Arrest of endosome acidification by bafilomycin A₁ mimics insulin action on GLUT4 translocation in 3T3-L1 adipocytes

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In insulin-sensitive fat and muscle cells, the major glucose transporter GLUT4 is constitutively sequestered in endosomal tubulovesicular membranes, and moves to the cell surface in response to insulin. While sequence information within GLUT4 appears to be responsible for its constitutive intracellular sequestration, the regulatory elements and mechanisms that enable this protein to achieve its unique sorting pattern under basal and insulin-stimulated conditions are poorly understood. We show here that arrest of endosome acidification in insulin-sensitive 3T3-L1 adipocytes by bafilomycin A₁, a specific inhibitor of the vacuolar proton pump, results in the rapid and dose-dependent translocation of GLUT4 from the cell interior to the membrane surface; the effects of maximally stimulatory concentrations of bafilomycin A₁ (400–800 nM) were equivalent to 50–65 % of the effects of acute insulin treatment. Like insulin, bafilomycin A₁

induced the redistribution of GLUT1 and Rab4, but not that of other proteins whose membrane localization has been shown to be insulin-insensitive. Studies to address the mechanism of this effect demonstrated that neither autophosphorylation nor internalization of the insulin receptor was altered by bafilomycin A₁ treatment. Bafilomycin-induced GLUT4 translocation was not blocked by cell pretreatment with wortmannin. Taken together, these data indicate that arrest of endosome acidification mimics insulin action on GLUT4 and GLUT1 translocation by a mechanism distal to insulin receptor and phosphatidylinositol 3-kinase activation, and suggest an important role for endosomal pH in the membrane dynamics of the glucose transporters.

Key words: endosomal pH, GDP-dissociation inhibitor, GLUT1, Rab4.

INTRODUCTION

A key metabolic effect of insulin is to increase glucose uptake in fat and muscle cells [1–4]. These insulin-sensitive tissues express at least two of the glucose transporter proteins: the ubiquitous GLUT1 at low levels and the fat/muscle-specific insulin-regulated GLUT4. Although the two transporters are highly similar in their deduced primary and secondary structures, they are located at different intracellular sites. In resting cells, GLUT1 is distributed to both plasma membranes and the cell interior, whereas GLUT4 is sequestered exclusively in intracellular storage sites. According to the currently accepted translocation model, originally proposed by Cushman and Wardzala [5] and Suzuki and Kono [6], insulin evokes a redistribution of both GLUT4 and GLUT1 from the interior of the cell to the plasma membrane, but the increased capacity of the cell to transport glucose is due mainly to GLUT4. Studies using chimaeric GLUT1–GLUT4 proteins expressed in both insulin-sensitive and insulin-unresponsive cell models in a transient or stable manner helped to identify structural motifs among the transporter molecules responsible for their differential sorting within the cell [7–11]. Thus both the N- and C-termini of GLUT4 contain sequence information responsible for sequestering the transporter into the cell interior, but to different subcompartments; the insulin-sensitive endosomal compartment recognizes the C-terminal 30 amino acids of GLUT4 [11]. However, the elements and mechanism(s) that recognize these sequences and facilitate the unique sorting pattern of GLUT4 (intracellularly in the basal state and on the plasma membrane under insulin-stimulated conditions) are largely unknown.

Electron microscopy analysis has shown that GLUT4 associates with endosomal–tubulovesicular structures in the *trans*-Golgi network in brown- and white-fat cells [12,13]. These and other morphological and biochemical data [14–16] suggest that the interaction of GLUT4 with endosomal, endosome-like or post-endosomal compartments may be part of the mechanism that maintains the unique distribution of GLUT4, resulting in a principally intracellular localization in the steady state. A typical characteristic of eukaryotic endosomes, along with other organelles (i.e. Golgi and lysosomes), is their acidification, which is maintained by a vacuolar class of proton-translocating ATPases [17–19]. Although the role of endosome acidification is not completely understood, it is important for several aspects of endocytic trafficking. Thus a well-established function of the low endosome pH is to dissociate lysosomally destined ligands from membrane-associated receptors [20]. In addition, endosome acidification has been shown to play a role in the recycling of internalized receptors back to the plasma membrane, or in the delivery of the endocytosed molecules to late endosomes or lysosomes [19,21].

In the present study we have investigated the role of endomembrane acidification in the intracellular sorting of GLUT4 and its insulin-regulated delivery to the cell surface in 3T3-L1 adipocytes. Towards this purpose, bafilomycin A₁, a selective inhibitor of the vacuolar proton pump [22], was used to arrest acidification of the endosomal/lysosomal system. We demonstrate here that, similar to insulin, bafilomycin A₁ induces rapid translocation of GLUT4 from the intracellular membrane fraction to the plasma membranes of 3T3-L1 adipocytes. These results suggest that modulation of adipocyte endosome acidification

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GDI, GDP-dissociation inhibitor; PI 3-kinase, phosphatidylinositol 3-kinase.

