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Edward Patrick Debold
Marquette University

Robert H. Fitts
Marquette University, robert.fitts@marquette.edu

Christopher W. Sundberg
Marquette University, christopher.sundberg@marquette.edu

Thomas M. Nosek
Case Western Reserve University

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Muscle Fatigue from the Perspective of a Single Crossbridge

Edward P. Debold

*Department of Kinesiology, University of Massachusetts,
Amherst, MA*

Robert H. Fitts

*Department of Biological Sciences, Marquette University,
Milwaukee, WI*

Christopher W. Sundberg

*Department of Physical Therapy, Marquette University,
Milwaukee, WI*

Thomas M. Nosek

*Department of Physiology and Biophysics,
Case Western Reserve University,
Cleveland, OH*

Abstract

The repeated intense stimulation of skeletal muscle rapidly decreases its force- and motion-generating capacity. This type of fatigue can be temporally correlated with the accumulation of metabolic by-products, including phosphate (P_i) and protons (H^+). Experiments on skinned single muscle fibers demonstrate that elevated concentrations of these ions can

reduce maximal isometric force, unloaded shortening velocity, and peak power, providing strong evidence for a causative role in the fatigue process. This seems to be due, in part, to their direct effect on muscle's molecular motor, myosin, because in assays using isolated proteins, these ions directly inhibit myosin's ability to move actin. Indeed, recent work using a single molecule laser trap assay has revealed the specific steps in the crossbridge cycle affected by these ions. In addition to their direct effects, these ions also indirectly affect myosin by decreasing the sensitivity of the myofilaments to calcium, primarily by altering the ability of the muscle regulatory proteins, troponin and tropomyosin, to govern myosin binding to actin. This effect seems to be partially due to fatigue-dependent alterations in the structure and function of specific subunits of troponin. Parallel efforts to understand the molecular basis of muscle contraction are providing new technological approaches that will allow us to gain unprecedented molecular detail of the fatigue process. This will be crucial to fully understand this ubiquitous phenomenon and develop appropriately targeted therapies to attenuate the debilitating effects of fatigue in clinical populations.

Key Words: Muscle Fatigue; Myosin; Troponin; Velocity; Power; Muscle Fiber

Muscle fatigue from high-intensity contractile activity is thought to be due in large part to 1) the accumulation of metabolic by-products acting to directly inhibit contraction and 2) a reduction in intracellular calcium (Ca^{2+}), levels acting to limit activation of the myofilaments.^{3,34} Ultimately, both of these intracellular changes contribute to fatigue by either directly or indirectly reducing the force- and motion-generating capacity of a myosin crossbridge.^{21,35} Thus, to fully understand the mechanisms underlying fatigue, it is crucial to elucidate how these biochemical changes in the intracellular environment inhibit the ability of myosin to proceed through the crossbridge cycle ([Fig. 1](#)).

In vivo magnetic resonance spectroscopy studies on exercising muscle demonstrated that the loss in maximal force during fatigue can be temporally correlated with the accumulation of metabolic by-products within the muscle cell, including inorganic phosphate (P_i) and hydrogen ions (H^+), the latter causing pH to decrease.^{10,18,95} Subsequent experiments that exposed skinned single muscle fibers to fatiguing concentrations of P_i (~30 mM) and H^+ (pH 6.2 to 6.5) showed that these ions dramatically reduce maximal isometric force,^{15,38,61} unloaded shortening velocity,¹³ and peak power,^{24,46,58} providing strong evidence that these ions play a causative role in the fatigue process. More specifically, because these experiments were

performed at saturating levels of free Ca^{2+} , and thus with a fully activated thin filament, it suggests that these depressive effects are due, in part, to a direct effect on the actomyosin crossbridge. In light of these observations, the field of fatigue has focused on determining which specific steps of the crossbridge cycle are affected by these fatigue agents. Fortunately, recent technological advances that have enabled the direct observation of the mechanics and kinetics of a single actomyosin crossbridge under fatigue-like conditions are providing the first opportunities to observe how a single crossbridge responds to these fatiguing ions.^{23,28} In addition, parallel advancements in single muscle fiber technology and transgenic manipulation of the contractile proteins are also providing important new insights into the molecular basis of the fatigue process.^{20,24,26,46,58-60} Characterizing the mechanisms of fatigue at the cellular and molecular levels is important because many of the most promising therapeutic interventions act at this level to attenuate the debilitating effects of fatigue in clinical populations.^{30,51,57,74,87}

