Structural disruption of the *trans*-Golgi network does not interfere with the acute stimulation of glucose and amino acid uptake by insulin-like growth factor I in muscle cells

Harinder S. HUNDAL,* Philip J. BILAN, Theodoros TSAKIRIDIS, André MARETTE† and Amira KLIP‡
Division of Cell Biology, The Hospital For Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8

The effects of insulin-like growth factor I (IGF-I) on glucose and amino acid uptake were investigated in fully differentiated L6 muscle cells, in order to determine whether the two processes are functionally related. Transport of both glucose and amino acid (methylaminoisobutyric acid, MeAIB) was activated rapidly in response to IGF-I. Stimulation reached a peak within 30 min and was sustained for up to 90 min. Maximal activation of either glucose or MeAIB transport was achieved at 3 nM IGF-I; the half-maximal activation (ED_{50}) of glucose transport was at 107 pM and that of MeAIB transport was at 36 pM. Stimulation of amino acid uptake occurred in the absence or presence of glucose, suggesting that this response is not secondary to increased glucose intake. Incubation of cells for 1 h with Brefeldin A (5 μg/ml), which disassembles the Golgi apparatus and inhibits the secretory pathway in eukaryotic cells, had no effect on the acute IGF-I activation of glucose and MeAIB transport. Moreover, Brefeldin A caused wide redistribution of the trans-Golgi antigen TGN38, as assessed by subcellular fractionation, without affecting the distribution of glucose transporters. The finding that the degree of activation, time response and sensitivity to IGF-I and Brefeldin A were similar for both glucose and MeAIB transport suggests commonalities in the IGF-I mechanism of recruitment of glucose transporters and stimulation of amino acid transport through System A. An integral trans-Golgi network does not appear to be required for the acute IGF-I stimulation of glucose or amino acid transport, even though stimulation of glucose transport occurs through recruitment of glucose transporters from intracellular stores in these cells. We propose that the donor site of glucose transporters (and perhaps of amino acid transporters) involved in the acute response to IGF-I lies beyond the trans-Golgi network, perhaps in an endosomal compartment in close proximity to the plasma membrane.

INTRODUCTION

There is current consensus that in skeletal muscle and adipose tissue the principal mechanism by which insulin acutely stimulates glucose transport consists of mobilization of an intracellular store of GLUT4 glucose transporters to the plasma membrane (PM) (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Douen et al., 1990; Hirshman et al., 1990). However, the nature of the signalling pathway and the identity of the cellular organelle(s) which participate in GLUT4 transporter storage and translocation in skeletal muscle still remain elusive. One morphological study (at the electron-microscope level, using immunogold labelling of GLUT4 molecules) has suggested that the internal pool includes the triad region (comprising the terminal cisternae of the sarcoplasmic reticulum and transverse tubules) (Friedman et al., 1991), whereas another study has proposed that GLUT4 molecules may accrue in the trans-Golgi network (TGN), where they await to be recruited to the PM in response to the insulin signal (Rodnick et al., 1992). However, a functional role for the TGN has not been established for the translocation of glucose-transporter-containing vesicles. Evidence does exist supporting the role of the Golgi apparatus (without specific implication of the TGN) in anterograde membrane traffic. This is largely based on studies with Brefeldin A (BFA), an isoprenoid fungal metabolite from *Eupenicillium brefeldianum* which rapidly inhibits protein traffic in the secretory pathway as a result of disassembly of the Golgi apparatus (Klausner et al., 1992). In normal rat kidney (NRK) cells BFA induces a rapid redistribution of the cisternal Golgi proteins into the endoplasmic reticulum and the TGN, to extend and mix with the endosomal system (Lippincott-Schwartz et al., 1991). After its dispersal to endosomes, TGN markers accumulate around the microtubule-organizing centre (Ladinsky and Howell, 1992). Functional implications have been studied in secretory cells, where BFA inhibits constitutive and regulated secretion of neutrotransmitters and hormones (Klausner et al., 1992).

We have observed that the insulin-induced translocation of GLUT4 to the PM can be reproduced in L6 muscle cells in culture in response to insulin and insulin-like growth factor-I (IGF-I) (Ramlal et al., 1988; Bilan et al., 1991, 1992a). The L6 muscle cell line studied was clonally selected for high fusion and expresses many of the morphological, biochemical and electrical properties of rat skeletal-muscle fibres (Yaffe, 1968; Shainberg et al., 1971). Differentiation of L6 myoblasts into myotubes in culture is associated with the appearance of muscle-specific proteins and the ability of these cells to respond to insulin (Mitsumoto et al., 1991). In L6 myotubes activation of glucose transport by both insulin and IGF-I is accompanied by translocation to the PM of

Abbreviations used: IGF-I, insulin-like growth factor I; PM, plasma membrane; TGN, trans-Golgi network; BFA, Brefeldin A; 2DG, 2-deoxyglucose; MeAIB, α-methylaminoisobutyrate; α-MEM, α-Minimal Essential Medium; HBS, Hepes-buffered saline.

