Cell Surface Labeling of Glucose Transporter Isoform GLUT4 by Bis-mannose Photolabel*

CORRELATION WITH STIMULATION OF GLUCOSE TRANSPORT IN RAT ADIPOSE CELLS BY INSULIN AND PHORBOL ESTER

(Received for publication, March 30, 1990)

Geoffrey D. Holman†, Izabela J. Kożka‡, Avril E. Clark‡, Carolyn J. Flower‡, John Saltis§, Alan D. Habberfield||, Ian A. Simpson§, and Samuel W. Cushman§

From the †Department of Biochemistry, The University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom and the §Experimental Diabetes, Metabolism, and Nutrition Section, Molecular, Cellular, and Nutritional Endocrinology Branch, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

A new impermeant photoaffinity label has been used for identifying cell surface glucose transporters in isolated rat adipose cells. This compound is 2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine. We have used this reagent in combination with immunoprecipitation by specific antibodies against the GLUT4 and GLUT1 glucose transporter isoforms to estimate the relative abundance of these two transporters on the surface of the intact adipocyte cell following stimulation by insulin and phorbol 12-myristate 13-acetate (PMA). In the basal state, GLUT4 and GLUT1 are both present at the cell surface but GLUT4 is more abundant than GLUT1. In response to insulin, GLUT4 increases 15-20-fold and GLUT1 increases ≈5-fold while 3-O-methyl-D-glucose transport is stimulated 20-30-fold. By contrast, PMA only induces a ≈4-fold increase in GLUT4 while GLUT1 increases ≈5-fold to the same level as seen with insulin. In addition, PMA stimulates 3-O-methyl-D-glucose transport ≈5-fold to only 13% of the insulin-stimulated state. Thus GLUT4 is the major glucose transporter isoform under all conditions, and it is selectively and markedly enriched in response to insulin but not PMA which increases GLUT1 and GLUT4 equally. Furthermore, stimulation of glucose transport activity correlates closely with the appearance of GLUT4 on the cell surface in response to both insulin and PMA but does not correlate with the sum of GLUT1 and GLUT4 appearance. These results suggest that GLUT4 may be inherently more active than GLUT1 due to a higher TK (turnover/Km).

Insulin is known to produce a very large stimulation of glucose transport activity in rat adipose cells. The stimulation of 3-O-methyl-D-glucose transport is usually 20-fold or greater (1-3). A large part of this stimulation is thought to be due to a translocation of glucose transporters from an intracellular pool to the plasma membrane (4-6). However, activation of transporters at the cell surface could play a role in increasing the magnitude of the stimulation (7, 8), and, indeed, a reduction in glucose transporter intrinsic activity has been reported to occur following isoproterenol treatment of insulin-stimulated adipose cells (9). The discrepancies observed between cytochalasin B-assayable glucose transporters in isolated plasma membrane fractions and glucose transport activity following stimulation by phorbol 12-myristate 13 acetate (PMA)1 (10, 11) could be due to low intrinsic activities of transporters in basal and PMA but not insulin-treated adipose cells. These comparisons are difficult, however, because the plasma membranes could be contaminated by low density microsomal membranes which contain high levels of glucose transporters (4-8). Such contamination would raise the apparent basal level of cytochalasin B-assayable glucose transporters and consequently give an underestimate of the insulin effect. In addition, adipose cells are known to contain two glucose transporter isoforms (12-14) which may contribute equally to the assessed cytochalasin B binding to plasma membranes but not to the glucose transport activity which is measured in the intact cell. Thus, methods for surface labeling of glucose transporters in intact cells should provide a more direct comparison with the observed transport rates. Surface labeling of 3T3-L1 adipocytes with [3H]borohydride has been shown to provide information on the translocation of the erythrocyte/brain glucose transporter isoform GLUT1 (15). An alternative is to use an impermeant bis-hexose photoaffinity label (16-19).

The new compound studied here is 2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) (20). The use of bis-mannose as an exofacial label is advantageous because these compounds are impermeable and photolabeling can be confined to the discrete plasma membrane pool of glucose transporters. In addition, ATB-BMPA is very stable in the dark and is readily soluble in physiological buffers. Finally, this approach to estimating the levels of glucose transporters on the cell surface is independent of the homogenization protocol. The problem of contamination of the plasma membranes, referred to above, is circumvented by this approach because microsomal glucose transporters will not be exposed to or bind the exofacial ligand during the photolabeling procedure. Thus, the cell physiology of insulin’s stimulation of glucose transport can be studied directly in the intact adipocyte cell.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† Present address: Baker Medical Research Institute, Prahran, Victoria, Australia.
|| Amgen Incorporated, Thousand Oaks, CA 91320.

