The Direct Molecular Effects of Fatigue and Myosin Regulatory Light Chain Phosphorylation on the Actomyosin Contractile Apparatus

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Abstract

Skeletal muscle, during periods of exertion, experiences several different fatigue-based changes in contractility including reductions in force, velocity, power output, and energy usage. The fatigue-induced changes in contractility stem from many different factors including alterations in the levels of metabolites, oxidative damage, and phosphorylation of the myosin regulatory light chain (RLC). Here, we measured the direct molecular effects of fatigue-like conditions on actomyosin unloaded sliding velocity using the in vitro motility assay. We examined how changes in ATP, ADP, Pi, and pH affect the ability of the myosin to translocate actin and whether the effects of each individual molecular species are additive. We found that the primary causes of the reduction in unloaded sliding velocity are increased [ADP] and lowered pH and that the combined effects of the molecular species are non-additive. Furthermore, since an increase in RLC phosphorylation is often associated with fatigue, we examined the differential effects of myosin RLC phosphorylation and fatigue on actin filament velocity. We found that phosphorylation of the RLC causes a 22% depression in sliding velocity. On the other hand, RLC phosphorylation ameliorates the slowing of velocity under fatigue-like conditions. We also found that phosphorylation of the myosin RLC increases actomyosin affinity for ADP suggesting a kinetic role for RLC phosphorylation. Furthermore, we showed that ADP binding to skeletal muscle is load dependent, consistent with the existence of a load dependent isomerization of the ADP bound state.
Introduction

Skeletal muscle exhibits several fatigue based changes in contractility (for review, see (9)). The physiological bases of fatigue induced changes in contractility have been shown to be due to several different factors including changes in excitation-contraction coupling, neuronal activity, and muscle contractility. The effects of fatigue on contractility are thought to result from several factors including alterations in the levels of metabolites (ATP, ADP, Pi, pH etc.).

Fatigue-like changes in metabolites in skeletal muscle fibers have been shown to cause reductions in muscle fiber velocity, force production, power output (for review, see (9, 17)). Myosin RLC phosphorylation, which occurs with repetitive twitch activations, may also play a role in the observed changes in muscle mechanics that occur during periods of exertion and fatigue (51). RLC phosphorylation appears to have a modulatory function in striated muscle fibers where both the magnitude and rate of tension development are enhanced, primarily at submaximal calcium levels ((51)). Furthermore, recent data suggest that phosphorylation also alters myosin kinetics under fatigue-like conditions, reducing myosin sliding velocity and increasing the affinity of myosin for ATP (18, 27, 50).

Taken together, these changes in muscle mechanics observed in muscle fiber studies suggest that fatigue-like changes in metabolites and phosphorylation affect one or more steps of the mechanochemical cycle of actomyosin. However, studying the direct molecular effects of fatigue and phosphorylation on the actomyosin contractile apparatus in muscle fibers can be extremely challenging due to several factors. Opposing effects have been observed in studies of living muscle fibers compared to skinned muscle fibers.
For example, whereas in living muscle fibers there is an increase in tension economy during fatigue, there is a decrease in tension economy in skinned muscle fibers (35). The effects of fatigue and phosphorylation are temperature dependent with major differences seen between studies of muscle fibers at 10-15°C and those conducted at more physiological temperatures (such as an enhancement of submaximal calcium activation in RLC-phosphorylated myosin, changes in lattice spacing, unloaded shortening velocity (for review, see (9)). With the exception of the jump plate technique implemented by Cooke and colleagues (40), studies in skinned muscle fibers, which allow for controlled examination of the effects of different biochemical conditions on actomyosin contractility, can suffer from disorganization of the myofilament proteins observed at physiological temperatures which may ultimately lead to changes in muscle mechanics. In addition, in the study of the direct molecular effects of fatigue and phosphorylation on the actomyosin contractile apparatus, it is difficult to separate molecular-based changes in the actomyosin interaction from broader, cellular-based changes in the muscle fiber and in the organization of the actomyosin lattice.

