The depressive effect of $P_i$ on the force-pCa relationship in skinned single muscle fibers is temperature dependent

E. P. Debold, J. Romatowski, and R. H. Fitts

Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin

Submitted 10 July 2005; accepted in final form 3 November 2005

Debold, E. P., J. Romatowski, and R. H. Fitts. The depressive effect of $P_i$ on the force-pCa relationship in skinned single muscle fibers is temperature dependent. Am J Physiol Cell Physiol 290: C1041–C1050, 2006. First published November 9, 2005; doi:10.1152/ajpcell.00342.2005.—Increases in $P_i$, combined with decreases in myoplasmic $Ca^{2+}$ are believed to cause a significant portion of the decrease in muscular force during fatigue. To investigate this further, we determined the effect of 30 mM $P_i$ on the force-Ca$^{2+}$ relationship of chemically skinned single muscle fibers at near-physiological temperature (30°C). Fibers isolated from rat soleus (slow) and gastrocnemius (fast) muscle were subjected to a series of solutions with an increasing free Ca$^{2+}$ concentration in the presence and absence of 30 mM $P_i$ at both low (15°C) and high (30°C) temperature. In slow fibers, 30 mM $P_i$ significantly increased the Ca$^{2+}$ required to elicit measurable force, referred to as the activation threshold at both low and high temperatures; however, the effect was twofold greater at the higher temperature. In fast fibers, the activation threshold was unaffected by elevating $P_i$ at 15°C but was significantly increased at 30°C. At both low and high temperatures, 30 mM $P_i$ increased the Ca$^{2+}$ required to elicit half-maximal force (pCa$^{50}$) in both slow and fast fibers, with the effect of $P_i$ twofold greater at the higher temperature. These data suggest that during fatigue, reductions in the myoplasmic Ca$^{2+}$ and increases in $P_i$ act synergistically to reduce muscular force. Consequently, the combined changes in these ions likely account for a greater portion of fatigue than previously predicted based on studies at lower temperatures or high temperatures at saturating Ca$^{2+}$ levels.

In addition to changes in $P_i$, myoplasmic free Ca$^{2+}$ concentration declines during fatigue, resulting from a decrease in the amount of Ca$^{2+}$ released from the sarcoplasmic reticulum (SR) (54), an effect that also may be mediated in part by $P_i$. It has recently been shown (2) that elevated levels of $P_i$ precipitate SR Ca$^{2+}$ and reduce the amount of Ca$^{2+}$ released by the SR. Therefore, to fully characterize the role of $P_i$ in fatigue, the effects of elevated $P_i$ must be examined at low Ca$^{2+}$ levels.

Both increases in $P_i$ and decreases in Ca$^{2+}$ can directly reduce muscle force, but when both ions change simultaneously, they are thought to act synergistically to cause even greater decreases in force. Several investigators (21, 31, 35, 39, 40) have demonstrated that, in addition to suppressing maximal Ca$^{2+}$-activated force, high $P_i$ depresses Ca$^{2+}$ sensitivity in muscle fibers. Martyn and Gordon (31) showed that adding 30 mM $P_i$ to skinned rabbit psoas fibers increased the Ca$^{2+}$ required to elicit half-maximal force (pCa$^{50}$). This finding has important implications for muscular fatigue because of the nature of the force-pCa relationship. This relationship is sigmoidal, with small changes in Ca$^{2+}$ resulting in large changes in force along the steep portion of the relationship (18). Therefore, in the later stages of fatigue, decreases in SR Ca$^{2+}$ release and $P_i$-induced depression of Ca$^{2+}$ sensitivity could combine to cause a precipitous drop in force. However, the single-fiber studies demonstrating a $P_i$-induced depressed Ca$^{2+}$ sensitivity were typically performed far below mammalian physiological temperatures, making it difficult to interpret the precise effects of elevated $P_i$ on the pCa-force relationship at in vivo muscle temperatures.

