The effects of phosphate and acidosis on regulated thin-filament velocity in an in vitro motility assay

Edward P. Debold, Thomas J. Longyear, and Matthew A. Turner

Department of Kinesiology, University of Massachusetts, Amherst, Massachusetts

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Debold EP, Longyear TJ, Turner MA. The effects of phosphate and acidosis on regulated thin-filament velocity in an in vitro motility assay. J Appl Physiol 113: 1413–1422, 2012. First published September 27, 2012; doi:10.1152/japplphysiol.00775.2012.—Muscle fatigue from intense contractile activity is thought to result from, in large part, from the accumulation of inorganic phosphate (Pi) and hydrogen ions (H+) acting to directly inhibit the function of the contractile proteins; however, the molecular basis of this process remains unclear. We used an in vitro motility assay and determined the effects of elevated H+ and Pi on the ability of myosin to bind to and translocate regulated actin filaments (RTF) to gain novel insights into the molecular basis of fatigue. At saturating Ca2+ levels, acidosis depressed regulated filament velocity (V_{RTF}) by ~90% (6.2 ± 0.3 vs. 0.5 ± 0.2 μm/s at pH 7.4 and 6.5, respectively). However, the addition of 30 mM Pi caused V_{RTF} to increase fivefold, from 0.5 ± 0.2 to 2.6 ± 0.3 μm/s at pH 6.5. Similarly, at all subsaturating Ca2+ levels, acidosis slowed V_{RTF}, but the addition of Pi significantly attenuated this effect. We also manipulated the [ADP] in addition to the [Pi] to probe which specific step(s) of cross-bridge cycle of myosin is affected by elevated H+. The findings are consistent with acidosis slowing the isomerization step between actomyosin ADP-bound states. Because the state before this isomerization is most vulnerable to P_i rebinding, and the associated detachment from actin, this finding may also explain the Pi-induced enhancement of V_{RTF} at low pH. These results therefore may provide a molecular basis for a significant portion of the loss of shortening velocity and possibly muscular power during fatigue.

fatigue; regulation; troponin; tropomyosin; ADP

IN RESPONSE TO INTENSE CONTRACTILE ACTIVITY, muscle temporarily loses much of its ability to generate force and power, in the process defined as fatigue (20). The associated accumulation of intracellular metabolites is thought to play a causative role in the fatigue process (6, 13, 21); however, the molecular mechanisms by which these metabolites inhibit the function of the contractile proteins remains unclear (6, 13, 20). Inorganic phosphate (Pi) is one of the metabolites that rapidly accumulates in the myoplasm, reaching concentrations as high as 30 mM in the latter stages of fatigue (4, 11, 73). This increase in [Pi] is thought to directly inhibit force generation by muscle because exposing a rested skinned muscle fiber to this level of Pi significantly reduces maximal isometric force (P0) (7, 8, 15, 29, 49). When Pi levels are this high, it is thought that Pi readily binds to myosin while it is strongly bound to actin and reverses the force-generating step, reducing force at the whole muscle level (9, 29, 66, 72). However, these effects are minimized at physiological temperatures (15), and Pi has little or no effect on shortening velocity (8, 15); therefore the increase in Pi concentration cannot account for the dramatic loss in shortening velocity (70) and would only explain a portion of the large decrement in peak power caused during fatigue (70).

Intense contractile activity also leads to the accumulation of H+ (i.e., acidosis or decreased pH) inside the myoplasm and represents one of the most well-known putative agents of fatigue (4, 11). Under certain conditions, the decline in pH can be correlated with the decline in force; therefore it was originally thought to play a role in the loss of force (11, 73). However, more recent evidence from muscle fibers indicates that it has only a minor depressive effect on P0 near mammalian physiological temperatures (54), leading some to challenge the magnitude of its role in fatigue (54, 57). However, a fatiguing level of acidosis can slow unloaded shortening velocity by ~30%, and, unlike the effects on force, this effect is independent of temperature, meaning the magnitude is similar at physiological temperatures (37). This depression of velocity extends into the force-velocity relationship, causing acidosis to significantly depress the peak power-generating capacity (37). Despite extensive investigations at the fiber level, the mechanism underlying the acidosis-induced depression in velocity remains unclear. This is due, in part, to the complexity of intact muscle making it difficult to determine which contractile proteins are affected and which specific step(s) in the cross-bridge cycle might be altered.

In vitro motility experiments utilizing isolated myosin molecules demonstrate that acidosis severely slows actin filament velocity (28, 39), suggesting that acidosis directly affects the function of actin and/or myosin. Velocity in this assay is thought to be dependent on the unitary step size of myosin (d) and the duration of the strongly-bound state (t_{on}) such that velocity = dl/dt_{on} (68). A single-molecule laser trap assay, in which single actomyosin interactions can be observed, was able to attribute the acidosis-induced decrease in velocity solely to a prolongation of t_{on} (14). Related single-molecule experiments suggested that this might be due to a slowing of the rate of ADP-release from the active sight of myosin (14). Therefore, this points to a specific step in the cross-bridge cycle of myosin that may be affected by acidosis to slow velocity of contraction. However, this is still not precise enough because both solution (61) and fiber (10) experiments have demonstrated that ADP-release from the active site of myosin is a two-step process (10, 61). In these more detailed models of the cross-bridge cycle (65, 66), the state following Pi release from the active site actomyosin is a transiently stable ADP-bound state referred to as AM'.ADP; this is followed by an isomerization that transitions actomyosin into the AM.ADP state from which ADP is subsequently released. The single-molecule experiments at low pH only suggested that one or both states is prolonged by acidosis but did not delineate which specific state might be prolonged. Resolving the specific step slowed by acidosis is particularly important for understanding...
fatigue because Pi is thought to preferentially rebind to the AM\textsuperscript{-}ADP state (72). Therefore, if acidosis slows the isomerization step of the cycle, then it may make myosin more vulnerable to the rebinding of Pi, which would lead to an increased reversal of the force-generating step and a synergistic reduction in force. Indeed, this mechanism may explain why a fatigue-like milieu that includes both Pi and H\textsuperscript{+} causes dramatic losses in the power-generating capacity of myosin in an in vitro motility assay (27).

