GLUT4 trafficking in insulin-stimulated rat adipose cells: evidence that heterotrimeric GTP-binding proteins regulate the fusion of docked GLUT4-containing vesicles

Cynthia M. FERRARA¹ and Samuel W. CUSHMAN

Experimental Diabetes, Metabolism, and Nutrition Section, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 10 Center Drive MSC 1420 (10/5N102), Bethesda, MD 20892-1420, U.S.A.

Agents that activate the G-protein G_i (e.g. adenosine) increase, and agents that activate G_s [e.g. isoprenaline (isoproterenol)] decrease, steady-state insulin-stimulated glucose transport activity and cell-surface GLUT4 in isolated rat adipose cells without changing plasma membrane GLUT4 content. Here we have further examined the effects of R_sG_s and R_iG_i ligands (in which R_s and R_i are G_s - and G_i -coupled receptors respectively) on insulin-stimulated cell-surface GLUT4 and the kinetics of GLUT4 trafficking in these same cells. Rat adipose cells were preincubated for 2 min with or without isoprenaline (200 nM) and adenosine deaminase (1 unit/ml), to stimulate G_s and decrease the stimulation of G_i respectively, followed by 0–20 min with insulin (670 nM). Treatment with isoprenaline and adenosine deaminase decreased insulin-stimulated glucose transport activity by 58%. Treatment with isoprenaline and adenosine deaminase also resulted in similar decreases in insulin-stimulated cell-surface GLUT4 as assessed by both bis-mannose photolabelling of the substrate-binding site and biotinylation of the extracellular carbohydrate moiety when evaluated under similar

experimental conditions. After stimulation with insulin in the absence of G_s and the presence of G_i agents, a distinct sequence of plasma membrane events took place, starting with an increase in immunodetectable GLUT4, then an increase in the accessibility of GLUT4 to bis-mannose photolabel, and finally an increase in glucose transport activity. Pretreatment with isoprenaline and adenosine deaminase before stimulation with insulin did not affect the time course of the increase in immunodetectable GLUT4 in the plasma membrane, but did delay both the increase in accessibility of GLUT4 to photolabel and the increase in glucose transport activity. These results suggest that R_sG_s and R_iG_i modulate insulin-stimulated glucose transport by influencing the extent to which GLUT4 is associated with occluded vesicles attached to the plasma membrane during exocytosis, perhaps by regulating the fusion process through which the GLUT4 in docked vesicles becomes exposed on the cell surface.

Key words: adenosine, exocytosis, G-proteins, isoproterenol, membrane fusion.

INTRODUCTION

It is well known that insulin stimulates glucose transport in isolated adipose cells by the translocation of GLUT4 from an intracellular pool to the plasma membrane [1,2]. A membrane-impermeant photolabel, 2-*N*-4-(1-*az*i-2,2,2-trifluoroethyl)benzoyl-1,3-bis(mannos-4-yloxy)-2-propylamine (ATB-BMPA), has been developed for specifically labelling cell-surface GLUT4 by binding to the GLUT4 substrate-binding site [3]. With this technique, stimulation with insulin results in 15–20-fold increases in glucose transport and cell-surface GLUT4 in adipose cells and 5–10-fold increases in these parameters in skeletal muscle from young lean rats [3–6]. Satoh et al. [5] further determined that the stimulation of glucose transport with insulin occurs through a specific sequence of plasma membrane-associated events: first an increase in plasma membrane-associated GLUT4 content, as assessed by membrane fractionation and Western blotting; secondly an increase in exposure of GLUT4 on the cell surface, as assessed by ATB-BMPA photolabelling; and finally an increase in glucose transport activity itself. In the presence of insulin, GLUT4 continuously cycles from the intracellular pool to the plasma membrane and back to the intracellular pool through a process in which the cell's entire complement of GLUT4 participates.

