Inhibition of shortening velocity of skinned skeletal muscle fibers in conditions that mimic fatigue

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1Institute of Human Performance and Rehabilitation, Center for Research and Technology Thessaly, and 2Department of Physical Education and Sport Science, University of Thessaly, Trikala, Greece; and 3Department of Biochemistry and Biophysics, Cardiovascular Research Institute, University of California, San Francisco, California

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Karatzaferi C, Franks-Skiba K, Cooke R. Inhibition of shortening velocity of skinned skeletal muscle fibers in conditions that mimic fatigue. Am J Physiol Regul Integr Comp Physiol 294: R948–R955, 2008. First published December 12, 2007; doi:10.1152/ajpregu.00541.2007.—The mechanisms responsible for the inhibition of shortening velocity that occurs during muscle fatigue have not been completely elucidated. Phosphorylation of the myosin regulatory light chain (RLC) occurs during heavy use; however, previous reports on its role in affecting velocity have been equivocal. To further understand the process of fatigue, we varied the levels of myosin RLC phosphorylation (from 10 to >50%) and the concentrations of protons (from pH 7 to 6.2) and phosphate (from 5 to 30 mM), all of which change during fatigue. We measured the mechanics of permeable rabbit psoas fibers at a temperature closer to physiological (30°C), using a temperature jump protocol to briefly activate the fibers at the higher temperature to preserve sarcomere homogeneity. Although lowered pH alone had an effect on velocity, it was the three factors together, i.e., high phosphorylation, low pH, and high phosphate, that acted synergistically to inhibit fiber velocity by ~40%. Our data demonstrate that in conditions that simulate physiological muscle fatigue, myosin phosphorylation does contribute to the inhibition of contraction velocity of fully activated fast muscle fibers.

IN SKELETAL MUSCLE FATIGUE, tension, shortening velocity and the rate of energy use are all inhibited (10, 32). Many studies have explored whether the buildup of the products of ATP hydrolysis [ADP, inorganic phosphate (P_i), and protons] directly affects the actomyosin interaction and may thus inhibit shortening velocity. Although several metabolites do affect fiber mechanics, they do not quantitatively account for the inhibition of shortening velocity observed in living fibers (see e.g., Refs. 10, 21, 32). Other factors to consider are any alterations in the structure and function of myosin per se, and specifically, the role of myosin light chain as a regulator of muscle mechanics. In fast fibers, both myosin heavy chain and myosin light chain composition have been found to be important determinants of unloaded shortening velocity (1), although the exact mechanism by which various isoforms of myosin light chain modulate velocity is not clear.

There is evidence that myosin regulatory light chain (RLC) phosphorylation can alter the interaction of myosin and actin and result in tension potentiation. In myosin, the RLC is phosphorylated by a calcium-activated kinase (MLCK), leading to increased RLC phosphorylation levels during sustained activity or fatigue of skeletal muscles. RLC phosphorylation was found to correlate with twitch tension potentiation after a tetanic contraction or during a train of twitches in vivo (26) and to increase tension in permeable fibers at low levels of calcium activation, providing one possible molecular mechanism for the correlation between myosin phosphorylation and twitch potentiation (25). The role of RLC phosphorylation in increasing twitch tension in vivo was further established in mice lacking MLCK, where twitch potentiation was almost totally eliminated (37).