In addition to exerting direct effects on actomyosin function, it is well documented that elevated levels of both Pi and H\textsuperscript{+} indirectly inhibit actomyosin through effects on the muscle regulatory proteins troponin (Tn) and tropomyosin (Tm) (16, 18, 19, 45, 46, 71). This depressive effect of Pi and H\textsuperscript{+} on Ca\textsuperscript{2+} sensitivity is believed to play a particularly important role in the latter stages of fatigue, when myoplasmic Ca\textsuperscript{2+} levels are reduced (1, 40). Although acidosis has only minor effects on Pi, at saturating Ca\textsuperscript{2+}, it significantly lowers force at submaximal Ca\textsuperscript{2+} levels, acidosis has also been shown to slow regulated thin filament velocity in a motility assay (58, 59, 69), but the mechanisms of this effect have yet to be elucidated.

Elevated Pi also depresses Ca\textsuperscript{2+} sensitivity of force in muscle, and there is strong evidence that this is due to a direct effect on myosin, such that Pi induces detachment from a state strongly bound to actin and thereby reduces the ability of these cross-bridges to activate the thin filament (16, 45, 46, 71). However, it is unclear how the effects of elevated Pi might be modulated or altered in the presence of elevated H\textsuperscript{+} as would be experienced during fatigue. Thus it is also important that we understand how elevations in both these ions affect the ability of myosin to bind to and translocate regulated actin filaments.

Therefore, in the present investigation, we examined the effects of acidosis and Pi, individually and in combination, on the ability of myosin to translocate actin filaments reconstituted with Tn/Tm (RTF) at varying levels of Ca\textsuperscript{2+} activation. The findings show that acidosis alone severely slows filament velocity at all Ca\textsuperscript{2+} levels, but this effect is dramatically and significantly attenuated in the presence of Pi. This suggests that elevated levels of these ions have antagonistic effects on one or more steps of the cross-bridge cycle of myosin. Our findings suggest that acidosis slows the isomerization of AM-ADP to AM-ADP and that this effect makes it more vulnerable to Pi rebinding and dissociation from its strong attachment to actin.

**MATERIALS AND METHODS**

**Proteins.** Myosin was isolated from chicken pectoralis muscle based on previous methods (41), with a high degree of purity based on SDS-PAGE gels (data not shown). Following this purification, myosin was stored in glycerol (50%, vol/vol) at 20°C to preserve its function for up to 6 mo. To remove inactive myosin molecules (i.e., deadheads) from the preparation, a further purification was done the day of each experiment as previously described (67). In this final purification, 200 μg/ml of myosin was mixed with an equimolar concentration of myosin and actin as well as 1 mM ATP. This solution was centrifuged at 400,000 g for 20 min at 4°C. Under these conditions, myosin molecules unable to hydrolyze ATP bind strongly to filamentous actin pellet, whereas the active myosin remains in the supernatant. Thus the supernatant was taken for the motility assays and the pellet discarded.

Actin was purified from the same chicken tissue as the myosin using well-established methods (52). The purified filaments were fluorescently labeled and stabilized overnight with tetrathymidroxyamine isothiocyanate (TRITC)/phallolidin (Sigma-Aldrich, St. Louis, MO) as previously described (17). Purified Tm and Tn (as a complex of TnI/TnC/TnT) from rabbit tissue were purchased from Life Diagnostics (West Chester, PA). The regulatory proteins (3–8 mg/ml) were stored in a buffer containing 50 mM NaCl, 10 mM Tris·HCl, 5 mM β-mercaptoethanol, 1 mM EMDTA with 0.09% NaN\textsubscript{3} at pH 7.4. This protein solution was split into experimental size aliquots and then flash-frozen using liquid nitrogen and stored at −80°C until the day of an experiment. Before an experiment, the Tn and Tm were combined with the filamentous actin within the experimental chamber (i.e., flowcell) to reconstitute regulated thin filaments, employing a previously described method (32).

**Solutions.** On the day of the experiment the myosin was diluted from the stock concentration of 25–35 mg/ml to 100 μg/ml in a high-salt myosin buffer (MB: 300 mM KCl, 25 mM Imidazole, 1 mM EGTA, 4 mM MgCl\textsubscript{2}, 1 mM DTT). Subsequent solutions for the motility assay were based on a low-salt actin buffer (AB: 25 mM KCl, 25 mM Imidazole, 1 mM EGTA, 4 mM MgCl\textsubscript{2}, 1 mM DTT). The motility buffers contained AB plus 2 mM ATP and 10 mM DTT, as well as an oxygen-scaevenging system consisting of glucose, glucose oxidase, and catalase to prevent photo-bleaching of the RTF. In addition, the assay buffer contained 1% methylcellulose to keep the filaments on the coverslip surface, and this was particularly crucial at low levels of free Ca\textsuperscript{2+}.