Here we used the in vitro motility assay to study the direct molecular the effects of fatigue-like metabolic molecular species (ATP, ADP, Pi, and pH) and myosin phosphorylation on the actomyosin contractile apparatus. The in vitro motility assay affords the ability to directly determine the molecular mechanism by which fatigue-like conditions affect the mechanochemistry of actomyosin at physiologically relevant temperatures and without the complicating factors of the myofilament array. We observed that fatigue-like metabolites cause a reduction in unloaded sliding velocity in both phosphorylated and dephosphorylated myosins. Both lowering pH and increasing
the concentration of ADP depressed sliding velocity independently, yet their combined effects were non-additive due to a transition from unloaded to loaded motility. Furthermore, we showed that phosphorylated myosin has a higher affinity for ADP than dephosphorylated myosin, reinforcing the notion that phosphorylation of the myosin RLC plays a direct role in tuning actomyosin kinetics (18, 20). Lastly, we showed that the ADP affinity of acto-myosin is load sensitive, suggesting that load sensitivity of ADP binding and release in skeletal muscle myosin is similar to the load sensitivity observed in non-muscle and smooth muscle myosins (30, 58, 59). This load sensitivity is higher for phosphorylated than dephosphorylated myosin, which is consistent with a kinetic scheme where there is a load sensitive isomerization of actomyosin in the ADP bound state (49).
Materials and Methods

Preparation of Proteins

Endogenously Dephosphorylated and Phosphorylated Rabbit Skeletal Myosin

All animal studies were conducted in accordance with institutional guidelines (University of Miami Miller School of Medicine #A3711-01). Phosphorylated and dephosphorylated rabbit skeletal myosin was isolated from rabbit fast skeletal muscle taken from freshly sacrificed New Zealand White rabbits as described previously (20). Purity of myosin preparations was determined by SDS-PAGE analysis and phosphorylation status examined on 8% polyacrylamide gel in the presence of 8 M urea (55).

Actin Purification and Labeling

Unlabeled actin was prepared from chicken pectoralis muscle acetone powder as described previously (20). The actin was suspended in actin buffer (25 mM KCl, 1 μM EGTA, 10 μM DTT, 25 μM imidazole, 4 μM MgCl₂). TRITC phalloidin labeled actin was prepared by reacting a 1:1 molar ratio mixture of TRITC phalloidin and actin in actin buffer overnight at 4°C.

In Vitro Motility Assays

The in vitro motility assays were performed as previously described (20, 21, 54) with some subtle modifications. Approximately 200 μg of myosin in 50% glycerol was precipitated in 1 ml of 10 mM DTT for one hour on ice and collected by centrifugation at 16,000 x g for 30 minutes at 4°C. The pellet was resuspended in 200 μl of myosin buffer (300 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl₂, 10 mM DTT). The
remainder of the protocol, including microscopy was identical to previous protocols published earlier (20, 21). All experiments were performed at 35°C with the temperature of the flow cell being regulated using a Bioptechs objective heater (Bioptechs Inc., Butler, PA).

**Motility Buffers**

All buffers were designed using balanced ions as determined using the Bathe program as described in (20) and WinMaxC (http://www.stanford.edu/~cpatton/maxc.html). All buffers had a total ionic strength of 150 mM, pCa 9, and contained 5 mM EGTA, 5 mM MgCl₂, 25 mM imidizole, 25 mM MES, 0.5% methyl cellulose, 2 mM dextrose, 160 units glucose oxidase, and 2 μM catalase. Both imidizole and MES were used to ensure effective buffering over the full range of pH values (at 35°C imidizole is effective from pH 6.29 to 7.29 while MES is effective from pH 5.49 to 6.49). A similar buffering scheme has been utilized in muscle fiber studies (27). In addition to the molecular species listed above, the standard buffer contained 5 mM ATP, 0.02 mM ADP, 2 mM Pi, pH 7.0 and the fatigue-like buffer contained 3 mM ATP, 0.3 mM ADP, 30 mM Pi, pH 6.2 (9). For the studies of the effects of individual molecular species on actin filament sliding velocity, standard buffer was prepared and only one molecular species (i.e. ATP, ADP, Pi, or pH) at a time was altered.

**Load Dependent Kinetics Measurements**
The procedure for measuring the load dependent kinetics was identical to the standard motility assay except the myosin was mixed with 1.25 μg/ml of alpha-actinin (Cytoskeleton Inc., Denver, CO) before being incubated in the flow cell.

