AMP-activated protein kinase activator A-769662 is an inhibitor of the Na⁺-K⁺-ATPase

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Submitted 9 January 2009; accepted in final form 13 October 2009

Benziane B, Björnholm M, Lantier L, Viollet B, Zierath JR, Chibalín AV. AMP-activated protein kinase activator A-769662 is an inhibitor of the Na⁺-K⁺-ATPase function in skeletal muscle cells. Short-term incubation of differentiated rat L6 myotubes with 100 µM A-769662 increased AMPK and acetyl-CoA carboxylase (ACC) phosphorylation in parallel with decreased Na⁺-K⁺-ATPase α₁-subunit abundance at the plasma membrane and ouabain-sensitive 86Rb⁺ uptake. Notably, the effect of A-769662 on Na⁺-K⁺-ATPase was similar in muscle cells that do not express AMPK α₁- and α₂-catalytic subunits. A-769662 directly inhibits the α₁-isozyme of the Na⁺-K⁺-ATPase, purified from rat and human kidney cells in vitro with IC₅₀ 57 µM and 220 µM, respectively. Inhibition of the Na⁺-K⁺-ATPase by 100 µM ouabain decreases sodium pump activity and cell surface abundance, similar to the effect of A-769662, without affecting AMPK and ACC phosphorylation. In conclusion, the AMPK activator A-769662 inhibits Na⁺-K⁺-ATPase activity and decreases the sodium pump cell surface abundance in L6 skeletal muscle cells. The effect of A-769662 on sodium pump is due to direct inhibition of the Na⁺-K⁺-ATPase activity, rather than AMPK activation. This AMPK-independent effect on Na⁺-K⁺-ATPase calls into question the use of A-769662 as a specific AMPK activator for metabolic studies.

AMPK is a metabolic master switch that regulates cellular energy homeostasis in response to metabolic or nonmetabolic stress (25, 28). AMPK is activated by an increase in the intracellular AMP-to-ATP ratio (AMP/ATP), driving a wide range of physiological effects in many tissues (24–26). A number of pharmacological agents activate AMPK in intact cells and tissues. These include biguanides metformin and phenformin (19, 44, 55) and thiazolidinediones (19, 30, 32), which affect the intracellular AMP/ATP ratio by inhibiting the mitochondrial respiratory chain (17, 19, 30, 41). Given the evidence that inhibition of mitochondrial respiration would have side effects unrelated to AMPK activation and that thiazolidinediones are potent peroxisome proliferator-activated receptor-γ activators (43), the adenosine analog 5-aminomimidazole-4-carboxamide-β-D-ribofuranoside (AICAR) is currently the most widely used AMPK activator. AICAR is taken up into the cell and converted by adenosine kinase to the monophosphorylated nucleotide, ZMP, which mimics the effect of AMP on AMPK activation (14). AICAR may also have AMPK-independent side effects (21, 33, 38, 46).

A small-molecule AMPK activator (A-769662) has been recently identified (13). A-769662 directly activates AMPK in cell-free assays through a mechanism that is distinct from AMP activation (45), and acts as a very potent pharmacological AMPK activator (8, 13, 20, 45). Administration of A-769662 to ob/ob mice decreases plasma glucose, body weight gain, and both plasma and liver triglyceride levels (13). These effects are beneficial when treating metabolic disorders such as type 2 diabetes and obesity. Thus, compound A-769662 holds promise in the search for a new class of drugs targeting AMPK, as well as an experimental tool for the study of the physiological effects of AMPK activation on a cellular level as well as whole body metabolism.

AMPK may regulate ion transport since AMPK activation inhibits the epithelial Na⁺ channel (2, 3, 7), O₂-sensitive K⁺ channel (16), cystic fibrosis transmembrane conductance regulator Cl⁻ channel (23, 29), and the renal-specific Na⁺, K⁺-2Cl⁻ cotransporter (18), prevents alkaline pH- and PKA-induced apical vacuolar H⁺-ATPase accumulation in epididymal clear cells (22), and mediates preconditioning in cardiac cells by regulating the activity and recruitment of sarcoplasmal ATP-sensitive K⁺ channel (48). Pharmacological activation of AMPK by AICAR reduces ouabain-sensitive Na⁺ transport in a lung epithelial cell line (H441), suggesting a role for AMPK in the regulation of the Na⁺-K⁺-ATPase (52, 53). Na⁺-K⁺-ATPase is an ubiquitously expressed energy-transducing ion pump that converts the energy of ATP into transmembrane ion gradients. The Na⁺-K⁺-ATPase activity is essential for the maintenance of electrolyte balance, preservation of membrane potential, and control of cellular volume in all tissues (5, 34). The effect of AICAR on sodium pump is compatible with recent evidence indicating that CO₂- and AICAR-induced AMPK activation in primary alveolar epithelial cells promotes a decrease in Na⁺-K⁺-ATPase activity and the pump endocytosis (50). This suggests that in response to AMPK activation, most of the energy-consuming processes in the cell, including Na⁺-K⁺-ATPase ion pumping function, which consumes roughly 25% of all cytoplasmic ATP (34), are switched off.
The effect of AMPK or AMPK-activating drugs on regulation of ion transport is equivocal. In rat cardiomyocytes, AICAR inhibits the Na\(^+\)H\(^+\) exchanger independently from activation of AMPK (38, 46). At the whole body level, infusion of AICAR in rats is associated with an acute fall in plasma K\(^+\) concentration ([K\(^+\)]) without an increase in K\(^+\) urinary excretion, concomitant with an increase in AMPK phosphorylation, suggesting a possible increase in skeletal muscle Na\(^+\)-K\(^+\)-ATPase or potassium channel activity (54). The effect of AICAR on plasma [K\(^+\)] was blunted in mice overexpressing a kinase-dead form of AMPK (54). However, the mechanism of whole body AICAR-induced potassium clearance and whether AMPK activation affects K\(^+\)-ATPase activity remains to be determined. Thus, the aim of the present study was to determine the effect of the novel AMPK activator A-769662 on Na\(^+\)-K\(^+\)-ATPase in cultured skeletal muscle cells to exclude an influence of systemic and nonspecific effects of known AMPK activators such as AICAR, biguanides, and thiazolidinediones.

