Ca\textsuperscript{++}-sensitizing mutations in troponin, P\textsubscript{i}, and 2-deoxyATP alter the depressive effect of acidosis on regulated thin-filament velocity

Thomas J. Longyear, Matthew A. Turner, Jonathan P. Davis, Joseph Lopez, Brandon Biesiadecki, and Edward P. Debold

Department of Kinesiology, University of Massachusetts, Amherst, Massachusetts; Department of Physiology and Cell Biology, Ohio State University, Columbus, Ohio

Submitted 17 October 2013; accepted in final form 17 March 2014

Longyear TJ, Turner MA, Davis JP, Lopez J, Biesiadecki B, Debold EP. Ca\textsuperscript{++}-sensitizing mutations in troponin, P\textsubscript{i}, and 2-deoxyATP alter the depressive effect of acidosis on regulated thin-filament velocity. J Appl Physiol 116: 1165–1174, 2014. First published March 20, 2014; doi:10.1152/japplphysiol.01161.2013.—Repeated, intense contractile activity compromises the ability of skeletal muscle to generate force and velocity, resulting in fatigue. The decrease in velocity is thought to be due, in part, to the intracellular build-up of acidosis inhibiting the function of the contractile proteins myosin and troponin; however, the underlying molecular basis of this process remains poorly understood. We sought to gain novel insight into the decrease in velocity by determining whether the depressive effect of acidosis could be altered by 1) introducing Ca\textsuperscript{++}-sensitizing mutations into troponin (Tn) or 2) by agents that directly affect myosin function, including inorganic phosphate (P\textsubscript{i}) and 2-deoxy-ATP (dATP) in an in vitro motility assay. Acidosis reduced regulated thin-filament velocity (V\textsubscript{RTF}) at both maximal and submaximal Ca\textsuperscript{++} levels in a pH-dependent manner. A truncated construct of the inhibitory subunit of Tn (TnI) and a Ca\textsuperscript{++}-sensitizing mutation in the Ca\textsuperscript{++}-binding subunit of Tn (TnC) increased V\textsubscript{RTF} at submaximal Ca\textsuperscript{++} levels but had no effect on V\textsubscript{RTF} at maximal Ca\textsuperscript{++} levels. In contrast, both P\textsubscript{i} and replacement of ATP with dATP reversed much of the acidosis-induced depression of V\textsubscript{RTF} at saturating Ca\textsuperscript{++}. Interestingly, despite producing similar magnitude increases in V\textsubscript{RTF}, the combined effects of P\textsubscript{i} and dATP were additive, suggesting different underlying mechanisms of action. These findings suggest that acidosis depresses velocity by slowing the detachment rate from actin but also by possibly slowing the attachment rate.

AFTER SEVERAL MINUTES OF REPEATED high-intensity contraction, muscle loses much of its ability to generate force and velocity (13, 14). While the mechanisms underlying the decrease in force have been intensively investigated, the mechanisms of the depression in velocity have received less attention and are therefore still poorly understood, particularly at the molecular level (1, 25, 34).

In response to fatiguing stimulation, the maximal shortening velocity of muscle decreases by 20 to 33% (13, 14). A significant portion of this loss is believed to be due to the accumulation of hydrogen ions (i.e., acidosis) acting to directly inhibit the function of contractile proteins (9, 15, 20, 26, 35). Specifically, acidosis is thought to 1) directly slow the kinetics of the actomyosin cross-bridge cycle (16, 35) and 2) decrease the ability of calcium (Ca\textsuperscript{++}) to activate the thin filament (19, 24).

Early hypotheses suggested that the decreased maximal shortening velocity in response to fatigue and the related observation of a slowed rate of relaxation were due to a slowing of the rate of cross-bridge detachment (21). Subsequent findings using skinned muscle fibers suggested a role for acidosis in this process, as it elicits a reduction in unloaded shortening velocity that is quantitatively similar to that observed during fatigue (6, 8, 35). Because the acidosis-induced decrements in velocity were observed at full Ca\textsuperscript{++} activation (i.e., pCa 4–5), it was suggested that acidosis directly inhibited the cross-bridge cycle (6, 8, 35). However, determining a direct effect at the cross-bridge level is extremely difficult within the highly complex environment of an intact muscle fiber where billions of myosin molecules interact with actin in the presence of a myriad of other proteins. Therefore, the effects of fatiguing levels of acidosis have been determined in an in vitro motility assay that used isolated myosin and actin (16). Decreasing pH from a neutral value (~7.0) to a value experienced during fatigue (6.8–6.2) causes a sharp reduction in actin filament velocity (V\textsubscript{actin}) in the absence of regulatory proteins, supporting the notion that acidosis directly affects myosin function (36). Our most recent work (17) suggests that acidosis slows an isomerization step in an ADP-bound state of the cross-bridge cycle (Fig. 1); however, it remains unclear whether this is the only step in the cycle affected by acidosis.

The other well-characterized effect of acidosis is its depressive effect on Ca\textsuperscript{++} sensitivity of the thin filament (19, 24). The limited previous investigations on shortening velocity have yielded contradictory results, with early work from the in vitro motility assay showing a significant decrease in Ca\textsuperscript{++} sensitivity (57, 58), whereas more recent work suggests that acidosis has no effect on Ca\textsuperscript{++} sensitivity (69).

