4-1-2014

Effects of Low Cell pH and Elevated Inorganic Phosphate on the pCa-Force Relationship in Single Muscle Fibers at Near-Physiological Temperatures

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Accepted version. *American Journal of Physiology: Cell Physiology,* Vol. 306, No. 7 (April 2014): C670-C678. DOI.

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Effects of low cell pH and elevated inorganic phosphate on the pCa-force relationship in single muscle fibers at near-physiological temperatures

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Abstract

Intense muscle contraction induces high rates of ATP hydrolysis with resulting increases in P_i, H^+, and ADP, factors thought to induce fatigue by interfering with steps in the cross-bridge cycle. Force inhibition is less at physiological temperatures; thus the role of low pH in fatigue has been questioned. Effects of pH 6.2 and collective effects with 30 mM P_i on the pCa-force relationship were assessed in skinned fast and slow rat skeletal muscle fibers at 15 and 30°C. At 30°C, pH 6.2 + 30 mM P_i significantly depressed peak force in all fiber types, with the greatest effect in type IIx fibers. Across fiber types, Ca^{2+} sensitivity was depressed by low pH and low pH + high P_i, with the greater effect at 30°C. For type IIx fibers at 30°C, half-maximal activation (pCa_{50}) was 5.36 at

pH 6.2 (no added P_i) and 4.98 at pH 6.2 + 30 mM P_i compared with 6.58 in the control condition (pH 7, no added P_i). At 30°C, n_2, reflective of thick filament cooperativity, was unchanged by low cell pH but was depressed from 5.02 to 2.46 in type IIx fibers with pH 6.2 + 30 mM P_i. With acidosis, activation thresholds of all fiber types required higher free Ca^{2+} at 15 and 30°C. With the exception of type IIx fibers, the Ca^{2+} required to reach activation threshold increased further with added P_i. In conclusion, it is clear that fatigue-inducing effects of low cell pH and elevated P_i at near-physiological temperatures are substantial.

Keywords

myofilament calcium sensitivity; fatigue; cross-bridge cycle

Introduction

the causes of muscle fatigue are complex and not completely understood (2, 12). It is characterized by a loss of power as a result of declines in force and velocity and may originate from central nervous system disturbances or peripheral factors within the skeletal muscles (12, 18). Understanding the etiology of muscle fatigue is critical, as it presents limitations to exercise performance and is clinically relevant in situations such as respiratory or cardiac failure (18).

During high-intensity exercise or a respiratory failure event, high rates of glycolysis and ATP hydrolysis result in a buildup of metabolites such as ADP, H^+, and P_i. These metabolites are thought to depress peak force by interfering with key steps in the cross-bridge cycle. Low cell pH is believed to depress force by interfering with the low-to-high force transition step (Fig. 1, *step 3*) and to depress velocity by slowing the ATP hydrolysis or ADP release step (Fig. 1, *steps 2* and *6*). It is hypothesized that elevating P_i accelerates the reverse rate constant of force generation (Fig. 1, *step 3*), depressing peak force (12). High P_i conditions have been shown to not alter (7) or to slightly increase (30) velocity. During high-intensity contractile activity, intracellular pH can reach values as low as 6.2 in amphibians (37), 6.3 in rats (24), and 6.4 in humans (15), while P_i increases to 30–40 mM in humans (3). Experiments in single muscle fibers were initially performed at low temperatures (5–20°C), where low cell pH (pH 6.2) and elevated P_i (30 mM) significantly depressed peak force at saturating (maximal) Ca^{2+} (5, 23, 30). Recent temperature jump-plate technology allowed single-fiber experiments to be conducted at physiological temperatures (30–35°C), and the depressive effects of low cell pH and elevated P_i on peak force were less pronounced (5, 7, 29).

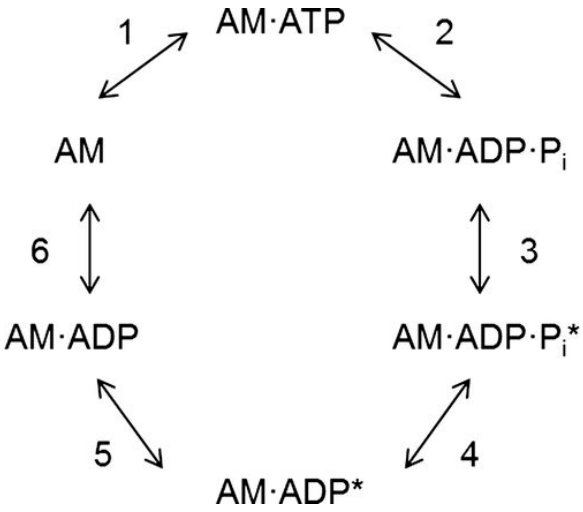

Fig. 1. Schematic of the cross-bridge cycle. A, actin; M, myosin; ∗, high-force bridge.

In a fatiguing event, myoplasmic free Ca^{2+} is not maximal, as sarcoplasmic reticulum (SR) Ca^{2+} release is depressed, in part due to Mg^{2+} inhibition of the ryanodine receptor and precipitation of Ca^{2+} with P_i in the SR (1,

2, 39, 40). During fatigue, the amplitude of the myoplasmic Ca^{2+} transient (pCa) is depressed and may reach <6.0 (1 μM) (1).

At 30°C, 30 mM P_i reduced peak force by 19% and 5% in type I and II fibers, respectively (7), while pH 6.2 reduced peak force by 12% and 4% in type I and II fibers, respectively (19). DeBold et al. (8) showed that elevated (30 mM) P_i depresses force at suboptimal Ca^{2+} concentrations at near-physiological temperatures. The reduction of myofilament Ca^{2+} sensitivity by P_i was more pronounced at 30°C than 15°C. Similar to P_i, the depressive effects of low cell pH on peak force are reduced at near-physiological temperatures, leading some to question the role of low cell pH in fatigue (2, 29, 33, 41). However, the effects of low cell pH have yet to be evaluated at suboptimal Ca^{2+} concentrations that are characteristic of fatigue. Therefore, the first aim of this study was to evaluate the effects of acidosis at suboptimal Ca^{2+} at 15 and 30°C.

