Use of Bismannose Photolabel to Elucidate Insulin-regulated GLUT4 Subcellular Trafficking Kinetics in Rat Adipose Cells

EVIDENCE THAT EOXYCITY IS A CRITICAL SITE OF HORMONE ACTION*

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The subcellular trafficking of tracer-tagged GLUT4 between the plasma membranes and low-density microsomes of rat adipose cells has been studied. Cell-surface GLUT4 have been initially tracer-tagged in the insulin-stimulated state with the [3H]bismannose photolabel 2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(omannos-4-xyloxy)-2-propylamine. The half-time for internalization of tracer-tagged GLUT4 when insulin is removed by collagenase treatment is similar to that observed for the decrease in immunodetectable GLUT4 in the plasma membranes and the decrease in glucose transport activity in the intact cells. In contrast, internalization of tracer-tagged GLUT4 also occurs when cells are maintained in the continuous presence of insulin even though the plasma membrane level of immunodetectable GLUT4 and plasma membrane glucose transport activity in the intact cells are unaltered. These data show, for the first time, that insulin has little, if any, effect on the rate constant for GLUT4 endocytosis, but instead, primarily increases the rate constant for exocytosis. Tracer-tagged GLUT4 that is returned to the low-density microsomes can be restimulated with fresh insulin to recycle to the plasma membranes and the decrease in glucose transport activity in the intact cells. Slot's group has shown that in brown adipose tissue (9) and heart muscle (10). SLOT et al. (11) found a smaller, approximately 12-fold, change in the recollection of GLUT4 and GLUT4 isoform in skeletal muscle.

Insulin now known to stimulate glucose transport in isolated rat adipose cells through a mechanism involving the translocation of glucose transporters, primarily the GLUT4 isoform, from a large intracellular pool to the plasma membrane (1–4). In rat adipose cells, insulin has been shown to produce increases in glucose transport activity and an increase in the concentrations of glucose transporters in the plasma membranes and low-density microsomes, respectively. This translocation has been clearly shown to be rapid, reversible, insulin concentration-dependent (1–3), and ATP-dependent (5). In addition, the membrane vesicles comprising the intracellular pool of glucose transporters have been partially purified from a crude microsomal membrane fraction containing components of the Golgi apparatus (6, 7). Convincing support for the translocation model has come from immunocytochemical localization of the GLUT4 isoform. Bornemann et al. (8) have shown insulin-dependent alterations in GLUT4 distribution in skeletal muscle. Slot et al. have used immunogold to localize GLUT4 glucose transporters in thin sections of brown adipose tissue (9) and heart muscle (10). Slot's group has shown that in brown adipose tissue in the basal state, GLUT4 are primarily localized to the trans-Golgi reticulum and tubulovesicular structures elsewhere in the cytoplasm, with <1% associated with the plasma membrane; after insulin stimulation, ~40% of the glucose transporters are located at the cell surface, and additional GLUT4 are enriched in the early endosomes (9). However, Smith et al. (11) found a smaller, ~12-fold, change in the redistribution of GLUT4 to the plasma membrane of white adipose cells.

Immunocytochemical studies suggest that glucose transporters are distributed in an insulin-regulated process similar to the receptor-mediated endocytosis that occurs in the processing of receptors such as the transferrin, asialoglycoprotein, and insulin receptors (12–15). However, measurements of the steady-state distribution of glucose transporters (1–4, 8–11) leave several mechanistic questions unanswered. For example, it is unknown whether the whole intracellular pool of glucose transporters is available for recycling to the plasma membrane. In addition, kinetic studies are needed to determine whether insulin regulates endocytosis or exocytosis.

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To address these issues, it is necessary to track individual-glucose transporter molecules as they recycle between subcellular compartments. Recently, we have developed a procedure for specifically labeling the plasma membrane pool of glucose transporters with the membrane-impermeant bismannosyl photolabel ATB-BMPA. Using this technique, we have shown that insulin produces a 15-20-fold increase in cell-surface GLUT4 and a 3-5-fold increase in cell-surface GLUT1 in both rat adipose cells (4, 16) and 3T3-L1 cells (17-19). We have previously used this photolabel to examine the kinetics of glucose transporter appearance and loss from the cell surface of rat adipose cells (16), but in the study described here, we have used the probe to examine the kinetics of glucose transporter cycling between subcellular membrane fractions and have kinetically analyzed the possible sites of insulin action within this cycle. The determination of the rates of internalization of photolabeled glucose transporters in the presence and absence of insulin, when combined with an analysis of time courses for stimulation of cycling by insulin, has allowed the construction of a kinetic scheme in which docking and fusion steps in membrane vesicle exocytosis are identified as important intermediate steps in the trafficking pathway and critical sites of hormone action.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fraction V bovine serum albumin was from Reheis Chemical Co.; type 1 crude collagenase was from CooperBiochemical, Inc. Rabbit polyclonal antiserum, prepared against a 20-amino acid peptide corresponding to the COOH-terminal sequence of GLUT4, was kindly supplied by Hoffmann-La Roche. Protein A and 3-O-methyl-[-U-14C]glucose were from Du Pont-New England Nuclear. ATB-2[3H]BMPA (specific activity, ~10 Ci/mmol) was prepared as described (20).

**Preparation of Rat Adipose Cells**—Male rats (CD strain, Charles River Laboratories, Inc.) were used. They were fasted standard NIH chow ad libitum for at least 6 days prior to study. The rats were anesthetized with a mixture of 70% CO2, 30% O2 and killed by decapitation between 7 and 8 a.m. The epididymal fat pads were removed, minced, and digested with collagenase as previously described (21). All incubations were carried out at 37 °C in a Krebs-Ringer bicarbonate HEPES buffer, pH 7.4, containing 10 mM sodium bicarbonate, 30 mM HEPES, 200 mM adenosine (KRBH buffer), and 1% (w/v) bovine serum albumin.

