Intracellular Delivery of Phosphatidylinositol (3,4,5)-Trisphosphate Causes Incorporation of Glucose Transporter 4 into the Plasma Membrane of Muscle and Fat Cells without Increasing Glucose Uptake*

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Significant progress has been made in recent years in understanding the intracellular signaling and traffic mechanisms regulating insulin-stimulated glucose uptake (1–3). However, the precise signals linking the occupied insulin receptor to the gain in active glucose transporters (GLUT4 isofrom) on the surface of muscle and fat cells are still incompletely mapped (2). GLUT4 cycles continuously to and from the plasma membrane, and at steady state, the majority of GLUT4 resides in intracellular compartments (4–7). Insulin stimulation rapidly promotes GLUT4 translocation to the cell surface (for reviews, see Refs. 8–10), a phenomenon engaging phosphatidylinositol (PI) 3-kinase. The experimental approaches showing a need for PI 3-kinase in GLUT4 translocation include the use of the pharmacological inhibitors wortmannin (11, 12) and LY294002 (13) and expression of both inhibitory (14, 15) and constitutively active (16) mutant constructs of PI 3-kinase. Microinjection of neutralizing antibodies to the catalytic subunit of PI 3-kinase (17) or of peptides encompassing the Src homology 2 domain of p85 (18) reduces insulin-stimulated GLUT4 translocation. These studies suggest that the class I p85/p110 PI 3-kinase is responsible for insulin-stimulated GLUT4 recruitment to the membrane (14, 15, 17). The enzyme phosphorylates the 3′-OH position of the inositol ring to generate the lipid products PI 3-phosphate, PI(3,4)P₂, and PI(3,4,5)P₃ in vitro (19). The main lipid produced in vivo in response to insulin stimulation is PI(3,4,5)P₃ (13, 20).

PI 3-kinase products bind to and promote the activation of several serine/threonine kinases, among which Akt/PKB appears to be required for GLUT4 translocation (15, 21–27). Thus, there is now general agreement that PI 3-kinase activation is necessary for the increase in GLUT4 at the plasma membrane (8, 13, 20, 28). However, it is not known whether the elevation in PI 3-kinase products, specifically PI(3,4,5)P₃, suffices to cause GLUT4 translocation or whether GLUT4 translocation suffices to increase glucose uptake. Stimuli such as interleukin 4 and clustering of integrin β₁ fail to increase glucose uptake even though they augment PI(3,4,5)P₃ levels (29, 30).

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The abbreviations used are: PI, phosphatidylinositol; AM, acetoxymethyl; BAPTA, 1,2-bis(aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)carboxyfluorescein; NBD, nitrobenzoxadiazoyl; PBS, phosphate-buffered saline; PIP, phosphatidylinositol phosphate; PI(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; FKB, protein kinase B.
We have shown previously that delivery of the acetoxy-methyl (AM) ester of PI(3,4,5)P₃ to 3T3-L1 adipocytes to increase intracellular levels of this lipid product of PI 3-kinase did not increase glucose uptake, although it could partially rescue the inhibitory action of wortmannin on stimulation of glucose uptake by insulin (31). However, the effect of PI(3,4,5)P₃-AM on GLUT4 localization and Akt activation was not analyzed. Here we demonstrate that PI(3,4,5)P₃-AM causes Akt phosphorylation and distribution of GLUT4 to the periphery of 3T3-L1 adipocytes. Moreover, using an additional strategy developed by Ozaki et al. (32) to deliver PI(3,4,5)P₃ into cells we examine the cell surface exposure of myc-tagged GLUT4 in L6 muscle cells. We find that carrier-mediated delivery of PI(3,4,5)P₃ causes Akt activation as well as translocation and incorporation of GLUT4 into the plasma membrane of L6 myoblasts. Yet, again, this increase in the GLUT4 myc content at the cell surface was not accompanied by an increase in 2-deoxyglucose uptake. We propose that PI(3,4,5)P₃ suffices to elicit translocation and insertion of GLUT4 at the plasma membrane. Because such action is insufficient to mimic the insulin-dependent gain in glucose uptake, we propose that input(s) additional to PI(3,4,5)P₃ are required for the culmination of increased transport of glucose into muscle and fat cells through the translocated transporters.