All assay buffer concentrations were determined using WinMaxC (56), with the most up-to-date binding constants. WinMaxC was also used to help determine the total ionic strength of each solution, which was held constant at 125 mM in each condition (except when excess MgADP was added, see below) by varying the level of KCl in the solutions. The pH of the assay buffers was set to 7.4, 6.8, or 6.5 by manipulating added HCl while monitoring a digital pH meter (Accumet Basic AB15; Fisher Scientific, Waltham, MA). The pH meter was calibrated daily according to the manufacturer’s instructions with three known standards (pH 4.0, 7.0, and 10.0), immediately before being used set at the pH on each solution. The MBs were made either with (30 mM) or without (0 mM) added Pi. This was assumed to be some level of contamination of Pi in the solutions even in the absence of added Pi, due to hydrolysis of ATP (8) and impurities such as PP\textsubscript{2} (2). However, the amount of such contamination was likely low (−0.5 mM) and consistent across conditions and therefore is unlikely to have significantly affected the results in our motility assay as confirmed previously in a motility assay (2).

In addition to Pi manipulation, experiments were also performed in which the [MgADP] was varied from 0 to 5 mM. Due to the additional ionic contribution of ADP and Mg\textsuperscript{2+} required, the total ionic strength of all solutions for this subset of experiments was increased to 160 mM. The free Ca\textsuperscript{2+} concentration in each condition was varied from pCa 10 to pCa 4 by varying the amount of CaCl\textsubscript{2} added to the solution, using the binding constants contained within WinMaxC (56).

**In vitro motility assay.** The in vitro motility assay was performed as previously described (17), with minor modifications. Briefly, 30 μl of 100 μg/ml of myosin in MB was added to a nitrocellulose coated flowcell, incubated for 30 s before 30 μl of 0.5 mg/ml BSA was added to cover any uncoated areas of the coverslip surface. After a 1-min exposure to BSA, 1 μM of the vortexed, unlabeled actin in AB was added to prevent any nonfunctional myosin from binding to the subsequently added labeled actin (i.e., an actin coat). To release any unlabeled actin from active heads, 45 μl of 1 mM ATP (in AB) was washed through the flowcell. Fluorescently labeled actin (60 μl of 20 mM TRITC) was then introduced into the flowcell, in the absence of ATP, and allowed to incubate with the myosin on the coverslip surface for 1 min. Following this step, 30 μl of 0.35 μM Tm and 0.75 μM Tn were added and allowed to incubate in the flowcell for 7 min,
resulting in the formation of reconstituted thin filaments. This method has been used previously and creates RTF capable of fully regulating the actomyosin interaction, as evidenced by the lack of filament movement at pCa levels above 9, while also showing strong Ca\textsuperscript{2+}-dependent increase in velocity, from pCa ~7 to 5 (32). After the 7-min incubation, the flowcell was washed with 30 μL AB to remove any unbound proteins. However, 100 nM Tn and 100 nM Tm were also added to the final assay buffer to ensure full and complete activation of the RTFs, as previously described (31).

Motion of the label-regulated actin filaments was visualized and recorded using the methods and equipment previously described (17). Briefly, the flowcell was placed on a Nikon Ti-U inverted microscope, with a ×100, 1.4NA CFI Plan Apo oil-coupled objective. An objective-mounted heater with a thermometer (20/20 Technologies, Wilmington, NC) was used to maintain the temperature at 30.0°C for all experiments. The coverslip surface was imaged using an intensified digital camera (Stanford Photonics, Palo Alto, CA), with a frame grabber (Epix, Buffalo Grove, IL) to capture the images using PIPER Control software (version 2.5.11, Stanford Photonics) running in MS Windows 7. Three 30-s videos were captured at 10 frames/s at three different locations in the flowcell.

**Video analysis.** RTF motion was tracked frame by frame using an automated tracking software program, CellTrak (MotionAnalysis, Santa Rosa, CA). Similar versions of this software have been used previously to track RTFs in the motility assay (30). Using this automated program, it was important to define the parameters of the analysis to calculate filament velocity. For example, noise that appears as tiny, short-lived specks of fluorescence must be eliminated by defining a lower limit to filament length of 0.5 μm. In addition, only filaments moving faster than 0.13 μm/s over the course of the 30-s recording were included in the analysis to determine filament velocity. At pCa levels 8–10, this criterion was not needed because there was no directed motion under these conditions at all levels of pH and P\textsubscript{i}. Typically each field of view would generate 50–100 filament velocities. The mean of these individual filament velocities was then combined with the other two fields to give the average RTF velocity for each condition. Thus each flowcell was counted as one observation, and at least three such observations were made for each condition at each pCa level.

In addition to calculating the average filament velocity for each condition, we also determined the fraction of filaments moving under each condition. This was done using a separate automated tracking program, LiTrackv7 (62), a plug-in for NIH ImageJ. Here we took a representative sample of each data set to efficiently determine the fraction moving under each condition, with filaments that moved less than 1.3 μm over the course of the 10-s time considered not to be moving. A 10-s segment for each 30-s video was used to minimize computer computational time. The LiTrackv7 program calculated the percentage of moving filaments by dividing the filaments defined as moving by the total number of filaments in a field of view.

