Knockout of the $\alpha_2$ but Not $\alpha_1$ 5'-AMP-activated Protein Kinase Isoform Abolishes 5-Aminimidazole-4-carboxamide-1-$\beta$-4-ribofuranoside- but Not Contraction-induced Glucose Uptake in Skeletal Muscle*

Received for publication, June 12, 2003, and in revised form, October 19, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M306205200

We investigated the importance of the two catalytic $\alpha$-isoforms of the 5'-AMP-activated protein kinase (AMPK) in 5-aminimidazole-4-carboxamide-1-$\beta$-4-ribofuranoside (AICAR) and contraction-induced glucose uptake in skeletal muscle. Incubated soleus and EDL muscle from whole-body $\alpha_2$- or $\alpha_1$-AMPK knockout (KO) and wild type (WT) mice were incubated with 2.0 mM AICAR or electrically stimulated to contraction. Both AICAR and contraction increased 2DG uptake in WT muscles. KO of $\alpha_2$ but not $\alpha_1$ abolished AICAR-induced glucose uptake, whereas neither KO affected contraction-induced glucose uptake. AICAR and contraction increased $\alpha_2$- and $\alpha_1$-AMPK activity in wild type (WT) muscles. During AICAR stimulation, the remaining AMPK activity in KO muscles increased to the same level as in WT. During contraction, the remaining AMPK activity in $\alpha_2$-KO muscles was elevated by 100% probably explained by a 2–3-fold increase in $\alpha_1$-protein. In $\alpha_1$-KO muscles, $\alpha_2$-AMPK activity increased to similar levels as in WT. Both interventions increased total AMPK activity, as expressed by AMPK-P and ACC-P, in WT muscles. During AICAR stimulation, this was dramatically reduced in $\alpha_2$-KO but not in $\alpha_1$-KO, whereas during contraction, both measurements were essentially similar to WT in both KO-muscles. The results show that $\alpha_2$-AMPK is the main donor of basal and AICAR-stimulated AMPK activity and is responsible for AICAR-induced glucose uptake. In contrast, during contraction, the two $\alpha$-isoforms seem to substitute for each other in terms of activity, which may explain the normal glucose uptake despite the lack of either $\alpha_2$- or $\alpha_1$-AMPK. Alternatively, neither $\alpha$-isoform of AMPK is involved in contraction-induced muscle glucose uptake.

The 5'-AMP-activated protein kinase (AMPK) is a multisubstrate serine/threonine protein kinase that is ubiquitously expressed and functions as an intracellular fuel sensor activated by depletion of high energy phosphor compounds (1, 2). Activation of AMPK initiates a complex series of signaling events, causing an increase in uptake and oxidation of substrates for ATP synthesis concurrent with decreasing ATP consuming biosynthetic processes such as protein (3, 4), lipid (1), and glycogen synthesis (5, 6).

Both human and rodent studies have shown that AMPK in skeletal muscle is activated during exercise in vivo (7–10) and during contraction in vitro (11–15) probably by several coincident mechanisms. These involve decreased ATP/AMP and PCr/Cr ratios (16, 17), decreased pH (16), and reduction of muscle glycogen content (6, 15) and substrate delivery (18–20). Therefore, it is tempting to ascribe a role for AMPK in muscle metabolism in response to exercise, and in particular investigators have hypothesized a role for AMPK in contraction-stimulated glucose uptake (11, 13, 21).

AMPK may also be activated by treatment with the adenosine analogue 5-aminimidazole-4-carboxamide-1-$\beta$-4-ribofuranoside (AICAR) in rat, mouse, and human skeletal muscle in vivo (6, 11, 21, 22) and in vivo in conscious rats (23). AICAR is taken up by the cell and is phosphorylated to ZMP, which mimics the activating effect of AMP on AMPK (2, 24) without affecting the adenosine nucleotide or phosphocreatine status of the cell (12, 21). Furthermore, AMPK can be activated by the antidiabetic drugs metformin (25–27) and rosiglitazone (27). Treatment with AICAR increases glucose uptake by an insulin-independent mechanism (11) that seems to depend on translocation of GLUT4 to the muscle surface membrane (28).

Activation of AMPK by exercise or drugs seems to have important therapeutic possibilities in the fight against conditions characterized by decreased insulin sensitivity such as type 2 diabetes and the metabolic syndrome. This view is supported by findings showing that short term activation of AMPK by AICAR increases insulin sensitivity of skeletal muscle (29). Long term treatment with AICAR has also been shown

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* This study was supported by Danish National Research Foundation Grant 504-14, the Danish Diabetes Association, the Novo Nordisk Foundation, European Commission Research and Technological Development Project QLG1-CT-2001-01488, INSERM, CNRS, and the French Ministry of Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Supported by a Hallas Møller fellowship from the Novo Nordisk Foundation.