**EXPERIMENTAL PROCEDURES**

**Antibodies and reagents.** A-769662 was a generous gift of Dr. Kei Sakamoto (University of Dundee, Dundee, UK). Specific anti-Na\(^+\)-K\(^+\)-ATPase 6H \(\alpha_1\)-subunit monoclonal antibodies were obtained from Dr. M. Caplan (Yale University, New Haven, CT). The McK1 antibody was generously given by K. Swope (Massachusetts Central Hospital, Boston, MA). Immunoprecipitation of total Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit was performed using the polyclonal antibody, anti-NK1, raised against purified rat kidney homogenate (10). The antibodies against phospho-Akt p-Ser\(473\), phospho-ERK1/2 (p-Thr\(202/204\)), phospho-AMPK p-Thr\(172\), phospho-acetyl-CoA carboxylase (ACC) p-Ser\(79\), and pan-\(\alpha\)AMPK were from Cell Signaling Technology (Beverly, MA). Kinase inhibitors Compound C, GF109203X, and the PKC activator phorbol 12-myristate 13-acetate (PMA) were from Calbiochem (La Jolla, CA). Streptavidin-agarose beads and EZ-link sulfo-NHS-SS-biotin were obtained from Pierce (Rockford, IL). Cell culture media and reagents were from Gibco (Invitrogen, Sweden). Human insulin (Actrapid) was from Novo Nordisk AS (Copenhagen, Denmark). Dimethyl sulfoxide (DMSO; Calbiochem) was used as a solvent for the protein kinase and phosphatase inhibitors. All other reagents were of analytical grade (Sigma, St. Louis, MO).

**Cell culture.** L6 cells (provided by Dr. Amira Klip, The Hospital for Sick Children, Toronto, ON, Canada) were grown in αMEM supplemented with 10% FBS, 1% penicillin and streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin), and 1% fungizone in 5% CO\(_2\)-95% O\(_2\) humidified air at 37°C. L6 cells were differentiated into myotubes for 6 days in αMEM supplemented with 2% FBS. Myotubes were serum starved 18 h before the start of the experiment. Differentiated L6 myotubes express only \(\alpha_1\)-subunit isoform of Na\(^+\)-K\(^+\)-ATPase (42).

**AMPK \(\alpha\) knockout cells.** Primary muscle cell cultures were derived from gastrocnemius and tibialis anterior muscles of 4-wk-old wild-type and AMPK\(\alpha_1\)-/-/-, AMPK\(\alpha_2\)-/-/- mice as previously described (40). Targeted disruption of AMPK\(\alpha_2\) loci was performed by injection of myoblasts with an adenovirus expressing the Cre recombinase (L. Lantier and B. Viollet, unpublished observations). Cells were grown supplemented with DMEM-F-12 containing 1% penicillin and streptomycin, 0.2% fungizone, 1% glutamine (Gibco), 10% FBS, and 2% Ultrasor G (Pall Life Sciences). The plates were precoated with 0.02% gelatin. Cells were differentiated into myotubes for 4 days in DMEM-F-12, 1% penicillin and streptomycin, 0.2% fungizone, 1% glutamine, and 2% horse serum (Gibco) in plates coated with Matrigel (BD Biosciences). On the day of the experiment, cells were serum starved for 4–5 h.

**Small interference RNA transfection of myotubes.** The small interference RNA (siRNA) sequences used to silence rat AMPK\(\alpha_1\) and \(\alpha_2\) were as follows: AMPK\(\alpha_1\) GCA UAU GCU GCA GGU AGA UdTdT and AMPK\(\alpha_2\) CGU CAU UGA UGA UGA GGC UdTdT (Qiagen) (30). A scrambled siRNA was used as a control in all experiments (seeded and grown as described in Cell culture). L6 cells were transfected with 100 nM siRNA using calcium phosphate (CellPfect Transfection kit; GE Healthcare) on days 2 and 4 of differentiation. siRNA-calcium phosphate precipitates were removed 16–18 h after the addition of siRNA. Experiments were performed 48–72 h after the last transfection.

**Cell incubation.** For the protein phosphorylation analysis, 6 day-differentiated myotubes were preincubated for 40 min either with 0.1% DMSO or with 100 μM A-769662 (AMPK activator). After the preexposure, cells were stimulated with insulin (120 nM) for 20 min or incubated for an additional 20 min. After treatment, cells were washed twice with ice-cold PBS and were harvested by being scraped into ice-cold lysis buffer. After the lysis, the resulting supernatant was centrifuged at 41,000 g for 75 min to pellet light-density microsomes, and AMPK activator. Experiments were performed 40 min after the addition of siRNA. Experiments were performed 48–72 h after the last transfection.