While it has been shown that both metabolites individually depress myofilament Ca^{2+} sensitivity at 15°C (20, 25), the collective effects of low cell pH and elevated P_i on the pCa-force relationship are unknown. Thus a second aim of this study was to assess the effects of pH 6.2 + 30 mM P_i on the pCa-force relationship at cold (15°C) and near-physiological (30°C) temperatures.

Methods

Ethical Approval

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

Solutions

Compositions of relaxing (pCa 9.0) and maximal activating (pCa 4.5) solutions were derived from a computer program utilizing the stability constants reported by Fabiato and Fabiato (9, 11), which include adjustments for temperature, pH, and ionic strength. All solutions contained (mM) 20 imidazole, 7 EGTA, 4 MgATP, and 14.5 creatine phosphate. P_i was added as K_2HPO_4 to yield a total concentration of 30 mM. Although no P_i was added to the control (0 mM) solution, resting P_i levels are ~0.5 and 0.7 mM in the fibers of fast and slow muscle, respectively, because of contamination from the hydrolysis and regeneration of ATP (30). Mg^{2+} was added in the form of $MgCl_2$ with a specified free concentration of 1 mM. Ionic strength was adjusted to 180 mM with KCl, and with the solution at 15 or 30°C, pH was adjusted to 6.2 or 7.0 with KOH. Ca^{2+} was added as $CaCl_2$. Various pCa solutions were made by mixing calculated volumes of pCa 4.5 and pCa 9.0 solutions (9, 11).

Single-fiber preparation

Male and female Sprague-Dawley rats were anesthetized with pentobarbital sodium (Nembutal; 50 mg/kg body wt ip), and the soleus (type I fibers), the deep region of the lateral head of the gastrocnemius (type IIa fibers), and the superficial region of the medial head of the gastrocnemius (type IIx fibers) were removed and placed in a 4°C relaxing solution. After the muscles were extracted, the rats were euthanized via a pneumothorax while still anesthetized. Muscles were dissected into small bundles (40–50 fibers) in relaxing solution, tied to glass capillary tubes, and stored in skinning solution [50% relaxing solution-50% glycerol (vol/vol)] at −20°C for ≤4 wk.

On the day of experimentation, fibers were isolated and studied as previously reported (8, 19). A muscle fiber was placed in 4°C relaxing solution in a glass-bottom stainless steel chamber and suspended between a force transducer (series 400A, Cambridge Technologies) and a servomotor (model 312C high-speed length controller, Aurora Scientific). Two chambers were maintained at 15°C by Peltier cells, and a third chamber was heated to 30°C by an electrically powered heating unit (42). While in relaxing solution (15°C), the fiber was briefly (30–40 s) exposed to 0.5% Brij 58 (Sigma) to disrupt the SR (27). An inverted microscope was used to view the fiber at ×40 magnification, and sarcomere length was adjusted to 2.5 μm (34). Sarcomere length was monitored and

adjusted throughout the experiment to maintain 2.5 µm. After determination of fiber length, fiber diameter was assessed from a digital image of the fiber obtained while it was briefly suspended in air. With use of Scion Image, three measurements of fiber width were made along the length of the fiber, and the average diameter was determined assuming a cylindrical shape (24).

Determination of single-fiber force characteristics

For determination of the pCa-force relationship, each fiber was subjected to a series of activating solutions ranging from pCa 7.0 to 4.5 at pH 7.0, pH 6.2, or pH 6.2 + 30 mM P_i at 15 or 30°C. For an individual fiber, the pCa-force relationship was analyzed as described in detail elsewhere (43). Briefly, force elicited at a given pCa was allowed to plateau and then expressed as a fraction of peak force, i.e., submaximal force/peak force at pCa 4.5 (P_r). Least-squares regression lines were fit to data points <50% of peak force and data points >50% of peak force. Activation threshold (AT), the pCa at initial force development, was defined as Ca^{2+} concentration, where $\log[P_r/(1 - P_r)] = -2.5$ (43). Half-maximal activation (pCa_{50}) was calculated as the mean intercept of least-squares regression lines with the line $y = 0$. The slope of the line fit to data above $P_r = 0.5$ was defined by n_1, and the slope of the line fit to data below $P_r = 0.5$ was indicative of thick filament cooperativity and defined by n_2 (8). The pCa-force curves in Figs. 6 and 7 were constructed with GraphPad Prism (San Diego, CA) and fitted with a four-parameter logistic curve.

Type I or IIa fibers were taken through control (pH 7 + 0 mM P_i) and experimental (pH 6.2 or pH 6.2 + 30 mM P_i) pCa-force curves at both temperatures. Fast type IIx fibers were not stable enough to maintain sarcomere uniformity through more than two pCa-force curve tests. At the onset and conclusion of each pCa-force curve test, peak force (pCa 4.5) at 15°C was measured. If a fiber's final peak force was <90% of the initial force, data for that fiber were eliminated. All fibers were exposed to the given conditions in a random order to control for order effects.

Myosin heavy chain composition and fiber typing

After the contractile measurements, fibers were solubilized in 10 µl of 1% SDS sample buffer and stored at −20°C. The myosin heavy chain (MHC) profile was obtained by running samples on 5–7.5% (wt/vol) Tris·HCl precast gels (Bio-Rad) and stained with the Silver Stain Plus kit (Bio-Rad). On the basis of their MHC profile, fibers were identified as type I, IIa, or IIx (Fig. 2). If fibers contained more than one MHC band, the fiber was typed on the basis of the predominant band. Such fibers (Fig. 2, *lane 1*) had maximal shortening velocities (V_o) and pCa-force relationships not significantly different from fibers with a single band (i.e., 1 MHC isoform).

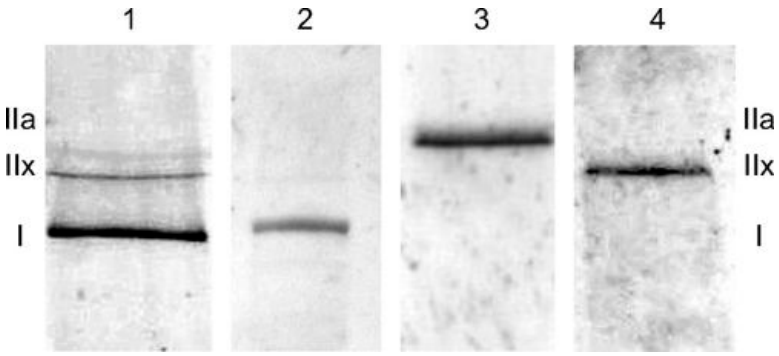

Fig. 2. Myosin heavy chain gel (7.5%). *Lane 1*, fiber with a predominant type I band and minor type IIa and IIx bands; *lanes 2, 3*, and *4*, type I, IIa, and IIx fibers, respectively.

