Effect of $P_i$ on unloaded shortening velocity of slow and fast mammalian muscle fibers

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Widrick, Jeffrey J. Effect of $P_i$ on unloaded shortening velocity of slow and fast mammalian muscle fibers. *Am J Physiol Cell Physiol* 282: C647–C653, 2002. First published November 21, 2001; 10.1152/ajpcell.00186.2001.—Chemically skinned muscle fibers, prepared from the rat medial gastrocnemius and soleus, were subjected to four sequential slack tests in Ca$^{2+}$-activating solutions containing 0, 15, 30, and 0 mM added $P_i$. $P_i$ (15 and 30 mM) had no effect on the unloaded shortening velocity ($V_o$) of fibers expressing type IIb myosin heavy chain (MHC). For fibers expressing type I MHC, 15 mM $P_i$ did not alter $V_o$, whereas 30 mM $P_i$ reduced $V_o$ to 81 ± 1% of the original 0 mM $P_i$ value. This effect was readily reversible when $P_i$ was lowered back to 0 mM. These results are not compatible with current cross-bridge models, developed exclusively from data obtained from fast fibers, in which $V_o$ is independent of $P_i$. The response of the type I fibers at 30 mM $P_i$ is most likely the result of increased internal drag opposing fiber shortening resulting from fiber type-specific effects of $P_i$ on cross bridges, the thin filament, or the rate-limiting step of the cross-bridge cycle.

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Muscle contraction occurs by the cyclic attachment and detachment of the thick filament protein myosin to the thin filament protein actin. This cross-bridge cycle is driven by the free energy of ATP hydrolysis, with myosin subfragment-1 serving as an ATPase when detached from actin and as a molecular motor when it reattaches to the thin filament (39). The force-generating step of the cycle occurs when the prepower stroke cross bridge, with the hydrolysis products ADP and $P_i$, still bound, isomerizes from a weakly bound, non- or low-force-generating conformation to a strongly bound, force-generating state (8). The release of $P_i$ from the cross bridge either triggers this isomerization (37) or occurs immediately after the cross bridge generates force (12, 25).

Intracellular $P_i$ can rise from a value of 1–6 mM at rest to >30 mM during exhaustive contractile activity (2, 6, 14, 29, 44). Elevated intracellular $P_i$ impairs the low- to high-force cross-bridge transition, resulting in a greater proportion of cross bridges in preforce-generating states (9). Consequently, peak Ca$^{2+}$-activated force ($P_o$) of slow and fast skinned muscle fibers declines exponentially as the intracellular $P_i$ concentration rises, with 30 mM $P_i$ sufficient to reduce $P_o$ by ~50% (1, 33, 35, 36, 38).

Although elevated intracellular $P_i$ causes a redistribution of cross-bridge states within the cross-bridge cycle, it is equivocal as to whether $P_i$ also alters the rates of transition between cross-bridge states. A number of investigators have shown that raising intracellular $P_i$ to 30 mM has no effect on the unloaded shortening velocity ($V_o$) of fast muscle fibers (7–9, 36), suggesting that at least in this fiber type, $P_i$ does not affect the overall cross-bridge cycling rate. The situation for slow fibers is less clear. A direct test of the effect of $P_i$ on the $V_o$ of slow muscle fibers appears to be limited to a single investigation that found moderate elevations in $P_i$ (10–20 mM) to have no affect on the $V_o$ of slow fibers obtained from the Antarctic fish *Notothenia neglecta* (1). More recently, Potma et al. (38) reported that, as $P_i$ rose to 30 mM, force declined by similar rates in slow and fast fibers even though ATPase activity declined more in the slow fibers. The authors reasoned that either cross bridges in the slow fibers produced two times the force as cross bridges in the fast fibers, which they considered unreasonable, or elevated $P_i$ reduced the cross-bridge detachment rate in the slow fibers. If this interpretation is correct, then high levels of intracellular $P_i$ would be expected to slow $V_o$ in this fiber type since $V_o$ is believed to be limited by the rate of cross-bridge detachment (26).

In the present study, we have reevaluated the effect of $P_i$ on the $V_o$ of slow vs. fast muscle fibers. Our research design differs from previous studies in that we have 1) studied low, moderate, and high physiological concentrations of intracellular $P_i$; 2) used fibers obtained from mammalian skeletal muscles; and 3) classified our fibers as slow or fast based on their myosin heavy chain (MHC) isoform content.