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fication may contribute to the mechanisms that sequester GLUT4 into the cell interior and trigger its externalization to the cell surface.

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EXPERIMENTAL

Materials

Human recombinant insulin was a gift from Eli Lilly (Indianapolis, IN, U.S.A.). Renaissance chemiluminescence detection kit was purchased from New England Nuclear (Boston, MA, U.S.A.); nitrocellulose transfer membranes (0.45 μ m pore size) were from Schleicher & Schuell (Keene, NH, U.S.A.); protein assay reagent was from Bio-Rad (Hercules, CA, U.S.A.); Acridine Orange was from Fisher Scientific (Pittsburg, PA, U.S.A.); horseradish peroxidase-coupled donkey anti-(rabbit IgG) and horseradish peroxidase-coupled anti-(mouse IgG) were from Boehringer Mannheim Corp. (Indianapolis, IN, U.S.A.). Bafilomycin A₁ and wortmannin were purchased from Sigma (St. Louis, MO, U.S.A.). C-terminal-directed polyclonal anti-GLUT4 (R1288), anti-GLUT1 (R480; a gift from Dr. Michael Czech, University of Massachusetts Medical Center, Worcester, MA, U.S.A.) and anti-[GDP-dissociation inhibitor-1 (GDI-1)] (R3357) [23] antisera, a polyclonal anti-(human Rab4) antiserum (R10502; a gift from Dr. Ira Mellman, Department of Cell Biology, Yale University School of Medicine, New Haven, CT, U.S.A.), and C-terminal-directed anti-(insulin receptor) (a gift from Professor Kenneth Siddle, Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge, U.K.) and anti-(transferrin receptor) (a gift from Dr. Jan Trowbridge and Dr. Sue White, Department of Cancer Biology, The Salk Institute, San Diego, CA, U.S.A.) monoclonal antibodies were utilized as IgG fractions purified on Protein A-Sepharose columns or as diluted crude antisera.

Cell cultures and cell treatment

3T3-L1 adipocytes were induced to differentiate as described previously [23]. Briefly, differentiation was initiated in confluent cells in 100 mm-diam. dishes by adding Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, 50 μ g/ml streptomycin sulphate, 5 μ g/ml insulin, 0.25 μ M dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. On the second day the medium was replaced with DMEM containing the above components except the last two. Thereafter the cells were maintained in DMEM containing 10% (v/v) fetal bovine serum and the antibiotics, with medium changes every 2–3 days. The cells were used between days 8 and 12 following induction of differentiation. Prior to experiments, adipocytes were serum-starved for 3 h in DMEM and then washed with PBS at room temperature. The cells were first treated or not with bafilomycin A₁ or wortmannin at the concentrations and for the time periods indicated in the Figure legends. The reagents were administered to cells in an 'intracellular buffer', containing 140 mM glutamic acid (potassium salt), 20 mM Hepes (pH 7.4), 5 mM MgCl₂, 5 mM EGTA and 5 mM NaCl. Control cells were treated with the same buffer. The cells were then stimulated or not with 100 nM insulin for 10 min at 37 °C, as indicated in the Figure legends, and subjected to subcellular fractionation.

Vital fluorescence microscopy

In some experiments, 3T3-L1 fibroblasts were seeded on glass coverslips (22 mm $\times$ 22 mm) and induced to differentiate as described above. Following serum starvation and treatment with bafilomycin A₁ for 30 min at 37 °C at the concentrations indicated in the Figure legends, the living cells were stained with Acridine Orange using previously described protocols [24–26]. Briefly, treated cells were further incubated at 37 °C for 10 min with 5 μ g/ml Acridine Orange in intracellular buffer. After three washes with the same buffer, the coverslips were mounted on slides by means of a mounting solution and were examined on a fluorescence microscope (Zeiss model LSM 310) using a dual filter for evaluation of both the orange and the green fluorescence of Acridine Orange (excitation at 488 and 520 nm, and emission at 515–530 and 580–630 nm).

