Does muscle creatine phosphokinase have access to the total pool of phosphocreatine plus creatine?

Hochachka, Peter W., and Mark K. P. Mossey. Does muscle creatine phosphokinase have access to the total pool of phosphocreatine plus creatine? Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R868–R872, 1998.—Two fundamental assumptions underlie currently accepted dogma on creatine phosphokinase (CPK) function in phosphagen-containing cells: 1) CPK always operates near equilibrium and 2) CPK has access to, and reacts with, the entire pool of phosphocreatine (PCr) and creatine (Cr). We tested the latter assumption in fish fast-twitch or white muscle (WM) by introducing [14C]Cr into the WM pool in vivo. To avoid complications arising from working with muscles formed from a mixture of fast and slow fibers, it was advantageous to work with fish WM because it is uniformly fast twitch and is anatomically separated from other fiber types. According to current theory, at steady state after [14C]Cr administration, the specific activities of PCr and Cr should be the same under essentially all conditions. In contrast, we found that, in various metabolic states between rest and recovery from exercise, the specific activity of PCr greatly exceeds that of Cr. Releasing of this unlabeled or “cold” Cr on acid extraction accounts for lowered specific activities. This unexpected and provocative result is not consistent with traditional models of phosphagen function.

creatine shuttles; muscle phosphagen; fish muscle phosphagen; muscle energetics; creatine compartmentalization

CREATINE PHOSPHOKINASE (CPK, EC 2.7.3.2 ATP:creatinine N-phosphotransferase) catalyzes the reversible reaction PCr + ADP + H⁺ → ATP + Cr, where PCr and Cr are phosphocreatine and creatine, respectively. The traditional and currently most widely accepted view of phosphagen function in skeletal and cardiac muscles in lower vertebrates (1, 17, 20, 25) and in mammals (12, 14, 15, 21, 24, 30) assumes 1) that the total acid-extractable pool of Cr + PCr (for convenience termed Crtot) occurs in aqueous solution and is fully accessible to CPK, 2) that solution chemistry “rules” apply globally in muscle cells in vivo, and 3) that the main CPK-phosphagen function is to “buffer” ATP concentrations during large-scale changes in muscle work and in ATP turnover rates. Although this view dominates the current literature (2, 5, 12, 17, 30) and extends to other phosphagen systems in invertebrate muscles (6, 28), alternative hypotheses consider 1) that the structural organization of phosphagen-containing cells physically constrains Crtot, 2) that solution chemistry rules may apply in vivo mainly to localized PCr/Cr pools, and 3) that intracellularly localized CPK isoforms in vivo create complex pathways of PCr and Cr metabolism (3, 9–11, 13, 18, 19, 22, 26, 27). An especially comprehensive current review on thinking in this area is edited by Saks and Ventura-Clapier (18).

We reasoned that the in vivo behavior of the total Cr and Cr predicted by each of these two views of phosphagen function differ substantially. We used a trout model system to examine the issue because, in fish, white muscle (WM) is uniformly fast twitch and is anatomically differentiated from slow-twitch fibers or red muscle. Our experimental strategy was to add exogenous [14C]Cr in vivo into the WM pool of Crtot; because of a high WM CPK activity, the traditional theory of phosphagen function would predict almost instantaneous reaction equilibration and similar or identical specific activities (SAs) of PCr and Cr under all metabolic conditions.

METHODS AND MATERIALS

Experimental animals. All fish experimental protocols followed the University of British Columbia Animal Care guidelines and were permitted under University Animal Care Certificates. Rainbow trout (Oncorhynchus mykiss) of both sexes (mean weight 469 g; SE = 36 g) were obtained from West Creek Trout Farms, Aldergrove, BC, and kept in aerated dechlorinated water in an outdoor circular holding tank until use.