A number of pharmacological agents affect insulin-stimulated glucose transport in adipose cells and thus seem to modulate the trafficking of GLUT4. The actions of agents mediated by G_s -coupled receptors (R_s) such as isoprenaline (isoproterenol; ISO) and G_i -coupled receptors (R_i) such as adenosine on insulin-stimulated glucose transport in rat adipose cells have been extensively investigated in our laboratory [7–10]. Kuroda et al. [8] studied the effects of ISO in the presence and the absence of adenosine on insulin-stimulated glucose transport activity in adipose cells. The removal of adenosine with adenosine deaminase (ADA) results in a 30% decrease in insulin-stimulated glucose transport activity. This decrease is prevented by the R_i agonists phenylisopropyladenosine, nicotinic acid and prostaglandin E_1 , suggesting that R_iG_i agents augment insulin-stimulated glucose transport. In contrast, the treatment of isolated adipose cells with ISO does not alter insulin-stimulated glucose transport activity in the presence of adenosine but induces a 50–70% decrease in the absence of adenosine.

These effects by R_iG_i and R_sG_s agents are paralleled by similar effects on cell-surface GLUT4, as assessed by ATB-BMPA photoaffinity labelling. However, the GLUT4 content of the plasma membranes is affected minimally, if at all, under the same conditions [10]. These results led to the hypothesis that the effects of adenosine and other R_iG_i agents on insulin-stimulated glucose

Abbreviations used: ADA, adenosine deaminase; ATB-BMPA, 2-*N*-4-(1-*az*i-2,2,2-trifluoroethyl)benzoyl-1,3 bis(mannos-4-yloxy)-2-propylamine; ISO, isoprenaline (isoproterenol); KRBH, Krebs–Ringer bicarbonate Hepes; R_s and R_i , G_s - and G_i -coupled receptors respectively.

¹ To whom correspondence should be addressed (e-mail cindy@grecc.ab.umd.edu). Present address: University of Maryland, Baltimore Veterans Administration Medical Center, 10 North Greene Street, GRECC (BT/18/GR), Baltimore, MD 21201-1524, U.S.A.

transport activity are to promote exposure of plasma membrane-associated GLUT4 on the cell surface, whereas the effects of ISO and other R_sG_s agents are to promote the accumulation of plasma-membrane-associated GLUT4 in occluded vesicles. Nevertheless, measurements of cell-surface glucose transporters with the use of a ligand that interacts with the substrate-binding site, as ATB-BMPA does, cannot distinguish between effects on GLUT4 positioning in the plasma membrane, i.e. between the cell surface and occluded vesicles, and effects on the activity of those GLUT4 molecules on the cell surface with exposure to substrate.

Thus the first purpose of this investigation was to establish a new method for assessing cell-surface GLUT4 independently of substrate binding and to use this method to distinguish between changes in GLUT4 positioning in the plasma membrane and cell-surface GLUT4 activity in response to R_iG_i and R_sG_s agents. Here we show that cell-surface GLUT4 can be detected and quantified by biotinylating the carbohydrate group on the first extracellular loop of the GLUT4 molecule by using biocytin hydrazide [11,12]. The use of this technique demonstrates clearly that ISO and adenosine modulate insulin-stimulated glucose transport activity by regulating the positioning of GLUT4 in the plasma membrane.

Changes in the positioning of GLUT4 in the plasma membrane are indicative of an effect on GLUT4 trafficking and specifically on the sequence of events in the plasma membrane in response to insulin, as previously described by Satoh et al. [5]. Thus the second purpose of this investigation was to determine how R_iG_i and R_sG_s agents affect the trafficking of GLUT4 in isolated adipose cells. Kinetic analysis of the time course of the response to insulin in the presence of activated G_i or G_s suggest that R_iG_i and R_sG_s agents regulate the fusion of docked GLUT4-containing vesicles with the plasma membrane.

