Phosphate and acidosis act synergistically to depress peak power in rat muscle fibers

Cassandra R. Nelson,1 Edward P. Debold,2 and Robert H. Fitts1

1Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin; and 2Department of Kinesiology, University of Massachusetts-Amherst, Amherst, Massachusetts

Am J Physiol Cell Physiol 307: C939–C950, 2014. First published September 3, 2014; doi:10.1152/ajpcell.00206.2014.—Skeletal muscle fatigue is characterized by the buildup of H+ and inorganic phosphate (Pi), metabolites that are thought to cause fatigue by inhibiting muscle force, velocity, and power. While the individual effects of elevated H+ or Pi have been well characterized, the effects of simultaneously elevating the ions, as occurs during fatigue in vivo, are still poorly understood. To address this, we exposed slow and fast rat skinned muscle fibers to fatigue-inducing levels of H+ (pH 6.2) and Pi (30 mM) and determined the effects on contractile properties. At 30°C, elevated Pi and low pH depressed maximal shortening velocity (Vmax) by 15% (4.23 to 3.58 fl/s) in slow and 31% (6.24 vs. 4.55 fl/s) in fast fibers, values similar to depressions from low pH alone. Maximal isometric force dropped by 36% in slow (148 to 94 kN/m²) and 46% in fast fibers (148 to 80 kN/m²), declines substantially larger than what either ion exerted individually. The strong effect on force combined with the significant effect on velocity caused peak power to decline by over 60% in both fiber types. Force-stiffness ratios significantly decreased with pH 6.2 + 30 mM Pi in both fiber types, suggesting these ions reduced force by decreasing the force per bridge and/or increasing the number of low-force bridges. The data indicate the collective effects of elevating H+ and Pi on maximal isometric force and peak power are stronger than what either ion exerts individually and suggest the ions act synergistically to reduce muscle function during fatigue.

A LOSS OF MUSCULAR FORCE AND POWER characterizes the state of fatigue. Several mechanisms may explain the drop in muscular performance during fatigue including central nervous system factors (27), inhibited sarcoplasmic reticulum (SR) Ca²⁺ release (1), and reduced cross-bridge force production (19). Elevations in metabolites such as hydrogen ions (H⁺) and inorganic phosphate (Pi) have been implicated in muscle fatigue due to their concomitant increase with the decrease in muscular force (46). Studies using NMR technology of exercising humans have demonstrated a strong inverse correlation between the drop in muscular force and the rise in H⁺ and Pi (6, 46). In later stages of fatigue, it has been demonstrated that muscle pH can drop from 7.0 to 6.2 and that Pi can exceed 30 mM (6, 23, 32, 42).

To determine the role of these metabolites in fatigue, the skinned fiber preparation has been widely used because it allows the composition of intracellular fluid to be directly controlled while maintaining the contractile proteins in their native sarcomeric structure. Evidence from permeabilized muscle fiber experiments demonstrates a temperature dependence, such that experiments performed at lower temperatures (5–20°C) show low cell pH or elevated Pi to significantly depress peak force (P0) while low cell pH but not elevated Pi reduces maximal shortening velocity (Vmax) (31, 39). More recently, temperature jump protocols allowing single fiber experiments at near-physiological temperatures (30°C) showed depressive effects of low cell pH or elevated Pi on P0, to be less pronounced (15, 28, 38) than at temperatures <25°C. However, at 30°C, the depressive effects of low cell pH on Vmax remained (15, 28).

Recently, we demonstrated low pH (6.2) plus high Pi (30 mM) to depress P0 at 30°C by 40–50% in type I and II fibers (37), while Karatzaferi et al. (26) reported a 50% decline in P0 in fast rabbit psoas fibers. They also observed a 20–40% drop in Vmax with the effect dependent on the degree of myosin light chain two phosphorylation (MLC2-P). While characterizing the effects of low pH plus high Pi on Vmax and Pmax is important, work capacity is dependent on peak power, which is obtained at intermediate velocities and forces (20). The independent effects of low pH and high Pi at low (<25°C) and near-physiological temperatures (30°C) on peak power are well known, but the effect of these ions acting together has only been studied in fast rabbit psoas fibers (26). Thus one goal of this work was to establish the effects of low pH plus high Pi on Vmax and the force-power relationship in slow as well as fast fibers.

