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The ubiquitous glucose transporter GLUT-1 belongs to the glucose-regulated protein family of stress-inducible proteins

(cellular stress response)

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ABSTRACT **In mammals, glucose transport is mediated by five structurally related glucose transporters that show a characteristic cell-specific expression. However, the rat brain/HepG2/erythrocyte-type glucose transporter GLUT-1 is expressed at low levels in most cells. The reason for this coexpression is not clear. GLUT-1 is negatively regulated by glucose. Another family of proteins, glucose-regulated proteins (GRPs), is also ubiquitously expressed and stimulated by glucose deprivation and other cellular stresses. We therefore hypothesized that GLUT-1 may be a glucose-regulated stress protein. This was tested by subjecting L_8 myocytes and NIH 3T3 fibroblasts to glucose starvation or exposure to the calcium ionophore A23187, 2-mercaptoethanol, or tunicamycin, all known to increase GRP levels. The mRNA for GLUT-1 was augmented by 50–300% in a time-dependent manner, similarly to the changes in GRP-78 mRNA. *Ex vivo* incubation of rat soleus muscles induced a marked and concomitant rise in the mRNA levels of GLUT-1 and GRP-78. Finally, calcium ionophore A23187 and 2-mercaptoethanol induced a 2- to 3-fold increase in the levels of the GLUT-1 protein and hexose uptake. In all instances in which GRP-78 and GLUT-1 responded to stress, the transcription of the cell-specific muscle/adipocyte-type insulin-responsive glucose transporter (GLUT-4) did not change. Thus, despite the lack of structural similarity, GLUT-1 and GRP-78 expression is regulated similarly, whereas the regulation of GLUT-4, which is structurally related to GLUT-1, is different. We propose that GLUT-1 belongs to the GRP family of stress proteins and that its ubiquitous expression may serve a specific purpose during cellular stress.**

Glucose transport in mammalian cells is mediated by a family of structurally related glycoproteins, the glucose transporters (GTs). GTs are usually expressed in a tissue-specific manner (1–3). In contrast, GLUT-1, the rat brain/HepG2/erythrocyte-type GT is ubiquitous, being expressed in most cells, often together with other tissue-specific GTs (1–3). The reason for the expression of more than one GT gene in a single cell is unknown. Certain GT genes seem to be regulated by extracellular signals. A physiologically important example is the effect of insulin on the expression of the muscle/adipose-type, insulin-responsive, tissue-specific transporter GLUT-4 *in vitro* and *in vivo*. Thus, in diabetic animals low levels of GLUT-4 mRNA are found, which can be corrected by insulin treatment (4–6). Recently, we and others have demonstrated that GLUT-1 mRNA levels in tissue culture are regulated by glucose. In absence of other factors like hormones, hexose concentrations above the normal range down-regulate the GLUT-1 mRNA levels, whereas glucose starvation up-regulates the mRNA (7–9). These findings motivated the comparison of the regulation of GLUT-1 expression to that of the stress proteins, glucose-regulated proteins (GRPs). GRPs were initially identified as proteins that are specifically synthesized in response to glucose deprivation (10). Subsequently, it was found that various agents that block protein glycosylation, disrupt the intracellular calcium stores, or induce through these or other means aberrant protein folding also enhance the synthesis of GRPs (11). It is believed that GRPs act as chaperones by binding to misfolded proteins to prevent aggregation (12–14). We have used various stimuli known to induce GRP transcription (11) to assess whether GLUT-1 expression and GRP expression are regulated coordinately.

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MATERIALS AND METHODS

Cells, Tissues, and Culture Conditions. L_8 skeletal muscle cells were grown and maintained as described (15). NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), 25 mM D-glucose, and 2 mM L-glutamine. Most experiments were performed in this medium. For glucose deprivation, cells were incubated in glucose-free DMEM supplemented with 10% FCS. Soleus muscles were isolated from young male albino rats (40–60 g) of the Sabra strain (Hebrew University). Their serum glucose concentration ranged between 8 and 10 mM. Muscles were removed and incubated as described (15), except that incubation temperature was 30°C. Following the incubation period, the muscles were frozen in liquid nitrogen. For analysis of fresh muscles they were removed and immediately frozen.

