Phosphate uptake in rat skeletal muscle is reduced during isometric contractions

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Abraham, Kirk A., and Ronald L. Terjung. Phosphate uptake in rat skeletal muscle is reduced during isometric contractions. J Appl Physiol 97: 57–62, 2004. First published February 27, 2004; 10.1152/japplphysiol.01294.2003.—During contractions, there is a net efflux of phosphate from skeletal muscle, likely because of an elevated intracellular inorganic phosphate (Pi) concentration. Over time, contracting muscle could incur a substantial phosphate deficit unless Pi uptake rates were increased during contractions. We used the perfused rat hindquarter preparation to assess [32P]Pi uptake rates in muscles at rest or over a range of energy expenditures during contractions at 0.5, 3, or 5 Hz for 30 min. Pi uptake rates were reduced during contractions in a pattern that was dependent on contraction frequency and fiber type. In soleus and red gastrocnemius, [32P]Pi uptake rates declined by 25% at 0.5 Hz and 50–60% at 3 and 5 Hz. Uptake rates in white gastrocnemius decreased by 65–75% at all three stimulation frequencies. These reductions in Pi uptake are not likely confounded by changes in precursor [32P]Pi specific activity in the interstitium. In soleus and red gastrocnemius, declines in Pi uptake rates were related to energy expenditure over the contraction duration. These data imply that Pi uptake in skeletal muscle is acutely modulated during contractions and that decreases in Pi uptake rates, in combination with expected increases in Pi efflux, exacerbate the net loss of phosphate from the cell. Enhanced uptake of Pi must subsequently occur because skeletal muscle typically maintains a relatively constant total phosphate pool.

P iT-1; P iT-2; phosphate efflux; Na-Pi; cotransport
at concentrations typical of rat plasma (3). Before use, the perfusate was warmed to 37°C and pH was adjusted to 7.40. Standard Pi concentration in Krebs-Henseleit is 1.1 mM, a concentration shown previously to effectively saturate the uptake process (1).

Rats were anesthetized with pentobarbital sodium, and surgery was performed to isolate the abdominal aorta and inferior vena cava. The hind feet and tail were tied with umbilical tape to concentrate flow to the hindlimb musculature. Once perfusion was established and the catheters secured, rats were killed with an overdose of pentobarbital sodium delivered into the carotid artery. Perfusion was maintained with a peristaltic pump, and the perfusion medium was equilibrated with 95% O2-5% CO₂ before entering the rat hindquarter.

The flow rate was increased gradually over the first 20 min. The initial 150–200 ml of venous effluent was discarded, after which the perfusion medium was recirculated. When perfusate flow and pressure reached steady values (~38 ml/min and ~60 mmHg perfusion pressure), the perfusion medium was replaced with one containing 0.12 μCi/ml [32P]Pi. This medium was then recirculated over the duration of the 30 min Pi uptake period. Perfusion pH was monitored periodically and, although it decreased over time, was always above 7.30. Every 10 min, perfusate samples were collected and centrifuged to isolate plasma for analysis of radioactivity and Pi concentration.

Before quick-freezing of the leg muscles, radioactivity was cleared from the extracellular space by switching the perfusate to one containing no [32P]Pi, for 8.5 min. This perfusate was not recirculated. We have shown previously that radioactivity in the venous effluent reduces to ~6% of initial over this washout period. As a result, the extracellular radioactivity constitutes only 1–3% of total radioactivity measured in the muscle, the exact value dependent on fiber type (1).

At the end of the washout (36 min), muscles were removed and quickly frozen with tongs cooled in liquid nitrogen. We sampled the soleus muscle (primarily slow-twitch red fibers), the red portion of the gastrocnemius (primarily fast-twitch red fibers), the white portion of the gastrocnemius (primarily fast-twitch white fibers), and the remainder of the gastrocnemius (mixture of fibers) (2).