Statistics

Data were analyzed with Sigma Stat (San Jose, CA) using an ANOVA followed by post hoc unpaired *t*-tests with a significance level of 0.05.

In a small population of type IIb fibers (n = 4), control and experimental data closely resembled data from type IIx fibers, such that there were no significant differences in the pCa-force relationship between type IIx and IIb fibers. Therefore, data from type IIb fibers are not included in the study. Additionally, there were no significant differences between male and female rat fibers in the pCa-force relationship in any fiber type, temperature, or condition, so data from both sexes were pooled.

Results

Temperature effects on peak force and pCa-force relationship

Representative force traces from slow and fast fibers at various Ca^{2+} concentrations are shown in Figs. 3 and 4. The time required for a fiber to reach peak force (dp/dt) was faster at higher temperatures. Increasing temperature from 15 to 30°C increased peak force (Fig. 5) and the slope of the pCa-force relationship (Figs. 6 and 7) in all fiber types at control conditions (pH 7 + 0 mM P_i). Myofibrillar Ca^{2+} sensitivity increased with increasing temperature in all fiber types, as indicated by significant increases in AT and pCa_{50} (Tables 1–3). The higher temperature elevated n_2, reflective of increased thick filament cooperativity, in type I and IIa, but not IIx, fibers in control conditions (Tables 1–3). More Ca^{2+} was required to initiate force in type IIx than type I fibers in control conditions, as indicated by significant fiber type differences in AT at 15 and 30°C (P < 0.01).

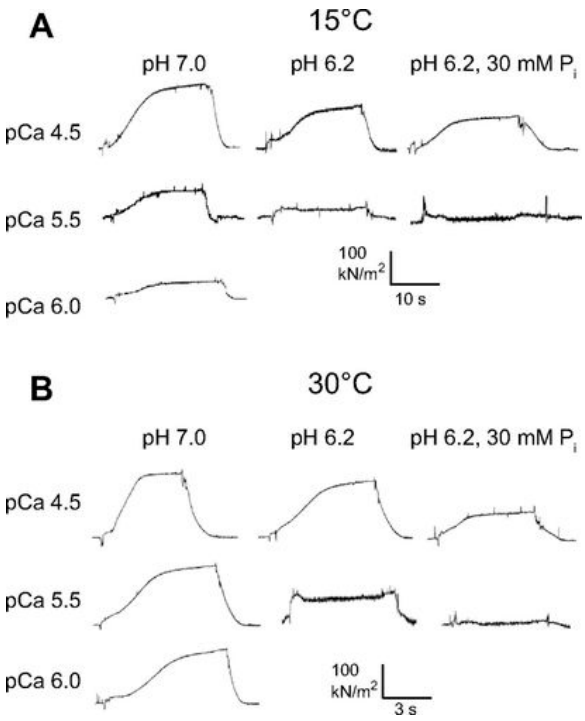

Fig. 3. Selected force records from a representative slow type I fiber at 15°C (*A*) and 30°C (*B*). Force records were obtained at pH 7, pH 6.2, and pH 6.2 + 30 mM P_i at pCa 4.5, 5.5, and 6.0. No force was observed at pCa 6.0 with pH 6.2 or pH 6.2 + 30 mM P_i conditions for either temperature.

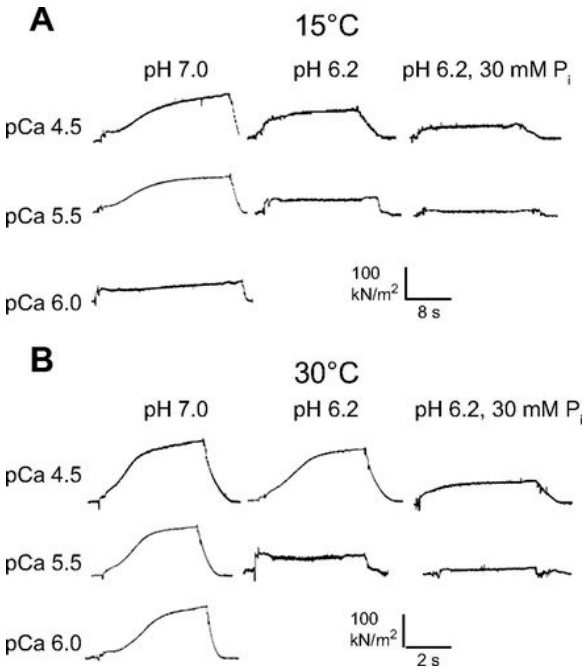

Fig. 4. Selected force records from a representative fast type IIx fiber at 15°C (*A*) and 30°C (*B*). Force records were obtained at pH 7, pH 6.2, and pH 6.2 + 30 mM P_i at pCa 4.5, 5.5, and 6.0. No force was observed at pCa 6.0 with pH 6.2 or pH 6.2 + 30 mM P_i conditions for either temperature.