The effect of acidosis on the force-pCa relationship is much less equivocal and is thought to result from acidosis directly affecting the ability of the muscle-regulatory protein troponin (Tn) to regulate and modulate the actomyosin interaction (50). One proposed mechanism suggests that acidosis affects Ca\textsuperscript{++} binding to Tn (via the Ca\textsuperscript{++}-binding subunit, TnC), possibly by protons directly reducing the affinity of Ca\textsuperscript{++} for its binding site (22, 44, 50). At very low levels of Ca\textsuperscript{++} activation (>pCa 6.0), unloaded shortening velocity can be limited by the rate of cross-bridge formation (46); therefore, inhibiting Ca\textsuperscript{++} activation acidosis could also limit velocity through this mechanism. This mechanism could be particularly important in the latter stages of fatigue when Ca\textsuperscript{++} release from the sarcoplasmic reticulum, and thus myoplasmic [Ca\textsuperscript{++}] is compromised (37).

To probe the effects of acidosis mediated by Tn, we took advantage of a recently characterized mutation in TnC (V43Q)
that has an increased sensitivity for Ca\(^{2+}\) and therefore causes a leftward shift in the force-pCa relationship at neutral pH (38). The location of this mutation in Tn crystal structure is shown in Fig. 2. Based on these prior observations, we hypothesized that, if the affinity of Ca\(^{2+}\) for TnC were increased, it might overcome the depressive effects of acidosis.

A competing view of the mechanism underlying the acidosis-induced decrease in Ca\(^{2+}\)-sensitivity posits a more prominent role for the inhibitory subunit of Tn (TnI) (2, 45). Various regions of TnI may play a role mediating the effects of acidosis; however, the COOH-terminal end has received considerable attention (11, 12, 68) because it contains the region thought to interact with actin, thus maintaining tropomyosin (Tm) in a position that helps to prevent myosin from strongly binding to actin in the absence of Ca\(^{2+}\) (65). Indeed truncation of the COOH-terminal residues beyond 156 in TnI, which is associated with the development of distal arthrogyrosis, increases contractile function in vitro, including increasing Ca\(^{2+}\) sensitivity of force (56). Similarly, the proteolytic cleavage of the analogous region in the cardiac isoform of TnI, which occurs during myocardial ischemia, results in an increased sensitivity to Ca\(^{2+}\) (27, 40). To explore this potential mechanism, we introduced a truncation mutant lacking a portion of the COOH-terminal end of TnI (see Fig. 2 for structural information). We hypothesized that removal of a portion of this region of TnI would weaken its affinity for actin at low Ca\(^{2+}\) and thus would attenuate the altered Ca\(^{2+}\) sensitivity and the depression in velocity of the thin filaments under acidic conditions.

To probe the direct effect of acidosis on the actomyosin interaction, we used two agents, inorganic phosphate (P\(_i\)) and 2-deoxy-ATP (dATP), both thought to increase the detachment rate by affecting one or more steps in the actomyosin cross-bridge cycle (Fig. 1). Elevating P\(_i\) increases the detachment rate from an actomyosin state with ADP bound in the active site (AM-ADP) by putatively reversing the Pi-release step (29) and therefore can probe the effects of shortening the lifetime of this state (29). Although less well characterized than the effects of P\(_i\), dATP is thought to accelerate both the rate of cross-bridge formation (i.e., the weak-to-strong binding transition) and ADP release from myosin (54, 55). An effect on ADP release may be particularly relevant to fatigue because this step

---

**Fig. 1.** Simplified consensus model of the cross-bridge cycle. The key steps of the ATPase cycle of myosin linked to the mechanical events. The first step, inorganic phosphate (P\(_i\)) release, is linked to actin strong binding and the movement of the lever arm that results in a unitary displacement (d). During this step, tropomyosin moves over the surface of actin to allow actomyosin binding, and this is thought to be the step regulated by Ca\(^{2+}\), i.e., the weak-to-strong transition (28). There are likely two actomyosin state with ADP bound in the active site (AM-ADP) states (60), with acidicosis thought to regulate the transition from the first (AM-ADP) to the second (AM-ADP) (17). We also believe that, when P\(_i\) is elevated, the powerstroke may not be reversed, but rather myosin dissociates from actin in a postpowerstroke state to account for P\(_i\)-induced increase in regulated thin-filament velocity (\(V_{RTF}\)) (17, 18). Thus there are likely many more steps than represented here, but for clarity we have reduced the key steps.

**Fig. 2.** Location of mutations within troponin (Tn) structure. A: rabbit skeletal crystal structure of the NH\(_2\)-terminal region of Ca\(^{2+}\)-binding subunit of Tn (TnC) shown with V43 labeled in blue, coordinates from Soman et al. (61). V43 is part of the hydrophobic patch in TnC thought to swing away from the center helix of TnC, allowing inhibitory subunit of Tn (TnI) to bind in the presence of Ca\(^{2+}\) (73). B: TnI (dark gray) binds to the NH\(_2\)-terminal of TnC (white) in the Ca\(^{2+}\)-activated state. The R156 TnI used in the present study represents a truncation mutant that eliminates the last 26 residues. The COOH terminus of TnI is not defined in the crystal structure, so the figure is truncated at residue 143. Both structural images were rendered with Polyview 3D. C: sequence of the COOH terminus of TnI for wild-type (WT) and for the TnI R156 variant illustrating the truncated residues.
is thought to limit unloaded shortening velocity (47), and it is also the step thought to be slowed by acidosis (16). Therefore, we hypothesized that substituting dATP for ATP would attenuate the depressive effects of acidosis on maximal velocity in the motility assay due to both its effects on ADP release and the rate of cross-bridge formation.

By using agents that affect specific steps in the actomyosin cross-bridge cycle and by introducing Ca\(^{2+}\)-sensitizing alterations into Tn, we aimed to gain novel insight into the molecular basis of the role of acidosis in depressed contraction velocity during fatigue.