**EXPERIMENTAL PROCEDURES**

**Materials**—Di-C₁₂-PI(3,4,5)P₃-AM was synthesized as described previously (31). All phosphoinositides, as well as the nitrobenzoxadiazole (NBD)-labeled or BodipyTM (Bodipy)-conjugated versions of PI(3,4,5)P₃, carrier (neomycin), and anti-PI(3,4,5)P₃ mouse IgG were from Echelon Biosciences (Salt Lake City, UT). BCECF-AM (31) and BA-PTA-AM were from Molecular Probes (Eugene, OR). Human insulin (Humulin) was obtained from Eli Lilly Canada, Inc. (Toronto, ON). Polyclonal anti-GLUT4 and monoclonal anti-GLUT4 serum (IP8) were from Biogenesis Inc. (Sanford, NH). Fluorescein isothiocyanate-conjugated and Cy3-conjugated goat-anti-rabbit secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Alexa 488-conjugated goat anti-mouse secondary antibody was from Molecular Probes. Polyclonal antibodies to Akt2 and Akt3, and Akt substrate peptide (Cross-tide) were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody to Akt1 was purchased from Cell Signaling (Mississauga, ON). 2-Deoxy-o-[³²P]ATP (6,000 Ci/mmol) was purchased from Amersham Biosciences. Anti-pS473Akt antibody was from Cell Signaling (Mississauga, ON). 2-Deoxy-o-[³²P]ATP was purchased from PerkinElmer Life Sciences. α-Phenylendiamine dihydrochloride reagent was from Sigma. All electrophoresis and immunoblotting reagents were purchased from Bio-Rad. All other reagents were of the highest analytical grade. All cell culture solutions and supplements were obtained from Invitrogen.

**Cell Culture**—3T3-L1 fibroblasts (a kind gift from Dr. G. Holman, University of Bath, UK) were grown in a monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 20% calf serum and 1% antibiotic solution (10,000 units/ml penicillin and 10 mg/ml streptomycin) in an atmosphere of 9% CO₂ at 37 °C. 3T3-L1 fibroblasts were differentiated into adipocytes as described previously (33). L6 muscle cells stably overexpressing GLUT4 tagged with a myc epitope at the first exofacial loop (L6GLUT4myc) were grown as described previously (15). Experiments were performed in monolayer in poly-lysine-coated 6-well plates. The cells were allowed to settle for 24 h and then serum starved for 3 h prior to treatment with 15 min with either 100 nm insulin or 150 μM PI(3,4,5)P₃-AM. Coverslips holding the cells were then immersed in 4% paraformaldehyde in PBS for 20 min at 4 °C. Excess parafomaldehyde was washed with 50 mM NH₄Cl in PBS for 5 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for 30 min and blocked with 5% goat serum in PBS for 30 min. Rabbit polyclonal antibodies against the GLUT4 C terminus (35) were used at a dilution of 1:250 to detect GLUT4 within the cells, and Cy3-conjugated goat anti-rabbit antibodies were used to label the primary antibodies. Fluorescence was detected using a Zeiss Laser Scanning Confocal Microscope 510. Immunofluorescent detection of GLUT4, Akt, PKB, and p-S473Akt in Plasma Membrane Lawns from Adipocytes Pretreated with PI(3,4,5)P₃-AM—Determination of GLUT4 distribution by immunofluorescence was performed after fixing and permeabilizing the cells. Briefly, fully differentiated 3T3-L1 adipocytes were trypsinized and resuspended in a low-density poly-lysine-coated 6-well plate. The cells were then permeabilized using 0.2% saponin in PBS at room temperature, fixed with 10 min blocking with 5% goat serum in PBS and incubation for 1 h at room temperature with anti-PI(3,4,5)P₃ mouse IgG. Coverslips were washed three times and reacted further with Alexa 488-conjugated anti-mouse IgG antibody. Images were obtained using a Zeiss Laser Scanning Confocal Microscope 510.

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Akt1, Akt2, and Akt3 were determined as described previously (33).

