Hyperosmolarity Reduces GLUT4 Endocytosis and Increases Its Exocytosis from a VAMP2-independent Pool in L6 Muscle Cells*

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Dailin Li‡§, Varinder K. Randhawa‡¶, Nish Patel‡, Michiko Hayashi‡, and Amira Klip‡¶

From the §Programme in Cell Biology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8 and the ¶Department of Biochemistry, University of Toronto, Toronto, Ontario M5G 1A8, Canada

The intracellular traffic of the glucose transporter 4 (GLUT4) in muscle cells remains largely unexplored. Here we make use of L6 myoblasts stably expressing GLUT4 with an exofacially directed Myc-tag (GLUT4myc) to determine the exocytic and endocytic rates of the transporter. Insulin caused a rapid (~3 min) gain, whereas hyperosmolarity (~0.5 M sucrose) caused a slow (~5 min) gain in surface GLUT4myc molecules. With prior insulin stimulation followed by addition of hypertonic sucrose, the increase in surface GLUT4myc was partly additive. Unlike the effect of insulin, the GLUT4myc gain caused by hyperosmolarity was insensitive to wortmannin or to tetanus toxin cleavage of VAMP2 and VAMP3. Disappearance of GLUT4myc from the cell surface was rapid (~2.5 min). Insulin had no effect on the initial rate of GLUT4myc internalization. In contrast, hyperosmolarity almost completely abolished GLUT4myc internalization. Surface GLUT4myc accumulation in response to hyperosmolarity was only partially blocked by inhibition of tyrosine kinases with erbstatin analog (erbstatin A) and genistein. However, neither inhibitor interfered with the ability of hyperosmolarity to block GLUT4myc internalization. We propose that hyperosmolarity increases surface GLUT4myc by preventing GLUT4 endocytosis and stimulating its exocytosis via a pathway independent of phosphatidylinositol 3-kinase activity and of VAMP2 or VAMP3. A tetanus toxin-insensitive v-SNARE such as TI-VAMP detected in these cells, might mediate membrane fusion of the hyperosmosmolarity-sensitive pool.

The glucose transporter 4 (GLUT4) is the predominant glucose transporter of muscle and adipose cells. In untreated adipocytes, GLUT4 recycles constitutively between the plasma membrane and intracellular loci (1, 2), with the steady-state distribution favoring the latter. Morphological and biochemical studies have detected GLUT4 in distinct but inter-related intracellular pools, including sorting endosomes, TGN, recycling endosomes, and specialized GLUT4 exocytic vesicles (3–6). GLUT4 endocytosis occurs via clathrin-coated vesicles, assisted by the GTPase dynamin. Thus, inhibition of clathrin-coated vesicle formation via K+ depletion (7), interference with dynamin-ampiphysin pairing (8), or expression of GTPase-deficient dynamin (9, 10), all prevent GLUT4 internalization in adipocytes. Little is known about the traffic of this transporter in muscle cells, despite the fact that muscle represents the largest in vivo site of glucose utilization.

Insulin shifts the subcellular distribution of GLUT4 resulting in a new steady state where a large fraction of GLUT4 resides at the plasma membrane of skeletal muscle (11–13), primary adipose cells (14, 15), L6 muscle cells in culture (16), and 3T3-L1 adipocytes (1). Studies in adipocytes indicate that this shift occurs primarily through the stimulation of GLUT4 exocytosis (1, 2), but whether or not insulin inhibits GLUT4 endocytosis is still debatable (1, 2, 17, 18). The contribution of exocytic and endocytic pathways to insulin action in muscle cells has not been explored.

Exposing 3T3-L1 adipocytes to hyperosmolar solutions also leads to the cell surface accumulation of GLUT4. It was postulated that this accumulation results from the stimulation of GLUT4 exocytosis by signals different from those elicited by insulin (19–21) through a so-called “alternative pathway.” However, the steady-state analysis used in those studies precluded the distinction of exocytic from endocytic traffic of GLUT4. The well-known effect of hyperosmolarity to disrupt clathrin function (22, 23) has prompted us to hypothesize that hyperosmolarity accumulates GLUT4 at the cell surface by inhibiting GLUT4 endocytosis. We have previously characterized a clone of L6 myoblasts stably expressing GLUT4 tagged with an exofacial myc epitope (GLUT4myc) (24), which could be used to more accurately measure GLUT4 endocytosis in response to different stimuli.

