Muscle cell depolarization induces a gain in surface GLUT4 via reduced endocytosis independently of AMPK

Nadeeja Wijesekara,1,2 Amanda Tung,1,2 Farah Thong,1 and Amira Klip1,2

1Programme in Cell Biology, The Hospital for Sick Children; and 2Department of Physiology, University of Toronto, Toronto, Ontario, Canada

Submitted 22 November 2005; accepted in final form 13 January 2006

Muscle cell depolarization induces a gain in surface GLUT4 via reduced endocytosis independently of AMPK. Am J Physiol Endocrinol Metab 290: E1276–E1286, 2006. First published January 17, 2006; doi:10.1152/ajpendo.00573.2005.—Contracting skeletal muscle increases glucose uptake to sustain energy demand. This is achieved through a gain in GLUT4 at the membrane, but the traffic mechanisms and regulatory signals involved are unknown. Muscle contraction is elicited by membrane depolarization followed by a rise in cytosolic Ca2+ and actomyosin activation, drawing on ATP stores. It is unknown whether one or more of these events triggers the rise in surface GLUT4. Here, we investigate the effect of membrane depolarization on GLUT4 cycling using GLUT4myc-expressing L6 myotubes devoid of sarcomeres and thus unable to contract. K+-induced membrane depolarization elevated surface GLUT4myc, and this effect was additive to that of insulin, was not prevented by inhibiting phosphatidylinositol 3-kinase (PI3K) or actin polymerization, and did not involve Akt activation. Instead, depolarization elevated cytosolic Ca2+, and the surface GLUT4myc elevation was prevented by dantrolene (an inhibitor of Ca2+ release from sarcoplasmic reticulum) and by extracellular Ca2+-chelation. Ca2+-calmodulin-dependent protein kinase-II (CaMKII) was not phosphorylated after 10 min of K+-depolarization, and the CaMK inhibitor KN62 did not prevent the gain in surface GLUT4myc. Interestingly, although 5′-AMP-activated protein kinase (AMPK) was phosphorylated upon depolarization, lowering AMPKα via siRNA did not alter the surface GLUT4myc gain. Conversely, the latter response was abolished by the PKC inhibitors bisindolylmaleimide I and calphostin C. Unlike insulin, K+-depolarization caused only a small increase in GLUT4myc exocytosis and a major reduction in its endocytosis. We propose that K+-depolarization reduces GLUT4 internalization through signals and mechanisms distinct from those engaged by insulin. Such a pathway(s) is largely independent of PI3K, Akt, AMPK, and CaMKII but may involve PKC.

glucose uptake; glucose transporter-4; calcium; adenosine 5′-monophosphate-activated protein kinase; potassium-induced depolarization

Muscle contraction derives energy from both endogenous (glycogen and triglycerides) and exogenous (circulating glucose and fatty acids) sources. The contracting muscle has elevated rates of glucose uptake, a response known to be independent of insulin. Subcellular fractionation studies show that this is associated with an increase in glucose transporter GLUT4 levels in isolated plasma membrane (PM) fractions of variable purity (13, 17). Furthermore, the intracellular pool providing such GLUT4 appears to be distinct from the insulin-sensitive pool (10, 13, 49). Despite intensive research on the subject (23, 45, 54, 55), relatively little is known about the mechanisms underlying GLUT4 dynamics in response to muscle contraction.

As well, there is little consensus regarding the signals regulating contraction-mediated glucose transport. Although studies suggest that this response is independent of phosphatidylinositol 3-kinase (PI3K) (38, 42, 73), the effector pathways invoked are diverse and include conventional protein kinase C (PKC) (28, 51), Ca2+-calmodulin-dependent protein kinase (CaMK) (71), and/or 5′-AMP-activated protein kinase (AMPK) (3, 21, 45, 65, 68).

Numerous physiological changes are associated with muscle contraction. The triggering event is membrane depolarization leading to a rise in cytosolic Ca2+ levels. Metabolic changes such as reduction in ATP and glycogen stores as well as cytosolic acidification also occur, and in vivo this is compounded by an elevation in blood flow, catecholamines, and additional circulating factors. It is not known which of these individual conditions acts as a stimulus to promote glucose transport into muscle cells. Indeed, some of these factors have the capacity to stimulate glucose uptake into skeletal muscle independently of contraction. In particular, pharmacological agents that induce Ca2+ release from the sarcoplasmic reticulum (SR) without causing muscle contraction increase glucose uptake into skeletal muscle (74).

Here, we hypothesize that membrane depolarization can elevate surface GLUT4 levels leading to glucose uptake and that this involves selective effects on GLUT4 exocytic and endocytic rates. This hypothesis cannot be easily approached experimentally in intact muscle due to the difficulty in dissecting GLUT4 traffic routes in this tissue. Instead, L6 myotubes stably expressing myc-tagged GLUT4 (GLUT4myc) (66) are a useful cellular model amenable to studying GLUT4 endocytic and exocytic traffic. The cells are responsive to insulin, which increases GLUT4 exocytosis without affecting its endocytosis, and are amenable to transfection, gene ablation via short interfering (si)RNA, and confocal fluorescence microscopy scrutiny (15, 25, 37, 40, 48). Moreover, L6 myotubes are devoid of sarcomeres and, hence, cannot mount a contraction response, allowing for a clean separation of the effects of depolarization and contraction.

