Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK)

Kay Barnes[1,*], Jean C. Ingram[1], Omar H. Porras[2], L. Felipe Barros[2], Emma R. Hudson[3], Lee G. D. Fryer[4], Fabienne Foufelle[5], David Carling[4], D. Grahame Hardie[3] and Stephen A. Baldwin[1]

[1]School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK
[2]Centro de Estudios Científicos CECS, Casilla 1469, Valdivia, Chile
[3]Biochemistry Department, Dundee University, Dundee DD1 5EH, Scotland, UK
[4]Cellular Stress Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, London W12 0NN, UK
[5]U465 INSERM, Centre de Recherches Biomédical des Cordeliers, 75270 Paris Cedex 06, France
*Author for correspondence (e-mail: K.Barnes@leeds.ac.uk)

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Summary

In the rat liver epithelial cell line Clone 9, the V_{max} for glucose uptake is acutely increased by inhibition of oxidative phosphorylation and by osmotic stress. By using a membrane-impermeant photoaffinity labelling reagent together with an isoform-specific antibody, we have, for the first time, provided direct evidence for the involvement of the GLUT1 glucose transporter isoform in this response. Transport stimulation was found to be associated with enhanced accessibility of GLUT1 to its substrate and with photolabelling of formerly 'cryptic' exofacial substrate binding sites in GLUT1 molecules. The total amount of cell surface GLUT1 remained constant. The precise mechanism for this binding site 'unmasking' is unclear but appears to involve AMP-activated protein kinase: in the current study, osmotic and metabolic stresses were found to result in activation of the α1 isoform of AMP-activated protein kinase, and transport stimulation could be mimicked both by 5-aminoimidazole-4-carboxamide ribonucleoside and by infection of cells with a recombinant adenovirus encoding constitutively active AMP-activated protein kinase. The effect of 5-aminoimidazole-4-carboxamide ribonucleoside, as for metabolic stress, was on the V_{max} rather than on the K_m for transport and did not affect the cell-surface concentration of GLUT1. The relevant downstream target(s) of AMP-activated protein kinase have not yet been identified, but stimulation of transport by inhibition of oxidative phosphorylation or by 5-aminoimidazole-4-carboxamide ribonucleoside was not prevented by either inhibitors of conventional and novel protein kinase C isoforms or inhibitors of nitric oxide synthase. These enzymes, which have been implicated in stress-regulated pathways in other cell types, are therefore unlikely to play a role in transport regulation by stress in Clone 9 cells.

Key words: Glucose, Transport, GLUT1, Stress, AMP-activated protein kinase

Introduction

Exposure of many mammalian cell types to metabolic stresses, such as hypoxia and inhibition of oxidative phosphorylation, or to osmotic stress results in an acute increase in the rate of glucose uptake. This adaptive response allows the cells to maintain or regain their ATP levels by increasing flux through the glycolytic pathway. In most cases the enhanced uptake of glucose is associated with an increase in the V_{max} for transport, with little or no change in the apparent affinity (K_m) for the substrate (Mercado et al., 1989; Barros et al., 2001). The cause of the increased V_{max} has been most intensively investigated in the rat liver epithelial cell line Clone 9, where it appears to involve an increase in the number of functionally active cell-surface transporters (Hamrahian et al., 1999). The total plasma membrane content of GLUT1, the only glucose transporter isoform so far detected in Clone 9 cells, is hardly altered by stress, suggesting that previously cryptic transport sites become 'activated'. However, the identity of the transporter(s) responsible for the increase in substrate uptake in stressed cells has not yet been definitively established.

The signal transduction pathways linking metabolic and osmotic stress to glucose transport are also unclear. However, a key feature of metabolic stresses such as hypoxia and exposure to inhibitors of oxidative phosphorylation is ATP depletion, which leads to the elevation of cellular AMP:ATP ratios and thus to stimulation of AMP-activated protein kinase (AMPK) activity (Hardie et al., 1998). A major function of AMPK appears to be as a protective device, switching off ATP-utilising biosynthetic pathways and switching on ATP-generating metabolic pathways so as to preserve ATP levels (Corton et al., 1994). Its activation not only by stress but also by physiological processes that are associated with stimulation of glucose uptake, such as muscle contraction (Winder and Hardie, 1996; Hutber et al., 1997), suggested that AMPK might also be involved in the regulation of transport (Baldwin et al., 1997). Evidence supporting such an involvement has come

from the use of the adenosine analogue 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which, following uptake into cells and conversion to the monophosphorylated derivative 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), mimics AMP in activating AMPK (Corton et al., 1995). Numerous studies have recently reported that AMPK activation by AICAR leads to translocation of the glucose transporter isoform GLUT4 to the plasma membrane and consequently to stimulation of glucose transport in skeletal muscle and cardiomyocytes (Merrill et al., 1997; Hayashi et al., 1998; Kurth-Kraczek et al., 1999; Russell et al., 1999; Hayashi et al., 2000). Similarly, we have shown that AICAR treatment of BHK cells leads to translocation of GLUT1 and concomitant stimulation of transport (Baldwin et al., 1997). In addition, Abbud and co-workers have more recently reported that AICAR stimulates glucose uptake in the GLUT1-containing cell line Clone 9, where transport stimulation does not appear to involve translocation of transporters (Abbud et al., 2000).

Although the studies described above suggest a role for AMPK in the signal transduction pathways involved in regulation of glucose transport by stress, AICAR has many cellular effects in addition to activation of this kinase (Kemp et al., 1999). A key objective of the present study was therefore to obtain additional evidence for the role of AMPK in the response of Clone 9 cells to stress. A second objective was to discover the identity of other components of the signalling pathways involved. These have so far been little investigated, although we have previously shown that stimulation of transport by metabolic and osmotic stresses involves neither the p38 MAP kinase pathway nor the enzyme phosphatidylinositol 3-kinase (PI 3-kinase), which plays an essential role in the stimulation of transport by insulin (Barros et al., 1995; Barros et al., 1997). The similar sensitivity of both insulin- and stress-induced transport stimulation to inhibition by ML-9, an inhibitor of several kinases, including myosin light chain kinase and protein kinase B (Smith et al., 2000), suggests that the pathways might share some elements (Barros et al., 1995). Recent studies have implicated not only protein kinase B but also atypical diacylglycerol-insensitive protein kinase C (PKC) isoforms ζ and λ as components downstream of PI 3-kinase in the insulin signalling pathway (Bandyopadhyay et al., 1997a; Standaert et al., 1997; Kotani et al., 1998; Hill et al., 1999; Bandyopadhyay et al., 2000). Similarly, conventional PKC isoforms have been implicated in the stimulation of glucose uptake by metabolic stress in L6 myotubes (Khayat et al., 1998), whereas the results of studies on H-2K^b myotubes suggest that stimulation of glucose uptake in skeletal muscle by exercise involves activation of nitric oxide synthase (NOS) by AMPK (Fryer et al., 2000). Although stimulation of transport by exposure of Clone 9 cells to alkaline pH has been reported not to involve phorbol-sensitive PKC isoforms (Hakimian and Ismail-Beigi, 1991), to date a possible role for PKC isoforms in the response to metabolic and osmotic stresses has not been investigated in these cells.

