Evidence against regulation of AMP-activated protein kinase and LKB1/STRAD/MO25 activity by creatine phosphate


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Submitted 12 July 2005; accepted in final form 1 November 2005

Taylor, Eric B., William J. Ellingson, Jeremy D. Lamb, David G. Chesser, Cori L. Compton, and William W. Winder. Evidence against regulation of AMP-activated protein kinase and LKB1/STRAD/MO25 activity by creatine phosphate. Am J Physiol Endocrinol Metab 290: E661–E669, 2006. First published November 8, 2005; doi:10.1152/ajpendo.00313.2005.—Muscle contraction results in phosphorylation and activation of the AMP-activated protein kinase (AMPK) by an AMPK kinase (AMPKK). LKB1/STRAD/MO25 (LKB1) is the major AMPKK in skeletal muscle; however, the activity of LKB1 is not increased by muscle contraction. This finding suggests that phosphorylation of AMPK by LKB1 is regulated by allosteric mechanisms. Creatine phosphate is depleted during skeletal muscle contraction to replenish ATP. Thus the concentration of creatine phosphate is an indicator of cellular energy status. A previous report found that creatine phosphate inhibits AMPK activity. The purpose of this study was to determine whether creatine phosphate would inhibit 1) phosphorylation of AMPK by LKB1 and 2) AMPK activity after phosphorylation by LKB1. We found that creatine phosphate does not inhibit phosphorylation of either recombinant or purified rat liver AMPK by LKB1. We also found that creatine phosphate does not inhibit 1) active recombinant α1β1γ1 or α2β2γ2 AMPK, 2) AMPK immunoprecipitated from rat liver extracts by either the α1 or α2 subunit, or 3) AMPK chromatographically purified from rat liver. Inhibition of skeletal muscle AMPK by creatine phosphate was greatly reduced or eliminated with increased AMPK purity. In conclusion, these results suggest that creatine phosphate is not a direct regulator of LKB1 or AMPK activity. Creatine phosphate may indirectly modulate AMPK activity by replenishing ATP at the onset of muscle contraction.

MUSCLE CONTRACTION RESULTS IN ACTIVATION OF THE AMP-activated protein kinase (AMPK) (36). Acute activation of AMPK initiates changes in cellular metabolism, enabling working skeletal muscle to better meet an energy challenge (7, 8, 35). These metabolic responses include increased glucose transport (11, 14, 19), increased fatty acid oxidation (19, 32), and decreased protein synthesis (3, 12, 24). Chronic activation of AMPK also results in adaptive transcriptional events (16), including mitochondrial biogenesis (2, 37, 40), which make skeletal muscle more robust when encountering subsequent energy challenges.

AMPK requires phosphorylation on its activation loop at Thr172 by an AMPK kinase (AMPKK) for activation (10, 29, 34). The tumor suppressor kinase LKB1 (STK-11) is a major AMPKK (9, 28, 38). Recently, generation of a skeletal muscle-specific LKB1 knockout mouse demonstrated that LKB1 is the major AMPKK in skeletal muscle (26). LKB1 requires association with the regulatory proteins Ste20-related adapter protein (STRAD) and mouse protein 25 (MO25) for full activity (4, 5, 9). An increase in the intracellular ATP-to-AMP ratio induces a conformational change in AMPK by interacting with 4 cystathionine β-synthase (CBS) domains on the γ-subunit (1, 27). This conformational change makes AMPK a suitable substrate for the constitutively active LKB1/STRAD/MO25 complex (LKB1). LKB1 activity is not increased by muscle contraction (25). AMPK activity has also been reported to be regulated by creatine-to-creatine phosphate and NAD-to-NADH ratios (22, 23).