† The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; ATB-BMPA, 2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; C12Es, nonaethylene glycol dodecyl ether.
The separate resolution of the cell surface exposure of glucose transporter isoforms requires immunoprecipitation of labeled glucose transporters using antibodies against the GLUT1 isoform or the recently discovered (21–28) GLUT4 isoform. James et al. (13) have shown that this latter isoform is only present in tissues that respond to insulin such as adipose tissue, heart, and skeletal muscle. We have therefore now used the ATB-BMPA photoprobe in combination with anti-GLUT1 and anti-GLUT4 antibodies to compare the proportions of the GLUT1 and GLUT4 glucose transporter isoforms on the cell surface with the increased glucose transport activity that occurs in response to either insulin or PMA treatment of intact adipose cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fraction V bovine serum albumin was from Sigma and was extensively dialyzed and filtered. Phloretin was from K and K Laboratories. Collagenase was from Worthington. Insulin was from Novo.

**Preparation of ATB-BMPA**—ATB-[2-3H]BMPA (specific activity \( \approx 10 \) Ci/mmol) was prepared from [2-3H]BMPA and 1-azi-2,2,2-trifluoroethyl-benzoic acid as described by Clark and Holman (20).

**Photolabeling of Rat Adipose Cells**—Rat adipose cells were isolated as described previously (2, 7) and after washing in a 1% albumin buffer (either albumin/HEPES buffer (2) or albumin/Krebs-Ringer bicarbonate buffer (23)) buffer containing adenosine (7) were resuspended at a density of 4 x 10^6 cells/ml in a Rayonet RPR 100 photochemical reactor (RPR 3000 lamp), with manual resuspension of the cells during the irradiation intervals. Following irradiation the cells were washed into polystyrene centrifuge tubes with 25 ml of 1% albumin-free HEPES buffer. After irradiation in 35-mm polycarbonate dishes. The samples were immediately irradiated for 3 X 20 s in a Rayonet RPR 100 photoreactor (RPR 3000 lamp), with manual resuspension of the cells during the irradiation intervals. Following irradiation the cells were washed into polystyrene centrifuge tubes with 25 ml of 1% albumin buffer at 18 °C. The cells were washed three times in this buffer and then resuspended and washed in homogenization buffer containing 10 mM Tris HCl, 0.5 mM EDTA, 255 mM sucrose, pH 7.2, at 18 °C. The cells were homogenized in 15 ml of this buffer and the plasma membranes were isolated as described previously using differential ultracentrifugation and either a sucrose (7) or a Ficoll (19) density step gradient.

In some experiments 1.0 ml of the 40% cell suspension (in 1% albumin buffer) was mixed with 333 µCi of ATB-[2-3H]BMPA in 500 µl of albumin-free HEPES buffer. After irradiation in 35-mm polystyrene dishes, these samples were washed as described above but using 10-ml washes. Following homogenization in 2.5 ml of Tris/EDTA/sucrose buffer, a combined plasma membrane/mitochondrial fraction was obtained by centrifugation at 16,000 X g for 20 min. This pellet was washed in Tris/PDTA/sucrose buffer a further three times and then solubilized in C6E6 (Boehringer Mannheim) for immunoprecipitation or in electrophoresis sample buffer for electrophoresis.

**Glucose Transport Measurements**—The rate constant for uptake of 50 µM 3-O-methyl-d-glucose in rat adipose cells was determined as described previously (2, 7).

**Electrophoresis**—The membrane samples were solubilized at room temperature and subjected to electrophoresis using 16 cm x 3-mm gels with 2.5-cm-wide sample wells in the stacking gel. The gels were run (generally overnight) in the Laemmli (29) discontinuous buffer system. The gels were stained with Coomassie Blue and then destained and sliced. The slices (in scintillation vials) were dried at 80 °C for 2-3 h and were then dissolved in 0.5 ml of alkaline hydrogen peroxide at 80 °C for a further 2 h. Scintillant was added and the radioactivity counted. The positions of the photolabeled peaks were compared with the positions of molecular weight markers (Sigma) in adjacent lanes. The variations of labeled peak positions relative to the albumin marker are shown in the figures, whereas the variations in the total migration distance allowed. The levels of radioactivity associated with each peak were obtained by summing the radioactivity in all the slices under the peak and subtracting a background radioactivity based on the average radioactivity of the slices on either side of the peak (15).