**Measurement of ouabain-sensitive \(86\)Rb\(^+\) uptake.** Na\(^+\)-K\(^+\)-ATPase transport activity was measured as ouabain-sensitive uptake of \(86\)Rb\(^+\), under conditions of initial rate, as previously described (1). Myotubes (day 6 of differentiation) were grown on six-well plates (Costar, Cambridge, MA) and were preincubated in serum-free αMEM with 1% DMSO or 40 μM AMPK inhibitor Compound C for 10 min at 37°C. After the preexposure, cells were stimulated with A-769662 (100 μM) for 40 min. Thereafter, myotubes were incubated in the presence or absence of ouabain (2 mM) and AMPK activators and/or inhibitors for 15 min. Indicated, Na\(^+\)-K\(^+\)-ATPase transport activity was determined after the addition of 50 μl of medium containing tracer amounts of \(^{86}\)RbCl (100 nCi/sample; GE Healthcare) for 10 min. Incubation was stopped by cooling on ice, and plates were washed three times with an ice-cold washing solution containing 150 mM choline chloride, 1.2 mM MgSO\(_4\), 1.2 mM CaCl\(_2\), 2 mM BaCl\(_2\), and 5m M HEPES, pH 7.4. Cells were lysed in 500 μl lysis buffer \(A\) and the radioactivity was measured by liquid scintillation. Protein content was determined in parallel using the BCA assay. Ouabain-sensitive \(^{86}\)Rb\(^+\) uptake was calculated as the difference between the mean values measured in triplicate samples incubated without or with 2 mM ouabain. Basal ouabain-sensitive \(^{86}\)Rb\(^+\) uptake was 7.8 ± 0.2 picomoles of Rb per microgram of protein per minute.

**Subcellular fractionation of L6 myotubes.** Subcellular fractionation of differentiated L6 myotubes was performed as previously described (4). The cells from 10-cm dishes (controls or A-769662-treated for 40 min) were gently scraped and placed on ice. All subsequent steps were carried out at 4°C. The cell pellet was resuspended in homogenization buffer (255 mM sucrose, 2 mM Na\(_2\)EDTA, 2 mM EGTA, 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A, and 2 mM HEPES; pH 7.4) and then homogenized using 15 strokes of a Dounce homogenizer (tight fitting). The homogenate was centrifuged at 19,000 g for 20 min, and the resulting supernatant was centrifuged at 41,000 g for 20 min to yield the high-density microsomes. The supernatant was centrifuged at 195,000 g for 75 min to pellet light-density microsomes. The pellet from the 19,000 g centrifugation was resuspended in 4 ml of homogenization buffer and layered on top of 7 ml Sucrose Cushion (1.12 M sucrose, 2 mM Na\(_2\)EDTA, 20 mM HEPES) and
centrifuged at 100,000 g for 60 min. The supernatant from the interphase was centrifuged at 40,000 g for 20 min, and the pellet corresponding to the plasma membrane was resuspended in homogenization buffer. Protein concentration was determined using a BCA protein assay kit.

Cell surface biotinylation. Myotubes (day 6 of differentiation) or wild-type and AMPK α knockout cells were preincubated in serum-free culture media in the absence or presence of 100 μM A-769662 for 40 min, washed three times with ice-cold PBS, and thereafter exposed to EZ-link sulfo-NHS-SS-biotin at a final concentration of 1.5 mg/ml in PBS at 4°C for 60 min with gentle shaking. Cell surface biotinylation was performed as described previously (1). After biotinylation, cells were harvested and lysed in ice-cold buffer A as described above, and cell lysates were subjected to streptavidin precipitation. After streptavidin precipitation, samples were analyzed by SDS-PAGE with subsequent Western blot with appropriate antibodies.

Metabolic labeling of myotubes with 32P, 33P, metabolic labeling was performed (1) to investigate in vivo phosphorylation of α-subunits of Na+-K+-ATPase. Myotubes (day 6 of differentiation) grown on 60-mm dishes were incubated for 3 h at 37°C in serum-free αMEM containing 32P (1 μCi/ml). A-769662 (100 μM) or PKC activator PMA (500 nM) was added during the last 40 min of incubation time. The incubation was terminated by cooling the culture dishes on ice and washing with the cells with ice-cold PBS. Myotubes were lysed in buffer A, and α-subunits were immunoprecipitated with polyclonal anti-NK1 rabbit antibodies. The bead pellets were mixed with Læmmli buffer (60 μl) (62.5 mM Tris·HCl, 2% SDS, 10% glycerol, and 10 mM DTT), separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Phosphoproteins were analyzed using Bio-Imaging Analyzer BAS-1800II (Fuji Photo Film, Japan), and quantification was performed using the Image Gauge software, version 3.4 (Fuji Photo Film). In each experiment, the amount of radioactivity incorporated into the α-subunit was corrected for the amount of the protein detected by the Western blot analysis. The quantitative data are reported as percentage of basal.

