

Acute Effectors of GLUT1 Glucose Transporter Subcellular Targeting in CIT₃ Mouse Mammary Epithelial Cells

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ABSTRACT: Lactogenic hormones cause intracellular targeting of glucose transporter 1 (GLUT1) for transport of glucose to the site of lactose synthesis in mammary glands. Our aim was to study the intracellular trafficking mechanisms involved in GLUT1 targeting and recycling in CIT₃ mouse mammary epithelial cells. Fusion proteins of GLUT1 and enhanced green fluorescent protein (EGFP) were expressed in CIT₃ cells maintained in growth medium (GM), or exposed to secretion medium (SM), containing prolactin. Agents acting on Golgi and related subcellular compartments and on GLUT1 and GLUT4 targeting in muscle and fat cells were studied. Wortmannin and staurosporine effects on internalization of GLUT1 were not specific, supporting a basal constitutive GLUT1 membrane-recycling pathway between an intracellular pool and the cell surface in CIT₃ cells, which targets most GLUT1 to the plasma membrane in GM. Upon exposure to prolactin in SM, GLUT1 was specifically targeted intracellularly to a brefeldin A-sensitive compartment. Arrest of endosomal acidification by bafilomycin A1 disrupted this prolactin-induced GLUT1 intracellular trafficking with central coalescence of GLUT1-EGFP signal, suggesting that it is *via* endosomal pathways. This machinery offers another level of regulation of lactose synthesis by altering GLUT1 targeting within minutes to hours. (*Pediatr Res* 63: 56–61, 2008)

The mammary gland is unique in its requirement for transport of free glucose into the cell to provide substrate for lactose synthesis (1). Glucose transporter 1 (GLUT1) is the main established isoform of glucose transporters expressed in mammary epithelial cells (MEC) (2–6). Hormonally regulated subcellular targeting of GLUT1 from the plasma membrane into the cell may have an important role for lactose synthesis in MEC during lactation (5,7,8).

This work is based on previous findings on GLUT1 targeting in MEC under hormonal stimulation, mimicking lactation (5,7). These findings were reconfirmed and expanded in our previous work on the model of GLUT1 targeting and recy-

cling in living MEC in culture that was used in this study (data not yet published). For this system, we constructed fusion proteins of GLUT1 and green fluorescent protein (GFP), and expressed them in CIT₃ mouse MEC in culture. CIT₃ cells are a nonneoplastic MEC line that is derived from Comma-1-D cells and exhibit polarized transport (9). Cells were maintained in growth medium (GM), or exposed to secretion medium (SM). Lactogenic hormones, namely prolactin, in SM changed subcellular targeting of GLUT1-GFP fusion proteins to an intracellular, mostly perinuclear, punctate pattern, as seen with native GLUT1. Our previous studies showed that GLUT1 targeting under this hormonal stimulation was a dynamic process. We demonstrated a basal constitutive GLUT1 recycling pathway between an intracellular pool and the cell surface, which targets most of the GLUT1 to the plasma membrane in maintenance GM, and specifically targets GLUT1 intracellularly upon exposure to SM containing prolactin.

The aim of this work was to further study the intracellular compartments involved in GLUT1 targeting, and to define the intracellular trafficking mechanisms involved in the basal recycling and in the intracellular targeting of GLUT1 in MEC. For this we used known agents acting on Golgi and related subcellular compartments and on GLUT1 and GLUT4 targeting in muscle and fat cells.

METHODS

Subcloning GLUT1 cDNA into GFP plasmid vectors. pEGFP-C1 and pEGFP-N1 GFP plasmid vectors (#6084-1 and #6085-1, respectively, enhanced GFP (EGFP) plasmid vectors, Clontech Laboratories Inc., Palo Alto, CA) were used. EGFP carries a red-shifted variant of wild-type GFP that contains two amino acid substitutions that has been optimized for brighter green fluorescence and higher expression in mammalian cells (10,11). GLUT1 cDNA (12) was recovered from pHepG2 using Bam H1 restriction digest or PCR. GLUT1 cDNA was subcloned into pEGFP-C1 and pEGFP-N1, respectively, to create N- and C-terminus fusion of GLUT1 to GFP. All recombinant vectors were sequenced to verify the correct orientation and exclude mutations.

