

# AMP-Activated Protein Kinase Activation by Adrenoceptors in L6 Skeletal Muscle Cells

## Mediation by $\alpha_1$ -Adrenoceptors Causing Glucose Uptake

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**AMP-activated protein kinase (AMPK), which functions as a sensor of cellular energy homeostasis, was phosphorylated after norepinephrine stimulation in L6 skeletal muscle cells. This effect was mediated by  $\alpha_1$ -adrenoceptors, with no stimulatory effects due to interactions at  $\alpha_2$ - or  $\beta$ -adrenoceptors.  $\alpha_1$ -Adrenoceptors are Gq-coupled receptors, and calcium but not phorbol esters could mimic the effect of  $\alpha_1$ -adrenergic stimulation; and we show that protein kinase C is not involved as an upstream signal to AMPK by  $\alpha_1$ -adrenergic stimulation and that the AMP-to-ATP ratio is unaltered after  $\alpha_1$ -adrenergic stimulation. We further show that glucose uptake mediated by  $\alpha_1$ - but not by  $\beta$ -adrenoceptors can be inhibited by AMPK inhibition. Acetyl-CoA carboxylase (ACC) is phosphorylated at Ser218 by AMPK, and  $\alpha_1$ - but not  $\beta$ -adrenoceptor stimulation results in phosphorylation of ACC at this residue. These results suggest a novel pathway where  $\alpha_1$ -adrenoceptor activation, independent of protein kinase C, leads to activation of AMPK in skeletal muscle, which contributes to  $\alpha_1$ -adrenoceptor-mediated increases in glucose uptake. *Diabetes* 55:682–690, 2006**

**A**MP-activated protein kinase (AMPK) has been described as a sensor of cellular and whole-body energy homeostasis and is present at high levels in tissues that regulate energy homeostasis, namely the liver, heart, adipose tissue, pancreas, brain, and skeletal muscle. AMPK is activated by hormonal and nutrient stresses that increase the AMP-to-ATP ratio after depletion of intracellular ATP levels, but some conditions (such as hyperosmotic stress) activate AMPK without ATP reductions (1). Structurally, AMPK is a heterotrimeric protein consisting of an  $\alpha$  catalytic subunit and  $\beta$ - and  $\gamma$ -regulatory subunits, and activation requires phosphorylation at Thr172 on the catalytic subunit by one or more upstream kinases (for more comprehensive review, refer

to 2). Recently two different upstream kinases have been identified, LKB1 (3–5) and CAMKK $\beta$  (6–8). Activation of AMPK activates pathways such as glucose transport, glycolysis, and  $\beta$ -fatty acid oxidation and inhibits pathways such as fatty acid and cholesterol synthesis through interactions with metabolic enzymes and proteins and effects on gene expression.

Adrenoceptors are classified into three main subtypes:  $\alpha_1$ -,  $\alpha_2$ -, and  $\beta$ -adrenoceptors, which couple to Gq (increase inositol 1,4,5-trisphosphate and diacylglycerol levels), Gi (inhibit cyclic AMP formation), and Gs (increase cyclic AMP formation) G-proteins respectively.  $\alpha_1$ - and  $\beta$ -adrenoceptors are found in skeletal muscle (9–15). However, the role of AMPK in adrenergically mediated responses in skeletal muscle has not been investigated to a great extent. With respect to AMPK, the  $\alpha$ -adrenoceptor agonist phenylephrine increases AMPK activity in isolated mouse soleus muscle (16), and other Gq-coupled receptors activate AMPK in transfected CHO-K1 cells (17). In adipose tissue,  $\beta$ -adrenoceptors activate AMPK in white adipocytes (18,19), 3T3-L1 adipocytes (20), and brown adipocytes (21).

Facilitation of glucose uptake in tissues important in glucose homeostasis, such as skeletal muscle, can be accomplished by adrenergic activation. There are many studies showing that epinephrine decreases glucose disposal, primarily by inhibiting insulin secretion via activation of  $\alpha_2$ -adrenoceptors. Epinephrine inhibits insulin-stimulated glucose uptake in skeletal muscle via  $\beta$ -adrenoceptors but in the absence of insulin, can increase glucose uptake in skeletal muscle (22). However, there is much evidence showing that increases of the sympathetic nervous system stimulate glucose uptake in skeletal muscle. One set of key experiments (23,24) shows that electrical stimulation of the ventromedial hypothalamus increases sympathetic activity, resulting in increased glucose uptake in skeletal muscle without alterations in plasma insulin levels. These effects are blocked by guanethidine but not by adrenal medullation, showing that norepinephrine and not epinephrine mediates this response (24). Other studies performed in vivo and in vitro show that  $\alpha$ - and  $\beta$ -adrenoceptor (12,15,25–29) activation increases glucose uptake in skeletal muscle via an insulin-independent pathway. Hence it is likely that circulating epinephrine has vastly different actions on glucose uptake as opposed to focally released norepinephrine at the synaptic clefts, which can reach high concentrations.

The present study aimed at investigating a possible adrenergic control of AMPK in L6 skeletal muscle cells.

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ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribose nucleoside; AMPK, AMP-activated protein kinase; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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We have studied adrenergic phosphorylation of AMPK with the focus on delineating which adrenergic subtypes are involved in the norepinephrine-mediated response and whether AMPK is involved in mediating a biological end point such as glucose uptake response to adrenergic agonists. We show that only  $\alpha_1$ -adrenergic activation phosphorylates and activates AMPK in these cells, and AMPK activation contributes to  $\alpha_1$ -adrenergic-mediated increases in glucose uptake.

## RESEARCH DESIGN AND METHODS

**Cell culture.** Rat L6 skeletal muscle cells were grown as described previously (12). To differentiate, cells were allowed to reach confluence, and the medium was changed to medium containing 2% FBS for 7 days, with medium changes every 2nd day. Experiments were restricted to cells from passages 2–15.

CHO-G4myc- $\alpha_{1A}$ AR (human  $\alpha_{1A}$ -adrenoceptor) and CHO-G4myc cells were grown in Dulbecco's modified Eagle's medium-Ham's F12 (1:1) containing 10% (vol/vol) heat-inactivated FBS, L-glutamine (2 mmol/l), gentamicin sulfate (80  $\mu$ g/ml), and fungizone (2.5  $\mu$ g/ml) under 8% CO<sub>2</sub> at 37°C. CHO-G4myc cells were selected with G418 (400  $\mu$ g/ml), whereas CHO-G4myc- $\alpha_{1A}$ AR cells were selected with both G418 and blasticidin S (5  $\mu$ g/ml). CHO-K1 and CHO- $\beta_2$ AR (human  $\beta_2$ -adrenoceptor) cells were grown in Dulbecco's modified Eagle's medium-Ham's F12 (1:1) containing 10% (vol/vol) heat-inactivated FBS, L-glutamine (2 mmol/l), penicillin (100 units/ml), and streptomycin (100  $\mu$ g/ml) under 8% CO<sub>2</sub> at 37°C. CHO- $\beta_2$ AR medium contained hygromycin B (30  $\mu$ g/ml).