Muscle stimulation. On introduction of [32P]Pi, left leg muscles were stimulated to contract isometrically at either 0.5, 3, or 5 Hz for 36 min (27.5 min with [32P]Pi, 8.5-min washout). These contraction protocols have been shown to increase metabolic rates by up to 3- to 30-fold above rest (17) and produce variable decrements in PCr content and, therefore, variable increases in Pi content in different muscles over 30 min of stimulation in situ (30). Twitch contractions were elicited with supramaximal square-wave pulses (6–8 V, 0.1-ms duration) delivered to the sciatic nerve. Resting muscle length was adjusted to produce maximal active tension, and muscle force was recorded by use of a Cambridge force transducer (Cambridge Technology, Watertown, MA) connected to a MacLab recording system (ADInstruments, Castle Hill, Australia). Right leg muscles were never stimulated to contract and served as resting controls.

Tissue analyses. Metabolites from muscle and plasma samples were extracted in cold 3.5% perchloric acid and neutralized with tri-n-octylamine and 1,1,2-trichlorotrifluoroethane (6). These samples were stored at ~80°C until analyzed.

To calculate Pi uptake, we determined the radioactivity in aliquots of muscle and plasma extracts using scintillation counting. The radioactivity in muscle (dpm/g) was then divided by the average specific activity of plasma Pi (dpm/μmol) to yield Pi uptake (in μmol Pi/g muscle) and then expressed as a rate per hour.

Tissue contents of adenine nucleotides and PCr were determined by reverse-phase and ion-exchange HPLC, respectively (37, 41). Additionally, fractions of HPLC effluent containing ATP and PCr were collected and counted to detect 31P incorporation into those organic phosphate pools. Plasma Pi concentration was determined by an enzymatic assay, as described previously (10). Because orthophosphate content in freeze-clamped muscle is elevated because of Pi generation during muscle freezing (5), resting muscle Pi content was estimated by using values for Pi obtained with 31P nuclear magnetic resonance spectroscopy (24). Pi content in contracting muscles was determined from the net change in organic phosphates added to the resting Pi value as follows: Pi = (PCr_diff) + (2 × IMP) + resting Pi, where PCr_diff is the difference in PCr contents between resting and contracted muscles.

Muscle water content was determined by drying a 150- to 200-mg portion of each mixed gastrocnemius section at 60°C. Metabolite concentrations and Pi uptake rates were calculated to a common water content of 76%, which is typical of rat skeletal muscle (29).

Western blotting. Protein isolation and processing were carried out as described previously (11), with slight modifications, because we used homogenized muscle rather than cultured cells. Frozen muscles were homogenized in a buffer of 5 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride, a protease inhibitor, and centrifuged for 10 min at 1,000 g. The supernatant was retained.

Total protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL). Protein (40 μg) from each fiber type and the heart was separated by SDS-PAGE (8% gel) and transferred to a nitrocellulose membrane. Blots were blocked for 1 h with milk and 0.2% Tween 20 and then incubated overnight at 4°C with a 1:2,000 dilution of rabbit anti-PiT-1 antiserum. Raised against a portion of human PiT-1 Na-Pi transporter ranging from amino acids 408–421, these polyclonal antibodies have been described previously (4). After three washes, the membranes were incubated in 50 mM Tris-HCl buffer, pH 7.5, with 0.2% Tween, milk, and goat anti-rabbit secondary antibody at room temperature for 1 h. After additional washing, the membranes were covered with a chemiluminescent substrate (ECL Plus, Amersham Pharmacia Biotech) and exposed to film for 15 to 2 min. Analysis of band densities was performed by use of NIH Image version 1.62.

Statistics. All data are expressed as means ± SE. Statistical differences in Pi uptake rates between resting and contracting muscles within the same animal and between muscles of different contraction frequencies across animals were determined by analysis of variance based on a 2 × 3 design with repeated measures on one factor. When statistical significance (P < 0.05) was detected between groups, Tukey’s post hoc test was used.

RESULTS

Pi uptake rates. Contractions caused reductions in Pi uptake rates that were dependent on stimulation frequency in a muscle region-specific manner (Fig. 1 and Table 1). In the soleus and red gastrocnemius, Pi uptake rates decreased with increasing contraction frequency, whereas in white gastrocnemius uptake rates declined to a common level, regardless of stimulation pattern.