Cell culture and medium. CIT₃ cells, kindly provided by M.C. Neville, Ph.D., are a nonneoplastic cell line derived from mouse MEC (after being selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions, and exhibit polarized transport) (9). Cells were maintained in GM, which is a nutrient-defined basal medium (DMEM/F12) (Invitrogen, Carlsbad, CA), containing 10 µg/mL insulin and 5 ng/mL EGF. To stimulate differentiation by lactogenic hormones, the media was changed to SM, by

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Abbreviations: EGFP, enhanced GFP (green fluorescent protein); GLUT1, glucose transporter 1; GM, growth medium; MEC, mammary epithelial cells; SM, secretion medium

adding prolactin 3 $\mu\text{g}/\text{mL}$ and hydrocortisone 3 $\mu\text{g}/\text{mL}$, and withdrawing EGF. Routine exposure to SM was 96 h before evaluating changes in GLUT1 subcellular targeting.

Transfection. Transient transfections were used to introduce the recombinant vectors carrying GLUT1-EGFP fusion proteins into the cells. Liposome-mediated transfection using LipoFectAmine Plus Reagent (#10964013, Invitrogen) was performed in 35-mm dishes, containing 5×10^5 cells per plate (60–80% confluent), according to manufacturer's instructions. Transient transfections were checked for fluorescent signal at 48–72 h, when maximal expression of the fluorescent signal was noted in 20–30% of the cells, based on previous experiments (not shown). Since in our previous experiments the behavior and intracellular distribution of the N- and C-fusion chimeras of GLUT1 to EGFP (EGFP-GLUT1 and GLUT1-EGFP, respectively) were consistently the same, further studies were carried out only with GLUT1-EGFP.

Fluorescent microscopy. Fluorescent signal was detected using OLYMPUS iX-70 epifluorescent microscope. Images were captured by an uncooled CCD camera (Optronics, DEI-750 CE Digital Output Model S60675). Exposure was adjusted in a linear manner and separate color channels were merged as indicated using Adobe Photoshop 5.0 software. For the study of changes taking place over time and under different conditions, cells were grown on round coverslips, and maintained in a 37°C chamber. Images were acquired sequentially to avoid crossover. Time-lapse images were captured by Snappy software and combined into a sequence using Macromedia Flash 4.0 software. Each condition was studied at least three times and results were consistent. Representative results are shown.

Inhibitors. Cells kept in GM or exposed to SM for 96 h were exposed to brefeldin A (5 μM), bafilomycin A1 (800 nM), wortmannin (1 μM), or staurosporine (2 μM). These optimal concentrations were determined based on the range of concentrations cited in other studies (referred to below) and pretesting different concentrations within that range (data not shown). Time-lapse images of the changes taking place in GLUT1-EGFP fluorescent signal subcellular targeting were recorded.

Brefeldin A is a macrocyclic lactone antiviral antibiotic synthesized from palmitate by fungi. It has the ability to change the morphology of some intracellular organelles of the central vacuolar system in eukaryotic cells, representing their steady-state structure, which is affected by the extent of membrane input and outflow. Brefeldin A causes rapid and reversible disassembly of the Golgi apparatus and its mixing with the endoplasmic reticulum (13,14).

Bafilomycin A1 is an antibiotic that originates from *Streptomyces griseus*. It is a specific inhibitor of vacuolar proton pump type H⁺-adenosine triphosphatase (V-ATPase) in animal cells (15) that causes arrest of endosomal acidification. It also inhibits the acidification of lysosomes and thus the degradation of proteins in them (16). Bafilomycin A1 not only slows bulk membrane flow, but causes additional inhibition of receptor recycling that is dependent on a peptide internalization motif on the cytoplasmic domain (17).

