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Trafficking of glucose transporters in 3T3-L1 cells

Inhibition of trafficking by phenylarsine oxide implicates a slow dissociation of transporters from trafficking proteins

Jing YANG, Avril E. CLARK, Roger HARRISON, Izabela J. KOZKA and Geoffrey D. HOLMAN*
Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, Avon, U.K.

We have compared the rates of insulin stimulation of cell-surface availability of glucose-transporter isoforms (GLUT1 and GLUT4) and the stimulation of 2-deoxy-D-glucose transport in 3T3-L1 cells. The levels of cell-surface transporters have been assessed by using the bismannose compound 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine (ATB-BMPA). At 27 °C the half-times for the appearance of GLUT1 and GLUT4 at the cell surface were 5.7 and 5.4 min respectively and were slightly shorter than that for the observed stimulation of transport activity ($t_{\frac{1}{2}}$ 8.6 min). This lag may be due to a slow dissociation of surface transporters from trafficking proteins responsible for translocation. When fully-insulin-stimulated cells were subjected to a low-pH washing procedure to remove insulin at 37 °C, the cell-surface levels of GLUT1 and GLUT4 decreased, with half-times of 9.2 and 6.8 min respectively. These times correlated well with decrease in 2-deoxy-D-glucose-transport activity that occurred during this washing procedure ($t_{\frac{1}{2}}$ 6.5 min). When fully-insulin-stimulated cells were treated with phenylarsine oxide (PAO), a similar decrease in transport activity occurred ($t_{\frac{1}{2}}$ 9.8 min). However, surface labelling showed that this corresponded with a decrease in GLUT4 only ($t_{\frac{1}{2}}$ 7.8 min). The cell-surface level of GLUT1 remained high throughout the PAO treatment. Light-microsome membranes were isolated from cells which had been cell-surface-labelled with ATB-BMPA. Internalization of both transporter isoforms to this pool occurred when cells were maintained in the presence of insulin for 60 min. In contrast with the surface-labelling results, we have shown that the transfer to the light-microsome pool of both transporters occurred in cells treated with insulin and PAO. These results suggest that both transporters are recycled by fluid-phase endocytosis and exocytosis. PAO may inhibit this recycling at a stage which involves the re-emergence of internalized transporters at the plasma membrane. The GLUT1 transporters that are recycled to the surface in insulin- and PAO-treated cells appear to have low transport activity. This may be because of a failure to dissociate fully from trafficking proteins at the cell surface. GLUT4 transporters appear to have a greater tendency to remain internalized if the normal mechanisms that commit transporters to the cell surface, such as dissociation from trafficking proteins, are uncoupled.

INTRODUCTION

The large insulin stimulations of glucose transport in adipose cells [1–3] and in 3T3-L1 cells [4] are now known to be due to the presence of the GLUT4 isoform of the glucose transporter. This isoform has been cloned and sequenced by five independent laboratories [5–9]. The study of the cellular-trafficking pathways of GLUT4 is of great importance in the understanding of the effect of insulin on the translocation of this isoform to the cell surface. A recent, very elegant, study by Slot *et al.* [10] has clearly shown that insulin produces a very large increase in GLUT4 in the plasma membrane of brown adipose tissue as detected by immunogold electron microscopy. The increase in plasma-membrane transporter was paralleled by a decrease in glucose transporter in a cytoplasmic pool associated with tubulo-vesicular structures. An alternative approach to studying glucose-transporter trafficking that we have developed is to use a cell-impermeant photoaffinity label. The photolabel, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA), is a bis-D-mannose derivative substituted with an (azitrifluoroethyl)benzoyl group. This only labels those transporters that are present at the cell surface of intact cells [11–14]. However, photolabelling of glucose transporters in light-microsome membranes can occur if the reagent has access to these, as it does in permeabilized cells (J. Yang, A. E. Clark, I. J. Kozka, S. W. Cushman & G. D. Holman, unpublished work) or in membrane fractions [16].

In experiments using the bismannose photolabel we have studied the kinetics of trafficking in rat adipocytes at 37 °C [16] and have found that GLUT1 and GLUT4 appeared rapidly on the cell surface with half-times of $\approx$ 2 min, which were slightly shorter than that for the stimulation of glucose transport. The losses of cell-surface GLUT1 and GLUT4 occurred with half-times of $\approx$ 12 min. These results suggested that the larger insulin stimulation of cell-surface availability of GLUT4 ($\approx$ 15–20-fold) compared with GLUT1 ($\approx$ 5-fold) was due to a lower rate of exocytosis of GLUT4 in the basal state. In addition to the cell-surface detection of transporters, Satoh *et al.* [17] have shown that the reagent can be used to monitor the internalization of transporters directly. Thus, after labelling, the cells can be maintained at 37 °C to allow cell processing to occur and then plasma membrane and light-microsome fractions can be isolated. By measuring transporter redistribution in this way we have obtained direct evidence that glucose transporters are recycled between the plasma membrane and the light microsomes, even in the continuous presence of insulin. In the presence of insulin the cell-surface level of transporters was shown to be constant, but photolabelled transporters were internalized and equilibrated

Abbreviations used: ATB-BMPA, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine; PAO, phenylarsine oxide; DMEM, Dulbecco's modified Eagle medium; KRH, Krebs–Ringer/Hepes; GLUT1 and GLUT4, glucose-transporter isoforms 1 and 4; $C_{12}E_9$, nona(ethylene glycol) dodecyl ether; G-protein, guanine-nucleotide-binding protein.

* To whom correspondence should be sent.