* Present address: Department of Anatomy and Physiology, University of Dundee, Dundee DD1 4HN, Scotland, U.K.
† Present address: Department of Physiology and Endocrinology, CRML, Laval University Hospital Research Centre, 2705 Boulevard Laurier, Ste-Foy, Québec, Canada G1V 4G2.
‡ To whom correspondence should be addressed.

GLUT1, GLUT3 and GLUT4 glucose transporters from an intracellular compartment (Bilan et al., 1992a,b; Mitsumoto and Klip, 1992). The L6 myotubes also display stimulation by insulin of amino acid uptake through System A (Hundal et al., 1992), which transports small neutral amino acids in a Na^+-dependent manner (Christensen, 1990). However, in contrast with our knowledge of stimulation of glucose uptake, the nature of amino-acid-transport System A activation and whether IGF-I can acutely stimulate amino acid transport in muscle cells is poorly defined. The protein(s) constituting this transport system have not been isolated, and there are currently no pharmacological or immunological tools to detect the presence of the constituents of this transport pathway.

In the present study we used differentiated L6 myotubes for two purposes: (*a*) to compare the regulation of amino-acid-transport System A and of glucose transport by IGF-I, in search for commonalities or differences that might reveal if stimulation of amino acid uptake can occur through recruitment of amino acid transporters; and (*b*) to investigate the possibility that the TGN participates in the IGF-I stimulation of glucose and/or amino acid transport, by assessing whether BFA can prevent these responses. This would enable us to test the hypothesis that vesicle recruitment through the secretory pathway participates in the IGF-I stimulation of glucose and amino acid uptake. We demonstrate that the time course of stimulation and sensitivity to IGF-I of glucose and amino acid uptake are virtually identical, and that neither response is altered when the TGN is disrupted by BFA, in spite of widespread dispersion of the TGN-specific antigen TGN38.

MATERIALS AND METHODS

Materials

Tissue-culture medium, fetal bovine serum and reagents were obtained from GIBCO. Cytochalasin B, 2-deoxy-D-glucose (2DG), α-methylaminoisobutyrate (MeAIB) and BFA were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). Recombinant human IGF-I was kindly given by Dr. M. Vranic (University of Toronto). 2-Deoxy-D-[^{3}H]glucose and α-[1-^{14}C]-methylaminoisobutyrate were purchased from DuPont (Boston, MA, U.S.A.).

Antibodies

A polyclonal antibody against TGN38 (Luzio et al., 1990) was used in the present study and was kindly supplied by Dr. J. P. Luzio (University of Cambridge). This antibody was recently shown to recognize both TGN38/41 isoforms of this TGN-specific protein (Reaves et al., 1992). Polyclonal antibodies specific for the GLUT1 (RaGLUTTrans) or the GLUT4 (IRGT) isoforms of glucose transporters were obtained from East Acres Biologicals (Southbridge, MA, U.S.A.). A polyclonal antibody specific for the mouse/rat GLUT3 transporter was kindly given by Dr. Ian Simpson (NIH, Bethesda, MD, U.S.A.).

Cell culture and incubations

Monolayers of L6 muscle cells were grown to the stage of myotubes as previously described (Koivisto et al., 1991; Mitsumoto and Klip, 1992) in α-Minimal Essential Medium (α-MEM) containing 5 mM glucose, in the presence of 2% fetal-bovine serum and 1% antimycotic/antibiotic solution (final concns. 100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B) at 37 °C in an atmosphere of air/CO_2 (19:1). The cells were grown in 3.5 cm-diameter dishes in 6-well plates for transport measurements. Cells were routinely deprived of serum for 5 h before uptake assays using serum-free α-MEM medium containing 25 mM glucose. At 1 h before uptake assays, they were also depleted of amino acids by using Hepes-buffered saline (HBS) (140 mM NaCl, 20 mM Hepes/Na, pH 7.4, 2.5 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$) containing 25 mM glucose and 5 mg/ml fatty-acid-free BSA. IGF-I or BFA was added to the incubations at the concentrations and times indicated in the Figure legends.