Wortmannin is an antifungal antibiotic that originates from *Penicillium fusiculosum*. It is a highly cell permeable specific inhibitor of phosphatidylinositol 3-kinase (PI3 K) that is necessary for insulin-stimulated glucose transport in myoblasts and adipocytes. In its presence GLUT1 and GLUT4 insulin-stimulated translocation to plasma membrane is inhibited (18–22).

Wortmannin also blocks the insulin independent GLUT1 constitutive basal trafficking pathway in muscle cells that involves PI3 K (21,23).

Staurosporine is an antibiotic that originates from *Streptomyces* species. It is a potent inhibitor of protein kinase C (PKC). Staurosporine decreases insulin-induced glucose uptake in fat and muscle cells (24) by inhibiting GLUT1 and GLUT4 trafficking to plasma membrane that is mediated via PKC-dependent pathways.

This study was approved by the Institutional Review Board as part of the research in Dr. Haney's laboratory at the ARS/USDA Children's Nutrition Research Center, Baylor College of Medicine. Only cell lines were studied.

RESULTS

Brefeldin A. CIT₃ transfected with GLUT1-EGFP and kept in SM for 96 h showed rapid diffusion of GLUT1-EGFP signal after treatment with 5 μM brefeldin A. The effect was fully seen after approximately 1.5–2.0 min. Upon withdrawal of brefeldin A, the process seemed to be fully reversible with reassembly of the green fluorescent signal of the GLUT1-EGFP perinuclear vesicles within 1.0–1.5 min (Fig. 1). As expected, brefeldin A had no significant visible effect on GLUT1-EGFP signal in GM (not shown).

Bafilomycin A1. CIT₃ transfected with GLUT1-EGFP and kept in SM for 96 h showed central coalescence of GLUT1-EGFP signal with the loss of peripheral vesicles after treatment with 800 nM bafilomycin A1 (Fig. 2). The effect was seen after approximately 1 h and was not reversible upon withdrawal of bafilomycin A1. Bafilomycin A1 had no visible effect on GLUT1-EGFP signal in GM (data not shown).

Wortmannin. In GM 1 μM wortmannin caused internalization of GLUT1-EGFP plasma membrane signal (Fig. 3). The effect was seen after approximately 1–2 h. No reversibility of the effect could be demonstrated upon withdrawal of the wortmannin. The same effect was seen in SM after approximately 1 h with no reversibility upon withdrawal of the wortmannin (Fig. 4).

Staurosporine. In GM 2 μM staurosporine caused internalization of GLUT1-EGFP plasma membrane signal (Fig. 5). In cells exposed to SM for 96 h, staurosporine caused central coalescence of GLUT1-EGFP signal and loss of peripheral vesicles signal (Fig. 6).