Data fitting

All curve fitting was done in SigmaPlot (Systat Software Inc., San Jose, CA) using a least squares fitting algorithm. Measurements of velocity versus ATP deviated from purely Michaelis-Menten kinetics since phosphate can act as a competitive inhibitor and affect velocity at low ATP (39). Thus the measurement of sliding velocity versus ATP was fit to a Hill curve:

\[ y = \frac{V_{\text{Max}} \cdot [ATP]^H}{K_M^H + [ATP]^H} \]  

(1)

where \( V_{\text{Max}} \) is the maximal sliding velocity, \( K_M \) is the concentration of ATP at which half maximal velocity is attained, and \( H \) is the Hill coefficient. Measurements of the effect of pH on velocity were fit to an analogous expression where \( K_M \) is replaced with the \( pH_{50} \) and [ATP] is replaced with the pH.

For studies with ADP, the data was fit to a competitive inhibitor model:

\[ V = \frac{V_{\text{Max}} [ATP]}{[ATP] + K_M \cdot (1 + \frac{[ADP]}{K_I})} \]  

(2)

where \( K_M \) was determined from the velocity dependence of ATP measurements, \( V_{\text{Max}} \) is the maximal velocity in the absence of ADP, and \( K_I \) is the inhibition constant.

For studies with Pi, the data were fit to a line.

Statistics
All experiments were repeated using at least two different myosin preparations. The filament velocity was determined by manual tracking using the freeware motility software, Retrac (http://mcl1.mcrl.ac.uk/Retrac). For each flow cell, the velocity and standard errors in the velocity were calculated for 15-30 sliding filaments over the course of 5-10 frames from at least two different areas of the flow cell (to ensure that there were no surface artifacts). Only moving filaments were counted in the average velocities.

When the data were fit to a model, parameter values and the errors in the parameters were determined from the least squares fit. A two-tailed t-test was used to examine the significance of the differences between velocities. The p value was calculated from the Student’s t-test distribution and corrected for multiple comparisons using the Holm t-test criteria when necessary.
Results

*Fatigue-like conditions reduce unloaded shortening velocity*

The unloaded sliding velocity of phosphorylated and dephosphorylated myosins were measured under both, standard (5 mM ATP, 0.02 mM ADP, 2 mM Pi, pH 7.0) and fatigue-like conditions (3 mM ATP, 0.3 mM ADP, 30 mM Pi, pH 6.2) (Fig. 1). Under standard conditions, phosphorylation of the RLC causes a 22% percent depression in sliding velocity ($V_{\text{dephos.}} = 7.0 \pm 0.4 \mu m/s$, $V_{\text{phos.}} = 5.5 \pm 0.3 \mu m/s$; $p<0.005$) (Fig. 1A). This is consistent with previous motility studies performed in the absence of exogenously added ADP and Pi (20). On the other hand, under fatigue-like conditions, phosphorylated myosin has a 28% higher sliding velocity than dephosphorylated myosin ($V_{\text{dephos.}} = 1.3 \pm 0.1 \mu m/s$, $V_{\text{phos.}} = 1.7 \pm 0.1 \mu m/s$; $p<0.02$) (Fig. 1B). Thus, fatigue-like conditions cause an 81% depression in sliding velocity in dephosphorylated myosin yet only a 69% depression in sliding velocity in phosphorylated myosin. In order to examine the molecular basis for the effects of fatigue metabolites and myosin phosphorylation, we examined the individual effects of ATP, ADP, Pi, and pH on unloaded sliding velocity by adding ATP, ADP, Pi, or lowering pH in the standard motility buffer.

*Fatigue-like reductions in the concentration of ATP has little effect on sliding velocity.*

During fatigue, the available cellular pool of ATP decreases from 5 mM to 3 mM (9). The effect of ATP on unloaded sliding velocity was measured for both phosphorylated and dephosphorylated myosins over a range of ATP concentrations (Fig. 2A). As ATP is added, the unloaded sliding velocity increases steadily reaching, the maximum near 0.5
mM ATP. Since buffer solutions also contained 0.02 mM ADP and 2 mM Pi, the data deviated from standard Michaelis-Menten kinetics at low concentrations of ATP (39). Consequently, the data were fit to Hill plots yielding the apparent $K_M$ for ATP determined at 50% of maximal velocity ($V = \frac{1}{2} V_{Max}$). There is no statistically significant difference between phosphorylated ($K_M = 225 \pm 25 \mu M$) and dephosphorylated ($K_M = 190 \pm 10 \mu M$) myosins ($p=0.21$), consistent with the values measured in muscle fiber studies under non-fatigue conditions (18). Furthermore, fatigue-like reductions in the concentration of ATP from 5 mM to 3 mM under fatigue-like conditions has little effect on sliding velocity since these values are within the plateau region of the curve (data not shown), indicating that physiological fatigue-based changes in ATP alone have little effect on sliding velocity, consistent with fiber studies (2, 16).