2DG and MeAIB transport

A dual-isotope technique was used to allow the simultaneous measurement of 2DG and MeAIB transport in the same population of cells, as previously described (Hundal et al., 1992). Briefly, after incubation with hormones or drugs, cell monolayers were rinsed with glucose-free HBS. Glucose and MeAIB uptakes were quantified by using 10 μM 2-deoxy[^{3}H]glucose (1 μCi/ml) and 10 μM [^{14}C]MeAIB (1 μCi/ml) for 10 min, unless otherwise stated. Non-specific uptake was determined by quantifying cell-associated radioactivity in the presence of 10 μM cytochalasin B (for 2-deoxy-D-glucose) and a 10 mM saturating dose of unlabelled MeAIB (for the amino acid). Uptake of 2-deoxy-D-[^{3}H]glucose and [^{14}C]MeAIB was terminated by rapidly aspirating the radioactive incubation medium, followed by three successive washes of cell monolayers with ice-cold 0.9% NaCl. Radioactivity associated with the cells was determined by cell lysis in 0.05 M NaOH, followed by liquid-scintillation counting. Total cell protein was determined by the Bradford (1976) method. Experiments were assayed in triplicate and performed at least three times. In preliminary studies we established that MeAIB (10 μM) transport in L6 myotubes was predominantly Na^+-dependent. The replacement of NaCl with choline chloride in the uptake medium decreased MeAIB uptake to 15% of the uptake rate in the presence of Na^+. When MeAIB uptake was measured at 4 °C, the rate was substantially diminished (by nearly 5-fold), indicating that transport of MeAIB must be dependent on the function of a carrier protein at the cell surface, rather than by simply entering cells by passive diffusion (a process less dependent on temperature). In preliminary experiments it was established that the rate of MeAIB uptake was linear for the first 20 min after exposure of L6 cells to 10 μM. Based on these preliminary studies and the results of Figure 1, we chose assay conditions of 10 min uptake at 10 μM MeAIB to measure initial rates of uptake through the System A amino acid transporter.

Subcellular fractionation of L6 myotubes and Western blots

Subcellular membrane fractions were isolated by a modification of a previously published method (Ramlal et al., 1988) as recently described in detail (Bilan et al., 1992a). This method yields initially a crude plasma-membrane fraction and a light microsomal fraction. The crude plasma membranes are further fractionated in discontinuous sucrose gradients. The membranes banding on top of 32% sucrose are enriched in plasma membranes, as revealed by their abundant content of the α_1-Na^+/K^+-ATPase subunit, a plasma-membrane marker (Bilan et al., 1992a). In contrast, the light microsomes are devoid of α_1-Na^+/K^+-ATPase subunit and are therefore considered as intracellular membranes. These microsomes are enriched in GLUT4 glucose transporters and contain the insulin-responsive pool of GLUT1, GLUT3 and GLUT4 transporters (Bilan et al., 1992b;

Mitsumoto and Klip, 1992; Sargeant et al., 1993). Membrane proteins (10 μg) were subjected to SDS/PAGE on 8%-polyacrylamide mini-gels and transferred to poly(vinylidene difluoride) (PVDF) membranes, which were incubated for 1 h at room temperature with buffer A (50 mM Tris/HCl, pH 7.4, 150 mM NaCl) containing 0.04% of the non-ionic surfactant Nonidet P40 and 3% BSA. PVDF membranes were incubated for 1 h at room temperature or overnight at 4 °C with anti-GLUT1 (1:2000), anti-GLUT3 (1:500) or anti-GLUT4 (1:1000) antibodies in buffer A, followed by 1:1000 horseradish-peroxidase-conjugated anti-rabbit IgG and detection by the enhanced chemiluminescence procedure. TGN38 was detected with anti-TGN38 antibody (1:1000), followed by ^{125}I-labelled Protein A (2 μCi/10 ml), exposure to Kodak X-Omat film at −80 °C and autoradiography.