**MATERIALS AND METHODS**

**Proteins.** Myosin and actin were purified from chicken pectoralis muscle as previously described (41, 49), with minor modifications described previously (18). Purified filaments were stabilized in the filamentous form and fluorescently labeled with tetramethylrhodamine isothiocyanate (TRITC)/phalloidin (Sigma-Aldrich, St. Louis, MO). Purified Tm was obtained from either rabbit psoas muscle or from human cardiac tissue (Life Diagnostics, West Chester, PA). Gel electrophoresis revealed that the human cardiac Tm was made up of roughly 60% of the α-isofrom and 40% β-isofrom, whereas the Tm from rabbit skeletal tissue was 50/50 α- and β-isofroms. Roughly half of the data were collected using each variant of Tm; however, a statistical comparison revealed that the different Tm had no effect on any of the variables tested (data not shown). Therefore, the two data sets were combined in the final analysis. The lack of a functional difference may be the result of there being similar portions of the α- and β-isofrom or due to the fact that even pure isofroms of α- and β-Tm dimers fail to display differences in biological activity (53).

Wild-type Tn (WT) subunits (TnI, TnC, and TnT) were isolated from rabbit psoas muscle as previously described (63). The TnC mutant was constructed by expressing the sequence (pET-24 plasmid) for rabbit fast skeletal TnC and purified as previously described (66). The WT and R156 truncated TnC were constructed in a pET-17b plasmid, expressed and purified as previously described (39).

**Solutions.** Purified myosin was diluted from a concentration of 25–35 mg/ml to 100 μg/ml in a high-salt myosin buffer (300 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl\(_2\), 1 mM DTT) before experimentation. The buffers for the motility assay were based on a lower-salt actin buffer (25 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl\(_2\), 1 mM DTT) and 2 mM ATP or 2-deoxy-ATP (Sigma-Aldrich). An oxygen-scavenging system was also added to prevent photobleaching of the actin filaments (57 mg glucose, 2.5 mg glucose oxidase, and 0.45 mg catalase), and methylcellulose (1% vol/vol) was added to these buffers to keep the filaments near the coverslip surface.

Buffer recipes were determined using WinMaxC (52). The total ionic strength at 95 mM, the Pi concentration was set to 15 mM. The pH was set to 7.4, 6.8, or 6.5 by manipulating added HCl. The buffer. It is important to note that the computer program for determining solution composition (52) takes into account the strong pH dependence of the chelating capacity of EGTA (3). The pH of the assay buffers was set to 7.4, 6.8, or 6.5 by manipulating added HCl. To simulate fatigue conditions, we added P_i, which, to maintain the total ionic strength at 95 mM, the P_i concentration was set to 15 mM. The Ca\(^{2+}\) concentration under each condition was varied from pCa 10 to pCa 5, carefully considering the pH dependence of the Ca\(^{2+}\)-binding affinities to all the other constituents in the solution.

**In vitro motility assay.** In vitro motility assays with reconstituted thin filaments were performed as previously described (17) using the equipment detailed in Debold et al. (18), with minor modifications. Briefly, myosin was loaded onto a nitrocellulose-coated coverslip surface at a saturating concentration of 100 μg/ml. This was followed by 0.5 mg/ml BSA to block any uncoated areas of the coverslip surface from interacting with actin. TRITC-labeled actin filaments were added to the flow cell in the absence of ATP but in the presence of 0.25 μM Tm and 0.75 μM Tn and incubated in the flowcell for 7 min to reconstitute regulated thin filaments, as previously detailed (32). The final assay buffer contained an additional 100 nM Tn and 100 nM Tm to ensure that the filaments remained fully regulated during experimentation (31). Reconstituted thin-filament motion was visualized using a Nikon Ti-U inverted microscope, with a ×100, 1.4 NA CFI Plan Apo oil-coated objective with the temperature maintained at 30.0°C for all experiments. For each flow cell, three 30-sec videos were captured at 10 frames/s and at three different locations within each flow cell.

**Video analysis.** The velocity of the reconstituted thin filaments was determined using an automated filament-tracking software program (CellTrak; MotionAnalysis, Santa Rosa, CA), as previously described for regulated thin filaments (30, 69). In an effort to eliminate the possibility of analyzing noise in the fluorescence signal, filaments <0.5 μm were eliminated from the analysis, and filaments with velocities <0.13 μm/s were considered to be stationary. A typical field of view generated 25–75 filament velocities, and the mean of these velocities was taken as the average velocity for a given field of view. The microscope slide was then moved to a new field of view within the flow cell twice more to generate a total of three recordings for each flow cell. For each condition tested, three to eight flow cells were used to generate the data, resulting in a total of 9–24 recordings contributing to the overall mean filament velocity for each condition.

**Statistical analyses.** Filament velocity at each pCa level was plotted and fit with a Hill equation: 

\[ V = V_{\text{max}}/[1 + 10^{p \text{Ca}_{50} - p \text{Ca}}] \]

where \( V \) is the average RTF velocity, \( V_{\text{max}} \) is the maximum velocity, \( n \) is the Hill coefficient, and \( \text{pCa}_{50} \) is the free \( [\text{Ca}^{2+}] \) (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 relationship using a curve-fitting routine within SigmaPlot 11.2 (Systat Software, San Jose, CA). Goodness-of-fit was evaluated using the \( R^2 \) value.

The data set of each day was fit separately to the above equation to generate individual parameters from the fits and the derived values used for inferential statistics, a methodology previously suggested as the optimum approach for comparing differences in the force or velocity-pCa relationships (28). The differences between maximal \( V_{\text{RTF}} \) and the parameters of the Hill fit were quantified using a two-way ANOVA, and a Tukey’s post hoc test was used to locate the differences. The effect of 15 mM P_i and dATP on the velocity-pCa relationship of filaments with WT Tn was determined by comparing the parameters of the Hill fit using a two-way ANOVA and Tukey’s post hoc tests. To compare the combined effects of the 15 mM P_i and dATP on maximal \( V_{\text{RTF}} \) velocities for filaments reconstituted with WT Tn, the data were analyzed using a three-way ANOVA (pCa x Pi x ATP type) with specific differences located using Tukey’s post hoc tests. All inferential statistics were performed in SigmaPlot/SigmaStat version 11.2, and the α-level for all statistical tests was set at 0.05.