Materials and Methods

Cells were obtained from the American Type Culture Collection (Rockville, MD). Bio-LC-ATB-BMPA (4,4′-*O*-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,trifluoroethyl)benzoyl]amino-1,3-propanediyl bis-D-mannose) was a kind gift from G. D. Holman, University of Bath. [^{3}H]2-deoxy-D-glucose (8.0 Ci mmol^{-1}), 3-*O*-[methyl-^{3}H]-D-glucose (81.5 Ci mmol^{-1}) and [γ-^{32}P]ATP were purchased from New England Nuclear (NEN) Life Science Products. The protein kinase C inhibitors Gö 6850, Gö 6976 and Ro 31-8220, and the nitric oxide synthase inhibitors N^G-monomethyl-L-arginine (L-NMMA) and L-N^G-nitro-L-arginine methyl ester (L-NAME) were from Calbiochem-Novabiochem Corporation. Myristoylated protein kinase Cζ pseudosubstrate peptide was from Biosource International. All other reagents were from Sigma-Aldrich Company Ltd., UK.

Adenoviral infection of Clone 9 cells

Clone 9 cells were grown to confluence in 10 cm dishes. The medium was then aspirated and the cells washed once in phosphate-buffered saline (PBS). In order to express a constitutively active form of AMPK, cells were infected with a recombinant adenovirus harbouring cDNA encoding residues 1-312 of AMPKα1, containing a mutation of threonine 172 to aspartic acid and bearing a myc-tag (Stein et al., 2000; Woods et al., 2000). An adenovirus in which the expression cassette contains the major late promoter but no exogenous gene was used to infect cells as a control. Adenovirus stocks were diluted in serum-free medium to give a multiplicity of infection of 10, and they were incubated with the cells for a period of 2.5 hours at 37°C. An equal volume of 2% fetal calf serum in medium was then added to the cells, which were incubated for a further period of 48 hours at 37°C. The cells were washed twice with PBS prior to exposure to stress in preparation for sugar transport assays or immunoblotting.

Cell culture and sugar transport assays

Clone 9 cells were cultured in 6-well plates or in 57 cm^2 dishes as described previously (Barros et al., 1995). In some instances cells were infected with recombinant adenovirus constructs as described above. Initial rates of radiolabelled sugar uptake were estimated in confluent cell cultures using a modification of a previously described protocol (Barros et al., 1995). For 3-*O*-methyl-D-glucose transport measurements, uptake was initiated by addition to each well of 0.5 ml 0.1 mM 3-*O*-[methyl-^{3}H]-D-glucose in Krebs Ringer Hepes buffer (KRH; 136 mM NaCl, 20 mM Hepes, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, pH 7.4) at 22°C, and the uptake was routinely measured for a period of 5 minutes. Preliminary experiments showed that uptake was linear for this period and did not exceed 20% of the equilibrium uptake value (data not shown). However, for determination of the kinetic parameters of transport, uptake was measured over a period of 30 seconds at 6°C to be certain that initial rates of uptake were being measured at all concentrations. Carrier-mediated uptake rates were calculated by subtracting non-specific uptake measured in the presence of 20 μM cytochalasin B from the initial rates. After stopping transport using three washes in 50 μM phloretin in ice-cold PBS, cells were lysed with 1% Triton X-100, and the radioactivity was measured by liquid scintillation counting. 2-Deoxy-D-glucose uptake measurements were performed in a similar fashion, except that 0.2 mM [^{3}H]2-deoxy-D-glucose was used and uptake was measured for 5 minutes at 37°C. Pre-incubations of cells with appropriate concentrations of stress-inducing agents (e.g. sodium azide) or inhibitors in KRHG (KRH plus 25 mM glucose) were carried out for 30-60 minutes at 37°C as indicated in the figure legends. Data are presented as the means±s.e.m. (n) (n is the number of experiments carried out). Significance was assessed using a Student's *t* test for two groups and was taken at $P<0.05$.

The AMPK assay

AMPK activity was measured as described previously (Hardie et al., 2000). Briefly, cell lysates were prepared from confluent uninfected

or adenovirus-infected Clone 9 cells, grown in 10 cm culture dishes by treatment with 0.5 ml lysis buffer (1% Triton X-100 in 50 mM Tris/HCl, pH 7.4 at 4°C, containing 0.25 M mannitol, 1 mM EGTA, 1 mM DL-dithiothreitol (DTT), 0.1 mM 4-(2-aminoethyl)benzenesulphonylfluoride (AEBSF), 1 mM benzamidine, 5 μg/ml soya bean trypsin inhibitor, 5 mM Na pyrophosphate and 50 mM NaF). Cells were scraped and transferred to a microfuge tube, and after centrifugation (13000 ***g***, 4°C for 3 minutes) they were immunoprecipitated using protein G-sepharose coupled to affinity-purified sheep antibodies against the α1 or α2 isoform catalytic subunits of AMPK or against a mixture of these antibodies (Woods et al., 1996). The immunocomplexes were centrifuged (18000 ***g***, 1 minute for 4°C), and the pellets washed with 5×1 ml ice-cold immunoprecipitation buffer (50 mM Tris/HCl, pH 7.4 at 4°C, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.1 mM AEBSF, 1 mM benzamidine, 5 μg/ml soya bean trypsin inhibitor, 5 mM Na pyrophosphate and 50 mM NaF) containing 1 M NaCl. After further washing with lysate buffer, the pellets were resuspended in 30 μl HEPES-Brij buffer (50 mM Na Hepes, pH 7.4, 1 mM DDT, 0.02% Brij-35) prior to the assay in reaction mixture (5 μl 1 mM [γ-^{32}P]ATP, 25 mM $MgCl_2$ [specific activity 250 to 500 cpm.pmol^{-1}], 5 μl 1 mM AMP in HEPES-Brij buffer, 5 μl 1 mM AMARA peptide [AMARAASAAASARRR] in HEPES-Brij buffer, 5 μl HEPES-Brij buffer; total volume 25 μl) at 30°C for 10 minutes. A 15 μl aliquot was then spotted onto P81 paper (Whatman). After stopping the reaction with 1% (v/v) phosphoric acid, radioactivity was measured by liquid scintillation counting. One unit of AMPK is the amount that incorporates 1 nmol phosphate into substrate peptide per minute.