Rounded-up Myoblasts upon Carrier-mediated Delivery of PI(3,4,5)P

Washes in PBS, fluorophore-conjugated anti-rabbit IgG was used to


tibody (Cell Signaling) (1:250) was prepared in blocking solution and

(1:250), 1% Triton X-100, 0.1% bovine serum albumin, 1 M NaCl, 1

|100 g/m of total protein from cell lysates. Antibody-coupled

beads were washed twice with ice-cold PBS and once with ice-cold lysis
buffer before use. The indicated enzymes were immunoprecipitated by

incubating with the antibody-bead complex for 2–3 h under constant
rotation at 4 °C. Immunocomplexes were isolated and washed four
times with 1 ml of wash buffer (25 mM HEPES, pH 7.8, 10% glycerol
(v/v), 1% Triton X-100 (v/v), 0.1% bovine serum albumin, 1 M NaCl, 1
mM diethioseitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM microcystin,
and 100 μM okadaic acid). Antibody (2 μg/condition) precomplexed to pro-
tein A- or protein G-Sepharose beads (20 μl of 100 mg/ml each) was
incubated with antigen (1 μg of total protein) for 4 h at 4 °C.

PI(3,4,5)P3-AM Elicits GLUT4 Translocation in 3T3-L1
Adipocytes—PI(3,4,5)P3-AM is a derivative of PI(3,4,5)P3 in which all of the negatively charged phosphates are masked by AM esters to allow the compound to cross cell membranes readily (31). Within the cytoplasm, PI(3,4,5)P3-AM is hydrolyzed to PI(3,4,5)P3 by endogenous intracellular esterases (31). 3T3-L1 adipocytes were treated for 15 min with 150 μM PI(3,4,5)P3-AM or 100 μM insulin, and then cellular localization of GLUT4 was assessed in permeabilized cells by indirect immunofluorescence using antibodies directed against the C

terminus of GLUT4 (Fig. 1A). Under basal conditions GLUT4 exhibited a perinuclear distribution with very low amounts at the cell periphery. Insulin and PI(3,4,5)P3-AM each caused a redistribution of GLUT4 to the cell periphery and a concomitant decrease in the level of this transporter in the perinuclear region. The redistribution of GLUT4 was visualized...
further by detection of the transporters on plasma membrane lawns (sheets) of 3T3-L1 adipocytes. Fig. 1B shows representative immunofluorescence images of GLUT4 associated with plasma membrane lawns. For the graph at the lower panel, fluorescence intensity was measured, and results shown are from three independent experiments (five fields in each). The mean intensity in control cells was assigned a value of 1, and for each treatment data are expressed as the mean ± S.E. -fold intensity. *, p < 0.05 compared with nontreated controls. Scale bar, 10 μm.

To determine whether the mobilization of glucose transporters seen in response to PI(3,4,5)P₂-AM was caused by nonspecific effects of acetate or formaldehyde (liberated upon hydrolysis of PIP₂-AM) we measured GLUT4 levels in plasma membrane fractions of 3T3-L1 adipocytes (41). Similarly, pretreatment of cells with phosphatidylinositol or the fatty acid palmitate did not increase the amount of GLUT4 associated with membrane lawns (Table I). Taken together, these results suggest that elevation of cellular PI(3,4,5)P₂ can specifically mobilize GLUT4 toward the membrane of 3T3-L1 adipocytes.

Table I

| Treatment          | GLUT4 associated with plasma membrane lawns |
|--------------------|--------------------------------------------|
| Control            | 1.00 ± 0.09                                |
| Insulin            | 3.67 ± 0.33*                               |
| PIP₃-AM            | 2.44 ± 0.27*                               |
| PI                 | 0.81 ± 0.08                                |
| BAPTA-AM           | 1.01 ± 0.04                                |
| BCECF-AM           | 0.81 ± 0.07                                |
| Palmitate          | 0.58 ± 0.04                                |

*a p < 0.05 compared with control.
assessed carrier-mediated delivery of PI(3,4,5)P₃ into these cells by fluorescence microscopy using fluorophore-conjugated PI(3,4,5)P₃ and indirect immunofluorescence utilizing anti-PI(3,4,5)P₃ IgG. Bodipy-conjugated PI(3,4,5)P₃ preincubated with carrier (neomycin) could be readily detected within 3T3-L1 adipocytes (Fig. 3A). Similarly, the intracellular delivery of phosphoinositides into detached, rounded-up L6GLUT4myc myoblasts was confirmed with 10 μM neomycin (Fig. 3A). Finally, cellular PI(3,4,5)P₃ content was detected in adhered L6GLUT4myc myoblasts using anti-PI(3,4,5)P₃ antibody (Fig. 3C). Cells treated with PI(3,4,5)P₃ and carrier showed a notably higher signal than control or carrier-only exposed cells. Collectively these experiments demonstrate the ability to deliver exogenous PI(3,4,5)P₃ using a carrier into 3T3-L1 adipocytes and L6 muscle cells.