**Exogenously added phosphate does not affect unloaded shortening velocity**

During fatigue, the concentration of Pi increases from 2 mM to 30 mM (9). The effect of Pi on unloaded sliding velocity was examined for both phosphorylated and dephosphorylated myosins (Fig. 2B). As can be seen from the data, Pi has no effect on unloaded sliding velocity for either phosphorylated or dephosphorylated myosins. The lack of effect of Pi on unloaded sliding velocity at saturating ATP is consistent with the data from skinned muscle fiber (10, 11, 13, 39) and *in vitro* studies (25).

**Acidosis decreases unloaded shortening velocity**

During periods of fatigue, the pH drops from 7.0 to 6.2 (46). The effects of alterations in pH on unloaded sliding velocity were measured for both dephosphorylated and
phosphorylated myosins (Fig. 3). As can be seen from the data, both phosphorylated and
dephosphorylated myosins showed significant reductions in sliding velocity when the pH
was lowered from pH 7.0 (standard pH) to 6.2 (fatigue pH), consistent with skinned fiber
studies (9, 14, 19, 36). These data were fit to Hill curves in order to obtain the pH_{50}, the
pH at which sliding velocity is reduced to 50% of the maximal sliding velocity. Both
phosphorylated and dephosphorylated myosins show indistinguishable pH_{50} values
(pH_{50}(dephos.) = 6.55 ± 0.02, pH_{50}(phos.) = 6.54 ± 0.03; p=0.54). Interestingly, at pH 7
phosphorylated myosin has a significantly lower sliding velocity than dephosphorylated
myosin (p<0.01), whereas at pH 6.2, there is no significant differences between the
sliding velocities of phosphorylated and dephosphorylated myosins (p=0.40).

Exogenously added ADP decreases velocity

During fatigue, ADP accumulates, increasing in concentration from 0.02 mM to 0.3 mM
(9). The effects of ADP accumulation on unloaded sliding velocity were examined for
both phosphorylated and dephosphorylated myosins (Fig. 4). As can be seen from both
the unnormalized (Fig. 4A) and normalized data (Fig. 4B), increasing [ADP] from 0.02
mM to 0.3 mM causes an 18% decrease in velocity for dephosphorylated myosin but a
34% decrease in sliding velocity for phosphorylated myosin. This is most likely due to
the approximately 2-fold higher affinity for ADP observed for phosphorylated myosin
compared to dephosphorylated myosin (K_{i}(dephos.) = 200 ± 30 μM, K_{i}(phos.) = 95 ± 10
μM; p<0.01). The K_{i} measured for dephosphorylated myosin is consistent with values
for skeletal muscle myosin measured in \textit{in vitro} motility assays (1) and muscle fibers (8,
9, 28, 48).
Load decreases the sensitivity of myosin to exogenous ADP

In order to examine the load sensitivity of ADP affinity for myosin, we introduced 1.25 μg/ml of alpha-actinin into the standard motility assay (see Materials and Methods) and examined the effect of exogenously added ADP (Fig. 4C and D). Alpha-actinin is a low affinity actin binding protein that transiently binds to translocating actin filaments in the \textit{in vitro} motility assay, exerting a frictional load that opposes the driving force of myosin (4, 20). As can be seen from the unnormalized (Fig. 4C) and normalized data (Fig. 4D), introducing a resistive load causes an approximately 10-fold decrease in the sensitivity of myosin to exogenously added ADP. For dephosphorylated myosin, the $K_I$ increases from 200 ± 30 μM in the absence of load to 5 ± 2 mM in the presence of load. For phosphorylated myosin, the $K_I$ increases from 95 ± 10 μM to 6 ± 4 mM in the presence of load.
Discussion

In this study, the *in vitro* motility assay was utilized to examine the direct molecular effects of fatigue-like conditions and myosin regulatory light chain phosphorylation on the unloaded sliding velocity of the actomyosin contractile apparatus. One of the major findings of this study was that under fatigue-like conditions, there was a significant reduction in unloaded sliding velocity for both phosphorylated and dephosphorylated myosins. While this result is consistent with fiber studies (for review, see (9)), using the motility assay we showed this effect can be ascribed directly to changes in the kinetics of the myosin motor, independent of fatigue-condition based changes in the myofilament lattice. In order to dissect the mechanism for these changes we examined independent effects of ATP, ADP, Pi, pH and phosphorylation on unloaded sliding velocity.

