty cycle) than skeletal myosin cross-bridges. This conclusion agrees with physiological data (Warsaw and Fay, 1983; Warsaw, 1987; Yamakawa et al., 1990), which suggest an increased duty cycle for smooth

ple, when the fraction of fast myosin (k) is 0.75, the predicted velocity of 0.41 V_{max} is achieved when $P_s = -3P_f$ (see Eq. 5 in Appendix). At this value, the propelling force of the faster cycling crossbridges (P_f , dotted line) is opposed by the compressive force of the slower cycling crossbridges (P_s , dashed line) so that the net force equals zero.

muscle myosin as a possible explanation for the ability of smooth muscle to generate comparable forces to skeletal muscle with far less myosin (Cohen and Murphy, 1979).

The in vitro motility assay has proven to be a valuable tool for studying interactions between myosin cross-bridges within a filament. The model proposed here may also be generally applied to the mechanics of whole muscles in which multiple myosin isoforms are expressed. In the developing rat soleus muscle, for example, both fast and slow myosin isoforms are simultaneously expressed. Correlation of maximum shortening velocity with myosin isoform composition suggests that the slower isoforms profoundly affect the muscle's maximum shortening velocity (Reiser et al., 1988) as is the case with actin velocity in the motility assay. Further studies will be needed, however, to address the question of why weakly bound cross-bridges (i.e., unphosphorylated smooth muscle myosin or pPDM-treated myosin) offer substantial resistance to actin filament motion. Direct measurement of the force produced by unphosphorylated smooth muscle cross-bridges interacting with a single actin filament may resolve this question (Kishino and Yanagida, 1988).

Appendix

Model for Cross-bridge Mechanical Interactions in Myosin Copolymers

A model has been developed to predict the velocity at which actin filaments move over myosin copolymers. The model's basic assumptions are described in the Discussion. Additional assumptions and details of the calculations are presented here.

Model Assumptions

(a) In a myosin copolymer consisting of fast (*f*) and slow (*s*) cycling cross-bridges, the cross-bridge force/velocity relationships (*P/V*) for the individual myosin species are assumed to be rectangular hyperbolas (Hill, 1938) and to exist for both positive and negative forces:

$$V_f = b_f \{ (P_o_f + a_f) / (P_f + a_f) \} - 1 \quad (1)$$

$$V_s = b_s \{ (P_o_s + a_s) / (P_s + a_s) \} - 1, \quad (2)$$

where *P_o* is the maximum positive cross-bridge force and *a* and *b* are constants that define the shape of the force/velocity relationship. For all equations and assumptions described in this Appendix, the term "weakly bound" can be substituted for "slow cycling." However, weakly bound bridges are assumed not to cycle and thus do not generate positive force. Therefore only a force/compression velocity relationship need be described (i.e., *P* < 0) for weakly bound bridges.

Additional assumptions for the cross-bridge force/velocity relationships are that fast and slow cycling cross-bridges: (a) generate comparable maximum forces, *P_{o_f}* = *P_{o_s}* (this is not a necessary assumption); (b) have maximum velocities (*V_{max}*) at zero force that are measured in the motility assay and normalized to the actin filament velocity using skeletal muscle myosin (*V_{max}* of Skeletal = 1.0; Smooth Phos. = 0.08; Smooth Unphos. = 0.014); (c) have force/velocity relationships with *a/P_o* values (i.e., curvatures) within the range of literature values (0.1–1.25). The exact value was selected by a least squares optimization routine to give the best fit of

the model to the observed actin filament velocity versus myosin filament composition data.

(b) If actin filaments are rigid, then any cross-bridge attached to an actin filament must move at the same velocity as the actin filament (*V_a*), regardless of the cross-bridge's inherent cycling rate. Therefore any fast and slow cycling cross-bridges that are attached to the same moving actin filament must have equal velocities:

$$V_a = V_s = V_f. \quad (3)$$

(c) Cross-bridges within a myosin filament are assumed to act mechanically in parallel. Therefore the mean cross-bridge force (*P̄*) equals the sum of the fast and slow cycling cross-bridge force contributions:

$$\bar{P} = kP_f + (1 - k)P_s, \quad (4)$$

where *k* and (1 - *k*) are the fractions of fast and slow cycling cross-bridges within the myosin copolymer, respectively.

