Phosphocreatine content of freeze-clamped muscle: influence of creatine kinase inhibition

Jeffrey J. Brault, Kirk A. Abraham, and Ronald L. Terjung
Department of Physiology, College of Medicine, Department of Biomedical Sciences, College of Veterinary Medicine, and Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211
Submitted 21 November 2002; accepted in final form 23 December 2002

Brault, Jeffrey J., Kirk A. Abraham, and Ronald L. Terjung. Phosphocreatine content of freeze-clamped muscle: influence of creatine kinase inhibition. J Appl Physiol 94: 1751–1756, 2003. The study of cellular energetics is critically dependent on accurate measurement of high-energy phosphates. Muscle values of phosphocreatine (PCr) vary greatly between in vivo measurements (i.e., by nuclear magnetic resonance) and chemical measurements determined from muscles isolated and quick-frozen. The source of this difference has not been experimentally identified. A likely cause is activation of ATPases and phosphotransfer from PCr to ADP. Therefore, rat hindlimb skeletal muscle was perfused either with or without 2 mM iodoacetamide, a creatine kinase inhibitor, and muscle was freeze-clamped either at rest or after contraction. Creatine kinase inhibition resulted in a decrease of 6 μmol/g higher PCr and lower creatine in the freeze-clamped soleus, red gastrocnemius, and white gastrocnemius. This PCr content difference was reduced when the initial PCr content was decreased with prior contractions. Therefore, the amount of PCr artifact appears to scale with initial PCr content within a fiber-type section. This artifact directly affects the measurement and, thus, the calculations of muscle energetic parameters from studies using isolated and frozen muscle.

SKELETAL MUSCLE FUNCTION SUCH as contraction and calcium regulation is critically dependent on the free energy (ΔG) available from ATP hydrolysis (ATP → ADP + Pi). The ΔG is determined by the ratio of the product and reactant concentrations of those metabolite pools readily available to react in solution. The free energy of ATP hydrolysis (ΔG_{ATP}) is defined as

\[ \Delta G_{ATP} = \Delta G^\circ + R \times T \times \ln \left( \frac{[ADP] \times [Pi]}{[ATP]} \right) \] (I)

where ΔG^\circ is the standard free energy, R is the gas constant, and T is absolute temperature. Intramuscular ATP is relatively abundant and considered readily available (“free”) to react in solution. Therefore, concentrations that are determined by various methods are similar and thought to be directly applicable to the calculations. On the other hand, the majority of ADP is bound, particularly to actin; therefore the total amount measured in acid-extracted muscle does not represent that which participates in the creatine (Cr) kinase (CK) reaction. It has not been possible to measure the free ADP (ADP_f) concentration, because its concentration within skeletal muscle is far below the sensitivity limits of measurements in vivo (nuclear magnetic resonance (NMR)). Rather, ADP_f concentration has been calculated by using the CK reaction (PCr + ADP_f + H^+ → Cr + ATP), because the equilibrium constant is known, the reaction is fast and near equilibrium under most conditions, and the relative concentrations of Cr and phosphocreatine (PCr) are high. In substituting this estimate of ADP_f into Eq. 1, it is apparent that changes in ΔG_{ATP} scale with changes in the Cr/PCr ratio and Pi. Therefore, accurate determinations of PCr, Cr, and Pi are vital for correct energetic measurements in skeletal muscle.

In contrast to measurements of ATP, striking differences are found in the measurements of skeletal muscle PCr and Pi depending on the method employed. For example, chemically determined PCr and Pi content in mammalian fast-twitch muscle from extracts of muscle quick-frozen at liquid nitrogen temperature expressed as the ratio of PCr/ATP (~2–3:1) and Pi/ATP (~0.8–1.0:1) (24, 26, 30, 33) is far different than corresponding values from NMR measurements in vivo (PCr/ATP ratio of ~3–4:1; Pi/ATP ratio of ~0.2–0.3:1) (1, 22, 26, 33), although this has not always been found (35). The cause of the systematically higher PCr and lower Pi values from NMR measurements in vivo have not been identified experimentally. A large quantity of bound intracellular Pi would make it “invisible” during NMR analyses and reconcile the difference for Pi; however, the same argument applied to PCr requires that the smaller value in muscle extracts must be attributed to a pool of PCr that is seen by NMR but is not extracted. This seems unlikely. On the other hand, the differences in Pi and PCr could be due to PCr hydrolysis during the process of muscle isolation (30) and/or quick-freezing (26). Calcium release during quick-freezing could activate actomyosin ATPase, hydrolyze ATP to ADP, and prompt phosphotransfer between PCr and ADP via the CK reaction. If this were the case, there would be little...
difference expected in ATP concentration, whereas there would be a decline in PCr and a stoichiometric increase in Cr and Pi.