![Fig. 1.](http://jap.physiology.org/)

**Fig. 1.** Pi uptake rates in soleus, red gastrocnemius, and white gastrocnemius at rest and during contractions at 0.5, 3, and 5 Hz. For resting values, data from resting muscles of all contraction groups were combined. *Significantly different (P < 0.05) from corresponding resting value. #Significantly different (P < 0.05) from 0.5-Hz value in soleus. Values are means ± SE.
Decreased uptake rates during contractions were not a result of lower PI1 transporter protein content, because PI1 protein expression was similar in rested and contracted muscles (n = 4). Average PI1 band densities for soleus were 3,504 ± 557 and 4,078 ± 331 arbitrary units for rested and contracted muscles, respectively. Values for red gastrocnemius were 3,680 ± 253 and 3,705 ± 145, respectively, and for white gastrocnemius were 1,853 ± 873 and 1,902 ± 326, respectively.

Muscle metabolite contents. Muscle metabolite levels measured in resting and stimulated muscles are shown in Table 1. Values for rested muscle are pooled data from the right legs of all rats, because there were no differences among contraction groups. There were no significant changes in metabolites in either the soleus or red gastrocnemius at the end of 30 min. However, all three stimulation frequencies resulted in lower PCr and higher P_i and IMP contents in white gastrocnemius.

Muscle force production. As shown in Fig. 2, muscle force production at 0.5 Hz gradually declined over 30 min to 90% of initial. Conversely, at 3- and 5-Hz stimulation, force declined rapidly over the first 5 min and leveled off at 38 and 27% of initial, respectively. Because the energy costs of contractions are directly related to force development as determined previously (17), we can estimate the energy demands of these contraction protocols. The integrated energy expenditures over the entire contraction duration are shown in Fig. 3.

**DISCUSSION**

The major finding in this study is that P_i uptake rates in all three muscle fiber types decreased during contractions in a manner that was dependent on contraction frequency and muscle fiber type. The response in highly oxidative fibers of decreased P_i uptake rates with increasing contractions frequency implies that energy expenditure may be important in determining P_i uptake (Figs. 1 and 3). This may also be true for the white gastrocnemius section, although not observed with our stimulation parameters. It is likely that even our lowest stimulation rate was excessive for the white muscles, because this muscle region has a relatively meager aerobic capacity compared with the red gastrocnemius and soleus (42). On the basis of observations of increased P_i efflux with increasing intracellular P_i concentration (35) and net loss of phosphate from contracting fast-twitch muscles (14, 27), we expected P_i uptake rates to increase during contractions and, therefore, at least partially compensate for phosphate loss due to elevated

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**Table 1. Metabolite contents and P_i uptake rates at rest and after 30 min contractions at 0.5, 3, and 5 Hz**

