The Role of Ca\(^{2+}\) in Insulin-stimulated Glucose Transport in 3T3-L1 Cells*  

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We have examined the requirement for Ca\(^{2+}\) in the signaling and trafficking pathways involved in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Chelation of intracellular Ca\(^{2+}\), using 1,2-bis(o-aminophenoxy)ethane-N\(_2\)N\(_3\)N\(_4\)N\(_5\)-tetraacetic acid (BAPTA-AM), resulted in \(>95\%\) inhibition of insulin-stimulated glucose uptake. The calmodulin antagonist, W13, inhibited insulin-stimulated glucose uptake by \(70\%\). Both BAPTA-AM and W13 inhibited Akt phosphorylation by \(70\%\). However, analysis of insulin-dose response curves indicated that this inhibition was not sufficient to explain the effects of BAPTA-AM and W13 on glucose uptake. BAPTA-AM inhibited insulin-stimulated translocation of GLUT4 by \(50\%\), as determined by plasma membrane lawn assay and subcellular fractionation. In contrast, the insulin-stimulated appearance of HA-tagged GLUT4 at the cell surface, as measured by surface binding, was blocked by BAPTA-AM. While the ionophores A23187 or ionomycin prevented the inhibition of Akt phosphorylation and GLUT4 translocation by BAPTA-AM, they did not overcome the inhibition of glucose transport. Moreover, glucose uptake of cells pretreated with insulin followed by rapid cooling to 4 °C, to promote cell surface expression of GLUT4 and prevent subsequent endocytosis, was inhibited specifically by BAPTA-AM. This indicates that inhibition of glucose uptake by BAPTA-AM is independent of both trafficking and signal transduction. These data indicate that Ca\(^{2+}\) is involved in at least two different steps of the insulin-dependent recruitment of GLUT4 to the plasma membrane. One involves the translocation step. The second involves the fusion of GLUT4 vesicles with the plasma membrane. These data are consistent with the hypothesis that Ca\(^{2+}\)-calmodulin plays a fundamental role in eukaryotic vesicle docking and fusion. Finally, BAPTA-AM may inhibit the activity of the facilitative transporters by binding directly to the transporter itself.  

Insulin stimulates glucose uptake in skeletal muscle and adipose tissue by stimulating the translocation of a facilitative glucose transporter, GLUT4, from an intracellular compartment to the cell surface. In recent years considerable progress has been made in our understanding of the downstream signaling pathways that are activated by insulin to mediate the translocation of GLUT4 to the cell surface. Upon insulin binding, the activated insulin receptor (IR)\(^{1}\) tyrosine kinase phosphorylates a number of downstream substrates, most notably the insulin receptor substrate (IRS) family of proteins, including IRS-1 and IRS-2 (1). Tyrosyl-phosphorylated IRS-1 and IRS-2 serve as docking stations for SH2 domain-containing proteins such as the class Ia (p85/p110-type) PI 3-kinase (1). Activation of PI 3-kinase is essential for insulin-stimulated GLUT4 translocation and glucose uptake (2) with generation of phosphoinositides 3,4,5-trisphosphate at the plasma membrane (PM) (3) serving to recruit and activate pleckstrin homology domain-containing proteins. Recent evidence indicates that the pleckstrin homology domain-containing Ser/Thr kinase Akt (otherwise called protein kinase B) plays a fundamental role in mediating insulin-stimulated GLUT4 translocation (4–6). A direct link between the insulin-signaling cascade and the more distal events associated with GLUT4 trafficking is yet to be identified. The precise nature of the insulin-responsive GLUT4 storage vesicle (GSV) and a detailed molecular description of how insulin promotes translocation of the GSV to the PM remain to be defined. In contrast, the mechanism by which GSVs dock and fuse with the PM is better understood, in part because of the similarity with synaptic vesicle trafficking in neurons. Both of these events involve the pairing of protein complexes in the vesicle compartment (v-SNARE, for vesicle membrane SNAP receptors) with cognate receptor complexes at the target membrane (t-SNARE, for target membrane SNAP receptors). Interactions between v-SNARE and t-SNARE proteins, as well as additional accessory proteins, are responsible for formation of the core complex, which is required for membrane docking and fusion (7). In adipocytes the core complex is comprised of the v-SNARE, VAMP2, and the t-SNAREs, syntaxin 4 and SNAP23 (8). In view of the similarity in molecular regulation between GLUT4 translocation in adipocytes and synaptic vesicle exocytosis in neurons, it has been suggested that GLUT4 translocation may represent a form of

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1 The abbreviations used are: IR, insulin receptor; IRS, insulin receptor substrate; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N\(_2\)N\(_3\)N\(_4\)N\(_5\)-tetraacetic acid (tетraacetoxyethyl)ester; 2-DOG, 2-deoxyglucose; GLUT4, glucose transporter; SNAP, soluble NSF attachment protein; v-SNARE, vesicle membrane SNAP receptors; t-SNARE, target membrane SNAP receptors; PM, plasma membrane; BSA, bovine serum albumin; KRP, Krebs-Ringer phosphate; HA, hemagglutinin; CHO, Chinese hamster ovary; PI, phosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
regulated exocytosis. Most regulated exocytic processes share several characteristic features. These include segregation of the cargo to be transported, increased delivery of this cargo to the cell surface in response to secretagogue, and the involvement of Ca\(^{2+}\) in the delivery process.