The protein assay

The amount of protein present was determined by the bicinchoninic acid method with bovine serum albumin as the standard (Smith et al., 1985).

Photolabelling

Dishes (4×10 cm diameter) of confluent Clone 9 cells were treated with or without (control) 5 mM sodium azide in KRHG for 30 minutes. The cells were then washed twice in KRH to remove the glucose before photolabelling with 500 μM Bio-LC-ATB-BMPA (Koumanov et al., 1998) by irradiation in open dishes twice for 1 minute periods using a Rayonet RPR 100 photochemical reactor (RPR-3000 lamps) at 22°C. After labelling, cells were washed three times in ice-cold KRH and then lysed in 200 μl 1% Triton X-100 containing 2 μM pepstatin A, 10 μM leupeptin and 100 μM AEBSF for 20 minutes on ice. Debris was removed from the lysates by centrifugation (100,000 ***g*** for 30 minutes), and biotinylated proteins from 1 ml (~1 mg protein) of the pooled lysate from each test condition were then precipitated by continuous mixing with 75 μl of a 50% (w/v) streptavidin-agarose bead slurry (Pierce and Warriner) overnight at 4°C. The precipitates were washed three times in 0.1% (w/v) Triton X-100 in PBS, twice in PBS and then eluted by heating at 95°C in 30 μl of sample buffer containing 2% (w/v) SDS and 10 mM DTT. Samples of the eluted protein (20 μl) and of the cell lysates (20 μg) were then subjected to western blotting to quantify GLUT1.

Western blotting and immunocytochemistry

For western blotting, proteins were electrophoresed on 10% (w/v) polyacrylamide/SDS gels and then transferred to nitrocellulose membranes. Blots were probed with affinity-purified rabbit antibodies against residues 477-492 of rat GLUT1 (0.5 μg/ml; [Davies et al., 1990]) or with mouse monoclonal antibodies to c-myc (0.25 μg/ml; Clone 9E10, Oncogene Research Products) overnight at 4°C, followed by goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase conjugate (1/40,000; Jackson ImmunoResearch Laboratories, Inc.), as appropriate, for 1 hour. In the case of AMPK, blots of Clone 9 cell lysates were probed with sheep antibodies specific for the phosphorylated form of the AMPK α subunit or with the antibodies specific for the α1 or α2 isoforms (Hardie et al., 2000), followed by an anti-sheep IgG horseradish peroxidase conjugate. The antigen was then visualised by addition of enhanced chemiluminescence detection reagent (Supersignal® Chemiluminescent substrate, Pierce Chemical Company). Staining intensity was quantified using a Bio-Rad Fluor-S gel documentation system and Multi-analyst software. Plasma membrane lawns were prepared and immunostained for GLUT1 as previously described (Barros et al., 2001). Images were obtained and quantified using a Zeiss LSM Pascal 5 Confocal microscope.

Results

Identification of stress-responsive glucose transporters in Clone 9 cells

As described in the Introduction, it has been hypothesised that stimulation of transport in Clone 9 cells by metabolic stress involves activation of cell-surface glucose transporters, possibly the GLUT1 isoform. To examine the nature of this putative activation process and to exclude the possibility that transport stimulation involves translocation of an unidentified transporter isoform to the cell surface, we used the membrane-impermeant photoaffinity labelling reagent Bio-LC-ATB-BMPA. This reagent and other bis-mannose derivatives have been used to identify and quantify cell-surface glucose transporters in many cell types, including adipocytes and skeletal muscle (Koumanov et al., 1998; Ryder et al., 2000). Clone 9 cells were treated with or without (control) 5 mM sodium azide for 30 minutes and then photolabelled with Bio-LC-ATB-BMPA. Biotinylated cell-surface proteins were separated from intracellular proteins using streptavidin-agarose as described in the Materials and Methods, and then the GLUT1 content of each fraction was assessed by immunoblotting with an isoform-specific antibody. The specificity of the cell-surface labelling was indicated by its almost complete inhibition in the presence of either 400 mM D-glucose or 20 μM cytochalasin B, a potent competitive inhibitor of GLUT1 (Fig. 1b, lanes 3 and 4). Moreover, streptavidin precipitates prepared from cells that had not been treated with Bio-LC-ATB-BMPA contained no detectable GLUT1 (Fig. 1b, lane 5).

Fig. 1 shows that although the total cellular content of GLUT1 was unaffected, the level of photolabelled cell-surface GLUT1 was increased 4.1±1.0 (4)fold following exposure of the cells to azide. However, as previously reported by ourselves and others (Shetty et al., 1993; Barros et al., 1995), quantification of GLUT1 by immunocytochemistry of plasma membrane lawns revealed no significant change in the total cell-surface concentration of this transporter (data not shown). In parallel experiments, exposure to azide increased the initial rate of 0.2 mM 2-deoxy-D-glucose uptake by 4.2±0.6 (4)fold. These results suggest that a substantial part of the effect of azide on glucose transport in Clone 9 cells stems from the unmasking of previously inactive GLUT1 molecules that were already present at the cell surface.

