Determination of the rates of appearance and loss of glucose transporters at the cell surface of rat adipose cells

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We have used an impermeant bis-mannose compound {2-*N*-[4-(1-*azi*-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine; ATB-BMPA} to photolabel the glucose transporter isoforms GLUT4 and GLUT1 that are present in rat adipose cells. Plasma-membrane fractions and light-microsome membrane fractions were both labelled by ATB-BMPA. The labelling of GLUT4 in the plasma membrane fraction from insulin-treated cells was ~3-fold higher than that of basal cells and corresponded with a decrease in the labelling of the light-microsome fraction. In contrast with this, the cell-surface labelling of GLUT4 from insulin-treated intact adipose cells was increased ~15-fold above basal levels. In these adipose cell preparations, insulin stimulated glucose transport activity ~30-fold. Thus the cell-surface labelling, but not the labelling of membrane fractions, closely corresponded with the stimulation of transport. The remaining discrepancy may be due to an approx. 2-fold activation of GLUT4 intrinsic transport activity. We have studied the kinetics of trafficking of transporters and found the following. (1) Lowering the temperature to 18 °C increased basal glucose transport and levels of cell-surface glucose transporters by ~3-fold. This net increase in transporters probably occurs because the process of recruitment of transporters is less temperature-sensitive than the process involved in internalization of cell-surface transporters. (2) The time course for insulin stimulation of glucose transport activity occurred with a slight lag period of 47 s and a $t_{1/2}$ of 3.2 min. The time course of GLUT4 and GLUT1 appearance at the cell surface showed no lag and a $t_{1/2}$ of ~2.3 min for both isoforms. Thus at early times after insulin stimulation there was a discrepancy between transporter abundance and transport activity. The lag period in the stimulation of transport activity may represent the time required for the ~2-fold stimulation of transporter intrinsic activity. (3) The decrease in transport activity after insulin removal occurred with a very high activation energy of 159 kJ·mol⁻¹. There was thus no significant decrease in transport or loss of cell-surface transporters over 60 min at 18 °C. The decrease in transport activity occurred with a $t_{1/2}$ of 9–11 min at 37 °C. Using the ATB-BMPA photolabel to monitor remaining cell-surface transporters, we have shown that the loss of GLUT4 and GLUT1 occurred with $t_{1/2}$ values of ~12 min. Thus the decrease in transport activity corresponds with the decrease in cell-surface transporters.

INTRODUCTION

The cloning and sequencing of the GLUT1 glucose transporter isoform described by Mueckler *et al.* [1] in 1985 has been rapidly followed by the discovery of structurally related isoforms (GLUT1–GLUT5) [2]. Of particular interest in relation to stimulation of glucose transport by insulin in adipose cells is the GLUT4 isoform, which is present only in insulin-sensitive tissues such as adipose, heart and muscle tissue. This isoform has been cloned and sequenced [3–6]. An increase in GLUT4 mRNA and protein has been shown to be associated with the differentiation of the adipocyte phenotype [7]. It is the concentration of this novel glucose transporter in adipose cells which is lowered in streptozotocin-induced diabetes [8,9]. Both the mRNA and the intracellular pool of this glucose transporter are strikingly lowered, whereas the level of the GLUT 1 transporter is unchanged. The large stimulations by insulin of glucose transport in adipose cells are clearly related to the presence of the GLUT4 isoform, but the structural features of this protein that make it acutely sensitive to insulin have not yet been determined. Structural features may be responsible for a unique cellular processing and consequently a unique distribution of this isoform within the cells of the insulin-sensitive tissues. One approach to examine the kinetics of the cellular trafficking is the use of an impermeant probe which can selectively label transporters as they appear and are removed from the cell surface.

