A-769662 activates AMPK β₁-containing complexes but induces glucose uptake through a PI3-kinase-dependent pathway in mouse skeletal muscle

Jonas T. Treebak,1,8 Jesper B. Birk,1,8 Bo F. Hansen,2 Grith S. Olsen,2 and Jørgen F. P. Wojtaszewski1
1Molecular Physiology Group, Copenhagen Muscle Research Centre, Department of Exercise and Sport Sciences, University of Copenhagen, Copenhagen, Denmark; and 2Novo Nordisk, Måleø, Denmark

Submitted 27 January 2009; accepted in final form 4 August 2009

Treebak JT, Birk JB, Hansen BF, Olsen GS, Wojtaszewski JF. A-769662 activates AMPK β₁-containing complexes but induces glucose uptake through a PI3-kinase-dependent pathway in mouse skeletal muscle. Am J Physiol Cell Physiol 297: C1041–C1052, 2009.—5'-AMP-activated protein kinase trimer composition. Extensor digitorum longus (EDL) muscles from 129S6/sv and C57BL/6 mice. Glucose uptake increased only in soleus from 129S6/sv when concentrations of A-769662 were 500 μM (15%, P < 0.05) and 1 mM (60%, P < 0.01). AMPK β₁ but not β₂-containing complexes were dose dependently activated by A-769662 in muscles from both genotypes (100% at 200 μM and 300–600% at 1 mM). The discrepancy between the A-769662-induced AMPK activation pattern and stimulation of glucose uptake suggested that these effects were unrelated. A-769662 increased phosphorylation of Akt in both muscles from both genotypes, with phosphorylation of T308 being significantly higher in soleus than in EDL in 129S6/sv mice (P < 0.01). In soleus from 129S6/sv mice, insulin receptor substrate 1-associated phosphatidylinositol 3 (PI3)-kinase activity was markedly increased with A-769662, and Akt phosphorylation and glucose uptake were inhibited by wortmannin while phosphorylation of acetyl-CoA carboxylase (S227) was unaffected. Thus, A-769662 activates β₁-containing AMPK complexes in skeletal muscle but induces glucose uptake through a PI3-kinase-dependent pathway. Although development of A-769662 has constituted a step forward in the search for AMPK activators targeting specific AMPK trimers, our data suggest that in intact muscle, A-769662 has off-target effects. This may limit use of A-769662 to specific AMPK trimers, our data suggest that in intact muscle, A-769662 would increase glucose uptake in skeletal muscle. However, no biological effects of AMPK activation by A-769662 in this tissue have been reported. We hypothesized that A-769662 would increase glucose uptake in skeletal muscle. We studied incubated soleus and extensor digitorum longus (EDL) muscles from 129S6/sv and C57BL/6 mice. Glucose uptake increased only in soleus from 129S6/sv when concentrations of A-769662 were 500 μM (15%, P < 0.05) and 1 mM (60%, P < 0.01). AMPK β₁ but not β₂-containing complexes were dose dependently activated by A-769662 in muscles from both genotypes (100% at 200 μM and 300–600% at 1 mM). The discrepancy between the A-769662-induced AMPK activation pattern and stimulation of glucose uptake suggested that these effects were unrelated. A-769662 increased phosphorylation of Akt in both muscles from both genotypes, with phosphorylation of T308 being significantly higher in soleus than in EDL in 129S6/sv mice (P < 0.01). In soleus from 129S6/sv mice, insulin receptor substrate 1-associated phosphatidylinositol 3 (PI3)-kinase activity was markedly increased with A-769662, and Akt phosphorylation and glucose uptake were inhibited by wortmannin while phosphorylation of acetyl-CoA carboxylase (S227) was unaffected. Thus, A-769662 activates β₁-containing AMPK complexes in skeletal muscle but induces glucose uptake through a PI3-kinase-dependent pathway. Although development of A-769662 has constituted a step forward in the search for AMPK activators targeting specific AMPK trimers, our data suggest that in intact muscle, A-769662 has off-target effects. This may limit use of A-769662 to study the role of AMPK in skeletal muscle metabolism.

AMP-activated protein kinase (AMPK) is ubiquitously expressed in the body and acts to control many important metabolic pathways, some of which are adversely affected in patients with the metabolic syndrome (17, 33, 41). Developing drugs that have the ability to reverse the disorders related to the metabolic syndrome is of great interest. One of the potential targets of such drugs is AMPK (36). AMPK is a serine/threonine (S/T) heterotrimeric kinase consisting of a catalytic α (α₁, α₂)-subunit and regulatory β (β₁, β₂)- and γ (γ₁, γ₂)-subunits. Long-term treatment of genetically modified animal models of obesity or Type 2 diabetes mellitus (T2DM) with the AMP analog 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), which activates AMPK, ameliorates these conditions by reversing hyperglycemia, hypertension, hypertriglyceridemia, and insulin resistance (6, 35, 40). AICAR also reduces hepatic glucose output, inhibits whole body lipolysis in T2DM patients, and stimulates glucose uptake in human skeletal muscle (5, 10). Furthermore, two of the most prescribed drugs to treat T2DM, metformin and rosiglitazone, activate AMPK in cell systems and in skeletal muscle in vivo most likely by inhibiting complex 1 in the mitochondrial respiratory chain (12, 18, 49). Thus, the potential for treating patients suffering from the metabolic syndrome using AMPK-stimulating drugs is great. However, metformin and rosiglitazone cause indirect activation of AMPK, and AICAR is known to have several off-target effects independent of AMPK activation (3, 15, 47). Recently, the thienopyridone A-769662 was described by Abbott Laboratories as a direct AMPK activator (9). A-769662 had beneficial effects on whole body metabolism in ob/ob mice treated for 5 days. A-769662 was primarily found to be taken up by the liver and exerted its effect here, reducing acetyl-CoA carboxylase (ACC) activity and suppressing gluconeogenic gene expression to reduce plasma and liver triglyceride content and plasma glucose levels, respectively (9). Later reports investigating A-769662 have contributed to the understanding of the mechanism by which A-769662 activates AMPK (14, 37, 38). AMPK is allosterically activated by A-769662 independent of AMP binding (37). Binding of A-769662 depends in part on S108 located in the carboxy radical binding domain of the β-subunit. Following A-769662 binding, S108 is autophosphorylated, resulting in the protection of the activating phosphorylation of T172 from upstream phosphatases, similar to the effect of AMP binding to the γ-subunit (37). Moreover, A-769662 appears only to activate β₁-containing complexes in cell-free assays (38). Since skeletal muscle is a metabolically important tissue in relation to whole body glucose disposal and since AMPK is highly expressed in skeletal muscle, we examined the effect of A-769662 in regard to AMPK activation and glucose disposal in isolated mouse skeletal muscles. Several different isoforms of the three AMPK subunits exist, and it is theoretically possible to make up 12 different trimer complexes. In human skeletal muscle we have previously found that only 3 of these 12 complexes contribute to total AMPK activity (45). Although expression of the various AMPK isoforms is known to be fiber type dependent in murine muscles (8, 31, 48) the trimeric composition is at present not known. Therefore, it was important to delineate the AMPK heterotrimeric complexes present in the muscles used [soleus and extensor digitorum

http://www.ajpcell.org 0363-6143/09 $8.00 Copyright © 2009 the American Physiological Society

