Mobilization of GLUT-4 from intracellular vesicles by insulin and K\(^+\) depolarization in cultured H9c2 myotubes

BO YU, LAURIE A. POIRIER, AND LAURA E. NAGY
Department of Nutrition, Case Western Reserve University, Cleveland, Ohio 44106–4906

Yu, Bo, Laurie A. Poirier, and Laura E. Nagy. Mobilization of GLUT-4 from intracellular vesicles by insulin and K\(^+\) depolarization in cultured H9c2 myotubes. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E259–E267, 1999.—The insulin-responsive glucose transporter, GLUT-4, moves from an intracellular compartment to the cell surface in response to insulin and/or muscle contraction. Treatment of H9c2 myotubes with insulin significantly increased uptake of 2-deoxyglucose. Depolarization of the myotubes by increasing extracellular [K\(^+\)], which mimics the initial phases of excitation-contraction coupling, also increased 2-deoxyglucose uptake. The K\(^+\)-but not insulin-evoked increase was blocked by dantrolene, an inhibitor of Ca\(^{2+}\) release from the sarcoplasmic reticulum. In contrast, wortmannin, an inhibitor of phosphatidylinositol 3-kinase 3-kinase, blocked insulin- but not K\(^+\)-stimulated 2-deoxyglucose uptake. Increased glucose uptake in response to insulin or K\(^+\)-depolarization was associated with increased GLUT-4 in plasma membranes and depletion of a population of small intracellular GLUT-4-containing vesicles. Similarly, in H9c2 cells transfected with c-myc-tagged GLUT-4, translocation of c-myc GLUT-4 to the cell surface was increased after stimulation with insulin or K\(^+\)-depolarization. Taken together, these data demonstrate that insulin and K\(^+\)-depolarization increase glucose uptake by recruiting GLUT-4 from intracellular vesicles to the plasma membrane of H9c2 myotubes via distinct signaling mechanisms.

skeletal muscle; vesicle trafficking; phosphatidylinositol 3-kinase; calcium

ADIPOSE TISSUE AND MUSCLE are important sites for glucose disposal. Because of its large mass, muscle utilizes the largest proportion of available glucose. One of the major factors regulating glucose uptake into the muscle is the quantity of glucose transporters on the cell surface. Glucose transport is mediated by a family of facilitative glucose transporters (GLUT-1 to GLUT-7) (18). In adipocytes and muscle, GLUT-1 mediates basal or nonstimulated transport, whereas GLUT-4, the insulin-recruitable glucose transporter, facilitates increased glucose transport in the presence of insulin (25). In the basal state, GLUT-4 is predominantly localized to intracellular vesicles, with relatively little GLUT-4 residing at the plasma membrane (25). On stimulation, these vesicles move to the plasma membrane where they dock and fuse, thereby increasing the number of GLUT-4 molecules on the cell surface. Decreased GLUT-4 translocation in response to stimulation contributes to impaired glucose homeostasis in individuals with diabetes, insulin resistance, and/or obesity (25).

The mechanisms for insulin-stimulated translocation to the cell surface are not completely understood, but it is currently accepted that insulin stimulation of phosphatidylinositol 3-kinase (PI 3-kinase) activity is required for GLUT-4 translocation (25). In muscle, contraction also rapidly stimulates the translocation of GLUT-4 to the cell surface (8). Three lines of evidence suggest that insulin and contraction increase GLUT-4 translocation via independent pathways. First, under certain pathophysiological conditions, such as diabetes, insulin resistance, or obesity, in which insulin-stimulated GLUT-4 translocation is impaired, contraction-stimulated GLUT-4 translocation in skeletal muscle is maintained (7, 8, 20). Second, the effects of insulin and exercise on GLUT-4 translocation are additive in skeletal muscle (8). Wortmannin, an agent which inhibits PI 3-kinase activity, blocks insulin-stimulated GLUT-4 translocation in skeletal muscle (8) as well as cultured L6 myotubes (3) but has no effect on contraction-mediated or Ca\(^{2+}\)-dependent GLUT-4 translocation in skeletal muscle (8). Conversely, contraction-mediated GLUT-4 translocation is Ca\(^{2+}\) dependent, but insulin stimulation proceeds independently of Ca\(^{2+}\) concentration in both skeletal muscle and cardiac myocytes (8). Finally, insulin and contraction appear to mobilize distinct intracellular GLUT-4 vesicles on the basis of vesicle density and sedimentation velocity (5). However, silver staining of vesicle proteins did not reveal any major differences in protein composition of the insulin- or contraction-sensitive vesicles (5).