Metabolic states. We administered [14C]Cr via an indwelling dorsal aorta catheter into trout with WM in differing metabolic states. Resting fish were held in black box metabolic chambers for 3 days to bring about a truly quiescent state (17, 20); for other metabolic states, fish were exercised in a Brett-type swim tunnel (8). Maximal swim speed with minimal or no WM contribution (Ucrit) was determined in a preliminary set of experiments with six individual trout and was 2.5 body lengths/s (SE = 0.3 body lengths/s). The exhausted state was defined by an inability to maintain 2 body lengths/s and a lack of struggling when removed from the swim tunnel (8). Because WM recruitment is minor at swimming speeds below Ucrit, fish were swum (or “sprinted”) at 120% of Ucrit for 7 min to ensure major WM contribution to swimming; longer swimming under these conditions would lead to major ATP depletion (8). By use of swim speeds above Ucrit, and by use of prolonged but lower speed swimming exercise to complete exhaustion (8), we were able to reproducibly identify and utilize two states as defined by Connett (5): a “buffering” condition in which muscle PCr levels are de-
creased while ATP levels remain constant and a "depleting" condition, where muscle [ATP] cannot be fully "buffered" by PCr, so concentrations of ATP decline. For the former condition, fish were sampled at 10 min after 7 min of swimming at 120% of Uₕex, whereas for the "depleting" metabolic condition, fish were sampled at 30 min after exhaustive swimming exercise (8). These muscle sampling protocols were difficult to design and for clarity are illustrated diagrammatically in Fig. 1, and the metabolite concentrations defining the various metabolic states of trout WM are summarized in Table 1.

<table>
<thead>
<tr>
<th>State</th>
<th>n</th>
<th>ATP</th>
<th>PCr</th>
<th>Cr</th>
<th>Cr₉ tot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>7</td>
<td>7.61 ± 0.19</td>
<td>22.2 ± 0.84</td>
<td>25.2 ± 1.1</td>
<td>47.4 ± 1.0</td>
</tr>
<tr>
<td>Sprint</td>
<td>4</td>
<td>6.45 ± 0.23</td>
<td>8.3 ± 1.9</td>
<td>36.9 ± 1.7</td>
<td>45.3 ± 1.3</td>
</tr>
<tr>
<td>Buffering</td>
<td>9</td>
<td>7.22 ± 0.13</td>
<td>19.8 ± 1.3</td>
<td>29.0 ± 1.6</td>
<td>48.7 ± 1.2</td>
</tr>
<tr>
<td>Exhausted</td>
<td>5</td>
<td>2.86 ± 0.82</td>
<td>2.2 ± 0.7</td>
<td>45.3 ± 1.3</td>
<td>47.5 ± 1.7</td>
</tr>
<tr>
<td>White muscle</td>
<td>6</td>
<td>3.52 ± 0.46</td>
<td>11.6 ± 1.1</td>
<td>38.0 ± 1.9</td>
<td>49.6 ± 1.5</td>
</tr>
</tbody>
</table>

Values in µmol/g are expressed as means ± SE. These metabolite concentrations, with corrections for modest decreases in extracellular fluid volume during exercise (16), are similar to those previously recorded for fish white muscle (8). Resting fish were held in black box metabolic chambers for 3 days to bring about a truly quiescent state (17, 20); for other metabolic states, fish were exercised in a Brett-type swim tunnel (8). Resting condition, muscle sampled on removal from black box metabolic chamber; sprint condition, muscle sampled at the end of 7 min at 120% maximal swim speed; buffering condition, muscle sampled at 10 min after sprinting; exhausted condition, muscle sampled immediately after complete exhaustion; depleting condition, muscle sampled at 30 min after complete exhaustion; PCr, phosphocreatine; Cr, creatine; Cr₉ tot, PCr + Cr. The conditions for Brett tunnel swimming protocols were similar to those used in previous studies; this was also the case for freeze clamping, acid extraction of tissue metabolites, and metabolite assays (17).