Although numerous studies have examined the role of Ca\(^{2+}\) in insulin-stimulated glucose transport, there remains little consensus concerning its overall role in this process. Investigations in L6 muscle cells, cardiac myocytes, and adipocytes failed to find a clear link between Ca\(^{2+}\) and glucose metabolism (9–12). In contrast, reduction of intracellular Ca\(^{2+}\) in rat adipocytes markedly inhibited insulin-stimulated glucose transport (13–15). Ca\(^{2+}\) may play a permissive role, or it may actively drive one or more of the steps involved in insulin-stimulated GLUT4 trafficking. In the latter case insulin may increase cytosolic Ca\(^{2+}\) by regulating the activity of a Ca\(^{2+}\) channel. In the former case there may be no change in cytosolic Ca\(^{2+}\) with insulin stimulation. Intuitively one might imagine at least two loci where Ca\(^{2+}\) might be involved in mediating the effects of insulin on glucose uptake. Firstly, Ca\(^{2+}\)/calmodulin has been implicated in mediating insulin activation of PI 3-kinase and Akt in rat hepatocytes and in 3T3-L1 adipocytes (16, 17). Secondly, several recent studies have reported a key role for Ca\(^{2+}\)/calmodulin in the late stages of vesicle docking/fusion (18–21). Thus, Ca\(^{2+}\) could be required both for the proximal signaling events of the insulin cascade and/or in the final stages of docking of GSVs with the plasma membrane.

In the present investigation we have re-evaluated the role of Ca\(^{2+}\) in insulin-stimulated glucose transport in 3T3-L1 adipocytes. To do this we have employed the membrane permeable form of the Ca\(^{2+}\)-chelating agent BAPTA-AM and the calmodulin antagonist W13. BAPTA-AM and W13 reduced insulin-stimulated glucose uptake by 95 and 60% and Akt phosphorylation by 75 and 70%, respectively. BAPTA-AM reduced GLUT4 translocation to the plasma membrane by 50% as determined by subcellular fractionation analyses. However, by using an antibody binding assay that measured insertion of glucose transporters into the membrane, we observed almost 100% inhibition of insulin-stimulated GLUT4 translocation in response to BAPTA-AM. We also found that treatment with ionophores prevented the inhibition of Akt phosphorylation and GLUT4 translocation by BAPTA-AM. These data indicate that Ca\(^{2+}\)/calmodulin is required for the efficient activation of Akt and are consistent with an obligate role for Ca\(^{2+}\) at a late post-docking stage in GLUT4 vesicle fusion.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—All reagents were from Sigma unless specified otherwise. All tissue culture medium was purchased from Life Technologies Inc., except fetal calf serum, which was obtained fromTrace Biosciences (Clayton, Australia). Bovine serum albumin was purchased from ICN (Costa Mesa, CA). Insulin was obtained from Calbiochem. The Ca\(^{2+}\) chelators, BAPTA-AM, BAPTA, and EGTA-AM, and ionophores, A23187 and ionomycin, were also from Calbiochem.

The calmodulin antagonist W13 was from Sigma. The polyclonal GLUT4 and IRAP antibodies have been described previously (22, 23). BCA reagent, used in protein assays, was from Pierce. The anti-phosphotyrosine monoclonal antibody (4G10) was provided by Dr. B. Druker (Oregon Health Sciences University, Portland, OR). Anti-IRS-1 polyclonal antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-p85 and anti-IRS-2 polyclonal antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). The monoclonal anti-influenza hemagglutinin (HA) epitope antibody was from Babco (Richmond, CA). Anti-phospho Ser473 Akt antibody was from Cell Signaling Technology Inc., except fetal calf serum, which was purchased from Life Technologies Inc., except fetal calf serum, which was obtained fromTrace Biosciences (Clayton, Australia). The Calbiochem. The Ca\(^{2+}\) chelators, BAPTA-AM, BAPTA, and EGTA-AM, and ionophores, A23187 and ionomycin, were also from Calbiochem.

**Cell Culture and Treatments—**3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described previously (24). CHO cells stably overexpressing the IR (CHO.IR cells) were cultured as described previously (25). In all experiments cells were serum-starved in Krebs-Ringer phosphate (KRP) buffer (12.5 mM HEPES, pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO\(_4\), 1 mM CaCl\(_2\), 0.4 mM NaH\(_2\)PO\(_4\), 0.6 mM NaNO\(_3\)) supplemented with 0.2% bovine serum albumin (BSA) for at least 2 h at 37 °C, and all further treatments were performed in the continued presence of this buffer except where indicated. Pretreatment and 2-DOG uptake were carried out in KRP buffer without Ca\(^{2+}\). In these experiments cells were rinsed in prewarmed KRP buffer as described above, except that it was without CaCl\(_2\) and supplemented with 5 mM EGTA, and incubated in this buffer for the duration of insulin stimulation and 2-DOG uptake. Where indicated, cells were incubated with BAPTA-AM (50 μM), made up in DMSO for 10 min followed by insulin (1 μM) for 15 min at 37 °C in the continued presence of BAPTA-AM. Incubation of cells in KRP with 0.2% BSA supplemented with 5 mM pyruvate gave comparable results (data not shown). In other experiments, cells were incubated with W13 (70 μM (17), made up in H\(_2\)O) for 20 min followed by insulin (1 μM) for 15 min at 37 °C. Following this, cells were rapidly cooled to 4 °C by washing with ice-cold KRP and incubated in the same buffer on ice. The cells were then incubated at 4 °C in the absence or the presence of BAPTA-AM, BAPTA, or EGTA-AM (all at 50 μM) or W13 (70 μM) for the times indicated, such that all cells were incubated at 4 °C for the same amount of time in experiments using the ionophore ionomycin (1 μM, made up in MeOH) was added simultaneously to the addition of BAPTA-AM. Treatment of cells with vehicle alone or in combination (at the appropriate final concentrations) was without effect in control experiments (data not shown).