EXPERIMENTAL

Materials

BSA fraction V was from Interger Co. (Purchase, NY, U.S.A.); type I crude collagenase was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); Protein A-Sepharose was from Pharmacia Biotech (Piscataway, NJ, U.S.A.); Thesit was from Boehringer Mannheim (Indianapolis, IN, U.S.A.); all other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rabbit polyclonal antisera for GLUT4, prepared against a 20-residue peptide corresponding to the C-terminal sequence, was kindly supplied by Hoffmann-La Roche (Nutley, NJ, U.S.A.). Anti-GLUT4 monoclonal antibody 1F8 was from East Acres Biologicals (Southbridge, MA, U.S.A.). Biocytin hydrazide was from Pierce Chemicals (Rockford, IL, U.S.A.). ^{125}I -labelled Protein A, ^{125}I -labelled sheep anti-mouse IgG anti-serum, 3-*O*-methyl-D-[U- ^{14}C]glucose and L-[^3H]glucose were from Dupont-New England Nuclear (Boston, MA, U.S.A.). ATB-[2- ^3H]BMPA (10 Ci/mmol) was kindly supplied by G. D. Holman (University of Bath, Bath, Avon, U.K.).

Preparation and incubation of adipose cells

Male rats (CD strain; Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were fed with standard NIH chow *ad lib*. for at least 5 days before study. The rats were anaesthetized with CO_2/O_2 (70:30) and killed by decapitation between 07:00h and 09:00h. The epididymal fat pads were removed and minced, then digested with collagenase as described previously [13]. All incubations were performed at 37 °C in a Krebs-Ringer bicarbonate Hepes (KRBH) buffer, pH 7.4, containing 10 mM

sodium bicarbonate, 30 mM Hepes, 200 nM adenosine and either 1% or 5% (w/v) BSA. The isolated adipose cells were diluted to approx. 12.5% (v/v) with KRBH buffer containing 200 nM adenosine, then incubated in the presence of 5% (w/v) BSA and 2.5 mM glucose. Diluted adipose cells were pretreated for 2 min without or with 200 nM ISO and 1 unit/ml ADA, then subjected to 0–20 min of maximal stimulation with insulin (670 nM).

Measurement of 3-*O*-methyl-D-glucose transport

After incubation, 150 μl aliquots of the isolated adipose cells from each condition were used for the assessment of 3-*O*-methylglucose transport, which was measured at a substrate concentration of 100 μM with a double-isotope (L-[^3H]glucose/3-*O*-methyl-D-[U- ^{14}C]glucose) technique, as described by Karnieli et al. [14].

Cell-surface photolabelling of GLUT4

After incubation, 3 ml of isolated adipose cells were photolabelled for 1 min with 300 μCi of ATB-[2- ^3H]BMPA by using a Rayonet photochemical reactor with 300 nm lamps (Southern New England Ultraviolet Co., Branford, CT, U.S.A.). Photolabelled cells were then washed with 20 mM Tris/HCl (pH 7.4)/1 mM EDTA/255 mM sucrose (TES) buffer at 18 °C, and centrifuged through silicon oil. GLUT4 was immunoprecipitated from the cell homogenates by the following procedure: The cells were solubilized for 20–30 min at room temperature in 2% (v/v) Thesit in PBS, pH 7.4. The cell lysate was then centrifuged at 150000 *g* for 10 min. The supernatant of each sample was incubated overnight at 4 °C with an immunocomplex consisting of polyclonal anti-GLUT4 antiserum and Protein A-Sepharose. The immunocomplexes were centrifuged at high speed; the supernatants were removed carefully. The complexes were washed, solubilized in sample buffer [2.3 M urea/1.5% SDS/15 mM Tris/HCl (pH 6.8)/100 mM dithiothreitol], and analysed by SDS/PAGE followed by gel slicing and counting, as described previously [3,5].

Quantification of GLUT4 in plasma membrane fractions

Plasma membranes were prepared from isolated adipose cells with the use of a method described previously [15]. GLUT4 concentrations were measured in the plasma membrane fractions by using SDS/PAGE procedures and Western blotting with an anti-(GLUT4 C-terminal peptide) antiserum and ^{125}I -labelled Protein A [16]. A Molecular Dynamics PhosphorImager (Sunnyvale, CA, U.S.A.) was used to quantify GLUT4. The areas of each band were corrected for background and expressed as a percentage of the arbitrary units for a membrane protein standard that was run on each gel.