In addition to directly inhibiting the crossbridge cycle, the metabolic by-products that are elevated during intense contractile activity are thought to indirectly affect contractile function by altering the ability of the muscle regulatory proteins (troponin [Tn] and tropomyosin [Tm]) to regulate actomyosin binding by making the thin filament less sensitive to Ca^{2+} .^{26,32,56,59} This mechanism is thought to play a particularly prominent role in fatigue at higher stimulation rates when the myoplasmic Ca^{2+} concentration is rapidly compromised and muscular force drops precipitously.^{3,49} Evidence from skinned single muscle fibers shows that when P_i and H^+ are increased to levels reached during fatigue, the force-calcium relationship is shifted to the right, such that more Ca^{2+} is required to activate the filaments.^{26,59} Although this effect is well established, recent insights into the basic mechanisms of muscle activation, in particular the calcium- and myosin-dependent motions of Tn⁸⁴ and Tm,³⁷ are opening a whole new area of understanding to explore the molecular basis of this effect during muscle fatigue. Therefore, a second key goal for the field of muscle fatigue has been to determine which structures and steps are altered to cause the reduction in thin filament activation and thus the loss of muscle function. Transgenic approaches that alter the structure of Tn are providing exciting insight into the specific structures and molecular motions underlying this process.²⁰ This

minireview highlights this recent work as well as the advances made using cutting edge biophysical approaches to determine the specific steps altered in the crossbridge cycle during fatigue. The review of these new findings progresses from observations at the single fiber level to the level of a single molecule.

Mechanisms Of Muscle Fatigue Identified From A Skinned Single Muscle Fiber

The skinned fiber preparation, in which the cell membrane is either mechanically peeled or chemically permeabilized, has provided valuable advancements in understanding how the metabolites that accumulate during fatigue disrupt key mechanical and biochemical steps in the crossbridge cycle. The advantage of this approach is that it permits precise control over the intracellular milieu to systematically study both the individual and the collective effects of metabolic by-products while leaving the contractile proteins in their intact sarcomeric state. In this section, we briefly review the individual and combined effects of elevated concentrations of P_i and H^+ on the crossbridge cycle, and thus on single fiber force, velocity, power, and contractile economy (i.e., fiber force/myofibrillar ATP turnover). It is important to note, however, that these ions also affect excitation-contraction coupling, and that other compounds accumulate during intense contractile activity (e.g., ADP, Mg^{2+} , oxidative free radicals, and extracellular K^+), which are also implicated in muscle fatigue.^{3,34} Here we focus primarily on recent discoveries of the effects of H^+ and P_i on contractile function from experiments conducted using the chemically skinned fiber preparation at near in vivo temperatures (30°C).

Effects of Intracellular Acidosis, H^+

During intense contractile activity, high rates of ATP hydrolysis and increased glycolytic flux generate hydrogen ions, H^+ , that cause a reduction in intracellular pH. In quiescent human skeletal muscle, intramuscular pH remains at ~7.0 but declines precipitously to between 6.5 and 6.2 during intense volitional contractions.^{10,95} These values, however, were measured via ^{31}P -MRS or muscle biopsies and represent the spatial average of a heterogeneous mixture of muscle fiber types. Thus, because the ATP hydrolysis rates differ considerably between fiber types,⁷⁶ more severe acidic states within individual

fibers, particularly fast type IIa or type IIX fibers, is highly likely. In saturating concentrations of free Ca^{2+} , a pH of 6.2 reduces peak isometric force of rat and rabbit fibers by 4%–18% at 30°C.^{46,66} This reduction is similar to the 10% decline observed in isolated living mouse fibers at 32°C,⁹³ but considerably lower than the ~30% reduction observed in skinned rat fibers at 15°C.^{13,15,46} The observation that acidosis depresses peak isometric force even under saturating Ca^{2+} conditions suggests that the hydrogen ion is acting directly on the cycling crossbridges. The acidosis-induced decrements in force may involve a reduction in the number and/or the force per crossbridge. This is difficult to determine at the single fiber level because the contractile properties represent the cumulative action of billions of individual crossbridges. Fortunately, we now have the ability to directly observe the effect of acidosis on a single actomyosin crossbridge, results that are discussed in the Molecular Basis of the Depression in Ca^{2+} Sensitivity during Fatigue section.

