Creatine uptake and creatine transporter expression among rat skeletal muscle fiber types

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Brault, Jeffrey J., and Ronald L. Terjung. Creatine uptake and creatine transporter expression among rat skeletal muscle fiber types. Am J Physiol Cell Physiol 284: C1481–C1489, 2003. First published February 5, 2003; 10.1152/ajpcell.00484.2002.—Total creatine (Crtotal = phosphocreatine + creatine) concentrations differ substantially among mammalian skeletal muscle. Because the primary means to add Crtotal to muscle is uptake of creatine through the sodium-dependent creatine transporter (CrT), differences in creatine uptake and CrT expression could account for the variations in Crtotal among muscle fiber types. To test this hypothesis, hindlimbs of adult rats were perfused with 0.05–1 mM [14C]creatine for up to 90 min. Creatine uptake rates at 1 mM creatine were greatest in the soleus (140 ± 8.8 nmol·h⁻¹·g⁻¹), less in the red gastrocnemius (117 ± 8.3), and least in the white gastrocnemius (97 ± 10.7). These rates were unaltered by time, insulin concentration, or increased perfusate sodium concentration. Conversely, creatine uptake rates were correspondingly decreased among fiber types by lower creatine and sodium concentrations. The CrT protein content by Western blot analysis was similarly greatest in the soleus, less in the red gastrocnemius, and least in the white gastrocnemius, whereas CrT mRNA was not different. Creatine uptake rates differ among skeletal muscle fiber sections in a manner reasonably assigned to the 58-kDa band of the CrT. Furthermore, creatine uptake rates scale inversely with creatine content, with the lowest uptake rate in the fiber type with the highest Crtotal and vice versa. This suggests that the creatine pool fractional turnover rate is not common across muscle phenotypes and, therefore, is differentially regulated.

Phosphocreatine; insulin; creatine turnover

THE NEAR EQUILIBRIUM CREATINE KINASE REACTION [phosphocreatine (PCr) + MgADP + H⁺ ↔ creatine + MgATP] is crucial for optimal energy utilization in skeletal muscle. PCr serves as a source of high-energy phosphates, thereby buffering changes in ATP content, especially at the onset of exercise and during intense contractions. Furthermore, the creatine kinase reaction enhances diffusion of these phosphates within muscle, which lessens the need for large nucleotide gradients (29). Although sustained muscle performance and exercise tolerance of animals with muscle creatine depletion are quite good, there are deficits in muscle performance at the onset of intense contractions (1, 16, 35). Furthermore, functional deficits in some patients with muscle inflammatory myopathy, dystrophy/congenital myopathy, and other neurovascular diseases (43) are exacerbated by the reduction in muscle PCr concentration, as oral creatine supplementation improves performance in many cases (42, 44, 45). Thus PCr and, by inference, total creatine (Crtotal = PCr + Cr) are essential for optimal function of mammalian skeletal muscle.

Because creatine is not synthesized by skeletal muscle, Crtotal content depends on the balance between cellular creatine loss and accumulation. Creatine loss from myocytes is generally considered a constant fractional process via the nonenzymatic cyclization of creatine and PCr to creatinine, which can pass freely through the cell membrane (46). Conversely, neither creatine nor PCr passes freely through the sarcolemma. Because the intracellular creatine concentration is maintained ~25- to 350-fold higher than the extracellular concentration (21, 25), creatine must be accumulated by active transport. This is achieved, in large part, through uptake by the high-affinity creatine transporter (CrT) driven by the cotransport of sodium and chloride ions (12, 20, 30). Both CrT mRNA (20, 31, 38, 40) and protein (19, 31, 47) are found in most mammalian tissues, with relatively high amounts in skeletal muscle, heart, and brain.