**RESULTS**

Decreasing the pH from 7.4 to 6.8 caused a large reduction in \( V_{\text{RTF}} \) at both saturating and subsaturating Ca\(^{2+}\) concentrations (Fig. 3). Decreasing the pH further to 6.5 caused even larger reductions in \( V_{\text{RTF}} \) at all [Ca\(^{2+}\)] from pCa 7.0 to 4.0 (Fig. 4), with the maximal velocity reaching only ~10% of the maximum value. In fact, \( V_{\text{RTF}} \) (0.21 ± 0.08 μm/s) at pH 6.5, even at saturating Ca\(^{2+}\) levels (pCa 4 and 5), was not significantly different from the threshold used to define filaments as moving (0.13 μm/s). At pH 6.8 most of the reduction in \( V_{\text{RTF}} \) occurred at saturating [Ca\(^{2+}\)], with little effect on the pCa_{50} (Table 1). In contrast, lowering the pH further to 6.5 caused a very dramatic rightward shift in the velocity-pCa relationship.
resulting in a greater than two-unit change in the pCa50 value (Table 2). In fact, the severity of the acidosis-induced depression in velocity likely contributed to the data being poorly fit ($R^2 = 0.22$) by the Hill equation.

Introducing the V43Q Ca$^{++}$-sensitizing mutation into TnC increased $V_{RTF}$ compared with filaments with WT Tn at every submaximal [Ca$^{++}$] from pCa 10 to 6.5 and at both high (7.4) and low pH (6.8) (Fig. 3). At pH 6.8, this resulted in an increase of 0.8 pCa units in the pCa50 value compared with WT filaments (Table 1), suggesting that the mutation increased the Ca$^{++}$ sensitivity for velocity. This effect occurred at both pH 7.4 and 6.8, indicating that it was pH independent. It is important to note that, although the presence of this mutation increased the Ca$^{++}$ sensitivity, acidosis still significantly decreased $V_{RTF}$ at every pCa level from 10.0 to 4.5 (Fig. 3). This suggests that the mutation did not reverse the depressive effect of acidosis despite sensitizing the filaments to Ca$^{++}$. We attempted to determine the effect at pH 6.5; however, the depression in $V_{RTF}$ at pH 6.5 was so severe that a reasonable fit to the Hill equation could not be generated (data not shown).

It is important to point out that, whereas filaments reconstituted with WT Tn did not move in the absence of Ca$^{++}$ (pCa 10, Fig. 3), some intermittent filament motion was detected for the filaments reconstituted with the V43Q TnC mutation (Fig. 3). This represented only a small percentage (~3%) of the total filaments, but it suggests a decreased ability to regulate actomyosin binding even in the absence of Ca$^{++}$.

Regulated thin filaments reconstituted with the truncated form of TnI (R156) responded nearly identically to filaments with WT Tn to increases in [Ca$^{++}$] at pH 7.4, with no difference in any $V_{RTF}$ from 10.0 to 5.0 (Fig. 3). However, at pH 6.8 the filaments with truncation at R156 moved significantly faster than the filaments with WT TnI at subsaturating [Ca$^{++}$] (pCa 7.5, 7.0, and 6.5, see Fig. 3), indicating a pH-dependent response to increases in Ca$^{++}$. The presence of the truncated TnI did not, however, alter the strong depressive effect of acidosis (pH 7.4 vs 6.8) at saturating Ca$^{++}$ (e.g., pCa 5.0), where the depression was similar to that observed for filaments with WT TnI (Fig. 3). Despite the individual differences at specific subsaturating [Ca$^{++}$], the value for the pCa50 was, like filaments with WT TnI, not affected by the decrease in pH from 7.4 to 6.8 (Table 1). We attempted to obtain filament velocities with this construct at pH 6.5, but, like the V43Q TnC mutation, the measureable velocities were so slow that reasonable fits to the Hill equation could not be obtained (data not shown).

**Influence of Pi, and dATP on $V_{RTF}$.** Acidosis had the strongest effect on $V_{RTF}$ at saturating Ca$^{++}$ levels (Fig. 4), suggesting that much of the depression in velocity was due to a direct effect on the actomyosin interaction. To test this idea, we sought to directly perturb actomyosin interaction using agents that alter myosin function. Our first candidate was Pi because it is thought to have little or no direct effect on Tn (48) but induces the detachment of myosin from actin in the strongly bound state (18, 29).

Indeed, at pH 7.4 the addition of 15 mM Pi increased $V_{RTF}$ by 10% at saturating Ca$^{++}$ levels but did not alter $V_{RTF}$ at subsaturating Ca$^{++}$ (Fig. 4). This is quantitatively similar to the 7% increase we observed previously using cardiac Tn (17).
Table 1. Parameter estimates of the Hill fits to velocity-pCa data