**Western blotting.** Cells were serum-starved overnight before each experiment on day 7 and exposed to drugs for times and concentrations indicated with the data. Cells were lysed directly by the addition of 65°C lysis buffer (62.5 mmol/l Tris, pH 6.8, 2% [vol/vol] SDS, 10% [vol/vol] glycerol, 50 mmol/l dithiothreitol, and 1% [vol/vol] bromophenol blue). The lysate was sonicated briefly followed by boiling for 3 min. Aliquots of samples (of same protein amount) were separated on 8 or 12% polyacrylamide gels and electrotransferred to Hybond-P polyvinylidene fluoride membranes (pore size 0.45  $\mu$ m; Amersham Pharmacia Biotech). Primary antibodies used were AMPK antibody (which detects the  $\alpha_1$ - and  $\alpha_2$ -isoforms of the catalytic subunit), phospho-AMPK antibody (Thr172), acetyl-CoA carboxylase (ACC) antibody (which detects endogenous levels of all isoforms of ACC), or phospho-ACC (Ser79) antibody (the isoforms of ACC that is expressed in skeletal muscle and L6 cells is ACC2 and hence the phosphorylation site in the rat sequence is at Ser218 and not Ser79, which is the equivalent site in ACC1). Primary antibodies were diluted 1:1,000, which were detected using a secondary antibody (horseradish peroxidase-linked anti-rabbit IgG) diluted 1:2,000 and enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). The blots were exposed to Hyperfilm ECL films (Amersham Pharmacia Biotech) and quantified on a Molecular Dynamics densitometer using ImageQuant NT software. Results are expressed as the ratio between the phosphorylated and total protein, with the ratio normalized in each experiment to that of control samples. All experiments were performed singly or in duplicate with *n* referring to the number of independent experiments performed.

In all experiments performed, 5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribo-nucleoside (AICAR) was used as a positive control. AICAR is widely used to investigate AMPK actions *in vivo* and *in vitro*. It is phosphorylated intracellularly to ZMP, which activates AMPK by mimicking AMP and also by promoting phosphorylation and activation of the upstream kinase, AMPK kinase (30–32).

**2-deoxy-[<sup>3</sup>H]-D-glucose uptake assay.** Glucose uptake in L6 cells was measured using the 2-deoxy-[<sup>3</sup>H]-D-glucose method (28) with modifications (12). To measure glucose uptake in CHO-G4myc- $\alpha_{1A}$ AR or CHO- $\beta_2$ AR cells, cells were seeded at  $5 \times 10^5$  cells per well and left to adhere overnight. Medium was replaced in the morning (to serum-free medium) for 4 h. For CHO-G4myc- $\alpha_{1A}$ AR cells, cells were washed in warm PBS, medium was replaced with glucose-free medium, and drugs were added for 50 min, after which 2-deoxy-[<sup>3</sup>H]-D-glucose (50 nmol/l) was added for 10 min. For CHO- $\beta_2$ AR cells, medium was replaced (to serum-free medium), and drugs were added for 2 h. After this, cells were washed in warm PBS, glucose-free medium was added, and drugs were re-added for 45 min, after which 2-deoxy-[<sup>3</sup>H]-D-glucose (50 nmol/l) was added for 15 min. Reactions were terminated by washing twice in ice-cold PBS, cells were digested (0.2 mol/l NaOH, 1 h, 60°C), and samples were transferred to scintillation vials with scintillant. When inhibitors were used, the time indicated with the results represents the time cells were pre-equilibrated with the inhibitors before agonists were added. All experiments were performed in duplicate with *n* referring to the number of independent experiments performed.

**AMPK activity.** L6 cells (day 7) were serum-starved overnight, medium was replaced, and cells were treated with drugs for 2 h. Cells were washed twice with ice-cold PBS and lysed in buffer A (20 mmol/l Tris-HCl [pH 7.5], 150 mmol/l NaCl, 1 mmol/l Na<sub>2</sub>EDTA, 1 mmol/l EGTA, 1% Triton, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l  $\beta$ -glycerophosphate, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, 1 mmol/l dithiothreitol, and 1 mmol/l phenylmethylsulfonyl fluoride) for 5 min on ice. After centrifugation (14,000g, 10 min, 4°C), the supernatant was assayed for protein content, and 200  $\mu$ g protein (in total volume of 200  $\mu$ l) was incubated overnight at 4°C with AMPK  $\alpha$ -subunit antibody at 1:80 dilution factor, followed by addition of 20  $\mu$ l 50% slurry of protein-A agarose beads (Upstate Biotechnology, Lake Placid, NY) for 2 h at 4°C. Immunoprecipitates were collected by centrifugation (18,000g, 1 min, 4°C); washed twice with 500  $\mu$ l buffer A and twice with 500  $\mu$ l buffer B (240 mmol/l HEPES, pH 7.4, and 480 mmol/l NaCl); and resuspended in 30  $\mu$ l reaction buffer (40 mmol/l HEPES, pH 7.0, 80 mmol/l NaCl, 0.8 mmol/l dithiothreitol, and 5 mmol/l MgCl<sub>2</sub>), which contained 100  $\mu$ mol/l SAMS peptide (HMRSAMSGHLVKKRR). The reaction was started by the addition of 10  $\mu$ l ATP buffer (75 mmol/l MgCl<sub>2</sub>, 500  $\mu$ mol/l free ATP, and 1  $\mu$ Ci/ml [ $\gamma$ -<sup>32</sup>P]ATP [3,000 Ci/mmol]). After 20 min at 30°C, the reaction was stopped by spotting 35- $\mu$ l samples on P81 Whatman filter papers, which were washed twice in 0.75% (vol/vol) orthophosphoric acid for 5 min and once in acetone for 5 min before drying and scintillation counting. AMPK activity is expressed as the amount of incorporated ATP (picomoles) per immunoprecipitated protein (relative to the amount of protein used for the immunoprecipitation) per minute. The final data are expressed as a percentage of the control values ( $3.2 \pm 1.9$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>).

