Mechanism of Action of A-769662, a Valuable Tool for Activation of AMP-activated Protein Kinase*

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We have studied the mechanism of A-769662, a new activator of AMP-activated protein kinase (AMPK). Unlike other pharmacological activators, it directly activates native rat AMPK by mimicking both effects of AMP, i.e. allosteric activation and inhibition of dephosphorylation. We found that it has no effect on the isolated α subunit kinase domain, with or without the associated autoinhibitory domain, or on interaction of glycogen with the β subunit glycogen-binding domain. Although it mimics actions of AMP, it has no effect on binding of AMP to the isolated Bateman domains of the γ subunit. The addition of A-769662 to mouse embryonic fibroblasts or primary mouse hepatocytes stimulates phosphorylation of acetyl-CoA carboxylase (ACC), effects that are completely abolished in AMPK-α1/−β�/−γδ/− cells but not in TAK1−/− mouse embryonic fibroblasts. Phosphorylation of AMPK and ACC in response to A-769662 is also abolished in isolated mouse skeletal muscle lacking LKB1, a major upstream kinase for AMPK in this tissue. However, in HeLa cells, which lack LKB1 but express the alternate upstream kinase calmodulin-dependent protein kinase kinase-β, phosphorylation of AMPK and ACC in response to A-769662 still occurs. These results show that in intact cells, the effects of A-769662 are independent of the upstream kinase utilized. We propose that this direct and specific AMPK activator will be a valuable experimental tool to understand the physiological roles of AMPK.

The AMP-activated protein kinase (AMPK)3 is a regulator of energy balance at both the cellular and the whole body levels.

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** The abbreviations used are: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminooimidazole-4-carboxamide riboside; AID, autoinhibitory domain; CaMKK, calmodulin-dependent protein kinase kinase; CBS, cystathionine β-synthase; GBD, glycogen-binding domain; GST, glutathione S-transferase; MEF, mouse embryonic fibroblast; OCT1, organic cation transporter-1; TAK1, TGFβ-activated kinase-1; TGFβ, transforming growth factor-β; TBS, Tris-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

autoinhibitory domain; CaMKK, calmodulin-dependent protein kinase kinase; CBS, cystathionine β-synthase; GBD, glycogen-binding domain; GST, glutathione S-transferase; MEF, mouse embryonic fibroblast; OCT1, organic cation transporter-1; TAK1, TGFβ-activated kinase-1; TGFβ, transforming growth factor-β; TBS, Tris-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

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models of obesity and insulin resistance such as the ob/ob mouse, the fa/fa rat, and the high fat-fed rat, AICAR was shown to reverse many of their metabolic abnormalities (25–28). At around the same time, the biguanide metformin was shown to activate AMPK in intact cells and in vivo (29). Metformin is currently the drug of first choice for the treatment of type 2 diabetes, being prescribed to at least 120 million people worldwide. The therapeutic effects of the drug are primarily on the liver, probably because hepatocytes express the organic cation transporter OCT1, resulting in more rapid uptake of the drug into hepatocytes than other cells (30,31). Recent studies involving mice in which AMPK could not be activated in liver due to a tissue-specific knock-out of the upstream kinase, LKB1, revealed that the anti-hyperglycemic effects of metformin were abolished, suggesting that the major effect of the drug is to repress gluconeogenesis via activation of liver AMPK (32). It is possible that an AMPK activator that was also effective in organs other than the liver would have additional efficacy.

Although metformin is relatively safe, it is not effective in all patients, perhaps due to variability in the efficiency of hepatic uptake by the OCT1 transporter (31). It does not activate AMPK or affect the phosphorylation or dephosphorylation of the kinase by upstream kinases and phosphatases in cell-free assays (33). However, metformin and the more potent biguanide, phenformin, have both been reported to be inhibitors of complex I of the respiratory chain (34,35), which suggests that they may activate AMPK indirectly by decreasing cellular ATP and increasing AMP. Significant changes in the cellular AMP:ATP ratio are indeed readily detected after treatment of cultured cells with phenformin (12), and decreases in ATP have recently been reported in primary rodent hepatocytes treated with metformin (36). Intestinal epithelial cells also express transporters of the OCT1 family (Oct1–3 (37)), and inhibition with metformin (36). Intestinal epithelial cells also express transporters of the OCT1 family (Oct1–3 (37)), and inhibition with metformin (36). Intestinal epithelial cells also express transporters of the OCT1 family (Oct1–3 (37)), and inhibition with metformin (36). Intestinal epithelial cells also express transporters of the OCT1 family (Oct1–3 (37)), and inhibition with metformin (36). Intestinal epithelial cells also express transporters of the OCT1 family (Oct1–3 (37)), and inhibition with metformin (36). Intestinal epithelial cells also express transporters of the OCT1 family (Oct1–3 (37)), and inhibition with metformin (36). It therefore seems quite likely that a drug that activated the AMPK system more directly would be more efficacious in the treatment of type 2 diabetes and the metabolic syndrome than the biguanides, while avoiding some of their side effects, which may be related to inhibition of the respiratory chain rather than to activation of AMPK per se. The results of a screen of >700,000 compounds designed to detect AMPK activators were reported recently (39). The thienopyridone A-592017 emerged from the initial screen, and after optimization, the more potent activator, A-769662, was developed. When administered to ob/ob mice, A-769662 had many of the effects expected for an AMPK activator, including decreases in plasma glucose and triglyceride, decreases in hepatic triglyceride, decreases in expression of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase and the lipogenic enzyme fatty acid synthase, and even decreases in weight gain (39). Although the utility of the compound as a drug may be limited by its poor oral availability, it does hold considerable promise as an experimental tool for the study of the downstream consequences of AMPK activation. However, the original study provided little information as to the exact mechanism of activation of AMPK by A-769662. We have therefore synthesized A-769662 and have now addressed its mechanism of action in cell-free assays and in intact cells.

