# Evidence of two mechanisms for the activation of the glucose transporter GLUT1 by anisomycin: p38(MAP kinase) activation and protein synthesis inhibition in mammalian cells

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1. Inhibitors of protein synthesis stimulate sugar transport in mammalian cells through activation of plasma membrane GLUT1, the housekeeping isoform of the glucose transporter. However, it has been reported that some of these compounds, in addition to their effect on protein synthesis, also activate protein kinases.

2. In the present study we have explored the role of these two effects on GLUT1 activation. In 3T3-L1 adipocytes and Clone 9 cells, stimulation of sugar transport by puromycin, a translational inhibitor that does not activate kinases, was not detectable until 90 min after exposure. In contrast, stimulation by anisomycin, a potent Jun-$NH_2$-terminal kinase (JNK) agonist, exhibited no lag phase. An intermediate response was observed to emetine and cycloheximide, weak activators of JNK.

3. The potency of anisomycin to stimulate transport acutely (30 min of exposure) was 5- to 10-fold greater than for its chronic stimulation of transport, measured after 4 h of exposure. The stimulation of transport by a low concentration of anisomycin (0·3 $\mu$M) was transient, peaked at 30–60 min and it was inhibited ($IC_{50} < 1$ $\mu$M) by SB203580, which indicates that its mediator is not JNK, but the homologous p38(MAP kinase) (p38(MAPK)). In contrast, the responses to 4 h exposure to 300 $\mu$M anisomycin or puromycin were refractory to SB203580.

4. Exposure to anisomycin resulted in rapid activation of p38(MAPK). Activation of both p38(MAPK) and GLUT1 by 0·3 $\mu$M anisomycin was cancelled by puromycin.

5. We conclude that the activation of GLUT1 in response to anisomycin includes two components: a delayed component involving translational inhibition and a fast, puromycin-inhibitable component that is secondary to activation of p38(MAPK).

Stimulation of glucose uptake, usually mediated by the 'housekeeping' isoform of the glucose transporter GLUT1, is an early event in the adaptive response of mammalian cells to metabolic stress (Widnell, Baldwin, Davies, Martin & Pasternak, 1990; revised by Ismail-Beigi, 1993). In some cell types, such as baby hamster kidney (BHK) cells, the stress-induced increase in glucose uptake correlates with the translocation of intracellular carriers to the cell surface (Widnell *et al.* 1990). In other cell types, such as Clone 9 cells, we and others have shown by a variety of techniques that stimulation of transport by stress results primarily from *in situ* activation of surface carriers (i.e. an increase in the catalytic activity of individual transporters) with little or no increase in their cell surface concentration (Shetty, Loeb, Vikstrom & Ismail-Beigi, 1993; Barros, Marchant & Baldwin, 1995). Activation of transporters may also contribute to the stimulation of glucose transport by insulin in cells such as adipocytes, although the effect is small in comparison to that resulting from insulin-induced translocation of transporters to the cell surface (Clark, Holman & Kozka, 1991).

The intracellular signalling pathways responsible for the activation of glucose transporters at the cell surface are largely unknown. However, we and others have recently shown that phosphatidylinositol (PI) 3-kinase, a necessary link in the stimulation of sugar uptake by insulin, is not involved in the stimulation of glucose transport by metabolic stress (Barros *et al.* 1995; Tsakiridis, Vranic &

Klip, 1995). These results point to the presence of multiple signalling pathways regulating glucose transport in mammalian cells. The fact that the response to metabolic stress is prevented by the kinase inhibitor ML-9 suggests that one of these pathways might involve MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) or perhaps one of its homologues (Barros *et al.* 1995).

An additional mechanism of GLUT1 modulation was proposed some years ago after the observation that anisomycin, an antibiotic routinely used as a protein synthesis inhibitor, induces a rapid increase in the catalytic activity of GLUT1 in 3T3-L1 adipocytes (Clancy, Harrison, Buxton & Czech, 1991; Czech, Clancy, Pessino, Woon & Harrison, 1992; Harrison, Clancy, Pessino & Czech, 1992). A model was proposed wherein a short-lived protein would act as a tonic inhibitor, keeping the carrier down-regulated in the basal state. Its rapid degradation after translational arrest would result in sugar uptake activation. Analogous results were also obtained in cultured human fibroblasts (Germinario, Manuel, Chang & Leckett, 1992) and L6 myotubes (Hayes, Biswas, Strout & Berger, 1993). However, an alternative possible mode of action of anisomycin is suggested by the recent discovery that this antibiotic is a potent activator of the group of serine kinases known as p45/55, c-Jun kinases (JNKs) or stress-activated protein kinases (SAPKs) (Cano, Hazzalin & Mahadevan, 1994; Kyriakis *et al.* 1994). These proteins are highly homologous to ERK and are activated by a variety of cellular stresses (Cano & Mahadevan, 1995).