Muscle contraction results in depletion of creatine phosphate, which is used as a temporary energy source to replenish ATP (18, 33, 39). Concentrations of creatine phosphate in skeletal muscle range from 5 to 40 mM (18, 33, 39). Creatine phosphate has been reported to inhibit AMPK activity. Furthermore, dietary administration of β-guanadinopropionionic acid results in creatine phosphate depletion and AMPK activation (40). Previously we demonstrated that long-chain acyl-CoA esters inhibit phosphorylation of AMPK by LKB1 (30). We hypothesized that creatine phosphate might allosterically regulate phosphorylation of AMPK by LKB1. We found that creatine phosphate does not inhibit phosphorylation of AMPK by LKB1. We also found that creatine phosphate does not inhibit recombinant or highly purified rat liver AMPK.

MATERIALS AND METHODS

Animal care. All procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Male Sprague-Dawley (SAS:VAF) rats (Sasco, Wilmington, MA) were housed in a temperature-controlled (21–22°C) room with a 12:12-h light-dark cycle. Rats were fed standard rat chow (Harlan-Teklad rodent diet, Madison, WI) and water ad libitum. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (48 mg/kg body wt).

Buffers. Buffer B consisted of 50 mM Tris·HCl, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 0.02% (wt/vol) Brij-35, 10% (vol/vol) glycerol, pH 7.4 at 4°C, 1 mM benzamidine, 1 μg/ml soybean trypsin inhibitor, 0.5 mM 4-[(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and 1 mM di-thiothreitol (DTT), which were added just before use; buffer C was like buffer B but without NaF, sodium pyrophosphate, benzamidine, soybean trypsin inhibitor, and AEBSF. AMPK phosphorylation buffer comprised 100 mM HEPES, 200 mM NaCl, 20% glycerol (vol/vol), 2 mM EDTA, 12.5 mM MgCl2, 0.5 mM ATP, 0.5 mM AMP, and 2.0 mM DTT, pH 7.0. AMARA phosphorylation buffer consisted of 40
mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl2, 0.2 mM ATP, 0.2 mM AMP, 0.33 mM AMARA peptide, and 0.05 μCi/μl [γ-32P]ATP, pH 7.0. Laemmli’s buffer was as previously described (15).

Materials. General reagents were obtained from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise stated. Creatine phosphate di-sodium and di-tris salts were obtained from Sigma-Aldrich Chemical. Recombinant LKB1/STRAD/MO25 lot no. 28144 (LKB1), LKB1tide, and protein phosphatase 2A (PP2A) were obtained from Upstate (Charleston, VA). His-bind nickel-binding resin was obtained from Novagen (Madison, WI). Polyethylene glycol 6000 (PEG) was obtained from Calbiochem (La Jolla, CA). Resins for chromatographic purifications were obtained from Amersham Biosciences (Piscataway, NJ).

Chromatographic purification of AMPK. AMPK from rat liver was purified as previously described by Hawley et al. (10). The same procedure was followed for isolation of gastrocnemius muscle AMPK with the following modifications. Muscles were stimulated to contract by electrical stimulation to activate AMPK. After activation of AMPK, muscles were excised and clamp-frozen at liquid nitrogen temperature, ground to powder by pestle and mortar at liquid nitrogen temperature, and then homogenized with a Brinkman Polytron PT 3000 in nine parts ice-cold buffer B to one part gastrocnemius muscle (vol/wt). The 2.5–6% PEG precipitation and chromatographic purification then proceeded identically to the liver purification. AMPK was stored at −95°C.

Generation and purification of recombinant AMPK. Bacteria expressing recombinant α1β1γ1 AMPK were prepared as previously described (6, 20, 21). Recombinant α2β2γ2 AMPK was prepared by an identical method. Recombinant α1β1γ1 and α2β2γ2 AMPKs were extracted and purified by nickel affinity chromatography as previously described (31). AMPK was concentrated in buffer C by use of Centricon Plus-20 centrifugal filter devices (Millipore, Bedford, MA) before use for AMPKK activity assays. Recombinant α1β1γ1 AMPK and recombinant α2β2γ2 AMPK were concentrated to 0.44 and 0.27 μg/μl, respectively.