PCr and Cr fractions were separated by high-performance liquid chromatography (HPLC) using modest modifications of published procedures (4). A volume of 250 µl of each extract was injected onto a Whatman Partisil 10SAX column using a Waters 625LC system. Initial solvent was 0.01 M phosphoric acid, pH 2.85, changing to 0.75 M KH₂PO₄ after 5 min, at a constant flow rate of 1.5 ml/min at 25°C. Total run time was 21 min. HPLC separation of PCr and Cr was excellent (see Fig. 2), and thus both metabolites could be efficiently collected for scintillation counting and SA calculations. Preliminary experiments with standards or known amounts of PCr and Cr and [14C]Cr were performed to determine a proper collection protocol. Typically, 6 ml were collected directly into scintillation vials for both the Cr and PCr fractions. These were dried for 24 h at 80°C, then suspended in ACS II scintillation cocktail (Amersham). The fractions were stirred with a magnetic stirrer for 15 min, then stored in the dark before quench-corrected counting in an LKB 1214 Rackbeta scintillation counter. All values are reported as means with SE. Student's t-test or one-way analysis of variance was used for statistical evaluation of the different data sets, and a probability value of 0.05 was accepted as the level of significance.

RESULTS

Establishing steady-state conditions. Cr concentrations in fish, although known to be stable under a variety of physiological conditions (7), were monitored simultaneously with measurements of the radioactivity of [14C]Cr in the plasma until the SA (disintegrations per minute per micromole plasma Cr) approached an asymptote (Fig. 2). This pattern was similar to that seen in earlier studies of Cr turnover in fish (7). It took ~2 h to reach an asymptote; stable plasma Cr concentrations and Cr turnover were operationally considered to represent physiological steady-state conditions, when muscle sampling was appropriate (Fig. 2).

Establishing different metabolic states in trout WM. As it was desirable to track the labeling patterns of PCr and Cr in the WM pool in different conditions, we took advantage of swimming and recovery to establish
to SA $^{14}$C-Cr were in the range of three to five in all three metabolic states examined. The lowest SA PCr/SA Cr values were found for muscle in the resting state; however, it should be emphasized that, because of the tissue volume required, these muscle samples could not be obtained by rapid biopsy sampling protocols (1, 8, 17, 20) and the PCr pool, compared with rapid sampling (20), was about one-half depleted during the collection of the tissue sample [significant PCr depletion can occur during sampling with only one or two tail flips (1, 8, 17, 20)]. Possibly for this reason, resting SA values did not statistically differ from any of the others. Any differences between the two remaining metabolic states were also minor and did not reach statistical significance. In all three metabolic states examined, the main result was a systematic and much higher SA of the PCr pool than of the Cr pool.

**DISCUSSION**

We consider that the power of this study arises from two sources. First, the experiments were performed in vivo under three realistic physiological conditions, and second, the experimental design allowed unequivocal testing of the traditional concept that the entire intracellular pool of PCr + Cr behaves as if in homogeneous solution in essentially all metabolic states. Our results clearly are not consistent with the assumption that CPK has access to, and interacts with, the entire pool of PCr and Cr in muscle cells. If the latter situation were the case, reaction equilibration would be established almost instantaneously and the SA of PCr would be the same as the SA of Cr; in none of the three metabolic states evaluated was this found. Instead, the SA of PCr always exceeded the SA of Cr. Empirically, the simplest explanation for lower SA of Cr compared with PCr involves the release, during acid extraction of freeze-clamped tissue, of a significant amount of unlabeled Cr that had not equilibrated during the in vivo protocol. Interestingly, similar data have been obtained by Savabi (19) working with an in vitro cardiac muscle prepara-

### Table 2. Specific activities for $^{14}$C-PCr and $^{14}$C-Cr for trout white muscle in three different metabolic states