1 The abbreviations used are: AMPK, 5'-AMP-activated protein kinase; AICAR, 5-aminimidazole-4-carboxamide-1-$\beta$-4-ribofuranoside; EDL, extensor digitorum longus; KO, knockout; WT, wild type; 2DG, 2-deoxy-d-glucose.

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to improve the metabolic status of obese KK-Ay-CETP mice (30) and diabetic ob/ob and db/db mice (31, 32) as well as obese Zucker rats (33), possibly by increasing the level of GLUT4 protein and various oxidative enzymes in skeletal muscle (34, 35).

In vitro studies of isolated mouse skeletal muscles overexpressing a kinase dead AMPK construct showed that AICAR- and hypoxia-stimulated glucose uptake was totally abolished, whereas contraction-stimulated glucose uptake was lowered by 30–40% (36). This clearly indicates dissimilarities between AICAR/hypoxia- and contraction-stimulated glucose transport in that AMPK is only partially responsible for contraction to stimulate muscle glucose transport. AMPK is a heterotrimeric enzyme complex consisting of a catalytic α-subunit and regulatory β- and γ-subunits. Two isoforms have been identified of the α-subunit (α1 and α2) and β-subunit (β1 and β2) and three isoforms of the γ-subunit (γ1, γ2, and γ3) (37–39). To date, it is not known which of the two catalytic α-isoforms convey the effects on glucose transport in skeletal muscle. It is also not known which of the two isoforms constitute the dominant isoform in skeletal muscle. These questions are important from a physiological point of view but also because design of drugs targeting AMPK may be facilitated if the precise α-AMPK isoform responsible for the desired metabolic effects is known.

To investigate this further, AMPK was activated by AICAR or contraction in muscles consisting of predominantly slow or fast twitch fiber types from mice that do not express either the α2- or α1-AMPK isoform. Here we show that AICAR stimulation of muscle glucose uptake requires the presence of the α2-isoform, whereas, in this respect, the α1-isoform is of no relevance. In contrast, neither of the two knockouts markedly decreases contraction-stimulated glucose uptake.

**MATERIALS AND METHODS**

**Targeting of the α2-Subunit Locus and Generation of α2-AMPK Knockout Mice**

A mouse 129-strain genomic library (Stratagene, La Jolla, CA) was screened with a specific mouse AMPK catalytic α2-subunit 500-bp fragment made by reverse transcription-polymerase chain reaction on liver messenger RNA using the forward 5′-AGGGCCCGACACACCCTAG-3′ and the reverse 5′-TGTTAGTCTCTGGCTTGAGA-3′ primers. One genomic clone encompassing a 14.5-kb genomic fragment was used to generate the targeting construct. This fragment contains the exons and the site of the previously reported IRES-2-isoform, whereas, in this respect, the α1-isoform is of no relevance. In contrast, neither of the two knockouts markedly decreases contraction-stimulated glucose uptake.

A 4-kb HincII-BglII 5′ homologous genomic fragment was blunt-ended with KpnI and linked into the 5′ NotI site of the previously reported IRES-γ-glut (40). This plasmid was Sall-digested, and the 3′ α1 homologous fragment, consisting of a 3-kb SacI-Sacl fragment, was inserted. The resulting targeting construct was linearized at the unique Xhol site and 40 μg was used to electroporate 1 × 10⁶ embryonic stem cells (ES cells; a kind gift from Anne K. Voell, Gottingen, Germany), which were cultured on mitomycin-treated embryonic fibroblast feeder layers. DNA from clones surviving G418 selection (200 μg/ml) was individually analyzed on Southern blot. DNA fragments were 7.5-kb and 13.5-kb for the 2-AMPK knockout mouse and its phenotype has recently been described elsewhere; for details, see Viollet et al. (42).

**Animals**

All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the Protection of Vertebrate Animals used for Experiments and Other Scientific Purposes (Council of Europe 123, Strasbourg, France, 1985). Four-month-old male and female α2-AMPK knockout (KO) and wild type (WT) mice as well as male α1-AMPK KO and WT mice were studied. Within each strain, KO and WT mice used for experiments were littermates produced by intercross-breeding using heterozygote parent animals. The genotype of the offspring was first determined by PCR on DNA extracted from a tail biopsy tested against positive control samples. The lack of α-AMPK protein was later verified by immunoblotting of samples from muscle tissue. The mice were maintained on a 12 h/12 h light-dark cycle and received standard rodent chow (Altromin 1324, Chr. Pedersen A/S, Ringsted, Denmark) and water ad libitum.