Recent reports (9, 12) have demonstrated that at saturating levels of Ca$^{2+}$, the depressive effects of high $P_i$ on $P_o$ are reduced at near-physiological temperatures. Coupland et al. (9) demonstrated in rabbit psoas fibers that the depressive effect of 25 mM $P_i$ on $P_o$ decreased as temperature increased from 2 to 32.5°C. Recent findings in our laboratory (12) have shown that the effect of 30 mM $P_i$ on $P_o$ is one-half as large in slow fibers and one-third as great in fast type II fibers at 30 vs. 15°C. A temperature-dependent decrease in the sensitivity to $P_i$ also was observed for measures of peak power output in slow type I fibers. It is, in fact, well established that muscle contractile properties are strongly influenced by temperature. Maximal force, shortening velocity, and peak power output increase as in vitro muscle temperature is raised from 10 to 35°C (46). In addition, it has been demonstrated (32, 50) that raising the temperature increases the Ca$^{2+}$ sensitivity of skinned single muscle fibers. Switzer and Moss (50) demonstrated that when the temperature of chemically skinned single fibers was raised...
from 10 to 15°C, there was a significant decrease in the amount of Ca\(^{2+}\) required to achieve pCa\(_{50}\). Maughan et al. (32) also observed an increase in the pCa\(_{50}\) of frog fibers as temperature increased from 5°C to 22°C. These studies demonstrated an increase in Ca\(^{2+}\) sensitivity at temperatures closer to physiological values, suggesting that myoplasmic free Ca\(^{2+}\) would have to decrease more than previously determined to explain the depressed muscular force production during fatigue.

The findings of increased Ca\(^{2+}\) and decreased P\(_i\) sensitivity at temperatures closer to physiological suggest that the role of P\(_i\) in muscle fatigue may be smaller than currently believed. However, the effect of elevated P\(_i\) on the force-pCa relationship or the fiber type dependence of this effect has not been examined at physiological temperatures. Therefore, the purpose of this study was to determine the effect of high P\(_i\) on the force-pCa relationship in both fast- and slow-twitch fibers at near mammalian physiological temperatures.

**METHODS**

**Single-fiber preparation.** Male Sprague-Dawley rats (Sasco, Madison, WI) were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), and the soleus, deep region of the medial head of the gastrocnemius (primarily type IIB fibers), and superficial region of the medial head of the gastrocnemius (primarily type IIB and IIX fibers) were removed and placed in a 4°C relaxing solution. The rats were subsequently killed via a pneumothorax while still heavily anesthetized. The protocol for animal care and disposal was approved by the Institutional Animal Care and Use Committee and followed the guidelines of the National Institutes of Health.

Each muscle was dissected into small bundles (~1 mm in width and up to 5 mm in length) in a relaxing solution, tied to glass capillary tubes, and placed in a skinnin solution. Bundles were stored in non-skinning solution [50% relaxing solution and 50% glycerol (vol/vol)] at 4°C for the first 24 h, after which the solution was replaced with fresh skinning solution and the bundles were stored at -20°C until use but not longer than 4 wk.

On the day of an experiment, fiber bundles were removed from the skinning solution and placed in a 4°C relaxing solution. An ~3-mm segment of a single fiber was isolated from a bundle and then transferred to an ~500-μl glass-bottomed chamber in a milled stainless steel plate. This plate was modified to incorporate a chamber that was cooled to 15°C by Peltier cells at one end and a second chamber less steel plate. This plate was modified to incorporate a chamber that elicits force, was calculated from data to be obtained on fast fibers at near-physiological temperatures.

The myosin heavy chain (MHC) composition was determined by SDS gel electrophoresis (57). After the contractile measurements were obtained, each fiber was solubilized in 10 μl of 1% SDS sample buffer and stored at -80°C. For establishment of the MHC profile, fibers were run on 5% polyacrylamide (wt/vol) gels and silver stained as described by Giulian et al. (22). Up to three isoforms of MHC type II (IIB, IIX, and IIb) have been identified in the rat; however, force-pCa relationships were not different among the type II fibers, and thus these data were grouped together for statistical analysis.

**Statistical analysis.** Data from slow type I fibers were subjected to a two-way (temperature × P\(_i\)) repeated-measures ANOVA with the α level set at 0.05 using Statistica version 5.1 (StatSoft, Tulsa, OK). Data from fast type II fibers were subjected to a one-way ANOVA and, subsequently, Tukey’s post hoc analysis with the α level set at 0.05. The use of two different experimental designs excluded direct comparisons between fiber types, but a qualitative comparison is addressed in the Discussion.
RESULTS

Chemically skinned single fibers were characterized on the basis of unloaded shortening velocity ($V_0$) using the slack test method (15) as previously demonstrated in our laboratory (57) and MHC composition as determined on SDS-PAGE gels (57). At 15°C without added Pi, $V_0$ did not differ between fiber types; however, the mean $V_0$ of the fast gastrocnemius fibers was 2.7-fold greater than that of the slow soleus fibers (Table 1), confirming a faster form of MHC present in the type II fibers.