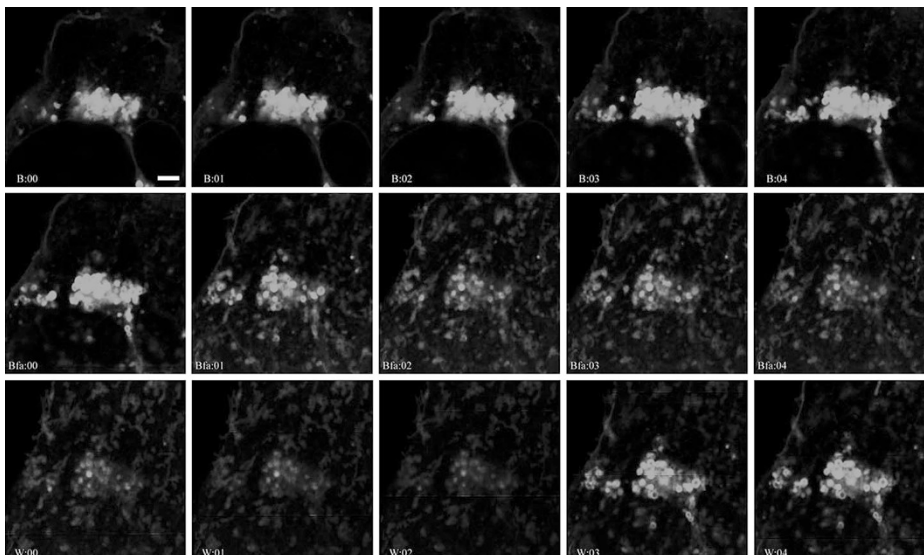


Figure 1. Brefeldin A causes reversible diffusion of the fluorescent signal of GLUT1-EGFP in SM. Numbers denotes frames taken every 30 s. B and Bfa marks the frames taken before and after the addition of brefeldin A. W denotes frames after withdrawal of brefeldin A. The figure plates are those were most changes occur, *i.e.* within the first 2 min after addition and withdrawal of brefeldin A. All images are high power images at 100 \times magnification. Bar = 1 μm .

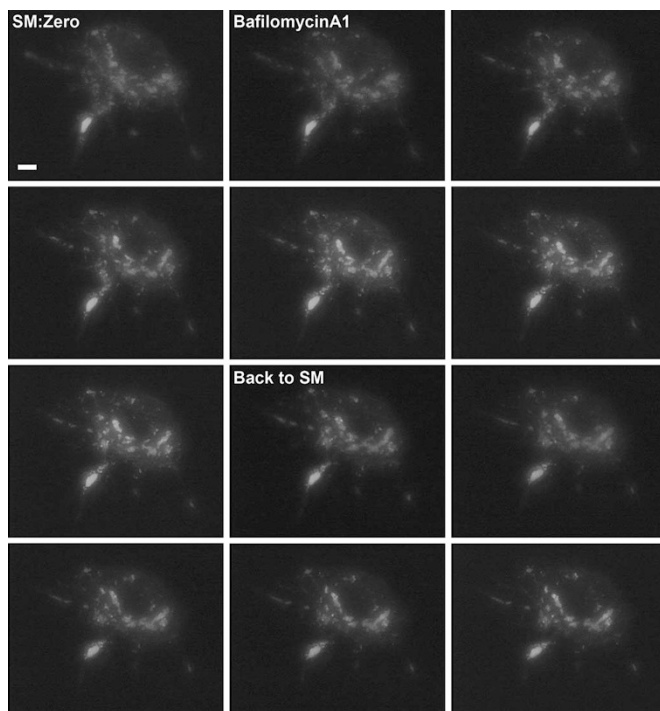


Figure 2. Bafilomycin A1 causes central coalescence of GLUT1-EGFP with loss of peripheral vesicles in SM. Time-lapse frames were captured every 5 min. The figure plates are from frames 20 min apart. All images captured with 1/15-s exposure time and are at 40 \times magnification. Bar = 5 μ m.

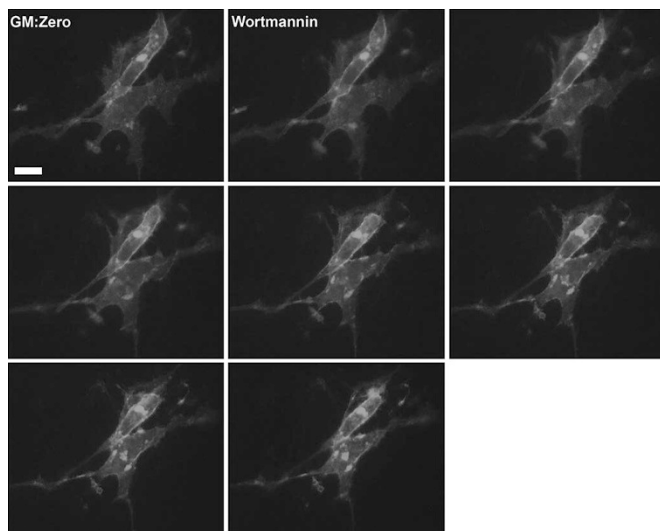


Figure 3. Wortmannin causes internalization of GLUT1 signal in GM. Time-lapse frames were captured every 5 min. The figure plates are from frames 20 min apart. All images captured with 1/2-sec exposure time and are at 60 \times magnification. Bar = 5 μ m.