**Methods**

Adult Sprague-Dawley rats (Simonsen Laboratories) were anesthetized with pentobarbital sodium, and the soleus and gastrocnemius muscles were excised and placed in chilled relaxing solution (4°C). The animals were then killed by pneumothorax. Small bundles of muscle fibers were dissected and placed in chilled relaxing solution (4°C) for 1–2 h prior to the beginning of the experiment. Microscopic analysis revealed that the bundles contained both slow and fast fibers. Shank tests and slack tests were performed as previously described (37). A repetition of each slack test was completed in order to ensure that the force-generating property of the fiber remained consistent from test to test.

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from the soleus and the superficial medial portion of the gastrocnemius. The fiber bundles were stored in a skinning solution (50% relaxing solution, 50% glycerol) maintained at 4°C for 24 h before being transferred to fresh skinning solution and stored at −20°C for up to 2 mo.

All relaxing and activating solutions contained 7.0 mM EGTA, 20.0 mM imidazole, 1 mM free Mg^{2+}, 4 mM MgATP, 14.5 mM creatine phosphate, and 15 U/ml creatine kinase. A 100 mM CaCl₂ standard solution (Calcium Molarity Standard, Corning, Corning, NY) was used to adjust the free Ca^{2+} concentration of the relaxing and activating solutions to pCa 9.0 and 4.5, respectively (where pCa = −log Ca^{2+} concentration). The following two activating stock solutions were made: one containing no added Pi and one containing sufficient K₂HPO₄ to raise the added Pi concentration to 30 mM. We have estimated that the actual Pi concentration is elevated during the subsequent period of unloaded shortening. Each fiber was subjected to an initial slack test in the 0 mM Pi solution, a second and a third slack test in the 15 and 30 mM Pi solutions, respectively, and a final control test with the 0 mM Pi solution.

In preliminary experiments, we obtained more reproducible results when solutions were stirred during fiber activation. Presumably, stirring prevents the accumulation of metabolites within the fiber that may alter contractile function. A jet of compressed air, directed through a 10-μl disposable pipette tip and obliquely across the solution surface, provided gentle agitation and stirring of the solution (28). To minimize evaporation, only the activating solutions were stirred, and then only during the few seconds required for fiber activation.

After the final slack test, the portion of the fiber suspended between the stainless steel troughs was removed and solubilized in an SDS sample buffer [containing 62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromphenol blue]. The sample was stored at −80°C. A Bio-Rad mini-Protein 3 electrophoresis system (Bio-Rad Laboratories, Hercules, CA) was used to determine the MHC isoform composition of each fiber. Fiber samples were denatured for 4 min at 95°C, and a portion of the solute was electrophoresed on a polyacrylamide gel consisting of a 7% separating gel with a 3.5% stacking gel (21). Electrophoresis was carried out at 70 V for 24 h (4°C). Samples of soleus and medial gastrocnemius muscles, prepared for electrophoresis as described by Bortolotto et al. (3), were used as MHC standards. The protein concentration of the standards was determined using the Bradford (5) protein assay. The gels were stained using the silver staining procedure described by Shevchenko et al. (41) modified in that the silver nitrate incubation was carried out at room temperature instead of 4°C.

Results are presented as means ± SE. Data were analyzed with a repeated-measures ANOVA (repeated effect of Pi concentration, group effect of MHC isoform composition). Significant differences between means were evaluated with Tukey’s post hoc test. Statistical significance was accepted at P < 0.05.
RESULTS

We isolated single muscle fiber segments from the soleus and the superficial medial gastrocnemius muscles to obtain slow and fast fibers for study. Although the vast majority of fibers from the soleus and medial gastrocnemius express the type I and type IIb MHC isoforms, respectively, the former muscle contains a small population of fibers (~15%) expressing the type IIa or IIx MHC isoform and the latter a small population (~8%) of fibers expressing the type IIx isoform (15). Therefore, each fiber segment that meets the criteria for a successful experiment (described below) was subsequently electrophoresed on a 7% SDS-PAGE to unequivocally classify the cell MHC isoform content.