The mitigated effects of pH on peak isometric force at near in vivo temperatures compared with colder temperatures has led some investigators to question the relative importance of acidosis in the fatigue process.^{66,68} However, the depressive effects of acidosis on crossbridge function during fatigue extend beyond the direct effects of H^+ on peak isometric force in saturating Ca^{2+} . For example, acidosis also decreases the sensitivity of the myofilaments to Ca^{2+} due, at least in part, to H^+ competitively inhibiting the binding of Ca^{2+} to troponin C.^{64,65} The reduced myofibrillar Ca^{2+} sensitivity manifests as a rightward shift in the force–calcium relationship and considerably larger reductions in peak isometric force in rat fibers contracting in pH 6.2 and submaximal Ca^{2+} conditions.⁵⁹ In addition, acidosis depresses the rate of tension development (k_{tr}) in skinned fibers under submaximal Ca^{2+} conditions as reflected by a reduced k_{tr} after a slack reextension maneuver at 15°C.⁵⁵ Given that the myoplasmic free Ca^{2+} decreases during high-intensity fatiguing contractions,^{2,49} the H^+ -induced decrements in crossbridge function under submaximal Ca^{2+} conditions are likely more representative of what occurs during fatigue in vivo.

From a human performance and clinical perspective, perhaps the most important question to consider is how these metabolites influence the ability of skeletal muscle to shorten under submaximal

loads and to generate power. In addition to the effect H^+ has on peak isometric force, intracellular acidosis reduces both the loaded and the unloaded fiber shortening velocities.^{42,43,46} For example, maximal rat fiber shortening velocities, determined by the slack test (V_0) and from extrapolation of the force–velocity curve (V_{max}), were reduced by ~30% and ~16%, respectively, in pH 6.2 and saturating Ca^{2+} conditions at 30°C⁴⁶ (Fig. 2A). The decrements in fiber shortening velocity are thought to be due to slowed myofibrillar ATPase activity,¹⁵ secondary to the H^+ -mediated reduction in the rate that ADP dissociates from the myosin head.²³ The effects of acidosis on both fiber force and velocity resulted in an 18%–34% reduction in peak power of rat fibers at 30°C⁴⁶ (Fig. 2B). Thus, severe acidosis (pH 6.2) plays an important role in fatigue by acting at multiple steps of the crossbridge cycle that lead to a reduced myofibrillar Ca^{2+} sensitivity and an impairment in fiber force, velocity, and power.

Effects of Inorganic Phosphate, P_i

Intramuscular concentrations of ATP in quiescent skeletal muscle (~5–6 mmol·kg⁻¹ w.w.) would be depleted rapidly during maximal-intensity contractile activity without the activation of creatine kinase and glycolysis.⁷⁵ However, buffering the fall in intracellular ATP via the creatine kinase reaction results in a rapid decline in phosphocreatine with concomitant increases in inorganic phosphate, P_i , that can reach >30 mM in human skeletal muscle.^{10,95} In saturating Ca^{2+} conditions, 25–30 mM P_i reduces peak isometric force of rat and rabbit fibers by 5%–19% at 30°C^{17,24} (Fig 2C). Similar to the temperature effects observed with acidosis, reductions in peak isometric force from 30 mM P_i at near in vivo temperatures are considerably less than the ~52% reduction found at 15°C.²⁴ Also similar to H^+ , elevated P_i causes a rightward shift in the force–calcium relationship, which exacerbates the reductions in peak isometric force at submaximal Ca^{2+} .²⁶ However, the observation that the rate of tension redevelopment in response to a slack reextension maneuver of an activated fiber (k_{tr}) is accelerated in the presence of P_i ⁹¹ but is unchanged or slowed in the presence of H^+ ⁵⁵ suggests that the mechanisms for the reduction in peak isometric force differ for P_i compared with H^+ . Although the mechanisms remain unresolved, it has been suggested that P_i inhibits peak isometric force by reducing the number of high-force crossbridges and/or the force per bridge.