<table>
<thead>
<tr>
<th>State</th>
<th>n</th>
<th>SA-PCr, dpm/µmol</th>
<th>SA-Cr, dpm/µmol</th>
<th>SA-PCr/SA-Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>7</td>
<td>985 ± 155</td>
<td>366 ± 96</td>
<td>3.10 ± 0.29</td>
</tr>
<tr>
<td>Buffering</td>
<td>9</td>
<td>1,216 ± 164</td>
<td>271 ± 48</td>
<td>4.74 ± 0.31</td>
</tr>
<tr>
<td>Depleting</td>
<td>6</td>
<td>1,033 ± 53</td>
<td>212 ± 10</td>
<td>4.95 ± 0.50</td>
</tr>
</tbody>
</table>

Values are means ± SE. In all cases, white muscle samples were taken 2 h after administration of labeled Cr (see Fig. 1), under steady-state plasma concentrations and turnover (Fig. 2). Resting condition, muscle sampled on removal of fish from black box metabolic chamber, recovering from catheterization for 3 days. [Two fish were allowed to recover for 6 days; the data, (not shown) from these animals were essentially identical to the ones given above for the resting condition.] Buffering condition, muscle sampled at 10 min after sprinting. Depleting condition, muscle sampled at 30 min after complete exhaustion, as described by Dobson et al. (8). For specific activity (SA) determinations, these muscle sampling conditions were chosen so as to have reasonable pool sizes of both PCr and Cr; the %PCr ranged from −47% at rest to −23% in the depleting condition (Table 1). 

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**Fig. 2.** Specific activity (SA) of plasma Cr following bolus administration of $^{14}$C-Cr via a dorsal aorta catheter in resting trout. Top inset shows constant plasma Cr concentration throughout manipulation. This $^{14}$C/Cr washout curve is similar to that previously reported in studies of Cr turnover in fish (7). The approach to asymptote at 2 h and the maintenance of constant plasma Cr concentrations were the two criteria used to identify physiological steady-state conditions; from previous studies (6), there is no indication that creatine turnover in fish changes under different physiological conditions. Thus muscle sampling occurred at 2 h after $^{14}$C-Cr administration for evaluating SA of phosphocreatine (PCr) and Cr in all 3 muscle metabolic states analyzed (see Tables 1 and 2). Bottom inset shows representative high-performance liquid chromatography (HPLC) indicating very efficient separation of PCr and Cr, which allowed the collection of pure aliquots for scintillation counting and comparing of WM PCr vs. Cr specific activities. See Ref. 4 and text for HPLC details. AU, arbitrary units; dpm, disintegrations/min.
tion. In Savabi’s in vitro studies, the heart muscle strips may have sustained interior anoxic (or ischemic) zones not easily accessible to exogenous labeled Cr; acid extraction could include such “cold” Cr pools and account for dilution of label (lower SA values). However, in our in vivo studies, all three metabolic conditions, especially those in postexercise hyperemia, are characterized by well-perfused epaxial muscle with aerobic metabolic pathways dominating contributions to energy production (17). Hence, such an explanation is not considered viable.

Rather, in our WM system (Table 2), and probably in heart preparations as well (13, 19), the data are more consistent with acid extraction leading to the release of a significant amount of Cr that normally does not easily equilibrate in intact cells with exogenously added \(^{[14C]}\text{Cr}\). It is presumed that such slowly equilibrating Cr must represent protein-bound (or otherwise compartmentalized) Cr, which could explain why it does not easily equilibrate with exogenous \(^{[14C]}\text{Cr}\) and would be consistent with directional Cr flux models of phosphagen function in muscles (3, 11, 26, 27). Such interpretations are also in agreement with parallel \(^1\text{H}-\)magnetic resonance spectroscopy (MRS) monitoring of PCr and Cr in human muscles. These recent studies appear to point to the existence of a pool of Cr that is not MRS “visible” in resting muscle but that appears in MRS spectra of muscle in ischemic fatigue (10, 23). In the present studies, \(^{[14C]}\text{Cr}\) seems to be able to equilibrate with PCr more readily than with Cr; in the MRS studies, essentially all of the PCr pool seems measurable, whereas a significant part of the Cr pool, at least in resting human muscle, appears to be MRS “invisible” (10, 23).

