Original Article

5-Aminoimidazole-4-Carboxamide-1-β-D-Ribofuranoside and Metformin Inhibit Hepatic Glucose Phosphorylation by an AMP-Activated Protein Kinase–Independent Effect on Glucokinase Translocation

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AMP-activated protein kinase (AMPK) controls glucose uptake and glycolysis in muscle. Little is known about its role in liver glucose uptake, which is controlled by glucokinase. We report here that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), metformin, and oligomycin activated AMPK and inhibited glucose phosphorylation and glycolysis in rat hepatocytes. In vitro experiments demonstrated that this inhibition was not due to direct phosphorylation of glucokinase or its regulatory protein by AMPK. By contrast, AMPK phosphorylated liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase without affecting activity. Inhibitors of the endothelial nitric oxide synthase, stress kinases, and phosphatidylinositol 3-kinase pathways did not counteract the effects of AICAR, metformin, or oligomycin, suggesting that these signaling pathways were not involved. Interestingly, the inhibitory effect on glucose phosphorylation of these well-known AMPK activators persisted in primary cultured hepatocytes from newly engineered mice lacking both liver α_1 and α_2 AMPK catalytic subunits, demonstrating that this effect was clearly not mediated by AMPK. Finally, AICAR, metformin, and oligomycin were found to inhibit the glucose-induced translocation of glucokinase from the nucleus to the cytosol by a mechanism that could be related to the decrease in intracellular ATP concentrations observed in these conditions. *Diabetes* 55:865–874, 2006

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ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; FBPase-2, fructose-2,6-bisphosphatase; Fru-1-P, fructose 1-phosphate; Fru-6-P, fructose 6-phosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; G6Pase, glucose-6-phosphatase; GKRP, glucokinase regulatory protein; Glc-6-P, glucose 6-phosphate; HBM, hepatocyte basal medium; PFK, 6-phosphofructokinase; PKA, protein kinase A; PKB, protein kinase B.

The control of glucose uptake depends on the relative rates of glucose transport and phosphorylation. In skeletal muscle and adipose tissue, glucose uptake is mainly governed by the translocation of the glucose transporter GLUT4 to the plasma membrane. In these tissues, the rate of glucose phosphorylation exceeds that of the transporter, and the concentration of intracellular glucose is low. In liver, glucose transport by GLUT2 is rapid and not limiting, and control is exerted by glucokinase. The relatively low affinity of glucokinase for glucose ($S_{0.5}$ ~7–10 mmol/l, Hill coefficient ~1.7) makes it well suited to adapt its rate to the variations of glycemia (1). In addition, glucokinase activity depends on the glucokinase regulatory protein (GKRP), which inhibits glucokinase and controls its intracellular distribution (1). At low glucose concentrations, glucokinase is bound to GKRP in the nucleus and translocates to the cytoplasm when glucose concentrations increase (2–4). The interaction between glucokinase and GKRP is modulated by fructose 6-phosphate (Fru-6-P) and fructose 1-phosphate (Fru-1-P), which promote the association or dissociation of the two proteins, respectively (5).

In muscle and adipose tissue, insulin stimulates glucose uptake by a protein kinase B/Akt substrate 160-mediated mechanism involving the recruitment of GLUT4 (6). By contrast, insulin does not exert short-term regulation of either GLUT2 or glucokinase in liver but stimulates glucose phosphorylation by the sterol regulatory element–binding protein-1c–mediated induction of glucokinase gene expression (7).

Exercise and hypoxia increase glucose uptake in skeletal muscle and heart by an insulin-independent stimulation of GLUT4 recruitment (8). Under these conditions, the AMP-activated protein kinase (AMPK) is activated and has been proposed to participate in this stimulation. AMPK is a heterotrimeric serine/threonine protein kinase composed of a catalytic subunit (α) and two regulatory subunits (β and γ) that acts as an energy and nutrient sensor in cells (9). AMPK is activated by an increased AMP-to-ATP ratio, as occurs in the absence of oxygen or in response to other stresses, leading to an inhibition of energy-consuming processes and a stimulation of ATP-producing pathways (9).

The most commonly used indirect activator of AMPK is 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

(AICAR), which is phosphorylated to AICAribotide, an analog of AMP. In skeletal muscle, the accumulation of ZMP leads to AMPK activation and stimulation of glucose uptake (10). Similarly, metformin, a biguanide used in the treatment of type 2 diabetes, activates AMPK and enhances glucose transport in skeletal muscle (11,12) by a mechanism generally believed to be independent of adenine nucleotides (13). Furthermore, AMPK activation by anoxia and oligomycin, due to a rise in AMP/ATP, also stimulates glucose uptake and glycolysis in heart by activating heart 6-phosphofructokinase (PFK) (14). The involvement of the LKB1-AMPK axis in the control of muscle glucose uptake was demonstrated both by the use of dominant-negative constructs of AMPK (15–17) and of knockout mice for AMPKα_2 (18) and for the upstream kinase LKB1 (19), which all abrogated the stimulation of glucose uptake induced by several of these AMPK activators.

Little is known about the role of AMPK in glucose phosphorylation and glycolysis in liver. Incubation of hepatocytes from starved rat with AICAR inhibited glucose phosphorylation, decreased fructose 2,6-bisphosphate (Fru-2,6-P_2) concentration, and inhibited glycolytic flux (20). This was recently confirmed by the demonstration that in vivo infusion of AICAR in dogs also induced a nearly complete inhibition of net hepatic glucose uptake (21). ZMP, which accumulates in hepatocytes, was found to directly inhibit glucokinase catalytic activity at high concentrations, but this was not sufficient to explain the inhibition of glucose phosphorylation by AICAR (20). The underlying mechanism could be related to AMPK. This hypothesis was tested in the present work by studying the effects of several AMPK activators on glucose phosphorylation in hepatocytes from normal rats and from mice lacking both α_1 and α_2 catalytic AMPK subunits in the liver.

RESEARCH DESIGN AND METHODS

Isolation, incubation, and culture of hepatocytes. Liver cells were prepared by the collagenase method (22) from starved male Wistar rats (200–300 g) or from postabsorptive male mice (25–30 g) after anesthesia with sodium pentobarbital (6 mg/100 g body weight) or ketamin/xylazin (8/1 mg per 100 g body weight), respectively. For incubations, hepatocytes (50 mg wet weight/ml) were preincubated for 15 min at 37°C in Krebs-Henseleit buffer saturated with O_2/CO_2 (1:19) containing the indicated concentrations of glucose as described (22). AICAR, metformin, oligomycin, or the vehicle were then added, and the cells were incubated for 20 min before the addition of [2-^{3}H]glucose (0.5 μCi/ml) or [3-^{3}H]glucose (0.25 μCi/ml) (Amersham Biosciences). After 20 min, samples were deproteinized in ice-cold 1 mol/l $HClO_4$ to measure ^3H_2O release and metabolite concentrations. For AMPK assay, the same experiments were carried out without radioactive tracers. Cells were collected by centrifugation, immediately frozen in liquid nitrogen, and stored at −80°C. For cultures, rat or mice hepatocytes were seeded for 4 h on type I collagen–coated dishes (2.10^4 cells per cm^2) and cultured overnight in a hepatocyte basal medium (HBM) supplemented with a cocktail of antibiotics and growth factors (Cambrex BioScience). Cultured hepatocytes were then incubated in basal HBM containing 10 mmol/l glucose in the presence of 1 mmol/l AICAR, 5 mmol/l metformin, 1 μmol/l oligomycin, or the vehicle for 4 h. The rate of glucose phosphorylation was measured during the last hour of incubation after addition of [2-^{3}H]glucose (0.25 μCi/ml).