**Preparation of Antibodies**—Rabbit antisera against the GLUT1 and GLUT4 glucose transporter isoforms were prepared using synthetic C-terminal peptides, EELFHLGADSVQ and STELEYLGPDEND, respectively, with CG and C linking moieties, respectively, as described by Davies et al. (30). The anti-GLUT1 antibody was affinity purified using alkali-stripped red cell membranes (31) while the anti-GLUT4 antibody was affinity purified using a peptide column in a manner similar to that described by Oka et al. (12). Peptide (CSTELEYLGPDEND, 5 mmoles) was coupled to Ristygel-4X (Pierce Chemical Co.) by shaking 2 ml of gel matrix in 0.1 M sodium borate buffer, pH 8.5. The gel was added to a small column and equilibrated with phosphate-buffered saline (154 mM NaCl, 19.5 mM sodium phosphate, pH 7.4). The column was then successively washed in phosphate buffer saline and 2 mM sodium chloride in 5 mM phosphate buffer, pH 7.2. Antibody was eluted with 3.5 mM sodium thiocyanate in 10 mM phosphate, pH 6.6, and immediately dialyzed against phosphate-buffered saline for 18 h. Finally, the sample was concentrated with polyethylene glycol. The yield of antibody was \( \approx 150 \) µg of antibody/ml serum.

**Immunoprecipitation**—Immunoprecipitation was carried out essentially according to the method described by Calderhead and Lienhard (15). Crude plasma membrane pellets (usually 150–300 µg) were solubilized in 600 µl of a solution containing 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 2 mM N-ethylmaleimide in phosphates buffer saline and 2% C6E6. Samples were mixed with 10 µl of affinity-purified antibody and 8 µl of protein A-Sepharose. The samples were then mixed at 0-4 °C either overnight or for 2-3 h. The pellets were then washed twice with 600 µl of the same buffer as above but containing 0.2% C6E6. Finally, antibody conjugate was released from the gel matrix with electrophoresis sample buffer.

**RESULTS**

**Cell Surface Photolabeling by ATB-BMPA**—A primary advantage of the diazirine photolabel over the previously used benzophenone photolabel is the rapidity of its activation (19). Fig. 1a compares the photolabeling achieved with 45 and 90 s of exposure of maximally insulin-stimulated adipose cells to the UV light. Maximum incorporation of label is achieved within 45 s. Cells did not appear to lyse nor was insulin-stimulated glucose transport activity impaired with 90 s of irradiation (data not shown). The photolabeled glucose transporter molecules run as a peak at \( \approx 50 \) kDa in our gel system. The peak is clearly separated from another labeled peak at 75 kDa. The labeling in the 75-kDa region results from nonspecific interactions with a protein of unknown identity. It probably corresponds to one of the major glycoprotein-type membrane proteins (18). The ratio of the photolabeling of this band compared with that of the glucose transporter band is approximately 2:1, as calculated by comparing the areas under the respective peaks. In some experiments the peak of the 75-kDa band was sharp and much higher than the glucose transporter band but the ratio of the areas of the two peaks remained 2:1. The average ratio was 2.2 ± 0.1 (mean ± S.E. n = 8). The efficiency of photolabeling observed in these experiments in which GLUT4 is the major isoform (see below) is similar to that observed when labeling GLUT1 in intact human erythrocyte (20) and both systems incorporate \( \approx 2500 \) dpm/pmol of cytochalasin B binding sites/mCi of ATB-BMPA. This suggests that GLUT1 and GLUT4 are labeled with similar efficiency although this comparison is difficult because of uncertainties concerning the number of cytochalasin B binding sites and the very different cell morphology found in adipocytes and erythrocytes.

Fig. 1b further demonstrates the specificity of ATB-BMPA labeling of the glucose transporter in insulin-stimulated rat adipose cells. Cytochalasin B (50 µM) gives an \( \approx 75\% \) inhibition of the incorporation of label into the glucose transport region of the gel. The photolabeling of cells in the insulin-stimulated state in the presence of cytochalasin B is only slightly higher than the labeling obtained in the basal state in the absence of cytochalasin B. Fig. 2 directly compares the
Cell Surface Labeling of Glucose Transporters

