The α2–5′AMP-Activated Protein Kinase Is a Site 2 Glycogen Synthase Kinase in Skeletal Muscle and Is Responsive to Glucose Loading

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The 5′AMP-activated protein kinase (AMPK) is a potential antidiabetic drug target. Here we show that the pharmacological activation of AMPK by 5-aminoimidazole-1-β-4-carboxamide ribofuranoside (AICAR) leads to inactivation of glycogen synthase (GS) and phosphorylation of GS at Ser 7 (site 2). In muscle of mice with targeted deletion of the α2-AMPK gene, phosphorylation of GS site 2 was decreased under basal conditions and unchanged by AICAR treatment. In contrast, in α1-AMPK knockout mice, the response to AICAR was normal. Fuel surplus (glucose loading) decreased AMPK activation by AICAR, but the phosphorylation of the downstream targets acetyl-CoA carboxylase-β and GS was normal. Fractionation studies suggest that this suppression of AMPK activation was not a direct consequence of AMPK association with membranes or glycogen, because AMPK was phosphorylated to a greater extent in response to AICAR in the membrane/glycogen fraction than in the cytosolic fraction. Thus, the downstream action of AMPK in response to AICAR was unaffected by glucose loading, whereas the action of the kinase upstream of AMPK, as judged by AMPK phosphorylation, was decreased. The fact that α2-AMPK is a GS kinase that inactivates GS while simultaneously activating glucose transport suggests that a balanced view on the suitability for AMPK as an antidiabetic drug target should be taken. *Diabetes* 53:3074–3081, 2004

The 5′AMP-activated protein kinase (AMPK) system is a sensor of cellular energy status that adjusts the supply of ATP to the demand for the nucleotide (1). Activation of α2-AMPK stimulates muscle glucose transport (2,3). Once glucose has been taken up and converted to glucose-6-phosphate (G6P), it can be stored as glycogen or metabolized by glycolysis to generate ATP. It has been reported that AMPK phosphorylates muscle glycogen synthase (GS) in cell-free assays at site 2 (Ser 7) (4). Thus, AMPK activation may under some conditions decrease the potential for glycogen synthesis. Recently, we and others showed that GS activity decreases in response to acute 5-aminoimidazole-1-β-4-carboxamide ribofuranoside (AICAR) treatment of muscle-like cells in culture (5), isolated and perfused skeletal muscle (6–8), and fast twitch, but not slow twitch, muscle in vivo (7). AICAR treatment leads to decreased gel mobility of GS in perfused muscle, which together with the decreased activity, is reversed by protein phosphatase treatment (6). These observations indicate that regulation of GS activity by AICAR involves phosphorylation of GS. Although it has been suggested that AICAR-induced GS deactivation is mediated by AMPK due to the negative correlation between α2-AMPK and GS activity (6), these data do not prove a causal relation. Thus, by studying muscle from α-AMPK knockout (KO) mice in the present study, we aimed to verify that AMPK is a muscle GS kinase in vivo.

AMPK activity decreases when muscle is exposed to fuel surplus. For example, glucose loading and glycogen accumulation suppress muscle AMPK phosphorylation/activation at basal conditions and during stimulation by AICAR or exercise (6,9–12). Although AMPK binds to glycogen through the glycogen-binding domain of the regulatory β subunit (13,14), the activity of purified rat liver AMPK was not changed by the addition of glycogen in cell-free assays (14). To further study the effect of fuel surplus on AMPK signaling, we also evaluated the influence of in vivo fuel surplus−induced suppression of AMPK activity on downstream targets such as acetyl-CoA carboxylase (ACC)-β and GS in rat skeletal muscle.

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ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-1-β-4-carboxamide ribofuranoside; AMPK, 5′AMP-activated protein kinase; CK, casein kinase; EDL, extensor digitorum longus; G6P, glucose-6-phosphate; GS, glycogen synthase; GSK, glycogen synthase kinase.

RESEARCH DESIGN AND METHODS

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, no. 123, Strasbourg, France, 1985).

Male Wistar rats (110–120 g; Taconic, Ry, Denmark), male and female α1-AMPK KO and wild type (WT) mice (age 4 months), and male α2-AMPK KO and WT mice were studied (2,15). Within each mouse strain, KO and WT mice used for experiments were littermates produced by intercross breading using heterozygote parent animals. The genotype of the offspring was determined by PCR on DNA extracted from tail biopsy (2).

The animals were maintained on a 12-h light/dark cycle and received standard rodent diet (Altromin no. 1324; Chr. Pedersen, Ringsted, Denmark) and water ad libitum.

Studies of isolated and incubated mouse muscle in vitro. Extensor digitorum longus (EDL; mainly IIa fibers) was obtained from anesthetized mice (6 mg pentobarbital/100 g body wt) and suspended by ligatures at resting tension (4–5 mN) in incubation chambers (Multimyograph system; Danish Myo-Technology, Aarhus, Denmark) in a Krebs-Henseleit buffer (30°C) containing 0.1% BSA, as previously described (2). AICAR (2 mmol/l, 40 min; Toronto Research Chemicals, Toronto, Canada) was added after 10 min of basal incubation. After being incubated, muscles were 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.