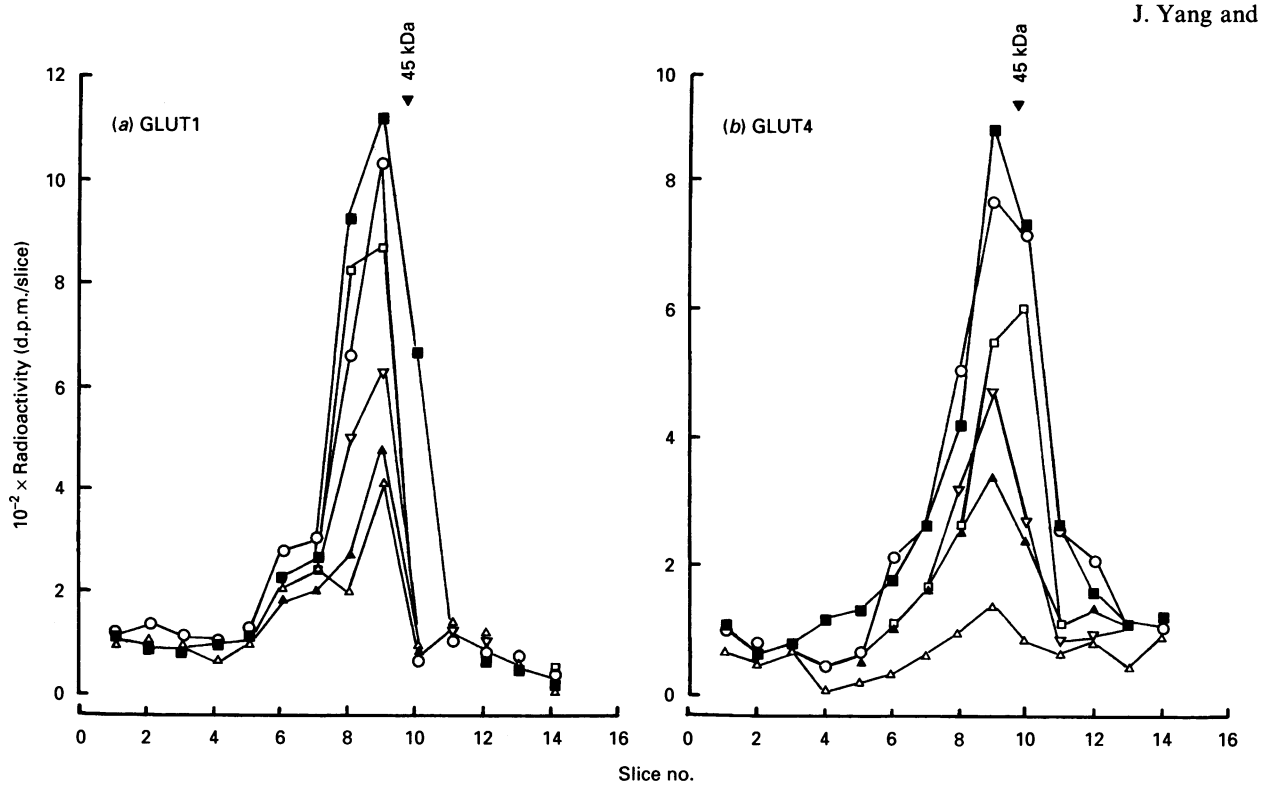

Fig. 1. Insulin stimulation of the cell-surface appearance of GLUT1 and GLUT4 in 3T3-L1 cells

3T3-L1 cells in 35 mm-diameter dishes were stimulated with 100 nM-insulin at 27 °C and then at zero time (△), 2.5 min (▲), 5 min (▽), 7.5 min (□), 12 min (○) and 30 min (■) the insulin was removed by washing twice in KRH buffer at 18 °C. The cells were then labelled at 18 °C with 100 μCi of ATB-BMPA in 250 μl of KRH buffer. After labelling the cells were washed four times in KRH buffer and then directly solubilized in $C_{12}E_9$ detergent buffer. GLUT1 (*a*) and GLUT4 (*b*) were then immunoprecipitated with anti-(*C*-terminal peptide) antibodies. The protein was then subjected to electrophoresis and the radioactivity was estimated by cutting out and counting the gel slices.

with the light-microsome pool. Studies on internalized ATB-BMPA-labelled GLUT4 have shown that insulin increases the rate of exocytosis of this isoform [17]. A similar conclusion was reached by Slot *et al.* [10] in their electron-microscopic study, which analysed the steady-state distribution of transporters in brown adipose tissue.

The kinetic studies suggest that some trafficking of GLUT1 and GLUT4 occurs via a common pathway, but that GLUT4 can be excluded from the common pathway in the absence of insulin and sequestered in a distinct vesicle pool. An approach to examining the possibly separate processing of the two transporters is to inhibit their trafficking. Phenylarsine oxide (PAO) is known to inhibit receptor endocytosis [18,19] and fluid-phase endocytosis in 3T3-L1 cells [20], but has been shown to inhibit the insulin stimulation of glucose transport in 3T3-L1 cells and in adipocytes [4,21] an exocytotic process [17]. Thus, in the study described here, we have kinetically monitored the insulin-stimulated translocation of GLUT1 and GLUT4 to the cell surface of 3T3-L1 cells and have, in addition, addressed the question of the site of action by PAO by monitoring the appearance and loss of these transporters in cells whose vesicle-processing pathways have been perturbed with this reagent.

MATERIALS AND METHODS

Materials

ATB-BMPA and ATB-[2-^{3}H]BMPA (sp. radioactivity ≈ 10 Ci/mmol) were prepared as described [11], 2-deoxy-D-[2,6-^{3}H]glucose was from Amersham International. Dulbecco's modified Eagle medium (DMEM) was from Flow laboratories. Foetal-bovine serum was from Gibco. Monocomponent porcine insulin was a gift from Dr. Ronald Chance, Eli Lilly Corp. Dexamethasone, isobutylmethylxanthine, Protein A–Sepharose and phenylarsine oxide were from Sigma. Nona(ethylene glycol) dodecyl ether ($C_{12}E_9$) was from Boehringer.

Cell culture

3T3-L1 fibroblasts were cultured in DMEM and differentiated to adipocytes by treatment with insulin, dexamethasone and isobutylmethylxanthine as described in [4,13]. Fully differentiated cells were washed with phosphate-buffered saline (154 mM-NaCl/12.5 mM-sodium phosphate, pH 7.4) and were then incubated for 2 h in serum-free medium containing 25 mM-D-glucose. This was followed by three washes in Krebs–Ringer/Hepes buffer (KRH buffer; 136 mM-NaCl/4.7 mM-KCl/1.25 mM-$CaCl_2$/1.25 mM-$MgSO_4$/10 mM-Hepes, pH 7.4) before use in experiments to determine 2-deoxy-D-glucose transport activity or cell-surface transporters.

Insulin stimulation experiments

Normally cell monolayers in 35 mm-diameter dishes were maintained at 37 °C with 100 nM porcine monocomponent insulin to produce fully stimulated cells. However, the half-time for stimulation of transport at 37 °C was found to be ≈ 3 min, which was considered to be too short a time for accurate comparisons with the time course of cell-surface photolabelling ($t_{\frac{1}{2}} \approx 2$ min). In rat adipocytes we were able to measure the time course for stimulation at 37 °C because the stimulation of these cells could be blocked at short time points by the addition of KCN. We have found, however, that the stimulation of transport in 3T3-L1 cells was not blocked by KCN. Instead, to compare the time course for insulin stimulation of transport and cell-surface availability of transporters in these cells we stimulated at 27 °C and then washed the cells at 18 °C before measuring

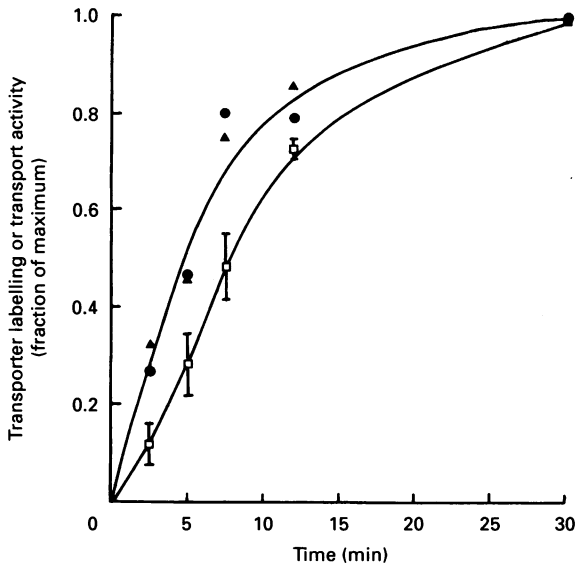

Fig. 2. Time course for insulin stimulation of cell-surface availability of glucose transporters and for stimulation of 2-deoxy-D-glucose transport

3T3-L1 cells were stimulated with 100 nM-insulin at 27 °C and then cell-surface GLUT1 (●), cell-surface GLUT4 (▲) and 2-deoxy-D-glucose transport activity (□) were determined at 18 °C. The immunoprecipitated transporters were subjected to electrophoresis and the total radioactivity associated with each transporter peak was determined. The levels of transporter labelling and transport activity at the indicated times were then calculated as a fraction of the maximum determined from the samples which were stimulated with insulin for 30 min. Two additional experiments showed the same result, and the average half-time for stimulation was calculated as described in the Materials and methods section.