3T3-L1 adipocytes were treated with carrier without or with PI(3,4,5)P₃, membrane lawns were generated, and Akt phosphorylation on Ser-473 was determined by immunofluorescence. Fig. 4A illustrates the results and indicates that Akt was phosphorylated in lawns from 3T3-L1 adipocytes pretreated with either insulin or PI(3,4,5)P₃ plus carrier, but not with carrier alone. Similarly, the ability of carrier-dependent delivery of PI(3,4,5)P₃ to activate Akt was also tested in L6 myoblasts, cells in which Akt is phosphorylated robustly by insulin (15, 34, 38, 45). Myoblasts were pretreated with either insulin or carrier with or without PI(3,4,5)P₃, and pS473Akt was detected by immunofluorescence in permeabilized myoblasts. Fig. 4B shows that insulin and PI(3,4,5)P₃ plus carrier, but not carrier alone, increased the level of pAkt in L6 myoblasts.

These results demonstrate that like the PI(3,4,5)P₃-AM (Fig. 2), carrier-mediated PI(3,4,5)P₃ delivery can activate Akt/PKB. Carrier-mediated Delivery of PI(3,4,5)P₃ Causes GLUT4myc Translocation in L6 Myoblasts—In further agreement with the results using PI(3,4,5)P₃-AM (Fig. 1, B and C), carrier-mediated delivery of PI(3,4,5)P₃ resulted in increased association of GLUT4 with plasma membrane lawns in 3T3-L1 adipocytes (Fig. 5A). Quantification of the fluorescence intensity derived from immunodetected GLUT4 is depicted in Fig. 5B, revealing that carrier-mediated PI(3,4,5)P₃ induced ~50% of the response seen with 100 nM insulin. The clonal line of L6 muscle cells used in this study stably expresses myc-tagged GLUT4. The exofacially facing myc epitope allows detection of membrane-inserted GLUT4 (15, 34, 38, 45). Hence, this system was used to ascertain whether PI(3,4,5)P₃ causes bona fide insertion of GLUT4 into the plasma membrane. Carrier-dependent delivery of various phosphoinositides was tested in this system. GLUT4myc appearance at the cell surface was assessed first by immunofluorescent detection of myc epitope exposure in intact (nonpermeabilized)
rounded-up myoblasts. Fig. 5C shows that in untreated control cells very little GLUT4myc was available at the surface of myoblasts examined by confocal fluorescence microscopy. Insulin treatment caused a clear increase in cell surface GLUT4myc, visualized as a rim of fluorescence in intact cells. PI(3,4,5)P3 plus carrier (10 μM each) also increased the amount of GLUT4myc at the cell surface, whereas the carrier alone (10 μM) or with PI(4,5)P2 (10 μM, not shown) had no effect on surface GLUT4myc. To complement this single-cell assay, the extent of GLUT4myc exposure at the surface of cell populations was determined on 3T3-L1 adipocytes and L6 myoblasts. As shown in Fig. 5, insulin elicited a 2.8-fold increase in GLUT4myc compared with the untreated control (Student’s t test). L6 myoblasts were incubated for 20 min with 100 nM insulin, PI(3,4,5)P3 plus carrier (10 μM each), or 10 μM carrier alone. Detection of anti-pS473Akt antibody via immunofluorescence and quantification is described under “Experimental Procedures.” Results are expressed as the mean ± S.E. relative to the untreated control. Lot CF-41-138 of PI(3,4,5)P3 was used in these studies, yielding p < 0.012 for PI(3,4,5)P3 versus control (Student’s t test). B, L6GLUT4myc myoblasts were incubated for 20 min with 100 nM insulin, PI(3,4,5)P3 plus carrier (10 μM each), or 10 μM carrier alone. Detection of GLUT4myc distribution. Further analysis of the mechanisms underlying these changes is beyond the scope of this study, but the results illustrate an insulin-like effect of PI(3,4,5)P3 plus carrier on the perinuclear compartment(s) of GLUT4myc, presumably as part of the mechanism that mobilizes transporters toward the cell surface.