**Cell Fractionation—**Following incubation with the appropriate agents, 3T3-L1 adipocytes were washed twice with ice-cold HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose) and homogenized in the same buffer supplemented with phosphatase and protease inhibitors (2 mM sodium orthovandate, 10 mM sodium fluoride, 1 mM tetrasodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 250 μM phenylmethylsulfonyl fluoride). Subcellular fractions were isolated by differential centrifugation as previously detailed (25) or by a modified protocol that gave comparable results. The modified protocol differed from that described (25) in the preparation of the PM fraction. In brief, centrifugation at 2,000 × g for 10 min was performed to remove mitochondria, nuclei, and unbroken cells. The resulting supernatant was then centrifuged at 18,000 × g for 20 min to pellet the crude PM fraction. This pellet was resuspended in HES buffer containing inhibitors and centrifuged again at 2,000 × g for 10 min to remove contaminating cellular material. This supernatant from this was then centrifuged again at 18,000 × g for 20 min to pellet the PM fraction. The high speed pellet (otherwise termed low density microsomal fraction) was prepared from the supernatant from the first 18,000 × g spin as described (25).

**Immunoblotting and Densitometry Analysis—**The protein content of all samples was determined using BCA reagent. The samples (10 μg) were subjected to SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride (Millipore Corporation, Bedford, MA), and the membranes were probed with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies. Antibody binding was detected by enhanced chemiluminescence according to the manufacturer’s instructions (Supersignal, Pierce). The protein bands were quantified by densitometry (GS-700 Imaging densitometer, Bio-Rad) using nonsaturated exposed x-ray films.

**Glucose Uptake Assays—**2-Deoxy-[\(^{14}\)C]glucose uptake was measured as described previously (26). In brief, 3T3-L1 adipocytes in 12-well plates were incubated in the absence or the presence of compounds, as indicated, in 500 μl of KRP with 0.2% BSA. The assay was initiated by adding 50 μl of 1 mM 2-deoxy-[\(^{14}\)C]glucose (20 μCi/mmole/KRP) and terminated after 1–2 min by washing cells rapidly three times with ice-cold PBS. The cells were solubilized in 1% Triton X-100, and 1/3 was quantitated by scintillation counting (Packard 1900CA liquid scintillation analyzer, Packard Instrument Co.). Glucose uptake was measured in duplicate in all treatments. Nonspecific uptake of 2-deoxy-[\(^{14}\)C]glucose was determined by the addition of cytochalasin B (50 μM) to the appropriate controls prior to the commencement of assays. Measurement of the transport of the nonmetabolizable glucose analogue 3-O-methylglucose (3-O-MG) was essentially as described above except that the assay was terminated after 45 s by washing cells three times in ice-cold PBS containing phloretin (100 μM).

**Plasma Membrane Lysosomal Assay—**The PM assay was performed essentially as described (27). In brief, 3T3-L1 adipocytes grown on glass cover slips were treated as indicated and then sonicated using a probe sonicator (Kontes Co., Vineland, NJ) to generate a lawn of PM fragme...
ments attached to the coverslip. The coverslips were then incubated in GLUT4-specific antiserum, followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody. Coverslips were washed with PBS, mounted onto glass microscope slides, and viewed using a 63×/1.4 Zeiss oil immersion objective on a Zeiss Axiovert fluorescence microscope equipped with a Bio-Rad MRC-600 laser confocal imaging system. Duplicate coverslips were prepared for each condition, and six random images of PM lawn were collected from each. The images were quantified using NIH 1.62 software.

Indirect Immunofluorescence of HA-GLUT4 Translocation—An exo-facial HA epitope-tagged GLUT4 construct containing a single HA epitope in the first exofacial loop between transmembrane domains 1 and 2 (kindly provided by Dr. Michael Quon, National Institutes of Health, Bethesda, MD) was inserted into the retroviral expression vector pBabeFugu and used to generate 3T3-L1 adipocytes stably expressing HA-GLUT4 as described (23). Following treatment of 3T3-L1 adipocytes stably expressing HA-GLUT4 cells were rinsed once in ice-cold PBS and then fixed in 2% paraformaldehyde with PBS for 15 min. Excess fixative was neutralized with 0.15 M glycine with PBS and blocked using 1% BSA with PBS for 30 min. The coverslips were incubated in anti-HA antibody (16B12) in 1% BSA with PBS for 1 h followed by a fluorescein isothiocyanate-conjugated secondary antibody in 1% BSA with PBS for 30 min. The coverslips were washed with PBS, mounted onto glass microscope slides, and viewed as described above.

**RESULTS**

The Ca2⁺ Chelator BAPTA-AM Inhibits Insulin-stimulated Glucose Uptake—It has been suggested that extracellular Ca2⁺ may play a role in insulin-stimulated glucose uptake in adipocytes (13). Consistent with this we observed a 30% decrease in insulin-stimulated 2-DOG uptake when cells were incubated in Ca2⁺ free buffer supplemented with 5 mM EGTA (Fig. 1A). To further investigate the role of Ca2⁺ in this process we performed experiments using the membrane-permeable form of BAPTA, namely BAPTA-AM, which is freely taken up into cells where it is hydrolyzed by cytosolic esterases and trapped intracellularly as the active chelator BAPTA. This reagent exchanges Ca2⁺ more than 100 times faster than other agents such as EGTA, because of the faster rates of association and dissociation. Pretreatment of 3T3-L1 adipocytes with BAPTA-AM for 10 min resulted in a dose-dependent inhibition of insulin-stimulated 2-DOG uptake with an IC50 of 15 μM (Fig. 1B). In all further experiments we used a BAPTA-AM concentration of 50 μM, at which we observed almost complete (~95%) inhibition of insulin-stimulated 2-DOG uptake (Fig. 1C). BAPTA-AM also caused significant inhibition of basal 2-DOG uptake (Fig. 1C). Identical results were obtained when cells were treated with BAPTA-AM in Ca2⁺-free buffer (basal, 10% ± 2; BAPTA-AM, 6% ± 1; insulin, 100%; insulin + BAPTA-AM, 9% ± 2; n = 4). Moreover, the nonesterified form of BAPTA or EGTA-AM had no significant effect on basal (control, 10% ± 2; BAPTA, 11% ± 3; EGTA-AM, 12% ± 2; n = 3) or insulin-stimulated 2-DOG uptake (control, 100%; BAPTA, 102% ± 5; EGTA-AM, 97% ± 3; n = 3). The inhibitory effect of BAPTA-AM did not involve an effect on intracellular ATP levels or hexokinase activity because it also inhibited insulin-stimulated transport of the nonmetabolizable glucose analogue 3-O-methylglucose (Fig. 1D).