Fig. 1. a, effect of irradiation on the photoincorporation of ATB-BMPA into plasma membrane proteins of insulin-stimulated rat adipose cells. Isolated cells were incubated at 40% cytocrit in 6.0 ml of incubation medium containing 0.1% albumin and 10 nM insulin for 20 min at 37°C. They were then mixed with 3.0 ml of albumin-free medium containing 250 μCi of ATB-[2-3H]BMPA in 9-cm polystyrene dishes and irradiated for 45 s (O) or 90 s (C). The plasma membranes were prepared and labeled proteins were analyzed as described under “Experimental Procedures.” b, cytochalasin B (cyb) inhibition of incorporation of ATB-[2-3H]BMPA into plasma membrane proteins of insulin-stimulated rat adipose cells. Cells were treated as in a except that insulin-stimulated adipose cells with (O) or without (O) 50 pM cytochalasin B in the incubation medium were irradiated for 2 × 30 s.

The labeling of the glucose transporters with ATB-BMPA in basal and maximally insulin-stimulated adipose cells. The labeling shown here in the glucose transporter region of the gel in the insulin-stimulated state is =6-fold greater than that observed in the basal state. Over five individual experiments, the labeling was 6.3 ± 1.0-fold higher in the insulin-treated cells. Labeling in the glucose transporter region of the gel in the basal state gives a slight peak above the normal gel background but most of this is probably unrelated to the presence of glucose transporters because it is not immunoprecipitated by either anti GLUT1 or anti GLUT4 antibodies (see below).

Photolabeling of the glucose transporters present on the cell surface of basal and PMA- and insulin-treated adipose cells results in the gel profiles shown in Fig. 3a. PMA treatment increases the labeling relative to that observed in basal cells; the incorporation of radiolabel is 36% of that observed in the insulin-treated cells without subtraction of the basal level and 25% with this subtraction.

We have also directly compared insulin treatment, PMA treatment, and PMA treatment with cytochalasin B present to compete with the photolabel, as shown in Fig. 3b. The peak from the cells treated with PMA plus cytochalasin B is reduced by 70% compared to that observed with PMA alone. Subtraction of the cytochalasin B-uninhibitable labeling from that obtained with both PMA and insulin treatments indicates that the exposure of glucose transporters on the cell surface in response to PMA is 38% of that observed in response to insulin (34 ± 4% over four experiments).

The time course for the appearance of photolabel reactive glucose transporters on the surface of the adipose cell in response to PMA is shown in Fig. 4. The stimulation by PMA is maximal at 8-15 min (two experiments) and this correlates well with the half-time for stimulation of glucose transport activity as observed by Simpson et al. (11).

Glucose Transport Activity—3-O-Methyl-D-glucose transport experiments carried out at 37°C show that PMA-treated adipose cells exhibit transport activities which are 10-15% of those found with insulin-treated cells. Basal cells exhibit transport activities which are 2-5% of those observed with insulin. The rate constants for influx of 50 μM 3-O-methyl-D-glucose are 0.164 ± 0.013 s⁻¹ (insulin, n = 16), 0.021 ± 0.002 s⁻¹ (PMA, n = 12), and 0.007 ± 0.001 s⁻¹ (basal, n = 11).

Fig. 5 shows the inhibition of 3-O-methyl-D-glucose transport by ATB-BMPA. The concentrations of ATB-BMPA which half-maximally inhibit the influx of 50 μM 3-O-methyl-D-glucose (the Kᵢ) are 247 ± 27 μM in the insulin-stimulated state, 312 ± 103 μM with PMA, and 247 ± 59 in the basal state (calculated from two experiments in each case). These data therefore show that the insulin and PMA treatments do not increase photolabeling through changes in the
Cell Surface Labeling of Glucose Transporters

FIG. 3. Effect of PMA treatment on the photoincorporation of ATB-BMPA into plasma membranes of rat adipose cells. a, isolated cells were incubated at 40% cytocrit in 6.0 ml of incubation medium containing 0.1% albumin without additions (○) or with 1 μM PMA (□) or with 10 nM insulin (■) for 20 min at 37 °C. They were then mixed with 3.0 ml of albumin-free medium with the same additions and with 333 μCi of ATB-[2-3H]BMPA in 9-cm polystyrene dishes and immediately irradiated for 3 × 20 s. The plasma membranes were prepared and the labeled proteins were analyzed as described under "Experimental Procedures." b, inhibition of photoincorporation of ATB-BMPA into plasma membrane proteins of PMA-treated rat adipose cells by cytochalasin B (cyb). The experiment was carried out as in a except that rat adipose cells were treated with 10 nM insulin (△), 1 μM PMA (●), or 1 μM PMA and 50 μM cytochalasin B (■).