**Fig. 4. Carrier-dependent delivery of PI(3,4,5)P3 causes phosphorylation of Akt in 3T3-L1 adipocytes and L6 myoblasts.**

A, 3T3-L1 adipocytes were incubated for 20 min with 100 nM insulin, PI(3,4,5)P3 plus carrier (10 μM each), or 10 μM carrier alone. Membrane lawns were generated, and Akt/PKB was detected by immunofluorescence using an anti-pS473Akt antibody. The lawn-associated fluorescent signal was quantified as described under “Experimental Procedures.” Results are expressed as the mean ± S.E. relative to the untreated control. Lot CF-41-138 of PI(3,4,5)P3 was used in these studies, yielding p < 0.012 for PI(3,4,5)P3 versus control (Student’s t test). B, L6 myoblasts were incubated for 20 min with 100 nM insulin, PI(3,4,5)P3 plus carrier (10 μM each), or 10 μM carrier alone. Detection of anti-pS473Akt antibody via immunofluorescence and quantification is described under “Experimental Procedures.” Results are expressed as the mean ± S.E. relative to the untreated control. Lot CF-41-138 of PI(3,4,5)P3 was used in this study yielding p < 0.08 for PI(3,4,5)P3 versus control (Student’s t test).

**DISCUSSION**

It is generally accepted that type IA PI 3-kinase(s) participates in the insulin-dependent translocation of GLUT4 to the perinuclear region (46, 47). We further assessed the effect of carrier-delivered PI(3,4,5)P3 on perinuclear GLUT4 and quantified these effects in L6 myoblasts. To this end, we examined the morphology of the GLUT4 pool around the nucleus of L6 myoblasts in the basal state and in response to insulin or PI(3,4,5)P3 plus carrier. The staining pattern of intracellular GLUT4myc in L6GLUT4myc myoblasts in three independent experiments is depicted in Fig. 6. In nonstimulated conditions, GLUT4myc assumed a polarized conical distribution at one pole of the nucleus in the majority (62.3 ± 14.7%) of the cells. In response to insulin, this distribution changed to cover a wider area of the nuclear perimeter and appeared even more expressed and less conical. The percent of cells that exhibited GLUT4myc cones was decreased to 9.7 ± 3.5 (p < 0.01 compared with control cells), and the percent of cells with GLUT4myc signal coverage exceeding half of the nuclear perimeter rose from 35.5 ± 14.1 to 63.5 ± 15.4. Strikingly, PI(3,4,5)P3 plus carrier, but not carrier alone, caused a similar change to insulin in GLUT4myc distribution. Further analysis of the mechanisms underlying these changes is beyond the scope of this study, but the results illustrate an insulin-like effect of PI(3,4,5)P3 plus carrier on the perinuclear compartment(s) of GLUT4myc, presumably as part of the mechanism that mobilizes transporters toward the cell surface.