An important property of muscle is the rate of tension development (dP/dt). This is particularly true of phasic contractions where contraction durations (and thus time for force development) are short (20). The dP/dt is thought to be limited by the forward rate constant of the transition from a low-force state (Fig. 1, state B) to the high-force state (Fig. 1, state C) and not SR Ca²⁺ release rate, Ca²⁺ diffusion, or binding to troponin-C (20). The sum of the forward and reverse rate constants (Fig. 1, step 3) determines the rate of force redevelopment (krd) of a fully active fiber following a slack-unslack procedure (3, 4, 19). At saturating levels of Ca²⁺, elevating Pi accelerates krd, and lowering pH does not change krd at 15°C (30, 41). The collective effects of low pH plus high Pi on krd at 15 or 30°C are unknown and were therefore evaluated in this study.

Fiber stiffness during activation is a reflection of the total number of cross bridges (low- and high-force states). A reduced fiber stiffness would suggest fewer bridges, while an increase in low-force bridges but no change in the total number of bridges should leave stiffness unaltered. Metzger and Moss (34) reported a fiber-type-dependent effect of low pH (6.2) on stiffness, in that stiffness was reduced at pH 6.2 in fast but not slow fibers at 15°C, suggesting a decreased number of cross-bridges.

Address for reprint requests and other correspondence: R. H. Fitts Marquette Univ., Dept. of Biological Sciences, 530 N. 15th St., Milwaukee, WI 53233 (e-mail: robert.fitts@marquette.edu).
provide evidence that elevations in H	extsuperscript{+} (15°C) and near-physiological (30°C) temperatures and added in the form of MgCl	extsubscript{2} with a specified free concentration of 1 added as K	extsubscript{2}HPO	extsubscript{4} to yield a total concentration of 30 mM. Mg	extsuperscript{2+} elevated Pi (5, 11). To our knowledge, the collective effects of (pH 7) and pH 6.2 with KOH. Ca	extsuperscript{2+} diameter was determined assuming a cylindrical shape (35).

**MATERIALS AND METHODS**

**Ethical approval.** All experiments and the protocol for animal care and disposal were approved by the Marquette University Institutional Animal Care and Use Committee.

**Solutions.** Compositions for solutions were derived using an iterative computer program using stability constants contained within Fabbri and Fabbri (18), adjusted based on the temperature, pH, and ionic strength of a given solution. Relaxing (pCa 9.0) and maximal activating (pCa 4.5) solutions contained the following (in mM): 20 imidazole, 7 EGTA, 4 Mg	extsubscript{2+}ATP, and 14.5 phosphate creatine. P	extsubscript{i} was added as K	extsubscript{2}HPO	extsubscript{4} to yield a total concentration of 30 mM. Mg	extsuperscript{2+} was added in the form of MgCl	extsubscript{2} with a specified free concentration of 1 mM. Ionic strength was adjusted to 180 mM for all solutions with pH 7 and pH 6.2 + 30 mM P	extsubscript{i} conditions.

Our results quantify the depression in velocity and power elicited by pH 6.2 + 30 mM P	extsubscript{i} in both type I and II fibers at low (15°C) and near-physiological (30°C) temperatures and provide evidence that elevations in H	extsuperscript{+} plus P	extsubscript{i} strongly depress peak fiber power and may decrease the force per cross bridge and/or increase the number of low-force cross bridges in slow and fast fibers.

**Single fiber force-velocity parameters** were determined by maximal activating the fiber and then stepping it to three submaximal shortening velocities (pCa 4.5) stiffness and k	extsubscript{0} tests. Slow and fast fibers were subject to control and fatigue conditions, and slow fibers were stable enough to perform given experiments at both temperatures. Fast fiber experiments were conducted either at 15 or 30°C. At the end of the experiment, a final contraction in maximal calcium was performed. If the fiber’s final peak force was <90% of the initial force, those data of the fiber were eliminated. All fibers were exposed to the control and fatigue conditions in a random order to control for order effects.