We have recently shown that cell-surface labelling with the bis-

mannose photolabel 2-*N*-[4-(1-*azi*-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine (ATB-BMPA) can provide quantitative information on the abundance of glucose transporter isoforms at the plasma membrane [10–12]. The technique allows a demonstration of the recruitment phenomenon without the need for subcellular fractionation. Using this technique we have recently shown that insulin results in an approx. 15-fold increase in cell-surface GLUT4 and an ~4-fold increase in cell-surface GLUT1 both in rat adipocytes [11] and in 3T3-L1 cells [12]. Thus we have now extended these studies on cell-surface labelling in order to examine the kinetics of the translocation of these glucose transporter isoforms. We have combined the use of the photolabel with the use of KCN treatment to arrest the translocation process [13–15]. Discrepancies between transport stimulation and transporter translocation have been proposed to be due to intrinsic activation of transporters [16–19]. The transport stimulation due to intrinsic activation is however shown here to be small when compared with the observed large changes in GLUT4 and GLUT1 transporter recruitment.

MATERIALS AND METHODS

Materials

Phloretin and fraction V BSA (which was extensively dialysed and filtered) were from Sigma. Collagenase was from Worthington. Insulin was from Novo. ATB-[2-³H]BMPA

Abbreviations used: ATB-BMPA, 2-*N*-[4-(1-*azi*-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine; C₁₂E₉, nonaethyleneglycol dodecyl ether; GLUT, glucose transporter isoform; PMA, phorbol 12-myristate 13-acetate.

(10 Ci/mmol) was prepared as described by Clark & Holman [10].

Glucose transport measurements

The rate constant for the uptake of 50 μ M-3-*O*-methyl-D-glucose was determined at 37 °C as previously described [20].

Insulin binding and dissociation measurements

Isolated adipocytes were incubated with 1 nM-¹²⁵I-insulin at 37 °C for 30 min in an albumin/Hepes buffer (140 mM-NaCl, 4.7 mM-KCl, 1.25 mM-Mg₂SO₄, 2.5 mM-CaCl₂, 2.5 mM-NaH₂PO₄, 10 mM-Hepes and 1% BSA, pH 7.4) containing 0.05% bacitracin. In the dissociation experiments the adipose cells were rapidly washed three times in albumin/Hepes buffer at ~12 °C. There was no detectable dissociation of insulin during this rapid washing. The non-specific cell-associated insulin was determined in the presence of 5 μ M-insulin and found to be less than 5% of the total bound insulin. All results were corrected for this non-specific binding. The cytocrit of the adipocyte suspensions was adjusted to 40%, and 100 μ l samples were transferred to 18–20 °C or 37 °C to initiate dissociation. At the indicated times 9 ml of albumin/Hepes buffer at 18 °C was added. The cells were isolated and the cell-associated radioactivity was determined. The addition of 20% trichloroacetic acid showed that 65% of the dissociated radioactivity at both 18 °C and at 37 °C was soluble.

Photolabelling of glucose transporters in rat adipose cells

Rat adipose cells were obtained from 170–190 g male Wistar rats as previously described [20]. Cells were suspended at a 40% cytocrit in 1% BSA/Hepes buffer at 37 °C. Following stimulation with insulin (1–10 nM as specified), 1 ml samples of cells were added to 333–500 μ Ci (as specified in the Figure legends) of ATB-[³H]BMPA in Hepes buffer without albumin in 35 mm-diam. polystyrene dishes and irradiated for 1 min in a Rayonet photochemical reactor using lamps emitting radiation of 300 nm. In some experiments, transport stimulation by insulin was arrested at the specified times by the addition of KCN (2 mM final concentration). Following irradiation, the cells were washed into polystyrene centrifuge tubes with 8 ml of albumin/Hepes buffer at 18 °C. The cells were rapidly washed three times in this buffer. In some experiments, crude plasma membrane samples were isolated from cells as described [11]. For direct immunoprecipitation experiments, cells were solubilized by vortex-mixing with 1.2 ml of detergent buffer containing 2% nonaethyleneglycol dodecyl ether (C₁₂E₉) with proteinase inhibitors (5 mM-EDTA, plus antipain, aprotinin, leupeptin and pepstatin A, each at 1 μ g/ml) in 5 mM-phosphate at pH 7.2.