Phosphorylation of the RLC is minimal in a single short activation but reaches 50% or greater during sustained activity (26, 27). During fatigue, levels of myosin phosphorylation increase to 50–80%, equivalent to the levels of phosphorylation studied presently (31). Twitch potentiation, which is produced by phosphorylation, remains evident even during fatigue (30, 31). By increasing the chances for the formation of strong binding states, RLC phosphorylation should also affect the dissociation rate of myosin from actin and thus should influence the velocity of contraction, especially when other factors affecting velocity are present, i.e., in fatigue. However, skinned fiber studies carried out at 10–15°C found no effect of RLC phosphorylation on the mechanics of fully activated fibers (25, 28). Results obtained in fully activated living fibers have been equivocal, with some investigators suggesting that myosin phosphorylation is associated with inhibition of energy output and velocity and others reaching the conclusion that it has no effect (2, 6, 7). The reason for this may lie within the differences of the various experimental protocols. Because previous work has suggested that changes in phosphate concentration and pH might play a role in the inhibition of muscle fibers, we chose to vary these two metabolites in conjunction with variation in the levels of myosin phosphorylation. The effects of both pH and phosphate concentration on fiber mechanics have been shown to be temperature dependent, so we also performed our experiments at a temperature closer to physiological than customarily used (4, 9, 14, 22, 33). To achieve more physiological temperatures, we measured force and velocities of our samples using a temperature jump protocol, allowing us to conduct measurements at 30°C and to vary experimental solutions in terms of pH and P_i content. We hypothesized that if RLC phosphorylation plays a role in altering fiber mechanics during fatigue, its contribution may be more evident in fatigue conditions and at higher temperatures. In this study, we have shown at 30°C that low pH, increased P_i concentration ([P_i]),

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and increased levels of myosin RLC phosphorylation work synergistically to inhibit the velocity of shortening.

METHODS

Muscle fiber preparation. Rabbit psoas fibers were harvested and chemically skinned in a solution containing the ingredients of the basic rigor buffer, as described below, with 3 mM additional EGTA and 50% glycerol (3). Animal care and euthanasia were performed following protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

"Dephosphorylated" fibers were obtained using the above basic procedure. "Phosphorylated" fibers (i.e., fibers with >50% phosphorylated RLCs) were produced by addition of phosphatase inhibitors (20 mM NaF and 20 mM phosphate) to the basic skinning and storage solutions along with 5 mM ATP. Both phosphate and fluoride inhibit the phosphatase that dephosphorylates the RLC, resulting in high levels of RLC phosphorylation. The initial level of phosphorylation in freshly skinned fibers was ~30–40%, and with the above treatment, it increased to >50% over ~1–2 wk of storage (phosphorylated fibers). On the other hand, from an initial level of ~30%, phosphorylation declined to <10% over a similar period of storage in the basic storage solution (dephosphorylated fibers).

Gel electrophoresis showed that the myosin RLC was the only protein that was phosphorylated using the above procedure (Fig. 1). The level of phosphorylation was assayed using isoelectric focusing gels stained with Pro-Q (Molecular Probes, Eugene, OR) and visualized using a Typhoon 9400 variable mode imager (Amersham Biosciences). The fluorescent stain used has a high affinity for phosphorylated serines/threonines, and it stained the phosphorylated light chain strongly. It also stained the dephosphorylated light chains, but with much less intensity (~25%). Staining of the dephosphorylated light chain did not present a problem, because the two forms of the light chain were separated on the gel by isoelectric focusing. Of particular importance is that no other band on the gel showed a significant change in either position or intensity (Fig. 1).

Muscle mechanics. For mechanical experiments, single fibers were dissected from a bundle of fibers and mounted between a solid-state force transducer and a rapid motor for changing fiber length, as described previously (3). Care was taken to measure the mechanics of phosphorylated and dephosphorylated fibers that were harvested from the same animal and had spent the same time in storage.

Most previous studies of skinned muscle fibers have been carried out at temperatures ≤15°C, because sarcomeres become rapidly disordered at higher temperatures. To alleviate this problem, we and others have employed brief temperature jumps from a cold activating solution, where the fibers are stable, to a warmer solution, where the measurements of interest are performed.