*Physiological fatigue-like changes in ATP and inorganic phosphate do not affect unloaded velocity*

Fatigue-like changes in the cellular levels of either ATP (5 to 3 mM) or Pi (2 to 30 mM) have no effect on the unloaded sliding velocity in the motility assay (Fig. 2). This result is consistent with fiber studies (2, 10, 11, 13, 39). Furthermore, the $K_M$ for ATP is unchanged when comparing phosphorylated and dephosphorylated myosins, consistent with the skinned fiber studies (18). It is important to note that although the accumulation of Pi had no effect on unloaded sliding velocity, it would be expected to play a role under loaded conditions, changing myosin force and power output (7, 10, 23, 39). While physiological reductions in ATP alone do not show any effect on sliding velocity, the 10-
fold reduction affinity of ATP for myosin (12) and the inhibition of phosphocreatine
resynthesis observed at low pH (16), could make changes in the cellular ATP levels more
significant in determining unloaded sliding velocity in intact muscle fibers.

*Acidosis decreases unloaded sliding velocity*

During fatigue, there is a significant acidosis of the muscle primarily due to increased
proton production from glycolysis (46). Here we showed that reductions in pH cause a
reduction in unloaded actin filament sliding in the *in vitro* motility assay particularly
below pH 6.7, consistent with skinned fiber (9, 14, 19, 36) and *in vitro* motility studies
(12). The reduction in pH from pH 7.0 to 6.2 decreases sliding velocity by ~91% and
~90% for dephosphorylated and phosphorylated myosins, respectively, showing that,
both myosins are similarly affected by changes in pH alone. Interestingly, Pate et al.
observed less pronounced effects of velocity depression by pH measured at higher
temperatures (30°C) in skinned muscle fibers (38). In contrast to this result, we and
others (12) observe a significant reduction in velocity at 30-35°C with acidosis alone.

The effects of pH in muscle fibers have been proposed to result from changes in
lattice spacing (57), constraints that are not present in the *in vitro* motility assay,
suggesting that while changes in the lattice spacing play a role in affecting the mechanics
of muscle fibers, there is also a direct molecular effect of acidosis on the actomyosin
contractile apparatus. Furthermore, acidosis causes a reduction in the force per
crossbridge (33). It is also possible that the observed differences in the effects of acidosis
stem from the presence of regulated thin filaments (38) in the skinned fibers whereas our
*in vitro* studies employ unregulated thin filaments. It has been suggested that direct
effects of the acidosis on the troponin complex (17, 34, 61) cause the observed shift in the intact fiber force-pCa relationship (15). Further *in vitro* motility studies utilizing regulated thin filaments could potentially help dissect the relative roles of acidosis on the thin filament regulatory apparatus and actomyosin.

*ADP inhibits unloaded sliding velocity*

Whereas decreasing pH affects both phosphorylated and dephosphorylated myosins similarly, increasing the ADP from 0.02 mM to 0.3 mM decreases sliding velocity 18% and 34% for dephosphorylated and phosphorylated myosins, respectively (Figs. 4A and 4B). This is primarily due to the fact that phosphorylated myosin has a 2-fold higher affinity for ADP than dephosphorylated myosin. It has previously been proposed that the principal effect of skeletal muscle myosin phosphorylation is the release of the myosin heads from the thick filament backbone which would increase the rate of attachment of myosin to actin (32). The data obtained here was collected using the *in vitro* motility assay where there was no intact thick filament backbone, revealing a direct kinetic role for RLC phosphorylation in addition to the observed structural role, consistent with the suggestion of Franks-Skiba et al. (18, 20).

*The effects of the individual molecular species involved in fatigue are non-additive*

Because both ADP and pH depressed velocity, one might expect that their combined effects would be additive. Surprisingly, this is not the case (Fig. 5) as the velocities of both phosphorylated and dephosphorylated myosins under fatigue-like conditions are significantly higher than their respective velocities at pH 6.2 in the absence
of other fatigue-like factors. Whereas at pH 6.2 there is no difference in sliding velocity between phosphorylated and dephosphorylated myosins, phosphorylated myosin has a significantly higher velocity than dephosphorylated myosin under fatigue-like conditions. As will be discussed below, the increased velocity observed under fatigue-like conditions for phosphorylated myosin could be due to differences in ADP affinity, the only observed kinetic difference between phosphorylated and dephosphorylated myosins.