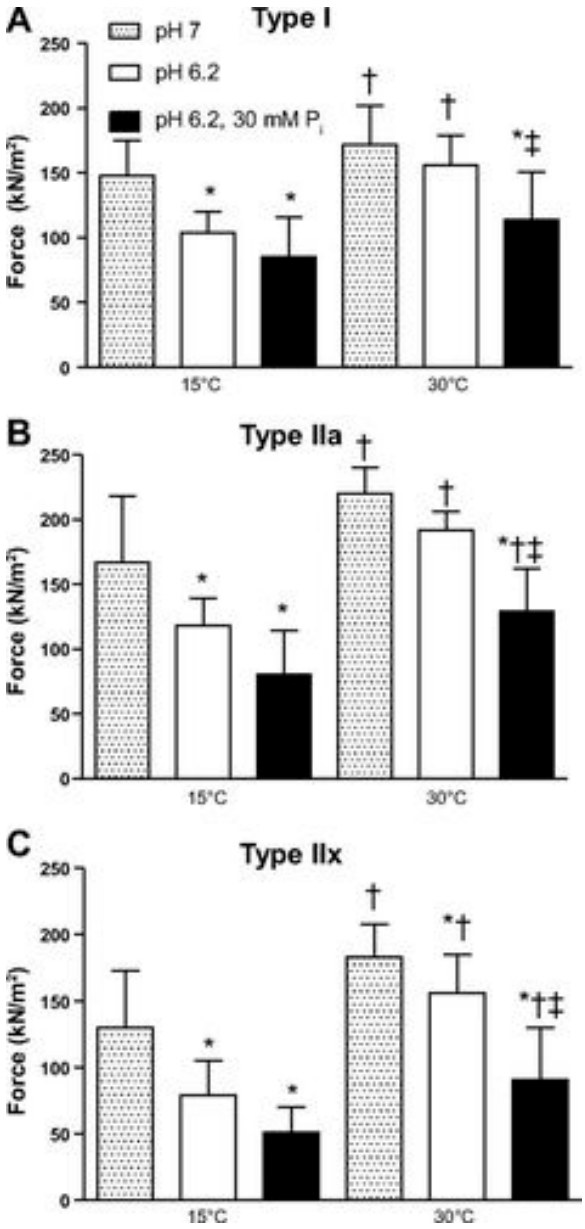

Fig. 5. Peak force (P_o) elicited at pCa 4.5 for type I (*A*), IIa (*B*), and IIx (*C*) fibers. Values are means ± SD. *Significantly different from pH 7 at the same temperature, $P < 0.05$. †Significantly different from comparable condition at 15°C, $P < 0.05$. ‡Significantly different from pH 6.2 at the same temperature, $P < 0.05$.

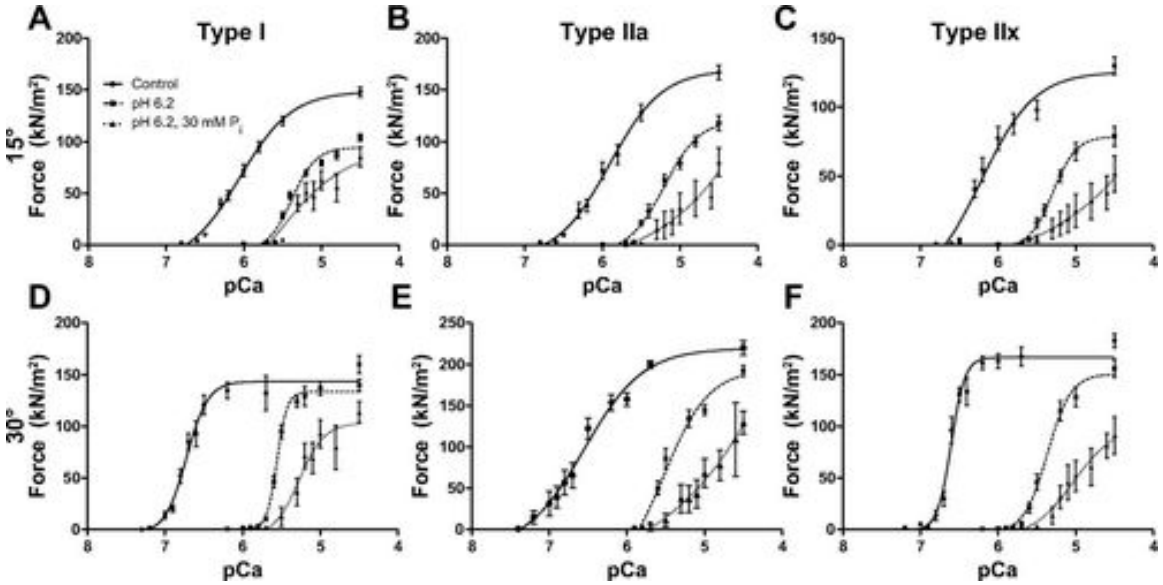

Fig. 6. Average pCa-force curves for type I (*A* and *D*), IIa (*B* and *E*), and IIx (*C* and *F*) fibers at 15°C (*A*–*C*) and 30°C (*D*–*F*). Each data set represents force (mean ± SE) at each Ca^{2+} concentration (in negative log units) from all fibers included in the experiment.