Studies of AICAR and glycogen using the perfused rat hindlimb model. Rats were subjected to 2 h of swimming in water maintained at 32–35°C, with weights attached to their tails (5.5% of body wt). In the 24 h preceding the swimming exercise, the rats' diet availability was restricted to 4 g (~45% of normal intake). After the exercise, the rats were fed lard and tap water ad libitum (low glycogen [LG] diet) or normal rat diet, tap water, and a 20% glucose drinking solution (high glycogen [HG] diet), until 3–6 h before perfusion. Rats were perfused 18–24 h after the swimming exercise.

Surgical procedure. On the day of the experiment, rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (4–5 mg/100 g body wt). Surgery for two-legged perfusion was performed as described by Ruderman et al. (16).

Perfusion procedure. All perfusions were carried out with cell-free medium consisting of Krebs-Ringer bicarbonate buffer solution, 4% BSA, 0.15 mmol/l pyruvate, and 4.2 IU/ml heparin. The medium was continuously gassed with 95% O_2 and 5% CO_2. When AICAR was included, it was present in the perfusate at a concentration of 2 mmol/l.

The 15-min preperfusion period was followed by perfusion with or without 2 mmol/l AICAR for 35 min. All perfusions were carried out with a nonrecycled perfusate flow using a flow rate of $0.5 \ ml \cdot min^{-1} \cdot g \ muscle^{-1}$. Muscle biopsies were taken from two different parts of the gastrocnemius muscle, the superficial white (WG) and the profound red (RG) parts, while the muscles were still being perfused. The biopsies were trimmed of connective tissue, blotted, freeze clamped with aluminum clamps cooled in liquid nitrogen, and stored at −80°C.

Muscle analyses

Glycogen. Muscle glycogen content was determined as glycosyl units after acid hydrolysis (17).

SDS-PAGE and Western blotting. Muscle lysates were prepared and subjected to SDS-PAGE as previously described (2,6). The following primary antibodies were used: antibodies recognizing GS when nonphosphorylated (Chemicon, Temecula, CA) or when phosphorylated on site 2 alone (Ser 7), site 2 + 2a (Ser 7 + 10), or site 3a + 3b (Ser 640 + 644), as previously described (18). Anti−α1-AMPK and −α2-AMPK antibodies have been described previously (19). The anti−pan α-AMPK and anti–phospho-specific ACC-β (Ser 218 rat/Ser 221 mouse) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). The anti–phospho-specific α-AMPK subunits (Thr 172) were obtained from Cell Signaling Technology (Beverly, MA).

Stoichiometry of site-specific protein phosphorylation using phospho-specific antibodies. We estimated the stoichiometry of the site-specific phosphorylation of GS as previously described (20). We estimated the total amount of GS by immunoblot in samples of muscle lysate and immunoprecipitates using the phospho-specific antibody. By comparing these two signals, an indication of the phosphorylation stoichiometry can be obtained, but only if the immunoprecipitation of the phosphorylated form of GS is 100% efficient. Thus, the immunoprecipitation efficiency was determined by immunoblot of muscle lysate and immunoprecipitates using the same phospho-specific GS antibody for immunoblot as was used for immunoprecipitation. These analyses were performed for the site 2 and site 2 + 2a GS phospho-specific antibodies using nonstimulated rat muscle. Immunoprecipitation was performed by binding the antibody to Sepharose G beads (Amersham Pharmacia, Bucks, U.K.) for 2 h. Before adding the muscle lysate, nonbound antibodies were removed by washing in lysate buffer. After an overnight incubation, immunoprecipitates were washed five times in lysate buffer before being subjected to SDS-PAGE.

Glycogen synthase activity. Muscle GS activity was measured in muscle lysates using a microtiter plate assay (Unifilter 350 plates; Whatman, Cambridge, U.K.). The assay ran in triplicates based on the original protocol described by Thomas et al. (21). GS activity was determined in the presence of 8 or 0.02 mmol/l G6P.

Subfractionation procedure. Muscles were homogenized (1:20, wt:vol) in buffer A (50 mmol/l HEPES [pH 7.5], 150 mmol/l NaCl, 20 mmol/l Na-pyrophosphate, 20 mmol/l β-glycerophosphatea, 10 mmol/l NaF, 2 mmol/l Na-orthovanadate, 2 mmol/l EDTA, 10% glycerol, 2 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 3 mmol/l benzamidine) for 20 s using a homogenizer (PT 3100; Brinkmann, Westbury, NY). Homogenates were rotated end over end for 20 min at 4°C and cleared by centrifugation (1,000g for 1 min). The supernatant was separated into a cytosolic fraction and a membrane-glycogen−enriched pellet by centrifugation at 200,000g for 30 min at 4°C. The pellet was resuspended in buffer A containing 1% Nonidet P-40. The protein content in the two fractions was measured by the bicinchoninic acid method (Pierce Chemical, Rockford, IL).