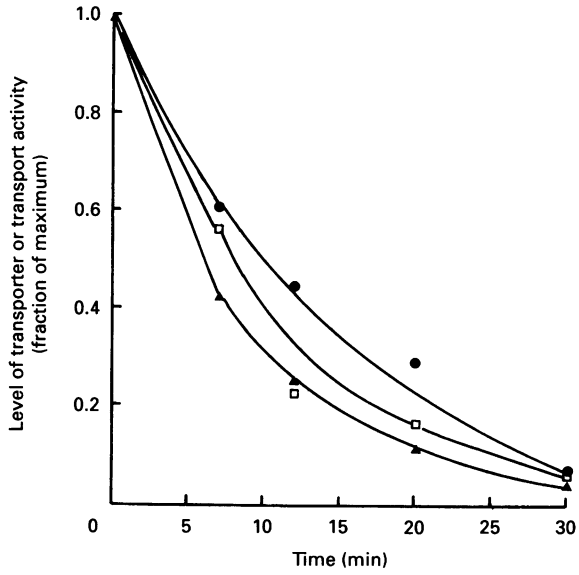

Fig. 3. Time course for the reversal of insulin stimulation

3T3-L1 cells in 35 mm-diameter dishes were stimulated with 100 nM-insulin for 30 min at 37 °C and then insulin was removed by washing twice with MES buffer and by maintaining the cells in this buffer for the indicated times. The cells were washed in KRH buffer at 18 °C and then photolabelled with 100 μCi of ATB-BMPA. After labelling, the levels of the GLUT1 (●) and GLUT4 (□) transporters were determined by immunoprecipitation and electrophoresis. Some cells were used for estimation of 2-deoxy-D-glucose transport activity (▲) as described in the Materials and methods section. The results shown are the means for two photolabelling experiments and for three experiments in the case of transport-activity determination.

transport and the amounts of photolabelled transporters. The uptake was measured by incubating the 35 mm dishes with 50 μM-2-deoxy-D-glucose in 1 ml of KRH buffer for 1–5 min at 18 °C. We have found that no further insulin stimulation occurs once insulin is removed and the dishes are washed at 18 °C. The rates of stimulation were determined from semi-log plots of $-\ln(1-f)$ against time, where f is the fraction of the maximum stimulation. Normally the slope of such a plot gives the activation rate constant, but because a lag phase occurred before the transport activation, the $t_{\frac{1}{2}}$ values were calculated according to the equation:

$$t_{\frac{1}{2}} = (\ln 2 - a)/b$$

where a is the intercept on the $-\ln(1-f)$ axis and b is the slope.

Reversal of insulin stimulation

Cell monolayers were stimulated with 100 nM-insulin at 37 °C for 30 min. The insulin stimulation was then reversed by washing cell monolayers twice with a MES buffer (136 mM-NaCl/4.7 mM-KCl/1.25 mM $MgSO_4$/1.25 mM-$CaCl_2$/10 mM-MES, pH 6.0) and then maintaining them in this buffer at 37 °C. At the times indicated in the Figure legends the dishes were washed twice in KRH at 18 °C and then either used for 2-deoxy-D-glucose-uptake determinations (as described above) or in cell-surface photolabelling experiments.

ATB-BMPA photolabelling

Plates (35 mm diameter) of the 3T3-L1 cells were washed in KRH buffer and were irradiated at 18 °C for 1 min in the presence of 100 μCi of ATB-[2-^{3}H]BMPA as described in [13,14]. The irradiated cells were washed four times in KRH buffer and solubilized in 1.5 ml of detergent buffer containing 2% $C_{12}E_9$, 5 mM-sodium phosphate and 5 mM-EDTA, pH 7.2, and with the proteinase inhibitors antipain, aprotinin, pepstatin and leupeptin, each at 1 μg/ml. After centrifugation at 20000 $g_{max.}$ for 20 min, the supernatant was carefully separated from unsolubilized pellet and the insoluble fat-cake and then subjected to immunoprecipitation to determine the amounts of photolabelled GLUT1 and GLUT4 transporter.

Cell fractionation

For each condition, two 35 mm dishes of fully-insulin-stimulated cells were photolabelled with 100 μCi each of ATB-[2-^{3}H]BMPA. The labelling was carried out with the lids on the 35 mm dishes so that radiation damage to the cells was minimized. After four washes of the dishes in KRH to remove excess label, the cells were then incubated at 37 °C for a further 60 min with insulin or with insulin and 20 μM-PAO. The cells from each dish were scraped into 0.5 ml for each dish of TES buffer (255 mM-sucrose/10 mM-Tris/HCl/0.5 mM-EDTA, pH 7.2). The pooled material from the two dishes were then homogenized at 900 rev./min in a 2 ml tight-fitting Teflon homogenizer (clearance $\approx$ 0.15 mm) with ten complete strokes. The homogenate was then centrifuged at 20000 $g_{max.}$ for 20 min. The pellet, containing the crude plasma-membrane fraction, was dissolved in 0.5 ml of 6%-$C_{12}E_9$ detergent buffer and diluted with 1 ml of 5 mM-sodium phosphate buffer. The 20000 $g_{max.}$ supernatant from above, containing the crude light-microsome fraction, was mixed with 0.5 ml of 6%-$C_{12}E_9$ detergent buffer. Both detergent-solubilized samples were kept at 0–4 °C for 30 min and then re-centrifuged at 20000 $g_{max.}$ for 20 min. The supernatants were then subjected to immunoprecipitation with anti-GLUT1 and anti-GLUT4 antibodies. In some experiments cells were