<table>
<thead>
<tr>
<th>pH</th>
<th>Tn</th>
<th>pCa50</th>
<th>Hill</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>WT</td>
<td>6.71 ± 0.07</td>
<td>2.02 ± 0.37</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>R156</td>
<td>6.76 ± 0.11</td>
<td>2.76 ± 0.49</td>
<td>0.82</td>
</tr>
<tr>
<td>6.8</td>
<td>WT</td>
<td>6.71 ± 0.07</td>
<td>1.76 ± 0.45</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>R156</td>
<td>6.78 ± 0.01</td>
<td>1.43 ± 0.41</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>V43Q</td>
<td>7.59 ± 0.18*</td>
<td>2.72 ± 1.36</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Values represent means ± SE for filaments reconstituted with wild-type (WT) troponin (Tn). Tn with a V43Q mutation in Ca⁺⁺-binding subunit of Tn (TnC) or Tn with the inhibitory subunit of Tn (TnI) truncated beyond residue 156. *Significantly different from WT.

although in the present study it reached statistical significance. The effect was stronger at pH 6.8, where Pi increased VRTF by 25% at saturating Ca⁺⁺ levels (pCa 5.5 to 4.5) but, again, had no effect on VRTF at subsaturating Ca⁺⁺ levels (Fig. 4). This Pi-induced increase in VRTF was even more pronounced at pH 6.5, where VRTF increased roughly 10-fold (Fig. 4), which is also qualitatively consistent with our previous observations using cardiac Tn (17). In fact, at subsaturating Ca⁺⁺ levels, where filaments exhibited no velocity in the absence of Pi, they began to move in the presence of the Pi. For example, at pCa 5.25, filaments went from displaying zero movement in the absence of Pi, to near maximal velocity in the presence of Pi. This latter result caused the acidosis-induced depression in pCa50 at pH 6.5 to be reversed by almost 2 pCa units (Table 2).

Replacing the ATP with dATP at pH 7.4 did not affect VRTF at low Ca⁺⁺ levels but significantly increased it from pCa 7.0 to 6.5 (Fig. 5), reaching a maximum of threefold at pCa 6.75 and representing a 18% increase at pCa 6.5. dATP also influenced the shape of the velocity-pCa relationship, resulting in a doubling of the steepness of the relationship, evidenced by the change in the Hill coefficient (Table 3). The effects of dATP at saturating [Ca⁺⁺] were qualitatively similar at pH 6.8 and 6.5 but larger in magnitude; in fact at pH 6.5 VRTF changed from zero at low Ca⁺⁺ levels to near maximal values in the presence of dATP. This drastic increase in VRTF caused by dATP attenuated the depressive effect of acidosis on the pCa50 value, increasing it by ~2 pCa units at pH 6.5 (Table 3).

In a separate set of experiments we systematically determined the effects of pH, dATP, and elevated Pi on VRTF at saturating Ca⁺⁺ levels (pCa 5.0). The magnitude of the increases in VRTF induced by Pi, and dATP at saturating Ca⁺⁺ levels were strikingly similar across all pH levels (Fig. 6). This suggested that the underlying mechanisms might be similar; therefore, we examined the combined effect of both of these changes on maximal VRTF in a separate set of experiments. Interestingly, the combined effects were much greater than those of either Pi, or dATP when added alone (Fig. 6). A three-way ANOVA (pH × ATP type × Pi) revealed that the effect of dATP was dependent on both pH and the presence of Pi. Specifically, the effect of dATP was significantly greater at the lower pH levels (6.8 and 6.5) then at pH 7.4. This is most evident when comparing the extremes of pH; for example, changing ATP for dATP increased VRTF by only 18% (ns) at pH 7.4 but almost doubled VRTF at pH 6.5.

The significant interaction between ATP × Pi indicates that the magnitude of the effect of dATP on VRTF depends on the presence of Pi. These findings, therefore, suggest that the combined effects of dATP and Pi may not be solely attributed to an additive effect, implying the existence of a synergistic component to the effects of Pi, and dATP. Thus it is clear that these two agents do not have the same effect on VRTF but rather act independently and possibly synergistically to increase VRTF.

In this ANOVA, the three-way interaction was not significant, indicating that interaction between Pi, and type of ATP was not dependent on the pH. This implies that any synergy between Pi, and dATP was similar in magnitude at each pH. In addition, post hoc tests revealed that the drop in pH caused a significant depression in VRTF under every condition, suggesting that acidosis caused a depression in VRTF even in the presence of Pi, or dATP, either separately or in combination.

DISCUSSION

The accumulation of H⁺ (i.e., acidosis) is thought to play a significant role in the process of skeletal muscle fatigue, in part, by reducing the velocity of contraction. Acidosis is thought to decrease velocity by slowing the kinetics of the cross-bridge cycle and decreasing the sensitivity of the thin filaments to Ca⁺⁺ (17). In the present investigation, we probed 1) the direct effect on the cross-bridge by using Pi, and dATP to alter the kinetics of the actomyosin interaction, and 2) the effect on Ca⁺⁺ sensitivity by introducing Ca⁺⁺-sensitizing mutations into Tn. Both Pi, and dATP increased VRTF at saturating [Ca⁺⁺], and both variants of Tn enhanced VRTF at subsaturating Ca⁺⁺ levels under acidic conditions but to a lesser extent than Pi, and dATP. Exploration of the mechanisms underlying these effects provides potentially novel insights into the molecular basis of the depressive effects of acidosis experienced during muscle fatigue.

Pi, and dATP can reverse the acidosis-induced depression in VRTF. The most pronounced effect of acidosis in the present study was the large depression in VRTF at saturating Ca⁺⁺ levels (Fig. 4). This suggests that much of the depression in velocity was due to a direct effect on the actomyosin interaction. This notion is supported by the observation that elevating Pi increased VRTF at saturating Ca⁺⁺ levels in a pH-dependent manner (Fig. 4). We have previously observed this same phenomenon using cardiac isoforms of Tn at twice the Pi concentration (17) and postulated that it is likely due to Pi rebinding to actomyosin in an ADP-bound state and inducing dissociation from a postpowerstroke state (18). The fact that we observe a similar response at 15 and 30 mM Pi suggests that the effect of Pi on VRTF is saturated below 15 mM.