Subcellular fractionation of 3T3-L1 adipocytes

After treatment and stimulation with insulin, cells were washed twice with cold PBS and once with HES buffer (20 mM Hepes, pH 7.4, 250 mM sucrose, 1 mM EDTA and 150 mM NaCl), and then scraped into HES buffer containing 1 $\times$ cocktail of protease inhibitors (1 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin and 1 mM benzamide) at 4 °C. The cells were then fractionated using previously characterized procedures and our established protocols for small-scale fractionation [23]. Briefly, adipocytes were homogenized in a motor-driven Teflon/glass homogenizer and centrifuged at 16000 *g* for 20 min in a Sorvall RC2-B centrifuge (SA600 rotor). The cells were then fractionated on a table-top Beckman TL-100 Ultracentrifuge at 4 °C to obtain intracellular membranes (composed of high- and low-density microsomes), plasma membranes and cytosol. Intracellular membranes were pelleted by centrifuging the 16000 *g* supernatant for 24 min at 200000 *g*. The plasma membrane fraction was collected from the interface of a 1.12 M sucrose cushion following centrifugation of the 16000 *g* pellet at 70000 *g* for 10 min and, after resuspension in HES buffer, was pelleted at 200000 *g* for 4 min. Particulate fractions were resuspended in HES buffer supplemented with 1 $\times$ protease inhibitor cocktail by passing through a 26.5 gauge needle nine times. Protein concentrations in the fractions were determined using the Bio-Rad protein assay. Aliquots of the fractions were analysed by SDS/PAGE and immunoblotting with the indicated antibodies.

SDS/PAGE and immunoblotting

Aliquots of proteins from subcellular fractions (15–75 μ g) were analysed by SDS/PAGE under reducing conditions using the method of Laemmli [27], with a 3% (w/v) stacking gel and a 10.5% (w/v) separating gel. Following electrophoretic separation, the proteins were electrotransferred to a nitrocellulose membrane. Membranes were blocked by incubation with 3% (w/v) non-fat dry milk and 2% (w/v) BSA in 30 mM Tris, pH 7.4, 150 mM NaCl and 0.05% Tween 20 (wash buffer). The blots were incubated for 16 h at 4 °C with the polyclonal anti-GLUT4 (R1288), anti-GLUT1 (R480) or anti-GDI-1 (R3357) anti-peptide IgGs, with anti-Rab4 antiserum (R10502) or with the monoclonal anti-(insulin receptor) IgG. Blots were then washed for 3 $\times$ 15 min with wash buffer. Bound antibodies were detected using horseradish peroxidase-bound anti-rabbit or anti-mouse immunoglobulins followed by chemiluminescence. Immunoreactivity was quantified by laser scanning densitometry (Molecular Dynamics) of the visualized protein by band-area integration. Two exposures of each blot were quantified to ensure that the exposures were within the linear range of the film.

In vitro phosphorylation of the insulin receptor

Partially purified insulin receptor preparations were derived from rat liver membranes as described previously [28]. Briefly, rat livers were homogenized in the presence of protease inhibitor cocktail, solubilized in 1% Triton X-100 and centrifuged (100 000 *g*, 1 h, 4 °C). The supernatant was applied on a wheat-germ-agglutinin-agarose column, and following extensive washings the insulin receptor was eluted with *N*-acetyl-D-glucosamine. Purified preparations (~15 µg of protein) were pre-incubated with bafilomycin A₁ for 30 min at the concentrations indicated in the Figure legends in 50 mM Hepes (pH 7.4), 2 mM MnCl₂ and 10 mM MgCl₂, and were then stimulated or not with 100 nM insulin for 20 min at room temperature. Phosphorylation was initiated by adding 25 µM [γ -³²P]ATP (2 µCi). After 10 min the reactions were terminated by adding Laemmli sample buffer [27], and the phosphoproteins were resolved on SDS/PAGE (7.5% gel). The gel was dried and subjected to autoradiography.

RESULTS

Bafilomycin A₁ stimulates relocation of GLUT4 from intracellular membranes to the adipocyte cell surface

To determine whether the mechanisms that sequester GLUT4 intracellularly or induce its movement to the cell surface in response to insulin are dependent upon acidification of the endomembrane system, 3T3-L1 adipocytes were treated acutely with bafilomycin A₁, a specific inhibitor of the proton-translocating vacuolar-type V-ATPases [18,22]. This proton pump is found in almost all eukaryotic cells, associated with membranes of several organelles, including clathrin-coated vesicles, the Golgi complex, lysosomes, synaptic vesicles, chromaffin granules and vacuoles of plants, fungi and yeast [19]. Following acute stimulation with insulin, the cells were fractionated to obtain intracellular membrane and plasma membrane fractions, and the levels of GLUT4 were determined by Western blotting analysis of the fractions utilizing anti-GLUT4 antibodies. As demonstrated in Figure 1, acute treatment of cells with bafilomycin A₁ (30 min at 37 °C; 800 nM) caused a large decrease in intracellular

Figure 1 Effect of bafilomycin A₁ on GLUT4 translocation in 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were pretreated with bafilomycin A₁ (BafA1; 800 nM) for 30 min at 37 °C, and were then stimulated or not with insulin (100 nM) for an additional 10 min at 37 °C. The cells were fractionated into intracellular membrane (IM) and plasma membrane (PM) fractions. Proteins (15 µg) were resolved by SDS/PAGE, transferred on to a nitrocellulose membrane and immunoblotted with anti-GLUT4 IgG (0.5 µg/ml). (A) Chemiluminescence detection of GLUT4 (a representative experiment is shown). (B) Results from densitometric scans (means ± S.E.M. of 4–10 independent cell fractionations). The effects of bafilomycin A₁ in decreasing IM and increasing PM GLUT4 levels were equivalent to 59 ± 6% and 55 ± 5% respectively of the maximal effects of insulin.