Type I and type II fibers were subjected to a series of solutions with increasing Ca$^{2+}$/H$^{1001}$ concentration in the presence and absence of 30 mM Pi at both low and high temperatures. Figure 1 depicts representative force records from a single type I fiber during each of the four conditions. In this fiber, elevating the temperature in the absence of Pi increased the amount of force elicited at any given Ca$^{2+}$/H$^{1001}$ concentration. This increased Ca$^{2+}$/H$^{1001}$ sensitivity at the higher temperature is apparent when comparing the force records in the 0 mM Pi condition of Fig. 1, A and B. At 15°C without added Pi, force was first generated at pCa 6.8; however, at 30°C, force production was initiated at pCa 7.2. Furthermore, at pCa 6.0 and 15°C without added Pi, the fiber produced 68% of peak force (peak force refers to the maximum force obtained at pCa 4.5 under each temperature and Pi), but at 30°C at the same free Ca$^{2+}$ concentration, the fiber produced 97% of peak force.

In contrast to the repeated-measures design utilized for type I fibers (because of the fragility of type II fibers at elevated temperatures, their data were obtained by employing an independent design), each fiber was exposed to either 0 or 30 mM Pi at either 15° or 30°C. Figure 2, A and B, shows sample force records of fast type II fibers from selected solution compositions. Similar to what was observed in a slow fiber, raising the temperature in the absence of added Pi caused an increase in Ca$^{2+}$/H$^{1001}$ sensitivity. At 15°C without added Pi, pCa 6.2 elicited roughly 50% of peak force, whereas at 30°C, the same free Ca$^{2+}$ concentration produced 95% of $P_0$.

Elevated Pi decreased force at all free Ca$^{2+}$/H$^{1001}$ concentrations at both 15° and 30°C. For example, at 15°C and with 30 mM Pi, the fiber shown in Fig. 1 did not produce measurable force until pCa 6.2 compared with pCa 6.8 without added Pi (Fig. 1A). Figure 1A also shows that at 15°C without added Pi, pCa 6.0 elicited 68% of $P_0$, whereas at the same Ca$^{2+}$/H$^{1001}$ concentration with 30 mM added Pi, the fiber produced just 24% of $P_0$. At 30°C without added Pi, pCa 6.4 yielded 90% of peak force, whereas the same pCa level elicited only 1% of peak force with 30 mM added Pi (Fig. 1B).

Table 1. Single-fiber characteristics in the absence of added Pi

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>n</th>
<th>Diameter, µm</th>
<th>$P_0$, kN/m²</th>
<th>$V_0$, 15°C</th>
<th>$V_0$, 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9</td>
<td>83.1 (11.9)</td>
<td>150 (29)</td>
<td>163 (40)</td>
<td>1.61 (0.34)</td>
</tr>
<tr>
<td>II</td>
<td>30</td>
<td>73.6 (8.8)</td>
<td>145 (38)</td>
<td>165 (19)</td>
<td>4.29 (0.74)*</td>
</tr>
</tbody>
</table>

Values displayed are means (SD); n, no. of fibers. $P_0$ is maximal isometric force, measured at pCa 4.5 without added Pi. $V_0$ is unloaded shortening velocity determined using the slack test method. Diameter was determined while the fiber was suspended in air. *$P < 0.05$ compared with the mean of type I fibers.

Figure 1. Selected force-pCa records from a slow type I fiber. The force records shown were obtained with 0 and 30 mM added Pi at 15°C (A) and 30°C (B) at pH 6.9. The fiber was moved from relaxing solution (pCa 9.0) to a separate chamber containing activating solution with the indicated free Ca$^{2+}$/H$^{1001}$ level. The force records at low Ca$^{2+}$/H$^{1001}$ (pCa 7.2–6.6) were recorded only long enough to illustrate the different rates and patterns of activation. Obtaining steady-state force levels at these pCa levels required up to a 1.5-min exposure time to the solution (see METHODS); thus the data shown were not used to derive force-pCa relationships. In 30 mM Pi, force traces below pCa 6.2 at 15°C and pCa 6.4 at 30°C did not produce measurable force and therefore are not shown.

AJP-Cell Physiol • VOL 290 • APRIL 2006 • www.ajpcell.org
Compared with the zero added Pi condition, the fast type II fiber exposed to 30 mM Pi produced less force at equivalent Ca$^{2+}$/H$^{+}$ concentrations at both low and high temperatures (Fig. 2). At 15°C, the effect of Pi was most pronounced at pCa 6.2 and 6.0, at which the fiber produced large amounts of force in 0 mM Pi but no measurable force in the presence of 30 mM Pi (Fig. 2A). At 30°C, the largest differences in Ca$^{2+}$-activated force responses caused by elevating Pi occurred at pCa 6.4 and 6.2 (Fig. 2B). At 30°C, pCa 6.2 elicited roughly 95% peak force, whereas at the same pCa level with 30 mM Pi present, no force was produced.