Kinetic analysis of time-course data

The time courses for the stimulation of glucose transport activity, plasma membrane GLUT4 content and cell-surface GLUT4 by insulin for each experiment were analysed by exponential curve-fitting ($y = ae^{-bx} + ce^{-dx}$) (Sigma Plot; Jandel Scientific, San Rafael, CA, U.S.A.). The half-times ($t_{1/2}$ values) to maximal stimulation with insulin and the initial rates of appearance or increase were then calculated. The initial rate of appearance was calculated from the slope of a line drawn tangent to the time-course curve at zero time.

Figure 1 Effects of insulin, and combined ISO addition and adenosine removal, on cell-surface GLUT4 in rat adipose cells as assessed with ATB-BMPA under the conditions used for biotinylation (a) and by biotinylation (b)

Isolated cells were treated with or without insulin (670 nM) for 20 min at 37 °C, followed by 10 min of treatment with or without ISO/ADA. (a) Upper panel: an aliquot of cells from each condition was washed with cold KRBH buffer and incubated with PBS at 4–10 °C for 30 min. The cells were then washed a second time with cold KRBH buffer and incubated again at 4–10 °C for 30 min. The cells were then photolabelled with ATB-[2-³H]BMPA as described in the Experimental section. Results are means $\pm$ S.E.M. for three independent experiments, presented as percentages of the maximal insulin response for each experiment. ⁺, significantly different from insulin in the presence of adenosine. Lower panel: data from a representative experiment. The graph represents the amount of ATB-[2-³H]BMPA in each gel slice. Symbols: $\square$, without insulin; $\blacklozenge$, with insulin; $\bullet$, with insulin, preceded by ISO/ADA. The positions of molecular mass markers are indicated. (b) Upper panel: an aliquot of cells from each condition was washed with cold KRBH buffer and incubated with 20 mM sodium periodate in PBS at 4–10 °C for 30 min. The cells were then washed a second time with cold KRBH buffer and incubated with biocytin hydrazide at 100 μ g/ml in KRBH buffer at 4–10 °C for 30 min. Biotinylated GLUT4 was then assessed as described in the Experimental section. Results are means $\pm$ S.E.M. for eight independent experiments, presented as percentages of the maximal insulin response for each experiment. ⁺, significantly different from insulin in the presence of adenosine. Lower panel: blot from a representative experiment.

Biotinylation of cell-surface GLUT4

For biotin labelling of GLUT4, isolated adipose cells were prepared as described previously. Diluted cells were incubated without insulin (basal), with 670 nM insulin for 20 min, or with 670 nM insulin for 20 min followed by 10 min with 200 nM ISO and 1 unit/ml ADA. After incubation, cells were washed with KRBH buffer at 4–10 °C and centrifuged through silicon oil. The cells were resuspended in fresh sodium metaperiodate (final concentration 20 mM) in PBS, pH 7.4, and then incubated in the dark for 30 min at 4 °C. This step caused the oxidation of cell-surface carbohydrate hydroxy groups to aldehyde groups [11,12]. The cells were washed three times with cold KRBH buffer and resuspended to 30% (v/v). Biocytin hydrazide was added to the cells to give a final concentration of 100 μ g/ml. Cells were then incubated for 30 min at 4 °C. The cells were washed again two or three times with cold KRBH buffer and GLUT4 was immunoprecipitated as described previously [5]. Immunoprecipitated GLUT4 samples were analysed further by SDS/PAGE and immunoblotting with ¹²⁵I-streptavidin to detect the biotinylated GLUT4. The samples were also immunoblotted with the

monoclonal anti-GLUT4 1F8 antibody and ¹²⁵I-labelled sheep anti-mouse antibody to control for potential differences in the total amount of GLUT4 (and adipose cells) in each sample. A Molecular Dynamics PhosphorImager was used to quantify the GLUT4 in each sample. Values are represented as percentages of the maximal stimulation with insulin.