Isolation and Analysis of the Cellular RNA. Total cellular RNA was isolated as described (16). The RNA (10–20 μg per lane) was size-fractionated by agarose gel electrophoresis and transferred to nitrocellulose paper for Northern blot analysis. For slot blot analysis, RNA was denatured with 6% formaldehyde and applied to nitrocellulose with the Schleicher & Schuell minifold II slot blot apparatus. The integrity of RNA was maintained during incubation, as assessed by visualization of ribosomal RNA by UV shadowing.

Hybridization Analysis. The filters were pretreated for 1–3 hr at 65°C in a solution of 1 M NaCl/1% SDS and hybridized to GLUT-1 (17), GLUT-4 (18), GRP-78 (19), or β-actin (20) probes. Hybridization was carried out at 65°C for 20–48 hr in a solution of 10% dextran/1 M NaCl/1% SDS. Filters were washed briefly at 65°C in 1× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate) and then washed four times at 65°C for 20 min, each in 0.1× SSPE/1% SDS (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA). Filters were dried and exposed to x-ray film at −70°C. At the

Abbreviations: dGlc, 2-deoxy-D-glucose; ER, endoplasmic reticulum; FCS, fetal calf serum; GT, glucose transporter; GLUT-1, brain/HepG2/erythrocyte-type GT; GLUT-4, muscle/adipocyte-type, insulin-responsive GT; GRP, glucose-regulated protein; HSP, heat shock protein; 2-ME, 2-mercaptoethanol.

high stringency conditions used, no cross-hybridization was observed between the GLUT-1 and GLUT-4 cDNAs.

Western Blot Analysis. L_8 cells (5×10^6) were incubated as described for each experiment. Cells were then harvested in cold phosphate-buffered saline (PBS) by scraping with a rubber policeman and homogenized for 2×10 sec in buffer containing 10 mM Hepes, 1 mM EDTA, and 0.25 M sucrose (pH 7.4) supplemented with several protease inhibitors. Homogenates were centrifuged at $12,000 \times g$ for 7.5 min at 4°C. Supernatants were centrifuged at $100,000 \times g$ for 60 min at 4°C, the membrane pellets were lysed in 1% Triton X-100 buffer containing 4 M urea, and the protein content was determined by the Bradford method (21). Proteins were separated on an SDS/polyacrylamide gel and analyzed by Western blot (22) with GLUT-1- and GLUT-4-specific rabbit antisera (gift of Hans G. Joost, University of Göttingen, Göttingen, F.R.G.) at a dilution of 1:200. Immunoreactive species were detected by using ^{125}I-labeled protein A.

Measurement of 2-Deoxy-D-Glucose (dGlc) Uptake in Myocytes. dGlc uptake rate was measured as described (15). Briefly, the cells in culture plates were rinsed eight times with 2 ml of PBS (pH 7.4); 1 ml of assay medium containing 0.5 μCi of [^{3}H]dGlc (1 Ci = 37 GBq), 0.1 mM unlabeled dGlc, and 0.25 μCi of [^{14}C]sucrose (the latter to determine extracellular