Immunoprecipitation. Myotubes were lysed in 0.5 ml ice-cold lysis buffer A. Insoluble material was removed by centrifugation (12,000 g for 10 min at 4°C). Aliquots of supernatant (300 μg of protein) were immunoprecipitated overnight at 4°C with 50 μl of polyclonal anti-NK1 rabbit antibodies. Immunoprecipitates were collected on protein G-agarose beads (Invitrogen Dynal, Oslo, Norway). Beads were washed four times in lysis buffer A and twice in PBS. Pellets were resuspended in Laemmli sample buffer.

Western blot analysis. Aliquots of cell lysate (30 μg of protein) or immunoprecipitates were resuspended in Laemmli sample buffer. Proteins were then separated by SDS-PAGE, transferred to PVDF membranes, blocked with 7.5% nonfat milk, washed with TBST (10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20) and incubated with appropriate primary antibodies overnight at 4°C. Membranes were washed with TBST and incubated with an appropriate secondary antibody. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

Na+-K+-ATPase purification. HEK 293 cells were grown until confluence, trypsinized, and harvested by centrifugation (160 g for 10 min). Cells were washed three times with ice-cold PBS. The final cell pellet was resuspended in 10–15 volumes of imidazol-sucrose buffer (25 mM imidazole, 1 mM EDTA, and 250 mM sucrose, pH 7.2) and homogenized by 20 strokes in glass-glass homogenizer on ice. The homogenate was centrifuged (9,000 g for 10 min at 4°C), and the pellet after this centrifugation was homogenized and centrifuged again (9,000 g for 10 min at 4°C). The two supernatants were combined and centrifuged (190,000 g for 45 min at 4°C). The pellet of crude membranes after this centrifugation was resuspended in the imidazol-sucrose buffer and was used as starting material for Na+-K+-ATPase purification as described previously (1, 27). Rat Na+-K+-ATPase holoenzyme was purified from rat kidney cortex as described previously (27). The Ethical Committee for North Region of Stockholm approved the study protocol. Quality of purification was verified by SDS-PAGE (7.5% gel) following Coomassie staining.

In vitro Na+-K+-ATPase activity. Purified Na+-K+-ATPase activity has been determined as described previously under Vmax conditions (1). For the concentration-response analysis, the samples were preincubated with the various compounds for 10 min at 37°C, before the reaction was started by the addition of a solution containing cold ATP and [γ-32P]ATP (3.3 nCi/μl). Analysis of concentration-response curves and the determination of half-maximal (50%) inhibitory concentration (IC50) were performed using GraphPad Prism software (San Diego, CA).

Statistical analysis. Data are presented as means ± SE. Comparisons between groups were performed using Student’s t-test. For multiple comparisons, one-way ANOVA with Sheffe’s correction was used. Significance was established at P < 0.05.

RESULTS

Dose dependence and time course of AMPK activation by A-769662 in L6 muscle cells. To determine the optimal conditions to obtain the maximal response of A-769662 on AMPK activation in L6 cells, we tested different concentrations (1, 10, 50, 100, and 250 μM) of A-769662 and different incubation times (10, 20, 40, 60, and 120 min). We found that 100 μM of A-769662 for 40 min in L6 cells gave a maximal increase of AMPK phosphorylation at Thr172 around twofold increase compared with basal phosphorylation (Fig. 1, A and C). The phosphorylation of AMPK substrate ACC at Ser79 exhibited a similar pattern (Fig. 1, B and D).

A-769662 activates AMPK signaling pathways in L6 muscle cells independently of insulin. To validate our experimental model, we assessed whether A-769662 alters AMPK and insulin signaling in L6 muscle cells. Incubation of differentiated L6 myotubes with 100 μM of A-769662 for 40 min increased AMPK phosphorylation at Thr172 compared with basal (Fig. 2A). Phosphorylation of the downstream AMPK substrate ACC at Ser79 exhibited a similar response in the presence of A-769662 (Fig. 2B). To prove that A-769662 was not affecting insulin signaling, phosphorylation of Akt at Ser473 and ERK at Thr202/Tyr204 was measured. A-769662 alone was without effect on phosphorylation of Akt and ERK, although insulin stimulated Akt and ERK/1/2 phosphorylation in the presence of A-769662 (Fig. 2D). These data indicate that, in L6 cells, A-769662 activates AMPK without interfering with the insulin signaling pathway.

A-769662 inhibits the Na+-K+-ATPase transport activity and cell surface abundance in L6 cells. To determine whether A-769662-induced AMPK activation affects the ion transport activity of the sodium pump in L6 cells, ouabain-sensitive 86Rb uptake, as a measure of Na+-K+-ATPase activity, cell surface biotinylation and subcellular fractionation, to assess abundance of the α1-subunit of the sodium pump in the plasma membrane, were performed in the absence or presence of A-769662. Incubation with 100 μM A-769662 for 40 min leads to a decrease of sodium pump activity (Fig. 3A), concomitant with a decrease of Na+-K+-ATPase abundance at the membrane surface compared with the basal condition (Fig. 3, B and C). These results provide evidence to suggest that A-769662 in-
hibits the sodium pump activity and decreases the abundance of Na\(^+\)-K\(^+\)-ATPase at the cell surface. Compounds C does not prevent A-769662 effect on AMPK in L6 cells. We studied the action of an inhibitor of AMPK, Compound C, on A-769662 effect in L6 cells. Compound C prevents the effects on O\(_2\)-sensitive K\(^+\) channel activity induced by the activation of AMPK by A-769662 (16). The effect of A-769662 on ouabain-sensitive \(^{36}\)Rb\(^+\) uptake and Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit abundance at the plasma membrane was unaltered by Compound C (Supplemental Fig. 1, A and B; supplemental data for this article can be found online at the American Journal of Physiology-Cell Physiology website).
However, Compound C was unable to prevent the A-769662-induced increase in phosphorylation of ACC at Ser79 (Supplemental Fig. 1D). These data indicate that A-769662 may abrogate the effect of compound C on AMPK inhibition.