**BAPTA-AM Inhibits GLUT4 Translocation**—We next examined the effects of BAPTA-AM on GLUT4 translocation to the cell surface using the PM lawn assay (Fig. 2). The morphology of the plasma membrane fragments was unaffected by BAPTA-AM. Insulin increased the level of GLUT4 at the PM by 4–5-fold. Pretreatment with BAPTA-AM inhibited insulin-stimulated GLUT4 levels in the PM lawns by ~50%. It has previously been reported that BAPTA-AM has no effect on GLUT4 translocation using this assay (28). However, quantification of GLUT4 in PM lawns was not performed in that study, in which case it is conceivable that a 50% inhibition may have been overlooked.

To further investigate the apparent inhibition of insulin-stimulated GLUT4 translocation by BAPTA-AM, we examined its effects on insulin-stimulated GLUT4 translocation by subcellular fractionation using differential centrifugation and immunoblotting (Fig. 3). In the absence of insulin very little GLUT4 was detected in the PM fraction obtained from basal cells. Consistent with the PM lawn data, insulin treatment resulted in a 5-fold increase in GLUT4 levels within the PM fraction (Fig. 3). Although BAPTA-AM alone had no significant effect on GLUT4 translocation, it caused a significant reduction...
BAPTA-AM resulted in complete inhibition of insulin-stimulated cell surface expression of GLUT4. These data implicates a role for Ca\(^{2+}\) in the fusion of GSVs with the plasma membrane and potentially resolve the discrepancy between the effects of BAPTA-AM on glucose transport and GLUT4 translocation as measured by the PM lawn technique or differential centrifugation (Figs. 1–3).

**BAPTA-AM Blocks Insertion of GLUT4 at the Plasma Membrane**—The above data suggest that although BAPTA-AM caused almost quantitative inhibition of insulin-stimulated glucose uptake, this reagent inhibited GLUT4 translocation by only 50%. This discrepancy is unlikely to reflect a technical limitation of our ability to quantify GLUT4 translocation because we observed quantitatively similar results using two different fractionation techniques. In view of recent findings implicating a role for Ca\(^{2+}\) at a post-docking step in vesicle transport (7), we reasoned that in the presence of BAPTA-AM, GSVs may dock at the PM but be blocked in their ability to fuse with the cell surface. Such docked vesicles may remain attached to the PM during preparation of PM fractions by mild sonication and labeled for GLUT4 as described under "Experimental Procedures." A, images from a representative experiment. B, quantitation of GLUT4 staining from six independent experiments showing the means ± S.E. *, p < 0.001 (insulin compared with insulin and BAPTA-AM).

To obtain more quantitative data we performed further experiments where the cell surface expression of HA-GLUT4 was quantified using a colorimetric assay (Fig. 4B) (30). Consistent with the immunofluorescence data, pretreatment with BAPTA-AM resulted in an almost total inhibition of insulin-stimulated cell surface expression of HA-GLUT4. These data implicate a role for Ca\(^{2+}\) in the fusion of GSVs with the plasma membrane and potentially resolve the discrepancy between the effects of BAPTA-AM on glucose transport and GLUT4 translocation as measured by the PM lawn technique or differential centrifugation (Figs. 1–3).

**BAPTA-AM Inhibits Insulin-stimulated Phosphorylation of Akt**—The above data implicate a role for Ca\(^{2+}\) at two different stages of the GLUT4 translocation process. Firstly, BAPTA-AM was shown to inhibit the translocation of GLUT4 to the cell surface by ~50%. Secondly, BAPTA-AM blocked the apparent insertion of GLUT4 into the plasma membrane. Collectively, these two effects may account for the almost complete inhibition of insulin-stimulated glucose transport by BAPTA-AM. The first effect of BAPTA-AM may involve a limitation in the availability of docking or fusion sites at the plasma membrane or an additional inhibitory effect of BAPTA-AM at a step between insulin binding to its receptor and that of GLUT4 docking at the PM. Pretreatment with BAPTA-AM had no significant effect on insulin-stimulated tyrosyl phosphorylation of the IR and IRS-1/IRS-2 (Fig. 5, top and middle panels). In contrast, BAPTA-AM reduced insulin-stimulated Akt phosphorylation by 75% (Fig. 5, bottom panel). Thus, chelation of intracellular Ca\(^{2+}\) with BAPTA-AM results in inhibition of insulin-stimulated Akt phosphorylation at a step that is distal to IR and IRS-1/IRS-2 phosphorylation.