The results obtained reveal that depolarization elicited in vitro by elevating extracellular K+ increases glucose uptake and surface GLUT4myc and that these effects are dependent on the intracellular Ca2+ concentration. We further explore signaling pathways downstream of Ca2+, compare these with insulin-derived signals, and examine the effect on the exocytic and endocytic rates of GLUT4myc.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Materials. Human insulin (Humulin R) was obtained from Eli Lilly Canada (Toronto, ON, Canada). Dinitrophenol (DNP), cytochalasin B (CB), cytochalasin D (CD), β-glucosidase, 2-deoxy-β-glucose, potassium glutamate, sodium glutamate, N-methyl-D-glucamine (NMG), gramicidin, all protease inhibitors, o-phenylenediamine dihydrochloride (OPD reagent), and wortmannin were obtained from Sigma Chemical (St. Louis, MO). BAPTA-AM, ionomycin, bisindolylmaleimide I (BIM), calphostin C, 1-[N-(O-bis-(5-isouquinolinesulfonyl)-N-methyl-l-tyrosyl]-4-phenylpiperazone (KN62), and dantrolene were purchased from Calbiochem (San Diego, CA). 2-Deoxy-D-[3H]glucose was purchased from PerkinElmer (Shelton, CT). Fura 2-AM was purchased from Molecular Probes (Invitrogen, Burlington, ON, Canada). Indinavir was obtained through a material transfer agreement from Merck (Rahway, NJ). Polyclonal antibody to the myc epitope was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AKT1, anti-phospho-Thr172AMPKα, anti-phospho-Thr286-CaMKII, and anti-phospho-Ser73- and phospho-Thr308-Akt antibodies were obtained from Cell Signaling. The sequence of the AMPKα1 siRNA used was GCA UAU GCU GCA GGU AGA UdTdT (nucleotides 738 to 756, acc. no. NM_019142), and the AMPKα2 siRNA used was CGU CAU UGA UGA GGC UdTdT (nucleotides 865–883, acc. no. NM_023991) (37), and the nonrelated control was AUU CUA UCA CCA GCG UCU CU (25). siRNA oligonucleotides were purchased from Dharmacon (Lafayette, CO). Calcium phosphate-based transfection reagent (CellPect transfection kit) was purchased from Amer sham Biociences (Baie d’Urfe, QC, Canada).

Cell culture. L6-GLUT4myc myoblasts were seeded onto glass coverslips (for Ca2+ measurements) or 12-well (for protein extraction) or 24-well (for all other assays) plates and were differentiated into myotubes in α-MEM supplemented with 2% (vol/vol) FBS and 1% (vol/vol) antibiotics/antimycotics. Cells were allowed to fully differentiate into myotubes and were deprived of serum for 3–5 h prior to any experimental manipulation.

Cell treatments. Unstimulated cells were incubated in HEPES-buffered saline (HBS) consisting of (in mM) 5 β-glucoside, 20 HEPES, 5 KCl, 140 NaCl, 1 CaCl2, and 2.5 MgSO4, pH 7.4. A DNP stock solution (0.5 M) was prepared in DMSO. DNP and insulin were diluted in the incubation buffer to final concentrations of 0.5 mM and 100 nM, respectively. K, Na, or NMG solutions contained (in mM) 5 glucose, 20 HEPES, 1 CaCl2, 2.5 MgSO4, and 40, 80, or 120 mM potassium glutamate, sodium glutamate, or NMG, respectively, pH 7.4. Osmolarity was adjusted to 300 mosM (determined by depression of the freezing point) with NaCl. All inhibitors were prepared in DMSO and diluted in HBS to indicated concentrations. The final concentration of DMSO never exceeded 0.1% (vol/vol). All stimulations were for 20 min unless indicated, and when the effect of an inhibitor was being investigated, the cells were pretreated with the inhibitor for indicated duration and then stimulated in the continuous presence of the inhibitor. Control cells were treated with DMSO only. Determination of 2-deoxy-D-[3H]glucose uptake in cells. 2-Deoxyglucose (2-DG) uptake was measured as described earlier (34). Briefly, at room temperature, 2-DG uptake was measured for 5 min in the respective ionic solution containing 10 μM nonradioactive 2-DG and 2-deoxy-[3H]glucose (1 μCi/ml). The reaction was stopped by two washes with ice-cold 0.9% NaCl (wt/vol) containing 25 mM β-n-glucose. When used, 100 μM indinavir was added to the transport solution only. Experiments with EGTA were performed in Ca2+-free HBS. A typical experiment analyzed the relevant conditions in triplicate to assess intraexperimental variation, and each experiment was repeated the number of times indicated in the figure legends, typically on different dates. For each experiment, the averaged uptake in unstimulated cells (basal) was ascribed a value of 1, and all results are expressed relative to this value.