**Glucose, Insulin, and AICAR Tolerance Tests**

Tolerance tests were performed on both α2- and α1-AMPK KO and corresponding WT mice. Glucose (2 g/kg), insulin (0.5 units/kg; Ac- trapid, Novo Nordisk, Bagsvaerd, Denmark), or AICAR (250 mg/kg; Toronto Research Chemicals Inc., Toronto, CA) was given intraperitoneally at time 0 (isotonic solutions). Tail blood was collected at −20, 20, 40, 60, 105, and 150 min, and blood glucose concentration was determined using a glucometer (Bayer, Leverkusen, Germany). Mice were semisemifasted before the glucose and AICAR tolerance test (received 40% of daily food intake 18 h prior to test) but were fed before the insulin tolerance tests. Area under the curve (AUC; mmol·min⁻¹) is calculated from the changes in blood glucose concentration at time point 20−150 min compared with basal value at time −20 min.

**Incubation of Isolated Muscles**

Soleus (mainly slow twitch fibers) and extensor digitorum longus (EDL; mainly fast twitch fibers) were obtained from anesthetized mice (6 mg of pentobarbital 100 g⁻¹ body weight) and suspended by ligatures at resting tension (4–5 milleinewton) in incubation chambers (Multi Myograph system; Danish Myo-Technology, Aarhus, DK) in a Krebs-Henseleit buffer at 30 °C (42).

AICAR Stimulation—AICAR (2 mM, 40 min, Toronto Research Chemicals Inc., Toronto, Canada) was added after 10 min of basal incubation.

Contraction—After 40 min of basal incubation, contraction was induced by electrical stimulation with a 10-s train (100-Hz, 0.2-ms impulse, −30 V) per min for 10 min. After incubation, muscles were harvested, washed in ice-cold Krebs-Henseleit buffer, blotted on filter paper, and quickly frozen with aluminum tongs precooled in liquid nitrogen and stored at −80 °C.

**2-Deoxy-D-glucose Uptake**

In some experiments, 2-deoxy-d-glucose (2DG) uptake was measured by adding 2-[1-14C]deoxy-d-glucose (1 mM) and [1-13C]mannitol (8 mM) (Amersham Biosciences) to the medium (specific activities of the two tracers in the medium were 0.128 and 0.083 μCi ml⁻¹, respectively). During AICAR incubation, 2DG uptake was measured during the last 5 min of muscle contraction and the first 5 min into recovery (42). Radioactivity in the supernatant was measured using liquid scintillation counting (Tri-Carb 2000; Packard Instrument Co.).

**Muscle Analyses**

Muscles were homogenized in ice-cold buffer (20 mM Tris base, 50 mM NaCl, 2 mM dithiothreitol, 50 mM NaF, 1% Triton X-100, 250 mM sucrose, 5 mM sodium pyrophosphate, 4 μg/ml leupeptin, 6 mM benzamidine, 500 μM phenylmethylsulfonyl fluoride, 50 μM soybean trypsin inhibitor, pH 7.4) for 20 s using a homogenizer (PT 3100; Brinkmann Instruments). Homogenates were rotated end over end for 1 h at 4 °C. Lysates were generated by centrifugation (17,500 × g) for 1 h at 4 °C. Lysates were quick frozen in liquid nitrogen and stored at −80 °C. Protein content in lysates was measured by the bicinchoninic acid method (Pierce).
\( \alpha\)-AMPK Knockouts and Muscle Glucose Uptake

Immunoblotting

Protein levels of the \( \alpha_1\) - and \( \alpha_2\)-AMPK subunits were determined by SDS-PAGE followed by immunoblotting using polyclonal antibodies generated in sheep as described by Woods et al. (43) (antibodies were kindly donated by D. G. Hardie, University of Dundee, UK). ACC\( \beta\) protein was accessed using horseradish peroxidase-conjugated streptavidin (DakoCytomatione, Glostrup, Denmark) as described by Wojtaszewski et al. (44). GLUT4 protein was detected in total crude membrane using polyclonal antibody raised against a peptide corresponding to the sequence in rat liver ACC (43) (antibodies were kindly donated by D. G. Hardie, University of Dundee, UK). ACC\( \beta\), internal ribosomal entry site; IRES, internal ribosomal entry site; \( \beta\)-geo, \( \beta\)-galactosidase and neomycin phosphotransferase fusion gene; pA, polyadenylation sequence; hatched vertical bar, deleted DNA; solid vertical bar, intron.