Measurements of glucose phosphorylation, glycolytic flux, and metabolites. The rate of glucose phosphorylation and glycolytic flux through PFK-1 were estimated by the detritiation of [2-^{3}H]glucose and of [3-^{3}H]glucose, respectively (22). The release of ^3H_2O was linear over time, and the rate was expressed as μmol of glucose detritiated · min^{-1} · g^{-1} (wet weight or protein). Hexose 6-phosphates, Fru-2,6-P_2 (23), and Fru-1-P (24) were determined by spectrophotometric assays as indicated. Adenine nucleotides were measured by high-performance liquid chromatography in neutralized-$HClO_4$ extracts (25).

AMPK assay. Total AMPK activity from hepatocyte extracts was assayed after precipitation with 10% (wt/vol) polyethylene glycol 6000 (26). One unit of AMPK activity corresponds to 1 nmol of product formed per min under the assay conditions.

Western blot analysis. Expression of glucokinase and AMPK and phosphorylation state of acetyl-CoA carboxylase (ACC) were monitored by SDS-PAGE immunoblots with anti-glucokinase (gift from J. Guinovart, Barcelona, Spain), anti-AMPKα_1 and AMPKα_2 (gifts from G. Hardie, Dundee, Scotland), and antiphospho-Ser79 ACC (Upstate) antibodies, respectively. Total ACC was determined using streptavidin linked to horseradish peroxidase (Bio-Rad), and loading control for glucokinase was assessed using anti–α-actin antibody (Santa Cruz).

In vitro phosphorylation. Purified recombinant bovine heart and rat liver PFK-2/fructose 2,6-bisphosphatase (FBPase-2) as well as purified recombinant human liver glucokinase and GKRP (all at 0.1 mg/ml) were incubated with 0.1 mmol/l [γ-^{32}P]MgATP (250 cpm/pmol) and AMPK purified from rabbit liver (500 mU/ml) and/or protein kinase A (PKA) (100 mU/ml) in a final volume of 25 μl as described (27). ^{32}P incorporation was measured by phosphorimaging after SDS-PAGE. Identification of phosphorylated peptides was obtained by tandem mass spectrometry analysis (MS/MS mode) after digestion of the phosphorylated proteins with trypsin and separation of peptides by reverse-phase narrow-bore high-performance liquid chromatography. The kinetic properties of PFK-2/FBPase-2 were determined on the same preparations phosphorylated with 1 mmol/l nonradioactive MgATP and purified AMPK or PKA for 40 min. PFK-2 and FBPase-2 activities were measured as described (27).

Generation of AMPK$\alpha_1\alpha_{2LS}^{-/-}$ knockout mice. To obtain a deletion of both catalytic subunits in the liver (AMPK$\alpha_1\alpha_{2LS}^{-/-}$), we first generated a liver-specific AMPKα_2-null mouse (AMPK$\alpha_2^{-/-}$) by crossing floxed AMPKα_2 mice (28) and AlfpCre transgenic line expressing the Cre recombinase under control of the albumin and α-fetoprotein regulatory elements (29). We then produced a liver-specific AMPKα_2 deletion on an AMPK$\alpha1^{-/-}$ background by crossing liver-specific AMPK$\alpha_2^{-/-}$ mice with AMPK$\alpha_1^{-/-}$ mice (18). Mice were genotyped by PCR on DNA extracted from a tail biopsy using specific primers for the Cre transgene and for the floxed AMPKα_2, the deleted AMPKα_1, and the wild-type AMPKα_1 allele.

Immunostaining and imaging. Isolated rat or mice hepatocytes were cultured overnight in complete HBM on type I collagen precoated glass coverslips. Hepatocytes were then incubated for 4 h in basal HBM with different concentrations of glucose and the same concentrations of AMPK activators as indicated above. The cells were then fixed for 15 min in 1% (wt/vol) paraformaldehyde/PBS, incubated for 10 min with 100 mmol/l glycine/PBS, permeabilized with 0.2% (vol/vol) Triton X-100/PBS, and blocked in 5% BSA/PBS (wt/vol). The fixed cells were incubated with primary glucokinase antibody for 1 h and then treated for 45 min with donkey anti-rabbit/AlexaFluor 594 (Molecular Probes). Coverslips were then air dried and mounted on glass microscope slides. Imaging was performed using a Zeiss Axiovert 200 fluorescence microscope and a Nikon Coolpix 995 digital camera. Nucleus and cytosolic quantification of glucokinase was performed on gray-scale converted images after laser-scanning confocal microscopy on a total of >100 cells in each group using the ImageJ 1.32 software (National Institutes of Health, Bethesda, MD).

Statistics. The results are expressed as means ± SE. The statistical significance of differences was calculated using Student's test.

RESULTS

Inhibition of glucose phosphorylation and glycolytic flux by AICAR, metformin, and oligomycin. In agreement with previous reports (20), AICAR activated AMPK (Fig. 1*A*), increased the phosphorylation state of ACC (Fig. 1*C*), and inhibited glucose phosphorylation (Fig. 1*E*) in suspensions of hepatocytes from starved rats in a dose-dependent manner. Metformin had similar effects (Fig. 1*B*, *D*, and *F*), although the maximal inhibition of glucose phosphorylation was less than with AICAR. Oligomycin also led to AMPK activation, ACC phosphorylation, and inhibition of glucose phosphorylation (Fig. 1*G–I*). These effects were also observed in hepatocytes from fed rats (data not shown). A plot of AMPK activity versus glucose phosphorylation rates (Fig. 1*J*) indicated that the relationship was not simple and suggested that elements other than AMPK were implicated.

