MYOSIN LIGHT CHAIN PHOSPHORYLATION INHIBITS
MUSCLE FIBER SHORTENING VELOCITY IN THE PRESENCE OF VANADATE

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ABSTRACT
We have shown that myosin light chain phosphorylation inhibits fiber shortening velocity at high temperatures, 30°C, in the presence of the phosphate analog, vanadate. Vanadate inhibits tension by reversing the transition into force generating states, thus mimicking a pre-power stroke state. We have previously shown that at low temperatures vanadate also inhibits velocity, but at high temperatures it does not, with an abrupt transition in inhibition occurring near 25°C (Pate et al, Biophys. J. 66:1554, 1994). Here we show that for fibers activated in the presence of 0.5 mM vanadate, at 30°C, shortening velocity is not inhibited in dephosphorylated fibers, but is inhibited by 37 ± 10% in fibers with phosphorylated myosin light chains. There is no effect of phosphorylation on fiber velocity in the presence of vanadate at 10°C. The $k_m$ for ATP, defined by the maximum velocity of fibers partially inhibited by vanadate at 30°C, is 20 ± 4 μM for phosphorylated fibers and 192 ± 40 μM for dephosphorylated fibers, showing that phosphorylation also affects the binding of ATP. Fiber stiffness is not affected by phosphorylation. Inhibition of velocity by phosphorylation at 30°C depends on the phosphate analog, with approximately 12% inhibition in fibers activated in the presence of 5 mM BeF$_3$ and no inhibition in the presence of 0.25 mM AlF$_4$. Our results show that myosin phosphorylation can inhibit shortening velocity in fibers with large populations of myosin heads trapped in pre-power stroke states, such as occurs during muscle fatigue.
INTRODUCTION

The nature of the pre-power stroke states is of considerable interest, and because they are transients in the cycle they are difficult to study during normal force generation. Inhibition of the actomyosin cycle by analogs of phosphate can populate these states by inhibiting the active cycle, producing lower tensions and velocities in fibers (5, 6, 9, 10, 27). Previous work by ourselves and by others has been carried out with fibers in which myosin light chains were in the dephosphorylated form. These studies agreed that at low temperatures the phosphate analogs inhibited both tension and velocity (5, 6, 9, 10, 27). We further found that in fibers, partially inhibited by addition of vanadate, the velocity of shortening is inhibited at low temperatures but not at high temperatures with an abrupt change in inhibition between 22 to 25 °C (24). The extreme steepness of this transition suggests that a highly cooperative interaction involving multiple subunits is responsible. The states populated by the phosphate analogs may mimic those populated by high phosphate during muscle fatigue (5, 6, 9, 10, 27). The mechanisms that affect actomyosin interactions during fatigue remain an area of active investigation. As described below, myosin becomes phosphorylated during fatigue. Here we investigated the possible roles of myosin phosphorylation in determining fiber velocity under conditions that may mimic fatigue as well as in the strange behavior of fiber velocity in the presence of vanadate, observed previously. We measured fiber mechanics in fibers partially inhibited by phosphate analogs at both high and low levels of myosin phosphorylation.

Previous investigations have identified one role for the function of myosin regulatory light chain phosphorylation in skeletal muscle. Phosphorylation of the myosin light chain is produced by a kinase activated by calcium-calmodulin, thus it increases when muscles are heavily used, for review see (29). The tension achieved in a muscle twitch depends on the history of activity of the muscle, with greater tensions achieved following a tetanic contraction or a train of twitches. Myosin phosphorylation also increases in either a tetanic contraction or in a train of twitches and the increased tension achieved in the post-tetanic twitch correlates well with the level of myosin light chain phosphorylation, for review see (29). Studies of permeable fibers found that phosphorylation of the light chain resulted in greater isometric tensions at low levels of calcium in partly activated muscle fibers, providing an explanation for the correlation between twitch potentiation and myosin phosphorylation observed in vivo (26, 33). There was no change in the tension achieved by fully activated muscle fibers. More recently a transgenic mouse with a
knockout of the kinase that phosphorylates the myosin light chain has shown definitively that activity induced twitch potentiation is the result of myosin phosphorylation (39).

Previous work has shown that the structure of the thick filament can be perturbed by phosphorylation of the regulatory light chain of myosin (19). A helical array of myosin heads bound to the core of the thick filament was first observed in resting frog muscles by both EM and x-ray diffraction, for review see (16). In permeable mammalian muscle, x-ray diffraction showed that the heads were disordered at low temperatures but bound in an ordered array to the thick filament at higher temperatures (37, 38). An ordered array has been seen in electron micrographs of myosin filaments from a variety of muscle types (18). Phosphorylation of the myosin regulatory light chain in relaxed myosin filaments from rabbit muscle disrupted their ordered array (19). This structural change provides a reasonable explanation for the ability of regulatory light chain phosphorylation to potentiate the tension of partially activated muscle fibers, described below.

The structural change induced in the thick filament by myosin phosphorylation provided an explanation for the observation that myosin phosphorylation increased the twitch tension but not the tetanic tension produced by living fibers (19). In the dephosphorylated fibers many of the myosin heads were bound to the core of the thick filament, where they were sterically unable to reach out to and interact with the actin filaments. In the phosphorylated fibers the myosin heads extend away from the core of the thick filament, where they more readily interact with the actin filament (19). This effect was prominent at low levels of activation where many heads were not interacting with actin, but in fully activated fibers, the effect was minimal because most myosin heads are participating in active cycles and thus few heads interact with the thick filament.

