A-769662 potentiates the effect of other AMP-activated protein kinase activators on cardiac glucose uptake

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AMPK is an important cellular sensor of the energy level. Its activation derives from an increase in the intracellular AMP-to-ATP ratio that typically occurs during metabolic stresses, such as myocardial ischemia or, more generally, O2 deprivation (3, 20). AMP stimulates AMPK allosterically via its binding to the AMPK γ-subunit and promotes its phosphorylation on Thr172, a residue located in the activation loop of the catalytic subunit. Both mechanisms participate in full activation of the enzyme, with the latter playing the main role. Liver kinase B1 (LKB1), the principal upstream AMPK kinase responsible for Thr172 phosphorylation under such circumstances, is constitutively active (1, 33). Actually, increased Thr172 phosphorylation mainly results from the fact that AMP binding prevents its dephosphorylation by protein phosphatases (11, 40). Recently, it has been shown that ADP behaved similarly to AMP in activating AMPK even if the role of ADP was not confirmed in vivo (7, 17, 27, 42).

Once activated, AMPK participates in the maintenance of energy homeostasis by switching off anabolic pathways that consume ATP and switching on alternative catabolic pathways that generate ATP (20, 40). One of the main roles of AMPK during myocardial ischemia is to promote glucose utilization by stimulating its uptake and anaerobic glycolysis, the sole way to produce ATP in the absence of O2 (26, 30). In this manner, AMPK promotes glucose-dependent ATP production protecting the heart against ischemic injury. Several genetic studies have demonstrated this concept (8, 31, 41, 43, 45). Ischemia-induced stimulation of glucose uptake and glycolysis is greatly inhibited in mice lacking AMPKα2, the predominant catalytic isoform in the heart, or expressing a dominant negative AMPK isoform (8, 31, 45). This decreased catabolic response leads to major ATP depletion and is accompanied by rapid and severe ischemic contracture, significantly increased apoptosis, augmented infarct size, and poor postischemic contractile recovery (8, 31, 45). The role of cardiac AMPK extends beyond the control of glucose metabolism under ischemic conditions. Indeed, AMPK activation by the antiadipic drug metformin or its analog phenformin has been shown to stimulate glucose uptake independently of insulin, with AMPK becoming an alternative pathway substituting for insufficient insulin response in insulin-resistant cardiomyocytes (4, 15). In addition to its insulin-mimetic action, AMPK is also able to (re)sensitize cardiomyocytes to insulin (4, 15, 25). Given its protective role, AMPK is now considered one of the most

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase composed of one catalytic (AMPKα) and two regulatory subunits (AMPKβ and AMPKγ) present under several isoforms (two isoforms for α and β and three isoforms for γ) (40). AMPK is an important cellular sensor of the energy level. Its activation derives from an increase in the intracellular AMP-to-ATP ratio that typically occurs during metabolic stresses, such as myocardial ischemia or, more generally, O2 deprivation (3, 20). AMP stimulates AMPK allosterically via its binding to the AMPK γ-subunit and promotes its phosphorylation on Thr172, a residue located in the activation loop of the catalytic subunit. Both mechanisms participate in full activation of the enzyme, with the latter playing the main role. Liver kinase B1 (LKB1), the principal upstream AMPK kinase responsible for Thr172 phosphorylation under such circumstances, is constitutively active (1, 33). Actually, increased Thr172 phosphorylation mainly results from the fact that AMP binding prevents its dephosphorylation by protein phosphatases (11, 40). Recently, it has been shown that ADP behaved similarly to AMP in activating AMPK even if the role of ADP was not confirmed in vivo (7, 17, 27, 42).

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promising targets in the treatment of cardiovascular disorders, such as ischemic heart disease and diabetic cardiomyopathy (20).

The therapeutic potential of AMPK has triggered the development of specific AMPK activators, among which compound A-769662 has recently emerged because of its selectivity and potential (9, 16, 35, 37). A-769662 binds directly to the AMPK β-subunit (37). Similar to AMP, it allosterically stimulates AMPK and prevents Thr172 dephosphorylation by protein phosphatases (9, 16, 35). Kim and colleagues (22) have recently demonstrated that cardiac pretreatment with A-769662 activates AMPK, preserves energy charge during ischemia, reduces infarct size, and allows a better recovery of contractile function during reperfusion. It is tempting to propose that part of A-769662’s protective outcome is due to A-769662-dependent stimulation of cardiac glucose uptake, which is responsible for ATP preservation during hypoxia/ischemia. However, it has been established, in skeletal muscle cells, that A-769662-mediated activation of AMPK is unable to stimulate glucose uptake (39). The aim of the present work was to reconcile these apparently inconsistent results. For this purpose, we evaluated the effect of A-769662 on glucose uptake in primary cultured cardiomyocytes under basal and different stimulating conditions, including phenformin, insulin, and ischemia-mimetic conditions.