AMPKK and AMPK activity assay. AMPKK activity was measured in a two-step assay as described previously (6, 10, 31). During the first step, AMPKK is phosphorylated and activated by LKB1. During the second step, phospho-Thr172 AMPK (P-AMPKK) phosphorylates AMARA peptide (10). Recombinant LKB1 (0.10 μg/μl) was diluted 1:19 in buffer C. Creatine phosphate was dissolved in water and then serially diluted in water or water with sodium acetate at a concentration 5× the desired final concentration during the first step of the assay. Recombinant α1β1γ1 AMPK was diluted 1:9 in water, and recombinant α2β2γ2 AMPK was diluted 1:19 in water. For the assay, 500 μl of polypropylene microcentrifuge tubes were loaded with 4 μl of AMPK phosphorylation buffer, 2 μl of diluted recombinant AMPKK preparation, and 2 μl of 5× creatine phosphate solution. Diluted LKB1 was added to start the first step of the reaction and incubated for 20 min at 30°C. After the first incubation, 15 μl of AMARA phosphorylation buffer was added to start the second step of the assay. After a 10-min incubation, the reaction was stopped by spotting a 1-cm² piece of Whatman P81 filter paper with 15 μl of the final reaction mix, waiting 20 s for complete absorption, and then placing the filter paper into a beaker with 100 ml of 1% phosphoric acid. At the conclusion of the assay, 5 × 90-s washes with 100 ml of 1% phosphoric acid were followed by a brief rinse with acetone. Filter papers were dried and then placed in 3 ml of ecolite scintillation fluid and counted for 1 min. For AMPKK assays using purified rat liver AMPK as a substrate, rat liver AMPK was dephosphorylated by incubation with 10 U/ml PP2A for 10 min at 30°C. PP2A was inactivated by the addition of 50 μM okadaic acid sodium salt dissolved in water to a final concentration of 200 nM.

Assays to measure the inhibitory effect of creatine phosphate on P-AMPKK were performed as above but with the following changes. Recombinant α1β1γ1 was diluted 1:24 in water. Recombinant α2β2γ2 was diluted 1:49 in water. LKB1 was diluted 1:4 in buffer C. To fully phosphorylate recombinant AMPK, 4 μl of AMPK phosphorylation buffer, 2 μl of water, 2 μl of diluted LKB1, and 2 μl of diluted recombinant AMPK were incubated at 30°C for 120 min (α1β1γ1) or 30 min (α2β2γ2). After incubation, 15 μl of AMARA phosphorylation buffer with creatine phosphate were added and allowed to incubate for an additional 10 min before the reaction was stopped and filter papers were spotted as above.

For assays of LKB1 activity against LKB1tide (17), 450 μl of AMPK phosphorylation buffer, 225 μl of 0.775 mM LKB1tide in water, 135 μl of water, and 4 μl of [γ-32P]ATP were combined, and 18 μl of this mixture were loaded into 500 μl of polypropylene microcentrifuge tubes. Tubes were loaded with 5× creatine phosphate solution as above, and the reaction was started with the addition of 2 μl of recombinant LKB1 diluted 1:4 and stopped by spotting filter papers and continuing as above.

For assays of AMPK from rat liver or muscle, 5 μl of AMPK from PEG precipitation, immunoprecipitation, or chromatographic purification were added to 5 μl of 5× creatine phosphate-sodium acetate in water and 15 μl of AMARA phosphorylation buffer and incubated for 10 min. The reaction was stopped by spotting filter papers as above. AMPK was immunoprecipitated as described previously by use of antibodies directed against the α1 and α2 subunits except that the final washes of the pellet just before the assay were performed in buffer C (13).

Statistics. The inhibitory effects of creatine phosphate at different concentrations on the phosphorylation of AMPK or LKB1tide by LKB1 and the phosphorylation of AMARA peptide by AMPK were compared by one-way analysis of variance. When main effects or interactions reached significance, the Newman-Keuls multiple-comparison test was used to determine the location. For all statistical tests, significance was set at P < 0.05. All statistical procedures were performed with the NCSS statistical program (Kaysville, UT). All data are reported as means ± SE.