Increases in intracellular Ca\(^{2+}\) concentration stimulate glucose transport in isolated skeletal muscle independent of contraction (8). The initial phases of excitation-contraction coupling can be elicited in vitro by increasing the extracellular concentration of K\(^+\), leading to membrane depolarization and release of intracellular Ca\(^{2+}\) stores (6). In isolated frog skeletal muscle, increasing concentrations of extracellular K\(^+\) increase glucose uptake (29). Despite the importance of muscle in maintaining glucose homeostasis, there is currently no muscle cell line appropriate for investigating the differential regulation of glucose uptake by insulin and membrane depolarization/contraction. The L6 cell line commonly used for studies of insulin stimulation of glucose uptake does not appear to exhibit Ca\(^{2+}\)-stimulated glucose uptake (14). Here we have characterized the regulation of glucose transport in H9c2 myotubes, a muscle cell line that exhibits many of the properties of skeletal muscle (13). As in skeletal muscle, both insulin and increasing extracellular K\(^+\) increased the translocation of GLUT-4 to the plasma membrane in cultured H9c2 myotubes. Using this unique model...
system, we have investigated the differential mechanisms for insulin- and K⁺-mediated increases in GLUT-4 translocation and glucose uptake.

**MATERIALS AND METHODS**

Materials. H9c2 muscle cells were from the American Type Culture Collection (ATCC, Rockville, MD). Cell culture reagents were obtained from Gibco (Grand Island, NY). Antibodies were obtained from the following sources: rabbit polyclonal anti-GLUT-4 (East Acres Biologicals, Cambridge, MA and Biogenesis, Sandown, NH), anti-GLUT-1 and anti-GLUT-3 (Chemicon, Temecula, CA), anti-GLUT-4 (used for immunosolations, a gift from S. Cushman), anti-VAMP-2 (Synaptic Systems, Gottingen, Germany), monoclonal antibodies to transferrin receptor (mAb 86.4, Zymed, South San Francisco, CA), and a monoclonal antibody to GLUT-4 (IRGT-2, a gift from P. N. Jorgensen, Novo Nordisk, Copenhagen, Denmark). Goat anti-rabbit and goat anti-mouse IgG (Fab fragments) coupled to horseradish peroxidase and protease inhibitor cocktail (Complete) were from Boehringer Mannheim (Indianapolis, IN), and those coupled to Texas-red and fluorescein isothiocyanate (FITC) were from Molecular Probes (Eugene, OR). Dynabeads-500 were obtained from Dynal (New York). All other reagent-grades chemicals were from Sigma (St. Louis, MO), Bio-Rad (Hercules, CA), or Fisher Biochemicals (Santa Clara, CA).

Cell culture. H9c2 cells were propagated as myoblasts in DMEM containing 10% fetal bovine serum (FBS) and penicillin-streptomycin in a 10% CO₂ incubator at 37°C. Differentiated H9c2 cells were propagated as myoblasts in DMEM containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (10% CO₂) in the presence or absence of 100 nM insulin. Cells were cultured overnight in DMEM containing 10% fetal bovine serum (FBS) and penicillin-streptomycin in a 10% CO₂ incubator at 37°C. Subconfluent H9c2 myoblasts were transfected with c-myc-tagged GLUT-4 (a gift from Y. Ebina) (11, 17) by use of the DOTAP liposomal transfection reagent (Gibco). c-myc GLUT-4 contains a 14-amino acid sequence of the human c-myc epitope inserted in the first exofacial loop of GLUT-4 (11). Transfected cells were selected by growth in 800 µg/ml G418. Cell lines whose expression level of the c-myc-tagged GLUT-4 was one- to fourfold that of endogenous GLUT-4 on Western blotting to determine the distribution of GLUT-4 and other vesicular proteins. Plasma membrane contamination was assessed by probing with antibody to the α-subunit of Na⁺/K⁺-ATPase. Although intracellular vesicles from untreated cells contained minimal plasma membrane contamination, plasma membrane was not completely separated by the 5,000 g centrifugation after insulin or K⁺ treatment. Therefore, plasma membranes were isolated from homogenates by centrifugation for 5 min at 200 g to remove nuclei. The resulting supernatant was then centrifuged at 16,000 g for 15 min. This pellet contained >97% of the α-subunit of Na⁺/K⁺-ATPase in all treatment groups.

