Biochemical and Biophysical Research Communications **276,** 16–22 (2000)
doi:10.1006/bbrc.2000.3417, available online at http://www.idealibrary.com on IDEAL®

AMP-Activated Protein Kinase Is Activated by the Stimulations of G_q-Coupled Receptors

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Received July 28, 2000

The AMP-activated protein kinase (AMPK) functions as a metabolic sensor that monitors cellular AMP and ATP levels. Platelet-activating factor (PAF) activates endogeneous AMPKα1 in Chinese hamster ovary cells expressing the PAF receptor coupled with both G_i and G_q, but its activity was not inhibited after treatment with islet-activating protein. Norepinephrine and bradykinin also activated AMPKα1 in cells expressing the G_q-coupled α_{1b}-adrenergic receptor and bradykinin receptor, respectively. Stimulations of the G_i-coupled α_{2A}-adrenergic receptor, fMet-Leu-Phe receptor, prostaglandin EP3α receptor, and G_s-coupled β_2-adrenergic receptor did not activate AMPKα1. AMPKα1 thus is activated specifically by stimulation of G_q-coupled receptors. G_q-coupled receptors transmit the signal for GLUT4 translocation and glucose uptake through an insulin-independent pathway. However, direct activation of AMPKα1 with treatment of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside did not trigger GLUT4 translocation nor stimulate glucose uptake in our cells. Thus, activation of AMPKα1 via G_q is not sufficient to trigger GLUT4 translocation or stimulate glucose uptake.

Key Words: **AMP-protein kinase; G_q; GLUT4; AICAR; PAF; norepinephrine; bradykinin.**

Abbreviations used: AMPK, AMP-activated protein kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; AMPKK, AMP-activated protein kinase kinase; GLUT4, glucose transporter type 4; CHO, Chinese hamster ovary; FCS, fetal calf serum; PAF, platelet activating factor; fMLP, fMet-Leu-Phe; IAP, islet-activating protein; KRHB, Krebs-Ringer/HEPES buffer; ANOVA, analysis of variance; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; PLCβ, phosphoinositide-specific phospholipase Cβ; ZMP, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranosyl 5′-monophosphate.

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AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase consisting of catalytic α-subunit and noncatalytic β- and γ-subunits (1, 2). Two isoforms of the α-subunit have been identified and were named as α1- and α2-subunits, which contain the kinase domain and also contribute to the AMP-binding site (3–5). The α1-subunit is widely distributed and appears to be almost ubiquitous, but distribution of the α2-subunit is comparatively limited. It is highly expressed in skeletal and cardiac muscle and in the liver (3, 6, 7).

AMPK phosphorylates many target proteins at serine residues which include 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, acetyl-CoA carboxylase, hormone-sensitive lipase, glycogen synthase, Raf-1 and endothelial NO synthase (1, 8, 9), and plays a key role in the regulation of carbohydrate, fat metabolism and gene expressions (1, 2). AMPK is activated by AMP or the phosphorylation of upstream kinase, AMPK kinase (AMPKK) (10), and is inhibited by both ATP and phosphocreatine (11). It has been reported that muscle contraction during exercise decreases phosphocreatine which inhibits AMPK in an allosteric manner, and subsequently AMPK is activated (11). When cells are exposed to stress (e.g., heat shock, hypoxia, metabolic toxicity, and starvation for glucose) such that AMP:ATP rises, AMPK is activated (12, 13). While the regulation of AMPK by intracellular signals is well-characterized, much less is known about its regulation by extracellular signals. One report indicates that stimulation of adipocytes by β-adrenergic catecholamines may activate AMPK (14), but the nature of any coupling between cell-surface receptor stimulation and AMPK regulation has not heretofore been characterized.