The present study was designed to ascertain whether protein synthesis inhibition or kinase activation is the mechanism by which anisomycin stimulates glucose uptake in mammalian cells. Experiments were carried out using 3T3-L1 adipocytes, a cell type in which the response of glucose transport to anisomycin has previously been characterized (Clancy *et al.* 1991; Harrison *et al.* 1992). In addition, we also investigated Clone 9 cells, an epithelial cell line that, in contrast to 3T3-L1 cells, is known to express only a single glucose transporter isoform, GLUT1 (Ismail-Beigi, 1993). Our results indicate that a large component of the stimulating effect of anisomycin is independent of protein synthesis inhibition and that this component involves p38(MAPK), a stress-sensitive member of the MAP kinase superfamily.

## METHODS

### Materials

Cells were obtained from the European Collection of Animal Cell Cultures. 2-Deoxy-D-[$^3$H]glucose (8·1 Ci mmol$^{-1}$) was purchased from DuPont-New England Nuclear. SB203580 (4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl) imidazole) was given by Dr John Lee, SmithKline Beecham, King of Prussia, PA, USA. Tissue culture reagents and standard chemicals were from Sigma.

### Cell culture and transport assays

Cells were grown and differentiated as described previously (Barros *et al.* 1995). Exposure to experimental conditions (e.g. addition of anisomycin) was preceded by a 4 h incubation in serum-free Dulbecco's modified Eagle's medium (DMEM) to reduce the basal rate of sugar transport. Experimental conditions were achieved by adding aliquots of 500× stock solutions. Anisomycin, emetine and cytochalasin B were dissolved in ethanol. Puromycin was dissolved in water. SB203580 was dissolved in dimethyl sulphoxide and added 60 min prior to exposure of cells to anisomycin and other agents. Control experiments showed that the vehicles used did not affect sugar transport. Uptake of 0·2 mM 2-deoxy-D-[$^3$H]glucose was measured in six-well plates for a period of 2 min at 37 °C 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) as described previously (Barros *et al.* 1995). Uptake was linear for up to 20 min in both cell types in the absence and presence of anisomycin and puromycin, confirming that intracellular phosphorylation of 2-deoxyglucose is not rate limiting under these conditions. Non-mediated uptake, estimated in the presence of 20 $\mu$M cytochalasin B, was usually lower than 20% of the basal uptake rate. It was not affected by up to 240 min of exposure to 300 $\mu$M anisomycin, and it was subtracted from the total uptake to obtain mediated uptake. For comparison with other studies, note that a well (35 mm diameter) contains approximately $10^6$ cells and 220 and 860 $\mu$g of protein for Clone 9 cells and mature 3T3-L1 adipocytes, respectively (Barros *et al.* 1995).

### Plasma membrane lawns

Isolated plasma membrane lawns from 3T3-L1 adipocytes were prepared and quantified as described previously (van den Berghe, Barros, van Mackelenbergh & Krans, 1996). By varying the gain of the confocal detection system, it was verified that under the conditions used, a linear relationship existed between the fluorescence detected and the amplitude of the signal as quantified using the imaging system.

### Determination of p38(MAPK) activity

After exposure to experimental conditions, 3T3-L1 adipocyte monolayers were washed twice with ice-cold phosphate-buffered saline (PBS; 10 mM $Na_2HPO_4$, pH 7·4, 150 mM NaCl), scraped off in PBS using a rubber policeman and sedimented at 200 *g* for 10 min at 4 °C. Cell cytosols were extracted with lysis buffer (50 mM Tris, pH 7·4, 150 mM NaCl, 10 mM sodium tetrapyrophosphate, 1 mM EDTA, 25 mM $\beta$-glycerophosphate, 100 $\mu$M sodium orthovanadate, 1% (w/v) Nonidet P-40, 0·5% (w/v) sodium deoxycholate, 0·1% (w/v) sodium dodecyl sulphate (SDS)) supplemented with 1 mM phenylmethylsulphonyl fluoride, 10 mM E-64, 1 $\mu$g ml$^{-1}$ pepstatin and 10 $\mu$g ml$^{-1}$ aprotinin. The cell extracts were clarified by centrifugation at 13 000 r.p.m. for 15 min at 4 °C. p38(MAPK) was immunoprecipitated from the supernatants using a 1 : 100 dilution of rabbit antiserum raised against the COOH-terminal peptide sequence of murine p38(MAPK) (ISFVPPPLDQEEMES) and protein A–agarose. The immunoprecipitates were washed twice using RIPA buffer and twice using assay buffer (25 mM Hepes, pH 7·4, 25 mM $\beta$-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 100 $\mu$M sodium orthovanadate). The immunoprecipitates were resuspended in assay buffer with either protein phosphatase 2A-inactivated MAPKAPK-2 (mitogen-activated protein kinase-activated protein kinase 2; p50; isolated from KB cells) (Freshney *et al.* 1994) and heat shock protein 27 (hsp27), or a glutathione-*S*-transferase fusion protein bearing the activating transcription factor 2 (GST-ATF2), as substrates. Assays were initiated by the addition of ATP (final