Figure 2 Dose-dependent effects of bafilomycin A₁ on GLUT4 translocation

Differentiated 3T3-L1 adipocytes were pretreated or not with the indicated concentrations of bafilomycin A₁ (BafA1) for 30 min at 37 °C, and were then stimulated or not with insulin (100 nM) for an additional 10 min at 37 °C, as indicated. Cells were fractionated into intracellular membrane (IM) and plasma membrane (PM) fraction. Portions of 15 µg of fraction proteins were resolved by SDS/PAGE, transferred on to a nitrocellulose membrane and immunoblotted with anti-GLUT4 IgG. (A) Chemiluminescence detection of GLUT4 on PM and IM (representative experiment). (B) Densitometric quantification (means ± S.E.M. of three independent cell fractionations).

GLUT4 levels (to 67 ± 8% of control; mean ± S.E.M.) and a proportionate increase in plasma membrane GLUT4 (175 ± 9% of control). The decrease in microsomal GLUT4 and increase in plasma membrane GLUT4 levels induced by bafilomycin A₁ were equivalent to 59 ± 6% and 55 ± 5% respectively (*n* = 10 independent cell fractionations) of the effects observed at a saturating insulin concentration (100 nM; 10 min stimulation) (Figure 1). Importantly, the effect of bafilomycin A₁ was not synergistic with the effect achieved at saturating concentrations of the hormone (Figure 1), and in fact the maximal effect of insulin was slightly reduced in the presence of bafilomycin A₁ (by ~10–15%; average of five independent cell fractionations) compared with the effect of insulin alone.

The effect of bafilomycin A₁ on GLUT4 localization was dose-dependent (Figure 2). Concentrations as low as 50 nM were up to 20% as effective as the maximally effective insulin concentration with regard to both the decrease in GLUT4 in intracellular membranes and the increase in plasma membranes. The effect peaked at 400 nM bafilomycin A₁, which was ~50% as effective as 100 nM insulin (Figure 2). Higher concentrations of bafilomycin A₁ (up to 800 nM) did not result in further increases in GLUT4 translocation (Figure 1). Together, these data demonstrate that bafilomycin A₁ can partially mimic the effects of insulin, and is capable of inducing relocation of GLUT4

Figure 3 Acridine Orange staining of bafilomycin A_1 -treated 3T3-L1 adipocytes

3T3-L1 adipocytes induced to differentiate on glass coverslips were treated or not with the indicated concentrations of bafilomycin A_1 (BafA1) for 30 min at 37 °C. The cells were then stained with Acridine Orange for 10 min at 37 °C and analysed in a fluorescence microscope with a dual filter for green and red fluorescence. Quenching of the granular orange fluorescence by bafilomycin A_1 was used as an indicator of the alkalinization of the endosomal/lysosomal system. The data were quantified by counting the individual orange granules of 10 randomly selected fields for each condition, and are expressed as percentages of the results with untreated control cells. The values are means $\pm$ S.E.M. of the observed fields in two independent experiments.

from intracellular pools to the plasma membrane when present at nanomolar concentrations.

To determine whether the effect of bafilomycin A_1 on GLUT4 translocation coincides with its effect of inhibiting endosome/lysosome acidification, we probed the cells with Acridine Orange. As demonstrated previously, this dye is 'acidotropic' and is taken up avidly by living cells, accumulating in acidified compartments and displaying orange fluorescence [25,26]. Alkalinization of the endocytic structure is accompanied by disappearance of the orange fluorescence [25,26]. In agreement with these findings, staining of control 3T3-L1 adipocytes with the dye for 10 min resulted in a granular appearance of the orange fluorescence associated with the acidified endosomes/lysosomes, whereas cytosol and nuclei showed green fluorescence [24–26] (Figure 3; and results not shown). Treatment of 3T3-L1 adipocytes with 20–800 nM bafilomycin A_1 for 30 min at 37 °C prior to Acridine Orange staining resulted in a dose-dependent quenching of the granular orange fluorescence (Figure 3), whereas the green fluorescence remained practically unaltered. These results imply that concentrations of bafilomycin A_1 similar to those that induce GLUT4 relocation to the adipocyte cell surface cause inhibition of acidification in the 3T3-L1 adipocyte endosomal/lysosomal system.