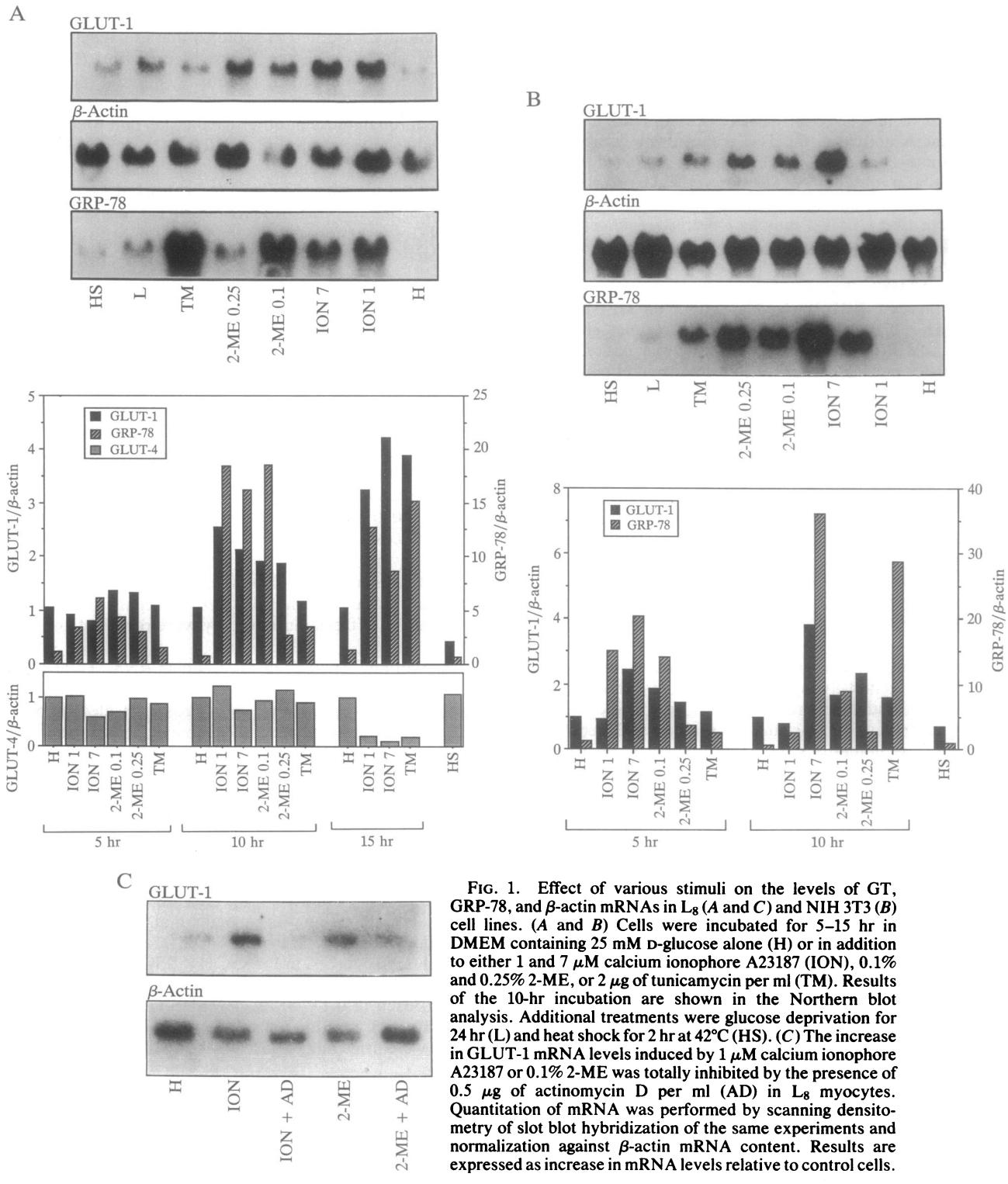

FIG. 1. Effect of various stimuli on the levels of GT, GRP-78, and β-actin mRNAs in L_8 (*A* and *C*) and NIH 3T3 (*B*) cell lines. (*A* and *B*) Cells were incubated for 5–15 hr in DMEM containing 25 mM D-glucose alone (H) or in addition to either 1 and 7 μM calcium ionophore A23187 (ION), 0.1% and 0.25% 2-ME, or 2 μg of tunicamycin per ml (TM). Results of the 10-hr incubation are shown in the Northern blot analysis. Additional treatments were glucose deprivation for 24 hr (L) and heat shock for 2 hr at 42°C (HS). (*C*) The increase in GLUT-1 mRNA levels induced by 1 μM calcium ionophore A23187 or 0.1% 2-ME was totally inhibited by the presence of 0.5 μg of actinomycin D per ml (AD) in L_8 myocytes. Quantitation of mRNA was performed by scanning densitometry of slot blot hybridization of the same experiments and normalization against β-actin mRNA content. Results are expressed as increase in mRNA levels relative to control cells.

space) in PBS was added to the plates. The assay was carried out in room temperature for 5 min and terminated by rinsing the cells fives times with 2 ml of ice-cold PBS. Under these conditions transmembranal transport is the rate-limiting step (15). All experiments were carried out in triplicate.