The objective of this study was to investigate how insulin and hyperosmolarity affect the recycling of GLUT4 in L6 muscle cells in culture, to examine how each stimulus modulates the endocytic and exocytic arms of GLUT4 traffic, and to compare the characteristics of the donor pools of GLUT4 in each instance. We show that both insulin and hyperosmolarity induce the subcellular redistribution of GLUT4 from intracellular loci to the plasma membrane in L6 myoblasts. Whereas insulin primarily stimulates GLUT4 exocytosis, hyperosmolarity largely prevents its endocytosis. Although inhibition of tyrosine kinases prevents significantly insulin-stimulated GLUT4 exocytosis, it does not affect the hyperosmosmolarity-induced block of GLUT4 internalization and only partially blocks hyperosmo-
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Lality-triggered GLUT4 accumulation at the cell surface. The results suggest that insulin draws GLUT4 from a specific pool that is affected by tetanus toxin and Wortmannin. In contrast, the accumulation of GLUT4 at the cell surface caused by hyperosmolality results from both reducing GLUT4 endocytosis and stimulating its exocytosis. Hyperosmolality is likely to draw GLUT4 from the recycling endosomal pool and/or an “alternative pool” that is insensitive to inhibition by tetanus toxin and Wortmannin.

Experimental Procedures

Materials—Human insulin (Humulin R) was purchased from Eli Lilly Canada Inc. (Toronto); o-phenylenediamine dihydrochloride (OPD) reagent was from Sigma-Aldrich (Oakville, Ontario). Mouse monoclonal anti-Myc antibody (9E10) in ascites fluid was a kind gift from Carmen de Hoog and Dr. Mike Moran (University of Toronto). Rabbit polyclonal anti-tetanus toxin-insensitive VAMP (TI-VAMP) antibody (49) was a generous gift from Dr. Thierry Galli (Institut Curie, Paris, France). Horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG and indocarbocyanine (Cy3)-conjugated goat anti-mouse IgG were obtained from Jackson ImmunoResearch (West Grove, PA). The mammalian expression vector for enhanced green fluorescence protein, pEGFP-N1, was purchased from Clontech (Palo Alto, CA); pcDNA3 was from Invitrogen (Carlsbad, CA). The light chain of tetanus toxin (TeTx) cDNA in pcDNA3 was obtained from Dr. Reiner Niemann (Medizinische Hochschule, Hanover, Germany), and the cDNAs for GFP-tagged VAMP2 and VAMP3 from Dr. William Trimble (University of Toronto). Maxi-prep ttip DNA purification columns and Effectene transfection kits were from Qiagen (Mississauga, Ontario). All DNA constructs used in transfections were prepared using Qiagen maxiprep columns according to the manufacturer’s recommendations.

Cell Culture—GLUT4myc cDNA was constructed by inserting the human c-Myc epitope (AEEQKLISEEDLLK, 14 amino acids recognized by the monoclonal antibody 9E10) into the first ectodomain of GLUT4 and subcloned into the pcX2 vector expression (25). A clone of L6 skeletal muscle cells transfected with GLUT4myc cDNA was selected in 12-well tissue culture plates. The following day, cell plates were rinsed 3 times with ice-cold K+-free or K+-plus Hepes buffer and incubated with the same buffer at 37 °C for 30 min. In the sucrose removal group, 0.45 m sucrose was present for the first hour of re-warming for endocytosis and removed for the last 30 min, and K+ (5 m) was always added.

Transfection of L6-GLUT4myc Cells—L6-GLUT4myc myoblasts were seeded at a density of 1 × 10⁴ cells/well onto 18-mm diameter glass coverslips in 12-well tissue culture plates. The following day, transfection was performed according to the Effective product manual, using 3 μl of the Effectene reagent per transfection condition (as indicated in the figure legends). After introducing DNA to cells for 5 h, the cultures were washed twice with PBS and maintained in culture medium for another 43 h until experimentation. These cells were deprived of serum in culture medium for 3 h at 37 °C prior to processing for immunofluorescence.

Immunofluorescence—After serum deprivation, cells were left untreated or treated with 100 nM insulin or 0.45 m sucrose at 37 °C for 30 min. Indirect immunofluorescence for GLUT4myc translocation was carried out on intact cells as described (28). All steps were performed at 4 °C unless otherwise indicated. Cells on coverslips were rinsed twice with ice-cold PBS prior to fixation with 0.5% paraformaldehyde in PBS for 30 min, then washed four times with PBS and incubated in the dark with secondary antibody Cy3-conjugated goat anti-mouse IgG, 1:1000 in PBS containing 3% goat serum for 1 h. The cultures were washed twice with PBS and incubated in the dark with secondary antibody Cy3-conjugated goat anti-mouse IgG, 1:1000 in PBS containing 3% goat serum for 1 h. The coverslips were mounted with 50 m glycerol in PBS for 10 min. The cultures were incubated with anti-Myc antibody 9E10 in HPMI for 1 h, washed four times with PBS and incubated in the dark with secondary antibody Cy3-conjugated goat anti-mouse IgG, 1:1000 in PBS containing 3% goat serum for 1 h. All subsequent steps were performed at room temperature. The coverslips were washed six times with PBS, fixed with 4% formaldehyde in PBS for 30 min, initiated at 4 °C, but immediately shifted to room temperature, followed by quenching with 0.1 m glycine in PBS for 10 min and three PBS washes of 5 min each at room temperature. The coverslips were mounted with 10 m of Antifade solution. Mounted coverslips were stored at 4 °C prior to analysis with a Leica TCS 4D fluorescence microscope. To detect TI-VAMP, cells on coverslips were triple labeled with 0.1% Triton X-100 for 20 min and re-warmed to 37 °C in K+-free Hepes buffer containing 0.45 m sucrose and/or K+ (5 m) Hepes buffer for another hour. The cultures were rinsed twice with pre-warmed K+-free or K+-plus (5 m) Hepes buffer and incubated with the same buffer at 37 °C for 30 min. In the sucrose removal group, 0.45 m sucrose was present for the first hour of re-warming for endocytosis and removed for the last 30 min, and K+ (5 m) was always added.