concentration 20 μM, 5 μCi [γ-$^{32}$P]ATP) and terminated by boiling in Laemmli buffer (composition: 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2·3% (w/v) SDS, 62·5 mM Tris-HCl (pH 6·8), 10 mM EDTA). Phosphorylated proteins were resolved by electrophoresis in 12·5% (w/v) SDS–polyacrylamide gels and autoradiography as described previously (Freshney *et al.* 1994).

Data are presented as the mean ± S.E.M. For uptake data, significance of differences was evaluated using Student's *t* test or analysis of variance (ANOVA) followed by Tukey–Kramer's test. For densitometry values, the Mann–Whitney *U* test was used as no normal distribution was assumed. Statistical significance is taken at $P < 0·05$.

## RESULTS

Anisomycin, in addition to its better known property of being an inhibitor of translation, is a strong activator of JNK/SAPKs, members of the MAP kinase family (Kyriakis *et al.* 1994; Cano *et al.* 1995). The latter property is not shared by puromycin, another inhibitor of protein synthesis (Edwards & Mahadevan, 1992). Figure 1 illustrates the time courses for the stimulation of sugar uptake in 3T3-L1 and Clone 9 cells by these agents. Consistent with previous reports (Clancy *et al.* 1991), the response to anisomycin was found to be fast, such that it could be detected within 30 min of exposure. In contrast, there was a marked lag before transport was increased in response to puromycin in both cell types. The difference is consistent with a two-phase response to translational inhibitors – a fast phase mediated via kinase activation and thus seen only with anisomycin, and a slower phase involving inhibition of translation and consequently seen with both anisomycin and puromycin. This notion is also supported by the observation that the responses to emetine (Fig. 1*A*) and cycloheximide (Fig. 1*B*; see also Clancy *et al.* 1991), translational inhibitors that activate JNK/SAPKs weakly (Kyriakis *et al.* 1994; Cano *et al.* 1994), were intermediate between those to anisomycin and to puromycin. The responses to emetine and cycloheximide were similar in both cell types (not shown). Note that at the concentrations used, all these inhibitors virtually abolish protein synthesis (Clancy *et al.* 1991; Germinario *et al.* 1992; Edwards & Mahadevan, 1992; Low, Ross & Grigor, 1994) and that biological effects due to translational arrest by puromycin have been demonstrated as early as 15 min after exposure (Low *et al.* 1994). Thus, the observed lag in transport stimulation by puromycin is not due to weaker or slower protein synthesis inhibition. The basal rate of sugar uptake in 3T3-L1 adipocytes can show important clonal variations. For comparison purposes, the standarized rate of sugar uptake into our cells (uptake rate/concentration of deoxyglucose; μl min$^{-1}$ (mg protein)$^{-1}$) was approximately 2, a value that is well within the range of those measured in other laboratories (e.g. 5, 4 and 1, in Sargeant & Paquet, 1993; van Putten & Krans, 1985; and Garcia de Herreros & Birnbaum, 1989, respectively).

Despite the large increase in sugar transport induced by anisomycin, the abundance of glucose transporters present at the plasma membrane of 3T3-L1 adipocytes did not change in response to the antibiotic (Fig. 2). In contrast, significant increases were detected in both GLUT1 and GLUT4 density in response to insulin (Fig. 2). Note that the plasma membrane lawn assay used at the light microscopy level only gives an average of surface GLUT density. In order to detect a possible redistribution of transporters between discrete domains of the cell membrane, the use of more powerful methods such as electron microscopy would be required.

