Ca²⁺ signals promote GLUT4 exocytosis and reduce its endocytosis in muscle cells

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Li Q, Zhu X, Ishikura S, Zhang D, Gao J, Sun Y, Contreras-Ferrat A, Foley KP, Lavandero S, Yao Z, Bilan PJ, Klip A, Niu W. Ca²⁺ signals promote GLUT4 exocytosis and reduce its endocytosis in muscle cells. Am J Physiol Endocrinol Metab 307: E209-E224, 2014. First published June 3, 2014; doi:10.1152/ajpendo.00045.2014.-Elevating cytosolic Ca²⁺ stimulates glucose uptake in skeletal muscle, but how Ca²⁺ affects intracellular traffic of GLUT4 is unknown. In tissue, changes in Ca²⁺ leading to contraction preclude analysis of the impact of individual, Ca2+-derived signals. In L6 muscle cells stably expressing GLUT4myc, the Ca²⁺ ionophore ionomycin raised cytosolic Ca²⁺ and caused a gain in cell surface GLUT4myc. Extraand intracellular Ca2+ chelators (EGTA, BAPTA-AM) reversed this response. Ionomycin activated calcium calmodulin kinase II (CaM-KII), AMPK, and PKCs, but not Akt. Silencing CaMKIIô or AMPK α 1/ α 2 partly reduced the ionomycin-induced gain in surface GLUT4myc, as did peptidic or small molecule inhibitors of CaMKII (CN21) and AMPK (Compound C). Compared with the conventional isoenzyme PKC inhibitor Gö6976, the conventional plus novel PKC inhibitor Gö6983 lowered the ionomycin-induced gain in cell surface GLUT4myc. Ionomycin stimulated GLUT4myc exocytosis and inhibited its endocytosis in live cells. siRNA-mediated knockdown of CaMKIIS or AMPKa1/a2 partly reversed ionomycin-induced GLUT4myc exocytosis but did not prevent its reduced endocytosis. Compared with Gö6976, Gö6983 markedly reversed the slowing of GLUT4myc endocytosis triggered by ionomycin. In summary, rapid Ca²⁺ influx into muscle cells accelerates GLUT4myc exocytosis while slowing GLUT4myc endocytosis. CaMKII8 and AMPK stimulate GLUT4myc exocytosis, whereas novel PKCs reduce endocytosis. These results identify how Ca2+-activated signals selectively regulate GLUT4 exocytosis and endocytosis in muscle cells.

GLUT4; cytosolic Ca²⁺, endocytosis; exocytosis; CAMKII; AMPK; PKC

GLUCOSE UTILIZATION BY EXERCISING MUSCLE involves the coordinated increase in vascular glucose delivery, enhanced glucose transport across the muscle fiber cell surface, and glucose metabolism (37). Glucose transport is a major regulated step in this process, and the number of GLUT4 glucose transporter units at the plasma membrane (PM) and t-tubule domains rapidly rises with muscle contraction. In the short term, this occurs through the mobilization of GLUT4-containing intracellular vesicles independently of new protein synthesis (10, 17, 30). The molecular mechanisms that elevate cell surface GLUT4 levels are vigorously debated. By contrast, insulin promotes GLUT4 exocytosis, as deduced from cultured muscle cells (14) and whole tissue imaging (26, 29, 40). Skeletal muscle cells offer the unique advantage of allowing measurements of GLUT4 endocytic and exocytic rates and to couple them to manipulations of signaling pathways (2, 19).

Three major effects of contraction have been proposed to lead to the downstream stimulation of glucose uptake into muscle: a rise in cytosolic Ca^{2+} , changes in the muscle energy charge, and mechanical stretch. Each of these in turn elicits intracellular signals that have been considered as possible mediators of the stimulated glucose influx. Signals triggered directly by the rise in intracellular Ca²⁺ include activation of calmodulin (CaM)-dependent kinases (e.g., CaMKK and CaM-KII) and conventional isoforms of protein kinase C (PKC). The paramount intracellular signal triggered by changes in the AMP/ATP ratio is activation of the AMP-activated protein kinase (AMPK). Signals triggered by mechanical stretch include the small G protein Rac1 and its downstream effector kinases PAK1/2 (8, 42). These pathways may also intersect one another to affect glucose uptake, as exemplified by the activation of AMPK by CaMKK (22, 49).

Numerous studies have examined the possible participation of each of the above signals in the stimulation of glucose uptake by muscle (16, 24, 37), but it is virtually unknown how they affect GLUT4 traffic. In particular, an increase in steadystate level of GLUT4 at the PM may arise from a stimulation of its mobilization to/insertion into the PM (exocytosis) or reduction in its internalization from the surface and return to intracellular stores (endocytosis). Given the complex architecture of skeletal muscle and the scarcity of tools to study the endogenous GLUT4 at a subcellular level in the tissue, it has not been possible to ascertain whether or how the individual signals normally activated by contraction affect GLUT4 dynamics.

To circumvent these difficulties, we generated muscle cell lines stably expressing a *myc*-tagged GLUT4 that allows for quantification of the rates of GLUT4 endocytosis and it subsequent exocytosis, relying on antibody detection of surface GLUT4 levels in nonpermeabilized cells (19, 46). By avoiding the complication of ongoing acto-myosin contraction, these noncontracting cells allowed us to study the consequence of the

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earliest signal arising during muscle contraction, i.e., a rapid elevation in cytosolic Ca^{2+} . Indeed, in skeletal muscle, small, caffeine-induced increases in the concentration of cytosolic Ca^{2+} that do not trigger contraction can stimulate glucose uptake (21, 52).

Here, we investigate how a rapid elevation in intracellular Ca^{2+} brought about by the Ca^{2+} ionophore ionomycin regulates GLUT4 traffic in L6-GLUT4*myc* myoblasts and examine the underlying signals controlling defined arms of GLUT4*myc* traffic. Ionomycin increased GLUT4*myc* exocytosis and reduced its endocytosis. In this context, CaMKII and AMPK were the major contributors to GLUT4*myc* exocytosis, whereas PKCs also regulated GLUT4*myc* endocytosis.

MATERIALS AND METHODS

Reagents and siRNAs. Ionomycin, Compound C, Gö6976, Gö6983, and blasticidin S-hydrochloride were from EMD Millipore (Billerica, MA). Human insulin (Humulin R) was from Eli Lilly (Toronto, ON, Canada). BAPTA-AM and okadaic acid were from Biomol (Plymouth Meeting, PA). Fluo 3-AM was from Invitrogen (Carlsbad, CA). STO-609 was purchased from Tocris Bioscience. The CN21 peptidic inhibitor of CaMKII, YGRKKRRQRRRKRPPKLGQIGRSKRV-VIEDDR, was provided by Dr. K. Ulrich Bayer (University of Colorado, Aurora, CO) as described (45), Horseradish peroxidase (HRP)-bound goat anti-rabbit IgG antibody and donkey anti-mouse IgM antibody were from Jackson ImmunoResearch Laboratories (West Grove, PA). Polyclonal antibodies to phospho (p)-AMPKa (Thr¹⁷²), p-CaMKII (Thr²⁸⁶), p-acetyl-CoA carboxylase (ACC, Ser⁷⁹), p-ERK1/2, p-p38 MAPK, p-JNK, and p-PAK1/2 (Thr⁴²³), p-Akt (Thr³⁰⁸), p-Akt (Ser⁴⁷³), and phospho-(Ser) PKC substrate antibody (no. 2261) were from Cell Signaling Technology (Danvers, MA). siRNA to CaMKIIS (GCU GAU GCC AGU CAU UGU AdTdT-), AMPKa1 (GCA UAU GCU GCA GGU AGA UdTdT-), and AMPKa2 (-CGU CAU UGA UGA UGA GGC UdTdT-) and nonrelated control (siNR) (-AAU AAG GCU AUG AAG AGA UAC dTdT-) were purchased from GenePharma (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), α-MEM, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were from Wisent (St-Bruno, QC, Canada). o-Phenylenediamine dihydrochloride (OPD), EGTA, DMSO, orthovanadate, protease inhibitor cocktail, rabbit polyclonal IgG to c-myc (epitope), mouse monoclonal IgM to α -actinin-1 (clone BM-75.2), and other chemicals were from Sigma Chemical (Oakville, ON, Canada).