RESULTS

Stimulation of glucose and MeAIB transport in L6 myotubes by IGF-I

The initial rate of MeAIB uptake was determined at various concentrations of MeAIB ranging from 10 to 600 μM and revealed a typical saturation curve (Figure 1). In preliminary experiments it was established that the rate of MeAIB uptake was linear for the first 20 min after exposure of L6 cells to 10 μM, 100 μM or 500 μM substrate (at 100 μM MeAIB uptake was 236, 614 and 1211 pmol/mg at 4, 10 and 20 min; at 500 μM the rate was 810 and 1560 pmol/mg at 10 and 20 min respectively). These conditions ensured the suitability of the uptake conditions to assess the kinetic parameters of MeAIB uptake. In cells exposed to 3 nM IGF-I for 30 min, the initial rates of uptake increased and approached saturation beyond 600 μM MeAIB (Figure 1a). The apparent increase in the $V_{max.}$ of transport was confirmed by a linear Hanes transformation of the data in Figure 1(a). A Hanes plot (in which the intercept on the abscissa gives $-K_m$ and the slope $1/V_{max.}$) was favoured over a conventional Lineweaver–Burk plot, since the distribution of errors involved in assessing the kinetic parameters is more uniform in the former (Price and Stevens, 1982). Transformation of the data using a least-squares regression analysis gave $V_{max.} = 115 \pm 6$ pmol/min per mg of protein and $K_m = 154 \pm 6$ μM for basal MeAIB uptake, and $V_{max.} = 197 \pm 9$ pmol/min per mg of protein, with no statistically significant change ($t = 2.229$, $P > 0.05$) in the K_m value at 180 ± 10 μM for IGF-I-treated cells (Figure 1b). The activation by IGF-I represented a 64% increase in the maximal transport capacity. The activation of 2DG uptake was also due to a $V_{max.}$ effect (basal $V_{max.} = 423 \pm 40$ pmol/min per mg of protein; $V_{max.}$ for IGF-I-treated cells $= 965 \pm 103$ pmol/min per mg of protein). K_m was not significantly altered ($t = 1.656$, $P > 0.1$), from 632 ± 64 μM in the basal state compared with 765 ± 32 μM in the IGF-I-stimulated state.

The time courses of response to IGF-I of glucose and amino acid uptake are shown in Figures 2(a) and 2(b) respectively.

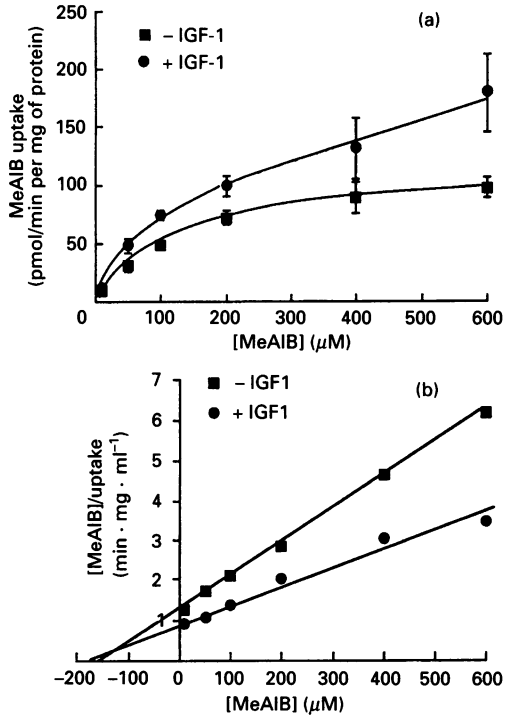

Figure 1 Effect of IGF-I on the transport kinetics of MeAIB in L6 myotubes

L6 myotubes were serum-deprived for 4 h in α-MEM containing 25 mM glucose, followed by amino acid depletion in HBS plus 25 mM glucose for 1 h before measurement of MeAIB uptake. MeAIB uptake was measured, as described in the Materials and methods section, in cells that had been exposed to 3 nM IGF-I during the last 45 min of the amino acid depletion period in the presence of different extracellular MeAIB concentrations (**a**). The data in (**a**) were transformed to give a linear graphical representation (**b**) allowing a more accurate assessment of the maximal MeAIB transport capacity ($V_{max.}$) and a substrate-concentration value at which transport was half-maximal (K_m). The values in (**a**) represent means ± S.E.M. from 4 separate experiments each performed in triplicate, whereas those in (**b**) are based on the mean uptake value from the 4 experiments performed.

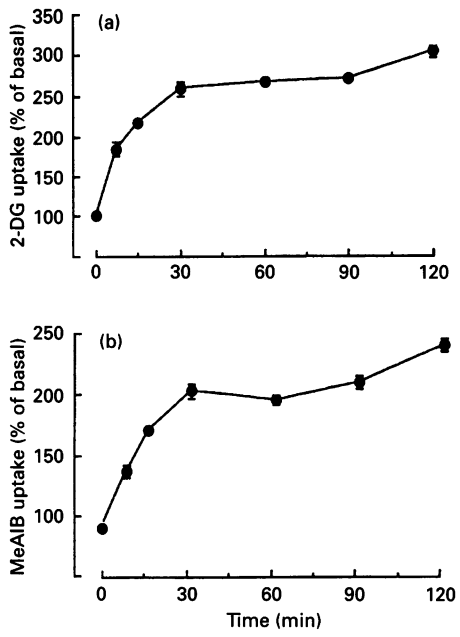

Figure 2 Time course of 2DG uptake (a) and MeAIB uptake (b) stimulated by IGF-I

L6 myotubes were depleted of serum for 4 h in α-MEM containing 25 mM glucose, followed by amino acid depletion in HBS plus 25 mM glucose for 1 h before simultaneous determination of 2DG and MeAIB uptake. L6 myotubes were incubated for various times with IGF-I (3 nM) in a manner so that the hormone-incubation period ended with the amino-acid-depletion period. The data are the means of three determinations from one experiment representative of three.