Fig. 1. Generation of \( \alpha_1\)-AMPK knockout mice. A, schematic representation (not to scale) of genomic structure of the \( \alpha_1\)-AMPK wild type allele (top), the \( \alpha_2\)-AMPK gene-targeting construct containing a promoterless IRES-\( \beta\)-geo cassette (middle), and the targeted gene (bottom). E, EcoRI digestion; IRES, internal ribosomal entry site; \( \beta\)-geo, \( \beta\)-galactosidase and neomycin phosphotransferase fusion gene; pA, polyadenylation sequence; hatched vertical bar, deleted DNA; solid vertical bar, intron. B, Southern blot analysis after EcoRI digestion of tail DNA from offspring derived from heterozygous intercrosses. Expected fragment sizes of the \( \alpha_1\)-AMPK wild type (+/+; 5.5 kb) and null (−/−; 5.0 kb) alleles after EcoRI digestion and hybridization with the indicated probe (solid horizontal bar in A) are shown. C, Western blot analysis of \( \alpha_1\)-AMPK and \( \alpha_2\)-AMPK proteins in liver and gastrocnemius muscle from wild type (+/+ and −/−) mice.

Serum Insulin

Mice were starved for 5 h (from 5:00 to 10:00 p.m.), samples were obtained from tail blood, and serum insulin was assessed using a rat enzyme-linked immunosorbent assay kit with a mouse insulin standard (Crystal Chem Inc., Chicago, IL).

Contraction Force

Contraction force was measured by a force transducer connected to one end of the muscle by the ligature. Force generated was registered on a computer and was expressed as the average force generated (above resting tension) during the 10 stimulation periods. The degree of "fatigue" was obtained by comparing the mean contraction force during stimulation trains 8–10 with the first stimulation train and is expressed as the percentage decrease in mean contraction force.

Calculations and Statistics

Control samples were added to both kinase activity assays and immunoblots, and assay-to-assay variation was accounted for by expressing the data relative to these samples. Data are expressed as means ± S.E. Statistical evaluation was performed by two-way analysis of variance for repeated measurements or Student’s tailed t test. When analysis of variance revealed significant differences, the Student-Newman-Keuls method was used as a post hoc test to correct for multiple comparisons. Differences between groups were considered statistically significant if \( p \) was <0.05.

RESULTS

Generation of \( \alpha\)-AMPK Knockout Mice—The description of the \( \alpha_2\)-AMPK KO mice is published elsewhere; for details, see Viollet et al. (42). In order to inactivate the AMPK catalytic \( \alpha_1\)-subunit gene, a replacement vector was constructed with 7 kb of ES-129-derived genomic fragment and a selection cassette deleting part of the catalytic domain of \( \alpha_1\)-AMPK from amino acids 97–157 (Fig. 1A). As shown in Fig. 1B, hybridization of EcoRI-digested genomic DNAs with the indicated 5′ external probe led to WT alleles of 5.5 kb and mutated alleles of 5.0 kb. Immunoblot analysis (Fig. 1C) of protein extracts prepared from \( \alpha_1\)-AMPK knockout liver and skeletal muscle
showed undetectable \(\alpha_1\)-protein and a level of \(\alpha_2\)-protein comparable with that observed in extracts from WT mice.

\(\alpha_1\), \(\alpha_2\), \(\alpha_3\)-, ACC\(\beta\)-, and GLUT4-protein Levels—Gene targeting for either the \(\alpha_2\) or \(\alpha_1\)-subunit resulted in complete lack of expression of these subunits at the protein level (Table I and Fig. 2). In the \(\alpha_2\) knockout muscles, a compensatory 200–300% increase in the protein level of the remaining \(\alpha_1\)-isoform was observed in EDL and soleus. In the \(\alpha_1\)-KO soleus muscles, a \(\sim 60\%\) increase in the remaining \(\alpha_1\)-isoform was observed, whereas no change was seen in EDL. The protein level of ACC\(\beta\) was also measured in the two muscles and was expressed to the same level in both of the two knockout strains (Table I and Fig. 2). The GLUT4 protein content was measured in the gastrocnemius muscle and was expressed to the same level in both of the two knockout strains (Table II and Fig. 2).

Phenotype of Knockout Mice—A detailed description of the \(\alpha_2\)-AMPK KO mouse phenotype has been published recently (42). In brief, these mice are hypoinsulinemic, glucose-intolerant, insulin-resistant, and AICAR-resistant (Fig. 3). In contrast, the \(\alpha_1\)-AMPK KO mouse has normal glucose tolerance and responds normally to insulin and AICAR (Fig. 3). Furthermore, body weight (data not shown), fasting glucose (Fig. 3, GTT), and serum insulin levels (\(\alpha_2\)-WT, 0.57 ± 0.07 ng/ml; \(\alpha_1\)-KO, 0.62 ± 0.12 ng/ml) are normal in \(\alpha_1\)-AMPK KO mice compared with WT.