Address for reprint requests and other correspondence: J. F. P. Wojtaszewski, Molecular Physiology Group, Copenhagen Muscle Research Centre, Dept. of Exercise and Sport Sciences, Univ. of Copenhagen, DK-2100 Copenhagen, Denmark (e-mail: jwojtaszewski@ifi.ku.dk).

First published August 5, 2009; doi:10.1152/ajpcell.00051.2009.
longus (EDL)]. Our hypothesis was that A-769662 would increase activity of specific AMPK trimers and glucose uptake in a fiber type- and dose-dependent manner in skeletal muscle.

**MATERIALS AND METHODS**

*Animals.* All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the protection of Vertebrate Animals used for Experiments and Other Scientific Purposes (Council of Europe 123, Strasbourg, France, 1985). Twelve- to sixteen-week-old female mice from three different strains were used: wild-type (WT) C57BL/6, WT 129S6/sv, and α1-AMPK knockout (KO) and WT mice (19) maintained on 129S6/sv background. All mice were bred and housed at Taconic (Lille Skensved, Denmark). WT littermates produced from hetero-hetero breeding were used as controls for the α1-AMPK KO mice. Mice were maintained on a 10:14-h light-dark cycle and received food and water ad libitum.

*In vitro incubation of isolated muscles.* The relative distribution of myosin heavy chain type 1 and type 2 fibers of EDL and soleus muscles from C57BL/6 and 129S6/sv mice have been studied previously (1, 2, 28). EDL, containing mainly type 2 fibers, and soleus, containing approximately equal amounts of type 1 and type 2 fibers, were quickly removed from anesthetized fed mice and incubated in Krebs-Henseleit buffer (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 24.6 mM NaHCO3 with addition of 8 mM mannitol, 2 mM pyruvate, and 0.1% BSA) at 30°C oxygenated with a gas containing 95% O2 and 5% CO2 (Multi Myograph System, Danish Myo-Technology). Incubations were carried out using the buffer to which DMSO (0.01–0.2%; Sigma-Aldrich), A-769662 (50μM–1mM; Novo Nordisk), AICAR (2 mM; Toronto Research Chemicals, Toronto, ON, Canada), wortmannin (0.5 μM; Sigma-Aldrich), and/or insulin (10 μU/ml; Actrapid, Novo Nordisk) was added. For experiments where skeletal muscles were induced to contract, stimulation was induced using the following protocol: one 10-s train (100 Hz, 0.2-ms impulse, ~30–40 V) (MultiStim System-D330, Harvard Apparatus, Scandanid) per minute for 10 min (43). Muscles used for signaling or AMPK activity measures were incubated for 120 min in the presence or absence of the compound indicated in the figure legends. Glucose uptake was evaluated as accumulation of 1 μCi [3H]2-deoxyglucose (GE Healthcare) using 8 mM [14C]magnesium (Perkin Elmer) as extracellular space marker. When glucose uptake was measured, muscles were incubated for 110 min followed by 10 min in the presence of the radioactively labeled tracers described above. After incubation, muscles were harvested, washed in ice-cold Krebs-Henseleit buffer, blotted on filter paper, and quickly frozen in liquid nitrogen and stored at −80°C. Subsequently, muscles were incubated at 80°C in 1 N NaOH to extract the accumulated intracellular [3H]2-deoxyglucose and [14C]magnesium. Radioactivity in the supernatant of lysate immunoprecipitated with protein G agarose, but not in the supernatant of lysate immunoprecipitated without antibody, was prepared with Laemmli buffer and heated for 3 min at 96°C and analyzed by SDS-PAGE and Western blotting using each of the seven antibodies recognizing the various AMPK subunit isoforms [α1, α2, β1, β2, and γ1 (the γ3 antibody used for immunoprecipitation was provided by Prof. D. G. Hardie, University of Dundee, UK, and the others were obtained as described below)] were immunoprecipitated (IP) from 300 μg of muscle lysate using specific antibodies and G protein-coupled agonist (Millipore) overnight at 4°C in IP buffer (50 mM NaCl, 1% Triton X-100, 50 mM NaF, 5 mM Na-pyrophosphate, 20 mM Tris-base (pH 7.5), 500 μM PMSF, 2 mM DTT, 4 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 6 mM benzamidine, and 250 mM sucrose). Samples of the IP, the post-IP (supernatant), and the pre-IP (supernatant of lysate immunoprecipitated with protein G agarose, but without antibody) were prepared with Laemmli buffer and heated for 3 min at 96°C and analyzed by SDS-PAGE and Western blotting using each of the seven antibodies recognizing the various AMPK subunit isoforms [α1, α2, β1, γ1 (provided by Prof. D. G. Hardie), β2, γ3 (Abcam, Cambridge, UK)], Forty micrograms of the IP and 20 μg of the post-IP and the pre-IP were loaded on the gels. The method for estimation of the AMPK trimer composition is described in Fig. 2. IP of the p85 subunit of the PI3-kinase was precipitated as described above using an antibody from Upstate Biotechnologies (no. 06–497, Billerica, MA).

**AMPK activity measurements.** β-Isomer-specific AMPK activity was measured on either β1- or β2-IPs from 200 μg of muscle lysate protein as described above. After an overnight incubation at 4°C, the IP was washed once in IP buffer, once in 480 mM HEPES (pH 7.0) and 240 mM NaCl, and twice in 240 mM HEPES (pH 7.0) and 120 mM NaCl, leaving no buffer with the agonist after the last wash. The reaction ran for 30 min at 30°C in a total volume of 30 μl containing 80 mM HEPES, 40 mM NaCl, 833 μM DTT, 200 μM AMP, 100 μM AMARA-peptide, 5 mM MgCl2, 200 μM ATP, and 2 μCi of [γ-32P]-ATP (Perkin Elmer). The reaction was stopped by adding 10 μl of 1% phosphoric acid to the reaction, after which 35 μl was spotted onto a piece of P81 filter paper, which was then washed 4 times 15 min in 1% phosphoric acid. The dried filter paper was analyzed for activity using liquid scintillation (Tri-Carb 2000).