A possible role for AMPK in the activation of glucose transport in Clone 9 cells by inhibition of oxidative phosphorylation and by osmotic stress

In other cell types, exposure to osmotic stress or elevation of

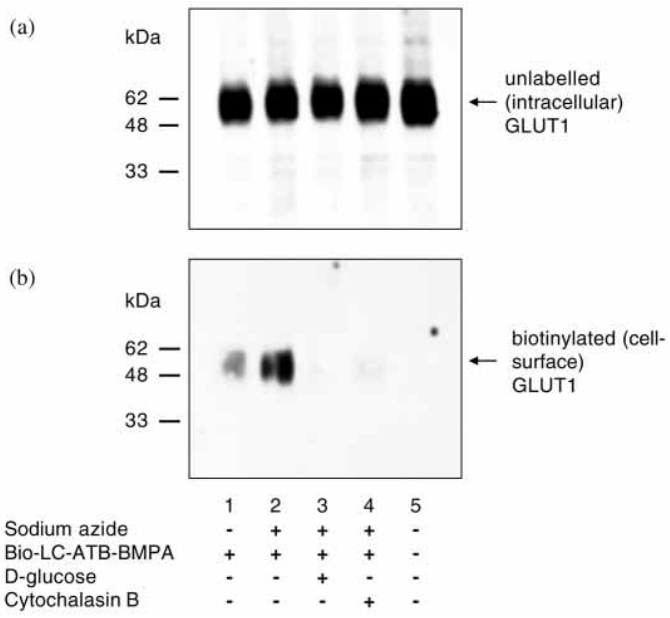

Fig. 1. Effect of metabolic stress on surface labelling of GLUT1 in Clone 9 cells by a membrane-impermeant photoaffinity reagent. Clone 9 cells were incubated with or without 5 mM sodium azide for 30 minutes, as indicated, then photolabelled with 500 μM Bio-LC-ATB-BMPA in the absence or presence of 400 mM glucose (lane 3) or 20 μM cytochalasin B (lane 4). A control sample (lane 5) was also UV-irradiated in the absence of Bio-LC-ATB-BMPA. Following cell lysis with 1% TX-100, samples of total lysate proteins (a) and of biotinylated cell-surface proteins isolated by adsorption to streptavidin agarose (b) were subjected to western blotting to detect GLUT1, as described in the Materials and Methods. The mobilities of the molecular mass markers are indicated on the left.

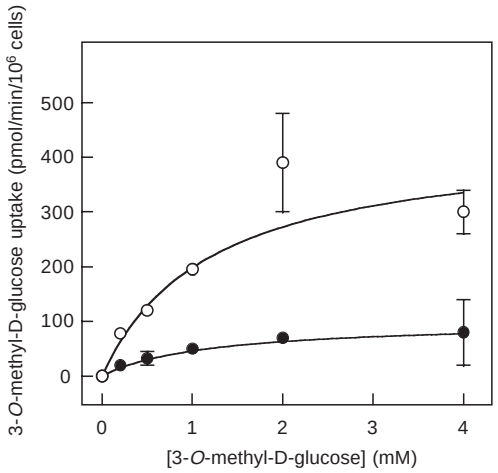

Fig. 2. Effect of AICAR exposure on the kinetic parameters of hexose uptake. Clone 9 cells were treated for 60 minutes with (open symbols) or without (closed symbols) 500 μM AICAR. Uptake of [^{3}H]3-*O*-methyl-D-glucose was then measured at 6°C in the presence of increasing concentrations of hexose as described in the Materials and Methods. Transport parameters were estimated by direct fitting of a rectangular hyperbola to the data using non-linear regression (Sigma Plot, Jandel). Data shown are means±s.e.m. (3).

cellular AMP:ATP ratios as a result of inhibition of oxidative phosphorylation has been reported to lead to the stimulation of AMPK activity (Hardie et al., 1998). A possible role for AMPK in the signal transduction pathways leading to stimulation of glucose uptake in Clone 9 cells was suggested by our finding that treatment of these cells with 500 μM AICAR for 60 minutes increased the initial rate of uptake of 0.1 mM 3-*O*-methyl-D-glucose at 6°C by 5.0±0.8 (3) fold. Similar results have recently been reported by another laboratory (Abbud et al., 2000). A detailed investigation of the kinetics of this phenomenon showed that, like inhibition of oxidative phosphorylation (Shetty et al., 1993; Barros et al., 1995) and exposure to hypertonic conditions (Barros et al., 2001), AICAR treatment increased the V_{max} for transport with little effect on K_m. In four paired experiments, one of which is illustrated in Fig. 2, exposure to 500 μM AICAR for 60 minutes increased the V_{max} for 3-*O*-methyl-D-glucose uptake from 114±9 (4) to 427±61 (4) pmol/minute/10^6 cells, whereas the K_m values of 2.6±0.8 (4) and 2.1±0.7 (4) mM measured in the control and AICAR-stimulated cells, respectively, were not significantly different. AICAR stimulation of transport also resembled that resulting from inhibition of oxidative phosphorylation and/or exposure to hypertonic conditions in that immunocytochemistry of plasma membrane lawns revealed no significant change in the total cell-surface concentration of GLUT1 (Fig. 3a,b). These findings confirmed, by an independent method, those recently reported by Abbud et al. (Abbud et al., 2000). The capacity of the plasma membrane lawn method to detect increases in cell surface GLUT1 was confirmed in our experiments by exposing the cells to $CoCl_2$ (Fig. 3c), a treatment known to increase both the expression of GLUT1 and the uptake of hexoses in Clone 9 cells (Behrooz and Ismailbeigi, 1997).

Given the similarity of the responses to AICAR, osmotic and

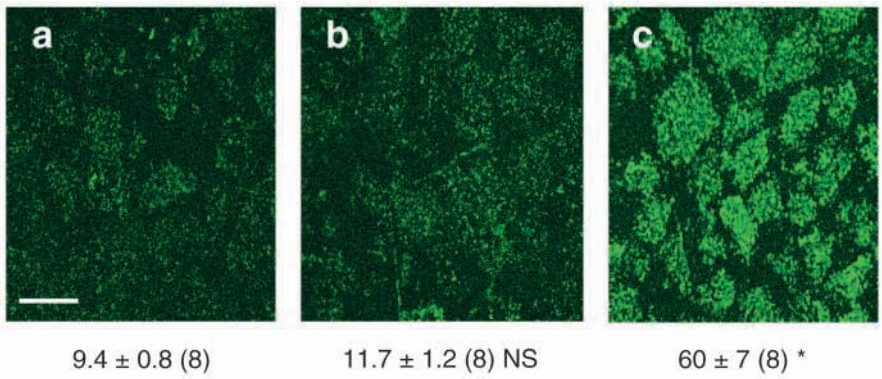

Fig. 3. Effect of AICAR exposure on the abundance of cell-surface GLUT1. Clone 9 cells were exposed for 60 minutes to buffer only (a) or to 500 μM AICAR (b) or for 24 hours to 250 μM $CoCl_2$ (c). Plasma membrane lawns were prepared, immunostained and quantified as described in the Materials and Methods. Data were pooled from two experiments in which eight fields were measured, each containing an average of 12 cells. The image intensity data (arbitrary units) shown beneath the images are means±s.e.m. [(8) number of fields]. *Significantly different (P<0.05) from the control (ANOVA plus Bonferroni's ad hoc test); NS, not significantly different from the control. Scale bar, 100 μm.