Creatine uptake rates measured in cell lines overexpressing the cloned CrT (14, 20, 32, 37), cultured myoblasts (13, 27, 33), giant sarcolemmal vesicles (47), and incubated muscle (51) reveal an apparent Michaelis-Menten constant (Km) for transport of 30–188 μM. Because this is near or below the typical plasma creatine concentration for mammals, it is probable that the transporter may be nearly saturated in vivo. This implies that creatine uptake would be proportional to the CrT content of the myocyte. In this context, variations in CrT content among fiber types, based upon Western blot analysis (31), predict that creatine uptake should be different among muscle phenotypes; the slow-twitch fibers of the soleus should exhibit a creatine uptake ~3.5-fold greater than the rate in the low-oxidative, fast-twist white muscle fibers. On the other hand, the fiber type with the highest Crtotal and vice versa. This suggests that the creatine pool fractional turnover rate is not common across muscle phenotypes and, therefore, is differentially regulated.
Cr\textsubscript{total} content is substantially different among muscle fiber types of nonprimate mammals (15, 31, 50). If creatine replacement (i.e., uptake) is obligated as a constant fraction of the Cr\textsubscript{total} pool present in the fiber sections, one would expect quite different creatine uptake rates among fiber types. On this basis, creatine uptake by the soleus fibers would be expected to be only 50–60% of the rate exhibited by the fast-twitch white fibers. Unfortunately, there is little information regarding the uptake of creatine among fiber types to evaluate which explanation is correct.

The purpose of this study was to quantitatively explore one critical process that could influence Cr\textsubscript{total} in skeletal muscle creatine uptake. We tested whether differences in creatine uptake and CrT expression could account for the observed difference in [Cr\textsubscript{total}]. Uptake rates were measured using the isolated perfused hindlimb of rats, which allows exquisite control of the extracellular environment while simultaneously perfusing all fibers types at a high flow rate through the intact vasculature. Creatine content, creatine transporter expression, and creatine uptake were measured in the same muscles.

METHODS

Animal Care

Male Sprague-Dawley rats (Taconic, Germantown, NY), weighing 350–400 g, were housed two per cage in a temperature (20–22°C)- and light-dark cycle (12:12 h)-controlled environment. All animals were provided unrestricted food and water except those perfused without insulin, which were fasted overnight before the experiment. This study was approved by the University of Missouri-Columbia Animal Care and Use Committee.

Hindquarter Perfusion

The standard perfusion medium consisted of 5% bovine serum albumin in Krebs/Henseleit buffer, 5 mM glucose, 100 μU/ml bovine insulin, and typical plasma concentrations of amino acids (4). In some experiments pertaining to insulin dependence, the perfusate contained either 0 or 1 μU of insulin per milliliter perfusate.

Immediately before use, the perfusate was filtered, warmed to 37°C, and adjusted to a pH of 7.40. A portion was used to prime the perfusion apparatus which included, in series, a peristaltic pump, a filter, a heating and oxygenating chamber supplied with 95% O\textsubscript{2}-5% CO\textsubscript{2}, and a bubble trap. The entire apparatus was located inside a Plexiglas cabinet maintained at 37°C. Perfusion pressure and temperature were monitored continuously throughout the experiment.

Rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and administered 100% oxygen during surgical preparation as described previously (18). The hind feet and tail were tied with umbilical tape to concentrate blood flow to the hindlimbs. After catheters were secured in the descending aorta and inferior vena cava and the flow was begun, the rats were humanely killed with an overdose of pentobarbital into the carotid artery.

The flow rate was increased gradually over ~20 min. The initial 150 ml of effluent were discarded to clear the system of essentially all red cells, after which the perfusate was recirculated. During this time, a fresh 300-ml volume of perfusate was prepared with 0.2 or 0.4 μCi [4-14]Ccreatine/ml perfusate (Moravek Biochemicals, Brea, CA) at a creatine concentration of 1.0 mM. For creatine concentration dependence, lower creatine concentrations were also used. For low-sodium perfusate, NaCl and NaHCO\textsubscript{3} of the Krebs/Henseleit buffer were replaced with equimolar concentrations of choline chloride and choline bicarbonate, respectively. Sodium nitroprusside (10 μM) was also added to prevent vasoconstriction. For high-sodium perfusate, NaCl was added directly to the perfusate. The perfusate was switched to this radiolabeled volume after the aortic perfusion pressure (~45 mmHg) was stable at a flow of 50 ml/min. After the initial void was cleared, the radiolabeled perfusate was recirculated throughout the experiment. Perfusion samples were collected every 15 min to verify perfusate concentrations.

