used as stubstituents on C_1 and C_2; namely H, CH_3, C_2H_5, and C_6H_5. They are differentiated to have approximately 1, 2, 3, and 5 Å in their diameters as spherical models. For simplified discussion, we consider that **2** has bulkier substituents on C_1 than on C_2. Thus the C_2 site will be more reactive than the C_1 site for dimerization. In this occasion discussion will be rather limited to the bulkiness of substituents on C_2. In cases where substituents are two C_6H_5 or one C_6H_5 and one H, monomer dianions are formed (Scheme 1). Dimerization of the radical anion occurs in cases where the substituents are two hydrogens or one H and one CH_3 (Scheme 2). In cases where the substituents are two CH_3, disproportionation occurs (Scheme 3). Therefore bulkiness of the substituents may control the progress of the reaction. Two alkyl groups are large in size for dimerization, and small for dianion formation, and may be suitable for disproportionation. For disproportionation reaction, the substituent must have a hydrogen to be abstracted. One CH_3 on C_2 of **1a** is substituted by C_2H_5 in order to investigate which hydrogen in two substituents, either CH_3 or C_2H_5, is easily abstracted. From analyses of a mixture of the products, it is concluded that the hydrogen-leaving power is about ten times stronger in CH_3 than in C_2H_5.

Acknowledgement

The authors wish to thank Mr. K. Kushida and A. Ono (Varian Instruments, Ltd.) for their kindness in measuring several 2D CH COSY spectra at 100.6 MHz.

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Intracellular pH and Inorganic Phosphate Effects on Skeletal Muscle Force

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INTRODUCTION

The molecular mechanisms of skeletal muscle fatigue are not fully understood. Intracellular pH (pHi) and inorganic phosphate (Pi) have been implicated as causes of peripheral fatigue. Both a rise in $[H^+]$ (decreased pHi) and elevated Pi result in the inhibition of force generation in skinned fibers (2) and sometimes in whole muscle preparations (1, 3). During exercise, an accumulation of H^+ and Pi occur simultaneously over time, which confounds the interpretation of the relative role of each with regard to skeletal muscle fatigue. The specific protonation state of Pi might also inhibit force generation, because the relative amount of Pi species ($H_2PO_4^-$ and HPO_4^{2-}) is dependent on pHi, and these amounts change over the physiologic range of pHi (pKa = 6.79) (3). In order to evaluate the role these metabolites have in muscle force, the effects of pHi and Pi on force have been dissociated by independently manipulating each.Changes in the amount of CO_2 in the perfusate (P_{CO_2}) manipulates pHi (1), and exogenous pyruvate lowers Pi (6). Performing simultaneous NMR spectroscopic and mechanical measurements can assess the effects of these treatments. We have compared the relationship between force and pHi in the presence and absence of inorganic phosphate and interpreted the relative contribution of Pi and pHi on muscle force generation.

METHODS

Isolated soleus from male Swiss Webster mice were attached to a Harvard Apparatus isometric force transducer and placed in a bath containing MOPS Ringers (11mM glucose, pH=7.4, 95% O_2, 5% CO_2) at 23°C. Tetanic force, as well as rise and relaxation time, were measured at optimum length every 10 minutes. Stimulation was at supramaximal voltage and fusion frequency for 1 second. The collateral muscle was placed in a custom designed NMR probe (5) and superfused from the same source of Ringers as the mechanics bath for ^{31}P-NMR spectra were acquired on a General Electric 7 Tesla GN300 at 121 MHz. Intracellular pH measurements were made using the chemical shift of Pi relative to PCr and the equation

$$pHi = 6.79 + \log\{(.89 - \delta)/(\delta - 3.19)\}.$$

Mechanical and metabolic measurements were repeated with control (11 mM glucose) and substrate (20mM pyruvate) solution (PYR), varying pHi by equilibration with 5%, 25%, and 50% CO_2, which resulted in P_{CO_2} values of 22, 174, and 266 torr respectively. All isometric contractions were bracketted by 11mM Glucose 5% CO_2 treatment to normalize data. Statistical analysis was performed using 2-way ANOVAS with relative force as the dependent variable, and pHi and Pi as independent variables. Paired comparisons of means were performed between each P_{CO_2} level and substrate treatment. Values of $p<.05$ were considered significant.

RESULTS

PYR lowered Pi levels from ~5 mM to <1 mM, confirming earlier results (6).

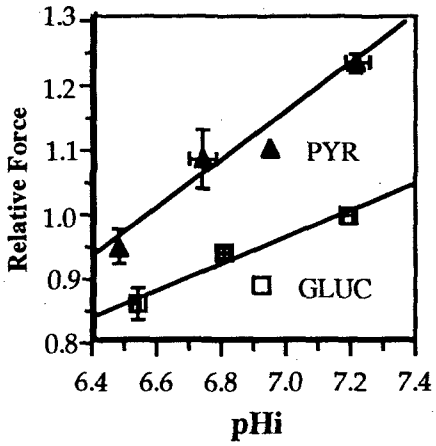
TABLE I: Force, Rise, and Relaxation Times in Soleus

	pHi	Force (g/g)	10-90 Rise Time (ms)	90-10 Relax Time (ms)
Gluc 5% CO_2	7.19 +.015	1747.9 +92.93 (n=36)	335.5 +12.92	205.28 +10.49
Gluc 25% CO_2	6.81 +.018	1712.6 +157.0 (n=8)	384.37 +25.08*	311.63 +24.71*
Gluc 50% CO_2	6.54 +.013	1644.4 +194.1 (n=6)	510.3 +59.70*	581.17 +63.2*
Pyr 5% CO_2	7.22 +.04	2110.0 +67.19 *# (n=32)	360.09 +27.68	413.44 +41.63* #
Pyr 25% CO_2	6.74 +.04	2013.0 +93.69 *# (n=10)	305.3 +28.04 #	495.2 +41.10* #
Pyr 50% CO_2	6.48 +0.0	1783.8 +85.85 (n=8)	355.75 +56.54 #	606.87 +66.85*

* p<.05 comparison to 11mM Gluc 5% O_2.