Glucose transporter type 4 (GLUT4) is expressed exclusively in adipocytes and skeletal muscle (15, 16). Translocation of GLUT4 from an intracellular pool to

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the plasma membrane is a major mechanism of glucose uptake not only in response to insulin in these tissues, but also in skeletal muscle during physical exercise (15–20). However, mechanisms responsible for the translocation of GLUT4 in response to physical exercise remain to be elucidated. It was reported that insulin and contraction of skeletal muscle use distinct intracellular signaling mechanisms to stimulate GLUT4 translocation and to increase glucose uptake (21–23).

We earlier reported that stimulation of G_q-coupled receptors triggers GLUT4 translocation and increases glucose uptake through an insulin independent pathway (24). For this we used our sensitive and quantitative method for GLUT4 translocation to measure c-myc epitope-tagged GLUT4 (GLUT4myc; G4myc) on the cell surface (25). We also reported that bradykinin might be one of the candidate molecules responsible for physical exercise-stimulated glucose transport via G_q-coupled bradykinin B_2 receptors in skeletal muscle (26). In skeletal muscle, AMPK is activated by muscle contraction and independently by insulin signaling (27–32) and may be a mediator of exercise-stimulated GLUT4 translocation and glucose uptake (30–32). In this study, we asked whether extracellular stimulations of heterotrimeric G_q-coupled receptors would induce the activation of AMPK and whether AMPK plays an important role in GLUT4 translocation and the stimulation of glucose uptake.

MATERIALS AND METHODS

Cell culture. Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium (Biological Industries) supplemented with 10% (v/v) fetal calf serum (FCS) (Life Technologies, Inc.). L6 cells, kindly provided by Dr. Amira Klip (The Hospital for Sick Children, Toronto, Canada), were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS and differentiated into myotubes, as described (26).

The parent cell lines used in this study were CHO-G4myc, a CHO cell line expressing GLUT4myc, constructed by inserting a human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4 (25); L6-G4myc, an L6 cell line expressing GLUT4myc.

Antibodies and peptides. Rabbit antibodies against anti-AMPK α1-subunit were raised against the peptides based on the amino acid sequences of AMPK α1-subunit for residues 339–358 (DFYLATSPPDSFLDDHHLTR) (3). "SAMS" peptide is a synthetic peptide substrate for AMPK (HMRSAMSGLHLVKRR) (33).

Establishment of stable cell lines expressing G_q-, G_i-, and G_s-coupled receptors. The human platelet-activating factor (PAF) receptor cDNA (34), the human adrenergic receptors α_{1b}-AR (35, 36), α_{2A}-AR (37) and β_2-AR (38) cDNAs, the human bradykinin B_2 receptor cDNA (39), the human fMet-Leu-Phe (fMLP) receptor cDNA (40) and mouse prostaglandin receptor EP3α cDNA (41) were subcloned into a mammalian expression vector, pCXN (42). These plasmids were cotransfected into CHO-G4myc cells with pSV2-bsr, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride (Funakoshi, Tokyo). Several independent clones expressing each receptor were established and designated as follows: CHO-G4myc-PAFR, CHO-G4myc-α_{1b}AR, CHO-G4myc-α_{2A}AR, CHO-G4myc-β_2AR, CHO-G4myc-BK_2R, CHO-G4myc-fMLPR, and CHO-G4myc-EP3α cells were CHO-G4myc cells stably expressing the PAF receptor, adrenergic receptors α_{1b}-AR, α_{2A}-AR, β_2-AR, bradykinin B_2 receptor, fMLP receptor, and prostaglandin E2 receptor EP3α, respectively; L6-G4myc-BK2R myoblasts were L6-G4myc myoblasts expressing the mouse bradykinin receptors (26, 43). Expression of β_2-AR was confirmed in a binding assay using L-[4-^{3}H]propranolol (NEN Life Science Products), and those of other receptors were as described previously (24, 26, 44).

Pretreatment with islet-activating protein (IAP). CHO cells were pretreated with or without IAP (100 ng/ml; Funakoshi) for 24 h at 37°C in Ham's F-12 medium with 10% fetal calf serum, then the CHO cells were incubated with Krebs-Ringer/HEPES buffer (KRHB) (25) for 20 min at 37°C, prior to PAF stimulation. Pretreatment with IAP abolishes G_i-coupled pathway(s).

