Light Chain Phosphorylation Regulates the Movement of Smooth Muscle Myosin on Actin Filaments

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ABSTRACT   In smooth muscles there is no organized sarcomere structure wherein the relative movement of myosin filaments and actin filaments has been documented during contraction. Using the recently developed in vitro assay for myosin-coated bead movement (Sheetz, M.P., and J.A. Spudich, 1983, Nature (Lond.)., 303:31–35), we were able to quantitate the rate of movement of both phosphorylated and unphosphorylated smooth muscle myosin on ordered actin filaments derived from the giant alga, Nitella. We found that movement of turkey gizzard smooth muscle myosin on actin filaments depended upon the phosphorylation of the 20-kD myosin light chains. About 95% of the beads coated with phosphorylated myosin moved at velocities beween 0.15 and 0.4 μm/s, depending upon the preparation. With unphosphory-lated myosin, only 3% of the beads moved and then at a velocity of only ~0.01–0.04 μm/s. The effects of phosphorylation were fully reversible after dephosphorylation with a phospha-tase prepared from smooth muscle. Analysis of the velocity of movement as a function of phosphorylation level indicated that phosphorylation of both heads of a myosin molecule was required for movement and that unphosphorylated myosin appears to decrease the rate of movement of phosphorylated myosin. Mixing of phosphorylated smooth muscle myosin with skeletal muscle myosin which moves at 2 μm/s resulted in a decreased rate of bead movement, suggesting that the more slowly cycling smooth muscle myosin is primarily determining the velocity of movement in such mixtures.

Muscle contraction is regulated by changes in Ca^{2+} concentration. In striated muscle, Ca^{2+} causes a conformational change in the troponin–tropomyosin complex which allows myosin filaments to move relative to actin filaments (1, 2). In smooth muscle cells, Ca^{2+} entry triggers the phosphoryla-tion of the 20-kD light chains of myosin by binding to calmodulin and activating myosin light chain kinase (1). A strong correlation exists between initiation of muscle contrac-tion and light chain phosphorylation in a number of smooth muscle tissues (3–7). In vitro, myosin light chain phosphory-lation activates the actin-activated MgATPase activity of smooth muscle myosin (8–12).

Movement of myosin along actin is believed to provide the force for contraction in all muscles (13). In striated muscle, where this concept was initially derived, the relative sliding of myosin filaments and actin filaments has been well docu-mented. Bipolar myosin filaments move symmetrically on oppositely oriented actin filaments and thereby pull the two Z-lines of each sarcomere together. Recently, Sheetz et al. (14, 15) have demonstrated that skeletal muscle myosin will propel polymer beads (1 μm in diameter) along actin cables of Nitella at a velocity comparable to that of skeletal myosin moving on actin in the muscle sarcomere. From those studies it is inferred that skeletal muscle myosin alone can move struc-tures unidirectionally on actin. The actin of Nitella is not unique in supporting such movement since movement has been observed with skeletal muscle actin filaments aligned by flow (16).

In smooth muscle, there are no well-defined sarcomeres (17). Smooth muscle myosin, however, does form filaments both in vivo and in vitro (18, 19). By analogy to striated muscle, force is believed to be generated by actin filaments. In this study we characterize the effect of myosin light chain phosphorylation on the rate of movement of smooth muscle

myosin on actin using the *Nitella*-based in vitro assay of Sheetz and Spudich (14) and Sheetz et al. (15). It was found that bead movement was dependent upon phosphorylation of the myosin and that the velocity was slower than that measured for rabbit skeletal myosin (14). Experiments in which phosphorylated smooth muscle myosin and skeletal muscle myosin were mixed demonstrated that smooth muscle myosin is able to retard the more rapid velocity of the skeletal muscle myosin. It was also found that unphosphorylated smooth muscle myosin can slow the movement of phosphorylated smooth muscle myosin.