Statistical analysis

Results were analysed with Student's *t* test and analysis of variance when appropriate. Differences were accepted as significant at the level of $P \leq 0.05$. Results are shown as means $\pm$ S.E.M.

RESULTS

Pretreatment of isolated adipose cells with ISO/ADA for 2 min to activate G_s maximally and minimize the activation of G_i , followed by the addition of insulin for 20 min, resulted in $57.9 \pm 4.4\%$ and $57.8 \pm 2.3\%$ decreases in maximal insulin-

Figure 2 Time course of insulin action on plasma membrane-associated GLUT4 in rat adipose cells

Isolated cells were prepared in the presence of 200 nM adenosine and either received no pretreatment or were pretreated for 2 min with ISO (200 nM) and ADA (1 unit/ml), followed by 0–20 min of insulin (670 nM). Plasma membranes were then prepared from the cells under each condition; GLUT4 was detected by SDS/PAGE and immunoblotting as described in the Experimental section. Results are means $\pm$ S.E.M. for five or six independent experiments. Symbols: $\square$, stimulation with insulin without pretreatment with ISO/ADA; $\blacklozenge$, stimulation with insulin with pretreatment with ISO/ADA respectively. Results in (a) are presented as percentages of the maximal response to insulin in the presence of adenosine; results in (b) are presented as percentages of the maximal response for each of the two respective conditions. Exponential curve fits are shown in (b); the solid and broken lines represent stimulation with insulin without and with pretreatment with ISO/ADA respectively.

stimulated glucose transport activity and cell-surface GLUT4 (assessed by ATB-BMPA photolabelling) respectively, but no change in the plasma membrane content of GLUT4 in comparison with stimulation with insulin alone. These results are similar to those observed by adding ISO/ADA either simultaneously with insulin or after maximal stimulation with insulin [7–10].

Figure 1 illustrates the results obtained with two different methods for detecting cell-surface GLUT4, labelling of the GLUT4 exofacial substrate-binding site with ATB-BMPA as previously described and biotinylation of the carbohydrate group on the extracellular loop of GLUT4. With the biotinylation method, cell-surface GLUT4 could be assessed independently of potential changes in GLUT4 substrate binding. However, because the method for biotinylation involved two incubations at 4 °C, we first determined whether the effect of treatment with ISO/ADA on insulin-stimulated GLUT4 translocation as assessed by photolabelling with ATB-BMPA could still be observed when isolated adipose cells were treated under the same conditions as cells that were biotinylated. As noted above,

Figure 3 Time course of insulin action on cell-surface GLUT4 measured with ATB-BMPA

Isolated cells were prepared in the presence of 200 nM adenosine and either received no pretreatment or were pretreated for 2 min with ISO (200 nM) and ADA (1 unit/ml), followed by 0–20 min of insulin (670 nM). Cells from each condition were then photolabelled with ATB-[2- 3 H]BMPA; GLUT4 was immunoprecipitated from each sample as described in the Experimental section. Results are means $\pm$ S.E.M. for three to six independent experiments. Symbols: $\square$, stimulation with insulin without pretreatment with ISO/ADA; $\blacklozenge$, stimulation with insulin with pretreatment with ISO/ADA. Results in (a) are presented as percentages of the maximal response to insulin in the presence of adenosine; results in (b) are presented as percentages of the maximal response for each of the two respective conditions. Exponential curve fits are presented in (b); the solid and broken lines represent stimulation with insulin without and with pretreatment with ISO/ADA respectively.