**W13 Inhibits Insulin-stimulated Phosphorylation of Akt and**
Adipocytes were incubated in the absence or the presence of BAPTA-AM (50 μM) for 10 min and subsequently treated with insulin (1 μM) for a further 15 min. A, cells on coverslips were rinsed in PBS, and indirect immunofluorescence was performed as described under “Experimental Procedures.” The panels show representative images from four independent experiments. B, cells grown in 24-well plates were rinsed twice in PBS, and quantitation of cell surface expression of HA-GLUT4 was determined by a colorimetric assay as described under “Experimental Procedures.” The data represent the means ± S.E. from four independent experiments with treatments performed in triplicate in each experiment. *, p < 0.001 (insulin compared with insulin and BAPTA-AM).

**FIG. 4.** Inhibition of insulin-stimulated cell surface expression of HA-GLUT4 by BAPTA-AM. 3T3-L1 adipocytes expressing HA-GLUT4 were incubated in the absence or the presence of BAPTA-AM (50 μM) for 10 min and subsequently treated with insulin (1 μM) for a further 15 min. A, cells on coverslips were rinsed in PBS, and indirect immunofluorescence was performed as described under “Experimental Procedures.” The panels show representative images from four independent experiments. B, cells grown in 24-well plates were rinsed twice in PBS, and quantitation of cell surface expression of HA-GLUT4 was determined by a colorimetric assay as described under “Experimental Procedures.” The data represent the means ± S.E. from four independent experiments with treatments performed in triplicate in each experiment. *, p < 0.001 (insulin compared with insulin and BAPTA-AM).

**FIG. 5.** BAPTA-AM has no effect on insulin-stimulated IR and IRS-1/IRS-2 phosphorylation but inhibits Akt phosphorylation. Adipocytes were incubated in the absence or the presence of BAPTA-AM (50 μM) for 10 min and subsequently treated with insulin (1 μM) for a further 15 min. The cells were homogenized, and subcellular fractions were prepared and subjected to SDS-PAGE and immunoblotting as described under “Experimental Procedures.” Individual panels show representative immunoblots from five independent experiments as follows. Top panel, pIR, antiphosphotyrosine immunoblot of the PM fractions showing a band at ~95 kDa, which corresponds to the tyrosyl-phosphorylated β-subunit of the IR. Middle panel, pIRS-1/2, antiphosphotyrosine immunoblot of the high speed pellet fractions showing a band at ~180 kDa, which corresponds to tyrosyl-phosphorylated IRS-1/IRS-2. Bottom panel, pAkt, antiphosphospecific Akt immunoblot of the cytosolic fractions showing a band at ~60 kDa, which corresponds to Akt phosphorylated at Ser473. Quantitation of protein bands was performed by densitometric analysis as described under “Experimental Procedures.” BAPTA-AM had no significant effect on insulin-stimulated tyrosyl phosphorylation of IR or IRS-1/IRS-2. Insulin-stimulated phosphorylation of Akt at Ser473 was inhibited by 75% ± 4% in the presence of BAPTA-AM (p < 0.001).

**Glucose Uptake**—Previous studies have suggested that the ubiquitously expressed Ca2+-binding protein calmodulin is required for the efficient activation of PI 3-kinase by insulin and subsequent activation of Akt (17). Consistent with this, we found that pretreatment of cells with the calmodulin antagonist W13 inhibited insulin-stimulated Akt phosphorylation by 70% (Fig. 6A) without affecting phosphorylation of the IR and IRS proteins (data not shown). In addition, W13 inhibited insulin-stimulated 2-DOG uptake by 60% (Fig. 6B).

Taken together, the above data suggest that Ca2+, presumably via its effects on calmodulin, plays an important role in the insulin-signaling cascade at the level of PI 3-kinase activation and are consistent with previous reports (17). It may also be inferred from the above data that inhibition of insulin-stimulated Akt phosphorylation by either BAPTA-AM or W13 may be at least partly responsible for the observed inhibition of GLUT4 translocation and glucose uptake. However, consistent with previous data (31), we observed a significant discrepancy between the dose-response curves for insulin-stimulated glucose transport and insulin-stimulated Akt phosphorylation in adipocytes (Fig. 7). These data indicate that at low concentrations of insulin (~5 nM), where glucose transport is almost at its maximum stimulation, Akt phosphorylation is only increased to a level that is 28% of that observed at maximum insulin stimulation. Because BAPTA-AM or W13 only decreased Akt phosphorylation by 70–75% at a maximum insulin concentration, it seems unlikely that this could account for the inhibitory effects of these compounds on insulin-stimulated GLUT4 translocation and glucose transport.

**Ionophores Prevent the Effects of BAPTA-AM on Akt Activation and GLUT4 Translocation but Not Glucose Uptake**—In an attempt to overcome the inhibitory effects of Ca2+ chelation with BAPTA-AM, we incubated cells with the Ca2+ ionophores A23187 or ionomycin. Incubation of cells with the ionophores (or vehicle) in the absence of insulin was without effect on Akt phosphorylation or GLUT4 translocation (data not shown). Simultaneous incubation of cells with either A23187 or ionomycin and BAPTA-AM prevented the inhibition of insulin-stimulated Akt phosphorylation by BAPTA-AM (Fig. 8A, upper panel). Moreover, treatment with either ionophore prevented the inhibition of insulin-stimulated GLUT4 translocation to the