**Photolabeling with ATB-BMPA**—Adipose cells were suspended at a 40% eotict in 1% albumin/KRBH buffer at 37 °C and stimulated with 0.7 nM insulin for 30 min. 3-ml samples of cells were added to 500 μl of KRBH buffer without albumin and containing 250 μCi of ATB-2[3H]BMPA in 55-mm polystyrene dishes. The dishes were irradiated (with the polystyrene lids on the dishes) for 1 min using a Rayonet photochemical reactor with 300 nm lamps. After photolabeling, the cells were washed with the respective membrane fraction from the starting sample. This was then divided by the number of starting cells in each sample determined from weighed samples of extracted triglyceride and by using previously determined typical triglyceride weights per adipose cell (25). No correction was made for immunoprecipitation efficiency, although it was checked regularly and found to be reproducibly ~85%. Protein was determined by the bicinchoninic acid assay (Sigma) using crystalline bovine serum albumin as the standard. Half-times for the changes in both immunodetectable GLUT4 and ATB-2[3H]BMPA-tagged GLUT4 were calculated as described above for changes in glucose transport activity.

**Analysis of Endocytic and Exocytic Rate Constants**—To obtain initial estimates of the endocytic and exocytic rate constants, we assumed the existence of only two pools of glucose transporters, one in the low-density microsomes and one in the plasma membranes. The rate of ATB-BMPA-tagged GLUT4 equilibrium would then be as follows (Equation 1):

\[
\frac{dT_p}{dt} = k_{en} \cdot \left(1 - T_p \right) + k_{ex} \cdot T_p
\]

where Tp is the fraction of glucose transporters in the plasma membrane, and k_e and k_n are the endocytosis and exocytosis rate constants, respectively (14, 26). Integration with Tp = Tp0 at t = 0 gives Equation 2.

\[
T_p = k_{en} \cdot \left(1 - \exp(-t(k_{en} + k_{ex}))\right) + T_p0 \cdot \exp(-t(k_{en} + k_{ex}))
\]

When Tp0 = 1 at t = 0, substitution gives Equation 3.

\[
T_p = k_{en} + k_{ex} \cdot \exp(-t(k_{en} + k_{ex}))
\]

Equations 2 and 3 can be used to analyze label redistribution under steady-state and non-steady-state conditions and is independent of the specific activity of the labeled glucose transporters; all that is required is that the total pool of glucose transporters is conserved. Under non-steady-state conditions, such as that occurring following insulin removal, the use of these equations assumes that k_e and k_n are instantaneously changed. In the insulin restimulation experiments, Tp0 is obtained directly as the fraction of label remaining in the plasma membranes following reversal. Equation 2 can also be used to analyze the non-steady-state redistribution of glucose transporters as detected by Western blotting. In the analysis of these data, Tp0 is the initial fraction of total immunodetectable GLUT4 in the plasma membranes. The rate constants were calculated by least-squares fitting (weighted for proportional error) to Equations 2 and 3 using the Fig P software (Biosoft).
RESULTS

Reversal of Effect of Insulin on Glucose Transport Activity

Insulin at a concentration of 0.7 nM is just sufficient to stimulate glucose transport activity to a maximum steady-state level in 30 min, with a t1/2 of 3–5 min as previously reported (data not shown) (27). To reverse this effect, we have added collagenase to digest the residual insulin as originally described by Kono et al. (5). Fig. 1 shows the results of an experiment in which the decrease in glucose transport activity was observed in collagenase concentrations from 0.1 to 5 mg/ml so that we could find an optimum concentration that would allow rapid reversal of the insulin response but would not itself stimulate this activity. 0.2 mg/ml collagenase restores the glucose transport activity to the basal state in ~60 min, consistent with the reversal obtained using alternative methods for reversing the insulin response including successive washes (20) and a low pH washing buffer (28), but somewhat faster than anti-insulin antisemur (27). Fig. 1 also shows that collagenase concentrations >0.5 mg/ml tend to raise basal glucose transport activities.

Fig. 2 illustrates not only the detailed time course of the reversal of insulin-stimulated glucose transport activity with 0.2 mg/ml collagenase and the stability of the maximum glucose transport response in adipose cells continuously exposed to 0.7 nM insulin, but also the time course of restimulation of glucose transport activity with readdection of insulin to cells originally stimulated by insulin and then reversed with collagenase. The decrease in insulin-stimulated glucose transport activity with collagenase occurs with a t1/2 of 11.8 ± 0.8 min (mean ± S.E., n = 3), while the stimulated activity observed in the continuous presence of insulin remains highly stable for at least 60 min. Addition of 0.7 μM insulin to cells that have been reversed with collagenase results in a restimulation of glucose transport activity to the maximum level observed in cells maintained in the continuous presence of insulin throughout the entire 120-min incubation. The t1/2 of the latter restimulation with 0.7 μM insulin (4.6 ± 1.0 min) is virtually identical to that of the initial stimulation with 0.7 nM insulin noted above. Addition of 0.7 μM insulin to cells continuously exposed to 0.7 nM insulin has no detectable further effect.

Because the data in Fig. 2 were obtained in the photolabeling experiments illustrated further in Figs. 3–5, the effects of UV irradiation on the time courses of the initial stimulation of glucose transport activity by insulin, the reversal of insulin-stimulated glucose transport activity by collagenase, and the restimulation of glucose transport activity by readdection of insulin after collagenase reversal were examined. Indeed, insulin stimulation was added specifically to the experimental protocol to determine if the rate of translocation of internalized photolabeled GLUT4 from the low-density microsomes to the plasma membranes in response to insulin would be influenced by the presence of UV photoactivable photolabel (see below). UV irradiation under the conditions employed here does not detectably influence the t1/2 for either the reversal by collagenase or the restimulation by insulin readdection, but does increase the t1/2 for the initial stimulation by insulin by ~30% when the latter immediately follows irradiation (data not shown).