**Skeletal muscle lysate preparation and Western blot analyses.** Skeletal muscle lysates from soleus and EDL muscles (~5–~9 mg, respectively) were prepared as previously described (42). Phosphorylation of TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1/4 (TBC1D1/TBC1D4) was measured using the phospho-Akt substrate (PAS) antibody (1:1000) (no. 9611, Cell Signalining Technology). Phosphorylation level of Akt on S473 (1:500, no. 9271) and AMPK on T172 (1:1000, no. 2531) were measured with phosphospecific antibodies (Cell Signaling Technology). Phosphorylation of ACC on S227 (1:1000, no. 07-303), the p85 subunit of the phosphatidylinositol 3 (PI3)-kinase (1:1000, no. 06-497), and T308 phosphorylation of Akt (1:500, no. 06-678) was evaluated using antibodies from Millipore (Billerica, MA). The α1-subunit of the Na+K+-ATPase (1:1000) was detected using an antibody from Developmental Studies Hybridoma Bank (no. a96F, Univ. of Iowa, Iowa City, IA). Immunoblot analyses were performed as previously described (44). In short, muscle lysates were adjusted to equal protein concentration and heated in Laemmli buffer (5 min, 96°C). Samples (30 μg) were loaded on self-cast 5–7.5% gels and transferred to polyvinylidene difluoride membranes (Immobilon Transfer Membrane, Millipore). Membranes were blocked (1 h at room temperature) in washing buffer (10 mM Tris-base, 150 mM NaCl, and 0.25% Tween 20) containing 2% low-fat milk protein. Membranes were then incubated with primary antibodies overnight at 4°C, followed by incubation (1 h at room temperature) with appropriate horseradish peroxidase-conjugated secondary antibody (1:3,000) (DAKO). Bands were visualized using a Kodak Image Station (2000MM, Kodak) and enhanced chemiluminescence (Millipore). Bands were quantified using Kodak ID 3.6 software.

**Results.** During the 10-s stimulation, the uptake of glucose was increased to 10.2±3.3% above the control condition (n = 5). The relative increase in glucose uptake was greater in the α1-AMPK KO mice (23.4±4.7%) than in WT mice (8.7±2.9%)(P < 0.05). The increase in glucose uptake in the α1-AMPK KO mice was blocked by incubation with DMSO (0.01–0.2%) (Fig. 1A), indicating that the observed increase in glucose uptake was dependent on AMPK activity. The increase in glucose uptake in both WT and α1-AMPK KO mice was blocked by incubation with AICAR (50 μM) and wortmannin (50 μM) (Fig. 1B). These results indicate that AMPK activity is important for the increase in glucose uptake that occurs during the 10-s stimulation.

**Discussion.** The increase in glucose uptake that occurs during the 10-s stimulation is dependent on AMPK activity. The increase in glucose uptake in the α1-AMPK KO mice is blocked by incubation with AICAR (50 μM) and wortmannin (50 μM). These results indicate that AMPK activity is important for the increase in glucose uptake that occurs during the 10-s stimulation.

**Conclusions.** The increase in glucose uptake that occurs during the 10-s stimulation is dependent on AMPK activity. The increase in glucose uptake in the α1-AMPK KO mice is blocked by incubation with AICAR (50 μM) and wortmannin (50 μM). These results indicate that AMPK activity is important for the increase in glucose uptake that occurs during the 10-s stimulation.
Insulin receptor substrate 1-associated PI3-kinase activity assay. Insulin receptor substrate 1 (IRS-1) was immunoprecipitated from 300 μg of lysate with a specific antibody kindly provided by Dr. Ken Siddle (Univ. of Cambridge, UK). After an overnight incubation at 4°C, the IP was washed twice in PBS (pH 7.5) with 1% NP-40 and 100 μM Na3VO4 and twice in Tris-buffered saline (pH 7.5) with 100 mM NaCl, 1 mM EDTA and 100 μM Na3VO4 and left with 50 μl of the last wash buffer. Ten microliters 100 mM MgCl2 and 10 μg 1-α-phosphatidylinositol (Sigma-Aldrich) were added, and the samples were left at room temperature for 15 min. The reaction was started by addition of 10 μl reaction mixture [8.25 mM Tris (pH 7.5), 825 μM EDTA, 6 mM MgCl2, 440 mM ATP, and 100 μM 32P-ATP (0.6 μCi) (Perkin Elmer)]. The reaction ran for 15 min at 30°C and was stopped by addition of 10 μl 5 N HCl. Then 180 μl methanol: chloroform (1:1) was added, and samples were shaken vigorously for 1 min and centrifuged for 90 s. Eighty microliters of the lower organic fraction were transferred to a new tube, and 50 μl were spotted onto a TLC Silica gel (Merck). The chromatographic separation ran for 45 min, at which the TLC gel was dried and exposed in a Phosphorimage Cassette for 48 h before scanning in a Molecular Dynamics STORM scanner (Struers Kebo Lab).

Insulin receptor phosphorylation assay. Chinese hamster ovary (CHO)-human insulin receptor cells were seeded in 12-well plates (125,000 cells/well) and grown until near confluence as described previously (16). Cells were stimulated for 60 min with 0, 0.01, 0.1, 1, 10, or 100 nM human insulin (Actrapid) or 0, 1, 10, 50, 200, or 1,000 μM A-769662 all in the presence of 0.2% DMSO for 60 min. Subsequently, cells were washed three times in ice-cold PBS and snap frozen by pouring liquid nitrogen into the wells. Cells were solubilized by addition of 100 μl lysis buffer. Activation of the insulin receptor was measured as phosphorylation of Y1158 on the insulin receptor using PhosphoELISA kits according to the manufacturer’s protocol (no. KHR9121, Invitrogen).

Statistical analysis. Data are expressed as means ± SE. Levene’s test of equality of variance was used to test whether groups had equal variances. Data were log-transformed if Levene’s test was significant. Statistical differences were assessed using two-way ANOVA with or without repeated measures where appropriate. In experiments where AICAR treatment was added as positive control, the effect of AICAR was evaluated separately using the basal/unstimulated muscles as controls. For post hoc testing, Tukey’s honestly significant difference test was applied. Statistical analyses were performed in SigmaStat 3.5 (Germany). Statistical significance was accepted at P < 0.05.