MATERIALS AND METHODS

Ethical information. This study was approved by the Animal Research Committee of Université catholique de Louvain and conformed with guidelines on animal experimentation in our institution.

Preparation and treatment of adult rat cardiomyocytes in primary culture. Adult cardiomyocytes from male Wistar rats were freshly prepared as previously described elsewhere (4). Cardiomyocytes were distributed equally in dishes coated with laminin and incubated at 37°C in medium 199 (Invitrogen, 5.5 mM glucose), 100 U/ml penicillin, and 100 µg/ml streptomycin for 1 h. Cells were then washed and incubated for another 1 h under the same conditions as before treatment. Details of the treatment are given in the figures. Hypoxia was induced by incubating cells at 37°C in an airtight Plexiglas chamber (Billups Rothenberg) in a 5% CO2-95% N2 atmosphere. The O2 level (0.5%) in the chamber was verified by a MAXO2 O2 analyzer (Maquet).

Preparation and treatment of perfused rat hearts. Hearts from adult male Wistar rats were excised and perfused retrogradely at 37°C under constant 70-mmHg pressure with Krebs-Henseleit buffer containing 110 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 11 mM glucose, 0.2 g/l mannitol, and 1.5 mM CaCl2 in equilibrium with a 95% O2-5% CO2 gas phase (13, 26). After a 15-min equilibration period and pretreatment in the presence or absence of A-769662 (100 µM), hearts were perfused with or without oligomycin (1 µM) for 10 min. At the end of the procedure, the hearts were immediately freeze clamped in liquid nitrogen and stored at −80°C. Frozen hearts were homogenized at 4°C in 9 volumes (vol/wt) of homogenization buffer that contained phosphatases and protease inhibitors (34, 45). Supernatants were stored at −80°C after centrifugation (10,000 g, 30 min, 4°C). For glucose uptake measurement in perfused hearts, [2,3-3H]glucose was added in the perfusion solution to reach a radioactive specificity of 0.2 µCi/ml, and samples of recirculating perfusate were taken every 5 min during 15 min. The accumulated tritiated water resulting from detrinitiated glucose was separated from the nontransported tritiated glucose by column chromatography and measured by a scintillation counter (4, 15).

Glucose uptake, glycolytic flux, and adenine nucleotide measurement in cultured cardiomyocytes. Glucose uptake and glycolytic flux were measured by the deitritation rate of [2-3H]glucose and [3-3H]glucose, respectively (4, 6, 15). In summary, tritiated glucose (0.2 µCi/ml, Perkin-Elmer) was added to the medium (including 5.5 mM glucose) 30 min before the end of the different treatments. Medium was then removed, and the tritiated water resulting from detrinitiated glucose was separated from the nontransported tritiated glucose by column chromatography and measured by a scintillation counter (4, 15). Purine nucleotides were measured in neutralized percllic acid extracts after their separation by HPLC (26).

Enzyme assay and phosphorylation state evaluation. The specific activities of α1-, α2-, β1-, and β2-containing AMPK heterotrimers were assayed after immunoprecipitation of 150 µg protein extracts with anti-α1, anti-α2 (a kind gift of B. Viollet, Institut Cochin, Paris, France), anti-β1 (Cell Signalling), and anti-β2 (Santa Cruz Biotechnology) antibodies, respectively. Activities were assayed in the presence of 0.2 µM AMP and measured by SAMS peptide phosphorylation (10).