Recycling of Glucose Transporters between Subcellular Membrane Fractions—Fig. 3 illustrates an SDS-PAGE analysis of the subcellular distribution of cell-surface ATB-BMPA-labeled GLUT4 in rat adipose cells under various steady-state conditions. For cells in the basal state, such an analysis shows that photolabeled and immunoprecipitated GLUT4 in the low-density microsomal fraction (Fig. 3B) is only ~2% of that observed in the plasma membrane fraction of fully insulin-stimulated cells (Fig. 3A). Similarly, the insulin-treated cells that have been immediately treated with 2 mM KCN to arrest the recycling process show low-density microsomal fraction (Fig. 3B) that is only 5% of that observed in the plasma membranes. The exclusion of the photolabel from the low-density microsomal pool in the basal state is due to the impermeable property of these photolabeling reagents (29). The low level observed in the insulin-stimulated state reflects the extent to which plasma membranes are excluded from the low-density microsomes during the subcellular fractionation procedure (22).

Fig. 3 also shows that the decrease in ATB-BMPA-tagged...
GLUT4 that occurs in the plasma membrane fraction when insulin-stimulated cells are treated with collagase is accompanied by an increase in ATB-BMPA-tagged GLUT4 in the low-density microsomal fraction. Fig. 2B shows that the amount of photolabeled GLUT4 recovered in the low-density microsomal fraction increases over 60 min to a level that is 15-fold higher than that observed in the sample from fully insulin-stimulated cells that have been immediately treated with KCN. In the low-density microsomal fraction isolated from cells maintained in the continuous presence of insulin, the ATB-BMPA-tagged GLUT4 recovered is 9-fold higher than the initial value.

The distribution of photolabeled GLUT4 among all three membrane fractions, determined from six experiments, is shown quantitatively in Table I. When the results are expressed in this quantitative fashion (see "Experimental Procedures"), any changes in the amount of ATB-BMPA-tagged GLUT4 in the plasma membrane fraction are fully accounted for by reciprocal changes in the low-density microsomal fraction, thus demonstrating a true transfer process. Table I also shows that only small amounts of cell-surface ATB-BMPA-labeled glucose transporter cofractionate with the high-density microsomal membrane fraction (from two experiments).

Fig. 4A demonstrates the results obtained by Western blotting plasma membrane fractions isolated from adipose cells that were incubated according to the experimental protocol described in the legend to Fig. 2. The plasma membrane fractions from cells maintained in the continuous presence of insulin retain high levels of immunodetectable GLUT4, as would be expected from the observed maintenance of a constant cellular glucose transport activity throughout this time (Fig. 2). The plasma membrane fractions from cells treated with collagenase to remove insulin lose immunodetectable GLUT4 with a half-life of 13.1 ± 1.3 min and reach a level that is 17 ± 2% of the initial value observed in the fully insulin-stimulated cells. The rate of decrease in immunodetectable GLUT4 in the plasma membrane fraction is similar to the rate of decrease in glucose transport activity noted above (t1/2 = 11.8 min) (Fig. 2). Insulin readdition restores the level of immunodetectable GLUT4 to the normal, fully stimulated level with a t1/2 of ~2.7 ± 0.2 min (Fig. 4A), which is slightly faster than the increase in glucose transport activity that occurs with a t1/2 of 4.6 min as noted above (Fig. 2).

In contrast to the results obtained by immunoblotting
GLUT4, the loss of ATB-BMPA-photolabeled GLUT4 occurs not only during insulin removal with collagenase, but also in adipose cells maintained in the continuous presence of insulin (Fig. 4B). In the plasma membrane fraction from the collagenase-treated cells, tracer-tagged GLUT4 decreases with a t\(_{1/2}\) of 9.4 ± 0.8 min to reach a fully reversed level at 17 ± 2% of its initial value, which is similar to that observed by Western blotting. ATB-BMPA-tagged GLUT4 found in the plasma membrane fraction isolated from cells maintained in the continuous presence of insulin decreases to a level ~45% of the initial value. The t\(_{1/2}\) for this decrease is 10.6 ± 1.5 min and is very similar to that obtained in the cells treated with collagenase to fully reverse insulin action. Also in contrast to the Western blotting results, addition of fresh insulin restores ATB-BMPA-tagged GLUT4 to a new steady-state level, also only ~45% of the initial, fully insulin-stimulated level. However, the latter occurs with a t\(_{1/2}\) of 2.7 ± 0.3 min, which is identical to that for the restoration of immunodetectable GLUT4 and slightly faster than that for the restimulation of glucose transport activity (Fig. 2).

Fig. 5A shows that the time course for the increase in immunodetectable GLUT4 observed in the low-density microsomal fraction is consistent with the corresponding decreases in immunodetectable GLUT4 in the plasma membrane fraction. The low-density microsomal membrane fractions from adipose cells maintained in the continuous presence of insulin show a constant level of immunodetectable GLUT4 throughout the time course. The time courses for the increases in ATB-BMPA-labeled glucose transporters that return to the low-density microsomes are shown in Fig. 5B. The increases occur both in the cells treated with collagenase and in the cells maintained in the continuous presence of insulin. Restimulation of the collagenase-reversed cells with insulin results in a decrease in low-density microsomal tracer-tagged GLUT4 with the same half-time as is observed for the increase in plasma membrane ATB-BMPA-tagged GLUT4.