Calculations and statistics. Control samples were analyzed at the same time as all activity assays, and assay-to-assay variation was accounted for by expressing the data relative to these samples. All Western blot analyses were performed within the linearity of the assay determined for each individual protein/antibody. Data are expressed as means ± SE. Statistical evaluation was performed by two-way ANOVA. When ANOVA revealed significant differences, a post hoc test was used to correct for multiple comparisons (Tukey's test). Differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

Treatment with AICAR decreases GS activity in mouse muscle. In incubated EDL muscle from WT mice, AICAR treatment lowered GS activity by 18–27% (Fig. 1A and D). This effect was maintained in the absence of α1-AMPK protein (Fig. 1A), but not in the absence of α2-AMPK protein (Fig. 1D). A significant elevation of GS activity was seen in nonstimulated muscles from the α2-AMPK KO EDL muscle compared with WT muscle (Fig. 1D). Muscle from WT and KO animals had similar total GS protein and total GS activity, which were not affected by AICAR treatment (Fig. 1).

AICAR treatment increases GS phosphorylation on site 2 in mouse muscle. GS site 2 phosphorylation was increased by AICAR treatment in WT mice (Fig. 1B). This effect was preserved in the absence of α1-AMPK protein (Fig. 1B) but was not apparent in muscle lacking the α2-AMPK protein (Fig. 1E). Also, the level of site 2 phosphorylation was decreased in nonstimulated muscle from the α2-KO mouse in line with the increased basal GS activity (Fig. 1E). There was no difference in phosphorylation on site 3a + 3b between WT and α1- or α2-KO muscle, and the phosphorylation was not affected by AICAR treatment (Fig. 1C and F). We have previously reported on an analysis of AMPK activity and phosphorylation in WT and KO muscle in response to AICAR treatment (2). Of interest for the present study are our findings that 1) the knockout of one α-AMPK isoform eliminates any AMPK activity for that particular α-AMPK isoform but maintains the remaining α-isoform AMPK activity in both basal and AICAR-induced conditions; 2) knockout of α2-AMPK reduces total α-AMPK phosphorylated on Thr 172 in basal conditions (to ~10% of WT) and during AICAR treatment (to ~5% of WT), whereas the lack of α1-AMPK does not change the total α-AMPK phosphorylation; and 3) the lack of α2-AMPK, but not α1-AMPK, decreases ACC Ser 221 phosphorylation in basal conditions (~33% of WT) and in response to AICAR (~19% of WT). The combined data from our current and previous
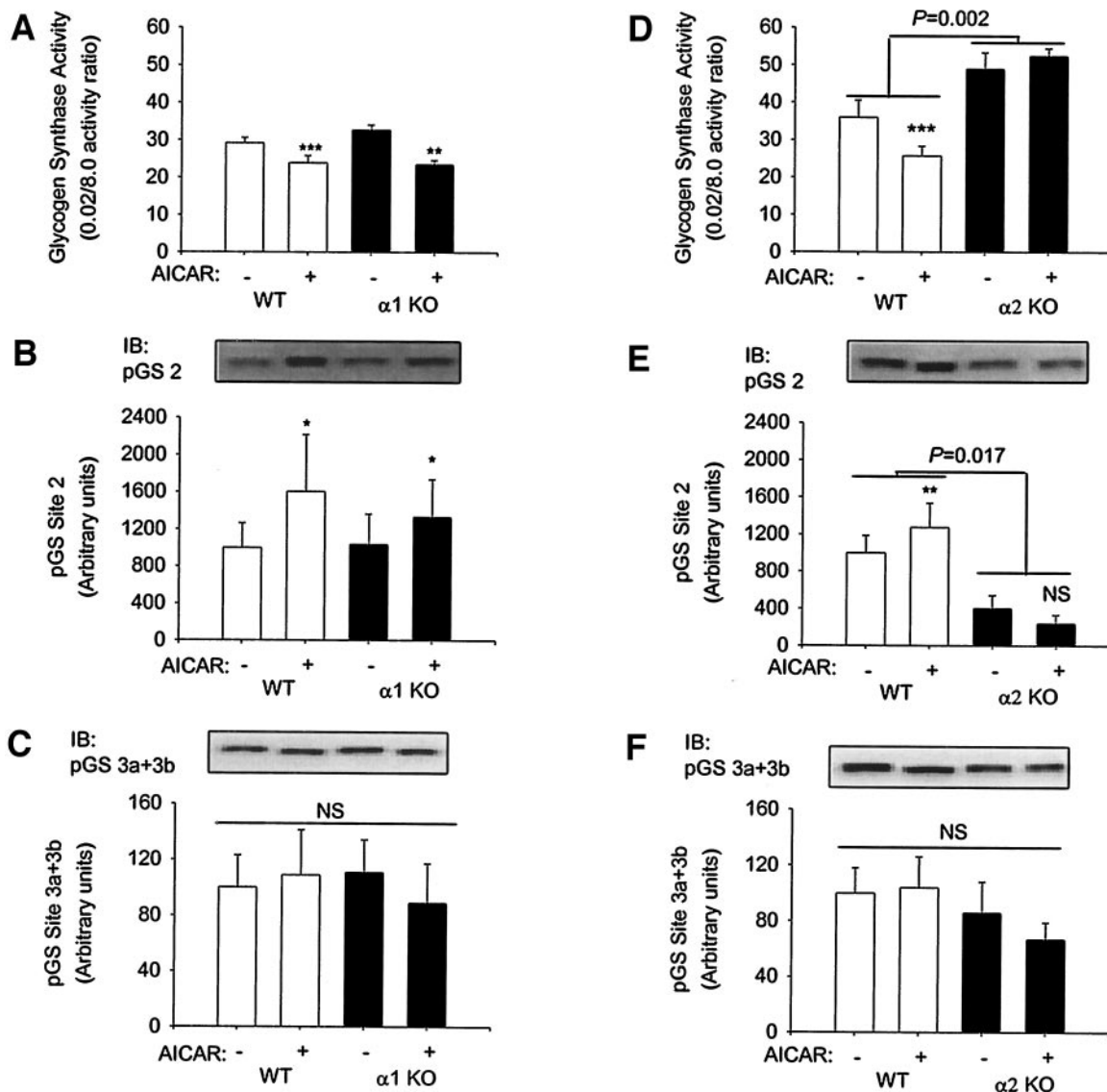
FIG. 1. GS 0.02/8.0 activity ratio (*A* and *D*) and GS phosphorylation (pGS) on site 2 (Ser 7; *B* and *E*), and site 3a + 3b (Ser 640 + 644; *C* and *F*) is measured in EDL muscle lysates from α1 or α2 KO mice. Muscles were incubated in vitro with or without AICAR. General differences between data obtained in α2 KO and WT are indicated by lines and *P* values. Data represent means ± SE, *n* = 6–8. Total GS activity or GS protein was not affected by AICAR and was not different between WT or KO animals. (Total GS activity for α1 and α2 strains, respectively: 29 ± 2 vs. 18 ± 2 nmol · min⁻¹ · mg protein⁻¹; GS protein for α1 and α2 strains, respectively: 44 ± 7 vs. 36 ± 6 AUs). *P < 0.05, **P < 0.01, ***P < 0.001 for nontreated (*B*) vs. AICAR-treated muscles. IB, immunoblot.