Table 1. Effect of exposure to sodium azide, AICAR or hypertonic sorbitol on the activity of the AMPK α1 and α2 isoforms in Clone 9 cells

	AMPK activity (units/mg protein)		
Treatment	AMPK α1	AMPK α2	AMPK α1 and α2
Basal	0.11±0.01	0.003±0.001	0.13±0.02
5 mM sodium azide	0.98±0.15	0.015±0.002	1.08±0.12
500 μM AICAR	0.56±0.11	0.011±0.005	0.61±0.13
0.4 M sorbitol	0.32±0.04	0.008±0.005	0.31±0.03

Clone 9 cells in triplicate dishes were exposed to 5 mM sodium azide, 500 μM AICAR or 0.4 M sorbitol for 60 minutes. AMPK α1 and/or AMPK α2 were then immunoprecipitated from cell lysates with the relevant antibodies, and the immunocomplexes were assayed for their ability to phosphorylate the AMARA peptide as described in the Materials and Methods. The data shown are means±s.e.m for three experiments.

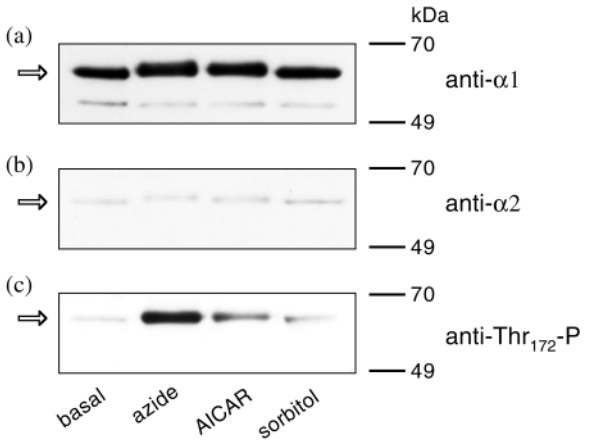

Fig. 4. AMPK α subunit isoforms and their phosphorylation in response to treatment of Clone 9 cells with sodium azide, AICAR and hypertonic sorbitol. Confluent dishes of cells were untreated (basal), exposed to 5 mM sodium azide (azide), 500 μM AICAR (AICAR) or 0.4 M sorbitol (sorbitol) for 60 minutes. Western blots of cell lysates were probed with antibodies against the α1 subunit of AMPK (a), against the α2 subunit (b) or with an antibody specific for the Thr_{172}-phosphorylated forms of the α1 and α2 AMPK subunits (c). The mobilities of molecular mass markers are indicated on the right. Arrows denote the position of the 63 kDa AMPK α subunits.

metabolic stress, we next sought to assess the possibility that AMPK is involved in the signal transduction pathways leading to stimulation of glucose uptake in Clone 9 cells by these stress stimuli. To this end we examined the effects of metabolic and osmotic stress on the activity of this enzyme. The effects on hexose uptake rates and on AMPK activity produced by exposure to 5 mM azide, 500 μM AICAR or hypertonic (0.4 M) sorbitol for 60 minutes were investigated in parallel using the same batch of cells. Following such treatments the initial rate of uptake of 0.1 mM 3-*O*-methyl-D-glucose increased 7.9±1.8-, 5.0±0.4- and 2.6±0.1-fold, respectively. Total AMPK activities in immunoprecipitates captured from lysates of the treated cells using a mixture of antibodies against both the α1 and α2 subunit isoforms of AMPK were 8.6±0.9-, 4.9±1.1- and 2.5±0.2-fold greater, respectively, than basal values (Table 1). Western blots of the lysates probed with antibodies specific for the phosphorylated form of the AMPK α-subunit revealed that changes in the phosphorylation state of the kinase paralleled the changes in enzymatic activity in each experimental condition, that is, phosphorylation of the α-subunit was lowest in untreated cells and highest in azide-treated cells (Fig. 4c). The slightly lower electrophoretic mobility of the bands stained in western blots by antibodies specific for the α1 and α2 subunits in samples from azide- and AICAR-treated cells compared with untreated cells, likewise reflects their phosphorylation (Fig. 4a,b). Measurement of AMPK activities in immunoprecipitates prepared using individual isoform-specific antibodies revealed that the α1 isoform accounted for most of the activity, both in the basal and stimulated states (Table 1). Western blotting confirmed that levels of the α1 subunit were greater than those of the α2 subunit in Clone 9 cells (Fig. 4a,b). These results differ from those of Abbud and colleagues, who reported that the predominant α-subunit in Clone 9 cells is α2 and suggested that changes in the activity of this subunit might be primarily responsible for the stimulation of glucose transport by AICAR (Abbud et al., 2000). We are uncertain of the reason for this discrepancy, but it does not reflect an inability of the antibodies used in the present study to precipitate the α2 subunits: in previous studies these were used to show that the α1 and α2 isoforms contribute equally to total AMPK activity in rat liver (Woods et al., 1996).