In the present work we have investigated the inhibition of velocity of permeable fast rabbit muscle fibers in three different phosphate analogs and at different temperatures and levels of myosin phosphorylation. This preparation may resemble the partially activated fibers, with a large population of myosin heads that are not interacting with actin in both cases, although this population is produced by different mechanisms. We find that myosin phosphorylation inhibits the velocity of fibers in the presence of the phosphate analog, vanadate, at high but not low temperatures. Thus, myosin phosphorylation eliminates the sharp transition in the inhibition of shortening velocity seen previously with dephosphorylated fibers,
and addition of vanadate inhibits velocity to approximately the same extent at all temperatures. Phosphorylation also reduces the $k_m$ for the maximum velocity as a function of [ATP], showing that it may affect fiber velocity by altering the binding of nucleotides at the active site of myosin. In contrast to vanadate, in the presence of the phosphate analog AlF$_4$, phosphorylation has no effect on velocity, and in the presence of BeF$_3$ it inhibits velocity by 12%.
MATERIALS AND METHODS

Fiber preparations.

Rabbits were sacrificed according to protocols approved by the Institutional Animal Care and Use Committee, and psoas fibers were harvested and chemically skinned as described previously (7). Control fibers were stored in a solution that contained 120 mM K-acetate, 5 mM MgCl₂, 5 mM EGTA, 50 mM MOPS, pH 7, 50% glycerol by volume. Fibers stored in the above buffer plus 5 mM ATP, 20 mM NaF, 20 mM K phosphate became phosphorylated over the course of approximately one week. The NaF and phosphate are inhibitors of the protein phosphatases involved in the dephosphorylation of a number of phosphorylated proteins, including the myosin light chain.

Determination of levels of protein phosphorylation.

The degree of phosphorylation of the myosin regulatory light chain was determined using isoelectric focusing gels, pH range 4 to 6. The phosphorylated light chain focuses at a more acidic pH. The gels were stained with a dye, Pro-Q (Molecular Probes, Eugene OR), which has increased specificity for phosphorylated serines and threonines, see Figure 1. The dye also stains some other proteins, but with lower intensity. The regulatory light chain of myosin is the only band seen to shift in the isoelectric focusing gels. As shown by the pattern for purified myosin, shown in lane 1, the dye also stains the dephosphorylated form of the light chain. Quantitation of the intensities of the dephosphorylated and phosphorylated light chains showed that the staining for the phosphorylated light chain was 4 times stronger, per micogram of light chain protein loaded, than for the dephosphorylated light chain. In the gel shown in Figure 1 the level of phosphorylation was <10% in the dephosphorylated fibers and 60 ± 15% in the phosphorylated fibers. These values compare favorably, with those obtained in living fibers that are relaxed, 5-10% and during moderate fatigue, ~50% (29). Gels run in SDS and stained with Pro-Q also showed no changes in intensity of any other proteins. The regulatory light chain of myosin is the only component of the myofibrillar array known to be phosphorylated in vivo in skeletal muscle fibers, and we conclude that it was the only protein to become phosphorylated in our skinned fiber preparations.


**Measurement of fiber mechanics**

For mechanical experiments, single fibers were dissected from a bundle of fibers and mounted in a solution between a solid-state force transducer and a rapid motor for changing fiber length. The apparatus allowed for rapid translation of the mounted fiber between 3 solutions. Three drops of solution, 140 µl each, were held by surface tension between a thin glass cover slip and one of three peltier units. The peltier units were mounted on a block that could be translated relative to the fiber, which was held stationary between a force transducer and a motor arm. The temperatures for the three peltier units could be set independently. The mounted fiber was first placed in a relaxing solution, where length and diameter were measured. The fiber was then activated in a second solution with appropriate levels of analogs at a low temperature, 5°C, and allowed to reach a plateau of isometric tension. At this temperature the fiber sarcomere arrangement was very stable. The fiber was then rapidly translated to a higher temperature solution (10-30°C) also with activating solution, where force reached its new maximum in approximately 200 milliseconds. Force was monitored for 1-2 seconds, during which a single force clamp was performed before the fiber was returned to the cold activating solution. The fiber was incubated at the lower temperature for 30 to 120 seconds, and then jumped again to the higher temperature for another measurement. The number of measurements made per fiber varied with the conditions. Fiber stability was checked by measuring a velocity close to the first one of a series, and discarding the fiber if this velocity was lower by more than 5%. In the absence of the phosphate analogs the number of velocities taken per fiber was usually 3-6 or less. In the presence of the analogs fiber stability increased and 6-8 measurements were possible. Fiber stiffness was measured by application of step increases in fiber length, complete in 0.5 msec. Tension was monitored following the step and peak tension was plotted against the step length.

Force velocity relationships were fit to the Hill equation as described previously (7). The fit defines two parameters, the maximum contraction velocity, the velocity extrapolated to zero tension; and $\alpha/Po$, which is related to the curvature, with lower values indicating more curvature.