**FIG. 6.** The calmodulin antagonist W13 inhibits insulin-stimulated Akt phosphorylation and glucose uptake. Adipocytes were incubated in the absence or the presence of W13 (70 μM) for 20 min and subsequently treated with insulin (1 μM) for 15 min. A, cells were homogenized, and subcellular fractions were prepared and subjected to SDS-PAGE and immunoblotting as described under “Experimental Procedures.” The panel shows a representative antiphospho-Ser473 Akt immunoblot of the cytosolic fraction. Insulin-stimulated phosphorylation of Akt was reduced by 70% ± 6% in four independent experiments (p < 0.001). B, 2-DOG uptake was measured in the final 2 min of treatment as described under “Experimental Procedures.” The results depict the means ± S.E. from four independent experiments. *, p < 0.001 (insulin compared with insulin and W13).
PM by BAPTA-AM, as determined by subcellular fractionation (Fig. 8A, lower panel). Consistent with previous reports in L6 cells (9), primary adipocytes (13, 14), cardiac myocytes (12), and isolated skeletal muscle (32) treatment with ionophores alone was without effect on basal glucose uptake (control, 11% ± 2; A23817, 10% ± 2; ionomycin, 11% ± 2; n = 4). However, the ionophores did cause a slight reduction in insulin-stimulated glucose uptake (control, 100%; A23817, 81% ± 5; ionomycin, 82% ± 4; n = 3). In marked contrast to the reversal of the BAPTA-AM inhibition of Akt phosphorylation and GLUT4 translocation by ionophores, treatment with ionophores did not reverse the inhibitory effects of BAPTA-AM on insulin-stimulated 2-DOG uptake (Fig. 8B).

**BAPTA-AM Inhibits GLUT4 and GLUT1 Transporter Activity**—The inability of ionophores to overcome the inhibitory effects of BAPTA-AM on insulin-stimulated glucose transport may be due to a direct effect of BAPTA-AM on the activity of the GLUT4 transporter. To investigate this possibility cells were treated with insulin at 37 °C for 15 min to stimulate translocation of GLUT4 to the cell surface. The cells were then rapidly cooled to 4 °C by washing in ice-cold buffer and maintained on ice to prevent further vesicular trafficking. The cells were then treated with BAPTA-AM for increasing times, and 2-DOG uptake was measured at 4 °C. As illustrated in Fig. 9A, the effects of insulin treatment prior to temperature shift were consistent with those seen earlier (Fig. 1), with insulin stimulating 2-DOG uptake 10-fold. Treatment of cells with BAPTA-AM resulted in a time-dependent inhibition of 2-DOG uptake. This inhibition was noticeable even at the earliest time point studied (0 min), where BAPTA-AM was added simultaneously with the 2-DOG. To test the specificity of the effects of BAPTA-AM at 4 °C, we performed similar experiments using the calmodulin antagonist W13 and the nonesterified membrane impermeant BAPTA or EGTA-AM (Fig. 9B). Consistent with the results above, 10 min of treatment with BAPTA-AM at 4 °C resulted in an approximately 35% reduction in maximal 2-DOG uptake (Fig. 9B). In contrast, W13, BAPTA, or EGTA-AM had no significant effect on glucose transport. These data indicate that BAPTA-AM may inhibit the activity of GLUT4 even when its presence at the PM is maintained by inhibiting endocytosis, under conditions that are independent of insulin-signaling or GLUT4 translocation. Moreover, the inhibition of GLUT4 transporter activity is specific to BAPTA-AM and appears to require access to the interior of the cell.

To determine whether the inhibition of glucose transporter activity by BAPTA-AM was specific to GLUT4, we investigated the effects of BAPTA-AM in CHO cells. These cells express high levels of the GLUT1 glucose transporter, but they do not express GLUT4 (33). In CHO.IR cells insulin stimulated 2-DOG uptake by almost 2-fold (Fig. 10A), and both basal and insulin-stimulated 2-DOG uptake were dramatically inhibited by pre-treatment with BAPTA-AM (Fig. 10A). Following insulin treatment and rapid cooling of cells to 4 °C, as described above, incubation with BAPTA-AM for 10 min resulted in a 30% reduction in maximal 2-DOG uptake, whereas treatment with BAPTA or EGTA-AM was without significant effect (Fig. 10B). These results are comparable with those observed in 3T3-L1 adipocytes, suggesting that the inhibition of GLUT4 and GLUT1 transporter activity by BAPTA-AM occurs in a similar fashion. Ionophores were unable to overcome the inhibition of 2-DOG uptake by BAPTA-AM in either 3T3-L1 cells or CHO.IR cells at 4 °C (data not shown).

**DISCUSSION**

In the current study we have investigated the role of Ca\(^{2+}\) in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. By chelating intracellular Ca\(^{2+}\) with BAPTA-AM or inhibiting calmodulin with W13, we find that Ca\(^{2+}\)/calmodulin is required at two stages of insulin-stimulated glucose uptake. Firstly, Ca\(^{2+}\)/calmodulin is involved in the translocation process that trig-
Ca²⁺ and Insulin Action

FIG. 9. Inhibition of GLUT4 transporter activity by BAPTA-AM but not W13, BAPTA, or EGTA-AM at 4 °C. A, adipocytes were incubated in the absence or the presence of insulin (1 μM) for 15 min at 37 °C. The cells were then rapidly cooled to 4 °C by washing in ice-cold buffer and incubating on ice. BAPTA-AM (50 μM) was added at the appropriate time, and 2-DOG uptake was measured for 1 min as described under “Experimental Procedures.” It is noteworthy that all cells were incubated at 4 °C for the same duration. The data depict the means ± S.E. of four independent experiments. *, p < 0.001 (absence compared with presence of BAPTA-AM).