RESULTS

Two GRPs, GRP-78 and GRP-94, are induced in cells by various stimuli, such as glucose deprivation, inhibition of N-glycosylation, and several other treatments that result in aberrant protein structure (11, 14, 23), the most rapid and effective stimuli being treatment with calcium ionophores, tunicamycin, and reducing agents such as 2-mercaptoethanol (2-ME) (24). We therefore examined the effect of these treatments on GLUT-1 gene expression. Calcium ionophore A23187 (1 and 7 μM), 2-ME (0.1% and 0.25%), and tunicamycin (2 μg/ml) increased the mRNA levels of GLUT-1 by 50–300%, in both L_8 myocytes (Fig. 1*A*) and NIH 3T3 fibroblasts (Fig. 1*B*). The response of GRP-78 mRNA levels to these stimuli in these cells was strikingly similar to that of GLUT-1 mRNA (Fig. 1 *A* and *B*). The increase occurred within 5–15 hr and was completely inhibited by actinomycin D (0.5 μg/ml) (Fig. 1*C*), suggesting an effect at the transcriptional level. Glucose starvation increased GLUT-1 and GRP-78 mRNAs with a slower time course, a lag period of 16–48 hr being observed (Fig. 2). Heat shock treatment, which induces another family of stress proteins, the heat shock proteins (HSPs) (25), does not increase GRP expression (refs. 11 and 12; Fig. 1 *A* and *B*). Treatment of the cells for 2 hr at 42°C resulted in a modest decrease of GLUT-1 mRNA levels (Fig. 1 *A* and *B*). Further incubation of the heat-shocked cells at 37°C for several hours did not reverse this effect (data not shown).

The L_8 myocytes contain, in addition to GLUT-1, low but detectable levels of GLUT-4 mRNA (Fig. 1*A*). The expression of GLUT-4 gene was tested in response to the stimuli that induced GRP-78 and GLUT-1. In contrast to the induction of GLUT-1, GLUT-4 mRNA levels were unaffected (Figs. 1*A* and 2).

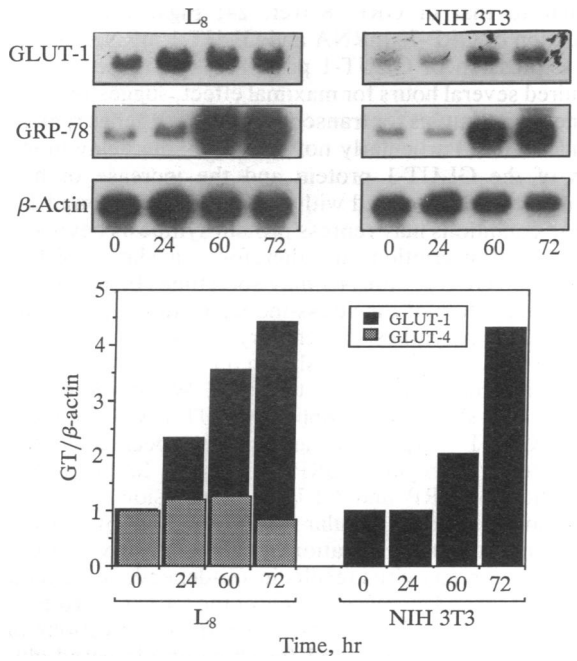

FIG. 2. Effect of glucose deprivation on mRNA levels of GT and GRP-78. L_8 and NIH 3T3 cell lines were incubated in glucose-free DMEM supplemented with 10% FCS for 24, 60, and 72 hr. Quantitation of GT mRNA was performed as described in the legend to Fig. 1.

Another stress condition known to increase GRP mRNA levels is tissue excision and *ex vivo* incubation (23). To evaluate the effect of this stimulus on GLUT-1 mRNA levels, we isolated rat soleus muscles and incubated them *ex vivo* for several hours (Fig. 3). Again, GLUT-1 and GRP-78 mRNA levels, which were quite low in freshly isolated muscles, increased dramatically within hours of incubation, without any change in the cell-specific GLUT-4 or β-actin mRNA levels. Addition of either serum, high concentrations of glucose, or insulin did not prevent the induction of either GLUT-1 or GRP-78 mRNA (Fig. 3).