V	extsubscript{0} was determined by imposing a series of rapid slack steps (100–400 μm) after the fiber was maximally activated in pCa 4.5 solution, as previously described (17, 28, 45). Fiber V	extsubscript{0} (μm/s) was determined from the slope of the least squares regression line of the plot of slack distance vs. the time required for the redevelopment of force.

Single fiber force-velocity parameters were determined by maximal activating the fiber and then stepping it to three submaximal isotonic loads, using custom-made software (SkinM), as previously described (28, 44). Force (as a percentage of peak) and corresponding shortening velocities were fit to the Hill equation (24) with the use of an iterative nonlinear curve-fitting procedure (Marquardt-Levenberg algorithm). Peak absolute fiber power was calculated with the fitted parameters of the force-velocity curve: P	extsubscript{max}, V	extsubscript{max}, and αP	extsubscript{0}, the parameter that specifies the curvature of the force-velocity relationship (where α is a constant with dimensions of force) (44). The normalized

Fig. 1. Schematic of the cross-bridge cycle. A, actin; M, myosin. *High-force cross bridge. Cross-bridge transitions are labeled with numbers and states labeled with letters in parentheses.
force-velocity and force-power curves were constructed by summat-
ing velocities or power values from 0 to 100% of P0, in increments of
1% (44).

Stiffness measurements were made using sarcomere length control
(SL Control), developed by Dr. Kenneth Campbell (7). Fibers were
vibrated at an amplitude equal to 0.05% of fiber length (mean fiber
length 2.20 mm) and a frequency of 2 kHz in both relaxing and pCa
4.5 solutions. Resting or passive stiffness (measured at pCa 9.0) was
subtracted from stiffness during a maximal contraction (pCa 4.5) so
the data reflect the stiffness due to the cross bridge and not passive
1% (44).

k0 Measurements were made directly following stiffness. During
maximal activation, the fiber was rapidly slackened and reextended, and
k0 was determined by fitting the redevelopment of tension to a single
exponential equation in SL Control. The duration of the slack was 20
ms at 15°C and 10 ms at 30°C. For fast fiber k0 measurements at 15
and 30°C, sarcomere length was laser clamped at 2.5 μm to prevent
sarcomere nonuniformity during tension redevelopment (33). Laser
clamp was not employed with slow type I fibers, as k0 values were not
different with and without the clamp (21).

To estimate the number of low-force bridges, we employed a
modification of the technique of Colombini et al. (9). The technique
measures cross-bridge stiffness (stiffness during activation − passive
stiffness) at P0, at different force values (% of P0), and with the fiber
in rigor. Colombini et al. achieved various levels of P0 by blocking
cross-bridge formation with N-benzyl-p-toluen sulphonamide (BTS),
while we reduced force by varying solution pCa between 7.0 and 4.5.
These experiments were carried out on a separate group of fibers (n =
6 for both slow and fast fibers) at 15°C. Both force and stiffness were
measured at 5- to 6-pCa points for each experimental group. At the
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The rigor solutions were similar to the activating solution described
above except that ATP and creatine phosphate were not added and
sufficient KCl was added to yield a total ionic strength of 180 mM
(34). The normalized data were plotted as force vs. stiffness (see Fig.
9) and best-fit line extrapolated to the y-intercept. The assumption is
that fiber stiffness at zero force (y-intercept) is attributed to low-force
cross bridges (8).

Myosin heavy chain composition and fiber typing. Following the
contractile measurements, fibers were solubilized in 10 μl of 1% SDS
sample buffer and stored at −20°C. The myosin heavy chain profile
was obtained by running samples on 7.5% (wt/vol) Tris·HCl precast
gels (Bio-Rad) and stained with the Silver Stain Plus kit (Bio-Rad).
Fibers were identified as type I, IIa, IIx, or IIb as previously described
(37). Type IIa and IIx fibers differed in their velocity and k0 values at
both 15 and 30°C. However, the depression in velocity, power, or
stiffness from pH 6.2 + 30 mM P1 was not different between the IIa
and IIx fiber types. Thus the data presented in this article for all
parameters except k0 combined IIa and IIx fibers into a single group
(no IIb fibers were included in this study). This also allowed for
comparisons to previously published data (15, 16, 28).