Cell culture and transfection. L6 myoblasts expressing GLUT4 with an exofacial *myc* epitope (L6-GLUT4*myc*) were cultured in α -MEM with 10% FBS (vol/vol), 1% antibiotics-antimycotic (vol/vol), and 2.5 µg/ml blasticidin as described (44). Transfection of siRNA was performed using jetPRIME reagent according to the manufacturer's protocol (Polyplus transfection). Cells were transfected with 100–200 nM of the indicated siRNA for 24 h and then cultured for an additional 48 h.

Determination of cytosolic Ca²⁺. Determination of cytosolic Ca²⁺ was performed essentially as described (32). Serum-deprived cells (3 h) grown on coverslips in α -MEM were washed three times with Ca²⁺-containing resting buffer (145 mM NaCl, 5 mM KCl, 2.6 mM CaCl₂, 1 mM MgCl₂, 5.6 mM D-glucose, and 10 mM HEPES, pH 7.4). Loading with 10 μ M Fluo 3-AM (10 mM stock in 20% pluronic acid in DMSO) was for 50 min at 37°C. Washed coverslips were mounted in a temperature-controlled chamber (37°C) on the stage of a Leica DM IRB microscope. Recording of fluorescence (excitation 490 nm, emission 530 nm) was initiated about 5 min prior to addition of ionomycin, and images were collected every 1.3 s with a PL APO ×40/1.25–0.75 objective using Meta Imaging Series 6.1 software by Molecular Devices (Sunnyvale, CA). For quantification, each cell was outlined using NIH ImageJ software, and the mean of fluorescence

intensity was calculated in n = 15-20 cells per condition. Intracellular Ca²⁺ is reflected as the percentage of fluorescence intensity relative to the resting condition prior to addition of ionomycin. The increase in fluorescence intensity is proportional to a rise in intracellular Ca²⁺ concentration.

Nucleotide measurements. L6-GLUT4myc cells (in 6-well plates) were serum deprived for 3 h followed by treatment with 1 μ M ionomycin for the times indicated. Cells were washed with ice-cold PBS and lysed in 0.4 M HClO₄ in PBS on ice for 10 min following centrifugation at 3,500 rpm for 10 min at 4°C. Supernatants were adjusted to pH 5–7 with 1 M K₂HPO₄. Samples were incubated on ice for an additional 10 min and recentrifuged as above. Supernatants were collected and nucleotide (AMP, ADP, and ATP) concentrations measured by HPLC (High Pressure Liquid Chromatograph, Agilent Technologies 1200 Series) in a 100- μ l injected sample volume. The flow rate of the mobile phase (60 mM KH₂PO₄:60 mM K₂HPO₄, 5:1 vol/vol containing 1% methanol) was 1 ml/min on a C₁₈ column (Kromasil). The detection wavelength was 259 nm, and total retention time was 20 min.

Cell lysates and immunoblotting. L6-GLUT4myc myoblasts (in 6-well plates) were lysed on ice with 300 μ l of RIPA buffer (100 mM NaCl, 0.25% wt/vol sodium deoxycholate, 1.0% wt/vol NP-40, 0.1% wt/vol SDS, 2 mM EDTA, 50 mM NaF, 10 nM okadaic acid, 1 mM sodium orthovanadate, protease inhibitor cocktail, and 50 mM Tris·HCl, pH 7.2) and processed as described (32). Lysates were immunoblotted using PVDF membranes with primary antibodies at 1:1,000 dilutions, except for actinin-1 antibody (1:10,000). Primary antibodies were detected with the appropriate HRP-conjugated secondary antibodies (1:5,000). Detection was completed with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA) and HyBlot CL autoradiography film from Denville Scientific, (Metuchen, NJ).

Steady-state surface GLUT4myc in myoblast monolayers. Cell surface GLUT4myc was detected by a colorimetric assay as described (47). Briefly, L6-GLUT4myc myoblasts (in 24-well plates) were treated with 1 µM ionomycin for 10 min in the continued presence of inhibitors, as required. The cells were washed twice with ice-cold PBS (supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+}) and then fixed for 15 min with 3% (vol/vol) paraformaldehyde (PFA), quenched 10 min with 100 mM glycine in PBS, blocked with ice-cold 5% (vol/vol) goat serum (GS), and reacted with polyclonal anti-myc antibody (1:500) in 5% GS for 1 h at 4°C. After washing six times with PBS, cells were incubated with HRP-bound goat anti-rabbit secondary antibody (1: 1,000) in 5% GS for 1 h at 4°C. Cells were washed six times with PBS and incubated with 1 ml/well of 0.4 mg/ml OPD and allowed to develop for 20-30 min at room temperature to obtain readings in the linear range. The reaction was stopped with 0.25 ml/well of 3 M HCl. Supernatant absorbance were measured at 492 nm. Background absorbance obtained from cells not incubated with anti-myc antibody was subtracted from all values.

GLUT4myc endocytosis. The disappearance of GLUT4myc from the PM was measured as previously described (1), with some modification. Briefly, Serum-deprived (3 h) L6-GLUT4myc myoblasts (in 24-well plates) were blocked with ice-cold 5% GS in PBS for 10 min and then reacted with anti-myc antibody solution (1:300) in 5% GS at 4°C for 1 h. The cells were washed extensively in ice-cold PBS and incubated with α -MEM prewarmed at 37°C for indicated times in the presence or absence of 1 μ M ionomycin (internalization stage). The cells were rapidly washed and fixed with 3% PFA in PBS (15 min), followed by quenching with 100 mM glycine in PBS (10 min) and blocking for 10 min with 5% GS. GLUT4myc bound to anti-myc antibody remaining at the cell surface was detected with HRPconjugated secondary antibody followed by color development using OPD, as described above. The results are expressed as fractions of the surface GLUT4myc level prior to internalization.

GLUT4myc exocytosis. GLUT4myc exocytosis was measured as described (19), with slight modification. After labeling of surface

GLUT4*myc* with anti-*myc* antibody and washing as in the endocytosis assay above, cells were incubated with α -MEM prewarmed at 37°C for 2 h, allowing labeled GLUT4*myc* internalization. Where necessary, cells were pretreated with inhibitors for the last 30 min of the internalization period. Cells were reexposed to 1 μ M ionomycin in the presence or absence of inhibitors and then rapidly washed in ice-cold PBS, fixed with 3% PFA, and quenched with glycine, as above. GLUT4*myc* bound to anti-*myc* antibody (reexiting to the cell surface) was detected with HRP-conjugated secondary antibody, followed by color development with OPD, as described above. The amount of GLUT4*myc* at cell surface following exocytosis was expressed as the fold difference over untreated controls.