<table>
<thead>
<tr>
<th></th>
<th>PCr, μmol/g</th>
<th>ATP, μmol/g</th>
<th>IMP, μmol/g</th>
<th>P_i, μmol/g</th>
<th>P_i uptake rate, μmol·g⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
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<tr>
<td>Rest</td>
<td>13.3 ± 0.8</td>
<td>4.57 ± 0.22</td>
<td>0.04 ± 0.02</td>
<td>3.6</td>
<td>2.47 ± 0.2</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>10.6 ± 1.3</td>
<td>5.17 ± 0.33</td>
<td>0.01 ± 0.01</td>
<td>6.1 ± 1.2</td>
<td>1.95 ± 0.2*</td>
</tr>
<tr>
<td>3 Hz</td>
<td>14.1 ± 0.5</td>
<td>4.68 ± 0.22</td>
<td>0.04 ± 0.02</td>
<td>5.2 ± 1.0</td>
<td>1.19 ± 0.2*</td>
</tr>
<tr>
<td>5 Hz</td>
<td>15.0 ± 2.4</td>
<td>5.60 ± 0.40</td>
<td>0.02 ± 0.01</td>
<td>6.6 ± 1.6</td>
<td>0.92 ± 0.2*</td>
</tr>
<tr>
<td><strong>Red gastrocnemius</strong></td>
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<tr>
<td>Rest</td>
<td>16.8 ± 0.5</td>
<td>6.93 ± 0.21</td>
<td>0.03 ± 0.01</td>
<td>2.3</td>
<td>1.42 ± 0.1</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>12.2 ± 1.8</td>
<td>6.50 ± 0.47</td>
<td>0.05 ± 0.03</td>
<td>6.5 ± 1.5</td>
<td>1.05 ± 0.2</td>
</tr>
<tr>
<td>3 Hz</td>
<td>16.1 ± 1.5</td>
<td>6.05 ± 0.34</td>
<td>0.08 ± 0.04</td>
<td>4.6 ± 0.9</td>
<td>0.72 ± 0.2*</td>
</tr>
<tr>
<td>5 Hz</td>
<td>15.5 ± 1.9</td>
<td>7.65 ± 0.39</td>
<td>0.13 ± 0.09</td>
<td>4.9 ± 1.1</td>
<td>0.63 ± 0.1*</td>
</tr>
<tr>
<td><strong>White gastrocnemius</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>23.2 ± 1.1</td>
<td>7.88 ± 0.28</td>
<td>0.05 ± 0.02</td>
<td>1.5</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>8.4 ± 3.1*</td>
<td>7.05 ± 0.37</td>
<td>0.34 ± 0.14*</td>
<td>17.6 ± 3.7</td>
<td>0.17 ± 0.03*</td>
</tr>
<tr>
<td>3 Hz</td>
<td>9.8 ± 1.6*</td>
<td>3.38 ± 0.46*</td>
<td>2.84 ± 0.56*</td>
<td>26.4 ± 2.8</td>
<td>0.12 ± 0.01*</td>
</tr>
<tr>
<td>5 Hz</td>
<td>7.9 ± 1.9*</td>
<td>3.88 ± 0.15*</td>
<td>3.18 ± 0.22*</td>
<td>25.9 ± 1.6</td>
<td>0.12 ± 0.02*</td>
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</table>

Values are means ± SE; n = 6 for all groups except rest, which are pooled data from all resting muscles (n = 18). PCr, phosphocreatine. Resting P_i values were derived from Kushmerick et al. (24) and contracted muscle P_i = PCrdiff + (2 × IMP) + resting P_i, where PCrdiff is the difference in PCr contents between resting and contracted muscles. *Significantly different (P < 0.05) from corresponding resting value.
efflux. However, our results indicate that a net loss of phosphate that may occur during contractions is not minimized, but rather exacerbated, by decreased Pi uptake.

Error analysis. Potential factors that may confound our measurement of Pi uptake are efflux of label from the muscle and dilution of the precursor pool specific activity that might occur by efflux of unlabeled Pi from the muscle during contractions. Obviously, any 32P label lost from the muscle via efflux would cause an underestimation of the uptake rate; however, we believe that this error is likely to be small. First, most of the Pi, taken up (50–70%) was incorporated into ATP and PCr. This keeps cellular Pi specific activity relatively low and, because these molecules cannot easily cross the plasma membrane, confines potential efflux of label to that in the Pi pool. Second, this cellular Pi specific activity was substantially lower than plasma Pi specific activity, averaging for resting muscle over the 30-min period only 0.3–2.5% that of the plasma, the exact value depending on fiber type. In contracting muscle, fractional loss of label would be even smaller, because the cellular Pi specific activity was even lower than that of resting muscle. Thus underestimation of Pi uptake due to loss of label in both resting and contracting muscle is likely to be small and would not significantly contribute to the large reduction in [32P]Pi taken up during contractions.