The former is thought to result from P_i accelerating the reverse rate constant of the low- to high-force state transition.⁶⁴ In addition, it has been suggested that P_i induces an unconventional power stroke where myosin detaches from actin early in the high-force state of the crossbridge cycle before the release of ADP and P_i .^{12,28,50} Although all possibilities may be at least partially responsible for the loss in fiber force, the latter is the only one currently able to explain the P_i -induced decline in fast fiber contractile economy.⁵⁰ The decrements in contractile economy, as a result of decreased fiber force but a maintained myofibrillar ATP hydrolysis rate, would accelerate the accumulation of metabolic by-products and ultimately the development of muscle fatigue.

In contrast to H^+ , elevated P_i does not inhibit maximal fiber shortening velocity (V_{max}) at near in vivo temperatures^{24,43} (Fig. 2C). However, muscle shortening under a load is more important for athletic prowess and the ability to perform daily activities, and peak fiber power is depressed by 18%–26% in 30 mM P_i at 30°C, a somewhat greater decline than peak isometric force alone²⁴ (Fig. 2C). This observation is explained by the 30%–38% increased curvature (quantified by the a/P_o ratio) in the force–velocity relationship, which results in less force generated for any given velocity.²⁴ Thus, high concentrations of P_i (~25–30 mM) seem to play an important role in muscle fatigue at the crossbridge level by inhibiting peak fiber force and power and by decreasing both the contractile economy and the myofibrillar Ca^{2+} sensitivity.

Combined Effects of P_i and H^+

Other than the first few seconds of contractile activity where the intracellular milieu becomes slightly more alkaline from the predominance of ATP generated via the creatine kinase reaction,¹ intracellular H^+ and P_i accumulate in concert during fatiguing contractions. Thus, in addition to studying the individual effects of these ions, it is also important to study their effects in combination to more closely mimic the fatigue environment in vivo.^{9,95} Given that H^+ and P_i seem to influence different states in the crossbridge cycle, it is not surprising that the combined effects of these ions act additively to inhibit contractile function at the crossbridge level. For example, in saturating Ca^{2+} , elevated H^+ (pH 6.2) and P_i (30 mM) depresses peak isometric force in rat slow and fast fibers by 36% and 46%,

respectively, at 30°C⁵⁹ (Fig. 2E). A surprising finding, however, is that the combined condition decreases peak force more than would be predicted from summation of the individual ion effects, which highlights the importance of studying the fatigue-induced effects of multiple metabolites in combination. This observation might be explained, in part, by the increased concentration of dihydrogen phosphate (H_2PO_4^-) that occurs in acidic (pH 6.2) compared with neutral conditions (pH 7.0).⁶¹ The dihydrogen phosphate species is more closely correlated to the decline in maximal force during in vivo fatigue compared with either the monohydrogen phosphate or the pH changes alone.^{18,48,95} However, whether dihydrogen phosphate is the dominant fatigue-inducing phosphate species or merely serves as a better marker of the totality of metabolic by-products accumulating within the intracellular milieu remains unknown.¹³

In addition to the combined effects that H^+ and P_i have on peak force, these ions also act to synergistically reduce myofibrillar Ca^{2+} sensitivity and peak fiber power. It was recently found in rat fibers that the combined condition of pH 6.2 and 30 mM P_i induced a considerably greater rightward shift in the force–calcium relationship compared with either ion alone.^{26,59} This observation is not surprising based on evidence that the mechanisms for the decreased myofibrillar Ca^{2+} sensitivity differ between P_i and H^+ .⁶⁴ Importantly, the shift in the force–calcium relationship is more pronounced at 30°C compared with 15°C, suggesting that the decrements in force from these ions may be more important than previously indicated by studies at saturating Ca^{2+} . Furthermore, pH 6.2 and 30 mM P_i conditions reduce peak power in rat and rabbit fibers by 55%–63% at 30°C^{43,58} (Fig. 2F). The large reduction in peak fiber power in this condition is likely explained by the reductions in fiber shortening velocity from H^+ and the inhibition in force from both H^+ and P_i . These reductions in fiber shortening velocity and peak power are further exacerbated when the myosin regulatory light chain (RLC) is phosphorylated.⁴³ The mechanisms for the additional depression with RLC phosphorylation is unknown but is important to identify because high-intensity contractile activity increases RLC phosphorylation.^{70,89}

Recently, Fitts has begun studying the effects of H^+ and P_i on the contractile properties of fibers isolated from muscle biopsies obtained from young (<30 yr) and old adult humans (>70 yr). The

preliminary data indicate that the combined pH 6.2 and 30 mM P_i conditions cause slightly lower reductions in maximal isometric force ($\sim 33\%$) and peak power ($\sim 50\%$) than previously reported for rat and rabbit fibers but did not differ between the fibers from the young and old adults.⁸⁰ These are the first findings to investigate the effects of H^+ and P_i on human muscle and provide direct evidence that these ions are essential mediators of human muscle fatigue.