Imaging steady-state surface GLUT4myc in single cells. L6-GLUT4myc myoblasts were grown on glass coverslips to about 60% confluence followed by serum deprivation for 3 h. Cells were treated for 10 min with 1 μ M ionomycin in 0.1% DMSO or with vehicle alone (Control). Labeling of surface GLUT4myc in nonpermeabilized cells was performed as described (41) and imaged using a Zeiss LSM 510 laser scanning confocal microscope. Images were scanned along the *z*-axis, and collapsed *xy* projections were assembled from optical stacks taken at 1- μ m intervals. The pixel intensity of each cell (23–34 cells per condition) was quantified by ImageJ software.

Imaging intracellular GLUT4myc-GFP in single cells. Subconfleunt L6 wild-type myoblasts grown on glass coverslips were transfected with GLUT4myc-GFP cDNA for 24 h before experimentation, as described (41). Serum-deprived (3 h) myoblasts were treated for 10 min with 1 μ M ionomycin in 0.1% DMSO or with vehicle alone. The cells were fixed with paraformaldehyde. Imaging and quantification of the green fluorescent protein (GFP) signal was performed the same as described above.

Transferrin recycling. L6-GLUT4myc myoblasts grown on glass coverslips to about 60% confluence were serum deprived for 3 h. Cells treated with vehicle or 1 μ M ionomycin were pulsed with transferrin-Alexa 546 (Tf-A546, 50 μ g/ml), in serum-free α -MEM supplemented with 1% BSA for 10 min at 37°C (41). Some coverslips were immediately processed (see below) for quantification of Tfn-A546 uptake, while others were chased with holotransferrin (human, 500 μ g/ml, Sigma-Aldrich, St. Louis, MO) for an additional 10 min at 37°C and then processed for determination of Tfn-A546 remaining in cells. After pulsing or chasing, cells on coverslips were placed on ice, washed twice with ice-cold PBS and then once with acid buffer (0.15 M NaCl, 0.1 M glycine, pH 3.0), and rinsed with PBS. Cells were then fixed with 4% paraformaldehyde in PBS at room temperature for 90 min. Imaging and quantification of the Tfn-A546 signal was performed as described above.

Statistical analysis. Results are presented as means \pm SE. Statistical analyses were carried out using Prism 6.0 software (San Diego, CA). Student's *t*-test was used to compare pairs of data, with Welch's correction for variance in homogeneity whenever needed. Data sets with three or more groups were compared using one-way or two-way analysis of variance with Tukey's post hoc analysis. A *P* value of <0.05 was considered statistically significant.

RESULTS

Ionomycin increases surface GLUT4 and activates CaMKII, AMPK, and PKC. In L6-GLUT4myc myoblasts, ionomycin (1 μ M) caused a very rapid increase in intracellular Ca²⁺ that remained elevated in the continued presence of the ionophore for more than 10 min (Fig. 1A). Concomitantly, ionomycin evoked a rise in surface GLUT4myc transporters in a dose- and time-dependent manner (Fig. 1B). With 1 μ M ionomycin, a maximum response was observed within 10 min (Fig. 1B). Ionomycin (2 μ M) elevated surface GLUT4myc further within the first 5 min (not shown), but longer incubation times with 2 μ M ionomycin dislodged some cells from the plates. Since stimulation with 1 μ M ionomycin for 10 min produced consistent results, these conditions were used thereafter. The gain in surface GLUT4*myc* induced by ionomycin was dependent on the rise in intracellular Ca²⁺ caused by Ca²⁺ influx from the medium, since it was fully prevented by the Ca²⁺ chelators EGTA or EGTA plus BAPTA-AM (Fig. 1*C*) without affecting basal levels of surface GLUT4*myc* (not shown). We did not use BAPTA-AM alone, because extracellular Ca²⁺ influx through ionomycin would rapidly saturate the intracellular Ca²⁺-buffering power of BAPTA. Ionomycin also caused a drop in intracellular ATP and markedly raised the AMP/ATP ratio by 10 min (Fig. 1*D*). The ATP consumption may be linked to activation of PM and microsomal Ca²⁺-ATPases working to counteract the rise in cytosolic Ca²⁺.

We next examined the signaling pathways triggered by ionomycin, keying on pathways that are activated during skeletal muscle contraction. Strong phosphorylation of CaMKII and AMPK in their activation loops on residues Thr²⁸⁶ and Thr¹⁷² occurred 5 and 10 min after ionomycin addition to the cells, respectively (Fig. 2, A and B). The downstream AMPK substrate ACC was also phosphorylated on Ser^{79} (Fig. 2B). Ionomycin also activated PKCs, since it stimulated strong phosphorylation of at least two PKC substrates of \sim 55 and ~36 kDa within 5 min (Fig. 2*C*). Ionomycin did not affect Akt phosphorylation on Thr³⁰⁸, whereas, it led to a small increase in phosphorylation on Ser⁴⁷³ of Akt. However, this effect was markedly weaker compared with insulin (Fig. 2D). In contrast, the low and site-selective phosphorylation of Akt by ionomycin would not be expected to have signaling consequences, as both sites on Akt must be phosphorylated to stimulate Akt kinase activity (34). Furthermore, ionomycin only weakly or did not stimulate phosphorylation of the MAPKs: ERK1/2, p38 MAPK, or JNK or Rac-activated PAK1/2 (results not shown).

Contribution of CaMKII, AMPK, and PKC to the gain in surface GLUT4. Analyzing the impact of AMPK, CaMKII, and PKC on the ionomycin-induced gain in cell surface GLUT4myc is challenged by the expression of different isoforms of each of these kinases. However, a combination of siRNA silencing and chemical inhibitors afforded valuable conclusions. CaMKIIS is the most abundant CaMKII isoform in L6 muscle cells (6), and siRNA-mediated knockdown of CaMKII δ , an average of 65 \pm 11% (Fig. 3A, top), significantly reduced the maximal response of the ionomycin-stimulated gain in steady-state surface levels of GLUT4*myc* by $37 \pm 11\%$ (Fig. 3A, bottom). The contribution of CaMKII to the ionomycin-stimulated GLUT4myc response was confirmed using the cell-penetrating peptidic inhibitor CN21 (45), which binds to the active site of all isoforms of CaMKII with high specificity. Preincubation of cells with CN21 reduced the GLUT4myc response to ionomycin by $32 \pm 4\%$ (Fig. 3B, bottom). Thus, CaMKII partly contributes to the ionomycin-induced gain in surface GLUT4myc.