AMPK assay. AMPK activity was measured, as described (29). Cells (10-cm plate or 6-well plate) were incubated in KRHB for 20 min at 37°C, then with the indicated concentrations of ligands for the indicated periods at 37°C. Cell lysates were prepared with buffer containing 1% Nonidet P-40 and immunoprecipitated with anti-AMPK α1-subunit antibody and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). The beads were washed three times with wash buffer (20 mM Tris–HCl, pH 8.0, 140 mM NaCl, 1% Nonidet P-40, and 1 mM dithiothreitol), and once with AMPK assay buffer (40 mM HEPES, 200 μM AMP, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM dithiothreitol, 5 mM $MgCl_2$, pH 7.0). The immunoprecipitates were resuspended in a 20 μl of assay mixture (40 mM HEPES, 200 μM AMP, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM dithiothreitol, 5 mM $MgCl_2$, 6.25 μM ATP, 0.2 mM "SAMS" peptide and 2 μCi [γ-^{32}P]ATP) and incubated for 10 min or indicated period at 30°C. The reaction was stopped by adding 10 μl of 250 mM EDTA. Twenty-five microliters of the supernatant was spotted onto Whatman P81 chromatography paper. The filter was washed four times in 75 mM phosphoric acid and air-dried. AMPK activity was assessed by measurement of incorporation of ^{32}P by scintillation counting (33).

Cell surface anti-c-myc antibody binding assay (GLUT4myc translocation assay). L6 myotubes in 24-well plates were incubated in 500 μl of KRHB for 20 min at 37°C, and then with the indicated concentrations of ligands for 10 min at 37°C. GLUT4myc translocation was measured, as described (26).

2-Deoxyglucose uptake measurement. L6 myotubes in 24-well plates were treated with the indicated concentrations of ligands for 10 min at 37°C. 2-Deoxyglucose uptake was measured, as described (25).

Statistical analysis. Data are presented as means ± SE. Statistical significance was ascertained by analysis of variance (ANOVA) followed by post hoc comparison using the Bonferroni/Dunn method.

RESULTS

Stimulation of heterotrimeric G-coupled receptors induces AMPK activity. Heterotrimeric GTP-binding proteins are associated with signal transduction from cell surface receptors, and abundant hormonal receptors specifically couple to G_q, G_i, and G_s. To determine if AMPK is involved in the signaling pathway of heterotrimeric GTP binding protein, we established CHO-G4myc cells expressing PAF receptors (CHO-G4myc-PAFR), which couple to G_q and G_i. As shown in Fig. 1, PAF activated endogenous AMPK by 2.1-fold in CHO-G4myc-PAFR cells. 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), a cell-permeable compound with a phosphorylated metabolite which activates AMPK, also activates AMPK in these cells (2.0-

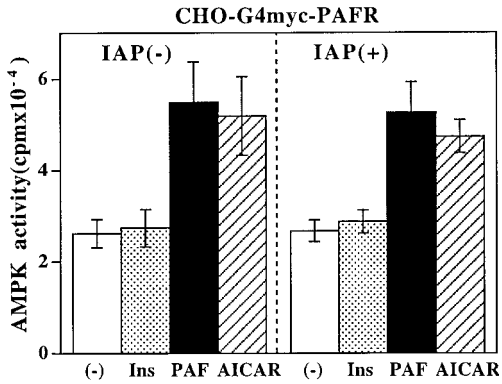

FIG. 1. Effects of IAP on the AMPK activity in CHO-G4myc-PAFR cells. CHO-G4myc-PAFR cells were treated with 3×10^{-7} M insulin (spotty bar), 2×10^{-8} M PAF (solid bar), 2×10^{-3} M AICAR (hatched bar), or buffer alone (open bar) for 10 min at 37°C after pretreatment with 100 ng/ml IAP or medium alone (−) for 24 h at 37°C, and assayed for AMPK activity as described under Materials and Methods. Values represent means ± SE of at least three determinations.

fold) (Fig. 1). Insulin did not increase AMPK activity in the cells, as previously reported (30, 45). The activation of AMPK with PAF was not inhibited by pretreatment of 100 ng/ml IAP (Fig. 1), which abolished the G_i coupling. Therefore, G_q probably mediates the activation of AMPK by PAF.

