Creatine Metabolism in Skeletal Muscle

I. CREATINE MOVEMENT ACROSS MUSCLE MEMBRANES*

COY D. FITCH AND ROBERT P. SHIELDS

From the Departments of Biochemistry, Medicine, and Pathology, University of Arkansas School of Medicine, Little Rock, Arkansas 72201

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SUMMARY

In studies in vitro, creatine-1-14C entered the extensor digitorum longus of young rats by a saturable process which had an apparent Vmax of 0.6 mmole per liter of intracellular water per hour and an apparent K0.5 of 5 × 10⁻⁴ M. Creatine entry by nonsaturable processes was negligible at physiologic external creatine concentrations. Anaerobiosis, 1 × 10⁻³ M 2,4-dinitrophenol, and cooling each reduced the intracellular accumulation of creatine-14C. The apparent Q10 for entry was 2.7. Loss of creatine from the muscle accelerated rapidly during incubation at 37°C and exceeded the rate of creatine entry, but no connection between entry and loss was apparent. The large creatine loss in vitro was considered to be an artifact because it accelerated so rapidly during incubation and because it is inconsistent with observations from earlier experiments in vivo which indicate that much of the creatine in skeletal muscle is trapped there.

A special mechanism for entry, the saturable process, and intracellular trapping of creatine provide a plausible explanation for the high creatine content of skeletal muscle.

Skeletal muscle depends on a uniquely high concentration of phosphorylcreatine to sustain contraction (1, 2), and yet creatine biosynthesis occurs predominantly in other tissues (3-7). Since muscle phosphorylcreatine concentrations are maintained in part by entry of newly synthesized creatine, an understanding of muscle biochemistry requires a description of the entry mechanism. Without creatine entry, phosphorylcreatine would be depleted by continuous irreversible creatinine formation and perhaps by outward flow of the intact creatine molecule. The creatinine loss approximates 2% each day (8); the loss by outward flow, if any, has not been measured accurately (9).

 Although creatine crosses various cell membranes (9-20), the mechanisms of creatine movement across muscle membranes have not been characterized previously. To study creatine movements in vitro, we chose the extensor digitorum longus because it is a long, thin, typical skeletal muscle with a tendon at each end; the two tendons eliminate problems encountered with cut muscle preparations.

EXPERIMENTAL PROCEDURE

Young male Sprague-Dawley rats were fed a purified diet (21) until they reached weights of 100 to 165 g. Then they were killed; their extensor digitorum longus muscles were removed rapidly, rinsed carefully for 5 sec in Krebs-Ringer bicarbonate solution (pH 7.4), blotted, weighed, and placed in separate beakers containing the incubation medium. For facilitation of the study of entry, creatine-1-14C (13,000 to 300,000 cpm per ml) was present in the incubation medium. For the study of creatine loss, muscle creatine was labeled by giving individual rats multiple intraperitoneal injections of creatine-1-14C (total dose, 20 to 25 µC; 2.62 µC per mole). These rats were killed 3 to 6 days after the last injection, and their extensor digitorum longus muscles were incubated in a medium containing predetermined amounts of nonradioactive creatine.

Following incubation, the muscles were rinsed for 10 sec in Krebs-Ringer bicarbonate solution, blotted, weighed, and homogenized in distilled water, with trichloracetic acid being added to yield final volumes of 5 ml and a trichloracetic acid concentration of 10%. A suitable sample of incubation medium was similarly treated. Protein precipitates were removed, and the supernatant fluids were extracted three times with ether. Appropriate samples of the extracted supernatant fluids then were transferred to planchets, dried, and counted with a thin window, continuous gas flow Geiger tube. An internal standard was used to correct for differences in self-absorption between samples.

Total water was measured by drying the muscles to a constant weight in an oven at approximately 105°C. Inulin space (extracellular water) was measured after incubating muscles at 37°C for 90 min in a 0.5% suspension of inulin-carboxyl-14C (30,000 cpm per ml) in Krebs-Ringer bicarbonate solution (pH 7.4). These measurements (Table I) confirmed previously reported values for the extensor digitorum longus (22, 23). The weight and total muscle water after incubation were used to calculate the volume of intracellular water during incubation (Table I). Intracellular water was calculated by subtracting the inulin space from total water.