RESULTS

The majority of GLUT4myc Is Located Intracellularly in Untreated L6 Myoblasts—The exofacial myc epitope of GLUT4myc allowed us to estimate the proportion of this protein exposed at the cell surface and its total cellular content by analyzing intact and permeabilized cells, respectively. The amount of myc epitope exposed at the surface of non-permeabilized cells was determined by a quantitative assay based on the...
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The other hand, accumulation of GLUT4myc to the cell surface by insulin triggered a rapid redistribution of GLUT4myc to the cell surface with a rapid redistribution of GLUT4myc to the cell surface with a half-life of 1 min. This rapid insulin response peaked at 15 min (Fig. 2). On the other hand, accumulation of GLUT4myc to the cell surface by hyperosmotic sucrose was a much slower process with a half-life of 20 min (Fig. 2). Hypertonic sucrose progressively augmented GLUT4myc presence at the cell surface, peaking after 1 h (Fig. 2).

Because phosphatidylinositol 3-kinase (PI3K) activation by by insulin is a prerequisite for the insulin-dependent translocation of GLUT4 to the cell surface in muscle and fat cells (29, 30), we investigated the involvement of PI3K in the hyperosmolarity-dependent GLUT4myc accumulation at the cell surface. In line with previous reports, the PI3K inhibitor wortmannin (100 nM) completely prevented the GLUT4myc gain at the cell surface produced by insulin. However, it did not inhibit the hypertonic sucrose-induced cell surface gain of GLUT4myc (Fig. 3). These results suggest that hyperosmolarity engages signaling pathways different from those involved in insulin action.

**Tetanus Toxin Does Not Block the Hyperosmolarity-depend-**

ent GLUT4myc Accumulation at the Cell Surface—SNARE proteins are required for membrane fusion in both neuronal and non-neuronal cells (31–33). In the final step of GLUT4 translocation, fusion of GLUT4-containing vesicles with the plasma membrane involves fusion complexes consisting of the t-SNARE proteins syntaxin 4 and SNAP23 on the target membrane, and v-SNAREs of the synaptobrevin family on the incoming vesicles (34–38). Tetanus toxin cleaves the synaptobrevin VAMP2 and VAMP3, rendering them inactive as fusogens (39). In L6-GLUT4myc myoblasts, insulin stimulates GLUT4myc translocation by 2-fold (24, 28, 40). We have recently reported that transient transfection of tetanus toxin light chain can inhibit the insulin-stimulated GLUT4myc translocation by 70% in L6-GLUT4myc myoblasts (40). In the present study, we compared the effects of tetanus toxin on...
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insulin- and hypertonic sucrose-induced cell surface accumulation of GLUT4myc. L6-GLUT4myc myoblasts were transiently transfected with cDNA encoding tetanus toxin light chain. Experiments were carried out 48 h after transfection. Following 30 min of treatment with insulin or hypertonic sucrose at 37 °C, cell surface GLUT4myc was labeled with anti-myc antibody at 4 °C and then detected by fluorescence microscopy on intact cells. Tetanus toxin markedly reduced the insulin-dependent gain in GLUT4myc at the cell surface (Fig. 4). Similarly, the level of surface GLUT4myc in unstimulated cells was not affected by tetanus toxin (Fig. 4): the basal-state levels of surface GLUT4myc were 0.98 ± 0.003 and 0.98 ± 0.005 in pcDNA3 and tetanus toxin-transfected cells, respectively, compared with a value of 1.00 assigned to basal, untransfected cells. These results suggest that insulin and hyperosmolarity draw GLUT4 from distinct intracellular pools that can be differentiated by their sensitivity to tetanus toxin. Moreover, the insulin-responsive pool appears to be static, because there is no contribution of tetanus toxin-sensitive GLUT4 to the basal state.