2-Deoxy-\(\beta\)-glucose Uptake—In both mouse strains, AICAR increased 2DG uptake by 40–50% in WT soleus and by 100–130% in WT EDL (Fig. 4, upper panels). Remarkably, knockout of the \(\alpha_2\)-subunit completely abolished the effect of AICAR on 2DG uptake in both EDL and soleus muscles. In contrast, knockout of the \(\alpha_1\)-subunit did not affect AICAR-induced 2DG uptake in either of the muscles (Fig. 4, upper panels).

Table I

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Genotype</th>
<th>Protein content</th>
<th>(\alpha_2)</th>
<th>(\alpha_1)</th>
<th>ACC(\beta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL</td>
<td>(\alpha_2)-WT</td>
<td>1.4 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(\alpha_2)-KO</td>
<td>ND</td>
<td>1.9 ± 0.3*</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>(\alpha_1)-WT</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(\alpha_2)-KO</td>
<td>ND</td>
<td>3.3 ± 0.5*</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>(\alpha_1)-WT</td>
<td>2.3 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(\alpha_1)-KO</td>
<td>2.3 ± 0.3</td>
<td>ND</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>(\alpha_2)-WT</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(\alpha_1)-KO</td>
<td>1.1 ± 0.1*</td>
<td>ND</td>
<td>1.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

In both mice strains, muscle contraction increased 2DG uptake by 200–270% in WT EDL and by 240–370% in WT soleus (Fig. 4, lower panels). Surprisingly, knockout of the \(\alpha_2\) isofrom significantly increased contraction-stimulated glucose uptake by \(\sim 25\%\) compared with WT in EDL, whereas the \(\alpha_2\)-KO did not affect contraction-stimulated glucose uptake in soleus. The \(\alpha_1\)-KO did not affect contraction-stimulated glucose uptake in EDL, but in soleus, the \(\alpha_1\)-KO decreased glucose uptake by \(\sim 25\%\) during contraction (Fig. 4, lower panels).

AMPK Signaling—In an effort to elucidate the underlying signaling events that might explain the effect of the \(\alpha_2\)-subunit knockout on glucose uptake, various measures of AMPK signaling were performed.

\(\alpha_1\) and \(\alpha_2\)-Immunoprecipitable AMPK Activity—In the WT muscles of both mouse strains, AICAR increased \(\alpha_2\)-AMPK and \(\alpha_1\)-AMPK activity in both muscles (Fig. 5). However, in the \(\alpha_2\)-KO mice, the remaining \(\alpha_1\)-AMPK activity in EDL increased with AICAR stimulation similarly to the WT muscles, and in the soleus the AICAR-induced increase in \(\alpha_1\)-AMPK activity tended (\(p = 0.08\)) to be larger than in the WT muscle (Fig. 5). Thus, although AICAR increased \(\alpha_1\)-AMPK activity in the \(\alpha_2\)-KO mice, this was not accompanied by increased glucose uptake. In \(\alpha_1\)-KO muscles, AICAR induced a normal increase in the remaining \(\alpha_2\)-AMPK activity (Fig. 5).

Contraction activated both \(\alpha_1\)-AMPK and \(\alpha_2\)-AMPK activity in the WT muscles (Fig. 6). Interestingly, in the \(\alpha_2\)-KO muscles, the remaining \(\alpha_1\)-AMPK activity was increased by \(\sim 100\%\) during contraction compared with WT muscles. In the \(\alpha_1\)-KO muscles, the remaining \(\alpha_2\)-AMPK activity was similar to the activity in WT muscles (Fig. 6).

Phosphorylation of \(\alpha\)-AMPK Thr\(^{172}\)—Next, we estimated the covalent activation of AMPK activity by measuring phosphorylation of \(\alpha\)-AMPK Thr\(^{172}\) (AMPK-P). The antibody used does not discriminate between the two \(\alpha\)-isoforms, and the data obtained therefore reflect the total (\(\alpha_1\) and \(\alpha_2\)) \(\alpha\)-Thr\(^{172}\) phosphorylation. In the WT muscles of both mouse strains, AICAR increased AMPK-P by 200–300% in both muscles (Fig. 7, upper panels). However, in the \(\alpha_2\)-KO mice, AMPK-P was reduced to very low levels, and only in the soleus did AICAR in fact cause

![Fig. 2. Representative immunoblots from \(\alpha_2\)-AMPK KO and WT mice (A) and \(\alpha_1\)-KO and WT mice (B), showing protein levels of \(\alpha_2\) and \(\alpha_1\)-AMPK isoforms and ACC\(\beta\) in lysates from EDL and soleus and GLUT4 in total crude membrane from the gastrocnemius muscle (Gast.).](image-url)
a small significant increase in AMPK-P. Interestingly, in neither of the two α2-KO muscles did AICAR stimulation result in values of AMPK-P that exceeded the basal values in WT muscles. In α2-KO muscles, AICAR induced similar increases in AMPK-P as in the corresponding WT muscles (Fig. 7, upper panels). These data indicate that α2-AMPK is the main donor of AMPK activity in both fast and slow skeletal muscle in the basal state and during stimulation with AICAR and probably largely explain why the stimulatory AICAR effect on glucose uptake is seen only in the α2-KO muscles.