(d) Assuming that actin filaments do not experience any significant viscous drag (see calculations for relatively large myosin coated beads in Sheetz and Spudich, 1983), then both the external force on the actin filament and *P̄* equal zero as the actin filament freely moves over myosin. Therefore from Eq. 4:

$$kP_f = (k - 1)P_s. \quad (5)$$

Thus for actin filament movement, the fast cycling cross-bridge population must generate a force that is equal and opposite to the internal load created by the compression or negative strain of the slower cross-bridge population.

Model Calculations and Predictions

To predict the resultant actin filament velocity for a given myosin copolymer, one must first determine the internal load (*P_i*) of the slowly cycling cross-bridge population. For any fraction of slow cycling cross-bridges, *P_s* can be obtained algebraically by solving Eq. 5 for *P_f* and substituting *P_f* into Eq. 1. After this substitution, Eqs. 1 and 2 are set equal to each other as in Eq. 3. These manipulations result in a quadratic equation that can be solved for *P_s*:

$$A_1 P_s^2 + A_2 P_s + A_3 = 0,$$

where:

$$A_1 = (b_s - b_f) (k - 1) / k,$$

$$A_2 = (b_s - b_f) (a_f + a_s(k - 1) / k) + b_f(P_o_f + a_f) - b_s(k - 1) / k (P_o_s + a_s), \text{ and}$$

$$A_3 = (b_s - b_f) a_f a_s + b_f(P_o_f a_s + a_s a_f) - b_s a_f (P_o_s + a_s),$$

with *A₁*, *A₂*, and *A₃* in terms of the force/velocity constants (*a* and *b*, where *b* = *V_{max}*[*a/P_o*]), maximum cross-bridge force, and fractions of fast and slow cycling cross-bridges.

Once the internal load (*P_i*) is determined for any mixture of fast and slow cross-bridges, the actin filament velocity (*V_a*) associated with this mixture is equal to *V_s* and calculated from Eq. 2.

The model predictions can also be obtained through graphical analysis (see Fig. 7 C). The shapes of the force/velocity relationships are critical to the predictions and may require revision once the techniques to measure the cross-bridge force/velocity relationship in the motility assay have been developed. For a sample prediction, the actin filament

velocity for a copolymer containing equal fractions of slow and fast myosin (i.e., $k = 0.5$) can be determined from the condition where the positive propelling force of the faster cycling bridges (P_f) equals the opposing negative force of the slower bridges (P_s). For other proportions, one scales along the force axis so that the P_f equals $[(k - 1)/k]P_s$. As the fraction of fast cycling cross-bridges is reduced, fewer fast cycling bridges share the slow cross-bridges' internal load. This results in an increased force per fast cycling cross-bridge and decreased cycling rate as predicted by the force/velocity relationship.

We thank Dr. Stephen Kron for his patience and assistance in the development of the motility assay and Drs. Ariano and Forehand for the use of their microscope and video camera.

This work was supported by grants from the National Institutes of Health HL35684 to D. Warsaw and HL38113 to K. Trybus. D. Warsaw is an Established Investigator of the American Heart Association.

Received for publication 5 December 1989 and in revised form 5 March 1990.

References

Brenner, B., M. Schoenberg, J. M. Chalovich, L. E. Greene, and E. Eisenberg. 1982. Evidence for cross-bridge attachment in relaxed muscle at low ionic strength. *Proc. Natl. Acad. Sci. USA.* 79:7288-7291.

Butler, T. M., and M. J. Siegman. 1985. High-energy phosphate metabolism in vascular smooth muscle. *Annu. Rev. Physiol.* 47:629-643.