Immunoisolation of GLUT-4 vesicles. GLUT-4-containing vesicles were immunoisolated from pooled small vesicle fractions isolated on glycerol gradients. M-500 Dynabeads (0.4 × 10^7) were coupled to anti-rabbit IgG by incubation at 37°C for 10 h. Beads were then washed twice in PBS and incubated with or without 100 nM insulin in PBS with 10 mM HEPES, pH 7.4, or 80 mM K⁺ (as above) for 10–30 min at 37°C. After treatment, cells were washed twice in ice-cold PBS and removed from the cell culture dish by gentle scraping with a cell scraper. Cells were centrifuged at 600 g for 5 min and then homogenized in a Wheaton glass homogenizer with the tight-fitting pestle (ceramitec 0.05) in homogenizing buffer (in mM: 150 NaCl, 1 EGTA, 0.1 MgCl₂, 10 HEPES, pH 7.4) with protease inhibitors. An S1 fraction was prepared by centrifugation at 5,000 g for 5 min. One or two milligrams of S1 protein were layered on a 10–25% glycerol gradient formed over a 50% sucrose pad. Samples were centrifuged at 60,000 g for 70 min (9) in an SW55 rotor, and 0.300-ml fractions were collected. A portion of each fraction was used for Western blotting to determine the distribution of GLUT-4 and other vesicular proteins. Plasma membrane contamination was assessed by probing with antibody to the α-subunit of Na⁺/K⁺-ATPase. Although intracellular vesicles from untreated cells contained minimal plasma membrane contamination, plasma membrane was not completely separated by the 5,000 g centrifugation after insulin or K⁺ treatment. Therefore, plasma membranes were isolated from homogenates by centrifugation for 5 min at 200 g to remove nuclei. The resulting supernatant was then centrifuged at 16,000 g for 15 min. This pellet contained >97% of the α-subunit of Na⁺/K⁺-ATPase in all treatment groups.

Western blotting. Equal volumes of fractions from gradient isolations were solubilized in SDS sample buffer without β-mercaptoethanol for 15 min at 37°C. Proteins were then separated on 4–18% polyacrylamide gels and transferred to...
PVDF for Western blot analysis. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween (TBS-T) for 2 h, washed twice with TBS-T, and then incubated with antibody overnight at 4°C. Antibodies were prepared in 1% BSA in TBS-T at 1:500 for GLUT-4 (polyclonal) and anti-GLUT-4 (monoclonal), 1:2,000 for the transferrin receptor (TfR) and cellubrevin, 1:10,000 for GLUT-3, and 1:1,000 for GLUT-1 and VAMP-2. Membranes were washed again in TBS-T with 5% nonfat dry milk and probed with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse IgG Fab fragments for 1 h. Unbound antibody was removed by washing, and membranes were incubated with enhanced chemiluminescence reagent. Immunoreactive protein quantity was assessed by scanning densitometry by use of exposures that were within the linear range of detectability for each of the proteins measured. Internal standards were included on each gel to normalize between blots.

Immunohistochemistry. For immunohistochemistry, cells were washed once in PBS and incubated with or without 100 nM insulin or 80 mM K+ as described in Separation of intracellular vesicles and plasma membrane. Cells were stimulated with agonist for 30 min and then fixed in freshly prepared 4% paraformaldehyde for 10 min on ice and then for 20 min at room temperature. Slides were then quenched in glycine and blocked for 1 h in 2% BSA and 5% fish gelatin with (for intracellular localization of GLUT-4) or without 0.02% saponin (for surface labeling). Slides were then incubated overnight at 4°C with antibody to GLUT-4 (1:200) or c-myc (9E10, 1:200) in blocking buffer. Slides were washed three times for 15 min in blocking buffer and then incubated for 1 h with Texas-red conjugated anti-rabbit IgG (1:200) or FITC-conjugated anti-mouse IgG (1:200) for 1 h in blocking buffer. Slides were finally washed three times for 15 min in blocking buffer, washed twice for 5 min in PBS, and mounted in Vectashield mounting medium. Cells were examined with a Bio-Rad confocal microscope under a ×60 lens. Multiple cells from at least five separate preparations were examined. Control experiments using only secondary antibody in the presence and absence of saponin were carried out with each secondary antibody used.

Statistical analysis. Data were presented as means ± SE and were compared by analysis of variance with the general linear model procedure on SAS for personal computers. Differences between treatment groups were then determined by least square means analysis. All data were tested for normal distribution. For some experiments, values were log transformed to obtain a normally distributed data set.