AMPK, acetyl-CoA carboxylase (ACC), and PKB/Akt phosphorylation were assessed on immunoblots of cell lysates with anti-phospho-Thr172 AMPK (Cell Signalling), anti-phospho-Ser79 ACC (Cell Signalling), and anti-phospho-Ser81/87 PKB/Akt (Cell Signalling), respectively. The phosphorylation state of Akt substrate of 160 kDa (AS160) was measured with anti-phospho-Akt substrate (Cell Signalling) antibody. The absence of effects of the different treatments on expression of AMPK, ACC, PKB/Akt, and AS160 was verified by immunoblot analysis with anti-AMPK (Cell Signalling), anti-ACC (Cell Signalling), anti-PKB/Akt (Upstate), and anti-AS160 (Upstate) antibodies, respectively. Band intensities were quantified by scanning films and processing image intensities with the ImageJ program (133 for Mac OS X). Data were normalized with an internal loading control (eukaryotic elongation factor 2, Cell Signalling) on the same gel after stripping. Each immunoblot is delimited by a solid rectangle. When present, vertical solid lines denote that the samples were run on the same gel but were not contiguous.

Cell death, apoptosis, and ROS measurements. At the end of oligomycin treatment, cardiomyocytes were incubated with 50 µM propidium iodide for 5 min to label dead cells (2). Percentages of dead cells were calculated from the number of dead (labeled) and living (unlabeled) cells observed under microscope (1,500 cells/condition and in triplicate for each experiment). Apoptosis was evaluated by TUNEL staining (DNA fragmentation labeling). Briefly, cardiac myocytes were fixed with 4% paraformaldehyde for 15 min at room temperature. After permeabilization with PBS-0.1% Triton, cells were incubated with a mix containing 10% TUNEL enzyme (Roche) and 90% Labeling Solution (Roche) for 1 h at 37°C. The percentage of apoptotic cells was calculated from the number of apoptotic nuclei (labeled) and nonapoptotic cells (unlabeled nuclei) observed under a microscope (1,500 cells/condition and in triplicate for each experiment). ROS production was evaluated by monitoring oxidation of the cell fluorescent probe 2’,7’-dihydrodichlorofluorescein diacetate (Invitrogen) (2).

Protein measurement and statistics. Protein content was estimated by Bradford’s method with BSA as the standard. Results are expressed as means ± SE. Statistically significant differences (P < 0.05) were assessed by one- or two-way ANOVA with Bonferroni’s post hoc test.
RESULTS

Effect of A-769662 on the AMPK signaling pathway in adult rat cardiomyocytes. Incubation of adult cardiomyocytes with A-769662 (100 μM) induced rapid (already maximum at 30 min) and robust AMPK phosphorylation on Thr172, which was maintained for at least 24 h (Fig. 1A). Stimulation of the AMPK signaling pathway was confirmed by the increase in phosphorylation of ACC, a bona fide AMPK substrate frequently assessed for its signaling (Fig. 1B). We also charted A-769662 dose-response curves (Fig. 1, C and D). Phosphorylation of both AMPK and ACC increased with the lowest concentration used (3 μM) and became statistically different at 10 μM. As A-769662 acts on AMPK via both allosteric stimulation and an increase in the phosphorylation state, ACC phosphorylation can be used, more accurately than AMPK phosphorylation, to evaluate the level of AMPK pathway activation (22). ACC phosphorylation reached a plateau at 100 μM (Fig. 1D), confirming that this concentration can be considered as an elevated one. These results, obtained in cultured cardiomyocytes from adult rats, also confirmed the AMPK activation observed in A-769662-treated mouse hearts (22).

Effect of A-769662 on basal and insulin-induced glucose uptake. We (4, 15) have previously shown that classical AMPK activators such as metformin, phenformin, and oligomycin can mimic insulin action in cultured cardiomyocytes, increasing glucose uptake in its absence. We first checked if A-769662 behaves in a similar way. We tested A-769662 at the concentration of 100 μM and followed the experimental protocol shown in Fig. 2A. Oligomycin, inhibitor of ATP synthase of the mitochondrial respiratory chain, served as a positive control of classical cellular AMP-raising AMPK activators. A-769662 and oligomycin augmented AMPK phosphorylation (Fig. 2B). Moreover, both activators induced similar phosphorylation of the AMPK downstream target ACC, revealing comparable stimulation of the AMPK pathway (Fig. 2C). In line with these results, oligomycin and A-769662 evoked equivalent phosphorylation of the AMPK substrate AS160 (Fig. 2E) and the GTPase-activating protein of Rab, which has been implicated in the translocation of glucose transporter 4 (GLUT4) to the plasma membrane (20, 32). In contrast, A-769662 did not increase glucose uptake, whereas oligomycin effectively stimulated this metabolic pathway (Fig. 2F).