RESULTS

A-769662 differentially induces glucose uptake in skeletal muscle from C57BL/6 and 129S6/sv mice. We tested the hypothesis that A-769662 would increase glucose uptake in isolated skeletal muscle. Initially we wanted to test whether the effect of A-769662 was dependent on either α1- or α2-AMPK subunits. Therefore, since our α1- and α2-AMPK KO mice have been congenically back-crossed to 129S6/sv and C57BL/6 backgrounds, respectively, we tested whether A-769662 would induce glucose uptake in WT mice from these two strains of mice. As can be seen in Fig. 1, A and B, A-769662 significantly increased glucose uptake in a dose-dependent manner but only in soleus from 129S6/sv mice at concentrations of 500 μM (~15%, P < 0.05) and 1 mM (~60%, P < 0.01). In both EDL and soleus from both mouse strains, AICAR significantly increased glucose uptake. Of note, we have previously found that AICAR-induced glucose uptake is dependent on α2-AMPK (19). On the basis of the finding that the bioavailability of A-769662 in muscle was low compared with other tissues (9), we wanted to ensure that the incubation time was optimal. Therefore, we performed a time course experiment and found that incubation with A-769662 resulted in increased glucose uptake at all time points investigated, with an apparent maximal effect after 2 h of incubation (Fig. 1C). All following incubation experiments were done for 2 h. AMPK is activated by an increase in the AMP/ATP ratio resulting from stimuli affecting the nucleotide status of the cell. Therefore, to test whether A-769662 activates AMPK by affecting nucleotide levels, we measured nucleotides and calculated the AMP/ATP and the PCr/(PCr + Cr) ratios. As shown in Fig. 1, D and E, incubation with A-769662 did not result in perturbations in the basal levels of these nucleotides whereas muscle contraction did.

A-769662-induced glucose uptake does not depend on α1-AMPK. As mentioned, the α1 AMPK KO mice are on a 129S6/sv background. Thus, to test whether α1-AMPK is necessary for A-769662 to induce glucose uptake, we incubated soleus from α1-AMPK KO and WT littermates ± 1 mM A-769662. As shown in Fig. 1F, α1-AMPK KO did not affect A-769662-induced glucose uptake. Unfortunately, the AMPK-α2 KO mice are on a C57BL/6 background and since glucose uptake in skeletal muscle from C57BL/6 WT mice does not increase in response to A-769662, we were not able to test whether α2-AMPK would be required for A-769662 to induce glucose uptake.

AMPK trimer distribution in soleus and EDL of C57BL/6 and 129S6/sv mice is similar. We next hypothesized that the differential response in glucose uptake was due to differences in the expression of different AMPK trimers in soleus and EDL from C57BL/6 and 129S6/sv mice. By immunoprecipitation and cross-blotting with antibodies directed against the different subunits of AMPK, we were able to delineate the AMPK trimer complexes present in EDL and soleus from 129S6/sv and C57BL/6 mice (Fig. 2, A–D). Both strains of mice expressed all seven AMPK subunit isoforms, but only five different complexes were detected: α1β2γ1, α2β2γ3, α3β1γ1, α1β2γ1, and α1β2γ1. Furthermore, we estimated the relative distribution of the different AMPK trimer complexes. As shown in Table 1, C57BL/6 and 129S6/sv mice overall have similar distribution of AMPK trimers within soleus and EDL with the α2β2γ1 complex contributing by far the most (~55–70%) to the total pool of AMPK trimers in both muscle types. Interestingly, and in contrast to human vastus lateralis muscle (4, 45), mouse skeletal muscle also contains β1-AMPK complexes. These complexes are not as heavily expressed as the β2-containing complexes. EDL from either genotype has only ~5% β1-trimers, whereas soleus contains ~18% β1-trimers. Taken together, it is unlikely that the differences observed in glucose uptake between muscles and mouse strains can be explained by differences in the distribution of the five AMPK trimers.

A-769662 induces activation of β1- but not β2-containing AMPK complexes. Since A-769662 was previously found to allosterically activate AMPK via a specific domain on the AMPK β-subunit (37), we next measured β1- and β2-AMPK-associated kinase activity to test the hypothesis that A-769662 caused differential activation of these complexes in soleus and EDL muscles from the two mouse strains. As expected, AICAR caused activation of AMPK β2-containing complexes ranging from 50% to 150%, with no differences between EDL and soleus or between C57BL/6 and 129S6/sv mice (Fig. 3, A
and B). However, $\beta_2$-AMPK complexes were not activated in response to A-769662 treatment in either soleus or EDL from either of the mouse strains (Fig. 3, A and B). This lack of $\beta_2$-AMPK activation was confirmed in another set of soleus and EDL muscles from C57BL/6 mice treated with A-769662 from which we immunoprecipitated $\beta_2$-AMPK and immuno-blotted with the phosphorylated T172 (pT172) AMPK antibody (Fig. 3E). In accordance with the considerably smaller expression of $\beta_1$ complexes, the activity associated with this isoform was also markedly lower compared with the activity associated with $\beta_2$. However, in both muscles from C57BL/6 and 129S6/sv mice, AMPK $\beta_1$-containing complexes were activated in a dose-dependent manner ranging from $\sim100\%$ at 200 $\mu$M A-769662 to 300–600\% at 1 mM A-769662 (Fig. 3, C and D). Of note, A-769662-induced $\beta_1$-AMPK activity was 100\% higher in soleus from C57BL/6 mice (9.4 $\pm$ 0.2 pmol$\cdot$min$^{-1}$$\cdot$mg$^{-1}$) compared with 129S6/sv (4.4 $\pm$ 0.4 pmol$\cdot$min$^{-1}$$\cdot$mg$^{-1}$), whereas $\beta_1$-AMPK activities in EDL from the two mouse strains were similar. Muscle contraction elicited a very weak pT172 phosphorylation associated with
Fig. 2. Delineation of AMPK trimers present in EDL and soleus from 129S6/sv and C57BL/6 mice. The α1, α2, β1, β2, and γ3 subunits of AMPK were immunoprecipitated from a pool of EDL or soleus (n = 10) prepared from the two genotypes of mice. A: EDL 129S6/sv. B: soleus 129S6/sv. C: EDL C57BL/6. D: soleus C57BL/6. The immunoprecipitate (IP) together with the supernatant (Post) and supernatant from control IP without antibody (Pre) were subjected to SDS-PAGE, and antibodies directed against all seven AMPK isoforms were used to cross-blot. By comparing the signal in the pre-to that of the post-IP (20 μg loaded for each), we were able to delineate the composition of the AMPK trimer complexes. In the following, the method used for estimating the AMPK trimer distribution will be explained using EDL from 129S6/sv as an example (A). From the α1 and α2 IPs it can be seen that they coimmunoprecipitate the same amount of β1 (~50% each). From the β1 IP it can be seen that β2 coimmunoprecipitates both α1 and α2, but only a very small fraction of each (<5%). Thus, ~5% of all complexes contain β1, and this 5% is evenly divided between α1 and α2 (~2–3%). From the α1 and α2 IPs it can be seen that α1 only coimmunoprecipitates a very small fraction of β2, while α2 coimmunoprecipitates the vast majority (~95%). From the β2 IPs it can also be seen that β2 on the other hand coimmunoprecipitates ~75% of both α1 and α2. Because ~95% of β2 is in complex with α2 and the remaining ~5% is coimmunoprecipitating 75% of α1, it is evident that muscle tissue has a much higher content of α2 than α1. From the IPs of α1, α2, β1, and β2, it can be seen that α1 and β1 do not co-IP any γ3, while α2 and β2 each coimmunoprecipitate all of it. Likewise, γ3 coimmunoprecipitates a small fraction of both α1 and 20–25% of β2 but does not coimmunoprecipitate any α1 or β1. This indicates that only one γ3 complex is present in the muscle lysates (α1β2γ3). We have indentified five complexes of which ~95% contains β2 and ~5% contains β1. Of the β2 complexes, γ3 is associated in a little less than 20%, so the α1β2γ3 complex must comprise ~20% (20% of 95% = 19%; i.e., ~20%) of all five complexes. The α1-subunit coimmunoprecipitated ~5% of β2, resulting in ~5% α1β2γ3 trimer complexes (5% of 95% = 4.75%; i.e., ~5%). Because ~5% of the complexes are α1β1γ3/α1β1γ3, the remaining ~70% of the complexes are α2β2γ3. Both muscles from both genotypes contain the same five AMPK trimers although in slightly different proportions (Table 1). Note that the γ3 blots from EDL muscle show two very close bands of which only the lower is γ3. In soleus muscle in which expression of γ3 is very low, the strong band in the γ3 blots is unspecific, but indistinguishable from the true γ1 band. The true γ1 band is only detectable in the α2, β2, and γ3 IP lanes. WB, Western blot.