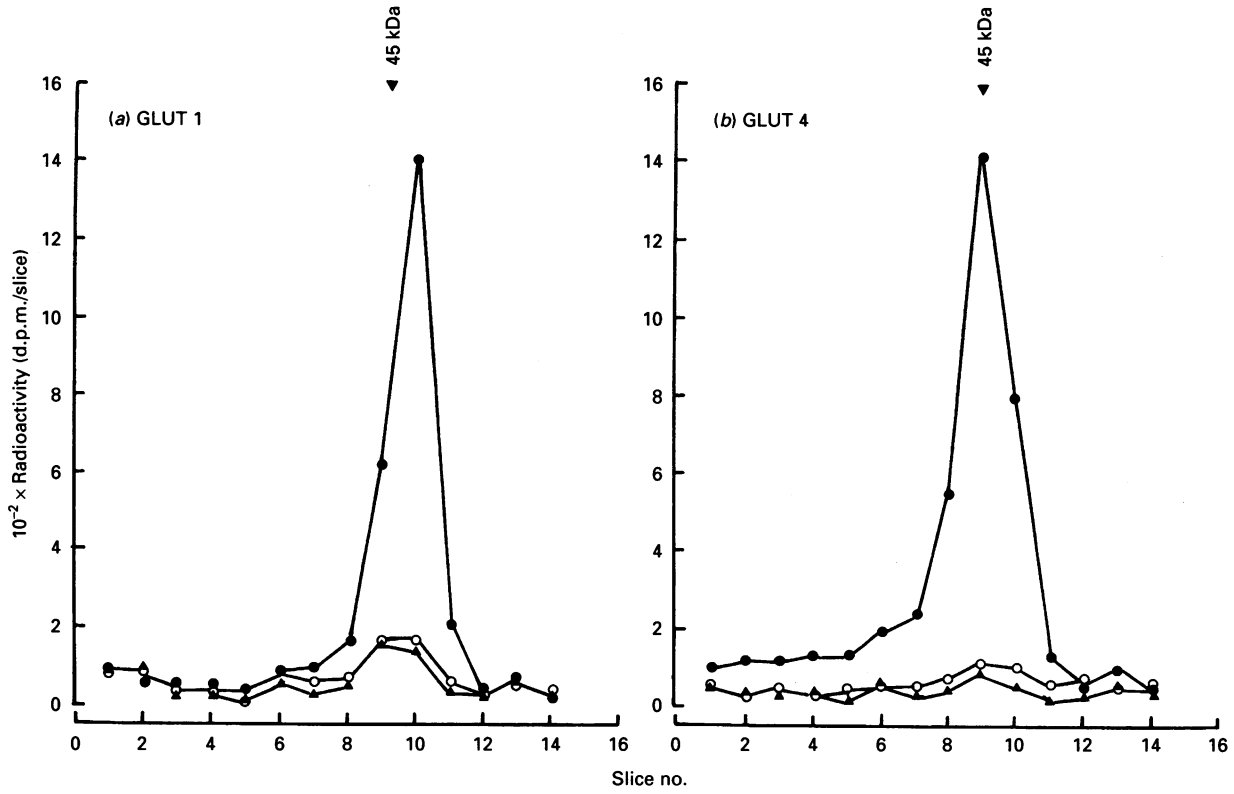

Fig. 4. Comparison of the effects of PAO and insulin on the cell-surface photolabelling of GLUT1 and GLUT4

3T3-L1 cells in 35 mm-diameter dishes were maintained either with no additions (○), with 100 nM-insulin (●) or with 10 μM-PAO (▲) for 30 min at 37 °C. The cells were then labelled with 150 μCi of ATB-BMPA. After labelling, GLUT1 (*a*) and GLUT4 (*b*) were immunoprecipitated with anti-(*C*-terminal peptide) antibodies. The protein was then subjected to electrophoresis and the radioactivity was estimated by cutting out and counting gel slices.

homogenized in a KCl buffer (150 mM-KCl/2 mM-$MgCl_2$/20 mM-Hepes, pH 7.4) and a 16000 $g_{max.}$ plasma-membrane and supernatant fraction were isolated as described in [22]. The results obtained were the same as those obtained using the Tes buffer.

Immunoprecipitation and electrophoresis

The detergent-solubilized samples, either from solubilized cells or from crude plasma-membrane or crude light-microsome fractions prepared as described above, were subjected to sequential immunoprecipitation with 30 μl of Protein A–Sepharose coupled with 100 μl of anti-GLUT1 or 50 μl of anti-GLUT4 antiserum. These antisera were raised against *C*-terminal peptides as described in [12,16]. After incubation for 2 h at 0–4 °C and washing of the immunoprecipitates three times with 1.0%- and once in 0.1%-$C_{12}E_9$ detergent buffer, the labelled glucose transporters were released from the antibody complexes with 10% (w/v) SDS/6 M-urea/10% (v/v)-mercaptoethanol electrophoresis sample buffer and subjected to electrophoresis on 10%-(w/v)-acrylamide gels. The radioactivity on the gel was extracted from gel slices and estimated by liquid-scintillation counting. The radioactivity in transporter peaks was corrected for background radioactivity which was based on the average radioactivity of the slices on either side of the peak [13].

RESULTS

Insulin stimulation of glucose transport and the availability of cell-surface transporters

The insulin stimulation of the cell-surface availability of glucose transporters was found to be very rapid at 37 °C ($t_{\frac{1}{2}} \approx 2$ min). This was considered to be too rapid for comparison with the stimulation of 2-deoxy-D-glucose transport activity at this temperature ($t_{\frac{1}{2}} \approx 3$ min). Instead, we followed the approach of Gibbs *et al.* [23], who studied the time course for insulin stimulation at 27 °C. At 27 °C the increase in cell-surface GLUT1 and GLUT4 occurred in parallel. Fig. 1 shows that a large increase above basal levels (≈ 45% of maximum) in cell-surface labelling of these two isoforms occurred with only 5 min of stimulation by 100 nM-insulin. The cell-surface levels of both isoforms then rose progressively and reached a maximum level of stimulation in ≈ 12 min. The cell-surface GLUT1 increases with $t_{\frac{1}{2}}$ of 5.7 ± 1.5 min (two experiments), which was similar to the rate of increase in cell-surface GLUT4 ($t_{\frac{1}{2}}$ 5.4 ± 0.7 min; two experiments). In Fig. 2 the time course for this stimulation of the cell-surface availability of transporters is shown to slightly precede the time course for insulin stimulation of 2-deoxy-D-glucose transport activity ($t_{\frac{1}{2}}$ 8.6 ± 1.5 min; three experiments).

Reversal of insulin action on glucose transport and loss of cell-surface glucose transporters

We examined the time course for the loss of cell-surface glucose transporters and compared this with the decrease in 2-deoxy-D-glucose transport activity (Fig. 3). The cells were fully stimulated by treatment with 100 nM-insulin for 30 min at 37 °C, and then the insulin was removed by washing and incubation with MES buffer at pH 6.0 for the indicated times. This procedure was developed by Gibbs *et al.* [24], who showed that this low-pH buffer resulted in insulin dissociation and a consequent decrease in transport activity. The rate of loss of 2-deoxy-D-glucose-