α-AMPK siRNA does not abolish the A-769662-induced decrease in the transport activity and cell surface abundance of Na⁺-K⁺-ATPase in L6 cells. We silenced AMPK by siRNA targeting α1- and α2-subunits of AMPK. The silencing of AMPK decreased the expression of the AMPK catalytic α-subunits by 60% (Fig. 4A), concomitant with a proportional decrease in phosphorylation at Thr172 and phosphorylation of ACC at Ser79 (Fig. 4, B and C). However, silencing of α-AMPK did not abolish the effect of A-769662 on the activity of the sodium pump (Fig. 4D) and the Na⁺-K⁺-ATPase cell surface abundance (Fig. 4E). These data further confirm that the effect of A-769662 on the sodium pump is independent of AMPK activation.

A-769662 inhibits the Na⁺-K⁺-ATPase transport activity and cell surface abundance in AMPK α knockout cells. Because we were not able to completely knock down AMPK α expression in L6 cells by siRNA, the decrease of activity...
and cell surface expression of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase could be due to the remaining 40% of AMPK signaling. To test our hypothesis, we utilized primary mouse muscle cells that do not express \(\alpha_1\)- and \(\alpha_2\)-subunits of AMPK. Wild-type cells express AMPK \(\alpha\), and A-769662 increased the phosphorylation of AMPK at Thr\textsuperscript{172} and ACC at Ser\textsuperscript{279} in these cells (Fig. 5, A–C). Conversely, AMPK \(\alpha\)-subunits were not expressed in AMPK \(\alpha\) knockout cells, and AMPK signaling toward ACC was totally ablated (Fig. 5, A–C), providing evidence to suggest that AMPK is the only kinase that phosphorylates ACC in skeletal muscle cells. Wild-type and AMPK \(\alpha\) knockout cells express the similar amount of \(\alpha_1\)-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (Fig. 5D). Thus, we assessed A-769662 effects on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in these cells. In AMPK \(\alpha\) knockout cells, A-769662 effectively decreased both the transport activity and cell surface abundance of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (Fig. 6, A and B), giving a similar pattern as wild-type cells. These data further confirm that the effect of A-769662 on the sodium pump is independent of AMPK activation.

**Effect of A-769662 and different AMPK activators on the activity of purified Na\textsuperscript{+}-K\textsuperscript{+}-ATPase.** To test whether A-769662 had a direct effect on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, we have measured enzymatic activity of purified rat kidney Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in the presence of A-769662, utilizing an \(\lbrack \gamma\rbrack\textsuperscript{32P}\)-ATP hydrolysis technique. Kidney Na\textsuperscript{+}-K\textsuperscript{+}-ATPase exclusively contains the \(\alpha_1\)-isoform (5). Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was inhibited 71% in the presence of 100 \(\mu\)M A-769662 (Fig. 7A). To determine whether inhibition on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by A-769662 is specific, we have measured Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in the presence of other AMPK activators, in concentrations previously described to increase AMPK activity in skeletal muscle cells (AICAR, 1 mM; metformin, 1 mM; rosiglitazone, 100 \(\mu\)M) (8, 19). These AMPK activators had no direct effect on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (Fig. 7A). These results clearly indicate that A-769662 promotes an inhibitory effect via direct interaction with Na\textsuperscript{+}-K\textsuperscript{+}-ATPase.

The IC\textsubscript{50} value was determined for the effect of A-769662 (Fig. 7B) on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase using \(\alpha_1\)-isoform-specific preparations purified from rat and human kidney cells. The well-characterized Na\textsuperscript{+}-K\textsuperscript{+}-ATPase inhibitor ouabain (34) was used as a reference compound. A-769662 inhibits the rat kidney enzyme with IC\textsubscript{50} similar to that observed for ouabain (57 \(\mu\)M for A-769662 and 14 \(\mu\)M for ouabain, respectively) (Fig. 7B). Contrary to rat enzyme, the human Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is less sensitive to A-769662 inhibition and, as expected (5) is more sensitive to ouabain with IC\textsubscript{50} for A-769662 of 220 \(\mu\)M and 15 \(\mu\)M for ouabain, respectively (Fig. 7C).