Contraction increased AMPK-P by 540–860% in WT muscles from both mouse strains (Fig. 7, lower panels). AMPK-P did not increase during contraction in α2-KO EDL, and in absolute values, the AMPK-P level in the contracted muscle was 73% lower compared with WT EDL. In contrast, in α2-KO soleus, contraction increased AMPK-P to a similar level compared with α2-WT soleus. In α1-KO muscles, contraction induced similar increases in AMPK-P as found in the corresponding WT muscles (Fig. 7, lower panels). These results indicate that the α2-isoform is the main donor of AMPK activity during contraction in EDL but apparently not in the soleus muscle and that α1-KO does not affect total AMPK-P at rest or during contraction.

Phosphorylation of ACCβ—Finally, we estimated total endogenous AMPK kinase activity (phosphorylation and allosteric effects) by measuring Ser227 phosphorylation of the AMPK substrate ACCβ (ACCβ-P) (Fig. 8, upper panels). During AICAR stimulation, these data are much in agreement with the AMPK-P data. Thus, in the WT muscles of both mouse strains, AICAR increased ACCβ-P markedly, and in α2-KO muscles, both the basal values and the AICAR-stimulated values were markedly smaller than in WT. Again, in neither of the muscles did AICAR stimulation result in ACCβ-P values that exceeded basal values in WT. In α1-KO muscles, AICAR induced similar increases in ACCβ-P as in the corresponding WT muscles (Fig. 8, upper panels). These data indicate that α2-AMPK is the main donor of kinase activity toward ACCβ-P in

**Fig. 3.** Glucose (GTT), insulin (ITT), and AICAR (ATT) tolerance tests were performed in α1- or α2-AMPK KO or WT mice by intraperitoneal injection of one of the agents (as described under “Materials and Methods”). The area under the curve (AUC, mU/min) was calculated as changes in blood glucose concentration at time points 20, 40, 60, 105, and 150 min after injection compared with basal blood glucose level. *, significantly different from WT (p < 0.05). Data are presented as means ± S.E., n = 14–16 for all groups.
the basal state and during AICAR stimulation.

Contraction increased ACCβ-P in WT EDL and soleus from both mouse strains. In contrast to the data obtained during AICAR stimulation, contraction increased ACCβ-P in both α2-KO and α1-KO muscles similarly to the corresponding WT muscles (Fig. 8, lower panels). This indicates that although α2-KO reduced AMPK-P in one muscle (EDL), the total endogenous AMPK activity was unaffected by both the α2-KO and the α1-KO in the two investigated muscles during contraction.

Muscle Glycogen Content—The potential of insulin, AICAR, and muscle contraction to stimulate glucose uptake is inversely correlated to muscle glycogen content (6, 47, 48). The α2-KO EDL had a decreased resting level of glycogen (~40%) whereas α2-KO soleus had a normal resting glycogen content (Table III). The α2-KO did not affect the resting level of glycogen in either of the two muscles. Compared with WT, glycogen levels were reduced to a similar extent in both α2-KO and α1-KO muscles after contraction (Table III).