The disconnection found between AS160 phosphorylation and glucose uptake under A-769662 treatment was confirmed in the perfused heart, where both oligomycin and A-769662 induced ACC and AS160 phosphorylation, although only oligomycin increased glucose uptake (Fig. 2, G–I).

Our previous studies (4, 15) also showed that classical AMPK activators increased the insulin response of cardiomyocytes. Likewise, oligomycin heightened the insulin-mediated phosphorylation of PKB, a key component of insulin signaling that regulates glucose transport (Fig. 2D) (5). AS160, also a substrate of PKB (and therefore shared with AMPK), was similarly overphosphorylated when oligomycin and insulin were combined (Fig. 2E). The increased

**Fig. 1.** Time course and dose response curves of A-769662 on AMP-activated protein kinase (AMPK; A and C) and acetyl-CoA carboxylase (ACC; B and D) phosphorylation. A and B: adult cardiomyocytes were incubated with A-769662 (100 μM) for time periods varying from 30 min to 24 h. pT172, phosphorylated Thr172; pS79, phosphorylated Ser79. C and D: adult cardiomyocytes were incubated with increasing concentrations of A-769662 (from 3 to 100 μM) for 1 h. Values are relative to A-769662 (100 μM) for 1 h and are means ± SE of at least 3 experiments. *P < 0.05 vs. untreated cells.
The insulin response was further confirmed by the presence of glucose uptake overstimulation (8-fold increment with oligomycin/H11001 insulin cotreatment vs. 3.3- and 2.9-fold for insulin and oligomycin, respectively; Fig. 2F). In contrast, AMPK activation by A-769662 (100 μM) had no insulin-sensitizing effect on either PKB or AS160 phosphorylation or on glucose uptake. To confirm that the absence of A-769662’s effect was not due to the relatively high dose used, we tested several lower A-769662 concentrations (Fig. 3). None of the concentrations analyzed increased insulin-mediated PKB/AS160 phosphorylation or glucose uptake stimulation. All these data (Figs. 2 and 3) were obtained using the frequently used insulin concentration of 1 nM. We then evaluated the action of A-769662 in the presence of oligomycin/H11001 insulin cotreatment.
Experiments. None of the A-769662-treated conditions was statistically different from untreated and insulin-treated cells. Values are means \( \pm \) SE of at least 3 experiments. None of the A-769662-treated conditions was statistically different from cells treated with insulin alone.

different concentrations of insulin (from 0.1 to 10 nM). A-769662 has no insulin-sensitizing action on cardiac glucose uptake whatever the insulin concentration used (data not shown).

Effect of A-769662 on the outcome of AMP-dependent AMPK activators. Next, we determined if A-769662 has a positive effect on AMPK signaling and its downstream metabolic targets under conditions that mimic ischemia, i.e., situations inhibiting the mitochondrial respiratory chain, which subsequently elevates AMP and ADP concentrations and activates AMPK. We first tested oligomycin by following the protocol shown in Fig. 4A. As shown in Fig. 2, oligomycin promoted the phosphorylation of both AMPK and ACC (Fig. 4, B and C). Interestingly, pretreatment with 100 \( \mu \)M A-769662 increased these oligomycin-dependent phosphorylations (Fig. 4, B and C). Consequently, the combination of both AMPK activators caused AS160 overphosphorylation and glucose uptake overstimulation (Fig. 4, D and E). Lower A-769662 concentrations (12.5 \( \mu \)M) similarly potentiated oligomycin’s effect on AMPK, ACC, AS160, and glucose uptake (data not shown).

Adult rat hearts were then perfused to ensure that AMPK overactivation can be demonstrated in intact tissues. Pilot experiments revealed that oligomycin and A-769662 induced maximal AMPK phosphorylation after 10 and 20 min of perfusion, respectively (data not shown). Hearts were then pretreated for 10 min with A-769662 before oligomycin was added for another 10 min period (Fig. 4F). As in cultured cardiomyocytes, A-769662 potentiated the effect of oligomycin on AMPK and ACC phosphorylation in perfused hearts (Fig. 4, G and H).