Statistics. Each fiber was treated as an independent observation.
Data were graphed and analyzed with Graph Pad Prism 5 (San Diego,
CA) using a two-way ANOVA followed by post hoc Tukey’s t-tests
with a significance level of 0.05.

RESULTS

This work shows that two important kinetic measurements,
velocity (V0) and the rate constant of tension development (ktr),
following a slack-unslack perturbation, evaluated at both 15
and 30°C, are highly temperature and fiber type dependent
(Fig. 2). ktr significantly increased in a fiber-type-dependent
fashion (I < IIa < IIx) at both temperatures (Table 1 and Fig.
2) and was significantly higher at 30°C compared with 15°C in
all fiber types. At 30°C compared with 15°C, V0 increased by
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(Fig. 2). ktr significantly increased in a fiber-type-dependent
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2) and was significantly higher at 30°C compared with 15°C in
all fiber types. At 30°C compared with 15°C, V0 increased by
4.2-, 3.4-, and 1.9-fold type I, IIa, and IIx fibers, respectively.
As temperature was elevated, fiber $k_{tr}$ increased by 11-, 10-, and 5.5-fold in type I, IIa, and IIX fibers, respectively. However, $k_{tr}$ was unaffected by pH 6.2 + 30 mM $P_i$ at 15 or 30°C in type I or II fibers. A trend but insignificant decrease in $k_{tr}$ ($P = 0.07$) was observed in type I fibers at 30°C (Table 1).

The effects of the low cell pH (6.2) and elevated $P_i$ (30 mM) condition on velocity ($V_o$ and $V_{max}$) in slow and fast fibers are summarized in Table 2 and Fig. 3. $V_o$ is typically higher than $V_{max}$, especially at higher temperatures due to sarcomere non-uniformity that occurs with loaded contractions (44). Temperature significantly increased velocity in type I and II fibers, while pH 6.2 + 30 mM $P_i$ significantly depressed $V_o$ in type I fibers at 15°C, type II fibers at both 15 and 30°C, and $V_{max}$ in both fiber types at both temperatures. Raising the temperature from 15 to 30°C blunted the pH + $P_i$ induced depression in velocity in slow but not fast fibers (Table 2). Composite force-velocity curves (Fig. 3) illustrate that fast fibers exhibit less curvature as evidenced by the significantly higher $a/P_o$ ratio (Table 2; $P = 0.007$ at 15°C and $P = 0.009$ at 30°C). For both fiber types, the $a/P_o$ ratio increased with an increase in temperature. Interestingly, pH 6.2 + 30 mM $P_i$ had no effect on $a/P_o$ in any condition, suggesting the force-velocity relationship was uniformly decreased under these conditions.

Temperature significantly increased peak power by six- to eightfold in type I and II fibers, while pH 6.2 + 30 mM $P_i$ conditions depressed peak power in both fiber types by ~60% at low and high temperatures (Fig. 4). Peak force ($P_o$), $V_{max}$, and peak power values from previous work in our laboratory (15, 28) in which the effects of pH 6.2 and 30 mM $P_i$ were studied individually are compared with the current study in Figs. 5–7. The individual ions significantly depressed force from control at 15°C, with the order $P_i > H^+$, while with both ions together, the inhibition was not different from $P_i$ alone. At 30°C, the high $P_i$ and high $P_i$ plus low pH conditions depressed type I force, while only the latter conditions inhibited type II fiber force (Fig. 5). Low cell pH significantly slowed $V_{max}$ in type I fibers at 30°C and type II fibers at both temperatures, while $P_i$ had no significant effect on velocity (Fig. 6). Except for the type I fibers at 15°C, the pH + $P_i$ induced depression in $V_{max}$ was not different than the depression of $V_{max}$ by pH 6.2 alone (Fig. 6). Individually, both pH 6.2 and 30 mM $P_i$ significantly depressed peak power from control in type I and II fibers at both temperatures (Fig. 7). At 30°C the pH 6.2 + 30 mM $P_i$ condition depressed peak power greater than either ion alone in both fiber types; however, at 15°C, the inhibition was not greater than that observed with higher $P_i$ alone ($P = 0.30$ and $P = 0.13$ in type I and II fibers, respectively; Fig. 7).