We recently improved our temperature jump protocols with the use of a new apparatus (14). The major improvement is the ease and rapidity with which the fiber can be switched between baths that have different temperatures and solutions. A fiber is initially activated at a low temperature (5°C), where fibers are exceptionally stable. After diffusive equilibration of Ca²⁺ and other relevant species, the fiber is rapidly transferred to an adjacent well containing an identical activating buffer, but at a higher temperature (10 or 30°C for a period of 2–3 s). The time spent between baths is <100 ms. Thermal equilibration across a 75-µm-diameter fiber is <1 ms. Thus tension rises rapidly, to the higher level of the greater temperature, and produces a mechanically stable fiber (Fig. 2). Both the maximal isometric tension and the rate of tension rise are reproducible for up to five activations at the higher temperatures (Fig. 2, A and B). During the period at the higher temperature, the tension is clamped at a selected fraction of the isometric value for 40 ms, during which fiber shortening velocity is measured (Fig. 2C). A series of such velocities obtained from multiple fibers is plotted to define the force-velocity relationships shown in Figs. 3 and 4. Reproducible measurements of tension and velocity were obtained at temperatures approaching physiological for mammals (30°C). In part, fiber stability is produced because the transition from a low-tension to a high-tension state occurs almost simultaneously throughout the fiber. Moreover, uniformity is better maintained because fibers are generating high tensions for only short periods of time, which is more equivalent to their in vivo use. The selected temperature of 30°C was the one at which reproducible measurements could be collected in a range of buffer conditions from pH 6.2, 30 mM P, to pH 7.0, 5 mM P.

Solutions. The basic rigor buffer contained 120 mM potassium acetate (KAc), 5 mM MgCl₂, 1 mM EGTA, and 50 mM MOPS, pH 7.0, or 50 mM MES, pH 6.2 (all from Sigma). A relaxing solution was achieved by addition of 20 mM creatine phosphate, 1 mg/ml creatine kinase, and 4 mM ATP. The ionic strength of the standard relaxing solution was ~0.19 M. Maximal activation (free Pca of 4.3) was achieved by addition of 1.1 mM CaCl₂, based on the calculations by Patton et al. (24) and our own calculations. When phosphate was raised to 30 mM, the KAc concentration was decreased to keep ionic strength constant as previously described (3). In the presence of high levels of P, it was necessary to increase total CaCl₂ concentration to 1.5 mM to achieve full activation. Because the buffers used to maintain pH change their pK for hydrogen ions, the pH was set at the appropriate temperature at which the solutions were to be used.

Data processing and statistical analysis. Fibers were first activated at 5°C, and a series of temperature jumps to 30°C were performed as described previously (11). Only one load clamp was performed during each temperature jump. The velocities of each fiber were measured in clamps to different loads. The fiber was periodically, usually every third clamp, measured at the same load to determine whether performance had deteriorated. Depending on the tension achieved in different conditions, the fibers remained stable for three to six clamps. Data obtained from a number of fibers were plotted together to define the force velocity curves shown in Figs. 3 and 4.

Force-velocity release data were fit to the Hill equation: 

\[ V = V_{\text{max}} \left( \frac{\alpha P_o}{P_o} \right) \left( 1 - \frac{P_o}{P_o + P_{\text{max}}} \right) \]

where \( V \) is the velocity at tension \( P \), \( V_{\text{max}} \) is the maximum contraction,
PHOSPHORYLATION AFFECTS VELOCITY OF CONTRACTION IN FAST FIBERS

In addition, a three-way factorial ANOVA was performed to examine the effects of phosphorylation (\(-P\), P level (5 vs. 30 mM), and pH (6.2 vs. 7.0)) for the velocity data collected at 30°C. A factorial design was employed because we were interested not only in assessing the significance of the effects of individual factors but in particular in determining the significance of the synergistic interactions of the three factors (for details, refer to Supplemental Material for this article, available online at the American Journal of Physiology-Regulatory, Integrative and Comparative Physiology website). The minimum significance level was set at \(P < 0.05\). A commercially available statistical software package was used (Statistica 6.0).