**Hexose Uptake into 3T3-L1 Adipocytes and L6 Myoblasts**—The above results show that carrier-mediated delivery of PI(3,4,5)P3 suffices to induce phosphorylation of Akt, changes in the perinuclear distribution of GLUT4myc, mobilization of GLUT4 to the cell surface, and insertion into the plasma membrane. It was therefore important to examine whether glucose uptake was stimulated under the same conditions. In L6 GLUT4myc myoblasts, GLUT4myc is highly overexpressed and is the transporter determining glucose uptake (39). Strikingly, carrier-introduced PI(3,4,5)P3 (10 μM, 20 min) had no effect on 2-deoxyglucose uptake in either 3T3-L1 adipocytes or L6GLUT4myc myoblasts (Fig. 7, A and B, respectively). Higher concentrations of the lipid-carrier complex (up to 50 μM) were also ineffective in stimulating glucose uptake. Pretreatments with carrier alone, with PI 3-phosphate, with a combination of PI 3-phosphate and PI(3,4,5)P3, or along with PI(4,5)P2 (not shown) were also without consequence on 2-deoxyglucose uptake. In contrast, 100 nM insulin caused an increase in hexose uptake of more than 7-fold and 2-fold in 3T3-L1 adipocytes and L6GLUT4myc myoblasts, respectively, the latter characteristic of the response of muscle cells in culture and isolated muscle preparations. To compare better the effect of insulin and exogenous PI(3,4,5)P3 on glucose uptake, cells were also stimulated with a submaximal concentration of insulin (1 nM). This concentration was shown previously to cause ~50% increase in GLUT4 associated with plasma membrane lawns in 3T3-L1 adipocytes (39), similar to the effect induced by PI(3,4,5)P3 (Fig. 5B). Likewise, 1 nM insulin induced an ~1.5-fold increase in surface GLUT4myc in L6GLUT4myc myoblasts (48), resembling the magnitude of the effect provoked by exogenous PI(3,4,5)P3 in these cells (Fig. 5D). Submaximal (1 nM) insulin stimulated glucose uptake nearly 4.5- and 1.5-fold in 3T3-L1 adipocytes (Fig. 7A) and L6GLUT4myc myoblasts (Fig. 7B), respectively. Yet, despite the similar levels of GLUT4 translocation achieved with insulin, exogenous PI(3,4,5)P3 had no effect on glucose uptake. These results suggest that insertion of GLUT4 into the plasma membrane in response to PI(3,4,5)P3 does not suffice to elicit stimulation of glucose uptake.
plasma membrane, based on the inhibitory action of pharmacological inhibitors and a dominant-negative mutant of the p85 subunit of the enzyme, in both adipose and muscle cells. Conversely, when the p110 catalytic subunit of class I PI 3-kinase is targeted to membranes in cultured adipocytes, surface GLUT4 increases.

In vitro, class IA PI 3-kinases can phosphorylate PI, PI 4-phosphate, and PI(4,5)P₂, resulting in the generation of PI 3-phosphate, PI(3,4)P₂, and PI(3,4,5)P₃, respectively. Thus, a fundamental question that arises is which of the specific PI 3-kinase product(s) mediates insulin action and in particular whether any one of these lipids suffices to elicit GLUT4 translocation to the plasma membrane and glucose uptake. Most studies have indistinctly measured glucose uptake or GLUT4 gain at the cell surface, yet these phenomena may require input from different products of the enzyme.

Several lines of evidence suggest that PI(3,4,5)P₃ is the major PI 3-kinase product required for the propagation of the insulin signal toward GLUT4. Overexpression of phosphatidylinositol phosphatases that decrease cellular PI(3,4,5)P₃ content reduce insulin-mediated GLUT4 translocation. This is the case for the 3'-phosphatase PTEN in one report (49), but not another (50). Conditional deletion of the PTEN gene from skeletal muscle increases the insulin response of glucose uptake in this tissue (51). However, because PTEN acts on several PIPs one cannot specifically assign a role for PI(3,4,5)P₃ versus PI(3,4)P₂ based on these observations. Like PTEN, the 5'-phosphatases SHIP and SKIP, which reduce PI(3,4,5)P₃ levels to elevate those of PI(3,4)P₂, impaired insulin-mediated GLUT4 translocation and glucose metabolism (52–54). Furthermore, SHIP2 knock-out mice exhibit enhanced insulin action (55). All of these studies support the notion that PI(3,4,5)P₃ is required for insulin action, but none shows that it is sufficient.

![PI(3,4,5)P₃ Causes GLUT4 Translocation but Not Glucose Uptake](http://www.jbc.org/)