The effect of the two potent inducers of GLUT-1 mRNA expression was examined at the protein level by Western blot analysis with antiserum raised against the human erythrocyte GT specific for the GLUT-1 protein (Fig. 4). Incubation of L_8 myocytes for 10 hr in the presence of the calcium ionophore A23187 or 2-ME resulted in a 2- to 3-fold increase of the GLUT-1 signal either immediately (2-ME) or after a recovery period (Fig. 4). The enhanced GLUT-1 protein expression persisted for at least 20 hr following the removal of the stimulant (Fig. 4, lanes 4–8). On Western blots the GLUT-1 protein occurred as a diffuse doublet of approximately 55 and 42 kDa, similar to the sizes observed in other studies (26, 27). The effect of calcium ionophore A23187 and 2-ME was evident only on the high molecular size band, presumably the glycosylated transporter. In contrast to these observations,

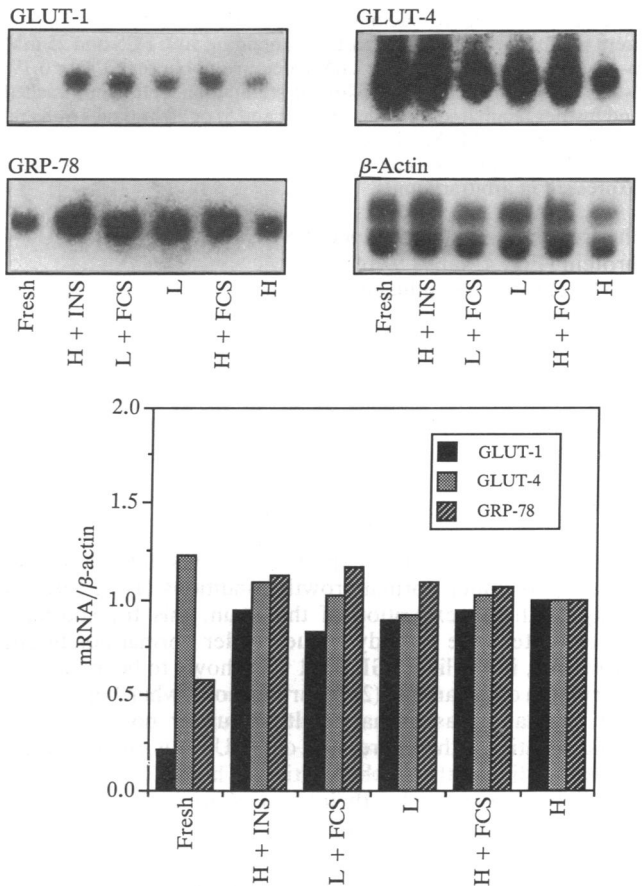

FIG. 3. GLUT-1, GLUT-4, GRP-78, and β-actin mRNA levels of rat skeletal muscle incubated *ex vivo*. Rat soleus muscles were incubated for 5 hr in DMEM containing 2.2 mM D-glucose without (L) or with 15% FCS (L + FCS) or containing 25 mM D-glucose without (H) or with 15% FCS (H + FCS) or 500 ng of insulin per ml (H + INS). For fresh muscle analysis, muscles were removed and immediately frozen. Quantitation of GT and GRP mRNAs was as described in the legend to Fig. 1.