RESULTS

To test the hypothesis that creatine phosphate inhibits the phosphorylation of AMPK by LKB1, we assayed the activity of recombinant LKB1/STRAD/MO25 (LKB1) against recombinant α1β1γ1 and α2β2γ2 AMPK in the presence of a creatine phosphate-dsodium salt at concentrations of 0, 5, 10, 20, 35, and 50 mM. Creatine phosphate-2Na⁺ reduced LKB1 activity toward recombinant α1β1γ1 AMPK at concentrations of 5, 10, 20, 35, and 50 mM (Fig. 1A) and recombinant α2β2γ2 AMPK at 10, 20, 35, and 50 mM (Fig. 1B). However, after accounting for the sodium added with the creatine phosphate by balancing with sodium acetate, the inhibitory effect was no longer present (Fig. 1, C and D). Thus, at a uniform sodium concentration of 100 mM, the replacement of two monovalent acetate anions with one divalent creatine phosphate anion resulted in a slightly increased LKB1 activity toward both α1β1γ1 and α2β2γ2 recombinant AMPK.

Both sodium chloride and sodium acetate alone markedly inhibit the phosphorylation of recombinant AMPK by recombinant LKB1 (Fig. 2). Tris salts show similar types of curves (data not shown). A smaller, but significant effect is seen with active recombinant AMPK that is fully phosphorylated.

Next, we tested the effect of creatine phosphate on the reactivation of a nonrecombinant AMPK by LKB1 (Fig. 3). Treatment of chromatographically purified rat liver AMPK with protein phosphatase 2A (PP2A) resulted in an almost complete loss of AMPK activity. AMPK was reactivated by adding recombinant LKB1 in the presence or absence of 50 mM creatine phosphate while controlling for sodium with
sodium acetate. Addition of LKB1 resulted in a significant reactivation of AMPK that was halted before full reactivation could occur. The addition of creatine phosphate had no effect on LKB1 activity. The same experiment was repeated with creatine phosphate di-tris with a Tris control, and creatine phosphate was found not to inhibit LKB1 activity (data not shown).

To determine whether LKB1 activity was inhibited against a substrate other than AMPK, we measured the activity of LKB1 against the peptide substrate LKB1tide at creatine phosphate concentrations of 0, 10, 20, 35, and 50 mM while controlling for sodium and total ionic strength with the addition of sodium acetate (Fig. 4A). Creatine phosphate at concentrations of 5, 10, 20, 35, and 50 mM inhibited LKB1 activity toward the peptide substrate LKB1tide. This result is in contrast to those we found against recombinant or liver heterotrimeric AMPK. To determine whether another small phosphomolecule could exert a similar inhibitory effect, we ran the identical assay but substituted a glucose 6-phosphate di-sodium salt (G-6-P) for the creatine phosphate di-sodium salt. G-6-P inhibited LKB1 activity similarly to creatine phosphate (Fig. 4B).

The results from the experiments utilizing the two-step assay to test LKB1 activity against AMPK suggested that creatine phosphate was not exerting an inhibitory effect against AMPK either. Accordingly, we first fully phosphorylated recombinant AMPK and then subsequently added 50 mM creatine phosphate (balanced with sodium acetate) concurrently with the addition of AMARA peptide. Creatine phosphate at 50 mM had a small activating effect on P-AMPK (Fig. 5A) and had no effect on P-AMPK (Fig. 5B).

To determine whether creatine alone or a combination of creatine and creatine phosphate would influence AMPK activity, fully phosphorylated recombinant AMPK was incubated in the presence of 30 mM creatine phosphate (balanced with sodium acetate) concurrently with the addition of AMARA peptide. Creatine phosphate at 50 mM had a small activating effect on P-AMPK. Creatine likewise had no effect on the phosphorylation of recombinant AMPK by recombinant LKB1 in either the presence or absence of creatine phosphate (data not shown).