RESULTS

Treatment of H9c2 myotubes with 1–100 nM insulin increased uptake of 2-deoxy-[3H]glucose; maximal stimulation was three- to fourfold above basal uptake at 10 and 100 nM insulin (Fig. 1A). Incubation of H9c2 myotubes with increasing concentrations of K+ also stimulated uptake of 2-deoxy-[3H]glucose (Fig. 1B). K+ at 10–20 mM increased 2-deoxy-[3H]glucose uptake by twofold; higher concentrations of K+ further increased uptake to fourfold greater than basal uptake (Fig. 1B). K+-evoked increases in glucose uptake in H9c2 myotubes were similar in sensitivity and magnitude to those previously reported in isolated skeletal muscle (28). In contrast, increasing extracellular K+ concentration had no effect on uptake of 2-deoxy-[3H]glucose in isolated rat adipocytes (Poirier and Nagy, unpublished observations). Transport of 3-O-[3H]methylglucose, a nonmetabolizable analog of glucose, measured over 2–10 s, was also stimulated by treatment with 100 nM insulin or 80 mM K+ for 15 min (Fig. 2A). The time course for stimulation of transport was similar in response to insulin or K+ (Fig. 2B). Activation of transport of 3-O-[3H]methylglucose was observed after 5 min of incubation with insulin or K+ (Fig. 2B). Transport was further increased in both insulin- and K+-treated cells from 10–30 min of treatment (Fig. 2B). In isolated adipocytes and skeletal muscle, insulin stimulation of glucose uptake is mediated via activation of PI 3-kinase (8). Pretreatment of H9c2 myotubes with 100 nM wortmannin had no effect on basal uptake of 2-deoxy-[3H]glucose but completely inhibited insulin stimulation of uptake (Fig. 3). K+-evoked increases in uptake were not inhibited by wortmannin (Fig. 3). In contrast, pretreatment of H9c2 myotubes with 25 µM dantrolene, an inhibitor of Ca2+ release from the sarcoplasmic reticulum, decreased K+-stimulated 2-deoxy-[3H]glucose uptake but had no effect on insulin-stimulated uptake (Fig. 4). Because of its limited solubility in aqueous solutions, we were unable to test whether higher concentrations of dantrolene would completely inhibit stimulation of 2-deoxyglucose by 80 mM K+. However, pretreatment of H9c2 myotubes with 25 µM dantrolene was able to completely inhibit stimu-
Uptake in response to 40 mM K\textsuperscript{+} was increased 1.94 \pm 0.19-fold over basal (n = 8) in the absence of dantrolene and 1.16 \pm 0.19-fold over basal (n = 7) in the presence of dantrolene. These data suggest that insulin and K\textsuperscript{+}-mediated depolarization increase glucose uptake by independent signaling mechanisms.

Increased 2-deoxy-[\textsuperscript{3}H]glucose uptake was associated with increased GLUT-4 in isolated plasma membrane fractions. Immunoreactive GLUT-4 was increased by 2.2 \pm 0.3-fold (P < 0.05, n = 11) in response to insulin and 1.8 \pm 0.2-fold (P < 0.05, n = 9) after K\textsuperscript{+} depolarization (Fig. 5A). H9c2 myotubes also express GLUT-1 and GLUT-3. Immunoreactive quantity of GLUT-1 in plasma membrane-enriched fractions increased by 1.4 \pm 0.3-fold (n = 9) and 1.5 \pm 0.2-fold (n = 8) in response to insulin and K\textsuperscript{+} treatment, respectively (Fig. 5A). Similarly, GLUT-3 at the plasma membrane increased by 1.3 \pm 0.1-fold (n = 8) and 1.2 \pm 0.1-fold (n = 7) in response to insulin and K\textsuperscript{+}, respectively (Fig. 5A).

The effects of 100 nM insulin and 80 mM K\textsuperscript{+} on uptake of 2-deoxy-[\textsuperscript{3}H]glucose were additive after stimulation for 10 min. However, when H9c2 cells were incubated with 100 nM insulin and 80 mM K\textsuperscript{+} together for 20 min, the effects were no longer additive (Fig. 6A).

