Influence of Inorganic Phosphate and pH on ATP Utilization in Fast and Slow Skeletal Muscle Fibers

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ABSTRACT The influence of P_i and pH was studied on myofibrillar ATP turnover and force development during maximally activated isometric contractions, in skinned single fibers from rabbit soleus and psoas muscle. ATP hydrolysis was coupled to the breakdown of NADH, which was monitored photometrically at 340 nm.

In psoas the depression by phosphate of force is twice that of ATP turnover, but in soleus force and ATP turnover are depressed equally by P_i. Most, but not all, of the ATPase and force values observed for a combination of high P_i and low pH could be explained by independent effects of P_i and pH.

The effects of P_i and pH on ATP turnover can be understood by a three-state cross-bridge scheme. Mass action of phosphate on the reaction from the actomyosin(AM)·ADP state to the AM·ADP·P_i state may largely account for the phosphate dependencies of ATPase activity found. Protons affect cross-bridge detachment from the AM·ADP state and the rate of the AM·ADP·P_i-to-AM·ADP transition. In this scheme, the effects of P_i and pH on cross-bridge kinetics appeared to be largely independent.

INTRODUCTION

During muscle contraction, energy turnover associated with the contractile apparatus takes place via the molecular interaction between the myosin heads and the active sites on the actin filament. This continuous process of formation and breakage of bonds, the cross-bridge cycle, is driven by the free energy change of the ATP hydrolysis reaction, of which P_i is one of the key products. With an increase in [P_i] the free energy of ATP hydrolysis decreases and product inhibition occurs, which is a commonly observed phenomenon for reversible enzyme reactions. Elevated levels of phosphate have been found to alter several parameters of contraction of both fast and slow skinned muscle fibers, such as isometric force, stiffness, tension transients, and calcium sensitivity (Hibberd et al., 1985; Nosek et al., 1987, 1990; Kawai et al., 1987; Chase and Kushmerick, 1988; Pate and Cooke, 1989b; Martyn and Gordon, 1992; Millar and Homsher, 1992; Iwamoto, 1995). Little effect on the unloaded shortening velocity and the shape of the force-velocity relation has been found (Cooke and Pate, 1985; Chase and Kushmerick, 1988).

Less is known about the influence of phosphate on energy utilization. The influence of P_i on the ATPase activity in slow muscle was not studied before, and for fast fibers data are available over a limited range of [P_i] (Webb et al., 1986; Kawai et al., 1987; Cooke et al., 1988; Bowater and Sleep, 1988). Whereas mechanical measurements reflect the faster reactions in the cross-bridge cycle, the rate of ATP hydrolysis provides information with regard to the slower steps. Hence, for the complete study of the cross-bridge kinetics, it is essential to measure both quantities. In the functioning filament array, the phosphate release step is thought to be involved in the transition to a high force-producing state, either directly (Hibberd et al., 1985) or after an isomerization (Dantzig et al., 1992).

The objective of this study is to compare the P_i dependence of isometric force and myofibrillar ATPase activity in slow and fast fibers, and to relate these observations to the current scheme of cross-bridge action. The effects of P_i concentrations between 0 and 30 mM were studied. To mimic the influence of prolonged fatigue and study possible interactions between the effects of P_i and pH (Dawson et al., 1986; Nosek et al., 1987), we also studied the effects at 30 mM P_i at pH 6.0. Our results indicate that in both fiber types P_i influences the (reverse) reaction from the force-generating to the no-force-producing state through mass action, whereas protons affect the detachment rate from the force-generating state and the reaction rate of the (forward) reaction from the no-force-generating to the force-generating state. To a first approximation, the kinetic effects of P_i and pH are independent, and the different effects on ATPase activity found in soleus and psoas can be explained by the fact that the proportions between the different rates for soleus differ from those for psoas, whereas the relative effects of P_i and pH on each of the rates could be similar in the two fiber types. Evidence will be presented suggesting that the decrease in force per force-producing cross-bridge with increasing phosphate concentration assumed for psoas (cf. Pate and Cooke, 1989a,b) is absent for soleus. The implications of these findings for muscle fatigue will be discussed.

MATERIALS AND METHODS

The experimental procedures and equipment were as described in detail previously (Potma et al., 1994a,b). In short, fiber bundles about 2 mm in

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diameter were obtained from psoas and soleus muscles of adult New Zealand white rabbits, which were anesthesized by injection of 9.6 mg · kg^{-1} fluanisone and 0.3 mg · kg^{-1} fentanyl citrate (Hypnorm; Janssen Pharmaceutica B.V., Beerse, Belgium), administered intramuscularly, and 60 mg · kg^{-1} sodium pentobarbitone in the ear vein, and exsanguinated via the carotid artery. The bundles were stored at −18°C for up to 2 months in relaxing solution containing 50% (vol/vol) glycerol (Goldman et al., 1984). Single fiber segments were isolated and immersed in relaxing solution with 1% (vol/vol) Triton X-100 (for 1 h at room temperature) to disrupt remaining membranes and remove ATPase activity of the sarcoplasmic reticulum. The 2–3-mm-long segments were mounted between a force transducer (AM801, Sensonor, Horten, Norway) and a micromanipulator by means of aluminium T-clips (Goldman and Simmons, 1984). The sarcomere length of the preparations was measured, in relaxing solution, by means of HeNe laser diffraction. The temperature was kept at 15 ± 1°C.

The composition of the relaxing, pre-activating, and activating solutions in which the fibers were incubated during the experiments was calculated using the equilibrium constants given by Fabiato (1981) and is listed in Table 1. The pH of the solutions was adjusted (at 15°C) with potassium hydroxide. The pH meter was calibrated using a phosphate buffer with an ionic stength of 200 mM (cf. Illingworth, 1981). Previous results indicate that 100 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethane-sulfonic acid provides adequate pH buffering at pH 6.0 (cf. Potma et al., 1994b; Chase and Kushmerick, 1988). For all activations it was ensured that the Ca^{2+} concentration was saturating by adding extra amounts of Ca^{2+} from a concentrated $CaCl_2$ stock during activation. Activating and pre-activating solutions with different P_i concentrations were made by mixing 0 mM P_i and 30 mM P_i solutions.

The ATPase activity of the fiber was measured by a coupled enzyme assay (cf. Glyn and Sleep, 1985). The ADP formed upon hydrolysis of ATP was resynthesized to ATP by an enzymatic coupling, which eventually resulted in the oxidation of NADH to NAD^+. This reaction sequence was catalyzed by pyruvate kinase and lactate dehydrogenase. NADH breakdown was determined photometrically from the absorbance at 340 nm of near-UV light. The ATPase activity was obtained by linear regression analysis of the absorbance signal (cf. Figs. 1 and 2). Force and absorbance signals measured in relaxing solution (pCa 9) served as a baseline for the active force and ATPase activity levels. [P_i] or pH of the relaxing solution did not significantly influence the passive force or the slope of the ATPase baseline.