Any [14C]creatine remaining in the extracellular space would contribute to an overestimate in the apparent creatine uptake into the cell. Therefore, before freeze-clamping the muscles, we cleared the extracellular space of the hindlimb tissues. Eight and a half minutes before the collection of tissues, the perfusate was switched to medium without creatine and was not recirculated. Timed samples of the venous effluent were initially collected to estimate the effective volume of perfusate radioactivity. The left lower hindlimb muscle sections were quick-frozen using aluminum tongs cooled in liquid nitrogen. Sections included the soleus (predominantly slow-twitch red fibers), deep lateral red gastrocnemius (predominantly fast-twitch red fibers), superficial medial white gastrocnemius (predominantly fast-twitch white fibers), and the remainder of the gastrocnemius (mixed fast-twitch fibers) (2). Frozen tissue samples were stored at −80°C until analyzed.

In Vivo Experiments

Rats were anesthetized with ketamine (100 mg/kg ip) and acepromazine (0.5 mg/kg ip), and a polyethylene (PE-50) catheter was placed in the right carotid artery and exteriorized at the back of the neck. After the rats regained consciousness (1–2 h later), they were given an ~1-ml oral dose of [4-14]Ccreatine (50 μmol creatine, 15 μCi (Moravek Biochemicals). Blood samples were collected in a heparinized syringe every 30 min via the carotid artery catheter. Samples were spun at 5,000 g, and the plasma was retained for analysis. After 2.5 h, the rats were anesthetized with pentobarbital (60 mg/kg ip), and hindlimb tissues were quick-frozen as described above. Plasma and frozen tissue samples were stored at −80°C until analyzed.

Metabolite and Creatine Uptake Rate Analyses

Metabolites from muscle sections were extracted in cold ethanolic (20% vol/vol) perchloric acid (3.5% wt/vol) (PCA) and neutralized with tri-n-octylamine and 1,1,2-trichlorotrifluoroethane (10). Perfusate and plasma samples were similarly extracted using PCA. Extracts were stored at −80°C until analyzed.

PCr and creatine concentrations were measured using ion-exchange HPLC as described by Wiseman et al. (52). PCr- and creatine-specific fractions were collected, and radioactivity was determined by liquid scintillation counting (quench corrected to disintegrations per minute).

Creatine uptake rates were calculated from the combined radioactivity (dpm/g) found in the Cr\textsubscript{total} pool (PCr + creatine) over time divided by the average perfusate- or plasma creatine-specific activity (dpm/nmol) for each animal.

To determine the muscle water content, a 150- to 250-mg portion of each gastrocnemius mixed fiber section was dried at 60°C to a stable weight. Metabolite concentrations and
uptake rates were calculated to a common water content of 76%, typical for rested rat skeletal muscle (24).

**RNA Analysis**

Total RNA was extracted from frozen muscle sections using TRIzol Reagent (Life Technologies, Grand Island, NY). Total RNA concentrations were quantified by absorbance at 260 nm, whereas RNA quality was assessed by separation on 5% acrylamide gel. The 99-nucleotide-long 18S rRNA probe, which protects an 80-nucleotide fragment, was transcribed from position 1,904 to 1,720 of the 3,427-base pair rat CrT cDNA insert kindly provided by Dr. R. D. Blakely (37). This probe sequence is located near the 3'-end of the coding region and is expected to hybridize both CrT mRNA transcripts described (20, 32, 37), which are likely alternative polyadenylations of the same coding region (39).

Total RNA (5 μg) from either skeletal muscle or yeast (used as a control) was analyzed using the RPA III Ribonuclease Protection Assay (Ambion). The 199-nucleotide-long CrT probe, which protects a 184-nucleotide fragment, was produced from position 1,904 of the 3,427-base pair rat CrT cDNA insert kindly provided by Dr. R. D. Blakely (37). This probe sequence is located near the 3'-end of the coding region and is expected to hybridize both CrT mRNA transcripts described (20, 32, 37), which are likely alternative polyadenylations of the same coding region (39).

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Protein Analysis

**Total protein isolation.** Frozen muscle sections were homogenized in 100 mM KCl and 50 mM imidazole (pH 7.0). After centrifugation, the precipitate was solubilized in sodium dodecyl sulfate and urea by incubation at 37°C for 2 h.