Experimental Procedures

Materials and Antibodies—A-769662 was synthesized as described previously (40). STO-609 was from Tocris (Ellisville, Missouri). Protein G-Sepharose and [γ-32P]ATP were from Amersham Biosciences (Little Chalfont, UK), Protease inhibitor mixture tablets from Roche Applied Science (Lewes, UK), precast SDS-polyacrylamide gels from Invitrogen (Paisley, UK), phosphocellulose P81 paper from Whatman, and iodonycin and phenformin were from Sigma (Poole, Dorset, UK). Anti Pan-AMPKα (anti-AMPK, catalog number 2532), phospho-AMPK (anti-pT172, catalog number 2535), pan-ACC (anti-ACC, catalog number 3661), and phospho-ACC (anti-pACC, catalog number 3662) antibodies were from Cell Signaling Technology (New England Biolabs, UK). Anti-TAK1 was from Santa Cruz Biotechnology (catalog number sc-7162). Anti-GST antibodies were purified as a by-product of production of antibodies against GST-LKB1 (41). Antibodies recognizing glutathione S-transferase (GST) were removed from the anti-GST-LKB1 antisera using an immobilized GST column. GST fusions of the rat α1 kinase domain (1–312, wild type, and T172D mutant) (42) and human CaMKKβ in bacteria (12) were expressed and purified as described. Rat α1 kinase domain (residues 1–310) and rat α1 kinase domain plus the autoinhibitory domain (residues 1–333) were amplified by PCR from a plasmid expressing the full-length α1 subunit (sense oligonucleotide, 5’-acctcggaattcgaggaagagaagcagccg-3’; 312 antisense oligonucleotide, 5’-tggttttctgcagagcagctgag-3’; 323 antisense oligonucleotide, 5’-gcgtctgaggtatttccttggtc-3’) and inserted into the EcoRI/Xhol sites of the pGEX6P2 vector (GE Healthcare). Positive clones were confirmed by DNA sequencing. Both GST recombinant proteins were expressed and purified as described (4). Recombinant protein phosphatase-2Cα was obtained as described (7).

Animals—All animal studies were approved by the University of Dundee Ethics Committee and performed according to the UK Animals (Scientific Procedures) Act 1986. Muscle-specific LKB1-deficient mice were generated, bred, and genotyped as described previously (43).

Preparations of AMPK and Upstream Kinases and AMPK Assays—Rat liver AMPK (a mixture of the α1 and α2 isoforms with β1 and γ1 (44)) was purified as far as the gel filtration step (45), and rat testis LKB1 complex was purified as described for the rat liver complex (6). AMPK activity was assayed as described previously (46). Immunoprecipitate assays of AMPK in cell lysates were performed as described previously (11).

Preparations and Assays of Other Kinases—The panel of 76 protein kinases was prepared and assayed in the presence and absence of A-769662 as described previously (47).

Creation of a GST-GBD Fusion and Assays of Glycogen Binding—The glycogen-binding domain (GBD) of the rat β1 subunit (residues 65–182) was amplified from a pcDNA3-rat β1 plasmid using primers incorporating SalI and EcoRI restric
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Scintillation Proximity Assay for AMP Binding—Human γ2 (CBS motifs 1–4) was expressed as a GST fusion as described previously (4). GST-γ2 was purified using a 5-ml GST FF column (GE Healthcare) followed by size exclusion chromatography as described previously (48). The protein was incubated with glutathione-coupled scintillation proximity assay yttrium silicate beads (GE Healthcare), preblocked with 5% gelatin from cold water fish skin (Sigma). The beads were washed with 50 mM sodium Heps, pH 7.4, 200 mM NaCl and resuspended to 10 mg/ml. A 96-well plate was set up with 0.1 mg of scintillation proximity assay beads and 120 μM [3H]AMP (GE Healthcare) and made up to 90 μl with buffer. The plate was shaken for 15 min at room temperature, and varying concentrations of A-769662 or AMP were added, to a final volume of 100 μl. The plate was shaken for 15 min, beads were allowed to settle, and the plate was read using a 1450 MicroBeta counter (PerkinElmer Life Sciences).