Fiber stiffness during activation, a reflection of the number of bound cross bridges (both low- and high-force states) was not different between fiber types or altered by the pH 6.2 + 30 mM $P_i$ condition at either temperature (Fig. 8). However, the force-stiffness ratio was significantly depressed across fiber types and temperatures in pH 6.2 + 30 mM $P_i$ conditions.

### Table 1. Rate of force development is unchanged by pH 6.2 + 30 mM $P_i$

<table>
<thead>
<tr>
<th>$n$</th>
<th>pH 7</th>
<th>pH 6.2 + 30 mM $P_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>11</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>Type IIa</td>
<td>11</td>
<td>6.2 ± 1.0*</td>
</tr>
<tr>
<td>Type IIX</td>
<td>11</td>
<td>23.1 ± 4.4**</td>
</tr>
<tr>
<td>Type II combined</td>
<td>22</td>
<td>16.1 ± 3.2*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$n$</th>
<th>pH 7</th>
<th>pH 6.2 + 30 mM $P_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>10</td>
<td>41.2 ± 5.4</td>
</tr>
<tr>
<td>Type IIa</td>
<td>7</td>
<td>66.9 ± 12.3*</td>
</tr>
<tr>
<td>Type IIX</td>
<td>6</td>
<td>128.4 ± 5.5**</td>
</tr>
<tr>
<td>Type II combined</td>
<td>13</td>
<td>95.3 ± 11.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, number of fibers studied where type I and type II fibers were isolated from 3 and 6 rats, respectively. Rate of force development ($k_{tr}$) is a rate constant with units s$^{-1}$. The $k_{tr}$ values at 30°C were all significantly higher than at 15°C ($P < 0.05$). There were no significant differences between pH 7 and pH 6.2 + 30 mM $P_i$ conditions in any fiber type. *Significantly different from type I fibers, $P < 0.05$. †Significantly different from type IIa fibers, $P < 0.05$.

### Table 2. Effect of pH 6.2 + 30 mM $P_i$ on velocity and force in type I and II fibers

<table>
<thead>
<tr>
<th>$n$</th>
<th>$V_o$, fl/s</th>
<th>$V_{max}$, fl/s</th>
<th>$a/P_o$</th>
<th>$V_{opt}$, fl/s</th>
<th>$P_{opt}$, kN/m$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I fibers, condition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15°C pH 7</td>
<td>14</td>
<td>1.89 ± 0.09</td>
<td>1.53 ± 0.09</td>
<td>0.07 ± 0.01</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>15°C pH 6.2, 30 mM $P_i$</td>
<td>14</td>
<td>1.00 ± 0.18*</td>
<td>1.16 ± 0.09*</td>
<td>0.08 ± 0.03</td>
<td>0.23 ± 0.01*</td>
</tr>
<tr>
<td>%Change</td>
<td>−47</td>
<td>−24</td>
<td>13</td>
<td>−26</td>
<td>−51</td>
</tr>
<tr>
<td>30°C pH 7</td>
<td>12</td>
<td>8.00 ± 0.74</td>
<td>4.23 ± 0.12</td>
<td>0.27 ± 0.06</td>
<td>1.25 ± 0.07</td>
</tr>
<tr>
<td>30°C pH 6.2, 30 mM $P_i$</td>
<td>12</td>
<td>6.11 ± 0.79</td>
<td>3.58 ± 0.17*</td>
<td>0.20 ± 0.09</td>
<td>0.91 ± 0.09*</td>
</tr>
<tr>
<td>%Change</td>
<td>−24</td>
<td>−15</td>
<td>−26</td>
<td>−27</td>
<td>−50</td>
</tr>
</tbody>
</table>

| Type II fibers, condition |
| 15°C pH 7 | 20 | 5.97 ± 0.78 | 3.37 ± 0.30 | 0.21 ± 0.04 | 0.85 ± 0.15 | 27.0 ± 2.4 |
| 15°C pH 6.2, 30 mM $P_i$ | 20 | 3.58 ± 0.50* | 2.31 ± 0.40* | 0.28 ± 0.07 | 0.83 ± 0.19 | 11.8 ± 1.5* |
| %Change | −31 | −40 | −25 | −12 | −56 |
| 30°C pH 7 | 13 | 13.48 ± 1.00 | 6.24 ± 0.56 | 0.56 ± 0.08 | 2.38 ± 0.15 | 43.8 ± 3.5 |
| 30°C pH 6.2, 30 mM $P_i$ | 13 | 8.76 ± 0.90* | 4.55 ± 0.48* | 0.59 ± 0.09 | 1.74 ± 0.15* | 25.4 ± 2.5* |
| %Change | −33 | −31 | 5 | −27 | −39 |