A-769662 and ouabain decrease Na\textsuperscript{+}-K\textsuperscript{+}-ATPase cell surface abundance in L6 cells. Since A-769662 inhibited Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and decreases its cell surface abundance, we tested the hypothesis that the inhibition of the sodium pump by compounds other than A-769662 would lead to a decrease in its cell surface abundance. Thus, we compared the effect of A-769662 and ouabain. Ouabain at a concentration of 100 \(\mu\)M inhibits the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase to the same extent as 100 \(\mu\)M A-769662 (Fig. 7B). At this concentration, both ouabain and A-769662 (40 min of incubation) exposure reduced the abundance of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase at the cell surface ~40% without modifying total Na\textsuperscript{+}-K\textsuperscript{+}-ATPase expression (Fig. 8A). As expected, A-769662 increased phosphorylation of AMPK at Thr\textsuperscript{172} and ACC at Ser\textsuperscript{79} after 40 min of stimulation; the effect was noticeable already after 10 min stimulation (Fig. 8, B and C). Ouabain was without effect on AMPK and ACC phosphorylation (Fig. 8, B and C); however, ouabain increased ERK1/2 phosphorylation at Thr\textsuperscript{202/Tyr\textsuperscript{204}} (Fig. 8D). A-769662 was without effect on ERK phosphorylation (Fig. 8D). These data indicate that the effect of A-769662 and ouabain on the cell surface abundance of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is unrelated to AMPK or ERK1/2 activation.

**Lack of phosphorylation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit in L6 muscle cells in response to A-769662.** Phosphorylation of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit triggers signals for Na\textsuperscript{+}-
AMPKα small interference (si)RNA does not abolish the A-769662-induced decrease in the transport activity and cell surface abundance of Na⁺/K⁺-ATPase. L6 cells were transfected with siRNA directed against AMPK α-subunits or scramble sequence as indicated in EXPERIMENTAL PROCEDURES. Transfected cells were incubated in the presence or absence of 100 μM A-769662 for 40 min, and the cell surface proteins were biotinylated with the cleavable biotinylation reagent sulfo-NHS-SS-biotin. A–C: aliquots of the total cell extract from each sample were separated on SDS-PAGE gel and immunoblotted with pan-AMPK (A), phospho-Thr172 AMPK (B), and phospho-Ser79 ACC (C) antibodies. Densitometric analysis of different proteins is shown as fold of basal. Data are means ± SE (n = 7). *P < 0.05 vs. basal. A representative Western blot image is shown at top of each panel. 

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Fig. 4. A representative Western blot image is shown at top of each panel. D: ouabain-sensitive ⁸⁶Rb⁺ uptake assay was performed as described in EXPERIMENTAL PROCEDURES. Results are means ± SE (n = 9–15). *P < 0.05 vs. basal. E: biotinylated proteins were recovered from cell extracts by precipitation on streptavidin-agarose. Na⁺/K⁺-ATPase α₁-subunit on the cell surface was detected by Western blot analysis using an antibody against the Na⁺/K⁺-ATPase α₁-subunit. Representative blot and densitometric analysis of cell surface Na⁺/K⁺-ATPase α₁-subunit are shown. Quantitative data are shown as means ± SE (n = 7–10). *P < 0.05 vs. basal.
K⁺-ATPase endocytosis and exocytosis (1, 11, 12). To determine whether A-769662 or ouabain promotes phosphorylation of Na⁺-K⁺-ATPase, myotubes were metabolically labeled with ³²P, for 3 h. Thereafter, cells were incubated with A-769662, ouabain, or the PKC activator PMA for 40 min. Neither A-769662 nor ouabain altered the pattern of total protein phosphorylation, while PMA notably increased protein phosphorylation (Supplemental Fig. 2A). We used a NK1 rabbit antibody, which has been previously reported to precipitate the Na⁺-K⁺-ATPase α₁-subunit. Background phosphorylation of Na⁺-K⁺-ATPase α₁-subunit was observed after 3 h of incubation of L6 myotubes in phosphorylation media. PMA increased total phosphorylation of Na⁺-K⁺-ATPase α₁-subunits 2.3-fold (Supplemental Fig. 2B). Phosphorylation of the Na⁺-K⁺-ATPase β-subunit was undetected (data not shown). To further explore the potential role of A-769662 or ouabain in Na⁺-K⁺-ATPase α₁-subunit phosphorylation, we utilized an antibody (McK1) that specifically recognizes the nonphosphorylated form of the enzyme on Ser₁₈, a residue that is a target for phosphorylation by protein kinase C (PKC). Phosphorylation of the α₁-subunit on Ser₁₈ is essential for Na⁺-K⁺-ATPase endocytosis (11, 12, 15). The use of McK1 as a tool to investigate phosphorylation of the α₁-subunit has been previously validated (9, 49). Ser₁₈ phosphorylation of the α₁-subunit of L6 cells was determined by immunoblot analysis with McK1. Incubation with A-769662 or ouabain was without effect on phosphorylation of Na⁺-K⁺-ATPase α₁-subunit, while PMA caused a decrease in McK1 immunoreactivity (Supplemental Fig. 2C), indicating PKC-dependent phosphorylation on Ser₁₈. These data provide evidence to suggest that the decrease in Na⁺-K⁺-ATPase
abundance in response to its inhibition by A-769662 or ouabain is independent from the pump subunit phosphorylation.

DISCUSSION

Activation of AMPK in peripheral tissues leads to an increase in skeletal muscle glucose uptake, glycolysis, fatty acid oxidation and inhibition of fatty acid synthesis and hepatic gluconeogenesis (26, 37). These highlight AMPK as an attractive target for the treatment of metabolic disorders. The discovery of a small-molecule direct activator of AMPK, the A-769662 compound, validated the role of AMPK in the treatment of metabolic disorders and provided evidence that AMPK activators may afford a feasible therapeutic approach for the clinical use.