Immunodetection of cell surface GLUT4myc. The amount of cell surface GLUT4myc was determined by an antibody-coupled colorimetric absorbance assay, as was previously described (66). Briefly, following stimulation, cells were exposed to polyclonal anti-myc antibody (1:100) for 60 min, fixed with 4% paraformaldehyde (PFA) for 10 min, and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:1,000) for 1 h. Cells were then washed six times, and 1 ml of OPD reagent (0.4 mg/ml o-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydrogen peroxide) was added for 30 min at room temperature. The reaction was stopped with 0.25 ml of 3 N HCl. Optical absorbance of the supernatant was measured at 492 nm. Background, as measured in samples incubated in peroxidase-conjugated anti-rabbit IgG alone (without primary antibody), was subtracted from all values. BAPTA-AM and EGTA experiments were performed in Ca2+-free HBS. Each experimental condition was tested in triplicate within each experiment, and each experiment was repeated the number times indicated in the figure legends, typically on different dates. For each experiment, the averaged reading in unstimulated cells (basal) was ascribed a value of 1, and all results are expressed relative to this value.

Detection of enzyme phosphorylation. Myotubes were stimulated for 10 min, and cell lysates were prepared from 12-well plates, as earlier described (60). The cells were lysed with 150 μl of 2X Laemmli sample buffer supplemented with 1 mM DTT, 1 mM Na3VO4, 100 mM okadaic acid, protease inhibitors (1 mM benzamidine, 10 μM E-64, 1 μM leupeptin, 1 μM pepstatin A, 0.2 mM PMSF), and 7.5% β-mercaptoethanol and heated for 30 min at 65°C. The lysates were then resolved by 10% SDS-PAGE and immunoblotted with antibodies to phospho-Thr172-Akt (1:1,000, 60 kDa), phospho-Thr286-CaMKII (1:1,000, 50 kDa), phospho-Thr172-AMPKα (1:1,000, 65 kDa), and α-actin-1 (1:1,000, 104 kDa). Immunoblots were scanned within the linear range and quantified using the computer software NIH Image J.

siRNA transfection. Cells were seeded in antibiotic-free medium and on day 4 were transfected with AMPKα1 and AMPKα2 siRNA (90 nM of each oligonucleotide sequence) using calcium phosphate-based transfection reagent (CellPect transfection kit) per the manufacturer’s instruction. Twelve to sixteen hours after transfection, cells were washed and the medium was changed to fresh α-MEM supplemented with 2% FBS. Experiments were performed 72 h posttransfection.

Determination of intracellular Ca2+. The intracellular Ca2+ concentration of myotubes was measured as previously described (50). Cells were seeded onto coverslips and loaded with 1 μg/ml fura 2-AM in HBS for 30 min at 37°C. Coverslips were washed twice with HBS and mounted in a thermoregulated chamber. After recording of baseline fluorescence in HBS, the cells were stimulated, and at the indicated times and the fura 2 fluorescence was recorded as the ratio of 340/380 nm. Corresponding intracellular Ca2+ concentrations were calculated per instructions by Molecular Probes, after determination of the minimum ratio obtained in the presence of 15 μM BAPTA-AM and 2.5 mM EGTA and the maximum ratio obtained in the presence of 1 μg/ml ionomycin.

Measurement of GLUT4myc externalization (recycling). The rate of GLUT4myc externalization was determined as described (15). Briefly, at 37°C, cells were stimulated while being exposed to polyclonal anti-myc antibody (1:200) for the times indicated. At each time point, cells were fixed with 3% PFA for 10 min, permeabilized with 0.1% Triton X-100 for 15 min, and lastly reacted with peroxidase-conjugated goat anti-rabbit IgG (1:1,000) for 1 h at 4°C to determine the amount of anti-myc antibody bound at the cell surface as well as that which had become labeled at the surface and subsequently internalized during the incubation time. Thus the amount of GLUT4myc labeled was determined using the OPD absorbance assay and was expressed as a percentage of total cellular GLUT4myc content. Total cellular GLUT4myc was determined by incubating the cells with the anti-myc antibody following permeabilization and then proceeding with the secondary antibody as above.
Measurement of GLUT4myc internalization (endocytosis). GLUT4myc internalization was measured as previously described (40), with slight modifications. Briefly, cells were stimulated with the respective stimulus to bring the transporters to the surface. Cells were rinsed with ice-cold PBS and reacted with the polyclonal anti-GLUT4 antibody (1:200) at 4°C for 1 h. The surface-labeled GLUT4myc was then allowed to internalize by rewarming the cells to 37°C in the presence or absence of the respective stimulus. At the indicated times, cell plates were placed on ice, washed three times with ice-cold PBS, fixed with 4% PFA for 10 min, and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:1,000) for 1 h at 4°C. The amount of GLUT4myc remaining on the cell surface at any time point after rewarming was determined using the OPD absorbance assay and was expressed as a percentage of the cell surface GLUT4myc level at 0 min of endocytosis.