Photolabelling of glucose transporters in membrane fractions isolated from rat adipose cells

Plasma membranes and light microsomes were isolated from control and insulin-treated (10 nM) rat adipose cells and suspended at 1 mg/ml in Tris/EDTA/sucrose buffer (10 mM-Tris/HCl, 0.5 mM-EDTA, 255 mM-sucrose, pH 7.2) as described [21]. Samples of 200 μ g were photolabelled with 200 μ Ci of ATB-BMPA by irradiating for 45 s. After irradiation, the samples were solubilized in 1 ml of C₁₂E₉ detergent buffer.

Immunoprecipitation of photolabelled glucose transporters

Rabbit antisera against the GLUT1 and GLUT4 glucose transporters were prepared using synthetic C-terminal peptides CEELFHPLGADSQV and CGSTELEYLGPDEND respectively, as previously described [10,11]. In most cases, 10–20 μ g of

affinity-purified antibodies was mixed with 8 μ l of Protein A-Sepharose. This conjugate was washed in 5 mM-phosphate buffer, and solubilized adipose cells or membranes in detergent buffer were added and mixed at 0–4 °C for 1.5–2 h. The supernatant from the first precipitation, generally with anti-GLUT1 antibody, was then subjected to further immunoprecipitation in the same way, but using antibody to GLUT4. The immunoprecipitates were washed four times with 1 ml of the same buffer but containing 0.2% C₁₂E₉. Finally, antibody was released from the gel matrix using electrophoresis sample buffer. In some cases immunoprecipitation was carried out using 100–150 μ l of antiserum conjugated to 20 μ l of Protein A-Sepharose. No loss in efficiency of precipitation was observed when using antiserum instead of purified antibody.

Electrophoresis

The solubilized membrane samples or the immunoprecipitated glucose transporters in electrophoresis sample buffer [10% (w/v) SDS, 6 M-urea and 10% (v/v) mercaptoethanol] were mixed with prestained 116 kDa and 29 kDa molecular mass marker proteins and were run (generally overnight) on 10% acrylamide gels in the Laemmli [22] discontinuous buffer system. Gels were washed for ~2 h in destain and then lanes were isolated by reference to the position of the prestained standards and were sliced. The slices (in scintillation vials) were dried at 80 °C for 2–3 h and then dissolved in 0.5 ml of H₂O₂ [containing 2% (v/v) NH₄OH] at 80 °C for a further 2 h. Scintillant was added and the radioactivity was counted. The positions of the photolabelled peaks were compared with the positions of molecular mass markers (Sigma) in adjacent lanes. The levels of radioactivity associated with each peak were obtained by summing the radioactivity in all slices comprising the peak and subtracting a background radioactivity which was based on the average radioactivity of the slices on either side of the peak. Cytochalasin B inhibition of the labelling of transporters recovered in the immunoprecipitates from basal cells was greater than 80% and in the cytochalasin B-treated condition there was no significant labelling above the background estimated as described above.

RESULTS

Labelling of subcellular membrane fractions

Table 1 shows the labelling of GLUT4 with ATB-BMPA in isolated plasma membranes and in low-density microsomal membranes. The labelling of isolated light-microsome fractions from basal cells was greater than the labelling of the light microsome fraction from insulin-treated cells. ATB-BMPA labelling of the plasma-membrane fraction from basal cells was ~3-fold less than that observed in the plasma-membrane fraction of insulin-treated cells. This relatively small change is likely to be a consequence of a contamination of the plasma-membrane fraction by light microsomes. The proportion of labelling of the plasma-membrane fraction from basal cells was only ~10% of the sum of the labelling obtained in both fractions. The sum of the labelling in the plasma-membrane and the light-microsome fractions was equal for insulin-treated and basal cells.

Cell-surface labelling and the use of KCN and low-temperature buffer to arrest glucose transporter cycling

As shown previously [11], insulin treatment increases the availability of cell-surface glucose transporters which can be labelled by the impermeant bis-mannose compound ATB-BMPA. Fig. 1 shows that the labelled glucose transporters ran as a peak at 50 kDa (45–60 kDa) on polyacrylamide-gel electrophoresis which is clearly distinguishable from a non-specifically labelled

Table 1. ATB-BMPA photolabelling of GLUT4 in membrane fractions isolated from rat adipose cells

Portions of 200 μ g of either plasma membranes or light microsomes from basal cells or cells treated with 10 nM-insulin were photolabelled with 200 μ Ci of ATB-BMPA; 1 ml of 2% C₁₂E₉ detergent buffer was then added and the labelled GLUT4 was immunoprecipitated and subjected to electrophoresis as described in the Materials and methods section. Radioactivity associated with the transporter was quantified by cutting and counting gel slices and results were calculated as a percentage of the radioactivity in the insulin-treated plasma membrane sample. The results are means $\pm$ S.E.M. from three experiments.