**Western blot.** Total protein concentrations were determined against bovine serum albumin standards using a biocinchonic acid protein assay (Pierce Chemical, Rockford, IL). Total protein samples (40 μg) were separated by electrophoresis on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. After being blocked with milk and 0.1% Tween 20, the membrane was incubated overnight with a 1:10,000 dilution of the primary antibody, a rabbit polyclonal antiserum raised against a bacterial fusion protein corresponding to a 50-amino acid nonconserved region of the CrT (37). The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was applied for 1–2 h. After being washed, membranes were covered with a chemiluminescent substrate (ECL Plus, Amersham Pharmacia Biotech) and exposed to film for 1–3 min. Band densities from the film were opticaly scanned.

Analysis of band densities was performed on a Macintosh computer using the gelplotting macro of the public domain National Institutes of Health (NIH) Image software program (version 1.62). Results are expressed as relative band density of the CrT mRNA to that of 18S rRNA.

**Statistics**

Repeated-measures analysis of variance was used to compare differences among fiber types with significance accepted at P < 0.05. Tukey’s procedure was used to calculate specific differences among means. Values are given as means ± SE.

**RESULTS**

**Creatine Uptake into Rat Hindlimb Muscle**

Venous effluent 14C radioactivity collected during the washout period decreased rapidly over time with 1.8% (± 0.1) of initial radioactivity remaining after 8.5 min (Fig. 1). This washout curve can be expressed as a double exponential (Fig. 1, inset), which suggests two distinct compartments were washed out at different rates (24). The initial rate of efflux (k1), which has a half-time of 0.5 min, likely represents the washout of the vascular compartment. The second, slower component (k2) has a half-time of 4.2 min and may be the washout of the interstitial space or some nonmuscle tissue or cells. This slower component is not likely an efflux from the intracellular muscle creatine pool because that rate of loss, which actually appears as creatine, has been measured to have a half-time of many days (7, 11, 33). Per fusate creatine concentrations had no significant effect on the washout rate (k1 or k2; data not shown); therefore, the data were pooled, and the single curve is presented in Fig. 1.

Muscles were excised after 8.5 min of washout to minimize the amount of extracellular [14C]creatine, which would confound intracellular [14C]creatine measurement in freeze-clamped muscle. Assuming the extracellular fluid volumes, as determined previously in the hindlimb perfusion (24), and equilibration of the vascular space with the interstitial space, perfusate radioactivity after 8.5 min of washout contributes <3% of the radioactivity measured in the muscle CrT total pools. Therefore, the measured 14C in the freeze-
clamped muscle is taken as a valid measure of creatine uptake.

Even though the duration for the extracellular washout was 8.5 min, we calculated the rate of uptake ending at 2.5 min into the washout period (e.g., the washout began at 57.5 min into a 60-min timed experiment). Venous effluent perfusate radioactivity and, consequently, creatine content at 2.5 min was <10% of initial. Thus we expect that minimal $[^{14}\text{C}]$-creatine uptake occurred after this time due to the small creatine gradient for uptake, which continued to diminish thereafter.

Although creatine uptake rates for each of the fiber types sections were reasonably linear with respect to time (Fig. 2; $r = 0.99$ for each fiber type section), experiments were typically performed for 1 h only.

Maximal creatine uptake rates, determined at 1 mM perfusate creatine (see below), differ among skeletal muscle fiber types, with the fastest rate ($140 \pm 8.8$ mmol h$^{-1}$ g$^{-1}$) found in the soleus and the slowest rate ($97 \pm 10.7$ mmol h$^{-1}$ g$^{-1}$) in the white portion of the gastrocnemius (Table 1). Surprisingly, these creatine uptake rates were inversely related to $\text{C}_{\text{r,\text{total}}}$ content of the skeletal muscle fiber types ($y = 208 - 2.85[C_{\text{r,\text{total}}}]$; $r = 0.99$). If these relative uptake rates reflect those necessary to maintain a steady-state intracellular creatine content in vivo, then the fractional turnover of the creatine pool is not uniform across fiber types, being 2.4 times faster in the slow-twitch soleus muscle than in the fast-twitch white portion of the gastrocnemius.