Statistical analysis. Statistical analyses were carried out using Prism 3.0 software (San Diego, CA). Two groups were compared using Student’s paired t-test and more than two groups were compared using analysis of variance (ANOVA) with Tukey’s post hoc analysis.

RESULTS

The muscle cell membrane maintains a membrane potential centered around the Nernst potential for K⁺ due to the dominance of K⁺ conductance over that of Na⁺ and the active removal of intracellular Na⁺ by the Na⁺/K⁺ pump. Membrane depolarization can be achieved by increasing extracellular K⁺ concentration (which would reduce its Nernst potential) or by increasing the conductance to Na⁺ ions. Because the muscle cell membrane is rather permeable to Cl⁻ ions, the elevations in extracellular K⁺ are preferably done in solutions where nonpermeating anions such as gluconate replace Cl⁻ to avoid net gains in salt and concomitant cell swelling.

As previously shown (35), replacement of extracellular NaCl with potassium gluconate causes membrane depolarization in L6 cells in a dose-dependent manner, and, as shown in Fig. 1A, 2-DG uptake into L6-GLUT4myc cells increases in parallel. In contrast, replacing extracellular Na⁺ with NMG⁺, a poorly permeating cation failed to increase 2-DG uptake into the cells (Table 1), confirming that the response is not the result of Na⁺ ion removal from the medium. Furthermore, replacement of potassium gluconate with sodium gluconate also failed to increase 2-DG uptake (Table 1), confirming that the response is due to the presence of K⁺ ions in the extracellular medium. Similar to K⁺ depolarization, addition of the Na⁺ ionophore gramicidin, which readily de polarizes cells in normal NaCl-containing medium, increased 2-DG uptake (Table 1). These results suggest that two distinct strategies that depolarize the muscle cell membrane increase glucose uptake regardless of the ionic mechanism achieved to elicit depolarization.

To verify that the increase in hexose uptake was due to a change in the rate of transmembrane uptake and not subsequent phosphorylation, we measured the effect of K⁺ depolarization on the influx of 3-O-methylglucose, a nonphosphorylatable hexose. As shown in Fig. 1B, the potassium gluconate solution elevated the uptake of this hexose, demonstrating an increase...
in the earliest step in glucose uptake. To assess the contribution of GLUT4 to hexose flux, 2-DG transport into L6-GLUT4myc cells was measured in the presence of indinavir, a drug that directly binds and specifically inhibits GLUT4 (53). As in previous observations, both basal and insulin-stimulated 2-DG uptake were inhibited by 70% in the presence of indinavir (Fig. 1C) (53). Similarly, 70% of K⁺ depolarization-induced 2-DG uptake was inhibited by indinavir, suggesting that GLUT4 is the primary glucose transporter contributing to K⁺ depolarization-stimulated glucose uptake.

Associated with glucose uptake was a gain in GLUT4myc on the PM provoked by K⁺ depolarization (Fig. 2A). The K⁺ depolarization-induced gain in surface GLUT4myc was additive to that of insulin (Fig. 2A), suggesting that the two stimuli may engage distinct and independent signaling pathways. Insulin activates class I, class II (C2α), and class IIβ phosphatidylinositol 3-kinases (PI3K), and extensive pharmacological and molecular studies support the participation of class I PI3K in insulin-stimulated glucose uptake (61). Moreover, the major product of class II PI3K, phosphatidylinositol 3-phosphate, also contributes to GLUT4 mobilization (30, 31, 43). Class I PI3K is inhibited by 100 nM wortmannin, whereas obliterating class II activity requires up to 10 times higher concentrations in intact cells (12). The participation of any PI3K in exercise-induced glucose uptake is controversial (52). To explore the possible participation of either class of PI3K in the depolarization-induced glucose uptake, L6 GLUT4myc myotubes were exposed to 100 nM or 1 μM wortmannin during depolarization and surface GLUT4myc was subsequently measured. Figure 2B shows that the K⁺ depolarization-induced gain in surface GLUT4myc was unaffected by the higher concentration of wortmannin, whereas the insulin effect was completely abolished (similar results were observed with 100 nM wortmannin; not shown). Similarly, although insulin caused a robust increase in the phosphorylation of the PI3K effector Akt, no change in Akt phosphorylation was observed upon K⁺ depolarization (Fig. 2C). Hence, the GLUT4 response induced by K⁺ depolarization does not appear to require the participation of either PI3K or Akt. A further distinction between the insulin and depolarization effects was noted upon disruption of actin dynamics. An intact actin cytoskeleton and PI3K-dependent actin remodeling are required for insulin-mediated GLUT4 translocation in muscle cells (48). Cytochalasin D, a specific inhibitor of actin filament assembly and a filament-severing agent, had no effect on the K⁺ depolarization-mediated gain in surface GLUT4myc while completely inhibiting the insulin effect (Fig. 2D). This result suggests that the gain in surface GLUT4 elicited by K⁺ depolarization proceeds in the absence of an intact actin network.