Fig. 6. The decrease in the transport activity and cell surface abundance of Na\(^+\)-K\(^+\)-ATPase is not prevented in AMPK\(\alpha\) knockout cells. Wild-type and AMPK\(\alpha\) knockout cells were incubated with A-769662 (100 \(\mu\)M for 40 min). A: ouabain-sensitive \(^{86}\)Rb\(^+\) uptake assay was performed as described in EXPERIMENTAL PROCEDURES. Results are means \(\pm\) SE \((n = 9–15), *P < 0.05\) vs. wild-type basal; \#P < 0.05 vs. AMPK\(\alpha\) knockout basal. B: biotinylated proteins were recovered from cell extracts by precipitation on streptavidin-agarose. Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit on the cell surface was detected by Western blot analysis using an antibody against the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit. Representative blots and densitometric analysis of cell surface Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit are shown. Quantitative data are shown as means \(\pm\) SE \((n = 7–10), *P < 0.05\) vs. basal, \#P < 0.05 vs. AMPK\(\alpha\) knockout basal.

Fig. 7. A-769662 inhibits the purified Na\(^+\)-K\(^+\)-ATPase. A: Na\(^+\)-K\(^+\)-ATPase purified from rat kidney was incubated with either A-769662 (100 \(\mu\)M for 40 min), 5-aminoimidazole-4-carboxamide-\(\beta\)-d-ribofuranoside (AICAR; 1 mM for 30 min), metformin (1 mM for 30 min), or rosiglitazone (100 \(\mu\)M for 30 min). The activity of Na\(^+\)-K\(^+\)-ATPase was assessed by measuring the release of \(^{32}\)P. Quantitative data are shown as means \(\pm\) SE \((n = 6), *P < 0.05\) vs. basal. B and C: Na\(^+\)-K\(^+\)-ATPase purified from rat kidney cortex (B) or human proximal tubular cells (C) was incubated with different concentrations of A-769662 and ouabain. Ouabain (2 mM or 10 \(\mu\)M) totally inhibits rat and human Na\(^+\)-K\(^+\)-ATPase, respectively. Quantitative data are shown as means \(\pm\) SE \((n = 6–12), *P < 0.05\) vs. basal.
management of type 2 diabetes (13). However, with any pharmacological treatment strategy, off-target effects may be observed. The results of the present study provide new information about mechanism of action of this AMPK activator and provide novel insight into effects of A-769662 on Na\(^+\)/K\(^+\)-ATPase.

We found that in rat L6 skeletal muscle cells, short-term incubation with A-769662 increased AMPK and ACC phosphorylation in parallel with decreased Na\(^+\)/K\(^+\)-ATPase \(\alpha_1\)-subunit abundance at the plasma membrane and ouabain-sensitive \(^{86}\)Rb\(^+\)-uptake. Notably, the effect of A-769662 on Na\(^+\)/K\(^+\)-ATPase was unaltered by siRNA AMPK silencing in rat muscle L6 cells and in primary AMPK \(\alpha_1\)-knockout mouse muscle cells. This finding provides evidence to suggest that A-769662 inhibits Na\(^+\)/K\(^+\)-ATPase independently from AMPK activation. A-769662 directly inhibits the \(\alpha_1\)-isoform of the Na\(^+\)/K\(^+\)-ATPase in preparation of purified enzyme isolated from rat and human kidney cells, under \(V_{\text{max}}\) condition at high (5 mM) ATP concentration, suggesting that A-769662 is not competing with ATP on the enzyme’s active site. Enzyme kinetic studies have shown that \(K_m\) of the Na\(^+\)/K\(^+\)-ATPase is low, suggesting that only extreme ATP depletion would alter its activity (6). The inhibitory effect of A-769662 on Na\(^+\)/K\(^+\)-ATPase activity is unique in comparison to other known AMPK activators. AICAR, metformin, and rosiglitazone, in concentrations previously described to increase AMPK activity in intact cells, were without effect on the activity of purified rat kidney Na\(^+\)/K\(^+\)-ATPase. Interestingly,
A-769662 displays a greater specificity to rat, as compared with the human α1 Na+/K+-ATPase isozyme (IC₅₀ 57 μM and 220 μM, respectively). Conversely, human α2 Na+/K+-ATPase is much more sensitive to ouabain (IC₅₀ 15 nM vs. 14 μM for rat kidney enzyme). Thus, A-769662 is unlikely to interact with Na+/K+-ATPase at the ouabain binding site.

AMPK activation in polarized epithelial cells from lung has been reported to decrease Na+/K+-ATPase activity and pump endocytosis (50, 52, 53). However, the molecular mechanism of Na+/K+-ATPase inhibition in lung cells involves activation of PKCζ downstream of AMPK, following PKCζ-dependent phosphorylation of the Na+/K+-ATPase α₁-subunit on Ser¹⁸ (15, 50). Here we report that Na+/K+-ATPase α₁-subunit phosphorylation was unaltered in response to AMPK activation by A-769662 in L6 cells (Supplemental Fig. 2, B and C). Thus, if AMPK activation affects the sodium pump in skeletal muscle cells, this effect is independent of changes in Na+/K+-ATPase phosphorylation. Moreover, AMPK activation is unlikely to inhibit Na+/K+-ATPase activity in skeletal muscle. Conversely, activation of the energy sensing AMPK may be a potential mechanism for contraction-induced activation of the sodium pump in skeletal muscle. Muscle contractions are a potent activator of AMPK (24, 37). Despite the associated energy consuming increase in ATP synthesis, the increase in Na+/K+-ATPase activity is essential for ion homeostasis during the contraction process. The active sodium pump preserves the ionic gradients, which are essential for maintaining the action potential and to allow for repeated contractions. Indeed, stimulation of L6 myotubes with AICAR or anoxia leads to AMPK-dependent activation of Na+/K+-ATPase, independently of Na+/K+-ATPase phosphorylation (B. Benziane, M. Björnholm, and A. V. Chibalin, unpublished observation). Thus, the effect of A-769662 on Na+/K+-ATPase overrides any AMPK-mediated activation that this compound may also exhibit.