Figures 1–6 are also available as QuickTime movies at <http://www.technion.ac.il/yehudit/Arik-0707/>.

DISCUSSION

We constructed fusion proteins of GLUT1 and GFP, and expressed them in CIT₃ mouse MEC to study GLUT1 targeting and recycling in living MEC in culture. In our previous studies (not shown here), we demonstrated a basal constitutive GLUT1 recycling pathway between an intracellular pool and

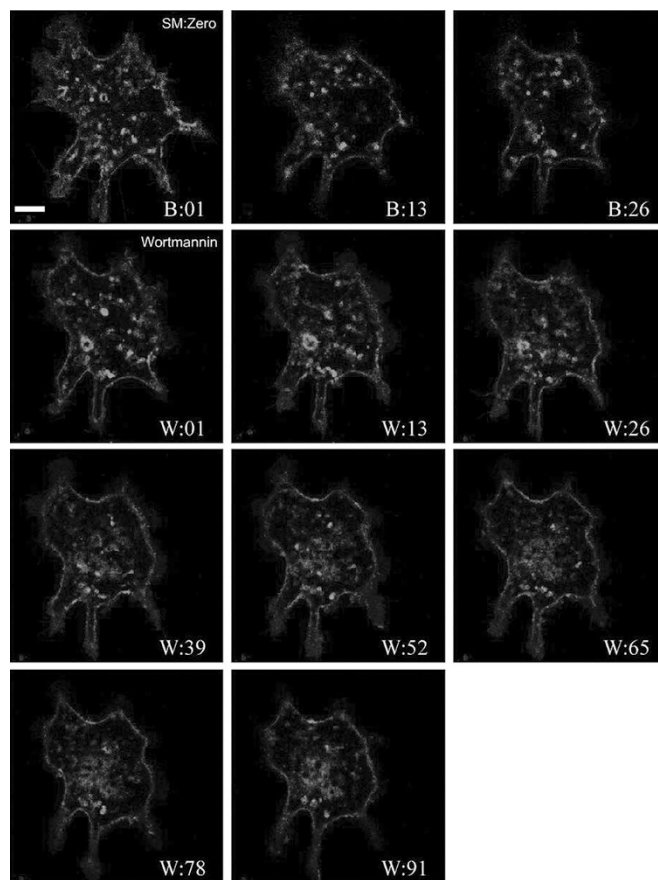


Figure 4. Wortmannin causes internalization of GLUT1 signal in SM. Time-lapse frames were captured every 1 min. The numbers denotes the time in minutes. *B* denotes before wortmannin was added to the medium. *W* marks the frames taken after the addition of wortmannin. All the images are high power images at 60 \times magnification. Bar = 5 μ m.

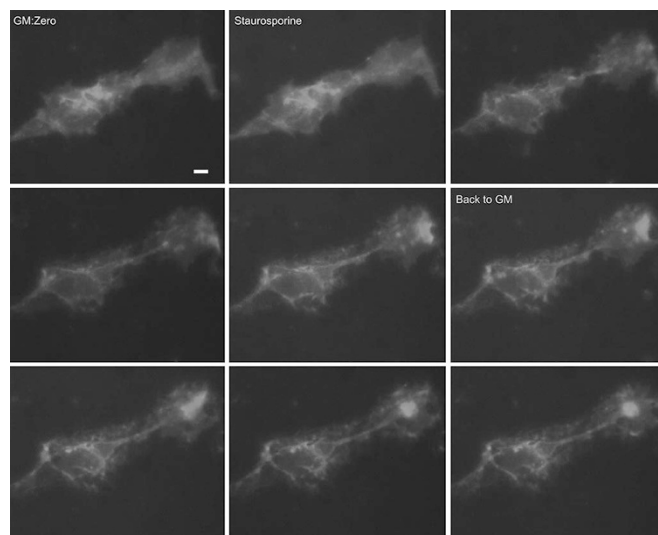


Figure 5. Staurosporine causes internalization of GLUT1-EGFP in GM. Time-lapse frames were captured every 2 min. The figure plates are from frames 22 min apart. All images captured with 2-s exposure time and are at 30 \times magnification. Bar = 5 μ m.