To investigate further the possible involvement of AMPK in the stimulation of glucose uptake by metabolic stress, Clone 9 cells were infected with recombinant adenovirus encoding a constitutively active (ca), myc-tagged mutant of AMPK (ca-AMPK). The initial rate of uptake of 0.1 mM 3-*O*-methyl-D-glucose uptake by unstressed cells infected with an adenovirus encoding ca-AMPK was 5.8±0.4-fold greater than that seen in uninfected cells (Fig. 5a). By contrast, infection of cells with an adenovirus lacking AMPK increased transport only 1.9±0.3-fold (Fig. 5a). Successful expression of the kinase was confirmed by staining western blots of cell lysates with antibodies to c-myc: a band with the expected size for ca-AMPK (35 kDa) was evident in cells infected with an adenovirus encoding myc-tagged ca-AMPK but not in uninfected cells or those infected with an adenovirus lacking AMPK (Fig. 5b). No changes in the total levels of GLUT1 expressed in the cells were detectable by western blotting following infection with either type of adenovirus (data not shown). Exposure to metabolic stress had little additional effect on transport in cells expressing ca-AMPK: the fold increase in the initial rate of 0.1 mM 3-*O*-methyl-D-glucose uptake following exposure to 5 mM azide was 5.4±0.3, 1.1±0.1 and 3.1±0.1 for uninfected cells, cells infected with adenovirus encoding ca-AMPK and cells infected with adenovirus lacking AMPK, respectively (Fig. 5a).

Effect of NOS and PKC inhibitors on stimulation of glucose uptake by metabolic stress or by AICAR

The effects of ca-AMPK expression and of AICAR on hexose uptake by Clone 9 cells, together with the observed stimulation of AMPK activity in these cells by both AICAR and stress stimuli, strongly suggested that this enzyme lies on the signal transduction pathway linking stress stimuli to GLUT1 activation. The targets of AMPK in Clone 9 cells have not been described, but the results of experiments performed on the muscle cell line H-$2K^b$ and on rat muscle (Fryer et al., 2000) identified NOS as a candidate. To probe the involvement of

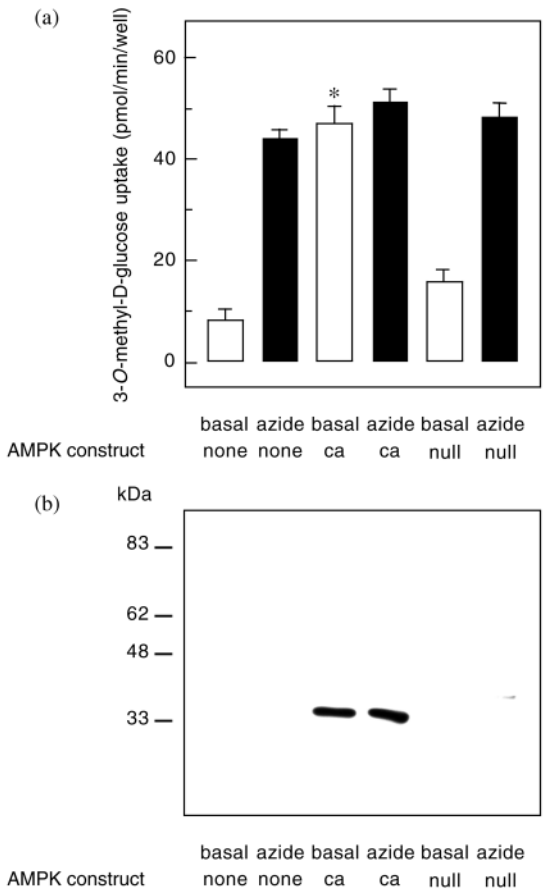

Fig. 5. Comparison of the effects of exposure to azide and of infection by an adenovirus encoding ca-AMPK, or lacking AMPK, on hexose transport. Clone 9 cells in triplicate culture dishes were maintained uninfected (none) or infected with adenovirus either encoding ca-AMPK (ca) or lacking the AMPK gene (null) for 48 hours. The cells were washed twice with PBS and treated for 30 minutes with (azide) or without (basal) 5 mM azide. Uptake of [^{3}H]3-*O*-methyl-D-glucose was then measured as described in the Materials and Methods. Results shown in (a) are means±s.e.m. (3). In parallel, western blots of cell lysates (10 μg protein) were prepared as described in the Materials and Methods section and stained with mouse antibodies to c-myc (b). The mobilities of molecular weight markers are indicated on the left. *This value is significantly different (P<0.05) from basal uptake rate in non-infected cells and from basal uptake rate in cells infected with adenovirus lacking the AMPK coding region.

nitric oxide in the stress response, we therefore examined the effects of the NOS inhibitors L-NMMA and L-NAME. Stimulation of glucose uptake by AICAR was not significantly inhibited by either compound (Table 2) when they were used at concentrations (400 μM and 2 mM, respectively) that prevented transport stimulation by AICAR in H-2K^b myotubes and in rat skeletal muscle strips (Fryer et al., 2000). Similarly, when used at these concentrations, L-NMMA and L-NAME decreased transport stimulation by azide by only approximately 30% (Table 2). These findings indicate that, in contrast to the situation in skeletal muscle, NOS probably does not lie on the pathway linking metabolic stress to transport stimulation in Clone 9 cells.

Stimulation of glucose uptake in L6 myotubes stressed by acute exposure to mitochondrial uncouplers has been associated with translocation to the plasma membrane and activation of conventional protein kinase C isoforms (Khayat et al., 1998). In addition, atypical protein kinase C isoforms have been implicated in the stimulation of glucose transport in adipocytes by insulin (Bandyopadhyay et al., 1997a; Standaert et al., 1997; Kotani et al., 1998; Bandyopadhyay et al., 2000). Although stimulation of transport by exposure of Clone 9 cells to alkaline pH has been reported not to involve phorbol-sensitive PKC isoforms, phorbol esters do stimulate transport (Hakimian and Ismail-Beigi, 1991). Protein kinase C isoforms therefore represent another set of signalling components potentially involved in the regulation of glucose uptake in Clone 9 cells in response to stress. To examine their involvement in the increase of glucose uptake provoked by metabolic stress or by AICAR in Clone 9 cells, the effects of a series of PKC inhibitors were therefore investigated.