The effect of bafilomycin A_1 on GLUT4 is exceedingly rapid

Estimation of the pH values in individual transferrin-containing endosomes revealed an elevation in pH (pH 7) following addition of bafilomycin A_1 to living cells within $\sim$ 2 min, the earliest time at which measurement was technically possible [21]. To determine whether bafilomycin A_1 -induced GLUT4 translocation parallels this rapid increase in pH, 3T3-L1 adipocytes were treated for as little as 5 min and up to 1 h. Following subcellular fractionation and resolution of the proteins by SDS/PAGE, GLUT4 levels were determined by immunoblotting. Figure 4 demonstrates that the effect of bafilomycin A_1 in inducing GLUT4 translocation to adipocyte plasma membranes can be observed as soon as 5 min

Figure 4 Effect of bafilomycin A_1 on GLUT4 translocation as a function of time

Differentiated 3T3-L1 adipocytes were pretreated with bafilomycin A_1 (400 nM) for indicated time periods (lanes 3–5) at 37 °C and were then stimulated (lane 2) or not (lane 1) with insulin for an additional 10 min at 37 °C. The cells were fractionated into intracellular membrane (IM) and plasma membrane (PM) fractions. Portions of 15 μ g of fractionated proteins were resolved by SDS/PAGE, transferred on to a nitrocellulose membrane and immunoblotted with anti-GLUT4 IgG. (A) Chemiluminescence detection of GLUT4 on PM and IM (representative experiment). (B) Densitometric quantification (means $\pm$ S.E.M. of three independent cell fractionations).

following treatment with the drug, and is sustained at the same level for at least 60 min, with no significant change in the increment within this period. These results demonstrate: (1) that the effect of bafilomycin A_1 in increasing translocation of GLUT4 to the plasma membrane reaches its maximum exceedingly rapidly; and (2) that this effect remains remarkably constant over a relatively long time period. Given the time course of the bafilomycin A_1 -induced inhibition of V-ATPase and increase in endosome pH [18,21], the results presented in Figures 1–4 are consistent with the idea that the arrest of endosome/lysosome acidification perturbs the retention mechanism that sequesters GLUT4 in intracellular storage locations, and induces its externalization to the cell surface.

Selectivity of the bafilomycin A_1 effect for insulin-regulatable membrane pools

3T3-L1 adipocytes express high levels of the ubiquitous glucose transporter GLUT1. Studies indicate that this glucose transporter also undergoes translocation in response to insulin, and that this effect is as great or greater than the effect on GLUT4 [1]. To examine whether bafilomycin A_1 is capable of inducing GLUT1 membrane relocation as well, 3T3-L1 adipocytes were treated acutely with the agent and, after subcellular fractionation, the amounts of GLUT1 in intracellular membrane and plasma

Figure 5 Bafilomycin A₁ induces GLUT1 translocation, similarly to insulin

Differentiated 3T3-L1 adipocytes were pretreated with bafilomycin A₁ (BafA1; 400 nM) for 30 min at 37 °C, and were then stimulated or not with insulin (100 nM) for an additional 10 min at 37 °C. The cells were fractionated into intracellular membrane (IM) and plasma membrane (PM) fractions. Fraction proteins (70 µg) were resolved by SDS/PAGE, transferred on to a nitrocellulose membrane and immunoblotted with anti-GLUT1 antiserum (1:500 dilution). (A) Chemiluminescence detection of GLUT1 (representative experiment). (B) Results of densitometric scans (means ± S.E.M. of five independent cell fractionations). The effects of bafilomycin A₁ in decreasing IM and increasing PM GLUT1 levels were equivalent to 58 ± 6% and 48 ± 5% respectively of the maximal effects of insulin.

membrane fractions were determined by immunoblotting with specific anti-GLUT1 antibodies. As illustrated in Figure 5, bafilomycin A₁ induces GLUT1 externalization from intracellular locations to the plasma membrane, similar to insulin. As with GLUT4, this effect was equivalent to 50–65% of the effect of insulin (Figure 5).