MATERIALS AND METHODS

Materials: Nitella axillaris was cultured from an original stock provided by Lincoln Taisz (University of California at Santa Cruz). In this protocol, 40 ml of chow (16 cups loam, 5 cups leaf mold, 6 cups fine sand, 4 cups steer manure, and 1 teaspoon bone meal), 15 ml of potting soil, and 60 ml H_2O were sterilized and then diluted in a 6" × 18" cylindrical tank filled with distilled water. After waiting for 2 d for particles to settle, 1-2 terminal internodes from a stock Nitella axillaris culture were added to the tank and it was placed under 100-ft candles of fluorescent (blue-green) illumination for 12-16 h/d. Cultures normally took 2-4 wk to mature.

Preparation of Proteins: All procedures were carried out at 4°C unless indicated otherwise. Turkey gizzard myosin was prepared as described previously (12). Turkey gizzard myosin light chain kinase was prepared according to the method of Adelstein and Klee (20) with the additional step of chromatography on a thiophosphorylated light chain Sepharose 4B affinity column, which removes trace amounts of phosphatase activity. Porcine brain calmodulin was prepared according to Klee (21). Phosphatases prepared from turkey gizzard smooth muscle were a generous gift of Dr. Mary D. Pato (University of Saskatchewan).

Nitella Dissection: The dissection was performed in 50-mm plastic petri dishes on a layer (2-3 mm thick) of Sylgard (Dow Corning, Midland, MI). First a cell was secured at both ends with pins (1-3 mm in length) of tungsten wire (0.003" in diameter) in a dissection buffer (10 mM KCl, 10 mM imidazole [pH 7.0], 4 mM MgCl₂, 2 mM EGTA, 50 mM sucrose, 1 mM ATP). Then the cell was opened with a transverse cut using micro-scissors. The cell was cut open along its whole length taking care to disturb as few chloroplasts as possible in the process. At the end of the lengthwise cut, another transverse cut was made and the central portion was pinned flat.

Phosphorylation of Myosin: Monomeric myosin was phosphorylated to intermediate levels in 0.6 M KCl, 50 mM Tris-HCl [pH 7.4], 4 mM magnesium acetate, 0.2 mM ATP, 0.1 mM EGTA, 0.2 mM CaCl₂, 0.2 mM dithiothreitol, 0.1 μM calmodulin, 50 nM myosin light chain kinase at 25°C using a myosin concentration of 10-20 μM. The phosphorylation reaction was terminated at various times by making an aliquot 2 mM with respect to EGTA and placing it on ice. The EGTA chelates calcium and thus inhibits myosin light chain kinase activity. Since there was no phosphatase present, the samples retained their level of phosphorylation throughout the course of the experiment. The final extent of phosphorylation was 1 mol/mol of light chain.

Myosin Bead Preparation: Covaspheres (Covalent Technology, Inc., Ann Arbor, MI) were diluted with eight parts of water, and one part of a myosin solution (10 times the final concentration) was added in 0.6 M KCl, 50 mM Tris-HCl [pH 7.4], 4 mM magnesium acetate, 0.2 mM dithiothreitol.

Myosin Bead Application: Myosin-coated beads were mixed 1:1 with dissection buffer containing 0.5 M sucrose. Samples were then drawn into a microcapillary and applied to a limited region of the dissected Nitella.

Quantitation of Bead Velocity: The active movement of the myosin-coated beads was markedly different from passive flow in that the beads moved without Brownian motion. A video tape of the bead movement was made and analysis of movement was performed on the replay where it was possible to freeze the motion. Positions of actively moving beads were marked in the frozen frame on clear plastic wrap placed on the screen. By advancing the tape and repeating the operation, an accurate position and time record of the bead movement was made. The maximum velocity was taken from measurements of uninterrupted movement over a distance of 10 μm or more.

RESULTS

When Covaspheres were reacted with 100 μg/ml of phosphorylated smooth muscle myosin, they moved on *Nitella*

actin cables at a velocity between 0.15 and 0.43 μm/s, depending on the preparation. The standard variation of velocities within a given preparation was only ±15-30%, and >95% of the beads that attached to the actin moved. If unphosphorylated myosin was used, then movement was observed only rarely (5 out of 150 beads that were followed) and then only at a very low velocity (~0.01-0.04 μm/s). In these studies, the amount of phosphorylated light chain in the unphosphorylated sample was <5% as measured by glycerol-urea gels. Bead motility thus depends upon phosphorylation.