The indolocarbozole compound Gö 6976, with IC_{50} values in the low nanomolar range (Martiny-Baron et al., 1993), selectively inhibits conventional protein kinase C isoforms but had no significant effect on the stimulation of transport resulting either from azide-induced metabolic stress (Fig. 6a) or from exposure of cells to AICAR (data not shown), even at inhibitor concentrations as high as 20 μM. Similarly, the bisindolylmaleimide compound Gö 6850 (GF 109203X; Bisindolylmaleimide I), which inhibits both conventional and novel PKC isoforms, the latter with IC_{50} values ≤250 nM (Martiny-Baron et al., 1993; Wilkinson et al., 1993), was without effect at concentrations up to 2 μM and inhibited azide-stimulated hexose transport by only ~30% even at 20 μM (Fig. 6b). Neither Gö 6976 nor Gö 6850 had any effect on the

Table 2. Effects of nitric oxide synthase inhibitors on stimulation of hexose uptake by sodium azide and AICAR in Clone 9 cells

	2-deoxy-D-glucose uptake (% of basal in absence of inhibitor)		3-*O*-methylglucose uptake (% of basal in absence of inhibitor)	
Treatment	Basal	5 mM azide	Basal	500 μM AICAR
No inhibitor[a]	100.0±6.5	470.7±24.0	100.0±41.9	285.5±41.5
400 μM L-NMMA[a]	68.3±3.6	324.6±5.6*	41.1±11.6	209.3±23.7
No inhibitor[b]	100.0±26.2	761.4±13.8	100.0±51.1	438.9±133.8
2 mM L-NAME[b]	77.4±6.1	554.7±7.1*	138.9±55.7	379.5±23.0

Clone 9 cells were treated with or without 400 μM L-NMMA or 2 mM L-NAME for 60 minutes. During the last 30 minutes of this period they were exposed either to 5 mM sodium azide or 500 μM AICAR or exposed to neither agent (basal). The uptake of [^{3}H]3-*O*-methyl-D-glucose or [^{3}H]2-deoxy-D-glucose was then measured as described in the Materials and Methods. [a,b]Separate batches of cells, which exhibited slightly differing fold-stimulations of transport by azide and AICAR, were used for experiments with L-NMMA and L-NAME. The results shown are the means±s.e.m for three experiments; *this value is significantly different (P<0.05) from azide-stimulated uptake rate in the absence of the inhibitor.

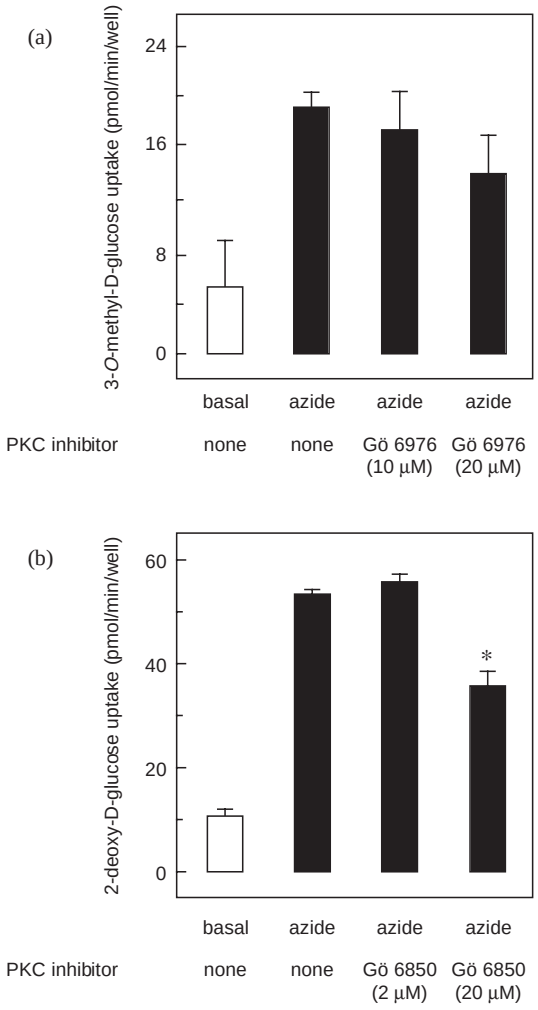

Fig. 6. Comparison of the effects of (a) Gö 6976 and (b) Gö 6850 on metabolic stress-stimulated hexose transport. Clone 9 cells in triplicate culture dishes were treated with or without the indicated concentrations of inhibitors for 45 minutes. During the last 30 minutes of this period, they were treated with (azide) or without (basal) 5 mM sodium azide. Uptake of (a) [^{3}H]3-*O*-methyl-D-glucose or (b) [^{3}H]2-deoxy-D-glucose was then measured as described in the Materials and Methods. Results shown are the means±s.e.m.; *this value is significantly different (P<0.05) from the azide-stimulated uptake rate in the absence of the inhibitor.

basal rate of hexose uptake into unstressed cells at concentrations up to and including 20 μM (data not shown). These findings indicate that, in contrast to the reported situation in L6 myoblasts (Khayat et al., 1998), neither conventional nor novel protein kinase C isoforms are likely to be involved in metabolic stress-regulated signal transduction pathways in Clone 9 cells.

Discussion

In Clone 9 cells, exposure to metabolic or osmotic stress leads to a rapid increase in the V_{max} for glucose uptake (Mercado et al., 1989; Barros et al., 2001). This increase is not paralleled by changes in the cell-surface levels of GLUT1, the only glucose transporter isoform so far detected in these cells. It has therefore been proposed that the increase in V_{max} stems from an activation of functionally 'cryptic' GLUT1 molecules already present at the cell surface (Hamrahian et al., 1999). This situation contrasts with the regulation of glucose transport in many other cell types, such as adipocytes, where, for example, insulin and osmotic stress stimulate transport via translocation of the GLUT4 glucose transporter isoform (and, to a lesser extent, GLUT1) to the cell surface from an intracellular location (Pessin et al., 1999). Using isoform-specific antibodies, it has been shown that Clone 9 cells lack not only the glucose transporter GLUT4 but also GLUT2 and GLUT3 (Shi et al., 1995). However, genome sequencing has recently led to the identification of several additional members of the mammalian GLUT family, some of which are known to be glucose transporters (Joost and Thorens, 2001). Moreover, one of these novel isoforms, GLUT8, has been reported to undergo translocation in response to insulin in blastocysts (Joost and Thorens, 2001). It follows that the enhanced rate of glucose uptake seen in Clone 9 cells following exposure to stress might reflect translocation of such an isoform or of another unidentified species. In the present study, we have for the first time been able to demonstrate that this is unlikely: using a membrane-impermeant photoaffinity-labelling reagent followed by isoform-specific immunodetection, we have shown that the stress-induced increase in glucose transport seen in Clone 9 cells closely correlates with increased exofacial labelling of a specific glucose transporter isoform, GLUT1. This finding strongly suggests that this isoform is responsible for the majority of stress-induced glucose uptake in Clone 9 cells. Such a conclusion is supported by the observation that the apparent affinity of the transport process for glucose (K_m for 3-*O*-methylglucose uptake of 2-3 mM; this paper) and its potent inhibition by cytochalasin B ($K_i \sim 10^{-7}$ M, [Hakimian and Ismail-Beigi, 1991]) are both characteristic of GLUT1.