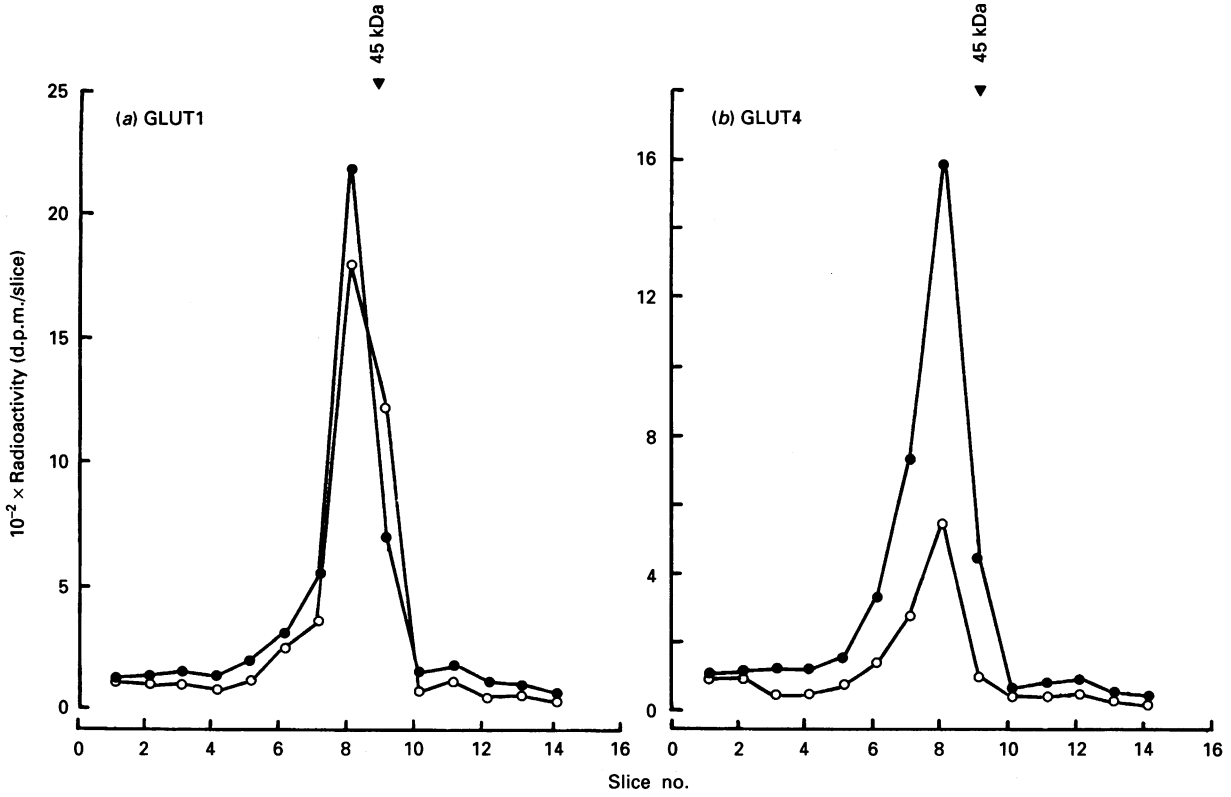

Fig. 5. Comparison of the effect of PAO on the availability of GLUT1 and GLUT4 in insulin-treated 3T3-L1 cells

3T3-L1 cells in 35 mm-diameter dishes were fully stimulated by treatment with 100 nM-insulin for 30 min at 37 °C. The cells were then maintained at 37 °C for a further 60 min in the presence (○) and the absence (●) of 20 μM-PAO. The cells were then photolabelled with 100 μCi of ATB-BMPA and then the GLUT1 (*a*) and GLUT4 (*b*) were immunoprecipitated with anti-(*C*-terminal peptide) antibodies. The protein was then subjected to electrophoresis and the radioactivity was estimated by cutting out and counting gel slices.

transport activity that we have observed (Fig. 3) agrees with their results. The calculated half-time for the decrease in 2-deoxy-D-glucose-transport activity was 6.5 ± 0.4 min (three experiments). This correlates well with loss of cell-surface GLUT1 ($t_{\frac{1}{2}}$ 9.2 ± 0.8 min; two experiments) and GLUT4 ($t_{\frac{1}{2}}$ 6.8 ± 1.7 min; two experiments). The decrease in cell-surface GLUT4 was slightly more extensive than for GLUT1, possibly reflecting the greater tendency of GLUT1 to be re-exocytosed in the absence of insulin.

Effect of PAO on glucose-transporter trafficking

A number of effects of PAO on the glucose-transport activity in basal 3T3-L1 cells have been noted. Frost & Lane [4] reported that PAO decreased the insulin stimulation of transport activity, but had no effect on the transport activity of basal cells. However, Gould *et al.* [25] reported that, at low concentrations of PAO (< 20 μM), a stimulation of the basal rate of transport occurred. Surprisingly, at concentrations of PAO where a transport stimulation was noted they found a decrease in cell-surface transporters (as detected by cell-surface labelling with NaB^3H_4 of galactose oxidase-treated cells) and a rise in plasma-membrane-associated GLUT1 as detected by Western blotting. We therefore examined the effect of 5–10 μM-PAO on basal transport activity and found a ≈ 1.5-fold increase in 2-deoxy-D-glucose transport. As shown in Fig. 4, 10 μM-PAO gave no detectable rise in either the concentration of GLUT1 or GLUT4. It is clear from this Figure, however, that any effect of PAO on basal levels of transporters is very small in comparison with the stimulatory effect of insulin.

Treatment of fully-insulin-stimulated 3T3-L1 cells with 20 μM-PAO for 60 min markedly decreased the cell-surface level of the GLUT4 isoform (Fig. 5*b*). However, the level of the GLUT1 isoform was only slightly decreased (Fig. 5*a*). The time course for the PAO-induced loss of cell-surface transporters is compared with the decrease in 2-deoxy-D-glucose-transport activity in Fig. 6. The loss of transport activity and GLUT4 occurred with half-times of 9.8 ± 1.6 and 7.8 ± 0.8 min respectively (two experiments), which were similar to the half-time for the decrease in transport activity that occurred when insulin was removed by the low-pH-washing procedure (Fig. 3). In contrast with this, the level of cell-surface GLUT1 remained high during the PAO treatment. During the PAO treatment the half-time for the decrease in cell-surface GLUT1 was greater than 60 min and was much longer than the half-time for the loss of GLUT1 that occurred when insulin was removed by the low-pH-washing procedure and which was only ≈ 9 min. To confirm that the decrease in GLUT4 was due to a redistribution of these transporters within the cell and not to an irreversible inhibition of transport [21], we have measured the binding of ATB-BMPA to the transporters in cells which had been permeabilized by treatment with 0.025% digitonin. Figs. 7(*a*) and 7(*b*) show that the total concentration of available GLUT4 was only slightly decreased (by ≈ 30%). This slight decrease may have occurred because the efficiency of labelling transporters present inside the cell may have been slightly less than for surface labelling. We also confirmed that neither GLUT1 nor GLUT4 was lost from the cell by Western-blotting crude membrane fractions obtained from cells treated with insulin and then 20 μM-PAO (J. Yang, A. E. Clark, R. Harrison, I. J. Kozka & G. D. Holman, unpublished work). The more striking effect of PAO treatment was to decrease just the GLUT4 at the cell surface. In the cells treated