	ATB-BMPA photolabelling (%)	Protein yield (% of total protein)
Insulin plasma membrane	100	39.3
Basal plasma membrane	34 $\pm$ 16	34.6
Insulin light microsomes	170 $\pm$ 65	60.7
Basal light microsomes	234 $\pm$ 82	65.4

Fig. 1. Cell-surface labelling of glucose transporters in intact adipose cells: effect of KCN addition before insulin treatment

Suspensions of basal rat adipose cells (●) or cells treated with 10 nM-insulin alone (▲) or with 2 mM-KCN followed by 10 nM-insulin (○) were photolabelled with 333 μ Ci of ATB-BMPA. The cells were then washed and homogenized and a crude membrane pellet was obtained. The labelled proteins were then separated by electrophoresis and the radioactivity was estimated by cutting and counting 3.5 mm gel slices. The positions of molecular mass marker proteins are indicated.

70 kDa peak. Insulin increased the labelling in the 50 kDa region of the gel by $\sim$ 7-fold compared with basal cells. Addition of 2 mM-KCN 2 min before the addition of insulin lowered this cell-surface labelling to near-basal levels.

Fig. 2 shows that the cell-surface labelling of glucose transporters was reversed to near-basal levels by washing insulin-treated cells in an insulin-free buffer for 40 min. Fig. 2 also shows the ATB-BMPA cell-surface labelling of insulin-treated cells which were washed free of insulin but maintained at 18 $^{\circ}$ C for 40 min. The Figure shows that the washed cells which were maintained at 18 $^{\circ}$ C retained the same level of transporters as

Fig. 2. Effect of temperature on the loss of cell-surface glucose transporters

Rat adipose cells were treated with 1 nM-insulin for 30 min at 37 $^{\circ}$ C and then either washed five times during 40 min in albumin/Hepes buffer without insulin at 18 $^{\circ}$ C (●) or 37 $^{\circ}$ C (▲), or maintained in albumin/Hepes buffer at 37 $^{\circ}$ C with 1 nM-insulin for 40 min (□). Cell suspensions were then photolabelled with 333 μ Ci of ATB-BMPA. The cells were then rewashed and homogenized, and a crude membrane pellet was obtained. The labelled proteins were then separated by electrophoresis and the radioactivity was estimated by cutting and counting 3.5 mm gel slices. The transporter region of the gel is shown. The position of the 45 kDa molecular mass marker protein is indicated.

those cells which were maximally stimulated by insulin. In addition, we have found that the inclusion of 2 mM-KCN in the insulin-free buffer at 37 $^{\circ}$ C only partially blocked the reversal process. Cells that were stimulated by insulin and then washed in an insulin-free buffer at 37 $^{\circ}$ C showed a 91 % decrease in glucose transport activity without KCN (from two experiments), but a decrease of 53 % in the continuous presence of KCN (from two experiments). The ATB-BMPA cell-surface labelling observed at 40 min after washing in this KCN buffer was 46 % of that obtained with cells maintained in the continuous presence of insulin.