**A** 129S6/sv EDL

<table>
<thead>
<tr>
<th>WB</th>
<th>α1</th>
<th>α2</th>
<th>β1</th>
<th>β2</th>
<th>γ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
</tr>
</tbody>
</table>

**B** 129S6/sv Soleus

<table>
<thead>
<tr>
<th>WB</th>
<th>α1</th>
<th>α2</th>
<th>β1</th>
<th>β2</th>
<th>γ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
</tr>
</tbody>
</table>

**C** C57Bl/6 EDL

<table>
<thead>
<tr>
<th>WB</th>
<th>α1</th>
<th>α2</th>
<th>β1</th>
<th>β2</th>
<th>γ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
</tr>
</tbody>
</table>

**D** C57Bl/6 Soleus

<table>
<thead>
<tr>
<th>WB</th>
<th>α1</th>
<th>α2</th>
<th>β1</th>
<th>β2</th>
<th>γ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
<td>IP</td>
<td>Post</td>
<td>Pre</td>
</tr>
</tbody>
</table>
Table 1. Relative distribution of AMPK trimer complexes present in 129S6/sv and C57BL/6 mice

<table>
<thead>
<tr>
<th></th>
<th>129S6/sv</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDL</td>
<td>Soleus</td>
</tr>
<tr>
<td>αβ2γ1</td>
<td>~70%</td>
<td>~55%</td>
</tr>
<tr>
<td>αβ2γ3</td>
<td>~20%</td>
<td>~&lt;2%</td>
</tr>
<tr>
<td>αγ2β3</td>
<td>~5%</td>
<td>~25%</td>
</tr>
<tr>
<td>αγ2γ1</td>
<td>~3%</td>
<td>~10%</td>
</tr>
<tr>
<td>αβγ1</td>
<td>~&lt;2%</td>
<td>~&lt;8%</td>
</tr>
</tbody>
</table>

Extensor digitorum longus (EDL) and soleus muscles (n = 10) from 129S6/sv and C57BL/6 mice were pooled, and composition of AMP-activated protein kinase (AMPK) trimers was estimated on the basis of the immunoprecipitation experiment shown in Fig. 2.

β1-AMPK immunoprecipitates, but unfortunately no pT172 signal from β1-AMPK immunoprecipitates could be obtained from muscles stimulated with A-769662 (data not shown). Collectively, since A-769662-induced AMPK activity in soleus from C57BL/6 mice was higher compared with 129S6/sv mice, and since glucose uptake only increased in soleus from 129S6/sv, this indicated that A-769662-induced glucose uptake in soleus in 129S6/sv mice occurred through an AMPK-independent mechanism. To evaluate whether changes in AMPK activity affected downstream targets of AMPK, we measured phosphorylation of T227 on ACC. Interestingly, A-769662 was able to stimulate Akt phosphorylation of the two Akt sites was much more potently increased in response to A-769662 in both muscles from both C57BL/6 and 129S6/sv mice incubated with A-769662. However, while glucose uptake only increased in soleus from 129S6/sv, this indicated that A-769662-induced glucose uptake was indeed increased in both muscles from both C57BL/6 and 129S6/sv mice incubated with A-769662. Wortmannin, the level of S227 phosphorylation on ACC was unaffected (Fig. 5E). Thus, wortmannin did not affect activation of AMPK.

A-769662 activates IRS-1-associated PI3-kinase in intact skeletal muscle. Since incubation with a combination of A-769662 and wortmannin resulted in complete inhibition of Akt phosphorylation and glucose uptake, we next investigated whether the PI3-kinase activity was increased by A-769662 in incubated soleus muscles from 129S6/sv mice. We measured IRS-1-associated PI3-kinase activity and found that activation of this lipid kinase was greatly induced by A-769662, even to a greater extent than with a maximal effective dose of insulin (Fig. 6A).

A-769662 does not activate the insulin receptor. To explore whether the PI3-kinase activation by A-769662 was mediated though an effect on the insulin receptor, we measured phosphorylation of one of the autophosphorylation sites (Y1158) on the insulin receptor. We performed this experiment in CHO cells overexpressing the human insulin receptor because this assay in our hands is not sensitive to explore phosphorylation of the insulin receptor in skeletal muscle. Whereas insulin caused an expected increase in autophosphorylation of Y1158, incubation with A-769662 had no effects in concentrations from 1 μM to 1,000 μM (Fig. 6, B and C). Therefore, A-769662 does not appear to affect the insulin receptor directly.