While the concentrations of Fru-1-P were not signifi-

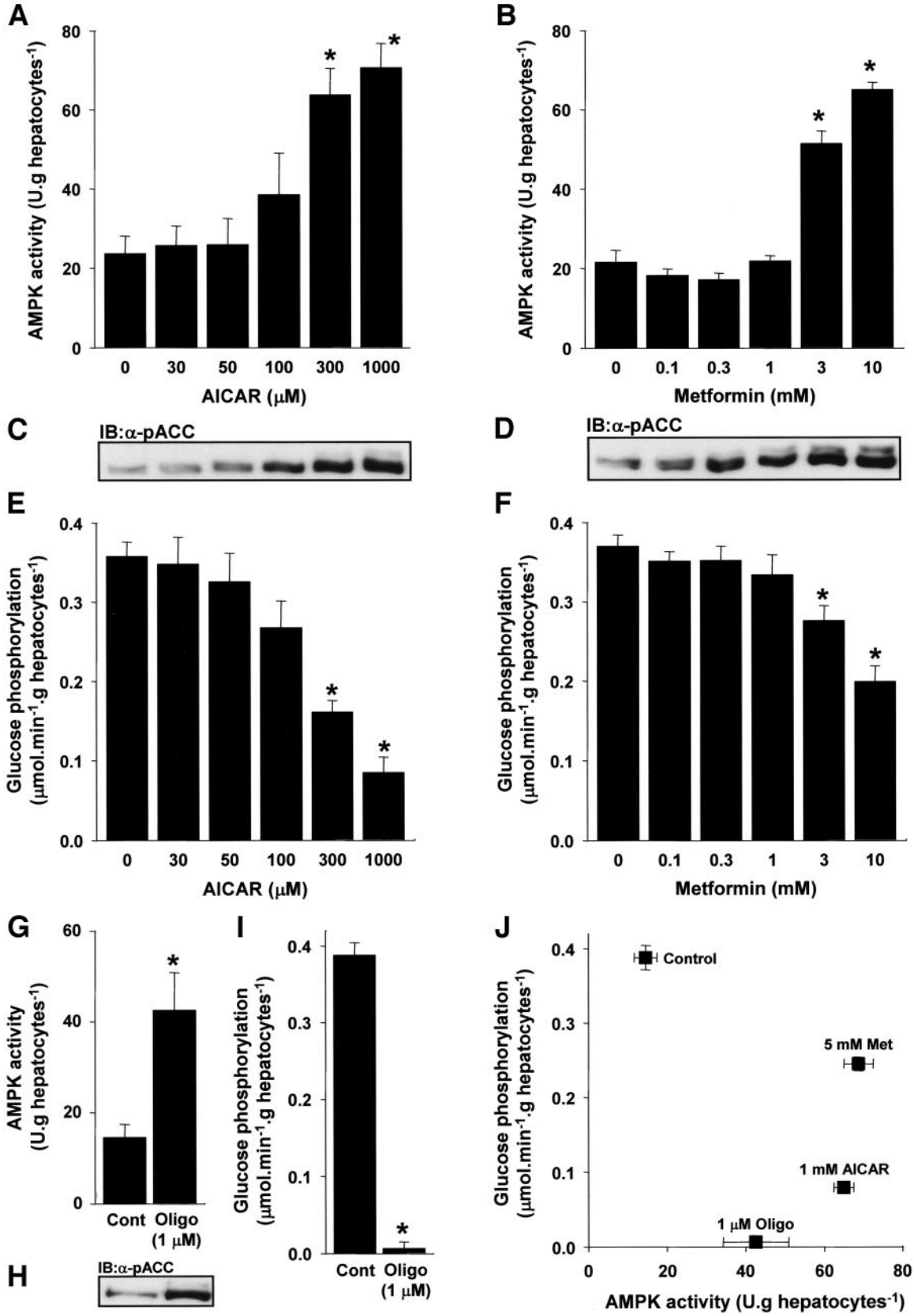


FIG. 1. Effects of AICAR, metformin, and oligomycin on AMPK activity (*A*, *B*, and *G*), ACC phosphorylation (*C*, *D*, and *H*), and glucose phosphorylation rate (*E*, *F*, and *I*) in isolated rat hepatocytes. Hepatocytes from starved rats were incubated for 40 min with 10 mmol/l glucose. The relationship between AMPK activities and glucose phosphorylation rates in the presence of AMPK activators was plotted in *J*; means ± SE ($n = 4$). *$P < 0.05$ compared with control vehicle.

TABLE 1
Effect of AICAR, metformin, and oligomycin on hexoses 6-phosphates, Fru-1-P, and Fru-2,6-P_2 concentrations and on glycolytic flux in rat hepatocytes

	Glc-6-P	Fru-6-P	Fru-1-P	Fru-2,6-P_2	Glycolytic flux (μmol · min^{-1} · g hepatocytes^{-1})
Control	119 ± 7	93 ± 5	28 ± 10	6.9 ± 0.7	0.18 ± 0.02
AICAR	46 ± 4*	53 ± 6*	39 ± 12	1.9 ± 0.2*	0.05 ± 0.00*
Metformin	80 ± 4*	53 ± 4*	23 ± 5	1.7 ± 0.4*	0.10 ± 0.01*
Oligomycin	27 ± 2*	27 ± 4*	13 ± 8	1.3 ± 0.2*	0.04 ± 0.01*

Data are means ± SE for three to four cell preparations. Measurements are given in nmol/g hepatocytes, unless otherwise indicated. *$P <$ 0.05 compared with control vehicle.

cantly modified, those of hexose 6-phosphates and Fru-2,6-P_2 and the rates of detritiation of [3-^{3}H]glucose were decreased in hepatocytes incubated with metformin, AICAR, and oligomycin, in increasing order of efficiency, indicating that the inhibition of glucose phosphorylation resulted in a subsequent inhibition of glycolytic flux (Table 1).

In vitro phosphorylation of glucokinase and liver PFK-2 by AMPK. The inhibition of glucose phosphorylation could be due to changes in the kinetic properties of glucokinase resulting from a direct phosphorylation of glucokinase and/or GKRP by AMPK. To test this hypothesis, purified glucokinase was incubated with purified AMPK and ^{32}P-labeled ATP. This led to a small and non linear ^{32}P-incorporation of <0.1 mol/mol of glucokinase, a value much lower than 0.8–1 mol/mol, the stoichiometry obtained with heart PFK-2, a known substrate of AMPK (14) (Fig. 2*A*). Moreover, a significant phosphorylation of GKRP alone or in combination with glucokinase by AMPK could not be demonstrated. These results suggest that glucokinase and GKRP are not good substrates for AMPK