In summary, H^+ and P_i contribute to fatigue, in large part, both by their direct inhibitory effects on the crossbridge and by reducing the sensitivity of the myofilaments to Ca^{2+} , the latter of which is exacerbated at near physiological compared with colder temperatures. Although the mechanisms remain unresolved, continued advancements in single fiber and molecular technology should make it possible to definitively identify the mechanism by which these ions individually and collectively inhibit maximal force, velocity, and power during fatigue.

Molecular Basis Of The Depression In Ca^{2+} Sensitivity During Fatigue

As discussed in the previous section, in addition to directly affecting the actomyosin interaction, elevated levels of H^+ and P_i are also thought to reduce muscle function indirectly by reducing the sensitivity of the thin filament to activation by Ca^{2+} .^{3,21} This reduction in Ca^{2+} sensitivity, well characterized in skinned single muscle fibers, is thought to play a particularly important role when myoplasmic Ca^{2+} concentration is compromised because of decreased release from the sarcoplasmic reticulum.^{3,49} The molecular mechanisms underlying the excitation-contraction (EC) coupling failure are still unclear, and this is the focus of a companion review in this issue. Interestingly, this decrease in myoplasmic $[Ca^{2+}]$ alone accounts for only a small portion of fatigue; rather, it is the decreased Ca^{2+} sensitivity caused by elevated P_i and H^+ , combined with the loss in Ca^{2+} that is thought to account for much of the loss in isometric force during fatigue.⁴ The large effect on the force results, in part, from the sigmoidal shape of the force-pCa relationship and suggests a strong role for the regulatory proteins Tn and Tm in the fatigue process (Fig. 3).

Brotto et al.²⁰ sought to determine the role of Tn and potential mechanisms underlying the decreased sensitivity in a study published in 2001. Working under the premise that fatiguing stimulation renders

the muscle hypoxic,⁵ they intermittently stimulated mouse diaphragm muscle under hypoxic conditions and examined the effect on force production and the muscle regulatory protein Tn. They found that fatigue occurred more readily, and to a greater extent under hypoxic versus normoxic conditions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis revealed that Tn was degraded when the muscle was fatigued under hypoxic but not normoxic conditions, with both the Ca^{2+} binding (TnC) and the inhibitory (TnI) subunits of Tn suffering truncations under these conditions. To determine whether these truncations were the source of the loss of force, they removed the truncated Tn and replaced it with full-length recombinant Tn. The results showed that the force-generating capacity recovered significantly in the fibers with the replaced Tn, providing strong evidence that the degradation of TnI/TnC plays a significant role in fatigue under hypoxic conditions, with the relevance to fatigue increased by the observations that even under normoxic conditions fatiguing stimulation can render a muscle hypoxic.⁵³

The authors speculate that the truncation of Tn may occur by a mechanism similar to that observed during myocardial ischemia when cardiac TnI is often truncated.⁸⁶ Ultimately, they suggest that the proteolytic cleavage of Tn may stem from the increase in, and subsequent damage from, reactive oxygen and nitrogen species, which have similar effects on the force versus calcium relationship of skinned muscle fibers.¹¹ Indeed, reactive oxygen species seem to target the regulatory proteins during fatigue.²² It is interesting to note that transgenic upregulation of heat shock protein 72 can ameliorate the effect of fatiguing stimulation under hypoxic conditions.⁷³ Elucidating the molecular motions and key regions of Tn altered by these truncations will be extremely important to understanding this mechanism.