To investigate the effect of creatine phosphate on nonrecombinant AMPK, we tested AMPK from both rat liver and skeletal muscle. We assayed a crude AMPK preparation from rat liver 2.5–6% PEG precipitates with creatine phosphate (balanced with sodium acetate) concentrations of 0, 10, 20, 35, and 50 mM (Fig. 7A). A statistically significant but small
inhibition was found with creatine phosphate concentrations of 20, 35, and 50 mM \((P < 0.05)\). Next, gastrocnemius PEG precipitates were tested under identical conditions, but a nearly maximal inhibition was found at 10 mM creatine phosphate (data not shown). To determine the range of the inhibitory effect, PEG precipitates from gastrocnemius muscle were assayed for AMPK activity in the presence of creatine phosphate at concentrations of 0.2, 0.5, 1, 2, and 10 mM (Fig. 7B). Addition of creatine phosphate resulted in significant inhibition of activity at concentrations of 0.5, 1, 2, and 10 mM \((P < 0.05)\). Because of the very different inhibitory effect of creatine phosphate on liver vs. skeletal muscle resuspended PEG precipitate AMPK preparations, we tested the effect of the small phosphomolecule G-6-P (balanced with sodium acetate) against the muscle preparation at concentrations of 0.2, 0.5, 1, 2, and 10 mM (Fig. 5C). Addition of G-6-P resulted in a potent inhibition of AMPK activity, with a significant effect appearing by 0.2 mM G-6-P \((P < 0.05)\). Maximal inhibition was greater with G-6-P than with creatine phosphate. When chromatographically purified gastrocnemius AMPK was tested, however, G-6-P, fructose 6-phosphate (F-6-P), or glucose 1-phosphate (G-1-P) had no effect on activity (data not shown). Thus the inhibitory effect of G-6-P was observed only in the less pure preparations.

Fig. 2. AMPKK activity of LKB1 and activity of recombinant \(\alpha_{2}\beta_{2}\gamma_{2}\) AMPK in the presence of 0, 10, 20, 40, 70, and 100 mM sodium chloride or sodium acetate. Concentrations shown do not include salts contributed by the buffer. \(A\): activation of recombinant \(\alpha_{2}\beta_{2}\gamma_{2}\) AMPK by LKB1. \(B\): activity of recombinant \(\alpha_{2}\beta_{2}\gamma_{2}\) AMPK after complete phosphorylation and activation by LKB1. All values were normalized to control values without salt. *Activity was significantly depressed from controls for both salts at all concentrations above 10 mM for LKB1 and above 40 mM for AMPK \((n = 3, P < 0.05)\).

Fig. 3. Activity of rat liver AMPK before and after treatment with protein phosphatase 2A (PP2A) and after partial reactivation by LKB1 in the presence of 0 or 50 mM creatine phosphate (CP). *AMPK activity was significantly different from that after reactivation by LKB1 in the presence of 0 mM CP \((n = 5, P < 0.05)\).
AMPK from liver and muscle PEG precipitates was isolated by immunoprecipitation. Separate immunoprecipitations were performed against both the $\alpha_1$ and $\alpha_2$ isoforms. Immunoprecipitates were assayed for AMPK activity in the presence of 0, 10, 20, 35, and 50 mM creatine phosphate (with sodium acetate). At all concentrations tested, creatine phosphate had no inhibitory effect on the AMPK activity of both the $\alpha_1$ (Fig. 8A) and $\alpha_2$ (Fig. 8B) immunoprecipitates from liver ($P < 0.05$).

Creatine phosphate had no inhibitory effect against $\alpha_1$ immunoprecipitates from gastrocnemius (Fig. 8C), whereas a small inhibitory effect beginning at a concentration of 10 mM creatine phosphate was found against $\alpha_2$ immunoprecipitates (Fig. 8D; $P < 0.05$).