Steady-state force values were plotted against the pCa for each fiber for each condition and analyzed using a linearized Hill plot. This analysis allowed for quantification of the effects of elevated Pi and increased temperature on the force-pCa relationship. The representative force-pCa relationships for a slow type I fiber under each experimental condition are plotted in Fig. 3. Without added Pi, increasing the temperature augmented force production at pCa 4.5 by roughly 20%. Increasing the temperature without added Pi also increased the Ca$^{2+}$ sensitivity of the fiber, causing a leftward shift in the force-pCa relationship; i.e., more force was generated at all levels of free Ca$^{2+}$. The addition of 30 mM Pi, however, depressed peak force and decreased the Ca$^{2+}$ sensitivity of the fiber at both 15 and 30°C. The decreased Ca$^{2+}$ sensitivity caused the force-pCa relationship to shift to the right (Fig. 3); thus the fiber produced less force at any given Ca$^{2+}$ level in the presence of added Pi. The mean data for both slow type I and fast type II fibers are expressed relative to control force (15°C, pCa 4.5, 0 mM Pi) in Figs. 4 and 5, respectively. These data demonstrate that temperature increased maximal force and Ca$^{2+}$ sensitivity, whereas Pi had the opposite effect, decreasing maximal force and Ca$^{2+}$ sensitivity in both fiber types. However, expressing the force relative to one condition (15°C, pCa 4.5, 0 mM Pi) made it difficult to compare the effects of phosphate between 15 and 30°C. Therefore, the effects of elevating Pi on the mean force-pCa relationship in which force is normalized to the force achieved at pCa 4.5 for each temperature and Pi level are shown in Figs. 6 and 7 for the slow type I and fast type II fibers, respectively. It is apparent from these data that elevated Pi...
levels caused a greater rightward shift in the force-pCa relationship at the higher temperature in both slow and fast fibers. The effects of temperature and 30 mM Pi on the force-pCa relationship of type I and type II fibers were statistically quantified by analyzing the parameters derived from the linearized Hill fit procedure (see METHODS). These data are summarized in Table 2 for type I fibers and in Table 3 for type II fibers. In type I fibers, increasing the temperature alone did not affect the pCa required to elicit activation threshold but significantly increased Po, pCa50, and n2. Conversely, 30 mM Pi caused the activation threshold and the pCa50 to occur at higher Ca2+ concentrations, with the effect significantly greater at 30°C than at 15°C.

Similar to type I fibers, increasing the temperature in type II fibers significantly increased pCa50 and n2 and had no effect on activation threshold. Elevating Pi in type II fibers significantly decreased pCa50 at both 15 and 30°C. Activation threshold was not affected by Pi at 15°C but was significantly lowered at 30°C. Thick filament cooperativity, as indicated by n2, was depressed by 30 mM Pi, in fast type II (Table 3) but not in slow type I fibers (Table 2). The independent design utilized in type II fibers did not allow for a statistical quantification of the interactive effects of Pi; however, types I and II fibers responded in a qualitatively similar manner. The temperature-dependent difference in the effect of Pi on pCa50 is depicted graphically in Fig. 8, which shows that in both type I and type II fibers, the Pi-induced shift in pCa was roughly twofold larger at the higher temperature.

A second set of experiments was performed at a neutral pH (7.0), because the true intracellular resting pH likely lies between 6.9 and 7.0 (56, 58). The findings at pH 7.0 followed closely the results at pH 6.9 for both type I and type II fibers (pH 7.0 data not shown). In slow type I fibers at pH 7.0, Ca2+ sensitivity, based on the value of pCa50, was reduced by a significantly greater amount by Pi at 30 vs. 15°C. Activation threshold and both fitting coefficients, n1 and n2, responded similarly at pH 6.9 and 7.0. In the fast type II fibers at pH 7.0, as at pH 6.9, the depressive effect of Pi on pCa50 and activation threshold was again more pronounced at the higher temperature. These results suggest that a 0.1-unit decrease in pH in this range has little or no effect on the depressive effects of Pi on Ca2+ sensitivity at either 15 or 30°C.
DISCUSSION

The effects of 30 mM Pi on the force-pCa relationship were examined in rat skinned single muscle fibers at low and near-physiological temperatures. We found that high intracellular Pi reduced Ca\(^{2+}\) sensitivity and that the effect was twofold greater at near-physiological temperatures. The Pi-mediated reduction in Ca\(^{2+}\) sensitivity was qualitatively similar in type I and type II fibers. These findings suggest that elevations in Pi with intense contractile activity may play a greater role in fatigue than previously believed based on experiments performed at lower temperatures.