RESULTS

We successfully measured the mechanics of fast psoas fibers at 30°C using an improved apparatus. We measured forces and velocities for two sets of permeable rabbit psoas fibers, one with low (<10%, dephosphorylated fibers) and one with high (>50%, phosphorylated fibers) RLC phosphorylation levels in solutions with different values of pH (7.0 and 6.2) and [Pi] (5 and 30 mM). Under conditions that mimic a nonfatigued muscle (pH 7.0, 5 mM Pi, 30°C, and dephosphorylated RLCs), the maximum velocity at zero load \(V_{\text{max}}\) was 6.6 ± 0.3 lengths/s and tension was 215 kN/m² (Fig. 3A, Table 1). These values are similar to previous observations made using permeable fibers (22) and were taken as control values for comparisons. Under these conditions, phosphorylated fibers had force-velocity relationships similar to control. When [Pi] was raised to 30 mM at pH 7.0, there was again no inhibition of the velocity of phosphorylated compared with dephosphorylated fibers (Fig. 3B, Table 1). The velocities of the phosphorylated fibers were slightly greater than those of dephosphorylated fibers at pH 7.0. When the pH was lowered to 6.2 with 5 mM Pi, the velocity of dephosphorylated fibers was inhibited relative to control, as

Fig. 2. A: tension is shown for a dephosphorylated fiber activated in a series of temperature jumps, pCa 4.3, 5 mM Pi, pH 7.0. The fiber was initially activated at 5°C and then switched to 30°C for 2–4 s, when tension suddenly increased (from ~0.05 to 0.24 N/m²). The fiber was then returned to a cold relaxing solution, where tension fell rapidly. The time scale was reduced 30X between the 2 bars on the axis, and the activation, first at 5°C and then at 30°C, was repeated 5 times. During activation, data collection rate was 1 s⁻¹ at 5°C and 10 s⁻¹ for a period of 2 s, which included the time spent in the 30°C solution. B: the rise in tension upon switching from 5 to 30°C is shown for the first (solid) and the last (dashed) activations presented in A. These 2 traces demonstrate the abruptness and reproducibility of the tension rise. C: fiber displacements during 4 tension clamps are shown. Each clamp, lasting 40 ms, was performed during a brief activation of a dephosphorylated fiber in 5 mM Pi, pH 7.0, at 30°C, with fiber length at 3.74 mm. Displacement was measured at 1-ms intervals (C), and the data between the 10th and 40th points were fitted by a straight line whose slope is the measure of velocity (solid lines). Velocities were 3.02, 3.08, 2.89, and 2.96 lengths/s, and tensions were 30, 29, 32, and 31% of isometric tension for clamps 1–4, respectively.
expected, but phosphorylation of the RLC caused a further inhibition of velocity (Table 1). In an activating solution designed to mimic fatigue (pH 6.2, 30 mM Pi), the velocity of dephosphorylated fibers was inhibited by 18% relative to control and the velocity of phosphorylated fibers was further inhibited to a total of ~40% relative to control, which was a slower velocity than that found for any of the other conditions at 30°C (Fig. 4, Table 1, and Supplemental Material). The main effect of myosin phosphorylation appeared to be on velocity. Myosin phosphorylation did not have an effect on fiber tension. Tension was of course lowered with increasing protons or phosphate (see DISCUSSION and Table 1).

The standard errors associated with the parameters $V_{\text{max}}$ and $a/P_{\text{o}}$ were used to determine whether the parameters found for two different curves were statistically different, as described in METHODS. The velocities were significantly different from the control fresh condition for three different conditions: pH 6.2, 5 mM Pi, phosphorylated ($P < 0.01$); pH 6.2, 30 mM Pi, dephosphorylated ($P < 0.05$); and pH 6.2, 30 mM Pi, phosphorylated ($P < 0.001$); the latter was the most affected (Table 1). The value of $V_{\text{max}}$ was significantly different between dephosphorylated and phosphorylated fibers in only one condition: pH 6.2, 30 mM Pi. There was no statistically significant effect of phosphorylation on $a/P_{\text{o}}$. The only value of $a/P_{\text{o}}$ that was different from other conditions was pH 7, 30 mM Pi, dephosphorylated, which was significantly greater, i.e., less curved, than what was found for fibers at pH 7.0, 5 mM Pi.