**Solutions**
The rigor buffer contained 120 mM KAc (potassium acetate), 5 mM MgCl₂, 1 mM EGTA, 5 mM potassium phosphate and 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7. A relaxing solution was achieved by addition of 20 mM creatine phosphate, 1 mg/ml creatine kinase and 4 mM ATP. Activating solution was obtained by addition of CaCl₂ to produce a pCa of 4.3. Stock solutions of 100 mM orthovanadate at pH 10 were prepared and boiled each day before use as described by Goodno and aliquots added directly to the activating solutions (15). The analogs AlF₄ and BeF₃ were achieved by adding 10-15 mM NaF and an appropriate concentration of AlCl₃ or BeCl₃. It was found that in order to achieve higher levels of inhibition by BeF₃, phosphate had to be omitted. For the other analogs the absence of phosphate decreased the inhibition produced by the analog by only a small amount, <10% and phosphate was included to provide a better comparison with the control solutions.

RESULTS

Most previous studies of fiber mechanics have been carried out at low temperatures, 5-15°C, because at higher temperatures the sarcomere pattern becomes rapidly disordered. This problem can be ameliorated by using temperature jumps to briefly activate fibers at the higher temperature (2, 14, 22, 35). Our previous work has shown that fibers could be activated for several seconds at temperatures of 30-35°C and maintain stable sarcomere diffraction patterns and mechanics (22). The apparatus used here is superior to that used previously, allowing more rapid transfer of fibers between temperatures. Using this apparatus we have found that up to 3-5 temperature jumps can be performed with less than 10% decrease in tension or velocity. This was critical for obtaining data in the absence of phosphate analogs. In the presence of the analogs the fibers generated much lower tensions and were stable for much longer.

Single fibers or pairs of fibers were dissected and mounted on the apparatus. Phosphorylated and dephosphorylated fibers were prepared as described in methods, and their mechanics compared. Mounted fibers were first observed in a solution containing relaxing solution, where sarcomere lengths were adjusted to 2.3-2.4 microns. They were then moved to a second solution where they were activated for two minutes at 5°C, allowing tension to reach a plateau and for diffusion of creatine kinase and creatine into the fiber. They were next moved briefly, 2 seconds, to a third solution that contained an activating solution at a higher temperature where
tension was measured and a load clamp to a lower tension was performed. Following the activation at the higher temperature they were returned to the 5ºC solution for 0.5-2 minutes, before being again activated at the higher temperature.

Fibers were activated in the presence and absence of vanadate and shortening velocity was measured as a function of vanadate concentration and temperature. In the experiments with vanadate, the vanadate was added to the activating solutions at both temperatures, so the fiber was first inhibited by vanadate at 5ºC before the jump to the higher temperature where velocity was measured. Tension was inhibited progressively as the concentration of vanadate was raised, with approximately 75% inhibition achieved by addition of 0.5 mM vanadate, in agreement with previous work (6, 9, 24, 36). Tension has been previously shown to vary linearly with the log [Vi] at 25 ºC (6, 9, 24, 36). Data obtained here at a slightly higher temperature, 30ºC, also varied linearly with log [Vi], with the same x intercept at zero tension, 1.6mM [Vi], but with a slightly steeper slope -0.47 ± 0.03 versus -0.32 ± 0.02 than was found previously (6, 9, 24, 36). Figures 2-4 show data obtained with 75% inhibition of tension by vanadate. Vanadate inhibited the tension of phosphorylated fibers by the same degree as dephosphorylated fibers at 30ºC, 74±7% and 79±5% respectively. As found previously, for dephosphorylated fibers the presence of vanadate inhibited velocity by about a factor of 2 over control (no vanadate) at 10ºC. At 10ºC the mechanics of the phosphorylated fibers resembled those of the dephosphorylated fibers in both the presence and absence of vanadate. As also observed previously for dephosphorylated fibers, velocity in the presence of vanadate was equal to control velocity for temperatures above 22ºC, see Figures 2-4 (24). The force-velocity curves of dephosphorylated and phosphorylated fibers were also similar at 30ºC in the absence of vanadate, see Figure 3. However, as described below, at 30ºC the shortening velocity of fibers in the presence of vanadate depended on the phosphorylation of the regulatory light chain of myosin.

Figure 2 shows the effect of vanadate and myosin phosphorylation on fiber velocity at 30ºC. The displacement of the fiber is shown as a function of time for a 40 millisecond load clamp to a tension close to 10% of isometric. The slopes of these curves were used to determine the velocities. The slopes are similar for dephosphorylated fibers and phosphorylated fibers in the absence of vanadate, and also for dephosphorylated fibers in the presence of vanadate. However, the slope of the displacement of the phosphorylated fibers was about 40% less in the presence of vanadate than for the three other cases. Figure 3 shows force velocity
relationships determined for the two fiber preparations in the presence and absence of vanadate. The dramatic observation shown in figures 2 and 3 is that myosin phosphorylation inhibited shortening velocity in the presence of vanadate. The degree of inhibition of fiber velocity is shown as a function of temperature in Figure 4, demonstrating that the abrupt transition in fiber velocities seen with dephosphorylated fibers is eliminated in the phosphorylated fibers.