the cell surface, which targeted most GLUT1 to the plasma membrane in maintenance GM. Upon exposure to SM-containing prolactin, GLUT1 was specifically targeted intra-

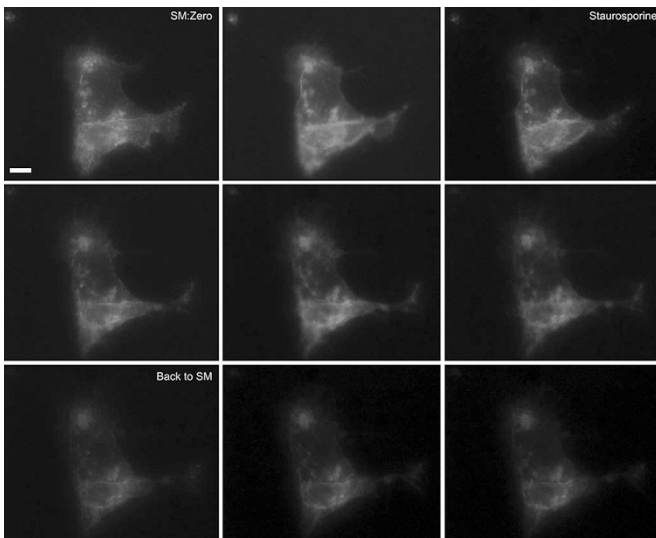


Figure 6. Staurosporine causes central coalescence of GLUT1-EGFP vesicles in SM. Time-lapse frames were captured every 2 min. The figure plates are from frames 18 min apart. All images captured with 1/2-sec exposure time and are at 60 \times magnification. Bar = 5 μ m.

cellularly. These changes occurred within hours and were in accordance with previous studies (5,7). GLUT1 intracellular targeting under the influence of lactogenic hormones was further characterized to brefeldin A sensitive vesicles that may be a subcompartment, derived from the cis-Golgi (7). The vesicular rather than static nature of this compartment well suits our current findings of dynamic transport system, which may be related to the cell's central vacuolar membrane traffic system (14).

The suggestion that GLUT1 does not solely act at the plasma membrane, but may also function in an intracellular organelle, conceptually complements the well-known insulin-regulated targeting of GLUT4 (25), and to a lesser extent of GLUT1, to their site of action, the plasma membrane, in fat and muscle cells. Sharing the same mechanisms of dynamic regulation by subcellular targeting raises the question, whether GLUT1 intracellular trafficking in MEC shares some of the endocytic and exocytic pathways involved in GLUT4 targeting in fat and muscle cells. Thus, we studied agents affecting the central vacuolar membrane trafficking system and the Golgi complex, and agents known to affect GLUT1 and GLUT4 targeting in muscle and fat cells.

It must be stressed that our findings are limited to mouse MEC, and more specifically to the CIT₃ cell line that we studied, and cannot be currently generalized or implied to humans or other mammals. The suggestion that glucose transporters other than GLUT1 may be more significantly involved in glucose regulation in MEC of other mammals during lactation (6,26) needs to be addressed.

Another limitation of this study is the issue of mammary cell-to-cell phenotypic variability in culture. Cell phenotype could vary in many ways that may influence GLUT1 targeting kinetics. This is an inherent limitation of a microscopic descriptive study. To decrease any possible selection bias we chose representative cells on a low power field before study-

ing them in high power. Although we repeated each experiment to verify reproducibility of our results, such selection bias cannot be fully excluded.