As an alternative to immunoprecipitation, we further purified gastrocnemius and liver AMPK from PEG precipitates by
column chromatography. AMPK chromatographically purified from liver (Fig. 9A) and gastrocnemius (Fig. 9B) was assayed for activity in the presence of creatine phosphate (with sodium acetate) at concentrations of 0, 10, 20, 35, and 50 mM. Creatine phosphate had a small inhibitory effect against purified gastrocnemius AMPK starting at a concentration of 10 mM (P < 0.05). In contrast, creatine phosphate had a small activating effect against AMPK purified from liver at concentrations of 20, 35, and 50 mM (P < 0.05). As a final test of the effect of using sodium acetate as a sodium control, we assayed purified liver AMPK for activity in the presence of 0, 10, 20, 35, and 50 mM creatine phosphate without controlling for the sodium added with the creatine phosphate (Fig. 9C). An extremely small inhibitory effect was found at concentrations of 20, 35, and 50 mM when a sodium control was not included (P < 0.05).

**DISCUSSION**

To date, relatively little information is available on effects of metabolites on the phosphorylation and activation of AMPK by upstream kinases. Previously, we reported that long-chain acyl-CoA esters inhibit phosphorylation of AMPK by LKB1/STRAD/MO25 (LKB1) (30). Creatine phosphate has been reported to inhibit AMPK (22). Therefore, we initially aimed to determine whether creatine phosphate also inhibited the phosphorylation of AMPK by LKB1. The results of the two-step assay used to measure LKB1 activity toward AMPK suggested that creatine phosphate was not inhibiting AMPK. Therefore, we also evaluated the effect of creatine phosphate on AMPK activity.

Our first experiments suggested that creatine phosphate was inhibiting LKB1 activity toward AMPK (Fig. 1, A and B). However, the creatine phosphate utilized was a di-sodium salt. Thus adding creatine phosphate to the reaction increased the Na⁺ molarity by double the increase in creatine phosphate molarity. During earlier efforts to purify the yet-to-be-identified AMPKK to homogeneity, we noticed that high salt concentrations led to a marked drop-off in AMPKK activity (data not shown). Therefore, experiments were performed to determine whether the apparent inhibition of LKB1 was specifically due to the creatine phosphate or the concomitant increase in Na⁺ and ionic strength. Our data clearly demonstrated that the activity of the recombinant LKB1 was markedly influenced by the salt concentration in the reaction medium. This finding suggested that the inhibition initially thought to be due to creatine phosphate could be due entirely to the salt contributed by the creatine phosphate preparation.

We controlled for the sodium added with creatine phosphate by balancing with sodium acetate. Sodium acetate was chosen for a sodium control after screening of several salts, including sodium citrate, sodium chloride, sodium sulfate, and Tris-HCl, for their independent effects on LKB1 activity. All salts reduced LKB1 activity. Acetate has a pKₐ of 4.76 and therefore does not interfere with the acid-base balance of the assay and requires very little HCl, and therefore the addition of chloride ion, to pH balance preparatory to the assay. Additionally,
Magnesium acetate is highly soluble in aqueous solutions. Thus the addition of acetate does not interfere with magnesium availability for the kinase reaction. The net effect of balancing creatine phosphate-2Na+/H10011 with sodium acetate was the addition of one creatine phosphate molecule and the subtraction of two acetate molecules for each incremental increase in creatine phosphate concentration.

Controlling for sodium completely abolished the inhibitory effect of creatine phosphate on the AMPKK activity of LKB1 (Fig. 1, C and D). Because the same effect was found for both recombinant αβ1γ1 and αβ2γ2 AMPK activity, the lack of inhibition was not isoform specific. These isoforms were not chosen for their biological relevance to any particular tissue but because different isoforms of each subunit could be tested with two constructs. We did not clone active complexes with the γ3 subunit. None have been reported by other groups. Because creatine phosphate did not inhibit reactivation of highly purified AMPK from rat liver (Fig. 3), the lack of an inhibitory effect is not an artifact limited to the use of recombinant AMPK as a substrate for LKB1. Additionally, no inhibitory effect was found when we used a creatine phosphate di-tris salt balanced with Tris-HCl. This result suggested that sodium acetate was not specifically masking an inhibitory effect of creatine phosphate (data not shown).