Table 2. Parameter estimates from Hill fits to velocity-pCa data for the effect of Pi

<table>
<thead>
<tr>
<th>pH</th>
<th>Pi</th>
<th>pCa50</th>
<th>Hill</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>0</td>
<td>6.71 ± 0.07</td>
<td>2.02 ± 0.37</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6.75 ± 0.04</td>
<td>1.83 ± 0.33</td>
<td>0.90</td>
</tr>
<tr>
<td>6.8</td>
<td>0</td>
<td>6.71 ± 0.07</td>
<td>1.76 ± 0.45</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6.34 ± 0.26</td>
<td>1.49 ± 0.46</td>
<td>0.81</td>
</tr>
<tr>
<td>6.5</td>
<td>0</td>
<td>4.55 ± 0.24*</td>
<td>4.57 ± 12.1</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6.24 ± 0.18†</td>
<td>1.32 ± 0.63</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Values represent means ± SE for filaments reconstituted with WT Tn. *Significantly different from pH 7.4 and 0 mM inorganic phosphate (Pi). †Significantly different from 0 mM Pi at pH 6.5.
The pH dependence of this effect, we have postulated, results from acidosis slowing the rate of ADP release from actomyosin, prolonging the AM-ADP state (16, 18). Prolongation of this AM-ADP state means that the cross-bridge spends more time in the AM-ADP state, which is the state vulnerable to Pi, rebinding (74); therefore the Pi-induced increase in $V_{RTF}$ is more pronounced at lower pH levels (see Fig. 1 for cross-bridge model). Thus the present data provide additional support for this hypothesis and demonstrate that the same effect is produced when an actin filament is reconstituted with fast skeletal Tn as with cardiac Tn.

An alternative hypothesis is that the increase in $P_i$ increases the population of weakly bound AM-ADP $P_i$ heads, putatively increasing the activation level of the thin filament. Indeed there is evidence to indicate that this can occur based on X-ray diffraction experiments in single fibers (5) and solution experiments on isolated thin filaments in the presence of weakly bound myosin S1 (42). On the basis of this mechanism, elevated $P_i$ should have increased the attachment rate, and thus $V_{RTF}$, at low $[Ca^{++}]$. However, $P_i$ did not enhance velocity at low $Ca^{++}$ levels at any pH (Fig. 4). Therefore, this effect, if acting in the present study, was not pronounced enough to affect our results. The much stronger effect of $P_i$ was observed at saturating $Ca^{++}$, which is consistent with $P_i$ increasing the detachment rate rather than affecting the attachment rate.

We also probed the direct effect of acidosis on the actomyosin interaction by substituting dATP for ATP in our buffers. This substrate was used because it has previously been shown to increase unregulated and regulated thin-filament velocity at neutral pH, and, unlike $P_i$, it does so without decreasing the force-generating capacity of muscle (55). In fact, dATP can even increase the force-generating capacity of isolated myosin (7). We did not observe an increase in $V_{RTF}$ at pH 7.4, which contrasts with the increases observed previously (55), but this is likely due to the fact that the dATP-induced increases in filament velocity are dependent on the presence of regulatory proteins on the thin filament (7). More importantly, we show for the first time that dATP can also significantly increase $V_{RTF}$ under acidic conditions, recovering roughly 50% of the loss in $V_{RTF}$ caused by decreasing the pH to 6.5 (Figs. 5 and 6). In fact, the enhancement of $V_{RTF}$ was significantly greater at pH 6.5 and 6.8 vs. pH 7.4, indicating that the magnitude of the effect of dATP is dependent on pH. Interestingly, the magnitude and the pH dependence of the increase in $V_{RTF}$ caused by dATP at saturating $[Ca^{++}]$ was quite similar to the effect caused by $P_i$ (Fig. 6). This suggested that $P_i$ and dATP might be acting through a similar mechanism; however, when both were combined, they had a largely additive effect on $V_{RTF}$, suggesting that they act through distinctly different mechanisms, a notion more consistent with previous findings.

Table 3. *Parameter estimates from Hill fits to velocity-pCa data for the effect of dATP*

<table>
<thead>
<tr>
<th>pH</th>
<th>ATP</th>
<th>pCa50</th>
<th>Hill</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>normal</td>
<td>6.71 ± 0.07</td>
<td>2.02 ± 0.37</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>2-deoxy</td>
<td>6.95 ± 0.06</td>
<td>4.31 ± 1.43</td>
<td>0.87</td>
</tr>
<tr>
<td>6.8</td>
<td>normal</td>
<td>6.71 ± 0.07</td>
<td>1.76 ± 0.45</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>2-deoxy</td>
<td>6.52 ± 0.02</td>
<td>4.47 ± 2.01</td>
<td>0.88</td>
</tr>
<tr>
<td>6.5</td>
<td>normal</td>
<td>4.55 ± 0.24</td>
<td>4.57 ± 12.1</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>2-deoxy</td>
<td>6.30 ± 0.03</td>
<td>4.09 ± 2.84</td>
<td>0.77</td>
</tr>
</tbody>
</table>