Cell Culture—Mouse embryonic fibroblasts (MEFs) from AMPK-α1+/+α2+/+ and AMPK-α1−/−α2−/− mice were generated as described previously (49). MEFs were cultured in standard Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, non-essential amino acids, and 1 mM sodium pyruvate. HeLa cells were cultured in minimum essential Eagle’s medium supplemented with 10% fetal bovine serum, non-essential amino acids, and 100 units/ml penicillin and 100 μg/ml streptomycin. MEFs from TAK1+/+ and TAK1−/− mice were generated (50) and cultured as for AMPK-α1−/−α2−/− MEFs. Cells cultured in 10-cm dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum were treated as described in the figure legends, rinsed with phosphate-buffered saline, and lysed in 500 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM sodium–β-glycerophosphate, 50 mM NaF, 1 mM dithiothreitol, with one EDTA-free protease inhibitor mixture tablet (Roche Applied Science) per 50 ml). The lysate was clarified by centrifugation and applied to a 5-ml GSTrap FF column (GE Healthcare) pre-equilibrated with sucrose buffer. Nonspecifically bound proteins were removed by extensive washing in wash buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol) and protein eluted in 20 mM reduced glutathione. Fractions containing GST-GBD were identified by protein assay and SDS-PAGE analysis. GST-GBD was dialyzed overnight into wash buffer with two buffer changes. To make the glycogen-Sepharose column, CNBr-activated Sepharose 4 Fast Flow (GE Healthcare) was washed with 10 volumes of cold 1 mM HCl. Glycogen was then directly coupled by incubation with 1 volume of 50 mg/ml bovine liver (Type IX) glycogen (Sigma-Aldrich) in 10 mM KH$_2$PO$_4$, pH 8.0, overnight at 4 °C. Excess glycogen was removed by washing the beads with 5 volumes of 10 mM KH$_2$PO$_4$, pH 8.0. Unreacted sites on the Sepharose were blocked by incubation with 1 volume of 0.1 M Tris-HCl, pH 8.0, at room temperature for 2 h. The beads were then washed with 6 × 3 volumes of 0.1 M Tris-HCl, pH 9.0, 0.5 M NaCl, and 6 × 3 volumes of 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl. The beads were finally resuspended and stored in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. For the binding assay, 50 μl of beads were incubated with 2 μg of protein in a final volume of 150 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl at 4 °C for 1 h. The glycogen-Sepharose beads were then pelleted at 13,000 rpm for 30 s, and 10 μl of supernatant were retained for analysis. The glycogen-Sepharose beads were then washed with 500 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl prior to resuspension in the original volume of the same buffer. The bead suspension prior to incubation (10 μl), the supernatant after incubation, and the resuspended pellet were analyzed on 4–12% Bis-Tris gels in a MOPS buffer system (Invitrogen). Proteins were transferred to a nitrocellulose membrane, which was blocked for 1 h at room temperature in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) + 5% nonfat milk powder. The membrane was washed in 4 × 10 ml of TBS. Anti-GST antibody (in 10 ml of TBS + 1% milk powder and 0.2% (v/v) Tween 20) was added and incubated for a further 1 h at room temperature. The membrane was washed 3 × 5 min with TBS + 0.2% v/v Tween 20 and 1 × 5 min in TBS. The membrane was scanned in the 680 channel of the Odyssey IR imager.
was continually gassed directly by bubbling with a mixture of 95% O_2, 5% CO_2. At the end of the incubation, muscles were quickly frozen in liquid N_2 and stored at −80 °C. Muscles were processed, and lysates were prepared as described previously (11).

Western Blotting (Cell Culture and Muscle Studies) — Equal amounts of cell lysates (10–30 µg of protein) were heated in SDS-PAGE sample buffer and analyzed by SDS-PAGE and electrotransfer to nitrocellulose membranes. The membranes were blocked for 30 min at room temperature in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween (TBS-T) containing 10% skimmed milk or 5% bovine serum albumin. The membranes were then incubated with the indicated antibodies in TBS-T containing 5% bovine serum albumin or 5% skimmed milk for 16 h at 4 °C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagent. Quantification of the bands was performed by digitalizing the ECL films using a Fuji LAS 1000 CCD camera and analysis of the intensities using the AIDA software.

RESULTS

A-769662 Regulates Heterotrimeric AMPK but Not Domains from the α or β Subunits — We initially tested the ability of A-769662 to activate the native αβγ complex of AMPK purified from rat liver, which is a mixture of the phosphorylated α1 and α2 catalytic subunit isoforms with β1 and γ1. Both A-769662 and AMP gave a significant stimulation of purified rat liver AMPK, although the former was 50-fold more potent (half-maximal effect (EC_{50}) at 116 ± 25 nM versus 6 ± 3 µM) and also gave a significantly greater stimulation (4.1 ± 0.5 versus 3.1 ± 0.5-fold) than AMP (in Fig. 1A, compare the open circles with those in Fig. 1B). The interactions between A-769662 and AMP were complex. In the presence of a saturating concentration of AMP (200 µM), increasing A-769662 up to 5 µM produced a small (1.4-fold) but significant (95% confidence interval 1.2–1.6-fold) additional activation (Fig. 1A, filled circles). However, in the presence of a saturating concentration of A-769662 (1 µM), increasing AMP up to 500 µM caused a small (30%) but significant inhibition (Fig. 1B, filled circles).

We next tested the ability of A-769662 to regulate the activity and/or ligand binding of various constructs derived from the α or β subunits. The compound had no effect on the activity of a T172D mutant of the α1 kinase domain that had been expressed in bacteria as a GST fusion (data not shown); because of the replacement of Thr-172 by an aspartate residue, this construct is constitutively active and does not require prior phosphorylation (42). Recently, it has been reported that a region just C-terminal to the kinase domain (residues 313–335 in human α1) represents an autoinhibitory domain (AID) (51). To assess whether A-769662 might relieve inhibition by the AID, we expressed the rat α1 kinase domain either with (1–333) or without (1–310) this region. Both were expressed as GST fusions in bacteria, purified on glutathione-Sepharose, and preincubated with MgATP in the presence or absence of recombinant CaMKKβ to phosphorylate Thr-172. As expected, the GST-α1 (1–310) construct, which contains the complete kinase domain but lacks the putative AID, was markedly activated by incubation with MgATP in the presence of CaMKKβ (Fig. 2A), and this correlated with phosphorylation of Thr-172 as assessed using an anti-pT172 antibody (Fig. 2B). Neither the dephosphorylated nor the phosphorylated 1–310 construct was activated by A-769662 (Fig. 2A). By contrast, the GST-α1