Figure 1 is a representative gel showing the identification of the MHC isoforms present in soleus and medial gastrocnemius muscle homogenates and in five of the single muscle fiber segments making up this study. Low levels of type IIa and IIx expression can be observed in the soleus (Fig. 1, lane 1) and medial gastrocnemius (Fig. 1, lane 2) homogenates, respectively. The three soleus fibers in Fig. 1 (lanes 3, 5, and 7) expressed solely the type I MHC isoform, whereas the two medial gastrocnemius fibers (lanes 4 and 6) expressed only the type IIb isoform. All of the soleus fibers reported in this study expressed the type I MHC isoform exclusively, whereas all of the medial gastrocnemius fibers expressed only the type IIb isoform.

The P_o and V_o of each fiber were determined in four separate slack tests conducted sequentially in activating solutions containing 0, 15, 30, and 0 mM P_i. Fibers were excluded from analysis if their P_o and V_o on the final slack test (0 mM P_i) deviated from the values obtained on the initial slack test (0 mM P_i) by >10 and 5%, respectively. These criteria assured that any change in fiber function observed on the second and third slack tests could be attributed to the addition of P_i to the activating solutions rather than to rundown or deterioration of the preparation.

Figure 2 shows representative force redevelopment records and slack test plots for type IIb and type I fibers. For clarity and brevity, only the force records for the longest slack step performed in the initial 0 mM P_i and in the 30 mM P_i solution are shown. Figure 2A shows that, compared with the 0 mM P_i condition, 30 mM P_i substantially reduced the P_o of the type IIb fiber but did not alter the time required for tension redevelopment. Consequently, the V_o of this fiber was similar at 0 and 30 mM P_i (Fig. 2B). Similar records for a type I fiber are presented in Fig. 2, C and D. Like the type IIb fiber, 30 mM P_i reduced P_o (Fig. 2C), but, unlike the fast fiber, 30 mM P_i slowed tension redevelopment (Fig. 2C) and reduced fiber V_o (Fig. 2D).

At 0 mM P_i, fibers expressing type IIb MHC produced 10% greater P_o per fiber CSA than the slow, type I fibers (Table 1). Addition of P_i to the Ca^{2+}-activating solution reduced the P_o of both groups of fibers, and removal of P_i on the fourth slack test resulted in the restoration of P_o to the initial value. Table 1 shows that 15 mM P_i depressed P_o to 58 ± 1% of the 0 mM P_i value for the type I fibers but only to 66 ± 2% of the 0 mM P_i value for the type IIb fibers. A further increase in the intracellular P_i to 30 mM had little additional effect on P_o of the type I fibers but further reduced the P_o of the type IIb fibers. Thus the relative effect of 30 mM P_i on P_o was similar in type I (56 ± 1% of the 0 mM value) and IIb (58 ± 3% of the 0 mM value) fibers.

DISCUSSION

The purpose of this study was to investigate the effect of intracellular P_i on the V_o of rat skeletal muscle fibers expressing either the slow, type I MHC isoform or the fast, type IIb isoform. We chose these particular isoforms since fibers expressing type I or IIb MHC represent the extremes in V_o for this species (4, 40). The primary finding of this study is that a high concentration of P_i had differential effects on the V_o of slow vs. fast skeletal muscle fibers. At 30 mM P_i, V_o was reduced by almost 20% in those fibers expressing the type I MHC isoform but was unchanged in fibers expressing type IIb MHC. The depressive effect of P_i on the V_o of slow fibers was not observed at 15 mM P_i. Thus the effects of P_i on V_o were evident only in slow fibers, and then only when the P_i concentration was increased to a relatively high level.

Our results for the type IIb fibers are in agreement with previous work conducted on this fiber type (7–9, 36). These studies have consistently shown that, at MgATP concentrations of ~4 mM, V_o is unaffected by intracellular P_i concentrations of up to 30 mM. We are aware of only one previous study that examined the effect of P_i on the V_o of slow muscle fibers. In that study, Altringham and Johnston (1) reported that 10 and 20 mM P_i did not alter the V_o of slow muscle fibers.
from the Antarctic fish N. neglecta in experiments conducted at 0°C and pH 7.4. The present results are not necessarily at odds with this previous work, since we observed that an intermediate Pi concentration of 15 mM had no effect on the \( V_0 \) of rat type I muscle fibers. Only when Pi was increased to 30 mM did we observe a depression in slow fiber \( V_0 \). This Pi concentration is 50% greater than the highest concentration examined by Altringham and Johnston (1). Whether differences between the results of Altringham and Johnston and the present study represent a species effect or a general effect of high concentrations of Pi on