FIG. 4. A time course for PMA stimulation of the photoincorporation of ATB-BMPA into plasma membrane proteins of rat adipose cells. Isolated cells were incubated at 40% cytocrit in 6.0 ml of incubation medium containing 0.1% albumin and 1 μM PMA for 0 min (○), 4 min (△), 8 min (○), and 15 min (□). They were then mixed with 3.0 ml of albumin-free medium containing 333 μCi of ATB-[2-3H]BMPA in 9-cm polystyrene dishes and immediately irradiated for 1 min. The plasma membranes were prepared and the labeled proteins analyzed as described under "Experimental Procedures.

FIG. 5. Inhibition of 3-O-methyl-D-glucose uptake into rat adipose cells by ATB-BMPA. The transport of 3-O-methyl-D-glucose was measured at various concentrations of ATB-BMPA, as described under "Experimental Procedures." The rate constant for uninhibited uptake (○) over the rate constant for inhibited uptake (△) is plotted against the ATB-BMPA concentration for basal (○), cells treated with 1 μM PMA (●), and cells treated with 10 nM insulin (△). Kₐ estimates (from two experiments) were obtained by nonlinear regression fitting to the Michaelis-Menten equation and the values are given under "Results."
GLUT1 followed by anti-GLUT4 and vice versa. The most striking observation is that the anti-GLUT4 antibody precipitates much more photolabeled glucose transporter than the anti-GLUT1 antibody in both the basal and insulin stimulated states, but especially the latter. In addition, similar results are obtained independent of the order of immunoprecipitation. Finally, doubling the amount of antibody does not increase the yield of photolabeled glucose transporters in the precipitates (data not shown).

These observations indicate that the GLUT4 isoform is the more abundant transporter species in the plasma membrane after insulin treatment of the adipose cell and accounts for 89.8 ± 1.2% (n = 4) of the total. In the plasma membranes from basal cells only low levels of both GLUT1 and GLUT4 are found. The amount of GLUT4 in basal cells is 15–20-fold less than in the insulin stimulated cells (4.9 ± 0.39%, n = 6) and the amount of GLUT1 is ≈5-fold less (19.9 ± 2.4%, n = 4). Thus the effect of insulin is to increase the GLUT4/GLUT1 ratio ≈4-fold by inducing a larger proportionate increase in GLUT4.

Fig. 7 illustrates that PMA and insulin increase GLUT1 by about the same amount. The PMA treatment gives a 4.7-fold increase in GLUT1 of 5.7 ± 0.2 (n = 2) which is similar to the 5.2-fold increase given by insulin (described above). PMA treatment increases GLUT4 by about 4-fold but this is only 18.5 ± 2.7% (n = 4) of the level in insulin-stimulated cells. Following treatment of cells with PMA, the ratio of GLUT4/GLUT1 is therefore similar to that found in basal cells. It is only insulin that increases this ratio.

**DISCUSSION**

The stimulation of glucose transport by insulin in the rat adipose cell is now known to involve translocation of glucose transporters from a large intracellular pool to the plasma membrane (4, 5). However, substantial quantitative discrepancies between the stimulation of glucose transport and the increase in glucose transporter concentration found in the plasma membranes have led to the suggestion that insulin, in addition to inducing translocation, also increases the intrinsic activity of the plasma membrane transporters. The quantitation of the insulin-stimulated increase in glucose transporter concentration has until now been based on measurements (either cytochalasin B binding or Western blotting) using isolated plasma membranes while the large stimulations of glucose transport activity have been measured in intact cells. While the discovery of the GLUT4 glucose transporter isoform readily explains why the original Western blotting studies using anti-human erythrocyte glucose transporter antisera were so discrepant (8), a substantial disparity remains between glucose transport activity and the translocation of the GLUT4 isoform as measured by Western blotting of isolated plasma membrane fractions (13, 14).

In addition, Muhlbacher et al. (10) have recently suggested that PMA stimulates the translocation of inactive glucose transporters.
transporters to the plasma membrane and that a subsequent activation step is required to mimic the full effect of insulin. These investigators also postulated the involvement of a protein kinase C in the translocation mechanism. Egan et al. (32) and Ishizuka et al. (33) have subsequently shown that PMA and insulin both stimulate the plasma membrane form of this enzyme. However, Obermaier-Kusser et al. (94) have gone on to suggest that a phosphatidyl inositol glycan second messenger system further activates the plasma membrane glucose transporters. The possibility that phorbol ester stimulates the translocation of a glucose transporter isoform which contributes to cytochalasin B binding but relatively little to glucose transport activity was not examined by these authors. The bis-mannose labeling procedure described here permits, in combination with immunoprecipitation by specific anti-GLUT4 and anti-GLUT1 antibodies, an examination of the contributions of translocation of GLUT4 and GLUT1 glucose transporter isoforms and their intrinsic activations to the stimulation of glucose transport in the intact cell.