To evaluate whether our creatine uptake rates measured in situ follow the rates apparent in vivo, rats were given $[^{14}\text{C}]$-creatine orally, and muscles were exsised 2.5 h later. Measured plasma creatine concentrations remained a constant 0.37 mM during the experiment. Rates of creatine uptake were calculated after correcting the muscle radioactivity for the $[^{14}\text{C}]$-creatine radioactivity that would be found in the extracellular space, using the plasma dpm/ml multiplied by the mannitol/inulin space of each fiber type (24). As illustrated in Fig. 3, the calculated creatine uptake rates were not significantly different from those measured in situ at a similar circulating creatine perfusate concentration ($0.32 \pm 0.01$ mM; cf., Fig. 4).

**Acute Modulation of Creatine Uptake**

The influence of plasma creatine concentration on muscle creatine uptake is illustrated in Fig. 4. Creatine uptake rates at plasma concentrations within the physiological range of 0.15–0.3 mM (25, 28, 34) approach maximal (~80% or greater). Exceptionally high creatine concentrations, up to ~1.0 mM, did not substantially further increase creatine uptake in all fiber types.

To investigate the role of the sodium gradient, the typical perfusate sodium (142 mM) was replaced by choline. Creatine uptake rates were ~60% slower in

Table 1. Creatine uptake and relevant metabolite parameters in the different skeletal muscle fiber sections

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>ATP, $\mu$mol/g</th>
<th>Phosphocreatine, $\mu$mol/g</th>
<th>Creatine, $\mu$mol/g</th>
<th>Total Creatine, $\mu$mol/g</th>
<th>Specific Activity, PCr/Cr Ratio</th>
<th>Uptake Rate, mmol h$^{-1}$ g$^{-1}$</th>
<th>Relative Creatine Uptake Rate, $%C_{\text{r,\text{total}}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>4.84 ± 0.24$^*$</td>
<td>13.7 ± 0.85$^+$</td>
<td>10.3 ± 0.41$^+$</td>
<td>24.0 ± 0.75$^+$</td>
<td>0.87 ± 0.07</td>
<td>140 ± 8.8$^+$</td>
<td>0.59 ± 0.05$^+$</td>
</tr>
<tr>
<td>Red gastrocnemius</td>
<td>7.11 ± 0.17</td>
<td>18.0 ± 0.71$^+$</td>
<td>13.4 ± 0.82$^+$</td>
<td>31.4 ± 1.06$^+$</td>
<td>0.87 ± 0.07</td>
<td>117 ± 8.3$^+$</td>
<td>0.37 ± 0.02$^+$</td>
</tr>
<tr>
<td>White gastrocnemius</td>
<td>7.31 ± 0.11</td>
<td>23.1 ± 0.80$^+$</td>
<td>16.0 ± 0.93$^+$</td>
<td>39.1 ± 1.46$^+$</td>
<td>1.03 ± 0.09</td>
<td>97 ± 10.7$^+$</td>
<td>0.25 ± 0.03$^+$</td>
</tr>
</tbody>
</table>

Values are means ± SE. Creatine uptake rates measured at 1 mM perfusate creatine for 1 h; $n = 9$ for all groups. $^*$Soleus value significantly different from red and white gastrocnemius value. $^+$Value significantly different (P < 0.05) among all fiber types.

**Fig. 2.** Time course of 1 mM perfusate $[^{14}\text{C}]$-creatine uptake in the different skeletal muscle fiber sections ($n = 5–9$/time point).

**Fig. 3.** Creatine uptake rates measured in vivo with oral administration of $[^{14}\text{C}]$-creatine ($n = 4$) compared with rates measured in situ using the isolated hindlimb preparation perfused at a similar creatine concentration ($0.32 \pm 0.1$ mM; $n = 9$).
the three fiber type sections when either all or 75% of perfusate sodium was exchanged (Fig. 5). This suggests that only 60% of creatine uptake at 1 mM extracellular creatine is creatine transporter dependent. Conversely, creatine uptake was not increased with an additional 50% sodium in the perfusate, suggesting that the normal sodium gradient does not limit creatine uptake.