### Table 1. Effect of Pi on peak Ca\(^{2+}\)-activated force and unloaded shortening velocity of single skinned muscle fibers expressing type I or type IIb MHC

<table>
<thead>
<tr>
<th>Slack test number:</th>
<th>1 0 mM</th>
<th>2 15 mM</th>
<th>3 30 mM</th>
<th>4 0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_o ), kN/m(^2)</td>
<td></td>
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<td></td>
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<tr>
<td>Type I MHC</td>
<td>148 ± 5 (114–181)</td>
<td>86 ± 3(^a) (66–110)</td>
<td>83 ± 4(^b) (65–110)</td>
<td>147 ± 5 (116–178)</td>
</tr>
<tr>
<td>Type IIb MHC</td>
<td>163 ± 8 (150–203)</td>
<td>108 ± 6(^b) (93–132)</td>
<td>94 ± 7(^b) (75–121)</td>
<td>158 ± 10 (136–206)</td>
</tr>
<tr>
<td>( V_o ), fiber length/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I MHC</td>
<td>1.59 ± 0.07 (1.30–1.98)</td>
<td>1.58 ± 0.08 (1.29–1.98)</td>
<td>1.29 ± 0.05(^a) (1.04–1.68)</td>
<td>1.58 ± 0.07 (1.25–1.94)</td>
</tr>
<tr>
<td>Type IIb MHC</td>
<td>6.84 ± 0.32 (5.57–7.70)</td>
<td>6.89 ± 0.33 (5.69–7.96)</td>
<td>6.88 ± 0.32 (5.76–7.70)</td>
<td>6.91 ± 0.34 (5.81–8.05)</td>
</tr>
</tbody>
</table>

Values are means ± SE for 12 fibers expressing type I myosin heavy chain (MHC) and for 6 fibers expressing type IIb MHC. Range is indicated in parentheses. \( P_o \), peak calcium-activated force; \( V_o \), unloaded shortening velocity. \(^a\) Significant difference (\( P < 0.05 \)) from slack test no. 1 within a given group of fibers.
skeletal muscle function is unknown. However, our data demonstrate that the \( V_o \) of mammalian slow muscle fibers is reversibly depressed by 30 mM Pi.

At the conclusion of the power stroke, the release of ADP and the subsequent binding of MgATP weakens the association between actin and myosin and promotes dissociation of the cross bridge (30). Thus elevations in intracellular ADP or reductions in the intracellular MgATP concentration impair cross-bridge transitions in intracellular ADP or reductions in the intracellular MgATP concentration impair cross-bridge dissociation and slow \( V_o \) (10, 22). Care was taken to ensure that the changes in fiber contractility observed in the present study were not the result of changes in nucleotide concentrations. This was thought to be particularly relevant in the present study since, at low concentrations of MgATP, high intracellular Pi has been shown to reduce the \( V_o \) of fast muscle fibers (36). We added creatine kinase to our experimental solutions to supplement the endogenous enzyme present in skinned fibers and ensure adequate buffering of ATP. The activating solutions were also gently stirred to disrupt any surface boundary layers and minimize metabolite buildup within the interior of the fiber (28). We assume that MgATP and ADP were adequately buffered in the fast fibers since, in agreement with the literature, their \( V_o \) was unaffected by the 30 mM Pi treatment (7–9, 36). We would argue that MgATP was adequately buffered in the slow fibers, too, since the rate of ATP hydrolysis in rat slow muscle fibers is only one-eighth to one-third that observed in rat fast, type IIb fibers (38, 40). Thus we believe that it is unlikely that the depressive effect of 30 mM Pi on the \( V_o \) of the slow fibers is caused by inadequate buffering of intracellular MgATP.

At physiological MgATP, the slowest step in the cross-bridge cycle is thought to be the isomerization of the cross bridge from the weakly bound to strongly bound state (13) or the dissociation of the postpower stroke cross bridge (42). A slowing of either of these steps would increase internal forces opposing fiber shortening and reduce \( V_o \). Although the mechanism responsible for the differential effect of 30 mM Pi on slow vs. fast fibers is not apparent from the present data, several possibilities appear most likely. A negative force opposing shortening could arise from weakly bound preforce-producing cross bridges, cross bridges that have completed their power stroke, or cross bridges formed during partial thin filament activation.