study strongly indicate that the α2-AMPK isoform is a GS site 2 kinase phosphorylating GS in response to AICAR.

Preexercise/diet treatment modulates muscle glycogen content in rats. The preexercise/diet treatment resulted in levels of muscle glycogen three- to fourfold higher in the HG compared with the LG group ($P < 0.001$). Further, glycogen was not affected by AICAR (in mmol/kg wet wt for WG and RG: LG basal 24.9 ± 1.6 and 18.5 ± 2.7, $n = 8$; HG basal 82.7 ± 7.0 and 80.0 ± 9.0, $n = 8$; LG AICAR 27.4 ± 2.2 and 24.2 ± 1.6, $n = 8$; HG AICAR 78.3 ± 6.1 and 61.8 ± 4.1, respectively, $n = 7$).

Glucose loading and AICAR decrease GS activity and induce GS phosphorylation in rat muscle. GS activity was decreased by glucose loading in the basal state and in the presence of AICAR (Fig. 2*A*). In addition, AICAR treatment decreased GS activity significantly (~40–60%). Possibly because of the very low GS activity in the HG

group of WG, no significant decrease was seen in response to AICAR in this muscle. Glycogen and AICAR did not affect total GS activity in either of the two types of muscle (Fig. 2). Similar to what was observed in the mouse EDL muscle, AICAR treatment of both RG and WG rat muscle induced phosphorylation of site 2 and site 2 + 2a, but not site 3a + 3b (Fig. 2*B–D*). This response to AICAR was independent of the prevailing muscle glycogen content. It was interesting that glucose loading per se led to increased phosphorylation of site 2 and site 2 + 2a, whereas site 3a + 3b was unaffected.