In the parental L6 muscle cells, depolarization by K⁺ has been associated with a rise in cytosolic Ca²⁺ (35). Similarly, as measured by the Ca²⁺ indicator fura 2-AM, the cytosolic Ca²⁺ concentration rose in the presence of elevated extracellular K⁺ in L6-GLUT4myc myotubes (Fig. 3A). Pretreatment of cells with dantrolene, an inhibitor of Ca²⁺ release from the SR (64), prevented the K⁺ depolarization-induced gain in surface GLUT4myc (Fig. 3B), suggesting that Ca²⁺ release from intracellular stores is relevant to bringing about the GLUT4 response. Importantly, dantrolene did not preclude the insulin response of GLUT4. EGTA, which chelates extracellular Ca²⁺, also completely abolished the K⁺ depolarization-induced gain in surface GLUT4myc (Fig. 3C). Surprisingly, the presence of extracellular EGTA also partially inhibited insulin-induced gain in surface GLUT4myc. This is in agreement with previous reports that insulin causes a local rise in Ca²⁺ beneath the PM and that Ca²⁺ chelation reduces insulin-mediated glucose transport (5, 6, 67, 70).

Recent findings suggest that AMPK may be activated in a Ca²⁺-dependent manner, given the activation of this enzyme by CaM kinase isofoms (19, 27, 69). Interestingly, K⁺ depolarization caused an increase in AMPK phosphorylation (Fig. 4A), although to a lesser extent than did DNP, a mitochondrial uncoupler and known activator of AMPK (47). As expected, stimulation with insulin did not alter the phosphorylation state of AMPK. To seek a causative role of AMPK in K⁺ depolarization-induced gain in surface GLUT4, the amount of AMPKα protein was reduced by using a combination of two siRNA oligonucleotide sequences targeting the α1 and α2 subunits (37). The combined siRNA lowered AMPKα expression by 85 ± 5.4% (P < 0.0001) compared with cells transfected with a nonrelated siRNA sequence (Fig. 4B). This reduction was associated with a 65 ± 9.7% (P < 0.05) drop in K⁺ depolarization-induced AMPK phosphorylation (Fig. 4C). Cells transfected with AMPKα siRNA displayed the normal insulin-induced gain in surface GLUT4myc, whereas the DNP-mediated response was completely abolished compared with the cells transfected with nonrelated siRNA (Fig. 4D). Surprisingly, the K⁺ depolarization-induced gain in surface GLUT4myc was unaffected by the reduction in AMPKα protein level. These results suggest two possibilities: that although AMPK phosphorylation is induced by K⁺ depolarization, this enzyme is not required for the K⁺ depolarization-mediated response of GLUT4 or that the 10–20% of remaining AMPKα expression is sufficient to mediate the K⁺ depolarization-induced effects.

We therefore explored whether CaMKII, a major CaMK isoform in muscle, is activated by K⁺ depolarization. Activation of this enzyme is reflected in its state of phosphorylation. As shown in Fig. 5A, although DNP robustly increased CaMKII phosphorylation, K⁺ depolarization, like insulin, failed to induce any response. Moreover, pretreatment with the non-isoform-specific CaMK inhibitor KN62, which binds to
the calmodulin-binding site of the enzyme (11), did not affect significantly either K⁺ depolarization- or insulin-induced gain in surface GLUT4myc (Fig. 5B). These results suggest that CaMKII is unlikely to be a downstream target of Ca²⁺/H⁺ mediating the K⁺ depolarization-induced responses.

In our search for the Ca²⁺ pathway target leading to the depolarization-induced rise in surface GLUT4, the participation of PKC was explored using PKC inhibitors. At low concentrations, BIM and calphostin C inhibit PKC by binding, respectively, to the ATP and diacylglycerol binding sites (2, 4). At a concentration of 1 μM, BIM completely abolished the K⁺ depolarization-elicited gain in surface GLUT4myc without affecting either basal or insulin-dependent levels of the transporter at the membrane (Fig. 5C). Similarly, at 250 nM, calphostin C did not alter either basal or insulin-stimulated amounts of surface GLUT4myc but completely inhibited the response to K⁺ depolarization (Fig. 5D). Therefore, PKC is a plausible mediator of the depolarization-elicited rise in surface GLUT4.