Three phosphate analogs have been employed extensively in previous investigations of fiber mechanics and myosin structure. These are thought to mimic different states in the kinetic cycle. We thus explored two additional analogs AlF₄⁻ and BeF₃⁻. The concentrations of the analogs and the composition of the buffer were chosen so that tension was inhibited by 75-80% to match that produced by 0.5 mM vanadate. The structure of the myosin catalytic domain in complex with ADP and AlF₄⁻ is thought to resemble an ADP•Pᵢ intermediate in the hydrolysis step (13). The addition of 250 μM AlF₄⁻ inhibited fiber tension by approximately 75% of control, and thus this concentration was used in subsequent studies. At 10°C velocity was also inhibited by approximately a factor of 2 in both dephosphorylated and phosphorylated fibers, data not shown. At 30°C, fiber velocity was inhibited to approximately 3.6 lengths/second for both phosphorylated and dephosphorylated fibers, see Figures 5 and 7. Thus, unlike the situation in vanadate, phosphorylation had no effect on velocity in the presence of AlF₄⁻. The structure of the myosin catalytic domain with bound ADP and BeF₃⁻ suggests that this complex resembles ATP rather than ADP•Pᵢ (13). Addition of 5 mM BeF₃⁻ to fibers inhibited tension by 75-80% of control, and inhibited velocity to 4.1 ± 0.2 and 3.6± 0.2 lengths/second for dephosphorylated and phosphorylated fibers respectively, see Figures 6 and 7. Thus in the presence of BeF₃⁻, phosphorylation inhibited fiber velocity, however the effect, 12 ± 5%, was smaller than that seen in vanadate. Thus fiber velocity depends on both the phosphate analog used and on the phosphorylation of the light chain, see Figure 7. In phosphorylated fibers the velocity was the same, ~ 3.6 lengths/second, in all the analogs. In dephosphorylated fibers the velocity was greatest in vanadate, 6.0 lengths/second, intermediate in BeF₃⁻, 4.1 lengths/second, and lowest in AlF₄⁻, where it was the same as in phosphorylated fibers, 3.7 lengths/second.

One hypothesis to explain the inhibition of fiber velocity by myosin phosphorylation in the presence of vanadate involves the release of myosin heads bound to the core of the thick filament by phosphorylation; allowing them to now bind weakly to actin and create a drag on filament motion. To further explore this hypothesis we measured the stiffness of the fibers in the
presence of vanadate to determine whether such weakly bound heads could be detected by their increased contribution to fiber stiffness. The stiffness of phosphorylated fibers, $2.7 \pm 4 \times 10^6$ N/m$^2$, was not significantly different from that for dephosphorylated fibers, $3.5 \pm 0.3 \times 10^6$ N/m$^2$, shown in Figure 8. The expected increase in fiber stiffness in the phosphorylated fibers is not observed. However, this result does not necessarily rule out a role for weakly bound heads, as discussed in more detail below.

To determine whether the binding of nucleotides in the presence of the phosphate analogs was altered by phosphorylation we measured velocities as a function of ATP concentration in the presence and absence of vanadate and at 30°C. In the absence of vanadate the $K_m$ was not significantly different between phosphorylated fibers, $168 \pm 20 \mu$M, and dephosphorylated fibers, $187 \pm 25 \mu$M. In contrast, in the presence of 0.5 mM vanadate, the $K_m$ for shortening velocity of phosphorylated fibers, $20 \pm 4 \mu$M, was 10 times lower than that of the dephosphorylated fibers, $192 \pm 40 \mu$M, see Figure 9. Thus, in low [ATP], phosphorylated fibers had a higher shortening velocity than dephosphorylated fibers. This indicates a tighter apparent binding of ATP for phosphorylated fibers than for dephosphorylated fibers in the presence of vanadate. In the presence of AlF$_4$ the $K_m$ was $17 \pm 7$ and $16 \pm 7 \mu$M in dephosphorylated and phosphorylated fibers respectively. In the presence of BeF$_3$ the $K_m$ was $288 \pm 87$ and $208 \pm 65 \mu$M in dephosphorylated and phosphorylated fibers respectively. These data suggest that the binding of ATP to the active heads is influenced by the phosphate analog bound to other heads in the fiber. It also shows that there is an effect of phosphorylation on ATP binding only in the case where there is an effect on shortening velocity.
DISCUSSION

Relation to previous work.

In order to better fit the crystal structures into the cycle it would be helpful to understand the properties of the states produced both in solution and in fibers. Understanding the properties of these states is also important, because, although they are transitory, they occupy an important place in the cycle. They are a necessary entry point to the force generating states, and they thus help control the flux through the power stroke (4). Most previous investigations of the effects of phosphate analogs on fiber function have been carried out at lower temperatures, 10-15°C, and with fibers in which the regulatory light chain was dephosphorylated. Addition of the analogs inhibits both tension and velocity, with greater inhibition of tension than velocity, particularly at lower levels of the analogs (5, 6, 9, 10, 27).

Our present results agree with previous work by ourselves and others, obtained under similar conditions, i.e. in fibers with dephosphorylated myosin regulatory light chains at low temperatures. Concentrations of the analogs that reduced isometric tensions to 25% of control values were selected. The concentrations required to produce this inhibition at 10°C were similar to those found previously (5, 6, 9, 27). Inhibition of tension to approximately 25% at 10°C was associated with inhibition of velocity to about 60% in both dephosphorylated and phosphorylated fibers. The new observation made here is that at higher temperatures the velocity depends on the phosphate analog used and on the state of phosphorylation of the regulatory light chain. At 30°C all of the phosphate analogs again inhibited tension to about 25% of control for either phosphorylated or dephosphorylated fibers, however the inhibition of velocity varied with the analog. In the presence of vanadate the velocity of dephosphorylated fibers was not inhibited but the velocity of phosphorylated fibers was inhibited by about 40% of that in the absence of vanadate. In the presence of AlF₄ or BeF₃, the velocities of dephosphorylated fibers were inhibited with a small further inhibition by phosphorylation in the presence of BeF₃, and no further inhibition in the presence of AlF₄ see figure 7.