As illustrated by Fig. 6, there were no significant differences in creatine uptake detected between 0, 100 μU, and 1 mU of insulin per milliliter of perfusate. Even though the insulin concentration typically used in the hindlimb perfusion (100 μU/ml) is higher than that normally found in rat plasma (25–60 μU/ml) (34, 36), we employed 1.0 mU/ml insulin to ensure a hyperinsulinemic response. Therefore, it does not appear that insulin has any direct effect on the creatine transport process.

Creatine Transporter Expression

The CrT mRNA abundance, as detected by the ribonuclease protection assay, was not different among fiber types (Fig. 7). Total CrT protein, as quantified by the predominant 55-kDa band, was greatest in soleus, less in the red gastrocnemius, and least in the white gastrocnemius (Fig. 8). The 70-kDa band that is routinely seen by others (31, 47) was weak and inconsis-

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Fig. 4. Creatine uptake rate as a function of perfusate creatine concentration (n = 4 except for 1.0 mM, which is n = 9).

Fig. 5. Creatine uptake rate as a function of perfusate sodium concentration (n = 2 at all concentrations except 142 mM, which is n = 9).

Fig. 6. Influence of perfusate insulin on creatine uptake rate (n = 3 for no insulin and 1 mU/ml insulin; n = 9 for 100 μU/ml insulin).

Fig. 7. Ribonuclease protection assay for the creatine transporter mRNA (n = 4). CrT, creatine transporter.
tent in our Western blots and could not be reasonably compared. We were able to analyze the 58-kDa band, which is thought to represent the CrT found in the sarcolemma (48). The 58-kDa band follows the same fiber section-specific expression pattern as the 55-kDa band, but only the values of the soleus and white gastrocnemius were significantly different from each other.

**DISCUSSION**

This is the first study to measure creatine uptake rates among three different skeletal muscle fiber types. The large differences in fiber Cr\textsubscript{total} concentration and the measured CrT content among these phenotypes permit an assessment of factors important to cellular creatine metabolism. Creatine uptakes measured previously in cultured myoblasts (13, 27, 33), cultured myotubes (13, 27), incubated whole muscle (17, 51), and giant sarcolemmal vesicles (47) have indicated that the apparent \( K_m \) of the CrT for creatine is fairly low, on the order of 50–120 \( \mu \text{mol/l} \). Our results are consistent, with near maximal rates of creatine uptake observed in the physiological range of 100–300 \( \mu \text{mol/l} \) creatine (75–140 nmol·h\(^{-1}\)·g\(^{-1}\)). Similar rates were measured in alert rats in vivo (cf., Fig. 3). These rates are significantly greater than those rates measured using muscle strips from young growing rats (51). This difference could be due to our delivery of the creatine through the capillaries. Even though the skeletal muscle phenotypes of all mammals are not identical, we suspect that these rates of creatine uptake are characteristic for mammalian muscle, including humans. As in previous work (13, 27, 33, 51), most of the creatine uptake was Na dependent and therefore assignable to the actions of the Na-dependent CrT. Similar to the observations of some (32, 47, 51), but in contrast to others (22, 33, 41), there was no influence of insulin on creatine uptake rate, implying that insulin has little if any direct membrane effect on creatine transport.

Rates of creatine uptake were greatest in the slow-twitch soleus muscle, less in the high-oxidative, fast-twitch section (red gastrocnemius), and least \( (P < 0.05) \) in the low-oxidative, fast-twitch section (white gastrocnemius; cf., Table 1). Generally, similar results were obtained in the in vivo experiments. These differences in creatine uptake rates across skeletal muscle fiber types are linearly correlated \( (r = 0.99) \) to muscle CrT protein content, estimated from Western blot analyses (cf., Fig. 9). Although the creatine transporter mRNA quantity was not different among fiber types (Fig. 8), CrT protein was greatest in the soleus, less in the red gastrocnemius, and less still in the white gastrocnemius (Fig. 7). These mRNA and protein results are in general accord with those of Murphy et al. (31), who found no differences in CrT mRNA but significantly more CrT protein in the red gastrocnemius and soleus than in the white gastrocnemius. The general correspondence between creatine uptake rates and CrT content among the fiber types appears consistent with uptake being proportional to the transporter content, especially because normal circulating plasma concentration is thought to nearly saturate the transporter. Furthermore, uptake of creatine by the transporter is driven by the electrochemical gradient of sodium. This gradient is well in excess of that needed to overcome the large negative creatine gradient into the cell. In fact, the absence of an enhanced creatine uptake in the presence of a hypernatremia (213 mmol/l) argues that the normal Na gradient is sufficient and does not limit creatine entry into the fiber. Thus, for a given circulating creatine concentration, it may be expected that creatine uptake should be proportional with muscle CrT content.