The purpose of this study was to evaluate whether chemical analysis of muscle PCr is confounded by the isolation and quick-freezing process due to the differences expected in ATP concentration, whereas there would be a decline in PCr and a stoichiometric increase in Cr and Pi.

The purpose of this study was to evaluate whether chemical analysis of muscle PCr is confounded by the isolation and quick-freezing process due to the differences expected in ATP concentration, whereas there would be a decline in PCr and a stoichiometric increase in Cr and Pi. We hypothesized that muscle collected after iodoacetamide treatment would have a higher PCr and lower Cr content and that this effect would be lessened when PCr content was decreased.

METHODS

Animal care. Male Sprague-Dawley rats (Taconic, Germantown, NY), weighing 325–375 g, were housed two per cage in a temperature- (20–22°C) and 12:12-h light-dark cycle-controlled environment. All animals were provided unrestricted food and water. 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 (3). Immediately before use, the perfusate was filtered (0.45 μm), 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% O2-5% CO2, 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 (14). The hind feet and tail were tied with umbilical tape to limit blood flow to the hindlimb tissues. After catheters were secured in the descending aorta and inferior vena cava and flow was begun, the rats were humanely killed with an overdose of pentobarbital into the carotid artery.

To determine the muscle water content, an 150- to 250-mg portion of each gastrocnemius mixed fiber section was dried at 60°C to a stable weight. Metabolite concentrations were calculated to a common water content of 76%, typical for rested rat skeletal muscle (20).

Statistics. Analysis of variance was used to identify main treatment effects, with significance accepted at P < 0.05. Values are given as means ± SE.

RESULTS

Preliminary experiments established that perfusion of the hindlimb for 10 min with medium containing 2 mM iodoacetamide was effective at inhibiting CK activity. As illustrated in Fig. 1, the typical >80% reduction in PCr that occurs with intense contraction conditions (5 Hz twitch for 30 s; Ref. 9) did not occur with prior exposure to iodoacetamide. Muscle PCr content remained high (~30 μmol/g) even though the muscle force profile was reasonably similar to stimulated muscle in the absence of iodoacetamide. Therefore, we had a useful perfusion condition that essentially eliminated CK activity.

As shown in Table 1, resting muscle PCr concentrations of 16–21 μmol/g, depending on the muscle fiber section, were higher by ~6 μmol/g when freeze-clamped with iodoacetamide. Cr concentrations in these same muscle sections were stoichiometrically lower. Thus total Cr (PCr + Cr) content of the muscles was not affected by iodoacetamide. Interestingly, these same iodoacetamide-treated muscles showed evidence of ATP degradation with lower ATP and higher IMP, AMP, and ADP concentrations. There were no changes in inosine, hypoxanthine, or adenine contents in the muscles (data not shown). The difference in total phosphate equivalents among the phosphate pools was determined for the mixed-fiber plantaris (Fig. 2). After
iodoacetamide treatment, phosphate is lower in the total nucleotide pool (\(\Delta[ATP] \times 3 - \Delta[ADP] \times 2 - \Delta[AMP] - \Delta[IMP]\); 4.45 ± 1.51 \(\mu\)mol/g) and \(P_i\) pool (2.73 ± 0.33 \(\mu\)mol/g) for a total decrement of 7.18 ± 1.38 \(\mu\)mol/g. This is in excellent stoichiometry with increased phosphate in the PCr pool (6.50 ± 1.32 \(\mu\)mol/g) and fructose 1,6 bisphosphate pool (0.58 ± 0.26 \(\mu\)mol/g) for a total increase of 7.08 ± 1.26 \(\mu\)mol/g.