G-6-P (Fig. 4A) inhibited LKB1 activity toward the peptide substrate LKB1tide similarly to creatine phosphate (Fig. 4B). This result suggested that the effect of creatine phosphate under these conditions was not due to any specific effect of creatine phosphate but rather a general effect of small phosphomolecules. We previously reported (30) that, although long-chain acyl-CoA esters inhibit phosphorylation of AMPK by LKB1, they stimulate phosphorylation of LKB1tide. These results indicate that 1) creatine phosphate does not specifically inhibit LKB1 under any conditions tested, and 2) as suggested in our previous report, LKB1tide is not a reliable substrate for measuring the AMPKK activity of LKB1.

In a previous study investigating the effect of creatine phosphate on AMPK activity, a stimulation of AMPK activity was noted in response to inclusion of creatine in the reaction mix (22). A concentration of 20 mM creatine was found to entirely overcome the inhibitory effects of both 20 and 40 mM creatine phosphate. In contrast, we saw no stimulatory effect of creatine on the activity of active, phosphorylated recombinant AMPK or the rate of phosphorylation of recombinant AMPK by LKB1 in either the presence or absence of creatine phosphate.

Because creatine phosphate from the first step of the AMPKK assay remains in the reaction mixture for the second step of the assay, creatine phosphate was expected to inhibit the second step of the assay. Because no inhibition was found, we speculated that creatine phosphate was not inhibiting AMPK either. Neither fully active recombinant αβ1γ1 AMPK nor fully active recombinant αβ2γ2 AMPK was inhibited by creatine phosphate. This result provided initial evidence that creatine phosphate does not inhibit AMPK (Fig. 5, A and B). Because the recombinant AMPK was prepared in a bacterial expression system, it was not contaminated with mammalian phosphatases, creatine kinase, or other enzymes that might specifically modify creatine phosphate. However, this experiment alone was not conclusive, because the lack of inhibition could have been an artifact of a bacterial expression system like improper folding of the recombinant AMPK.

Creatine phosphate inhibited AMPK PEG precipitates from liver vs. gastrocnemius muscle to markedly varying degrees (Fig. 7, A and B). The much greater inhibition found on the muscle AMPK preparation suggested that a contaminant in either preparation was interacting with the creatine phosphate and confounding the results. If a phosphatase or other enzyme is present in one or both preparations, the magnitude of inhibition would be affected. Because controls that included sodium acetate in the creatine phosphate stock behaved like the creatine phosphate stock without sodium acetate, the sodium acetate did not mask the inhibitory effect of creatine phosphate.
were nonspecifically interacting with the creatine phosphate to produce an inhibitory effect by increasing the free phosphate concentration, a small phosphomolecule like G-6-P would be expected to produce similar results. The more potent inhibitory effect of G-6-P compared with creatine phosphate in the muscle preparation suggested that the inhibition was not specifically due to creatine phosphate. To remove potential contaminants, AMPK was further purified from the PEG precipitates.

Additional purification of AMPK from PEG precipitates by immunoprecipitation eliminated the inhibitory effect of creatine phosphate against liver α1 and α2 AMPK and muscle α1 AMPK. The result that immunoprecipitation resulted in a loss of inhibition suggests that the inhibition found on the much more crude AMPK PEG precipitates was the result of a contaminant. A small inhibitory effect against α2 muscle AMPK persisted. Although a small inhibition of muscle α2 AMPK remained, inhibition of liver α2 AMPK was not found. This indicates that the remaining inhibition of skeletal muscle but not liver AMPK was due to a contaminant from skeletal muscle rather than a direct effect of creatine phosphate on the α2 AMPK heterotrimer. These results strongly support data from the recombinant AMPK experiments, suggesting that creatine phosphate does not inhibit AMPK. Our finding that creatine phosphate failed to inhibit AMPK immunoprecipitated from rat liver PEG precipitates contradicts the earlier report that creatine phosphate inhibits AMPK (22). The reasons for our divergent results are unclear; however, the earlier study does not indicate which form of creatine phosphate was used or whether a control was used for the salt added with the creatine phosphate.