Creatine was determined enzymatically by the method of Tanzer and Gilvarg (24) as modified by Bernt, Bergmeyer, and Möllering (25).
Creatine Metabolism in Skeletal Muscle. I

Table I

Some characteristics of rat extensor digitorum longus muscle

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of the muscle (mg)</td>
<td>35-67b</td>
</tr>
<tr>
<td>Increase in weight during incubation (%)</td>
<td>16.1 ± 0.33 (49)c</td>
</tr>
<tr>
<td>Total water before incubation (% of wet weight)</td>
<td>76.8 ± 1.8 (6)c</td>
</tr>
<tr>
<td>Total water after incubation (% of wet weight)</td>
<td>79.3 ± 0.77 (12)c</td>
</tr>
<tr>
<td>Inulin (extracellular) space (% of wet weight)</td>
<td>25.1 ± 2.8 (12)c</td>
</tr>
<tr>
<td>Muscle creatine (mg/g, wet weight)</td>
<td>3.96 ± 0.24 (6)c</td>
</tr>
<tr>
<td>Serum creatine (mg/100 ml)</td>
<td>1.83 ± 0.23 (6)c</td>
</tr>
</tbody>
</table>

* A transverse section from each of six muscles at the point of greatest thickness revealed an oval surface which measured 1.63 ± 0.18 mm in least diameter and 3.06 ± 0.14 mm in greatest diameter.

b Range of values.

c Mean ± S.E. The number of determinations is given in parentheses.

Fig. 1. Creatine entry into skeletal muscle. Individual muscles were incubated at 37° under O2-CO2, 95:5, in 10 ml of Krebs-Ringer bicarbonate solution (pH 7.4) containing the specified concentrations of creatine-1-14C. Separate experiments were done to obtain each point and the average of three or more experiments at each time interval is shown. The apparent extracellular space after incubation for 30 min is less than it is after longer incubation periods, and in calculation of the distribution ratios at 30 min an extracellular space of 18.6% of muscle (wet weight) was used.

RESULTS

Creatine entry into the extensor digitorum longus is illustrated by the data in Fig. 1. A distribution ratio greater than 1 for the radioactive creatine (counts per min per ml of intracellular water/counts per min per ml of medium) was reached with an external creatine concentration of 0.1 mM, which is about the physiologic extracellular concentration (Table I). This entry rate would be sufficient to replace approximately 4% of the total amount of creatine in muscle each day. Much lower distribution ratios were observed with the higher external creatine concentrations, but with both the physiologic and the high concentrations the increases in distribution ratios were reasonably linear for at least the 1st hour. On the basis of these observations, a 1-hour incubation period was used in subsequent studies to provide easily measurable distribution ratios.

Saturation of the creatine entry process is shown in Fig. 2. Entry other than by the saturable process may occur, but, with the external creatine concentrations used in these studies, nonsaturable entry would be only a small part of the total. From our data, the $K_D$ (26) estimated for the nonsaturable component is less than 0.05 hour⁻¹. Since the value for $K_D$ is small and difficult to determine accurately, the Lineweaver-Burk plot was made without a correction for nonsaturable entry. This plot of the data gives an apparent $V_{max}$ of 0.6 mmole of creatine entering per liter of intracellular water per hour and an apparent $K_m$ of 5 × 10⁻⁴ M.

Fig. 2. Effect of external creatine concentration on creatine entry. Individual muscles were incubated for 1 hour at 37° under O2-CO2, 95:5, in 10 ml of Krebs-Ringer bicarbonate solution (pH 7.4) containing predetermined amounts of creatine-1-14C. The mean, the number of experiments, and the standard error are shown for each concentration.

To complement the studies on creatine entry, the movement of radioactive creatine out of the extensor digitorum longus was measured. In two of these experiments, creatine concentra-
tions also were measured enzymatically (24, 25). Both the enzymatic measurements and the measurements of radioactivity yielded the same values for creatine loss. The loss was small at the 15- and 30-min time intervals, but subsequently the rate of loss accelerated sharply (Fig. 3) and greatly exceeded the rate of loss that would be predicted from the $K_o$ for nonsaturable creatine entry. In fact, the rate of loss between 30 and 180 min exceeded the $V_{max}$ for the saturable entry of creatine in vitro. By the end of the 1st hour the accelerated loss of creatine could cause an error of about 5% in the measurements of creatine entry; at incubation periods of 90 min and longer the error in measuring creatine entry might be much greater. Because of the uncertainty about the distribution ratios obtained from the longer periods of incubation, the appearance of a steady state could cause an error of about 5% in the measurements of creatine entry; at incubation periods of 90 min and longer the error in measuring creatine entry might be much greater. Because of the uncertainty about the distribution ratios obtained from the longer periods of incubation, the appearance of a steady state after 90 min in Fig. 1 is difficult to evaluate. It may only represent an artifact of too long an incubation period.

The effects of conditions that interfere with metabolism are shown in Figs. 4 through 6. As would be expected if entry occurs by an energy-requiring process, anaerobiosis, poisoning by $10^{-3}$ M 2,4-dinitrophenol (Fig. 4), and cooling each reduced the distribution ratios to low values. In showing the effect of cooling on entry (Fig. 5), the amount of radioactive creatine in vitro.