Nonlabeled ATB-BMPA inhibits 3-O-methyl-D-glucose transport with a $K_i$ of 250 $\mu$M in basal, PMA-treated, and insulin-treated rat adipose cells. The $K_i$ for ATB-BMPA inhibition of GLUT1 glucose transport activity in human erythrocytes is $\approx 300 \mu$M and therefore these results suggest that GLUT4 and GLUT1 have the same affinity for ATB-BMPA. Since the efficiency of labeling and of immunoprecipitation of GLUT4 and GLUT1 are similar the photolabeling technique can give a good approximation of the relative cell surface concentrations of GLUT4 and GLUT1.

Zorzano et al. (14) have suggested that GLUT1 is the major isoform present in the membranes from basal adipose cells. In our experiments, the labeling of GLUT4 and GLUT1 is very low in the basal state. In addition, the observed ratio of GLUT4/GLUT1 will be dependent on the efficiencies of labeling and immunoprecipitation which are similar (see "Results") but may not be exactly the same for the two isoforms. Bearing in mind these reservations concerning the accuracy of the technique, the results still suggest that GLUT4 is present in at least a 2-fold higher concentration than the GLUT1 glucose transporter isoform in the basal state. In five separate experiments the GLUT4/GLUT1 ratio was never less than this value. These results obtained with basal rat adipose cells are thus very different to those obtained in 3T3-L1 adipocytes where GLUT1 clearly predominates at the cell surface in the basal condition (35). This difference is presumably related to the much higher abundance of GLUT1 in these cells where it exceeds that of GLUT4; in rat adipose cells, GLUT1 is only present in the cell at about 2–5% of the GLUT4 abundance (12).

It seems probable that the basal level of GLUT1 in the plasma membranes of rat adipose cells may vary depending on the age and size of the cells. This may account for the discrepancy between the reports of some authors who show that basal and insulin-stimulated cells exhibit the same 3-O-methyl-D-glucose transport kinetic behavior (1–3, 36) and others who suggest that the kinetic characteristics are different (37). The GLUT1 $K_m$ value for 3-O-methyl-D-glucose exchange transport in erythrocytes (38) and the oocyte expression system (39, 40) is 5-fold higher than the GLUT4 $K_m$ in insulin-stimulated rat adipose cells (2, 36). Thus, in most cases where the $K_m$ value for 3-O-methyl-D-glucose exchange transport in basal adipose cells has been examined, the GLUT1 $K_m$ makes only a very small contribution to the operational $K_m$. The major kinetic feature of the stimulation of glucose transport by insulin remains the large $V_{max}$ increase which we have not shown correlates with an 20-fold increase in GLUT4 in the plasma membrane.
Using the ATB-BMPA photoprobe we have now shown that the extent to which PMA stimulates the appearance of glucose transporters on the cell surface is smaller than the effect of insulin on glucose transporter appearance but greater than PMA's corresponding stimulation of glucose transport activity. The labeling of glucose transporters agrees reasonably well with the extent of phorbol ester stimulation of the appearance of glucose transporters in the plasma membrane fraction as assessed by cytochalasin B binding (11). We have also shown here that the time course for PMA stimulation of photolabeling roughly parallels that for stimulation of glucose transport activity (11) suggesting that the arrival of glucose transporters at the cell surface is immediately accompanied by their participation in the glucose transport process without a significant lag.