*Indicates significantly different from pH 6.5 in 2-deoxy ATP (dATP). †Indicates significantly different from pH 7.4 and 6.8 with normal ATP. §Indicates significantly different from pH 7.4 in dATP.
example, Pi is thought to increase $V_{\text{RTF}}$ by preferentially re-binding to actomyosin in an AM-ADP state before the state from which ADP is released (the AM-ADP state) (10, 64, 74). Once rebound to Pi, myosin quickly dissociates from a post-power stoke state, thereby reducing the strongly bound lifetime and thus increasing $V_{\text{RTF}}$ (17, 18). In contrast, dATP is thought to increase both the weak-to-strong binding transition (54, 55). An increased rate of the weak-to-strong binding transition could underlie the strong effect of dATP that persists in the presence of 15 mM Pi (Fig. 6). Assuming that at 15 mM Pi, the effect on the detachment rate is saturated, as is the case for the effect of Pi on force (51), only an increase in the attachment rate (i.e., weak-to-strong binding) could enable $V_{\text{RTF}}$ to be increased in the additive manner observed (Fig. 6). This would suggest that acidosis, not only slows the detachment rate, but also slows the rate of cross-bridge attachment (i.e., the weak-to-strong binding transition). The slowed attachment rate could result from a slowing of any step in the cross-bridge cycle subsequent to ATP-induced dissociation to a step immediately before the formation of the strong binding to actin. This would certainly include the step involving the hydrolysis of ATP, which we have previously hypothesized is slowed by acidosis (16).

The increased in $V_{\text{RTF}}$ could also be due to an increased rate of dADP release from myosin because this is thought to be the step that limits unloaded shortening velocity (47, 59, 75). The increased rate of ADP release may result from the subtle structural alterations to ATP reducing the affinity of myosin for dADP hastening its release from actomyosin and consequently increasing velocity (55). Paradoxically, speeding the release of ADP from the AM-ADP state might be expected to decrease the number of strongly attached cross-bridges and thus force; however, this effect must be compensated for by the increased rate of the transition from weak-to-strong binding to maintain or even increase force (54).

The increased rate of weak-to-strong binding might also explain the distinctly different effect dATP had on the shape of velocity-pCa relationship compared with Pi (Fig. 5). For example, dATP increased the steepness of the curve evidenced by an increase in the Hill coefficient. This effect likely contributed to restoration of the pC_{50} value at pH 6.5 from 4.55 to 6.30 (Table 3). This increase in the weak-to-strong binding transition would increase the number of strongly bound cross-bridges, which in turn would enhance the activation of the thin filament via strong binding (28). If the increased rate of the weak-to-strong transition underlies our observations, it would be consistent with previous findings demonstrating that dATP can speed the overall rate of ATP hydrolysis (55) and increase maximal isometric force in muscle fibers (7).

Regulatory proteins play a minor role in the acidosis-induced depression of $V_{\text{RTF}}$. We found that acidosis decreases $V_{\text{RTF}}$ at both subsaturating and saturating Ca$^{++}$ concentrations in a manner that is qualitatively similar to previous observations using cardiac isoforms of Tn (17, 57, 58, 69). This similarity was somewhat unexpected based on previous findings showing that acidosis can have a more pronounced effect on the Ca$^{++}$ sensitivity of force in cardiac vs. skeletal muscle (2). This may be due the differential factors regulating force and velocity as discussed below and suggests that, unlike force, the structural differences between cardiac and skeletal Tn do not seem to dictate the acidosis sensitivity of $V_{\text{RTF}}$ in vitro.

With the use of WT skeletal Tn, decreasing the pH from 7.4 to 6.8 did not significantly alter the apparent value for pC_{50}, suggesting that the Ca$^{++}$ sensitivity was not altered; however, at certain subsaturating [Ca$^{++}$], the decrease in velocity was significant (Fig. 3). Therefore, the decrease in the apparent value for pC_{50} may not fully reflect that acidosis had a depressive effect on the ability of Ca$^{++}$ to bind to TnC and activate the thin filament. Indeed decreasing the pH to 6.5 elicited a more pronounced rightward shift in the velocity pCa (Fig. 3). This finding suggests that acidosis does indeed decrease the Ca$^{++}$ sensitivity of $V_{\text{RTF}}$ but that it requires the pH to be below 6.8 to elicit a significant change in the apparent pC_{50}.

The present findings are consistent with acidosis primarily slowing the detachment rather than the attachment rate because elevated Pi, which increases the detachment rate (64), reversed much of the acidosis-induced depression in $V_{\text{RTF}}$ (Fig. 4). This is further supported by the observation that filaments reconstituted with the Ca$^{++}$-sensitizing variants of Tn (Fig. 2) and WT Tn filaments responded similarly to decreasing pH and had no effect on the pronounced depression in $V_{\text{RTF}}$ at saturating Ca$^{++}$ levels.

The actomyosin detachment rate may not, however, be the only factor governing filament velocity, particularly at low Ca$^{++}$ levels. Filaments reconstituted with both the V43Q mutation in TnC and the truncated variant of TnI (R156) showed increased $V_{\text{RTF}}$ at subsaturating Ca$^{++}$ levels at low pH compared with filaments with WT Tn (Fig. 3). For example, filaments reconstituted with the V43Q TnC mutation demonstrated an apparent increase in Ca$^{++}$ sensitivity compared with filaments with WT Tn under normal pH and retained this increase at pH 6.8. The increased velocity at subsaturating Ca$^{++}$ suggests that the filament experienced a greater level of activation at any given subsaturating [Ca$^{++}$], which would lead to more cross-bridges attaching to and moving the thin filament. This is consistent with previous work in muscle fibers demonstrating that at low [Ca$^{++}$] (typically pCa 6.0 or below) unloaded shortening velocity can be limited by the rate of cross-bridge attachment (46). Therefore, the increased velocity at subsaturating conditions suggests that acidosis also affects the attachment rate of myosin at low [Ca$^{++}$].

However, it is important to note that acidosis still significantly depressed $V_{\text{RTF}}$ in the presence of this mutation (V43Q). This finding suggests that, although the mutation to TnC sensitizes the filaments to Ca$^{++}$, it does not reverse the effects of acidosis and implies that this amino acid is not involved in the putative mechanism that allows acidosis to affect the ability of Ca$^{++}$ to bind to TnC (23, 50).