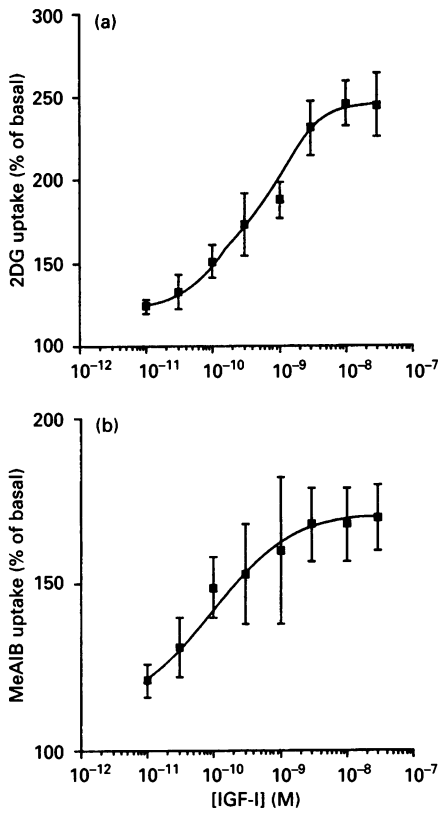

Figure 3 Dose–response relationships of 2DG uptake (a) and MeAIB uptake (b) stimulated by IGF-I

L6 myotubes were incubated in serum-free α-MEM containing 25 mM glucose for 4 h. This was followed by a 1 h amino acid depletion in HBS containing 25 mM glucose. The cells were incubated with IGF-I for the last 45 min of the amino-acid-depletion period in the presence of 5 mg/ml fatty-acid-free BSA before measurement of 2DG and MeAIB uptake. Each curve is representative of 6–8 similar experiments, and the points show the S.E.M. of these experiments.

Uptake of both nutrients was rapidly stimulated 7 min after exposure to 3 nM IGF-I, achieving a new plateau value by 30 min. This new plateau value was sustained for up to 90 min of exposure to IGF-I, after which the initial rates of uptake appeared to begin a second phase of hormonal stimulation for both substrates.

The sensitivity to IGF-I of glucose and amino acid uptake was assessed from dose–response curves after a 45 min hormone incubation for the simultaneous stimulation of 2DG and MeAIB uptake. This incubation period was chosen since at this point the stimulated uptake rates of both substrates has reached a stable plateau with a dose of 3 nM IGF-I. Figure 3 illustrates the averaged results of 6–8 independent IGF-I dose–response experiments, where 2DG and MeAIB uptakes were measured simultaneously in the same cells (Figures 3a and 3b respectively). The averaged results were then analysed by Hanes transformation to calculate the responsiveness and sensitivity to IGF-I of each process. The maximal response of 2DG transport to IGF-I stimulation was 2.5-fold, whereas the MeAIB transport response was 1.7-fold. The mean ED_{50} values for the stimulation of 2DG and MeAIB uptake were 1.07×10^{-10} M and 3.57×10^{-11} M respectively, as defined by Hanes transformation. The R coefficients of the Hanes plots of the averaged experiments were close to unity for both 2DG and MeAIB uptake. From independent experiments, the slope of the line for 2DG was 0.0041 ± 0.0006 and for MeAIB it was 0.0059 ± 0.001. The difference between these values corresponded to a Student's t value of 1.56 and $P > 0.1$, indicating that the ED_{50} values are not statistically different.

The possibility was entertained that the stimulation of MeAIB uptake could be due to an increase in driving force caused by a higher energy level in IGF-I-stimulated cells, which have an augmented glucose uptake. Therefore, experiments were carried out where IGF-I was presented to cells in the presence of 0, 5 and 25 mM glucose, and MeAIB uptake was subsequently determined. The fold stimulation of amino acid uptake by IGF-I under these conditions was 1.45 ($n = 2$), 1.51 ($n = 2$) and 1.67 ($n = 6$) respectively. There were no statistically significant differences among these stimulations when analysed by ANOVA. These results suggest that stimulation of amino acid uptake by IGF-I does not depend on the concomitant elevation of glucose intake.