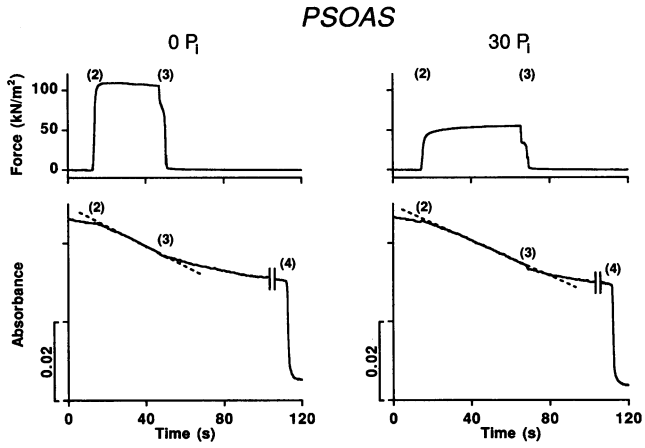


FIGURE 1 Recordings of force production (*upper traces*) and absorbance (*lower traces*) for a psoas fiber under control conditions (*left panels*, 0 mM added P_i, pH 7.1) and with 30 mM added P_i (*right panels*). When the preparation was immersed in the activating solution (pCa < 4.8) inside the 30-μl measuring chamber (*2*), active isometric force developed, and the absorbance decreased at a faster rate (*dashed line*). After the fiber was moved back into relaxing solution (pCa 9), both the force and the slope of the absorbance signal returned to their initial (baseline) values (*3*). The change in slope of the NADH absorbance signal was a measure of ATPase activity. Finally, 0.5 nmol ADP was injected into the measuring chamber to calibrate the absorbance signal (*4*). Dimensions of the fiber segment: length, 2.15 mm; diameters, 70/80 μm.

Because there was a large difference in ATP turnover between psoas and soleus fibers two different setups were used, which were both described previously (Potma et al., 1994a,b). Both setups consisted of two 80-μl troughs and a measuring chamber, in which the ATPase activity was measured. The volumes of the measuring chambers used for psoas and soleus were 30 and 4 μl, respectively. The solution inside the measuring chambers was stirred continuously. In the 30-μl chamber this was achieved via a moving membrane and in the 4-μl chamber via a syringe that injected

TABLE 1 Composition of the solutions (mM)

Solution name	$MgCl_2$	Na_2ATP	EGTA	HDTA	CaEGTA	KH_2PO_4	KProp
0 mM P_i, pH 7.1							
Relaxing	7.77	5.90	5.0	—	—	—	84.5
Pre-activating	7.67	5.90	0.5	4.5	—	—	84.7
Activation (pCa 4.7)	7.63	5.95	—	—	5.0	—	84.6
30 mM P_i, pH 7.1							
Relaxing	9.18	5.89	5.0	—	—	30.0	0.91
Pre-activating	9.09	5.89	0.5	4.5	—	30.0	1.10
Activation (pCa 4.8)	9.04	5.93	—	—	5.0	30.0	1.06
0 mM P_i, pH 6.0							
Relaxing	6.59	8.33	5.0	—	—	—	118.4
Pre-activating	6.58	8.33	0.5	4.5	—	—	118.4
Activation (pCa 3.6)	6.58	8.86	—	—	5.0	—	116.7
30 mM P_i, pH 6.0							
Relaxing	6.88	8.37	5.0	—	—	30.0	65.3
Pre-activating	6.87	8.37	0.5	4.5	—	30.0	65.3
Activation (pCa 3.7)	6.87	8.88	—	—	5.0	30.0	63.7

In addition, all solutions contained 1 mM free Mg^{2+}, 5 mM MgATP, 100 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethane-sulfonic acid (BES), 10 mM phosphoenol pyruvate, 4 mg/ml pyruvate kinase (470 units/mg, Sigma Chemical Co., St. Louis, MO), 0.24 mg/ml lactate dehydrogenase (710 units/mg, Sigma), 5 mM sodium azide, 10 μM oligomycin B, 0.8 mM NADH, and 0.2 mM p^1, p^5-di(adenosine-5′)pentaphosphate. Relaxing solutions contained 20 mM ethylene glycol-bis(β-amino-ethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pre-activating solutions 0.5 mM EGTA and 19.5 mM hexan-diamin-otetraacetic acid (HDTA), and activating solutions 20 mM CaEGTA. Potassium propionate (KProp) was added to adjust ionic strength to 200 mM.

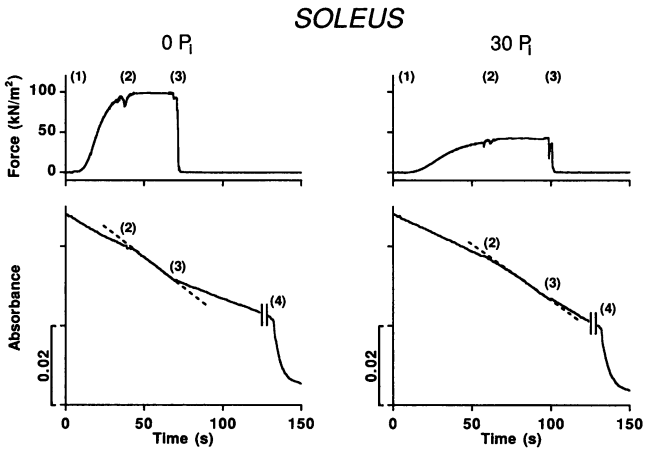


FIGURE 2 Recordings of force production (*upper traces*) and absorbance (*lower traces*) for a soleus fiber in solutions with 0 mM added P_i (*left*) and with 30 mM added P_i (*right*). To avoid touching of the walls, soleus fibers were first transferred from relaxing solution into a trough that contained activating solution (*1*) before they were placed in the 4-μl measuring chamber (*2*). Subsequently, the fibers were immersed in relaxing solution again (*3*), and 0.05 nmol ADP was injected (*4*). As the fiber was placed in the measuring chamber (*2*) NADH breakdown was accelerated (*dashed line*), and after it was taken out again (*3*) the slope of the absorbance signal returned to its original value. ATPase activity followed from the change in slope of the NADH absorbance signal. Dimensions of the fiber segment: length, 1.85 mm; diameters, 70/130 μm.

and aspirated a small volume of 0.4 μl. UV light from a 75-W xenon arc lamp passed through the chamber beneath the preparation. The transmitted light was monitored by two UV-enhanced photodiodes at 340 and 400 nm, the latter of which provided a reference signal, independent of NADH concentration. The absorbance signal was calibrated at the end of each recording by adding 0.05 and 0.005 μl (for the 30- and 4-μl chambers, respectively) of 10 mM ADP by means of a stepper-motor controlled injector. The force and absorbance signals were filtered at 1 kHz and 2.5 Hz, respectively (−12 dB/oct). The data were recorded on a chart recorder and on a computer at a sampling rate of 5 Hz.