The non-additive effects of ADP and pH can explained by the differences between loaded and unloaded kinetics. Under unloaded conditions, the velocity of myosin in the motility assay is limited by myosin cross-bridge detachment (47). Thus, exogenously added ADP has a depressive effect on velocity, similar to what was observed in studies of the effect of ADP on velocity seen here (Fig. 4) and elsewhere (1, 8, 9, 28, 48). On the other hand, under loaded conditions, myosin sliding velocity is no longer limited by detachment of myosin from actin but rather the amount of force that can be generated by the ensemble of myosins working against the imposed load, which results from (strongly or weakly) attached, non-force producing myosin cross-bridges (12). In this case, the velocity can be described by:

\[ V = V_{\text{Max}} \times \frac{[F_d(F_L) - F_L]}{F_d} \]  

(Eq. 3)

where \( V_{\text{Max}} \) is the maximal sliding velocity in the absence of a load, \( F_d(F_L) \) is the average driving force of the bed of myosin (which is a function of load (24)), and \( F_L \) is the imposed load.

It has been proposed that acidosis effects several biochemical transitions within the myosin biochemical cycle (12, 29), reducing the force per crossbridge (33) while increasing the population of weakly bound (29), dragging cross-bridges, introducing a
load into the motility assay (60). Under loaded conditions the detachment limited model for motility no longer applies. Consistent with this notion, fatigue-like behavior in skinned fiber studies can be mimicked by agents that populate weak-binding states in the absence of acidosis (18, 27, 50).

Since under loaded conditions ADP causes an increase in force (22, 35), the addition of ADP to the motility assay under acidotic conditions will increase the net myosin driving force and thus sliding velocity, according to Eq. 3. Thus, the competing effects of the load introduced into the motility assay with acidosis (12) along with the enhancement of force with ADP accumulation, explain the non-additive effects of the individual molecular species involved with fatigue observed here. Consistent with this idea, Godt and Nosek showed that effects of the different molecular species involved with fatigue are additive over the range of pH 6.65-7 (19) but not below pH 6.65 (36). Similarly, as can be seen in figure 3, the effects of acidosis became appreciable once the pH dropped below 6.7. Since acidosis introduces a load into the motility assay and thus causes a transition from unloaded to loaded kinetics, it is not surprising that the effects of acidosis were additive over the range that acidosis is not appreciable (Fig. 3). Moreover, the higher affinity of phosphorylated actomyosin for ADP should increase the driving force, explaining the greater recovery of velocity for phosphorylated myosin under fatigue-like conditions compared to the dephosphorylated myosin.

*Phosphorylated myosin biochemistry is more sensitive to load*

Here (Fig. 1) and previously (20), we showed that phosphorylation causes a 20% reduction in unloaded velocity in the absence of exogenously added ADP and Pi at pH
7.4 This difference in sliding velocity was primarily due to an increase in the myosin duty cycle with phosphorylation (20). In the current work, we show that phosphorylated myosin has a 2-fold higher affinity for ADP, suggesting a biochemical basis for the reduced velocity and increased duty cycle of phosphorylated myosin under unloaded conditions. Consistent with this notion, Patel et al. (41) showed that phosphorylation slows the rate of transition from a strongly bound state to a weakly bound state (i.e. the detachment rate).

The myosin RLC binds the elongated alpha-helical neck region of the myosin molecule. In addition to its role as a force generator (43), the neck region has also been proposed to serve as a molecular load sensor, transmitting load directly to the active site, altering myosin kinetics (37).

Previous motility data was consistent with a stiffening of the load-sensing myosin lever arm upon phosphorylation (20). Since ADP release is the load sensitive step of the myosin biochemical cycle (26, 59), one would expect greater load sensitivity to ADP release upon phosphorylation. We therefore introduced a load into our experiment by adding alpha-actinin to the motility assay surface and assayed the dependence of velocity on exogenously added ADP. We can define a load sensitivity parameter, \( \psi \), as:

\[
\psi = \frac{K_f(\text{Loaded})}{K_f(\text{Unloaded})}
\]

where a greater value of \( \psi \) is indicative of a motor with a greater load sensitivity.