![FIGURE 1. Effects of A-769662 on the native rat liver AMPK complex. A, activation of native rat liver AMPK (a mixture of the α1 and α2 isoforms with β1 and γ1) by A-769662 in the presence (filled symbols) and absence (open symbols) of 200 µM AMP. The data were fitted to the equation Activity = Basal + (((Activation × Basal) − Basal) × [A-769662])/(EC_{50} + [A-769662])). The circles are the experimental data, and the solid line is the theoretical curve obtained using the parameters estimated (in the absence of AMP: Basal = 25 ± 3 nmol-min^{-1}·mg^{-1}; Activation = 4.1 ± 0.5-fold; EC_{50} = 116 ± 25 nM; in the presence of AMP: Basal = 54 ± 4 nmol-min^{-1}·mg^{-1}; Activation = 1.4 ± 0.1-fold; EC_{50} = 250 ± 230 nM; all estimates ± standard error of the mean). B, activation of rat liver AMPK by AMP in the presence (filled symbols) and absence (open symbols) of 1 µM A-769662. The data were fitted as for A. The parameters estimated (in the absence of A-769662: Basal = 24 ± 4 nmol-min^{-1}·mg^{-1}; Activation = 3.1 ± 0.5-fold; EC_{50} = 6 ± 3 µM; in the presence of A-769662: Basal = 102 ± 4 nmol-min^{-1}·mg^{-1}; Activation = 0.69 ± 0.03-fold; all estimates ± standard error of the mean).
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A) Effect of A-769662 on GST-α1 constructs

B) Phosphorylation of GST-α1 by CaMKKβ

C) Effect of A-769662 on GBD:glycogen binding

FIGURE 2. Effects of A-769662 on recombinant constructs derived from the α and β subunits. A, effects of phosphorylation of GST fusions of the α1 kinase domain (1–310) or the α1 kinase domain plus the autoinhibitory domain (1–333) by CaMKKβ on AMPK activity and lack of effect of A-769662. The designated construct was incubated with MgATP with or without CaMKKβ as described under “Experimental Procedures,” and AMPK activity was measured in the presence and absence of 1 μM A-769662. B, phosphorylation of GST fusions of the α1 kinase domain or the α1 kinase domain plus the autoinhibitory domain by CaMKKβ. The designated construct was incubated as for A, and samples were subject to Western blotting with anti-GST and anti-pT172 antibodies. C, a GST fusion of the β1 glycogen-binding domain (GST-GBD) was incubated with bovine liver glycogen covalently attached to Sepharose and the concentration of A-769662 indicated on the right for 10 min. The glycogen-Sepharose was removed by centrifugation, and the load, the supernatant (SN), and the glycogen-Sepharose pellet (P, resuspended to the original volume with buffer) were analyzed by Western blotting using anti-GST antibody. The two smaller polypeptides detected using this antibody may represent slight degradation of the fusion protein.

(1–333) construct that contains the putative AID was not activated by incubation with MgATP in the presence of CaMKKβ, although phosphorylation of Thr-172 still occurred (Fig. 2B). These results confirm the proposal that the region from residues 311–333 in rat α1 (equivalent to 313–335 in human) acts as an autoinhibitory domain (51) and show that the presence of this domain does not prevent phosphorylation of Thr-172. They also show that A-769662 does not activate AMPK by relieving inhibition of the kinase domain by the AID (Fig. 2A).

The β subunits of AMPK complexes contain a GBD that causes the kinase to associate with glycogen particles in intact cells (52, 53). Although a crystal structure for this domain has been determined in the presence of a model polysaccharide, β-cyclodextrin (54), its regulatory significance remains unclear. Nevertheless, we developed an assay to test whether A-769662 might cause dissociation of this domain from glycogen. The GBD from β1 (residues 65–182) was expressed in E. coli as a GST fusion and purified on glutathione-Sepharose. When bovine liver glycogen was covalently attached to Sepharose, incubated with the GST-GBD fusion, and then centrifuged, all of the fusion protein was recovered in the pellet (P) and none in the supernatant (SN). Note that with this preparation of the GST-GBD fusion, there was a slight degradation of the protein so that it migrated as a closely spaced triplet. Interaction of the GST-GBD with glycogen-Sepharose was not affected by the presence of 0.5 or 5 μM A-769662 (Fig. 2C). As controls for the specificity of this binding assay, we used GST without the fused GBD or Sepharose without the attached glycogen, and in both cases, all of the GST or GST-GBD fusion appeared in the supernatant (not shown). We also used a double mutant version of the GST-GBD in which Trp-100 and Trp-133 of the GBD were mutated to alanine and glycine, respectively (it has been shown that these mutations prevent binding of the GBD to glycogen (53)). Once again, all of the GST-GBD fusion appeared in the supernatant (not shown).