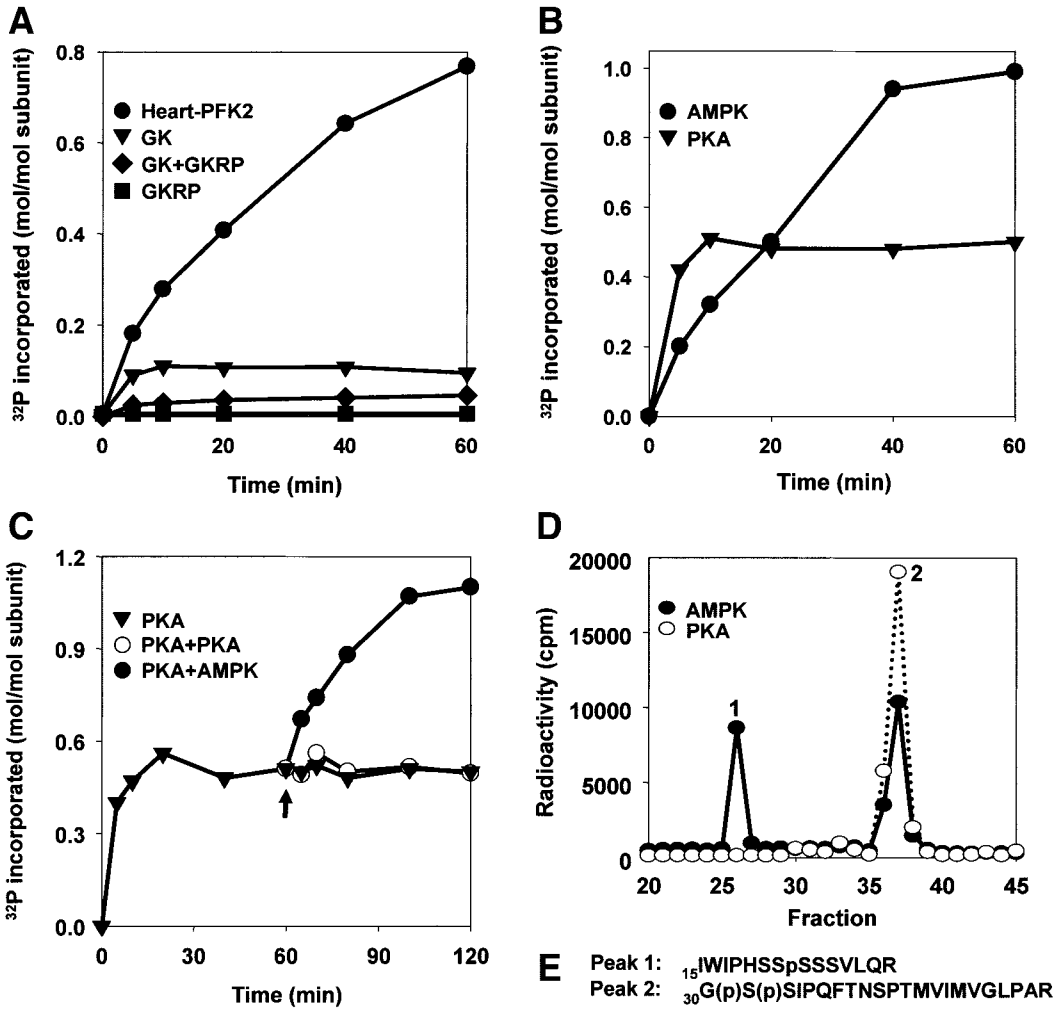


FIG. 2. In vitro phosphorylation of rat liver glucokinase or PFK-2/FBPase-2 by AMPK. *A*: Purified recombinant heart PFK-2, liver glucokinase, and/or GKRP were incubated in presence of [γ-^{32}P]MgATP with purified AMPK. *B* and *C*: Purified recombinant liver PFK-2/FBPase-2 was incubated with purified AMPK and/or with PKA. High-performance liquid chromatography profiles (*D*) and MS/MS sequencing of ^{32}P-labeled peptides of PFK-2/FBPase-2 (*E*) were determined after trypsin digestion. One typical experiment is presented and similar results were obtained in two other preparations.

TABLE 2
Effects of in vitro phosphorylation by AMPK or PKA on kinetic properties of liver PFK-2 and FBPase-2

	PFK-2			FBPase-2			PFK-2–to–FBPase-2 ratio
	K_m Fru-6-P (μmol/l)	V_{max} (mU/mg protein)	V_{max}/K_m	K_m Fru-2,6-P_2 (μmol/l)	V_{max} (mU/mg protein)	V_{max}/K_m	V_{max}/K_m (×10)
Control	38 ± 8	27.4 ± 1.4	7.3	126 ± 16	7.5 ± 0.4	0.60	12.2
AMPK	53 ± 8	23.4 ± 1.1*	4.4	90; 88	5.2; 5.1	0.58	7.5
PKA	1,127 ± 323*	20.3 ± 1.5*	0.2	216 ± 37*	11.6 ± 0.5*	0.54	0.3

Data are means ± SE for three determinations; otherwise, individual values are given. *$P < 0.05$ compared with control.

and that glucokinase inhibition was unlikely to be due to phosphorylation.

The inhibition of glycolytic flux by AICAR, metformin, and oligomycin could result from phosphorylation of liver PFK-2/FBPase-2 by AMPK. Liver PFK-2/FBPase-2 is a substrate of the cyclic AMP-dependent PKA, which inactivates PFK-2 and activates FBPase-2 by phosphorylating Ser32. This explains the effect of glucagon to decrease Fru-2,6-P_2 concentrations in hepatocytes (30). AMPK was found to phosphorylate liver PFK-2/FBPase-2, and the extent of phosphorylation was about twice that observed with PKA (Fig. 2*B*). In addition, AMPK was able to further phosphorylate liver PFK-2/FBPase-2 that had already been maximally phosphorylated by PKA (Fig. 2*C*), suggesting that others sites were phosphorylated by AMPK. Indeed, after digestion by trypsin and analysis of the phosphorylated peptides by mass spectrometry, Ser22 was found to be phosphorylated by AMPK but not by PKA (Fig. 2*D* and *E*). A radioactive peptide containing Ser32 was also separated. It contained a single phosphate after phosphorylation by either PKA or AMPK. Fragmentation of this peptide did not allow one to distinguish between Ser32 and Ser33. Finally, we showed that phosphorylation of liver PFK-2/FBPase-2 by AMPK led to minimal changes in the kinetic of PFK-2 with no effect on FBPase-2, in contrast with the effects of PKA (Table 2). It is concluded that although liver PFK-2/FBPase-2 is a substrate for AMPK, phosphorylation has no significant effect on the kinetic properties of the enzyme.

Absence of AMPK involvement in the inhibition of glucose phosphorylation. We used several known inhibitors to test whether the inhibition of glucose phosphorylation by AICAR, metformin, and oligomycin could be mediated by other intracellular signaling pathways. Our results indicated that the effects of the AMPK activators persisted in the presence of inhibitors of endothelial nitric oxide synthase, phosphatidylinositol 3-kinase, extracellular-signal regulated kinases, p38-mitogen-activated protein kinase, and c-Jun-N-terminal kinase, suggesting that these signaling pathways were not involved (data not shown).