**Statistical analyses.** A two-way ANOVA (pH × P\textsubscript{i}) was performed in SigmaPlot 11 (Systat Software, San Jose, CA) to analyze the effects of pH and P\textsubscript{i} on RTF velocity at saturating levels of free Ca\textsuperscript{2+} (pCa 4.0), and a Tukey’s HSD post hoc test was used to locate differences. The same analysis was used to determine the effect of ADP and P\textsubscript{i} on RTF velocity at pCa 4.0. The α-level for this and all subsequent statistical tests was set at 0.05. For the Ca\textsuperscript{2+} -dependent experiments, RTF velocity was plotted as a function of pCa and was fit with a Hill equation: $V = V_{max}[1 + 10^{([pCa - pCa_0]/\text{Hill coefficient})}]$, where $V$ is RTF velocity, $V_{max}$ is the extrapolated maximum velocity, $n$ is the Hill coefficient, and pCa\textsubscript{0} is the free [Ca\textsuperscript{2+}] (in-log units) at which RTF velocity is half the maximum value. The curve fits to the data were generated for the velocity vs. pCa and fraction moving vs. pCa using the curve-fitting feature within SigmPlot 11. The goodness-of-fit was evaluated using the $R^2$ value. To compare the parameters of the fit across conditions, 95% confidence intervals (CI) were determined based on the number of observations and the standard error of the estimate of the parameter and differences, with nonoverlap indicating significance.

**RESULTS**

**Effect of P\textsubscript{i} and pH on maximal velocity.** RTF velocity was measured in the in vitro motility assay in either the absence (0 mM) or presence of (30 mM) added P\textsubscript{i} at a free [Ca\textsuperscript{2+}] of pCa 4.0. As expected from prior observations (58, 59), decreasing the pH in the absence of added P\textsubscript{i} caused a significant slowing of RTF velocity. When the pH was lowered from 7.4 to 6.8, RTF velocity decreased by over 50%, and by ~90% when comparing pH 7.4 to 6.5 (Fig. 1, white bars). In contrast, 30 mM P\textsubscript{i} increased RTF velocity at each pH level (Fig. 1, black bars). The P\textsubscript{i}-induced increase in velocity was insignificant at pH 7.4 (4.6 ± 0.3, 0 mM Pi vs. 4.9 ± 0.2 μm/s, 30 mM Pi, means ± SE), but at pH 6.8 and 6.5 the increase was significant, increasing velocity over 60% at pH 6.8 (2.1 ± 0.1 vs. 3.5 ± 0.2 μm/s for 0 and 30 mM Pi, respectively) and by fivefold at pH 6.5 (from 0.5 ± 0.1 vs. 2.6 ± 0.2 μm/s for 0 and 30 mM Pi, respectively). It is important to note, however, that, although Pi increased RTF velocity at any given pH, it did not fully restore the low-pH velocities back to values observed at pH 7.4 in the absence of Pi. Thus Pi does not appear to completely reverse the acidosis-induced depression in velocity, but it does dramatically attenuate the depressive effects of acidosis on filament velocity.

To probe the mechanisms underlying the P\textsubscript{i}-induced increase in $V_{RTF}$ at low pH we also increased [ADP] as well as the [P\textsubscript{i}] concentration in the motility assays. Because our previous work suggested that acidosis prolongs an AM.ADP state (14), we raised the level of ADP to increase population of this state at pH 7.4 and then subsequently added P\textsubscript{i} to try to reproduce the P\textsubscript{i}-induced increase in $V_{RTF}$ at low pH. In agreement with previous observations (25), raising the [ADP] to mM levels drastically reduced $V_{RTF}$ (Fig. 2); however, the addition of 30 mM Pi did not significantly alter $V_{RTF}$ at either 2 or 5 mM ADP. Because the addition of ADP to actomyosin is thought to populate the AM.ADPPi state (10, 61), this finding strongly suggests that acidosis prolongs the AM'.ADP

![Fig. 1. Actin velocity ($V_{act}$) at pCa 4. Peak regulated actin filament (RTF) velocities at each level of pH in the absence (open bars) and presence (shaded bars) of 30 mM added inorganic phosphate (Pi). The values represent the means ± SE RTF velocity at pCa4 and 2 mM ATP. *Significantly different from pH 7.4 and 0 mM added Pi; #significantly different from 0 mM and 30 mM added Pi, compared within each pH level; ¶Pi-induced increase in velocity was significantly greater at pH 6.5 vs. pH 6.8. The temperature was set at 30°C.](http://jap.physiology.org/)
demonstrated strong Ca$^{2+}$ that the RTFs were fully regulated at pCa 10 (Fig. 4A) and we systemati-
cally manipulated the pH, the Pi, and the [Ca$^{2+}$] under similar conditions (25, 30, 31, 69). We then systemati-
ically established, in our control conditions (pH 7.4, 0 mM added Pi), these ions on the velocity-pCa relationship using RTF. We 
regulate the actomyosin interaction, we measured the effects of 
both pH 6.8 and 6.5; however, due to the increased variability 
due to the poor fit to the Hill equation ($R^2 = 0.23$) and increased 
error around the parameter estimates. This made some of the 
comparisons of the parameter estimates across pH levels in-
significant despite having a large magnitude (Table 1).

At pH 7.4, the addition of 30 mM Pi, did not significantly affect the maximal RTF velocity, the value for pCa$_{50}$, or the Hill 
coefficient of the velocity-pCa relationship. However, at both 
pH 6.8 and 6.5, the added Pi significantly increased RTF 
velocity at pCa levels from 7.0 to 4.0. The Pi-induced increase in 
RTF velocity was most dramatic at pH 6.5, with most of the 
the nearly stopped filaments displaying Ca$^{2+}$-dependent velocities that increased up to 2.5 μm/s in the presence of Pi, at saturating 
Ca$^{2+}$. Interestingly, the increased velocity did not appear to result from an increased sensitivity to Ca$^{2+}$ or cooperativity, 
as neither parameter was significantly altered by the increased 
Pi. This was most evident when the normalized velocities were plotted as a function of the pCa level, where the velocity-pCa 
curves in the presence of Pi, superimpose on the curves in the 
absence of added Pi, (Fig. 5).