AICAR induces phosphorylation of AMPK and ACC. α-AMPK Thr 172 phosphorylation was decreased by glucose loading (Fig. 2*E*), as was the ability of AICAR to induce this phosphorylation. This effect was also observed for α2-AMPK activity (6) measured in vitro. Thus, the data confirmed the notion that α2-AMPK contributes to the
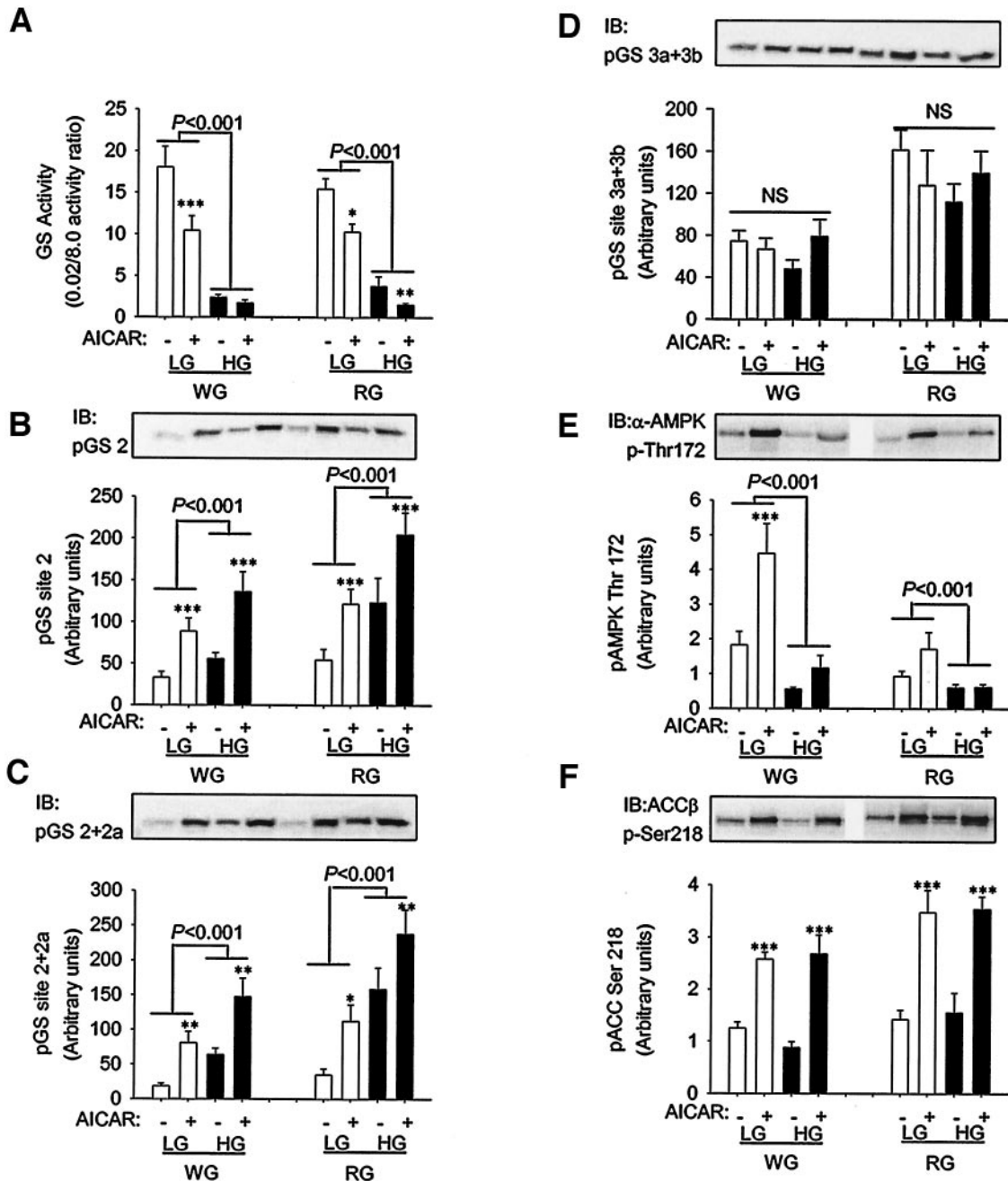
FIG. 2. GS 0.02/8.0 activity ratio (*A*); GS phosphorylation (pGS) on site 2 (Ser 7; *B*), site 2 + 2a (Ser 7 + 10; *C*), and site 3a + 3b (Ser 640 + 644; *D*), α-AMPK Thr 172 phosphorylation (pAMK; *E*); and ACC-β phosphorylation (pACC) on Ser 218 (*F*) measured in lysates from rat white gastrocnemius (WG) and red gastrocnemius (RG) muscle. General differences between data obtained in muscle high glycogen (HG) compared with low glycogen (LG) content are indicated by lines and *P* value. Data represent means ± SE, *n* = 7–8. Total GS activity was not affected by AICAR or glycogen (average 18 ± 1 and 32 ± 2 nmol · min^{-1} · mg protein^{-1} in WG and RG respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for nontreated (*B*) vs. AICAR-treated muscles. IB, immunoblot.

majority of AMPK activity in rodent skeletal muscle during AICAR treatment. The degree of ACC-β Ser 218 phosphorylation was increased by AICAR treatment, but nonstimulated and AICAR-stimulated phosphorylation was unaffected by muscle glycogen content (Fig. 2*F*).

Stoichiometry of site 2 and 2a GS phosphorylation. Under the conditions used, the efficiency of the pGS site 2 and 2 + 2a antibodies for immunoprecipitation were close to 100% for both antibodies (Fig. 3). We estimated the amount of total GS in the phosphorylated GS immunoprecipitates and compared that with the total GS in lysates.

When corrected for the immunoprecipitation efficiency in the individual experiments, this value served as an estimate of the phosphorylation stoichiometry and was ~31 ± 5% for GS site 2 and only 1 ± 0.2% for site 2 + 2a. These measures were performed in perfused, nonstimulated rat WG muscle with normal glycogen content.