To assess the role of AMPK, we used newly engineered mice that were totally deficient in the α_1 isoform and whose expression of the α_2 isoform was specifically deleted in the liver by Cre-recombinase technology ($AMPK\alpha_1\alpha_{2LS}^{-/-}$). We first confirmed that AMPK activation and inhibition of glucose phosphorylation by AICAR, metformin, and oligomycin were indeed observed in hepatocytes from wild-type mice (Fig. *3*). As expected, expression of both isoforms of AMPK as well as AMPK activation could not be detected in hepatocytes from $AMPK\alpha_1\alpha_{2LS}^{-/-}$ mice (Fig. 3*A*–*C*). The residual small apparent activity of AMPK after polyethylene glycol fractionation in hepatocytes from $AMPK\alpha_1\alpha_{2LS}^{-/-}$ mice was nonspecific, since no significant activities were detected after immunoprecipitation of both isoforms of AMPK (data not shown). Furthermore, the lack of AMPK activation was confirmed by the absence of ACC phosphorylation (Fig. 3*B*). Basal glucose phosphorylation was significantly reduced in $AMPK\alpha_1\alpha_{2LS}^{-/-}$ hepatocytes (Fig. 3*C*) and could have resulted from the apparent decreased expression of liver glucokinase in this condition (Fig. 3*D*). More importantly, the inhibition of glucose phosphorylation by the AMPK activators persisted in hepatocytes from the $AMPK\alpha_1\alpha_{2LS}^{-/-}$ mice and was similar to that observed in wild-type mice, thus demonstrating that the inhibition of glucose phosphorylation by AICAR, metformin, and oligomycin did not require AMPK.

Mechanism of inhibition of glucose phosphorylation. The effects of AICAR, metformin, and oligomycin on glucose phosphorylation were tested at various concentrations of glucose in suspensions of hepatocytes from starved rats (Fig. 4*A*). In control experiments, the relationship between the rates of glucose phosphorylation and glucose concentrations was sigmoidal (apparent $S_{0.5}$ ~14 mmol/l), as expected (22). Metformin, AICAR, and oligomycin, in the same increasing order of efficiency as in Fig. 1, decreased the affinity for glucose (apparent $S_{0.5}$ ~22 mmol/l, 40 mmol/l, and 57 mmol/l, respectively) without significantly affecting the calculated V_{max} (~1.2 μmol · min^{-1} · g $hepatocytes^{-1}$). This inhibition of glucose phosphorylation is reminiscent of the apparent competitive inhibition exerted by Fru-6-P, which inhibits glucokinase by promoting its nuclear binding to GKRP (5). Therefore we studied the intracellular distribution of glucokinase in primary cultures of rat hepatocytes. As expected, increasing glucose concentrations induced a translocation of glucokinase from the nucleus to the cytosol in control conditions. Interestingly, this translocation was greatly impaired in hepatocytes incubated with AICAR, metformin, and oligomycin at both 10 and 25 mmol/l glucose (Fig. 4*B*), thus suggesting that the inhibition of glucose phosphorylation resulted from an impairment of glucokinase translocation in response to glucose. We repeated these experiments with hepatocytes from wild-type or $AMPK\alpha_1\alpha_{2LS}^{-/-}$ mice (Fig. 4*C*). No difference was observed between both strains of mice in the nuclear-to-cytoplasmic ratio whatever the concentration of glucose (Fig. 4*D*). However, we confirmed that the total glucokinase content was significantly reduced in the $AMPK\alpha_1\alpha_{2LS}^{-/-}$ compared with wild-type mice (−23%, $P < 0.05$; Fig. 4*E*). More importantly, our results clearly demonstrated that the inhibition of glucokinase translocation persisted in this AMPK-deficient model, thus ruling out a role for AMPK in this phenomenon.

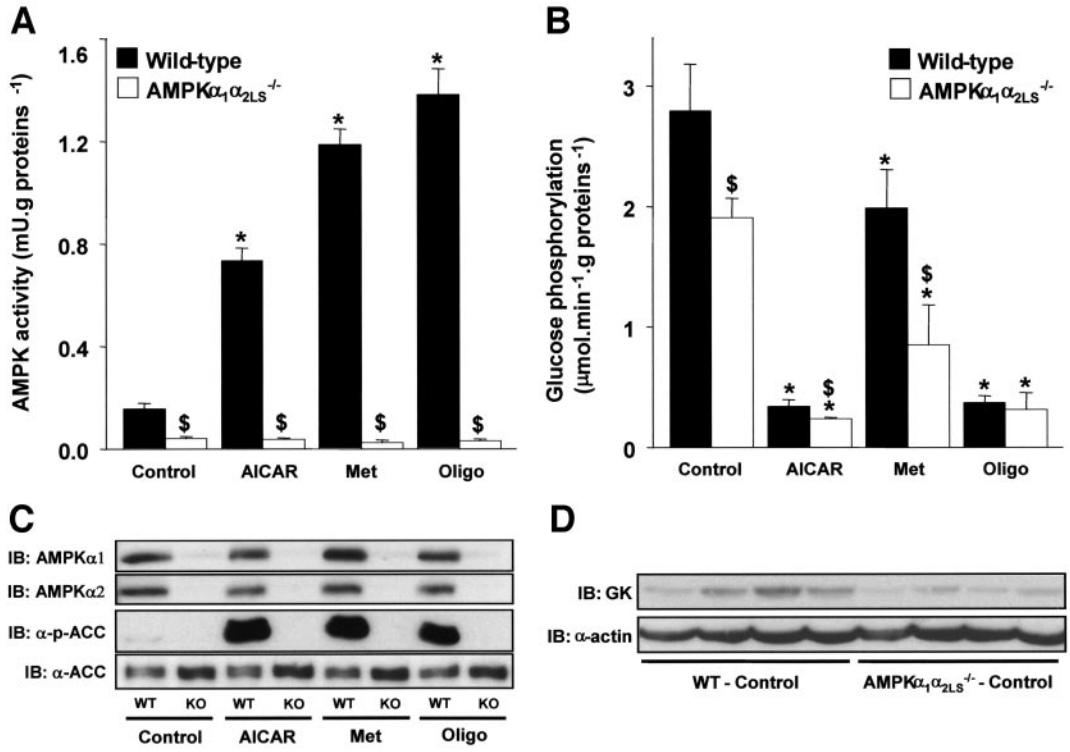


FIG. 3. Effects of AICAR, metformin, and oligomycin on AMPK activity (*A*), AMPK content and ACC phosphorylation (*C*), and glucose phosphorylation rate (*B*) in cultured hepatocytes from wild-type and AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice. Cultured hepatocytes were incubated for 4 h with 10 mmol/l glucose in the presence of 1 mmol/l AICAR, 5 mmol/l metformin, and 1 µmol/l oligomycin or vehicle; means ± SE (n = 5). *\$$P < 0.05$ compared with control-vehicle or wild type, respectively. *D*: glucokinase content in cultured hepatocytes from wild-type and AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice incubated as described above in presence of vehicle.

Finally, we investigated whether a fall in ATP concentration could be involved in the inhibition of glucose phosphorylation by the AMPK activators. Metformin, AICAR and oligomycin induced a significant decrease in ATP concentration in rat hepatocytes (Fig. 5*A*). This was also demonstrated in mouse hepatocytes, the fall in ATP being more pronounced in AMPK$\alpha_1\alpha_{2LS}^{-/-}$ compared with wild-type mice (Fig. 5*B*). Therefore, a clear relationship between ATP concentration and the rate of glucose phosphorylation was seen in the presence of metformin, oligomycin or the vehicle in hepatocytes from rat and from both wild-type and AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice (Fig. 5*C* and *D*). In the presence of AICAR, the relationship differed and was in line with an additive inhibitory effect of ZMP on glucokinase catalytic activity (20). Therefore, we suggest that the fall in ATP concentration could explain the inhibition of glucose-induced translocation of glucokinase by AICAR, metformin, and oligomycin (Fig. 6).