**AMP-to-ATP ratio and ATP level measurement.** L6 cells (day 7) were serum-starved overnight, new medium was added for 2 h, and cells were treated with drugs for 30 min. Cell extracts were isolated and the AMP-to-ATP ratio measured as previously described (21), except that ATP levels were measured in duplicate using a commercial kit (ATP determination kit time stable assay; Biaffin, Kassel, Germany). Results are expressed as the ratio of AMP to ATP and also as nanomoles ATP per milligram protein.

**Data analysis.** All results are expressed as means  $\pm$  SE of *n*. Data were analyzed using nonlinear curve fitting (GraphPad PRISM version 3.03) to obtain pEC<sub>50</sub> values, where appropriate. Statistical significance was determined using paired Student's *t* test. *P* values  $\leq 0.05$  were considered significant.

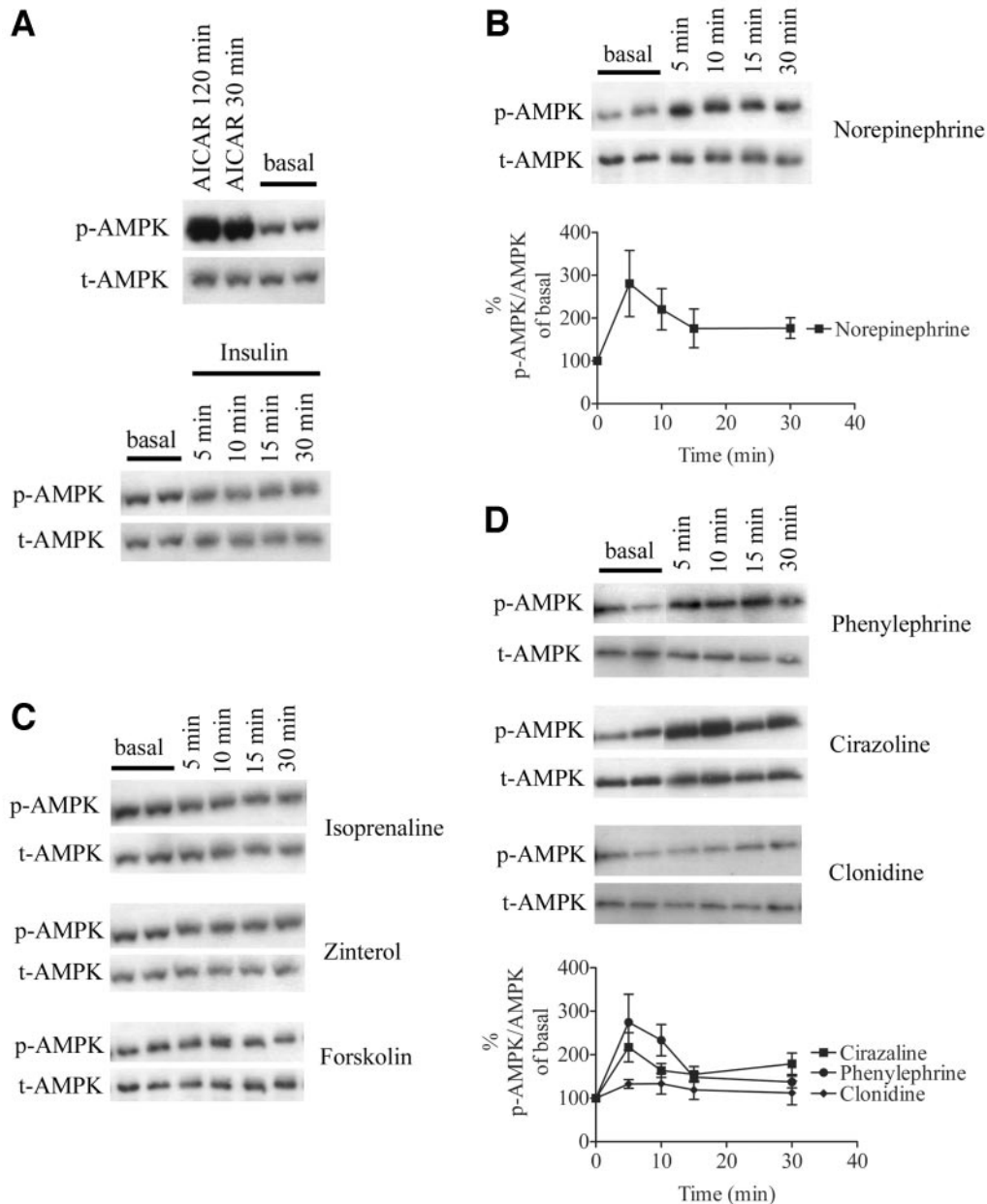
**Drugs and reagents.** The following were gifts: the AMPK inhibitor compound C was a gift obtained from Merck Research Laboratories (Rahway, NJ). Zinterol hydrochloride was obtained from Bristol-Myers Squibb (Noble Park, Victoria, Australia). CHO-G4myc- $\alpha_{1A}$ AR, CHO-G4myc, CHO-K1, and CHO- $\beta_2$ AR cells were provided by Prof. Roger J Summers (Monash University, Melbourne, Victoria, Australia).

Drugs and reagents were purchased as follows: rosiglitazone (Alexis Biochemicals, Lausen, Switzerland); 2-deoxy-[<sup>3</sup>H]-D-glucose (12 Ci/mmol<sup>-1</sup>; Amersham Biosciences, Buckinghamshire, U.K.); G66976 and G66783 (Cal-Biochem, La Jolla, CA); 2,4-dinitrophenol (Merck Schuchardt OHG, Hohenbrunn, Germany); insulin (Actrapid) (Novo Nordisk, Bagsvaerd, Denmark); [ $\gamma$ -<sup>32</sup>P]ATP (3,000 Ci/mmol) (PerkinElmer Sverige, Upplands Väsby, Sweden); A23187, cirazoline, (-)-isoprenaline, LY294002, (-)-norepinephrine, phenylephrine, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical, St. Louis, MO); AICAR (Toronto Research Chemicals, North York, Ontario, Canada); and SAMS peptide (Upstate Biotechnology).

All cell culture media and supplements were obtained from Gibco-BRL Life Technologies (Gaithersburg, MD). All antibodies were obtained from Cell Signaling Technology (Beverly, MA). All other drugs and reagents were of analytical grade.