In contrast, the small but significant increase in $V_{\text{RTF}}$ at subsaturating [Ca$^{++}$] observed for filaments reconstituted with the truncated TnI (R156) was only observed at low pH (Fig. 3). Indeed at pCa 7.5 $V_{\text{RTF}}$ was significantly greater under acidic conditions vs. normal pH for this construct (Fig. 3). These observations indicate a pH-dependent effect on $V_{\text{RTF}}$ and suggest that the final 26 residues of fast skeletal Tnl contain a pH-sensitive domain. Again, at the cross-bridge level, this increase in $V_{\text{RTF}}$ at subsaturating Ca$^{++}$ could be due to either an increase in the rate of weak-to-strong binding transition of myosin (i.e., cross-bridge formation) or an increased rate of detachment of myosin from the strongly bound state (for model see Fig. 1). If the increase were due to an acceleration of the
rate of detachment of myosin, then we would have expected \( V_{\text{RTF}} \) to also be increased at saturating \( \text{Ca}^{2+} \) levels with this variant of TnI, but this was not evident in the data (Fig. 3). Therefore, the findings suggest that the increase in \( V_{\text{RTF}} \) at subsaturating \( \text{Ca}^{2+} \) is due to an increase in the rate of the weak-to-strong binding transition.

At the molecular level, the removal of the final 26 residues may weaken the contacts of TnI with actin and thus weaken the inhibition of actomyosin binding in the absence of \( \text{Ca}^{2+} \) (65). Further support for this notion comes from the observation that filaments with R156 TnI are not completely stopped in the absence of \( \text{Ca}^{2+} \) (Fig. 3). However, our findings suggest that this would have to occur in a pH-dependent manner.

Thus, although the major effect of acidosis is on the actomyosin interaction, the findings from these mutations suggest that, at subsaturating levels of \( \text{Ca}^{2+} \), there is a small but potentially important role for Tn in the acidosis-induced decrease in \( V_{\text{RTF}} \).

Comparison with the effects on the force-pCa relationship. The rightward shift in the velocity-pCa relation we observed at pH 6.5 is qualitatively similar to the acidosis-induced rightward shift in the force-pCa relationship seen by others (19, 24, 48, 50). However, isometric force (71) and unloaded shortening velocity (67) are thought to be governed by distinctly different steps in the cross-bridge cycle (67, 70); thus it is likely that distinctly different mechanisms underlie the rightward shift in the velocity-pCa relationship observed in the present study.

Maximal isometric force is thought to be limited by the force per cross-bridge and the duty cycle of myosin (i.e., percent of the ATPase cycle spent strongly bound to actin) (72). Although there is some evidence to suggest that acidosis may decrease the force per cross-bridge (43), most evidence suggests that the rightward shift in the force-pCa relationship is predominantly caused by decreased activation of the thin filament (19, 44, 48, 50). By inhibiting activation of the thin filament, force is reduced due to a slowing of the rate at which myosin progresses from a weakly to strongly bound state, leading to a decrease in the number of force-generating cross-bridges (4, 28). In contrast, unloaded shortening velocity is thought to be most strongly governed by the rate of detachment of myosin from actin, rather than the weak-to-strong binding rate (33, 67). Thus it appears that, even though acidosis produces a similar rightward shift in the force and velocity-pCa relationships, the underlying mechanisms of the two effects are likely distinctly different.

It is of course possible that these mechanisms may overlap due to the ability of myosin strong binding to influence thin-filament activation (62). For example, increasing the detachment rate would decrease the number of strongly bound cross-bridges and thus decrease thin-filament activation. This decrease in thin-filament activation would lead to a slowing of the rate of attachment of subsequent cross-bridges (i.e., negative cooperativity), which should be reflected in the Hill coefficient (n). However, in the present data, acidosis did not have a significant effect on n (Table 1), suggesting that a change in the cooperative binding does not play a major role in the acidosis-induced depression of \( V_{\text{RTF}} \).

Conclusions. The most pronounced effect of acidosis occurred at saturating \( \text{Ca}^{2+} \) levels, suggesting that it slows \( V_{\text{RTF}} \) through a direct effect on the actomyosin interaction. Much of this effect can be attenuated by either increasing the detachment rate and/or increasing the rate of the weak-to-strong binding transition with Pi and dATP. To a much lesser extent, the acidosis-induced decrease in \( V_{\text{RTF}} \) may be mediated by an effect on Tn because \( \text{Ca}^{2+} \)-sensitizing variants of Tn attenuated some of the decrease in filament velocity but only at subsaturating \( \text{Ca}^{2+} \) levels.

GRANTS
This work was supported by an AHA Scientist Development Grant (09SDG2100039) to E. Debold.

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

AUTHOR CONTRIBUTIONS
Author contributions: T.J.L., J.P.D., J.L., B.J.B., and E.P.D. conception and design of research; T.J.L., M.A.T., and J.L. performed experiments; T.J.L., M.A.T., and E.P.D. analyzed data; T.J.L., M.A.T., J.P.D., B.J.B., and E.P.D. interpreted results of experiments; T.J.L. and E.P.D. prepared figures; T.J.L. and E.P.D. drafted manuscript; T.J.L., J.P.D., B.J.B., and E.P.D. edited and revised manuscript; T.J.L., M.A.T., J.P.D., J.L., B.J.B., and E.P.D. approved final version of manuscript.

REFERENCES
Acidosis and Regulated Filament Velocity • Longyear TJ et al.


43. Pate E, Cooke R. Addition of phosphate to active muscle fibers probes actomyosin states within the powerstroke. Pflügers Arch 414: 73–81, 1989.