DISCUSSION

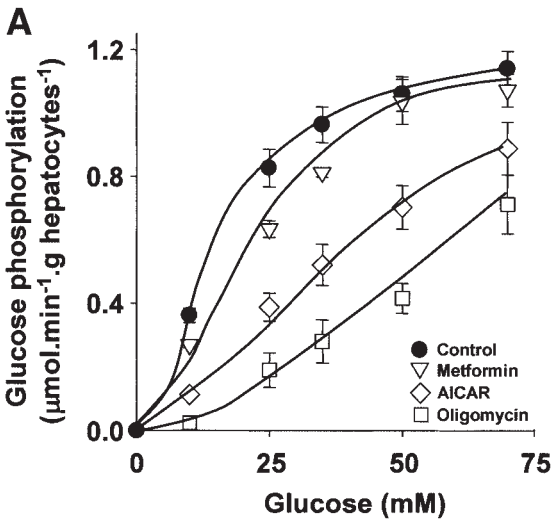
In the present work we report that, by contrast with muscle, AICAR, metformin, and oligomycin inhibit liver glucose phosphorylation by an AMPK-independent mechanism. Although all these agents did activate AMPK, this enzyme is clearly not involved in the inhibition of glucose phosphorylation because this event persisted in hepatocytes from mice deficient in both isoforms of the AMPK catalytic subunits in the liver.

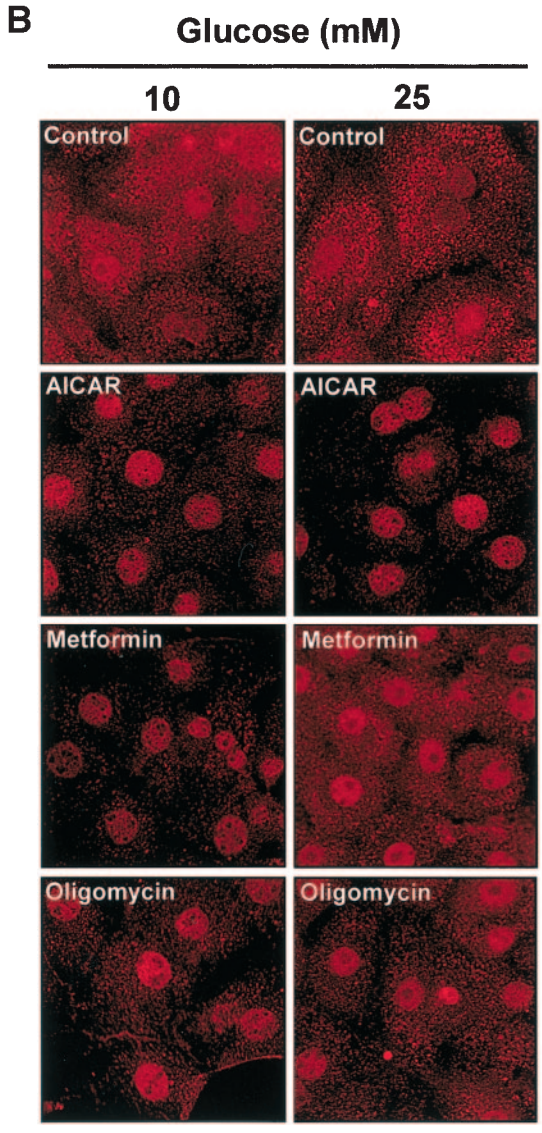
AMPK-independent effects of AICAR, metformin, and oligomycin. Taken together with the ZMP-mediated inhibition of fructose-1,6-bisphosphatase by AICAR and the resulting inhibition of gluconeogenesis (25), our results add to the previous caveats concerning the use of AICAR to study the consequences of AMPK activation. Indeed, all enzymes whose activities are influenced by AMP are also potential targets for ZMP. In turn, metformin, and oligomycin, by their respective inhibition of the mitochondrial respiratory-chain complex I (31) and of the F_0-F_1-ATP synthase, could also exert AMPK-independent effects that may be responsible for the inhibition of glucose phosphorylation.

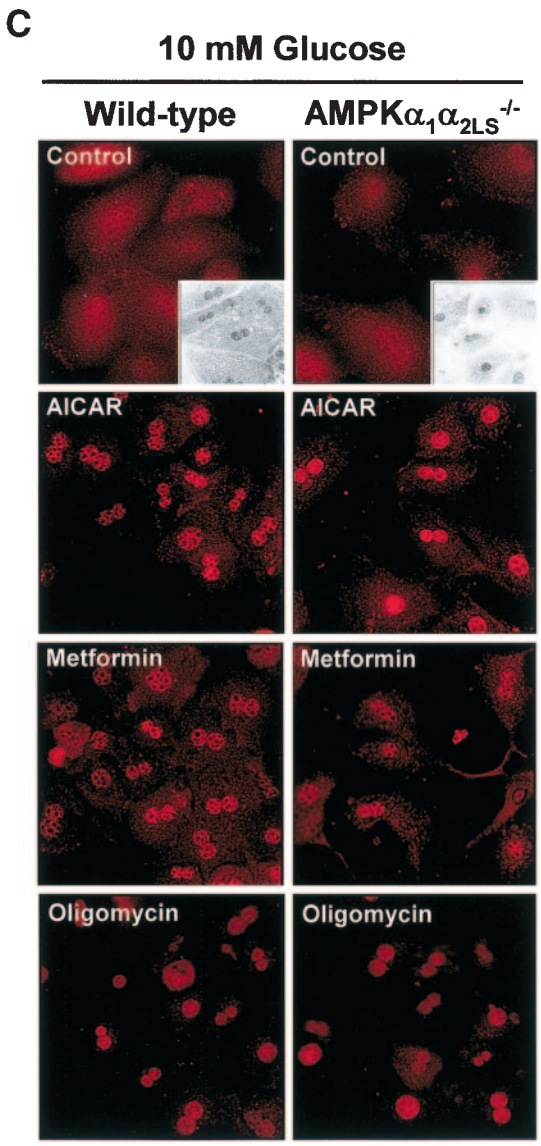
AICAR and metformin are generally believed to activate AMPK without changing the concentration of adenine nucleotides (9). However, our results indicate that this can be questionned, at least in liver and for the concentrations used, since both compounds induced ATP depletion. This confirms previous observations showing that concentrations of AICAR >0.5 mmol/l decreased intracellular ATP in hepatocytes (32,33) and liver (21). The exact mechanism of this fall is not fully understood, but our preliminary results (34) suggest that AICAR, like metformin (31), could inhibit liver mitochondrial oxidative phosphorylation by an AMPK-independent mechanism.