Inhibition of the Na+/K+-ATPase in L6 cells, by either A-769662 or ouabain, leads to a decrease in the Na+/K+-ATPase cell surface abundance, which most likely is due to increased endocytosis of pump units. This may reflect a common mechanism of removing nonfunctional pump molecules from the cell surface with a subsequent dissociation of the inhibitor from the Na+/K+-ATPase in intracellular compartments. After the dissociation, the Na+/K+-ATPase could return to the cell surface via a mechanism involving constitutive exocytosis or it may undergo lysosomal degradation. Low doses of ouabain stimulate sodium pump endocytosis in a manner analogous to hormone and receptor-mediated sodium pump endocytosis (31, 35, 36). However, receptor-mediated endocytosis of rodent Na+/K+-ATPase requires phosphorylation of the α₁-subunit at Ser¹⁸ (11, 15). Neither A-769662 nor ouabain increases α₁-subunit Ser¹⁸ phosphorylation of the sodium pump in L6 muscle cells (Supplemental Fig. 2, B and C). Thus, A-769662 and ouabain appear to promote sodium pump endocytosis via an alternative pathway, independent of Ser¹⁸ phosphorylation. The mechanism by which inhibition of Na+/K+-ATPase triggers a decrease in cell surface abundance remains to be elucidated.

The AMPK-independent inhibition of the Na⁺/K⁺-ATPase constitutes one of several reported side effects of A-769662. This compound also inhibits nonproteolytic components of the 26S proteasome by an AMPK-independent mechanism (39). The proteasome contains six Rpt ATPases that are required for the unfolding of protein substrates and their translocation to the proteolytic chamber (51). Thus, the AMPK activator A-769662 appears to also be a general ATPase inhibitor. However, the ability of A-769662 to inhibit Na⁺/K⁺-ATPase under Vₘₐₓ conditions at high ATP concentrations contradicts the hypothesis. The ability of A-769662 to directly inhibit distinct ATPases beyond the Na⁺/K⁺-ATPase requires further verification.

Hypothetically, the effect of A-769662 on AMPK activity in living cells and tissues could consist of two components. One component is the direct activation of AMPK by A-769662, due to binding of the compound directly to the AMPK molecule. Another component could be through an increase in the cellular ATP concentration, which may then decrease the AMP/ATP ratio, due to inhibition of the sodium pump (and, possibly, other ATPases). The net effect of these processes, acting in opposition, may mask the effect of A-769662 to activate AMPK in intact cells or at the whole body level. Indeed, inhibition of Na⁺/K⁺-ATPase by ouabain in rat parotid acinar cells decreases AMPK activity, due to a decrease in the AMP/ATP ratio (47). However, we did not observe any effect of ouabain on AMPK and ACC phosphorylation in L6 muscle cells (Fig. 8, B and C). Conversely, A-769662 activates AMPK by an AMP-independent mechanism (45). The physiological consequences, as well as the relationship between direct effect of A-769662 on AMPK activity and possible indirect effects arising from changes in the AMP/ATP ratio, require further investigation.

In conclusion, the AMPK activator A-769662 directly inhibits activity and decreases the cell surface abundance of the sodium pump in skeletal muscle cells. These effects are similar in magnitude to that of the Na⁺/K⁺-ATPase inhibitor ouabain. Furthermore, the effect of A-769662 on Na⁺/K⁺-ATPase is independent of AMPK activation. Our findings suggest that the physiological effects of the AMPK activator A-769662 may also be due to inhibition of the sodium pump and alterations in both ion homeostasis and trans-membrane currents. This AMPK-independent effect on Na⁺/K⁺-ATPase challenges the experimental and clinical utility of A-769662 as a specific AMPK activator for metabolic studies.

ACKNOWLEDGMENTS

We thank Dr. Kei Sakomoto for the generous gift of A-769662. We thank Dr. Michael Caplan and Dr. Kathleen Sweadner for the kind gift of anti-Na⁺/K⁺-ATPase α₁- and α₂-subunit antibodies. We are grateful to Dr. Geoff Holman and Dr. Jens Peter Andersen for helpful discussions and critical reading of the manuscript.

GRANTS

This work was supported by grants from the Swedish Research Council, Swedish Foundation for Strategic Research, the Novo-Nordisk Foundation, and the Commission of the European Communities (contract no. LSHM-CT-2004-512013 EUGENEHEART and contract no. LSHM-CT-2004-005272 EXGENESIS).

DISCLOSURES

No conflicts of interest are declared by the author(s).

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