The αt subunit of the Na\(^{+}\)-K\(^{+}\)-ATPase does not associate with the p85 subunit of PI3-kinase. Unpublished observations from Dr. Alexander Chibalin and coworkers show that A-769662 can inhibit the Na\(^{+}\)-K\(^{+}\)-ATPase in CHO cells overexpressing the human insulin receptor because this assay in our hands is not sensitive to explore phosphorylation of the insulin receptor in skeletal muscle. Whereas insulin caused an expected increase in autophosphorylation of Y1158, incubation with A-769662 had no effects in concentrations from 1 μM to 1,000 μM (Fig. 6, B and C). Therefore, A-769662 does not appear to affect the insulin receptor directly.

Phosphorylation of Akt in soleus from 129S6/sv mice by A-769662 is wortmannin sensitive. Activation of the PI3-kinase in skeletal muscle can be inhibited by wortmannin. Therefore, to investigate the potential mechanism by which Akt phosphorylation was increased, soleus from 129S6/sv mice were incubated ± 1 mM A-769662 ± 0.5 μM wortmannin. As a control, other 129S6/sv soleus muscles were incubated ± 10 μM/ml insulin ± 0.5 μM wortmannin. Wortmannin completely ablated both A-769662- and insulin-induced Akt phosphorylation on both S473 and T308 (Fig. 5, A and B). Interestingly, A-769662 was able to stimulate Akt phosphorylation to a similar extent as a maximal insulin stimulus. Importantly, wortmannin completely blunted A-769662- and insulin-induced glucose uptake (Fig. 5C). To further investigate the potential signaling pathway by which A-769662 causes glucose uptake, we investigated whether phosphorylation of the Akt substrates TBC1D1/TBC1D4 was affected by stimulation with A-769662 using the PAS antibody. We found that PAS phosphorylation was significantly increased in soleus incubated with A-769662 but was fully inhibited by the addition of wortmannin (Fig. 5D). In the muscles incubated with A-769662 plus wortmannin, the level of S227 phosphorylation on ACC was unaffected (Fig. 5E). Thus, wortmannin did not affect activation of AMPK.

A-769662 activates IRS-1-associated PI3-kinase in intact skeletal muscle. Since incubation with a combination of A-769662 and wortmannin resulted in complete inhibition of Akt phosphorylation and glucose uptake, we next investigated whether the PI3-kinase activity was increased by A-769662 in incubated soleus muscles from 129S6/sv mice. We measured IRS-1-associated PI3-kinase activity and found that activation of this lipid kinase was greatly induced by A-769662, even to a greater extent than with a maximal effective dose of insulin (Fig. 6A).

A-769662 does not activate the insulin receptor. To explore whether the PI3-kinase activation by A-769662 was mediated through an effect on the insulin receptor, we measured phosphorylation of one of the autophosphorylation sites (Y1158) on the insulin receptor. We performed this experiment in CHO cells overexpressing the human insulin receptor because this assay in our hands is not sensitive to explore phosphorylation of the insulin receptor in skeletal muscle. Whereas insulin caused an expected increase in autophosphorylation of Y1158, incubation with A-769662 had no effects in concentrations from 1 μM to 1,000 μM (Fig. 6, B and C). Therefore, A-769662 does not appear to affect the insulin receptor directly.

The αt subunit of the Na\(^{+}\)-K\(^{+}\)-ATPase does not associate with the p85 subunit of PI3-kinase. Unpublished observations from Dr. Alexander Chibalin and coworkers show that A-769662 can inhibit the Na\(^{+}\)-K\(^{+}\)-ATPase (personal communication). In addition, it has been shown in some (11, 21, 29, 30, 50) but not all models (23, 24) that inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase by ouabain increases PI3-kinase activity, possibly through an association between p85 and the αt subunit of the Na\(^{+}\)-K\(^{+}\)-ATPase, leading to increased Akt phosphorylation. This effect can be inhibited by wortmannin (30). Therefore, we tested the hypothesis that the αt subunit of the Na\(^{+}\)-K\(^{+}\)-ATPase coimmunoprecipitates with the regulatory p85 subunit of the PI3-kinase when stimulated with A-769662. However, as shown in Fig. 7, although we were able to completely immunoprecipitate all p85 from soleus from 129S6/sv, the αt subunit of the Na\(^{+}\)-K\(^{+}\)-ATPase did not coimmunoprecipitate.

D I S C U S S I O N

In this study we have investigated the effect of A-769662 on glucose uptake in incubated soleus and EDL muscles from two different genotypes of mice. One surprising finding was that A-769662-induced glucose uptake occurred in a muscle type-dependent manner in 129S6/sv mice via a PI3-kinase-dependent pathway. Owing to the lack of a suitable genetic model (e.g., a muscle-specific αt/α2-AMPK KO), we cannot completely exclude the possibility that AMPK is involved in
A-769662-induced glucose uptake. However, the indirect evidence for lack of involvement of AMPK in this process was that A-769662 clearly activated β1-AMPK-containing complexes in both soleus and EDL from both C57BL/6 and 129S6/sv mice, but glucose uptake increased only in soleus from 129S6/sv mice. Furthermore, when A-769662-induced glucose uptake was inhibited by wortmannin, activation of the AMPK system as indicated by phosphorylation of ACC was intact. Finally, α1-AMPK appears dispensable for A-769662 to induce glucose uptake since in α1-AMPK KO mice, glucose uptake still occurs in response to A-769662. In our model system, stimulation with A-769662 results in increased IRS-1-associated PI3-kinase activity and downstream phosphorylation of Akt which can be inhibited by wortmannin. On the basis of available data in the literature (30) and since A-769662 does not affect the activity of the insulin receptor, we initially thought that this activation occurred through an association between the β1-subunit of the Na⁺-K⁺-ATPase and the regulatory p85 subunit of the PI3-kinase, and that this signal in turn would be transduced through the PI3-kinase/Akt signaling pathway to ultimately result in glucose transporter 4 (GLUT4) molecules entering the plasma membrane. However, we were...
unable to see any association between p85 and the α₁-subunit of the Na⁺-K⁺-ATPase. Thus, at present, we are uncertain regarding the mechanism by which A-769662 activates the PI3-kinase. However, p85 contains several domains capable of protein-protein interactions (39), and it may be that signaling complexes could be formed in response to A-769662 stimulation allowing specific downstream signaling events to occur. Since the response to A-769662 on glucose uptake is mediated through PI3-kinase signaling, and since we observed a significant phosphorylation of Akt in all muscles, it is not clear why glucose uptake was not increased in both soleus and EDL from both C57BL/6 and 129S6/sv mice. One possible explanation could be that to induce glucose uptake through an Akt-mediated mechanism, a certain absolute threshold of Akt phosphorylation is required. Alternatively, however, it is possible that the ability of A-769662 to induce glucose uptake through phosphorylation of Akt depends on both the genetic makeup and the metabolic properties of the muscle. In line with this hypothesis, A-769662-induced T308 and S473 phosphorylation in the soleus and EDL from C57BL/6 mice increased similarly at 200 μM but did not increase any further at 1 mM. Thus, it may be that the genetic background of this mouse strain does not allow for the required level of Akt activation to induce glucose uptake in response to A-769662. On the other hand, Akt phosphorylation in 129S6/sv mice was dramatically increased at 1 mM of A-769662 in both soleus and EDL, but only in the more oxidative soleus did Akt phosphorylation of T308 reach a level high enough to increase glucose uptake, a level comparable to a maximal insulin stimulus (Fig. 5, A and B). Alternatively, the PI3-kinase-dependent glucose transport may be dependent on signaling components unrelated to Akt.