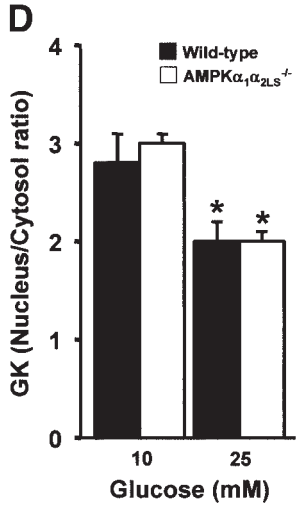
Mechanism of inhibition of glucose phosphorylation. Net glucose uptake in the liver depends on the relative rates of glucokinase and glucose-6-phosphatase (G6Pase). Inhibition of net glucose uptake could thus result from an inhibition of glucokinase or from an increased rate of G6Pase, or a combination of both. However, there is no evidence for an increased flux through G6Pase since both metformin and AICAR inhibit gluconeogenesis (25,35), thus suggesting that the inhibition of glucose uptake is only due to a decrease in glucose phosphorylation by GK.

Our results indicate that glucokinase inhibition resulted from its sequestration in the nucleus, which is known to result from its binding to GKRP (1–4). Several AMPK-independent mechanisms can be proposed to account for









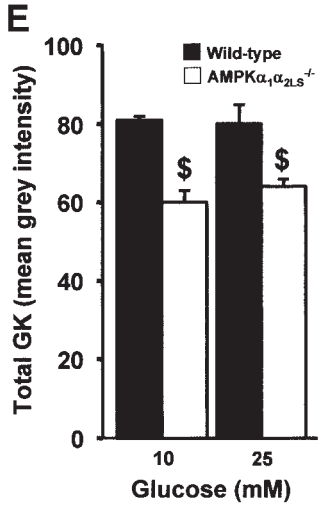


FIG. 4. Effects of AICAR, metformin, and oligomycin on glucose-induced nuclear translocation of glucokinase in hepatocytes from rat or wild-type and AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice. The glucose phosphorylation rates of rat hepatocytes incubated with different concentrations of glucose in the presence of 1 mmol/l AICAR, 5 mmol/l metformin, and 1 µmol/l oligomycin or vehicle are represented in *A*; means ± SE (n = 4). Images of stained cells are representative of three different preparations of cultured hepatocytes from rat (*B*) or wild-type and AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice (*C*) incubated with AMPK activators as indicated in the legend of Fig. 3. The nucleus-to-cytoplasmic ratio (*D*) and the quantification of total glucokinase (*E*) were measured at 10 and 25 mmol/l glucose on gray-scale converted images (see *inserts*) after laser-scanning confocal microscopy and on a total of more than 100 cells in each group; means ± SE (n = 3). *\$$P$ < 0.05 compared with 10 mmol/l glucose or wild-type mice, respectively.

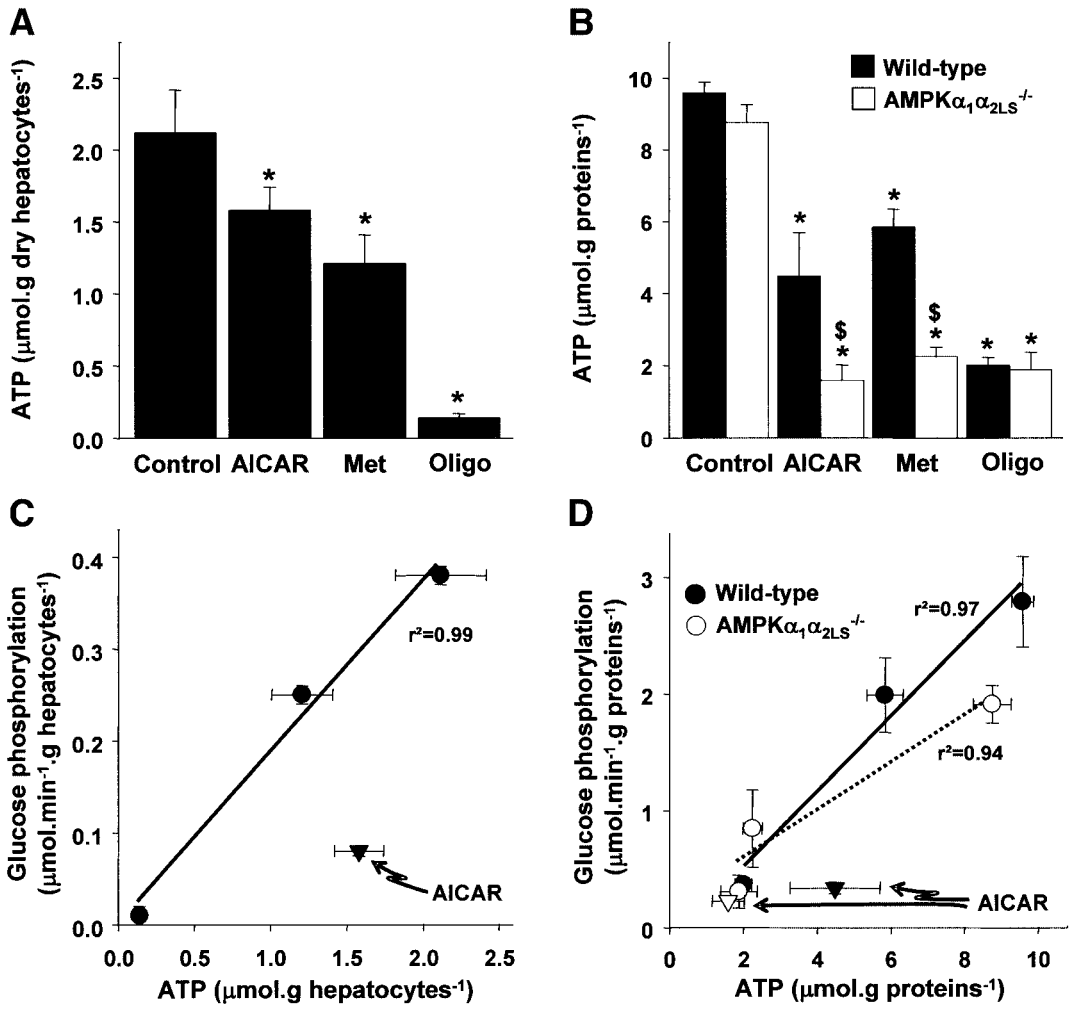


FIG. 5. Effects of AICAR, metformin, and oligomycin on intracellular ATP concentrations in hepatocytes from rat or wild-type and AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice. Hepatocytes isolated from rat (*A*) or wild-type and AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice (*B*) were incubated with AMPK activators as indicated in the legend of Figs. 1 and 3, respectively. The relationship between ATP concentrations and glucose phosphorylation rate in the presence of metformin, oligomycin, or the vehicle was plotted in *C* or *D*; means ± SE (n = 5). *\$$P$ < 0.05 compared with control or wild-type vehicle, respectively.

this inhibition. GKRP binding to glucokinase, which is competitive with glucose, is favored when the GKRP is bound to Fru-6-P, and is inhibited when the ligand site is occupied by Fru-1-P (5). However, we could not demonstrate significant changes in Fru-1-P concentration. Furthermore, Fru-6-P can also be ruled out because its concentration decreased in hepatocytes incubated with AICAR, metformin, and oligomycin.