A-769662 Does Not Displace AMP from the γ Subunit—The interactions between activation by AMP and A-769662 shown in Fig. 1 suggested that the binding sites for these compounds might overlap. To test this, we expressed a GST fusion of the four CBS motifs from human γ2 in bacteria and purified the fusion protein on glutathione-Sepharose (4). We bound the protein to scintillation proximity beads and incubated with [3H]AMP at 120 μM, a concentration that should yield close to maximal binding. We then examined whether unlabeled AMP or A-769662 could displace the labeled AMP. Fig. 3A shows that unlabeled AMP displaced a large amount of the labeled AMP, leaving a small residual radioactivity that represents the background using this method. The dissociation constant for AMP estimated from this curve was 80 ± 15 μM (S.E.), which is close to the value of 60 μM estimated previously using a different binding assay (4). By contrast, although high concentrations of A-769662 appeared to cause a small drop in the radioactivity in the scintillation proximity assay, it clearly did not displace [3H]AMP significantly over the concentration range where it caused activation of AMPK (Fig. 3A).

Effects of AMP and A-769662 on Dephosphorylation by Protein Phosphatase-2Ca—To test whether A-769662, like AMP (7), inhibited dephosphorylation of Thr-172, AMPK purified

| CaMKKβ: | A-769662: |
|---|---|
| - | + |
| - | - |
| + | + |
| + | - |

| anti-GST | anti-pT172 |
|---|---|
| 1 | 2 |
| 3 | 4 |
| 1-310 | 1-333 |

| GST-GBD | SN | P | A-769662 |
|---|---|---|---|
| 0 μM | | | |
| 0.5 μM | | | |
| 5 μM | | | |
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A) Displacement of [3H]AMP from γ2 subunit

B) Effects on dephosphorylation of Thr-172

FIGURE 3. A and B, displacement of [3H]AMP by unlabeled AMP or A-769662 from a GST fusion with the twin Batten domains of human γ2 (A) and effects of A-769662 on dephosphorylation of AMPK (B). A, a GST-γ2 fusion (CBS1–4 (4)) was bound to scintillation proximity beads coated with glutathione, incubated with 120 nM AMP, and increasing concentrations of [3H]AMP (open circles) or A-769662 (filled circles). Bound radioactivity was determined with a scintillation counter. Data for AMP binding were fitted using GraphPad Prism to the equation Radioactivity = (Maximum (labeled AMP)/(labeled AMP) + [unlabeled AMP] + K_MAMP) + Background. The curve is the theoretical curve generated using the best-fit parameters obtained (Maximum = 5200 cpm; Background = 1800 cpm; K_MAMP = 60 ± 15 μM (±S.E.)). B, purified rat liver AMPK was incubated with protein phosphatase-2Cα (9 ng) without Mg^2+ or other addition (open circles) or with 5 mM MgCl_2 in the absence (filled circles) or presence of 200 μM AMP (triangles) or 1 μM A-769662 (inverted triangles). Samples were withdrawn at various times and analyzed by Western blotting using anti-pT172 antibody. Intensities of the phosphorylated bands were determined using Li-Cor Odyssey infra-red scanner, and the results were expressed as the percentage of zero time intensity.

Effect of A-769662 on Other Protein Kinases—We screened A-769662 at 10 and 50 μM in cell-free assays against a panel of protein kinases other than AMPK; data obtained using 10 μM A-769662 are shown in Fig 4. Although one or two kinases appeared to be slightly stimulated at 10 μM, this may just be experimental variation because none of these were stimulated significantly at 50 μM A-769662 (not shown). The majority of the kinases were not significantly affected by 10 μM A-769662, including two kinases that are members of the AMPK-related kinase family (BRSK2 and MARK3). A number of kinases were marginally inhibited, but in only two cases, PIM1 and PIM3, was this by more than 50%. Thus, at a concentration that is 10 times the concentration that is saturating for AMPK activation in cell-free assays, the effects of A-769662 on the activities of other protein kinases were negligible or small.

Phosphorylation of Acetyl-CoA Carboxylase in Response to A-769662 Requires AMPK—To test whether effects of A-769662 in intact cells were dependent on AMPK, we utilized immortalized fibroblasts from wild-type and double AMPK knock-out (AMPK−/−/α2−/−) MEFs. Fig. 5A shows that expression of the full-length α1 and α2 isoforms of the catalytic subunits of AMPK were readily detectable by Western blotting in the wild-type MEFs but were not detectable in the double knock-out MEFs, either with isoform-specific antibodies (anti-α1 and -α2) or with a phosphospecific antibody that recognizes both isoforms (anti-pT172). A polypeptide of slightly smaller size was detected by Western blotting, even after prior immunoprecipitation, using anti-α1 antibody in the knock-out MEFs but not the wild-type MEFs. Since the gene targeting strategy was designed to delete 60 amino acids from the kinase domain that are critical for kinase activity (residues 97–157), this polypeptide may represent a truncated α1 subunit with this internal deletion. Whatever the identity of this polypeptide, AMPK activity was undetectable in the double knock-out MEFs after immunoprecipitation from the extracts (not shown). Pilot experiments revealed that in wild-type MEFs, phosphorylation of acetyl-CoA carboxylase (ACC) was maximal after treatment for 15 min with 100 μM A-769662, and this increase was sustained for at least 1 h (not shown). Fig. 5B shows that, in wild-type MEFs, there was an increase in the phosphorylation of AMPK at concentrations up to 300 μM A-769662, although the effect was smaller than that seen with 2 mM phenformin. No signal was obtained in the double knock-out MEFs after immunoprecipitation from the extracts (not shown). The phosphorylation of ACC in response to A-769662 in wild-type MEFs was saturated at 100 μM, when the level of phosphorylation was essentially identical to that seen in response to 2 mM phenformin. Significantly, the phosphorylation of ACC in response to both A-769662 and phenformin was undetectable in the double knock-out MEFs, even after a long exposure (Fig. 5B), and this was not due to a reduced expression of ACC protein. This confirms that the effect of both A-769662 and phenformin on the phosphorylation of ACC in MEFs is completely dependent on AMPK.
FIGURE 4. Effect of A-769662 (10 μM) on the activities of a panel of 76 protein kinases. Assays were performed in duplicate, and the results are shown in rank order as the percentage of activity when compared with a control without compound, ± standard deviation. Abbreviations of kinases are as presented elsewhere (47).
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A) Detection of AMPK-α1 and α2 in wt and ko MEFs