Three analogs of phosphate have been shown to bind tightly to the myosin ADP complex and to mimic the myosin-ATP or myosin-ADP-Pi complexes. The crystal structures of the myosin catalytic domain have been obtained in the presence of all of these analogs (12, 13, 28).
position and coordination of BeF₃ in complex with myosin-ADP suggests that it is a mimic of the myosin-ATP state (13). A similar argument suggests that vanadate and AlF₄ are mimics of a transition state that occurs during the hydrolysis step (13, 28). Our results suggest that these analogs populate states with different properties. Although both vanadate and AlF₄ are mimics of a transition state, with similar crystal structures, their effect in this assay are different, with addition of AlF₄ inhibiting velocity in dephosphorylated fibers while addition of vanadate does not.

The chemistry of vanadate is complex with oligomerization producing both tetrameric and decameric species, especially at lower pH and at higher concentrations (1). The decavanadate species has been shown to bind to myosin at the P-loop, which also binds to nucleotides (34). We believe that there are several arguments suggesting that monomeric orthovanadate is responsible for the inhibition in fiber velocity observed here and by others, not the oligomeric species. Vanadate was prepared at high pH, 10, and boiled, a process known to depolymerize the oligomers (15). It was diluted directly into the experimental solution, at low concentration where oligomers are not likely to form, and used within a few minutes, which will not provide time for oligomerization. In addition, waiting for many minutes, during which oligomers could form if they were going to, did not alter the inhibition produced in either tension or velocity. While we believe that these arguments are strong, the complex chemistry of vanadate should be kept in mind when interpreting the results. Although the concentration of decavanadate may be small, it binds to myosin with much higher affinity than monomeric vanadate (34), and its possible role in these studies cannot be ruled out completely.

**The role of myosin light chain phosphorylation in skeletal muscle.**

In smooth muscles myosin light chain phosphorylation plays a major role in controlling the activation of the muscle, however in skeletal muscle its role is only to modulate the mechanics of active muscles, for review see (3, 29). The observation that post tetanic twitch potentiation correlates with the level of myosin phosphorylation first suggested a functional role in skeletal muscle (29). Post tetanic twitch potentiation is defined as the increase in twitch force that occurs for a short time following a tetanic contraction. Myosin phosphorylation also increases following a tetanic contraction because the myosin light chain kinase is activated by calcium, leading to the hypothesis that potentiation may be the result of myosin phosphorylation. Potentiation of twitch tension also occurs during a train of twitches, a phenomenon known as staircase, which has been attributed to myosin phosphorylation. In collaboration with Stull and Persechini, we
were the first to show that myosin phosphorylation increased tension in permeable fibers at low levels of activation, thus providing a molecular mechanism for the correlation between myosin phosphorylation and twitch potentiation (26).

Subsequent work in a number of laboratories has explored the mechanical phenomenon associated with myosin phosphorylation in greater depth (11, 21, 25, 30-32). The increase in fiber force has been shown to be the result of a faster rate of attachment of the myosin heads to actin (21, 32). In addition it slows the rate of relaxation of partially activated but not fully activated skinned rabbit fibers (25). Both effects are thought to be due to the disruption of the myosin thick filament, which facilitates the interaction of the myosin heads with the actin filament.

Several observations suggest that myosin phosphorylation may have a greater effect on fiber function at higher temperatures. This is to be expected, if phosphorylation exerts its effect through disordering the ordered array of myosin heads bound to the thick filament, because the array is more stable at higher temperatures, see discussion below. Both post tetanic twitch potentiation and the potentiation that occurs during staircase have been found to be much greater at higher temperatures (17), with twitch potentiations following a tetanus of 20% at 20 °C and 60% at 37 °C (30). The greater effect of myosin phosphorylation at the higher temperatures is consistent with the hypothesis that it exerts its effect through disordering of the ordered array of the myosin heads bound to the core of the thick filament, as this ordered array is also more stable at higher temperatures (38).

Here we suggest that myosin phosphorylation has an additional role in inhibiting shortening velocity in the presence of a phosphate analog. We also find that this effect is seen at higher temperatures and not at lower temperatures, a temperature dependence compatible with a mechanism involving its disordering of the ordered array of the thick filament. This observation suggests that myosin phosphorylation may play a role in the inhibition of fiber velocity that occurs during fatigue.

**What is the mechanism by which myosin phosphorylation inhibits fiber velocity?**

A schematic representation of the cross bridge cycle is shown in Figure 10. Active heads are cycling through States 1-6, with States 4-6 producing tension. State 7 represents the ordered array of myosin heads bound to the core of the thick filament. The addition of the phosphate
analogs would populate the non-force generating states, 1-3, and could also populate State 7 to varying degrees in the dephosphorylated fibers but not in the phosphorylated fibers.