As an alternative to immunoprecipitation, liver and muscle AMPK were purified beyond the PEG precipitation by column chromatography. Creatine phosphate exerted a slight activating effect on highly purified AMPK from rat liver. Although a slight inhibitory effect remained toward muscle AMPK, chromatographic purification of muscle AMPK markedly reduced the substantial amount of inhibition found toward muscle PEG precipitates. These results concur with the results found with recombinant and immunoprecipitated AMPK. The extremely small inhibition of purified liver AMPK found when sodium acetate was not utilized as a sodium control demonstrates that the absence of a creatine phosphate-mediated inhibitory effect was not being caused by the presence of sodium acetate. Additionally, the substantial inhibition found against crude AMPK PEG precipitates was present with the sodium acetate control. Furthermore, although G-6-P potently inhibited AMPK in skeletal muscle PEG precipitates, G-6-P had no inhibitory effect on chromatographically purified skeletal muscle AMPK (data not shown). It is possible that addition of G-6-P to less pure preparations resulted in perturbations in ATP or AMP in the reaction mix, thus causing apparent decreases in activity without necessarily inhibiting AMPK directly.

Even if creatine phosphate does not inhibit AMPK or LKB1 directly, it most certainly is important for the regulation of the cellular AMP/ATP ratio, the classic regulator of AMPK activity. In the initial stages of muscle contraction, creatine phosphate can be utilized to buffer ATP depletion caused by an elevated rate of ATP utilization. At rest, when creatine phosphate concentration is elevated in the muscle, ATP is maintained at a high concentration, whereas AMP is at a low concentration. With a low rate of ADP production, the adenylate kinase equilibrium is in favor of ADP and little AMP is formed. At the onset of muscle contraction, creatine phosphate is rapidly hydrolyzed to creatine by creatine kinase to convert ADP to ATP. As the creatine phosphate concentration declines, the consequent rise in free ADP shifts the equilibrium of the adenylate kinase reaction so that AMP begins to accumulate in the cell. The consequent increase in the AMP/ATP ratio results in activation of AMPK by LKB1. Without creatine phosphate to initially buffer ATP depletion, ADP and thus AMP would accumulate more rapidly. The higher rate of AMP production would result in earlier activation of AMPK. Likewise, the absence of creatine phosphate would be expected to result in AMPK activation during very brief periods of muscle contraction. Hence, creatine phosphate is likely an indirect regulator of AMPK activity.

In summary, we found that creatine phosphate does not inhibit phosphorylation of recombinant or purified rat liver AMPK by LKB1. Inhibition of LKB1 activity against the peptide substrate LKB1tide was not specific to creatine phosphate. Inhibition of rat liver AMPK by creatine phosphate was completely abolished with increasing AMPK purity. G-6-P inhibited crude muscle AMPK from 2.5 to 6% PEG precipitates more potently than creatine phosphate. Furthermore, creatine phosphate inhibition of rat gastrocnemius muscle AMPK was greatly reduced with increased AMPK purity. We found that creatine phosphate did not inhibit I) active recombinant α1β1γ1 or α2β2γ2 AMPK, 2) α1 and α2 AMPK complexes immunoprecipitated from rat liver PEG precipitates, and 3) AMPK chromatographically purified from rat liver. Creatine phosphate may indirectly affect AMPK activity by buffering ATP depletion and AMP formation at the onset of skeletal muscle contraction. In conclusion, these results provide evidence that creatine phosphate is not a specific regulator of LKB1/STRAD/MO25 or AMPK activity.

GRANTS

This research was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-41438.

REFERENCES