Because hyperosmolarity-induced GLUT4myc accumulation still occurs when VAMP2 and VAMP3 are completely cleaved by tetanus toxin (40), we examined whether tetanus toxin-insensitive VAMP (TI-VAMP) is expressed in L6 muscle cells. TI-VAMP was first described in epithelial cells and found to form apical SNARE complexes with syntaxin 3 and SNAP23, suggesting its involvement in apical exocytosis in epithelial cells (49). By indirect immunofluorescence, TI-VAMP was detected in L6 muscle myoblasts and found to be distributed in perinuclear and punctate cytoplasmic locations (Fig. 5). Interestingly, the majority of TI-VAMP was found outside of the region of VAMP2 or VAMP3 judged from the fluorescence of transfected VAMP2-GFP or VAMP3-GFP (Fig. 5). The possible role of TI-VAMP in mediating fusion of the basal state recycling GLUT4 compartment and/or the hyperosmosality-drawn compartment will have to be rigorously tested in the future.

Additive Effect of Insulin and Hyperosmolarity on Surface GLUT4myc—If insulin and hyperosmolarity draw GLUT4 from different intracellular pools, their effects should be additive. A 40-min stimulation by insulin increased the presence of GLUT4myc at the cell surface by 2.3 ± 0.1-fold, and 30 min of exposure to hyperosmolarity increased cell surface GLUT4myc by 2.1 ± 0.1-fold (Fig. 6). When insulin was given for the first 10 min, followed by the combined stimuli of insulin and sucrose for 30 min, cell surface GLUT4myc increased 3.2 ± 0.1-fold (Fig. 6). This higher level of GLUT4myc present at the cell surface is significantly different from the effect of either insulin or hyperosmolarity alone. In contrast, when hyperosmolarity preceded insulin addition, the maximum effect equaled that achieved by hyperosmosality alone (Fig. 6). This result is consistent with the notion that hyperosmolarity has the additional effect of causing insulin resistance (see “Discussion”).

Hyperosmolarity Inhibits GLUT4myc Retrieval from the Cell Surface—The cell surface GLUT4myc accumulation brought about by insulin or hyperosmolarity can result from either the stimulation of GLUT4myc exocytosis or the inhibition of its endocytosis. To record GLUT4myc internalization, cells were stimulated with insulin at 37 °C, reacted with anti-myc antibody at 4 °C to label cell surface GLUT4myc and then rewarmed to allow endocytosis in the absence or presence of insulin or hypertonic sucrose. At the indicated times, cells were chilled again and the myc antibody-labeled GLUT4myc remaining on the surface was detected by the densitometric assay. The amount of GLUT4myc remaining at the cell surface at defined time points was expressed as a percentage of cell surface GLUT4myc level at 0 min of endocytosis. GLUT4myc was found to be rapidly internalized following insulin removal with a t1/2 of ≈3 min (Fig. 7A). Hypertonic sucrose retained GLUT4myc at the cell surface for the entire time tested (2–60 min) (Fig. 7A). A similar observation was made when using 0.6 M mannitol instead of 0.45 M sucrose as hyperosmolar challenge (data not shown). In the continued presence of insulin, cell surface-labeled GLUT4myc attained a slightly higher value by 10 min after initiation of its internalization (Fig. 7A). To identify whether this difference resulted from insulin-induced
Experimental Procedures. Shown are cell images from one typical experiment.

Expression in permeabilized cells was detected with anti-TI-VAMP antibody coupled to the indirect immunofluorescence assay as described under "Experimental Procedures." Shown are cell images from one typical experiment. Bar, 5 μm.

GLUT4 re-exocytosis or a delayed inhibition of its endocytosis rate, we measured the extent of re-exocytosis of the myc antibody-labeled GLUT4myc. After labeling the insulin-recruited GLUT4myc transporters at the plasma membrane, GLUT4myc internalization was allowed to proceed at 37 °C for 20 min in the absence of the hormone, so that ~80% of the myc antibody-labeled GLUT4myc was internalized. Insulin was then reintroduced for 10 or 20 min. Under these conditions, the amount of surface GLUT4myc was reduced by ~35–40% of the level attained by internalization for 30 or 40 min in the absence of insulin (Fig. 7A, inset) and this increase was equivalent to the difference between the endocytic curves measured in the continuous presence and absence of insulin (Fig. 7A). Therefore, anti-myc-labeled GLUT4myc is recycled back to the cell surface in the presence of insulin.

We then compared the rates of GLUT4myc internalization in basal and insulin-stimulated cells. Surface GLUT4myc was labeled with anti-myc antibody in unstimulated cells. GLUT4myc was found to be internalized rapidly in the basal state with a half-time of ~1.5 min (Fig. 7B). The presence of insulin caused a minor delay in the rate of GLUT4 internalization at 10–60 min after the initiation of internalization (Fig. 7B) in a similar pattern as shown in Fig. 7A. However, the initial rates of internalization for the first 5 min, when ~50% to 60% of GLUT4myc disappeared from the cell surface, were identical under all conditions, i.e. in the basal state, in the presence of insulin during re-warming, and in insulin-prestimulated cells (Fig. 7C).