Contraction Force—In both mouse strains, electrical stimulation for 10 min induced a mean contraction force during stimulation of ~32 millinewtons in EDL and ~95 millinewtons in soleus and was not affected by KO of either the α2- or α2-AMPK isoform. At the end of the stimulation period, contraction force was reduced with ~85 and ~60% in EDL and soleus, respectively, and this was not affected by KO of either the α1- or the α2-isoform.
DISCUSSION
In the present study, we have shown that knockout of the \( \alpha_2 \)-isoform of AMPK completely abolished AICAR-induced glucose uptake in mouse skeletal muscle, whereas knockout of the \( \alpha_1 \)-isoform had no effect in this respect. The effect of \( \alpha_2 \)-isoform knockout on AICAR-induced glucose uptake was independent of muscle fiber type and was not caused by a decrease in muscle GLUT4 protein content but was associated with a dramatic decrease in AMPK signaling. Thus, the \( \alpha_2 \)-isoform delivered the vast majority of total AMPK activity in the basal state and during AICAR stimulation. In contrast, contraction-induced glucose uptake was essentially unaffected by knockout of either \( \alpha \)-isoform of AMPK, and knockout caused basically unaltered AMPK signaling during contraction. Our data extend the findings reported by Mu et al. (36), in which a muscle-specific overexpression of a kinase-dead form of the AMPK catalytic \( \alpha \)-isoform completely abolished AICAR-induced glucose uptake, whereas contraction-induced muscle glucose uptake was only partially reduced by 30–40%. Based on the three tolerance tests, we were also able to show that the \( \alpha_2 \)-KO mouse is glucose-intolerant and AICAR- and insulin-resistant, whereas no altered phenotype was seen regarding these parameters in the \( \alpha_1 \)-KO mouse. The glucose intolerance and insulin resistance are probably due to an elevated plasma catecholamine level, as reported previously (42). AICAR resistance, as observed in the whole body \( \alpha_2 \)-KO mice, was also reported in muscle-specific kinase-dead mice (36), and it therefore seems, at least in part, to depend on a reduced glucose uptake in skeletal muscles.

It is interesting to note that knockout of the \( \alpha_2 \)-isoform led to
a 200–300% increase in protein content of the α1-isoform in the soleus and EDL muscles, whereas knockout of the α1-isoform only increased α2-AMPK expression moderately and only in the EDL. The up-regulation of one α-isoform when the other is missing indicates a compensatory counteraction to restore AMPK activity. Therefore, the vast increase of α1-protein in α2-KO muscles supports the concept that the α2-AMPK activity in general is the most important contributor of AMPK activity in mouse skeletal muscles. This is further strengthened by the fact that only the α2-KO mouse was AICAR-resistant, whereas the α1-KO mouse was not. Furthermore, the relative activity of the isoforms can also be judged by estimations of activity/phosphorylation of downstream AMPK targets and/or by using antibodies recognizing identical phosphorylated sequences in the two α-isoforms (e.g. the anti-phospho-α-AMPK Thr^172 antibody). These prerequisites are fulfilled in the present study, thereby giving us the opportunity to estimate the relative kinase activity of α2-AMPK and α1-AMPK based on AMPK Thr^172 and ACCβ Ser^227 phosphorylation.

It is apparent that α2-KO dramatically decreased basal as well as AICAR-induced α-AMPK Thr^172 phosphorylation in both muscles, whereas α1-KO had no effect in this respect. Moreover, although α1-AMPK activity increased normally in response to AICAR stimulation in the α2-KO muscles, the increase in α-AMPK Thr^172 phosphorylation did not, in fact, exceed unstimulated levels found in the WT muscles (Fig. 7). This would suggest that α2 is the most prominent isofrom phosphorylated in response to AICAR treatment, either as the result of a more abundant protein expression or as a result of a higher sensitivity for phosphorylation by the upstream kinases.

Basically similar results were obtained when assessing endogenous AMPK activity by ACCβ Ser^227 phosphorylation. Again, the total AMPK activity, as judged by ACCβ Ser^227 phosphorylation, was reduced in the α2-KO muscles (Fig. 8). In accordance with the α-AMPK Thr^172 phosphorylation, the knockout of the α1-isoform did not cause any alteration in basal or AICAR-stimulated ACCβ Ser^227 phosphorylation. Together, these findings reinforce the conclusion that α2-containing AMPK complexes are the main contributors of AMPK activity during AICAR stimulation in mouse muscles and therefore necessary for increasing glucose uptake during this type of stimulation.

It is worth noticing that, in the α2-KO muscles, AICAR increased α1-AMPK activity to the same level as in WT muscles, albeit the protein level of the α1 in the α2-KO muscles was increased 200–300%. This could indicate that AICAR did not accumulate to the same extent in α2-KO as in WT muscles or that the increased amount of α1-protein is located in subcellular compartments that are unavailable for activation by AMPK kinase during AICAR stimulation. Whereas the limited amount of tissue from the incubated muscles precluded direct measurements of the ZMP levels, the fact that α2-AMPK activity in α2-knockout muscles increased with AICAR stimulation similarly to WT still indicates that if α1-AMPK activation was an important event in AICAR-stimulated glucose uptake, then glucose transport should have increased in the α2-knockout muscles.