To confirm that AMPK is activated by stimulation of G_q-coupled receptors, we established CHO-G4myc cells expressing G_q-coupled α_{1b}-adrenergic receptors (CHO-G4myc-α_{1b}AR) and G_q-coupled bradykinin B_2 receptors (CHO-G4myc-BK_2R), respectively. Treatment of AICAR activated AMPK in these cells (Fig. 2A). Norepinephrine, a ligand of α_{1b}-adrenergic receptors, stimulated AMPK activity in CHO-G4myc-α_{1b}AR cells by 2.0-fold, and bradykinin also activated AMPK in CHO-G4myc-BK_2R cells by 2.0-fold (Fig. 2A). Although treatment with AICAR increased AMPK activity in the parent CHO-G4myc cells, the activation of AMPK by norepinephrine or bradykinin was not observed in CHO-G4myc cells which do not express those receptors (Fig. 2B). Bradykinin and AICAR activated AMPK in a dose-dependent manner in CHO-G4myc-BK_2R cells, but the parent CHO-G4myc cells did not respond to any applied concentrations of bradykinin (Fig. 2C). Bradykinin- and AICAR-activated AMPK activities reached a maximum in about 10 min, and the time course of AICAR-stimulated AMPK activity was almost the same as that of bradykinin in CHO-G4myc-BK2R cells (Fig. 2D). In AMPK assay from crude extract, the final concentration of ATP is widely used as 200 μM (33). AMPK activities in *in vitro* assays after immunoprecipitation of crude extract were not different between 6.25 μM and 200 μM of ATP concentrations in the assay mixture (data not shown). After incubation of CHO-G4myc-BK_2R cells for 60 min with buffer alone without glucose (control), AMPK activity also increased slightly (Fig. 2D). This is possibly due to the glucose-starvation which was reported to activate AMPK (46).

We next asked if the stimulation of G_i- or G_s-coupled receptors would result in increases in AMPK activity. We established CHO-G4myc cells stably expressing G_i-coupled α_{2A} adrenergic receptors (CHO-G4myc-α_{2A}AR), fMLP receptors (CHO-G4myc-fMLPR), prostaglandin E2 receptors EP3α (CHO-G4myc-EP3α), and G_s-coupled β_2 adrenergic receptors (CHO-G4myc-β_2AR), respectively. Norepinephrine, a ligand of α_{2A} adrenergic receptors, did not activate AMPK in CHO-G4myc-α_{2A}AR cells (Fig. 3). Treatments with either fMLP in CHO-G4myc-fMLPR cells or prostaglandin E2 in CHO-G4myc-EP3α cells did not increase AMPK activity (Fig. 3). Isoproterenol, a ligand of β_2 adrenergic receptors, did not activate AMPK in CHO-G4myc-β_2AR, while activation of AMPK by AICAR was observed in the cells (Fig. 3). The stimulation of G_s-coupled prostaglandin E_2 receptor EP4 by prostaglandin E_2 did not activate AMPK in CHO-G4myc cells expressing prostaglandin E_2 receptors EP4 (unpublished data). Incubation with forskolin, an activator of adenylate cyclase, or 8-bromo-cAMP, a cell-permeable cyclic AMP analog, did not lead to the activation of AMPK (data not shown).

Taken together, these results show that stimulation of G_q-coupled receptors, but not G_i- or G_s-coupled receptors activates AMPK in CHO cells.