ISO/ADA resulted in a 58 % decrease in insulin-stimulated cell-surface GLUT4 assessed with ATB-BMPA when incubations and labelling were performed at 37 °C. When the experimental incubations included treatment with ISO/ADA at 37 °C, followed by two incubations at 4 °C as described in the Experimental section, cell-surface GLUT4 in the basal state increased approx. 6-fold compared with the previous experimental conditions at 37 °C. In spite of this increase in basal cell-surface GLUT4, stimulation with insulin resulted in a significant increase in cell-surface GLUT4 compared with the basal condition, and treatment of insulin-stimulated adipose cells with ISO/ADA resulted in an approx. 40 % decrease in cell-surface GLUT4 compared with stimulation with insulin alone (Figure 1a). When the results were adjusted for the increase in labelling in the basal state, the effect of ISO/ADA on insulin-stimulated cell-surface GLUT4 was an approx. 65–70 % decrease, which was similar to the effect observed at 37 °C.

A comparison of the results for ATB-BMPA photolabelling under the experimental conditions used for biotinylation with the results for biotinylation demonstrates that similar effects of

Figure 4 Time course of insulin action on glucose transport activity

Isolated cells were prepared in the presence of 200 nM adenosine and either received no pretreatment or were pretreated for 2 min with ISO (200 nM) and ADA (1 unit/ml), followed by 0–20 min of insulin (670 nM). Cells from each condition were then assayed for glucose transport activity as described in the Experimental section. Results are means $\pm$ S.E.M. for eight to ten independent experiments. Symbols: $\square$, stimulation with insulin without pretreatment with ISO/ADA; $\blacklozenge$, stimulation with insulin with pretreatment with ISO/ADA. Results in (a) are presented as percentages of the maximal response to insulin in the presence of adenosine; results in (b) are presented as percentages of the maximal response for each of the two respective conditions. Exponential curve fits are presented in (b); the solid and broken lines represent stimulation with insulin without and with pretreatment with ISO/ADA respectively.

ISO/ADA treatment on insulin-stimulated cell-surface GLUT4 were observed with these two methods. As illustrated in Figure 1b, cell-surface GLUT4 in the basal state was elevated compared with ATB-BMPA labelling at 37 °C, which was similar to ATB-BMPA labelling after incubations at 4 °C. Stimulation with insulin resulted in a significant increase in cell-surface GLUT4 compared with basal values. Similarly to the effect on ATB-

BMPA labelling of cell-surface GLUT4, treatment of insulin-stimulated adipose cells with ISO/ADA decreased the overall amount of cell-surface GLUT4 detected by the biotin label by approx. 30 %. When the results were adjusted for the increase in labelling in the basal state, the effect of ISO/ADA on insulin-stimulated cell-surface GLUT4 measured by biotinylation was an approx. 50 % decrease, which is similar to the effect observed with ATB-BMPA at 37 °C. Thus a decrease in the incubation temperature resulted in a quantitative but not a qualitative change in the relationship between basal, insulin-stimulated and ISO/ADA-treated cell-surface GLUT4.

The effect of ISO in the absence of adenosine on insulin-stimulated cell-surface GLUT4 and glucose transport activity suggests an alteration in the proportion of plasma-membrane-associated GLUT4 in the occluded and fully cell-surface-exposed states (i.e. docked and/or fused vesicles). Steady-state measurements do not, however, distinguish between an accumulation of docked vesicles during exocytosis and vesicles budding from the plasma membrane during endocytosis. Computer modelling of the subcellular trafficking of GLUT4 during stimulation with insulin predicts a primary effect of ISO/ADA treatment on exocytosis and suggests that an increased proportion of plasma-membrane-associated GLUT4 in occluded vesicles would be detectable during time-course experiments [17]. A second set of experiments was therefore designed to determine the effect of treatment with ISO/ADA on the time course of maximal stimulation with insulin of glucose transport activity, cell-surface GLUT4 and plasma-membrane-associated GLUT4. In these experiments, isolated adipose cells were pretreated for 2 min without or with ISO/ADA before the addition of insulin. This period was sufficient to achieve the maximal stimulation of G_s by ISO as well as the removal of any stimulation of G_i by adenosine.