To examine the effects of the three independent factors and their interaction (phosphorylation, pH, and Pi) on mean velocity between the various conditions at 30°C, we used factorial ANOVA (see Supplemental Material for details). Apart from the main effects of pH [$F(1,297) = 20.465, P < 0.01$] and phosphorylation [$F(1,297) = 3.9171, P < 0.05$], we also found that not only was the interaction of Pi level and pH (Pi × pH) significant [$F(1,297) = 8.4008, P < 0.01$] but also the interaction of the three factors (Pi × pH × phosphorylation) was significant [$F(1,297) = 7.6237, P < 0.01$]. No other main effects or interactions were significant (for statistical table, refer to Supplemental Material and Supplemental Fig. S2).

The force-velocity curves also define the power produced by the muscle fiber. The power is equal to force × velocity. The fits of the Hill equation to the data were used to compute the power produced as a function of the load on the fiber, shown in Fig. 5. The power was inhibited by 11% in dephosphorylated fibers by increasing phosphate from 5 to 30 mM. This is in agreement with the 15% inhibition seen by previous workers upon a slightly greater change in phosphate, from 0 to 30 mM, indicating that in fast-twitch fibers, added phosphate has only a small effect on power (9). There was no significant inhibition of power in phosphorylated fibers when phosphate was increased. When the pH was lowered to 6.2, the power was not inhibited in dephosphorylated fibers. An inhibition of 18–34%, for the same change in pH, has been previously observed for fast-twitch rat fibers that were presumed to be dephosphorylated (15). We do not have an explanation for this discrepancy; however, a different species was used, our solutions contained 5 mM Pi, and the level of phosphorylation was not determined in the above work. The power was significantly inhibited (55%) when both the pH was decreased and the phosphate concentration was raised (Fig. 5). The effect of phosphorylation was to cause a further depression in the maximum power to 70% inhibition relative to control. Phosphorylation also inhibited power at pH 6.2, 5 mM Pi, but to a lesser extent (22%) than at the higher phosphate level. The inhibition of power by phosphorylation seen at pH 6.2 at either 5 or 30 mM Pi is due to its effect on the velocity.

In summary, there was a progressive decrease in velocity as the pH was first lowered and the phosphate concentration was raised, and this decrease in velocity was amplified by myosin phosphorylation at the low pH conditions. This amplification was seen at the higher temperature and did not occur at 10°C (Fig. 4B, Table 1). The inhibition of velocity resulted in a corresponding decrease in power, which was greatest when pH
was lowered, phosphate was raised, and myosin was phosphorylated.

The fits of the Hill equation to the force-velocity relationships defined a parameter $a/P_o$ that describes the curvature of the relationship. Greater values of $a/P_o$ are associated with straighter, e.g., less concave, force-velocity relationships, which would lead to increased power generated at a given force and thus to an increase in the ability of the muscle to perform work. Although $a/P_o$ tended to be slightly smaller in phosphorylated fibers than in dephosphorylated fibers, the difference was not statistically significant (Table 1).

### DISCUSSION

Our results show that in conditions imitating fatigue in vivo, the phosphorylation of the myosin RLC could influence muscle mechanics by inhibiting the velocity of contraction. In addition to increasing the tension of partially activated fibers at low levels of calcium (25, 26, 28, 29), we presently have shown that RLC phosphorylation inhibits the velocity of fully activated fibers at low pH and high [Pi]. These three conditions all coexist during fatigue of fast skeletal muscle fibers. This inhibition does not occur at 10$^\circ$C, in agreement with previous work (25, 28, 29). The effect of this inhibition also alters the peak power that the muscle can produce (see Fig. 5). Both elevated phosphate and decreased pH inhibit peak power, with significant inhibition of peak power occurring at pH 6.2, 30 mM Pi. This power output is further inhibited by myosin phosphorylation. This inhibition of power by phosphorylation is produced primarily by the inhibition of velocity.