## RESULTS

**$\alpha_1$ -Adrenoceptor but not  $\alpha_2$ - or  $\beta$ -adrenoceptor activation increases AMPK phosphorylation in L6 cells and recombinant CHO-K1 cells.** The AMPK activator AICAR phosphorylated AMPK in L6 cells ~sixfold over basal levels (*n* = 55–58), whereas insulin (*n* = 8) had no effect (Fig. 1A). The endogenous adrenergic ligand norepinephrine phosphorylated AMPK, with an approximate two- to threefold increase in the *p*-AMPK-to-AMPK ratio (Fig. 1B). This effect was still present after 2 h of norepinephrine stimulation (~twofold increase in *p*-AMPK-to-AMPK ratio; data not shown). To determine which adrenergic receptor subtype(s) mediated the norepinephrine effect, L6 cells were stimulated with different subtype-specific adrenergic agonists. The  $\beta$ -adrenoceptor agonist

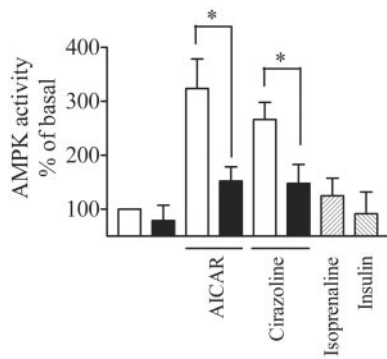


**FIG. 1.** Representative blots of phosphorylated (Thr172) and total AMPK content in differentiated L6 cells after AICAR (2 mmol/l) and insulin (1  $\mu$ mol/l) (A); norepinephrine (1  $\mu$ mol/l,  $n = 7$ ) (B); isoprenaline (10  $\mu$ mol/l,  $n = 7$ ), zinterol (10  $\mu$ mol/l,  $n = 8$ ), and forskolin (1 mmol/l,  $n = 7$ ) (C), and phenylephrine (10  $\mu$ mol/l,  $n = 5$ ), clonidine (10  $\mu$ mol/l,  $n = 5$ ), and cirazoline (10  $\mu$ mol/l,  $n = 11$ ; and 10 mmol/l,  $n = 5$ ) (D) treatment. Graphs show the ratio of phosphorylated AMPK to total AMPK. Data represent means  $\pm$  SE obtained from  $n$  single experiments. Data are presented as a percentage of the response to control cells at time 0 min.

isoprenaline (even after 2 h of incubation; data not shown) and the  $\beta_2$ -adrenoceptor agonist zinterol were without effect (Fig. 1C). The adenylate cyclase activator forskolin was also without effect (Fig. 1C). Phenylephrine, an  $\alpha_1/\alpha_2$ -adrenoceptor agonist, phosphorylated AMPK to a similar extent as norepinephrine (Fig. 1D). This effect was mediated by  $\alpha_1$ -adrenoceptor activation because the  $\alpha_1$ -adrenoceptor agonist cirazoline phosphorylated AMPK (Fig. 1D, which was sustained for up to 2 h; data not shown), whereas the  $\alpha_2$ -adrenoceptor agonist clonidine was without effect (Fig. 1D). These phosphorylation studies correlated well with AMPK activity measurements. AICAR and cirazoline increased AMPK activity, which was inhibited by the AMPK inhibitor compound C, whereas isoprenaline and insulin, which did not phosphorylate

AMPK, did not increase AMPK activity (Fig. 2). The increases in AMPK activity after  $\alpha_1$ -adrenergic stimulation are not due to alterations in the AMP-to-ATP ratio or to decreased ATP content because cirazoline failed to significantly affect the AMP-to-ATP ratio or ATP levels compared with the positive controls rosiglitazone and dinitrophenol, which increase the AMP-to-ATP ratio and significantly reduced ATP levels (Tables 1 and 2).

To determine whether the effect of  $\alpha_1$ - and  $\beta_2$ -adrenoceptors on AMPK phosphorylation was confined to muscle cells, we have used CHO-K1 cells transfected with either the human  $\alpha_{1a}$ -adrenoceptor (Fig. 3A) or human  $\beta_2$ -adrenoceptor (Fig. 3B), which are the predominant subtypes expressed in L6 cells (12,15). The human  $\alpha_{1a}$  rather than the  $\alpha_{1b}$ -adrenoceptor (as used by Kishi et al. [17]) was



**FIG. 2.** AMPK activity measurements in L6 cells after AICAR (2 mmol/l, 2 h), cirazoline (10  $\mu$ mol/l, 2 h), isoprenaline (10  $\mu$ mol/l, 2 h), and insulin (1  $\mu$ mol/l, 2 h) treatment in the absence/presence of compound C (1  $\mu$ mol/l, 30 min prestimulation). □, control; ■, plus compound C. Histograms represent means  $\pm$  SE of four experiments. Asterisks represent statistical difference as analyzed by paired Student's *t* test (\**P* < 0.05).

used because the  $\alpha_{1b}$ -adrenoceptor is exclusively expressed in liver, whereas the  $\alpha_{1a}$ -adrenoceptor is detected in skeletal muscle (10,12). In both cell systems AICAR but not insulin phosphorylated AMPK (~threefold increase after 30 min or 2 h of stimulation,  $n = 4-8$ ) as observed in the L6 cells above. In CHO $\alpha_1$  cells, norepinephrine and cirazoline phosphorylated AMPK ~fourfold (Fig. 3A). In CHO $\beta_2$  cells, norepinephrine, isoprenaline, and forskolin had no effect on basal AMPK phosphorylation levels (Fig. 3B). In CHO-K1 and CHO-G4myc cells, isoprenaline and cirazoline did not phosphorylate AMPK (data not shown). **AMPK is involved in glucose uptake mediated by  $\alpha_1$ - but not  $\beta$ -adrenoceptor activation.** We examined whether cirazoline can increase glucose uptake in L6 cells via AMPK by using an AMPK inhibitor, compound C. AICAR-stimulated but not insulin-stimulated glucose uptake was inhibited by compound C (Fig. 4B), and compound C inhibited the phosphorylation of AMPK by AICAR (Fig. 4A). Norepinephrine-mediated glucose uptake is via both  $\alpha_1$ - and  $\beta_2$ -adrenoceptors in L6 cells (12). Compound C partially inhibited glucose uptake by norepinephrine, but this effect was not statistically different (Fig. 4C; *P* = 0.16, paired Student's *t* test), which could be representative of norepinephrine using both  $\beta$ - and  $\alpha$ -adrenoceptors to increase glucose uptake. Isoprenaline-mediated glucose uptake was not inhibited by compound C (Fig. 4D), but compound C significantly inhibited cirazoline-mediated glucose uptake (Fig. 4D) and cirazoline-mediated AMPK phosphorylation (Fig. 4A).