Figures 2, 3 and 4 illustrate the time course of insulin action without or with pretreatment with ISO/ADA. Similar to previous results from this laboratory, no effect of ISO/ADA was observed on the steady-state level of GLUT4 in plasma membranes as assessed by immunoblotting (Figure 2a) [7–10] or on the time course of GLUT4 appearance in the plasma membranes (Figure 2b). The $t_{1/2}$ values and initial rates of plasma-membrane-associated GLUT4 appearance were also similar for maximal stimulation with insulin without and with pretreatment with ISO/ADA (Table 1). In contrast, pretreatment with ISO/ADA significantly decreased not only the steady-state level of cell-surface GLUT4 (Figure 3a) but also the $t_{1/2}$ to maximal stimulation and the initial rate of appearance of GLUT4 on the adipose cell surface, as assessed by ATB-BMPA photoaffinity labelling (Figure 3b, Table 1). Significant decreases in the steady-state stimulation of glucose transport activity (Figure 4a) and the $t_{1/2}$ of and initial rate for the stimulation of glucose transport activity with

Table 1 Kinetic analysis of insulin-stimulated plasma-membrane-associated GLUT4 events, calculated by fitting of an exponential curve

* Significantly different from the value for insulin alone. The numbers in parentheses are the numbers of experiments.

Event	Insulin		ISO/ADA/insulin	
	$t_{1/2}$ (min)	Initial rate (min ⁻¹)	$t_{1/2}$ (min)	Initial rate (min ⁻¹)
Plasma membrane GLUT4	2.8 $\pm$ 0.4 (5)	0.28 $\pm$ 0.05	2.6 $\pm$ 0.7 (6)	0.35 $\pm$ 0.08
Cell-surface GLUT4	3.1 $\pm$ 0.6 (6)	0.26 $\pm$ 0.04	5.5 $\pm$ 0.6* (3)	0.12 $\pm$ 0.02*
Glucose transport activity	3.2 $\pm$ 0.5 (10)	0.23 $\pm$ 0.02	5.1 $\pm$ 0.6* (8)	0.14 $\pm$ 0.02*

insulin (Figure 4b, Table 1) were also observed with treatment with ISO/ADA.

DISCUSSION

Previous investigations from this laboratory demonstrated that R_1G_1 agents such as adenosine increase the maximally insulin-stimulated glucose transport activity, whereas R_sG_s agents such as ISO decrease it. Under conditions of maximal (insulin plus adenosine) or decreased (insulin plus ISO in the absence of adenosine) insulin-stimulated glucose transport activity, changes in cell-surface GLUT4 as assessed by photolabelling with a ligand for the substrate-binding site parallel the changes in glucose transport activity. The present investigation demonstrates that the detection of cell-surface GLUT4 by biotinylation of the exofacial carbohydrate moiety of GLUT4 also parallels the changes in glucose transport activity. Thus the present results suggest that, in ISO-treated adipose cells in the absence of adenosine, some of the GLUT4 molecules are found in occluded vesicles associated with the plasma membrane with neither the substrate-binding site nor the first extracellular domain of the protein exposed on the cell surface. The present study also demonstrates that the mechanisms of action of R_sG_s and R_1G_1 agents on glucose transport activity and the extent to which membrane-associated GLUT4 molecules are associated with occluded vesicles seem to occur primarily via alterations in the kinetics of GLUT4 exocytosis.

To determine whether the treatment of insulin-stimulated adipose cells with ISO/ADA results in the occlusion of GLUT4 or an alteration in the substrate-binding site, we compared cell-surface labelling of GLUT4 with the use of the ATB-BMPA photolabel with biotinylation of the carbohydrate group on the first extracellular loop. Both labelling procedures were performed by using the same incubation protocol. Biotinylation of GLUT4 was increased by stimulation of isolated adipose cells with insulin; treatment of the insulin-stimulated cells with ISO/ADA decreased biotinylation. These results demonstrate directly that treatment of insulin-stimulated cells with ISO/ADA results in a decrease in cell-surface GLUT4 rather than a change in substrate binding. The present investigation therefore directly confirms the hypothesis first proposed by Vannucci et al. [10] that R_sG_s and R_1G_1 agents such as ISO and adenosine respectively modulate the amount of GLUT4 in plasma-membrane-associated occluded vesicles.