Two other treatments, known to activate AMPK via increased AMP concentrations, were tested in combination with A-769662 in cultured cardiomyocytes. Hypoxia, which is very close to the ischemic condition, and phenformin, the biguanide analog of the antidiabetic drug metformin, which inhibits the mitochondrial respiratory chain complex I (29), both induced AMPK and ACC phosphorylation and stimulated glucose uptake (Fig. 5). Similarly to what occurred with oligomycin, A-769662 enhanced the action of these two AMPK activators on the three targets studied (Fig. 5). A similar synergistic outcome of A-769662 was observed with metformin. Indeed, we (4) have previously shown that a high concentration (between 1 and 10 mM) of metformin was necessary to activate AMPK and stimulate glucose uptake in cardiomyocytes. Here, 1 mM of metformin only slightly increased (~30%) glucose uptake (Fig. 6). However, in the presence of A-769662, glucose uptake was more than doubled by 1 mM metformin after overstimulation of the AMPK pathway (Fig. 6). Similarly, A-769662 significantly increased glucose uptake stimulation by 5 mM metformin (control: 0.09 \( \pm \) 0.02 \( \mu \)mol·h\(^{-1}\)·mg\(^{-1}\), metformin: 0.29 \( \pm \) 0.05 \( \mu \)mol·h\(^{-1}\)·mg\(^{-1}\), and metformin + A-769662: 0.48 \( \pm \) 0.02 \( \mu \)mol·h\(^{-1}\)·mg\(^{-1}\), \( P \leq 0.05\)).

In addition to glucose uptake, we also checked if A-769662 could increase the glycolytic response to classical AMPK activators. As for glucose uptake, A-769662 preincubation intensified the increase in glycolytic flux induced by oligomycin or phenformin without affecting the basal rate (Fig. 7).

Inasmuch as A-769662 intensified the impact of different AMPK activators that are known to function via modification of adenine nucleotide concentrations, we verified that it did not indirectly influence AMPK via amplification of AMP and ADP concentrations. When cardiomyocytes were treated with oligomycin, both AMP and ADP concentrations were elevated whereas ATP was decreased, resulting in a threefold increase of the (AMP + ADP)-to-ATP ratio (Fig. 8A). A-769662 cotreatment did not amplify this ratio. In fact, it tended to reduce the (AMP + ADP)-to-ATP ratio.

Specific action of A-769662 on different AMPK\(\alpha\) and AMPK\(\beta\) subunits. It has been previously reported that A-769662 specifically targets the AMPK \(\beta_{1}\)-subunit in hepatocytes and extensor digitorum longus muscle (37). Therefore, we wondered whether the sensitizing effect of A-769662 on cardiac AMPK activation was subunit specific in cardiomyocytes. We assayed specific AMPK activities by immunoprecipitation of AMPK\(\alpha_{1}\), \(\alpha_{2}\), \(\beta_{1}\), and \(\beta_{2}\)-isoforms. As expected, oligomycin activated AMPK\(\alpha_{1}\) (4-fold increase vs. control)-, AMPK\(\alpha_{2}\) (10-fold)-, AMPK\(\beta_{1}\) (6-fold)-, and

![Diagram](https://via.placeholder.com/150)
AMPK\textsubscript{\alpha2} (5-fold)-containing heterotrimers (Fig. 8, B–E). Treatment with A-769662 did not activate AMPK\textsubscript{\alpha1}, or amplify oligomycin-dependent AMPK\textsubscript{\alpha1} activation (Fig. 8B). Using mesenchymal stem cells, which are known to exclusively express the AMPK \textsubscript{\alpha1}-isoform without any detectable AMPK \textsubscript{\alpha2}-isoform, we (12) have previously shown that A-769662 could be a potent activator of AMPK\textsubscript{\alpha1}. The only way to explain the absence of AMPK\textsubscript{\alpha1} activation by A-769662 in cardiomyocytes would be the lack of AMPK\textsubscript{\alpha1}/\textsubscript{\beta1}-containing heterotrimers in these cells. In contrast, A-769662 strongly
enhanced activity and oligomycin-mediated activation of AMPK heterotrimers containing \( \alpha_2 \)- or \( \beta_1 \)-subunits, reaching a maximum activation of \( \pm 40 \)-fold (vs. control; Fig. 8, C and D). These results suggest that AMPK\( \beta_1 \) preferentially binds to AMPK\( \alpha_2 \) in cardiomyocytes. Finally, A-769662 seemed to slightly augment (1.7-fold) AMPK\( \beta_2 \)-containing heterotrimer activity (Fig. 8E). Because of the latter unexpected result, we checked possible cross-contamination of AMPK\( \beta_1 \) immunoprecipitates (Fig. 8F). Whereas AMPK\( \beta_2 \) was absent in the AMPK\( \beta_1 \) immunoprecipitate, a small but significant quantity of AMPK\( \beta_1 \) was found in the AMPK\( \beta_2 \) immunoprecipitate. This contamination could explain the minor increase of A-769662-mediated AMPK activation that occurred after AMPK\( \beta_1 \) immunoprecipitation.