As a descriptive morphologic study, we did not deal with cells' ability to synthesize and secrete lactose in culture, which is not solely dependent on lactogenic hormones and may be influenced by other factors, such as the intracellular matrix. However, if these cells had expressed lactose, the issue of cell-to-cell phenotypic variation would have been minimized.

This work forms a continuum with previous *in vivo* (5) and *in vitro* (7) studies, thus further supporting our results within their limited scope. Our methodological approach can be applied to other primary MEC to generalize the conclusions.

In MEC taken from lactating rabbit, brefeldin A caused dissociation of trans- but not medial-Golgi marker enzymes (27). Previously, we demonstrated in fixed CIT₃ cells that under the influence of prolactin and hydrocortisone GLUT1 is sequestered within the cell, and is diverted from normal sorting pathways to a brefeldin A-sensitive compartment (7). Morphologically, brefeldin A caused loss of the perinuclear compartment of GLUT1, which may represent Golgi-derived vesicles (7). In living CIT₃ cells transfected with GLUT1-EGFP and kept in SM, brefeldin A caused disruption of the Golgi stacks with diffusion of the fluorescent signal of GLUT1, merging with, but not staining, the endoplasmic reticulum. Upon withdrawal of brefeldin A, the process seemed fully reversible with reassembly of the perinuclear fluorescent signal of GLUT1-EGFP (Fig. 1). The full effect was seen within 2–3 min [not 30 min as previously described (27)], and was reversible within 1 min upon withdrawal of the brefeldin A.

In fat and muscle cells, GLUT4, and to a lesser extent GLUT1, is constitutively sequestered in the endosomal tubulovesicular system, and moves to the cell surface in response to insulin. In muscle cells arrest of endosomal acidification by bafilomycin A1 results in rapid dose-dependent translocation of GLUT4 from the cell interior to the plasma membrane surface, mimicking insulin effect. This insulin-like effect of bafilomycin A1 causes redistribution of GLUT1 and Rab4, a regulatory component of the secretory and endocytic system, as well (28). The mechanism by which arrest of endosomal acidification by bafilomycin A1 causes translocation of GLUT4 and GLUT1 is distal to the insulin receptor and phosphatidylinositol 3-kinase (PI3 K) activation. Endosomal pH is important in membrane dynamics and in the hormonally regulated intracellular sorting machinery of glucose transporters. In CIT₃ cells kept in SM bafilomycin A1 caused central coalescence of GLUT1-EGFP and the loss of peripheral vesicles (Fig. 2). The effect was not reversible upon withdrawal of bafilomycin A1. We conclude that in living mouse MEC prolactin causes intracellular targeting of GLUT1 by altering rates of GLUT1 exocytosis and endocytosis. Arrest of endosomal acidification by bafilomycin A1 disrupts this recycling process *via* endosomal compartments. The importance of endosomal pH to the prolactin-induced hormonally regulated sorting of GLUT1 in MEC, thus shares characteristics with GLUT4 and GLUT1 insulin-dependent intracellular redistribution in adipocytes.

Wortmannin is considered a specific inhibitor of PI3 K that is necessary for insulin-stimulated glucose transport in myoblasts and adipocytes. In the presence of wortmannin GLUT1 and GLUT4 insulin-stimulated translocation to plasma membrane was inhibited (18–22). Wortmannin also blocks a constitutive basal GLUT1 trafficking pathway in muscle cells that involves PI3 K but is independent of insulin, resulting in sequestration of GLUT1 in a perinuclear compartment (21,23). This seems to be a ubiquitous pathway used by other cell types for basal glucose uptake by GLUT1, as shown in fibroblasts (29,30). GLUT1 protein appears to recycle between an intracellular site and the plasma membrane, and PI3 K seems to have an important functional role in this recycling by regulating membrane protein traffic. By inhibition of PI3 K, wortmannin causes selective blockade of this protein recycling with accumulation of glucose transporters in intracellular location in fibroblasts (29). In CIT₃ mouse, MEC wortmannin caused internalization of GLUT1-EGFP plasma membrane signal in GM (Fig. 3). We were not able to demonstrate reversibility of the effect upon withdrawal of the wortmannin. Similar effects of internalization and central coalescence were seen in SM (Fig. 4). It seems that the effects of wortmannin on GLUT1 in mouse MEC are independent of prolactin, and are related to blockade of the constitutive basal GLUT1 trafficking pathway. These findings further support recycling of GLUT1 by exocytosis and endocytosis. Study of the effects of wortmannin on the endosomal system and GLUT4 recycling compartments in adipocytes (31) supports the role of endosomal derived vacuoles and endosomal recycling compartments, sensitive to wortmannin, for this process.