AMPK in the cytosolic and membrane-glycogen fractions. The membrane-glycogen fraction contained α-AMPK protein corresponding to only 4 ± 5% of that in the cytosolic fraction (*n* = 6; $P < 0.001$) (Fig. 4*A*); this pattern was not significantly changed by AICAR treatment.
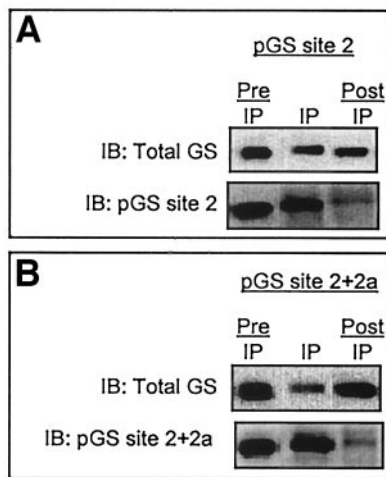
FIG. 3. Estimation of the phosphorylation stoichiometry based on Western blot analysis. Samples of the pre- and post-immunoprecipitation extract as well as the immunoprecipitate (IP) itself was loaded on gels and immunoblotted using an antibody recognizing the nonphosphorylated form of GS (A and B, top) or an antibody recognizing the phosphorylated form of GS (pGS; A and B, bottom). Two phospho-specific GS antibodies were used: pGS site 2 (A) and pGS site 2 + 2a (B). Based on 3–6 independent experiments (n = 2 or 3), it was estimated that 31 ± 5% of total GS is phosphorylated on site 2 and that only 1 ± 0.2% of total GS is phosphorylated on site 2 + 2a in nonstimulated, perfused rat white gastrocnemius muscle. IB, immunoblot.

Overall, the distribution pattern of Thr 172−phosphorylated α-AMPK (Fig. 4B) reflected the α-AMPK distribution, and the Thr 172−phosphorylated α-AMPK was increased by AICAR in both fractions (2.6-fold in the cytosolic fraction [$n = 6$, $P = 0.004$] and 2.4-fold in the membrane-glycogen fraction [$n = 6$, $P = 0.06$]). When the Thr 172−phosphorylated α-AMPK was expressed relative to the content of α-AMPK in each individual sample, the α-AMPK in the membrane-glycogen fraction was phosphorylated to a higher extent than in the cytosolic fraction (Fig. 4C). (In the basal condition, this was observed in five of the six preparations [$P = 0.2$], whereas it was observed

in all preparations from AICAR-treated muscles [$P < 0.001$, $n = 6$].)

DISCUSSION

The present data provide evidence that AMPK is a GS kinase in skeletal muscle, phosphorylating site 2 in response to AICAR stimulation and leading to inactivation of GS. Using the α-AMPK isoform−specific KO mouse, we showed that the effect of AICAR is dependent on the α2, and not the α1, isoform of AMPK. Also, whereas phosphorylation and activation of total AMPK in response to AICAR was decreased by glucose loading, the phosphorylation of the downstream targets (ACC-β Ser 218 and GS site 2) was normal.

We found that ~33% of GS was phosphorylated on site 2 in nonstimulated rat muscle with normal glycogen content. Although this is an approximate estimate, our data imply that a substantial fraction of GS becomes phosphorylated on site 2 in response to AICAR. In contrast, the amount of GS phosphorylated on both sites 2 and 2a appears to be a very small proportion of the total enzyme, even after AICAR treatment. This observation was surprising, because it has been thought that casein kinase (CK) 1 is constitutively active and catalyzes site 2a phosphorylation as soon as site 2 has been phosphorylated (22). The phosphorylation of GS sites 3a + 3b was not altered in response to AICAR. Because these sites are believed to be involved in a hierarchical phosphorylation starting with a CK-2−induced priming phosphorylation on site 5, followed by phosphorylation on sites 4, 3c, 3b, and 3a by glycogen synthase kinase (GSK)-3, our data likely indicate that phosphorylation on sites 3c, 4, and 5 is also not changed. Sites 2, 2a, and 3 are believed to be the main regulatory sites determining GS activity. Because only sites 2 and 2a are phosphorylated in response to AICAR, and full GS activity after AICAR treatment is restored by protein phosphatase treatment (6), then either site 2 is the determinant for AICAR-induced GS inactivation or other sites (e.g., GS site 1a, 1b, or other as-yet undefined sites)
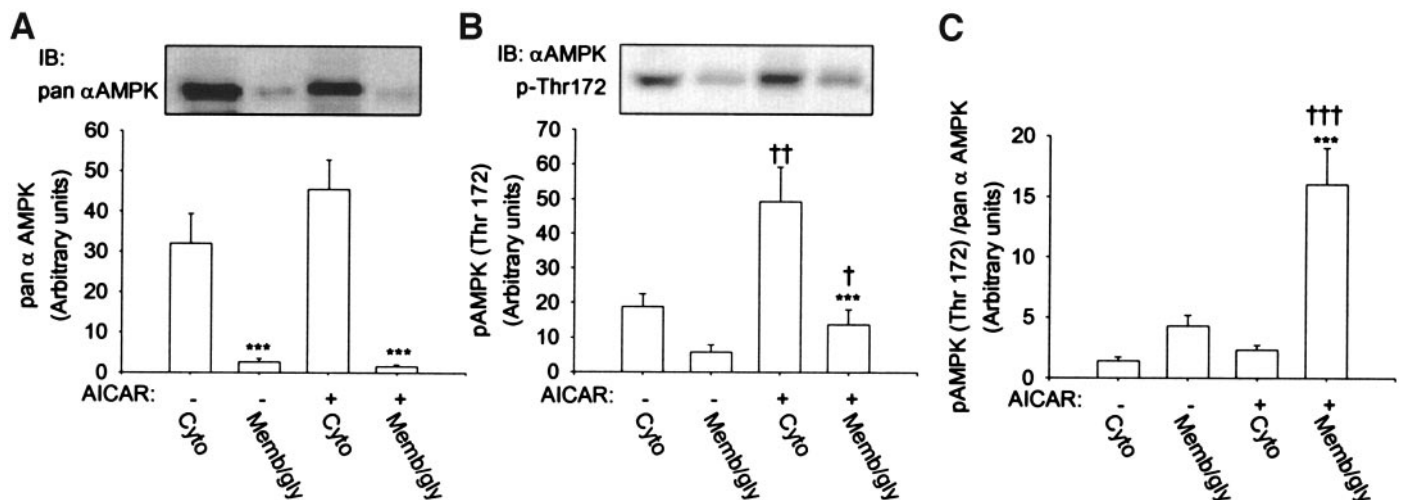