Another factor that could confound our experiment is whether muscle contractions changed the specific activity of the precursor pool of Pi, that is used for uptake by the muscle. For example, if the greater efflux of Pi that is expected to cross the sarcolemma during contractions (14, 16) were to dilute the specific activity of the interstitial pool of Pi, the same actual rate of Pi uptake into the myocyte would carry fewer counts of 32P and appear as a reduction in Pi uptake. We believe that the problem is real but that it does not confound our fundamental findings. The specific activity of the interstitial pool of Pi is determined by the relative rates of influx from the plasma (labeled Pi) and muscle fibers (“cold” Pi). Estimates of the rates of Pi influx from the plasma into the interstitial space of muscle can be obtained from fractional extractions for K+ (0.55–0.60 in slow-twitch muscle; Refs. 33, 36) and the knowledge that Pi exchanges at 52% of the rate as K+ (15). Thus we used a fractional exchange value of 0.3 min⁻¹ for soleus and red gastrocnemius, which fits within measurements of capillary exchange of 0.25/min and 0.38/min of the plasma pool for Gd-diethylenetriamine-pentaacetic acid and 51Cr-labeled EDTA, molecules larger than K+ (22, 39). For white gastrocnemius, we used a fractional exchange value of 0.1 min⁻¹, the same exchange value reported for Gd-diethylenetriamine-pentaacetic acid in fast-twitch muscle (39). We have calculated that the specific activity of the interstitial Pi pool for rested muscle is 82, 90, and 72% that of plasma for soleus, red gastrocnemius, and white gastrocnemius, respectively.¹ Thus, because we used plasma specific activity for our calculations of Pi uptake, there appears to be a systematic underestimate of the actual rate; however, the relatively similar interstitial specific activities across muscle fiber sections indicate that the error does not confound the fundamental observation of differences in Pi uptake rates across muscle fiber sections. Furthermore, if we include values for the increased rates of Pi efflux from contracting muscles measured by Hilton and coworkers (14, 16), we estimate that the specific activity of the interstitial pool of Pi decreased to ~76–87% of plasma Pi specific activity in red gastrocnemius, with a negligible decrease in soleus. This 5–15% error in interstitial Pi specific activity for red gastrocnemius could not reasonably account for the 26–56% reductions in 32P present in the muscle during contractions (Table 1). On the other hand, the error contributed by dilution of the specific activity (26–63%) is larger in the white gastrocnemius but still does not eliminate all of the measured reduction in 32P observed during the three contraction conditions (65–75%). Thus, whereas correcting for the error introduced by Pi efflux from the muscle would lessen the magnitude of the reduction in Pi uptake, it would not reasonably eliminate the basic observation that muscle contractions reduce Pi uptake. Thus we believe that the fundamental observation that Pi uptake is reduced during muscle contractions is valid. This implies that Pi uptake is modulated in an acute manner.

Potential mechanisms. Although the mechanism mediating the decrease in Pi uptake during contractions is unclear, there are several possibilities. First, Pi uptake rate may be altered by postranslational modification of Na-Pi transporters. PiT-1 and PiT-2 contain multiple consensus sequence phosphorylation sites for protein kinase C (PKC) (11, 20), which is activated in a contraction frequency-dependent manner in skeletal muscle (7, 34). PKC activation has been shown to reduce type III Na-Pi cotransporter-mediated Pi uptake in cultured human embryonic kidney cells (11) and increase Pi uptake in cultured fibroblasts (20), so the effect of PKC activation seems to be dependent on cell type. Second, there may be acute modulation of the number of Na-Pi transporters in the plasma membrane. Removal of functional transporters from the sarcolemma via endocytosis would reduce Pi uptake. Precedent for this comes from studies of Na-Pi transport in the brush border membrane of kidney cells that have shown internalization of Na-Pi transporters after stimulation by parathyroid hormone (12, 19). This response is also mediated by PKC (12). Third, an increase in intracellular Pi concentration might be expected to reduce transport activity by inhibiting the release of Pi from the Na-Pi transporter inside the sarcolemma. This would require that this release step is rate limiting for the overall Pi transport process. Unfortunately, the molecular mechanisms of Na-Pi cotransport are not well described. Finally, lower rates of Pi uptake could be caused by a reduced energetic driving force for Pi uptake due to the depressed sodium gradient and membrane depolarization that occur during action potentials. This seems an unlikely explanation, however, because the membrane is substantially depolarized for only ~3 ms during each action potential (25), which, at a stimulation rate of even 5 Hz, would be ~1.5% of the time. Also, the favorable concentration gradient for sodium is slightly greater than the opposing Pi gradient; thus, with the assumption that two sodium ions are transported with each H2PO4⁻, the free energy derived from the sodium gradient is sufficient even when the membrane is completely depolarized. Even in this case, Pi uptake becomes