**Creatine Transporter Protein**

Several aspects of the simple relationship between creatine uptake and muscle CrT protein content deserve careful evaluation. First, it is known that not all of the creatine taken up by myocytes occurs through the Na-dependent CrT. A fraction of the creatine taken into the muscle was sodium independent (~40%; cf.,
Fig. 5), an observation found with cultured smooth muscle and fibroblasts (13), cultured myoblasts (27, 33), and giant sarcolemmal vesicles (47). Because the extracellular-to-intracellular creatine gradient is ~25 to 350-fold against entry by simple diffusion, another transport process is implicated. This other process may not necessarily be specific for creatine but must derive energy either directly by ATP hydrolysis or cotransport of another molecule down its favorable electrochemical gradient. Calcium and magnesium are both potential candidates, as has been suggested by the work of Dai et al. (12), because the positive charge and concentration gradient of these ions could provide energy for transport. Interestingly, this non-Na-dependent creatine uptake process could account for the presence of creatine in muscle of a patient with a genetic deficiency of the Na-dependent CrT (53). Alternatively, there could be an excess accumulation of [14C]creatine within the cell caused by a creatine-for-creatine exchanger. If this process exists, excess radioactivity would accumulate within the cell, because the incoming and cellular creatine exist at very different specific activities. Thus any efflux of intracellular creatine would carry out less radioactivity than came with equimolar creatine entry into the cell. This would be a futile process and would not contribute to any net uptake of creatine within the myocyte. It is presently unclear whether this process may have contributed to the non-Na-dependent uptake of [14C]creatine in our experiments.

Second, it has recently been shown that a large fraction of the CrT protein, identified immunologically at 55 and 70 kDa, is localized to the mitochondrial fraction of the sarcolemmal fraction (47, 48). If this mitochondrial component of the CrT is not involved in direct creatine uptake from the plasma, then it is inappropriate to compare the 55-kDa major content of CrT among the fiber types to the measured creatine uptake rates, as we did in Fig. 9. Rather, if the inference from the work of Walzel et al. (47, 48) is correct, then it is most appropriate to relate creatine uptake rates to the content of the 58-kDa CrT protein across fiber types. Interestingly, this provides an instructive comparison. As also illustrated in Fig. 9, there remains an excellent correlation (r = 0.99) with a value of 56 nmol·h⁻¹·g⁻¹ as the y-intercept, the rate expected at zero CrT protein content or, to state it differently, the rate of non-Na-dependent creatine uptake. This prediction is remarkably similar to the measured Na-free creatine uptake rates of ~40–60 nmol·h⁻¹·g⁻¹ for the three fiber types. Thus our results are consistent with the expectation derived from the work of Walzel et al. (47, 48) that the 58-kDa band of the CrT is the important form of the transporter. The merits of this inference requires that the non-Na-dependent creatine uptake process is common across fiber types, a proposition that could be tested once the nature of this other process is characterized.

Third, recent work by Boehm and coworkers (8) indicates that the plasma membrane CrT content of heart vesicles can be varied, in a manner consistent with changes in creatine uptake, without changes in total CrT protein content of the tissue. This raises the possibility that externalization of CrT protein to the sarcolemmal membrane from an internal site could be a means of regulating creatine uptake.

Finally, the CrT protein can be phosphorylated (49, 55), and differences in phosphorylation are related to changes in creatine uptake in plasma vesicles (55). Thus modulation of creatine uptake may involve acute control of CrT phosphorylation-dephosphorylation, similar to that observed for other members of this transport protein family (5). Whether these and/or other processes are important in controlling creatine uptake in skeletal muscle awaits evidence from future investigations.