Values are means ± SE; $n$, number of fibers studied where type I and type II fibers were isolated from 4 and 6 rats, respectively. $V_o$, maximal shortening velocity determined from slack test; $V_{max}$, maximal unloaded shortening velocity determined from the Hill plot test; $a/P_o$, unitless parameter describing curvature of the force-velocity relationship; $V_{opt}$ and $P_{opt}$, velocity and force at peak power *Significantly different from pH 7, $P < 0.05$. At both pH 7 and pH 6.2, 30 mM $P_i$, all values at 30°C were significantly higher than 15°C.
suggesting an increase in the number of low-force bridges and/or reduced force of the high-force state. Type II fibers had a higher force-stiffness ratio than type I fibers in control but not pH 6.2 + 30 mM Pi conditions, implying that type II fibers either elicit more force or less stiffness per cross bridge in the nonfatigued state (Fig. 8).

To further evaluate the effects of pH 6.2 + 30 mM Pi on the relative force-per-cross bridge, we employed a technique of Colombini et al. (9), described in MATERIALS AND METHODS, in Fig. 9. The y-intercept of these plots approximates the relative percentage of low-force cross bridges. In type I and II fibers, pH 6.2 + 30 mM Pi conditions resulted in a plot with a higher y-intercept, implying a higher percentage of low-force cross bridges. The difference in the pH 7 (1%) vs. pH 6.2 + 30 mM Pi (7%) intercept was not significantly different in type II fibers but showed a trend toward a higher intercept (P = 0.19).

DISCUSSION

The objective of this study was to determine the combined effects of high H+ and Pi on slow and fast fiber function and to provide a better understanding of how these ions alter the cross-bridge cycle. Additionally, to our knowledge, the results provide the first report of k_tr in the fast fiber subtypes IIa and IIx at 30°C. Fiber k_tr is thought to reflect the sum of the forward and reverse rate constants of the weak to strong binding step (Fig. 1, step 3) (3). Our finding of a 3.7-fold lower k_tr (at 15°C) in the type IIa vs. IIx fiber suggests that the weak to strong binding transition is considerably slower in the IIa fiber and in fact closer to the rate observed in the slow type I fiber (Fig. 2). Interestingly, increasing temperature accelerated k_tr considerably more in the slow type I and fast type IIa fiber than in fast IIx fibers. Apparently, the forward rate constant of the weak to strong binding transition is less temperature sensitive in the fast IIx fiber (12).

Regarding muscle fatigue, it is known to be in part caused by H+ and Pi inhibition of force and power (19). The individual effects of these ions are well known, but the collective effects have been less studied (19, 37). Our results demonstrate that the pH 6.2 + 30 mM Pi condition significantly inhibits peak fiber force, velocity, and power in type I and II fibers at cold
The depressive effects of pH 6.2 or 30 mM Pi on Po are significantly attenuated at higher temperatures (Fig. 5). We have shown that at submaximal Ca\(^{2+}\) concentrations characteristic of fatigue, both pH 6.2 alone and 30 mM Pi alone and pH 6.2 + 30 mM Pi significantly depressed Po at 15 and 30°C (37). Here, we emphasize that although the effects of pH 6.2 or 30 mM Pi on Po at 30°C and saturating Ca\(^{2+}\) (pCa 4.5) are minimal, when the metabolites are elevated simultaneously, a 36 and 46% depression in Po in type I and II fibers, respectively, is apparent. The synergism of the low pH and high Po condition could be in part caused by an increase in the H\(_2\)PO\(_4^-\) from which is >90% of the total Pi at pH 6.2 while only ~60% at pH 7.0. While controversial, there are data supporting the hypothesis that the deprotonated form of Pi is the primary causative agent in muscle fatigue (20).