The molecular mechanism responsible for the inability of most of the GLUT1 molecules present at the Clone 9 cell surface to be labelled by Bio-LC-ATB-BMPA or to transport glucose in the basal state (Hamrahian et al., 1999) remains unclear. However, catalytically inactive GLUT1 molecules, which are similarly refractory to labelling by exofacial affinity reagents, have also been detected in adipocytes, where they appear to be 'unmasked' in response to anisomycin (Harrison et al., 1992). Interestingly, measurements of the binding of the transport inhibitor cytochalasin B to Clone 9 cell plasma membranes have revealed the presence of fewer binding sites in non-stressed cells when compared with those in cells exposed to sodium azide (Shi et al., 1995). Cytochalasin B binds to the endofacial conformation of the substrate-binding site in facilitative glucose transporters (Devés and Krupka, 1978): if the binding sites in Clone 9 cells represent GLUT1 molecules, it follows that both the exofacial and endofacial conformations of the substrate-binding sites of these transporters are somehow masked in the catalytically inactive form of the protein proposed to exist in non-stressed cells. It has been proposed that such masking stems from the binding of an inhibitory protein, but its identity is not known (Shi et al., 1995).

The signal transduction pathways linking metabolic and osmotic stresses to the activation of cell-surface GLUT1 in Clone 9 cells similarly remain unclear. However, our findings

and those of others (Abbud et al., 2000) that AICAR not only activates AMPK but also stimulates glucose transport in a manner akin to metabolic stress suggested that this enzyme might be involved in the regulation of transport by stress stimuli in these cells. This hypothesis is strengthened by two additional findings from the present study: (a) that expression of constitutively active AMPK mimics the effect of stress on transport; and (b) that following exposure to sodium azide or hypertonic sorbitol solutions, AMPK activity is increased in parallel with the stimulation of hexose uptake. Moreover, as described in the Introduction, several groups have recently provided evidence for a role for AMPK in stress-induced translocation of the GLUT4 glucose transporter isoform in skeletal muscle and heart.

Although our data show that AMPK is likely to be involved in the stimulation of transport not only by AICAR but also by metabolic and osmotic stress, the relevant downstream targets of AMPK in Clone 9 cells have not yet been identified. AMPK is known to phosphorylate and activate endothelial nitric oxide synthase (eNOS) during ischaemia in rat hearts (Chen et al., 1999). Similarly, in mouse H-2K^b muscle cells, activation of AMPK by AICAR has been shown to stimulate NOS activity, and NOS inhibitors completely block the stimulation by AICAR of glucose uptake in these cells (Fryer et al., 2000). Because 8-Br cGMP also stimulates transport in these cells, and inhibition of guanylate cyclase by LY83583 blocks stimulation of transport by AICAR, it has been proposed that activation of AMPK in muscle cells stimulates GLUT4-mediated glucose transport by activation of NOS coupled to downstream signalling components, including cGMP (Fryer et al., 2000). However, this mechanism is unlikely to be responsible for stimulation of transport by stress in Clone 9 cells because transport in these cells is not affected by 8Br-cGMP and is stimulated, rather than inhibited, by LY83583 (Prasad et al., 1999). Furthermore, in the present study we have shown that the NOS inhibitors L-NMMA and L-NAME do not prevent hexose transport stimulation induced by exposure of cells either to azide or to AICAR.

Conventional PKCs have been reported to be involved in transport stimulation by mitochondrial uncouplers in other cell types (Khayat et al., 1998), and the atypical isoform PKCζ is involved in the response to ischaemic hypoxia in rat cardiomyocytes (Mizukami et al., 2000). In an attempt to identify possible downstream components of stress-regulated signal transduction pathways in Clone 9 cells, we therefore investigated the role of PKC isoforms in the regulation of glucose transport. The lack of effect of small molecule inhibitors indicates that conventional and novel PKC isoforms are unlikely to play a role in the response to stress in these cells. It was not possible to use a similar approach to investigate the potential involvement of atypical PKC isoforms because the most widely used small molecule inhibitor of these enzymes, the bisindolylmaleimide compound Ro 31-8200 (Standaert et al., 1997), has recently been shown to be an equally effective inhibitor of AMPK in vitro (Davies et al., 2000). We found that treatment of Clone 9 cells with this compound in vivo at a concentration of 10 μM completely prevented both the activation of AMPK and the stimulation of hexose transport induced by exposure to AICAR or to sodium azide (data not shown). The membrane-permeable, myristoylated PKCζ pseudosubstrate peptide (myr-SIYRRGARRWRKL) has been employed as a more specific inhibitor of the atypical PKC isoforms (Bandyopadhyay et al., 1997b; Standaert et al., 1997; Bandyopadhyay et al., 1999). However, although we found that this peptide, used at a concentration of 40 μM, completely prevented the stimulation of transport by azide and inhibited that by AICAR by 65%, the peptide was found to weakly inhibit the activity of purified AMPK in vitro, with an IC_{50} of approximately 100 μM (data not shown). No definitive conclusions can therefore be drawn about the involvement of atypical PKC isoforms in the response of Clone 9 cells to stress.

In summary, our findings suggest that metabolic stress stimulates activation of cell-surface GLUT1 in Clone 9 cells via a pathway that involves AMPK. The relevant downstream effectors of this enzyme remain unclear, but are unlikely to include either NOS or conventional and novel PKCs. After submission of this manuscript, Xi et al. reported that p38 MAP kinase mediates AICAR-stimulated glucose transport in Clone 9 cells (Xi et al., 2001). However, we have previously found that although activation of this enzyme, for example by anisomycin, can stimulate hexose uptake, the kinase is not involved in the stimulation of uptake either by azide or by osmotic stress (Barros et al., 1997; Barros et al., 2001). Further investigations will therefore be required in order to understand the mechanism by which transporter activation occurs and to identity additional components of the signalling pathway involved in the response to stress.

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