Activations of AMPK in L6 myotubes. We then determine if stimulation of G_q-coupled receptors would induce AMPK activation in other cells. Bradykinin activated AMPK in L6-G4myc myotubes stably expressing bradykinin B_2 receptors (L6-G4myc-BK_2R), as observed in CHO-G4myc-BK_2R cells, while the parent L6-G4myc myotubes (which do not express the receptors) did not respond to bradykinin (Fig. 4A). The fold-increase of AMPK activation by bradykinin in L6-G4myc-BK_2R myotubes (1.5-fold) is less than that in CHO-G4myc-BK_2R cells (2.0-fold). AICAR also induced a significant increase in AMPK activity in both L6 myotubes, but insulin did not affect the AMPK activity (Fig. 4A).

GLUT4 translocation and glucose transport by bradykinin or AICAR. Other investigators reported that AMPK activation might be important for contraction-induced glucose transport in rat skeletal muscle and ischemia-induced glucose transport in rat cardiac muscle, which is not inhibited by wortmannin (30, 32, 47). In these studies, AICAR increased glucose uptake and triggered GLUT4 translocation in skeletal muscle via a wortmannin-insensitive pathway. We earlier developed a highly sensitive and quantitative method to detect directly c-myc epitope-tagged GLUT4 on the cell surface (25). Using this system, AICAR did not trigger