Low cell pH and elevated Pi have been hypothesized to depress force at the same step of the cross-bridge cycle but by
different mechanisms (Fig. 1, step 3) (19). It is believed that H+ slows the forward rate constant while Pi accelerates the reverse rate constant of this step. Our data on \( k_{tr} \) and stiffness support this hypothesis. We observed no effect of pH 6.2 + 30 mM Pi conditions on \( k_{tr} \) in slow type I or either fast fiber type at 15 or 30°C. It is known that individually, Pi increases \( k_{tr} \) while low cell pH has no effect (30, 41). Pi is thought to increase \( k_{tr} \) by accelerating the reverse rate constant of step 3 (Fig. 1), shifting the distribution of the cross bridges toward the low-force state (Fig. 1, state B) (41). Metzger and Moss (30) showed pH 6.2 alone to have no effect on \( k_{tr} \) at saturating Ca\(^{2+}\) (pCa 4.5) but depress \( k_{tr} \) at submaximal Ca\(^{2+}\). They suggested that low cell pH depressed the forward rate constant of force generation (Fig. 1, step 3) at submaximal but not maximal Ca\(^{2+}\) levels, with the former condition reducing the force of the strongly bound cross bridges (30). Our data show that low pH blunts the stimulatory effect of Pi on \( k_{tr} \), suggesting either an inhibition of the forward rate constant and/or fewer bridges transitioning from the low- to high-force state.

Peak-activated stiffness of slow and fast fibers was unchanged by the pH 6.2 + 30 mM Pi condition and, consistent with the findings of others, was independent of temperature (22). Because Po was depressed by the pH 6.2 + 30 mM Pi condition, the force-stiffness ratio decreased in both fiber types.
at 15 and 30°C. Since stiffness of a contracting fiber is thought to reflect the number of attached cross bridges (34), the reduced ratio could be interpreted as an increase in the number of low-force bridges (Fig. 1, state B) and/or less force per high-force cross bridge (Fig. 1, state C). A possibility exists that a Ca\textsuperscript{2+}/H11001\textsuperscript{2+}-dependent increase in stiffness due to titin contributed to total fiber stiffness and to the reduced force-stiffness ratio in the fatigue condition (29). However, these possibilities seem unlikely given the extremely small stretch amplitudes used to measure stiffness (<1 nm per half sarcomere). Stiffness due to components other than the cross bridge (presumably titin) has been shown to contribute little or no detectable tension with stretch amplitudes \(<10\) nm per half sarcomere, and even with much larger stretches, stiffness due to titin was \(<2\%\) of the total activated fiber stiffness (2).

Although not significant, we observed a trend toward an increased number of low-force bridges in type II fibers (Fig. 9). The force vs. stiffness plot, obtained by activating with various levels of Ca\textsuperscript{2+}, extrapolated to the y-intercept, provides an estimate of the percentage of low-force cross bridges. The

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**Fig. 7.** Power (W/l) in type I (A and C) and II (B and D) fibers at 15 and 30°C. Values are means ± SE, \(n > 12\) fibers per group with the number of rats studied shown in table 2. The relative power unit of W/l is equivalent of kN·m\textsuperscript{2}-fl\textsuperscript{1}·s\textsuperscript{1}. Data for pH 6.2 from Knuth et al. (28) and data for pH 7, 30 mM P\textsubscript{i}, obtained from Debold et al. (15). *Significantly different from pH 7, \(P < 0.05\). #Significantly different from pH 6.2, \(P < 0.05\). +Significantly different from pH 7, 30 mM P\textsubscript{i}, \(P < 0.05\).

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**Fig. 8.** Stiffness (A and B) and the force-stiffness ratio (force/stiffness) (C and D) at pCa 4.5 in type I and II fibers at 15 and 30°C. Values are means ± SE, \(n = 9\) and 12 slow and fast fibers, respectively, at each temperature from a total of 4 rats. *Significantly different from pH 7, \(P < 0.05\). #Significantly different than type I fibers, \(P < 0.05\). All values in D at 30°C are significantly higher than the values in C, \(P < 0.05\).
This is in agreement with Westerblad and Lannergren (43), support the hypothesis that elevating H⁺ alters the force-stiffness plot. Nonetheless, the data in type II fibers indicate that the ratio between stiffness and force is a constant. Colombini et al. (9) developed this technique using BTS to determine the number of bridges.