Psoas fibers were transferred from a trough with relaxing solution (low calcium concentration, high calcium buffering) into a trough with pre-activating solution (low calcium concentration, low calcium buffering, same [P_i] and pH as the activating solution tested) after which activation took place inside the measuring chamber, which contained activating solution (saturating calcium concentration). To prevent soleus fibers from touching the sides of the very narrow chamber (width 0.4 mm), they were activated before being transferred into the 4-μl chamber, in a trough that contained the same (activating) solution as the measuring chamber. For soleus the low EGTA, pre-activating solution was not used. For both psoas and soleus, relaxing solutions were used that had the same pH as the activating solution used for the contraction under study. For activations at low [P_i] ($\leq$5 mM) relaxing solutions with 0 P_i were used, and for high [P_i] ($\geq$10 mM) relaxing solutions with 30 mM P_i were used.

After the first activation (0 P_i, pH 7.1) fiber length was readjusted such that sarcomere length was 2.4 μm, and the fiber dimensions were measured. After that, sarcomere length usually remained stable throughout the experiment. Thereafter the activation at 0 P_i and pH 7.1 was repeated (first control), followed by two or three isometric activations in solutions at different pH and/or with added P_i, and again a control contraction. ATPase activity and force were corrected for fiber deterioration by linear interpolation between control values. The intermediate isometric results were normalized to the interpolated values. The experiments were stopped when the force during a control activation was less than 80% of the force during the first activation, and all measurements from the former control activation onward were discarded.

Isometric contractions were performed at seven phosphate concentrations ranging between 0 and 30 mM. Moreover, a possible interaction between the effects of P_i and pH was tested in paired experiments in which the influence of a combination of high P_i and low pH was compared to the effects of high P_i or low pH alone. In the latter set of experiments the three conditions under study, i.e., 30 mM P_i, pH 7.1; 0 P_i, pH 6.0; and 30 mM P_i, pH 6.0 were tested in random order, preceded and followed by controls (0 P_i, pH 7.1).

The rise of phosphate concentration inside the measuring chamber during the measurements (due to the ATP hydrolyzed) was small. Typically about 0.3 nmol for psoas and 0.03 nmol for soleus (i.e., about 0.01 mM in both cases) of P_i was formed during contraction (cf. Figs. 1 and 2).

Values are given as means ± SEM. All statistical statements are based on two-tailed Student's *t*-tests ($p < 0.05$). For the combined experiments at high P_i and low pH, paired *t*-tests were performed.

RESULTS

The protocols used during the experiments for psoas and soleus fibers are illustrated in Figs. 1 and 2. In these figures, the force and absorbance signals from a control experiment without added phosphate (*left panels*) and with 30 mM added phosphate (*right panels*) are shown. Psoas fibers (Fig. 1) were transferred from pre-activating solution into the measuring chamber, where active force developed. Soleus fibers (Fig. 2) were first transferred from relaxing solution into a trough in which activation took place, before they were brought into the measuring chamber. The fact that the activating solution inside this activating trough was not stirred and the high calcium buffering of the relaxing solution carried with the fiber resulted in a slower force development in soleus fibers than in psoas fibers. Whereas, with respect to 0 P_i solution, force development was slowed down in 30 mM P_i solution, it was accelerated in the solutions with pH 6.0. The fast force development in pH 6.0 solutions was probably due to the reduced Ca^{2+} buffering capacity of EGTA at low pH (this was also reflected in a relatively slow relaxation when the fiber was returned to relaxing solutions with pH 6.0). The deceleration of force rise at high [P_i] can be explained by a decrease in free Ca^{2+} concentration (cf. Table 1) and in calcium sensitivity (Brandt et al., 1982; Nosek et al., 1990; Fryer et al., 1995). The steady decline in absorbance when there was no preparation inside the measuring chamber, i.e., the baseline, was caused by a contaminating ATPase in the enzymes used and by NADH bleaching under UV light. When a fiber entered the measuring chamber, a faster decline in absorbance occurred, resulting from the actomyosin ATPase inside the fiber. From their respective measuring chambers psoas and soleus fibers were returned to relaxing solution, and force and absorbance signals returned to their baselines. The ATPase activity measured in relaxing solution (pCa 9), which was used to correct the Ca^{2+}-activated ATPase activity, appeared to be independent of P_i and pH and amounted to 7 ± 2% and to 8 ± 3% of the average ATPase under control conditions, for psoas and soleus, respectively (n = 13 for both fiber types). It could originate from residual membrane-bound ATPases or, more likely, from Ca^{2+}-independent myosin ATPase activity.

The average isometric ATPase activity, A_0, and force level, F_0, during the first control contractions, for psoas fibers were 0.41 ± 0.03 mM/s and 130 ± 5 kN/m^2 (n = 13), respectively. For soleus fibers A_0 and F_0 amounted to 0.050 ± 0.003 mM/s and 110 ± 5 kN/m^2 (n = 13). Assuming a myosin concentration of 0.2 mM (e.g., Glyn and Sleep, 1985) the ATPase activities found are equivalent to a rate of ATP turnover per myosin head of 2.0 ± 0.2 per second for psoas and of 0.25 ± 0.02 per second for soleus.

Effects of P_i

Comparing Figs. 1 and 2 it can be seen that the influence of phosphate in psoas and soleus fibers is different. At 30 mM P_i the relative decreases in ATPase activity and force in soleus are similar, but in psoas the reduction in ATPase activity is less than half of the reduction in force. The P_i dependencies of isometric ATPase activity and force for the complete set of experiments in which [P_i] was varied are summarized in Fig. 3. This figure shows normalized isometric ATPase activity and force as a function of the P_i concentration for psoas and soleus. ATPase activity and force decrease steadily as [P_i] is increased. For soleus fibers the decrease in ATPase activity and force is the same: for all P_i concentrations investigated, normalized ATPase activity and force were not significantly different. However, for psoas fibers ATPase activity decreases less than force. For 2, 10, 20, and 30 mM P_i the difference between ATPase activity and force is significant. From the error bars in Fig. 3 it can be seen that ATPase activity shows a larger variability than corresponding average force values. This is probably due to small variations in temperature during the experiments, because ATPase activity is more sensitive to temperature than force.

If it is assumed that the force produced by individual cross-bridges in a half-sarcomere is additive, economy can be expressed as follows (cf. Schramm et al., 1994):

$$\text{Economy} = \frac{\text{Force per cross-sectional area/length of a half-sarcomere}}{\text{ATPase per fiber volume/Avogadro's number}}$$

Under control conditions, economy for psoas and soleus fibers amounts to 0.44 ± 0.04 and to 3.1 ± 0.2 pN · s per molecule of ATP hydrolyzed. Therefore, during isometric contractions, soleus is 7 times more economical than psoas. Whereas the economy of soleus is not affected by P_i concentration, psoas fibers become less economical as [P_i] is increased.