Fig. 3 shows that KCN addition after insulin treatment did not alter the cell-surface availability of the GLUT4 glucose transporters. Additional data showed that GLUT1 labelling was unaltered by this treatment. Since transporter recruitment was blocked when the KCN treatment was carried out before insulin addition, this experiment shows that additional glucose transporters were not recruited to the cell surface during the 1 min photolabelling procedure. Further evidence that recycling of glucose transporters in insulin-treated cells and in basal cells must be slower than the time required for photolabelling is the observation that the light-microsome pools of transporters were not labelled during the 1 min photolabelling period. If turnover was significant during this period, then a large proportion of transporters would be labelled at the plasma membrane and would return to the light-microsome pool. In the present study, we found that the light-microsome fraction from intact basal cells contained no ATB-BMPA-labelled glucose transporter peak, whereas the light-microsome fraction from insulin-treated cells showed an ATB-BMPA photolabelled glucose transporter peak which was only $\sim$ 4 % of that obtained in the plasma-membrane fraction. Similar results were obtained with the impermeant benzophenone-bis-hexose photolabel [21]. Thus, although a large pool of intracellular transporters is present in adipocytes and transporters can bind the photolabel if they have

Fig. 3. Immunoprecipitation of GLUT4 from ATB-BMPA-cell-surface-photolabelled rat adipose cells: effect of KCN addition after insulin treatment

Suspensions of rat adipose cells were treated with 10 nM-insulin for 20 min at 37 °C (□) or treated with insulin and then treated for 2 min with 2 mM-KCN (●). Suspensions of these cells were then photolabelled with 500 μ Ci of ATB-BMPA. Cells were then washed and directly solubilized in $C_{12}E_9$ detergent buffer. Following immunoprecipitation with anti-GLUT4 antibody, the protein was subjected to electrophoresis and the radioactivity was determined by cutting and counting 7 mm gel slices.

access to it (Table 1), these transporters do not visit the cell surface during short incubation periods. Recycling of transporters becomes significant after 40–60 min at 37 °C, and

both the ATB-BMPA-labelled transporters [23] and the benzophenone-bis-hexose-labelled transporters [21] are recycled to the light microsomes even in the continuous presence of insulin.

Insulin stimulation of glucose transporter translocation to the cell surface

Since we observed the complete block of glucose transport inactivation at 18 °C and the complete block of activation by KCN, buffer at this temperature in the presence of KCN was routinely used to arrest the cellular processing of transporters. Use of this technique had the advantage that when stimulation of transport activity and appearance of cell-surface transporters (see below) were arrested after short time periods, subsequent assays of this activity did not have to be performed immediately. In addition, these assays could be carried out at a temperature which was independent of the activation temperature. The arrest of both activation and inactivation by KCN was adequate for assays performed at 18 °C within 10–20 min of the addition of KCN.

Using the above procedure, we have compared the ATB-BMPA cell-surface labelling of glucose transporters obtained after stimulation at 18 °C and 37 °C (Fig. 4). The ATB-BMPA labelling of GLUT4 and GLUT1 in basal cells maintained at 18 °C was ~1.5-fold and ~3.9-fold higher respectively than in cells maintained at 37 °C (from three experiments). Cells maintained at 18 °C also showed an ~4-fold elevation of basal transport activity. The insulin stimulation of the labelling of both isoforms and the maximal stimulation of glucose transport activity were only slightly decreased (by ~20%) by lowering the temperature to 18 °C. At 18 °C the labelling of GLUT4 in insulin-treated cells was 7.4-fold higher than that in basal cells, whereas the GLUT4 labelling at 37 °C was 14.8-fold higher than in basal cells (from three experiments). In these experiments the increases in GLUT1 labelling above basal levels were 2.8-fold at 18 °C and 10.7-fold at 37 °C.

Fig. 4. Comparison of cell-surface levels of glucose transporters in adipose cells incubated at 18 °C and 37 °C

Rat adipose cells were treated at 18 °C or 37 °C for 30 min in the presence (I) or absence (B) of 10 nM-insulin. Cell suspensions were then treated with 2 mM-KCN and photolabelled with 500 μ Ci of ATB-BMPA or used to assay 3-O-methyl-D-glucose (3-O-MG) transport. Photolabelled cells were washed and directly solubilized in $C_{12}E_9$ detergent buffer, and transporters were immunoprecipitated with anti-GLUT4 and anti-GLUT1 antibodies. The protein was then subjected to electrophoresis and the radioactivity was determined by cutting and counting gel slices. The results are means $\pm$ S.E.M. from three experiments.