**Fig. 4.** Effect of anaerobiosis and of 2,4-dinitrophenol on creatine entry. Individual muscles were incubated for 1 hour at 37° in 10 ml of Krebs-Ringer bicarbonate solution (pH 7.4) containing 0.1 mM creatine-$1^{14}$C. For the study of the effect of anaerobiosis, N$_2$-CO$_2$, 95:5, replaced the usual gas phase of O$_2$-CO$_2$, 95:5. For the study of the effect of dinitrophenol, the medium contained $10^{-3}$ M 2,4-dinitrophenol, and the gas phase was O$_2$-CO$_2$, 95:5. The means, numbers of experiments, and standard errors are shown.

**Fig. 5.** Effect of temperature on creatine entry. Individual muscles were incubated for 1 hour at various temperatures under O$_2$-CO$_2$, 95:5, in 10 ml of Krebs-Ringer bicarbonate solution (pH 7.4) containing 0.1 mM creatine-$1^{14}$C. The mean, the number of experiments, and the standard error are shown for each temperature.

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**Fig. 6.** Effects of external creatine concentration and of anaerobiosis on creatine loss. Individual muscles containing creatine-$1^{14}$C were incubated for 1 hour in 10 ml of Krebs-Ringer bicarbonate solution (pH 7.4) containing predetermined amounts of nonradioactive creatine. The gas phase was either O$_2$-CO$_2$, 95:5, or N$_2$-CO$_2$, 95:5. The means, the numbers of experiments, and the standard errors are shown.

Despite the simultaneous occurrence of creatine entry and loss, no connection between the two processes was apparent. Anaerobiosis for 1 hour did not greatly affect loss (Fig. 6) but inhibited entry (Fig. 4); and changing the external creatine concentration from 0.1 to 3.0 mM had no effect on loss (Fig. 6) but significantly increased entry (Fig. 2). Moreover, at 37° creatine loss began to accelerate (Fig. 3) before a change in the rate of intracellular accumulation of creatine-$1^{14}$C from the medium was detectable (Fig. 1).

**DISCUSSION**

**Mediation of Creatine Entry**—All of our observations point to a mediated entry process that is capable of moving creatine into muscle against a concentration gradient. Some of the observations, on the other hand, are incompatible with certain other modes of entry. Saturation of the entry process shows that creatine does not enter by simple diffusion, and the dissociation of entry from loss, in vitro, disproves restricted diffusion through small pores in the membranes (27). Also, the dissociation of entry from loss shows that there is not a direct exchange of intracellular creatine for extracellular creatine.

An energy-dependent concentrative entry process would be most consistent both with the requirement for oxygen and with the inhibition of creatine-$1^{14}$C accumulation by dinitrophenol and cooling, but additional information on the state of creatine in skeletal muscle is needed to prove that a concentration gradient exists between skeletal muscle and plasma. If intracellular creatine is retained by phosphorylation, by binding, by compartmentalization, or by a combination of these possibilities, the gradient observed between muscle and plasma could be more apparent than real. However, whether concentrative or
not, mediated entry in vivo would be important in maintaining a high creatine content since skeletal muscle membranes evidently do not permit significant entry by diffusion ($K_D < 0.05 \text{ hour}^{-1}$).

**Retention of Intracellular Creatine**—For normal plasma creatine concentrations to maintain the creatine content of skeletal muscle, either mediated entry in vivo must be greater than the rate in vitro, or creatine loss in vivo must be less than the loss in vitro. Both suppositions are possibly true, but available evidence supports the latter. In the rat, the half-life in vivo of body creatine, which presumably represents the true half-life of muscle creatine (9), is 30 to 35 days (10, 11). Such a long half-life indicates that most of the creatine is trapped in skeletal muscle until lost by creatinine formation. If this interpretation of the studies in vivo is correct, then the large loss of creatine in vivo which we observed must be an artifact. The acceleration of creatine loss also is best explained as an artifact caused by conditions in vitro.

Trapping of at least some of the creatine in skeletal muscle has long been known to occur (13). Phosphorylcreatine comprises 60% or more of the total creatine content of rat skeletal muscle (1, 8, 28), and it would not be expected to penetrate muscle membranes (13). Also, binding of most of the nonphosphorylated creatine to an unidentified substance in muscle membranes (13) also may operate only to permit entry. The loss of creatine then would depend on the presence of a separate outflow process. If the latter process were to have a low affinity for creatine, it could permit little outflow under physiologic conditions in vivo, but, under conditions in vitro favoring net breakdown of phosphorylcreatine, it could account for a large creatine loss. Two observations which support the existence of a separate process for the outflow of creatine are the acceleration of creatine loss after the 30-min time interval in vitro and the dissociation of entry from loss.

Although the ways by which creatine normally is retained in muscle are incompletely understood, a special mechanism for entry plus intracellular trapping provide a plausible explanation for the high creatine content of skeletal muscle.

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**REFERENCES**

17. **Thomas, J., Biochem. J.,** 64, 335 (1956).
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