Combined effects of P_i and pH

During fatigue, pH and the P_i concentration may change concomitantly. To mimic this condition and to study possible interactions between P_i and pH, we measured the effects of a combination of 30 mM added P_i and pH 6.0 and compared these, in paired experiments, with the effects of 30 mM P_i (at pH 7.1) and pH 6.0 (at 0 P_i), separately. In Fig. 4 the results of these experiments for psoas and soleus are shown. Obviously, the interventions have a different influence in psoas and soleus, especially on ATPase activity. For psoas, ATPase activity at 30 mM P_i, pH 7.1; 0 P_i, pH 6.0; and 30 mM P_i, pH 6.0 is decreased with respect to the standard conditions. However, for soleus at 0 P_i, pH 6.0, ATPase activity is higher than A_0, and at 30 mM P_i, pH 6.0 it is not significantly different from A_0.

For each set of paired measurements the product of the normalized ATPase activities (or forces) at 30 mM P_i, pH 7.1 and at 0 P_i, pH 6.0 was calculated and compared to the

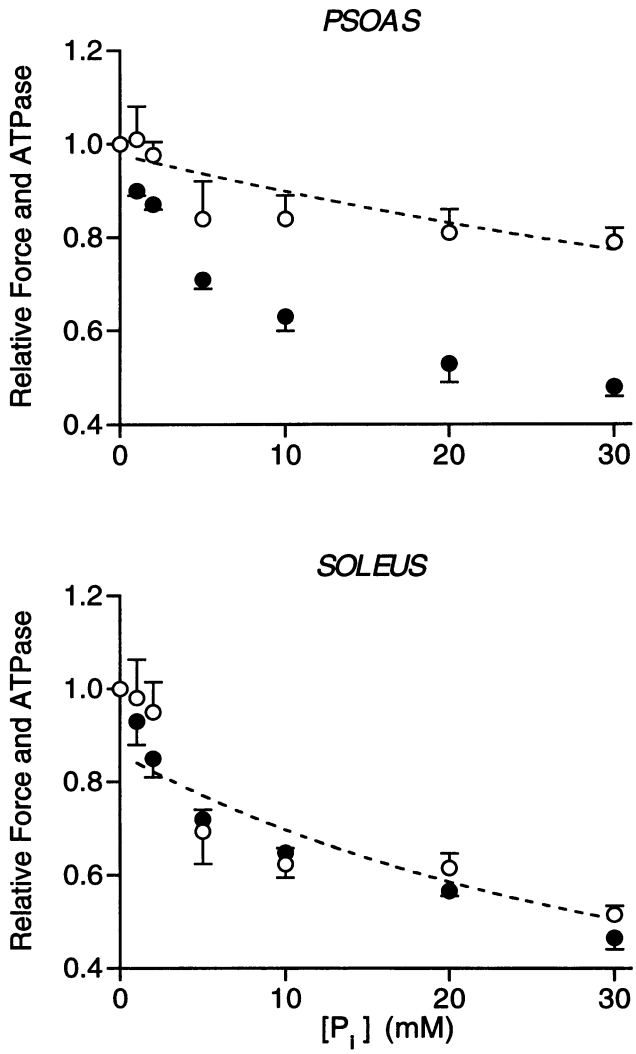


FIGURE 3 Isometric ATPase activity and force as a function of P_i concentration in psoas (*upper panel*) and soleus (*lower panel*) fibers. ATPase activity (○) and force (●) were normalized to control values with no phosphate added to the solution. Data points represent means ± SEM of 5 or more observations. The dashed lines represent the best fits to the ATPase data points (weighed by their respective SEMs) based on the three-state cross-bridge model, as described in the Appendix. It is assumed that mass action of phosphate alone is responsible for the influence of [P_i] on ATPase activity.

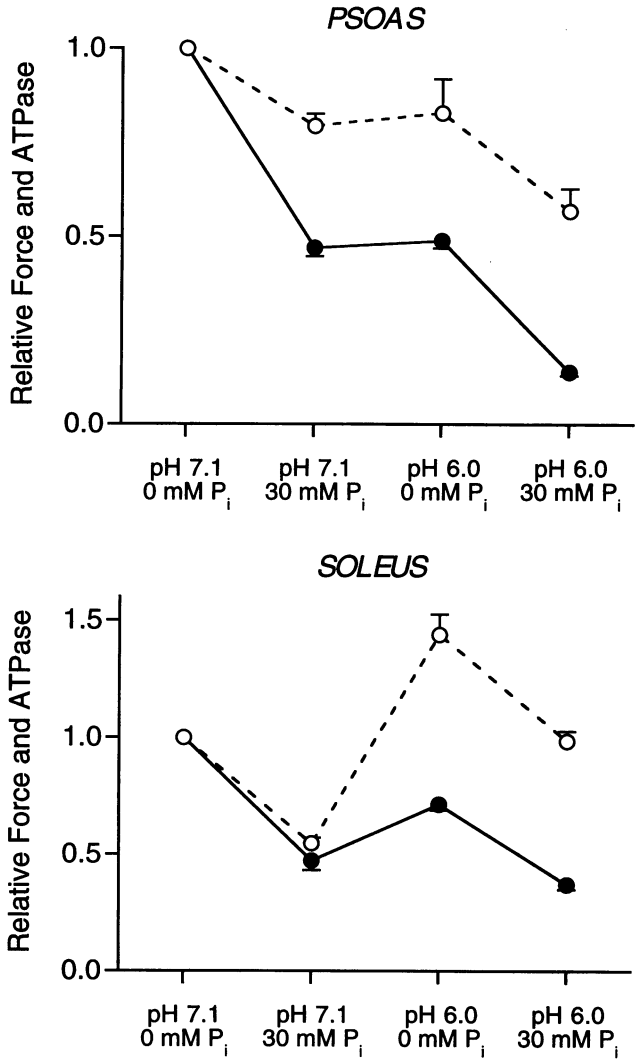


FIGURE 4 The effects on ATPase activity and force of a combination of high [P_i] and low pH compared to the individual effects of high [P_i] and low pH, for psoas (*upper panel*) and soleus (*lower panel*). Isometric ATPase activity (○) and force (●) for the different (P_i, pH) combinations (*abscissa*) were normalized to control values with zero added P_i and pH 7.1. Data points represent means ± SEM (for psoas $n = 9$; for soleus $n = 5$).

value found for the combined interventions 30 mM P_i, pH 6.0 in a paired t-test ($p < 0.05$). For psoas the ATPase found for the combined intervention, $(0.57 \pm 0.07) \cdot A_0$, was not significantly different from the product of the ATPase activities, i.e., $(0.66 \pm 0.08) \cdot A_0$. Force for the combined intervention, $(0.14 \pm 0.01) \cdot F_0$, however, was significantly different from the product of forces, $(0.23 \pm 0.02) \cdot F_0$ ($n = 9$). For soleus both ATPase, $(0.98 \pm 0.04) \cdot A_0$, and force, $(0.37 \pm 0.02) \cdot F_0$, for the combined intervention were not significantly different from the product of the ATPase activities, $(0.78 \pm 0.05) \cdot A_0$, and the force product, $(0.34 \pm 0.04) \cdot F_0$ ($n = 5$), respectively. However, for soleus the independence of the effects on ATPase activity of the interventions is a borderline case, because in an unpaired t-test for soleus the ATPase activity of the combined intervention was significantly different from the product.