To determine if phosphorylation and dephosphorylation would reversibly modulate the ability of myosin to move the beads, the same preparation of unphosphorylated myosin was repeatedly phosphorylated and dephosphorylated as described in Sellers et al. (22) prior to addition to beads. In the presence of calmodulin and calcium, the kinase phosphorylated the 20-kD light chains and such myosin moved at 0.17 μm/s (Fig. 1). After treatment with EGTA and the catalytic subunit of smooth muscle phosphatase I, which removed >90% of the light chain phosphate, no bead movement was observed. The same myosin sample was then phosphorylated and then dephosphorylated again and a corresponding modulation of bead movement was observed (Fig. 1). Thus bead movement is regulated specifically by the phosphorylation-dephosphorylation process. Movement could also be obtained by directly phosphorylating myosin which was already attached to the beads.

One possible cause for the lack of movement of unphosphorylated myosin-coated beads is that unphosphorylated myosin does not bind to the beads. This was directly examined by electron microscopy. Beads were mixed with either phosphorylated or unphosphorylated myosin exactly as described in Materials and Methods and then diluted into *Nitella* dissection buffer. After 5-10 min, an aliquot was added to an electron microscopic grid and negatively stained. All of the
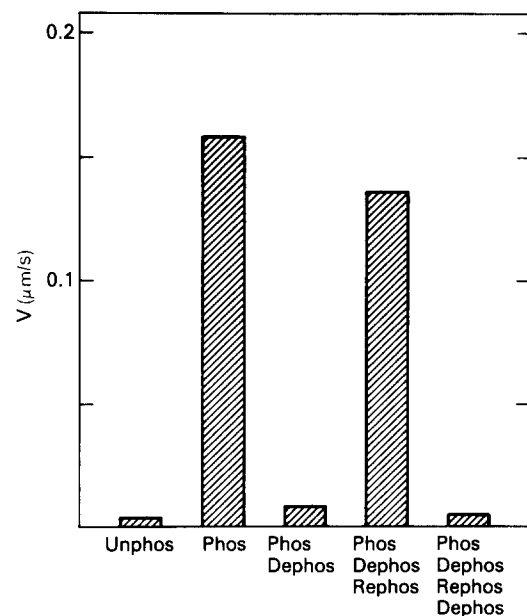