Because pH and Ca$^{2+}$ affect the number of moving filaments as well as velocity, we also quantified the percentage of moving filaments under each condition at pCa 4 (Fig. 3). Decreasing the pH from 7.4 to 6.8 had a less pronounced effect on percentage of moving than velocity, with the percentage of moving decreasing by less than 10% in the absence of added Pi. The effect on the percentage moving was much more pronounced when the 
pH was decreased to 6.5, where only ~25% of the filaments moved in the absence of Pi. Interestingly when Pi was added, the percentage of moving filaments significantly increased at both pH 6.8 and 6.5, with the increase at pH 6.5 being significantly greater than at 6.8. In fact, increasing the Pi 
caused the percentage of filaments moving to almost fully 
recover to that observed under the control conditions (pH 7.4, 0 mM added Pi) at both pH 6.8 and 6.5.

Effects of Pi and pH on the pCa velocity relationship. To determine whether pH and Pi affect the ability of Tn/Tm to 
regulate the actomyosin interaction, we measured the effects of 
these ions on the velocity-pCa relationship using RTF. We 
established, in our control conditions (pH 7.4, 0 mM added Pi), that the RTFs were fully regulated at pCa 10 (Fig. 4A) and demonstrated strong Ca$^{2+}$-dependent activation at pCa levels from ~7 to 4. The observed values for pCa$_{50}$ and the Hill coefficients ($n$) were in good agreement with prior reports under similar conditions (25, 30, 31, 69). We then systematically 
manipulated the pH, the Pi, and the [Ca$^{2+}$] to determine whether and how these ions affected Ca$^{2+}$ activation of the 
RTF (Fig. 4). In the absence of free Ca$^{2+}$ (pCa 10), RTF 
velocity was zero, indicating that the reconstituted filaments 
were fully regulated in the presence and absence of Pi, and at 
every pH. Decreasing pH alone slowed RTF velocities at 
saturating Ca$^{2+}$ levels (pCa <5.0) in a pH-dependent manner. However, the decreased pH caused the pCa$_{50}$ to increase by 0.5 
and 0.8 pCa units at pH 6.8 and 6.5, respectively (Table 1), 
suggesting that acidosis might induce an enhancement of Ca$^{2+}$ 
sensitivity of the RTF. This effect was similar in magnitude at 
both pH 6.8 and 6.5; however, due to the increased variability 
and poor fit of the Hill equation, it did not reach significance at 
6.5 and 0 mM Pi. The same decreases in pH caused only minor 
changes in the Hill coefficient at pH 6.8 and large, but not 
significant, changes in the Hill coefficient at pH 6.5. These 
observations suggest that the acidosis might increase Ca$^{2+}$ 
sensitivity of velocity in RTF while not significantly altering the 
cooperative nature of activation. At pH 6.5, the severity of 
the effects of acidosis on filament velocity likely contributed 
to the poor fit to the Hill equation ($R^2 = 0.23$) and increased 
error around the parameter estimates. This made some of the 
comparisons of the parameter estimates across pH levels in-
significant despite having a large magnitude (Table 1).

To more fully characterize the effects of Pi, and H$^+$ on RTF 
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absence of added Pi, (Fig. 5).

Because pH and Ca$^{2+}$ affect the number of moving filaments as well as velocity, we also quantified the percentage of moving filaments under each condition as a function of added free Ca$^{2+}$ (Fig. 6). Decreasing the pH from 7.4 to 6.8 had a mild effect on the fraction moving, decreasing it from ~80% to about 65–70% at saturating pCa levels (5.0–4.0). However, decreasing the pH further to 6.5 had a much greater effect on 
the fraction moving; in fact only about 25% of the filaments 
moved even at saturating Ca$^{2+}$ levels. As with the velocity-
pCa analysis, the Hill fit to fraction moving vs. pCa showed 
that pCa$_{50}$ was slightly increased from 7.4 to 6.8, suggesting an increased sensitivity; however, this occurred despite a decrease in the Hill coefficient (Table 2). Added Pi had no effect on the 
fraction moving at pH 7.4 but increased it at pH 6.8 at every 
pCa from 8.0 to 4.0, with the difference between 0 and 30 mM 

Fig. 2. Maximal RTF velocity as a function of MgADP concentration. Peak 
velocity was recorded as the average of all fields recorded at pCa4. Open bars 
display the means ± SE of the velocity in the absence (open bars) and the 
presence (shaded bars) of 30 mM added Pi. Error bars indicate SE; *signifi-
cantly different from pH 7.4 and 0 mM added Pi. The temperature was set at 
30°C.

Fig. 3. Fraction moving at pCa 4. Percentage moving filaments at each level of 
pH in the absence (open bars) and presence (shaded bars) of 30 mM added Pi. 
The values represent the means ± SE. *Significantly different from pH 7.4 and 
0 mM added Pi; #significantly different from 0 mM added Pi, compared at each 
pH level; ¶P$_i$-induced increase was significantly greater at pH 6.5 vs. pH 6.8. 
The temperature was set at 30°C.
being ~20% from pCa 6.5 to 4.0. At pH 6.5, this effect was even more dramatic with the added P_i, causing the fraction moving to increase from less than 25% in the absence of P_i to values over 60% at the highest Ca^{2+} concentrations in the presence of P_i. In general, the parameters of the Hill fits to the fraction moving mirrored those of the fits to the velocity-pCa coefficients (data not shown).