After depressing PCr content to 4–6 \(\mu\)mol/g, by prior muscle contractions, iodoacetamide reduced the magnitude of the higher PCr and lower Cr to 1–5 \(\mu\)mol/g whereas total Cr remained essentially unchanged (Table 1). A significant difference in ATP content was found only in the soleus as a result of iodoacetamide with prior contractions.

**DISCUSSION**

To our knowledge, this is the first study to experimentally establish that PCr degradation via CK occurs with tissue sampling, even with great care to rapidly resect and quick-freeze the muscle. When CK was inhibited by iodoacetamide in resting muscle, PCr concentration was ~6 \(\mu\)mol/g greater and Cr was ~6 \(\mu\)mol/g lower than when CK was not inhibited. Because total Cr remains constant, this lower PCr content indicates that an energy-utilizing process was activated, a suggestion that has been made by Meyer et al. (26) and Soderlund and Hultman (30) and is consistent with the data of Curtin and Woledge (8). Therefore, an artifact exists in the isolation and freezing process that consumes high-energy phosphates and substantially effects chemical measurement of PCr and Cr. Interestingly, the observation of a greater PCr content (~5.5 \(\mu\)mol/g) in the muscle of CK-deficient compared with wild-type mice (33) could characterize the same process as demonstrated here.

It is curious that the higher PCr amount in the presence of iodoacetamide was similar among the different skeletal muscle fiber sections. This implies that the total \(P_i\) exchange in the absence of iodoacetamide was similar for each of the muscles. The precipitating event for this hydrolysis is likely Ca\(^{2+}\) release from the sarcoplasmic reticulum as a result of tissue damage (30), rapid cooling (21), or freezing (13). This in turn would activate ATPases and hydrolyze ATP to ADP (26, 30). Given the high capacity of CK activity and the high equilibrium constant, there is expected to be a rapid decrease in PCr content with little to no change in ATP content (6). If the ATP hydrolysis rate was the limiting process, then the amount of the PCr artifact might be expected to vary with fiber type (26), because significant differences in the rates of sarcoplasmic re-

![Graph showing Phosphocreatine content and muscle freezing](image-url)
ticulum Ca^{2+} ATPase (16) and myosin ATPase exist among the different fiber types (4, 5). Obviously, this was not the case. Two alternatives may be pertinent. First, there may not be a sufficient difference in ATPase rates, relative to the variation in artifact (e.g., due to differences in collection time and/or freezing rate), to manifest a difference in PCr artifact among the fiber types of this study. Second, the extent of activation (e.g., the amount of calcium released) may be such that a fixed amount of ATP hydrolysis occurs in spite of different rates. During contractions when the PCr is low, the freeze artifact is expected to be smaller, possibly related to a reduced sensitivity of the involved ATPase(s) in the extreme cellular environment. Furthermore, in the presence of the high demand for ATP resynthesis, the CK rate should be influenced by the PCr concentration available to hydrolyze. Thus, the lower the initial PCr content (or PCr/Cr ratio), the smaller should be the change in PCr content that occurs during the quick-freezing process. Indeed, there can be no difference in PCr in muscles frozen with and without CK activity when the initial PCr content is zero. We confirmed this expectation, but not with absolute precision in all fiber sections. The size of the PCr artifact remained 20–30% of the existing PCr pool within the muscle, at the time of the isolation and freezing, regardless of whether the muscle was resting or during intense contractions, in two of the muscle sections (soleus and white gastrocnemius), but not in the third (red gastrocnemius). We cannot account for the larger (42%) PCr artifact in the red gastrocnemius suggests that this PCr value may be aberrant. Nonetheless, we interpret our findings to support the expectation that the magnitude of the PCr artifact is directly proportional to the PCr pool within the muscle.

Fig. 2. Difference in phosphate equivalents between muscle treated with iodoacetamide and muscle in its absence (plantaris; n = 8). F1,6BP, fructose 1,6 bisphosphate. See text for calculation of the composite change in phosphate.