FIGURE 1 Effect of reversible phosphorylation-dephosphorylation of smooth muscle myosin on the rate of bead movement. Unphosphorylated myosin was phosphorylated, dephosphorylated, and rephosphorylated as described by Sellers et al. (12). After each manipulation a sample was mixed with beads and its motility assayed.
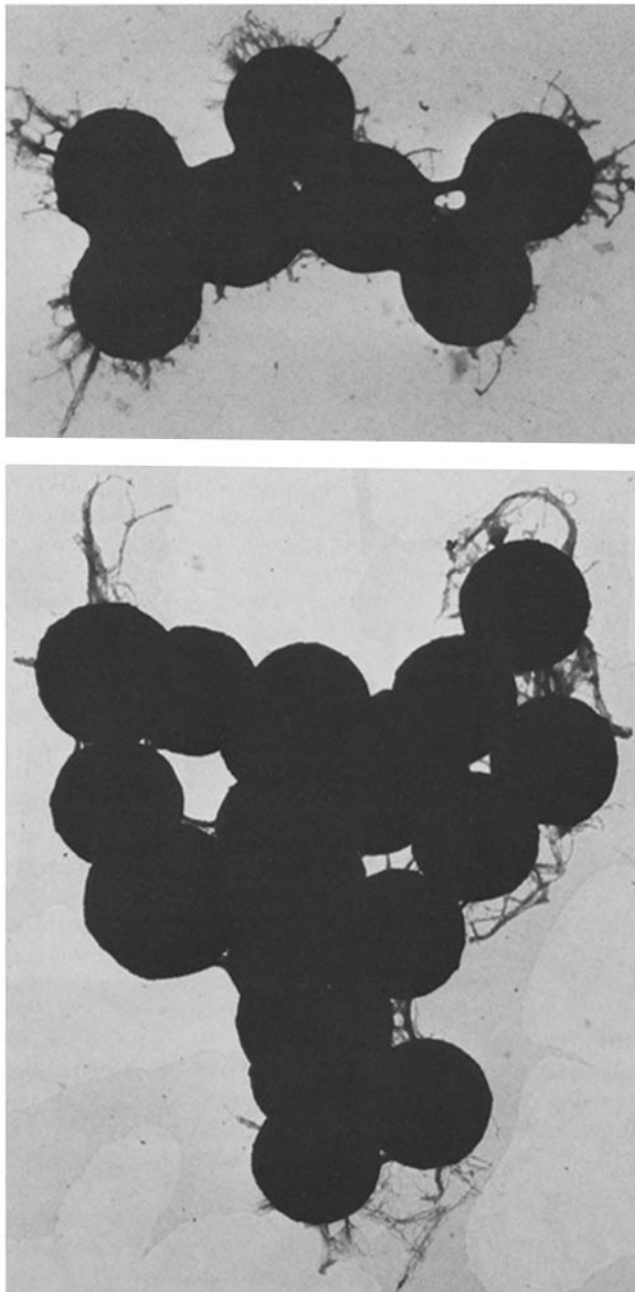
FIGURE 2 Phosphorylated (*upper panel*) and unphosphorylated (*lower panel*) smooth muscle myosin beads were prepared as described in Materials and Methods (100 μg/ml final myosin concentration) and incubated for 1 h. The myosin beads were then mixed 1:1 with dissection buffer containing 0.5 M sucrose, and then deposited into an excess of dissection buffer, as is done for the *Nitella*-based motility assay (see Materials and Methods). These myosin beads were then placed on an electron microscope grid and negatively stained with 1% uranyl acetate. ×19,000.

beads or bead aggregates had one or more myosin filaments bound (Fig. 2). There was no difference between the appearance of phosphorylated or unphosphorylated myosin-treated beads.

No free myosin filaments were observed in the background. Attempts to directly quantitate the amount of myosin binding to beads by sedimentation is complicated since the myosin filaments formed upon dilution into the coupling buffer also sediment even in the absence of beads. Little myosin was

bound to the beads in 0.5 M NaCl and these beads did not move on the actin substratum.

Only a slight concentration dependence of bead velocity has been observed with other myosins assayed in this system (15, 23). Similar results were found for beads coated with phosphorylated smooth muscle myosin (Fig. 3). In fact, no significant change in bead velocity is observed with an increase in myosin concentration from 10 to 100 μg/ml. At lower myosin concentrations, the bead movement dropped abruptly to zero. Bead velocity, therefore, shows no dependence on myosin concentration at myosin concentrations >10 μg/ml.

The maximal velocity of smooth muscle myosin beads varied from one preparation to another, but in general was ~10-fold lower than that observed with skeletal muscle myosin–coated beads. Smooth muscle myosin in vivo is reported to exert a larger force per crossbridge than does skeletal muscle myosin which may be related to its slower cycling rate (24). To determine if one myosin would predominate in determining the velocity of movement, phosphorylated smooth muscle myosin was mixed with skeletal muscle myosin prior to formation of thick filaments and prior to addition of beads. The total myosin concentration was maintained at 100 μg/ml. Smooth muscle myosin and skeletal muscle myosin do form co-polymers (25). As seen in Fig. 4, the velocity of bead movement was essentially that of smooth muscle myosin for a 1:1 mixture of smooth and skeletal myosins. The addition of even a small fraction of smooth muscle myosin to skeletal muscle myosin caused a dramatic inhibition of bead velocity.