In CHO $\alpha_1$  cells, cirazoline increased glucose uptake in a concentration-dependent manner ( $pEC_{50}$  8.4  $\pm$  0.2; max-

**TABLE 1**  
AMP-to-ATP ratios in differentiated L6 cells after various treatments

Treatment	AMP-to-ATP ratio
Control	0.23 $\pm$ 0.05
Cirazoline (10 $\mu$ mol/l)	0.29 $\pm$ 0.04
Insulin (1 $\mu$ mol/l)	0.21 $\pm$ 0.04
Dinitrophenol (0.5 mmol/l)	0.58 $\pm$ 0.09*
Rosiglitazone (10 $\mu$ mol/l)	0.53 $\pm$ 0.09†

Data are means  $\pm$  SE from six experiments performed in duplicate. Statistical differences were determined using paired *t* test where \**P* < 0.01 and †*P* < 0.05.

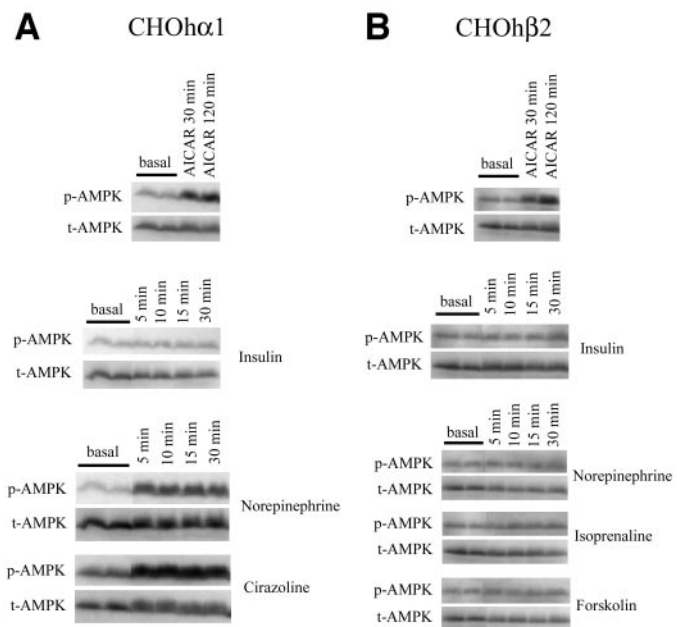
**TABLE 2**  
ATP amount in differentiated L6 cells after various treatments

Treatment	ATP amount (nmol/mg protein)
Control	9.19 $\pm$ 1.30
Cirazoline (10 $\mu$ mol/l)	7.28 $\pm$ 2.12
Insulin (1 $\mu$ mol/l)	6.71 $\pm$ 2.12
Dinitrophenol (0.5 mmol/l)	3.79 $\pm$ 1.22*
Rosiglitazone (10 $\mu$ mol/l)	2.95 $\pm$ 0.87†

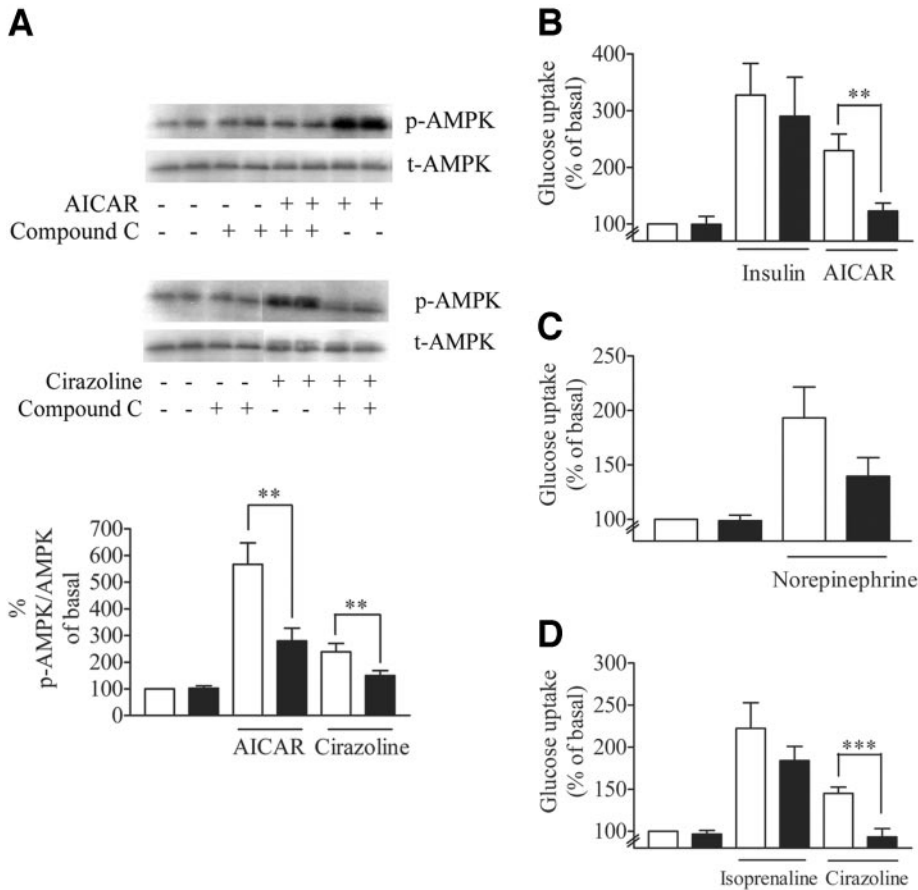
Data are means  $\pm$  SE from five experiments performed in duplicate. Statistical differences were determined using paired *t* test where \**P* < 0.05 and †*P* < 0.01.

imum increase 260  $\pm$  11% over basal; hill slope 0.86;  $n = 4$ ; data not shown). AICAR, insulin, and cirazoline increased glucose uptake, but compound C only inhibited AICAR and cirazoline-mediated increases in glucose uptake (Fig. 5A). In CHO $\beta_2$  cells, isoprenaline increased glucose uptake in a concentration-dependent manner ( $pEC_{50}$  7.7  $\pm$  0.4; maximum increase 131  $\pm$  4% over basal; hill slope 0.90;  $n = 4$ ; data not shown). AICAR, insulin, and isoprenaline increased glucose uptake, but only AICAR-stimulated glucose uptake was inhibited by compound C (Fig. 5B).

**Calcium, but not protein kinase C (PKC), is involved in AMPK phosphorylation.**  $\alpha_1$ -Adrenoceptors are Gq-coupled receptors, and their activation results in increased phosphatidylinositol turnover, activation of phospholipase C, and increased intracellular calcium levels. To investigate whether the effect of  $\alpha_1$ -adrenoceptor stimulation could be mimicked by PKC activation and increases in calcium levels, L6 cells were treated with either TPA (activator of conventional and novel PKCs; 1  $\mu$ mol/l) or A23187 (calcium ionophore; 1  $\mu$ mol/l). A23187 but not TPA was able to significantly phosphorylate AMPK (~twofold increase over basal) in the time period examined (Fig. 6).