In Fig. 5 the data are represented as relative isometric tension cost, i.e., the ratio of normalized ATPase activity and normalized force (where tension cost under control conditions, $tc_0 = 1$). In absolute terms tension cost for psoas is 6.9 ± 0.8 times tension cost for soleus, under control conditions. For psoas, all three interventions increase tension cost, for soleus tension cost was not affected by P_i concentration. Under mimicked fatigue conditions, the increase in tension cost (and consequently the decrease in economy) was larger in psoas ($tc = (4.1 \pm 0.5) \cdot tc_0$) than in soleus ($tc = (2.7 \pm 0.2) \cdot tc_0$), mostly because of the large force reduction for psoas.

DISCUSSION

The major findings of this study are: 1) in soleus isometric ATPase activity and force are decreased in proportion as [P_i] is increased, whereas in psoas ATPase activity is decreased less than force; 2) for low pH as well as for a combination of high [P_i] and low pH, ATPase activity is decreased less than force in both fiber types; and 3) the effects of P_i and pH are not always independent.

Comparison with previous studies

The values obtained for isometric ATPase activity and force at pH 7.1 and zero P_i added (130 ± 5 kN/m^2 and 2.0 ± 0.2 per myosin head per second for psoas, and 110 ± 5 kN/m^2 and 0.25 ± 0.02 per myosin head per second for soleus, respectively) correspond well with previous results (Gibbs and Gibson, 1972; Wendt and Gibbs, 1973; Glyn and Sleep,

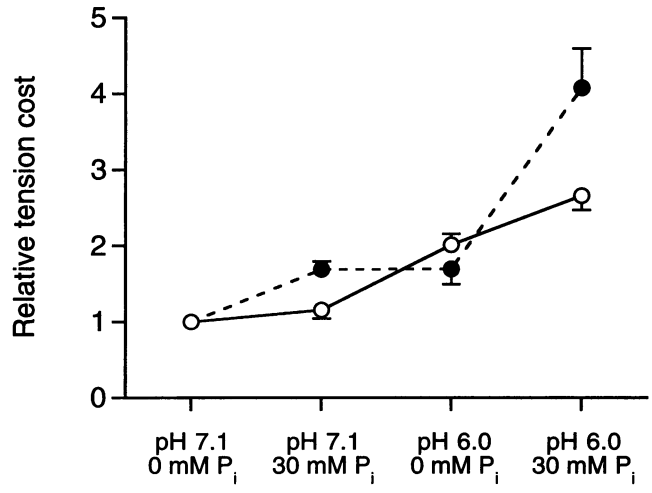


FIGURE 5 The effects on relative tension cost of high [P_i], of low pH, and of their combination, for psoas and soleus fibers. Isometric tension cost for psoas (●) and soleus (○) were normalized to control values with zero added P_i and pH 7.1. Data points represent means ± SEM, number of observations as in Fig. 4.

1985; Kawai et al., 1987; Cooke et al., 1988; Barclay et al., 1993; Potma et al., 1994b).

Effects of P_i

Previously studies were performed on the effects of P_i on isometric force in skinned fibers from fast muscle (Cooke and Pate, 1985; Pate and Cooke, 1989b; Millar and Homsher, 1990; Dantzig et al., 1992; Martyn and Gordon, 1992) and from slow muscle (Altringham and Johnston, 1985; Nosek et al., 1990; Stienen et al., 1992; Millar and Homsher, 1992; Fryer et al., 1995). The influence of P_i on ATPase activity in fast fibers was reported by Webb et al. (1986), Kawai et al. (1987), Cooke et al. (1988), and Bowater and Sleep (1988).

To compare these findings to our data phosphate concentrations inside the preparations have to be estimated. By means of ^{31}P-NMR we found that our control solution, which used phosphoenol pyruvate as the ATP-regenerating system (instead of PCr, used in most other studies), contained a contaminating $[P_i]$ of 0.09 mM. Moreover, during contraction P_i accumulates in the fiber because of ATP hydrolysis. The average additional P_i concentration is proportional to the ATPase activity (i.e., 0.41 and 0.050 mM/s for psoas and soleus, respectively) and the square of fiber diameter (86 ± 4 μm and 87 ± 3 μm, for psoas and soleus, respectively). Computation of this $[P_i]$ (cf. Stienen et al., 1990; Pate and Cooke, 1989b) using a diffusion constant for P_i at 15°C of 2.4×10^{-10} m^2/s (cf. Yoshizaki et al., 1982) showed that the average values of additional $[P_i]$ due to ATP hydrolysis were 0.4 and 0.05 mM, inside psoas and soleus fibers, respectively. After correction of the nominal P_i concentration for P_i accumulation and contamination a logarithmic function was fitted to the measured ATPase and force values, weighed by their SEMs. The values found with no P_i added to the solution were not taken into account, because a small error in the estimate of the P_i accumulation would have a disproportionally large impact on the fit, especially in the case of soleus. The following relations were obtained for psoas: relative ATPase = $(1.04 \pm 0.03) - (0.17 \pm 0.03) \times \log[P_i]$ ($R = 0.98$); relative force = $(0.97 \pm 0.01) - (0.33 \pm 0.02) \times \log[P_i]$ ($R = 0.99$); and for soleus: relative ATPase = $(0.98 \pm 0.05) - (0.31 \pm 0.04) \times \log[P_i]$ ($R = 0.98$); relative force = $(0.95 \pm 0.03) - (0.30 \pm 0.02) \times \log[P_i]$ ($R = 0.99$).