All three of the phosphate analogs used here bind with great affinity to the protein in the same place as would be occupied by the gamma phosphate of ATP. Their release from myosin is very slow, in the order of minutes, compared to the cycling rate of active cross-bridges (5, 6, 27). During shortening, which is measured over 40 msec, very few heads change between active and trapped populations. Therefore, in a fiber whose tension has been inhibited by 80%, a large fraction of the myosin heads, probably 80%, are trapped in States 2 and 3, and possibly State 7, by bound phosphate analog. Active force and shortening are produced by the remaining 20% of heads that have not bound the phosphate analog and are cycling through States 1-6. Shortening velocity is not altered by myosin phosphorylation in the absence of the phosphate analogs, suggesting that the properties of the active heads do not depend on myosin phosphorylation. However in the presence of the analogs, the velocity depends both on the phosphate analog and on the state of myosin phosphorylation. Thus, the velocity generated by the active heads in the partially inhibited fibers must be in some way determined by the interaction between the active heads and the heads trapped in states where they are weakly bound and/or unbound to actin. Such interactions could occur between different myosin molecules, or could occur between an active myosin head, and its trapped partner.

A reasonable hypothesis is that the inhibition of velocity is produced by a drag exerted on filament sliding by non-force generating heads that bind weakly to actin, State 3 in Figure 10. Myosin heads that are not generating force can be sequestered by binding to the backbone of the myosin filament in an ordered array, State 7. Yu and co-workers measured the formation of this ordered array in fully relaxed permeable muscle fibers by observing the intensity of the myosin layer lines in X-ray diffraction patterns (38). They found that the array was more ordered at higher temperatures. Levine and co-workers showed that myosin phosphorylation reduced the fraction of myosin heads bound to the core of the thick filament and increased the fraction of myosin heads in a disordered configuration in the space around the thick filament (19). Thus in the phosphorylated fibers the non-force generating heads could more readily bind to the thin filaments than in the dephosphorylated fibers. The binding of such heads has been previously shown to inhibit filament sliding (8). The difference between phosphorylated and dephosphorylated fibers would be greater at higher temperatures, where the more stable array
would play a greater role. Xu et. al. also found that the phosphate analog vanadate was more effective at promoting the formation of the ordered array than was BeF$_3$, a result compatible with this hypothesis (38). However, the data of Xu et. al. do not explain why there is a sharp transition in fiber velocity in the presence of vanadate at about 22 °C. Their data do not show a cooperative transition in the stability of the ordered array of myosin heads, which becomes progressively more ordered with increasing temperature, (38). However, the interactions between myosin heads and the thick and thin filaments in the activated fibers are complex, and cooperative behavior could result from changes in lattice spacing, or from more complex interactions between active heads and the ordered and disordered heads that are not generating force. Thus the absence of a cooperative transition in the temperature dependence of the cross bridge order-disorder equilibrium in relaxed fibers (38) does not preclude the steep transition in shortening velocity observed here. In addition, the stiffness of phosphorylated fibers would be expected to be greater if more myosin heads were bound to the thin filament, and the stiffness of phosphorylated and dephosphorylated fibers in the presence of vanadate was the same, see Figure 8. However, this result does not rule out a role for the weakly bound heads. It is possible that the population of weakly bound heads does increase in the phosphorylated fibers, but is not detected by increased stiffness. The contribution from the weakly bound heads may be small compared to that from the strongly bound active heads. Alternatively, the inhibition of velocity could arise from an interaction between a strongly bound active head, and its weakly bound partner. Although the hypothesis discussed above is parsimonious, arising from known actions of myosin phosphorylation, more work will be required to determine its validity.

A Second hypothesis is that the phosphorylated fibers could be slower than dephosphorylated fibers in the presence of vanadate due to a slower binding of ATP and/or release of ADP at the end of the power stroke. The data of Figure 9 show that the apparent affinity for ATP is in fact greater in phosphorylated fibers than in dephosphorylated fibers. The maximum shortening velocity is about 2 fold reduced in the phosphorylated fibers. If the rate of binding of ATP to the rigor actomyosin complex was unchanged, but the maximum velocity was reduced by a factor of two, the value of $K_m$ would be expected to also be reduced by a factor of 2. In the absence of vanadate the $K_m$ for the dependence of velocity on [ATP] is independent of phosphorylation within experimental error, and is approximately 180 μM. If the maximum shortening velocity is inhibited by a factor of 0.6 the $K_m$ would be expected to be about 100 μM, which is 5 times...
greater than the $K_m$ observed for the phosphorylated fibers of 20 µM. Thus the apparent affinity for ATP is considerably greater in the phosphorylated fibers in the presence of vanadate.

The values of $K_m$ observed in the dephosphorylated and phosphorylated fibers shows that the binding and possibly release of nucleotides have been affected by phosphorylation, but only in the presence of vanadate. First, we conclude that the inhibition of velocity observed in phosphorylated fibers is not produced by a slower rate of binding of ATP as the affinity for ATP is actually greater in the phosphorylated fibers. Second, the observation that phosphorylation does not alter the apparent binding of ATP in the absence of vanadate suggests that the binding of nucleotides by the active myosin heads is not affected by phosphorylation. This observation does not support the hypothesis that the conformation of the nucleotide site is affected by the distant phosphorylation via direct communication through the head. Thus the inhibition of fiber velocity by phosphorylation and the lower $K_m$, both seen in the presence of vanadate most probably arise from an interaction between the active heads and the inactive heads that have bound ADP•Vi. Although the site of phosphorylation is on the regulatory light chain, some 10nm from the active site it could affect nucleotide binding by altering interactions between myosin heads. These interactions most probably occur between heads on the same molecule, but the possibility that they occur between heads on distant molecules cannot be excluded. Communication between the active site and a phosphorylated serine on the regulatory light chain has been observed previously for two-headed smooth muscle myosin (20).