Interestingly, PMA and insulin give an equal stimulation of GLUT1 but not GLUT4 translocation. The observed cell surface labeling of GLUT4 in PMA-treated adipose cells is 18% of that obtained with insulin-treated cells and correlates, more closely than the total cell surface labeling does, with the observed stimulation of glucose transport activity (shown in this study to be 13% of the rate obtained with insulin-treated cells). The combined immunoprecipitated photolabel using anti-GLUT4 and anti-GLUT1 antibodies is roughly equal to the observed total labeling in unprecipitated samples from the PMA-stimulated cells. The total is ~30% of that observed with insulin and thus is only slightly less than the 30–40% observed without precipitation. The discrepancy between the labeling and the observed transport rates in basal and PMA-stimulated cells may be because GLUT1 and GLUT4 have low turnover numbers in the basal and PMA-stimulated states. In addition, part of the discrepancy may simply be due to the extra manipulations employed to obtain photolabeling which may increase the concentration of cell surface transporters in the basal and PMA-stimulated states. However, a major part of the discrepancy between ligand binding (cytochalasin B and, in our case, ATB-BMPA) and glucose transport activity that has been noted (10, 11) seems to be due to the stimulation by PMA of the apperance of GLUT1 in the plasma membrane and the possible relatively small contribution of these glucose transporters to the glucose transport rate.

In general, the most relevant parameter in comparing the intrinsic activities of glucose transporters at low substrate concentrations is the TK value. The TK is the turnover number divided by the $K_a$ and has the units of an association constant (39). The TK multiplied by the concentration of transporters is equal to the $V_m/K_m$ ratio which is easily determined from the permeability or uptake rate constant obtained at low substrate concentrations. The TK is thus of physiological interest because it is the membrane permeability which limits the steady-state supply of intracellular glucose for metabolism. In addition, the TK is a useful parameter in comparing transporter isoforms because (like the $V_m/K_m$ ratio) it has the same value for exchange and net transport experiments even though the turnover numbers and $K_a$ values in these experiments may be different. Thus, since the insulin- and PMA-stimulated increases in the 3-O-methyl-d-glucose transport rate constants are similar to the increase in GLUT4 but not GLUT1, then it follows that the intrinsic activity of GLUT4 (TK4) is probably greater than the intrinsic activity of GLUT1 (TK1).

In conclusion, we have shown that the cell surface labeling technique with the bis-mannose photolabel can provide quantitative information on the abundance of glucose transporter isoforms at the plasma membrane and allows a demonstration of the recruitment phenomenon without the need for subcellular fractionation. Indeed, we have shown here that an ~20-fold increase in the recruitment of the GLUT4 glucose transporter isoform can explain most of insulin's stimulation of glucose transport activity. In addition, the technique has demonstrated that PMA and insulin stimulate GLUT1 but not GLUT4 translocation equally so that additional factors other than protein kinase C activation must be required to give the full insulin stimulation of GLUT4 translocation. The bis-mannose photolabel ATB-BMPA should be a useful tool to further explore these aspects of the glucose transporter translocation mechanism.

Acknowledgments—We are grateful to the Science and Engineering Research Council and the Medical Research Council (United Kingdom) for financial support. We thank Dr. G. E. Lienhard and D. M. Calderhead for their advice on immunoprecipitation and for discussions on the analysis of the intrinsic activities for GLUT1 and GLUT4. We also thank Carole Bramble (Bath) and M. J. Zarnowski and D. R. Yver (Bethesda) for their skilled technical assistance.