**Fiber type dependence.** Because of the different experimental designs used, strict statistical comparisons could not be made for type I and type II fibers; however, qualitative statements can be made regarding the response to Ca\(^{2+}\) at different temperatures and levels of Pi. The differences observed in the

**Table 2. Mean force-pCa parameters for soleus type I fibers**

<table>
<thead>
<tr>
<th></th>
<th>0 mM Pi</th>
<th>30 mM Pi</th>
<th>0 mM Pi</th>
<th>30 mM Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCa(_{50})</td>
<td>6.06 (0.18)</td>
<td>5.72 (0.14)</td>
<td>6.69 (0.13)</td>
<td>6.03 (0.11)</td>
</tr>
<tr>
<td>Activation threshold</td>
<td>7.12 (0.12)</td>
<td>6.83 (0.39)</td>
<td>7.28 (0.17)</td>
<td>6.62 (0.21)</td>
</tr>
<tr>
<td>(n_1)</td>
<td>1.53 (0.61)</td>
<td>2.00 (1.62)</td>
<td>1.69 (0.61)</td>
<td>1.86 (0.44)</td>
</tr>
<tr>
<td>(n_2)</td>
<td>2.56 (0.46)</td>
<td>2.72 (1.52)</td>
<td>4.07 (1.19)</td>
<td>4.19 (1.65)</td>
</tr>
</tbody>
</table>

Data were collected at pH 6.9. The values were obtained from linearized Hill plots of the force-pCa curve and represent means (SD). pCa\(_{50}\) and activation threshold are shown in negative log units (pCa). *Significantly different from 0 mM Pi. †Significant temperature \(\times\) Pi interaction. ‡Significantly different from 15°C.

**Table 3. Mean force-pCa parameters for gastrocnemius type II fibers**

<table>
<thead>
<tr>
<th></th>
<th>0 mM Pi</th>
<th>30 mM Pi</th>
<th>0 mM Pi</th>
<th>30 mM Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCa(_{50})</td>
<td>6.15 (0.08)</td>
<td>5.87 (0.11)</td>
<td>6.53 (0.04)</td>
<td>5.90 (0.06)</td>
</tr>
<tr>
<td>Activation threshold</td>
<td>6.60 (0.16)</td>
<td>6.66 (0.34)</td>
<td>6.56 (0.12)</td>
<td>6.19 (0.11)</td>
</tr>
<tr>
<td>(n_1)</td>
<td>2.46 (1.16)</td>
<td>1.95 (0.67)</td>
<td>3.22 (1.59)</td>
<td>2.74 (0.62)</td>
</tr>
<tr>
<td>(n_2)</td>
<td>6.00 (3.09)</td>
<td>2.22 (1.10)</td>
<td>11.95 (5.82)</td>
<td>7.72 (2.52)</td>
</tr>
</tbody>
</table>

Data were collected at pH 6.9. The values were obtained from linearized Hill plots of the force-pCa curve and are means (SD). pCa\(_{50}\) and activation threshold are shown in negative log units (pCa). *Significantly different from 0 mM Pi. †Significant temperature \(\times\) Pi interaction. ‡Significantly different from 15°C.

**Fig. 7.** Mean normalized force-pCa curves for fast type II fibers. Po, normalized to the level obtained in pCa 4.5 (pH 6.9) at 15°C (A) and 30°C (B) at both concentrations of Pi. Each data point represents the mean (SD) force at each pCa level. Each curve represents data from a separate group of fibers (○, no added Pi; ◦, 30 mM Pi added). The number of fibers was 7 for all groups except the 30°C, 30 mM Pi condition, which numbered 9 fibers. The lines are derived from best-fit Hill equations.

**Fig. 8.** Pi-induced depression of Ca\(^{2+}\) required to elicit half-maximal force (pCa\(_{50}\)). Bar graphs show the absolute change in the pCa\(_{50}\) induced by 30 mM Pi at 15 and 30°C for slow type I (A) and fast type II fibers (B). The values in negative log units are the differences in mean pCa\(_{50}\) values shown in Tables 2 and 3.
force-pCa relationship between slow- and fast-twitch muscle at 15°C agree with previous findings from rat skinned fibers, demonstrating that fast fibers activate at a higher free Ca\(^{2+}\) concentration but display a greater degree of cooperative binding than slow fibers (10, 14, 47). These fiber type-dependent differences in the force-pCa relationship have been attributed to differences in troponin C (TnC) isoforms and cooperativity (37, 49). The fiber type differences were maintained at higher temperatures, suggesting that the different isoforms of TnC were affected similarly by the increase in temperature and that any fiber type differences in cooperativity were maintained at the higher temperature.