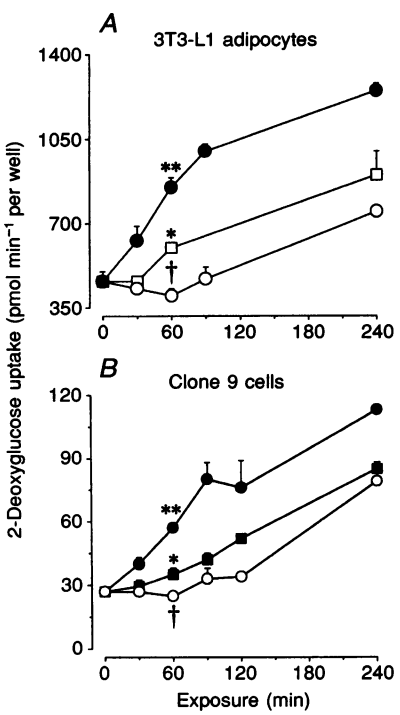


**Figure 1. Time course of the stimulation of 2-deoxyglucose uptake by protein synthesis inhibitors**

Cells were exposed to 300 μM anisomycin (●), 100 μg ml$^{-1}$ puromycin (○), 10 μg ml$^{-1}$ emetine (□) or 500 μM cycloheximide (■) for the times indicated. Uptake of 0·2 mM 2-deoxyglucose was measured as described in Methods. *A*, 3T3-L1 adipocytes. *B*, Clone 9 cells. Mean + S.E.M. (3 separate experiments). Similar results were obtained in at least three other experiments for each cell type. * Significantly different from basal (not shown) or puromycin-treated cells; ** significantly different from puromycin-, emetine- and cycloheximide-treated cells; † not significantly different from basal value (ANOVA, Tukey–Kramer's test).

It has been reported that anisomycin is more potent in its activation of JNK/SAPKs than in its inhibition of protein synthesis (Cano *et al.* 1994). If the rapid phase in the stimulation of transport by anisomycin involves kinase activation, while the slow phase involves inhibition of translation, then these two phases should differ in their sensitivity to anisomycin. One would predict that stimulation of transport measured after longer times of exposure, when the putative component resulting from translational arrest becomes relatively more important, would exhibit a lower sensitivity to anisomycin than for the early phase of transport stimulation. Figure 3 shows that this is indeed the case. The stimulation of 2-deoxyglucose uptake, measured 30 min after exposure to anisomycin, displayed a 5- to 10-fold higher sensitivity to the antibiotic than the corresponding response measured after a 240 min exposure. Moreover, when the cells were stimulated with a lower concentration of anisomycin, expected to elicit only the high affinity component of the stimulation (see arrows in Fig. 3), the time course of the stimulation was changed (Fig. 4). Uptake rates peaked at 30–60 min and returned to basal levels by 240 min. This is in marked contrast with the monotonic profiles observed with 300 $\mu$M anisomycin (Fig. 1). The decrease in uptake seen after 60 min exposure to anisomycin was not due to antibiotic depletion as no further stimulation could be obtained by re-addition of 0·3 $\mu$M anisomycin at 180 or 210 min (data not shown). Surprisingly, the stimulation of transport by 0·3 $\mu$M anisomycin virtually disappeared in the presence of 100 $\mu$g ml$^{-1}$ puromycin, with inhibitions (mean ± S.E.M. with the number of separate experiments in parentheses) of 96 ± 8% (4) and 91 ± 6% (3) in adipocytes and Clone 9 cells, respectively. In single experiments in 3T3-L1 adipocytes and Clone 9 cells, 10 $\mu$g ml$^{-1}$ emetine inhibited 70 and 65% of the stimulation of sugar uptake by 0·3 $\mu$M anisomycin, respectively.