Butler, T. M., M. J. Siegman, and S. U. Mooers. 1986. Slowing of cross-bridge cycling in smooth muscle without evidence of an internal load. *Am. J. Physiol.* 251:C945-C950.

Chalovich, J. M., L. E. Greene, and E. Eisenberg. 1983. Crosslinked myosin subfragment-1: A stable analogue of the subfragment-1 · ATP complex. *Proc. Natl. Acad. Sci. USA.* 80:4909-4913.

Clark, T., P. K. Nagai, C. Sutherland, U. Groschel-Stewart, and M. P. Walsh. 1986. Vascular smooth muscle caldesmon. *J. Biol. Chem.* 261:8028-8035.

Cohen, D. M., and R. A. Murphy. 1979. Cellular thin filament protein contents and force generation in porcine arteries and veins. *Circ. Res.* 45:661-665.

Dillon, P. F., M. O. Aksoy, S. P. Driska, and R. A. Murphy. 1981. Myosin phosphorylation and the crossbridge cycle in arterial smooth muscle. *Science (Wash. DC).* 211:495-497.

Edman, K. A. P. 1979. The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibers. *J. Physiol. (Lond.).* 291:143-159.

Eisenberg, E., T. L. Hill, and Y. Chen. 1980. Cross-bridge model of muscle contraction: quantitative analysis. *Biophys. J.* 29:195-227.

Fay, F. S., D. D. Rees, and D. M. Warsaw. 1981. The contractile mechanism in smooth muscle. In *Membrane Structure and Function*. E. E. Bitar, editor. John Wiley & Sons, Inc. New York. 4:79-130.

Greene, L. E., J. R. Sellers, E. Eisenberg, and R. S. Adelstein. 1983. Binding of gizzard smooth muscle myosin subfragment 1 to actin in the presence and absence of adenosine 5'-triphosphate. *Biochemistry.* 22:530-535.

Hai, C.-M., and R. A. Murphy. 1988. Regulation of shortening velocity by crossbridge phosphorylation in smooth muscle. *Am. J. Physiol.* 255:C86-C94.

Harada, Y., A. Noguchi, A. Kishino, and T. Yanagida. 1987. Sliding movement of single actin filaments on one-headed myosin filaments. *Nature (Lond.).* 326:805-808.

Hellstrand, P., and R. J. Paul. 1982. Vascular smooth muscle: relations between energy metabolism and mechanics. In *Vascular Smooth Muscle: Metabolic, Ionic, and Contractile Mechanisms*. M. S. Crass III and C. D. Barns, editors. Academic Press, Inc., New York. 1-35.

Hill, A. V. 1938. The heat of shortening and the dynamic constants of muscle. *Proc. R. Soc. Lond. Ser. B.* 126:136-195.

Kamm, K. E., and J. T. Stull. 1985. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* 25:593-620.

Kishino, A., and T. Yanagida. 1988. Force measurements by micromanipulation of a single actin filament by glass needles. *Nature (Lond.).* 334:74-76.

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

Kron, S. J., Y. Y. Toyoshima, T. Q. P. Uyeda, and J. A. Spudich. 1990. Assays for actin sliding movement over myosin coated surfaces. *Methods Enzymol.* In press.

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

Moos, C. 1973. Actin activation of heavy meromyosin and subfragment-1 ATPases; steady state kinetic studies. *Cold Spring Harbor Symp. Quant. Biol.* 37:137-143.

Murphy, R. A. 1980. Mechanics of vascular smooth muscle. *Handb. Physiol.* II:325-351.

Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *Methods Enzymol.* 85:164-181.

Paul, R. J., E. Gluck, and J. C. Ruegg. 1976. Cross bridge ATP utilization in arterial smooth muscle. *Pfluegers Arch. Eur. J. Physiol.* 361:297-299.

Pemrick, S., and A. Weber. 1976. Mechanism of inhibition of relaxation by N-ethylmaleimide treatment of myosin. *Biochemistry.* 15:5193-5198.