L6 muscle cells express more $\alpha 1$ than $\alpha 2$ subunits of AMPK (32), and simultaneous siRNA-mediated knockdown of both isoforms achieved a 61 ± 6% reduction in their combined expression, determined using a pan- α AMPK antibody (Fig. 3*B*, *top*). AMPK $\alpha 1/\alpha 2$ knockdown dropped by 31 ± 4% the ionomycin-induced gain in cell surface GLUT4*myc* relative to the response of cells treated with nontargeting siRNA oligomers (Fig. 3*B*, *bottom*). In addition, the AMPK catalytic site inhibitor Compound C lowered the GLUT4*myc* response to



Fig. 1. Ionomycin stimulated the steady-state level of surface GLUT4*myc* in a Ca²⁺-dependent manner. *A*: L6-GLUT4*myc* myoblasts were grown on glass coverslips and processed for live cell imaging of changes in intracellular Ca²⁺ as described in MATERIALS AND METHODS. Arrow indicates addition of 1 μ M ionomycin. Shown is the change in relative Fluo 3 fluorescence (Δ F/F) in response to ionomycin (black line) compared with baseline (gray line) over 15 min and is representative of \geq 3 experiments. *B*: L6-GLUT4*myc* myoblasts in 24-well plates were serum deprived for 3 h prior to treatment with 0.5 or 1.0 μ M ionomycin or vehicle (control). Cells were processed for steady-state cell surface GLUT4*myc* at indicated times, as described in MATERIALS AND METHODS (*n* = 4-6, ***P* < 0.01, ##*P* < 0.0001). *Bar graph*: fold change in cell surface GLUT4*myc* caused by ionomycin (1 μ M, 10 min). In another condition, cells were pretreated with 20 μ M BAPTA-AM for 30 min before being given EGTA and vehicle or ionomycin. Illustrated are cell surface GLUT4*myc* levels calculated as % \pm SE of maximum ionomycin response (*n* = 5, ##*P* < 0.001). *D*: cells were treated for varying times (0, 1, 2, 5, 10 min) with 1 μ M ionomycin and processed for adenine nucleotide quantification as in MATERIALS AND METHODS. Shown is the mean ratio of AMP/ATP (*n* = 3, **P* < 0.05).

ionomycin by $35 \pm 4\%$ (Fig. 3*B*, *bottom*). The effectiveness of Compound C in blocking activation of AMPK by ionomycin was confirmed by its marked inhibition of ACC phosphorylation (Fig. 3*B*, *top*). Hence, like CaMKII, AMPK contributes, albeit only in part, to the gain in surface GLUT4 elicited by ionomycin.

As illustrated in Fig. 1, intracellular AMP levels rose compared with ATP in ionomycin-treated muscle cells, and this might explain the activation of AMPK (likely via LKB1); however, calcium-calmodulin dependent CaMKK1/2 are also upstream kinases of AMPK in skeletal muscle (21, 22, 48). The participation of the calcium-dependent upstream kinase CaMKK in the ionomycin-elicited signaling to AMPK was explored using its inhibitor STO-609 (Fig. 3*B*, *top*). STO-609 lowered the maximal level of ionomycin-induced phosphorylation of Thr¹⁷² on AMPK, but it also reduced the basal pT172 AMPK levels. Although the fold stimulation of p-Thr¹⁷² AMPK in the presence of STO-609 remained high at 3.0 ± 0.7 vs. 2.3 ± 0.5 in control cells, the action of ionomycin on AMPK was reduced. This effect of STO-609 on AMPK activation is similar to the effect of AMPK knockdown (see Fig. 5*C*, below). STO-609 also inhibited the maximal steady-state

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Fig. 2. Ionomycin stimulates activation of CaMKII, AMPK, and PKC. Serum-deprived L6-GLUT4myc myoblasts were treated for 5 or 10 min with 1 μ M ionomycin or vehicle (10 min, control). Cell lysates were prepared in duplicate with RIPA cell lysis buffer and immunoblotted in duplicate. *A*: phospho (p)-T286 CaMKII, *B*: p-T172 AMPK and p-S79 ACC, *C*: p-PKC substrates, *D*: p-T308, and S473 Akt. Actinin-1 immunoblots are loading controls. In *D*, an insulin-treated sample (Ins) served as a strong positive control for activated p-Akt. Gels are representative of \geq 3 independent experiments.

gain in cell surface GLUT4*myc* by 48 \pm 7%, to a similar degree as that achieved by siAMPK and Compound C (Fig. 3*B*, *bottom*). Thus, in response to ionomycin, AMPK activation was probably triggered by both calcium (CaMKK)- and AMP-dependent mechanisms, in both cases reducing GLUT4 translocation. Interestingly, the combination of STO-609 and Compound C inhibited the ionomycin-induced gain in cell surface GLUT4*myc* more than either inhibitor alone (Fig. 3*F*). This action of STO-609 is consistent with the observations that overexpressed constitutively active CaMKK α stimulated glucose uptake independently of AMPK in skeletal muscle (48).

Because of the numerous members in the PKC family, the contribution of the PKC family kinases was tested using class-specific inhibitors. The inhibitor of the conventional and novel PKC isoforms (α , β 1, β 2, γ , δ , ϵ , η , θ), Gö6983, reduced the GLUT4*myc* response to ionomycin by $26 \pm 2\%$ (Fig. 4A). In contrast, an inhibitor of conventional PKC isoforms (α , β 1, β_{2} , γ), Gö6976, did not prevent the ionomycin-induced gain in cell surface GLUT4myc. However, Gö6976 raised basal GLUT4myc cell surface levels. thereby significantly lowering the fold change in the GLUT4myc response induced by ionomycin (Fig. 4B). Because Gö6976 + ionomycin did not give ahigher GLUT4myc response than ionomycin alone, one interpretation is that conventional PKCs play a role in the full response to ionomycin. Furthermore, Gö6976 and Gö6983 had effects on the gain in surface GLUT4myc caused by the conventional and novel PKC activator phorbol 12-myristate 13-acetate (PMA; Fig. 4, C and D) that were qualitatively similar to their respective effects on ionomycin. Both Gö6976 and Gö6983 inhibited serine phosphorylation of the major ionomycin-stimulated substrates of PKC [detected by antiphospho-(Ser)-PKC substrates], suggesting that conventional PKC isoforms phosphorylate abundant PKC substrates (Fig. 4E). To ensure that PKC inhibitors had not negated the increase in cytosolic Ca²⁺ caused by ionomycin, we measured the effect of Gö6983 on the fluorescent signal of the Ca²⁺ indicator Fluo 3-AM. Indeed, Gö6983 had negligible effects on baseline and ionomycin-stimulated Ca²⁺ levels, thus PKC inhibition affected ionomycin actions downstream of Ca²⁺ influx (Fig. 4F).

We next examined the potential cross-talk among AMPK, CaMKII, and PKC activation by ionomycin. AMPK activation was independent of CaMKII, since siCaMKIIδ (which lowered the phosphorylation of CaMKII) did not affect basal or ionomycin-stimulated p-Thr¹⁷² AMPK (Fig. 5A). In addition, CN21 did not inhibit basal or ionomycin-stimulated p-Thr¹⁷² AMPK (Fig. 5B). In turn, CaMKII activation was independent of AMPK or its upstream CaMKK, since silencing the AMPK catalytic subunits or treatment with STO-609 did not appreciably affect the fold stimulation of p-Thr²⁸⁶ CaMKII by ionomycin (Fig. 5, C and E). However, the basal level of p-Thr286 CaMKII was reduced by those treatments (Fig. 5, C and E). Moreover, Compound C treatment did not affect basal or ionomycin-stimulated p-Thr286 CaMKII (Fig. 5D), suggesting that signals other than AMPK contribute to the ionomycin-induced activation of CaMKII. Last, Gö6976 and Gö6983 did not affect basal or ionomycin-stimulated levels of p-Thr²⁸⁶ CaMKII and p-Thr¹⁷² AMPK (Fig. 5F), indicating that neither conventional nor novel PKC isoforms signal through CaMKII or AMPK.

The results of Fig. 5 illustrated that three separate signaling pathways regulate GLUT4 traffic in response to a rise in cytosolic Ca²⁺ by ionomycin. These pathways did not converge at the level of AMPK, CaMKII, or PKC activation and prompted us to determine whether they would therefore be additive in their inhibition of the rise in surface GLUT4*myc*. We first tested if the CaMKII inhibitor CN21 further inhibited the effect of RNAi-mediated AMPK α 1/ α 2 knockdown. Indeed, the combination had an additive inhibitory effect on the ionomycin-induced gain of GLUT4*myc* at the cell surface (Fig. 6A). In addition, the PKC inhibitor Gö6983 in combination with siCaMKII δ or siAMPK α 1/ α 2 had additive inhibitory effects on the ionomycin-induced gain of surface GLUT4*myc* (Fig. 6, *B* and *C*).