**DISCUSSION**

The present study examined, for the first time, the combined effects of elevated levels of P_i and H^+ on the ability of myosin to bind to and translocate regulated actin filaments. Acidosis strongly depressed regulated filament velocity at all Ca^{2+} levels; however, the addition of P_i restored a significant portion of the RTF filament velocity as well as the percentage of filaments moving under acidic conditions. This suggests that H^+ and P_i may exert competing effects on the same step of the cross-bridge cycle, with H^+ slowing but P_i speeding the detachment rate. These findings therefore may provide a molecular basis for the loss of contractile velocity and power associated with fatigue.

**Acidosis slows maximal and submaximal RTF velocity.** Decreasing pH from 7.4 to 6.8 and 6.5, in the absence of added P_i, depressed RTF velocity at saturating free Ca^{2+} levels compared with pH 7.4, which qualitatively agrees with previous observations in the in vitro motility assay, performed at the same temperature as the present study (58, 59, 69). This slowing of RTF velocity could potentially be due to a direct effect of acidosis on the actomyosin interaction and/or an indirect effect mediated through Tn/Tm. Our previous work, using unregulated actin, affords us the ability to compare the magnitude of the direct and indirect effects of acidosis (17). The drop in RTF velocity caused by decreasing pH from 7.4 to 6.8 (~50%) is very similar in magnitude to the decrease in unregulated filaments observed with the same drop in pH (17). This suggests that most of the depression in velocity is attributable to a direct effect on the actomyosin interaction. However, the decrease in filament velocity caused by dropping the pH from 7.4 to 6.5 was more pronounced with RTF (~95%) than with unregulated actin (~67%). This implies that, in addition to a direct effect on actomyosin, a portion of the depressive effect of acidosis is mediated through Tn/Tm at pH 6.5. Previous work suggests that Tm (23), TnC (19, 53), and TnI (47) all may be involved in mediating the depressive effects of acidosis.

It is interesting to note that the depressive effects of acidosis were significantly more severe when comparing 7.4 vs. 6.5 than 7.4 vs. 6.8. This implies that a threshold value is crossed below a certain pH, and the effects on contractile function become more severe and would imply a greater role in fatigue as the drop in pH becomes more severe. This also suggests that the mechanisms involved are more complex than a simple pH-dependent change in the Gibbs-free energy of ATP hydrolysis (ΔG_ATP) because decreasing the pH from 7.4 to 6.5 only decreases the ΔG_ATP ~10% (24), yet RTF velocity is decreased by ~90% (Fig. 1).

One of the most well-characterized effects of acidosis is its ability to depress Ca^{2+} sensitivity of force (3, 12, 19, 44, 48, 51, 53, 63). Decreasing pH from 7.0 to 6.5 in muscle fibers can decrease the pCa_{50} value of the force-pCa relationship by 0.61 units at 30°C (63). In the present study, we observed acidosis to exert the opposite effect on the velocity-pCa relationship with a decrease in pH 7.4 to 6.8 causing an increase in Ca^{2+} sensitivity (Table 1). A similar magnitude change was observed at pH 6.5 but was not significant due to the severity of the effects on velocity increasing the relative error. The observation of increased Ca^{2+} sensitivity at pH 6.8 therefore contrasts with prior work examining the effects of acidosis on the force-pCa relationship in muscle fibers (3, 12, 19, 44, 48, 51, 53, 63). The contrasting impact on Ca^{2+} sensitivity is likely due to known differences in the behavior of the force-pCa and velocity-pCa relationships, where it is observed that the velocity-pCa relationship is much more sensitive to Ca^{2+} and less cooperative than the force-pCa relationship (31). The putative mechanism for the acidosis-induced decrease in Ca^{2+} sensitivity seen when examining the force-pCa relationship is thought to involve free H^+ interfering with Ca^{2+} binding to TnC (53). However, because we observed the opposite effect of acidosis on Ca^{2+} sensitivity, it suggests that either the

Table 1. Parameter estimates of the Hill fits to velocity-pCa data

<table>
<thead>
<tr>
<th>Conditions</th>
<th>P_i</th>
<th>Hill coeff.</th>
<th>pCa_{50}</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>0</td>
<td>2.40 ± 0.70</td>
<td>6.15 ± 0.07</td>
<td>0.83</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>30</td>
<td>2.12 ± 0.50</td>
<td>6.25 ± 0.07</td>
<td>0.83</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>0</td>
<td>2.26 ± 0.84</td>
<td>6.66 ± 0.09*</td>
<td>0.64</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>30</td>
<td>2.09 ± 0.45</td>
<td>6.71 ± 0.06*</td>
<td>0.86</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>0</td>
<td>0.72 ± 0.58</td>
<td>6.96 ± 0.50</td>
<td>0.23</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>30</td>
<td>1.46 ± 0.42</td>
<td>6.82 ± 0.10*</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Mean Hill fit parameters derived from fits to the data in Figure 2. Numbers displayed are means ± SE. Significant differences were determined by constructing 95% confidence intervals around the parameter estimate. *Significantly different from pH 7.4 and 0 mM inorganic phosphate (P_i). R^2 calculated from least-squares value.
The acidosis-induced prolongation of the AM. ADP lifetime, at 30°C (14) could exert these opposing effects on thin filament activation. Because this is a strongly bound state, prolonging its lifetime would activate the thin filament more strongly at any given submaximal [Ca ++], as observed previously in motility assays (35) and in single muscle fibers (64). In fact, the increased Ca ++ sensitivity and tendency for a decreased Hill coefficient observed in the present study (Table 1) are quite similar to the pattern of effects caused by adding strong-binding, rigor-like NEM-S1 myosin molecules to single muscle fibers at low levels of Ca ++ activation (22, 64). Therefore through its effects on myosin, acidosis may exert a similar effect on RTF velocity in the motility assay.