Fig. 5. Time courses for insulin stimulation of glucose transport and for the appearance of cell-surface transporters

Rat adipose cells were treated with 2 nM-insulin at 37 °C for the indicated times. Cell suspensions were then treated with 2 mM-KCN and either used in transport assays (●) or photolabelled with 500 μ Ci of ATB-BMPA. Following labelling, cells were washed and directly solubilized in $C_{12}E_9$ detergent buffer. Transporters were then immunoprecipitated with anti-GLUT4 (□) and anti-GLUT1 (■) antibodies. The protein was then subjected to electrophoresis and the radioactivity was estimated by cutting and counting gel slices. The results in (a) were calculated as a fraction of the fully stimulated level of transport or labelling, with subtraction of the levels of activity found in basal cells, and are means $\pm$ S.E.M. from three to five experiments. The maximum levels were similar to those in Fig. 4. In (b) the lines were derived from regression (weighted for relative error) of the combined GLUT1 and GLUT4 labelling or transport. The half-times given in the Results section were from analysis of three to five separate experiments.

The stimulation of 3-*O*-methyl-D-glucose transport in rat adipose cells by 2 nM-insulin was rapid at 37 °C (Figs. 5a and 5b). However, the time course for stimulation was not consistent with a single rate constant for activation. Instead, the log plot of these data (Fig. 5b) had a positive intercept on the time axis. This suggests that there is a lag time for activation. The lag time, calculated as the intercept on the time axis, was 47.1 ± 9.5 s (from five experiments). As the intercept on the time axis was positive, the $t_{1/2}$ was not calculated from the slope of this plot in the normal way, but was calculated as $t = (\ln 2 - c)/m$, where c is the intercept on the y axis and m is the slope. The $t_{1/2}$ was 3.2 ± 0.2 min (from five experiments).

We have compared the time course for the insulin stimulation of glucose transport activity with that for the increase in cell-surface GLUT1 and GLUT4. Figs 5(a) and 5(b) show that, unlike the stimulation of glucose transport activity, the appearance of cell-surface transporters occurred with no significant

lag, and also that this arrival of transporters at the surface very slightly preceded transport stimulation. The half-times for the recruitment of transporters that can be photolabelled were calculated to be 2.32 ± 0.30 min for GLUT1 and 2.17 ± 0.18 min for GLUT4 (from three experiments). The experimental errors involved in determining changes in cell-surface transporters were found to be greater than those involved in the determination of transport activity. Although the mean values (from three to five experiments) for the transporter levels and the transport rate were different, at some time points the S.E.M. values for these estimates overlapped. The maximum difference between transporter appearance and transport stimulation (which was only ~ 2 -fold) occurred only at short times after insulin addition. This small difference was less evident when insulin concentrations higher than 2 nM were used to stimulate the cells (A. E. Clark, G. D. Holman & I. J. Kozka, unpublished work).

Loss of glucose transporters from the cell surface following insulin removal

Fig. 6(a) shows the return of 3-*O*-methyl-D-glucose transport activity to basal levels when insulin was removed. This inactivation was very sensitive to lowering of the temperature, and the process was completely inhibited at 18–20 °C. The half-times for loss of transport activity increased sharply from ~ 9 min at 37 °C to ~ 15 min at 34 °C, ~ 30 min at 30 °C and ~ 100 min at 26 °C, with no detectable loss of transport activity at 18–20 °C. The activation energy calculated from these data was very high ($159 \text{ kJ} \cdot \text{mol}^{-1}$). At 37 °C the decrease in cell-associated insulin paralleled the transport inactivation (Fig. 6b). At 18–20 °C, cell-associated insulin decreased more slowly and the receptors remained partially occupied even after 40 min at this temperature.

Using the ATB-BMPA photolabel, we have also examined the time course for loss of cell-surface GLUT1 and GLUT4 after insulin removal and have compared this with the time course for the decrease in transport activity at 37 °C. Fig. 6(c) shows that the decrease in the availability of both cell-surface GLUT4 and GLUT1 followed the decrease in 3-*O*-methyl-D-glucose transport activity. The decrease in transport activity occurred with a $t_{1/2}$ of 10.9 ± 1.4 min (from three experiments), whereas the loss of cell-surface GLUT4 occurred with a $t_{1/2}$ of 12.3 ± 3.0 min (from three experiments) and the decrease in GLUT1 occurred with a $t_{1/2}$ of 11.7 ± 2.7 min (from two experiments).