The exact signals that result in these changes in the trafficking of GLUT4 when insulin-stimulated adipose cells are treated with ISO, an R_sG_s agent, in the absence of adenosine, an R_1G_1 agent, are unknown. Previous investigations in this laboratory excluded changes in GLUT4 phosphorylation in mediating the inhibition of insulin-stimulated glucose transport activity by ISO/ADA [16,18]. An association between insulin-stimulated glucose transport activity, GLUT4 and G-proteins has been suggested to occur in cardiac myocytes [19]; a direct interaction between G-proteins and GLUT4 in adipose cells has also been proposed, with potential intermediate steps between G-protein activation and the glucose transporter response [8,10,20]. However, interactions between G-proteins and the vesicle docking and fusion proteins and/or the vesicle budding and fusion machinery must now also be considered [21].

Given direct evidence for the accumulation of occluded GLUT4 in the plasma membrane of adipose cells in response to treatment with ISO/ADA, we attempted to assess whether these occluded GLUT4 molecules are part of the exocytosis and/or endocytosis pathways. Our approach was based on comparing the measured time courses of insulin's stimulating actions on

plasma-membrane-associated GLUT4, cell-surface GLUT4 and glucose transport activity with the respective time courses predicted from a series of kinetic models of subcellular trafficking of GLUT4 reported by Holman et al. [17]. These investigators predicted that if the treatment of insulin-stimulated adipose cells with ISO/ADA results in an increase in the number of GLUT4 molecules in occluded vesicles associated with exocytosis, then the lag between the appearance of plasma-membrane-associated GLUT4 and the increase in glucose transport activity would probably be greater than with insulin alone. However, if treatment with ISO/ADA results in an increase in the amount of GLUT4 in occluded vesicles during endocytosis, then the time course for stimulation of glucose transport activity with insulin would probably reach maximum before the time course for stimulation of plasma-membrane-associated GLUT4 with insulin; the latter would probably be slower with treatment with ISO/ADA than with insulin alone. On the basis of these predictions, the observations in the present study strongly suggest that the treatment of insulin-stimulated adipose cells with ISO/ADA results primarily in an increase in the number of GLUT4 molecules in occluded vesicles associated with exocytosis.

Thus the results of this investigation suggest a revision of the current model of the stimulation of glucose transport with insulin. Treatment of insulin-stimulated adipose cells with ISO/ADA results in an enhanced occlusion of plasma-membrane-associated GLUT4 and a delay in the stimulation of cell-surface GLUT4 and glucose transport activity with no effect on the time course for plasma-membrane-associated GLUT4. These results suggest that the effect of R_sG_s and R_1G_1 agents seems to be on GLUT4 exocytosis, possibly a regulation of the fusion of docked GLUT4-containing vesicles with the cell surface.

Although the present results are consistent with the concept that an effect of ISO/ADA on GLUT4 exocytosis can account fully for the inhibition of steady-state insulin-stimulated glucose transport activity, it is also possible that ISO/ADA secondarily affects the rate of GLUT4 endocytosis. Further investigation of this possibility would be difficult because it requires a method for separating plasma membranes from transient occluded vesicles so that the kinetics for GLUT4 movement from the former to the latter could be studied. It is hypothesized that these transient GLUT4 endocytosis vesicles have short half-lives, thus rendering the experiments difficult and the likelihood that GLUT4 would accumulate in such vesicles very low.

In conclusion, R_sG_s ligands such as ISO, and R_1G_1 ligands such as adenosine, significantly alter the kinetics of GLUT4 trafficking. The inhibition of insulin-stimulated glucose transport activity by activated G_s seems to occur via a trapping of GLUT4 in occluded vesicles associated with the plasma membrane during exocytosis, thus slowing the fusion process by which plasma membrane-bound GLUT4 molecules are exposed on the cell surface. Activated G_i presumably has the opposite effect. In both cases, it is not an effect on substrate binding.

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