REFERENCES

1. Vinten, J., Gliemann, J., and Østerlind, K. (1976) J. Biol. Chem. 251, 794–800
2. Taylor, L. P., and Holman, G. D. (1981) Biochim. Biophys. Acta 642, 325–335
3. Gliemann, J., and Rees, W. D. (1983) in Curr. Top. Membr. Transp. 18, 339–370
4. Cushman, S. W., and Wardzala, L. J. (1980) J. Biol. Chem. 255, 4758–4762
5. Sussuki, K., and Kono, T. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2542–2545
6. Simpson, I. A., and Cushman, S. W. (1986) Annu. Rev. Biochem. 55, 1059–1089
7. Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karmeli, E., Sands, L. B., and Cushman, S. W. (1988) Biochim. Biophys. Acta 763, 295–307
8. Joost, H. G., Weber, T. M., and Cushman, S. W. (1988) Biochem. J. 249, 163–167
9. Joost, H. G., Weber, T. M., Cushman, S. W., and Simpson, I. A. (1987) J. Biol. Chem. 262, 11261–11267
10. Muhlbacher, C., Karmeli, E., Schaff, P., Obermaier, B., Moshack, J., Rattenbuber, E., and Haring, H. U. (1988) Biochem. J. 249, 865–870
11. Simpson, I. A., Habberfield, A. D., Holman, G. D., Cushman, S. W., and Saltis, J. (1989) Diabetes Suppl. 1, Abstr. 168
12. Oka, Y., Asano, T., Shishasaki, Y., Kasuga, M., Kamazawa, Y., and Takaku, F. (1988) J. Biol. Chem. 263, 13432–13439
13. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) J. Biol. Chem. 263, 185–189
14. Zorzano, A., Wilkinson, W., Kottiar, N., Thiodis, G., Wadzinkski, B. E., Ruoho, A. E., and Pilch, P. F. (1989) J. Biol. Chem. 264, 12368–12363
15. Calderhead, D. M., and Lienhard, G. E. (1988) J. Biol. Chem. 263, 12171–12174
16. Holdaus, G. D., and Midgley, P. J. W. (1985) Carbohydr. Res. 135, 337–341
17. Holman, G. D., and Rees, W. D. (1987) Biochim. Biophys. Acta 897, 393–400
18. Holman, G. D., Parkar, B. A., and Midgley, P. J. W. (1986) Biochim. Biophys. Acta 855, 115–126
19. Holman, G. D., Karim, A. R., and Karim, B. (1988) Biochim. Biophys. Acta 946, 75–84
20. Clark, A. E., and Holman, G. D. (1990) Biochim. J. 269, 615–622
21. James, D. E., Strube, M. I., and Mueckler, M. (1989) J. Biol. Chem. 264, 83–87
22. Charroin, M. J., Brosius, F. C., Alper, S. L., and Lodish, H. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2535–2539
23. Birnbaum, M. J. (1989) Cell 57, 305–315
24. Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. T., Cornellius, P., Fekela, P. H., and Lane, M. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 3150–3154
25. Fukumoto, H., Kayano, T., Buse, J. B., Edwards, Y., Pilch, P. F., Bell, G. I., and Seino, S. (1989) J. Biol. Chem. 264, 7776–7779

Downloaded from http://www.jbc.org/ by guest on March 21, 2020
26. Kahn, B. B., Charron, M. J., Lodish, H. F., Cushman, S. W., and Flier, J. S. (1989). *J. Clin. Invest.* **84**, 404-411
27. Berger, J., Biawas, C., Vicario, P. P., Strout, H. V., Saperstein, R., and Pilch, P. F. (1989). *Nature* **340**, 70-72
28. Sivitz, I., DeSautel, S. L., Kayano, T., Bell, G. T., and Pessin, J. E. (1989). *Nature* **340**, 72-74
29. Laemmli, U. K. (1970). *Nature* **227**, 680-685
30. Davies, A., Meeran, K., Cairns, M. T., and Baldwin, S. A. (1987). *Biochem. J.* **256**, 515-520
31. Schroer, D. W., Frost, S. C., Kohanski, R. A., Lane, M. D., and Lienhard, G. E. (1996). *Biochem. Biophys. Acta* **885**, 317-326
32. Egan, J. J., Saltis, J., Wek, S. A., Simpson, I. A., and Londos, C. (1990). *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1052-1056
33. Ishizuka, T., Cooper, D. K., Hernandez, H., Buckley, D., Standaert, M., and Farese, R. V. (1990). *Diabetes* **39**, 181-190
34. Obermaier-Kusser, B., Muhlbacher, C., Seffer, E., Friel, R., Machiraju, F., Schmidt, F., and Haring, H. U. (1989). *Biochem. J.* **256**, 515-520
35. Calderhead, D. M., Kitagawa, K., Tanner, L. I., Holman, G. D., and Lienhard, G. E. (1990). *J. Biol. Chem.* **265**, 13800-13808
36. Whitesell, R. R., and Gliemann, J. (1979). *J. Biol. Chem.* **255**, 5276-5283
37. Whitesell, R. R., Regen, D. M., and Abumrad, N. A. (1989). *Biochemistry* **28**, 6937-6943
38. Barnett, J. E. G., Holman, G. D., and Munday, K. A. (1973). *Biochem. J.* **131**, 211-221
39. Gould, G. W., and Lienhard, G. E. (1989). *Biochemistry* **28**, 9447-9452
40. Keller, K., Strube, M., and Mueckler, M. (1989). *J. Biol. Chem.* **264**, 18884-18889
Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester.
G D Holman, I J Kozka, A E Clark, C J Flower, J Saltis, A D Habberfield, I A Simpson and S W Cushman

*J. Biol. Chem.* 1990, 265:18172-18179.

Access the most updated version of this article at [http://www.jbc.org/content/265/30/18172](http://www.jbc.org/content/265/30/18172)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/265/30/18172.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/30/18172.full.html#ref-list-1](http://www.jbc.org/content/265/30/18172.full.html#ref-list-1)