**Fig. 5.** Carrier-dependent delivery of PI(3,4,5)P₃ causes GLUT4 translocation and insertion at the plasma membrane. A and B, GLUT4 association with plasma membrane lawns of 3T3-L1 adipocytes. 3T3-L1 adipocytes grown on glass coverslips were serum starved and treated for 20 min with 100 nM insulin, or carrier with or without PI(3,4,5)P₃ (10 μM each). Plasma membrane lawns were then prepared and reacted with anti-GLUT4 antibody as described under “Experimental Procedures.” Shown are images representing three independent experiments (A) and the quantitation of the fluorescent signal intensity in these experiments, as detailed under “Experimental Procedures” (B). C, immunofluorescent detection of GLUT4myc in intact, rounded-up L6 myoblasts. L6 GLUT4myc cells stably expressing GLUT4myc were detached from the substratum using Ca²⁺- and Mg²⁺-free PBS for 15 min. Dislodged cells were suspended in HEPES-buffered RPMI and seeded on glass coverslips. These rounded-up L6 myoblasts were allowed to attach for 10–20 min prior to 20 min treatment with 100 nM insulin, PI(3,4,5)P₃ plus carrier (10 μM each), or carrier alone. Surface GLUT4myc was then detected by indirect immunofluorescence using an anti-myc antibody in nonpermeabilized cells as described under “Experimental Procedures.” Results show representative cells of at least five experiments. Scale bar, 10 μm. D, quantification of surface GLUT4myc in L6 myoblast monolayers. L6 myoblast monolayers were exposed for 20 min to 100 nM insulin or PI(3,4,5)P₃ plus carrier (10 μM each), or carrier alone. The cells were then rinsed, and surface GLUT4myc was detected on intact cells using anti-myc antibody followed by secondary antibody coupled to horseradish peroxidase, as described under “Experimental Procedures.” GLUT4myc levels at the surface of untreated cells are given a value of 1.0, and all other values are expressed relative to this value. Nonspecific antibody binding was measured by anti-mouse IgG alone and was subtracted from all experimental values. Results are the mean of 21 independent experiments, each performed in triplicate, using PI(3,4,5)P₃ lots CF-I-178, CF-II-47, CF-41-138 and CF-66-107 (along with carrier). **, p < 0.001, *, p < 0.05 compared with control, by analysis of variance.
Here we undertook a direct approach to analyze the role of PI(3,4,5)P3, in insulin action. Two methods were used to deliver PI(3,4,5)P3 across the negatively charged lipid bilayer. The first one was the use of an AM derivative of PI(3,4,5)P3 (31), and the second one was the introduction of unmodified PI(3,4,5)P3 along with a polycationic carrier (32). We have reported previously that intracellular delivery of PI(3,4,5)P3, AM alone did not elevate glucose uptake in 3T3-L1 adipocytes, but it could partly restore insulin-stimulated glucose transport inhibited by the PI 3-kinase inhibitor wortmannin (31). We concluded that PI(3,4,5)P3 generation is a required, but not sufficient input for the stimulation of glucose uptake by insulin. The present study was designed to assess the role of inositol phospholipids in the mobilization of GLUT4 to the cell periphery and its full insertion into the cell membrane, a process required for insulin stimulation of glucose uptake. The following conclusions can be made from our findings: (i) Intracellular delivery of PI(3,4,5)P3 results in mobilization of intracellular GLUT4 to the cell periphery. This effect was observed using two different PI(3,4,5)P3 delivery methods in two different insulin-sensitive cell lines, 3T3-L1 adipocytes and L6 myoblasts. Increased peripheral localization of GLUT4 was evident by formation of a peripheral “rim” of the transporter in 3T3-L1 adipocytes and L6 myoblasts and by increased GLUT4 detection on plasma membrane lawns derived from 3T3-L1 adipocytes treated with PI(3,4,5)P3-AM. The intracellular compartment(s) of GLUT4 was concomitantly modified by the exogenous PI(3,4,5)P3, evinced by a morphological change in the perinuclear GLUT4 distribution from conical to crown-like in L6 myoblasts. (ii) Carrier-mediated delivery of PI(3,4,5)P3 caused surface insertion of the mobilized GLUT4 molecules, as demonstrated by a gain in surface myc epitope detected in nonpermeabilized L6 myoblasts expressing GLUT4myc upon challenge with PI(3,4,5)P3. (iii) GLUT4 mobilization and surface insertion induced by PI(3,4,5)P3 correlated with the plasma membrane migration and activation of Akt2/PKBβ in 3T3-L1 adipocytes and L6 myoblasts. This kinase is thought to be involved in GLUT4 traffic in response to insulin (15, 25, 34). (iv) Strikingly, and despite the “insulin mimetic” effects induced by carrier-dependent PI(3,4,5)P3 delivery into L6 myoblasts, glucose uptake was not stimulated.