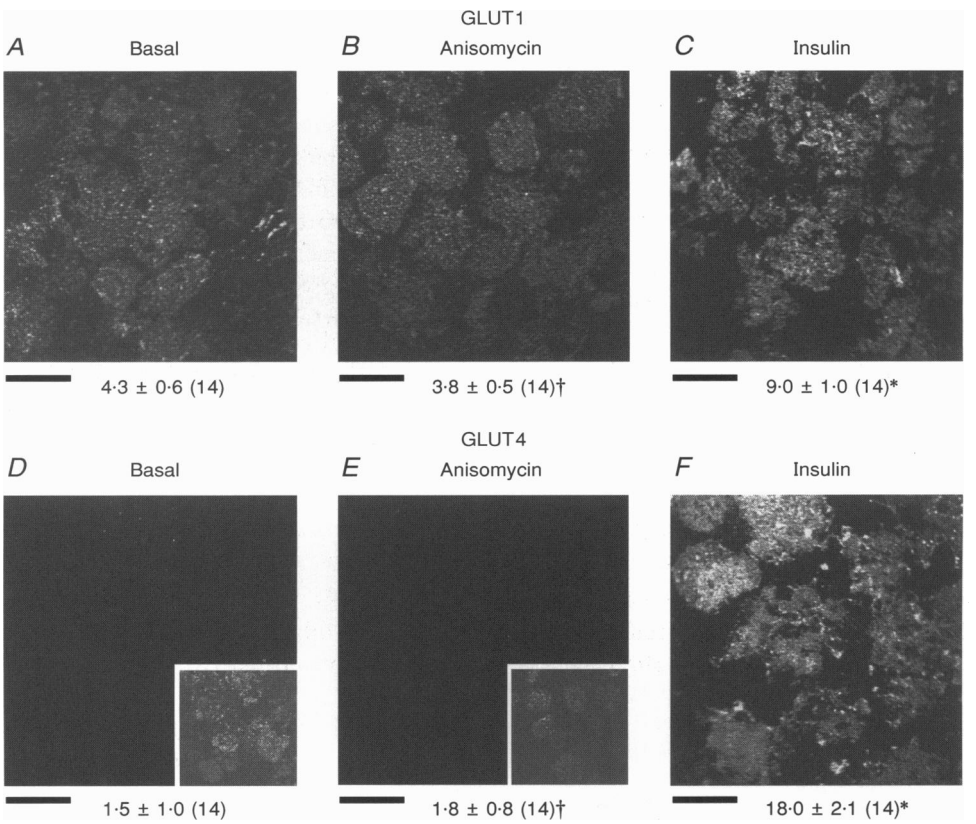


**Figure 2. Effect of anisomycin on the abundance of plasma membrane (PM)-associated glucose transporters in 3T3-L1 adipocytes**

Cells were exposed for 90 min to DMEM only (*A* and *D*), DMEM plus 300 $\mu$M anisomycin (*B* and *E*) or 1 $\mu$M insulin (*C* and *F*). PM lawns were then prepared, stained for GLUT1 (*A–C*) or GLUT4 (*D–F*), and quantified as described in Methods. Microscope detection parameters were kept the same for all figures except for the insets which show the whole of panels *D* and *E* scanned at a higher gain in order to demonstrate that the lack of staining did not reflect the absence of cell membranes on the coverslips. Scale bars are 25 $\mu$m. The densities of GLUT1 and GLUT4 present at the plasma membrane are given under each experimental condition, in arbitrary units, as means ± S.E.M. (14 lawns from two separate experiments). * Significantly different from basal value; † not significantly different from basal value (Mann–Whitney *U* test).

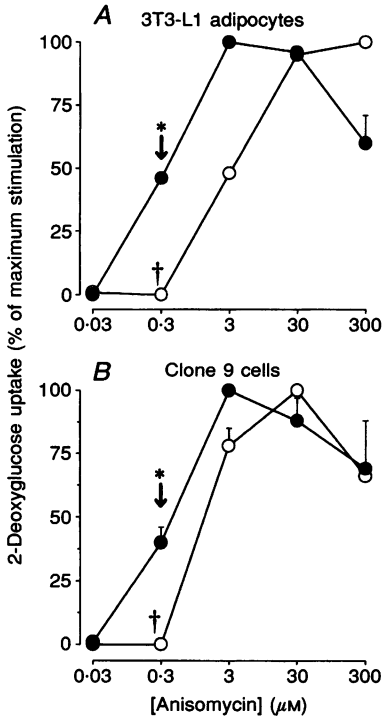


**Figure 3. Dose–response curves of the early and late stimulation of 2-deoxyglucose uptake by anisomycin**

Cells were exposed for 30 min (●) or 240 min (○) to increasing concentrations of anisomycin, without varying the volume of vehicle used. Uptake of 0·2 mM 2-deoxyglucose was measured as described in Methods. *A*, 3T3-L1 adipocytes. *B*, Clone 9 cells. Values are mean + S.E.M. (3 separate experiments). Data are the average of two experiments done in triplicate for each cell type. * Significantly different from either basal value or cells exposed to anisomycin for 240 min; † not significantly different from basal value (ANOVA Tukey–Kramer). Arrows indicate the concentration of anisomycin chosen for the experiment illustrated in Fig. 4.

In order to investigate the effects of protein synthesis inhibitors on the functional properties of GLUT1, the uptake of 2-deoxyglucose was measured in the presence of an increasing concentration of unlabelled 3-*O*-methyl-D-glucose. As this sugar is a good substrate for GLUT1 but is not metabolized inside the cell (Baldwin, 1993), this type of experiment allows an accurate estimation of the apparent affinity of the transporter for sugars in the form of the experimental parameter $IC_{50}$. A similar approach with unlabelled 2-deoxyglucose is not feasible due to its toxicity at high concentrations. Figure 5 shows that in both cell types, protein synthesis inhibitors did not significantly affect the affinity of GLUT1 for 3-*O*-methyl-D-glucose. Therefore, if it is assumed that no changes occur in the substrate specificity of GLUT1, it can be concluded that both kinase activation and translational inhibition act by increasing the capacity ($V_{max}$) of the transporter for sugars.