What is the possible mechanism responsible for the inhibition of velocity? Previous studies have indicated that the increase in tension observed in partially activated fibers may be caused by an effect of myosin phosphorylation on the structure of the thick filament (17). Structural studies have revealed that myosin heads in resting muscle fibers are either ordered in a linear array (18), and it is plausible that this effect also could be involved in the inhibition of velocity observed presently. Disruption of the array would produce a greater population of myosin heads available to bind to actin in non-force-generating states. Such binding has been shown previously to inhibit filament velocity in an in vitro assay (8). The hypothesis also would have to involve an effect of myosin phosphorylation on the ability of these heads to exert a drag on filament velocity at lower pH and higher [Pi]. The mechanism of this effect is unknown but could possibly involve interactions between the heads of myosin or between the RLC and the core of the thick filament. This hypothesis remains highly speculative, and an alternate hypothesis is discussed below.

An alternative hypothesis to explain the inhibition of velocity could be that the kinetics of the actomyosin interaction are directly altered by pH, Pi, and myosin RLC phosphorylation. We recently showed that myosin phosphorylation also inhibits filament velocity in fibers that are partially inhibited by the phosphate analog vanadate (11). The $K_m$ for the dependence of the maximum contraction velocity on ATP concentration was also lower for phosphorylated fibers in the presence of vanadate. This observation indicates that the apparent binding of ATP may be greater in phosphorylated fibers. In this respect, the phosphorylated fibers would resemble slow-twitch fibers, which also have a greater apparent affinity for ATP than do fast-twitch fibers (23). These results suggest that the affinity for nucleotides could be altered under some conditions by myosin phosphorylation and that this might also account for the slower velocity. Although the phosphorylation occurs at a site that is distant from the site of ATP binding, there is evidence in smooth muscle myosin that these two sites can communicate. The spectral properties of fluorescent probes placed adjacent to the phosphorylated serine are altered by the binding of nucleotides (19).

Our results do not determine which of the above mechanisms could account for the inhibition of velocity or whether another mechanism entirely is responsible. For example, a decrease in filament spacing has been shown to inhibit velocity (20) and also to mimic the effect of myosin phosphorylation on tension in partially activated fibers (36). The two mechanisms

### Table 1. Experimental conditions and Hill parameters of the force-velocity relationship under different conditions

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Phosphorylation, +/-</th>
<th>pH</th>
<th>[Pi], mM</th>
<th>$P_o$, kN/m²</th>
<th>$a/P_o$, μm</th>
<th>$V_{max}$, length/s</th>
<th>Relative $P_o$, %</th>
<th>Relative $V_{max}$, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>–</td>
<td>7.0</td>
<td>5</td>
<td>215±10</td>
<td>0.36±0.06</td>
<td>6.6±0.3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>7.0</td>
<td>5</td>
<td>192±23</td>
<td>0.30±0.08</td>
<td>6.9±0.5</td>
<td>89</td>
<td>105</td>
</tr>
<tr>
<td>30</td>
<td>–</td>
<td>7.0</td>
<td>30</td>
<td>152±22</td>
<td>0.77±0.10</td>
<td>6.2±0.2</td>
<td>71</td>
<td>94</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>7.0</td>
<td>30</td>
<td>165±12</td>
<td>0.64±0.18</td>
<td>6.8±0.5</td>
<td>77</td>
<td>103</td>
</tr>
<tr>
<td>30</td>
<td>–</td>
<td>6.2</td>
<td>5</td>
<td>210±9</td>
<td>0.51±0.11</td>
<td>5.9±0.5</td>
<td>97</td>
<td>89</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>6.2</td>
<td>5</td>
<td>205±19</td>
<td>0.46±0.14</td>
<td>4.7±0.3</td>
<td>95</td>
<td>72</td>
</tr>
<tr>
<td>30</td>
<td>–</td>
<td>6.2</td>
<td>30</td>
<td>103±9</td>
<td>0.45±0.07</td>
<td>5.4±0.2</td>
<td>48</td>
<td>82</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>6.2</td>
<td>30</td>
<td>106±12</td>
<td>0.38±0.06</td>
<td>3.9±0.1</td>
<td>49</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>6.2</td>
<td>30</td>
<td>41±8</td>
<td>0.12±0.02</td>
<td>1.2±0.05</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>6.2</td>
<td>30</td>
<td>42±9</td>
<td>0.14±0.02</td>
<td>1.1±0.1</td>
<td>20</td>
<td>17</td>
</tr>
</tbody>
</table>