The K⁺ depolarization-induced elevation in surface GLUT4 may result from an increase in GLUT4 exocytosis, a reduction...
in its endocytosis, or both. To determine whether K⁺ depolarization can boost the rate of GLUT4myc exocytosis, the time course of GLUT4myc appearance on the cell surface was detected in a live-cell recycling assay. The half-times for labeling of all GLUT4myc at the cell surface with anti-myc antibody in basal, insulin, and K⁺ depolarization-stimulated states were 90, 30, and 60 min, respectively (Fig. 6A). This result indicates that K⁺ depolarization increases the rate of GLUT4 exocytosis, although not to the same extent as insulin. Next, we examined whether K⁺ depolarization led to a reduction in GLUT4 endocytosis by measuring the surface GLUT4myc remaining at different times after internalization of labeled GLUT4myc. Both in basal and in insulin-stimulated states, GLUT4myc was internalized rapidly and to a similar extent (Fig. 6B). The half-time of GLUT4myc internalization in basal, insulin, and K⁺ depolarization-stimulated states was 3.5, 4, and 8 min, respectively. Under K⁺ depolarization, GLUT4myc was largely retained at the cell surface, so that 60 ± 4% of the amount labeled remained associated with the surface after 20 min (compared with 43 ± 5% in the basal state). Therefore, the K⁺ depolarization-induced increase in surface GLUT4 levels is prominently associated with a reduction in GLUT4 endocytosis.

**DISCUSSION**

Several studies in isolated rat or frog skeletal muscles show that membrane depolarization can stimulate glucose uptake into muscle (9, 22, 24, 63). Our results show for the first time that muscle cell depolarization without contraction increases glucose uptake in L6-GLUT4myc myotubes. The effects are comparable regardless of the mechanisms used to achieve depolarization (high extracellular K⁺ or gramicidin). A study in the cardiomyocyte-derived cell line H9c2 also showed elevated glucose uptake and GLUT4 in response to depolarization (75). Therefore, it is plausible that the trigger of skeletal muscle contraction already has the ability to signal for an increase in glucose uptake into muscle fibers, presumably in anticipation of elevated energy requirement during contraction.

In the L6 cell system, depolarization increased the cytosolic Ca²⁺ levels measured by integrating the fura 2 signal over the entire myotube. As this is a measurement of the whole cytosolic Ca²⁺ concentration, it is not surprising that the increase observed was small (150 nM) compared with the change observed in contracting skeletal muscle (up to 300 nM in slow-twitch and 2 μM in fast-twitch muscles) (8). Although it is conceivable that depolarization may cause local transient Ca²⁺ spikes, possibly near the membrane, modest changes in cytosolic Ca²⁺ levels may suffice to trigger the response of glucose uptake. Indeed, landmark studies from Holloszy's group [Wright et al. (71) and Yoon et al. (74)] have shown that glucose uptake into skeletal muscle can be induced upon elevation of cytosolic Ca²⁺ concentration below the contraction threshold, using caffeine or W7 to elicit release of the ion from the SR. In the cell culture, we further demonstrate the need for both extracellular Ca²⁺ and Ca²⁺ from intracellular stores to induce a gain in surface GLUT4. These observations suggest that Ca²⁺-induced Ca²⁺ release may be the mechanism whereby depolarization increases cytosolic Ca²⁺ levels. L6 myotubes do not develop transverse tubules even though they express dihydropyridine receptors, typical of the tubules in the mature muscle (44). In the latter tissue, Ca²⁺-induced Ca²⁺ release becomes relevant when the voltage-gated Ca²⁺ chan-

---

**Fig. 3.** K⁺ depolarization-mediated responses require Ca²⁺ efflux from intracellular stores. A: K⁺ depolarization induces elevation of cytosolic Ca²⁺. Cells were seeded on coverslips and loaded with 1 μg/ml fura 2-AM for 30 min. After recording of baseline fluorescence, cells were stimulated with 120 mM potassium gluconate. At indicated times, fura 2 fluorescence was recorded at 340/380 nm, and cytosolic Ca²⁺ concentrations were calculated from the ratio of fluorescence intensities. Results are expressed as means ± SE of 3 independent experiments. B and C: dantrolene and EGTA prevent K⁺ depolarization-induced gain in surface GLUT4myc. Myotubes were pretreated without or with 25 μM dantrolene (B) or 2.5 mM EGTA (C) for 15 min and then incubated for 20 min in HEPES-buffered saline, 120 mM potassium gluconate, or 100 nM insulin in HEPES-buffered saline in the absence (open bars) or presence (filled bars) of the chelators/inhibitors. Cell surface GLUT4myc was measured and expressed as means ± SE of 3 to 4 independent experiments. EGTA experiments were done in Ca²⁺-free solutions. *P < 0.05 vs. basal. All values are normalized relative to their respective basal control.
nels are dissociated from the Ca$^{2+}$ release channels of the SR (58). Otherwise, the mechanical communication between the T-tubular voltage-gated Ca$^{2+}$ channels and SR Ca$^{2+}$ release channels is the predominant means of increasing cytosolic Ca$^{2+}$ (58).