Two distinct but highly homologous groups of stress-activated MAP kinases have been identified so far in mammalian cells: JNK/SAPKs and p38(MAPK). The synthetic compound SB203580 can inhibit the latter with high specificity in the low micromolar range; most importantly, it does not affect the activity of JNK/SAPKs and several other kinases and phosphatases (Cuenda *et al.* 1995). Figure 6 shows that SB203580 inhibited the rapid stimulation of glucose transport by 300 μM anisomycin (measured at 30 min) with high affinity ($IC_{50} < 1$ μM) in both cell types. Similar inhibition of transport stimulation by 0·3 μM anisomycin was also observed (not shown). In contrast, the responses to 300 μM anisomycin and 100 μg ml$^{-1}$ puromycin, measured after 240 min exposure, were largely resistant to inhibition by this agent (Fig. 6). Failure to inhibit the late responses was probably not due to degradation or inactivation of the SB compound, as no

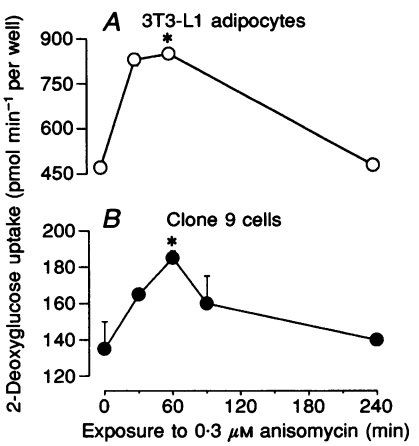


**Figure 4. Time course of 2-deoxyglucose uptake stimulation by a low concentration of anisomycin**

Cells were exposed to 0·3 μM anisomycin for the times indicated. Uptake of 0·2 mM 2-deoxyglucose was measured as described in Methods. *A*, 3T3-L1 adipocytes. *B*, Clone 9 cells. Values are mean + S.E.M. (3 separate experiments). Similar results were obtained in two other experiments for each cell type. * Significantly different from basal values (Student's *t* test).

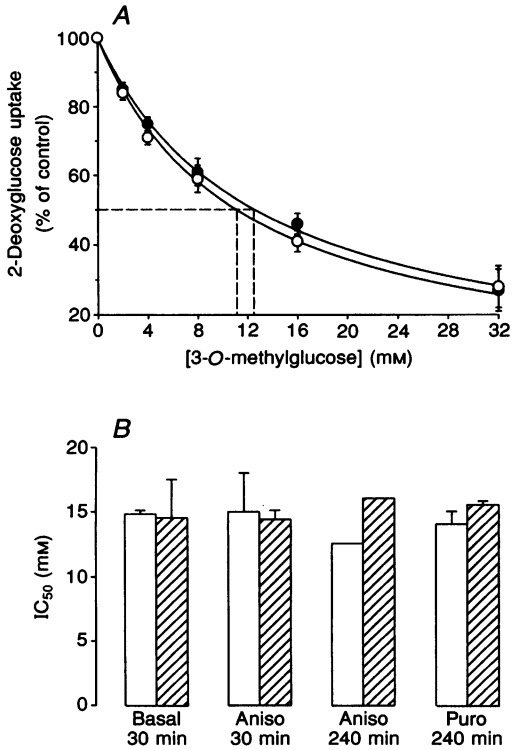


**Figure 5. Competition of 2-deoxyglucose uptake by 3-*O*-methyl-D-glucose in cells exposed to protein synthesis inhibitors**

*A*, 3T3-L1 adipocytes were exposed to 300 $\mu$M anisomycin (●) or control buffer only (○) for 240 min. Uptake of 0·2 mM 2-deoxyglucose was then measured in duplicates in the presence of various concentrations of unlabelled 3-*O*-methyl-D-glucose (0, 2, 4, 8, 16, 32 mM) as described in Methods. In this experiment, anisomycin stimulated basal sugar uptake by a factor of 4·3. The concentrations necessary to inhibit 50% of 2-deoxyglucose uptake ($IC_{50}$), are indicated as vertical lines in the figure and were determined by non-linear regression assuming competitive inhibition of a saturable process, using the program Enzfitter (Sigma). Values are mean ± S.E.M. *B*, summarized data. 3T3-L1 adipocytes (□) and Clone 9 cells (▨) were exposed to 300 $\mu$M anisomycin (Aniso) for 30 or 240 min, or to 100 $\mu$g ml$^{-1}$ puromycin (Puro) for 240 min. Values are mean + S.E.M. (3 separate experiments) except for 300 $\mu$M anisomycin (240 min) that correspond to a single determination for each cell type. There were no significant differences between groups (ANOVA).

inhibition was obtained after its re-addition at 180 min (not shown).