\# p<.05 paired comparisons between substrate treatments.

Figure 1: Relative Tetanic Force vs. pHi



PYR significantly increased force at all pH levels (Table I). Lowering pHi consistently decreased tetanic force in control and PYR conditions. In addition, there was a steeper force-pHi relationship in PYR treatment ($y = -1.46 + .37x$, $R^2 = .979$), in comparison with the dependence of force on pHi in controls ($y = -0.48 + 0.21x$, $R^2 = 0.966$). Figure 1 shows pHi effects on force at both Pi levels. Although it is apparent from Figure 1 that the presence of Pi does have an effect on the force-pHi relationship, tetanic force was more highly correlated to pHi than to either phosphate species (Table II). Rise and relaxation times (Table I) declined in low pHi conditions. In PYR, rise times shortened, whereas relaxation lengthened.

TABLE II: Total, Diprotonated, and Monoprotonated Phosphate Levels in Soleus

	pHi	Total Pi (μg/g)	H_2PO_4 (μg/g)	HPO_4^- (μg/g)
Gluc 5% CO_2	7.19 +.015	6.76 +.73 (n=22)	1.91 +.22	4.85 +.52
Gluc 25% CO_2	6.81 +.018	7.74 +2.10 (n=4)	3.68 +.95	4.06 +1.16
Gluc 50% CO_2	6.54 +.013	6.74 +.05 (n=2)	4.25 +.02	2.48 +.07
Pyr 5% CO_2	7.22 +.04	2.64 +1.01 (n=2)	0.69 +.22	1.96 +.78
Pyr 25% CO_2	6.74 +.04	1.69 (n=1)	0.88	0.81
Pyr 50% CO_2	6.48 +0.0	N.D.	N.D.	N.D.

Data presented in mean ± SE

DISCUSSION

Decreased [Pi] increased force at all P_{CO_2} levels, and did enhance the dependence of force generation on pHi. The effect of pHi on force concurs with skinned fiber data (2), but does not agree with results from hypercapnic manipulation in perfused muscle preparations (1). There are several possible explanations for this discrepency. One possibility is that there is inadequate O_2 delivery to the isolated muscle preparation. However, since the mouse soleus is small (~1 mm in diameter), there is little chance of O_2 limitation during hypercapnia because the diffusional distances are less than 500μm. It is also possible that at low pHi, a 1 second tetanic stimulation was not sufficiently long enough for the muscle to attain maximum force . However, longer stimulations did not appreciably increase force (data not shown). Force measured at 1 second tetani was not significantly different from that of longer tetani. Hence, the results observed give an accurate presentation of the effects pHi has on tetanic force in isolated whole muscle.

It has been suggested that a specific protonation state of phosphate is correlated to muscle force (3). The diprotonated species ($H_2PO_4^-$) is thought to lower force by binding more tightly to actomyosin crossbridges. Because force production is correlated with the release of Pi from the crossbridge, the higher binding affinity of $H_2PO_4^-$ would result in a decrease in force. As pHi decreases, there is a greater proportion of $H_2PO_4^-$ present, indicating that this phenomenon is an indirect effect of pHi on skeletal muscle force. We examined the dependence of force on pHi, HPO_4^{2-}, and $H_2PO_4^-$. The slopes of each regression line show a higher correlation between force and pHi (R^2=.966 in normal [Pi]) (Figure 1) than between force and either phosphate species ($H_2PO_4^-$, R^2=.138; HPO_4^{2-}, R^2=.035) alone (Table II). In these experiments, the primary effect of pHi on force does not appear to be mediated by a specific species of phosphate.

Table I shows that pHi and Pi influence other properties during tetanic stimulation. Proton accumulation could alter kinetics of force development (rise and relaxation times) in several ways. Enzymes, including Ca^{2+}-ATPase, actomyosin ATPase, and those in glycolysis, such as phosphofructokinase, are inhibited when pHi falls. The decline in enzymatic activity inhibits the speed at which a muscle can develop force or relax after stimulation. Low Pi causes faster rise yet slower relaxation times by different mechanisms which are not fully understood. Faster kinetics are expected since the phosphate release step in the crossbridge cycle would be more favorable due to lower Pi. However, this hypothesis can not explain why relaxation rates are significantly slower than in control conditions. It would seem plausible that Ca^{2+} uptake at the sarcoplasmic Ca^{2+}-ATPase would be enhanced by the increase in ΔG for ATP breakdown caused by low free Pi. Yet this is in direct opposition to our observations. If phosphorylation of the Ca^{2+}-ATPase is necessary to stimulate Ca^{2+} transport, this type of upregulation is not as effective as in normal conditions because free Pi has been depleted.

Ca^{2+} would remain in the sarcoplasm for a longer period of time, and relaxation would slow. It seems that there are two independent mechanisms responsible for the effect of low [Pi] on rise and relaxation.

By maintaining muscles in a resting state, our experimental design avoids introducing factors arising from constant stimulation, specifically, a concomitant rise of proton and phosphate levels. The independent diminution of Pi in this preparation allows the examination of the relative contribution of pHi on force generation. In so doing, we conclude that the relation between skeletal muscle force and pHi is affected by the depletion of Pi.

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