In summary, A-769662 acts mainly on the heterotrimeric forms containing AMPK \( \beta_1 \) and \( \alpha_2 \)-isoforms in adult rat cardiomyocytes.

**Effect of A-769662 on cardiomyocyte survival.** Finally, we estimated the putative protective effect of A769662-mediated AMPK overactivation on cardiomyocyte survival in hypoxia-mimetic conditions, i.e., long-term oligomycin treatment. Such treatment increased cell death and apoptosis (Fig. 9, A and B). Cotreatment with A-769662 prevented this increment in cardiomyocyte mortality and apoptosis. The protection afforded by A-769662-dependent AMPK activation could be related to significant increased glucose utilization (Fig. 4E) and to a concomitant decrease in oligomycin-mediated ROS production (Fig. 9C).

**DISCUSSION**

In this study, we evaluated the ability of the specific AMPK activator A-769662 to stimulate cardiac glucose uptake under different conditions and its capacity to cooperate with other classical AMPK activators.

**AMPK activation by A-769662 does not fuel either basal or insulin-stimulated cardiac glucose uptake.** Classical AMPK activators, such as metformin, phenformin, and oligomycin, induce AS160 phosphorylation and stimulate glucose uptake in cultured rat cardiomyocytes (4, 15). They also increase the cardiomyocyte response to insulin (4, 15). Here, we confirmed these features with oligomycin. In contrast, although it activates AMPK and enhances AS160 phosphorylation, A-769662 does not stimulate cardiac glucose uptake or sensitize cardiomyocytes to insulin. The disconnection between AS160 and glucose uptake under A-769662 treatment was also demonstrated in the perfused heart. We (15) have previously demonstrated such disconnection between AS160 phosphorylation and stimulation of glucose uptake under insulin treatment when we concomitantly blocked the insulin negative feedback loop.
involving the mammalian target of rapamycin (mTOR)/p70 ribosomal S6 protein kinase (p70S6K) pathway using rapamycin (15).

The inability of A-769662 to stimulate glucose uptake in an AMPK-dependent manner has already been established in skeletal muscle (37, 39). An explanation of this incapacity could be found in A-769662’s narrow specificity. A-769662 only targets cardiac heterotrimeric forms containing α2/β1-subunits, whereas classical AMPK activators impact AMPK heterotrimers more broadly via an elevation of the cellular AMP level. Our study revealed that the activation of α2/β1-containing AMPK is sufficient to phosphorylate AS160 but not to induce glucose uptake. AS160 is clearly not the sole element regulating glucose uptake. Indeed, AMPK-dependent regulation of glucose transport involves other mechanisms, including cytoskeleton reorganization, fusion of vesicles containing GLUT4 with the plasma membrane, and GLUT4 endocytosis (5, 36, 38, 44). We speculate that heterotrimeric forms of AMPK different from α2/β1 could regulate at least part of these mechanisms.

It is reasonable to propose the same explanation for the lack of A-769662’s insulin-sensitizing action that we highlighted in the present study. Our work pointed out the incapacity of A-769662 to enhance insulin-induced PKB/AS160 phosphorylation and glucose uptake stimulation. We (15) have previously implicated at least two different mechanisms in the ability of classical AMPK activators to increase the insulin response. Indeed, we incriminated AMPK-mediated inhibition of the insulin negative feedback loop involving the mTOR/p70S6K pathway in both PKB and AS160 overphosphorylation. We (15) have also demonstrated that this mechanism cannot explain the glucose uptake overstimulation observed in cardiomyocytes incubated with insulin and classical AMPK activators. A recent
study (18) hypothesized the participation of an AMPK-mediated decrease in plasma membrane cholesterol facilitating GLUT4 insertion in this phenomenon. Whatever the exact nature of all these mechanisms, part of them should be under the control of AMPK heterotrimers containing subunits other than \(\beta_1\). This hypothesis is reinforced by the fact that A-769662 is unable to inhibit the insulin-mediated stimulation of p70S6K (data not shown), whereas other AMPK activators induce efficient p70S6K suppression (15).