Rate constants for the endocytosis (k\(_{\text{en}}\)) and exocytosis (k\(_{\text{ex}}\)) of GLUT4 were estimated from the ATB-BMPA-tagged and immunodetectable GLUT4 internalization and restimulation data shown in Fig. 4 using Equations 2 and 3 (see "Experimental Procedures"). This analysis assumes that adipose cells comprise only two pools of glucose transporters, a plasma membrane pool and an intracellular pool. However, this may be a marked oversimplification (see below). The results are outlined in Table I. The estimate of k\(_{\text{en}}\) for the internalization of ATB-BMPA-tagged GLUT4 in the continuous presence of insulin is not significantly different from that following insulin removal. In contrast, the exocytosis rate constant is ~3-fold lower following insulin removal than in the presence of continuous insulin, but ~9-fold lower than with insulin restimulation. Comparing the rates of redistribution of immunodetectable GLUT4 during insulin removal and insulin restimulation shows that k\(_{\text{en}}\) is increased ~25-fold by insulin, again without a significant change in k\(_{\text{ex}}\). Combining all the estimates of k\(_{\text{en}}\) under conditions of insulin removal and comparing these with the combined values obtained from insulin stimulation and continuous insulin indicates an ~10-fold increase in k\(_{\text{ex}}\) in response to insulin without a significant alteration in k\(_{\text{en}}\).

While this paper was in revision, Jhun et al. (30) reported a study using a similar approach based on photolabeling rat adipose cells with a [3H]bisglucose derivative. In this instance, however, these investigators labeled cells only during the steady state, either basal or maximally insulin-stimulated. They subsequently analyzed their results using the same simple two-pool model as used here and concluded that insulin stimulates glucose transport by decreasing k\(_{\text{ex}}\) by 2.8-fold and increasing k\(_{\text{en}}\) by 3.3-fold. While our own results cannot rule out a small decrease (perhaps 30–50%) in k\(_{\text{ex}}\) in response to insulin, we believe that Jung et al. missed the primary and major effect of insulin to increase k\(_{\text{en}}\) by at least 10-fold, and probably more like 20–25-fold, for the main reason that their "basal" cells were actually in a partially stimulated state. First, insulin typically stimulates 3-O-methylglucose transport in adipose cells isolated from the epididymal fat pads of 150–200-g male

\[ \text{Fig. 4. GLUT4 content of plasma membrane fraction during continuous glucose transport response to insulin and reversal and restimulation of glucose transport response to insulin in rat adipose cells. A, total GLUT4 as assessed by Western blotting; B, cell-surface GLUT4 as assessed by ATB-[2-3H]BMPA photolabeling. See legends to Figs. 2 and 3 for experimental procedures. Results are the means ± S.E. of the individual values obtained in three representative experiments (see "Experimental Procedures")}. \]

\[ \text{Fig. 5. GLUT4 content of low-density microsomal membrane fraction during continuous glucose transport response to insulin and reversal and restimulation of glucose transport response to insulin in rat adipose cells. A, total GLUT4 as assessed by Western blotting; B, cell-surface GLUT4 as assessed by ATB-[2-3H]BMPA photolabeling. See legends to Figs. 2 and 3 for experimental procedures. Results are the means ± S.E. of the individual values obtained in three representative experiments (see "Experimental Procedures")}. \]
Evidence for Intermediate States in Insulin Stimulation of Glucose Transporter Translocation—Clark et al. (16) have shown that the appearance of GLUT4 at the adipose cell surface that can be photolabeled with ATB-BMPA precedes the increase in glucose transport activity. These results are consistent with those of Karnieli et al. (27), who showed that the rate of increase in the number of cytochalasin B-binding sites in the plasma membrane fraction isolated from insulin-stimulated cells is greater than the rate of increase in glucose transport activity observed in intact cells. Similarly, Gibbs et al. (32), using a technique involving borohydride labeling of GLUT1, showed a fast rate of appearance of this isoform, while Yang et al. (33) showed that the rates of appearance of both GLUT1 and GLUT4, detected by using the ATB-BMPA photolabeling technique, are greater than the rate of onset of fully activated glucose transport activity in 3T3-L1 cells. As shown in Fig. 6, we have extended these observations here by comparing the rate of appearance of GLUT4 detectable by Western blotting with that of GLUT4 detectable by ATB-BMPA labeling. At 37 °C in cells maintained in the presence of adenosine, the rates of appearance of GLUT4 as detected by these methods show only a small difference. However, the rates of appearance of cell-surface GLUT4 detected by both methods precede the rate of increase in glucose transport activity (Fig. 6A). The rate of increase in immunodetectable GLUT4 under these conditions has a $t_{1/2}$ of $\sim 1.5$ min, and any difference between the rates of appearance of immunodetectable GLUT4 and GLUT4 accessible to the ATB-BMPA photolabel is difficult to resolve.

To improve the resolution of precursor intermediate states in the insulin-stimulated subcellular trafficking, we have carried out the comparisons at 20 °C (Fig. 6B). At 20 °C, the half-times for the rates of appearance of GLUT4 detected by ATB-BMPA photolabeling ($t_{1/2} \sim 9$ min) and by Western blotting ($t_{1/2} \sim 5$ min) are both faster than the rate of increase in glucose transport activity, which occurs with a $t_{1/2} \sim 12$ min. The difference in the half-time for appearance of GLUT4 that could be photolabeled by ATB-BMPA and the half-time for the rate of increase in glucose transport activity is similar to that observed by Yang et al. (33), who showed that in 3T3-L1 cells at 27 °C, the $t_{1/2}$ values for these increases are 5.7 and 8.6 min, respectively.