**FIG. 3.** **A:** In CHO $\alpha_1$  cells, AICAR (2 mmol/l) but not insulin (1  $\mu$ mol/l) phosphorylates AMPK ( $n = 4-8$ ). AMPK is phosphorylated by norepinephrine (1  $\mu$ mol/l) and cirazoline (1  $\mu$ mol/l). Blots are representative of four experiments performed. **B:** In CHO $\beta_2$  cells, AICAR (2 mmol/l) but not insulin (1  $\mu$ mol/l), norepinephrine (1  $\mu$ mol/l), isoprenaline (10  $\mu$ mol/l), or forskolin (1 mmol/l) phosphorylates AMPK. Blots are representative of three experiments performed.



**FIG. 4. A:** Representative blots of the effect of compound C (1  $\mu\text{mol/l}$ , 5 min preincubation) on AICAR (2 mmol/l, 2 h;  $n = 5$ ) and cirazoline (10  $\mu\text{mol/l}$ , 2 h;  $n = 4$ ) phosphorylation of AMPK. Effect of the AMPK inhibitor compound C (1  $\mu\text{mol/l}$ , 5 min preincubation) on insulin- (1  $\mu\text{mol/l}$ ,  $n = 4$ ) and AICAR- (2 mmol/l,  $n = 7$ ) (B), norepinephrine- (10  $\mu\text{mol/l}$ ,  $n = 4$ ) (C), and isoprenaline- (10  $\mu\text{mol/l}$ ,  $n = 5$ ) and cirazoline- (10  $\mu\text{mol/l}$ ,  $n = 6$ ) (D) mediated increases in glucose uptake. □, control; ■, plus compound C. Histograms represent means  $\pm$  SE of  $n$  experiments performed in duplicate. Asterisks represent statistical difference as analyzed by paired Student's  $t$  test (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

Both treatments, however, were able to increase glucose uptake (A23187 1  $\mu\text{mol/l}$ ,  $207 \pm 13\%$  over basal,  $n = 3$ ; TPA 1  $\mu\text{mol/l}$ ,  $211 \pm 23\%$  over basal,  $n = 7$ ). TPA also had no effect on basal AMPK phosphorylation levels in CHO $\alpha_1$  cells (data not shown).

Glucose uptake in L6 cells in response to insulin, AICAR, or cirazoline is inhibited by Gö6983 (inhibits novel, conventional, and atypical PKC isoforms) but not by Gö6976 (inhibits novel and conventional PKC isoforms), showing an involvement of atypical PKCs (Fig. 7A). To determine whether PKCs are upstream of the signal to AMPK, two different approaches were used. First, downregulation of conventional and novel PKCs can be achieved with long-term stimulation of cells with TPA (33). After downregulation of these PKC isoforms, cirazoline (as well as AICAR) was still able to phosphorylate AMPK to the same level as cells not prestimulated with TPA (Fig. 7B), indicating that conventional and novel PKCs are not involved in the cirazoline and AICAR response. Second, AMPK phosphorylation by AICAR or cirazoline was not inhibited by either Gö6976 or Gö6983 (Fig. 7C), suggesting that no isoforms of PKC are involved in an upstream mechanism of AMPK.

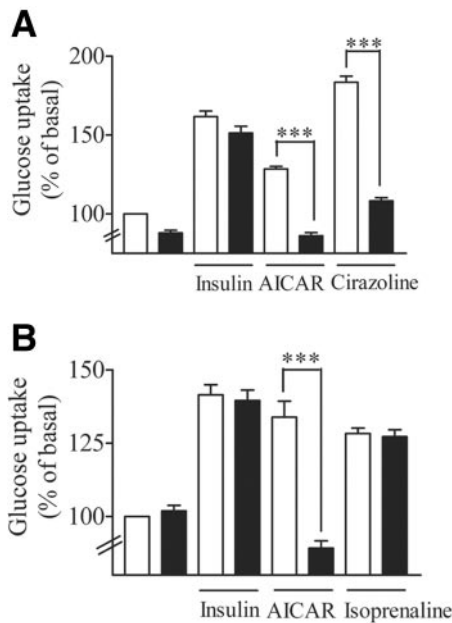
**$\alpha_1$ -Adrenoceptor activation phosphorylates ACC at Ser218 in L6 cells.** One downstream target of AMPK is phosphorylation of ACC2 at Ser218. In L6 cells, AICAR and cirazoline but not isoprenaline or insulin (data not shown) phosphorylated ACC2 at Ser218, and this was inhibited largely by compound C (Fig. 8).

**DISCUSSION**

Insulin-stimulated glucose uptake is severely compromised in type 2 diabetes, and recently, there has been great

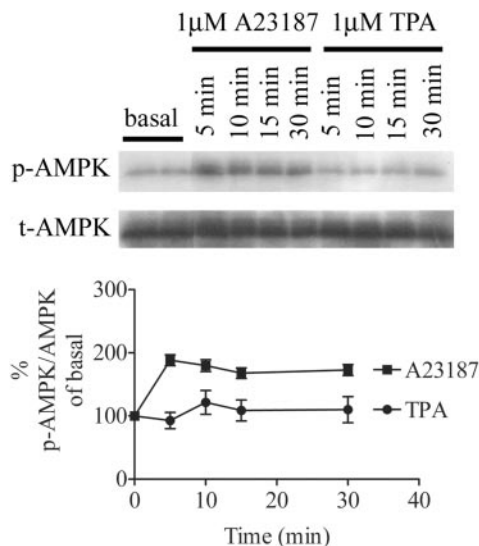
focus on the potential of AMPK in the treatment of type 2 diabetes. Insulin and AICAR both increase glucose uptake in skeletal muscle but use two distinct signaling pathways to mediate this effect that probably converge at some point. Phosphatidylinositol 3-kinase is necessary for insulin-stimulated but not AICAR-stimulated glucose uptake in skeletal muscle, whereas AMPK is necessary for AICAR-stimulated but not insulin-stimulated glucose uptake (34–36 and D.S.H., T.B., unpublished data). The effects of AICAR, at least in skeletal muscle, are due to interactions directly resulting from AMPK activation because glucose uptake by AICAR is abolished in skeletal muscle from AMPK inactive mutant mice (37), and overexpression of a dominant-negative AMPK in rat skeletal muscle abolishes AICAR-mediated increases in glucose uptake (38,39). Of interest are the recent discoveries that hormonal and nutritional stresses have the ability to elicit their effects via AMPK, including leptin (16) and adiponectin (36,40,41). Leptin activates AMPK in skeletal muscle via two pathways: directly on skeletal muscle and also by actions in the hypothalamus to increase  $\alpha$ -adrenergic sympathetic activity to activate AMPK in skeletal muscle (16). To this extent, we have investigated the role of the endogenous ligand norepinephrine on AMPK in L6 skeletal muscle cells.