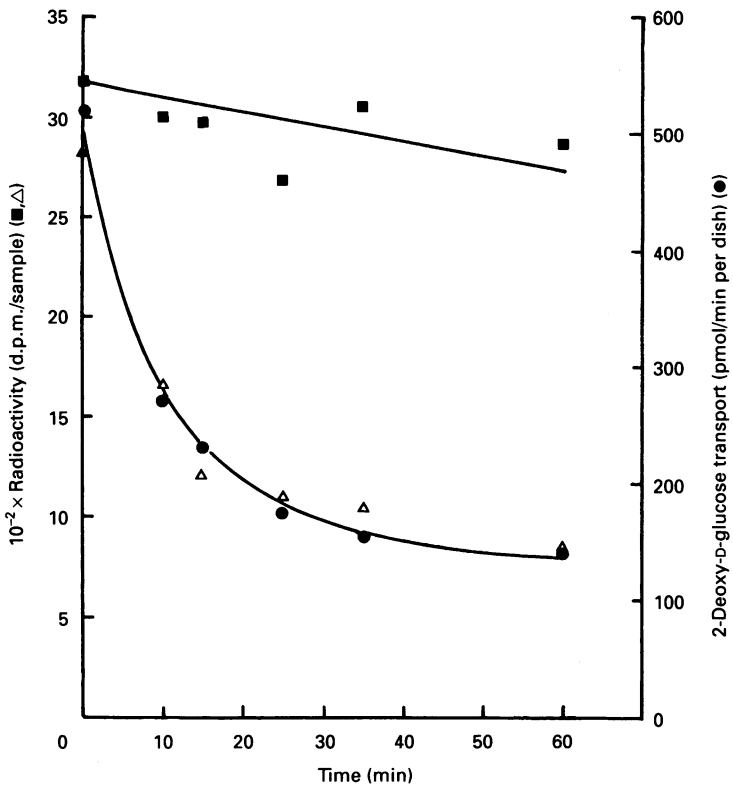

Fig. 6. Time course for the PAO-induced loss of cell-surface transporters and transport activity

PAO treatment of fully-insulin-stimulated 3T3-L1 cells was carried out as described in Fig. 5. The total radioactivity associated with photolabelled GLUT1 (■) and GLUT4 (△) was compared with the loss of 2-deoxy-D-glucose transport activity (●). The results are the means for two photolabelling experiments and, in the case of the transport-activity measurements, three experiments.

with insulin alone, ≈ 60% of the available transporter was at the cell surface. In the cells treated with PAO, ≈ 20% of the available transporters were at the surface (two experiments). In addition we have found, by measuring ATB-BMPA photolabelling in permeabilized cells, that the total available GLUT1 concentration was only slightly decreased after PAO treatment. Approx. 60% of the available GLUT1 transporters were at the surface both in the presence and in the absence of PAO.

A possible interpretation of this differential effect on GLUT1 and GLUT4 cell-surface availability is that, in the presence of PAO, both transporters are removed from the cell surface at the same rate, but their recycling is differentially perturbed. Thus both GLUT1 and GLUT4 could be endocytosed even in the presence of PAO, but inhibition of GLUT4, but not GLUT1, re-emergence and re-exposure to the photolabel at the cell surface could then have resulted in the observed decrease in its detected surface concentration.

To examine the possibility that both transporters were internalized in the presence of PAO, we labelled the cell-surface transporters in fully-insulin-stimulated cells with ATB-BMPA and then treated them with PAO and insulin for a further 60 min. We then homogenized the cells and separated a crude plasma membrane fraction from the light-microsome fraction. After 60 min in the continuous presence of insulin, the light-microsome level of photolabelled GLUT1 and GLUT4 was increased above that observed at zero time. This indicates that internalization of transporters occurred even in the continuous presence of insulin (Table 1). The percentages of photolabelled transporter found in the light-microsome fraction are consistent with the results of Calderhead *et al.* [13], who determined by Western blotting that ≈ 50% of the total recovered GLUT1 and GLUT4 were present in the light-microsome pool of basal cells and that this was decreased to ≈ 25% in insulin-treated cells. The light-microsome association of GLUT1 was slightly less than that which occurred for the GLUT4 isoform. This probably reflects the greater tendency of GLUT1 to be re-exocytosed under these conditions.

Table 1 also shows that the internalization of GLUT1 and GLUT4 also occurred in cells treated with PAO. However, in PAO-treated cells the additional GLUT4, above that found with insulin alone, which was lost from the cell surface was not recovered in the light microsomes, but partitioned with the plasma-membrane fraction during homogenization. The total recovered photolabel was unaltered by the PAO treatment. Western blotting confirmed that neither GLUT4 nor GLUT1 internalization to the light-microsome fraction was increased by the PAO treatment compared with insulin alone (J. Yang, A. E. Clark, R. Harrison, I. Kozka & G. D. Holman, unpublished work). Fig. 8 shows that ≈ 35% of GLUT4 transporters that were photolabelled in the insulin-stimulated state were transferred to the light-microsome fraction after 60 min. This proportion was not altered by the PAO treatment.

DISCUSSION

Cushman & Wardzala [26] and Suzuki & Kono [27] proposed in 1980 that glucose transporters, like receptors and other membrane proteins, were translocated from light-microsome vesicles to the plasma membrane of adipose cells in response to insulin. Recently two different, but complementary, approaches to investigating this membrane-trafficking phenomenon have provided very strong support for their hypothesis. Slot *et al.* [10] have shown that, in brown adipose tissue, immunodetectable GLUT4 was localized to a cytosolic-vesicle pool with very low transporter levels in the plasma membrane of basal cells. Upon insulin stimulation, the GLUT4 located in the plasma membrane was increased 40-fold. The second approach that has provided strong supportive evidence for the translocation hypothesis has involved use of the bis-D-mannose photolabel ATB-BMPA in trafficking kinetic experiments [16,17]. Satoh *et al.* [17] have shown, in trafficking experiments using the ATB-BMPA photolabel, that transporters are recycled between plasma-membrane and light-microsome pools. Fully-insulin-stimulated cells were photolabelled with ATB-BMPA and then insulin was removed by a collagenase treatment. Labelled transporters were shown to be transferred to the light-microsome membranes. Equilibration of cell-surface-labelled transporters with the light-microsome pool also occurred when cells were maintained, after labelling, in the continuous presence of insulin. In the presence of insulin, the level of transporters in the light microsomes reached a lower steady-state level than that which was reached after insulin removal. These experiments have thus provided direct evidence that transporters move between the plasma-membrane and light-microsome pools. Furthermore, re-stimulation experiments have directly shown that internalized transporters are re-exocytosed to the plasma membrane. Insulin stimulation was shown to be due to an enhancement of exocytosis rather than due to an inhibition of the endocytosis of transporters [16,17].

Use of the ATB-BMPA photolabelling reagent has also allowed an assessment of the magnitude of translocation to be made in intact adipose cells. Insulin has been shown to increase cell-surface GLUT4 15–20-fold, whereas GLUT1 is only increased ≈ 5-fold. The use of this 'in situ' labelling procedure