Fig. 2. Stimulation of 3-O-[\textsuperscript{3}H]methylglucose by insulin and K\textsuperscript{+} depolarization in H9c2 myotubes. A: cells were stimulated with and without 100 nM insulin or 80 mM K\textsuperscript{+} for 15 min, and uptake of 100 \mu M 3-O-[\textsuperscript{3}H]methylglucose was measured over 2–10 s. Values represent means \pm SE; n = 7–10. Uptake for insulin and K\textsuperscript{+}-treated cells was greater than basal, P < 0.05. B: cells were treated with insulin or K\textsuperscript{+} for 5–30 min, and transport of 100 \mu M 3-O-[\textsuperscript{3}H]methylglucose was measured at 5 s. Values represent means \pm SE; n = 6–10, except at 30 min, when n = 4. Insulin and K\textsuperscript{+} significantly increased transport compared with basal, P < 0.05, and transport was significantly lower at 5 min compared with all other time points (P < 0.05); however, there was no significant interaction between treatments and time of incubation.

Fig. 3. Inhibition of insulin-stimulated glucose uptake by wortmannin. H9c2 cells were preincubated for 30 min with or without 100 nM wortmannin and then stimulated with or without 10 nM insulin or 80 mM K\textsuperscript{+} for 20 min in the continued presence or absence of wortmannin. Uptake of 2-deoxy-[\textsuperscript{3}H]glucose was measured over 10 min. Values represent means \pm SE; n = 5. Values with different superscripts are significantly different, P < 0.05.

Fig. 4. Inhibition of K\textsuperscript{+}-mediated glucose uptake by dantrolene. H9c2 cells were preincubated for 5 min with or without 25 \mu M dantrolene chloride and then stimulated with or without 10 nM insulin or 80 mM K\textsuperscript{+} for 20 min in the continued presence or absence of dantrolene. Uptake of 2-deoxy-[\textsuperscript{3}H]glucose was measured over 10 min. Values represent means \pm SE; n = 6–7. Values with different superscripts are significantly different, P < 0.05.
This saturation in uptake was paralleled by changes in the translocation of GLUT-4 to the plasma membrane. GLUT-4 in plasma membrane-enriched fractions was increased to a greater degree in response to 10 nM insulin and 40 mM K\(^+\) in combination (2.12 ± 0.29-fold over basal) compared with single treatments after 15 min of stimulation (1.45 ± 0.07-fold for insulin and 1.49 ± 0.16-fold for K\(^+\), \(P < 0.05\), \(n = 10\)) (Fig. 5B). However, responses were no longer additive after 30 min of treatment (Fig. 5B), with insulin alone increasing GLUT-4 in plasma membrane-enriched fractions by 1.7 ± 0.3, K\(^+\) by 1.5 ± 0.2, and the combination by 1.6 ± 0.3 (\(n = 8\)).

Translocation to the cell surface was also qualitatively visualized by immunohistochemistry in H9c2 cells transfected with c-myc-tagged GLUT-4. In unstimulated cells, the intracellular distribution of GLUT-4 and c-myc GLUT-4 was similar, with staining observed in large perinuclear vesicles, as well as smaller punctate staining throughout the cell body (Fig. 7). Uptake of 2-deoxy-[\(^{3}H\)]glucose in c-myc GLUT-4-transfected H9c2 cells was 21.5 ± 3.6 pmol·mg protein\(^{-1}\)·10 min\(^{-1}\) in basal cells and was increased to 36.2 ± 4.4 and 62.5 ± 7.9 pmol·mg protein\(^{-1}\)·10 min\(^{-1}\) (\(n = 6\), \(P < 0.01\) compared with basal) by 10 nM insulin and 80 mM K\(^+\), respectively. In unstimulated cells, surface labeling with anti-c-myc antibody was minimal (Fig. 8A). After stimulation with 100 nM insulin for 30 min, large areas of the surface of the H9c2 myotubes were accessible to dense surface labeling with anti-c-myc antibodies (Fig. 8B). After K\(^+\) depolarization, surface labeling with anti-c-myc was also observed, but staining was confined to smaller, discrete regions on the myotube surface (Fig. 8C).