The first 4 columns present the experimental conditions: temperature; phosphorylation of the regulatory light chain (RLC), denoting dephosphorylated fibers with a minus sign and phosphorylated fibers with a plus sign; pH, and inorganic phosphate concentration ([Pi]), respectively. The fifth column shows isometric tension ($P_o$); values are means ± SE (n = 10–20). The sixth column reports the $a/P_o$ parameter, derived from the fit of the Hill equation with standard errors, determined from the fit. The seventh column shows the maximum shortening velocity ($V_{max}$), also derived from the fit to the data, with standard errors. The eighth and ninth columns report the $P_o$ and $V_{max}$ values relative (%) to dephosphorylated fibers at pH 7.0 and 5 mM Pi. The velocities are significantly different from the control fresh condition (first row) for 3 different conditions: pH 6.2, 5 mM Pi, phosphorylated (P < 0.01); pH 6.2, 30 mM Pi, dephosphorylated (P < 0.05); and pH 6.2, 30 mM Pi, phosphorylated (P < 0.001).
discussed above are not mutually exclusive, and both could be 
operating. Although either mechanism could explain why ve-
locity could be slowed, neither gives a satisfactory explanation 
of why this inhibition occurs only synergistically with the other 
conditions. In summary, our current data do not define a unique 
mechanism, and more work is required to establish the exact 
mechanism responsible for the inhibition of velocity.

It is of interest to note that the inhibition of shortening 
velocity by myosin phosphorylation was observed only at 
30°C, and not at 10°C (Fig. 4, Table 1). The lack of an effect 
on velocity at 10°C agrees with earlier work on myosin RLC 
phosphorylation (25, 28, 29). This observation may be related 
to a known structural effect of temperature on the thick 
filament array. In permeable mammalian muscle, studies of 
X-ray diffraction patterns have shown that myosin heads are 
disordered at low temperatures but ordered at higher tempera-
tures (34, 35). Thus, if the effect of myosin phosphorylation 
on fiber velocity is related to a disruption of the structure of the 
thick filament array, this effect would be expected to be more 
evident at high temperatures, as observed presently. Other 
studies have also reported a strong effect of temperature on 
phenomena attributed to RLC phosphorylation. Both postte-
stanic twitch potentiation and the potentiation that occurs during 
staircase have been found to be greater at higher temperatures 
(16). Myosin phosphorylation also has been found to have a 
greater effect on tension of partially active fibers at 23°C than 
at 15°C (27). We conclude, on the basis of our and other 
observations, that the effects of myosin phosphorylation would 
be more pronounced at higher temperatures. Still, the kinetics 
hypothesis cannot be excluded, since any effects that phos-
phorylation might have on kinetics could be masked by the 
pronounced temperature effect on the myosin array.

The present results highlight the complexity of fatigue, since 
we observed that RLC phosphorylation inhibited velocity only 
when other fatigue conditions were present (low pH, high [P_i]). 
Two studies have shown that the potentiation in twitch ten-
sions, attributed to RLC phosphorylation, can be produced by 
low levels of phosphorylation in fatigued muscle (18, 30). 
Although the velocity effects found presently may not be 
directly related to those results, together these and our obser-
vations suggest that the effects of RLC phosphorylation may 
be more pronounced in fatigued muscle fibers compared with 
with fresh fibers. This could be related to a higher population 
of myosin heads in non-force-generating states in fatigued fibers, 
due to changes in metabolites as well as to a lower level of

![Fig. 4. Force-velocity relationships for fibers activated at 30 mM P_i, pH 6.2, 
and 10 or 30°C. Lines and symbol descriptions are as defined in Fig. 3. A: at 
30°C, phosphorylation inhibited velocity by ~25% relative to the correspond-
ing dephosphorylated fibers (pH 6.2, 30 mM P_i). B: at 10°C, phosphorylation 
did not produce a difference in velocity.](http://ajpregu.physiology.org/)