In contrast to the effect of AICAR, contraction-induced glucose uptake was largely unaffected by KO of either isofrom. The question is whether the lack of effect of either KO on glucose transport during contraction is due to compensation of the...
other α-isoform for the one missing or whether AMPK activity is not important for stimulating contraction-induced muscle glucose transport. In contrast to the findings during AICAR stimulation (Fig. 5), lack of α2-AMPK was compensated for by an increase in α1-AMPK activity during contraction in both muscle types (Fig. 6). Still, in the muscles lacking the α2-isoform, α-AMPK Thr172 phosphorylation was markedly reduced in the EDL but not in the soleus. Thus, as judged by α-AMPK Thr172 phosphorylation, the lack of α2-AMPK was not fully compensated for in the EDL, whereas it was in the soleus. Nevertheless, glucose transport was not decreased compared with WT in either muscle (Fig. 4). However, if one considers phosphorylation of ACCβ, a downstream target of AMPK, a better index of total endogenous AMPK activity, then compensation for the lack of the α2-AMPK isoform was essentially complete during contraction in both muscle fiber types (Fig. 8).

In this context, however, it should be kept in mind that other kinases/phosphatases might also regulate ACCβ phosphorylation (49). Furthermore, it has recently been demonstrated that during prolonged exercise, a dissociation of AMPK activity and ACCβ phosphorylation occurred (44). Thus, it is not certain whether ACCβ phosphorylation is a good index of total endogenous AMPK activity, and therefore it is not possible with certainty to conclude from our data whether endogenous AMPK activity was altered or not during contractions in the two knockout strains.

Interestingly, in the AMPK kinase-dead mice, contraction force during electrical stimulation was reduced in both soleus and EDL (50), showing that total lack of AMPK activity does play a role for contraction force. In incubated rat muscle, it has been shown that a decrease in contraction force is associated with a decrease in glucose uptake (14), but it remains unknown whether the reduced contraction force in the kinase-dead mouse (50) is important for the reported reduced glucose uptake. In our α1- and α2-KO muscles, contraction force was not different from the WT muscles, and a similar degree of fatigue was observed. Thus, in our knockout models, differences in glucose uptake are not explained by differences in force production.

Glucose transport and AMPK signaling were essentially normal in α1-KO muscles at rest and during contractions, although there was a slight decrease in contraction-induced glucose transport in the α1-KO soleus. Muscle glycogen was not increased in the α1 KO muscles, and therefore the decrease in the contraction-induced glucose transport in the soleus cannot be explained by an inhibitory effect of increased muscle glycogen on glucose uptake as has been shown previously (15, 48, 51). In contrast, in the α2-KO muscles, glycogen was significantly decreased in the EDL, offering a plausible explanation for the slight increase in contraction-induced glucose transport of the α2-KO EDL muscles compared with WT.

Activation of AMPK with AICAR in resting muscle is by many investigators considered a viable surrogate for activation of AMPK during muscle contraction. Interestingly, in contrast to contraction, AICAR stimulation of resting muscle was not sufficient to increase glucose transport in α2-AMPK muscles despite an increase in α1-AMPK activity. Thus, AICAR stimulation of glucose uptake in resting muscle is totally dependent upon α1-AMPK activity, whereas during muscle contraction, lack of α2-AMPK activity clearly can be compensated for. However, it should be borne in mind that electrical stimulation of skeletal muscles to intermittent titanic contractions as in the present study is a quite demanding protocol, causing considerable decrease in the contraction force over the 10-min stimulation period. Therefore, findings from such experiments may not be directly transferable to more moderate in vivo exercise conditions.

In conclusion, we have shown that knockout of the α2-isoform of AMPK completely abolished AICAR-induced glucose uptake in mouse skeletal muscle of different fiber types, whereas knockout of the α1-isoform had no effect in this respect. We also showed that the α2-isoform delivered the vast majority of total AMPK activity in the basal state and during AICAR stimulation. In contrast, contraction-induced glucose transport was essentially unaffected by knockout of either α-isoform of AMPK, possibly explained by essentially unaltered signaling downstream of AMPK during contraction. Alternatively, neither α-isoform is involved in contraction-stimulated muscle glucose uptake. In either case, we cannot exclude the possibility that an up-regulation of parallel signaling mechanisms to AMPK compensates for the lack of AMPK isoforms. Finally, AICAR-induced muscle glucose transport cannot be used as a substitute for muscle contraction, because it is dependent only on the α2-isoform, whereas neither α-isoform of AMPK is essential for contraction-induced muscle glucose uptake. The present data also indicate that the α2-isoform of AMPK is the preferable target when designing new drugs aiming at increasing peripheral glucose uptake both due to the importance of this isoform in AICAR-induced glucose uptake and because of the preferential expression in skeletal muscles (37, 39).

Acknowledgments—We thank professor D. Grahame Hardie for the kind donation of α-AMPK antibodies and Anne K. Voss for the kind donation of ES cells. Betina Bolmgren, Ann-Christina Henriksen, and Vigdis Hoel Christie are acknowledged for skilled technical assistance.

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