To investigate further the proposed selective action of bafilomycin A₁ on membranes and proteins shown to be affected by insulin, most probably in relation to GLUT4 membrane dynamics, we examined possible bafilomycin A₁-induced changes in the localization patterns of the small GTP-binding protein Rab4 and its escort protein GDI-1. Previous studies in fat cells and adipocytes in culture have demonstrated the insulin-dependent redistribution of Rab4 from intracellular membranes to the cytosol, whereas the membrane partitioning of GDI-1 remained unaltered by similar insulin treatment [23,29–33]. 3T3-L1 adipocytes were treated acutely with bafilomycin A₁ and, following subcellular fractionation and SDS/PAGE, the levels of GDI-1 and Rab4 were assessed by immunoblotting. As illustrated in Figure 6(A), the populations of GDI-1 associated with the intracellular membranes and plasma membranes remained unchanged following treatment with insulin or bafilomycin A₁, in agreement with our previous studies [23]. Conversely, the amount of Rab4 present on adipocyte intracellular membranes was decreased upon bafilomycin A₁ treatment (Figure 6B). Similar to the effects of insulin [29–32], bafilomycin A₁ caused solubilization of intracellular Rab4, as indicated by the lack of a proportionate increase in Rab4 on the plasma membrane (Figure 6B) and its quantitative recovery in the cytosolic fraction ([32]; and results not shown). The specificity of the bafilomycin A₁ effect for insulin-regulatable intracellular membrane proteins was further suggested by results demonstrating no detectable differences in the SDS/PAGE patterns of Coomassie Blue-stained intracellular membrane and plasma membrane proteins derived from bafilomycin A₁-treated and control 3T3-L1 adipocytes (results not shown). Taken together, these results are consistent with the notion that bafilomycin A₁ selectively affects the dynamics of membrane pools that are also affected by insulin.

Figure 6 Effects of bafilomycin A₁ on membrane distribution of GDI-1 and Rab4

Differentiated 3T3-L1 adipocytes were pretreated with bafilomycin A₁ (BafA1; 400 nM) for 30 min at 37 °C and were then stimulated or not with insulin (100 nM) for an additional 10 min at 37 °C. The cells were fractionated into intracellular membrane (IM) and plasma membrane (PM) fractions. Fraction proteins (50 µg) were resolved by SDS/PAGE and transferred on to a nitrocellulose membrane. The membrane was cut into two, and the upper part was immunoblotted with anti-GDI-1 serum (1:20000 dilution) (A), while the lower part was immunoblotted with anti-Rab4 serum (1:1000 dilution) (B). Shown are chemiluminescence detection (A and B) in representative experiments out of three with similar results, and the results of densitometric scans (means ± S.E.M. of three independent experiments) (B). Rab4 levels in the IM fraction upon bafilomycin A₁ and insulin treatment were 64 ± 8% and 53 ± 6% respectively of the control levels.

The effect of bafilomycin A₁ in inducing GLUT4 translocation is distal to the insulin receptor and phosphatidylinositol 3-kinase (PI 3-kinase)

The insulin-mimicking effects of bafilomycin A₁ on membrane dynamics, documented in Figures 1 and 2, is likely to result from the activation of the intracellular signalling pathway that leads to translocation of GLUT4 from intracellular sites to the cell surface. Two crucial molecular elements with unequivocal roles in signalling the acute insulin effect on GLUT4 relocation are the insulin receptor itself and PI 3-kinase [34–36]. Essential early events in the insulin-regulated signalling circuit are insulin receptor autophosphorylation and its subsequent internalization following ligand binding [34,36]. To determine whether stimulation of these insulin receptor functions by treatment of adipocytes with bafilomycin A₁ could account for the observed effect on GLUT4 translocation, two types of experiments were performed. We first analysed whether bafilomycin A₁ could interact with the insulin receptor to stimulate insulin receptor autophosphorylation. Incubation of purified insulin receptor preparations with bafilomycin A₁ failed to activate insulin receptor autophosphorylation (Figure 7, compare lane 1 with lanes 3 and 4). By contrast, insulin receptors were readily autophosphorylated upon insulin treatment under the conditions of the experiment (Figure 7, lanes 1 and 2), and the presence of bafilomycin A₁ did not affect this activation (Figure 7, lanes 5 and 6). Next, in order to determine whether bafilomycin A₁, like insulin, accelerates the internalization of insulin receptors, we analysed the levels of insulin receptors on both the plasma membranes and

Figure 7 Effect of bafilomycin A_1 on insulin receptor autophosphorylation *in vitro*

Wheat-germ-agglutinin-purified insulin receptor preparations were pre-incubated with bafilomycin A_1 (BafA1) at the indicated concentrations for 30 min, and were then stimulated or not with 100 nM insulin (Ins) for 20 min at room temperature. Phosphorylation was initiated by adding [γ - 32 P]ATP, and reactions were terminated 10 min later by adding Laemmli sample buffer. Following separation of the proteins by SDS/PAGE, the phosphorylated 95 kDa insulin receptor (IR) was detected by autoradiography. Shown is an autoradiogram from a representative experiment out of three with similar results. The apparent increased intensity in lane 6 was not reproducible from experiment to experiment.

the intracellular membranes in bafilomycin A_1 -treated 3T3-L1 adipocytes by Western blotting with anti-(insulin receptor) antibodies. As demonstrated in Figure 8, levels of insulin receptors present in the plasma membrane fractions were slightly but reproducibly decreased (10%) following insulin stimulation (37 °C, 10 min) due to insulin-induced insulin receptor internalization, in agreement with previous studies [37,38]. By contrast, the insulin receptor levels detected in plasma membrane fractions of bafilomycin A_1 -treated adipocytes (30 min, 37 °C) were indistinguishable from those of control cells (Figure 8, compare lanes 1 with lanes 3–5).