K+ Depletion Causes a Gain in GLUT4myc at the Cell Surface and Prevents GLUT4myc Internalization—Disassembly of clathrin lattices by K+ depletion results in an inhibition of the endocytosis of LDL and transferrin receptors in fibroblasts and hepatocytes (22, 41, 42). It also causes the accumulation of GLUT4 at the cell surface in adipocytes (7). In the present study, we explored whether inhibition of clathrin-dependent endocytosis by K+ depletion mimics the hyperosmolarity effects on GLUT4myc traffic. For the GLUT4myc externalization assay, cells were incubated with K+-free Hepes buffer for 2 h prior to detection of cell surface GLUT4myc with the anti-myc antibody coupled to the densitometric assay. K+ depletion was found to increase cell surface GLUT4myc by 3.6–1.5-fold (Fig. 8A).

Next, the effect of K+ depletion on GLUT4myc internalization was analyzed. K+ depletion was carried out during GLUT4myc labeling with anti-myc antibody at 4 °C for 1 h and continued during re-warming in the presence of hypertonic sucrose for another hour. Hypertonic sucrose was added during this period to maintain the transporters at the cell surface while the intracellular K+ was being depleted. The cells were then incubated in K+-free, iso-osmotic (sucrose-free) buffer for 30 min, and under these conditions, GLUT4myc remained at the cell surface (Fig. 8D). A control experiment confirmed that removal of sucrose in K+-containing medium allowed full internalization of GLUT4myc (Fig. 8D). These results suggest that K+ depletion, like hyperosmolarity, retains GLUT4myc at the cell surface.

Tyrosine Kinases Are Not Involved in Hyperosmolarity-induced GLUT4myc Retention at the Cell Surface—It has been reported that tyrosine kinases participate in hyperosmolarity-induced GLUT4 externalization in 3T3-L1 adipocytes (19), based on steady-state measurements of GLUT4 accumulation at the cell surface, which do not differentiate between exocytic and endocytic events. Therefore, we examined the effect of the tyrosine kinase inhibitor erbsatin analog (erbstatin A) on GLUT4myc exocytosis and endocytosis in the presence of hypertonic sucrose in L6 muscle cells. Inhibition of tyrosine kinases by erbsatin A (40 μg/ml) prevented significantly insulin-stimulated GLUT4myc translocation (data not shown). Hyperosmolarity caused an increase in protein tyrosine phosphorylation, which was prevented by erbsatin A (Fig. 9A). However, the tyrosine kinase inhibitor only partially blocked the hypertonic sucrose-induced GLUT4myc externalization (Fig. 9A). The same results were obtained when another tyrosine kinase inhibitor, genistein, was used (Fig. 9B). Interestingly, erbstatin A did not affect the hypertonic sucrose action on GLUT4myc internalization (Fig. 9C), suggesting that tyro-
sine kinases are not involved in hyperosmolarity-induced GLUT4myc retention at the cell surface. Because erstatin A partially inhibited GLUT4myc externalization, the accumulation of GLUT4myc at the surface caused by hypertonic sucrose appears to result partly from erstatin A-insensitive GLUT4myc retention and partly from stimulation of erstatin A-sensitive GLUT4myc exocytosis.

**DISCUSSION**

GLUT4 is a determinant of insulin sensitivity in muscle and fat cells. In the L6 skeletal muscle cell line, GLUT4 expression occurs after differentiation from myoblasts into myotubes (26). We have previously reported that expression of GLUT4myc in L6 myoblasts leads to the segregation of the protein to a GLUT4-specific pool, conferring insulin sensitivity to glucose uptake (24). This conclusion is based on the finding that, in L6-GLUT4myc myoblasts, the intracellular GLUT4myc compartment contains the majority of the insulin-regulatable amino peptidase but less than half of the GLUT1, and the sensitivity of glucose uptake to insulin is markedly improved. Indeed, we confirm in the present study that 90% of the GLUT4myc resides intracellularly.2 Upon insulin or hypertonic sucrose stimulation, 30% of the total cellular GLUT4myc is redistributed to the cell surface within 30 min. These results demonstrate that, as with previous observations in 3T3-L1 adipocytes, GLUT4myc is vastly retained in the intracellular pool in the basal state and is redistributed to the cell surface in response to insulin and hyperosmolarity in L6-GLUT4myc myoblasts.