The data from this study support the idea that the proximal signaling events in response to A-769662 to induce glucose uptake involve the PI3-kinase. Using the PAS antibody to investigate the phosphorylation level of TBC1D1/TBC1D4 in soleus from 129S6/sv in response to A-769662, we found that TBC1D1/TBC1D4 phosphorylation was significantly elevated from both C57BL/6 and 129S6/sv mice. One possible explanation could be that to induce glucose uptake through an Akt-mediated mechanism, a certain absolute threshold of Akt phosphorylation is required. Alternatively, however, it is possible that the ability of A-769662 to induce glucose uptake through phosphorylation of Akt depends on both the genetic makeup and the metabolic properties of the muscle. In line with this hypothesis, A-769662-induced T308 and S473 phosphorylation in the soleus and EDL from C57BL/6 mice increased similarly at 200 μM but did not increase any further at 1 mM. Thus, it may be that the genetic background of this mouse strain does not allow for the required level of Akt activation to induce glucose uptake in response to A-769662. On the other hand, Akt phosphorylation in 129S6/sv mice was dramatically increased at 1 mM of A-769662 in both soleus and EDL, but only in the more oxidative soleus did Akt phosphorylation of T308 reach a level high enough to increase glucose uptake, a level comparable to a maximal insulin stimulus (Fig. 5, A and B). Alternatively, the PI3-kinase-dependent glucose transport may be dependent on signaling components unrelated to Akt.

The data from this study support the idea that the proximal signaling events in response to A-769662 to induce glucose uptake involve the PI3-kinase. Using the PAS antibody to investigate the phosphorylation level of TBC1D1/TBC1D4 in soleus from 129S6/sv in response to A-769662, we found that TBC1D1/TBC1D4 phosphorylation was significantly elevated

---

**Table 2. Phosphorylation of ACC in response to A-769662 and AICAR in EDL and soleus from 129S6/sv and C57BL/6 mice**

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>200 μM</th>
<th>1 mM</th>
<th>2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDL</td>
<td>Soleus</td>
<td>EDL</td>
<td>Soleus</td>
</tr>
<tr>
<td>129S6/sv</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>100±25</td>
<td>123±35</td>
<td>80±17</td>
<td>132±25</td>
</tr>
<tr>
<td>A-769662 (200 μM)</td>
<td>85±22</td>
<td>348±82</td>
<td>116±30</td>
<td>350±76</td>
</tr>
<tr>
<td>A-769662 (1 mM)</td>
<td>156±17</td>
<td>573±69</td>
<td>111±23</td>
<td>398±90</td>
</tr>
<tr>
<td>AICAR (2 mM)</td>
<td>362±79</td>
<td>847±173</td>
<td>424±90</td>
<td>932±154</td>
</tr>
</tbody>
</table>

All values are means (± SE) and related to the degree of acetyl-CoA carboxylase (ACC) phosphorylation in the basal EDL from 129S6/sv mice (n = 6). AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside. **Different from basal within muscle: *P < 0.05, †P < 0.01. ††Different from EDL within genotype and treatment condition: *P < 0.05, †P < 0.01. **Different from 200 μM within muscle: #P < 0.05.

---

**Fig. 4.** Incubation of EDL and soleus from C57BL/6 and 129S6/sv mice with A-769662 results in phosphorylation of Akt. Muscles (n = 6) were incubated ± A-769662 (200 μM or 1 mM) or ± AICAR (2 mM) for 2 h. Samples were subjected to SDS-PAGE probing for Akt phosphorylation of T308 and S473. A and B: S473 phosphorylation in EDL and soleus from C57BL/6 and 129S6/sv mice. C and D: T308 phosphorylation in EDL and soleus from C57BL/6 and 129S6/sv mice. **P < 0.01, different from basal within muscle. ††P < 0.01, different from EDL within genotype and treatment condition. #P < 0.05 and ##P < 0.01, different from 200 μM within muscle.
and that this response was fully inhibited by wortmannin. This finding supports the hypothesis that A-769662-induced glucose uptake may involve the PI3-kinase-Akt-TBC1D1/TBC1D4 signaling axis which is also thought to be mediating insulin’s effects on glucose uptake in skeletal muscle (7, 25, 26, 43, 46).

One other novel finding of the present investigation was that stimulation with A-769662 in intact muscle activated $\beta_1$- but not $\beta_2$-AMPK-containing heterotrimeric complexes. Previous investigations have also found selective $\beta_1$-activation by A-769662 when investigating recombinant AMPK trimers in vitro (37, 38). From those studies it was not clear whether the different recombinant AMPK trimers used would play a relevant role within a certain tissue. Since we initially thought that the differences observed in regard to A-769662-induced glucose uptake between muscles and genotype could be explained by differences in AMPK trimer expression, we decided to dissect out the composition and distribution of the AMPK trimers expressed in soleus and EDL muscles from C57BL/6 and 129S6/sv mice. We found that skeletal muscle from C57BL/6 and 129S6/sv mice express the same five AMPK trimers. Furthermore, there is no difference in trimer distribution between the two genotypes in soleus or in EDL muscles.