Effect of BFA on glucose and amino acid transport in L6 cells

As outlined in the Introduction, we hypothesized that BFA might prevent IGF-I stimulation of glucose and/or amino acid uptake, if these responses were mediated by vesicle recruitment through the secretory pathway. As a pre-requisite to these experiments, we investigated the ability of BFA to disrupt the TGN in L6 myotubes. To this aim we isolated subcellular membrane fractions from control and BFA-treated L6 myotubes and assessed the subcellular distribution of TGN38, a transmembrane glycoprotein localized to the TGN (Luzio et al., 1990). In control untreated cells, the TGN38 protein was recovered predominantly in the light-microsomal fraction isolated by subcellular fractionation (Figure 4a). The origin of this fraction has been determined to be intracellular, based on the high levels of GLUT4 glucose-transport content (Figure 4b) and the virtual absence of α_1-Na^+/K^+-ATPase (Bilan et al., 1992a,b). Conversely, the TGN38 antigen was virtually undetectable in the isolated plasma-membrane fraction (Figure 4a), which is rich in GLUT1 and GLUT3 glucose transporters (Figure 4b), confirming previous observations. Treatment of myotubes with 5 μg/ml BFA for 1 h significantly decreased the TGN38 content of the intracellular membranes without increasing its abundance in the plasma membrane. This is likely to be the result of dispersion of the TGN, which is then distributed evenly among all membrane fractions isolated. In contrast, BFA treatment did not alter the distribution of the glucose transporters (Figure 4b).

Structural disruption of the TGN by BFA was corroborated by immunofluorescence using the anti-TGN38 antibody. Exposure of L6 myotubes to 5 μg/ml BFA for 1 h markedly affected the localization of the immunoreactive TGN38 protein, which changed from a perinuclear clustering to a dispersed cytosolic localization (A. Marette, Y. Mitsumoto and A. Klip, unpublished work). These observations suggest that, under the conditions chosen, BFA can effectively disrupt the TGN.

These same conditions of incubation with the fungal metabolite were then applied to studies of glucose and amino acid uptake. Table 1 shows that addition of 5 μg/ml BFA for 60 min to L6 myotubes did not affect the basal rate of uptake of 2DG, suggesting that the resting rate of transport is not determined by a continuous recycling of glucose transporters from the TGN to the cell surface within this time frame. If glucose-transporter recycling is ongoing in the course of the 60 min, it must be from a BFA-insensitive pool of transporters, possibly endosomes. Similarly, BFA was unable to prevent the ability of IGF-I to stimulate 2DG uptake. For these experiments, the drug was

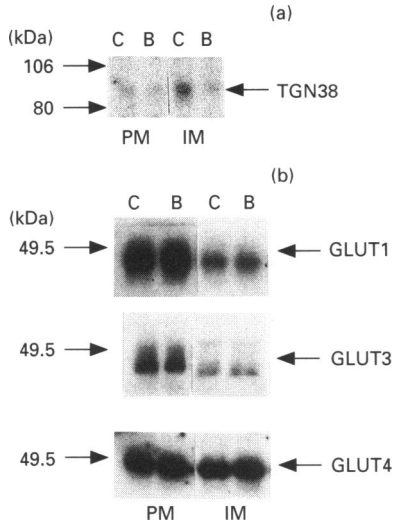

Figure 4 Effect of BFA on the subcellular distribution of TGN38 (a) and the GLUT1, GLUT3 and GLUT4 glucose transporters (b)

L6 myotubes grown in 10 cm-diameter culture dishes were incubated in serum-free α-MEM containing 25 mM glucose for 4 h. This was followed by a 1 h amino-acid-depletion in HBS containing 25 mM glucose. BFA (5 μg/ml) was added from an ethanolic stock solution at the beginning of the 1 h amino-acid-depletion period. Control cell dishes (C) received an equal volume of ethanol instead of BFA (B). Cells were harvested and subjected to subcellular fractionation as outlined in the Materials and methods section. Plasma membranes (PM) and light microsomes (IM) were isolated and analysed on Western blots by using specific antibodies as outlined in the Materials and methods section. The distribution of the TGN38 antigen and of the GLUT1, GLUT3 and GLUT4 glucose transporters is illustrated.