FIG. 10. Inhibition of GLUT1 transporter activity by BAPTA-AM but not BAPTA or EGTA-AM at 4 °C. A, CHO.IR cells grown in 12-well plates were incubated in the absence or the presence of BAPTA-AM (50 μM) for 10 min and subsequently treated with insulin (1 μM) for a further 15 min. 2-DOG uptake was measured for 2 min as described under “Experimental Procedures.” The data represent the means ± S.E. of four independent experiments. *, p < 0.001 (basal compared with BAPTA-AM); **, p < 0.001 (insulin compared with BAPTA-AM). B, CHO.IR cells were incubated in the absence of insulin (1 μM) for 15 min and then cooled rapidly to 4 °C by washing in ice-cold buffer and incubating on ice. The cells were incubated for a further 10 min in the absence or the presence of BAPTA-AM (B-AM), BAPTA (B), or EGTA-AM (E-AM) (all at 50 μM except W13, which was at 70 μM), and 2-DOG uptake was measured for 1 min as described under “Experimental Procedures.” The data depict the means ± S.E. of four independent experiments. *, p < 0.001 (absence compared with presence of BAPTA-AM).

...of the Ca²⁺/calmodulin system. This may involve the insulin signaling pathway, because we observed a significant decrease in Akt activation in response to BAPTA-AM or W13. Secondly, there is a requirement for Ca²⁺ at a post-docking stage of GLUT4 vesicle translocation to the PM, presumably involving membrane fusion. In addition to the above findings we also present evidence that BAPTA-AM inhibits the activity of mammalian facilitative glucose transporters (GLUT1 and GLUT4) independently of Ca²⁺, possibly by binding to the glucose-binding site in the transporter.

In the present report we have made three separate observations supporting a role for Ca²⁺/calmodulin in insulin-stimulated glucose transport. First, exclusion of Ca²⁺ from the extracellular buffer resulted in a 30% decrease in insulin-stimulated glucose uptake. Second, the Ca²⁺ chelator BAPTA-AM inhibited insulin-stimulated GLUT4 translocation and the fusion of GLUT4 vesicles with the plasma membrane. Third, the calmodulin antagonist W13 inhibited insulin-stimulated glucose uptake by 60%. A number of other studies have also suggested a role for Ca²⁺ in insulin-stimulated glucose transport. Incubation of adipocytes with ionomycin in the absence of extracellular Ca²⁺ or with the Ca²⁺ chelator Quin 2-AM inhibited insulin-stimulated glucose transport (13–15). There is also accumulating evidence to implicate calmodulin in this process. The calmodulin antagonist CGS 9343B and the Ca²⁺-dependent calmodulin protein kinase II inhibitor KN 62 reduced insulin-stimulated glucose uptake in rat skeletal muscle (34, 35). In contrast a number of studies have failed to find a role for Ca²⁺ in insulin-stimulated glucose uptake (9–12, 36). For example, removal of extracellular Ca²⁺ or chelation of intracellular Ca²⁺ using fura-2 had no effect on basal or insulin-stimulated glucose transport in cardiac myocytes (12).

One possibility for this controversy may be that many of the agents that are used to chelate intracellular Ca²⁺ do so with a large variation in efficiency because of differences in association/dissociation constants for Ca²⁺ (12, 37). For example, BAPTA has >100-fold higher association and dissociation rates than EGTA (37). A second problem is that many of the techniques used to chelate Ca²⁺ may not have accessed all of the intracellular pools of Ca²⁺. It has recently been shown that Ca²⁺ plays an important role in vesicle fusion and that the Ca²⁺− is released from the lumen of the vesicle during the actual fusion process (18–20, 37). Hence, in this instance it is necessary to use fast chelators such as BAPTA or chelators that can cross all lipid membranes. A third factor is the use of reagents at suboptimal concentrations and cell models with limited responses (see Ref. 14 for a discussion).

One of the steps in the insulin-stimulated accumulation of GLUT4 at the cell surface that requires Ca²⁺ is the actual translocation process itself. In view of the complex nature of this process, we have been unable to pinpoint the precise locus of the Ca²⁺-dependent step. However, it may involve the insulin signal transduction pathway because we observed a significant decrease in insulin-stimulated Akt phosphorylation in the presence of either BAPTA-AM or W13. This appeared to be a specific effect because upstream signaling events including...
IR and IRS-1/2 phosphorylation were intact. However, further scrutiny of this step makes it unlikely that this is the sole cause of the defect in GLUT4 translocation. The dose-response curves of Akt phosphorylation and glucose uptake are significantly different, and the inhibition of insulin-stimulated Akt phosphorylation observed with either BAPTA-AM or W13 was only partial. One possibility is that a specific intracellular pool of Akt may be more potently inhibited by BAPTA-AM or W13. However, we were unable to find any evidence for this by measuring Akt phosphorylation in different subcellular fractions. Nevertheless, these data clearly implicate an important role for Ca\(^{2+}\)/calmodulin in the full activation of Akt, and this is likely to be relevant to other downstream events. This is consistent with previous reports showing that activation of PI 3-kinase by insulin is inhibited by calmodulin antagonists (17) or by inhibition of Ca\(^{2+}\) influx (16). Direct interactions between calmodulin and IRS-1 and also between calmodulin and PI 3-kinase provide a potential explanation for the above effects (38, 39).

The inhibition of GLUT4 translocation by BAPTA-AM accounted for a 50% loss in GLUT4 at the PM, as determined by either the PM lawn assay or subcellular fractionation. However, the insulin-stimulated appearance of GLUT4 at the cell surface, as determined by cell surface binding, was totally blocked. Taken together, these data highlight the potential unsuitability of subcellular fractionation and plasma membrane lawns to unambiguously detect GLUT4 insertion at the plasma membrane. We surmise that the GLUT4 vesicles are docked at the PM but are unable to subsequently fuse and integrate into the cell surface membrane. It is known that docking complexes, involving the SNARE proteins, when formed do so with very high affinity (40). It is therefore likely that docked vesicles would remain attached to the PM during isolation of these membranes in vitro.\(^2\) This conclusion is supported by recent findings from in vitro vesicle fusion reactions where it has been shown that BAPTA, but not EGTA, inhibits endosome-endosome (37), endosome-lysosome (20), intra-Golgi membrane (19), and vacuole membrane fusion at a post-docking step (18). Similarly, calmodulin antagonists inhibited vesicle fusion, and this effect was reversed by the addition of excess calmodulin (18, 19). Hence, there is now considerable evidence to suggest a potential role for Ca\(^{2+}\)/calmodulin in many different, if not all, vesicle fusion events in eukaryotic cells. Therefore the fusion of GLUT4 vesicles with the PM would appear to represent another example of Ca\(^{2+}\)/calmodulin-regulated fusion.