If our findings have general application, then the chemically determined PCr and Cr measurements, typically presented in many animal or human studies, do not represent the true in vivo values. This can have several implications. First, it is apparent that any calculation of ADP_{i} would be incorrect because of the large error in the PCr/Cr ratio used. Recalculated ADP_{i} values, corrected for the PCr artifact described herein, would be considerably lower for resting muscle but increase over a wider-fold range as PCr content declines experimentally within the muscle. Thus any reliance on the uncorrected value(s) of ADP_{i} would be misleading. Fortunately, typical interpretations of results in those studies are based on the general pattern or direction of response, not on absolute value(s) of ADP_{i}. Thus even though errors are found in published work, the interpretation of results in the affected studies remain generally unchanged (e.g., Refs. 10, 24). Second, the observed phenomenon reported here may help clarify previous results implicating changes in and/or the distribution of PCr and Cr pools within muscle. For example, some studies involving Cr depletion in rats (25, 31) have reported a surprisingly low PCr/Cr ratio in the quick-frozen muscle. As predicted by Meyer et al. (25), these aberrant values can be reconciled if a freezing artifact is considered. Recalculations of PCr and Cr contents to account for the expected PCr hydrolysis return the PCr/Cr ratios to values more consistent with the expected energy state of the rested muscle (25). Similarly, the general inconsistency of low PCr/Cr ratios often observed with oral
Cr supplementation (15, 17) could be reconciled, if the variability in PCr hydrolysis that occurs during quick-freezing were similar in magnitude to the induced change in muscle PCr brought about by Cr supplementation. Thus true PCr changes would be lost in the measurement variability. It is unlikely, however, that the observed phenomenon reported here can clarify previous results that implicate the existence of different pools of PCr and/or Cr within muscle. For example, evidence for a large discrete pool of Cr, suggested by differential radiolabeling of Cr and PCr in fish muscle (19), is further strengthened if their results for rested muscle are recalculated taking into account the \( \sim 6 \mu\text{mol/g} \) PCr isolation/freezing artifact. Similarly, there is little quantitative relationship between the PCr that was degraded during freezing and the putative PCr pool contained within the mitochondria (29, 34). The \( \sim 6 \mu\text{mol/g} \) PCr measured here for the white gastrocnemius is far greater (more than sixfold) than a liberal estimate of the mitochondrial PCr pool, on the basis of reasonable estimates of mitochondrial volume (11) and a maximal mitochondrial Cr concentration of 20 mM (34). Finally, it is important to emphasize that the artifact characterized in this study pertains to data obtained by using chemical determinations of PCr and Cr. In this regard, studies employing \( ^{31}\text{P-NMR spectroscopy} \) for high-energy phosphates are not confounded; in fact, this in vivo method identified the inconsistencies in PCr measurement that our study helps reconcile experimentally.

Similar to the findings demonstrated in isolated perfused hearts (12, 18), we confirmed that CK was inhibited by iodoacetamide in skeletal muscle in our case by eliminating PCr degradation during contractions (cf. Fig. 1). In contrast, iodoacetamide does not effectively inhibit mechanical function at moderate work intensity or ATPases in vitro in the heart (12, 18). ATP hydrolysis also proceeds in skeletal muscle of this study, because there was a net loss of ATP and an increase in ADP, AMP, and IMP contents in iodoacetamide-treated muscles, compared with muscles in the absence of iodoacetamide (Fig. 2). This loss of high-energy bonds from ATP, presumably caused by the lack of contribution from PCr, represents a net ATPase activity of \( \sim 2.5 \mu\text{mol/g} \), an amount seemingly less than the \( \sim 6 \mu\text{mol/g} \) represented in the PCr that was retained. Whether this represents a reduction in ATPase rate with iodoacetamide is unclear. Fortunately, this does not impact on the calculation of the isolation/freeze artifact, because evidence of the artifact is solely due to differences in PCr content.

In summary, PCr content of inactive muscle was appreciably higher when isolated and frozen in the presence of iodoacetamide, a CK inhibitor. This difference was lessened as PCr was reduced by prior muscle contractions. Our results provide quantitative evidence for an isolation/freeze artifact that occurs in the chemical measurement of PCr, Cr, and P; in all fiber types. This artifact directly affects the measurement and, thus, the calculations of muscle energetic parameters from studies using isolated and frozen muscle.

We gratefully acknowledge the excellent technical assistance of Hong Song and Jackie Love. This study was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-21617.

REFERENCES