**TABLE II**

| Inoculation conditions | Parameter measured | Rate constants $k_{on}$/$k_{off}$ $min^{-1}$ | $min^{-1}$ | $k_{on}$/$k_{off}$ |
|------------------------|--------------------|---------------------------------------------|-------------|------------------|
| Insulin ATB-BMPA       | 0.059 ± 0.036      | 0.012 ± 0.002                               |
| Insulin removal         | 0.044 ± 0.005      | 0.003 ± 0.001                               |
| Continuous insulin     | 0.047 ± 0.007      | 0.032 ± 0.006                               |
| Insulin ATB-BMPA       | 0.112 ± 0.039      | 0.106 ± 0.025                               |
| Insulin immobilization | 0.060 ± 0.027      | 0.077 ± 0.013                               |

**Discussion**

In 1980, Suzuki and Kono (2) and Cushman and Wardzala (1) independently proposed that glucose transporters in rat adipose cells were mainly localized in an intracellular pool in the basal state and that insulin produced a shift in the distribution of glucose transporters to the cell surface. Since this proposal, the hypothesis has been extensively supported by measurements of steady-state distributions of glucose transporters (1-5, 8-11). However, the direct demonstration that tracer-tagged glucose transporters redistribute to the plasma membrane in response to insulin has previously not been studied in detail. Oka and Czech (34) photolabeled intact cells in the basal state with cytochalasin B in the presence of 4,6-O-ethylene-β-glucose to inhibit labeling of the cell-surface glucose transporters such that labeling was restricted to the low-density microsomal glucose transporter pool. They were then able to show that the cytochalasin B tracer-tagged glucose transporters moved to the cell surface in response to insulin stimulation and were then associated with the plasma membrane fraction of the cells.

Our own studies involving the use of the impermeant photolabel ATB-BMPA have shown that insulin increases the cell-surface availability of GLUT4 in rat adipose cells by 15-20-fold (4, 16, 35). An advantage of the bismannose photolabel ATB-BMPA is that it is an impermeable reagent and does not have access to the glucose transporters located in the intracellular pool. Thus, the discrete plasma membrane pool of glucose transporters can be selectively labeled, and the transfer of tracer-tagged glucose transporters to the low-density microsomes can be followed. By using benzophenone derivatives of bismannose photolabels.
nose and bisglucose, we have shown that glucose transporters in rat adipose cells that were initially labeled in the plasma membranes are internalized to the low-density microsomes following 40 min at 37 °C even in the presence of insulin (35). A similar internalization of ATB-BMPA-tagged glucose transporters was observed following incubation of 3T3-L1 cells with insulin for 60 min at 37 °C (19). Our previous studies have therefore already suggested that glucose transporters recycle in the presence of insulin. We now show here that by using the bis-hexose photolabeling approach, the time courses for internalization and recycling between subcellular membrane fractions can be determined in rat adipose cells.

While our previous studies suggested that glucose transporters recycle in the presence of insulin, this study documents this phenomenon in detail. GLUT4 tagged with the bismannose tracer on the surface of the adipose cell in the insulin-stimulated state moves from the plasma membranes to the low-density microsomes with a $t_{1/2}$ of 10.6 min in the continuous presence of insulin. At the same time, a constant insulin-stimulated steady-state distribution of immunodetectable GLUT4 is maintained between these two membrane fractions. If the movement of tracer-tagged GLUT4 is indicative of the movement of unlabeled GLUT4, then GLUT4 must be rapidly and continuously recycling in the continuous presence of insulin. The best evidence that tracer-tagged GLUT4 and unlabeled GLUT4 move similarly is the similarity in $t_{1/2}$ values for their internalization when insulin is removed by collagenase treatment (9.4 and 13.1 min, respectively) and for their resumption back to the plasma membrane when insulin is readded to collagenase-treated cells (2.7 and 2.7 min, respectively). The latter two experimental circumstances are the only ones in which both tracer-tagged GLUT4 and unlabeled GLUT4 are expected to move together.

The internalization of tracer-tagged GLUT4 in the continuous presence of insulin ultimately results in the achievement of an apparent new steady-state distribution of labeled GLUT4, with the plasma membranes retaining ~45% of the initial value and the low-density microsomes accounting for the other ~55%. It is highly significant that exactly this same steady-state distribution is achieved when adipose cells labeled in the insulin-stimulated state are reversed with collagenase and then resuspended with the readdition of insulin. These observations are consistent with the possibility that ATB-BMPA-tagged GLUT4 fully equilibrates with the entire intracellular pool of glucose transporters. The specific activity of the ATB-BMPA-tagged glucose transporters thus decreases, and only an ~30% proportion of these return to the plasma membrane on resumption. The level of ATB-BMPA-tagged GLUT4 distributed between the plasma membranes and the low-density microsomes in the resuspended state is therefore equal to the steady-state distribution of GLUT4 between these fractions detected by Western blotting (24) or by cytochalasin B binding (27) with isolated membranes obtained by subcellular fractionation of cells in the fully insulin-stimulated state.

These data also provide for the first time direct evidence that the site of insulin action in stimulating glucose transporter translocation lies in the exocytosis leg of the recycling process. By simple inspection alone, the close similarity of the rates of insulin-stimulated distribution of GLUT4 in the continuous presence of insulin ($t_{1/2} = 10.6$ min for tracer-tagged GLUT4) and with insulin removal ($t_{1/2} = 13.1$ min for immunodetectable GLUT4, 9.4 min for tracer-tagged GLUT4, and 11.8 min for glucose transport activity) shows that insulin does not significantly decrease the rate of endocytosis of glucose transporters. Thus, insulin must markedly increase the rate of their exocytosis. In a more formal manner, application of the simple two-pool analytical procedure also suggests that insulin increases the rate of exocytosis of GLUT4 without significantly changing the rate of endocytosis (see “Results”).