A further crucial element linking insulin receptor signalling to GLUT4 translocation is activated PI 3-kinase and its lipid products [1,36]. Inhibition of PI 3-kinase activity by the fungal metabolite wortmannin blocks translocation of GLUT4 in response to insulin [39]. Therefore, to determine whether activation of PI 3-kinase is required for bafilomycin A_1 -induced GLUT4 translocation, 3T3-L1 adipocytes were pretreated with 100 nM wortmannin for 30 min, conditions shown to completely block the insulin-stimulated increase in GLUT4 translocation ([39,40]; and results not shown). The cells were then incubated in the presence of bafilomycin A_1 or insulin, and the levels of GLUT4 in plasma membrane and intracellular membrane fractions were

Figure 8 Effect of bafilomycin A_1 treatment on plasma membrane insulin receptor levels in 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were pretreated or not with the indicated concentrations of bafilomycin A_1 (BafA1) for 30 min (lanes 3–5) at 37 °C. Where indicated, a 10 min stimulation with insulin was conducted at 37 °C. Plasma membrane proteins (65 μ g of protein), prepared by subcellular fractionation, were resolved by SDS/PAGE, then transferred on to a nitrocellulose membrane and immunoblotted with monoclonal anti-(insulin receptor) antibody. Shown is chemiluminescence detection of a representative Western blot, and results from densitometric scans (means $\pm$ S.E.M. of three independent experiments).

Figure 9 Effect of wortmannin on bafilomycin A_1 -induced GLUT4 translocation

Differentiated 3T3-L1 adipocytes were incubated in intracellular buffer in presence or absence of wortmannin (Wort; lane 4) for 30 min at 37 °C. The incubation was prolonged for an additional 30 min at 37 °C in the presence or absence of bafilomycin A_1 (BafA1; lanes 3 and 4), followed by 10 min stimulation with insulin as indicated (lane 2). The cells were then fractionated into intracellular membrane (IM) and plasma membrane (PM) fractions. Fractionated proteins (15 μ g) were resolved by SDS/PAGE, transferred on to a nitrocellulose membrane and immunoblotted with anti-GLUT4 IgG. Shown are chemiluminescence detection in a representative experiment (A) and the results of densitometric scans (B) (means $\pm$ S.E.M. of three independent cell fractionations).

analysed by immunoblotting. As demonstrated in Figure 9, wortmannin pretreatment did not prevent the translocation of GLUT4 induced by bafilomycin A_1 . Together, these results indicate that the effect of bafilomycin A_1 in activating GLUT4 translocation to the plasma membrane in 3T3-L1 adipocytes is distal to both insulin receptor activation and PI 3-kinase activation.

DISCUSSION

Current evidence suggests that the actual target of the insulin-receptor-induced chain of signalling events that culminates in GLUT4 relocation on the fat/muscle-cell surface is the GLUT4 vesicle itself, and several recent studies support this assumption. Thus GLUT4 vesicles derived from insulin-stimulated fat-cell intracellular membranes display changes in density [41], consistent with insulin-induced changes in membrane protein/lipid composition of these vesicles. Although the exact nature of intracellular GLUT4 vesicles and the different membrane pools that contain GLUT4 have not yet been fully elucidated, morphological studies visualize GLUT4 membranes essentially as populations of endosomes, endosome-like or post-endosomal

structures sequestered intracellularly in resting insulin-sensitive cells [12–16].

Previous studies on the role of endosome acidification in the effects of insulin on glucose transport and glucose transporter translocation in rat adipocytes used acidotropic agents such as chloroquine, NH_4Cl and monensin, and yielded conflicting results [42,43]. Inhibition, no change or stimulation (140% increase in basal 3-*O*-methylglucose uptake induced by chloroquine) of basal or insulin-regulated glucose transport or transporter activity have been reported [42,43]. A typical problem with this sort of experiment is that the weak bases, in addition to their effects on the acidity of the endosome compartment, may perturb essential events along the insulin-receptor-initiated signalling chain that could result in abrogation of the final biological response. Bafilomycin A_1 selectively inhibits V-ATPase, but not F_1F_0 - or E_1E_0 -ATPases at concentrations up to $1\text{ }\mu\text{M}$, making this macrolide antibiotic a valuable tool for studying the role of the vacuolar proton pump and intravesicular acidity in membrane trafficking events [18,22,44]. The main focus of the present study was to analyse whether an arrest of endosome/lysosome acidification, achieved through specific inhibition of a H^+ -ATPase by bafilomycin A_1 , could modulate the mechanisms that retain GLUT4 in the intracellular membrane compartment in the basal state or induce its movement to the plasma membranes upon insulin stimulation of 3T3-L1 adipocytes. The key finding we document here is that bafilomycin A_1 treatment of intact cells caused a rapid, dose-dependent depletion of the intracellular GLUT4 pool and a corresponding increase in GLUT4 on adipocyte plasma membranes. To the best of our knowledge, this study provides the first information concerning a potential role of endosomal pH in the mechanisms underlying unique GLUT4 sorting under basal and insulin-induced conditions through the use of specific V-ATPase inhibition by bafilomycin A_1 .