Numerous studies have shown that the actin-activated MgATPase of gizzard smooth muscle myosin is correlated (though not linearly) with phosphorylation of the myosin light chains (22, 26). To examine the correlation between bead velocity and extent of phosphorylation, samples of myosin that contained various intermediate levels of phosphorylation were prepared by phosphorylation of myosin at high ionic strength where the myosin is monomeric (22). Under these
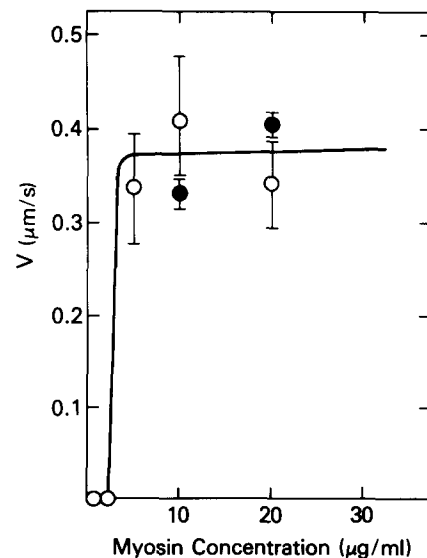

FIGURE 3 Concentration-dependence of velocity of phosphorylated smooth muscle myosin. Fully phosphorylated myosin concentration was varied as indicated in the bead reaction mixture and motility was subsequently assayed. Error bars indicate the SDs. The different symbols represent two different preparations of smooth muscle myosin.
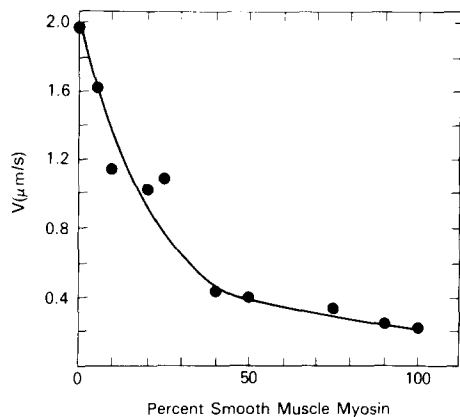
FIGURE 4 Velocity of mixtures of phosphorylated smooth muscle myosin and rabbit skeletal muscle myosin. Phosphorylated smooth muscle myosin and rabbit skeletal muscle myosin were mixed in varying ratios with beads at a total myosin concentration of 100 µg/ml and the velocity was determined.
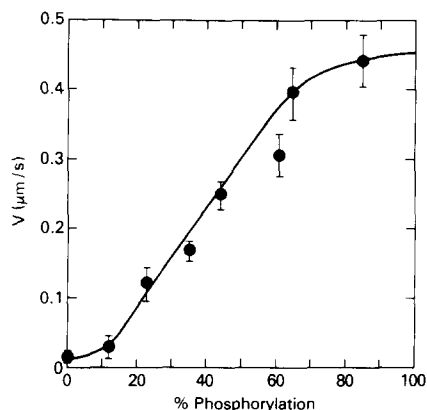

FIGURE 5 Correlation between velocity and intermediate phosphorylation levels. Myosin was phosphorylated to intermediate levels of incorporation at high ionic strength as described in Materials and Methods and the resulting samples were assayed for motility at a myosin concentration of 100 µg/ml. For >20% phosphorylation, >90% of the beads which settled and attached to the actin cables moved. For <20% phosphorylation, however, <10% of the beads which settled and attached moved, and the data graphed are the average rate of movement of all the beads which settled and attached. The line drawn through the data points does not represent a theoretical or statistical fit.

conditions, the two heads of gizzard myosin have been shown to be phosphorylated randomly at equal rates (22). At myosin concentrations of 100 µg/ml, bead velocity showed a graded dependence on myosin phosphorylation with half-maximal velocity at ~40% phosphorylation (Fig. 5). The intermediate velocities observed would not be predicted from the concentration dependence data shown in Fig. 3 where the velocity was essentially "all or none." It appears that unphosphorylated smooth muscle myosin can retard the velocity of phosphorylated myosin.