Wilson et al. (1995) showed that the pooled data from the studies on fast muscle (normalized by setting the force obtained in the presence of 5 mM P_i to 100%) produce a linear relationship between isometric force and $\log[P_i]$, with a slope of −41.4% per decade. Our data are in good agreement with this finding, because if we normalize to the value at 5 mM P_i, a decline of 44 ± 2% per decade is observed. Comparisons of the P_i dependence of isometric force in fast and slow fibers showed that fast fibers can be more sensitive to P_i than slow ones (Millar and Homsher, 1992; Stienen et al., 1992), less sensitive (Fryer et al., 1995), or (as in this study) equally sensitive (Nosek et al., 1990). The data from the latter study, on rabbit psoas and soleus, quantitatively showed excellent agreement with our findings. The difference found with the study of Fryer et al. can be attributed to species and/or muscle fiber type, because they used extensor digitorum longus and soleus from rat. Their soleus data are quantitatively very similar to ours, but the rat extensor digitorum longus appears to be more sensitive to P_i than rabbit psoas. Some of the apparently lower-force inhibition in rabbit soleus fibers found by Stienen et al. (1992) and by Millar and Homsher (1992) may be explained as follows. In our study, especially in the case of soleus, the intracellular $[P_i]$ in solutions with zero added P_i is substantially lower than in these studies, because of the lower level of P_i contamination. Moreover, P_i accumulation inside their fibers may have been higher because their solutions were not stirred. As a consequence there will be a difference in $[P_i]$ and thus force, at which the results are normalized.

The effect of P_i on ATPase activity that we observed in psoas fibers is in good agreement with previous comparisons of the ATPase at a low and a higher P_i in rabbit psoas by Webb et al. (1986) and Cooke et al. (1988). Quantitative agreement with the studies of Kawai et al. (1987) and Bowater and Sleep (1988), in which the ATPase for a range of phosphate concentrations between 0 and 20 mM was measured, is excellent.

Combined effects of P_i and pH

In our earlier study on the pH effects on isometric ATPase activity and force (Potma et al., 1994b) the lowest pH investigated was 6.4. After renormalization on pH 7.3, the values for 0 P_i, pH 6.0 found in this study can be compared with these previous findings. Whereas for psoas ATPase activity was constant between pH 6.4 and 7.9, a 20% decrease was seen from pH 6.4 and 6.0. This observation agrees with that of Cooke et al. (1988), who found in skinned fibers from rabbit psoas muscle that, as pH was increased from 6 to 7, ATPase was increased by about 25%, with almost all of the change occurring between pH 6 and 6.5 (10°C). The values we found for force for psoas and for ATPase activity and force for soleus at pH 6.0 all are logical continuations of the trends seen between 7.9 and 6.4. Moreover, the force values found at pH 6.0 show good quantitative agreement with those of Chase and Kushmerick (1988) for both fiber types.

For each pH value between 7.9 and 6.0, normalized tension costs for psoas and soleus fibers were not significantly different (unfortunately in Potma et al., 1994b, it was erroneously stated that the values at pH 6.4 were significantly different), and the tension costs found at pH 6.0 agree with the trend seen between pH 7.9 and 6.4. Consequently, an important observation of Potma et al. (1994b) also holds for pH 6.0, namely that normalized tension cost shows essentially the same increase with decreasing pH in fast and slow fibers.

Our finding that the depression of isometric force by a combination of high P_i and low pH in psoas was larger than expected on the basis of the effects of the interventions applied separately agrees with the findings of Nosek et al. (1987, 1990), Cooke and Pate (1985), and Cooke et al. (1988). Chase and Kushmerick (1988) and Pate and Cooke (1989b) found that the effects on force of P_i and pH were independent. The differences may be accounted for by the smaller pH decrease used by Pate and Cooke (from 7 to 6.2) and the smaller P_i increase applied by Chase and Kushmerick (up to 15 mM instead of 30 mM). Moreover, mimicking the conditions of Chase and Kushmerick, Nosek et al. (1990) found that in psoas P_i had a stronger effect at pH 6.0 than at pH 7.1. For soleus fibers, this synergistic influence of P_i and pH on force was not found by Nosek et al. (1990) or in this study. In agreement with our observation for psoas fibers, Cooke et al. (1988) found that the effects on ATPase activity of a P_i change (from 3 to 20 mM) and a pH change (from 7 to 6) were independent.

The new elements provided by our comparative study, the effect of P_i on ATPase in soleus and the combined effects of P_i and pH on ATPase in psoas and soleus, will be discussed below.

Implications on the cross-bridge level

The basis of the metabolic effects on myofibrillar energy utilization and force resides in the kinetic scheme for the actomyosin ATPase activity. During muscle contraction there exists a cyclic interaction between myosin and actin, the forming and breaking of cross-bridges, which is driven by the energy available from hydrolysis of ATP into ADP and P_i. It is not firmly established which transition limits the overall cycling rate, i.e., the steady-state ATPase rate. During a maximal isometric contraction, for fast muscle the rate-limiting step is considered to be an isomerization after P_i release. In slow muscle it is likely that a number of steps contribute to the steady-state ATPase, and no single species will predominate during isometric contractions (Millar and Homsher, 1992). Caged ATP experiments in the presence of P_i (Hibberd et al., 1985) and comparisons of the influence of P_i on stiffness and force (Hibberd et al., 1985; Kawai et al., 1987; Martyn and Gordon, 1992; Iwamoto, 1995) indicate that P_i affects the (reversible) transition of attached cross-bridges from the force-producing AM · ADP state, to the no-force or low-force AM · ADP · P_i state. The observation that force decreases linearly with the logarithm of P_i is compatible with models in which a major force-producing state occurs subsequent to P_i release. Based on thermodynamic considerations, the model of Pate and Cooke (1989a) predicts, for a maximally activated isometric contraction, linear relationships between force and log [P_i] and between ATPase and log [P_i].

The main finding of the present study is that, whereas the effects of P_i on force are similar in soleus and psoas fibers, the inhibition by P_i of ATPase activity in soleus is about twice as strong as in psoas and equal to the inhibition of force. The influence of the ligand concentrations on the free energy of hydrolysis of MgATP is given by $\Delta G^{ATP} = \Delta G^0 + RT \ln([ATP]/[ADP][P_i])$, where ΔG^0 is the standard free energy, R is the Boltzmann constant, and T is the absolute temperature. Raising [P_i] will lower the free energy of hydrolysis of MgATP and therefore decrease the ability of the cross-bridges to perform work. For a certain increase in [P_i], ΔG^{ATP} and thus the work the cross-bridges can perform are decreased by the same amount in soleus and psoas. Because the length of the cross-bridge stroke probably is not affected by P_i, our finding that average force goes down by the same fraction in both fiber types seems plausible.

A decrease in ATPase activity with increasing P_i can be caused by a decrease in the number of force-producing (AM · ADP) cross-bridges (resulting from an increase of the rate of the AM · ADP to AM · ADP · P_i transition) and/or by a decrease of the forward rate at which AM · ADP cross-bridges are detached. For psoas, a substantial decrease of the latter detachment rate is unlikely, because P_i has a very small (or no) influence on unloaded shortening velocity (Cooke and Pate, 1985; Pate and Cooke, 1989b) and on the rate of tension convergence upon photoliberation of caged ATP (Hibberd et al., 1985). To explain that for psoas ATPase activity is affected less than force, Pate and Cooke (1989a,b) hypothesized that P_i has its greatest effect on highly strained cross-bridges, which dominate isometric tension.