Regulation of GLUT4 endocytosis and exocytosis. We have developed methods to independently measure the exocytosis or endocytosis of GLUT4*myc* in muscle cells (19). To assess exocytosis, anti-*myc* antibody is allowed to internalize bound to GLUT4*myc* for 2 h at 37°C to populate the transporter storage pools. Excess anti-*myc* is then washed away before stimulation with ionomycin and determination of labeled GLUT4*myc* that reaches the cell surface over



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Fig. 3. Silencing or inhibiting CaMKII or AMPK reduces the gain in surface GLUT4*myc*. L6-GLUT4*myc* myoblasts were transfected with siRNA oligomers targeted to (*A*) CaMKII δ or (*B*) AMPK α 1/ α 2, or a nontargeting control (siNR) for 72 h. Cells were processed as in Fig. 1*B* with ionomycin (1 μ M, 10 min) or vehicle (control), and, where used, inhibitors were added during the last 30 min of serum deprivation and included during treatment with ionomycin: (*C*) CaMKII inhibitor CN21 peptide (5 μ M); (*D*) AMPK inhibitor Compound C (10 μ M, CC); (*E*) CaMKK inhibitor STO-609 (10 μ M, STO); (*F*) STO-609 + Compound C coincubation. *A* and *B*: cell lysates were immunoblotted in duplicate with anti-pan-CaMKII or anti-pan- α -subunit AMPK to monitor effectiveness of knockdown relative to siNR. *D* and *E*: immunoblotting for p-ACC or p-AMPK demonstrated effectiveness of Compound C or STO-609, respectively. Shown are representative gels of \geq 3 experiments. *Graphs*: steady-state levels of cell surface GLUT4*myc* presented as fold change from control (control: siNR or vehicle), 4 independent siRNA experiments, or 3 independent experiments with inhibitors. Statistical analysis by 1-way ANOVA with comparisons as indicated (**P* < 0.05, ***P* < 0.01, ##*P* < 0.0001).

time. Hence, this assay effectively measures GLUT4*myc* exocytosis and not overall GLUT4*myc* recycling. To assess endocytosis, anti*myc* antibody is bound to the cell surface at 4°C and washed away, and then the cells are quickly rewarmed to 37°C with/without stimulation with ionomycin, allowing GLUT4*myc* endocytosis to proceed. Antibody-bound GLUT4*myc* remaining at the cell surface over time is quantified. Ionomycin rapidly stimulated the exocytosis of GLUT4*myc*, reaching maximal response within 10 min (Fig. 7A). The fold response of GLUT4*myc* exocytosis to ionomycin was greater than the fold change measured at steady state (Fig. 3), because the former detects only GLUT4*myc* that exits the intracellular stores to the cell surface, and the assay is not influenced by ongoing GLUT4*myc* endocytosis.



Fig. 4. Inhibiting conventional and novel PKC classes reduces the gain in surface GLUT4*myc*. L6-GLUT4*myc* myoblasts were serum deprived for 3 h and preincubated with Gö6976 (*A*, *C*) or Gö6983 (*B*, *D*) (1 μ M) prior to treatment with ionomycin (*A*, *B*: 1 μ M, 10 min, *n* = 5), PMA (*C*, *D*: 1 μ M, 10 min, *n* = 4) or (vehicle control). *A–D*: steady-state cell surface GLUT4*myc* was performed as in Fig. 1*B*. *E*: cell lysates were immunoblotted with anti-p-PKC substrate antibody. Proteins of approximate sizes (indicated) were phosphorylated in response to ionomycin and inhibited by either PKC inhibitor. *F*: cytosolic Ca²⁺ measurements (as in Fig. 1*A*) in cells preincubated for 30 min with Gö6983 (gray line) or DMSO vehicle (black line). Baseline Fluo 3 fluorescence of cells was captured prior to exposure to ionomycin followed by continuous monitoring of fluorescence ≤15 min, as described in MATERIALS AND METHODS and Fig. 1*A*. **P* < 0.05, ***P* < 0.01, ##*P* < 0.0001.

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Fig. 5. CaMKII and AMPK are stimulated in parallel by ionomycin and not affected by PKC inhibitors. L6-GLUT4*myc* myoblasts were transfected with siRNA to CaMKII δ , AMPK α 1/ α 2, or a nontargeting control (siNR) and processed as in Fig. 3. Prior to ionomycin stimulation, cells were pretreated with CN21 peptide, Compound C (CC), STO-609 (STO), Gö6983, or Gö6976 as in Fig. 3. *A*–*E*: cell lysates were immunoblotted for CaMKII-pan, p-T286 CaMKII, AMPK α -pan, p-T172 AMPK α , as indicated. GAPDH and actinin-1 immunoblots were loading controls. In *B*, all lanes were from the same immunoblot. Results shown are representative of \geq 3 independent experiments.

In addition to stimulating the rate of GLUT4*myc* exocytosis, ionomycin slowed down the rate of GLUT4*myc* endocytosis, such that 10 min after its addition the remaining surface GLUT4*myc* level was significantly higher than that in unstimulated cells (Fig. 7B). In all subsequent experiments, GLUT4*myc* exocytosis or endocytosis was measured in control cells (rewarming without stimulus) and cells treated with ionomycin at the 10 min time point that followed rewarming to 37°C.

CaMKIIô knockdown caused a marked reduction (57 \pm 19%) in ionomycin-stimulated GLUT4*myc* exocytosis compared with cells treated with nontargeting siRNA, yet it did not affect GLUT4*myc* constitutive exocytosis in control cells (i.e., basal exocytosis) (Fig. 8A). Buttressing these results, the CaM-KII inhibitor CN21 reduced GLUT4*myc* exocytosis to nearly the same degree (39 \pm 5%; Fig. 8B). In a similar fashion, AMPK α 1/ α 2 knockdown (50%, as shown) and treatment with Compound C or STO-609 reduced the ionomycin-stimulated GLUT4*myc* exocytosis by 33 \pm 4, 27 \pm 9, or 43 \pm 9%, respectively (Fig. 8, *C*–*E*). These experiments reveal that both CaMKII and AMPK contribute to the increased rate of GLUT4*myc* exocytosis evoked by ionomycin.

Gö6983 reduced GLUT4*myc* exocytosis by $20 \pm 3\%$ (Fig. 9A). On the other hand, Gö6976 slightly raised the basal

levels of GLUT4*myc* in the exocytosis assay without apparent effect on the total response to ionomycin. However, the fold change of the response to ionomycin in the presence of Gö6976 (2.4 ± 0.3) was lower than that in the absence of inhibitors (3.1 ± 0.2) (Fig. 9*B*). Therefore, both conventional and novel PKC isoforms may play modest roles in basal and ionomycinstimulated GLUT4*myc* exocytosis.

The signals regulating GLUT4*myc* endocytosis in response to ionomycin were next investigated. siRNA-mediated knockdown of CaMKII (59 \pm 7%) or AMPK (68%, as shown) had no effect on the slowing of GLUT4*myc* endocytosis by ionomycin (Fig. 10, *A* and *B*). Similarly, Compound C or STO-609 did not affect ionomycin-regulated GLUT4*myc* endocytosis (Fig. 10, *C* and *D*). Hence, neither CaMKII nor AMPK played a role in the ionomycin-dependent reduction of GLUT4*myc* endocytosis.