Norepinephrine stimulated AMPK phosphorylation in L6 skeletal muscle cells via  $\alpha_1$ -adrenoceptors, but not  $\beta$ - or  $\alpha_2$ -adrenoceptors.  $\alpha_1$ -Adrenoceptor activation increases AMPK activity in mouse soleus muscle (16) and in CHO cells transfected with the  $\alpha_{1a}$ -adrenoceptor (this study) and  $\alpha_{1b}$ -adrenoceptor (17). This later study showed that Gq- ( $\alpha_{1b}$ -adrenoceptor and bradykinin 2 receptor) and not Gi- ( $\alpha_{2a}$ -adrenoceptor) or Gs- ( $\beta_2$ -adrenoceptor) coupled

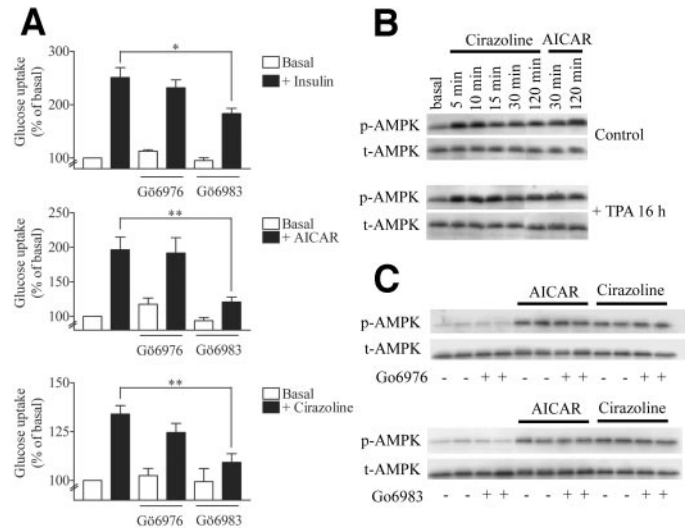


**FIG. 5. A:** Effect of compound C (1  $\mu\text{mol/l}$ ) in CHO $\alpha_1$  cells on insulin- (1  $\mu\text{mol/l}$ ), AICAR- (2  $\text{mmol/l}$ ), or cirazoline- (1  $\mu\text{mol/l}$ ) mediated increases in glucose uptake. **B:** Effect of compound C (1  $\mu\text{mol/l}$ ) in CHO $\beta_2$  cells on insulin- (1  $\mu\text{mol/l}$ ), AICAR- (2  $\text{mmol/l}$ ), or isoprenaline- (10  $\mu\text{mol/l}$ ) mediated increases in glucose uptake.  $\square$ , basal;  $\blacksquare$ , plus compound C. Histograms represent means  $\pm$  SE of four to seven experiments performed in duplicate. Asterisks represent statistical difference as analyzed by paired Student's *t* test (\*\**P* < 0.001).

receptors cause activation of AMPK. Forskolin, 8-bromo-cAMP, and insulin were also without effect (17), and we also observed similar results in CHO cells. Other Gq-coupled receptors, such as the angiotensin type 2 receptor in rat vascular smooth muscle cells (42) and the histamine (H1) and thrombin (PAR) receptors in human umbilical vein endothelial cells (43), also activate AMPK. The data presented in this study are consistent with the notion that Gq-coupled receptors have the ability to phosphorylate AMPK. However, this is not a general phenomenon that only occurs after stimulation of Gq-coupled receptors



**FIG. 6. Representative blots of the effect of TPA (1  $\mu\text{mol/l}$ , *n* = 4) and A23187 (1  $\mu\text{mol/l}$ , *n* = 4) on AMPK phosphorylation in L6 muscle cells. The histograms are means  $\pm$  SE of four experiments performed in duplicate.**

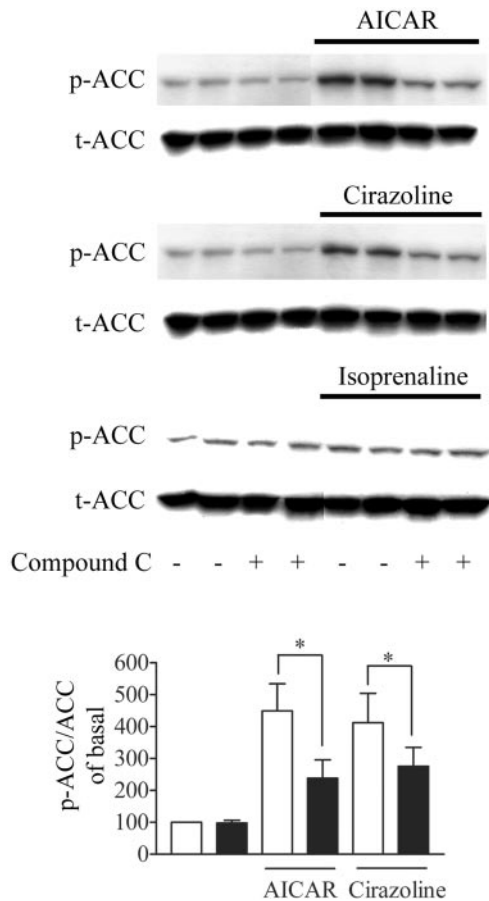


**FIG. 7. A:** Effects of PKC inhibition (cells were pretreated with the PKC inhibitor Gö6976 or Gö6983 [1  $\mu\text{mol/l}$ , 5 min] before agonist stimulation) on insulin- (1  $\mu\text{mol/l}$ , *n* = 4), AICAR- (2  $\text{mmol/l}$ , *n* = 4) or cirazoline- (10  $\mu\text{mol/l}$ , *n* = 4) induced glucose uptake. **B:** Representative blot showing the effect of TPA treatment (100  $\text{nmol/l}$ , 16 h) on the ability of cirazoline (10  $\mu\text{mol/l}$ ) and AICAR (2  $\text{mmol/l}$ ) to phosphorylate AMPK (*n* = 4). **C:** Representative blots showing the effect of PKC inhibition by Gö6976 or Gö6983 (1  $\mu\text{mol/l}$ , 5 min) on the ability of AICAR (2  $\text{mmol/l}$ , 2 h, *n* = 3) or cirazoline (10  $\mu\text{mol/l}$ , 2 h, *n* = 3) to phosphorylate AMPK. The histograms are means  $\pm$  SE of four experiments performed in duplicate. Asterisks represent values that are significantly different (\**P* < 0.05, \*\**P* < 0.01) from samples of agonists itself.