The maximal increase in surface GLUT4myc caused by exogenously delivered PI(3,4,5)P3 is smaller than the response elicited by insulin. It is possible that the efficiency of the PI(3,4,5)P3 delivery systems is insufficient to mimic quantitatively the rise in PI(3,4,5)P3 caused by the hormone, although it suffices to mobilize GLUT4 to a significant extent. Indeed, for most of the end points measured (GLUT4 translocation, Akt translocation and/or phosphorylation) the response to PI(3,4,5)P3 was smaller than the insulin effect. It is also very likely that the duration of the PI(3,4,5)P3 produced in response to the hormone is critical for accurate signal transduction to metabolic end points. Indeed, longer duration or higher magnitude of plasma membrane localization of the PI(3,4,5)P3 subunits of PI 3-kinase (57). Finally, it is possible that signals additional to PI(3,4,5)P3 are required for the full extent of translocation. These could involve carefully balanced levels of other phosphoinositides such as PI(3,4)P2, PI 3-phos-
phate or PI 5-phosphate (58, 59) or signals complementary to the PI 3-kinase pathway. It must be stressed that the exogenously delivered PI(3,4,5)P_3 may be metabolized to some of those forms during the course of the loading period, and hence the biological responses observed here cannot be uniquely ascribed to PI(3,4,5)P_3. Alternatively, a PI 3-kinase independent pathway has been proposed in adipocytes to contribute to the full mobilization of GLUT4, involving the sequential engagement of Cbl, CAP, and the small GTPase TC10 (60, 61). However, this signaling cascade does not appear to operate in muscle cells (62), and the exact role of TC10 in adipocytes is still being defined (63).

The significant though partial translocation of GLUT4 to the plasma membrane caused by PI(3,4,5)P_3 was not accompanied by a parallel increase in 2-deoxyglucose uptake. Based on insulin dose-response curves of GLUT4 translocation and hexose uptake, we calculate that a partial increase in uptake should have been detectable for the extent of GLUT4 translocation by PI(3,4,5)P_3 in both cell types, if translocation alone sufficed to stimulate glucose uptake. The lack of stimulation of glucose uptake is not the result of lack of sensitivity of the assay because increases in 2-deoxyglucose uptake as small as 10% can be detected with statistical accuracy. Rather, it is possible that a threshold of PI(3,4,5)P_3 is required to stimulate glucose uptake (64), similar to a threshold proposed for eliciting glucose uptake (64), similar to a threshold proposed for eliciting GLUT4 translocation (56). Alternatively, again, other phosphoinositides might be required to impart glucose uptake properties to the translocated transporters in response to insulin. Interestingly, and coincident with the results reported here, Maffucci et al. (58) recently showed GLUT4 translocation in response to individual, different phosphoinositides without concomitant stimulation of glucose uptake. Here we show that adding PI 3-phosphate along with PI(3,4,5)P_3 also failed to provoke an increase in glucose uptake (Fig. 7). Hence, it is possible that in parallel to the PI 3-kinase lipid products, insulin generates additional inputs required for the stimulation of glucose uptake through the translocated GLUT4. These could include so far unidentified downstream targets of the serine kinase activity of proteins phosphorylated by PI 3-kinase or PI 3-kinase-independent signals (17, 65). Regardless of the identity of the PI(3,4,5)P_3-independent signal, it is clear that exogenously delivered PI(3,4,5)P_3 can cause a significant GLUT4 mobilization to the surface and insertion into the plasma membrane. Future work should focus on identifying the nature of the PI(3,4,5)P_3 complementary signal that is required to stimulate glucose uptake.

In conclusion, exogenous delivery of PI(3,4,5)P_3 to muscle and fat cells in culture causes a mobilization and membrane insertion of GLUT4, partly mimicking the response to insulin. The exogenous lipid also remodels the perinuclear GLUT4 pool and fat cells in culture causes a mobilization and membrane insertion of GLUT4 to the plasma membrane. Future work should focus on identifying the nature of the PI(3,4,5)P_3 complementary signal that is required to produce GLUT4 translocation but not glucose Uptake.
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Intracellular Delivery of Phosphatidylinositol (3,4,5)-Trisphosphate Causes Incorporation of Glucose Transporter 4 into the Plasma Membrane of Muscle and Fat Cells without Increasing Glucose Uptake

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