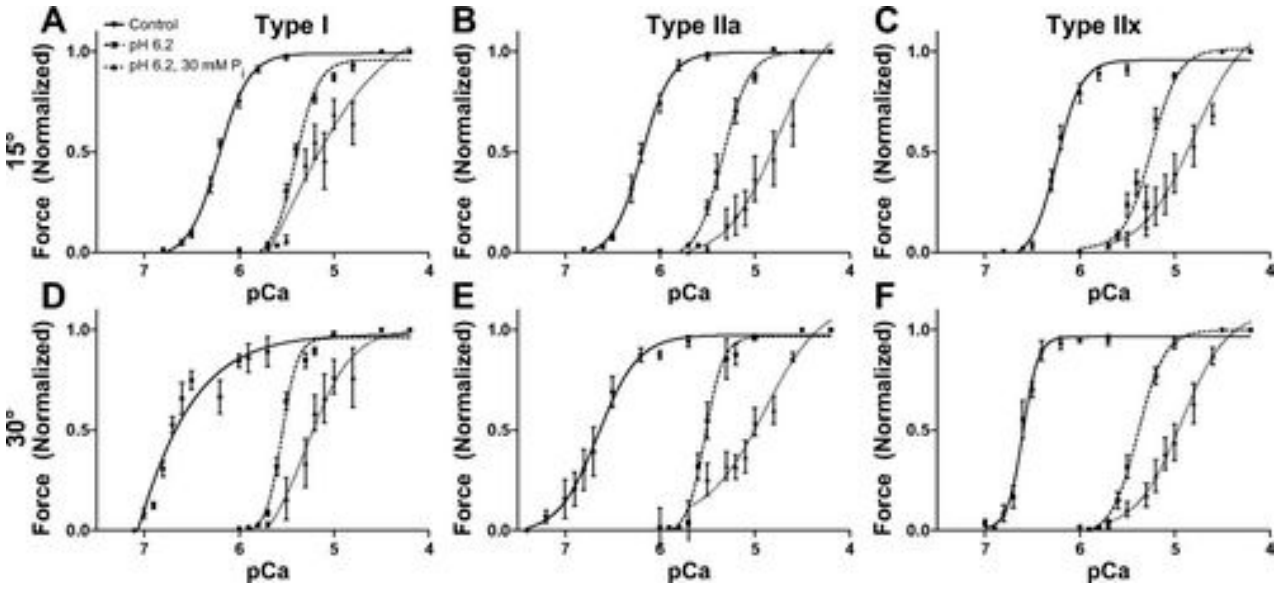

Fig. 7. Mean normalized pCa-force curves for type I (*A* and *D*), IIa (*B* and *E*), and IIx (*C* and *F*) fibers at 15°C (*A*–*C*) and 30°C (*D*–*F*). Maximal isometric force (P_o) normalized to the level obtained in pCa 4.5 at both temperatures in all conditions is plotted against pCa. Values are means ± SE.

Table 1. Type I fiber force characteristics

	15°C			30°C		
	pH 7.0	**pH 6.2**	**pH 6.2 + 30 mM P_i**	**pH 7.0**	**pH 6.2**	**pH 6.2 + 30 mM P_i**
n	19	19	10	20	18	10
pCa_{50}	6.06 ± 0.26	5.40 ± 0.09*	5.18 ± 0.35*‡	6.77 ± 0.11†	5.56 ± 0.09*†	5.16 ± 0.36*‡
AT	6.92 ± 0.20	6.08 ± 0.22*	5.75 ± 0.16*‡	7.33 ± 0.22†	6.09 ± 0.21*	5.85 ± 0.29*‡
n_1	2.15 ± 0.80	1.86 ± 1.29	1.84 ± 1.89	1.84 ± 1.58	2.23 ± 1.00	2.08 ± 1.29
n_2	2.99 ± 0.93	3.51 ± 1.22*	3.68 ± 3.16	4.02 ± 1.63†	4.50 ± 1.72†	3.96 ± 3.55

Values are means ± SD. Data were obtained from linearized Hill plots of the pCa-force curve. *n*, Number of fibers. pCa_{50} and activation threshold (AT) are shown in negative log units.
*Significantly different from pH 7.0 at the same temperature, $P < 0.05$.
†Significantly different from comparable condition at 15°C, $P < 0.05$.
‡Significantly different from pH 6.2 at the same temperature, $P < 0.05$.

Table 2. Type IIa fiber force characteristics

	15°C			30°C		
	pH 7.0	**pH 6.2**	**pH 6.2 + 30 mM P_i**	**pH 7.0**	**pH 6.2**	**pH 6.2 + 30 mM P_i**
n	17	9	7	7	7	7
pCa_{50}	5.96 ± 0.29	5.31 ± 0.13*	4.90 ± 0.25*‡	6.73 ± 0.30†	5.49 ± 0.11*†	5.04 ± 0.31*‡
AT	6.90 ± 0.27	6.11 ± 0.27*	5.77 ± 0.24*‡	7.21 ± 0.28†	5.99 ± 0.18*	5.61 ± 0.31*‡
n_1	2.00 ± 0.87	1.69 ± 0.52	2.44 ± 1.47	1.27 ± 0.77	3.32 ± 1.59*†	2.26 ± 0.89
n_2	2.85 ± 1.13	3.59 ± 1.79	3.22 ± 1.71	5.03 ± 2.05†	5.40 ± 2.20†	4.59 ± 2.36

Values are means ± SD. Data were obtained from linearized Hill plots of the pCa-force curve. *n*, Number of fibers. pCa_{50} and AT are shown in negative log units.
*Significantly different from pH 7.0 at the same temperature, $P < 0.05$.
†Significantly different from comparable condition at 15°C, $P < 0.05$.
‡Significantly different from pH 6.2 at the same temperature, $P < 0.05$.

Table 3. Type IIx fiber force characteristics

	15°C			30°C		
	pH 7.0	**pH 6.2**	**pH 6.2 + 30 mM P_i**	**pH 7.0**	**pH 6.2**	**pH 6.2 + 30 mM P_i**
n	17	14	10	13	11	6
pCa_{50}	6.16 ± 0.25	5.34 ± 0.11*	4.89 ± 0.29*‡	6.58 ± 0.12†	5.36 ± 0.09*	4.98 ± 0.27*‡
AT	6.70 ± 0.18	5.87 ± 0.23*	5.81 ± 0.31*	7.03 ± 0.20†	5.97 ± 0.18*	5.89 ± 0.37*
n_1	2.11 ± 1.40	2.75 ± 1.49	2.21 ± 0.94	2.17 ± 1.30	3.50 ± 1.90*	3.22 ± 1.86
n_2	4.43 ± 2.30	5.57 ± 3.10	2.59 ± 0.94*‡	5.02 ± 1.87	5.03 ± 3.07	2.46 ± 1.21*‡

pH and P_1 effects on peak force

At saturating Ca^{2+} (pCa 4.5), the depressive effects of pH 6.2 on force were less pronounced at 30°C than 15°C in all fiber types, such that peak force was significantly depressed in all fiber types at 15°C but only in the fast type IIx fibers at 30°C. In pH 6.2 + 30 mM P_i, peak force was significantly depressed from control in all fiber types at both temperatures, with the greatest effects in type IIx fibers at 15°C (61% force depression) and 30°C (50% force depression) (Fig. 5).

pH and P_1 effects on pCa-force relationships

At suboptimal Ca^{2+}, pH 6.2 and pH 6.2 + 30 mM P_i significantly reduced force in all fiber types at both temperatures. At 15°C and submaximal Ca^{2+} concentration pCa 5.5 (5 µM), low cell pH depressed force by 74% in slow fibers (Fig. 3*A*) and 86% in fast type IIx fibers (Fig. 4*A*) compared with control. At 30°C and pCa 5.5, pH 6.2 depressed slow fiber force by 41% (Figs. 3*B* and 8) and fast type IIx fiber force by 73% (Figs. 4*B* and 8). In pH 6.2 + 30 mM P_i, no force was generated at pCa 6.0 at either temperature (Figs. 3 and 4), while at pCa 5.5, force was reduced by 91, 95, and 98% in type I, IIa, and IIx fibers, respectively, at 30°C compared with control (Fig. 8).