There is a curious similarity in the variation of $K_m$ between dephosphorylated and phosphorylated fibers in the presence of vanadate and between fast and slow fibers. The magnitude of $K_m$ has the same correlation with muscle velocity. At low [ATP], the slow fibers actually had a faster shortening velocity than the fast fibers, and a lower $K_m$ for half-maximal velocity (23). Thus the similarities between fast and slow muscle types and between dephosphorylated and phosphorylated fibers in the presence of vanadate suggests a similar mechanism may account for the slower fiber velocity seen in slow, type I, muscle and in phosphorylated fast, type II, muscle. Shortening velocity is thought to be related to a slower release of ADP in the slow fibers suggesting that the slower velocity of the phosphorylated fibers may be related to a slower release of ADP.

Although we favor the second of the two hypotheses posed above, that phosphorylation affects nucleotide binding and release, rather than the first, that velocity is inhibited by the drag created
by non-force generating heads, both explanations for our results have merit. In fact both hypotheses may involve interactions of non-force heads with force generating heads. Ultimately structural studies will be needed to discern the combined effects of RLC-Pi and Vi on cross bridge structure, thick filament structure and cross bridge-thin filament interactions.

Conclusions.

Our most important conclusion is that myosin regulatory light chain phosphorylation is more complex than previously thought. In addition to its known role in potentiating the tension of fibers at low levels of activation, at temperatures above 22°C, it influences the velocity of fibers that are partially inhibited by the phosphate analog vanadate, and to a lesser extent by BeF₃. It also decreases the $K_m$ for velocity measured in the presence of vanadate as a function of ATP, demonstrating that the binding of nucleotides is affected by phosphorylation. This may account for the slower velocity seen in fibers with phosphorylated myosin light chains. Because the states populated by the phosphate analogs studied here are thought to mimic those that are also populated by the high levels of phosphate that build up during muscle fatigue, our results suggest that phosphorylation of the myosin regulatory light chain, which also occurs during fatigue, may be playing a role in the inhibition of fiber velocity that is observed in vivo. Recent work in our laboratory, unpublished, suggests that this is the case with myosin phosphorylation producing a small inhibition of fiber velocity at 30°C in high phosphate and low pH. Thus myosin phosphorylation may play a role in the inhibition of fiber velocity observed during muscle fatigue, a phenomenon that has not been fully explained by previous investigations.