Fig. 5. Wortmannin (Wmn) inhibits A-769662-induced Akt phosphorylation and glucose uptake in soleus from 129S6/sv mice without affecting activation of AMPK. Soleus ($n=10$) were incubated ± 1 mM A-769662 ± 0.5 $\mu$M wortmannin for 2 h. A subset of muscles ($n=4$) were incubated ± 10 mU/ml insulin ± 0.5 $\mu$M of wortmannin. A and B: samples were subsequently subjected to SDS-PAGE probing for Akt phosphorylation of S473 (A) and T308 (B). C: in another set of muscles, glucose uptake in response to ± 1 mM A-769662 ($n=14$) or 10 mU/ml insulin ($n=4$) in the absence or presence of 0.5 $\mu$M wortmannin was evaluated. D and E: in addition, the muscle lysates used for Akt phosphorylation measurements were also immunoblotted for phosphorylation of TBC1D1/D4 using the phospho-Akt substrate (PAS) antibody (D) and for phosphorylation of S227 on acetyl-CoA carboxylase (ACC) (E). F: representative blots. B, basal; A, A-769662. **P < 0.01, different from basal.
However, the two β₁-containing trimers (α₁β₁γ₁ and α₂β₁γ₁) as well as the activity associated with these complexes constitute only a very small fraction of the total pool of AMPK trimer complexes/activity, i.e., <5% in EDL and <18% in soleus from both C57BL/6 and 129S6/sv mice for the expression of the trimer complexes, and ~1–2% in EDL and 8–10% in soleus from both C57BL/6 and 129S6/sv mice for basal activity. The existence of β₁-containing complexes is in line with previously published data from mouse skeletal muscle showing association between β₁- and α₁/α₂-subunits (8, 48). However, the functional relevance of α₂β₁γ₁- and α₁β₁γ₁-AMPK trimers in skeletal muscle from mice is not known at present. Evidence from whole body α₂-AMPK KO mice, however, shows that deficiency of α₂-AMPK results in decreased protein expression of β₁ (23–32%), β₂ (60–66%), and γ₁ (24–37%)–AMPK in skeletal muscle (20). This suggests that AMPK complexes containing any of these three subunits may play important roles in regulating metabolic processes in mouse skeletal muscle. Thus, since we found strong activation of β₁–AMPK complexes with A-769662 in C57BL/6 mice and at concentrations that did not induce glucose uptake in 129S6/sv mice, it is likely that A-769662 has effects on metabolism in skeletal muscle unrelated to regulation of GLUT4 mobilization. Nevertheless, human vastus lateralis muscle does apparently not contain any β₁-AMPK-containing heterotrimers (45), and this therefore questions the relevance of using A-769662 to study AMPK in mouse skeletal muscle with the intention of transferring knowledge to human skeletal muscle. Of note, the AMPK complex composition in human liver is different from skeletal muscle. Human liver contains some (~15%) β₁–AMPK complexes (unpublished observations by the authors) and it is likely that A-769662 may have significant and AMPK-dependent biological effects in this tissue and in other tissues expressing β₁–AMPK trimers.

In this study we observed the greatest increase in AMPK activation and glucose uptake using relatively high concentrations of A-769662. Previous studies that have used this compound to study the effects on metabolism in cells and phosphorylation of AMPK and ACC in skeletal muscle have used lower (1–300 μM) concentrations (9, 13, 14, 34, 37, 38). However, on the basis of the relatively poor bioavailability of A-769662 in skeletal muscle compared with liver (9), and since our experimental model involved incubated intact skeletal muscles rather than primarily in vitro studies like those previously reported (14, 37), we wanted to create as high a concentration gradient across the sarcolemma as possible. Therefore, in our model system the half-maximal effect of A-769662 on AMPK activation was considerably higher (i.e., >500 μM) compared with results obtained in cell-free assays (i.e., ~116 ± 25 nM) (14). Since we only measured AMPK activity at 200 and 1 mM A-769662, it is not possible to say whether

---

**Fig. 6.** A-769662 activates the phosphatidylinositol 3 (PI3)-kinase but does not lead to phosphorylation of Y1158 of the insulin receptor (IR). A: soleus muscles (n = 4–8) from 129S6/sv mice were incubated with 1 mM A-769662 or 10 nM/ml insulin, and IR substrate 1 (IRS-1)-associated PI3-kinase activity was measured as described in MATERIALS AND METHODS. B and C: Chinese hamster ovary (CHO) cells overexpressing the human insulin receptor were stimulated with different concentrations of insulin (B) or A-769662 (C), and cell lysates were used to measure phosphorylation of Y1158 of the insulin receptor (n = 3–4). *P < 0.05 and **P < 0.01, different from basal. †P < 0.05, different from A-769662.
incubation with 1 mM created a maximal response. However, on the basis of the calculations of the relative change in AMPK activity going from unstimulated to stimulation with 200 μM and 1 mM of A-769662, it is clear that the dose-response relationship is linear in this interval. Thus, we believe that the concentration range of A-769662 used in the present study has been appropriate.

It was previously reported that in β1-AMPK KO mice, ACC phosphorylation in EDL muscle in response to A-769662 was abolished (38). Therefore, in addition to β2-AMPK activity we also measured ACC S27 phosphorylation in soleus and EDL incubated with A-769662. Interestingly, and in contrast to previously published data (38), we found that ACC phosphorylation only increased in soleus and not EDL from C57BL/6J mice (Table 2). At present the reason for this discrepancy is not clear. Although we do not know the absolute amount of specific AMPK trimers in soleus compared with EDL, we found that soleus contains more β1-containing AMPK trimers compared with EDL in relative terms (<18% compared with <5%, respectively; Table 1). Thus, our data, although correlative, support the idea that A-769662-induced ACC phosphorylation is β2-AMPK dependent. Importantly, however, and in contrast to previous findings (38), our findings in EDL suggest that β1-AMPK-containing complexes can be activated by A-769662 without any resulting increase in ACC phosphorylation.

In summary, we have investigated the effect of A-769662 on glucose uptake and AMPK activation. We found that A-769662-induced glucose uptake is dependent on genotype and muscle fiber type, increasing only in soleus from 129S6/sv mice. We also showed that the effect on glucose uptake is likely mediated by the PI3-kinase/Akt/TBC1D1/TBC1D4 signaling pathway. Thus, although we do not know the mechanism by which the PI3-kinase is activated by A-769662, our data suggest that A-769662 has off-target effects as previously shown (34). This should be taken into consideration in future studies involving A-769662. However, we also show that A-769662-induced AMPK activation in an intact muscle preparation is exclusively associated with β2-AMPK complexes. Since skeletal muscle is an important tissue for maintaining whole body glucose homeostasis and since no AMPK complexes in human skeletal muscle contain β1, development of future drugs targeting AMPK in skeletal muscle should acknowledge this tissue-specific expression of AMPK trimer complexes.

ACKNOWLEDGMENTS

We acknowledge skilled technical assistance from Thomas E. Jensen, Betina Bolmgren, and Pia Jensen. We appreciate the kind gift of various AMPK isoform-specific antibodies from Professor D. Grahame Hardie (Dundee University, UK), and we thank Dr. Benoit Viollet for providing mouse D-1-beta-D-ribofuranoside acutely stimulates skeletal muscle 2-deoxyglucose uptake in healthy men. Diabetes 56: 2078–2084, 2007.