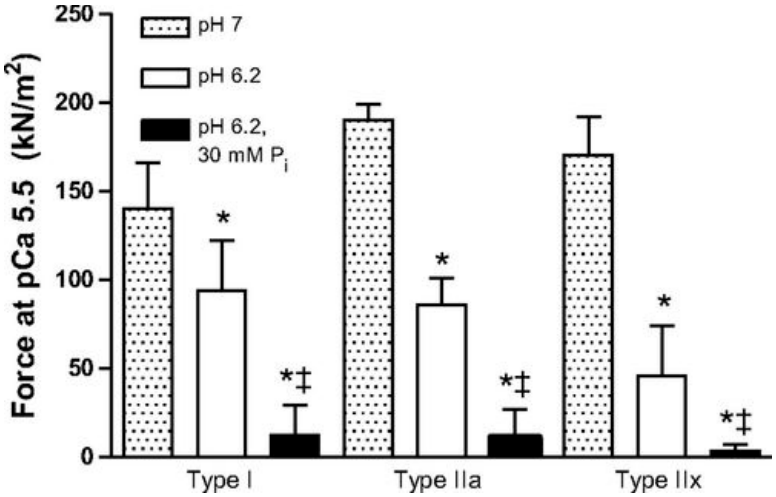

Fig. 8. Force at suboptimal Ca^{2+} concentration (pCa 5.5 or 5 µM) for all fiber types in pH 7, pH 6.2, and pH 6.2 + 30 mM P_i at 30°C. Values are means ± SD. *Significantly different from pH 7, $P < 0.05$. ‡Significantly different from pH 6.2, $P < 0.05$.

At pH 6.2, the pCa-force relationship in all fiber types was significantly shifted to higher free Ca^{2+} levels for a given percentage of P_o, indicative of reduced myofibrillar Ca^{2+} sensitivity, with a greater shift at 30°C (Figs. 6 and 7). This resulted in lower pCa_{50} values; for example, in type I fibers, the low pH-induced change in pCa_{50} was 0.66 unit at 15°C and 1.21 units at 30°C (Fig. 9). In pH 6.2 + 30 mM P_i, the pCa-force relationship showed an even greater reduction in myofibrillar Ca^{2+} sensitivity than in low pH alone at both temperatures, with larger effects at 30°C (pCa_{50} change of 0.88 unit at 15°C and 1.61 units at 30°C in type I fibers) (Fig. 9). Under low cell pH conditions, pCa_{50} was significantly lower in type IIx than type I and IIa fibers at 30°C (Tables 1–3).

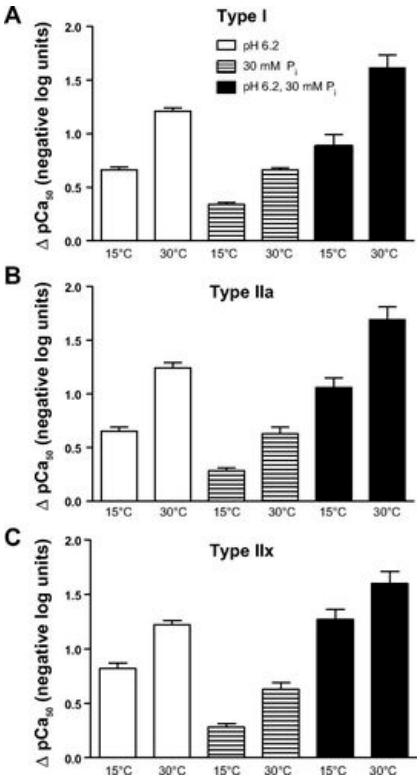

Fig. 9. Absolute change in pCa_{50} from control induced by acidosis (pH 6.2), elevated P_i (30 mM), and pH 6.2 + 30 mM P_i at 15 and 30°C for type I (*A*), IIa (*B*), and IIx (*C*) fibers. Values (means ± SE) are differences in mean pCa_{50} values in Tables 1–3. Data for elevated (30 mM) P_i alone are from Debold et al. (8).

In pH 7 and 6.2 at 15°C, n_2 was significantly higher in type IIx than type I fibers. Elevating both H^+ and P_i selectively reduced n_2 in type IIx fibers (Table 3) at 15 and 30°C, such that fiber type differences seen at 15°C in the control condition and in pH 6.2 were no longer apparent. Fibers generated force at lower Ca^{2+} concentrations (higher AT) in type I and IIa than type IIx fibers at pH 7, and low pH increased the Ca^{2+} required to initiate force (AT) in all fiber types at both temperatures. The pH effect on AT was significantly exacerbated by addition of 30 mM P_i in type I and IIa, but not type IIx, fibers, which resulted in no fiber type differences in AT in pH 6.2 + 30 mM P_i (Tables 1–3).

To better illustrate temperature effects, the pCa-force relationship was normalized to peak force for each condition (Fig. 7). The shift in the pCa-force curve induced by low cell pH and low pH + P_i is greater at 30°C (Fig. 7, *D–F*) than 15°C (Fig. 7, *A–C*) in all fiber types. The reduction of myofilament Ca^{2+} sensitivity induced by pH and pH + P_i was more pronounced at higher temperatures, as quantified by the greater decrease in pCa_{50} (Fig. 9). The pH effect was significantly larger than the P_i effect (Fig. 9) (8) in all fiber types at both temperatures. The effects of pH 6.2 + 30 mM P_i on the change in pCa_{50} were additive at both temperatures in type IIx and IIa fibers but only at 30°C in type I fibers (Fig. 9).

Discussion

We have shown that low cell pH (6.2) reduces myofibrillar Ca^{2+} sensitivity in all fiber types, as indicated by a significantly depressed AT and pCa_{50}, and the effects are greater at near-physiological temperatures. Prior to this study, the effects of low cell pH on force at suboptimal Ca^{2+} concentrations characteristic of fatigue at near-physiological temperatures (30°C) were unknown. At 15 and 30°C, pH 6.2 + 30 mM P_i further depresses AT in type I and IIa fibers and pCa_{50} and peak force in all fiber types more than pH 6.2 or 30 mM P_i alone. Low cell pH did not change n_2, suggesting that acidosis did not alter thick filament cooperativity; however, in combination with P_i, n_2 was depressed in fast type IIx fibers at 15 and 30°C. These findings characterize the individual and collective roles of low cell pH and elevated P_i in force depression at near-physiological temperatures and implicate a critical role of H^+ and P_i in mediating fatigue.