Elevation of the endosomal pH (pH 7) has been shown to occur within less than 2 min following exposure of HepG2 cells to bafilomycin A_1 ; it is also highly efficient, since subsequent addition of weak bases did not result in further increases in endosomal pH [21]. In fact, the time course of bafilomycin A_1 -induced GLUT4 translocation from intracellular stores to plasma membranes observed in the present study (Figure 4) corresponds well with the reported rapid effect of this agent in increasing endosomal pH. Similarly, the maximally activating concentration of bafilomycin A_1 with regard to GLUT4 translocation is similar to the bafilomycin A_1 concentration found to be effective in blocking endosomal acidification in 3T3-L1 adipocytes (Figure 3) and in other living cells of several types [21,24–26,45]. Together, these results are consistent with the notion that the altered glucose transporter membrane dynamics result from the specific effect of bafilomycin A_1 on V-ATPase.

Studies on bafilomycin A_1 -treated CHO and HepG2 cells have demonstrated that elevated endosome pH has no significant effect on, or slightly slows down, the recycling pathway [21,45]. In fact, our studies also demonstrated that bafilomycin A_1 treatment of 3T3-L1 adipocytes does not significantly alter the relative amounts of immunoreactive transferrin receptor associated with intracellular membrane or plasma membrane fractions (results not shown). These results, together with the unchanged localization patterns of other proteins analysed here (Figures 6 and 8), imply a selective insulin-like effect of bafilomycin A_1 on endomembranes harbouring the glucose transporters, rather than a stimulation of the bulk flow of membrane proteins to the cell surface. Our results further indicate that the bafilomycin A_1 -affected glucose transporter pools are distinct from the constitutively recycling compartment that contains transferrin receptors. Previous studies have suggested the presence of two or more

distinct pools of intracellular GLUT4 in insulin-sensitive cells, and on several occasions limited co-localization of GLUT4 and endosome marker proteins, i.e. transferrin receptor or clathrin, has been documented [15,16,46–48].

Unlike insulin-stimulated GLUT4 translocation, the translocation of GLUT4 induced by bafilomycin A_1 treatment of cells proceeds independently of insulin receptor autophosphorylation (Figure 7). Furthermore, we found that plasma membrane insulin receptor levels were unaltered in bafilomycin A_1 -treated 3T3-L1 adipocytes (Figure 8), suggesting that accelerated insulin receptor internalization could not account for the observed insulin-like effect of the agent. Also in contrast with the effect of insulin, the effect of bafilomycin A_1 in inducing GLUT4 translocation seems to operate via a pathway independent or downstream of PI 3-kinase and its lipid products, as the fungal PI 3-kinase inhibitor wortmannin did not arrest bafilomycin A_1 -induced GLUT4 translocation (Figure 9). Together these results indicate that the mechanism whereby bafilomycin A_1 triggers GLUT4 translocation is distal to insulin receptor or PI 3-kinase activation, and are consistent with the notion that endomembrane alkalinization causes the observed GLUT4 membrane dynamics. These data further imply that low endosomal pH may play a role in the recognition mechanisms that retain GLUT4 intracellularly and/or induce its externalization.

Is it possible that the insulin-induced signalling pathway initiated with the insulin receptor and culminating with GLUT4 relocation on the fat/muscle-cell surface includes inhibition of the vacuolar proton pump as one of the final events of the cascade? The lack of additive effects of insulin and bafilomycin A_1 on GLUT4 translocation (Figure 1) is suggestive of a common mechanism of action of these two agents. Notably, studies indicating that V-ATPase is subject to intracellular regulation by signalling molecules thought to be important in insulin-regulated glucose transport and GLUT4 translocation to the plasma membrane, such as protein kinase C [19,49,50], add further support to this hypothesis. In fact, the ability of insulin to increase the intracellular pH has long been known [51], although no direct data are yet available regarding the action of insulin on V-ATPase or on the pH of GLUT4-containing vesicles. The results in the present study indicate that such a mechanism is possible, the elucidation of which will require rigorous experimentation. In any case, the present data demonstrate a bafilomycin A_1 -induced change in GLUT4 membrane dynamics, and indicate that endomembrane acidification may play a role in the intracellular mechanisms that are responsible for sorting GLUT4.

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