In contrast, Gö6983 markedly reversed the inhibition of GLUT4*myc* endocytosis from the cell surface by $45 \pm 5\%$ (Fig. 11A). In addition, Gö6976 slowed the basal rate of GLUT4*myc* endocytosis and reversed a fraction of the ionomycin-inhibited GLUT4*myc* endocytosis, since the net inhibition of endocytosis in the presence of ionomycin plus Gö6976 (44 \pm 2.5%) was slightly reduced compared with the net inhibition seen with ionomycin (55 \pm 2.6%) (Fig. 11*B*). Therefore, novel and CYTOSOLIC Ca2+ REGULATES GLUT4 TRAFFIC IN MUSCLE CELLS



Fig. 6. CaMKII, AMPK, and PKC operate in parallel to regulate GLUT4*myc* traffic. L6-GLUT4*myc* myoblasts were transfected with siRNA to CaMKII δ , AMPK α 1/ α 2, or a nontargeting control (siNR), as indicated, and processed as in Fig. 3. Prior to ionomycin stimulation, cells were pretreated with CN21 (*A*; 5 μ M, 30 min) peptide or Gö6983 (*B*, *C*; 1 μ M, 30 min). *A–C*: steady-state cell surface GLUT4*myc* was measured on cell monolayers from 3, 4, or 8 independent experiments, respectively, and presented as percent maximal response to ionomycin (relative to siNR or control). Statistical analysis by 1-way ANOVA with comparisons as indicated (**P < 0.01, #P < 0.001, #P < 0.0001).

perhaps conventional PKC isoforms play a role in basal and ionomycin-inhibited GLUT4*myc* endocytosis.

Intracellular distribution of GLUT4myc in response to ionomycin. The gain in surface GLUT4myc caused by ionomycin was confirmed and visualized in single cells by using confocal fluorescence microscopy. Ionomycin induced a marked (>6-fold) gain in GLUT4myc at the surface of nonpermeabilized L6-GLUT4myc myoblasts (Fig. 12A). Confocal fluorescence microscopy also allowed us to visualize the effect of ionomycin on the intracellular distribution of the transporter by using GLUT4myc-GFP transiently expressed in L6 myoblasts. Dotted lines were drawn outside the imaged cells to define their perimeters. Whereas in control cells GLUT4myc-GFP is largely located in the perinuclear region, ionomycin induced its partial dispersion across the cytosol and toward the cell periphery (Fig. 12*B*). Of more than 50 ionomycin-treated cells, \sim 80% had a GLUT4*myc*-GFP distribution qualitatively represented by the ionomycin-treated cells illustrated in Fig. 12*B*, whereas 90% of ~50 control cells had a GLUT4*myc*-GFP distribution qualitatively represented by the control cells in Fig. 12*B*. The ionomycin effect on GLUT4*myc* was specific for this membrane protein, since transferrin (Tfn) receptor recycling, measured by the uptake and release of the Tfn-Alexa 546 fluorescent conjugate, was not regulated by ionomycin (Fig. 12*C*).

DISCUSSION

A rapid rise in cytosolic Ca^{2+} triggers muscle contraction, and the ensuing stimulation of skeletal muscle glucose uptake



Fig. 7. Ionomycin stimulates GLUT4*myc* exocytosis and reduces its endocytosis. Exocytosis (*A*) and endocytosis (*B*) rates of GLUT4*myc* were measured as described in MATERIALS AND METHODS. Means of 5 or 6 experiments were plotted as fold response compared with vehicle-treated controls. **P* < 0.05, ##*P* < 0.001, ##*P* < 0.0001.

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Control STO lonomycin lonomycin +STO

Cell surface GLU (fold over conti

Fig. 8. Ionomycin-induced exocytosis of

GLUT4*myc* requires CaMKII and AMPK. *A*, *C*: representative immunoblots with anti-pan-

CaMKII or anti-pan- α -subunit AMPK demonstrates CaMKII δ or AMPK α 1/ α 2 knockdown,

respectively. GLUT4myc exocytosis was mea-

sured after 10 min in control (DMSO vehicle of

siNR) or ionomycin-treated cells as described (Fig.

7). A: siCaMKII δ -treated cells (n = 7). B: cells pretreated with CN21 (n = 4). C: siAMPK α 1/

 α 2-treated cells (n = 4). D: cells pretreated with

Compound C (CC, n = 9). E: cells pretreated

with STO-609 (STO, n = 5). *P < 0.05, **P <

0.01, #P < 0.001, ##P < 0.0001.

through GLUT4 has been studied for years (18). PKC, AMPK, and CaMKII are the major signaling kinases implicated by many studies in regulating contraction-stimulated glucose uptake. However, the signaling pathways and the mechanisms that control GLUT4 traffic in response to contraction are still largely debated. In part this is due to the difficulty in deconstructing the individual events that constitute the complex phenomenon of muscle contraction.



Fig. 9. Ionomycin-induced exocytosis of GLUT4*myc* is partly regulated by PKC. GLUT4*myc* exocytosis was measured after 10 min in control (DMSO vehicle) or ionomycin-treated cells as described (Fig. 7). A: Gö6983 (n = 6). B: Gö6976 (n = 8) pretreatments. ##P < 0.0001.

The L6-GLUT4*myc* skeletal muscle cell line offers a versatile cell culture system to study GLUT4 traffic, in which GLUT4 determines the rate of glucose uptake (38). In these cells, insulin accelerates the rate of GLUT4 vesicle exocytosis to the cell surface, whereas a decrease in the energy charge of the cell by uncoupling mitochondria with DNP reduce GLUT4 endocytosis (1).

L6-GLUT4myc muscle cells do not contract; hence, they offer the possibility to study the effect of Ca²⁺-triggered signals independently of mechanical work. Here, we show that a sudden rise in cytosolic Ca²⁺, using the Ca²⁺ ionophore ionomycin, increases the steady-state levels of GLUT4myc at the muscle cell surface in cell monolayers or single cells, using ELISA-based and imaging approaches, respectively. Ionomycin achieves this by accelerating GLUT4myc exocytosis and slowing GLUT4myc endocytosis without largely affecting the recycling of other membrane proteins, such as the transferrin receptor (measured by transferrin uptake and release). We directly implicate CaMKII and AMPK along with contributions from conventional and novel PKC isoforms in the regulation of GLUT4myc exocytosis by Ca²⁺ and further implicate novel PKC in the Ca2+-induced retardation of GLUT4myc endocytosis in muscle cells. These combined mechanisms effectively achieve GLUT4 mobilization to, and increased permanence at the muscle cell membrane.

Several kinases mediate the ionomycin-dependent increase in surface GLUT4 traffic. Ionomycin evoked a rapid and sustained rise in cytosolic Ca^{2+} , and the signaling kinases CaMKII and AMPK were strongly activated within 5 and 10 min. The activation of CaMKII and AMPK was evinced by phosphorylation of their respective T-loop residues Thr²⁸⁶ and Thr¹⁷², respectively. In addition, ionomycin-stimulated activation of PKC isoforms revealed by phosphorylation of major PKC substrates, which was blocked by the PKC inhibitors Gö6976 and Gö6983.