Perrie, W. T., and S. V. Perry. 1970. An electrophoretic study of the low-molecular-weight components of myosin. *Biochem. J.* 119:31-38.

Reiser, P. J., C. E. Kasper, M. L. Greaser, and R. L. Moss. 1988. Functional significance of myosin transitions in single fibers of developing soleus muscle. *Am. J. Physiol.* 254:C605-C613.

Sellers, J. R. 1985. Mechanism of the phosphorylation-dependent regulation of smooth muscle heavy meromyosin. *J. Biol. Chem.* 260:15815-15819.

Sellers, J. R., M. D. Pato, and R. S. Adelstein. 1981. Reversible phosphorylation of smooth muscle myosin, heavy meromyosin, and platelet myosin. *J. Biol. Chem.* 256:13137-13142.

Sellers, J. R., J. A. Spudich, and M. P. Sheetz. 1985. Light chain phosphorylation regulates the movement of smooth muscle myosin on actin filaments. *J. Cell Biol.* 101:1897-1902.

Sheetz, M. P., and J. A. Spudich. 1983. Movement of myosin-coated fluorescent beads on actin cables in vitro. *Nature (Lond.).* 303:31-35.

Siegman, M. J., T. M. Butler, S. U. Mooers, and R. E. Davies. 1976. Rigor and resistance to stretch in vertebrate smooth muscle. *Am. J. Physiol.* 231:1509-1514.

Siegman, M. J., T. M. Butler, S. U. Mooers, and R. E. Davies. 1980. Chemical energetics of force development, force maintenance, and relaxation in mammalian smooth muscle. *J. Gen. Physiol.* 76:609-629.

Somlyo, A. V., Y. Goldman, T. Fugimori, M. Bond, D. Trentham, and A. P. Somlyo. 1988. Cross-bridge kinetics, cooperativity, and negatively strained cross-bridges in vertebrate smooth muscle. *J. Gen. Physiol.* 91:165-192.

Stone, D. B., and S. C. Prevost. 1973. Characterization of modified myosin at low ionic strength. Enzymatic and spin-label studies. *Biochemistry.* 12:4206-4211.

Taussky, H. H., and E. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* 202:675-685.

Toyoshima, Y. Y., S. J. Kron, E. M. McNally, K. R. Niebling, C. Toyoshima, and J. A. Spudich. 1987. Myosin subfragment-1 is sufficient to move actin filaments in vitro. *Nature (Lond.).* 328:536-539.

Trybus, K. M. 1989. Filamentous smooth muscle myosin is regulated by phosphorylation. *J. Cell Biol.* 109:2887-2894.

Trybus, K. M., and L. Henry. 1989. Monoclonal antibodies detect and stabilize conformational states of smooth muscle myosin. *J. Cell Biol.* 109:2879-2886.

Trybus, K. M., and S. Lowey. 1984. Conformational states of smooth muscle myosin. Effects of light chain phosphorylation and ionic strength. *J. Biol. Chem.* 259:8564-8571.

Warsaw, D. M. 1987. Force-velocity relationship in single isolated toad stomach smooth muscle cells. *J. Gen. Physiol.* 89:771-789.

Warsaw, D. M., and F. S. Fay. 1983. Cross-bridge elasticity in single smooth muscle cells. *J. Gen. Physiol.* 82:157-199.

White, H. D. 1982. Special instrumentation and techniques for kinetic studies of contractile systems. *Methods Enzymol.* 85:698-708.

Wolledge, R. C., N. A. Curtin, and E. Homsher. 1985. *Energetic Aspects of Muscle Contraction*. Academic Press, London. 52-56.

Work, S. S., and D. M. Warsaw. 1988. Detection of surface movements in smooth muscle cells: Digital video microscopy. *Comput. Biol. Med.* 18:385-393.

Yamakawa, M., D. E. Harris, F. S. Fay, and D. M. Warsaw. 1990. Mechanical transients of isolated single smooth muscle cells: effects of lowering temperature and extracellular calcium. *J. Gen. Physiol.* 95:697-715.