The number of cross bridges in weakly bound, preforce-producing states is increased in the presence of Pi (32, 36), and it is now thought that these cross bridges experience negative forces as fibers contract (11, 27, 43). In fast muscle fibers, these weakly bound cross bridges have been estimated to detach when strain reaches ~3 nm (27). In these fibers, detachment is presumably unaffected by Pi, since \( V_o \) is independent of the intracellular Pi concentration (present data and Refs. 7–9 and 36). Our findings are consistent with a model in which the dissociation of weakly bound cross bridges is impaired by high levels of Pi in fibers expressing slow, but not fast, MHC. This implies that, at 30 mM Pi, preforce-producing cross bridges in slow muscle fibers would experience greater strain before detachment than similar cross bridges in fast fibers. This strain would oppose fiber shortening and slow \( V_o \).

As an alternative, Pi may modulate the transition or isomerization of the weakly to strongly bound cross-bridge state. The rate of this transition appears to vary greatly between slow and fast fibers, being 30–40 times slower in the former (33). Consequently, this transition is one of the slowest steps of the entire cross-bridge cycle of fibers expressing slow MHC (33). Our finding of a depressive effect of 30 mM Pi on slow fiber \( V_o \) could be explained if elevated Pi slowed this transition to the point where it became the new rate-limiting step of the cross-bridge cycle. As this transition is slowed, there would be an accumulation of weakly bound cross bridges that, as discussed above, would work to slow fiber shortening. Note that this mechanism does not require Pi to differentially affect slow vs. fast fibers (although that possibility is not excluded). Consider a situation where Pi slowed the transition from weakly to strongly bound cross bridges to a similar extent in slow and fast fibers. This would reduce \( V_o \) in the slow fibers but would have little effect on \( V_o \) of the fast fibers since, in the latter, this transition is initially far from rate limiting (33).

At the conclusion of the power stroke, the release of ADP and the subsequent binding of MgATP weakens the association between actin and myosin and promotes dissociation of the cross bridge (30). Pate and Cooke (36) have concluded that Pi competes with MgATP at the myosin nucleotide binding site. Thus, as the MgATP concentration is lowered and Pi rises, more myosin heads exist with Pi bound to the active site. These cross bridges are slow to detach, increase negative strain, and lower \( V_o \) (36). As argued above, it seems unlikely that the depressive effect of 30 mM Pi on the \( V_o \) of the slow fibers was caused by inadequate buffering of intracellular MgATP. However, one would predict a slowing of \( V_o \) if the affinity of the nucleotide-binding site for Pi was greater in slow vs. fast fibers. If this is the case, then impaired cross-bridge detachment in the slow fibers would increase negative strain and slow \( V_o \), even at the 4 mM MgATP concentration used in the present investigation.

Under conditions of partial thin filament activation, unloading shortening slows at slack steps greater than ~75 nm/half-sarcomere (31, 34). Thin filament activation can be reduced by raising Pi, an effect that is more pronounced in slow vs. fast fibers (23, 24, 32). This raises the possibility that the effect of Pi on thin filament activation is responsible for the reduced \( V_o \) of the slow fibers. The present study was not designed to rigorously investigate biphasic shortening. However, Metzger (31) studied fast fibers under conditions of partial thin filament activation and found that raising Pi actually eliminated the low-velocity phase of unloaded shortening. This argues against a role of Pi in slowing unloaded shortening, but whether similar results would be observed for slow fibers is not clear.

To summarize, we have demonstrated that, at physiological levels of MgATP, 30 mM Pi reversibly de-
presses $V_o$ in slow, but not in fast, mammalian skeletal muscle fibers. In contrast, most cross-bridge models predict that $V_o$ is independent of $P_i$ concentration. Although our results on fast fibers are in agreement with these models, our slow fiber data are not, a discrepancy most likely because previous cross-bridge models have been developed almost exclusively on data from fast muscle fibers. The present data are in agreement with recent work reporting functionally important differences in the cross-bridge cycle of slow vs. fast muscle fibers in the presence of elevated intracellular $P_i$ (33, 38).

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