![Fig. 5. Power produced by the fibers under the 8 different conditions explored 
at 30°C, shown as a function of the load on the fiber. The power was calculated 
from the fits to the force-velocity curves and is expressed as watts per square 
meter of fiber for a fiber length of 1 mm. There is little change in the power 
until both phosphate level is raised and pH is lowered. The effect of the 
slower velocity is to depress power for fibers at pH 6.2, with a greater effect 
at higher phosphate concentration. Curves for control fibers are gray; curves for phosphorylated fibers are black. Conditions are indicated by 
curves with solid lines (pH 7.0, 5 mM P_i), long dashes (pH 7.0, 30 mM P_i), 
medium dashes (pH 6.2, 5 mM P_i), and short dashes (pH 6.2, 30 mM P_i).](http://ajpregu.physiology.org/)
activation due to inhibition of calcium release. This could result in increased drag and decreased velocity.

Previous work has shown that there is a strong correlation between shortening velocity and tension economy, i.e., slower fibers have higher tension economies, whereas the inhibition of velocity during fatigue coincides with an increase in tension economy (5, 7). Thus, although we did not measure tension economy, based on the known correlation between velocity and ATPase kinetics, our results may also indicate that myosin phosphorylation could play a role in tension economy, thus contributing to energy conservation during fatigue of fast-twitch muscle fibers.

Previous results on myosin phosphorylation are dependent on fiber type. Much of the previous work with living fibers has been done with mouse extensor digitorum longus, which is composed of type 2a and type 2b fibers. Twitch potentiation was found only in type 2b and not in the more oxidative type 2a or in type 1. The present work was done with rabbit psoas fibers, which are composed almost exclusively of type 2d fibers (12). Although we have no results with other fiber types, it is likely that our results are most pertinent to type 2 fibers, since type 1 fibers do not show an increase in tension economy during fatigue.

In addition, our study points to important methodological considerations for future work with permeable fibers. First, our results highlight the importance of conducting measurements in conditions that simulate more closely the physiological environment. Moreover, we observed (not shown) that it takes some time for permeable fibers to fully dephosphorylate after dissection. Thus, depending on collection method and timing of use after sample collection, uncontrolled partial phosphorylation of myosin light chains might have contributed to past conflicting results in the literature about the role of myosin light chain content on determining velocity of contraction.

Our results also may help reconcile previous contradictory in vivo observations. Crow and Kushmerick (6, 7) showed that the inhibition of shortening velocity occurred at the same time as the increase in myosin phosphorylation, suggesting that RLC phosphorylation inhibited shortening velocity. However, Butler et al. (2) activated muscles with two sequential tetanic activations and found early in the second activation, when RLC phosphorylation was high, that the velocity of shortening, measured at 5% load, was actually faster by 11%, concluding that RLC phosphorylation did not inhibit velocity. Based on our results, it is possible that in the study of Butler et al., early in the second tetanic activation the velocity would not be inhibited by myosin RLC phosphorylation because changes in the concentrations of intracellular metabolites had not occurred.

**Perspectives and Significance**

We conclude that myosin RLC phosphorylation can contribute significantly to the inhibition of velocity of fully activated fibers at low pH and high [P_i]. This helps explain the inhibition of velocity observed during fatigue and also may be involved in the increased tension economy, which contributes significantly to the adaptation of a muscle to decreased energy supply. The effect of phosphorylation is only evident at higher temperatures, highlighting the importance of studying muscle fibers under conditions close to physiological. The underlying mechanism of velocity inhibition by phosphorylation could involve an increase in the drag force exerted by non-force-generating myosin heads bound weakly to actin, or it could involve an alteration in the binding and release of nucleotides. Our data do not differentiate between these two alternatives, and more work is needed to establish the exact mechanism responsible for the inhibition of velocity.

**REFERENCES**