Fortunately, recent advances in our understanding of the basic regulation of contraction are revealing much of the detail of this process. For example, we now know that in the absence of Ca^{2+} , the C-terminus of the inhibitory subunit of Tn (TnI) stabilizes Tm in a position that sterically blocks myosin strong binding to actin.³⁹ Ca^{2+} binding to TnC exposes a hydrophobic patch in the N-terminus, which causes the C-terminal region of TnI to switch from being tightly bound to actin to being associated with the N-terminus of TnC.⁸⁴ Tm then

moves to occupy a position that allows myosin to bind to actin, initially weakly and then transitioning to a strongly bound state, which fully activates the thin filament.⁵⁴ It is this weak-to-strong binding transition of myosin to actin (P_i -release step in Fig. 1) that is thought to be the step in the crossbridge cycle regulated by Ca^{2+} .³⁹ Because myosin strong binding is required to fully activate the thin filament, the loss of apparent Ca^{2+} sensitivity with fatigue could be due, in part, to a direct effect on myosin. Alternatively, as evidence from single muscle fibers suggests, this acidosis-induced decrease in Ca^{2+} sensitivity may be partially due to H^+ competing with Ca^{2+} for binding to TnC.⁶⁵ There is also strong evidence of a role for TnI in the acidosis-induced decrease in Ca^{2+} sensitivity from cardiac muscle,^{8,19,78,94,96} although it is still not clear which domains in Tn, specific amino acids, and molecular motions mediate this effect. There is also evidence that all three subunits of Tn and possibly Tm³⁶ might contribute to the acidosis-induced depression in Ca^{2+} sensitivity,^{60,65,94} but the most compelling evidence implicates either TnC⁶⁵ or TnI,⁶⁴ or even the intricate communication between the two subunits.⁸ Thus, the new findings from studies investigating the basic mechanisms underlying activation of the thin filament represent exciting new areas to probe our understanding of how fatiguing agents act to decrease Ca^{2+} sensitivity.

Effect Of Fatiguing Agents On The Mechanics And Kinetics Of A Single Crossbridge

How Does Acidosis (Low pH) Slow the Velocity of Contraction?

As described earlier, previous findings in skinned single muscle fibers strongly suggest that elevated levels of H^+ and P_i help cause fatigue by directly inhibiting the force- and the motion-generating capacity of myosin.^{15,61} On the basis of these findings, the authors hypothesized specific steps in myosin's crossbridge cycle that might be affected by these ions to explain the loss of function. A simplified model of a conventional crossbridge cycle,⁶⁷ including these steps, is shown in Figure 1 (green pathway). Early work on isolated myosin in solution suggested steps where H^+ exchange occurred, and thus the steps that might be affected by acidosis.^{6,14} These included the step associated with ATP hydrolysis^{14,47} and the P_i -release step.¹⁴ Because

these steps occur either off actin or concomitant with the strong binding,⁷ slowing them would decrease the rate of attachment and therefore be consistent with a decrease in maximal isometric force observed in skinned fibers at lower temperatures.^{15,31,45} The same mechanisms are likely to be responsible for the decrements of maximal isometric force at near physiological temperatures but are just less pronounced because acidosis exerts a reduced effect at higher temperatures.^{46,66,93}

The effect of acidosis on unloaded shortening velocity was also thought to be minimized at physiological temperatures based on work in intact muscle fibers.⁹³ However, it is difficult to reduce the intracellular pH to the levels experienced during fatigue in vivo^{85,95} in this type of preparation. Therefore, investigators turned to the skinned muscle fiber preparation where they observed that decreasing the pH from 7.0 to 6.2 reduces unloaded shortening velocity by ~20%–30% even near physiological temperatures (30°C).^{42,43,46} Consistent with the reduction in unloaded shortening velocity, in an in vitro motility assay (a measure analogous to unloading shortening velocity), Debold et al.²³ observed that reducing the pH from 7.4 to a value reached during fatigue (6.4) slowed the actin filament velocity by roughly 67% at 20°C. A similar reduction in velocity is observed at 30°C,²⁷ suggesting that the effect on velocity is not as temperature dependent as the effect on force. Importantly, because this assay only includes myosin and unregulated actin filaments, it confirmed that acidosis directly affects myosin's ability to move actin.