One of the assumptions of the analysis we have used is that following insulin removal, $k_{en}$ and $k_{ex}$ are immediately changed. However, this may not be the case if insulin is not instantaneously removed by the collagenase treatment. Indeed, Quon and Campfield (26), also using a two-pool model, suggested that the rate of insulin dissociation from its receptor determines the rate of net reversal of insulin-stimulated glucose transport. This may be the case in the early experiments described by Karnieli et al. (27), which were computer-simulated by Quon and Campfield. However, we have previously reported that insulin is removed from rat adipose cells with a $t_{1/2}$ of <5 min under conditions of collagenase treatment (3). In addition, we have now shown that if the reversal rate is delayed because of residual insulin or residual stimulus, then one would expect to see a more rapid internalization of label than of net loss of immunodetectable plasma membrane GLUT4 since the recycling of the former would be reduced by dilution within the intracellular vesicle pool. Because we have determined similar rates of internalization of ATB-BMPA-tagged GLUT4 and of net loss of immunodetectable GLUT4 following insulin removal, we conclude that both GLUT4 net endocytosis and ATBBMPA-tagged GLUT4 exchange with unlabeled intracellular GLUT4 are both dependent on a slow endocytosis rate constant and are not rate-limited by insulin dissociation from its receptor.

The experimental approach we have taken may also underestimate the magnitude of insulin's stimulation of $k_{ex}$. First, as discussed above with reference to $k_{en}$, the change in $k_{ex}$ following insulin removal may not be instantaneous. Second, the reversal of insulin action may not have been complete, and the cells may not have returned to a true basal state. This may be due to a small but significant collagenase stimulation of GLUT4 translocation (see “Results”) and possible UV radiation damage of the cells (although the cells were protected by irradiation through polystyrene lids on the dishes, and as noted below, various control experiments appear to rule out radiation damage as a significant variable). Third, the estimate of photolabel remaining in the plasma membrane is dependent on good subcellular fractionation techniques, and the plasma membrane fraction may be partly contaminated by photolabel that had transferred to the low-density microsomal fraction. Other evidence also suggests that insulin produces a much larger stimulation of $k_{ex}$ than estimated here. In experiments in which glucose transporter endocytosis in basal and insulin-treated rat adipose cells has been blocked by incubation with a major histocompatibility complex class I peptide, the increases in glucose transport activity can be used to estimate exocytosis rate constants. These experiments show that insulin increases $k_{ex}$ by ~20-fold over basal levels.

An apparent anomaly arises in comparing the rate of internalization of tracer-tagged GLUT4 in the continuous presence of insulin with the rate of reappearance of photolabeled GLUT4 in the plasma membranes stimulated by readdiation of insulin to collagenase-treated cells, however, in so far as the latter ($t_{1/2} = 2.7$ min) is much faster than the former ($t_{1/2} = 10.6$ min) then one would expect to see a more rapid internalization of label than of net loss of immunodetectable plasma membrane GLUT4 since the recycling of the former would be reduced by dilution within the intracellular vesicle pool. Because we have determined similar rates of internalization of ATB-BMPA-tagged GLUT4 and of net loss of immunodetectable GLUT4 following insulin removal, we conclude that both GLUT4 net endocytosis and ATB-BMPA-tagged GLUT4 exchange with unlabeled intracellular GLUT4 are both dependent on a slow endocytosis rate constant and are not rate-limited by insulin dissociation from its receptor.

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Therefore, \( t_{1/2} = \ln{(2/k_{ee} + k_{en})} \) for both the recycling time course and the insulin stimulation time course. Because \( k_{ee} \) and \( k_{en} \) have insulin-stimulated values in both these experiments, it follows that the \( t_{1/2} \) values would be expected to be the same.

However, since the \( t_{1/2} \) values in these experiments are not equal, we have examined several possibilities that may account for the discrepancy. First, the possibility that irradiation may have damaged the cells can be considered. Indeed, we have found that adipose cells are not protected by irradiating them through the plastic lids of the Petri dishes, then irradiation markedly slows the reversal of the insulin response. Nevertheless, in this regard, we note that the recycling \( t_{1/2} \) (10.6 min) and the insulin stimulation \( t_{1/2} \) (2.7 min) which are compared were both carried out in irradiated cells. Although it could be argued that the latter \( t_{1/2} \) is determined after cells have recovered from irradiation, control experiments (see "Results") demonstrate that acute irradiation slows the \( t_{1/2} \) for the initial insulin stimulation in fresh cells by no more than 50%. We have further considered the possibility that irradiation may have slowed the endocytosis rate constant \( (k_{en}) \). A direct comparison of changes in glucose transporter transport by insulin is expected to be modified by cell-culture treatment (when one would expect that internalization would be mainly dependent on \( k_{en} \)) in irradiated and nonirradiated cells shows no significant difference. An irradiation effect on \( k_{en} \) is possible. However, this would have to be transitory because if \( k_{en} \) were reduced but \( k_{ee} \) were not, then we would have observed an abnormally low steady-state distribution of label and not one that corresponds to the distribution of glucose transporters as detected in cytochalasin B-binding experiments (27) and in Western blotting experiments (24) in nonirradiated cells.\(^5\)

While we cannot totally exclude the possibility that the discrepancy between the insulin stimulation \( t_{1/2} \) and the recycling \( t_{1/2} \) is due to cell damage, we think it is important to also consider the possibility that the effect is due to the recycling of glucose transporters through intermediate states in the cytosol as illustrated on the right of Fig. 7. In their analysis of immunocytochemical studies on the subcellular distribution of GLUT4 in brown adipose tissue, Slot et al. (9) identified some 11 separate locations of immunodetectable GLUT4, eight cytoplasmic and three associated with the plasma membrane. In their description of the translocation model for insulin stimulation of glucose transport, Karnieli et al. (27) postulated that there may be four plasma membrane intermediate states in the subcellular trafficking pathway. These intermediate precursor states are also shown in Fig. 7. It is most unlikely that recycling through all these intermediates will be a simple kinetic process. Therefore, the method we have used to determine the apparent \( k_{en} \) and \( k_{ee} \), although useful, almost certainly oversimplifies the recycling process. We have examined the kinetic predictions of a system (Fig. 7) in which two intracellular compartments, the endosomes and the specialized "secretory" tubulovesicular compartment, are involved. It is clear from computer simulations of this system that the involvement of a second intracellular compartment allows a rapid initial stimulation of translocation by insulin, but a slower recycling of glucose transporters in the continuous presence of insulin. This is because the tubulovesicular compartment could rapidly dock and fuse with the plasma membrane without endocytosis, contributing significantly to the stimulation \( t_{1/2} \) (as it does when there is only one intracellular pool) (Equations 2 and 3). However, with two intracellular pools, the recycling in the continuous presence of insulin would depend on endosome processing and recycling, which could markedly slow the turnover \( t_{1/2} \) that maintains a steady-state distribution of glucose transporters.