DISCUSSION

In rat adipose cells, the large stimulation by insulin of glucose transport has been shown to involve translocation of transporters from an intracellular pool to the plasma membrane [24–26]. Using the bis-mannose photolabel ATB-BMPA, we have shown a good correlation between stimulation of transport by insulin or the phorbol ester phorbol 12-myristate 13-acetate and the translocation of the GLUT4 isoform [11]. In that study and the present study we observed 15–20-fold stimulation of translocation by insulin. The small amount of cell-surface labelled glucose transporters in basal cells is not due to an inability of transporters in the light-microsome pool to react with the label, since we have shown here that when transporters in a light-microsome fraction of membranes have access to the photolabel then photoincorporation of the probe occurs. This demonstrates that the low cell-surface labelling of GLUT4 in basal cells is a consequence of the inaccessibility of the transporter due to its sequestration inside the cell. The experiments in which we have labelled the GLUT4 transporter in membrane fractions also provide a demonstration of the difficulties involved in quantifying the extent to which transporter translocation occurs by examining

Fig. 6. Time courses of the decrease in (a) glucose transport activity, (b) cell-associated insulin and (c) cell-surface glucose transporters

(a) Rat adipose cells were fully stimulated by treatment with 1 nM-insulin for 30 min at 37 °C and then the rate of deactivation of 3-*O*-methyl-D-glucose transport was measured after incubation at 18–20 °C (□), 26 °C (△), 30 °C (■), 34 °C (▲) and 37 °C (●). In each case, 2 mM-KCN was added and then transport was measured at 37 °C. Results are the means $\pm$ S.E.M. of 7–11 observations from three experiments. (b) Cell suspensions were fully stimulated by treatment with 1 nM-¹²⁵I-insulin for 30 min at 37 °C and then the rate of loss of cell-associated insulin was measured at 18–20 °C (□) and 37 °C (■) as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three observations from one experiment. (c) Cell suspensions were fully stimulated by treatment with 1 nM-insulin for 30 min at 37 °C and then the rate of loss of cell-surface transporters at 37 °C was measured. Cell suspensions were treated with 2 mM-KCN and then labelled with 500 μ Ci of ATB-BMPA. Cell suspensions were then washed and directly solubilized in C₁₂E₉ detergent buffer and transporters were immunoprecipitated with anti-GLUT4 (●) and anti-GLUT1 (□) antibodies. The protein was then subjected to electrophoresis and the radioactivity was estimated by cutting and counting the gel slices. The results were calculated as a fraction of the insulin-stimulated level, with subtraction of the levels of activity found in basal cells. Results are means $\pm$ S.E.M. from two or three experiments. Half-times for loss of cell-surface transporters were calculated from semi-log plots of these data.

membrane fractions. The labelling of the plasma membrane fraction of basal cells was $\sim 10\%$ of the sum of the labelling in plasma-membrane and light-microsome fractions, and most of the observed labelling could therefore have been due to a contamination of the plasma-membrane fraction by light-microsome membranes. Such contamination would thus lead to an underestimate of the real insulin-stimulated increase in transporters in the plasma membrane of the intact cell.

It has been shown by Kono's group [27,28] that reagents such as KCN and dinitrophenol, which lower cellular ATP, block the stimulation of glucose transport by insulin. We have shown here that KCN also blocked the appearance of glucose transporters at the cell surface. If an intrinsic activation of glucose transport is part of the stimulatory mechanism, then this result rules out the possibility that the involvement of ATP is only at the intrinsic activation step. If this were the case, then surface labelling would not have been blocked by KCN treatment. However, we cannot distinguish between the possibility of ATP involvement at just the translocation step and an alternative in which ATP is required at both the translocation and the intrinsic activation steps.