As A-769662 is ineffective in stimulating glucose uptake and increasing insulin sensitivity in an AMPK-dependent manner in tissues such as skeletal and cardiac muscles, the decrease in the plasma glucose level and in body weight gain in diabetic ob/ob mice treated with this compound should be explained by its action on other tissues, such as the liver (9).

A-769662 potentiates the effect of classical AMPK activators, including phenformin and ischemia-mimetic conditions. Our data show that A-769662, despite its inability to stimulate...
glucose uptake by itself, promotes overstimulation of the AMPK signaling pathway, including AS160 and glucose uptake, when combined with AMP-dependent AMPK activators, namely, oligomycin, hypoxia, phenformin, and metformin. Similar A-769662-dependent overstimulation occurs on cardiac glycolysis. Amplification of AMPK activation by A-769662 only involves \( \alpha_2 \beta_1 \)-containing heterotrimers and is independent of increased AMP concentration. As it has been previously shown that A-769662 and AMP are not able to act in an additive allosteric way on AMPK activity (35), we hypothesize that A-769662 binding to AMPK \( \beta_1 \)-subunit acts synergistically with AMP, increasing their inhibitory action on protein phosphatases. We cannot exclude an effect of A-769662 directly on LKB1-dependent phosphorylation. However, a parallel study (14) has shown that a combination of A-769662 with 5-aminomimidazole-4-carboxamide ribonucleotide (an AMP-related AMPK activator) synergistically stimulates AMPK in C2C12 muscle cells and primary mouse hepatocytes without any significant increase in LKB1 activity.

The sensitizing action of A-769662 on antidiabetic-related AMPK activators, such as phenformin and metformin, could be helpful in treating insulin-resistant hearts. Indeed, such a combination could be used in vivo to evoke significant cardiac AMPK activation and glucose uptake stimulation, which are known to be hardly attainable with metformin alone.

Concerning myocardial infarction, several studies (8, 24, 31, 45) have shown that AMPK is activated during ischemic episodes and that this activation is heart protective. However, we (26) have also previously reported that cardiac AMPK is not activated maximally during ischemia, indicating that a significant percentage of AMPK remains available for pharmacological overactivation. Our results disclosed that A-769662 induces such overstimulation in cultured cardiomyocytes and perfused hearts. The fact that it preserved cardiomyocytes from oligomycin-induced cell death and apoptosis could be related to the compound’s protective action, as reported by Kim and coworkers (22) in ischemia-reperfused mouse hearts. The disappearance of A-769662-mediated cardiac protection in the ischemic heart of mice overexpressing the dominant negative form of AMPK reveals that it acts via AMPK (22). The potentiation effect of A-769662 only involves the AMPK catalytic \( \alpha_2 \)-subunit, in agreement with evidence showing that LKB1/AMPK\( \alpha_2 \) are particularly important for the regulation of cardiac metabolism under ischemia or ischemia-mimetic treatments (8, 19, 45). Glucose uptake is required to produce ATP via anaerobic glycolysis and is essential to decrease ischemia-reperfusion injury (21). Therefore, its overstimulation certainly participates in the protective action of A-769662 in hypoxia/ischemia. However, other downstream AMPK targets could be involved. First, we showed that A-769662 reduced oligomycin-mediated ROS production. We could speculate on the existence of an A-769662-mediated decrease in ROS production in ischemic-reperfused hearts. Indeed, AMPK activation has been associated to the reduction in ROS production during myocardial ischemia (23). Second, it has been proposed recently that A-769662/AMPK also protect the ischemic heart by inhibiting mitochondrial permeability transition pore opening (28).

Conclusions. In the present study, we showed that A-769662 alone is unable to stimulate glucose uptake or to play an insulin-sensitizing role. However, its intensifying effect on other AMPK activators makes it a potentially useful participant in the beneficial role of AMPK during myocardial infarction and in the insulin-resistant heart.

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DISCLOSURES
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EFFECT OF A-769662 ON CARDIAC GLUCOSE UPTAKE

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REFERENCES