Theoretically, the inhibition of a signalling event can affect distal events whether the event is active (i.e. its activity is changed by the agonist) or just permissive (its activity does not vary but is required in a supportive, constitutive role). Our measurement of p38(MAPK) in 3T3-L1 adipocytes supports the former scenario (Fig. 7). Exposure to anisomycin rapidly increased the activity of p38(MAPK). At 30 min of exposure, 0·3 and 300 $\mu$M anisomycin induced a similar degree of activation. However, at 240 min, the effect of 300 $\mu$M anisomycin was stronger. The latter might have been caused by 'superinduction' of p38(MAPK) due to translational inhibition at high concentrations of anisomycin

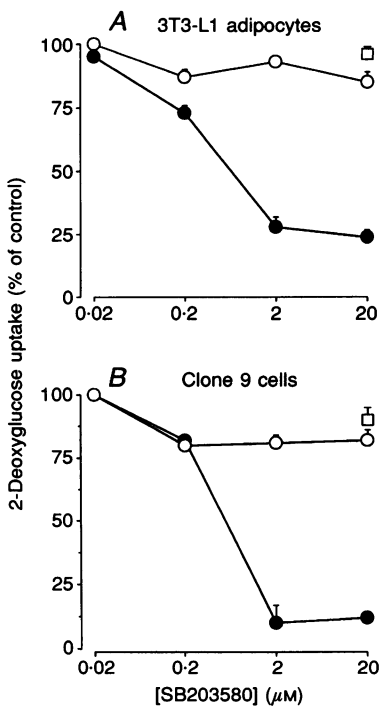


**Figure 6. Effect of SB203580 on the stimulation of 2-deoxyglucose uptake by anisomycin and puromycin**

Cells were pre-incubated with increasing concentrations of SB203580. After 60 min, anisomycin or puromycin were added to a final concentration of 300 $\mu$M or 100 $\mu$g ml$^{-1}$, respectively. For anisomycin-treated cells, uptake of 0·2 mM 2-deoxyglucose was measured as described in Methods after a further 30 min (●) or 240 min (○). For puromycin-treated cells (□), uptake was measured after 240 min. *A*, 3T3-L1 adipocytes. *B*, Clone 9 cells. Values are mean + S.E.M. (3 separate experiments). Similar results were obtained in at least two other experiments for each cell type.

(Mahadevan & Edwards, 1991). In addition, puromycin strongly inhibited the activation of the kinase by anisomycin. As shown above, these three features were also present in the activation of GLUT1 by anisomycin.

## DISCUSSION

Our data suggest that the fast stimulation of glucose transport by anisomycin in two types of mammalian cells is mediated by the activation of p38(MAPK). In previous reports, it has been shown that anisomycin increases the catalytic activity of the glucose transporter GLUT1 (Clancy *et al.* 1991; Harrison *et al.* 1992; Germinario *et al.* 1992; Hayes *et al.* 1993). Clancy *et al.* (1991), by directly comparing the uptake of 2-deoxyglucose and 3-*O*-methylglucose in 3T3-L1 adipocytes, demonstrated that the effect of anisomycin is solely due to transport stimulation and that intracellular sugar phosphorylation does not play a role in uptake stimulation. We have repeated such experiments with identical results in both 3T3-L1 adipocytes and Clone 9 cells (not shown). In addition, our own measurements in 3T3-L1 adipocytes indicate that exposure to anisomycin does not change the number of GLUT1 or GLUT4 carriers at the cell surface (Fig. 2). Therefore, stimulation of transport does not involve translocation of transporters from the cell interior to the surface in this case.

The activation of GLUT1 by anisomycin was found to be composed of an early component due to kinase activation and a delayed component, possibly resulting from translational arrest. Puromycin, and to a lesser degree emetine and cycloheximide, elicited only the late component, which was detectable only after a lag phase of more than 60 min and was insensitive to the p38(MAPK) inhibitor SB203580. It has been proposed that translational arrest stimulates glucose uptake as the result of the rapid degradation of a putative short-lived protein that tonically inhibits GLUT1 (Czech *et al.* 1992). In the context of such a hypothesis, the delay in the response to puromycin presumably reflects the time required for the concentration of the putative regulatory protein to fall to threshold value below which tonic inhibition of transport is released.

The component of transport stimulation resulting from kinase activation is better revealed at low concentrations of anisomycin, where the latter is not an effective inhibitor of translation. This component is more rapid in onset than that due to translational arrest and, unlike the latter, it is transient. Moreover, it can be blocked by SB203580, a specific inhibitor of p38(MAPK), with an $IC_{50} < 1\ \mu M$, which is consistent with the reported *in vitro* $IC_{50}$ of p38(MAPK) inhibition by SB203580 (0·6 $\mu M$; Cuenda *et al.* 1995). The specificity of SB203580 for the enzyme allows us to propose the presence of a signalling link between p38(MAPK) and GLUT1. This hypothesis is also supported by the similar sensitivity to anisomycin and insensitivity to puromycin, and of the rapid activation of GLUT1 and the enzyme. The actual mechanism by which anisomycin activates the stress-activated kinases is currently unknown, so the inhibitory effect of puromycin described here may prove to be a useful tool for elucidating such activation mechanisms.