To maximize the stability of the preparation, skinned fiber experiments have predominantly been performed at lower, nonphysiological temperatures (≤15°C) (4, 22, 23, 25). Under these conditions, low cell pH significantly depressed force at suboptimal and saturating Ca^{2+} concentrations (14, 19). When jump-plate technology emerged and fibers were set up at cold temperatures and studied at near-physiological temperatures (≥25°C), the depressive effects of low pH on peak force were reduced (29). This observation led to the hypothesis that the contribution of low pH or H^+ to fatigue was minimal at physiological temperatures. However, Allen and Westerblad (1) showed that the amplitude of the Ca^{2+} transient declined with fatigue, reaching <1 μM (pCa 6.0). Thus fatigue is more accurately mimicked in experiments carried out at submaximal Ca^{2+}. An important finding in this study was that low cell pH significantly contributed to force depression at submaximal Ca^{2+}, with a more pronounced effect at near-physiological temperatures (30°C).

Effects of temperature on P_o and the pCa-force relationship

Our results show that peak force increased in all fiber types with temperature. This is consistent with the report of Ranatunga and Wylie (32) that peak force of the rat soleus and extensor digitorum longus muscles increased by nearly twofold as temperature increased from 10 to 35°C. Davis and Epstein (6) proposed that a local unfolding within the cross-bridge secondary/tertiary structure might cause a greater force generation with rising temperature. Ca^{2+} binding to troponin C is enhanced at higher temperatures (36). Therefore, less Ca^{2+} was required to develop force, as evidenced by a temperature-sensitive increase in pCa for AT and pCa_{50} in all fiber

types. The temperature-induced shift in the pCa-force relationship toward lower free Ca^{2+} levels is consistent with previous findings in our laboratory (8) and others (21, 36) and results from a temperature-induced increase in myofibrillar Ca^{2+} sensitivity. The forward rate constant of force generation (Fig. 1, *step 3*) is greatly accelerated by increasing temperature (44). Consequently, more high-force cross bridges are formed at a given suboptimal Ca^{2+} at high (30°C) than low (15°C) temperatures.

The myofibrillar Ca^{2+} sensitivity of force development is fiber type-dependent, with fast fibers activating at a higher free Ca^{2+} but with a greater degree of cooperative binding (12, 13). Our results confirmed this, as AT values are higher (less Ca^{2+}) in slow type I than fast type IIx fibers at 15 and 30°C. Thick filament cooperativity, quantified by n_2, is temperature-sensitive, with binding enhanced at higher temperatures (8, 35, 36). We observed this to be true for slow type I and fast type IIa, but not fast type IIx, fibers. DeBold et al. (8) reported significant increases in n_2 with temperature in type I and II fibers but did not subdivide type II fibers into types IIa and IIx. Because type IIx fibers have a high n_2 compared with type I or IIa fibers at 15°C, additional cooperative binding reserve may be less in type IIx fibers, making any increase with temperature difficult to detect.

Effects of pH and P_i on P_o

Consistent with the findings of others (19, 29), we found that low cell pH (6.2) depresses peak force less at saturating Ca^{2+} concentrations (pCa 4.5) at higher than lower temperatures, in that low pH had no significant effect on peak force of type I and IIa fibers and only a modest effect on peak force of type IIx fibers at 30°C. Our finding that low pH depresses P_o in fast type IIx fibers suggests that either the number of cross bridges or the force per bridge remained depressed with increasing temperature (25). Knuth et al. (19) observed no pH effect on peak force of fast fibers at 30°C, but fibers were not subdivided into types IIa and IIx, and the lack of a low pH-induced decline in force may have resulted from a high percentage of type IIa fibers. It has been proposed that elevated H^+ inhibits the forward rate constant of force generation (Fig. 1, *step 3*) (12). Since acidosis and temperature affect this step, the effects should be additive, with temperature reducing the force-depressive effects of low pH. This was the case, but to a lesser extent, in type IIx fibers.

In muscle fatigue, decreasing cell pH is accompanied by an increase in P_i up to 30 mM (3). Karatzaferi et al. (17) found that 30 mM P_i at 30°C depressed peak force by ~25% in fast fibers, while DeBold et al. (7) observed a 19 and 5% decline in type I and II fibers, respectively. The collective effects of low cell pH and elevated P_i on peak force on a given fiber type have been less studied. Potma et al. (31) showed that, at 15°C and pH 6.0 + 30 mM P_i, peak force was depressed by ~63 and ~86% in rabbit soleus and psoas fibers, respectively. Karatzaferi et al. reported peak force reductions of 81 and 52% at 10 and 30°C, respectively, in rabbit psoas fibers (a muscle composed primarily of fast fibers) exposed to pH 6.2 + 30 mM P_i. Under the same conditions, we found a 44, 41, and 50% reduction of peak force in type I, IIa, and IIx fibers, respectively, at 30°C, with greater declines at 15°C. Elevated H^+ and P_i are hypothesized to depress peak force by different mechanisms, with H^+ depressing the forward rate constant and P_i accelerating the reverse rate constant of force generation (Fig. 1, *step 3*); thus it follows that the combined effects of low cell pH and elevated P_i on peak force would be additive (22).

Effects of pH and P_i on pCa-force relationship

We demonstrate a greater rightward shift (i.e., increased Ca^{2+} for a given percentage of P_o) in the pCa-force curve at 30°C than 15°C as a result of low cell pH, implicating low pH as a more critical mediator of fatigue than previously believed on the basis of experiments carried out at supramaximal Ca^{2+} concentrations (19, 29). With low pH or low pH + P_i, temperature does not affect pCa_{50}, an effect not observed in control conditions, where temperature elevates pCa_{50} in all fiber types. While elevating temperature can attenuate the effects of low pH and P_i on P_o at supramaximal Ca^{2+} concentrations, it does not have an effect on force at suboptimal Ca^{2+} concentrations. One possible explanation for this observation is that the inhibition of force resulting from the competitive inhibition by H^+ of Ca^{2+} binding to troponin C effectively negates the increased myofibrillar Ca^{2+} sensitivity induced by increasing temperature (36, 38).