B) Phosphorylation of ACC in wt and ko MEFs

C) Phosphorylation of AMPK and ACC in wt and ko hepatocytes

FIGURE 5. Effects of A-769662, phenformin, and AICAR in wild-type (wt) and double knock-out (ko) (AMPK-α1−/− α2−/−) mouse embryonic fibroblasts (MEFs) and primary mouse hepatocytes. A, wild-type and double knock-out MEFs were analyzed by Western blotting with the indicated AMPK antibodies or with anti-β-actin as a loading control. In one experiment (second panel from top), AMPK-α1 was immunoprecipitated (IP) with anti-α1 antibody prior to Western blotting. B, wild-type or double knock-out MEFs were incubated with the indicated concentrations of A-769662 or 2 mM phenformin for 1 h, and lysates were analyzed by Western blotting using the indicated phosphospecific or non-phosphospecific antibodies. Results are presented as means ± S.E. of duplicates and are representative of two or more experiments. Blots were quantified by densitometry, and phosphorylation of ACC is presented in arbitrary units (AU). C, primary hepatocytes from wild-type and double knock-out mice were incubated with the indicated concentrations of A-769662 or 500 μM AICAR for 4 h, and lysates were analyzed by Western blotting using the indicated phosphospecific or non-phosphospecific antibodies.

To study this in a more physiological context, we also examined the effect of A-769662 on phosphorylation of AMPK and ACC in primary hepatocytes derived from wild-type and double AMPK knock-out (AMPK-α1−/−α2−/−) mice (Fig. 5C). The results were similar to those in the MEF cells in that A-769662 at concentrations up to 200 μM, and AICAR at concentrations up to 500 μM, produced a marked phosphorylation of ACC that was abolished in cells from the double knock-out mice. A small increase in AMPK phosphorylation in response to A-769662 was also observed, although it was much smaller than the increase in response to AICAR.

Phosphorylation of AMPK and ACC in Response to A-769662 Requires an Upstream Kinase but Is Independent of the Upstream Kinase Utilized—To determine whether the phosphorylation and activation of AMPK and the phosphorylation of ACC in intact cells in response to A-769662 requires the upstream kinase LKB1, we examined the effects of A-769662 in muscle tissue isolated from wild-type mice or from mice with a muscle-specific deletion of LKB1 (43). Fig. 6A shows an experiment where we incubated isolated wild-type (LKB1+/+) extensor digitorum longus muscle ex vivo with various concentrations of A-769662 and compared its effect with those of 2 mM AICAR. This showed that the effect of A-769662 on phosphorylation of Thr-172 on AMPK was smaller than that of AICAR and was most evident at 300 μM. However, the effect on ACC phosphorylation was already evident at 30 μM and was saturated at 100 μM. Fig. 6B shows the effect of 100 μM A-769662 or 2 mM AICAR on LKB1+/+ or LKB1−/− muscles. Both agents stimulated phosphorylation of AMPK and ACC in the wild-type muscle, but phosphorylation of both proteins was abolished in muscles lacking LKB1.

To determine whether A-769662 would activate AMPK in cells expressing an alternate upstream kinase, we studied HeLa cells, which lack expression of LKB1 but do express CaMKKβ (12). Fig. 7A shows that A-769662 caused a small but significant activation and phosphorylation of AMPK in these cells, whereas ionomycin (which activates AMPK via increased Ca2⁺ and activation of CaMKKβ (12)) caused a larger effect on both parameters. However, both A-769662 and ionomycin caused a large increase in phosphorylation of ACC. The effect of phenformin was not significant in these cells, as reported previously (6). To test whether the effects of A-769662 and ionomycin were mediated by CaMKKβ, we also performed these experiments in the presence of the CaMKK inhibitor STO-609. This inhibitor reduced the effects of both agents on the phosphorylation and activation of AMPK. It caused a particularly large inhibition of the effect of ionomycin, although inhibition was not complete because some stimulation by ionomycin was still evident. Moreover, a robust phosphorylation of ACC in response to A-769662 was still observed in the presence of STO-609 (Fig. 7A).

Phosphorylation of AMPK and ACC in Response to A-769662 Does Not Require TAK1—To test whether phosphorylation and activation of AMPK and ACC required the presence of TAK1, we studied TAK1+/+ and TAK1−/− MEFs. Although the effects of A-769662 on AMPK phosphorylation were small in these cells, there were clear increases in the phosphorylation of ACC, and this was identical when the TAK1+/+ and TAK1−/− MEFs were compared (Fig. 7B).