Implications for Muscle Creatine Turnover

It is interesting to note that the creatine uptake rates are inversely related (r = 0.99) to the CrT total concentration within the fiber types. Regardless of the absolute value obtained under various experimental conditions, creatine uptake by the soleus was generally 45–70% greater than the uptake by the white gastrocnemius. Strikingly, the creatine content of the soleus is ~40% less than that of the white gastrocnemius muscle (cf., Table 1). Accepting that these rates are applicable to steady-state conditions in vivo (cf., Fig. 3), muscle creatine turnover must occur at different rates among the fiber types. This difference is apparent whether one considers the CrT total, free creatine, or the PCr pools within the muscles. Further, complexities from the potential compartmentation of creatine, as suggested by Hochachka and Mossey for fish muscle (23), do not appear to complicate creatine metabolism for mammalian muscle. As given in Table 1, we observed that the specific activities of muscle PCr and creatine were very similar, with their ratios approaching unity. This argues that the entire chemical pools of PCr and creatine are freely exchangeable and, for purposes of creatine turnover, can be considered as one pool of CrT total. It is important to recall that creatine uptake is thought to be the only process whereby the myocyte obtains creatine. This argues that creatine uptake is not the sole determinant of intracellular creatine content in skeletal muscle. Rather, degradation of creatine and loss from the cell must be viewed as an important regulator of intracellular creatine content. For example, the differences in CrT total content could be reconciled if the fractional loss of creatine from the soleus were 2.4-fold higher than that from the white gastrocnemius. How this could occur is unclear because creatine is thought to spontaneously and nonenzymatically degrade to creatinine, which can freely diffuse out of the cell (6). This would make creatine degradation proceed as a constant fraction of the CrT total pool. Thus there would be a large amount of creatine degraded in the white gastrocnemius and a small amount in the soleus. Interestingly, in vitro experiments demonstrate that this spontaneous degradation process is favored at high temperature and low pH (26).
It is not known, however, whether differences in temperature (or pH) or differences in an enzymatic pathway that converts creatine to formaldehyde, hydrogen peroxide, and ammonium (54) could contribute to putative differences in \( C_{\text{total}} \) degradation among the fiber types.

**Experimental Considerations**

Our calculation of uptake rates assumes that essentially all the \(^{14}\text{C}\) creatine taken into the cell remains intracellular for the duration of the experiment. Any efflux as either labeled creatinine or creatine would cause an understimation of creatine uptake. Significant \(^{14}\text{C}\) loss of creatine to creatinine during the 1-h experiments seems unreasonable because basal turnover of creatine is exceptionally low, at only a few percent per day (7, 11, 33, 46). Furthermore, there is little additional chemical drive for creatine loss from the cell, because the uptake rate over an hour would increase intracellular \( C_{\text{total}} \) <1%. This is not expected to accelerate creatine loss because large fractions of PCr may be dephosphorylated to creatine during intense contractions and yet no measurable creatine is lost from the cell (3, 9). Finally, recognizing that the incoming \(^{14}\text{C}\) creatine mixes freely with intracellular creatine, any fractional loss of creatine or creatinine that does occur would be at a very low specific activity compared with the incoming \(^{14}\text{C}\) creatine. Similarly, additional factors that could confound appropriate measurement of creatine uptake in alert animals in vivo should not be a problem. For example, the potential error introduced by the inability to clear the muscle extracellular pool of \(^{14}\text{C}\) creatine in vivo before freezing was minimized by subtracting this contribution, using reasonable estimates of extracellular volume in each of the fiber types and the assumption that it equilibrates with the plasma creatine pool. Thus we believe that the measured creatine uptake rates obtained in our study are an accurate characterization of the creatine transport processes for the different skeletal muscle fiber types.

In conclusion, creatine uptake rates differ among skeletal muscle fiber types in a manner reasonably assigned to the 58-kDa band of the creatine transporter. However, creatine uptake scales inversely with total creatine content of the fiber phenotype. The greatest creatine uptake rate was observed in the fiber type with the smallest creatine content (soleus), whereas the opposite was true for the white gastrocnemius. Thus the fiber type with the highest \( C_{\text{total}} \) content exhibits the lowest uptake rate or, to state it differently, the lowest creatine replacement rate. This suggests that creatine turnover is not common across muscle phenotypes, where creatine degradation is not a simple fractional loss of the total creatine pool. This implies that differential regulation of creatine degradation occurs among skeletal muscle fiber types.

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