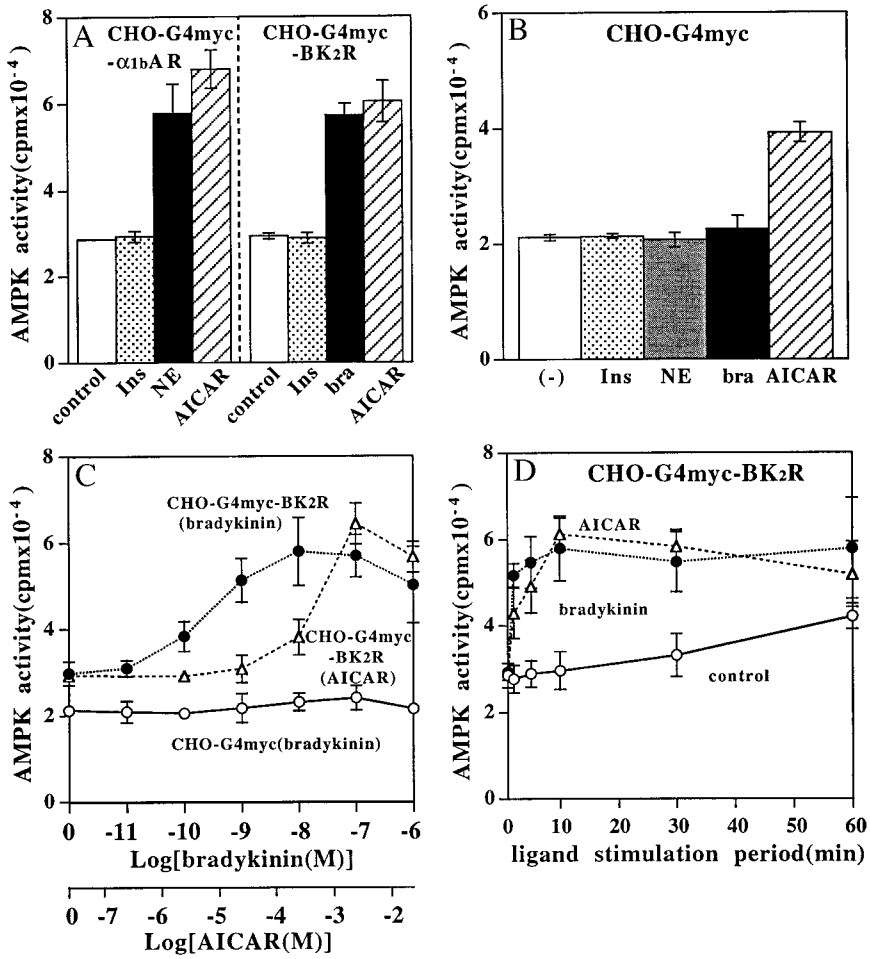

FIG. 2. AMPK activity in CHO-G4myc cells expressing G_q-coupled receptors. (A) CHO-G4myc cells expressing G_q-coupled α_{1b}-adrenergic receptors (CHO-G4myc-α_{1b}AR) and G_q-coupled bradykinin B_2 receptors (CHO-G4myc-BK_2R) were treated with 3×10^{-7} M insulin (dotted bar), 10^{-6} M norepinephrine (solid bar), 10^{-7} M bradykinin (solid bar), 2×10^{-3} M AICAR (hatched bar) or buffer alone (open bar) for 10 min at 37°C, and assayed for AMPK activity as described under Materials and Methods. (B) CHO-G4myc cells were treated with 3×10^{-7} M insulin (dotted bar), 10^{-6} M norepinephrine (shaded bar), 10^{-7} M bradykinin (solid bar), 2×10^{-3} M AICAR (hatched bar), or buffer alone (open bar) for 10 min at 37°C, and assayed for AMPK activity as described. (C) CHO-G4myc-BK_2R cells were incubated with various concentrations of bradykinin (-●-) or AICAR (△-△) for 10 min at 37°C, and assayed for AMPK activity as described. CHO-G4myc cells were also incubated with various concentrations of bradykinin (○-○) for 10 min at 37°C, and assayed for AMPK activity. (D) CHO-G4myc-BK2R cells were treated with 10^{-7} M bradykinin (-●-), 2×10^{-3} M AICAR (△-△) or buffer alone (○-○) for the indicated period at 37°C, and assayed for AMPK activity as described. Values represent means ± SE for at least three determinations.

GLUT4 translocation or stimulate glucose uptake in L6-GLUT4myc myotubes, yet insulin did do so (Figs. 4B and 4C). Bradykinin induced the translocation of GLUT4myc and stimulated glucose uptake in L6-GLUT4myc-BK_2R myotubes (Figs. 4B and 4C). The same data was obtained in CHO-GLUT4myc-BK_2R cells (data not shown). Taken together, these observations suggest that AMPK activation is not sufficient for bradykinin-stimulated glucose transport in cultured cells.

DISCUSSION

In the present study, we found that stimulation of the well-characterized G_q-(neither G_i- nor G_s-)coupled receptors, α_{1b}-adrenergic receptor and bradykinin receptor, activated AMPKα1, and external receptor-coupled signals to activate AMPKα1 exists in CHO cells and L6 myotubes.

Muscle contraction triggers GLUT4 translocation and stimulates glucose uptake via an insulin-independent pathway (19, 20, 48). We reported that bradykinin directly triggers GLUT4 translocation and stimulates glucose uptake via G_q in culture cells and proposed that bradykinin is a candidate molecule for the exercise/contraction-stimulated glucose transport in skeletal muscle (26). A well-known pathway that is mediated by G_q is the activation of phosphoinositide-specific phospholipase Cβ (PLCβ) (49, 50). We found that GLUT4 translocation by bradykinin is mediated