The elevation in cytosolic Ca$^{2+}$ could facilitate the activation of signaling molecules that leads to the increase in glucose uptake. AMPK is a key signaling molecule implicated in insulin-independent glucose transport (20). Recent suggestions regarding a Ca$^{2+}$-dependent activation of this enzyme led us to investigate its role in depolarization-induced glucose uptake (19, 27, 69). Although AMPK was phosphorylated during depolarization, gene silencing of AMPK α-subunits was without effect on the depolarization-induced gain in PM GLUT4. AMPK plays a variety of roles in regulating energy control, and therefore it may participate in alternative responses elicited by depolarization (7). It is possible that AMPK may become important for glucose uptake beyond a certain activation threshold and/or once muscle contraction has ensued. AMPK is best known to be activated in response to a reduction in the

---

**Fig. 4.** AMPK is not required for the gain in surface GLUT4myc induced by K$^{+}$ depolarization. A: AMPK is phosphorylated during K$^{+}$ depolarization. Myotubes were stimulated for 10 min in HEPES-buffered saline, 120 mM potassium gluconate, 100 nM insulin in HEPES-buffered saline, or 0.5 mM dinitrophenol (DNP) in HEPES-buffered saline. Lysates were resolved by SDS-10% PAGE and immunoblotted with antibody to phospho-Thr172-AMPK (62 kDa) or antibody to α-actinin-4 (104 kDa) to assess sample loading. Representative immunoblots are shown, with position of weight marker standard proteins indicated at right. Immunoblots were scanned within the linear range and quantified with NIH Image J software. Quantified values represent means ± SE from 6 independent experiments. *P < 0.05 vs. basal. B and C: AMPK gene silencing via siRNA. B: myotubes were transfected with nonrelated siRNA (NR) or combination of 2 siRNA sequences directed against the 2 α-subunits of AMPK (AMPKα1/2 siRNA) for 72 h. Lysates were resolved by SDS-10% PAGE and immunoblotted with anti-AMPK or anti-α-actinin-4. Representative immunoblots are shown, with position of weight marker standard proteins indicated at right. C: transfected myotubes were incubated in 120 mM potassium gluconate for 10 min. Lysates were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-Thr172-AMPK or anti-α-actinin-4 antibodies. Immunoblots were scanned within the linear range and quantified using NIH image J. Quantified values represent means ± SE of 3–5 experiments and are expressed relative to cells transfected with NR siRNA. All values are normalized according to α-actinin-4 content. *P < 0.001, **P < 0.05 vs. cells transfected with NR siRNA. D: reduction in AMPKα1/2 does not affect K$^{+}$ depolarization-induced gain in surface GLUT4myc. Transfected cells were incubated in HEPES-buffered saline, 120 mM potassium gluconate, 100 nM insulin in HEPES-buffered saline, or 0.5 mM DNP in HEPES-buffered saline for 20 min. Cell surface GLUT4myc was measured and expressed as means ± SE of 4 independent experiments. *P < 0.05 vs. NR-transfected, DNP-stimulated cells; **P < 0.05 vs. respective basal. Open bars, NR siRNA transfected cells; filled bars, AMPKα1/2 siRNA transfected cells. All values are normalized relative to respective basal control.
ATP-to-AMP ratio (18). Therefore, it is possible that the cellular ATP level needs to be compromised to achieve the necessary threshold of AMPK activity. DNP is a mitochondrial uncoupler that can markedly reduce cellular ATP levels and is an established activator of AMPK (47). Accordingly, through the use of AMPK α-subunit gene silencing, we observed that AMPK is required for the DNP-induced stimulation of glucose uptake. We have previously shown (32) that the response of glucose uptake to DNP is partially Ca²⁺-dependent. These results suggest that both Ca²⁺ and changes in the ATP/AMP ratio, in combination, may contribute to AMPK activation in response to DNP.

CaMKs are calmodulin-dependent kinases that are activated in the presence of Ca²⁺ (57). The involvement of CaMKII in depolarization-induced glucose uptake was explored because this is a major isoform expressed in skeletal muscle proposed to be involved in the stimulation of muscle glucose uptake (1, 8, 71). The lack of phosphorylation of CaMKII in depolarized L6 myotubes suggests that this enzyme is not activated, at least at 10 min of stimulation. Half-maximal activation of CaMKII occurs at a concentration of 0.5–1 μM free Ca²⁺ (26). Thus the intracellular Ca²⁺-raised in 10 min of depolarization (80 nM) may have been insufficient to cause CaMKII phosphorylation. Alternatively, CaMKII phosphorylation may have been transient and missed by the single-time determination. However, the lack of effect of the CaMK inhibitor KN62 suggests that this family of enzymes is unlikely to participate in the depolarization-induced gain in surface GLUT4. Nonetheless, a more thorough investigation is required to systematically test the possible participation of each of the CaMK isoforms, including myosin light-chain kinase and eukaryotic elongation factor-2 kinase.

Because conventional PKC isoforms are directly activated by elevated cytosolic Ca²⁺ (46) and K⁺ depolarization of frog
skeletal muscle caused accumulation of diacylglycerol and PKC translocation and activation (59), we investigated the possible contribution of the PKC family to depolarization-mediated glucose transport. Here, we report that two PKC inhibitors, at concentrations that target conventional and novel but spare atypical PKC, obliterate the depolarization-stimulated gain in surface GLUT4. Further experiments are required to confirm PKC involvement and identify the participating isoform(s). Because Ca^{2+} chelation and PKC inhibition had similar outcomes, it is likely that the two targeted signals work in series. Intriguingly, in earlier work we failed to see an increase in glucose uptake in the parental line of L6 myotubes exposed to the Ca^{2+} ionophores A23187 (33) or ionomycin (36). In the parental line, GLUT1 contributes significantly to glucose uptake (53), possibly obscuring any effect that cytosolic Ca^{2+} might have had on the endogenous GLUT4 (those cells showed low response to insulin and no response to hyperosmolarity or depolarization, unlike the GLUT4/myc-expressing line used in the present study).