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References


Figure 1. Isoelectric focusing gel of two fiber preparations used in these studies. Lane 1 shows purified myosin with regulatory light chains (RLC) in the dephosphorylated form. Lanes 2 and 3 show control and phosphorylated fiber preparations with myosin regulatory light chains in the dephosphorylated and phosphorylated forms respectively. The gel was stained with Pro-Q, which stains phosphorylated serines/threonines selectively. However Pro-Q also stains some unphosphorylated proteins, but with less intensity. In particular it stains the dephosphorylated regulatory light chain with one forth the intensity as it does the phosphorylated form. The gel shows that the regulatory light chains of the fibers shown in lane 3 are 60 ± 15% phosphorylated, while those of the fibers shown in lane 2 are less than 10% phosphorylated. No other band on the gel displays a change in intensity or in position, demonstrating that the procedure to phosphorylate the fiber preparations was specific for the regulatory light chain of myosin.
Figure 2. Fiber displacements are shown for tension clamps performed in the presence and absence of 0.5 mM vanadate. The y-axis shows the position of the motor arm to which the fiber is attached as a function of time. In each case the tension was clamped to approximately 10% of isometric. The circles show the data obtained in the absence of vanadate and the squares show the data obtained in the presence of vanadate. The open symbols show data obtained for fibers in which the myosin regulatory light chain is dephosphorylated. The filled symbols show the data for fibers in which the regulatory light chains are phosphorylated. The data between 10 and 40 milliseconds was fit by a straight line providing estimates of fiber velocity, shown by the solid lines. A series of velocities obtained from several fibers were used to construct force-velocity curves shown in Figures 3, 5 and 6. Fiber lengths were 3.2 millimeters, 30 °C.
Figure 3. Force velocity curves at 30°C in the absence, open circles, and presence, closed circles, of vanadate, for fibers in which the regulatory light chains are dephosphorylated, A and phosphorylated, B. Force clamps to varying levels of force were performed as described in Figure 2, and the velocities measured from a series of fibers were plotted to obtain the force velocity relations shown. The data were fit to the Hill equation as described in Pate et al. (24), defining two parameters, the maximum shortening velocity extrapolated to tension = 0, $V_{\text{max}}$, and $\alpha/P_0$, which is related to the curvature, with lower values indicating more curvature. In dephosphorylated fibers the fits define $V_{\text{max}} = 5.4 \pm 0.2$ lengths/sec, $\alpha/P_0 = 0.35 \pm 0.05$ in the absence of vanadate; $V_{\text{max}} = 6.0 \pm 0.2$ lengths/sec, $\alpha/P_0 = 0.20 \pm 0.04$, in 0.5 mM vanadate; In phosphorylated fibers the fit defines, $V_{\text{max}} = 6.3 \pm 0.3$ lengths/sec, $\alpha/P_0 = 0.20 \pm 0.06$, in the absence of vanadate; $V_{\text{max}} = 3.8 \pm 0.3$ lengths/sec, $\alpha/P_0 = 0.26 \pm 0.06$, in 0.5 mM vanadate.
Figure 4. The inhibition of fiber shortening velocity by vanadate. The velocity obtained in the presence of vanadate is shown relative to the velocity in the absence of vanadate. The data obtained by Pate et al. in 1994, with dephosphorylated fibers show that vanadate inhibits velocity at low but not at high temperatures, open circles solid line. Upon phosphorylation of the myosin light chains, vanadate inhibits velocity at all temperatures, closed circles, dashed line. More recent data obtained with dephosphorylated fibers resembles that obtained in 1994, open squares.
Figure 5  Force velocity curves for fibers in 250 μM AlF₄, 30°C. Dephosphorylated fibers, open circles, solid line; phosphorylated fibers, closed circles, dashed line. The data were fit to the Hill equation, as described in Pate et. al. The fits defined: dephosphorylated fibers, $V_{\text{max}} = 3.7 \pm 0.15$ lengths/sec, $\alpha/Po = 0.36 \pm 0.06$; phosphorylated fibers, $V_{\text{max}} = 3.6 \pm 0.15$ lengths/sec, $\alpha/Po = 0.32 \pm 0.05$. 
Figure 6. Force velocity curves for fibers in 5 mM BeF$_3$ 30°C. Dephosphorylated fibers, open circles, solid line; phosphorylated fibers, closed circles, dashed line. The data were fit to the Hill equation, as described in Pate et. al. (24). The fits define: dephosphorylated fibers, $V_{\text{max}} = 4.1 \pm 0.2$ lengths/sec, $\alpha/Po = 0.5 \pm 0.1$; phosphorylated fibers, $V_{\text{max}} = 3.6 \pm 0.2$ lengths/sec, $\alpha/Po = 0.4 \pm 0.1$. 
Figure 7. The values of the maximum contraction velocity, $V_{\text{max}}$, are plotted for dephosphorylated fibers, open bars, and phosphorylated fibers, shaded bars, in four conditions: in the absence of phosphate analogs, control, and in the presence of the three phosphate analogs studied here. Force velocity curves defining $V_{\text{max}}$ are shown in Figures 3, 5 and 6. All data were obtained at 30°C. For each analog the concentration was chosen to give 25 ±5% of the control tension obtained in the absence of the analogs; concentrations are given in the legends to Figures 3, 5 and 6.
Figure 8. The stiffness of muscle fibers activated in 0.5 mM Vi at 30°C was determined by step changes in fiber length. The figure shows the tension reached as a function of the length of the stretch: dephosphorylated fibers, open circles, solid line; phosphorylated fibers closed circles, dashed line. The stiffness is determined from the slope of the linear fit to the data. The initial isometric tensions were 52 ± 5 kN/m², dephosphorylated and 47 ± 6 kN/m², phosphorylated. The values of the Young's modulus were: dephosphorylated fibers, 3.5 ± 0.3 x10⁶ N/m²; and phosphorylated fibers, 2.7 ± 0.4 x10⁶ N/m².
Figure 9
The maximum contraction velocity of rabbit psoas fibers activated in 0.5 mM vanadate at 30°C as a function of [ATP]; dephosphorylated fibers, open circles, solid line; phosphorylated fibers, filled circles, dashed line. Values of $V_{\text{max}}$ were determined by extrapolation of force-velocity curves to zero tension, each obtained at a specific [ATP], in which force clamps to varying levels of force were performed as described in Figure 2. The solid and dashed lines are the fit of the Michaelis-Menten equation to the data, defining values for velocity at saturating ATP of 6.4 ± 0.5 lengths/sec and $K_m$ of 192 ± 40 μM for the dephosphorylated fibers; and velocity at saturating ATP of 3.7 ± 0.4 lengths/sec and $K_m$ of 20 ± 4 μM for the phosphorylated fibers.
Figure 10. Cross bridge states in muscle fibers. States 1-6 represent the series of cycling states found in active muscle with the 3 states in the lower row producing force. State 1 shows myosin bound to ATP following dissociation from actin. The transition from State 1 to 2 involves nucleotide hydrolysis, with ADP.P\textsubscript{i} favoring the bent pre-power stroke conformation. In State 3 myosin is bound weakly to actin and is not generating force. Both Myosin ATP and Myosin ADP.P\textsubscript{i} can bind weakly to actin, and these are shown together as State 3. An isomerization leads from State 3 to State 4 which does generate force. The release of Pi leads to a stronger bond in State 5, and the release of ADP leads to the even stronger rigor bond at the end of the power stroke in State 6. State 7 shown in the top row is the state observed in relaxed muscle with the myosin heads bound in an ordered array around the thick filament. Here we hypothesize that this state is also found in active fibers partially inhibited by vanadate. The state of the nucleotide bound to myosin in State 7 has not been determined conclusively, but myosin is probably in the pre-power stroke conformation with ADP.P\textsubscript{i}. Myosin phosphorylation destabilizes State 7. Partially inhibition of active fibers by a phosphate analog, would result in increased populations of states that mimic States 1-3 and possibly State 7.