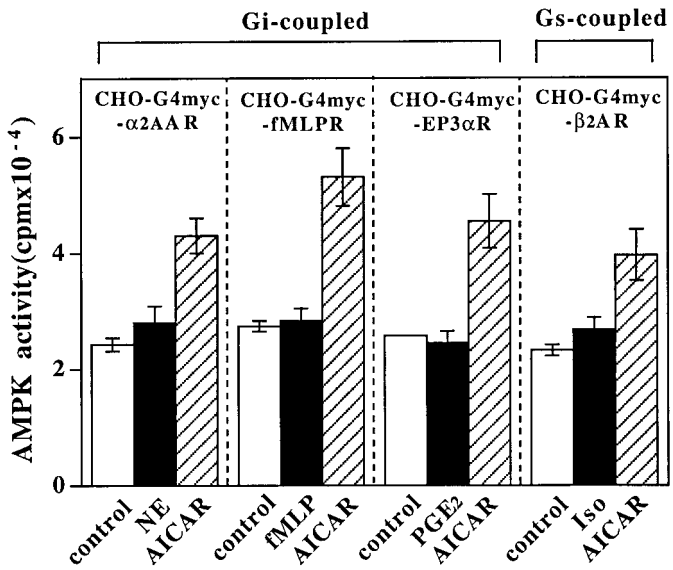

FIG. 3. AMPK activity in CHO-G4myc cells expressing G_i- or G_s-coupled receptors. CHO-G4myc cells expressing G_i-coupled α_{2A} adrenergic receptors, fMLP receptors, prostaglandin E_2 receptors EP3α, and G_s-coupled β_2 adrenergic receptors were treated with each ligand (solid bar), 2×10^{-3} M AICAR (hatched bar) or with buffer alone (open bar) for 10 min at 37°C, and assayed for AMPK activity as described. Values represent means ± SE for at least three determinations.

independently by the activation of PLCβ (26). Our data demonstrate that GLUT4 translocation triggered by bradykinin, which is independent of insulin signaling, is not mediated via AMPK.

AICAR is an analog of adenosine that is taken up into the cell and is phosphorylated to form 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranosyl 5′-monophosphate (ZMP). ZMP mimics the effects of AMP on AMPK, causing allosteric activation and promoting phosphorylation and activation by the upstream kinase, AMPKK (1, 51, 52). AICAR triggers GLUT4 translocation and increases glucose uptake in skeletal muscle in organ culture in the absence of insulin via a wortmannin-insensitive pathway (30, 31). Therefore, we considered the possibility that bradykinin increases the activity of AMPK α1 which subsequently triggers GLUT4 translocation and stimulates glucose uptake in L6 muscle cells expressing bradykinin B_2 receptors. However, we found that AICAR did not trigger GLUT4 translocation and stimulate glucose uptake in L6 myotubes, while AICAR increased AMPK activity. Therefore, AMPK activation is not sufficient to trigger GLUT4 translocation and stimulation of glucose uptake. However, the roles of AMPK in glucose transport might depend on the cell type. GLUT4 translocation are observed parallel to the activation of AMPK in skeletal muscle when treated with AICAR, but the direct evidence of the relationship between the activation of AMPK and glucose uptake (GLUT4 translocation) has not been reported.

In rat epididymal fat cells, isoproterenol gives rise to a stimulation of AMPK via β-adrenergic receptors and cell-permeable cyclic AMP analogue also activates AMPK (14). This result is incompatible with our data