In adipocytes and skeletal muscle, intracellular GLUT-4 is localized to distinct vesicular compartments that can be distinguished by their size and density (22, 25). One possible mechanism by which insulin and K\(^+\) depolarization could differentially increase glucose uptake would be to stimulate the translocation of GLUT-4 from different vesicular compartments. Separation of intracellular vesicles from H9c2 myotubes on glycerol velocity gradients revealed that GLUT-4 was distributed to two compartments of different sizes. The smaller vesicular compartment contained the majority of the
GLUT-4 in unstimulated H9c2 cells (Fig. 9). Light microsomes isolated from rat adipocytes by standard methods of differential centrifugation migrate like the small vesicle fraction of the H9c2 myotubes on glycerol velocity gradients (Poirier and Nagy, unpublished observation). Immunoreactive GLUT-4 quantity in the small vesicle compartment in H9c2 cells was 3.0 ± 0.4-fold greater than in large vesicles (n = 12). In contrast, TfR was more evenly distributed between the small and large vesicle fractions (Fig. 9); the ratio of immunoreactive TfR in small vesicles to large vesicles was 1.3 ± 0.2 (n = 11). Both VAMP-2 and cellubrevin were highly enriched in the large vesicles, with very little immunoreactivity found in the small vesicle compartment (Fig. 9); 16 ± 4% (n = 4) and 20 ± 3% (n = 8) of intracellular VAMP-2 and cellubrevin, respectively, were localized to the small vesicle fraction. Immunoisolation of GLUT-4-containing vesicles from the small vesicular compartment was carried out to determine whether these proteins were resident in GLUT-4 vesicles. A percentage of 76 ± 9% (n = 5) of GLUT-4 from the small vesicle fractions was immunoisolated with antibody to GLUT-4 (Fig. 9). Similarly, TfR communoisolated with GLUT-4 vesicles, but VAMP-2 and cellubrevin were not detected in the immunoisolated GLUT-4 vesicles (Fig. 9).

Treatment of H9c2 myotubes with 100 nM insulin or 80 mM K⁺ for 30 min resulted in a loss of immunoreactive GLUT-4 from the small vesicular compartment (Fig. 10). GLUT-4 in the small vesicles was decreased by 46.5 ± 6.7% and 43.7 ± 8.7% in insulin and K⁺-treated cells, respectively, compared with untreated cells. TfR was also decreased after insulin and K⁺ treatment [47 ± 6.7% (n = 5) and 46 ± 14% (n = 3), respectively]. There was no apparent mobilization of GLUT-4 from the large vesicle fractions, with GLUT-4 immunoreactivity increasing to 1.2 ± 0.2-fold (n = 6, P < 0.05 vs. basal).
DISCUSSION

Uptake of glucose into differentiated H9c2 myotubes was increased three- to fivefold by stimulation with insulin or increasing concentrations of extracellular K\(^+\). Increased uptake was associated with a shift of GLUT-4 from intracellular storage compartments to the cell surface. This is the first description of K\(^+\)-evoked depolarization increasing GLUT-4 translocation to the cell surface and glucose uptake in cultured muscle cells. The intracellular signaling mechanisms utilized by insulin and K\(^+\)-dependent depolarization were distinct: insulin action requires the activity of PI 3-kinase and is independent of intracellular Ca\(^{2+}\) release, whereas K\(^+\)-mediated responses do not require PI 3-kinase activity but are dependent on the release of intracellular Ca\(^{2+}\). Treatment of H9c2 myotubes with dantrolene, which inhibits the release of Ca\(^{2+}\) from the sarcoplasmic reticulum, blocked K\(^+\)-mediated increases in glucose uptake. Dantrolene also prevents K\(^+\)-stimulated increases in glucose uptake (29), as well as hyperglycemia-induced increases in glucose uptake in skeletal muscle (21). Moreover, the responses in H9c2 myotubes to K\(^+\) depolarization are consistent with data from skeletal muscle indicating that contraction-dependent translocation of GLUT-4 is independent of PI 3-kinase activity but requires the release of Ca\(^{2+}\) (8).

Stimulation of glucose uptake was associated with a twofold increase in the plasma membrane localization of GLUT-4 protein. This change in translocation was smaller than the often three- and fourfold increases in glucose uptake observed after stimulation with maximal insulin or extracellular K\(^+\). However, a more moderate translocation of two other glucose transporters to the plasma membrane fraction was also observed after stimulation with either insulin or K\(^+\) depolarization. It is likely that the 20–30% increase in GLUT-3 and 40–50% increase in GLUT-1 at the cell surface observed after stimulation with insulin or K\(^+\) depolarization also contributed to the total increase in glucose uptake. Increased quantity of all three glucose transporters in the large vesicles at the cell surface after insulin stimulation has also been reported for L6 myotubes (32).