In the current study we have not specifically addressed whether insulin regulates intracellular Ca\(^{2+}\) levels. However, our data may contribute to the ongoing discussion related to this question. Several studies failed to detect an insulin-dependent change in intracellular Ca\(^{2+}\) in various cell types including cultured myotubes, cardiac myocytes, and adipocytes (9–12). In contrast, using the fluorescent Ca\(^{2+}\) indicators Indo-1 and FIP18, it has recently been shown that in intact single skeletal muscle fibers near membrane, but not global, Ca\(^{2+}\) concentrations are increased in response to insulin (41). Moreover, L-type Ca\(^{2+}\)-channel blockers, which have previously been shown to inhibit insulin-stimulated glucose transport (42), prevented this response (41). Our studies are consistent with this because we observed a modest decrease in insulin-stimulated glucose transport upon removal of extracellular Ca\(^{2+}\). In addition, the GLUT4 vesicles themselves may contribute to the near membrane increase in Ca\(^{2+}\). In the case of endosome-endosome (37), endosome-lysosome (20), intra-Golgi membrane (19), and vacuole membrane fusion (18), it has been concluded that Ca\(^{2+}\) is released from the lumen of the vesicles, thus promoting fusion. This conclusion was based on the observation that vesicle fusion was inhibited in the presence of EGTA-AM but not EGTA (20, 37). In contrast, the nonesterified version of BAPTA was able to inhibit fusion, presumably because of its fast Ca\(^{2+}\) exchange rate (18–20, 37). However, in these studies it was necessary to use very high concentrations of EGTA-AM, probably because of the very high intraluminal Ca\(^{2+}\) levels in these organelles. By analogy we surmise that GLUT4 vesicles may also contain Ca\(^{2+}\) that is released into the cytoplasm when the vesicles are docked at the PM. These findings may explain a number of previous observations concerning the role of Ca\(^{2+}\) in insulin action. Firstly, they may explain why elevated Ca\(^{2+}\) alone is not capable of triggering GLUT4 translocation. The elevation in Ca\(^{2+}\) may only be involved at a late stage when the vesicles have docked with the membrane. Secondly, they may explain the difficulty in overcoming these effects with ionophores. A burst in the release of intraluminal vesicular Ca\(^{2+}\) triggers this event rather than a global increase in intracellular Ca\(^{2+}\). Thirdly, they may explain why slow Ca\(^{2+}\) chelators such as EGTA fail to inhibit this process and emphasizes the utility of the fast Ca\(^{2+}\) chelator BAPTA. We have previously shown that in streptolysin O-permeabilized adipocytes insulin-stimulated GLUT4 translocation is preserved in the presence of EGTA (27). However, this slow Ca\(^{2+}\) chelator would not have accessed the lumen of the GLUT4 vesicles, thus potentially explaining the inability of this reagent to inhibit GLUT4 translocation. Finally, we have been unable to recapitulate the effects of BAPTA-AM using EGTA-AM. It is possible that there may be difficulty in reaching concentrations high enough to chelate the intraluminal calcium levels inside these vesicles. Additionally, the hydrolyzing esterases may not be present in the lumen of these vesicles.

In the present study we were able to reverse the BAPTA-AM inhibition of insulin-stimulated Akt phosphorylation and GLUT4 translocation with ionophores. However, this was not the case for glucose transport. Our observation that BAPTA-AM inhibited basal glucose transport was also of note. Three additional observations suggest that this effect may represent a separate, Ca\(^{2+}\)-independent, effect of BAPTA-AM to inhibit the transport activity of the transporter itself. First, glucose transport was sensitive to BAPTA-AM at 4 °C. Second, glucose transport in CHO.IR cells was inhibited by BAPTA-AM. These effects were unaltered by ionophores. Third, molecular modeling studies indicate that many low energy conformations of BAPTA are capable of presenting a glucose-type arrangement of oxygen atoms (data not shown). This effect is similar to that described for the glucose transport inhibitor cytochalasin B, which is a rigid macrocycle (43). Because of the above, we propose that in aqueous buffers BAPTA is able to assume a conformation that resembles the d-isomer of glucose, which may therefore allow it to act as a competitor of glucose binding. This may make this reagent less suitable for in vivo experiments than previously realized.

In conclusion, we suggest that Ca\(^{2+}\)/calmodulin does play an important role in insulin action on glucose transport. First it is involved in some process of the GLUT4 translocation process, and second it is involved in the fusion of GLUT4 vesicles with the PM. Based on recent studies it is tempting to speculate that the molecular regulation of both of these processes may be linked, possibly at the PM. Klip and co-workers have recently shown that key elements of the insulin signal transduction pathway including PI 3-kinase assemble at unique sites at the PM (44). Importantly, they have also shown that GLUT4 vesicles...
Ca$^{2+}$ and Insulin Action

icles appear to insert at these same sites. It will therefore be of interest to determine whether calmodulin, which has been reported to interact with PI 3-kinase (39), is also concentrated at such sites and whether BAPTA-AM or other relevant inhibitors can prevent the assembly of these structures.

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The Role of Ca\textsuperscript{2+} in Insulin-stimulated Glucose Transport in 3T3-L1 Cells
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