The model shown in Fig. 7 would also account for the observation of intermediate and partially occluded forms of the glucose transporter that may be precursor states in the stimulation leg of the pathway. At present, it is not entirely clear how abundant the occluded forms of the glucose transporter are. Therefore, the reduced abundance and high concentration in the plasma membrane at early times following insulin stimulation (Fig. 6) may be, to some extent, influenced by the use of KCN to arrest recycling (36). However, several observations suggest that consideration of occluded and partially occluded glucose transporters (Fig. 7) is required. First, because insulin stimulation is rapid but recycling of glucose transporters is relatively slow, one would expect the bulk of the glucose transporters to be localized to the plasma membrane unless some glucose transporters translocate to the plasma membrane and back again without participating in glucose transport. Second, a rapid onset of residence of glucose transporters in the occluded and partially occluded states is required to account for the observation that the \( t_{1/2} \) values for the appearance of GLUT4, as detected by Western blotting of plasma membrane fractions and by ATB-BMPL photolabeling, are faster than those for glucose transport stimulation. ATB-BMPL might label partially occluded glucose transporters at the point where occluded vesicles or tubulovesicular elements fuse with the plasma membrane. At this point, glucose transporters might be exposed to photolabel as well as transport substrate, but not fully participate in glucose transport because of association with trafficking proteins (39). The small (1.5-2-fold) discrepancy that we have noted in comparing glucose transport activities and photolabeled GLUT4 levels in the basal and insulin-stimulated states (4, 18, 37) is likely to be due to a proportion of the glucose transporters being present in this catalytically inactive state in basal adipose cells. Third, the observation that the steady-state distribution of glucose transporters at 20 °C, when endocytosis is markedly reduced (16),\(^6\) is similar to that which occurs at 37 °C when glucose transporters recycle through the endosomes suggests that glucose transporters may emerge at the cell surface and be returned to the low-density microsomes via a mechanism that does not involve the normal endocytosis route.

The model that we have described in this study extends our previous model of glucose transporter translocation and has identified exocytosis steps in subcellular trafficking that will be the focus for further studies on the mechanisms for insulin stimulation of glucose transport. In addition, the identified intermediate states provide a mechanistic basis for the glucose transporter intrinsic activity changes previously proposed to have been observed in rat adipose cells in response to adenosine and isoproterenol (38-40) or during specific altered metabolic states (41) and in 3T3-L1 cells (42, 43).

\(^{1}\) Jhun et al. (30) reported a \( t_{1/2} \) of 3.24 ± 0.51 min for the internalization of tracer-tagged GLUT4 in the continuous presence of insulin, compared to the value reported here of 10.6 ± 1.5 min. Their \( t_{1/2} \) for GLUT4 recycling is thus more consistent with the \( t_{1/2} \) for insulin stimulation than is ours. Possible sources of this difference include differences in the photolabelling and/or UV lamps. However, as discussed above, our UV irradiation conditions do not appear to significantly influence the GLUT4 cycling process as measured here. Another potential source of difference is the possibility that the apparent label internalization rates are influenced by the processing of the samples after labelling.

\(^{2}\) Jhun et al. report in their Fig. 5 that at zero time, the label recovered in their low-density microsomal fraction is approximately one-third of that recovered in the plasma membranes in the insulin-stimulated state. This suggests that their subcellular fractionation procedure and/or their failure to arrest cycling leads to an overestimate of internalization. In our studies, we have used KCN to prevent cycling during the processing of the cells and have observed only 5% of labeled GLUT4 in the low-density microsomal fraction at zero time.

\(^{3}\) K. C. Appell, S. W. Cushman, and L. A. Simpson, unpublished data.
FIG. 7. Kinetic development of hypothetical mechanism of insulin's stimulatory action on glucose transport (based on original hypothetical mechanism described by Karnieli et al. (27) and the immunohistochemical data recently reported by Slot et al. (8)). Ooccluded, partially occluded, and fully functional glucose transporters are defined by their detectability by Western blotting in plasma membranes, their susceptibility to photolabeling with ATB-BMPA, and their ability to transport 3-O-methylglucose. Fully functional transporters are detectable by immunoblotting, are labeled by ATB-BMPA, and do transport 3-O-methylglucose.

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Note Added in Proof—Czech and Buxton (44) have recently reported that GLUT4 tagged by trypsin cleavage is internalized less in the insulin-stimulated state than in the basal state. They concluded that insulin inhibits the internalization process. Their data, but not their conclusions, are entirely consistent with those of Yang and Holman (31) and with the data in Fig. 5. In all these studies, less GLUT4 is internalized with insulin present. However, we suggest that this effect of insulin occurs because more GLUT-4 is recycled in the insulin-stimulated state.