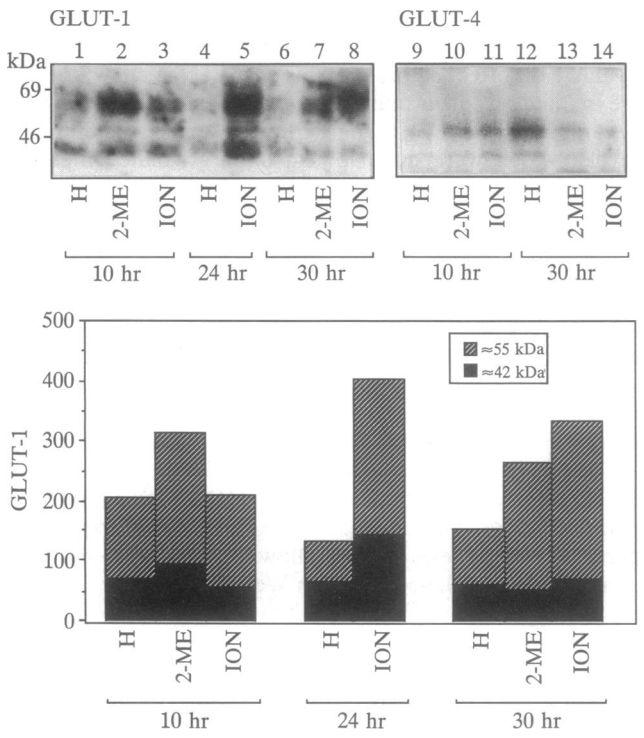

FIG. 4. Western blot analysis of the GLUT-1 and GLUT-4 proteins in response to calcium ionophore A23187 and 2-ME. L_8 cells were incubated for 10 hr in DMEM containing 10% FCS and 25 mM D-glucose (H) with or without 1 μM calcium ionophore (ION) or 0.1% 2-ME (lanes 1–3 and 9–11). Following stimulation, cells were washed and further incubated for 14 hr (lanes 4 and 5) or 20 hr (lanes 6–8 and 12–14) in fresh DMEM with glucose and FCS alone. Quantitation of the GLUT-1 protein was performed by scanning densitometry and is expressed in arbitrary units.

the treatments did not induce the expression of the GLUT-4 protein (Fig. 4, lanes 9–14).

The GT protein induced under these conditions appears to be functional: following treatment with calcium ionophore or 2-ME the rate of hexose transport increased 2- to 3-fold (Fig. 5). The uptake was completely inhibited by 5 μM cytochalasin B, indicating that the observed transport was a facilitated one even in the presence of the stimulants (data not shown).

DISCUSSION

GLUT-1 is ubiquitously expressed in constitutively detectable levels under normal growth conditions (1–3). Interestingly, with the exception of the brain, this transporter is barely detectable in body tissues under normal conditions. However, in the liver, GLUT-1 was shown to be induced by starvation of the animal (28). Furthermore, when hepatocytes are maintained as primary cultures under normal glucose concentrations, the expression of GLUT-1 is enhanced (28). This is in line with the observation of higher GLUT-1 levels in cell lines compared to their tissue of origin (1). The present study demonstrated that GLUT-1 can be further increased under various conditions that induce the other known GRPs, GRP-78 and GRP-94 (11). These conditions seem to have in common the ability to disrupt the structure of newly formed proteins (11, 14, 23). GLUT-1 responded to the stimuli regardless of the tissue origin, the type of cell line, or the constitutive levels of the coexpressed tissue-specific GT. This latter characteristic was most clearly evident by the remarkable induction of GLUT-1 in soleus muscles in the presence of very high levels of GLUT-4 mRNA. The response pattern of the GLUT-1 gene to various stimuli was