Early studies investigating the role of pH at suboptimal Ca^{2+} concentrations in skinned fibers were conducted at room temperature (22–23°C) or lower (10–15°C) (10, 15, 25) and a pH range of 6.2–7.4. Hermansen and Osnes (15) showed no significant effect of pH on the pCa-force curve at pH 6.5 vs. pH 7.0 at room temperature in rabbit soleus fibers, and at the same temperature, Fabiato and Fabiato (10) reported that pH 6.2 shifted pCa_{50} ~0.35 unit (~1 μM) compared with pH 7.0 in frog semitendinosus. Metzger and Moss (25) reported a similar 0.35 pCa unit (~1 μM) pCa_{50} shift from pH 7 to pH 6.2 at 15°C in rat soleus fibers. Our data show a larger H^+-induced shift in pCa_{50} than previously reported, with pH 6.2 shifting the pCa_{50} of type I fibers 1.21 units at 30°C and 0.66 unit (~3 μM) at 15°C. An explanation for the differences between studies is not readily apparent but could relate to sample size, which was considerably larger in our study, and slight differences in temperature. At 15°C, even small differences in temperature would result in significant changes in pCa_{50} (6, 36). Finally, in mammalian fast muscle, Palmer and Kentish (28) describe a 3.63 μM shift in pCa_{50} in pH 6.2 at 25°C, a value comparable to the 4.11 μM shift we observed in type IIx fibers at 30°C.

P_i alone (30 mM) reduced pCa_{50} more at 30°C (0.66 unit in type I fibers) than at 15°C (0.34 unit) compared with control (8) (Fig. 8). Our study has shown a greater depressive effect on myofibrillar Ca^{2+} sensitivity induced by low cell pH than P_i at 15 and 30°C. A novel result of this study is that the effects of low pH + P_i on myofibrillar Ca^{2+} sensitivity are additive at both temperatures (Tables 1–3, Fig. 8). The purpose of investigating the collective effects of low cell pH and elevated P_i was to more closely mimic in vivo fatigue in the skinned fiber preparation. We chose pH 6.2 and 30 mM P_i to represent the "worst-case scenario" in fatigued muscle (3, 24). Moopanar and Allen (26) showed that when mouse flexor digitorum brevis fibers were fatigued using 400-ms, 100-Hz tetani at 37°C, the Ca^{2+} concentration required for 50% of peak force increased by 200 nM. This is a considerably smaller shift than we show in skinned fibers in pH 6.2 + 30 mM P_i (~7 μM or 1.61 pCa units at 30°C in type I fibers). With isolated single living fibers contracting in vitro, the diffusion (intracellular-extracellular) gradient would have been high; thus it seems unlikely that pH fell to 6.2 or that P_i reached 30 mM. This would in part explain the smaller differences in function than we show in the worst-case scenario.

The rightward shift of the pCa-force curve to higher free Ca^{2+} levels as a result of low pH and low pH + P_i increased at both temperatures and in a fiber-type manner: type IIx > type IIa > type I. With high-intensity exercise, fast fibers depend more on glycolysis and, thus, produce more H^+ and P_i than slow fibers (13). This, in combination with the observation that fast fibers are more sensitive to the fatiguing effects of these ions (Tables 1–3), in part explains the increased fatigability of fast type IIx vs. slow type I fibers.

Thick-filament cooperativity assessed by n_2 is significantly depressed by 30 mM P_i at 15°C, but not 30°C, in fast fibers (8). The temperature dependence was attributed to the P_i-induced decline in the number of high-force cross bridges in fast fibers at 15°C, but not 30°C (8). Interestingly, pH 6.2 + 30 mM P_i depressed n_2 in type IIx fibers at 15 and 30°C. A possible explanation for this is that the collective effects of low pH and elevated P_i counter the elevated temperature acceleration of the low- to high-force state (Fig. 1, *step 3*) and shift the distribution of cross bridges more to a low-force or unbound state (16). Thus the decline of n_2 in the low-pH, high-P_i condition may have resulted from fewer bound cross bridges, which would reduce not only peak tension, but also the ability for one bridge to influence the binding of another.

Acidosis significantly increased the amount of Ca^{2+} (lower pCa) required to initiate the development of force (AT) in all fiber types and at both temperatures. Debold et al. (8) observed a similar effect with 30 mM P_i, except in type II fibers at 15°C, where AT was unaltered. The more pronounced effect of low pH than high P_i on AT is likely due to the competitive inhibition of H^+ on Ca^{2+} binding to troponin C (38).

In this study, we determined that, at Ca^{2+} levels characteristic of fatigue, low pH significantly depressed force at low (15°C) and near-physiological (30°C) temperatures and that, in combination, low pH and elevated P_i significantly depressed myofibrillar Ca^{2+} sensitivity and P_o to a greater extent than low pH or elevated P_i alone

(8). In fast type IIx fibers, low pH + P_i significantly depressed thick filament cooperativity, an effect primarily attributed to increased P_i, while low cell pH had a strong depressive effect on the Ca^{2+} required for initial force development (AT) in all fiber types. Coupled to our previous observation that maximal shortening velocity (V_o) and peak power are significantly depressed by low pH (19) and that peak power is significantly depressed by elevated P_i (7), it is clear that the fatigue-inducing effects of low cell pH and elevated P_i on cross-bridge function are substantial.

Acknowledgments

We thank Dr. Jeff Widrick for help with solutions.

Grants

This work was supported by a Marquette University Way Klinger Fellowship to R. H. Fitts.

Disclosures

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

Author Contributions

C.R.N. and R.H.F. are responsible for conception and design of theresearch; C.R.N. performed the experiments; C.R.N. analyzed the data; C.R.N.and R.H.F. interpreted the results of the experiments; C.R.N. prepared thefigures; C.R.N. drafted the manuscript; C.R.N. and R.H.F. edited and revisedthe manuscript; C.R.N. and R.H.F. approved the final version of the manu-script.

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