Consistent with differential mechanisms of action, the effects of insulin and K\(^+\) depolarization were additive after short-term stimulation (see Fig. 6). Effects of insulin stimulation and contraction on glucose uptake and GLUT-4 translocation have been found to be additive in some, but not all (8), experiments using isolated skeletal muscle. However, in cultured H9c2 cells, additivity was not observed after longer periods of activation. Saturation of the response could occur either at a convergent point in the signaling cascade or in the process of GLUT-4 translocation from its intracellular storage site to the plasma membrane. Incubation of H9c2 myotubes under hypertonic conditions (450 mM sucrose for 10 min) completely depleted the small intracellular GLUT-4 vesicles (Yu and Nagy, unpublished observations) compared with the 40–50% mobilization seen with insulin or K\(^+\). This suggests that saturation of GLUT-4 translocation and glucose uptake is not limited by the pool of intracellular GLUT-4 that can be mobilized. Activation of PI 3-kinase or increases in intracellular Ca\(^{2+}\) concentrations are not likely to be the final step in the signaling events leading to translocation of GLUT-4 to the plasma membrane. However, it is not clear whether these two signaling pathways converge before activation of GLUT-4 translocation. One of the downstream targets of PI 3-kinase involved in the recruitment of GLUT-4 to the plasma membrane after insulin activation of adipocytes and L6 muscle cells is protein kinase B (PKB or Akt) (27, 28). However, PKB/Akt does not appear to be involved in contraction-stimulated translocation of GLUT-4 in skeletal muscle (15). Another downstream target of PI 3-kinase is protein kinase C\(\zeta\) (PKC-\(\zeta\)) (2). PKC-\(\zeta\) appears to be activated in response to increased phosphoinositides and involved in GLUT-4 translocation in adipocytes and L6 myotubes (1, 2). However, PKC-\(\zeta\) is an atypical PKC isoform, which lacks the Ca\(^{2+}\)-binding domain of the typical PKC family members and is therefore not regulated by changes in the concentration of Ca\(^{2+}\). Therefore, it is not likely that insulin and Ca\(^{2+}\) signals both act via PKC-\(\zeta\). Further studies will need to be carried out to determine whether, and at what site, these two pathways converge.

In skeletal and cardiac muscle and adipocytes, intracellular GLUT-4 resides in tubulovesicular structures and small vesicles of an average diameter of 50–70 nm (22). In H9c2 myotubes, intracellular GLUT-4 was distributed to small and large vesicles on velocity gradients. As in isolated rat adipocytes (9), the small vesicles were highly enriched with GLUT-4 relative to the larger vesicles. Upon insulin stimulation or K\(^+\)-mediated depolarization, small vesicles containing GLUT-4 were depleted by 50% (Fig. 8), similar to the insulin-induced mobilization of small GLUT-4 vesicles (9) or light microsomes (25) in adipocytes. This mobilization is consistent with the hypothesis that small GLUT-4-containing vesicles represent a “GLUT-4 storage vesicle,” which can be mobilized in response to stimuli that recruit GLUT-4 to the plasma membrane (22).

In contrast to the marked mobilization of GLUT-4 from the small vesicular compartment, GLUT-4 was not mobilized from the large vesicular fraction in the H9c2 myotubes (Fig. 8). The large vesicular fraction appears to be a heterogeneous population of vesicles. In addition to colocalization of TfR, VAMP-2, and cellubrevin (Fig. 7), these vesicles also colocalize with rab5, a small GTP-binding protein associated with early endosomes, and the dihydropyridine receptor, a Ca\(^{2+}\) channel present in the transverse tubules (t-tubules) of skeletal muscle (Yu and Nagy, unpublished observations). The presence of minor plasma membrane contaminants (see MATERIALS AND METHODS) or t-tubule membranes (see next paragraph) in the large vesicle
fraction could account for the small increase in GLUT-4 in the large vesicle fraction after insulin stimulation or K⁺ depolarization. Further resolution of these different components will be necessary to determine whether insulin and/or K⁺ depolarization affects the distribution of GLUT-4 within this compartment.

Although the small intracellular GLUT-4-containing vesicles appear to represent a specialized vesicular compartment, the nature and derivation of these vesicles are unclear. When GLUT-4 is transfected into a heterologous cell line such as Chinese hamster ovary or PC-12, it accumulates in a small vesicular compartment, suggesting that GLUT-4 is sorted to a specialized intracellular compartment that is present in a number of cell types (9, 10). These small GLUT-4-containing vesicles are endocytically derived; cell surface-labeling CHO cells transfected with c-myc-tagged GLUT-4 indicate that GLUT-4 initially passes through an endosomal compartment and then accumulates as small vesicles (30). In rat adipocytes, surface biotinylation experiments have also shown that GLUT-4 cycles with other endosomal proteins, such as the Tfr, between the plasma membrane and light microsomes (12). Indeed, GLUT-4 vesicles in light microsomes of rat adipocytes contain 60% of intracellular Tfr (12). Although the Tfr is found in GLUT-4 vesicles in rat adipocytes, it is a relatively minor component of these vesicles (12). Similar to the distribution in rat adipocytes, the Tfr in H9c2 myotubes was distributed to both large and small vesicles, with no significant enrichment in either population. Tfr immunoisolated with GLUT-4-containing small vesicles. Moreover, Tfr in the small vesicular compartment was decreased in response to both insulin and K⁺ depolarization, similar to the insulin-stimulated recruitment of Tfrs from light microsomes in isolated rat adipocytes (12). These data suggest that, whereas the Tfr is not excluded from small GLUT-4-containing vesicles in H9c2 myotubes, it is also not specifically enriched in these insulin and depolarization-sensitive small vesicles.