A major goal of this study was to map the impact of depolarization on mechanisms of GLUT4 traffic. Insulin increases cell surface GLUT4 level largely by increasing GLUT4 exocytosis (40, 56). An intact actin network, PI3K and Akt are required for GLUT4 translocation to the PM in response to the hormone (48). Conversely, we demonstrate here that the gain in surface GLUT4 induced by depolarization is not prevented by wortmannin at concentrations that inhibit both classes I and II PI3K. Moreover, K^{+} depolarization did not increase Akt activity, which is, however, robustly activated by insulin. In mature muscle, the participation of PI3K and Akt in contraction-induced glucose uptake is debatable (52), but most studies agree that isolated skeletal muscle mounts an increase in glucose uptake during contraction that is independent of these two enzymes. Our results suggest that at least the triggering event of muscle contraction does not involve either signaling molecule. In addition, the depolarization-mediated gain in cell surface GLUT4 does not require an intact actin network, consistent with the observation that PI3K activation is a prerequisite for insulin-dependent actin remodeling in muscle cells (48). Accordingly, in our search for differences in the mechanisms that underlie the gain in surface GLUT4 in response to depolarization and insulin, we compared the contribution of the two arms of GLUT4 traffic, exocytosis and endocytosis. The results revealed that, during depolarization, the gain in GLUT4 on cell surface is achieved primarily by reducing the rate of endocytosis with a small increase in the rate of exocytosis. This behavior is radically opposite to that elicited by insulin. In contrast, a previous study shows that, in primary cardiomyocyte cultures, electrical stimulation only promotes GLUT4 exocytosis (72). The divergence of these observations is likely due to differences in the tissue and cellular systems utilized and the mode of stimulation.

Although the observed effects may be specific to GLUT4, there is a possibility that the reduction in GLUT4 endocytosis is due to a generalized effect of depolarization on the membrane and thus on vesicular traffic. Hypertonic sucrose retains GLUT4 on the PM by disrupting the clathrin-coated pit formation and, hence, preventing GLUT4 endocytosis (40). Depolarization may function in a similar manner to retain GLUT4 molecules. In addition, the depolarization-mediated gain in cell surface GLUT4 does not require an intact actin network, consistent with the observation that PI3K activation is a prerequisite for insulin-dependent actin remodeling in muscle cells (48). Accordingly, in our search for differences in the mechanisms that underlie the gain in surface GLUT4 in response to depolarization and insulin, we compared the contribution of the two arms of GLUT4 traffic, exocytosis and endocytosis. The results revealed that, during depolarization, the gain in GLUT4 on cell surface is achieved primarily by reducing the rate of endocytosis with a small increase in the rate of exocytosis. This behavior is radically opposite to that elicited by insulin. In contrast, a previous study shows that, in primary cardiomyocyte cultures, electrical stimulation only promotes GLUT4 exocytosis (72). The divergence of these observations is likely due to differences in the tissue and cellular systems utilized and the mode of stimulation.
GLUT4 molecules responsible for the depolarization-induced glucose transport originate from the same pool of transporters that maintain basal glucose uptake (i.e., the recycling endosomes). This idea resonates with the increase in surface transferrin receptor, a marker of the endosomal recycling compartment, observed in contracting muscle (39) and with immuno-fluorescence studies, suggesting that contraction mobilizes the transferrin receptor-associated pool of GLUT4 (49). In contrast, insulin induces translocation of a distinct pool of GLUT4 transporters largely devoid of transferrin receptors (14, 29).

In summary, depolarization-induced glucose transport produces its effects independently of the insulin-signaling pathway and an intact actin network. By contrast, the depolarization signal transduction may require participation of cytosolic Ca\(^{2+}\) and PKC isoforms but not AMPK and CaMKII. The depolarization-induced gain in surface GLUT4 is achieved largely by slowing down the rate of GLUT4 endocytosis. Further studies should identify the molecular mechanisms underlying such events.

ACKNOWLEDGMENTS

We thank Dr. P. J. Bilan for helpful advice and careful reading of the manuscript.

GRANTS

This work was supported by a grant from the Canadian Diabetes Association to A. Klip. Studentship funding for N. Wijesekara and A. Tung was provided by grants to A. Klip from the Canadian Diabetes Association and the Canadian Institutes of Health Research. F. Thong was supported by postdoctoral fellowships from the Natural Science and Engineering Research Council of Canada and the Canadian Institutes of Health Research.

REFERENCES

MEMBRANE DEPOLARIZATION-INDUCED GAIN IN SURFACE GLUT4