For soleus, however, we found that ATPase activity and force were equally depressed. If P_i would not affect the detachment rate in soleus too, then with increasing P_i, the number of force-producing cross-bridges should decrease twice as much in soleus as in psoas. Because the effect on force is the same in both fiber types, this would imply that these bridges in soleus have an entirely different strain distribution at low P_i than in psoas. Alternatively, for soleus the detachment rate of force-producing cross-bridges may be decelerated by P_i, but a twofold reduction would be required for P_i to have a similar effect on the strain distribution in soleus and psoas. On the basis of our present findings we cannot decide which of these possibilities provides the predominant explanation of the differences between slow and fast fibers. An important role may be played by a major difference in kinetics, namely that whereas in psoas detachment from the force-producing state is very slow compared to the other transitions, in soleus this may not be the case (Millar and Homsher, 1992). Irrespective of the exact mechanism, an important conclusion from our experiments is that the hypothesis put forward by Pate and Cooke does not apply for soleus.

At low [P_i], a decrease in pH probably slows down the forward reaction from the AM · ADP · P_i to AM · ADP state and accelerates detachment of AM · ADP cross-bridges (Metzger and Moss, 1990a,b; Cooke et al., 1988; Potma et al., 1994b). If pH would have an influence on the backward rate from AM · ADP to AM · ADP · P_i, it would influence the cross-bridge cycle at high [P_i], but this might

not be detectable at low P_i. As explained in more detail in the Appendix, the independence of the effects of P_i and pH on ATPase which is found in psoas and, to a lesser degree, in soleus, argues against a substantial influence of pH on the backward rate from AM · ADP to AM · ADP · P_i. The synergistic influence of P_i and pH on force that was found in psoas but not in soleus may also be a consequence of a very different strain distribution of force-producing cross-bridges at low P_i in soleus in comparison with psoas.

In the Appendix a quantitative analysis, in terms of the kinetics of a three-state cross-bridge model, is shown of the effects of P_i and pH, seperately and in combination, in soleus and psoas fibers, assuming that P_i affects the transition from the AM · ADP to the AM · ADP · P_i state but has no effect on detachment from the force-producing AM · ADP state. The rate constants obtained at 30 mM P_i, pH 7.1 and at 0 P_i, pH 6.0 give a satisfactory prediction of the ATPase activity at 30 mM P_i, pH 6.0. These calculations also suggest that the different influences of P_i and pH on ATPase found for soleus and psoas are explained by a difference in proportions between the rates with respect to each other rather than a very different influence of P_i or pH on the rates per se.

Relevance to muscle fatigue

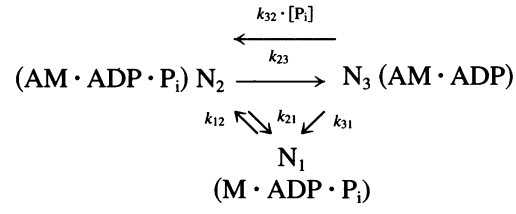
When a muscle fiber maintains a high level of activity for an extended time a number of metabolites, such as P_i, H^+ and MgADP, may accumulate, resulting in impaired contractile function (e.g., Dawson et al., 1978; Edman and Matiazzi, 1981; Crow and Kushmerick, 1982; Westerblad et al., 1991; Nagesser et al., 1992). Maximum contraction velocity, peak tetanic tension, twitch tension, and rate of MgATP hydrolysis all decline, along with slower tension transients and relaxation rates (Dawson et al., 1978; Edman and Matiazzi, 1981; Crow and Kushmerick, 1982). To investigate the mechanisms of fatigue on the level of the contractile proteins the influence of the metabolites on the energetics and mechanics of contraction was studied in skinned fiber preparations (Cooke and Pate, 1985; Kawai et al., 1987; Chase and Kushmerick, 1988; Nosek et al., 1990; Potma et al. 1994b; Fryer et al., 1995). Cooke et al. (1988) showed for glycerinated rabbit psoas fibers that the decrease in isometric force and ATPase activity due to increased levels of both phosphate and protons is similar in magnitude to that observed in living fibers, from which they concluded that end-product inhibition by P_i and H^+ is a major determinant of the fatigue process.

Resting slow-twitch fibers contain more P_i (5–10 mM) than fast-twitch fibers (1–3 mM), and during fatiguing stimulation the $[P_i]$ in fast and slow fibers can approach 50 and 25 mM, respectively (cf. Fryer et al., 1995). Because force depends on $\log[P_i]$, force inhibition by phosphate accumulation in fast muscle will be larger than in slow muscle. Moreover, because the actomyosin ATPase activity in slow muscle is much lower, accumulation of P_i and H^+ will be less in slow fibers, if the extrusion mechanisms are equally effective. In this study we have shown that with an increase in $[P_i]$ (isometric) economy in fast fibers goes down, but in slow fibers it remains constant. Finally, concurrent increases in $[P_i]$ and $[H^+]$ result in a higher increase in tension cost for fast than for slow fibers. This difference is exacerbated by the synergistic effect of P_i and pH on force, which occurs in fast-twitch but not in slow-twitch fibers. All of these findings are in agreement with the observation that slow muscle is less susceptible to fatigue than fast muscle (e.g., Westerblad et al., 1991).

APPENDIX

Influence of P_i and pH on the rate constants in a three-state model

Provided that P_i only affects the back rate of the transition between the AM · ADP · P_i and AM · ADP states through mass action, in a three-state cross-bridge model (Scheme 1) the effects of P_i and pH on the reaction rate constants can be derived. These effects can be obtained from the effects of P_i and pH on ATPase activity and force found in this study, and rate constants obtained previously (cf. Potma et al., 1994a,b):



(Scheme 1)

where M is myosin, AM is actomyosin, N_1 is detached cross-bridges, N_2 is no-force-producing cross-bridges, N_3 is force-producing cross-bridges, k_{12} is the attachment rate, k_{21} is the reverse attachment rate, k_{23} is the rate of myosin isomerization and/or P_i release, $k_{32} \cdot [P_i]$ is the reverse rate of myosin isomerization and/or P_i release, and k_{31} is the rate of ADP release and/or cross-bridge detachment. Solving the differential equations for this scheme (cf. Potma et al., 1994b) for the isometric steady state, the ATPase activity per myosin head as a function of $[P_i]$ is given by the following (hyperbolic) relation:

$$\text{ATPase} = k_{31} \cdot N_3 = \frac{k_{12}k_{23}k_{31}}{k_{12}k_{23} + k_{12}k_{31} + k_{21}k_{31} + k_{23}k_{31} + (k_{12}k_{32} + k_{21}k_{32}) \cdot [P_i]}$$

For maximally activated fibers, neither a P_i nor a pH dependence for cross-bridge attachment has been reported (cf. Iwamoto, 1995, who found that calcium and P_i affect different steps in the actomyosin ATPase cycle). Therefore we assume that k_{12} and k_{21} are independent of P_i and pH, and use values from the literature. Because for psoas the maximum shortening velocity (Cooke and Pate, 1985; Pate and Cooke, 1989b) and the rate of the tension transient after photolysis of caged ATP (Hibberd et al., 1985) are independent of P_i, k_{31} is probably independent of P_i for psoas. The modeling for soleus was performed under the same (simplifying) assumption, i.e., a P_i-independent k_{31}. With k_{31} from our earlier studies (Potma et al., 1994a,b), the forward and reverse rate constants, k_{23} (s^{-1}) and k_{32} ($mM^{-1}\ s^{-1}$), can be derived from the equation given above by fitting the ATPase as a function of P_i.