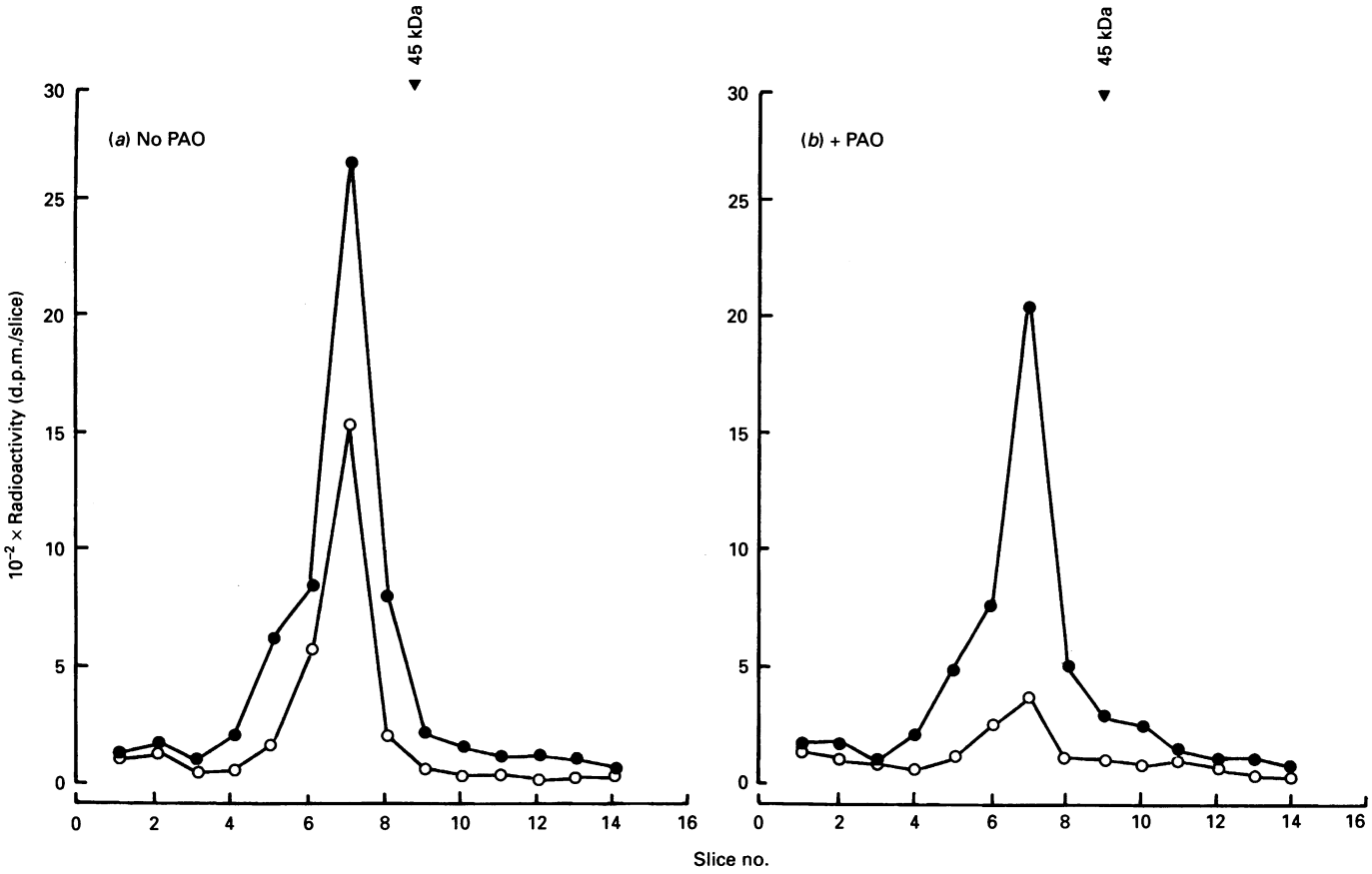

Fig. 7. Experiment to determine whether the PAO-induced decrease in cell-surface GLUT4 is due to a redistribution to the cell interior

3T3-L1 cells in 35 mm-diameter dishes were fully stimulated by treatment with 100 nM-insulin for 30 min at 37 °C. The cells were then maintained at this temperature for a further 50 min in the absence (*a*) or in the presence (*b*) of 20 μM-PAO. The cells were then either photolabelled directly with 100 μCi of ATB-BMPA (○) or were permeabilized in the presence of 100 μCi of ATB-BMPA by treatment with 0.025% digitonin for 8 min at 18 °C and then irradiated (●). GLUT4 was then immunoprecipitated with anti-(*C*-terminal peptide) antibody. The protein was then subjected to electrophoresis and the radioactivity was estimated by cutting out and counting gel slices.

Table 1. Internalization of glucose transporters in 3T3-L1 cells

Fully-insulin-stimulated 3T3-L1 cells were photolabelled with ATB-BMPA and then either homogenized immediately (0 min) or maintained at 37 °C with insulin (60 min) or insulin + 20 μM-PAO (60 min) before homogenization. Crude plasma-membrane and light-microsome fractions were prepared and solubilized in $C_{12}E_9$ detergent buffer. GLUT1 and GLUT4 were then immunoprecipitated and subjected to electrophoresis. The radioactivity in the transporter peaks was estimated, and the results are expressed as a percentage of radioactivity recovered in the light-microsome fraction compared with the sum of the radioactivity recovered in the plasma-membrane and the light-microsome fractions.

			Transporter...	Percentage of radioactivity in light-microsome fraction			
				GLUT1		GLUT4	
Addition	Time (min)	Expt.		Individual results	Mean ± S.E.M.	Individual results	Mean ± S.E.M.
Insulin	0	1		13.7	11.0 ± 0.5	17.1	14.0 ± 0.8
		2		9.8		10.9	
		3		13.1		14.4	
		4		11.0		13.2	
Insulin	60	1		38.1	27.9 ± 4.5	39.0	35.6 ± 5.3
		2		29.8		31.1	
		3		27.5		42.0	
		4		16.2		18.2	
Insulin + PAO	60	1		21.9	23.2 ± 1.8	33.3	32.0 ± 1.2
		2		29.3		35.4	
		3		20.6		29.8	
		4		20.9		29.5	

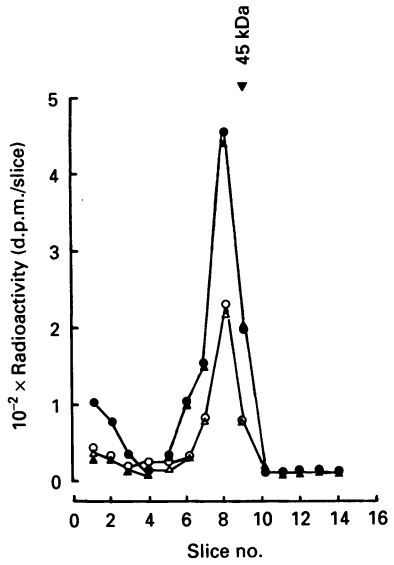

Fig. 8. Redistribution of photolabelled GLUT4 to subcellular membrane fractions of 3T3-L1 cells

3T3-L1 cells in 35 mm-diameter dishes were fully insulin-stimulated by treatment with 100 nM-insulin for 30 min at 37 °C. They were then photolabelled with 100 μCi of ATB-BMPA and then maintained with (circles) and without (triangles) 20 μM-PAO for a further 60 min. Crude plasma membrane (closed symbols) and light-microsome membranes (open symbols) were prepared from two dishes for each condition. These fractions were solubilized in $C_{12}E_9$ detergent buffer. GLUT4 was then immunoprecipitated with anti-(*C*-terminal peptide) antibody and subjected to electrophoresis; radioactivity was estimated by cutting out and counting gel slices.

has circumvented the need to subject the cells to subcellular fractionation, a procedure that is considered to underestimate the magnitude of the translocation effect, owing to the cross-contamination of plasma-membrane and light-microsome fractions. The photolabel has been used to compare the time courses for appearance and loss of transporters in rat adipocytes with the time course for a change in glucose-transport activity [16]. This study has shown that, on insulin addition, the arrival of GLUT1 and GLUT4 transporters at the cell surface slightly precedes the increase in transport activity, but that the loss of these transporters from the cell surface on insulin removal correlated well with transport. We have confirmed here that these effects also occur in 3T3-L1 cells. The loss of transporters from the cell surface occurs with half-times that are very similar to that for the internalization of other membrane proteins and receptors [28–30], suggesting that this internalization step occurs by fluid-phase endocytosis [31]. The high activation energy for reversal of transport stimulation and the almost complete cessation of glucose-transporter internalization at 18 °C [16] are also consistent with this possibility.