FIG. 4. Western blot analyses of pan α AMPK (A), Thr 172 phosphorylated αAMPK (pAMPK [Thr 172]; B), and the ratio between pAMPK(Thr 172) and pan-α AMPK (C). After perfusion (without or with AICAR) of rats with normal muscle glycogen content, white gastrocnemius muscles were fractionated into a membrane-glycogen (Memb/gly) and a cytosolic (Cyto) fraction. Data represent means ± SE, $n = 6$. ***$P < 0.001$ for cytosolic vs. membrane-glycogen fraction. Differences between nontreated and AICAR-stimulated values for pAMPK (Thr172): ††$P < 0.01$ for cytosolic fraction; †$P = 0.06$ for membrane-glycogen fraction; differences between nontreated and AICAR-stimulated values for pAMPK (Thr172)/pan αAMPK: NS for cytosolic fraction, †††$P < 0.001$ for membrane-glycogen fraction.

are major regulators of GS activity. Taken together with the data showing that site 2 on GS is an AMPK target in vitro (4), the present data convincingly show that GS is also an AMPK target in vivo. However, it remains possible that in vivo AMPK action on GS is indirectly regulated through action of other GS phosphatases or kinases.

The indication of an important role of site 2 (and to a lesser extent site 2a) in regulating GS activity in intact muscle tissue contrasts with observations made using overexpression of mutated GS in COS cells (23), where site-specific Ser-Ala mutations in site 2 and/or site 2a led to only a minor increase in GS activity (23). Of note is the observation that GS overexpressed in COS cells displayed activity in the absence of the glycogen core protein, glycogenin, whereas GS from tissues seems to be in a 1:1 association with this protein (24). Whether this difference influences the regulation of GS activity by phosphorylation at these sites remains to be evaluated.

Confocal microscopy analysis on rat soleus and tibialis anterior muscles using the same α1- and α2-AMPK antibodies as in the present study revealed a clear dotted pattern of both isoforms inside the fibers (C. Prats, T. Ploug, J.F.P.W., unpublished observations). We have not yet extended these analyses to cells other than the myocytes within the muscle tissue, but the presence of α1-AMPK inside the myocytes excludes the trivial explanation for the lack of an α1-AMPK KO GS phenotype being due to the lack of α1-AMPK expression.

AMPK is a promising antidiabetic drug target (25); however, the possibility that decreased GS activity may influence glycogen synthesis in vivo needs to be considered. In fact, AICAR treatment leads to a normalization of muscle glycogen synthesis in insulin-resistant muscle (26). A likely mechanism for this phenomenon is that AICAR, in addition to phosphorylating GS, also activates glucose transport (2), in turn increasing cellular levels of G6P (7), which may overrule inhibitory phosphorylation of GS by allosteric activation. Both activation of glucose transport and induction of GS phosphorylation by AICAR appear to be dependent on the α2-AMPK isoform, but the contributions of the different isoforms of the regulatory subunits of AMPK (β/γ) have not yet been fully elucidated (27,28). If different α2-AMPK heterotrimeric complexes regulate glucose transport and GS phosphorylation, new pharmacological drugs might be developed to target only glucose transport, thus circumventing possibly inappropriate effects on GS activity.