The molecular basis of actin filament velocity is well described by a simple detachment-limited model in which the actin filament velocity is equal to the size of myosin's power stroke (d) divided by the time it spends strongly attached to actin (t_{on}) (i.e., velocity = d/t_{on}).⁴¹ Therefore, the decrease in velocity caused by low pH could be due to either a decrease in d or an increase in t_{on} . The advent of the single molecule laser trap assay³³ now enables researchers the ability to directly determine these single molecule parameters and therefore determine the molecular basis of the acidosis-induced decrease in velocity. Using this assay, Debold et al.²³ found that acidosis had no effect on myosin's power stroke but prolonged t_{on} by roughly threefold, an amount that could quantitatively account for the 67% decrease in velocity in the motility assay at 20°C. This mechanism

was pursued further because, as [Figure 1](#) shows, a prolongation of t_{on} could result either from a slowing of the rate of ADP release from myosin or from a prolongation of the rigor state. Subsequent experiments in the single molecule laser trap assay, in which the concentration of ATP was manipulated to determine the ADP and rigor lifetime, suggested that t_{on} was prolonged because of a slowed rate of ADP release from myosin.²³ Furthermore, more recent work, in which the ADP concentration was manipulated, suggested that acidosis actually slows the ADP-bound isomerization step,²⁷ i.e., the step preceding ADP release²⁵ (see [Fig. 1](#)). Thus, these findings provide the first direct evidence of the specific step in the crossbridge cycle that likely underlies the decrease in contraction velocity during muscle fatigue.

How Does P_i Reduce Muscular Force?

This same single molecule laser trap assay has been used to provide insight into how elevated levels of P_i act to reduce muscular force.²⁸ At the molecular level, force is the product of the force per crossbridge (F_{uni}) and the number of attached crossbridges. The number of attached crossbridges is determined by the amount of time myosin spends strongly bound to actin, which is in turn dependent on its duty ratio (the percentage of the crossbridge cycle spent strongly bound to actin).⁸⁸ The prevailing model⁸³ suggests that when elevated, P_i rebinds to myosin in the state strongly bound to actin but before the ADP-isomerization step ([Fig. 1](#)). Subsequently, this induces the rapid reversal of myosin's power stroke and detachment from actin. Thus, in this model, P_i acts to decrease myosin's duty ratio, and in isometrically contracting muscle, this leads to a decrease in the number of strongly bound crossbridges and therefore the amount of force. This model is supported by observations in skinned single muscle fibers demonstrating that P_i depresses maximal isometric force in a concentration-dependent manner.¹⁶ However, as indicated earlier, the isometric force in muscle fibers represents the collective action of more than a billion myosin molecules cyclically interacting with the actin filament, making it difficult to determine how a single crossbridge behaves when P_i rebinds to myosin.

Motivated by this challenge, Debold et al.²⁸ recently used a three-bead laser trap assay to directly observe the effect of elevated P_i on myosin's force-generating capacity. Because of myosin's low

duty ratio (~4%), which is likely decreased further by P_i , the investigators increased the myosin concentration such that a miniensemble (~6) of myosin molecules were available to bind to the actin filament. A glass bead held in a laser trap assay behaves as if attached to a linear spring;⁸¹ therefore, this assay serves as a piconewton (pN) force transducer. In the absence of P_i , the miniensembles of myosin generated a distribution of displacements of the actin filament, with a high frequency of low force events (0.2–1 pN) interspersed with occasional high-force events (3–6 pN) (Fig. 4). When the P_i was elevated to a level reached during fatigue (30 mM), the high-force events in the assay were completely absent and only low force events remained. This effect caused an 80% reduction in the average force in the presence of P_i . Consistent with observations in muscle fibers, force in this miniensemble laser trap assay was dependent on the concentration of P_i . These observations are consistent with P_i reducing force by accelerating myosin's detachment from actin, thus providing support for the notion that P_i causes fatigue by "knocking" myosin heads off actin.

The next question to answer was which specific step(s) in the crossbridge cycle does P_i rebind to and how does it cause myosin's detachment from actin. Existing hypotheses suggest that P_i rebinds to the preisomerization ADP-bound state,⁹² induces a reversal of myosin's power stroke, and then detaches from actin.⁸³ Reversing the power stroke would negate the displacement of the actin filament, and thus, a prediction of this model is that elevating P_i should reduce actin filament velocity in the motility assay. Debold et al.²⁷ tested this prediction by simultaneously lowering the pH (which prolongs the ADP-bound state, see Fig. 1) and raising the P_i in a motility assay. Surprisingly, they observed that velocity increased in the presence of P_i at low pH, with velocity more than doubling when P_i was raised to 30 mM at pH 6.5.²⁷ To explain these data, they proposed an alternative model in which P_i does not reverse myosin's power stroke but rather induces detachment from a post-power stroke state, creating a branch in the conventional pathway of the crossbridge cycle (Fig. 1, pink pathway). Computer simulations based on this novel branch in the crossbridge cycle were able to accurately reproduce this observation as well as the effect of P_i on force.^{27,28}