Table 1 Effects of BFA on 2DG uptake and MeAIB uptake in L6 myotubes

L6 myotubes were incubated in serum-free α-MEM containing 25 mM glucose for 4 h. This was followed by a 1 h amino acid depletion in HBS containing 25 mM glucose. BFA (5 μg/ml) was added at the beginning of the amino-acid-depletion period. Control cells received an equal volume of ethanol instead of BFA. Cells were exposed to IGF-I (3 nM) for the last 45 min of the amino-acid-depletion period. Results are means $\pm$ S.E.M. from 4 separate experiments each performed in triplicate. The statistical analysis (Student's t test) of the Basal versus IGF-I-stimulated transport in each case is given in the lower panel.

	Uptake (pmol/min per mg of protein)			
	2-DG		MeAIB	
Transport	−BFA	+BFA	−BFA	+BFA
Basal	8.46 $\pm$ 0.92	8.18 $\pm$ 0.70	7.69 $\pm$ 0.42	8.45 $\pm$ 0.47
+IGF-I	13.63 $\pm$ 1.5	13.56 $\pm$ 1.06	13.39 $\pm$ 1.01	11.54 $\pm$ 0.57
Student's t	2.035	4.094	5.229	3.834
P	< 0.025	< 0.005	< 0.001	< 0.005

added for 60 min, with IGF-I present during the last 45 min of this incubation. IGF-I was fully capable of stimulating 2DG uptake under these conditions, and the fold response was identical with that observed in cells not exposed to BFA.

We have previously demonstrated that IGF-I stimulates 2DG uptake through recruitment of glucose transporters present in a light-microsomal fraction isolated upon subcellular fractionation of L6 myotubes (Bilan et al., 1992a). The lack of effect of BFA on the response of 2DG uptake to IGF-I suggested that the intracellular pool of glucose transporters was not affected by the fungal metabolite. This was indeed demonstrated by the lack of effect of BFA on the subcellular distribution of GLUT1, GLUT3 or GLUT4 glucose transporters (Figure 4). These proteins retained their original distribution after exposure to BFA, suggesting that neither the intracellular nor the plasma-membrane pools of glucose transporters reside on membranes of TGN origin.

The effect of BFA on amino acid uptake was also investigated. In the presence of BFA, basal MeAIB uptake was marginally elevated but this difference was not statistically significant (Student's $t = 1.206$). The response of MeAIB uptake to IGF-I was slightly lower in BFA-treated than in untreated myotubes, but again this difference was not statistically significant (Student's $t = 1.195$). As a consequence of the small increase in the basal rate and the lower stimulated uptake, the fold increase in MeAIB transport activity in response to IGF-I was lower in BFA-treated cells. However, the stimulation by IGF-I was still highly statistically significant ($P < 0.005$). Moreover, the apparent sensitivity of MeAIB uptake to BFA was not obvious at a higher dose of BFA. In two separate experiments (each performed in triplicate) 50 μg/ml BFA had no effect on either basal or IGF-I-stimulated MeAIB uptake (basal uptake 6.97 versus 6.32 pmol/min per mg of protein in the absence and presence of BFA respectively; IGF-I-stimulated uptake 9.21 versus 9.00 pmol/min per mg of protein in the absence and presence of BFA respectively). Hence it is apparent that BFA did not alter the acute stimulatory effect of IGF-I on amino acid uptake through System A.

DISCUSSION

The ability of L6 muscle cells to respond to low concentrations of insulin and IGF-I can be attributed to the expression of their respective receptors in the PM (Beguinot et al., 1985; Bilan et al., 1992a). IGF-I is a more potent stimulator of glucose transport in these cells than is insulin through its own receptor (Bilan et al., 1992a). In addition, there is evidence that IGF-I is able to bind to the insulin receptor of L6 cells with greater efficacy than insulin itself (Burant et al., 1987). However, IGF-I binding to the insulin receptor does not result in potentiation of insulin action on glucose transport, indicating that the intracellular signals that emanate from the two receptors (or hybrid receptors) converge on a common signalling pathway or end-point (Lamphere and Lienhard, 1992).

In the present study we determined parallelisms in the dose–response and time course of stimulation of glucose and amino acid uptake by IGF-I, suggesting that similar mechanisms may underlie each response. The fold stimulation of glucose transport was somewhat higher than that of MeAIB transport when a maximal dose of IGF-I was used. Differences in the fold stimulation do not necessarily imply differences in the underlying mechanism, since these relative values depend in part on the basal rate of transport for each substrate.

The results presented also indicate that the stimulation of amino acid uptake is not secondary to the increased glucose intake, since IGF-I was able to stimulate MeAIB uptake to the same extent in glucose-rich as in glucose-depleted medium. However, the stimulation of amino acid uptake could be secondary to increases in the driving force created by the ionic transmembrane gradients. In rat skeletal muscle, the rapid stimulation of amino acid uptake by insulin is not prevented by ouabain, and it has been proposed that this response to the

hormone does not involve a change in the transmembrane ionic gradient (Guma et al., 1988).