¹ The rate of plasma Pi influx into the interstitium was calculated by multiplying the estimated fractional exchange of Pi (0.3 min⁻¹ or 0.1 min⁻¹; Refs. 33, 36, 39) by the total plasma Pi pool size, which was determined from the plasma Pi concentration (1.2 mM) and the known blood flow to each muscle region in the perfused rat hindquarter that would occur at our total perfusion rate (13). The rate of resting muscle Pi efflux into the interstitial Pi pool was taken to be the same as the rate of Pi uptake, as would be the case during steady state.
energetically limited only when intracellular Pi concentration rises to ~25 μmol/g. Furthermore, it is expected that the sodium gradient is well maintained during the stimulation protocols employed here, as recent calculations by Nielsen and Clausen (31) estimate that the Na\(^+\)-K\(^+\)-ATPase in skeletal muscle can keep pace with the influx of Na\(^+\) at excitation frequencies approaching 55 Hz, which is more than 10-fold greater than the highest frequency employed here. Whether these or other mechanisms contribute to the downregulation of P\_i uptake during contractions awaits confirmation in future investigations.

**Metabolic responses across fiber types.** In analyzing both force and metabolic profiles of the three muscle regions at different contraction frequencies, it is clear there was a heterogeneity among fiber types in response to muscle stimulation. The white gastrocnemius section showed large declines in PCR at all contraction frequencies and loss of ATP to IMP at both 3 and 5 Hz (Table 1), indicative of an extremely taxed metabolic response. Accordingly, P\_i uptake rates were reduced to a similar level during all three contraction protocols. This is in contrast to the soleus and the red gastrocnemius section, because both PCR and ATP contents were essentially at resting values at the end of all of the contraction frequencies. This result is somewhat surprising and differs from results published previously (30), in which declines in PCR of 23 and 41% were measured at 30 min at the higher frequencies of 3 and 5 Hz, respectively. However, in that study, force declined only 25–30% from initial force, as opposed to the 60–70% reduction in the present study. Because the energetic cost of contractions is dependent on the extent of force developed (17), the lower PCR values of Meyer and Terjung (30) may be explained by greater metabolic demand during those conditions.

Conversely, the extent of fatigue of our muscles at the higher frequency conditions would require a relatively low energy expenditure for the entire muscle, consistent with an expected modest change in PCR. Nonetheless, the highly oxidative fibers of the red portion of the gastrocnemius are expected to contribute significantly to force development, even with large reductions in total force of the whole muscle (42). Although the absence of PCR changes in the red gastrocnemius remains unexplained, it is not likely that PCR values remained unchanged during the entire 30-min contraction period. Rather, large decreases in PCR occur in these fibers early in the contraction period when force development is high (30, 40). Thus it is reasonable to expect that the intracellular P\_i concentrations were elevated in these muscles during at least the initial phase of contractions. As a result there would be a drive for an increased efflux of P\_i from the muscle, much like that reported by Hilton and coworkers (14, 16).

In conclusion, P\_i uptake rates are decreased in skeletal muscle during contractions in a manner seemingly dependent on energy expenditure. These results imply that acute modulation of the P\_i uptake process occurs during contractions. Additionally, reduced P\_i uptake, coupled with potentially high rates of P\_i efflux, would serve to further induce a net phosphate loss from contracting muscle, a loss that would require replacement subsequent to contractions.

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