The similar data obtained for mouse 3T3-L1 adipocytes and rat Clone 9 epithelial cells suggest that the activation of GLUT1 via p38(MAPK) may be a conserved signalling event. Moreover, while this work was being carried out, it was reported that in human KB cells, the stimulation of sugar uptake by anisomycin can be blocked by 25 $\mu M$ SB203580 (Gould, Cuenda, Thomson & Cohen, 1995). It is

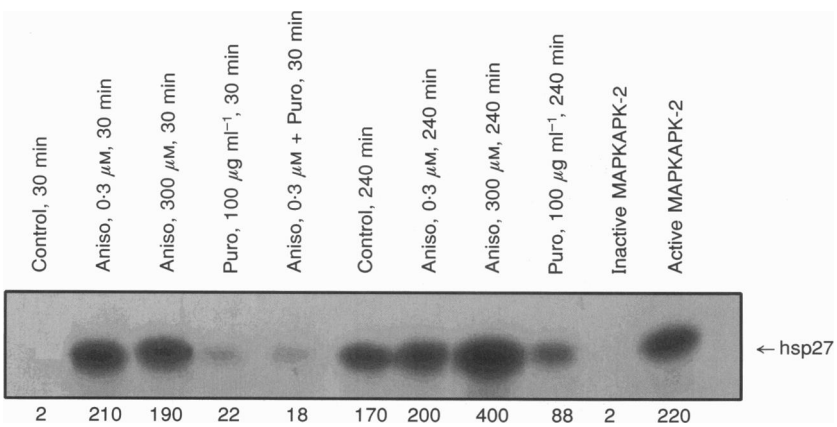


**Figure 7. Effect of protein synthesis inhibitors on the activity of p38(MAPK) in 3T3-L1 adipocytes**

After exposure to the indicated experimental conditions, cell lysates were prepared and their p38(MAPK) was immunoprecipitated as described in Methods. These precipitates were then used to phosphorylate hsp27. Each lane corresponds to $10^7$ adipocytes. Inactive MAPKAPK-2 was obtained by exposure to protein phosphatase 2A (PP2A) while active MAPKAPK-2 was obtained by exposure to activated p38(MAPK). The numbers correspond to the quantification of the signal in each lane by digital scanning. A similar result was obtained in a separate experiment using GST-ATF2 as substrate.

therefore possible that a similar pathway is present in KB cells. However, it is not known yet whether the effect of anisomycin in KB cells is due to *in situ* activation or translocation of transporters, nor whether in KB cells SB203580 inhibits anisomycin-stimulated sugar transport with a potency comparable to its inhibition of the kinase.

In theory p38(MAPK) could activate GLUT1 either by direct phosphorylation or by means of a downstream kinase such as MAPKAPK-2. The former seems to be the case for NHE-1, one of the isoforms of the $Na^+$–$H^+$ exchanger. This transporter, known to be activated by phosphorylation, can be phosphorylated *in vitro* by p38(MAPK), probably in a consensus Ser–Pro motif (Kusuhara, Han, Ulevitch & Berk, 1995). As GLUT1 is predicted to expose two such motifs to the cytosol (residues 148–149 and 265–266), direct phosphorylation of the transporter by the kinase is possible in principle. GLUT1 also displays two intracellular Arg–X–X–Ser sites for phosphorylation by MAPKAPK-2 (residues 92–95 and 223–226). However, some evidence against the involvement of this kinase has been provided in *Xenopus* oocytes where injecting activated MAPKAPK-2 had no effect on glucose transport, whereas injecting MAP kinase (ERK), or its upstream activator MEK (MAPK/ERK kinase), induced a 2·5-fold stimulation (Merral, Plevin, Stokoe, Cohen, Nebreda & Gould, 1993).

In summary, we have presented evidence that two distinct mechanisms mediate the *in situ* activation of GLUT1 in mammalian cells by anisomycin: inhibition of protein synthesis and activation of p38(MAPK). The latter may be important for cell metabolism and survival as many physiological and pathological stimuli known to activate the enzyme, including heat shock, UV radiation, inflammatory cytokines, and osmotic stress (Cano *et al.* 1994; Freshney *et al.* 1994), are also known to stimulate glucose transport (Fischer, Thomas, Rose & Kammermeier, 1992; Baldwin, 1993).


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