The samples used in this experiment consisted of mixtures of unphosphorylated, monophosphorylated, and diphosphorylated myosin since they were derived from a time course of phosphorylation. A simpler experiment to examine whether unphosphorylated myosin retards the movement of phosphorylated myosin was performed by mixing various amounts of totally unphosphorylated myosin with fully (i.e., di-) phos-
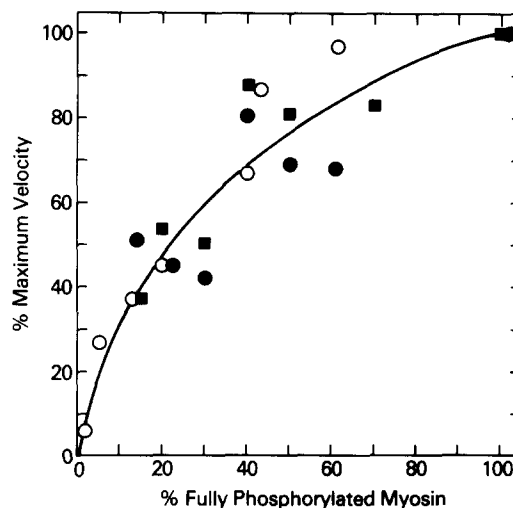

FIGURE 6 Correlation between velocity and ratio of active to inactive myosin. Closed symbols represent the velocity of mixtures of diphosphorylated and unphosphorylated myosins at a concentration of 100 µg/ml. Greater than 90% of the beads that settled and attached to the actin cables moved. The squares and circles represent two independent experiments. Open symbols represent the data from Fig. 5 corrected for percent of diphosphorylated myosin. The line through the data point was drawn by eye and does not represent a theoretical or statistical fit.

phorylated myosin prior to formation of thick filaments, at a constant total myosin concentration of 100 µg/ml. In this experiment, intermediate velocities are also seen with 50% maximal velocity occurring at 25% phosphorylated and 75% unphosphorylated myosin. Since the time course of phosphorylation in Fig. 4 was carried out under conditions where the phosphorylation of the two heads occurs randomly at equal rates, the amount of diphosphorylated myosin in each of these samples can be calculated from the total incorporation of phosphate since the amount of diphosphorylated myosin is equal to X^2, where X is the fractional phosphorylation (see reference 22 for detailed explanation). When the data in Fig. 5 are corrected for percent diphosphorylated myosin (open circles in Fig. 6), it agrees reasonably well with the data obtained from mixing fully phosphorylated and fully unphosphorylated myosin. This may suggest that monophosphorylated myosin, like unphosphorylated myosin, is not active in supporting bead movement, but does not imply that they bind to actin identically. Thus, it appears that the velocity of bead movement is modulated by the ratio of unphosphorylated myosin to diphosphorylated myosin when these mixtures are added to beads. More work will be required to determine the behavior of monophosphorylated myosin.

DISCUSSION

The unidirectional translocation of smooth muscle myosin–coated beads on *Nitella* actin is dependent upon phosphorylation of the myosin light chains. The occasional slow movement produced by the unphosphorylated myosin can possibly be explained as a result of contamination by phosphorylated myosin or perhaps attributed to damage of the unphosphorylated myosin resulting in a loss of regulation. Even 5–10% of such myosin could produce some movement. Both phosphorylated and unphosphorylated myosin seem to bind to the beads in a filamentous form and thus the lack of movement

with unphosphorylated myosin is not due to lack of binding.

The myosin concentration-dependence of velocity indicates that the bead movement produced by phosphorylated myosin is essentially "all or none." That is, above a myosin concentration of 10 μg/ml, the velocity is constant. Below this concentration, velocity is essentially zero. This may reflect a critical concentration for myosin filament formation, or, as suggested by the experiments of Sheetz et al. (15), may reflect the need for a minimum density of myosin on the bead surface. The electron microscopic visualization of filaments bound to the beads suggests that with smooth muscle myosin the former may be the case. Since the beads are essentially without inertia, such a finding is not surprising. Thus, the rate of movement measured in the in vitro motility assay is analogous to the shortening velocity of unloaded muscle in that it is independent of the number of myosin heads contributing to the movement.