Whatever the explanation, the simple experimental strategy used in this study leads to an unequivocal conclusion: namely, that in vivo fast-twitch type muscles, despite the occurrence of CPK at very high concentrations compared with other enzymes, the enzyme does not have access to, and does not interact with, the total intracellular pool of PCr and Cr. That is why the CPK reaction cannot fully equilibrate \(C_{\text{tot}}\) with exogenously added \(^{[14C]}\text{Cr}\) within the time frame of the protocol.

Perspectives

There are two kinds of implications of this work. The first is practical, affecting interpretations of metabolite data and metabolic regulation; the second is more theoretical and affects interpretations of in vivo (so-called 3-D or structured) metabolism. In the first case, for over two decades metabolic biochemists have assumed that the CPK reaction is near equilibrium under all conditions. By quantitatively measuring the concentrations of \(^1\text{H}\), PCr, Cr, ATP, and \(P_i\), these workers would use the equilibrium constant of the CPK reaction to calculate free ADP concentrations. This approach has been particularly widespread in \(^{31P}\)-MRS studies of muscle rest-work transitions because in vivo ADP concentrations are below the MRS sensitivity threshold and because a popular metabolic regulation model assumes ADP as a key controlling metabolite (30). To be valid, all such calculations would require that CPK is interacting with the total pool of PCr + Cr. If the results of the present study are generally applicable, they seriously challenge the above key underlying assumption of many previous (especially \(^{31P}\)-MRS) studies of muscle metabolic regulation. However, it is important to add this caveat: our data are completely compatible with the assumption that CPK function in vivo is near equilibrium under all conditions. The key point is that CPK does not “see” the total PCr + Cr pool, so CPK holds only a part of the in vivo pool near equilibrium. From anything we know about CPK, local PCr + Cr pools presumably must be at or near equilibrium. The only kind of model that can compatibly incorporate all of the above information requires binding of a significant amount of WM Cr and requires that the bound Cr is not readily equilibrated through CPK with the rest of the PCr + Cr pool. This is somewhat analogous to similar data on adenylate binding and compartmentation (29).

A second consequence of this study concerns theoretical models of in vivo CPK function. As emphasized above, over the last three to four decades metabolic biochemists have favored either a “solution chemistry” model of CPK function or various “creatine shuttle” models, all of which require precise CPK isozyme binding (usually postulated at sites of ATP utilization and sites of ATP production) and some sort of 3-D directionality to CPK function (18). The data in the present study are more compatible with the latter than the former views of CPK function in vivo, if only because the latter models require localized and obviously compartmentalized CPK, PCr, and Cr, not to mention the adenylates (29). Such a situation could help to explain why not all Cr is rapidly equilibrated by CPK in fish WM in vivo. However, it is important to emphasize that our experimental approach could yield no specific information on either the nature or directions of any Cr shuttling pathways or indeed on the identity of Cr binding. Nevertheless, because the total pool size of PCr + Cr is large, in the 45–50 mM range in fish WM (Table 1) as found in other studies (8, 17), it is clear that the concentration of Cr binding sites must also be quite high (at least in the mM range). Although the identity of some Cr binding sites are known (18), their summed in vivo concentrations are far lower than would seem to be required to explain our data. Thus the mystery of the missing Cr binding sites represents a potentially fruitful point of departure for future work.

In summary, it appears that under intact, realistic physiological conditions, order and structure are imposed on Cr metabolism in muscle, thus preventing simple solution-like behavior of \(C_{\text{tot}}\) and CPK. At the least, this means that the use of the near-equilibrium nature of the CPK reaction in estimating in vivo free ADP concentrations should be reconsidered.

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