Although mechanical work from Ca^{2+} -triggered contraction is expected to place a demand on cellular ATP in muscle, L6-GLUT4*myc* myoblasts cannot contract in response to ionomycin (or other depolarizing agents), due to a lack of sarcomere contractile units. Thus, the rise in cellular AMP (Fig. 1*D*) and activation of AMPK that we observed may have resulted from excessive activity of the Ca²⁺-ATPase in the face of a large ionomycin-induced influx of Ca²⁺. Indeed, contracting skeletal muscle fibers can partly activate AMPK by influx of Ca^{2+} independently of the energy demand by the contractile apparatus (4, 39). In our study, the CaMKK inhibitor STO-609 lowered basal and ionomycin-stimulated levels of p-Thr¹⁷² AMPK, although there was still a normal fold increase in p-Thr¹⁷² AMPK following ionomycin plus STO-609 treatment compared with the fold change in the absence of the inhibitor. These results implicate both calcium sensing and AMP in the regulation of basal and ionomycin-stimulated AMPK activity. The AMPK contribution to the gain in surface GLUT4 evoked by ionomycin was substantiated by inhibition through Compound C or knockdown of the AMPK catalytic α-subunits. STO-609 also lowered the total levels of ionomycin-stimulated AMPK and in doing so may have elicited its effect on GLUT4myc traffic. However, combined treatment of L6-GLUT4myc myoblasts with STO-609 and Compound C led to greater inhibition of the ionomycin-induced gain in cell surface GLUT4myc compared with either inhibitor alone. One study has reported that constitutively active CaMKKa can stimulate muscle glucose uptake independently of AMPK (48), whereas others have reported that the caffeine-evoked rise in cytosolic calcium required a CaMKK-AMPK pathway to stimulate muscle glucose uptake (22). Our results support a role for CaMKK in the GLUT4 response to ionomycin that may be both AMPK dependent and independent. Future studies using CaMKK siRNA-mediated knockdown would be needed to shed more light on the role of CaMKK in calcium-dependent stimulation of GLUT4 traffic and glucose uptake in muscle cells.

We found that, in parallel to the activation of AMPK, CaMKII and conventional/novel PKC isoforms contribute to the ionomycin-induced steady-state gain of GLUT4*myc* at the cell surface. Input by CaMKII was supported by the inhibitory effect of the CN21 peptidic inhibitor of CaMKII and by siRNA-mediated knockdown of the abundantly expressed CaMKII δ (6).

With the caveat of whether PKC inhibitors are exclusively selective for isoform classes, we propose the involvement of conventional and novel PKC in GLUT4 traffic. There are four isoforms of conventional PKC and four of novel PKC, and E220

Fig. 10. Ionomycin inhibits endocytosis of GLUT4*myc* and does not require CaMKII or AMPK. *A*, *B*: representative immunoblots with anti-pan CaMKII or anti-pan- α -subunit AMPK demonstrates CaMKII δ or AMPK α 1/ α 2 knockdown, respectively. GLUT4*myc* endocytosis was measured after 10 min rewarming in control (DMSO vehicle of siNR) or ionomycintreated cells as described (Fig. 7). *A*: siCaMKII δ -treated cells (*n* = 5). *B*: siAMPK α 1/ α 2 cells (*n* = 3). *C*: Compound C-pretreated cells (*n* = 7). **P* < 0.05, ***P* < 0.01; ns, not significant.



input by PKC was assessed using the widely used and classspecific inhibitors Gö6976 and Gö6983 (53). Gö6976, an inhibitor of conventional PKC, raised the basal steady-state cell surface levels of GLUT4*myc*. In addition, Gö6976 inhibited a portion of the ionomycin-stimulated gain in cell surface GLUT4*myc*, because the fold change of the response to ionomycin was significantly inhibited in the presence of Gö6976. The conventional and novel PKC inhibitor Gö6983 did not alter the basal steady-state cell surface levels of GLUT4*myc* and significantly lowered the ionomycin-induced response of GLUT4*myc* by ~25%.

Thus, AMPK, CaMKII, and PKC, which are known to have important roles in contraction-stimulated skeletal muscle glucose uptake, each provides partial input into the GLUT4 traffic response to a rise in cytosolic Ca^{2+} in muscle cells (37).

Ionomycin regulates the exocytic rate of GLUT4 traffic. GLUT4-containing vesicles dynamically recycle, fuse, and internalize from the PM in nonstimulated muscle cells (14). Insulin stimulates the rate of GLUT4 exocytosis in L6GLUT4*myc* muscle cells, isolated cardiomyocytes, and mature skeletal muscle (19, 23, 28, 36, 54), whereas it has very little to no effect on GLUT4 endocytosis in skeletal muscle cells or tissues (1). On the other hand, there is much less known about the arms of GLUT4 traffic regulated by contraction or contraction-related stimuli.

Here, we have capitalized on our recently developed assays that measure the exocytosis or endocytosis of a cohort of cell surface antibody-loaded GLUT4*myc* proteins to demonstrate that ionomycin can regulate both the exocytic and the endocytic arms of GLUT4*myc* traffic in muscle cells. Specifically, inhibition of CaMKII and AMPK signaling using small-molecule inhibitors or siRNA-mediated knockdown of these kinases only interfered with the ability of ionomycin to regulate the exocytosis of GLUT4*myc*, leaving unaffected the regulation of GLUT4*myc* endocytosis by ionomycin. By use of genetic and molecular approaches, the involvement of CaMKII and AMPK has been previously implicated in contraction-stimulated glucose uptake in mouse skeletal muscle (31, 49,



Fig. 11. Ionomycin-regulated endocytosis of GLUT4*myc* is partly reversed by inhibition of PKC. GLUT4*myc* endocytosis was measured as in Fig 7 in control or ionomycin-treated cells with Gö6983 (A, n = 6) or Gö6976 (B; n = 6) pretreatments. In B, the net gain of ionomycin-stimulated cell surface GLUT4*myc* was calculated as the difference between the fold changes of the cells treated with Gö6976 and Gö6976 + ionomycin. *P < 0.05, **P < 0.01, #P < 0.0001.

51), and our study supports the role of these kinases in stimulating GLUT4 vesicle translocation. Last, PKC activation also regulated the exocytosis of GLUT4*myc* to a small degree. Overall, these results show that multiple pathways feed into the regulation of GLUT4 vesicle movement toward the muscle cell surface in response to a rise in intracellular Ca^{2+} .

Ionomycin regulates the endocytic rate of GLUT4 traffic. We previously studied two conditions that occur during muscle contraction, i.e., depolarization of the cell membrane and reduction in intracellular ATP. Nigericin or K⁺-dependent depolarization modestly stimulated GLUT4myc recycling to the membrane and markedly reduced its endocytosis (47). Similarly, mitochondrial uncoupling by dinitrophenol (DNP) significantly slowed GLUT4myc endocytosis, effectively raising surface GLUT4myc (1), but the mediating intracellular signals that regulate GLUT4 endocytosis or exocytosis in response to depolarization or mitochondrial uncoupling were not identified. Intriguingly, the simultaneous activation of AMPK and PKC, using AICAR plus PMA, reduced GLUT4 internalization from the PM (1), demonstrating a need to determine the signals that regulate GLUT4 cycling in response to defined stimuli.