DISCUSSION

The results in this report provide new information about the mechanism of action of A-769662 and show that the compound is a useful experimental tool to study the downstream consequences of AMPK activation in intact cells and in vivo. A-769662 activated the native αβγ complex of AMPK purified from rat liver extremely potently in cell-free assays, with a half-
maximal effect at 120 nm. This is even lower than the EC₅₀ of 800 nm reported for A-769662 by Cool et al. (39), although this may be due to differences in the preparation of AMPK used and/or the assay conditions, because our estimated EC₅₀ for the natural activator, AMP, was also lower than that reported by Cool et al. (39) (8 versus 56 μM). The ability of A-769662 to directly activate AMPK both in cell-free assays and in intact cells makes it unique among currently known cell-permeable activators. Other activators, such as AICAR, metformin, and the thiazolidinediones, do not activate AMPK directly in cell-free assays, and either are pro-drugs that are converted to active components inside the cell (e.g. AICAR, which is converted to the AMP analogue ZMP (21)) or work even more indirectly, e.g. by inhibiting the respiratory chain (metformin) or by triggering release of adiponectin from adipocytes (thiazolidinediones) (3).

Our results also suggest that A-769662 does not act by binding to any of the known ligand-binding sites on the α, β, or γ subunits and must utilize a novel binding site. A-769662 had no effect on the activity of the isolated kinase domain from the α₁ isoform, either using a T172D mutant that does not require prior phosphorylation (not shown) or after phosphorylation of Thr-172 on the wild-type kinase domain by CaMKK (Fig. 2A). Neither did A-769662 relieve inhibition of the phosphorylated kinase domain by the autoinhibitory domain (residues 311–325).

FIGURE 6. Effects of A-769662 and AICAR on mouse extensor digitorum longus muscle. A, isolated extensor digitorum longus muscle from LKB1⁺/⁺ mice were incubated for 1 h in the presence or absence of the indicated concentrations of A-769662 or 2 mM AICAR. Extracts were prepared, and phosphorylation of ACC and AMPK was analyzed by Western blotting. Blots were quantified by densitometry, and phosphorylation of ACC is presented in arbitrary units (AU) ± S.E. (n = 3–5). B, as A but using 100 μM A-769662 and LKB1⁻/− and LKB1⁻/⁻ muscle.
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333) previously identified by Pang et al. (51) (Fig. 2A). An incidental finding that came out of these experiments was that the presence of the AID did not prevent the phosphorylation of Thr-172 by CaMKKβ (Fig. 2B) or LKB1 (not shown), although it did completely prevent activation by these upstream kinases. The AID of AMPKα1 and -α2 aligns with, and show some sequence similarity with, the ubiquitin-associated domains in the AMPK-related kinases (56). Indeed, Pang et al. (51) have modeled the interaction between the kinase domain and the AID of α1 based on the structure of the kinase and ubiquitin-associated domain of the AMPK-related kinase MARK2. However, the functions of the ubiquitin-associated domain in the AMPK-related kinases and the AID in the α subunits of AMPK appear to be different because Jaleel et al. (56) found that although the ubiquitin-associated domains did not inhibit the AMPK-related kinases, they were required for their phosphorylation by the LKB1 complex. By contrast, we now report that although the AID in the AMPKα subunits is not required for phosphorylation by either CaMKKβ or LKB1, it does completely prevent the activation caused by these phosphorylation events. A-769662 did not cause dissociation of the glycogen-binding domain from glycogen (Fig. 2C), making it unlikely that the compound binds to the glycogen-binding site on this domain. We also found using a scintillation proximity assay that A-769662 did not displace [3H]AMP from the isolated Bateman domains on the γ2 subunit significantly under conditions where unlabeled AMP clearly did (Fig. 3A). This was a little surprising because A-769662 mimics not just one but two of the effects of AMP on the AMPK system, i.e. (i) allosteric activation (Fig. 1) and (ii) inhibition of dephosphorylation (Fig. 3B). Our finding that A-769662 did not significantly displace AMP from the isolated Bateman domains suggests that the binding sites for these two ligands must be different, although they may produce a similar change in conformation.

Our studies in intact cells reinforce the idea that A-769662 is a specific and direct activator of AMPK and are also consistent with the idea that it has a dual effect, causing bothallosteric activation and inhibition of dephosphorylation. Increased phosphorylation of ACC by A-769662 in mouse embryo fibroblasts and primary mouse hepatocytes was completely dependent on the expression of the two catalytic subunits of AMPK (Fig. 5), showing that, at least when measuring ACC phosphorylation, the compound is completely dependent on AMPK for its effects.

It was noticeable in all of our intact cell studies with A-769662 that although the effects on AMPK phosphorylation were often quite small, the effects on ACC phosphorylation were generally larger. For example, although phenformin or AICAR had much larger effects than A-769662 on AMPK phosphorylation in MEF cells and primary hepatocytes (Fig. 5), the effects of these agents on ACC phosphorylation were similar. Related observations were made in skeletal muscle (Fig. 6) and in HeLa cells (Fig. 7A). The most likely explanation for these apparent differences is that phosphorylation of ACC is such a sensitive marker of AMPK activation that maximal phosphorylation of ACC occurs when only a small proportion of AMPK has been phosphorylated. The concentrations of AICAR and phenformin chosen for this study were designed to give maximal phosphorylation and activation of AMPK in these cells and are likely to be greater than those required to give maximal ACC phosphorylation. An additional explanation in the case of the effects of the Ca2+ ionophores in HeLa cells (Fig. 7A) is that calcium ions activate phosphorylation by the upstream kinase CaMKKβ (12–14) but do not cause allosteric activation of AMPK. The phosphorylation of ACC in response to A-769662 would reflect a combination of allosteric activation and increased phosphorylation, but the allosteric effect on AMPK is lost during preparation of the extracts and is not reflected in the kinase assays. By contrast, the effect of increased intracellular Ca2+ is entirely mediated by increased phosphorylation, and the effect on AMPK activity would be fully preserved in the extract. A third potential explanation for these apparent differences is that A-769662 might be able to activate dephosphorylated AMPK, but we could obtain no evidence that this was the case. Although the compound did cause some activation of purified AMPK after treatment with protein phosphatase-2Cα (data not shown), the degree of activation of the treated and untreated kinase was the same (4-fold), and A-769662 did not alleviate the large inactivation caused by protein phosphatase treatment. Therefore, the small activation of the protein phosphatase-treated enzyme was most likely due to activation of the small residual amount of phosphorylated kinase left in the preparation.