Given that stimulation of hexose transport in L6 cells can be fully accounted for by translocation of glucose transporters from an intracellular light-microsomal pool to the plasma membrane (Ramlal et al., 1988), we investigated whether this traffic could be interfered with by use of BFA, and if so whether amino acid uptake would be similarly affected. BFA effectively blocks traffic of secretory (Fugiwara et al., 1988; Hendricks et al., 1992), lysosomal (Lippincott-Schwartz et al., 1991) and membrane proteins (Shite et al., 1990), through its pharmacological action on components of the Golgi complex. This compound, when administered to L6 myotubes, resulted in a dispersion of the TGN, based on morphological and biochemical redistribution of the specific TGN antigen, TGN38. However, under the same experimental conditions, BFA failed to interfere with the stimulation by IGF-I of either glucose or amino acid uptake. Whereas the TGN38 no longer migrated with the light-microsomal fraction upon subcellular fractionation of BFA-treated myotubes, the intracellular glucose-transporter pool remained in this fraction.

The inability of BFA to prevent stimulation of glucose transport by IGF-I in the face of substantial TGN disruption contests the view that glucose transporters are directly recruited from the TGN to the plasma membrane. A role for the TGN as a donor site of the GLUT1 protein was proposed by Blok et al. (1988) for insulin-treated 3T3-L1 adipocytes, based on electron-microscopy observations of the subcellular distribution of immunogold-labelled GLUT1 proteins. Similarly, Rodnick et al. (1992) proposed that the TGN may represent the site of intracellular GLUT4 transporter storage in skeletal muscle, based on immunodetection by electron microscopy of this protein in perinuclear regions. It is possible to reconcile these observations with the results of the present study by proposing that the TGN contains a substantial amount of glucose transporters, which then furnish a select pool of vesicles that become the target for insulin or IGF-I signals, providing transporters to the PM. This view is supported by immunofluorescence and confocal microscopy observations made in L6 myotubes, indicating that GLUT4 glucose transporters are largely localized to a perinuclear region (Y. Mitsumoto and A. Klip, unpublished work). Moreover, this scenario would be consistent with a recent suggestion that two intracellular pools of glucose transporters exist in 3T3-L1 adipocytes, only one of which is in direct exchange with the PM (Robinson and James, 1992). The regulated transporter-containing vesicles may be localized in close proximity to the PM, and may only require membrane fusion for the insertion of extra copies of glucose transporters into the PM. This pathway would be predicted to be BFA-insensitive. Precedence for this statement are the observations by Klausner et al. (1992) and Hunziker et al. (1992), who reported that the function of the endocytic pathway (with respect to endocytosis, endosomal acidification and lysosomal enzyme function) was not affected by BFA despite morphological changes caused to endosomes and lysosomes. Recently, Rosa et al. (1992) also demonstrated that BFA inhibits the formation of constitutive secretory vesicles, but does not prevent secretion from pre-formed vesicles. In spite of these arguments, it cannot be ruled out at present that structural disruption of the TGN may not affect its function in intracellular traffic.

The results show that in L6 myotubes stimulation of MeAIB uptake by IGF-I had a similar time course of activation and hormone-sensitivity compared with that of glucose transport. Such observations may denote a similar mechanism of activation of both membrane transport processes by IGF-I. If System A transporters were translocated to the PM by acute IGF-I or insulin action, the mechanism could be envisaged to involve the vesicle system that carries glucose transporters to the PM. Using BFA, we were unable to inhibit selectively stimulation of glucose or amino acid uptake by BFA. However, the results presented appear to exclude the direct involvement of endoplasmic-reticulum-to-Golgi vesicle traffic and of the TGN in glucose-transporter recruitment and System A amino acid transport stimulation by IGF-I, based on the inability of BFA to prevent IGF-I-mediated activation of glucose and amino acid uptake. When this work was being completed, an exciting new communication by Kong et al. (1993) reported the identification of a cDNA sequence cloned from the pig kidney-derived LLC-PK_1 cell line, which when expressed in COS-7 cells increased uptake of MeAIB. The sequence codes for a polypeptide of 673 amino acid residues, and has strong resemblance to the Na^+-dependent glucose transporter of epithelia. Antibodies to the new protein, however, are not yet available, and furthermore it is not known if this amino acid transporter is expressed in non-epithelial cells. When immunological tools are generated, it will be feasible to investigate in a direct way the possibility of recruitment of amino acid transporters in response to hormones and growth factors.

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