**Insulin Stimulates GLUT4 Exocytosis and Hyperosmolarity Inhibits Its Internalization**—GLUT4myc undergoes rapid internalization upon insulin removal. Half of the surface-labeled GLUT4myc is internalized within 3 min. Even in the presence of insulin, the rate of GLUT4myc internalization is not appreciably slowed down, having approximately the same $t_{1/2}$ of 3 min. These results suggest that insulin does not regulate GLUT4 internalization in L6-GLUT4myc myoblasts. This contrasts with observations made in fat cells where a small proportion of insulin-induced gain in surface GLUT4 appears to be due to inhibition of GLUT4 endocytosis (1, 18). The 3-min half-time measured for GLUT4myc internalization in L6 myoblasts is very similar to the 4.2- or 3-min half-time for GLUT4 or insulin-regulatable amino peptidase endocytosis, respec-

2 In a previous report, a shorter incubation time with 0.1% Triton X-100 resulted in incomplete cell permeabilization and underestimation of the intracellular content of GLUT4myc (24).

**FIG. 7.** Hyperosmolarity inhibits endocytosis of GLUT4myc, whereas insulin has little effect on the endocytosis. Confluent, quiescent L6-GLUT4myc myoblasts were incubated in the presence or absence of 100 nM insulin at 37 °C for 30 min, reacted with anti-Myc antibody at 4 °C for 1 h to label cell surface GLUT4myc and then re-warmed to allow endocytosis in the absence or presence of 100 nM insulin or 0.45 M sucrose for 2–60 min. At the indicated times, cells were chilled and the Myc antibody-labeled GLUT4myc remaining on the surface was measured by the OPD optical densitometric detection assay as described under "Experimental Procedures." The amount of GLUT4myc remaining on the cell surface at any time point after re-warming was expressed as a percentage of the cell surface GLUT4myc level at 0 min of endocytosis. 

A, time course of GLUT4myc internalization. Here surface GLUT4myc was labeled with anti-Myc antibody in cells pre-stimulated with insulin. Inset, confluent, quiescent L6-GLUT4myc myoblasts were stimulated with 100 nM insulin at 37 °C for 30 min, reacted with anti-Myc antibody at 4 °C for 1 h to label cell surface GLUT4myc and then re-warmed to allow endocytosis in the absence of insulin for 20 min. Insulin was then added for 10 or 20 min. Cells were chilled again and the Myc antibody-labeled GLUT4myc remaining on the surface was measured by the OPD optical densitometric detection assay as described under “Experimental Procedures.” Shown are the means ± S.E. of two to five separate experiments each performed in triplicate. B, time course of GLUT4myc internalization. Here surface GLUT4myc was labeled with anti-Myc antibody in insulin-unstimulated cells. Shown are the means ± S.E. of two to five separate experiments each performed in triplicate. C, after being re-stimulated with insulin at 4 °C for 1 h to label cell surface GLUT4myc, cells were re-warmed to allow endocytosis in the absence or presence of insulin for 5 min. Shown are the means ± S.E. of two to three separate experiments each performed in triplicate. $p > 0.05$ by ANOVA with post-testing. Surface GLUT4myc was labeled with anti-Myc antibody in cells pre-stimulated with insulin (open bars) or un-stimulated cells (filled bars).
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GLUT4 internalizes via clathrin-coated pits (7, 44). K+-depletion and hypertonic shock are two strategies known to perturb the formation of clathrin coats (22, 23, 41) by preventing the interaction between clathrin and adaptors proteins (22). We demonstrate here that K+-depletion mimics hyperosmosality by causing a gain in GLUT4myc at the cell surface and preventing GLUT4myc endocytosis. These results strongly support the concept that hyperosmosality accumulates GLUT4 at the cell surface, at least in part, through inhibition of GLUT4 endocytosis.

Hyperosmosality is a stress stimulus that activates a tyrosine kinase pathway (19, 45), and tyrosine kinase activity is required for the surface gain in GLUT4 in 3T3-L1 adipocytes (19). However, it is unlikely that this effect is related to the retention of GLUT4 at the cell surface, because an inhibitor of the tyrosine kinases, erbstatin A, was unable to prevent the inhibition of GLUT4myc endocytosis by hyperosmosality in muscle cells (Fig. 9C). Erbstatin A and genistein prevented the hyperosmosality-induced GLUT4 externalization by only ~50% (Fig. 9C). Chen et al. (19) reported complete prevention of hyperosmosality-induced GLUT4 translocation by inhibition of tyrosine kinases. Therefore, it is conceivable that GLUT4 endocytosis is not blocked by hyperosmosality in 3T3-L1 cells.