Effect of temperature on force-pCa relationship. The increased Ca\(^{2+}\) sensitivity caused by increasing temperature from 15 to 30°C is consistent with the findings of Sweitzer and Moss (50) and others (4, 27, 32), who observed increases in Ca\(^{2+}\) sensitivity when temperature was raised. Sweitzer and Moss (50) observed an increase in the value of pCa_{50} in rabbit psoas fibers when temperature was increased from 10 to 15°C. The pCa_{50} was even higher in a subset of fibers tested at 22°C. Maughan et al. (32) observed a similar phenomenon in frog semitendinosus fibers when comparing force-pCa relationships derived at 5, 16, and 22°C.

The present results, however, contradict the findings of several investigators (23, 47, 48) who observed an increase in temperature to decrease Ca\(^{2+}\) sensitivity in mechanically skinned single fibers. The reason for this discrepancy is not readily apparent but may be related to the use of different fiber preparations. The SR remains intact in mechanically skinned fibers. Therefore, caffeine is added to the bathing solution to induce Ca\(^{2+}\) release, and sodium azide is added to inhibit Ca\(^{2+}\) sequestration by mitochondria. Increasing the temperature would presumably increase the activity of the SR pumps, which would act to suppress Ca\(^{2+}\) levels during activation. Thus the actual free Ca\(^{2+}\) concentration at higher temperatures may have been lower than expected, causing the appearance of decreased Ca\(^{2+}\) sensitivity at higher temperatures.

Goldman et al. (24) increased temperature in chemically permeabilized rabbit psoas fibers by laser-pulse jumps from 10 to 30°C and also observed decreased Ca\(^{2+}\) sensitivity with the temperature rise. Sweitzer and Moss (50) suggested that differences in the method of fiber fixation to the force transducer may have contributed to the discrepancy in the findings. Sweitzer and Moss employed a mechanical fixation technique similar to that used in the present study and determined a minimal amount of fiber compliance. Authors who have observed decreased Ca\(^{2+}\) sensitivity with an increase in temperature typically have used glue combined with t-clips, which Sweitzer and Moss (50) suggested may allow for greater compliance, particularly during high-force contractions such as those at higher temperatures. The increased compliance could increase sarcomere shortening, which has been associated with a decreased Ca\(^{2+}\) sensitivity acting through a reduced Ca\(^{2+}\) affinity for TnC (29). Furthermore, if the shortening was great enough, it would place the fiber on the ascending limb of the force-length relationship and therefore decrease the degree of cooperativity because of myosin strong binding. These mechanisms may, in part, account for why the present findings differ from those demonstrating decreased Ca\(^{2+}\) sensitivity at higher temperatures.

As demonstrated previously (45, 46), increasing the temperature increased P_o of single muscle fibers. Temperature may increase force by either increasing the force per cross bridge or the number of force-producing cross bridges. Most evidence attributes the temperature-induced increase in maximal isometric force to a kinetically mediated increase in the number of strongly bound cross bridges (3, 30, 59). Sinusoidal analysis performed over a wide range of temperatures has determined that the Q_{10} of the forward rate constant of force generation is much greater than that of the backward rate constant (53, 59). These findings imply that although the overall speed of myosin ATPase is increased at higher temperatures (28), the cross bridge spends a greater proportion of the cross bridge cycle strongly bound to actin (i.e., an increased duty cycle), thereby increasing P_o.

A temperature-induced increase in the number of strongly bound cross bridges may help to explain the observed temperature-induced increase in Ca\(^{2+}\) sensitivity. The large leftward shift in the force-pCa relationship coupled with a nearly twofold increase in n_t caused by increasing temperature suggests that at the higher temperature, cooperative binding of myosin to actin was enhanced (49, 50). This notion is supported by the observation that increased temperature enhanced TnC affinity for Ca\(^{2+}\), thus facilitating myosin binding to actin (50). Second, a larger number of strongly bound cross bridges would promote the movement of tropomyosin (Tm) on neighboring thin-filament regulatory units (A7TmTn) off the myosin binding sites on actin, thus stabilizing Tm in the “open state” (25).