The results obtained with LKB1−/− muscle (Fig. 6) suggest that an upstream kinase is necessary for the effect of A-769662 on ACC phosphorylation to be observed, presumably because dephosphorylated AMPK is not activated by the compound. However, the effect appears to be independent of the particular upstream kinase being utilized. Thus in HeLa cells, which do not express LKB1 but do express CaMKKβ (6, 12), A-769662 still promoted phosphorylation of both AMPK and ACC in a similar manner to the Ca2+ ionophore, ionomycin. Interestingly, although the CaMKK inhibitor STO-609 greatly reduced phosphorylation of ACC in response to ionomycin, a substantial phosphorylation of ACC in response to A-769662 remained (Fig. 7). A likely explanation of these results is that ionomycin acts by increasing Ca2+ and thus activating CaMKKβ and, although this would increase phosphorylation of Thr-172 on AMPK, there would be no concomitant allosteric activation of the kinase. By contrast, A-769662 acts both by inhibiting dephosphorylation of Thr-172 and by causing allosteric activation of AMPK, and even the very low basal activity of CaMKKβ in STO-609-treated cells without ionomycin may be sufficient to observe these effects. This would also explain why the effect of STO-609 on ACC phosphorylation in response to A-769662 was less than its effect on the response to ionomycin.

It was recently reported that the TAK1 could activate the S. cerevisiae homologue of AMPK, the SNF1 complex, when expressed in the yeast, and could also phosphorylate Thr-172 and activate mammalian AMPK in cell-free assays (15). Our findings that phosphorylation of AMPK and ACC by A-769662 was identical in TAK1+/+, and TAK1−/− mouse embryo fibroblasts do not support an important role for TAK1 as an upstream kinase in these cells. However, they do not rule out
the possibility that it could act as an upstream kinase for AMPK in other cell types or in other circumstances.

We would suggest that A-769662 is superior to other AMPK activators, including the nucleoside AICAR and the biguanide drugs, metformin and phenformin, for studies of the downstream actions of AMPK in intact cells and in vivo. AICAR is taken up into cells by adenosine transporters (57) and is converted by adenosine kinase into the mono-phosphorylated nucleotide ZMP, which mimics the effects of AMP on AMPK activation, albeit 50-fold less potently than AMP itself (21). One problem with the use of AICAR is that ZMP has been found to regulate other AMP-sensitive enzymes such as glycogen phosphorylase (58) and fructose-1,6-bisphosphatase (59), which is not the case with A-769662 (39). Another problem is that, although not itself an agonist or an antagonist of adenosine receptors, it does compete with adenosine for re-uptake into cells. In some in vitro systems, this can cause effects that are due to increased accumulation of adenosine outside cells, with consequent binding to adenosine receptors (57). Although the biguanide drugs, metformin and phenformin, activate AMPK when incubated with intact cells (29), they have no effect on AMPK or its phosphorylation or dephosphorylation in cell-free assays (33). The mechanism by which they activate AMPK has not been completely elucidated, but it has been reported that their entry into cells is catalyzed by organic cation transporters such as OCT1 (31) and that the drugs then accumulate in mitochondria (driven by the membrane potential across the inner mitochondrial membrane, which would favor uptake of cations), where they inhibit complex I of the respiratory chain (34, 35). This may in turn produce an increase in the cellular AMP:ATP ratio that activates AMPK. An increase in the AMP:ATP ratio has indeed been demonstrated in the case of phenformin (12), although it has been more difficult to detect in the case of the less rapidly and potently acting biguanide, metformin, possibly because this was studied in cells that do not express OCT1 (33, 60). This is supported by the fact that using primary hepatocytes, which do express OCT1, metformin treatment resulted in a decrease in ATP content (36). If this proposed mechanism of action for the biguanides is correct, they may be no more specific as pharmacological activators of AMPK than are other metabolic poisons such as the mitochondrial ATP synthase inhibitor, oligomycin (33). As a good example of the shortcomings of these widely used AMPK activators, it has recently been shown that AICAR, metformin, and oligomycin all inhibit glucose phosphorylation in isolated rodent hepatocytes by inhibiting the translocation of glucokinase from the nucleus to the cytoplasm. However, all three agents were just as effective in this regard in isolated hepatocytes from double knock-out (AMPK-α1−/−α2−/−) mice as in those from wild-type controls, showing that these effects do not require AMPK and are probably mediated by ATP depletion (36). Unlike AICAR, metformin, or oligomycin, A-769662 is a direct activator of AMPK in cell-free assays, and it is certainly the most potent and specific pharmacological activator of AMPK available at present. This drug will be a valuable experimental tool to study the physiological roles of AMPK. A more complete understanding of the mechanism by which it activates AMPK may also facilitate the design of novel AMPK activators that could be used to treat patients with metabolic disorders.

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