Computing the load sensitivity parameter for data taken in the absence (Figs. 4A and 4B) and presence of a frictional load (Figs. 4C and 4D), we saw that \( \psi(\text{dephos.}) \) is approximately 20 and \( \psi(\text{phos.}) \) is approximately 60, indicating that phosphorylated myosin is more load sensitive.
Studies of the load dependence of ADP affinity presented here show that ADP affinity is extremely load sensitive with an approximately 10-fold lower affinity for exogenously added ADP under load. This fact requires that the ADP exchangeable state precedes the load dependent transition. A lowering of affinity for exogenously added ADP under load is consistent with a kinetic scheme in which there is a load dependent isomerization from a moderate-affinity ADP-bound state where ADP is exchangeable, to a state in which the affinity of myosin for ADP is low and thus exogenously added ADP has little effect. It is possible that loading the myosin causes a closing of the active site, preventing ADP release and making the myosin less sensitive to exogenously added ADP (37).

Load sensitivity may be conveyed via prevention of an isomerization step that leads to the low affinity state and ADP is released. Such an isomerization has been inferred from the crystal structures and EM reconstructions of several non-muscle myosins and smooth muscle myosins in which ADP is added to rigor myosin (For review, see (37)), all of which complete a working stroke in 2 steps (58, 59). Structural evidence for the isomerization has not been directly observed for skeletal muscle myosin (44); however, optical trapping, kinetic, and fluorescence data have all suggested the existence of multiple ADP bound states (1, 6, 49, 56), consistent with our data. It is an intriguing possibility that this state is a common feature of all myosins and that structural studies have not observed this transition because of the large free energy change associated with ADP dissociation from actomyosin(37).

Relation to fiber studies
Our data, showing a decrease in unloaded sliding velocity with phosphorylation (Fig. 1) in the absence of fatigue-like conditions and an increase in velocity with phosphorylation under fatigue conditions, contrasts with fiber studies in which no change (5, 18, 42, 50, 52) or a decrease (18, 50) in velocity was observed. In a two state model, with attachment rate, \( f \), detachment rate, \( g \), and duty cycle \( f/(f+g) \), it has been proposed that the increased mobility of the myosin heads upon phosphorylation due to myosin heads detaching from the thick filament backbone increases \( f \) (32, 53). There is also evidence however, that phosphorylation acts to additionally decrease \( g \) (41). Our data, which shows a slowing of velocity and an increased duty cycle (20) with myosin phosphorylation, supports the notion that phosphorylation of the RLC causes a decrease in \( g \). Since the \textit{in vitro} motility assays employed here utilized monomeric myosin lacking a thick filament backbone any observed changes in actomyosin mechanics can be assigned to molecular based changes in the actomyosin interaction, independent of the effects that have been proposed to be due to the thick filament backbone (31, 32, 62). In muscle fibers where release of the myosin heads from the thick filament backbone with phosphorylation also increases \( f \), there will be more complex effects of decreasing \( g \) and increasing \( f \) on the duty cycle. Since velocity is inversely proportional to the duty cycle, the competing effects on \( f \) and \( g \) can potentially explain the different effects on velocity observed in fiber studies (5, 18, 42, 50, 52). Future studies using thick filaments in motility assays will help to clarify the relative effects of phosphorylation on attachment and detachment of myosin crossbridges.

Our observation that ADP release from phosphorylated myosin is more load sensitive suggests that under fatigue-like (loaded) conditions, ADP release would be
slowed, increasing the duty cycle. The resulting increase in the driving force (Eq. 3), provides a potential explanation for the greater recovery of velocity in the \textit{in vitro} motility assay for phosphorylated myosin under fatigue-like conditions compared to the dephosphorylated myosin.

\textbf{Perspectives and significance}

It has long been known that muscle fatigue is associated with the accumulation of metabolic end products and an increase in myosin RLC phosphorylation. \textit{In vitro} motility assay experiments can provide insight into the complex molecular mechanism of muscle fatigue because they provide a means to quantify force and velocity in a simple, well-defined system. While motility assays provide important information about the molecular-based changes in the actomyosin interaction, the ultimate characteristics of muscle under fatigue conditions involves contributions from a host of factors including changes in the myofilament array, changes in neuronal excitation, calcium handling, and cellular energetics (reviewed in (16)).