In addition, a temperature-induced increase in the flexibility of Tm, allowing for greater exposure of the myosin binding sites on actin, also may have contributed to the increased cooperativity (25). The fact that the activation threshold was unaltered by temperature (30 vs. 15°C) in the present study indicates that the minimal Ca\(^{2+}\) level required for the initiation of binding to TnC was unaltered by temperature, and therefore the increased Ca\(^{2+}\) sensitivity was due mainly to enhanced cooperativity following the initial activation.

Effects of Pi on force-pCa relationship. The force-inhibiting effects of Pi at saturating levels of Ca\(^{2+}\) (pCa 4.5) are greater at cold compared with near-physiological temperatures (8–10, 13). Coupland et al. (9) found a 25 mM increase in Pi to reduce the P_o of rabbit psoas fibers by 50% at 10°C but by only 16% at 30°C, whereas we observed a 30 mM increase in Pi to reduce peak force by 54 and 50% in type I and type II fibers, respectively, at 15°C and by 19 and 5%, respectively, at 30°C (12). This has been attributed to the finding that the forward rate constant of force generation is much more temperature sensitive than the reverse rate constant (59). Therefore, at the higher temperature, the equilibrium constant more strongly favors force generation in the presence of high Pi. The reduced effect at near-physiological temperatures was more apparent in fast type II than in slow type I fibers. This is consistent with the hypothesis that high Pi reduces the number of high-force cross bridges as well as the force per bridge in fast fibers but only the latter in slow fibers. At near-physiological temperatures, we hypothesize that the main effect of high Pi would be a reduction in the force per cross bridge.

Elevating Pi significantly increased the free Ca\(^{2+}\) level required for activation and half-maximal peak force (lower pCa_{50}) at both temperatures, in both fiber types, and depressed n_t in type II but not type I fibers. The Pi-induced reduction in fast fiber n_t was less at the higher temperature, a finding
consistent with our hypothesis that $P_i$ reduction in the number of high-force cross bridges in fast fibers is less at the higher temperature. The observed decrease in $Ca^{2+}$ sensitivity (rightward shift in the $pCa$-force relationship shown in Figs. 6 and 7) is in agreement with previous work (21, 31, 35, 40). Although the mechanism of this effect is unknown, several theories have been proposed. Using fluorescently labeled derivatives of TnC, El-saleh (16) demonstrated that elevated $P_i$ inhibited $Ca^{2+}$ binding to TnC in skeletal muscle. Palmer and Kentish (40), in contrast, suggested that the effects of $P_i$ are more accurately described by kinetic alterations in the cross-bridge cycle. Using fluorescently labeled TnC probes, they observed that $P_i$ had no effect on $Ca^{2+}$ binding to TnC. Palmer and Kentish attributed most of the $P_i$-induced depression of $Ca^{2+}$ sensitivity to a functional antagonism between $Ca^{2+}$ and $P_i$, with $Ca^{2+}$ promoting the transition from a weak to strong binding state and $P_i$ enhancing the reverse step (strong to weak transition). Therefore, at low free $Ca^{2+}$ levels, high $P_i$ has a more powerful effect on reducing force than it does at saturating $Ca^{2+}$ levels.

Assuming that $n_2$ is indicative of the degree of strong cross-bridge binding, the lack of a $P_i$ effect on $n_2$ in the slow fiber suggests that the $P_i$-induced decline in $P_i$, in this fiber type was mediated by a reduced force per cross bridge rather than by fewer cross bridges. At $30°C$, we propose that the main effect on both slow and fast fibers is a reduced force per strong cross bridge. However, fiber stiffness, another measure of strong binding, has been shown to be less sensitive to $P_i$ than force (31), implying that there is a strongly bound non-force-generating state. The existence of this state is further supported by work using caged $P_i$ compounds (11), which suggests this state is one in which actomyosin contains both $P_i$ and ADP and thus is not sensitive to increasing the $P_i$. Thus elevated $P_i$ may depress the activation threshold and $pCa_{50}$ by pushing force-generating cross bridges from an ADP-bound state back into a strongly bound but non-force-generating state with $P_i$ bound. Thus stiffness may have been increasing before force in the presence of high $P_i$ and less-than-saturating $Ca^{2+}$. If this is the mechanism, it is approximately twofold stronger at the higher temperature, which is evident in Figs. 6 and 7.

The $P_i$ release from myosin and the force-generating step may be regulated by $Ca^{2+}$ (31), and the sensitivity of these steps to $P_i$ depends on the fiber type and temperature. The fiber type-dependent effect on force is similar to that observed when the pH is lowered in skinned muscle fibers at $15°C$ (34) and $30°C$ (unpublished observations), suggesting that decreases in $pH$ and increases in $P_i$ have similar effects on the regulation of the cross-bridge cycle.