It is interesting to note that the percentage of filaments moving was affected differently than the velocity by the acidosis. For example, comparing pH 7.4 to 6.8, the velocity reduced much more than the percentage moving at pH 6.8 (Figs. 4 and 5). This suggests that the velocity of most of the filaments was uniformly slowed by acidosis, rather than acidosis having a strong deactivating effect on the thin filaments. Thus, under these conditions, it would seem that acidosis is having the greatest effect on the rate-limiting step of velocity, thought to be the ADP-release rate (60), rather than limiting the activation of the thin filament and cross-bridge formation. The effects on percentage moving were also different from the effects on velocity at pH 6.5. Here most of the filaments were stopped, even at pCa 4.0 (Fig. 2), which again could be due to incomplete activation of the thin filament or a slowing of the steps in the cross-bridge cycle that limit velocity. However, because the addition of Pi increased the percentage moving from 10 to over 60% as well as greatly increasing the velocity under these conditions, it suggests again that the filaments were activated but stalled in a state that limited velocity (e.g., AM'. ADP). Under these conditions, it is likely that Pi is re-binding to the AM'.ADP state and speeding velocity by inducing dissociation from a strongly-bound state (i.e., reducing t ca). Alternatively, the elevated Pi could somehow have increased activation of the thin filament, i.e., a calcium-like effect; however, if this were the case, elevated Pi should have also activated the RTF in the absence of Ca ++. This was not observed in the present study at either the high or low pH (Figs. 4 and 6), suggesting that Pi was not directly activating the thin filament.

Pi enhances RTF velocity at low pH. The most novel finding of the present study is the observation that elevated levels of Pi reverse much of the acidosis-induced depression in RTF velocity and the depression in percentage of moving filaments. This was observed at virtually all levels of filament activation, from pH 7 to 4 (Figs. 4 and 5). In contrast, at pH 7.4, elevated levels of Pi did not significantly alter maximal RTF velocity or percentage of moving filaments at maximal or submaximal levels of Ca ++. The lack of a Pi-induced effect at normal pH is in agreement with prior observations utilizing both the in vitro motility assay (25) and the skinned single muscle fiber preparation (36, 42, 55), all performed at 30°C as in the present study. Our conclusion that Pi enhances velocity at low pH seemingly contrasts with the conclusions from skinned single muscle fibers, where increasing Pi from 5 to 30 mM at pH 6.2 was shown to slow maximal unloaded shortening velocity (V max) by 12% (36). However, comparing the relative magnitude of the decrease in V max (57%) in fibers (36) when going from the control value (pH 7.0, 5 mM Pi) to the fatigue-like conditions (pH 6.2 and 30 mM Pi) reveals a change nearly identical to the relative decrease in V actin (53%) caused by decreasing pH and adding 30 mM Pi in the present investigation (Fig. 1). The consistency between the data sets is further
supported by the strikingly similar effects of Pi on velocity at the high pH levels, where elevated Pi has no significant effect on velocity (36). Considering the effects from 0 to 30 mM Pi at low pH in both fiber and in vitro motility experiments therefore suggests that elevated levels of Pi may act to enhance rather than depress unloaded shortening velocity.

Elevated levels of Pi did not have a significant effect on either the pCa50 or the Hill coefficient, suggesting that it did not alter Ca\(^{2+}\) sensitivity or the nature of cooperative binding under the unloaded conditions of the motility assay (Table 1). These findings are also consistent with prior in vitro motility studies (25) and qualitatively similar to observations in single muscle fibers (42). The absence of a Pi-induced depression of Ca\(^{2+}\) sensitivity on the velocity-pCa relationship, however, contrasts with the strong depressive effects of Pi on the force-pCa relationship, well-characterized in single muscle fibers (45). The different effect of Pi on the force-pCa vs. velocity-pCa relationship, well-characterized in single muscle fibers (38, 43); however, because V\(_{\text{actin}}\) and unloaded shorting velocity are thought to be predominantly limited by the duration of the strongly bound state (34, 50, 60, 68), we focused on determining the effects of acidosis on the biochemical transitions governing t\(_{\text{on}}\).

Our previous work in the motility and single molecule trap assay suggested that acidosis prolongs one or both of the strongly bound AM.ADP states (14). In addition we also have demonstrated that Pi can enhance the velocity of unregulated actin filaments depressed by acidosis (17). This observation was consistent with a simple detachment-limited model of the strongly bound state (34), where acidosis prolongs t\(_{\text{on}}\) by slowing ADP release from actomyosin, whereas Pi shortens t\(_{\text{on}}\) by inducing detachment from a strongly bound postpower stroke state (17). However, this simple model of the cross-bridge cycle of myosin assumes that there is only one AM.ADP state, and there is strong evidence from both solution kinetics (61) and single muscle fibers (10) that there are two AM.ADP states in the cross-bridge cycle separated by an isomerization step (65). Figure 7 depicts a schematic of the cross-bridge showing where the isomerization step takes place as well as the associated biochemical changes.