Current models for the docking and fusion of vesicles with the plasma membrane require the interaction of vesicle proteins, such as members of the synaptobrevin family, VAMP-2, and/or cellubrevin, with proteins on target membranes, such as syntaxin (23). Consistent with this hypothesis, treatment of adipocytes and 3T3-L1 adipocytes with tetanus toxin, which specifically cleaves synaptobrevin family members, prevents insulin stimulation of GLUT-4 translocation (16, 26). VAMP-2 and cellubrevin coimmunoisolate with intracellular GLUT-4 vesicles in 3T3-L1 adipocytes (31), and VAMP-2 coimmunoisolates with GLUT-4 vesicles in isolated rat adipocytes (4). GST fusion proteins for VAMP-2, but not cellubrevin or VAMP-1, inhibit insulin-stimulated GLUT-4 translocation in 3T3-L1 adipocytes (16). In H9c2 myotubes, VAMP-2 and cellubrevin colocalized predominantly with the endosomal/large vesicle fraction (Fig. 9). Although a small quantity of VAMP-2 and cellubrevin was found in the small vesicle fraction, we were unable to detect VAMP-2 or cellubrevin in immunoisolated small GLUT-4 vesicles (Fig. 9B). In 3T3-L1 adipocytes, although 73% of GLUT-4 protein immunoisolated from a light microsomal fraction, only 16% of the VAMP-2 and 55% of the cellubrevin immunoisolated with GLUT-4 vesicles (30). This very low quantity of VAMP-2 and cellubrevin relative to GLUT-4 in the small GLUT-4 vesicles suggests that very little VAMP-2/cellubrevin is needed to dock with target proteins on the plasma membrane (i.e., significantly less protein machinery is needed for docking and fusion relative to the quantity of GLUT-4 cargo in the vesicle).

Alternatively, novel VAMP isoforms may be expressed in H9c2 myotubes and/or other GLUT-4-expressing cells. The recent cloning of a novel VAMP isoform, VAMP-5, that is expressed in differentiated myotubes as well as heart and skeletal muscle, supports the latter hypothesis (33).

GLUT-4 translocation to the plasma membrane in response to insulin or K⁺ depolarization was assessed by isolation of plasma membrane-enriched fractions and by following the translocation of a c-myc-tagged GLUT-4 to the cell surface. Whereas the immunohistochemical methods used to assess c-myc-tagged GLUT-4 translocation are only qualitative, both methods are consistent in demonstrating a translocation of GLUT-4 to the cell surface. Interestingly, the apparent distribution of c-myc-tagged GLUT-4 on the cell surface differed in response to insulin and K⁺ depolarization treatment; insulin stimulation revealed c-myc-tagged GLUT-4 in large patches across the myotube surface, whereas after K⁺ treatment, GLUT-4 was more evenly distributed across the surface of the myotube in small, discrete regions. Using subcellular fractionation techniques, Roy and Marette (24) demonstrated that GLUT-4 is primarily distributed to t-tubules in rat skeletal muscle after exercise. Insulin also increases GLUT-4 in isolated t-tubule fractions, as well as plasma membrane, in rat skeletal muscle (19). A t-tubule system has been reported in H9c2 myotubes; the system is highly branched and continuous with the plasma membrane (13). This t-tubule system may therefore convey some type of structural/positional information for the localization of GLUT-4 vesicle fusion with the cell surface after K⁺ depolarization. The distinct distribution of GLUT-4 on the surface of the H9c2 myotubes in response to insulin and K⁺ depolarization suggests that this unique cell culture model will be useful for investigation of the specialized mechanisms for signal transduction, as well as GLUT-4 vesicle trafficking, involved in the stimulation of glucose uptake in response to insulin stimulation and K⁺-mediated depolarization.

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