**Role of $P_i$ in fatigue.** An increased $P_i$ is thought to be responsible for a portion of the loss in muscular force and power during fatigue (1, 2, 20). Recently, however, studies that have examined the effect of elevated $P_i$ on $P_i$ have demonstrated that the depressive effects on peak force are minimized at near-physiological temperatures (9, 12). These findings at $30°C$ suggest that the role of $P_i$ in muscular fatigue may have been overestimated previously based on findings obtained at lower temperatures. The reduced effect of $P_i$, however, was observed at $pCa$ 4.5 and thus may not be applicable to fatigue, where inhibition of SR $Ca^{2+}$ release is known to reduce the amplitude of the intracellular $Ca^{2+}$ transient (20, 54, 55). Myoplasmic $Ca^{2+}$ is reduced most during the later stages of fatigue, when $P_i$ would be at its highest concentration. Thus, to assess the role of elevated $P_i$ in fatigue more definitively, we determined its effects at suboptimal $pCa$ levels. The results of the present study confirm the reduced effect of $P_i$ on $P_i$ at high vs. low temperatures at saturating free $Ca^{2+}$ levels but demonstrate an increased depressive effect of $P_i$ on $Ca^{2+}$ sensitivity at near-physiological temperatures ($30°C$).

These results imply that during the later stages of high-intensity contractile activity, compromised $Ca^{2+}$ release along with high $P_i$ act synergistically to effect large reductions in force and, presumably, power output. For example, if $Ca^{2+}$ were reduced from $pCa$ 5.0 to 6.0 (a typical drop in free intracellular $Ca^{2+}$ observed after fatiguing stimulation of intact fibers) at physiological temperature, in the presence of $30mM P_i$, force would be reduced by $50\%$ in slow type I fibers and by $90\%$ in fast type II fibers.

The extent and rate of fatigue observed in vivo is dependent on several factors, including the fiber type and stimulation frequency (20). The results of the present study could potentially explain the pattern of force decline observed in intact fibers stimulated at a high frequency (54). In these experiments, tetanic force typically declines soon after stimulation begins, and then force is maintained or declines slowly for several minutes before demonstrating a rapid decline to $−20−30\%$ of initial force (1). The initial decline in tetanic force ($−15\%$) that occurs in the early stages of fatigue has been ascribed in part to an increase in $P_i$ with no change in SR $Ca^{2+}$ release or myoplasmic $Ca^{2+}$. Once $Ca^{2+}$ release becomes compromised, the combined effects of elevated $P_i$ and decreased free intracellular $Ca^{2+}$ would cause force to drop steeply as observed during the final phase of fatigue.

The steeper force-$pCa$ relationship observed in fast type II fibers, combined with the effects of $P_i$, likely contributes to the increased susceptibility to fatigue of fast type II fibers (20). For example, the present findings predict that in response to a change from $pCa$ 5.0 to 6.0, the $P_i$-induced drop in force would be twice as large for type II as for type I fibers. Furthermore, because of their accelerated rate of ATP utilization, fast fibers would accumulate fatiguing levels of $P_i$ more rapidly than slow fibers. In addition, phosphate also is believed to diffuse into the SR through an anion channel and to form a precipitate of $Ca^{2+}$-Pi in the SR, thereby reducing the free $Ca^{2+}$ available for release (43), an effect that would be more rapid and more pronounced in fast fibers because of their increased rate of ATP hydrolysis. During the initial stages of fatigue, tetanic $Ca^{2+}$ increases (54), but as $P_i$ accumulates, SR $Ca^{2+}$ release and intracellular $Ca^{2+}$ concentration decrease (43). The reduced $Ca^{2+}$, $P_i$-induced rightward shift of the $pCa$-force relationship and direct effects of $P_i$ on the cross bridge combine to induce large declines in muscular force and power output. These data help to establish the importance of the combined effects of elevations in $P_i$ and decreases in $Ca^{2+}$ in eliciting fatigue during intense contractile activity at near-physiological temperatures and offer possible explanations for the increased susceptibility of fast fibers to fatigue from intense contractile activity. It can be postulated that the fatigue-inducing effects of high $P_i$ during in vivo exercise are likely greater than predicted from studies at cooler temperatures.

**GRANTS**

This work was supported by the National Aeronautics and Space Administration Grant NAG9-1156 (to R. H. Fitts) and an American Heart Association Predoctoral Fellowship (to E. P. Debold) from the Northland Affiliate.