For low $[P_i]$, where $k_{32} \cdot [P_i]$ is negligible, we have shown before that the influence of pH on ATPase activity, force, stiffness, and the rate of tension redevelopment after unloaded shortening can be explained by

changes in k_{31} and k_{23} (Potma et al., 1994b). It is not necessary to incorporate an influence of pH on the force per AM · ADP cross-bridge, and thus the effect of pH on k_{31}, the rate constant of detachment from the force-producing state, is proportional to the effect of pH on isometric tension cost. The influence of pH on k_{23} then follows from the effect of pH on ATPase activity.

As a first approximation we modeled the influence of a combination of high $[P_i]$ and low pH as if the effects on the rate constants of the two interventions were independent. It should be noted that this implies that k_{32} would be independent of pH. Using the effects on the rates of the interventions applied separately, their combined influence on the ATPase activity can be predicted and compared to the actual values found.

Psoas fibers

The values of k_{12} and k_{21} for psoas fibers, at 15°C, can be estimated from values at 20°C, given by Zhao and Kawai (1993), assuming a Q_{10} of 2: $k_{12} = 75\ s^{-1}$, $k_{21} = 64\ s^{-1}$. The detachment rate k_{31} at pH 7.1 was obtained previously (Potma et al., 1994a): $k_{31} = 2.8\ s^{-1}$. In the top panel of Fig. 3 the dashed line shows the hyperbolic fit, corresponding to mass action of P_i. This fit ($R = 0.92$) of the ATPase activity as a function of $[P_i]$ was performed after correction for P_i accumulation inside the fiber due to ATP hydrolysis (cf. Discussion). Means were weighed by their SEMs, and the ATPase value found at zero added P_i was not used. The rate constants that correspond to this fit are $k_{23} = 15\ s^{-1}$ and $k_{32} = 0.09\ mM^{-1}\ s^{-1}$. Force per force-producing AM·ADP cross-bridge decreased by a factor of 1.7 between 0 and 30 mM P_i (cf. Pate and Cooke, 1989a,b). As pH decreases from 7.1 to 6.0, k_{31} increases, with tension cost, from 2.8 to 4.7 s^{-1}, and k_{23} decreases from 15 to 5 s^{-1}. Force per AM · ADP cross-bridge (by choice) is not affected by pH. Finally, solving the (forward) problem with the rates found at 0 P_i, pH 7.1; 30 mM P_i, pH 7.1; and 0 P_i, pH 6.0, the predicted ATPase activity at 30 mM P_i, pH 6.0 was 0.61 times the "predicted" ATPase activity at 0 P_i, pH 7.1. The measured value at 30 mM P_i, pH 6.0 amounted to $0.57(\pm 0.06) \cdot A_0$. The agreement between these values reflects the independence of the effects of P_i and pH on ATPase. Force per AM · ADP cross-bridge, however, is decreased by a factor of 2.4, reflecting the synergistic effect of P_i and pH on force.

Soleus fibers

For soleus fibers, values of the rate constants k_{12} and k_{21} were estimated from values given by Wang et al. (1994) at 20°C, assuming a Q_{10} of 2: $k_{12} = 3\ s^{-1}$, $k_{21} = 6.9\ s^{-1}$. The detachment rate at pH 7.1 was derived from Potma et al. (1994b): $k_{31} = 0.32\ s^{-1}$. The hyperbolic function shown by the dashed line in the bottom panel of Fig. 3 ($R = 0.94$) corresponds to rate constants: $k_{23} = 3\ s^{-1}$ and $k_{32} = 0.03\ mM^{-1}\ s^{-1}$. It should be noted that k_{23} is poorly resolved by the fitting procedure, but this does not affect our general conclusions. Force per AM · ADP cross-bridge is decreased by a factor of 1.2 between 0 and 30 mM P_i, but this decrease may be due to the rather poor fit at low $[P_i]$. Between pH 7.1 and 6.0, k_{31} increases by the same fraction as tension cost, from 0.32 to 0.64 s^{-1}, and k_{23} decreases from 3 to 2 s^{-1}. Force per AM · ADP cross-bridge is, again, not affected by pH. The predicted and measured ATPase activities at 30 mM P_i, pH 6.0 amount to 0.88 and to $0.98(\pm 0.04)$ of their respective values at 0 P_i, pH 7.1, indicating that the effects of P_i and pH on ATPase in soleus may not be completely independent, analogous to the situation in cardiac muscle (Ebus et al., 1994). Force per AM · ADP cross-bridge is decreased by a factor of 1.2, basically showing that both P_i and pH hardly affect this quantity in soleus fibers.

Limitations of the model

In general, the kinetics for soleus are less well defined, but qualitatively the relative effects on the rate constants seem to be similar to those for psoas. Furthermore, it should be noted that there are limitations to the three-state scheme presented, which is the simplest scheme to explain our results, but naturally does not give a complete description of cross-bridge kinetics. For example, the transition denoted by k_{31} consists of a number of steps: an isomerization that precedes ADP release, ADP release, ATP binding, cross-bridge detachment, and ATP hydrolysis. The individual rates of these transitions cannot be determined on the basis of our experiments, but k_{31} will mainly be determined by the slowest transition.

Moreover, the relation between ATPase and P_i in both fiber types is better fitted by a logarithmic function than by a hyperbola, indicating that higher order effects other than mass action show up at low P_i. From the values obtained for k_{23} and k_{32} a phosphate dissociation constant (k_{23}/k_{32}) follows of 165 ± 40 mM in psoas and 100 ± 30 mM in soleus. These values are at the high side of the range found in fibers previously (Webb et al., 1986; Bowater and Sleep, 1988; Dantzig et al., 1992; Millar and Homsher, 1992; Wang et al., 1994) but are less than found in solution studies (cf. Bowater and Sleep, 1988). These limitations indicate the necessity of a strain-dependent model to fully account for all of the data available in the literature. Nevertheless we would like to emphasize that especially the results in soleus fibers indicate that mass action of P_i plays an important kinetic role.

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