By contrast, the fall in ATP that is observed with the AMPK activators might be crucial for the control of glucokinase activity, because ATP is a substrate of glucokinase (in vitro half saturation with 0.5 mmol/l) and exerts an effect similar to that of Fru-1-P, decreasing the affinity of GKRP for Fru-6-P (5). A drop in ATP concentration is therefore expected to increase the affinity of GKRP for glucokinase and to prevent translocation to the cytosol. Although little is known about a putative ATP binding site on GKRP, we suggest that ATP could affect the conformation of GKRP and its binding to glucokinase. In addition, it has been proposed that GKRP could act as a nuclear chaperone, which carries glucokinase into the nucleus via a piggy-back mechanism, and that the subsequent glucokinase translocation to the cytosol might be energy dependent (36,37). Therefore we speculate that the glucose-mediated translocation of glucokinase to the cytosol could be an ATP-dependent process that is impaired in our conditions.

Beside GKRP in the nucleus, liver PFK-2/FBPase-2 has been recently identified as a new intracellular binding partner for glucokinase (38). Indeed, the nonphosphorylated form of PFK-2 could act as a cytoplasmic anchor for glucokinase, whereas the phosphorylated form would favor its nuclear sequestration (39). While AMPK could phosphorylate Ser32, the residue involved in the PFK-2–mediated inhibition of glucose phosphorylation by glucagon (39), the control of glucokinase distribution by an AMPK-mediated phosphorylation of PFK-2 is irrelevant in our conditions. Indeed, the effect of AICAR, metformin, and oligomycin on glucokinase subcellular localization was not modified in AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice.

Role of AMPK in the regulation of glucokinase expression. Our observation of a lower glucokinase protein content in hepatocytes from AMPK$\alpha_1\alpha_{2LS}^{-/-}$ mice could suggest that AMPK favors glucokinase expression. This is clearly at variance with the report by Foretz et al. (40) showing that expression of a constitutively active form of AMPK in the liver decreases glucokinase mRNA and protein content. We have no simple explanation for this

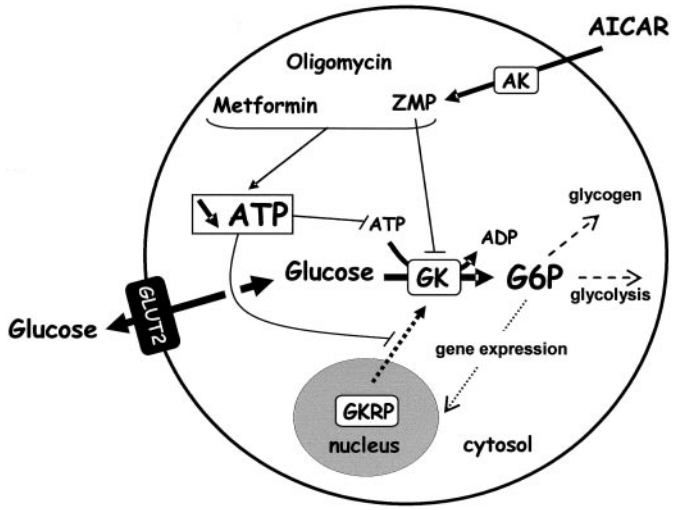


FIG. 6. Mechanism of inhibition of liver glucose phosphorylation by AMPK activators. Metformin, AICAR, and oligomycin decrease ATP concentration, which leads to inhibition of glucose phosphorylation by preventing glucokinase translocation to the cytosol.

discrepancy and we speculate that several unrelated factors could control glucokinase expression, depending on the model used. However, the fact that glucokinase protein content was also reduced in livers from starved and refed AMPK-null mice, independently of any significant difference in insulin level and glucokinase mRNA compared with wild-type mice (M. Foretz and B. Viollet, unpublished results), could suggest an accelerated turnover of glucokinase. The elucidation of the underliyng mechanisms would require a thorough analysis of the various factors involved in the control of glucokinase gene expression and glucokinase protein turnover.

Pathophysiological and therapeutical implications. Most tissues respond to anoxia by increasing their glycolysis, which is the sole pathway able to produce ATP under these conditions. Here we report a different situation, namely a negative Pasteur effect, i.e., a decrease in glycolytic flux induced by the lack of ATP. By contrast with skeletal muscle, the liver seldom suffers from hypoxia, and this situation is unlikely to have physiological implications. However, this peculiar response due to the remarkable properties of glucokinase and its regulatory protein could hasten preexisting pathological conditions leading to irreversible cell damage. Indeed, any pathological condition that decreases liver ATP should inhibit glycolysis and hence ATP supply, thereby worsening the situation in a vicious circle. For example, liver hypoxia is a frequent consequence of failure of the right ventricle and depletion of adenine nucleotides was observed in acute pathological conditions such as paracetamol-induced hepatotoxicity (41).

AICAR has been proposed as a potential therapeutic agent in relation with its hypoglycemic effects (42,43) or its putative interest to combat iatrogenic hypoglycemia (44). However, as reported here, AICAR also induced intracellular ATP depletion that could be detrimental for hepatocytes. Furthermore, prolonged administration of AICAR has also been shown to induce apoptosis (45), a side effect that does not advocate the safe use of this compound. One may wonder whether the inhibition of liver glucose phosphorylation by metformin has some bearing on the clinical effects of this antidiabetic drug since the concentration used in this study largely exceeds the therapeutic range.

Finally, it has been recently proposed that specific glucokinase activators could be potent tools to reduce hyperglycemia in type 2 diabetic patients, partly by stimulating liver glucose uptake (46,47). However, the role of glucokinase in the control of glucose homeostasis is not only quantitative but also qualitative due to the glucose-induced transcriptional effects that result from the intrahepatic accumulation of Glc-6-P (48) and should also be taken into account. Indeed, while overexpression of liver glucokinase or use of glucokinase activators decrease glycemia by stimulating liver glucose uptake and glycogen synthesis in animal models of type 2 diabetes (46,47,49), it may also induce long-term deleterious effects due to the transcriptional effects of glucose on lipogenic enzymes gene expression, thus promoting lipogenesis and/or steatosis (50).

In conclusion, by using a new powerful knockout model to investigate the involvement of AMPK in the regulation of liver metabolic pathways, we demonstrated that inhibition of glucose phosphorylation by AICAR, metformin, and oligomycin was due to an AMPK-independent impairment of the glucose-induced translocation of glucokinase following ATP depletion.

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