In the present study, we designed experiments to enable us to delineate whether the state before (AM*.ADP) and/or following the isomerization step is prolonged by acidosis. To do this, we took advantage of two well-characterized properties of actomyosin in the ADP-bound state. First, evidence from both

Table 2. Parameter estimates of the Hill fits to fraction moving vs. pCa data

<table>
<thead>
<tr>
<th>Conditions</th>
<th>fit parameters</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>pCa50 = 6.35 ± 0.05</td>
<td>0.89</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>pCa50 = 6.70 ± 0.08</td>
<td>0.75</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>pCa50 = 6.92 ± 0.40</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Mean Hill fit parameters derived from fits to the data in Figure 3. Numbers displayed are means ± SE. Significant differences were determined by constructing 95% confidence intervals around each parameter estimate. *Significantly different from pH 7.4 and 0 mM Pi. R² calculated from least-squares value.

Fig. 7. A schematic model of the cross-bridge cycle. Link between mechanical and kinetic events in the cross-bridge cycle. AM*.ADP, unitary step size of myosin, which occurs concomitant with P, release. This is followed by the 2 kinematics involved in the release of ADP, one of which is susceptible to P, rebinding. AM*.ADP, which is indicated with an asterisk. The binding of ATP induces dissociation from the strongly bound state, whose duration is t\(_{\text{rigor}}\). It is t\(_{\text{on}}\) that is thought to be the predominant factor limiting RTF velocity (34).
solution kinetics (61) and single muscle fibers (10) demonstrates that elevating ADP alone populates only the AM.ADP and not the AM’.ADP state because the energy barrier is too high to reverse the isomerization step under these conditions (61). By increasing the [ADP], we specifically elevated only the population of the AM.ADP state. Second, oxygen exchange experiments demonstrated that P_i preferentially rebinds to the AM’.ADP state and rapidly causes dissociation from actin (72).

Our observation that increasing the [ADP] alone, at pH 7.4, slowed V_{RTF} (Fig. 2) is consistent with previous observations (25). At a molecular level, this suggests that ADP readily rebinds to the AM state and reforming the AM.ADP state thereby prolongs the strongly bound state and slows filament velocity. However, our observation that elevated levels of P_i did not reverse the slowing of V_{RTF} induced by elevated ADP strongly suggests that P_i does not readily rebind to the AM.ADP state and therefore confirms that this is not the state prolonged by acidosis. Rather, because P_i dramatically increased V_{RTF} at low pH, it suggests that acidosis prolongs the AM’.ADP state, implying also that acidosis slows the isomerization step rather than the actual release of ADP from actomyosin. Therefore the P_i-induced increase in V_{RTF} under acidic conditions results from acidosis increasing the AM’.ADP population and P_i readily rebind to the AM’.ADP state to form the AM.ADP.P_i state. The AM.ADP.P_i is thought to be in rapid equilibrium with the M.ADP.P_i state, and therefore strongly bound cross-bridges detach, reducing force (29) and leading to a decrease in the duration of the strongly bound state. Thus velocity is increased by the addition of P_i due to an increased detachment rate from a postpower stroke state (17).

Because this mechanism would act to decrease the number of strongly bound cross-bridges, it would also be predicted that acidosis would make myosin more susceptible to P_i rebinding and thus augment the P_i-induced reduction in force (29). This idea is supported by the observation that combined elevations in P_i and H^+ dramatically reduce the force- and power-generation capacity of myosin in a loaded motility assay (27). Their combined effect is much more pronounced than the effect of P_i alone on the force-velocity and force-power relationships in single muscle fibers (15). Thus it would appear that any P_i-induced increase in shortening velocity that might attenuate the effects of fatigue would likely be offset by a decrease in force and ultimately the power-generating capability of muscle.

This isomerization step (AM’.ADP ↔ AM.ADP) is also thought to be slowed when myosin is working against a resistive load (72). Thus the idea that P_i preferentially rebinds to the AM’.ADP state may also explain why elevated levels of P_i affect isometric force in fibers (7, 8, 15) but not unloaded shortening velocity or actin filament velocity in the motility assay (2, 17, 33).

Although this mechanism is consistent with the present findings and several other observations (26) including those from single muscle fibers (36), it is certainly not the only possible explanation for the P_i-induced increase in velocity. More complex mechanisms involving a greater number of states in the cross-bridge cycle could certainly be devised to capture the pH and P_i-dependent effects on velocity. At present we chose to explore only the simplest model that was most consistent with our own data as well as with the observations from others examining the effects of pH and P_i.

**Conclusions.** By varying the free Ca^{2+}, P_i, and H^+, we have simulated some of the crucial intracellular changes experienced during the later stages of fatigue in response to intense contraction activity (11, 40) to gain insight into the underlying molecular mechanisms of these effects. The present results suggest that most of the acidosis-induced inhibition of RTF velocity is primarily due to a direct effect on actomyosin because it is significant in the presence and absence of the regulatory proteins.

Our results suggest that acidosis slows a specific step in the cross-bridge cycle of myosin. By slowing the isomerization between the AM’.ADP ↔ AM.ADP, acidosis prolongs the strongly bound state and therefore accounts for the acidosis-induced slowing of filament velocity. When combined with elevations in P_i, the slowing of this transition makes actomyosin more vulnerable to P_i rebinding and therefore is consistent with the dramatic losses in myosin force- and power-generating capacity when both ions are elevated (27), as would occur during the latter stages of fatigue. Therefore combining the direct effects of these ions on actomyosin with their indirect effects mediated through Tn/Tm may help to explain the dramatic losses in the force-velocity and force-power relationships in fatigued muscle (70).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: E.P.D. and M.A.T. conception and design of research; E.P.D., T.J.L., and M.A.T. performance of research; E.P.D., T.J.L., and M.A.T. analysis and interpretation of data; E.P.D., T.J.L., and M.A.T. preparation of manuscript; E.P.D. and T.J.L. revising the manuscript for important intellectual content. All authors reviewed the final version of manuscript.

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