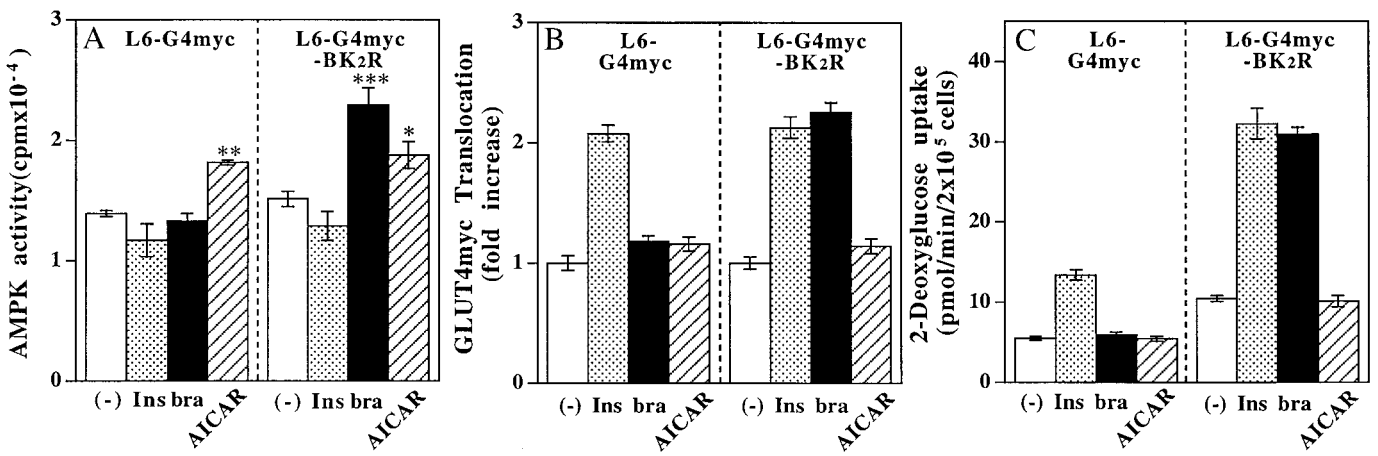

FIG. 4. The AMPK activity, GLUT4myc translocation and glucose uptake by insulin, bradykinin and AICAR in L6-G4myc myotubes and L6-G4myc-BK2R myotubes. (A) The parent cells (L6-G4myc myotubes) and those expressing bradykinin B_2 receptors (L6-G4myc-BK2R myotubes) were incubated with 10^{-7} M insulin (dotted bar), 10^{-7} M bradykinin (solid bar), 2×10^{-3} M AICAR (hatched bar) or with buffer alone (open bar) for 10 min at 37°C. AMPK activity was determined as described under Materials and Methods. (B) The parent cells (L6-G4myc myotubes) and those expressing bradykinin B_2 receptors (L6-G4myc-BK$_2$R myotubes) were stimulated with 10^{-7} M insulin (dotted bar), 10^{-7} M bradykinin (solid bar), 2×10^{-3} M AICAR (hatched bar) or with buffer alone (open bar) for 10 min at 37°C. The GLUT4myc translocation was measured as described. (C) The parent cells (L6-G4myc myotubes) and those expressing bradykinin B_2 receptors (L6-G4myc-BK$_2$R myotubes) were stimulated with 10^{-7} M insulin (dotted bar), 10^{-7} M bradykinin (solid bar), 2×10^{-3} M AICAR (hatched bar) or with buffer alone (open bar) for 10 min at 37°C. The 2-deoxyglucose uptake was measured as described. Values represent means ± SE of at least three determinations, significantly different from basal activity: *$P < 0.01$; **$P < 0.001$; ***$P < 0.0005$.

in CHO cells. Adipocytes might have a different regulation of AMPK by extracellular stimulation.

Direct activation of AMPKα1 by AICAR treatment did not trigger GLUT4 translocation or stimulate glucose uptake in our cell systems. These results suggest that activation of AMPKα1 by norepinephrine or by bradykinin determines the fate of glucose taken into the cells via G_q-coupled receptors rather than determine the signaling pathway for GLUT4 translocation or stimulation of glucose uptake. Insulin binds to its receptor, stimulates glucose uptake and switches on anabolic pathways. However norepinephrine or bradykinin binds to G_q-coupled receptors, stimulates glucose uptake, activates AMPKα1 and switches on the catabolic pathways to supply energy sources.

ACKNOWLEDGMENTS

We thank Drs. J. Miyazaki, M. Nakamura, T. Shimizu, A. Hirasawa, G. Tsujimoto, J. F. Hess, H. Kurose, R. J. Lefkowitz, F. Boulay, F. Ushikubi, S. Narumiya, S. Yokoyama, and H. Higashida for kindly providing the plasmids and Dr. Y. Asahi for assistance. M. Ohara provided comment on the manuscript. This work was supported by research grants from the Ministry of Education, Science, Sports, and Culture of Japan (to Y.E. and to K.K.), by a grant from Naito Foundation, by a grant from Japan Diabetes Foundation, by a grant from Kato Memorial Bioscience Foundation, and by a grant from Seirankai-prize (to K.K.).

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