Staurosporine is a potent inhibitor of protein kinase C (PKC). In fat and muscle cells, insulin increases glucose uptake *via* a PKC-dependent pathway. Staurosporine inhibits GLUT1 and GLUT4 trafficking to plasma membrane in adipocytes (24). PKC is involved in insulin signaling in other cell types as well, *i.e.* fibroblasts (32), resulting in GLUT1 recruitment to plasma membrane. But PKC is also involved in translocation of GLUT3 to plasma membrane in activated platelets in response to thrombin (33), suggesting a common PKC activation-mediated signaling pathway for recruitment of glucose transporters to the cell surface to increase glucose uptake (34). In CIT₃ mouse MEC in GM staurosporine caused internalization of GLUT1-EGFP plasma membrane signal (Fig. 5). In SM, it caused central coalescence of GLUT1-EGFP and loss of peripheral vesicles (Fig. 6). These findings support the role of PKC in the sorting machinery controlling GLUT1 recycling in MEC, as in many other cells. However, since staurosporine had similar effects in GM and SM, we cannot establish a role for PKC in the prolactin-regulated GLUT1 intracellular trafficking and targeting.

It should be noted that both our GM and SM contained insulin, thus limiting our conclusions regarding possible mechanisms involved in the effects of wortmannin and staurosporine on GLUT1 intracellular targeting.

Further studies, aimed directly at studying the roles of PI3 K and PKC in GLUT1 intracellular trafficking in MEC, possibly using medium deprived of insulin, are needed. Further works will also have to define the underlying mechanisms that

allow relatively rapid changes in GLUT1 subcellular targeting in response to prolactin. Possibly, this is related to phosphorylation or dephosphorylation reaction. Alternatively, glycosylation may play a significant role (7). The marked effects of bafilomycin A1 seen with the arrest of endosomal acidification merits further study regarding the role of subpopulations of endosomes and endosomal recycling compartments in GLUT1 basal recycling (31) and intracellular targeting in SM. Colocalization studies using Rab proteins (*e.g.* Rab4, Rab5, and Rab11) involved in the regulation of transport through distinct domains on the endosomes have shown that endosomes are organized in compartments within the same continuous membrane, which cooperatively generate a recycling continuum (35).

In summary, we demonstrated a basal constitutive GLUT1 membrane-recycling pathway between an intracellular pool and cell surface in CIT₃ mouse MEC, which targets most of GLUT1 to the plasma membrane in GM, and is common to other cell types (29). It is responsible for maintaining basal glucose uptake and may be regulated by PI3 K and PKC, thus blocked by wortmannin and staurosporine. However, in MEC there is also hormonally regulated cell type-specific, developmental stage-specific sorting machinery for GLUT1 intracellular targeting in lactation. Upon exposure to prolactin, GLUT1 is specifically targeted intracellularly to a brefeldin A-sensitive compartment (7), most likely *via* endosomal pathways, thus disrupted by bafilomycin A1. This quick mechanism that supplies free glucose intracellularly for lactose synthesis in the Golgi offers another level of regulation of lactose synthesis by altering GLUT1 targeting within minutes to hours, as demonstrated *in vivo* (5). The rapid responsiveness of GLUT1 targeting suggests that this machinery does not require new protein synthesis, and may support glucose transport as a rate-limiting step for lactose synthesis during lactation.

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