REFERENCES
1. Cushman, S. W., and Wardzala, L. J. (1980) J. Biol. Chem. 255, 4758-4762
2. Suzuki, K., and Kono, T. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2542-2545
3. Simpson, I. A., and Cushman, S. W. (1986) Annu. Rev. Biochem. 55, 1009-1089
4. Holman, G. D., Kozka, I. J., Clark, A. E., Flower, C. J., Saltis, J., Habberfield, A. D., Simpson, I. A., and Cushman, S. W. (1990) J. Biol. Chem. 265, 18172-18179
5. Kono, T., Suzuki, K., Dansey, L. E., Robinson, F. W., and Blevins, T. L. (1981) J. Biol. Chem. 256, 6400-6407
6. Zorzano, A., Wilkinson, W., Kotlar, I. J., Theodis, G., Wardzinski, B. E., Rubbo, A. E., and Pich, P. F. (1989) J. Biol. Chem. 264, 12558-12564
7. James, D. E., Brown, R., Navarro, J., and Pich, P. F. (1988) Nature 333, 283-285
8. Bortiemann, A., Plough, T., and Schmalbruch, H. (1992) Diabetes 41, 215-221
9. Slot, J. W., Guze, H. J., Gigenack, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. 113, 123-135
10. Slot, J. W., Guze, H. J., Gigenack, S., James, D. E., and Lienhard, G. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7815-7819
11. Smith, B. M., Charron, M. J., Shah, N., Lodish, H. F., and Jarett, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6893-6897
12. Goldstein, A. J., Brown, M. S., Anderson, R. G. W., Russel, D. W., and Schneider, W. J. (1985) Annu. Rev. Cell Biol. 1, 1-39
13. Stoecugrii, W., Guze, H. J., and Stroas, G. J. (1987) J. Cell Biol. 104, 1281-1288
14. Tannen, L. I., and Lienhard, G. E. (1987) J. Biol. Chem. 262, 5975-5980
15. Sonne, O. (1988) Physiol. Rev. 68, 1129-1150
16. Clark, A. E., Kozka, I. J., and Holman, G. D. (1991) Biochem. J. 278, 235-241
17. Calderhead, D. M., Kitagawa, W., Tannor, L. I., Holman, G. D., and Lienhard, G. E. (1990) J. Biol. Chem. 265, 13800-13805
18. Kozka, I. J., Clark, A. E., and Holman, G. D. (1991) J. Biol. Chem. 266, 11726-11731
19. Yang, J., Clark, A. E., Kozka, I. J., Cushman, S. W., and Holman, G. D. (1992) J. Biol. Chem. 267, 10393-10399
20. Clark, A. E., and Holman, G. D. (1990) Biochem. J. 265, 13800-13809
21. Weber, T. M., Joost, H. G., Simpson, I. A., and Cushman, S. W. (1988) in The Insulin Receptor (Kahn, C. R., and Harrison, L. C., eds) Vol. II, pp. 171-187, Alan R. Liss, Inc., New York
22. Simpson, I. A., Yver, D. R., Hassin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B., and Cushman, S. W. (1988) Biochem. Biophys. Res. Commun. 165, 391-397
23. Whitesell, R. E., and Glimann, J. (1979) J. Biol. Chem. 254, 5276-5283
24. Nishimura, H., Saltis, J., Habberfield, A. D., Garty, N. B., Greenberg, A. S., Cushman, S. W., Loduz, C., and Simpson, I. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11500-11504
25. Cushman, S. W. and Salans, L. B. (1982) Lipid Res. 19, 269-273
26. Quinn, M. J., and Campfield, L. A. (1991) J. Theor. Biol. 150, 95-107
27. Karnieli, E., Zarnowski, M. J., Hassin, P. J., Simpson, I. A., Salans, L. B., and Cushman, S. W. (1981) J. Biol. Chem. 264, 4772-4777
28. Gibson, E. M., Allard, W. J., and Lienhard, G. E. (1986) J. Biol. Chem. 261, 16597-16603
29. Midgley, P. J. W., Parkar, B. A., and Holman, G. D. (1988) Biochem. Biophys. Res. Commun. 165, 391-397
30. Juhn, B. H., Rampal, A. L., Liu, H., Latcha r, M., and Jung, C. Y. (1992) J. Biol.
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(References)

31. Yang, J., and Holman, G. D. (1993) J. Biol. Chem. 268, 4600-4603
32. Gibbs, E. M., Lienhard, G. E., and Gould, G. W. (1988) Biochemistry 27, 6981-6983
33. Yang, J., Clark, A. E., Harrison, R., Kozka, I. J., and Holman, G. D. (1992) Biochem. J. 281, 809-817
34. Oka, Y., and Czech, M. P. (1984) J. Biol. Chem. 259, 8125-8133
35. Holman, G. D., Karim, A. R., and Karim, B. (1988) Biochim. Biophys. Acta 948, 75-84
36. Nishimura, H., Zarnowski, M. J., and Simpson, I. A. (1993) J. Biol. Chem., in press
37. Palfreyman, S. W., Clark, A. E., Denton, R. M., Holman, G. D., and Kozka, I. J. (1992) Biochem. J. 284, 275-281
38. Joost, H. G., Weber, T. M., Cushman, S. W., and Simpson, I. A. (1997) J. Biol. Chem. 262, 11261-11267
39. Kuroda, M., Honnor, R. C., Cushman, S. W., Londos, C., and Simpson, I. A. (1987) J. Biol. Chem. 262, 245-253
40. Vannucci, S. J., Nishimura, H., Satoh, S., Cushman, S. W., Holman, G. D., and Simpson, I. A. (1992) Biochem. J., 286, 325-330
41. Kahan, B. B., Shulman, G. I., DeFronzo, R. A., Cushman, S. W., and Rosselli, L. (1991) J. Clin. Invest. 87, 561-570
42. Harrison, S. A., Buxton, J. M., Clancy, B. M., and Czech, M. P. (1991) J. Biol. Chem. 266, 19438-19449
43. Harrison, S. A., Clancy, B. M., Pessino, A., and Czech, M. P. (1992) J. Biol. Chem. 267, 3763-3768
44. Czech, M. P., and Buxton, J. M. (1993) J. Biol. Chem. 268, 9187-9190