Our studies have shown a direct effect of fatigue metabolites and RLC phosphorylation on the unloaded velocity of the actomyosin contractile apparatus, which should be considered when interpreting skinned and intact muscle fiber studies. Future \textit{in vitro} motility studies that incorporate regulated thin filaments (3), filamentous myosin (45), and external loads (4) will help provide a more complete description of the underlying molecular basis for muscle fatigue.
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References


Figure Legends

Figure 1: Fatigue-like conditions reduce actin filament sliding velocity. (A) Comparison of the unloaded sliding velocities of RLC dephosphorylated and phosphorylated myosins under basal conditions. The velocity of phosphorylated myosin ($V = 5.5 \pm 0.3 \mu m/s$) is significantly reduced compared to the dephosphorylated myosin ($V = 7.0 \pm 0.4 \mu m/s ; p<0.005$). (B) Comparison of the unloaded sliding velocities of dephosphorylated and phosphorylated myosins under fatigue-like conditions shows that fatigue-like conditions cause a reduction of sliding velocity for both dephosphorylated and phosphorylated myosins. In contrast to the results under basal conditions, the velocity of phosphorylated myosin ($V = 1.7 \pm 0.1 \mu m/s$) is significantly increased compared to dephosphorylated myosin ($V = 1.3 \pm 0.1 \mu m/s ; p<0.02$).

Figure 2: Fatigue-like reductions in the concentration of ATP and phosphate have little effect on sliding velocity. (A) The effects of ATP concentration on unloaded sliding velocity. Reducing the available concentration of ATP from basal (5 mM) to fatigue-like conditions (3 mM) has no effect on sliding velocity (data not shown). Also, there is no significant difference in the $K_M$ between phosphorylated ($K_M = 225 \pm 25 \mu M$) and dephosphorylated myosins ($K_M = 190 \pm 10 \mu M; p=0.21$). Data fit to the Hill equation (see text for details). (B) The effects of changing the concentration of inorganic phosphate (Pi) on unloaded sliding velocity. A line with zero slope was fit to the data for illustrative purposes. The addition of Pi has little effect on the unloaded sliding velocity of either phosphorylated or dephosphorylated myosins. Thus the accumulation of Pi from basal
levels (2 mM) to fatigue-like levels (30 mM) has little effect on unloaded sliding velocity.

Figure 3: Acidic conditions decrease actin filament velocity. Changing the pH from basal levels (7.0) to fatigue-like levels (6.2) causes a significant reduction in sliding velocity. The data were fit to the Hill equation and it was shown that there is no difference in the pH50 for either phosphorylated (pH50 = 6.54 ± 0.03) or dephosphorylated myosins (pH50 = 6.55 ± 0.02; p=0.54).

Figure 4: Exogenous ADP decreases actin filament velocity. The (A) unnormalized and (B) normalized data are shown with fits to a competitive inhibitor model (Eq. 2). Changing the concentration of ADP from basal (0.02) to fatigue-like levels (0.3 mM) causes a significant depression in unloaded sliding velocity for both phosphorylated and dephosphorylated myosins. The inhibition constant for phosphorylated myosin ($K_I = 95 ± 10 \mu M$) is significantly higher than dephosphorylated myosin ($K_I = 200 ± 30 \mu M$; p<0.01), demonstrating that phosphorylated myosin has an approximately 2-fold higher affinity for ADP than dephosphorylated myosin. (C and D): Load decreases the sensitivity of actin filament velocity to exogenously added ADP. A frictional load was introduced into the motility assay using the low affinity actin binding protein, alpha-actinin. The (C) unnormalized and (D) normalized data were fit to Eq. 2. Loading the myosin causes a significant reduction in the ability of ADP to depress actomyosin sliding. Loading both the phosphorylated ($K_I = 6 ± 4$ mM) and dephosphorylated ($K_I = 5 ± 2$ mM) myosins causes a lowering of the affinity for exogenously added ADP.
Figure 5: The combined effects of the individual molecular species involved in fatigue are non-additive. Fatigue-like changes in pH (6.2) alone causes a significant depression in sliding velocity in the absence of other fatigue-like ionic molecular species. At pH 6.2, there is no significant difference in the unloaded sliding velocity between phosphorylated and dephosphorylated myosins (p=0.40). On the other hand, the combined effect of all of the molecular species together (at pH 6.2) has an increased velocity compared to acidosis alone. This suggests that effects of the individual molecular species involved in fatigue are non-additive. Furthermore, phosphorylated myosin has a greater recovery of velocity than dephosphorylated myosin (p<0.05) under fatigue like-conditions when compared to the effects of acidosis alone.
Figure 1
Figure 4
Figure 5