Reduced GLUT4 endocytosis in muscle cells is emerging as a recognized response to energy demand, since it occurs in cardiomyocytes challenged with inhibitors of the mitochondrial electron transport or oxidative ATP synthesis (i.e., metformin, oligomycin) (54, 55) or skeletal muscle cells with the mitochondrial uncoupler DNP (1), and all these treatments are known to activate AMPK (1, 54, 55). Ionomycin slows down GLUT4myc endocytosis and also activates AMPK; however, none of the three strategies we used to inhibit AMPK (Compound C, AMPK knockdown, or indirectly STO-609) interfered with the reduction in GLUT4myc endocytosis. In an apparent inconsistency with our observations, Fazakerley et al. (13) found that the AMPK activators AICAR and A-769662 did not increase the rate of exocytosis of HA-tagged GLUT4 introduced into L6 cells via a retroviral strategy, and instead they reduce GLUT4 endocytosis. However, that study similarly found that insulin also reduces GLUT4 endocytosis and does not stimulate its exocytosis, an observation that strikingly differs with all of our studies in L6 myoblasts or myotubes, where insulin promotes GLUT4 exocytosis and does not alter

its endocytosis (1, 15, 28, 47). Also countering the notion that AMPK activation per se could reduce GLUT4 endocytosis, we have observed that AICAR alone does not slow down GLUT4 internalization, but instead this effect is achieved only when the PKC activator PMA is added on top of AICAR (1). It is also noteworthy that oligomycin and metformin may act on other pathways in addition to AMPK, as oligomycin raises cytosolic Ca²⁺ (56), and others have demonstrated that metformin-stimulated glucose uptake in L6 muscle cells may require PKC rather than AMPK activation (43). Furthermore, the net gain in surface GLUT4*mvc* in response to K^+ -dependent depolarization, in our past study, was achieved independently of AMPK or CaMKII input, yet it was ascribed to a slow and modest increase in cytosolic Ca²⁺ and possibly conventional and novel PKC isoforms (47). Overall, we hypothesize that PKC signaling can regulate GLUT4 endocytosis by two modes, one independent of AMPK (stimuli: ionomycin, hyperosmolar K^+) and one in conjunction with AMPK (stimuli: oligomycin, metformin). However, exclusive AMPK activation may only regulate GLUT4 exocytosis.

In contrast to the lack of participation of CaMKII or AMPK in the regulation of GLUT4 endocytosis by ionomycin, lowering PKC activity tapered GLUT4myc endocytosis. The inhibitor of conventional plus novel PKCs, Gö6983, did not affect basal GLUT4myc endocytosis and reversed the ionomycindependent slowing of GLUT4myc endocytosis by \sim 45%. The conventional PKC inhibitor Gö6976, while slowing down the basal GLUT4myc endocytosis (consistent with the rise in basal steady-state cell surface levels of GLUT4myc induced by Gö6976; Fig. 3), did not affect the ionomycin response compared with control. Thus, one might argue that conventional PKCs may also effectively reduce the net inhibition of GLUT4myc endocytosis by ionomycin. These results suggest that at least novel PKC isoforms are involved in stimulusdependent control of GLUT4myc endocytosis, but future studies should identify the specific PKC isoforms regulating GLUT4myc endocytosis. It will equally require investigation to discern how novel PKCs (which are not Ca²⁺-dependent) are activated by ionomycin. PM depolarization activates phospholipase C, leading to generation of diacylglycerol (which stimulates conventional and novel PKCs) in rat skeletal muscle, isolated muscle fibers, and cultured primary rat myotubes (3, 9,

Α Surface GLUT4myc



В GLUT4myc-GFP distribution





8.

6

4

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12, 35). Indeed, we have found input by the novel PKC ϵ in the gain in surface GLUT4myc through a muscarinic acetylcholine receptor-dependent mechanism in carbachol-stimulated C₂C₁₂ myotubes (33). In addition, a rise in intracellular Ca^{2+} can activate phospholipase C activity in Golgi membranes (25). In this context, calphostin C, a competitor of diacylglycerol binding to PKCs, partially inhibits contraction-stimulated glucose transport in wild-type (50) and PKC α knockout mice (20). In the present study, PMA (a diacylglycerol mimetic) elevated the steady-state cell surface level of GLUT4myc, and this response was fully inhibited by Gö6983 but only partly affected by Gö6976. Overall, these observations support input by conventional and novel PKCs in the regulation of GLUT4 traffic.

Coincubation of Gö6983 with CaMKIIδ or AMPKα1/α2 knockdown cells produced additive inhibition of ionomycininduced GLUT4myc abundance at the PM (Fig. 6) and is consistent with PKC regulating the endocytic arm of GLUT4 traffic while either CaMKII or AMPK regulates GLUT4 exocytosis.

Overall, AMPK, CaMKII, and novel PKC have significant roles in regulating GLUT4 traffic. In addition to these signals, there may be regulation of GLUT4 by cytosolic Ca²⁺, including the possibility that its action on calmodulin also influences the motor protein Myo1c, implicated in GLUT4 traffic (6, 7). Further studies are required to understand the full scope of calcium signaling in the regulation of GLUT4 traffic.

It is intriguing that earlier studies with ionomycin showed it could not stimulate glucose uptake in isolated rat adipocytes and skeletal muscle if ionomycin remained with the cells during the glucose uptake assay (11, 27). However, if ionomycin was first washed out of isolated muscle preparations 15 min prior to measurement of glucose uptake, uptake was significantly stimulated (27). We speculate that ionomycin may be a weak inhibitor of GLUT4 transport function, since we have observed that ionomycin inhibits 2-deoxy-[³H]glucose uptake in L6-GLUT4myc myoblasts by 20% when it is present in the glucose uptake assay (results not shown). Therefore, washout from isolated muscle preparations might relieve the inhibition of transport by ionomycin (27). Future studies will be required to formally implicate ionomycin as an inhibitor of glucose transport. However, by following GLUT4 traffic, we bypassed its confounding effects on glucose uptake, which allowed us to better understand the role of Ca^{2+} in regulating GLUT4 traffic.

In summary, ionomycin provoked a rapid increase in cytosolic Ca2+ in muscle cells and strongly activated CaMKII, AMPK, and conventional and novel PKC classes. Ionomycin also stimulated the rate of GLUT4 exocytosis while simultaneously slowing down the rate of GLUT4 endocytosis. Both AMPK and CaMKII, long-standing kinases known to regulate contraction-stimulated muscle glucose uptake, were shown to control the stimulation of GLUT4myc exocytosis in response to the Ca²⁺ ionophore but not its endocytosis. However, novel and potentially conventional PKC are involved in ionomycinregulated GLUT4myc endocytosis. Future studies are needed to reveal how GLUT4 endocytosis is regulated by Ca²⁺ signaling and PKCs, as this may provide an avenue to elevate glucose uptake in muscle of diabetic individuals. Our results highlight a so far underappreciated regulation of GLUT4 traffic by Ca²⁺-derived signals that stimulate GLUT4 exocytosis and slow down GLUT4 endocytosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Q.L., X.Z., S.I., D.Z., J.G., Y.S., A.C.-F., and K.P.F. performed experiments; Q.L., X.Z., S.I., D.Z., J.G., Y.S., A.C.-F., K.P.F., S.L., P.J.B., A.K., and W.N. analyzed data; Q.L. and Y.S. prepared figures; Q.L., X.Z., S.I., D.Z., J.G., Y.S., A.C.-F., K.P.F., S.L., Z.Y., P.J.B., A.K., and W.N. approved final version of manuscript; S.I., Z.Y., P.J.B., A.K., and W.N. conception and design of research; S.I., A.C.-F., S.L., P.J.B., A.K., and W.N. interpreted results of experiments; P.J.B. drafted manuscript; P.J.B., A.K., and W.N. edited and revised manuscript.

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