The present findings are likely to have physiological significance with regard to exercise, given that exercise is a powerful activator of AMPK in skeletal muscle (29,30). AMPK is a regulator of energy flux, and thus from a teleological point of view, it seems reasonable that activation during exercise inhibits the energy-consuming glycogenesis by increasing the phosphorylation of GS. However, the regulation of GS in response to exercise depends on both exercise duration and intensity and involves local and humoral factors (31). Our own unpublished observations in rat indicate that GS activity decreases initially during muscle contraction (first 3–5 min), concurrently with elevated site 2 phosphorylation. However, during continued contractile activity, GS activity then increases despite a continued elevated site 2 phosphorylation. In agreement with this observation, 10 min of contractile activity increased GS activity similarly in control muscle and muscle from the AMPK dead mouse (32) and from the two KO models used in the present study (S.B.J., J.N.N., J.B.B., G.S.O., D.G.H., B.F.H., E.A.R., J.F.P.W., unpublished observations). During exercise in humans, site 2 on GS is phosphorylated (J.N.N., J.B.B., D.G.H., B.F.H., E.A.R., J.F.P.W., unpublished observations); further, as we have previously reported, there is a negative correlation between AMPK and GS activity during exercise in humans (33), suggesting that AMPK via site 2 phosphorylation may inhibit GS activity. Taken together, these observations indicate that AMPK may act as a site 2 GS kinase during exercise, but that the resultant effect on GS activity depends on a multitude of other local and humoral factors. More work is needed to define the role of AMPK in regulating GS activity during exercise.

In line with previous observations (5,6,9–12), phosphorylation and activation of AMPK depends on fuel availability, either glucose in the extracellular medium or muscle glycogen. However, the present data suggest that regulation of some downstream targets of AMPK (ACC-β and GS) is not compromised by glucose loading when muscles are treated with AICAR. The fact that AICAR-induced signaling downstream of AMPK is well maintained during conditions of fuel surplus suggests that hyperglycemia in insulin-resistant states will not compromise AMPK action. The bulk of ACC-β and AMPK is distributed in the cytosol (34,35), and measurements in whole muscle lysates may give rise to faulty conclusions about the AMPK activity positioned in specific locations. In fact, our fractionation data suggest that AMPK in the membrane-glycogen fraction has enhanced sensitivity for activation by the upstream kinase. Whether this is due to glycogen or to another component in this fraction awaits further study.

GS activity is regulated by glycogen through a negative feedback mechanism (36,37). The mechanism for this effect is not clear, but it may be related to changes in kinases and/or phosphatases acting on GS. GS phosphorylation of site 2 and site 2 + 2a, but not of site 3a + 3b, was elevated by glucose loading, an observation that likely explains the lower GS activity in these muscles. Of note was that the same phosphorylation pattern has been seen in patients with glycogen phosphorylase deficiency (J.N.N., J.B.B., D.G.H., B.F.H., E.A.R., J.F.P.W., unpublished observations). The lack of changes in site 3a + 3b phosphorylation is in agreement with the observation that GSK-3 activity is unchanged in muscle of these patients and is not affected by glucose loading in healthy human and rodent skeletal muscle (33,38; J.N.N., J.B.B., B.F.H., E.A.R., J.F.P.W., unpublished observations). Measurements of AMPK phosphorylation and activity in total muscle lysates showed that total AMPK activity was suppressed under conditions of fuel surplus, suggesting that AMPK does not play a role in the increased phosphorylation of site 2 under these conditions. However, the phosphorylation of AMPK was elevated in the membrane-glycogen fraction, so it remains possible that this specific subfraction of AMPK could be responsible for the increased phosphorylation of site 2. Further studies are required to address these issues. Finally, in mouse, G_M (muscle glycogen–targeting subunit of protein phosphate

1) is important for maintaining glycogen storage and GS activity, likely because of the action of the associated phosphatase, protein phosphatase 1 (39,40). G_M also seems to play an important role in the increase in GS activity after glycogen depletion in human muscle cells (41). Thus, G_M-directed phosphatase activity may be an important regulator of GS phosphorylation. Of note is that phosphatase treatment of GS from glycogen-loaded muscles does not restore GS activity to the same degree as GS obtained from glycogen-lowered muscles (6), suggesting that phosphatase action is compromised, perhaps also in the intact cell, and that this may account for a GS site-specific action of phosphatases.

In conclusion, the present study has provided strong evidence that $\alpha2$- rather than $\alpha1$-containing AMPK complexes function as a GS kinase in intact skeletal muscle that upon activation by AICAR induces phosphorylation of GS on site 2 (and subsequently to a limited extent on site 2a, probably by CK-1), but not site 3a + 3b. As judged by AMPK phosphorylation, the action of the upstream kinase is suppressed by glucose loading, whereas the downstream action of AMPK is not, which may relate to the possibility that AMPK itself may become a better substrate for the upstream kinase when colocated with glycogen. Finally, we have provided evidence that the influence of glycogen on GS activity involves changes in phosphorylation on site 2 (and, to a lesser extent, on site 2a), but not on site 3a + 3b.

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