Surprisingly, the reversal of the insulin effect on glucose transport was not completely blocked by KCN treatment at 37 °C, and at 40 min approximately half of the transporters were lost from the cell surface even in the continuous presence of KCN. Thus the activation of glucose transport by insulin appears to be more sensitive to ATP depletion than the inactivation process.

We have confirmed earlier studies [28,29] that showed that activation of transport occurs more slowly at 18 °C but reaches a final level of stimulated activity that is only slightly less than that which occurs at 37 °C, and that the basal transport activity rises ~ 4 -fold on transfer of adipose cells to 18 °C. In addition, we have observed that the availability of cell-surface transporters that were cell-surface-photolabelled increased markedly on transfer to 18 °C. This increase in translocation and the associated increase in glucose transport activity probably occurs because internalization of transporters (see below) is more sensitive than

the exocytotic stimulatory process to a lowering of temperature.

The observed slight lag period in the time course for activation of glucose transport was similar to the lag period for activation reported by others [30,31]. However, there was no significant lag period in the appearance of cell-surface GLUT4 or GLUT1. Both of these transporters appeared at the cell surface with a $t_{1/2}$ of ~ 2.3 min at 37 °C. This is consistent with the observations of Karnieli *et al.* [32], who have shown that the appearance of cytochalasin B-binding sites in plasma-membrane fractions isolated from insulin-treated adipose cells preceded transport stimulation. In addition, Gibbs *et al.* [33] showed that the arrival of GLUT1 at the surface of 3T3-L1 cells preceded the stimulation of transport. These experiments appear to provide evidence for a two-step process in the complete activation of glucose transport. However, it seems unlikely that in the basal state all of the glucose transporters are inactive, since the glucose transporters in the light microsomes can be reconstituted into phospholipid vesicles, where they show glucose transport activity [25]. In addition, the discrepancy between GLUT4 transporter abundance and the transport rate is only ~ 1.5 –2-fold in the basal state and in PMA-treated cells [11] and (as shown here) at short times after insulin addition to the adipose cells. Thus only a small proportion of the plasma membrane transporters is likely to have low intrinsic transport activity, and it may be these transporters that require a subsequent covalent or structural activation by insulin. Alternatively, a small proportion of the plasma membrane pool of transporters may be occluded from participating in the transport process and may require an additional insulin-dependent movement to a location where these transporters are operative [17].

The inactivation of glucose transport and the loss of cell-surface-available transporters was very sensitive to temperature and was markedly decreased by lowering the temperature to 18 °C. No decrease in glucose transport activity or in cell-surface transporters occurred even after 40–60 min at 18 °C. Approximately half of the radioactive insulin which had bound to the cells was dissociated from the cells during this period, so

that it is unlikely that the maintenance of a residual stimulation by insulin could account for the maintenance of full glucose transport activity. However, partial occupancy of the insulin receptors may be sufficient to maintain the full stimulatory effect [34]. Others who have examined the relationship between dissociation of insulin and the maintenance of the stimulation of transport have shown that dissociation and inactivation occur in parallel [35] or have shown (as we have) that transport activity persists under conditions in which insulin dissociates from its receptor [14,36]. The time course for return of stimulated transport activity to basal levels at 37 °C and the time course for the loss of cell-surface GLUT4 and GLUT1 were similar, with $t_{1/2}$ values of approx. 10–12 min. The change in glucose transporters in the plasma membrane due to insulin stimulation was ~15-fold and ~5-fold for GLUT4 and GLUT1 respectively. The difference in stimulation ratio (~3-fold) may occur because GLUT4 has a greater tendency to be located intracellularly in the basal state [12]. A relatively fast endocytosis and/or a slow exocytosis of GLUT4 compared with GLUT1 in the basal state could account for this distribution. Our measurements of the transporter internalization rates following insulin removal indicate that the difference in the stimulation ratio is probably not due to the faster endocytosis of GLUT4, and it therefore seems that the exocytosis of GLUT4 may be slower than that of GLUT1 in the basal state.

The impermeant photolabel ATB-BMPA will be useful in the further investigation of translocation kinetics, since it will be possible to directly measure transporter recycling to the light microsomes. In addition, it will be possible to investigate effects of mediators and inhibitors on translocation kinetics.

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