The Nitella system provides a simple and reproducible means to measure the factors affecting velocity of movement of a particular myosin. It was found that phosphorylated smooth muscle myosin–coated beads moved with about a 10-fold lower velocity than did skeletal muscle myosin–coated beads (15) and this difference is also seen with unloaded shortening velocity of intact smooth and skeletal muscle fibers (27, 28). Siemankowski et al. (27) have recently proposed that the dissociation rate of ADP from actomyosin–ADP is the kinetic step which limits unloaded shortening velocity. In this regard, it is interesting to note that the dissociation rate of ADP from gizzard acto-S-1 (27, 29) is considerably slower than that from fast skeletal muscle myosin. Whether an exact correlation exists between bead velocity and ADP dissociation rate must await further study.

Mixtures of fully phosphorylated smooth muscle myosin and skeletal muscle myosin resulted in bead movement at intermediate velocities. It appears that the more slowly cycling phosphorylated smooth muscle myosin is predominant in determining the overall velocity of such mixtures. This is consistent with data from mechanical studies which suggest that smooth muscle myosin exerts a higher force per crossbridge than does skeletal muscle myosin (24).

A surprising finding was that the presence of unphosphorylated myosin slows the velocity of movement of phosphorylated myosin when mixed on the beads. There are several possible explanations for this. Unphosphorylated myosin does bind to actin in the presence of ATP even though its Mg-ATPase is not greatly activated by such binding (30). These slowly cycling, unphosphorylated crossbridges may retard the velocity of the rapidly cycling, phosphorylated myosin much in the same way that phosphorylated smooth muscle myosin retards the velocity of the faster cycling skeletal muscle myosin. These unphosphorylated crossbridges may be analogous to those operating during the so-called "latch" state observed under some conditions in smooth muscle fiber preparations. Latch occurs after 15–20 min of contraction when the level of myosin phosphorylation and shortening velocity returns to near resting levels, yet the tension or force remains elevated (4, 6). Thus, under these conditions phosphorylation correlates not with tension but with shortening velocity, and unphosphorylated crossbridges can support tension. There is some controversy as to whether latch occurs ubiquitously in smooth muscles and with what biochemical change it correlates. Thus, care must be exercised before drawing too close a parallel between our data and that derived

from fiber work (3, 5).

Another possible explanation for the graded velocities seen with the mixtures is that the presence of unphosphorylated myosin in a filament lowers the cycling rate of neighboring phosphorylated myosins by directly affecting the MgATPase activity. We feel that this is not likely, however, since there is no evidence for this from studies of the actin-activated MgATPase activity of mixtures of phosphorylated and unphosphorylated smooth muscle myosin (22).

The intermediate levels of myosin phosphorylation in Fig. 5 were generated during a time course of phosphorylation which results in mixtures of unphosphorylated and of mono- and diphosphorylated myosin, whereas the levels in Fig. 6 (closed symbols) were obtained by mixing fully phosphorylated and fully unphosphorylated myosin. When the data in Fig. 5 are corrected for percent diphosphorylated myosin, however, they are similar to those in Fig. 6. This suggests that both heads of myosin must be phosphorylated before the molecule can participate in movement. This is in agreement with the observation that both heads of smooth muscle myosin must be phosphorylated before actin can activate the MgATPase activity of either head (22, 26). More studies will be required before it can be stated with certainty that monophosphorylated myosin is not active in supporting movement.

These studies indicate that measurements of bead velocity can be used to probe the regulation and mechanisms of smooth muscle contraction. All of the experiments described were carried out in the presence of EGTA, indicating that Ca^{2+} is not required for bead movement. This is in agreement with studies of skinned smooth muscle fibers where either a calcium-insensitive myosin light chain kinase is used or where the myosin is prephosphorylated with gamma-S-ATP which is resistant to phosphatases and allows for tension measurements to be made in the absence of calcium (31). Evidence exists, however, that a second regulatory system involving Ca^{2+} may operate in smooth muscle (32). The nature of this system is not known at present but may involve direct Ca^{2+}-binding to the myosin (33) or thin filament regulatory proteins (34, 35). The Nitella system should prove useful in determining whether Ca^{2+} plays a secondary or modulatory role by interacting directly with smooth muscle myosin.

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