The curvature of the force-velocity relationship, defined by the ratio of force per number of bridges, has been shown to change in a fiber-type-dependent manner at 30°C by pH 6.2 or 30 mM Pᵢ. Knuth et al. (28) found pH 6.2 to depress a/Pₒ in type I fibers and increase a/Pₒ in type II fibers, while Debold et al. (15) observed 30 mM Pᵢ to decrease a/Pₒ in both fiber types. Collectively, the data suggest that the depression in peak fiber power in pH 6.2, 30 mM Pᵢ conditions is not fiber type or temperature dependent and, at 30°C, was significantly more than the power depression by low pH or high Pᵢ alone. Taken with the observation that peak stiffness was unchanged, this suggests that the effects of pH 6.2, 30 mM Pᵢ are synergistic, supporting the hypothesis H⁺ and Pᵢ inhibit muscle fatigue.

Furthermore, we suggest that the declines in Pₒ observed with fatigue are in part due to the pH 6.2 + 30 mM Pᵢ condition, reducing the force per high-force cross bridge and/or increasing the number of low-force cross bridges. This, combined with the low cell pH prolongation of the time in the AM·ADP state of the cross-bridge cycle (14), thereby depressing velocity, implicates these ions as significant mediators of skeletal muscle fatigue.

APPENDIX

Description of microsystem. The experiments described in this article utilized a novel microsystem which is a modification of a system first described by Karatzafert et al. (24). The system allows rapid exposure of the fiber to different temperatures between 5 and 35°C. Figure A1 shows a top and side view of a machinist sketch of the system. Initially, the system is placed on a setup plate (Fig. A2A) that contains an adjustable well (Fig. A2B). When the well is in the up position, the force and position transducers are both submerged in the well filled with relaxing solution (Fig. A2C). After the fiber ends are attached to the transducers, the well is lowered with the well height adjuster (Fig. A2B) and the Pelletier unit moved to the right with the position adjuster (Fig. A2C) so that the fiber is positioned in the first test position (Fig. A1B). The fiber is suspended between the bottom of the Pelletier post and the glass coverslip in 100 μl of relaxing solution (10°C). In this position, the fiber can be viewed and sarcomere length measured and adjusted as it is directly over the inverted microscope objective (Fig. A1B). The fiber can be rapidly exposed to different temperatures and/or activating solutions by moving the four station Pelletier unit to positions 2, 3, or 4 (Fig. A1, A and B). In position 1, the fiber can be immersed in solution containing fluorescent compounds monitored by epifluorescence, and laser clamp of sarcomere length can be performed. For the latter, a laser beam is diffracted up through the fiber by positioning a mirror in one of the objective ports, and the first order diffraction pattern is measured with a diode paced directly above the top of the slit in the port. This system was used in the experiments described in this article (Table 1) for measuring fast fiber kₒ. Sarcomere length was clamped at 2.5 μm, the fiber was activated (pCa 4.5) and then rapidly slackened and unslacked, and kₒ was measured during redevelopment of force with sarcomeres clamped at 2.5 μm (Fig. A1C). The clamp was removed during the slack-unslack maneuver (Fig. A1C).

Fig. 9. Low-force cross-bridge percentage determined by force vs. stiffness plot. Force and stiffness elicited at a range of free Ca²⁺ concentrations were normalized to rigor force and stiffness in pH 7 and pH 6.2, 30 mM Pᵢ conditions at 15°C in 6 type I (A) and 6 type II (B) fibers from a total of 3 rats. The points from each fiber are means (±SE) fit with a line and extrapolated to the ordinate, crossing a point that has stiffness but no force. Graphs show the complete range of points obtained. Insets: best fit lines, zoomed in where the lines cross the y-axis.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

Fig. A2. A: machinist sketch of setup plate, setup well, and well height adjuster. Measurements shown in inches. B: setup well and well height adjuster in isolation. C: microsystem, setup plate, and setup well in same frame.