Now that the essential predictions of the translocation hypothesis have been confirmed, questions concerning the mechanistic details of the trafficking pathways can be addressed. The transporter-immunolocalization study of Slot *et al.* [10] has provided evidence that the GLUT4-trafficking pathway involves clathrin-coated vesicles. The association of this transporter in clathrin vesicles was increased by insulin treatment of the cells. Other trafficking proteins that may be involved include small guanine-nucleotide-binding proteins (G-proteins). It is now known that these are intricately involved in many vesicle-trafficking events [32]. G-protein involvement in glucose-transporter trafficking has been clearly implicated by the discovery by Baldini *et al.* [33] that the non-hydrolysable GTP analogue guanosine 5′-[γ-thio]triphosphate stimulates translocation of GLUT4 from the light-microsome pool to the plasma membrane of rat adipocytes. Other proteins that are likely to be involved in the trafficking include adaptins. These have been implicated in the enhancement of receptor association with clathrin [34], and their involvement in the trafficking of other membrane proteins is likely. A reasonable interpretation of some features of the trafficking kinetics of glucose transporters in 3T3-L1 cells that we have observed here is that a dissociation from trafficking proteins occurs before the transporters can fully participate in transport.

The observed lag between transporter appearance at the cell surface and the full stimulation of transport [16,17,23,35; the present study] may occur because transporters are still associated with trafficking proteins at early time points after the addition of insulin to the cells. Thus translocation may precede transport activation, owing to the requirement for a dissociation from these proteins. The effects of PAO on the trafficking of membrane proteins have generally been reported to be due to inhibition of fluid-phase endocytosis by this reagent [18–20]. However, the resolution of an effect on endocytosis or exocytosis is difficult. It is possible, for example, that receptors are internalized and reappear at the surface, but then fail to dissociate from the trafficking proteins because this process is blocked by PAO. Receptor abundance at the surface would remain high, owing to a single cycle of internalization and re-exocytosis, and the whole cycling process would halt. The net result would be that endocytosis would appear to be inhibited. There is evidence against the possibility that a simple block of endocytosis occurs. PAO has been shown [25] to decrease surface transferrin receptors, as measured by transferrin binding to whole cells, but to increase the level of plasma-membrane-associated receptors measured in an isolated membrane fraction. It was proposed [25] that this effect may have occurred because recycled vesicles containing the receptors could not fuse with the plasma membrane, but remained associated with it, during subcellular fractionation. An alternative explanation is that vesicles do fuse with the plasma membrane, but that the receptors are associated with trafficking proteins and therefore not fully available for ligand binding. These two proposals are not mutually exclusive, and a combination of both these effects may occur.

We have shown here that PAO has distinct effects on the trafficking of GLUT1 and GLUT4. The abundance of GLUT1 at the cell surface remained high when fully-insulin-stimulated cells were treated with PAO. However, the surface level of GLUT4 decreased with a time course for redistribution similar to that which occurred after insulin removal by washing in a low-pH buffer. This suggests they were internalized by the fluid-phase-endocytosis route. In contrast with the above results (which were obtained from surface labelling, which was carried out after PAO treatment) we have shown that, when photolabelling was carried out in fully-insulin-stimulated cells and cells were subsequently treated with PAO, then both GLUT1 and GLUT4 were internalized to the light-microsome pool.

An additional observation concerning the transport activity of GLUT1 in PAO-treated cells also suggests that, although the GLUT1 surface-level is constant, recycling of this isoform may have occurred. We have calculated that the GLUT1 contribution to transport is about one-third of that of GLUT4 in fully-insulin-stimulated cells [R. W. Palfreyman, A. E. Clark, R. M. Denton, G. D. Holman & I. J. Kozka, unpublished work]. Yet when the insulin stimulation was reversed with PAO, the fall in transport paralleled the decrease in GLUT4 and the remaining cell-surface GLUT1 appeared to make only a minimal contribution to transport. Thus GLUT1 may be internalized and recycled to the surface, but remain associated with trafficking proteins, which

suppress its transport capability. This association with trafficking proteins may be similar to that which occurs in the lag phase of insulin activation [16,17,23,35; the present study] and may account for the low contribution of cell-surface GLUT1 to the transport activity in basal cells [13,37]. Harrison *et al.* [38] have proposed that GLUT1 activity is suppressed in transfected 3T3-L1 cells. We have shown [14] that it is difficult to remove GLUT1 from the cell surface after a chronic insulin treatment, but that this cell-surface GLUT1 appears to have low transport capability. The location of transporters of suppressed activity may be in surface-attached vesicles. These may be early intermediates in the insulin-stimulated trafficking pathway [17]. If some of the surface-attached vesicles are surface-exposed, then glucose transporters in these vesicles would be photolabelled, but would possibly not be able to participate in transport if they were still associated with trafficking proteins.

If the effect of PAO is mainly due to an inhibition of exocytotic vesicle fusion with the plasma membrane and/or inhibition of transporter dissociation from trafficking proteins, then this inhibition could also account for the decrease in cell-surface GLUT4 that is detectable with the ATB-BMPA photolabel. GLUT4 transporter vesicles may have a greater tendency to remain internalized and not fully surface-exposed if the normal mechanisms that commit transporters to the cell surface, such as dissociation from trafficking proteins, are uncoupled. A proportion of the GLUT4 transporters which are removed from surface accessibility to the photolabel in PAO-treated cells appear to be fractionated with the plasma membrane. The percentage of transporters recovered in the light microsomes was similar to that obtained with insulin alone. The effect requires further investigation, but it may be similar to that observed with transferrin receptors, where a PAO-induced failure of recycled vesicles to fuse with the plasma membrane was proposed [25]. A similar effect was observed when insulin-stimulated rat adipocytes were treated with isoproterenol [39].

The greater tendency of GLUT4 to remain internalized also accounts for the low levels of this isoform at the cell surface of basal cells [12,13,40].

In conclusion, we have shown, by measuring trafficking kinetics of glucose-transporter isoforms in 3T3-L1 cells, that there is a lag between transporter appearance and participation in transport. We have provided evidence that the GLUT1 and GLUT4 isoforms are recycled even in the presence of insulin. In addition, we have shown that GLUT1 and GLUT4 exhibit different trafficking properties when processing is perturbed with PAO. The above properties of translocation indicate an involvement of a slow dissociation from trafficking proteins during recycling. This proposal implicates an involvement of unspecified trafficking proteins, and further resolution of the proposed mechanism will depend on the identification and characterization of these proteins.

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