**Insulin and Hyperosmosality Draw GLUT4 from Different Intracellular Pools**—GLUT4 accumulated at the cell surface with very different time courses in response to insulin and hypertonic sucrose. We speculate that there may be an insulin-regulated exocytic GLUT4 pool in L6 muscle cells, which can be rapidly mobilized by insulin and that hyperosmosality draws GLUT4 from an alternative pool of GLUT4 and/or the recycling endosomes. Supporting this concept that insulin and hyperosmosality draw GLUT4 from different intracellular pools in L6 muscle cells is their differential sensitivity to tetanus toxin.

VAMP2 and VAMP3 are expressed in muscle and fat cells (46–48) and reside in different GLUT4 pools in both muscle and fat cells (5, 40, 48). As shown earlier (40) and confirmed here, tetanus toxin reduced the insulin-dependent GLUT4myc translocation in L6 myoblasts. Our previous study also demonstrated that the reduction can be rescued by the toxin-resistant mutant VAMP2 but not by the toxin-resistant mutant VAMP3, suggesting that VAMP2 but not VAMP3 is required for GLUT4 vesicle fusion with the plasma membrane in response to insulin (40). We now show that expression of tetanus toxin does not alter GLUT4myc externalization caused by hyperosmosality. These results support the concept that insulin and hyperosmosality recruit GLUT4 from different intracellular pools, one requiring VAMP2 and another one that is tetanus toxin-insensitive. Thus, neither VAMP2 nor VAMP3 are the fusogenic v-SNARE for the incorporation of GLUT4 vesicles from recycling endosomes into the plasma membrane. It is conceivable that a tetanus toxin-insensitive VAMP such as TI-VAMP (49) could mediate fusion of the recycling endosome with the plasma membrane in muscle cells. Indeed, TI-VAMP was detected in L6-GLUT4myc myoblasts, and its localization was partially distinct from that of VAMP2 or VAMP3. The possible role of TI-VAMP in fusion of the hyperosmosality-drawn pool is under investigation.

The action of insulin and hyperosmosality on GLUT4myc externalization was partly additive. A previous study failed to observe an additive effect in 3T3-L1 adipocytes where the cells

Fig. 8. K+-depletion mimics hyperosmosality in causing a gain in GLUT4myc at the cell surface and preventing GLUT4myc endocytosis. A, assay of cell surface GLUT4myc: confluent, quiescent L6-GLUT4myc myoblasts were incubated with K+-free or K+-plus (5 mM) Hepes buffer at 37 °C for 2 h. For the group of K+-depletion with sucrose, cells were incubated with K+-free Hepes buffer at 37 °C for 2 h and 0.45 M sucrose was added for the last 30 min. GLUT4myc exposed at the cell surface was reacted with anti-Myc antibody and coupled to the OPD optical densitometric detection assay as described under “Experimental Procedures.” Shown are the means ± S.E. of three separate experiments each performed in triplicate; *, p < 0.05 versus basal by ANOVA with post-testing. B, GLUT4myc endocytosis assay: confluent, quiescent L6-GLUT4myc myoblasts were treated in three different manners (Sucrose, Sucrose-removal, and K+-depletion) as shown schematically. All conditions were stimulated with 100 nM insulin at 37 °C for 30 min, reacted with anti-Myc antibody in K+-free or K+-plus (5 mM) Hepes buffer at 4 °C for 1 h. Cells were re-warmed to 37 °C in K+-free Hepes buffer containing 0.45 M sucrose or K+-plus (5 mM) Hepes buffer for another hour. Cells were then incubated with K+-free, iso-osmolar Hepes buffer or K+-plus (5 mM) Hepes buffer at 37 °C for 30 min. In the sucrose removal group, 0.45 M sucrose was present for the first hour of re-warming for endocytosis and removed for the last 30 min, and K+ (5 mM) was always added. Cells from all conditions were chilled again and the Myc antibody-labeled GLUT4myc remaining on the cell surface at defined time points was expressed as a percentage of the cell surface GLUT4myc level at 0 min of endocytosis. Shown are the means ± S.E. of three to five separate experiments each performed in triplicate; *, p < 0.05 versus sucrose removal by ANOVA with post-testing.

GLUT4 myoblasts were treated in three different manners (Sucrose, Sucrose-removal, and K+-depletion) as shown schematically. All conditions were stimulated with 100 nM insulin at 37 °C for 30 min, reacted with anti-Myc antibody in K+-free or K+-plus (5 mM) Hepes buffer at 4 °C for 1 h. Cells were re-warmed to 37 °C in K+-free Hepes buffer containing 0.45 M sucrose or K+-plus (5 mM) Hepes buffer for another hour. Cells were then incubated with K+-free, iso-osmolar Hepes buffer or K+-plus (5 mM) Hepes buffer at 37 °C for 30 min. In the sucrose removal group, 0.45 M sucrose was present for the first hour of re-warming for endocytosis and removed for the last 30 min, and K+ (5 mM) was always added. Cells from all conditions were chilled again and the Myc antibody-labeled GLUT4myc remaining on the cell surface at defined time points was expressed as a percentage of the cell surface GLUT4myc level at 0 min of endocytosis. Shown are the means ± S.E. of three to five separate experiments each performed in triplicate; *, p < 0.05 versus sucrose removal by ANOVA with post-testing.
were pretreated with hyperosmolar solution prior to exposure to insulin (20). We also failed to observe any additive effect when we incubated L6-GLUT4myc myoblasts in the same manner. The lack of additivity under these latter conditions may be due to the inhibition of insulin signaling by hyperosmolarity at the level of Akt, as reported previously (20). In contrast, treating L6 muscle cells with insulin, followed by the addition of hyperosmolar solution, caused a further increase in the surface