because in adipose tissue, Gs-coupled receptors ( $\beta$ -adrenoceptors) are capable of phosphorylating and activating AMPK (18–21). Additionally in brown adipose tissue where  $\alpha_1$ -adrenoceptors increase glucose uptake in  $\beta_3$ -adrenoceptor knockout mice (44),  $\alpha_1$ -adrenoceptor activation did not phosphorylate AMPK (21). These results in adipose tissue are opposite to what we observe in L6 skeletal muscle cells and CHO cells, which may suggest that in adipose tissue, regulation of AMPK by G-protein-coupled receptors differs from other cell types.

AMPK can be activated by two different upstream kinases, LKB1 (3–5) and CAMKK $\beta$  (6–8), via different activators, such as decreased ATP and increased AMP-to-ATP ratios for LKB1 and alterations in calcium metabolism for CAMKK. Because  $\alpha_1$ -adrenoceptor activation leads to phospholipase C activation, which results in the hydrolysis of phosphatidylinositol (4,5)-bisphosphate to produce inositol-1,4,5-phosphate that releases calcium from intracellular stores, and diacylglycerol, which can activate PKC, it is plausible to hypothesize that  $\alpha_1$ -adrenoceptors may activate AMPK via calcium. The calcium ionophore A23187 activates AMPK in LKB1 $^{-/-}$  embryo fibroblasts, NIH3T3, and Hela cells (6–8), and treatment of L6 cells with A23187 phosphorylated AMPK, making it likely that  $\alpha_1$ -adrenoceptor activation of AMPK is via CAMKK because  $\alpha_1$ -adrenoceptor activation did not decrease ATP levels or alter the AMP-to-ATP ratio in L6 cells. Further investigation of  $\alpha_1$ -adrenergic activation of AMPK is currently being investigated.

Activation of AMPK is also not secondary to changes in PKC activity despite PKC being implicated in  $\alpha_1$ -adrenoceptor mediation of glucose uptake (12,29). The phorbol ester TPA (which activates conventional and novel PKC isoforms) did not phosphorylate AMPK, and downregulation of conventional and novel PKCs with long-term TPA



**FIG. 8.** Representative blots showing the effect of AMPK inhibition on the ability of AICAR (2 mmol/l, 2 h), cirazoline (10  $\mu$ mol/l, 2 h), or isoprenaline (10  $\mu$ mol/l, 2 h) to phosphorylate ACC. Cells were pre-treated with the AMPK inhibitor compound C (1  $\mu$ mol/l, 5 min) before agonist stimulation. □, control; ■, plus compound C. Histograms represent means  $\pm$  SE of four to six experiments performed in duplicate. Asterisks represent statistical difference as analyzed by paired Student's *t* test (\**P* < 0.05).

treatment (33) or the use of PKC inhibitors did not affect the ability of cirazoline (or AICAR) to phosphorylate AMPK, suggesting that PKCs are not involved in phosphorylation of AMPK (PKC inhibition was also ineffective in inhibiting AICAR activation of AMPK in rat papillary muscle [45]). However,  $\alpha_1$ -adrenoceptor- and AICAR-mediated glucose uptake is very dependent on atypical PKCs, which are presumably downstream of AMPK on glucose uptake effects. These results agree with other studies investigating the interaction between AMPK and PKC. In L6 cells, AICAR treatment activated atypical PKCs, but this effect was downstream of its activation of AMPK, hence raising the possibility that atypical PKCs may be a final activator of glucose uptake for varying agonists, independent of their initial signaling pathways (46). Additionally, dinitrophenol activation of glucose uptake but not AMPK activation was sensitive to calcium chelation and PKC inhibition, suggesting that AMPK activation may be independent of calcium and PKC (47)

Focally released norepinephrine (which can reach very high levels locally in skeletal muscle) and circulating epinephrine (low levels in the blood stream and acts at many different target tissues) may have completely different effects on skeletal muscle glucose uptake and AMPK activation in vivo. Circulating epinephrine inhibits glucose

uptake in skeletal muscle by insulin-dependent mechanisms, whereas norepinephrine released from sympathetic nerves (including under conditions of stress such as cold exposure and exercise) increases glucose uptake via insulin-independent mechanisms (rev. in 48). The regulation of AMPK in skeletal muscle is also G-protein dependent, with Gq-coupled receptors activating AMPK (this study; 16). To investigate the role of AMPK of adrenergically mediated glucose uptake, we have used the AMPK inhibitor compound C. This is a potent and small molecule inhibitor of AMPK that acts as a reversible inhibitor of AMPK by binding to the ATP-binding site on AMPK (49). Compound C inhibited AICAR-mediated but not insulin-mediated glucose in all cell types investigated here, and inhibited AICAR-mediated phosphorylation of AMPK, showing that its effects are mediated via AMPK inhibition.  $\alpha_1$ - but not  $\beta_2$ -adrenoceptor-mediated glucose uptake was inhibited by compound C, which suggests that glucose uptake mediated by  $\alpha_1$ -adrenoceptors is attributed to AMPK activation. Additionally, TPA-mediated glucose uptake was not inhibited by compound C in L6 cells (data not shown), which correlates with the inability of TPA to phosphorylate AMPK.

One function of AMPK activation is to provide energy to cells by stimulating the use of alternate fuels. AMPK phosphorylates and inactivates ACC2 at Ser218, which is a rate-limiting step in the conversion of acetyl-CoA to malonyl-CoA, resulting in increased free fatty acid oxidation. PKA can also phosphorylate ACC2 but at distinct residues from those phosphorylated by AMPK (rev. in 50).  $\alpha_1$ -Adrenoceptor and AICAR (but not isoprenaline) stimulation resulted in the phosphorylation of ACC2 at Ser218.

In summary,  $\alpha_1$ -adrenoceptor activation, but not  $\alpha_2$ - or  $\beta$ -adrenoceptor activation, increases AMPK phosphorylation and activity in L6 skeletal muscle cells through a pathway independent of PKC. Although both  $\alpha_1$ - and  $\beta_2$ -adrenoceptors are able to increase glucose uptake in these cells, AMPK is only involved in  $\alpha_1$ -adrenoceptor-mediated glucose uptake.

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