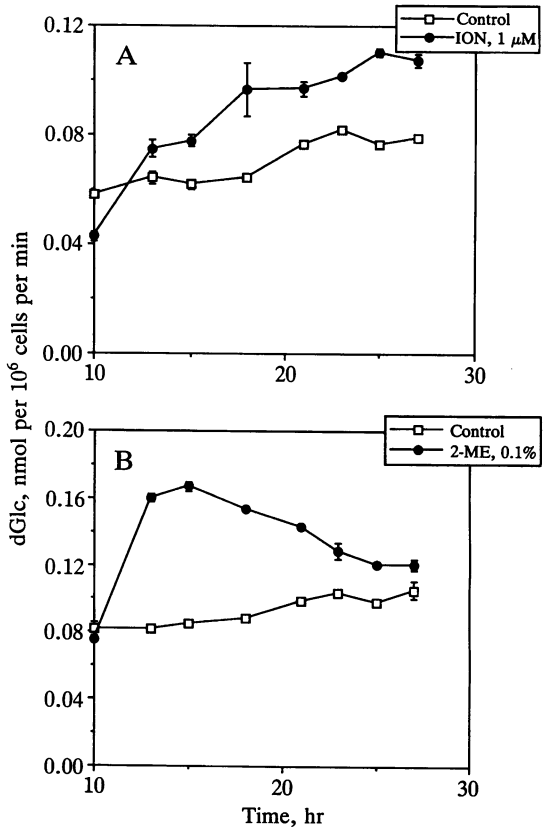

FIG. 5. Hexose uptake of L_8 myocytes in response to calcium ionophore A23187 and 2-ME. L_8 cells were incubated for 10 hr in DMEM containing 10% FCS and 25 mM D-glucose (H) with or without 1 μM calcium ionophore (*A*) or 0.1% 2-ME (*B*). Following stimulation, the cells were washed and further incubated in fresh DMEM with 25 mM glucose and FCS alone for the times indicated. Since both stimuli affect the rate of cell proliferation, each experiment was individually controlled according to cell number at the end of the experiment.

similar to that of GRP-78 (ref. 24; Figs. 1 and 3). The induction of GRP-78 mRNA and GLUT-1 mRNA as well as the expression of GLUT-1 protein under these conditions required several hours for maximal effect, suggesting that the immediate stimulus for transcription of both genes remains to be identified. Particularly noteworthy is the delay in induction of the GLUT-1 protein and the increase of hexose transport in cells treated with calcium ionophore and 2-ME. These conditions may repress protein synthesis beyond their effect on transcription, and therefore translation of the accumulated GLUT-1 mRNA may sometimes be possible only after elimination of the stress-inducing stimuli and recovery of the protein synthesis machinery.

The GLUT-1 response shown in this study is not an unspecific reaction of cells to stress. When another major stress, heat shock, was applied, GLUT-1 was not induced. Thus, GLUT-1 expression seems to be specifically induced by factors that regulate GRP expression. Interestingly, the induction of GRP and GLUT-1 expression seems to be coordinated during cellular transformation by oncogenes (29–32). Thus, transformation of chicken embryo fibroblasts by Rous sarcoma virus results in *src*-dependent induction of GRP-78 (29) and increased levels of the GLUT-1 protein (31). Likewise, mouse fibroblasts transformed by various oncogenes such as *src*, *ras*, and *fps* show an increased glucose uptake that is correlated with elevated levels of the GLUT-1 mRNA (30–32). At least in one examined case (29, 31), the time course of GLUT-1 induction was similar to that of GRP-78 induction.

The GLUT-1 protein is structurally homologous to the other GT proteins, including GLUT-4 (1–3), but shows no structural homology to GRP-78 or GRP-94. The latter proteins are related to the family of the HSPs. Yet, despite the structural similarity, GRPs and HSPs are not regulated in a coordinated manner. Similarly, neither do GLUT-1 and GLUT-4 respond to the same stimuli. However, similar stimuli seem to coinduce the structurally unrelated GLUT-1 and GRP-78 proteins. The basis for this similarity in the regulation pattern is not clear. We could not observe a significant homology between the putative promoter area of the GLUT-1 gene and the 300-base-pair 5′ upstream region of the GRP-78 gene that enables inducibility by calcium ionophore A23187 (33). However, the critical ionophore-responsive sequences have not yet been delineated; we may therefore have overlooked motifs common to the regulatory regions of the two genes. Furthermore, such common regulatory elements may reside outside the presently available sequences.

GRPs are induced by the presence of malfolded proteins in the endoplasmic reticulum (ER) and are bound to prefolded and malfolded proteins that accumulate in the ER (14). It was therefore suggested (12–14) that GRPs may act as chaperones in the assembly of exocytotic proteins, both during normal synthesis and under conditions that promote protein denaturation and aggregation. Although regulated by the concentration of glucose in the culture medium, none of the GRPs described so far is involved in glucose transport through the plasma membrane (34). Our hexose uptake results indicate a role for GLUT-1 in recovery from cellular stress: following treatment with calcium ionophore A23187 or 2-ME the transport of glucose was enhanced severalfold and the enhanced uptake was sustained for at least 20 hr. In principle, this function could be fulfilled by any one of the tissue-specific GTs, but evolutionary or economic considerations may have forced many cell types to rely upon only few genes under conditions endangering cell survival. Our findings suggest that GLUT-1 is one of these genes.

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