**Fig. 10. Model of intracellular GLUT4 traffic pathways.** In untreated L6-GLUT4myc myoblasts, 10% of GLUT4myc resides at the cell surface (1). In response to hyperosmolarity, 25% of GLUT4myc recycles to plasma membrane through translocation from an alternative pool and/or the recycling pool (2) and remains at the cell surface due to inhibition of endocytosis. The exocytic event stimulated by hyperosmolarity is PI3K-independent and tetanus toxin-insensitive. Approximately 20% of the GLUT4myc translocates from a GLUT4-specific exocytic pool to the plasma membrane in response to insulin that requires the participation of PI3K and VAMP2 (3). Approximately 45% of the GLUT4myc remains intracellularly in stimulated cells (4).

**Fig. 9.** Inhibition of tyrosine kinase blocks the GLUT4myc gain at the cell surface but does not prevent the inhibition of GLUT4myc internalization induced by hyperosmolarity. A, confluent, quiescent L6-GLUT4myc myoblasts were treated with 40 μg/ml erbstatin A for 20 min prior to and during subsequent 30 min of incubation with 0.45 M sucrose. C, cells were left untreated with sucrose; S, cells were treated with sucrose. EA, erbstatin A. (a) Steady state level of cell-surface GLUT4myc: GLUT4myc exposed at the cell surface was reacted with anti-Myc antibody and coupled to the OPD optical densitometric detection assay as described under “Experimental Procedures.” Shown are the means ± S.E. of 8 to 11 separate experiments each performed in triplicate; *, p < 0.05 versus erbstatin A-untreated basal cells by ANOVA with post-testing; **, p < 0.05 sucrose versus erbstatin A + sucrose by ANOVA with post-testing. (b) Steady state level of cell-surface GLUT4myc (Fold increase over control basal).

**Fig. 8.** GLUT4myc endocytosis assay: confluent, quiescent L6-GLUT4myc myoblasts were stimulated with 100 nM insulin at 37 °C for 30 min, and reacted with anti-Myc antibody at 4 °C for 1 h in the absence or presence of 40 μg/ml erbstatin A. Cells were re-warmed to 37 °C to allow endocytosis for 1 h in the absence or presence of 0.45 M sucrose and 40 μg/ml erbstatin A. Cells were chilled again and the Myc antibody-labeled GLUT4myc remaining on the surface was analyzed by the OPD optical densitometric detection assay as described under “Experimental Procedures.” The amount of GLUT4myc remaining on the cell surface at defined time points was expressed as a percentage of the cell surface GLUT4myc level at 0 min of endocytosis. Shown are the means ± S.E. of three separate experiments each performed in triplicate.
GLUT4myc compared with the effect of either stimulus alone. Hypertonic sucrose must draw GLUT4myc from an alternative GLUT4 pool and/or recycling endosomal pool to account for the appearance of additional GLUT4myc at the cell surface, and as shown in Fig. 7, also retains GLUT4myc at the cell surface. Hence, hyperosmolality causes a compounded gain of GLUT4 molecules at the cell surface.

Lastly, insulin and hyperosmolality engage different signals in their action. PI3K activation is required for insulin-dependent GLUT4 translocation in fat and muscle cells (29, 30, 50–53). In contrast, the hyperosmolality-induced accumulation of GLUT4 at the cell surface is not prevented by the PI3K inhibitor wortmannin in 3T3-L1 adipocytes (19).3 Here we demonstrate that PI3K activity is not required for GLUT4myc externalization caused by hyperosmolality in L6 myoblasts.

Current models suggest that in 3T3-L1 adipocytes the increased amount of GLUT4 at the surface in response to insulin is due to recruitment of GLUT4 from a putatively static exo-

3 Small inhibition was seen in one study using wortmannin concentra-
tions of 100 nM or higher, which might have inhibited other pathways (21).
Hyperosmolarity Reduces GLUT4 Endocytosis and Increases Its Exocytosis from a VAMP2-independent Pool in L6 Muscle Cells
Dailin Li, Varinder K. Randhawa, Nish Patel, Michiko Hayashi and Amira Klip

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