These findings are therefore providing unique insight into the molecular basis of muscle fatigue, and new theories are emerging as a result of these findings. Indeed, based on these observations, it seems that the depressive effects of acidosis on contraction velocity may be somewhat offset by the effects of elevated P_i because the acidosis-induced depression in velocity is attenuated under these conditions.^{25,27} However, this would likely come at the expense of force generation and ultimately power as P_i would dramatically decrease the number of crossbridges generating force. Indeed, this hypothesis is supported by the observation that in a loaded in vitro motility assay, a fatigue-like milieu (pH 6.2, 30 mM P_i , 0.3 mM ADP) dramatically reduces myosin force- and power-generating capacity⁴⁰ to a degree that is strikingly similar to the magnitude of the effect of fatigue on the force-velocity relationship of intact muscle.⁹⁰

Can Fatigue Be Attenuated in Clinical Populations?

Recent reports on cardiac muscle have revealed exciting new developments for treating heart failure with pharmacological agents that can either activate myosin or sensitize the thin filament to Ca^{2+} .^{44,52} The translation of these ideas and compounds to skeletal muscle research represents an innovative approach to potentially treat skeletal muscle weakness and fatigue in a host of clinical populations.⁶³ In fact, this line of research represents an emerging area of commercial development.⁷⁴ The agents that target myosin⁹⁷ and Tn⁶⁹ are particularly attractive because they exert their effects without increasing intracellular Ca^{2+} levels and, therefore, reduce the potential for unwanted side effects from upstream targets.⁴⁴ In addition, because P_i and H^+ are thought to directly affect myosin and the muscle regulatory proteins, these classes of compounds would target two of the principal mechanisms of muscle fatigue and therefore represent the crucial first steps toward the development of a treatment that might attenuate fatigue in clinical populations and the elderly. Motivated by this goal, Longyear et al.⁵¹ recently demonstrated that replacing ATP with the ATP analog, 2-deoxy-ATP, can significantly attenuate the depressive effects of acidosis on filament velocity and Ca^{2+} sensitivity⁵¹ in the in vitro motility assay. Thus, this may represent a promising agent not only to potentially treat chronic heart failure, as is being explored,⁶² but also, based on our results, to help attenuate skeletal muscle fatigue. In addition,

because the effects of 2-deoxy-ATP on the steps of the crossbridge cycle have already been determined,^{71,72} this line of inquiry will also help further identify the specific steps in the crossbridge cycle that are affected by P_i and H^+ .

Conclusions

The mechanisms underlying fatigue have been studied for more than a century, and this work has provided immense insight into this highly conserved phenomenon of skeletal muscle. Nuclear magnetic resonance spectroscopy and biochemical assays have provided insights into the biochemical changes that occur in muscle during the fatigue process.¹⁰ Leveraging this information, single muscle fiber work helped demonstrate that the metabolic by-products directly inhibit the force- and the motion-generating capacity of muscle during fatigue.³⁸ Indeed, it is now clear that elevations in H^+ and P_i are major contributors to the loss in force and velocity.³⁵ More recently, investigators have taken advantage of advances in single molecule biophysical techniques to gain unprecedented insight into the specific steps in the crossbridge cycle that are affected during fatigue.^{23,25,28} These include the step that controls the velocity of contraction (ADP release) and the step that accelerates detachment and therefore reduces force (P_i -induced detachment). Similarly, advances in molecular biology are helping demonstrate the role TnC and TnI play in the decreased Ca^{2+} sensitivity,²⁰ which is particularly important when Ca^{2+} release from the SR is compromised.³ Thus, it is an exciting time to study muscle fatigue because technological advances are enabling us to answer longstanding questions in ways thought to be impossible just a few decades ago. Continuing these efforts will be crucial for both improving our basic understanding of the fatigue process, and for the development of pharmaceutical therapies to attenuate fatigue in individuals with chronic conditions (e.g., congestive heart failure and chronic obstructive pulmonary disease), for whom fatigue dramatically limits quality of life. Exciting recent developments at the molecular level are pointing toward promising new approaches to attenuate muscle fatigue that occurs from the accumulation of metabolites.⁵¹

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